Melittin based on silica nanoparticles for *Agrobacterium tumefaciens* inhibition

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An effective method for *Agrobacterium tumefaciens* inhibition using silica nanoparticles (NPs) as the carrier and melittin as the antibacterial drug is developed. The diameters of the inhibition zones of *A. tumefaciens* treated with different concentrations of melittin-conjugated silica NPs, pure silica NPs, gentamicin and free melittin are determined. The results indicate that the pure silica NPs have no impact on *A. tumefaciens*. The melittin-conjugated silica NPs have strong antibacterial activity against *A. tumefaciens*. In addition, the antibacterial activity of the melittin-conjugated silica NPs is dose dependent. The diameter of the inhibition zone caused by the melittin-conjugated silica NPs is almost the same as the positive control. Compared with the free drug molecules, silica NPs as an ideal carrier can gather a large number of drug molecules on their surface so as to greatly enhance the antibacterial activity.

1. Introduction: *Agrobacterium. tumefaciens* is a gram-negative bacteria commonly found in soil and survives by relying on the nutrients penetrated by root tissues, and which specially infects the injured areas of plants and induces a bacterial disease. Therefore the inhibition of *A. tumefaciens* is essential in agricultural production [1, 2]. Currently, antibiotics mainly include penicillins, cephalosporins and macrolides. Although these antibiotics mentioned above are widely used for bacterial inhibition, some negative effects of antibiotics should be considered [3]. In recent years, the antibacterial strategy based on antimicrobial peptides has attracted great interest because of their small molecular weight, high heat stability and a wide antimicrobial spectrum against gram-negative and -positive bacteria, viruses, fungi and parasites [4]. It is widely agreed that antimicrobial peptides may interact with the cell membranes of bacteria, resulting in the formation of transmembrane ion channels. Transmembrane ion channels can directly cause the leakage of cell contents and cell death [5]. Although the advantages mentioned above are obvious, one of the main drawbacks is that the bare melittin involved in the antibacterial process may easily be cleaved by some enzymes *in vivo*. It is thus highly desirable to develop stable and effective antibacterial methods.

With the development of nanotechnology in recent years, some nanomaterials such as silica nanoparticles (NPs) play important roles in bioanalysis, separation science, and disease diagnosis and treatment [6–9]. Especially, their applications to drugs delivery have attracted much attention because of their facile modification, good biocompatibility and high surface-to-volume ratio [10–13]. In addition, melittin is one of antimicrobial peptides with a good antibacterial effect. In this Letter, we report on the preparation of melittin-conjugated silica NPs for *A. tumefaciens* inhibition.

2. Experiments: Carboxyl-modified silica NPs (COOH-SiNPs) were prepared by the reverse microemulsion method. Immobilisation melittin onto COOH-SiNPs was performed using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) method. 500 µl of COOH-SiNPs (~1 mg) was washed by centrifuging twice with 0.1 M MES buffer (pH 5.5). The pellet was then resuspended in 500 µl of 0.1 M MES buffer (pH 5.5). One milligram of EDC and 2.5 mg of Sulfo-NHS were immediately added into the NPs solution. The solution was then incubated for 15 min at room temperature with gentle shaking, followed by centrifuging and washing with 0.1 M MES buffer (pH 5.5) three times. The NPs were resuspended in 500 µl of 0.1 M PBS buffer (pH 7.4). 500 µl of melittin diluted in PBS at a concentration of 1 mg/ml was then immediately added to the NPs solution. The solution was then incubated for 4 h at room temperature with gentle shaking, followed by centrifuging and washing with a 0.1 M PBS buffer (pH 7.4) three times. The NPs were resuspended in a 0.1 M PBS buffer (pH 7.4) and stored at 4°C until used. For protein content determination we adopted the Coomassie brilliant blue (CBB) G-250 method.

The disk diffusion method was used to determine the antibacterial activity of the melittin-conjugated silica NPs. The size and uniformity of the COOH-SiNPs were measured with a JEM100CXII transmission electron microscope (Japan). Particle size distribution and zeta potentials were determined using a Zetasizer (Nano-ZS, Malvern Instruments, Malvern, UK). Absorbance was measured using a UV–VIS spectrophotometer (TU-1901, China). The shape of *A. tumefaciens* was observed with a scanning electron microscope (HitachiS-3400 N).

3. Results and discussions: The antibacterial strategy of melittin based on silica NPs is described in Fig. 1.

Preparation methods of silica NPs are mainly based on Stöber method and the reverse microemulsion method (W/O). Although the Stöber method is relatively simple and can be carried out in only a few hours [14], it is limited by the non-uniformity of the products obtained, filtration and further separation for the products. The silica NPs were prepared using the reverse microemulsion method (W/O), which is a robust and efficient method for NPs preparation. The microemulsion yields monodisperse particles in the nanometre range. The size of the COOH-SiNPs was measured using a transmission electron microscope (TEM). As shown in Fig. 2, the NPs were uniform in shape with average diameter of ~60 nm (the total number of silica NPs measured by the SIS image processing software is 60 and the size of measured silica NPs is 60 ± 3.2 nm). The functionalised NPs were very well dispersed in aqueous solution and no aggregation was observed because of the electrostatic force between the NPs.

These distributions were measured with the Zetasizer Nano ZS instrument operating in the standard (on default) backward scattering mode. In this case, the position of the major peak of the scattered intensity distribution (68 ± 1.05 nm) is bigger than the average diameter determined by the TEM method (60 ± 3.2). The data shown are the average of three batches. The polydispersity index of the COOH-SiNPs was 0.04 ± 0.01.

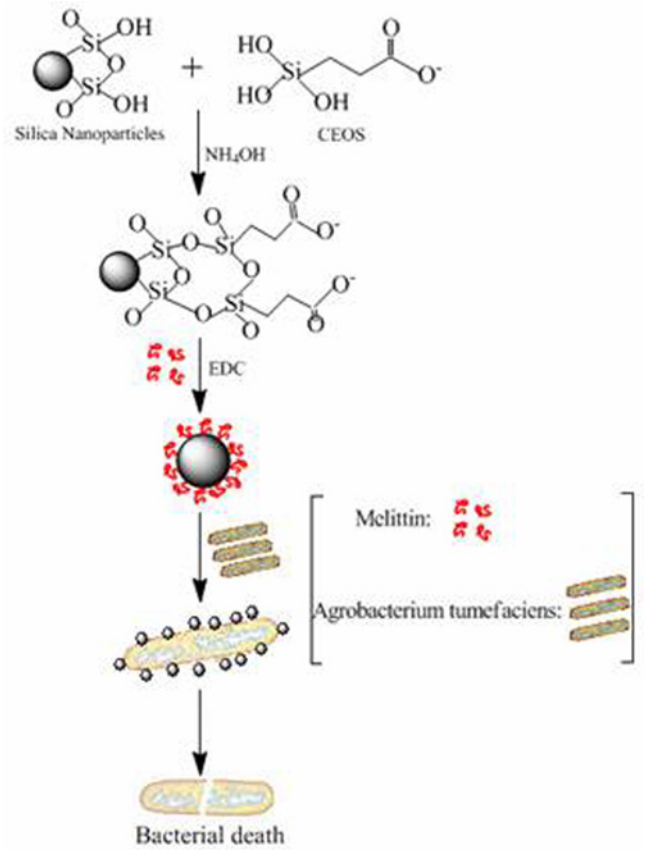

Figure 1 *Antibacterial strategy of melittin based on silica nanoparticles*

The modified carboxyl on the surface of the silica NPs was confirmed using a Zetasizer. The pure silica NPs and COOH-SiNPs provide a large number of carboxyls and oxhydryls on their surface, respectively. Hence the degree of deprotonation of the COOH-SiNPs is obviously stronger than the pure silica NPs in the natural condition. As shown in Fig. 3, the COOH-SiNPs and pure silica NPs posses surface charges of −45.7 and −25.3 mV, respectively. Therefore we conclude that the silica NPs can be easily functionalised with carboxyls.

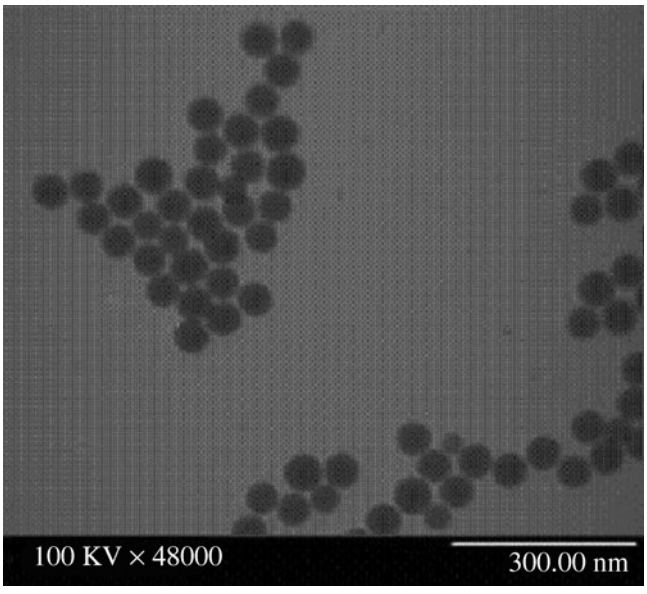

Figure 2 *TEM image of the carboxyl-modified silica nanoparticles*

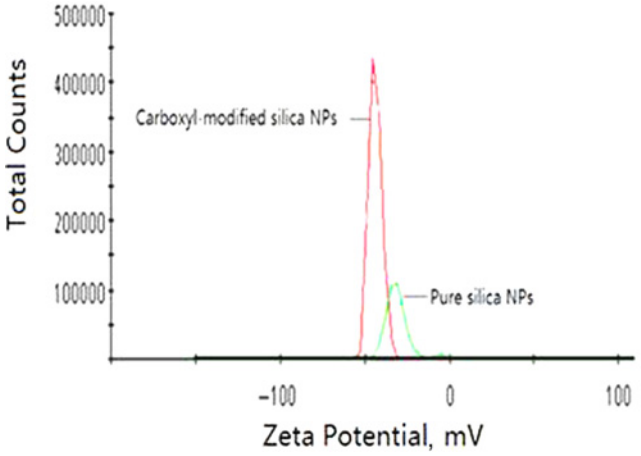

Figure 3 *Zeta potential of the carboxyl-modified silica NPs and the pure silica NPs*

CBB G-250 is a red dye with the maximum absorption wavelength at 465 nm, which binds preferentially and quickly to the hydrophobic regions of protein resulting in the absorbance enhancement at 595 nm. The calibration curve shows a good linear relationship between A and C in the range of 0–100 µg. The regression equation is $A_{595} = 0.0085C + 0.0464$ ($R^2 = 0.9993$). The number of immobilised melittin molecules on the silica NPs can be quantitatively calculated from the regression equation of standard bavine serum albumin. Hence we calculate that about 247 µg of melittin is immobilised on 1.0 mg of the surface of the silica NPs.

To demonstrate the antibacterial activity of the melittin-conjugated silica NPs against *A. tumefaciens* and the good biocompatibility of pure silica NPs, *A. tumefaciens* was treated with 5.0 mg/ml of melittin-conjugated silica NPs, 4.0 mg/ml of pure silica NPs, 1.0 mg/ml of free melittin and 1.0 mg/ml of gentamicin, respectively. As discussed above, about 247 µg of melittin can be immobilised on 1.0 mg of the surface of the silica NPs. About 1.0 mg of melittin conjugated on 4.0 mg of the surface of the silica NPs. The diameter of the inhibition zones caused by the melittin-conjugated silica NPs, free melittin and gentamicin are about 26.6 ± 0.9, 22.5 ± 0.5 and 19.1 ± 0.8 mm, respectively (Table 1). The results indicate that the melittin-conjugated silica NPs have strong antibacterial activity against *A. tumefaciens* and are more effective than the positive controls (gentamicin and melittin) under the same concentration. In addition, no inhibition zone was observed in the negative control. We believe that pure silica NPs show no antibacterial effect and can serve as a good drug carrier because of their good biocompatibility.

Table 1 Diameter of inhibition zone of melittin-conjugated silica NPs compared with gentamicin and melittin determined by disc diffusion method[a]

Test sample[b]	Inhibition zone diameter, mm
melittin-conjugated silica NPs[c]	26.6 ± 0.9
melittin	22.5 ± 0.5
gentamicin	19.1 ± 0.8
silica NPs	—

[a]Diameter of inhibition zone including disc diameter of 6 mm represents means ± standard deviations for triplicate experiments ($p < 0.05$)
[b]Concentration of melittin-conjugated silica NPs, melittin and gentamicin used was 10 µg/well
[c]Melittin-conjugated silica NPs (the mass of melittin on the silica NPs surface is calculated)
— no inhibition zone

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Table 2 Diameter of inhibition zone of different concentrations of melittin-conjugated silica NPs compared with gentamicin and melittin

Inhibition zone diameter, mm[a]					
Melittin-conjugated silica NPs[b]				Melittin	Gentamicin
500 μg/ml	250 μg/ml	125 μg/ml	50 μg/ml	500 μg/ml	500 μg/ml
19.5 ± 0.6	15.4 ± 0.3	9.75 ± 0.9	7.1 ± 0.2	13.5 ± 0.4	12.8 ± 0.3

[a]Diameter of inhibition zone including disc diameter of 6 mm represents means ± standard deviations for triplicate experiments ($p < 0.05$)
[b]Melittin-conjugated silica NPs (the mass of melittin on the silica NPs surface is calculated)

The antibacterial activity of the melittin-conjugated silica NPs against bacteria was demonstrated to be dose dependent (Table 2). A dramatic increase in the diameter of the inhibition zone was observed as the melittin-conjugated silica NPs concentration increased from 50 to 500 μg/ml (these concentrations were calculated based on the mass of melittin on the surface of the silica NPs). The diameter of the inhibition zone caused by the melittin-conjugated silica NPs between the concentrations of 125 and 250 μg/ml is almost the same as the positive control. In this work, we selected gentamicin and free melittin as the positive control. Gentamicin is one of the aminoglycoside antibiotics, which is usually used for the treatment of bacterial infections, and, in particular, those caused by gram-negative bacterial infections. In addition, gentamicin has the advantages of high heat stability and a wide antimicrobial spectrum. Hence we chose gentamicin as one positive control. As shown in Fig. 4, it is clear that the antibacterial effect of melittin-conjugated silica NPs is better than gentamicin and free melittins.

To visually observe the antibacterial effect of melintin-conjugated silica NPs, the shape of *A. tumefaciens*, *A. tumefaciens* treated with the pure silica NPs and *A. tumefaciens* treated with the melittin-conjugated silica NPs were characterised by SEM. As shown in Fig. 5, the micrograph by SEM showed that the surface of *A. tumefaciens* cells treated with the pure silica NPs was smooth and showed a typical

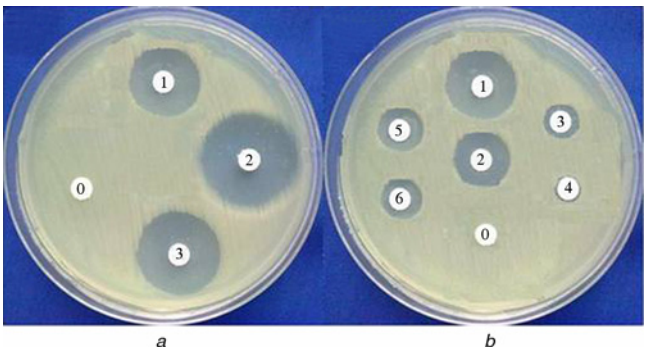

Figure 4 *Inhibition zones of A. tumefaciens treated with the presterilised filter papers impregnated with 10 μl of different types of drugs*
a Zones of inhibition of the melittin-conjugated silica NPs compared with gentamicin and melittin
From No. 0 to No. 3 represents 4.0 mg/l of pure silica NPs, 1.0 mg/l of free melittin, 5.0 mg/l of melittin-conjugated silica NPs and 1.0 mg/l of gentamicin, respectively
b Inhibition zones of different concentrations of melittin-conjugated silica NPs compared with gentamicin and melittin
From No. 1 to No. 4 represents 500 μg/ml, 250 μg/ml, 125 μg/ml and 50 μg/ml of melittin-conjugated silica NPs, respectively
No. 5 and No. 6 represent 500 μg/ml of melittin and gentamicin, respectively
No. 0 represents 500 μg/ml of pure silica NPs

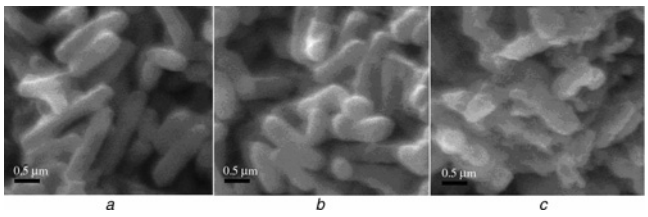

Figure 5 *SEM images of A. tumefaciens*
a A. tumefaciens treated with deionised water
b A. tumefaciens treated with 160 μg/ml of pure silica NPs
c A. tumefaciens treated with 200 μg/ml of melittin-conjugated silica NPs

characteristic of being rod shape, which was almost the same as the pure *A. tumefaciens* cells. We believe that the pure silica NPs have no significant impact on bacterial cells and their good biocompatibility is further confirmed; whereas cells treated with the melittin-conjugated silica NPs suffered from severe damage. Melittin molecules enriched on the surface of the silica NPs resulting in a large number of misshapen and fragmentary bacterial cells can be seen from the micrographs. Hence the antibacterial effect of melittin-conjugated silica NPs is very effective.

4. Conclusion: We prepared melittin-conjugated silica NPs for *A. tumefaciens* inhibition. The size of the COOH-SiNPs is about 60 nm. About 247 μg of melittin can be immobilised on 1.0 mg of the surface of silica NPs. According to the determination of inhibition zones, the pure silica NPs have no impact on *A. tumefaciens*. The melittin-conjugated silica NPs have strong antibacterial activity against *A. tumefaciens*, and are more effective than the positive controls (gentamicin and melittin) under the same concentration of 1.0 mg/ml. In addition, the antibacterial activity of the melittin-conjugated silica NPs against bacteria is dose dependent. We believe that antimicrobial peptides based on silica NPs is an effective method for *A. tumefaciens* inhibition.

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6 References

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