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Research Article

Combined Poly(Lactide-Co-Glycolide) Microspheres Containing Diphtheria Toxoid for a Single-shot Immunization

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Abstract. To develop a single-shot vaccine containing diphtheria toxoid (DT) with a sufficient immune response, poly(lactide-co-glycolide) (PLGA) microspheres were prepared by water-in-oil-in-water double emulsification and solvent extraction techniques using low or high-molecular-weight PLGA (LMW-MS or HMW-MS). Stearic acid (SA) was introduced to HMW-MS (HMW/SA-MS) as a release modulator. Mean particle sizes (*dvs*, μm) varied between the prepared microspheres, with LMW-MS, HMW-MS, and HMW/SA-MS having the sizes of 29.83, 110.59, and 69.5 μm, respectively; however, the protein entrapment and loading efficiency did not vary, with values of 15.2–16.8 μg/mg and 61–75%, respectively. LMW-MS showed slower initial release (~2 weeks) but faster and higher release of antigen during weeks 3~7 than did HMW-MS. HMW/SA-MS showed rapid initial release followed by a continuous release over an extended period of time (~12 weeks). Mixed PLGA microspheres (MIX-MS), a combination of HMW/SA-MS and LMW-MS (1:1), demonstrated a sufficient initial antigen release and a subsequent boost release in a pulsatile manner. Serum antibody levels were measured by ELISA after DT immunization of Balb/c mice, and showed a greater response to MIX-MS than to alum-adsorbed DT (control). A lethal toxin challenge test with MIX-MS (a DT dose of 18 Lf) using Balb/c mice revealed complete protection, indicating a good candidate delivery system for a single-shot immunization.

KEY WORDS: PLGA; single-shot vaccine; diphtheria toxoid; stearic acid; immunization.

INTRODUCTION

An important current issue in vaccination is the need for new adjuvants and efficient delivery systems (1,2). The development of a single-shot vaccine, which would eliminate the necessity of multiple shots for complete immunization, and therefore prevent drop-outs among subjects, is currently a major focus for the World Health Organization; single-shot vaccines would lead to greater compliance among patients and would be more cost-effective (3–5). The encapsulation of an antigen into microspheres is one promising approach to developing new single-shot vaccines that could protect the entrapped antigen and control its release rate (6). Diphtheria toxoid (DT) is a formaldehyde-detoxified diphtheria toxin used for the treatment of diphtheria, a bacterial disease caused by *Corynebacterium diphtheria*. An alum-adsorbed combined diphtheria-tetanus-pertussis vaccine is currently available on the market and requires multiple recall injections with different vaccination schedules: in Korea and the USA, four doses at 2, 4, 6, and 15–18 months of age are recommended; in the European Union, three doses at 3, 5, and 12 months of age are recommended. Protection against tetanus and diphtheria relies on the production of toxin-neutralizing antibodies; pertussis immunity is rather complex, involving not only antibodies against several antigens such as filamentous hemagglutinin, pertussis toxin, and other surface proteins, but also T cell-mediated responses (7–9).

Poly(lactide-co-glycolide) (PLGA), a biodegradable and biocompatible polymer approved by the FDA, has been widely used for many years for the controlled release of protein-based vaccine antigens. One of the main benefits of PLGA microspheres is the ability to vary the degradation rate from several days to over a year by selecting polymers with a particular lactide-glycolide ratio and molecular weight (MW). The combined use of different PLGA microspheres for a single-shot immunization gave us insight into the control of antigen release at predetermined time intervals in a pulsatile

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manner, thereby mimicking two or three boosting injections (4,10). Versatile techniques used to attain a pulsatile release pattern have been reported: the combination of small- and large-sized microspheres (11), the combination of high- and low-MW polymers or two or more batches of microspheres with different rates of antigen release (4,12), and double-walled microparticles consisting of hydrophilic and hydrophobic polymers (13). In addition, PLGA nanoparticles can be used to modulate immune responses against encapsulated antigens due to their ability to efficiently target antigen-presenting cells and to facilitate appropriate processing and presenting of the antigens to T cells (14–16). Therefore, PLGA microencapsulation might be a promising approach for vaccine formulations, with the potential benefits of reducing the number of inoculations as well as enhancing immune responses.

However, single-shot vaccines often induce less immunization than do two or three consecutive doses of a conventional alum-adsorbed vaccine. This failure is mostly attributed to inadequate properties of the delivery systems, such as the particle size control, payload of the antigen, protein denaturation, and insufficient release of the antigen. Among them, release of the antigen is the most important factor in eliciting sufficient immune responses. Specifically, the significance of initial antigen release has been well-recognized; higher initial release induces faster and greater immune responses (17,18). If the primary immunization is strong enough, boosting immunity is higher as well, despite the lesser secondary stimulus. Higher initial antigen release stimulates hormonal immunity and cell-mediated immunity, and consequently appears vital for repeated stimulation of the memory B cell population and for maintaining antibody (Ab) titers over longer periods of time.

In the present study, DT-containing PLGA microspheres were fabricated with different MWs of PLGA: high-MW PLGA microspheres (HMW-MS) and low-MW PLGA microspheres (LMW-MS). Stearic acid (SA), a long-chain fatty acid, was introduced to HMW-MS (designated HMW/SA-MS) as a release modulator to increase the initial DT release. Further, mixed PLGA microspheres (MIX-MS) were prepared by combining LMW-MS and HMW/SA-MS in order to induce a pulsatile release pattern. The physicochemical properties of various DT-loaded MS and their *in vitro* DT release profiles were characterized. Serum Ab levels were measured and direct challenge tests were carried out using Balb/c mice for DT immunizations.

MATERIALS AND METHODS

Materials

Two types of PLGA (50:50 lactide-to-glycolide) with a MW of 50,000~75,000 (high-MW, capped; HMW) and 5000 (low-MW, uncapped; LMW), and SA were supplied by Sigma-Aldrich Company (St Louis, MO, USA). Polyvinyl alcohol (PVA, MW 13,000–23,000, 87–89% hydrolyzed) and sodium dodecyl sulfate (SDS) were supplied by Bio-rad (Hercules, CA, USA). Ethyl acetate (EA) was supplied by Duksan Science (Seoul, Korea). Diphtheria toxoid (DT, MW 62,000) was supplied by Dongshin Pharm. Co., Ltd. (Gyeonggi-do, Korea). The micro-bicinchoninic acid (BCA) assay reagent was supplied by Pierce (Rockford, IL, USA).

Preparation of DT-Loaded PLGA Microspheres

PLGA microspheres were fabricated using a previously described water-in-oil-in-water (w/o/w) double emulsification and solvent extraction technique with a slight modification (19). Briefly, a solution of DT in a pH 7.4 phosphate buffer (W_1, internal aqueous phase) containing 2% PVA solution as a stabilizer was emulsified with 6% (*w*/*v*) polymer in ethyl acetate by homogenization (X-520D, CAT, Germany) at high speed (approximately 13,000 rpm) in an ice bath for 5 min. This resulting W/O emulsion was then emulsified using a magnetic stirrer at 600 rpm in a 2.5% (*w*/*v*) PVA solution (W_2, outer aqueous phase). This W/O/W emulsion was agitated for 10 min. The organic solvent was extracted with a 5% (*v*/v) isopropyl alcohol solution. Microspheres were harvested by centrifugation (Union55R, Hanil, Korea), washed and freeze-dried (Ecospin, Hanil, Korea). SA was included in HMW-MS at 5% (*w*/w) to prepare the HMW/SA-MS.

Physicochemical Characterization of DT-Loaded Microspheres

The particle size of the microspheres was determined by laser diffractometry. Briefly, lyophilized microspheres were dispersed in phosphate-buffered saline (PBS) at pH 7.4 using a bath-type sonicator (Model 2210, Branson Ultrasonics, Danbury, CT, USA) at an output power of 90 W for 10 min. The dispersed sample was analyzed using a Malvern sizer/E (Malvern Ins., UK) which consisted of helium-neon laser ($\lambda = 633$ nm) as the light source, a 300-mm optic lens, and photo sensitive detectors at angles ranging from 0.026° to 32.5°. The particle size was expressed as volume-surface mean diameter (d_{vs}) in micrometers. The size distribution was expressed as a span value, which was calculated using the equation, $(D_{90} - D_{10})/D_{50}$, where D_{10}, D_{50}, and D_{90} are the particle diameters at 10, 50, and 90% cumulative volumes, respectively. The protein contents of the microspheres were assayed by a micro-BCA method previously reported (20,21). Briefly, 20 mg of microspheres were digested in 5 mL of 0.1 N NaOH solution containing 5% (*w*/*v*) SDS by shaking overnight on a vortex mixer (Genie-2, Sci Ind, Inc., NY, USA) until the complete dissolution of the microspheres. The sample was centrifuged (3500 rpm, 10 min) and the supernatant was analyzed to determine the DT concentration using the BCA micro-assay with UV/VIS spectrophotometry at 562 nm. Drug entrapment (μg/mg) was determined as the ratio of the weight of encapsulated DT to the total weight of microspheres. The loading efficiency (%) was expressed as the ratio of actual DT loaded to the theoretical DT loaded (20). Each sample was assayed in triplicate. The morphology of microspheres was observed using scanning electron microscopy (SEM; JEOL 35CF Scanning Electron Microscope, Tokyo, Japan). Microspheres were dropped onto a copper grid using a double-sided tape and coated with platinum under a vacuum.

In vitro Release of DT-Loaded PLGA Microspheres

PLGA microspheres (60 mg) were suspended in 5.0 mL PBS at pH 7.4 and retained in an orbital shaking incubator (Jeio-tech, SI900R, Korea) at 37°C with successive shaking (250 rpm). At predetermined time intervals, release medium was withdrawn and replaced with 2 mL of fresh PBS solution, then centrifuged at 3500 rpm for 10 min. Protein concentration in the supernatant was analyzed by the BCA micro-assay. Release profiles were drawn in terms of cumulative release (%) *versus* incubation time.

Measurement of Levels of Serum Ab After DT Immunization

Animals

Female Balb/c mice aged 7–8 weeks were obtained from Hanlim, Ltd. (Kyunggi-do, Korea). The mice were housed under conditions that included a controlled light cycle and controlled temperature (23°C). Tap water and standard laboratory chow were available *ad libitum*. All animal experiments were performed in accordance with the "Principles of Laboratory Animal Care" (NIH publication number 85-23, revised 1996) and were permitted by the Institutional Animal Care and Use Committee of Chung-Ang University (Seoul, Korea).

Immunization Protocol

The female Balb/c mice were pooled into four groups ($n = 5$). The mice were injected subcutaneously in the left side of the abdomen with 75 Lf of DT-loaded PLGA microspheres suspended in saline. As a positive control, 25 Lf of alum-adsorbed DT was injected three times at 2-week intervals. Blood samples were collected from the mice at weeks 2, 4, 6, 8, 10, 12, 16, 20, and 24 following primary immunization. Test serum was obtained by centrifugation and kept at − 20°C until analysis by the enzyme-linked immunosorbent assay (ELISA).

ELISA of DT

The ELISA was performed using 96-well ELISA microtiter plates (Immuno Plate U96 Polysorp. Nunc) which were coated with 1 μL/well of DT dissolved in Tris-buffered saline (TBS, pH 7.4) and incubated overnight at 4°C. The plates were washed several times with TBS containing 0.05% Tween 20 and blocked by incubation for 2 h at 37°C with 200 μL of 1% bovine serum albumin in TBS in each well. The sera obtained from mice were diluted to 1:1 in TBS, and 50 μL of diluted samples were loaded into each well of the microtiter plates. Fifty microliters of goat anti-mouse horse radish peroxidase were added to each well to form conjugates. Then, 2,2-azino-di-(3-ethyl-benzthiazoline 6-sulfonate) containing 0.1% H_2O_2 was added to each well as a substrate solution, and the plates were incubated again at room temperature for 10 min. The ELISA plates were read in a plate reader at 415 nm (22,23).

Direct Challenge Test

A direct challenge protocol was performed according to a previously reported method (24). Balb/c mice were randomly divided into four groups ($n = 5$) and the mice were injected subcutaneously with 0.1 mL of 10 LD_{50} diphtheria toxin in normal saline. This test was performed 6 weeks after the immunization of the mice with PLGA microspheres or alum-adsorbed DT: group 1 received 6 Lf of MIX-MS (MIX-MS_Low), group 2 received 18 Lf of MIX-MS (MIX-MS_High), group 3 received 6 Lf of alum-adsorbed DT three times at 2-week intervals (positive control group), and group 4 received saline (not immunized; negative control group). The mortality of all groups was monitored for the next 5 days.

Statistical Data Analysis

All data are expressed as mean ± standard deviations (SD). Statistical significance was determined using the two-sample Student's t test with $p < 0.05$ as the minimal level of significance.

RESULTS

Characterization of DT-Loaded PLGA Microspheres

DT-loaded PLGA microspheres were prepared by modified w/o/w double emulsification and solvent extraction techniques. As shown in Table I, the particle size and size distribution of the microspheres varied based on composition. The low-MW PLGA microspheres (LMW-MS) had the smallest particle size (29.83 μm, span value 1.05). PLGA microspheres containing SA as a release modulator (HMW/SA-MS) exhibited smaller size (69.5 μm, span value 1.21) than SA-free conventional PLGA microspheres (HMW-MS; 110.59 μm, span value 1.36). However, the protein entrapment and loading efficiency did not vary, with the values of 15.2–16.8 μg/mg and 61–75%, respectively. HMW/SA-MS showed the highest protein entrapment and loading efficiency, and LMW-MS showed slightly smaller values than did the others.

In vitro Release of DT-Loaded PLGA Microspheres

The *in vitro* release of DT-loaded PLGA microspheres was examined in PBS at pH 7.4 (Fig. 1). LMW-MS showed a lower initial release of antigen than did the others, releasing less than 10% in the first 3 weeks, followed by a gradual increase to 50% over the next 4 weeks. In contrast, HMW-MS showed a high initial burst release (approximately 30%) during the first week, no release for the following 7 weeks, and then slightly increased release for the next 4 weeks, resulting in less than 40% release in total. The inclusion of SA in HMW-MS (HMW/SA-MS) modified the release pattern: this formulation showed the greatest initial burst release (approximately 45%) during the first week, no release in the following week, slightly increased release for 6 weeks, and gradually increased release for the next 4 weeks, resulting in more than 70% release in total.

The release of antigen was further modified by the combination of HMW/SA-MS and LMW-MS (1:1). These

Table I. Characteristics of DT-loaded LGA Microspheres

Microspheres	Particle size distribution		Protein entrapment (μg/mg)	Loading efficiency (%)
	Particle size (d_{vs}, μm)	Span value*		
LMW-MS	29.83 ± 0.54	1.05	15.18 ± 0.13	61.02 ± 0.76
HMW-MS	110.59 ± 3.39	1.36	16.04 ± 0.09	69.98 ± 0.55
HMW/SA-MS	69.5 ± 1.21	1.21	16.75 ± 0.15	75.16 ± 1.22

Values represent means ± SD ($n = 3$)
* calculated using the equation: $(D_{90} - D_{10})/D_{50}$, where D_{10}, D_{50}, and D_{90} are the diameters at 10, 50, and 90% cumulative volumes, respectively
DT diphtheria toxoid, *PLGA* poly(lactide-co-glycolide), *MW* molecular weight, *LMW-MS* low-MW PLGA (5 kDa) microspheres, *HMW-MS* high-MW PLGA (50–75 kDa) microspheres, *HMW/SA-MS* high-MW PLGA (50–75 kDa) microspheres including 5% (*w*/w) stearic acid

mixed PLGA microspheres (MIX-MS) showed a pulsatile release pattern overall: a high initial burst release (approximately 30%) on day 1, no release for 2 weeks, a boost release (approximately 20%) for a week, no release for the next 3 weeks, then a continuously increasing release for the next 6 weeks, resulting in 90% release in total. This behavior could satisfy the pre-requisite of an ideal single-shot vaccination, which demonstrates a sufficient initial antigen release and a subsequent boost release in a pulsatile manner, i.e., on-and-off mode.

Immunogenicity of DT-Loaded PLGA Microspheres

Figure 2 shows the anti-DT Ab responses induced by DT-loaded PLGA microspheres using Balb/c mice. The positive control group received 25 Lf of alum-adsorbed DT *via* three consecutive subcutaneous injections at 2-week intervals. The other treatment groups received 75 Lf of DT-loaded LMW-MS, HMW/SA-MS, or MIX-MS as a single subcutaneous injection. The control group showed a stable anti-DT Ab response, in which a rapid onset of high Ab levels was observed and the Ab levels were maintained for an acceptable period of time. Both HMW/SA-MS and LMW-MS induced an insufficient response compared with that of the control; Ab levels showed a rapid decline after 8 weeks, and subsequently decreased by approximately half at week 24. In addition, particularly during the first 6 weeks, the Ab levels of LMW-MS were significantly lower ($p < 0.05$) than those of the control group. However, the response of the MIX-MS group was greater than that of the control group throughout the whole period of observation. Especially at 16–24 weeks, the MIX-MS group showed a significantly higher response ($p < 0.05$) than did the control. Even though the sole use of either HMW/SA-MS or LMW-MS was less effective than the alum-adsorbed DT, the combination of both MS formulations (MIX-MS) produced an effective immune response.

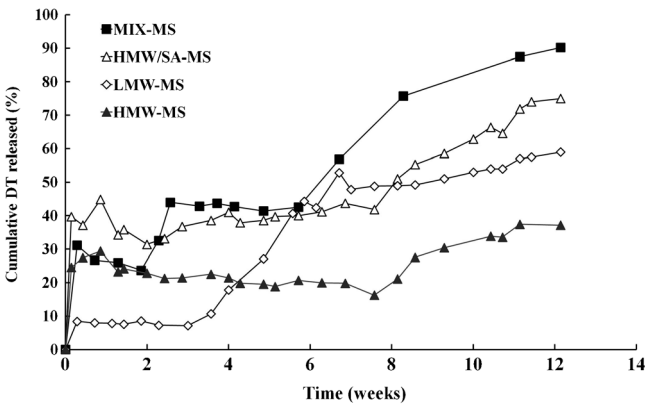

Fig. 1. Cumulative release profile of DT-loaded PLGA microspheres

Toxin Challenge Test in Mice

Figure 3 shows the results from the direct challenge study with Balb/c mice. The positive control (alum-adsorbed DT-treated) group received three consecutive subcutaneous injections of 6 Lf at 2-week intervals. The other groups received a single subcutaneous injection. The positive control group exhibited partial immunity with one mortality in 48 h, while the negative control (saline-treated) group exhibited total mortality in 24 h. The MIX-MS group showed dose-dependent survival. No mice survived at 24 h with a dose of 6 Lf (MIX-MS_Low), whereas a dose of 18 Lf (MIX-MS_High) provided complete protection from the lethal toxin. These results indicate that MIX-MS with a sufficient dose of antigen could be a good candidate for single-shot vaccinations to acquire protective immune responses.

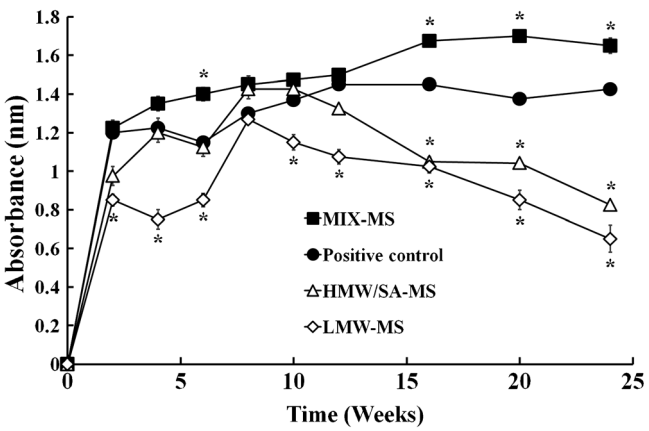

Fig. 2. Serum antibody levels measured by ELISA after DT immunization using Balb/c mice. Data are expressed as mean ± SD ($n = 5$). Statistical analysis was performed using the two-sample Student's *t* test (*$p < 0.05$ *versus* positive control)

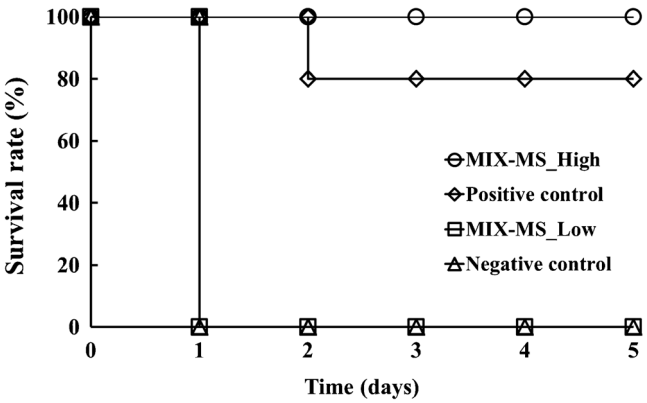

Fig. 3. Kaplan-Meier survival curves for direct toxin challenge test in Balb/c mice ($n = 5$ for each group). The formulations were subcutaneously injected to the mice as follows: MIX-MS_Low and MIX-MS_High were the combined formulations of LMW-MS and HMW/SA-MS at a 1:1 ratio, and administered once at 6 and 18 Lf doses, respectively. The positive control group received 6 Lf of alum-adsorbed DT three times at 2-week intervals. The negative control group received saline

DISCUSSION

PLGA microspheres have been widely used due to their benefits of being non-immunogenic polymers with long safety records (25,26). However, conventional PLGA microspheres have problems, such as low initial antigen release and insufficient immunogenicity. In this study, we focused on the importance of initial antigen release and the attainment of an ideal release pattern by the combination of different PLGA microspheres. Composition and characteristics of the microspheres are summarized in Table II. LMW-MS showed slower initial release for the first 2 weeks but faster and higher release of antigen during weeks 3~7 than did HMW-MS. HMW/SA-MS showed rapid initial antigen release with higher initial immune response and continuous antigen release after 7 weeks. As a result, we expected that HMW/SA-MS would efficiently work to induce an adequate immune response because it showed a 70% release in total for 12 weeks with a gradual release pattern. However, unfortunately, the sole use of either LMW-MS or HMW/SA-MS was unsatisfactory to yield enough response: although Ab production was increased to the similar level of positive control for the first 8–10 weeks, the titers went down significantly afterward. This failure might be due to the inconsistency between *in vitro* and *in vivo* circumstances including the test period: *in vitro* release experiment for 12 weeks could not adequately simulate the *in vivo* condition of 6 months. In addition, polymer degradation in the biological environment might have been quite different from that of the *in vitro* condition. Thus, we tried a combination of HMW/SA-MS and LMW-MS (MIX-MS) and fortunately obtained a pulsatile release pattern, resulting in a sufficient and long-lasting antigen response compared with that of alum-adsorbed DT.

The immunogenicity of PLGA microspheres is affected by various factors, such as the particle size, surface properties, antigen content, and release kinetics. Small-sized microspheres (typically less than 10 μm) are taken up by phagocytic antigen-presenting cells and efficiently stimulate primary responses or T cell-mediated immune response, while larger particles (generally greater than 10 μm) can provide an extracellular depot for secondary immune responses by way of B cell stimulation (5). Polymer solubility and solidification rate influence the particle size and encapsulation efficiency of PLGA microspheres. Compared with HMW polymers, LMW polymers are more soluble, and thus permit shrinking of the droplet before solidification, resulting in smaller-sized particles (27,28). Smaller size is helpful in increasing the specific surface area for effective antigen diffusion and in increasing the amount of antigen located at or near the surface (28,29). If the loading efficiency is similar, smaller particles possibly retain the antigen molecules by more densely packing them on the surface. Additionally, if the diffusion distance encountered by the particles is short, the antigens trapped in the core diffuse out rapidly. While LMW polymers slowly solidify to produce more porous microspheres, HMW polymers tend to rapidly solidify to encapsulate the peptides during microsphere formation (27,28). LMW PLGA particles with higher micro-porosity have been reported to increase the accessible peptide within the polymer matrix (28,30). LMW PLGA particles also exhibit more rapid degradation rates than do HMW PLGA particles because of their lower glass transition temperature (Tg, around 39°C) compared to that of HMW PLGA (over 50°C), resulting in increased antigen release. Low Tg causes softening of the polymer matrix at 37°C; thus, LMW PLGA microspheres release entrapped proteins near the periphery of the particle matrix more rapidly (31). It has been further reported that Tg of PLGA decreases with decreased lactide content in the copolymer composition and decreased molecular weight (32).

Controlling the surface-located antigen has been suggested as one of the major ways to increase the initial antigen release and immunogenicity of microspheres (33,34). Since

Table II. Composition and Characteristics of DT-loaded PLGA Microspheres

PLGA MS	Composition	*In vitro* DT release	*In vivo* immune response
HMW/SA-MS	High-MW PLGA (50–75 kDa) microspheres complexed with stearic acid	Rapid initial release with high amount of surface antigen; continuous release after 7 weeks.	Insufficient response with a rapid decline after 8 weeks.
LMW-MS	Low-MW PLGA (5 kDa) microspheres	Slower initial release than that of HMW-MS; continuous release for 3–7 weeks.	Significantly lower response than that of alum-adsorbed DT, showing a rapid decline after 8 weeks.
MIX-MS	Combination of HMW/SA-MS and LMW-MS (1:1)	Mutual compensation by both microspheres, resulting in a pulsatile release pattern.	Sufficient and long-lasting response throughout the whole period of 24 weeks.

high initial immunogenicity produces a large number of memory cells, strong and persistent immunogenicity can be obtained even if there is little additional stimulation afterwards. Just as the antigen encapsulated in the microspheres, the antigen adsorbed on the surface of the microspheres affects immunogenicity (34,35). It is well known that the hydrophobicity of PLGA microspheres stimulates the immune system; more hydrophobic materials easily access to antigen-presenting cells and allow easier phagocytosis by macrophages (36,37). However, the high hydrophobicity of PLGA is suspected to induce protein aggregation. Studies have reported that hydrophilic substitution of PLGA could be beneficial for immunization, as it is advantageous for antigen release and *in vivo* safety (38,39). Polyethyleneoxide (PEO)-combined PLGA has shown the potential to minimize the biphasic release and possibly the aggregation of proteins. Tetanus toxoid-containing microspheres prepared with this modified polymer induced an immune response in mice comparable to that of the commercial adsorbate vaccine and even slightly superior to that of intact PLGA microspheres (39).

The adjustment of hydrophobic nature during the microencapsulation process was tested in this experiment. An addition of SA increased the initial antigen release and further improved continuous antigen release from the PLGA microspheres. SA is a long-chain fatty acid that contains free

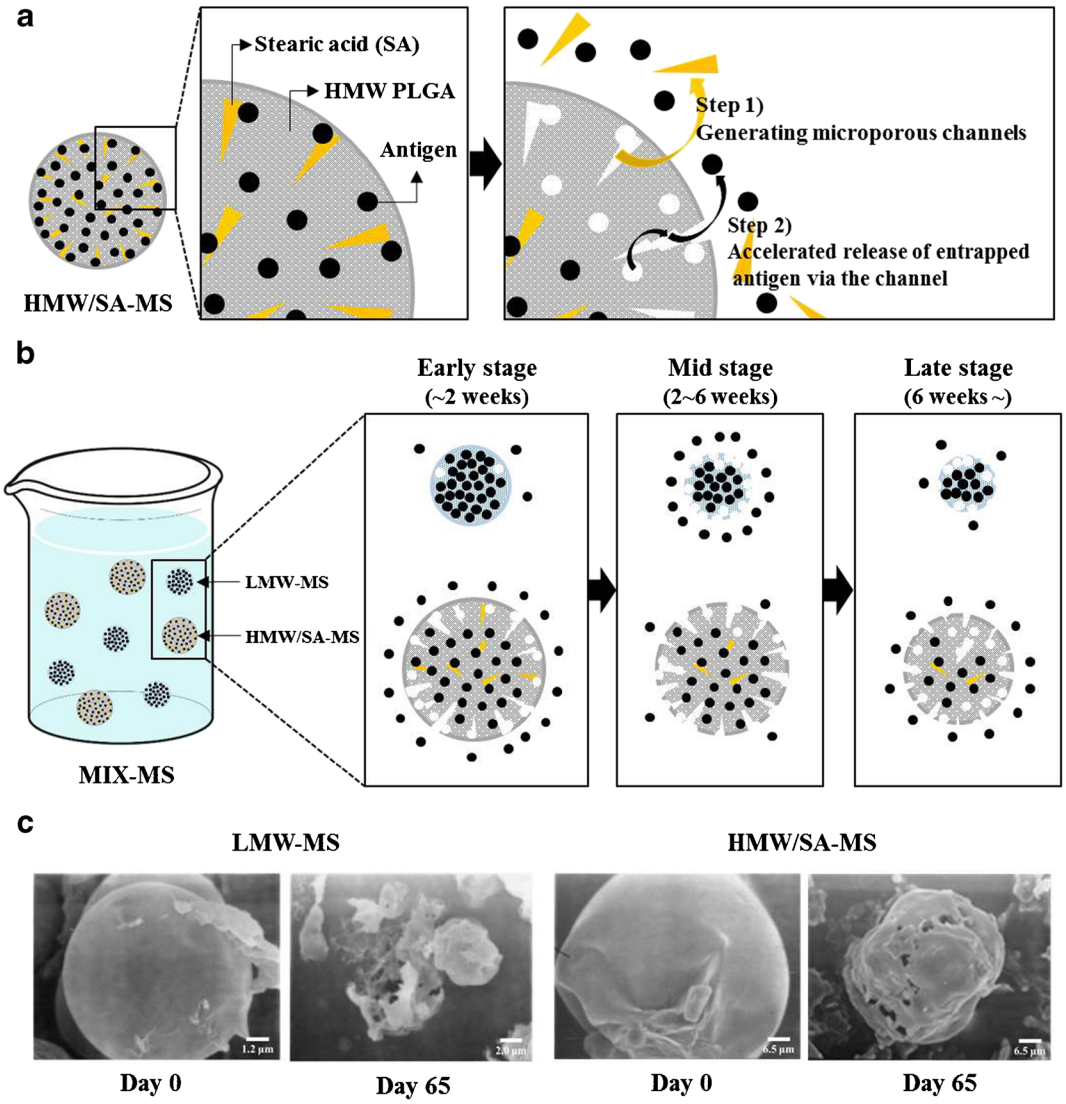

Fig. 4. Antigen release mechanisms of **a** HMW/SA-MS and **b** MIX-MS, and SEM images of **c** LMW-MS and HMW/SA-MS. **a** HMW/SA-MS consisted of hydrophobic HMW PLGA and relatively hydrophilic SA as a release modulator, in which SA is mainly located in the vicinity of the surface to generate microporous channels for solute diffusion, resulting in accelerated release of the encapsulated antigen. **b** MIX-MS, the combination of LMW-MS and HMW/SA-MS at a 1:1 ratio, compensates for the limitations of the two types of MS. In the early stage (~2 weeks), surface antigen mainly partitioned in HMW/SA-MS diffuses out easily and rapidly; in the mid stage (2~6 weeks), antigen release is mainly governed by LMW-MS; in the late stage (after 6 weeks), continuous release from HMW/SA-MS is dominant, while LMW-MS still contributes a small portion of the released drug. **c** At day 0, both LMW-MS and HMW/SA-MS showed the perfectness in their shapes; at day 65, LMW-MS was mostly collapsed in structure, while HMW/SA-MS maintained its spherical shape with pore generation on the surface

carboxyl terminal group. Due to this polar group, SA reveals relative hydrophilicity and can be placed at the organic solvent/water interface in double emulsion-solvent extraction method. This type of interfacial partitioning behavior during PLGA particle formation has been reported earlier (33), confirming that the surfactant and antigen molecules were accumulated at the interface between organic and aqueous phases. As depicted in Fig. 4a, SA complexed with PLGA efficiently and mainly anchored in the vicinity of the surface due to its relative hydrophilicity compared with that of the ester-terminated PLGA. In the biological environment, by melting down this fatty acid, microporous channels for solute diffusion are generated, resulting in accelerated release of the encapsulated antigen. This also provides a microenvironment for polymer denaturation to some extent. Chu et al. showed that the addition of SA improved the encapsulation efficiency, but also accelerated the *in vitro* release of huperzine A from microspheres (38). They also observed that an increased amount of small molecules reduced the Tg of PLGA. By adding SA, the Tg of the microspheres was reduced from 42.4 to 40.3°C (SA/drug, 1:2) and to 39.4°C (SA/drug, 1:1). In addition, the increased amount of hydrophilic carboxylic groups improved the hydrophilicity of the microspheres, which lead to accelerated swelling of the polymeric matrix and easier diffusion of huperzine A.

Release of an antigen from the PLGA microspheres may occur in either a continuous or a pulsatile pattern (40). Antigen at or near the microsphere surface is released initially upon microsphere hydration and the consequent dissolution of the antigen. This initial release is followed by a second diffusional phase of release, wherein either the antigen continuously diffuses out of the microsphere while the polymer degrades (continuous release) or diffusion of the antigen is hindered by the polymer, preventing release until significant polymer degradation has occurred (pulsatile release). The present study proposed the feasible development of a single-shot vaccine by the combination of different DT-loaded PLGA microspheres. In practice, the combination of HMW/SA-MS and LMW-MS at a 1:1 ratio (MIX-MS) showed a pulsatile release pattern, resulting in a repeated burst release at around 2–3 and 6–8 weeks, followed by continuous antigen release for 12 weeks. Since the surface of HMW/SA-MS is relatively less hydrophobic than that of HMW-MS, it was able to increase entrapment of the antigen at the surface, and thus release it rapidly after immersion into the dissolution media. Meanwhile, HMW/SA-MS degraded slowly compared to LMW-MS. Based on the cumulative DT release profile, as shown in Fig. 4b, we concluded that the mutual compensation in drug release between two types of MS was as follows: in the early stage (~ 2 weeks), surface antigen mainly partitioned in HMW/SA-MS diffused out easily and rapidly; in the mid stage (2~6 weeks), antigen released from both HMW/SA-MS and LMW-MS occurred simultaneously, but was mainly governed by LMW-MS; in the late stage (after 6 weeks), continuous release from HMW/SA-MS was dominant, while LMW-MS still contributed a small portion of the released drug. Degradation of the polymers also proceeded concomitantly. Fig. 4c showed the SEM images of the microspheres: in the beginning (day 0), both LMW-MS and HMW/SA-MS showed the perfectness in their shapes; in the late stage (day 65), LMW-MS was mostly collapsed in the structure, while HMW/SA-MS maintained its spherical shape with pore generation on the surface, indicating continuous antigen release. *In vivo* experiments further supported this release behavior. MIX-MS induced the greatest immune response at 24 weeks, which was superior to the response induced by three consecutive doses of the alum-adsorbed DT. These results were closely related with the survival rate of mice in the direct challenge test. MIX-MS with a dose of 18 Lf (MIX-MS_High) provided complete protection in the lethal toxin challenge, indicating it as a good candidate for single-shot injections to acquire protective immune responses. However, MIX-MS with a dose of 6 Lf (MIX-MS_Low) protected no mice. An optimal injection dose is required for the successful development of a single-shot vaccination. Since the present immunization protocol has been studied with a limited number of animals, further studies on larger populations are needed to establish an efficient immunization protocol. Additionally, possibility of boosting immune responses through the modification of microspheres with specific ligands and/or the employment of other adjuvants should be addressed in the future.

CONCLUSION

DT-containing PLGA microspheres were successfully fabricated with different MWs of polymer and with the inclusion of SA as a release modulator. The combination of HMW/SA-MS and LMW-MS at a 1:1 ratio (MIX-MS) demonstrated a sufficient initial antigen release and a subsequent boost release in a pulsatile manner. MIX-MS with a sufficient antigen dose showed a capacity to elicit complete and long-lasting immunogenicity after a single injection. Thus, we conclude that MIX-MS is a promising candidate delivery system for a single-shot immunization.

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