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Antimicrobial activity of Ag nanoclusters encapsulated in porous silica nanospheres

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Abstract

Silica nanospheres encapsulating silver nanoclusters (denoted as Ag-SiO_2) have been synthesized *via* a one-pot microemulsion method and their antibacterial activities have been investigated in detail. ICP-AES analysis indicated that the silver ions were continually released from the silica spheres in aqueous solution. The antibacterial properties of Ag-SiO_2 against both Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* were evaluated using minimum inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and the modified Kirby–Bauer method. The results show that the Ag-SiO_2 exhibits excellent and durable antimicrobial activities. The growth of both Gram-negative *E. coli* and Gram-positive *S. aureus* was completely inhibited during 48 h culture period. Moreover, the tested silver nanoclusters show higher activity against bacteria than the silver nanoparticles. This newly designed Ag-SiO_2 may offer a rapid and constant antimicrobial solution for practical applications.

Keywords: D. SiO_2; Silver clusters; Antimicrobial activity

1. Introduction

It is a well known fact that ionic silver is an excellent antibacterial agent and the release of silver ions by the nanoparticles has been proposed, and these ions can interact with many vital enzymes and inactivate them. The bacterial cells in contact with silver nanoparticles take in silver ions [1], which inhibit several functions in the cell and damage the cells. Simultaneously, many studies have revealed that silver nanoparticles can be used as effective growth inhibitors in various microorganisms and silver nanoparticles possess high antibacterial activity against both Gram-positive and Gram-negative bacteria [2,3]. More importantly, the effective biocidal concentration of silver nanoparticles is at a nanomolar level in contrast to a micromolar level of silver ions [4,5]. Nevertheless, naked Ag nanoparticles are chemically highly active, and are easily oxidized in air, resulting generally in loss of dispersibility and even antibacterial activity [6,7]. It is thus crucial to develop protection strategies to chemically stabilize the naked Ag nanoparticles against degradation during the use [8]. These strategies include the use of different polymers to stabilize Ag nanoparticles by the formation of nanocomposites. Such nanocomposites can effectively enhance the antibacterial activity and stability of Ag nanoparticles and the release of silver ions can be sustained for a long time, so that this silver species will be of great potential for antibacterial application. However, they often require complex preparation methods with high cost, and accompany biocompatibility problems [9].

Porous silica nanoparticles have attracted much attention in the fields of selective separation [10,11], drug delivery [12], and enzyme immobilization due to their good biocompatibility [13], processability, extremely high stability, and their facile synthetic methods. Further developments in the synthesis and modification of nanosized porous silica nanoparticles have created new possibilities for their biomedical applications [14–16]. In particular, the encapsulation of inorganic materials by growing porous silica stabilizes and simultaneously introduces additional functions to the nanoparticles [17]. For example, metal oxides,

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metal sulfides, and metals, can be incorporated directly into the porous silica nanopartilces *via* the microemulsion method, resulting in a core–shell nanostructure that the porous silica shell protects the inorganic materials [14]. The porous silica shell offers the advantage of being biocompatible and able to continuously release the encapsulated inorganic materials [3]. However, bacteria killing became less effective due to the slow release of silver ions from the encapsulated silver nanoparticles.

It should be noted that smaller particles exhibit higher solubility than larger particles according to the Kelvin equation. So, it is reasonable that smaller Ag particles have higher silver ions releasing speed due to the larger specific surface area and thus the effectiveness of smaller silver particles in killing bacteria will be higher than that of larger silver particles. Silver clusters are extremely small that they are considered as a bridge between metal atom and metal nanoparticles [18]. So, uniformly dispersed silver clusters in porous silica will be of great potential for their antibacterial application. The porous silica shell rendered the Ag nanoclusters dispersible in aqueous solution and prevented the active silver from aggregation. The silver nanoclusters were used as the "reservoir" of antimicrobial silver ions. Additionally, the existence of a thin ceramic SiO_2 matrix endows the composite different advantages towards their practical applications such as good biocompatibility, processability, and rapid and consistent release of silver ions.

We have recently developed a one-pot method for the synthesis of porous silica encapsulated silver nanoclusters, which are characterized by a central cavity and a composite wall. It was found that the composites exist as uniform nanospheres and the particle size of these nanospheres is about 40 nm with a central cavity ~15 nm. The presence of silver clusters was confirmed by TEM and XRD after calcination of the powder [19]. The main objective of this report was to explore the antibacterial activity of the Ag-SiO_2 against Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*).

2. Materials and methods

2.1. Reagents

Brij 58(GR) was purchased from ACROS Organics. Silver nitrate ($AgNO_3$), TEOS (tetraethyl orthosilicate), and cyclohexane were all analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. Sodium borohydride ($NaBH_4$, 96%) and ammonium hydroxide (NH_4OH, 28 wt%) were purchased from Sinopharm Chemical Reagent Co. Ltd. Ethanolamine ($NH_2CH_2CH_2OH$, 99%) was purchased from Alfa Aesar. All the chemicals were used as received without further purification. Water used in all experiments was prepared by passing through an ultra pure purification system.

2.2. Instrumentation and measurements

TEM images were taken on a FEI Tecnai F30 at an acceleration voltage of 300 kV. The weight content of silver in Ag-SiO_2 nanospheres was determined by atomic emission spectroscopy with inductively coupled plasma (ICP-AES). The measurements were made on the spectrometer OPTIMA 2000 DV (Perkin-Elmer, USA). An Agilent 8453 UV–vis diode-array spectrophotometer was used for the record of UV–vis spectra of suspensions of silica encapsulated Ag clusters.

2.3. Synthesis of Ag nanoclusters encapsulated in porous silica nanospheres

10 g of Brij 58 was added to 50 mL of cyclohexane and the mixture was heated to 50 °C under stirring until the solution was clear, followed by the addition of 4.5 mL of 1.0 M aqueous silver nitrate and 30 min stirring. Then, 1.5 mL of ethanolamine was added and the stirring was kept for 1 h. Next, 1 g of $NaBH_4$ was added, followed by the addition of 1 mL of 10^{-3} M NaCl solution. After 3 h stirring, 10 mL of ammonium hydroxide was added to the solution and stirring was continued for 20 min. Finally, 6 g of TEOS was added to the reaction system and the sol–gel process was allowed to proceed for 2 h at 50 °C. Then, the silica encapsulated silver clusters were precipitated out by adding 2-propanol, followed by two times wash with mixture of 2-propanol and water (3:1 in volume) by centrifugation. The solid samples were dried at 100 °C for 8 h and then calcined at 400 °C for 2 h under air stream.

2.4. Bacterial culture

E. coli (ATCC 25922) and *S. aureus* (ATCC 6538P) were selected as model Gram-negative and Gram-positive bacteria, respectively. Bacteria cells were grown overnight in LB medium (Luria–Bertani broth) at 37 °C and then harvested during the exponential growth phase by centrifugation. *E. coli* or *S. aureus* cells were washed twice to remove residual growth medium constituents and re-suspended in sterile saline solution (0.85% NaCl). The bacterial suspensions employed for the tests contained 2.5×10^6 colony forming units (CFU) per mL.

2.5. Modified Kirby–Bauer technique

50 μL *E. coli* or *S. aureus* cells suspensions (2.5×10^6 CFU/mL) were coated on the surface of LB-agar plates (LB liquid media with 1.5% agar). The sterilized filter papers with diameter of 20 mm were dipped into the suspension of materials and then dried in air. After that, these filter papers were placed on the plates cultured with bacteria. The zone of inhibition was observed and measured after 24 h incubation at 37 °C. The test was carried out in triplicate to ensure reproducibility.

2.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) for Ag-SiO_2 nanospheres or silica encapsulated silver nanoparticles

2 mL of Ag-SiO_2 or silica encapsulated silver nanoparticles suspensions with different concentrations (3.2, 2.4, 2.0, 1.6, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 mg/mL) were added to 2 mL

bacteria suspensions (concentration 2.5×10^6 CFU/mL). Then the mixtures were incubated at 37 °C with shaking at 200 rpm for 24 h. The turbidities of bacteria were observed every 12 h and clear tube demonstrated no bacteria growth. The minimal concentration of the Ag-SiO_2 nanospheres in the tube remaining clear after 24 h is MIC. In order to determine the MBC of Ag-SiO_2, equal volume of 20 μL solution from the clear tubes in the MIC test was further coated on the surface of LB-agar plates. Then the bacteria colonies were observed after 24 h incubation at 37 °C, and MBC is the minimal concentration of Ag-SiO_2 when no bacteria grows or the number of bacteria colonies is less than 5. Negative control tube contained only LB broth. Positive control tube contained LB and tested bacteria.

2.7. Antibacterial kinetic test and growth inhibition study

For the kinetic test, suspensions of Ag-SiO_2 (at concentrations of 0.4, 0.6, and 0.8 mg/mL, 0.45 at% silver-doped silica) were prepared in each sterilized flask and inoculated with an equivalent volume of *E. coli* or *S. aureus* suspension. Then the bacteria suspensions were incubated at 37 °C with shaking at 200 rpm. The initial time of adding Ag-SiO_2 particles to LB bacteria suspension was taken as zero, and at set time intervals, the number of viable cells were determined by the colony counting method. The kill percentage of bacteria was calculated by (Ncontrol–Nsample)/Ncontrol × 100%, where Ncontrol and Nsample represent the number of viable cells in the cell suspensions with control (0.85% NaCl) and the sample, respectively. The evaluation was run in triplicate and all the antibacterial tests were repeated at least for three times.

For growth inhibition study, the bacteria (*E. coli* and *S. aureus*) were cultured to a mid-log phase in LB broth and re-dispersed in 2 mL LB broth to obtain a concentration of 5.0×10^6 CFU/mL. Then 2 mL suspensions of Ag-SiO_2 (at concentrations of 0.2, 0.4, 0.6, 1.0, and 1.2 mg/mL) were added in the suspension of bacteria and incubated at 37 °C with shaking at 220 rpm. The growth behavior of bacteria was observed every 12 h.

2.8. Silver content in Ag-SiO_2

0.5 mL solution of HF was added to 20 mg of Ag-SiO_2, followed by the addition of aqua regia. Then, the solution was diluted with water to volume in a 100 mL flask and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analysis for the solution was conducted at once. The silver weight content in Ag-SiO_2 was 0.45 at%.

2.9. Silver ions release properties of Ag-SiO_2

0.05 g of Ag-SiO_2 was added to 10 mL sterile water and the solution was stirred for 1.5 h at 37 °C, followed by high speed centrifugation. The supernatant was poured out and the solid sample was transferred to 10 mL sterile water and treated with the same procedure for three times. Then, the four supernatants were used for ICP-AES analysis.

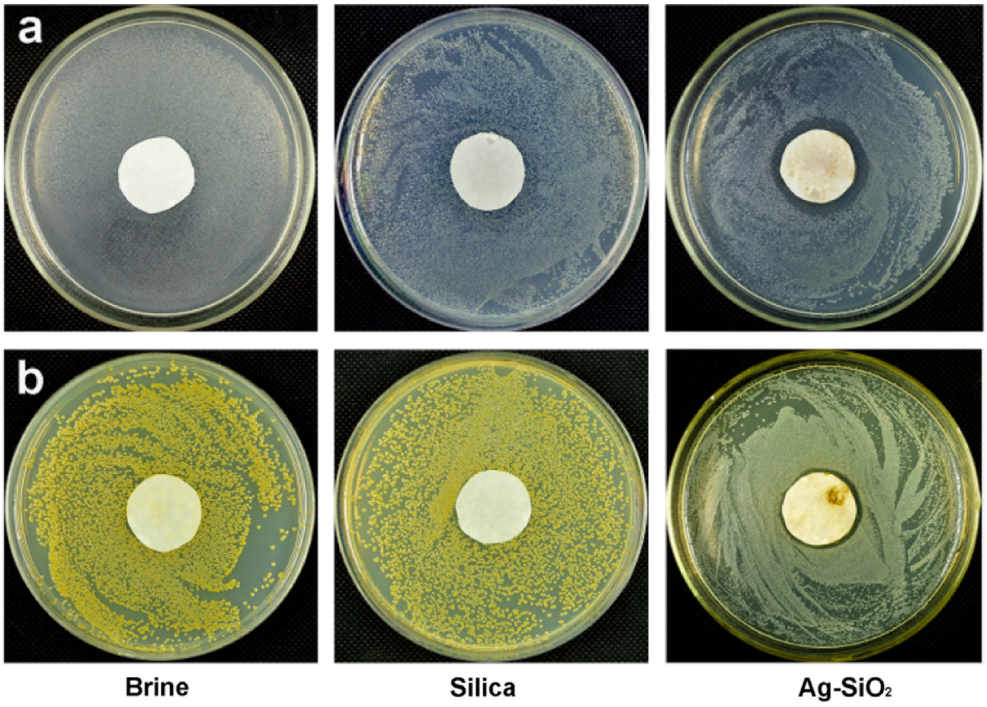

Fig. 1. Antibacterial ability of Ag-SiO_2. The modified Kirby–Bauer tests were employed to evaluate antibacterial activities toward Gram-negative bacteria *E. coli* (a) and Gram-positive bacteria *S. aureus* (b). Photographs were taken after incubation for 24 h at 37 °C. The cell concentration was 2.5×10^6 per mL.

3. Results and discussion

We investigated the antibacterial ability of the Ag-SiO_2 for both Gram-negative *E. coli* and Gram-positive *S. aureus* by using a modified Kirby–Bauer method. Suspensions of Ag-SiO_2 were dripped onto the filter paper and then the filter paper was gently placed on a lawn of *E. coli* and *S. aureus* in a Luria–Bertani (LB) agar plate. After 24 h of incubation, the Ag-SiO_2 led to a clear zone of inhibition (Fig. 1a and b), with average diameters of 28.6 and 22.8 mm for *E. coli* and *S. aureus*, respectively. The difference in diameter suggested that the Ag-SiO_2 had better antibacterial effects against Gram-negative *E. coli* than Gram-negative *S. aureus* since the membranes of Gram-positive bacteria are thicker and more stable than those of Gram-negative bacteria [10]. Sterile water and silica nanoparticles without encapsulated silver nanoclusters were also tested to confirm that the growth inhibition observed was caused by silver and not the silica materials or other species.

Furthermore, we employed the methods of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) to quantitatively evaluate the antimicrobial ability of the Ag-SiO_2. Based on the turbidity of the cell suspension, the MIC is defined as the lowest or minimum concentration of an antimicrobial material that inhibits the visible growth of a microorganism in artificial media after a fixed incubation time. Serially diluted suspensions of Ag-SiO_2 were incubated with equal volumes of *E. coli* and *S. aureus* solutions, respectively and the bacterial growth was monitored. After 24 h of incubation, we found that the MIC was 0.2 mg/mL and 0.3 mg/mL for *E. coli* and *S. aureus*, respectively (Table 1).

Table 1
Antibacterial activity (MIC) of Ag-SiO_2 against *E. coli and S. aureus* in 24 h.

Bacteria	Ag-SiO_2 (mg/mL)						
	0	0.05	0.1	0.2	0.3	0.4	0.5
E. coli	+	+	+	−	−	−	−
S. aureus	+	+	+	+	−	−	−

LB liquid media was clear before incubation with 2.5×10^6 of *E. coli* or *S. aureus*; "−"no growth; and "+"growth.

To further confirm the antimicrobial efficacy of the materials, the clear solutions resulted from the incubation for MIC evaluation were dripped onto the LB liquid media and incubated them again for 24 h to determine the MBC of Ag-SiO_2, which is the minimal concentration of Ag-SiO_2 when no bacteria grows or the number of bacteria colonies is less than 5. As shown in Fig. 2, the formation of colonies for *E. coli* was fully inhibited in the LB agar plates containing Ag-SiO_2 with a silver dosage of 0.72 μg/mL (0.4×0.0045 mg/mL). Meanwhile, the value for *S. aureus* was 1.08 μg/mL (0.6×0.0045 mg/mL), which is far smaller than the reported results [20], indicating the excellent activities of the silica encapsulated silver clusters.

The antibacterial kinetic test can provide the effectiveness for silver ions release of the Ag-SiO_2 in aqueous solution and the related antibacterial efficacy. Suspensions of Ag-SiO_2 (at concentrations of 0.4, 0.6, and 0.8 mg/mL, 0.45 at% silver-doped silica) were placed in equal volumes of aqueous *E. coli* and *S. aureus* solutions, respectively, and contacted for periods of time. Then, the mixtures were dripped onto the Luria–Bertani (LB) agar plate and incubated for 24 h.

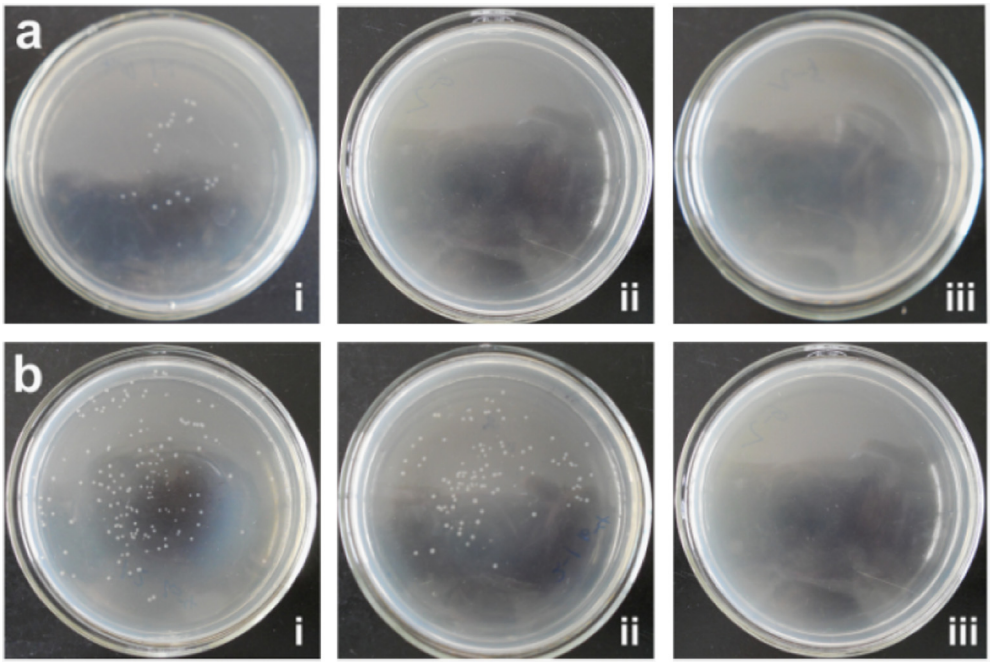

Fig. 2. Petri dishes with LB agar incubated with different concentrations of Ag-SiO_2 toward *E. coli* ((a-i) 0.2, (a-ii) 0.3 and (a-iii) 0.4 mg/mL) and *S. aureus*. ((b-i) 0.3, (b-ii) 0.4, and (b-iii) 0.6 mg/mL), showing variable numbers of colonies when supplemented with different concentrations of Ag-SiO_2.

The formation of colonies was examined by the plate counting method. As shown in Fig. 3, the kill percentage of bacteria increases rapidly and approaches an asymptotic value, reflecting the quick silver ions release and very fast kill rate of Ag-SiO_2 due to its unique structure. We soaked the Ag-SiO_2 in sterile water and measured the concentration of Ag^+ in the solution via ICP-AES analysis. The data confirmed the rapid and consistent release of Ag^+ from Ag-SiO_2 (Table 2), suggesting the high concentration of silver ions in the solution and strong antibacterial activity of Ag-SiO_2.

According to the Kelvin equation, smaller particles exhibit higher solubility than larger particles, leading to higher silver ions releasing speed and thus higher effective biocidal concentration. We tested the antibacterial activity of both Ag-SiO_2 and silica encapsulated silver nanoparticles with diameter of about 6 nm against test strains. As shown in Fig. 4, MIC values of Ag-SiO_2 for *E. coli* and *S. aureus* are both smaller than that of silica encapsulated silver nanoparticles, reflecting the higher activity of silver nanoclusters against bacteria than that of the silver nanoparticles.

We also evaluated the antibacterial properties of the Ag-SiO_2 for both Gram-negative *E. coli* and Gram-positive *S. aureus* by measuring the microbial durability of bacteria incubated with this material. Bacteria (either *E. coli* or *S. aureus*) were cultured in LB liquid media containing Ag-SiO_2 with different concentrations, and the bacterial growth was monitored. We found that Ag-SiO_2 of 0.2% could delay Gram-negative *E. coli* growth up to 1 day, and the growth of both Gram-negative *E. coli* and Gram-positive *S. aureus* was completely inhibited during 48 h culture period when the concentration of Ag-SiO_2 was increased to 0.5% (Table 3), suggesting the long-term antibacterial activity of Ag-SiO_2 to bacteria suppression. This result is in agreement with the consistent release of Ag^+ from Ag-SiO_2 (Table 1).

LB liquid media was clear before incubation with 2.5×10^6 of *E. coli* or *S. aureus*; "−"no growth; "+"growth.

Fig. 5 shows the UV–vis spectra and photograph image of the Ag-SiO_2 aqueous suspensions for different time storages. Actually, naked Ag nanoparticles are easily oxidized and/or aggregated in air and then their antibacterial activities will reduce. The yellow color of Ag nanoparticles colloidal solutions will gradually change to dark brown and the extinction peak of Ag NPs shifts bathochromicly with a large decrease in intensity due to the aggregation of Ag nanoparticles. On the contrary, the yellow color of the Ag-SiO_2 suspension did not have any change during 5 days storage and the extinction peak of corresponding UV–vis spectra was almost the same, reflecting the high stability and resistance to oxidation/aggregation of the Ag clusters encapsulated in porous silica compared to conventional Ag nanoparticles.

We ascribe this high stability to the confinement of the silica pore for the Ag clusters and this confinement prevented the aggregation of Ag clusters. Considering the fact that the aggregation states of Ag nanoparticles are critical to their antibacterial activity, it is reasonable to propose that the high stability of the Ag-SiO_2 endows them with a long-term antibacterial activity.

Table 2
Consistent release properties of Ag-SiO_2.

Time	1	2	3	4
Ag^+ (μg/mL)	42.78	12.28	12.52	11.21

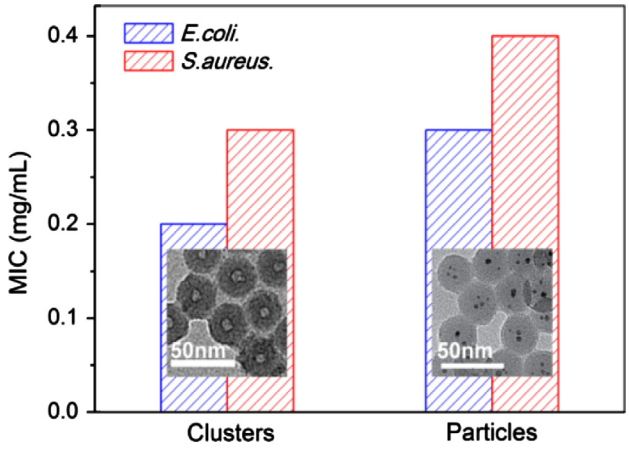

Fig. 4. Antibacterial activity of Ag-SiO_2 and silver nanoparticles against test strains.

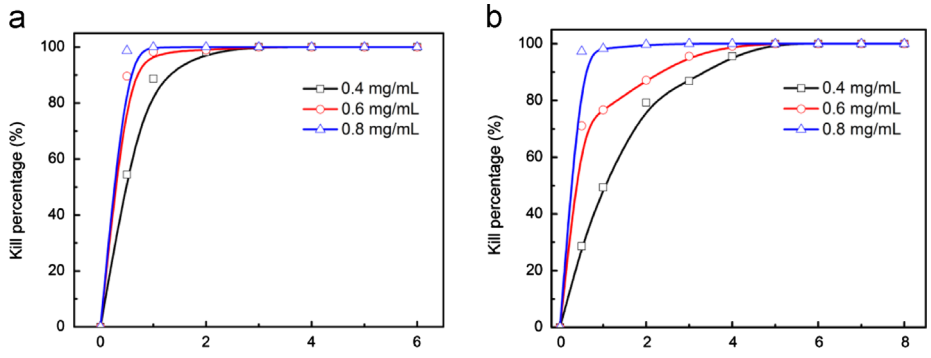

Fig. 3. Plot of kill percentage of bacteria versus contact time (h) of Ag-SiO_2 on *E. coli* (a) and *S. aureus* (b). The kill percentage of bacteria was calculated as $(A-B)/A \times 100$ (where A is the number of surviving *E. coli* or *S. aureus*. colonies in the blank solution and B is the number of surviving *E. coli* or *S. aureus*. colonies in Ag-SiO_2 suspensions).

Table 3
Growth of Gram-negative and Gram-positive bacteria with different concentrations of Ag-SiO_2.

Material added (mg/mL)	*E. coli*				*S. aureus*			
	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h
Control	+	+	+	+	+	+	+	+
0.1	−	+	+	+	+	+	+	+
0.2	−	−	+	+	−	+	+	+
0.3	−	−	−	−	−	−	+	+
0.5	−	−	−	−	−	−	−	−
0.6	−	−	−	−	−	−	−	−

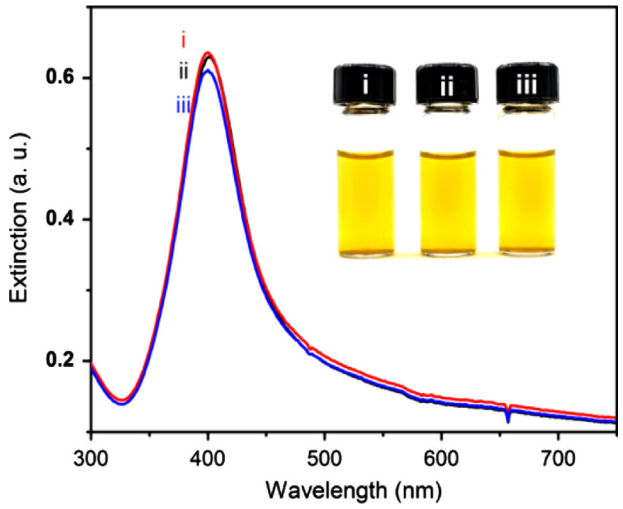

Fig. 5. The UV–vis spectra of Ag-SiO_2 aqueous solution with a surface plasmon resonance band: red line (i), new synthesis; black line (ii), stored for 5 days in light; and blue line (iii), stored for 5 days in dark. Inset: photos of the corresponding Ag-SiO_2 aqueous solution under different conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In summary, the Ag-SiO_2 presented excellent and durable antimicrobial activities in strongly killing both Gram-negative *E. coli* and Gram-positive *S. aureus*. More importantly, these silica nanospheres encapsulating silver nanoclusters released silver ions rapidly and consistently and showed substantially higher antibacterial activity than silica nanospheres encapsulating silver nanoparticles. It is expected that such efficient antimicrobial hybrid inorganic materials could find potential clinical and environmental applications.

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