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Functionalization of Polyvinyl Alcohol Composite Film Wrapped in $a_m$-ZnO@CuO@Au Nanoparticles for Antibacterial Application and Wound Healing

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Abstract

Over recent years, researchers have been intensively investigating advanced antibacterial agents. However, the drug resistance in bacteria remains to be a challenging problem for wound healing. Therefore, it is urgent to find a novel effective antibacterial therapy to enhance wound healing. Herein, we reported a new antibacterial film (PVA@$a_m$-ZnO@CuO@Au) which showed heat, reactive oxygen species (ROS), Zn$^{2+}$, and Cu$^{2+}$-induced disinfection. An antibacterial efficacy of 98.7% against $E.\ coli$ and 97.5% against $S.\ aureus$ was obtained by PVA@$a_m$-ZnO@CuO@Au film under red laser irradiation for 10 min, with the combined effect of photothermal, photodynamic, and released Zn$^{2+}$ and Cu$^{2+}$. In vivo wound healing and tissue compatibility studies were carried out over a period of 10 days on skin wounds. Fast healing was observed in PVA@$a_m$-ZnO@CuO@Au film treated wounds with minimum scarring. PVA@$a_m$-ZnO@CuO@Au film also possessed sustained antibacterial properties due to the large embedded depth of $a_m$-ZnO@CuO@Au NPs. The technique and resulting
materials have large potential for applications in wound healing.

Keywords: wound healing; antibacterial; disinfection; photothermal; photodynamic

1. Introduction

The variety and rapid variability of pathogenic microorganisms make antibacterial therapy face enormous challenges [1-3]. The problem of drug resistance has been worsened in recent years. For instance, the emergence of "superbugs" resistant to several antibiotics has caused alarm and even panic around the world [4,5]. Therefore, there is an urgent need to develop new effective antibacterial methods. Nanometer antibacterial material is a kind of functional material, which is prepared by making antibacterial agent into nanometer scale or connecting antibacterial agent with nanometer carrier [6-8]. It has been reported that Zn$^{2+}$ and Cu$^{2+}$ are widely used as antibacterial agents due to their low toxicity and high biosafety [9-11]. However, disadvantages of discoloration, narrow antibacterial spectrum, poor long-term durability, poor heat resistance and stability have hindered their further development. In contrast, nanomaterials (NMs) such as copper oxide (CuO) and zinc oxide (ZnO) nanoparticles (NPs) can overcome these problems to some extent [12-13]. Recently, with the development of nanotechnology, a series of low-dimensional structure CuO@ZnO hybrid NMs has emerged, which provided great space for the development of CuO and ZnO-based antibacterial materials with lower toxicity and longer effective time [14,15]. Thus, it has been the hotspot to improve the antibacterial activity of NMs by the synergistic effect generated via coupling hybrid nanomaterials.

Antimicrobial phototherapies, such as photothermal and photodynamic, have broad-spectrum antibacterial properties and show great potential for the treatment of bacterial infections [16,17]. Based on nanotechnology, the form of nano-antibacterial technology by NMs with photothermal conversion and photodynamic effects have drawn increasing attention [18]. Upon the excitation by light source at specific wavelength, heat
and reactive oxygen species (ROS) are generated, which can kill the bacteria as a result of protein denaturation, enzyme inactivity and imbalance of metabolic activity [19]. The damage mechanism of ROS to bacteria can be mainly concluded into two pathways: (1) destroying the plasma membrane permeability to make some substances flow out, or affecting the metabolic activities of the bacteria; (2) breaking and disaggregating DNA strands, generating stable oxidation products [20-22]. Photo antibacterial technology has high level of sterilization, good safety and lower risk in causing the emergence of drug-resistant bacteria [23,24]. Therefore, it is a new therapy with broad application prospects, and this technology will make a breakthrough with the emergence of new photothermal agents and photosensitizers.

It is necessary for wound healing to minimize infections at wound sites [25,26]. Furthermore, it is quite important to choose NMs with good biocompatibility to participate in antibacterial activity to accelerate wound healing. Wound healing is a complex, dynamic, and sequential process associated with coagulation, inflammation, angiogenesis, tissue formation, and tissue remodeling. Various photothermal agents such as Au nanoparticles (AuNPs), transition metal sulfides, carbon nanotubes (CNTs), and graphene oxide (GO) have been reported [27-30]. Among them, AuNPs are preferred due to their high absorption ability in the red light region and excellent biocompatibility. Meanwhile, a strong photothermal conversion capability can be achieved through adjusting their morphology and size to make the plasmon resonance absorption peak move to the red light region, thereby increasing the local temperature and killing bacteria [31,32]. CuO and ZnO NPs have been considered as powerful photosensitizers among various semiconductor materials [33,34]. However, the application of CuO and ZnO NPs as photocatalysts is hampered by two constraints: limited light utilization conferred by a wide band gap, and low quantum efficiency due to the recombination of photo-generated electrons and holes. Coupling CuO or ZnO NPs with AuNPs has been envisioned as an effective strategy to overcome these barriers. Hence, an approach to obtain uniform
CuO@ZnO hybrid NMs with AuNPs in each CuO@ZnO hybrid NMs by the in situ reduction process is needed.

Fig. 1. Schematic illustration of PVA antibacterial film embedded with $a_m$-ZnO@CuO@Au NPs for antibacterial wound dressings.

Taking the above development and their limitations into consideration, we utilized a reported strategy to prepare a hybrid multi-shelled hollow materials with binary compositions of ZnO@CuO@Au NPs, based on copper-aided oxidative degradation of an amorphous ($a_m$) zeolitic imidazolate frameworks (ZIFs). Subsequently, the $a_m$-ZnO@CuO@Au NPs were embedded in polyvinyl alcohol (PVA) film (PVA@$a_m$-ZnO@CuO@Au) to achieve great antibacterial activity. In contrast to cancer therapy, simultaneous antibacterial therapy has seldom been reported. By taking advantage of the photothermal and photodynamic effects of $a_m$-ZnO@CuO@Au NPs, angiogenesis of Zn$^{2+}$ and Cu$^{2+}$, and moist conditions, the materials offered antibacterial activity and tissue regeneration capability at the same time (as shown in Fig. 1). The different antibacterial characteristics of PVA@$a_m$-ZnO@CuO@Au film and subsequent
biocompatibility were systematically assessed both \textit{in vitro} and \textit{in vivo}.

2. Results and discussion

2.1 Fabrication and characterization of the $a_m$-ZnO@CuO@Au NPs

A hybrid multi-shelled nanoparticles ($a_m$-ZnO@CuO@Au NPs), based on copper-aided oxidative degradation of an amorphous zeolitic imidazolate frameworks ($a_m$-ZIFs), is illustrated in Fig. S1. Firstly, $a_m$-ZIFs in rhombic dodecahedral form was synthesized by directly mixing zinc nitrate aqueous solution with ICA methanolic solution at room temperature for 45 min. Next, CuNPs with an average size of 3.1 ± 0.4 nm and AuNPs with an average size of 2.9 ± 0.6 nm were introduced to $a_m$-ZIF ($a_m$-ZIF@Cu@Au NPs) through an in situ reduction method by using Cu(acac)$_2$ as a Cu source, HAuCl$_4$ as a Au source and tetrabutylammonium borohydride (R-NBH$_4$) as a reducing agent. Finally, the obtained $a_m$-ZIF@CuO@Au NPs was calcined in 280 °C tube furnace to form $a_m$-ZnO@CuO@Au NPs. TEM was employed to investigate the shape features, surface morphology and size determination of the as-synthesized $a_m$-ZIF NPs, $a_m$-ZIF@Cu@Au NPs, and $a_m$-ZnO@CuO@Au NPs. As shown in Fig. 2A, $a_m$-ZIF NPs have a unique sphere structure with the average size of 550 nm. By the in situ synthesis of $a_m$-ZIF@Cu@Au NPs, the NPs was covered with AuNPs of varying sizes (Fig. 2B). As shown in the TEM images (Fig. 2C,a,b), by calcining $a_m$-ZIF@Cu@Au NPs at 280 °C, well-defined multi-shelled hollow NPs with an average hydrodynamic diameter around 425 nm were produced. Fig. 2C,c,d shows a HR-STEM image of $a_m$-ZnO@CuO@Au NPs, and the corresponding Au, Zn, and Cu element maps are shown in Fig. 2D, respectively. The corresponding EDX spectrum (Fig. S2A) shows characteristic energy lines of Cu, Zn, Au, and O arising from the CuO, ZnO, and AuNRs. Additionally, the XRD pattern (Fig. S2B) further supports the formation of $a_m$-ZnO@CuO@Au hybrid structures.
Fig. 2. (A) TEM images of am-ZIF NPs. (B) TEM images (a,b) and HR-TEM (c) of am-ZIF@Cu@Au NPs. (C) TEM images (a,b) and HR-TEM (c,d) of am-ZnO@CuO@Au NPs. The lattice spacing of 0.23 nm is attributed to the facet of Au. (D) TEM images (a) of am-ZnO@CuO@Au NPs and their corresponding EDS elemental mapping images (b-e). The lattice fringes can be resolved, which are attributed to ZnO and CuO, respectively. (E) The SEM images (a) of am-ZnO@CuO@Au NPs and their corresponding EDS elemental mapping images (b-g).

2.2 Fabrication and characterization of the PVA@am-ZnO@CuO@Au NPs film

After radical polymerization, the functional am-ZnO@CuO@Au NPs were embedded in a PVA film. As shown in Fig. S3, the surface of the pure PVA film was flat. After the incorporation of functional am-ZnO@CuO@Au NPs, many NPs were found to be incorporated into the PVA film and their uniform distribution was confirmed by EDS
elemental mapping (Fig. 2). The corresponding EDX spectrum (Fig. S4) shows characteristic energy lines of Cu, Zn, Au, and N arising from the $a_m$-ZnO@CuO@Au NPs. The PVA@$a_m$-ZnO@CuO@Au film showed diffraction peaks matching the free $a_m$-ZnO@CuO@Au NPs structure, further proving successful loading of $a_m$-ZnO@CuO@Au NPs into the PVA film. Furthermore, we compared the mechanical properties of pure PVA and composite PVA film containing different amount of $a_m$-ZnO@CuO@Au NPs (PVA@$a_m$-ZnO@CuO@Au 1-3) with a thickness about 0.1 cm. As shown in Table S1, PVA@$a_m$-ZnO@CuO@Au film exhibited higher tensile strength than pure PVA. Similarly, there was an increase of 25% of the Young’s modulus of the PVA-based film due to the incorporation of $a_m$-ZnO@CuO@Au NPs.

2.3 Photodynamic performance of the PVA@$a_m$-ZnO@CuO@Au film

ESR spin trapping and spin labeling are two recognized techniques for the detection of short-lived free radicals and paramagnetic species [35,36]. Here, we select 5-tertbutoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) as a spin trap for the hydroxyl radical (·OH), 4-oxo-2,2,6,6-tetramethylpiperidine (4-oxo-TEMP) for detection of singlet oxygen ($^{1}\text{O}_2$). Fig. 3A and Fig. S5 shows the ESR spectra obtained from solutions containing various spin probes and $a_m$-ZnO NPs, $a_m$-ZnO@CuO NPs, $a_m$-ZnO@Au NPs, $a_m$-ZnO@CuO@Au NPs, and PVA@$a_m$-ZnO@CuO@Au 3 film during irradiation with simulated red laser. No ESR signal was observed for spin probes $a_m$-ZnO NPs. In contrast, upon irradiation for 10 min in the presence of $a_m$-ZnO@Au NPs, $a_m$-ZnO@CuO@Au NPs, and PVA@$a_m$-ZnO@CuO@Au film, we clearly observed a four-line spectrum and there-line spectrum which was the characteristic spectrum for the BMPO/·OH and 4-oxo-TEMP/$^{1}\text{O}_2$ adduct, respectively. Compared to the EPR signal intensity of $a_m$-ZnO NPs, $a_m$-ZnO@CuO NPs, and $a_m$-ZnO@Au NPs, the BMPO/·OH and 4-oxo-TEMP/$^{1}\text{O}_2$ signal intensity of $a_m$-ZnO@CuO@Au NPs increased significantly, suggesting that deposition of AuNPs onto ZnO and CuO significantly enhanced
photogeneration of ·OH and \(^1\text{O}_2\). Interestingly, when \(a_m\)-ZnO@CuO@Au NPs were embedded into PVA film, the BMPO/·OH and 4-oxo-TEMP/\(^1\text{O}_2\) signal intensity of PVA@\(a_m\)-ZnO@CuO@Au 3 film was similar to \(a_m\)-ZnO@Au NPs.

**Fig. 3.** (A) ESR spectra obtained from solutions containing BMPO/4-Oxo-TEMP and PVA@\(a_m\)-ZnO@CuO@Au film after irradiated with red laser irradiation. (B) The expected reaction mechanism for the enhancement effect on generating ROS. (C) The irradiation time-dependent temperature elevation of different samples. (D) IR images of different samples (a, \(a_m\)-ZnO@CuO@Au NPs solution; b, pure PVA film; c-e, PVA@\(a_m\)-ZnO@CuO@Au 1-3 film) under red laser irradiation. (E) Water contact angles of pure PVA film and PVA@\(a_m\)-ZnO@CuO@Au film. (F) Cumulative amounts of Cu\(^{2+}\) released from PVA@\(a_m\)-ZnO@CuO@Au film with and without red laser irradiation.

The mechanism of ROS generation induced by the photodynamic \(a_m\)-ZnO@CuO@Au NPs is schematically displayed in **Fig. 3B**. For \(a_m\)-ZnO@CuO@Au NPs, the observed enhanced ROS generation may result from the following effects of AuNPs: (1) enhancing light absorption because of the SPR of Au; (2) changing the band gap of ZnO/CuO and thereby promoting reactivity of photoinduced charge carriers, and (3) increasing the efficiency of electron transport and charge carrier separation \cite{37,38}. This indicated that increased absorption of light through the SPR of AuNPs did play a role in the enhanced photoactivity observed for the \(a_m\)-ZnO@Au and \(a_m\)-ZnO@CuO@Au HNSs. The
photo-induced electrons transfer from AuNPs to ZnO/CuO conduction band after exposure to red laser irradiation. The transferred electrons in the conduction band and the holes in the valence band continuously induce a series of photochemical reactions to generate ROS at a\textsubscript{m}-ZnO or CuO surfaces in the aqueous solution. On the contrary, on the surface of pristine AuNPs, red laser irradiation produced photoelectrons and Auger electrons, which subsequently participated in ROS-generating reactions. However, a\textsubscript{m}-ZnO@CuO and a\textsubscript{m}-ZnO did not play exclusively separate roles in ROS generation during photochemical reactions, and the simple stacking of each effect from Au and a\textsubscript{m}-ZnO@CuO was insufficient to explain the great enhancement in the observed ROS yield.

2.4 Photothermal performance of the PVA@a\textsubscript{m}-ZnO@CuO@Au NPs film

The absorption spectra of a\textsubscript{m}-ZnO@CuO@Au NPs and PVA@a\textsubscript{m}-ZnO@CuO@Au film showed a strong broad red light absorption (Fig. S6). We initially investigated the temperature changes of am-ZnO@CuO@Au and PVA@a\textsubscript{m}-ZnO@CuO@Au film under 2.0 W/cm\textsuperscript{2} of 635 nm laser irradiation. The temperature of a\textsubscript{m}-ZnO@CuO@Au NPs increased to 56.4 °C after 5 min of irradiation, confirming the fast and high-efficient photothermal conversion capacity of am-ZnO@CuO@Au NPs. The temperature of the PVA@a\textsubscript{m}-ZnO@CuO@Au film could be accurately regulated to increase from 13.5 °C (PVA@a\textsubscript{m}-ZnO@CuO@Au 1) to 27.2 °C (PVA@a\textsubscript{m}-ZnO@CuO@Au 3) under irradiation for 10 min, while pure PVA film increases only increased by 3.2 °C (Fig. 3C). A thermal imaging camera was used to record the temperature variation and the results further confirmed the successful converting red light to heat (Fig. 3D).

Wound dressings should be mechanically robust and hydrophilic to obtain fine permeability, which was beneficial for wound healing [39]. Therefore, we measure the tensile strength and contact angle of pure PVA film and PVA@a\textsubscript{m}-ZnO@CuO@Au film. When we placed the water on the fibers, the water contact angle was 52.4° (wettability)
(Fig. 3E). Contact angle measurement revealed that the addition of \( a_m\)-ZnO@CuO@Au to PVA film (PVA@\( a_m\)-ZnO@CuO@Au 1, PVA@\( a_m\)-ZnO@CuO@Au 2, and PVA@\( a_m\)-ZnO@CuO@Au 3) retained the hydrophilicity of PVA film. The results of water absorption experiments for PVA film and PVA@\( a_m\)-ZnO@CuO@Au film are shown in Table S2. With the increase of \( a_m\)-ZnO@CuO@Au NPs content, the water absorption of PVA@\( a_m\)-ZnO@CuO@Au film was increased compared with pure PVA film. This was because the addition of functionalized \( a_m\)-ZnO@CuO@Au NPs contributed to the slightly increase of cross-linking density of PVA composite.

2.5 Zn\(^{2+}\) and Cu\(^{2+}\) Releasing from the PVA@\( a_m\)-ZnO@CuO@Au film

Fig. 3F and Fig. S7 exhibits the cumulative concentrations of Cu and Zn ions released from the different film (PVA@\( a_m\)-ZnO@CuO@Au 1-3) to the PBS (pH 7.4) at 37 °C. The PVA@\( a_m\)-ZnO@CuO@Au 1-3 film showed a similar release behavior with slow release of Cu\(^{2+}\) and Zn\(^{2+}\). PVA@\( a_m\)-ZnO@CuO@Au 3 showed the biggest release while PVA@\( a_m\)-ZnO@CuO@Au 1 was the smallest, consistent with the embedded \( a_m\)-ZnO@CuO@Au NPs contents. The Zn\(^ {2+}\) and Cu\(^{2+}\) were formed by the dissociation of ions from the \( a_m\)-ZnO@CuO@Au NPs. To explore the effects of laser on the release of Zn\(^{2+}\) and Cu\(^{2+}\), PVA@\( a_m\)-ZnO@CuO@Au 3 was monitored with or without 635 nm laser. As shown in Fig. 3F and Fig. S7, the release of Zn\(^{2+}\) and Cu\(^{2+}\) under laser illumination were enhanced. The enhanced release of Zn\(^{2+}\) and Cu\(^{2+}\) under laser illumination was probably because of the photothermal conversion function of PVA@\( a_m\)-ZnO@CuO@Au film, which may occur during the volume shrinkage process owing to the burst release of soluble materials in film carrier.

2.6 In intro antibacterial assessment of PVA@\( a_m\)-ZnO@CuO@Au film

For wound healing, the antibacterial properties of biomaterials are a major standard for defining an eligible wound dressing [40]. To assess this ability, the antibacterial efficacy
of PVA@am-ZnO@CuO@Au film against E. coli (Fig. 4A) and S. aureus (Fig. 4B) was measured with or without 635 nm laser irradiation. Consequently, the PVA@am-ZnO@CuO@Au film exhibited low antibacterial properties in the dark against both bacteria, which was enhanced with increasing am-ZnO@CuO@Au NPs content. However, the antibacterial properties were improved enormously, and the PVA@am-ZnO@CuO@Au film possessed excellent antibacterial abilities under laser irradiation. In addition, as shown in Fig. 4C,D, the antibacterial ability of the PVA@am-ZnO@CuO@Au film increased with the increasing am-ZnO@CuO@Au NPs content under 635 nm laser irradiation, when the antibacterial rate even reached 98%, while the dark group only reached 53%. This discrepancy may be related to release Zn²⁺/Cu²⁺ to damage bacterial cell membranes, causing the cytoplasm to leak, resulting in antibacterial properties. However, 635 nm laser irradiation can induce am-ZnO@CuO@Au NPs to generate heat and abundant ROS, and accelerate Zn²⁺/Cu²⁺ release, resulting in a boost in antibacterial efficacy (Fig. 1). We next confirmed the photodynamic therapy efficacy of PVA@am-ZnO@CuO@Au film at low laser power doses. A significant enhancement in antibacterial efficacy was observed when the PVA@am-ZnO@CuO@Au film was illuminated by red laser (0.1 W/cm²) for only 10 min, which was predominantly ascribed to the rapid production of intensive ROS from PVA@am-ZnO@CuO@Au NPs after irradiation (Fig. S8A,B).

To understand the antibacterial mechanism, the interaction of PVA@am-ZnO@CuO@Au film with S. aureus and E. coli bacteria were studied under 635 nm laser irradiation by SEM. Extensive membrane damage was observed in E. coli and S. aureus treated with PVA@am-ZnO@CuO@Au film with 635 nm laser irradiation (Fig. 4E). In comparison, with pure PVA film, the typical morphology of S. aureus and E. coli with a smooth surface and spherical and rod shape were observed, respectively. The damaged morphology and cell rupture could be attributed to the binding and internalization of PVA@am-ZnO@CuO@Au film. To further verify its antibacterial
efficacy, Cy5.5/FITC staining was used to observe the adhesion of bacteria. As shown in Fig. 4F, the results indicated that as the $a_m$-ZnO@CuO@Au concentration increased, the number of adherent bacteria decreased (red is *E. coli* and green is *S. aureus*), which was consistent with the above results. Taken together, TEM and bacterial fluorescence images were clear evidence for the antibacterial effects of PV A@$a_m$-ZnO@CuO@Au film under 635 nm laser irradiation.

![Image of bacterial adhesion and fluorescence](image)

**Fig. 4.** Antibacterial efficacy (A,B) and antibacterial rate (C,D) of PV A@$a_m$-ZnO@CuO@Au film against *E. coli* (A,C) and *S. aureus* (B,D) without and with red laser (2.0 W/cm$^2$) irradiation. (E) SEM micrographs of *E. coli* and *S. aureus* treated with PV A@$a_m$-ZnO@CuO@Au film after laser irradiation for 10 min. (F) The adhered *E. coli* and *S. aureus* with laser irradiation were stained by Cy 5.5 and FITC.

### 2.7 In vitro cytotoxicity assessment of PV A@$a_m$-ZnO@CuO@Au film
The cytotoxicity of PVA@a_m-ZnO@CuO@Au film was evaluated with or without 635 nm laser irradiation [41]. Fig. 5A shows low-level biological toxicity of PVA@a_m-ZnO@CuO@Au film through culturing with NIH-3T3 fibroblast cells, even at relatively high concentrations (PVA@a_m-ZnO@CuO@Au 3). The cells were then stained with both propidium iodide (PI) and calcein AM to identify dead and live cells, respectively (Fig. 5B). The full screen green fluorescence in the region without 635 nm laser treatment, illustrated that PVA@a_m-ZnO@CuO@Au film did not cause cancer cell death without 635 nm laser irradiation. After 635 nm laser irradiation, a slightly inhibition ratios against NIH-3T3 cells occurred (Fig. S9), because of heat and ROS produced from the a_m-ZnO@CuO@Au NPs by 635 nm laser irradiation. It showed that short periods of laser could affect the cell growth, but not completely. In in vivo animal experiments, the NIH-3T3 cells proliferation of PVA@a_m-ZnO@CuO@Au film was evaluated, as shown in Fig. S10. PVA@a_m-ZnO@CuO@Au film was suitable for growth, attaining a significant level of increase in cell proliferation after 5 and 7 days of culture.

Platelet aggregation and adhesion are important ways to induce activating blood coagulation process, as well main examination indexes [42]. Platelet adhesion experiments utilized the ability of platelets to aggregate and adhere. SEM was used to observe the adhesion of platelets on the surface of the materials, and study its ability in enriching platelets. As shown in Fig. 5C, the number of adherent platelets of PVA@a_m-ZnO@CuO@Au film were significantly increased compared with pure PVA film. This result could be attributed to the strong electrostatic interaction of PVA@a_m-ZnO@CuO@Au film surface with platelets, which would affect platelet adhesion and activation, and activate platelets. Therefore, platelets on the surface of the PVA@a_m-ZnO@CuO@Au film can activate adjacent platelets to aggregate on the surface of the film, causing a series of blood coagulation reactions, thereby promoting blood coagulation and accelerating wound healing.
RBCs have been widely used in biosafety evaluations of various biomedical materials, as blood-contact biomaterials will inevitably interact with RBCs [43]. In this work, we studied the effect of PVA@am-ZnO@CuO@Au film on the morphology and lysis of RBCs to determine the interaction of the PVA@am-ZnO@CuO@Au film with RBC membrane structure. Fig. 5D shows RBCs in the presence of PVA@am-ZnO@CuO@Au film. Overall, the PVA@am-ZnO@CuO@Au film appeared to affect the RBC morphology in a concentration-dependent manner. Specifically, a few RBCs became crenated in PVA@am-ZnO@CuO@Au 3. The results showed that the PVA@am-ZnO@CuO@Au film had a slight interaction with the RBC membrane.

Hemolysis testing determines the solubility of the blood cells in contact with foreign materials and has been a powerful *in vitro* test to evaluate the hemolysis property of
wound dressing [44]. It is known that for the materials having potential applications as drug or implanted devices, a hemolysis rate of lower than 5% is required. It is seen from Fig. 5E that PVA@ZnO@CuO@Au film hemolysis percentages were far below the demanded level of 5%. As shown in Fig. 5F, the morphology of red blood cells (RBCs) did not undergo obvious changes in the presence of PVA@ZnO@CuO@Au film as compared to the negative and positive controls. These results indicated that the PVA@ZnO@CuO@Au film were safe for blood-contact applications and were suitable for wound healing.

2.8 In vivo evaluation of wound healing

To evaluate the wound healing capacity on diabetic wounds during the healing process, PVA@ZnO@CuO@Au film was applied to wound sites. The groups treated with PVA@ZnO@CuO@Au film with 635 nm laser irradiation exhibited enhanced healing after 10 days of implantation, compared to control (pure PVA only) and PVA@ZnO@CuO@Au film without red laser irradiation (Fig. 6A). Specifically, the PVA@ZnO@CuO@Au film without red laser irradiation showed better healing potential than the controls with wound areas of 77 ± 3.1% (PVA@ZnO@CuO@Au), 60 ± 2.6% (PVA@ZnO@CuO@Au 2), and 38 ± 3.2% (PVA@ZnO@CuO@Au 3) of the initial wound size at day 10 (Fig. 6B). We suspect that the PVA@ZnO@CuO@Au film released Cu²⁺/Zn²⁺ and generated heat/ROS to the wound areas to facilitate healing processes. Interestingly, the wounds treated with PVA@ZnO@CuO@Au 3 with red laser irradiation showed complete closure at day 10. Thus, combining an appropriate dressing material (PVA@ZnO@CuO@Au film) with 635 nm laser irradiation could enhance the wound healing process in vivo.
Fig. 6. (A) Photographs of the wound on the mice at 10 days. (B) Corresponding wound area at 10 days. (C) Histological photomicrographs of wound surface at different magnifications (a control; b, pure PVA film+red laser; c-e, am-ZnO@CuO@Au 1-3 film+red laser). (D) Body weight versus the number of days after am-ZnO@CuO@Au NPs treatments. (E) Histological photomicrographs of major organs after am-ZnO@CuO@Au NPs treatments.

Histological examination (H&E) of the entire wound healing process was done over a period of 10 days to reveal the healing activity of PVA@am-ZnO@CuO@Au film with red laser irradiation [45]. As shown in Fig. 6C, the wound biopsies from PVA@am-ZnO@CuO@Au film with red laser irradiation showed complete epidermal cover over the wound. Whereas, the pure PVA showed epidermal covering only at the
edges of the wound. A decrease in the period of epithelization of the treated wounds compared to pure PVA could be attributed to an increase in the rate of wound healing. However, in the deeper tissues, the pure PVA group showed granulation tissue with dilated blood vessels along with fibroblast proliferation differ from the PVA@am-ZnO@CuO@Au film with red laser irradiation, which showed more prominent fibrosis, collagenization, and small blood vessels. Therefore, it can be concluded that the biopsies at 10th wounding day showed good healing with a complete epidermal cover, and replacement of the granulation tissue with fibrosis and collagenization as part of the healing response in PVA@am-ZnO@CuO@Au film with red laser irradiation. Here, PVA@am-ZnO@CuO@Au 3 with red laser irradiation showed a greater degree of fibrosis. Pure PVA showed a more delayed healing response with granulation tissue still persisting.

2.9 In vivo cytotoxicity evaluation of PVA@am-ZnO@CuO@Au NPs

The US Centers for Disease Control and Prevention reports that NPs can penetrate the skin and into other organs from the respiratory system [42]. Therefore, the biocompatibility of am-ZnO@CuO@Au NPs needed to be evaluated in vivo. Body weight and behaviors of the treated mice were monitored to evaluate in vivo toxicities of am-ZnO@CuO@Au NPs on a daily basis. Treatment with am-ZnO@CuO@Au NPs did not induce any visible adverse reaction on the behaviors or body weight of mice during the 10 days (Fig. 6D). Finally, the pathological figures of major organs (Heart, liver, spleen, lung, and kidneys) were demonstrated by histology at day 10. As shown in Fig. 6E, no apparent damages were detected among organs, especially in the liver, spleen, and kidneys, during the entire time period.

3. Conclusion

In conclusion, a new type of PVA film embedded with functional inorganic
at-ZnO@CuO@Au NPs was prepared and characterized. It showed significant antibacterial efficacy against S. aureus (99.80%) and E. coli (97.5%) within 10 min after application, on account of the combined action of PTT, PDT, and Zn\(^{2+}\) and Cu\(^{2+}\) under 635 nm laser irradiation. The volume variation of this film can be controlled by the red laser to alter the rate of release of Zn\(^{2+}\) and Cu\(^{2+}\) to the physiological environment. In addition, this study demonstrated that the as-fabricated PVAm@at-ZnO@CuO@Au film had excellent biocompatibility, high stability at physiological conditions. In vivo wound therapeutic outcome also demonstrated that the film accelerated wound healing compared with the control groups. Therefore, the PVAm@at-ZnO@CuO@Au film is promising for use in clinical treatment of wound infection.

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**Conflict of interests**

The authors declare no conflict of interest.

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