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Cationic polypeptide micelle-based antigen delivery system: A simple and robust adjuvant to improve vaccine efficacy

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ABSTRACT

Modern subunit vaccines with purified or recombinant antigens are important alternatives to the traditional vaccines. However, there remains a big challenge to elicit potent antibody production and CD8 T cell response. Nanoparticle-based antigen delivery systems have emerged as an innovative strategy to improve the efficacy of subunit vaccines. The present study reported self-assembled cationic micelles based on poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) hybrid polypeptides as a simple and potent vaccine delivery system. The results showed that the PEG-PLL-PLLeu micelles spontaneously encapsulated OVA antigens with great loading capacity (LC = 55%) and stability. More importantly, the polypeptide micelle formulations robustly enhanced vaccine-induced antibody production by 70–90 fold, which could be due to their capability of inducing dendritic cell maturation, enhancing antigen uptake and presentation, as well as promoting germinal center formation. Furthermore, the polypeptide micelles could simultaneously encapsulate OVA and polyriboinosinic: polyribocytidylic acid (PIC), a TLR3 agonist, to synergistically augment tumor specific cytotoxic-T-lymphocyte (CTL) response. Hence, the polypeptide micelle-based antigen delivery system could be a robust adjuvant to enhance vaccine-induced immune responses.

1. Introduction

With the development of vaccine technology, subunit vaccines based on recombinant antigens or purified pathogen subunits have emerged as an important alternative to the traditional vaccines. However, their weak immunogenicity remains the major limitation for future clinical applications [1]. Immunological adjuvants are a group of compounds that have been applied as an effective strategy to increase vaccine-induced humoral and cellular immune responses [2]. Current adjuvants include two major categories: immune potentiators, such as bacteria toxins and Toll-like receptor (TLR) agonists, which directly stimulate immune cells, and delivery systems that facilitate antigen uptake and presentation [3]. More recently, the combination of delivery systems and immunopotentiators has been reported to synergistically enhance vaccine efficacy, therefore being considered as the next generation adjuvants [1,3,4].

A successful adjuvant candidate should be safe, biodegradable, and capable of co-delivering both antigens and immune potentiators [5]. In the past decade, nanoparticle-based vaccine delivery platforms, such as liposomes and biodegradable polymeric nanoparticles, have been reported as an innovative strategy for vaccine and adjuvant development [6,7]. Previous studies have shown that nanoparticles could effectively encapsulate antigens and facilitate antigen uptake by antigen presenting cells both *in vitro* and *in vivo* [8,9]. Moreover, nanoparticles could be engineered to stimulate dendritic cell (DC) maturation and antigen presentation [10,11], thereby enhancing vaccine-induced humoral and cellular immune responses [12,13]. In addition, nanoparticles have been shown to augment the immunopotency of immunopotentiators [14–16].

Polymeric micelles are core-shell nanoparticles generated by spontaneous self-assembly of individual amphiphilic polymeric molecules [17,18]. Till date, polymeric micelles have been demonstrated as an effective platform for drug and gene delivery due to the great stability and biocompatibility as well as high loading capacity [19,20]. More recent studies showed that micelles could also act as an effective antigen delivery system. Boudier et al. prepared pH-sensitive polymethacrylic acid-b-polyethylene oxide (PMAA2100-b-POE5000)/poly-L-lysine (PLL)

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micelles for antigen peptide delivery. The *in vitro* study showed that these polyion complex micelles not only effectively loaded antigen peptides, but also facilitated their uptake and release in DCs. Moreover, these polyion complex micelles significantly promoted DC maturation, indicating their immunostimulatory effect [21,22]. Jain et al. compared the immunogenicity of hepatitis B surface antigen (HBsAg) formulated by poly-lactic acid (PLA) or PEG-PLA-PEG. The results showed that PEG-PLA-PEG micelles were much more potent than PLA nanoparticles to enhance and prolong HBsAg-induced mucosal antibody responses through both intranasal and oral immunization [23,24]. These data suggested polymeric micelles as a potential adjuvant candidate for vaccine development. Unfortunately, the preparation of micelle-formulated vaccines remains complicated, and their immunopotency need to be further optimized.

We previously described self-assembled cationic micelles based on poly(ethylene glycol)-b-poly (L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) hybrid polypeptides as a potent gene vector to enhance DNA transfection both *in vitro* and *in vivo* [25]. In the present study, the PEG-PLL-PLLeu micelles were demonstrated as simple and potent vaccine delivery systems that could spontaneously encapsulate ovalbumin (OVA) with high loading capacity and excellent particle stability. We investigated the immunoregulatory effects of the polypeptide micelles both *in vitro* and *in vivo*. The results showed that the polypeptide micelles significantly promoted DC maturation as well as antigen uptake and presentation. Moreover, the polypeptide micelles dramatically enhanced vaccine-elicited germinal center (GC) formation and antibody production *in vivo*. Furthermore, the polypeptide micelles could simultaneously encapsulate OVA and polyriboinosinic: polyribocytidylic acid (PIC), a TLR3 agonist, to synergistically augment tumor-specific cytotoxic T-lymphocyte (CTL) response.

2. Material and methods

2.1. Materials

O-(2-aminoethyl)-O′-(2-methyl) polyethylene glycol (PEG-NH_2, Mw = 2000) was purchased from Sigma-Aldrich (MA, USA). L-leucine (LLeu) and ε-benzyloxycarbonyl-L-lysine (LLZ) were purchased from GL Biochem Ltd. (Shanghai, China) and recrystallized from ethyl acetate three times. Triphosgene was purchased from J&K Scientific Ltd. (China) and recrystallized from diethyl ether before use. N-carboxyanhydride of ε-benzyloxycarbonyl-L-lysine (LLZ-NCA) and N-carboxyanhydride of L-leucine (LLeu-NCA) were prepared as previously reported [28]. Hydrogen bromide 33 wt.% solution in glacial acetic acid was purchased from ACROS Organics (China). Ovalbumin (OVA, grade V) was purchased from Sigma (MO, USA). Recombinant mouse GM-CSF, IL-4 and IL-2 were purchased from Peprotech (Rocky Hill, USA). Mouse IL-6, IL-5, and IFN-γ ELISA kits were obtained from Biolegend (CA, USA). Fluorochrome-labeled anti-mouse monoclonal antibodies (CD8, CD4, CD40, CCR7, CD86) were purchased from eBioscience (CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgG2b was purchased from Southern Biotechnologies (AL, USA). The OVA-transfected B16 melanoma cell line (B16-OVA) was kindly provided by Dr. Chunfeng Qu (Chinese Academy of Medical Sciences & Peking Union Medical College). Six- to eight-week-old C57BL/6J mice were obtained from Guangdong Province Laboratory Animal Center (Guangzhou, China), and maintained in the institutional animal care facility. All animal protocols were approved by Institutional Animal Care and Usage Committee of Shenzhen Institutes of Advanced Technology.

2.2. Micelles synthesis and characterization

The PEG-PLL-PLLeu copolymers were synthesized by ring opening polymerization of N-carboxyanhydride (NCA) as previously reported [25]. PEG-PLLZ copolymers first were synthesized by ring opening polymerization of LLZ-NCA using PEG-NH2 as initiator. Next, the PEG-PLLZ-PLLeu copolymers were synthesized by further ring opening polymerization of LLeu-NCA initiated by PEG-PLLZ. By changing the feed molar ratio of PEG-NH2 to LLZ-NCA and LLeu-NCA (20:40 and 30:40), two PEG-PLLZ-PLLeu copolymers (PEG-$PLLZ_{20}$-$PLLeu_{40}$ and PEG-$PLLZ_{30}$-$PLLeu_{40}$) were synthesized (Supplementary material, Table s-1). PEG-PLL_{20}-$PLLeu_{40}$ (20:40) and PEG-PLL_{30}-$PLLeu_{40}$ (30:40) copolymers were then obtained by the deprotection of PEG-PLLZ-PLLeu, followed by the dialysis against 0.1 wt.% ammonia solution and then distilled water for 48 h. The final products were collected by freeze-dry, and the structure of the copolymers was analyzed by ^{1}H NMR spectra using Bruker 400 MHz nuclear magnetic resonance instrument.

To prepare micelle-formulated nanovaccines, 20:40 and 30:40 polypeptides were dissolved in UltraPure water (1 mg/ml) followed by sonication for 1 h to form cationic polypeptide micelles. The resulted micelle solution was then gently mixed with equal volume of OVA solution (0.2 mg/ml) in UltraPure water at 4 °C for 15 min to allow the encapsulation of OVA by the micelles. In some experiments, PIC (a TLR3 agonist) was incorporated into the nanovaccines to generate polypeptide micelle-PIC (PMP) vaccines (micelles: OVA: PIC = 10: 2: 1, w/w/w, Table 1). The particle size and ζ potential of the micelles or nanovaccines were determined by photon correlation spectroscopy (PCS) using Nano-ZS ZEN3600 (Malvern Instruments).

To determine the stability of nanoparticles, the micelles or nanovaccines were suspended in different medium at 4 °C, and the particle size was measured at different time points as indicated. To determine the OVA encapsulation efficiency (EE) and loading capacity (LC) of the micelles, nanoparticles were ultra-centrifuged at 100,000 ×*g* for 30 min, and the amount of unbound OVA in supernatant was determined using Bio-Rad Protein Assay Kit (BioRad, CA, USA). The EE and LC calculated using the following equations: EE = (total protein − unbound protein) / total protein × 100% [26]; LC = (total protein − unbound protein) / total dry weight of nanovaccine × 100% [27].

2.3. Agarose electrophoretic mobility shift assays (AEMSA) of PMP vaccine

Polypeptide micelle-PIC (PMP) vaccines were prepared by gently mixing the polypeptide micelles, OVA and PIC in water (micelles: OVA: PIC = 10:2:1, w/w/w). After incubation at room temperature for 15 min, the soluble PIC or PMP vaccines were run on 1% agarose in TAE buffer at 90 V for 30 min, and PIC was visualized by Gel Red (Biotium, CA, USA) staining.

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

Mouse BMDCs were generated as described previously [28]. In brief, bone marrow cells were isolated from C57BL/6J mouse femur and tibia, and then cultured in X-vivo 15 medium (Lanza, MD, USA) supplemented with GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) at 37 °C for 6 days to acquire immature DC. The immature DCs were

Table 1
Characterization of polypeptide micelles and micelle vaccines.

Compositions (mass ratio)	Compositions (mass ratio)	Size[a] (nm)	ζ-potential[a] (mV)	EE[b]
20:40	20:40 Polypeptides	98 ± 2	61 ± 5	–
20:40 + OVA	Polypeptides: OVA (5:1)	121 ± 3	35 ± 2	>95%
20:40 + OVA + PIC	Polypeptides: OVA: PIC (5:1:0.5)	133 ± 4	33 ± 2	>95%
30:40	30:40 Polypeptides	95 ± 1	60 ± 4	–
30:40 + OVA	Polypeptides: OVA (5:1)	120 ± 4	34 ± 2	>95%
30:40 + OVA + PIC	Polypeptides: OVA: PIC (5:1:0.5)	128 ± 5	33 ± 2	>95%

[a] The size and ζ potential were measured in water.
[b] EE (encapsulation efficiency) = (total protein − unbound protein) / total protein × 100%.