# Smaller sizes of Ag-PVP Nanoparticles Control Inflammatory Responses, and Reduce CD80 and CD86 Expression Levels, in Macrophages Infected with *Chlamydia trachomatis*

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## ABSTRACT

*Chlamydia trachomatis* is an important cause of sexually transmitted infections that can manifest itself as either acute cervicitis, pelvic inflammatory disease, and more commonly as a chronic asymptomatic infection. One of the most important factors affecting the potential effectiveness of *Chlamydia* control program is the timing of inflammation. Early intervention strategies that can reduce excessive inflammatory responses could benefit control efforts in reducing the disease severity. Previously we reported that poly-vinyl pyrrolidone (PVP)-coated silver (Ag) nanoparticles (Ag-PVP) at 5-10 nm reduced pro-inflammatory cytokine (IL-6 and TNF) production levels by up to 75% in mouse J774 macrophages infected with *C. trachomatis*. Here we hypothesize that the anti-inflammatory actions of Ag-PVP maybe dependent on the nanoparticle size. We tested three different sizes of Ag-PVP (10, 20 and 80 nm) for their abilities to reduce pro-inflammatory cytokines in macrophages infected with *C. trachomatis*. Our results show by cytokine specific ELISAs that all sizes of Ag-PVP reduced IL-6 and TNF as elicited from macrophages infected with live *C. trachomatis,* with the 10 nm size exhibiting the greatest anti-inflammatory effect. Our MTT assay shows that the anti-inflammatory effect of Ag-PVP is not due to cell death at the concentration used. However, at higher concentrations all tested Ag-PVP sizes were toxic to cells. We also demonstrated by flow cytometry the ability of Ag-PVP (5-10 nm) to reduce the expression levels of CD80 and CD86 co-stimulatory molecules on infected macrophages. This interesting finding suggests their potential to control not only pro-inflammatory cytokines produced by innate immune cells but also those produced by *C. trachomatis* activated T cells during the adaptive immune response. Overall our data imply that low concentration and smaller size Ag-PVPs are more effective as regulators of the inflammatory response to *C. trachomatis* and that further investigations can be made using these nanoparticles to combat inflammation induced by this bacterium.

**Key words:** *Chlamydia,* CD80, CD86, silver nanoparticles, cytokines, inflammation, and macrophage.

## 1. INTRODUCTION

*Chlamydia trachomatis* is an obligate intracellular bacterial pathogen responsible for sexually transmitted infections worldwide [1, 2]. Like other infectious organisms, *Chlamydia* infection of macrophages allows the secretion of various inflammatory mediators to mitigate the infection. Inflammatory cytokines enhance the clearance of infection in two major ways: 1) directly by producing toxic molecules against the pathogen, and 2) indirectly by attracting or activating phagocytic cells or by activating the adaptive immune response. However, because of the persistence nature of *C. trachomatis* infection, inflammatory mediators will be produced massively and contribute to the disease manifestations [3]. Therefore, it is critical to balance the levels of inflammatory mediators released during early infection.

Surface coated silver nanoparticles have been found to exhibit different biological properties [4]. One of the main advantages of using nanoscale materials in biomedical science research is that they can easily cross physiological barriers such as the blood-brain barrier and the tight epithelial junctions of mucosal surfaces [5]. However, one possible disadvantage of nanoscale materials is their potential for indiscriminate entry into various organs and become toxic to the cells [6-8]. Despite the wide application of silver nanoparticles, there is a serious

lack of information how nanoparticle size influence its effects. Here we tested three different sizes of Ag-PVP (10, 20 and 80 nm) for their abilities to reduce pro-inflammatory cytokines in macrophages infected with *C. trachomatis*. We also demonstrated the ability of Ag-PVP (5-10 nm) to reduce the expression levels of co-stimulatory molecules (CD80 and CD86) on infected macrophages. Here we present our data and discuss the role of Ag-PVP as regulators of the inflammatory response during *C. trachomatis* infection.

## 2. MATERIALS AND METHODS

### 2.1 Cell lines and culture

Mouse J774 macrophages were obtained from the American type culture collection (ATCC, Rockville, MD) and cultured in Dulbecco Modified Eagle Medium (DMEM) (ATCC) supplemented with 10% heat-inactivated FBS, 1 µg/mL antibiotic and antimycotic (Invitrogen). All cells were maintained at 37 °C in a humidified incubator containing 5% $CO_2$.

### 2.2 Preparation of Ag-PVP nanoparticles

Ag-PVP at 80 and 20 nm were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA) and Ag-PVP at 5-10 nm was synthesized following the method of Elechiguerra et al [6].

### 2.3 Infection of J774 macrophages

*C. trachomatis* MoPn Nigg II was purchased from ATCC (ATCC #VR-123) and propagated in HeLa cells monolayers in MEM/H supplemented with 10% FBS. To determine the effect of Ag-PVP on the production level of TNF and IL-6, J774 ($10^6$ cells/well) cells were infected with infectious particle of *C. trachomatis* ($10^4$ IFU/well) for two days and then infection media were replaced with media containing various concentrations of Ag-PVP. As a control the cells were stimulated with *E. coli* LPS (1 µg/mL). Cell-free-supernatants were collected after 24 hours following centrifugation at 450 x g for 10 min at 4 ºC and stored at -80ºC until used. These time point and MOI were found to be the optimum conditions based on our previous studies.

### 2.4 Cytokines

All reagents and antibodies for cytokine ELISAs were purchased from BD Biosciences and ELISAs were performed according to the manufacturer suggested protocol.

### 2.5 Cell cytotoxicity assay

One of the disadvantages of nanomaterials in biomedical sciences application is their toxicity. Therefore, we evaluated the cytotoxicity effect of Ag-PVP on J774 macrophages. J774 macrophages ($10^4$ cells/well) were plated in 96-well plates and after 24 hours incubations, various concentrations of Ag-PVP were added to the cells. The cells were incubated for an additional 24 hours and then treated with the MTT reagent. The plates were covered and left at 37 ºC in a humidified incubator containing 5% $CO_2$ for 3 hours. The absorbance in each well was measured at 570 nm using a TECAN Sunrise ELISA plate reader (TECAN US Inc., Durham, North Carolina). Percent cell viability was calculated by extrapolating the optical density reading compared to normal cells (control).

### 2.6 Flow cytometry

Studies using the murine model of *C. trachoamtis* genital tract suggested that T cells responses may also cause collateral tissue damage [4]. The activation of CD80 and CD86 on antigen presenting cells is required for T cells to be fully activated [10], thus we tested the effect of Ag-PVP on the activation of CD80 and CD86 on *C. trachomatis* infected macrophages. Macrophage J774 cells ($10^6$ cells/well) were left uninfected or infected with *C. trachomatis* ($10^4$ IFU/well) and after a 2 day infection, the media were replaced with fresh media supplemented with or without Ag-PVP (3.2 g/mL). After 24 hours post-Ag-PVP incubation, the cells were harvested and immunostained with anti-mouse CD80 and CD86. FACS data was acquired using BD FACS Canto II flow cytometer (BD Biosciences). Dead cells were excluded from analysis and at least $10^5$ viable cells were acquired for each sample. The FACS data was analyzed using FlowJo (Tree Star Inc, Ashland, Oregon, USA).

### 2.6 Statistics analysis

For inhibition studies all the data are expressed as mean ± SD and data were analyzed by using the two-tailed unpaired Student's *t*-test and $P < 0.005$ was considered significant. For cytoxicity studies the data are expressed as the mean ± SD of three experiments.

## 3. RESULTS

### 3.1 All sizes of Ag-PVP reduced IL-6 and TNF as elicited from macrophages infected with live *C. trachomatis*.

During *C. trachomatis* invasion of macrophage TNF and IL-6 are expected to be secreted, therefore, we measured their levels in *C. trachomatis* infected macrophage. Comparing with uninfected cells, infected macrophages exhibited significant level of these cytokines (Fig. 1A and B). However, when Ag-PVP was added the levels of these cytokine were reduced in a dose dependent manner. Comparing with the cells that didn't get Ag-PVP, all sizes of Ag-PVP reduced TNF and IL-6 significant ($P < 0.005$) at the concentrations of 3.2 and 6.25 µg/mL (Fig. 1A and B). Comparing among different sizes of Ag-PVP the 10 nm gave greatest anti-inflammatory effect supporting the usefulness of smaller size Ag-PVP to inhibit cytokines induced during early *C. trachomatis* infection. In the other hand, when J774 cells were stimulated with LPS only the level of IL-6 was reduce in dose dependent manner (Fig. 2C and D) and this attributed to the difference secretion profile between the two cytokines. When we compare the inhibition effects among the different sizes of Ag-PVP, 10 and 20 nm exhibited the greatest anti-inflammatory effect when LPS used as the stimulant (Fig. 1C and D).

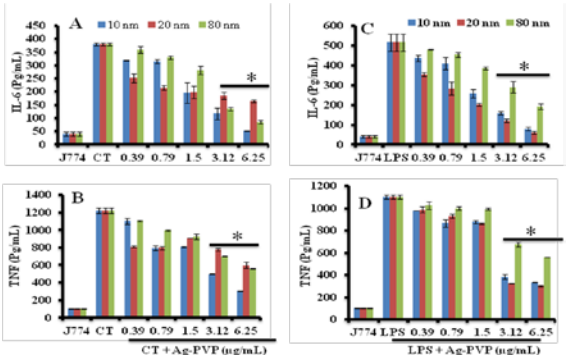


**FIGURE 1: All sizes of Ag-PVP reduced IL-6 and TNF, with the 10 nm size exhibiting the greatest anti-inflammatory effect.** J774 mouse macrophage cell line ($10^6$ cells/mL) were infected with live *C. trachomatis* ($10^4$ IFU/mL) (A and B) or LPS (1 mg/mL) (C) in the presence and absence of various concentrations of Ag-PVP.

### 3.2 The decreased level of TNF and IL-6 is not due to cell death.

Despite the many benefits of nanotechnology, some studies indicate that certain nanoparticles may cause adverse effects because of their small size and unique properties. Therefore, we next asked whether the decreased inflammatory response observed is due to a toxic effect of Ag-PVP. Here we evaluated decreased cell viability under the influence of Ag-PVP ranging from 0.39-200 µg for 24 hours. More than 80 % of cells survived at Ag-PVP concentrations ranging from 0.39-3.12 (Fig. 2), suggesting that these concentrations are not toxic to the cells. More specifically it implies that the anti-inflammatory effect observed at 3.12 µg/mL is not due to increased cell death. Moreover, comparing toxicity level of the different sizes of Ag-PVP at 3.12 µg/mL, the 10 nm exhibited 99.5 % cell viability whereas 20 nm and 80 have exhibited lower cell viability, 80.2 % and 86%, respectively. This implies that the smaller size nanoparticles are more advantageous in minimizing the toxicity. Our result shows that regardless of the sizes, higher concentration of Ag-PVP is toxic to the cells, where the cell viability decreases gradually as we increased the Ag-PVP concentration (Fig. 2). One plausible explanation for such differences is small size and lower concentration makes them highly mobile in lieu of aggregating in one location, thus became less toxic.

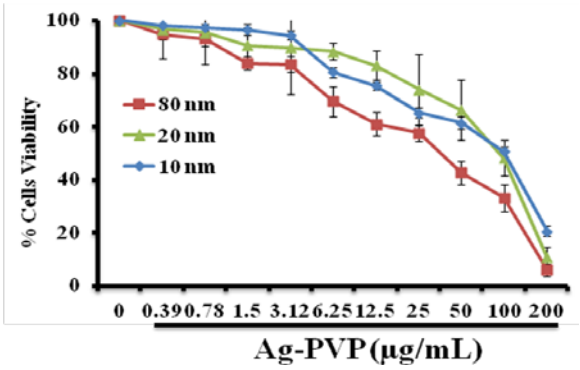


**FIGURE 2: MTT assay shows that the anti-inflammatory effect of Ag-PVP is not due to cell death at the concentrations used.** The viability of J774 cells was treated with various sizes of silver nanoparticles at a concentration range of 0.39-200 µg/mL is expressed as percentage of untreated cells. The values are the mean of three experiments.

### 3.3 Ag-PVP reduced the expression of CD80 and CD86

In most eukaryotic cells activation of Toll like receptors (TLR) trigger the production of inflammatory mediators, and the expression of co-stimulatory molecules, which are essential for the induction of adaptive immune responses. Thus, finally we asked whether Ag-PVP at 10 nm is effective in inhibiting the expression level of these molecules. Our FACS analysis revealed that CD80 and CD86 are expressed by J774 in response to live *C. trachomatis*, however, the presence of Ag-PVP reduced their expression (Fig. 3A). The expression of CD80 and CD86, along with MHC molecules allows the presentation of microbial peptide to activate naïve CD4 T cells to produce more pro-inflammatory mediators to the site of infection.

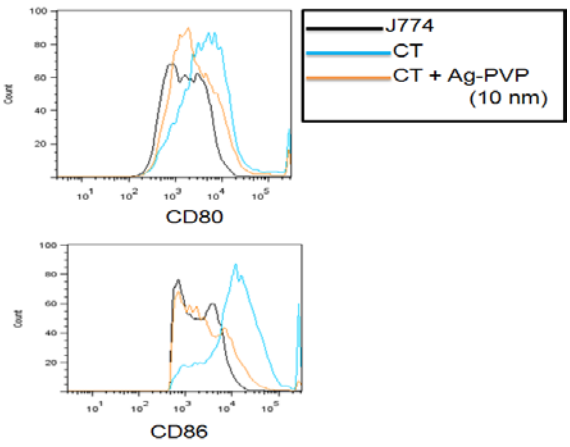


**FIGURE 3: Ag-PVP reduced the expression of CD80 and CD86.** Macrophage cells were treated with live *Chlamydia trachomatis* ($10^4$ IFU) in the presence and absence of Ag-PVP (3.12 µg/mL). The expression levels of CD80 and CD86 were determined using Flow Cytometry.

## 4. DISCUSSION

Inflammatory mediators released by *C. trachomatis*-infected macrophages initiate inflammation [10]. The inflammation of an infected tissue has several beneficial effects in combating infection. It recruits cells and molecules of innate immunity out of the blood and into the tissue where they are needed to destroy the pathogen directly. In addition, it increases the flow of lymph bearing microbes and antigen-bearing cells nearby lymphoid tissues, where they will activates lymphocyte and initiates adaptive immune response. However, over production of inflammatory mediators promote infiltration of immune cells that can release protease that damage other cells [2-4]. Thus, it is important to control excess inflammatory molecules during *C. trachomatis* infection.

Silver nanoparticles have been investigated as anti-inflammatory molecules in various infectious models [6]. Previously we reported that Ag-PVP at 5-10 nm reduced pro-inflammatory cytokine (IL-6 and TNF) production levels by up to 75% in mouse J774 macrophages infected with *C. trachomatis*. Since the effect of nanoparticles may be size dependent here we tested three different sizes of Ag-PVP (10, 20 and 80 nm) for their abilities to reduce pro-inflammatory cytokines in macrophages infected with *C. trachomatis.* At 3.12 µg/mL concentration, small size (10 nm) Ag-PVP exhibited a better anti-inflammatory effect than did the 20 and 80 nm sizes. At this concentration, the anti-inflammatory effects are shown in the order of 10 nm > 20 nm > 80 nm, which supports our hypothesis. Our MTT assay revealed that the effect of Ag-PVP at 3.12 and 6.25µg/mL is not due to the killing of cells. It is also interesting to see that Ag-PVP (5-10 nm) reduced the expression levels of CD80 and CD86 on infected macrophages. This finding suggests that Ag-PVP has the potential to control not only pro-inflammatory cytokines produced by innate immune cells but also those produced by *C. trachomatis* activated T cells during the adaptive immune response. In summary, our data implies lower concentration and smaller size Ag-PVP nanoparticles are may be effective as regulators of the inflammatory response to *C. trachomatis*.

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