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

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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 respect 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.

Graphical Abstract

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Keywords

controlled release; pH-sensitive particles; viral infections; subunit vaccines; herpes simplex viruses

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.

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 MWs and different isoelectric points (IEP) (Lysozyme: Mw=14.3kDa, IEP=11; Myoglobin: Mw=17kDa, IEP=7.0; α -Lactalbumin: Mw=14kDa, 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 ¹HNMR). 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 μ l 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 μ l 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 μ g/ml) or solute OVA control (2000, 5, 1, 0.2 μ g/ml) in 100 μ l 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 μ l) 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 μ g/ mouse, TLRs: 10 μ g 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 IFN γ measurement using flow cytometry

Spleens were processed as described in Supplementary Information. 2×10^6 splenocytes were plated in 96-well plates with 100 μ l DC wash media containing gD protein (20 μ g/ml) and incubated for 4 h at 37°C. Media (100 μ l) 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 (eBiosciences). 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 IFN γ antibody (clone XMG1.2) for 20 min at 4 °C. Cells were analyzed by flow cytometry on a BD LSRII flow cytometer.

2.7 Statistical Analysis

Two-tailed unpaired Student's t test was used to compare two groups. $P < 0.05$ was considered to be statistically significant. All the experiments were repeated two to three times independently.

Additional information available as online supplementary Information.

3. Results and Discussion

3.1 Characterization of blend particles

Three random copolymers, BMA-AA (BA3, molar ratio of 3:1), BMA-DMAEMA (molar ratio of 3:1 (BD3) and 1:1 (BD1)) were designed (Supplementary Fig. S1). For BA3, the Mw of BA3 is 111 kD, and PDI is 1.9. For BD3 and BMA-DMAEMA BD1, Mw = 1174kD, PDI = 1.2 and Mw = 140kD, PDI = 1.7 were obtained, respectively. With higher BMA content and molecular weight, random copolymer BD3 was more hydrophobic than BD1.

The composition of blend particles (DA1-5) was determined by ^1H NMR (Supplementary Fig. S2). The particles contained PLGA and BMA copolymers with a weight ratio around 1:1 for all types of blend particles (DA1-5) (Fig. 1a). The molar ratio of BA3 and BD3 in BMA copolymers decreased from DA1 to DA5 (Fig. 1b). DA1 contained BA3 only while DA5 contained pure BD3. The composition of blend particles containing BD1 were similar to those contained BD3. The loading of lysozyme (LSZ), myoglobin (MGB) or α -lactalbumin (α -LA) ranged from 2 to 30 μg per mg particle (Table 1). The difference in loading may be due to the difference of interaction between polymers and proteins, which resulted in the different degree of loss during the fabrication process of particles. For example, the overall charge of LSZ is positive when pH is lower than 7.0. The loading of LSZ in DA4 particles higher than those containing DA5 because DA5 had higher fraction of positively charged DMAEMA, which would weaken the electrostatic attraction between LSZ and polymer matrix and result in the loss of LSZ in aqueous solutions.

For particles without proteins (Fig. 2a,e), DA1 and DA2 particles, which have higher ratios of AA, showed negative surface charge (-25 to -35 mV) at neutral pH, while DA3, with an equal ratio of AA and DMAEMA, exhibited relatively neutral surface charge at all three pHs. DA4 and DA5 exhibited a zeta potential near neutral at pH 7.4 and reached positive zeta potential around 5-10 mV at pH 4.6. Blend particles encapsulated with the three model proteins had similar trends in terms of zeta potential to unloaded particles. The values, however, were slightly different depending on the protein (Fig. 2 b-h). For example, the

overall charge of lysozyme (LSZ) is positive within the pH range of between 4.6 and 7.4. The presence of LSZ increased the zeta potential of particles (Fig. 2b). We also recognize some interesting behaviors of particles containing BD1 and LSZ (Fig. 2f). The zeta potential of those particles did not increase but decrease compared to particles without LSZ at pH 4.6. It is possible that the presense of LSZ changed the fraction of AA and DMAEMA on the surface of particles. It was possible that less DMAEMA side chains were present on the surface of particles in the presence of proteins.

DA1, DA2 and DA3 particles had relative smaller diameters (~210 nm and ~450 nm, respectively) and narrower size distribution at all three pHs (pH4.6, 6 and 7.4) than other types of particles, which indicated that those three types of particles dispersed well in solution with fewer aggregations (Fig. 3). DA4 and DA5 exhibited severe aggregation and sedimentation when the pH approached neutral, though both types of particle returned to a relatively uniform size distribution at the acidic pHs for particles loaded with LSZ and MGB proteins (Fig. 3 b,c,f,g). The size distribution of different particles corroborate with the zeta potential at different pH environments.

Our results demonstrate that there are complex interactions between proteins and polymer network through electrostatic interactions, which would have an effect on the colloidal stability of particles, the release of proteins and loading of proteins. Additionally, a fraction of proteins are nearly the surface since the incorporation of proteins changed the zeta potential.

3.2 In vitro release of protein from blend particles in citric acid- Na_2HPO_4 buffer

Figures 4 summarizes the *in vitro* release of three model proteins (LSZ, MGB and α -LA) from particles containing BA3 and either BD3 or BD1 in three pH environments (pH 4.6, 6 and 7.4). The desirable particle formulation for protein antigens we aim to achieve is the one which exhibits a high level of protein release at acidic pHs and a minnimal release at pH 7.4. We examined the total release of three protein in 5 days (Fig. 4) and also the initial burst release rate within three hours (Supplementary Fig. S3). For all the particles but BD1-containing particles loaded with α -LA, 75% protein were released within the first hour. The initial release and total release showed similar dependence on pH and composition of particles. Therefore, we focused on our discussions on the total release.

LSZ (MW=14.3 kDa, IEP=11) protein exhibits an overall negative charge within the range of pH 7.4 and 4.6. DA1 ~ DA5 particles that contained BD1 showed about 20 to 60% release at pH 7.4 while those particles containing BD3 showed about 30% to 0% release. Additionally, LSZ was released from BD3-containing particles nearly as efficiently as from BD1 containing particles at pH 4.6 and 6 (Fig. 4a, d). Therefore, BD3-containing particles were more favorable for LSZ. Within BD3 containing particles, the total release of LSZ increased from DA1 to DA5 at pH 4.6 (from 60% to 95%), but decreased at pH 7.4 (from ~30% to 0%) (Fig. 4a). At pH 6, the total release had a concave quadratic trend, and had a maximum value with DA3. Therefore, both BD3-containing DA3 and 4 particles can be ideal formulations for LSZ.

MGB (Mw=17kDa, IEP=7.0) protein exhibited a slightly negative charge at pH 7.4 and positive charge at pH 6 and pH 4.6. BD1 and BD3 containing particles showed the similar trend of MGB release at all three pHs (Fig. 4b, e). However, BD1 containing particles exhibited a less degree of release at pH 7.4 but more favorable release at pH 6 and 4.6. Therefore BD1 containing particles are more desirable. Within BD1 containing particles, DA2, 3 and 4 showed similar level of release (15 ~ 20% total protein) at pH 7.4, but DA3 gave a highest level of release at pH 6 and 4.6 (Fig. 4e). Therefore BD1-containing DA3 particles are the ideal choice for MGB.

α -LA (Mw=14kDa, IEP=4.3) protein exhibits overall negative charge at pH 7.4 and 6 and nearly neutral at pH 4.6. BD3-containing particles showed a significant protein release from 20 to 75% at pH 7.4 while a low to moderate release at acidic pHs as BD3 fraction increased to 100% (DA5) (Fig. 4c). In comparison, for BD1 containing particles, DA2-DA5 particles maintained 30%-40% at pH 7.4 but increased to 50%-80% at acidic pHs (Fig. 4f). Therefore, BD1-containing DA2 to 5 can be a good candidate formulation for α -LA.

The *in vitro* release data demonstrate that it is possible to optimize the composition of particles to maximize the protein release at endosomal environment and minimize the release at the extracellular environment. Meanwhile, the polydispersity and stability of particles were taken into consideration. Polydispersity index (PDI) was used to evaluate the particle aggregation (Supplementary Fig. S4). Aggregation of particles significantly affects the size of particles, which would change the efficiency of particles in accessing to the target cells *in vivo*, the efficiency of uptake and antigen presentation [19-21]. It also leads to poor stability and homogeneity of products during the storage. Considering the extracellular environment (pH 7.4) and the long-term storage condition as 4 °C refrigerator in DPBS buffer, blend particles with lower PDI at pH 7.4 were preferred.

For the LSZ protein, the desirable particles based on the release profile, BD3-containing DA3 and 4 particles, had similar PDI so both are ideal formulations. For MGB, BD1-containing DA3 particles had the ideal release profile, however, their PDI was about 0.6, which indicated the formation of aggregations at pH 7.4. The suboptimal particles can be BD1-containing DA2 or DA4 particles. Or we can modify copolymers to increase BMA content of BA3. At pH 7.4, both proteins and copolymers approach to neutral. Higher hydrophobic content can facilitate the retaining of MGB. At acidic pH, the repulsion between positive polymer chains (DMAEMA) and between positive polymer and positive protein can enhance the release of MGB. As for whether the overall surface charge of particles at pH 7.4 is favorable for the stability of particles, it has to be experimentally tested. For α -LA protein, from the point of view of release profile, BD1-containing DA2 to 5 can be a good candidate formulation for α -LA, however, DA2 and 3 had more desirable profile of size distribution. We did notice that the PDI of DA2 and 3 was still relatively large, which was expected based on the zeta potential of these two particles. Based on what we have learned from our results, we could adjust the ratio of BMA to DMAEMA to 3:2. The decrease of DMAEMA in BD1 would result in a slightly negatively charged surface and increase the stability of particles, while retaining the negatively charged protein at pH 7.4, and not introducing a dramatic effect on the release at acidic pHs where α -LA approaches to neutral.

The protein size and solubility in a solvent also affect the release kinetics and the total release amount. All three model proteins we chose in this study have similar molecular weights ranging 14-17 kDa. Thus, the influence of protein size was not a factor in our results. The diffusion of protein from the particle interior can be considered as a mass transport in porous media. Porosity, constrictivity and tortuosity of the polymeric matrix may influence the diffusion coefficient of proteins [22]. Although there were no significant changes observed in terms of particle diameter at different pH environments, the polymer chains with same charges may exclude each other, thus increasing the porosity as well as the average pore size of the polymer matrix. The burst release of proteins and the difference of zeta potential between protein-loaded particles and unloaded particles indicated that protein molecules were in part distributed on or near the particle surface. Therefore, the diffusion of proteins within the interior of particles is not expected to be the determining factor for the overall release.

One additional factor we should also consider is the loading of proteins. Our unpublished results showed that the loading of proteins within particles can affect the subsequent immune responses. Therefore, the desirable formulations based on the profiles of release and size distribution have to take into consideration the loading of proteins. We recognize that it is quite a daunting task to achieve optimal conditions for all the objectives, that is, optimal release, loading and colloidal stability. Our strategy demonstrates that the utilization of the electrostatic interactions between charged polymers and proteins offers a promising approach.

3.3 In vitro OVA antigen-presentation and cytokine production with blend particles

We next examined how the composition of blend particles affected the antigen presentation by dendritic cells (DC2.4 cells) to T cells *in vitro*. OVA has a IEP closed to α -LA protein. Therefore, based on the results of *in vitro* release of α -LA, BA3 and BD1 copolymers were used to fabricate OVA-blend particles. OVA was successfully incorporated into all blend particles with a protein loading ranging from 34-48 μ g OVA / mg particle. OVA-loaded particles showed a similar trend of average size (Fig. 5a), size distribution (PDI) (Fig. 5b) and pH-dependence of zeta potential (Fig. 5c) and *in vitro* release (Fig. 5d) to those of model protein α -LA when composition changed from DA1 to DA5. All the blend particles at 0.2 μ g OVA/ml dose more effectively stimulated CD8⁺ T cell *in vitro* compared to soluble OVA control (Fig. 5e). There was an increasing trend of CD8⁺ T cell stimulation from DA1 to DA5. Cells incubated with DA4 and DA5 particles resulted in significantly higher IL-2 secretion (5000 and 7000 pg/ml for DA4 and DA5, respectively) than DA1, DA2 and DA3.

Although different particle types exhibited varying size and zeta-potential in pH 7.4, there were no significant differences in terms of DC2.4 cell viability and particle uptake after 4 h incubation at 37 °C (Supplementary Fig. S5). DA1 had a relatively higher amount of uptake in DC2.4 cells, yet the cytokine secretion with DA1 particle was the lowest compared to other particles. Therefore, the difference in antigen presentation efficiency was mainly due to the release of protein from the pH-responsive copolymer blend particles, and less dependent on the uptake of particles.

These results corroborate well with the initial release of OVA in the five types of particles (Fig. 5d, inset). Since the particles were only incubated with DC2.4 cells for 4 h before the removal of excess of particles and the additional of T cells. The initial release of proteins was expected to be critical for *in vitro* T cell stimulation. For the first three hours, DA2–5 released little (less than 5%) at pH 7.4 but 15 ~ 40% at acidic pHs; DA5 released the highest amount at acidic pHs in comparison with other particles; DA1 released the highest amount at pH7.4 (extracellular environment, 60%). Based on the soluble OVA control at different concentrations, soluble OVA was inefficient in cross-presentation and stimulating T cells even at 2 mg/ml, which was much greater than the amount released into the extracellular environment from particles before being taken up by cells. Therefore, the efficiency of T cell stimulation was dependent on the degree of release of OVA from particles intracellularly. It is essential to control the composition of particles to minimize the release extracellularly but maximize the release intracellularly in order for maximum CD8⁺ T cell stimulation.

3.4 The effect of particle compositions on immune responses in vivo

Next we examined whether the release of antigen from blend particle systems affect immune responses in an animal model. HSV-2 is a member of the herpes virus family that infect the human mucosa. Currently there are no effective vaccines against HSV-2 yet. The HSV-2 gD protein has a theoretical pKa of 7.6 calculated by ExPASy tool [23, 24], which is close to myoglobin (MGB) used in the controlled release studies. BA3 and BD1 were used to fabricate particles of DA1-5. Similar trends of size (Fig. 6a), size distribution (Fig. 6b), pH-dependence of zeta potential (Fig. 6c) was observed between gD- and MGB-DA particles. The pH-dependent gD release from gD-loaded particles was consistent with that of MGB-loaded particles except BD5. gD-loaded DA5 particles released less than 10% at pH 7.4 and 40% total proteins at acidic pHs compared to more than 60% for MGB-loaded DA5 particles (Fig. 6d). This difference was likely due to that the MW of gD was nearly four times greater than MGB.

We then examined the ability of DA1, 3 and 5 particles to induce antibody responses and primary antigen-specific T cell responses in mouse spleens. The antigen-specific IgG level in the serum was quantified using ELISA. Particle DA3 achieved the highest antigen-specific IgG titer among all three formulations after 5 d post boost immunization (Fig. 6e). The antigen-specific CD4⁺ and CD8⁺ T cells in spleen were quantified by detecting intracellular IFN γ ⁺ T cells. A significant higher level of IFN γ ⁺ CD4⁺ T cells (3.7%) was detected in DA3-treated mice than DA5 ($p < 0.05$) and DA1-treated mice (Fig. 6f). Though the difference in the level of IFN γ ⁺ CD8⁺ (2.9%) T cells within three groups was not statistically significant, there was a trend that a higher level in DA3-treated mice than other two groups (Fig. 6g).

The three particles have similar sizes (Fig. 6a). In comparison with *in vitro* release data, the responses of antibody and CD4⁺ correlated well with the release at pH 6.0. In contrast, CD8⁺ T cell responses were less composition-dependent. Based on our *in vitro* CD4⁺ T cell antigen presentation by using model antigen OVA (Fig. 5e) and the release of OVA from particles (Fig. 5d), higher release of OVA at acidic pHs correlated well with a higher level

of stimulating T cells. So gD-loaded DA5 particles were expected to induce higher CD8 T cell responses. However, *in vivo*, CD8⁺ T cell responses received the help from CD4⁺ T cells. Collectively, DA3 particles showed a slightly higher level of CD8⁺ T cell responses than DA1 and 5 particles. Our results indicate that optimization of the release of antigens at different pH environments enables optimized immune responses. Considering the particle stability and the strength of immune responses, DA3 is the most promising candidate as HSV-2 gD antigen delivery vehicles.

4. Conclusions

This study focused on modulating the release of proteins with varying isoelectric points by using a blend of charge polymers and neutral polymers. By tuning the composition of the polymer blend, we were able to modulate the release of proteins at different pH environments *in vitro*, and obtain optimal or at least suboptimal formulations for target proteins. The *in vitro* and *in vivo* data demonstrated that with optimized composition, particles containing OVA or HSV-2 gD were able to achieve higher levels of immune responses. This study provides a design framework for the composition of blend particles with respect to individual proteins in order for a better control over the induced humoral and cellular immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
MW	molecular weight
PDI	polydispersity index
DPBS	Dulbecco's Phosphate Buffered Saline

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Figure 1. Characterization of blend particles composition by using nuclear magnetic resonance (NMR)

a. The particles contained PLGA and pH responsive copolymers with a weight ratio around 1:1 for all types of blend particles (DA-1 to DA-5). BMA-X represents the total amount of two copolymers, poly[(butyl methacrylate)-co-(dimethylaminoethyl methacrylate)] (DMAEMA) and poly[(butyl methacrylate)-co-(acrylic acid)] (AA). **b.** The molar ratio of DMAEMA to AA in particles decreased from DA-1 to DA-5. DA-1 contained AA only while DA5 contained pure DMAEMA.

Figure 2. Characterization of zeta potential of particles of BA3/BD3 (a,b,c,d) or BA3/BD1 (e,f,g,h) formulation using dynamic light scattering (DLS)

a. e. blank particles (Blank); **b. f.** lysozyme (LSZ); **c. g.** myoglobin (MGB); **d. h.** α -lactalbumin (α -LA). The zeta potential of particles was measured at room temperature in a 10 mM citric acid and sodium phosphate buffer with a pH of 4.6, 6.0 or 7.4. Values are the mean zeta potential from triplicates $\pm$ s.e.

Figure 3. Characterization of hydrodynamic diameter of particles of BA3/BD3 (a,b,c,d) or BA3/BD1 (e,f,g,h) formulation using DLS

a. e. blank particles (Blank); **b. f.** lysozyme (LSZ); **c. g.** myoglobin (MGB); **d. h.** α -lactalbumin (α -LA). The size of particles was measured at room temperature in a 10 mM citric acid and sodium phosphate buffer with a pH of 4.6, 6.0 or 7.4. Values are the mean size from triplicates $\pm$ s.e. * indicates the severe aggregations, and the measurements were not accurate.

Figure 4. In vitro release of each model protein from blend particles of BA3/BD3 (a,b,c) or BA3/BD1 (d,e,f) formulation

a.d. lysozyme (LSZ); **b.e.** myoglobin (MGB); **c.f.** α -lactalbumin (α -LA). Total release fraction of each model protein after 5 days incubation at 37 °C were measured using BCA assay. Values are the mean of duplicates $\pm$ s.e.

Figure 5. Characterization and *in vitro* protein release of ovalbumin (OVA) – loaded particles and CD8⁺ T cell stimulation mediated by particles

a. the hydrodynamic diameter; **b.** PDI; **c.** zeta potential of particles; **d.** total release fraction of OVA after 5 days incubation (left) and initial release (%) of OVA within 3 hours; **e.** CD8⁺ T cell activation characterized by the level of IL-2. Dendritic cells (DCs) were co-incubated with a given amount of particles containing the indicated amount of OVA protein for 4 h. The particles were then washed away and the DCs were co-incubated with class I OVA peptide-specific CD8⁺ T cell hybridoma cells. IL-2 secretion in media was utilized to evaluate the activation of CD8⁺ T cells. Cells incubated with DA4 and DA5 particles resulted in significantly higher IL-2 secretion (5000 and 7000 pg/ml for DA4 and DA5, respectively) than DA1, DA2 and DA3. Values are the mean of triplicates $\pm$ s.e. Student's t test was used to compare different groups. * $p < 0.05$, ** $p < 0.0001$ were considered as statistically significant.

Figure 6. Characterization and protein release of gD-loaded blend particles, and primary antibody and T cell responses *in vivo* mediated by particles

a. the hydrodynamic diameter; **b.** PDI; **c.** zeta potential of particles; **d.** total release fraction of HSV-2 gD protein after 5 days incubation at 37 °C; **e.** gD-specific antibody level in serum; **f.** gD-specific IFN γ ⁺ CD4⁺ T cells; **g.** gD-specific IFN γ ⁺ CD8⁺ T cells. Mice were immunized with particles containing 5 μ g of gD antigen through subcutaneous administration at weeks 0 and 2. 5 d post boost, Sera and spleen were isolated for the assays of antibody and T cell responses. The gD-specific CD4⁺ and CD8⁺ T cells in spleen were quantified by detecting intracellular IFN γ -positive T cells. Values are the mean of four mice $\pm$ s.e. Student's t test was used to compare different groups. * $p < 0.05$ is considered as statistically significant.

Table 1Protein loadings in blend particles (μg protein / mg particle)

Particle composition	BA3/BD3					BA3/BD1				
	DA-1	DA-2	DA-3	DA-4	DA-5	DA-1	DA-2	DA-3	DA-4	DA-5
Lysozyme (LSZ)	16.17 $\pm$ 0.12	28.31 $\pm$ 0.29	30.06 $\pm$ 0.54	11.97 $\pm$ 0.11	4.01 $\pm$ 0.09	3.23 $\pm$ 0.83	2.94 $\pm$ 0.42	5.51 $\pm$ 0.11	4.41 $\pm$ 0.06	1.96 $\pm$ 0.08
Myoglobin MGB	15.07 $\pm$ 0.83	9.04 $\pm$ 0.17	14.07 $\pm$ 0.46	20.36 $\pm$ 1.98	10.41 $\pm$ 0.02	23.04 $\pm$ 1.75	20.03 $\pm$ 1.15	10.31 $\pm$ 0.59	8.17 $\pm$ 1.29	1.87 $\pm$ 0.38
α -lactalbumin (α -LA)	7.05 $\pm$ 0.14	9.03 $\pm$ 0.19	5.13 $\pm$ 0.15	5.92 $\pm$ 0.16	9.47 $\pm$ 0.32	24.42 $\pm$ 0.01	22.64 $\pm$ 0.09	19.69 $\pm$ 0.09	25.43 $\pm$ 0.35	21.00 $\pm$ 0.13

Note: data are mean $\pm$ s.e.