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Efficient antimicrobial silk composites using synergistic effects of violacein and silver nanoparticles

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ABSTRACT

Violacein, a natural violet biopigment with efficient bioactivities from Gram-negative bacteria, possesses good affinity to silk fiber and complexes with silver. In this paper, a new approach involving the surface modification of silk fabrics with violacein for the in-situ synthesis of silver nanoparticles (SNPs) was developed. Violacein is used to modify silk material. Subsequently, silk containing bio-violacein was in situ assembled by silver ions and formed SNPs. Functional silk composites (FSC) containing bio-violacein and SNPs were obtained with effective synergistic antimicrobial effects. FSC were characterized by FT-IR spectroscopy, UV–visible absorption spectroscopy, and scanning electron microscopy/energy dispersive spectroscopy, and X-ray diffraction. Exhaustion and amount of violacein on silk fabric were 65.82% and 0.16 g/g, respectively. SNPs were small particles with irregular shapes and sizes < 60–70 nm. Antimicrobial activities of the FSC were evaluated against *S. aureus, E. coli, and C. albicans*. The silk fabric with violacein possessed good antimicrobial activity against *S. aureus*, with a bacterial reduction of 81.25%. FSC with violacein combined with SNPs integration exhibited good synergistic properties as excellent antimicrobial activities against *S. aureus*, *E. coli*, and *C. albicans*, with microbial reductions of 99.98%, 99.90%, and 99.85%, respectively. FSC not only exhibited the enhanced antimicrobial effects but also exhibited a broadened antimicrobial range.

1. Introduction

Silk is a biologically-derived and protein polymer purified from domesticated silkworm (*Bombyx mori*) cocoons and has been demonstrated to possess excellent properties, including biocompatibility, robust mechanical strength in various material formats, and controllable rates of biodegradation to nontoxic products in vivo [1–3]. Growing interest in silk materials, including silk films, composites, and coatings, hydrogels and nanoparticles, is found in applications for biological tissues, drug delivery, surgical sutures, hemostatic bandage materials, and implanted materials [4–7]. With the growing global threat of multidrug-resistant microbes as well as an increasing public desire for natural products, it is imperative to develop alternative, natural, antimicrobial materials [8–10]. The infection management in surgical operations remains a major challenge because of the limitations of conventional, systemic, antibiotic delivery [11–13]. Some antibiotic substances and new nanomaterials have been employed to modify material surfaces [14,15]. Silk materials subjected to antimicrobial treatments have shown the feasibility of inhibiting bacterial growth in wounds and reducing infection rates [16,17]. These demonstrate that silk represents a novel, customizable, antibiotic platform for focal delivery of antibiotics using a range of material formats. The silk fiber coating is the main method for the preparation of composite materials [8,18]. However, the polymer coated might adversely affect some fiber properties, including silk biocompatibility and flexibility. Thus, fabrications of new silk composites using natural products, which retain resistance to biological activity as well as their distinctive physical and handling properties, should be significant research areas.

Violacein, a violet biopigment produced by *gram-negative* bacteria, has attracted much attention due to its pharmacological properties and bioactivities [19,20]. Violacein has a number of bioactivities, including broad-spectrum antimicrobial activities, strong bactericidal and cytotoxic activities in human colon cancer cells, antileishmanial, antiulcerogenic, antiviral, antibiotic, antitumoral, and anti-*Trypanosoma cruzi* activities [21–23]. Silver nanoparticles (SNPs) have special property in different fields, such as sensor [24–26]. SNPs have gained much attention in biomedical science and have been widely used to functionalize a range of materials and surfaces. SNPs exert antimicrobial activity against a broad spectrum of bacterial, yeast, fungi, and viral species, including several antibiotic resistant bacterial strains

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[27,28]. Unlike antibiotics, silver does not provoke microbial resistance and is broadly used in wound management. Therefore, a broad therapeutic window for SNPs is expected [29,30]. Violacein is a dimeric structure composed of 5-hydroxyindole, oxindole, and 2-pyrolidone subunits formed by condensation of two modified tryptophan molecules [31,32]. It possesses good affinity to silk fibers and complexes with silver ions.

In this study, silk fabric was firstly modified with a bio-violacein, and the altered fabric then in situ complexed with SNPs. The resulting antimicrobial fabric was then characterized to confirm the presence and distribution of violacein and SNP on the surface. The fabric's antimicrobial activities was evaluated against gram-negative (*E. coli*), gram-positive (*S. aureus*), and *C. albicans* bacterial strains and it was found that the functional silk composites possessed enhanced antimicrobial activity from synergistic effects of violacein combined with SNPs.

2. Experimental

2.1. Materials

Bombyx mori silk satin was obtained from Zhejiang Jiaxin Silk Co., Ltd. (Jiaxing, China). Violacein was obtained from Jiangsu Yiming Biomaterials Co., Ltd. (Taixing, China). All other chemicals used were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). All reagents were used as received without further purification. Double-distilled water was used to prepare aqueous solutions.

2.2. Modification of silk fabric with violacein and in situ assembly of silver nanoparticles

Violacein was then used to modify silk fabric according to an established dyeing method. Violacein, 0.25 g, was dissolved in 20 mL of ethanol. Silk fabric, 5.0 g, was immersed in water, 80 mL, and the violacein ethanol solution slowly added with vigorous stirring. The modification process was conducted in a PYROTEC-2000 dyeing machine (Roaches International, Ltd., West Yorkshire, UK). The bath temperature was increased from room temperature to 85 °C at a rate of 1 °C/min before being held at 85 °C for 60 min. All modified samples were rinsed with double-distilled water and then dried at room temperature.

Violacein-modified silk fabric, 1 g, was treated with silver-ammonia solution (10 mmol/L), 50 ml, at 30 °C for 15 min with the agitation. The resulting material was then dried at room temperature and the sample impregnated in a vitamin C solution (3 mmol/l), 50 ml at 30 °C for 15 min; vitamin C served as a silver ion reducing agent. Finally, the sample was dried at room temperature. Here, a silver-ammonia solution was used instead of the usually-used $AgNO_3$ in order to prepare more uniform SNPs. In the silver ammonia solution, silver ions could more slowly release. The functional silk composites containing bio-violacein and silver nanoparticles were obtained.

2.3. Characterization of the FSC containing violacein and SNPs

FT-IR spectra of the modified silk fabric were measured using a Nexus-670 FTIR-Raman spectrometer (Nicolet Analytical Instruments, Madison, WI, USA). Violacein's UV–visible absorption was recorded on a HITACHI U-2910 spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The visual appearance of silk fiber was captured using a Canon EOS 5D digital camera (Canon Inc., Tokyo, Japan).

Scanning electron microscopy/energy dispersive spectroscopy (SEM-EDS; SEM, JSM-5600LV, JEOL Ltd., Tokyo, Japan; EDS, IE 300 ×, Oxford, England) was used to characterize fiber surface morphology and measure fiber surface element composition. Fiber silver content was determined by Prodigy inductively coupled plasma-atomic emission spectroscopy (Leeman Labs, Inc., Hudson, NH, USA).

Exhaustion (*E*) and exhaustion amount (*Gq*) of violacein on the fabric was calculated by measuring the absorbance of residual violacein at 593 nm, according to Eqs. (1) and (2), respectively.

$$E(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \tag{1}$$

$$Gq(mg/g) = \frac{C \times V \times E}{W} \tag{2}$$

where A_0 and A_1 are the absorbance of violacein ethanol solution at λ_{max} before and after modification, respectively, C (mg/mL) is the violacein concentration, V (mL) is the volume of violacein solution, and W (g) the fabric weight.

The reflectance spectra and absorption color yield (K/S) of violacein-modified and unaltered, control fabric were measured using a Datacolor SP 600^+ spectrophotometer (Datacolor, Lawrenceville, NJ, USA). The tristimulus values X, Y, and Z of the samples were measured under illuminant D_{65} using the 10° standard observer in the visible spectrum of 360–700 nm. The reflectance at the wavelength of maximum absorption (λ_{max}) was used to calculate the absorption color yield of samples using the Kubelka-Munk Eq. (3) shown below:

$$K/S = \frac{(1 - R)^2}{2R} \tag{3}$$

where K is the substrate absorption coefficient, S the substrate scattering coefficient and R the sample reflectance at λ_{max}.

2.4. Antimicrobial activities of FSC

Antimicrobial activities of the modified silk fabrics against *S. aureus* (a gram-positive bacterium), *E. coli* (a gram-negative bacterium), and *C. albicans* were evaluated by a "standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions" (ASTM E2149–01). This method is designed to evaluate the resistance of antimicrobial-treated specimens to microbial growth under dynamic contact conditions. *S. aureus*, *E. coli*, and *C. albicans* were used as test organisms. Silk fabrics (0.75 g of each) that were modified with violacein only, with violacein and SNPs, and unaltered (the latter as a control) were cut into small pieces and transferred to individual 250 ml Erlenmeyer flasks containing a working bacterial dilution 50 ml in volume. All flasks were loosely capped, placed in an incubator and shaken at 37 °C and 120 rpm using an incubator-shaker for 1 h. After a series of dilutions of the bacterial sample solutions using buffer solution, 1 ml of dilution, 1 ml was plated onto nutrient agar. The inoculated plates were incubated at 37 °C for 2 d and the surviving cells counted. Antimicrobial activity was expressed in terms of percentage reduction of the organism after contact with the test specimen compared with the number of bacterial cells surviving without such silk samples. The percentage reduction was calculated using Eq. (4).

$$\text{Reduction of bacteria } (\%) = (B - A)/B \times 100 \tag{4}$$

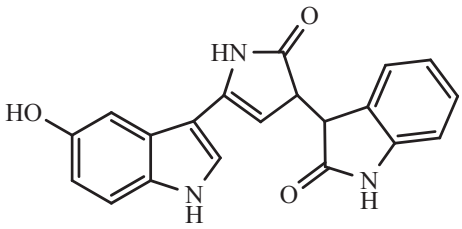
where A and B are the surviving cells (colony forming unit (CFU)/ml) for the flasks containing test samples and without samples, respectively. R (%) is the percentage reduction.

3. Results and discussion

3.1. Modification of silk fabric with violacein

Violacein is a natural bisindole derivative and chemical structure was shown in Scheme 1. It is soluble in organic solvents, such as ethanol and acetone, and disperses in water. The UV–visible absorption spectrum of violacein in ethanol solution was recorded on a HITACHI U-2910 spectrophotometer. The λ_{max} of violacein in the visible range occurred at 594 nm.

Silk as a natural protein polymer has good affinity to violacein.



Scheme 1. Chemical structure of violacein.

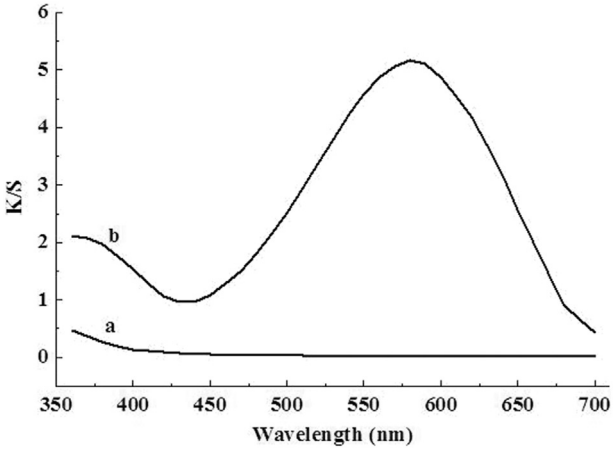


Fig. 1. Absorption color spectra of modified and control fibers: a. control silk fibers, and b. modified silk fibers.

According to experimental protocols, the calculated *E* and *Gq* of violacein on silk fabrics were 65.82%, and 0.16 g/g, respectively. Violacein-modified and unmodified (control) fabrics were characterized by obtaining their reflectance spectra and absorption color yield (Fig. 1). The results indicated that control fabric showed no absorbance from 350 to 700 nm while modified fabric possessed a strong absorption peak from 500 nm to 650 nm, with λ_{max} at 594 nm. This was in agreement with the absorption peak of violacein biopigment in ethanol solution and was attributed to π-π* and n-π* transition of the violacein bisindole conjugated system. Thus, this means that the silk fabric have been successfully modified with violacein.

The structural confirmation of violacein incorporated onto the silk fabric was conducted by FT-IR spectroscopy of violacein-modified and unmodified samples (Fig. 2). A new shoulder peak observed around 3451 cm^{-1} on modified fabric was the characteristic peak of –OH. The shoulder peak at 2854.3 cm^{-1} was the characteristic peak of –CH– on violacein. Peaks at 1702 cm^{-1} and 1695.9 cm^{-1} were attributed to the combination of carbonyl stretching in violacein and silk macromolecules. A peak at 1642 cm^{-1} was the characteristic peak of –C = C– stretching in violacein's benzene ring. Finally, a peak around 1105.6 cm^{-1} on the modified sample was the characteristic peak of -N-C = stretching of the violacein structure. These observations indicated the successful incorporation of violacein onto silk fibers by the dyeing process.

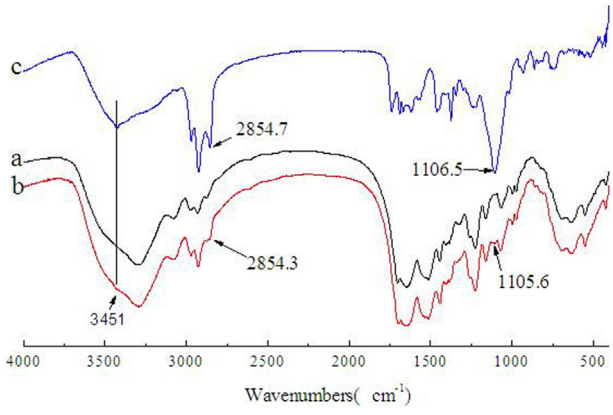


Fig. 2. FT-IR spectra of the samples: a. control silk sample, b. sample modified with violacein, and c. violacein.

3.2. Assembly of silver nanoparticles in situ on the silk modified with violacein

Silver has been known as an effective disinfectant for many years and is being used in many forms today in the treatment of infectious diseases [27,30]. Macromolecules of violacein-modified silk possess many bisindole groups. The amino groups of bisindoles can easily complex silver ions. Here, a silver-ammonia solution was used as a silver source and vitamin C used as a reducing agent. The complex silver ions on the violacein were in situ reduced. The self-assembly process of silver nanoparticles in situ on the silk containing *violacein* was shown in Scheme 2.

SEM images of control, violacein-modified, and violacein and SNP-modified fibers were obtained (Fig. 3). The surfaces of degummed and violacein-modified fibers appeared smooth, which indicated that violacein molecules were evenly permeated into the fibers and fixed in the macromolecules by intermolecular forces. The surfaces of violacein-modified fibers loaded with SNPs by a redox reaction showed small particles of irregular shape and with sizes below 60–70 nm. These SNPs exhibited excellent dispersity on the fiber surfaces. These observations confirmed that FSC containing violacein and SNPs were successfully obtained.

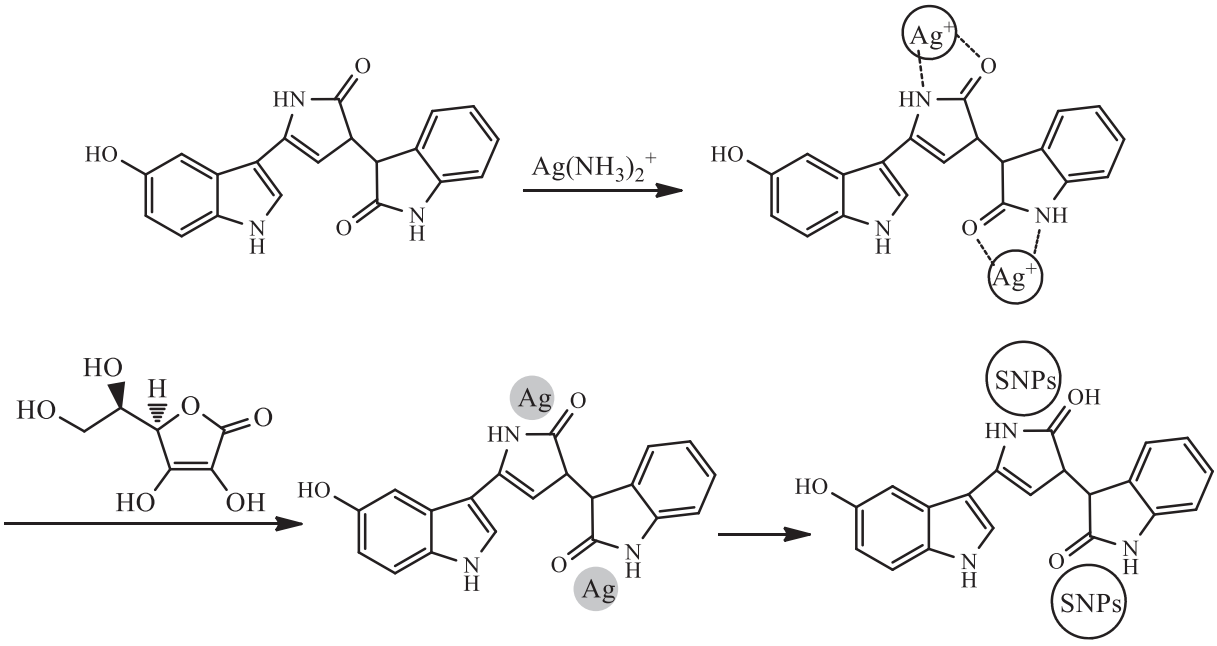
The silver content in the FSC was determined by Prodigy inductively couple plasma-atomic emission spectrometry and was found to be 8.98 mg/g. EDS was used to characterize and measure the FSC surface element composition. An EDS spectrum of FSC is shown in Fig. 4. The FSC surface elemental content analysis indicated that the surface silver content was 17.9% (*w*/w). The silver content of the sample surface was much higher than that of the silk matrix, which demonstrated that SNPs were mainly self-assembled on the surface of FSC.

The visual appearance of silk fibers was captured by digital photography (Fig. 5). Degummed fiber was white while violacein-modified fiber was a bright blue-purple and the FSC containing violacein and SNPs was brown.

X-ray diffraction (XRD) patterns of violacein-modified and the FSC containing violacein and SNPs are shown in Fig. 6. Diffraction peaks at 20.32 (with cell constants of d = 4.3669 Å) and 24.56 (d = 3.6216 Å) are indexed to silk crystal. The diffraction peaks in 38.06 (d = 2.3623 Å) and 44.20 (d = 2.0473 Å) are indexed to adherent SNPs [28,29].

3.3. Antimicrobial assay of the functional silk composites

Many biochemical properties of materials are dependent on their surface chemical structure. The antimicrobial activities of the control and FSC containing violacein and SNPs were evaluated against *S. aureus*, *E. coli*, and *C. albicans* by ASTM E2149–01; the results are summarized in Fig. 7. The antimicrobial properties of the control sample were poor, with microbe count reductions in all three species at < 10%. After violacein modification, the silk fabrics showed good antimicrobial activity against *S. aureus*, with an 81.25% reduction in microbes. The silk modified violacein has certain antibacterials property to *C. albicans*. But there was no improvement against *E. coli*. When violacein-modified fibers were further assembled with SNPs, the antimicrobial properties against *S. aureus*, *E. coli*, and *C. albicans* were all excellent, with microbe reductions at 99.98%, 99.90%, and 99.85%, respectively. The silk fabrics modified only with silver nanoparticles



Scheme 2. Self-assembly process of silver nanoparticles in situ on the silk

showed good antimicrobial activity against *S. aureus*, *E.* with 99.84% reduction in microbes and against *E. coli*, with 90.10%, respectively. There was almost no improvement against *C. albicans*. These results clearly indicated that there were synergistic effects between the presence of both violacein and SNPs that significantly improved the antimicrobial properties of the FSC. The new composite have important potential application in the biomaterials and functional composite fields.

The results of the antimicrobial activities are also expressed with the format of log reduction as in Fig. 8. The antibacterial activity value of test (△lg CFU/mL) is calculated as following:

$$\triangle \lg \mathrm{CFU/mL} = \lg C - \lg T$$

where, C and T is the average concentration (CFU/mL) of bacterial cells of control and modified samples, respectively.

The synergistic effect of both violacein and SNPs was consistent with the reduction rate. FSC with violacein combined with SNPs integration exhibited excellent antimicrobial activity.

The cytotoxicity study of silver nanoparticles, violacein and their combinations have been reported by many references. Webster et al. [33] demonstrate that no significant toxicity is observed for Ag nanoparticles around 10 nm over a range of 0.5–40 μg/mL. Caries et al. [34] point out that the cytotoxic effect of Ag nanoparticles is diameter-dependent, which is enhanced by decreasing the nanoparticle diameter. Baygar et al. [35] coat silk sutures with Ag nanoparticles, which do not induce any significant adverse effect on cell viability of the 3 T3 fibroblasts. Similar results are reported by another group [36]. When the absorbable PLGA sutures are functionalized with silk sericin and silver, the cell viability and proliferation do not result in significant effect by the presence of silver. In general, the silver nanoparticles have weak cytotoxicity.

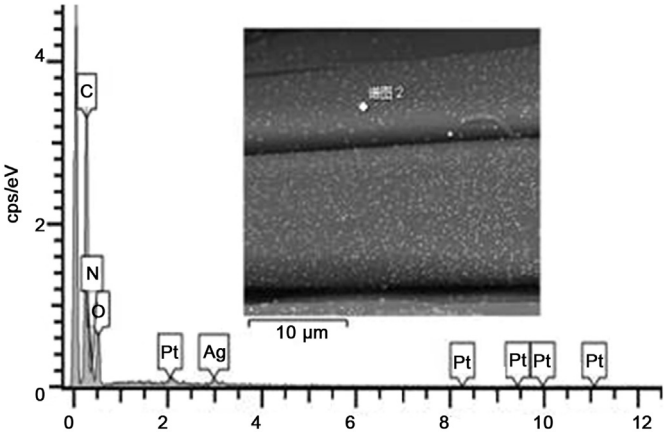


Fig. 4. EDS spectra of the modified silk fibers.

Bromberg et al. [37] investigate the growth inhibition and pro-apoptotic activity of violacein in Ehrlich ascites tumor. Complete hematology, biochemistry and histopathological analysis of liver and kidney indicate that daily doses of violacein up to 1000 μg/kg for 35 days are well tolerated and did not cause hematotoxicity nor renal or

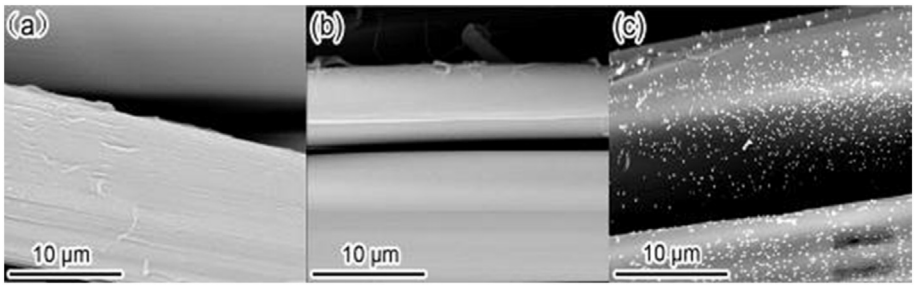


Fig. 3. SEM images of (a) degummed silk fiber, (b) violacein-modified silk fibers, and (c) violacein and SNP-modified silk fibers.

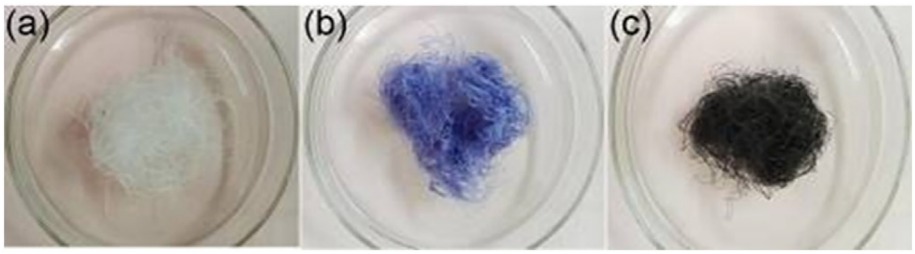


Fig. 5. Appearance of the silk fibers. (a) degummed silk fiber, (b) violacein- modified silk fibers, and (c) violacein and SNPs-modified silk fibers.

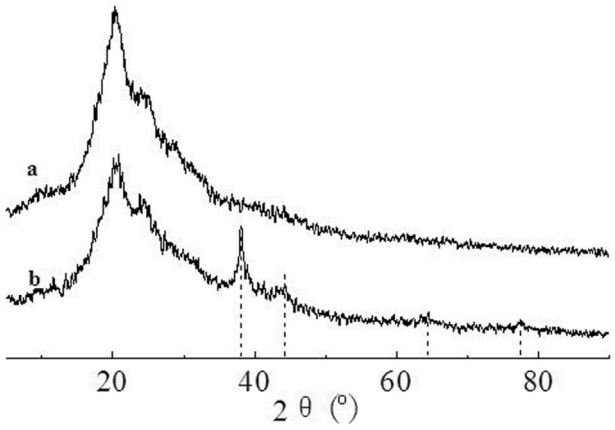


Fig. 6. XRD of violacein and SNP-modified silk fibers: a. modified silk with vio. and b. modified silk with vio. and SNP.

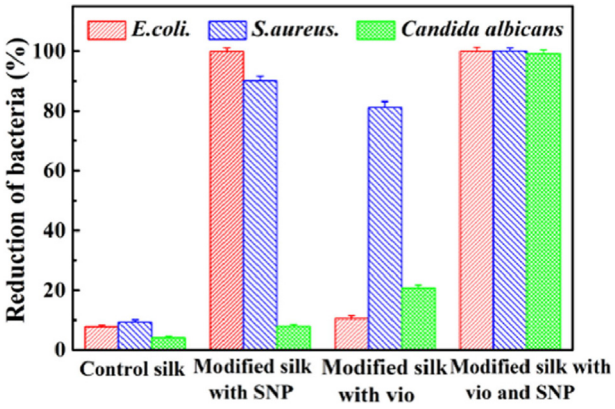


Fig. 7. Antimicrobial activities of the control and violacein and SNP-modified samples.

hepatotoxicity when administer to mice. Berti et al. [38] study the cytotoxicity of violacein in different physicochemical experimental conditions. Results also show that the viability of HCT-116 colon cancer cell is dependent on the violacein concentration. For violacein-DMSO at the concentration of 1 μM, the HCT116 cell growth is inhibited by 15% only. Rahul et al. [39] measure the in vitro cytotoxicity of microbial pigments and confirmed that violacein has low toxicity to Trypanosoma brucei gambiense bloodstream forms, the IC_{50} of which is 3.9 μg/mL. They also measure the in vitro cytotoxicity of violacein to other cells. Violacein shows weak cytotoxicity to blood mononuclear cells and to Hela cervical and MCF7 breast cancer cell lines, with IC_{50} values different from 98 to 158 μg/mL. The effect of nanoparticles on violacein's cytotoxicity is further studied and results show that when violaceion is combined with Ag or Au nanoparticles, the cytotoxicity is not affected. So the weak cytotoxicity of violacein and its combination with metal nanoparticles provide an idea about its safety to be used as a drug. From the above literatures, the silk modified with violacein and silver nanoparticles have weak cytotoxicity.

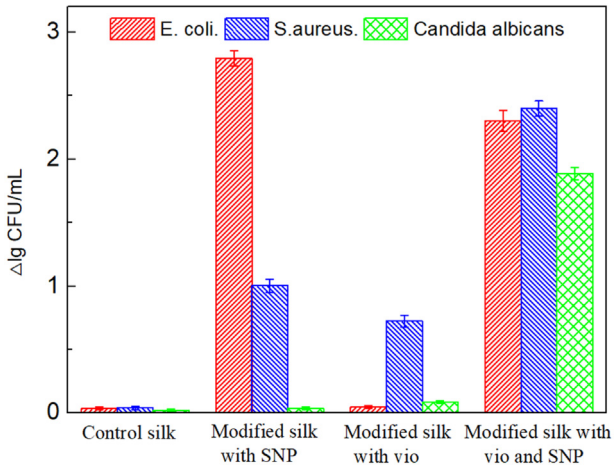


Fig. 8. Antibacterial activities of the control and violacein and SNP-modified samples with the log reduction.

4. Conclusions

Violacein and SNPs were successfully bound onto silk fabric and the novel functional silk composites were obtained. The exhaustion (E) and amount (Gq) of violacein on the fibers were 65.82%, and 0.16 g/g, respectively. Violacein was found to have good affinity for these fibers and also to effectively complex with silver ions. The self-assembly process of silver nanoparticles in situ on the modified silk with *violacein* was completed. The SNPs produced on the silk fabric modified with *violacein* were small particles of irregular shape and sizes below 60–70 nm. The functional silk composites containing violacein and SNPs possessed strong synergistic effects and exhibited excellent antimicrobial activities against *S. aureus*, *E coli*, and *C. albicans*. The novel FSC possess great potential applications in the advanced materials.

Acknowledgements

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Declaration of Competing Interest

There are no conflicts to declare.

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