

# Evaluation of protective efficacy using a nonstructural protein NS1 in DNA vaccine–loaded microspheres against dengue 2 virus

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**Abstract:** Dengue virus results in dengue fever or severe dengue hemorrhagic fever/dengue shock syndrome in humans. The purpose of this work was to develop an effective antidengue virus delivery system, by designing poly (*dl*-lactic-co-glycolic) acid/polyethylene glycol (PLGA/PEG) microspheres using a double-emulsion solvent extraction method, for vaccination therapy based on locally and continuously sustained biological activity. Nonstructural protein 1 (NS1) in deoxyribonucleic acid (DNA) vaccine–loaded PLGA/PEG microspheres exhibited a high loading capacity (4.5% w/w), yield (85.2%), and entrapment efficiency (39%), the mean particle size 4.8  $\mu$ m, and a controlled in vitro release profile with a low initial burst (18.5%), lag time (4 days), and continued released protein over 70 days. The distribution of protein on the microspheres surface, outer layer, and core were 3.0%, 28.5%, and 60.7%, respectively. A release rate was noticed to be 1.07  $\mu$ g protein/mg microspheres/day of protein release, maintained for 42 days. The cumulative release amount at Days 1, 28, and 42 was 18.5, 53.7, and 62.66  $\mu$ g protein/mg microspheres, respectively. The dengue virus challenge in mice test, in which mice received one dose of 20  $\mu$ g NS1 protein content of microspheres, in comparison with NS1 protein in Al(OH)<sub>3</sub> or PBS solution, was evaluated after intramuscular immunization of BALB/c mice. The study results show that the greatest survival was observed in the group of mice immunized with NS1 protein–loaded PLGA/PEG microspheres (100%). In vivo vaccination studies also demonstrated that NS1 protein–loaded PLGA/PEG microspheres had a protective ability; its steady-state immune protection in rat plasma changed from 4,443  $\pm$  1,384 pg/mL to 10,697  $\pm$  3,197 pg/mL, which was 2.5-fold higher than that observed for dengue virus in Al(OH)<sub>3</sub> at 21 days. These findings strongly suggest that NS1 protein–loaded PLGA/PEG microspheres offer a new therapeutic strategy in optimizing the vaccine incorporation and delivery properties of these potential vaccine targeting carriers.

**Keywords:** dengue virus, adjuvant, NS1 protein–loaded PLGA/PEG microspheres, vaccination

## Introduction

The spread of dengue virus throughout the tropics represents a major, rapidly growing public health problem, with an estimated 2–5 billion people at risk of dengue fever and the life-threatening disease, severe dengue.<sup>1,2</sup> A safe and effective vaccine for dengue is urgently needed.<sup>3–5</sup> There are four serotypes of dengue virus (DEN1–DEN4) that are endemic in most areas of Southeast Asia, Central and South America, and other subtropical regions.<sup>6</sup> Illnesses range from inapparent infection to dengue fever, or, in severe cases, potentially fatal dengue hemorrhagic fever/dengue shock syndrome. These symptoms are usually followed by rash, lymphadenopathy, arthralgia, or myalgia. In

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some cases, the infection may lead to the more severe dengue hemorrhagic fever with plasma leakage. Better disease management, vector control, and improved public health measures will help reduce the current disease burden.

Vaccination remains the most hopeful preventive measure, but vaccine development has been slowed by fears that immunization might predispose individuals to the severe form of dengue infection. Dengue vaccine has been in development for more than 30 years. Several attempts have been made to develop safe and effective DEN vaccines, but at present, there are no safe and effective vaccines which induce protective immunity to all serotypes of DEN.<sup>2,7,8</sup> The ideal vaccine must be able to induce protective response to all dengue serotypes simultaneously. The level of attenuation in each dengue vaccine component can be increased, if needed, by introducing additional attenuating mutations that have been well characterized. Recent advances in recombinant deoxyribonucleic acid (DNA) technology have made it possible to explore a novel approach for developing vaccines.<sup>4</sup> Natural infection or vaccination with native and recombinant proteins may induce an immune response to the surface envelope E-protein, which has been shown to be protective to superinfection with homologous serotype of the virus.

Recent advances in particle therapeutics for vaccine applications have provided diverse groups of synthetic particles with defined cellular and biological functions. Lipid and polymeric particles, as well as virus and virus-like particles, have been used to facilitate antigen delivery, with concurrent delivery of antigens and adjuvant serving to enhance what would otherwise be limited and short-lived responses to subunit vaccines.<sup>7</sup> The benefits of particle-based carriers include long-term release of antigens, oriented antigen and/or adjuvant presentation, multivalent presentation, and targeting.<sup>9–11</sup> Using microspheres for immunotherapy includes uptake by APC, targeting, shielded trafficking of cargo, codelivery of immunomodulatory agents and antigens, and sustained release of antigen, eliminating the need for repeated doses of the vaccine. The tunability of particles makes it possible to make particles in discrete sizes and geometries. Surface functionalization makes it possible to orient pathogen-relevant danger signals on the particle surface. This also enables multivalent presentation of PAMPs or antigens, mimicking repetitive presentation by live pathogens and leading to enhanced antigenicity through receptor cross-linking and immune cell activation. It is hoped that sustained release of antigen from particles can induce strong protection, eliminating the need for repeated doses of the vaccine (prime-boost).

The goal of this study is not only to develop a novel dengue virus vaccine by using the locally isolated DEN-2 ribonucleic acid (RNA) virus (PL046 strain) as the source of DEN-2 nonstructural protein 1 (NS1) antigen, but also to develop NS1 protein-loaded poly (*dl*-lactic-co-glycolic) acid/polyethylene glycol (PLGA/PEG) microspheres as a vaccine adjuvant, which might enhance the efficiency of the vaccine (adjuvant effect) while conferring long-term protection. The ultimate aim was to produce an effective, single-dose formulation based on currently used vaccines.

## Material and methods

### Materials

#### NS1 gene-containing plasmid DNA vaccine (NS1 protein)

Immunoaffinity-purified NS1 was prepared from lysates of DEN-2 RNA virus (PL046 strain) that had been cultured in the Department of Microbiology and Immunology, National Defense Medical Center, Taiwan.<sup>12</sup> Briefly, the Taiwanese local DEN-2 strain PL046, isolated from dengue fever patient, was used for the cloning of the *NS1* gene. Virus propagation was carried out in C6/36 cells utilizing RPMI-1640 medium containing 5% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA). Plasmid DNA was produced in bacteria by using double-banded CsCl preparations. The purified proteins were analyzed with protein assay reagent (Bio-Rad, Hercules, CA, USA) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to determine the protein concentration and purity. The purity was quantified by CCD software (AlphaEase Version 2.02, Alpha Innotech, San Leandro, CA, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich. PLGA 50:50 (Resomer® RG 502; molecular weight [Mw]: 12,000; inherent viscosity: 0.24 dL/g) was obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany). Bicinchoninic acid solution (BCA), copper (II) sulfate, SDS, and phosphate buffered saline (PBS) were obtained from Sigma Aldrich. PEG (Mw 6,000), and poly(vinyl pyrrolidone) (PVP) (Mw 40,000) were obtained from Aldrich Chemical Co (Gillingham, UK). Dichloromethane (DCM), acetone, tetrahydrofuran, and methanol were supplied by BDH (Poole, UK). All materials were used as supplied.

#### Preparation of microspheres

The preparation of microspheres was carried out as adapted by previous papers by a w/o/w (water-in-oil-in-water) solvent evaporation method.<sup>13,14</sup> Briefly, a 1.0 mL solution of 30 mg/mL NS1 protein 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; they 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 NSI protein loading of microspheres

Accurately weighed 8–10 mg of freeze-dried microspheres were treated with 1 mL of acetone by shaking on an IKA VXR Basic Vibrax Small Shaker (Rose Scientific Ltd., Alberta, Canada).<sup>15</sup> The sample was centrifuged. BCA assay was used to determine the BSA concentration in the supernatant against a series of BSA standards obtained from placebo microspheres treated under the same conditions. Each sample was assayed in triplicate.

The entrapment% (E%) and encapsulation efficiency (EE%) of the protein were calculated from the equations indicated below:

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

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

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

### 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>15</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 was redispersed in 1 mL of 2% (w/v) SDS solution and agitated for 4 hours at room temperature using an IKA Vibrax shaker. The samples were centrifuged 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 binding protein by 2% SDS is based on proteins weak binding to PLGA microspheres, causing them to digest into extended polypeptide chains and freeing them from association with PLGA.

#### Treatment with 5% SDS

The above sedimented samples 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 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 by 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 Zeta Plus (Brookhaven Instruments Corp, Holtsville, NY, USA). Each sample was analyzed at least five times to give an average value and standard deviation for particle zeta potential.

### Microspheres morphology

The microspheres were characterized by scanning electron microscopy using a Hitachi S-3000N microscope (Hitachi,

Tokyo, Japan). Samples were mounted on a carbon adhesive tab and sputter coated (10–15 nm) with gold palladium (60/40 alloy).

## In vitro NS1 protein release from microspheres

A series of tubes containing triplicate microsphere samples (15 mg) were 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 µm low protein-binding Aerodisk HT) samples were analyzed by BCA assay. 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 microspheres mass as above. All protein quantities were expressed as a percentage of the initial protein present based on the theoretical load and the initial microspheres 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 Center, Taiwan. 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: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 our previous report.<sup>12</sup> In brief, for challenge experiments, the BALB/c inbred strain mice was purchased from National Laboratory Animal Facility, National Science Council, Taipei, Taiwan, and were housed at the animal facility at the National Defense Medical Center under barrier conditions.

For immunization experiments, the pD2NS1 was produced in bacteria by using double-banded CsCl preparations. Groups of 3- to 4-week-old female C3H mice were randomly divided into four groups of ten. Groups 1 and 2 served as controls (PBS only) and received one dose of NS1 protein solution (intraperitoneal injections of 0.1 mL of PBS containing 20 µg of virus). Groups 3 and 4 received 20 µg NS1 protein-loaded PLGA/PEG microspheres, and 20 µg NS1 protein-loaded alum intramuscularly injected into the mouse thighs, respectively. The required dose of freeze-dried microspheres was weighed out and resuspended in saline immediately before administration. The characteristics of the NS1 protein-loaded PLGA/PEG microspheres used for immunization are presented in Table 1. One week after the final boost, mice were intravenously challenged with  $5 \times 10^9$  PFU/mice plaque-forming units of PL046 in 100 µL PBS. After virus challenge, the mice were monitored daily for morbidity and mortality, for 3 weeks. A group of 10 mice injected intravenously from the tail base with 20 µg of recombinant NS1 in CFA served as protein control. Mice mortality was monitored daily for 21 days. Levels of antigen bioactivity were also determined in BALB/c mice. Serum was collected at Week 0, 1, 2, and 3. For the bioactivity studies, the antigens containing NS1 protein were coated in the micro-enzyme-linked immunosorbent assay (ELISA) plate, and the bioactivity of the antigens was evaluated using antigen-specific antibodies and analyzed by ELISA.

## ELISA

The concentrations of serum anti-NS1 were determined as described elsewhere.<sup>12</sup> In brief, sera were collected by retro-orbital sinus puncture from pD2NS1-immunized, pcDNA- or PBS-treated mice at different times, beginning 1 week after final immunization, and analyzed for the presence of NS1-specific antibodies. For ELISA detection of anti-NS1 antibody, the 96-well microtiter plates were precoated with recombinant NS1 protein. One milligram recombinant NS1 protein in 100 µL PBS was coated in each well and incubated overnight. After incubation with 200 µL of 5% powdered

**Table 1** The characteristics of NS1 protein-loaded microspheres prepared by the water-in-oil-in-water

Formulations factor			Yield %	Average size (µm)	Zeta potential (mV)	E %	EE %	NS1 protein distribution		
PLGA % (w/v)	PVP % (w/v)	PLGA: PEG						BCA %	2% SDS %	5% SDS %
4	10	2:1	$85.2 \pm 1.9$	$4.8 \pm 0.5$	$-23.8 \pm 0.6$	$4.5 \pm 0.1$	38.9	$3.0 \pm 0.3$	$28.5 \pm 2.4$	$60.7 \pm 4.5$

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

**Abbreviations:** BCA, Bicinchoninic acid solution; E, entrapment; EE, encapsulation efficiency; PLGA, poly (D,L-lactic-co-glycolic) acid; PVP, poly(vinyl pyrrolidone); SDS, sodium dodecyl sulfate; PEG, polyethylene glycol; NS1, nonstructural protein 1.

milk in PBS on each well for 1 hour to prevent nonspecific binding, 50  $\mu$ L of serial dilutions of test sera were added to each well and incubated overnight at 4°C. After the samples were washed with PBS/Tween 20, bound proteins were detected with HRP-conjugated goat antimouse IgG (1:2,000; Cappel Laboratories, Malvern, PA, USA). Color was generated by adding 2, 2-azino-bis (ethylbenzthiazoline sulfonic acid), and the absorbance at 450 nm was measured on an ELISA reader (MRX II, DYNEX Technologies, Chantilly, VA, USA). For this experiment, a control mouse IgG Mab (H25B10; ATCC CRL-8017) was used as the reference.

The nonspecific antibody-binding sites were blocked with 5% skim milk in PBS, and the membranes were reacted with anti-DEN-2 mAb. The resulting blot was treated with horseradish peroxidase-conjugated goat antimouse immunoglobulin (Cappel) and developed either with enhanced chemiluminescence reagent (Amersham/GE Healthcare, Little Chalfont, UK) or by alkaline phosphatase-conjugated secondary antibody treatment with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

## 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

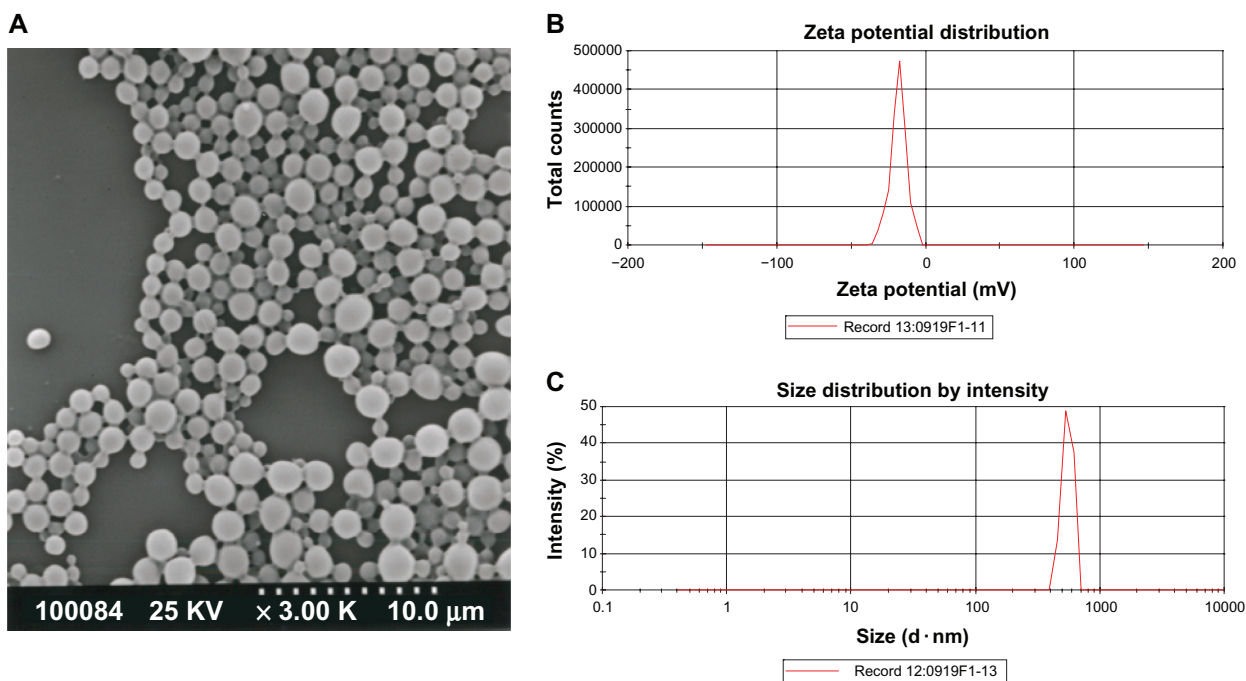
nonparametric modification of the Newman–Keuls test was used for subsequent pair-wise comparisons. The effects of DEN-loaded microspheres were compared using a one-way analysis of variance. Results were considered statistically significant if  $P < 0.05$ .

## Results and discussion

### Characteristics of NSI protein-loaded PLGA/PEG microspheres

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

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. Microspheres of  $<5$  microns disseminate into systemic lymphoid tissues, where antigen release would be expected to induce a circulating antibody response.<sup>16–18</sup> In contrast, microspheres  $>5$  microns remain in the IgA inductive environment of the Peyer's patches, where antigen recognition would stimulate a disseminated



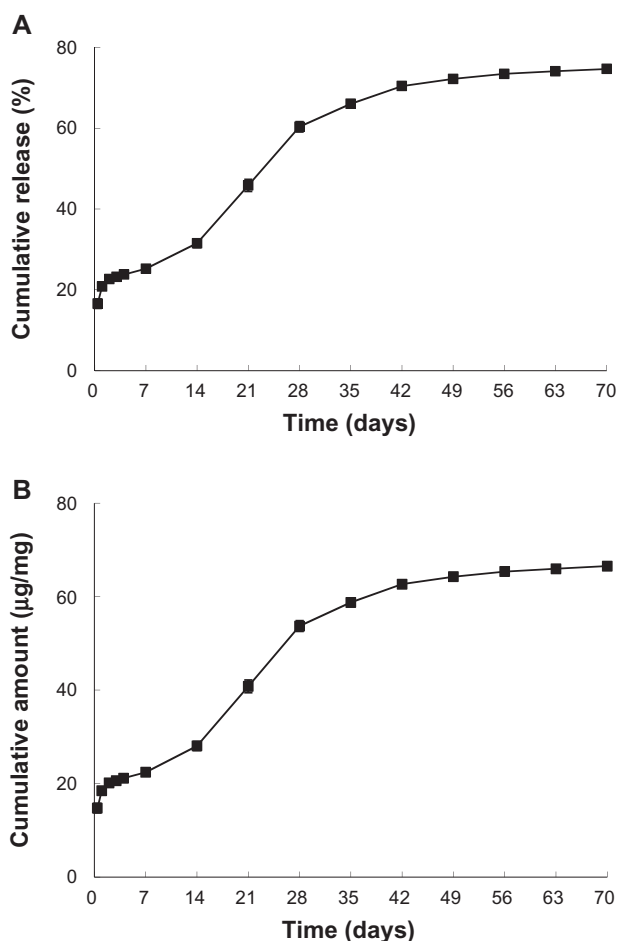
**Figure 1** The SEM of NSI protein-loaded PLGA/PEG 2:1 microspheres (A), zeta potential (B), and size distribution (C).

**Abbreviations:** NSI, nonstructural protein I; PLGA/PEG, poly (D-lactic-co-glycolic) acid/polyethylene glycol; SEM, scanning electron microscopy.

mucosal IgA immune response. Eldridge et al<sup>19</sup> reported 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 microns. The size range achieved by this project, therefore, is ideal for maximizing mucosal and systemic immune response.

The zeta potential of blank BSA PLGA microspheres was found to be  $-23.8 \pm 1.7$  mV. The low mean zeta potential value with high zeta deviation for the PLGA/PEG 2:1 formulation shows that certain PEG inclusion may be moderating the surface charges of the microspheres. 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.<sup>20</sup> Ahsan et al<sup>20</sup> reported that the further the potential is from zero or greater the absolute value of the zeta potential of the particle, the more effective 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 NS1 protein 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 NS1 protein 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 the in vitro NS1 protein release study. The cumulative release of NS1 protein, in terms of % w/w, provided an index of delivery efficiency; it had an upper limit of 70.4% of the NS1 protein load at 42 days. A steady release rate was noticed to be  $1.07 \mu\text{g}$  NS1 protein/mg microspheres/day of NS1 protein release maintained for 42 days. The cumulative release amount at Day 1, 28, and 42 was 18.5, 53.7, and  $62.66 \mu\text{g}$  NS1 protein/mg microspheres, respectively. The initial burst release of NS1 protein-loaded PLGA/PEG microspheres was estimated from the cumulative release of 24 hours to be 20.8%, followed by a short lag phase. Smaller particles are generally formed with higher impact. The tortuous polymeric diffusion pathways are varied in smaller particles.<sup>21</sup> This ultimately leads to a sustained diffusion of protein from the particles. Our NS1 protein-loaded microspheres, formulations prepared with a PLGA:PEG ratio of 2:1, with smaller average diameter, showed the most suitable release pattern among the formulations studied. The



**Figure 2** Cumulative release % (A) and amount (B) of NS1 protein from PLGA/PEG 2:1 microspheres.

**Abbreviations:** NS1, nonstructural protein 1; PLGA/PEG, poly (D,L-lactic-co-glycolic) acid/polyethylene glycol.

cumulative amount of NS1 protein shows 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 an extra amount of hydrophobic polymer with PEG might have caused more appropriate polymeric networks to deliver the dengue virus for a prolonged period of time, among the experimental formulations. In most of the studies previously described using PLGA microspheres for protein delivery, the protein had been adsorbed onto the surface of microspheres.<sup>15</sup> An anticipated advantage of sustained release is single-inoculation therapy rather than treatment with prime and boost injections.

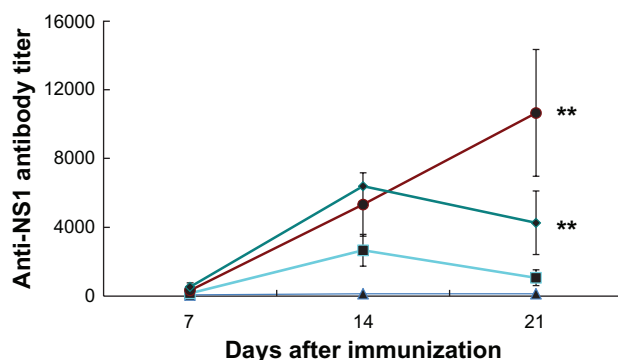
The importance of understanding release and degradation kinetics, as well as metabolic products, is also exemplified by the potential for particle degradation to influence the immune response. Particle stability is a major determinant of drug-release rate.<sup>22,23</sup> Since immune responses to antigens are

very specific, any changes to the structure and conformation of the antigens destroy their antigenic ability. It is, therefore, always essential to release the protein in its native conformation. The release of aggregated or denatured protein from the microsphere may result in an unwanted immune response. As an example, hydrolyzed acidic metabolites (glycolic, lactic) have the potential to alter pH and cause inflammation. The most notable aspect of the current studies is the entrapped effect and controlled-release effect of potent active NS1 protein achieved through the presentation of NS1 protein inside PLGA/PEG 2:1 microspheres. The NS1 protein-loaded PLGA/PEG microspheres delivery system, and its prospects for achieving a long-term with multiple phase release in one single-dose vaccine, is promising.

## Animal study

### NS1 protein-loaded microspheres immunization and DEN challenge in mice

To study the NS1 protein-specific antibody response elicited by pD2NS1 immunization, pooled sera from groups of DNA-immunized and/or virus-challenged mice were collected and tested by Western blotting using DEN-infected cell lysates as antigens (data not shown). As shown in Figure 3, no NS1-specific antibody responses could be detected in any immunized groups after injections. These results were different from the NS1 protein-loaded PLGA/PEG microspheres that elicited a strong antibody response after one boosting. However, much stronger antibody responses were noticed in NS1 protein-loaded PLGA/PEG microsphere-immunized mice after the virus challenge, indicating a priming effect of DNA immunization in an antigen-specific manner. In addition to Western analysis by using the whole



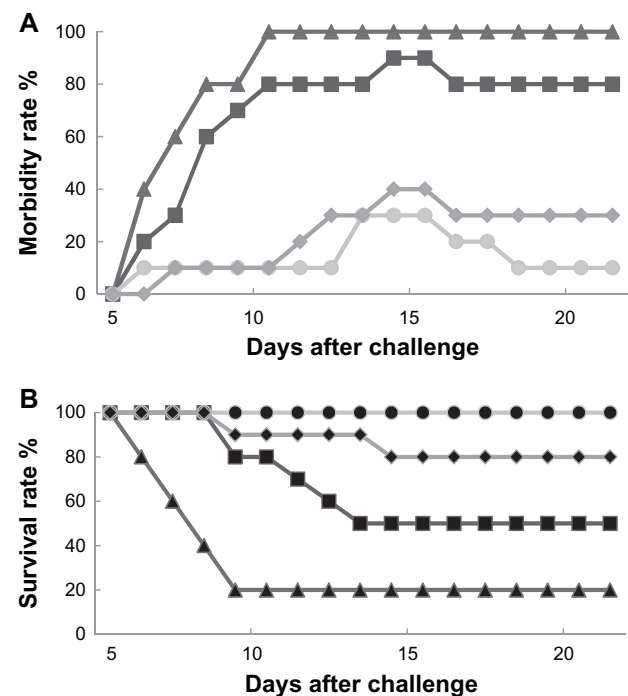
**Figure 3** Dynamics of serum anti-NS1 IgG titer with time by ELISA assay.

**Notes:** ▲, PBS control group; ■, NS1 protein solution (20 µg per mouse); ●, 20 µg NS1 protein-loaded PLGA/PEG microspheres; ◆, 20 µg NS1 protein in Al(OH)<sub>3</sub>. \*\**p* < 0.01.

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; NS1, nonstructural protein 1; PLGA/PEG, poly (*dl*-lactic-co-glycolic) acid/polyethylene glycol; PBS, phosphate buffered saline.

DEN-infected cell lysates as antigens, we also employed purified recombinant NS1 protein as an antigen to detect the DNA-induced antibody response by ELISA. The results shown in Figure 3 indicate a substantial increase of anti-NS1 titers from mice 1 to 3 weeks after the final pD2NS1 immunization, but not in control mice.

To assess the efficacy of NS1 protein-loaded PLGA/PEG microspheres immunization against DEN infection, the mice were checked daily for survival rates for the differential experimental groups at 21 days postchallenge, and the results are shown in Figure 4. The greatest survival was observed in the group of mice immunized with 20 µg NS1 protein-loaded PLGA/PEG microspheres and 20 µg NS1 protein-loaded alum, in which 10 out of 10 tested mice survived the live DEN challenge. The second best survival rate was observed in the group of mice immunized with 20 µg DEN with alum, whose survival rate was 80%. In contrast, the PBS solution alone group failed to confer a comparable level of protection on the immunized mice.



**Figure 4** Evaluation of morbidity (A) and survival (B) rates of C3H mice immunized with pD2NS1 by lethal virus challenges.

**Notes:** Each group of mice (*n* = 10) was injected intramuscularly with DNA vaccines (20 µg per mouse) or PBS, beginning at 3 weeks old. One week after the third immunization, mice were challenged intravenously with  $5 \times 10^6$  plaque-forming units of a Taiwanese DEN-2 strain PL046. Mice were checked every day after viral challenges to observe the pathological symptoms. The morbidity rate of challenged mice is determined by the percentage of mice that developed hind leg paralysis. ▲, PBS control group; ■, NS1 protein solution (20 µg per mouse); ●, 20 µg NS1 protein-loaded PLGA/PEG microspheres; ◆, 20 µg NS1 protein in Al(OH)<sub>3</sub>.

**Abbreviations:** NS1, nonstructural protein 1; PLGA/PEG, poly (*dl*-lactic-co-glycolic) acid/polyethylene glycol; PBS, phosphate buffered saline; DEN-2, dengue virus type 2.

To further investigate the potential protective role of anti-NS1 antibody induced by DNA vaccination, we performed the challenge assay on newborn mice from DNA-immunized dams. Female ICR mice with NS1-DNA vaccination were subsequently mated with male ICR ones. The newborn mice from the DNA-immunized dams were then challenged subcutaneously with NGC-N viruses and the mouse mortality was monitored daily for 3 weeks. Only if all of the newborn mice from the same dam survived was the immunization scored as protective.

Alum, the standard adjuvant used in the majority of immunization regimens, is a trivalent aluminum salt. It was first tested as an adjuvant in a tetanus toxin vaccine. 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. 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. Antigen uptake and CD8+ T-cell activation in DCs treated with soluble antigen, or particles with surface-attached (latex or PLGA particles) or encapsulated antigen (PLGA particles) were reported.<sup>24</sup> Antigen encapsulation resulted in increased cellular uptake of antigen and induced T-cell responses at 1,000-fold lower antigen concentrations than free antigen, and tenfold lower concentrations than surface-attached antigen. While MHC class I presentation of particle-encapsulated antigen remained 80% effective at 96 hours postintroduction, there was no detectable presentation by cells treated with soluble or particle surface-coated antigens. The mechanism of antigen delivery was postulated to influence cross-presentation. Support for this mechanism is the finding that while macropinocytosis of soluble antigen leads to poor MHC class I presentation by APCs, phagocytosis of particle-packaged antigen enhances cross presentation, leading to potent CTL responses.

These results clearly illustrate that intramuscular immunization with the NS1 protein-loaded PLGA/PEG microspheres could protect mice from a live DEN challenge. The NS1 protein 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 NS1 protein vaccine, although equipped with no untoward reactions, has a high survival rate and delivery simplicity. These factors would contribute to a low cost for the vaccination, which is suitable for developing countries where DEN infection epidemics and endemics are problems of public health concern.

## Conclusion

In conclusion, the NS1 protein-loaded PLGA/PEG microspheres resulted in a controlled release rate of NS1 protein over 70 days. Physicochemical characterization data and the release result of the NS1 protein-loaded PLGA/PEG microspheres obtained in this study may be relevant in optimizing the incorporation of the PLGA/PEG microspheres formulation into vaccine delivery. To evaluate the potential of DNA vaccine against dengue infection, we characterized the protective efficacy and immune responses of mice intramuscularly injected with plasmid encoding DEN-2NS1. Intravenously challenged by lethal DEN-2, mice vaccinated with NS1-DNA exhibited a delayed onset of paralysis, a marked decrease of morbidity, and a significant enhancement of survival. Also a moderate increase of NS1-specific antibody titer from immunized mice measured by ELISA was noted.

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## Disclosure

The authors report no conflicts of interest in this work.

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