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# Nasal vaccination with r4M2e.HSP70c antigen encapsulated into N-trimethyl chitosan (TMC) nanoparticulate systems: Preparation and immunogenicity in a mouse model

Mehran Dabaghian [a,b], Ali Mohammad Latifi [a], Majid Tebianian [b], Hamid NajmiNejad [c], Seyyed Mahmoud Ebrahimi [a,*]

[a] Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, P.O. Box 14155-3651, Tehran, Iran
[b] Department of Biotechnology, Razi Vaccine and Serum Research Institute (RVSRI), P.O. Box 31975/148, Karaj, Tehran, Iran
[c] Yazd University of Medical Sciences and Health Services, Department of Genetics and Molecular Medicine, Yazd, Iran

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ABSTRACT

In this study, the potential of N-trimethyl chitosan (TMC) nanoparticles as a carrier system for the nasal delivery of the r4M2e.HSP70c, as an M2e-based universal recombinant influenza virus vaccine candidate, was investigated in mice. The anti-M2e specific cellular and humoral immune responses were assessed and the protective efficacy against a 90% lethal dose (LD90) of influenza A/PR/8/34 (H1N1) in a mice model was evaluated.

Our results showed that the intranasal immunization of mice with r4M2e.HSP70c+TMC rather than the control groups, r4M2e+TMC, r4M2e and PBS (Phosphate buffer saline), significantly elevated both longevity and serum level of the total M2e-specific IgG antibody with a significant shift in the IgG2a/IgG1 ratio toward IgG2a, induced a Th1 skewed humoral and cellular immune responses, increased IFN-γ, IgG, and IgA in the bronchoalveolar lavage fluid (BALF), and promoted the proliferation of peripheral blood lymphocytes with lower morbidity and mortality rate against viral challenge.

In conclusion, based on evidence to our finding, nasal vaccination with r4M2e.HSP70c antigen encapsulated into N-Trimethyl Chitosan (TMC) nanoparticulate system showed to induce a long lasting M2e-specific humoral and cellular immune responses and also provided full protection against a 90% lethal dose (LD90) of the influenza virus A/PR/8/34 (H1N1). It seems, protective immunity following intranasal administration of r4M2e could be resulted by the cooperation of both adjuvants, TMC and HSP70c.



## 1. Introduction

The mucosal tissues of the upper respiratory tract are the main portal entry of influenza virus, and the mucosal immune system provides the first line of defense against this pathogen [1]. So, the intra-nasal route of vaccination could lead to enhanced mucosal immune responses [2]. Up to now, most commercially licensed vaccines against influenza infection have been designed for parenteral administration excluding FluMist Nasovac, which is a live-attenuated influenza vaccine inoculated intranasally [3].

All commercially available influenza vaccines are based on the immunodominant antigens, hemagglutinin and neuraminidase (HA, NA) [4]. However, these proteins are highly variable across the strains; therefore, these vaccines induce a subtype-specific immune response and are unable to provide protection against different influenza variants or strains challenge [5,6]. Hence, development of new influenza vaccines with the ability to provide cross-clade protection is very tempting. The extracellular domain of M2 (M2e) has 24 amino acid residues and is highly conserved among human influenza A strains [7]. To increase its low immunogenicity different studies have utilized various adjuvant formulations [8–11].

As immune adjuvants are crucial part of viral vaccine construction, particularly those that are designed to elicit T cell responses. Heat shock protein 70 (HSP70), as a nature and Th1 cytokine-like adjuvant, was reported having capacity to induce potent CD8+ cytotoxic T lymphocyte (CTL) and IFN-$_\gamma$ responses against their associated antigenic peptides [12–15]. Thus, it seems linkage of M2e to HSP70 can represent a potential approach for increasing the potency of vaccines, especially intending to stimulate CD8+ CTL.

* Corresponding author.
*E-mail address:* smebrahimi@shirazu.ac.ir (S.M. Ebrahimi).

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We previously demonstrated that four tandem repeats of influenza A virus M2e genetically fused to the C-terminus of the Mycobacterium tuberculosis HSP70 (mHSP70c), as a recombinant 4M2e.HSP70c fusion protein, provided full protection against the lethal dose of multiple influenza A virus strains in mice and chicken via the intramuscular route [16,17].

Encouraged by these findings and due to the advantages of mucosal vaccination, we have attempted to encapsulate r4M2e. HSP70c in a suitable antigen carrier which can be given intranasally as a mucosal vaccine.

Potential of self-administration, lower costs, decreased the risk of transmission of infection by needle, and suitability for mass vaccination are some benefits of mucosal vaccination over conventional vaccination [18]. Furthermore, regular ways of administering vaccines, intramuscular or intradermal, lead to secretion of serum immunoglobulin G (IgG) but they are meager stimulators of local immune response and secretory IgA (S-IgA) production at the mucosal sites [4].

The main antibody of the mucosal surfaces, S-IgA, has been shown to have a great potential in the intracellular and extracellular neutralization of influenza virus [19]. Moreover, anti-HA S-IgA has been found to elicit cross-protective immunity more effectively than serum IgG [20]. However, to date, scant attention has been devoted to the extent and nature of anti-M2e S-IgA role in protecting the body against influenza infection.

Moreover, respiratory tract is also the site of entry for influenza virus [21–23]. Nasal-associated lymphoid tissue (NALT) represents mucosal immunity in nasal mucosa and therefore plays an important role in the induction of local and systemic immune responses to antigens and microorganisms [24]. NALT as a part of mucosa-associated lymphoid tissue (MALT) is composed of lymphocytes, follicle-associated epithelium (FAE), and epithelial Micro fold cells(M cells) [24].

In order to induce local and systemic immune responses, inhaled antigens and microorganisms from the airways need to gain access to the sub epithelial lymphoid tissues [21].

But, due to multiple factors including the epithelial barrier, mucociliary clearance, and enzymatic and chemical destruction, soluble antigens are usually not sufficient to mount the protective immune responses following intranasal administration [25].

Hence, to circumvent these restrictions, encapsulating antigens into suitable carriers is favorable [26]. Chitosan and its derivatives share properties which make it desirable to be used as an antigen carrier [27]. Chitosan (CS) is a non-toxic, biodegradable, and biocompatible amino polysaccharide, which is obtained by *N*-deacetylation of chitin, the natural polymer extracted from crustacean shells or the mycelium of fungi [28].

Despite these promising properties, chitosan is soluble in acidic PH and its solubility in neutral and alkaline pH is low and it may change its structure; therefore, sub epithelial space with physiologic PH (neutral) may be harmful to chitosan stability [20]. To improve solubility, a methylated derivative of chitosan, *N*, *N*, *N*-trimethyl chitosan (TMC), has been developed [29]. TMC has the capacity to induce broad antibody response [30,31], promotes the maturation of DCs [26,32] and offers higher solubility in physiological pH than chitosan [33]. Furthermore, TMC has the potential to interact with the mucin on mucosal surface and prolongs the nasal residence time of the encapsulated antigen on mucosal sites which is commonly known as the mucoadhesion property of TMC [27,34]. Given these premises, TMC has been widely exploited as a nasal carrier for antigens, e.g. tetanus toxoid [35], HBsAg [36].

Here, an effort has been made to formulate r4M2e.HSP70c recombinant protein with N-TMC nanoparticles via ionotropic gelation method by Tripolyphosphate (TPP) for Influenza A intranasal vaccination. Then the potential of N-trimethyl chitosan (TMC) nanoparticles as a carrier system for the nasal delivery of the r4M2e.HSP70c, as an M2e-based universal recombinant influenza vaccine candidate, was immunologically investigated in 6–8 weeks old female Balb/C mice.

## 2. Materials and methods

### 2.1. Peptide

Recombinant 4 M2e (r4M2e) and 4 M2e.HSP70c (r4M2e. HSP70c) fusion protein with the M2e consensus sequence of human influenza A virus that circulated before the pandemic of 2009 (H1N1, H2N1, H3N2; amino acid sequence: SLLTEVETPIRNEWGCRCNDSSD) was expressed, purified and prepared as a vaccine candidate from an E. coli expression system as previously described [17]. Recombinant proteins were tested for the presence of endotoxin by E-TOXATETM (limulus amebocyte lysate) test kit (Sigma, USA). Endotoxin-levels were below 0.05 EU/ml.

### 2.2. Nanoparticle preparation

The formulation of r4M2e.HSP70c+TMC and r4M2e+TMC nanoparticles were carried out based on ionotropic gelation of *N*, *N*, *N*-trimethyl chitosan (TMC), as previously described [37]. Briefly, different concentrations (0.5, 1, 1.5, and 2 mg/ml) of Trimethyl Chitosan (kindly provided by dr. Maryam Iman, Baqiatallah university of medical science [38] were prepared in distilled water and filtered through 0.45 mm membrane filters. The r4M2e. HSP70c or r4M2e recombinant protein (10 mg/ml) was added to sodium tripolyphosphate (TPP) solution (1mg/ml). Thereafter, 200 µl of r4M2e.HSP70c+TPP or r4M2e+TPP solutions (1mg/ml) were added drop wise to various TMC solution while stirring at room temperature for 1 h. Aliquots of 1 ml of the resulting antigen- loaded TMC NPs suspensions were centrifuged for 15 min at 10,000*g* in the presence of glycerin. To obtain antigen loaded nanoparticles for physicochemical analysis and i.n. immunization, the supernatants were decanted and the pellet were suspended in PBS (Phosphate buffer saline).

### 2.3. Characterization

#### 2.3.1. Morphology

The size and zeta potential of the synthesized nanoparticles were determined by dynamic light scattering (DLS) using the zeta sizer SZ3000 (Malvern instrument, Worcestershire, UK.) [39].

Moreover, the morphology of the NPs was studied using transmission electron microscopy (TEM) according to a reported procedure [40]. The nanoparticle aqueous dispersion (50 µl) was dropped on the grid and air dried. Then nanoparticles were stained with a solution of phosphotungstic acid at 1% (w/v) (pH 7.3) for 5 min and then grids were washed and left to dry. Electron micrographs were acquired using an electron microscope JEOL 1400 MET operating at 80 kV.

#### 2.3.2. Loading efficacy

To determine the amount of entrapped protein in the NPs, difference between the total amount added to the loading solution and the amount of non-entrapped protein remaining in the supernatant was calculated. The level of r4M2e.HSP70c or r4M2e peptides in the supernatants were measured by the BCA protein assay [41]. Aliquots of the resulting NPs suspension were centrifuged for 20 min at 18,000*g* and 10 °C and the supernatants were then separated from the NPs. The encapsulation efficacy (EE) of r4M2e.HSP70c or r4M2e loaded N-TMC NPs were calculated from the following equations:

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