PLA-PEG as a Delivery System for a *Chlamydia trachomatis* Subunit Vaccine

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ABSTRACT

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infections (STIs) but as yet there is no available vaccine to combat this infection. Different strategies have been employed to meet this challenge, and one such strategy is the development of a *Chlamydia* vaccine using nanoparticle-delivery systems. Previously we reported the successful encapsulation and characterization of a model protein, bovine serum albumin (BSA) in poly (lactic acid)-b-Poly (ethylene glycol) (PLA-PEG) nanoparticles. Here we encapsulated a peptide derivative of *C. trachomatis* major outer membrane protein (MOMP), (named M278) in PLA-PEG and characterized it *in vitro* followed by immunogenicity studies *in vivo*. We hypothesize that M278 encapsulated in PLA-PEG will provide for its slow controlled release and thus enhance its capacity to induce immune responses in mice. M278 was encapsulated in PLA-PEG by the double emulsion method (water/oil/water). Scanning Electron microscopy (SEM) analyses revealed PLA-PEG-M278 to be coagulated particles and in the conformation of what have been referred to as a PEGylated "brush" with rough outer surfaces. The zetasizer measurement showed the sizes of the PLA-PEG-PBS and PLA-PEG-M278 to be ~ 200-272 nm. These findings demonstrate that the encapsulation process had little effect on changing the properties and size of the nanoparticles, which are essential for maintaining the integrity and delivery potential of the particles. The protein encapsulation efficiency of PLA-PEG-M278 was ~ 60%, while the *in vitro* release study showed a slow and continuous release of the M278 protein from PLA-PEG nanoparticles. Three groups of BALB/c mice (PLA-PEG-PBS, PLA-PEG-M278 and M278), received three immunizations at two-week intervals and two weeks after the last immunization sera were collected for systemic antibody analyses. PLA-PEG-M278 immunized mice produced higher IgG and IgG1 M278-specific antibodies as compared to the M278 immunized mice. Our data indicates the successful encapsulation and characterization of M278 in PLA-PEG and, more importantly, that PLA-PEG enhanced the capacity of M278 to induce antibody responses in mice. These findings suggest that the PLA-PEG-M278 holds considerable promise as a nanovaccine against *C. trachomatis,* and warrants efficacy studies in mice

Key words: *Chlamydia trachomatis*, bacteria, Major outer membrane protein, poly (lactic acid)-b-Poly (ethylene glycol), antibody.

1.0 INTRODUCTION

Genital-tract infection with the obligate intracellular bacterium, *Chlamydia trachomatis*, poses a significant risk, especially in women. Infection with *Chlamydia* can result in acute salpingitis and pelvic inflammatory disease (PID), whose long-term consequences include chronic pain, ectopic pregnancy and infertility. According to the World Health Organization, there are an estimated 4 to 5 million new cases of chlamydial infections each year in the USA alone. Among urban adolescent females, the incidence rate can be as high as 30%. The annual costs of treating and caring for 4 million patients with genital *Chlamydia* infection might be as high as $3 billion. Worldwide it is estimated that more than 500 million people still are at high risk of infection. Over 140 million persons are infected and about 6 million are blind in Africa, the Middle East, Central and South-East Asia, and countries in Latin America [1].

Nano-particulate delivery systems belong to a category of adjuvants that facilitate antigen uptake by antigen presenting cells (APCs) or by increasing the influx of professional APCs into the injection site. PLA-PEG microparticles and nanoparticles are extensively studied as drug delivery vechicles. They offer the advantages of controlled, sustained drug release, sub-cellular size and biocompatibility. Di-block nanoparticles like Poly(D,L-lactide-co-glycoide) (PLGA) and PLA-PEG provide controlled/sustained release properties, subcellular size, and biocompatibility with tissues and cells [2 and 3], and they are well established carrier systems with high potential for delivery of bioactive macromaolecules, including peptides, proteins and nucleic acid vaccines.

Recently we reported that a peptide derivative of *C. trachomatis* major outer membrane protein (MOMP), (named M278) induces strong systemic and mucosal immune response [2]. However, there are very few studies that have employed PLA-PEG as a delivery system for vaccine candidates [4]. This study was designed to encapsulate M278 in PLA-PEG and characterized it *in vitro* followed by immunogenicity studies *in vivo*. We hypothesize that M278 encapsulated in PLA-PEG will provide for its slow and controlled release, and thus enhance its capacity to induce immune responses in mice. Findings of this study are presented in this manuscript.

2.0 MATERIALS AND METHODS

2.1 Preparation of Nanoparticles

M278 was encapsulated in PLA-PEG Diblock polymer nanoparticles by a modified water/oil/water double emulsion–evaporation technique [2]. Briefly 500 mg of the PLA-PEG was emulsified in 10 mL of ethyl acetate. To the primary emulsion, 2 mg of M278 was added, sonicated, followed by addition of polyvinyl alcohol (PVA) in distilled water (1%). The resulting double emulsion was gently stirred overnight at room temperature (RT) to evaporate the organic solvents resulting in formation of M278-encapsulated in PLA-PEG (PLA-PEG-M278) nanoparticles (NPs). Nanoparticles were harvested by centrifugation, washed three times in distilled water and lyophilized in the presence of 5% trehalose, and then stored at -80^{o}C in a sealed container until used. Sterile PBS was used in the primary emulsion formation to prepare PLA-PEG-PBS blank nanoparticles to serve as a negative control.

2.2 PLA-PEG-M278 Nanoparticles Size and Zeta Potential Determinations

The particle sizes of PLA-PEG-PBS and PLA-PEG-M278 NPs were measured by dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, UK). Samples of PLA-PEG-PBS and PLA-PEG-M278 were suspended in distilled water, sonicated for 5 min, and then placed in a disposable cuvette for size and zeta potential measurements. Each sample was measured in triplicates for each preparation of NPs and is reported as the average.

2.3 Scanning Electron Microscopy (SEM)

The morphology of the freeze-dried NPs: PLA-PEG-PBS and PLA-PEG-M278 was investigated by SEM (Zeiss EVO 50 VPSEM). The NPs were mounted on metal pegs using conductive double-sided tape, and sputter coated with a gold layer prior to SEM analyses.

2.4 *In vitro* Release of M278 from PLA-PEG NPs and Ultra Violet Visualization (UV-Vis)

The release of the M278 peptide from PLA-PEG-M278 was determined as described [1]. Briefly, PLA-PEG-M278 and PLA-PEG-PBS (50 mg each) were each suspended in PBS containing 0.01% sodium azide. Suspensions were incubated at 37^{o}C and at various time intervals (0.5 to 7.5 h, and days 1–21) supernatants were collected by centrifugation at 12,000 rpm for 5 min and kept at -20^{o}C until used. The Micro BCA protein assay was used to quantify M278 in supernatants and the absorbance was read at 570 nm using a microplate reader (TECAN US Inc., Durham, NC).

The absorbance spectra of M278 and NPs were determined using the DU 800UV/Vis spectrophotometer program. NPs were diluted in 1 mL deionized water and the absorbance for each spectrum wavelength was observed and used to determine whether M278 was encapsulated within the PLA-PEG NPs.

2.5 Mouse Immunization

Female BALB/C mice (5-6 weekold) were purchased from Charles River Laboratory (Raleigh, NC). Animals were housed according to Alabama State University IACUC guidelines, under standard pathogen-free environmental conditions at ambient temperature, and supplied with food and water. Three groups of mice, each containing 4 mice per group, were immunized subcutaneously as follows: Group-1 (PLA-PEG-PBS), Group-2 (PLA-PEG-M-278) and Group-3 (M-278) all at (50 ug/dose) in 100 uL of sterile PBS (pH 7.4). Mice received a total of 3 immunization at 2-week intervals. Two weeks after the last immunization animals were euthanized by CO_2 inhalation (Figure 1).

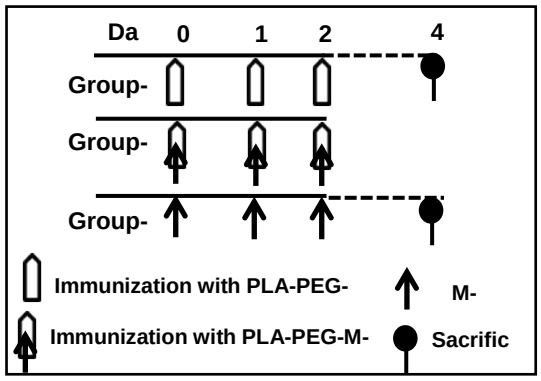

Figure 1. Scheme of immunization experiments in BALB/C mice.

2.6 Serum Antibody Determination

ELISA was employed to determine the antibody level in sera collected from groups of mice To determine the anti-M278 antibody response, 100 µL of purified M278 antigen was coated in ELISA plate at a 1 µg/mL concentration. For antibody concentrations, serial dilutions (1:250, 1:500, and 1:1000) of the samples were made in antibody buffer (2% bovine serum albumin in PBS with 0.05% Tween 20), and ELISAs were performed essentially as already reported by us [2] using HRP-conjugated goat anti-mouse Ig (1:4000) secondary antibody (Southern Biotech Associates (SBA), Birmingham, AL) and TMB as substrate. Absorbance was read at 450 nm using a Tecan plate reader. Isotype determination kits (SBA) were used for antibody isotype determination as described above.

3.0 RESULTS AND DISCUSSIONS

3.1 SEM, Zetasizer and Zeta potential

SEM was employed to assess the morphological characteristics of PLA-PEG-PBS and PLA-PEG-M278. PLA-PEG-PBS images appeared to be coagulated particles and in the conformation of what has been referred to as a PEGylated "brush" with a rough outer surface (Figure 2A). This may be attributed to the hydrophobic and hydrophilic domains of the NP, which will have separate dispositions following lyophilization. Magnification of this brush structure of the PLA-PEG M-278 sample led to the discovery of a web-like matrix that contained grape-like structures dispersed throughout the image (Figure 2 B).

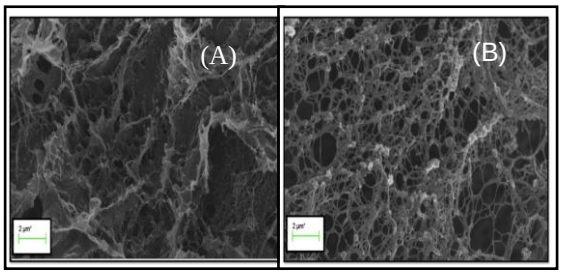

Figure 2. (A) SEM photograph of PLA-PEG-PBS, (B) PLA-PEG-M278.

The average size of the PLA-PEG-PBS, and PLA-PEG M278 NPs was 272 nm (Figure. 3 A) for both particles. The zeta potential value is one of the most important particle characteristics because it can affect both particle stability and particle mucoadhesion. The zeta potentials of PLA-PEG-PBS and PLA-PEG-M278 were 30 mV and 29 mV, respectively (Figure. 3 B). These findings demonstrate that the encapsulation process had little effect on changing the properties and size of the NPs, which are essential for maintaining the integrity of the particles.

3.2 Cummulative release of M278 from PLA-PEG NPs and UV-Vis

The *in vitro* release profile of M278 from PLA-PEG-M278 was one of a triphasic release pattern (Figure 4A). The first phase corresponds to the initial fast release in the first 8 h (18-21%). This kind of release is advantageous in case of vaccine delivery, as it gives a primary immunization effect.

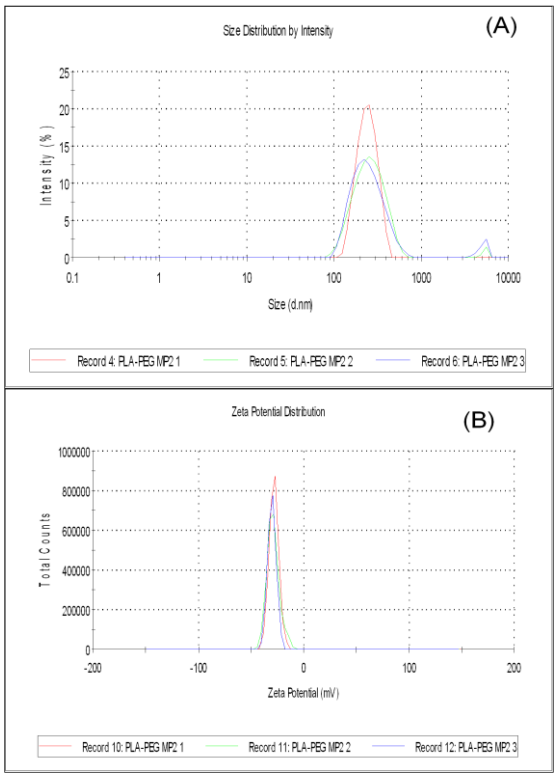

Figure 3. Size distribution (A) and zeta potential (B) of PLA-PEG-M278 NPS. NPs were diluted with distilled water, sonicated, and filtered to obtain homogeneous suspensions before analyses.

The second phase demonstrates a constant continuous release of M278 that was controlled by diffusion. The third phase release began by day 3, and was probably dominated by the PLA-PEG degradation release due to the PLA core which is responsible for generating a booster effect in an *in vivo* release system. PLA-PEG-M278 NPs showed a 31-34% cumulative M278 release by day 20.

It is possible that during the course of encapsulation of protein in NPS, absorbance of proteins to the outer surface of NPs can occur, which could result in the burst effect. To ensure that M278 was successfully encapsulated, we performed UV-Vis analysis which revealed the absorbance spectrum of M278 at a wavelength of ~285 for proteins. Essentially no absorbance was attained for PLA-PEG-M278 PLA-PEG-PBS, thus verifying the successful encapsulation of M278 (Figure 4 B).

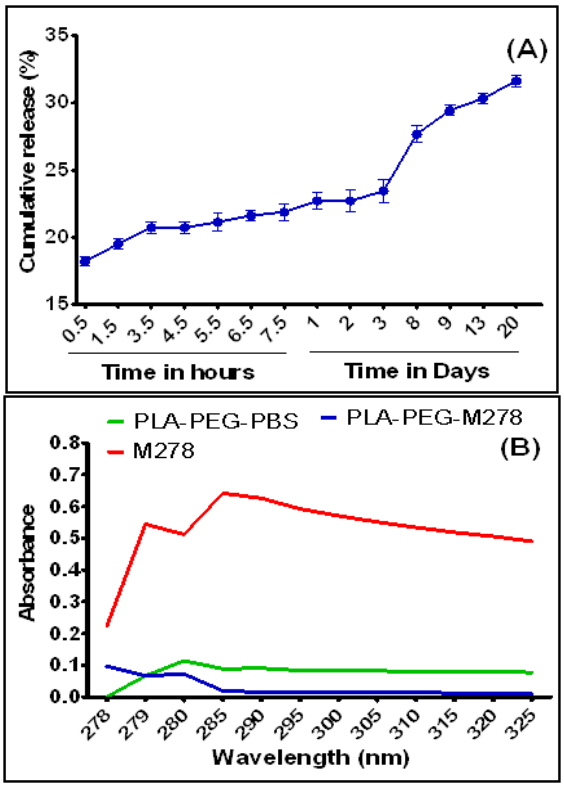

Figure 4. (A) *In vitro* release of the M278 peptide from PLA-PEG-M278 NPs. Release of M278 from PLA-PEG-M287 was performed in PBS (pH 7.4). Data represent the mean +/- SD of triplicate experiments. (B) Comparative analyses of peak variations of M278, PLA-PEG-M278 and PLA-PEG-PBS. UV-visible spectra of M278 (red), PLA-PEG-PBS (green) and PLA-PEG-M278 (blue) were performed in deionized water at 25^{o}C.

3.3 Serum Antibody

To study the serum antibody immune responses, serum samples were collected from mice on days 42 post-immunizations and analyzed for the presence of M278 specific antibodies. The levels of IgG and IgG1 isotype antibodies were significantly enhanced in sera from PLA-PEG-M278 immunized mice compared to those produced in the M278 or PLA-PEG-PBS immunized mice (P < 0.001). This enhanced antibody response may be attributed to the slow release of M278 from PLA-PEG to bolster the antibody immune reponses (Figure 5).

4.0 CONCLUSION

Overall, our study shows the successful encapsulation and characterization of M278 peptide in PLA-PEG NPs. Physiochemical characterization studies confirmed M278 encapsulation in PLA-PEG as being small, stable, and with slow release properties.

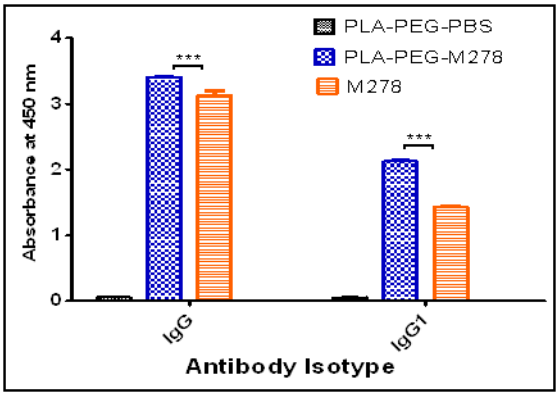

Figure 5. Serum antibody profiles in immunized groups of mice. Pooled sera from immunized mice were used to detect IgG and IgG1 antibody isotypes by ELISA.

More importantly, PLA-PEG enhanced the capacity of M278 to induce antibody responses in mice. These findings suggest that the PLA-PEG-M278 holds considerable promise as a nanovaccine against *C. trachomatis*, and warrants efficacy studies in mice.

5.0 REFERENCES

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