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Disintegration and cancer immunotherapy efficacy of a squalane-in-water delivery system emulsified by bioresorbable poly (ethylene glycol)-block-polylactide☆

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ABSTRACT

Vaccine adjuvant is conferred on the substance that helps to enhance antigen-specific immune response. Here we investigated the disintegration characteristics and immunotherapy potency of an emulsified delivery system comprising bioresorbable polymer poly(ethylene glycol)–polylactide (PEG–PLA), phosphate buffer saline (PBS), and metabolizable oil squalane. PEG–PLA-stabilized oil-in-water emulsions show good stability at 4 °C and at room temperature. At 37 °C, squalane/PEG–PLA/PBS emulsion with oil/aqueous weight ratio of 7/3 (denominated PELA73) was stable for 6 weeks without phase separation. As PEG–PLA being degraded, 30% of free oil at the surface layer and 10% of water at the bottom disassociated from the PELA73 emulsion were found after 3 months. A MALDI-TOF MS study directly on the DIOS plate enables us to identify low molecular weight components released during degradation. Our results confirm the loss of PLA moiety of the emulsifier PEG–PLA directly affected the stability of PEG–PLA-stabilized emulsion, leading to emulsion disintegration and squalane/water phase separation. As adjuvant for cancer immunotherapeutic use, an HPV16 E7 peptide antigen formulated with PELA73 plus immunostimulatory CpG molecules could strongly enhance antigen-specific T-cell responses as well as anti-tumor ability with respected to non-formulated or Alum-formulated peptide. Accordingly, these advances may be a potential immunoregulatory strategy in manipulating the immune responses induced by tumor-associated antigens.

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1. Introduction

A vaccine contains an agent dubbed antigen that resembles a disease-causing pathogen (e.g. bacterium, virus, or toxin) to elicit adaptive immune responses. Vaccines have been commonly applied to induce prophylactic immunity against infectious pathogens [1]. For therapeutic ends, there is a trend to generate information derived from immunology to develop therapeutic vaccines or immunotherapy technology against pathogen-associated cancers [2], and immune dysfunctions such as chronic inflammation and autoimmune disease [3]. Ideally, cancer immunotherapy utilizes tumor-associated antigens to produce robust immunity and anti-tumor efficacy. However, recombinant protein or epitope peptide-based immunotherapies have faced limited clinical success caused by the relatively low immunogenicity, and hence require the incorporation of adjuvants to elicit efficient cytotoxic T lymphocyte (CTL) activities against tumor cells [4,5].

Emulsions have been widely used in the immunotherapy and vaccine development to enhance immune responses to co-administered antigens [6]. In contrast to water-in-oil (W/O) emulsions, which foster local reactions at the injection site, O/W emulsions have the advantages of low oil content and high injectability when performing vaccination [7]. Regarding the mechanisms of adjuvant action, O/W emulsions possess high efficiency to the induction of an early and strong cytokine- and chemokine-rich environment at the site of injection, and beneficial of modulation

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of genes involved in leukocyte migration and antigen presentation [8,9]. We have previously studied on the engineering of amphiphilic bioresorbable polymers as a promising strategy for the delivery of vaccine antigens and/or immunostimulatory molecules [10,11]. Ideally, bioresorbable polymeric emulsifiers, with a hydrophobic block that is degradable, show bulk degradation and further resorb *in vivo*. This allows stabilization of emulsion particles during storage, however, disintegration of the system post-injection. Immunogenicity studies in mice by using ovalbumin and influenza as a model showed that bioresorbable polymers-stabilized emulsions are able to induce potent antibody responses [10–13].

The degradation rate of a bioresorbable polymer implant has been shown to correlate with cell vitality, cell growth and host response [14], it is relatively important to evaluate the degradation characteristics as well as the final products of the designed polymer for those applications in biomedical use. To the best of our knowledge, the detail degradation characteristics of PEG–PLA were not fully investigated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technology. In addition, whether degradation of polymeric emulsifier can turn on the emulsion disintegration during storage and post-injection is still unknown. In this study, we plan to study the relation between degradation of polymer and disintegration of emulsion. Moreover, it is interesting to study on whether the bioresorbable polymer-based vaccine adjuvant can enhance the cell-mediated responses elicited by peptide antigen so as to be used as an immunotherapy tool in suppressing tumor growth. Degradation of PEG–PLA was carried out in pure water at 25 °C and 37 °C selected to mimic the usual storage conditions and the post-administration stage, and followed by analytical techniques such as gel permeation chromatography (GPC) and MALDI-TOF mass spectrometry. *In vivo* distribution was investigated in mouse model to elucidate the targeting delivery of antigen-loaded systems bearing fluorescence. The immunogenicity studies in mice were investigated by assessing the anti-tumor T-cell responses as well as the effectiveness of these responses in inducing tumor regression. These results were compared with those obtained from conventional aluminum-based mineral salts (Alum) adjuvant and immunostimulatory CpG oligodeoxynucleotides.

2. Materials and methods

2.1. Materials

DL-Lactide was purchased from Aldrich (Seelze, Germany) and recrystallized from ethyl acetate. Polyethylene glycol 2000 monomethyl ether ($MePEG_{2000}$) was supplied by Fluka (Buchs, Switzerland) and used as received. Tin(II) 2-ethylhexanoate ($SnOct_2$), phosphate buffer saline (PBS), squalane, α-cyano-4-hydroxycinnamic acid (CHCA), sodium trifluoroacetate (Na-TFA), dimethyl sulfoxide-*d6* (DMSO-*d6*) were purchased from Sigma (St. Louis, Missouri, USA). All solvents were of analytical grade. AB-type diblock copolymer PEG–PLA was synthesized by ring-opening polymerization of DL-lactide in the presence of $MePEG_{2000}$ and $SnOct_2$, as described previously [11].

2.2. Degradation of PEG–PLA

5 mg of PEG–PLA was dissolved in the eppendorf tube filled with 100 μL of distilled, deionized water. The tubes were placed either at room temperature (25 °C) or in a circulating water bath at 37 °C. At predetermined time points, three specimens were collected and lyophilized before being subjected to analyses.

2.3. Measurements

GPC was performed by using a setting composed of an isocratic pump, a refractive index (RI) detector, and two size exclusion columns connected in series, one PLgel 5 μm guard column (7.5 × 50 mm), and one PLgel 5 μm mixed-D column (7.5 × 300 mm). The mobile phase was tetrahydrofuran (THF) and the flow rate was 1.0 mL/min. Data were expressed with respect to polystyrene standards (Varian, Inc., Amherst, MA, USA). ^{1}H NMR spectra was recorded at room temperature with a Varian VXR 300 MHz spectrometer (Varian, Palo Alto, CA, USA) using DMSO-d_6 as a solvent and tetramethylsilane as a shift reference. Mass spectra were acquired by using the Micromass® MALDI micro MX™ Time of Flight Mass Spectrometer (Waters®, Milford, MA, USA) in the reflection mode. 1 μL aliquot of degradation samples were premix with 1 μL of 0.2% TFA/acetonitrile and mixed with CHCA as the matrix and Na-TFA as the dopant. 1 μL aliquot of sample solutions were spotted on a MALDI sample plate and air-dried to form a thin matrix/analyte film. The degraded samples without the matrix were deposited onto a plate containing porous silicone spots (Waters® MassPREP™ DIOS-target™ Plate, Milford, MA, USA) to monitor the low molecular weight components.

2.4. Polymer-stabilized emulsions

20 wt.% of PEG–PLA/PBS was mixed with squalane oil in three oil/aqueous weight ratios: 3/7, 5/5 and 7/3 (named PELA37, PELA55 and PELA73, respectively). The mixtures were then emulsified using a Polytron® PT 3100 homogenizer (Kinematica AG, Swiss) at 6000 rpm for 5 min. The resulting emulsions were served as a stock for further characterizations such as the stability, the dispersion type, and the particle size. The stability of emulsions was recorded by placing each sample at 4 °C, 25 °C and 37 °C, and then noted the visual aspects. The dispersion types of the emulsions were measured by using an ES-51® conductivity meter (HORIBA, Kyoto, Japan). The emulsion was re-dispersed in PBS and then applied to monitor the particle size by optical microscope (Olympus DP70, Olympus Inc., Tokyo, Japan) and by laser light scattering technique (Brookhaven 90 plus particle size analyzer, Brookhaven Instruments Limited, NY, USA).

2.5. Peptides and cell line

Peptide used in this study is an H-2D^b-restricted (RAHYNIVTF; RAH) CTL epitope derived from the human papillomavirus (HPV) type 16 E7 protein, and was synthesized in-house by solid phase method using an automated peptide synthesizer (Prelude™, Protein Technologies Inc., Tucson, AZ, USA), employing the Fmoc group for α-amino group protection. The purity was >90% for the peptide.

Murine epithelial cell line transformed with the oncogenes Ras and HPV-16 E6 and E7, TC-1 (ATCC number: CRL-2785™), was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA).

2.6. Mice and ethic statement

Six-to-eight week-old female C57BL/6 mice were obtained from the National Laboratory Animal Center. All mice were housed at the Laboratory Animal Facility of the NHRI, Miaoli County, Taiwan. All animal studies were approved by the NHRI Institutional Animal Care and Use Committee (NHRI-IACUC-098010-A).

2.7. Immunization and T-cell immunity

Mice were immunized s.c. with 30 μg of RAH peptide in PBS or formulated with 300 μg/dose of aluminum phosphate suspension (ADJU-PHOS®, Brenntag AG, Frederikssund, Danish) or 10 μg/dose of CpG (5′-TCC ATG ACG TTC CTG ACG TT-3′ with all phosphorothioate backbones; synthesized by Invitrogen Taiwan Ltd.) or PELA73 or PELA73/CpG combination adjuvant. The PELA73-containing formulations were investigated by re-dispersing 100 μL of stock PELA73 emulsion into 900 μL of vaccine bulk before injection.

Seven days after injection, spleen from the immunized mice was collected and cell suspensions were harvested followed by resuspended in ACK lysis buffer (consisting of 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 1 min. Single cell suspensions (5×10^5 cells) were re-stimulated in triplicate in the presence or absence of 10 μg/mL target RAH peptide. Interferon (IFN)-γ- and interleukin (IL)-4-secreting cells were analyzed by enzyme-linked immunosorbent spot (ELISPOT) assay (eBioscience, San Diego, USA) and the concentration of released cytokine in culture medium was quantified by enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, Minnesota, USA).

For ELISPOT analysis, cells and medium were decanted from the capture antibody coated plates after culturing in a 5% CO_2 incubator at 37 °C for 3 days. After washing, biotinylated detection antibody was added to the plates and incubated at room temperature for 2 h and then the antibody solution was decanted. After another 45 min of incubation at room temperature with Avidin–HRP conjugate, freshly prepared AEC substrate solution (Sigma, Saint Louis, MO, USA) was added and allowed to develop color at room temperature for 40 min. By monitoring development of spots, the substrate reaction was stopped by washing wells 3 times with distilled water. The spots were then counted by using an automated ELISPOT plate reader (Cellular Technology Ltd., Shaker Heights, OH, USA). The numbers of cytokine-secreting splenocytes were calculated as the average of spots in the triplicate stimulant wells. Cell-depleted supernatants were analyzed by ELISA development kit using paired antibodies following the manufacturer's instructions. The assay was developed by adding aqueous tetramethylbenzidine substrate solution (TMB, NeA-Blue®, Clinical Science Products, Inc. Mansfield, MA, USA), and the reaction was stopped in 2 N H_2SO_4. Plates were read at 450 nm on an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

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