

# Development of *Yersinia pestis* F1 antigen-loaded microspheres vaccine against plague

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**Abstract:** *Yersinia pestis* F1 antigen-loaded poly(DL-lactide-co-glycolide)/polyethylene glycol (PEG) (PLGA/PEG) microspheres were produced using a water-in-oil-in-water emulsion/solvent extraction technique and assayed for their percent yield, entrapment efficiency, surface morphology, particle size, zeta potential, in vitro release properties, and in vivo animal protect efficacy. The *Y. pestis* F1 antigen-loaded microspheres (mean particle size 3.8  $\mu$ m) exhibited a high loading capacity (4.5% w/w), yield (85.2%), and entrapment efficiency (38.1%), and presented a controlled in vitro release profile with a low initial burst (18.5%), then continued to release *Y. pestis* F1 antigen over 70 days. The distribution (%) of *Y. pestis* F1 on the microspheres surface, outer layer, and core was 3.1%, 28.9%, and 60.7%, respectively. A steady release rate was noticed to be 0.55  $\mu$ g *Y. pestis* F1 antigen/mg microspheres/day of *Y. pestis* F1 antigen release maintained for 42 days. The cumulative release amount at the 1st, 28th, and 42nd days was 8.2, 26.7, and 31.0  $\mu$ g *Y. pestis* F1 antigen/mg microspheres, respectively. The 100 times median lethal dose 50% (LD<sub>50</sub>) of *Y. pestis* Yokohama-R strain by intraperitoneal injection challenge in mice test, in which mice received one dose of 40  $\mu$ g F1 antigen content of PLGA/PEG microspheres, F1 antigen in Al(OH)<sub>3</sub>, and in comparison with F1 antigen in Al(OH)<sub>3</sub> vaccine in two doses, was evaluated after given by subcutaneous immunization of BALB/c mice. The study results show that the greatest survival was observed in the group of mice immunized with one dose of F1 antigen-loaded PLGA/PEG microspheres, and two doses of F1 antigen in Al(OH)<sub>3</sub> vaccine (100%). In vivo vaccination studies also demonstrated that F1 vaccines microspheres had a protective ability; its steady-state IgG immune protection in mice plasma dramatic increased from 2 weeks (18,764 $\pm$ 3,124) to 7 weeks (126,468 $\pm$ 19,176) after vaccination. These findings strongly suggest that F1-antigen loaded microspheres vaccine offer a new therapeutic strategy in optimizing the vaccine incorporation and delivery properties of these potential vaccine targeting carriers.

**Keywords:** PLGA, immunological, protective responses

## Introduction

*Yersinia pestis* is a Gram-negative bacterium and the causative agent of plague. In man, plague can occur in three forms: bubonic, septicemic, and pneumonic. Bubonic plague arises following transmission by flea vectors, causing the swelling of lymph nodes in the victim, typically in the axillae or groin to form buboes. If detected in time, this infection is susceptible to intensive antibiotic therapy and individuals should recover. Occasionally, septicemic plague infection can occur without the appearance of buboes; this is a more insidious presentation, providing fewer triggers-to-treat and a vague general syndrome. Pneumonic plague can develop as a secondary syndrome in infected individuals or else can develop as a primary infection following exposure to an aerosol of *Y. pestis* organisms from an infected individual in close contact.

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In the first decade of the 21st century, 56 persons were reported to have the disease in the United States, of which seven died. Worldwide, 21,725 persons were affected, with 1,612 deaths, for a case-fatality rate of 7.4%.<sup>1</sup>

Vaccines against bubonic plague have been available as killed whole-cell preparations since the mid-19th century and generally have been demonstrated efficacious against the bubonic form of the disease.<sup>2-4</sup> Protecting against the pneumonic form of the disease has been possible only with live attenuated strains of *Y. pestis*.<sup>5</sup> Subunit antigens of *Y. pestis* were identified as long ago as the mid-twentieth century,<sup>3-9</sup> but it was only with the advent of recombinant DNA technology that these could be fully exploited with consistent production of pure, stable recombinant proteins.<sup>6,7,10-12</sup>

The field of nanotechnology has growing applicability to medical biotechnology, including drug and vaccine delivery.<sup>9,13-17</sup> Polymeric particles made from inert materials or biodegradable polymers such as poly-L-lactide (PLA)<sup>18,19</sup> or poly-L-lactide-co-glycolides (PLGAs)<sup>20-23</sup> allow drug encapsulation within a hydrophobic core or absorption to the hydrophilic shell.<sup>23,24</sup> This encapsulation processes can be manipulated to encapsulate drugs or vaccines within the interior.<sup>25,26</sup> Particles of different sizes might influence the immune response to the passenger antigen.<sup>27</sup>

Here we describe the encapsulation of *Y. pestis* F1-antigen onto PLGA/PEG microspheres in order to determine whether this delivery system will enhance immunogenicity in mice. The size of microspheres, encapsulation efficiency, in vitro release profiles, and release pattern were reported. The formulation was further evaluated for in vivo animal protect efficacy. The goal of the study is developed *Y. pestis* F1 antigen-loaded PLGA/PEG microspheres, which might enhance the efficiency of the *Y. pestis* F1 antigen (adjuvant effect) while conferring long-term protection. The ultimate aim was to produce an effective, single-dose formulation based on the currently used vaccines.

## Methods

### Materials

*Y. pestis* F1, the F1 antigen, was purified from the culture supernate of *Y. pestis* grown at 37°C in BHI broth medium (Difco, Detroit, MI, USA). The F1 was extracted by 30% ammonium sulfate precipitation overnight at 4°C, collected the protein by centrifugation, and resuspended in 0.1 × volume of phosphate-buffered saline (PBS), 30% ammonium sulfate precipitation overnight at 4°C again. The protein was collected by centrifugation and resuspended in 0.05 × volume of water, and dialyzed against water, analyzed by SDS-PAGE, then

freeze-dried and weighed. PLGA 50:50 was obtained from Boehringer Ingelheim (Ingelheim, Germany), Resomer RG 502 (molecular weight [Mw]: 12,000; inherent viscosity: 0.24 dL/g). Bicinchoninic acid solution (BCA), copper (II) sulfate, sodium dodecyl sulfate (SDS) and PBS were obtained from Sigma-Aldrich (St Louis, MO, USA). Polyethylene glycol (PEG) (Mw 6,000), poly(vinyl pyrrolidone) (PVP) (Mw 40,000) were obtained from Sigma-Aldrich. Dichloromethane (DCM), acetone, tetrahydrofuran and methanol were supplied by BDH (Poole, UK). All materials were used as supplied.

## Characterization of protein integrity

### Preparation of microspheres

The preparation of microspheres was conducted using minor modifications of a previously published water-in-oil-in-water (w/o/w) solvent evaporation method.<sup>19,28</sup> Briefly, 30 mg *Y. pestis* F1 in 1 mL PBS buffer was added to 5 mL of DCM containing 300 mg PLGA/PEG 2:1 using a homogenizer (20,000 rpm for 3 minutes) to provide the primary emulsion. The resulting emulsion was then mixed at high speed with 30 mL of continuous phase solution containing 10% (w/v) PVP as an emulsion stabilizer. The resulting w/o/w multiple emulsion was stirred with a magnetic stirrer for 16 hours under ambient conditions to extract DCM. The microspheres were cleaned by centrifugation and resuspension in distilled water three times to remove PVP and were then freeze-dried. The final products were stored in desiccators below 4°C. At least three lots of microspheres were prepared for each formulation. The production yield was calculated by the following equation:

$$\text{Yield (\%)} = \frac{W_d}{[\text{PLGA} + \text{protein}] \times 100} \quad (1)$$

where  $W_d$  is the weight of dry microspheres.

### Determination of *Y. pestis* F1 loading of microspheres

Accurately weighed 8–10 mg of freeze-dried microspheres were treated with 1 mL of acetone and shaken for 2 minutes at 120 rpm.<sup>29</sup> The sample was centrifuged. BCA assay was used to determine the F1 concentration in the supernatant against a series of F1 standards obtained from placebo microspheres treated under the same conditions. Each sample was assayed in triplicate.

The entrapment% (E%) and encapsulation efficiency (EE%) of protein were calculated from the equation indicated below:<sup>30</sup>

$$E\% = (W_i)/(W_m + W_i) \times 100 \quad (2)$$

$$EE\% = (E\% \times W_m)/W_i \times 100 \quad (3)$$

where  $W_t$  is the determination of protein amount,  $W_m$  is the weight of microspheres, and  $W_i$  is the initial added amount of protein.

### Distribution of F1

#### Analysis of surface protein

The amount of protein associated with the surface of protein-loaded microspheres was estimated using two approaches for comparison as described previously.<sup>31</sup>

#### Treatment with BCA reagent

The analysis of surface protein by BCA is based on the displacement of adsorbed proteins by a negatively charged detergent that binds to hydrophobic regions of protein molecules, causing them to unfold into extended polypeptide chains and freeing them from association with other molecules. Microspheres (5–6 mg) were suspended in 0.5 mL PBS and mixed with BCA reagent (2 mL BCA solution, 40 mL copper [II] sulfate). The samples were shaken for 2 minutes at room temperature. The microspheres were sedimented by centrifuging and the supernatant was analyzed for protein content using the BCA procedure. At least three samples of microspheres were assayed for each formulation.

#### Treatment with 2% SDS

The above sedimented sample were redispersed in 1 mL of 2% (w/v) SDS solution and agitated for 4 hours at room temperature using an IKA Vibrax shaker (Sigma-Aldrich). The samples were centrifuged at 4,000 g for 5 minutes and the supernatant was analyzed for protein using a BCA assay. At least three samples of microspheres were assayed for each formulation. The analysis of weak binding protein by 2% SDS is based on proteins binding to PLGA microspheres, causing them to digest into extended polypeptide chains and freeing them from association with by PLGA.

#### Treatment with 5% SDS

The analysis of reserved protein by 5% SDS is based on the PLGA microspheres digest into polypeptide chains and frees them from association with by PLGA. The above sedimented sample were redispersed in 1 mL of 5% (w/v) SDS solution and agitated for 16 hours at room temperature using an IKA Vibrax shaker. The samples were centrifuged at 4,000 g for 5 minutes and the supernatant was analyzed for protein using a BCA assay. At least three samples of microspheres were assayed for each formulation.

### Particle size

Particles were ultrasonicated to produce well-dispersed microspheres in deionized water and measured using a dynamic light-scattering particle-size analyzer (LB-500, Horiba, Kyoto, Japan). Average particle size was expressed as volume mean diameter in  $\mu\text{m}$ . Each sample was analyzed in triplicate.

### Zeta potential

Microspheres were dispersed in deionized water using an ultrasonicator. The particle zeta potential was recorded using ZetaPlus (Brookhaven Instruments Corporation, Holtsville, NY, USA). Each sample was analyzed at least five times to give an average value and standard deviation for particle zeta potential.

### Microsphere morphology

The microspheres were characterized by scanning electron microscopy (SEM) using an AMR1000. Samples were mounted on a carbon adhesive tab and sputter coated (10–15 nm) with gold palladium (60/40 alloy).

### In vitro *Y. pestis* F1 release from microspheres<sup>28</sup>

A series of tubes containing triplicate microsphere samples (5 mg) was suspended in 1 mL of sterile-filtered buffer (100 mM sodium phosphate, 10 mM sodium chloride, 0.03% polysorbate 80, pH 7.4), then incubated at 37°C under static conditions for 0.25, 1, 2, 3, 4, 7, 14, 21, 28, 35, 42, 56, 63, and 70 days. The samples were then centrifuged (4 minutes; 17,500 g). The release medium was replaced with a fresh buffer. Filtered (0.2  $\mu\text{m}$  low-protein-binding Aerodisk HT) samples were assayed using the BCA method. The pH of the medium was confirmed to be  $7.2 \pm 0.2$  through the study duration. Unreleased protein was recovered by extraction of the remaining microsphere mass as described above. All protein quantities are expressed as a percentage of the initial protein present based on the theoretical load and the initial microsphere mass.

### Animal study

#### Immunization and challenge experiments in mice

Studies in laboratory animals were designed and conducted in AAALAC-accredited animal facilities in accordance with the regulations and guidelines of the National Science Animal Centre, Taiwan, Republic of China. The mice were housed in pairs in polypropylene cages, with free access to food and water. The vivarium was maintained on a 12-hour

light:12-hour dark cycle, with a room temperature of  $22^{\circ}\text{C}\pm 1^{\circ}\text{C}$  and relative humidity level of  $50\%\pm 5\%$ . All studies were in compliance with the rules set forth in the Guide for the Care and Use of Laboratory Animals. Animal immunization and challenge experiments were adapted from previous report.<sup>31–33</sup> In brief, for challenge experiments, the BALB/c inbred strain mice were purchased from National Laboratory Animal Facility, National Science Council, Taipei, Taiwan, Republic of China, and were housed at Animal Facility of National Defense Medical Center under barrier condition.

The characteristics of the F1-loaded PLGA/PEG microspheres used for immunization are presented in Table 1. For immunization experiments, each group of ten mice were immunized. In the protection experiments, on Day 0, a PBS solution as control (group A), a 40  $\mu\text{g}$  dose of F1 antigen encapsulated in 0.1 mL PLGA/PEG microspheres (group B), and F1 antigen in  $\text{Al}(\text{OH})_3$  (group C) were given subcutaneously in a single dose to groups of ten mice. Two 40  $\mu\text{g}$  doses of F1 antigen in  $\text{Al}(\text{OH})_3$  (group D) were administered subcutaneously to the mice on Days 0 and 16. Following administration, body mass, and food intake were measured weekly.

To test the efficacy of the F1 antigen vaccines, immunized mice were challenged with *Y. pestis* Yokohama-R L00702 100 times the median lethal dose 50% ( $\text{LD}_{50}$ ) ( $10^3$  colony-forming units) by intraperitoneal injection at 45 days after the first immunization. All mouse care and procedures were in accordance with institutional policies for animal health and well-being, and all challenges adhered to Biosafety Level 3 practices. Mice morbidity rate and survival rate were monitored daily for 10 days after challenge for development of symptoms. Animals that succumbed to challenge were autopsied, livers and spleens were scored for enlargement, and any evidence of abnormality was noted. The immune response of anti-F1 IgG antibody titer was also determined in BALB/c mice. Serum was collected at Week 5.

## Immunoanalysis

For the bioactivity studies, the containing F1 antigens were coated in the micro-enzyme-linked immunoassay (ELISA)

plate and the anti-F1 IgG antibody titer was evaluated using antigen-specific antibodies and analyzed by ELISA. Briefly, 96-well microtiter plates were coated with F1 antigens diluted to 5  $\mu\text{g}/\text{mL}$  in PBS buffer (pH 9.6) and incubated overnight at  $4^{\circ}\text{C}$ . Nonspecific binding was blocked with 1% BSA in PBS buffer. Pooled test sera were diluted in twofold serial dilutions across the plates and incubated at  $37^{\circ}\text{C}$  for 1 hour. After five washes by 0.01 M PBS buffer containing 0.05% Tween-20 (PBST), horseradish peroxidase-labeled goat antimouse IgG (Roche, Basel, Switzerland) diluted 1:1000 in PBST was added to each well and incubated for 30 minutes at  $37^{\circ}\text{C}$ . The plates were washed three times in PBST buffer, and 100  $\mu\text{L}$  of 0.01% peroxidase substrate 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well. The reaction was stopped by the addition of 50  $\mu\text{L}$  of 2.5 M  $\text{H}_2\text{SO}_4$  per well, and then optical density was read at 450 nm with an ELISA plate reader (Bio-Rad, Hercules, CA, USA). Titers were expressed as the reciprocal of the dilution that intercepted the baseline (mean of negative control well plus three standard deviation).

## Cytokine level measurement

In order to measure the IFN- $\gamma$ , TNF- $\alpha$ , and IL-4, sera were collected from the four groups of animals after 35 days of postprimary immunization before challenge with *Y. pestis* Yokohama-R L00702. The levels of three cytokines IFN- $\gamma$ , TNF- $\alpha$ , and IL-4 were measured by cytokine-specific BD OptEIA™ Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Concentration of specific cytokine was calculated from standard curve and data were presented as mean concentration of cytokine (pg/mL) of triplicate wells.

## Statistical analysis

Survival after each treatment was analyzed using Kaplan–Meier survival curves. Nonparametric Kruskal–Wallis statistics were used to determine overall treatment effect using the day of death as the nonparametric variable. The nonparametric modification of the Neuman–Keuls test

**Table 1** The characteristics of *Yersinia pestis* F1-loaded microspheres prepared by the w/o/w

Formulations factor			Yield %	Average size ( $\mu\text{m}$ )	Zeta potential (mV)	E %	EE %	<i>Yersinia pestis</i> F1 distribution		
PLGA % (w/v)	PVP % (w/v)	PLGA:PEG						BCA %	2% SDS %	5% SDS %
4	10	2:1	$85.2\pm 1.9$	$3.8\pm 0.8$	$-23.8\pm 0.6$	$4.5\pm 0.1$	38.1	$3.1\pm 0.2$	$28.9\pm 3.4$	$60.7\pm 4.5$

**Notes:** Mean  $\pm$  standard error resulted from sample number  $n=5$ . Entrapment% = (protein weight/microspheres weight)  $\times$  100; Yield = [microspheres weight/(*Yersinia pestis* F1 weight + PLG polymer weight)]  $\times$  100.

**Abbreviations:** BCA, bicinechonic acid; E, entrapment; EE, encapsulation efficiency; PEG, poly(ethylene glycol); PLGA, poly(DL-lactide-co-glycolide); PVP, poly(vinyl pyrrolidone); SDS, sodium dodecyl sulfate; w/o/w, water-in-oil-in-water.



was used for subsequent pairwise comparisons. The effects of F1-loaded PLGA/PEG microspheres were compared using a one-way analysis of variance. Results were considered statistically significant if  $P < 0.05$ .

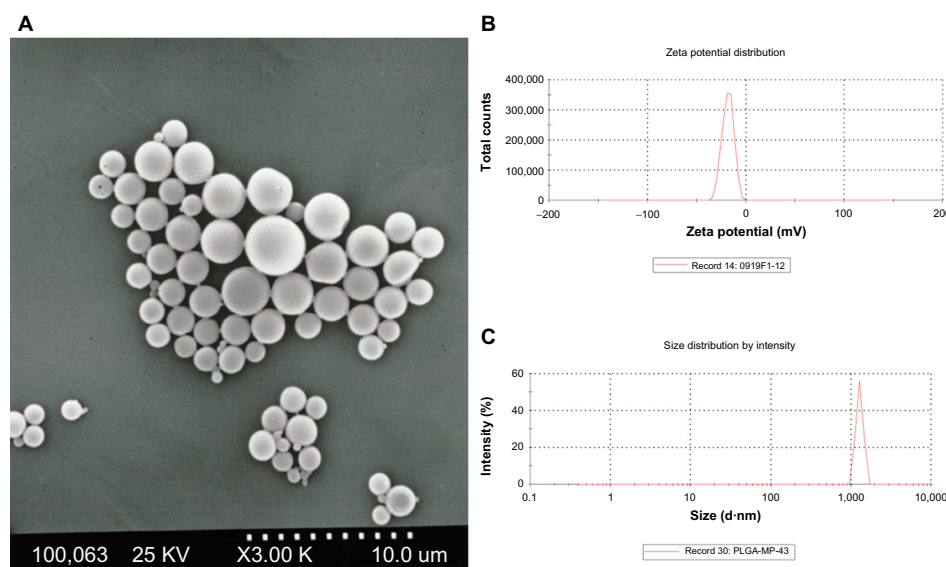
## Results and discussion

### Characteristics of *Y. pestis* F1-loaded PLGA/PEG microspheres

The characteristic of *Y. pestis* F1-loaded PLGA/PEG microspheres presented in Table 1, using the PLGA/PEG 2/1 and adding salt in the primary phase to prepare the *Y. pestis* F1-loaded microspheres for the delivery of antigens and/or adjuvants. The surface of *Y. pestis* F1-loaded PLGA/PEG microspheres is characteristically smoothly (Figure 1). There were no substantial variations in terms of surface roughness observed on the surfaces of the particles.

Particles have been shown to traffic to the draining lymph node in a size-dependent manner. An attractive strategy would be to deliver the vaccine-loaded microspheres to a subset of draining lymph nodes corresponding to a specific site or organ to minimize systemic toxicity.<sup>8,34</sup> Rao et al<sup>22</sup> showed that lymphatic uptake and node retention of particles was inversely related to size and hydrophobicity, and directly related to the anionic charge on the particles. The 50 nm particles were ideal for sustained regional delivery into the lymphatics for prevention/treatment of oligometastases. Florence et al<sup>35</sup> already demonstrated that particles uptake was by way of the Peyer's patches and other elements of the gut-associated lymphoid tissue. Modification of the particle

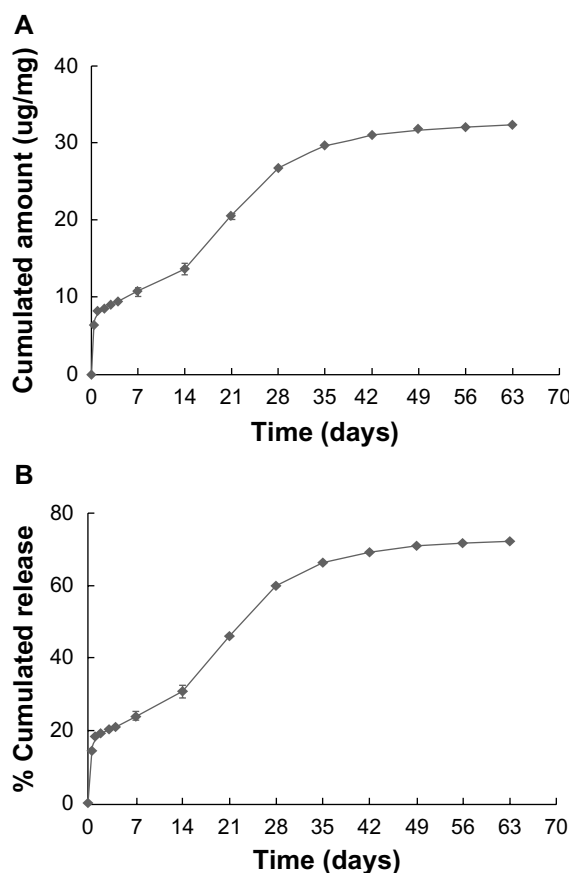
surface with specific ligands has indicated widespread uptake by nongut-associated lymphoid tissue, following their binding to and internalization by enterocytes. In a separate study, carboxylated polystyrene nanoparticles of various sizes, normalized for equal doses of conjugated antigen, were used to study size-dependent immunogenicity. Particle sizes of microspheres play an important role during uptake through the M-cells present in the Peyer's patches of the intestines and by antigen presenting cells that mediate immune responses. When microspheres of sizes  $< 5 \mu\text{m}$  extravasate from the Peyer's patches within macrophages which disseminate into systemic lymphoid tissues, where antigen release would be expected to induce a circulating antibody response.<sup>36</sup> Also, large particles (500–2,000 nm) are engulfed by peripheral APCs at the site of injection, while small nanoparticles (20–200 nm) are internalized in macrophages residing in lymph nodes. Smaller nanoparticles were independently able to diffuse across the interstitium and enter the lymphatic system, while delayed transport of larger nanoparticles to lymph nodes supports a requirement for cell-based transport. In contrast, microspheres with sizes higher than  $5 \mu\text{m}$  remain in the IgA inductive environment of the Peyer's patches, where antigen recognition would stimulate a disseminated mucosal IgA immune response. It has been suggested by many researchers, including Eldridge et al<sup>13</sup>, that the most attractive possibility of concurrent circulating IgG and secretory IgA immunity is achievable through oral immunization with vaccine-microspheres spanning the size range of 1–10  $\mu\text{m}$ . The size range achieved by this project, therefore, is ideal for



**Figure 1** SEM of *Yersinia pestis* F1 antigen-loaded PLGA/PEG 2:1 microspheres (A), zeta potential (B), and size distribution (C).  
**Abbreviations:** PEG, poly(ethylene glycol); PLGA, poly(DL-lactide-co-glycolide); SEM, scanning electron microscopy.

maximizing mucosal and systemic immune response. Rosli et al<sup>37</sup> demonstrated that the transgene protein expression and IgA antibody responses at local mucosal sites suggest immunological response to a potential oral DNA vaccine formulated within the microsphere carriers. Thomas et al<sup>38</sup> reported that the surface charge of microspheres as indicated by the zeta potential has a significant effect on its uptake by phagocytic cells of the immune system. They reported that the further the potential is from zero or greater the absolute value of the zeta potential of the particle, the more effective are their uptake. Zeta potential can also be used as an indicator of the suspension stability. The higher the absolute value, the greater the repulsive forces between the particles; therefore, the potential for coagulation and flocculation is at the minimum. The high zeta potential, therefore, increases the chances of uptake of the microspheres through the Peyer's patches. Since the goal of this project is to induce immune response to the encapsulated *Y. pestis* F1 antigens, it is important that the particle sizes and zeta potential of the microspheres are within the range that makes their potential phagocytosis by antigen presenting cells possible. The *Y. pestis* F1 antigens formulation with zeta potential of  $-23.8$  mV was ideal for vaccine delivery and was chosen for the in vivo studies.

Figure 2 summarizes the findings of in vitro *Y. pestis* F1 release study. The cumulative release of *Y. pestis* F1 antigen, in terms of %w/w, provided an index of delivery efficiency; it had an upper limit of 70.4% of the *Y. pestis* F1 antigen load at 42 days. The initial burst release of *Y. pestis* F1 microspheres was estimated from the cumulative release of 24 hours to be 16.5%, followed by a sustained diffusion of F1 antigen from the particles. A steady release rate was noticed to be  $0.55 \mu\text{g}$  *Y. pestis* F1 antigen/mg microspheres/day of *Y. pestis* F1 antigen release maintained for 42 days. The cumulative release amount at day 1, 28, and 42 was 8.2, 26.7, and  $31.0 \mu\text{g}$  *Y. pestis* F1 antigen/mg microspheres, respectively. Our *Y. pestis* F1-loaded microspheres, formulations prepared with a PLGA:PEG ratio of 2:1 with a smaller average diameter, showed the most suitable release pattern among the formulations studied. The cumulative amount of the *Y. pestis* F1 show the release of the antigen was extended throughout the study period. Continuous release of antigens has been found to be useful, since it leads to continuous exposure to low quantities of antigens. Thus, incorporation of extra amounts of hydrophobic polymer with PEG might have caused more appropriate polymeric networks to deliver the F1 antigen for a prolonged period of time among the experimental formulations. In most of the studies previously described using PLGA microspheres for protein delivery,



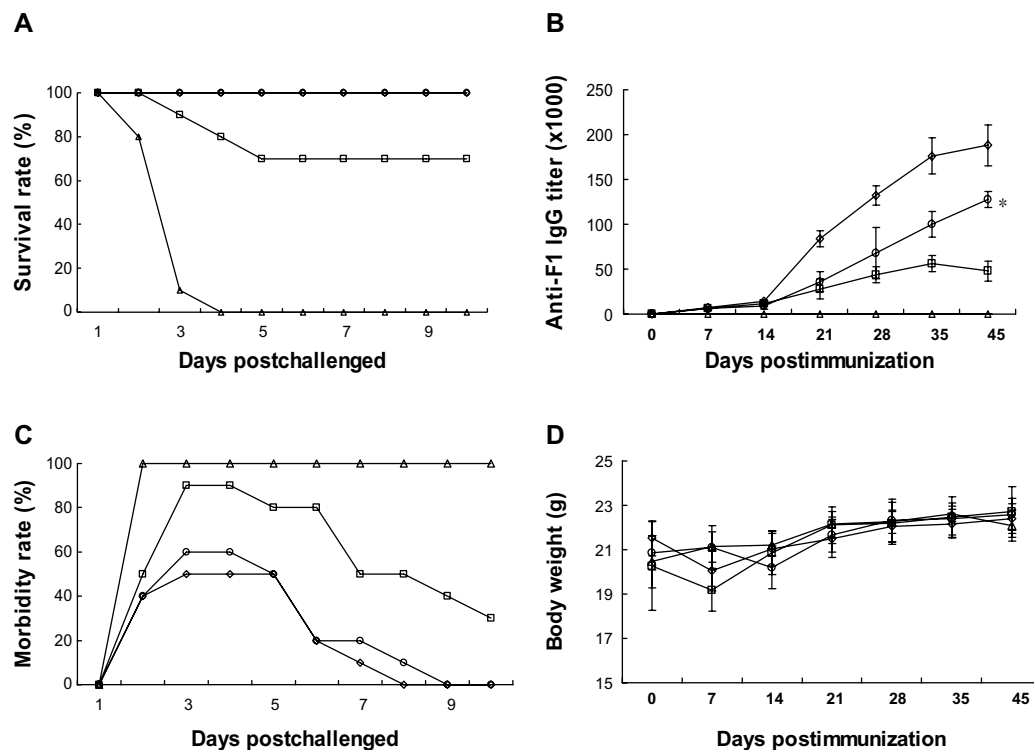
**Figure 2** Cumulative release amount (A) and % (B) of *Yersinia pestis* F1 antigen from PLGA/PEG 2:1 microspheres.

**Abbreviations:** PEG, poly(ethylene glycol); PLGA, poly(DL-lactide-co-glycolide).

the protein had been adsorbed onto the surface of microspheres.<sup>26,39–43</sup> The most notable aspect of the current studies is the entrapped effect and controlled release effect of potent active *Y. pestis* F1 antigen achieved through the presentation of *Y. pestis* F1 inside PLGA/PEG 2:1 microspheres. The *Y. pestis* F1-loaded microsphere delivery system offers the prospect of achieving a long-term, multiple phase release, single dose vaccine in the future.

## Animal study

To assess the efficacy of *Y. pestis* F1-loaded PLGA/PEG microspheres immunization against *Y. pestis* infection, the mice were checked daily for survival rates for the differential experimental groups at 10 days postchallenge, and the results are shown in Figure 3A. The greatest survival was observed in the group of mice immunized with  $40 \mu\text{g}$  *Y. pestis* F1-loaded PLGA/PEG microspheres and two doses of  $40 \mu\text{g}$  *Y. pestis* F1-loaded Alum, in which ten out of the ten tested mice survived the *Y. pestis* challenge. The second best survival rate was observed in the group of mice immunized with  $40 \mu\text{g}$  F1 antigen with Alum one dose, whose survival rate was 70%.



**Figure 3** Evaluation of survival rate (A), the *Yersinia pestis* F1-specific IgG antibody response (B), morbidity rate (C), and body weight changed (D) of BALB/c mice immunized with *Yersinia pestis* F1 antigen and by *Yersinia pestis* Yokohama-R L00702 100 times LD<sub>50</sub> (10<sup>3</sup> cfu) challenges.

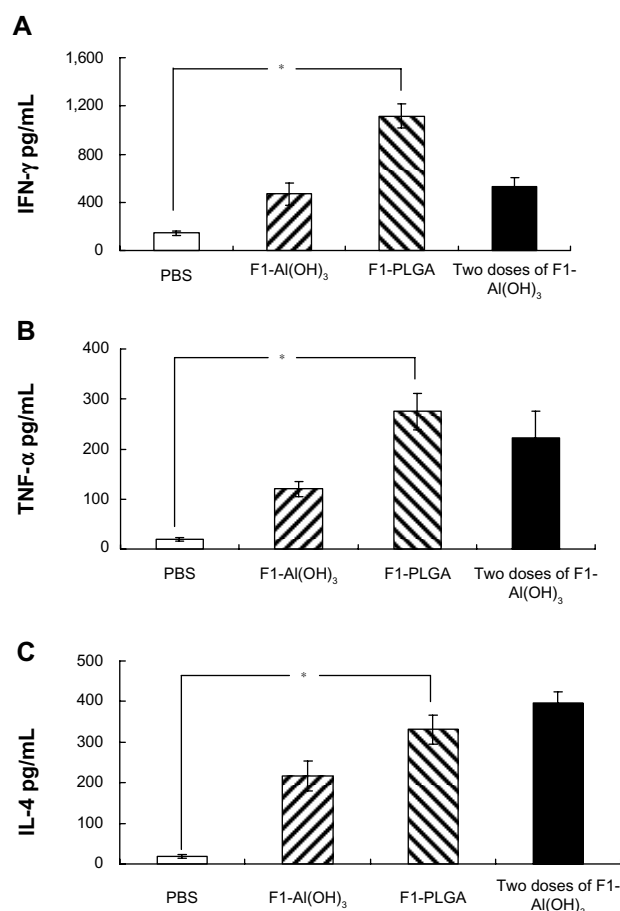
**Notes:** Each group of mice (n=10) was subcutaneous injected with F1 subunit vaccines (40 μg per mouse) or PBS, beginning at 3 weeks old. One week after immunization, mice were intraperitoneally challenged with 100 times LD<sub>50</sub> of a Yokohama-R L00702. Mice were checked every day after challenges to observe the pathological symptoms. The morbidity rate of challenged mice is determined by the percentage of mice. PBS control group (Δ), one dose F1 in Al(OH)<sub>3</sub> (40 μg per mouse) (□), one dose 40 μg F1-loaded PLGA/PEG microspheres (○), and two doses of 40 μg F1 in Al(OH)<sub>3</sub> (◇). Each point is the mean of values from ten mice. Results are represented as the mean ± standard deviation, n=10. Significant differences in the different tissues were detected by comparing them with the group treated with media (PBS) (\*P<0.05).

**Abbreviations:** cfu, colony-forming units; LD<sub>50</sub>, median lethal dose; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); PLGA, poly(DL-lactide-co-glycolide).

In contrast, the PBS solution alone group failed to confer a comparable level of protection on the immunized mice. Mice immunized with the F1-loaded microspheres vaccine showed full protection (100% survival). These efficacies were better than those mice immunized with F1-Al(OH)<sub>3</sub> one dose only (70% survival;  $P<0.05$ ). These results suggest that the F1-loaded microspheres vaccine can contribute to protective immunity against plague. Figure 3B show that the *Y. pestis* F1-specific IgG antibody response elicited by *Y. pestis* F1 immunization. As shown in Figure 3B, the F1-loaded PLGA/PEG microspheres showed higher specific antibody responses than one dose of F1-Al(OH)<sub>3</sub> immunized groups after injections. The F1 vaccines microspheres had a protective ability; its steady-state IgG immune protection in mice plasma dramatically increased from 2 weeks (18,764±3,124) to 7 weeks (126,468±19,176) after vaccination, and this was threefold higher than that observed for one dose of F1 vaccine in Al(OH)<sub>3</sub> at 45 days. However, much stronger antibody responses were noticed in *Y. pestis* F1-loaded microspheres immunized mice after the *Y. pestis* Yokohama-R strain L00702 challenge, indicating a priming effect of *Y. pestis*

F1-loaded microspheres immunization in an antigen-specific manner. Mice morbidity was observed; tremors, thicker fur, impatience, weakened activity, poor eating habits, and other signs of illness and death were recorded (Figure 3C). No significantly different body weights were recorded across groups (Figure 3D).

To assess the types of Th cell responses elicited by the F1 antigen vaccines, cytokine responses were measured after 35 days of postprimary immunization by cytokine-specific BD OptEIAM Set. The specific IFN-γ, TNF-α, and IL-4 were significantly enhanced as were IFN-γ, TNF-α, and IL-4, particularly by the F1-loaded microspheres-immunized and F1-Al(OH)<sub>3</sub> groups. Figure 4 showed that F1-Al(OH)<sub>3</sub> group resulted in higher IL-4 level, and relatively lower IFN-γ than F1-loaded microspheres group; which may result from F1-Al(OH)<sub>3</sub> induced exogenous soluble antigens humoral immune response, or Th<sub>2</sub> pathway. F1-loaded microspheres presented in the IL-4 and IFN-γ level, which may result from exogenous soluble antigen via phagocytosis induced Th1, and Th2 pathway reactions. F1-Al(OH)<sub>3</sub> and F1-loaded microspheres group detected TNF-α expression, which may



**Figure 4** Cytokine responses by mice show enhanced IFN- $\gamma$ , TNF- $\alpha$ , and IL-4 type cell responses.

**Notes:** (A) IFN- $\gamma$  cytokine responses, (B) TNF- $\alpha$  cytokine responses, and (C) IL-4 cytokine responses were measured by cytokine-specific ELISPOT and sandwich ELISA, respectively. Each point is the mean of values from ten mice. Results are represented as the mean  $\pm$  standard deviation,  $n=10$ . Significant differences in the different tissues were detected by comparing them with the group treated with media (PBS) (\* $P<0.05$ ).

**Abbreviations:** ELISA, enzyme-linked immunoassay; ELISPOT, enzyme-linked immunosorbent spot; PBS, phosphate-buffered saline.

be associated with the activation of antigen-presenting cells. These results suggest that both F1 antigen-containing vaccines stimulated specific IFN- $\gamma$ , TNF- $\alpha$ , and IL-4-type cytokines.

Only a few adjuvants are licensed for human use,<sup>14,44</sup> Alum, the most commonly used, is a trivalent aluminum salt. It was first tested as an adjuvant in a tetanus toxin vaccine in 1926. Injection of alum causes macrophage and leukocyte migration to the injection site. Proposed mechanisms of action include entrapment/adsorption of antigens within mineral deposits for either sustained antigen release (depot effect) or enhanced antigen uptake by APCs.<sup>15,26</sup> Alum is engulfed by macrophages, and is reported to persist inside the cell for several months. The depot effect of alum has been argued against based on continued adjuvant effects following excision of the injection site. Particulates and crystals stimu-

late the immune system to induce inflammatory responses. Conversely, nanometer- to micrometer-sized crystal, sphere, and hydrogel forms of aluminum salts have been used as vaccine adjuvants to enhance antibody responses in animals and humans.<sup>42,44</sup>

Parent et al<sup>45</sup> suggest that a plague vaccine capable of inducing cell-mediated and humoral immunity would be effective against pneumonic plague. Antigen encapsulated into PLGA microspheres is good for oral administration because the polymer decreases the antigen degradation at the acid condition; also, oral delivery may result in high IgA levels. Shen et al<sup>46</sup> and Schliehe et al<sup>47</sup> reported that soluble antigen, or PLGA particles with surface-attached or PLGA particle encapsulated antigen, resulted in antigen uptake and CD8+ T-cell activation in dendritic cells, which increased cellular uptake of antigen and induced T-cell responses at 1,000-fold lower antigen concentration. Kuo-Haller et al<sup>16</sup> reported that using ovalbumin delivered orally or intranasally via polymer particles to mice was effective. Repeated prime/boost at a single site results in high ovalbumin-specific antibody levels in the serum for mice immunized orally (IgA) and intravaginally (IgA and IgG) after 3 months. Nayak et al<sup>48</sup> demonstrated that rotavirus (RV) loaded in PLA and PLGA polymer particles (size ranges between 1 and 8  $\mu$ m) were evaluated in mice by oral, intranasal, and intramuscular routes of administration. They reported that between pre- and postimmunized sera, immunoglobulin A (IgA) and immunoglobulin G (IgG) titer rose  $\geq 4$ -fold. PLGA encapsulated RV microparticles elicited better antibody response through the intranasal route (90%), and when administrated through oral route, PLA encapsulated RV microparticles showed 83.3% improved response.

These results clearly illustrate that intraperitoneal immunization with the *Y. pestis* F1-loaded PLGA/PEG microspheres could protect mice from a live *Y. pestis* challenge. The *Y. pestis* F1 antigen incorporation, physicochemical characterization data, and animal results obtained in this study may be relevant in optimizing the vaccine incorporation and delivery properties of these potential vaccine targeting carriers. In designing a vaccine, besides safety and immunogenicity, several factors were also considered, such as ease of administration and production cost. The microsphere-based retained antigen *Y. pestis* F1 antigen vaccine, although equipped with no untoward reactions, has a high survival rate and delivery simplicity. These factors would contribute to a low cost of the vaccination, which is suitable for developing countries where *Y. pestis* infection epidemics and endemics are problems of public health concern.



## Conclusion

In conclusion, the *Y. pestis* F1 antigen-loaded PLGA/PEG microspheres using the w/o/w emulsion solvent evaporation technique was prepared successfully. Study results show that microspheres resulted in a controlled release of *Y. pestis* F1 antigen over 70 days. To evaluate the potential of *Y. pestis* F1 vaccine against *Y. pestis* infection, we characterize the protective efficacy and immune responses of mice subcutaneously injected with *Y. pestis* F1-loaded PLGA/PEG microspheres exhibited a marked decrease of morbidity, and a significant enhancement of survival. Also, a dramatic increase of F1-specific antibody titer from immunized mice measured by ELISA was noted. Physicochemical characterization data and the release result of the *Y. pestis* F1-loaded PLGA/PEG microspheres obtained in this study may be relevant in optimizing the incorporation of PLGA/PEG microspheres formulation into vaccine delivery in the near future.

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## Disclosure

The authors declare no conflicts of interest in this work.

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