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Construction and Antibacterial Study of Targeted Peptide Nanodrug Delivery System

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Abstract: *The discovery of broad-spectrum antibiotics has greatly reduced human mortality due to bacterial infections, but the widespread use of broad-spectrum antibiotics in the past 20 years has led to the emergence of multiple drug resistance (MDR) pathogens, making bacterial infection modern serious problems facing medicine. In addition, the abuse of broad-spectrum antibiotics can also cause diseases such as intestinal flora imbalance, colitis, and candidiasis. Therefore, it is of great significance to develop new targeted antibacterial drugs, which can effectively treat bacterial infections while reducing the generation of drug-resistant bacteria and avoiding the imbalance of microbiota in patients. Based on this, we designed a temperature-responsive nano-drug delivery system containing antimicrobial peptides. The system uses gold nanostars as carriers to couple targeted antimicrobial peptides and loads them into thermosensitive hydrogels. It can form a gel according to the increase in the ambient temperature, and the photothermal effect is stable. In vitro antibacterial experiments show that the system has an excellent killing ability for StaphylococcuS.aureus. The method reported in this article hopes to provide a certain reference value for future nanoparticle antibacterial research.*

Keywords: *Targeted Peptide Nanodrug, Photothermal therapy, Temperature sensitive hydrogel*

1. Introduction

S. aureus, the pathogen of gram-positive bacteria, is a common cause of surgical site infection (SSI). Chronic wound infection caused by it is a global medical and health problem. In addition, antimicrobial resistance (AMR) pathogens caused by traditional treatment methods such as the use of antibiotics have been widely spread in 22 countries. In the United States, AMR bacteria infect 2 million people every year. These infections cause health care costs of 20 billion dollars [1-2]. For people with low immunity such as newborns, HIV patients, diabetes patients [3] and cancer patients treated by chemotherapy [4], this bacterial infection will be more serious and fatal [5]. The World Health Organization classifies AMR bacteria as an imminent danger [6]. Therefore, it is urgent to develop new antibiotics that can avoid bacterial resistance to establish effective treatment measures for wound bacterial infection.

There are two main ways for bacteria to develop antibiotic resistance. One is through the internal mechanism: (1) genetic strategy, gene mutation and horizontal gene transfer (HGT) to obtain exogenous DNA encoding the determinants of drug resistance, and produce enzymes that make antibiotics lose their antibacterial activity, or change the target of antibiotic action [7]; (2) reduce the intake of antibacterial drugs. This behavior can be triggered by the high expression of the bacterial drug efflux pump, and the drugs will be discharged out of the cell before entering the target site of the bacterial cell. It can also change the permeability of the cell membrane, reducing the entry of antibiotics into the cell [8]. The other is drug resistance through biofilm protection. The main function of biofilm is to gradient reduce the concentration of antibiotics and antibacterial agents in the biofilm, slow down drug penetration, and reduce the diffusion rate of antibacterial drugs, so that most drugs can not completely penetrate the bottom of the biofilm, and can not eliminate the biofilm [9]. In vitro and in vivo experiments show that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of biofilm bacteria are usually 10~1000 times [10-12] of planktonic bacteria. Therefore, biofilm plays an important role in the obstinacy of wound bacterial infection.

The antibacterial activity of metals or metal compounds has been fully reported, and metal nanoparticles such as gold [13], silver [14], copper [15], titanium [16], and zinc [17] have been proven to play an antibacterial role in wound biofilm infection. Gold nanoparticles are highly sought after by scientists because of their excellent photothermal properties, antibacterial properties, drug-loading capacity, and biocompatibility. Compared with macro Au, Au NPs under micro conditions have more

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unique physical and chemical characteristics, such as photothermal properties. It is reported that PTT will not induce bacterial drug resistance, but can also play a combined antibacterial role [18]. Au NPs functionalized with polyethylene glycol (PEG) or polystyrene (PS) can kill S. aureus by virtue of their hydrophilicity or hydrophobicity. After NIR irradiation, their antibacterial ability can be improved by about 17% [19].

Some antimicrobial peptides have the function of targeting gram-positive bacteria. Antimicrobial peptides (AMPs) are defense tools in the innate immune system, which can provide an immediate response after pathogen invasion [20]. AMPs can reduce the risk of bacterial resistance and have a strong antibacterial effect. They are candidate drugs to replace antibiotics. However, AMPs have high cytotoxicity, short half-life, and poor permeability to biofilm, resulting in the very limited clinical application of AMPs [21]. Therefore, modification of AMPs to obtain more effective and safer antibacterial drugs is a hot spot of current research.

In this paper, we designed a temperature-sensitive nano drug loading system: using gold nanostar as the carrier, the targeted antibacterial peptide, and its coupling were combined to form AuNs PEG AMPs nanoparticles, and AuNs PEG AMPs were embedded into Pluronics F127/F68 temperature sensitive hydrogel to form AuNs PEG AMPs Gel, which can quickly gel under body temperature and help to attach to skin wounds. It was found that the nano-drug loading system realized the sustained release of AuNs PEG AMPs nanoparticles, and also showed better biosafety. In addition, the system also has the function of the temperature response to light and heat, and has the effect of biofilm destruction, providing a promising drug-loading platform for wound bacterial infection treatment and photothermal treatment.

2. Results and Discussion

First of all, we used HEPES one-step synthesis method to prepare gold nano star nanocarrier. Next, target antibacterial peptides were synthesized by solid phase synthesis of Fmoc and labeled with near-infrared fluorescent dyes. Then, SH-PEG and antibacterial peptides were coupled with gold nanostar by using the characteristics of the gold sulfur bond to prepare AuNS PEG AMPs nanomaterials. Finally, the nanomaterial was added to the F127/F68 thermosensitive hydrogel system, so that a temperature-sensitive nano drug loading system was established. There is a tendency to deform into the solid hydrogel at 20 ℃, and the gelling temperature is lower than the body surface temperature (about 37 ℃).

Good light temperature rising performance and repeatable light temperature rising ability can better play the role of photothermal synergistic antibacterial. The aqueous solution of AuNs PEG AMPs has good photothermal properties, but the photothermal properties of the nanoparticles in the solid gel form need to be studied. Therefore, the 808 nm laser was set under the same conditions in this experiment to test the single photothermal experiment, repeated photothermal experiment, and 7-day photothermal experiment of AuNs PEG AMPs Gel nano drug carrier system. The results of a single photothermal experiment are shown in Figure 1-a. After gelling, the AuNs PEG AMPs Gel drug-carrying system still has the good photothermal ability, and the material temperature rises more than 25 after 6 min of NIR irradiation ℃; The results of repeated photothermal experiments are shown in Figure 1-b, and the AuNs PEG AMPs Gel drug loading system has the ability to repeatedly raise temperature under NIR irradiation, indicating that the material's photothermal performance is repeatable; The results of the 7-day photothermal experiment are shown in Figure 1-c. During this period of time, the AuNs PEG AMPs Gel drug loading system still has the NIR light heating capacity, and the photothermal capacity does not weaken with the extension of time, indicating that the photothermal performance of this material is stable.

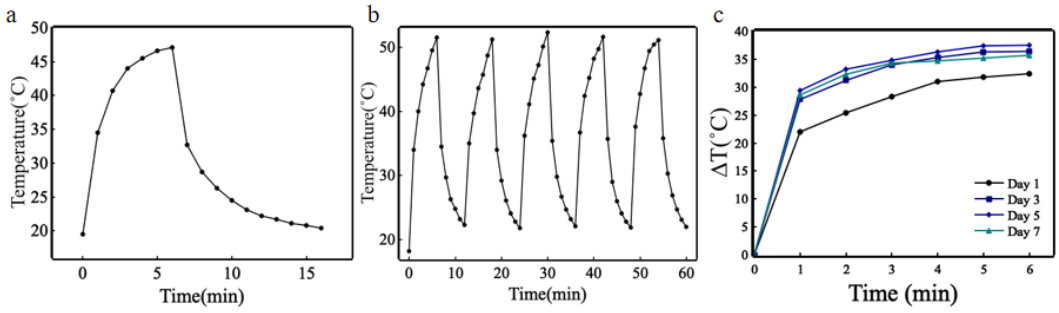

Figure 1: a) Single heating and cooling curve of AuNs-PEG-AMPs-Gel; b) Multiple heating and cooling curves of AuNs-PEG-AMPs-Gel; c) Solar thermal stability of AuNs-PEG-AMPs-Gel in seven days

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The ability of drug release will determine the duration and effect of the drug on the wound. Therefore, we conducted an in vitro drug release experiment on the drug loading system under the simulated environment with 37℃ 150 rpm and PBS as a buffer solution, the AuNs PEG AMPs Gel drug loading system can rapidly release AuNs PEG AMPs nanoparticles within 0-6 hours. As shown in Figure 2, this nanosystem can release about 53.2% of the nanoparticles within 4h. This ability to rapidly release drugs mainly achieves a rapid bactericidal effect in antibacterial treatment; The total amount of nanoparticles released by AuNs PEG AMPs Gel drug loading system was 79.9% in 12 hours, after which the drug release rate slowed down significantly, and the total amount of nanoparticles released was 92.4% in 48 hours at the end of the experiment. The sustained release effect of the drug can play an antibacterial and bacteriostatic role in the late stage of the antibacterial treatment.

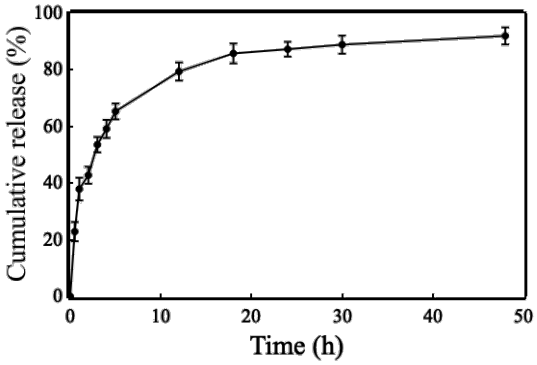

Figure 2: AuNs-PEG-AMPs-Gel drug release curve

In this drug carrier system, targeting antimicrobial peptides is a key factor in the antibacterial process. Based on this, we focused on the in vitro multi-strain antibacterial coating experiment of AuNs PEG AMPs and AuNs PEG AMPs Gel to explore their antibacterial ability and targeting. In terms of anti-StaphylococcuS.aureus, the experimental results of AuNs PEG AMPs and AuNs PEG AMPs Gel are shown in Figure 3 and Figure 4. AuNs PEG AMPs can effectively kill StaphylococcuS.aureus, and the anti-StaphylococcuS.aureus performance of AuNs PEG AMPs nanomaterials is enhanced after adding temperature-sensitive hydrogels.

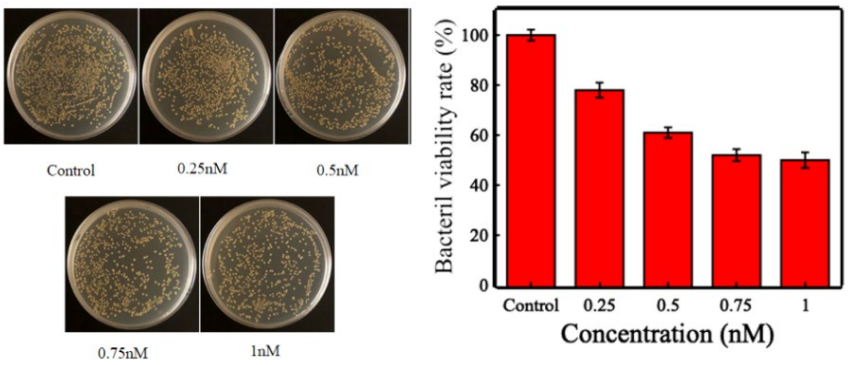

Figure 3: AuNs-PEG-AMPs anti- S. aureus coating test results

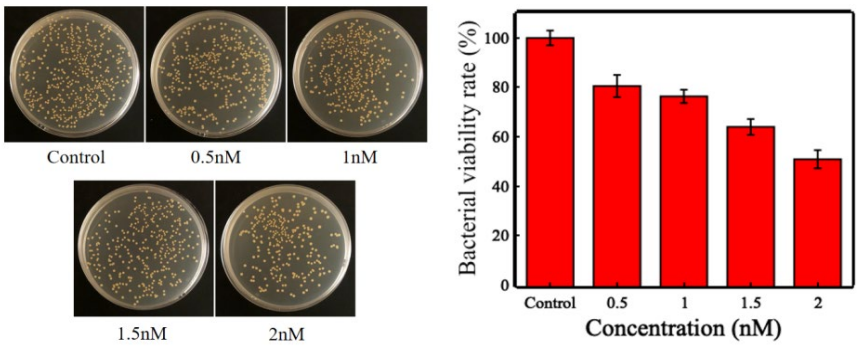

Figure 4: AuNs-PEG-AMPs-Gel anti- S. aureus coating test results

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Because there is a targeting domain (GLFVD) in the selected antimicrobial peptide sequence, which can target and identify StaphylococcuS.aureus, we speculate that the killing ability of the drug carrier system against Escherichia coli is weak. After analyzing the results of the coated plate experiment of Escherichia coli, we found that neither AuNs PEG AMPs nor AuNs PEG AMPs Gel could effectively kill Escherichia coli (Fig. 5 and Fig. 6), which verified our conjecture.

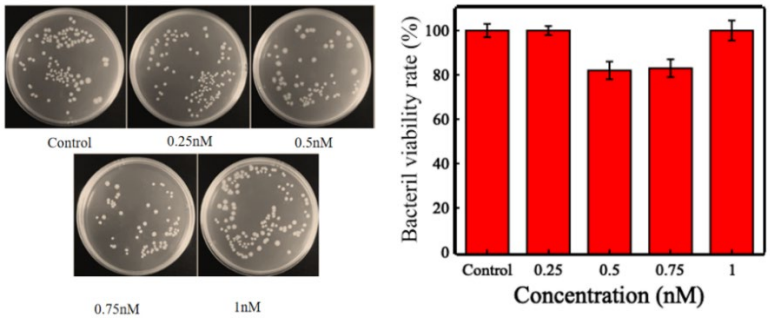

Figure 5: AuNs-PEG-AMPs anti-E.coli coating test results

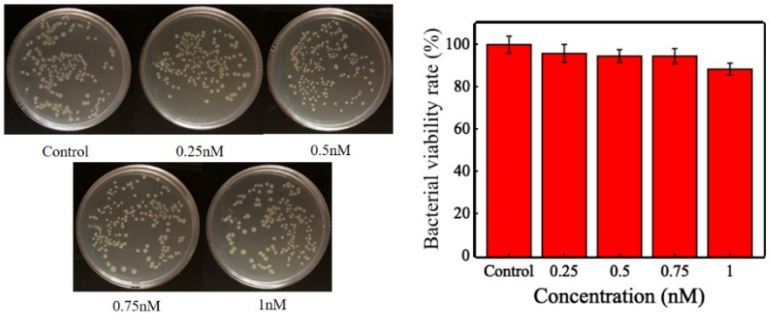

Figure 6: AuNs-PEG-AMPs-Gel anti-E.coli coating test results

In addition, because the gold nanostar in the drug-carrying system has very excellent photothermal performance, we added AuNs PEG AMPs and AuNs PEG AMPs Gel to the staphylococcuS.aureus bacterial solution respectively and then irradiated them with 808nm laser under the same conditions. The experimental results are shown in Figure 7 and Figure 8. The drug carrier system shows excellent photothermal performance, heats up rapidly in a short time, and kills StaphylococcuS.aureus greatly.

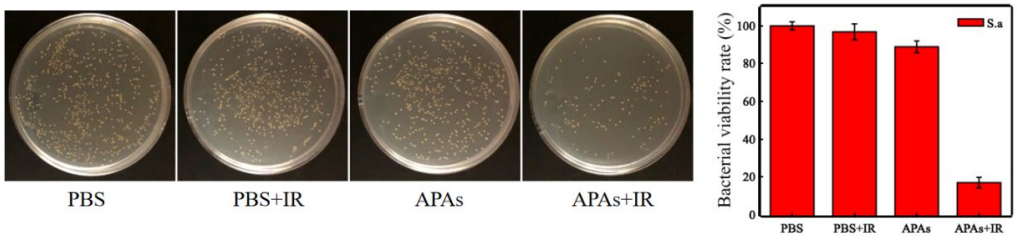

Figure 7: AuNs-PEG-AMPs photothermal resistance S. aureus coating test results

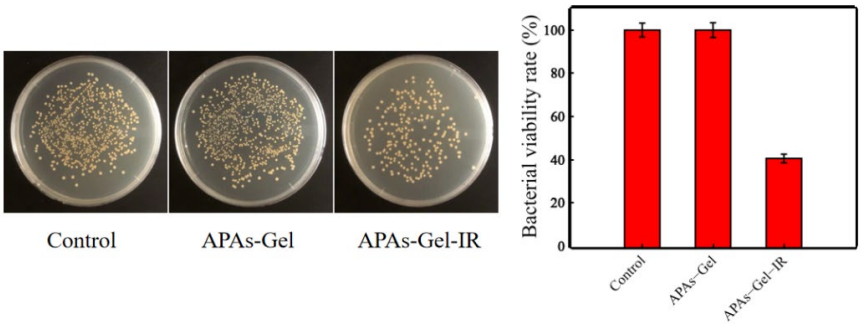

Figure 8: AuNs-PEG-AMPs-Gel photothermal resistance S. aureus coating test results

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3. Conclusion

In summary, we designed a targeted polypeptide nano drug loading system AuNs PEG AMPs Gel based on the main line of enhancing the antibacterial ability of targeted antimicrobial peptides AMPs through nano drug loading system. Through characterization and in vitro experiments, we conducted a series of studies on the synthesis, antibacterial performance, biofilm destruction inhibition ability and biological security of AuNs PEG AMPs Gel. We found that AuNs PEG AMPs and AuNs PEG AMPs Gel have good targeted antibacterial ability against S. aureus, which solved the problem of off-target antibacterial effect caused by a high concentration of AMPs. The photothermal effect produced by the irradiation of a 1.8 W/cm^2 808 nm laser can enhance the effect on S The killing effect of aureus can improve the comprehensive antibacterial ability. At the same time, AuNs PEG AMPs Gel has good biological safety, which solves the problem of high cytotoxicity of AMPs. It is hoped that our research can help the clinical S Aureus leads to the problem of antibiotic resistance in wound infection, providing a new solution and a complete preparation method.

4. METHODS AND EXPERIMENTS

4.1. Materials.

808 nm laser (MDL-N-808-5 W) was purchased from Changchun Xinrui Laser Technology Co., Ltd. HPLC (LC-8A) was purchased from Shimadzu Co., Ltd. Cyanine 7(Cy7), AuNs and peptide sequence (NH_2-GLFVDKGKRWWKWWRRGC-COOH) were synthesized in our own laboratory. Pluronics F127 and Pluronics F68 (Sigma).

4.2. Preparation of AuNS.

Before the start of the experiment, all vessels needed to be used were washed thoroughly with aqua regia (HNO_3: HCl = 1:3) in their current formulation, after which it was rinsed repeatedly with double distilled water. After completing the preparation, 1g of $AuCl_4 \cdot 4H_2O$ was taken into a washed glass bottle and added to 100 mL of ultrapure water and vortexed in the dark to obtain a chloroauric acid solution with a concentration of 40 mM and stored at 4 °C in the dark. 3 g of HEPES powder was weighed, transferred to a pear-shaped flask, precisely added 85.2 ml of ultrapure water using a 5 mL pipette, dissolved by stirring under sonication, and used for further use. 0.4 g of NaOH was weighed, transferred to a 15 mL centrifuge tube, 10 mL of ultrapure water was added, and a NaOH solution with a concentration of 1 M was obtained after vortexing, from which 4.8 mL in HEPES solution was removed and stirred well. This mixed solution was subjected to pH determination using a corrected pH meter, which could be added dropwise in small amounts using the remaining NaOH solution to adjust the solution pH to around 7.4, resulting in a HEPES buffer.

4.3. Preparation of AuNS-PEG-AMPs.

In this paper, the Fmoc solid phase synthesis method is used to artificially synthesize the targeted antibacterial peptide, with MBHA resin as the solid phase carrier. The synthesis steps are as follows:

1) Weigh 200 mg of resin and put it into the reactor. Add an appropriate amount of N, N-dimethylformamide (DMF) to completely immerse the resin. After magnetic stirring for 2 hours, the resin will be fully swelled and drained. Add 20% piperidine DMF solution (v/v) to cut the amino protective group, stir for 5 min, drain, repeat for 8 times, and rinse with DMF for 3 times. Use a glass tube to absorb a small amount of resin, and then drop ninhydrin, pyridine and phenol solutions respectively. Use a drying gun to heat for a few seconds, and judge the removal of amino protective group of the resin by color reaction. If the solution quickly turns blue purple, it indicates that the protective group has been removed.

2) Weigh 5 times (0.4 mmol/g × 0.2 g) molar amount of Fmoc Cys OH is put into a 5 mL glass bottle, and an equal molar amount of condensation agent (HOBt, HBTU) is added to dissolve it in 4 mL DMF, and 400 μL Organic base DIEPA, shake and dissolve until the solution is clear, add it into the reactor with resin, and stir the first amino acid in the sequence for 2 h (other amino acids react for 45 min). Absorb a small amount of resin with a glass tube for color reaction. The resin is colorless. Drain the reactor and rinse it with DMF three times.

3) Repeat the above protective group removal, color reaction and step 2) to complete the synthesis of the targeted antibacterial peptide GLFVDK (Mtt) GKRWWKWWWRRGC, retain the Fmoc protective

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group of the last amino acid, wash it with isopropanol and n-hexane respectively for three times after being pumped dry, and continue to pump dry until the resin is dried. Weigh about 60 mg of resin, and put the rest into a refrigerator at - 20 ℃ for sealing. Add the above 60 mg resin into the reactor, add appropriate amount of DMF, soak and stir for 30 min to make it fully swollen again. Drain the reactor, use the solution of VDCM: V acetic acid=9:1, add it into the reactor and stir it. After reaction for 3 minutes, drain it and repeat for 6 times; Use the solution of VDCM: VTFA: VTIS=94:1:5, add it into the reactor, stir it, react for 5 min, then drain it, and repeat for 9 times. The DMF was flushed for 5 times and drained. At this time, the Mtt protective base had been removed.

4) Weigh Cy7 (12 mg), HOBt (72 mg), EDC (96 mg), add DMF (1.2 mL) and DIEA (30 μL) After full ultrasonic dissolution, it is added into the reactor and stirred to avoid light reaction. After 12 h, the resin and solution in the reactor were blue and black, and the synthesis of targeted antibacterial peptide GLFVDK (Cy7) GKRWWKWWWRRGC was completed. Rinse repeatedly with DMF until the solution is colorless. Use 20% piperidine DMF solution (v/v) to remove the final Fmoc protective group, and the resin will change from blue black to pink. After the reaction, wash it with DMF, isopropanol and n-hexane for three times respectively, and then put it into a vacuum drying oven for further drying after being pumped dry.

5) Take H_2O (50 μL), TFA (1.88 mL), TIS (20 μL) And EDT (50 μL) Shake it evenly to complete the preparation of polypeptide cutting solution. Put the fully dried resin into a 5mL glass bottle to avoid light reaction. After 3 hours, the reaction solution will be took out and filtered, the volatile component strong acid nitrogen will be blown away, and anhydrous ice ether will be dropped at a constant rate to separate out white flocs, and then washed repeatedly with anhydrous ice ether, and centrifuged at 3000 rpm at 4 ℃ for 10 minutes. Collect the precipitate, blow nitrogen until it is completely dry, obtain crude AMPs, and weigh them.

6) Dissolve the crude product in 2 mL ultrapure water and 2 mL acetonitrile mobile phase, centrifuge at 8000 rpm at room temperature for 10 min, collect the supernatant, 0.22 μM Organic membrane filtration. High performance liquid chromatography (HPLC) was used to perform gradient elution with ACN mobile phase containing 0.1% TFA and ultrapure water. At 220 nm and 749 nm, the main peak products were collected in the dark to complete purification and separation. After the collected solution is subjected to spin evaporation and freeze-drying in the dark, a pure blue powder AMPs is obtained. A small amount of AMPs is taken to identify the molecular weight of peptides, and the rest are stored in the dark at - 80 ℃.

4.4. Preparation of AuNs PEG AMPs Gels

First, prepare AuNS by HEPES one-step synthesis method, weigh 1g SH PEG (Mw=5000 Da), completely dissolve it in 1mL ultrapure water, and obtain 1mg/mL SH PEG aqueous solution. Add to 10 mL of AuNs solution (0.2 nM) of 5 μL SH-PEG solution (1 mg/mL), shaking reaction in dark for 30 min. Weigh 0.5 mg of pure AMPs, add it to this reaction solution, and shake it away from light. After 12 h, centrifuge the reaction solution at 13000 rpm at 4 ℃ for 15 min. Collect the precipitate, resuspension it with ultrapure water, collect the precipitate by repeated centrifugation, finally add 1 mL ultrapure water, resuspension it to obtain 2 nM AuNs PEG AMPs solution, and place it in a 4 ℃ refrigerator for sealing and storage in the dark. 2 nM AuNs PEG AMPs solution 2 mL, add 0.52 g F127, 0.08 g F68, fully stir and put into 4 ℃ refrigerators for 12 h. 2 nM AuNs PEG AMPs Gels were obtained.

4.5. Photothermal performance test of AuNs PEG AMPs Gel drug loading system

At 4 ℃ Take down the drug loading system loaded with 2 nM AuNs PEG AMPs 200 μL. Transfer to a 0.5 mL centrifuge tube, fix the 808 nm NIR laser and centrifuge tube, adjust the power to 1.8 W/cm^2, and the duration of single irradiation is 6 min.

Single photothermal experiment: use a thermal imager to record the temperature changes of temperature rise and natural cooling, once a minute.

Repeated photothermal experiment: repeat the single photothermal experiment for five consecutive times to observe whether the AuNs PEG AMPs Gel drug loading system has the capability of repeated temperature rise.

Photothermal experiment on the 7th day: The first single light temperature rise experiment of AuNs PEG AMPs Gel drug loading system was recorded as D1, and the photothermal experiment was conducted on D3, D5 and D7 respectively. To observe whether the photothermal capacity of AuNs PEG

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AMPs Gel drug loading system is stable for a long time.

In vitro drug release performance of AuNs PEG AMPs Gel drug delivery system

Start the thermostatic shaker and set the parameter to 37 ℃. 150 rpm, preheating for 30 min. Start the thermostatic water bath and set the temperature to 37 ℃. Preheat for 30 min. Prepare 10 mL PBS buffer solution into a 15 mL centrifuge tube, seal the centrifuge tube containing PBS into a preheated thermostatic water bath, and heat PBS to 37 ℃. For standby, the validity of PBS after heating is 12h. After expiration, pour it into the waste liquid tank, prepare a new PBS buffer solution, and repeat the above steps.

Take 1 mL of AuNs PEG AMPs Gel with a concentration of 0.2 nM and transfer it to a 10 mL centrifuge tube ℃ After the gel is completely formed in the water bath pan, slowly add 3 mL 37 ℃ PBS buffer to put into the shaker. At this moment, the time point is recorded as 0 h. The liquid level is completely separated from the gel. The PBS buffer acts as the drug-release solution. Take out 1 mL of the release solution at the following time points (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 18 h, 24 h, 30 h, 48 h), and slowly replenish the same amount of fresh 37 ℃ PBS. Divide the released liquid into 96 holes, 200 μL for each hole. Use the microplate reader to measure the OD value at 740 nm wavelength. According to the concentration standard curve of AuNs PEG AMPs, the cumulative drug release at this time point was calculated.

Antibacterial coating test of AuNs PEG AMPs and AuNs PEG AMPs Gel

Use sterilized sterile ultrapure water to prepare 2 nM, 1.5 nM, 1 nM, 0.5 nM AuNs PEG AMPs aqueous solution for standby.

Anti S.aureus coated plate test: take the logarithmic growth period S.aureus broth, fresh TSB broth diluted to 1×10^8 CFU/ mL. Each concentration (2 nM, 1.5 nM, 1 nM and 0.5 nM) AuNs PEG AMPs solution/AuNs PEG AMPs Gel solution and sterile PBS were taken 100 μL. Add to 1.5 mL sterile centrifuge tube, and add 10^8 CFU/mL S. aureus bacterial solution 100 μL respectively. Shake well and put in 37 ℃ Incubate in a 250 rpm shaking table, take it out and transfer it to the ultra clean workbench after 4 hours, dilute it 80000 times with TSB broth, and take 100 μL on the TBA agar plate, apply evenly with the applicator, and then put in 37 ℃ In the biochemical incubator, there are three parallel samples in each group. After 12 hours, count the number of colonies.

Test of anti E. coli coated plate: take E Coli bacterial liquid, fresh LB broth diluted to 1×10^8 CFU/ mL. Each concentration (2 nM, 1.5 nM, 1 nM and 0.5 nM) AuNs PEG AMPs solution/AuNs PEG AMPs Gel solution and sterile PBS were taken 100 μL. Add to 1.5mL sterile centrifuge tube, and add 10^8 CFU/mL E. coli bacterial solution 100 μL respectively. Shake well and put in 37 ℃ Incubate in a 250 rpm shaking table, take it out and transfer it to the ultra clean workbench after 4 hours, dilute it 80000 times with LB broth, and take 100 μL put on LB agar plate, and the applicator is put into 37 ℃ In the biochemical incubator, there are three parallel samples in each group. After 12 hours, count the number of colonies.

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