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Tailoring polymeric hybrid micelles with lymph node targeting ability to improve the potency of cancer vaccines

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ABSTRACT

It has been widely accepted that lymph nodes (LNs) are critical targets of cancer vaccines and particles sized between 10 and 100 nm with a neutral or negative surface charge are preferred for lymphatic transfer after subcutaneous or intradermal injection. However their limited uptake by antigen presenting cells (APCs) and inadequate retention within LNs undoubtedly restrains their strength on activating T cell immunity. Here, we address this issue by tailoring the physicochemical properties of polymeric hybrid micelles (HMs), which are self-assembled from two amphiphilic diblock copolymers, poly-(ethylene glycol) phosphorethanolamine (PEG-PE) and polyethylenimine-stearic acid conjugate (PSA) via hydrophobic and electrostatic interactions. We successfully encapsulate melanoma antigen peptide Trp2 and Toll-like receptor-9 (TLR-9) agonist CpG ODN into HMs with a size of sub-30 nm. Their surface characteristics which are found closely related to their *in vivo* kinetics can be modulated by simply adjusting the molar ratio of PEG-PE and PSA. Our results demonstrated the optimized HMs with an equal mol of PEG-PE and PSA can potently target proximal LNs where their cargos are efficiently internalized by DCs. Furthermore, HMs mediated Trp2/CpG delivery system greatly expands antigen specific cytotoxic T lymphocytes (CTLs) and offers a strong anti-tumor effect in a lung metastatic melanoma model.

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1. Introduction

A major challenge in the development of subunit vaccines is the efficient delivery of antigen/adjuvant to secondary lymphoid organs, since antigen presentation and initiation of T cell-mediated immune responses occur primarily at these locations [1]. In lymph nodes, antigens are captured by dendritic cells (DCs), processed within these cells, and then presented on their surface in complex with major histocompatibility complex (MHC) I molecules. This surface complex of MHC-antigen is recognized by T cells, which then initiate cellular immune responses [2]. Thus it is also critical for antigen uptake by DCs after they transport to lymph node to augment required immune response.

Various particles have been developed to encapsulate or conjugate vaccine antigens [2–4] and they can protect antigen and adjuvant from degradation, improve their delivery to DCs and lymph nodes, and prolong their contact with the immune system [5–7]. Furthermore, actively targeting these particles to DCs has proven effective at activating cellular immunity by the particles to recognize surface receptors specifically on DCs, such as mannose [8], Fc receptor(FcR) [9], and DEC-205 [4,10]. Another particularly attractive approach is to target particles directly to draining lymph node (DLN) by modulating their physico-chemical characteristics [11–13], including shape, charge and size [3]. DLNs contain large numbers of immature DCs and T cells, supporting quite efficient internalization of vaccine antigen by substantial DLN-resident DCs, followed by presentation to T cells [14]. Indeed, injection of vaccine particles directly into DLNs has been shown to elicit robust immune responses [6,15]. Particles with a small size around 10–100 nm tend to target LN more efficiently than large size nanoparticles [3,16]. PEGylation of particles was also an alternative successful approach to enhance lymphatic trafficking and reduced retention at the injection site [12,17,18], since the PEG coating can reduces the interaction of particles with collagen fibres and glycosaminoglycans in the interstitium and hydrophilic particles can move through the interstitial water channels more effectively than hydrophobic

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carriers [16].

However, PEGylated modification of particles in a chemical-covalent way might make particles damaged and their biologic cargos especially proteins would be inactivated at some extent. What's more, PEGylation can reduce retention of particles in DLNs and antigen uptake by DCs [19]. It can also raise toxicity issues; for example, increasing the PEG ratio in PEGylated 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) led to greater liver tropism, where the cationic particle may cause systemic toxicity [20]. Using an alternative to PEG, we reported the self-assembly of cationic micelles from the amphiphilic copolymer polyethylenimine-stearic acid conjugate (PSA) [21]. By subcutaneous injection of cationic PSA micelles near the inguinal lymph nodes of mice, results suggested that the positive charge of the particles facilitated retention for more than 3 days in DLNs as well as cellular trafficking in DCs. On the other hand, the positive charge also seemed to hamper the particles' ability to migrate to DLNs far from the injection site. This was probably due to strong interactions with negatively charged proteins or cells around the injection site [22]. These findings highlight the challenge of optimizing the properties of particle-based antigen delivery systems to simultaneously maximize DLN targeting and uptake by dendritic cells while also minimizing unwanted interactions with other cells and tissues.

To address these challenges, we tried to combine PSA with another amphiphilic diblock copolymer, poly-(ethylene glycol) phosphoethanolamine (PEG-PE), to generate polymeric hybrid micelles (HMs) in a self-assembled manner and expected to exploit their respective advantages while avoiding their disadvantages so that to activate T cell immunity. PEG-PE spontaneously aggregates into micelles in water, and these micelles are often used to carry small hydrophobic drug [23]. The hydrophilic and hydrophobic segments in PEG-PE and PSA are of similar sizes but opposite charges, suggesting that the HMs should stably assemble via hydrophobic and electrostatic interactions (Fig. 1). This hybridization ingeniously enabled PEG introduced into cationic micelles in a physical way without any complicate chemical conjugation. The physico-chemical properties of these hybrid micelles can be adjusted simply by changing the mol ratio of the two diblock copolymers, eliminating the need to alter the copolymer architecture.

Herein, we describe the design and synthesis of these HMs, and we tried to systematically examine how their physico-chemical properties effect on their ability to target DLNs and be taken up by DCs residing there. We loaded the micelles with melanoma antigen Trp2 and Toll-like receptor 9 (TLR-9) agonist CpG ODN that trigger an innate immune response involving production of pro-inflammatory and Th1 cytokines that promote CTL and activate $CD4^+$ Th1 T cells [24,25]. The ability of this co-delivery system to elicit antigen-specific CTL activity in mice as well as *in vivo* antitumor effect in lung metastatic melanoma inoculated in mice was finally investigated.

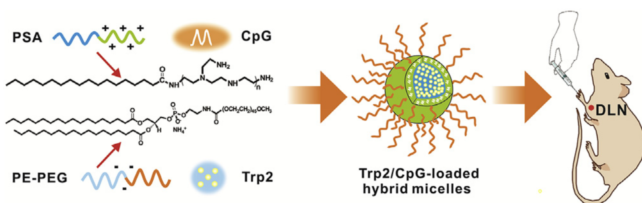

Fig. 1. Schematic representation of melanoma antigen peptide Trp2 and Toll like agonist CpG ODN co-delivered in hybrid micelles. Hybrid micelles were prepared by mixing cationic PSA with anionic PEG-PE, and then loaded with Trp2 by dialysis method. These Trp2-loaded micelles were simply mixed with CpG ODN to allow adsorption. The co-delivery system was injected into the forepaw of mice and assessed for its ability to migrate to the brachial DLN as well as to be up-taken by DCs and induce CTL activity.

2. Material and method

2.1. Materials

1,2-distearoyl-***sn***-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) and DSPE-PEG2000-NHS were purchased from Avanti Polar Lipids. 5′-SPO_3-CpG ODN_{1826} (5′-TCCATGACGTTCCTGACGTT-3′) and Texas Red or Cy5-labeled CpG ODN_{1826} were purchased from Sangon Biotech (Shanghai). $Trp2_{180-188}$ peptide (SVYDFFVWL) and FITC-labeled $Trp2_{180-188}$ were obtained from Kaijie Peptide Company (Chengdu, China). The fluorescent dye 1,1’-dioctadecyl-3,3,3′,3’-tetramethylindodicarbocyanine (DiD) perchlorate was obtained from Life Technologies (Carlsbad, CA, USA). 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) was obtained from Sigma-Aldrich.

2.1.1. Cells and animals

Murine melanoma cell line B16F10 (syngeneic with C57BL/6) and murine dendritic cell line DC 2.4 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). B16F10 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, Invitrogen, Carlsbad, CA) and DC 2.4 cells were maintained in RPMI-1640. In both cases, the medium was supplemented with 10% fetal bovine serum (Invitrogen), 100 U mL^{-1} penicillin (Invitrogen) and 100 μg mL^{-1} streptomycin (Invitrogen).

Female C57BL/6 mice 6–8 weeks old and BALB/c nude male mice 6–8 weeks old were obtained from the Laboratory Animal Center of Sichuan University (Chengdu, China). All animal experiments were approved by the Institutional Animal Care and Ethics Committee of Sichuan University.

2.2. Preparation and physico-chemical characterization of Trp2-loaded micelles

Trp2 peptide was assembled using a dialysis method into PEG-PE micelle (PPM), PSA micelle(PPM) or hybrid micelles(HMs) that had previously been prepared using the following mol ratios of PSA to PEG-PE 15:85 (HM15), 50:50 (HM50) and 85:15 (HM85). In all cases, the mass ratio of micelles to peptide was 4:1. The encapsulation efficiency (%) of Trp2 in micelles was determined using reverse-phase HPLC (1260 Infinity, Agilent Technologies, Wilmington, NC, USA).

The size distribution and zeta potential of Trp2-loaded micelles were analyzed using dynamic light scattering and electrophoretic light scattering. Measurements were collected on a photon correlation spectrometer equipped with a 50-mV laser (Zetasizer Nano ZS90, Malvern Instruments, UK). The surface morphology of Trp2-loaded HM50 (HM50-Trp2) and HM50 loaded with Trp2 and CpG (HM50-Trp2-CpG) was investigated by negative-stain transmission electron microscopy (H-600, Hitachi, Japan). Micelles (0.5 mg/mL) were allowed to adsorb onto a copper grid for 60 s, then the bulk solution was removed and the grid was stained for 30 s with phosphotungstic acid (1%, w/v). Excess dye was removed and the grids were analyzed.

2.3. Evaluation of micelle-mediated cargo delivery into cells

DiD-loaded PEG-PE micelle (PPM), PSA micelle (PSAM) or HMs were prepared by film hydration and then added to cultures of DC 2.4 cells in the absence of serum. After 1.5-h incubation, cells were washed and fixed with 4% paraformaldehyde, followed by staining

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