Physio-structural and Immunological Characterization of a PLGA 85/15 Encapsulated *Chlamydia* Recombinant MOMP Nanovaccine Candidate

Rajnish Sahu, Saurabh Dixit, Skyla Duncan, Shree R. Singh and Vida A. Dennis*
Center for NanoBiotechnology & Life Sciences Research,
Alabama State University, 915 South Jackson Street, Montgomery, AL, 36104, USA

ABSTRACT

The quest for vaccine development against *Chlamydia trachomatis* (CT), the leading cause of bacterial sexually transmitted diseases worldwide, has escalated to reduce its occurrence and global economic burden. Recently, nanovaccines have emerged as attractive candidates due to flexibility in their formulation, biodegradability, biocompatibility and adjuvant properties. Nanovaccines applicability depends on their physical properties for their immunogenicity, which is vital for biosafety and therapeutic efficacy studies. We have developed a nanovaccine against CT employing its recombinant MOMP (major outer membrane protein) by encapsulating it within PLGA [poly (D, L-lactic-co-glycolic acid) 85/15 nanoparticles. Physio-structural characterizations included Zeta-sizing, Zeta-potential, DSC (Differential Scanning Calorimetry), UV spectroscopy and *in vitro* release of rMOMP. Encapsulated rMOMP nanoparticles were small (~200 nm), thermally stable (90ºC) and negatively charged (-13 mV). UV spectroscopy showed minimal presence of rMOMP on the outer surface of encapsulated nanoparticles, indicating its successful encapsulation. Moreover, rMOMP was slowly released from within PLGA nanoparticles over a 31-day period, suggesting its attractiveness as a vaccine candidate. Encapsulated rMOMP was devoid of endotoxin contamination as assessed by the Polymyxin B inhibition assay using mouse J774 macrophages. Of significance was the observation that encapsulated rMOMP activated mouse dendritic cells (DCs), the major antigen presenting cells, for induction of Th1 immune responses, which are critical for protection against CT. Exposure of mouse DCs to encapsulated rMOMP at different concentrations revealed marked increase in the production of the Th1 cytokines, IL-6 and IL-12p40. Our data warrants efficacy studies in mouse for assessing the overall potential of our nanovaccine as a candidate against CT.

Key words: *Chlamydia trachomatis*, MOMP, poly (D, L-lactic-co-glycolic acid), dendritic cells, cytokines.

1.0 INTRODUCTION

In pursuit of a vaccine against *Chlamydia trachomatis* (CT), the most reported bacterial sexually transmitted infection globally, our lab focused on biodegradable polymeric nanoparticles to provide efficient delivery of biomolecules. We have developed several CT nanovaccines employing PLGA encapsulated with its recombinant MOMP or peptides and shown them to enhance immune responses [1, 2]. Previously, we reported that full rMOMP encapsulated within PLGA (50:50, Lactide:Glycolide) nanoparticles elicited robust Th1 immune responses *in vitro* and *in vivo* [1]. Moreover, we observed that PLGA (50:50) was a key factor in providing the slow release of rMOMP to potentiate Th1 immune responses. Our goal in the present study is to develop a nanovaccine by encapsulating full rMOMP into PLGA (85:15) which has a slower release profile of its encapsulated antigen and as such will potentially induce more robust Th1 immune responses. Our previous studies have shown that PLGA nanoparticles are non-toxic and do not trigger specific immune responses and are well tolerated in mice, which make them a novel delivery system [1, 2].

2.0 MATERIALS AND METHODS

2.1 Preparation of nanoparticles

Recombinant MOMP was encapsulated in PLGA (85:15) nanoparticles by a modified water/oil/water double emulsion–evaporation technique essentially as described [1, 2, 3]. Briefly, 300 mg of PLGA was emulsified in dichloromethane followed by addition of rMOMP (2 mg), homogenization and then addition of 1% Polyvinyl Alcohol (PVA). The resulting double emulsion was gently stirred overnight at room temperature (RT) to allow evaporation of the organic solvents, harvested by ultracentrifugation, washed and lyophilized in the presence of 5% trehalose. Sterile PBS was used in the primary emulsion formation to prepare PLGA-PBS nanoparticles to serve as a negative control.

2.2 Zeta-sizer and zeta-potential

Nanoparticles were suspended into water and transferred to cuvette. The particle sizes were analyzed by using Zeta sizer Nano-ZS (Malvern Instruments, UK) [1, 2, 3]. Each sample was measured three times and reported as the mean size in diameter.

2.3 Differental scanning calorimetry (DSC)

Nanoparticles were analyzed for temperature stability using DSC (Toledo DSC 833e, Mettler, Columbus, Ohio). Nanoparticles were weighed and heated at the rate of 20^0C per minute from 30^0C to 120^0C under nitrogen and then

cooled from 120^{0}C to 30^{0}C at same rate to determine their respective stability.

2.4 Ultraviolet spectroscopy

Nanoparticles were suspended into distilled water and scanned using Nanodrop 2000c to access the availability of protein onto surface [1, 3].

2.5 Encapsulation efficiency

Nanoparticles were weighed, suspended into solubilization buffer (0.1N NaOH and 2% SDS) and kept on a shaker overnight at RT to allow complete solubilization. The suspensions were centrifuged at high speed and supernatants were collected for protein estimation. The encapsulated protein was measured using micro BCA kit. PLGA-PBS was used for background correction. Encapsulation efficiency was calculated using the following formula [EE = 100 x (A –B)/A] (A is total amount of rMOMP and B is un-encapsulated rMOMP) [1, 3].

2.6 *In vitro* release of rMOMP from PLGA nanoparticles

Nanoparticles were suspended in sterile PBS containing 0.01% sodium azide and suspensions were kept at 37^{0}C. Supernatants were collected every 24 hours for 31 days by centrifugation and stored at -20^{0}C until used [1, 2, 3].

2.7 Stimulation of mouse J774 macrophages with nanoparticles and endotoxins activity

Mouse J774 macrophages were obtained from the American Type Culture Collection (Waldorf, MD, USA) and propagated in Dulbecco's modified Eagle's culture medium at 37°C in a humidified 5% CO_2 atmosphere. Mouse J774 macrophages (1×10^6/mL) were seeded in 12-well plates and stimulated with rMOMP and encapsulated PLGA-rMOMP (0.01 µg/mL) in the presence and absence of polymyxin B (10 µg/mL) for 24 hours to determine the presence of any endotoxin activity. We elected to use PLGA-rMOMP at 0.01 µg/mL for this study because this concentration elicited higher production of cytokines by macrophages as compared to naked rMOMP. Stimulation of macrophages with LPS (0.01 µg/mL) and PLGA-PBS served as positive and negative controls, respectively. Cell free-culture supernatants were collected by centrifugation after 24 hours and used for interleukin (IL-6) cytokine detection by specific cytokine ELISA kits as reported [1, 2, 3].

2.8 Isolation and culture of dendritic cells

Mouse bone-marrow cells were flushed from femurs (five to six mice) with sterile RPMI media containing glutamax (Gibco Invitrogen, Carlsbad, CA), RBCs were lysed using ACK lysing reagent (Invitrogen) and washed twice. Cells were grown for 7 days in tissue-culture petri dishes at 37^{0}C in a humidified 5% CO_2 atmosphere with change of media on days 3 and 6. The culture medium consisted of RPMI glutamax supplemented with, 10% heat-inactivated fetal bovine serum (Gibco), 50 µM β-merceptoethanol, 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) and antibiotic/antimycotic (Invitrogen) [4]. Dendritic cells (DCs) were harvested on day 7 and cell counts were adjusted at 0.50×10^6 cells/culture tube.

2.9 Encapsulated rMOMP dose response studies

DCs were stimulated for 24 hours using various concnetrations of rMOMP and encapsulated PLGA-rMOMP (1, 0.1 and 0.01 µg/mL). LPS (0.01 µg/mL) and PLGS-PBS were used as positive and negative controls, respectively. Cytokines (IL-6, IL-12p40 and IL-10) were quantified in cell free-culture supernatants using ELISA kits [1, 2, 3].

3.0 RESULTS AND DISCUSSIONS

3.1 Physio-structural characterizations

Physio-structural techniques were used in the morphological assessments of encapsulated PLGA-rMOMP and PLGA-PBS nanoparticles. UV-visible spectrum analysis revealed minimal absorption of protein on the surface of PLGA-rMOMP nanopartilces at 280 nm wavelength, which validates the encapsulation of rMOMP into PLGA (Figure 1).

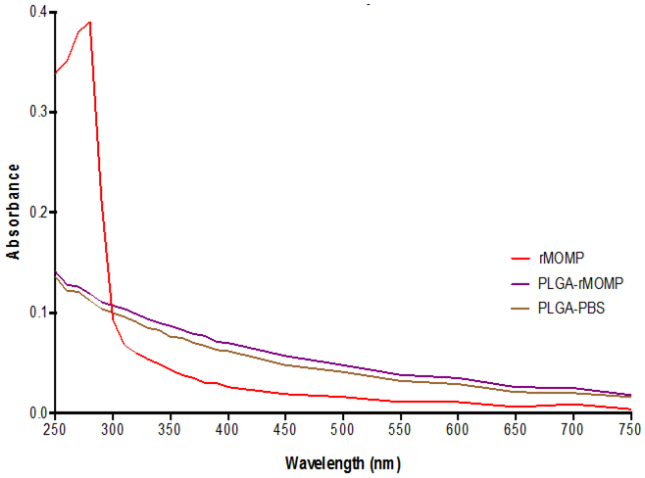

Figure 1: UV-Vis spectral scan of nanoparticles. PLGA-rMOMP, PLGA-PBS and rMOMP were resuspened in distilled water and measured spectrophotometrically.

In contrast, rMOMP showed maximal protein absorption at the same wavelength. Nanoparticles were overall small in size with zeta-sizer measurments of (223±3.41) and (208±2.30) for PLGA-PBS and PLGA-rMOMP. Zeta potentials for PLGA-PBS and PLGA-rMOMP were (-12.8±0.32) and (-9.15±0.13) (Figure 2). The importance of surface charge indicates the stability of nanoparticles. DSC revealed the temperature stability of PLGA-rMOMP and PLGA-PBS nanopartilces as 94^0C and 93^0C, respectively. The high thermal stability of PLGA-rMOMP indicates that encapsulation did not cause any adverse effect on the physical state of PLGA (Figure 3).

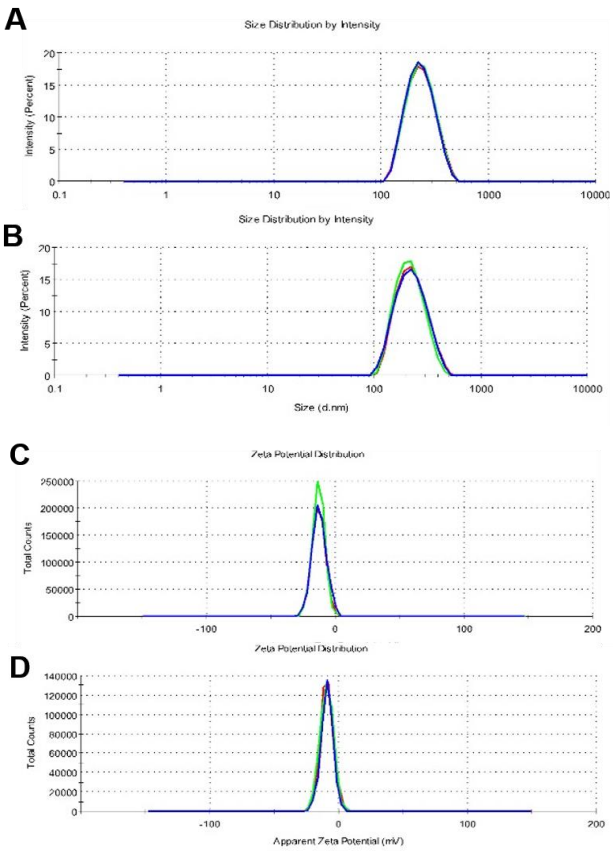

Figure 2: Zeta-Sizer and Zeta-potenial studies. (A) PLGA-PBS and (B) PLGA-rMOMP (zetasizer); (C) PLGA-PBS and (D) PLGA-rMOMP (Zeta-Potential). Nanoparticles were resuspended in distilled water and analyzed using zeta-sizer nano-ZS.

3.2 *In vitro* release of encapsulated rMOMP from PLGA nanoparticles

A high encapsulation efficiency of 90% was observed via modified double emulsion evaporation technique for nanoparticles. A sustained slow release was observed over a peiod of 31 days with 98% of total encapsulated rMOMP. This indicates a slow release profile which is highly suitable for vaccine delivery (Figure 4).

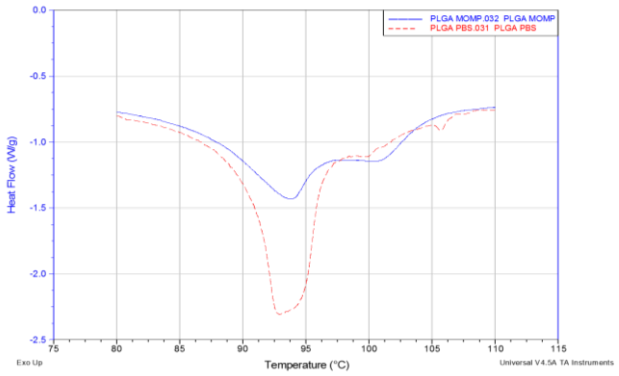

Figure 3: Differential Scanning Calorimetry. Approximately, 10 mg of nanoparticles were heated at the rate of 20°C per min from 30 to 120°C under nitrogen and then cooled from 120 to 30°C at the same rate, using a differential scanning calorimeter.

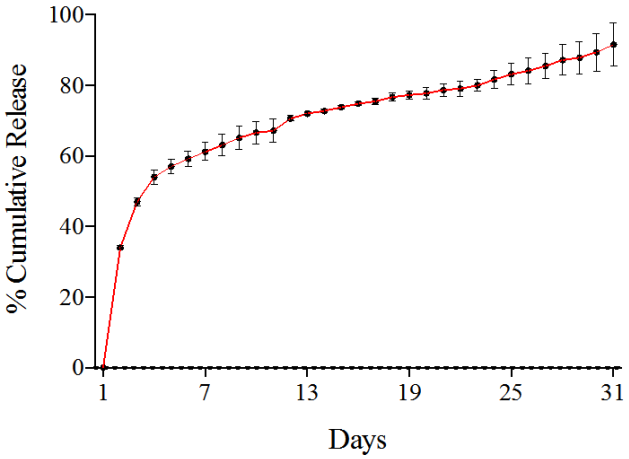

Figure 4: *In vitro* release of rMOMP from PLGA nanoparticles. Release of rMOMP from PLGA nanoparticles over a period of 31 days was performed by resuspensions of nanoparticles in sterile PBS followed by spectrophotometry measurement of the rMOMP protein.

3.3 Stimulation of macrophages and endotoxins activity

We first documented that PLGA-rMOMP nanoparticles were free of endoxin activity prior to use for immunological studies. Thus, macrophages were stimulated with PLGA-rMOMP in the presence and absence of of polymyxin B (PB) followed by quantification of IL-6 in supernatants. Our results show that both PLGA-rMOMP and naked rMOMP induced production of IL-6 by macrophages, which was not attributed to contamination by endotoxin since no differences were seen in IL-6 levels in the presence or absence of PB. In contrast, IL-6 induced by LPS stimulation of macrophages was completely abrogated in the presence of PB (Figure 5).

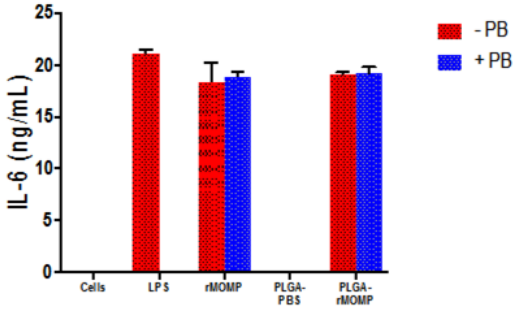

Figure 5: Encapulated rMOMP is free of endoxtin activity. Macrophages were stimulated with LPS, rMOMP and PLGA-rMOMP in the presense and absense of Polymyxin B (PB). Cell free-culture supernatants were collected and used for detection of IL-6 by ELISA.

3.4 Encapsulated rMOMP dose-response studies

Previously we reported that PLGA (50:50) potentiated the capacity of rMOMP to induce the production of cytokines in mouse J774 macrophages [1]. Herein, we investigated whether a similar effect would occur with rMOMP encapsulated within PLGA (85:15) nanoparticles. Mouse DCs were stimulated with various concentrations (1, 0.1 and 0.01 µg/mL) of naked rMOMP and PLGA-rMOMP nanoparticles to determine the optimum differential concentration between the two stimulants required for DCs stimulation. We observed that the 0.01 µg/mL concentration of PLGA-rMOMP induced levels of the Th1 cytokines (IL-6 and IL-12p40) comparable to the levels induced by the 1 and 0.1 µg/mL concentrations (Figure 6A and B). On the other hand, IL-6 and IL-12p40 levels induced by rMOMP were concentration dependent with lesser production at the lower concentration. These findings confirm the slow release of encapsulated rMOMP and its potentiation by PLGA (85:15). Of interest, PLGA-rMOMP and rMOMP dose-dependently induced less IL-10 (Th2 cytokine) (Figure 6C). PLGA-PBS did not induce any cytokine production, confirming that PLGA alone is non-stimulatory but has the capacity to potentiate immune responses.

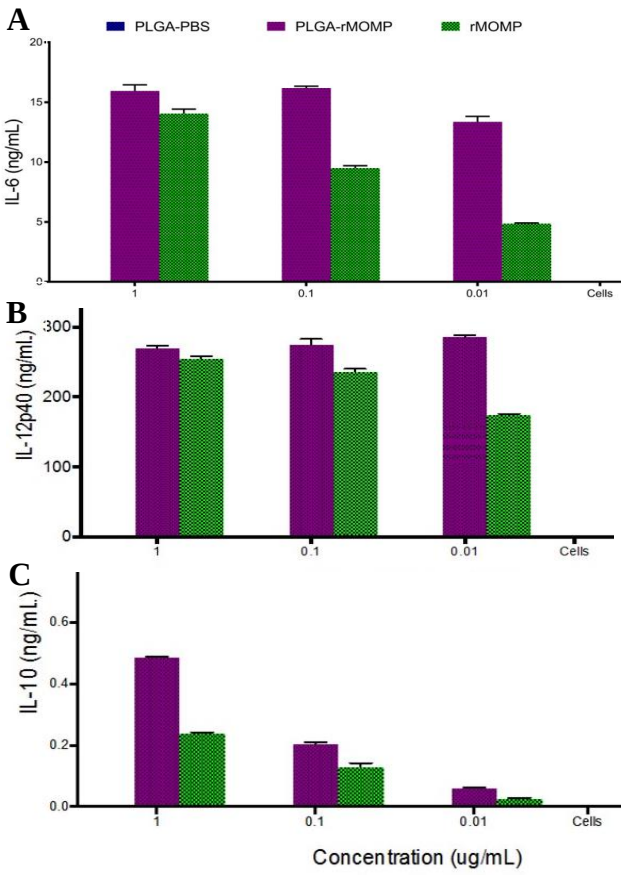

Figure 6: Dose response analysis of cytokine production by DCs stimulated with encapsulated and naked rMOMP. IL-6 (A), IL-12p40 (B) and IL-10 (C) were measured by ELISA from cell free supernatants collected at 24 hour after DCs stimulation.

4.0 CONCLUSION

Overall, our results indicate that our PLGA-rMOMP nanovaccine is stable after encapsulation and well tolerated under heat, which provides sustained release of encapsulated rMOMP. Stimulation of DCs by nanovaccine revealed that a very low concentration of encapsulated rMOMP was stimulatory when compared with higher doses of naked rMOMP, suggesting the slow release of rMOMP. Our nanovaccine triggered higher levels of IL-6 and IL-12p40 and lower levels of IL-10 upon activation of DCs. This suggest PLGA-rMOMP polarization towards a Th1 rather than Th2 immune response, which corroborates our previous reports [1, 2]. Collectively, these data suggests that our PLGA-rMOMP nanovaccine stimulates DCs and elicits enhanced Th1 immune responses which are important in host protection against chlamydial infections.

REFERENCES

[1] Fairley SJ, Singh SR, Yilma AN, Waffo AB, Subbarayan P, Dixit S, Taha MA, Int Journal of Nanomedicine 2013;8: 2085-99.
[2] Taha M, Singh SR, Dennis VA. Nanotechnology. 2012; 23, 325101.
[3] Dixit S, Singh SR, Yilma AN, Agee RD 2nd, Taha M, Dennis VA. Nanomedicine. 2014; 10(6) 1311-21.
[4] Nam TN, Akihiro K, et al. PNAS. 2010, 107 (46) 19961-19966.

ACKNOWLEDGEMENTS

This work was supported by funding from The National Institute Of Allergy And Infectious Diseases of the National Institutes of Health under Award Number R21AI111159 and NSFCREST (HRD-1241701).
*Corresponding author, vdennis@alasu.edu.