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# Folate-Functionalized Nanoparticles for Targeted Anti-Chlamydial Drug Delivery

Tayson T. Lin
Department of Immunology and Microbiology
Wayne State University School of Medicine
540 East Canfield Street
Detroit, Michigan 48201 USA

Faculty Advisor: Dr. J. A. Whittum-Hudson

## Abstract

*Chlamydia trachomatis* causes sexually transmitted infections that target reproductive organs. In 2010, over one million chlamydial infections were reported to the Centers for Disease Control. However, the actual count is probably higher because many people living with Chlamydia are often unaware of their infections. Azithromycin is the most common antibiotic treatment today for acute chlamydial infections. Unfortunately, when Chlamydiae deviate from their regular development cycle and enter a reversible state called persistence, the pathogen becomes incredibly resilient to antibiotics. Furthermore, persistent infections are also associated with other serious complications such as reactive arthritis and pelvic inflammatory disease. By unknown mechanisms, persistent Chlamydiae can wake from the persistent state and revert back to an actively infectious state; thus, knowledge about antibiotic efficacy on infections recovering from persistence is critical for designing improved drug treatments. This study explores the use of folate-targeted, biodegradable poly(d-L-lactide-*co*-glycolide) (PLGA) nanoparticles as improved anti-chlamydial drug delivery systems. Folate-conjugated nanodevices have already been utilized for the targeted delivery of therapeutic and imaging agents to cells with over-expressed folate receptors such as cancer cells or inflammatory cells of arthritis patients. A cell culture model was used to compare the efficacy of targeted folate-functionalized mPEG-PLGA-Nanoparticles (FA-NP Combo) to non-targeted mPEG-PLGA-Nanoparticles (NP Combo) for delivering Azithromycin (AZ) and Rifampicin (RIF) drug combos to chlamydial infections which have up-regulated folate receptor expression. A third treatment consisting of free AZ and RIF combo (Free Combo) was also conducted in parallel to the nanoparticle treatments. Cells were infected with *C. trachomatis* (serovar K/UW-31) and serial dilutions of FA-NP, NP, or Free Combo drugs were added to duplicate wells. In order to induce persistent infection, Penicillin G was added to the infection medium. Morphological analyses consisting of chlamydial inclusion size and inclusion counts were then used to assess viability of Chlamydiae recovering from persistence after drug treatment. Results showed infections treated with FA-NP Combo had significantly less chlamydial inclusions compared to cells pulsed with non-targeting NP Combo or Free Combo drugs. Furthermore, 2-D analyses of FA-NP Combo treatment group also showed significantly smaller chlamydial inclusion sizes. Building upon morphological analyses, transcript levels of key genes associated with chlamydial viability (16S ribosomal RNA primary transcripts) were assessed using Reverse Transcription-qPCR (RT-qPCR) after acutely or persistently infected cells were treated with FA-NP, NP or Free Combo drugs. In these experiments, FA-NP Combo outperformed its non-targeted counterparts and reduced 16S rRNA primary transcripts by statistically greater amounts. Together with this set of combined results, this project demonstrates molecularly the improved efficacy of folate-functionalized nanoparticles for targeted combination antibiotic delivery to acute and persistent infections. Furthermore, morphological data on targeted nanoparticles' improved therapeutic effect on infections recovering from persistence unlocks new knowledge on targeted nanoparticle performance during a phase of the chlamydial developmental cycle that is little understood by the scientific community.

**Keywords: Nanoparticles, Folate-Targeted, Chlamydiae**

## 1. Introduction

*Chlamydia trachomatis* is a major global health concern and causes more STDs than any other pathogen in the world[1]. Without proper treatment, Chlamydiae can cause serious complications such as infertility and ectopic pregnancies. In addition to reproductive damage, ocular infection by *C. trachomatis* may lead trachoma, one of the leading causes of preventable, infectious blindness in the world. Global estimates of Chlamydia induced trachoma now stand at 40 million people, of whom 8.2 million have permanently lost their sight[2]. Ideally, infection prevention is the body's best defense, but formulation of improved anti-chlamydia treatments could prove equally vital for a healthier global society.

The chlamydial biphasic developmental cycle provides insight to the barriers for effective treatment[3]. During an active infection, the cycle starts when metabolically inert but infectious chlamydial elementary bodies (EBs) attach onto and enter the host cell. Once inside, the EBs start to develop within chlamydial inclusions (vacuoles) where they differentiate into metabolically active, noninfectious reticulate bodies (RBs). After 18 to 24 hrs, the RBs reorganize back into EBs; and within 48-72 hrs, the expansion of mature chlamydial inclusions is followed by extrusion of infectious EBs. In special circumstances, the cycle can deviate from its normal pathway as Chlamydiae enter a reversible state called persistence in which large aberrant RBs (aRBs) are observed. Persistent Chlamydiae demonstrate unique transcription profile as genes involved in growth and cytokinesis are severely attenuated[4,5]. Moreover, persistent infections are believed to be associated with reactive arthritis in the joints since phenotyping of Chlamydiae found in the synovial tissue of reactive arthritis patients demonstrated gene expression characteristic of persistent infections[4,5].

One of the major obstacles for successful treatment lies in drug delivery. In order for anti-chlamydial therapies to work, they are required to penetrate three membranes (the host cell membrane, the chlamydial inclusion membrane, and finally the bacterial membrane) before delivering their payload. To overcome this obstacle, biodegradable poly(d-L-lactide-*co*-glycolide) (PLGA) nanoparticles were tested as potential drug delivery candidates because fluorescent 6-coumarin tagged nanoparticles were found to rapidly traffic into the inclusions of Chlamydia infected-HEp2 cells[6]. Subsequent experiments treated infected cells with nanoparticles containing Azithromycin (AZ) and Rifampicin (RIF) as recent studies suggested that using a combination of AZ and RIF is more effective for treatment of persistent Chlamydial infections[7]. The results were promising as nanoparticle encapsulated drug treatment (NP) showed a significant reduction in chlamydial inclusion size and numbers in both acute and persistent infections when compared to free combination drugs at the same concentrations – free combination drugs (Free Combo) are antibiotics not encapsulated within nanoparticles[6]. Supplementing morphological analyses, effects of nanoparticle or free combination drug treatment were examined at the molecular level as the transcription of key chlamydial genes associated with viability (16S ribosomal RNA primary transcripts) was quantified using RT-qPCR (McPharlin *et al*, unpublished data). The results mirrored that of the morphological experiments as nanoparticles outperformed free combination drugs, reducing 16S rRNA primary transcripts by greater amounts. In terms of clinical significance, these findings demonstrated, quantitatively and qualitatively, nanoparticles' promising ability as anti-chlamydial drug delivery systems.

Nanoparticle potential can be optimized through folate functionalization. Folate-functionalized nanoparticles (FA-NP) have recently become a scientific phenomenon in terms of targeted drug delivery to cells with over-expressed folate receptors such as cancer cells or inflammatory cells of arthritis patients[8]. Furthermore, *in vitro* experiments elucidated the potential of folate-functionalized nanoparticles for enhancing the absorption of drugs with poor oral bioavailability such as the anti-cancer drug, paclitaxel[9].

Similar to cancer and inflammatory cells, chlamydial infected cells also demonstrate increased expression of folate receptors. This discovery was elucidated with RT-qPCR showing up-regulated transcript levels of three principle isoforms of folate receptors (alpha, beta and gamma) in both human and mouse cell lines infected with Chlamydia (Gerard *et al*, unpublished data). Building upon these findings, targeted folate-functionalized and non-targeted nanoparticles both loaded with AZ were tested *in vivo* ($n = 4$ mice/treatment). The results were promising as infected mice treated with targeted nanoparticles had significantly greater AZ levels ($p < 0.05$) in their tissues (knees, genital tract, and liver) when compared to their non-targeted counterpart at 72 hr after intravenous delivery (Whittum-Hudson *et al*, manuscript in preparation). Furthermore, free AZ was not detected in any tissue after the same time.

Previous research constructed the platform for this project by identifying folic acid as an appropriate targeting molecule for anti-chlamydial therapies. This study took the next step in targeted nanoparticle research and compared the drug delivery potential of folate-functionalized nanoparticles against non-functionalized counterparts in acute and persistent infections. Adding another dimension to the project, the laboratory also evaluated the efficacy of targeted nanoparticles on Chlamydiae recovering from persistence. By methods still unknown to the Chlamydia scientific community, persistent Chlamydiae can wake from a state of differential gene expression and revert back to

an actively infectious state. A portion of this project was dedicated to simulating these events *in vitro* by the removal of penicillin G from infection medium after persistence was induced. If infections successfully revert back to active infections, this reversion correlates to high chlamydial viability as the aRBs escape persistence and transform into infectious EBs. However, if infections fail to recover from persistence after the addition of targeted folate-functionalized nanoparticles, this is strong evidence supporting the treatment's success at killing and clearing Chlamydia.

Similar to the experiments described before, the success of different treatment groups was gauged morphologically and molecularly as targeted folate-functionalized mPEG-PLGA-nanoparticles (FA-NP Combo) or non-targeted mPEG-PLGA-nanoparticles (NP Combo) containing AZ and RIF were administered to infected cells; methoxypoly(ethylene glycol), or mPEG, served as a folate linker in targeted nanoparticles and was also integrated in non-targeting nanoparticles as an appropriate control. For treatment of Chlamydiae recovering from persistence, morphological analyses were used to evaluate targeted nanoparticle's effectivness at reducing inclusion size and numbers. RT-qPCR measuring primary transcripts of 16S rRNA quantified chlamydial viability by molecular techniques in treated acute or persistent infections. The goal of this project was to determine whether FA-functionalized nanoparticles are the better drug delivery system for reducing chlamydial load. If so, use of targeted nanoparticles can amount to fundamental savings in public health costs for chlamydial treatments. More importantly, these discoveries might further support targeted nanoparticles to be the gateway to curing antibiotic-resilient persistent infections.

## 2. Methods

### 2.1. *C. trachomatis* Elementary Bodies Purification

*C. trachomatis* serovar K (UW-31) was the pathogen used in this project. Standardized Chlamydia stocks were obtained by growth in HEp2 cells (human lung epithelial cells, Manassas, VA) and purified using previously published methods[6]. HEp2 cells were cultured in Iscove's Modified Dulbecco's Medium (DMEM) (Mediatech, Herndon, VA) with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 0.2 µg of gentamicin/ml (Life Technologies, Carlsbad, CA) and 2 mM L-glutamine (SigmaAldrich, St. Louis, MO). Upon purification, all aliquots were stored at -80°C. Concentrations of infectious stocks (inclusion-forming units, [IFU]/ml) were determined by standard methods with McCoy cells grown in Eagle's Minimum Essential Medium with Earle's salts (EMEM; Mediatech) with the same supplements as above.

### 2.2. Nanoparticle Fabrication

Nanoparticles were formulated using a single-step surface functionalizing technique for polymeric nanoparticles[10]. PLGA polymers (32 mg) along with antibiotics AZ and RIF (ranging from 50 to 100% w/w of polymer) were first dissolved in chloroform. An oil-in-water emulsion was formed by sonicating (Sonicator XL, Misonix, NY) the polymer-antibiotic solution in 2.5% w/v aqueous Polyvinyl Alcohol (PVA) solution for 5 min over ice bath. For targeted nanoparticles, polylactide-polyethylene glycol-folate (PLA-mPEG-FA) was dissolved in chloroform and added drop-wise to the above emulsion with stirring; when PLA-PEG-FA is introduced in the oil/water emulsion, the PLA block partitions into the polymer containing organic phase and PEG-FA block partitions into the aqueous phase. Removal of the organic solvent results in the formation of nanoparticles with FA on the surface. For formulation of non-targeted nanoparticles, PLA-mPEG without FA ligand was used.

The emulsions were then stirred for 18 hrs at ambient conditions followed by 2 hrs under vacuum to remove the residual chloroform. Targeted and non-targeted nanoparticles were recovered by ultracentrifugation and washed with deionized water to remove any unencapsulated antibiotics and residual PVA solution. The nanoparticle suspension was lyophilized to obtain the final products.

### 2.3. Nanoparticle Characterization

To determine AZ and RIF loading within nanoparticles, targeted and non-targeted nanoparticles samples were extracted with methanol for 15 hrs. The extract was centrifuged and the antibiotics within the supernatant were isolated using high-performance liquid chromatography (HPLC) and their concentrations were quantified with UV/Vis spectroscopy at wavelength of 210 nm for AZ and 238 nm for RIF (Whittum-Hudson *et al*, manuscript in preparation).

Table 1 Nanoparticle Drug Loadings

| Formulation | % AZ Loading | % RIF Loading | Ratio AZT:RIF |
|---|---|---|---|
| FA-mPEG-NP | 1.715 | 0.128 | ≈ 13:1 |
| mPEG-NP | 2.883 | 0.272 | ≈ 11:1 |

Table 1 characterizes nanoparticle formulations used in this project. Due to the limited control of fabrication techniques used, targeted and non-targeted nanoparticles drug ratios are similar, but not equal; thus serial dilutions nanoparticles used in this project were normalized to one antibiotic, AZ. For Free Combo treatments, the drug ratio was 12 AZ: 1 RIF − the average drug ratio of targeted and non-targeted nanoparticles. Free Combo antibiotics were formulated with Azithromycin (Astatech) and Rifampicin (Sigma) suspended in medium. Nanoparticle drug conjugates were weighed and sonicated in medium for 30 seconds on the day of treatment.

## 2.4. Nanoparticle Drug Release

In vitro drug release from nanoparticles was determined in phosphate−buffered saline (PBS, 0.15 M, pH 7.4) containing 0.1% w/v Tween 80. Nanoparticle suspensions were placed in several 1.5-ml centrifuge tubes in a water bath shaker set at 100 rpm and 37°C (Precision, Thermo Scientific, Marietta, OH). At predetermined time intervals, a set of three tubes were centrifuged at 21,000 x g for 10 min and the supernatants were analyzed for AZ and RIF concentrations by HPLC (Whittum-Hudson *et al*, manuscript in preparation).

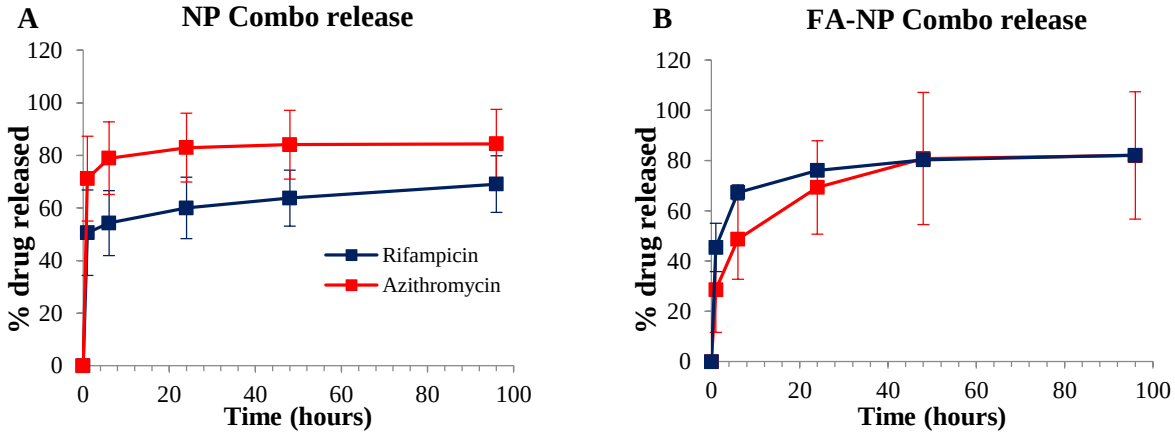


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Figure1. *In vitro* drug release from non-targeted nanoparticles (A) and targeted folate-functionalized particles (B) Nanoparticles were suspended in phosphate−buffered saline (PBS, 0.15 M, pH 7.4) containing 0.1% w/v Tween 80 suspensions and incubated in a water bath shaker set at 100 rpm and 37°C.

## 2.5. Morphological Experiments during Recovery from Persistent Infection Sample Preparation

For count analyses, McCoy cells were seeded in 96-well microtiter plates (BD Falcon) while area analyses utilized HEp2 cells cultured in four well-chamber slides (Lab-Tek). Cells in seeded plates or chamber slides were allowed to adhere at 37°C in 5% $CO_2$ atmosphere. Once cells reached confluency, growth medium was aspirated and cells were infected with *C. trachomatis* at a multiplicity of infection (m.o.i.) of 3 IFU/cell. Infected McCoy cells in 96-well plates were centrifuged at 1200 x g for 1 hr at RT followed by 1 hr incubation at 37°C. Infected HEp2 cells in chamber slides were incubated at 37°C for 1 hr with rocking every 15 minutes to ensure homogenous inoculum distribution. Following incubation, excess inoculum was aspirated and growth medium containing 1 µg/ml cycloheximide (Sigma), 0.06% glucose (Sigma) and penicillin G (100 U/ml) was applied. Treatment began at t = 24 hrs as FA-NP Combo, NP Combo or free combo drugs in serial dilutions were added to persistently infected McCoy or HEp2 cells. At t = 48 hrs, infection medium (which contained penicillin G and any drugs not taken up by cells)

was aspirated; this was followed immediately by a wash with Dulbecco's DPBS (Mediatech) to remove residual Penicillin G or antibiotics. Fresh growth medium containing no cycloheximide or Penicillin G replenished the wells – the absence of Penicillin G allows the persistent Chlamydiae to revert back into an active infection (recovery from persistence). Morphological analyses consisting count and size analyses began 36 hrs later at t = 84 hrs.

### 2.6. Quantifying the Inclusion Numbers and Size

Infected McCoy or HEp2 cells were fixed with absolute methanol for 10 minutes and stained for immunofluorescence with chlamydia-specific antibodies labeled with fluorescein isothiocyanate (FITC). This antibody detects chlamydial bacteria and the inclusions in which they develop. For each antibiotic dose, chlamydial inclusions in stained McCoy cells were counted. For size analyses, pictures of stained HEp2 cells in each treatment group were taken using ImagePro Plus V6.3 (MediaCybernetics, Bethesda, MD). Inclusion size was measured as area ($\mu m^2$). The mean area was plotted as a function of antibiotic concentration. Size and count data were analyzed graphically and statistically using SigmaPlot V11.2 (Systat Software, San Jose, CA).

### 2.7. Acute and Persistent Sample Preparation for Molecular Experiments

HEp2 cells were cultured in six well plates (Corning, Thermo Fisher Scientific) and inoculum was applied to confluent monolayers followed by 1hr incubation. For acute samples, infected cells were pulsed with serial dilutions of FA-NP, NP or Free Combo drugs after incubation. In persistent samples, infected cells were cultured with infection medium containing Penicillin G for 24 hrs before initializing drug treatment[6]. Forty-eight hours after introduction of antibiotics, cells were scraped off the plates, centrifuged and stored at -80°C. Total nucleic acids from collected samples were obtained by hot phenol/chloroform extractions and pure RNA was isolated via extensive RNase-free DNase I treatment; small quantities of DNase-treated samples were subjected to PCR amplifying chlamydial 16S rDNA followed by agar electrophoresis to detect residual DNA[4]. With confirmation of DNase treatment success, standard reverse transcription was used to convert RNA into cDNA. Next, qPCR was performed using SYBR green (Applied Biosystems, Warrington, UK) with a PE BioSystems model 7500 sequence detector as described[4]. Chlamydial 16S ribosomal RNA (rRNA) primary transcripts was amplified and normalized to host 18S rRNA. Data were analyzed using v1.7 Sequence Detection Software from PE BioSystems. Normalized $\Delta$Ct values and their corresponding fold changes ($2^{-\Delta\Delta Ct}$) were calculated with values from treated samples compared to the positive control (infection without treatment) to quantify relative reduction in chlamydial 16S rRNA primary transcripts. Graphical and statistical analyses on molecular data were conducted in SigmaPlot V11.2.

## 3 Results

### 3.1 FA-NP Improved Drug Delivery to Cells Recovering from Persistent Infections

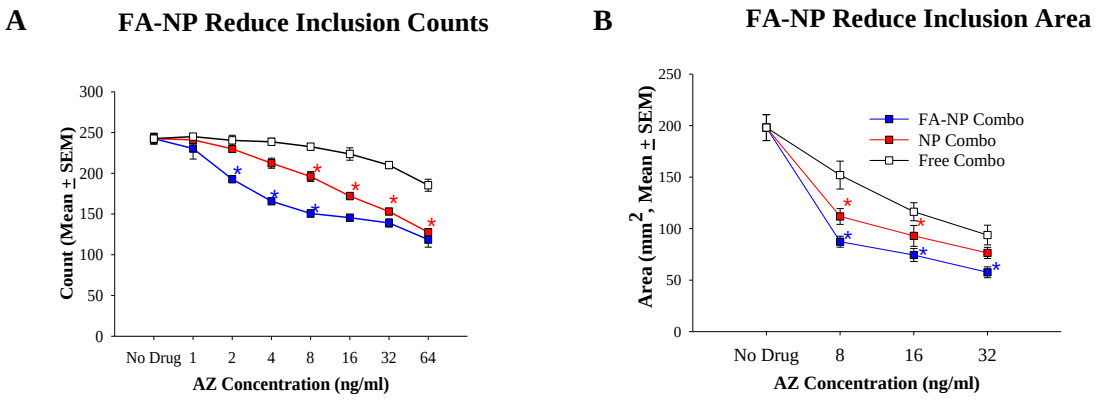


Figure 2. Inclusion count (A) and Inclusion Size (B) in Recovering from Persistent Infections
Chlamydial inclusion size and numbers were compared among treatments as a measure of pathogen viability.

Note RIF concentrations were approximately 10-fold lower than those of AZ. The AZ concentration alone is given for clarity of axis labels.

(A), FA-NP Combo, NP Combo, and Free Combo treatments' effect on chlamydial inclusion numbers. Free Combo had little effect on reducing inclusion numbers from concentrations 0 to 8 ng/ml AZ. On the other hand, non-targeted nanoparticles demonstrated significantly less inclusion numbers when compared to Free Combo at $\geq$ 8ng/ml of AZ (Red * = $p < 0.05$, Student's t-test between NP Combo and Free Combo Treatments). (B), 2-D inclusion area experiments mirror inclusion count analysis results as NP outperformed Free Combo treatment with significant reductions in inclusion sizes (Red * = $p < 0.05$). Overall, targeted FA-NP Combo proved to be the best treatment and reduced inclusion counts/sizes by significantly greater amounts when compared to non-targeted counterparts (Blue * = $p < 0.05$, Student's t-test between FA-NP Combo and NP Combo).

## 3.2. Improved Drug Delivery to Acute and Persistent Chlamydial Infections with Targeted Nanoparticles

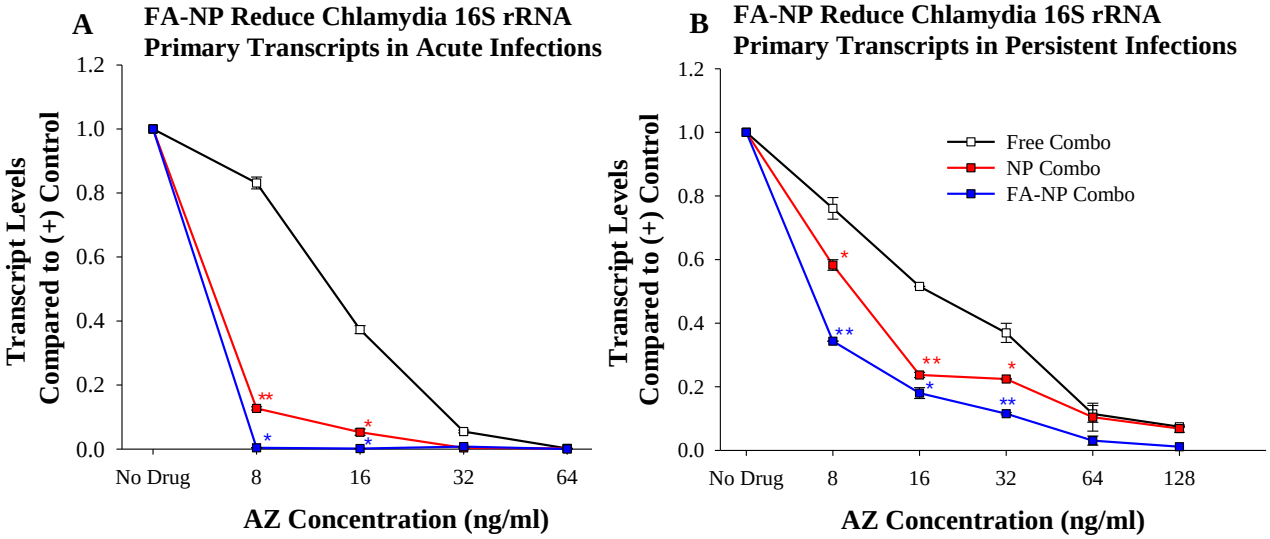


Figure 2. Molecular Results for Persistence and Acute Infections
The amounts of Chlamydial 16S rRNA primary transcripts were quantified using RT-qPCR. Transcript levels were compared among treatments as a measure of pathogen viability. Again, the AZ concentration alone is given for clarity of axis labels.

FA-NP Combo had the best performance with significantly lower chlamydial 16S rRNA primary transcript levels than non-targeted drugs in both acute and persistent infections (Blue * = $p < 0.05$, **= $p < 0.01$, Student's two sample t-test between FA-NP Combo and NP Combo). FA-NP Combo and NP Combo cleared acute and persistent infections at much lower drug concentrations when compared to free drugs.

## 4. Discussion

This project's genesis was founded upon years of research in nano-scale Chlamydia treatment. The promising drug delivery potential of nanoparticles was first effectively illuminated with fluorescent microscopy showing 6-coumarin-labeled nanoparticle accumulation within chlamydial inclusions[6] − evidence that nanoparticles were successful in penetrating the multiple membrane barriers as mentioned before. In a similar study, treatment with nanoparticles containing AZ and RIF resulted in smaller chlamydial inclusion numbers/sizes than unencapsulated combination antibiotics (McPharlin *et al*, unpublished data).

In this study, improved performance by non-targeted nanoparticles containing AZ and RIF drug combos when compared to free drugs for treating acute, persistent and recovering from persistent infections supported previous

findings. This was shown by greater reduction in chlamydial inclusion numbers/sizes as well as attenuation of chlamydial 16S rRNA primary transcript levels for NP Combo drugs compared to Free Combo drug treatment ($p < 0.05$ or $p < 0.01$, at most drug concentrations administered).

Overall, folate functionalized nanoparticles with combination drugs was the most effective treatment for reducing Chlamydia load. FA-NP Combo treatment groups have significantly smaller inclusion sizes/numbers and 16S rRNA primary transcript expression when compared to non-targeted nanoparticles in all infection types tested ($p < 0.05$ or $p < 0.01$, at most drug concentrations). These findings expands upon initial *in vitro* molecular studies elucidating up-regulated folate receptor gene expression in Chlamydia-infected cells, and reinforces *in vivo* experiments documenting greater antibiotics levels in tissues of infected mice treated with targeted nanoparticles (Whittum-Hudson *et al*, manuscript in preparation).

Based on the information gathered, targeted nanoparticles have promising potential for treating chlamydia in all the phases of its dynamic life cycle. Special emphasis is placed on the ability of targeted nanoparticle to effectively treat persistent Chlamydiae that are not only resilient to antibiotics, but also associated with other serious complications such as reactive arthritis. In the next major step, *in vivo* mouse studies will evaluate how targeted nanoparticles containing both antibiotics behave in infected animals with functional immune responses as opposed to basic *in vitro* settings. If these experiments prove successful, the project findings will play a significant role in formulating future anti-chlamydial therapies that utilize less antibiotics. This amounts to fundamental savings in public health costs and will help facilitate more effective anti-chlamydial treatment worldwide.

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