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Synthesis of triblock amphiphilic copolypeptides with excellent antibacterial activity

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ABSTRACT

Antibacterial peptides as a novel antimicrobial agent have overwhelmingly caught researchers' attention with unique superiority. Here, we report a series of triblock copolypeptides K_{10}-*b*-F_n-*b*-K_{10} synthesized by sequential NCA ring-opening polymerization. The obtained triblock copolypeptides have good antibacterial efficacy against both *S. aureus* and *E. coli*. The hemolysis test has been conducted and it suggests that the triblock copolypeptides have low cytotoxicity and good biocompatibility. Furthermore, we present a systematic study on their self-assembly behavior and confirm that they can self-assemble into vesicles. Hence, such triblock copolypeptides have potential applications in antibacterial therapy and biomedical application field.

1. Introduction

Considering the abuse of antibiotics in humans and animals [1,2], multi-resistant bacteria have become a serious problem which is a threat to the health of humans. Recently, antimicrobial peptides (AMPs) [3–5] have become a research hotspot due to their excellent properties, such as high antibacterial activity, broad antimicrobial spectrum [6], and unique antimicrobial mechanism. They have different antibacterial mechanisms compared with traditional antibiotics due to their unique structures. Natural AMPs are usually composed of hydrophilic (positively charged amino acid residue, such as lysine and arginine) and hydrophobic amino acid residues (such as phenylalanine and leucine). AMPs kill bacteria via pore-forming mechanism [7,8]: AMPs are absorbed onto the membrane (negative charge) of bacteria via electrostatic interaction, and hydrophobic amino acid residuals insert into the lipid membrane, thereby resulting in pore formation. This kind of bactericidal mechanism makes it difficult for pathogens to develop resistance against AMPs [9]. Nevertheless, some obstacles [10,11] have restricted the wide application of AMPs, including fluctuating efficacy, exorbitant cytotoxicity to human cells, time consumption, and expensive production. Instead, N-carboxyanhydride (NCA) ring-opening polymerization [12,13] is an effective and economical method to obtain peptides in large quantities. In our previous work [14,15], we successfully synthesized some AMPs, including random and diblock copolypeptides with excellent antibacterial activity via NCA ring-opening polymerization. Furthermore, some other peptide-based materials are prepared through this method [16–18].

Amphiphilic multi-block polypeptides, including antimicrobial peptides, are significantly important biomaterials which can self-assemble into nanoparticles with various morphologies due to their intermolecular interaction. In the past few decades, several studies on vesicles [19,20], micelles [21,22] and nanofibers [23–25] self-assembled by amphiphilic polypeptide-based materials have been reported. These kinds of multi-block polypeptide nanoparticles can be widely used in drug delivery [26–28], surgical repair [29–31] and tissue engineering [32–34] due to their excellent biodegradability and biocompatibility [35].

In this work, a series of amphiphilic triblock copolypeptides composed of lysine (K) and phenylalanine (F) amino acid residues were synthesized by sequential NCA ring-opening polymerization, including K_{10}-*b*-F_{10}-*b*-K_{10}, K_{10}-*b*-F_{20}-*b*-K_{10} and K_{10}-*b*-F_{30}-*b*-K_{10}. The antimicrobial activity against both $Gram^+$ (*Staphylococcus aureus*) and $Gram^-$ (*Escherichia coli*) bacteria and the toxicity to red blood cells were evaluated. Furthermore, their self-assembly behavior was also systematically studied by dynamic light scattering (DLS), transmission electron microscope (TEM), and critical vesicular concentration (CVC).

2. Experimental

2.1. Material

N-ε-benzyloxycarbonyl-L-lysine and L-phenylalanine were purchased

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from Shanghai Hanhong Chemical Corporation, China. Isobutylamine, triphosgene, and trypsin were purchased from Aladdin Industrial Corporation, China. Hexane, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and acetone were purchased from Tansoole Corporation. Hydrogen bromide (30% in acetic acid) was purchased from J&K Corporation. *E. coli* (ATCC35218) and *S. aureus* (ATCC29213) were purchased from Nanjing Bianzhen Biological Technology Co., Ltd. DMSO was dried using molecular sieves. Other chemicals were used as they were purchased.

2.2. Preparation of amino acid NCA monomers

Z-lysine NCA monomers were synthesized according to the following protocols. Briefly, N-ε-benzyloxycarbonyl-L-lysine (30.000 g, 107.14 mmol) were suspended in 200 mL of THF in a 500 mL flask. Then, 13.8 g triphosgene were added to the solution and reacted at 55 °C for 4 h with a transformation from suspension to clarified liquid. The solution was dropped into 1000 mL of hexane and filtered. Subsequently, the solid was dissolved in THF to repeat the precipitation process twice. Finally, the obtained white powder was dried at 50 °C under vacuum. Yield: ~75%. Phenylalanine–NCA monomers were prepared with similar methods. Yield: ~68%.

2.3. Synthesis of the poly(Z-Lys)$_{10}$

Exactly 10.0 g Z-lysine NCA monomers were dissolved in 50 mL of dried DMSO in a dried flask followed by the addition of 0.239 g isobutylamine as the initiator. The solution was stirred for 10 mins and then react under high vacuum for 12 h at room temperature. Afterward, the solution was precipitated in 1000 mL water to obtain white powder and then filtered. The powder was washed by water several times. Finally, the product was dried in vacuum at 50 °C for 24 h to obtain the poly(Z-Lys)$_{10}$. Yield: ~85%.

2.4. Synthesis of diblock copolypeptides poly(Z-Lys)$_{10}$-b-Phe$_n$

Precisely 3.0 g poly(Z-Lys)$_{10}$ were dissolved in 20 mL of dried DMSO, and 2.123 g phenylalanine NCA monomers were subsequently added into the solution and reacted in vacuum for 12 h at room temperature. Then, 3.409 g Z-lysine NCA monomers (3.409 g, 11.140 mmol) were added to the solution and reacted in vacuum for another 12 h at room temperature. The mixed solution was precipitated in 1000 mL water to obtain a solid product, filtered, washed with water several times, and then dried in vacuum at 50 °C for 24 h to obtain a predicted product of poly(Z-Lys)$_{10}$-*b*-Phe$_{10}$-*b*-(Z-Lys)$_{10}$. Yield: ~85%.

Triblock copolypeptides poly(Z-Lys)$_{10}$-*b*-Phe$_{20}$-*b*-(Z-Lys)$_{10}$ and poly(Z-Lys)$_{10}$-*b*-Phe$_{30}$-*b*-(Z-Lys)$_{10}$ were synthesized via similar methods of poly(Z-Lys)$_{10}$-*b*-Phe$_{10}$-*b*-(Z-Lys)$_{10}$.

The three triblock copolypeptides (2.0 g) were dissolved in 10 mL HBr (30% in acetic acid) and reacted for 4 h at room temperature. Then, three solutions were dropped to 200 mL acetone in three beakers. The precipitates were washed by acetone several times to remove the residual acid. Finally, the products were dialyzed against deionized water in three dialysis tubes for 2 days and dried in vacuum at 50 °C to obtain the final product K$_{10}$-*b*-F$_n$-*b*-K$_{10}$.

2.5. Antibacterial measurement and antibacterial mechanism

To prepare the bacteria–saline suspensions, *E. coli* and *S. aureus* were grown at 37 °C for 18 h in LB broth, and 1 mL bacteria suspensions were centrifuged at a speed of 4000 r/min. Subsequently, both bacteria were washed with saline several times. Finally, 1 mL saline were added to form bacteria–saline suspensions and then diluted to 10^6 CFU/mL for antibacterial test.

A series of K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ solution (100 μL) with different concentrations (ranging from 1000 μg/mL to 4 μg/mL) were prepared by two-fold dilution with saline. Then, the diluted bacteria–saline suspensions (100 μL) were added to each peptide solution and the mixtures were cultured for 2 h at 37 °C. Thereafter, the mixtures were diluted 10, 10^2, 10^3, 10^4, and 10^5 times with saline. Each diluted mixture (100 μL) was dropped to plates, mixed with 5 mL LB agar, and cultured at 37 °C for 48 h. Bacteria–saline suspension without K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ served as the control. Finally, the colony numbers were counted, and the results were the averages of three independent experiments.

In addition, TEM was used to observe the morphology changes of bacteria before and after treatment with K$_{10}$-*b*-F$_{20}$-*b*-K$_{10}$ solution.

2.6. Self-assembly of triblock copolypeptides

K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ vesicles were prepared according to the following protocols: 5.0 mg of K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ were directly dissolved in 5 mL of deionized water and then stirred for 2 h.

2.7. Hemolysis assays

To prepare the cell–PBS suspension, fresh goat blood was centrifuged and washed thrice with PBS to obtain pure red blood cells. Then, 1.5 mL red blood cells were added to 30 mL PBS solution to form a cell–PBS suspension (5%, v/v). A series of K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ solution at different concentrations (ranging from 2000 μg/mL to 8 μg/mL) were prepared by two-fold dilution with PBS solution. Exactly 100 μL cell–PBS suspensions were treated with 100 μL diluted K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ solution for 1 h at 37 °C. The cell–PBS suspensions with 0.1% Triton X-100 were determined as the positive control, and the absorbance value of supernatant was analysed at 576 nm. The results were the average of three independent trials.

2.8. Determination of critical vesicular concentration (CVC)

The critical vesicular concentration (CVC) refers to the lowest concentration of copolymers where they can self-assemble into vesicles. Different concentrations (ranging from 500 μg/mL to 0.5 μg/mL) of K$_{10}$-*b*-F$_n$-*b*-K$_{10}$ vesicle solutions were prepared by two-fold dilution with deionized water. Then, pyrene (3.0 mg), serving as the probe to detect the formation of vesicles, was dissolved in 25.0 mL of acetone. Eleven centrifuge tubes were prepared and each was added with 10 μL of pyrene/acetone solution. After kept open overnight to remove acetone, 4.0 mL of vesicle solutions at each concentration were added into centrifuge tubes with continuous stirring for 12 h. Each sample was measured by fluorescence spectroscopy (excitation at 334 nm and emission from 350 to 500 nm). The intensity at 371.9 nm was chosen as the evaluate indexs. Then, the graph (the intensity *vs.* the log of each concentration) was drawn with two linear fitting lines. The CVC value was determined by calculating the intersection of two linear fitting lines.

2.9. Characterization

2.9.1. ^{1}H NMR

Bruker AV 400 MHz spectrometer was applied to record the ^{1}H NMR spectra with DMSO-d_6 or D_2O as solvent and TMS as standard at room temperature.

2.9.2. Dynamic light scattering

Nano-ZS 90 Nanosizer (Malvern Instruments, Ltd., Worcestershire, UK) was used to study the DLS of triblock copolypeptides vesicles at a fixed scattering angle of 90°.

2.9.3. ζ-Potential

ζ-Potential studies were performed at 25 °C on a Zeta Sizer Nano series instrument (Malvern Instruments, Malvern, UK) equipped with a multipurpose titrator (MPT-2).

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