Supplementary Information for

Antimicrobial polymer-siRNA polyplexes as a dual-mode platform for the treatment of wound biofilm infections

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Experimental methods

Materials. All chemicals and solvents for syntheses were purchased from Fisher Scientific and Sigma-Aldrich and used without further purification unless otherwise stated. The chemicals were used as received. Dichloromethane (DCM) and tetrahydrofuran (THF) were used as solvent for chemical synthesis and dried per standard procedures. All siRNA (si_scramble, sense strand, 5'-UUCUCCGAACGUGUCACGU-3'; si_MMP9, sense strand, 5'-GCA UAA GGA CGA CGU GAA U-3'; Cy3-labeled scramble) were purchased from Sigma-Aldrich. All reagents/materials were purchased from Fisher Scientific and used as received.

Bacteria and mammalian cell lines. The following bacteria strains were used for this study: MRSA IDRL-6169, RFP-expressing MRSA IDRL-12570 and bioluminescent MRSA USA300 NRS384 strain (SAP 231). Overnight cultures of the bacteria were prepared by transferring isolated colony from the agar plate to culture tubes with sterile media broth. The bacterial cultures were then incubated overnight at 37 °C with aeration and agitation (275 rpm) until desired growth phase. Cultures were harvested by centrifugation and washed with 0.85% sodium chloride solution three times. Concentrations of resuspended bacterial solutions were determined by optical density measured at 600 nm. RAW 264.7 cells and NIH-3T3 cells were purchased from ATCC. eGFP-expressing RAW 264.7 (RAW 264.7:eGFP) cells were generous gift from Prof. Michelle Farkas (University of Massachusetts, Amherst). All cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (Fisher Scientific, SH3007103) were used for cell culture. CyQUANT™ LDH Cytotoxicity Assay Kit was purchased from ThermoFisher Scientific.

Preparation and Characterization of PONI-C11-TMA/siRNA polyplexes. PONI-C11-TMA was synthesized and prepared following the previously reported literature.[1] Polyplexes were formed through simple co-incubation. Briefly, polymer stocks were prepared in Milli-Q water and added to sterile tubes followed by addition of siRNA at varied N/P ratios. The solutions were mixed and incubated at ambient temperature for 10 min. Complete media (10% FBS, 1% antibiotic) was added to the PONI-C11-TMA/siRNA polyplexes. To optimize siRNA encapsulation, 10 nM siRNA was mixed with PONI-C11-TMA at different N/P ratios and subjected to electrophoresis on 1% agarose gel. To evaluate protection against RNase A digestion, polyplexes were incubated with RNase A and then denatured with SDS. Heparin solution was added to displace siRNA from the polyplexes, and siRNA was subjected to electrophoresis on agarose gel. Size and zeta potential were measured by DLS and morphology of the polyplexes was visualized via transmission electron microscopy (TEM).

Encapsulation efficiency determination. The extent of siRNA loading in polyplexes was measured using the Quant-iT RiboGreen RNA Assay Kit. Total siRNA content is the amount of encapsulated and non-encapsulated/free siRNA in the polyplexes. The difference between free and total siRNA was used to calculate the amount of siRNA encapsulated within the polyplexes. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The % encapsulation efficiency was calculated using the formula below.

% siRNA encapsulation efficiency = [(Amount of total siRNA in the polyplex) - (Amount of free siRNA in the polyplex)] / (Amount of total siRNA in the polyplex) × 100%

Biofilm penetration study of polyplexes. GFP-expressing MRSA biofilms were prepared using the preparation method described below for biofilms. The polyplexes were prepared using Cy5.5-tagged PONI-C11-TMA with scrambled_siRNA (N/P ratio 40). Biofilms were then treated for 1 h at 37 °C. The penetration profile study was performed using the Nikon A1 resonant scanning confocal with TIRF module. Images were processed using NIS-Elements.

Evaluation of antimicrobial efficacy for minimum inhibitory concentrations (MIC), minimum bactericidal c determination (MBC), and biofilms. Bacteria from stock agar plates were inoculated in TSB broth at 37 °C until they reached the log phase. Grown bacteria were harvested by centrifugation and washed with 0.85% sodium chloride solution three times. Concentrations of resuspended bacteria were determined by optical density measured at 600 nm. Bacterial culture was diluted in M9 (5% TSB) media to an OD 600 of 0.001 and 50 µL of bacterial culture was added to 96-well plate with media-only control. Materials varying concentrations were added and incubated in the shaker for overnight. Next day, the visual growth of culture was checked, and the wells without visual growth were determined as MIC. Then, 10 µL of subculture in each well was plated over TSA plates and incubated at 37 °C overnight. The plates without visual colony growth were determined as MBC and the images of the plates were taken. All these experiments were done with triplicates and repeated twice separately.

Similarly, seeding solutions were made in TSB to an OD 600 of 0.1. 100 µL of the seeding solutions were added to each well of a microtiter well plate. TSB medium without bacteria was used as a negative control. Plates were covered and incubated at room temperature under static conditions for desired periods. Planktonic bacteria were removed by washing with phosphate-buffered saline (PBS) three times. Treatment materials (polyplexes, polymer only, siRNA only) were diluted prior to use to obtain the desired testing concentrations. The biofilms were treated with materials for 3 hours at 37 °C. To determine bacteria viability in biofilms, the testing solutions were removed, and washed with PBS. Fresh PBS was then added and the remaining bacteria from biofilms were redispersed through sonication for 20 min and mixing with a pipet. Solutions containing redispersed bacteria were then plated onto tryptic soy agar plates, and colony-forming units were counted after incubation at 37 °C overnight.

Biocompatibility of polyplexes. Briefly, macrophage RAW 264.7 (ATCC CRL-1658) cells were cultured in Dulbecco's modified Eagle medium (DMEM, ATCC 30–2002) with 1% antibiotics and 10% bovine calf serum in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h in a 96-well plate. The cells were treated with the materials (polyplexes, polymer only, siRNA only) with controls in DMEM. After 18 h incubation, media containing materials was removed, and cells were washed with phosphate-buffered saline (PBS). LDH cytotoxicity assay (CyQUANT™ LDH Cytotoxicity Assay) was performed to determine mammalian cell viability following the manufacturer's instructions.

% Cytotoxicity = {[Treated LDH activity – Spontaneous LDH activity] / [Maximum LDH activity – Spontaneous activity]} x 100

Hemolysis of polyplexes. Human whole blood (pooled, mixed gender) was purchased from BioIVT Elevating Science and processed as soon as received. Red blood cells were collected through centrifugation at 5000 rpm for 5 min followed by washing 4 times with PBS buffer and then diluted in PBS to a final concentration of approximately 5% (v/v). Polyplexes, polymer and siRNA only were serially diluted using PBS and incubated in 96-well plates (200 µL/well). The blood cell suspension (20 µL/well) was added to each well and the plates were incubated at 37 ˚C for 1 h while shaking at 150 r.p.m. PBS and Triton X-100 (1%) served as negative and positive controls, respectively. After incubation period, the mixture was centrifuged at 3000 r.p.m. for 7 minutes and 120 µL of supernatant was transferred to a new 96-well plate. The absorbance was recorded at 560 nm in each well, and hemolysis was calculated using the following formula:

$Hemolysis = \{[OD560_{sample} - OD560_{PBS}]/[OD560_{Triton} - OD560_{PBS}]\} \times 100$

Confocal Laser Scanning Microscopy (CLSM) for evaluation of cellular uptake or knockdown. Confocal laser scanning microscopy (CLSM) imaging was performed using a Nikon A1 Spectral Detector Confocal Microscope. Cells were seeded in a 35 mm glass bottom culture dish (MatTek, MA) a day before the experiments. On the day of treatment, cells were washed with PBS followed by replacement with

complete media (10% FBS, 1% antibiotic) containing either PONI-C11-TMA/si_eGFP (50 nM) for knockdown or PONI-C11-TMA/Cy3-siRNA (25 nM) for cellular uptake followed by another 24 h of incubation at 37°C. After removing medium, the cells were washed with PBS, stained with Lysotracker (Invitrogen), and visualized for fluorescence imaging using the confocal microscope. Images were processed and analyzed using the NIS-Elements Advanced Research software (Nikon).

Imaging Flow Cytometry for knockdown evaluation. Cells were seeded for 24 h prior to treatments. On the day of the experiment, cells were washed with PBS and incubated with PONI-C11-TMA/si_eGFP in complete media followed by another 24 h of incubation at 37°C. The cells were trypsinized, harvested, and resuspended in PBS for flow cytometry analysis on Amnis ImageStream Mark II Imaging Flow Cytometer (Luminex). At least 1000 events were analyzed for each sample according to the standard instrumentation protocol from Amnis.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for evaluation of MMP9 knockdown efficacy in vitro and in vivo. RAW 264.7 cells (2×10^5 cells/well) were cultured in a 6-well plate for 24 h prior to the experiment. On the day of the experiment, cells were washed with PBS and treated with PONI-C11-TMA/si_MMP9 polyplexes (siRNA dose of 50 nM at N/P 40) for 48 h. At the end of incubation, cells were washed with PBS and then harvested with TRIZol reagent. For *in vivo* samples, tissues were harvested once mice were sacrificed. The wound sections were washed with PBS and homogenized with Trizol reagent. RNA extraction was performed using the Pure Link RNA Mini kit (Ambion) following the manufacturer's protocol. Superscript IV reverse transcriptase (Invitrogen) was used for conversion of approximately 100 ng of RNA to cDNA, along with RNaseOut, also following the manufacturer's instructions. RT-PCR was performed on cDNA as prepared above using a CFX Connect Real Time System with iTag Universal SYBR Green Supermix (Biorad). All primers were purchased from Integrated DNA Technologies (Caralville, Iowa). The following sequences were used: MMP9 Forward (5′-TTG GTC CAC CTG GRR CAA CT-3′); β-actin Forward (5′-GAT CAG CAA GCA GGA GTA CGA-3′). At least three biological replicates were performed for each control group, and three technical replicates were used for each biological replicate. All MMP9 mRNA measurements were normalized to β-actin.

Ethics statement. C57BL/6 mice were supplied by Jackson Laboratory. Mice were housed in sterile cages with a 12-hour light/ 12-hour dark cycle. Mice were allowed to acclimatize for at least a week before any of the procedures were performed. All animal experiments were performed following the authorized protocol (IACUC Protocol ID 2648) and the policies issued by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

Generation and treatment of biofilm-infected murine skin wound defects. Mice were anesthetized using isoflurane and meloxicam was subcutaneously administered for pain management. The skin on the dorsum of the mouse was shaved and disinfected with alternating povidone-iodine and alcohol swabs, thrice. Subsequently, a sterile 5-mm circular full-thickness skin wound was created using a skin puncture biopsy tool (Acuderm Inc., Fort Lauderdale, FL). Using a micropipette, 10^8 CFU of bioluminescent MRSA USA300 NRS384 strain (SAP 231)[2] in saline (10 μL) was inoculated onto the wound bed. To prevent secondary bacterial contamination and allow visualization of the wound bed, semi-occlusive transparent Tegaderm® (3M, St. Paul, MN) was affixed over the wound using Vetbond®. Biofilm was allowed to form for 24 h and infection was tracked through IVIS imaging of the luminescence signal from the bacteria. Mice were then separated into four groups of four to receive one of the following: a) PBS (100 μL), b) polymer only (100 μL, 20 μM), c) polyplexes (100 μL, 20μM at NP 40), and d) vancomycin (100 μL, 20 mg/kg in PBS). The treatment was administered topically, once a day for four days. 24 h after the last treatment, the mice were sacrificed through CO_2 asphyxiation. A 3-mm circular full-thickness skin sample from the inner infection area was collected using a skin biopsy punch and then collected for

either further H&E staining or homogenized for quantitative bacterial colony counting and measurement of MMP9 levels described above. Photographs and IVIS images were taken daily, and weight, wound size, and purulence scores were noted every day. For H&E staining, it was conducted as previously described.[3]

Supplementary Figures

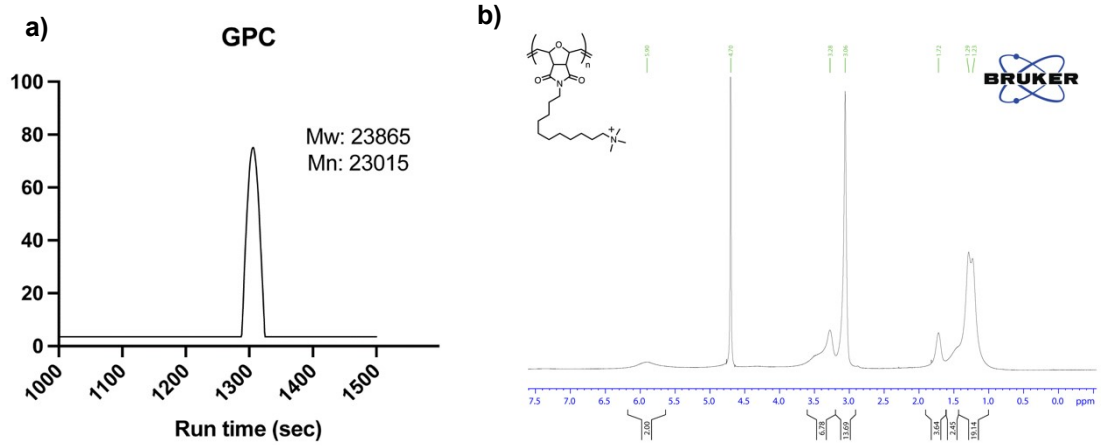

Figure S1. Characterization of synthesized PONI-C11-TMA by a) THF-GPC using PMMA standard. Mw: 23,865 g/mol, Mn: 23,015 g/mol b) ^{1}H NMR spectra in D_2O

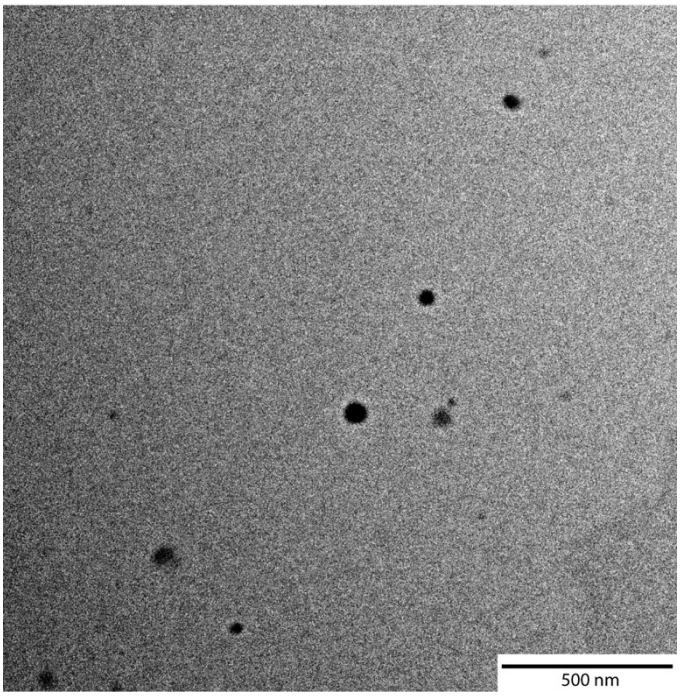

Figure S2. Representative transmission light microscopy (TEM) micrographs of polyplexes at N/P 40.

N/P ratio	10	20	30	40

PONI-C11-TMA (nmol)	0.1125	0.225	0.33	0.435
siRNA (nmol)	0.015	0.015	0.015	0.015
Mol ratio	7.5	15	22	29

Table S1. Formulation conditions for PONI-C11-TMA with siRNA at varying N/P ratios. All values represent a preparation of polyplexezs at working concentration, with a final volume of 0.5 mL.

	PONI-C11-TMA_siRNA polyplexes	PONI-C11-TMA polymers	siRNA_scaramble
MRSA (IDRL-6169)	160 nM	160 nM	Not Active

Table S2. Minimal inhibitory concentrations (MIC) of polyplexes, polymers and siRNA against MRSA (IDRL-6169)

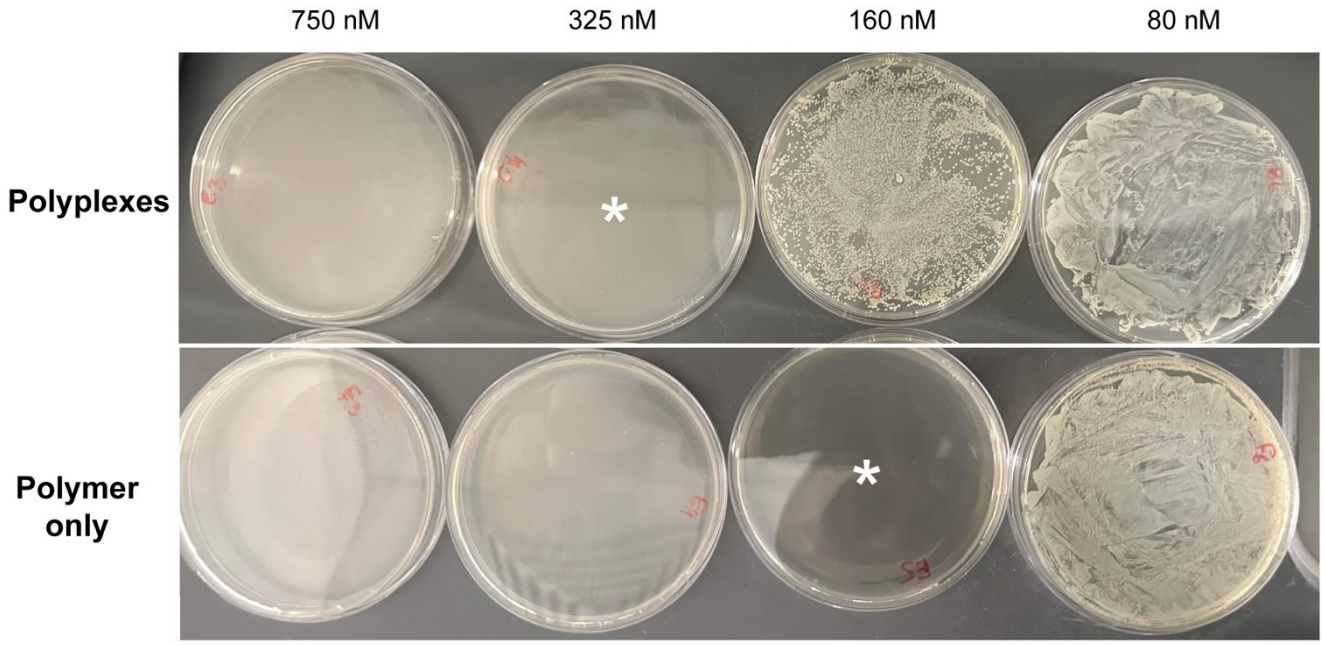

Figure S3. Images of agar plates with colonies to determine minimal bactericidal concentrations (MBC) at serially diluted concentrations. The plate with no visual growth of colonies was determined as MBC (*marked plates).

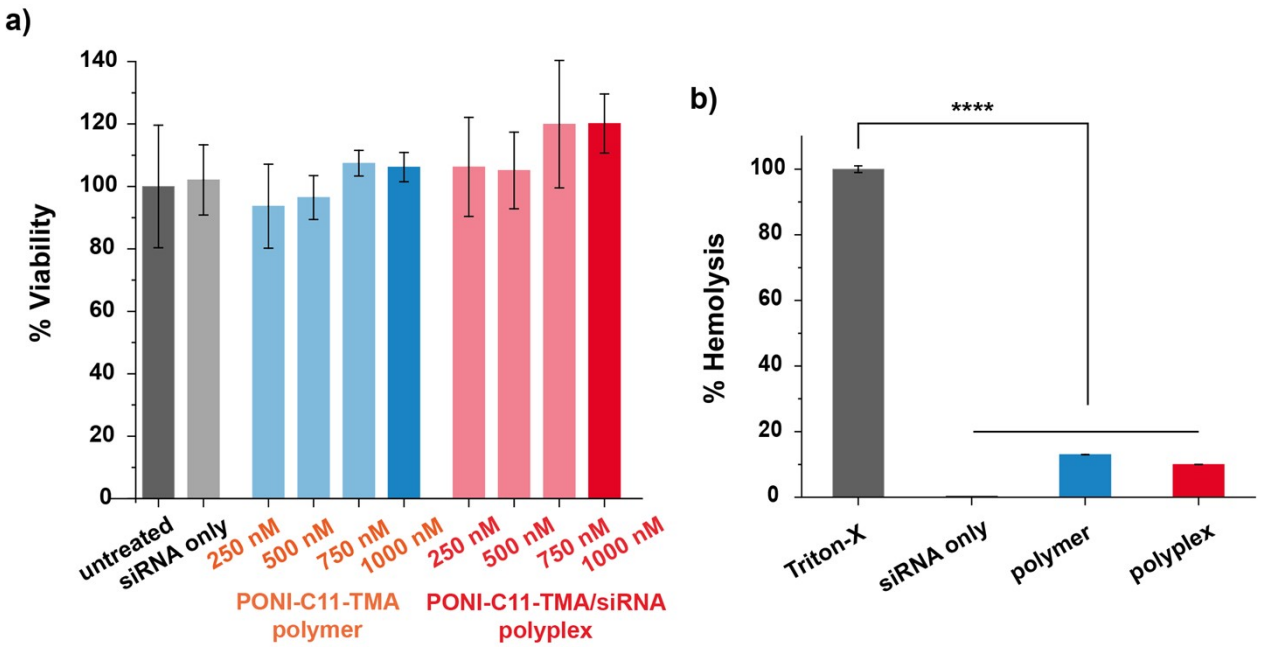

Figure S4. Biocompatibility of polyplexes with si_scramble. a) Viability of RAW 264.7 cells after 24 h exposure to indicated polymer and polyplexes at varied concentration of polymers. b) Evaluating hemolytic activity of the polyplexes at N/P 40 and its individual components siRNA only and polymer only, compared with lysing agent Triton-X. Error bars represent standard deviation (SD) of at least three experimental replicates (Data are presented as mean ± SD, one-way Anova and Tukey multiple comparisons, ****$p < 0.001$).

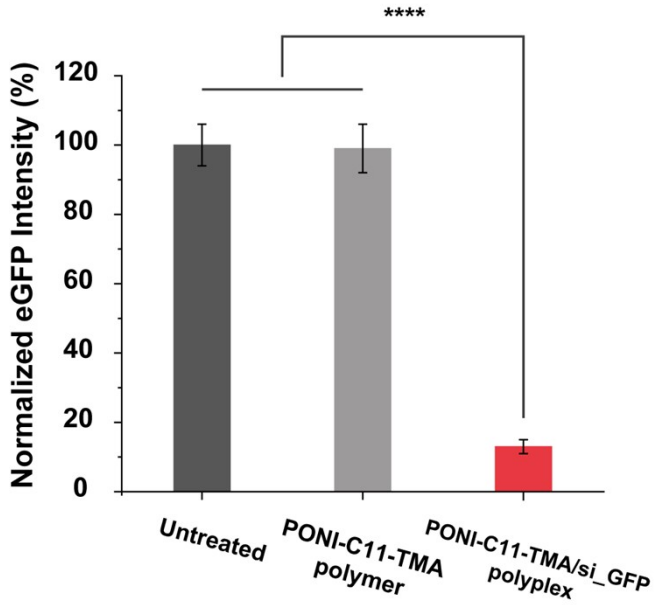

Figure S5. Quantification of eGFP knockdown in RAW 264.7:eGFP cells following treatment with **PONI-C11-TMA**/si_eGFP (50 nM of siRNA) polyplexes at N/P 40 quantified by flow cytometry (Data are presented as mean ± SD, one-way ANOVA, and Tukey multiple comparisons, ****$p < 0.001$).

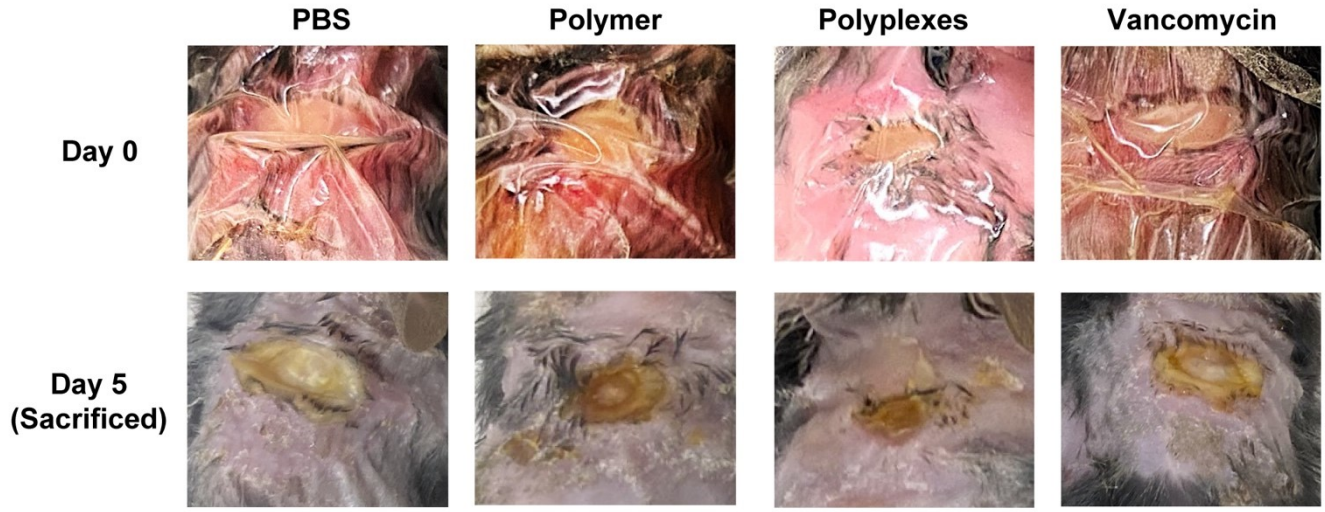

Figure S6. Wound images of mice taken at day 0 and day 5 of each treatment group respectively

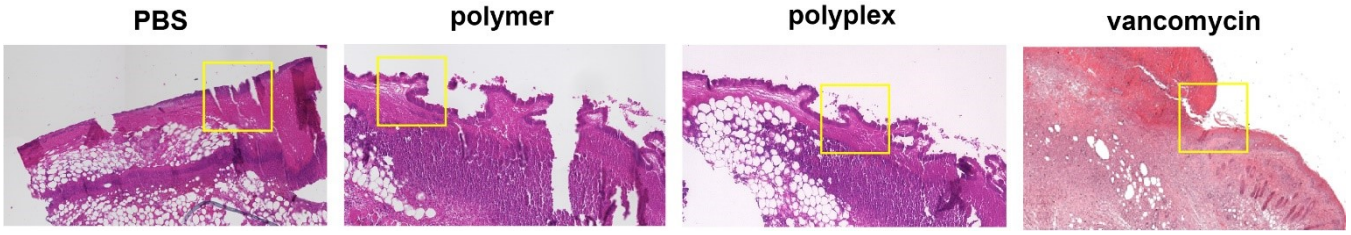

Figure S7. Representative H&E stained histology sections of wound treated with indicated treatments.

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1 A. Gupta, R. F. Landis, C.-H. Li, M. Schnurr, R. Das, Y.-W. Lee, M. Yazdani, Y. Liu, A. Kozlova and V. M. Rotello, *J. Am. Chem. Soc.*, 2018, **140**, 12137–12143.

2 R. D. Plaut, C. P. Mocca, R. Prabhakara, T. J. Merkel and S. Stibitz, *PLoS One*, 2013, **8**, e59232.

3 C. J. Bell, N. Gupta, K. D. Tremblay and J. Mager, *Mol. Reprod. Dev.*, 2022, **89**, 337–350.