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NANOMEDICINE

Bioreducible alginate-poly(ethylenimine) nanogels as an antigen-delivery system robustly enhance vaccine-elicited humoral and cellular immune responses

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ABSTRACT

Although polysaccharide nanogels have emerged as a novel antigen delivery system for vaccine development, whether modulating the redox sensitivity of nanogels could improve vaccine efficacy remains unclear. In the present study, we generated bioreducible cationic alginate-polyethylenimine (PEI) nanogels as a novel vaccine delivery system. Briefly, nanogels were prepared by the electrostatic interaction of negatively charged alginate sodium with branched PEI_{2k}, followed by disulfide cross-linking to generate bioreducible nanogels (AP-SS). The AP-SS nanogels demonstrated great antigen-loading capacity and minimal cytotoxicity. The *in vitro* study showed that reducible AP-SS nanogels not only facilitated antigen uptake by mouse bone marrow dendritic cells (BMDCs), but also promoted intracellular antigen degradation and cytosolic release. Moreover, AP-SS nanogels significantly enhanced both MHC class I and II antigen presentation by BMDCs. Compared with the non-reducible nanogels, AP-SS nanogels more potently enhanced vaccine-induced antibody production and $CD8^+$ T cell-mediated tumor cell lysis. Hence, the bioreducible alginate-PEI nanogels could serve as a potent adjuvant to improve vaccine-elicited humoral and cellular immune responses.

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1. Introduction

Vaccination is not only the most cost-effective approach to prevent infection around the world, but also a novel therapeutic strategy to treat cancer and chronic infectious diseases [1]. The immune responses induced by vaccination mostly include two parts: the humoral immunity mediated by secreting antibodies, and the cellular immunity mediated by $CD8^+$ T cells and NK cells [1]. Although current vaccines can successfully induce protective antibody responses, it remains a big challenge to effectively evoke protective $CD8^+$ T cell responses essential for anti-viral, anti-tumor, and anti-parasite immunities [2]. Generating antigen-specific $CD8^+$ T cell immunity requires the presentation of antigenic peptides by MHC class I molecules (also called “cross presentation”), which unfortunately are poor for synthetic proteins or subunit antigens. Hence, an effective adjuvant must be applied to augment vaccine-triggered immune responses, especially $CD8^+$ T cell response [3]. Recently, vaccine delivery systems based on organic or inorganic nanoparticles have been reported as a novel strategy to improve vaccine efficacy [4,5]. In particular, nanoparticles prepared from biodegradable polymers are extremely of interest due to their great biocompatibility and effective clearance from the body [6–8]. Previous studies showed that nanoparticle-based vaccine delivery system prevented antigen degradation, increased antigen uptake by antigen presenting cells (APCs), and promoted dendritic cells (DCs) maturation [9]. Hence, nanoparticle-based vaccine delivery systems hold great potential in developing highly effective adjuvants with minimal adverse effects.

Nanogels are nanosized three-dimensional polymeric networks that can be prepared from either synthetic polymers, such as poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL), or natural polymers, such as polysaccharides [10]. Among them, polysaccharide-based nanogels appear to be very attractive due to their great biocompatibility and the abundance of raw materials [8,10,11]. Nochi et al. [12] developed cationic pullulan nanogels as an intranasal vaccine delivery system, which significantly enhanced vaccine-induced systemic and mucosal antibody production. Debache et al. [13] showed that intranasal or intraperitoneal immunization with chitosan nanogel-formulated NcPDI vaccines more effectively protected mice from *Neospora caninum* tachyzoites infection than free NcPDI did. These data suggested that engineered polysaccharide nanogels could be a promising tool to improve vaccine efficacy [14].

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The immunopotency of nanogels has been shown to be regulated by their physicochemical properties, such as surface charge, size, shape, and hydrophobicity [10]. More recent studies suggested that the efficiency of nanovaccines might be enhanced by promoting nanoparticle degradation and antigen release [15,16]. The bioreducible nanoparticles are stimuli-responsive nanoparticles prone to disassembly in the presence of reductants, such as γ-glutamyl-cysteinyl-glycine (GSH) and cysteine, and then quickly release their payloads [17,18]. The cytosol and cell nucleus are reducing conditions with GSH hundred fold higher than the extracellular fluid does. Moreover, the late endosomes also demonstrate a reducing environment (mostly caused by cysteine) essential for antigen processing [19]. The different redox potentials between the inside and outside of cells allow the bioreducible nanoparticles to effectively deliver drug to intracellular compartments, while maintaining a great stability in extracellular spaces [20]. The bioreducible nanoparticles have been reported as a potent tool to enhance gene transfection and anti-cancer effect of chemotherapeutic agents by promoting intracellular drug/gene release [21–23]. More recently, Hirosue et al. [16] reported that the conjugation of OVA peptides onto poly(propylene sulfide) nanoparticles with disulfide bonds more significantly enhanced MHC class I presentation than that with non-reducible bonds. However, whether modulating the redox sensitivity of nanogels could enhance vaccine-induced humoral and cellular immunity remains unclear.

In the present study, we prepared bioreducible cationic alginate-PEI nanogels for antigen delivery. The bioreducible nanogels demonstrated great antigen loading capacity and minimal cytotoxicity. We further evaluated their immunoregulatory effects both *in vitro* and *in vivo*. Compared with non-reducible nanogels, the bioreducible nanogels more dramatically enhanced vaccine-induced antibody production and $CD8^+$ T cell-mediated tumor cell lysis, which could be due to their strong capability of promoting intracellular antigen processing and cytosol release as well as MHC class I/II antigen presentation.

2. Materials and methods

2.1. Materials

Polyethylenimine (Mw = 2000, PEI_{2K}), alginate sodium (low viscosity, 100–300 cP), and suberic acid bis(N-hydroxysuccinimide ester) (DSS) were purchased from Sigma-Aldrich (MO, USA). The cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was purchased from Toronto Research Chemicals, Inc. Ovalbumin (OVA, grade V), aluminum hydroxide gel (Alum), and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich (MO, USA). DQ™ ovalbumin (DQ-OVA) and 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Invitrogen (CA, USA). Fluorescence-labeled anti-mouse CD4, CD8, CD40 and CD86 monoclonal antibodies were purchased from eBioscience (CA, USA). Recombinant mouse GM-CSF, IL-2 and IL-4 were obtained from PeproTech (NJ, USA). Mouse cytokine and IgG ELISA kits were purchased from BioLegend (CA, USA). CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit was purchased from Promega (WI, 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/6 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 the Institutional Animal Care and Usage Committee of Shenzhen Institutes of Advanced Technology.

2.2. Preparation and characterization of nanogels

Alginate-PEI nanogels were prepared by a two-step procedure. Firstly, alginate sodium and PEI_{2K} were dissolved in 0.05 M MES buffer solution (pH = 5.5) to generate alginate solution (1 mg/ml) and PEI_{2K} solution (1 mg/ml), respectively. Secondly, the alginate solution was added dropwise into the PEI_{2K} solution with magnetic stirring for 4 h at room temperature to allow the formation of AP nanogels. The unbound PEI_{2K} was removed by dialysis using a dialysis membrane (MW = 8000–14,000 Da) in distilled water for 3 days. Thirdly, the AP nanogels were cross-linked with DTSSP (dissolved in distilled water 1 mg/ml) or DSS (dissolved in DMSO, 20 mg/ml) with a mass ratio of AP to cross-linkers at 10:1 to generate bioreducible alginate-PEI-DTSSP nanogels (AP-SS) and non-reducible alginate-PEI-DSS nanogels (AP-CC), respectively. The reaction was carried out at room temperature overnight, followed by dialysis with a dialysis membrane (MW = 8000–14,000 Da) in distilled water for 3 days. The cross-linked nanogels were then filtered and stored at 4 °C for future use. The morphologies of nanogels were observed by transmission electron microscope (TEM) using a JEM-100CXII microscope operating at an acceleration voltage of 100 kV. The TEM samples were prepared by dropping the sample solution onto a copper grid precoated with a layer of Formvar film, and then stained by 1% (w/v) phosphotungstic acid solution (pH 6.4–7.0).

To prepare nanovaccines, OVA solution was treated using Detoxi-Gel Endotoxin Removal Gel (Thermo Scientific, IL, USA) to remove endotoxin (<0.1 EU/ml), then mixed with nanogels at different mass ratios as indicated. The mixture was incubated at 4 °C for 30 min to allow OVA encapsulation by nanogels. The particle size, PDI, and ζ potential of nanogels and nanogel-OVA vaccines were determined by photon correlation spectroscopy (PCS) using Nano-ZS ZEN3600 (Malvern Instruments) at 25 °C. To evaluate the stability of nanogels was suspended in ultrapure water at 4 °C for up to 8 weeks, and the particle size of nanogels was measured at different time points as indicated.

Protein encapsulation efficiency and loading capacity of different nanogels were determined as described previously [16]. Briefly, nanogels were mixed with OVA at different mass ratios at 4 °C for 30 min, followed by ultra-centrifugation at 100,000 $\times g$ for 30 min. The amount of unbound OVA in supernatants was determined using Bio-Rad BCA Protein Assay Kit (BioRad, CA, USA), and OVA encapsulation efficiency (EE) and loading capacity (LC) of nanogels were calculated using the following equation: EE = (total protein − unbound protein) / total protein × 100%, LC = (total protein − unbound protein) / total weight of nanovaccines × 100%.

2.3. In vitro cytotoxicity of nanogels

Mouse splenocytes were seeded in a 96-well plate at 2×10^5 cells/well in RPMI1640 medium with 10% FBS, and cultured with 10–100 μg/ml of nanogels at 37 °C for 24 h. The LDH activity in supernatants was quantified using Cytotox96 Non-Radioactive Cytotoxicity Assay Kit according to manufacturer's instruction, and cell viability was calculated using the following formula: viability (%) = $(1 - OD_{exp} / OD_{max}) \times 100\%$.

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

Mouse BMDCs were generated as described previously [24]. In brief, bone marrow cells were isolated from C57BL/6 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 DCs. The immature DCs were stimulated with 50 μg/ml of nanogels or 2 μg/ml of LPS for 24 h, and then labeled with FITC-anti-mouse CD40 and FITC-anti-mouse CD86 monoclonal antibodies. The expressions of CD40 and CD86 on BMDCs were measured using a Beckman Coulter Quanta SC cytometer (Beckman Coulter, CA, USA).

2.5. Antigen uptake

Immature BMDCs or Raw 264.7 mouse macrophages were cultured with soluble or nanogel-absorbed OVA-FITC (2.5 μg/ml, BD Biosciences,

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