Electronic Supplementary Material (ESI) for Biomaterials Science.

Electronic Supplementary Information

Bacteria-Responsive Biopolymer-Coated Nanoparticles for Biofilm Penetration and Eradication

Yingying Wang[†] and Anita Shukla[*,‡]

[†]*Department of Chemistry, Brown University, Providence, Rhode Island 02912, USA*

[‡]*School of Engineering, Center for Biomedical Engineering, Brown University, Providence, Rhode Island 02912, USA*

E-mail: anita_shukla@brown.edu

1 Characterization of *V. vulnificus* Enzyme Production

1.1 Gelatin Hydrolysis Test

Sterile LB with 3% (w/v) gelatin was prepared and allowed to solidify in culture tubes (4 mL per tube) at 4 °C. Several *V. vulnificus* colonies were isolated from an agar plate culture and transferred to the gelatin medium by piercing the solid gelatin. Controls of non-inoculated media were included. The cultures tubes were incubated at 37 °C for 24 h. Following this incubation, the culture tubes were placed vertically in an ice bath for 30 min, and the phase of medium was examined. Liquid medium in *V. vulnificus* inoculated media confirmed gelatin hydrolysis and gelatinase production.

1.2 Hyaluronidase Detection

LB broth (6 g in 120 mL of water) containing 1% (w/v) agarose was autoclaved at 121 °C for 15 min. The molten medium was allowed to equilibrate to 50 °C prior to the addition of 40 mL of filter-sterilized bovine serum albumin (BSA, 5% (w/v) in water) and 40 mL of filter-sterilized HA (2 mg/mL in water) both equilibrated to 50 °C. This BSA and HA containing LB agar (LBHA) was poured into petri dishes (2-3 mm thick). After solidification, plates were stored at 4 °C. Overnight cultures of *V. vulnificus* were diluted 1:500 (v/v) with LB, and 20 μL of this bacterial suspension was innoculated on LBHA plates. These plates were incubated at 37 °C and observed daily until bacterial growth was observed ($\sim$48 h). At this point, the plate was flooded with 2 M acetic acid, which binds HA and albumin to form a white precipitate. Hyaluronidase production was confirmed if a clear zone was observed surrounding the region of bacterial growth. Non-inoculated controls were included.

2 Quantification of Encapsulation Efficiency and Drug Loading Capacity

Encapsulation efficiency (EE%) and drug loading capacity (DL%) of the different NP formulations was determined by measuring the absorbance of unloaded Doxy in washing supernatant solutions. Absorbance was measured at 340 nm using a Cytation 3 plate reader (BioTek, Winooski, VT) using 96-well UV-transparent microplates. Absorbance values were used to quantify mass of Doxy by comparing with a Doxy standard curve (Fig. S1†). EE% and DL% were obtained using the following equations:

$$EE\% = \frac{Initial\ Doxy\ mass - Free\ Doxy\ mass\ in\ supernatant}{Initial\ Doxy\ mass} \times 100\%$$

$$DL\% = \frac{Initial\ Doxy\ mass - Free\ Doxy\ mass\ in\ supernatant}{Final\ NP\ mass} \times 100\%$$

3 Assessing Hemolysis

RBC hemolysis was determined by incubating 100 μL of free Doxy and Doxy-GNPs, CS-Doxy-GNPs, or HA-CS-Doxy-GNPs at Doxy concentrations of 1.56 to 200 μg/mL with 100 μL of 5% BRBCs or HRBCs in 96-well plates at 37 $^{\circ}C$ for 2 h. Blank NPs at concentrations ranging from 31.25 to 4000 μg/mL were also tested, along with 0.1% (v/v) Triton X-100 and 1$\times$ PBS as the positive controls (PCs) and negative controls (NCs), respectively. After incubation, the plates were centrifuged at 1000 rpm for 5 min. A 100 μL aliquot of the supernatant from each well was transferred to a 96-well plate. The absorbance of the supernatant was measured at 540 nm (Abs_{540}). Normalized hemolysis was calculated using the following equation:

$$Hemolysis\ (\%) = \frac{Abs540_{sample} - Abs540_{NC}}{Abs540_{PC} - Abs540_{NC}} \times 100$$

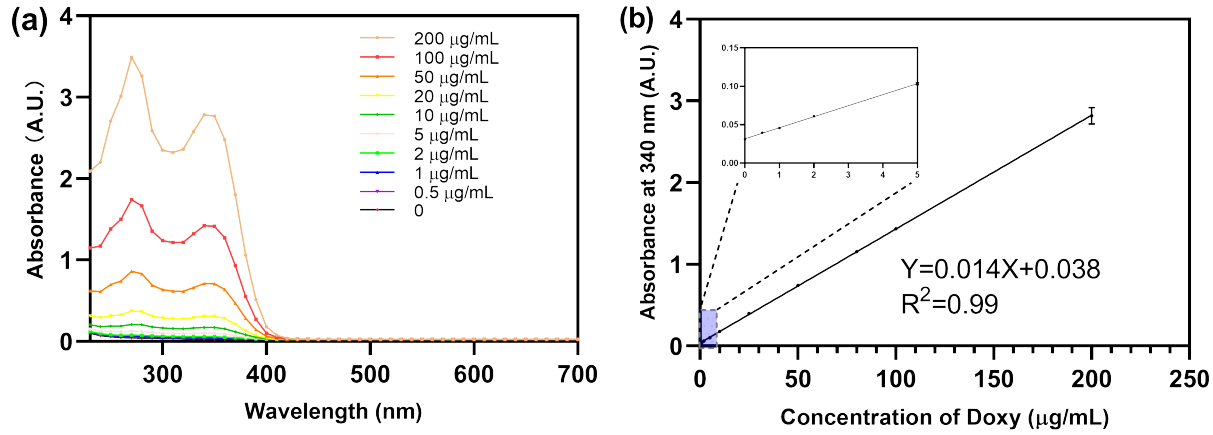

Fig. S1: Spectral scan of Doxy and standard curve. (a) Absorbance spectra of Doxy aqueous solutions at varying concentrations from 0.5 to 200 μg/mL. (b) Doxy absorbance at 340 nm as a function of Doxy concentration. Inset: Absorbance at 340 nm versus Doxy concentration from 0.5 to 5 μg/mL. Data are shown as mean $\pm$ standard deviation; n=3).

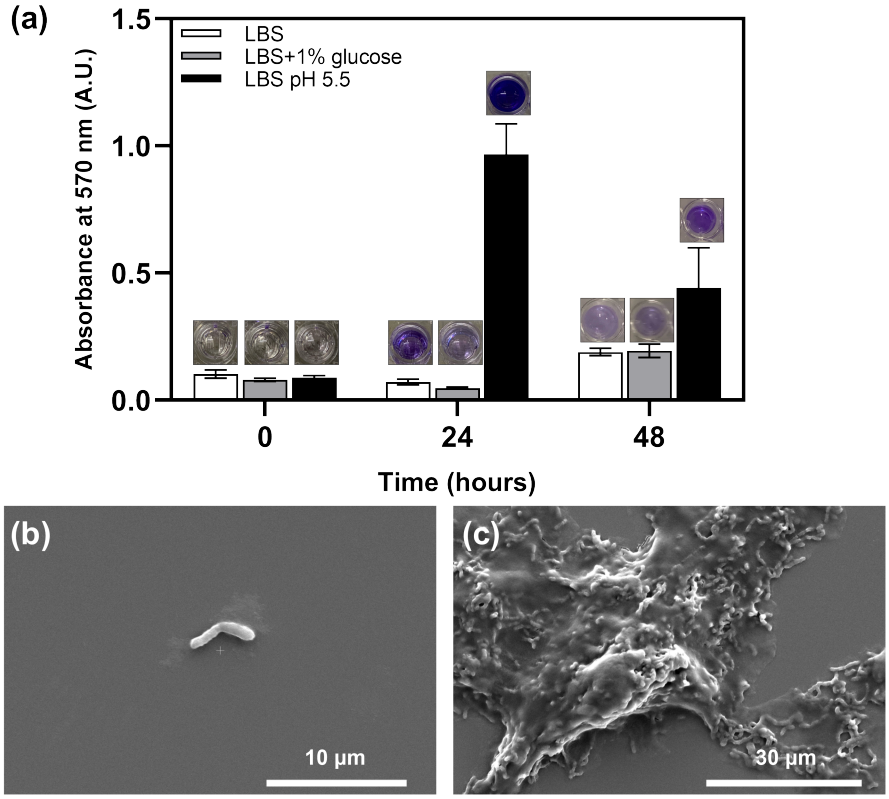

Fig. S2: Formation of *V. vulnificus* biofilms at different culture conditions over time. (a) Crystal violet staining was used to assess the formation of *V. vulnificus* biofilms in LBS, LBS with 1% (w/v) glucose, and LBS at pH 5.5. Absorbance at 570 nm was read to measure biofilm biomass. Images following crystal violet extraction are shown for each condition. SEM images of (b) planktonic *V. vulnificus* and (c) *V. vulnificus* biofilm on silicon wafers. Scale bar: 10 μm and 30 μm in (b) and (c), respectively. SEM images are representatives of at least three repeats.

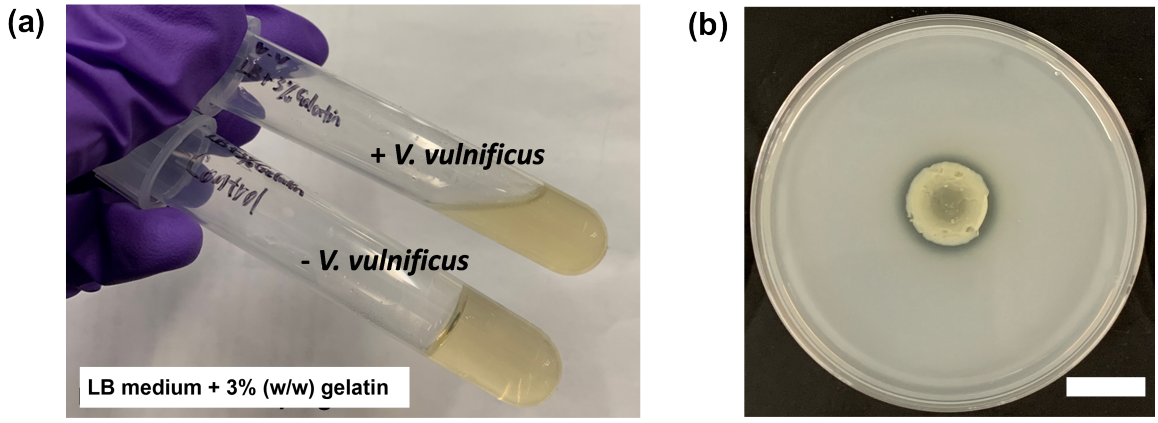

Fig. S3: Characterization of the *V. vulnificus* enzyme production. (a) Gelatin hydrolysis test with (+) and without (-) *V. vulnificus*. Flowing media indicates gelatinase production in the + *V. vulnificus* condition. (b) A plate method for the detection of hyaluronidase. A clearance zone indicates *V. vulnificus* hyaluronidase production. Scale bar: 2 cm. Data are representative of at least three independent tests.

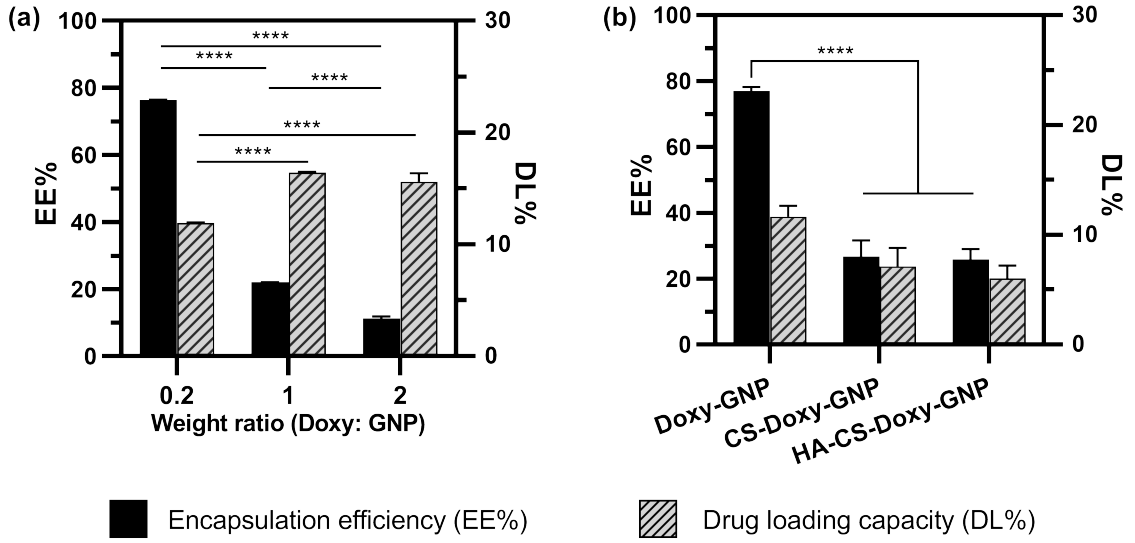

Fig. S4: Encapsulation efficiency (EE%) and drug loading (DL%) of (a) Doxy-GNPs with a ratio of Doxy to GNPs of 0.2, 1.0, and 2 (w/w), and (b) Doxy-GNPs, CS-Doxy-GNPs, and HA-CS-Doxy-GNPs with a ratio of Doxy to GNPs of 0.2 (w/w). Results are shown as mean ± standard deviation; n = 3. Statistical significance ($^{****}p < 0.0001$) between groups was determined using two-way ANOVA with Tukey's post hoc analysis.

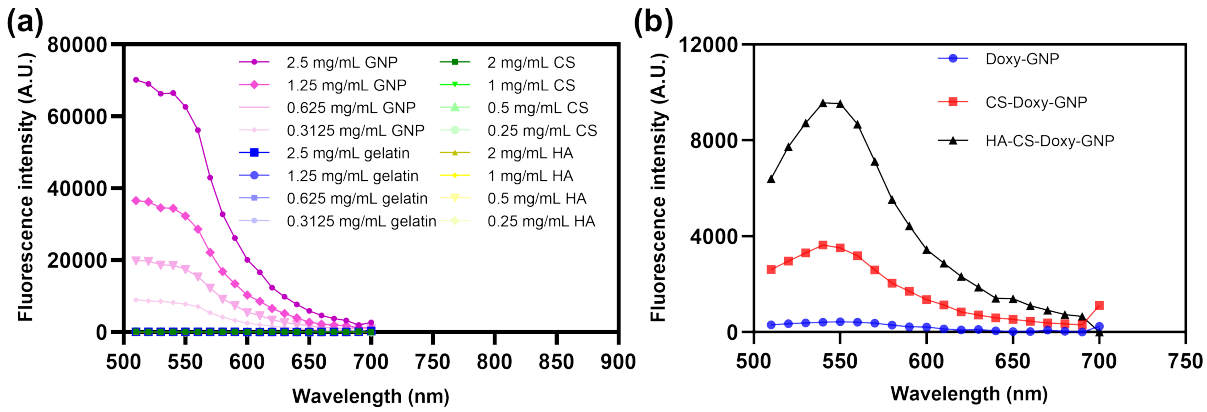

Fig. S5: Fluorescence spectra of (a) blank GNPs, gelatin, CS, and HA at different concentrations, and (b) 100 μg/mL Doxy loaded NPs upon expsure to an excitation wavelegnth of 480 nm.

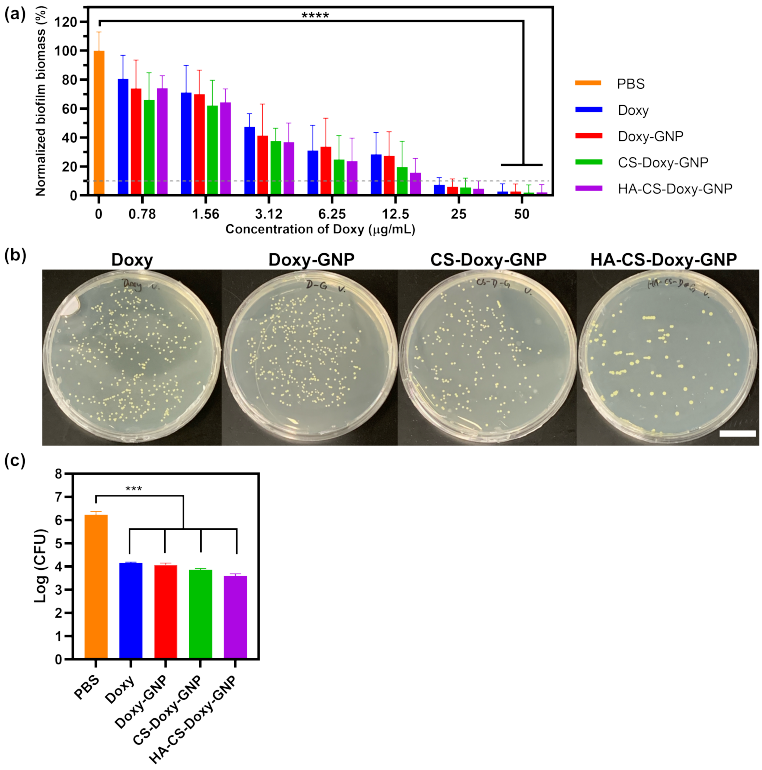

Fig. S6: Effect of NP treatment on preformed *V. vulnificus* biofilms. (a) Normalized biofilm biomass was determined after treatment of 48 h aged, preformed *V. vulnificus* biofilms with varying concentrations of free Doxy or Doxy loaded NPs at equivalent Doxy concentrations. (b) Photographs of bacterial colonies formed on agar and (c) quantified CFU following *V. vulnificus* biofilm treatment with PBS, 50 μg/mL Doxy, and Doxy loaded NPs at an equivalent Doxy concentration for 24 h. Scale bar: 2 cm. Results are shown as mean $\pm$ standard deviation. Statistical significance ($^{***}p < 0.001$; $^{****}p < 0.0001$) between the no treatment control and the $MBEC_{90}$ or between groups was determined using one-way ANOVA with Tukey's post hoc analysis; n=3. The photographs of bacterial colonies on agar are representatives of at least three repeats.

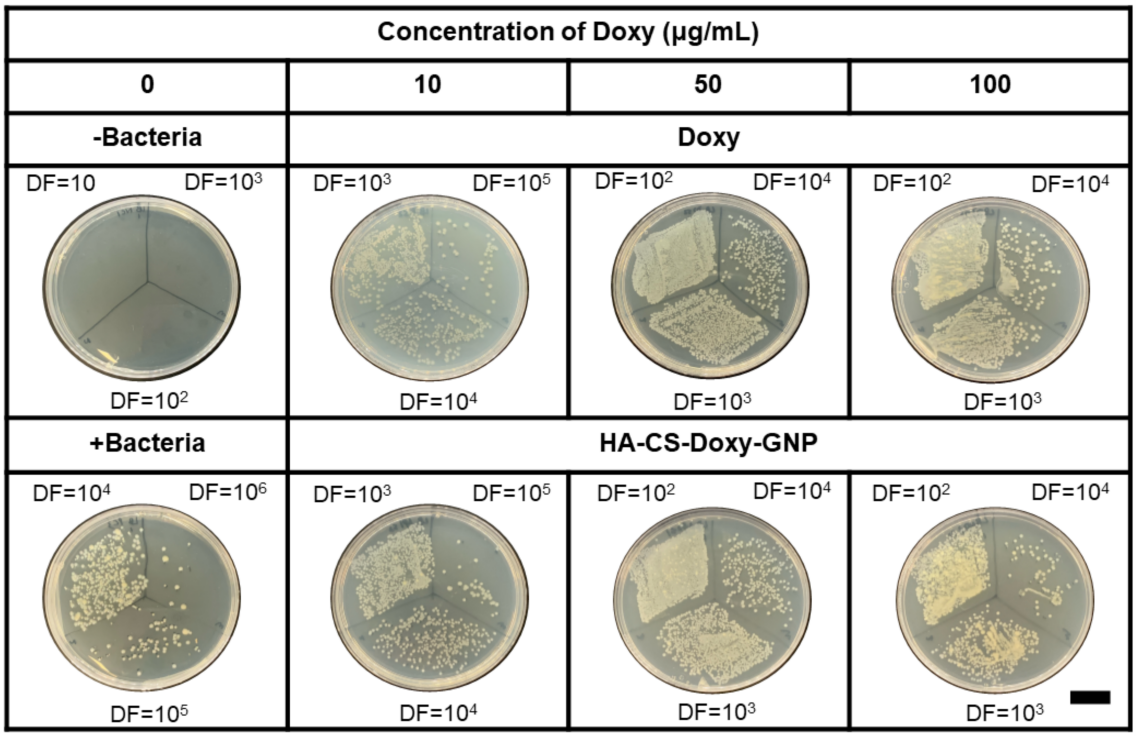

Fig. S7: Colonies formed on LB agar following free Doxy or Doxy loaded NP treatment of an *ex vivo* porcine skin *V. vulnificus* infection model. Dilution factor (DF) = final solution volume/volume of stock solution. Scale bar: 2 cm. Images of CFU on agar are representative of at least three repeats.

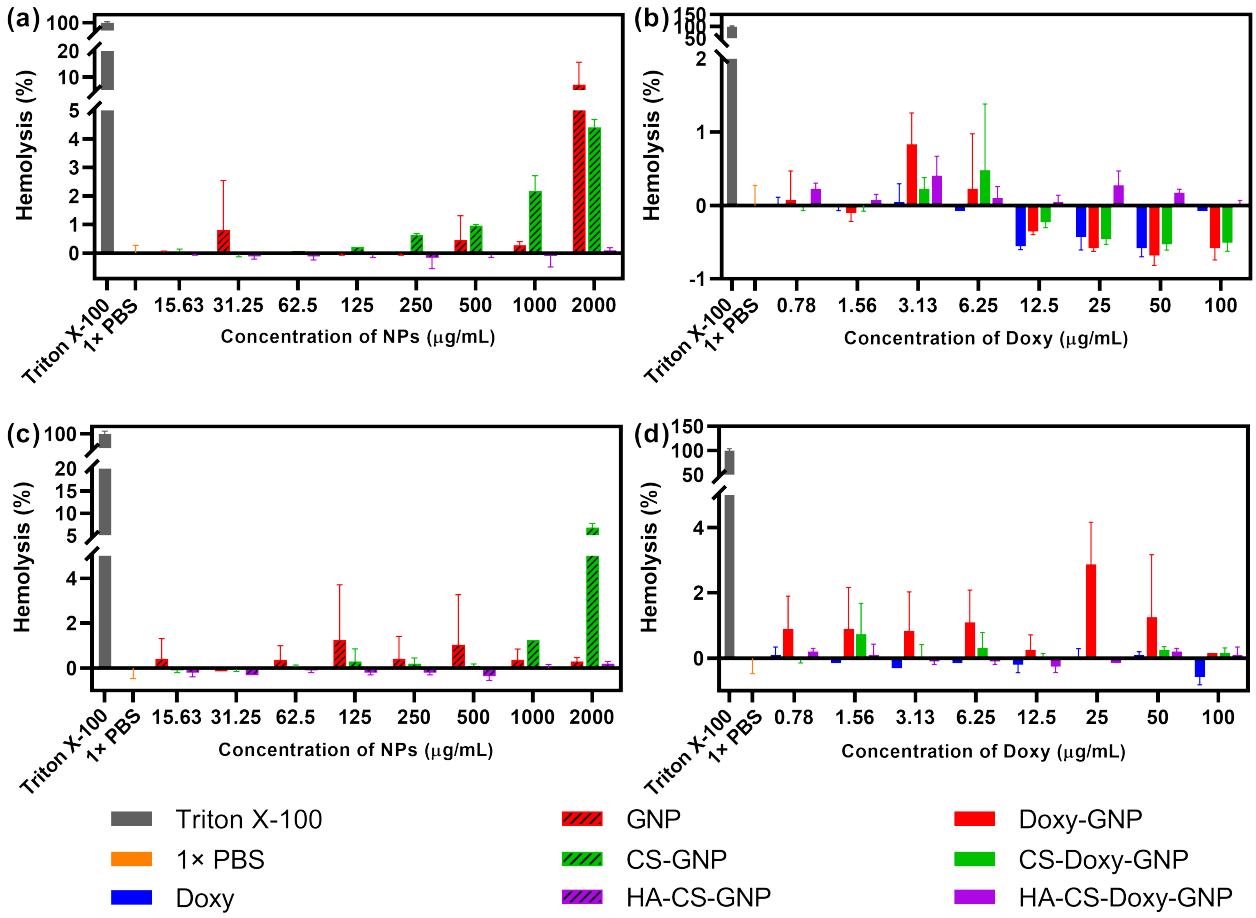

Fig. S8: Hemolysis of BRBCs and HRBCs exposed to free Doxy, Doxy loaded NPs, and blank NPs. BRBCs exposed to (a) blank NPs and (b) Doxy or Doxy loaded NPs at different Doxy concentrations. HRBCs exposed to (c) blank NPs and (d) Doxy or Doxy loaded NPs at different Doxy concentrations. Results are shown as mean ± standard deviation; n=3.