Sustained Release of Functional Interleukin-10 Encapsulated within Poly (lactic acid)-b-Poly (ethylene glycol) Nanoparticles Down-regulates Cytokines in Mouse Macrophages Exposed to *Chlamydia trachomatis*

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ABSTRACT

Inflammation which is induced by the presence of cytokines and chemokines is an integral part of Chlamydial infection. *Chlamydia trachomatis* (CT), the bacterial pathogen responsible for this sexually transmitted infection worldwide, causes severe inflammation including, but not limited to, pelvic inflammatory disease, ectopic pregnancy and infertility in women. It can be regulated using effective alternative therapeutics, including anti-inflammatory molecules. We showed that interleukin-10 (IL-10) can down-regulate the secretion of inflammatory cytokines triggered by CT in macrophages. A major problem with IL-10 is its short biological half-life thus requiring high dosages for biomedical applications. Our goal in this study was to encapsulate IL-10 within the biodegradable polymer, PLA-PEG (Poly (lactic acid)-b-Poly (ethylene glycol) nanoparticles in to prolong its half-life. IL-10 was successfully encapsulated in PLA-PEG by the double emulsion method, followed by physiochemical characterizations and functional studies. Results from Ultra Violet (UV) visible and Fourier Transform-Infrared Spectroscopy (FT-IR) revealed successful encapsulation of IL-10 within PLA-PEG. Encapsulated IL-10 had an average size of ~ 100 to 200 nm, with an encapsulation efficiency > 71 %. Temperature stability of encapsulated IL-10 was up to 89°C as shown by differential scanning calorimetry analysis. Additionally, the *in vitro* release study revealed an initial burst followed by the slow and continuous release of IL-10 from PLA-PEG nanoparticles. The anti-inflammatory effect of encapsulated IL-10 was tested using various concentrations (1-100 ng/mL) over a 24-hour time-point in mouse J774 macrophages exposed to the recombinant major outer membrane protein (rMOMP) of CT. Encapsulated IL-10 reduced the levels of IL-6 in macrophages in a time- and concentration-dependent fashion, correlating with its stability and slow release capacity. Our data shows successful encapsulation of IL-10 and that PLA-PEG can prolong the half-life of IL-10. More importantly, encapsulated IL-10 is functional by down-regulating IL-6 in macrophages exposed to rMOMP at relatively low dosages.

Key words: *Chlamydia trachomatis*, (Poly (lactic acid)-b-Poly (ethylene glycol), IL-6, IL-10.

1.0 INTRODUCTION

During a Chlamydia-induced inflammatory response, the presence of damaged cells activates macrophages to release a series of pro-inflammatory mediators (IL-1, IL-6, TNF-α, etc.), that contribute to the chronic inflammation associated with the disease[1]. Consequently, Interleukin-10 (IL-10), an anti-inflammatory cytokine, has been investigated as a therapeutic agent for such autoimmune and inflammatory diseases. The short biological half-life of IL-10 limits its usage and requires large, and frequent, dosage administration. Here in this study, we encapsulated IL-10 in PLA-PEG nanoparticles to extend its biological half-life. We chose PLA-PEG polymers because they are extensively used to develop nano-encapsulating therapeutic materials for targeted delivery potential, low toxicity and controlled release applications due to their biocompatibility and bioadhesive properties. Thus, the combination PEGylated-PLA IL-10 nanoparticle therapeutic approach could provide extending the biological half-life of IL-10 for therapeutic applications. In this study, we explored the hypothesis that encapsulated IL-10 will enhance IL-10 anti-inflammatory properties by inhibiting inflammatory mediators that are produced by mouse J774 macrophages after their exposure to rMOMP of CT.

2.0 MATERIALS AND METHODS

2.1. Preparation of nanoparticle

IL-10 was encapsulated in PEG-b-PLA Diblock polymer nanoparticles by a modified water/oil/water double emulsion-evaporation technique essentially as described [2-4]. Briefly 300 mg of PLA-PEG was emulsified in Ethyl acetate followed by addition of 1 mg of monkey recombinant IL-10, homogenization and addition of 1% Polyvinyl Alcohol (PVA). The resulting double emulsion was gently stirred overnight at room temperature (RT) to evaporate 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 PLA-PEG-PBS nanoparticles to serve as a negative control.

2.2. Encapsulation efficiency

The encapsulation efficiency of encapsulated IL-10 was performed as described [2-4]. Briefly, lyophilized encapsulated IL-10 (20 mg) was added to 1 mL of 0.1 N NaOH containing 2% SDS, shaken overnight at RT, the supernatant was collected by centrifugation at 13, 680×g for 5 min and then stored at -20°C. The Micro BCA protein assay was used to quantify free IL-10 in supernatants and the absorbance was read at 570 nm using a microplate reader (TECAN US Inc., Durham, NC). Background readings were corrected by subtracting the optical density (OD) values of supernatants from the encapsulated PBS negative control nanoparticles. The IL-10 encapsulation efficiency (EE) was calculated using the formula: EE = A-B)/A ×100 (A is the total IL-10 amount and B is the free IL-10 amount). These measurements were performed three times.

2.3. UV visible spectra

Encapsulated nanoparticles (5 mg each) and recombinant IL-10 (1 mg/mL) for spectroscopy were prepared by dissolving them in DNAse-RNAse free water, and the UV visible spectra were taken using Beckman Coulter DU 800 UV/Vis spectrophotometer (VWR Lab shop, Batavia, IL, USA) [3-4].

2.4. Fourier Transform-Infrared (FT-IR)

FT-IR spectra were recorded for encapsulated nanoparticles in attenuated total reflectance (ATR) mode using an IR spectrophotometer (Thermo Fisher Nicolet 380 FT-IR) [2-4]. The spectra were obtained with 64 scans/sample ranging from 4,000 to 1,000 $cm^{-1}$and a resolution of 4 cm^{-1}. The sample chamber was purged with dry N2 gas.

2.5. Differential Scanning Calorimetry (DSC)

Temperature stability experiments were carried out using DSC (Toledo DSC822e; Mettler, Columbus, Ohio) as described [2-3]. Encapsulated nanoparticles (10 mg each) were heated at a 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.

2.6. *In vitro* IL-10 release

The release of IL-10 from the encapsulated IL-10 was determined as described [2-3]. Encapsulated nanoparticles (20 mg each) were suspended in PBS containing 0.01% sodium azide (500 µL) and incubated at 37°C. At various time-intervals (1, 2, 4 h, and days 1-60), supernatants were collected by centrifugation at 13, 680 × g for 5 min and stored at -20°C until assayed. The released IL-10 was measured using the Micro BCA assay as calculated from the standard curve. Absorbance reading of the encapsulated PBS control nanoparticles was subtracted from those of the encapsulated IL-10 and the results are expressed as accumulative release over the entire 60-day assessment period.

2.7. *In vitro* stimulation of macrophages with encapsulated nanoparticles

The anti-inflammatory effect of the encapsulated IL-10 nanoparticles was investigated by exposing these nanoparticles to macrophages in the presence and absence of rMOMP. Mouse J774 macrophages were propagated in DMEM medium which consisted of glutamine, 10% heat-inactivated fetal bovine serum, and 1 µg/mL antibiotic and antimycotic and incubated at 37°C in a humidified incubator containing 5% CO2. Macrophages (1 x 10^6 mL/well) were cultured in 12-well plates and media containing encapsulated nanoparticles at various concentrations (0-100 ng/ml) in the presence and absence of rMOMP at 10 µg/mL were added followed by incubation for an additional 24h. Unstimulated cells and PLA-PEG-PBS served as negative controls Cell-free supernatants were collected by centrifugation and stored at -20°C until used.

2.8. Cytokines measurement

Cytokine enzyme-linked immunosorbent assays (ELISAs) were used to quantify concentrations of mouse IL-6 in cell-free supernatants using Opti-EIA kits [2-4].

3.0 RESULTS

3.1 Characterization studies

Our results revealed that IL-10 was encapsulated in PLA-PEG with an encapsulation efficiency of 71 %. We next conducted UV visible spectra to confirm that IL-10 was completely encapsulated and not absorbed on the surface of PLA-PEG. We used naked IL-10 (positive) and PLA-PEG-PBS (negative) as controls. As shown in Figure 1, a peak absorbance at 280 nm wavelength (UV visible region for protein) (red box) for IL-10 alone indicates the presence of IL-10 in the sample solution. However, encapsulated IL-10 nanomaterial shows no peak absorbance at the same wavelength, indicating successful IL-10 encapsulation within PLA-PEG nanoparticles (purple box). Similar absorbance pattern was observed for PLA-PEG-PBS (green box).

We then employed FT-IR to identify variations in chemical functional groups within naked IL-10, PLA-PEG-IL-10 and PLA-PEG-PBS for further validation of the successful encapsulation of IL-10. We observed unique peak shifts at wavelengths 3383.81, 1635.16 and 1080.03 (cm-1) for IL-10 which were absent in PLA-PEG-IL-10 and PLA-PEG-PBS spectra (Figure 2). Overall, the FT-IR provided

additional evidence of the encapsulation of the IL-10 in PLA-PEG.

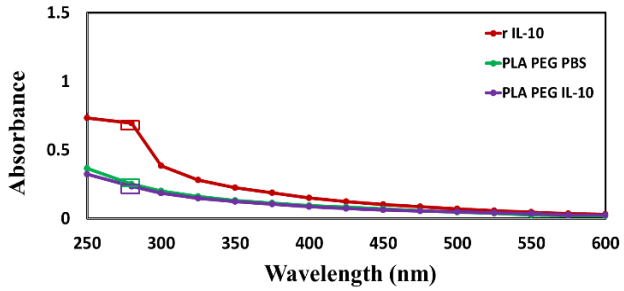

Figure 1. UV visible spectra to determine the encapsulation of IL-10. Nanoparticles (5 mg dissolved with 1 mL of DNAse-RNAse free water) and 1 mg/mL of free IL-10 were analyzed by UV visible spectrum.

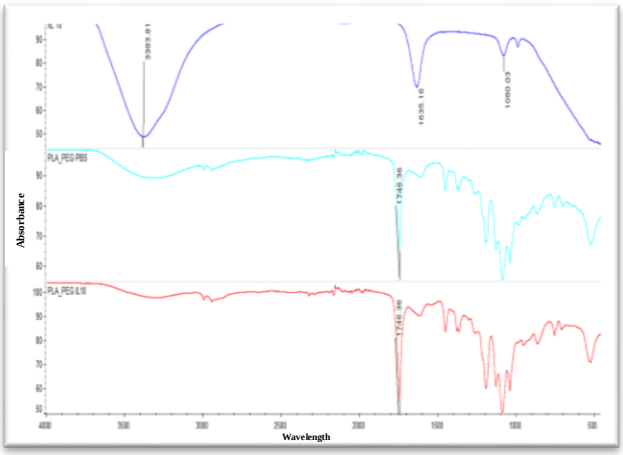

Figure 2. FT-IR spectra showing various functional groups and differences in the absorption spectra for nanoparticles. The FT-IR spectrum was recorded with 64 scans/samples ranging from 4000 to 1000 cm-1 and a resolution of 4 cm-1 at ambient temperature

We also performed DSC analysis to study the physical state of encapsulated IL-10 which could affect its *in vitro* release patterns. The DSC thermograms of PLA-PEG- PBS (control) and PLA-PEG-IL-10 are depicted in Figure 3. The melting temperature (Tm) of PEG is ~ 40ºC. The melting temperature of PLA has shifted from typical value of 150ºC due to the broadening effect indicative of interaction between the two monomers and lower crystallinity of PLA components. Furthermore, the melting endothermic peaks for PLA-PEG-PBS appeared at 93.3°C (green) and for PLA-PEG-IL-10, 88.59°C (blue), (Figure 3), suggesting no significant encapsulation effect on the thermal stability of the PLA-PEG-IL-10 nanoparticles.

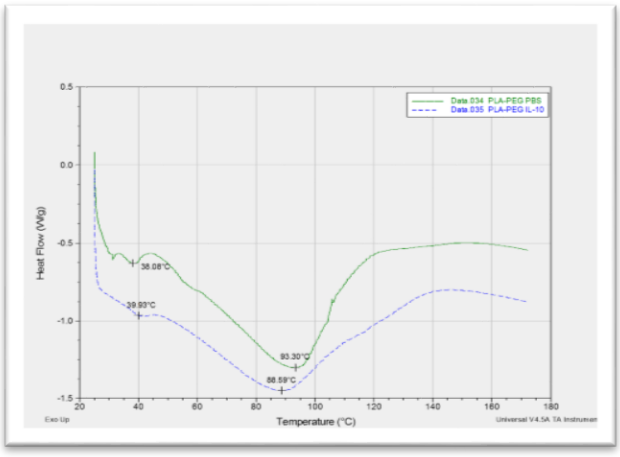

Figure 3. Thermal stability of IL-10 encapsulated within PLA-PEG nanoparticles. Approximately, 10 mg of PLA-PEG-IL-10 was accurately weighed into an aluminum pan, sealed, and then heated at the rate of 20°C per min from 30 to 120°C under nitrogen and then cooled from 120 to 30°C at the same rate, using a differential scanning calorimeter. Shown are peaks for PLA-PEG-PBS (green) and PLA-PEG-IL-10 (blue), respectively.

Lastly, the controlled release of the encapsulated IL-10 was investigated for up to 60 days. Our results show the gradual release of IL-10 up to day 60 (Figure 4) thus highlighting that IL-10 encapsulated in PLA-PEG is an effective and safe delivery system.

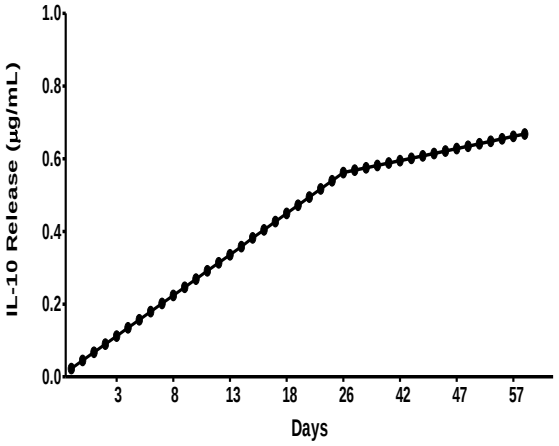

Figure 4. *In vitro* release pattern of IL-10 from PLA-PEG nanoparticles over a 60-day period. Release of IL-10 from nanoparticles was performed by encapsulated protein in PBS (pH 7.4). At each time-point, supernatants were obtained by centrifugation at 13, 680 × g for 5 min, and the IL-10 content was measured spectrophotometrically. Shown is the average cumulative release of IL-10 over a period of 60 days and the experiment was performed three times.

3.2 *In vitro* bioactivity of PLA-PEG-IL-10

The highly immunogenic MOMP of CT contributes to its inflammation by stimulating macrophages to secrete pro-inflammatory cytokines such as IL-6 that plays a pivotal role in the pathogenesis of the disease. Herein, we used IL-6 as a marker of inflammation to determine the anti-inflammatory effect of encapsulated IL-10 on its secretion from macrophages stimulated with rMOMP. Our data shows that all concentrations of IL-10 alone and encapsulated IL-10 (1-100 ng/mL) reduced the levels of IL-6 in mouse J774 macrophages post 24 hr treatment as compared to rMOMP (positive control) and PLA-PEG-PBS concentrations (negative control) (Figure 5).

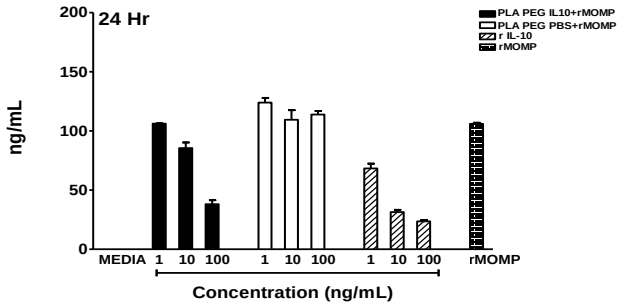

Figure 5. Dose-dependent study showing down-regulation of IL-6 by encapsulated and naked IL-10 in mouse J774 macrophages. Macrophages (10^6 cells/mL) were stimulated with rMOMP and incubated for 24 hr with different concentrations of PLA-PEG-IL-10 (1 to 1000 ng/mL) and PLA-PEG-PBS. IL-6 in cell free-supernatants was quantified by specific ELISA.

4.0 DISCUSSION AND CONCLUSIONS

IL-10 is a pleotropic anti-inflammatory cytokine as it regulates the expression of multiple inflammatory mediators elicited from a variety of cell types [5]. Its regulatory effect has been proven against bacterial inflammation [6] including CT [7] and other inflammatory diseases [8]. However, the effective therapeutic potential of IL-10 is limited due to its very short biological half-life which necessitates frequent administrations in biomedical applications. In this study, we encapsulated IL-10 in the polymeric biodegradable PLA-PEG nanoparticles, which have a very slow release property, in an attempt to prolong its biological half-life for therapeutic applications. IL-10 was successfully encapsulated in PLA-PEG as proven by the characterization studies (Figures1-4). Encapsulated IL-10 exhibited thermal stability and maintained its functionality by dose-dependently down-regulating the expression of the inflammatory cytokine IL-6 in macrophages, in response to the highly immunogenic rMOMP of CT.

Overall, our results presented here clearly demonstrate that IL-10 encapsulated in PLA-PEG nanoparticles is an effective delivery system for IL-10 as it prolonged its biological half-life, provided its sustained slow release while maintaining its functional anti-inflammatory properties *in vitro*. The immunotherapeutic effect of encapsulated IL-10 on inflammatory responses provides proof of concept for IL-10 application in bacterial inflammatory and autoimmune diseases.

5.0 REFERENCES

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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).

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