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Cationic micelle based vaccine induced potent humoral immune response through enhancing antigen uptake and formation of germinal center

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ABSTRACT

Nanoparticles have been proven to be an effective vaccine delivery system that can boost immune responses to subunit vaccines. Herein, we developed and characterized a cationic polymeric polyethylene glycol$_{2000}$-poly ϵ-caprolactone$_{2000}$-polyethylenimine$_{2000}$ (mPEG$_{2000}$-PCL$_{2000}$-g-PEI$_{2000}$) micelle as a potent vaccine delivery system to boost the immune response *in vivo*. The micelles that we developed exhibited great antigen-loading capability and minimal cytotoxicity *in vitro*. Meanwhile, micelles facilitated OVA antigen uptake by dendritic cells both *in vitro* and *in vivo*. More importantly, a micelle-formulated OVA vaccine could significantly promote anti-OVA antibody production by 190-fold and potently enhance T cell proliferation and the secretion of IL-5 and IFN-γ. We attributed these effects to its ability to promote antigen uptake, antigen deposition, and germinal center formation. In conclusion, the mPEG$_{2000}$-PCL$_{2000}$-PEI$_{2000}$ micelle that we developed has potential as potent vaccine delivery system to induce Th2 immune response.

1. Introduction

Vaccination remains one of the most effective strategies for preventing infectious diseases and saves millions of lives each year [1,2]. Traditional vaccines composed of live or attenuated pathogens have been proven efficient at evoking robust immune responses and controlling several types of infections [3]. Nevertheless, safety issues related to these vaccines have seriously limited their clinical applications. To improve safety, a new generation of vaccines (subunit vaccines) composed of recombinant or purified proteins have been developed to be safe and effective vaccines [4]. Unfortunately, most of these recombinant antigens are poorly immunogenic and therefore require adjuvants or carriers to boost the immune response. To date, alum (aluminum hydroxide), an adjuvant that has been clinically approved by FDA, mainly induces humoral immune responses, but fails to evoke potent $CD4^+$ Th1 cell or $CD8^+$ cytotoxic T cell immune responses [5], which are very important for preventing or controlling infections. Additionally, previous studies also claimed that alum can induce inflammation at injection sites [6]. In past several decades, particulate (nano- or micro-particles)-formulated vaccines have emerged as one of the most promising strategies to tune and boost immune responses [7,8]. Notably, nanoparticles have received considerable attention for inclusion in vaccine formulations because of the following favorable characteristics. First, nanoparticle-formulated antigens with a particle size in the range of 0.1–1 μm are more robustly recognized and processed by dendritic cells (DCs) compared with soluble antigens [9]. Several previous studies have shown that formulating antigen into nanoparticles can increase antigen uptake by DCs and enhance antigen cross-presentation, thereby enhancing both the strength and quality of T cell immune responses. Second, nanoparticles with the capability to co-deliver antigen and adjuvants (such as PIC, CpG, or R847) to the same DC can enable in the generation of effective immune responses [10–12]. Third, nanoparticle surfaces can be decorated with different molecules, such as specific target ligands or cell penetrating peptides, allowing them to achieve bioactive targeting [13]. Finally, the remarkable *in vitro* and *in vivo* biodegradable and biocompatible properties of polymeric nanoparticles make such particles well suitable for *in vivo* investigations [14], which is the foremost requirement for vaccines.

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More recently, polymeric micelles, as a type of nanoparticles, have been extensively studied for vaccine delivery system. For examples, Eby et al. reported that OVA-conjugated PDS-PEG-bl-PPS micelles target APCs in lymph nodes and the spleen. In immunized mice, micelles formulated with CpG enhanced antigen-specific immune responses better than a soluble OVA/CpG mixture, as indicated by enhanced anti-OVA titers, greater IFN-γ production, and reduced IL-10 levels [15]. Boudier et al. developed a novel pH-sensitive micelle based on $PMAA_{2100}$-b-POE_{5000}/poly-L-lysine, which induced dendritic cell (DC) maturation and greatly improved the secretion of TNF-α and IL-6, supporting its potential application in immunotherapy [16]. All these results indicated that using polymeric micelles as an antigen carrier might have great potential application in a vaccine delivery system.

Previously, we successfully developed a series of PEG-PLL-PLLeu cationic polypeptide micelles with a positive charge that significantly improved gene transfection efficiency both *in vitro* and *in vivo* [17]. In a vaccine delivery system, PEG-PLL-PLLeu micelles (PEG-$PLLZ_{20}$-$PLLeu_{40}$ and PEG-$PLLZ_{30}$-$PLLeu_{40}$) significantly induced DC maturation and enhanced antigen presentation *in vitro*, and also robustly improved OVA-specific immune responses [18]. More recently, we reported that cationic mPEG-PCL-PEI micelles significantly improved *in vitro* gene transfection efficiency in B16-F10 and 293T cells and inhibited tumor growth in both B16F10 subcutaneous tumor and lung metastasis models [19,20]. Herein, we hypothesized that the cationic $mPEG_{2000}$-PCL_{2000}-g-PEI_{2000} micelles also spontaneously encapsulated ovalbumin (OVA) and had potential as a potent vaccine delivery system. The particle size, surface zeta potential, OVA encapsulation efficiency, and *in vitro* cytotoxicity on L929 cells and splenocytes were characterized. Moreover, the effect of micelle-formulation on T cell proliferation, antigen-specific antibody production, and germinal center formation were further investigated *in vivo*.

2. Materials and methods

2.1. Materials

Stananous octoate [$Sn(Oct)_2$], mPEG (molecular weight 2000 or 5000), PEI, and acryloyl chloride were obtained from Sigma–Aldrich (St. Louis, MO, USA). Triethylamine, chloroform, dichloromethane, and petroleum ether were purchased from Chengdu KeLong Chemicals (Chengdou, China). Recombinant mouse GM-CSF and IL-4 were obtained from Peprotech (Rocky Hill, NJ, USA). Mouse IL-5 and IFN-γ ELISA kits were purchased from Biolegend (San Diego, CA, USA). Fluorochrome-labeled anti-mouse monoclonal antibodies (PE-CD11c, FITC-CD40, FITC-CD83, Alexa-Fluor 488-GL7, and Brilliant Violet 421-B220) were purchased from Biolegend. EndoFit Ovalbumin (endotoxins <1 EU/mg) was purchased from InvivoGen (San Diego, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2b, or IgG2c were obtained from Southern Biotechnologies (Birmingham, AL, USA). The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-ltetrazolium bromide (MTT) was obtained from Beyotime Institute Biotechnology (Shanghai, China). Alum was purchased from Pierce Biotechnology (Rockford, IL, USA). C57BL/6J mice that were 6–8 weeks of age were obtained from Wenzhou Medical University Laboratory Animal Center (Wenzhou, China), and were maintained at our institutional animal care facility.

2.2. Micelle development and characterization

Briefly, $mPEG_{2000}$-PCL_{2000}-g-PEI_{2000} polymers were successfully synthesized using a previously reported method [19]. First, $mPEG_{2000}$-PCL_{2000} was synthesized by ring-opening polymerization of ϵ-CL and mPEG at 140 °C using $SnOct_2$ as a catalyst. Next, the crude product was dissolved in dichloromethane and precipitated three times with petroleum ether, before being oven dried for 48 h to yield the $mPEG_{2000}$-PCL_{2000} block polymer. Thereafter, 0.5 mol $mPEG_{2000}$-PCL_{2000} block polymer that was obtained as dissolved into anhydrous dichloromethane, followed by the dropwise addition of 0.6 mol acryloyl chloride to the reaction at 40 °C for 6 h to obtain —C═C terminated $mPEG_{2000}$-PCL_{2000} block polymers. Finally, $mPEG_{2000}$-PCL_{2000}-g-PEI_{2000} block polymer was synthesized by coupling the PEI_{2000} onto —C═C terminated $mPEG_{2000}$-PCL_{2000} block polymers *via* a Michael addition reaction in chloroform, similar to a method published previously [19].

As a consequence of the amphiphilic property of the $mPEG_{2000}$-PCL_{2000}-g-PEI_{2000} block polymer, the obtained $mPEG_{2000}$-PCL_{2000}-g-PEI_{2000} block copolymer self-assembled into micelles (2 mg/ml) in aqueous solution as the temperature was increased up to 50 °C. After cooling to ambient temperature, cationic $mPEG_{2000}$- PCL_{2000}-PEI_{2000} (2 mg/ml) was gently mixed with an equal volume of OVA water solution (0.4 mg/ml) for 10 min to obtain OVA-loaded $mPEG_{2000}$-PCL_{2000}-PEI_{2000} micelles. The size and zeta-potential of blank micelles or OVA-loaded $mPEG_{2000}$-PCL_{2000}-PEI_{2000} micelles (micelle-vaccines) were detected by laser diffraction (zeta plus zeta potential analyzer, Brookhaven Instruments Corp., Holtsville, NY, USA). To determinate the OVA encapsulation efficiency (EE) and loading capacity (LC) of the micelle, micelle-vaccines were ultracentrifuged at 100,000 × *g* for 30 min, and the amount of unbound OVA in the supernatant was measured using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The EE and LC were calculated using the following equations: EE = (total protein − unbound protein)/total protein × 100% [21]; LC = (total protein − unbound protein)/(total dry weight of micelle vaccine) × 100% [22].

The stability of micelle/OVA formulations as a function with time was determined by measuring the size changes of the vaccine suspended in different medium at 37 °C. To investigate to the release behavior of OVA from the complexes of micelle/OVA, the micelle/OVA formulation was prepared as previously described, and incubated at 37 °C under mild agitation (120 rpm/min). At different time points, 500 μl aliquot of release medium was removed and centrifuged at 100,000 × *g* for 30 min. Blank micelle was also used as blank control. The amount of protein released present in the supernatant were measured by using a Bio-Rad Protein Assay Kit.

2.3. Bone marrow dendritic cell (BMDC) culture and stimulation

Mouse BMDCs were generated as described previously [23]. Briefly, bone marrow cells were isolated from C57BL/6J mouse femur and tibia, and then were cultured *ex vivo* in X-*vivo* 15 medium (Lanza, MD, USA) containing GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) at 37 °C for 6 days to yield immature DCs. Immature DCs were treated with medium (Med), empty micelles (20 μg/ml), or CpG (10 μg/ml) for 24 h. The DCs were harvested and incubated with FITC-anti-mouse-CD40 and PE-anti-mouse-CD83 monoclonal antibodies on ice for 30 min. The expression levels of CD40 and CD83 on BMDCs were detected by flow cytometry (FACSCalibur, BD, San Jose, CA, USA).

2.4. Antigen uptake by BMDCs and the intracellular localization of antigen in BMDCs

Immature DCs were cultured with free FITC-OVA (2.5 μg/ml, BD Biosciences) or micelle-formulated FITC-OVA at 37 °C for 30 min. After washing with PBS, the uptake of OVA-FITC by BMDCs was determined by detecting OVA-FITC-positive cells using flow cytometry. BMDCs (1×10^6 cells/ml) were cultured with free OVA-FITC or