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In situ gelling nasal inserts for influenza vaccine delivery

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Abstract

Purpose: The objective of this study was to investigate the potential of rapidly gelling nasal inserts as vaccine delivery system. *Methods*: Nasal inserts were prepared by freeze-drying hydrophilic polymer solutions containing influenza split vaccine. In vitro vaccine release from polymer solutions and inserts and the vaccine hemagglutination activity were determined. In vivo immunization studies in mice and rats were performed with nasal solutions and nasal inserts. *Results*: The in vitro release of proteins (vaccine) from polymeric solutions and inserts was incomplete because of the high molecular weight of the proteins. The release rate was controlled by the polymer (Lutrol® F68 > PVP 90 > HPMC K15M > Carbopol® > chitosan ≥ carrageenan = xanthan gum) because of differences in solution viscosity and possible polymer–protein interactions. Xanthan gum, a negatively charged polymer with intrinsic adjuvanticity, enhanced the serum IgG as well as the nasal IgA response in in vivo studies with nasal vaccine solutions. Poly-L-arginine and cationic lipid were the best performing adjuvants. Solutions containing vaccine with xanthan gum and cationic lipid were effectively stabilized with 0.4 M NaCl. *Discussion*: The specific activity of the major vaccine protein, hemagglutinin, was not significantly affected by the addition of polymers and the freeze-drying process during insert preparation. The addition of cationic lipid as adjuvant decreased the hemagglutination activity, which strongly indicated inhibition of the protein binding site to erythrocytes. Inserts prepared from xanthan gum and cationic lipid stabilized with NaCl showed a reduced protein activity but were superior to the cationic lipid alone. *Conclusion*: Rat immunization with solid nasal inserts based on xanthan gum containing the influenza vaccine, with or without an additional cationic lipid adjuvant, resulted in similar IgG levels as the pure nasal liquid vaccine formulation.

Key words: *Bioadhesive drug delivery systems; enhancers; extended release; influenza vaccine; nasal inserts; nasal vaccination*

Introduction

Mucosal routes of immunization are attractive alternatives to parenteral immunization because they may not require trained personnel for administration and lead to a potentially higher patient compliance. It is widely accepted that the majority of the invading pathogens enter the body through mucosal surfaces[1]. Thus, mucosal sites have a potential as first-line defense against entering pathogens (mucosal immunity). The nasal mucosa is under investigation because of its high vascularization, accessibility, circumvention of gastrointestinal degradation, and hepatic first-pass metabolism[2]. The nose offers the additional advantage that the vaccine can be delivered to the appropriate site, the nasal associated lymphoid tissue (NALT)[3] with minimal dilution. Nasal secretions are known to contain immunoglobulins (IgA, IgG, IgM, IgE), protective proteins such as complement as well as neutrophils and lymphocytes in the mucosa[4]. The nasal mucosa and its associated lymphoid structure do not only allow serum and local (nasal) immune response but they are also inductive sites of the mucosa-associated lymphoid tissue (MALT) and thus convey good secretory immune response at distant mucosal sites such as the intestine, lung, and vagina[5–7].

Continuous antigen delivery was long believed to result in immune tolerance induction, but controlled delivery of

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antigens has been shown to induce mature immune response[8]. Continuous antigen delivery alone may not be sufficient to induce an immune response but requires appropriate adjuvants[9].

Influenza viruses are capable of causing recurrent annual epidemics and more serious pandemics, which spread rapidly. The severity of the spreading and the resulting pharmaco-economic impact are influenced by the high antigenetic variability of the virus, the emergence of new strains, and the degree of preexisting protective immunity in the exposed population[10]. Currently, annual intramuscular vaccinations with formaldehyde-inactivated virus particles, split or subunit vaccines, are applied to control the morbidity and mortality associated with influenza outbreaks.

Numerous nasal drug delivery systems for vaccines are under investigation including emulsions[11,12], liposomal preparations[13,14], virosomes[15], proteosomes[16], gels and polymeric solutions[17,18], and polymeric microparticles[19–21]. In 2000, a commercial virosome-based nasal influenza vaccine spray using a nontoxic natural variant of the *Escherichia coli* heat-labile toxin as adjuvant was introduced (Nasalflu® Berna, Berna Biotech AG, Basel, Switzerland). However, this vaccine was withdrawn by the company after the first season of commercialization because of potential toxicity problems. Lately, another nasal influenza vaccine spray for humans, FluMist™ (MedImmune Inc., Gaithersburg, MD, USA), based on live attenuated influenza virus strains, has been approved by the Food and Drug Administration (FDA) and launched in the United States.

Despite intensive research and approval of the first nasal vaccine preparations for human use, current nasal vaccine delivery systems still suffer many disadvantages, such as low immunogenicity, danger of toxicity (especially with toxin-based adjuvants), complex production processes, and vaccine protein instabilities, which hinder broad application to a wide variety of vaccines.

In situ gelling nasal inserts have previously been described as a promising extended drug delivery system[22,23]. This solid dosage form is prepared by freeze-drying of drug-containing polymer solutions and consists of a sponge-like hydrophilic polymer matrix, in which the drug is embedded. The insert allows for easy dosing with a high potential for systemic administration. Once in contact with the highly vascularized nasal mucosa, the polymer sponge rapidly takes up water and forms a gel from which the drug is released in a controlled fashion. The use of bioadhesive polymers ensures a prolonged nasal residence time for an extended release application.

The aim of this study was to incorporate influenza split vaccine under preservation of the hemagglutinin activity into nasal inserts based on hydrophilic polymers and to investigate solutions and inserts of various polymers and enhancers/adjuvants with respect to their effect on the serum and local mucosal immune response.

Materials and methods

Materials

Vaccine

Monovalent split inactivated influenza vaccine NIB16 (H1N1) (reassortant of the two A/H1N1 strains, A/Tawaïn/1/86 and X31, produced by propagation on chicken eggs, purified, split, and inactivated by formaldehyde at Sanofi Pasteur batch #SVA2219) in phosphate-buffered saline (PBS) buffer at a concentration of 250 µg hemagglutinin/mL. The vaccine also contained an equivalent amount of nucleoprotein and traces of matrix proteins.

Polymers

ι-Carrageenan (Genuvisco® carrageenan type TPH-1, Copenhagen Pectin A/S, Lille Skensved, Denmark); polyacrylic acid (Carbopol® 971P NF, BFGoodrich, Cleveland, OH, USA); hydroxypropyl methylcellulose (HPMC, Methocel® K15M, Colorcon Ltd., Dartford, UK); polyvinyl pyrrolidone (PVP, Kollidon® 90F) and polyoxypropylene–polyoxyethylene block copolymer poloxamer 188 (Lutrol® F68, BASF AG, Ludwigshafen, Germany); xanthan gum (Keltrol® F, Kelco International, Tadworth, UK).

Enhancers

Sodium lauryl sulfate (SDS, Texapon® K12, Henkel KGaA, Düsseldorf, Germany); poly-L-arginine hydrochloride (molecular weight 70,000–150,000), protamin sulfate, and sodium glycocholate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); cationic lipid and chitosan (Protosan™ UP G213, 86% deacetylation, Pronova Biomedical, Oslo, Norway); Eudragit® RL 30D (ammonio methacrylate copolymer, type A, USP; Röhm GmbH & Co. KG, Darmstadt, Germany). All other excipients were of pharmaceutical grade. Purified water was used as a solvent if not otherwise stated.

Preparation of various vaccine-containing nasal drug delivery systems

Vaccine solutions

The vaccine solution was diluted with purified water to a hemagglutinin concentration of 45 µg/200 µL = 225 µg/mL. The enhancers (0.5%, w/v) and the polymer (0.5% or 2%, w/v) were added and stirred until uniformly distributed.

Inserts

Aliquots (V = 1500 or 200 µL for in vitro and V = 20 µL for in vivo characterization) of the above prepared vaccine solutions with/without polymer and enhancers were placed into blister moulds (in vitro) or into silicon tubing (in vivo, Rotilabo Silicon Tubing TR60 2 mm/4 mm, Carl Roth GmbH + Co., Karlsruhe, Germany) and frozen at −25°C for 1 hour. The samples were then freeze-dried (0.25 mbar for 24 hours with increasing shelf temperature −15°C to 0°C and a final drying for 2 hours at +15°C and 0.01 mbar) (Gamma 2–20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The inserts were stored in a desiccator until use.

Viscosity measurements

All investigated solutions were left unagitated overnight and were then equilibrated to 22°C for 1 hour in a water bath (Haake G with thermostat Haake D8, Haake Meßtechnik GmbH und Co., Karlsruhe, Germany) before measurement. The viscosity of the polymer solutions was determined with a rotational viscosimeter (Rheostress RS 100, Haake Meßtechnik GmbH und Co.) in the controlled rate mode at a shear rate of 5/s over a time interval of 150 seconds at 22°C utilizing a plate-cone-geometry 20 mm/4 mm (n = 3).

In vitro drug release

A self-made diffusion cell was used for drug release studies mimicking the humidity properties of the nasal mucosa (Figure 1). It consisted of a release medium container (polypropylene reaction tube 1.5 mL with conic bottom; Plastibrand®, Brand GmbH & Co. KG, Wertheim, Germany) with a small magnetic stirrer (length = 10 mm) inside. The lid of the vial was cut open from the top and a regenerated cellulose filter membrane (pore size 0.45 µm, d = 13 mm; RC-fleeze supported, Sartorius AG, Göttingen, Germany) was fixed between the lid and the rim of the vial. A small hole was made in the vial just below the membrane to allow the escape of air during refilling of the vial. A needle (20G; Sterican® 0.90 × 40 mm, B. Braun Melsungen AG, Melsungen, Germany) was inserted through a second hole in the lower third of the vial and was fixed there with superglue (UHU® Sekundenkleber, UHU GmbH, Bühl, Germany). The vial was filled with approximately 1.5 mL preheated (37°C) phosphate buffer pH 6.0 (USP XXVI). The filter membrane was wetted but not submersed, and the exact amount of medium was determined by weighing. The solutions (V = 200 µL) or inserts (V = 200 µL) corresponding to a hemagglutinin dose of 45 µg were exactly weighed and placed on top of the filter membrane through the opening in the lid and the release system was closed with Parafilm® 'M' sealing film (American National Can Co., Chicago, Illinois, USA) to avoid evaporation of release medium. The whole assembly was then continuously stirred with 750 rpm (Variomag multipoint HP 15, H+P Labortechnik AG, Oberschleißheim, Germany) at 37°C. Samples of 0.5 mL were taken at predetermined time points and replaced by fresh medium. The protein content of the samples was analyzed using a commercial colorimetric protein assay (Micro BCA Protein Assay, Pierce, Rockford, IL, USA; sample volume 0.5 mL, incubation time 120 minutes) with UV detection (λ = 562 nm; UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). The actual drug loading of the inserts was determined by complete dissolution of inserts in phosphate buffer pH 6.0 (USP XXVI) followed by the Micro BCA Protein Assay. A standard curve was run for each assay, which was prepared by dilution of the original vaccine solution (250 µg hemagglutinin/mL) and was based on the hemagglutinin and not the total protein content. All measurements were performed in triplicate (mean ± SD). Placebo inserts were also investigated to quantify the Ultraviolet (UV)-interference of the polymers. The placebo-value was subtracted from the value of the drug-loaded inserts at each time point.

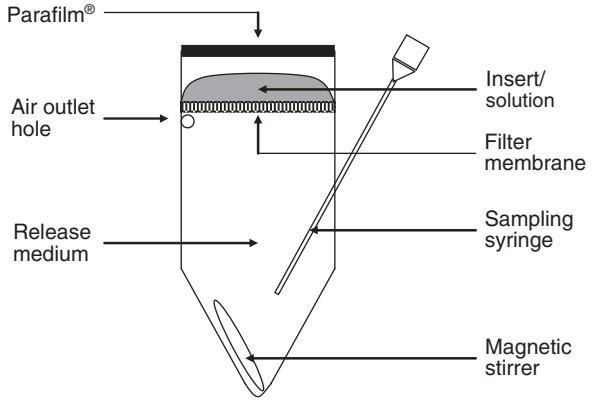


Figure 1. Design of the diffusion cell for drug release studies of nasal solutions and inserts containing influenza vaccine.

The adsorption of protein to the release system was determined by incubating a solution of the vaccine in release buffer (concentration 45 µg hemagglutinin/vial) in the complete assembly under drug release test conditions and measuring the protein concentration after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 hours by the Micro BCA Protein Assay (n = 3). The protein recovery decreased with time and reached a constant value after 2 hours of 69.2 ± 4.1%.

A mass balance study was performed at the end of each experiment. The residual amount of protein above the filter membrane was determined by dissolving the remaining gel in 5 mL of release medium and analysis using the Micro BCA Protein Assay. The sum of the

adsorbed, released, and remaining protein was calculated and expressed as percent of the original protein loading of the test sample (45 μg hemagglutinin).

Moisture sorption of the inserts

Inserts (V = 1.5 mL) were exactly weighed and placed in a closed vessel above a saturated ammonium chloride solution (79% relative humidity) at room temperature. The moisture sorption was determined as weight increase of the insert over time (6, 12, 24, 48, and 72 hours) normalized to the initial dry polymer weight of the inserts, which was kept constant for all inserts (n = 3).

Hemagglutination activity assay

Inserts were reconstituted in the original volume (200 μL) of water. The solutions or reconstituted inserts were then diluted 1:10 with PBS and the protein content was determined before the hemagglutination test using a commercial colorimetric protein assay (Micro BCA Protein Assay, Pierce; sample volume 0.5 mL, incubation time 120 minutes) with UV detection (λ = 562 nm; UV-2101 PC, Shimadzu Deutschland GmbH). The protein content was adjusted to 0.5 μg/25 μL by further dilution.

About 25 μL of these solutions were given into the first well of a 96-well plate (V-bottom, Greiner Bio-One BioScience Division, Frickenhausen, Germany), and a series of 1 + 1 dilutions of each sample was prepared in PBS. About 25 μL of an erythrocyte suspension [1% (v/v) in PBS, from specific pathogen-free chicken supplied by the Institute for Poultry Diseases, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany] and another 25 μL of PBS was then added to each well. After 40 minutes of incubation at ambient conditions the agglutination of the erythrocytes was evaluated visually. The HA titer was defined as the reciprocal value of the highest dilution (given as the $\log_2$) of the sample, which still resulted in agglutination of the erythrocytes, visually detected by the reduced flow of the blood cells (n = 3). A negative (buffer) and a positive control (vaccine) was run with the sample on each plate.

In vivo immunization studies

All in vivo vaccine studies were performed at Sanofi Pasteur (Marcy l'Etoile, France). The tested formulations were prepared at the Freie Universität Berlin and shipped in cooled containers for the studies. In addition to the influenza vaccine (hemagglutinin 4.5 μg/20 μL), the liquid formulations contained 0.5% (w/v) polymers with (0.5%, w/v)/without enhancers. These concentrations corresponded to 100 μg of polymer and enhancer in the application volume of 20 μL. Nasal inserts were prepared by freeze-drying 20 μL—aliquots of these solutions. The controls (vaccine solution, vaccine solution with cationic lipid) were provided by Sanofi Pasteur.

Animals

Outbred OF1 female mice (Iffa-Credo, St. Germain-sur-l'Arbresle, France) and female Sprague-Dawley rats (Iffa-Credo) were housed in an accredited facility and were cared for under the guidelines of Sanofi Pasteur-France local Ethics Committee.

Immunization schedule and samplings

Groups of six OF1 mice were immunized twice at a 3-week interval by the intranasal route without anesthesia with 20 μL of the liquid formulation containing a defined amount of the monovalent influenza vaccine NIB16 (A/H1N1) corresponding to 4.5 μg of hemagglutinin. Animals were killed 2 weeks after the last immunization, and antibody levels were determined in both serum and nasal secretions.

Groups of five rats were immunized twice at a 3-week interval by introduction of a nasal insert (V = 20 μL) into one nostril under anesthesia. The inserts were pushed into the nostril until they were out of sight, using thin slightly bent forceps. Each insert contained a defined amount of the monovalent influenza vaccine NIB16 (A/H1N1) corresponding to 4.5 μg of hemagglutinin. Two control groups received intranasally 50 μL per nostril (total 100 μL) of liquid influenza vaccine alone (4.5 μg hemagglutinin) or vaccine mixed with cationic lipid (4.5 μg hemagglutinin + 200 μg cationic lipid). Animals were killed 2 weeks after the last immunization and blood was sampled for antibody titration.

Antigen-specific ELISA assays

Sera from immunized animals were analyzed using ELISA for the presence of NIB16-specific IgG. In addition and only in the mouse study, NIB16-specific IgA and total IgA ELISA antibody responses were measured in nasal wash samples. Briefly, 96-well flat-bottom plastic plates (Dynex Technologies Inc., Chantilly, VA, USA) were coated overnight at 4°C with the influenza monovalent NIB16 antigen at 100 ng/100 μL (hemagglutinin protein) per well in 0.05 M carbonate buffer pH 9.6. Plates were then blocked for 1 hour with 150 μL of PBS pH 7.1 containing 0.05% Tween 20 and 1% (mouse ELISA) or 2% (rat ELISA) (w/v) powdered skim milk (DIFCO™, Becton Dickinson Diagnostic Systems, Sparks, MD, USA).

All further incubations were carried out in the final volume of 100 μL, followed each time by four washings with PBS pH 7.1 containing 0.05% Tween 20. Serial dilutions of the sera in blocking buffer were added to the

plates and incubated for 90 minutes at 37°C. The plates were then washed with PBS pH 7.1 containing 0.05% Tween 20. Peroxydase conjugates diluted in blocking buffer were added and incubated for 90 minutes at 37°C. The conjugates used were specific for either mouse IgG (diluted at 1/5000, Ref: A3673) or mouse IgA (diluted at 1/500, Ref: A4789) (both Sigma-Aldrich Chemie GmbH) or rat IgG (diluted at 1/20,000, Ref: 112-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The plates were further washed and incubated in the dark for 20 minutes at room temperature with 3,3´5,5´-tetramethyl benzidine (TMB). The reactions were stopped with 100 µL of HCl 1N.

The absorbance was measured at 490–650 nm with an automatic plate reader (VERSAmax, Molecular Devices France, St. Grégoir, France or Labystems M96V). The mean value of the blanks (the signals obtained in wells incubated with all reagents except serum or secretion) was subtracted from the data. The NIB16-specific IgG and IgA titers were calculated from the regression curve obtained with a reference sera or nasal secretion originating from the same animal species present on each ELISA plate. The quantity of total mouse IgA in mouse nasal wash secretions was quantified with the known protein concentration of the purified mouse IgA (Ref: 02-6500, ZYMED Laboratories Inc., San Francisco, CA, USA), defined by the supplier. The antibody titers were calculated, for the optical density value range of 0.2–3.

IgG serum response results are given in arbitrary units (UA*) ($\log_{10}$). IgA mucosal responses were expressed as normalized specific activity (NSA) with NSA = [anti-NIB16 IgA titers (UA*)/total IgA titers (ng/mL)] × 1000.

Results and discussion

The aim of this study was to investigate the potential of in situ gelling nasal inserts as nasal vaccine delivery system in vitro as well as in vivo. The sponge-like inserts are prepared by freeze-drying aqueous solutions of the vaccine and a hydrophilic polymer. The polymer forms the carrier matrix of the insert. Before the actual study with inserts, it was necessary to identify vaccine-compatible polymers and enhancers/adjuvants. Thus, the effect of additives (i.e., the polymers to form nasal inserts and enhancers to increase the mucosal uptake of the vaccine proteins) on the solution properties and on the immune response of nasally applied influenza vaccine solution was tested.

Compatibility of the vaccine with solutions of polymers and enhancers and in vitro vaccine release from polymer solutions

The vaccine solution in pH 7.4 buffer is a slightly turbid, colloidal solution because of the high molecular weight of the proteins. The major protein hemagglutinin (approximately 1500 amino acid residues and molecular weight of 240 kDa) is a trimer composed of identical monomers, each consisting of two chains linked by disulfide bonds[24,25]. It is negatively charged at neutral pH values. The vaccine also contains an equivalent amount of nucleoprotein (75 kDa, positively charged at neutral pH-values) and traces of matrix proteins.

The vaccine–hydrophilic polymer solutions were prepared by directly dissolving the polymer in the vaccine solution in order to avoid further dilution of the vaccine. The dissolution of hydrophilic polymers in the vaccine solution was not problematic for negatively charged (Carbopol®, carrageenan, xanthan gum) or neutral polymers (Lutrol® F68, PVP 90, HPMC K15M). An exception was the positively (oppositely) charged chitosan, which is also known to act as an absorption enhancer for proteins[26,27]. The addition of chitosan (0.5%, w/w) to influenza vaccine resulted in an increased turbidity.

Commonly used negatively charged absorption enhancers (SDS, Na-glycocholate) also did not interact with vaccine protein (Table 1). All positively charged

Table 1. Properties of various enhancers and visual appearance (turbidity) of vaccine solutions with and without enhancers (vaccine protein concentration 45 µg/200 µL) at 22°C.

Enhancer (0.5%, w/w)	Charge	Charge density, 10^{-3}/Da	MW	Comments	Visual appearance
No enhancer	—	—	—	—	+
SDS	Negative	3.47	288	Surfactant	+
Na-Glycocholate	Negative	2.05	488	Surfactant	+
Cationic lipid	Positive	—	—	Liposomes	++++
Poly-L-Arginine	Positive	6.41	95,000	Protein	+++
Chitosan	Positive	5.28	—	85% DA[a]	+++
Protamin sulfate	Positive	5.05	4800	Protein	++
Eudragit® RL	Positive	3.88	150,000	Nanoparticles	+++

Turbidity: + = slight, ++ = medium, +++ = strong, ++++ = slight precipitation.
[a]Deacetylation.

enhancers, including chitosan, increased the visually observed turbidity of the vaccine solution, indicating the formation of complexes. This shielding of the protein's negative charge and thus an increase in its hydrophobicity could be a mechanism for potential absorption enhancement, because lipophilic membranes are usually impermeable for highly charged molecules. Observations supporting this theory have already been made for the gastrointestinal uptake of the highly charged anticoagulant heparin, which was successfully delivered when bound to polycationic polymeric nanoparticles[28]. Although the exact mechanism of absorption was not identified, the importance of the polycationic nature of the polymer was stressed. Condensation of larger, highly charged drugs by electrostatic complexation for improved drug absorption was also discussed in the literature[29,30].

For large molecules, such as the influenza vaccine antigens, aggregation and precipitation of the formed complexes on the mucosal surface could enhance the immune response by particulate uptake through M-cells of the NALT[31].

The neutral polymer HPMC K15M was chosen as carrier for the liquid formulations containing different enhancers (except chitosan, which by itself forms inserts) because of its good potential to form nasal inserts, its relatively high viscosity compared with other polymers [0.5% (w/w) polymer solution: chitosan 5.6 ± 4.1 mPa.s, PVP 90 10.9 ± 3.6 mPa.s, carrageenan 31.2 ± 5.7 mPa.s, Lutrol® F68 (25%, w/w) 33.1 ± 4.3 mPa.s, HPMC K15M 55.9 ± 2.6 mPa.s, xanthan gum 498.8 ± 9.5 mPa.s, Carbopol® 730.7 ± 10.5 mPa.s] and its compatibility with the charged enhancers (negatively charged polymer was not compatible with positively charged enhancers). In addition, the higher viscosity of the HPMC K15M solutions could result in a longer nasal residence time.

Viscosity measurements showed that the addition of the enhancers Na-glycocholate, protamine sulfate, and Eudragit® RL 30D did not affect the viscosity of the HPMC K15M solution (Table 2). However, SDS, cationic lipid, and poly-L-arginine increased the HPMC K15M solution viscosity approximately by a factor of two. The viscosity-enhancing effect of SDS on HPMC K15M was previously described and related to adsorption of the polymer onto SDS-micelles[32]. The cationic lipid is known to form liposomes in aqueous solution. Positively charged liposomes were shown to increase the viscosity of a Carbopol® 974P NF gel likely because of electrostatic interactions[33]. HPMC K15M was also shown to adsorb onto the surface of liposomes[34]. Thus, a viscosity increase with a similar mechanism as for SDS could be possible. Poly-L-arginine is a polymer with a molecular weight of 95,000 Da and may thus increase the HPMC K15M solution viscosity by itself and due to increased polymer chain entanglements.

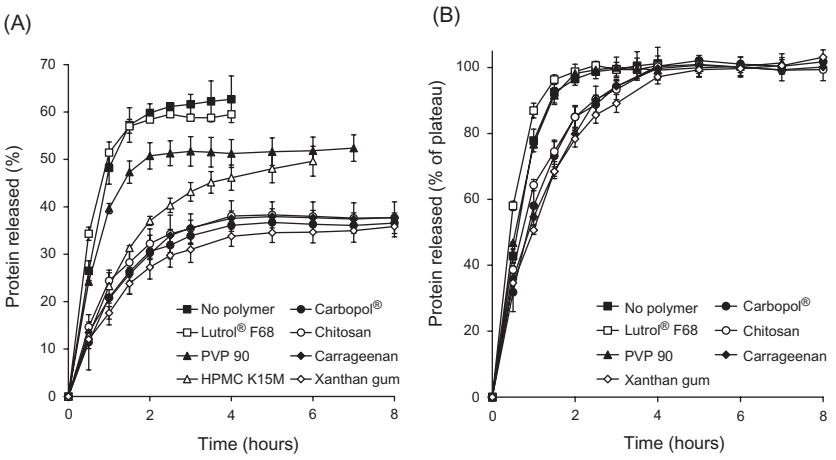
Table 2. Viscosity of HPMC K15M solutions 0.5% (w/w) containing different enhancers 0.5% (w/w) (22°C, $n = 3$).

Enhancer	Viscosity, mPa.s
None	55.9 ± 2.6
Na-glycocholate	52.8 ± 5.3
Protamine sulfate	54.2 ± 2.3
Eudragit® RL 30D	58.3 ± 4.7
SDS	102.1 ± 3.1
Cationic lipid	107.4 ± 4.0
Poly-L-Arginine	111.2 ± 7.2

After the compatibility studies, the vaccine release was investigated first from polymer solutions. Polymer solutions containing influenza vaccine are an intermediate product during the manufacture of in situ gelling nasal inserts by freeze-drying and were used for the in vitro and in vivo screening of potential polymers and enhancers/adjuvants.

The protein was released from different polymer solutions (Figure 2A). The protein detection method was universally applicable to all proteins and did not differentiate between hemagglutinin, nucleoprotein, and the matrix protein traces. Because of adsorption to the filter membrane, a maximum of only approximately 70% of the vaccine proteins was released into the medium. Most of the formulations did not reach 70% but leveled off earlier. This indicated incomplete drug release from the formulations. Mass balance studies showed that the missing protein remained above the filter membrane. The amount of protein adsorbed to the filter and released and recovered above the filter added up to 100%.

The release from Lutrol® F68 solutions was rapid and reached a plateau after 2.5 hours and was similar to polymer-free vaccine solutions (Figure 2A). PVP 90 showed a slightly retarded release with a slightly lower plateau. This retardation effect was even stronger for HPMC K15M because of its higher viscosity. These three polymers (Lutrol® F68, PVP 90, HPMC K15M) were of neutral character. No electrostatic interaction with the major vaccine proteins (hemagglutinin negatively charged, nucleoprotein positively charged) would be expected. As a result, the total protein amounts released from these solutions were similar to the pure vaccine solution. Carbopol®, carrageenan, and xanthan gum showed similar protein release profiles (Figure 2A). All three leveled off at approximately 35–38% protein released. This low value may be related to the negative charge of these polymers. The missing amount of protein corresponded to the nucleoprotein fraction of the vaccine. Incomplete release of this fraction because of electrostatic binding to the polymers may have



Polymer	Time to plateau (hours)	Protein released at plateau (%)	Mass balance (%)
No polymer	2.5	61.9 ± 4.0	97.7 ± 5.8
Lutrol® F68	2.5	60.3 ± 5.2	112.9 ± 6.7
PVP 90	2.5	51.7 ± 5.3	111.1 ± 6.7
HPMC K15M	>6.0	46.4 ± 3.2	112.1 ± 5.2
Carbopol®	3.0	36.5 ±1.0	119.2 ± 4.3
Xanthan gum	4.0	34.8 ± 2.2	115.0 ± 4.7
Carrageenan	4.0	37.7 ± 1.4	107.6 ± 4.4
Chitosan	4.0	37.9 ± 2.8	92.0 ± 5.0

Figure 2. In vitro protein release and mass balance of nasal solutions [polymer content: 0.5% (w/v) [25% Lutrol® F68], V = 200 µL, vaccine dose: 45 µg hemagglutinin, n = 3]. (A) 100% = total protein content of inserts, (B) 100% = protein released at plateau.

occurred. The slow protein release from chitosan solutions with a plateau at 38% was attributed to electrostatic interactions between the polymer and the hemagglutinin.

The protein release data from different polymer solutions was also plotted by taking the protein amount released at the plateau as the 100% value to compare the release rates from the various solutions (Figure 2B). HPMC K15M was not replotted, because it did not reach a plateau within the investigated 8 hours. Two groups of polymers were identified. Solutions of Lutrol® F68 and PVP 90 reached 100% release (= plateau) within 2 hours, similar to the polymer-free vaccine solutions. The slightly enhanced release from Lutrol® F68 solutions may be attributed to the surfactant properties of the polymer, thus solubilizing the protein. The second group of polymers (carrageenan, xanthan gum, chitosan, Carbopol®) reached the plateau (100%) within 4 hours. Also HPMC K15M belongs into this group. The prolonged release is related to the viscosities of the solutions and/or electrostatic interactions between proteins and polymers.

In vivo performance of polymer solutions with/ without enhancers

The serum immune response to influenza vaccine solution with different polymeric carriers at day 35 post primary application on day 1 and boost on day 22 was measured as specific IgG serum titers (Figure 3A). All polymers except Carbopol® led to increased IgG titers. With the exception of xanthan gum, the average titers (including responders and nonresponders) of the polymers chitosan, PVP 90, carrageenan, Lutrol® F68, and HPMC K15M were below that of the pure vaccine. However, the pure vaccine control was not prepared and shipped together with the other formulations; a direct comparison may therefore not be appropriate.

A comparison of the different polymers revealed the following order of IgG immune response: xanthan gum > chitosan > carrageenan = PVP 90 = Lutrol® F68 > HPMC K15M > Carbopol®. Solutions applied to the nasal cavity are usually very rapidly removed by the ciliary clearance[35]. The solutions are transported to the back of the throat, from where they are swallowed. Swallowing of the influenza vaccine would inactivate

the proteins and result in no immune response. Bioadhesive formulations can prolong the nasal residence time. This could be the reason for the superior IgG serum response for xanthan gum and chitosan solutions compared with the other polymers. In addition, xanthan gum has intrinsic adjuvant properties and has been described as a murine lymphocyte activator[36]. A similar increase in local and serum immune response after nasal application of an influenza virus vaccine in solution with gellan gum, an extracellular anionic polysaccharide, and chitosan was observed[17]. The comparatively low IgG response for HPMC K15M may result from the clearance of the formulation from the nasal cavity before the release of the vaccine because of less-pronounced bioadhesive properties of the polymer and slow drug release compared with PVP 90 and Lutrol® F68. No correlation was found between the in vitro drug release results and the in vivo behavior.

The mucosal specific IgA response, determined in nasal washes, showed similar results as the serum IgG titers (Figure 3B). Xanthan gum was again the most promising polymer. PVP 90 also showed a raised IgA immune response compared with the pure vaccine solution.

Next, the effect of various enhancers was investigated with HPMC K15M solutions because of their good compatibility (no precipitation) with this uncharged polymer (Figure 3). The enhancers Na-glycocholate and insoluble Eudragit® RL nanoparticles formulated with HPMC K15M revealed no enhancing effect (enhancement factor f = titer HPMC K15M with enhancer/titer HPMC K15M without enhancer = 1.0 and 2.3, respectively) (Figure 3A). The formulation containing SDS could not be administered quantitatively because of its high viscosity and was therefore not measured. Protamin sulfate, poly-L-arginine, and cationic lipid given with HPMC K15M increased the serum titers by a factor f = 39.8, 417, and 457, respectively, compared with vaccine solutions containing HPMC K15M alone. Best enhancement of the mucosal specific IgA response was also observed for poly-L-arginine and cationic lipid (Figure 3B).

The enhancing effect of bile salts described in the literature is often explained by their ability to damage the mucosal membrane, thus destroying its barrier function[37]. Additional inhibition of proteolytic enzymes (e.g., leucin aminopeptidase) has been discussed for Na-glycocholate[38]. The poor performance of Na-glycocholate in the present study may be explained as follows: the vaccine proteins were no substrate for the inhibited protease or the large size of the vaccine proteins (colloidal size range) still prevented the uptake even though the membrane had become more porous.

The cationic enhancers, on the other hand, may also aggregate and condense the colloidally dispersed, high

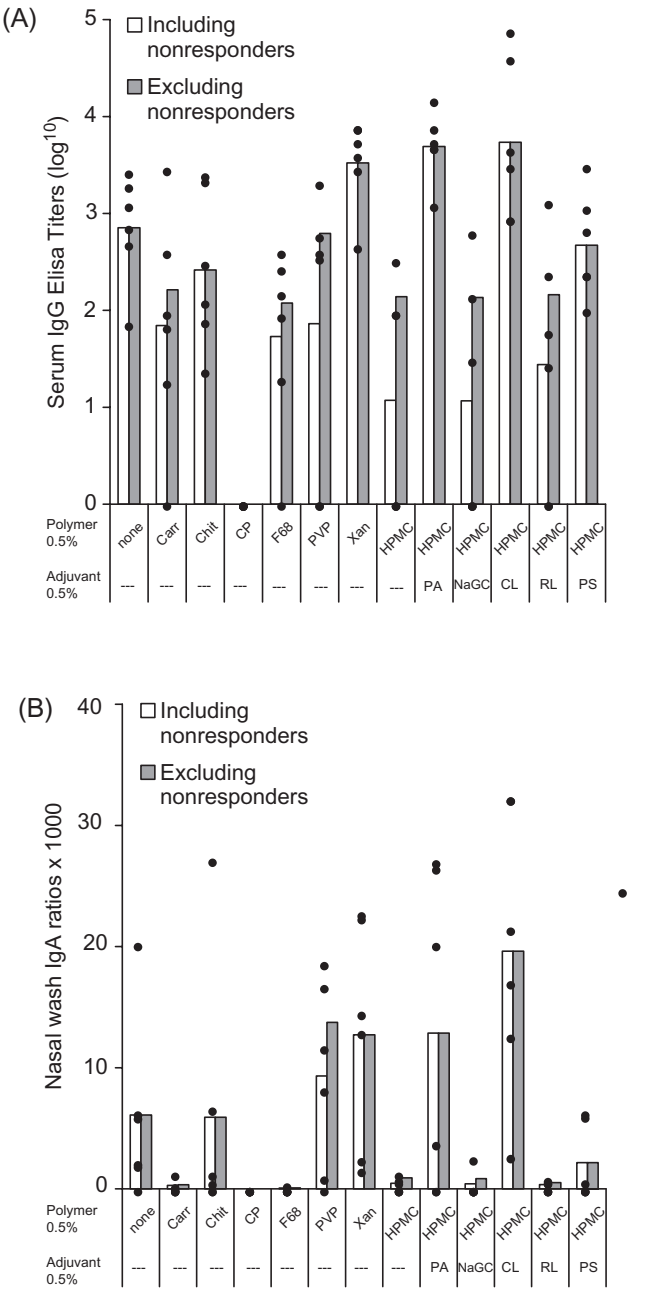
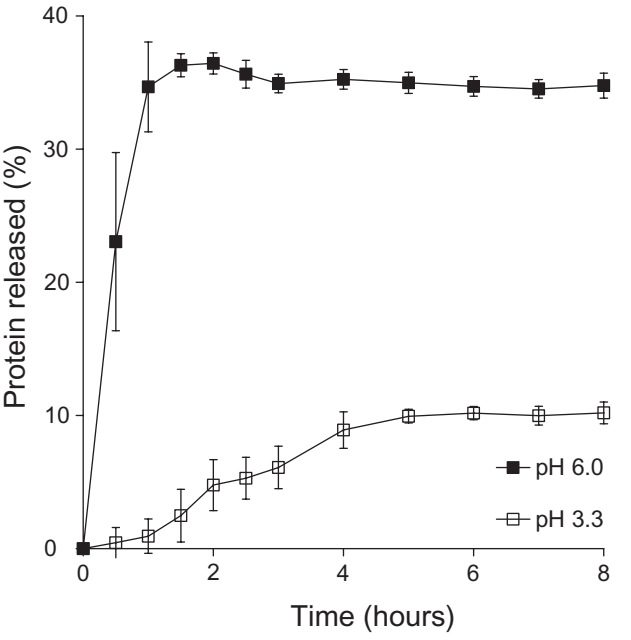


Figure 3. (A) Serum immune response (IgG ELISA titer) and (B) local immune response (normalized specific activity of IgA in nasal washes) to vaccine solutions with various polymers (0.5%, w/v) and enhancers (0.5%, w/v) in mice (V = 20 µL, dose = 4.5 µg hemagglutinin, points represent the data of individual animals). The antibody responses were measured 2 weeks after the second immunization of a regimen comprising two immunizations 3 weeks apart. (carr, carrageenan; Chit, chitosan; CP, Carbopol®; F68, Lutrol® F68; PVP, PVP 90; Xan,xanthan gum; HPMC, HPMC K15M; PA, poly-L-arginine; NaGC, sodium glycocholate; CL, cationic lipid; RL, Eudragit® RL 30D; PS, protamine sulfate).

molecular weight antigens as previously discussed, and thus allow uptake by M-cells. A minimum positive charge density seemed to be required because the studies of cationic enhancers with different charge density

revealed that the one with the lowest charge density (Table 1), Eudragit® RL nanoparticles, were the least effective enhancer. The enhancing effect of N-trimethylchitosan for nasally administered ^{14}C-mannitol in rats depended on its degree of quarternization as a measure of charge density[39]. A minimum of 22% quaternization was required for effective enhancement.

As mentioned above, vaccine solutions containing Carbopol® did not elicit an immune response (Figure 3A and B). This led to the assumption that the vaccine was not released from the solution or at least not in the active form. A 0.5% (w/w) Carbopol® solution in water had a pH of 3.3, whereas all other polymers were in the pH range of 6.4–7.5 (chitosan pH 4.8). Conformational changes of the hemagglutinin around pH 5 have previously been described and might be responsible for the inability of the Carbopol® solutions to elicit an immune response[40]. The Carbopol®-vaccine solution was more turbid than the pure vaccine solution and also less viscous than the pure Carbopol® solution, which indicated electrostatic interactions. To substantiate these assumptions, vaccine was released from identical Carbopol® solutions using as release medium phosphate buffers of pH 6.0 and 3.3, which were both adjusted to the same osmolality (0.82 osmol/kg). The lower pH resulted in a much slower vaccine release compared with pH 6.0, thus explaining the missing immune response for Carbopol®-containing vaccine solutions (Figure 4).



Sample	Release medium pH	Release medium osmol/kg	Protein released after 8 hours (% ±SD)	Mass balance (%)
Solution	6.0	0.84	34.9 ± 0.7	103.8 ± 4.3
Solution	3.3	0.82	10.2 ± 0.8	104.1 ± 4.3

Figure 4. In vitro protein release and mass balance of Carbopol® 971 solutions at different pH of the release medium [polymer content: 0.5% (w/v), V = 200 µL, vaccine dose: 45 µg hemagglutinin, n = 3].

Combination of polymers and enhancers for insert formation

Xanthan gum was chosen as polymer and cationic lipid as adjuvant for further studies because of their good performance in the in vivo studies using nasal solutions. A simultaneous dissolution of xanthan gum and cationic lipid resulted in precipitation because of their opposite charge. pH-adjustment was not suitable to avoid precipitation, because precipitation of the xanthan gum/cationic lipid was only prevented at pH < 1 and not up to pH 12, where the cationic lipid itself precipitated. Salts (low molecular weight electrolytes) have been shown to disrupt interpolyelectrolyte complexes of DNA and poly(*N*-alkyl-4-vinylpyridinium) cations because of shielding of the charges[41]. Thus, salt addition was investigated for the stabilization of mutual solutions of xanthan gum and cationic lipid. Turbidity of a solution of xanthan gum and cationic lipid could not be prevented, but sufficiently high concentrations of NaCl (0.4 mol/L) or $CaCl_2$ (0.2 mol/L) could avoid precipitation (Table 3). No precipitation occurred during 10 minutes centrifugation at 600 × g at room temperature and after 2 days storage at 4°C when dissolving both cationic lipid and xanthan gum in the vaccine solution in the presence of 0.2 mol/L $CaCl_2$.

Table 3. Stabilization of solutions of cationic lipid (0.25%, w/v) and xanthan gum (0.25%, w/v) by salt addition (pH of salt solutions: NaCl pH 6.6, $CaCl_2$ pH 6.0).

C_{Na+}, mol/L	C_{Ca2+}, mol/L	Visual appearance (precipitation)	Centrifugation (600 × g, 10 minutes) Sediment	Centrifugation (600 × g, 10 minutes) Supernatant
—	—	Strong	Strong	Clear
0.1	—	Medium	Medium	Turbid
0.2	—	Low	Low	Turbid
0.4	—	None	None	Turbid
—	0.1	Low	Low	Turbid
—	0.2	None	None	Turbid
—	0.4	None	None	Turbid

In situ gelling nasal inserts were prepared by freeze-drying the salt-stabilized vaccine solutions containing xanthan gum and cationic lipid. Because of the relatively high salt content, the susceptibility of the nasal inserts to moisture sorption at elevated humidity was investigated (Figure 5). The weight increase because of moisture uptake increased substantially with $CaCl_2$ because of the hygroscopic nature of this salt (Figure 5). The weight increase with $MgCl_2$ was lower

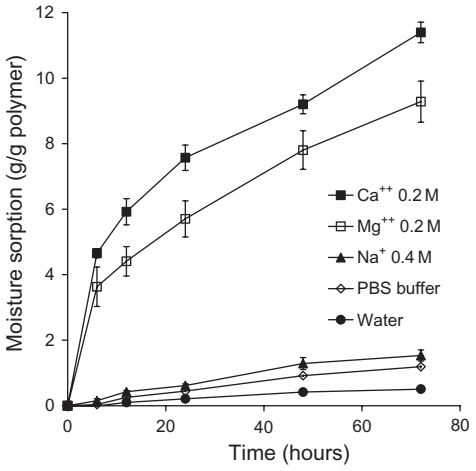
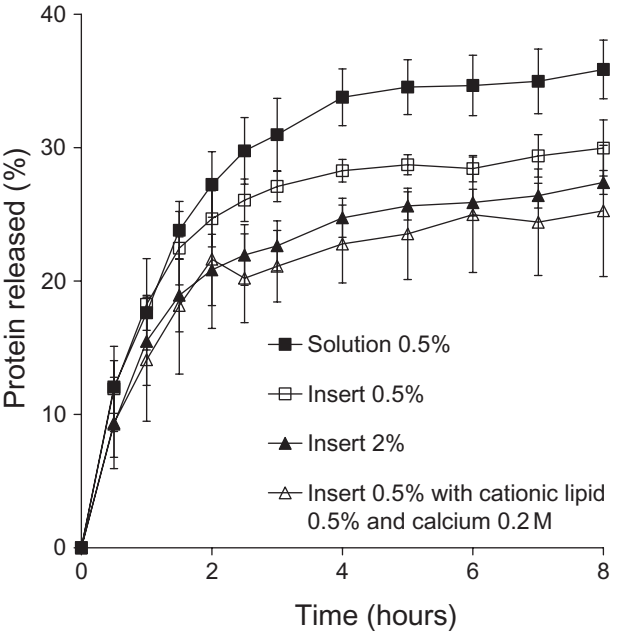


Figure 5. Moisture sorption of xanthan gum inserts with/without cationic lipid (CL 0.5%, w/v) and stabilizing chloride salts prepared in PBS buffer or demineralized water during storage in humid air [polymer content: 0.5% (w/v), $V = 1.5$ mL, 22°C, 79% r.h., $n = 3$].



Sample	Composition	Concentration (%) (w/v)	Protein released after 8 hours (% ± SD)	Mass balance (%)
Solution	Xanthan gum	0.5	35.9 ± 2.2	115.0 ± 4.7
Insert	Xanthan gum	0.5	29.9 ± 2.1	100.3 ± 4.4
Insert	Xanthan gum	2.0	27.4 ± 0.9	98.2 ± 4.4
Insert	Xanthan gum	0.5	25.3 ± 4.9	99.6 ± 4.2
	Cationic lipid	0.5		
	$CaCl_2$	0.2 M		

Figure 6. In vitro protein release and mass balance of xanthan gum solutions and inserts [polymer content: 0.5% or 2% (w/v), $V = 200$ µL, vaccine dose: 45 µg hemagglutinin, $n = 3$].

but was still high. NaCl, a nonhygroscopic salt, did not enhance the moisture sorption. The lowest moisture sorption was observed for inserts prepared in purified water. The presence of the cationic lipid had no effect on the moisture sorption.

Solid nasal inserts were formed by freeze-drying vaccine-xanthan gum (0.5% and 2%, w/w) solutions. In contrast to vaccine–polymer solutions, protein release from the inserts required water uptake and hydration of the polymer matrix before the diffusion of the proteins. The release was therefore slightly lower for inserts compared with the corresponding solution (Figure 6). However, the hydration was rapid. The time until the inserts were completely moistened (color change from dry white to wet creamy, opaque) was only 4.7 ± 0.4 minutes for inserts containing xanthan gum 0.5% + cationic lipid 0.5% + NaCl 0.4 M. The delay in drug release because of transformation from the dry to the wet is thus very short. This is one of the advantages of these rapidly gelling inserts. The final polymer concentration in the rehydrated insert was higher than that of the solutions; thus the inserts presented a higher diffusional barrier, which resulted in the lower plateaus of protein release. Increasing the polymer content of the solution before freeze-drying and adding the cationic lipid stabilized with $CaCl_2$ further reduced the protein release rate (Figure 6).

Effect of preparation, additives, and storage on the hemagglutination activity

It is important to maintain the specific activity of the protein during their incorporation into dosage forms and during storage in order to deliver a therapeutically/immunologically active protein. The agglutination of erythrocytes is a measure of the specific activity of the influenza vaccine hemagglutinin. The success of an influenza vaccination is measured in a serum hemagglutination inhibition test using the fact that antihemagglutinin antibodies prevent erythrocyte clotting in the presence of hemagglutinin.

None of the additives alone (polymers, enhancers, stabilizing salts) exhibited a hemagglutination activity. The vaccine solution had a HA titer of $\log_2$ 6.3 (Table 4). Boiling the vaccine solution for 1 minute resulted in a total loss of the activity because of heat denaturation of the hemagglutinin, whereas freeze-drying of the vaccine without additives preserved its activity. Inserts prepared by freeze-drying of vaccine–xanthan gum or vaccine–HPMC K15M solutions generally maintained the hemagglutination activity or led only to minor reductions (xanthan gum). Preparation of nasal inserts by freeze-drying is a mild preparation method and did

Table 4. Influence of boiling and freeze-drying of the vaccine solution and storage time of inserts (polymer content 0.5%, w/v) (over-dried silica gel in a desiccator at room temperature) on the hemagglutination activity of the vaccine (protein concentration 45 μg/200 μL).

Composition	Sample type	Storage time, months	HA Titer $\log_2$
Untreated	Solution	0	6.3 (6,6,7)
Boiled	Solution	0	0.0 (0,0,0)
Freeze-dried	Powder	0	6.0 (6,6,6)
Xanthan gum	Insert	0	5.7 (5,6,6)
Xanthan gum	Insert	2	6.0 (6,6,6)
HPMC K15M	Insert	0	6.3 (6,6,7)
HPMC K15M	Insert	2	6.0 (6,6,6)

Table 5. Influence of different chloride salts, cationic lipid, and xanthan gum on the hemagglutination activity of the vaccine in solutions and inserts (protein concentration 45 μg/200 μL).

Composition (%, w/v)				
Formulation	Ion, mol/L	Cationic lipid, %	Xanthan gum, %	HA Titer $\log_2$
Solution	—	—	—	6.3 (6,6,7)
Solution	Ca^{2+}, 0.2	—	—	6.7 (6,7,7)
Solution	Na^{+}, 0.4	—	—	9.0 (9,9,9)
Solution	—	0.5	—	2.7 (2,3,3)
Solution	Ca^{2+}, 0.2	0.5	—	2.0 (2,2,2)
Solution	Ca^{2+}, 0.2	0.5	0.5	0.0 (0,0,0)
Insert	Ca^{2+}, 0.2	0.5	0.5	0.0 (0,0,0)
Solution	Na^{+}, 0.4	0.5	0.5	5.0 (5,5,5)
Insert	Na^{+}, 0.4	0.5	0.5	4.7 (4,5,5)

not affect the specific activity of the incorporated vaccine. Storage of inserts over 2 months at 4°C had no effect on the hemagglutination activity.

Addition of NaCl or $CaCl_2$ to the vaccine led to an increased hemagglutination activity, especially for the NaCl (Table 5). The reason for this effect remained unclear. Addition of the cationic lipid reduced the hemagglutination titer strongly because of the previously observed electrostatic interaction. The lipid probably blocked the active center of hemagglutinin and thus prevented binding of the erythrocytes to the protein. The hemagglutination activity of the vaccine was further reduced by simultaneous addition of cationic lipid and calcium chloride and disappeared completely when xanthan gum was also added for both solution and insert. In contrast, NaCl in combination with cationic lipid and xanthan gum increased the hemagglutination activity compared with the cationic lipid alone for both solution and insert. About 0.4M NaCl was chosen as stabilizer for xanthan gum and cationic lipid solutions based on results obtained during storage and handling under ambient conditions and with the hemagglutination assay.

In vivo performance of nasal inserts

In vivo vaccination studies with nasal inserts were performed in rats, the mouse nostrils being too small to receive a solid insert. Serum IgG response was measured on day 37 post primary immunization and boost on day 22. Polymeric nasal inserts based on xanthan gum, HPMC K15M, and chitosan without additives resulted in a reduced immune response compared with the pure vaccine solution (Figure 7). However, both controls, pure vaccine and vaccine with cationic lipid, were not prepared and shipped together with the other formulations; a direct comparison may therefore be inappropriate. In case of xanthan gum, the immune response was mainly reduced because of one nonresponding rat in the group (n = 5). As already seen in mice with nasal polymer solutions (Figure 3), xanthan gum inserts elicited higher IgG levels than HPMC K15M. Chitosan is often described to enhance the immune response[17,27]. In this regard, its weaker effect on IgG immune response compared with xanthan gum and especially HPMC K15M remained unclear. Addition of cationic lipid as adjuvant significantly increased the immune response to HA in HPMC K15M inserts but not in xanthan gum inserts (when the nonresponding animal was excluded) (Figure 7). Xanthan gum may not be the most appropriate matrix for the cationic lipid, which could have interacted with the oppositely charged xanthan gum rather than with the vaccine proteins. Poly-L-arginine did not show a significant

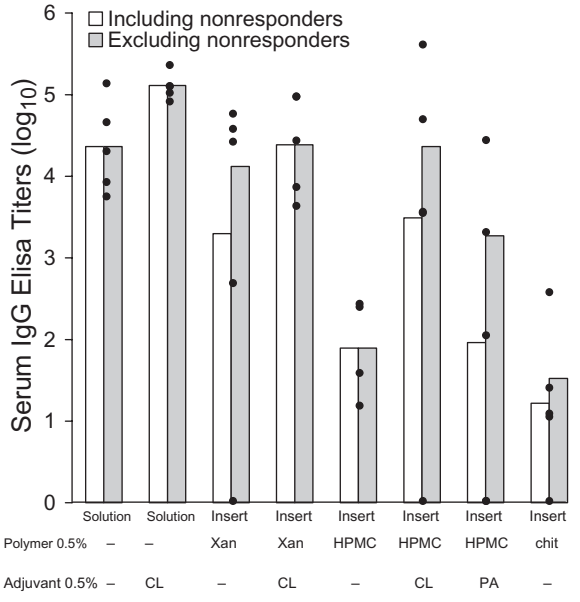


Figure 7. Serum immune response (IgG ELISA titer, arbitrary units) to vaccine solutions and inserts (polymer and adjuvant each 0.5%, w/v) in rats (V = 20 μL, dose = 4.5 μg hemagglutinin, points represent the data of individual animals). The antibody responses were measured 2 weeks after the second immunization of a regimen comprising two immunizations 3 weeks apart. (Abbreviations as in Figure 3).

enhancing effect in nasal HPMC K15M inserts, although it was expected from the in vivo solution studies in mice. The missing enhancement may be related to the low stability of poly-L-arginine during storage of the inserts (supplier suggests storage temperature <0°C).

In situ gelling nasal inserts were suitable for the delivery of influenza vaccine. Although a direct comparison to pure nasal vaccine was not possible, xanthan gum inserts with cationic lipid as adjuvant provoked a similar immune response. This may not be an advantage for influenza vaccine, which is relatively stable in aqueous solutions. However, incorporation into xanthan gum inserts with adjuvant may increase the storage stability of antigens, which are unstable in solution while simultaneously eliciting a similar immune response as the antigen solution.

Conclusion

Influenza split vaccine was successfully incorporated into nasal inserts without loss of the hemagglutinin-specific activity. The in vitro protein release from polymeric vaccine solutions was incomplete, likely because of electrostatic binding of fractions of the vaccine (hemagglutinin positively charged, nucleoprotein negatively charged). In vivo solution studies revealed serum and local mucosal immune response in mice. Xanthan gum had immune response-enhancing properties. Among the adjuvants tested, cationic lipid and poly-L-arginine showed the highest potential. In vivo immunization studies revealed that vaccine-loaded nasal inserts provoked an immune response. Nasal inserts have a potential as nasal vaccine delivery system, although more investigations are necessary to elucidate the different performance of various polymers and enhancers and to further optimize the choice of materials.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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