Antimicrobial peptide-loaded gold nanoparticle-DNA aptamer conjugates as highly effective antibacterial therapeutics against *Vibrio vulnificus*

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*Vibrio vulnificus* causes fatal infections in humans, and antibiotics are commonly used in treatment regimens against *V. vulnificus* infection. However, the therapeutic effects of antibiotics are limited by multidrug resistance. In this study, we demonstrated that an antimicrobial peptide (AMP), HPA3P^His, loaded onto a gold nanoparticle-DNA aptamer (AuNP-Apt) conjugate (AuNP-Apt-HPA3P^His) is an effective therapeutic tool against *V. vulnificus* infection *in vivo* in mice. HPA3P^His induced bacterial cell death through the disruption of membrane integrity of *V. vulnificus*. The introduction of AuNP-Apt-HPA3P^His* into V. vulnificus*-infected HeLa cells dramatically reduced intracellular *V. vulnificus* by 90%, leading to an increase in the viability of the infected cells. Moreover, when *V. vulnificus*-infected mice were intravenously injected with AuNP-Apt-HPA3P^His, a complete inhibition of *V. vulnificus* colonization was observed in the mouse organs, leading to a 100% survival rate among the treated mice, whereas all the control mice died within 40 hours of being infected. Therefore, this study demonstrated the potential of an AMP delivered by AuNP-Apt as an effective and rapid treatment option against infection caused by a major pathogen in humans and aquatic animals.

The emergence and spread of multidrug-resistant bacteria are global concerns. Overcoming antibiotic resistance is an urgent and imperative issue. A major hurdle in the elimination of pathogens in a host is the poor uptake of many antibiotics by infected host cells, which results in intracellular persistence of bacteria and failure of antimicrobial therapy¹-³.

*Vibrio vulnificus*, a halophilic Gram-negative bacillus, is a highly virulent, opportunistic pathogen that causes gastroenteritis, primary sepsis, and wound infection in humans. Infection by *V. vulnificus* commonly occurs via ingestion of contaminated seafood and an exposed open wound, and its incidence is significantly rising⁴. *V. vulnificus* spreads rapidly and causes extensive tissue damage, leading to a mortality rate of over 50% among infected patients with sepsis⁵. *V. vulnificus* infects a host via gastrointestinal tract⁶, and disrupts tight junctions of intestinal cells, leading to its intracellular colonization in the intestine intestinal epithelium⁷. Combinational antibiotics therapy is the most common treatment regimen against *V. vulnificus* infection⁸,⁹. However, resistance to antibiotics and inefficient delivery of antibiotics contribute to the high fatality rate observed following *V. vulnificus* infection¹⁰,¹¹,¹². Therefore, innovative approaches for developing alternative antibacterial agents are in demand.

Diverse classes of antibacterial compounds have been designed for effective treatment of fatal bacterial infections. Among them, antimicrobial peptides (AMPs), which are a crucial part of the host defense system with

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solutions containing either SDS or TFE (Fig. 1A). These CD results suggest that HPA3P His is capable to bind to potential-sensitive dye 3,3′-
their intact structure and function, into diverse types of cells20–22. In addition, the elimination of intracellular ing increase in fluorescence24. Once the fluorescence intensity stabilized,
any other compounds affect the potential of the bacterial membrane, the dye releases into the buffer, result-
clinical application of AMPs 17. To overcome these limitations, we developed an AMP delivery system using gold
three bacterial species tested in this study (Table 1). When compared with the MIC and MBC of antibiotics for
vulnificus (Table 1), and AuNP-Apt His itself
His-tagging to HPA3P did not alter the MIC and MBC against
vulnificus, their bactericidal activities against
ative bacteria (Table 1). For The C-terminal end of HPA3P was tagged with hexahistidine (His) to load HPA3P and showed similar or variable MICs and MBCs for other gram-negative or -pos-
tory concentrations (MICs) and minimal bactericidal activity (MBC). Among them, HPA3P exhibited the lowest
V. vulnificus, their bactericidal activities against
V. vulnificus, S. typhimurium, and S. aureus (Sa). cMBC: minimal bactericidal concentration against Vv, St, and Sa.
Table 1. Antibacterial activities of synthetic peptides and and antibiotics. aM.W: molecular weight.
bMIC: minimal inhibitory concentration against Vibrio vulnificus (Vv), Salmonella typhimurium (St), and Staphylococcus aureus (Sa). bMIC: minimal bactericidal concentration against Vv, St, and Sa.

to bacterial membranes. Next, the ability of HPA3PHis to permeabilize intact membranes was analyzed using the membrane
potential-sensitive dye 3,3′-dipropylthiadicarbocyanine iodide (DiSC3-5). Should antimicrobial peptides or any other compounds affect the potential of the bacterial membrane, the dye releases into the buffer, resulting
increase in fluorescence34. Once the fluorescence intensity stabilized, V. vulnificus MO6-24/0 were treated with HPA3PHis at 0.25X, 0.5X, and 1X of the MIC. Treatment with HPA3PHis resulted in an immediate increase in fluorescence in a concentration-dependent manner, suggesting that HPA3PHis has membrane-depolarizing activity (Fig. 1B). We also examined whether the integrity of the bacterial membrane was disrupted by HPA3PHis, using SYTOX Green and propidium iodide (PI). These dyes cannot pass through intact membranes, but will bind to DNA upon membrane damage by antimicrobial agents, resulting in an increase in fluorescence. The
fluorescence intensity of SYTOX Green increased after treatment with HPA3P\textsuperscript{His}, indicating the bacterial membrane was disrupted (Fig. 1C). We further confirmed the disruption of \textit{V. vulnificus} membranes integrity by PI staining and flow cytometry. As shown in Fig. 1D, 21.22% of the bacteria in the \textit{V. vulnificus} MO6-24/0 control group stained positive for PI. The groups treated with HPA3P\textsuperscript{His} showed the increased PI fluorescent signal in a concentration-dependent manner compared to the control (Fig. 1D). Collectively, these data indicate that HPA3P\textsuperscript{His} induces bacterial cell death through a membranolytic mechanism.

Effective delivery of HPA3P\textsuperscript{His} in mammalian cells by AuNP-Apt\textsuperscript{His} conjugates. HPA3P\textsuperscript{His} were loaded on AuNP-Apt\textsuperscript{His} by simple mixing, followed by incubation for 10 min to generate AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His} composite (Fig. 2A). The binding capacity assay indicated efficient association of HPA3P\textsuperscript{His} with AuNP-Apt\textsuperscript{His}, where approximately 50–60% of the HPA3P\textsuperscript{His} in the reaction mixture bound to AuNP-Apt\textsuperscript{His} (Fig. 2B). The particle size distribution data from the dynamic light scattering (DLS) assay demonstrated that the average diameters of AuNP-Apt\textsuperscript{His} and AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His} were 83.0 ± 1.3 nm and 874.7 ± 232.8 nm, respectively (Fig. 2C). The zeta potential (ζ) of AuNP-Apt\textsuperscript{His} and AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His} was −39.32 mV and −25.23 mV, respectively, indicating that the formation of AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His} complex decreases the negative charge on the AuNP-Apt\textsuperscript{His} surface, and this decrease facilitates the cellular uptake of particles\textsuperscript{25,26}. To examine the effectiveness of intracellular delivery of HPA3P\textsuperscript{His} by AuNP-Apt\textsuperscript{His}, HeLa cells were incubated with AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His} for 10 min, following which immunostaining of histidines and confocal laser scanning microscopic analyses were performed. As shown in Fig. 2D, strong fluorescent signals were detected in AuNP-Apt\textsuperscript{His}-HPA3P\textsuperscript{His}-incubated cells, whereas
much weaker fluorescence was observed in cells incubated with HPA3P His alone. These results indicate that AuNP-Apt His composites efficiently deliver HPA3P His into mammalian cells and that HPA3P His alone does not effectively penetrate cells.

Efficient bactericidal action of AuNP-Apt His-HPA3P His leading to survival of V. vulnificus-infected host cells. To examine the antibacterial effect of AuNP-Apt His-HPA3P His, V. vulnificus-infected HeLa cells were incubated with AuNP-Apt His-HPA3P His for 10 or 30 min. The viable intracellular V. vulnificus cells were extracted from HeLa cells and analyzed by colony-forming assay. Compared with that of buffer- or AuNP-Apt His-incubated cells, the number of viable V. vulnificus cells in the AuNP-Apt His-HPA3P His-treated cells decreased by 90% in 10 min (Fig. 3A). In contrast, only 20% or 40% decrease in viable V. vulnificus cells after for 10 or 30 min of treatment with HPA3P His was observed, respectively (Fig. 3A). HPA3P His and AuNP-Apt His, HPA3P His did not exhibit cytotoxicity against HeLa cells and mouse erythrocytes up to 10 µM as determined by cell viability and hemolysis assays, respectively (Fig. 3B and C). In addition, we determined the effect of AuNP-Apt His-HPA3P His on V. vulnificus-infected host cell survival. V. vulnificus-infected HeLa cells were incubated with buffer, AuNP-Apt His, HPA3P His, or AuNP-Apt His-HPA3P His, and viable HeLa cells were measured up to 4 h. Nearly all the infected HeLa cells incubated with buffer, AuNP-Apt His, or HPA3P His were dead at 4 h after infection (Fig. 3D). In contrast, the treatment of infected HeLa cells with AuNP-Apt His-HPA3P His completely prevented their death (Fig. 3D). Thus,
these results suggest that AuNP-AptHis conjugates improve the penetrability of HPA3PHis, leading to the effective bactericidal action of HPA3PHis.

**Prevention of death of V. vulnificus-infected mice by AuNP-AptHis-HPA3PHis treatment.** We extended our study to test the effectiveness of AuNP-AptHis-HPA3PHis in vivo in mice. To induce septicemia and wound infection caused by the invasion of *V. vulnificus* into tissues and vasculature, *V. vulnificus* was subcutaneously inoculated into the skin of mice. The spreading of *V. vulnificus* in the organs of mice, including spleen, inguinal lymph node, and liver, was monitored for 4 h after inoculation. As shown in Fig. 4A, *V. vulnificus* sufficiently colonized in these organs as early as 30 min. Using this mouse model, the in vivo therapeutic effect of AuNP-AptHis-HPA3PHis was examined. Mice were infected with *V. vulnificus*, followed by a single intravenous injection with buffer, AuNP-AptHis, HPA3PHis, or AuNP-AptHis-HPA3PHis at 2 h after inoculation. Then, the survival rate of the mice was observed for 120 h. All infected mice injected with buffer, AuNP-AptHis, or HPA3PHis died before 42 h after infection (Fig. 4B). In sharp contrast, all the infected mice injected with AuNP-AptHis-HPA3PHis survived until 120 h (Fig. 4B). To ensure that the survival of mice was due to the inhibition of *V. vulnificus* by HPA3PHis delivered by AuNP-AptHis, the number of viable *V. vulnificus* cells was measured in the spleen, inguinal lymph node, and liver homogenates of these mice at 18 h after inoculation. There was no
Figure 4. Complete protection from mortality induced by *Vibrio vulnificus* infection in mice by systemic administration of AuNP-Apt<sub>His</sub>-HPA3Phis (A) Distribution of *V. vulnificus* in the organs of mice after subcutaneous inoculation of *V. vulnificus* (5 × 10⁴ CFU). The mice were sacrificed at indicated time points. Each data point represents the mean ± SEM of two mice. (B) Schematic illustration of *in vivo* mice experiment is presented at the top. The percent survival of *V. vulnificus*-infection mice after treatment with buffer, AuNP-Apt<sub>His</sub> (5 nM), HPA3Phis (2 mg/kg), or AuNP-Apt<sub>His</sub>-HPA3Phis (~1 mg/kg of AMP loaded on 5 nM of AuNP-Apt<sub>His</sub>) until 120 h after infection has been presented. Five mice were used for each group. (C) The *in vivo* bactericidal efficacy of AuNP-Apt<sub>His</sub>-HPA3Phis was confirmed by examining the viable *V. vulnificus* counts in the spleen, inguinal lymph node (ILN), and liver isolated from the *V. vulnificus*-infected mice treated with buffer, AuNP-Apt<sub>His</sub>, HPA3Phis, or AuNP-Apt<sub>His</sub>-HPA3Phis. Four mice were used for each group, and mice were sacrificed 18 h after *V. vulnificus* infection. Data are expressed as the number of CFUs/g organ. Asterisks indicate statistically significant values (*p < 0.05; **p < 0.01; ***p < 0.001).
colonization of *V. vulnificus* in the AuNP-AptHis-HPA3PHis-treated mice organs, whereas the organs from the mice injected with buffer, AuNP-AptHis, or HPA3PHis showed significant colonization of *V. vulnificus* (Fig. 4C).

**Discussion**

In this study, we demonstrated that AuNP-AptHis-HPA3PHis is a potent antimicrobial agent against *V. vulnificus* infection, supported by its rapid and highly effective bactericidal action in host cells. *V. vulnificus* is a representative pathogenic strain that evades the host defense systems and replicates rapidly, resulting in mortality among humans and aquatic animals. Its infection contributes to 95% of seafood-related mortality in humans. A high dose of combinational antibiotics, such as doxycycline + ceftazidime, is a common primary therapeutic regimen against *V. vulnificus* infection. However, studies on antibiotic sensitivity of *V. vulnificus* isolates from the US, Europe, and Asia indicate that substantial portions of the isolates show resistance to various antibiotics. This emergence of multidrug-resistant *V. vulnificus* imposes the need to seek alternative therapeutic approaches.

Naturally occurring AMPs are produced as gene-encoded precursor proteins by all living organisms from prokaryotes to humans to protect from infections and inhibit competing microbes in bacteria. The interaction of amphipathic AMPs with bacterial membrane disrupts the integrity of the membrane, leading to rapid membrane permeabilization and lysis of bacteria. Structural and functional diversities of AMPs along with their rapid mode of action render AMPs attractive pharmacological agents that could overcome antibiotic resistance. Few AMPs are already in the market and various AMPs are currently under clinical trial as antimicrobial agents. However, the currently used AMPs are largely limited to topical applications due to their rapid proteolytic degradation and clearance as peptides in addition to their ineffective *in vivo* systemic delivery.

Although researchers have mainly focused on identifying bactericidal AMPs and their derivatives in the last decade, limited efforts have been made for innovative development to enhance the effectiveness of AMPs in living systems. AMPs, being cationic or amphiphilic, can readily bind to serum proteins and are degraded by proteolytic enzymes in the blood plasma, followed by rapid clearance from circulation, resulting in loss of their antimicrobial activity. To prevent AMP proteolysis, elaborative chemical modifications of AMPs, such as generation of prodrug, cyclization of AMPs, and tagging or blocking of amino- or carboxyl ends of AMPs, have been attempted. More recently, various AMP formulations with nanocarriers, such as lipids and polymers, were evaluated to increase the efficacy of AMPs; however, usually the process of loading AMPs is complex and their *in vivo* effectiveness was minimal or not confirmed. Currently, only a few reports on the development of bactericidal AMPs against *V. vulnificus* infection are available.

To the best of our knowledge, we are the first to develop an AMP-derived antibacterial agent with high efficacy in *V. vulnificus*-infected mammals. A single intravenous (IV) injection of AuNP-AptHis-HPA3PHis in *V. vulnificus*-infected mice efficiently and rapidly eliminates *V. vulnificus* in the host, as evidenced by no viable *V. vulnificus* in the organs of mice at 18 h after injection (Fig. 4C). All the infected mice survived after the administration of AuNP-AptHis-HPA3PHis, which was in sharp contrast to 0% survival rate among control mice (Fig. 4B). The intravenous injection of HPA3PHis alone failed to prevent death of *V. vulnificus*-infected mice, resulting in 100% mortality (Fig. 4B). These results indicate that this robust bactericidal activity observed is mainly attributable to the conjugation of HPA3PHis to AuNP-AptHis. The functional contribution of AuNP-AptHis is that it enables the efficient intracellular delivery of HPA3PHis to the host in addition to the increase in stability of HPA3PHis by protecting HPA3PHis from proteolysis. Another advantageous property of this system lies in its simplicity to be employed in a wide range of applications, where AuNP-AptHis conjugates can load any type of AMPs after His-tagging by simple mixing for few minutes at room temperature. Moreover, AuNP-AptHis conjugates were effective after a single administration, and thus, this longer lasting efficacy is a useful parameter that can help in avoiding short-term repetitive administration and reducing overall cost for therapy. Additionally, AuNP-AptHis-HPA3PHis did not exhibit any evident host toxicity (Figs 3B, C and 4B). Their efficient intracellular uptake and effective bactericidal activity would contribute to reduced dosing regimen, which renders minimizing possible toxic response. Earlier studies reported that AuNPs exhibit little toxicity, which is largely dependent on the particle size. 15-nm AuNPs used in this study have been known to be non-cytotoxic. Thus, these advantageous properties of AuNP-AptHis-derived AMP delivery system render it a promising antibacterial therapeutic candidate. To facilitate the development of AuNP-aptamer-AMP conjugate-based therapeutics, pre-clinical studies that prove the safety of AuNP-aptamer composites as well as the assessment of economic feasibility for large-scale production of AMPs are necessary. In addition, massive bactericidal screening of other candidate AMPs using HPA3PHis as a reference would contribute to development of more effective AuNP-aptamer-AMPs as antimicrobial therapeutics.

**Methods**

**Peptide synthesis and purification.** The peptides were synthesized using the 9-fluorenlymethoxycarbonyl (Fmoc) solid-phase method on Rink amide 4-methyl benzhydrylamine resin (Novabiochem; 0.55 mmol/g) with a Liberty microwave peptide synthesizer (CEM Co., Matthews, NC, USA). The peptides were prepared according to a previously described method.

**Antibacterial assay.** *V. vulnificus* MO6-24/O, *S. typhimurium* and *S. aureus* cells were cultured at 37°C in cation adjusted Mueller-Hinton broth (CA-MHB; BD Difco™, Detroit, MI, USA). MIC and MBC of each AMP was measured in microdilution assays according to Clinical and Laboratory Standards Institute (CLSI) recommendations. In brief, serial two-fold dilutions of 0.5–64 μM of each AMP were added to the CA-MHB media containing cultures of mid-log phase bacteria (5 × 10⁵ CFUs/ml). The cultures were grown for an additional 16–24 h at 37°C. After incubation, MIC and MBC were determined as the lowest concentrations of AMP inhibiting bacterial growth based on the OD₆₀₀ measurements or required to kill 99.9% of the test inoculum, respectively.
Circular dichroism (CD) spectroscopy. CD spectroscopy was performed using a Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan), with a quartz cell with a 1.0-mm path length. Spectra were measured at wavelengths ranging from 190 to 250 nm. CD spectral data of the peptide at a fixed concentration of 40 μM were recorded in Dulbecco’s phosphate-buffered solution (DPBS), 30 mM sodium dodecyl sulfate (SDS) solution, or 50% 2,2,2-trifluoroethanol (TFE) solution.

Membrane depolarization assay. The effect of HPA3PHis on bacterial membrane depolarization was measured using the membrane potential-sensitive dye DiSC3-5. V. vulnificus MO6-24/0 was cultured in LBS medium at 30 °C and then washed three times with buffer A (DPBS containing 20 mM glucose). The bacteria were resuspended to an OD600 of 0.05 in buffer A with 0.1 M KCl added and then DiSC3-5 was added to a final concentration of 0.1 μM. This mixture was incubated for 15 min to stabilize the level of fluorescence. Different concentrations, 0.25X, 0.5X, and 1X of the MIC of HPA3PHis and DPBS were added. The fluorescence was continuously measured for 10 min at an excitation wavelength of 622 nm and emission wavelength of 670 nm.

SYTOX Green uptake assay. V. vulnificus MO6-24/0 were grown in LBS medium at 30 °C, washed, and then resuspended in DPBS to 2 × 10^8 CFU/ml. After adding 1 μM SYTOX Green, the bacteria were incubated for 15 min in the dark. HPA3PHis was added at 0.25X, 0.5X, and 1X of the MIC to the bacterial suspensions. Changes in fluorescence as a result of the interactions between SYTOX Green and bacterial DNA were measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm.

Flow cytometric analysis of bacterial membrane disruption. The disruption of V. vulnificus membrane by the peptide was analyzed by flow cytometry. V. vulnificus MO6-24/0 were cultured in LBS medium and harvested by centrifugation at 6000 g for 10 min. After washing with DPBS, the bacteria were resuspended to an OD600 of 0.2, and then treated with 0.5X, 1X, or 2X of the MIC of HPA3PHis and DPBS for 5 min at 30 °C with shaking at 140 rpm. After the treatment, the bacteria were harvested and incubated with 10 μg/ml of PI for 20 min. Bacterial cell staining with PI was measured using a CytoFLEX flow cytometer (Beckman, Brea, CA, USA).

Preparation of AuNP-Apt conjugates and AuNP-Apt-AMP complex. Citrate-stabilized AuNPs (15 nm diameter) were purchased from BBI Life Science (UK). His-tag DNA aptamers were conjugated to AuNPs by a previously described method and the AuNP-Apt-AMP complex was prepared according to a previously described procedure. The size and ζ-potential of AuNP-Apt-AMP were investigated using a dynamic light scattering spectrophotometer (ELSZ-1000; Otsuka Electronic Korea, Seongnam, Korea).

Binding capacity assay. Binding capacity assay between AMPs and AuNP-Apt conjugates was performed according to a previously described method.

Mammalian cell culture. HeLa cells were cultured in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Welgene) and 1% penicillin–streptomycin (Welgene) at 37 °C/5% CO2.

Visualization of peptide delivered by AuNP-Apt conjugates to mammalian cells. HeLa cells (5 × 10^4/well) were seeded on 10-mm cover slips and incubated with the AuNP-Apt-AMP complex and control reagents for 10 min. The final concentration of AMP and AuNP-Apt conjugate was 0.5 μM and 1 nM, respectively. The cells were fixed and immunostained as previously described.

Cytotoxicity assay. The cytotoxicity of AMP against HeLa cells was determined as previously described.

Viable intracellular bacterial count assay. HeLa cells (5 × 10^4/well) were cultured for 18–24 h in 24-well culture dishes. HeLa cells were infected with V. vulnificus MO6-24/0 cells for 30 min at a multiplicity of infection (MOI) of 20. The infected cells were washed with DMEM containing 50 mg/ml gentamicin (Gibco, Invitrogen, Carlsbad, CA, USA) and PBS. Then, the cells were treated with AuNP-Apt-AMP complex or control reagents for 30 min. The HeLa cells were washed with PBS and lysed with PBS containing 1% (v/v) Triton X-100, and intracellular bacteria were plated onto LBS plates to determine the numbers of CFUs.

V. vulnificus-infected mammalian cell viability assay. HeLa cells (1 × 10^4/well) were seeded in 96-well culture dishes. After 18–24 h, the HeLa cells were treated with buffer or infected with V. vulnificus for 30 min at a 1:20 MOI. The cells were washed with 50 mg/ml gentamicin (Gibco, Invitrogen). Next, the cells were incubated for 0, 0.5, 1, 2, and 4 h with AuNP-Apt-AMP complex or control reagents. The infected cells were harvested by trypsin-ethylenediaminetetraacetic acid, and viable HeLa cells were measured by trypan blue exclusion assay.

Animals. Animal studies were performed on specific-pathogen-free, 6-week-old, female ICR mice (DBL, Eumsung, Korea) weighing 18–20 g. Mice were housed in a room under a 12 h reversed light cycle, humidity of 30–40%, and temperature of 22 ± 1 °C. The experiment protocols were approved by the Chung-Ang University Support Center for Animal Experiments, and all methods were performed in accordance with the guidelines and regulations.

Hemolysis assay. Fresh red blood cells (RBCs) from ICR mice (DBL) were collected and washed three times with PBS. The various concentrations (0.1 to 10 μM) of peptides were incubated with 8% (v/v) washed RBCs for 1 h at 37 °C. The samples were centrifuged at 800 × g for 10 min, and the absorbance of the supernatants was measured at 540 nm by microplate reader (ASYS UVM 340; Biochrom, Cambridge, UK). As a negative or a positive control, PBS or 0.1% (v/v) Triton X-100 were used, respectively. The percentage of hemolysis was calculated by the following equation: % hemolysis = [(A_{peptide}−A_{PBS})/(A_{0.1% Triton X-100−A_{PBS}})] × 100.
**In vivo mice experiment.** V. vulnificus MO6-24/O cells grown overnight in LBS medium at 30°C were inoculated and subsequently harvested when bacterial growth reached mid-log phase and washed with PBS. Mice were deprived of food and water for 4–18 h before inoculation. Then, mice were subcutaneously injected with 100 µl of PBS containing 1–5 × 10^7 CFU of V. vulnificus. Two hours after bacterial challenge, V. vulnificus-infected mice were intravenously injected with AuNP-AptHis-HPA3PHis complex and control reagents. Mice were monitored for 120 h after injection and immediately killed when they were moribund.

**Viable bacterial count in murine organs.** At 18 h after bacterial challenge, mice of each group were sacrificed and the spleen, inguinal lymph node, and liver were removed. The organs were weighed, homogenized, and dissolved in LBS. The homogenates were plated on LBS agar and the plates were incubated at 30°C overnight. Results were expressed as the number of CFUs/g.

**Statistical analysis.** Data were presented as means ± SEM. Student’s t-test (Excel; Microsoft Corp., Redmond, WA, USA) was used, and p < 0.05 was considered statistically significant.

**Availability of materials and data.** The authors declare no restrictions on the availability of materials or information.

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Additional Information
Competing Interests: The authors declare that they have no competing interests.

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