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Programming the composition of polymer blend particles for controlled immunity towards individual protein antigens

Xi Zhan [a], Hong Shen [b,*]

[a] Department of Biological Structure, University of Washington School of Medicine, Seattle, WA 98195, USA
[b] Elsa Biologics, LLC, Box 25725, WA 98165, USA

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ABSTRACT

In order for a more precise control over the quality and quantity of immune responses stimulated by synthetic particle-based vaccines, it is critical to control the colloidal stability of particles and the release of protein antigens in both extracellular space and intracellular compartments. Different proteins exhibit different sizes, charges and solubilities. This study focused on modulating the release and colloidal stability of proteins with varied isoelectric points. A polymer particle delivery platform made from the blend of three polymers, poly(lactic-*co*-glycolic acid) (PLGA) and two random pH-sensitive copolymers, were developed. Our study demonstrated its programmability with respective to individual proteins. We showed the colloidal stability of particles at neutral environment and the release of each individual protein at different pH environments were dependent on the ratio of two charge polymers. Subsequently, two antigenic proteins, ovalbumin (OVA) and Type 2 Herpes Simplex Virus (HSV-2) glycoprotein D (gD) protein, were incorporated into particles with systematically varied compositions. We demonstrated that the level of *in vitro* $CD8^+$ T cell and *in vivo* immune responses were dependent on the ratio of two charged polymers, which correlated well with the release of proteins. This study provided a promising design framework of pH-responsive synthetic vaccines for protein antigens of interest.

1. Introduction

Proteins are one of the most common antigens used in vaccination. Polymeric particles have been applied for protein antigen delivery for decades [1–5]. Antigen processing and presentation occur within both endolysosomal and cytosolic compartments of antigen-presenting cells (APCs), which result in the association of antigen fragments with major histocompatibility complex (MHC) molecules for the presentation to T cells. It has been shown that the release of antigen from delivery vehicles at the intracellular compartments of APCs leads to more efficient access to the antigen presentation pathway than antigen release in the extracellular environment [2]. Thus, controlling the release of antigens in the extracellular space (pH = 7.4) and intracellular compartments (endosome/lysosome, pH = 4–6) of APCs is critical for both the quality and quantity of the immune response. A number of pH-responsive polymers, such as poly(propyl acrylic acid), polyethylenimin and chitosan, have been used to enhance the release of drugs or biologics from endosomal environments [6–11]. Previous studies have demonstrated that by using pH-responsive polymers, the intracellular targeting efficacy of MHC molecules can be enhanced both *in vitro* and *in vivo*, and T cell activation is promoted [3,12,13].

Different protein antigens exhibit different solubility, size, and charge at different pH environments [14], all of which may affect their release from a delivery system. We hypothesized that we could tune the release of protein antigens by carefully controlling the interaction of protein antigens with polymeric matrix at both extracellular environment and acidic intracellular environments. We aim to develop a versatile delivery platform, whose compositions can be easily tuned for the desirable release profile and colloidal stability with respective to each individual protein antigen.

Abbreviations: PLGA, poly(lactic-*co*-glycolic acid); PAA, 2-propylacrylic acid; BMA, butyl methacrylate; DMAEMA, 2-(dimethylamino)ethyl methacrylate; AIBN, 2,2′-azobis(2-methylpropionitrile); DCM, dichloromethane; PVA, polyvinyl alcohol; DOX, doxorubicin; LSZ, lysozyme; MGB, myoglobin; α-LA, α-lactalbumin; OVA, ovalbumin; HSV-2 gD, type 2 Herpes Simplex glycoprotein D; IEP, isoelectric points; BCA, bicinchoninic acid; ELISA, enzyme-linked immunosorbent assay; TMB, tetramethylbenzidine; TLR, Toll-like receptor; MPL, monosphosphoryl lipid A; IFNγ, interferon gamma cytokine; NMR, nuclear magnetic resonance spectroscopy; GPC, gel permeation chromatography; DLS, dynamic light scattering; SEM, scanning electron microscope; M_w, molecular weight; PDI, polydispersity index; DPBS, Dulbecco's phosphate buffered saline.

* Corresponding author. Tel.: +1 2064913482.
E-mail address: hong.shen@elsa-biologics.com (H. Shen).

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Three polymers, poly(lactic-*co*-glycolic acid) (PLGA) and two random pH-sensitive copolymers (poly[(butyl methacrylate)-*co*-(acrylic acid)] (BMA-AA) and poly[(butyl methacrylate)-*co*-(dimethylaminoethyl methacrylate)] (BMA-DMAEMA)) were chosen to fabricate particles. The two pH-sensitive polymers, BMA-AA and BMA-DMAEMA, have isoelectric point at 4.2 and 8.3, respectively [15,16]. The carboxyl group in BMA-AA and the tertiary amine group in BMA-DMAEMA polymer becomes protonated as the pH shifts from neutral to low pH. Three model proteins with similar M_ws and different isoelectric points (IEP) (Lysozyme: M_w = 14.3 kDa, IEP = 11; Myoglobin: M_w = 17 kDa, IEP = 7.0; α-Lactalbumin: M_w = 14 kDa, IEP = 4.3. [17]) were effectively incorporated into these particles.

Our results demonstrate that each model protein in different compositions of blend particles exhibited distinct release profiles in buffers with pH 4.6, 6 or 7.4. The desired release profile and colloidal stability for a target antigen was achieved by optimizing the ratio of two pH-responsive polymers. This strategy was applied to formulate particles containing ovalbumin (OVA) and type 2 Herpes Simplex Virus (HSV-2) gD proteins. We showed that T cell responses *in vitro* and *in vivo* were dependent on the ratio of two pH responsive polymers.

2. Materials and methods

2.1. Materials

Tetrahydrofuran (THF) and dichloromethane (DCM) were supplied by EMD Chemicals Inc., NJ. Anhydrous acrylic acid (AA), butyl methacrylate (BMA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), 2,2′-azobis(2-methylpropionitrile) (AIBN), poly(vinyl alcohol) (PVA), lysozyme from chicken egg white (LSZ), myoglobin from equine skeletal muscle (MGB), α-lactalbumin from bovine milk (α-LA) and ovalbumin (OVA) grade VII were purchased from Sigma–Aldrich, MO. HSV-2 gD recombinant protein was obtained from DevaTal, Inc., NJ. Poly(lactic-*co*-glycolic acid) (PLGA, 50:50, IV = 0.55–0.75dL/g) was from LACTEL (DURECT Corporation, AL). Citric acid and sodium phosphates were from J. T. Baker and Fisher Scientific, respectively. All cell culture reagents were purchased from Life Technologies, NY.

2.2. Fabrication and characterization of blend particles

The free radical polymerization of acrylic acid (AA), butyl methacrylate (BMA) or 2-(dimethylamino)ethyl methacrylate (DMAEMA), butyl methacrylate (BMA) was adapted from a procedure reported in 1999 [18] and was detailed in Supplementary information.

The blend particles were fabricated by using double emulsion method as described previously [3]. 50 mg PLGA and dipolymers were dissolved in 1 ml DCM with a weight ratio as 1:1 (PLGA: dipolymers). The molar ratio of BMA-AA to BMA-DMAEMA was varied from 1:0, 3:1, 1:1, 1:3 and 0:1 for particle DA1 to DA5. Protein (LSZ, MGB, α-LA, OVA or HSV-2 gD) were encapsulated in the polymeric particles under sonication. Detailed methods can be found in Supplementary Information. The actual molar ratio of copolymers incorporated into blend particles was determined by NMR (Bruker AV500 ^{1}H NMR). The size distribution and zeta potential of the particles were characterized by a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) at room temperature in 10 mM citric acid and sodium phosphate buffer.

2.3. In vitro *release of protein in citric acid-Na_2HPO_4 buffer*

The *in vitro* release kinetics of three model proteins (LSZ, MGB and α-LA) was studied in citric acid-Na_2HPO_4 buffer at a pH as 4.6, 6.0 or 7.4. Blend particles (DA1 to DA5) containing lysozyme (LSZ), myoglobin (MGB) or α-lactalbumin (α-LA) were suspended in the buffer at a concentration of 3.3 mg/ml in the total volume of 1.2 ml. The particle suspensions were incubated in 37 °C incubator. At different time points (0.5, 3, 24, 48, 72 or 120 h), 200 μl of each particle suspension was collected and centrifuged at 13,200 rpm for 10 min. Bicinchoninic acid (BCA) protein assay (Pierce® BCA Protein Assay Kit, Thermo Scientific, IL) was used to measure the protein amount in both supernatants and pellets. The loading efficiency of proteins in each particle type was calculated based on the feed amount and the total of protein mass in supernatants and pellets.

2.4. In vitro *antigen presentation with blend particles*

DC2.4 cells (a gift from K.L. Rock, University of Massachusetts Medical School) were plated in round bottom 96-well plate in 100 μl media with a density as 5×10^4 cells/well, and incubated at 37 °C overnight. Appropriate amount of OVA-blend particles (OVA concentration 0.2 μg/ml) or solute OVA control (2000, 5, 1, 0.2 μg/ml) in 100 μl media were added to DC2.4 cells. After 4 h at 37 °C, B3Z T cells (a gift from N. Shastri, University of California, Berkeley) in DC2.4 media (100 μl) were also added at a density of 10^5 cells/well and co-cultured with DC2.4 cells for 24 h at 37 °C. The supernatants were then collected and used to determine the levels of cytokines by enzyme-linked immunosorbent assay (ELISA).

2.5. Mice

6–8 weeks old female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Three types of particles with varied compositions (DA1, DA3, DA5), containing HSV-2 gD protein, were mixed with particles containing TLR ligands, lipid A detoxified (MPL, Salmonella Minnesota R595, Avanti Polar Lipids, Inc) and CpG oligonucleotide 1826 (CpG 1826, TriLink BioTechnologies, CA), and then injected into mice subcutaneously at weeks 0 and 2 (n = 4 mice/group, gD: 5 μg/mouse, TLRs: 10 μg CpG 1826, 16 ng MPL/mouse). 5 days post-boost, the serum and spleens from each group were collected to examine the antibody and T cell response. All procedures used in this study complied with federal guidelines and institutional policies, and were approved by the University of Washington Institutional Care and Animal Use Committee.

2.6. In vitro *stimulation of splenocytes for intracellular cytokine IFNγ measurement using flow cytometry*

Spleens were processed as described in Supplementary Information. 2×10^6 splenocytes were plated in 96-well plates with 100 μl DC wash media containing gD protein (20 μg/ml) and incubated for 4 h at 37 °C. Media (100 μl) containing GolgiPlug/GolgiStop (BD Biosciences) was then added and cells were incubated for an additional 6 h. Cells were then stained with the fixable viability dye eFluor® 780 following the manufacturer's protocol (eBioscience). The cells were then incubated with anti-CD16/CD32 antibody to block Fc receptors, and stained with APC anti-mouse CD3 (clone 145-2C11), PerCp-Cy5.5 anti-mouse CD4 (clone RM4-5), FITC anti-mouse CD8 (clone 53-6.7) antibodies at 4 °C for 20 min. After fixation and membrane permeabilization with Cytofix/Cytoperm (BD Biosciences), cells were incubated with PE anti-mouse intracellular IFNγ antibody (clone XMG1.2) for 20 min at 4 °C. Cells were analyzed by flow cytometry on a BD LSRII flow cytometer.