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Développement de nouveaux systèmes de délivrance de vaccins à base de polysaccharides

Kévin Blanchard

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Développement de nouveaux systèmes de délivrance de vaccins à base de polysaccharides

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RÉSUMÉ

La vaccination, particulièrement chez les espèces animales, demeure toujours un outil efficace de préventions des maladies infectieuses. Les adjuvants sont des composants généralement indispensables dans la formulation des vaccins de par leurs rôles de vecteurs de l'antigène ainsi que de stimulateurs du système immunitaire. En effet, les antigènes seuls, pour la plupart, ne permettent pas d'induire une protection satisfaisante. Les propriétés uniques du chitosane, polymère naturel biocompatible et biodégradable, offrent un matériau de choix pour l'élaboration de nouvelles générations d'adjuvant tel que des nanoparticules ou des hydrogels.

Les travaux de cette thèse ont portés sur l'élaboration d'adjuvants à base de chitosane chez l'animal. La préparation de solutions visqueuses de chitosane ($0,2 \leq C_p \leq 0,75$ % (w/v)) en association avec différents types d'antigènes à savoir une souche atténuée de bactéries vivantes atténuées, de virus vivants atténués ou inactivés ainsi qu'une protéine recombinante purifiée ont permis d'obtenir une réponse immunitaire chez les différentes espèces animales étudiées. Par ailleurs, le chitosane, par l'inspection des animaux durant les essais ainsi que post-mortem, a démontré une bonne innocuité ainsi qu'une résorbabilité satisfaisante. Dans le cadre du développement d'un système de relargage retardé d'antigènes, nous avons débuté l'élaboration d'un système permettant de prélever et d'injecter, via un système classique seringue/aiguille, une solution visqueuse gélifiante en conditions physiologiques (150 mM, 37°C). La diffusion plus lente d'un antigène associé à ce matériau a pour objectif d'améliorer la protection des animaux en stimulant de manière prolongée les différents acteurs du système immunitaire.

Mots-clés : chitosane ; vaccin ; adjuvant ; solutions visqueuses ; nanoparticules ; hydrogels ; stérilisation

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ABSTRACT

Vaccination, especially in animal species, remains already an efficient tool in the prevention of infectious diseases. The carrier and immunostimulant properties of adjuvant allow increasing the action of antigen which, alone, is not enough capable to induce a long and strong immune response in host. The unique properties of chitosan, a biocompatible and biodegradable natural polymer, offer a choice material to elaborate new generations of adjuvant such as nanoparticles or hydrogels.

This PhD works was focus on the development of chitosan-based adjuvant for animal species. The preparation of chitosan-based viscous solutions, with a polymer concentration from 0.2 to 0.75 % (w/v) mixed with different kind of antigens such as live attenuated bacteria, live attenuated or inactivated virus and a recombinant protein allowed obtaining an immune response in the studied animals. Moreover, the observation of animals during the protocol or in post-mortem inspections indicated a satisfying safety and resorbability. *In vitro* experiments were also conducted developing a syringeable and injectable *in situ* gelling chitosan-based hydrogel containing a model protein, destined to standard injection system. The slow release of antigen in the host should interact with the immune system longer increasing the final protection against diseases.

Key-words: chitosan; vaccine; adjuvant; viscous solutions; nanoparticles; hydrogels; sterilization

RERMERCIEMENTS

INTRODUCTION GÉNÉRALE

Les adjuvants ainsi que la formulation finale des vaccins sont des éléments importants pour induire une réponse immunitaire permettant la protection d'un individu contre un pathogène donné, sans toutefois générer d'effets secondaires indésirables. Ainsi, il faut toujours envisager un compromis entre efficacité et innocuité.

La vaccination des espèces animales est tout d'abord un enjeu de santé publique, puisqu'elle permet la prévention d'épidémie intra ou inter-espèces (ex : zoonose) ou encore d'une contamination de la chaîne alimentaire. Il s'agit également d'un enjeu économique puisque la vaccination est souvent le seul moyen de lutter contre une maladie donnée, par exemple, dans le cas de l'élevage d'animaux de production, où aucun traitement n'existe.

La formulation de vaccins dans le domaine vétérinaire est un travail complexe du fait de la variété des espèces animales (animaux de compagnies ou animaux d'élevages), des voies d'administrations des vaccins (orale, intramusculaire, sous-cutanée...) ainsi que des habitudes de travail des vétérinaires ou des éleveurs. En ajoutant un certain nombre de contraintes telles que la stérilisation, la stabilité au stockage ou encore le procédé d'industrialisation, la mise au point d'un vaccin efficace représente un véritable défi.

Depuis plusieurs dizaines d'années maintenant, les progrès technologiques dans les domaines de la biochimie et de l'immunologie ont permis le développement d'antigènes de mieux en mieux définis comme les protéines recombinantes. L'administration de ces antigènes sous-unitaires permet d'accroître la sécurité du vaccin en s'affranchissant des risques d'infection provenant de la reprise d'activité potentielle des virus ou bactéries atténués, classiquement utilisés. Cependant, l'immunogénicité de ces antigènes recombinants est insuffisante pour générer une réponse immunitaire capable de protéger un individu contre l'agent pathogène. Par ailleurs, les adjuvants classiques tels que les sels d'aluminium, couplés avec des antigènes sous-unitaires, ne permettent pas de stimuler à la fois les réponses humorale et cellulaire garantes d'une protection optimum de l'hôte.

La société MERIAL, filiale du groupe SANOFI, est un acteur mondial de la santé animale expert dans le développement et la fabrication de médicaments et vaccins destinés aussi bien aux animaux de compagnie que de production. Dans le cadre d'une

prospection de nouvelles technologies adjuvantes permettant de répondre à la fois à la demande en innocuité et en efficacité, le chitosane apparaît aujourd'hui comme un candidat à fort potentiel. Le chitosane a suscité de très nombreuses recherches dans le domaine biomédical avec des applications telles que la régénération tissulaire ou la délivrance de principes actifs avec des résultats prometteurs.

Le chitosane est un polysaccharide dérivé de la chitine, un polymère naturel extrait de la carapace de crustacés ou des parois cellulaires de champignons. Il s'agit d'un copolymère constitué d'unités N-acétyl-D-glucosamine et de D-glucosamine dont la liaison glycosidique est de type $\beta \rightarrow 1-4$. Il est considéré comme un matériau particulièrement adapté pour l'élaboration d'adjuvant sur la base de sa biocompatibilité, biodégradabilité ainsi que de sa non-toxicité. Le chitosane, seul polysaccharide cationique naturel connu à ce jour, soluble en milieu aqueux acide faible, peut être utilisé pour élaborer des dispositifs de délivrance ayant des formes physiques variées comme des solutions, des hydrogels ou encore des systèmes micro ou nanoparticulaires. La maîtrise des paramètres physico-chimiques intrinsèques du polymère, tels que le degré d'acétylation (fraction molaire d'unités N-acétyl-D-glucosamine) ou la masse molaire ainsi que sa mise en forme est une expertise du laboratoire *Ingénierie des Matériaux Polymères* (IMP) de Lyon, plus particulièrement de l'équipe *Matériaux à l'interface avec les Sciences de la Vie*, dirigée par le Professeur Laurent David.

Ce manuscrit rapporte les travaux issus de la collaboration entre MERIAL et le laboratoire IMP mutualisant, respectivement, compétences dans le domaine du développement de vaccins et développement de matériaux biocompatibles. L'objectif du projet de thèse étant de développer des systèmes de délivrances de vaccins compatibles avec différentes natures d'antigène (bactéries, virus, protéines) destinés à différentes espèces animales à savoir : volaille, chien et porc.

Le manuscrit de thèse est divisé en trois parties chacune destinée à la publication dans des revues internationales à comité de lecture. Tout d'abord, un état de l'art des vaccins expérimentaux utilisant le chitosane, sous ses différentes formes, comme adjuvant dans des modèles *in vivo*. Le deuxième chapitre est consacré à l'utilisation du chitosane, dans le cadre de la collaboration avec MERIAL, portant sur les différents essais *in vivo* réalisés. Enfin, le dernier chapitre est consacré aux travaux portant sur l'élaboration d'une solution visqueuse, prélevable et injectable, permettant la formation d'un gel physique de chitosane en conditions *in vivo*. Ce dernier aspect constitue un axe

de recherche innovant sur l'élaboration de matériaux structurés à des fins de délivrance de biomolécules.

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LISTE DES ABREVIATIONS

Ab	Antibody
APCs	Antigen-Presenting Cells
API	Active Pharmaceutical Ingredient
ASC	apoptosis-associated speck-like protein
ASTM	American Standard Test Method
CAV-2	Canine Adenovirus of Type 2
CCID	Cell Culture Infection Dose
CD	Cluster of Differentiation
CDV	Canine Distemper Virus
CHC	Chitosan Hydrochloride
CMC	Carboxy-Methyl chitosan
CMIS	Common Mucosal Immune System
Cp	Concentration en Polymère
CPE	Cytopathic Effect
CpG	Cytosine-Phosphate Guanine
CPV	Canine Parvovirus
CS	Chitosan
CS HMW	Chitosan High Molecular Weight
CS LMW	Chitosan Low Molecular Weight
DA	Degree of Acetylation
DCs	Dendritic Cells
DES	Diethyl Squarate
DQ	Degree of Quaternization
DS	Dextran Sulfate
E.coli	<i>Escherichia coli</i>
FAVN	Fluorescent Antibody Virus Neutralisation
FITC	Fluorescein 5(6)-Isothiocyanate
G'	Storage modulus
G''	Loss modulus
GP	Glycerol-Phosphate
HA	Hemagglutinin
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromaography
HYA	Hyaluronic Acid
IFN	Interferon
IL	Interleukin
IR	Infrared
LAIV	Live Attenuated Influenza Vaccine
LCs	Langerhans Cells
LN	Lymph nodes
MALLS	Multi-Angle-Laser-Light-Scattering
MDCK	Madin-Darby Canine Kidney
MHC	Major Histocompatibility Complex
Mn	Number-Average Molar Mass
Mv	Viscosimetry-Average Molar mass

M_w	Weight-Average Molar Mass
MWCO	Molecular-Weight-Cut-Off
NA	Neuraminidase
NALP3	Nacht Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein 3
NLRP3	NOD-like receptor family, pyrin domain containing 3
NMR	Nuclear Magnetic Resonance
OMS / WHO	World Health Organization
OVA	Ovalbumin
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PDI	Polydispersity Index
PECs	Polyelectrolyte Complexes
PEG	Polyethylene Glycol
PI	Polydispersity Index
Pi2	Parainfluenza type 2
Pm	<i>Pasteurella multocida</i>
PS	Polystyrene
QuilA	Quillaja A
R_g	Radius of gyration
RPMIc	Roswell Park Memorial Institute Cell
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel electrophoresis
SEC	Size Exclusion Chromatography
SFC	Spot Forming Cells
SLS	Static Light Scattering
SNPs	Silica Nanoparticles
SpaA	Surface protective antigen A
SPF	Specific-Pathogen-Free
TEM	Transmission Electron Microscopy
TMC	Trimethyl Chitosan
TPP	Tripolyphosphate
TT	Tetanus toxoid

CHAPITRE 1

ETAT DE L'ART : LE CHITOSANE COMME POLYSACCHARIDE POUR L'ELABORATION DE SYSTEMES DE DELIVRANCE DE VACCINS

- Chapter 1 -

*State of the art: chitosan, a versatile polysaccharide for vaccine
delivery systems*

INTRODUCTION AU CHAPITRE 1

Un vaccin est une formulation s'appuyant principalement sur deux composés : l'antigène (ou principe actif) spécifique de la maladie contre laquelle l'immunité est recherchée et l'adjuvant permettant le transport de l'antigène jusqu'aux cellules du système immunitaire ainsi que l'activation de ce dernier.

Les dernières décennies ont vu l'émergence de nouvelles générations d'antigènes (vaccins sous-unitaires, vectorisés, recombinants...) offrant une sécurité supérieure aux vaccins traditionnels (virus ou bactéries atténués...). Cependant, ces nouveaux antigènes requièrent l'utilisation d'un adjuvant pour induire une réponse immunitaire optimale, assurant une protection efficace de l'hôte. La catégorie d'adjuvants la plus répandue actuellement sur le marché est celle à base de sels d'aluminium. Ils permettent une stimulation du système immunitaire dans la plupart des cas mais elle est souvent partielle, limitée à la réponse humorale, la réponse cellulaire étant absente. De plus, ils sont l'objet de controverses régulières quant à leurs possibles effets secondaires.

L'objectif de ce premier chapitre bibliographique est de dresser l'état de l'art de l'utilisation du chitosane comme système de délivrance de vaccins à visée prophylactique. Le chitosane est un biopolymère issue de la chitine, un polymère naturel extrait généralement à partir de carapaces de crustacés, d'endosquelette des calmars ou de la paroi cellulaire de champignons.

La section « *chitosan : a unique cationic semi-natural polymer* » est consacrée au rappel des propriétés physico-chimiques et biologiques du polymère dans le cadre d'applications vaccinales. Nous abordons dans un premier temps (sections II.1 et II.2) des aspects généraux sur le chitosane et la présentation des différents dérivés classiquement utilisés dans le cadre de système de délivrance. Nous traitons dans la section II.3 la caractérisation des paramètres intrinsèques du chitosane à savoir le degré d'acétylation (DA) et la masse molaire. Le DA est un facteur prépondérant dans les diverses applications du polymère. Il peut être calculé par de nombreuses méthodes (dosages, FT-IR, UV-Vis...) mais nous soulignons les travaux de Hirai *et al.* permettant, par RMN du proton, une détermination rapide de sa valeur. La masse molaire est classiquement déterminée par chromatographie d'exclusion stérique couplée avec un système de détection par diffusion statique de la lumière. Nous abordons finalement

dans la section II.4 les propriétés biologiques du polymère à savoir sa biodégradabilité, ses propriétés mucoadhésives ainsi que ses propriétés antibactériennes. La biocompatibilité est un facteur clé dans la formulation de matériaux à fin médicale, puisque celui-ci ne doit pas induire d'effet néfaste chez l'hôte, ces derniers pouvant compromettre les bénéfices recherchés. Concernant la formulation de vaccin, il a été montré qu'un faible DA était en faveur d'une meilleure biodégradabilité tandis que l'influence de la masse molaire n'est pas encore clairement statuée. Les propriétés mucoadhésives, due à la présence des groupements cationiques, sont capables d'améliorer le temps de résidence du polymère sur les surfaces mucosales. Enfin, l'activité antimicrobienne du chitosane doit être mesurée au cours de la préparation de vaccins à base de bactéries vivantes atténuées. En effet, le chitosane ayant des propriétés bactériostatiques peut avoir un effet délétère sur l'activité immunogène des bactéries utilisées.

La section III intitulée « *chitosan-based vaccine delivery system* » est consacrée à l'utilisation du chitosane dans des applications vaccinales. Cette revue présente les principaux modes d'administration (intramusculaire, sous-cutané, oral...) et les différents antigènes (protéines, virus, bactéries) vectorisés ou adjuvés par les différentes formes physiques du chitosane.

Les études rapportées portent sur des vaccins prophylactiques dans le cadre d'expérimentations *in vivo*. La première partie (section III.1) est consacrée aux solutions visqueuses de chitosane utilisées dans le cadre de vaccins anti-grippaux. Il a été montré que le chitosane, contrairement à l'aluminium, est capable d'induire une réponse immunitaire locale favorisant ainsi une meilleure protection de l'hôte. La deuxième partie (section III.2) présente les hydrogels à base de chitosane. Les études portant sur cette forme sont plus restrictives et plus récentes, le principal effet recherché étant un effet dépôt pour un relargage de l'antigène différé dans le temps visant à stimuler plus longtemps le système immunitaire. La dernière partie (section III.3) est consacrée aux systèmes particuliers à base de chitosane, particulièrement les nanoparticules. Un très grand nombre d'études portant sur l'évaluation de nouvelles générations d'adjuvants ont pour objet les systèmes particuliers. En effet, leurs formes et leurs tailles offrent des mimés biologiques de virus ou de bactéries permettant, lorsqu'ils sont couplés à des antigènes, de favoriser la reconnaissance des agents infectieux par le système immunitaire.

Le chitosane, du fait de ses propriétés physico-chimiques, permet l'élaboration de particules par un certain nombre de méthodes (gélification ionique, complexation, émulsification...). La méthode la plus explorée pour des expériences *in vivo* est la gélification ionique du chitosane par le tripolyphosphate de sodium. Les résultats chez l'animal montrent que ces systèmes particuliers induisent une réponse immunitaire avec diverses natures d'antigènes ou voies d'administration. La formulation d'un vaccin représente un véritable challenge puisque l'influence de nombreux paramètres tels que la nature de l'antigène, la voie d'administration, la forme de l'adjuvant, les interactions adjuvant/antigène, etc. sont déterminant vis-à-vis de la réponse immunitaire.

La section IV intitulée « *specfications for highly immunogenic chitosan-based vaccine delivery systems* » propose une vue d'ensemble de l'activité immunologique du chitosane depuis sa prise en charge par les cellules du système immunitaire jusqu'à l'induction de la réponse immunitaire. La principale difficulté aujourd'hui, dans la compréhension du mécanisme d'action du chitosane est la multiplication des études avec différents chitosanes. En effet, l'influence du DA, de la masse molaire, la diversification des modifications chimiques, des antigènes, etc. ne permettent pas d'établir clairement l'influence de chaque paramètre sur la réponse immunitaire finale.

La conclusion revient sur les acquis observés dans les études citées mais présente également les limites actuelles du polymère dans la préparation de vaccins. En effet, un certain nombre de critères tels que la stabilité au stockage ou la stérilisation demeurent des paramètres critiques non investis limitant une rapide mise sur le marché des vaccins à base de chitosane. Deux axes de travail doivent être désormais privilégiés: (1) la compréhension de mécanisme d'action du chitosane et l'influence de ses paramètres intrinsèques; (2) la mise au point de méthodes permettant la fabrication industrielle des formes adjuvantes choisies.

State Of The Art: Chitosan, A Versatile Polysaccharide For Vaccine Delivery Systems

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ABSTRACT:

New vaccines require efficient delivery systems or adjuvants to induce a protective immune response with an optimum safety for the patients. Chitosan, a bio-sourced polysaccharide, has good safety records and many of its properties can be tuned via an adaptation of its molar mass and degree of acetylation. Thus, the versatility of chitosan allows the development of many different formulations from solutions, through hydrogels, to nanoparticles. This review provides a multidisciplinary overview on chitosan-based vaccine delivery systems, focusing on the pertinent parameters involved in the establishment of an appropriate immune response, to fight various infectious diseases.

KEYWORDS:

Chitosan; vaccine; hydrogels; nanoparticles; solutions; adjuvant

I. INTRODUCTION

Recently, the Ebola hemorrhagic fever outbreak reminded us the urgency to provide efficient solutions, easily workable, to respond to potential pandemic diseases.¹ Vaccine constitutes a strategic medical tool allowing an individual, but also a collective, protection against infectious diseases.² To fight high mortality diseases such as malaria, tuberculosis or AIDS, vaccines need to be efficient and as accessible as possible to avoid the use of restrictive and costly treatments, if existing. Prevention through vaccine campaigns has shown the ability to eradicate smallpox disease³ and poliomyelitis is expected to end for next years.⁴

Most vaccines for human or veterinary uses are based on live attenuated, inactivated or killed organisms.⁵ But, to meet the need of an improved safety and efficacy, other technologies have emerged such as recombinant protein subunit or DNA vaccines. Unfortunately, in many cases subunit antigens were not efficient enough to induce, alone, a potent immune response.⁶

The weakness of the antigen should be counter-balanced by an appropriate adjuvant that should achieve the transport and delivery of antigens to immune cells and the stimulation of both arms of immunity to induce a humoral and a cellular response.⁷ Brito and O'Hagan defined the concept of adjuvant as: '*we know what it should do but not what it is*'.⁸ The literature offers each year many reviews about research on vaccine adjuvants with various advices to their adjuvantivity.^{5,6,8-12}

Future challenges for vaccine research and development are numerous. The following advances could be quoted as the more important: (1) Antigen dose-sparing in order to response to the increasing worldwide demand in vaccine protection¹³ ; (2) needle-free administration to reduce injuries and contamination risks¹⁴ ; (3) induction of strong and long lasting immunity for new antigens ; (4) avoid the cold supply chain with preservation of the vaccine performances¹⁵ ; (5) highly safe adjuvants based on biocompatible materials. To win these challenges, many research strategies are in progress. For several years now, administration via mucosal or intradermal routes demonstrated a high potential to avoid parenteral injection.¹⁴ Moreover both routes induced interesting immune responses: local and systemic response for mucosal vaccine¹⁶ and low dose of antigen for intradermal.¹⁷ A mix carrier and immunostimulant

has proved to induce strong and modulable immunity.⁷ Despite all these solutions, a strong adjuvant system remains to be designed to ensure efficient vaccines.⁸

The primary requirement to develop a new adjuvant is the safety issue. Polysaccharides, from natural sources, such as alginates or hyaluronic acid,¹⁸ were investigated and proved safe. Chitosan, a polysaccharide derived from chitin, appeared a good candidate due to its biocompatibility, biodegradability and absence of toxicity, the main safety requirements for vaccine development.^{19,20} Chitosan is a versatile material with a unique cationic character that allowed the development of a wide range of formulations from a simple solution to complex nanoparticles, as well as smart *in situ* gelling hydrogels. Mucosal adhesion is also claimed for chitosan,²¹ particularly attractive for developing delivery system for mucosal administration. The ability of polymers to interact with various biological materials from bacteria to DNA adds another advantage for vaccine development.²²

The goal of this review is to present the state-of-the-art on chitosan and chitosan derivatives-based vaccine delivery systems. Chitosan has been widely exploited and reviewed for medical applications such as tissue regeneration or drug delivery,²² but vaccine applications were not fully explored. In the first part of this contribution, we provide an overview of the impact of the intrinsic parameters of chitosan on its physico-chemical and biological properties. In the second part, we present chitosan-based vaccine delivery systems and their elaboration processes with a systematic analysis of the impact of the polymer physico-chemical properties on its adjuvanticity.

II. CHITOSANE: A UNIQUE CATIONIC SEMI-NATURAL POLYMER

II.1. General aspects

Chitosan is a semi-natural polymer obtained from partial deacetylation of chitin, a natural polysaccharide extracted from crustacean shell,²³ cephalopod endoskeleton²⁴ or the cell walls of fungi.²⁵ Chitosan is a linear copolymer of *N*-acetyl-D-glucosamine and D-glucosamine linked by a β -1,4-glycosidic bond (Fig. 1). Apart from its molar mass, another important intrinsic parameter defines this group of macromolecules, namely the degree of acetylation, DA, i.e. the molar percentage of remaining N-acetyl groups in the polymer chain.^{26,27} It is commonly admitted that up to DA=60-70% the polysaccharide is known as chitosan and as chitin above. Chitosan differs from chitin by its ability to dissolve in weakly acidic media via the protonation of the $-NH_2$ moieties.

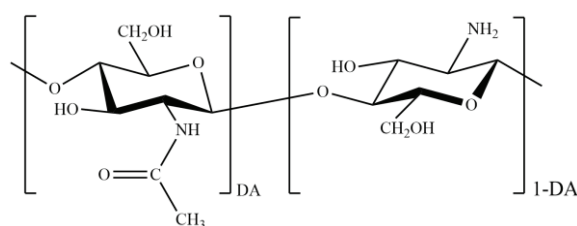


Figure 1 : Chemical structure of chitosan

Chitosan is a cationic polyelectrolyte whose solubility is mainly affected by its DA, its molar mass and also the pH of the solution. Sorlier *et al.* showed that the value of the intrinsic pK_{A0} of chitosan varied between 6.46 and 7.32,²⁸ when DA increased from 0 % to 70 %, allowing chitosan to be soluble under these pH values, with most acids (usually hydrochloride or acetic acid).^{28,29} Moreover, the physico-chemical properties of chitosan varied with DA in a patterned way. Indeed, three distinct DA domains were identified : (1) a polyelectrolyte domain for DA below 20 % in which chitosan is highly hydrophilic; (2) a transition domain between DA = 20 % and 50 % where hydrophilic and hydrophobic interactions counterbalance each other ; (3) a hydrophobic domain for DAs over 50 % in which inter-chain interactions are favoured.³⁰ The impact of DA on the physico-chemical properties of chitosan is well illustrated by the fact that chitosan at DA around 40% and higher remains soluble at neutral-pH.^{31,32} Anyhow the solubilisation of chitosan, irrespective of DA, requires a weakly acidic pH to ensure the protonation of the amines. Rheological investigations have shown that the viscosities of chitosan aqueous

solutions increased sharply with increasing polymer concentration and exhibited a pseudoplastic non-Newtonian behaviour.³³⁻³⁶

Chitosan was widely explored in many field such as biomedical,^{22,37} cosmetics,³⁸ food,³⁹ agriculture⁴⁰ and wastewater treatment.^{41,42}

II.2. Chitosan derivatives for delivery system

Chitosan salts were obtained directly from the corresponding acidic solution by water elimination via freeze-drying⁴³ or spray-drying.⁴⁴ The resulting solubility in acid-free media was increased, offering a ready-to-use water-soluble polymer. The salt nature influenced the release properties in case of drug delivery as observed by Huanbutta *et al.*⁴⁵

Chitosan was chemically modified either on the hydroxyl or amine moieties. A variety of derivatives^{26,27,46-48} could thus be elaborated to improve its solubility in neutral and alkaline media, its chelating properties, antibacterial activity or enhancing its adsorption properties. Carboxy-methyl chitosan (CMC) and trimethyl chitosan (TMC), the most frequently used chitosan derivatives in delivery systems, were fully reviewed (see Table 1).

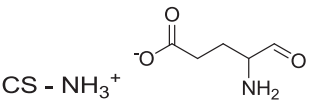
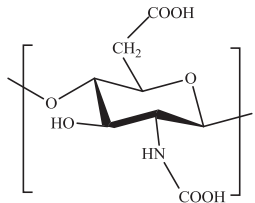
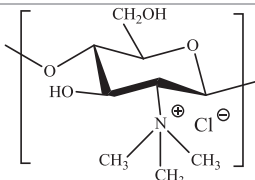
Chitosan derivative	Chemical structure of modified unit	References
<i>Chitosan salts</i>		
Chitosan hydrochloride (CHC)	CS - NH ₃ ⁺ Cl ⁻	Signini <i>et al.</i> ⁴³ Signini <i>et al.</i> ⁴⁹ Rauw <i>et al.</i> ⁵⁰
Chitosan glutamate	 CS - NH ₃ ⁺	Colonna <i>et al.</i> ⁵¹ Huanbutta <i>et al.</i> ⁴⁵ Svindland <i>et al.</i> ⁵²
<i>Modified chitosan</i>		
Carboxy-methyl chitosan (CMC)	 example : <i>N,O</i> -Carboxymethyl chitosan	Upadhyaya <i>et al.</i> ⁵³ Review Upadhyaya <i>et al.</i> ⁵⁴ Review
Trimethyl chitosan (TMC) or quaternized chitosan		Domard <i>et al.</i> ⁵⁵ Boonyo <i>et al.</i> ⁵⁶ Mourya <i>et al.</i> ⁵⁷ Review Sadeghi <i>et al.</i> ⁵⁸

Table 1 : Chitosan derivatives commonly use in delivery system

II.3. Intrinsic parameters of chitosan: from characterisations to properties

Chitosan intrinsic parameters will influence its physico-chemical and biological properties. The degree of acetylation, the molar mass and the distribution of acetyl group were necessary to characterise, in order to evaluate their influence on chitosan formulation process and biological behaviours.

II.3.1. Degree of acetylation determination

The DA can be determined by various analytical techniques such as potentiometric titration⁵⁹ and many spectroscopic methods including Infrared,⁶⁰ Raman,⁶¹ UV,⁶² NMR (¹H, ¹³C, ¹⁵N) ⁶³⁻⁶⁸ spectroscopies and, recently, with HPLC.⁶⁹ ¹H NMR is a method of choice because of its accuracy and rapidity. Contrary to IR or UV, NMR does not require calibrations curves or polymer reference with known DA. Moreover, it is the only method proposed by the American Standard Test Method (ASTM) International for the DA calculation of chitosan based on the Vårum *et al.* method.⁶⁵ Hirai *et al.* also proposed an efficient method based on ¹H NMR spectroscopy⁶³ without treatment of chitosan, contrary to Vårum. The mole fraction of *N*-acetyl-D-glucosamine groups was calculated based on the below relation using integral intensities as described in Figure 2.

$$\text{DA (\%)} = \frac{\frac{1}{3} \times I_{\text{CH}_3}}{\frac{1}{6} \times I_{\text{H}_2-\text{H}_6}} \times 100$$

Equation 1 : Determination of the degree of acetylation of chitosan using the Hirai method.

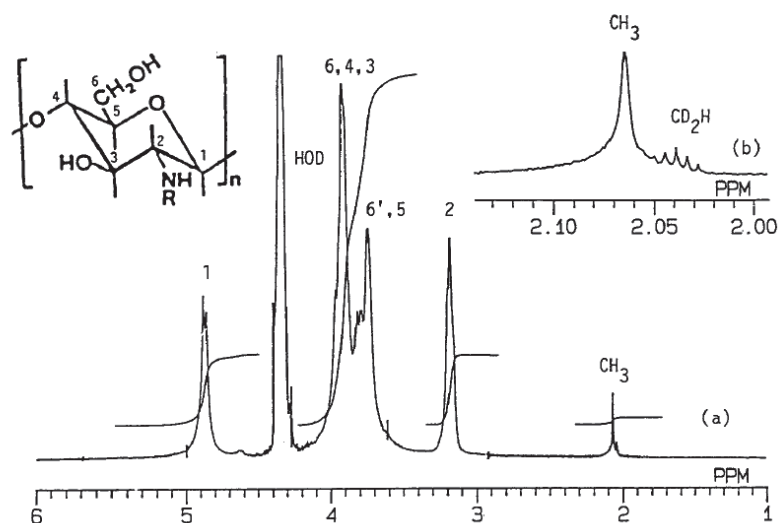


Figure 2 : 400 MHz ^1H NMR spectrum of chitosan in $\text{CD}_3\text{COOD}/\text{D}_2\text{O}$ at 70°C . From ⁶³ with permission.

II.3.2. Molar mass determination

Molar mass can be determined using various methods, such as viscosity,^{70,71} static light scattering or size-exclusion chromatography.^{72,73} Viscosity measurement, using capillary viscometer, determines the viscosimetric-average molar mass M_v using the Mark-Houwink equation $[\eta] = K \times M_v^a$. Based on many works reported in the literature, Kasaai⁷¹ proposed general equations for the determination of K and a for chitosan otherwise, K and a constants could be chosen in reference works⁷¹ or determinate by light scattering method⁷⁴ or by end-group analysis.⁷⁰ They were strongly depended on the experimental conditions: nature of solvent, temperature and chitosan parameters (DA, M_w). Static light scattering (SLS), via the Zimm method or coupled with size exclusion chromatography (SEC), was used to determine the weight-average molar mass M_w and the radius of gyration R_g . To calculate M_w , it is essential to determine the refractive index increment (dn/dc), depending on the physico-chemical properties of chitosan samples and experimental conditions.^{30,75} Sample preparation for light scattering experiments is crucial, as aggregates can form via intermolecular interactions and alter the average M_w .⁷⁶ SEC is a common method to determine the weight-average molar mass and the mass distribution of the polymer. SEC can be coupled with a refractometer and a multi-angle-laser-light-scattering (MALLS) detector to determine M_w , M_n , the polydispersity index, the radius of gyration and the actual concentration.⁷⁷

Experimental conditions and sample preparation, mainly solvent choice and the filtration of the polymer also interfere with the quality of the results.

II.4. From intrinsic properties to biological effects

As demonstrated previously, chitosan is not a single polymer, but there are many different chitosans, according to the molar mass and degree of acetylation. Though the mechanisms of chitosan biological activities are not fully identified, one can speculate that the cationic charges on the polymer chain would interact with most biological materials, mainly negatively charged. Many works demonstrated that physico-chemical properties of chitosan influenced its biological properties as reviewed by Aranaz *et al.*⁷⁸ We only review here pertinent aspects for the vaccine field. Biocompatibility, biodegradability, mucosal adhesion and antimicrobial properties were explored.

II.4.3. Safety and biocompatibility

The biocompatibility of an adjuvant is a prerequisite condition to develop any vaccine delivery system and thus, properties such as cytocompatibility, biodegradability and non-toxic properties were looked for.¹⁰

Biodegradability is an important aspect to take into account for the elaboration of a vaccine delivery system, because it should be eliminated from the host after the end of its action. Chitosan is degraded *in vivo* through the action of enzymes such as lysozyme or chitinases.¹⁹ The elimination of the polymer via the renal clearance needs a low molar mass, estimated for chitosan at around 10 kg.mol⁻¹.⁷⁹ By measuring the chitosan concentration in plasma after oral administration, Chae *et al.* suggested that the intestinal adsorption of the polymer decreased with the use of a high molar mass chitosan (> 230 kg.mol⁻¹).⁸⁰ The enzymatic degradation by lysozyme mainly depends on the degree of acetylation, which seems to be the major parameter impacting *in vitro* the depolymerisation kinetics.⁸¹⁻⁸³ Verheul *et al.* studied the degradation by lysozyme of chitosan and TMC for DAs ranging from 11 to 55%.⁸⁴ They showed that higher DAs allowed a faster and more significant depolymerisation of the polysaccharides. Hong *et al.* demonstrated that a sufficiently cross-linked chitosan hydrogel prevented the lysozyme degradation over 18 days.⁸⁵ Conversely, Mawad *et al.* introduced lysozyme in chitosan films to control the depolymerisation rate and reported that chitosan degradation was 3 times superior with lysozyme-loaded films than without enzyme.⁸⁶ *In vivo* studies reported similar results confirming the influence of DA on chitosan

degradation. Zhou *et al.* reported that acetylated chitosan microspheres were degraded faster in lysozyme media in case of *in vitro* experiments.⁸⁷ They also conducted *in vivo* experiments showing that particles implanted in rats and rabbits, were well tolerated and did not induce undesired reactions.⁸⁷ Moreover, the final formulation of chitosan as hydrogel or nanoparticles will influence the kinetics and the mechanism of degradation of chitosan *in vivo*.⁸⁸

The absence of toxicity of the materials used for delivery is essential to ensure the safety of the formulations. Toxicity was investigated by observing the local or general negative effects of increasing quantities of material administered to a host. Chitosan demonstrated a low toxicity in many cases for medical application such as wound healing,⁸⁹ tissue regeneration⁹⁰ or drug delivery system.⁹¹ For a long time now, chitosan toxicity was evaluated through different administration routes and formulations with *in vitro* or *in vivo* experiments. Baldrick⁸⁸ reported the absence of toxicity of chitosan by oral administration, recently confirmed by Lagarto *et al.* with a well-tolerated single oral dose of 2000 mg/kg in mice.⁴⁴ In a rabbit model, Rao *et al.* implanted chitosan films intramuscularly by surgery without any negative effects.⁹² In a study concerning human trial with a ground chitosan-based hydrogel injected through i.m. route, Neimert-Andersson *et al.* concluded that the polymer was safe and well tolerated by patients.⁹³ Minami *et al.* reported that subcutaneous administration induced anorexia above a 50 mg/kg and mortality above 150 mg/kg in dogs,⁹⁴ but not for other species.^{88,95} Considering that a standard dose of a vaccine formulation injected to humans or animals is around 1 mL (or less) containing from 0.5 to 2 % (w/v) of chitosan, a patient would receive approximately 5 to 20 mg of chitosan. Taking into account the weight of a child or young animal, the quantity of matter delivered would definitely be under the risk-value announced previously. Unfortunately, chitosan intrinsic parameters values were generally not or only partially detailed in many cases: consequently it is not possible to evaluate chitosan toxicity behaviour as a function of DA or M_w .

II.4.4. Mucosal adhesion properties

Mucosal surfaces, first gateway for pathogens, are very interesting administration routes for vaccine delivery due to the activation of specific mucosal immune system. Adjuvants with good mucoadhesive properties favoured residence time in the depot site and potentially enhanced the crossing of the mucosal barriers.^{16,96} The mucosal

adhesion of chitosan was mainly attributed to its cationic nature, as illustrated by its interactions with mucin, a negatively charged glycoprotein present on mucosal surfaces, driven by electrostatic interactions.⁹⁷⁻⁹⁹ Lerh *et al.* found that chitosan had superior mucoadhesive properties compared to non-ionic or anionic natural polymers¹⁰⁰ and highly acetylated chitosans showed a lower capacity to interact with mucin.⁹⁷ Moreover, the adhesion of the polysaccharide on mucosal surfaces was favoured by high molar masses, as a result of increased viscosities of the solutions and multiple attachment points. Dhawan *et al.* confirmed the mucoadhesive properties of chitosan microspheres synthesized through different methods described in the literature, with potential application in vaccine delivery.¹⁰¹ Results showed that the preparation method influenced the adhesive properties: better chitosan-mucin interactions were observed via emulsification and ionotropic gelation. They concluded that the zeta potential of the particles was the main factor affecting the amount of absorbed mucin, decreasing with a decrease in zeta potential.

In addition to its good mucosal adhesiveness, chitosan also exhibits a permeation enhancing effect, allowing the delivery of active compounds through the mucosal barrier.^{78,102} The interactions between chitosan, thanks to its positive charges, and the epithelial membrane opened the tight junction by a reorganization of associated proteins as proposed by Shipper *et al.*^{103,104} Many works reported this opening via *in vitro* and *in vivo* experiments with chitosan or its derivatives as reviewed by Benediktsdóttir *et al.*¹⁰⁵ Fundamental mechanism inducing this property is not completely elucidated and needs further explorations.

II.4.5. Antimicrobial activity of chitosan

Chitosan antimicrobial activity was reported many times in the literature, as reviewed by Kong *et al.*,¹⁰⁶ particularly for medical¹⁰⁷ or food¹⁰⁸ applications. Today, the mechanisms of action underlying the antibacterial effect still remain unexplained. Nonetheless, several factors were identified to play a key role in the antimicrobial activity such as the intrinsic parameters of chitosan, the nature of microorganism or experimental conditions. The degree of acetylation of chitosan greatly impacted the interactions with bacteria bearing anionic molecules on their surface.¹⁰⁶ Indeed, electrostatic interactions, similarly to the mucosal adhesion, mainly drove adhesion of chitosan on bacterium membrane. Mellegård *et al.* obtained a better antimicrobial

activity with a low DA (DA = 16 %) on various bacteria,¹⁰⁹ which is consistent with other works.^{110,111} Liu *et al.* reported the impact of the chitosan concentration on the antimicrobial activity: *Escherichia coli* (*E.coli*), a gram-negative bacterium, was killed with high polymer concentrations (starting to 200 ppm) regardless of molar mass.¹¹² They also showed that low molar mass chitosan, (below 7.10^4 g.mol⁻¹), inhibited bacterial growth, starting at 50 ppm polymer concentration. The influence of the molar mass on the antimicrobial activity of chitosan was studied many times, but the reported results were conflicting. For exemple, Zheng *et al.* reported that, for *E. coli*, low molar masses (< 48.5 kg.mol⁻¹) improved the antibacterial activity irrespective of the chitosan concentration.¹¹³ In the same study, with *Staphylococcus aureus*, a gram-positive bacterium, the authors observed the opposite behaviour: an enhancement of the antimicrobial activity with increasing molar masses (> 129 kg.mol⁻¹). Younes *et al.* also found that low DA and low M_w improved the antibacterial activity of chitosan with *E.coli*.¹¹⁰ Conversely, No *et al.* found that high molar mass (> 470 kg.mol⁻¹) chitosans induced stronger antibacterial activity, for *E. Coli* than oligomers (< 22 kg.mol⁻¹).¹¹⁴

Despite these discordant results, two main explanations were proposed. First, chitosan induced an impermeabilizing effect of the bacteria membrane via the formation of a complex between the cationic charges of chitosan and the anionic charges of bacteria. Thus, the bacteria were covered in a chitosan layer preventing the circulation of nutritive elements thus inducing the death of the pathogen. Second, the penetration of chitosan into the bacterium inducing a disturbance in the metabolism of the cell.^{106,113} Other effects were also observed and cell membrane disruption was proved many times.¹¹⁵⁻¹¹⁷ Chung *et al.* investigated the morphology of bacteria in presence of chitosan, with transmission electron microscopy (TEM). Results (Figure 3) clearly showed that chitosan damaged the outer membrane of *E. coli* and so induced its death.¹¹¹

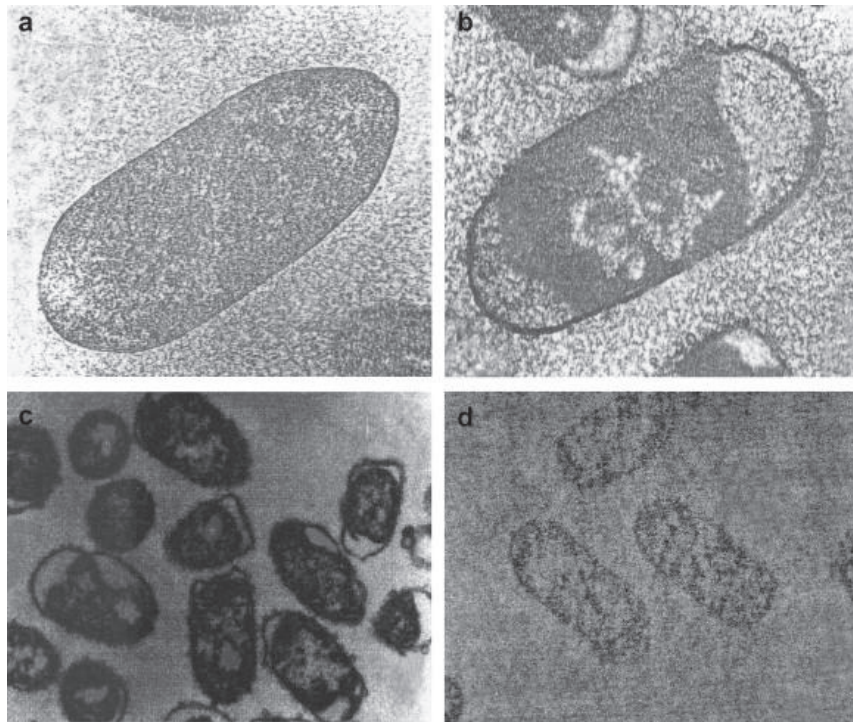


Figure 3 : TEM micrographs of intact cells of Escherichia coli when *E.coli* was exposed to chitosan (2500 mg/l, DA = 5%) after 0 hr (a), 2 hr (b), 4 hr (c), 6 hr (d) respectively. From ¹¹¹ with permission.

In order to use chitosan in vaccine delivery systems, particularly in the case of live attenuated bacteria vaccine, it will be imperative to preserve the viability of the pathogen within the chitosan matrix, to ensure a maximal efficacy of the vaccine.

III. CHITOSAN-BASED VACCINE DELIVERY SYSTEM

Four main formulations of chitosan can be found: solutions, hydrogels, micro and nanoparticles. The few published reviews on chitosan-based materials for vaccine delivery systems focused on particulate formulations applied to mucosal applications avoiding solutions and hydrogels.¹¹⁸⁻¹²⁰ One objective of this review is to provide a wide scope on the potentialities of chitosan as a promising material for vaccine delivery.

III.1. Chitosan-based solutions as vaccine adjuvants

Table 2 references studies focused on chitosan solution-based systems for vaccine delivery, reporting *in vivo* experiments for the last fifty years.

Chitosan specifications	Formulation	Antigen	Administration route	Animal model	Results	Ref.		
					Humoral immune response	Cellular immune response	Challenge	
Chitosan glutamate Protosan UP G210	0.5 % (w/v) chitosan in saline at pH 6	Influenza surface glycoproteins HA and NA	Intranasal	Female BALB/c mice	Systemic IgG ↑ Local IgA, IgG, IgM ↑	Not studied	No	Bacon <i>et al.</i> (2000) ¹²¹
Chitosan DA = 11,5 %	0.5 % (w/v) chitosan	H. pylori bacteria	Oral	BALB/c mice	IgG2a ↑ IgG1 ↑	Th1 ↑ (IL-2, IL-12, IFN-γ) Th2 ↑ (IL-4, IL-10)	Yes	Xie <i>et al.</i> (2007) ¹²²
(1) Chitosan DA = 7 % MW = 270 kg/mol	1.0 % (w/v) in 1% acetic acid and saline solution	Ovalbumin	Intranasal	Female BALB/c mice	IgG ↑ IgA ↑	Not studied	No	Boonyo <i>et al.</i> (2007) ⁵⁶
(2) Chitosan DA = 4 % MW = 500 kg/mol								
(3) TMC with DQ = 20, 40 and 60 % from chitosan (1)								
Chitosan Protosan G213	1.5 % (w/v) in DPBS	β-galactosidase	Subcutaneous	Female C57BL/6 mice	IgG ↑ IgG1 ↑ IgG2a ↑	Not studied	No	Zaharoff <i>et al.</i> (2007) ¹²³
Chitosan derivative DA = 25 %	0.5 % (w/v) chitosan in glutamate buffer	Inactivated influenza virus Influenza subunit	Intramuscular	BALB/c mice	Hemagglutination inhibiting antibodies ↑ Hemagglutination neutralizing antibodies ↑	Not studied	Yes	Ghendon <i>et al.</i> (2008) ¹²⁴
Chitosan DA = 25 % (1) MW = 300 kg/mol (2) MW = 10 kg/mol	(1) 0.5 % (w/v) chitosan in glutamate buffer (2) 0.05 % (w/v) chitosan in glutamate buffer	Inactivated influenza virus Influenza subunit	Intramuscular	BALB/c mice	HI antibodies ↑ HN antibodies ↑	CD3 ↑ NK ↑ CD 25 ↑	No	Ghendon <i>et al.</i> (2009) ¹²⁵
						I-AK (MHC II) ↑ H-2Db (MHC I) ↑		

Chitosan specifications	Formulation	Antigen	Administration route	Animal model	Results	Ref.		
					Humoral immune response	Cellular immune response	Challenge	
(1) Chitosan DA ≤ 17 % MW = 350 kg/mol	(1) 0.5% (w/v) in 0.5 % acetic acid pH (4.0)	His-GnRH-TR peptide	Subcutaneous	Male Sprague-Dawley rat	IgG ↑ IgG1 ↑ IgG2a ↑	Not studied	No	Sáenz <i>et al.</i> (2009) ¹²⁶
(2) Chitosan DA ≤ 25 % MW = 70 kg/mol	(2) 0.5% (w/v) in 0.5 % acetic acid pH (4.0)							
(3) Phosphorylated chitosan	(3) 0.5% (w/v)							
Chitosan hydrochloride DA = 5 – 30 %	0.5 % (w/v) chitosan in PBS	Live Newcastle Disease vaccine	Oculo-nasal	SPF white Leghorn chicken	no adjuvant effect	IFN-γ ↑	No	Rauw <i>et al.</i> (2010) ⁵⁰
Chitosan hydrochloride DA = 5 – 30 %	0.5 % (w/v) chitosan in PBS	Live Newcastle Disease vaccine and rHVT-ND	Oculo-nasal	Isa Brown layer chicken	IgG ↑ HI ↑	IFN-γ ↑	Yes	Rauw <i>et al.</i> (2010) ¹²⁷
Chitosan (Sigma)	0.4 % (w/v) chitosan in sodium acetate buffer pH 5	M1 protein (<i>Influenza subunit</i>)	Intranasal	Female BALB/c mice	IgG ↑ IgA ↑	IFN-γ ↑	Yes	Sui <i>et al.</i> (2010) ¹²⁸
TMC from Chitosan DA = 8 % MW = 120 kg/mol	Concentration mentioned TMC in PBS	Ovalbumin or Diphtheria toxoid	Intradermal	Female BALB/c mice	IgG1 ↑ IgG2a ↑	IFN-γ ↑ IL-4 ↑	No	Bal <i>et al.</i> (2010) ¹²⁹
Chitosan glutamate DA = 25 %	0.5 % (w/v) chitosan in glutamate buffer	Inactivated poliovaccine	Intramuscular	BALB/c mice	Neutralizing antibodies ↑	Not studied	No	Ghendon <i>et al.</i> (2011) ¹³⁰
Chitosan glutamate	0.5 % (w/v) chitosan	Influenza subunit	Intranasal	Female BALB/c mice	IgA ↑ IgG ↑ IgG1 ↑ IgG2a ↑ HI ↑ SRH ↑	(IFN-γ; IL-2) ↑ (IL-4; IL-5; IL-10) ↑ IL-17 ↑	No	Svindland <i>et al.</i> (2011) ⁵²

Chitosan specifications	Formulation	Antigen	Administration route	Animal model	Results	Ref.	
					Humoral immune response	Cellular immune response	Challenge
Protosan UP G213 DA = 10-25 % MW = 200 – 600 kg/mol	1.5% (w/v) chitosan in DPBS	Ovalbumin	Subcutaneous	C57BL/6 mice	IFN- γ \uparrow (mix with IL-12 immunostimulant) CD4+ and CD8+ T-cell responses	Heffernan <i>et al.</i> (2011) ¹³¹	
Protosan UP CL213	1 mg	Ovalbumin	Intraperitoneal Subcutaneous	Female C57BL/6, BALB/c, IL-1R1 ^{-/-} and NLRP3 ^{-/-} mice	IL-1 β \uparrow IL-1 α \uparrow IL-12p70 \uparrow IFN- γ \uparrow IL-17	Mori <i>et al.</i> (2012) ¹³²	No
(1) Chitosan (Sigma) DA = 24 % MW = 150 kg/mol (2) Chitosan (Chitopharm S) DA = 19 % MW = 80 kg/mol	(1) 0.5% (w/v) in PBS (2) 0.5% (w/v) in PBS	Ovalbumin	Subcutaneous	C57BL/6 mice	IFN- γ \uparrow CD4+ and CD8+ T cells \uparrow	Scherließ <i>et al.</i> (2013) ¹³³	No
N-trimethylaminoethylmethacrylate chitosan (TMAEM-C)	0.5 % (w/v) TMAEM-C in HEPES buffer	Ovalbumin	Intranasal	Female BALB/c mice	IgG \uparrow IgG1 \uparrow IgG2a \uparrow sIgA \uparrow	Liu <i>et al.</i> (2014) ¹³⁴	No
Ultrapur chitosan (Golden-shell biochemical Inc.)	50 mg in 500 μ L	CTB-UE protein (<i>H. Pylori subunit</i>)	Oral	SPF Balb/c Male	IFN- γ \uparrow IL-17 \uparrow	Xing <i>et al.</i> (2015) ¹³⁵	No

Table 2 : Chitosan-based solutions used in vaccine formulations.

III.1.1. Chitosan solutions as versatile adjuvants

Chitosan was investigated in various studies, demonstrating its ability to enhance protein antigenicity. Boonyo *et al.* mixed chitosan and OVA before inoculation via intranasal administration in mice.⁵⁶ They examined the influence of chitosan molar mass (270 and 500 kg.mol⁻¹) and the influence of the degree of quaternization (DQ) of TMC on the immune response, determined by IgG and sIgA titrations. The results indicated that every chitosan formulation induced a better systemic or local immune response than the antigen without adjuvant. Chitosan with high molar mass induced a higher IgG and IgA response than medium molar masses. TMC with DQ = 40% induced the best immune response with the highest IgG and sIgA antibody titres. Aluminium control group induced good IgG response but did not elicit IgA secretion. Zaharoff *et al.* mixed chitosan with β -galactosidase, a model antigen protein, to evaluate its adjuvanticity in mice via subcutaneous administration.¹³⁶ Chitosan glutamate was solubilised in PBS at 1.5% (w/v) which was higher than the concentration usually injected around 0.5% (w/v), as reported in Table 2, in order to obtain a high viscosity to achieve the gel depot. Fluorescent-labelled proteins were monitored *in vivo* to evaluate the sustained release from chitosan solutions. They reported that more than 60 % of the initial protein load remained at the injection site 7 days after administration (Figure 4). In the presence of chitosan, antigen-specific IgG antibody titers were increased five-fold and antigen-specific CD4⁺ proliferation six-fold. Chitosan was found to be equivalent to Freund's adjuvant and superior to alum with antigen-specific antibody titers over six-fold higher.

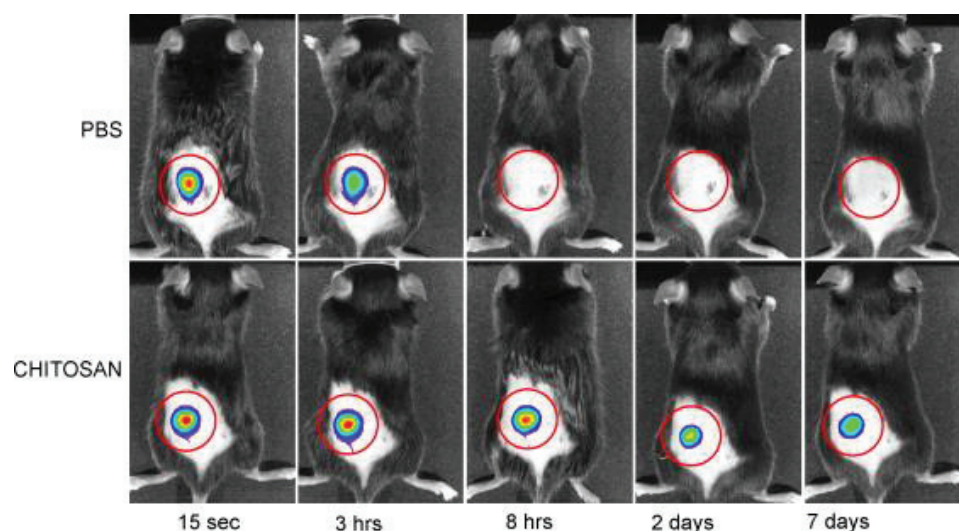


Figure 4 : Chitosan maintained a depot of β -galactosidase. Spatiotemporal distributions of a single subcutaneous administration of a fluorescently-labeled model antigen acquired via non-invasive fluorescence imaging. From ¹³⁶ with permission.

Rauw *et al.* investigated an animal model other than mice and confirmed the adjuvant properties of the chitosan solutions.⁵⁰ Chitosan solutions were mixed with live Newcastle disease virus vaccine before oculo-nasal administration in specific-pathogen-free (SPF) white Leghorn chicken. They reported the ability of chitosan to enhance the antigen-specific cell-mediated immune response in the spleen. Chitosan was also reported to offer best protection in challenge tests for Newcastle disease when administered before *in ovo* vaccination with a turkey herpes recombinant virus expressing the fusion gene of NDV (rHVT-ND).¹²⁷

Despite a lack of information on the preparation of chitosan-based solutions, the materials seemed easy to prepare and were used in many studies as reported in Table 2. We also note that crucial information such as DA or M_w were not provided, or simply based on imprecise manufacturers' data. When these informations were reported, chitosans with DAs less than 30 % and with a molar mass range between 70 and 500 kg.mol⁻¹ were selected. The impact of chitosan intrinsic parameters on the quality and intensity of the immune response is thus difficult to establish and would require a systematic investigation. Nevertheless, chitosan was able to induce satisfying immune response whatever, the molar mass, as suggested by Sáenz *et al.*¹²⁶ They reported the induction of both IgG1 and IgG2a antibodies with low and high M_w . Studies also

demonstrated that chitosan allowed an enhanced immune response using various biological materials such as live attenuated virus, bacteria (*H. Pyroli*),¹²² protein as ovalbumin or influenza subunit. Mori *et al.* demonstrated that, contrary to alum, chitosan did not inhibit IL-12 cytokines secretion and enhanced the cell-mediated immune response.¹³² Moreover, when chitosan was associated with CpG as immunostimulant, the responses were equivalent or superior to those with alum and CpG for the IL-1 β , IL-12, IFN- γ , IL-17 and IgG1 production. It is interesting to note that the association of both chitosan and CpG induced significant better IgG antibody titers than separately. Baudner *et al.* reported similar effects with a mixture of chitosan microparticles and LTK-63 adjuvant against *C meningococcus* bacterium.¹³⁷

III.1.2. Chitosan solutions as adjuvant for influenza virus vaccine

Bacon *et al.* prepared chitosan solutions mixed with two antigenic surface glycoprotein of the influenza virus (hemagglutinin (HA) and neuraminidase (NA)) in order to evaluate the adjuvant properties of the polymer.¹²¹ The vaccine was administrated by the intranasal route in female BALB/c mice. The systemic immune response was evaluated by serum IgG antibody titration. The local respiratory immune response was determined by the IgA titration in nasal secretions and IgA, IgG and IgM secreted from nasal and pulmonary lymphocytes were also evaluated. The antigens alone did not induce any strong systemic nor local immune responses, while chitosan significantly enhanced the IgG and IgA production. Moreover, the intranasal administration of chitosan lead to a better local response than alum adjuvanted formulations injected subcutaneously. After three injections, the IgA titers in nasal secretions from animals immunized with the chitosan-adjuvanted formulations were superior to those of the alum reference group that only induced a week immune stimulation.

In similar experiments, Ghendon *et al.* administrated intramuscularly to BALB/c mice a subunit influenza virus mixed with chitosan.¹²⁴ They demonstrated that the chitosan concentration influenced antibody titres, reaching an optimum for 0.5% (w/v). They reported that chitosan significantly increased the immune response in comparison with chitosan-free formulations, three to six times greater depending on the vaccine. Moreover, challenge test with the administration of the viral strain confirmed the protective effectiveness of chitosan based vaccines. After the second administration,

chitosan offered a complete protection of mice after a viral challenge using a concentration of 1,000-fold higher than the median lethal dose compared to a 30 % protection for non-adjuvanted formulation. Svindland *et al.*⁵² and Sui *et al.*¹²⁸ also reported good results with the intranasal administration of chitosan adjuvanted influenza vaccine in mice.

III.2. Chitosan hydrogels as efficient sustained release vaccine delivery system

Peppas *et al.* defined hydrogels as three-dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids.¹³⁸ Hydrogels were largely investigated in various applications as reviewed by Amhed¹³⁹ and particularly in biomedical¹⁴⁰ fields as drug delivery or tissue engineering.¹⁴¹⁻¹⁴³ Hydrogel formation can occur via chemical or physical assembly of the parent polymer. Thanks to its cationic behaviour and copolymer structure, chitosan allowed the design of hydrogels through both synthetic routes. Although chitosan-based hydrogels were intensively explored for medical applications as reviewed by Berger *et al.*¹⁴⁴ or Bhattarai *et al.*,¹⁴⁵ the vaccine field was investigated only within the last ten years (recapitulated in Table 3) and appeared to be an open research area.

Delivery System	Formulation of hydrogel	Antigen	Adjuvant	Administration route	Model	Results	References
<i>Physical Hydrogel</i>							
Thermo-sensitive chitosan hydrogel	Chitosan (no information) Glycerol 2-phosphate disodium hydrate	Ovalbumin	No	Subcutaneous	Male C57B1/6 and OT-I and OT-II transgenic mice	CD8 ⁺ and CD4 ⁺ T cells expansion ↑ IgG ↑	Gordon <i>et al.</i> (2008) ¹⁴⁶
Silica nanoparticles loaded Thermo-sensitive chitosan hydrogel	Chitosan (DA= 15-25% ; Mw= 50-190 kg.mol ⁻¹) Glycerol 2-phosphate disodium hydrate	Ovalbumin	Quil A	Subcutaneous	Female C57B1/6 and OT-I and OT-II transgenic mice	IgG ↑ CD8 ⁺ and CD4 ⁺ T cells expansion ↑	Gordon <i>et al.</i> (2010) ¹⁴⁷
Liposomes and cubosomes loaded thermo-sensitive chitosan hydrogel	Chitosan (no information) Glycerol 2-phosphate disodium hydrate	Ovalbumin	Quil A	Subcutaneous	Male C57B1/6 and OT-I and OT-II transgenic mice	CD8 ⁺ and CD4 ⁺ T cells expansion ↑ IFN-γ ↑ IgG ↑	Gordon <i>et al.</i> (2012) ¹⁴⁸
Thermo-sensitive chitosan derivatives hydrogel	Chitosan (DA = 5% Mw = 780 kg.mol ⁻¹) derivative (HTTC) α,β-glycerol-phosphate	Split H5N1 antigen	No	Intranasal	Female BALB/c mice	IgG, IgG1; IgG2a; IgA; HI ↑ IFN-γ ; IL-4 ↑ CD8 ⁺ T cells expansion ↑	Wu <i>et al.</i> (2012) ¹⁴⁹
Chitosan-based hydrogel for microneedles	Chitosan 10 % (w/v) : pH = 6	Ovalbumin	No	Intradermal	Male Sprague-Dawley rat	IgG ↑	Chen <i>et al.</i> (2013) ¹⁵⁰
Thermo-sensitive chitosan hydrogel	Chitosan (DA = 15-25%; Mw = 50-190 kg.mol ⁻¹) 1% (w/v) Methyl cellulose 0.5% (w/v) Glycerol 2-phosphate disodium hydrate 9% (w/v)	Ovalbumin	(a) Quil A (b) MPL	Subcutaneous	Female C57B1/6 and OT-I and OT-II transgenic mice	CD8 ⁺ and CD4 ⁺ T cells expansion ↑ IgG ↑ IFN-γ ↑	Kojarunchitt <i>et al.</i> (2015) ¹⁵¹

Delivery System	Formulation of hydrogel	Antigen	Adjuvant	Administration route	Model	Results	References
Two-components cross-linkable chitosan hydrogel	Part 1 : Chitosan / Hydroxyapatite Part 2 : TPP / Chondroitin sulfate	Ovalbumin	(a) Montanide ISA50 (b) Montanide ISA61 (c) Freund's Adjuvant (complete or incomplete) (d) aluminium hydroxide gel (e) Quil A	Subcutaneous	Male BALB/c mice	OVA-antibodies ↑ (with adjuvant)	Chua <i>et al.</i> (2015) ¹⁵²
Chemical hydrogel							
Chitosan-based hydrogel : Viscogel™	Chitosan hydrochloride Diethyl squarate	Act-HIB	No	Subcutaneous Intramuscular	Female mice	IgG1 ↑; IgG2a ↑ IL-2; IL-4; IL-6 ; IFN-γ ; TNF ; IL-17A ; IL-10 ↑	Neimert-Andersson <i>et al.</i> (2011) ¹⁵³
Chitosan-based hydrogel : Viscogel™	Chitosan hydrochloride Diethyl squarate	Act-HIB	No	Subcutaneous Intramuscular	Healthy volunteers of both sexes, ages 22-50	No significant results	Neimert-Andersson <i>et al.</i> (2014) ⁹³

Table 3 : Chitosan-based hydrogels for vaccine delivery systems. Thermo-sensitive systems were the most used due to the injection comfort and rapid gel formation.

III.2.3. Preparation of physical hydrogels

Physical hydrogels resulted from the establishment of various non-covalent interactions between polymer chains.¹⁵⁴ The main interactions involved are summarized in Figure 5: (a) hydrophobic interactions, leading to block aggregation with increasing temperature; (b) electrostatic interactions with an oppositely charged moiety (a small molecule or polymer); (c) Low energy hydrogen-bonds.

In case of chitosan hydrogels, the size of the cross-linker, the global charge of the polysaccharide and cross-linker mainly affected the kinetic of the reaction. Many other parameters such as the molar mass, the DA and the concentration of chitosan but also the concentration of the cross-linking agent and the duration of the reaction affected the final characteristic of the hydrogel.¹⁴⁴

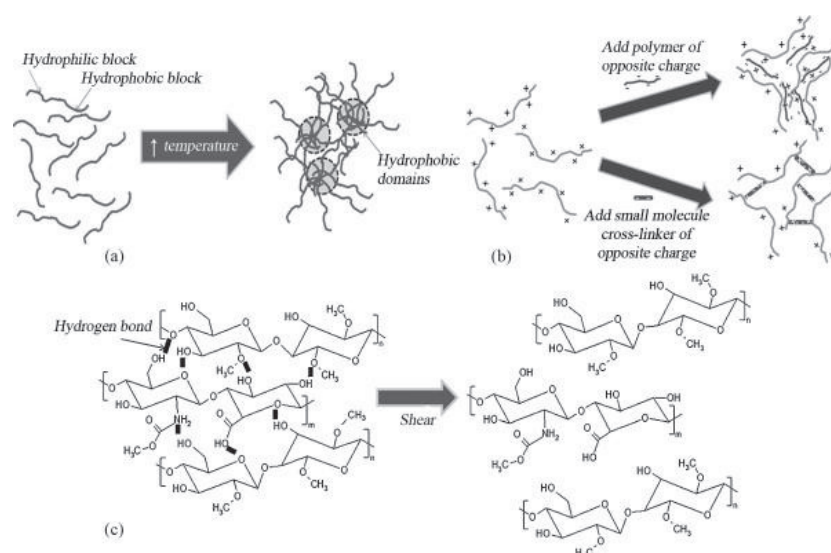


Figure 5 : Mechanisms of preparation of physical hydrogel (a) physical gelation driven by hydrophobic interactions (b) physical gelation based on charge interactions with an oppositely-charged polymer or an opposite-charged small molecule (c) physical gelation via hydrogen bonding interactions. From ¹⁵⁴ with permission.

III.2.4. Chemical hydrogel preparation

Chemical hydrogels were obtained via a large variety of chemical reactions : radical polymerization, high energy irradiation or by enzymatic processes.¹⁴⁰ Common cross-linkers were reviewed by Bhattarai *et al.* (Figure 5 in section III.2).¹⁴⁵ Genipin was frequently exploited with chitosan in nanoparticles^{155,156} or hydrogels¹⁵⁷ based delivery systems. Genipin proved to be less toxic *in vivo* than glutaraldehyde, offering a cross-linking agent meeting the requirements of biomedical applications.

III.2.5. Vaccine delivery system based on chitosan physical hydrogel

Thermo-sensitive hydrogels materials (or thermo-responsive, temperature-sensitive) undergo a sol-gel transformation, upon a thermal stimulation. Thus, the formulation, liquid at room temperature and thus injectable, gelled at body temperature leading to a 3D depot implanted without surgery.¹⁴⁷ Benefits of these systems according to Vermonden *et al.* were: an increase in contact time of the vaccine in the host organism, favouring the interactions with the immune system; a protection of the antigen against degradation; an enhancement of the immune response and a decrease in the number of injections and in the antigen dose.¹⁴¹ In tissue engineering applications, Chenite *et al.* added glycerol-phosphate (GP) salts to elaborate an *in situ* gelling chitosan solution in order to deliver growth factors.¹⁵⁸ The addition of GP salts had two effects: an increase in the solubility of the system for a pH value range between 6.8 to 7.2 and a temperature-triggered gelation process. Various studies in biomedical applications using glycerophosphate-based chitosan hydrogels were conducted, as reviewed by Zhou *et al.*

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Gordon *et al.*, with this technology, modified the retention of ovalbumin loaded into silica nanoparticles (SNPs).¹⁴⁷ *In vitro* experiments, carried out for 14 days, showed that the cumulative release of OVA was around 90 % from SNPs in PBS, indicating that the protein was not permanently entrapped in particles. Once SNPs were incorporated within a chitosan gel, only 35 % of OVA were released, under its soluble form, and 15 % entrapped into SNPs demonstrating that proteins were maintained during an extended period compared to PBS. *In vivo* experiments reported that mixing chitosan-based hydrogel with SNP influenced the chitosan final immune response. CD₄⁺ T cell expansion and OVA-specific antibody titres were greater than for SNP alone, showing the beneficial effect of the hydrogel. A depot effect inducing sustained release was assumed to enhance *in vivo* the immune stimulation. In other experiments, Gordon *et al.* observed an immune response by the subcutaneous injection of an OVA-loaded *in situ* forming hydrogel of chitosan, contrary to chitosan nanoparticles tested in the same conditions.¹⁴⁶ Wu *et al.*¹⁴⁹ administrated hydrogel¹⁶⁰ loaded with H₅N₁ vaccine via the intranasal route. Fluorescence imaging, using a labelled antigen, showed a significantly improved retention in hydrogel with 6 times more antigen remaining in the nasal cavity of the mice after 2 h than in PBS (Figure 6).

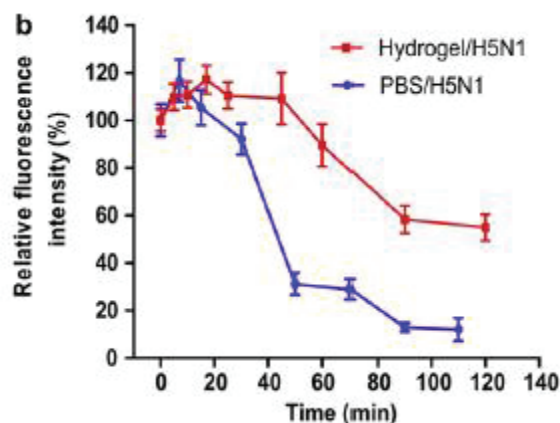


Figure 6 : Relative fluorescence intensity detected after intranasal administration of NHS-Cys5 labeled H5N1 in PBS or hydrogel at different time intervals. From ¹⁴⁹ with permission.

Immunological results showed a high mucosal response (sIgA) and a humoral immune response (IgG) superior to MF59, a commercial adjuvant, prepared and injected in the same conditions. With hydrogels, both the humoral and cellular immune responses were enhanced, with a better production of IL-4 and IFN- γ than with the unadjuvanted antigen and similar to MF59. Finally, the chitosan hydrogel stimulated the CD8+ T cell immune memory, offering an increased protection against future infection. Recently, Adams *et al.*¹⁶¹ and various authors, reviewed by Lofthouse¹⁶², demonstrated the beneficial effect of sustained release of proteins to activate the immune system in similar experiments.

Bhattarai *et al.* by grafting polyethylene glycol (PEG) on chitosan obtained a thermo-sensitive chitosan derivative PEG-g-Chitosan.¹⁶³ The amount of grafted PEG impacted the BSA release by alteration of the solubilisation kinetics of the PEG-g-Chitosan derivative. Total dissolution was attained within 3 weeks for 45% grafted chitosan, and thus the total release of protein. They also confirmed the absence of denaturation of the released protein.

Recently, Chua *et al.* proposed a system based on physical cross-linking of chitosan to prepare a composite hydrogel.¹⁵² A two component preparation, with a chitosan/hydroxyapatite mixture containing OVA/adjuvant and a tripolyphosphate (TPP)/ chondroitin sulphate mixture was injected via a co-injection system (Figure 7) forming a gel at the injection site. TPP and chondroitin sulphate were used as cross-linkers for chitosan, according to many authors.^{164,165} Hydrogels with OVA did not induce better response than OVA alone, while the immune response was drastically

higher in the presence of montanide ISA61. This hydrogel system allowed an effective protection for one year.

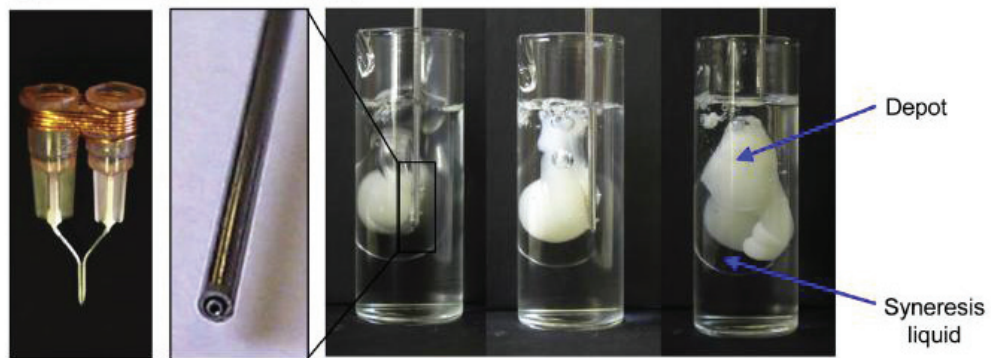


Figure 7 : Co-injection system containing chitosan/ hydroxyapatite and TPP/Chondroitin sulfate. The gel was spontaneously formed at the exit of the concentric arrangement of needles. From ¹⁵² with permission.

III.2.6. Vaccine delivery system based on chitosan chemical hydrogel

Neimert-Andersson *et al.* proposed a chemical hydrogel, called ViscoGel™, made with chitosan hydrochloride mixed with diethyl squarate (DES) (Figure 8) for 3 days at 40°C.¹⁵³ De Angelis *et al.* proposed a possible structure (Figure 9) of the chemical bridges responsible for the formation of the gel with the reaction of the amine group of chitosan and the DES.¹⁶⁶ Then, the gel was ground to allow injection in animals.

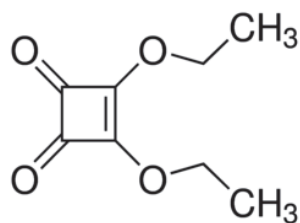


Figure 8 : Chemical structure of diethyl squarate

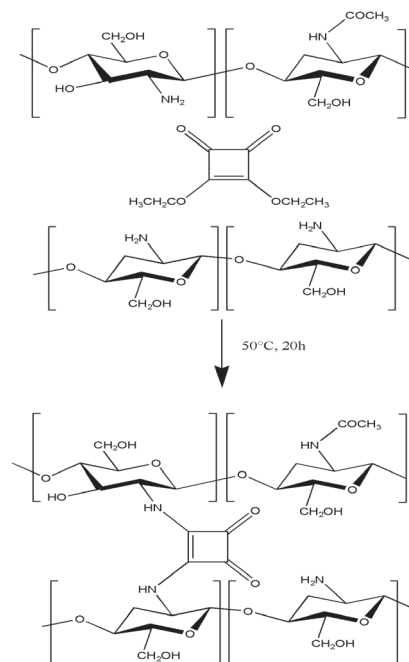


Figure 9 : Possible structure of chitosan-based hydrogel cross-linked with diethyl squarate. From ¹⁶⁶ with permission

ViscoGel™, was mixed with vaccine against *Haemophilus influenza* type b (Act-HIB®) and injected in mice. It did not induce side nor toxic effects after implantation and was totally eliminated after three weeks. ViscoGel™/Act-HIB® induced both cellular and humoral immune responses, superior to Act-HIB® alone in serum in both s.c. and i.m. administration routes. Notable results were the reduced antigen dose down to ten-fold with a similar immune response between 2 µg for Act-HIB® alone and 0.2 µg for ViscoGel™/Act-HIB®. One interesting observation in their pre-clinical evaluations on mice was that the immune competent cells rapidly infiltrated the hydrogel material,¹⁵³ this infiltration being probably responsible for the cell-mediated response. Neimart-Andersson *et al.* conducted a human clinical trial based on their promising preliminary results with chitosan hydrogel. ViscoGel™ safety was evaluated for various dose levels and reported to be satisfying for vaccine development.⁹³ Conversely to their pre-clinical evaluations, no adjuvant effect was detected in humans with an antigen loaded chitosan hydrogel. The anti-HIB antibody production in the chitosan group was similar to the group without chitosan for each antigen dose and at every time point monitored after vaccination. Nevertheless, they reported a beneficial effect of chitosan that allowed the production of IFN-γ normally inhibited by Act-HIB alone. To explain this effect, it was proposed that the slow release of Act-HIB® from the hydrogel modified the antigen presentation kinetics to immune cells.

III.3. Chitosan-based micro/nanoparticles for vaccine delivery system

Chitosan based micro and nanoparticles were intensively investigated over the last twenty years as vaccine delivery system.¹⁶⁷ Amidi *et al.*¹¹⁹ and Arca *et al.*¹²⁰ already reviewed chitosan-based delivery systems for protein and vaccine antigen, focusing on particle carriers. Consequently, we only review here studies over the last five years. Moreover, we focus on studies presenting *in vivo* experiments for prophylactic vaccines.

III.3.1. Preparation of chitosan based particles

Various methods were proposed to elaborate chitosan-based micro or nanoparticles. Several reviews reported these systems for drug delivery system such as Agnihtori *et al.*¹⁶⁸ or Sinha *et al.*¹⁶⁹. Specific methods to elaborate chitosan-based particles for vaccine delivery system were addressed by Amidi *et al.*¹¹⁹ and Islam *et al.*¹⁷⁰. We summarized these methods in table 4 and discussed advantages and disadvantages in the case of vaccine development.

Methods	Principe	Use in vaccine delivery		References
		Advantages	Disadvantages	
Ionic gelation	Tripolyphosphate (TTP) or small anionic molecule mixed with chitosan solution under stirring	<ul style="list-style-type: none"> - high encapsulation efficiency on protein - range size (from 100 to 1000 nm) 	<ul style="list-style-type: none"> - Low stability in physiologic media - collection of nanoparticles (use of glycerol bed) 	Calvo <i>et al.</i> ¹⁷¹ Bugnicourt <i>et al.</i> ¹⁶⁴ Antoniou <i>et al.</i> ¹⁷² Haung <i>et al.</i> ¹⁷³
Precipitation/coacervation	Precipitation of chitosan by addition of salt or coacervation by droplet in an alkaline media. Particles are washed several times with water and collected.	<ul style="list-style-type: none"> - no solvent - no cross-linker - good encapsulation yield - large range size (nano to microparticles) 	<ul style="list-style-type: none"> - low synthesis reproducibility 	Berthold <i>et al.</i> ¹⁷⁴ Koppolu <i>et al.</i> ¹⁷⁵
Polyelectrolyte complexation	Dilute anionic polymer (alginate, dextran sulfate, heparin...) mixed with dilute chitosan under magnetic stirring at room temperature	<ul style="list-style-type: none"> - no solvent - no cross-linker - no surfactant - controlled surface charge - high adsorption efficiency - range size (from 200 to 1000 nm) 	<ul style="list-style-type: none"> - limited lower size (not under 200 nm) - low concentration 	Luo <i>et al.</i> ¹⁷⁶ Sæther <i>et al.</i> ¹⁷⁷ Weber <i>et al.</i> ¹⁷⁸ Delair ¹⁷⁹ Liu <i>et al.</i> ¹⁸⁰
Spray-drying	Chitosan solution is sent with air flux through a nozzle equipping spray dryer system. Chitosan obtained is kept under powder form or cross-linked.	<ul style="list-style-type: none"> - high encapsulation efficiency 	<ul style="list-style-type: none"> - range size(> 1µm) and dispersity - cross-linker agent 	Illum <i>et al.</i> ¹⁸¹ Mi <i>et al.</i> ¹⁸² Estevinho <i>et al.</i> ¹⁸³
Emulsification-based synthesis methods	Preparation of water-in-oil emulsion followed with various steps (depending on authors): <ul style="list-style-type: none"> - ionic gelation - chemical gelation - both ionic and chemical gelation - solvent evaporation - coalescence 	<ul style="list-style-type: none"> - good encapsulation yield - large range size (nano to microparticles) 	<ul style="list-style-type: none"> - organic solvent - surfactant 	Brunel <i>et al.</i> ¹⁸⁴ Kofuji <i>et al.</i> ¹⁸⁵

Table 4: Elaboration methods of chitosan-based particles for delivery systems. Advantages and disadvantage in case of pharmaceutical and vaccine applications.

Ionic gelation with TPP was confined to the production of dispersions at low particle solid content, up to 0.2 % (w/v). Recently, Sipoli *et al.* proposed a process to elaborate dispersions at 0.5 % (w/v) concentration, using controlled experimental conditions in a tank reactor.¹⁸⁶ Nevertheless, storage stability is an important limitation of this method. Chitosan/ TPP nanoparticles, as reported by various authors, preserved their colloidal properties only at low pH.¹⁷³ These restricted conditions are not compatible with most biological environments. Indeed, isotonic conditions and

physiological pH are required for injection in host to avoid local undesired reactions and pain after injection.

Until now, chitosan-based polyelectrolyte complexes (PECs) were prepared in dilute conditions (below than 0.5 % (w/v)) to obtain submicronic particles as mentioned by Luo *et al.*¹⁷⁶ or Delair.¹⁷⁹ Costalat *et al.* overcame this limitation by dialysing a mixture of chitosan, dextran sulphate solutions and sodium chloride to screen the attractive electrostatic interactions.¹⁸⁷ They reached a particle concentration in water as high as 3 % (w/v) with high stability, up to two years at 4°C. With a size range similar to viruses and a fair capability to adsorb proteins, PECs appeared to be an interesting strategy to prepare a carrier material for vaccine delivery. The colloidal stability in phosphate buffered saline (PBS) buffer was established for various concentrations at room temperature and 37°C by Costalat *et al.*¹⁸⁸

III.3.2. Influence of the particle size in the immune response

Kreuter defined nanoparticles as solid particles ranging in size from 10 nm to 1000 nm (1µm) and microparticles from 1 µm to 1000 µm (1 mm).¹⁸⁹ They consisted in the macromolecular materials in which the active pharmaceutical ingredient (API) was dissolved, entrapped, or encapsulated, and/or to which the active material was adsorbed or attached. Leleux *et al.* summarized studies on the impact of the particle-size (made from various polymers) on the immune system and they pointed out that the particle size could strongly affect the interaction with the immune cell and their biodistribution in the host.¹⁹⁰

Foged *et al.* studied the uptake of negatively charged polystyrene (PS) nanoparticles by dendritic cells (DCs).¹⁹¹ Particles with a diameter of 0.5 µm or below were preferably uptaken by DCs. After the surface charge inversion with proteins or polypeptides, the authors showed that a positive charge enhanced the uptake of large size particles by DCs. Manolova *et al.* reported that the trafficking of PS nanoparticles to lymph nodes (LN) was dependant of their size, when injected to mice.¹⁹² Small particles from 20 to 200 nm were freely transported to LN whereas particles with diameters between 500-2000 nm were carried by DCs. They concluded that only small particles could specifically target LN-resident cells. Shima *et al.* conducted *in vivo* experiments to observe the size effect of poly(γ-glutamic acid) NPs on cellular uptake and maturation of DCs.¹⁹³ They observed a higher activation of DCs with small particles, 40 nm, compared to 100 and 200 nm particles also that a quicker migration to LN for the lower size NPs.

As previously, it was concluded that the decrease in particle size induced a lower uptake by DCs but that the 40 nm NPs were found to stimulate DCs maturation in LN. Characteristics of particles, particularly their sizes, directly influenced the primary immune response inducing an adaptive response.¹⁹⁴ Interactions between particles and antigen-presenting cells will be favoured at the injection site for larger sized particles and in LN for smaller sizes. These associations allow to carry the antigen and to activate DC maturation. Stano *et al.* injected NPs 30 nm or 200 nm in diameter, associated with OVA antigen in mice via the intranasal route.¹⁹⁵ They showed that larger NPs enhanced the magnitude of CD4+ T cells responses for mucosal and systemic humoral immune responses. IgG and IgA antibody titers also increased with 200 nm OVA-loaded NPs compared to 30 nm particles.

III.3.3. Interactions between antigen and particles in the immune response

According to Zhao *et al.*,¹⁶⁷ antigen-particle interactions are divided into four main groups (Figure 10) : conjugation, encapsulation, adsorption or mix (with no established interactions)

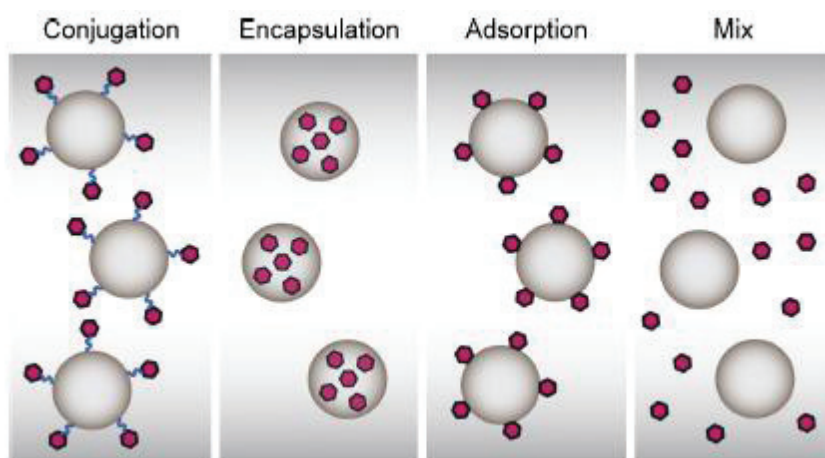


Figure 10 : Main group of interactions between particle and antigen. From¹⁶⁷ with permission.

Several studies examined the influence of carriers / antigens interactions in order to elaborate the most efficient adjuvant system.¹⁹⁶ Encapsulation and adsorption were the most widely used assembly processes. For encapsulation, the antigen was added to the polymer solution before particle formation. Main claims for encapsulation of antigen were to use particles as a tank to inject simultaneously a carrier and a sustained release system¹⁹⁷ ensuring also the protection of the antigen against degradations.¹⁹⁸ For the adsorption process, the antigen was added to pre-formed particles and the final

assembly featured a structure inspired by viruses (nanoparticles) or a bacteria (microparticles), positively impacting the quality of the immune response.¹⁹⁹ Many parameters can influence the carrier loading capacity such as the concentration ratio of antigen to particles, the media for assembly, particle size and zeta potential. For example, using chitosan-based polyelectrolyte complexes, Drogoz *et al.* reported HIV-1 p24 sorption yields ranging from 25 to 75 % depending on the sorption buffer.²⁰⁰

Although less explored, conjugated systems were successfully investigated. Slütter *et al.* reported that TMC conjugated with ovalbumin (TMC-OVA nanoconjugates) offered best immunizations in mice with higher IgG titers compared to an OVA solution, a mixture of OVA/TMC and OVA loaded TMC NPs.²⁰¹ DCs maturation was also drastically increased with TMC-OVA demonstrating a strong initiation of the adaptive immune response. Finally, conjugation was found to maintain the immunogenicity of the antigen, as demonstrated by Western blot. Conjugation could be used to elaborate antigen loaded NPs decorated with a targeting species as proposed by Jung *et al.*²⁰² They reported a DCs-targeting peptide conjugated to chitosan nanoparticles, encapsulating OVA as model antigen. Mice were subcutaneously immunized and OVA-specific antibody titration showed that the best immune response was obtained for OVA loaded peptide-NPs with higher IgG antibody titers. IgG1 and IgG2a, markers for Th2 and Th1 type immune response, was also found to be higher than with OVA or OVA loaded blank NPs.

III.3.4. Chitosan based particles for prophylactic vaccine delivery system

Chitosan specifications	Formulation		Antigen	Administration route	Animal model	Results		Ref.	
	Synthesis Method	Particle Size				Antigen-Particle Interaction	Humoral immune response		Cellular immune response
TMC DQ = 20 % from Chitosan DA = 8% $M_w = 120 \text{ kg.mol}^{-1}$	(A) Conjugation antigen-chitosan (B) ionic gelation (TPP)	(1) 28 nm (2) 312 nm	(1) Conjugation (2) Mix	Ovalbumin	Intranasal	Female BALB/c mice	IgG ↑ IgA ↑ Not studied	No No	Slütter <i>et al.</i> (2010) ²⁰³
TMC DQ = 15 % from Chitosan DA = 8 % $M_w = 120 \text{ kg.mol}^{-1}$	Ionic gelation (TPP or CpG)	300 nm	Encapsulation	Ovalbumin	Intranasal	Female BALB/c mice	IgG ↑ IgA ↑ IgG1/ IgG2a ratio ↑ (depending on cross-linker)	IFN-γ ↑ (with CpG) No	Slütter <i>et al.</i> (2010) ²⁰⁴
TMC DQ = 20 % from Chitosan DA = 8% $M_w = 120 \text{ kg.mol}^{-1}$	Ionic gelation (TPP)	280 nm	Encapsulation	Ovalbumin	Intramuscular	Female BALB/c mice	IgG ↑	Not studied	Slütter <i>et al.</i> (2010) ²⁰¹
TMC DQ = 20 % from Chitosan DA = 8% $M_w = 120 \text{ kg.mol}^{-1}$	Ionic gelation (TPP)	from 219 to 344 nm	Encapsulation	OVA or Diphtheria toxoid	Intradermal	Female BALB/c mice	IgG1 ↑ IgG2a ↑	IFN-γ ↑ IL-4 ↑	Bal <i>et al.</i> (2010) ¹²⁹
TMC DQ = 15 % from Chitosan DA = 8% $M_w = 120 \text{ kg.mol}^{-1}$	(1) Ionic gelation (TPP) (2) coating PLGA NPs	(1) 280 nm (2) 450 nm	Encapsulation	Ovalbumin	(Y) Intranasal (Z) Intramuscular	Female BALB/c mice	IgG ↑ sIgA ↑	Not studied	Slütter <i>et al.</i> (2010) ²⁰⁵
Chitosan DA ≤ 15 % $M_w = 190 \text{ kg.mol}^{-1}$	Coating poly-(ε-caprolactone) NPs	125 nm	(1) Encapsulation (2) Adsorption	Influenza A H1N1 hemagglutinin recombinant protein	(Y) Intranasal (Z) Intramuscular	Female BALB/c mice	(Y) & (Z) HI ↑; IgG, IgG1, IgG2a ↑ (Y) IgA ↑	(Y) IFN-γ ↑ (Y) & (Z) IL-4 ↑	Gupta <i>et al.</i> (2011) ²⁰⁶
Chitosan acetate	Coacervation (sodium sulphate)	not mentioned	Mix	Inactivated poliovaccine	Intramuscular	BALB/c mice	Neutralizing antibodies ↑	Not studied	Ghendon <i>et al.</i> (2011) ¹³⁰

Chitosan specifications	Formulation		Antigen	Administration route	Animal model	Results		Ref.
	Synthesis Method	Particle Size				Antigen-Particle Interaction	Humoral immune response	
<p>(1) Thiolated-TMCS (1) 5 (2) 6 D_{thiol} (%) 25 DQ (%) 17 DA (%) 17 M_w (kg.mol⁻¹) 21 5</p>	(A) Chemical cross-linking (thiolated-hyaluronic acid) (B) PECS (hyaluronic acid)	from 226 to 352 nm (depending on formulation)	Encapsulation Ovalbumin	(Y) Intranasal (Z) Intradermal	Female BALB/c mice	(Y) IgG ↑ (Z) IgG, IgG1↑	Not studied No	Verheul <i>et al.</i> (2011) ²⁰⁷
<p>(2) TMCS identical to (1) without thiolation Chitosan DA = 20 % M_w = 71.3 kg.mol⁻¹</p>	Complexation (antigen)/ coacervation (sodium sulphate)	153 nm	(1) Adsorption (2) Encapsulation Plasmid DNA for swine influenza	Intramuscular	Female BALB/c mice	IgG ↑	T cells proliferation ↑ No	Zhao <i>et al.</i> (2011) ²⁰⁸
<p>Chitosan DA = 20 % M_w = 71.3 kg.mol⁻¹</p>	Ionic gelation (TPP)	370 nm	Encapsulation Newcastle disease virus (NVD)	(Y) Oral (Z) Intranasal	SPF chickens	IgA ↑ IgM ↑	T cells immune response enhanced Good Safety No mortality	Zhao <i>et al.</i> (2012) ²⁰⁸
<p>(1) TMC DQ = 23.8 % from Chitosan DA = 8 % M_w = 120 kg.mol⁻¹</p>	Complexation (antigen)	from 69 to 88 nm	Complexation Hepatitis B surface antigen	(Y) Intranasal (Z) Intramuscular	Female BALB/c mice	(Y) IgG, IgG1, IgG2a ↑ IgA ↑ (Z) IgG, IgG2a ↑	Not studied No	Tafaghodi <i>et al.</i> (2012) ²⁰⁹
<p>(2) Chitosan no information</p>	Ionic gelation (TPP)	from 314 to 418 nm	Encapsulation Ovalbumin	(Y) Intranasal (Z) Intradermal	Female BALB/c mice	(Y) IgG, IgG1↑ (Z) IgG, IgG1↑	Not studied No	Bal <i>et al.</i> (2012) ²¹⁰
<p>Chitosan DQ = 15 % from Chitosan DA = 8 % M_w = 120 kg.mol⁻¹</p>	Complexation (antigen)/ Ionic gelation	not studied	Encapsulation Chimeric protein HP14/30	Intranasal	Female guinea pigs	IgA ↑	Not studied No	Hausner <i>et al.</i> (2013) ²¹¹
<p>Chitosan DA = 5 % M_w = 45 kg.mol⁻¹</p>	Coating Gold NPs	40 nm	Adsorption Tetanus toxoid	Oral	Swiss albino mice	IgG ↑ IgA ↑	Not studied No	Barhate <i>et al.</i> (2013) ²¹⁷
<p>Chitosan Low viscous (Sigma-Aldrich, USA)</p>	Complexation (antigen DNA)	from 512 to 1675 nm	Complexation Recombinant plasmid DNAs : (Influenza protection)	Intranasal	Female BALB/c mice	IgG ↑ IgA ↑	IFN-γ ↑ Protection > 60 %	Sawaengsak <i>et al.</i> (2014) ²¹²

Chitosan specifications	Formulation		Antigen	Administration route	Animal model	Results		Ref.	
	Synthesis Method	Particle Size				Antigen-Particle Interaction	Humoral immune response		Cellular immune response
Chitosan DA = 42/74/106 $M_w = 10 \text{ kg.mol}^{-1}$	Ionic gelation (TPP) / alginate coating	from 280 to 1000 nm	Adsorption	Measles antigen	Oral	Male BALB/c mice	IgG ↑ IgA ↑ Not studied	No No	Biswas <i>et al.</i> (2015) ²¹³
Chitosan DA = 8.2% $M_w = 10 \text{ kg.mol}^{-1}$	Ionic gelation (TPP)	560 nm	(1) Conjugation (2) Adsorption	(1) Targeting peptide (2) Ovalbumin	Subcutaneous	Female BALB/c mice	IgG, IgG1, IgG2a ↑ Not studied	No	Jung <i>et al.</i> (2015) ²⁰²
Maleimide-N-trimethylaminoethyl methacrylate chitosan Chitosan DA = 5 %	Ionic gelation (TPP)	Not studied	(1) Conjugation (2) Encapsulation	(1)(2) Ovalbumin	Intranasal	Female Balb/c mice	IgG ↑ IgA ↑ HI ↑	No	Liu <i>et al.</i> (2015) ²¹⁴
Maleimide-N-trimethylaminoethyl methacrylate chitosan Chitosan DA = 5 %	(A) Precipitation coacervation (sodium sulphate) (B) chitosan coated alginate pre-gel	from 400 to 5800 nm	Adsorption	Protective antigen of anthrax	Intranasal	SKH1 Female mice C57BL/6Nc r female mice	IgG, IgG1, IgG2a ↑ IgA ↑	No	Bento <i>et al.</i> (2015) ²¹⁵
Maleimide-N-trimethylaminoethyl methacrylate chitosan	Ionic gelation (TPP)	140 nm	(1) Conjugation (2) Encapsulation	Influenza A (H1N1) antigen	Intranasal	Female BALB/c mice	IgG, IgG1, IgG2a ↑ IgA ↑	No	Liu <i>et al.</i> (2015) ²¹⁶

Table 5 : Chitosan-based micro or nanoparticles vaccine delivery systems used *in vivo* experiments.

Micro or nanoparticles systems were the most studied chitosan-based vaccine delivery technologies. Various preparation methods were explored to produce materials with the best control over particle size using processes avoiding, as much as possible, organic solvent or surfactant. TPP-induced gelation of chitosan is the most widely used process for nanoparticles production, because it is an easy procedure to implement and reproducibly produces nanoparticles complying with pharmaceutical requirements for vaccine development. Moreover, this method proved compatible with various derivatives of chitosan such as hydrochloride salt,²¹⁸ TMC²¹⁹ or *N*-trimethylaminoethylmethacrylate.²¹⁴ Unfortunately, an important limitation is the stability in physiological media for injection and the compatibility with biological materials needing to reach neutral pH to be active. This limitation is less important in case of intranasal administration which required lower pH value, around 5.5 to 6.5. Chitosan particles were largely used to explore mucosal vaccination and, as for solution, the immune responses were elicited with the advantageous induction of local immune responses.

From our survey it appears that both the degree of acetylation and molar mass of chitosan were not systematically mentioned. When the information was provided in the various works reviewed, molar mass and DA of chitosan were inferior to 150 kg.mol⁻¹ and lower than 20 %, respectively. Vila *et al.* reported results with the preparation of nanoparticles via ionic gelation and discussed the impact of chitosan molar mass on the immune response.²¹⁸ With chitosans of $M_w = 23, 38$ or 70 kg.mol⁻¹ they obtained NPs similar in sizes, zeta potentials and tetanus toxoid (TT) loading efficiencies. But, by intranasal administration they reported that low molar masses provided a higher immune response at earlier times, whereas high M_w led to the best immune response in the long term. In all cases, IgG and IgA antibody titers were higher than formulation without NPs. The influence of M_w was also studied by Biswas *et al.*, comparing three chitosans to produce alginate coated chitosan nanoparticles for measles vaccine.²¹³ Molar masses from 42 kg.mol⁻¹ to 106 kg.mol⁻¹ impacted the final particle mean size from 432 to 1003 nm (with alginate coated NPs). Nevertheless, no important differences were observed in the obtained immune responses, every chitosan-based formulation offered satisfying IgG and IgA antibody responses in mice. Systems based on chitosan 42 kg.mol⁻¹ induced a better immune response, probably because of the lower size of the final system and higher zeta potential, favouring mucosal adhesion. *In vitro* studies, led by Xu and Du, showed the influence of DA and M_w on protein encapsulation and release,

with nanoparticles produced via ionic gelation.²²⁰ An increase in M_w from 10 to 210 kg.mol⁻¹ induced a 3 fold increase in loading efficacy and a reduction of BSA release from 74 to 18 %. A slow antigen release was targeted to develop a single-injection vaccine able to induce an antigen-specific immunological memory and reduce the cost linked to a multi-step vaccination procedure.²²¹ Nevertheless, these results need confirmation *in vivo*. In similar *in vitro* experiments, Gan and Wang reported that other parameters influenced the encapsulation and release of protein, such as chitosan concentration, chitosan/ TPP mass ratio or BSA concentration.²²² Conversely, Vila *et al.* reported that chitosan molar mass did not influence protein loading when chitosan NPs were associated with TT protein.²¹⁸ Hagenaars *et al.*²²³ and Verheul *et al.*²²⁴ observed that the DQ of TMC had no impact whereas an increase in its DA decreased the adjuvant properties of NPs. To explain this, the authors evoked the fast enzymatic degradation of high DA - TMCs (as exposed in section II.4.1 and their lower toxicity which did not induce a strong 'danger' signal. They finally concluded that the lower physical stability of reacylated- TMCs was the most important factor causing a low immune response.

To the best of our knowledge, no study addressed the impact of the DA of chitosan on the final immune response. As established before (section II.4) DA greatly impacts the biological properties of the polysaccharide, hence one can assume that it is also the case in the vaccine field. On the basis of the studies reported in Table 4 and also on other reviews^{119,120} the induction of a potent immune response depends on many parameters and it would be tedious and highly time consuming to analyse the influence of each one. We summarized all this parameters in Figure 11 in case of chitosan-based vaccine preparations.

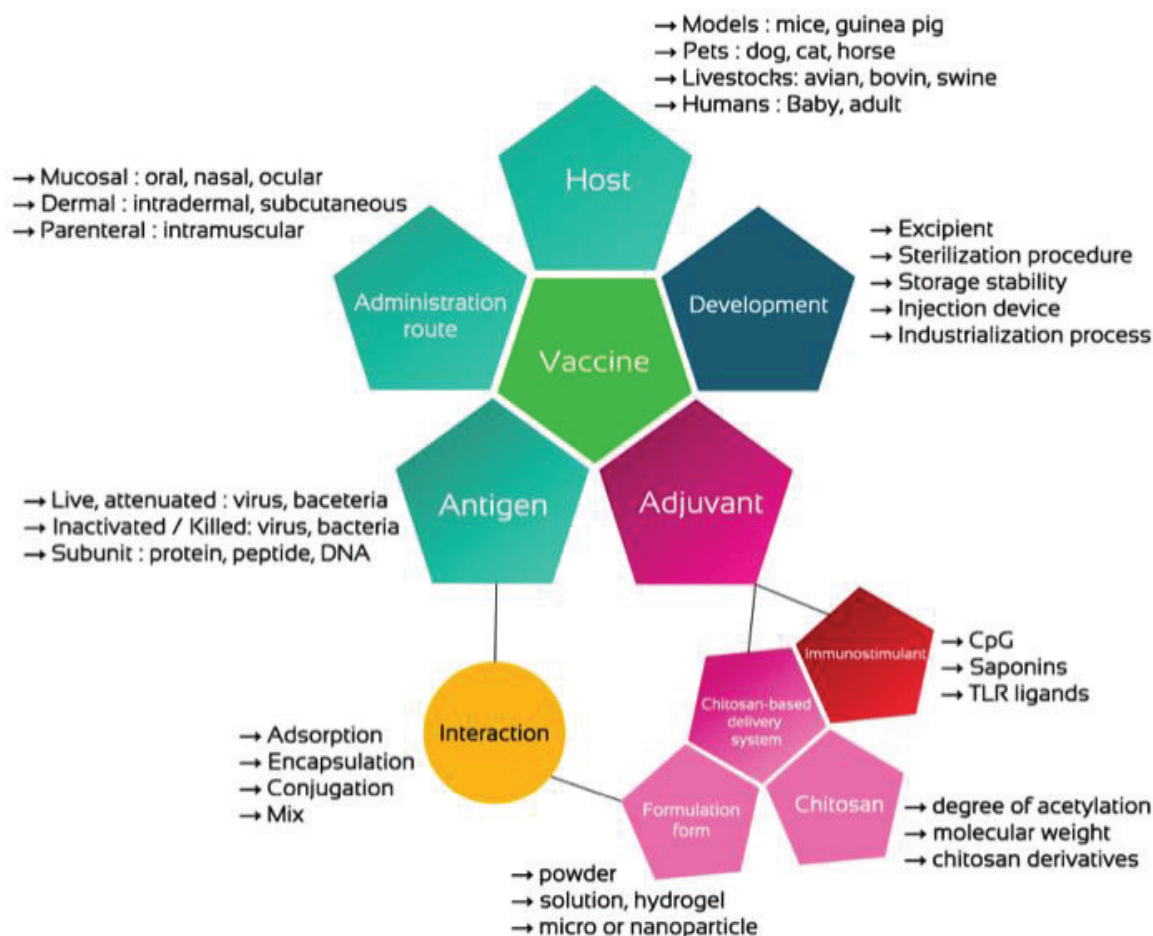


Figure 11: Key parameters for the elaboration of chitosan-based vaccine system. Adjuvant is an essential component to enhance immune response. It is selected depending on various final parameters such as host, administration route, antigen and viability for industrial development. Adapted from ¹¹ with permission.

III.3.5. Administration routes of chitosan-based particles

III.3.5.1 Mucosal administration

Mucosal routes, principally intranasal and oral, are advantageous administration pathways in terms of practicability and of immune protection. No needle being required, no trained personal is needed and the risk of accidental contamination is limited. Several mucosal vaccines are already on the market, for influenza, polio or cholera diseases.⁹⁶ Chitosan was investigated for mucosal vaccine and particularly using particles as reviewed by Illum *et al.*,²²⁵ Van der lubben *et al.*,²²⁶ Kang *et al.*²¹ or Islam *et al.*¹⁷⁰ Illum *et al.* were among the pioneers to report results on chitosan-based nasal delivery system of influenza vaccine.²²⁵ They observed a similar immune response as the other studies reported until now; IgG and IgA antibody titers were sufficient to achieve

the right level of protection. Serum antibody levels were as strong as subcutaneous vaccination and level of specific antibody IgA were found to be superior to classical administration routes. Results were similar in many studies such as Liu *et al.* who recently prepared nanoparticles conjugated to OVA as model antigen.²¹⁴ Moreover, they observed another advantage previously noticed about the intranasal injection: the induced production of IgA antibody at nasal, buccal or vaginal mucosal sites and in the lungs also. In other works, Liu *et al.* conjugated Influenza A (H₁N₁) antigen to NPs and observed a similar immune response with a higher IgA titer compared to alum-based vaccine.²¹⁶ Dissemination of antigen-specific lymphocytes was due to the common mucosal immune system (CMIS)²¹ allowing the induction of specific IgAs in other mucosal surfaces, though the response remained stronger at the vaccine-exposed mucosa.²²⁷

Kang *et al.* summarized several studies using chitosan microspheres to induce an immune response via the intranasal route.²¹ Conclusions and explanations were consistent with Amidi *et al.*¹¹⁹ or Islam *et al.*¹⁷⁰ As discussed previously, the mucoadhesive properties of chitosan enhanced the vaccine residence time at the administration site, opening tight junctions of mucosal barrier and facilitating transport of vaccine system, particularly via paracellular pathways.

III.3.5.2 Intradermal administration

The immunological interest of the intradermal routes lays in the potential to reach antigen-presenting cells as Langerhans cells, dermal dendritic cells, the first actors of immunity.²²⁸ It was demonstrated many times that injection in skin induced more efficient immune response with an antigen dose-sparing effect.²²⁹ Moreover, intradermal administration offered an easier practicability, with the help of new delivery devices.¹⁷ Microneedles²³⁰ and other systems as liquid jet injectors or solid injectors²³¹ are currently under development to provide safe administration equipments. The intradermal vaccination could eliminate or reduce syringe needle injuries,²³² whose treatment induced a cost over US\$ 500 million.^{233,234}

Chitosan-based particles, from various preparation methods, were used for the immunization of model animals via the intradermal route. Verheul *et al.* prepared chitosan-based nanoparticles covalently linked with hyaluronic acid (HYA) for intradermal immunization in mice.²⁰⁷ Thiolated-TMC mixed with OVA, reacting with thiolated- HYA, lead to a particulate system with size ranging between 230 and 340 nm,

a positive surface charge and loading efficiency superior to 30 %. The boost was injected 3 weeks after the prime and the humoral immune response was measured. They showed that covalently formed NPs induced a higher IgG antibody titer than OVA alone, contrary to non-thiolated TMC and HYA used to prepare nanoparticles by polyelectrolyte complexation, which induced similar responses as OVA alone. The superior immunization results obtained with the covalently linked NPs what attributed to their better stability in physiological media compared to PECs. Bal *et al.* prepared nanoparticles by the TPP-induced ionic gelation with OVA as model antigen.²¹⁰ Injected to mice twice at three week intervals, the serum IgG antibody titers were significantly higher in case of NPs injection than with OVA alone, with a predominance of IgG1, indicating a humoral immune response. It was reported that no IgA antibodies were observed through the intradermal administration route. In other works, Bal *et al.* reported, in mice, the induction of both cellular and humoral immune responses using chitosan nanoparticles for vaccination via the intradermal route.¹²⁹

IV. SPECIFICATIONS FOR HIGHLY IMMUNOGENIC CHITOSAN-BASED VACCINE DELIVERY SYSTEMS

The mode of action of chitosan-based adjuvants mainly depends on the administration routes,²³⁵ the adjuvant design and the antigen nature. Inducing a strong immune response requires the stimulation of various components of the immune system such as antigen presenting cells, innate and adaptative immunity.¹¹

To induce an infection, a pathogen needs to cross the natural barriers such as the oral, intranasal, vaginal or rectal mucosa. In these tissues, local immuno-competent cell can get activated and induce an immune response.^{227,236} To be efficient, vaccine delivery systems have to go through the same barriers as pathogens. For instance, at the mucosal level, they should not be washed away by the mucociliary clearance, constantly eliminating mucus, but remain in contact with the surface of the mucosa to penetrate it and reach the local immuno-competent cells. Kang *et al.* focused their review work on the ability of chitosan to be an efficient mucosal adjuvant.¹¹⁹ As discussed previously, the ability of chitosan to open tight junction facilitates the transport of antigen (section II.4.4) and its mucosal adhesion properties enhances the residence time at mucosal surface. Slütter *et al.* reported that chitosan solution mixed with OVA enhanced the antigen diffusion across Calu-3 cell monolayer.²⁰³

To target the immune system, the delivery of the active compound should occur in specific organs, cells types (such as antigen-presenting cells (APCs)), or cell compartment to initiate a protective immune response.¹¹ Dendritic cells were cited among the most efficient APCs and their interactions with adjuvants were regularly investigated. Chitosan nanoparticles were reported to interact with DCs and induce their maturation, contrary to solutions alone.²³⁷ Jung *et al.* used chitosan nanoparticles decorated with peptide to specifically target DCs.²⁰² *In vitro* experiments with fluorescent-labelled systems (flow cytometry, confocal laser scanning microscopy) showed the targeting properties of the peptide-NPs compared to blank NPs on DCs. *In vivo* experiments also confirmed that functionalized NPs induced the best protection as discussed before (section III.3.3). Koppolu *et al.* studied the influence of chitosan particle sizes on the uptake by DCs, subsequent activation and antigen presentation.²³⁸ They found that particles were uptaken by all macrophages, but antigen uptake was size-dependant with best results for diameters of 1µm. The activation of macrophages was strongly enhanced in the presence of particles. MHC I, CD80, CD86 and CD40 together with MHC II and CD54 (to a lesser extent) expression increased from 2 to 10-fold. Finally, they observed that antigens with particles increased the proliferation of antigen-specific CD8+ and CD4+ T cells, compared to antigen alone. These results underlined a more efficient antigen presentation and processing for the particulate formulation than for the solution form of the antigen.

When an antigen loaded adjuvant is uptaken by APCs, the cell migrates to the closest lymph node to trigger the adaptive immune response.²³⁹ In rats, Liu *et al.* observed the transport of labelled OVA-loaded chitosan NPs (NPs-OVA).²¹⁴ The high level of NPs-OVA in cervical lymph nodes 10 min after administration indicated a fast migration, contrary to OVA alone which was observed 1 hour after injection. Li *et al.* demonstrated, *ex vivo*, the superior uptake of OVA associated to chitosan NPs by Langerhans cells (LCs).²⁴⁰ In the same paper, *in vivo* experiments with subcutaneous vaccination in mice followed by fluorescent scanning microscopy reported that migration of antigen to lymph nodes was higher for NPs-OVA than other systems.²⁴⁰

It is important that delivery systems should be able to transport the antigen to immune cells, i.e. to release it with the least denaturation possible.²⁴¹ Various methods were used to control the integrity of antigen after its association with chitosan formulations: for example Slütter *et al.*²⁰⁵ used SDS-PAGE analysis to confirm the

integrity of OVA extracted from chitosan NPs or Sayin *et al.* with tetanus toxoid antigen.²⁴² The agarose gel electrophoresis method was also used to confirm the intact structure of antigen,²⁰⁸ using DNA to protect against swine influenza virus. Gupta *et al.* with H1N1 hemagglutinin (HA) protein also confirmed by Western blot the conservation of the antigen initial structure after association with particulate systems.²⁰⁶

The last crucial point to induce a strong and long lasting protection is to stimulate both the humoral and the cell-mediated immune response. For each formulation (solution, hydrogel or particles) Chitosan proved that it was possible to meet this requirement as seen from Table 2, Table 3 and Table 5. Bacon *et al.* earlier reported a chitosan-based delivery system using a chitosan solution mixed with hemagglutinin (HA) and neuraminidase (NA), to protect mice against influenza.¹²¹ They showed that the IgG titer was equivalent to alum after the administration of the last boost and the IgA antibody production was also enhanced in the presence of polymer contrary to alum, which did not stimulate a local immune response. Another study by Zaharoff *et al.* demonstrated that both arms of immunity were stimulated when mice were immunized subcutaneously with β -galactosidase in a chitosan solution. Moreover with the group that received the chitosan solution as adjuvant, the antigen-specific serum IgG titer was superior to that from the alum group and to antigen alone.¹³⁶

Chitosan derivatives were also able to induce an immune response as reported by Bal *et al.* The intradermal administration of TMC solutions or TMC-based particles mixed with OVA induced a higher production of IgG antibodies in mice than antigen alone.¹²⁹ They also reported that these systems were able to induce dendritic cell maturation with the upregulation of, CD83 and CD86, markers of the cellular response also that MHC-II, IL-12 and IL-6 of the humoral immune response.

V. CONCLUSIONS

Chitosan, because of its biocompatibility, biodegradability, bioadhesiveness and low toxicity, offers many advantages for the elaboration of safe and efficient vaccine delivery systems. This review underlines the versatility of the polymer, because of its particular physico-chemical properties and also to its adaptability to various processing technologies. Hence, a great variety of physical forms can be manufactured such as viscous solutions, hydrogels or micro/nanoparticles, to carry different nature of

antigens (live attenuated bacteria or virus and protein). The multitude of formulations enabled the administration of vaccines via different routes and chitosan was particularly attractive for a mucosal immunization strategy. Chitosan and its derivatives were found to induce both humoral and cell-mediated responses in animal models, offering a competitive material compared to commercial products for the elaboration of vaccine adjuvants.

In some studies using chitosan-based materials, we could note that the polymer was not fully characterized, particularly in chitosan-based solutions or hydrogels. Indeed, the intrinsic parameters of chitosan are known to be important factors in the final biological properties of administrated delivery systems. Moreover, interactions between chitosan and antigens were not investigated in detail, such as virus or bacteria compatibility with the polymer. Considering these two aspects, a complete characterization would improve the understanding of the mechanism of action of chitosan. The elucidation of structure-property relationships will be an important part of the success in vaccine applications in the future years.

Before bringing chitosan-based vaccines to the market, some aspects remain perfectible such as sterilization and storage stability. Sterilization is a prerequisite prior any administration in hosts and the development of a procedure leading to a controlled chitosan material should be investigated. From now, various methods such as ethylene oxide, γ -radiation or saturated steam sterilization were reported to alter the polymer properties (molecular weight, degree of acetylation) and consequently the biological properties. In addition, storage stability of chitosan products should be explored in order to provide an improved shelf-life. Indeed, vaccines generally require a two year stability at low temperature; nowadays no existing system offers this long-term stability. Various factors could be scrutinized, both extrinsic (temperature, pH...) or intrinsic (purity, M_w , DA, water content), and they require an attentive study.

To overcome this limitation, research should focus on the control of chitosan-based products from the raw material to the storage stability and the comprehension of the role of chitosan formulation in the induction of immune response. Nevertheless, chitosan remains an attractive biomaterial for the development of safe and efficient adjuvants in response to the actual and future challenges in human and veterinary vaccine area.

VI. ACKNOWLEDGMENT

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CONCLUSION DU CHAPITRE 1

Ce premier chapitre met en lumière la très grande variété de matériaux à base de chitosane élaborés dans le cadre d'applications vaccinales. Les trois formes rapportées sont les solutions, les hydrogels et les nano ou microparticules. Employées dans de nombreuses études utilisant différentes natures d'antigène, ces trois formes ont permis dans tous les cas d'obtenir une activité adjuvante.

Les solutions à base de chitosane ont été les premières explorées, principalement dans le cadre de vaccins administrés par voie orale. Cette forme a notamment montré de bons résultats pour des vaccinations antigrippales par voie intramusculaire, avec des protections similaires aux vaccins de référence à base d'aluminium. Par ailleurs, le chitosane délivré par voies mucoales a également permis d'induire une protection locale, grâce à une réponse immunitaire efficace contre des agents pathogènes transmis par ces voies ci. La facilité de préparation et la stabilité des solutions de chitosane offre ainsi un bon candidat adjuvant, en plus des propriétés immunostimulantes du polymère. Cependant, peu d'études rapportent l'utilisation de chitosane par voie intramusculaire, voie traditionnellement employée pour la vaccination.

Les particules à base de chitosane ont été particulièrement étudiées en raison de leur capacité de présentation et de leur forme, proche des systèmes biologiques (virus, bactéries). La méthode de préparation la plus répandue est la gélification ionique. Dans des conditions opératoires définies, le chitosane est mis en présence de sels de tripolyphosphate (TPP) formant ainsi un réseau structuré tridimensionnel. Ce type de matériaux a démontré de bonnes capacités de chargement en antigènes et une stimulation du système immunitaire satisfaisante. Les limites actuelles demeurent la stabilité dans le temps ainsi que la possibilité de produire en quantité importante, restreignant leurs utilisations pour des applications industrielles.

Les hydrogels de chitosane sont des matériaux plus récemment explorés dans le cadre d'applications vaccinales. La formation des hydrogels peut être réalisée par voie physique ou chimique mais nécessite toujours l'ajout d'autres composés avec une biocompatibilité encore peu connue. Ces systèmes présentent l'avantage de délivrer l'antigène sur un période de temps plus longue que les solutions ou les particules, offrant ainsi un « réservoir » d'actif. Les réponses immunitaires sont prometteuses, avec des productions d'anticorps allant jusqu'à un an. Cependant, il est encore difficile de

déterminer le rôle exact du chitosane dans ces matériaux puisque d'autres composés (adjuvants, agents de réticulation) sont également présents dans les formulations.

Le chitosane, sous ses différentes formes, permet d'obtenir une réponse immunologique avec une biocompatibilité excellente. En effet, aucun cas d'effet secondaire n'a été rapporté dans la littérature lors de son administration sous forme de vaccins. Les avantages offerts par les solutions à base de chitosane nous ont conduits à étudier leur efficacité lors de l'élaboration de vaccins expérimentaux destinés à différentes espèces animales, tels que la dinde, le chien ou le porc. Les résultats de ces recherches sont l'objet du chapitre deux du manuscrit. Bien qu'efficaces pour induire une protection des animaux, la biocompatibilité de certains adjuvants actuellement disponibles n'est plus satisfaisante. Dans ce contexte, des vaccins contre le « *rouget du porc* » ont été préparés avec des solutions visqueuses, nanoparticules ou un mélange des deux à base de chitosane. Par ailleurs, certains antigènes seuls ne sont pas assez efficaces et les adjuvants actuels ne sont pas satisfaisants aussi bien en termes d'efficacité que d'innocuité. Dans cette situation, des vaccins contre le « *choléra des poules* » ont été préparés avec des résultats encourageants. Enfin, un modèle d'antigène peu purifié, comme la majorité des actifs destinés aux animaux, a été utilisé afin de déterminer le pouvoir adjuvant des solutions de chitosane en comparaison d'une référence commerciale. Dans notre cas, les vaccins testés chez le chien ont apporté des résultats d'innocuité satisfaisants et des réponses immunitaires variables selon la maladie visée.

Les travaux rapportés dans le chapitre deux permettent ainsi d'évaluer le potentiel du chitosane pour des applications vétérinaires avec des essais réalisés directement sur l'espèce animale visée.

CHAPITRE 2

LES SOLUTIONS VISQUEUSES DE CHITOSANE COMME PLATEFORME ADJUVANTE POUR L'ELABORATION DE VACCINS VETERINAIRES

- Chapter 2 -

*Controlled chitosan-based solutions as adjuvant platform for
various veterinary vaccine candidate*

INTRODUCTION AU CHAPITRE 2

Le deuxième chapitre du manuscrit de thèse rapporte les résultats et discussions liés à la préparation et à l'administration de systèmes de délivrance de vaccins à base de chitosane (solution visqueuse particulièrement) dans le cadre d'expérimentations *in vivo* chez différentes espèces animales.

La section II « *Materials and Method* » rapporte la préparation des vaccins, les protocoles d'administration chez les différentes espèces animales ainsi que les résultats d'innocuités, immunologiques ou de protections obtenus. Trois espèces animales sont concernées dans ce chapitre, à savoir la dinde (représentative des espèces aviaires), le chien (espèces canines) et le porc (espèces porcines).

Les sections II.1 et II.2 sont consacrées à la préparation du polysaccharide ainsi qu'à l'élaboration de solutions visqueuses stériles de chitosane prêtes à l'emploi. En effet, afin d'obtenir des adjuvants à base de chitosane stables à pH physiologique (pas de précipitation) et en contact avec les différents antigènes, plusieurs chitosanes (variations des DA et M_w) ont été testés. Par ailleurs, nous proposons une méthode pour stériliser, par autoclave, une solution de chitosane sans entraîner de dégradations du polymère (conservation du DA et de la M_w).

La section II.3 traite de l'élaboration des vaccins adjuvantés avec des solutions de chitosane pour les modèles dinde et chien, solution de chitosane, nanoparticules (NPs) et solution de chitosane/ NPs pour le porc. Les vaccins destinés aux dindes sont constitués de bactéries vivantes atténuées (antigène) de *Pasteurella Multocida* (*Pm*) modifiée et de chitosanes ($M_w = 200 \text{ kg.mol}^{-1}$; DA = 25 ou 50 %; $C_p = 0,2 \text{ \% (w/v)}$). Les vaccins destinés aux chiens sont constitués de virus vivants atténués (antigène) de Canine Adenovirus de type 2 (CAV-2), virus inactivés de Rage et de chitosane ($M_w > 500 \text{ kg.mol}^{-1}$; DA > 40 %; $C_p = 0,4 \text{ \% (w/v)}$). Les vaccins destinés aux porcelets sont constitués de protéines purifiées SpaA (antigène) et de solution de chitosane ($M_w > 500 \text{ g.mol}^{-1}$; DA > 40 %; $C_p = 0,75 \text{ \% (w/v)}$), de NPs ($C_{NPs} = 3 \text{ \% (w/v)}$) et d'un mélange de solution visqueuse de chitosane ($M_w > 500 \text{ g.mol}^{-1}$; DA > 40 %; $C_p = 0,75 \text{ \% (w/v)}$) / NPs ($C_{NPs} = 3 \text{ \% (w/v)}$).

La section II.4 décrit les protocoles expérimentaux pour l'évaluation *in vitro* des vaccins. Les évaluations *in vitro* permettent de déterminer l'influence du chitosane sur

les différents antigènes utilisés ainsi que la stérilité des échantillons de chitosane avant administration chez l'animal. En effet, il est important que le chitosane n'ait pas d'action délétère sur les propriétés immunogènes des actifs, ce qui compromettrait son utilisation dans des essais *in vivo*. La stérilité des préparations est également un aspect essentiel puisque que toutes contaminations extérieures pourraient entraîner d'une part des effets secondaires chez l'animal (infections, etc.) mais également perturber l'analyse des résultats obtenus.

La section II.5 est consacrée aux protocoles expérimentaux des essais *in vivo* chez les différentes espèces animales. Les évaluations *in vivo* (section II.6) de l'activité des vaccins inoculés chez la dinde sont réalisées par le biais d'un *challenge* c'est-à-dire par l'exposition de l'animal à une souche virulente du pathogène pour lequel la protection est recherchée. Pour les essais sur chiens et cochons, l'activité des vaccins injectés est évaluée par immuno-monitoring, en suivant l'évolution de la concentration sérique en des anticorps spécifiques (IgG) ou interféron gamma (IFN- γ).

La section III « *Results and Discussions* » est dédiée à la présentation des résultats issus de la section II et à leurs discussions. La section III.1 décrit la méthode de préparation d'une solution de chitosane stérilisée par autoclave sans modification de la masse molaire ou du DA. La préparation de solutions visqueuses de chitosane ne permet pas la stérilisation par filtration discriminante à 0,22 μm destinée aux liquides fluides. D'autres procédés tels que la stérilisation par oxyde d'éthylène ou par irradiation ne sont, également, pas compatibles par risque de contaminations ou de dégradation du polymère. La stérilisation par autoclave est la seule méthode envisageable. Cependant, les solutions de chitosane subissent une importante dégradation (diminution de la masse molaire) lors de cycles classiques (20 min, 121°C). La modification des étapes de préparation de la solution via deux méthodes permettent d'obtenir un chitosane sans modification majeure de ses paramètres intrinsèques.

La section III.2 est consacrée à la compatibilité du chitosane avec des antigènes de natures bactériennes ou virales. Dans le cadre du vaccin à base de *Pm*, un chitosane de DA autour de 25% présente la meilleure compatibilité, c'est-à-dire qu'il permet la survie des bactéries constitutives du vaccin. Pour la protection contre CAV-2, nous montrons que le chitosane de haut DA (> 40 %) est compatible avec les virus, c'est-à-dire qu'il n'entraîne pas d'effet délétère susceptible de diminuer l'immunogénicité de ces derniers.

Dans la section III.3, les résultats d'innocuité et d'efficacité des différents essais réalisés sont rapportés. Tout d'abord, l'innocuité des diverses solutions de chitosane évaluées est satisfaisante, aucun effet secondaire n'a été observé quelles que soient la voie d'administration et le modèle animal. Les résultats sur l'efficacité des préparations vaccinales montrent que le vaccin aviaire induit une protection de l'ordre de 90 % chez les animaux si le DA est de 25 % tandis qu'à DA = 50 % la protection est faible et la survie des animaux chute à 30 %. Le vaccin canin CAV-2 permet d'atteindre une réponse immunitaire humorale et cellulaire équivalente au vaccin de référence à base d'aluminium. Par contre, le vaccin Rage adjuvanté avec le chitosane n'induit pas une réponse immunitaire suffisante, selon les termes de l'Organisation Mondiale de la Santé (OMS) pour permettre une protection des chiens. Le vaccin sous-unitaire destiné à la protection contre la maladie du « Rouget du porc » administré chez des porcelets permet d'obtenir une réponse immunitaire satisfaisante, mais perfectible, dans le cas de la solution de chitosane. Concernant l'utilisation de NPs aucun effet adjuvant n'a été détecté, le mélange solution/ NPs permet l'obtention d'une réponse immunitaire mais inférieure à la solution seule.

La section IV, conclusions du chapitre 2, fait ressortir que sur l'ensemble des pathologies et des modèles animaux testés, les solutions de chitosane sont de bons candidats-adjuvants pour de futures applications vaccinales. En effet, les solutions de chitosane sont simples à préparer, présentent une excellente innocuité et sont compatibles avec l'utilisation de pathogènes vivants, ainsi que d'immunogènes sous-unitaires. La modification des paramètres intrinsèques du chitosane, DA et masse molaire, permet de moduler ses propriétés physico-chimiques et biologiques offrant ainsi une plateforme à fort potentiel pour la préparation d'adjuvant. Les réponses immunitaires bien qu'étant parfois en deçà de certaines valeurs visées, particulièrement pour le modèle Rage, laissent un important potentiel quant à l'exploitation du chitosane comme système de délivrance de vaccins par exemple par voie mucosale, comme le montrent les bons résultats obtenus sur le modèle dinde.

Controlled Chitosan-Based Solutions As Adjuvant Platform For Various Veterinary Vaccine Candidate

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ABSTRACT:

Chitosan is a semi-natural biocompatible polymer used as adjuvant in the development of vaccine delivery system. One challenge, before administration in animals, is to elaborate sterile materials in order to avoid infections. Sterilization of chitosan solutions via filtration is a key issue and we report here a method to elaborate ready-to-use sterile chitosan solutions by autoclaving, with a restricted degradation of the polymer. The molar mass and degree of acetylation of the polymer were measured by SEC and ¹H NMR spectroscopy, respectively. Chitosan adjuvants were then assessed in various animal-species such as turkeys, dogs and pigs, associated to different antigen natures: live attenuated bacteria; live attenuated viruses, an inactivated virus and a recombinant protein. The immunogenicity and efficacy of the vaccines were measured in challenge animal models or via serological monitoring. Protection of turkeys with a live attenuated bacteria coupled with chitosan at DA = 25% provided a protection of 90% of the challenged animals. Viruses and protein antigens induced an immune response satisfying for further developments, respectively in dog and pig species.

KEY WORDS:

Recombinant protein; virus; bacteria; vaccine delivery; chitosan; sterilisation.

I. INTRODUCTION

The vaccination of animal species is a major issue both for public health and economical aspects.¹ In companion animals, vaccination allows to protect against diseases which can be contracted in kennels for example, or contaminate humans such as rabies. In livestock farming, the prevention of epidemic diseases allowed avoiding the contamination, reducing treatment costs by decreasing the use of pharmaceutical products if existing or by the culling of animals. To reach this target, the need of a safe and efficient adjuvant platform remains a challenge. Moreover, the ideal adjuvant, highly valuable would be compatible with various kinds of antigen (bacteria, virus and protein) administrable in several animal species via different administration routes (intramuscular, ocular...).

Chitosan is a polysaccharide, obtained from the partial deacetylation of chitin, a natural and abundant polymer extracted from crustacean shells² or cell walls of fungi.³ Chitosan is a copolymer composed by N-acetyl-D-glucosamine and D-glucosamine units linked by a $\beta\rightarrow 1,4$ -glycosidic bond. The protonation of amino groups along the chains, when dissolved in acid aqueous media, allowed the preparation of various physical forms, from solutions to nanoparticles.⁴ Chitosan is a biocompatible, biodegradable and non-toxic polymer⁵ responding to the criteria for the elaboration of adjuvants in the vaccine development.⁶

Sterilisation is a prerequisite for the preparations of parenteral injections. Chitosan is known to be degraded under classical sterilisation processes as gamma irradiation, ethylene oxide or autoclaving.⁷ Moreover, the high viscosities of the solutions were not compatible with a sterilizing filtration through 0.22 μm pore membranes.⁸ The decrease in viscosity⁹ and yellowing¹⁰ during sterilization treatments were reported many times and may impede pharmaceutical applications. To overcome these disadvantages, we designed a sterilization procedure via autoclaving preventing the polymer degradation and allowing an easy preparation of ready-to-use sterile chitosan solutions for vaccine formulations against diseases for which solutions exist (Rabies, CAV-2...) or not (*Pasteurella multocida*). *In vitro* experiments were conducted to formulate efficient and applicable chitosan-based vaccines, compatible with sensitive antigen. *In vivo*

experimentations indicated that chitosan was a good candidate for veterinary vaccines in various animal species.

II. MATERIALS AND METHODS

II.1. Chitosan preparation

II.1.1. Purification of chitosan

Chitosan, batches 113 and 114, were purchased from Mahtani Chitosan PVT Ltd. (India). Purification was carried out before use to ensure the elimination of all residues and provide a pharmaceutical grade. The chitosan powder was dissolved at 5 g/L in an aqueous acetic acid solution for one night at room temperature under mechanical stirring. The mixture was filtered, under pressure (3 bars), successively through 3, 0.8 and 0.45 μm pore size membranes (Millipore, France) and precipitated by adding a solution of ammonium hydroxide (28% (w/v)) The precipitate was washed several times with deionised water until neutral pH. Neutralised chitosan was freeze-dried and finally stored at room temperature.

II.1.2. Modification of the degree of acetylation of chitosan

The degree of acetylation was modified according to the procedure by Vachoud *et al.*¹¹ Chitosan was dissolved at 10 g/L (w/v) in a hydro-alcoholic mixture made of deionised water and 1,2-propanediol at a 1:1 (v/v) ratio. Acetic anhydride was added to reach the desired degree of acetylation. Chitosan was precipitated, washed several times until elimination of alcohol, then freeze-dried and stored. DA values and the absence of 1,2-propanediol were confirmed by ¹H NMR. Chitosans at DAs 25% and 50% were prepared for the preparation of vaccines.

II.1.3. Sterilization of chitosan solutions for vaccine delivery

Purified chitosan was placed in a heat resistant bottle, suspended or dissolved in de-ionized water or in weakly acidic media, according to the tested procedure, at a concentration of 1% (w/v). Chitosan solutions were sterilised using a laboratory autoclave (model K7, Getinge) in standard experimental conditions: 121°C for 20 min at pressure of 2.1 bars.

II.2. Chitosan analysis

II.2.1. Molar mass determination

The weight-average molecular weight (M_w) was measured by size exclusion chromatography (SEC) (3000 and 6000 PW TSK gel columns, 7.8 mm inner diameter and 30 mm length) coupled on line with a differential refractometer (Optilab T-rEX, Wyatt Technology) and a multi-angle-laser-light-scattering (MALLS) spectrophotometer (Dawn EOS, Wyatt Technology) equipped with a 5 mW He/Ne laser operating at $\lambda = 632.8$ nm. Analyses were performed using a micro-batch mode using K5 flow cell. A degassed 0.2 M acetic acid / 0.15 M sodium acetate buffer (pH 4.5) was used as eluent. The flow rate was maintained at 0.5 mL/min. Chitosan powder was dissolved in the same solvent at 0.1 % (w/v) and 100 μ L were injected after filtration on a 0.45 μ m pore size membrane.

II.2.2. Determination of the degree of acetylation

Chitosan powder samples were dissolved in diluted acidic D₂O and the of acetylation was determined using ¹H NMR spectroscopy (Bruker, 400 MHz) according to the method proposed by Hirai *et al.*¹²

II.3. Elaboration of vaccines

II.3.1. Preparation of *Pasteurella multocida* vaccines

Experimental live attenuated *Pasteurella multocida* modified strain was mixed with 0.4 % (w/v) chitosan solutions at 1:1 (v/v) ratio. pH was adjusted at 6.4 with 1 N sodium hydroxide (NaOH) solution. Chitosan with medium molar mass and DA = 25 % was used for group 1, DA = 50 % for group 2. Final antigen concentration was 3.0×10^8 CFU/mL.

II.3.2. Preparation of canine vaccines

The following antigens: Rabies, Canine Distemper Virus (CDV), Canine Adenovirus type 2 (CAV2), Parainfluenza type 2 (Pi2) and Canine Parvovirus (CPV) were similar to the commercial products. Rabies was mixed with 0.8 % (w/v) chitosan (DA = 45%) solution and finally diluted to 0.4 % (w/v) with phosphate buffered saline (PBS) buffer. pH was adjusted at 7.3 with 1N NaOH solution. CDV, CAV2, Pi2 and CPV as a freeze-dried were reconstituted with a 0.4% (w/v) chitosan solution adjusted at pH 7.3 with 1N NaOH solution.

II.3.3. Preparation of *Erysipelothrix rhusiopathiae* vaccines

Recombinant SpaA protein, corresponding to the P65 protein of *Erysipelothrix rhusiopathiae* (the most represented and immunogenic protein of Ery.) was produced in *Escherichia coli* system with a molecular weight of 49 kDa. SpaA was mixed with a 1.5 % (w/v) sterile chitosan solution at a 2:3 (v/v) ratio. The preparation was finally diluted to 0.75 % (w/v) with a PBS buffer for the first group [solution+SpaA]. pH was adjusted at 6.5 with 0.1N NaOH solution. For the second preparation [NPs /SpaA], vaccine was elaborated using chitosan-based nanoparticles synthesised from the method proposed by Schatz *et al.*¹³. The protein solution was added to sterile nanoparticles at 6 % (w/v) at 2:3 (v/v) ratio under moderate stirring for one night at 5°C. pH was adjusted to 6.2 with 0.1N NaOH and PBS was added to be in physiological conditions and reach a final concentration at 3 % (w/v). For the last group [solution + NPs/SpaA], we elaborated a composite system using antigen loaded chitosan particles (at 1:15 ratio (v/v)) dispersed within chitosan solution at ratio 16:9 (v/v). pH was adjusted to 6.2 and PBS was added to reach a final concentration of the system at 0.75 % (w/v) for the chitosan solution and 3% (w/v) for the nanoparticles. For all vaccine preparation, the final antigen concentration was fixed at 50 µg/mL.

II.4. In vitro evaluations of chitosan activities

II.4.1. Sterility of chitosan materials

The sterility of chitosan materials was analyzed before administration to animals. Sterility tests were conducted according to recommended procedures of the European Pharmacopeia. Materials were incubated for 14 days within 30 and 35°C in thioglycolate medium. Bacterial growth was regularly checked. The sterility was determined via visual control, in case of bacterial development, it would have been considered as contaminated.

II.4.2. Chitosan and bacteria compatibility

Fresh cultures of *P. multocida* were re-suspended in 0.4 % (w/v) chitosan solutions at various degree of acetylation. Mixtures were incubated at room temperature during 4 hours. Bacteria viability was evaluated at different time points (0, 1, 2 and 4 hours) by the dot dilution methodology for scoring (10^{-5} , 10^{-6} , 10^{-7}).

II.4.3. Chitosan and virus compatibility

Vero cells were infected with suspensions of live attenuated canine virus (CDV) in the presence of chitosan-based solutions (high DA = 45 %; MMW = 150 and HMW = 550 kg.mol⁻¹). Mixtures were incubated at 37°C for 2 hours to measure the cytopathic effect (CPE) of the virus. The titre obtained by the Karber method was expressed by the logarithm of the inverse of the viral dilution causing a 50 per cent CPE.

II.5. Immunization and sampling of turkeys, dogs and pigs

All animals were managed similarly, with due regard for their well-being and in compliance with Merial Ethics Committee, other local applicable regulations and requirements, and International laws and ethics.

II.5.1. Immunization of turkeys

Thirty three week-old conventional turkeys were divided into three treatment groups ($n=10$). All the animals (without the control group) were each inoculated via ocular administration at day 0 by eye-drop (one eye) of 0.1 mL of vaccine prepared as described in section II.3.1. Safety was evaluated by daily monitoring and the observation of local reactions at the depot site.

II.5.2. Immunization and sampling of dogs

Fourteen three month-old specific-pathogen-free (SPF) dogs were divided into two treatment groups ($n=7$). Each dog was inoculated via subcutaneous injection at day 0 and day 28. Each dog received the same antigen dose for prime and boost vaccination in 1 mL with vaccines elaborated as described in II.3.2. Rabies vaccine was only injected at day 28 without boost. Safety was evaluated by a daily health monitoring and local reaction observations at injection sites. Humoral and cell-mediated immunity monitoring were conducted via blood samples on dry or heparinised tubes from day -1 to 84 for IgG, IFN- γ secretion and memory B cells titration.

II.5.3. Immunization and sampling of pigs

Twenty-one seven-weeks-old *Erysipelothrix rhusiopathia* negative pigs were divided into four treatment groups ($n=6$ for vaccine groups and $n=3$ for control group). Each pig was inoculated via intramuscular injection at day 0 and day 21 with the same antigen dose. The pigs of groups 1, 2 and 3 received for each injection 1 mL of the vaccines, elaborated and described in section II.3.3. The adjuvant safety was evaluated

by monitoring the animal general conditions and local reactions. Post-mortem inspections to observe lesions or residual products at the injection site were also conducted. Heparinised blood samples were collected from all pigs at day 20, 42 and 49 for the analysis of the antibody and B memory cells responses.

II.6. Evaluation of immunization

II.6.1. Challenge in turkeys

All the animals were challenged at day 21 after the inoculation of the vaccine preparations. 0.1 mL of a virulent strain of *Pasteurella multocida* was administrated by eye-drop (on eye) using a 1-mL needleless syringe. The clinical signs, kinetics of mortality and mortality rates, were observed for each group before and after challenge.

II.6.2. CAV-2 specific neutralizing IgG response in dogs

A CAV-2 specific seroneutralization assay has been developed. First, a constant amount of CAV-2 was incubated *in vitro* with several dilutions of the sera, and were then inoculated on cells susceptible to CAV-2 virus (MDCK cells). The infection of MDCK was determined by observation of a cytopathic effect. The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells.

II.6.3. CAV-2 specific IFN- γ response by ELIspot in dogs

The dog IFN- γ ELIspot assay was performed with a commercial ELIspot kit (R&D Systems, EL781). 500×10^3 peripheral blood mononuclear cells (PBMCs) were directly added in wells and were restimulated by CAV-2 active ingredient in RPMIc for 48h at $37^\circ\text{C} + 5\% \text{CO}_2$. As control, 500.10^3 PBMCs remained not restimulated and were cultured in RPMIc alone. The method was considered as quantitative. One spot obtained on the nitrocellulose support was considered as one IFN- γ secreting cell. The proportion of CAV-2-specific IFN- γ -producing cells in PBMCs was calculated as follows: Number of IFN- γ -spot forming cells (IFN- γ -SFC) obtained upon CAV-2 specific-restimulation - Number of IFN- γ -SFC upon control condition. Data was expressed as number of IFN- γ -SFC per number of PBMCs.

II.6.4. Rabies specific neutralizing antibody IgG response by FAVN in dogs

The principle of the fluorescent antibody virus neutralisation (FAVN) test is the *in vitro* neutralisation of a constant amount of rabies virus by the several dilutions of the

titrated sera before inoculating cells susceptible to rabies virus (BHK-21 cells). The serum titre was the dilution at which 100 % of the virus was neutralised in 50% of the wells. The infection of BHK-21 cells was monitored by immuno-fluorescent staining of rabies virus. This titre was expressed in IU/ml by comparing it with the neutralising dilution of an internal control dog serum origin under the same experimental conditions.

II.6.5. Measurement of SpaA-specific IgG response by ELISA in pigs

A sandwich ELISA assay was used to quantify SpaA-specific IgG response in porcine sera. ELISA plates were coated at +4°C with 100µl/well of recombinant SpaA protein in carbonate buffer. The antibody coated plates were washed three times and unoccupied sites clocked with PBS added of 5% milk for 1h at 37°C. Serial dilutions of the sera were distributed in SpaA-coated plate and incubated for 1h30 at 37°C. After 3 washes, 100µl/well of anti-porcine IgG conjugated to peroxydase (Jackson Immunoresearch) was added and plates were incubated 1h at 37°C. Peroxydase substrate was finally added for 30 min at room temperature (RT) in the dark. The enzymatic reaction was blocked by addition of 50µl of 1N H₂SO₄. Optical density was measured at 450 and 630 nm with an ELISA reader.

III. RESULTS AND DISCUSSION

III.1. Sterilization procedure to provide controlled chitosan-based solutions

The high viscosity of chitosan solution depending on the concentration and M_w , did not allowed sterilisation by filtration through 0.22 µm membrane because the polymer blocked the membrane.⁸ According to the European Pharmacopeia, autoclaving, or saturated steam under pressure, is preferred over the other methods to sterilise aqueous solutions. Unfortunately, as reported by No *et al.* chitosan solutions were sensitive to thermal treatments inducing the polymer degradation.⁹ Other methods were investigated to sterilise chitosan such as gamma or beta ray irradiations or gas (using ethylene oxide) treatments as reported by Yang *et al.* on chitosan dried powder¹⁰ and Lim *et al.* on chitosan films.¹⁴ Sterilisation of chitosan powder via autoclaving according to Yang *et al.* appeared to be a promising method because no change of the molar mass or DA was observed,¹⁰ but the aspect of the product changed from light to dark yellow, not satisfying for a pharmaceutical application. Juan *et al.* reported a similar method by the suspension of chitosan powder in water followed by sterilisation via autoclave.⁷ No

information on the chitosan colour was reported but the preservation of M_w and DA was claimed. The inconvenient of this method was to add the acid, sterilised too, in sterile conditions to obtain a final sterile chitosan solution.

In this work, we used the autoclaving method with a standard procedure: 121°C during 20 min. Three preparation procedures (as described in section II.1.3) were tested to determine would be best at preserving the molar mass of chitosan. In the first method (group A), chitosan was dissolved under moderate magnetic stirring by adding a stoichiometric amount of glacial acetic acid, with respect to the free amino functions. Then, the solution was sterilised. Second (group B), a suspension of chitosan in water was sterilised and then the solubilisation of polymer was achieved by the addition of acetic acid (stoichiometric amount) in sterile conditions. Third (group C), a suspension of chitosan in diluted acetic acid (stoichiometric amount) solution was sterilised and then the solubilisation was achieved at room temperature under magnetic stirring.

The chitosan solutions from group A evolved from colourless to yellow and, moreover, insoluble residues appeared after sterilisation. This modification could be attributed to the Maillard reaction, as suggested by Muzzarelli.¹⁵ The results showed a decrease in the molar mass down to 60 % of the initial value and the DA, remained unaltered, whatever the procedure. Similar modifications of the M_w were reported by No *et al.* showing classical effects of autoclaving on the chitosan properties.⁹ The depolymerisation of chitosan could be attributed to the acid hydrolysis of some glycosidic bonds as reported by Värüm *et al.* using HCl acid¹⁶ and Holme *et al.* in case of the depolymerisation of solid chitosan¹⁷ or chitosan salts solutions.¹⁸ In similar conditions to group A, acetic acid was also used to prepare low molar mass chitosan at high temperatures (from 65 to 85°C) such as reported by Tsao *et al.*¹⁹ The results from group B showed that no significant modification of the DA and the molar mass occurred during the sterilization of the solutions. It can be assumed that chitosan in suspension did not undergo hydrolysis since the solubilisation took place after the addition of acid post-sterilization. No mechanism was proposed in the literature to explain the preservation of chitosan during thermal treatments in water; nevertheless, one can speculate that in absence of acid, the glycosidic bonds cannot be hydrolysed especially in heterogeneous conditions. In group C, after sterilization, the chitosan molar mass slightly increased by 16 %. The presence of the acid in the chitosan suspension did not

induce any degradation of the polysaccharide but probably a slight intermolecular association explaining the increase in weight-average molecular weight M_w . The general aspect of solutions was satisfying for the elaboration of vaccine without change of the colour, i.e. colorless and limpid, or the apparition of insoluble materials as in group A.

Group	Initial DA ^a	Final DA ^b	Initial M_w ^c	Final M_w ^d	molar mass % difference
A	1.9	2.0	540 ± 25	203 ± 6	- 62.4
B	1.9	2.2	540 ± 25	545 ± 11	Not significative
C	1.9	2.1	540 ± 25	624 ± 4	+ 15.6

Table 1: Intrinsic parameters of chitosan before and after the sterilisation of chitosan at 1% (w/v) via autoclaving method were evaluated. The molar mass difference (expressed in %) between initial and final M_w was calculated via the following relationship: % difference = [(initial M_w - final M_w) / (initial M_w) × 100]. Experiments were conducted on three samples.

^a degree of acetylation before autoclaving (in %)

^b degree of acetylation after autoclaving (in %)

^c molar mass M_w before autoclaving (kg.mol⁻¹)

^d molar mass M_w after autoclaving (kg.mol⁻¹)

Our study demonstrated the advantage to avoid the solubilisation of chitosan before the sterilisation step. Moreover, the method used for group C avoided the addition of the acetic acid under aseptic conditions, reducing the risk of contamination of the sample. This last procedure allowed preparing ready-to-use chitosan solutions.

III.2. In vitro experiments

III.2.1. The compatibility between chitosan and *Pasteurella multocida*

When added to chitosan, the bacterial population should be maintained stable, with long shelf life, until the administration of the vaccine. Indeed, the bacterial population should be sufficient to induce a protection of animals, against virulent pathogen. In our case, live attenuated bacteria based-vaccine needed to maintain a bacterial population above 90 % of the initial amount.

In this experiment, the viability of bacteria in chitosan solutions was determined in order to elaborate an active vaccine formulation. Chitosans (DA from 2 to 50 %) and *Pasteurella multocida* were mixed to prepare systems similar to *in vivo* experiments. The bacterial viability was established from 0 to 4 hours. The results, reported in table 2, demonstrated that chitosans at low (2 %) and high (50 %) DAs were not compatible

with live attenuated bacteria, with a decrease of 92 % and 88 %, respectively, of the initial population. However, a DA of 25 % had no impact on the bacterial counts.

Times (h)	Control group		DA 2 %		DA 25 %		DA 50 %	
	Bacteria count	Bacteria ratio	Bacteria count	Bacteria ratio	Bacteria count	Bacteria ratio	Bacteria count	Bacteria ratio
0	4.1E+09	1	1.1E+09	1	2.3E+09	1	2.1E+09	1
1	6.7E+09	1.613	8.1E+08	0.738	2.4E+09	1.059	1.2E+09	0.566
2	8.1E+09	1.698	3.9E+08	0.360	3.1E+09	1.382	5.0E+08	0.236
4	7.5E+09	1.823	8.8E+07	0.080	2.1E+09	0.926	2.6E+08	0.123

Table 2: Evaluation of the compatibility between chitosan solutions and an experimental live attenuated *P. multocida* bacteria strain. Results indicate the bacterial population after the incubation at various times. Bacteria ratio was calculated with the following relationship: $r = (\text{amount of bacteria at } t_x / \text{amount of bacteria at } t_0)$.

As reviewed by Kong *et al.*,²⁰ chitosan has antimicrobial properties, and the viability of bacteria was mainly dependant on the degree of acetylation. Low DAs were reported to have a good antibacterial activity by interaction between the high density of chitosan positive charges and the membrane of the bacterium.²¹ Two main explanations were proposed: (1) the formation of an impermeable layer around bacteria membranes by chitosan, blocking the transport of nutriments in the microorganism conducting to the death; (2) the penetration of chitosan in the bacteria disturbing the maturation of bacteria because of interactions with DNA.²⁰

In case of DA = 50 %, we expected to obtained a lower antibacterial activity than DA = 2 % and 25 %. No similar results were reported in the literature for high degree of acetylation. Indeed, recent experiments led with chitosan at various DA by Younes *et al.* in the presence of four gram-negative bacteria as *Pasteurella multocida*,²² showed that the polymer antibacterial activity decreased with increasing DAs. Both DA at 41 and 42 % provided a lower activity than DA = 24 % itself lower than DA = 2 % evaluated in the study. In an other study, Mellegård *et al.* observed the same tendency with chitosans at DA = 16 and 48 %. The lower DA offered a greater antibacterial activity on *E.coli*.²¹ Nevertheless, it is important to note that experimental conditions such as the nature and the initial amount of bacteria, the chitosan concentration, the pH and the culture medium affected the final activity. Anyway, the reason why, contrary to the above

studies, our chitosan at DA 25 % had a lower antibacterial activity than the sample with DA = 50 % still remains unclear, though this was reproducibly observed.

Finally, results for medium DA were satisfying for the elaboration of chitosan-based vaccines: at DA = 25 %, the bacterial population remained stable for 4 h. Despite the low compatibility of chitosan at DA = 50 % with *Pm* bacteria, it was selected for *in vivo* assessment due to the facility to elaborate a homogenous chitosan solution at neutral pH and because of *in vitro* and *in vivo* experiments were conducted in parallel, avoiding to predict the obtained *in vivo* results. Chitosan with the low DA was removed from *in vivo* assessment because of the difficulty to prepare a homogenous material at the pH of administration, chitosan turned from solution to gel at pH about 6.0.

III.2.2. Compatibility of chitosan with live attenuated virus

The viability of live attenuated virus into chitosan solutions was evaluated and, as for bacteria, was a selective criterion in the elaboration of an efficient vaccine.

Chitosan solutions at 0.4 % (w/v) were used to reconstitute a lyophilized live attenuated canine virus and incubated at 37°C for 2 hours. In this experiment, Canine Distemper Virus (CDV), was used as model for other canine virus, particularly the Canine Adenovirus of type 2 (CAV-2) used for the *in vivo* evaluations of chitosan-based solution in dogs. The infectivity was evaluated by determining the CCID 50 corresponding to the 50% *cell culture* infective dose²³ with a control group (in buffer solution) and with two chitosans of different molar masses.

The results, reported in figure 1, indicated that both high and medium M_w chitosans were included between the confidence interval of the control group. The viral population was not modified in presence of chitosan-based solutions demonstrating the ability to use the polymer as adjuvant candidate for live attenuated virus.

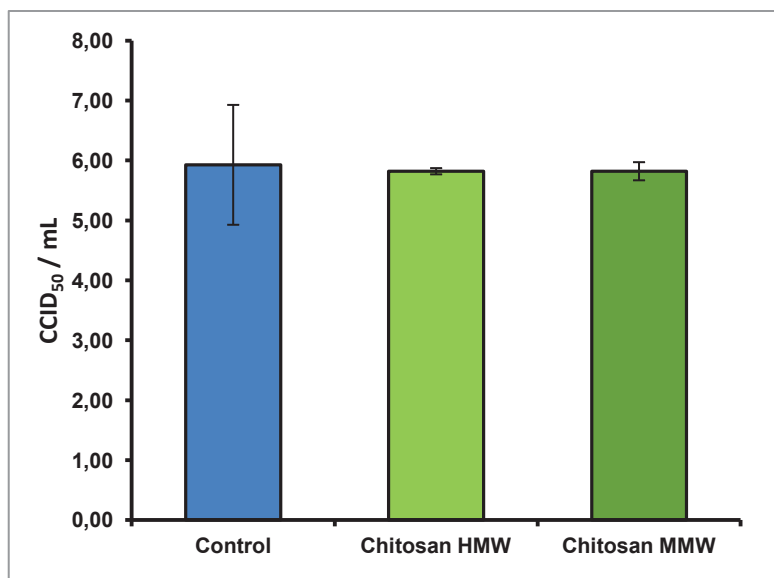


Figure 1 : Evaluation of the compatibility of chitosan (DA=45%) solutions mixed with the canine distemper virus, a live attenuated canine virus. *CCID 50* (50% cell culture infective dose). Control group was free of chitosan.

To our knowledge no information on chitosan compatibility with live attenuated virus was reported about mammal viruses. So, our results demonstrated, for the first time, the compatibility of chitosan with a high degree of acetylation to be used in presence of virus. Wang *et al.* reported the use of chitosan as adjuvant in case of influenza vaccination using live attenuated virus.²⁴ Even if they did not describe the compatibility between chitosan and the live attenuated antigen, their results were in favour of compatibility as their results showed protection in vaccinated animals.

III.3. In vivo experiments

III.3.3. Live attenuated bacteria in turkey

A live bacteria attenuated by genetic modifications was used to induce immunity against *Pm* bacteria, the pathogen agent of fowl cholera.²⁵ A control group, without vaccination, was used to check the virulence of the challenge strain. Two groups of animals received the vaccine, formulated with a chitosan solution with a DA of 25 or 50 % (as described in section II.3.1) and administered via the ocular route. On day 21, turkeys were challenged with a virulent strain of *Pasteurella multocida*.

No undesired effect was observed after the intra-ocular inoculation of young turkeys with chitosan-based vaccines. No apparent irritation signs such as conjunctiva

or corneal oedema were observed and the general health condition was satisfying. All animals survived until day 20 before challenge, highlighting the safety of the administered chitosan solutions.

First, the control group confirmed the virulence of the challenge strain with a high mortality level in animals. The mortality and morbidity, reported in table 3, indicated an influence of the degree of acetylation on the protection against virulent bacteria with a rate of surviving animals up to 90% in the presence of the lower DA at 25%. Chitosan-based formulation with DA = 50 % was not effective as it displayed low level of protection against virulent strain with a rate of surviving animals up to 30%.

Group	Efficacy results on day 35				
	# on D21	# of deaths	# of sick turkey at day 35	Protection against mortality	Relative protection against morbidity
Chitosan antigen DA=25 % +	10	1	0	90 %	88.9 %
Chitosan antigen DA=50 % +	10	7	0	30 %	22.2 %
Control group	10	9	0	-	-

Table 3 : Protection by live attenuated *Pm* chitosan-based vaccine immunization after lethal challenge *Pm* in turkeys on day 35.

A good safety record of chitosan-based solutions was observed by Di Colo *et al.*²⁶ by the administration of chitosan hydrochloride and *N*-carboxymethylchitosan (Cp = 1% (w/v); Mw = 1490 kg.mol⁻¹; DA = 10 %) in eyes of rabbits. In other study, chitosan hydrochloride (Cp = 0.5 % (w/v); DA = 10–25 %) was also used by Rauw *et al.*²⁷ via oculo-nasal administration and they did not report any side effects after vaccination. Anyhow, to the best of our knowledge, no work reported any animal protection against *Pm* bacteria with chitosan solutions.

In this study, the lowest DA offered the best protection after challenge indicating a good immune protection after vaccination compared to the higher DA. It was reported by Sogias *et al.*²⁸ that chitosan featured a better mucosal adhesion properties at low DA. In acidic conditions, the positive charges along the chitosan chain favoured electrostatic interactions with the mucus layer at the eye surface, allowing adhesion.²⁹ We can assume that the retention time due to a stronger adhesion of chitosan at DA = 25 % was longer than DA = 50 %. Moreover, it was reported, in case of mucosal administration,

that a higher viscosity favoured the retention time of the mixture allowing the antigen to go through the natural barriers and interact with immune cells.³⁰ In our work, though we did not conduct viscosity measurements, the fluidities of both chitosan solutions at DA = 25 and 50 % appeared similar, suggesting that viscosity could not account for the differences in protections observed between the two DAs. We can assume that the good level of animal protection after challenge obtained with the low DA chitosan-based vaccine could be attributed to the better preservation of bacteria in this system. *In vitro* experiments of section II.2.1 clearly support this assumption, as with DA = 50 % the decrease in the bacterial titer was about of 76 % after 2 h (approximate time between preparation and administration).

III.3.4. Live attenuated virus in dogs

Live attenuated virus CAV-2 mixed with chitosan solution at 0.4% (w/v) (as described in section II.3.2) was administrated in three month-old dogs, at day 0 (prime) and day 28 (boost) via subcutaneous route. Control group, vaccinated with alum-based adjuvant in similar conditions, was used as positive reference. Safety, humoral and cellular immune responses were monitored until day 84.

No side effect was observed via subcutaneous immunization of dogs after prime/boost vaccinations. The dogs' general health was good and they presented a normal development. No local reaction was observed at the injection site and the palpation indicated a good resorption of polymer.

The serological monitoring (Figure) indicated that chitosan-adjuvanted preparation enhanced a humoral immune response as strong as alum, until day 84. Boost vaccinations at day 28 did not induce an increase in the specific IgG antibody titers for both adjuvants. It was demonstrated, by Wang *et al.*,²⁴ that a chitosan (DA, Mw unknown) solution (final concentration at 0.2 % (w/v)) could enhance the immunogenicity of live attenuated influenza vaccine (LAIV). IgG, IgG2a and IgG1 antibody titers were found to be significantly higher than LAIV via intranasal administration in mice. In our case, the injections were subcutaneous and, to our knowledge, no work ever reported the protection of animals with chitosan-adjuvanted live attenuated virus via this route.

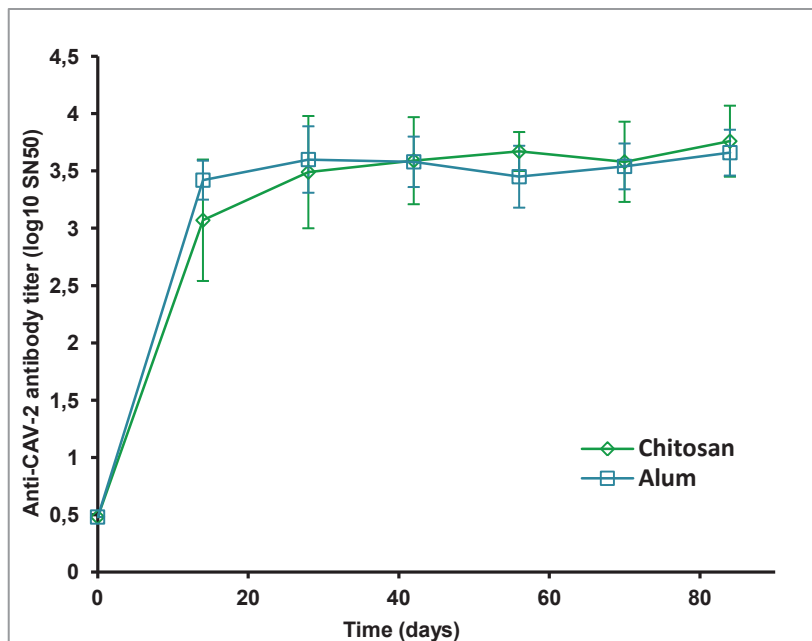


Figure 2 : Seroneutralizing titres after subcutaneous vaccination with live attenuated CAV-2. Total anti-CAV IgG before the prime vaccination and subsequent after prime and boost vaccinations. Mean titres \pm SD of seven dogs are shown. Chitosan DA = 45 % and Mw = 550 kg.mol⁻¹ mixed with CAV-2 $\geq 10^{2.5}$ DIC₅₀

The number of IFN- γ secreting cells measured at day 35 in both chitosan and alum adjuvanted CAV-2 showed a comparable cellular immune response in both animal groups.

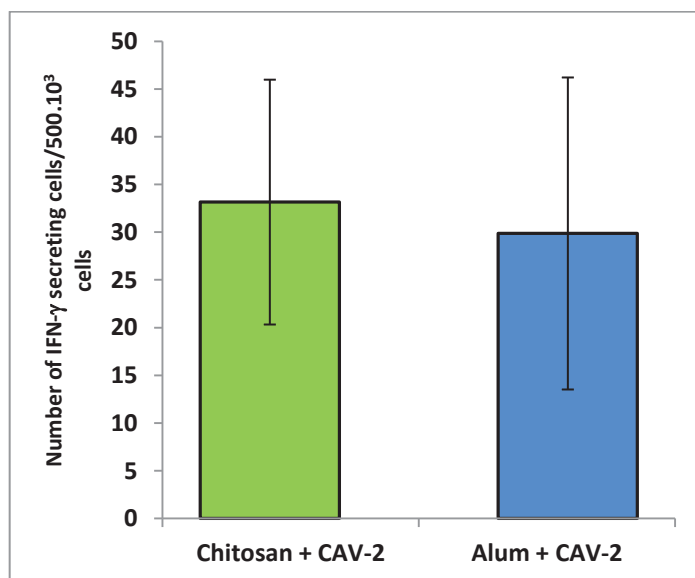


Figure 3 : IFN- γ titers after subcutaneous vaccination with CAV-2 antigen formulated with Chitosan or Alum.

III.3.5. Inactivated virus in dogs

Inactivated Rabies virus was mixed with chitosan solution (as described in section II.3.2) for administration to three month-old dogs, via subcutaneous route without boost. A positive control group was vaccinated in similar conditions with alum (close to the commercial product). Safety, humoral and cellular immune responses were monitored until day 84.

The serological monitoring (Figure 4) showed that the humoral immune response of the chitosan-based vaccine was lower than the positive control at the onset of immunity and also in the long term immune response. Anti-rabies antibody titers increased up to a maximum at day 14, then decreased until the day 56. One month after the vaccination, antibody titers in the chitosan group dropped lower than the recommended value of the World Health Organization (WHO) for the rabies protection i.e. 0.5 UI/mL.³¹

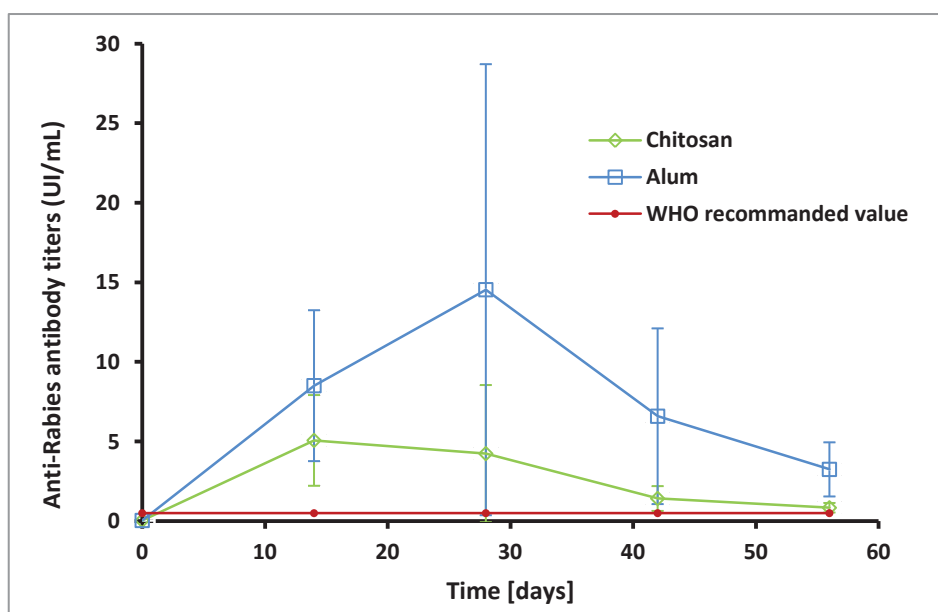


Figure 4 : Serological monitoring of antibody titres after subcutaneous vaccination with live Rabies antigen. WHO set the protection of animal above 0.5 UI/mL. Means titres \pm SD of seven dogs are shown. Chitosan at DA= 45 and Mw = 550 kg.mol⁻¹.

The results showed that chitosan viscous solutions were not able to induce a sufficient and prolonged immune response in case of rabies vaccination. Alum, used as reference, achieved the animal protection, thanks to a better stimulation of the immune system. Three mechanisms of action were classically attributed to alum in the induction of an immune response³²⁻³⁴: (1) the formation of a depot at the injection site offering a

sustained release effect; (2) the association of antigen with alum producing a particulate system more easily uptaken by the antigen presenting cells (APCs); (3) the immunostimulation of immunity by the induction of inflammation activating APCs.

The first mechanism appears as the most disputed and recent investigations by Hutchison *et al.*³⁵ and Gupta *et al.*³⁶ reported that the formation of a depot was not necessary for the induction and set up of the immune response. Nevertheless, based on this first explanation, Zaharoff *et al.* investigated chitosan viscous solutions and reported,³⁷ with fluorescence imaging, that the administration of chitosan via the subcutaneous route allowed the retention of a model protein at the injection site in mice. The results showed that more than 60 % of the initial amount of antigen was maintained into chitosan after 7 days following vaccination. Despite the fact that chitosan parameters were not reported, we could assume that in our case the viscosity of the injected solution was not sufficient to induce a depot effect, because of a lower concentration, contrary to Zaharoff. Moreover, the mucoadhesive properties of chitosan were probably not effective in case of subcutaneous administration than mucosal decreasing the retention of polymer.

Secondly, the association of antigen with alum was suggested to promote the formation of a particulate system allowing the absorption by APCs. Several factors affected this proposition such as the chemical nature of the aluminum salt, the properties of the antigen, the preparation of antigen- material adjuvant and, finally, the environmental parameters (pH, buffer composition).³⁸ In our case, no experiment was conducted to confirm the formation of particulate materials but Bekale *et al.* demonstrated that chitosan solutions were able to form particles with proteins.³⁹ The low immune response induced in dogs could be attributed to the fact that particles obtained from chitosan solution and rabies antigen were not efficient because of a non-optimal size or an altered antigen presentation. We could also presume that the formation of complexes did not occur so decreasing the adjuvanticity of the polymer.

The elucidation of the immunological mechanism of action of alum remains an intensive research field. The latest proposition suggested that alum induced caspase-1 activation, initiating IL-1 β and IL-18 release.⁴⁰ These latter interleukins were shown to be pro-inflammatory cytokines, playing various biological roles in the immune and inflammatory responses.^{41,42} Then, Li *et al.* suggested that the secretions of IL-1 β , IL-18 and

IL-33 were mediated by the NLRP3 protein (or NALP3) and ASC (apoptosis-associated speck-like protein, an adaptor molecule).⁴³ They reported that alum induced the formation of the NLRP3 inflammasome, but also other adjuvant such as QuilA or chitosan particles, suggesting a common mechanism for particulate systems. Mechanism of action was not clearly established concerning the relationship between inflammasome and alum adjuvanticity, as stated by De Gregorio *et al.*,⁴⁴ and remains under investigation. Concerning chitosan, Bueter *et al.* demonstrated that chitin (in particulate form) was not able to activate NLRP3-inflammasome contrary to particulate chitosan, showing the influence of the degree of acetylation of the polymer.^{45,46}

In our case, the low immunization performances of the chitosan-formulated virus could be attributed to the fact that, contrary to alum, no particle, in optimal configuration, was formed between rabies inactivated virus and chitosan. Moreover we used a chitosan with a DA close to 50 %, a high DA probably deleterious to the induction of an immune response, according to the work by Bueter *et al.* cited above.

III.3.6. Recombinant protein loaded chitosan formulations in pigs

In this protocol, chitosan-based nanoparticles were elaborated, via the complexation of oppositely charged polyelectrolytes, in order to provide a homogeneously distributed material mimicking the particular shape of infectious agents such as virus or bacteria.⁴⁷ This kind of system was naturally considered to target antigen presenting cells and to facilitate the recognition and presentation of antigen compared to the recombinant protein alone.⁴⁸ Moreover, the mix of antigen loaded particles with a chitosan solution was also envisioned in order to obtain a sustained release system, one of the mechanism associated to alum suggested to improve the immune response.⁴⁹

Chitosan-based solutions, chitosan-based submicronic particles and mixtures of chitosan-based solution and particles were prepared (as described in section II.3.3) and mixed with a recombinant protein, SpaA, from *Erysipelothrix rhusiopathiae* bacteria. The prepared vaccines were administered in pigs via the intramuscular route at day 0 and 21, and the humoral response was monitored for 49 days after the first injection.

No side effect was observed via intramuscular immunization of pigs after prime and boost vaccination. General health of pigs was satisfying and they presented a normal development. Post-mortem inspection at injection sites did not revealed lesions, oedema nor residues. These first results showed that chitosan at DA = 45 % was eliminated in less than 49 days without deleterious effects on animal health and it could be used for a parenteral application, contrary to the recommendation in literature.^{50,51} Indeed, chitosan with high DA was known to be degraded faster than low DA and the accumulation of the amino sugars produced an inflammatory response responsible of the formation of local undesired reactions.⁵²⁻⁵⁴

The anti-SpaA IgG titres were measured in sera before the second vaccination and twice after. All formulations induced a higher specific antibody titres compared to the control group with spaA in PBS buffer (historical data not shown). The results obtained were variable depending on chitosan-based formulations with a best protection for the [solution+SpaA] group. In this group, all the animals were seroconverted at day 42 and 49 while for [NPs/SpaA] and [Solution+NPs/SpaA] groups, not every pig responded to vaccination. We also note that the [Solution+SpaA] group was the only one to have antibody titers reaching the threshold of 2.2 Log₁₀ required to consider the animals as protected against *Erysipelothrix rhusiopathiae*. However, the best combination of adjuvants and SpaA antigen tested in the laboratory raised antibody titers up to 4 logs which were not reached with our formulations (historical data not shown).

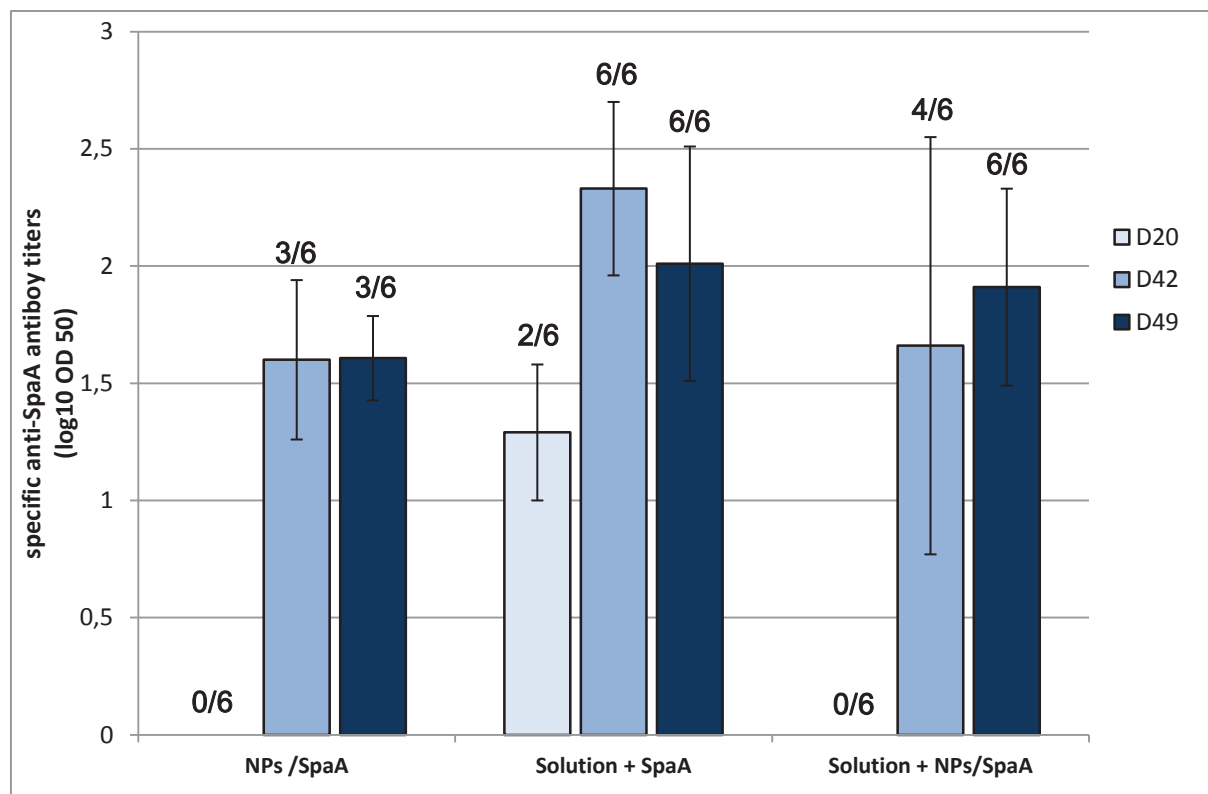


Figure 5 : Antibody titres after intramuscular vaccination with SpaA. Total anti-SpaA IgG before the boost vaccination and two subsequent after boost vaccination. Mean titres \pm SD of responding pigs are shown.

The chitosan-based particles used in this study were previously used as vaccine delivery system by adsorbing the p24 HIV-1 capsid protein on the surface of submicronic particles.^{55,56} Both studies reported to stimulate an immune response via the enhancement of IgG, IFN- γ and IL-4 production after subcutaneous vaccination in mice. In our experiment, nanoparticles (group [NPs/SpaA]) were not found to improve the immune response compared to the antigen alone (data not shown). Indeed, as mentioned by Islam *et al.*,⁶ various works demonstrated an enhanced protection with chitosan-based particulate vaccines. However, our results are close to those of Gordon *et al.* who obtained no immune response after subcutaneous injection in mice of OVA loaded chitosan-based particles (obtained from precipitation/coacervation method) contrary to the chitosan-based hydrogel they used.⁵⁷

The results of [solution+SpaA] group are consistent with other studies led by Ghendon *et al.* with influenza subunit antigen via intramuscular administration using chitosan-based solution.^{58,59} In our case, we used a high degree of acetylation (DA= 45%), whereas other investigators used chitosan with a DA lower than 30 % (more often 20%) and various molar masses (from 10 to 500 kg.mol⁻¹). A higher DA was

favourable for the formulation of the vaccine at physiological pH, contributing to the biocompatibility of the material. Moreover, our final concentration of the injected chitosan solution was 0.75 % (w/v), higher than classically used (0.5 % (w/v)). As mentioned previously, a higher viscosity of chitosan solution is favourable to obtain a sustained release effect³⁷ suggested to promote a prolonged immune response.⁶⁰

With the mix system [solution+NPs/SpaA] the enhancement of the protection was intermediate between those obtained with the [NPs/SpaA] and the [solution+SpaA] groups. This result can probably be explained considering that a fraction of the antigen macromolecules contained in the chitosan solution adsorbed on the particles and thus became inefficient, with respect to the observed low adjuvant activity of the [NPs/SpaA] system. To improve performances of the mixed delivery system, we could investigate other materials for the nanoparticulate delivery system keeping the chitosan solution as an *in situ* gelling matrix, because of its biocompatible, biodegradable and adjuvant properties. As an example, inorganic particles such as silica⁶¹ or gold⁶² could be considered as they were successfully used in vaccine applications.

Mixing adjuvants was also reported to be an advantageous strategy to improve the performance of vaccine as suggested by Guy.⁶³ For instance, the combination of chitosan with immunostimulant, like CpG in the vaccination against *Helicobacter pylori*, or the mix of LTK 63,⁶⁴ a mutant *E.coli* enterotoxin, with chitosan microparticles induced a superior immune response than when the adjuvant was administrated separately.⁶⁵

To improve the performance of chitosan, other administration routes could be investigated and particularly the intranasal route. Indeed, it was reported earlier⁶⁶ that the use of chitosan in mucosal vaccination allowed the elicitation of effective immune responses. Islam *et al.*⁶ reviewed the use of chitosan microparticles as nasal and oral vaccine carriers. As said previously, the bioadhesive properties of chitosan increased the retention time of formulation and this polysaccharide can open the tight junctions of the epithelial barrier facilitating the transport of antigen to immune cells.

Injectable *in situ* forming chitosan hydrogels have been investigated for many years now and appeared to be interesting alternatives to chitosan viscous solutions. Hydrogels were reported to be a promising system for the sustained release of active compound as reviewed by Vermonden *et al.* for protein delivery.⁶⁷ Gordon *et al.*

reported in many studies^{57,61,68} the use of chitosan and glycerol-phosphate salts to elaborate adjuvant material able to undergo a sol-gel transition at body temperature. They demonstrated that the presence of hydrogel favoured a higher immune response compared to antigen alone or loaded particles and stimulated both the cellular and the humoral immunity.

IV. CONCLUSION

In this paper, we reported a method to sterilise chitosan solutions without degradation, i.e. preserving the intrinsic parameters of the polysaccharide. Hence, it was possible to prepare ready-to-use sterile solutions of chitosan with perfectly controlled physico-chemical properties. Then, the adjuvant capacity of the chitosan solutions was demonstrated as it enhanced the immune responses obtained for various antigens, in different animal species. The chitosan-based solutions were mixed with live attenuated bacteria for avian species, live attenuated and inactivated viruses for canine species and a recombinant protein for pigs. The association of each antigen with chitosan induced immune responses. For turkey immunization, we demonstrated the influence of the degree of acetylation in the protection of challenged animals. A medium DA (25 %) was advantageous with a protection level up to 90 % contrary to 30 % with a high DA (50%). In dogs, a high DA induced an immunological protection in case of CAV-2 vaccination. Chitosan solutions were equivalent to the reference i.e. alum hydrogel. We obtained a promising immune response in case of Rabies vaccine, which requires further investigations to establish a long-lasting protective immunity. The activation of the immunity in pigs against the *Erysipelothrix rhusiopathiae* bacterium was effective using a protein as antigen, but remained also perfectible.

Chitosan-based solutions appeared to be promising materials for the veterinary fields due their ease of preparation, their good safety records and the relative low-cost of polymer.

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CONCLUSION DU CHAPITRE 2

Le chapitre deux rapporte la préparation et l'évaluation du chitosane comme adjuvant dans des vaccins expérimentaux. Différents modèles animaux ont été testés, la dinde, le chien et le porc, en présence d'antigènes respectivement de nature bactérienne, virale et protéique.

D'une part, le polymère présente une innocuité satisfaisante chez toutes les espèces puisque aucun effet secondaire n'a été constaté. Par ailleurs, des inspections post-mortem du site d'injection sur les porcs ont été conduites montrant une bonne résorbabilité, puisqu'aucune trace de fibrose ou de résidu lié au vaccin n'a été observée.

D'autre part, les résultats expérimentaux des essais *in vivo* sont encourageants puisque une protection ou une réponse immunitaire ont été obtenues chez toutes les espèces et pour des natures d'antigène très variées. Chez la dinde, le vaccin à base de chitosane (DA = 25 % et Mw = 220 kg.mol⁻¹) contre la bactérie *Pasteurella multocida* a permis de protéger 90 % de la population après l'administration d'une souche pathogène. Concernant le modèle chien, les essais d'immunisation contre l'adénovirus canin de type 2 ont montré une réponse équivalente entre le vaccin à base de chitosane et la référence commerciale à base d'aluminium. Les essais réalisés sur le modèle rage ont montré quant à eux une réponse immunitaire plus faible que la référence et moins durable dans le temps. En effet, le seuil de protection pour les animaux, défini par l'OMS à 0,5 UI/mL d'anticorps neutralisants, n'a pas été maintenu avec le vaccin à base de chitosane. Cependant, la modularité des performances adjuvantes du chitosane, en fonction de sa masse molaire et de son DA, permettent d'envisager des réponses immunitaires potentiellement supérieures aux actuelles. Enfin, chez le porc, la comparaison des différents matériaux à base de chitosane évalués a révélé que la solution à base de chitosane permet d'induire une réponse immunitaire. Cette réponse est inférieure aux vaccins commerciaux mais offre une meilleure innocuité. Les nanoparticules ou les mélanges nanoparticules/solution de chitosane n'ont pas permis d'obtenir de réponse immunitaire satisfaisante.

Ces trois études montrent le potentiel du chitosane comme adjuvant de vaccins. Cependant, il est difficile de tirer une conclusion générale des propriétés du chitosane puisque différents polymères ont été élaborés pour des raisons d'applicabilités. Par ailleurs, la nature de l'antigène et le site d'injection sont d'autres facteurs influençant

fortement la réponse immunologique. Dans ces travaux la voie mucosale a permis d'obtenir les résultats les plus satisfaisants tandis que les voies sous-cutanée (chez le chien) et intramusculaire (chez le porc) ont montré des performances perfectibles.

Par ailleurs, la littérature rapporte des exemples d'hydrogel à base de chitosane permettant d'induire une bonne réponse immunitaire chez l'hôte, grâce à l'effet « réservoir » induisant un relargage prolongé dans le temps de l'antigène. Sur ces bases bibliographiques, nous avons envisagé l'administration d'hydrogels de chitosane comportant l'antigène à délivrer. Les solutions visqueuses de chitosane, utilisées dans les formulations précédemment, ne conduisent pas à la formation d'un hydrogel physique de polymère *in vivo*. Ainsi, nous avons conçu des formulations de solutions de chitosane capables de gélifier *in situ*, car des hydrogels ne peuvent être injectés directement au moyen de seringues et d'aiguilles conventionnelles. Cette étude sera l'objet du chapitre 3 de ce manuscrit.

CHAPITRE 3

HYDROGELS PHYSIQUE DE CHITOSANE INJECTABLES ET GELIFIANTS *IN SITU* POUR LA DELIVRANCE DE PROTEINE : PREPARATION ET CARACTERISATION

- Chapter 3 -

*Chitosan-based injectable in situ gelling physical hydrogels for
protein delivery: preparation and characterisation*

INTRODUCTION AU CHAPITRE 3

Le troisième chapitre de ce manuscrit de thèse rapporte les résultats et discussions liées à la préparation et la caractérisation *in vitro* d'un système de délivrance de vaccins à base d'une solution de chitosane gélifiante en conditions *in vivo*.

L'administration de solutions de chitosane gélifiables *in vivo* pour la délivrance de principes actifs, particulièrement de vaccins, nécessite actuellement l'utilisation d'agents de réticulation dont la biocompatibilité n'est pas encore satisfaisante. Ainsi, la préparation de solutions injectables et gélifiantes, en conditions physiologiques, à base de chitosane seulement demeure un challenge.

La section « *Materials and Methods* » concerne la préparation et la caractérisation des systèmes injectables à base de solutions de chitosane gélifiantes en conditions physiologiques. Les sections II.1, II.2 et II.3 décrivent les produits utilisés ainsi que la modification du chitosane et sa caractérisation. En effet, différents chitosanes ont été utilisés pour la fabrication des particules (CS-NPs) et des hydrogels. La section 2.4 rapporte la préparation des CS-NPs utilisés comme vecteurs de protéines, ici l'ovalbumine (OVA), celle-ci utilisée comme antigène modèle. Les sections 2.5 et 2.6 sont consacrées à l'élaboration des solutions gélifiantes de chitosane et à leur procédé de gélification en conditions *in vivo* (tampon PBS, pH = 7,4, température de 37°C). Les sections II.7 et II.8 sont consacrées à la caractérisation des nanoparticules (mesure de taille) et des interactions CS-NPs et OVA (rendement d'adsorption, stabilité). Les sections II.9 et II.10 décrivent, respectivement, l'analyse par rhéologie des matériaux à bases de chitosane (solutions et hydrogels) et le relargage de l'OVA (marqué par un agent de fluorescence) dans des conditions proches *in vivo*.

La section III « *Results and Discussion* » expose les résultats obtenus à partir des expériences décrites dans la section II. La section III.1 porte sur la caractérisation du chitosane. Dans la section III.2 nous montrons que les CS-NPs à 6 % (w/v) dans l'eau sont stables durant 125 jours à 5 et 37°C avec des tailles moyennes de 660 nm et 540 nm, respectivement. La stabilité des particules permet leur conservation avant l'ajout de l'antigène, offrant ainsi un temps de travail confortable. Dans la section III.3, l'adsorption d'OVA sur les CS-NPs a été suivie sur une période de 24 h et de 30 jours. Les rendements d'association sont supérieurs dans tous les cas à 60 %. Ces résultats

montrent une bonne stabilité des suspensions colloïdales sous leurs formes « nues » ou « décorées » (c'est-à-dire en présence ou non de protéines). Dans la section III.4 nous mettons en évidence que des mélanges de chitosanes de masses molaires différentes, mais de degré d'acétylation équivalent, permettent d'accroître la concentration en polymère des solutions tout en conservant une viscosité compatible avec une injection dans l'organisme. Ainsi, l'utilisation d'une matrice polymère constituée d'un chitosane de haute masse molaire ($> 500 \text{ kg.mol}^{-1}$) et de faible masse molaire ($< 10 \text{ kg.mol}^{-1}$) permet l'élaboration de systèmes injectables et gélifiants en conditions physiologiques. L'étude de la viscosité a permis de déterminer une valeur seuil maximum de 100 Pa.s pour laquelle, sous faible taux de cisaillement, il est inconfortable ou impossible de prélever la solution de chitosane avec un système d'administration classique de vaccin, à savoir une seringue équipée d'une aiguille. Les formulations pouvant être utilisées pour la préparation de solutions gélifiantes injectables sont les suivantes : $C_{\text{HMW}} = 1 \text{ \% (w/v)}$ and $C_{\text{LMW}} = 1 / 1.5 / 2 / 3 \text{ \% (w/v)}$; $C_{\text{HMW}} = 1.25 \text{ \% (w/v)}$ and $C_{\text{LMW}} = 1 / 1 / 1.5 / 2 \text{ \% (w/v)}$; $C_{\text{HMW}} = 1.5 \text{ \% (w/v)}$ and $C_{\text{LMW}} = 1.5 / 2 \text{ \% (w/v)}$. L'incorporation de l'OVA seule ou de CS-NPs chargée d'OVA modifie la viscosité initiale des solutions de chitosane mais ne perturbe pas de leur gélification. Enfin, la section 3.5 est l'objet des résultats et discussions du relargage de la protéine modèle à partir d'hydrogels de chitosane. La présence de deux chitosanes est moins favorable à la rétention de l'antigène seul dans la matrice, en comparaison avec une formulation contenant un seul chitosane de haute masse molaire. L'ajout de l'OVA sur les NPs est également moins favorable à la rétention de la protéine. Cependant, les résultats sont encourageants puisque de faibles pourcentages de relargage ont été observés, moins de 5 % sur des durées de 48 heures pour tous les systèmes évalués.

Ces travaux ouvrent des perspectives permettant d'envisager la modulation des propriétés des solutions gélifiantes en modifiant le degré d'acétylation du chitosane et sa masse molaire ou en étudiant des mélanges de chitosanes de façon plus systématique. L'influence du DA sur les propriétés de gélification pourraient permettre de modifier la zone de solubilité du chitosane. Par ailleurs, le protocole en conditions physiologiques pour l'étude du relargage des protéines reste éloigné des conditions *in vivo* d'un vaccin.

La formulation d'une solution gélifiante *in situ* à base de chitosane seulement reste un défi technologique. Les premiers éléments apportés par ce chapitre 3 permettent de donner des orientations prometteuses pour atteindre un tel système.

Chitosan-Based Injectable *In Situ* Gelling Physical Hydrogels For Protein Delivery: Preparation And Characterisation

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ABSTRACT

The elaboration of injectable *in situ* gelling chitosan physical solutions as antigen delivery devices is reported. The aim is to prolong the exposure of immune cells to the antigen, with sustained-released materials so as to improve the vaccine residence time at the depot site. To be administered by injections, we selected an *in situ* gelling liquid matrix in which nanoparticles containing the model antigen were dispersed. This liquid matrix was a chitosan solution designed to gel at physiological pH, in the absence of any chemical cross-linker. The formation of the physical hydrogels in physiological physico-chemical conditions was confirmed by analysing their viscoelastic properties. A mixture of high and low molar mass chitosans was required to obtain a syringeable and injectable *in situ* gelling chitosan-solution. FITC-labelled OVA, was used to determine the release properties of chitosan-based hydrogels elaborated.

KEYWORDS

Chitosan; hydrogels; vaccine delivery system; protein release; syringeable formulation

I. INTRODUCTION

Most vaccines in human or veterinary fields require repeated administrations to induce a strong and long-lasting immune response. The protection of a growing worldwide population orders new challenges in vaccine development such as dose-sparing and decrease of injuries due to the mishandling of needle.¹ Moreover, latest advances in biotechnology offer safer antigens, such as sub-unit proteins, but they require an adjuvant to elicit a satisfying immune response.² The elaboration of an all-in-one prime-boost vaccine, i.e. a device allowing the delivery of the antigen dose required for an efficient protection via a single injection, appears to be a response to these challenges by using new biocompatible and immune-stimulant materials.³ Natural polymers (alginates, hyaluronan, cellulose...) were studied in biomedical applications⁴⁻⁶ and offer the possibility to design safe and various physical forms, such as hydrogels⁷ or particles.⁸

Chitosan (CS) is a polysaccharide obtained from the partial deacetylation of chitin, a natural polymer extracted from crustacean shells or cell walls of fungi.⁹ The polymer is composed of *N*-acetyl-*D*-glucosamine and *D*-glucosamine units linked by a $\beta\rightarrow 1,4$ -glycosidic bond and the molar fraction of the *N*-acetyl-*D*-glucosamine in the polymer chain is referred to as the degree of acetylation (DA).⁹ Its extended properties such as biocompatibility,¹⁰ biodegradability, mucoadhesion¹¹ or immuno-adjuvantivity¹² have conducted many teams to use the polymer in various medical applications.¹³ Wound healing,¹⁴ tissue engineering¹⁵ and drug delivery systems¹⁶ were among the most explored and offer today various chitosan-based technologies such as micro/nanoparticles¹⁷ or hydrogels.^{18,19} Chitosan was also used in the vaccine field with promising results particularly via mucosal administration as reviewed by Islam *et al.*²⁰

The combination of various technologies that have emerged over the years in the vaccine formulation domain, leads to a new generation of carrier adjuvant able to promote a longer release of antigen, favourable for the stimulation of the immune system. *In vitro* results reported by Gordon *et al.* showed that the release of protein from OVA loaded silica particles (NPs/OVA) embedded within a chitosan hydrogel was slower than from NPs/OVA dispersed in PBS.²¹ Therefore, the embedded formulation of the

antigen was more efficient at inducing an immune response in mice than NPs/OVA or OVA in PBS.

Chitosan hydrogel could be prepared via physical or chemical synthesis routes.¹⁹ Most experiments reported the used of chitosan thermo-sensitive systems using additive compounds such as glycerol phosphate²² or PEG.²³ These 'smart' materials were able to modify their structure from a fluid solution at room temperature to a gel at body temperature after their implantation.²² An immune response was obtained in a mouse model by Wu *et al.* using a chitosan derivative/ α,β -glycerol-phosphate salt material in the presence of split H₅N₁ as antigen.²⁴ Recently, Chua *et al.* administrated to mice, an OVA loaded chitosan-based hydrogel (using tripolyphosphate and chondroitin sulphate as cross-linkers).³ In association with Montanide ISA 50 as immunostimulant, high antibody titres were observed over one year. The good immune response was clearly attributed to the presence of the hydrogel allowing a long lasting release of the antigen essential for the elaboration of 'one shot' vaccine delivery systems.

Many of the reported chitosan-based hydrogels were obtained with cross-linking agents, the biocompatibilities of which, was not fully known and should be further investigated to avoid deleterious secondary effects. For example, Molinaro *et al.* reported a severe oedematous response after injection of chitosan-based thermosensitive hydrogel via subcutaneous route in rat paw.²⁵

The design of a safe one-shot vaccine device devoid of cross-linker remains an unmet need and it is still a challenge to obtain a syringeable and injectable chitosan-based *in situ* forming hydrogel for antigen delivery. In this context, we report here on the elaboration of a syringeable and injectable chitosan-based, *in situ* gelling hydrogel, exempt of cross-linker, designed for protein delivery. Our approach consisted in mixing nanoparticles, as adjuvant carrier, in the presence of a model protein onto their surface, with an *in situ* forming chitosan hydrogel to induce a sustained release effect of particles.

II. MATERIALS AND METHODS

II.1. Materials

Chitosan from squid pen, batch 113, was purchased from Mahtani Chitosan PVT Ltd (India) and was purified before use as described in the next section (*purification of chitosan*). Glacial acetic acid; ammonium hydroxide; 1,2-propanediol; nitrite sodium; ovalbumin (OVA) grade V and dextran sulphate (DS 10K) were purchased from Sigma-Aldrich (France).

II.2. Modification of chitosan intrinsic parameters

II.2.1. Purification of chitosan

The chitosan powder was dissolved at 5 g.L⁻¹ in an aqueous acetic acid solution overnight at room temperature under mechanical stirring. The chitosan solution was filtered, under pressure at 3 bars, successively through 3, 0.8 and 0.45 µm pore size membranes (Millipore, France). Then, the polymer was precipitated by adding a concentrated solution of ammonium hydroxide, separate by centrifugation (10 000 rpm, 10 min) and washed several times with deionised water until neutral pH. Finally, the neutralised chitosan was freeze-dried and store at room temperature.

II.2.2. Acetylation of chitosan

The modification of the degree of acetylation was carried out according to Vachoud *et al.*²⁶ Chitosan was dissolved at 10 g.L⁻¹ in a hydro-alcoholic mixture of deionised water and 1,2 propanediol. Acetic anhydride was added to reach the desired degree of acetylation. Chitosan was precipitated, washed several times until elimination of alcohol, then frozen and lyophilised.

II.2.3. Depolymerisation of chitosan

Low molar mass chitosans were obtained according to Allan *et al.* controlled nitrous deamination allowing chain scissions.^{27,28} To determine the necessary time to obtain the targeted molar mass, we conducted a kinetics study of the depolymerisation reaction. A 20 mL chitosan solution at 0.5 % (w/v) in a pH 4.5 acetate buffer was prepared and the dissolution was conducted overnight at room temperature. Before the reaction, 12 Eppendorf tubes were prepared with 100 µL of ammonium hydroxide solution and 400 µL of deionised water to receive 1 mL aliquots at different time points. A 10 g.L⁻¹ sodium nitrite solution was added to the chitosan solution to obtain a

nitrite/glucosamine unit molar ratio of 0.2. Precipitates were centrifuged and washed with deionised water, then the molar mass of each chitosan aliquot was analysed. The depolymerisation kinetics was then established and used to prepare the targeted low molar mass chitosans, using similar experimental conditions.

II.3. Characterizations of chitosan

II.3.4. Determination of the degree of acetylation

The degree of acetylation (DA) of chitosan was calculated from ^1H nuclear magnetic resonance (NMR) spectroscopy according to Hirai *et al.*²⁹ 10 mg of chitosan were dissolved in 1 mL of D_2O containing 5 μL of 12 M HCl. The spectra were recorded on a Bruker spectrometer (400 Hz) at 30°C. The DA was deduced from the ratio of the area of the methyl protons of the *N*-acetyl-D-glucosamine residues to that of all of the H_2 to H_6 protons of both D-glucosamine and *N*-acetyl-D-glucosamine residues.

II.3.5. Determination of the molar mass

The weight-average molar mass (M_w) and the polydispersity index (PI) were measured by size exclusion chromatography (SEC) (3000 and 6000 PW TSK gel columns, 7.8 mm inner diameter and 30 mm length) coupled on line with a differential refractometer (Optilab T-rEX, Wyatt Technology) and a multi-angle-laser-light-scattering (MALLS) spectrophotometer (Dawn EOS, Wyatt Technology) equipped with a 5 mW He/Ne laser operating at 632.8 nm. Analyses were performed using a microbatch mode using K5 flow cell. A degassed 0.2 M acetic acid / 0.15 M sodium acetate buffer (pH 4.5) was used as eluent. The flow rate was maintained at 0.5 mL/min. Chitosan was dissolved in the same solvent at 0.1% (w/v) and 100 μL were injected after filtration on a 0.45 μm pore size membrane.

II.4. Preparation of the chitosan-based materials

II.4.6. Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared as described previously by Schatz *et al.*³⁰ Briefly chitosan was dispersed at 0.1% (w/v) in Versol[®] water. Solubilisation was achieved by adding a stoichiometric amount of acid corresponding to free amino groups. Ionic strength was adjusted to 50 mM with sodium chloride and a pH of 4.0 was obtained with 0.1 M hydrochloric acid. Dextran sulphate was solubilised directly in Versol[®] water, 50 mM NaCl was added to obtain the desired ionic strength and pH was

adjusted at 4.0. Both solutions were filtered through 0.22 μm membranes (Millipore, France) before use. Colloidal PECs were formed in non-stoichiometric conditions at a molar charge ratio $R (n^+/n^-) = 2$, at room temperature using chitosan as starting solution. The solution containing dextran sulphate was added in one shot to the starting solution, under a constant magnetic stirring of 750 rpm. The final volume of the particle dispersion was 800 mL with a solid content of 0.1% (w/w). Because of non-stoichiometric conditions, the polymer in excess was not completely consumed, thus, a low amount of free polymer still remained in solution. To remove it, particles were separated from the continuous phase by centrifugation at 9,000 rpm for 60 min at 20°C. The supernatant was discarded carefully, then the pellet re-suspended in Versol® water to reach a solid content of 6 % (w/v) and finally stored at 4°C.

II.4.7. Preparation of chitosan-based injectable solutions

Chitosan was solubilised in water by adding the stoichiometric amount of acetic acid to ensure protonation of the amino groups of D-glucosamine units to obtain a pH = 4.5. The polymer was dissolved overnight at room temperature under magnetic stirring. The administration of materials required to be close to a physiological pH, between 6.5 and 7.5. We chose to adjust the pH of the solution to 6.0, below the onset of the neutralisation of the chitosan solution so as to avoid gelation on storing. To prepare chitosan solution at pH = 6, we added three-fold less acetic acid than the required stoichiometric quantity and mixed until the entire solubilisation of the polymer. Finally, the pH was adjusted with glacial acetic acid or 1N sodium hydroxide. The preparation of solutions with one or more chitosans was realised in the same conditions. The pH of samples before gelation was measured with a pH-meter (model PHM210, MeterLab, Radiometer Analytical).

II.4.8. Preparation of chitosan-based hydrogels

A specific system was elaborated into the laboratory (Figure) in order to be close to the physiological conditions of gelification and allowing the characterization of obtained materials. In brief, the chitosan solution was introduced into a cavity formed by two dialysis membranes each one maintained between two Teflon rings with a height of 2 mm and with an internal radius of 25 mm. The final volume of the chitosan solution was 2 mL. The assemblage was finally closed with a screw-nut locking system. The system containing the chitosan solution was let into a PBS solution at 37°C for 24 hours

under gentle magnetic stirring to reach the complete gelation of the polymer. The selected dialysis membrane (Spectra/Por® membrane from Carl Roth) was made of regenerated cellulose with a molecular-weight-cut-off (MWCO) of 6-8 kg.mol⁻¹ and prepared before used as recommended by the manufacturer.

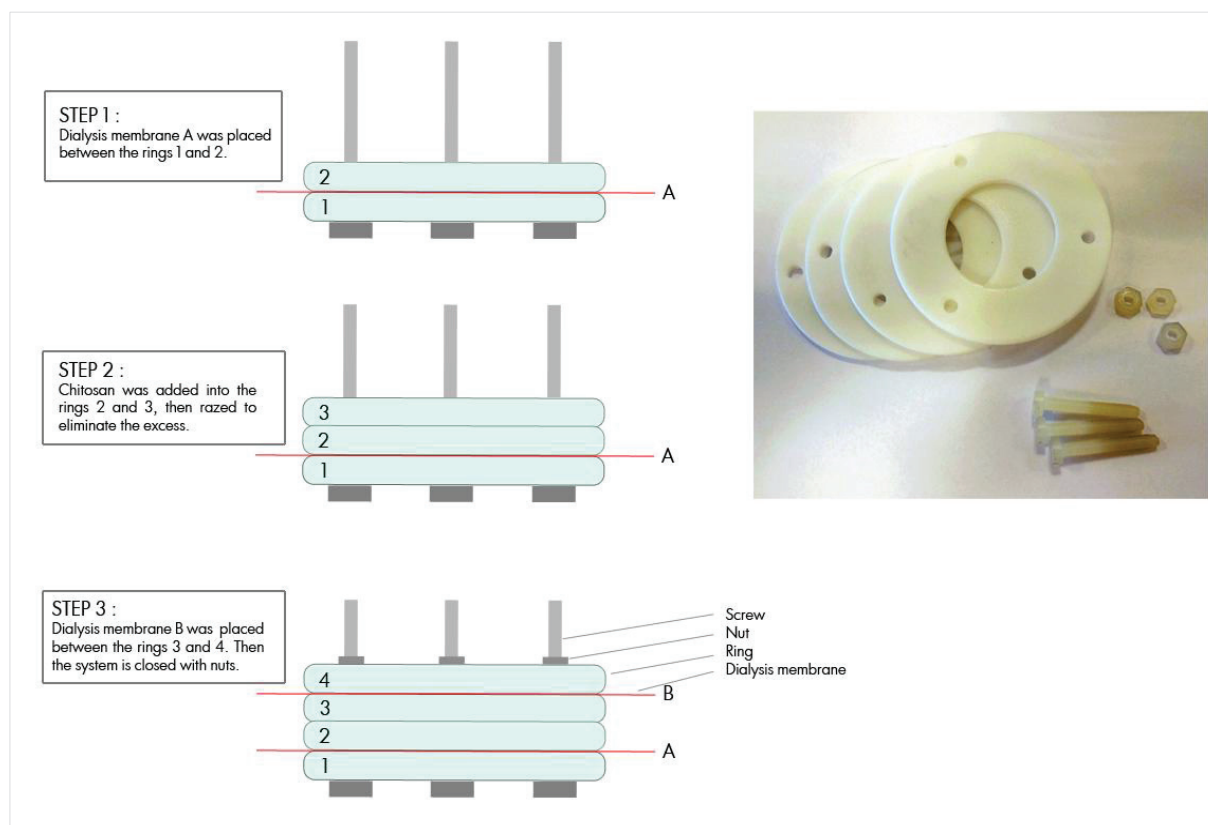


Figure 1 : Gelation system to prepare chitosan hydrogels *in vitro* conditions; the obtained tablets were directly usable for rheological measurements.

II.5. Characterisation and stability of nanoparticles

The mean sizes and the polydispersity indexes (PDI) of the prepared NPs were determined by dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Orsay, France) equipped with a 10 m/W He/Ne laser beam operating at $\lambda=633\text{nm}$ (at 173° scattering angle). The particles were diluted in water at 0.02 % (w/v) and the measurements (n=5) were performed at 25°C. The storage stability of nanoparticles (at 4 and 37°C) was evaluated for one month by macroscopic observations and size analysis.

II.6. Ovalbumin sorption onto nanoparticles and stability

The sorption process consisted in mixing volumes of particle dispersion and ovalbumin (OVA) solution, in Versol® water, to reach the desired pair of concentration (0.5, 1 and 3 % for the NPs and 25, 50, 100, 300 and 500 µg/mL for OVA), under moderate end-overhead stirring at room temperature. The OVA and NPs concentrations were obtained by dilution of the initial particle dispersion and protein with the same buffer. NPs/OVA particles were centrifuged 10 min at 25,000 rpm to remove residual particles. The amount of proteins loaded onto nanoparticles was deduced from the titration of free OVA in the supernatant, carefully separated from the pellet. The free protein was titrated using µBCA or BCA Protein assays according to the manufacturer's instructions, calibrated via serial dilutions in the same experimental buffer. The sorption yield was calculated as follows:

$$\text{Sorption yield (\%)} = \frac{[\text{OVA}]_{\text{input}} - [\text{OVA}]_{\text{residual}}}{[\text{OVA}]_{\text{input}}} \times 100$$

where $[\text{OVA}]_{\text{input}}$ is the OVA concentration titrated in the control sample (i.e., all the reactants were present but not the particle suspension) of the original OVA solution for each independent experiment; $[\text{OVA}]_{\text{residual}}$ is obtained from titration of the supernatant, taking into account the background signal from a blank experiment in which all the reactants were present but the protein. The stability of the NPs/OVA, stored at 4 or 37°C, was evaluated by measuring at different times the yield sorption of proteins onto nanoparticles.

II.7. Rheological studies of chitosan materials

The rheological properties of chitosan materials were performed on a controlled stress rheometer (AR 2000EX, TA Instruments). The temperature was fixed at 25°C for all experiments. The shear viscosity of solutions was measured with a cone / plate geometry with an angle of 0.0698 rad and radius of 25 mm and the size gap was 2000 microns. The shear rates ranged from 0.005 to 100 s⁻¹ and the value of the viscosity η_0 was evaluated at low shear rates from the extrapolated Newtonian plateau. The dynamic shear was measured with plate-plate geometry with a radius of 25 mm. The storage modulus (G') and loss modulus (G'') were measured as a function of the angular frequency. The values of the strain amplitude were verified to ensure that all measurements were performed within the linear viscoelastic regime, where the G' and

G'' are independent of the strain. The applied strain for the characterisation of gel was 0.8 %.

II.8. Protein release from chitosan-based hydrogel

We used a model protein, OVA, labelled with a fluorescent probe, fluorescein 5(6)-isothiocyanate, derived from fluorescein functionalized with an isothiocyanate reactive group reacting with proteins.³¹ FITC-OVA was prepared as described by Krauel *et al.*³² Briefly, 20 mg of FITC was dissolved in 10 mL of carbonate buffer (50 mM, pH 9.5) and 100 mg of OVA powder was added to the solution. The solution was stirred in the dark at 4°C for 20 hr. Unbound FITC was removed by dialysis against deionised water, with repeated washing, using a regenerated cellulose membrane (Spectra/Por® membrane, MWCO =6-8 kg.mol.⁻¹)

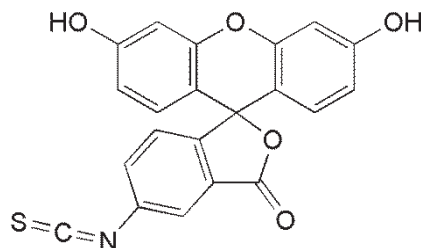


Figure 2 : Chemical structure of fluorescein 5(6)-isothiocyanate (FITC)

Labeled protein alone or sorbed FITC-OVA onto nanoparticles surface were introduced into chitosan *in situ* gelling solutions. The mixture was gelled by incubation during 24 hr, in the dark, at 37°C in PBS buffer (pH = 7.4). Then, the hydrogel was placed in a Petri dish and 2 mL of PBS buffer were added. Samples were placed in oven at 37°C. The supernatants were collected and replaced by a fresh PBS buffer at regular times for seven days. It was centrifugated and 200 μ L were placed into well of black plate for fluorescent analysis.

The released (FITC-OVA) in supernatants, from the physical chitosan hydrogel, was determined by spectrofluorometry (excitation wavelength = 490 nm, emission wavelength = 540 nm) using a multi-mode Synergy Mw microplate reader (BioTek Instruments) at 25°C. The fluorescence intensity of FTIC-OVA solutions prepared in PBS buffer was measured as function of the FTIC-OVA concentration, in the range from 0.5 to 20 μ g/mL. The calibration curve (6 points) was obtained with a correlation coefficient above 0.99. Percent of protein released was determined by dividing the amount of

released protein by total incorporated into hydrogel. For all the release studies, the starting amount of protein was fixed at 50 µg/mL for all groups.

III. RESULTS AND DISCUSSIONS

III.1. Materials

The purified chitosan (CS HMW), batch 113, used as starting material in all experiments, had a degree of acetylation of $8.7 \pm 0.8 \%$ and a weight-average molar mass $620 \pm 7 \text{ kg.mol}^{-1}$. The chitosan for the preparation of CS-DS nanoparticles had a DA = 53 % and $M_w = 107 \text{ kg.mol}^{-1}$. The depolymerised chitosan (CS LMW) used for the gel preparation had a DA = 9 % and $M_w = 7.9 \text{ kg.mol}^{-1}$. Dextran sulphate for the particle preparation featured $M_w = 8.7 \text{ kg.mol}^{-1}$ and a degree of sulfation, determinate as described by Schatz *et al.*³⁰, $T_s = 2.4$.

III.2. Chitosan-based nanoparticles

The use of nanoparticles as subunit vaccine delivery system has been explored for many years now and were shown to be easily uptaken by antigen presenting cells (APCs) in comparison with the soluble antigen alone.³³ The literature offers various methods to prepare CS nanoparticles like emulsification or ionic gelation but each of these methods use solvent and cross-linker, which may be a supplementary source of toxicity or secondary effects not favourable for an industrial exploitation.³⁴ Polyelectrolyte complexes from the electrostatically driven assembly of oppositely charged polymer provides particles via a simple to implement method. In the case of chitosan, the use of dextran sulphate to form particles was successfully investigated in our laboratory.^{30,35} Polymers were solubilised in water, with no additive, and colloidal dispersions formed spontaneously by the addition of the dextran solution into the chitosan solution, under specific experimental conditions (depending on chitosan DA and M_w , pH, ionic strength, polymer concentration). This elaboration method was satisfying for pharmaceutical application and robust as mentioned in a previous patent.³⁶ Colloidal stability is an important factor in the development of vaccine preparation using NPs as antigen carrier. Indeed, in order to reach the desire activity, adjuvant should be stable (without modification of its parameters) until the preparation of the vaccine.

The nanoparticle stability during storage was evaluated by measuring the particle mean size evolution with time in Versol[®] water at 6 % (w/v), at two storing temperatures: 4 and 37°C. Over the time frame of the experiment, no flocculation was observed, the particle size remained around 750 nm for 20 days, in accordance with previous results (Figure 3). Weber *et al.* reported, a similar stability (superior to 10 days) of particles ($d_h > 600$ nm ; PDI >0.3) in water by using the same preparation method of nanoparticles.³⁷ Stability of NPs at 6 % (w/v) in water at 37°C was monitored over 125 days and the particles size remained at 540 ± 10 nm.

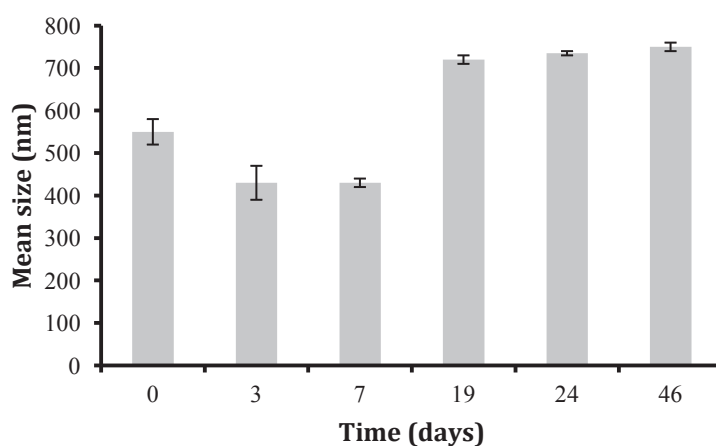


Figure 3: Storage stability of CS-based NPs at 6% (w/v) in water at 5°C during 46 days after the preparation.

We noted that the sample preparation by dilution of concentrated particles could disturb the system and induce its flocculation (observed in few cases) affecting the size measurement.

III.3. OVA sorption and stability of NPs/OVA particles

Ovalbumin was used as model protein for the elaboration of vaccine delivery system because it is a low-cost material, well-characterized, abundant and well purified. The sorption was carried out in Versol[®] water onto chitosan nanoparticles at 3% (w/v) and monitored over 24 hr. For the protein concentration of 25 $\mu\text{g/mL}$, the sorption yield reached 100 % but it remained around 80 % for concentrations of 100, 300 and 500 $\mu\text{g/mL}$ (Figure 4). Polexe *et al.* reported similar adsorption kinetics with IgAs in PBS media.³⁸ After 24 hr of incubation, they showed that the sorption yields were dependent on the antibody (Ab) concentration: the lowest protein inputs allowing the highest immobilization yields, in a similar trend as observed in the present work. It is

interesting to note that no flocculation was observed in the experimental conditions tested, showing a good stability of the system.

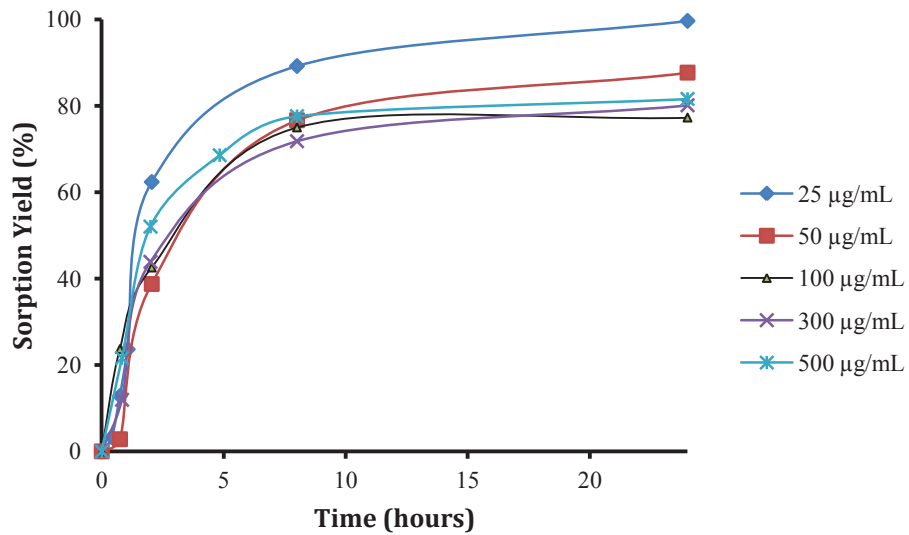


Figure 4 : Adsorption kinetics of OVA onto CS-NPs at 3 % (w/v) in water media during 24h at room temperature under moderate stirring.

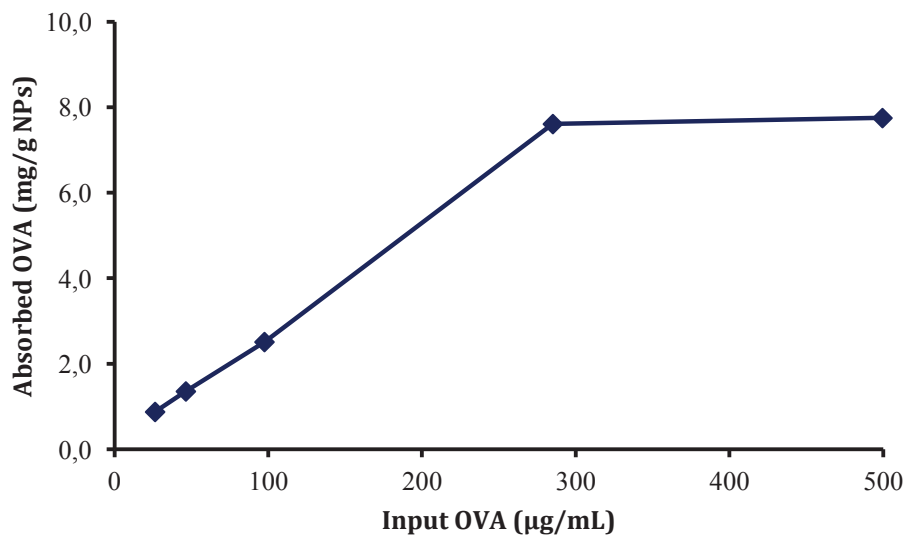


Figure 5: Protein loading onto chitosan-based nanoparticles at 3% (w/v) in water media at various concentrations at the pseudo-plateaus value of the kinetics curves.

The plot of the sorption values at the pseudo-plateau as a function of time, Figure 5, suggested that the OVA sorption at the chitosan nanoparticle interface depended on the initial protein concentration up to a saturation of the available binding sites at around $300 \mu\text{g}\cdot\text{mL}^{-1}$, corresponding to $7.7 \text{ mg}\cdot\text{g}^{-1}$ (protein/particle). Nagaoto *et al.* reported a high OVA adsorption onto chitosan-based NPs at 4 % (w/v) formed by a ionic gelation induced with sodium sulphate. OVA was added into the NPs suspension and reached a protein loading about $446 \text{ mg}\cdot\text{g}^{-1}$.³⁹ In our case, the sorption value remained satisfying because vaccine applications need for a low dose of antigen, most part of time around $50 \mu\text{g}\cdot\text{mL}^{-1}$.

The stability of the OVA particle assembly was investigated by assaying the protein release on storing in water, at various time points over 30 days and we checked the general aspect. Samples were shaken to determine if sedimentation or flocculation phenomenon occurred.

No flocculation, nor important sedimentation were observed demonstrating a good colloidal stability for 30 days. As seen in Figure 6, the stability of the systems was obtained for various protein concentrations. We also conducted experiments at 37°C with similar results in terms of stability and sorption yield.

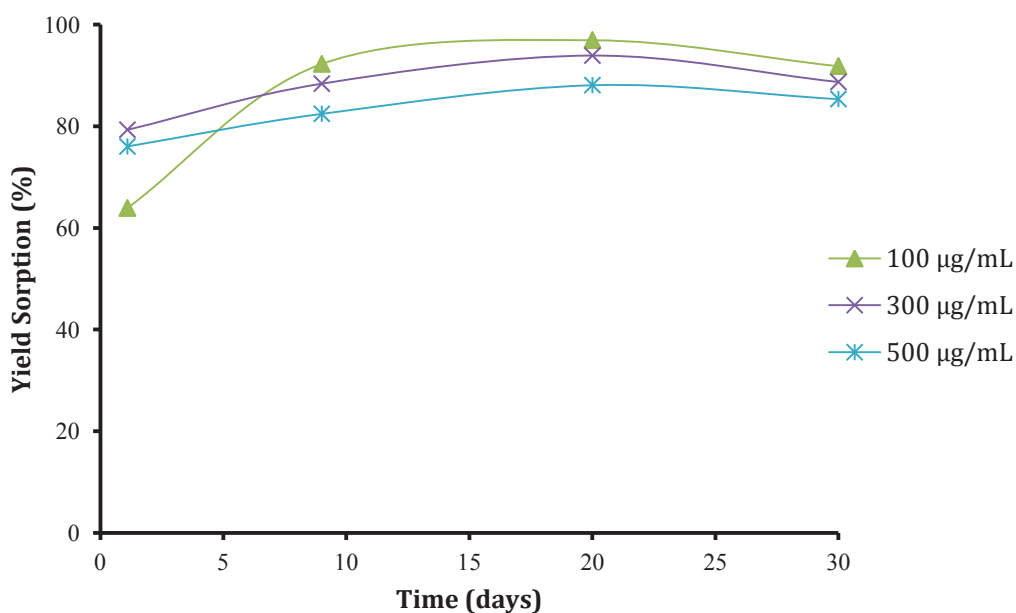


Figure 6 : Sorption stability of OVA onto CS-NPs at 3% (w/v) in water media during 30 days at 5°C .

The sorption yield was superior to 80 % after ten days of incubation for OVA concentration at 100, 300 and 500 µg/mL. We observed a good stability of these systems during 30 days at 5°C in water media.

These results were satisfying for *in vitro* assessments of the next vaccine delivery systems elaborated, i.e. that NPs/OVA could be elaborated in advance, their initial sorption yields remaining constant. Moreover, in case of *in vivo* evaluation of NPs/OVA alone, suspensions could be stored at 5°C for thirty days, at least.

III.4. Rheological studies

III.4.1. Chitosan solutions

The viscosity of a chitosan solution is dependent on many parameters such as its intrinsic properties (DA or M_w) or experimental conditions (concentration, pH, ionic strength, and temperature). High molar mass chitosan solutions with various DAs at varying concentrations and pH were investigated to define the viscosity range allowing the total suction the polymer. Rheological measurements were conducted as described in section II.6.

The polymer concentration was the major experimental parameters driving the viscosity of the system at low shear rates as shown in figure 7. Similar results were reported by Montembault *et al.* with a chitosan at DA = 36.7 %.⁴⁰ The increase in pH from 4.5 (solubilisation in standard conditions) to 6.0 did not impact the system excepted when the chitosan concentration was at 3 % (w/v), with a viscosity about ten times superior for the high pH. For other concentrations, the moderate modification of the viscosity did not affect the practicability of the solution.

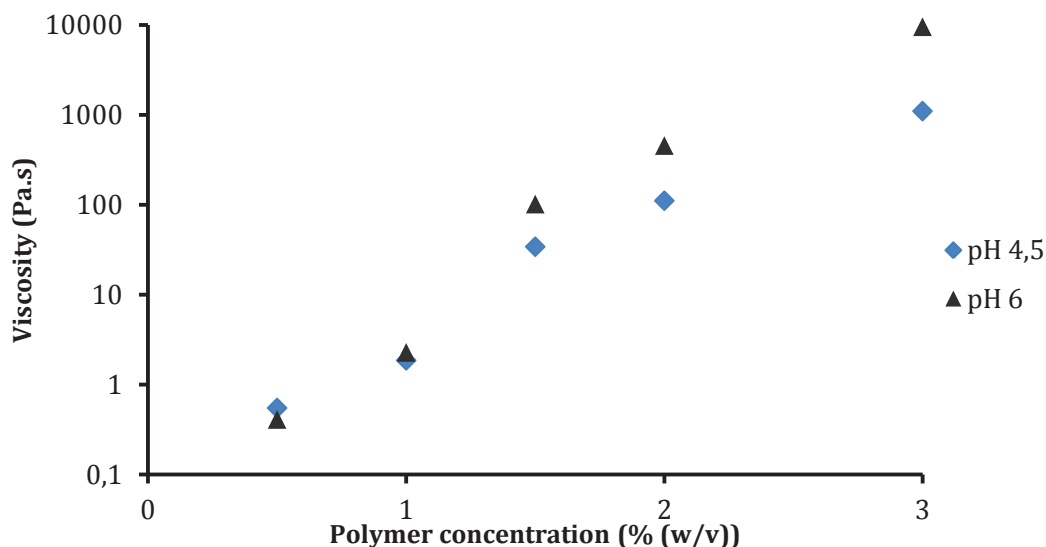


Figure 7 : Zero shear rate viscosity of chitosan (DA = 9 % and $M_w > 500 \text{ kg.mol}^{-1}$) solutions (pH = 4.5 and 6) at various concentrations, at room temperature.

As reported in Figure 8, the DA did not appear as a predominant factor impacting the zero shear rate viscosity for solution between 0.5 and 3 %. Montembault *et al.* reported similar results with chitosans at DA from 5 to 52 %, ⁴¹ i.e. they showed that no major modification of the η_0 was attributed to the modification of the DA at the same polymer concentration.

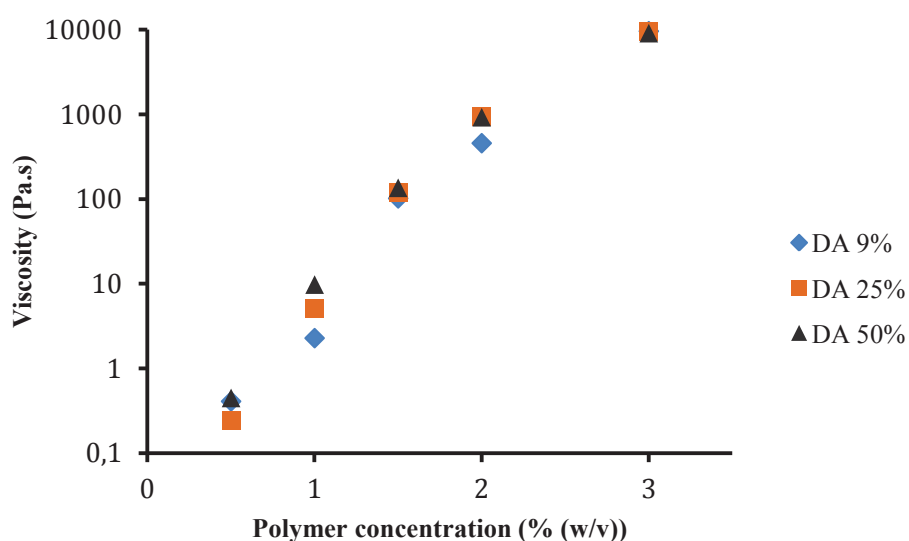


Figure 8 : Influence of DA on the zero share rate viscosity of chitosan solutions at various concentrations placed at room temperature.

The first step to elaborate a fully injectable vaccine delivery system was to provide a syringeable chitosan-based solution. The syringeability (the suction of the solution into a syringe through a needle) is a key factor for the applicability and the facility of use of a formulation. Indeed, most vaccines can be found as liquid forms in vials or as lyophilized powder that should be solubilised. In order to evaluate the syringeability, we analysed both the viscosity and the syringeability (by manual evaluation) of chitosan solutions. To elaborate the matrix precursor of the physical hydrogel we selected, on the basis of preceding work, a high molar mass chitosan at low DA = 9%, to favor chain entanglement and gelation at physiological pH by neutralization of $-NH_3^+$ groups.⁴⁰

We selected a viscosity range for which the syringeability was satisfying, i.e. that the suction time was not superior to five seconds. Moreover, after suction and injection no bubbles were produced, thus avoiding potential defects in the gel homogeneity. An 18G needle with a 2-mL syringe, classically employed for the intramuscular vaccination of pigs, horses and cows, were used to conduct these experiments.

As seen in Figure 9, the syringeability zone was below a zero shear rate viscosity of 60 Pa.s, corresponding to solutions with a chitosan concentration below 1.5% (w/v). At this value and above, it was impossible to take the sample from the vial, thus excluding all the formulation with a viscosity superior to this value in further experiments. In order to elaborate a homogenous and cohesive physical hydrogel, as shown in Figure 11, it was necessary to have a system with a concentration higher than 1.25% (w/v), otherwise only a poorly defined and inhomogeneously structured physical hydrogel was formed (Figure 12). In the latter case, the obtained material after 24 hr of incubation was a mixture composed of both solution and gel.

The chitosan concentration range to obtain a physical hydrogel from an injectable chitosan-based solution was really narrow, within 1.25 - 1.50 % (w/v) with high molar mass and DA < 20 %. To overcome this restricted range of concentration, and keeping the objective to form a physical hydrogel from low viscosity solutions, we used a mixture of chitosans of high and low molar masses.

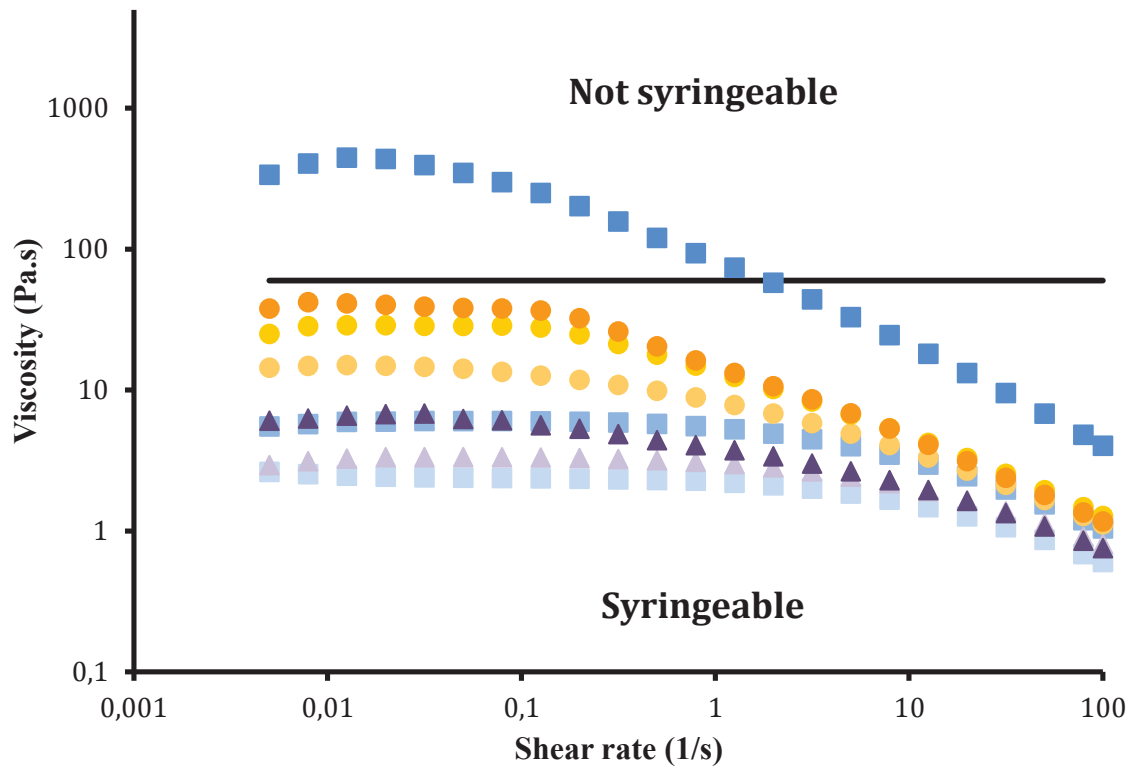


Figure 9: Viscosity of chitosan-based solutions at pH = 6.

- (□) $C_{(HMW)} = 1.0 \%$ (w/v)
- (■) $C_{(HMW)} = 1.25 \%$ (w/v)
- (■) $C_{(HMW)} = 2.0 \%$ (w/v)
- (▲) $C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v)
- (▲) $C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 2.0 \%$ (w/v)
- (●) $C_{(HMW)} = 1.25 \%$ (w/v) ; $C_{(LMW)} = 1.0 \%$ (w/v)
- (●) $C_{(HMW)} = 1.25 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v)
- (●) $C_{(HMW)} = 1.25 \%$ (w/v) ; $C_{(LMW)} = 2.0 \%$ (w/v)

We observed that mixtures, of high and low molar mass chitosans, allowed the elaboration of systems with a final concentration up to 3.25 % (w/v) with a possible syringeability (Figure 9), but for suction time superior to five seconds above 3 % (w/v). To elaborate an *in situ* gelling hydrogel, chitosan solutions within the concentration range from 1.5 to 2.5 % (w/v) were selected. The next step of this work was to check that these mixtures of both low and high molar mass chitosans were able to form homogenous chitosan hydrogels in physiological conditions.

Since the gelling matrix will contain the vaccine, we investigated the addition of OVA and/or NPs to the chitosan solutions, so as to evaluate their impact on the zero shear rate viscosity and the gelation process. To ensure the gel formation, we selected a low molar mass chitosan concentration of 1.5 % (w/v) and a high molar mass chitosan

concentration at 1 % (w/v). The protein concentration was set at 100 $\mu\text{g}/\text{mL}$ (superior vaccine dose reported in the literature with OVA).⁴² The NPs concentration was 1 % (w/v) associated with OVA at 100 $\mu\text{g}/\text{mL}$ using the method described in section 2.6 , omitting the centrifugation step.

The mixture of both low and high molar mass chitosans presented a low viscosity, Figure 10. The addition of NPs associated or not with OVA slightly altered the gelling matrix viscosity. On the contrary, the addition of OVA alone resulted in an increase in the gelling matrix viscosity that could be attributed to increase interactions between the protein negative charges and the positive charges of the chitosan matrix. Hence, OVA alone could be considered as a cross-linking agent of chitosan via its negative charges. Though this system was above the syringeability threshold, we kept it on the basis of the high potential of the gelling matrix system as drug/vaccine delivery system.

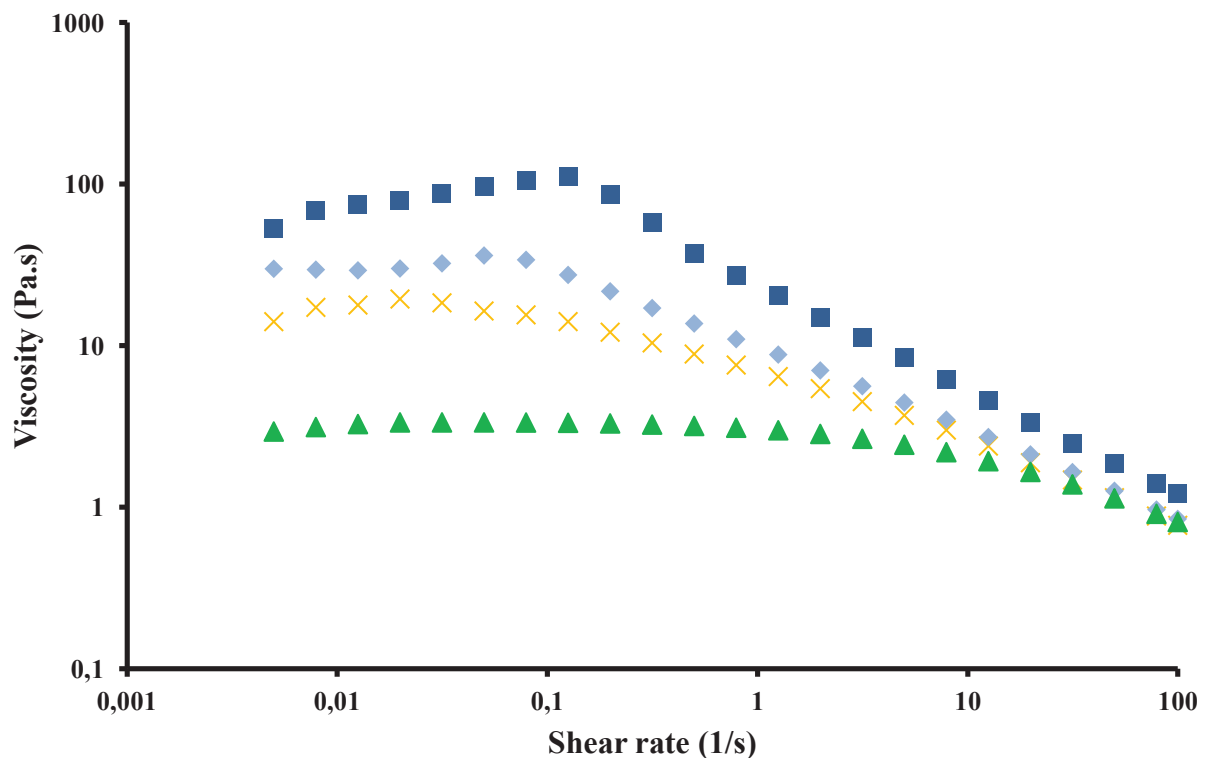


Figure 10 : Viscosity of syringeable chitosan-based solutions with a pH adjusted at 6.0.

(▲) Chitosan matrix at $C_{(\text{HMW})} = 1 \%$ (w/v) ; $C_{(\text{LMW})} = 1.5 \%$ (w/v),

(✕) NPs $C_{(\text{NPs})} = 1 \%$ (w/v) in chitosan matrix,

(■) OVA $C_{(\text{OVA})} = 100 \mu\text{g}/\text{mL}$ in chitosan matrix,

(◆) OVA loaded NPs $C_{(\text{OVA})} = 100 \mu\text{g}/\text{mL}$ and $C_{(\text{NPs})} = 1 \%$ (w/v) in chitosan matrix.

III.4.2. Chitosan-based hydrogel

Most injectable chitosan hydrogels reported in the literature, were based on a temperature-induced sol-gel transition requiring the use of an additive in the formulation like glycerol phosphate as reported by Gordon *et al.*,⁴³ Wu *et al.*²⁴ (using a chitosan derivative) and Kojarunchitt *et al.*⁴⁴ This strategy led to efficient *in situ* gelling chitosan hydrogels as reviewed by Zhou *et al.* with gelling time from 10 sec to 140 min.²² Chitosan was often reported as a safe material but, as discussed previously, the biocompatibility could decrease in the presence of cross-linkers. Therefore, the elimination of any kind of additive appeared to us as a prerequisite for the elaboration of a safe vaccine delivery system.

The physical hydrogels were elaborated as described in section 2.4.3. Chitosan solutions were introduced in the gelation device and incubated at 37°C in PBS buffer for 24hr. Then, the hydrogels were stored in a Petri dish with a little amount of water, before rheological measurements. For each hydrogel, G' and G'' moduli were measured over a frequency-range of 100 to 0.05 rad.s⁻¹.

The formed hydrogels were homogenous, as seen in Figure 11 and cohesive enough to be easily handled. The polymer concentration range was 1.25% (w/v) and above. Below this value the gels were not cohesive enough to be investigated, Figure 12.

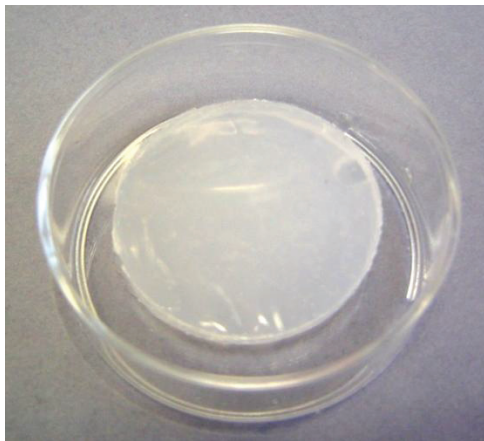


Figure 11 : Chitosan-based hydrogel from low and high molar mass chitosan ($C_{LMW} = 1\%$ and $C_{HMW} = 1\%$) after 24 hr at 37°C in PBS buffer.

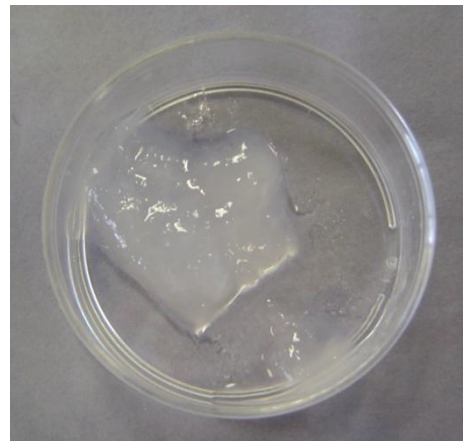


Figure 12 : Chitosan-based hydrogel from high molar mass chitosan ($C_{HMW} = 1\%$) after 24 hr at 37°C in PBS buffer.

From the rheological measurements of hydrogels, the storage moduli G' were ten times higher than the loss moduli G'' (Figure 13), confirming, according to Almdal *et al.*,⁴⁵ that the sol-gel process indeed occurred in PBS at 37°C. G' and G'' moduli logically increased with the chitosan concentration in solutions indicating an improved cohesiveness of the gels. In case of chitosan at concentration at 1% (w/v) the measurement was not possible because no physical gel was formed.

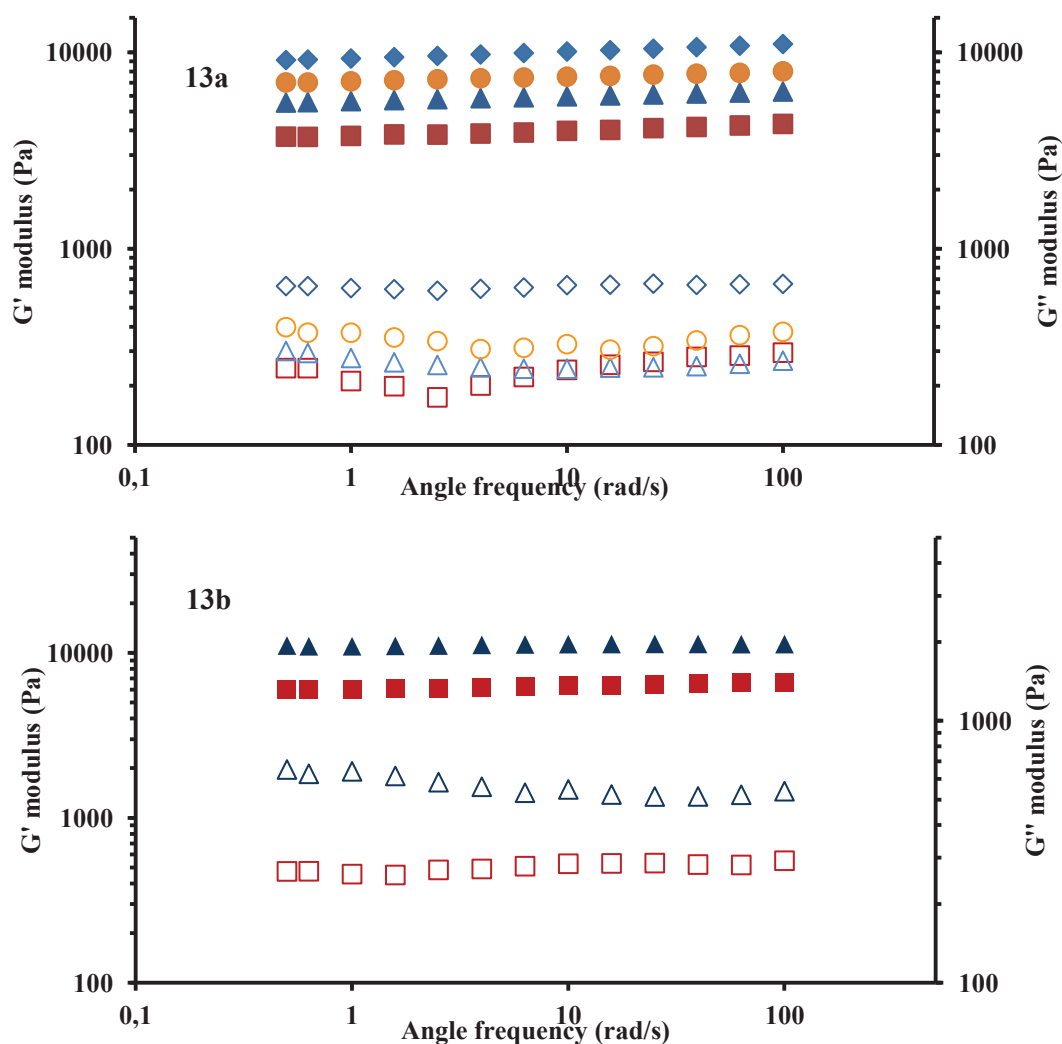


Figure 13a: Influence of the polymer concentration on the variation of G' and G'' moduli versus frequency for chitosan hydrogels obtained in PBS buffer. Measurements were conducted at room temperature.

- G' [$C_{(HMW)} = 1.25\%$ (w/v)]
- G'' [$C_{(HMW)} = 1.25\%$ (w/v)]
- ▲ G' [$C_{(HMW)} = 1.50\%$ (w/v)]
- △ G'' [$C_{(HMW)} = 1.50\%$ (w/v)]
- G' [$C_{(HMW)} = 2.00\%$ (w/v)]
- G'' [$C_{(HMW)} = 2.00\%$ (w/v)]
- ◆ G' [$C_{(HMW)} = 2.50\%$ (w/v)]
- ◇ G'' [$C_{(HMW)} = 2.50\%$ (w/v)]

Figure 13b: Variation of G' and G'' moduli versus frequency for chitosan physical hydrogels from chitosan-mixtures. Rheological measurements were conducted at room temperature.

- G' [$C_{(HMW)} = 1.0\%$ (w/v) ; $C_{(LMW)} = 1.5\%$ (w/v)]
- G'' [$C_{(HMW)} = 1.0\%$ (w/v) ; $C_{(LMW)} = 1.5\%$ (w/v)]
- ▲ G' [$C_{(HMW)} = 1.0\%$ (w/v) ; $C_{(LMW)} = 2.0\%$ (w/v)]
- △ G'' [$C_{(HMW)} = 1.0\%$ (w/v) ; $C_{(LMW)} = 2.0\%$ (w/v)]

Rheological measurements evidenced that the hydrogels from a mixture of high and low Mw chitosans displayed a lower G' (6300 ± 200 Pa) than for the mono-component hydrogels $G' = 10\,000 \pm 600$ Pa, for identical chitosan concentrations of 2.5 % (Figure 13). To obtain similar values for the conservation modulus, the concentration of the bi-component system should be increased to 3 % to account for the low DP chitosan, keeping constant the high molar mass chitosan.

The bi-component strategy was used to obtain hydrogels with various combinations of chitosans such as $C_{\text{HMW}} = 1$ % (w/v) and $C_{\text{LMW}} = 1 / 1.5 / 2 / 3$ % (w/v); $C_{\text{HMW}} = 1.25$ % (w/v) and $C_{\text{LMW}} = 1 / 1 / 1.5 / 2$ % (w/v); $C_{\text{HMW}} = 1.5$ % (w/v) and $C_{\text{LMW}} = 1.5 / 2$ % (w/v). For vaccine applications, mechanical properties have a limited importance as the vaccine delivery system has not a structural function, as it would be for tissue engineering, but is a depot site for antigens. Therefore, the lower visco-elastic properties observed for the hydrogels elaborated from a mix of chitosans with two different molar masses are a minor issue. The advantage of the bi-component strategy was to provide a syringeable and injectable chitosan-based *in situ* gelling hydrogel with adequate viscoelastic behaviours to act as a sustained release device.

The incorporation of active compounds, OVA alone or OVA loaded onto nanoparticles was investigated to observe their impact on the rheological behaviours of the final hydrogels. First, the addition of protein alone or protein loaded NPs did not prevent the gelation of the chitosan-based solutions containing two chitosans and the final macroscopic aspect of hydrogels was similar as the one shown in Figure 11.

The G' and G'' moduli of the bi-component system composed of two chitosans ($C_{\text{HMW}} = 1.0$ % (w/v) and $C_{\text{LMW}} = 1.5$ % (w/v)) were measured in the presence or not of NP or OVA, figure 14. The addition of NPs alone into the chitosan matrix did not modify G' though the addition of particles noticeably induced an increase in the solution viscosity, Figure 10. The addition of OVA alone or loaded onto the surface of NPs increased G' , indicating that the protein acted as a cross-linker agent. G' moduli up to $13,800 \pm 700$ Pa with OVA alone and up to $9,500 \pm 500$ Pa for OVA loaded NPs were observed. These results correlated with previous observations, i.e. that the loading of OVA onto NPs surface reduced the availability of interaction between OVA and the chitosan matrix inducing the lower G' value.

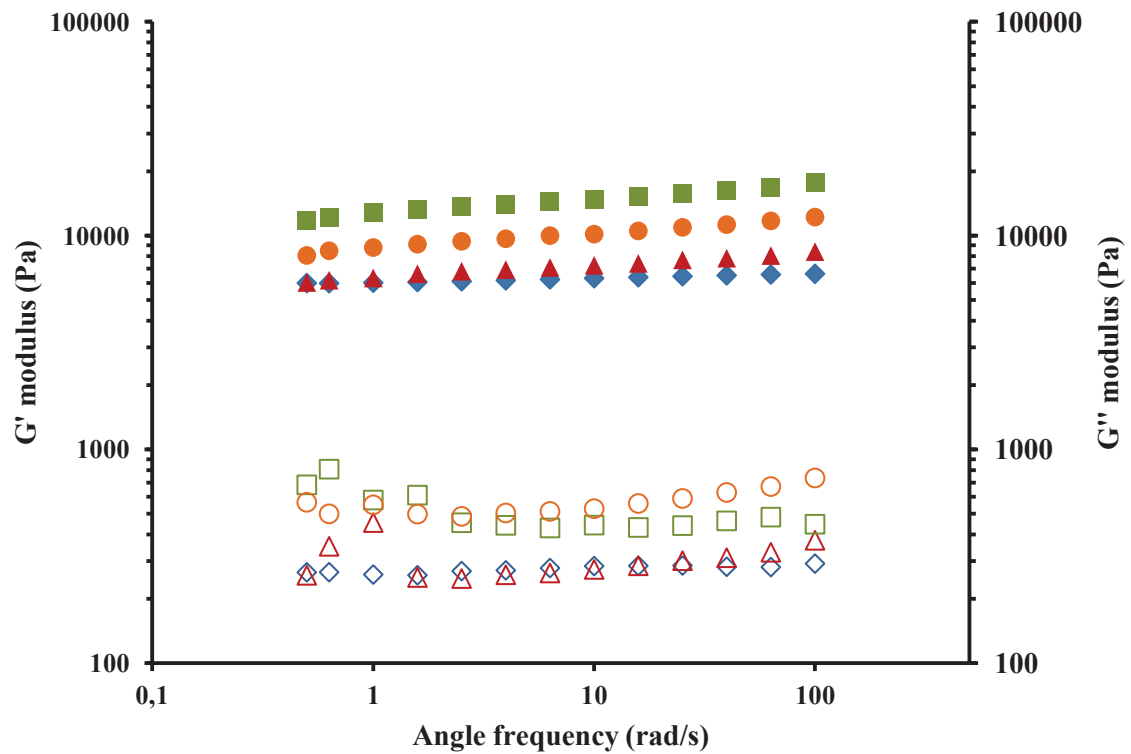


Figure 14 : Influence of the addition of active compounds on the variation of G' and G'' moduli versus frequency for chitosan hydrogels obtained in PBS buffer. Measurements were conducted at room temperature.

- ◆ G' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v)]
- ◇ G'' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v)]
- ▲ G' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(NPs)} = 1.0 \%$ (w/v)]
- △ G'' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(NPs)} = 1.0 \%$ (w/v)]
- G' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(OVA)} = 100 \mu\text{g/mL}$]
- G'' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(OVA)} = 100 \mu\text{g/mL}$]
- G' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(NPs)} = 1.0 \%$ (w/v) ; $C_{(OVA)} = 100 \mu\text{g/mL}$]
- G'' [$C_{(HMW)} = 1.0 \%$ (w/v) ; $C_{(LMW)} = 1.5 \%$ (w/v) ; $C_{(NPs)} = 1.0 \%$ (w/v) ; $C_{(OVA)} = 100 \mu\text{g/mL}$]

III.5. Protein release from chitosan-based hydrogel

To induce a long-lasting and efficient immune protection in the host, a high antibody response and the setting up of the memory immunity; it is required to boost the immune system by repeated injections of vaccine. To overcome this limitation because it could be the source of various disadvantages such as the risk of mishandling,⁴⁶ stress in host,⁴⁷ or the requirement of supplementary vaccine doses, a prime-boost vaccine delivered in a unique injection appeared to be a potent alternative.

In our work, both the antigen alone and the NP loaded antigen were added into the chitosan matrix to design this type of release system. We used OVA-FITC, as fluorescent labelled model antigen, incorporated into chitosan solution or adsorbed onto CS-NPs. Systems were gelled as described in section II.2.3. Hydrogels were immersed into PBS and placed at 37°C, OVA-FITC concentration was determined by fluorescent measurements at various times.

Chitosan hydrogels containing OVA-FITC featured a yellow colour, attributed to the fluorescence agent, Figure 15, but the gelation occurred and the general aspect was similar as reported previously in section III.3.2.

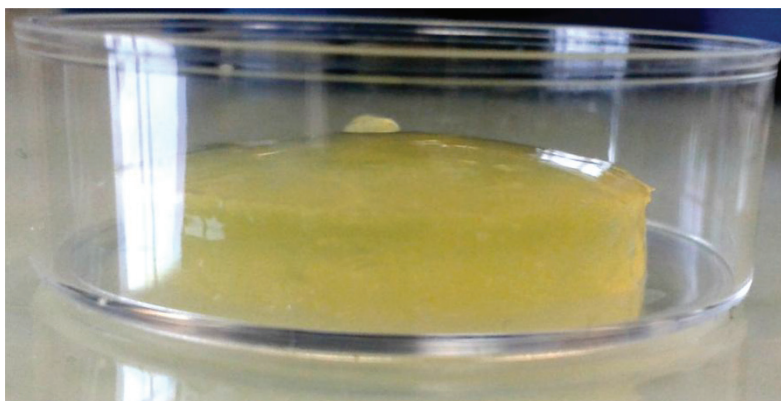


Figure 15 : OVA-FITC loaded chitosan hydrogel after incubation at 37°C in PBS during 24h

The investigated systems are reported in table 1:

System reference	Chitosan HMW concentration (% (w/v))	Chitosan LMW concentration (% (w/v))	NPs concentration (% (w/v))	OVA-FITC ($\mu\text{g/mL}$)
A	2	-	-	50
B	1	1	-	50
C	1	1.5	-	50
D	1	1	1	50
E	2	-	1	50

Table 1 : Investigated systems for the release study of protein from chitosan hydrogels

Sample E did not form any gel neither after 24hr nor 48hr of immersion in PBS, despite numerous assays. Hence, the presence of NPs prevented the gelation of the mono-component chitosane solution contrary to bi-component systems. Investigations should be led to understand the influence of NPs onto the gelation process. Moreover, for samples A and D at time 168 hours (7days), it was not possible to report results because the volume of PBS was too low for sampling, despite the closing of Petri dish.

The protein release was impacted by the final formulation, Figure 16. Sample A provided the best protein retention, with a cumulative release of 1% after 48 hr. Using a mixture of chitosan in the gelling matrix favoured the release which was not correlated to the low molar mass chitosan content in the formulation (B and C). Finally, the loading of OVA onto NPS surface further increased the release of the protein from the chitosan matrix. After 48 hr, 2.8 μg was released for NPs/OVA compared to 2.0 for OVA alone. It could be attributed, as suggested previously, to a lower charge density of OVA, onto NPs, decreasing interactions with the chitosan matrix and thus decreased the retention of protein in system.

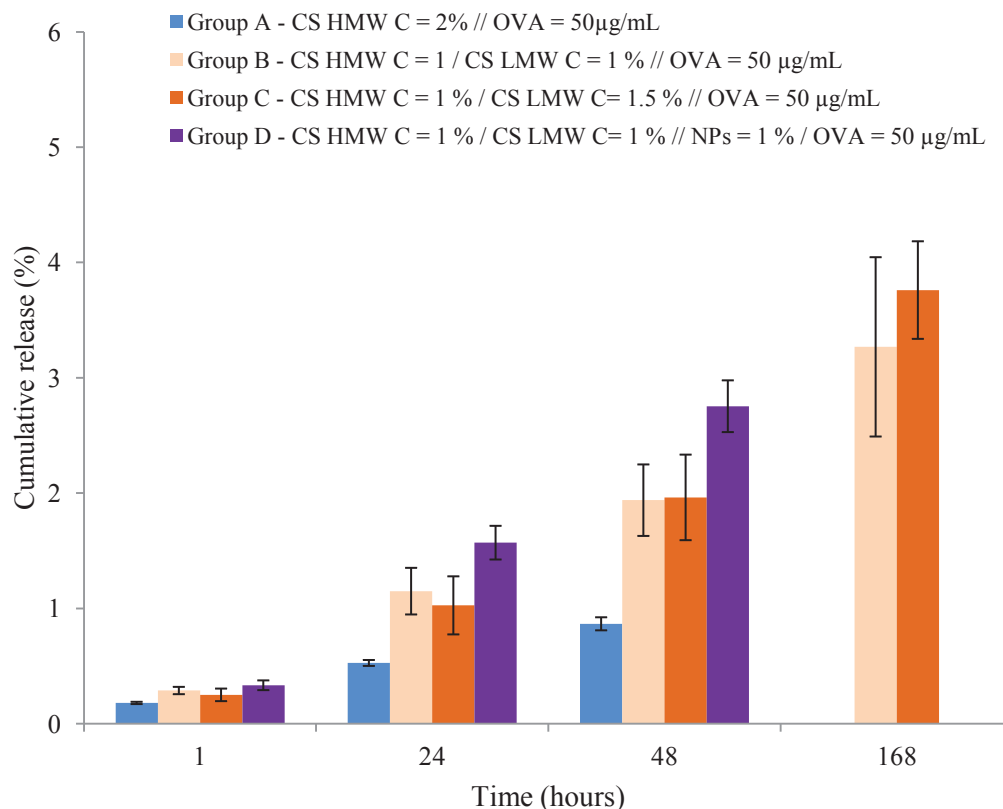


Figure 16 : Cumulative release OVA-FITC from various chitosan-based hydrogel formulations. Data showed was the mean \pm s.d. of three experiments.

Despite these differences, the release of protein was low, in each case below 5 % after 7 days of incubation. No major degradations of the hydrogels were observed during the *in vitro* experiments, which can account for the slow release of OVA. Gordon *et al.*, in similar *in vitro* experiments, reported that less than 10 % (between 5 and 10 %) of the initial amount of OVA was released from a chitosan/glycerol-phosphate hydrogel until ten days.⁴³ Their results showed that the OVA release occurred within the first 4 days, with an initial burst release the first 48 hours. Then, no release was observed until the end of the study. They assumed that the electrostatic interactions between proteins and chitosan, confirmed with Raman experiments, together with the low degradation of the material *in vitro* (no mechanical and biological degradations) could account for the low level of release. It is likely that these assumptions also apply in our case. But one has to take into account the limitations of the *in vitro* model i) absence of mechanical solicitations arising from the movement of the host that could induce cracks within the gel, improving the release; ii) the absence of enzymatic and mechanical erosions of the material that would happen *in vivo* and increase the release. For instance, Köping-

Höggard *et al.*,⁴⁸ reported that chitosanase, an enzyme causing the degradation of chitosan, permitted the release of all pDNA associated with chitosan. Also one has to take into account that these *in vitro* experiments were carried out after the gelation had occurred which is not representative of the *in vivo* situation in which the formulation will be administrated as a viscous matrix that will gel with time.

A particular feature of our system is the slow gelation kinetics, compared with the chitosan/glycerol-phosphate hydrogel that gelled in less than ten minutes (depending on experimental parameters).²² Here, two options are possible. i) First, one can consider this slow gelation as an advantage for what we want: a prime-boost vaccination with one single administration. The slow gelation would allow the partial release of the antigen from the viscous solution for the prime, so-called burst effect as observed by Bhattarai *et al.*,¹⁹ and the boost would take place after completion of the gelation step; ii) Second is to consider this slow gelation as a disadvantage that is even more critical taking into account that *in vivo*, gelation might be disturbed by movements of the host. Hence that will require further investigation on the gelation kinetics and to envision ways to reduce the gelation time, for instance by increasing the pH of the gelling matrix.

IV. CONCLUSION

In this work we reported the first *in situ* gelling system for vaccine delivery containing only chitosan and no cross-linker. Composite matrixes were envisioned, composed of a chitosan hydrogel with dispersed chitosan nanoparticles, for enhancing the delivery of the active protein. The nanoparticles obtained by polyelectrolyte complexation of chitosan and dextran sulphate remained stable for 125 days in water for a solid content of 6%. The immobilization yields of the model protein was in the 66 % to 95 %, depending on the initial protein input. The OVA sorption stability was of 30 days in water at 5 and 37°C

A major achievement of this work was to evidence that mixtures of chitosan, notably different in molar mass, could lead to solutions with moderate viscosities, hence easily administered by injection, that could gel in physiological conditions. Using one single high molar mass chitosan was limiting in term of concentration range to obtain a syringeable preparation (i.e. the zero share rate viscosity of the chitosan-based solutions below than 100 Pa.s, ideally less than 60 Pa.s. Thus, the use of a mixture of two chitosans differing in molar masses allowed reaching a final chitosan concentration, in initial solution, from 1.5 % (w/v) to 3.25 % able to form an hydrogel in simulated *in vivo* conditions (PBS buffer pH 7.4, 37°C). The incorporation of OVA (loaded or not onto chitosan-based nanoparticles) into chitosan solutions was successful. The release properties of chitosan-based hydrogel were investigated. OVA-FITC was prepared and incorporated into chitosan matrix or loaded onto CS-NPs surface. Less than 5 % of protein was liberated after 48 hr for all systems tested.

Further investigation on this system are still needed in particular to take advantage of the slow gelling kinetics that could provide the prime vaccination, the boost being ensured by the slow release from the formed gel.

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CONCLUSION GÉNÉRALE

Le premier chapitre, de ce manuscrit présente un état de l'art de résultats *in vivo* obtenus chez différents modèles expérimentaux ayant reçu des vaccins utilisant le chitosane comme adjuvant. Pour ces applications, le chitosane a été formulé sous trois grandes familles à savoir les solutions visqueuses, les hydrogels et les particules (de taille nano ou micrométrique). Le chitosane a été étudié à de nombreuses reprises avec différentes natures d'antigènes, de modèles vivants ou voies de d'administrations. Les résultats ont montré que le polymère permet, dans la plupart des cas, de solliciter les deux bras de l'immunité c'est-à-dire de produire à la fois réponse humorale et une réponse cellulaire. Une spécificité du chitosane est sa capacité à induire une réponse immunitaire locale par administration mucosale, contrairement aux adjuvants classiques à base d'aluminium par exemple, ce qui explique les nombreux exemples d'application trouvés dans la littérature. Concernant les autres études, la diversification des paramètres étudiés (antigènes, formes physiques, etc.) ne permet pas de mettre en évidence de tendances claires sur la réponse immunitaire par d'autres voies d'administration ou en utilisant des antigènes sous-unitaires différents de l'ovalbumine, très largement étudiée. Il apparait que pour l'exploitation du polysaccharide pour des candidats vaccins vétérinaires utilisant des voies ou des antigènes peu étudiés, le champ d'investigation est ouvert : aucune donnée comparable n'existe.

L'objectif premier de ces travaux de thèse était de recueillir des données sur l'innocuité du chitosane chez différentes espèces animales et par différentes voies d'injections. Pour tous les modèles animaux étudiés, dinde, chien et porc aucun effet néfaste n'a été observé, quelle qu'aient été les voies d'administration envisagées : oculaire (mucosale), sous-cutanée et intramusculaire. Des essais complémentaires menés sur le cobaye, puis cheval, mais non présentés dans ce manuscrit, ont également démontré une sécurité maximale du chitosane avec des effets locaux, après administration, équivalent à une injection de solution saline témoin.

Le deuxième objectif de ce travail était d'évaluer les capacités adjuvantes de formulations à base de chitosane dans divers cas concrets correspondants à des produits actuellement commercialisés par la société Merial. Ces travaux ont constitué le deuxième chapitre de ce document. Les résultats chez le modèle aviaire sont encourageants puisqu'un chitosane a été identifié permettant la protection de dindes à

hauteur de 90 % contre la bactérie *Pasteurella multocida*. La formulation était une solution visqueuse de chitosane administrée par voie mucosale. Des tests *in vitro* ont montré que la survie des bactéries constitutives du vaccin était dépendante du degré d'acétylation du chitosane. Chez les modèles chien et porc, respectivement, pour des vaccins à base de virus et d'une protéine purifiée, les résultats immunologiques sont en deçà des recommandations pour l'obtention d'une bonne protection des animaux. En effet, chez le chien la vaccination contre la rage en présence de chitosane ne permet pas d'obtenir une réponse suffisamment longue dans le temps. Cependant les résultats sont encourageants, puisque la réponse immunitaire est existante. Sur le modèle chien, toujours, les résultats immunologiques dans le cadre d'un vaccin contre l'adénovirus de type 2 (CAV-2) montrent une efficacité similaire à l'adjuvant base d'aluminium utilisé comme référence. La nature de l'antigène semble être à l'origine de cette différence. En effet l'obtention d'une réponse immunitaire pour un vaccin inactivé (Rage) nécessite un adjuvant plus performant que pour un vaccin atténué (CAV-2). Dans le cas des vaccins chez le porc, l'évaluation des formes solutions visqueuses, nanoparticules (NPs) et solutions visqueuses associées aux NPs a été menée. Les résultats ont montré que la solution visqueuse permettait l'obtention d'une réponse immunitaire supérieure aux autres adjuvants à base de chitosane testés. Cependant, la réponse est inférieure, en termes d'efficacité, au vaccin de référence (adjuvant sous forme d'émulsion) mais présente une innocuité supérieure. Différentes pistes d'amélioration citées dans le deuxième chapitre sont proposées pour l'obtention de réponses immunitaires supérieures chez les chiens et le porc telles que l'utilisation de solutions gélifiantes *in vivo*, le couplage du chitosane avec des immunoadjuvants ou encore le changement de la voie d'administration.

Le dernier chapitre ouvre sur l'élaboration de matériaux susceptibles d'améliorer la réponse immunitaire pour des vaccins à base d'antigènes sous-unitaires. En effet, un des mécanismes souvent admis pour l'induction d'une réponse immunitaire est l'effet dépôt de l'adjuvant qui permet de localiser l'antigène au point d'administration et de permettre un relargage dans le temps. Dans le cas des solutions visqueuses de chitosane, cet effet n'est peut-être pas assez important pour induire une réponse immunitaire d'intensité compatible avec une protection efficace de l'hôte contre le pathogène. Pour augmenter cet effet dépôt, une possibilité serait d'utiliser des solutions gélifiantes *in situ*. Cependant, les systèmes gélifiants existants ne sont pas entièrement satisfaisants puisqu'il est nécessaire d'associer au chitosane d'autres composés dont la

biocompatibilité est encore peu connue. Malgré cela, ces systèmes sont très attractifs conceptuellement car ils sont fluides et donc facilement prélevables à la seringue à température ambiante (20 à 25°C) et leur transition sol-gel s'effectue en augmentant la température à 35°C ou plus, températures corporelles des mammifères (39°C environ pour le porc et 38,5°C pour le chien). L'administration directe d'hydrogels physiques de chitosane sous sa forme finale n'est pas envisageable puisqu'ils sont impossibles à prélever à la seringue. Des études rhéologiques ont montré que des formulations à base de deux chitosanes de masses molaires différentes permettent d'obtenir un système gélifiant en conditions physiologiques (37°C dans un tampon phosphate salin, PBS). Par ailleurs, l'étude du relargage des protéines a permis d'observer que moins de 5 % des protéines étaient libérées sous 48h suggérant une diffusion lente à travers le matériau. Ces formulations présentent l'avantage d'être assez fluides pour être prélevées à la seringue munie d'une aiguille et sans l'utilisation d'agent de réticulation pour la formation d'un gel. Ces systèmes restent cependant perfectibles, puisque les vitesses de gélification sont relativement lentes dans le cadre d'applications vaccinales. En effet, les gels ont été obtenus à l'issue de 24 h d'incubation contre des systèmes pouvant gélifier en moins de dix minutes à l'aide d'additif. Afin d'améliorer les matériaux proposés la modification du degré d'acétylation et de la masse molaire du chitosane permettrait de tirer avantage des différentes propriétés physico-chimiques induites. L'augmentation du DA permet d'obtenir une gamme de solubilité plus étendue tandis que la modification de la masse molaire permet de faire varier la viscosité de la solution initiale pour une concentration similaire en polysaccharide. Une augmentation de la vitesse de gélification avec le chitosane seul, dans les connaissances actuelles, ne semble pas être envisageable. Cependant, le chitosane peut être couplé avec d'autres polymères naturels (tels que l'alginate ou l'acide hyaluronique), connus pour leur bonne biocompatibilité, pour former des hydrogels. Cette alternative nécessiterait le développement de nouveaux systèmes d'injections adaptés aux méthodes de travail des éleveurs ou vétérinaires dans le domaine vétérinaire.

Dans le cadre de l'élaboration de nouveaux systèmes de délivrance de vaccins à destination d'espèces animales, les résultats *in vivo* obtenus sont encourageants et offrent des données initiales importantes pour la société Merial permettant de futures exploitations du chitosane.

