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Antigen-loaded polymeric hybrid micelles elicit strong mucosal and systemic immune responses after intranasal administration

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ABSTRACT

Increasing attention has been paid to nasal delivery. Subunit vaccines based on antigenic proteins or polypeptides offer good safety. However, lack of delivery efficiency, particularly for nasal immunization, is a big issue. Here we designed a highly tunable polymeric hybrid micelle (PHM) system offering good vaccine efficacy after nasal administration. PHMs are formulated from two amphiphilic diblock copolymers, polycaprolactone-polyethylenimine (PCL-PEI) and polycaprolactone-polyethyleneglycol (PCL-PEG), the ratio of which determines PHM physicochemical properties. Citraconic anhydride-modified ovalbumin (Cit-OVA), as model antigen, was incorporated into PHMs via electrostatic interaction, giving antigen-loaded micelles of around 150 nm in size. Their surface characteristics which are found closely related to their in vivo kinetics can be modulated by adjusting the mass ratio of PCL-PEG and PCL-PEI. PHM/Cit-OVA complexes containing PCL-PEI and PCL-PEG in a 1:1 mass ratio induced strong immune responses in nasal mucosa and serum in vivo without causing obvious toxicity, and Cit-OVA was efficiently internalized by dendritic cells. These results demonstrate the promise of this multifunctional polymeric delivery system for nasal vaccination.

1. Introduction

Successful as some vaccination strategies may be, the development of vaccine still faces great challenges, due to the viral diversity and high mutation rate of infectious diseases like influenza, HIV/AIDS and cancer. Compared with inactivated or attenuated whole-cell vaccine, vaccines containing a fragment of attenuated pathogen such as a protein or polypeptide may offer greater safety. These so called subunit vaccines represent a new generation of vaccine candidates. Meanwhile, as the primary method for protecting against these diseases up-to-date, vaccination via intramuscular, subcutaneous or intradermal injection plays an outstanding role in evoking systemic immune responses, but may trigger adverse reactions that reduce patient compliance, such as pain, nausea, headache, fever and allergic reactions [1–4], and it cannot induce sufficient mucosal antibodies, which are essential for prevention of diseases infected through mucosa. To reduce such adverse reactions, an increasing number of studies have sought to develop effective vaccines that can be delivered nasally.

Nasal immunization has drawn people's attention owing to its capability in inducing mucosal immune responses and good compliance. The U.S. Food and Drug Administration approved the first intranasal vaccine called FluMist® that works against influenza in 2003 [5]. Nasal administration is an attractive route for immunization because it does not require needles and syringes, so it can be used for large-scale immunization. The nasal mucosa is easily accessible and highly vascularized, and it contains abundant T and B cells, dendritic cells, macrophages and lymphoid tissues such as nasal-associated lymphoid tissue (NALT) [6,7]. Nasal administration can induce both mucosal and systemic immune responses, more importantly, it is necessary for vaccine against infectious pathogens that gain nasal access to the body and could elicit neutralizing antibodies at the site of entry to prevent their invasion. Therefore, intranasal vaccination with subunit vaccines is of great immune potential.

Despite its advantages, intranasal vaccination presents several challenges: enzymes in the nasal cavity may degrade the antigen carried by the vaccine, and cilia movement may shorten vaccine intranasal residence time, resulting in poor antigen uptake. Most importantly, subunit vaccines on their own often elicit only weak immune responses so they are not transported efficiently to adjacent lymph nodes [8]. Therefore, nasal immunization usually requires the use of vaccine adjuvants or delivery systems to protect the antigen immunogenicity and increase the antigen delivery efficiency.

Several polymers have been used to prepare nasally administered vaccines, including methacrylate [9], oligomannose [10], alginate [11], and chitosan and its derivatives [12]. A Suitably derivatized polyethylenimine (PEI) appears to induce stronger antiviral immune

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responses than polymers mentioned above, and diverse PEI forms work as potent mucosal adjuvants for viral glycoprotein antigens [13]. PEI is difficult to degrade, and its cytotoxicity which correlates with its molecular size and number of charges [14] requires attention. This highlights the need for less toxic PEI derivatives that provide good adjuvant activity, so further studies are needed to synthesize new polymers with lower toxicity [15].

In previous work from our laboratory, we modified PEI using cyclodextrin to form a cationic bioadhesive material with a reduced charge density on the polyamine backbone, resulting in reduced cytotoxicity [16]. What's more, polymeric hybrid micelle system (PHMs) has been successfully applied in the delivery of small molecule nucleic acid for melanoma therapy after intravenous injection [17]. We also showed that PEI could be conjugated to polycaprolactone (PCL) to obtain amphiphilic PCL-PEI, which can self-assemble into micelles. Meanwhile, different mass ratio of polycaprolactone-polyethyleneglycol (PCL-PEG) was added to form PHMs that could adjust the positive charges of PCL-PEI, so PHM system with highly tunable property could be used as potent vaccine carrier for nasal administration. Here we developed PHMs containing PCL-PEI and PCL-PEG as a novel vaccine system for nasal administration. PHMs were prepared in different mass ratios of PCL-PEI and PCL-PEG to examine the relationship between surficial properties of the micelles and the nasal retention time, cellular uptake and lymph node transport.

We loaded PHMs with the model antigen ovalbumin (OVA) after modifying it with citraconic anhydride (Cit-OVA). OVA, as a widely used model antigen in vaccine development, are negatively charged at physiological condition. Due to the limited charge densities, OVA complexes inefficiently with cationic polymers such as PCL-PEI via electrostatic interactions. Modifying OVA with citraconic anhydride (Cit-OVA) could decrease its pI value [18], making electrostatic encapsulation more efficient.

In our study, we report the design and synthesis of PCL-PEI and PCL-PEG polymer and the fabrication of a polymeric hybrid micelle system-based intranasal delivery platform for vaccine. We tried to systematically examine how their physico-chemical properties such as surface charges and PEG density effect on their ability to cellular uptake and immune effect. The in vitro experiments of Cit-OVA loaded PHMs with different surface electrical properties were carried out in DC 2.4 cells and Madin-Darby canine kidney (MDCK) cells as a model for nasal epithelium. The ability of these PHMs to deliver antigen to lymph nodes and elicit immune responses was investigated in mice immunized intranasally.

2. Materials and methods

2.1. Materials and animals

Branched polyethylenimines (PEI) with molecular weights of 2000 (PEI2k), ε-caprolactone and stannous (II) octoate (SnOct), Citraconic anhydride and OVA (albumin from chicken egg white) were obtained from Sigma-Aldrich (USA). 4-Nitrophenyl chloroformate (NPC) was obtained from Aladdin (China). ε-caprolactone was purified by vacuum distillation from calcium hydride (CaH_2). Benzyl alcohol and dichloroform were distilled before use. Methoxy-poly (ethylene glycol) (mPEG) with the molecular of 5000 (mPEG 5k-NH_2) was purchased from JianKai (Beijing, China). All organic solvents used for study were of analytical grade. CCK-8 Cell Proliferation Assay Kit was obtained from KeyGEN BioTECH.

BALB/c (female) mice 6–8 weeks old were obtained from Institute of laboratory animals of Sichuan academy of medical sciences & Sichuan provincial people's hospital (Chengdu, China) and housed in a specific pathogen free, light-cycled and temperature-controlled facility. All experiments were approved by the Institutional Animal Care and Ethics Committee of Sichuan University.

2.2. Synthesis and characterization of PCL-PEI and PCL-PEG

The synthesis routing of PCL-PEI and PCL-PEG was according to Hanmei Li et al. [19]. The scheme consists of three steps including the synthesis of poly(ε-caprolactone) (PCL-OH), the activation of PCL-OH with NPC and the coupling of NPC-activated PCL with PEI or mPEG 5k. In brief, Monohydroxy-terminated PCL-OH was first synthesized by the ring-opening polymerization of ε-caprolactone using benzyl alcohol as an initiator and SnOct as a catalyst (0.1% moles of ε-caprolactone). Then PCL-OH was activated with NPC to afford PCL-NPC. PCL-PEI was obtained by reacting PCL-NPC with PEI 2k. PCL-PEG was synthesized by reacting PCL-NPC with mPEG 5k-NH_2 (Scheme S1). The chemical structure of PCL-PEI and PCL-PEG were confirmed using ^{1}H NMR spectrometry. The molecular of PCL-PEG and PCL-PEI were determined using gel permeation chromatography (GPC).

2.3. Modification of OVA

To make OVA more negatively charged at physiological pH and therefore help it complex more efficiently with cationic PEI derivatives, we modified the primary amino groups in OVA with citraconic anhydride.

OVA (102.1 mg) was dissolved in 0.5 M $NaHCO_3$ buffer (pH 9.0, 20 ml). The solution was stirred at 4°for 30 min, and citraconic anhydride (41.4 mg) was added slowly. After stirring for 2 h, the mixture was purified by dialysis for 72 h in a bag with a molecular weight cut-off of 2 kDa. The final product (Cit-OVA) was obtained as white powder after lyophilization. The zeta potentials of OVA and Cit-OVA (500 μg/ml) were measured using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Efficiency of the citraconic anhydride modification was assessed based on ion exchange chromatography on an AKTA chromatography system (GE Health Care, USA).

2.4. Preparation of PHMs and PHM/Cit-OVA

Empty PHMs were prepared by solvent exchange. Briefly, copolymer mixture (10 mg), composed of PCL-PEI and PCL-PEG mixed in mass ratios of 1:0, 3:1, 1:1, or 1:3, were dissolved in 2 ml tetrahydrofuran (THF). Then 10 ml of 5 mM HEPES buffer was added slowly to the THF solution, and the mixture was moderately stirred at room temperature for 15 min. Finally the mixture was rotary-evaporated under vacuum at 37 °C to remove organic solvents, leaving unloaded PHMs.

To investigate how the proportion of cationic segments affects PHM properties, we prepared PHMs with different mass ratios of PCL-PEI and PCL-PEG; the corresponding PHMs varied from weight content of 25% PCL-PEI (PHM25) to 100% PCL-PEI (PHM100). As expected, the net cationic charge of PHMs can be modulated simply by changing the mass ratios of PCL-PEI: PCL-PEG.

The PHM formulations were then loaded with OVA or Cit-OVA at different concentrations in order to optimize final complexes size, polydispersity index (PDI) and encapsulation efficiency. The desired amount of OVA or Cit-OVA solution was then mixed with an equal volume of PHM solution, the mixture was vortexed gently for 10 s, and incubated at room temperature for 30 min to allow particles to form. Size distribution and zeta potential of the resulting loaded PHMs were measured using a Zetasizer Nano ZS90. The presence of Cit-OVA inside PHMs was confirmed using the BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Encapsulation efficiency was determined by comparing the amount of Cit-OVA remaining in the supernatant after centrifuging PHM/Cit-OVA solution (13,225 g, 10 min) with the amount of Cit-OVA used to prepare the PHM/Cit-OVA.

2.5. Cell culture

Murine dendritic cells DC 2.4 and Madin-Darby canine kidney