Polyphenylene carboxymethylene (PPCM), the active component of the topical contraceptive Yaso-GEL, exhibits potent antimicrobial activity against Neisseria gonorrhoeae in preclinical studies

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ABSTRACT

Introduction Polyphenylene carboxymethylene (PPCM) is a condensation polymer that has both contraceptive and antimicrobial activity against several sexually transmitted viruses including HIV, herpes simplex virus, Ebola virus and SARS-CoV-2 in preclinical studies. PPCM, both as an active pharmaceutical ingredient (API) and in a vaginal gel formulation (Yaso-GEL), has an excellent safety profile. Here, we evaluated the efficacy of PPCM against Neisseria gonorrhoeae in vitro and in a gonorrhoea mouse model.

Methods The minimal inhibitory concentration (MIC) of PPCM was determined against 11 N. gonorrhoeae strains by agar dilution and a microtitre plate-based method. In vivo efficacy was tested in a murine model of N. gonorrhoeae genital tract infection by applying Yaso-GEL, PPCM incorporated in 2.7% hydroxethylcellulose (HEC), or the HEC vehicle vaginally prior to challenge with N. gonorrhoeae. Vaginal swabs were quantitatively cultured over 5 days to assess efficacy.

Results PPCM MIC against N. gonorrhoeae ranged between 5–100 µg/mL (agar dilution) and 50–200 µg/mL (microtitre plate method). PPCM/HEC gel applied vaginally prior to bacterial challenge resulted in a concentration-dependent inhibition of infection. Yaso-GEL containing 4% PPCM prevented infection in 100% of mice. Incubation of N. gonorrhoeae with PPCM increased membrane permeability, suggesting PPCM directly compromises N. gonorrhoeae viability, which may be a mechanism by which PPCM inhibits N. gonorrhoeae infection.

Conclusions Yaso-GEL containing the API PPCM showed significant activity against N. gonorrhoeae in vitro and in vivo in a female mouse model. These data support further development of Yaso-GEL as an inexpensive, non-hormonal and non-systemic product with both contraceptive and antimicrobial activity against gonorrhoea and other common sexually transmitted infections (STIs). Such multipurpose prevention technology products are needed by women in all economic, social and cultural circumstances to prevent unintended pregnancy and STIs.

BACKGROUND

Over 82 million Neisseria gonorrhoeae infections are estimated to occur globally each year.¹ The primary site of gonococcal infection is the urethra in males and the cervix and/or urethra in females; nasopharyngeal and rectal infections are also common. Women and neonates born to infected mothers suffer the most serious morbidity and mortality associated with gonorrhoea. Both acute and silent N. gonorrhoeae cervical infections can ascend to cause pelvic inflammatory disease, which is associated with ectopic pregnancy, infertility and chronic pelvic pain. Maternal N. gonorrhoeae is associated with premature rupture of membranes, low birthweight babies and a failure to thrive and transmission during delivery can result in acute conjunctivitis.² Individuals with gonorrhoea are more susceptible to HIV and may also transmit HIV more readily due to higher levels of HIV transcripts in their genital fluids.³

Control of gonorrhoea is limited to safe-sex counselling and the identification and treatment of infected individuals and their sexual contacts. Treatment, however, is seriously threatened by the emergence of antibiotic-resistant N. gonorrhoeae strains, particularly in low-to-middle-income countries where the diagnosis is often empirical and antibiotic use is less regulated.³ Several candidate gonorrhoea vaccines are under development; however, no

WHAT IS ALREADY KNOWN ON THIS TOPIC

• Control of gonorrhoea is seriously threatened by the emergence of multidrug-resistant Neisseria gonorrhoeae. Polyphenylene carboxymethylene (PPCM), the active component of Yaso-GEL, is both a contraceptive and antimicrobial compound with activity against other sexually transmitted pathogens.

WHAT THIS STUDY ADDS

• PPCM has potent activity against multiple strains of N. gonorrhoeae in vitro and when used prophylactically in a mouse model.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

• This study supports further development of PPCM as a safe, inexpensive and non-hormonal multipurpose technology that offers women protection from several sexually transmitted infections including gonorrhoea.
vaccine is yet licensed for gonorrhea. While a gonorrhea vaccine is a desirable public health tool to prevent disease and curb the evolution of antibiotic resistance, multipronged interventions will always be needed to protect individuals in areas where vaccines are not available and to provide an alternative for those with vaccine hesitancy.

One approach for preventing sexually transmitted infections (STIs) in women and transgender females is the use of vaginally applied microbicides. Advantages of this strategy include low production costs and giving the female partner control over their use. Multipurpose prevention technology products (MPTs) that have both microbicidal and contraceptive activity are also highly desirable. The addition of contraceptive activity to an STI prevention product is expected to increase its acceptability and actual use. Polyphenylene carboxymethylene (PPCM), the active pharmacological ingredient of Yaso-GEL, is a condensation polymer of mandelic acid and an inexpensive topical contraceptive agent. PPCM also has microbicidal activity against sexually transmitted viruses, including HIV, herpes simplex virus (HSV), Ebola virus11 and SARS-CoV-2. Importantly, PPCM has a good safety profile in preclinical toxicity assays and is not cytotoxic or damaging to epithelial cells. Here we evaluated the in vitro and in vivo efficacy of PPCM as a topical microbicidal against N. gonorrhoeae to more fully define the protective potential of Yaso-GEL against STI pathogens.

METHODS

Materials

PPCM is a condensation polymer with a molecular weight of 3900 g/mol and a polydispersity index of 1.4 and is licensed to Yaso Therapeutics. PPCM is highly soluble in water and stable. PPCM was synthesised by Wilmington PharmaTech (Newark, Delaware, USA) under cGMP for Yaso Therapeutics. PPCM agar solutions were produced by the addition of PPCM to supplemented GC agar (36 g GC medium base, 5 g Bacto agar per liter of dH2O) containing Kellogg’s Supplement I13 and 12 μM Fe(NO3)3 as described. Yaso-GEL is a 4% (40 mg/mL) PPCM aqueous gel, produced by Dow Development Labs (Petaluma, California, USA) for Yaso Therapeutics. PPCM HEC (hydroxyethyl cellulose) gel of 2 mg/mL was prepared by combining 20 mg PPCM and 27 mg HEC in 10-mL endotoxin-free phosphate-buffered saline (PBS) and stirring for 10–15 min until a 2.7% gel was formed. Gynol-II (DPT Laboratories) is a commercially available spermicidal gel that contains 2% nonoxynol-9 (N-9).

Strains and growth conditions

N. gonorrhoeae strains used in this study were two laboratory strains (FA1090, MS11), two ceftriaxone-resistant strains (H041, F89), four well-characterised WHO reference strains and three N. gonorrhoeae isolates isolated from the USA between 2014 and 2019 (Table 1). N. gonorrhoeae was cultured in 7% CO2 at 37°C on supplemented GC agar. GC agar with antibiotic selection (vancomycin, colistin, nystatin, trimethoprim and streptomycin (GC-VCN15S agar)) and heart infusion agar were used to isolate N. gonorrhoeae and murine commensal microbiota from murine vaginal mucus as described. All bacterial culture media were from Difco (Becton Dickinson).

Minimal inhibitory concentration against N. gonorrhoeae

For agar dilution assays, twofold decreasing concentrations of PPCM were added to cooled (55°C) GC agar and 6 mL of each concentration were poured into a separate well of a 6-well tissue culture plate. Control wells consisted of media without antibiotics. Well-isolated colonies of the N. gonorrhoeae strain tested were harvested from GC agar plates after 20- to 22-hour incubation and suspended in GC broth (GCB) to a concentration of 107 colony-forming units (CFU) per millilitre. Ten microlitres of each suspension (ca. 105 CFU) were spotted onto the agar in each well with up to five spots per well. Plates were scored for growth after 24-hour incubation. Each strain was tested in triplicate within each of the two independent experiments.

For the microtitre plate-based assay, N. gonorrhoeae colonies were harvested from GC agar plates after 20-hour incubation and suspended in 10 mL of supplemented GCB. Bacterial suspensions were passed through a 1.2-μM filter to remove bacterial aggregates, and the optical density at 600 nm (OD600) was adjusted to 0.08. Filtered suspensions were then incubated in T25 tissue culture flasks at 150 rpm for 3 hours at 35.5°C, after which the OD600 of the cultures was adjusted again to 0.08 (−10 CFU/mL). The suspension was then diluted 1:50 in GCB, and 100 μL (−103 CFU) was added to wells of a microtitre plate containing 100 μL of serial dilutions of PPCM in supplemented GCB or supplemented GCB without PPCM. The microtitre plates were incubated at 24 hours at 35.5°C in 7% CO2, after which 5 μL from each well were inoculated onto GC agar plates. The plates were scored for growth after 24-hour incubation. Two independent iterations of the assay were conducted with three technical replicates tested in each assay. The MIC of Yaso-GEL (4% PPCM) against N. gonorrhoeae was tested in microtitre plates similarly, using a positive displacement pipette to dilute the gel. For both the agar dilution and microtitre plate-based assays, the MIC was the lowest concentration of PPCM from which no N. gonorrhoeae strains were isolated, and each assay was performed

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Table 1: Minimal inhibitory concentration (MIC) of PPCM against the N. gonorrhoeae strains used in this study as determined by agar dilution and microtitre plate assays

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>Agar dilution</td>
</tr>
<tr>
<td>FA1090*</td>
<td>STM*</td>
</tr>
<tr>
<td>MS11*</td>
<td>STM*</td>
</tr>
<tr>
<td>H041 (WHO X)†</td>
<td>PEN-G, TET*, AXI, CIPR, CFXr, CROr</td>
</tr>
<tr>
<td>F89†</td>
<td>PEN-G, TET*, CFXr, CROr, CIPr</td>
</tr>
<tr>
<td>WHO Ft</td>
<td>susceptible</td>
</tr>
<tr>
<td>WHO Gt</td>
<td>PEN-G, TET*, CIPr</td>
</tr>
<tr>
<td>WHO Kn</td>
<td>PEN-G, TET*, CIP, CFXr</td>
</tr>
<tr>
<td>WHO Lt</td>
<td>PEN-G, TET*, CIP, CRO, CFXr</td>
</tr>
<tr>
<td>CONUS-9542†</td>
<td>TET*, CIPr</td>
</tr>
<tr>
<td>CONUS-3668†</td>
<td>PEN*, CIPr</td>
</tr>
<tr>
<td>CONUS-6364†</td>
<td>PEN*, CIPr</td>
</tr>
</tbody>
</table>

*Commonly used laboratory N. gonorrhoeae strains.
†Multidrug-resistant, ceftriaxone-resistant N. gonorrhoeae strains.21 28
‡US isolates; from the USU GC Resistance Repository and Reference Laboratory isolated in 2017 (CONUS-9542), 2019 (CONUS-3668) and 2014 (CONUS-6364).
AZL, azithromycin; CFX, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; HLR, high-level resistant; I, intermediate susceptible; LLR, low-level resistant; N/A, not available; PEN, penicillin; PPCM, polyphenylene carboxymethylene; R, resistant; STM, streptomycin; TET, tetracycline.

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twice on different days, with each strain tested in triplicate in each experiment.

Cell permeability assay

The effect of PPCM on cellular membranes was assessed using the SYTO9/propidium iodide (PI) counterstain assay as follows. *N. gonorrhoeae* strain MS11 was harvested from GC agar plates after 19-hour incubation and suspended in GCB to an OD<sub>600</sub>=0.08. Aliquots of the suspension were incubated with PPCM (100 μg/mL) or no PPCM for 6 hours. At hourly time points, aliquots from each culture were quantitatively cultured for *N. gonorrhoeae* or incubated in the dark with PI and SYTO9 (1:1 mix from LIVE/DEAD kit) using 100 μL of bacterial suspension with 0.2 μL of PI and SYTO9. After 30-min incubation at room temperature, the stained suspensions were examined under fluorescent microscopy at 40× using the green and blue filters. Photos were taken in three to five different fields of triplicate samples from each preparation at each time point, and stained cells were counted using Image J. The per cent permeability was calculated as the [(no of compromised cells divided by the total number) × 100], with the green signal showing total membranes and the red signal showing compromised membranes. Differences were analysed by ordinary two-way analysis of variance (ANOVA) using computations that assume that all rows are sampled from populations with the same scatter (SD). Differences in the number of CFU recovered over time were measured by repeated measures ANOVA (GraphPad Software, La Jolla, California, USA). Differences ≤0.05 were considered significant. The assay was performed three times and with three technical replicates in each assay.

In vivo efficacy testing

The method used to test PPCM efficacy against *N. gonorrhoeae* is described in the online supplemental material file, and a schematic of the protocol is shown in online supplemental figure S1. Briefly, female BALB/c mice were randomised and treated with Premarin and antibiotics to promote the susceptibility to *N. gonorrhoeae*. Two days after Premarin treatment was initiated, mice were anaesthetised and inoculated vaginally with 30 μL of different concentrations of PPCM in 2.7% HEC, 2.7% HEC alone (vehicle control), Gynol-II, Yaso-GEL or a placebo gel using a positive displacement pipette. Thirty seconds later, mice were challenged vaginally with 10 μL of a PBS suspension containing 10<sup>7</sup> CFU of piliated *N. gonorrhoeae* strain MS11. Vaginal swabs were quantitatively cultured for *N. gonorrhoeae* on days 1, 2, and 5 postinoculation. The percentage of culture-positive mice at each time point was plotted as a Kaplan-Meier curve, and the results for each group were compared by the log-rank test with Bonferroni correction. The average numbers of CFU isolated per vaginal swab suspension from each experimental group were compared over time by repeated measures ANOVA. A total of 67 mice were used in this study, with experimental groups consisting of 5–7 mice/group. Sample size was determined based on our previous study that showed 6–8 mice/group was adequate to detect a significant difference (p<0.05) in percentage of mice colonised for the moderately active microbicidal methylcellulose.15

Animal studies declaration

Animal experiments were conducted at the Uniformed Services University, a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under protocol MIC-20-013, which was approved by the University’s Institutional Animal Care and Use Committee.

RESULTS

PPCM in solution and formulated gel has potent activity against *N. gonorrhoeae* in vitro and in vivo

MIC of PPCM was determined against 11 *N. gonorrhoeae* strains that differ in antibiotic susceptibility (table 1). Using the agar dilution method, the MIC of PPCM ranged from <5 to 100 μg/mL. MICs were also determined using a microtitre plate method in which logarithmic-phase bacteria were incubated with decreasing concentrations of PPCM. PPCM also showed activity in this assay, with MIC values 2- to 10-fold higher (50–200 μg/mL) compared with the agar dilution method. We also determined that the MIC of Yaso-GEL ranged between 0.31 and 0.63 mg/mL against strain MS11 using the microtitre plate–based method (figure 1). We conclude that PPCM in aqueous solution and in Yaso-GEL are highly inhibitory against *N. gonorrhoeae*, with no differences in PPCM susceptibility observed among a diverse set of *N. gonorrhoeae* strains.

Based on these results, we next tested the in vivo efficacy of 2 mg/mL against *N. gonorrhoeae* strain MS11, which is 10× the in vitro MIC against this strain. For these experiments, mice were vaginally inoculated with PPCM mixed with 2.7% HEC to create a viscous formulation that would be retained longer in the vagina or HEC alone prior to the *N. gonorrhoeae* challenge. We tested the commercial spermicide Gynol-II in parallel, which contains 2% of N-9, which is a detergent that kills *N. gonorrhoeae*, but unlike Yaso-GEL, is highly toxic to host cells.16 No *N. gonorrhoeae* was recovered from any of the seven mice in the 2 mg/mL PPCM-HEC gel-treated group 24 hours after challenge or at any time point through 5 days; in comparison, 100% (6/6) of mice in the vehicle control group were culture-positive for 2 consecutive days following bacterial challenge (p=0.0008) and 50% (3/7) were culture-positive on day 5 (figure 2A). The difference in the number of recoverable CFUs/100 mL vaginal swab suspension over time was also significantly lower in PPCM-treated mice versus HEC-treated mice (p≤0.0001) (figure 2B). Fifty per cent (3/6) of mice in the Gynol-II group were culture-positive on days 1, 2 and 5 postchallenge, which was significantly higher than the PPCM-treated group (p=0.04) as was the average number of CFUs/mL recovered over time (p=0.03) (figure 2A,B, purple lines).

To determine the lowest effective dose of PPCM in vivo, we pretreated mice with PPCM-HEC containing 0.25–2.0 mg/mL PPCM. Pretreatment with 1.0 and 0.5 mg/mL PPCM-HEC was significantly more effective than the HEC vehicle alone as shown by the percentage of culture-positive mice over time (p=0.03 and p=0.05, respectively) (figure 2C, gold and light blue lines) and the average number of CFUs/mL recovered (p=0.07 and p=0.004, respectively) (figure 2D). Mice treated with the 0.25 mg/mL dose showed an initial drop in the percentage of culture-positive mice and CFUs/mL recovered on day 1 but were similar to the HEC vehicle control for both parameters on subsequent time points (green line, figure 2C and D). The 2 mg/mL dose did not perform as well as in the first experiment, with 40% (2/5) of the mice colonised over 5 days (p=0.19 vs vehicle control) compared with 0% (0/7) mice in the first study (figure 1C, red line) and the difference in the recoverable bioburden approaching, but not achieving statistical significance (p=0.07). We next tested the in vivo efficacy of Yaso-GEL. The results showed Yaso-GEL to be highly effective compared with the placebo gel in reducing both the percentage of culture-positive mice over time (p=0.002).


**Figure 1** Yaso-GEL exhibits potent activity against *Neisseria gonorrhoeae* in vitro. The minimum inhibitory concentration of Yaso-GEL, which contains PPCM at a concentration of 40 mg/mL, was determined by incubating 10^5 CFUs of *N. gonorrhoeae* strain FA1090 with twofold dilutions of the formulated gel in a microtitre plate (moving right to left on the plate as shown above) for 24 hours. Five-microlitre aliquots were then spotted onto GC agar, incubated for 24 hours, and scored for growth. The lowest dilutions of Yaso-GEL completely inhibited *N. gonorrhoeae* ranged from 1:16 to 1:32, which corresponds to PPCM concentrations of 0.16–0.32 mg/mL. The placebo gel had no effect at any dilution tested. CFU, colony-forming units; PPCM, polyphenylene carboxymethylene.

**DISCUSSION**

Protection from STIs and pregnancy protection are both critical aspects of women’s health. Currently there are no options that provide dual protection that is under the control of the female partner. In the last decade, the need for dual protection that is woman-controlled has become widely recognised. This unmet need has evolved into an active pipeline for the development of MPTs, defined as an innovative class of products that deliver varied combinations of HIV prevention, other STI prevention and contraception. Currently available MPT methods are limited. Both male and female condoms function as MPTs, but their use has been limited by dissatisfaction/discomfort, cost and availability. Early in vitro and limited clinical studies suggested that N-9 might offer some protection against gonorrhea as well as contraception. Unfortunately, the cytotoxic effects of N-9 can actually damage host genital epithelial cells and may even increase the risk of HIV infection. *N. gonorrhoeae* is very sensitive to N-9 in vitro, which is not surprising since it is a surfactant that is cytotoxic and damages cell membranes. In our study, an N-9-containing spermicide was used as a positive control but was not as effective as PPCM in the mouse model.

Many newer vaginal MPT products that are currently in the development pipeline focus on hormonal contraceptives combined with HIV prevention, with relatively little attention to other important STIs. While the extreme morbidity and mortality linked with HIV are widely feared, other STI pathogens, particularly HPV, HSV, *Trichomonas vaginalis*, *Chlamydia trachomatis*, and *N. gonorrhoeae* pose a greater infection risk to many women and their partners. Gonorrhea is particularly problematic not only because of significant global incidence and serious health sequelae, but worsening antibiotic resistance has severely limited treatment options. Only one vaginal product, a recently marketed vaginal contraceptive, is in clinical trials for *N. gonorrhoeae* and *C. trachomatis* prevention.
Figure 2  PPCM and Yaso-GEL are effective in preventing experimental *Neisseria gonorrhoeae* genital tract infection. The in vivo efficacy of PPCM formulated in 2.7% HEC or Yaso-GEL in preventing *N. gonorrhoeae* infection was tested by applying the test compounds vaginally followed by the challenge with *N. gonorrhoeae* strain MS11. Panels on the left (A, C and E) show the percentage of culture-positive mice on days 1, 2 and 5 postchallenge, and panels on the right (B, D, F) show the average number of *N. gonorrhoeae* CFU recovered from each group for each of three different experiments. In the first experiment, 2% PPCM was significantly effective versus the HEC vehicle when comparing: (A) the percentage of culture-positive mice at each time point (p=0.008) and (B) the number of CFUs recovered per millilitre of vaginal swab suspension over time (2 mg/mL PPCM, p<0.0001) (n=6–7 mice/group). PPCM was also significantly more effective against *N. gonorrhoeae* than Gynol II, which contains the spermicidal detergent N-9, for per cent colonised (p=0.04) and CFUs recovered per millilitre (p=0.03). No significant difference in the percentage of mice colonised was detected in the Gynol II-treated and the HEC control groups (p=0.64); the difference in CFUs recovered per millilitre was at a level of p=0.56. (C) Comparison of the percentage of infected mice given decreasing doses of PPCM (n=5 mice/test group, 7 mice in the HEC group) showed that the 1.0 and 0.5 mg/mL doses were more effective than HEC alone (p=0.04 and p=0.05, respectively). The 2 mg/mL treatment and the 0.25 mg/mL treatment showed no significant difference compared with the vehicle control (p=0.07 and p=0.19, respectively). (D) The average number of CFUs recovered per millilitre overtime was significantly lower in the 1 mg/mL and 0.5 mg/mL treatment groups compared with HEC alone (p=0.007 and p=0.03, respectively), but not in the 2 mg/mL dose and 0.25 mg/mL dose (p=0.07 and p=0.19, respectively). In a third experiment, Yaso-GEL was tested similarly; results showed the gel to be highly effective compared with the placebo gel in reducing (E) the percentage of culture-positive mice over time (p=0.002) and (F) the colonisation load (p=0.0005) (n=7 mice/group). While Yaso-GEL appeared more effective than Gynol-II, the differences were not statistically different for the percentage of mice colonised over time (p=0.11) or log10 CFU recovered (p=0.15). In all panels, bars represent SE of the mean. CFU, colony-forming units; HEC, hydroxyethyl cellulose; N-9, nonoxynol-9; PPCM, polyphenylene carboxymethylene.
In this study, we evaluated the ability of PPCM to prevent infection by N. gonorrhoeae. PPCM (aka SAMMA) is a unique (non-sulfated/non-sulfonated) polyanion developed by the Topical Prevention of Conception and Disease (TOPCAD) at Rush University Medical Center and the University of Illinois, Chicago. The MPT potential of PPCM was recognised by TOPCAD because it demonstrated significant contraceptive activity as well as anti-infective activity against multiple pathogens.

PPCM, like other polyanions tested to date, is a non-cytotoxic, broad-spectrum antimicrobial agent, with activities against HIV-1, HSV-1, HSV-2, papillomavirus, N. gonorrhoeae and C. trachomatis. The increased bacterial membrane permeability observed in N. gonorrhoeae exposed to PPCM, accompanied by a reduction in the number of recoverable bacteria suggests PPCM directly impacts gonococcal viability. This direct mode of action may explain PPCM-mediated inhibition of N. gonorrhoeae in vitro and in the murine model. Other mechanisms may contribute to PPCM-mediated prevention of infection. Many STI pathogens initiate infection by attaching to heparan sulfate or other receptors on the host cell surface. For example, polyanions such as PPCM prevent viral infection by binding to the viral envelope to block attachment to the host cell. Some N. gonorrhoeae adherence and invasion pathways use heparan sulfate glycoprotein receptors, but whether PPCM can prevent N. gonorrhoeae infection by blocking these interactions was not tested in our study.

In summary, PPCM formulated into Yaso-GEL continues to show significant promise as an MPT product that is non-hormonal, safe, inexpensive, stable and can be accessed when needed. An intervention with combined gonorrhoea prevention and contraceptive activity is particularly important for at-risk populations in low- and middle-income countries. Further clinical development is warranted.

Figure 3  PPCM increases the permeability of cellular membranes. The effect of PPCM on gonococcal cellular membrane integrity and viability was assessed over a 6-hour incubation period. Bacterial suspensions were exposed to PPCM or not exposed, and aliquots were taken at hourly time points and stained with SYTO9/propidium iodide (PI) (panel A) or quantitatively cultured (panel B). The per cent permeability was calculated using the formula

\[
\frac{\text{No of Compromised}}{\text{No of Total}} \times 100
\]

with the green signal showing total membranes and the red signal showing compromised membranes. (A) The percentage of non-compromised cells in samples with PPCM significantly decreased over time compared with samples without PPCM (p<0.0001; ordinary two-way ANOVA), with the most dramatic decrease seen between 3 and 6 hours; (B) a significant reduction in the average number of CFUs recovered from samples incubated with PPCM versus no PPCM occurred over time (p=0.001; repeated measures ANOVA), with a >1 log reduction occurring between 4 and 6 hours. The results are shown for three assays performed in triplicate. ANOVA, analysis of variance; CFUs, colony-forming units; PPCM, polyphenylene carboxymethylene.
REFERENCES


Supplemental Material

**in vivo efficacy testing protocol**

Female BALB/c mice (6 to 8 weeks old; Charles River) in anestrus or the diestrous stage of the reproductive cycle were acclimated to the animal facility for 10 days after which stained vaginal smears were prepared and examined using a light microscope to identify the stage of estrous. Mice in anestrus or the diestrous stage of the estrous cycle were randomized into cages (3-5 mice/cage) and inoculated intraperitoneally (IP) with 0.5 mg of Premarin 2 days prior to bacterial inoculation (d-2), the morning of bacterial inoculation (d0), and two days post-bacterial inoculation (d2) as shown in Fig. S1. Trimethoprim (TMP) was administered orally (0.4 g/L drinking water) on the first day of Premarin treatment; mice were also given 3.6 mg/kg of streptomycin (STM) by IP injection on days -2 through day 1. STM was provided in the drinking water along with TMP (5 g STM and 0.4 g TMP per liter of water) starting on day 2 until the end of the experiment. On day 0, mice were anesthetized with a mixture of ketamine/xylazine and 30 μL of PPCM formulated in 2.7% HEC, 2.7% HEC alone, Gynol-II®, or Yaso-GEL™ were applied vaginally using a positive displacement pipette. Thirty seconds later, mice were challenged with 10 μL of a PBS suspension containing 10^5 CFU of Ng strain MS11, which is a dose that infects 80-100% of mice. Vaginal mucus was collected on days 1, 2 and 5 post-inoculation using a sterile rayon swab (Puritan Medical Products Company, LLC; Ref. #25-800 R 50) and quantitatively cultured for Ng on GC-VCNTS agar. A small portion of sample was also cultured on HIA plates to monitor the presence of potentially inhibitory facultatively anaerobic commensal microbiota, which might cause clearance that is unrelated to the treatment. No inhibitory commensal flora were isolated from any of the mice this study. Differences in the percentage of culture-positive mice at each time point and the average number of CFU recovered per milliliter of vaginal swab suspension over time were compared between experimental groups using the Log-Rank test with Bonferroni correction and a repeated measures ANOVA, respectively. p values < 0.05 were considered significant. For each experiment, 5-7 animals were tested in each experimental group for a total of 68 animals used in this study. Sample size was based on historical data that show 5-7 animals is sufficient for detecting a significant difference in the percentage of mice colonized over time for nonoxynol-9 versus no treatment. The study was not blinded and no data points were excluded from the study.

![Figure S1: Schematic of in vivo efficacy testing of PPCM or Yaso-GEL™ against N. gonorrhoeae.](image)