Responsive Polymer-Coated Gelatin Nanoparticles to Combat Bacterial Biofilms

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Statement of Purpose: It's estimated that ~80% bacterial infection in humans involve biofilm formation.[1] Biofilm protects the resident bacteria, contributing to the growth of antimicrobials-resistance. Nanoparticles (NPs) that target specific microbial and biofilm features show great potential to eradicate the biofilm.[2] However, developing smart drug delivery system (DDS) which response to multiple bacteria stimuli, thereby increasing specificity to biofilm, remains a challenge. Here, we developed a NP DDS that responds to both bacterial enzymes and pH. We report a hyaluronic acid (HA) and chitosan (CS) coated doxycycline (Doxy) loaded gelatin nanoparticle (GNP) and investigate its efficacy to combat bacterial biofilm (**Fig. 1A**). Under neutral physiological conditions, net negative charge of the coated NPs is expected to enhance blood circulation times when introduced systemically. At biofilm infection site, bacterial hyaluronidases will degrade the outmost HA layer exposing the underlying CS layer. The NPs become positive charge and more prone to attaching on bacteria inside the biofilm. The acid biofilm microenvironment will trigger the CS barrier layer to swell providing access of bacterial gelatinases to the GNP core. Degradation of the GNP will increase Doxy release from the NP, leading to efficient bacteria death and biofilm eradication.

Methods: A two-step desolvation method was used to prepare GNPs. Doxy-loaded GNPs were synthesized by adding lyophilized GNP to Doxy solution (weight ratio of 5:1). Then cationic CS (1 mg/mL) and anionic HA (1 mg/mL) were coated on GNP (1 mg/mL) sequentially by layer by layer coating method at pH 6. Size, zeta potential, and morphology were examined using dynamic light scattering and scanning electron microscopy (SEM). The responsive release properties of drug loaded NPs were studied at pH 7.4 and 5, and with and without gelatinase and hyaluronidase. To test the antibacterial and antibiofilm efficacy of these NPs, *Vibrio vulnificus* (ATCC 27562), a gram-negative pathogen which can cause severe wound and septicemic infections, was used. Microdilution assays were used to determine NP efficacy against planktonic *V. vulnificus*. Biofilm penetration was investigated by confocal microscopy, and biofilm inhibition and eradiation were studied by crystal violet staining, colony counting, and live/dead staining with Syto 9 and propidum iodide via live/dead biofilm viability kit.

Results: By optimizing fabrication conditions (e.g., polymer concentration, assembly time, and pH), GNP, Doxy-GNP, CS-Doxy-GNP and HA-CS-Doxy-GNP were successfully prepared (**Fig. 1A** top right shows a representative SEM image). As shown in **Fig. 1B**, the average hydrodynamic diameter increased with additional polymer coating layers, from ~215 nm for Doxy-GNP to 243 nm and 292 nm for CS-Doxy-GNP and HA-CS-Doxy-GNP, respectively. Additionally, zeta-potential confirmed successful coating, showing charge reversal between uncoated NPs and CS versus HA and CS coated NPs. The encapsulation efficiency and drug loading capacity of HA-CS-Doxy-GNPs was ~8% and 6%, respectively. Hyaluronidase treatment led to the most rapid Doxy release kinetics from HA-CS-Doxy-GNP at the conditions tested, comparable to what was observed in *V. vulnificus* conditioned media which was expected to express hyaluronidase, gelatinase and reduced pH.[4] (**Fig. 1C**). For planktonic *V. vulnificus*, the visible growth of bacteria was completely inhibited by HA-CS-Doxy-GNP with a minimal inhibitory concentration of ~4.15 µg/mL. The penetration ability of DDS is important in biofilm eradication process. As shown in **Fig. 1D**, HA-CS -GNP was able to penetrate pre-formed *V. vulnificus* biofilms. The biofilm eradication effect of HA-CS-Doxy-GNP was shown in **Fig. 1E**, mature *V. vulnificus* biofilm was removed after 24 h incubation.

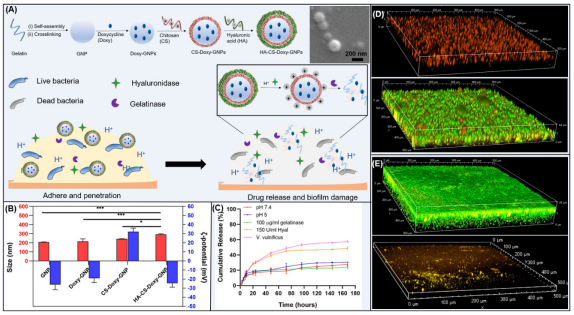

Figure 1. (A) Schematic of responsive NP synthesis and degradation. SEM of HA-CS-Doxy-GNP (top right). (B) Hydrodynamic diameter and zeta potential for NPs. Statistical analysis uses one-way analysis of variance with Tukey-Kramer post hoc analysis. Data was considered statistically significant for $p < 0.05$ (note: *$p < 0.05$, ***$p < 0.001$). (C) Normalize cumulative release of Doxy from HA-CS-Doxy-GNP under different conditions. (D) *V. vulnificus* biofilm (top) and penetration of HA-CS-GNP in biofilm during 24 h incubation (bottom). Red=biofilm; green=NPs. (E) *V. vulnificus* biofilm treated with PBS (top) and HA-CS-Doxy-GNP after 24 h incubation (bottom). Green=live bacteria; red=dead bacteria.

Conclusions: We have developed a dual-response HA-CS-Doxy-GNP and demonstrated its antibacterial and antibiofilm properties *in vitro*. To the best of our knowledge, this is the first report of a polymer-coated NP that combines pH, hyaluronidase and gelatinase responsiveness to combat bacterial biofilms. This responsive DDS could be used to deliver multiple drugs including those aimed specifically at biofilms (e.g., antibiofilm peptide) or signaling molecules for infection detection (e.g., fluorescent dyes) for the detection and treatment of broad-spectrum bacteria and biofilms.

References: [1]Verderosa, A. Front.Chem.,2019;28:7: 824. [2] Chen H. Nanoscale,2018;10:20946-20962. [3] Liu Y. Chem.Soc.Rev.,2019;48:428-44. [4] Jones, M. Infect. Immun., 2009;77:1723-1733.