Susceptibility of *Chlamydia trachomatis* to the excipient hydroxy-ethylcellulose: pH and concentration dependence of antimicrobial activity

Ali A. Abdul Sater, David M. Ojcius, and Matthew P. Meyer*

Running head: Anti-chlamydial effects of hydroxyethyl cellulose

School of Natural Sciences, University of California, Merced, California 95344

*To whom correspondence should be addressed:

Dr. Matthew Meyer, School of Natural Sciences, University of California, P.O. Box 2039, Merced, CA 95344. Telephone: (209) 228-2982. Fax: (209) 228-4053. E-mail: mmeyer@ucmerced.edu.
Abstract

Hydroxyethyl cellulose (HEC) is used as a neutral excipient in microbicides against sexually-transmitted pathogens. However, HEC inhibits infection of cervical epithelial cells by *Chlamydia trachomatis* at pH 5 in a concentration-dependent manner. At pH 7, infection is inversely dependent on the concentration of HEC, possibly due to pH-dependent calcium sequestration.
*Chlamydia trachomatis* causes pelvic inflammatory disease, ectopic pregnancy, and reproductive disability and is the most common bacterial sexually transmitted infection in the world (4). However, chlamydial infection is asymptomatic in most men and women infected with the pathogen (9). Since new preventative antimicrobials could prevent chlamydial infection, there is much effort to develop microbicides and related compounds (7,20). Recently, a number of reports have highlighted the *in vitro* anti-chlamydial properties of polysaccharide-based chemotherapeutics and excipients (2,8,10,19). Studies on the basis of the antimicrobial properties of polysaccharides highlight the need for excipient-only controls in testing the effectiveness of new antimicrobials, and contribute to our understanding of the molecular mechanism of chlamydial adhesion to polysaccharides or glycoproteins on the surface of host cells. The studies reported below are designed to interrogate the potential anti-chlamydial effects of the common polysaccharide-based excipient, hydroxyethyl cellulose (HEC) as a function of both concentration and pH.

Excipients are an inherent part of drug delivery systems for both topical vaginal medications and contraceptives and fall into four principal classes: antioxidants, preservatives, acidifying agents, and gelling agents (8). Gelling agents are frequently polysaccharides, which are also adhesins through which bacterial pathogens bind to host cells. As such, these molecules can have competitive inhibitory effects on bacterial adhesion. However, it is possible that these excipients can play a more complex role in host-pathogen interactions. Although a number of potential host-cell adhesins have been proposed for the initial stages of mammalian infection by *C. trachomatis* (3,14,18), it appears possible that membrane-localized calcium concentrations could also play a role in promoting infection.

In the current study, three experimental parameters were adjusted regarding the effects of a commonly used excipient, hydroxyethyl cellulose, upon infection of cervical epithelial cells (HeLa cells) by the lymphogranuloma (LGV/L2) serovar of *C. trachomatis*. HeLa 229 cells were chosen as a model host system, since they are immortalized cervical epithelial cells and are germane to *in vitro* modeling of vaginal infection by *C. trachomatis*. The effects of HEC concentration, average molecular weight, and
pH were explored in order to test the hypothesis that HEC monomer concentration is the primary determinant of antimicrobial potency. Both HeLa 229 cells and the LGV/L2 strain of *C. trachomatis* were obtained from American Type Culture Collection (ATCC; Manassas, VA). The serovar D of *C. trachomatis* was a gracious gift from Dr. Deborah Dean (CHORI, Oakland; and University of California, Berkeley). HeLa cells were maintained in a humidified incubator at 37 °C with 5% CO$_2$ in Dulbecco modified Eagle medium Glutamax-1 (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum. *C. trachomatis* was grown and the multiplicity of infection (MOI) was calculated as previously described (1). The excipient solutions were prepared by dissolving 20 mg of the HEC polymers in 10 ml of deionized water with stirring and while heating. Two average molecular weights of 1.3 MDa and 90 KDa were used (Aldrich, Milwaukee, WI). The resulting solution was then diluted 1:1 with 0.2 M phosphate (pH 7) or 0.2 M acetate (pH 5) to give a final concentration of 1 mg/ml. This solution was diluted 1:100 in 0.1 M phosphate (pH 7) or 0.1 M acetate (pH 5) to give a final concentration of 10 µg/ml. The solutions were then filtered through sterile 0.22 µm PVDF filters (Fisher Scientific, Houston, TX). One control consisted of adding 20 µl of sterile deionized water to 80 µl of chlamydiae to a final MOI of 0.75. The two other controls were buffered using 20 µl of sterile 100 mM acetate or phosphate buffer at pH 5 or 7, respectively, added to the same volume of chlamydiae. The concentrations of HEC listed in Figures 1 and 2 are final concentrations after dilution into the chlamydial stock. The buffer concentrations were chosen so that the resulting pH during chlamydial infection was indistinguishable from the stock excipient solution.

The potency of HEC solutions as antimicrobials was tested by mixing 20 µl of the (5X) HEC solution with 80 µl of *C. trachomatis* such that the final MOI was 0.75. This mixture was incubated at room temperature for 20 minutes and then added to 6-well plates of HeLa cells grown to a density of approximately 5 × 10$^5$ cells/well (~ 70 % confluency). The plates were incubated at 37 °C for 24 hrs. The media were then aspirated from the wells, the cells fixed with chilled methanol for 8-10 min., washed with PBS, and stained using Hoechst stain and FITC-conjugated chlamydial antibody (Argene,
Varilhes, France). The percent of infected cells was quantified by counting chlamydial inclusions in several fields of a fluorescence microscope (Leica Microsystems, Bannockburn, IL).

Figure 1 illustrates a dose-dependent response of *C. trachomatis* LGV/L2 inclusion formation versus HEC polymer concentration at pH 5. These concentrations of HEC also inhibit the infection by the more clinically-relevant serovar D at each pH tested (data not shown). Interestingly, the effectiveness of the excipient is not directly proportional to the concentration of individual HEC monomer units in the infection assay. This finding is in contrast to the possibility that there could be a concentration dependence based on individual monomer concentration or polymer concentration. In fact, because the monomer is not specifically hydroxyethyl-substituted at a single site, different monomer sites might be anticipated to yield different affinities for binding sites on chlamydiae. However, substitution is expected to be stochastic and both the large and small HEC polymers should have the same distribution of substitution. Surprisingly, Figure 2 shows an inverse polymer concentration dependence at neutral pH. The lowest final polymer concentration (2 µg/ml of 1.3 MDa HEC) attenuates *C. trachomatis* LGV/L2 infection by 80 % of the control. Notably, the phosphate and acetate buffer controls show similar infection percentages. Thus the observed effect is unlikely to be due simply to the hydronium ion concentration.

Buffered pH levels used in the current study were chosen to provide a point of comparison with the work of Lampe, et al. While studies performed at pH 4 also demonstrated inhibition for the LGV/L2 serovar (data not shown), the effect was less pronounced than at pH 5 or 7.

The results obtained for the infection of HeLa cells by *C. trachomatis* at pH 5 (Figure 1) could be explained in terms of chlamydial binding to host-cell glycolipids and glycoproteins. Using 125I-labeled chlamydiae, bacteria were observed to bind to phosphatidylethanolamine, asialo-GM1 and asialo-GM2 moities (6). Specifically, it is thought that *C. trachomatis* binds to the GalNAcβ1-4Galβ1-4Glc pattern. HEC utilizes a repeating Glcβ1-4Glc unit that is nonspecifically substituted with hydroxyethyl or hydroxyethoxyethyl repeats. The extent of substitution is constant between the two polymers used in this study. Given the similarity of GalNAcβ1-4Galβ1-4Glc and Glcβ1-4Glc in
structure and charge neutrality, it is possible that HEC acts as a competitive inhibitor for host recognition sites on the chlamydial surface. At each pH, two comparisons can be made regarding monomer concentration. For the measurements at 200 µg/mL HEC, there is a distinct and statistically significant difference between the inhibition efficiencies. This implies that the effect observed is not proportional to monomer concentration. Furthermore, Fig. 2 shows statistically significant differences between infection percentages for loadings of both 2 µg/mL and 200 µg/mL. Likewise, at each pH, there are four different polymer concentrations. The nonlinear dependence of inhibition on HEC polymer concentration implies that the polymer with an average molecular weight of 1.3 MDa can inhibit more host recognition sites on the cell membrane of chlamydiae than the smaller 90 KDa polymer; since 2 µg/ml 90 KDa HEC is 22 nM in concentration, while 200 µg/ml 1.3 MDa HEC is 1.5 nM. Thus, while the larger polymer is present at <10% the concentration of the smaller polymer, it has slightly more inhibitory activity.

The percent inhibition at pH 7 is counter to what might be expected, since the inhibitory activity of HEC varies inversely and nonlinearly with polymer concentration. This effect could be explained by the [Ca$^{2+}$] requirements of chlamydial infection. It has been reported that extracellular [Ca$^{2+}$] facilitates both attachment and endocytosis of chlamydiae (5,13). While a participation of locally high concentrations of calcium in invasion by C. trachomatis has not been shown yet, microcrystalline calcium phosphate can mediate the attachment of adenovirus to HeLa cells. Thus, coprecipitation of adenovirus with calcium phosphate elicited a > fourfold increase in infectivity over bare adenovirus (17). In the adenovirus studies, it was hypothesized that the microcrystalline calcium phosphate destabilized the membranes of HeLa cells. Similarly, HeLa cells treated with basic calcium phosphate crystals and a luciferase reporter plasmid showed luciferase activity of about 25% of that achieved with LipofectAmine (15).

Since membrane localization of calcium likely occurs during infection by C. trachomatis, a hypothetical role of HEC in aiding infection can be envisioned. Two processes are operative during the precipitation of supersaturated solutions: nucleation and crystal growth. Nucleation is the formation of a
small collection of highly organized molecules or ions that is capable of forming a suitable template for further crystal growth. The entity formed in this process is known as the critical nucleus, which is defined by its equivalent tendency to disperse into solution or aggregate into morphologically defined crystals (11). It has been shown that the presence of HEC facilitates the nucleation of calcium phosphate in supersaturated solutions at pH 7.4 (12). Interestingly, when the calcium carrying capacity of HEC was studied as a function of pH, it was found that HEC promoted the mineralization of calcium phosphate at pH 7 and consequently released calcium phosphate at pH 4.8 with no significant hysteresis upon repeated cyclings of pH change, implying that HEC does not undergo chemical degradation at low pH (16). The pH dependence of HEC-promoted mineralization indicates that increased local membrane calcium phosphate would not be expected in the experiments in Fig. 1, but could be attained for the results shown in Fig. 2. While the results gathered in the current study are not conclusive, the tendency for HEC to promote nucleation in a concentration-dependent manner may explain the results observed in Fig. 2.

While the current study was initially designed to characterize the anti-chlamydial effects of a common class of excipients, the collected data have also provided insight into factors that mediate the in vitro infectivity of C. trachomatis. While it appears likely that neutral polysaccharides such as HEC are capable of competitively interfering with adhesion, the current study emphasizes the necessity for proper buffering in vaginal excipients. The current study also highlights a heretofore neglected property of excipient components: that of providing a template for calcium phosphate nucleation.

**Acknowledgements.** These studies were funded by the University of California.
Fig 1. Number of epithelial cells infected by the LGV/L2 serovar of *C. trachomatis* in the presence of two concentrations of hydroxyethyl cellulose (HEC) of two different average molecular weights at pH 5. All samples except the water control are buffered by 0.1 M acetate (pH 5). The averages and standard deviations are representative of results from four replicates in two separate experiments. P-values for relevant comparisons: *: 0.52; **:0.11.

Fig 2. Number of epithelial cells infected by the LGV/L2 serovar of *C. trachomatis* in the presence of two concentrations of hydroxyethyl cellulose (HEC) of two different average molecular weights at pH 7. All samples except the water control are buffered by 0.1 M phosphate (pH 7). The averages and standard
deviations are representative of results from two separate experiments. P-values for relevant comparisons: *: 0.14; **: 0.10.
References


