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Targeted antigen delivery to dendritic cell via functionalized alginate nanoparticles for cancer immunotherapy

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ABSTRACT

The purpose of the present study was to identify an "easy-to-adopt" strategy to enhance immune responses using functionalized alginate (ALG) nanoparticles (MAN-ALG/ALG = OVA NPs), which were prepared by $CaCl_2$ cross-linking of two different types of ALG. The mannose (MAN) modified ALG (MAN-ALG) was used for dendritic cell targeting. The other component, composed of ovalbumin (OVA), a model antigen, is conjugated to ALG (ALG = OVA) via pH sensitive Schiff base bond. Grafting of alginate was demonstrated by FT-IR and ^{1}H NMR, while the morphological structure, particle size, Zeta potential of MAN-ALG/ALG = OVA NPs were measured using TEM and DLS. The OVA releasing behavior of MAN-ALG/ALG = OVA NPs was determined as a function of pH. Antigen uptake was examined by flow cytometry and confocal laser scanning microscopy *in vitro* using mouse bone marrow dendritic cells (BMDCs). The results showed that MAN-ALG/ALG = OVA NPs facilitated antigen uptake of BMDCs and cytosolic release of the antigen. Significant up-regulation of cytokine secretion and expression levels of the surface co-stimulatory molecules were also observed in MAN-ALG/ALG = OVA NPs-treated BMDCs, compared to free OVA. *In vivo* bio-distribution study using Cy7 (a near-infrared fluorescence dye) labeled MAN-ALG/ALG = OVA NPs showed efficient *in vivo* trafficking of the nanoparticles from the injection site to the draining lymph nodes. Moreover, MAN-ALG/ALG = OVA NPs were found to enhance cross-presentation of OVA to B3Z T cell hybridoma *in vitro*. Subcutaneous administration of MAN-ALG/ALG = OVA NPs also induced major cytotoxic T lymphocytes (CTL) response and inhibition of E.G7 tumor growth in C57BL/6 mice. In summary, we report here that the MAN-ALG/ALG = OVA NPs have the potential as a potent nanovaccine for cancer immunotherapy.

1. Introduction

Developing new and effective cancer therapy is one of the most important areas in modern medicine and cancer immunotherapy has emerged as a novel therapeutic strategy [1,2]. Dendritic cells (DC) are the most potent antigen-presenting cells (APCs) that play a critical role in antigen capture, processing and presentation in immunotherapy to induce adaptive immunity [3–5]. Classically, extracellular antigens are taken up by DC and presented via the MHC class II (MHC-II) molecules to activate $CD4^+$ T cells-mediated humoral immunity. However, when antigens are delivered into the cytoplasm of DC, the antigens would be degraded in the proteasomes and presented by major histocompatibility complex class I (MHC-I) molecules, which engender cellular immunity and induction of antigen-specific cytotoxic T lymphocytes (CTLs) [6]. This cellular immunity is essential for effective immune cells-based cancer therapy.

Many peptides and proteins have been used as antigens to generate antigen-specific immune responses. Peptides and proteins in their soluble form are usually safe and well tolerated, although they induce low levels of immune responses because of poor uptake by antigen

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presenting cells. In addition, adverse physiologic conditions such as enzyme degradation, nonspecific interaction with other molecules in the extracellular matrix also interfere with the antigen presentation process. A promising approach to enhance potency of proteins and peptides vaccine is by biomaterial-based antigen delivery [7–9]. Indeed, antigen-loaded nanoparticles (*e.g.*, liposomes, polymeric carriers) have been used as cancer vaccination because they could protect the antigens from degradation. Encapsulation of antigens in nanoparticles also increased DC antigen uptake and these nanosystems can release antigens in a controlled manner, leading to enhanced cellular and humoral immune response. One example was reported by Ma et al., in which a model antigen, ovalbumin (OVA), was co-packaged with NH_4HCO_3 in pH-responsive poly(D,L-lactic-co-glycolic acid) (PLGA) NPs. Rapid intracellular release of OVA was detected and the OVA encapsulated pH-responsive PLGA NPs could be observed in the endosomes and lysosomes. Because of the acidic microenvironment of these compartments (pH 6.5 and 5.0 for endosomes and lysosomes, respectively), the pH-responsive PLGA NPs then generate NH_3 and CO_2, which disrupt lysosomes and release OVA into the cytoplasm to induce antigen-specific $CD8^+$ T lymphocyte activation [10].

Natural polysaccharides have been widely used as vehicles of antitumor drugs, genes and proteins *etc.* [11,12]. Alginate, a polyanionic copolymer of mannuronic and guluronic sugar residues, is a typical anionic polymer with carboxyl groups in the molecular chain. It exhibits excellent biocompatibility and biodegradability. Since alginate forms hydrogel with multivalent cations in mild condition, it has been extensively utilized in a variety of biomedical applications such as cell encapsulation and drug delivery [13–15]. Ma et al. had prepared bioreducible cationic alginate-polyethylenimine (PEI) nanogels (AP-SS nanogels) as a novel vaccine delivery system. They found that AP-SS nanogels significantly enhanced both MHC class I and II antigen presentation of bone marrow-derived DC (BMDC), *in vivo* antigen-specific antibody production and $CD8^+$ T cell-mediated tumor cell lysis [16].

In the present study, mannose (MAN) modified alginate (MAN-ALG) was used for DC targeting. Model antigen, OVA, was conjugated to ALG (ALG = OVA) via pH sensitive Schiff base bond and the MAN-ALG/ALG = OVA nanoparticles (MAN-ALG/ALG = OVA NPs) were prepared by $CaCl_2$, which crosslinks MAN-ALG and ALG = OVA. MAN-ALG/ALG = OVA NPs-delivered OVA uptake and intracellular releasing kinetics was assessed *in vitro* as well as DC maturation and cross-presentation. *In vivo* trafficking of MAN-ALG/ALG = OVA NPs to the draining lymph nodes and antitumor effects were also investigated in order to evaluate the potential of MAN-ALG/ALG = OVA NPs for cancer immunotherapy.

2. Materials and methods

2.1. Materials, cell lines and animals

Sodium alginate (ALG, viscosity: 160 mpa·s, 20 °C, 1% aqueous solution) was supplied by Qingdao Crystal Rock Biology Development Co., Ltd. (Qingdao, China). Fluorescein isothiocyanate (FITC), tetrabutylammonium (TBA) hydroxide Sodium periodate and 2-chloro-1-methylpyridinium iodide (CMPI) and 4-Aminophenyl α-D-mannopyranoside (MAN) were purchased from Aladdin (Shanghai, China). Ovalbumin (OVA) and Calcium chloride anhydrous were purchased from Sigma (Shanghai, China). Near-Infrared Cyanine 7 dyes and FITC were used to label OVA. The methods of Cy7 labeled OVA (OVA-Cy7) and FITC labeled OVA (OVA-FITC) were prepared according to previously reported method [17]. All the other chemicals were of analytical grade.

RPMI-1640 medium was purchased from HyClone (USA), and fetal bovine serum (FBS) was obtained from Gybico (Australia). The anti-mouse monoclonal antibodies of CD40, CD80, CD86, and CCR-7 were all obtained from eBiosciences (USA). Recombinant murine IFN-γ, TNF-α, IL-4, IL-12p70 and granulocyte macrophage colony stimulating factor (GM-CSF) were obtained from PeproTech (USA). The cell counting kit-8 (CCK8) was obtained from Beyotime Biotechnology (China). LysoTracker Red, BCA protein quantification kits, and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Distilled deionized water was used throughout the experiments and the pH of distilled deionized water was adjusted to 7.0–7.5 with 0.01 mol/L NaOH.

E.G7-OVA cells, the murine lymphoma cell line EL4 expressing chicken OVA, were kindly provided by Dr. Lanxia Liu from Institute of Biomedical Engineering, Chinese Academy of Medical Science & Peking Union Medical College (Tianjin, China) and were cultured in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 units/mL penicillin-streptomycin and 10% fetal bovine serum (FBS).

Female C57BL/6 and BALB/c mice purchased from Vital River, Peking, China, were placed in a specific pathogen-free (SPF) environment with a consistent room temperature and humidity. All animal procedures were reviewed and ethically approved by Center of Tianjin Animal Experiment ethics committee and authority for animal protection (Approval No.: SYXK (Jin) 2011-0008).

2.2. Synthesis and characterization of the functional ALG

2.2.1. Preparation of OVA modified ALG (ALG = OVA)

Covalent linkage of OVA to ALG via a pH-sensitive Schiff base bond was performed as followed. Partially oxidized sodium alginate was prepared according to a previously reported method [18]. The oxidation reaction was carried out at room temperature during 24 h. In a dark bottle, ALG was diluted to 1% in deionized water and then sodium periodate aqueous solution was added under stirring. The ratios between repetitive unit of ALG and sodium periodate were 10: 1. The reaction was stopped after 24 h by the addition of ethylene glycol (20 mL) to reduce the excess periodate. The reaction mixture was stirred for 2 h at RT. After reaction, the resultant solution was dialyzed using dialysis bag (MWCO, 1000) against deionized water with several changes of water. The dialyzate was then lyophilized to obtain oxidized sodium alginate. The degree of oxidation was determined by the hydroxylamine hydrochloride method [19].

To form Schiff's base between aldehyde group in oxidized sodium alginate and amino group in OVA, oxidized sodium alginate and OVA were respectively dissolved in water (the total volume was 50 mL). The solution mixture was continuously stirred at room temperature for 24 h. The graft copolymer was purified by precipitation with the addition of amount of $CaCl_2$. The production was again dispersed in water and excess EDTA was added to remove Ca^{2+}, and then, the production was dialyzed using dialysis bag (MWCO, 3400) against deionized water with several changes of water. The final product ALG = OVA was obtained after freeze-dried. The chemical structure of ALG = OVA was confirmed by ^1H NMR spectroscopy (Varian Mercury 400, USA) and IR spectroscopy (Spectrum Instruments Co., Ltd. Brook Germany).

2.2.2. Preparation of MAN modified ALG (MAN-ALG)

Tetrabutylammonium-alginate (ALG-TBA) was first synthesized following previous Yuan's work [20]. Five hundred milligram (1.190 mmol) ALG-TBA was dissolved in 50 mL of dimethylformamide (DMF) which was dried by calcium hydride distillation (CaH_2). Then, 152.1 mg CMPI (0.595 mmol) was added to activate the ALG-TBA in nitrogen at 0 °C temperature. One hour later, 4-Aminophenyl α-D-mannopyranoside (0.357 mmol) was added to react at room temperature for 24 h. Then, the reaction mixture precipitated in absolute ethanol, repeatedly dissolved and precipitated for three times. Then, MAN-ALG solution was transferred into a dialysis bag (MWCO 3400) to dialyze against distilled water for 2 days. The final product MAN-ALG was obtained after freeze-dried. The chemical structure of MAN-ALG was confirmed by ^1H NMR spectroscopy and IR spectroscopy. The