RAPID COMMUNICATION

Ability of Chitosan Gels to Disrupt Bacterial Biofilms and Their Applications in the Treatment of Bacterial Vaginosis

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ABSTRACT: Recurrence of bacterial vaginosis is attributed to the inability of various formulations to disrupt bacterial biofilms. A negatively charged polysaccharide matrix coats the bacterial communities in the biofilm and restricts the penetration of antibiotics. Therefore, bacteria in the deeper segments of the biofilm persist and perpetuate the infection. In this study, we have tested the efficacy of two bioadhesive polymers, cationic chitosan and anionic polycarbophil, to disrupt *Pseudomonas aeruginosa* biofilms grown in the Center for Disease Control bioreactor as well as on the 96-well plates. The biofilms were treated with various concentrations of polycarbophil and chitosan at pH 4 or 6. Biofilm integrity following various treatments was evaluated by crystal violet stain and laser confocal microscopy employing Syto9 (live-cell stain) and propidium iodide (dead-cell stain). These studies demonstrated that chitosan gel disrupts the *P. aeruginosa* biofilm more effectively than does polycarbophil; and this effect is independent of the pH and charge densities on either polymers. © 2013 U.S. Government.

Keywords: biofilm; bacterial vaginosis; chitosan; carbopol; hydrogels; mucosal drug delivery; microscopy; absorption

INTRODUCTION

Bacterial vaginosis (BV) is the most common cause of vaginal infections in women during pregnancy and is also associated with preterm labor.[1] BV is caused by the disruption of normal bacterial flora, which results in the overgrowth of anaerobic bacteria in the vaginal cavity. Normal flora predominated by lactobacilli generate lactic acid and maintain vaginal pH around 4, which is unfavorable for the growth of BV causing anaerobic bacteria such as *Gardnerella vaginalis*.

Application of triple sulfa creams, erythromycin, tetracycline, acetic acid gel, and povidone iodine vaginal douches can only resolve BV symptoms temporarily.[3] The most successful treatment regimens for BV include oral or topical administration of metronidazole.[4] Although metronidazole oral tablets and 0.75% metronidazole vaginal gel were equally effective in reducing BV symptoms in the short run, the long-term follow up was shown to have increased BV recurrences.[4,5]

The failure of the current formulations to prevent BV recurrence is believed to be due to their inability to disrupt bacterial biofilms, which are layers of bacteria proliferating on physiological tissues as well as on inanimate surfaces. A negatively charged polysaccharide matrix that restricts the penetration of antibiotics coats the bacterial communities in a biofilm. Therefore, bacterial cells in the deeper quarters of the biofilm can survive the antibiotic treatment, perpetuate the biofilm survival, and cause hard-to-treat health conditions such as BV.[6,7] The polymeric matrix

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provides extra protection to the bacteria under hazardous environmental conditions.[8,9] Bacteria in the biofilms are known to exhibit 1000 times greater resistance to antibiotics than their planktonic conterparts.[10] Therefore, it is imperative to incorporate agents that are capable of reducing biofilm integrity so that the antimicrobials in the formulation could exhibit their optimal effect.

Currently marketed vaginal metronidazole gels such as metrogel is a polyacrylic acid gel based formulation.[11] The objective of the current work is to test the hypothesis that chitosan gels are more effective in disrupting the bacterial biofilms than the polyacrylic acid based gels and may potentiate the action of metronidazole incorporated in the gel formulation.

MATERIAL AND METHODS

Chemicals and Reagents

Freeze-dried *Pseudomonas aeruginosa* was purchased from ATCC (Manassas, Virginia) and stored at −80°C in 100 μL aliquots for further use. Various ingredients to prepare bacterial cultures were obtained from Fisher Scientific (Fair Lawn, New Jersey). Syto®9 and propidium iodide were obtained from Invitrogen (Invitrogen, Carlsbad, California). Carbopol® 934P NF polymer (polycarbophil) was a gift from Lubrizol Corporation (Wickliffe, Ohio). Medium molecular weight chitosan with 85% deacetylation was purchased from Sigma–Aldrich (St. Louis, Missouri). Other chemicals and reagents were obtained from Sigma–Aldrich.

Growing Biofilms in a 96-Well Plate

A freshly thawed *P. aeruginosa* aliquot was incubated with 5 mL tryptic soy broth (TSB) at 37°C for 24 h and the culture was diluted to 160 mL with sterile TSB. Each well in a 96-well plate was inoculated with 250 μL of the above preparation and incubated upon shaking at 37°C for 48 h. Every 8–10 h during the incubation period, the spent nutrients were removed from the wells and replaced with fresh TSB.

Biofilm Cultures in CDC Bioreactor

CDC Bioreactor (Biosurface Technologies, Bozeman, Montana) was used to simulate the environment that promotes biofilm growth. All the components of the CDC bioreactor were autoclaved and setup according to manufacturer's protocols. The entire system was sealed from the environment by 0.22 μm filters. An isolated colony of *P. aeruginosa* was inoculated into 0.3 mg/mL agar broth and incubated upon continuous shaking at 37°C overnight. A 1 mL aliquot from this culture was introduced into the bioreactor (Biosurface Technologies) housing glass or polycarbonate coupons. During the batch phase (first 24 h following the inoculation), the contents of the bioreactor were maintained under constant stirring. Then during the continuous phase, which extends 48 h after the batch phase, 0.1 mg/mL TSB (Fluka, St. Louis, Missouri) was delivered via ISCO TRIS peristaltic pump (Teledyne Isco Inc., Lincoln, Nebraska) through bubble traps into the bioreactor at a flow rate of 20 mL/h and the bioreactor was maintained at 37°C under constant stirring for 48 h.

Biofilm Treatment with Chitosan and Polycarbophil Gels

Various percentages of medium molecular weight chitosan (Sigma–Aldrich) gels were prepared with 1% glacial acetic acid in distilled water. Similarly gels containing various percentages of polyacrylic acid (Sigma–Aldrich) were prepared in distilled water. The pH of chitosan and polyacrylic acid gels was adjusted to either 4 or 6. The biofilms adhered to the well-bottoms of the 96-well plate were treated with either chitosan gel (0.125%, 0.25%, 0.5%, or 1%) or carbopol gel (0.125%, 0.25%, 0.5%, or 1%) at pH 4 or 6. Next, the biofilms were rinsed with distilled water, stained with crystal violet, and the extent of their disruption was quantified.

The biofilms grown on polycarbonate coupons in the bioreactor were treated for various lengths of time with chitosan or polycarbophil gels maintained at pH 4 or 6. Subsequently, the coupons were rinsed with distilled water, stained with crystal violet. Similarly, the glass coupons treated either with chitosan or polycarbophil gels at pH 4 or 6 were gently rinsed twice with distilled water, treated with live/dead cell stains, and imaged.

Evaluation of Biofilm Integrity

Following the treatment with polymeric gels, the wells were gently rinsed and the residual cells adhering to the bottom of each well were stained with 25 μL of 0.3% crystal violet at 37°C for 10 min. The wells were rinsed thoroughly with sterile water, a 100 μL aliquot of 0.5 M monobasic sodium phosphate in 50% ethanol was added to each well, the contents of the wells were mixed thoroughly, and the resultant stained biofilms were detected using a micro-plate reader at 540 nm. The absorbance value obtained from each well is expected to correlate with the intact bacterial cells in the biofilm. The biofilms grown on the polycarbonate coupons were stained similarly with crystal violet, imaged by a digital camera, and the intensity of crystal violet stain was determined using ImageJ software (http://imagej.nih.gov/ij/).

The biofilms grown on the glass coupons on the other hand were treated with syto9 that stains live cells and propdium iodide that stains dead cells. The biofilms were rinsed thoroughly after the treatment with live/dead markers and imaged using inverted