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PERSPECTIVE

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Constitutionally isomeric antimicrobial peptides: Candidates for antibacterial treatment with morphology-dependent efficacy

Yujia Lu, Yiyang Shao, and Yin Wang*

Shanghai Frontiers Science Center of Drug Target Identification and Delivery, Engineering Research Center of Cell & Therapeutic Antibody, National Key Laboratory of Innovative Immunotherapy, School of Pharmaceutical Sciences, Shanghai Jiao Tong University, Shanghai 200240, China

*Correspondence: yinwang@sjtu.edu.cn

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Antimicrobial peptides (AMPs) have been used as a substitute for antibiotics due to the special antibacterial mechanism synergistically driven by their positive surface charge and hydrophobic domains. However, the relationship between the amino acid sequence and the antimicrobial efficacy remains unclear. In this review, we summarized the recent advances in constitutionally isomeric AMPs, which regulated the antibacterial efficiency by changing the position of certain amino acid residues. We also briefly illustrated the application of constitutionally isomeric peptides templated with inorganic nanoparticles in antibacterial treatments. At the end, we provided future outlooks and potential challenges faced in this research area. We hope that our efforts could bring more insight into the rational design of AMPs and how subtle changes in peptide sequence would affect the resultant bioactivity.

INTRODUCTION

Other than cancer, bacterial infection is among the world's most threatening diseases due to its susceptibility and high contagiousness. Its clinical manifestations include cough, fever, vomiting, diarrhea, ulcers, etc., based on the infection type. If bacterial infection is not treated in time and effectively, patients may suffer from chronic diseases, sepsis, and organ failure, which may ultimately lead to death. At present, the first-line drugs against bacterial infection are still antibiotics. However, years' overuse of antibiotics during the treatment has caused the emergence of multi-drug resistant bacteria. To make matters worse, bacteria can form biofilms through secreting extracellular matrix (e.g., polysaccharide, protein, DNA, etc.) to protect themselves from the antibiotics. Such phenomena significantly hinder the efficacy of antibiotic treatments, bringing a new challenge to combat bacterial infections. Thus, novel drugs that can overcome the multi-drug resistance and surpass the structural barrier of bacteria or biofilms are in urgent need.

In recent years, peptides have gained increasing attention for their wide range of applications in biomedical fields. Due to some distinct merits, such as simplicity of synthesis, good biocompatibility and biodegradability, and low immunogenicity, peptides stand out as carriers for drug delivery to improve the water solubility and change the pharmacokinetics of the drugs. In addition, it is easy to incorporate specific targeting groups into the peptide sequences during the synthetic process, achieving targeted delivery of drugs that possess high systemic toxicity. Moreover, by regulating the peptide sequences or changing the assembling conditions, peptides could be engineered into different morphologies, i.e., nanofibers, nanoribbons, or nanotwists, etc. With such good tunability and moldability, peptide-based delivery systems with different nanostructures could easily regulate the biodistribution of drugs, ultimately influencing the bioactivity of the drugs in the body.[1,2]

Apart from being applied as drug-delivery carriers, peptides themselves could also be used as drugs.[3] Numerous researches have reported peptides being used in cancer, anti-inflammation, bacteria elimination, bone generation, cardiac protection, and so on. Among them, antimicrobial peptides (AMPs) used as a substitute for antibiotics have received growing attention during the past few years. AMPs mainly consist of hydrophobic amino acid residues and cationic ones. Researchers have found that the hydrophobic parts could augment the insertion of AMPs into the bacterial membrane. However, the introduction of the fatty acid chain at the N terminus might facilitate the aggregation of the AMPs due to the increase in hydrophobicity, resulting in a decrease in anti-bacterial efficacy.[4] Meanwhile, the cationic residues could bind with the negatively charged membranes through electrostatic interactions, which interfere with the bacterial membrane's integrity and ultimately result in the fracture and apoptosis of bacteria. Compared with antibiotics, benefiting from AMPs' distinct antimicrobial mechanism, it is scarcely possible for bacteria to develop multi-drug resistance because it is difficult to alter or repair the constituents on bacteria's membrane in a time-consuming and laborious metabolic way. Moreover, along with the investigation on natural AMPs, synthetic AMPs with the capability of self-assembling have long been studied and exhibit stronger antibacterial capacity. The cationic residues of AMPs could be displayed on the surface of these assemblies in a higher density, which not only enhances the stability against proteolysis but also amplifies the antimicrobial ability than those unassembled counterparts. However, the relationship among the amino acid sequences of AMPs, assembled morphologies, and the resultant bactericidal efficiency still remains ambiguous.

CONSTITUTIONALLY ISOMERIC PEPTIDE ASSEMBLIES FOR ANTIBACTERIAL TREATMENTS

Constitutionally isomeric peptides, a class of peptides with the same amino acid constituents but different sequences which possess different biological activities,[5,6] could well explain the issue mentioned above. Zhou and co-workers reported a pair of constitutionally isomeric tripeptide amphiphiles (PAs), each containing two glycine residues (G) and one Alanine residue (A).[7] Both were capped with a hydrophobic *n*-butylazobenzene group at the N terminus. Under transmission electron microscopy (TEM), the morphologies of these PAs altered from left-handed nanotwists to untwisted nanofibers when switching A from the N terminus to the C terminus (Figures 1A & C). Despite the difference in morphologies, both PAs exhibited a dose-dependent antibacterial ability against *E. coli* and methicillin-resistant *S. aureus* (MRSA). Notably, they could also disrupt biofilms generated by MRSA or multi-drug resistant *P. aeruginosa*. Furthermore, both PAs experienced a reversible assembly-disassembly process when being exposed to stimuli such as light or host-guest chemistry between the *n*-butylazobenzene group and β-cyclodextrin (β-CyD) (Figures 1B & D). With such properties, the antimicrobial and antibiofilm efficacy of PAs could be on-demand modulated upon certain stimuli (Figure 1E).

Based on the previous study, The same group further capped these constitutionally isomeric PAs with a hydrophilic *N*-methyl-d-glucamine residue at the C terminus, forming three azobenzene-glycopeptides (AGPs).[8] TEM images revealed that those AGPs could form nanotwists, nanoribbons, and nanofibers by adjusting the position of A in the peptide sequence (Figure 1F). Based on the results of X-ray diffraction (XRD), although these AGPs were similar in structure, the internal molecular packing behavior was slightly different, which resulted in the changes in morphologies. However, even though these AGPs formed different nanostructures, they possessed similar biofilm inhibition and elimination capacity against MRSA. Compared with PAs mentioned above, except for the stimuli including UV irradiation and host-guest chemistry, these AGPs could also undergo a reversible assembly-disassembly process when raising the temperature because of the change in the intermolecular interaction. TEM images and circular dichroism (CD) spectroscopy confirmed that the assemblies of AGPs collapsed upon heating, UV irradiation, and the addition of β-CyD, while they all gradually recovered after removing the corresponding stimuli (Figure 1G). Such properties endowed

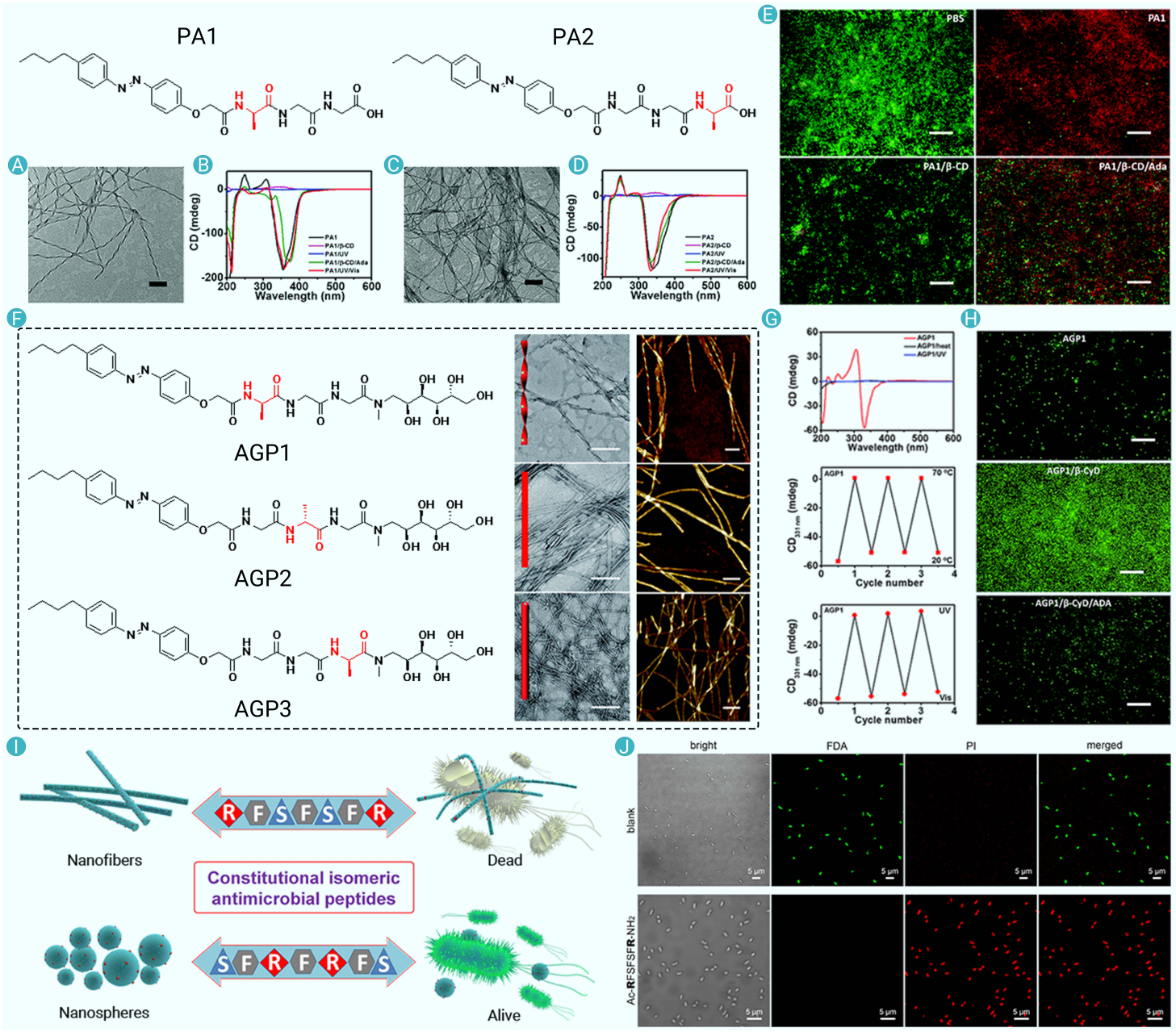

Figure 1. Molecular structures, TEM images of (A) PA1 and (C) PA2. The scale bars are 200 nm. CD spectra of 0.7 mM of (B) PA1 and (D) PA2 in PBS (pH=7.4) upon light and host-guest chemistry stimuli. (E) Fluorescence microscopy imaging of *S. aureus* biofilms incubated with 0.35 mM of PA1, PA1/β-CD or PA1/β-CD/Ada at 37 °C for 24 h after staining with LIVE/DEAD™ BacLight™ Bacterial Viability Kit. The scale bars are 20 μm. (F) Molecular structures, TEM, and AFM images of AGP1, AGP2, and AGP3 assemblies. The scale bars are 200 nm. (G) The reversible assembly-disassembly behavior of 0.6 mM of AGP1 upon heat and UV stimuli. (H) Fluorescence microscopy imaging of the formed *S. aureus* biofilms in the presence of 0.6 mM of AGP1, AGP1/β-CyD, or AGP1/β-CyD/ADA for 24 h at 37 °C after SYTO9 staining. The scale bars are 20 μm. (I) Schematic illustration of different self-assembled morphologies of Ac-RFSFSFR-NH_2 and Ac-SFRFRFS-NH_2 and their morphology-dependent antibacterial activity. (J) Laser scanning confocal microscope (LSCM) imaging *E. coli* incubated with 150 μM of Ac-RFSFSFR-NH_2 after staining with FDA and PI. Live bacteria are stained green and dead bacteria are stained red in all fluorescence images. Adapted with permission from ref 7, 8, and 9. Copyright 2019 Royal Society of Chemistry. Copyright 2019 Royal Society of Chemistry. Copyright 2022 American Chemical Society.

them with the potential to be used as stimuli-responsive switchable antimicrobial agents. The bactericidal efficacy of these AGPs was consistent with the existence of their assemblies, which was significantly hindered once they disassembled upon certain stimuli (Figure 1H). In summary, the change in the amino acid position could cause great variation in assembling behavior. At the same time, the reversible turn-on/off ability to eliminate bacteria of these isomeric AGPs might give new insight into combating muti-drug resistance. Compared to antibiotics, these AGPs might be an on-demand weapon to treat acute bacterial infections because their turn-on/off ability allows them to kill bacteria without being applied to the infection sites in a long run, which greatly reduces the possibility of developing drug resistance.

Unlike the peptide isomers reported by Zhou and co-workers displaying comparable antimicrobial capacity though differed in amino acid sequence, Li and co-workers found out that subtle changes in amino acids' position could lead to dramatic differences in antibacterial efficacy. A pair of isomeric AMPs with the same composition of arginine (R), serine (S), and phenylalanine (F) residues but different orders was designed.[9] Both of them were capable of self-assembling into specific nanostructures (Figure 1I). Under TEM, nanofibers were observed for Ac-RFSFSFR-NH_2 while the dominant morphology turned into irregular nanospheres when placing the two R residues in the middle of the sequence (Ac-SFRFRFS-NH_2). Besides, their apparent potentials changed along with the change in morphology. Ac-RFSFSFR-NH_2 possessed an extremely high apparent potential while Ac-SFRFRFS-NH_2 was nearly neutral. As the positive potential of AMPs plays a

crucial role when binding with the negatively charged bacteria membrane, those two isomeric peptides with distinct positive charges thus generated significantly different antibacterial efficacy. The high surface potential of nanofibers formed by Ac-RFSFSFR-NH_2 reinforced the binding efficiency to the surface of bacteria and disrupted the cell membrane, which ultimately induced a more drastic bacteria death with a minimal inhibitory concentration (MIC) of only 150 μM against *E. coli* and *S. aureus* (Figure 1J). In sharp contrast, nanospheres formed by Ac-SFRFRFS-NH_2 was incapable of eliminating bacteria effectively due to its low surface charge. It didn't reach MIC until 1200 μM or 1300 μM against *E. coli* and *S. aureus*, respectively. Overall, this study emphasized how subtle changes in amino acid sequences could be utilized to modulate the antimicrobial efficacy of constitutionally isomeric AMPs. The antibacterial efficacy of Ac-RFSFSFR-NH_2 greatly surpassed the other one while it still showed an ideal biocompatibility. However, although Ac-RFSFSFR-NH_2 showed strong antibacterial efficiency in vitro, whether it is still effective in vivo remains unknown. Further experiments on animals are urgently needed. Besides, the balance between the antibacterial efficacy and the biocompatibility of AMPs might raise a new challenge. Through the alternation of the peptide sequence, a more in-depth investigation on the balance between potent antibacterial ability of AMPs and excellent biosafety is also in urgent need.

ISOMERIC PEPTIDE-INORGANIC NANOPARTICLE HYBRIDS FOR ANTIBACTERIAL TREATMENTS

Besides the direct application of constitutionally isomeric AMPs, peptide isomers decorated with inorganic nanoparticles were also able to kill bacteria. Joshi and co-workers designed two short peptide amphiphiles (sPAs), FYA and YFA, and further modified the surface with silver nanoparticles (AgNPs).[10] Based on TEM images, those sPAs could self-assemble into straight and twisted nanofibers, respectively. Moreover, the introduction of AgNPs could further affect the assembling behaviors of sPAs to a different extent and result in great changes in their morphologies. Compared with AgNPs or sPAs alone, those AgNPs-sPAs hybrids exhibited a broad spectrum and enhanced bacteria inhibition capacity through the release and a higher cellular uptake of AgNPs. The research again highlighted that small alterations in peptide sequence could greatly impact the structure of peptide-nanoparticle hybrids, resulting in distinct efficacy. Inspired by this study, other AMP-based hybrids might also be a novel platform to synergistically treat bacterial infections.

CONCLUSION AND PERSPECTIVE

In conclusion, we summarized the latest research on the application of constitutionally isomeric peptides in antibacterial treatments. The studies revealed that subtle changes in the position of amino acids led to significant changes in the assembled morphologies because of the variation in the inter/intramolecular interactions. Meanwhile, the morphology would further impact their resultant antibacterial efficiency. Furthermore, the application of AMPs or peptide-based hybrids exhibited a broad spectrum antibacterial and antibiofilm efficacy, which offered new insights into combating multi-drug resistant bacterial infection.

However, potential challenges might hinder the further application of these AMPs or hybrids in clinic. The major one remains in the balance between the antimicrobial efficacy and the biocompatibility of these AMPs. Being positively charged, AMPs can enhance their binding to the bacterial membrane and exert their bactericidal ability, but those cationic AMPs may exhibit unexpected cytotoxicity. Nevertheless, screening the surface charge to improve the biocompatibility would attenuate their antibacterial activity. Thus, the further application of AMPs highly depends on finding the balance between their bactericidal capacity and biosafety. Moreover, although it is confirmed that variation in the amino acid sequence of constitutionally isomeric AMPs could regulate their antibacterial activity, whether changing the sequence would improve or impair the activity remains unknown. The relationship between peptide sequence design at the molecular level and the resultant property requires more detailed investigation to facilitate the *de novo* design of AMPs. Besides, among all the research, the antibacterial assays were conducted in vitro. Further studies on their antibacterial efficacy in vivo are needed. In addition, the introduction of inorganic nanoparticles such as AgNPs to peptide isomers also exhibited antibacterial activity. Hence, other peptide-inorganic nanoparticle hybrids or peptide-based conjugates with specific stimuli-responsive properties might offer some new insights into synergetic antibacterial treatments. Collectively, despite the promising antimicrobial efficiency of these constitutionally isomeric AMPs, further exploration is still needed to give a more detailed elucidation on their molecular design, assembled morphologies, and the resultant antibacterial efficacy.

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AUTHOR CONTRIBUTIONS

Yujia Lu was responsible for the literature review, drafting of the initial manuscript, and figure organization. Yiyang Shao contributed to the literature review and engaged in discussion. Yin Wang was responsible for supervising and revising the manuscript. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.