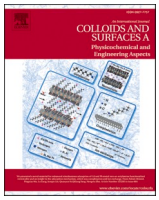
Colloids and Surfaces A: Physicochemical and Engineering Aspects 651 (2022) 129708

Contents lists available at ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

journal homepage: www.elsevier.com/locate/colsurfa



Fabrication of colloidal silver-peptide nanocomposites for bacterial wound healing

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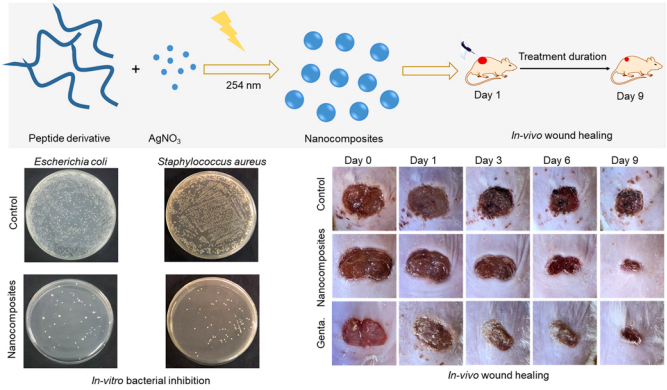
HIGHLIGHTS

- Peptide-silver nanocomposites were fabricated via an in-situ photochemical reduction method.
- The nanocomposites have shown antibacterial efficiency against Escherichia coli and *Staphylococcus aureus* at the *in-vitro* level.
- The nanocomposites have shown a promising wound healing efficiency and no side effects were observed in biosafety tests.

GRAPHICAL ABSTRACT

A short peptide-based functional compound was used to fabricate silver-peptide nanocomposites without any toxic chemical reducing agents. These nanocomposites have shown promising in-vitro antibacterial activity and wound disinfection at in-vivo level.



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https://doi.org/10.1016/j.colsurfa.2022.129708
Received 8 May 2022; Received in revised form 23 June 2022; Accepted 12 July 2022
Available online 16 July 2022

ARTICLE INFO

Keywords:
Short peptide
Metal-composites
Antibacterial
In-vivo wound healing
Wound disinfection

ABSTRACT

Increased antibiotic resistance has become a real healthcare challenge for humanity. Several novel approaches for the formation of nanomaterials have been developed to tackle different bacterial strains and wound disinfection. Short peptide modulated metal nanomaterials have gained attention because of their biocompatibility and ability to reduce metal ions *in-situ* to form nanoparticles. Here we have used a short peptide conjugate as a reducing and capping agent to make stabilized silver-peptide composite nanoparticles under mild UV light exposure. This photochemical method is one of the green approaches and reduces the toxic effects of chemicals that are often used in traditional synthetic strategies. These composite nanoparticles demonstrated promising activity against *Escherichia coli* and *Staphylococcus aureus* at an *in-vitro* level and have the ability to cure wounds disinfections *in-vivo*. Furthermore, these composite nanoparticles show excellent biocompatibility, and no side effects were observed in liver enzymes and organs, in a histopathology study. Such a simple strategy of sustainable supramolecular chemistry could help develop novel nanomaterials as potential antibiotic agents of the future.

1. Introduction

The conventional strategies for treating bacterial wound infections are based on using antibiotics and disinfectants worldwide [1]. Unfortunately, due to the overuse of antibiotics, many bacterial strains have recently developed resistance against the marketed antibiotics, [2] which has become a real threat to humanity and a challenge for the scientific community. Interestingly, silver metal has been considered and used as an antibacterial and inflammatory agent for as long as the existence of humanity [3]. However, silver ions have shown side effects like accumulation in normal tissues and organs and the ability to diffuse back and forth at a cellular level [4]. Nanotechnology could potentially overcome the aforementioned challenges to make the best use of silver metal as an antimicrobial agent. Therefore, silver ions (Ag^1) could be reduced first by chemical and physical methods to elementary (Ag^0) form, which is considered the least toxic [5]. At the same time, the nanoparticles formed from these synthesis methods are less stable and demonstrate a propensity to random aggregation, and also the use of toxic chemical reagents could have a potential hazardous threat to normal tissues and organs. To avoid this toxicity effect and aggregation issue, biocompatible extracts of plants and microorganisms could have limited advantages as reducing agents to make silver-based nanomaterials [6,7].

By using plants and microorganisms to reduce the silver ions for the synthesis of metal nanomaterials, researchers have opted for it as a green approach. Still, it is difficult to understand the physicochemical interaction during the capping process and the random aggregation of metal nanoparticles remains a challenge [8,9]. In the quest to understand the interactions between reducing agent and silver metal and to find a solution of random aggregation, several different reducing and stabilizing agents for metal nanomaterials, including silk fibres [10], polymers [11], imidazole [12], graphene oxide and polysaccharides[13] have been reported. To further understand the physicochemical properties and tackle the stability concerns, metal nanoparticles need stabilizing and capping agents to be stabilized, and peptides/proteins could be the best choice because of their biodegradable and biocompatible properties [14]. Recently, amino acids and self-assembling short peptides have been used to reduce the metal ions *in-situ* with the help of UV light and capable of stabilizing the nanoparticles with known physical and chemical interactions between two components [15,16]. The presence of aromatic amino acid residues like tyrosine, phenylalanine, and tryptophan in the sequence of short peptides could potentially reduce metal ions through a photoionization method where an electron could be released from the aromatic group due to UV light [15].

Self-assembly of biomolecules like peptides and proteins is a natural process. Several weak molecular interactions, including electrostatic interactions, hydrogen bonding, pi-pi stacking, and cation-pi interactions, are playing a central role in forming the biomaterials for several biomedical applications [17,18]. Dipeptide diphenylalanine (FF), a motif of amyloid-beta 42, emerged as a simple molecule to undergo self-assembly to form diverse nanomaterials for several different applications in healthcare and light-harvesting in the energy sector [19]. Interestingly, not only dipeptides but single amino acids with aromatic groups have demonstrated the ability to form self-assembled biomaterials, which have shown antimicrobial properties [20]. The nano-silver wires were fabricated due to the reduction of silver ions by FF dipeptide assembled nanotubes [21]. FF based ordered nano assemblies showed antibacterial properties because of the membrane disruption ability and gene regulations of bacteria [22]. Interestingly, it has been reported that short peptide derivatives can phase separate into unstable liquid droplets (coacervates). These liquid droplets are enriched in water content and able to sequester the small client molecules and macromolecules like DNA for drug and gene delivery. Most of these ordered assembled systems cannot make spherical-shaped nanomaterials and spherical nanoparticles may have more penetration to disrupt the bacterial membrane with enhanced uptake [23], which could be improved by using supramolecular nanomaterials and phase separation materials [24]. To enhance the antibacterials activity of phase separating molecules, we used the short peptide building blocks as reducing and capping agents for forming silver-peptide nanocomposites for *in-vitro* and *in-vivo* antibacterial activity which is not investigated so far until date.

Herein, we report a short peptide building block composed of two diphenylalanine FF and linked through a disulfide linker (FFssFF) and have shown a capability for liquid-liquid phase separation to form the liquid droplets (coacervates) [25]. We used the FFssFF as a reducing and capping agent for the formation of colloidal Ag/FFssFF composite nanoparticles through a photoionization method, and non-covalent interactions play a role in the formation of the composite nanoparticles. Interestingly, these colloidal nanocomposites demonstrated the potential activity against two bacterial strains of gram-positive and gram-negative bacteria. Furthermore, the nanocomposites show promising wound healing and disinfection properties at an *in-vivo* level, and no side effects were noticed in the blood and liver of animals. We strongly believe that these nanocomposites could play a potential role in designing new short peptide-based supramolecular antimicrobial agents to meet the requirements of the current era.

2. Experimental section

2.1. Materials

Nutrient Broth and silver nitrate salt ($AgNO_3$) were purchased from Fisher Scientific. The commercial antibiotic drug Gentamicin (batch No. E009) in cream form was purchased from Ray Pharma private limited. Nutrient Agar, formalin, ethanol, and PBS buffer were purchased from Sigma-Aldrich. The lyophilized powder of FFssFF peptide derivative was custom synthesized by PEPMIC private limited. All reagents were used

as received or otherwise stated.

2.2. Preparation of Ag/FFssFF nanocomposites

We used the solution phase method of mixing two components and then UV light irradiation for certain time as reported previously [15]. The preparation of Ag/FFssFF composite nanoparticles was carried out using a 500 μL (1 mg/mL) solution of peptide in dH_2O and 60 μL $AgNO_3$ solution (100 mM), followed by the addition of 200 μL 10 mM PBS and 240 μL dH_2O. The final concentration of FFssFF and silver nitrate in the working solution was 0.5 mg/mL and 6 mM, respectively. The working solutions were then serially irradiated under UV light (254 nm) for different time points of 2, 5, 10, 20, and 30 min. The control synthesis system was considered as exposure to normal visible light for a maximum time. The reaction was visually observed for any precipitation and changes in color. After optimization of the reaction, the synthesized product was collected and stored in the dark at 4 °C for further experiments.

2.3. Instrumentation for characterization

The hydrodynamic size and ζ-potential of as-synthesized Ag/FFssFF composite in distilled water were measured by a zeta-potential analyzer (ZS-90, Malvern UK) at 25 °C. The UV/Vis spectra were recorded on a UV/VIS spectrophotometer (AE-S90-MD, UK) using a 1 mm quartz cuvette. The surface structure and morphological characteristics of the product were investigated by using a scanning electron microscope (JEOL-EM-6700) and transmission electron microscope. The elemental mapping of the product was obtained through High-resolution transmission electron microscope (HR-TEM) images on a model equipped with energy dispersive spectroscopic (EDS) at 200 kV.

2.4. In-vitro antibacterial activity (determination of colony-forming units)

Frozen cultures of *Escherichia coli* (ATCC-15224) and *Staphylococcus aureus* (ATCC-25923) were refreshed on a nutrient agar medium and kept in an incubator at 37 °C for 24 h. With a sterilized wire loop, a single colony from the refreshed cultures was introduced to the 20 mL nutrient broth medium contained in the 50 mL centrifuge tubes and kept for 12 h at 37 °C in a shaker incubator at 180 RPM. The broth cultures were washed with PBS and diluted to the optical density of $OD_{600} = 0.1$ (1×10^8 colony-forming unit (CFU) per mL) for further experiments. In 96 well plates, the broth cultures were further diluted to 1×10^5 CFU/mL, which were used for *in vitro* assays. The bacterial strains *Escherichia coli* and *Staphylococcus aureus* were divided into three treatment groups control group (no treatment), Ag/FFssFF group (treated with Ag/FFssFF material) and FFssFF (treated with FFssFF peptide). For *in vitro* antibacterial evaluation, a series of different concentrations of the Ag/FFssFF (0, 6.25, 12.5, and 18.75 μg/mL) and FFssFF (0, 100, 200, and 400 μg/mL) were tested. The treatments were allowed to withstand for 2 h, and afterwards, 100 μL from the treatment groups was dispensed to the already prepared nutrient agar plates. Since we used a turbidimetric method to determine the MIC of both viable and nonviable bacterial cells, the plates were incubated at 37 °C until the appearance of the matured colonies, which were counted.

2.5. Disc diffusion assay

A standard disc diffusion assay was carried out to further confirm the sensitivity of the bacterial strains to Ag/FFssFF, with minor modifications [26]. Briefly, from the standardized broth cultures of bacterial strains (1×10^5 CFU/mL), 100 μL was dispensed on the culture plates, and a uniform lawn was prepared using sterilized bend glass rods. The sterilized filter discs of 6 mm were impregnated with 12.5 μg/mL of Ag/FFssFF and 400 μg/mL of FFssFF. The filter discs were then gently placed on bacterial cultured media plates. The buffer PBS was used as a negative control, and the zone of inhibitions was measured after 24 h.

2.6. In vivo wound healing

A previously described procedure was used to determine the *in vivo* wound healing potential [27]. All the protocols were approved by the Ethical Review Board with reference No. 7441. Male Balb/c mice were purchased from the National Institute of Health, Islamabad. A surgical procedure was used to develop a wound on the back of the mice after the anesthesia procedure. The *Staphylococcus aureus* bacteria $\sim 1 \times 10^8$ CFU were inoculated to generate an infected skin wound model. All infected mice were randomly chosen and placed in three treatment groups and one control group ($n = 3$). The infected wound models were treated with silver-peptide composite nanoparticles (Ag/FFssFF), and commercial antibiotic Gentamycin (Genta), whereas, the negative control was untreated. The final concentration of Ag/FFssFF was 12.5 μg/mL. The wounds of all groups were photographed and retreated after every 24 h.

2.7. Histopathology

A tiny slice of a tissue sample from the wound infected region was sliced and homogenized in 1 mL PBS, then cultured on a nutrient agar medium for 24 h at 37 °C to determine the bacterial load in the wound area. All mice were euthanized on the ninth day for histopathological investigation, and the main organs were preserved in formalin and paraffined for histology and H&E staining.

2.8. SEM of treated bacterial cells

Following the treatment, microorganisms from all treated groups were collected and centrifuged for 3 min in PBS before being fixed overnight at 4 °C. After 3x washing with PBS, bacterial cells were dehydrated for 15 min using an ethanol solution (30 %, 50 %, 70 %, 90 %, and 100 %). Finally, bacterial cells were placed on silicon wafers, dried, and sputtered with gold for field emission scanning electron microscopy (FE-SEM) images.

2.9. In vivo toxicity assessment

To investigate whether the bacterial treatment with Ag/FFssFF nanocomposites has manifested any toxicity, the blood biochemical parameters and tissues were investigated in treated mice. At the end of *in-vivo* treatment, all mice were euthanized, and blood was taken for the chemistry of blood experiments. Gentamycin was used as a positive control, whereas blank mice were considered as a negative control. Eight major blood parameters were examined, whereas the major organs, including the heart, liver, spleen, lungs, and kidney, were examined for histopathology [27].

3. Results and discussion

3.1. Fabrication and characterization of colloidal Ag/FFssFF nanocomposites

We use a small peptide derivative (FFssFF), a nonpolar sticker diphenylalanine (FF) linked with a disulfide polar spacer that showed liquid-liquid phase separation through a multitude of weak molecular interactions [25]. The aromatic amino acids have shown an ability to reduce the metal ions [21,28], and therefore, we considered the FFssFF molecule as a reducing agent with mild UV exposure through a photochemical reduction method [15] - a green synthetic strategy for the formation of metal composites nanomaterials. The lyophilized powder of FFssFF (1 mg/mL) was dissolved in Milli-Q and mixed with a 100 mM concentration of $AgNO_3/AgBF_4$ under a mild vortex. After mixing these two components, the pH was raised to 7.2 by using phosphate buffer saline 10 mM in 1:1 to mixture volume and irradiated this mixture with

UV light 254 nm for 10 min (Fig. 1A). The conditions were optimized by varying the concentration of both components and UV exposure time and distance from the UV lamp, which resulted in different sizes of nanocomposites revealed by dynamic light scattering (DLS) measurement and surface zeta potential, as shown in Table S1. The color of Ag/FFssFF composite nanoparticles was changed from transparent to light grey-reddish (Fig. 1B), and the intensity of color depends on the concentration of mixing components, UV exposure time, and energy. Silver nanoparticles are usually characterized by UV–VIS spectrophotometer to see the characteristic plasmon peak at 410 nm [29]. Interestingly, the characteristic peak of surface plasmon resonance of silver composite nanoparticles was not clear at 410 nm, which may attribute to the trapping of silver nanoparticles inside the peptide building blocks; in other words, the peptide could have a strong binding affinity with silver in its internal core. At higher concentrations of the $AgNO_3$ and FFssFF 2.0 mg/mL, nanocomposites appeared to be unstable, and resultantly random aggregation was observed. Furthermore, UV light exposure time could have played a role in enhancing the rate of reduction reaction of silver ions. Fig. 1B indicates the reduction of Ag^+ by the peptide (FFssFF) and resultantly converting it into composite supramolecular nanoparticles (Ag/FFssFF) in aqueous media [30].

To further evaluate the physicochemical properties of *in-situ* synthesized nanoparticles, we selected a sample where 10 min of UV exposure time was used for morphological investigation. The size of the nanocomposites was found to be 118 nm, and the surface zeta potential was determined to be + 29.9 mV by dynamic light scattering (DLS), as shown in Supplementary Fig. S1–2. This zeta potential indicates that the charge on the surface of nanocomposites is positive, attributed to the positive charges of amine functionality of FFssFF and the stability of nanocomposites in solution form. The scanning electron microscope (SEM) and transmission electron microscope (TEM) were used to reveal the shape of Ag/FFssFF nanocomposites. Interestingly, silver-peptide nanocomposites exhibit the spherical shape observed by both techniques, as illustrated in Fig. 1C and Supplementary Fig. S3. However, the particle size is not well in agreement with the size determined by DLS, which we reasoned that the colloidal nature of Ag/FFssFF nanocomposites and bigger-sized nanocomposites could have been wiped off during the preparation of TEM samples. Furthermore, the EDX spectra reveal sharp peaks of Ag, confirming the presence of silver, whereas other peaks may originate from the chemistry of FFssFF, as shown in supplementary information S3.

3.1.1. *In-vitro antibacterial activity*

To assess the antibacterial activity of Ag/FFssFF colloidal nanocomposites, we selected the two commonly used bacterial strains, *Escherichia coli* and *Staphylococcus aureus,* for *in-vitro* antibacterial assays. The photographs in insets of Fig. 2A and B reveal the optical absorbance of the broth cultures when treated with the Ag/FFssFF in the concentration range of 6.25 μg/mL to 18.75 μg/mL. With increasing concentration of FFssFF in the Ag/FFssFF treatment of *Escherichia coli* and *Staphylococcus aureus,* respectively, the optical absorbance or turbidity of the culture decreases significantly compared to the control (untreated) cultures. The growth of the bacterial strains was significantly inhibited at 12.5 and 18.75 μg/mL for both *Escherichia coli* and *Staphylococcus aureus*.

A decrease in the culture absorbance is associated with the susceptibility of the bacteria of tested compounds [31]. To support this result, we conducted a percentage survival experiment by using 100 μL of Ag/FFssFF (12.5 μg/mL) and treated with *Escherichia coli* and *Staphylococcus aureus* separately. The treated sample with Ag/FFssFF revealed a percentage survival rate of 2.9 % and 2.1 % for *Escherichia coli* and *Staphylococcus aureus*. A CFU method was used to assess the inhibition of the growth of both bacterial strains, and it is shown in Fig. 3C and D that there is a significant difference in the growth of both strains as compared to control Fig. 3A, B. Furthermore, the bacterial samples from the respective four petri dishes were used for the SEM images. Surprisingly, the membrane disruption is not clearly observed in SEM images in a treated group. However, we emphasized that the growth inhibition is may happened because of the Ag^+ and reactive oxygen species (ROS) generated from the Ag/FFssFF nanocomposites [32]. There are several mode of actions to kill the bacteria by using nanotechnology has been reported and silver ions and ROS are commonly found in metal based nanomaterials and polymeric modified metal nanoparticles [33–35].

To further validate our results of *in-vitro* antibacterial activity, we used standard disc diffusion assay to further confirm the sensitivity of the bacterial strains to Ag/FFssFF nanocomposites. Briefly, from the

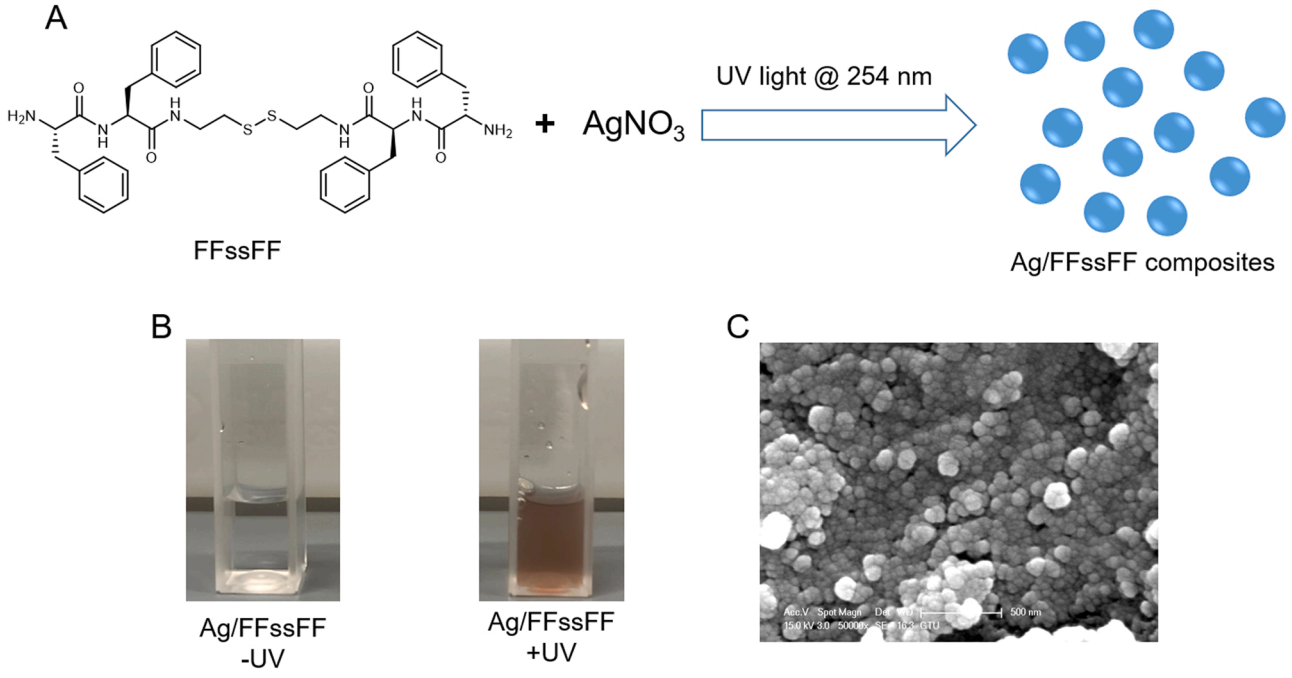

Fig. 1. Schematic diagram for formation of Ag/FFssFF composite nanoparticles (A), photographs of Ag/FFssFF mixture before and after UV light exposure (B), SEM image of Ag/FFssFF nanocomposites (C).

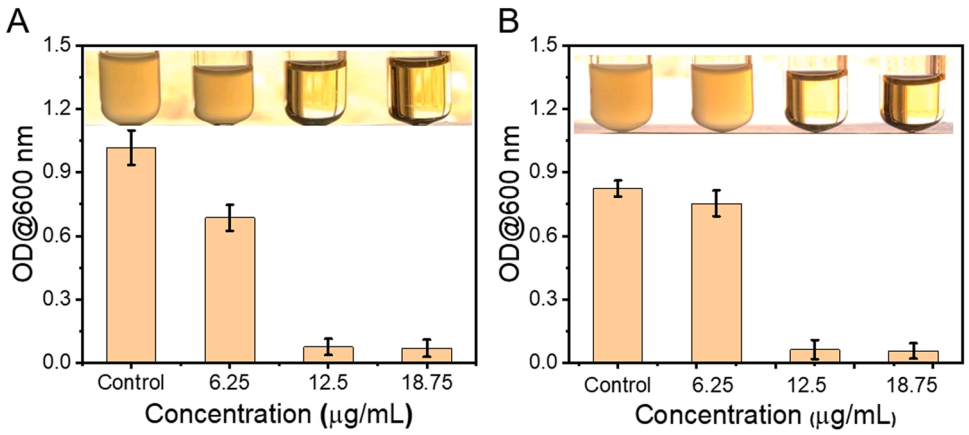

Fig. 2. *In-vitro* antibacterial activity of Ag/FFssFF nanocomposites. Ag/FFssFF activity against Escherichia coli and Staphylococcus aureus, respectively (A and B). Insets are a respective pictorial representation of samples incubated with Ag/FFssFF nanocomposites of Escherichia coli and Staphylococcus aureus.

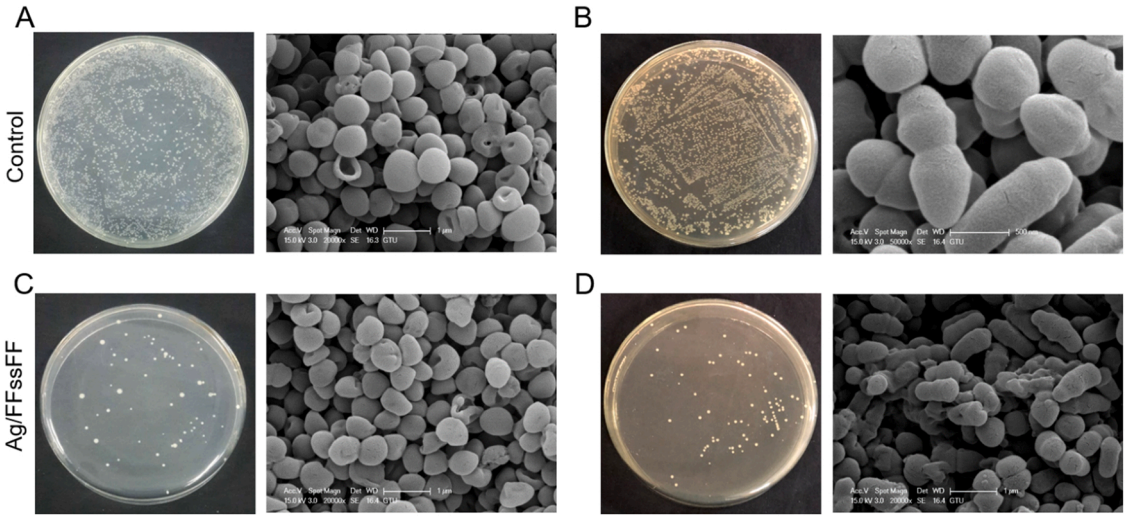

Fig. 3. Colony Forming Units assay and SEM of bacterial cells in treated and untreated samples, (A and B) left panel photographs of petri dishes to show growth of bacterial cells (*Escherichia coli* and *Staphylococcus aureus*) as a control or untreated group and right panel represent the SEM images of respective bacterial cells. (C and B) left panel photographs of petri dishes to show the inhibition in growth of bacterial cells (*Escherichia coli* and *Staphylococcus aureus*) for Ag/FFssFF or treated group, and the right panel represent the SEM images of respective bacterial cells of the treated groups.

standardized broth cultures of bacterial strains (1×10^5 CFU/mL), 100 μL was dispensed on the culture plates, and a uniform layer of bacteria was prepared using sterilized bend glass rods. The sterilized filter discs of 6 mm were impregnated with 12.5 μg/mL of Ag/FFssFF and 400 μg/mL of FFssFF (Supplementary information S5). The filter discs were then gently placed on bacterial cultured media plates. The buffer PBS was used as a negative control, and the zone of inhibitions was measured after 24 h. We found a significant difference in the inhibition zone of Ag/FFssFF compared to control and FFssFF samples.

3.2. *In vivo wound healing*

After determining the antibacterial activity through both *in-vitro* assays, we used the colloidal Ag/FFssFF nanocomposites for bacterial wounds healing in Balb-c mice. Fig. 4A shows the excision of skin and creation of the wounds by *Staphylococcus aureus* at day 0 and then we used the nanocomposites to disinfect the bacterial wounds for their healing.The anesthetized mice were shaved on the dorsal side and excised to prepare a circular wound ~ 1 cm. The photographs of wounds indicate the *in vivo* wound healing and antibacterial potential of the Ag/FFssFF nanocomposites (Fig. 4B). The experimental setup comprised one treatment group (Ag/FFssFF) and Gentamycin group as positive control and one negative control, and we used three mice for each group during the treatment process. On selective days of treatment, the size of wound was measured and plotted against treatment days in Fig. C. The decreasing in the diameter of the wounds was observed, and their relative percentage was calculated as shown in Fig. 4B and C. There has been a significant reduction 3-fold in the wound area on the 9th day for Ag/FFssFF nanocomposites as compared to control group and comparable to the standard marketed antibiotic Gentamycin. Interestingly, we continuously monitored the bacterial survival on same predefined days for the treatment which showed no bacterial colonies on the 6th and 9th days of the treatment (Fig. 4D and E). Henceforth, it was evident that the Ag/FFssFF nanocomposites significantly enhanced the wound healing by inhibiting the growth of the microorganisms at the wound site. The *in-vitro* and *in-vivo* results both show the antibacterial activity of nanocomposites and ability to enhance the wound healing process.

3.3. *Biosafety evaluation*

After observing the successful wound healing ability of Ag/FFssFF colloidal nanocomposites, we assessed the toxicity of Ag/FFssFF nanocomposites through blood biochemistry profile and biosafety experiments of liver enzymes. Interestingly, no significant fluctuation in blood

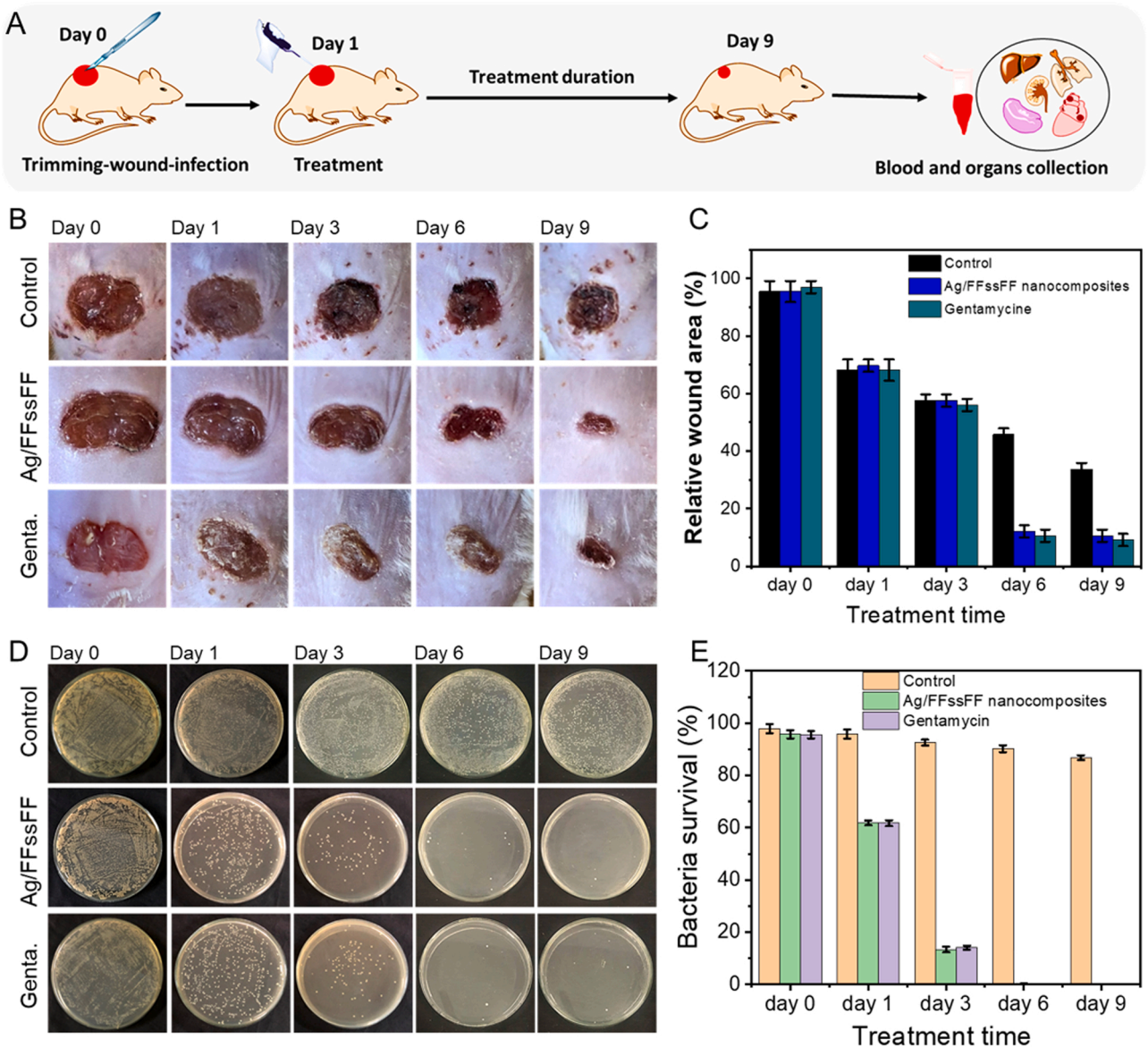

Fig. 4. *In-vivo* bacterial wound healing, (A) schematic illustration of treatment protocol, (B) representative photographs of bacterial infections (*Staphylococcus aureus*) in the mice as a control, treated with Ag/FFssFF nanocomposites and treated with Gentamycin, (C) relative percentage wound area in all groups at the different day of treatments and relative area treated infection was decreased 3 fold as compared to control, (D) bacterial growth by CFU assay at everyday treatment in all groups, (E) percentage bacterial survival at different treatment days calculated from (D) and significant inhibition in bacterial survival is shown as compared to control.

parameters was observed, which validates the biocompatibility and biosafety of the nanocomposites. The samples were collected on the completion of *in-vivo* treatment on day 9. The blood biochemical parameters were studied, including ALT, AST, ALP, TP, ALB, GLOB, Urea, and Creatinine, as indicated in supplementary information S6a. Similarly, further toxicity assessment was carried out by studying the major organs, including the Heart, Liver, Spleen, Kidney, and Lungs, for any histopathological changes, as indicated in supplementary information S6b. No major changes were observed through the H&E sections of the major organs as compared with the untreated group. The normal blood biochemistry and histopathology revealed that the Ag/FFssFF nanocomposites are safe, biocompatible, and non-toxic to organs and blood chemistry.

4. Conclusion

In summary, we developed the facile strategy to reduce and stabilize the silver nanoparticles *in-situ* by mixing short peptide derivative and silver salt with UV light irradiation which resultantly formed the Ag/FFssFF nanocomposites. The short peptide conjugate is not only involved in the reduction of metal ions but also may acts as a stabilizing agent for silver nanocomposites depending on the concentration of both components used for preparation of composites. This design attributes the importance of green synthesis of nanoparticles which demonstrated promising antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and proved a wound healing agent at an *in-vivo* level. At an *in-vitro* level, Ag/FFssFF nanocomposites exhibited promising activity against *Escherichia coli* and *Staphylococcus aureus* and disinfected wounds at the *in-vivo* level for healing process. The excellent biocompatibility and non-toxicity profiles in organs and blood chemistry made the Ag/FFssFF nanocomposites a potential supramolecular material for antibacterial applications.

CRediT authorship contribution statement

MO and MA conceived the work and designed the experiments. A.A, SA, MO conducted the experiments. AK, AA, and MA helped in analyzing the data. ND performed the SEM and TEM. ZS provided the guidance and necessary help for animal experiments. All authors were involved in writing the manuscript and discussed it before submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgment

M.O acknowledges the support from Prof. Chunying Chen. M.A. acknowledges the support from Prof. Xuehai Yan for his comments and suggestion on the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2022.129708.

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