Characterization of rMOMP-187 Peptide Encapsulated in PLGA 50:50 Nanoparticles

Murtada Taha, Shree R. Singh, Chelsea Butler, Elijah Nyairo, Vida A. Dennis*

Centre for Nanobiotechnology and Life Science Research, Alabama State University, 1627 Hall St, Montgomery, AL 36104.

ABSTRACT

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial pathogen in the world, with an estimated more than 100 million clinically diagnosed cases occurring annually. However, this major human pathogen currently has no effective vaccine. Thus, the ultimate goal of this project is to contribute in the efforts of developing an effective mucosal vaccine against *C. trachomatis*. In the present study a peptide derivative of MOMP corresponding to amino acids 187–344(rMOMP-187) was encapsulated in PLGA 50/50 nanoparticles and characterized using Fourier Transform Infrared Spectroscopy (FTIR) which showed that the spectra of rMOMP-PLGA50/50 nanoparticles (rMP-PLGA50/50) corresponded to that of PLGA nanoparticles alone, while the Zetasizer measurement showed the size of the rMOMP-PLGA to be about 110 nm. Initial *in vitro* characterization of the rMOMP-PLGA nanoparticles demonstrated its ability to elicit significant production levels of interleukin (IL)-6 cytokine and nitric oxide (NO) by mouse J774 macrophages in a dose dependent fashion. Toxicity study of PLGA 50/50 to J774 macrophages revealed minimal toxicity at concentrations between 7.8 to 250 µg/mL. Overall, our study shows the successful encapsulation and characterization of rMOMP-187 in PLGA 50/50 and its lack of toxicity to mammalian cells, thus suggesting its potential usefulness as a vaccine delivery system against *C. trachomatis*.

Keywords: *C.trachomatis*, MOMP, PLGA, IL-6, NO

1 INTRODUCTION

Chlamydia trachomatis, a Gram negative intracellular bacterium, is a major human health pathogen due to its role as the leading cause of bacterial sexually transmitted diseases in the World. To date, there is no efficacious vaccine available against this human pathogen. Development of a vaccine against *C. trachomatis* was initiated several years ago without any success, in part, because of our poor understanding of the regulation of the immune response in the female genital tract, the lack of adjuvants that target vaccines to the genital mucosa, our limited knowledge of which *C. trachomatis* antigens induce protective immune responses (1,2,3) and also due to hypersensitivity effect that results upon re-exposure when whole organisms were used as vaccine (4). Recently vaccine development efforts are being directed towards subunit or multimeric vaccines. The MOMP of *C. trachomatis* is one of the best understood and most desirable proteins for use as a vaccine candidate (5). However, purified MOMP and other subunits have been evaluated in several animal models with limited success, perhaps due to ineffective delivery systems or lack of effective adjuvants to boost mucosal immunity (2). Thus, safe and effective delivery systems are required to protect and deliver rMOMP to antigen-presenting cells and to the genital mucosal inductive sites or the associated secondary lymphoid tissues. PLGA is well characterized and approved for human use by the FDA because of its long term safety (6,7), which is a clear advantage for antigen delivery. PLGA nanoparticles offer a great flexibility with respect to the manipulation of physicochemical properties of the polymer and the range of antigens and immunomodulators that they can accommodate (8).

2 MATERIALS AND METHODS

2.1 Preparation of nanoparticles

A w/o/w double emulsion–evaporation technique was used; 344µg of rMOMP-187 was injected into 500 µl of chloroform containing PLGA. This primary emulsion was homogenized using sonicator for 15 s. The primary emulsion was further emulsified in 2 mL 9% polyvinyl alcohol (PVA) in phosphate buffered solution (PBS) for 20s. The resulting emulsion was then added drop wise to 8 mL of 9% PVA/PBS and was left to stir over night at 4 ^{o}C to allow the polyvinyl alcohol to evaporate. Nanoparticles were harvested by centrifugation at 41,000 rpm, for 1 min., washed three times in distilled water and lyophilized overnight using freeze dryer. Lyophilized Nanoparticles were kept at -80 °C in a sealed container.

2.2 Fourier Transform-Infrared (FT-IR)

Fourier Transform-Infrared (FT-IR) spectra were recorded for neat rMOMP-187, PLGA50/50 and the rMP-PLGA50/50 nanoparticles blends in attenuated total reflectance (ATR) mode using an IR spectrophotometer. The spectra were obtained with 64 scan per sample ranging from 4000 to 400 cm^{-1} and a resolution of 4 cm^{-1}. The sample chamber was purged with dry N_2 gas.

2.3 Particle size measurement

The particle sizes of PLGA50/50 and rMP-PLGA50/50 nanoparticles were measured by dynamic light scattering using a Zetasizer Nano- ZS. Samples of rMP-PLGA50/50 nanoparticles were suspended in distilled filtered water, sonicated, and then placed in a disposable cuvette for size measurements. Each sample was measured three times for triplicate preparations of nanoparticles and is reported as the mean.

2.4 *In vitro* protein release

The release of the peptide from the P50/50 was determined following the method of Bouissou et al (9). Briefly, rMP-PLGA50/50 or PLGA50/50 were suspended in 200 µL of 1 x PBS containing 0.01% sodium azide. The suspension was incubated horizontally at 37°C and at various time intervals (30 minutes, 8, 24, 48, and 72 h supernatants were collected by centrifugation at 12,000 rpm for 5 min and kept at -20 ^{o}C till used. The released peptide was measured using NanoDrop ND-1000 at 280 nm.

2.5 Cytotoxicity assay

Cytotoxicity of PLGA50/50 was measured using the MTT dye reduction assay in mouse J774 cells. Mouse J774 macrophages was seeded in 96-well plates at the density of 1.0×10^5 viable cells/well in 50 µL of complete media and incubated overnight at 37°C under 5% CO_2 to allow cell attachment. 50 µL of PLGA50/50 was added to the cells in concentrations ranging from 7.8 to 1000 µg/mL. After 24 h, 15 µL of MTT dye solution were added into each well, and the cells were incubated for 4 hours at 37°C under 5% CO_2. To stop the reaction, 100 µL of solubilization solution were added to each well and plates incubated for at least 1h at room temperature in the dark. Absorbance at 570 nm was measured using a microplate reader.

2.6 Dose response and kinetics of cytokine production

For the dose-response studies, 1×10^{-6} /mL J774 macrophages were cultured in 12 well plates and incubated at 37°C in a humidified atmosphere (5% CO2 and 95% air) for 24 h, then stimulated with various concentrations of rMP-PLGA50/50 (1, 5, 10, 20 and 40 µg/mL), and cell free supernatants were collected after 24 h post-stimulation. For the study of the kinetics of cytokines production, macrophages were stimulated with rMP-PLGA50/50 or PLGA50/50 (10 µg/mL). Cell-free supernatants were collected after 2, 24, 48, and 72 h post-stimulation.

2.7 Cytokine measurements

Sandwich ELISA was used to measure cytokine secretions in culture supernatants of mouse J774 macrophages according to the manufacturer's protocol.

2.8 Statistical analysis

All the data are expressed as means ± standard deviation of samples run in triplicate. Data were analyzed by ANOVA single factor using differences between the means of two treatments. *P* values ≤ 0.05 were considered significant.

3 Results and Discussion

3.1 Fourier Transform-Infrared (FT-IR) analysis

The FTIR spectra of the rMOMP-187, PLGA50/50 and rMP-PLGA50/50 are shown in Fig. 1. The carbonyl band of rMOMP-187 appears at 1625.3 cm^{-1}, and that of PLGA50/50 at1742 cm^{-1} while that of rMP-PLGA50/50 after encapsulation was shifted to overlapped at 1742 cm^{-1} with that of PLGA50/50. Overlapping of the CH stretching band of PLGA50/50 and that of rMP-PLGA50/50 at 2942.2 cm^{-1} was also observed. The hydroxyl band of rMOMP-187 at 3288.2 cm^{-1} disappeared after encapsulation. Typical bands for ester carbonyl stretch (C=O) at 1747 cm^{-1}, C-O stretch at 1128 cm^{-1} and C-O-C group at 1046 cm^{-1} were found for both PLGA50/50 and rMP-PLGA50/50. The disappearance of the bands at 3288.2 cm^{-1}, 2027.9 cm^{-1} and 1626.3 cm^{-1} may indicate interaction between the peptide and PLGA50/50 or the presence of peptide in the nanoparticle composition.

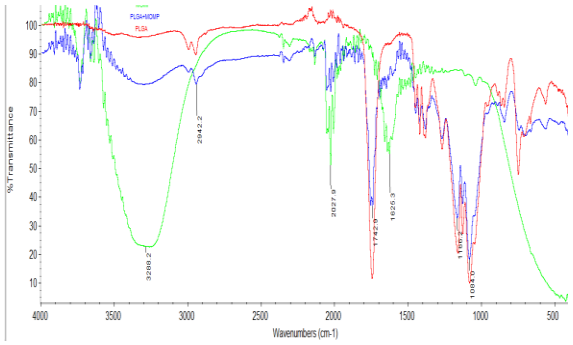

Figure 1: Infrared spectra of blank PLGA50/50, rMOMP-187, rMOMP-187 within PLGA50/50 nanoparticles.

3.2 Size measurements

Particle size has been demonstrated to play an important role in determining the level of cellular and tissue uptake by antigen presenting cells (10), which will directly affect the encapsulation efficiency and the release of the rMOMP187 peptide from rMP-PLGA50/50 nanoparticles. The mean particle size of rMP-PLGA50/50 is shown in Fig. 2 which corresponds to 110 nm. It is possible that the formation of rMP-PLGA50/50 in this size range facilitates their uptake by J774 macrophages, thereby enhancing the release and the immune responses.

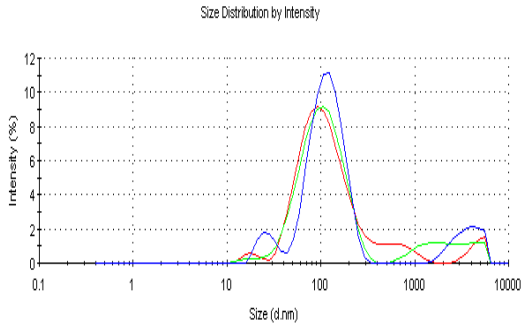

Figure 2: The mean particle size of rMP-PLGA50/50 nanoparticles using Zetasizer Nano techniques.

3.3 *In vitro* release of the peptide from PLGA50/50 Nanoparticles

Fig. 3 shows the release of the rMOMP-187 from PLGA50/50. The nanoparticles initially showed a rapid or burst release up to 8 h, followed by a decrease in the release up to 48 h, before it start to increase at 72 h (Fig. 3). The release of the drugs or a peptide from nanoparticles generally occur through different mechanisms, our *in vitro* peptide release study showed a triphasic pattern with an initial burst followed by a decrease then an increase release over a 72 h period.

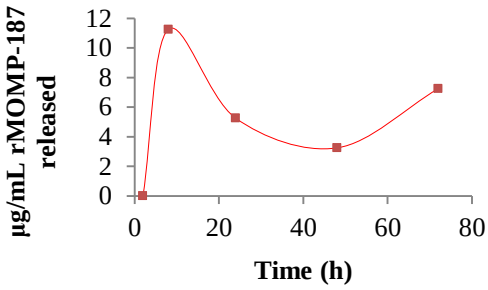

Figure 3: *In vitro* release of rMOMP-187 from PLGA50/50 nanoparticles.

3.4 Cytotoxicity studies of PLGA50/50 nanoparticles

MTT assay was used to estimate the *in vitro* cytotoxicity of PLGA50/50 to mouse J774 macrophage. As shown in Fig. 4 the cell viability of J774 macrophages incubated with different concentrations of PLGA50/50 for 24 h was above 80% for a dose as high as 500 µg/mL (Fig. 4). Overall, there were no adverse effects on cell viability by PLGA50/50, thus suggesting the safety of this delivery system for vaccine studies.

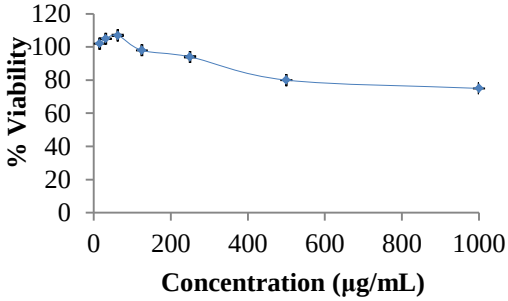

Figure 4: MTT assay for cytotoxicity of PLGA50/50 to J774 macrophage cells after incubation for 24 h.

3.5 Dose response and kinetics of IL-6 production

IL-6 plays an important role in the differentiation of B cells into antibody-producing plasma cells, which are important components of the host response to chlamydial infection, and tend to correlate with resistance to MoPn (11). Fig. 5 illustrates the results of dose response and kinetic studies of IL-6 produced by J774 macrophages in response to rMP-PLGA50/50 and PBS-PLGA50/50

stimulations. IL-6 was detectable at stimulant concentrations as low as 1 µg/mL (Fig. 5A). rMP-PLGA50/50 stimulated production of IL-6 in a dose-dependent manner (Fig. 5A).

For time kinetics study, the cytokine concentrations were determined at 2, 24, 48 and 72 h after addition of PLGA50/50 or rMP-PLGA50/50 to the cell cultures. The concentrations of IL-6 increased rapidly within the first 24 h of stimulation with rMP-PLGA50/50. After this time, IL-6 concentration declined gradually (Fig. 5B). IL-6 reached its peak values at 24 h (Fig. 5B).

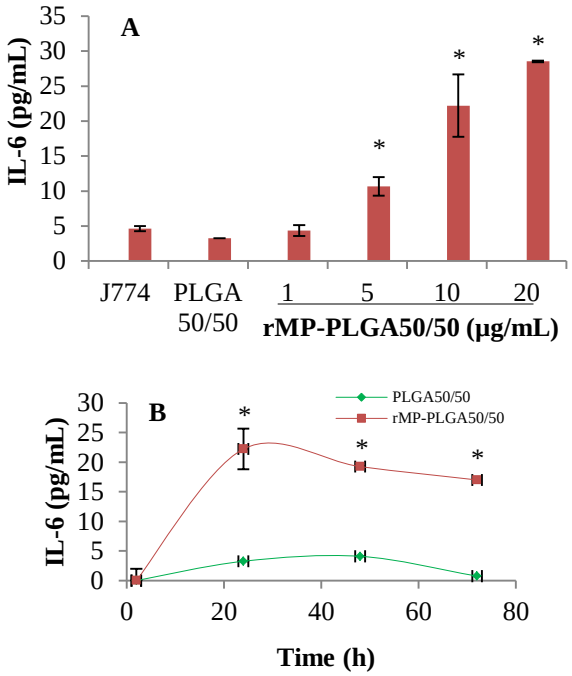

Figure 5: Dose response and time kinetics analysis of rMP-PLGA50/50 required to induce optimal level of IL-6 production.

3.6 Dose response and kinetics of nitric oxide (NO) production

The role of NO in defense against *Chlamydia* has been reported by several researchers (12,13,14). Neither medium nor P50/50 alone induced significant production of NO at all examined time-points. NO was detectable at stimulant concentrations as low as 1 µg/mL of rMP-PLGA50/50 (Fig. 6A). The optimum concentration of rMP-PLGA50/50 induced NO production was found to be 5 µg/mL. However, higher concentration such as 10 and 20 µg/mL produced less of NO (Fig. 6A). This may have been due to the very slow release of the peptide from the nanoparticles, which was insufficient to induce high production levels of NO in cells.

For kinetics study, NO was determined at 2, 24, 48 and 72 h after addition of PLGA50/50 or rMP-PLGA50/50 to the cell cultures. The concentrations of NO studied increased sharply at 24 h, before starting to decline at 48 and continued to increase again at 72 h (Fig. 6B). The concentrations of NO reached its peak values at 72 h (Fig. 6B).

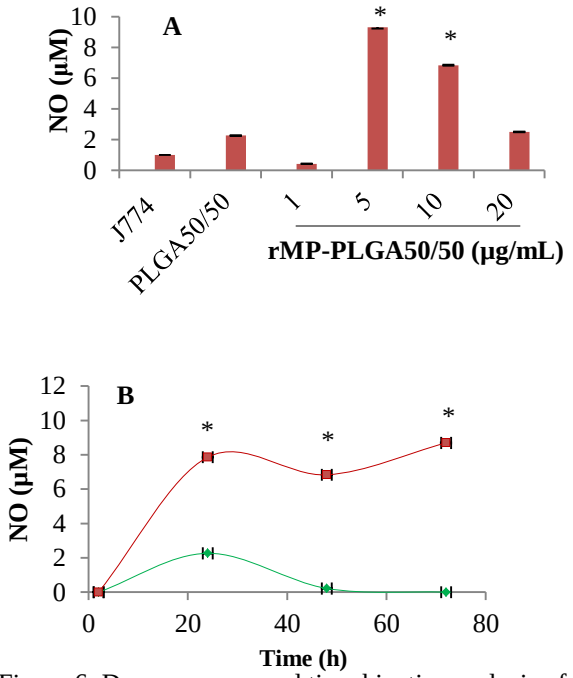

Figure 6: Dose response and time kinetics analysis of rMP-PLGA50/50 required to induce optimal level of NO production.

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