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RESEARCH ON NANODELIVERY SYSTEMS FOR NASAL VACCINE

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LIST OF PUBLICATIONS

Residence time and uptake of porous and cationic maltodextrin-based nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles. ***Le et al; Int J Pharm. 2018 Aug 29.***

Porous nanoparticles with self-adjuvanting M2e-fusion protein and recombinant hemagglutinin provide strong and broadly protective immunity against influenza virus infections. ***Bernasconi et al; Front. Immunol, 2018 Aug 21.***

Porous and cationic maltodextrin nanoparticles are more efficient vectors for protein delivery into nasal mucosal cells than cationic or anionic nanoparticles. ***Le et al; Int J Pharm, Revision.***

Porous and cationic maltodextrin nanoparticles carrying CTA1-DD adjuvant induce protection from virus infection and transmission. ***Le et al; submitted.***

Review: Nanotechnology solutions for mucosal vaccine. ***Le et al; Submitted.***

ABBREVIATIONS

(except of publications)

CME	clathrin-mediated endocytosis
CavE	caveolin-mediated endocytosis
BSA	bovine serum albumin
PEG	polyethylene glycol
PLGA	poly(lactic-co-glycolic acid)
Tf	transferrin
EGF	epidermal growth factor
LDL	low-density lipoprotein
DOX	doxorobucin hydrochloride
HA	hemagglutinin
NA	neuraminidase
IIV	inactivated influenza vaccine
LAIV	live attenuated influenza vaccine
WIV	whole inactivated virus vaccines
TIV	trivalent inactivated vaccine
QIV	quadrivalent inactivated vaccine
NK cells	natural killer cells
CT	cholera toxin
MHC	major histocompatibility complex

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AIM AND OUTLINE OF THE THESIS

Aim of the thesis

This thesis was 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, France.

The work was a part of 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 Nils Lycke from the University of Gothenburg. The aim of this project is to develop a universal influenza vaccine. UniVac Flu project used the adjuvanted and targeted the conserved antigen M2 as a construction: CTA1-3M2e-DD. This antigen contains three parts: the non-toxic cholera toxin subunit A as the vaccine adjuvant, the extracellular portion M2e as the universal flu antigen, and the dimer of the D fragment of the staphylococcal protein A for targeting B cells. Therefore, to optimize the mucosal vaccine efficacy we combined CTA1-3M2e-DD with the nanoparticle technology. This vaccine was evaluated for the induction of protective immunity after intranasal administration and we addressed the protection from influenza virus transmission (the process by which influenza viruses spread between mice) with Pr. Peter Staeheli and co-workers, from the University of Freiburg.

We also investigated the oral administration of the nanoparticle vaccine in collaboration with Maria Rescigno *et al.*, form the European Institute of Oncology in Milan. The immune response of antigen loaded nanoparticle through intradermal vaccination was investigated by Linda Klavinskis and co-workers, from the University of King's college London.

In our laboratory, we compared different nanocarriers to find the best candidate for intranasal influenza vaccine. Overall, the aim of this thesis is the evaluation of different nanocarriers used as nasal delivery system to search a suitable vehicle for the nasal vaccine.

Outline of the thesis

This thesis is organized in four principal chapters:

The PART I is a general introduction about nanomedicine and influenza vaccine. This chapter is arranged in two main sections:

- The first section focuses on nanomedicines and their applications. This section contains a review, which discusses different nanotechnology approaches to improve the efficacy of the mucosal vaccine.

  Publication 1: **"Nanotechnology solutions for mucosal vaccine".** MQ. Le, R. Carpentier, D. Betbeder (submitted).

- In the second section, the main features of Influenza virus and vaccines are described.

The PART II presents the results on the nanoparticles evaluation for nasal vaccine:

- In the first part, we present an article to evaluate the best type of nanoparticles captured by mucosa cells *in vivo* and *in vitro*.

  Publication 2: **"Residence time and uptake of porous and cationic maltodextrin-based nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles."** MQ. Le, R. Carpentier, I. Lantier, C. Ducournau, I. Dimier-Poisson, D. Betbeder. International Journal of Pharmaceutics (accepted).

- In the second part, the results about the evaluation of the 5 nanoparticles as nasal protein delivery system was reported.

  Publication 3: **"Porous and cationic maltodextrin nanoparticles are more efficient vectors for protein delivery into nasal mucosal cells than cationic or anionic nanoparticles".** MQ. Le, R. Carpentier, I. Lantier, C. Ducournau, I. Dimier-Poisson, D. Betbeder. International Journal of Pharmaceutics (revision).

- In the third part, we present the study about the inhibition of H3N2 influenza virus transmission using our intranasal influenza vaccine delivered by NP.

Publication 4**: "Porous and cationic maltodextrin nanoparticles carrying CTA1-DD adjuvant induce protection from virus infection and transmission".** MQ. Le, L. Ye, V. Bernasconi, R. Carpentier, NY. Lycke, P. Staeheli, D. Betbeder (submitted).

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

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

ABSTRACT

Nasal administration has great advantage for stimulating the immune system, such as stimulating local and systemic protective immunity. However, delivery systems and adjuvants are often necessary to improve the efficacy of the intranasal vaccine. We applied nanoparticle technology to deliver a universal influenza vaccine via the nasal route in a European FP7 project called UniVacFlu.

We evaluated different nanoparticles to search the best nanocarrier for an intranasal vaccine. Here we compared 5 types of nanoparticles with different surface charges (anionic or cationic) and various inner compositions as potential vectors: cationic and anionic liposomes, cationic and anionic PLGA (Poly Lactic co-Glycolic Acid) nanoparticles and zwitterionic maltodextrin nanoparticles (cationic surface with an anionic lipid core: NPL). We first quantified their nasal residence time after nasal administration in mice using *in vivo* live imaging and NPL showed the longest residence time. *In vitro* endocytosis on mucosal cells (airway epithelial cells, macrophages and dendritic cells) using labeled nanoparticles were performed by flow cytometry and confocal microscopy. Among the 5 nanoparticles, NPL were taken up to the greatest extent by the 3 different cell lines and the endocytosis mechanisms of NPL were characterized. In order to compare different nanoparticles as vaccine carriers, antigen loading and cell delivery were evaluated. In this study, we compared the loading and delivery of labeling ovalbumin with airway mucosa cells (airway epithelial cells, macrophages and dendritic cells) by flow cytometry. Our data showed that NPL were the best candidate that can payload with highest amount of protein and eventually the most efficient cellular protein delivery capacity. Taken together, our study revealed that among 5 nanoparticles, NPL were the best nanocarrier that own longer nasal residence time, efficiently uptake and deliver protein into airway epithelium. NPL were then selected as nanocarrier for the UniVac Flu project.

The flu antigens CTA1-3M2e-DD and HA were formulated with NPL. The CTA1-3M2e-DD is an adjuvanted antigen composed of the A1 subunit of cholera toxin and a conserved epitope of influenza A virus (M2e), as well as the dimer of the synthetic analogue of Staphylococcus aureus protein A (DD) used to target B cells. To improve antigenic effect, recombinant HA from H1N1 was combined with CTA1-3M2e-DD. These formulations were evaluated in mice

by the UniVacFlu consortium. We observed that CTA1-3M2e-DD and HA loaded into NPL could be a promising universal intranasal influenza vaccine.

Keywords: nanoparticles, intranasal vaccine delivery, influenza, mucoadhesive

RÉSUMÉ

L'administration nasale a un grand avantage pour stimuler l'immunité protectrice locale et systémique. Cependant, des systèmes d'administration et des adjuvants sont souvent nécessaires pour améliorer l'efficacité du vaccin intranasal. Nous avons appliqué la technologie des nanoparticules en tant que système universel de délivrance de vaccins contre la grippe dans le projet européen FP7 appelé UniVacFlu.

Nous avons évalué différentes nanoparticules (NP) pour rechercher le meilleur nanovecteur. Pour cela, nous avons comparé 5 types de nanoparticules avec différentes charges de surface (anioniques ou cationiques) et diverses compositions internes comme vecteurs potentiels: des liposomes cationiques ou anioniques, des NP de PLGA cationique ou anionique (poly acide lactique co-glycolique) et une NP cationique composée de maltodextrine fonctionnalisée par un agent cationisant avec un cœur de lipides anioniques (NPL). Nous avons d'abord quantifié leur temps de résidence nasale après l'administration nasale chez la souris en utilisant l'imagerie *in vivo* et les NPL ont montré le plus long temps de résidence. *L'*endocytose *in vitro* sur des cellules muqueuses (cellules épithéliales des voies respiratoires, macrophages et cellules dendritiques) en utilisant des nanoparticules marquées a été réalisée par cytométrie de flux et microscopie confocale. Parmi les 5 nanoparticules, les NPL ont été majoritairement captées par 3 lignées cellulaires différentes représentatives d'un épithélium respiratoire et les mécanismes d'endocytose ont été caractérisés. Afin d'évaluer le meilleur vecteur en tant que véhicules, le chargement d'antigènes et la délivrance intracellulaire ont été évalués dans des cellules de la muqueuse des voies respiratoires (cellules épithéliales des voies aériennes, macrophages et cellules dendritiques) par cytométrie de flux. Nous montrons que les NPL sont les meilleurs candidats capables de délivrer la plus grande quantité de protéines dans les cellules. Pris ensemble, notre étude a révélé que parmi 5 nanoparticules, la NPL était le meilleur nanovecteur en termes de temps de résidence nasale, d'endocytose par les cellules et de délivrance de protéines dans l'épithélium des voies respiratoires. Les NPL ont donc été sélectionnées comme nanovecteurs pour le projet UniVac Flu.

Les antigènes de la grippe CTA1-3M2e-DD et HA ont été formulés avec les NPL. Le CTA1-3M2e-DD est un antigène chimérique adjuvanté et ciblé. Il est composé de la sous-unité A1 de la toxine du choléra et un épitope conservé du virus grippal A (M2e), ainsi que le dimère de l'analogue synthétique de la protéine A de Staphylococcus aureus (DD) utilisé comme agent

de ciblage des lyphocytes B. Pour améliorer l'effet antigénique, l'HA recombinant de H1N1 a été combinée avec CTA1-3M2e-DD. Ces formulations ont été évaluées chez la souris par le consortium UniVacFlu. Les résultats ont montré que CTA1-3M2e-DD et HA chargé dans les NPL formeraient un vaccin intranasal prometteur contre la grippe.
Ce travail de thèse montre que les NPL sont des nanovecteurs d'intérêt pour le vaccin nasal.

Mots - clés : nanoparticules, délivrance, administration intranasale, vaccin, grippe

RÉSUMÉ DE LA THÈSE

Préambule

Ce travail a été réalisé sous la direction du Professeur Betbeder au sein de l'équipe Nanomédecine de l'unité Inserm LIRIC-U995 de l'Université de Lille. Nous avons évalué différentes nanoparticules comme un système d'apport de protéines dans la muqueuse nasale. Ces études sont importantes pour développer un nouveau système de délivrance nano-technologique pour une application dans le domaine du vaccin, et plus particulièrement pour le développement d'un vaccin universel contre la grippe administré par les voies muqueuses. Ce travail fait partie d'un consortium européen appelé UniVacFlu. Le projet UniVacFlu a été financé par le septième programme-cadre de l'Union européenne FP7 / 2007/2013. Le consortium UniVacFlu est coordonné par le professeur Lycke de l'Université de Göteborg et est composé de plusieurs partenaires: l'Université de Gand, l'Université de Fribourg, l'Institut européen d'oncologie de Milan et le Kings College de Londres. Notre rôle au sein du consortium UniVacFlu est (i) de comparer différentes nanoparticules pour définir le meilleur nanovecteur pour un vaccin nasal contre la grippe, (ii) de préparer et de caractériser les formulations vaccinales (nanoparticules et antigènes), et (iii) de fournir des formulations à nos partenaires.

Introduction

La vaccination est l'un des moyens les plus efficaces pour contrôler les maladies infectieuses telles que la grippe. Actuellement, il existe deux formulations du vaccin antigrippal: le vaccin antigrippal inactivé et le vaccin antigrippal vivant atténué. Le plus grand défi pour le vaccin antigrippal est la variabilité élevée et l'évolution rapide du virus qui résulte d'une adaptation constante du vaccin pour correspondre aux souches circulantes. Un autre obstacle pour un vaccin efficace est le délai nécessaire pour la fabrication du vaccin et l'approbation par les autorités réglementaires. En outre, les groupes à haut risque, tels que les personnes âgées ou les personnes immunodéprimées peuvent ne pas répondre de manière optimale à la vaccination en raison d'une fonction immunitaire diminuée. Les limites mentionnées mettent en évidence la nécessité de technologies innovantes et un système de délivrance d'antigènes, servant de plateforme vaccinale, pourrait améliorer la production et la disponibilité des vaccines, tout en induisant de manière durable une immunité protectrice. Différentes voies d'administration et différentes stratégies de vaccination pourraient améliorer la réponse immunitaire des vaccins traditionnels. En outre, les vaccins ciblant des régions conservés, universelles, du virus entraînent une immunité plus large et plus durable. Ces vaccinations «universelles» sont donc vivement souhaitables (Kunisaki and Janoff 2009).

Le projet UniVacFlu a pour but le développement d'un vaccin intranasal universel contre la grippe basé sur l'épitope grippal conservé M2e. En outre, pour vaincre la potentielle faible immunogénicité de l'antigène M2e, un adjuvant innovant (CTA1-DD), a été fusionnée à 3 répétitions M2e en tandem, pour donner la protéine de fusion antigénique adjuvantée : CTA1-3M2e-DD. CTA1 correspond à la toxine cholérique détoxifiée. CTA1 est lié à la région C-terminale d'un dimère du fragment D de la protéine A de *Staphylococcus aureus* (DD). DD lie les fragments Fc et Fab des immunoglobulines présentes sur les cellules B permettant ainsi la liaison du complexe CTA1-DD aux cellules (Lycke 2004a). En outre, afin de créer une réponse antigénique plus large, CTA1-3M2e-DD a été associée avec l'HA recombinant de H1N1.

Les nanoparticules (NP) sont des transporteurs conçus pour prolonger le temps de résidence intranasale des antigènes et les protéger contre la dégradation. Selon cette idée, le vaccin universel contre la grippe sera une combinaison nanotechnologique de NP, de CTA1-3M2e-DD et d'HA administrée par voie nasale. Différents nanovecteurs ont été étudiés dans notre

laboratoire pour sélectionner le système approprié pour le développement de la grippe intranasale.

De nombreuses études récentes ont montré que les nanoparticules améliorent significativement l'administration de candidats vaccins dans la muqueuse nasale (Bernocchi, Carpentier, and Betbeder 2017). Cependant, une meilleure compréhension de l'interaction des nanoparticules avec les cellules muqueuses sont essentielles pour l'administration de médicaments ainsi que pour les applications vaccinales.

Par conséquent, étudier comment les caractéristiques physicochimiques des nanomatériaux influencent leurs interactions avec les cellules sont essentielles pour obtenir des applications cliniques efficaces. Les interactions des NP avec les cellules dépendent du matériau, de la taille, de la forme (Blanco, Shen, and Ferrari 2015), de la charge de surface et de la chimie de surface des NP. Par ailleurs, la physiologie cellulaire (état de différenciation, taux de prolifération, morphologie ...) et le type cellulaire, ainsi que leurs interactions avec milieux physiologiques (sels, protéines, agglomérats ...), influencent aussi les mécanismes d'interaction des nanoparticules avec des cellules. En outre, les NP sont utilisées comme système de délivrance pour le vaccin nasal, pour améliorer la délivrance de l'antigène aux cellules immunitaires tout en limitant la clairance muqueuse. D'autres avantages comprennent leur capacité à protéger des antigènes contre la dégradation enzymatique et le transport à travers la barrière muqueuse. Cependant, alors que de nombreux systèmes de délivrance nanoparticulaires sont décrits, aucune comparaison objective, dans des conditions équivalentes de dose, de modèle… n'a été faite; il est donc difficile de déterminer le vecteur idéal.

Dans cette étude de thèse, nous avons étudié l'effet de la composition et de la charge de surface des nanoparticules sur leurs interactions avec les cellules de la muqueuse nasale, en comparant des liposomes anioniques et cationiques, des nanoparticules anioniques et cationiques d'acide co-glycolique poly-lactique (PLGA NP) et les NPL zwitterionique (nanoparticules poreuses à base de maltodextrine avec un cœur composé lipide anionique). Dans cette étude, nous avons déterminé l'influence de la charge et de la composition interne de ces 5 types de nanoparticules sur le temps de résidence nasale *in vivo* et leur absorption *in vitro* par les cellules de la muqueuse respiratoire. Dans cette comparaison, différentes propriétés telles que le chargement d'antigènes et la délivrance intracellulaire doivent être évaluées dans des cellules épithéliales des voies respiratoires, macrophages et cellules dendritiques.

BUT DE LA THESE

Afin de sélectionner le nanovecteur approprié pour délivrer le vaccin intranasal contre la grippe, nous avons comparé différentes nanoparticules utilisées pour l'application de vaccins. Ces nanoparticules ont été synthétisées et comparées en termes d'interaction avec la muqueuse nasale *in vivo* et *in vitro* et d'efficacité de chargement et de délivrance d’antigènes dans les cellules.

Résultats et discussion

De nombreuses études ont montré les avantages de l'utilisation des nanoparticules en tant que système de délivrance intranasal de vaccin : capacité à protéger les antigènes contre la dégradation enzymatique, amélioration de la délivrance intracellulaire, extension du temps de résidence dans la cavité nasale. Cependant, une meilleure compréhension de l'interaction des nanoparticules avec les cellules muqueuses est essentielle pour l'administration de médicaments ainsi que pour les applications de vaccins.

Nous avons étudié l'effet de la charge de surface et de la composition des nanoparticules sur leurs interactions avec les cellules de la muqueuse nasale sur 5 types de nanoparticules cationiques et anioniques. L'étude du temps de résidence nasale *in vivo* de ces 5 types de nanoparticules a montré que les PLGA NP et les NPL ont le temps de résidence le plus long. Ceci est en accord avec les expériences précédentes qui ont décrit une interaction plus forte entre les composés cationiques et la muqueuse des voies aériennes que les composés anioniques ou neutres (Marasini, Skwarczynski, and Toth 2017). En effet, l'augmentation du temps de résidence peut être attribuée aux fortes interactions électrostatiques entre les nanoparticules cationiques et les fractions sialiques et les acides anioniques des glycosaminoglycanes portés par les mucines à la surface des cellules des voies respiratoires. De façon inattendue, les liposomes cationiques ont montré un comportement complètement différent, même s'ils possédaient une charge positive de surface similaire aux PLGA NP cationiques et aux NPL.

Dans cette étude, les NPL montrent le temps de résidence nasale le plus long et en quantité plus importante comparé aux 4 autres nanoparticules. Ceci pourrait être dû à la présence de lipides anioniques dans le noyau de ces nanoparticules (Paillard et al. 2010). Dans une autre étude, nous avons constaté que les NPL augmentaient le temps de résidence nasale d'une protéine encapsulée et que ces NPL ont été observées dans la première couche de la muqueuse nasale après administration nasale (Bernocchi et al. 2016). Selon cette conclusion, nous avions émis l'hypothèse que NPL pourraient être plus efficacement absorbées par les cellules sous-jacentes à la couche de mucus. Afin de confirmer cela, nous avons étudié l'endocytose cellulaire des 5 types de nanoparticules dans des lignées cellulaires représentatives de la muqueuse des voies respiratoires (Kiyono and Fukuyama 2004) par cytométrie en flux et microscopie confocale. Fait intéressant, alors que les NPL ont montré une endocytose initiale rapide (dans les 2 premières heures), les autres types de nanoparticules étaient progressivement captées pendant 24h. Comme prévu, les nanoparticules anioniques étaient endocytosées dans une moindre

mesure par rapport aux cationiques (Le Broc-Ryckewaert et al. 2013). Ces résultats étaient confirmés dans les 3 lignées cellulaires en utilisant une autre sonde fluorescente: Rhodamine DHPE (Dihexadécanoyl-sn- glycéro-3-phosphoéthanolamine). L'efficacité relativement faible de l'endocytose des liposomes par rapport aux PLGA NP et aux NPL pourrait être attribuable à leur stabilité *in vitro* et *in vivo*. Cependant, nous avons confirmé leur stabilité colloïdale.

Comprendre les mécanismes d'endocytose des nanoparticules est donc vital pour développer des applications thérapeutiques efficaces. En pratique, les voies d'endocytose des 5 nanoparticules pourraient être médiées par des mécanismes non-phagocytaires, la taille de toutes les nanoparticules est inférieure à 1µm. En raison de l'endocytose relativement faible des liposomes et PLGA NP par rapport aux NPL, nous avons concentré notre étude sur mécanismes d'endocytose de ces NPL. Nous avons observé un profil similaire pour toutes les lignées cellulaires: l'inhibition des voies dépendantes des clathrines, des cavéolines et de la micropinocytose a diminué l'endocytose des NPL. La dynamine est impliquée dans de nombreuses voies d'endocytose, y compris les voies dépendantes des clathrines et des cavéolines, et favorise la scission de vésicules d'endocytose intracellulaires (Sahay, Alakhova, and Kabanov 2010). L'inhibition des mécanismes dépendants de la dynamine a confirmé que les clathrines et les cavéolines étaient impliquées dans l'endocytose des NPL. Nos résultats suggèrent que l'endocytose des NPL était une combinaison de plusieurs voies. Dans une étude la voie de la clathrine s'est révélée être la principale voie d'absorption cellulaire des nanoparticules de chitosan dans les cellules Caco-2 (Ma and Lim 2003). D'autres expériences ont suggéré que la voie de la clathrine et la macropinocytose étaient impliquées dans l'absorption de nanoparticuoles de cholestéryl-pullulane (CHP) (Jiang et al. 2013). Nous avons constaté que les NPL entraient dans les cellules épithéliales, les macrophages et les cellules dendritiques via une combinaison de plusieurs voies. Ce comportement pourrait être due à la composition et la structure poreuse de ces nanoparticules, ce qui permet aux NPL d'exposer à la fois les charges cationiques (polysaccharides) et anioniques (lipides), permettant ainsi de fortes interactions avec les composants de la membrane cellulaire.

L'étude du temps de résidence nasale et de l'endocytose de 5 NP a montré que les NPL étaient le meilleur candidat pour délivrer des antigènes dans les cellules épithéliales des voies respiratoires en raison de son long temps de résidence et de ses capacités d'endocytose. Selon ces résultats prometteurs, nous avons continué à comparer ces 5 nanoparticules différentes en termes de capacité d'association et de délivrance intracellulaire de protéines. Le but de cette étude est de déterminer le meilleur système de délivrance pour les vaccins administrés par voie nasale.

En conséquence, nous avons comparé l'efficacité de la charge protéique des 5 types de NP. Seul le NPL a montré 100% de charge en protéines à des rapports massiques de 1/10 et 3/10 (OVA / NP). Les PLGA NP et les liposomes n'ont pas atteint ce niveau de chargement de protéines, comme attendu (Colletier et al. 2002). Ceci est probablement dû à la structure des nanoparticules: les NPL sont poreuses et la surface d'interaction avec les protéines est plus grande que les nanoparticules non poreuses comme le PLGA NP et les liposomes où les interactions protéiques sont limitées à la surface des nanoparticules. Avec les liposomes, nous avons observé une augmentation de la taille et une diminution du potentiel zêta qui s'approchait du potentiel zêta de la protéine libre, démontrant clairement une association de l'OVA avec ces NP à leur surface. Cette association a atteint la saturation puisque l'augmentation du rapport OVA / NP a conduit à une diminution de l'efficacité d'association. Pour augmenter la charge protéique, l'OVA aurait pu être piégé dans les liposomes comme décrit précédemment mais l'efficacité de chargement reste faible (Watson, Endsley, and Huang 2012) et les efforts pour augmenter l'efficacité de la charge protéique conduisent à une hétérogénéité de taille incompatible avec les applications pharmaceutiques (Xu, Khan, and Burgess 2011). Avec les PLGA NP, nous n'avons pas observé de grandes modifications de la taille et du potentiel zêta lorsqu'ils sont associés à des protéines. Cependant, les nanoparticules de PLGA sont des particules sphériques (Sanna et al. 2012) et il est probable que les protéines interagissent uniquement avec la surface des nanoparticules. De plus, l'insertion d'une protéine au cours de la synthèse de PLGA NP pourrait dénaturer sa forme native (van de Weert, Hennink, and Jiskoot 2000) et donc son immunogénicité ou sa fonction. Par conséquent, la méthode de post-chargement est le processus préféré pour associer des protéines aux PLGA NP.

Nous avons ensuite évalué la délivrance de protéines dans 3 lignées cellulaires représentatives du tissu muqueux des voies respiratoires: les cellules épithéliales, les cellules dendritiques et les macrophages. Pour garder la cohérence, la même quantité de nanovecteurs a été utilisée tout au long de la comparaison. Dans ces lignées cellulaires, les 5 types de nanoparticules ont délivré la protéine OVA. Cependant, le NPL étaient beaucoup plus efficaces à cet égard que les 4 autres types de nanoparticules testés. La différence est apparue dans les 30 premières minutes pour les cellules épithéliales et dendritiques et après seulement 2h dans les macrophages. Cela implique que l'utilisation de NPL pour la délivrance de protéines ne nécessitera pas un temps d'exposition trop long du nanovecteur avec des cellules ou des tissus. (Bernocchi et al. 2016).

La cinétique de la délivrance protéique par les 5 types de nanoparticules a varié entre les types cellulaires, et notamment les macrophages. Dans ces cellules, on ne peut pas exclure que la

phagocytose et la pinocytose non-spécifiques se produisent en combinaison avec l'endocytose, conduisant à une libération constante et non saturée de la protéine sur 24 heures. Ceci est soutenu par la délivrance de protéines par les PLGA et les liposomes qui étaient très similaires à l'endocytose de la protéine seule (augmentation de 1,5 fois).

A notre connaissance, il s'agit de la première étude qui compare plusieurs types de nanocarriers actuellement pour la délivrance de protéines dans les voies respiratoires. Nous avons précédemment montré que l'administration intranasale d'un extrait total de *Toxoplasma.gondii* chargé dans NPL a fourni une protection contre un challenge oral de ce parasite (Dimier-Poisson et al. 2015) et que NPL augmentent également le temps de résidence nasal de protéines (Bernocchi et al. 2016). Ces données soulignent le potentiel de NPL en tant que nanovecteur pour la délivrance de vaccins administrés par voie nasale.

Nous avons décidé d'utiliser les NPL comme nanosystème de libération d'un vaccin intranasal contre la grippe. Les antigènes de la grippe CTA1-3M2e-DD et HA ont été formulés avec NPL. La construction de l'antigène CTA1-3M2e-DD a été préparée par l'association de l'adjuvant muqueux CTA1-DD et du domaine M2e, un épitope conservé de la grippe. La protéine de fusion CTA1-3M2e-DD combine donc un adjuvant et un antigène conservé de grippe A pour générer un vaccin anti-grippal universel.

Différents antigènes(Ag) de la grippe ont été combinés aux NPL pour préparer la formulation à des rapports de 1/5 (m/m). Le CTA1-3M2e-DD, l'hémagglutinine (HA) ou l'adjuvant CTA1-DD ont été chargés dans ces NPL. L'analyse des formulations 1/5(m/m) a montré une augmentation de la taille des formulations, ce qui signifie que certaines formulations NPL-Ag ont été partiellement agglomérées.

Lorsque l'antigène a été incubé aux NPL, nous avons obtenu seulement une légère diminution de la charge positive des nanoparticules montrant que CTA1-3M2e-DD a été chargé avec succès dans la NPL. Un résultat similaire a été obtenu avec le chargement de deux antigènes différents dans les NPL.

Afin d'évaluer si un antigène était libre, nous avons analysé la formulation par électrophorèse en gel de polyacrylamide en conditions non- dénaturantes (PAGE natif)**.** Au rapport 1/5, les protéines CTA1-3M2e-DD et CTA1-DD étaient totalement liées au NPL alors que seulement 70% de l'HA était associé. Ces résultats peuvent être attribués à l'utilisation de détergents utilisés pour solubiliser l'HA. Cela peut former des micelles qui empêchent l'association d'HA aux NPL. Cependant, la plus grande partie de l'HA est liée aux NPL et ces résultats suggèrent qu'à un rapport de 1/5 (m/m), 3 antigènes différents ont été associés avec succès aux NPL. Ces

formulations ont été testées par nos partenaires du consortium UniVacFlu pour une analyse *in vivo* d'une vaccination intranasale contre le virus de la grippe.

Après immunisations intranasales de souris avec CTA1-3M2e-DD ou CTA1-3M2e-DD / NPL, les souris immunisées avec CTA1-3M2e-DD / NPL démontraient l'efficacité protectrice de cette formulation contre la souche peu virulente X47 du virus de la grippe (H3N2), comparées aux souris ayant CTA1-3M2e-DD. De plus, les souris immunisées par voie intranasale avec le HA + CTA1-M2e-DD / NPL ont été entièrement protégées contre une infection provoquée par le virus agressif PR8 (A / Puerto Rico / 8/34 (H1N1), alors que la formulation HA / NPL, CTA1-M2e-DD / NPL ou CTA1-M2e-DD seule n'a pas protégé les souris. Dans ce projet, nous avons montré que la co-intégration de deux antigènes cibles spécifiques de la grippe, le CTA1-3M2e-DD et l'HA dans les NPL était plus efficace contre les infections par le virus de la grippe que l'un ou l'autre des composants utilisés seuls, le vecteur combiné HA + CTA1-3M2e-DD / NPL présente une immunogénicité améliorée et une meilleure protection virale après immunisations intranasales.

Ensuite, nous avons appliqué ces NPL comme nanosystème de délivrance d'antigène pour développer un vaccin intranasal contre le virus de la grippe Udorn (H3N2). Dans cette étude, nous avons évalué l'immunogénicité, l'efficacité protectrice et la transmission du virus en utilisant des formulations vaccinales composées de Udorn split virus seul ou en mélange avec les nanoparticules NPL et / ou CTA1-DD et administrées par voie nasale.

Les charges et l'hydrophobie des NPL facilitent les interactions électrostatiques avec les antigènes chargés négativement. De plus, leur structure poreuse permet aux NPL d'être chargées avec une grande quantité d'antigènes. Les antigènes provenant du virus Udorn inactivé et éclaté (split Udorn) et le CTA1-DD utilisé dans notre étude s'associent aux NPL comme le démontre nos PAGE natifs. Fait important, les NPL peuvent très efficacement délivrer split Udorn dans les 3 lignées cellulaires de la muqueuse nasale. L'amélioration de la délivrance des antigènes dans les cellules présentatrices d'antigènes font des NPL un système idéal de délivrance.

L'étude *in vivo* a montré que l'administration intranasale de split Udorn / NPL augmente légèrement la réponse des anticorps IgG spécifiques d' Udorn par rapport aux souris ayant reçu Udorn seul. L'ajout du CTA1-DD améliore encore le niveau d' IgG spécifiques produits.

Dans le cas d'infection par des virus respiratoires tels que la grippe, une attention particulière devrait être accordée au nez et aux poumons en tant qu'organes de transmission et de points d'entrée de ces virus respiratoires (Kohlmeier and Woodland 2009). Dans cette étude, nous avons comparé nos vaccins antigrippaux en termes d'inhibition virale dans le museau et les

poumons des souris infectées. Corrélées à la sécrétion d'IgG spécifiques d' Udorn, les souris recevant split Udorn / NPL ont montré une diminution de la réplication du virus dans le museau et les poumons des souris, alors que celles vaccinées par Udorn n'étaient pas protégées contre l'infection virale. L'effet additionnel du CTA1-DD a été retrouvé. Nos données ont démontré que l'utilisation d'un adjuvant et d'un nanosystème d'administration (CTA1-DD + NPL) a un fort effet sur la clairance du virus dans le museau et les poumons des souris infectées. Alors que les souris immunisées avec split Udorn fragmenté ne pouvaient pas complètement inhiber la propagation (transmission) du virus, par contre les souris recevant split Udorn / NPL n'ont montré aucune transmission du virus. Il n'est pas surprenant d'observer que les souris vaccinées avec split Udorn / CTA1-DD / NPL ont aussi complètement bloqué la transmission du virus Udorn. Ces résultats ont démontré que même si le nanovecteur peut améliorer l'efficacité du vaccin, il n'est pas suffisant pour induire une réponse immunitaire complètement protectrice.

Il a été démontré qu'un vaccin antigrippal recombinant à base de nanoparticules et d'HA nécessitait un adjuvant à base de saponine afin d'induire des réponses protectrices contre les souches de la grippe A (H3N2) (Smith et al. 2017). De même, des liposomes modifiés au PEG contenant l'adjuvant CRX-601 et du méthylglycol chitosan amélioraient la réponse immunitaire sublinguale des souris vaccinées contre la grippe (Oberoi et al. 2016). Ces études ont démontré l'intérêt de la combinaison d'un nanosystème de delivrance, du choix des antigènes et d'un adjuvant pour améliorer l'efficacité d'un vaccin antigrippal.

Conclusion

Les nanoparticules sont des outils prometteurs pour la vaccinantion intranasale grâce à leurs capacités de la délivrance de protéines dans la muqueuse, de stabilisation des protéines et d'augmentation du temps de contact (résidence) avec les épithéliums. Le développement d'un vaccin muqueux à base de nanoparticules a nécessité la présence de nanovecteurs capables d'associer des antigènes, de pénétrer dans les cellules et de les libérer dans les cellules. Parmi les 5 types de nanoparticules étudiés dans cette comparaison, nous démontrons que les NPL sont le meilleur candidat potentiel pour l'application du vaccin muqueux. De plus, des données *in vivo* ont montré que les NPL administrées par voie intranasale amélioraient l'efficacité du vaccin universel contre la grippe testé. Ce travail de thèse a démontré l'intérêt de l'utilisation de nanoparticules cationiques à base de maltodextrine et lipidées.

La vaccination muqueuse présente des avantages significatifs par rapport à la vaccination parentérale, y compris l'absence d'aiguille, l'induction d'une immunité systémique et muqueuse et un faible coût. Malgré ces avantages, actuellement, seuls un vaccin nasal et huit vaccins oraux sont homologués pour une administration à l'homme, ciblant les cinq agents pathogènes suivants: la grippe, le poliovirus, le choléra, salmonella typhi et le rotavirus. De plus, la plupart des vaccins homologués pour l'administration par voie muqueuse sont à base de virus vivants atténués qui présentent le risque de retrouver leur pouvoir pathogène. Il n'y a toujours pas de vaccins muqueux (oral, intranasal…) contre de nombreuses autres maladies infectieuses telles que le paludisme, le VIH, la leishmaniose, l'hépatite B et C. Cette étude de thèse contribue à l'amélioration des nanosystèmes de délivrance pour le vaccin muqueux. Basés sur cette étude, d'autres vaccins muqueux à base de nanoparticules pourraient être développés à l' avenir.

PART I: INTRODUCTION

1. Nanoparticle based drug delivery system

The application of nanotechnology in drug delivery has gained increasing interest over the past few decades. Nanoparticles (NPs) are made of organic or inorganic materials to deliver drugs into the body for the treatment of various diseases. NPs such as polymeric micelles, quantum dots, liposomes, polymer-drug conjugates, dendrimers, biodegradable nanoparticles, silica, etc. are few examples of nanoparticulate materials undergoing preclinical development, or already used in clinic (Kabanov and Alakhov 2002; Duncan 2003; Farokhzad and Langer 2006; Davis, Chen, and Shin 2008; Peer et al. 2007; Tasciotti et al. 2008). These nanomaterials can deliver low molecular weight compounds, proteins and nucleotides to target disease cells or tumors to maximize clinical benefit while limiting untoward side effects. Such NPs are also expected to drastically improve drug delivery for treatment diseases.

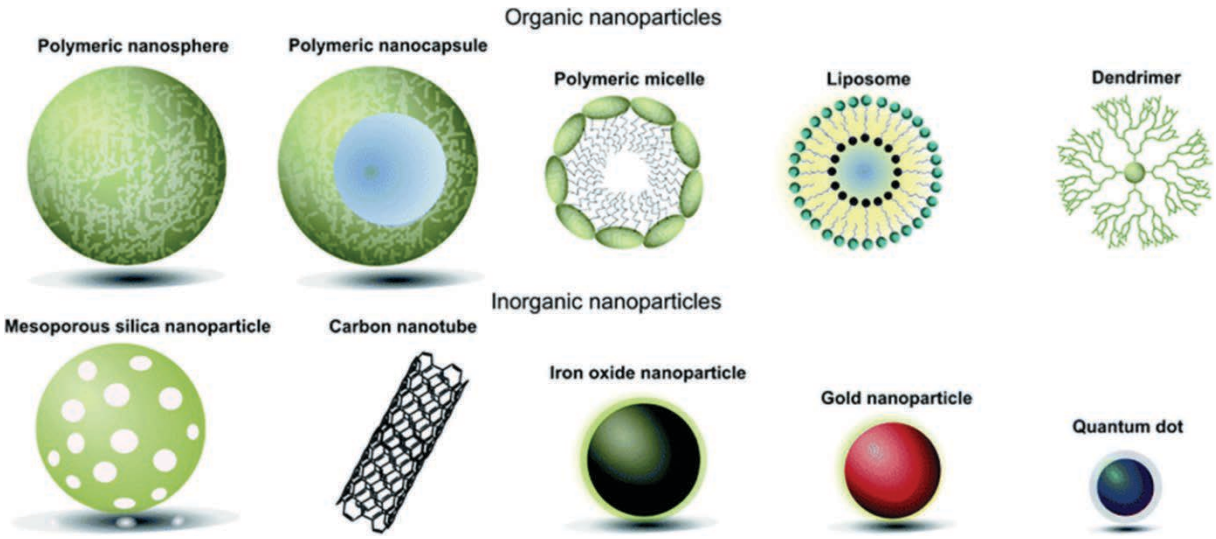

Figure 1: Representation of several types of nanoparticles used in biomedical applications

Nanoparticles require site-specific cellular entry to deliver their cargo to subcellular position. NPs can employ multiple pathways for cellular entry.

1.1. Endocytosis of nanoparticles

Macromolecules and particles can be taken up by eukaryotic cells from the surrounding medium by an active process called endocytosis. Endocytosis involves multiple stages. First, the material is enclosed in an area of plasma membrane, which then buds off inside the cell to form a vesicle named as endosomes (or phagosomes in case of phagocytosis). Second, the endosomes deliver the cargo to various specialized cellular compartment, which enables sorting of materials towards different destinations. Finally, the cargo is delivered to

intracellular compartments, recycled to the extracellular milieu or delivered across cells (a process known as “transcytosis” in polarized cells). Depending on the cell type, as well as the proteins, lipids, and other molecules involved in the process, endocytosis can be classified into several types (Doherty and McMahon 2009; Kumari, Mg, and Mayor 2010). Five main different mechanisms of endocytosis are: phagocytosis, clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (CavE), clathrin/caveolae-independent endocytosis, and macropinocytosis. Some authors may consider the last four mechanisms subtypes as process of pinocytosis. Compared to phagocytosis, which takes place mainly in professional phagocytes, pinocytotic mechanisms are more common and occur in many cell types (Fig 2) (Salatin and Yari Khosroushahi 2017; Sahay, Alakhova, and Kabanov 2010).

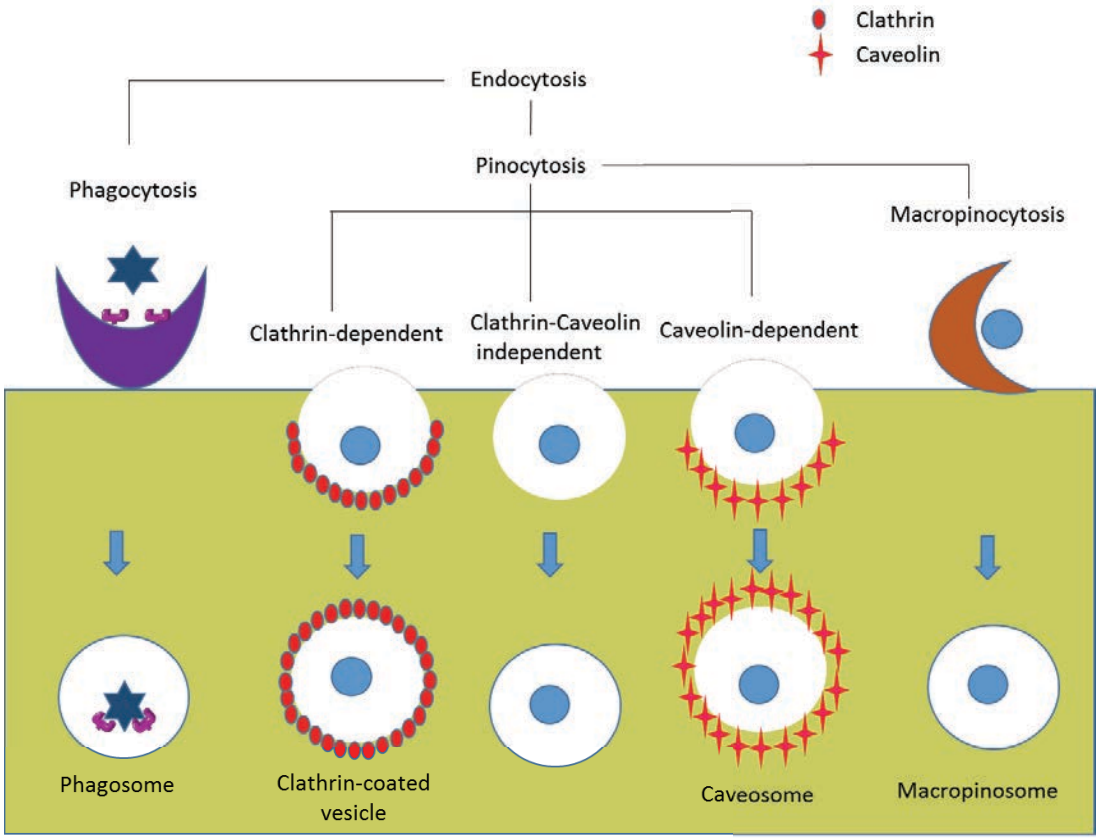

Figure 2: Schematic presentation of endocytosis mechanisms

1.2. Parameters affecting endocytosis

1.2.1. Surface chemistry of nanoparticle

The very first requisite for an optimal surface chemistry is that the NPs stay dispersed in biological media. Proteins tend to bind to NPs surface (Lynch, Dawson, and Linse 2006). Proteins can form a corona that can quickly cover the entire NPs surface, even at low

concentrations (Lundqvist et al. 2011; Monopoli et al. 2011). An effective approach to limit protein fouling is to coat the surface with a polymer brush layer that generates repulsive steric forces (Leckband and Israelachvili 2001). The modification of NPs by non-ionic polymers helps to particle stability through steric stabilization and limits the direct interaction between cells and phagocytic system (Storm et al. 1995). Polyethylene glycol (PEG) is a neutral and hydrophilic material that can be grafted on the surface of particles leading to high blood circulation time due to low opsonization and escape from capture by the phagocytic system (Gref et al. 1995). PEGylated NPs are often referred as "stealth" nanoparticles (Vasir and Labhasetwar 2008). The modification of surface of NPs by PEG avoids the adsorption of proteins and recognition of them by macrophages. Therefore, these modified NPs can easily escape the blood stream and can accumulate in tumours (Czeczuga 1975). In another study, bovine serum albumin (BSA) loaded inside porous cationic polysaccharide NPs or lipids inhibit the complement activation of these nanoparticles. This study demonstrated that protein binding in/on NP was able to generate stealth NPs and may represent an alternative to the use of PEG (Paillard et al. 2010). The modification of NPs with neutral polysaccharides is also another strategy to create stealthness as the polysaccharides can interact with particular receptors on the cell membrane and tissue surface thus caused active targeting (Lemarchand, Gref, and Couvreur 2004).

Nanoparticle with targeting

Nanoparticle's targeting is normally achieved by decorating the particles with moieties that bind preferentially to specific receptors on cells surface. Nevertheless, this is strongly dependent on the identification of cellular/tissue-specific markers. Cellular specificity combined with endocytosis ensures that once the NPs reach their target, they are readily internalized. For this reason, as the receptor of transferrin (Tf) is highly present on cell surface, receptor mediated endocytosis of transferrin NP is widely used to deliver drugs within cells (Tanaka, Fujishima, and Kaneo 2001; Iinuma et al. 2002). For example, poly(lactic-co-glycolic acid) (PLGA) nanoparticles covered by Tf were shown to be selectively uptaken by tumors cells *in vitro* and *in vivo* (Chang et al. 2012). Furthermore, the coating of these NP by Tf highly increased their plasma retention in mice and rats. At the cellular level, the increased uptake by F98 glioma cells of Tf-PLGA suggests interaction between Tf and the overexpressed Tf-receptor at the surface of F98 cell plasma membranes. These studies open a new concept: Tf is used to cover NP in order to get stealth and targeted NP. Other receptors have been widely used

to improve endocytosis such as epidermal growth factor (EGF) receptors (Kirpotin et al. 2006; Zeng, Lee, and Allen 2006; Lee et al. 2007; Mi, Zhao, and Feng 2013), albumin receptors (Schmid et al. 2007; Kratz 2008), folate receptors (Pan et al. 2002; Zhang, Huey Lee, and Feng 2007; Han et al. 2009), and low-density lipoprotein (LDL) receptors (Chnari et al. 2006).

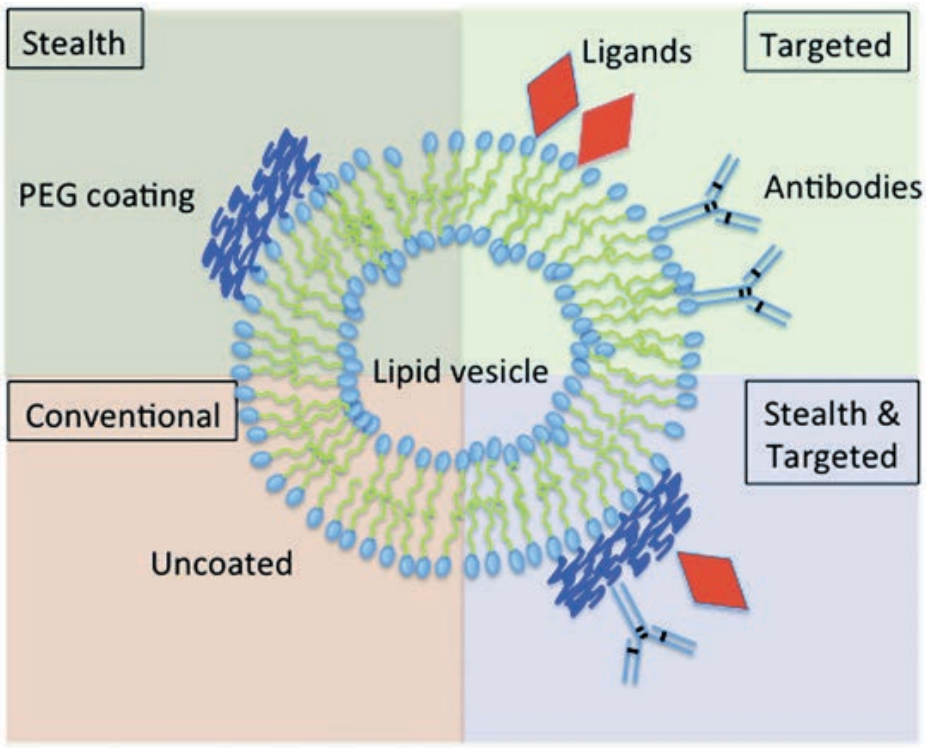

Figure 3: Schematic representation of surface modified liposomes.

1.2.2. Size of the nanoparticle

Some entry mechanisms are very specific when it comes to acceptable cargo size, whereas others are less. For example, phagocytosis has been believed for quite some time to be associated uniquely with the uptake of large particles (>1 μm). However, there are several reports that show the phagocytosis of nanometer-sized particles such as viruses (Clement et al. 2006; Ghigo et al. 2008), and polymer NPs (Lunov et al. 2011), as well as quantum dots (Fischer et al. 2010). Nevertheless, it is worth noting that this endocytic mechanism is strongly linked to protein opsonization, which can induce aggregation and hence a loss in size control. Similarly, macropinocytosis can be exploited for the uptake of large particles (up to 1 mm) (Swanson 2008). However, because macropinocytosis is a cargo-unspecific uptake mechanism, it can accommodate a range of cargo sizes and types of materials and often operates in conjunction with other entry mechanisms (Reynwar et al. 2007; Nam et al. 2009; Thurn et al. 2011). Eukaryotic cells can use clathrin-mediated entry to internalize particles with sizes

ranging from 10 nm to 300 nm (Ehrlich et al. 2004). For caveolae mediated endocytosis formed caveosomes have diameters 50–80 nm with a neck 20–40 nm (Kurzchalia and Parton 1999; Pelkmans and Helenius 2002). Even though this would suggest a tight limit for cargo size (Wang et al. 2009). They observed that this route can facilitate the entry of NPs up to 100 nm in diameter. For clathrin-independent the uptake of particles is observed for NP larger than 100 nm (Mayor and Pagano 2007).

1.2.3. Effect of nanoparticle's charge

The modification of NPs with positively charged functional groups facilitates electrostatic interaction with negatively cell membrane (He et al. 2010; Lankoff et al. 2013). Hence, generally cationic NPs have higher uptake than negatively charged (Harush-Frenkel et al. 2007). The internalization of NPs with negative charge surface decreases by increasing the surface charge, while an increase of uptake of cationic NPs is observed while increasing the charge density (He et al. 2009). The permeability of positively charged drug carriers to gastrointestinal mucous barrier is higher than neutral and negatively charged NPs because of the presence of negatively charged proteins in outer surface of gastrointestinal epithelial cells (El-Shabouri 2002). The cationic nature of chitosan-based NPs facilitates the electrostatic interaction with cell membrane and subsequently cellular internalization (Tahara et al. 2009; Duceppe and Tabrizian 2010; Amidi et al. 2010). However, in some cases, surface modified by negative charge can enhance the uptake of nanoparticles. By increasing the negative surface charge of carboxymethyl dextran-modified iron oxide NPs, these particles are highly taken via non- specific pathways (Ayala et al. 2013). Super paramagnetic iron oxide NPs coated with anionic silica have a cellular uptake 3-fold higher than cationic one (Prijic et al. 2010). Citrate groups grafted on NP giving a negative surface charge of NPs, increases the stability on NPs in serum-free culture medium and subsequently their cellular internalization (Kolosnjaj-Tabi et al. 2013).

1.3. Examples of nanoparticle as drug delivery system in clinics

Nanotechnology has provided the possibility of delivering drugs to specific cells using NPs (Ranganathan et al. 2012). Targeted drug delivery is intended to reduce the side effects of drugs with concomitant decrease in dose and treatment expenses (Tiwari et al. 2012). Nanoscale particles/molecules are developed to improve the bioavailability and pharmacokinetics of therapeutics. Currently, there are several commercialized drug products such as liposomes (and

virosomes), polymer NPs, protein–drug conjugates, antibodies or metal-based nanoformulations…

Liposomes are used as drug carriers or drug-delivery systems based on their ability to encapsulate hydrophilic molecules in their aqueous inner space as well as hydrophobic molecules in their phospholipid bilayer membranes. There are many products in the market employing liposomes as drug delivery systems. Doxil®, a liposome formulation containing doxorobucin hydrochloride (DOX), is the first FDA-approved nano drug delivery system based on PEGylated liposome technology. DOX is located within the hydrophilic core of the liposomes in the form of DOX–sulphate complex (Veronese and Harris 2002). Sequus Pharmaceuticals, USA originally developed Doxil® in 1995 as an intravascular injection for the treatment of advanced ovarian cancer, multiple myeloma and HIV-associated Kaposi's sarcoma. Ambisome® liposome contain Amphotericin B for injection is a sterile, non-pyrogenic freeze-dried product for intravascular infusion. Ambisome® liposomes (100 nm) provides prolonged circulation in the plasma, along with *in vivo* stabilization. It is a product of Astellas Pharma USA approved in 1997. Ambisome® is approved for the treatment of serious, life-threatening fungal infections including leishmaniasis, aspergillosis, blastomycosis, coccidioidomycosis in febrile, neutropenic patients. Ambisome® is also prescribed for the treatment of invasive systemic infections caused by Aspergillus, Candida, or Cryptococcus in patients those cannot tolerate conventional Amphotericin B therapy (Bulbake et al. 2017).

Polymer-based nanoformulations such as Genexol®, Paclitaxel in 20–50 nm micelles (Oerlemans et al. 2010) composed of block copolymer poly(ethylene glycol)- poly(D,L-lactide). Genexol® are used for metastatic breast cancer treatment.

Protein–drug conjugates such as Abraxane®, the NPs (130 nm) formed by albumin with conjugated paclitaxel (Saif 2013). Abraxane® is used for metastatic breast cancer, non-small-cell lung cancer treatment.

Metal-based nanoformulations such as Feraheme™ (Ferumoxytol), superparamagnetic iron oxide NPs coated with dextran with hydrodynamic diameter 50 nm. Feraheme™ are used for treatment of iron deficiency anemia in adults with chronic kidney disease (Weissig, Pettinger, and Murdock 2014).

1.4. Conclusion

Currently, nanomedicine is undergoing of attention led by using NPs to deliver drugs to cells. Such NPs are engineered so that they target specifically diseased cells, improving efficacy, decreasing side effects, and overall improving human health. This technique reduces the side effects of drugs in the body. However, despite many advantages of nanomedicine, there are a number of potential drawbacks for using these nanodrug delivery vehicles. The disadvantages of NPs as drug delivery system are, for example: immunogenicity induction, poor site-specific accumulation, production cost… (Blanco, Shen, and Ferrari 2015). By successively addressing each of these barriers, nanoparticle based drug delivery system could revolutionize the way we treat disease of humans in the future.

REVIEW

Many viruses and bacteria infect humans through mucosal surfaces, such as those in the lungs, gastrointestinal tract and reproductive tract. To fight these pathogens, scientists are developing vaccines that can create the efficient defense at mucosal surfaces. Vaccines can be delivered via mucosal role but they are quickly cleared away before it can provoke an immune response. To overcome that, many strategies have developed to protect the antigens and to target immune cells to generate a strong immune response in mucosal surfaces. Some current approaches for mucosal vaccine are presented in the review herein.

PUBLICATION 1: NANOTECHNOLOGY SOLUTIONS FOR MUCOSAL VACCINE

Nanotechnology solutions for mucosal vaccine

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Abstract

The vast majority of human pathogens infect at the mucosal surfaces. Preventing infection at these sites by mucosal vaccines is a promising approach for vaccine development. However, to date only a few vaccines have applied for mucosal use. It has also been difficult to induce a mucosal response consistently after the immunization of vaccine antigen by the mucosal route because of their rapid elimination by mucus turn over or inactivation by mucosal enzymes. Other potential limitations include lack of interaction of antigen with M cells and other mucosal cells that are responsible for antigen uptake or processing. It is necessary to investigate the specific immunization strategies to improve their efficacy. Application of nanotechnology to create mucoadhesive particle that can prolong mucosal residence time, target the mucosal immune system and incorporate mucosal adjuvants maximizing immune response is key strategy to improve the effectiveness of mucosal vaccines. This review summary new nanotechnology approaches to mucosal immunization have now been proposed to address these issues.

1. Introduction

Due to the vast surface area, the mucosal surfaces of the gastrointestinal tract, urogenital, respiratory tracts and the ocular cavities are major routes of entry into the body for the pathogens [1, 2]. The majority of current vaccination methods target the systemic immune system and elicit only a weak or no mucosal immune response [3, 4]. In contrast, mucosal immunization with an appropriate vaccine delivery vehicle induces both protective mucosal and systemic immune responses, leading to a dual layer of protection against pathogens [5]. In addition, compared to parenteral routes, mucosal vaccine offers advantages such as ease of administration, decreased costs, avoidance of needle-stick injuries and reduced risk of transmission of blood-borne diseases. Moreover, this needle- and syringe-free delivery helps to reduce medical waste [6]. Therefore, mucosal vaccines are highly desirable.

Despite these numerous advantages, the development of mucosal vaccines has been slow. Only a handful of mucosal vaccines have been approved for clinical use in humans, the best-known example being the Sabin polio vaccine, which is given orally and absorbed in the digestive tract [6]. Others include oral vaccines against *Salmonella typhi*, *Vibrio cholera*, and rotavirus and a nasal vaccine against influenza virus [7-9]. Challenges for mucosal immunization are: dilution of mucosal vaccine in the mucosal fluids, proteolytic degradation and fast mucosal clearance that might limit effective capture by the mucosal immune system [7].

Different strategies can improve mucosal vaccines such as the use of an efficient antigen delivery system associated or not to an adjuvant. Nano-delivery system may protect antigen from degradation, overcome the mucus barrier and potentially target the mucosal inductive sites. These nanocarriers can further be functionalized with targeting moieties to deliver the vaccine to microfold cells (M-cells) or antigen presenting cells (APCs). We will focus this review on the interest of using different nanocarriers to improve the efficacy of vaccines.

2. Mucosal immune system

Mucosal immune responses that occur at mucosal membranes of the intestines, the urogenital tract and the respiratory system, i.e., surfaces that are in contact with the external environment. The mucosal immune system can be divided into two general compartments known as inductive and effector sites. Inductive sites are areas where antigen sampling leads to initial activation of immune cells, while at effector sites, T and B cells are activated **(Figure 1)** [10]. The main inductive sites consist of specific lymphoid tissues, known as mucosa-associated

lymphoid tissue (MALT) as well as their surrounding regional lymph nodes [10, 11]. The MALT contains T-cell zones, B cell-enriched areas containing a high frequency of surface IgA-positive (sIgA+) B cells and a subepithelial area with APCs for the initiation of specific immune responses. The MALT is covered by a follicle-associated epithelium (FAE) that consists of M-cells, columnar epithelial cells which play a central role in the initiation of mucosal immune responses. These cells are specialized in the transport of antigens/microorganisms from the lumen to underlying dendritic cells (DCs) located in the subepithelial dome region (SED) [12, 13]. DCs present antigens to CD4+ T cells in the interfollicular regions (IFRs). Activated CD4+ T cells can subsequently support class-switch recombination and somatichypermutation in naïve B cells in the germinal center, resulting in the generation of IgA-expressing B cells or IgA+ B cells. Retinoic acid plays a key role allowing IgA+ B cells to traffic to mucosal effector sites. At these sites, IgA+ B cells mature into IgA producing plasma cells [14]. In addition to T-cell dependent IgA responses, T-cell independent IgA class switching can also take place by a direct action of DCs on B cells [15]. Finally, upon binding to the polymeric immunoglobulin receptor (pIgR) expressed on epithelial cells, IgA is translocated from the lamina propria into the lumen as secretory IgA (sIgA) [16, 17]. Mucosal vaccines can also induce systemic IgG. Due to dual protection, mucosal vaccination could be a promising strategy against pathogens infection. An optimum mucosal vaccination strategy should aim to protect mucosal surface against pathogen infection (e.g., sIgA, sIgG), cytolytic T lymphocytes (e.g., CD8+) and T-helper lymphocytes (e.g., CD4+, Th17) and a protective systemic response [18].

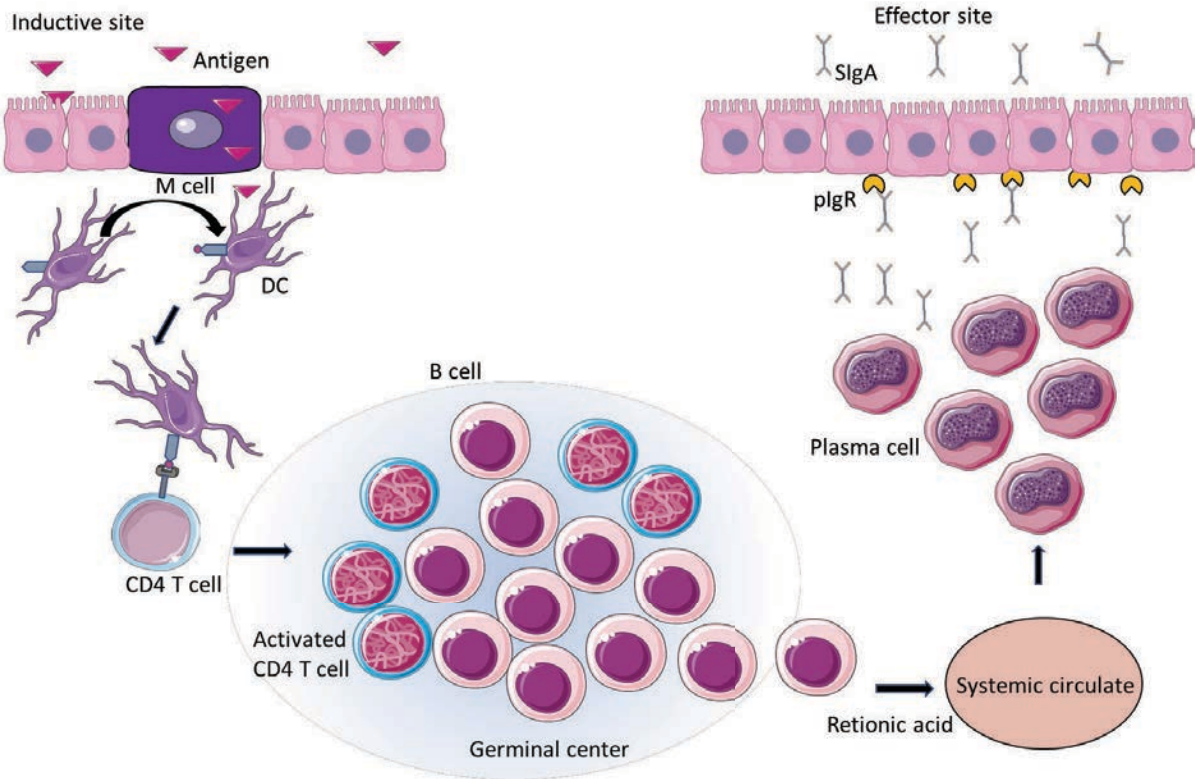

Figure 1: Induction of mucosal immune responses. At inductive site, M cells sample antigens and transfer them to dendritic cells (DCs). DCs take up antigen, present processed antigen to T cells. Activated T cells stimulate B cells to become of IgA-expressing B cells in germinal center. IgA-expressing B cells enter systemic circulate to traffic to mucosal effector sites and become plasma cells. Plasma cells produce polymeric IgA molecules that are translocated as sIgA following binding to the polymeric Ig receptor (pIgR).

3. Strategies for mucosal vaccines

To enter into contact with mucosa cells, nanovaccines must cross the mucosa barrier mainly composed of mucous.

3.1. Mucin and mucoadhesive properties

Mucus membranes are the moist surfaces largely covering the mucosal surface such as the gastrointestinal and respiratory tracts. The main components of all mucus gels are mucin glycoproteins, lipids, inorganic salts and water [19]. Mucins are large molecules (0.5–40 MDa in size, 3–10 nm in diameter), formed by mucin monomers linked together via disulfide bonds [20]. These mucin fibers reversibly crosslink and entangle with each other, forming the mucus layer with shear-thinning properties [21]. In addition, mucins are proteins glycosylated with a

dense glycan coverage [22, 23]. Most mucin glycoproteins have a high sialic acid and sulfate content, resulting in a strongly negative surface. Thus, the charge repulsion greatly increases the rigidity of mucin fibers [20]. Meanwhile, the mobility of positively charged particles in mucus decreases due to electrostatic interaction and thus they are easily to be trapped in mucus. Glycosylated regions of mucins are separated by "naked" protein regions, which are internal cysteine-rich domains along the peptide backbone [24]. Similar with the electrostatic interaction, the hydrophobic interaction increases the mucoadhesion of hydrophobic particles and macromolecules **(Figure 2**) [25].

The most common mucoadhesive materials include chitosan (CS), maltodextrin, alginate, hyaluronic acid, carboxymethyl cellulose, hydroxyethyl cellulose, pectin, etc [26-28] **(Figure 3).** Mucoadhesion has been commonly applied to improve the residence time of particles at the mucosal surface and, hence, the uptake of particulate vaccines when administered by the nasal or pulmonary routes [29]. Mucoadhesive particles have been widely explored as promising strategy for mucosal vaccine.

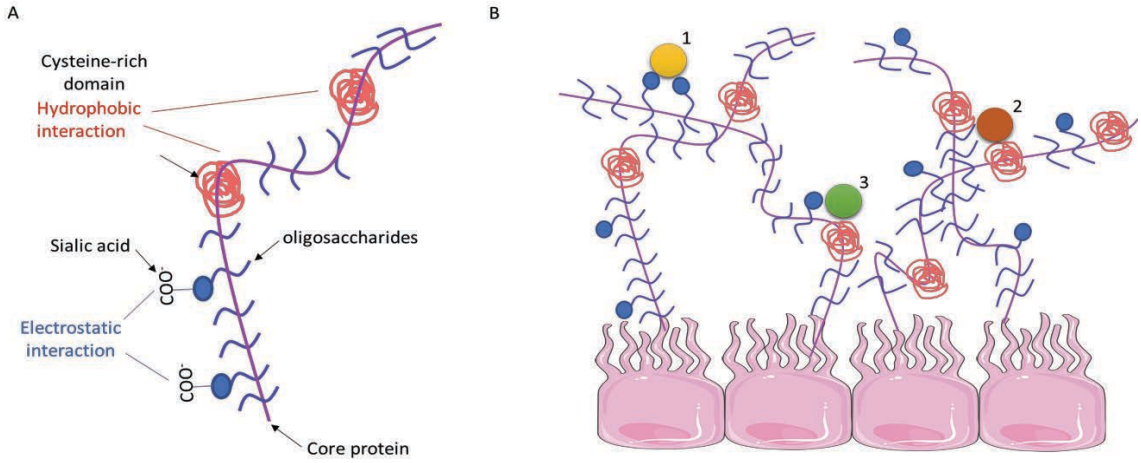

Figure 2: Mucin and mucoadhesive interaction. A: Structure of mucin and their potential mucoadhesive components. B: Examples mucin and nanoparticle interaction: 1- nanoparticle interact with mucin via electrostatic interaction. 2- nanoparticle interact with mucin via hydrophobic interaction. 3-nanoparticle interact with mucin via both electrostatic and hydrophobic interactions.

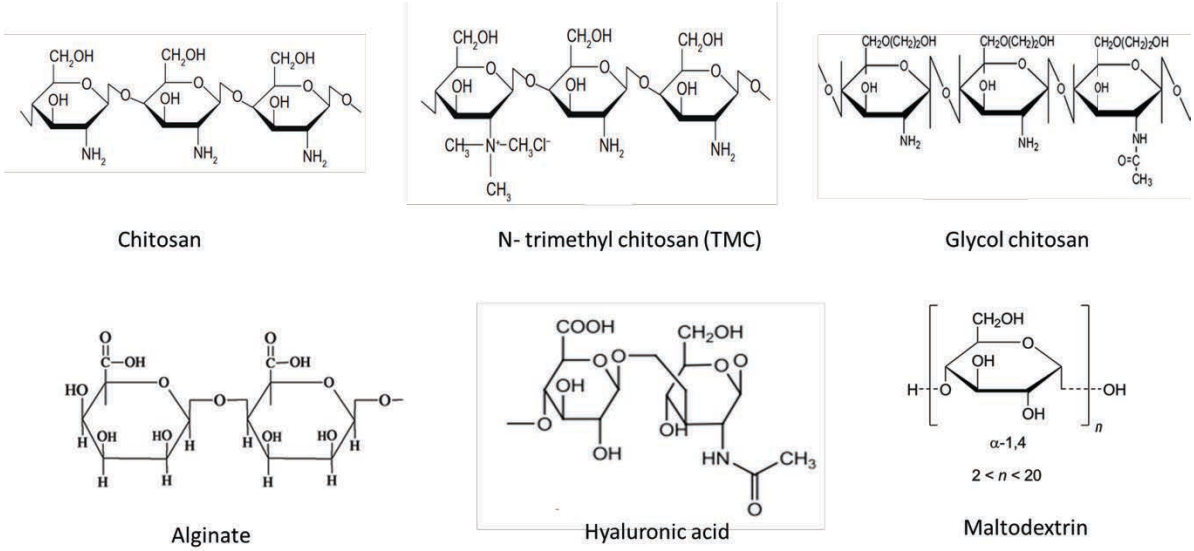

Figure 3: Mucoadhesive materials

3.1.1. Mucoadhesive nanoparticles

Chitosan is a cationic polymer obtained by deacetylation of chitin [30]. Its mucoadhesive property can overcome the mucociliary clearance, thus increasing the residence time of the formulation. CS also promotes para-cellular transportation through the nasal mucosa [31]. Therefore, CS is used as an adjuvant for mucosal vaccination [32-38]. To induce specific mucosal immunity, CS was employed as a mucosal gene carrier. The previously constructed multi-epitope gene vaccine against mycobacterium bovis bacillus Calmette-Guérin (BCG) was combined to CS. The gene vaccine delivered in a CS formulation via mucosal route remarkably enhances specific sIgA level and mucosal IFN-γ(+) T cell response [39]. The adsorption of negatively charged human serum albumin (HSA) onto the surface of the CS nanoparticles has been shown to enhance transfection efficacy. A strong systemic and mucosal immune response was observed after vaccination using HSA-loaded CS-DNA (HSA-CS-DNA) complexes [40].

A drawback of CS, however, is its limited aqueous solubility at neutral and basic pH. CS derivatives with improved solubility over a wide pH range have been developed. Among them, trimethyl chitosan (TMC) is widely used for nasal vaccines [41, 42]. Niels Hagenaars and colleagues synthesized different TMC NPs with varying degrees of quaternization (DQ, 22–86%), O-methylation (DOM, 0–76%) and acetylation (DAc 9–54%). These TMC NPs were formulated with whole inactivated influenza virus (WIV). Increase degree of quaternization strongly enhances the immunogenicity and induces protection against viral challenge after

intranasal vaccination [43]. The adjuvant effect of TMC NPs is also significantly reduced by reacetylation of TMC, whereas the DQ and DOM lightly influence the adjuvanticity of TMC [43].

Glycol chitosan (GC) is a CS derivative conjugated with ethylene glycol branches, water soluble at a neutral/acidic pH values [44]. GC NPs showed the lowest nasal clearance rate and better mucosal cellular uptake as compared to CS NPs [45]. Moreover, GC NPs induced stronger immune response as compared to CS NPs.

Other polysaccharides such as maltodextrin, alginates and hyaluronic acid have been considered as potential mucosal vaccine deliver carriers.

Porous maltodextrin-based nanoparticles (NPL) are composed of a cationic maltodextrin matrix enclosing lipid in their core **(Figure. 4).** NPL are highly endocytosed by airway epithelial cells and significantly improve protein delivery in cells. Moreover, its mucoadhesive properties can promote after nasal administration protein delivery in airway mucosa cells [46]. Furthermore, NPL-*Toxoplasma gondii* formulations after nasal administration protect animals against chronic and congenital *Toxoplasmosis* [47, 48]. These studies provide evidence of the potential of NPL for the development of new vaccines against a wide range of pathogens.

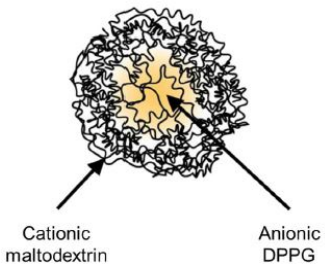

Figure 4: Representation of porous maltodextrin-based nanoparticles (NPL).

Alginates are safe and inexpensive natural polymers with high mucoadhesive properties [49]. It consists of varying proportions of 1, 4-linked β-D-mannuronic acid (M), α-L-glucuronic acid (G). They are known to have a wide variety of uses, particularly as thickeners or emulsion stabilizers in manufacturing of processed foods and pharmaceutical formulations. They have a unique property of forming gel in the presence of divalent cations such as calcium [50]. Vaccine enclosed alginate microparticles have been demonstrated to be effective for oral immunization in several animal species [51]. Sodium alginate microparticles were tested as a delivery system

for oral vaccines. The oral or subcutaneous administration of ovalbumin encapsulated in alginate microspheres were equally effective in stimulating the systemic immune response in mice. These studies suggest that alginate microparticles could be used as mucosal delivery systems [52].

Hyaluronic acid is a naturally polysaccharide made of D-glucuronic acid and N-acetyl-D-glucosamine. O'Hagan *et al.,* have described a composition which contains a selected antigen in combination with a hyaluronic acid polymer. It was concluded that the vaccine composition may display enhanced immunogenicity as compared to antigen alone [53]. Singh *et al.* studied hyaluronic microspheres as a delivery system for flu antigen and observed an adjuvant effect after nasal administration [54].

3.1.2. Improvement of the mucoadhesivity of NP

Poly(lactic-co-glycolic) acid (PLGA), a biocompatible and biodegradable polymer with sustained release property, is extensively used for the therapeutic delivery of proteins and peptides including vaccines. However, due to its poor mucoadhesiveness, PLGA has limited use in mucosal vaccination. The half time of clearance of non- mucoadhesive formulations from the human nasal cavity is only about 20 min [40]. Such a rapid clearance time may not allow sufficient retention for antigen to be taken up by APCs in the nasal-associated lymphoid tissue (NALT). Incorporation of mucoadhesive polymers such as CS to the delivery system can overcome such limitations and increases absorption of protein and peptides across the mucosal barrier [55, 56]. Moreover, these particular polymers can prolong their residence time in the nasal cavity [57-59].

HBsAg loaded PLGA microparticles were prepared and coated with CS and TMC. It was showed that surface coated particles greatly induced anti-HBsAg titer as compared to plain PLGA microparticles and the outcome as higher with TMC-coated PLGA microparticles [60]. In another study, Dilip Pawar *et al*., demonstrated that surface coating of PLGA NP with GC could induce significantly higher systemic and mucosal immune response compared to PLGA and CS-PLGA NPs [61].

To enhance mucoadhesive properity, poly(lactic acid) (PLA) nanospheres was modified by GC or alginate. Systemic and mucosal immune responses were investigated after the intranasal administration of *Streptococcus equi* (S. equi) antigens associated by adsorption or encapsulation to PLA nanospheres coated by GC or alginate. The modified nanoparticles

induced a mixed Th1 and Th2 response, being therefore potential carriers for the delivery of S. equi antigens [62].

Coating liposomes with CS to improve mucoadhesive property has also been studied. Anionic liposomes (AL) associated to CS-DNA complexes was developed [63]. It was showed that AL-CS-DNA NPs can enhance the delivery of the anti-caries DNA vaccine pGJA-P/VAX into nasal mucosa and induced a significantly higher level of sIgA than the CS/DNA.

Nasally delivered mucoadhesive liposome using an inactivated H5N3 virus as a model antigen was conducted [64]. Mucoadhesive liposomes were developed using 2 polysaccharides: tremella (T) or xanthan gum (XG). XG is derived from *Xanthomonas campestris*, and T is extracted from *Tremella fuciformis* (TreE). These mucoadhesive liposomes are more suitable for nasal delivery which elicited higher sIgA and serum IgG after two vaccinations in chicken.

3.2. MALT targeting

Nanovaccine must reach the immune cells of the MALT. The main targets are M cells, DCs and macrophages

3.2.1. M- cells targeting

Although M cells are the natural antigen sampling cells, they compose less than 1% of all intestinal epithelial cells and are difficult to reach in the NALT [65]. In order to improve vaccine uptake, M cells can be specifically targeted, which may provide an opportunity to enhance activation of the mucosal immune system by increasing vaccine delivery across the intestinal epithelial layer to the inductive MALT underneath [66].

The M cells express a wide range of carbohydrate markers with diverse glycoconjugate profiles [67], which perhaps allows M cells to interact with a broad range of microbes [68]. For example, while Ulex europaeus agglutinin-1 (UEA-1), the α-L-fucose residue- specific lectin, recognizes M cells and goblet cells lining the mouse Peyer's patch [69, 70]. Particle mediated delivery vehicles can be targeted to M-cells by surface modifications with lectins that naturally bind to M cell surfaces through α-L-fucose residue **(Figure 5)** [66, 71]. Orally delivered polystyrene microspheres covalently coupled to Ulex europaeus agglutinin-1 (UEA-1) lectin resulted in selective binding and rapid uptake by Peyer’s patch M-cells, in comparison with other non-M cell specific lectins [72]. In another study, LTA (Lotus tetragonolobus from

Winged or Asparagus pea) was explored as the potential of α-l-fucose targeting. LTA grafted PLGA nanoparticles loaded with HBsAg elicited strong mucosal and systemic response against Hepatitis B [73].

Secretory immunoglobulin-A but not IgG or IgM adheres to the apical membrane of M cells promoting its uptake by M cells [74-76]. In addition, sIgA has favourable properties for the development as a vaccine formulation component due to its endogenous nature, non-pathogenic origin and the lack of interspecies variation in its interaction with M cells. Dectin-1 has been described as a small type II transmembrane protein of the C-type lectin family. Its presence on M cells was shown to play a role in sIgA capture and internalisation **(Figure 5)** [77]. According to these findings, chitosan dextran sulphate (CS-DS) NPs loaded with pertussis toxin (PTX) and sIgA was developed for the intranasal delivery of proteins. The *in vivo* uptake of sIgA-loaded CS-DS NPs showed a preferential uptake of NPs probably by M-cells. The results of this study indicated the potential application of sIgA for improving a nasal vaccine delivery system [78].

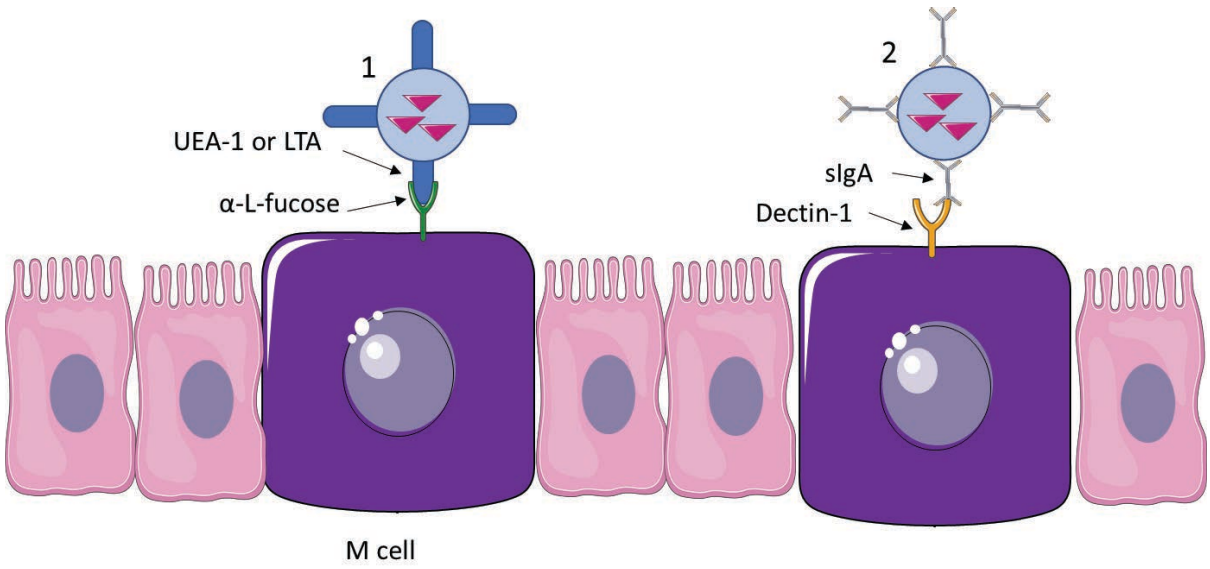

Figure 5: M cell targeting. 1- Nanoparticle conjugated UEA-1 or LTA for α-l-fucose on M cell recognition. 2- Nanoparticle conjugated sIgA for Dectin-1 on M cell recognition.

3.2.2. Dendritic cells or macrophages targeting

Given the essential role of APCs, and especially DCs, in the initiation of adaptive immune responses, the potential to directly target has been considered. However, most bioconjugate vaccines have been tested through parenteral routes of administration. DEC-205 receptor

belongs to C-type lectin receptors (CLRs) which expressed primarily by DCs was conjugated to CS NPs to deliver antigen to DCs **(Figure 6a)** [79]. The plasmid DNA encoding N protein (highly conserved part from SARS-CoV) was selected antigen for this delivery system. Intranasal administration this formulation led to the detection of augmented levels of N protein specific systemic IgG and nasal IgA antibodies.

The mannose receptor is also one kind of CLRs primarily present on the surface of macrophages and immature dendritic cells [80, 81]. The receptor recognizes terminal mannose, N-acetyl-glucosamine and fucose residues on glycans attached to proteins [82] found on the surface of some microorganisms. Grafting a manose motif to CS could improve receptor-mediated endocytosis of the carrier **(Figure 6b)** [83-85]. Mannosylated chitosan (MCS) as a delivery system had been employed to deliver genes into APCs [86-89]. A vaccine delivery system based on mannosylated chitosan microspheres (MCMs) was studied. The dermonecrotoxin (BBD) collected from Bordetella bronchiseptica antigens were loaded in MCMs or chitosan microspheres (CMs). The results showed that mannose moieties in the MCMs induced immune-stimulating effects through mucosal delivery. These outcomes were probably the result of specific interaction between mannose groups in the MCMs and mannose receptors on the macrophages [90]. Wenjun Yao *et al.,* used a mannose ligand to modify CS, which enhanced the delivery efficiency of CS via mannose receptor-mediated endocytosis [91]. A DNA vaccine was condensed with mannosylated CS (MCS) or CS NP to form the vaccines against prostate tumor. These vaccine formulations were intranasally administered in a mice prostate carcinoma model to evaluate the efficacy on inhibition of the growth of tumor cells. Immunization with DNA formulated MCS showed significant higher level of IgG than DNA conjugated formulations lead to efficiently suppress the growth of tumor cells.

A water soluble, linear copolymer of D-mannose and D-glucose, namely glucomannan (GM), was explored as a ligand for mannose receptors [92]. Glucomannosylated CS NP entrapped bovine serum albumin (BSA) were found to uptake RAW 264.7 via both mannose and glucose transporter-mediated endocytosis. GM modification nanoparticle resulted in significantly higher systemic (serum IgG titer), mucosal (sIgA) and cell-mediated immune responses in comparison with non-modified CS NPs in BALB/c mice. In another study, glucomannosylated bilosomes (GM- bilosomes) was studied for oral vaccine. Thin film hydration method was used for the preparation of BSA loaded GM-bilosomes [93]. The immune response observed for GM-bilosomes was similar to alum adsorbed BSA (BSA-AL) administered through

intramuscular route. More importantly, GM-bilosomes induced mucosal and cell mediated immune response which was failed via intramuscular administration. GM-bilosomes could be considered as promising carrier and adjuvant system for oral mucosal immunization and efficiently exploited for oral delivery of other candidate antigens.

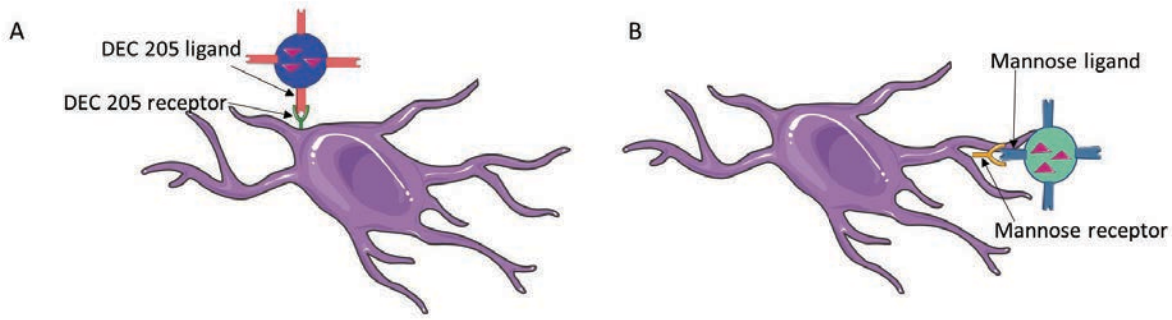

Figure 6: Dendritic cells or macrophage targeting. A) Nanoparticle conjugated DEC 205 ligand for DC recognition. B) Nanoparticle conjugated mannose ligand for mannose receptor on DC recognition.

3.3. Mucosal adjuvant

Mucosal adjuvants improve the immunogenicity of an antigen by binding to a receptor or target immune cell [94].

Cholera toxin (CT) and the E. coli heat-labile enterotoxin (LT) bind to ganglioside receptors present on enterocytes, DCs, macrophages as well as B and T-lymphocytes [94, 95]. Furthermore, they are immunogens that induce antigen-specific sIgA and serum IgG antibody responses; and when co-administered with a protein antigen they can potentially enhance the mucosal and serum antibody response to induce long-term memory to the antigen [94]. The adjuvant activity of these toxins may result from improved antigen uptake as well as inducing the secretion of various cytokines [94]. Due to their high degree of toxicity these toxins are not considered as useful adjuvant although efforts have been made to detoxify them [96].

Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR). They recognize molecules that are generally shared by pathogens but distinct from host molecules or pathogen-associated molecular patterns (PAMPs). TLRs are expressed on the membranes of leukocytes

including dendritic cells, macrophages, natural killer cells, cells of the adaptive immunity (T and B lymphocytes) and non immune cells (epithelial and endothelial cells, and fibroblasts) [97- 98]. Our current knowledge in the area of the TLRs and their ligands, should also be applied to the development of safe and effective mucosal adjuvants [99].

Lipopolysaccharides (LPS) are large molecules found in the outer membrane of gram-negative bacteria. It binds the TLR4 receptor which expressed in many cell types, but especially in monocytes, dendritic cells, macrophages and B cells, which promotes the secretion of pro-inflammatory cytokines and nitric oxide [100]. Since LPS is too toxic to be used as an adjuvant, the nontoxic derivative of the lipopolysaccharide (LPS) from *Salmonella minnesota* known as monophosphoryl lipid A (MPLA) was developed **(Figure 7a).** MPLA has been used as a vaccine adjuvant most effectively when incorporated into liposomes [101]. Ovalbumin (OVA) and MPLA were incorporated in PLGA NPs induced a stronger IgG and IgA immune response than that induced by OVA in PBS solution or OVA incorporated into PLGA NPs. These findings demonstrated that co-delivery of OVA and MPLA in PLGA NPs induced both systemic and mucosal immune responses. This system therefore could be a suitable strategy for oral vaccination [102].

Imiquimod (TLR7 agonist) combined to CS NPs to help antigens overcome mucosal barriers **(Figure 7b).** [103]. The study used of a multifunctional nanocarrier consisting of a lipophilic immunostimulants in the core, and a polymeric corona made of CS, intended to associate antigens and facilitate their transport across the nasal mucosa. The recombinant hepatitis B surface antigen (HB), was selected as antigen for the validation of the concept. The nanocapsules containing imiquimod elicited a protective immune response characterized by increasing IgG levels over time and specific immunological memory. This study suggested the capacity of the nanocapsules to modulate the systemic immune response upon nasal vaccination.

Cytidine-phosphate– Guanosine (CpG) motifs are unmethylated dinucleotides commonly found in bacterial DNA but not in mammalian genomes that have been found to be immunostimulatory. CpG motifs are considered PAMPs due to their abundance in microbial genomes but their rarity in vertebrate genomes [94, 95, 104]. The CpG is recognized by TLR9, which is expressed only in B cells and plasmacytoid dendritic cells (pDCs) in humans **(Figure 7c).** [105]. Strong adjuvant effect on cellular and humoral immunity was observed [95, 104, 106]. Alignani D *et al*., demonstrated that orally administered OVA/CpG induces a specific

immune response against OVA in mice [106].

Five potential immunopotentiators including LPS, PAM_3CSK_4, CpG, the NOD-like receptor 2 ligand - muramyldipeptide (MDP) and the non-toxic beta subunit of cholera toxin (CTB) were conducted their adjuvant effect after nasal administration [107]. Results showed that co-encapsulation of an additional immune-modulator with the antigen into TMC NPs can further improve the immunogenicity of the vaccine. However, the strength and quality of the response depends on the immunopotentiator as well as the route of administration.

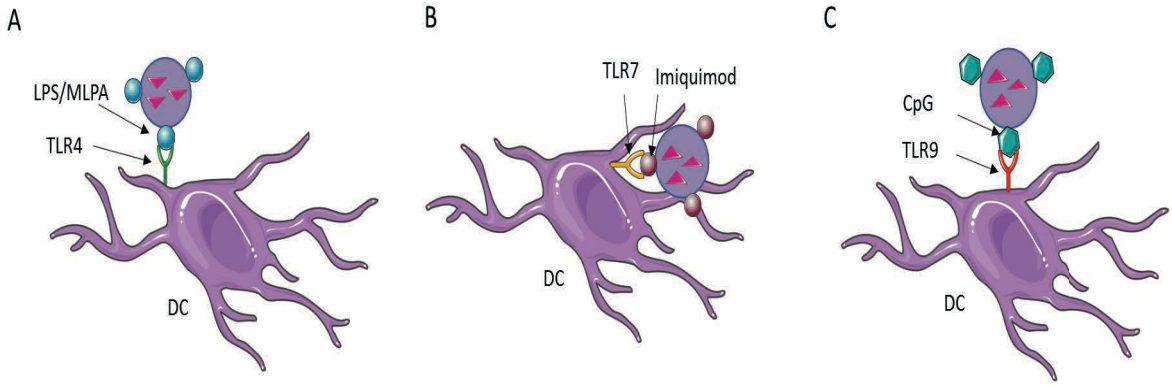

Figure 7: Mucosal adjuvants. A) Nanoparticle associated LPS or MLPA for TLR4 on DC recognition. B) Nanoparticle associated imiquimod for TLR7 on DC recognition. C) Nanoparticle associated CpG for TLR9 on DC recognition.

4. Conclusion

Different strategies exist for the delivery and presentation of vaccine antigen to the mucosal immune system. Effective mucosal immunization will require (a) overcoming physiological barriers at mucosal routes, (b) targeting and delivering antigen into mucosal epithelium for appropriate processing of antigens that lead to specific T and B cell activation. Mucoadhesive NPs associated with mucosal targeting or adjuvant aim to bypass the mucus layer and present antigens to the target cells could be optimistic solutions for mucosal vaccine. This review gives evidences of the interest of applying nanotechnologies as both delivery system of antigens and targeting immune cells in mucosa.

ABBREVIATIONS

Antigen presenting cell (APC), microfold or membranous cells (M-cells), polylactic-co-glycolic acid (PLGA), polylactic acid (PLA), an adjuvant that uses squalene (MF59), Adjuvant System 03 (AS03), gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), larynx-associated lymphoid tissue (LALT), secretory immunoglobulin A (sIgA), dendritic cells (DCs), subepithelial dome region (SED), cluster of differentiation 40 (CD40), cluster of differentiation 4 (CD4), cluster of differentiation 8 (CD8), transforming growth factor beta (TGF-β), interleukin (IL), lymphocyte B (B cells), lymphocyte T (T cells), T helper (Th), polymeric immunoglobulin receptor (pIgR), chitosan (CS), Bacillus Calmette–Guérin (BCG), chitosan DNA (CS-DNA), human serum albumin (HSA), HSA-loaded chitosan DNA (HSA-CS-DNA), N-trimethyl chitosan (TMC), degrees of quaternization (DQ), degrees of O-methylation (DOM), degree of acetylation (DAc), inactivated influenza virus (WIV), glycol chitsan (GC), hepatitis B virus antigen (HBsAg), chitosan PLGA (CS PLGA), anionic-chitosan-liposome DNA (AL-CS-DNA), fusion DNA vaccine against PAc and glucosyltransferase I of Streptococcus mutans (pGJA-P/VAX), Zwitterionic porous maltodextrin-based nanoparticles (NPL), maltodextrin nanoparticles (NP^{+}), Ulex europaeus agglutinin-1 (UEA-1), α-L-fucose (LTA), Cholera toxin (CT), chitosan dextran sulphate (CS-DS), pertussis toxin (PTX), mannosylated chitosan (MCS), mannosylated chitosan microspheres (MCMs), *Bordetella bronchiseptica* dermonecrotoxin (BBD), chitosan microspheres (CMs), plasmid Gastrin-releasing peptide (pGRP), Glucomannan (GM), bovine serum albumin (BSA), murine macrophage cell line (RAW 264.7), cholera toxin (CT), heat-labile enterotoxin (LT), toll like receptors (TLRs), pattern recognition receptors (PRR), lipopolysaccharide (LPS), cluster differentiation 14 (CD14), lymphocyte antigen 96 (MD2), monophosphoryl lipid (MPLA), ovalbumin (OVA), CpG oligodeoxynucleotides (CpG ODN), toll-like receptor 2 agonist (Pam_3CSK_4), muramyldipeptide (MDP), cholera toxin B subunit (CTB).

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2. Influenza and vaccination

2.1. Influenza virus

2.1.1. Influenza virology

Influenza viruses belong to the family Orthomyxoviridae and they are categorized into four types: A, B, C and D (Palese 2004). Influenza A viruses, together with influenza B viruses, are responsible for human seasonal epidemics and pre-pandemic outbreaks. Moreover, they cause respiratory illness in humans with the potential for severe issues. Annual outbreaks cause 3–5 million severe cases and between 250,000 and 500,000 deaths (Horimoto and Kawaoka 2005).

Each influenza A virus is further classified based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), into subtypes. At present, 18 HA and 11 NA subtypes have been described infecting in birds and mammals. In addition to influenza A viruses, two evolutionary diverging influenza B virus lineages have been reported: the Yamagata and the Victoria lineages (Rota et al. 1990). Influenza A viruses responsible for seasonal influenza epidemics belong to the H1N1 and H3N2 subtypes and, together with influenza B viruses, are responsible for millions of infections each year (Freidl et al. 2014).

Influenza viruses A, B and C are very similar in overall structure. The virus particle is around 100 nm in diameter and usually spherical. The viral particles of all influenza viruses are similar in composition (Bouvier and Palese 2008). These are made of a viral envelope containing surface glycoproteins HA and NA. The central core contains the viral RNA genome (vRNA) and other viral proteins that package and protect this RNA. There are seven or eight pieces of segmented negative-sense RNA, each piece of RNA containing either one or two genes, which code for a protein (Bouvier and Palese 2008) such as: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, nuclear export protein (NEP), RNA polymerase (PA, PB1 and PB2) (Ghedin et al. 2005).

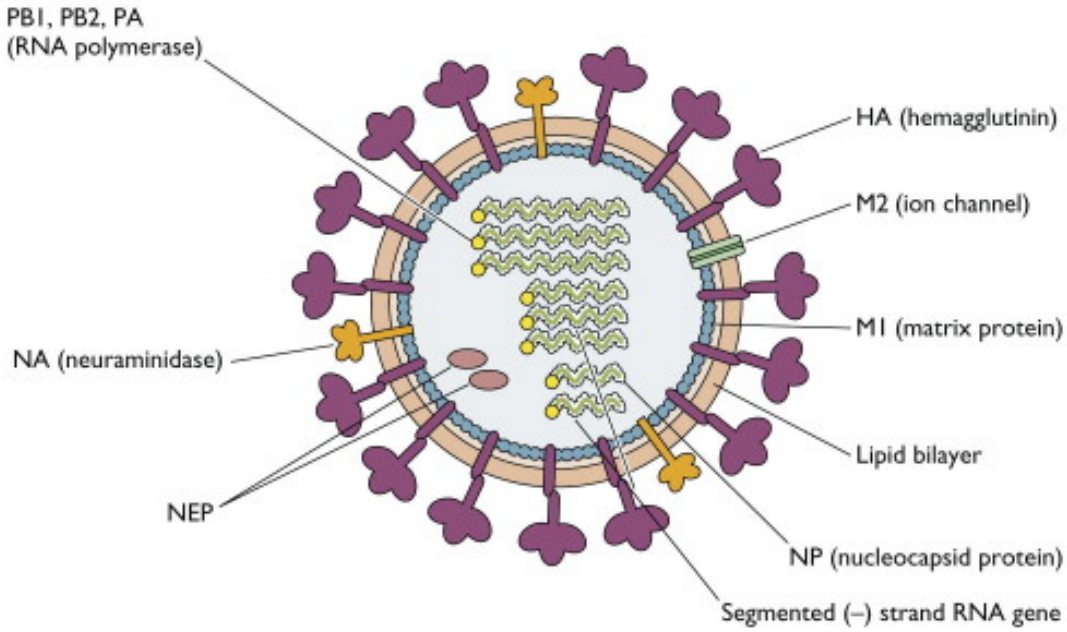

Figure 4: Schematic presentation of Influenza A virus. Hemagglutinin (HA), neuraminidase (NA), matrix protein 2 (M2), matrix protein 1 (M1), nucleoprotein (NP) and others ribonucleoproteins (PB1, PB2 and PA). Eight native-senses RNA lay in the capsid. Nulear export protein (NEP).

2.1.2. Mechanism of replication

Influenza viruses can replicate only in living cells. Influenza infection and replication is a multi-step process: first, the virus bind to the cell, then deliver its genome (Smith and Helenius 2004). HA bind to sialic acid sugars on the surfaces of epithelial cells facilitate the endocytosis of virus (step 1) (Wagner, Matrosovich, and Klenk 2002). After internalization into the host cells, the virus localize 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 (Edinger, Pohl, and Stertz 2014; Lakadamyali, Rust, and Zhuang 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 and viral RNA (vRNAs) are delivered in the cytoplasm (Jing et al. 2008) (step 2). The M2 ion channel is blocked by amantadine drugs, preventing infection (Pinto and Lamb 2006).

In the nucleus, the vRNAs serve as templates for the production of two forms of positive-sense RNA: viral mRNA (messenger RNA) and cRNA (complementary RNA). The synthesis of mRNA is catalyzed by the viral RNA-dependent RNA polymerase (comprising the three

subunits PA, PB1 and PB2) (step 3a). These mRNA then are exported into the cytoplasm for viral proteins translation (step 4). The cRNAs remain in the nucleus and are the template for synthesis of further negative-sense genomic vRNA segments for packaging into progeny virions (step 3b) (Cros and Palese 2003). The new proteins are either secreted through the Golgi apparatus to reach the cell surface (in the case of HA and NA, step 5b) or moved back into the nucleus to bind vRNA (step 5a). The vRNA and viral core proteins leave the nucleus and reach the cell surface at HA and NA location (step 6). The mature virus collect HA and NA in the new virus surface then buds off from the cell (step 7) (Nayak, Hui, and Barman 2004). After the release of new influenza viruses, the host cell dies.

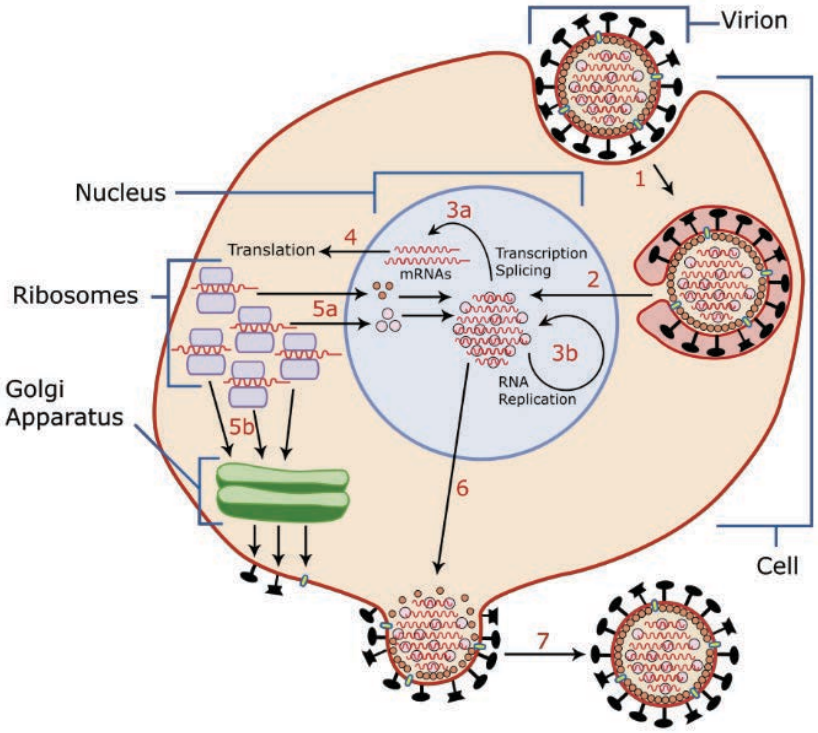

Figure 5: Influenza virus replication mechanism

2.2. Influenza vaccines

2.2.1. Conventional vaccine against influenza

Vaccination is one of the most effective means for public health control of infectious diseases such as influenza. Currently, there are two types of the influenza vaccine: the inactivated influenza vaccine (IIV) and the live attenuated influenza vaccine (LAIV).

Inactivated influenza vaccines

Inactivated influenza vaccines are the most commonly products for influenza vaccination due to relatively low production costs, safety, and effectiveness. There are four types of inactivated vaccines: whole inactivated virus vaccines (WIV), split virus vaccines, subunit vaccines, and virosomal influenza vaccines (Soema et al. 2015). Typically, whole-virus killed vaccines are produced in 9-11-day-old pathogen-free embryonated chicken eggs, chemically inactivated with formaldehyde or β- propiolactone, concentrated and purified. In split virus vaccines, the virus envelope is disrupted by detergent treatment to exhibit all viral proteins (Neurath et al. 1971). Concerning subunit vaccines, it is required the purification steps to separate the nucleocapsid and lipids from the surface proteins HA and NA (Laver and Webster 1976). Virosomal vaccines are lipid based vaccines made of phospholipids and antigens to build a viral nanoparticle. Split virus and subunit vaccines are more frequently used as seasonal influenza vaccines in human due to their less reactogenicity as compared to the whole-virus products (Beyer et al. 2011).

Most human seasonal trivalent inactivated vaccine/quadrivalent inactivated vaccine (TIV/QIV) are available as single dose vaccines for people ≥9 years of age administered via an intra muscular (i.m.) injection. Current guidelines recommend that children 6 months old to <9 years old receive an extra dose administered 4 weeks after the first vaccination. Efficacy of existing vaccines is highly dependent on the age group, with much lower efficacy in children, the elderly, and adults with underlying conditions (Swayne et al. 2011; Sandbulte et al. 2015).

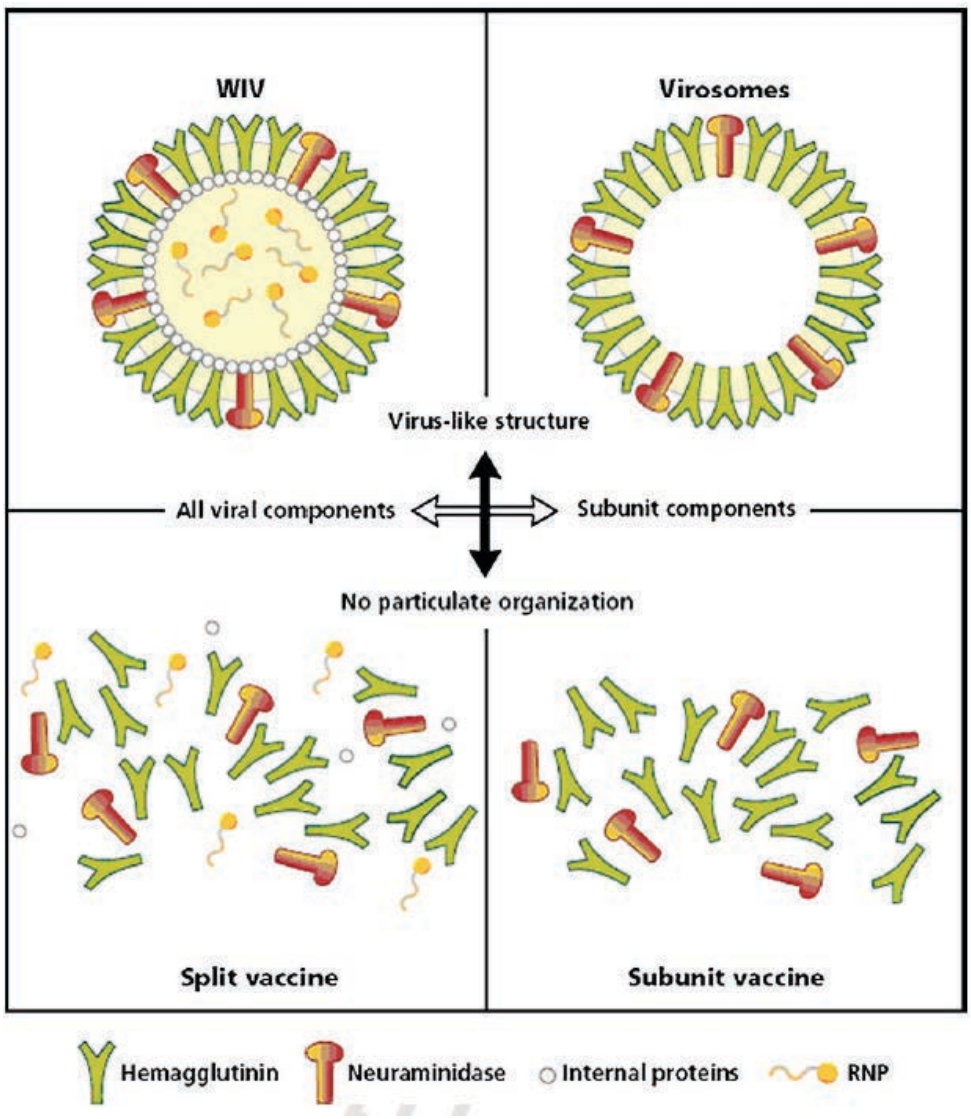

Figure 6: Schematic presentation of composition and spatial organization of WIV, split, subunit and virosome vaccines (Hagenaars et al. 2008)

Live-Attenuated influenza Vaccines

Live-attenuated influenza vaccines are available in several countries for use in human and horses. LAIVs for human use were independently obtained after serial passage in eggs resulting in viruses with cold-adapted, temperature-sensitive mutations that prevented their growth at temperatures higher than 35 ^{0}C and thus restricting virus replication to the nasal cavity (Maassab, Heilman, and Herlocher 1990). LAIVs have an advantage over inactivated products because they mimic a natural route of infection but with very low rate of adverse reactions. In contrast to inactivated products, vaccination with live products provides both humoral and cell-mediated immunity. They can induce mucosal IgA responses in the upper respiratory tract, hence providing more comprehensive cross-reactive and longer-lasting immune responses

(Loving et al. 2013; Hoft et al. 2017). These vaccines are not recommended for use in immunocompromised people because of their inherent risk of developing disease. Other perceived risks associated with the use of LAIVs are vaccine strain reversion and recombination with circulating field strains. However, the different LAIV systems have been consistently safe and stable. An intranasal cold-adapted LAIV produced by MedImmune (FluMist) was licensed in the U.S. in 2003. A quadrivalent version of FluMist received approval in 2012.

2.2.2. Strategies for influenza vaccines

The greatest problem for vaccine manufacturing for both animal and human influenza is the high variability and rapid evolution of the virus. Another obstacle is the timeline for influenza vaccine manufacture. From strain selection to vaccine distribution it takes at least 6 months and in some instances, the distribution and antigenic make up of circulating viruses at the end of the season differs from the selected vaccine strain, which could result in a mismatch. Moreover, high-risk groups, such as the elderly or immunocompromised people may not respond optimally to vaccination due to declined immune function (Kunisaki and Janoff 2009; Lambert et al. 2012).

The limitations discussed above require the need for new strategies that could improve vaccine efficiency and induce long-lasting broadly protective immunity. Alternative routes of delivery and different vaccination approaches could improve immune response to traditional vaccines. Moreover, vaccines that target conserved regions of the virus lead to broader, longer-lasting immune response, termed “universal” vaccines, are also studied these days.

2.2.2.1. Adjuvants for influenza vaccine

The role of adjuvant

Fundamentally, adjuvants improve the ability of the host immune system to recognize the administered antigen as foreign and respond to it. Adjuvants are used to boost responses in populations with poor immune responses. Another advantage is that adjuvants can accelerate responses to the vaccine, for example during a pandemic. The inclusion of adjuvants can enable dose sparing, both for routine and pandemic vaccines. A final use is to enable mucosal delivery of vaccines. Mucosal surfaces are much harder to vaccinate for a number of reasons such as mucus and proteolytic enzymes barriers to antigen. Specific adjuvants may be required to protect the antigen in this environment and to induce a local immune response. One adjuvant

that was licensed for this purpose was the heat labile enterotoxin of Escherichia coli, which was included in Nasalflu, but this has been withdrawn (Gluck, Gebbers, and Gluck 1999).

Adjuvants in licensed influenza vaccines

Alum is the oldest and most widely used adjuvant. Though it should be noted that the description alum, which strictly refers to potassium alum $KAl(SO4)_2$ only, often covers a broad range of aluminium salts, including sodium, potassium and ammonium alum.

MF59 is an oil-in-water adjuvant which contains squalene, polysorbate 80 and sorbitan trioleate. Squalene was chosen as the oil component is a naturally oil found in large quantities in human tissues.

AS03 is an oil-in-water adjuvant, developed by GSK as part of a broader Adjuvant System which has multiple members (Garcon and Di Pasquale 2017). AS03 contains squalene, DL-α-tocopherol and polysorbate 80. Variants of AS03 have been produced, based on the amounts of these contents.

AF03 is an oil-in-water adjuvant developed by Sanofi Pasteur. AF03 contains squalene, montane 80 PH and eumulgin B1- PH. The manufacture of AF03 is slightly different to MF59 and AS03, using phase inversion temperature emulsification process (Klucker et al. 2012).

In general, adjuvanted influenza vaccines have a good safety profile and improve the immune response to vaccine antigens. The addition of an adjuvant may not address the problems with the current generation of influenza vaccines. However, novel adjuvants may also help in the drive for a universal influenza vaccine by stabilizing antigens, boosting responses to recombinant antigens, or redirecting the immune response towards either a local or a cellular response (Tregoning, Russell, and Kinnear 2018).

2.2.2.2. Nucleic acid influenza vaccines

Nucleic acid vaccines were studied decades ago but have already been widely used for infectious diseases. Nucleic acid vaccination is a technique for protecting against disease by injection with antigenic DNA (as a plasmid) or RNA (as mRNA). In contrast to recombinant bacteria or virus vaccines, nucleic acid vaccines consist only of DNA or RNA, uptaken by cells and delivered to the cytoplasm or nucleus and transformed into protein antigen. There are several reasons why nucleic acids are attractive candidate vectors for the development of vaccines for infectious diseases. Firstly, nucleic vaccines are relatively inexpensive and easy

to manufacture. In contrast to recombinant bacteria or viruses, the vaccine composition is simple and has immunological advantages. Unlike live-attenuated viruses, which can revert in to their pathogenic form, nucleic acid vaccines cannot replicate inside the body. Moreover, many target proteins can be produced in one time leading to the induction of a wider range of immune response types (Khalid et al. 2017).

To produce a DNA vaccine, a DNA copy is made from the viral RNA segment coding for the antigen of interest (i.e. an influenza protein), which is then inserted into a plasmid. The plasmid is administered into the host allowing antigen production in cells of the host, which results in an immune response against the antigen. An influenza DNA plasmid encoding for H5N1 HA, NP and M2 proteins loaded into cationic liposome induced antibody and T cell responses after intramuscular injections in a clinical study (Smith LR et al. 2010). The vaccine has showed to induce HA antibody titers comparable to titers induced by a subunit vaccine. While influenza DNA vaccines are a promising concept, several issues regarding safety have to be considered such as concern for genome integration, or the high doses and devices needed (e.g., electroporation) (Soema et al. 2015).

The mRNA vaccine technology overcomes the challenges of DNA approach (Schlake et al. 2012). It offers advantages in speed and precision of antigen design which is particularly important for emerging infections, such as potential pandemic influenza (Partridge et al. 2010). Moreover, mRNA vaccines induce both cellular and humoral response (Midoux et al. 2015).

At 105–106 Da in size, mRNA requires a delivery system to be taken up by the host cells. This can be accomplished by nanoparticulate vehicles that encapsulate mRNA and then deliver mRNA to the cytoplasm of the target cells. Many synthetic materials have been discovered as a nano-delivery system for mRNA vaccine such as lipid nanoparticle or polymer based nanoparticle (Table 1).

Materials	Application	mRNA delivered	Route of administer	Ref
Ionizable LNP	Zika vaccine	prM-E	Intradermal	Pardi N et al. 2017
Ionizable LNP	Influenza vaccines	H7 and H10 haemagglutinins	Intramuscular or intradermal	Bahl K et al. 2017
Ionizable LNP	HIV therapy	VRC01 light and heavy chain	Intravenous	Pardi N et al. 2017
PSA nanomicelle	HIV vaccine	HIV-1 gag	Subcutaneous	Zhao M et al. 2016
CP 2k conjugate	HIV vaccine	HIV gp120	Intranasal	Li M et al. 2016
Modified dendrimer nanoparticle	Influenza, Ebola or Toxoplasma gondii vaccine	Haemagglutinin, Ebola glycoprotein and six T. gondiispecific antigens	Intramuscular	Chahal JS et al. 2016

Table 1: Selected synthetic materials for mRNA vaccines

CP 2k, cyclodextrinpolyethyleneimine 2k; gag, group-specific antigen; gp120, envelope glycoprotein gp120; HIV, human immunodeficiency virus; LNP, lipid nanoparticle; PLGA, poly(lactic-co-glycolic acid); PSA, polyethyleneimine-stearic acid.

Reports of mRNA based vaccination described the use of mRNA encoding the influenza virus nucleoprotein these vaccines generated a cytotoxic T cell response in mice (Khalid et al. 2017). There are ongoing clinical trials for mRNA vaccines for influenza, Zika and HIV (Khalid et al. 2017). Recently, lipid nanoparticles (LNP)-formulated, with mRNA vaccines, encoding HA proteins of H10N8 or H7N9 induced strong immune responses in mice, ferrets, and nonhuman primates. Furthermore, a single dose of H7N9 mRNA loaded into LNP protected mice from a lethal challenge and decreased lung viral titers in ferrets. The study showed that LNP-

formulated mRNA vaccines can induce protective immunogenicity against influenza infection (Bahl K et al. 2017).

For infectious diseases with high mutation rates such as influenza, mRNA vaccine can be produced reliably allowing rapid response to emergence of pandemic strains. Therefore, mRNA vaccines are of great strategies for the prevention of influenza virus infection (Petsch et al. 2012).

2.2.2.3. Universal influenza vaccines

The “universal” influenza vaccine approaches attempt to overcome the problem of the highly changing nature of influenza viruses. The objective of these vaccines is to induce cross-protection, which depends on stimulating both humoral and cell-mediated arms of the immune system. These “universal” vaccines are based on the concept of developing broadly protective effect against conserved viral epitopes. The primary goal of these strategies is to avoid annual vaccine updates and decreasing the need for re-vaccination. To develop universal influenza vaccine, researchers try to target either the highly conserved epitopes of the HA (the HA stalk domain) or the extracellular domain of the M2 protein (M2e), or target internal proteins like the nucleoprotein (NP) and the matrix protein (M1) to induce cross-protective T-cell response (Rajao and Perez 2018).

2.3. Intranasal universal influenza vaccine – UniVac Flu Project

Influenza vaccines are administered mostly by intramuscular route which has some limitations. Poor T cell response and no mucosal response is induced. Intranasal route for influenza vaccine administration, on the contrary, offers several advantages. Firstly, this route use is needle-free and painless. The vaccine activates the immune cells in the nasal-associated lymphoid tissue (NALT) effector sites including larynx- and bronchus-associated lymphoid tissues (Davis 2001). Hence, intranasal route can also exert defensive activities at the prime sites of influenza virus infection. Moreover, the vaccine applied intranasally may also reach the blood circulation and the systemic immune response is stimulated in spleen and peripheral lymph nodes (Neutra and Kozlowski 2006). Thus, both mucosal and systemic immune responses can be expected after the intranasal immunization of appropriate vaccine formulation (Svindland et al. 2012).

Currently available influenza vaccines are made of either inactivated whole viruses or virus subunit or split products (Wong and Webby 2013). However, while these vaccines are less reactogenic they confer limited immunogenicity and require either high antigenic dose or a

booster dose or adjuvants (Centers for Disease and Prevention 2012; Neuzil et al. 2006). One approach to improve influenza vaccines is to include adjuvants that boost the immune response. Cholera toxin (CT) has been known as the potential adjuvant for mucosal vaccine. Cholera toxin is protein complex secreted by the bacterium vibrio cholera (Yusuf 2015; Faruque and Nair 2002). CT is a complex made of six protein subunits: a single copy of the A subunit (part A, enzymatic), and five copies of the B subunit (part B, receptor binding), expressed as AB_5. The five B subunits form a five-membered ring. The A subunit has two important segments. The A1 portion of the chain (CTA1) payload that ADP-ribosylates G proteins, while the A2 chain (CTA2) links the CTA1 subunit to the pentameric CTB subunits (De Haan and Hirst 2004).

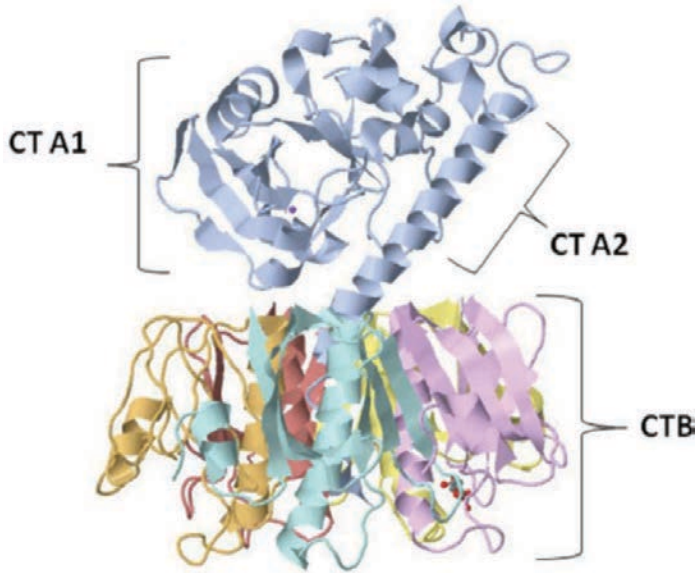

Figure 7: Crystal structure of cholera toxin. The heterodimeric CTA protein subunit (blue) is composed of CTA1 and CTA2. The cholera toxin B subunit is composed of five identical polypeptide subunit chains (yellow, purple, red, orange, and turquoise), each with membrane receptor GM1ganglioside binding capacity (Odumosu et al. 2010).

However, CT can induce toxicity. To circumvent the problem with toxicity of CT, a non toxic derivative of CT, termed CTA1 was developed (Agren et al. 1997). The CTA1 has strong ADP-ribosyl transferase activity and is thought to act on several G proteins. 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 Fc and Fab fragments of immunoglobulin present on B cells, thereby allowing binding of the complex CTA1-DD (Agren et al. 1997; Lycke 2004a; Eriksson, Schon, and Lycke 2004).

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, Schon, and Lycke 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, Schon, and Lycke 2004).

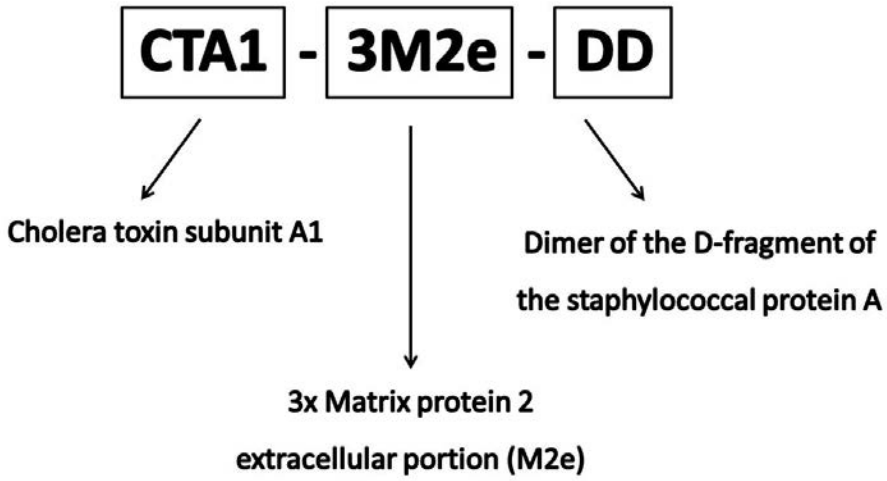

Figure 8. Adjuvanted antigen CTA1-3M2e-DD. The fusion protein is composed by three parts: the adjuvant CTA1, subunit A1 of the cholera toxin; the antigen M2e, extracellular domain of the conserved porous channel of influenza A and a targeting portion DD, dimer of the D fragment of the staphylococcal protein A.

To overcome weak immunogenicity of the M2e-based universal vaccine, this innovative adjuvant- CTA1-DD, has been therefore fused to 3M2e, to give CTA1-3M2e-DD (Figure 5). Mucoadhesive nanocarrier systems created from starch, hyaluronic acid and chitosan offer a promising approach for the development of mucosal vaccines (Illum et al. 2001; Singh, Briones, and O'Hagan 2001). Such carriers can be designed to prolong their residence time in the nasal cavity, to protect entrapped antigens against degradation, to enhance uptake by M-cells, and to target the antigens more specifically to APC (Soane et al. 1999; Soane et al. 2001; Bernocchi et al. 2016; Dimier-Poisson et al. 2015). According to this idea, cationic porous maltodextrin based-NPs (NPL) were applied as vaccine delivery system to deliver CTA1-3M2e-DD + HA into nasal mucosa. This vaccine formulation was challenged with H3N2 and H1N1 influenza virus strains to investigate the protective effect. Recently, we observed that this vaccine was able to protect against live flu viruses infection. This study demonstrated that

CTA1-3M2e-DD + HA loaded NPL could be potential universal influenza vaccine (Bernasconi *et al.*, Journal Frontiers in Immunology, submitted).

PART II: RESULTS

Several strategies have been explored using nanoparticles for the mucosal delivery of antigen such as: protection to the antigen, enhanced antigen absorption, extended residence time in the mucosa. Hence, a better understanding of the interaction of nanoparticles with mucosal cells is essential for drug delivery as well as vaccine applications. Therefore, investigating how the physicochemical characteristics of nanomaterials influence their interactions with cells is a critical issue to obtain effective clinical applications.

In this study, we investigated the effect of nanoparticles' surface charge and inner composition on their interactions with nasal mucosa cells, by comparing anionic and cationic liposomes, anionic and cationic Poly-Lactic co-Glycolic Acid nanoparticles (PLGA NP) and cationic NPL (cationic porous maltodextrin-based nanoparticles with an anionic lipid core).

PUBLUCATION 2: RESIDENCE TIME AND UPTAKE OF ZWITTERIONIC NANOPARTICLES IN THE NASAL MUCOSA: COMPARISION WITH ANIONIC AND CATIONIC NANOPARTICLES

Residence time and uptake of zwitterionic nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles

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Keywords : nanoparticles; zwitterion; nasal; vaccine; mucosa

ABSTRACT

Different types of biodegradable nanoparticles (NP) have been studied as nasal mucosa cell delivery systems. These nanoparticles need to strongly interact with mucosa cells to deliver their payload. However, while many studies are promising, few simultaneous comparisons with other systems have been made and it is therefore difficult to determine the best candidate. Here we compared 5 types of nanoparticles with different surface charge (anionic or cationic) and various inner compositions as potential vectors: cationic and anionic liposomes, cationic and anionic PLGA (Poly Lactic co-Glycolic Acid) NP and zwitterionic maltodextrin NP (cationic on their surface with an anionic lipid core: NPL). We first quantified their nasal residence time after nasal administration in mice using *in vivo* live imaging and NPL showed the longest residence time. *In vitro* endocytosis studies on mucosal cells (airway epithelial cells, macrophages and dendritic cells) using labeled nanoparticles were performed by flow

cytometry and confocal microscopy. Among the 5 nanoparticles, NPL were taken up to the greatest extent by the 3 different cell lines and the endocytosis mechanisms were characterized. Taken together, we observed that the nanoparticles' cationic surface charge is insufficient to improve mucosal residence time and cellular uptake and that the zwitterionic NPL are the best candidates to interact with airway mucosal cells.

1. Introduction

Many recent studies have shown that nanoparticles significantly improve the delivery of drugs and vaccines into the nasal mucosa [1]. The advantages of using nanoparticles as a delivery system include their ability to protect drugs or antigens against enzymatic degradation. Moreover, these nanosystems also improve intracellular delivery by transporting an encapsulated drug (or vaccine) across the membrane barrier, while they may also extend residence time in the nasal cavity [2, 3]. Hence, a better understanding of the interaction of nanoparticles with mucosal cells is essential for drug delivery as well as vaccine applications. Therefore, investigating how the physicochemical characteristics of nanomaterials influence their interactions with cells is key to achieving effective clinical applications. Nanoparticle interactions with cells may depend on nanoparticle material [4], size [5], shape [6], surface charge [7] and surface chemistry [8]. However cell physiology (differentiation state, proliferation rate, morphology…) and cell type, together with their interactions with physiological media (salts, proteins, agglomerates…), make the understanding of the mechanisms of the nanoparticle interactions with cells very challenging. This much is evident from the relatively small number of publications about the interactions of nanoparticles with the airway epithelium [9-11].

In this study, we investigated the effect of nanoparticles' surface charge and inner composition on their interactions with nasal mucosa cells, by comparing anionic and cationic liposomes, anionic and cationic Poly-Lactic co-Glycolic Acid nanoparticles (PLGA NP) and zwitterionic NPL (cationic porous maltodextrin-based nanoparticles with an anionic lipid core).

Firstly, nano-liposomes are spherical, self-closed structures formed by a single lipid bilayer with an aqueous phase inside and may be either anionic or cationic. Liposomes are able to deliver material into cells or even inside particular cellular compartments [12]. Liposomes modified with sterylglucoside have been shown to be useful as vectors of nasally administered insulin [13]. Liposomes as nasal vaccine delivery systems have been well studied [14, 15].

Secondly, PLGA is one of the most successful biodegradable polymers used widely as a nanoparticle based drug delivery system [7]. PLGA coated with chitosan improved tetanus protein delivery in the blood *via* the intranasal route [16].

Finally, zwitterionic porous maltodextrin-based nanoparticles (NPL) have been shown to be a promising adjuvant for intranasal vaccination [17, 18].

In this study, we determined the influence of the charge and inner composition of these 5 types of nanoparticles on the *in vivo* nasal residence time and their *in vitro* uptake by airway mucosal cells.

2. Results

2.1. Nanoparticle synthesis and characterization

The size and zeta potential of the 5 types of nanoparticles were characterized **(Table 1 and Figure 1)**. Three cationic nanoparticles were prepared: liposomes, PLGA NP and NPL, all exhibited a zeta potential of between +31 mV and +37 mV. NPL are porous zwitterionic nanoparticles as they have a cationic surface but an anionic core, they therefore are cationic nanoparticles. Anionic nanoparticles were also prepared, with anionic liposomes having the greater negative charge of about -65mV, while anionic PLGA NP exhibited a negative charge of -32 mV. The nanoparticles all possessed a size ranging from 75 to 110 nm, and all types had a low polydispersity index (PDI) demonstrating that the size of nanoparticles was homogenous in solution. In addition, all these nanoparticles were spherical in shape [17, 19, 20].

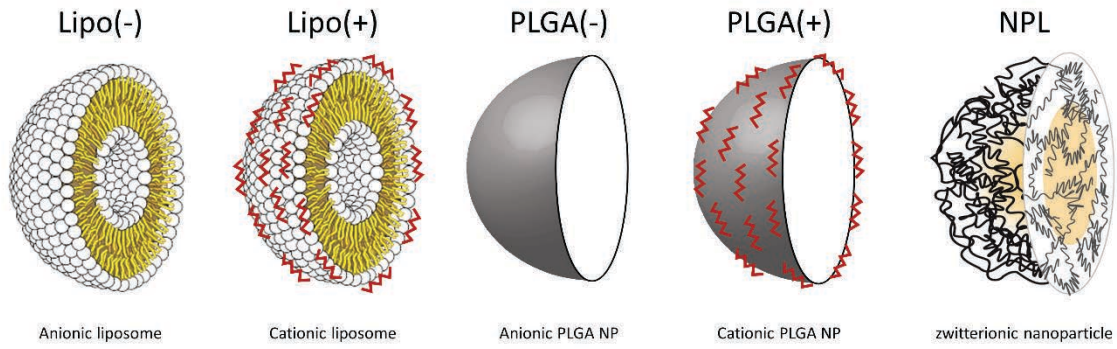

Figure 1. Representation of the 5 types of nanoparticles. Cut view showing the surface and the core of the nanoparticles. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

	Size (z-average) (nm)	PDI	Zeta-potential (mV)
Lipo(-)	75.91	0.290	-65.9 ± 7.86
Lipo(+)	77.85	0.174	+31.0 ± 13.2
PLGA(-) NP	88.66	0.233	-32.6 ± 7.77
PLGA(+) NP	110.70	0.146	+32.7 ± 12.2
NPL	102.50	0.181	+37.7 ± 7.87

Table 1. Size and zeta potential of the 5 types of nanoparticles. The size and the zeta potential of the 5 types of nanoparticles were measured by dynamic light scattering and by electrophoretic mobility, respectively. The PDI (polydispersity index) represents the homogeneity of the size of nanoparticles. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

2.2. Nasal residence of the nanoparticles

Labeled batches of NP (20 µg) having the same fluorescence were used (**Supplemental Figure 1**). Mice were imaged at different time points **(Figure 2)** and the kinetics of the nasal residence time is presented in **Figure 3**.

Cationic and anionic liposomes and anionic PLGA NP were quickly eliminated from the nose (30 min), while cationic PLGA NP and NPL had the longer residence times in the nose of 6h after administration. Comparing the area under curve data showed that NPL remained in the nose at higher doses compared to cationic PLGA NP (612 vs 359 a.u.²) while the residence times of these two nanoparticle types were similar.

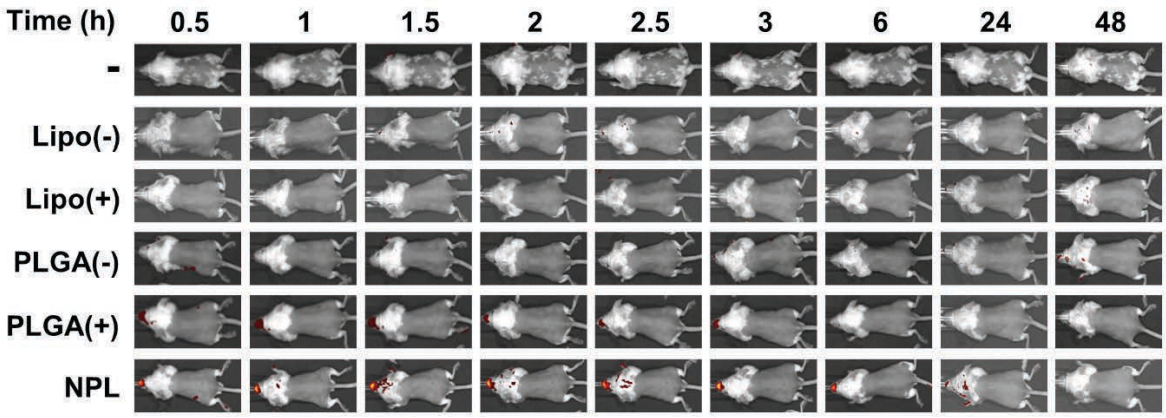

Figure 2. Biodistribution of the 5 types of nanoparticles. Mice were treated with PBS (-) or one of the 5 types of nanoparticles labeled with DiI. The mice were imaged at different time points with the In Vivo Imaging System IVIS® Spectrum (n = 3 per group). Images are representative of 3 independent experiments. -: vehicle control, Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

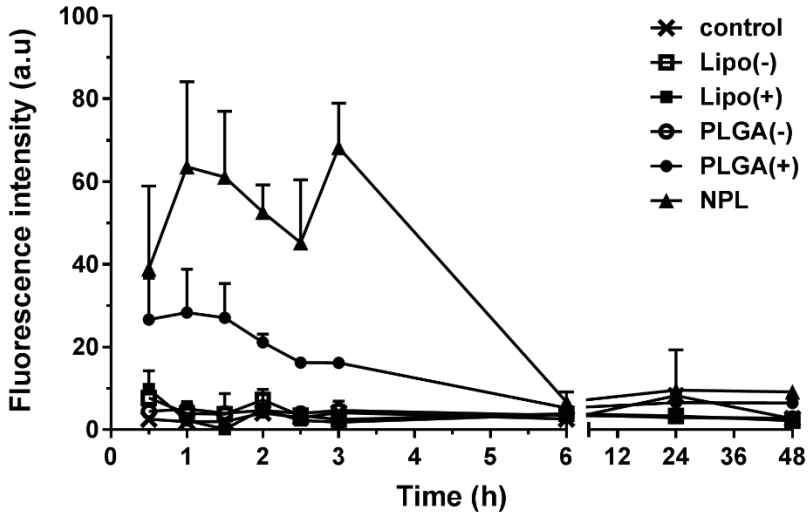

Figure 3. Evaluation of the nasal residence time of the 5 types of nanoparticles. The nasal fluorescence of nanoparticles from the images of the figure 2 were quantified and the mean fluorescence intensity +/-SD is reported on the graph for each nanoparticle. -: vehicle control, Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

2.3. Endocytosis of nanoparticles in H292 airway epithelial cells

Epithelial cells are arranged in tightly packed layers that line the surfaces and cavities of tissues and organs throughout the body. The first barriers that nanoparticles face once nasally instilled are mainly the epithelial cells and some specialised cells such as M cells [21, 22]. We evaluated the uptake of the nanoparticles in H292 airway epithelial cells producing mucus using flow cytometry and confocal microscopy [23]. The uptake of all 5 nanoparticles increased over time meaning that nanoparticles accumulated in the cells, without reaching a steady-state under the conditions tested. After 24h of treatment with the 5 types of nanoparticles, the anionic and cationic liposomes were slowly taken up by epithelial cells and only 1% of cells were positive. PLGA NP were endocytosed 5 to 8 times more than liposomes, respectively, with 17% of

positive cells for anionic PLGA and 36% of positive cells for cationic PLGA. Finally, we observed that NPL were strongly taken up (46-fold more than liposomes) with more than 90% of positive cells. Indeed, NPL were quickly endocytosed since after only 30min 40% of cells were positive (**Figure 4a**). These results were confirmed by confocal microscopy **(Figure 4b)** and corroborated using a different fluorescent probe (**Supplemental Figure 3**).

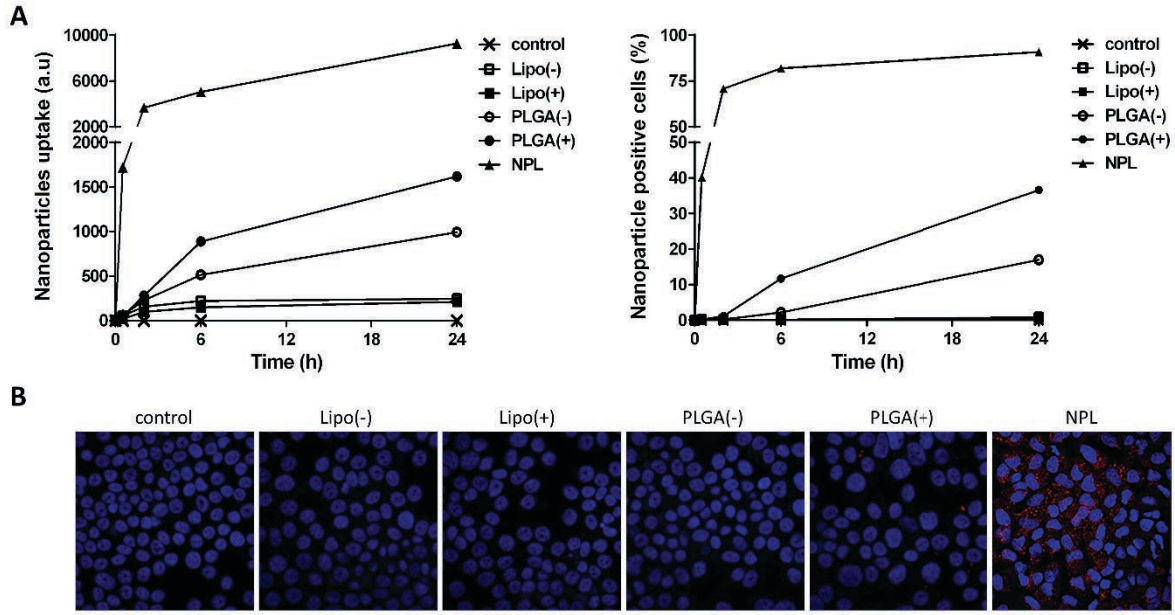

Figure 4. Uptake of the 5 types of nanoparticles in H292 airway epithelial cells. A) Kinetics of the NP uptake by flow cytometry. The NPs were labeled with DiI then incubated with cells for 30 min, 2h, 6h and 24h, then analyzed by flow cytometry. Nanoparticle uptake (left) and percentage of positive cells for labeled-NP(right). B) Confocal microscopy images of cellular uptake. The cells were incubated for 2h with NP labeled with DiI (red) then cells were processed for confocal imagery with a staining of the nuclei by Hoescht 33342 (blue). Original magnification: 40x. Representative graphs and images of three independent experiments. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

2.4. Endocytosis of nanoparticles in SRDC dendritic cells

Different subsets of antigen presenting cells (APC) are present in the nose-associated lymphoid tissue (NALT). Dendritic cells have a key role in nanoparticle uptake [5, 24]. After 24h of treatment, the uptake of liposomes by SRDC was low, but nevertheless higher than in the airway epithelial cells. The endocytosis of PLGA NP was faster than liposomes with a better uptake of cationic PLGA NP (25% of positive cells) compared to the anionic PLGA NP (11% of positive cells). Again, NPL showed remarkable endocytosis with 57% of positive cells after 6h incubation. Interestingly, we found that the uptake of NPL at 30 min was very high (16

times better than cationic liposomes) (**Figure 5a**). These results were confirmed by confocal microscopy **(Figure 5b)** and corroborated using another fluorescent probe (**Supplemental Figure 3**).

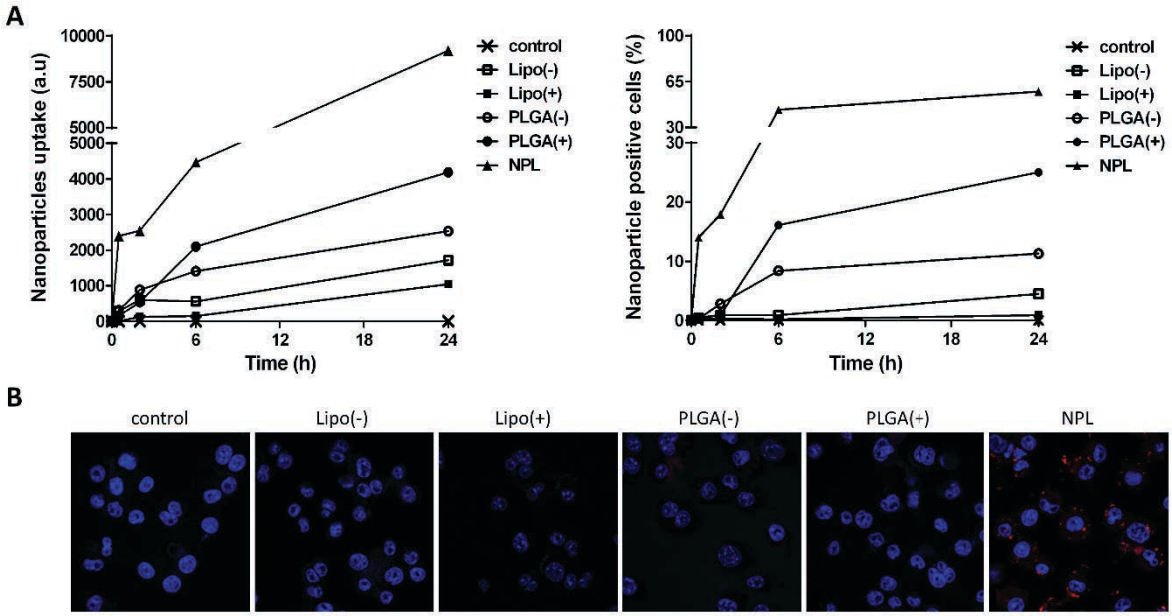

Figure 5. Uptake of the 5 types of nanoparticles in SRDC dendritic cells. A) Kinetics of the NP uptake by flow cytometry. The NPs were labeled with DiI then incubated with cells for 30 min, 2h, 6h and 24h, then analyzed by flow cytometry. Nanoparticle uptake (left) and percentage of positive cells for labeled-NP (right). B) Confocal microscopy images of cellular uptake. The cells were incubated for 2h with NP labeled with DiI (red) then cells were processed for confocal imagery with a staining of the nuclei by Hoescht 33342 (blue). Original magnification: 40x. Representative graphs and images of three independent experiments. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

2.5. Endocytosis of nanoparticles in THP-1 macrophages

As the presence of macrophages in the airways is highly documented we evaluated the ability of macrophages to uptake the NP [25]. Once again, kinetics were comparable and NPL showed the highest endocytosis rates (98% of positive cells at 24h), with a 65-fold increase *vs.* liposomes and a 5-fold increase *vs.* PLGA (+). The endocytosis rates were constant, except for NPL that showed rapid initial uptake (93% of cells were positive after 2h) that continued more slowly thereafter **(Figure 6a)**. These results were confirmed by confocal microscopy studies **(Figure 6b)** and corroborated using another fluorescent label (**Supplemental Figure 3**).

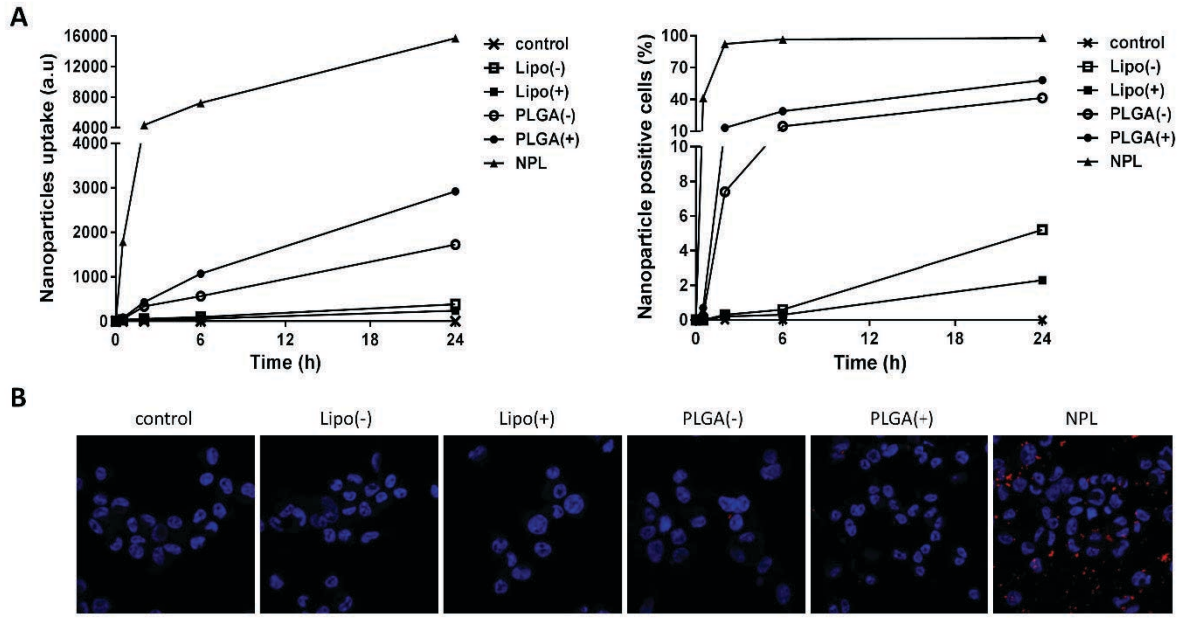

Figure 6. Uptake of the 5 types of nanoparticles in THP-1 macrophages. A) Kinetics of the NP uptake by flow cytometry. The NPs were labeled with DiI then incubated with cells for 30 min, 2h, 6h and 24h, then analyzed by flow cytometry. Nanoparticle uptake (left) and percentage of positive cells for labeled-NP (right). B) Confocal microscopy images of cellular uptake. The cells were incubated for 2h with NP labeled with DiI (red) then cells were processed for confocal imagery with a staining of the nuclei by Hoescht 33342 (blue). Original magnification: 40x. Representative graphs and images of three independent experiments. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).

2.6. Endocytosis mechanism of NPL

Owing to their higher uptake, we focused the study of the endocytosis mechanisms on the zwitterionic NPL in order to understand why this nanoparticle is so effectively endocytosed. Cells were pretreated with known inhibitors of specific pathways of endocytosis **(Table 2)**. As shown in **Figure 7,** the inhibition of the caveolae pathway gave approximately a 24% decrease in the uptake of NPL by H292 cells and a decrease of about 33% and 52% in SRDC and THP-1 cells, respectively. Clathrin inhibition had a significant effect on NPL internalization in SRDC (60-71% of inhibition) and in THP-1 cells (about 53% of inhibition), but had a more moderate effect in H292 cells (32% of inhibition). Dynamin was likely one of the main actors in NPL endocytosis by H292 and THP-1 as dynasore inhibited 53% and 58%, respectively, of the nanoparticle uptake by these cell lines. However, dynasore had a lesser effect on SRDC with 29% of inhibition. Macrophagocytosis may also be a vital pathway of NPL entry into

SRDC and THP-1, since amiloride exhibited inhibition of 54% and 44 %, respectively. As expected, amiloride impaired only 36% of the nanoparticle uptake by the non-phagocytic H292 epithelial cells. Lastly, ATP depletion by NaN_3 pretreatment resulted in an *ca.* 45% decrease in NPL uptake by H292 and THP-1 cells, while it showed stronger inhibition in SRDC with a 60% decrease, confirming the energy-dependent nature of the endocytosis mechanisms. Taken together, these data clearly showed that the cellular uptake pathways of NPL is mediated by a combination of several cellular endocytosis mechanisms.

Inhibitors	**Final concentration**	**Endocytosis pathways**
Nystatin, Filipin	20 µg/ml 10 µg/ml	Caveolae
Chlopromazine (CPZ), Phenylarsine oxide (PAO)	10 µg/ml 1 µg/ml	Clathrin
Dynasore	26 µg/ml	Dynamin
Amiloride	23 µg/ml	Macropinocytosis
Sodium azide (NaN_3)	600 µg/ml	ATP inhibitor

Table 2. Inhibitors of endocytosis. The final concentration and the targeted endocytosis pathways of the endocytosis inhibitors used in the study.

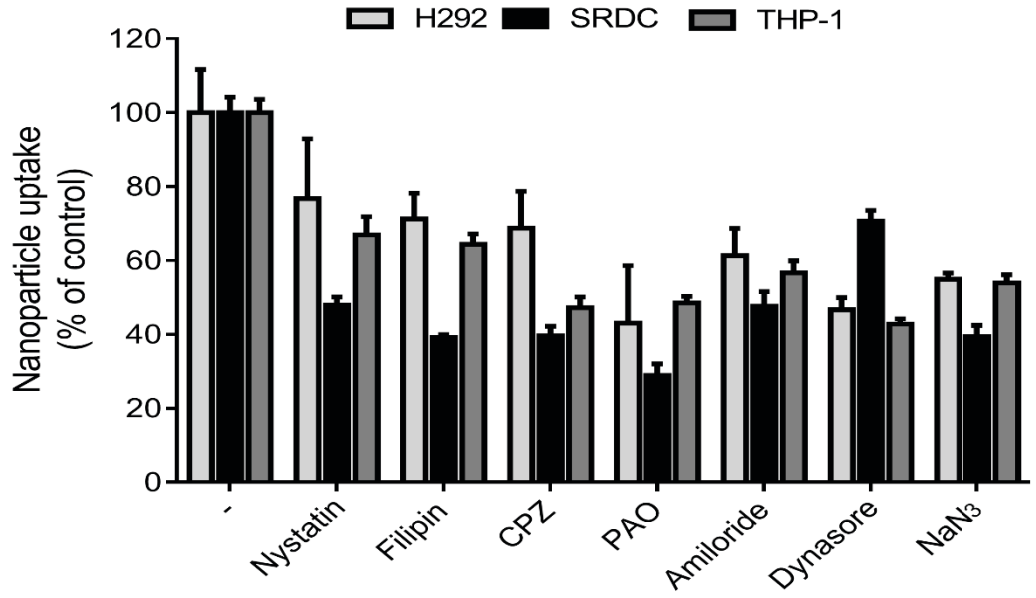

Figure 7. Mechanism of endocytosis of NPL in H292, SRDC and THP-1 cells. The cells were pre-treated with different endocytosis inhibitors following the table2 then DiI-labeled NPL were added to cells for 30 min at 37°C. Cells were then washed and immediately anaylsed by flow cytometry. Data represent the mean fluorescence intensity +/- SEM (n = 3) and untreated cells were set to 100% of NPL uptake. Representative graphs of three independent experiments performed in triplicates. - : untreated cells, CPZ: chlorpromazin, PAO: Phenyl Arsine Oxide.

3. Discussion

An increasingly large number of studies have examined nasal delivery of drugs and vaccines using nano-carriers such as micelles, polysaccharide nanoparticles [2], liposomes or PLGA nanoparticles, but little is known of their ability to interact with mucosal cells in the airway. In this study, we compared 5 types of nanoparticles commonly used as nano-carriers for intra-nasal administration: they were either anionic or cationic (liposomes and PLGA NP), while NPL are zwitterionic (a cationic surface with an anionic core). The aim of this study was to correlate the characteristics of the nanoparticles with their ability to be efficiently endocytosed by airway epithelial cells. Many studies have described the potential of different types of NP for mucosal delivery, but they were generally administered at very high doses: while positive results were obtained, no comparison was made with other delivery systems. This comparative study was set up with the same dose of each of the nanoparticle types (1µg/cm²), and each had the same fluorescence. In the literature, due to their poor endocytosis, liposomes and PLGA NP have generally been studied at higher doses, up to 100 fold more than used in this study [26-28].

Our study of *in vivo* nasal residence time of these 5 nanoparticle types showed that cationic PLGA NP and NPL had the longer residence times **(Figure 2)**. This is in accordance with previous experiments that described a stronger interaction between cationic compounds and the airway mucosa than anionic or neutral compounds [2, 5, 29]. Indeed, increased residence times might be attributed to the strong electrostatic interactions between cationic nanoparticles with anionic sialic and acidic moieties of glycosaminoglycan contained in the mucin and on the surface of airway cells [30]. Unexpectedly, the cationic liposome nanoparticles showed completely different behavior, even though they possessed the same positive charge density as cationic PLGA NP and NPL.

In this study the longer nasal residence time of NPL compared to the 4 other nanoparticles **(Figure 3)** might be due to the presence of anionic lipids inside the core of these nanoparticles [31]. In another study we found that NPL increased the nasal residence time of an encapsulated protein and that these NPL were observed in the first layer of the nasal mucosa cells after nasal administration [32]. According to this finding, we hypothesized that NPL could be more efficiently taken up by the cells underlying the mucus layer. In order to confirm this, we studied the cellular uptake of the 5 nanoparticle types by cell lines representative of the airway mucosa [24, 33].

Flow cytometry and confocal studies showed that the endocytosis was consistent among the 3 cell lines for the 5 types of nanoparticles. Interestingly, while NPL showed a rapid initial endocytosis (in the first 2 hours), the other types of nanoparticles were more slowly and continuously taken up over the 24h experiment. As expected, anionic nanoparticles were taken up to a lesser extent than the cationic ones [34, 35] **(Figures 4, 5 and 6; Supplemental Figure 3)**.

Despite their common use in nanomedicine (DOXIL®), we observed that liposomes are poorly taken up by the cells compared to the other types of nanoparticles. These results were confirmed in the 3 cells lines using another fluorescent probe: Rhodamine DHPE (Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine) (**Supplemental Figure 2**). The relatively poor endocytosis efficiency of liposomes compared to PLGA NP and NPL could be attributable to their stability *in vitro* and *in vivo*. However, we confirmed that the colloidal properties and the fluorescent labeling of the liposomes were both stable (**Supplemental Figure 3**).

Cell membranes have many negatively charged groups on the surface. It is expected that positively charged nanoparticles will thus be better associated *via* ionic interactions and that

their subsequent endocytosis is thus facilitated [6, 36, 37]. In our study, we found that the uptakes of the cationic liposomes and cationic PLGA NP were clearly lower than that of the cationic NPL in all 3 cell lines representing the nasal mucosa. These *in vitro* data confirmed the *in vivo* results and supported our hypothesis. The greater residence time of NPL might be due to their muco-adhesive property (acting as a zwitterion) that may increase subsequent cellular uptake. Furthermore, it has been shown that polysaccharides have a high attraction towards mucosal surfaces covering the nasal, pulmonary and gastrointestinal tract [38].

Cell membranes are impermeable to macromolecules [39, 40]. Nanoparticles, like viruses, are taken up *via* endocytosis [41-45]. Understanding the endocytosis mechanisms of nanoparticles is thus vital for developing efficient therapeutic applications. Practically, the endocytosis pathways of the 5 nanoparticles might be mediated *via* non-phagocytic mechanisms since the size of all nanoparticles is less than 1 µm. According to the relatively poor uptake of liposomes and PLGA NP compared with NPL, we focused our investigation of the cellular uptake mechanisms on NPL. We observed a similar profile for all the cell lines: inhibition of clathrin, caveolae and micropinocytosis pathways inhibited the uptake of NPL **(Figure 7)**. Dynamin is involved in many endocytosis pathways, including clathrin and caveolae pathways [46], and promotes the scission and release of intracellular endocytic vesicles. Inhibiting dynamin-dependent mechanisms provided confirmation that clathrin and caveolae were indeed involved in the endocytosis of NPL. Interestingly, depleting ATP by NaN_3 did not completely block the uptake of NPL in the 3 cell lines tested, though the dose of inhibitor used was sub-optimal to protect cells from mortality.

Our results demonstrate that NPL endocytosis was significantly inhibited by all the inhibitors tested, suggesting that NPL enter these 3 cell lines by a combination of all the pathways. The clathrin pathway has been shown to be the major route for cellular uptake of chitosan nanoparticles in Caco-2 cells [47]. Other experiments suggested that clathrin-mediated endocytosis and macropinocytosis were involved in the uptake of cholesteryl pullulan (CHP) nanoparticles [48]. Interestingly, we found that zwitterionic NPL entered epithelial cells, macrophages and dendritic cells *via* a combination of several pathways. This behavior might be due to the composition and the porous structure of these nanoparticles, which allows the NPL to expose both cationic (polysaccharide) and anionic (lipid) charges together with the lipophilic core, thus enabling strong interactions with cell membrane components.

4. Conclusion

Taken together, this comparison of 5 types of nanoparticle has demonstrated that zwitterionic NPL is the best candidate for delivering drugs into airway epithelial cells owing to their long residence time and efficient endocytosis. Further studies are needed to confirm the utility of zwitterionic nano-carriers for improving the uptake of drugs administered *via* the nasal mucosa.

5. Material and Methods

5.1. Material

Maltodextrin was purchased from Roquette (France), DPPG (1,2-dipalmitoyl-snglycero-3-phosphatidylglycerol) and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) came from Lipoid (Germany), while PLGA (Resomer RG503H) was from Evonik (Germany). Non-essential amino acids, RPMI 1640, Iscove's Modified Dulbecco's Medium (IMDM), fetal calf serum (FCS), phosphate buffered saline (PBS), Hoescht 33342, DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate), trypsin, NaOH, L-glutamine and absolute ethanol were purchased from ThermoFisher Scientific (France). Epichlorhydrin (1-chloro-2,3-epoxypropane), $NaBH_4$, GTMA (glycidyl-trimethyl-ammonium chloride), deacetylated chitosan (50-190kDa), PD-10 Sephadex G25 desalting column, nystatin, filipin (caveolae-mediated endocytosis inhibitors), chlorpromazine (CPZ) and phenylarsine oxide (PAO) (clathrin-mediated endocytosis inhibitor), amiloride (macropinocytosis endocytosis inhibitor), dynasore (dynamin endocytosis inhibitors) and NaN_3 (mitochondrial inhibitor) were from Sigma Aldrich (France).

The human muco-epidermoid bronchiolar carcinoma cell line NCI-H292 (H292) was generously donated by Dr J.M. Lo-Guidice (Lille, France). The human monocytic cell line THP-1 was provided by Dr. F. Nesslany (Lille, France). The $CD4^{-}CD8\alpha^{+}CD205^{+}CD11b^{-}$ immortalized murine spleen dendritic cell line SRDC was obtained from Pr. I. Dimier-Poisson (Tours, France)[49].

5.2. Methods

5.2.1. Cell culture

The H292 and THP-1 cells were maintained in RPMI supplemented with 10 % (v/v) heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2mM L-glutamine at 37°C

in a humidified, 5% CO_2 atmosphere. The SRDC were cultured in IMDM supplemented with 5% (v/v) heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2mM L-glutamine at 37°C in a humidified, 5% CO_2 atmosphere. Cells were seeded 3 days before treatment at a density of 1.5 x 10^5 cells per well in 8-well glass chamber slides (LabTekII, Thermo Scientific Nunc Lab) or 5 x 10^5 cells per well in 6-well plates.

5.2.2. Nanoparticle synthesis

Anionic and cationic liposome synthesis

For anionic liposomes, 35 mg of lipids (DPPC 80% and DPPG 20%) were dissolved in 2 ml of ethanol. Dissolution was performed for 5 minutes under stirring at 150 rpm. Dissolved lipids were then injected into ultrapure water (aqueous phase) under stirring at 150 rpm. For cationic liposome, 35 mg of DPPC dissolved in ethanol was injected into ultrapure water (aqueous phase) under stirring at 150 rpm following by the addition of 10% chitosan (w/w of lipid) at 80^{o}C. No surfactant was added at any step of the synthesis. Residual organic solvents were eliminated under vacuum evaporation.

Anionic and cationic PLGA NP synthesis

Anionic PLGA NP were produced by nanoprecipitation at room temperature [50]. The PLGA polymer was dissolved at 10 mg/mL in an acetone/ethanol mixture (85:15) comprising the organic phase. Dissolution was performed for 5 minutes under stirring at 150 rpm. Dissolved PLGA were then injected into ultrapure water (aqueous phase) under stirring at 150 rpm. No surfactant was added at any step of the synthesis. Residual organic solvents were eliminated under vacuum evaporation. For cationic PLGA NP, the dissolved PLGA was injected into ultrapure water containing 10% chitosan (w/w of PLGA) under stirring at 150 rpm. Chitosan (10 mg/ml) was prepared in water containing 5% acid acetic (v/v).

NPL synthesis

The NPL were prepared as described previously [31]. Briefly, maltodextrin was dissolved in 2 N sodium hydroxide with magnetic stirring at room temperature. They were reticulated and cationised using epichlorhydrin and GTMA to obtain hydrogels that were neutralized with acetic acid and sheared using a high pressure homogenizer. The nanoparticles thus obtained were purified by tangential flow ultra-filtration (Centramate Minim II, PALL, France) using a 300 kDa membrane (PALL, France) and mixed with DPPG above the gel-to-liquid phase transition temperature to produce NPL.

5.2.3. Characterization of nanoparticles

The size (Z-average) and zeta potential of the nanoparticles were measured with the zetasizer nanoZS (Malvern Instruments, France) by dynamic light scattering and by electrophoretic mobility analysis, respectively. The nanoparticles were measured in triplicate at a concentration of 100 µg/ml in ultrapure water.

5.2.4. Labeling of nanoparticles

The 5 types of nanoparticles were labeled with the DiI (NP/DiI) previously diluted in ethanol at 1mg/ml. For the labeling of the cationic and anionic liposomes, DiI was added in ethanol together with the lipids to a final concentration of 0.1% (w/w of lipid). For the labeling of the cationic and anionic PLGA NP, DiI was added during nanoparticle synthesis in the organic phase with a final concentration of 0.1% (w/w of PLGA) [34]. The labeling of NPL was performed by mixing the ethanol solution of DiI with premade NPL nanoparticles at a final concentration of 0.5% (w/w of DPPG) [32]. The DiI loading was confirmed by gel permeation studies on a PD-10 Sephadex G25 desalting column. The nanoparticles were kept in the dark at 4 °C before use. Batches having the same fluorescence intensity at the same concentration of nanoparticles were used for the studies.

5.2.5. Nanoparticle endocytosis

Cells were plated for 3 days in 6-well plates and treated for different exposure times (0.5, 2, 6, and 24h) with 10 µg of NP/DiI. The cells were then analyzed by flow cytometry with a BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA). For confocal microscopy, the cells were plated for 3 days in 8-chamber slides. After three washes with PBS, cells were treated for 2h with 1 µg of different NP/DiI and washed again with PBS. After that, cells were fixed for 10 min with 4% (v/v) paraformaldehyde at 4°C and nuclei were stained with Hoescht 33342. Cells were viewed with a LSM710 Zeiss confocal microscope (Zeiss).

5.2.6. Treatment with endocytosis inhibitors

Cells were pre-incubated for 15 min at 37°C with various endocytosis inhibitors: nystatin (20 µg/ml), filipin (10 µg/ml), chlorpromazine (CPZ 10 µg/ml), phenylarsine oxide (PAO 1 µg/ml), amiloride (0.023 mg/ml), dynasore (26 µg/ml), NaN_3 (600 µg/ml). NPL/DiI were added for 30 min then cells were collected by trypsin and nanoparticle endocytosis was measured by flow cytometry.

5.2.7. In vivo nasal residence studies after nasal administration

Non-anesthetized mice (n=3) received intra-nasally 20 µg (11µl per nostril) of fluorescently-labeled nanoparticles. The five 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 and 48h following administration, and one control mice was also imaged with each treatment group at each time point. Acquisitions and analyses of images thus obtained were performed with the PerkinElmer Living Image software (version 4.2). Mice were maintained under pathogen-free conditions in our animal house. Experiments were carried out in accordance with the guideline for animal experimentation (EU Directive 2010/63/EU) and the protocol was approved by the local ethics committee at Tours University (CEEA VdL).

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Conflict of interest

The authors declare no conflict of interest.

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Supplemental data :

Residence time and uptake of zwitterionic nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles

by Le MQ. et al.

1. Supplemental Material and Methods

1.1. Supplemental material

Rhodamine-DHPE (1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine), hereafter abbreviated as Rhod, was from Life Technologies, France.

1.2. Supplemental Methods

1.2.a. Control of the fluorescence of the DiI-labeled nanoparticles for in vivo *experiments*

Batches of nanoparticles having the same fluorescence intensity at the same concentration were used as described in the material and methods. Additionally, for *in vivo* experiments, drops of 10µL containing serial dilutions of DiI-labeled nanoparticles (from 9µg to 0µg) were loaded onto a glass slide. The fluorescence of the nanoparticles was determined with the In Vivo Imaging System IVIS® Spectrum (PerkinElmer, Waltman, USA).

1.2.b. Stability study of the anionic and cationic DiI-labeled liposomes

DiI-labeled liposomes were synthesized in water and the size, the zeta potential and the fluorescence of anionic and cationic liposomes were analyzed as described in the material and methods section. Immediately after the synthesis batches of liposomes were divided into 2 groups, each receiving an extra volume of 11.11% of either water or 10xPBS followed by homogenization. Each batch were divided again into 2 groups stored either at 4°C or 37°C. The size, the zeta potentials and the fluorescence were analyzed at the specified time points from 0.5 to 24 hours.

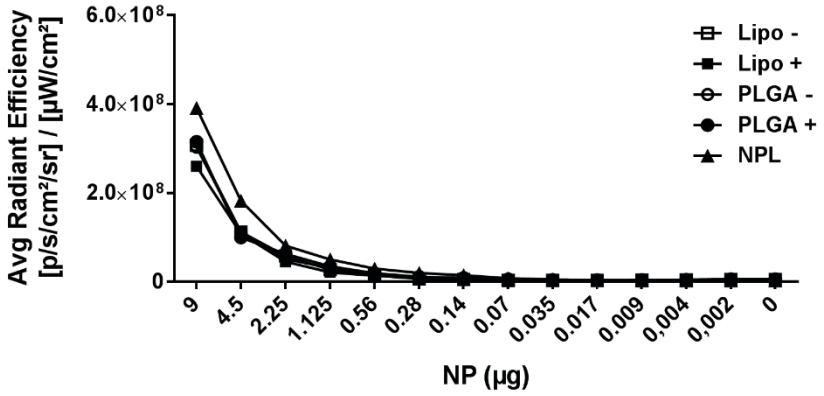
1.2.c Labeling and characterization of the Rhod-labeled nanoparticles

The labeling of the 5 types of nanoparticles with Rhod was performed following the same protocol as for DiI-labeling: the 5 types of nanoparticles were labeled with the Rhod previously diluted in ethanol at 1mg/ml. For the labeling of the cationic and anionic liposomes, Rhod was added in ethanol together with the lipids to a final concentration of 0.1% (w/w of lipid). For the labeling of the cationic and anionic PLGA NP, Rhod was added during nanoparticle synthesis in the organic phase with a final concentration of 0.1% (w/w of PLGA). The labeling of NPL was performed by mixing the ethanol solution of Rhod with premade NPL

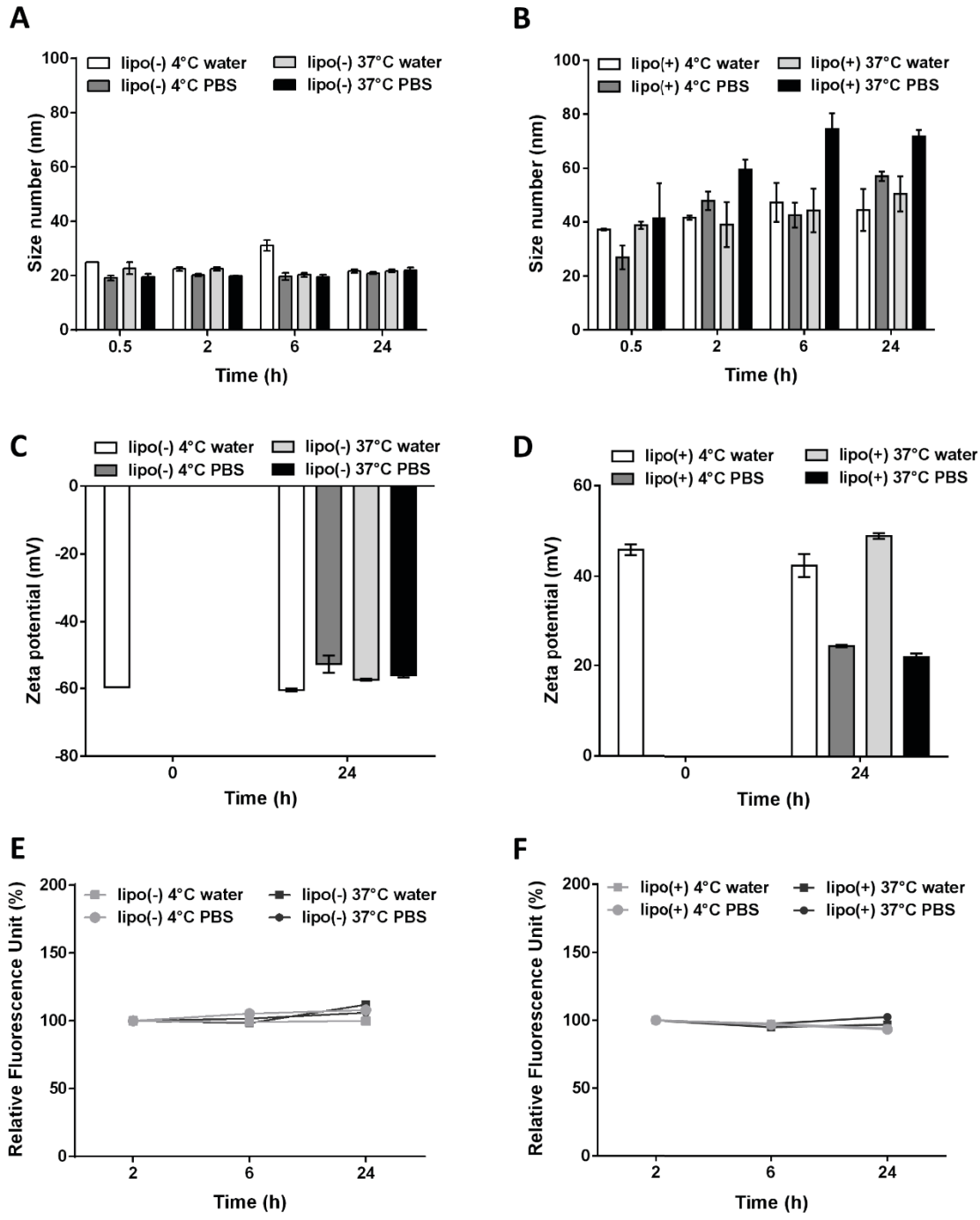
nanoparticles at a final concentration of 0.5% (w/w of DPPG). The Rhod loading was confirmed by gel permeation studies on a PD-10 Sephadex G25 desalting column. The nanoparticles were kept in the dark at 4 °C before use. The size and the zeta potential of all nanoparticles were measured with the zetasizer nanoZS (Malvern Instruments, France) as described in the material and methods. Batches having the same fluorescence intensity at the same concentration of nanoparticles were used for the studies.

1.2.d. Endocytosis of the Rhod-labeled nanoparticles

Cells were plated for 3 days in 6-well plates, as described in the material and methods, and treated for different exposure times (0.5, 2, 6, and 24h) with 10 µg of Rhod-labeled nanoparticles. The cells were then analyzed by flow cytometry with a BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA).

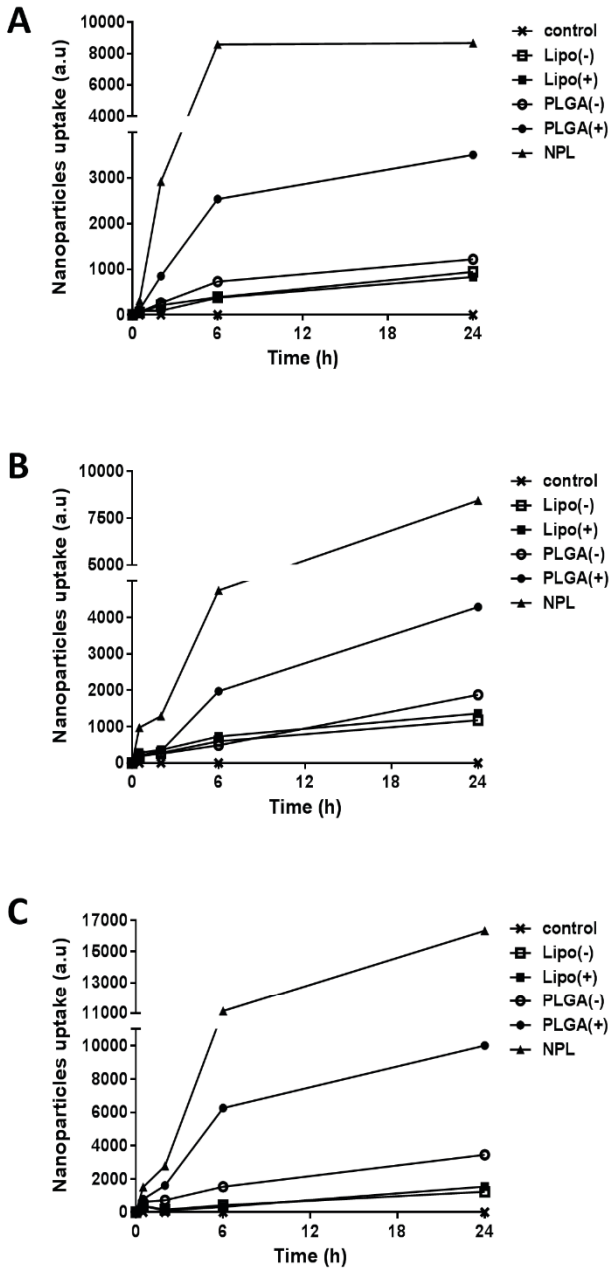


Supplemental Figure 1 : Control of the fluorescence of the DiI-labeled nanoparticles for *in vivo* experiments. Before the *in vivo* experiment, the fluorescence of nanoparticles was analyzed with the In Vivo Imaging System IVIS® Spectrum. The fluorescence of each of the 5 types of nanoparticles were very similar, as shown above. Representative data of 4 experiments. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).



Supplemental Figure 2 : Stability of the liposomes. Liposomes were synthesized in water then water or PBS (final concentration 1x) was added and they were stored either at 4°C or at 37°C for 24h. The size (A-B), the zeta potentials (C-D) and the relative fluorescence (E-F) of anionic (A, C, E) and cationic (B, D, F) liposomes were analyzed. No shrinkage (meaning a size decrease) nor destabilization was observed (A-B). The zeta potential remained consistent with the original charge of the liposomes (C-D) and the fluorescence intensities were constant throughout the experiment (E-F), demonstrating the stability of the liposomes in buffer at 37°c. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL:

zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core).



Supplemental Figure 3 : Endocytosis of Rhod-labeled nanoparticles. The endocytosis of the 5 types of Rhod-labeled nanoparticles were analyzed by flow cytometry in NCI-H292 airway epithelial cells (A), in SRDC dendritic cells (B) and in macrophage-derived THP-1 cells (C). Consistently with the DiI-labeled nanoparticles, liposomes were slowly taken over the 24h experiment, PLGA NP exhibited an intermediary endocytosis profile, while NPL were quickly endocytosed. Lipo(-): anionic liposome, Lipo(+): cationic (chitosan-coated) liposome, PLGA(-): anionic PLGA nanoparticle, PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, NPL:

zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core). Rhod: Rhodamine-DHPE (1,2-Dihexadecanoyl-s

Conclusion

This study demonstrated that among the 5 different nanoparticles, NPL is the best candidate that owns longer nasal residence time and efficiently deliver drugs into airway epithelial cells. Nanocarriers administered intra-nasally may improve the delivery of antigens to immune cells while limiting their mucosal clearance. Other advantages include their ability to protect antigens against enzymatic degradation and the transport across the mucous barrier. In order to select the appropriate antigen delivery systems for intranasal vaccine, different properties such as antigen loading and cell delivery must be evaluated. In the next study, we compared the loading and delivery of antigens with airway mucosa cells (airway epithelial cells, macrophages and dendritic cells). The study was performed on anionic and cationic liposomes, anionic and cationic PLGA and NPL (cationic on their surface and anionic in their porous core).

PUBLICATION 3: ZWITTERIONIC NANOPARTICLES ARE MORE EFFICIENT VECTORS FOR PROTEIN DELIVERY INTO NASAL MUCOSAL CELLS THAN CATIONIC OR ANIONIC NANOPARTICLES

Zwitterionic nanoparticles are more efficient vectors for protein delivery into nasal mucosal cells than cationic or anionic nanoparticles

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Keywords: nanoparticles; zwitterion; nasal; vaccine; protein delivery

ABSTRACT

Different types of biodegradable nanoparticles (NP) have been studied as delivery systems for proteins into nasal mucosal cells, especially for vaccine applications. Such a nanocarrier must have the ability to be loaded with proteins and to transport this payload into mucosal cells. However, comparative data on nanoparticles' capacity for protein loading, efficiency of subsequent endocytosis and the quantity of nanocarriers used are either lacking or contradictory, making comparisons and the choice of a best candidate difficult. Here we compared 5 types of nanoparticles with different surface charge (anionic or cationic) and various inner compositions as potential vectors: the zwitterionic NPL (cationic maltodextrin NP with an anionic lipid core), cationic and anionic PLGA (Poly Lactic co-Glycolic Acid) NP, and cationic and anionic liposomes. We first quantified the protein association efficiency and NPL associated the largest amount of ovalbumin, used as a model protein. *In vitro*, the delivery of fluorescently-labeled ovalbumin into mucosal cells (airway epithelial cells, dendritic cells and macrophages) was assessed by flow cytometry and revealed that the NPL delivered protein

to the greatest extent in all 3 different cell lines. Taken together, these data underlined the potential of the zwitterionic NPL as efficient protein delivery systems to mucosal cells.

1. Introduction

Nasal vaccination is an attractive strategy for the induction of mucosal and systemic immunity [1, 2]. Indeed, most pathogenic infections start at a mucosal surface and the induction of both local and distal mucosal immunity offers the possibility of neutralizing them at their point of entry. However, despite this clear advantage, as well as the practicality of needle-free administration, intranasal vaccination remains challenging owing largely to inefficient uptake and rapid clearance of the antigen.

Nanoparticles (NP) have been tested as delivery systems for nasal vaccines and shown to improve the delivery of antigens to immune cells while limiting their mucosal clearance. Other advantages include their ability to protect antigens against enzymatic degradation and their ability to transport antigens across the mucous barrier [3, 4]. However, while many nanoparticulate delivery systems are described, no direct comparison between available types has so far been published, making it difficult to choose the ideal nanocarrier for this application.

Natural and synthetic biodegradable materials are generally used as components of nanoparticles. Polyester derivatives such as poly lactic-co-glycolic acid (PLGA), lipid-based nanoparticles such as liposomes, and polysaccharides such as chitosan, starch, alginate or dextran are the main candidates under evaluation [4, 5]. Maltodextrin-based nanoparticles (NPL) have been studied as delivery systems of antigens and drugs administered via the nasal route [6]. These zwitterionic NP are made from cationic maltodextrin with an anionic phospholipid core. NPL can deliver antigens to the mucosal cells [7]. After intranasal administration of a NPL-based vaccine, an immune protection was observed after an oral challenge [8, 9]. NPL also increase the nasal residence time of proteins [10]. These studies strongly support the potential for NPL to be an excellent vaccine delivery system. PLGA is composed of two polymers: lactic acid and glycolic acid, linked by ester bonds easily metabolized by the body and auto-degradable. PLGA has been approved by the American Food and Drug Administration and European Medicine Agency (EMA) for human applications [11] and it has been demonstrated that PLGA nanoparticles (PLGA NP) are of interest for vaccines [12-16]. Liposomes are spherical, self-closed structures consisting of one or several phospholipid bi-layers enclosing an aqueous phase [17]. Due to their lipid structure, they are bioavailable and able to entrap both hydrophilic and hydrophobic drugs – in addition, liposomes could have an adjuvant effect [18].

In order to compare different antigen delivery systems, different properties such as antigen loading capacity and cell delivery must be evaluated [19]. In this study, we compared the loading and delivery of antigens using three types of airway mucosal cells (airway epithelial cells, macrophages and dendritic cells). The study was performed on NPL (cationic on their surface and anionic in their porous core), on anionic and cationic PLGA and on anionic and cationic liposomes.

2. Material and methods

2.1. Materials

Maltodextrin was purchased from Roquette (France) while DPPG (1,2-dipalmitoyl-snglycero-3-phosphatidylglycerol) and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) were from Lipoid (Germany). Cell culture media (RPMI 1640 and IMDM), fetal calf serum (FCS), non-essential amino acids, trypsin, L-glutamine, phosphate buffered saline (PBS), NaOH, ethanol, DAPI (4,6-diamidino-2-phenylindole), DiI (1,10-dioctadecyl-3,3,30,30-tetramethylene) and Micro BCA Protein Assay Kit were purchased from ThermoFisher Scientific (France). PLGA (50:50, Acid copolymer : Resomer RG503H) was from Evonik (Germany). Epichlorhydrin (1-chloro-2,3-epoxypropane), GTMA (glycidyl-trimethyl-ammonium chloride), $NaBH_4$, chitosan, PD-10 Sephadex G25 desalting column, ovalbumin (OVA) and Fluorescein-5-isothiocyanate (FITC) were all purchased from Sigma-Aldrich (France).

The human muco-epidermoid bronchiolar carcinoma cell line NCI-H292 (hereafter H292) was supplied by Dr J.M. Lo-Guidice (University of Lille, France). The human monocytic cell line THP-1 was donated by Dr. F. Nesslany (Pasteur Institute of Lille, France). The CD4–CD8α+CD205+CD11b– murine spleen dendritic cell line SRDC line was obtained from Pr. I. Dimier-Poisson (University François-Rabelais of Tours, France)[20].

2.2. Cell culture

The H292 and THP-1 cells were maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL Penicillin, 100 mg/mL streptomycin and 2mM L-glutamine at 37^{o}C in humidified 5% CO_2 atmosphere. SRDC were cultured in IMDM supplemented with 5% heat-inactivated FCS, 100 U/mL Penicillin, 100 mg/mL streptomycin and 2mM L-

glutamine at 37°C in humidified 5% CO_2 atmosphere. Cells were seeded 3 days before treatment at a density of 1.5 x 10^5 cells per well in 8-well glass chamber slides (LabTekII, Thermo Scientific Nunc Lab), or at 5 x 10^5 cells per well in 6-well plates.

2.3. Nanoparticles synthesis

Zwitterionic NPL synthesis

NPL were prepared as described previously [21]. Maltodextrin was dissolved in 2N sodium hydroxide with magnetic stirring at room temperature. To introduce the positive charge, epichlorhydrin and GTMA were added to make cationic polysaccharide gels. The gels were then neutralized with acetic acid and sheared using a high-pressure homogenizer (LM20, Microfluidics, France). The nanoparticles thus obtained were purified by tangential flow ultra-filtration (AKTA flux 6, GE Healthcare, France) using a 750 kDa membrane (GE Healthcare, France) and mixed with DPPG above the gel-to-liquid phase transition temperature to produce NPL.

Cationic and anionic PLGA NP

Anionic PLGA NP were produced by nanoprecipitation at room temperature [22]. The PLGA polymer was dissolved at 10 mg/mL in an acetone/ethanol mixture (85:15) composing the organic phase. Dissolution was performed for 5 minutes under stirring at 150 rpm. Dissolved PLGA was then injected into ultrapure water (aqueous phase) under stirring at 150 rpm. No surfactant was added at any step of the synthesis. Residual organic solvents were eliminated under vacuum evaporation. For cationic PLGA NP, dissolved PLGA was then injected into ultrapure water with 10% of chitosan (w/w of PLGA) (aqueous phase) under stirring at 150 rpm.

Cationic and anionic liposomes

Anionic liposome: 35 mg of lipid (DPPC 80% and DPPG 20%) were dissolved in 2 ml of ethanol. Dissolution was performed for 5 minutes under stirring at 150 rpm. Dissolved lipids were then injected into ultrapure water (aqueous phase) at 80°C under stirring at 150 rpm.

Cationic liposome: 35 mg of DPPC dissolved in ethanol was injected into ultrapure water (aqueous phase) at 80^{o}C under stirring at 150 rpm, chitosan (10w/w of lipid) was added after

10 minutes. No surfactant was added at any step of the synthesis. Residual organic solvents were eliminated under vacuum evaporation.

2.4. Characterization of nanoparticles and formulations

The size (hydrodynamic diameter in Z-average) of nanoparticles and formulations was measured by dynamic light scattering in pure water. The zeta potentials were measured by electrophoretic mobility in pure water. Measurements were carried out in triplicate using a zetasizer nanoZS (Malvern Instruments, France).

2.5. Labeling of ovalbumin

Ovalbumin (OVA) was labeled with FITC according to a previously described protocol [8]. Briefly, FITC was added to proteins solubilized in 0.1 M bicarbonate buffer (pH 9.5) at a ratio of 1/10 (w/w), and the solution was stirred for 6 h in the dark at room temperature. The labeled protein was purified by gel filtration on a PD-10 Sephadex desalting column (Sigma–Aldrich).

2.6. Post-loading of protein onto nanoparticles

The loading of different nanoparticles with OVA was performed by mixing both components in solution at room temperature for 1h at an OVA/NP ratio of 1/10 or 3/10 (w/w).

2.7 Characterization of the association of protein with NP

The protein association of OVA with NP was evaluated using native polyacrylamide gel electrophoresis (native PAGE). Formulations were supplemented with a non-denaturing buffer (Tris–HCL 125 mm (pH 6.8), 10% glycerol and 0.06% bromophenol blue) and run on a 10% acrylamide-bisacrylamide gels stained by the silver nitrate method. Gels were scanned and quantify using the ImageJ software. Under these conditions, non-associated protein enters the gel while NP-associated protein does not.

2.8. Protein delivery by nanoparticles in cells

Cells were plated for 3 days in 6-well plates and treated for different times (0.5, 2, 6, and 24h) with 1µg of OVA-FITC either in a free state, or associated with nanoparticles at a 1/10 (w/w) ratio. The cells were then analyzed by flow cytometry with a BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA).

3. Results

3.1. Fabrication and protein association characterization

The five types of nanoparticles were synthesized and their size and zeta potential were characterized **(Figures 1 and 2)**. The 2 anionic nanoparticles prepared: anionic PLGA NP (PLGA(-)) and anionic liposome (Lipo(-)), exhibited a zeta potential of -23 and -52mV, respectively. Cationic nanoparticles were also prepared by covering nanoparticles with chitosan. PLGA(+) had a zeta potential of +38mV while Lipo(+) had a zeta potential of +25mV. NPL are porous, zwitterionic nanoparticles with a cationic surface and an anionic, porous core – they therefore appeared as cationic nanoparticles with a zeta potential of +45mV. The nanoparticles all possessed a Z-average size ranging from 57 to 161 nm. In addition, all these nanoparticles were spherical in shape [8, 23, 24].

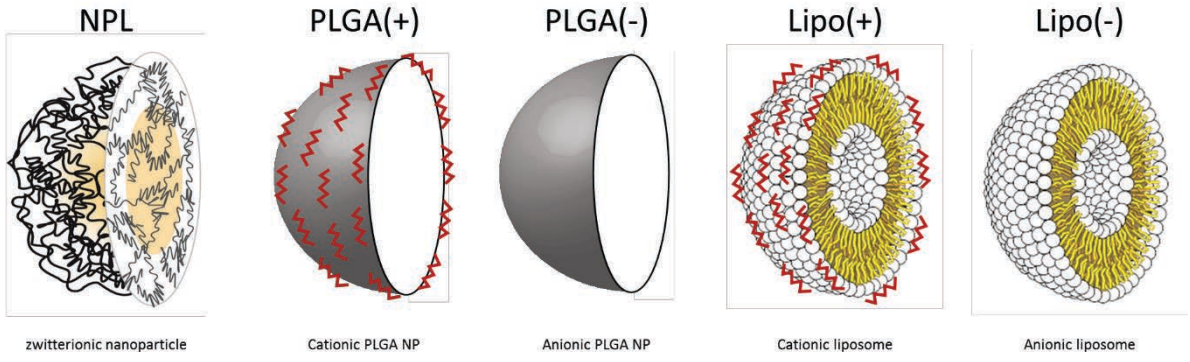

Figure 1. Representation of the 5 types of nanoparticles. Cross sectional view showing the surface and the core of the nanoparticles. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-) : anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome.

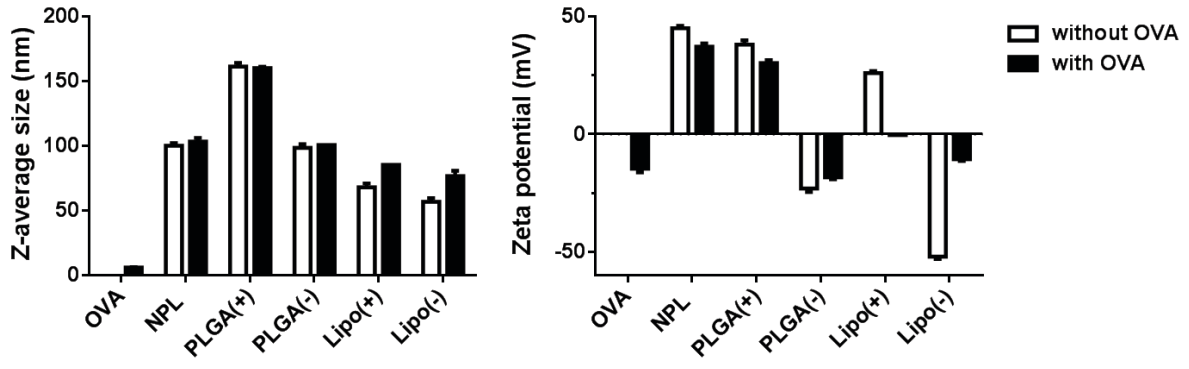

Figure 2. Size and zeta potential of the 5 types of nanoparticles and their formulations with OVA. The nanoparticles' size (left) and the zeta potential (right) of ovalbumin (OVA), the 5 types of nanoparticles and their formulations with OVA at a mass ratio of 1/10 (OVA/NP), were measured by dynamic light scattering and electrophoretic mobility, respectively. The graphs represent the mean +/- SD of three independent measurements. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-): anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome.

The OVA appeared as a 6nm-protein with a negative surface charge of -15mV. The association of OVA to NPL did not change the nanoparticles' size and only a slight decrease (-8mV) of the zeta potential was observed, meaning that proteins were associated inside these porous nanoparticles. The formulations OVA/PLGA(+) and OVA/PLGA(-) showed a slight decrease of their respective surface charges (+30mV and -18mV) and both PLGA formulations retained their size. Finally, the OVA formulations with the liposomes increased their size by around 20nm and drastically modified the zeta potential : 0mV for Lipo(+) and -10mV for Lipo(-).

This suggested an association of OVA with the 5 types of nanoparticles and this was confirmed by native PAGE (**Table 1**). At a mass ratio OVA/NP of 1/10, nanoparticles with a positive surface charge associated 90% of the OVA, and even 100% for NPL. By contrast anionic nanoparticles associated only 10% (Lipo(-)) to 50% (PLGA(-)) of the OVA. To highlight differences in the protein loading capacity of the nanoparticles, particularly the cationic NP, a mass ratio OVA/NP of 3/10 was also examined. NPL still associated 100% of the OVA. The loading reached 70% for PLGA(+) but fell to only 10% for Lipo(+). Regarding the anionic nanoparticles, the loading of OVA with the mass ratio 3/10 was null for Lipo(-) and 20% for PLGA(-).

Hence, the ratio of 1/10 (w/w) of the OVA/NP was used in the following formulation experiments.

Formulations	OVA/NP ratio (w/w)	
	1/10	3/10
OVA/NPL	100%	100%
OVA/PLGA(+)	90%	70%
OVA/PLGA(-)	50%	20%
OVA/Lipo(+)	90%	10%
OVA/Lipo(-)	10%	0%

Table 1. Loading efficacy of OVA with the 5 types of nanoparticles. Native PAGE were run with the OVA/NP formulations at 1/10 and 3/10 mass ratios and non-associated proteins were determined by gel densitometry. The percentages of association with the NP were calculated by comparison with the 100% OVA reading and are reported in the table. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-): anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome.

3.2. Comparison of the protein delivery by nanoparticles in H292 airway epithelial cells

Epithelial tissue covers the outside of the body and lines organs and cavities. The first barriers that nanoparticles face once nasally instilled are primarily the airway epithelial cells and some specialised cells such as microfold (M) cells [25, 26]. We evaluated the delivery of protein by the 5 types of nanoparticles into H292 airway epithelial cells using flow cytometry for 24h [27]. The endocytosis of OVA alone increased for 2h then constantly decreased until 24h, where intracellular OVA was no longer detectable. Nevertheless, even the maximum amount of endocytosed OVA was still low. OVA delivery by NPL was rapid and 14 times more efficient than protein alone at 2h, whereupon the delivery reached a steady-state. The kinetics of protein delivery by PLGA(+) was comparable to those of the PLGA(-) until 6h then a decrease was observed. PLGA(-) showed a similar profile to NPL (an initial and rapid protein delivery within 2h followed by a steady-state), but were only 3 times more efficient than OVA alone. The delivery of OVA by Lipo(+) was firstly as efficient and quick as PLGA NP then decreased after 2h. With Lipo(-), the OVA delivery increased during the first 2h, being roughly half as efficient as Lipo(+), then decreased to reach a similar value to the OVA alone. Finally, at 24h, NPL had delivered OVA 10 times more efficiently than PLGA(+) and Lipo(+) and 4 times more than PLGA(-) while OVA alone or associated with Lipo(-) was not detectable

(**Figure 3**). Together, all the 5 types of nanoparticles delivered OVA into the cells but major differences in the kinetics of the delivery and in its efficiency were highlighted.

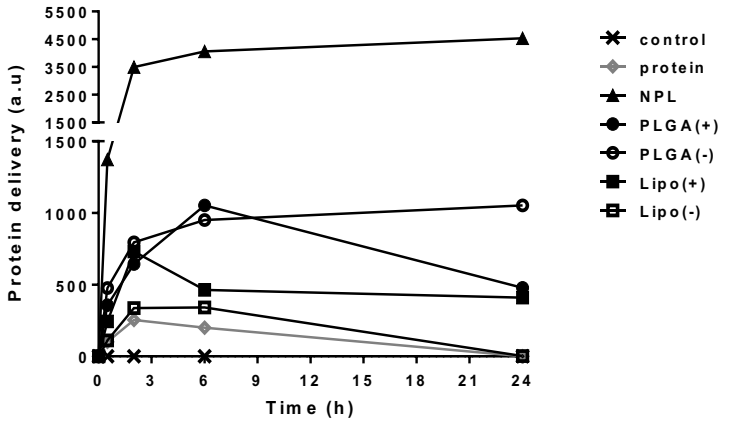

Figure 3. Protein delivery by the 5 types of nanoparticles in H292 airway epithelial cells. Ovalbumin (OVA) was labeled with fluorescein and cells were treated with OVA (protein, grey line) or OVA/NP formulations (1/10 w/w ratio). The kinetics of protein delivery by the 5 types of nanoparticles (or the protein endocytosis for non-formulated OVA) were evaluated by flow cytometry. Representative graph of three independent experiments. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-): anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome, Control : untreated cells.

3.3. Comparison of the protein delivery by nanoparticles in SRDCs dendritic cells

Nasal- or nasopharynx- associated lymphoid tissue (NALT) represents the immune system of nasal mucosa and is a part of mucosa-associated lymphoid tissue. The NALT is specifically enriched in dendritic cells (DCs) [28-30] that pick up the antigens from M-cells, or directly through the epithelial layer, for subsequent antigen presentation and immune response triggering [31]. OVA alone was slowly and weakly endocytosed for 2h before reaching a steady-state. By comparison, the 5 types of nanoparticles showed a rapid initial delivery of OVA into DC during the first 30 min. At 30 min, NPL displayed the most efficient protein delivery (235 times more than OVA) followed by Lipo(-) (x90), Lipo(+) (x60), PLGA(-) (x20) then PLGA(+) (x15). After 30 min, the 5 types of nanoparticles behaved differently: NPL and Lipo(-) reached a steady-state, while protein kept being delivered until 6h for the other NP. At 24h, NPL had delivered OVA 16 times more efficiently than non-associated protein, while the 4 other types of nanoparticle showed an increase limited to x4 for PLGA(-) to x7 for PLGA(+), while liposomes had only intermediary values (**Figure 4**). This comparison meant that NPL was once more the most efficient nanoparticle-based protein delivery system into DC.

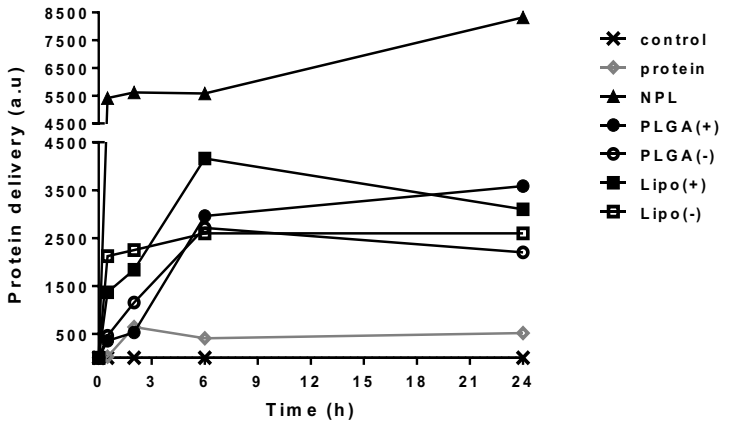

Figure 4. Protein delivery by the 5 types of nanoparticles in SRDC dendritic cells. Ovalbumin (OVA) was labeled with fluorescein and cells were treated with OVA (protein, grey line) or OVA/NP formulations (1/10 w/w ratio). The kinetics of protein delivery by the 5 types of nanoparticles (or the protein endocytosis for non-formulated OVA) were evaluated by flow cytometry. Representative graph of three independent experiments. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-): anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome, Control : untreated cells.

3.4. Comparison of the protein delivery by nanoparticles into THP-1 macrophages

As the presence of macrophages in the airways has been documented [32], we examined the ability of the 5 types of nanoparticles to deliver proteins into macrophages. Compared to the other cell types in this study, the kinetics of protein delivery (and endocytosis of OVA alone) were more homogenous in THP-1, though the rate slightly decreased after 6h. After 30 min, the delivery of OVA by the 5 types of nanoparticles was equivalent to the endocytosis of OVA alone. After 2h, differences appeared and remained constant until 24h, when NPL were 3 times more efficient than OVA alone while the 4 other types of nanoparticles were only by 1.5-1.7 times better at delivering OVA into THP-1 (**Figure 5**). This again demonstrated that NPL was the most efficient nanoparticle in delivering OVA into macrophages.

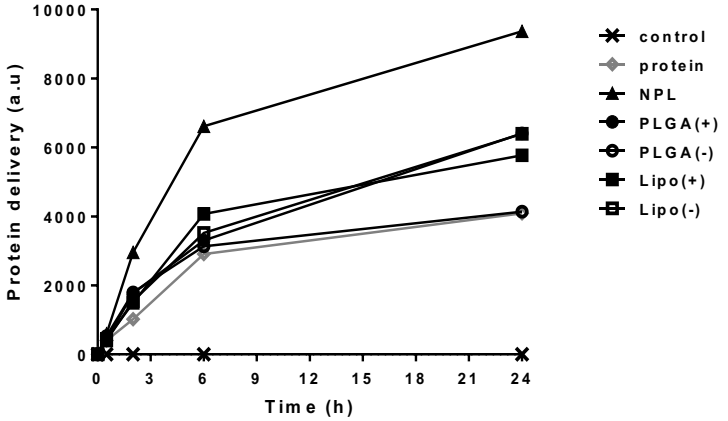

Figure 5. Protein delivery by the 5 types of nanoparticles into THP-1-derived macrophages. Ovalbumin (OVA) was labeled with fluorescein and cells were treated with OVA (protein, grey line) or OVA/NP formulations (1/10 w/w ratio). The kinetics of protein delivery by the 5 types of nanoparticles (or the protein endocytosis for non-formulated OVA) were evaluated by flow cytometry. Representative graph of three independent experiments. NPL: zwitterionic nanoparticle (cationic maltodextrin-based porous nanoparticle with anionic lipid core), PLGA(+): cationic (chitosan-coated) PLGA nanoparticle, PLGA(-): anionic PLGA nanoparticle, Lipo(+): cationic (chitosan-coated) liposome, Lipo(-): anionic liposome, Control : untreated cells.

4. Discussion

The vast majority of pathogens invade at the mucosal surfaces in the body and mucosal vaccination is a promising strategy for the induction of protective, mucosal-specific immunity. The nasal route is advantageous because of the presence of the relatively accessible nasal associated lymphoid tissue (NALT). However, nasally administered antigens are quickly cleared from nasal mucosal surfaces and delivery systems that protect antigens from this rapid clearance are needed [2, 33]. The challenge is developing a delivery system that protects antigens from clearance and degradation, and improves their delivery to cells [34]. An increasing number of nanocarriers have been used in nasal vaccine applications such as micelles, liposomes, polysaccharide nanoparticles and PLGA nanoparticles [5]. Nonetheless, no comparison of the vaccine efficiency of these nanocarriers has yet been published.

We previously compared the *in vivo* nasal residence time and the endocytosis of 5 types of nanoparticles: the zwitterionic NPL, cationic and anionic PLGA NP, cationic and anionic liposomes, and showed that zwitterionic NPL had the longest residence time and were the most efficiently endocytosed by the 3 cell lines used in this study (*Residence time and uptake of*

zwitterionic nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles. Le et al., submitted publication). Here we compared the antigen loading and the cellular delivery efficacy of these 5 types of nanoparticles, using OVA as a model protein.

Many studies have evaluated the protein loading efficiency of nanocarriers as a priority condition for their use as a vaccine delivery system. Accordingly, we compared the protein loading efficacy of the 5 types of NP. Only the zwitterionic NPL showed 100% of protein loading at mass ratios of 1/10 and 3/10 (OVA/NP). PLGA NP and liposomes did not reach this level of protein loading [35-37] (**Figure 1 and Table 1**). This is likely due to the structure of the nanoparticles: NPL are porous and the surface of interaction with protein is larger than non-porous nanoparticles like PLGA NP and liposomes where protein interactions are limited to the nanoparticles' surface. With liposomes, we observed an increase of the size and a decrease of the zeta potential that approached the zeta potential of the free protein, clearly demonstrating an association of OVA with these NPs at their surface. This association reached saturation since increasing the OVA/NP ratio led to a decrease of efficiency (**Table 1**). To increase the protein loading, OVA should have been entrapped in the liposomes as previously described but the loading efficiency remains low [35, 38] and efforts to increase the protein loading efficiency lead to size heterogeneity incompatible with pharmaceutical applications [39]. With PLGA NP, we did not observe large modifications of the size and the zeta potential when associated with proteins. However, PLGA NP are spherical particles [24] and it is likely that the proteins only interact with the nanoparticles' surface. Indeed, the insertion of a protein during the synthesis of PLGA NP might denature its native form [40, 41]. Therefore, the post-loading method is the preferred process to associate proteins to PLGA NP.

We then evaluated the protein delivery in 3 cell lines representative of the airway mucosal tissue: epithelial cells, dendritic cells and macrophages. To keep consistency, the same amount of nanocarriers was used all along the comparison. In these cell lines, all of the 5 types of nanoparticles delivered the OVA protein. However, the zwitterionic NPL was much more efficient in this regard than the 4 other types of nanoparticles tested. The difference appeared in the first 30 minutes for epithelial and dendritic cells and after only 2h in macrophages. This implies that using NPL for protein delivery will not require an overly long exposure time of the nanocarrier with cells or tissues (*Residence time and uptake of zwitterionic nanoparticles in the nasal mucosa: comparison with anionic and cationic nanoparticles. Le et al., submitted publication)*[8, 10].

The kinetics of protein delivery by the 5 types of nanoparticles varied between the cell types, and especially in macrophages. In these cells, one cannot preclude that non-specific phagocytosis and pinocytosis occurred in combination with endocytosis, leading to a constant, non-saturated protein delivery over 24h. This is supported by the protein delivery by PLGA NP and the liposomes that were very similar to the protein endocytosis (1.5 time increase).

To our knowledge, this is the first study to compare several types of nanocarriers currently under investigation for the delivery of proteins in airways. We have previously shown that intranasal administration of a total extract of *Toxoplasma.gondii* loaded into NPL afforded protection against this parasite by oral challenge in both chronical and congenital contexts [8, 9], and that NPL also increased the nasal residence time of proteins [10]. Taken together with the current study, these data underline the potential for NPL as a nanocarrier for the delivery of nasally-administered vaccines.

Conclusion

The development of a nasally-administered, nanoparticle-based vaccine requires nanocarriers capable of being loaded with antigens and that they enter the mucosal cells in order to deliver these antigens into cells. Among the 5 types of nanoparticles tested here, our comparison demonstrates that zwitterionic, porous NPL nanoparticles are the best candidate for protein loading and protein delivery. Studies should now be performed *in vivo*, taking appropriate account of the specific biological environment.

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Conflict of interest

The authors declare no conflict of interest.

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Comments

Cationic porous maltodextrin based-NPs (NPL) have received particular interest for the delivery of macromolecules via nasal route. In previous studies, we have already demonstrated that amongst the 5 nanoparticles tested, NPL owns the longest nasal residence time, higher capacity of cellular uptake and protein delivery so this delivery system was used as nano delivery system to develop an intranasal influenza vaccine.

In the next study, we evaluated the ability of nanoparticle-base influenza vaccine to protect animals from transmission of the virus to naïve animals.

The immunogenicity of intranasal vaccine formulations made of split Udorn virus alone or admixed with the nanoparticles NPL and/or CTA1-DD (a demonstrated mucosal adjuvant) was evaluated in mice. The viral antigen loading and delivery by NPL were performed in nasal mucosal cell models. Moreover, the immunogenicity and the inhibition of the virus transmission protective efficacy were examined.

PUBLICATION 4: ZWITTERIONIC NANOPARTICLES CARRYING CTA1-DD ADJUVANT INDUCE PROTECTION FROM VIRUS INFECTION AND TRANSMISSION

Zwitterionic nanoparticles carrying CTA1-DD adjuvant induce protection from virus infection and transmisison

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Abstract

Influenza vaccines administered intramuscularly exhibit poor mucosal immune responses at the respiratory mucosa which is the prime site of the infection. Intranasal vaccination is a potential another route for vaccine delivery which has been demonstrated as effectively inducing protective immune responses in both systemic and mucosal compartments. The major influenza vaccines are based on subunit/split influenza vaccines which is relatively low immunogenicity and thus required proper adjuvant and/or delivery vehicle for immunogenicity enhancement. Herein we demonstrated a stronger protection against influenza virus by split

Udorn virus co-delivered intranasally with zwitterionic nanoparticles (NPL) carrying the mucosal adjuvant, CTA1-DD. NPL were firstly evaluated for their ability to load and deliver antigens into nasal mucosa cells. Data showed that at mass ratio 1:5, all the antigens were loaded into NPL. Moreover, NPL significantly enhanced virus delivery into three different cell lines, airway epithelial cells, macrophages and dendritic cells. Compared to the flu virus vaccination, intranasal vaccination with NPL associated split Udorn was slightly enhanced the specific humoral immune response thus decreased the virus infection and the addition of CTA1-DD greatly improve this effect. Furthermore, mice received NPL carrying split Udorn and/or CTA1-DD did not spread virus to naïve mice. These results demonstrate an efficient intranasal vaccine delivery system utilizing a combination of a porous NPL and mucosal adjuvant CTA1-DD to achieve a robust influenza virus protection.

1. Introduction

Influenza is a highly contagious devastating respiratory disease which causes public health and socio-economic problems worldwide [1]. Intranasal route for influenza vaccine administration has become promising strategy. This route is needle-free and painless. Moreover, antigens activated the immune cells at the nasal-associated lymphoid tissue homing at the respiratory immune effector sites including larynx- and bronchus-associated lymphoid tissues [2] that exert their defensive activities at the prime sites of influenza virus infection. Furthermore, the vaccine antigens applied intranasally may also reach the blood circulation and the systemic immune response is stimulated in spleen and peripheral lymph nodes [3]. Thus, both mucosal and systemic immune responses can be expected after the intranasal immunization of appropriate vaccine formulation [4].

Currently available influenza vaccines are made of either inactivated whole viruses or the virus subunit/split products [5]. The inactivated vaccine is highly immunogenic but often cause adverse reactions particularly in infants and children [6] while the subunit/split vaccines confer limited immunogenicity and require either high antigenic dose or a booster dose [7, 8]. Cholera toxin (CT) bind to ganglioside receptors present on enterocytes, DCs, macrophages as well as B and T-lymphocytes [9, 10]. It can induce antigen-specific sIgA and serum IgG antibody responses and when co-administered with a protein antigen it can potentially enhance the mucosal and serum antibody response to induce long-term memory to the antigen. Due to its high degree of toxicity, CT is not considered as a useful adjuvant although efforts have been to limit its toxicity [11]. Consequently, non-toxic derivative of CT, termed CTA1 was developed [12] and to improve its adjuvant property, 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 Fc and Fab fragments of immunoglobulin present on B cells, thereby allowing binding of the complex CTA1-DD to the B-cell receptors [12-14]. CTA1-DD has adjuvant effects comparable to those of CT, and it enhances a wide range of immune responses following intranasal immunizations, including antibody, CD4+ T cell and cytotoxic CD8+ T cell responses [15]. Candidate vaccines containing CTA1-DD have been shown to enhance immunity to HIV-1, Chlamydia trachomatis, H. pylori, M. tuberculosis, rotavirus and influenza A virus [16].

Mucoadhesive nanodelivery systems created from starch, hyaluronic acid and chitosan offer a significant potential for the development of mucosally administered antigens [17-19]. Such carriers can be designed to prolong their residence time in the nasal cavity [20-22] to protect

entrapped antigens against degradation, to enhance uptake by M-cells, and to target the antigens more specifically to antigen presenting cells (APCs). Zwitterionic porous maltodextrin based-NPs (NPL) have received particular interest for the delivery of macromolecules via nasal route. Data showed that NPL increased the nasal residence time of a carried protein in the first layer of the nasal mucosa cells after nasal administration [22]. Furthermore, zwitterionic NPL has been successfully used as a nanocarrier for mucosal vaccines [19, 23].

In this study, we evaluated immunogenicity of intranasal vaccine formulations comprised of split Udorn virus alone or admixed with the nanoparticles NPL and/or CTA1-DD (a demonstrated mucosal adjuvant) [24]. The viral antigen loading and delivery by NPL were performed in nasal mucosal cell models. Moreover, the immunogenicity and the inhibition of the virus transmission protective efficacy in a mouse model were examined.

2. Materials and Methods

2.1. Animals

Six to eight weeks old BALB/c and DBA/2J mice were purchased from Janvier Laboratories (Strasbourg, France). Mice were kept under specific-pathogen-free conditions in the local animal facility. All experiments were performed in accordance with the guidelines of the Federation for Laboratory Animal Science Associations (FELASA) and the national animal welfare body. Experiments were in compliance with the German animal protection law and were approved by the local animal welfare committee.

2.2. NPL synthesis

NPL were prepared as described previously [25]. Maltodextrin was dissolved in 2N sodium hydroxide with magnetic stirring at room temperature. To introduce the positive charge, epichlorhydrin and GTMA were added to make cationic polysaccharide gels. The gels were then neutralized with acetic acid and sheared with a high-pressure homogenisor (LM 20, Microfluidics, France). The nanoparticles thus obtained were purified by tangential flow ultra-filtration (AKTA flux 6, GE Healthcare, France) using a 750 kDa membrane (GE Healthcare, France) and mixed with DPPG above the gel-to-liquid phase transition temperature to produce NPL.

2.3. Virus preparation

Udorn virus were inactivated following incubating with β-propiolactone (β-BPL) for 16 hours at 4 ℃, then subsequently incubated at 37℃ for 2 hours to facilitate hydrolysis of β-BPL.

Inactivated virus were stored at -80°C. To split Udorn virus, the virus was incubated with 10% Trixton X-100.

2.4. Protein labeling and loading into nanoparticles

The split Udorn virus was covalently labeled with FITC according to the following protocol: FITC was added to split Udorn virus, solubilized in 0.1 M bicarbonate buffer (pH 8.3), and the solution was mixed for 6 h in the dark at room temperature. The labeled virus was purified by gel filtration on a PD-10 Sephadex column. The concentration of the labeled protein was then evaluated using the Micro BCA Protein Assay Kit following the supplier's instructions. Proteins were post-loaded with pre-made sterile NPL by mixing both components at RT for 1 h obtaining 1:5 (w/w) protein:NPL formulations.

2.5. Characterization of the nanoparticle and formulations

The hydrodynamic diameter (Z-average) of nanoparticles was measured by dynamic light scattering in pure water. The zeta potential was measured by electrophoretic mobility in pure water. Measurements were carried out in triplicate using a zetasizer nanoZS (Malvern Instruments, France).

2.6. Characterization of the association of proteins/split virus to NPL

The protein association of split Udorn virud to NP was evaluated using native polyacrylamide gel electrophoresis (native PAGE). Formulations were supplemented with a non-denaturing buffer (Tris–HCL 125 mm (pH 6.8), 10% glycerol and 0.06% bromophenol blue) and run on a 10% acrylamide-bisacrylamide gels stained by the silver nitrate method. Gels were scanned and quantify using the ImageJ software. In these conditions non-associated proteins enter the gel while NP-associated proteins do not.

2.7. Split Udorn delivery by NPL

H292 and THP-1 cells were maintained in RPMI supplemented with 10 % heat inactivated foetal calf serum (FCS), 100 U/mL Penicillin, 100 mg/mL streptomycine and 1% L-glutamine at 37^{o}C in humidified 5% CO2 atmosphere. SRDC were cultured in IMDM supplemented with 5% heat inactivated FCS, penicillin (100 U/ml)/streptomycin (100 mg/ml), L-glutamine (2 mM) at 37°C in humidified 5% CO_2 atmosphere. Cells were seeded 3 days before treatment at a density of 1.5 x 10^5 cells per well in 8-well glass chamber slides (LabTekII, Thermo Scientific Nunc Lab) or 5 x 10^5 cells per well in 6-well plates.

Cells were plated for 3 days in 6-well plates and treated for 3 h with 1µg of split Udorn-FITC either free or associated to nanoparticles at a 1/5 ratio (w/w). The cells were then collected by trypsin analyzed with flow cytometry with a BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA).

2.8. Vaccination of mice

Six to eight weeks old female BALB/c mice (purchased from Janvier Labs) were immunized with 10 µl (5 µl per nostril) containg 1 µg of split Udorn alone or admixed with the nanoparticles NPL and/or CTA1-DD by intranasal route (i.n.) for three times, 10 days apart. Ten days after the first and the second boost immunizations, serum samples were collected for measuring the levels of anti-HA specific antibody by ELISA.

2.9. Detection of anti-Udorn antibodies in serum

To determine Udorn-specific antibody titers, microtiter plates (MaxiSorp, Nunc) were coated with split Udorn virus, and incubated overnight at 4°C. After washing, the plates were blocked for 2h with 5% BSA in PBS. After blocking, a series of 1/3 dilutions of the different serum samples were loaded on the peptide-coated plates. The bound antibodies were measured with a horseradish peroxidase-labeled antibody directed against mouse IgG diluted 1/2000 (Thermo Fisher Scientific, USA). After washing, the microtiter plates were incubated for 20 min with TMB substrate (Tetramethylbenzidine, Life technologies). The reaction was stopped by adding 0.5M H_2SO_4 and absorbance was measured at 450 nm. The endpoint titers are defined as the highest dilution resulting in an O.D. value two-fold that of background (pre-immune serum).

2.10. Virus transmission study

Six to eight weeks old female BALB/c mice were immunized with 10µl containing 1 µg of split Udorn, split Udorn/NPL, split Udorn/CTA1-DD/NPL and OVA/NPL by intranasal route for three times 10 days apart. Five weeks after the second immunization all immunized BALB/c mice were infected intranasally with 20 µl of $3x10^4$ PFU H3N2 Udorn virus (A/Udorn/307/1972 (H3N2)) and mice were used as index animals. At 24 h post infection index mice were one to one co-housed with uninfected 6-weeks-old DBA/2 mice as contact mice in a clean cage. The snouts and the lungs of the index and contact mice were collected on day 4 post co-housing and viral loads were determined by viral titration assay.

2.11. Viral titration

MDCK cells were maintained in DMEM (Sigma Aldrich), 10% FCS (Biochrom KG, Germany) medium, kept at 37 °C in 5% CO_2 and passaged twice weekly using trypsin (Sigma Aldrich, Sweden). MDCK cells were grown to a confluent cell layer in 12-well plates for plaque assay. Snouts and lungs were homogenized in 800 µl cold PBS using FastPrep® spheres, tubes and homogenizer for 3 times at 6.5 m/s for 20s. Afterwards samples were centrifuged for 10 min at 9,000 rpm at 4°C and supernatants were performed a 10-fold dilution in OptiMEM (Thermo Fisher Scientific) supplemented with 0.3% BSA. A volume of 450 µl diluted samples were added on confluent MDCK cells for 2h at RT. After removing the inoculum, cells were overlaid with 1.5% Avicel (FMC BioPolymer) in DMEM supplemented with 10% BSA for 72h. Cells were then fixed with 3.7 % formaldehyde and stained with 1% crystal violet solution for 15 min. Plaques were used to calculate virus titers which is measured in plaque-forming units (pfu) per ml.

2.12. Statistics

Data were analyzed by one-way and two-way ANOVA using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA).

RESULTS

1. Characterization of NPL and formulations

NPL is about 38 nm in size while split Udorn is 111 nm. The combination of the split Udorn with NPL at mass ratio 1:5 created the formulation with the size as the NPL alone. The binding of protein antigen was further confirmed by zeta potential. NPL carried positive charge, about 38 mV and split Udorn owned the negative charged (-20 mV). Interestingly, the antigen associated with nanoparticles exhibited a positive charge implying that split Udorn was sucessfully absorbed inside the NPL **(Figure 1**).

We also loaded NPL with the split Udorn and CTA1-DD. The split Udorn/CTA1-DD/NPL (mass ratio 1:5) showed a size as small as NLP. Lower or decreasing PDI after incorporation protein antigen with NPL emphasized the homogenous of the formulation solutions due to absorbance protein antigen inside NPL. Moreover, the zeta potential remianed positive after combined antigens into NPL confirmed the sucessful loading **(Figure 1).**

In addition to size and zeta study, native PAGE was performed to verify the loading of protein antigens into NPL. Split Udorn was completely associated into NPL as no free protein was observed while the combination of two different protein antigens into NPL resulted in 90% of loading efficacy. Data strongly demonstrated that all protein antigens were associated into NPL at mass ratio 1:5 of the formulation **(Figure 2 and Table 1).**

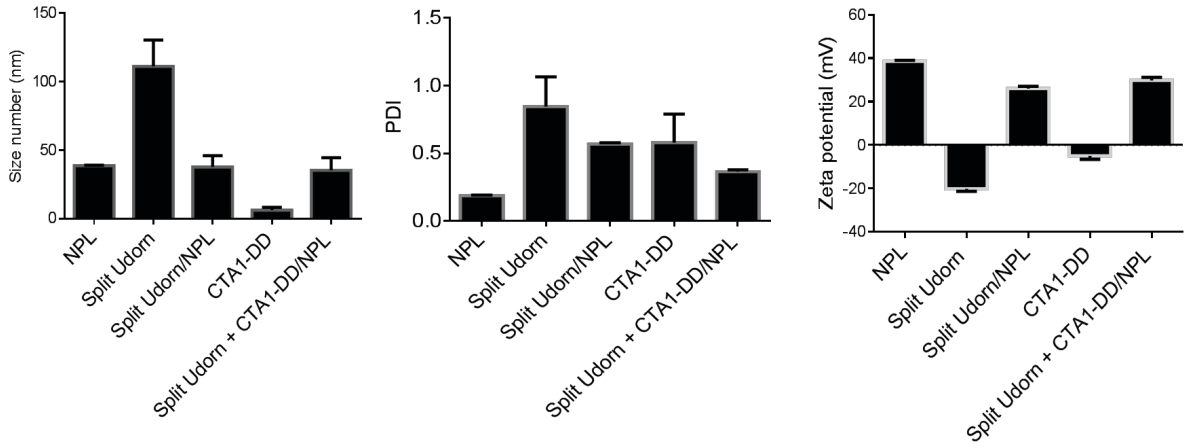


Figure 1: **Size, PDI and zeta potential of NPL, split Udorn, CTA1-DD and the related formulations**. The size (left), PDI (middle) and the zeta potential (right) of NPL, split Udorn, CTA1-DD, split Udorn/NPL, split Udorn/CTA1-DD/NPL were measured by dynamic light scattering and electrophoretic mobility, respectively. The graphs represent the mean +/- SD of three independent measurements.

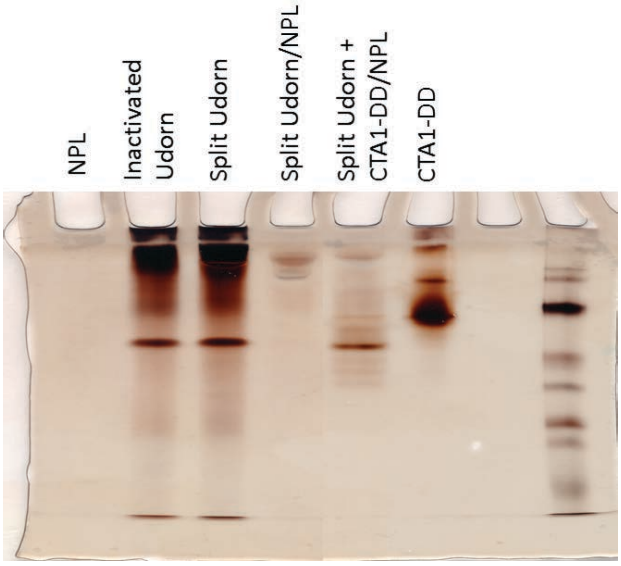


Figure 2: **Characterization of the split Udorn/NPL and split Udorn/CTA1-DD/NPL.** The association of antigens into NPL was characterized by native polyacrylamide gel (PAGE) electrophoresis using formulation at 1:5 (w:w) ratios. Unbound proteins were revealed by silver nitrate staining.

Formulations	Percentage of loading
Split Udorn /NPL	100%
Split Udorn + CTA1-DD/NPL	90%

Table 1: **Loading efficacy of split Udorn and split Udorn/CTA1-DD with NPL.** The percentages of association to the NP were calculated over the 100% protein alone input and reported on the table.

3. Cellular delivery split Udorn by NPL

We investigated the delivery of split Udorn by NPL into 3 different cell lines that dominated in nasal mucosa: airway epithelial cells, macrophage and dendritic cells. Data showed that associated split Udorn virus into NPL significantly increased the uptake of split Udorn by H292 airway epithelial cells, THP-1 macrophage and SRDC dendritic cells. The deliverywas increased 4 times in H292 and SRDC while it was 5 times in the case of THP-1 macrophages **(Figure 3).**

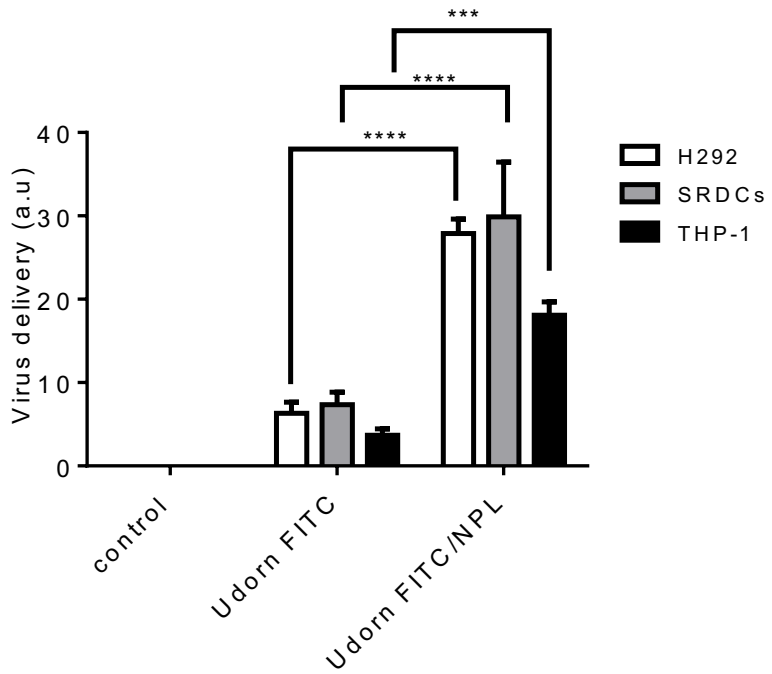


Figure 3: **Split Udorn delivery by NPL in H292 airway epithelial cells, SRDC dendritic cell and THP-1 macrophage**. Split Udorn was labeled with fluorescein and cells were treated with split Udorn or split Udorn/NPL (1/5 mass ratio). The protein delivery by NPL were evaluated by flow cytometry after 3 h of incubation. Statistical analysis: Two-way ANOVA; asterisks indicate p-values: $p < 0.0001$ (****) and $p<0.001$ (***).

4. Udorn-specific antibody titers after intranasal vaccination

To investigate the suitability of vaccines for intranasal vaccination, we compared the serum responses of mice after intranasal vaccination. Mice were immunized intranasally with split Udorn alone, split Udorn loaded NPL, or split Udorn loaded NPL conjugated with CTA1-DD. The levels of HA-specific serum IgG of mice vaccinated with different formulations were shown in **Figure 4**. The Udorn-specific IgG antibody titers in sera of immunized mice with were significantly increased after 2 booster immunizations except the negative control OVA/NPL. A mixture of split Udorn and NPL greatly enhanced the Udorn-specific IgG antibody titers in sera of mice as compared to the OVA/NPL group of control. Moreover, split Udorn/NPL have a trend to enhance the secretion of IgG levels as compared to mice vaccinated with split Udorn. Importantly, the second booster induced a further raise in the IgG levels of mice vaccinated with split Udorn/CTA1-DD/NPL than those achieved split Udorn. Altogether, these results underlined the immunostimulating effect of NPL and CTA1-DD upon intranasal administration.

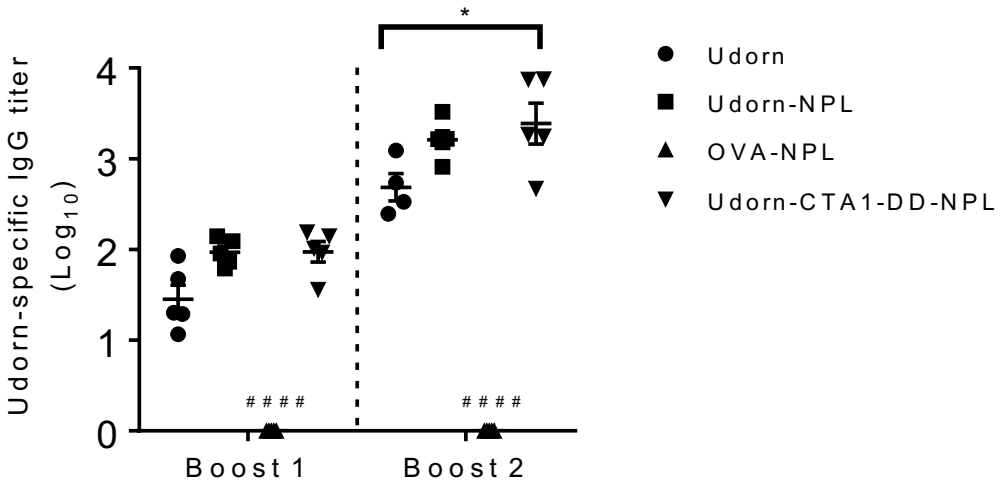


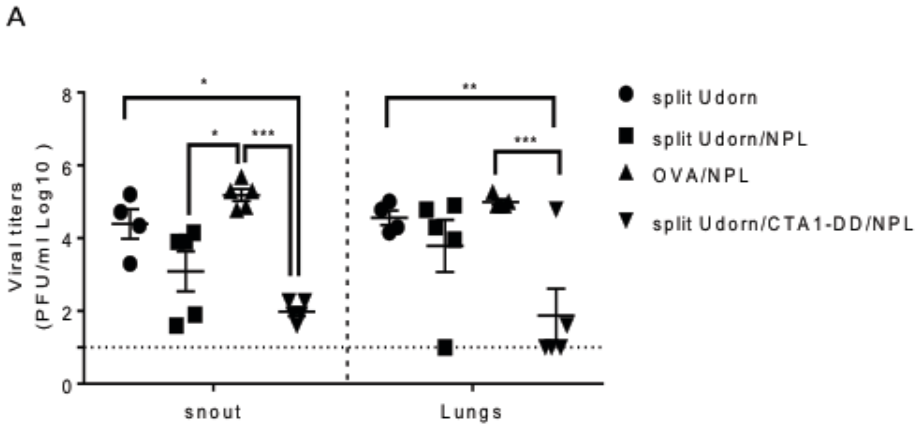
Figure 4: **Serum Udorn specific IgG titers from vaccinated immunized mice**. Udorn specific IgG titers were measured by ELISA assay in serum from mice immunized i.n. with split Udorn or split Udorn/NPL or OVA/NPL or split Udorn/CTA1-DD/NPL. Statistical analysis: Two-way ANOVA; asterisks indicate p-values: $p<0.05$ (*). The hash indicate difference between OVA/NPL to other groups; p-values: $p<0.0001$ (####).

5. Protection against virus transmission

The snout and lung are the local microenvironment in which primary immune protection against respiratory pathogens such as influenza virus takes place. For this reason, we determined whether intranasal administration of all vaccines was sufficient to induce mucosal immunity and protection against influenza virus infection. We challenged immunized mice intranasally with $3*10^4$ PFU/animal of H3N2 Udorn virus 2 weeks after the last of three immunizations. Snout and lung viral titers were determined 4 days after challenge. Virus transmission was assessed by monitoring the influenza virus titres in the snouts and lungs of both index and contact mice.

Mice immunized with split Udorn/NPL or split Udorn/CTA1-DD/NPL exhibited significantly lower viral titers in the snout as compared to OVA/NP group. We also found more reduced viral titer in the snouts of the contact mice co-housed with index mice immunized with split Udorn/NPL. However, protection against infection in the index mice was not significant different between split Udorn alone and split Udorn/NPL. Association of CTA1-DD to the formulation split Udorn/NPL significantly enhanced the virus clearance in both snouts and lungs of index mice. In contrast, immunization with split Udorn was not likely to block virus infection **(Figure. 5a).**

To determine whether the reduction of viral titer in index mice by split Udorn/NPL with or without CTA1-DD could limit virus transmission, index mice were co-housed with contact or naïve mice. Though mice vaccinated with split Udorn were poorly protected from virus infection, they were protected from virus transmission. Importantly, mice receiving split Udorn/NPL with or without CTA1-DD showed 100% of virus transmission prevention **(Figure. 5b).**



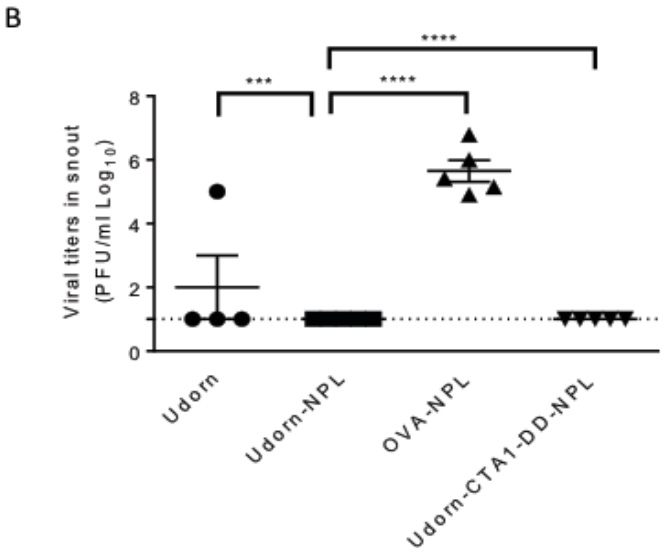


Figure 5: Viral transmission from vaccinated immunized index mice. A) The viral titers in index mice's snout and lungs B) The viral titers in contact mice's snout. Statistical analysis: One-way or two-way ANOVA; asterisks indicate p-values: p<000.1 (****), p<0.001, p < 0.01 (**) and p<0.05 (*).

Discussion

Zwitterionic nanoparticles (NPL) are composed of a cationic maltodextrin matrix enclosing lipid in their core. NPL are highly endocytosed by airway epithelial cells and significantly improve protein delivery in cells [22]. Furthermore, NPL based vaccine protects animals against chronic & congenital infection [19, 23]. Providing evidence of the potential for NPL to be excellent nanoparticle-associated vaccines against a wide range of pathogens. CTA1-DD has been used for mucosal vaccines [16]. CTA1-DD was originally developed to target the B cell receptor, but its adjuvant effect also depends on DCs and can enhance the priming of CD4+ T cells in a complement-independent manner [24, 26]. This study aimed to evaluate the combined effect of NPL and CTA1-DD as vaccine delivery system and mucosal adjuvant for influenza vaccine respectively.

The positive charged and hydrophobic of NPL facilitate electrostatic interactions between NPL and the negatively charged antigen. Moreover, their porous structure allow NPL to be loaded with high amount of antigens [19]. The antigen of split Udorn and CTA1-DD used in our study were shown to remain largely associated with the NPL showing through Native PAGE (Fig 1

and 2, Table 1). Importantly, NPL can very efficiently delivered split Udorn in 3 main cell lines of the nasal mucosa (Fig 3). The improving uptake and processing of the encapsulated antigen by APCs make NPL an ideal nano delivery systems.

In vivo study showed that intranasal administration of a split Udorn/NPL slightly enhance Udorn specific IgG antibody response as compared to the mice received split Udorn alone. Adding CTA1-DD into the split Udorn/NP formulation significantly improve the level of Udorn specific IgG to level greater than those achieved with split Udorn alone.

In the case of infections with respiratory viruses such as influenza, particular attention should be paid to the snout and lung as the targets for mucosal immunization because they are the entry points for respiratory viruses [27]. In this study, we compared our influenza vaccines in term of virus inhibition through viral titer in snout and lungs of infected mice. Correlated with the secretion of Udorn specific IgG, mice receiving split Udorn/NPL showed a trend to decrease virus replication in snouts and lungs of index mice while those achieved split Udorn seem could not be exhibited the protection from virus infection. Additional effect was found when CTA1-DD was employed. Our data demonstrated that the using an adjuvanted nano delivery system (CTA1-DD + NPL) evoked strong effect in virus clearance in snout and lungs of infected mice. While mice immunized with split Udorn could not completely inhibit virus spread, mice receiving split Udorn/NPL showed 100% block virus transmission. It is not surprising to observe mice achieved split Udorn/CTA1-DD/NPL completely blocked the transmission of Udorn virus to contact mice. These results demonstrated that even though the nanocarrier can enhance the vaccine efficacy, it is not sufficient to induce strongest immune response. Using an adjuvant is necessary to create significant protection against the influenza virus. It was demonstrated that recombinant HA nanoparticle-based influenza vaccine required a saponin-based adjuvant in order to induce protective responses of intranasal vaccine against influenza A(H3N2) strains [28]. Polyethylene glycol (PEG) modified liposomes containing CRX-601 adjuvant and methylglycol chitosan increased the murine sublingual immune response to influenza vaccination [29]. These studies demonstrated the interest of combination of a nanodelivery system and an adjuvant in order to improve influenza vaccine efficacy.

Conclusion

Intranasal administration NPL and/or CTA1-DD co-delivery with split Udorn enhanced immune response and virus inhibition capacity, compared to intranasal administration of

soluble (conventional) influenza vaccine. Taken together, our study that NPL carrying CTA1-DD and split Udorn could be a promising intranasal influenza vaccine.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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PART III: DISCUSSION

Nasal vaccination is an attractive strategy for the induction of mucosal-specific immunity (Ogra, Faden, and Welliver 2001; Zaman, Chandrudu, and Toth 2013). Nanoparticles have been proposed as delivery systems for vaccine applications (Bernocchi, Carpentier, and Betbeder 2017; Zhao et al. 2014). Natural and synthetic biodegradable materials are used as carriers of proteins, peptides and nucleotides based vaccines. The nanomaterials used in nasal vaccine delivery are mainly polyesters derivatives such as poly (lactic-co-glycolic acid; PLGA), polysaccharides such as chitosan, starch, alginate and dextran, lipid based nanoparticles such as liposome (Bernocchi, Carpentier, and Betbeder 2017; Marasini, Skwarczynski, and Toth 2017).

Influenza virus owns the ability to seasonally shift. Therefore every year, vaccine should be adapted to the new emerging strains. Hence it raises a question of the development of a universal flu vaccine: it could ideally protect from the changes due to the drift and shift of influenza virus and also confer long-lasting protection. Nanoparticles increasingly play a substantial role in vaccine design, thanks to their ability to improve antigens stability and to enhance their delivery. According to that idea, the aim of this thesis work is the selection of an appropriate nanocarrier for intranasal vaccines. We tested 5 nanoparticles widely used for nasal vaccine including: NPL, cationic/anionic PLGA, cationic/anionic liposome nanoparticles. These 5 different nanoparticles were successfully synthesized and characterized. An ideal nanoparticulate vaccine should be able to load efficiently the antigens in a stable manner, to cross the mucus barrier and to deliver the antigen to the mucosal immune cells. In this thesis we evaluated every barrier that have to cross the nanovaccines to understand the physico-chemical characteristics necessary for such application.

1. Interaction of nanoparticles with nasal mucosa

1.1. Nasal residence time

Several approaches have been explored using nanoparticles for the mucosal delivery of antigen such as: protection to the antigen, enhanced antigen absorption, extended residence time in the mucosa. Despite the excellence of mucosal delivery, limitations compared with injection still restricts its application and development (Gonzalez-Paredes, Torres, and Alonso 2017; Niu et al. 2016; Niu et al. 2017). The relatively low delivery efficiency is resulted from complex and strong physiological barriers present in mucosal routes (Ensign, Cone, and Hanes 2012). These obstacles include the environment with varying pH and numerous enzymes, which challenges

the bioactivity of agents. Moreover, the sticky mucus layer and intact epithelium act as permeation and absorption barrier, respectively.

Since the protective mucus strongly hinders the accessibility of drugs or vaccines to epithelia, mucoadhesive nanoparticles were applied to overcome the mucus layer and deliver drug or vaccine into mucosal cells or to improve their uptake by macrophages and dendritic cells. Therefore polymers (e.g. chitosan and TMC) have been employed in literature to extend the nasal residence of antigens. Even the simple conjugation of OVA with chitosan can slow the protein clearance in the nasal cavity, while the unconjugated antigen is almost completely cleared from the nose within 2 hours (Slutter et al. 2010). Hence the use of muco-adhesive nanoparticles to attain a sustained antigen release is reasonable.

According to this idea, we compared 5 different nanoparticles in term of nasal residence time aiming to search for the nanocarrier that owns mucoadhesive property and stay longer in the nasal mucosa. Our data demonstrated that cationic PLGA and NPL have the higher nasal residence time in mice and that NPL were observed in higher quantity. In another study, we found that NPL increased the nasal residence time of an encapsulated protein. Moreover, NPL were observed in the first layer of the nasal mucosa cells after nasal administration (Bernocchi et al. 2016).

Indeed, increased residence times might be attributed to the strong electrostatic interactions between cationic nanoparticles with anionic sialic and acidic moieties of glycosaminoglycan contained in mucins and on the surface of airway cells (Plotkowski et al. 2001). To increase mucoadhesive property, nanoparticles have been covered by mucoadhesive materials, such as chitosan, TMC or glycol chitosan (Asane et al. 2008; Richardson, Kolbe, and Duncan 1999).

1.2. Uptake by nasal mucosa cells

Epithelial cells are formed in tightly packed layers that cover the surfaces and cavities of tissues and organs throughout the body. The first barriers that nanoparticles face once nasally instilled are mainly the epithelial cells and some specialized cells such as M cells (Rivera et al. 2016; Weitnauer, Mijosek, and Dalpke 2016). Moreover, different subsets of antigen presenting cells (APC) are present in the nose-associated lymphoid tissue (NALT). Dendritic cells have a key role in nanoparticle uptake (Kiyono and Fukuyama 2004; Marasini, Skwarczynski, and Toth 2017). It is important to evaluate the uptake of nanoparticle by nasal mucosa cells as these are the cells that will take up nanoparticles after intranasal administration.

The nanoparticle NPL was showed to be efficiently taken up by airway epithelial cells (Bernocchi et al. 2016). Moreover, following either the polysaccharidic or the lipidic part, similar uptake kinetics for both NPL components were observed (Bernocchi et al. 2016).

Study about the cellular uptake of the 5 nanoparticle types by cell lines representative of the airway mucosa showed that the endocytosis was consistent among the 3 cell lines: airway epithelial cells, macrophages and dendritic cells. NPL were quickly internalized by the cells while the other types of nanoparticles were more slowly and continuously taken up over 24h (Publication 2, Le *et at.*).

Cell membranes have many negatively charged groups on the surface. It is expected that positively charged nanoparticles will thus be better associated via ionic interactions and that their subsequent endocytosis is thus facilitated (Blanco, Shen, and Ferrari 2015; Ma et al. 2013; Xiao et al. 2011). Unexpectedly, the cationic liposome and PLGA nanoparticles showed completely different behavior, even though they possessed the same positive charge density as NPL.

Studies about endocytosis mechanism showed that NPL entered epithelial cells, macrophages and dendritic cells via a combination of several pathways. These results might explain why the cellular uptake of NPL was higher.

2. Nanoparticle as protein delivery system

2.1. Protein/antigen loading

Evaluation of the protein loading efficiency of the nanoparticles is critical for an appropriate vaccine formulation.

Ovalbumin, as model of antigen was used to evaluate the loading capacity of NPL (Bernocchi et al. 2016). Size and zeta potential analysis as well as Native-PAGE revealed that the formulation of OVA:NPL at mass ratios of 1:3 led to the complete association of the OVA to the NPL.

The loading efficacy of OVA into the 5 nanoparticles was conducted. Again amongst the 5 nanoparticles, NPL show the highest capacity of OVA loading. This is due to the structure of NPL, they are cationic on their surface, porous and filled with anionic lipids inside. They possess both cationic and anionic charge so that they can interact with both the cationic and

anionic amino acids via electrostatic interaction. These interactions could facilitate the association between OVA and NPL (Publication 3, Le *et al.*).

The protein loading could depend on the type of antigen. In a study about the loading of *Toxoplasma gondii* extract (TE) into NPL, it was observed that from a 1:1 ratio of NPL/TE (w/w), total loading of TE was observed (Dimier-Poisson et al. 2015). Though TE is a complex mixture of antigens with heterogeneous molecular weights.

It is interesting to note that at to our knowledge this percentage of loading (100 mg of proteins for 100 mg of nanoparticles) has not been described earlier and is far higher than that observed with hollow silica mesoporous nanoparticles (Mahony et al. 2014). These data demonstrate the interest of such nanoparticle.

2.2. Protein delivery

A good nasal delivery system need to cross the mucus barrier and deliver the antigen to immune cells. Amongst the 5 nanoparticles tested, again NPL was found to be the best delivery system in the 3 cell lines (airway epithelial cells, macrophage and dendritic cells) (Publication 3, Le *et al.*).

These outcomes may come from the ability to higher cellular uptake of the NPL. Indeed, to deliver antigens, NPL have to be uptaken by the cells then deliver their payload. Furthermore, loading OVA inside the porous structure of NPL lead to minimize the change in size of the formulation and influence on endocytosis ability of NPL. In contrary, OVA was bound to the surface of PLGA NPs and liposome and eventually that's might interfere for the internalization. That might explain why the uptake of these nanoparticles by cells was not efficient.

In another study, *Toxoplasma gondii* extract was successfully delivered into the airway epithelial cells by NPL while free TE failed to be really detected in the cells (Dimier-Poisson et al. 2015). Interestingly, delivery of TE by NPL did not seem to be influenced by the presence of serum (Dimier-Poisson et al. 2015). NPL were also able to efficiently deliver the antigens into airway epithelial cells, macrophages and DCs. This delivery induced the activation of these cells via the NF-kB pathway, through TLR2 and TLR4 signaling and the production of pro-inflammatory cytokines (Dimier-Poisson et al. 2015).

Altogether, due to the high capacity of endocytosis, NPL exhibited efficient cellular protein delivery. We demonstrated that amongst the 5 nanoparticles tested, NPL owns the longest nasal

residence time, higher capacity of cellular uptake and protein delivery so this delivery system was used for the influenza vaccine project.

Although this study clearly showed the interest of NPL further studies should be conducted. For example we observed that NPL can stay up to 6 h in the nasal mucosa (Publication 2, Le *et al*.) however we don't know what cells really have uptaken NPL. The fate of OVA/NPs should be investigated to clarify whether the OVA was delivered into the nasal mucosa cells or not.

Though *in vitro* data demonstrated that PLGA and liposomes nanoparticles were less efficient than NPL in term of nanoparticle uptake and protein delivery, there could be different outcomes for *in vivo* data. Particularly, the cationic PLGA and liposomes nanoparticles were coated by chitosan, a well-known molecule that have ability to open tight junction of the epithelia barrier. Once they bypass the tight junctions, the cationic PLGA and liposome nanoparticle have more chance to deliver antigens into the APCs underlying the epithelium.

More importantly, to evaluate the vaccine efficacy, the immune response after vaccine administration is a critical issue. The humoral and cellular response of these OVA/NPs after nasal administration should be tested. Furthermore, elucidation of the mechanism that modulate the innate and adaptive immune system by these OVA/NPs is needed.

3. Nanoparticle as influenza vaccine delivery system

The flu antigen CTA1-3M2e-DD and HA were formulated with NPL. The construct of antigen CTA1-3M2e-DD were prepared by the conjugation of the mucosal adjuvant CTA1-DD and the M2e domain, a conserved epitope of influenza. CTA1-3M2e-DD fusion protein consists an adjuvant and a conserved region of influenza A virus in a potential candidate for a universal anti-influenza vaccine. The current opinion in the flu field is that we would need more than one antigen in order to generate a universal flu vaccine and only one antigen is probably not sufficient. Yassine H.M. *et al.* 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). Recently, Deng *et al.* demonstrated that incorporating both the HA stalk domains and M2e induces strong long-lasting immunogenicity, fully protecting the mice against challenges by different influenza A viruses (Deng et al. 2018). Hence, the use of hemagglutinin, a key antigen of influenza vaccines, seems to be necessary to develop a universal influenza vaccine. In our project,

recombinant HA originated from influenza H1N1 (A/Puerto Rico/8/34 (H1N1)) was together with CTA1-3M2e-DD loaded into NPL.

Different flu antigens were combined to NPL to make the formulation at ratios 1:5 (w/w). CTA1-3M2e-DD, hemagglutinin (HA) or adjuvant CTA1-DD. The dynamic light scattering analysis showed at ratio 1:5, an increase of the formulation size was observed meaning that some NPL-Ag were partially agglomerated. When antigen was incubated with NPL, we obtained only a slight decrease of the positive charge of the nanoparticles showing that CTA1-3M2e-DD was successfully loaded into NPL. Moreover, two different antigens were also successfully loaded into NPL (Table 2). In order to assess if some antigen was free, we analyzed the formulation by native PAGE (Figure 9). At ratio of the formulation 1:5, the protein CTA1-3M2e-DD and CTA1-DD was totally bound to NPL while 70 % of HA was associated. These results may be attributed to the use of detergents (Empigen® BB). Indeed, we used a detergent to solubilize HA, this can form micelles that prevent the HA association to the NPL. However most of the HA is bound to the NPL (Table 3). Taken together these results suggest at ratio 1:5, 3 different antigens were successfully associated to NPL.

These formulations were sent to our partners in the consortium for *in vivo* analysis against viral challenge.

Protein/Formulation	Size (nm)	PDI	Zeta potential (mV)
NPL	45.6	0.22	+43.3
HA	1.72	0.65	-11.37
CTA1-3M2e-DD	238.6	0.37	-11.86
CTA1-DD	7.36	0.43	-9.35
HA/NPL	83.67	0.21	+24.46
CTA1-3M2e-DD/NPL	472.3	0.54	+30.5
CTA1-3M2e-DD + HA /NPL	241.2	0.39	+26
CTA1-DD+HA/NPL	43.63	0.46	+29.1

Table 2: Size and zeta potential of protein antigens, nanoparticles and of the formulation (antigen:NPL). Nanoparticles were mixed with antigens at a ratio of the antigen:NPL (1:5, w/w). Size and zeta potential are measured in triplicate by dynamic light scattering on a zetasizer. PDI: Polydispersity index. Results represented by mean data.

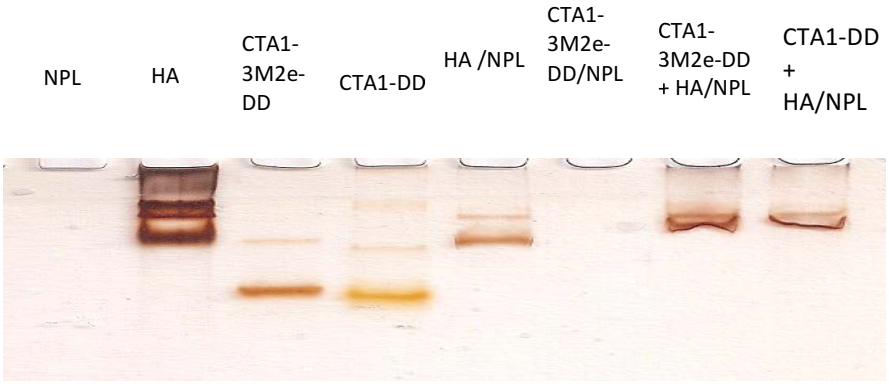

Figure 9: Characterization of the formulations by Native-PAGE

Following intranasal immunizations of mice with CTA1-3M2e-DD or CTA1-3M2e-DD /NPL, we found that mice immunized with CTA1-3M2e-DD/NPL got an enhanced protective efficacy against a challenge with the X47 influenza virus strain (H3N2) as compared to mice received CTA1-3M2e-DD. Furthermore, mice immunized intranasally with the combined HA+ CTA1-3M2e-DD/NPL vector were fully protected against a challenge infection with the aggressive PR8 virus (A/Puerto Rico/8/34 (H1N1), whereas non of the HA/NPL, CTA1-3M2e-DD /NPL or CTA1-3M2e-DD alone immunized mice were protected (Bernasconi *et al.*, Journal Frontiers in Immunology, submitted).

Protein	% Protein binding
HA	70%
CTA1-3M2e-DD	100%
HA + CTA1-3M2e-DD	100%
HA + CTA1-DD	100%

Table 3: Protein loading efficiency by NPL

We have previously reported that nasal immunizations with NPL preparations could stimulate significant protection against Toxoplasma *gondii* in mice (Dimier-Poisson et al. 2015). In another study, NPL was able to totally load both CTA1-DD and split Udorn and this vaccine formulation induced a stronger protection against influenza virus (Publication 4, Le *at al.*). In this project, we have shown that co-incorporation of two influenza specific target antigens, the CTA1-3M2e-DD and recombinant HA into NPL is more broadly effective against influenza virus infections than either component used alone. Thus, the combined HA+CTA1-3M2e-DD/NPL vector exhibited improved immunogenicity and enhanced virus protection following intranasal immunizations.

However, we failed to show the beneficial function of NPL with adjuvanted antigen, the CTA1-3M2e-DD or HA+CTA1-3M2e-DD. In fact, this study used high dose of antigen (5 µg per dose) and 3 immunizations that leads to maximum immune response so that we could not see the additional effect of NPL. Indeed, lower dose of antigen (1 µg per dose) showed increase level of T cell proliferation in spleen and mesenteric lymph nodes (mLN) by CTA1-3M2e-DD /NPL as compared to CTA1-3M2e-DD alone (data not shown).

In the end of this project, we found that HA+ CTA1-3M2e-DD/NPL could be a potential universal influenza vaccine. However, further studies should be done in future to obtain an efficient influenza vaccine with a low dose of flu antigens. The vaccine should be challenged with other strains of influenza virus to fulfill the concept of universal influenza vaccine. Moreover, instead of using the whole HA from H1N1, the stalk domain, the conserved region from HA can be applied as a flu antigen. Other strategies to improve vaccine efficiency should be conducted. CTA1-DD contains the c-terminal region of a dimer of the D-fragment of the protein A from *Staphylococcus aureus* (DD) which targets B-cells. However, once the adjuvanted antigen CTA1-3M2e-DD was loaded into the porous structure of NPL, it could be a question that whether the B-cell targeting still keeps its function or not. Indeed, the CTA1-DD could be covered by NPL and no longer be recognized by B-cells. Importantly, it has been shown that NPL are not able to open tight junctions (Bernocchi et al. 2016) so the main vaccine antigen is delivered into nasal mucosa cells, such as epithelial cells, M cells or dendritic cells. It could be a potential strategy to improve this vaccine efficiency if the targeting moiety is recognized by these cells and constructed to appear in the surface of NPL.

NPL carrying vaccine antigen can be targeted to M-cells by surface modifications with lectins that naturally bind to M-cell surfaces through α-L-fucose residue (Neutra 1999; Mabbott et al. 2013). Besides, NPL could be covalently coupled with sIgA that has been demonstrated to be captured by Dectin-1, a small type II transmembrane protein of the C-type lectin family presence on M cells (Rochereau et al. 2013).

In order to target DCs present in nasal mucosa, NPL can be conjugated with a mannose motif that is recognized by mannose receptor of DCs (Raghuwanshi et al. 2012). NPL otherwise can be decorated with a cell surface marker such as DEC-205 that is primarily expressed by DCs and should facilitate the uptake and delivery vaccine antigen into DCs (Park et al. 2008; Diebold et al. 1999; Yeeprae et al. 2006).

The nanocarrier can be co-delivered with an adjuvant that can improve the ability of the host immune system to recognize the administered antigen as foreign and respond to it. Moreover, in our study we found that though the NPL enhanced the vaccine delivery however, it was not sufficient to induce stronger immune response and virus protection. Recruitment of an adjuvant was necessary to create significant protection effect against influenza virus (Publication 4, Le *et al.*). To induce the vaccine efficiency, NPL can co-deliver the adjuvanted and targeted antigen. Currently, there are several adjuvants licensed for human vaccine such as alum, MF59, AS03 AF03. However, these adjuvants are mostly applied for non mucosal vaccine.

Other adjuvants for nasal vaccine are still under development such as toll like receptors (TLR) ligands. Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR) which are expressed on the membranes of leukocytes including DCs, macrophages, natural killer cells, cells of the adaptive immunity (T and B lymphocytes) and non immune cells (epithelial and endothelial cells, and fibroblasts) (Delneste, Beauvillain, and Jeannin 2007). Many of the other TLRs have also been targeted to boost vaccine responses, for example, topical application of imiquimod, a TLR7 agonist (Hung et al. 2016), agonists of TLR3 (rintatolimod) (Overton et al. 2014) and TLR9 (CpG oligodeoxynucleotides) (Cooper et al. 2004) have also been tried. Co-delivery the vaccine formulation with these TLR ligands could be another strategy to improve nasal vaccine efficacy.

Taken together, this thesis study demonstrated that NPL could be a promising nano carrier for intranasal influenza vaccine. Other strategies can be applied to improve the vaccine efficiency such as targeting the vaccine formulation to nasal mucosa cells or co-delivery with a mucosal adjuvant should be investigated in future.

PART IV: CONCLUSION AND PERSPECTIVES

Nanoparticles are promising tools for protein delivery in the mucosa, useful for their protein stabilization function and more advantageous than other administrations. The development of a nanoparticle-based mucosal vaccine requires nanocarriers able to load antigens, to enter the cells and to deliver the antigens into cells. Among the 5 types of nanoparticles: cationic/anionic PLGA NPs, cationic/anionic liposomes and NPL, we observed that NPL are the best candidate. Furthermore, *in vivo* data showed that NPL improved the efficacy of the universal influenza vaccine developed by the consortium. In order to further improve the efficacy of a mucosal vaccine, NPL could be modified to target the mucosal cells such as M cells, DCs or co-delivered with a mucosal adjuvant that can activate the innate immune system, hence maximizing the immune response.

Mucosal vaccination has significant advantages compared with parenteral vaccination including needle-free, the induction of systemic and mucosal immunity and low cost. Despite these advantages, currently, one nasal and eight oral vaccines are licensed for administration to humans, targeting the following five pathogens: influenza, poliovirus, cholera, Salmonella typhi and rotavirus. Furthermore, most of the licensed vaccines for mucosal delivery are based on live attenuated viruses which carry the risk of recovering their pathogenicity. There are still no mucosal vaccines against infectious diseases such as malaria, HIV, leishmaniasis, Hepatitis B and C. This thesis study contributes to select the appropriate nano delivery system for mucosal vaccine. Based on this study, other mucosal vaccines applying nanoparticle system should be developed in future.

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