

Biodegradable Peptide Polymers as Alternatives to Antibiotics Used in Aquaculture

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Abstract

The pressure of antimicrobial resistance has forced many countries to reduce or even prohibit the use of antibiotics in feed. Therefore, it is in urgent need to develop alternatives to antibiotics to control infectious diseases in feed and aquaculture. To address this long-lasting challenge, we prepared peptide polymers that display potent and broad-spectrum activity against common pathogenic bacteria in aquaculture, low hemolysis and low cytotoxicity, and doesn't induce bacteria to develop resistance or cross resistance to antibiotics. The optimal peptide polymer demonstrates strong *in vivo* therapeutic potential in an adult zebrafish infection model. Moreover, the optimal peptide polymer is biodegradable by enzyme into single amino acids and dipeptides to totally lose antibacterial activity and, therefore, will not cause antimicrobial selective pressure. Our study suggests that peptide polymers are promising alternatives to antibiotics in aquaculture and open new avenues to address the global challenge of antimicrobial resistance.

Introduction

With the increasing demand of animal protein nutrition, intensive animal production is growing very fast. To meet the production volume requirement in aquaculture, poultry and livestock, Food and Drug Administration (FDA) approved the use of antibiotics in feed in 1950s¹. Since then, food animal industry more and more relies on the use of antibiotics^{2, 3}. The global consumption of antimicrobials, majorly antibiotics, in food animals was 131,109 tons in 2013, and estimated to be 200,235 tons by 2030⁴. However, antibiotic residues in food animals

will be transmitted to the human through the food chain, destroying human microbiome and inducing bacteria to develop drug resistance (Fig 1a)⁵⁻⁷. It is also noteworthy that many antibiotics are eventually discharged into the environment especially for aquaculture⁸⁻¹¹. A recent investigation reveals a high level content of antibiotics, such as sulfonamides, in the surface water in different countries, which will cause serious antimicrobial resistance problems (Fig 1b)¹²⁻¹⁴. Antibiotic residues in water will also spread in the ecosystem, further exerting selective pressure on environmental microorganisms and accelerating the occurrence and evolution of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs), which will cause a great potential threat to animal and human health (Fig 1a). It is estimated that drug-resistant bacteria will cause about 10 million deaths per year by 2050, if no effective action is taken immediately¹⁵. As early as 2006, the European Union announced a total ban on the use of antibiotics in feed. Subsequently, the United States, Japan, China and other countries successively promulgated various policies to reduce or even prohibit the use of antibiotics in feed¹⁶⁻²⁰. It is a consensus that it is imperative to prohibit the massive use of antibiotics all over the world in food animals, including aquaculture.

In order to replace the use of antibiotics in aquaculture, multiple strategies have been actively explored such as vaccines^{21, 22}, biological control²³⁻²⁶, antimicrobial peptides^{27, 28}, medicinal herbs extract^{29, 30} and nanoparticles^{31, 32}. However, none of these strategies meet the requirement for antibiotic substitution up to date. It is highly desired that antibiotic substitutes in aquaculture have broad spectrum activity, potent activity against drug-resistant bacteria, low cost in production, excellent biocompatibility, and most critically fully biodegradability to give degraded products that completely lose antibacterial activity and will not cause antimicrobial selective pressure in organisms and the environments. It's worth mentioning that degradable antibacterial polymers have been studied, such as the acrylate copolymers degradable in aqueous solution into lower molecular weight oligomers that have decreased antibacterial activity³³, antibacterial polyionenes degradable rapidly at pH 10 and slowly under neutral or acidic conditions³⁴, and antibacterial polycarbonate degradable under physiological conditions³⁵. However, degradable antimicrobials as need for antibiotic substitution in aquaculture refer to antimicrobials that are fully degradable to very basic components such as single amino acid in relative short period, rather than degradable to large fragments because large fragments can still have moderate antibacterial activity to cause antimicrobial resistance and antimicrobial selective pressure. Colistin is a representative host defense peptide (HDP) that has ever been used as an animal feed additive for many years. At present, colistin has been banned as a feed additive in China and Thailand because colistin cannot be completely degraded by enzymes existing in the environment and animals, and the use of colistin in feed is found to accelerate the dissemination of the first mobile mechanism of colistin resistance, termed MCR-1, in

animals and then in human beings^{17, 36, 37}. Up to date, no successfully replacement of antibiotics is available in aquaculture.

Synthetic mimics of HDP have shown broad-spectrum antibacterial activity³⁸⁻⁵⁰, and can be promising candidates of antibiotic substitutes if ideally degradable into individual residues without antimicrobial activity. In this work, we design antibacterial peptide polymers that are composed of lysine and serine in different proportions and are easily synthesized in minutes via the fast ring-opening polymerization on amino acid *N*-carboxyanhydride (NCA) using lithium hexamethyldisilazide (LiHMDS) as the initiator (Fig 1c)^{51,52}. Among these peptide polymers, α -Poly-L-lysine composed entirely of lysine is well known to have antibacterial activity but very limited application as an antimicrobial agent due to its significant cytotoxicity. Therefore, we replace part of the positively charged lysine residue with the hydrophilic serine residue to reduce the cytotoxicity of resulting peptide polymers and the acute toxicity to zebrafish, while having the minimal impact on antibacterial activity. The optimal peptide polymers meet all the desired properties of antibiotic substitutes in aquaculture, such as exhibiting excellent antibacterial activity against common pathogenic bacteria in aquaculture and significantly improving the survival rate of zebrafish after *Vibrio anguillarum* infection, and do not induce bacteria to develop antibacterial resistance. Moreover, the peptide polymers are biodegradable into individual amino acids and dipeptides that have no antibacterial activity and, therefore, will not cause antimicrobial resistance and antimicrobial selective pressure in organisms and the environment. Aforementioned advantages, all together, imply potential application of these peptide polymers as substituents of antibiotics in aquaculture.

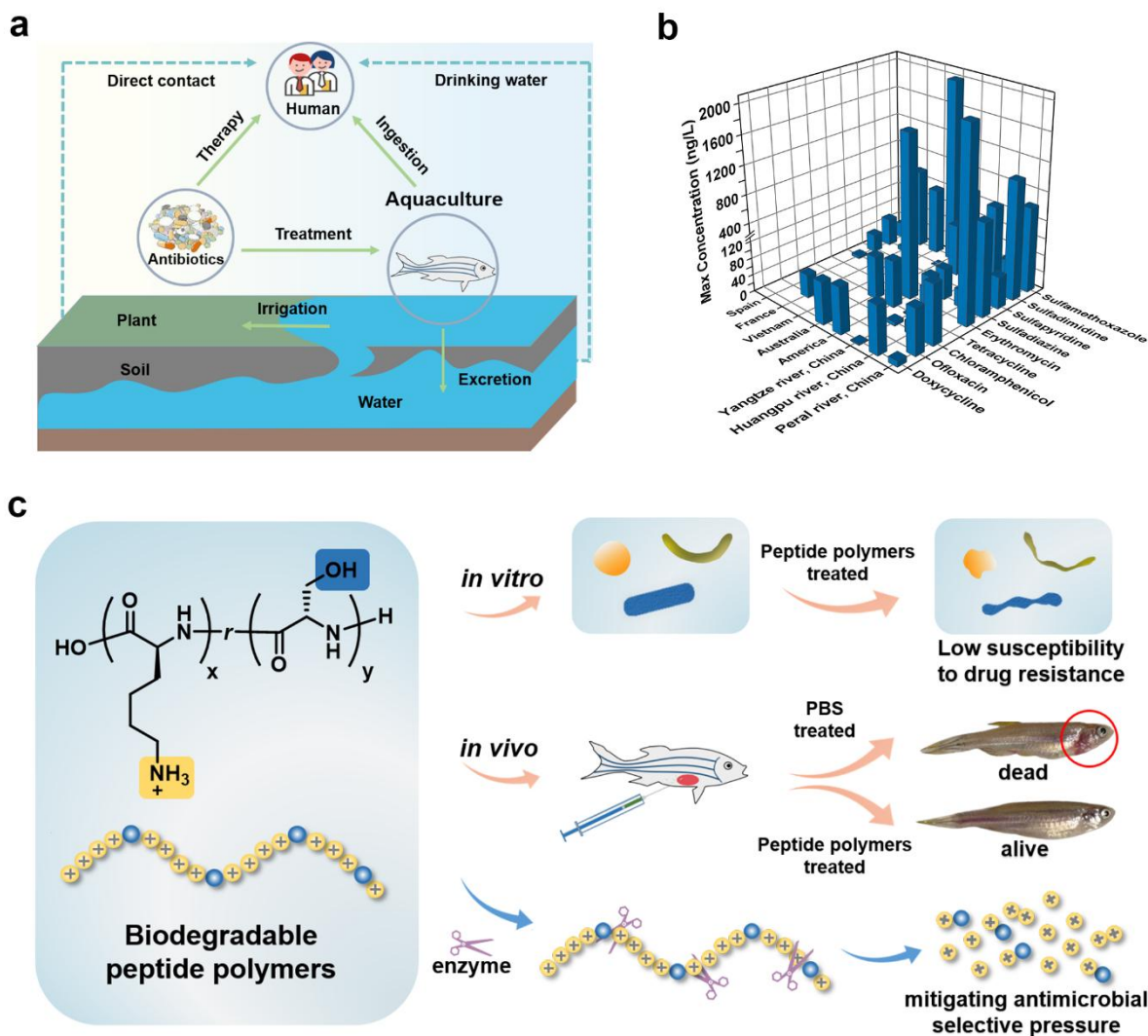


Fig 1. a) The massive use of antibiotic in aquaculture results in antibiotic contamination in the environment, antimicrobial resistance and antimicrobial selective pressure. b) Maximum concentration of antibiotic residues in surface water of various countries. c) Biodegradable peptide polymers as alternatives to antibiotics by displaying potent antibacterial activity *in vitro* and *in vivo*, low susceptibility to induce drug resistance and antimicrobial selective pressure.

Results

Peptide polymers (K_xS_y) were synthesized from the ring-opening polymerization on a mixture of $N\epsilon$ -*tert*-butyloxycarbonyl-L-lysine (Boc-L-Lys) NCA (Fig S1) and *O*-(*tert*-butyl)-L-serine (*t*Bu-L-Ser) NCA (Fig S2) at variable ratios using LiHMDS as the initiator, followed by treatment with trifluoroacetic acid to remove protecting groups (Fig 2a). The obtained peptide polymers have narrow dispersities ($\mathcal{D} = 1.1-1.2$) and similar chain length ($DP = 19-22$) according to the characterization by gel permeation chromatography (GPC) (Fig 2b, GPC traces in Fig 2c and Fig S9-13). By modifying the C-terminus of peptide polymers with *tert*-butyl benzylamine and analyzing with nuclear magnetic resonance (NMR), we further determined the DP (similar to the result from GPC analysis) and the ratio of lysine to serine in the final peptide polymers (Fig 2b, 2d and Fig S3-7).

Antibacterial studies showed that three peptide polymers (K₂₁ (α -Poly-L-lysine), K₁₉S₂, K₁₈S₄) had potent activities against multiple food animal-associated pathogens, including *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) as representative common pathogenic bacteria, *Vibrio alginolyticus* (*V. alginolyticus*), *Vibrio anguillarum* (*V. anguillarum*) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) as common pathogenic bacteria in aquaculture, *Salmonella pullorum* (*S. pullorum*), *Salmonella typhimurium* (*S. typhimurium*) and *Salmonella anatum* (*S. anatum*) as common pathogenic bacteria in poultry, *Mannheimia haemolytica* (*M. haemolytica*), *Streptococcus suis* (*S. suis*) and *Salmonella choleraesuis* (*S. choleraesuis*) as common pathogenic bacteria in livestock (Fig 2e). The minimum inhibitory concentration (MIC) of three most active peptide polymers (K₂₁, K₁₉S₂, K₁₈S₄) were in the range of 0.78-50 μ g/mL. In addition, all these peptide polymers showed negligible (less than 0.2%) hemolysis on human red blood cells at a peptide polymer concentration up to 2000 μ g/mL (Fig 2f). Peptide polymers K₁₈S₄, K₁₆S₆ and K₁₃S₈ all displayed low cytotoxicity against mammalian cells even at a peptide polymer concentration of 200 μ g/mL, using COS-7 as a model (Fig 2g). K₁₈S₄ displayed the best overall performance and was selected as the optimal peptide polymer for further studies.

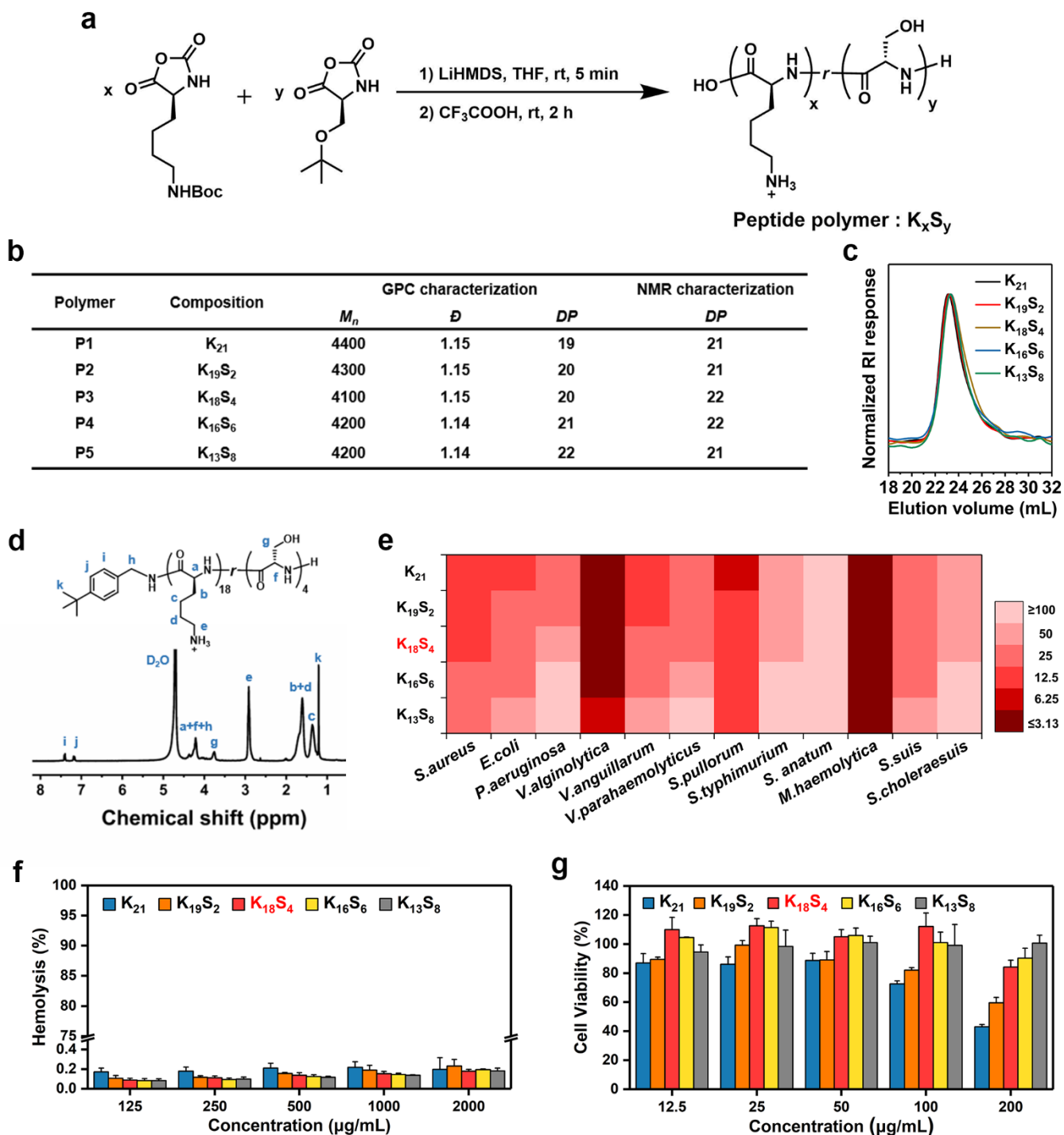


Fig 2. a) Synthesis of peptide polymers. b) GPC characterization of peptide polymers at the sidechain NHBoc protected stage using DMF as the mobile phase. M_n is the number average molecular weight. \mathcal{D} is the dispersity index; DP is the degree of polymerization. NMR characterization was conducted on deprotected peptide polymers bearing N-terminal *tert*-butyl-benzylamine. c) GPC trace of peptide polymers. d) Representative NMR spectrum of peptide polymers. e) Antibacterial activity of peptide polymers against common pathogenic bacteria in aquaculture, poultry and livestock. f) Hemolysis of peptide polymers on hRBCs, all data were presented as the mean \pm SD (n = 3). g) Cytotoxicity of peptide polymers on COS-7 cells, all data were presented as the mean \pm SD (n = 3).

We continued to examine the antibacterial activity of $K_{18}S_4$ on common pathogenic bacteria in aquaculture,

using aquaculture antibiotics thiamphenicol and sulfadimethoxine for comparison (Fig 3a). K₁₈S₄ displayed potent activities against all these pathogens with MIC in the range of 3.13-25 µg/mL; K₁₈S₄ was also bactericidal with minimum bactericidal concentration (MBC) identical to its MIC for most bacteria. The overall antibacterial activity of K₁₈S₄ was superior to thiamphenicol and sulfadimethoxine, especially for MBC. We also examined the activity of K₁₈S₄ against fungi, though fungi are not major pathogens in aquaculture. K₁₈S₄ displayed potent activity against *C. neoformans* (MIC=1.56-3.13 µg/mL for all three strains) comparable to amphotericin B, but no obvious activity against *C. albicans* at a concentration up to 200 µg/mL (Table S1). In the acute toxicity test on adult zebrafish, the LC₅₀ (the median lethal concentration) value of K₁₈S₄ was 98.14 µg/mL (Fig 3b, Table S2), which was much higher than the MIC and MBC values of K₁₈S₄ and indicated selective antibacterial properties of K₁₈S₄. Compared with α-Poly-L-lysine (LC₅₀ of K₂₁: 19.61 µg/mL), the acute toxicity of K₁₈S₄ to adult zebrafish was also significantly reduced (Table S2). A further time-killing kinetics study on K₁₈S₄ against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* and *V. anguillarum* (a representative of the common pathogenic bacteria in aquaculture) revealed a quick killing of bacteria at 2×MIC concentration of K₁₈S₄, such as the complete killing of *V. anguillarum* within 30 minutes (Fig 3c). The bacteria-killing of K₁₈S₄ was much faster than that of conventional antibiotic thiamphenicol, indicating advantages of K₁₈S₄ in treating aquaculture-encountered bacterial infections.

To examine if bacteria can develop resistance to the peptide polymers, we treated Gram-positive *S. aureus* and Gram-negative *E. coli* with K₁₈S₄ at 0.5×MBC concentration continuously, and found that none of the bacteria developed resistance against K₁₈S₄ even after continuous treatment for 888 and 1152 generations of *S. aureus* and *E. coli*, respectively (Fig 3d, Fig S14-15). In sharp contrast, *S. aureus* and *E. coli* developed resistance toward antibiotics quickly, displaying 824-fold resistance against norfloxacin for *S. aureus* and 64-fold resistance against ampicillin for *E. coli*. Moreover, cross-resistance was observed on the norfloxacin-treated 264th generation *S. aureus* against gentamycin and thiamphenicol, and on the ampicillin-treated 504th generation *E. coli* against penicillin and kanamycin (Fig 3e), which reflects the serious problem of cross-resistance among antibiotics used in aquaculture. Fortunately, cross-resistance was not observed on K₁₈S₄-treated 888th generation *S. aureus* and 1152th generation *E. coli* against antibiotics commonly used in aquaculture (Fig 3d), which indicates a superior advantage of peptide polymers as potential substitutes of antibiotics in aquaculture, minimizing the antimicrobial resistance in the environments.

Even if an antibacterial agent, such as K₁₈S₄, shows low tendency to induce antimicrobial resistance, full degradability is still highly desired for antibiotic substitutes in aquaculture. The massive use and subsequent

discharge of the antimicrobial agents to the environment may cause antimicrobial selective pressure and disruption of balance of the complicated microbial system in the environment, which is a big concern in massive use of antibiotics in aquaculture. Therefore, an ideal substitution of antibiotics in aquaculture should be fully degradable to individual residues and thus lose antibacterial activity completely after degradation. $K_{18}S_4$ was completely degraded within 6 hours in the presence of protease type XXIII from *Aspergillus oryzae*, as confirmed by NMR analysis on the disappearance of the characteristic peaks for α -CH group on the peptide backbone at 4.27 ppm and 3.82 ppm (Fig 3f). The obtained mixture after 6 hours was analyzed by HRESI-MS and was identified as individual amino acid and dipeptide (Fig S16), which have no antibacterial activity, indicating that biodegradable $K_{18}S_4$ can greatly reduce the antibacterial resistance and antimicrobial selective pressure in the environments (Fig 3g). In addition to enzymes produced by microorganisms, $K_{18}S_4$ can also be degraded by trypsin and chymotrypsin that are widely distributed in the digestive tract. Depending on the enzyme for the treatment the degradation products were dipeptides or tripeptides, all of which have no antibacterial activity (Fig S17c-e). ϵ -Poly-L-lysine as a FDA approved antibacterial food additive showed slightly better antibacterial activity than $K_{18}S_4$, but slower bacteria-killing (Fig S17a-b). Although ϵ -Poly-L-lysine was degradable by specific enzymes produced by few specific types of microorganisms⁵³, it was highly stable in the presence of many common enzymes such as trypsin and chymotrypsin, and maintained the original antibacterial activity after treatment with trypsin and chymotrypsin (Fig S17c-d). Therefore, ϵ -Poly-L-lysine is not a suitable antibiotic substitute in aquaculture.

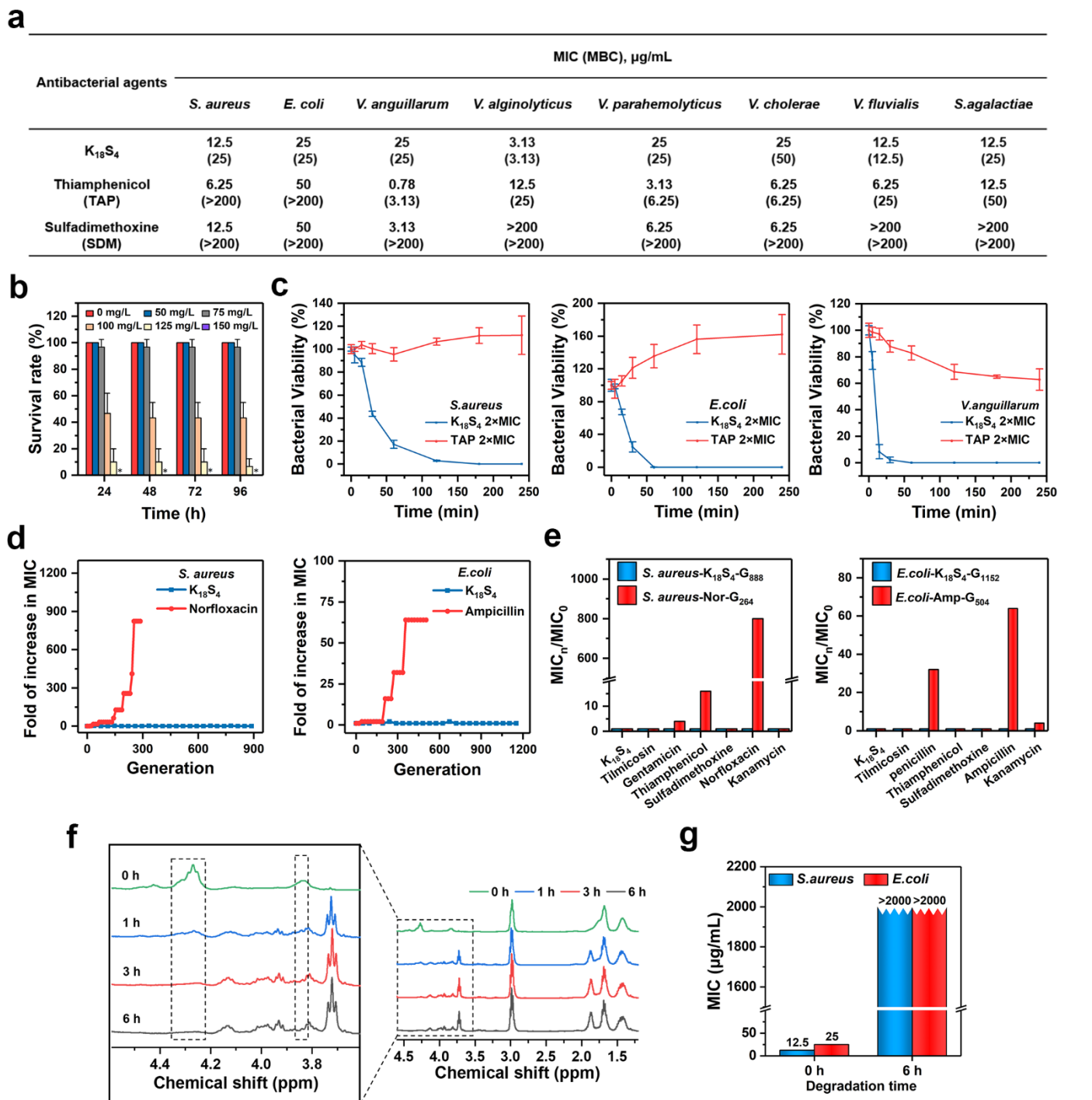


Fig 3. a) MIC and MBC values of K_{18}S_4 and antibiotics against common pathogenic bacteria in aquaculture. MIC: the minimum inhibitory concentration, MBC: the minimum bactericidal concentration. b) Acute toxicity of K_{18}S_4 on adult zebrafish, * means the survival rate is 0. c) Killing kinetics of K_{18}S_4 against *S. aureus*, *E. coli* and *V. anguillarum* at $2\times\text{MIC}$. d) Antimicrobial resistance assay on *S. aureus* and *E. coli* treated continuously with K_{18}S_4 or antibiotics. e) The changes of MIC values for antibiotics against *S. aureus*- K_{18}S_4 -G₈₈₈, *S. aureus*-norfloxacin-G₂₆₄, *E. coli*- K_{18}S_4 -G₁₁₅₂ and *E. coli*-ampicillin-G₅₀₄, which are treated with K_{18}S_4 or norfloxacin for 888, 264, 1152, and 504 generations, respectively, at $0.5\times\text{MBC}$. f) NMR spectrum of K_{18}S_4 over time in the presence of protease XXIII from *Aspergillus oryzae* for biodegradability study. g) The MIC value change of K_{18}S_4 before and after enzymatic degradation.

We also synthesized a morpholino naphthalimide fluorophore-tethered green fluorescent peptide polymer,

Dye-K₁₈S₄, for antimicrobial mechanism study using time-lapse fluorescent confocal imaging (Fig 4, Fig S8). After *S. aureus* was incubated with Dye-K₁₈S₄ and propidium iodide (PI, red fluorescence) for around 450s, a small amount of Dye-K₁₈S₄ began to accumulate in the cytoplasm without enrichment on the cell membrane beforehand (Fig 4b). At about 1260s, a burst of Dye-K₁₈S₄ and PI into the cytoplasm indicated critical damages on the cell membrane of *S. aureus* (Fig 4a, 4b). The observed membrane damage was at the late stage of the bacteria-polymer interaction when *S. aureus* was killed by the peptide polymer. Since the Dye-K₁₈S₄ was not enriched on the cell membrane, the membrane damage may not be caused by Dye-K₁₈S₄ directly from the outside of the bacteria. Fluorescence intensity analysis across *S. aureus* cells, at 120s and 1500s after incubation, echoed above observation that Dye-K₁₈S₄ did not enrich either on the cell membrane or in the cytoplasm at an early stage, but enriched inside the cell at a late stage (Figure 4c). Interestingly, incubation of *E. coli* and *V. anguillarum* with Dye-K₁₈S₄ and PI both led to immediate enrichment of Dye-K₁₈S₄ on the cell membrane, and then obvious enrichment of Dye-K₁₈S₄ and PI into the bacteria after incubation for a longer time (Fig 4d, 4g). Fluorescence intensity analysis across *E. coli* and *V. anguillarum*, both echoed the two stages that Dye-K₁₈S₄ firstly accumulated on the cell membrane of *E. coli* and *V. anguillarum*, and then entered the cells (Fig 4e-f, 4h-i).

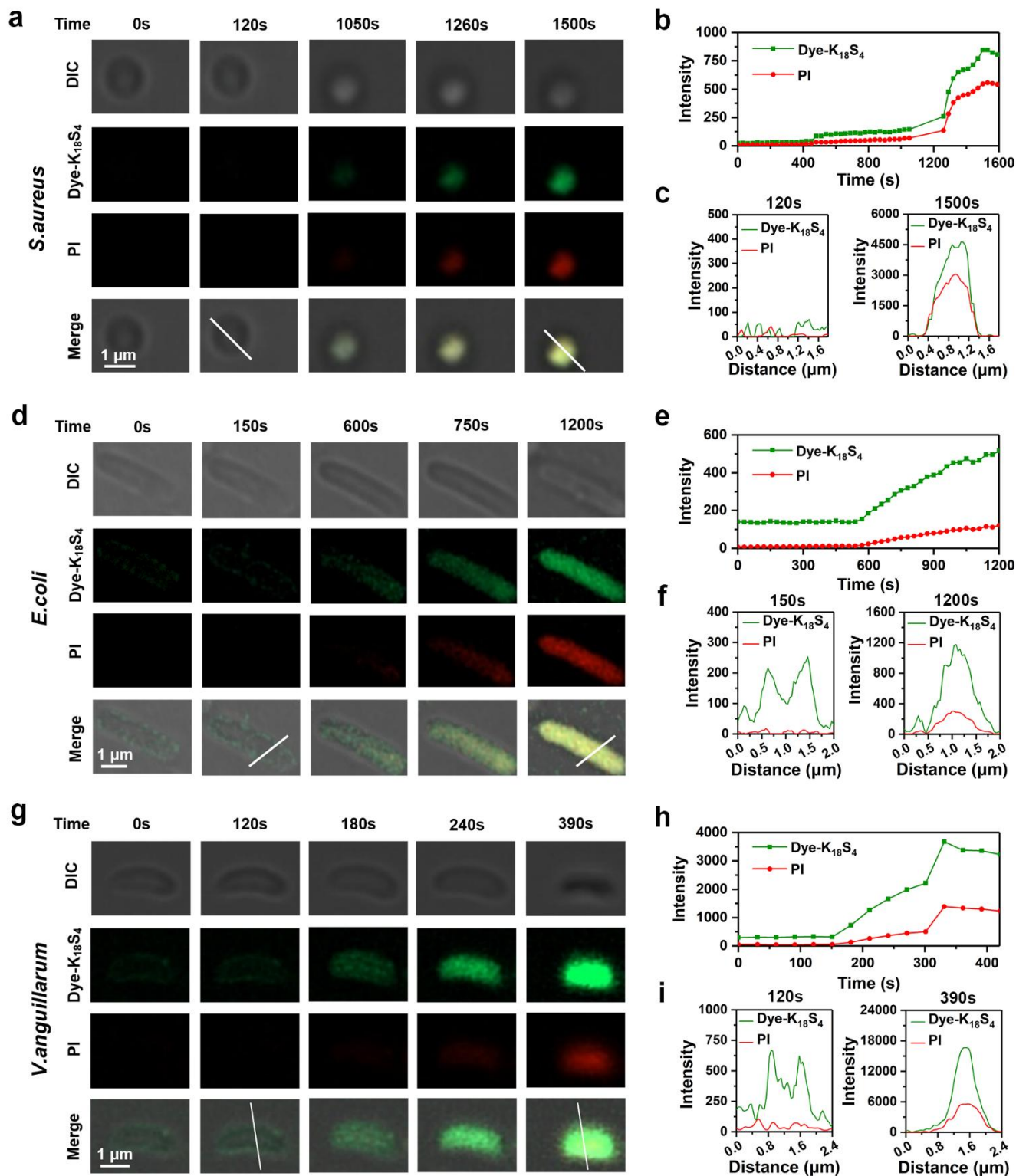


Fig 4. Time-lapse fluorescent confocal imaging of a) *S. aureus*, d) *E. coli* and g) *V. anguillarum* incubated with Dye-K₁₈S₄ (5×MIC) in the presence of propidium iodide. Fluorescence intensity of Dye-K₁₈S₄ and propidium iodide versus time in ROI (region of interest) of b) *S. aureus*, e) *E. coli* and h) *V. anguillarum*. Fluorescence intensity profiles of Dye-K₁₈S₄ and propidium iodide across c) *S. aureus* at 120s and 1556s, f) *E. coli* at 150s and 1200s and i) *V. anguillarum* at 120s and 390s.

In further outer membrane permeability assays at the early stage of bacteria-polymer interaction, K₁₈S₄ was

found to cause significant permeability changes to the outer membranes of *E. coli* and *V. anguillarum*, similar to polymyxin B (Fig 5a, 5b). Adding exogenous LPS, a main component of the outer membrane, to K₁₈S₄ showed a LPS dose-dependent antibacterial activity against *E. coli* and *V. anguillarum* (Fig 5c). These results are consistent to aforementioned observation of K₁₈S₄ enrichment on the outer membrane of *E. coli* and *V. anguillarum* (Fig 4d, 4g). Therefore, the antibacterial mechanism of K₁₈S₄ against Gram-negative *E. coli* and *V. anguillarum* may be explained by the strong interaction on cell membrane. Interestingly, K₁₈S₄ only caused mild depolarization on cytoplasmic membrane of Gram-positive *S. aureus* (Fig 5d) at the early stage of bacteria-polymer interaction, which may not be enough to pose an adequate threat. So we speculated that there may still be other antibacterial mechanisms.

Oxidative stress is involved in the antibacterial process of a variety of cationic antibacterial peptides^{54, 55}. Membrane perturbation and DNA binding are both common ways to cause the increase of intracellular reactive oxygen species (ROS) level⁵⁶. In addition to the observed mild membrane polarization at the early stage of bacteria-polymer interaction, K₁₈S₄ could strongly bind to DNA at even low N:P ratio of 1:1, which suggests a possible generation of ROS within bacteria after K₁₈S₄ treatment (Fig S18). So, we used 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) as the probe to determine the ROS level in bacteria. After K₁₈S₄ treatment on bacteria for 1 hour, the ROS level of *S. aureus*, *E. coli* and *V. anguillarum* all increased significantly compared to normal bacterial cells. (Fig 5e-g). By adding ROS scavenger N-acetyl-l-cysteine (NAC) to inhibit the increase of ROS level in bacteria, the MBC of K₁₈S₄ increased by only 2 times against *E. coli* and *V. anguillarum*, but 8 times (changing from 25 µg/mL to 200 µg/mL) against *S. aureus* (Fig 5h). This result indicated that ROS played an important role in the antimicrobial process of K₁₈S₄ against *S. aureus*. Among the possible ROS related antibacterial mechanisms, the increase of intracellular ROS level after the peptide polymer entered the bacteria could cause the damage of bacterial membrane due to membrane lipid peroxidation^{57, 58}. Characterization on the morphological changes of bacteria by transmission electron microscope (TEM) and scanning electron microscope (SEM) showed membrane lysis and cytoplasm outflow of *S. aureus*, *E. coli* and *V. anguillarum* after treatment with K₁₈S₄ (Fig 5i, 5j). Altogether, although K₁₈S₄ has a certain interaction with the cell membranes of Gram-positive and Gram-negative bacteria, the antimicrobial mechanisms of K₁₈S₄ against Gram-positive and Gram-negative bacteria are different. We speculate that K₁₈S₄ has a mild interaction with the bacterial membrane at the early stage of bacteria-polymer interaction during K₁₈S₄ entering *S. aureus*; then membrane depolarization and subsequent strong interaction between K₁₈S₄ and DNA induce an increase in intracellular ROS level, which cause the peroxidation of cell membrane and ultimately destruction of the bacterial membrane. For Gram-negative

bacteria, we speculate that $K_{18}S_4$ first binds with LPS to change the permeability of the outer membrane, and could stick between the outer membrane and the inner membrane before $K_{18}S_4$ disrupts the bacterial membrane, similar to precedent reports on antibacterial peptides and their mimics^{59, 60}. Finally, the bacterial membrane is destroyed by the strong interaction between $K_{18}S_4$ and bacterial membrane, resulting in death of Gram-negative bacteria.

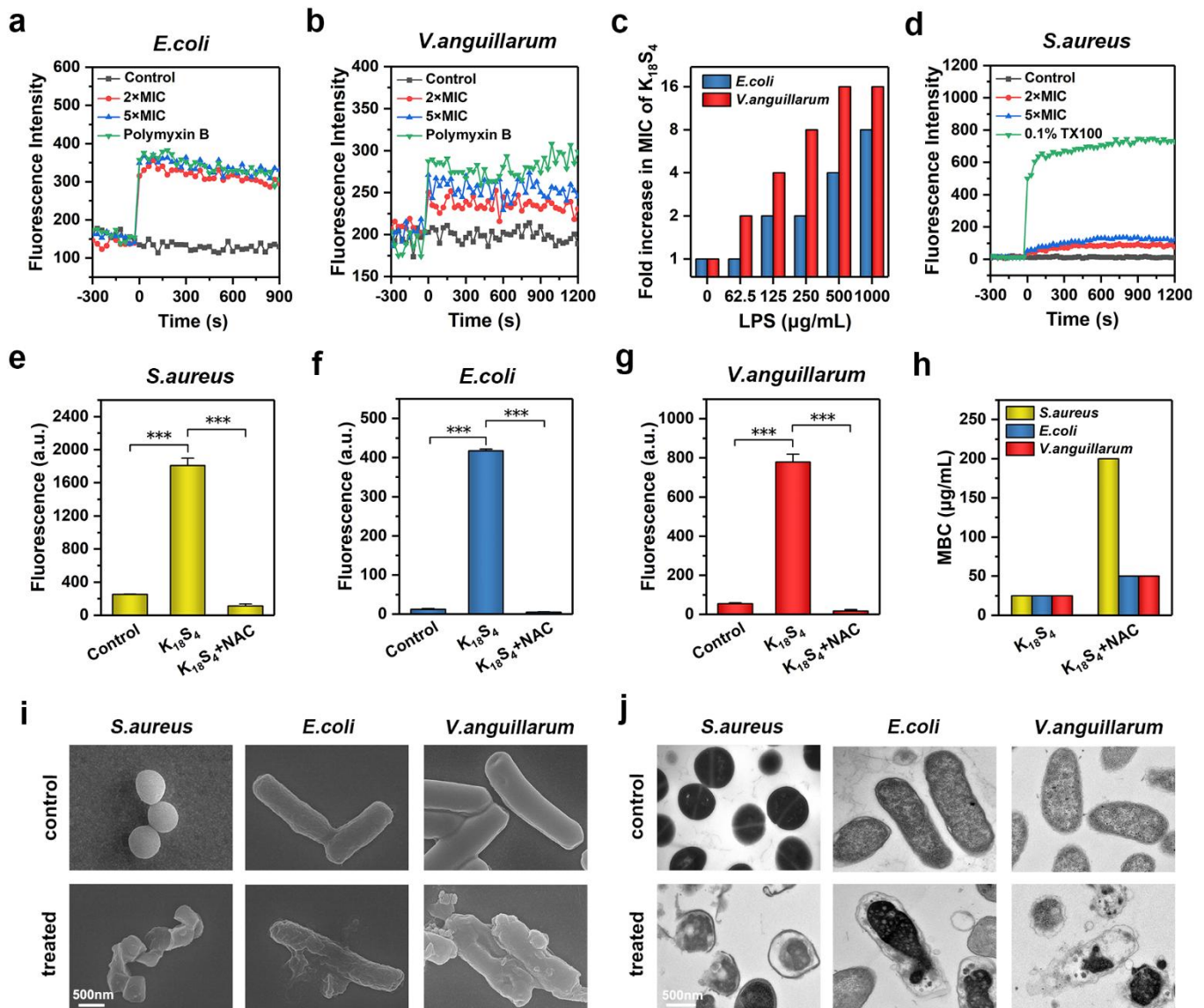


Fig 5. Outer membrane permeability of a) *E. coli* and b) *V. anguillarum* induced by $K_{18}S_4$. c) Exogenous addition of purified LPS from *E. coli* inhibited the antibacterial activity of $K_{18}S_4$ against Gram-negative bacteria *E. coli* and *V. anguillarum* in a dose-dependent manner. d) Cytoplasmic membrane depolarization of *S. aureus* induced by $K_{18}S_4$. Intracellular ROS intensity produced by e) *S. aureus*, f) *E. coli* and g) *V. anguillarum* in the presence of PBS, $K_{18}S_4$ (5×MIC), and the mixture of $K_{18}S_4$ (5×MIC) and ROS quencher NAC (7.5 mM). *** p < 0.001. h) The MBC values of $K_{18}S_4$ against *S. aureus*, *E. coli* and *V. anguillarum* with or without NAC (7.5 mM). i) SEM and j) TEM characterization on *S. aureus*, *E. coli* and *V. anguillarum* with or without $K_{18}S_4$ incubation.

Encouraged by the potent and broad-spectrum *in vitro* antibacterial activity of K₁₈S₄, we continued to evaluate the therapeutic potential of K₁₈S₄ *in vivo* using an adult zebrafish infection model. Administration of antimicrobial agents by direct injection has proven as a rapid and effective method in aquaculture and has been recognized as a practical drug administration strategy^{61, 62}. So, zebrafish were intraperitoneally injected with *V. anguillarum* (4×10⁶ CFU (colony forming unit)), and a single dose of K₁₈S₄ (20 mg/kg) was administered intraperitoneally at 30 minutes post infection. Then the survival rate of zebrafish was recorded every 12 h for 7 days (Fig 6a). Administration of K₁₈S₄ increased the survival rate of infected zebrafish from 16.8% in the PBS-treated control group to 83.3% (Fig 6b). We also observed substantially reduced bacterial load in liver, spleen and kidney after administration of K₁₈S₄ (Fig 6c). The infected zebrafish treated with PBS showed obvious symptoms of hemorrhagic sepsis; whereas, zebrafish treated with K₁₈S₄ were similar to healthy zebrafish and showed no symptoms of hemorrhagic sepsis (Fig 6d). Furthermore, intraperitoneal administration of K₁₈S₄ did not affect the liver and kidney function of healthy zebrafish (Fig 6e). These results altogether suggested that K₁₈S₄ has promising therapeutic potential and favorable biosafety *in vivo* as potential antibacterial substitute of antibiotics in aquaculture.

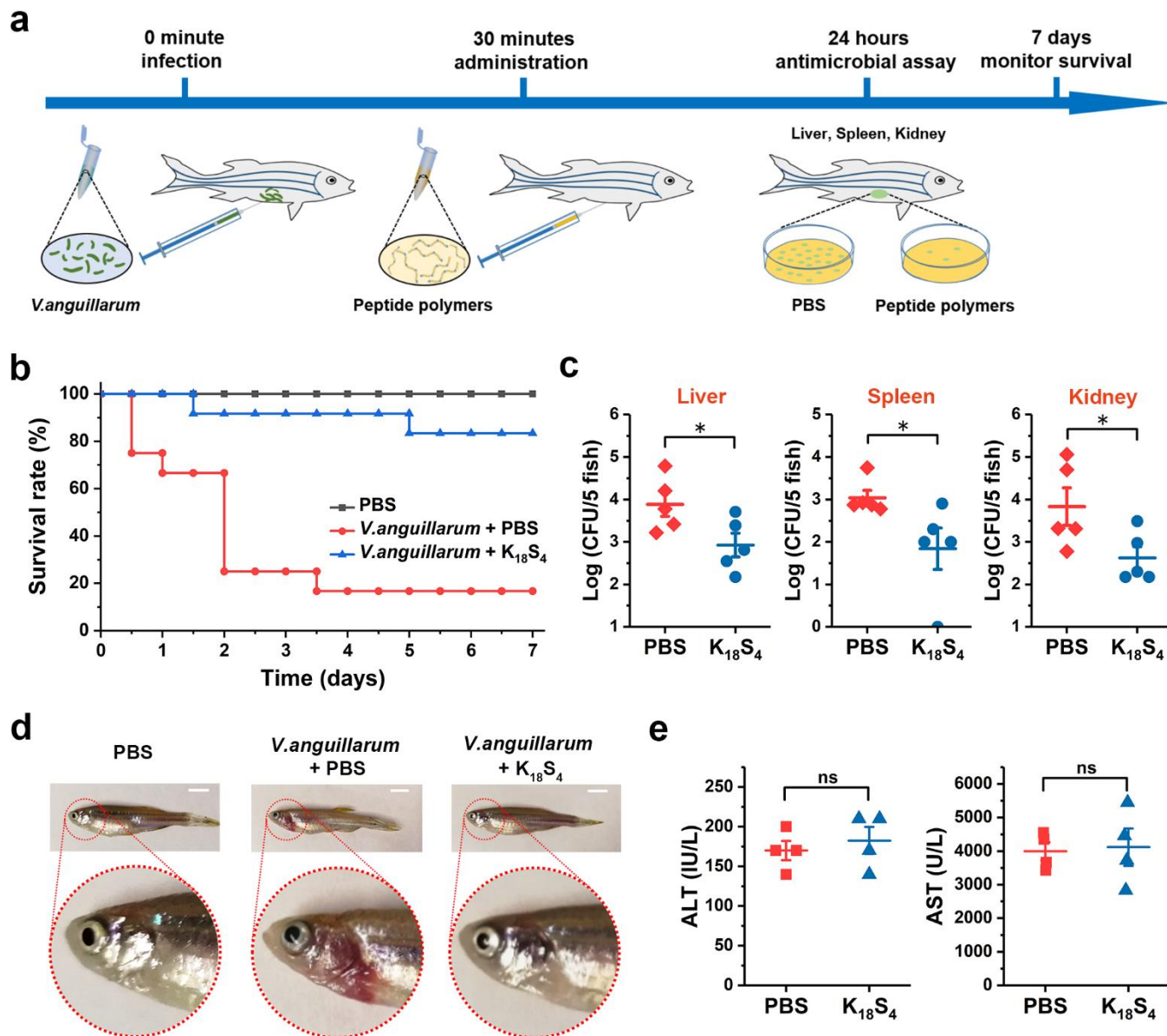


Fig 6. a) Schematic diagram of the *in vivo* anti-infectious study. b) Effect of $K_{18}S_4$ on survival rate of zebrafish infected with *V. anguillarum*. c) Colony forming unit (CFU) of *V. anguillarum* in liver, spleen and kidney of zebrafish after treatment with PBS or $K_{18}S_4$ for 24 hours. (*) represents $p < 0.05$ (Student's t test). d) Symptoms of uninfected zebrafish, *V. anguillarum*-infected zebrafishes treated with PBS or $K_{18}S_4$. Scale bar: 5mm. e) Serum biochemistry analysis of AST and ALT at 24 hours post-treatment with $K_{18}S_4$ or PBS. Each data point was collected from blood of 10 fishes due to the limited amount of blood in each fish. ns (not significant) represents $p > 0.05$.

Discussion

Finding antibiotic substitute in aquaculture is urgent and challenging. Promising substitute requires not only superior overall antibacterial performance but also complete degradability to ideally individual residues to totally lose antimicrobial activity and, therefore, cause no antimicrobial resistance and antimicrobial selective pressure. In this study, we prepared peptide polymers to explore their potential in addressing the long-lasting challenge of

antimicrobial resistance and antimicrobial selective pressure and urgent need to replace the use of antibiotics in aquaculture. The optimal peptide polymer exhibits potent and broad-spectrum activity against common pathogenic bacteria in aquaculture and low cytotoxicity *in vitro*, as well as strong therapeutic potential *in vivo*. Moreover, bacteria didn't develop resistance to peptide polymers or cross resistance to antibiotics after bacteria were treated repeatedly with peptide polymers. The biodegradability of peptide polymers enabled their enzymatic degradation to single amino acids and dipeptides, which has no antibacterial activity and will not induce antimicrobial resistance and antimicrobial selective pressure. These results altogether suggest that peptide polymers are promising alternatives to antibiotics in aquaculture.

Methods

Synthesis of N ϵ -*tert*-butyloxycarbonyl-L-lysine NCA (Boc-L-Lys NCA) monomer: Boc-L-Lys NCA was prepared according to literature with slight modification^{63, 64}. N ϵ -*tert*-butyloxycarbonyl-L-lysine (10.0 g, 40.6 mmol) and α -pinene (16.6 mL, 107.4 mmol) were suspended in anhydrous THF (0.3 M) in a round bottom flask cooled with an ice-water bath, followed by the addition of triphosgene (5.4 g, 17.9 mmol) in anhydrous THF under N₂. The reaction was stirred at 50 °C for 2 hours, and then concentrated to remove the solvent. The obtained mixture was dissolved in ethyl acetate (80 mL) and washed with ice water (3 \times 80 mL) and cold brine (1 \times 80 mL). The collected organic layer was dried over anhydrous MgSO₄, filtered and concentrated to give a crude product. The crude product was purified from recrystallization for three times using mixed solvent of ethyl acetate/hexane (1/2, v/v) in a glovebox with N₂ protection to afford pure NCA monomer as a white solid (3.9 g, 35.3%). ¹H NMR (400 MHz, CDCl₃): δ 6.92 (br, 1H), 4.66 (br, 1H), 4.33 (dd, J = 6.4, 4.4 Hz, 1H), 3.14 (s, 2H), 1.99-2.05 (m, 1H), 1.80-1.89 (m, 1H), 1.5-1.56 (m, 4H), 1.45 (s, 9H).

Synthesis of O-(*tert*-butyl)-L-serine NCA (*t*Bu-L-Ser NCA) monomer: *t*Bu-L-serine NCA⁶⁵ was prepared from O-(*tert*-butyl)-L-serine (5 g, 31 mmol), by using the similar method as the Boc-L-lysine NCA, to give a white solid (3.8 g, 65.5%). ¹H NMR (400 MHz, CDCl₃): δ 6.53 (br, 1H), 4.42 (dd, J = 4, 2.4 Hz, 1H), 3.72-3.66 (m, 2H), 1.17 (s, 9H).

Synthesis of peptide polymers: The synthesis of K₁₈S₄ was described below and other peptide polymers were synthesized in a similar way. The polymerization was carried out in a nitrogen purged glovebox. Firstly, Boc-L-Lys NCA (108.9 mg, 0.4 mmol) and *t*Bu-L-Ser NCA (18.7 mg, 0.1 mmol) were dissolved in anhydrous THF (7 mL) in a dried reaction vial equipped with a magnetic stir bar. Then a solution of LiHMDS (16.7 mg, 0.1 mmol) in anhydrous THF (1 mL) was added to the reaction and the reaction was stirred at room temperature for 5 minutes. After the completion of the polymerization, the reaction solution was poured into 80 mL hexane to precipitate out

a white flocculent solid. The solid was then collected from centrifugation, dissolved in THF (1 mL), and precipitated out again using hexane (80 mL). This cycle was repeated for three times to obtain the side-chain protected peptide polymers. Polymers at the side-chain protected stage were characterized by GPC using DMF as the mobile phase. N-Boc and *tert*-butyl protecting groups were removed by incubating polymers in 2 mL trifluoroacetic acid (TFA) for 2 hours under gentle shaking. Removing TFA gave a yellow oil, which was dissolved in methanol (1 mL) and then precipitated out as a white flocculent solid by adding methyl *tert*-butyl ether (40 mL) to the mixture. The deprotected peptide polymers were collected by centrifugation. After three cycles of dissolution/precipitation, the collected white precipitate was dissolved in deionized water and lyophilized to obtain the final peptide polymers for subsequent research.

Synthesis of N-terminal functionalized peptide polymers: The chain length of peptide polymers can be further determined by N-terminal functionalization⁵¹. After completion of the reaction for normal peptide polymer synthesis, *tert*-butyl benzylamine (5 equiv. of initiator) was added into the reaction mixture and the reaction was stirred for 12 hours. N-terminal functionalized peptide polymers were obtained according to the above purification and deprotection operation. All deprotected N-terminal functionalized peptide polymers were characterized by ¹H NMR.

Synthesis of Dye-K₁₈S₄: 2-(3-aminopropyl)-6-morpholino-1H-benzo[de]isoquinoline-1,3(2H)-dione was synthesized as described in the previous literature^{66, 67}. The synthesis of Dye-K₁₈S₄ was similar to the N-terminal functionalized peptide polymers except that *tert*-butyl benzylamine was replaced with 2-(3-aminopropyl)-6-morpholino-1H-benzo[de]isoquinoline-1,3(2H)-dione (5 equiv. of initiator) during the N-terminal functionalization process. And Dye-K₁₈S₄ was obtained according to the above purification and deprotection operation. The deprotected Dye-K₁₈S₄ was characterized by ¹H NMR.

Microorganisms and culture: All strains were from American Type Culture Collection (ATCC), China Center of Industrial Culture Collection (CICC), China General Microbiological Culture Collection Center (CGMCC) and China Veterinary Culture Collection Center (CVCC), respectively. *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC9027, *Vibrio alginolyticus* CICC10484, *Vibrio cholerae* CICC23794, *Streptococcus agalactiae* CICC10465, *Mannheimia haemolytica* CVCC3832, *Streptococcus suis* CVCC3311, *Salmonella choleraesuis* CVCC2139, *Salmonella pullorum* CVCC1879, *Salmonella anatum* CICC21498 and *Salmonella typhimurium* CVCC2220 were cultured at 37 °C. *Vibrio anguillarum* CICC24712, *Vibrio parahaemolyticus* CGMCC1.1616, *Vibrio fluvialis* CICC21612 were cultured at 30 °C. *Vibrio* was cultured in 2216E medium and other strains were cultured in Luria-Bertani (LB) broth.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Measurement: The definition of MIC is the lowest concentration of an antimicrobial agent that completely inhibits the growth of microorganisms. The MIC was measured using a standard serial microdilution method.

Briefly, 50 μL of antimicrobial agent solution in MH medium was added in each well of a 96-well plate. Overnight cultured bacteria were washed and diluted in MH medium to a cell density at 2×10^5 CFU/mL. Then 50 μL of the suspension was added into each well. The final concentration of the peptide polymers in the 96-well plate was in the range of 3.13-200 $\mu\text{g/mL}$. MH medium only and bacteria in MH medium were used as the blank control and the positive control, respectively, in the same 96-well plate. The 96-well plate was incubated at 37 $^\circ\text{C}$ or 30 $^\circ\text{C}$ for 9 hours, and the absorbance at 600 nm was measured on a Molecular Devices SpectraMax M2 precision microplate reader. The percentage of bacteria cell growth was calculated from

$$\% \text{ cell growth} = \frac{A_{600}^{\text{sample}} - A_{600}^{\text{blank}}}{A_{600}^{\text{control}} - A_{600}^{\text{blank}}} \times 100$$

The experiment was repeated three times independently.

The definition of MBC is the minimum drug concentration required to kill 99.9% of the tested microorganisms. After finishing the MIC measurement, 2 μL of bacteria suspension from each well in the 96-well plate was transferred to an agar plate. Then the agar plate was incubated for 18 hours and the CFU was counted by naked eyes to determine the MBC values.

Hemolysis activity: The hemolytic activity was measured using human red blood cells. The blood was washed with Tris-buffered saline (TBS, pH=7.2) three times to get red blood cells (RBCs), and RBCs were resuspended in TBS to achieve 5% (v/v). Serial dilution of peptide polymer solution was done in a 96-well plate like in MIC operation. TBS was used as a blank control, and Triton X-100 (0.1% v/v in TBS) was used as the positive control. After adding 50 μL RBC suspension, the 96-well plate was incubated at 37 $^\circ\text{C}$ for 1 hour. Subsequently, the plate was centrifuged at 3700 rpm for 5 minutes and 80 μL of the supernatant was transferred into another 96-well plate. The absorbance of supernatant was determined at 405 nm using a microplate reader. The experiment was repeated three times independently. The percentage of hemolysis was calculated from

$$\% \text{ Hemolysis} = \frac{A_{405}^{\text{sample}} - A_{405}^{\text{blank}}}{A_{405}^{\text{control}} - A_{405}^{\text{blank}}} \times 100$$

Cytotoxicity assay: The cytotoxicity of peptide polymers was performed according to literature⁶⁸. Firstly, COS-7 was cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 $^\circ\text{C}$ and 5% CO_2 to the appropriate density. After cells were diluted to 5000 cells/mL, 100 μL of cell suspension was added to each well of a 96-well plate and the plate was incubated at 37 $^\circ\text{C}$ for 24 hours. After removing the old medium from each well, 100 μL DMEM containing peptide polymers was added to each well by a 2-fold dilution method to obtain a concentration in the range of 6.25–200 $\mu\text{g/mL}$. After incubation for 24 hours, 10 μL of MTT solution (5 mg/mL) was added to each well, followed by incubating the plate in the dark for another 4 hours. After removing the suspension from each well, 150 μL of DMSO was added to each well, and the plate was shaken in a shaker for 20 minutes to fully dissolve the purple solid. The absorbance at 570 nm was recorded using a Molecular Devices SpectraMax M2 precision microplate reader. DMEM medium only and DMEM medium containing growing cells were used as

the blank and positive controls, respectively. The experiment was repeated three times independently. The percentage of cell viability was calculated from

$$\% \text{ cell viability} = \frac{A_{570}^{\text{sample}} - A_{570}^{\text{blank}}}{A_{570}^{\text{control}} - A_{570}^{\text{blank}}} \times 100$$

Bacteria killing kinetics: *S. aureus*, *E. coli* and *V. anguillarum* cultured to the stationary phase were diluted with MH medium to a concentration of 2×10^5 CFU/mL for use. The suspension was mixed with K₁₈S₄ or thiamphenicol solution in an equal volume at a final concentration of $2 \times \text{MIC}$, and then the mixture was incubated at 37 °C or 30 °C. An aliquot of 20 µL of the bacteria suspension was taken out at 0, 10, 30, 60, 120, 240 minutes during the incubation and diluted to a suitable bacterial concentration and then spread on an agar plate. Finally, the agar plates were incubated at 37 °C or 30 °C for 18 hours, and the percentage of survival colonies at different time points can be calculated after counting CFU.

Drug resistance test: *S. aureus*, and *E. coli* cultured to the stationary phase were diluted with MH medium to a concentration of 2×10^5 CFU/mL for use. K₁₈S₄ or antibiotic was added to the bacteria suspension to a final concentration of $0.5 \times \text{MBC}$. The resulting mixture was incubated at 37 °C for 24 hours under shaking at 200 rpm. Then the suspension was diluted 400 times with MH medium again, and another round of co-cultivation of antibacterial agent with bacteria was carried out. The cycle was repeated every 24 hours and the change of MIC and MBC value was detected every 4 days. The concentration of K₁₈S₄ and antibiotics was adjusted and maintained at $0.5 \times \text{MBC}$ according to test results. The growth generation of bacteria treated with K₁₈S₄ or antibiotic was calculated according to the following bacterial growth kinetics.

Bacterial growth kinetics: *S. aureus* and *E. coli* cultured to the exponential period were diluted with MH medium to a concentration of 2×10^5 CFU/mL. Then the working suspension was treated with K₁₈S₄ or antibiotic at a final concentration of $0.5 \times \text{MBC}$ and the mixture was cultured at 37 °C under gentle shaking. During the cultivation process, 10 µL of the mixture was taken out each time and streaked on the agar plate at different time points. Then the agar plates were cultured at 37 °C for 18 hours. Finally, the bacterial growth rate can be calculated by counting the CFU on the agar plate.

Enzymatic biodegradation: The peptide polymer (4 mg/mL) was mixed with protease (0.2 mg/mL protease XXIII from *Aspergillus oryzae*, or 0.1 mg/mL trypsin, or 0.1 mg/mL chymotrypsin) in PBS (0.01M, pH=6.5 for protease XXIII, pH=7.2 for trypsin and chymotrypsin) and the mixture was incubated at 37 °C (for trypsin and chymotrypsin) or 45 °C (for protease XXIII), respectively. Aliquots were taken out at time intervals and heated at 100 °C for 10 minutes to inactivate the enzyme. Then the MIC of degradation solution was measured. The

degradation products were characterized by NMR to determine the time required for full degradation. The components of the final degradation products were determined by ESI-MS.

Scanning electron microscopy (SEM) imaging: *S. aureus*, *E. coli* and *V. anguillarum* cultured to the exponential period were diluted with MH medium to a concentration of 2×10^7 CFU/mL. Then the bacteria suspension was mixed with an equal volume of $K_{18}S_4$ solution, and the final polymer concentration was $5 \times \text{MIC}$. The mixture was cultured in an incubator for a period of time until approximately 90% of the bacteria died. After centrifugation at 4000 rpm for 5 minutes, the supernatant was carefully discarded, and the remained bacteria were mixed well by adding 1 mL PBS. The bacteria were collected again by centrifugation. Then 1 mL PBS buffer containing 4% glutaraldehyde was added, and the sample was fixed overnight at room temperature. After centrifugation, the supernatant was removed and the collection was washed with 1 mL PBS, followed by gradual dehydration with different concentrations of ethanol (30, 50, 70, 80, 90, 95 and 100%). The dehydrated bacteria were dispersed in 20 μL anhydrous ethanol and pipetted onto a gold-plated silicon wafer for observation by SEM.

Transmission electron microscope (TEM) imaging: The preparation of samples is similar to SEM characterization. *S. aureus*, *E. coli* and *V. anguillarum* treated with $K_{18}S_4$ ($5 \times \text{MIC}$) were collected by centrifugation and fixed overnight with 2.5% glutaraldehyde. After being washed with PBS for three times, *S. aureus*, *E. coli* and *V. anguillarum* were fixed with 1% OsO_4 in PBS for 1.5 hours. Then samples were washed two times with PBS and dehydrated with a series of graded ethanol solutions (30% - 100%). The embedding liquid and acetone were mixed at a ratio of 1:1 and 3:1 (v/v) to treat the dehydrated sample for 1 hour, respectively. Then samples were treated with pure embedding liquid overnight and slices with a thickness of 70-90 nm were obtained with LEICA EM UC7 ultra-thin microtome. Finally, the slices were stained with 2% uranyl acetate and lead citrate for 10 minutes and observed by transmission electron microscope.

Time-lapse fluorescent confocal imaging: *S. aureus*, *E. coli* and *V. anguillarum* cultured to the exponential period were diluted with MH medium to a concentration of 2×10^7 CFU/mL. Then 10 μL of this suspension was dropped on the dish with glass bottom. After standing for 5 minutes, Dye- $K_{18}S_4$ ($10 \times \text{MIC}$) and PI (40 μM) was mixed in a ratio of 1/1 (v/v) and 10 μL of the mixture was dropped into the bacteria solution. Finally, three channels were selected in the confocal microscope, the bright field of differential interference contrast (DIC), 488 nm (detecting Dye- $K_{18}S_4$) and 562 nm (detecting PI), and images were collected at 30s intervals.

Outer membrane permeability studies: *E. coli* and *V. anguillarum* cultured to the exponential period were washed three times with HEPES buffer (5 mM, pH=7.4, containing 5 mM glucose) and diluted to 10^7 CFU/mL.

Then the bacteria suspension were incubated with the membrane potential-sensitive fluorescent dye 1-N-Phenyl-naphthylamine (10 μ M), and the fluorescence value at an excitation wavelength of 350 nm and an emission wavelength of 420 nm was recorded by SpectraMax®M2 plate reader until it was stable. Finally, K₁₈S₄ was added at the final concentration of 2 \times MIC and 5 \times MIC, and the fluorescence value was recorded using polymyxin B as positive control.

LPS binding assays: *E. coli* and *V. anguillarum* cultured to the stationary phase were diluted with MH medium to 2×10^5 CFU/mL. A two-fold dilution of K₁₈S₄ was prepared in a 96-well plate with MH medium containing different concentrations of LPS (0-2000 μ g/mL) purified from *E. coli*. Then 50 μ L of the bacteria suspension was added to mix with the 50 μ L of the peptide polymers-LPS solution. MIC values were obtained after the 96-well plate was incubated for 9 hours.

Cytoplasmic membrane depolarization studies: *S. aureus* cultured to the stationary phase was washed three times with HEPES buffer (5 mM, pH=7.4, containing 20 mM glucose) and diluted to 10^7 CFU/mL. Then the bacteria suspension was incubated with the membrane potential-sensitive fluorescent dye 3,3'-Dipropylthiadicarbocyanine Iodide (0.8 μ M) for 1 hour until the dye was incorporated into the cell membrane. After adding KCl (0.1M) to balance the charge inside and outside of the cytoplasm, the fluorescence value at an excitation wavelength of 622 nm and an emission wavelength of 670 nm was recorded by SpectraMax® M2 plate reader until it was stable. Finally, K₁₈S₄ was added at the final concentration of 2 \times MIC and 5 \times MIC, and the fluorescence value was recorded with Triton X-100 as the positive control.

Intracellular ROS assay: *S. aureus*, *E. coli* and *V. anguillarum* cultured to the stationary phase were washed three times with PBS. Then the bacteria were resuspended in PBS to 2×10^8 CFU/mL for *S. aureus* and 2×10^7 CFU/mL for *E. coli* and *V. anguillarum*. The bacteria suspension was then mixed with the solution of ROS sensitive fluorescent probe DCFH-DA (40 μ M in PBS) at a ratio of 1/1 (v/v). The mixture was incubated at 37 °C or 30 °C for 30 minutes, and then the bacteria were harvested by centrifugation and washed with PBS for three times to remove the extracellular DCFH-DA. An aliquot of 90 μ L of bacteria suspension was placed in a 384-well plate, and then 10 μ L of K₁₈S₄, K₁₈S₄+NAC (7.5 mM) or PBS was added, respectively. The 2',7'-Dichlorofluorescein (DCF) fluorescence intensity at an excitation wavelength of 488 nm and an emission wavelength of 530 nm was recorded by SpectraMax® M2 plate reader.

DNA binding assay: Agarose gel electrophoresis was used to evaluate the ability of K₁₈S₄ to bind with DNA. According to the different N/P ratio (0, 0.5, 1, 2 and 3, respectively), 5 μ L of different concentration gradient

polymers were added to 5 μL DNA solution (140 $\mu\text{g}/\text{mL}$) and incubated for 30 minutes at room temperature. Here N represents the amino group of the polymer side chain, and P represents the phosphate ion in DNA. The resulting samples mixed with ethidium bromide in Tris-borate-EDTA buffer and analyzed by electrophoresis using 1% agarose gel at 120 V for 30 minutes. DNA bands were visualized using a Gel Documentation and Image Analysis System (SAGECREATION, China).

Acute toxicity test of adult zebrafish: The acute toxicity test of adult zebrafish was conducted according to the OECD standard test method⁶⁹. All animal experiments conformed to regulatory standards according to protocols approved by the Animal Care Committee of East China University of Science and Technology. Wild type AB zebrafish (weighing $0.4 \pm 0.1\text{g}$) were fed in the laboratory for 2 weeks to adapt to the environment. The feeding environment was at 28 $^{\circ}\text{C}$ and the circadian rhythm was 14:10 per day. At the beginning of the experiment, the zebrafish were randomly exposed to 2 L exposure solution contained peptide polymers at the different concentration. Three repeating groups were used for each concentration and each repeating group contained 10 fish. In order to maintain the oxygen content in the water, the exposure solution was changed every 24 hours. There was no feeding or oxygen supply during the whole experiment. The dead individual was removed immediately. The survival of adult zebrafish was then calculated at 24, 48, 72 and 96 hours. The median lethal concentration (LC_{50}) was calculated by probit regression analysis in the IBM SPSS Statistics 26.0 software.

Zebrafish survival assay: Adult zebrafish were randomly divided into three groups (10 fish per group): PBS group, (*V. anguillarum* + PBS) group and (*V. anguillarum* + K_{18}S_4) group. *V. anguillarum* cultured to the exponential period was washed three times with PBS and then was resuspended in PBS to 4×10^8 CFU/mL. Subsequently, inoculation was performed by intraperitoneal injection of 10 μL *V. anguillarum* suspension or 10 μL PBS for the experimental groups and the control group, respectively. After 30 minutes, PBS and K_{18}S_4 (20 mg/kg) were treated for (*V. anguillarum* + PBS) group and (*V. anguillarum* + K_{18}S_4) group, respectively. The survival and the symptoms of infection were observed during the 7-days observation period.

Antimicrobial assay *in vivo*: *V. anguillarum* cultured to the exponential period was washed three times with PBS, then the bacteria were resuspended in PBS to 4×10^8 CFU/mL. Inoculation was performed by intraperitoneal injection of 10 μL *V. anguillarum* suspension for 50 zebrafish. After 30 minutes, 50 zebrafish were divided into two groups and treated with K_{18}S_4 (20 mg/kg) or equal volume of PBS, respectively. 24 hours later, all zebrafish were anesthetized with 0.3% tricaine and then euthanized. The heart, liver and spleen were taken out by aseptic operation, and the tissues of five fish in each group were mixed into one sample due to the small size. Finally, the

tissues were homogenized with PBS containing 0.1% TX100 and plated onto agar for CFU count.

Serum biochemical index: 80 adult zebrafish were randomly divided into two groups, and each was injected intraperitoneally with K₁₈S₄ (20 mg/kg) or equal volume of PBS. After 24 hours, all zebrafish were anesthetized with 0.3% tricaine and then euthanized. After euthanasia, zebrafish was quickly dried and the eyeballs were removed immediately by tweezers. The blood was carefully collected with a pipette when it filled the eye socket according to the literature⁷⁰. The blood sample of 10 zebrafish in the same group was mixed into one sample due to the limited amount of blood in each fish. After standing at room temperature for 2 hours, the serum was obtained by centrifugation at 4000 rpm for 5 minutes, and then the serum was transferred to another centrifuge tube and analyzed with a serum biochemical detection instrument.

Statistical analysis: The data of statistical analysis was performed by Origin software. Significance between the two groups was determined by *t*-test. All mean ± standard deviation was indicated by the error bars.

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Author contributions

R.L. directed the whole project. P.M. and R.L. conceived the idea, proposed the strategy, designed the experiments, evaluated the data, and wrote the manuscript together. P.M. performed the majority of the experiments. Y.W. performed the exploration of polymerization methods and the characterization of peptide polymers. W.J. and Y.C. participated in the animal study; N.S. performed the cytotoxicity assays; M.Z. conducted the time-lapse fluorescent confocal imaging; J.X. and Z.Q. conducted the scanning electron microscope imaging. All authors participated in the discussion of the results and commented on the manuscript.

Competing interests

The authors declare the following competing financial interest (s): R.L. and P.M. are co-inventors on a patent application covering reported materials and application on alternatives to antibiotics in aquaculture. All remaining authors declare no competing interests.

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