An Effective Chitosan Based Hydrogel that Improves the Efficiency of Intranasal Vaccination

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Abstract

Intranasal vaccination has been developed as an efficient alternative strategy of parenteral vaccination. Though intranasal vaccination has limitations as well, the dominances of it including activating significant mucosal responses; requiring no professionals to administrate; having almost no side effect, etc. is tempting. This research is aimed to improve the effect of intranasal vaccination for H3N2 split antigens through thermo-sensitive chitosan hydrogel (HTCC/H3N2). To prepare the thermal sensitive hydrogel, chitosan was reacted with quaternary ammonization reagent glycidyl trimethyl ammonium chloride (EPTAC), and mixed with αβ-mixtured-glycerolphosphate (αβ-GP) to initiate the ionic crosslinking between the amino groups. Afterwards, HTCC/H3N2 was rationally tailored to gelate at 37 ˚C within 10 min, which prolonged the antigen retention time for 4 times, increase the intra-mucosal delivery of antigens for 3-fold, and ultimately give rise to robust serum IgG levels (illustrating the systemic immunization strength) and intranasal IgA concentrations (demonstrating mucosal immunization strength), with 5-fold and 8-fold increase, compared with fluidic H3N2 via Intranasal administration (H3N2/IN). These results demonstrate the booming future of the thermo-sensitive hydrogel as efficient antigen delivery system.

Keywords: Chitosan quaternary ammonium salt; Hydrogel; Vaccine delivery system; Mucosal immunization; H3N2.

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List of Abbreviations

Abbreviation	Name
GAS	Group A Streptococcus
LCP	Lipid core peptide
DOTAP	1,2-dio-leoyl-3-trimethylammonium-propane
DC-chol	3β-[N-(N',N'-dimethylaminoethane)-carbamoyl
OVA	Ovalbumin
NE	Nanoemulsion
HPMCP	thiolated hydroxypropyl-methyl Cellulose phthalate
PLG	Poly (lactide co-glycolides)
PLA	poly lactic acid
$NaHCO_3$	Sodium bicarbonate
CO_2	Carbon dioxide
CHP	Cholesteryl-group-bearing pullulan
EPTAC	Glycidyl trimethyl ammonium chloride
HTCC	N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride
αβ-GP	αβ-mixtured-glycerolphosphate
$-NH_2$	Ammonium group
KBr	Potassium bromide
FTIR	Fourier transform infrared spectroscopy
QD	Quaternization degree
$AgNO_3$	Silver nitrate
$NH_4Fe(SO_4)_2 \cdot 12\ H_2O$	Ammonium iron(III) sulfate
NH_4SCN	Ammonium thiocyanate
N_2	Nitrogen
SEM	Scanning electron microscope

PBS	Phosphate buffered saline
ELISA	Enzyme linked immunosorbent assay
BSA	Bovine serum albumin
H_2SO_4	Sulfate acid
OD	Optical density
NH-C=O	Amide
IgA	Immunoglobulin A
IgG	Immunoglobulin G

Table 1: List of abbreviation

1 INTRODUCTION

1.1 Introduction of Vaccination

Vaccination has been proved to be a most triumphant medical intervention towards infectious pandemics, chronic diseases and cancer, which has reduced morbidity and mortality significantly [1]. A research done by the World Health Organization shows that every $1 invested in immunization which means parenteral vaccination in the context, returns an estimated $16 in health-care savings. [2]

Up-to-date commercial vaccinations centered on administrating via parenteral pathways, including subcutaneous or intramuscular injections. However, these strategies suffered from the following limitations. Above all, parenteral vaccination is not effective enough since it fails to stimulate mucosal immune responses which are the first sites of entry for most pathogens invade the organism, and bestow potent immune protections [3]. Furthermore, the injection of vaccines must be executed by medical-trained professionals, hence in back countries, some people do not have chances to receive vaccination [4]. For instance, it is insufficient health care and professional coverages that lead to the result of 12.9 million infants did not receive any vaccinations in 2016 worldwide. Moreover, cross-contamination caused by loosely sterilized injections has become sterilization is a severe problem, resulting in millions of deaths all over the world. Last but not least, various kinds of adverse side effects may occur including tissue edema, irritability etc. [5]. Therefore, to improve the sanitary conditions of people, especially those who live in back countries, the limitations of vaccinations must be eliminated.

1.2 Nasal Vaccination and Challenges

With various superiorities, nasal vaccinations which simultaneously stimulate systemic and mucosal responses by delivering antigens through nasal mucosa, have been regarded as a promising approach. To begin with, nasal vaccination activates mucosal responses which generate mucosal-specific antibodies in the nasal and remote mucosal sites, such as pulmonary,

gastrointestinal and vaginal tissues, which confers major mucosal protections [3]. Only through mucosal sites administration can mucosal protections be elicited. Besides, practical use is also a dominance of nasal vaccination since it does not require professionals and can be administrated by needle-free devices, which will be a competent method to prohibit contamination and extend immunization coverage in devious places [4]. In addition, the adverse side effect caused by parenteral vaccination such as tissue edema, irritability can be decidedly diminished by nasal vaccination. With these dominances, nasal vaccination can immensely improve the immunization and health condition of the world [6].

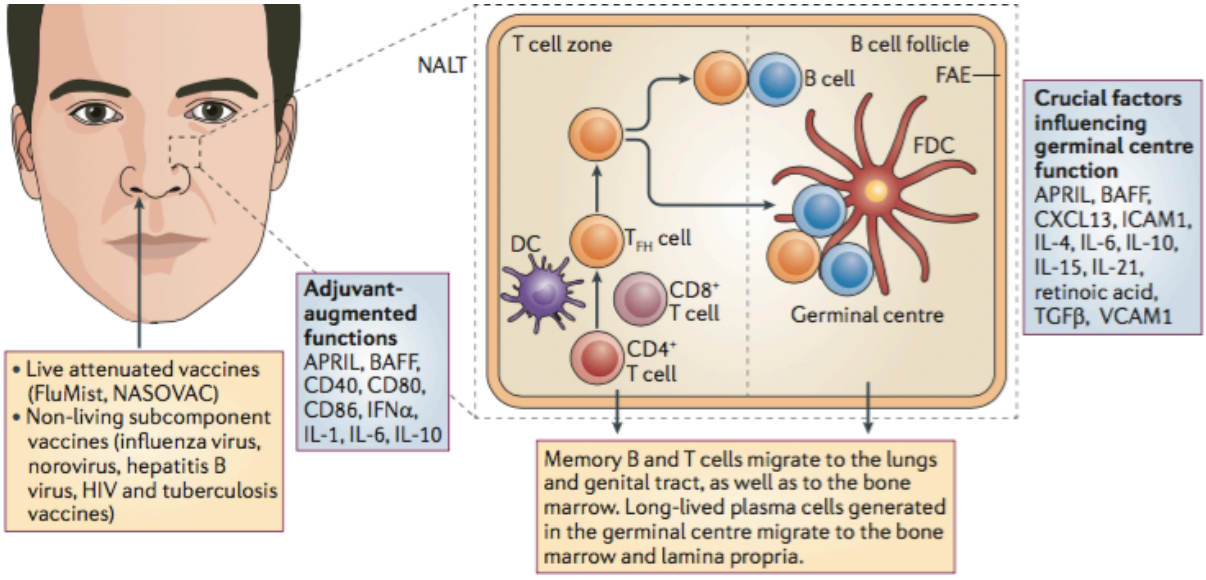


Fig. 1. Intranasal vaccination stimulates immune responses in the nasopharynx-associated lymphoid tissue [7].

For efficient nasal vaccinations, current research is required to gribble with the following challenges. First and most importantly, the tight conjunction of the epithelial cells that envelope the surface of mucosal is extremely hard to break through if the antigens need to be delivered through the mucosa, which may be supreme restraint of mucosal vaccination. The mucosal cilium and the secreta that covers the surface of mucosal forms the mechanical defend system. For instance, the nasal ciliary beating frequency of human can reach up to 500 times per minute, which makes it very arduous for the antigens to stay on the nasal mucosa. Therefore, the natural barrier shields the antigens' entry.

On the other hand, the secreta which is a gel-like liquid that covers the mucosa will also be refreshed continuously deal with the movements of the cilium, and significantly shorten the

time that antigens stay on the mucosa. The secreta on nasal mucosa can be cleared completely in 20 minutes. If the antigens cannot stay on mucosa long enough, the outcome of the vaccination will decline to a large extent or even be eliminated. Both the natural barrier and the mucociliary clearance of mucosa will create severe difficulties [8].

In addition, employing well-defined antigens and getting excellent biosafety profile, modern vaccination suffers poor immune potentiation in the end [3]. Though intranasal vaccination adjuvants like cholera toxin and heat-labile enterotoxin had been proved to be compelling when activating immunity, they were proved to be toxic [9]. As a result, there is a compelling need for adequate mucosal vaccine adjuvant which covers the following features:

1). Potent antigen delivery at mucosal sites;

2). Long antigen retention;

3). Robust systemic and mucosal immune activations.

1.3 Intranasal Antigen Delivery System

As mentioned, rational designed intranasal delivery system is the key to prevent infectious diseases, which is demanded to felicitate the sub-mucosal antigen delivery, prolong the antigen retention and activate the potent engagement of immune systems [10]. Current research focused on the development of liposomes, emulsions, biodegradable particles and hydrogels to stimulate systemic and mucosal immunity [11].

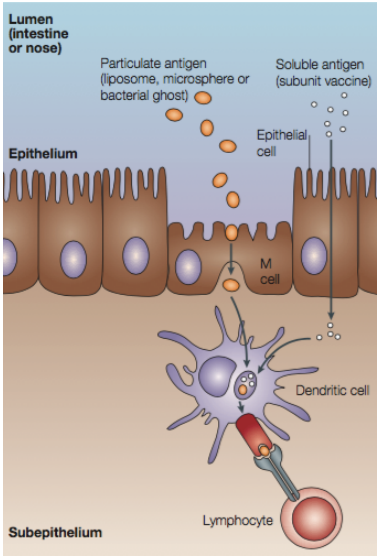


Fig. 2. Immunization by mucosal routes [12].

1.3.1 Liposome

Liposome is a phospholipid bilayer built up by phospholipid and cholesterol, with a hydrophilic head and a hydrophobic tail. When diffused in an aqueous system, the phospholipid can exist in various kinds of patterns such as micelle, bilayer, and liposome.

To deal with Group A Streptococcus (GAS) which has no corresponding vaccines, scientists encapsulated GAS epitope into lipid core peptide and forms a self-adjuvanting carrier that was delivered via nasal mucosa. As a peptide-based vaccine delivery system, lipid core peptide (LCP) is capable of activating immune responses efficiently against Schistosoma [13, 14], hookworm [14, 15], cancer and malaria [16].

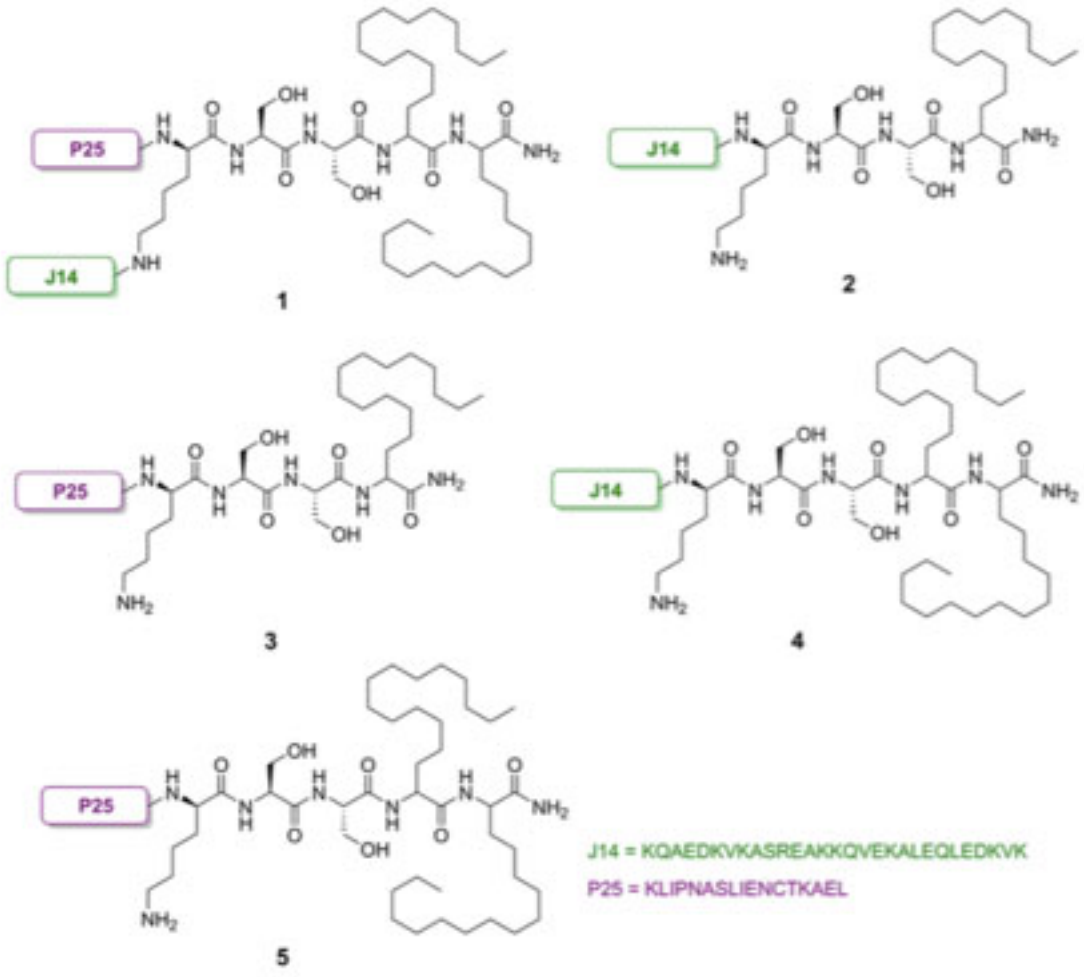


Fig. 3. Schematic representation of peptide (P25 and J14) [17].

Results of animal experiments showed that mucosal and systemic immune responses were both induced while IgA and IgG (IgG1 and IgG2a) production was also stimulated. The effects lasted permanently, which high levels antibody (IgA and IgG) were detected even after five months [17].

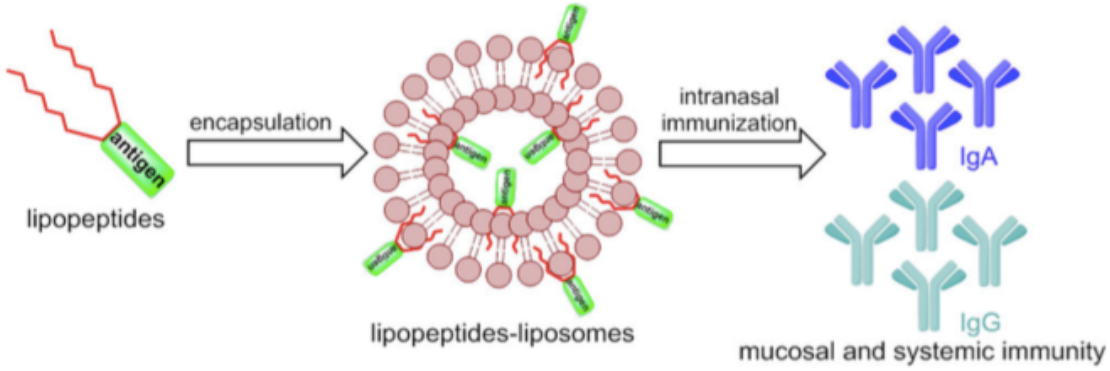


Fig. 4. Schematic representation of lipopeptides and antigens encapsulated into liposomes and stimulate mucosal and systemic immune responses [17].

A cationic liposome comprised of 1,2-dio-leoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] (DC-chol) (DOTAP/DC-chol liposome) were used to carry ovalbumin (OVA). The intranasal administration stimulated OVA-specific IgA production and enhanced serum IgG1 levels. The result demonstrated that the cationic DOTAP/DC-chol liposome induces TH2 immune responses [18]. The increase of IL-4 and CD11c+ dendritic cells reinforce that DOTAP/DC-chol liposomes derive antigen-specific immune responses. Though OVA was only a antigen model, this research illustrate the future of liposome material employed as a mucosal adjuvant against infectious diseases [19].

Though liposome has an excellent capacity to adhere to the nasal mucosa and hence to be absorbed, the possible rupture and dissociation of liposomes on the mucosal sites may become serious concerns for the vast applications of liposomes in intranasal delivery.

1.3.2 Emulsion Adjuvant

As a sort of intranasal antigen delivery system, emulsion adjuvants are composed by oil-in-water emulsions and biodegradable oils which diminishes the toxicity broaden the range of practicable antigens [20].

Nanoemulsion(NE), a nanoscale oil-in-water emulsion, induces potent systemic and mucosal responses. The local inflammatory effects can be eliminated when NE was delivered via nasal mucosa [21]. The animal tests showed the rise of T_H1 immune responses and IL-17, along with the decline of IL-4, IL-5 and IG1. The data illustrated that NE is capable to redirect entrenched T_H2 immune responses and obtain balanced responses [22]. Therefore, NE may be employed to treat diseases associate with T_H2 immunity.

As a tolerated NE adjuvant in animals and humans, the mixture of $W_{80}5EC$ and antigen ($W_{80}5EC$-Ag) is capable of activating potent and balanced immune response[23, 24]. The NE action on epithelial cells and dendritic cells exhibited that the epithelial cells were engulfed by the DCs. Results showed that the exposure of epithelial cells and dendritic cells to NE stimulated the uptake of antigen. In all, $W_{80}5EC$-Ag balanced systemic and mucosal responses and strengthened antigen production [25].

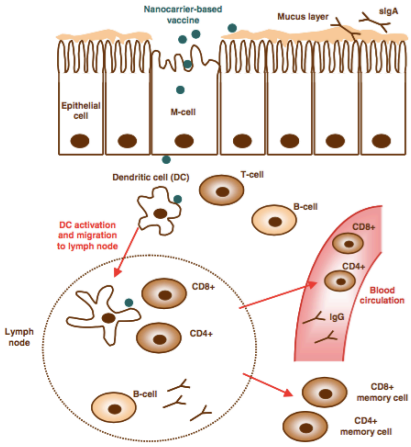


Fig. 5. Development of cellular and humoral immune responses following mucosal administration of nanocarrier-based vaccines [25].

Despite of the capacity to activate robust immune responses, the retention of emulsion adjuvant is not permanent enough [10].

1.3.3 Biodegradable Microsphere

Biodegradable microsphere has been used in oral vaccine researches and proven to be a potential nasal delivery carrier [26]. The most promising vaccine sustained-release system is macromolecule polymer and capsule, found by synthetic or natural biodegradable microsphere.

The mannan-decorated mucoadhesive thiolated hydroxypropyl-methyl cellulose phthalate (HPMCP) microspheres (Man-THM) are incorporated with ApxIIA. The Apx toxin which creates pores in the host cell membrane is one of the most significant causes of pleuropneumoniae [27]. Mucoadhesive thiolated HPMCP microspheres could be target to mannose receptors and pathogen recognition receptors antigen presenting cells in the respiratory immune system.

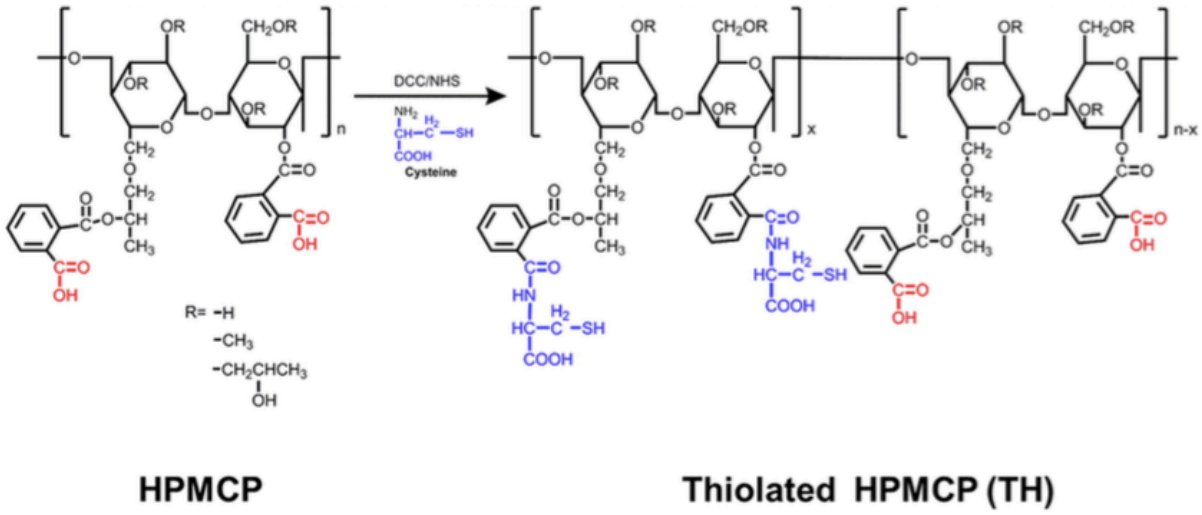


Fig. 6. The reaction scheme for the synthesis of thiolated HPMCP (TH) [28].

Results reinforced that the Man-THM augmented mucosal sIgA, IgG and IgA immune responses. The safety of this adjuvant by the high survival rate of vaccinated mice is also a remarkable dominance [28]. Thus, Man-THM is capable for protecting organisms from pathogenic bacteria infection.

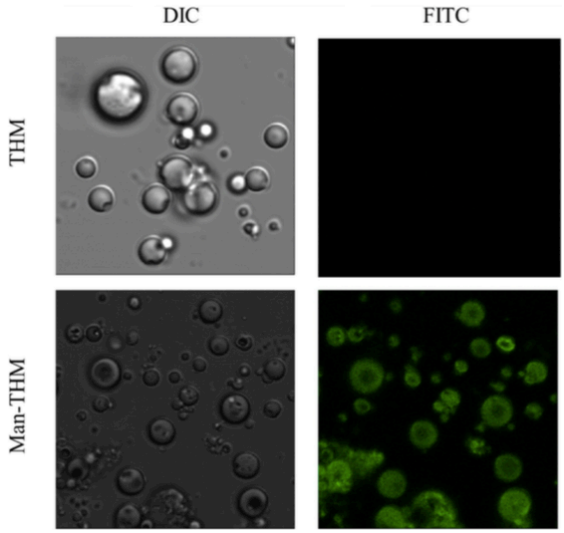


Fig. 7. Confirmation of the surface decoration of mannan in Man-THM. FITC-conjugated mannan was used to create the FITC-Man-THM and the surface fluorescent signal was monitored by CLSM [28].

As synthetic materials, Poly (lactide co-glycolides) (PLG) and poly lactic acid (PLA) have favorable biodegradability and biocompatibility. These materials are further developed and improved. Employed as intranasal delivery system, surface-functionalized, pH-sensitive PLGA microparticles were promoted. Sodium bicarbonate ($NaHCO_3$) and HBsAg were enveloped into PLGA microparticles while mucoadhesive polymer chitosan and mannose receptor were placed on the surface of PLGA microparticles. $NaHCO_3$ reacts with lysosomes and produces carbon dioxide (CO_2) bubbles, which erupted the PLGA microparticles shell and compel the release of HBsAg rapidly [29]. The velocities of CO_2 and HBsAG release were lowered when the pH was raised to approximately 7.4. Four types of microparticles including PLGA, MN-PLGA, CS-PLGA and MN-CS-PLGA were synthesized through the procedure. This illustrated that the residence time of the MN-CS-PLGA microparticles and stronger humoral and cell-mediated immune responses are strengthened. Therefore, PLGA microparticles associated with chitosan and mannan is an efficient tool to deliver HBsAg via nasal mucosa [30].

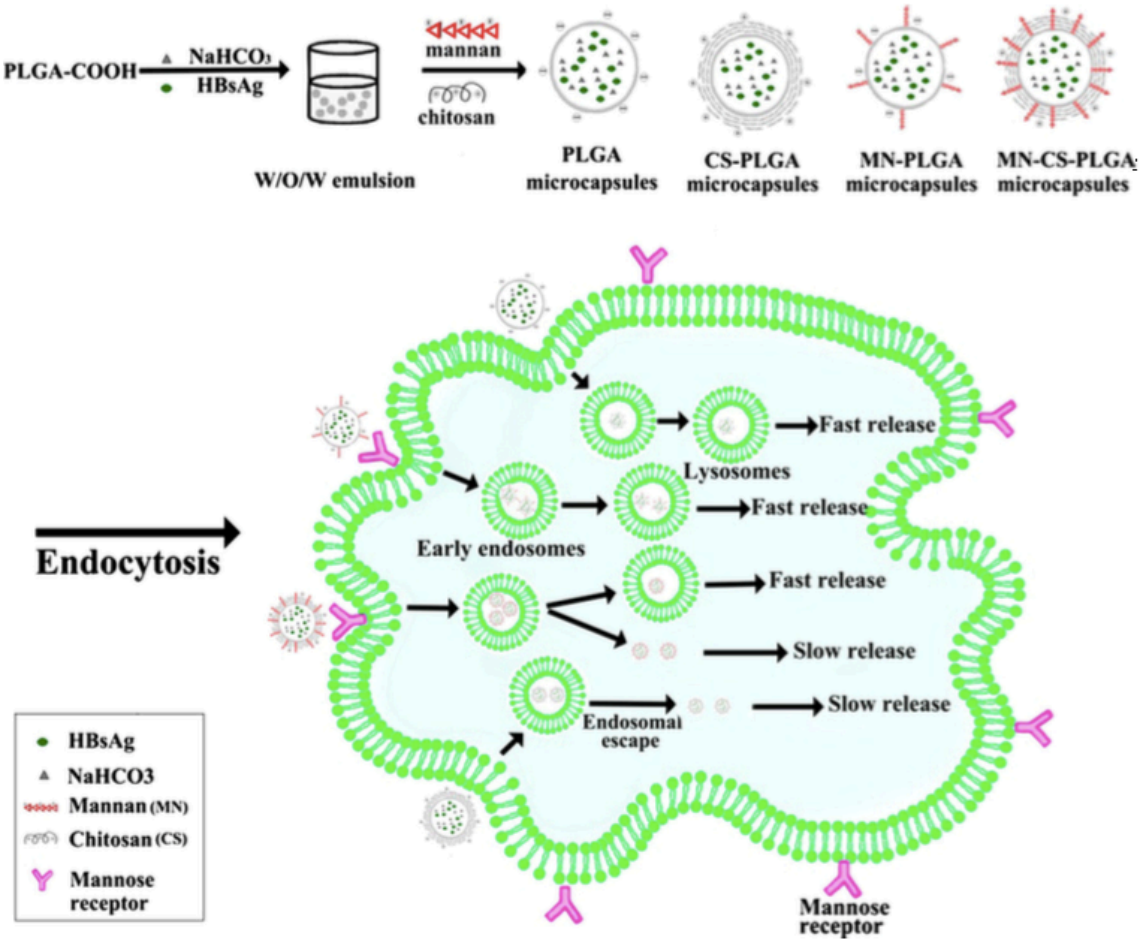


Fig. 8. A thematic diagram showing intracellular macrophage uptake and intracellular antigen release patterns of pH-responsive PLGA, MN-PLGA, CS-PLGA and MN-CS-PLGA microparticles [30].

1.3.4 Hydrogel

Using water as dispersing medium, macromolecule hydrogels are steric macromolecular reticulate structures which are hydrophilic and can absorb much water or liquid have great biocompatibility and elasticity. Thermal-sensitive hydrogels can freeze at a particular temperature. The intermolecular force between the hydrogel and the bioactive substances is weak. Hence the bioactive substances can be conserved permanently without losing activity. However, hydrogels are most synthetic materials; therefore, their biodegradability and biocompatibility are lower than natural materials, which limits their clinical usage.

For instance, the cholesteryl-group-bearing pullulan (CHP) forms nanogels by self-assembly in water, which envelops proteins by acquiring chaperon-like activity and mostly hydrophobic interactions [31]. Altogether, the CHP and the protein construct an antigen delivery system. The Clostridium botulinum was encapsulated in CHP and delivered via nasal mucosal. This induced robust IgA and IgG antigen responses and the nanogel particles can target to mucosa membranous cells and be uptake by dendritic cells remarkably. The results suggested that CHP merit further development as a universal protein-based antigen-delivery carrier for intranasal vaccination [32].

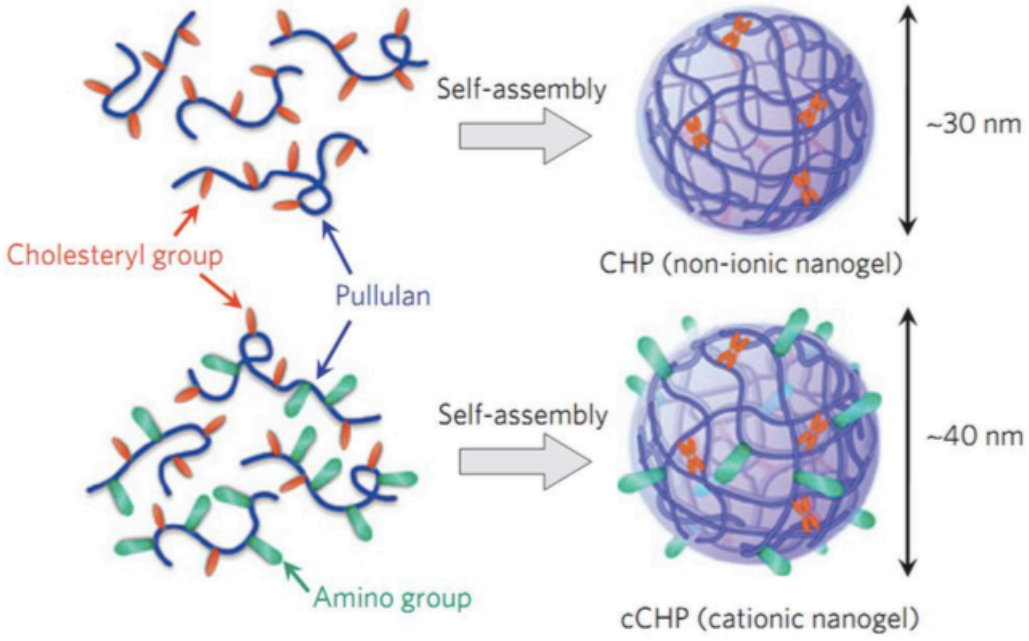


Fig. 9. Schematic preparation of nanogel made from pullulan (CHP: cholesteryl-group-bearing pullulan) [32].

1.4 Chitosan and its Derivate Function as Thermal-Sensitive Intranasal Vaccination

To deal with the limited biological responses and biocompatibility of synthetic materials, scientists have begun to search for natural substances to synthesize thermal-sensitive hydrogels. Chitosan, the product of chitin which is a kind of natural polysaccharide after deacetylation, has favorable tissue-compatibility and biodegradability [33].

As a kind of natural cation polysaccharide which is limited in quantity, the exceptional features of chitosan make it a broadly investigated and employed substance in the field of nasal delivery and immune. Formed by chitosan and delivered via nasal mucosa, OVA antigen is mixed with chitosan and forms a nanoparticle mixture. This adjuvant has high stability and thiol sensitivity. The superior immunogenicity is also a significant dominance compared to non-stabilized particles including (IgG, IgG1, IgG2, IgA) [34].

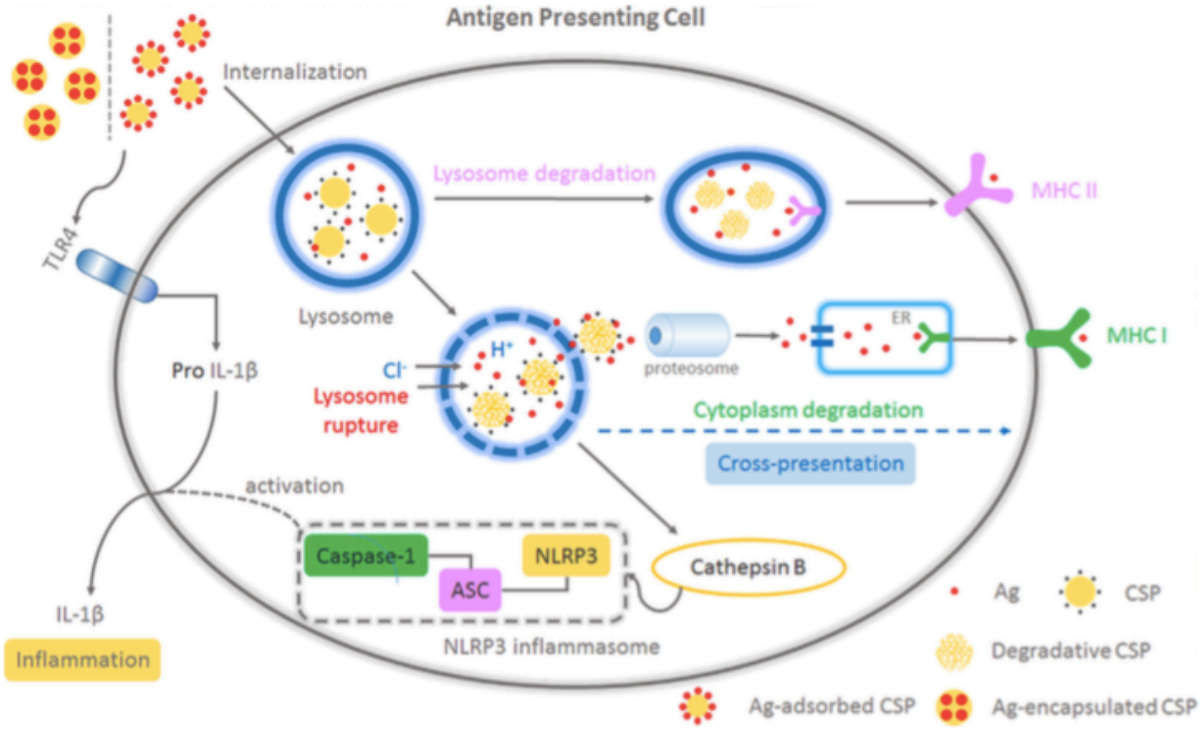


Fig. 10. Model of CSP-induced NLRP3 inflammasome activation and cross-presentation (Ag: antigen; CSP: chitosan particle.) [35].

Composing a nanoparticle adjuvant, the H1N1 virus is encapsulated in chitosan. This adjuvant can stimulate robust systemic and mucosal responses when administrated intranasally or intramuscularly [36]. Besides, the cellular immune responses are also activated. Though chitosan has adequate hydrophilicity, its solubility is minimal and can only dissolve in acidic conditions which may damage the medicines carried by chitosan [35].

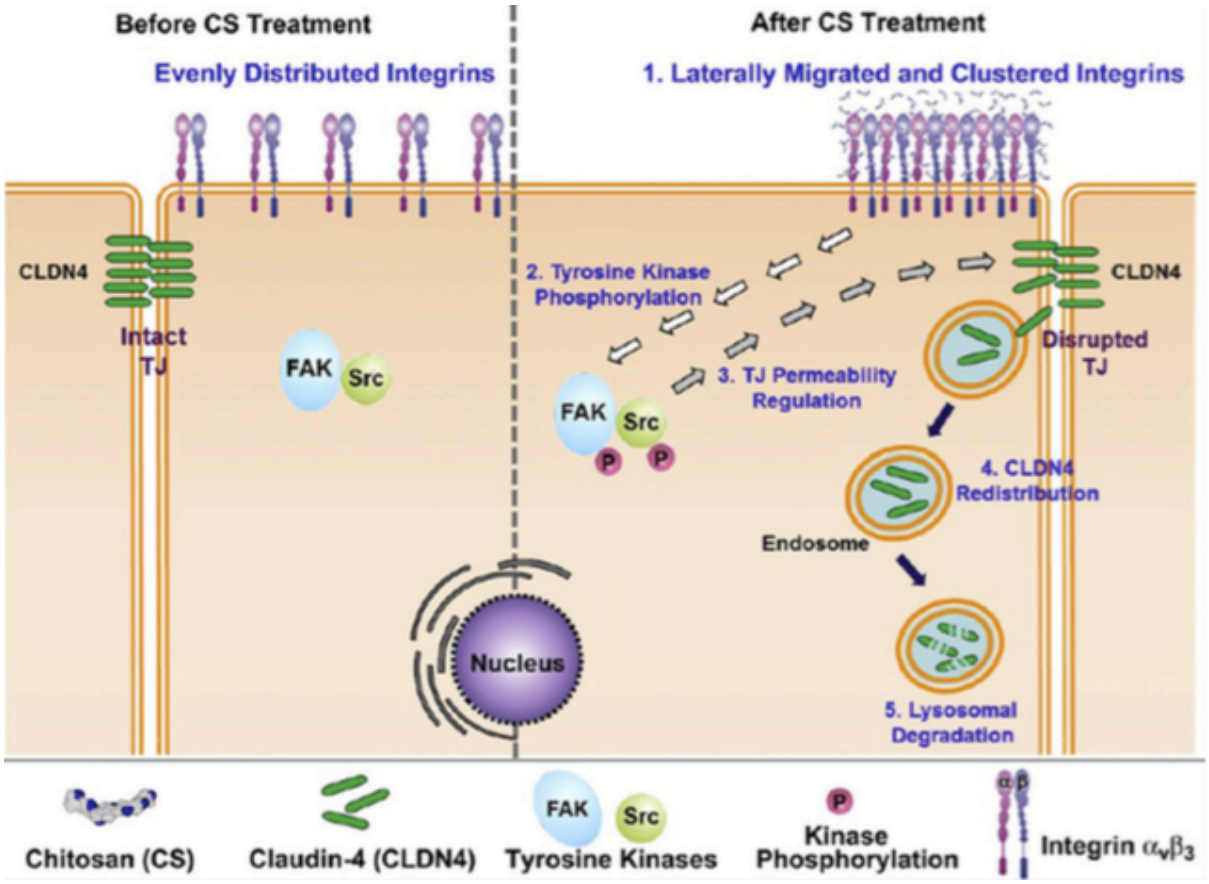


Fig. 11. The signaling mechanism of CS-mediated TJ opening [37].

Accordingly, chitosan-based hydrogel harbors promising potential for the efficient intranasal antigen delivery system.

To improve the solubility of chitosan, increase the positive charges, mucosal adhesion, hygroscopicity and retention characteristic, a quaternary ammonium salt of chitosan is synthesized. The ammonium group of chitosan reacts with glycidyl trimethyl ammonium chloride (EPTAC) and forms N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC). HTCC is then incorporated with αβ-mixtured-glycerolphosphate (αβ-GP) and interacts with it through coulombic interaction. The mixture is a novel thermal-pH-sensitive

hydrogel which is a liquid at room-temperature. If the proportion of GP and HTCC is proper, the liquid can transform to a non-liquid gel after being heated for a certain time. Better results can be obtained if this innovative hydrogel is used to deliver drugs through nasal mucosa.

1.5 The Purpose of this Research

Though nasal delivery of small molecules drugs has been successful, the development of intranasal vaccination has just yet begun. However, few were put into clinical applications, which illustrates the significant difference between the delivery of small molecules drugs and vaccines. Despite of their efficiency, some mucosal vaccinations suffer limited security and stability. Therefore, this research targets to develop an intranasal vaccination adjuvant that is effective, safe and stable, and impel the application of self-administrated vaccine.

As a cationic material, chitosan can break through the conjunction between epithelial cells and facilitate the delivery of medications. Traditional chitosan based adjuvants fail to resolve the problem of secreta mucociliary clearance efficiently. Besides, the cross-linking agent used like glutaraldehyde when synthesizing these adjuvants are high toxic. Thus, using αβ-GP as cross-linking agent, this thermal-sensitive hydrogel would be an ideal intranasal delivery system. The adjuvant would have these features: potent antigen delivery at mucosal sites; long antigen retention; robust systemic and mucosal immune activations. Not only can this new type of vaccination solve most limitations of traditional vaccinations, but also promote the efficiency of parenteral vaccinations significantly. The application of self-administrated vaccine promoted can improve the sanitary condition of back countries and save more lives.

2 The Synthesize of HTCC Hydrogel

2.1 Materials and Reagents

Chitosan ((MW 1.5×10^6, degree of deacetylation is 95%) was purchased from Putian Zhongsheng Weiye Co. Ltd. EPTAC was acquired from Dongying Guofeng Fine Chemical Co. Ltd. (Shandong, China). (Fujian, China). αβ-GP was obtained from Kaiyuan Pharmaceutical & Chemical Co. Ltd. (Shanxi, China). Isopropyl, acetone, etc. (AR) were provided by Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China). Silver Nitrate, ammonium iron(III) sulfate, ammonium thiocyanate, etc. (AR) were also purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China). H3N2 influenza vaccine (split virion) was obtained from Hualan biological engineering, INC. (Henan, China). 96-well ELISA plates were provided by Corning Inc. (Shanghai, China).HRP-conjugated goat anti-mouse secondary antibody (IgG、IgG1、IgG2a、IgA) was procured from Abcam plc. (Shanghai, China).

2.2 Synthesis and Characterization of HTCC With Different Quaternization Degree

2.2.1 Synthesize of HTCC

The principal of the synthetic process is to use quaternization reagent EPTAC reacts with the active ammonium group ($-NH_2$), and decorates it with quaternary ammonium group. The HTCC was prepared by using the following method. A certain amount of chitosan powder was dissolved in isopropanol and moved to a three-neck round bottom flask. The mixture was stirred and heated at a particular temperature of precisely 80 ˚C.

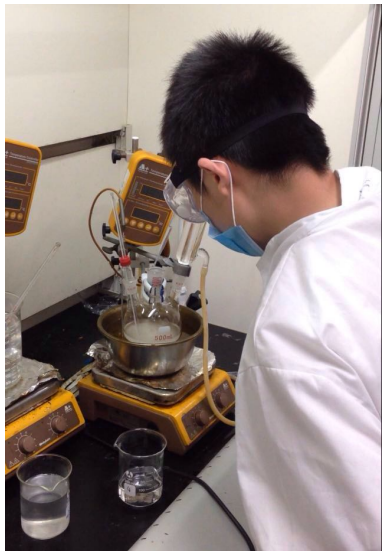


Fig. 12. Add EPTAC into chitosan-isopropanol suspensions dropwise.

A certain amount of quaternization reagent EPTAC was dissolved in deionized water, put into a constant pressure liquid funnel. The EPTAC solution was added into the chitosan-isopropanol suspensions dropwise. The mixture was heated at 80 ˚C for 7 hours before experiencing suction filtration. The HTCC participation was washed by cold water once and cold acetone twice. 4 ˚C cold acetone was added to the HTCC precipitation which was stirred overnight. The next day, the HTCC precipitation were cleaned by cold acetone for another three times until it was collected. The HTCC product was produced after it was dried at 60 ˚C in a drying oven.

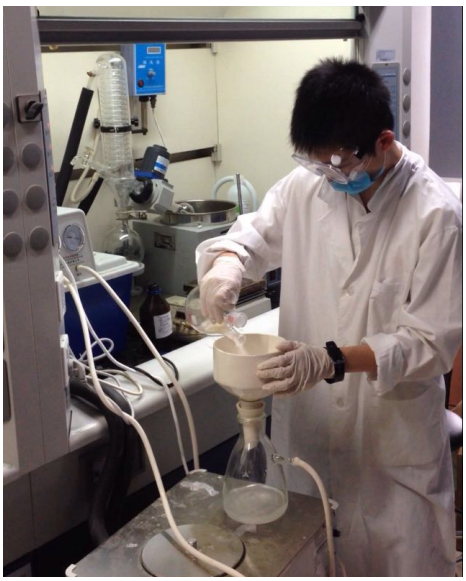


Fig. 13. Filtrate and wash the HTCC mixture.

2.2.2 HTCC Infrared Spectrometry

After it was mixed with dry potassium bromide (KBr) and performed, chitosan quaternary ammonium salt powder's infrared spectrogram was examined and recorded under a Fourier transform infrared spectroscopy(FTIR) spectrometer.

2.2.3 HTCC Quaternization Degree Titration

According to the reaction equation, whenever a quaternary ammonium group was introduced, an Cl^- ion would also be imported. Therefore, by measuring the Cl^- ion content of HTCC, the degree of being displaced by quaternary ammonium group which is the HTCC quaternization degree (QD) in chitosan can be calculated. The Cl^- ion content was measured by Volhard method. In allusion to the glutinous system of chitosan, the determination approach was improved so that the terminal point could be estimated handily. The procedure was as follows:

A certain amount of chitosan quaternary ammonium salt specimen was weighed accurately, and 20 mL of 0.1 mol/L acetic acid solution was added. The liquid was stirred under room-temperature for 1 hour to ensure that HTCC is sufficiently dissolved. 2 mL of concentrated nitrate acid was added to the specimen before 100 mL of ethyl alcohol was added to form flocculent precipitate so that it would be easier to observe the color change of the terminal point. 20 mL of 0.1 mol/L silver nitrate ($AgNO_3$) standard solution was added, so that the Cl^- in the solution formed white participation.

Fig. 14. Weigh certain amount of specimen.

After that, 5 mL of dibutyl phthalate (DBP) was added before the rubber stopper was placed and the container was considerable oscillated. At last, 2 mL of ammonium iron(III) sulfate ($NH_4Fe(SO_4)_2 \cdot 12\ H_2O$) indicator was added, and the remaining Ag^+ was back titrated by using 0.1 mol/L ammonium thiocyanate (NH_4SCN) standard solution until the solution emerged light reddish brown. The QD was calculated by the following equation:

$$QD = (V \cdot c/1000)/[V \cdot c/1000+W_1(1-DD)/M_1+(W_1 \cdot DD-W_2)/M_2]\times 100\%$$

The V and c in this equation represents the volume (mL) and concentration (mol/L) of $AgNO_3$; DD serves as the degree of deacetylation of the specimen; M_1, M_2 represents the relative molecular mass of N-acetylglucosamine unit and N-deacetylglucosamine unit respectively; W_1 is the mass of the sample, and W_2 can be calculated as follows:

$$W_2 = V \cdot c \cdot M_3/1000$$

M_3 is the relative molecular mass of the chitosan quaternary ammonium salt.

2.3 Preparation of HTCC Hydrogel

Certain amount of HTCC was weighed and transferred into a 25 mL flask, 5 mL of 0.2 mol/L lactic acid was mixed with HTCC. The mixture was stirred for 30 min under room-temperature before 3 mL of deionized water was added and the concoction was blended for another 2.5 h to ensure that HTCC is sufficiently dissolved.

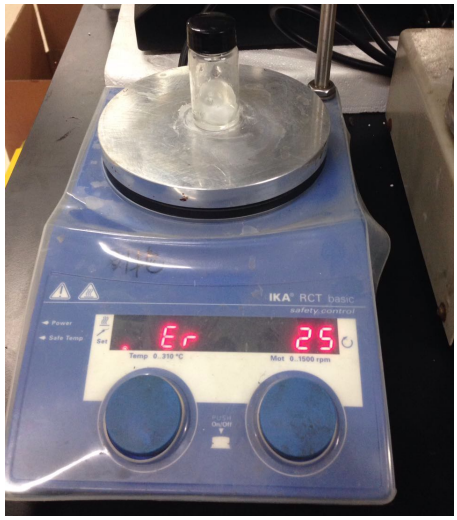


Fig. 15. The HTCC-lactic acid mixture was stirred.

A particular concentration of αβ-GP aqueous solution was added dropwise to the HTCC acidic solution to procced ionic-crosslinking in an ice bath. After 10 min of reaction, the HTCC hydrogel was produced.

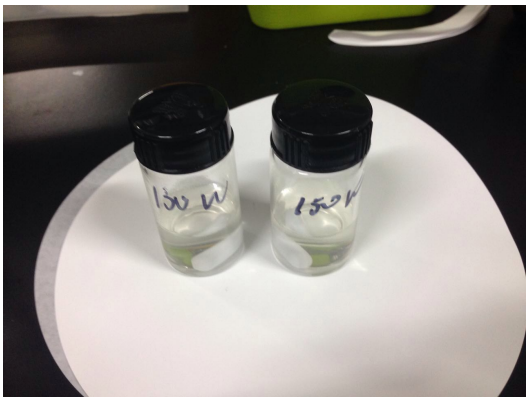


Fig. 16. HTCC hydrogel product.

2.4 Characterization and Optimization of HTCC Hydrogel

2.4.1 Morphology of HTCC Hydrogel

The steric reticulate structure of the chitosan quaternary ammonium salt hydrogel was constructed by hydrogen bonds, charges and hydrophobic interactions. Small pieces of dry HTCC hydrogel with various QD that had the same volume were froze in -196 ℃ liquid nitrogen (N_2) rapidly. The solid hydrogel was cut with a knife and the section of it received treatments including ion sputtering and spray-gold. After that, the gel's steric structure was observed with scanning electron microscope (SEM).

Fig. 17. Scanning electron microscope.

2.4.2 Rheological Properties of HTCC Hydrogel

The morphology of the hydrogel is between solid and liquid, which it has both viscidity and elasticity. Before gelatinization, the system emerges solution state while viscidity feature dominates. When gelatinization occurs, the hydrogel appears solid state, and the elasticity characteristic of the system seizes the principal. The curve that describes the hydrogel system's elasticity modulus G' and viscidity modulus G'' vary with time or temperature need to be determined. The time or temperature corresponds to the intersection point of elasticity modulus G' and viscidity modulus G'' is the time or temperature when the system transforms from liquid to solid, which is called gelation time or temperature.

The procedure of HTCC hydrogel transforming from solution state to gel state can be detected accurately by using a rheometer. The gelation time or temperature and shear viscidity of the HTCC hydrogel can also be calculated accurately with the help of a rheometer. The method of determining the rheological properties of the hydrogel is as follows:

First, the air compressor was turned on, and the circulation water and rheometer's main engine was started. After that, operations which were required according to the operating rules including initialization and zeroing the equipment, etc. Approximately 2 mL of the sample was placed on a 4 ° and 40 mm diameter cone plate.

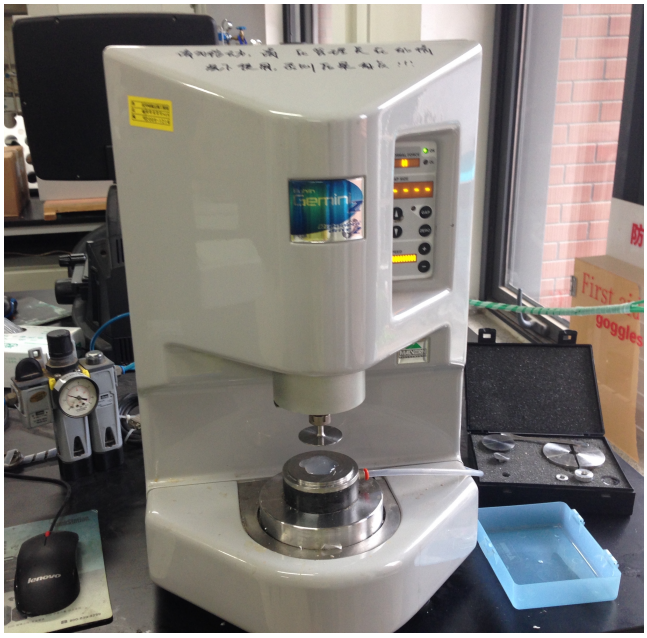


Fig. 18. Determination of rheological properties of HTCC hydrogel.

The separation distance was fixed as 150 μm before the deading was closed. Finally, the viscidity mode and vibrate mode were used to detect the viscidity and gelation time of HTCC hydrogel according to the preset program. While measuring the viscidity, the temperature was set at 20 ˚C, and the shearing rate was emplaced at 40 r/s; when detecting the gelation time, the temperature was initialized at 37 ˚C, the vibrating frequency was arranged at 1 Hz, and the shear stress was set as 0.03 Pa.

2.5 Estimation on the Antigen Retention: In Vivo Animal Imaging Study

The fluoresce labeled H3N2 vaccine was made followed manufacture's procedure. Briefly, dissolve ~10 mg of the protein in 1 mL of 0.1 M sodium bicarbonate buffer pH 8.3. Then, dissolve the amine-reactive dye in DMSO or DMF at 10 mg/mL. While stirring the protein solution, slowly add 50–100 μL of the reactive dye solution from step 2. Incubate the reaction for 1 hour at room temperature with continuous stirring. Equilibrate a 10 × 300 mm gel filtration column (Sephadex G-25 column or equivalent matrix) with PBS. Separate the conjugate on the gel filtration column. Store the conjugates under the same conditions used for the parent protein.

Fluorescence probe Cy7-SE was used to mark the H3N2 spilt vaccine, which was mixed with HTCC hydrogel with different QD. The mixture was delivered to mice by using nasal drip before the antigen retention of mice were analyzed by a Cri Maestro In-vivo imaging System. This procedure was used to evaluate the variation of the antigen retention of HTCC hydrogel with different QD. The specific operating procedure was as follows:

HTCC hydrogel with different QD was synthesized by using the method provided in 2.2.1. The Cy7-H3N2 was diluted until the HA concentration was 375 μg/mL. The diluted Cy7-H3N2 was then mixed with HTCC hydrogel according to the volume proportion of 3:7. BALB/c mice were given 10 μL dosage for each naris. The mice were put into isoflurane anesthesia box and lightly anesthetized for 5 min before they were transferred into a Cri Maestro In-vivo imaging System for detecting the fluorescence magnitude in the mice's nasal cavity. After the detection, the mice were placed in a warm and ventilated environment for their natural vivification.

Fig. 19. The Cri Maestro In-vivo imaging System.

This anesthesia-detect-vivification procedure was repeated to detect the fluorescence magnitude in the mice's nasal cavity at predetermined times. Carestream Molecular Imaging Software was used to analyze the fluorescence magnitude in the mice's nasal cavity quantitatively. This software was also used to overlay the X-ray and fluorescence image to exhibit the fluorescence change in the mice's nasal cavity intuitively.

2.6 Determination of Antigen Transmucosal Release *In Vitro*

The mucosa membranes of BALB/c mice were removed from both sides of the nasal septum and washed with phosphate buffered saline (PBS). With cilia side up, the mucosa sheet was placed on static Franz diffusional cells (diameter 5 mm, receptor volume 6 mL). 6 mL PBS (pH 7.4) was added to the receptor while 0.5 mL hydrogel/H3N2 or other delivery system was dropped on the cilia side. The concentration of the antigen was 150 μg/L (HA) initially. The water bath was kept at 37.0 ˚C while the receptor solution was stirred continuously at 300 rpm. 150 μL of the receptor medium was displaced by the fresh medium at 1, 2, 3, 4 and 6 h. The accumulative release of H3N2 antigens was sampled at predetermined times while micro-BCA method was used to examine the protein concentration of H3N2.

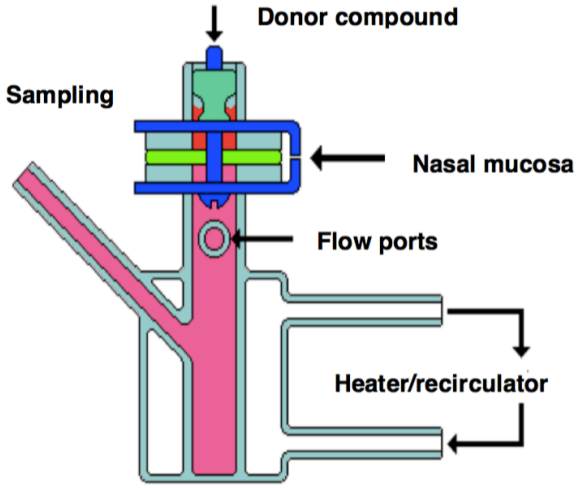


Fig. 20. Figure Schematic diagram of penetration measuring device.

2.7 Mucosal Antibody Responses

6-8 weeks old balb/c inbred strain female mice were distributed to 5 per group. They were immunized at day 0 and day 28. The serum and nasal wash was collected on day 28. The antigen-specific IgG and IgA in the serum after immunization was detected by the method enzyme linked immunosorbent assay (ELISA). The specific operating procedure was as follows:

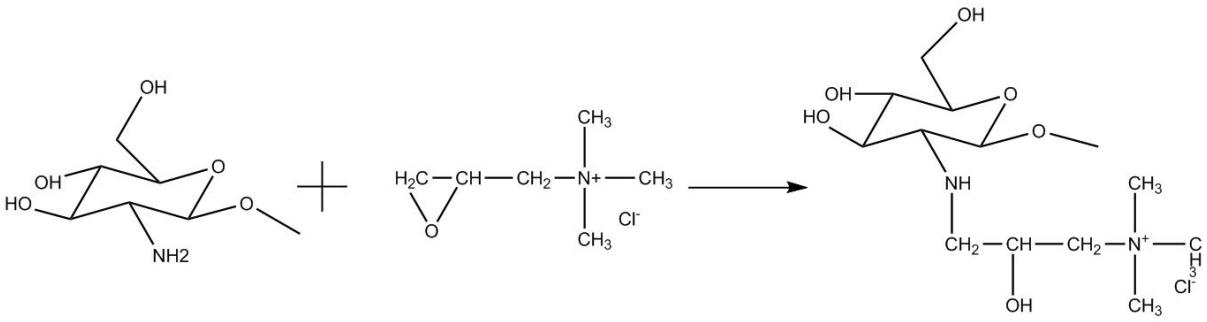
96-well plates were coated by spilt H3N2 with 2 μg/mL antigen concentration overnight at 4 ˚C in a calorstat. The volume of the coating buffer was 100 μL. The coating buffer was removed,

and PBST (0.01 mol/L, pH 7.2~7.4 PBS which contained 0.05% Tween-20) was used to wash the plates for three times. The plates were blocked with 300 μL of 0.5% bovine serum albumin (BSA) and put in a calorstat for 1 h at 37 ˚C. The confining liquid was disposed while the plates were washed with PBST for three times. 100 μL of serum sample was diluted serially by PBST that contains 0.1%BSA and reacted for 30 min in a calorstat at 37 ˚C. This caused that the H3N2 influenza antibody in the serum specifically bound to the coating antigen. After the diluent was discarded, the plates were washed for four times using PBST. 100 μL of HRP-conjugated goat anti-mouse IgG or IgA secondary antibody (1:10000 dilution) was added. The reaction lasted 30 min at 37 ˚C in a calorstat, so that the DON-HRP-Conjugated specifically bound the Fc fragment of the serum-specific antigen. The secondary antibody was disposed, and the plates were washed six-time using PBST. 200 μL of TMB (3,3',5,5'-Tetramethylbenzidine) color liquid was added to quantify HRP. After 20 min of reaction, 50 μL of 1 mol/L sulfate acid (H_2SO_4) was added to terminate the chromogenic reaction. A microplate reader was used to detect optical density (OD) at 450 nm with a reference wavelength which equaled to two times of the OD value of negative serum.

3. Results & discussion

3.1 Characterization of HTCC

Chitosan is only soluble in acidic solution, which confines the application scope of it. Therefore, the synthesize of hydrosoluble chitosan ramifications has become a research focus, and chitosan quaternary ammonium salt is one of them. There are hydroxyl groups and ammonia groups in chitosan molecules so that that quaternary ammoniation can take place at the hydroxyl groups or the ammonia groups. Since the chitosan quaternary ammonium salt has large steric hindrance and strong hydratability, the hydrogen bond between chitosan molecules can be sufficiently weaken which increases the water solubility of chitosan ramifications.



Chitosan EPTAC HTCC

Fig. 21. Synthesis route of the chitosan quaternary ammonium salt.

A chitosan quaternary ammonium salt can be synthesized by applying Menshutkin reaction which transfers the ammonia group to a quaternary ammonium salt or conjuncts the ammonia group with a small molecule quaternary ammonium salt [38]. With appropriate biocompatibility, water solubility, moisture absorbability, moisture retentivity, etc., the chitosan quaternary ammonium salt has favorable features. This paper reports on the synthesize of a chitosan quaternary ammonium salt using EPTAC and chitosan. To obtain an intelligent thermal-pH sensitive hydrogel, the conditions were optimized. EPTAC is a water soluble small molecule, while chitosan is an insoluble macromolecule. However, chitosan is soluble in

isopropanol, so that in this study, the mixed solvent system composed of water and isopropanol was used as the reactive solvent.

The sector of HTCC hydrogel that was froze in liquid nitrogen and observed with a SEM. As shown in Figure 21, the structure of the froze hydrogel was poriferous.

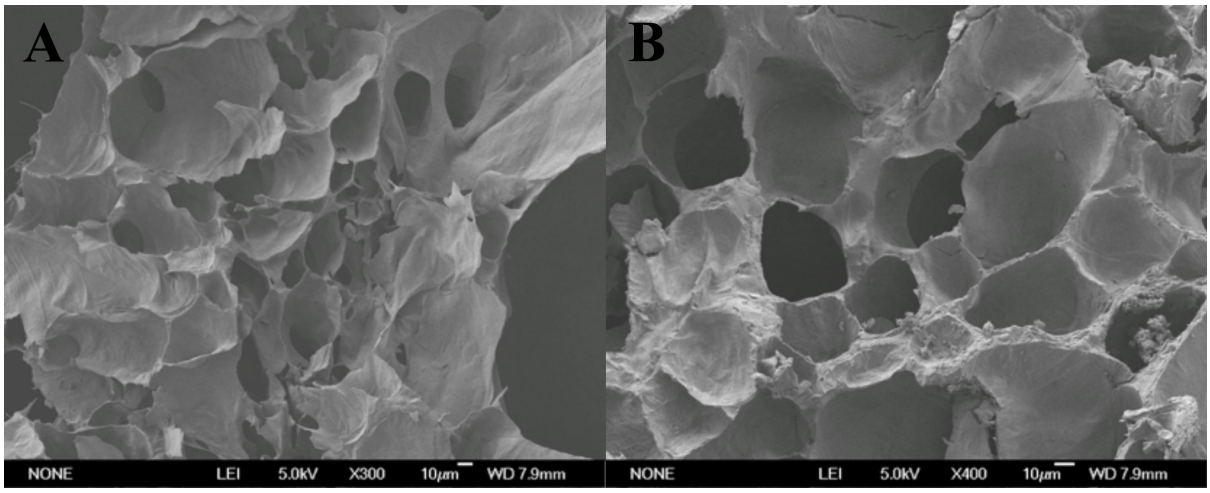


Fig. 22. SEM images of gel state HTCC hydrogel.

The product was characterized by an infrared (IR) spectrometer. As Figure 22 shows, the IR spectrogram of chitosan molecule had strong stretch vibration absorption spectra of O-H and N-H around $3450\ cm^{-1}$. The absorption spectra corresponded to the hydroxyl group and ammonia group of the N-deacetylglucosamine unit. There was also a absorption peak of ammonia group around $1610\ cm^{-1}$. Since the DD of the chitosan molecule used was 95%, there were three characteristic absorption bands of amide (NH-C=O), at the position of $1657\ cm^{-1}$ (amide I), $1551\ cm^{-1}$ (amide II) and $1323\ cm^{-1}$ (amide III). After the ammonia group was substituted, the magnitude of the characteristic absorption band at 1610 cm^{-1} decreased. The bending vibration absorption band of C-H of $-CH_3$ decreased at 1490 cm^{-1}, and the magnitude of the saturated C-H band at 2800 cm^{-1} increased. The reason why this occurred is that the induction of quaternary ammonium group introduced $-CH_3$ as well. Besides, the bending vibration absorption band of the O-H of the hydroxyl group at 1030 cm^{-1} and 1074 cm^{-1} was also strengthened. This demonstrated that the hydroxypropyltrimethyl ammonium chloride was linked to the ammonia group of chitosan.

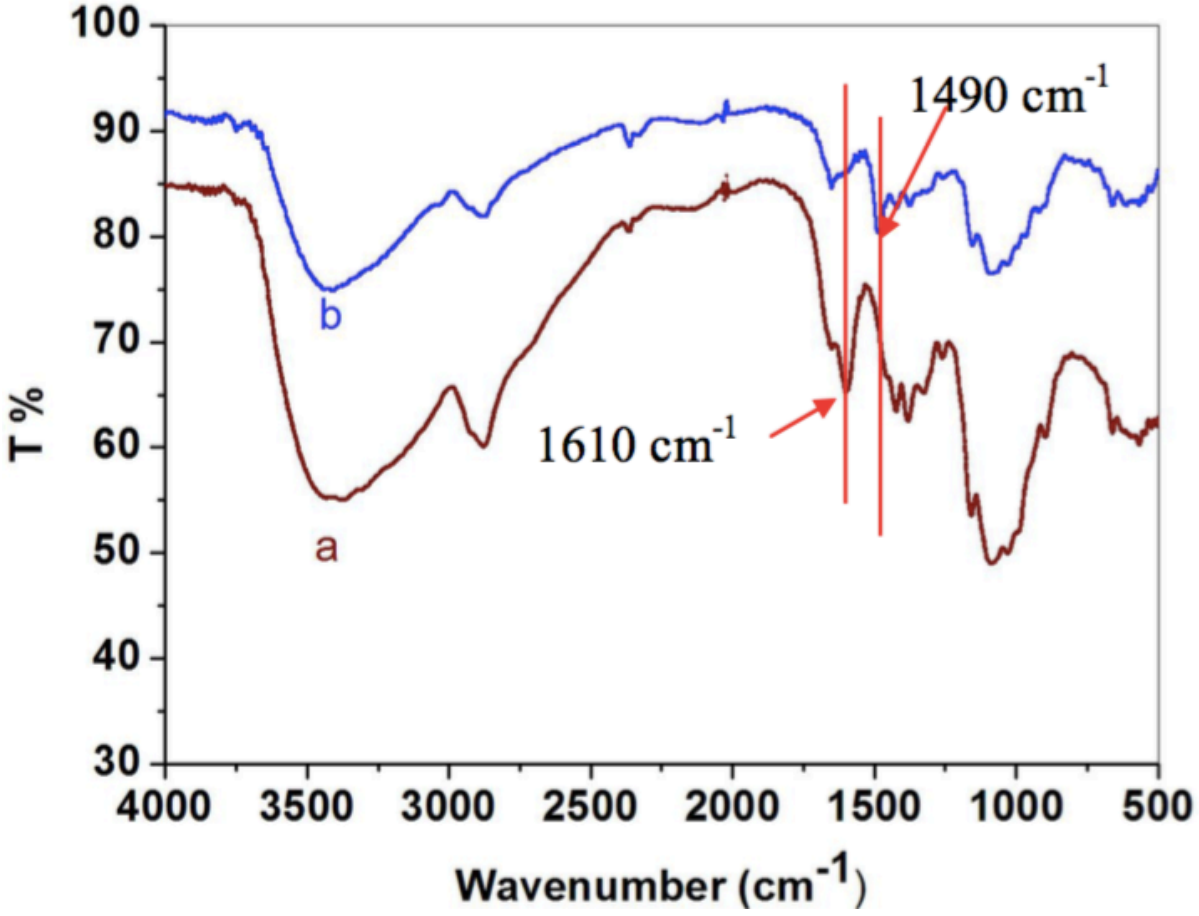


Fig. 23. IR spectrum of chitosan (a) and HTCC (b).

3.2 HTCC Hydrogel with the Optimized Quaternization Degree

The quaternary ammonization can improve the water solubility of chitosan, and make it capable of dissolving in weak acid solution. However, to eliminate the influences of the solution, reactants with same concentration and concentrated lactic acid (0.3M) were used to ensure that HTCC hydrogel with different QD can completely dissolve. The Same technique was applied to synthesize the HTCC hydrogel with different QD.

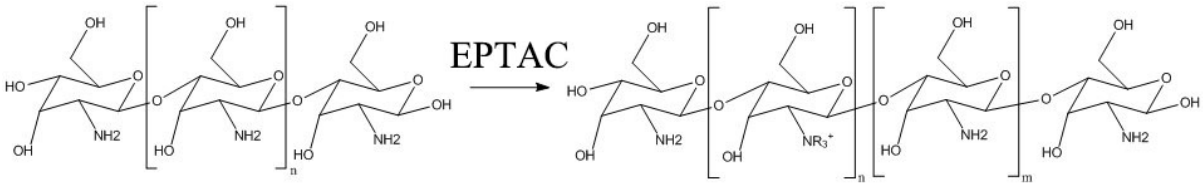


Fig. 24. Synthesize of HTCC with different QD.

The gelation time and viscosity were measured by a rheometer. It could be known from Table 2 that as the QD of HTCC hydrogel increased, the viscosity decreased so that the gelation time was prolonged. As the temperature in nasal cavity is approximately 37 ℃, and the gelation time need to be limited in a small range around 10 min. The viscosity of 0% group was too high, of 60% and 80% group were too low with inappropriate gelation time, while their gelation temperature were not appropriate at 37 ℃, 21% and 41% group was selected for further experiments.

QD	Viscosity/Pa·s	Gelation time (min)
0%	10.2 ± 2.7	16.7 ± 5.3
21%	9.2 ± 4.3	8.9 ± 3.8
41%	2.5 ± 0.4	9.8 ± 4.1
60%	1.3 ± 0.3	27.9 ± 6.9
80%	0.7 ± 0.1	65.3 ± 6.9

Table. 2. Viscosity and gelation time of HTCC hydrogel with various QD.

The reason why this occurred was that the higher the QD was, the stronger the cationic characteristic of the material was. Therefore, the solubility of this material was enhanced in acidic solution; the electrostatic repulsive-force was strengthened; the cross-linked chitosan backbone became more stretched, resulted in the reduction of its viscosity. Besides, the hydrophilicity of this cationic material was appropriate, hence the gel phase was hard to be formed through hydrophobic interaction when the temperature rises. As a result, the gelation time at 37 ˚C was prolonged as the QD of HTCC hydrogel increased.

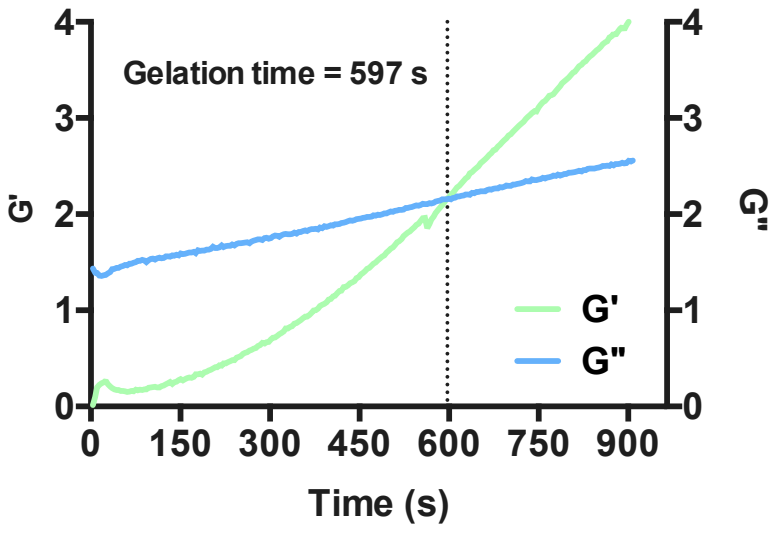


Fig. 25. The of gelation time of HTCC/GP system.

3.3 Antigen Deposition and Retention in Nasal Cavity

Fluorescence probe Cy7-SE was applied to estimate the retention time of spilt H3N2 antigen in nasal cavity. The 21%, 41% QD hydrogel-Ag and PBS-Ag were administrated intranasally. According to Figure 24, there was only about 13% of fluorescence labeled antigen 2 h after the PBS/antigen was administrated. On the contrary, strong signal of fluorescence labeled antigen was detected 2 h after the mice were treated with hydrogen/antigen. Moreover, for at least 4 h, quantitative date provided by the Fluorescence probe Cy7-SE showed that about 40% antigens remained in nasal cavity of 41% HTCC-Ag. However, no signal of fluorescence labeled antigen delivered by PBS remained in nasal cavity, demonstrating significant dominance of HTCC-Ag delivery system.

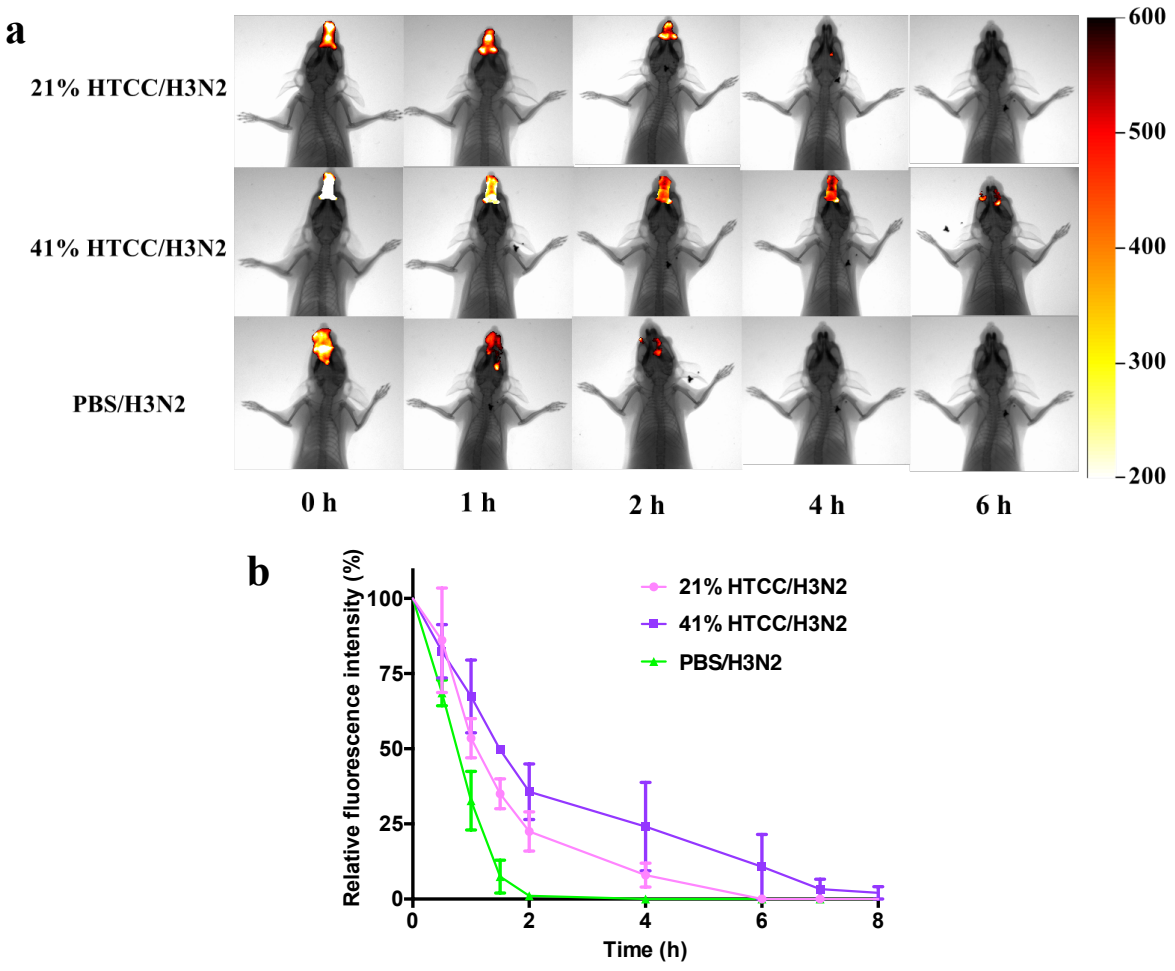


Fig. 26. Illustration of antigen residence time in nasal cavity (a) and nasal antigen single decrease (b).

The thermal sensitivity of HTCC hydrogel is probably the main reason why the antigen retention was prolonged. The hydrogel/antigen mixture was transferred to gel state at body temperature after it was administrated. The tight conjunction of epithelial cells was opened while the antigen was sent into the nasal tissues. Meanwhile, the mucociliary clearance frequency was significantly reduced, resulting in the reduction of mucociliary clearance period. In addition, since the mucosal surface was negatively charged, the positive charges of HTCC are capable of providing strong mucoadhesion to the hydrogel. Hence, with these excellent characteristics, HTCC hydrogel can perpetuate the residence time of antigens to a large extent.

3.4 Mucosal Penetration of Antigens

Franz diffusional cells were used to simulate the effect of antigens passing through the mucosa quantitatively. In this research, nasal mucosa of mice was placed on the Franz diffusional cells while the HTCC hydrogel was added to the upper layer of the mucosa to proceed infiltration, which simulates the process of the H3N2 antigen entering the inner layer of nasal mucosa. According to Figure 27, the hydrogel groups had shown their significant dominate of infiltration capacity of antigens in the first 30 min. Compare to the 3% of infiltration of PBS group, the 41% hydrogel group had already had 14% of antigen that penetrated the mucosa. In 5 h, more than 30% of antigen passed through the mucosa of 41% hydrogel group, while only less than 8% of antigen of PBS group entered the receptor fluid. Hence, the optimization of QD of chitosan quaternary ammonium salt had been accomplished. The the outstanding features of 41% hydrogel-Ag group had been exhibited, therefore, it could be used for further animal experiments. The reason why the HTCC have such significant superiority is that the cationic feature of this material can help to open the tight conjunction of epithelial cells. By introducing a quaternary ammonium group, the cationic characteristic of chitosan was largely improved. The positive charge effect of HTCC hydrogel is capable of increasing the permeability of epithelial cells, so that the antigens can penetrate the mucosa. However, the PBS does not have this feature, so that the amount antigen that passed through the mucosa of PBS group was relatively small.

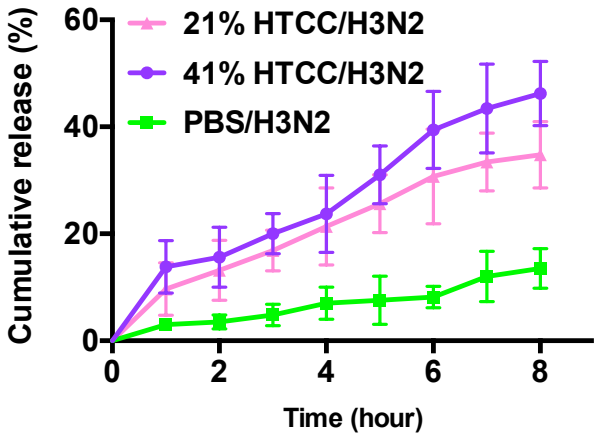


Fig. 27. In vitro simulation of antigen penetrating through nasal mucosa.

3.5 Mucosal and Systemic Antibody Secretion

Intranasal immunization is capable of inducing mucosal immune responses, which play an important role in defending the organism. As the lymphocytes relate to nasal mucosal immunization are stimulated in nasal cavity, they enter lymph and blood circulation, and are brought to all parts of the organism. This ensures the mucosa tissue of the organism can form a net of immunization responses, which is known as common mucosal immune system. Both systemic and mucosal immunization can be induced. Since the strategy system established has the dominances of longer antigen residence time, strong capacity of facilitating antigens to penetrate through the mucosa, it is anticipated that the hydrogel also harbored the potential to simultaneous orchestrate systemic and mucosal immune activation. Immunoglobulin A (IgA) is the main responding molecule of mucosal responses of the respiratory tract. Recently, researches about IgA have become more and more significant [39]. The IgA is a critical immune factor on the surface of mucosa.

Figure 28 exhibits the titer level of mice's nasal lavage after being administrated with the different delivery system including: H3N2/Alum formulation via intramuscular injection (which is the commercial formulation that served as positive control of systemic immune activation); H3N2/IM: Fluidic H3N2 alone via intramuscular injection; HTCC/IN: HTCC/H3N2 formulation via intranasal administration; H3N2/IN: Fluidic H3N2 alone via intranasal administration; PBS: Negative control.

Results showed that intranasal immunization could induce mucosa-specific IgA antibody, while injection-vaccination hardly induce mucosa-specific IgA (titer <20), as same as the blank group. Figure 26 also demonstrated that simply delivering H3N2 antigen could induce IgA antibody, which was more than the ones induced by most of the other delivery system including Alum/IM. The IgA induced by HTCC/IN was 24 times of PBS, and 12 times of Alum/IM.

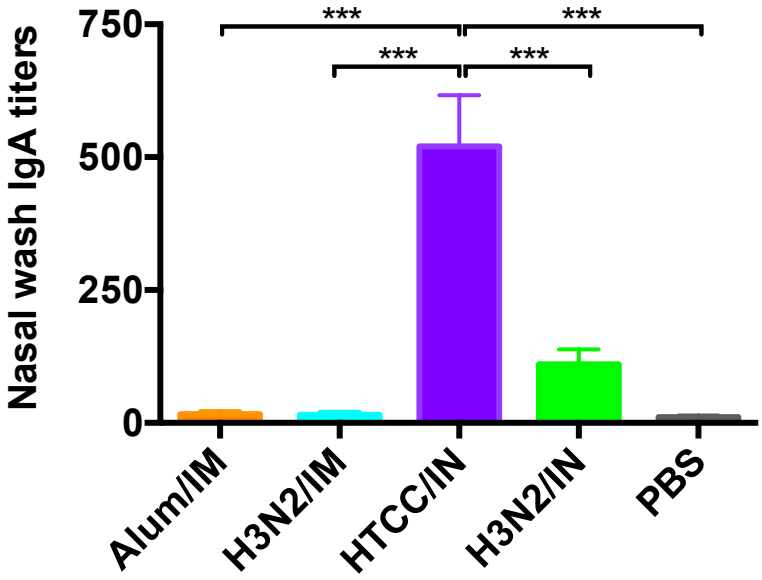


Fig. 28. IgA secretion.

The results of IgG induction, which illustrated the secretion of numerous amount of antibody produced by systemic immunity. The HTCC/IN group showed the strong activation of IgG (titer≈277), while the blank group exhibited almost no sigh of IgG induction (titer=4). Though the IgG induced by Alum/IM was also favorable, the toxicity of the Alum adjuvant had strongly confined the application of Alum/IM while this adjuvant failed to activate mucosal immune engagement. In comparison, the IgG induced by HTCC/IN was 70 times of PBS, and 1.2 times of Alum/IM. Hence, the effect of HTCC/IN is significantly potent.

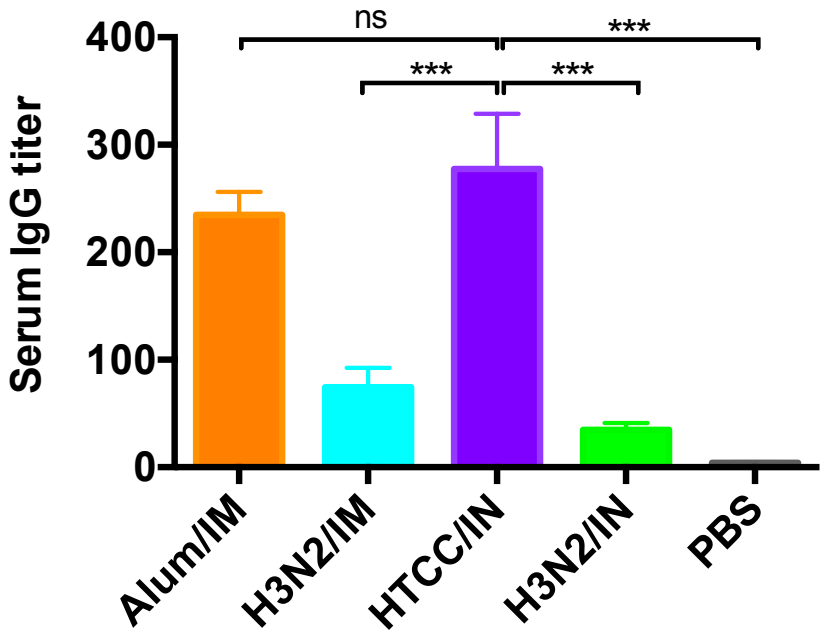


Fig. 29. IgG secretion.

Research has shown that chitosan is capable of stimulating epithelial cells through mucosal epithelium [40]. The permanent stimulation effect of HTCC hydrogel to the epithelial cells might activate the induction of IgA. In a word, the HTCC hydrogel stimulated significantly strong IgA activation, which may provide strong protection against influenza diseases.

4 Conclusion

A potent intranasal vaccination delivery system which can eliminate most limitations of parenteral vaccination can be synthesized by this method efficiently. The HTCC hydrogel based vaccine can be administrated effortlessly while the effect of HTCC-Ag vaccine has the characteristics of: Potent antigen delivery at mucosal sites; Long antigen retention; Robust systemic and mucosal immune activations. The delivery system was applied to delivery H3N2 spilt antigen, and animal experiments showed that the HTCC-Ag preparations can induce IgA effectively. Based on the previous result, vivo imaging was applied, and the results illustrated that the antigen residence time in nasal cavity was remarkably prolonged. Contrast with PBS based intranasal delivery system, the antigen residence time was improved by 4 times. In comparison with H3N2/IN which is the normal form of intranasal vaccination, the HTCC/IN system induced 8 times of IgA which demonstrates the intensity of mucosal immunization and 5 times of IgG which illustrates the intensity of systemic immunization. Based on these magnificent results, the booming future of HTCC hydrogel delivery system can be envisaged.

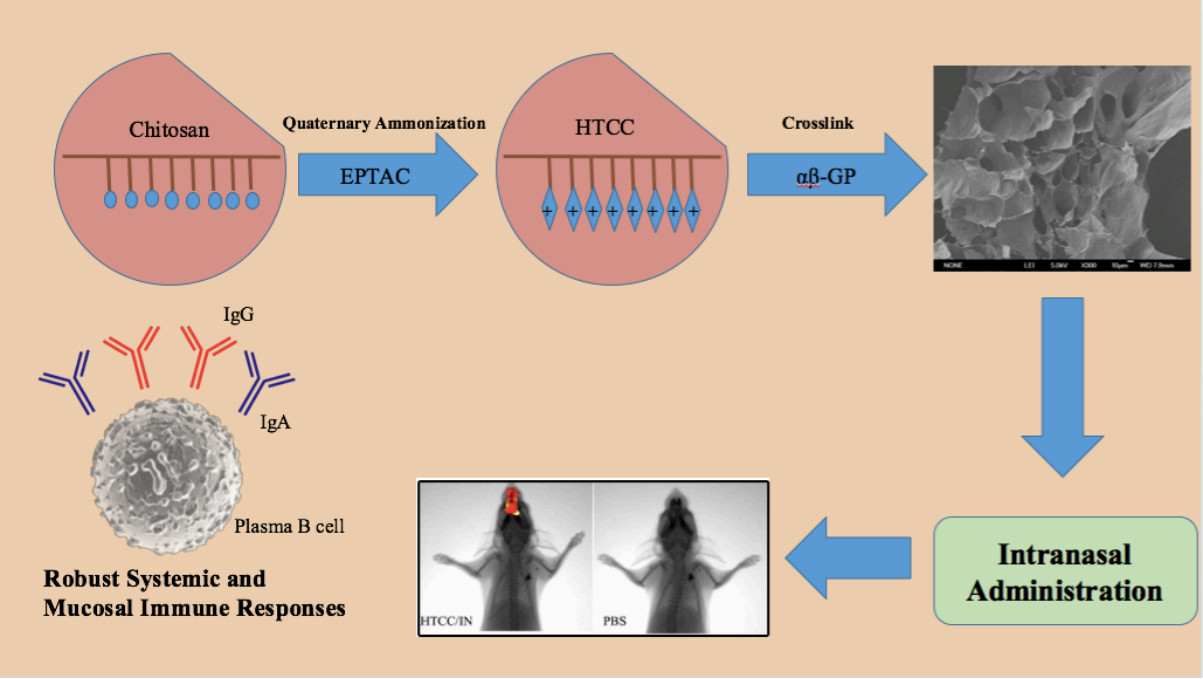


Fig. 30. Scheme of Mode of Action of Intranasal Vaccination based on Chitosan Hydrogel.

As intranasal vaccination is capable of inducing immune activation at distant mucosal sites, it can be applied as the delivery system of HPV and HIV vaccines. Moreover, not only can this delivery system be used for vaccine delivery, but also for other medications including psychotropic medications, anesthetics, insulin, etc. The HTCC hydrogel system is stable, and easy to be administrated, so that it can be used in rural areas with poor medical environment.

Despite of the bright future of this delivery system, problems may occur through its development. Though HTCC hydrogel exhibits significant potentiality of intranasal delivery system, further researches need to be done for HTCC-Ag vaccination's clinical applications. This research was proposed to treat the H3N2 influenza in Hong Kong recently. However, none of the chitosan ramifications based vaccines have been included in a licensed vaccine product, hence, lots of efforts need to be made. For instance, to improve the hydrogel delivery system so that it is able to hold different types of antigens which are capable of inducing cross-immunity. The industrialized production of the HTCC hydrogel will also be a notable issue.

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