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Poly (lactic acid)-poly (ethylene glycol) nanoparticles provide sustained delivery of a *Chlamydia trachomatis* recombinant MOMP peptide and potentiate systemic adaptive immune responses in mice

Saurabh Dixit, Ph.D^a, Shree R. Singh, Ph.D^a, Abebayehu N. Yilma, Ph.D^{a,1}, Ronald D. Agee II, MS^{a,2}, Murtada Taha, Ph.D^{a,3}, and Vida A. Dennis, Ph.D^{a,*}

^aCenter for NanoBiotechnology Research (CNBR), Alabama State University, Montgomery, AL USA

Abstract

PLA-PEG [poly (lactic acid)-poly (ethylene glycol)], a biodegradable copolymer, is underexploited for vaccine delivery although it exhibits enhanced biocompatibility and slow release immune-potentiating properties. We document here successful encapsulation of M278, a *Chlamydia trachomatis* MOMP (major outer membrane protein) peptide, within PLA-PEG nanoparticles by size (~73–100 nm), zeta potential (–16 mV), smooth morphology, encapsulation efficiency (~60%), slow release pattern, and non-toxicity to macrophages. Immunization of mice with encapsulated-M278 elicited higher M278-specific T-cell cytokines [Th1 (IFN- γ , IL-2), Th17 (IL-17)] and antibodies [Th1 (IgG2a), Th2 (IgG1, IgG2b)] compared to bare M278. Encapsulated-M278 mouse serum inhibited *Chlamydia* infectivity of macrophages, with a concomitant transcriptional down-regulation of MOMP, its cognate TLR2 and CD80 co-stimulatory molecule. Collectively, encapsulated-M278 potentiated crucial adaptive immune responses, which are required by a vaccine candidate for protective immunity against *Chlamydia*. Our data highlights PLA-PEG's potential for vaccines, which resides in its slow release and potentiating effects to bolster immune responses.

Keywords

Chlamydia trachomatis; bacteria; PLA-PEG nanoparticles; antibody; cytokines

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Corresponding author: Vida A. Dennis, Ph.D, Center for NanoBiotechnology Research (CNBR), Alabama State University, 1627 Hall Street, Montgomery, AL, 36104; Phone: (334)-229-8447, vdennis@alasu.edu.

¹Global Institute of Public Health, New York University, New York, NY

²College of Podiatric Medicine, Western University of Health Sciences, Pomona, CA

³Department of Natural Sciences, Albany State University, Albany, GA

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Background

Biodegradable polymers typically employed for fabrication of nanoparticles provide controlled release, low toxicity, high encapsulation efficiency, sub-cellular size and bio-distribution *in vivo*¹. They are also excellent delivery systems for bioactive macromolecules, including peptides and proteins² and can be fabricated to evoke immune responses, either by direct stimulation of antigen presenting cells (APCs) or delivering antigens to specific cellular compartments³. Reportedly, PLA-PEG [poly (lactic acid)-poly (ethylene glycol), a biodegradable copolymer, can control release of proteins over several weeks⁴, which is ideally attractive for vaccine delivery⁵. PLA-PEG sustained release, sub-cellular size, and enhanced biocompatibility facilitate uptake of antigens by APCs and their influx to the injection site⁶. Encapsulation within PLA-PEG has tremendous benefits *in vivo* including protecting the integrity and activity of biomolecules while augmenting their immune-potentiating effects. This copolymer apparently enhances the therapeutic activity of encapsulated drugs^{5,7} and consequently is more commonly used for drug delivery. Conversely, it is not widely exploited for vaccine delivery⁸, in spite of its aforementioned inherent properties, which are desirable for vaccines.

Chlamydia trachomatis, the most frequently reported bacterial sexually transmitted infection worldwide, causes considerable morbidity and socioeconomic burdens⁹. Currently, there is no approved vaccine against *Chlamydia*, perhaps due to ineffective delivery systems or formulations that do not bolster immune responses to achieve long-lasting protective immunity against this intracellular pathogen. The major outer membrane protein (MOMP) of *C. trachomatis* is highly immunogenic and hence frequently employed with conventional adjuvants for vaccine development. Nevertheless, these vaccine formulations have only afforded partial protection against *Chlamydia* infections^{10–12}. Adjuvants although commonly used for vaccine studies suffer from side effects, ineffectiveness to certain antigens, poor ability to elicit cell-mediated immune responses¹³, inconsistencies in producing humoral immunity, and instability to freezing and drying, which may restrict their applicability against intracellular pathogens¹⁴. Thus, there is a necessity to explore alternative nonconventional delivery systems for MOMP, such as biodegradable nanoparticles, which can stimulate long-lasting protective immune responses.

In the present study, we employed PLA-PEG as a delivery system for M278¹⁵, a *C. trachomatis* recombinant MOMP peptide, and hypothesized that encapsulation of M278 will provide for its sustained slow release to potentiate and bolster immune responses in mice. First, by *in vitro* studies we determined the physiochemical characteristics of encapsulated-M278 (size, zeta potential, morphology, absorbance and chemical compositions), encapsulation efficiency, release pattern, and toxicity to macrophages. Next, we compared adaptive immune response outcomes triggered by encapsulated-M278 with that of bare M278 in immunized mice by quantifying M278-specific cellular (Th1, Th2 and Th17 cytokines, and chemokines) and serum antibodies (Th1 and Th2). Finally, we determined immune serum-mediated inhibition of *Chlamydia* infectivity of macrophages, and the ensuing effect on the mRNA transcriptional expression of MOMP, its cognate TLR2, and the CD80 co-stimulatory molecule. Herein, we present and discuss our findings.

Methods

Fabrication of nanoparticles

Recombinant MOMP-278 (M278) was cloned as previously reported¹⁵ and encapsulated in PLA-PEG nanoparticles using a modified water/oil/water double emulsion evaporation technique^{16,17} and then lyophilized in the presence of 5% trehalose (as a stabilizer) to obtain encapsulated-M278 (PLA-PEG-M278). Phosphate buffered saline (PBS) was similarly encapsulated in PLA-PEG to serve as a negative control (PLA-PEG-PBS). All lyophilized nanoparticles were stored at -80°C in a sealed container until used.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The morphology of nanoparticles was assessed using SEM (Zeiss EVO 50 VPSEM) and TEM (Zeiss EM10 TEM) as recently published^{16–18}. For SEM, nanoparticles were mounted on metal pegs using conductive double-sided tape and sputter coated with a gold layer prior to examination. For TEM, nanoparticles were dispensed in distilled water and added to formvar/carbon grids prior to microscopy analysis.

Zetasizer and zeta potential measurements

The mean sizes and zeta potentials of nanoparticles were measured using a zetasizer Nano-ZS instrument (Malvern Instruments, UK)^{16–18}. Each nanoparticle sample was measured in triplicates.

Fourier transform-infrared (FT-IR) spectrometry

Chemical analyses of the functional groups present in nanoparticles were determined using FT-IR^{16,18}. The spectra were obtained with 64 scans per sample ranging from 4000 to 500 cm^{-1} and a resolution of 4 cm^{-1} .

Ultraviolet visualization (UV-Vis)

UV-Vis was employed to ascertain M278 encapsulation within PLA-PEG as recently described^{17,18}. Nanoparticles and bare M278 were each diluted in deionized water and their absorbance and spectral wavelengths were assessed using the DU 800UV/Vis spectrophotometer (Beckman Coulter, Fullerton, CA).

Encapsulation efficiency

The encapsulation efficiency (EE) was extrapolated from measurements of the total M278 encapsulated within PLA-PEG as described previously^{16,17} and calculated as: $\text{EE} = \frac{A-B}{A} \times 100\%$, where A is the total M278 amount, B is the free M278 amount. These measurements were performed three times.

In vitro release of encapsulated-M278

Release of encapsulated-M278 was determined as reported^{16, 17}. Briefly, nanoparticles (100 mg each) were suspended in PBS containing 0.01% sodium azide and incubated at 37°C . At predetermined time-intervals (up to 20 days), tubes were centrifuged, and the supernatants were removed followed by replenishment of fresh PBS to nanoparticles. The Micro BCA

protein assay was used to quantify M278 in collected supernatants with absorbance readings at 562 nm using a microplate reader.

Cell viability assay

The effect of nanoparticles on mouse J774 macrophages viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay and the Cell-Titer 96 Cell Proliferation Assay kit (Promega) as described¹⁹. Absorbance at 570 nm was measured using a microplate reader.

Mice immunizations

Female 6–8 weeks old BALB/c mice (Charles River Laboratory, Raleigh, NC) were used for this study. The animal studies were performed following a protocol approved by the Alabama State University Institutional Animal Care and Use Committee (IACUC). Mice were housed under standard pathogen-free environmental conditions at ambient temperatures of 25°C, and provided with sterile food and water *ad libitum*. Four groups of mice (n=6) received three subcutaneous immunizations at two-week intervals with PBS, M278 and nanoparticles (PLA-PEG-PBS or PLA-PEG-M278) (Figure 1). The PLA-PEG-M278 mice each received 50 µg/100 µL of encapsulated-M278 in PBS; those in the PLA-PEG-PBS group received an equivalent weight of nanoparticles in 100 µL of PBS. Mice in the M278 group received 50 µg/100 µL of purified M278; those in the PBS group received 100 µL of PBS only. Two weeks following the last immunization (day 42), all mice were sacrificed to collect spleen and serum samples for cellular and antibody analyses, respectively.

T-cells purification and stimulation

Spleens were collected from groups of immunized mice and pooled per group in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Single spleen cell suspensions were obtained using gentleMACS™ Dissociator (Miltenyi Biotec, Auburn, CA), filtered through 40-µm nylon mesh strainer (BD Biosciences), and washed prior to red blood cells lyses using ACK lysing solution (Gibco). Cells were incubated with anti-CD 90.2-conjugated magnetic beads and total T cells were isolated by positive selection over MACS columns (Miltenyi Biotec). Purity of T cells was greater than 95% as assessed by flow cytometry employing PE-conjugated anti-CD 90.2 antibodies (data not shown). Naïve single spleen cell suspensions were treated with Mitomycin-C (25 µg/mL) for 30 min at 37°C in a 5% CO₂ humidified atmosphere to serve as antigen presenting cells (APCs).

Purified T-cells and APCs at 2×10^6 /mL (1×10^6 T cells and 1×10^6 APCs) co-cultures were stimulated with purified M278 (10 µg/mL) in 24-well plates and incubated at 37°C in a 5% CO₂ humidified atmosphere. The selected 10 µg/mL of M278 was based on previous titration studies in our laboratory. Cell free-culture supernatants were collected by centrifugation after 48 hr and stored at –80°C until used.

Quantification of cytokines and chemokines

Cytokines and chemokines were quantified in cell free-culture supernatants using multiplex ELISA as described^{17,19–21}. Only those cytokines and chemokines that were statistically

significant ($P < 0.05$) above the detection limit (3.2 pg/mL) are reported in this study. The multiplex ELISA was repeated twice and each sample was run in triplicates.

Quantification of serum antibodies

Sera collected from groups of mice were pooled (per group) and used to detect M278-specific antibodies as previously described^{11,17} except that 1 µg/mL of purified M278 was used to coat plates in the present study.

Serum inhibition assay

Serum inhibition assay was used to investigate the functional role of immune serum on *C. trachomatis* infectivity of (or multiplication in) mouse J774 macrophages. Macrophages were plated at 10^6 /mL/well in 24-well plates and infected with live *C. trachomatis* (10^5 inclusion forming unit, IFU/well) in antibiotic-free DMEM medium in the presence or absence of pooled immune serum (1:100 dilution). The plates were incubated at 37°C in a 5% CO₂ humidified atmosphere, and after 48 hr supernatants were removed by centrifugation, and cells were washed three times with PBS prior to extraction of total RNA using Qiagen RNeasy kit.

Quantitative real-time PCR (qRT-PCR)

TaqMan qRT-PCR was employed as described²⁰ to assess the mRNA gene transcripts of MOMP (as a marker of *C. trachomatis* infectivity of macrophages), TLR2 (*C. trachomatis* cognate receptor) and the CD80 co-stimulatory molecule. TaqMan qRT-PCR was conducted using TaqMan RNA-to-Ct 1-step kit and TaqMan gene expression assay (for TLR2, CD80 and GAPDH) according to the manufacturer's instructions (Life Technologies). MOMP primers and probe were designed using primer express software (Life Technologies). All values were normalized with respect to the GAPDH "housekeeping" gene mRNA levels. Results are presented as the fold increase over control (unstimulated cells) using the CT method²⁰.

Immunofluorescence staining (IFA)

We conducted IFA to confirm *C. trachomatis* infectivity of mouse J774 macrophages. Macrophages (2.5×10^4 cells/well) were seeded in 8-well chamber slides and infected with live *C. trachomatis* (2.5×10^3 IFU/well) in the absence or presence of pooled immune serum (1:100 dilution). After 48 hr, macrophages were washed, fixed with 2% paraformaldehyde and blocked with 10% normal goat serum. Macrophages were stained with FITC-conjugated anti-*C. trachomatis* polyclonal antibody (1:2000 dilution) as reported²² and PE-conjugated macrophage surface marker ER-MP58 (1:100 dilution) for 1 hr, washed, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) combined with an anti-fade mounting solution (Life Technologies). *Chlamydia* was visualized in macrophages using a Nikon Eclipse Ti Confocal Microscope (Nikon Instrument, Melville, NY).

Statistical analysis

Data were analyzed by one- or two-way analysis of variance (ANOVA) followed by Tukeys post-hoc test or the two-tailed Mann Whitney test using GraphPad Prism 5 Software. Significance was established at $P < 0.001 = ***$, $P < 0.01 = **$ and $P < 0.05 = *$.

Results

Physiochemical properties of nanoparticles

SEM and TEM microscopy techniques were employed to assess the morphology and size of nanoparticles. By SEM analysis, PLA-PEG-PBS appeared to be coagulated particles and in the conformation of what is referred to as PEGylated “brush” with rough outer surface²³ (Figure 2, A). Magnification of this brush structure within PLA-PEG-M278 revealed a web-like matrix that contained grape-like structures dispersed throughout (Figure 2, B). Additionally, TEM images of PLA-PEG-PBS (Figure 2, C) and PLA-PEG-M278 (Figure 2, D) revealed their appearances to be spherical and smooth in shape with nanosizes < 100 nm.

As particle size plays an important role in determining the level of cellular and tissue uptake²⁴, we employed zetasizing, which revealed PLA-PEG-PBS and PLA-PEG-M278 were 83 and 73 nm, respectively (Figure 2, E, F), thereby corroborating the TEM results. Next, we measured the zeta potential, which is one of the most essential particle characteristics affecting particle stability. The zeta potentials of PLA-PEG-PBS (Figure 2, G) and PLA-PEG (Figure 2, H) were -10 mV and -16 mV, respectively. These findings demonstrate that encapsulation of M278 did not adversely change the property and size of the nanoparticle, which are essential for maintaining its integrity and stability.

FT-IR and UV-Vis analyses

FT-IR further identified variations in chemical functional groups within and between PLA-PEG nanoparticles. Appearances of typical characteristic peaks for -C-H stretch (at 2883 cm^{-1}), C-O stretch (at 1452 cm^{-1}) -C(O)-O-C-(ester C-O) group formation (at 1342 cm^{-1}) and the presence of the ester carbonyl (CO) group (at 1751 cm^{-1}) were observed within the PLA-PEG-PBS nanoparticles (Figure 3, A). After encapsulation, the positions of some characteristic peaks shifted towards lower frequency for -C-H stretch (to 2880 cm^{-1}) and towards higher frequency for the -C(O)-O-C-(ester C-O) group (to 1352 cm^{-1}). This shift in the spectra after encapsulation may indicate a chemical interaction between M278 and PLA-PEG. The presence of M278 within PLA-PEG-M278 is evident with the -NH peak (protein amide band/amines) frequency (at 1650 cm^{-1}) (Figure 3, B) that is absent from within PLA-PEG-PBS. This finding provides additional evidence of successful encapsulation of M278 within PLA-PEG nanoparticles.

To rule out the possibility of a burst effect, which can result from adsorption of protein to the outer surface of nanoparticles during fabrication, we next compared their absorbance spectra with that of bare M278. As shown in Figure 3, C, UV-Vis of bare M278 showed protein absorption at a wavelength of ~ 285 nm. In contrast, minimal absorption was seen on the outer surface of nanoparticles, further validating the successful encapsulation of M278.

Encapsulation efficiency and release profile of M278 from PLA-PEG

A modified water/oil/water double emulsion method was employed for fabrication of encapsulated-M278, resulting in ~60 % encapsulation efficiency. The cumulative release of encapsulated-M278 was one of a biphasic release pattern (Figure 3, *D*) whereby phase one was rapid and lasted up to day 1 (18–21%), followed by a gradual release over a 20-day period. Only 32% of M278 was released the entire time-period, thus confirming its sustained slow release profile.

Cytotoxicity effect of PLA-PEG nanoparticles on mouse macrophages

Toxicity is of major concerns when using nanoparticles in biomedical applications, including even biodegradable polymers. Given the significance of macrophages in innate immunity and *Chlamydia* replication in macrophages, we tested the toxicity of nanoparticles against mouse J774 macrophages. Both dose and time-dependent toxicity studies were conducted by exposing macrophages to concentrations of nanoparticles ranging from 2 to 0.1 mg/mL over a 24, 48 and 72 hr time-period. At all examined time-points, all concentrations of nanoparticles do not affect the viability of macrophages (Figure 3, *E* and *F*), which indicate they are non-toxic to macrophages, and substantiate their safety and biocompatibility for *in vivo* studies.

Cellular adaptive immune responses

Next, we compared potentiating of T cell-specific cellular immune responses by PLA-PEG-M278 with that of bare M278 in mice (on day 42 post-immunization). Co-cultures of purified T cells and APCs were re-stimulated with M278 to quantify cytokines and chemokines by multiplex ELISA. Compared to bare M278, immunization with PLA-PEG-M278 significantly augmented ($P < 0.001$) M278-specific T-cell responses including Th1 (IFN- γ , IL-2), Th17 (IL-17), IL-3, GM-CSF, M-CSF and LIF cytokines (Figure 4, *A-G*) along with the CXCL10 chemokine (Figure 4, *H*). IL-4 (Th2 cytokine) was also secreted, albeit of lower level and significance ($P < 0.05$) (Figure 4, *I*). In contrast, T-cells from bare M278-immunized mice produced significantly ($P < 0.001$) higher IL-5 (Th2 cytokine) as compared to PLA-PEG-M278 immunized mice (Figure 4, *J*). Control groups of mice (PBS and PLA-PEG-PBS) did not produce T cell-specific responses (Figure 4, *A-J*). Our results show that encapsulated-M278 potentiates adaptive T cell-specific immune responses in mice.

Antibody adaptive immune responses

We also investigated the capacity of encapsulated-M278 to potentiate antibody immune responses in mice by quantifying M278-specific serum antibodies on day 42 post-immunization by ELISA. Immunization with PLA-PEG-M278 elicited significantly ($P < 0.001$) higher IgG, IgG2a (Th1) as well as IgG1 and IgG2b (Th2) antibodies compared to bare M278 immunized mice (Figure 5, *A*). Moreover, the antibody response was predominantly Th2-driven with IgG1/IgG2a and IgG2b/IgG2a ratios of 6.7 and 14, respectively.

We further determined isotype-specific antibody titers by performing serial 2-fold dilutions of sera and observed that PLA-PEG-M278 induced higher isotype-specific antibody titers than did bare M278 with fold differences of 4, 4, 16 and 64, respectively for IgG, IgG1, IgG2a and IgG2b (Figure 5, B), again suggesting more of a Th2-driven response. Control mice (PBS and PLA-PEG-PBS) did not produce serum-specific antibodies (Figure 5, A and B). These findings confirm the encapsulated-M278 potentiating effect also on antibody adaptive immune responses in mice.

In vitro serum inhibition studies

Because of the high levels of serum antibodies triggered by PLA-PEG-M278 in mice, we hypothesized, that immune serum might function to inhibit *Chlamydia* infectivity and/or replication in macrophages. Therefore, we infected mouse J774 macrophages with *C. trachomatis* in the absence or presence of immune serum followed by RNA extraction to quantify MOMP mRNA gene transcript, as a marker of infectivity. Expression of MOMP was significantly reduced ($P < 0.001$) in macrophages exposed to serum from PLA-PEG-M278 immunized mice (Figure 6, A), suggesting, perhaps, less *C. trachomatis* infectivity. In contrast, MOMP expression was significantly enhanced ($P < 0.001$) in macrophages exposed to serum from other immunized groups (PLA-PEG-PBS, PBS and M278; Figure 6, A), which may suggest more *C. trachomatis* infectivity of macrophages.

Given that TLR2 is critical for *Chlamydia*-mediated host cell activation and MOMP signaling, we assessed in parallel the ensuing impact of reduced MOMP expression on TLR2 mRNA gene transcripts in macrophages. TLR2 expression was reduced ($P < 0.001$) in macrophages exposed to PLA-PEG-M278 immune serum as compared to other groups (PLA-PEG-PBS, PBS and M278) of mice (Figure 6, B). Moreover, we similarly observed significant reduction ($P < 0.05$) in the expression of the CD80 co-stimulatory molecule (Figure 6, C).

To confirm infectivity, IFA was used for direct visualization of *C. trachomatis* within macrophages. As shown in Figure 6, columns 1, 2, 3 and 4, respectively reflect staining for nuclear (blue fluorescence), macrophage surface (red fluorescence), anti-*Chlamydia* antibody (green fluorescence) and their overlay images (yellow fluorescence). Our results demonstrate that cells alone did not contain *Chlamydia* (Figure 6, D). However, marked infectivity of cells was seen in the presence of PLA-PEG-PBS immune serum (Figure 6, E), and less infectivity when cells were exposed to PLA-PEG-M278 immune serum (Figure 6, F). Cells exposed to M278 immune serum (Figure 6, G) and PBS (Figure 6, H) were also markedly infected with *Chlamydia*, thus underscoring the inhibition of *Chlamydia* infectivity of macrophages by encapsulated-M278 mouse immune serum.

Discussion

Problems associated with conventional adjuvants for vaccines are protein denaturation and low bioavailability *in vivo*²⁵. PLA-PEG offers an attractive alternative to conventional adjuvants as a vaccine-delivery system because it can augment the immunogenicity of proteins by providing their controlled slow release to the immune system^{1,2,26}. Moreover, PLA-PEG can enhance the protein loading capacity, reduce its burst effect, prevent

degradation, and increase the circulation time and bioavailability of proteins without altering their spatial configuration^{27,28}. In the present study, we exploited PLA-PEG immune-potentiating and controlled slow release properties as a vaccine-delivery system for M278, a *C. trachomatis* MOMP peptide. We show (i) successful M278 encapsulation, its *in vitro* physiochemical characterization and release profile (ii) that encapsulated-M278 triggered higher specific cellular and antibody adaptive immune responses in mice, as compared to bare M278, and (iii) that encapsulated-M278 immune serum markedly inhibited *Chlamydia* infectivity of macrophages, with concomitant transcriptional down-regulation of MOMP, its cognate TLR2, and the CD80 co-stimulatory molecule.

Encapsulating proteins in biodegradable polymers has become of major concerns because heat generated during sonication and organic solvents may adversely affect their biological functions. Here we employed ethyl acetate, an organic solvent of PLA, which in the presence of PEG acts as a surfactant in an ethyl acetate-water system²⁹ and PVA as the emulsifying agent for fabricating stable PLA-PEG nanoparticles. PLA-PEG-M278 nanoparticles had a -16 mV zeta potential, 73–100 nm size and ~60% encapsulation efficiency, thus corroborating the > -10 mV zeta potential and $> 50\%$ encapsulation efficiency attained for other PLA-PEG nanoparticles fabricated with ethyl acetate^{28–30}. The inability of PLA-PEG-M278 to affect macrophages viability may have resulted from their non-toxic properties or alternatively to their rapid uptake and processing by macrophages, henceforth greater viability. Indeed, subcellular sizes of fabricated PLA-PEG, as reported herein, can facilitate uptake of antigens by APCs⁶. In addition, the PEGylated brush structures visualized via SEM, most likely contributed to augmenting PLA-PEG-M278 stability by forming a “sheath” like system due to the hydrophobic and hydrophilic domains of the nanoparticle having separate dispositions following lyophilization. Reportedly, this brush structure can prevent adsorption of biological components that trigger the uptake of particulates by macrophages, and aid in the stability of proteins^{30,31}.

An initial burst release of encapsulated-M278 followed by a gradual slow release of 30–32% over a 20-day period was observed and is consistent with previous reports whereby hepatitis antigen encapsulated within PLA-PEG exhibited a 40% cumulative release over a 42-day period⁸. We did not investigate the mechanism(s) of encapsulated-M278 release but it may have resulted from degradation of the nanomatrix and diffusion and/or collaborative processes²⁷. Most studies attribute an initial burst release to outer protein adsorption³², however our UV-Vis data supports M278 encapsulation, and further suggests that its initial burst and sustained release may result from the overall degradation kinetics of PLA-PEG. Further support of our data comes from an elegant pharmacokinetics study conducted in mice, which documented that PLA-PEG prolonged the half-life of the antiretroviral drug zidovudine (AZT) delivery over time by increasing its $T_{\max}$ ³³. These investigators also reported that, due to the slow release of AZT from within PLA-PEG, its metabolic breakdown was slower resulting in an increase in both its main half-life and bioavailability³³. Performing pharmacokinetics studies of PLA-PEG-M278 in mice are of future investigations in our lab.

Immunization with encapsulated-M278 potentiated M278-specific adaptive immune responses when compared with bare M278. Specifically our observation of heightened

levels of Th1 cytokines including IL-2 and especially IFN- γ , is congruent with marked production of IFN- γ by PLA-PEG²⁶. Increased levels of IFN- γ and IL-2 are of significance as they are associated with protection against *Chlamydia* and other intracellular pathogens including *Leishmania major* and *Mycobacterium tuberculosis*^{34–35}. IFN- γ activates Th1 CD4 and CD8 effector T cells that are required for a successful *Chlamydia* vaccine¹³ as well as for antigen presentation and lysosome activity in macrophages³⁶. Additionally, IFN- γ has several anti-chlamydial actions in clearing infections by inducing indoleamine-2, 3-dioxygenase (IDO), which lowers the intracellular tryptophan pool necessary for chlamydial metabolism³⁷ and limits chlamydial growth by depleting its intracellular iron³⁷. The impact of IFN- γ for *Chlamydia* resistance is underscored by lack of protective immunity in IFN- γ receptor-deficient mice¹⁷, and for resistance to *Chlamydia* infections in humans³⁸.

IL-17 production by encapsulated-M278 immune T-cells adds credence to our findings given that it can synergize with IFN- γ for inhibition of *Chlamydia* lung infections³⁹. Similarly, a *Chlamydia* vaccine formulated with MOMP, PmpG, PmpE/F-2 and the DDA/TDB adjuvant conferred protection against chlamydial infections, with heightened IFN- γ , IL-17 and TNF- α production by CD4⁺ T cells³⁵. Noteworthy of mention is triggering of CXCL10 and GM-CSF by encapsulated-M278, which are essential for stimulating cell growth, differentiation of precursors needed by immune cells⁴⁰, and for their contribution to clearance of *Chlamydia* infections in mice^{41–43}. Elevated levels of CXCL10 and CCL5 have been noted in the genital tract of *Chlamydia*-infected mice⁴¹, and mice deficient in their putative CXCR3 and CCR5 receptors, respectively are unable to clear their *Chlamydia* genital tract infections⁴¹. Studies also show that topical application of GM-CSF followed by transcutaneous immunization with MOMP protected mice against genital and respiratory tract chlamydial infections, and protection was afforded by IFN- γ and serum antibody⁴². Likewise, GM-CSF combined with UV-inactivated EBs protected mice against *Chlamydia* infection⁴³. Overall, our findings support that encapsulated-M278 potentiates T cell-mediated immune responses in mice, especially those that are prerequisites for protection against *C. trachomatis*.

Encapsulated-M278 triggered high M278-specific Th1 (IgG2a) and Th2 (IgG1 and IgG2b) antibodies in mice, which are congruent with other studies of PLA-PEG as a delivery system for tetanus toxoid and hepatitis B antigens^{8,32}. Seemingly, IgG1 and IgG2b are associated with protection against *Chlamydia*^{44,45} although the precise contribution of antibody to *Chlamydia* protection is still not completely understood. Chief amongst our findings was encapsulated-M278 serum-mediated inhibition of *Chlamydia* infectivity and/or multiplication in macrophages. We did not explore the definitive role of antibodies in the serum-mediated inhibition of *Chlamydia* infectivity of macrophages but nonetheless, strong evidence suggests that antibodies could be involved in resistance to reinfection⁴⁶ or in blocking attachment of *C. trachomatis* to cells⁴⁷. In support of this notion is that *C. trachomatis* pre-coated with IgG2b antibodies and administered intra-vaginally to mice afforded them reduced infection rates⁴⁸. In the present study, the reduction in MOMP and TLR2 expression in infected macrophages exposed to encapsulated-M278 serum are indicative of less *Chlamydia* infectivity of macrophages. Conversely, as TLR2, a cognate *C. trachomatis* receptor is critical for *Chlamydia*-mediated host cell-activation and MOMP

signaling⁴⁹; the presence of more *Chlamydia* in macrophages would correlate with higher TLR2 and MOMP expressions as observed in macrophages exposed to bare M278 or control sera. The low level of CD80 expression, that is critical for T cell-activation, also agrees with results obtained in CD80/CD86 knockout mice, which are resistant to a primary *C. trachomatis* infection⁵⁰. Therefore, encapsulated-M278 immunization elicited functional immune serum due to the transcriptional down-regulation of MOMP and TLR2, as markers of *C. trachomatis* infectivity.

In summary, this is the first report, to our knowledge, to employ PLA-PEG as a vaccine-delivery system for *C. trachomatis* MOMP or its peptide derivative. We demonstrate that immunization with encapsulated-M278 promotes enhanced adaptive immune responses and serum-mediated inhibition of *Chlamydia* infectivity in macrophages, which are all desirable for protection against this pathogen. Our data further provides compelling evidence that PLA-PEG holds strong promise as a vaccine-delivery system for not only *C. trachomatis* but also other intracellular pathogens. The potential of PLA-PEG for vaccine delivery undeniably resides in its inherent potentiating and controlled slow release effects to bolster immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic of immunization experiments in mice.

Figure 2.

Physiostructural characterization of nanoparticles. The size and morphology of nanoparticles were determined using SEM (A and B) and TEM (C and D) where one drop of the nanoparticles was deposited on a formvar grid or metal stub, respectively. Size (E and F) and zeta potential (G and H) of nanoparticles were evaluated by the Zetasizer.

Figure 3. Physicochemical characterization, release and cytotoxicity studies. FT-IR (A and B) and UV-Vis spectra (C) confirmed successful encapsulation of M278 within PLA-PEG. *In vitro* release of encapsulated-M278 over a 20-day period (D). Cytotoxicity of nanoparticles to mouse J774 macrophages over a 72 hr time-period (E and F).

Figure 4.

Immunization with encapsulated-M278 potentiates specific cellular immune responses in mice. Responses were assessed on day 42 post-immunization where purified T cells (1×10^6 /mL) were co-cultured with antigen presenting cells (APCs, 1×10^6 /mL) and re-stimulated with M278 (10 μ g/mL) for 48 hr. Cytokines (A-G, I-J), and chemokines (H) in cell free-culture supernatants were quantified by multiplex ELISA. Significance between groups was established at $P < 0.001 = ***$, $P < 0.01 = **$ and $P < 0.05 = *$.

Figure 5.

Immunization with encapsulated-M278 potentiates specific antibody immune responses in mice. Serum was collected from mice on day 42 post-immunization and used to determine IgG, IgG1, IgG2a and IgG2b antibodies by ELISA (A). To determine antibody titers (B), two-fold serial dilutions of serum were made and the endpoint titer was considered the last dilution with readings higher than the mean + 3 standard deviations of negative controls. Significance between groups was established at $P < 0.001 = ***$.

Figure 6.

Serum-mediated inhibition of *Chlamydia* infectivity of macrophages. Mouse J774 macrophages ($10^6/\text{mL}$) were infected with *C. trachomatis* (CT; 10^5 IFU, Inclusion Forming Units) and incubated for 48 hr in the absence and presence of mouse immune serum (1:100 dilution). qRT-PCR was used to quantify mRNA expression of MOMP (A), TLR2 (B) and CD80 (C). Immunofluorescence microscopy visualization of *Chlamydia* in macrophages (D-H). Columns 1, 2, 3 and 4, respectively reflect staining for nuclear (blue fluorescence), macrophage surface (red fluorescence), anti-*Chlamydia* antibody (green fluorescence) and their overlay images (yellow fluorescence).