Biomimetic Hemoglobin-Naftifine Nanoparticles to Overcome the Innate Immune Evasion of *S. aureus*

**Abstract**

*Staphylococcus aureus* can evade innate immune clearance through limited innate immune responses and high bacterial immune resistance. Here, we report a robust nanotherapeutic strategy to overcome immune evasion of *S. aureus* using red blood cell membrane (RBCM) coated hemoglobin (Hb)-naftifine nanoparticles (Hb-Naf@RBCM). Under infectious microenvironment, Hb-Naf@RBCM changed bacterial membrane lipid composition and responsively initiated lipid peroxidation on *S. aureus* to promote neutrophil chemotaxis. Oxygen carried by Hb-Naf@RBCM enhanced the impaired neutrophil respiratory burst killing *S. aureus* under hypoxic condition. Hb-Naf@RBCM also sensitized *S. aureus* to immune clearance via naftifine-induced carotenoid pigment biosynthesis inhibition, ferric Hb-induced bacterial H2S downregulation, persisters bacteria suppression, and biofilm inhibition. This dual strategy reduced *S. aureus*-related immune resistance and enhanced the immunotherapeutic response. Treatment with nanoparticles exhibited excellent therapeutic outcome in vitro and in mouse models of *S. aureus* infection. Our results established a novel immunological strategy for overcoming innate immune evasion against *S. aureus*.

**Introduction**

*Staphylococcus aureus* (*S. aureus*) is a clinically major human pathogen that poses a serious public health threat. The emergence of multidrug resistance along with a steady decline in the discovery of new antibiotics increase susceptibility to *S. aureus* infections. Novel prevention and treatment strategies against multidrug resistant *S. aureus* are urgently needed. The innate immune system provides inspiration for developing new immunotherapies to overcome multidrug resistance. However, *S. aureus* has developed multiple escape mechanisms to evade host immune systems. Importantly, an arsenal of secreted factors help *S. aureus* avoid detection by the first-line responders, such as neutrophils. Worse, hypoxic infectious microenvironment limits the oxygen supply for neutrophil respiratory burst, which generates reactive oxygen species (ROS) required for killing certain bacteria. In addition, *S. aureus* produces the golden carotenoid pigment (staphyloxanthin) and hydrogen sulfide (H2S), two important general defense system that protect *S. aureus* from host oxidant killing. To this end, a refined immunological strategy to overcome multiple innate immune evasion mechanisms of *S. aureus* is needed.

Lipid peroxides (LPO) produced from polyunsaturated fatty acids (PUFA) plays an important role in leukocyte recruitment. *S. aureus* does not synthesize but can incorporate PUFA, which is abundant in human cell membrane including red blood cell membrane (RBCM). Responding to oxidative and acidic conditions, hemoglobin (Hb), an endogenous protein, can generate highly reactive oxidants capable of damaging most biological substrates of bacteria, including lipids, nucleic acids, and amino acids*.* For lipids, Hb can exhibit a pseudo-peroxidase-like enzymic activity and catalyze lipid peroxidation. As a natural oxygen carrier, Hb is widely developed as oxygen therapeutics to correct oxygen deficit in a variety of clinical setting. Interestingly, ferric Hb can clear H2S. Naftifine, a US Food and Drug Administration (FDA)-approved antifungal drug, inhibits the biosynthesis of staphyloxanthin and does not function as an antibiotic against *S. aureus*, thereby sensitizing *S. aureus* to host oxidant killing together with Hb.

Here, we report an RBCM-coated Hb-Naf nanoparticles (Hb-Naf@RBCM), capable of regulating innate immune system and impairing bacterial immune resistance against *S. aureus*. The cores of the NPs were made of Hb and naftifine via a self-assembly process and it was subsequently coated with RBCM. The NPs exhibited good biocompatibility and preferentially accumulated in the infected tissues through the EPR effect. Due to its responsiveness to both low pH and overloaded hydrogen peroxide in the infected tissues, the NPs generated highly reactive oxidants in the infected tissue, including ferryl Hb. Ferryl Hb damaged most biological substrates of bacteria, including lipids, nucleic acids, and amino acids. *S. aureus* incorporated PUFA from the RBCM coating of the NPs. After that, PUFA in the bacterial membrane was oxidized into LPO via NPs-induced lipid peroxidation. The resulting LPO in the infected tissues promoted the chemotaxis of leukocytes including neutrophils. Oxygen carried by the NPs alleviated hypoxia in hypoxic infected tissues and enhanced the respiratory burst of neutrophils to generate more ROS against *S. aureus*. Naftifine blocked the biosynthesis of staphyloxanthin, a carotenoid pigment shielding *S. aureus* from oxidant killing. Ferric Hb generated during NPs-induced lipid peroxidation inhibited bacterial H2S, which protects *S. aureus* from oxidative stress. Inhibition of staphyloxanthin and H2S sensitized *S. aureus*, especially persisters, to oxidant killing induced by both Hb and neutrophils. Hb-Naf@RBCM showed striking therapeutic benefits in treating several *S. aureus* infection models due to its strong bacterial immune resistance inhibition ability and innate immune system regulation ability.

**Hb-Naf@RBCM NPs inhibited biosynthesis of naftifine and downregulated bacterial H2S level**

The Hb-Naf NP was first constructed through the assembly of naftifine and hemoglobin. The NP was then coated with RBCM using an extrusion process to produce Hb-Naf@RBCM NP, with a hydrodynamic diameter of approximately 60 nm, as confirmed by dynamic light scatter (DLS) and transmission electron microscope (TEM) imaging. The NP was stable in DI water at 4°C for 48 hours. Its zeta potential was −21.3 mV, distinct from that of hemoglobin alone (-6.5 mV). The Hb-Naf@RBCM NPs had a negative charge potential of -27.5 mV, similar to that of RBCM. The loading content of Hb in Hb-Naf@RBCM was calculated to be 41.5%, with a naftifine content of 8.2%. The Hb-Naf@RBCM NPs demonstrated excellent biocompatibility in vitro.

*S. aureus* protects itself from host oxidant killing through the production of the golden carotenoid pigment (staphyloxanthin) and H2S, its main defense mechanisms. The protective effects of staphyloxanthin and H2S are similar and synergistic, and their production increases in response to H2O2. When one of the defense mechanisms is lacking, the other compensates, as demonstrated by the increase in staphyloxanthin production in H2S-inhibited cells and H2S production in staphyloxanthin-inhibited cells in the presence of H2O2. Therefore, it is essential to develop new antimicrobial agents that can target both defense mechanisms effectively.

The Hb-Naf@RBCM NPs successfully suppressed the production of staphyloxanthin in various strains of *S. aureus* (Newman, MRSA ATCC33591, and USA300). The application of naftifine, Hb-Naf, and Hb-Naf@RBCM caused the colorless *S. aureus* due to a significant reduction in the synthesis of the carotenoid pigment.

Using the classic lead acetate reactivity test for H2S detection, as well as H2S-specific twisted internal charge transfer (TICT)–based and monobromobimane-based fluorescent probes, we showed that Hb-Naf@RBCM NP could effectively lower the H2S level in *S. aureus* strains.

**Hb-Naf@RBCM NP responsively induced lipid peroxidation on *S. aureus***

The above results suggested Hb-Naf@RBCM NP has the potential to sensitize *S. aure*us to oxidant killing by inhibiting biosynthesis of staphyloxanthin and downregulating bacterial H2S level. Given Hb can responsively generate highly reactive oxidants capable of damaging most biological substrates of bacteria, including lipids, nucleic acids, and amino acids, we first examined whether Hb-Naf@RBCM NP could affect the growth of *S. aureus*. Growth of *S. aureus* strains with different treatments was monitored under infected tissues-mimicked conditions (pH 6.5, 150 μM H2O2). Whereas growth of the *S. aureus* treated with Hb or naftifine alone was unaffected and *S. aureus* treated with Hb-Naf NP exhibited limited growth inhibition, Hb-Naf@RBCM exhibited relatively strong growth inhibition ability on *S. aureus*. But after adding H2S donor NaHS, the growth inhibition induced by Hb-Naf@RBCM was attenuated, which suggested H2S can protect *S. aureus* from NP-induced oxidant killing. We also overserved evident membrane disruption and deformation on Notably, compared to Hb-Naf NP, Hb-Naf@RBCM NP showed higher *S. aureus* growth inhibition and more serious membrane deformation. These results demonstrated RBCM also contributed to the damage caused by Hb-Naf@RBCM. *S. aureus* cannot synthesize but can incorporate PUFA, which is abundant in RBCM. PUFA in RBCM incorporated into *S. aureus* cell membrane made *S. aureus* more suspectable to oxidative stress.

As such, we wondered whether the damage caused by Hb-Naf@RBCM NPs specifically correlated with lipid peroxidation, under which oxidants such as free radicals attack lipids containing carbon-carbon double bond, especially PUFA and may ultimately rupture the membrane. To test this hypothesis, we first evaluated the ROS level in *S. aureus* with different treatments. We observed stronger ROS fluorescence in *S. aureus* treated with Hb-Naf@RBCM compared to Hb-Naf. Then, we assayed DNA, RNA, and protein oxidation levels in *S. aureus* with different treatments. Both Hb-Naf NP and Hb-Naf@RBCM NP treatments significantly increased the DNA, RNA, and protein oxidation level and there was no significance difference between Hb-Naf NP and Hb-Naf@RBCM. Then we assayed lipid peroxidation in *S. aureus* using the lipophilic dye C11-BODIPY and MDA assay kit. Consistent with the hypothesis that the damage caused by Hb-Naf@RBCM NP specifically correlated with lipid peroxidation, we observed that *S. aureus* treated with Hb-Naf@RBCM NP exhibited larger fluorescence intensities and higher MDA level than *S. aureus* treated with Hb-Naf.

Interestingly, both growth inhibition and increasing MDA level were low pH and high H2O2 level responsive. The results indicated the antimicrobial ability of Hb-Naf@RBCM is responsive to infectious microenvironment.

**Hb-Naf@RBCM NPs promoted neutrophil chemotaxis and potentiated innate immune clearance**

Given that lipid peroxidation in wound sites enhances long-range immune detection, we investigated whether Hb-Naf@RBCM NPs can enhance neutrophil chemotaxis by inducing LPO accumulation in *S. aureus*. Using a transwell assay, we evaluated the recruitment of neutrophils to the bacterial membrane of three strains of *S. aureus* with different treatments. Our results showed that treatment with Hb-Naf@RBCM NPs significantly increased neutrophil recruitment compared to treatment with Hb-Naf NPs. However, when we added the lipid radical scavenger liproxstatin-1, Hb-Naf@RBCM-induced neutrophil recruitment was suppressed. These findings indicate that the enhanced neutrophil chemotaxis is dependent on lipid peroxidation induced by Hb-Naf@RBCM.

We next determined whether Hb-Naf@RBCM could render *S. aureus* more susceptible to immune clearance in human blood. We measured the viability of three strains of *S. aureus* cells with different treatments mixed with whole blood or neutrophils which play a central role in the innate immune response and a critical role in bacterial killing. The results suggested Hb-Naf@RBCM made *S. aureus* more susceptible to immune clearance in human blood via blocking the biosynthesis of staphyloxanthin, downregulating bacterial H2S level, and changing bacterial membrane lipid composition.

To evaluate the impact of Hb-Naf@RBCM on *S. aureus* immune clearance by the human innate immune system, we studied the viability of three strains of *S. aureus* treated with different treatments when mixed with human whole blood or neutrophils. Neutrophils play a crucial role in the innate immune response and bacterial killing. Our results indicated that Hb-Naf@RBCM made *S. aureus* more susceptible to immune clearance in human blood by blocking staphyloxanthin biosynthesis, inhibiting bacterial H2S, and altering the lipid composition of the bacterial membrane.

We investigated the oxygen-carrying capacity of Hb-Naf@RBCM in response to *S. aureus'* use of a hypoxic microenvironment, which weakens the respiratory burst of neutrophils. We added Hb-Naf@RBCM to deoxygenated PBS in a closed system and observed a rise in dissolved oxygen levels, while the control group showed no increase. This was further confirmed through a hypoxia probe via CLSM in 3T3 cells, where Hb-Naf@RBCM was able to alleviate cellular hypoxia.

We then investigated if Hb-Naf@RBCM could enhance the impaired respiratory burst of neutrophils in hypoxia. Our findings showed that the fMLP-stimulated extracellular superoxide anion levels and opsonized zymosan (OZ)-induced intracellular total ROS generation levels were significantly reduced in hypoxia but increased after adding Hb-Naf@RBCM. This suggests that Hb-Naf@RBCM can enhance the impaired respiratory burst of neutrophils in hypoxia. Moreover, the antimicrobial ability of whole blood or neutrophils with different treatments on *S. aureus* in hypoxia was also improved by adding Hb-Naf@RBCM, overcoming immune evasion by promoting neutrophil recruitment, increasing the sensitivity of *S. aureus* to oxidative killing, and enhancing neutrophil respiratory burst.

**Hb-Naf@RBCM NPs reduced persisters and disrupted biofilm formation**

*S. aureus* has an innate characteristic of surviving normally lethal antibiotic treatment without acquiring genetic resistance, this is due to the presence of persister bacteria, a subpopulation that has slow metabolism and high tolerance. Persister bacteria contribute to the development and spread of antibiotic resistance, and so far, no persister-specific treatments have been approved by the FDA. Utilizing potentiating agents to make persister bacteria more susceptible to the innate immune system is a promising approach to addressing this challenge.

*S. aureus* persisters, which were able to survive normally lethal antibiotic exposure (ciprofloxacin challenge for 3 hours), generated higher levels of staphyloxanthin and hydrogen sulfide (H2S). Our findings showed that Hb-Naf@RBCM NPs can effectively reduce the levels of both staphyloxanthin and H2S in persisters, thus increasing the ability of whole blood or neutrophils to combat both persisters (ciprofloxacin challenge for 3 hours) and stationary-phase *S. aureus*. Additionally, Hb-Naf@RBCM NPs inhibited the formation of *S. aureus* biofilm, as confirmed by crystal violet assays.

**The therapeutic efficacy of the Hb-Naf@RBCM NPs in *S. aureus*-induced pneumonia model and *S. aureus*-induced peritonitis model**

The distribution of Hb-Naf NPs and Hb-Naf@RBCM NPs was studied in mice with MRSA (ATCC33591) thigh infections using an in vivo imaging system (IVIS). The mice were injected with Cy5.5-labeled NPs and then sacrificed 6 hours or 24 hours after injection for ex vivo imaging. The results showed that compared to Hb-Naf NPs, Hb-Naf@RBCM NPs had higher accumulation in the infected thigh at both 6 hours and 24 hours after injection, due to the RBCM-enhanced permeability and retention (EPR) effect. These findings demonstrate the targeting ability of the NPs to infected tissues.

The in vivo antimicrobial efficacy of Hb-Naf@RBCM NPs was tested in *S. aureus* pneumonia (MRSA ATCC33591), peritonitis (MRSA ATCC33591), and bacteremia (USA 300) models. For the pneumonia model, *S. aureus* was administered intranasally to induce lung infections in mice. One hour later, the infected mice were treated with free Hb, free naftifine, Hb-Naf NPs, and Hb-Naf@RBCM NPs. Hb-Naf@RBCM NPs showed the highest antimicrobial efficacy, resulting in a reduction of more than three orders of magnitude in the colony-forming unit (CFU) count. Hb-Naf NPs showed moderate efficacy, while free naftifine had very limited efficacy due to its inability to directly act as an antibiotic agent against *S. aureus* and the hypoxic microenvironment at the infection site that limited host oxidant killing. Free Hb had no effect on bacterial load.

For the peritonitis model (MRSA ATCC33591), the different treatments were administered intravenously 6 hours after infection. Compared to treatment with free naftifine, Hb-Naf NPs resulted in a modest survival benefit, while Hb-Naf@RBCM NPs, with their antimicrobial and immunomodulatory abilities, resulted in 100% mouse survival after two weeks, demonstrating an excellent therapeutic outcome. The CFUs in various organs were measured 12 hours after bacterial infection. Hb-Naf@RBCM NPs showed higher antimicrobial efficacy compared to Hb-Naf NPs due to higher NP accumulation, changes in bacterial membrane lipid composition, and promotion of neutrophil recruitment. Both Hb-Naf@RBCM NPs and Hb-Naf NPs significantly improved the therapeutic efficacy compared to free naftifine and free Hb in all the tested organs.

**The therapeutic efficacy of the Hb-Naf@RBCM NP in *S. aureus*-induced blood infection model**

Further evaluating the therapeutic effectiveness of Hb-Naf@RBCM NPs, a *S. aureus* bacteremia model was employed. *S. aureus* (USA 300) was administered via the tail vein to induce mouse blood infection and sepsis. Treatments including free naftifine, free Hb, Hb-Naf NPs, and Hb-Naf@RBCM NPs were given intravenously 6 hours after infection. Hb-Naf@RBCM NPs showed the greatest effect in clearing *S. aureus*, resulting in higher survival rates compared to free naftifine, free Hb, and Hb-Naf NPs. The bacterial burden in blood and major organs was measured 12 hours after infection. Results showed that Hb-Naf@RBCM NPs significantly reduced bacterial levels in most organs and blood compared to free naftifine and Hb-Naf NPs. Histopathological analysis was also performed to assess therapeutic benefits. In septic mice infected with *S. aureus*, sections stained with haematoxylin and eosin (H&E) revealed typical multiple organ injury. However, Hb-Naf@RBCM showed significant reduction in multiple organ injury, while other treatments had limited efficacy.

The safety of Hb-Naf NPs and Hb-Naf@RBCM NPs was assessed in healthy mice through three consecutive administration every other day. Tissue histology, inflammatory cytokine levels, and biochemical analysis showed no significant harm to the organs, indicating that both types of NPs are safe for use.

**Conclusion**

We have developed a nanotherapeutic strategy to overcome the immune evasion of *S. aureus* by reducing bacterial immune resistance and enhancing the immunotherapeutic response. The combination of Hb, naftifine, and RBCM effectively weakens *S. aureus*, including persisters, by blocking staphyloxanthin biosynthesis, reducing bacterial H2S levels, and altering the bacterial membrane composition. Hb-Naf@RBCM responsively triggered lipid peroxidation, leading to enhanced neutrophil chemotaxis, and also relieved hypoxia by delivering oxygen, which strengthened the respiratory burst of neutrophils against *S. aureus*. Our results demonstrate remarkable therapeutic outcomes both in vitro and in vivo, with minimal side effects. These findings offer a promising approach for overcoming bacterial immune evasion and reducing the risk of antibiotic resistance emergence or spread.

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