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Preparation and anti-bacterial properties of a temperature-sensitive gel containing silver nanoparticles

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The purpose of this study was to prepare a temperature-sensitive gel containing silver nanoparticles and to investigate its anti-bacterial properties *in vitro*. The aqueous gel was prepared using Pluronic F127 (18-22%) and Pluronic F68 (3-9%) in a cold method to obtain a proper gelation temperature at 37 °C. Viscoelastic properties of the system were measured by rheological measurements and the physicochemical properties were evaluated by MJ-22 Dial-reflex metaloscope and Zetasizer Nano ZS90. The *in vitro* antimicrobial activity was evaluated by a disk diffusion test, minimum inhibitory concentration, and minimum bactericidal concentration. A temperature-sensitive gel containing silver nanoparticles with 20 wt% F127 and 6 wt% F68 had suitable fluidity at 25 °C and was semi-solid at 37 °C. Silver nanoparticle size averaged 78.0 nm. The gel optimized formulation achieved a suitable viscosity. The MIC and MBC of the gel ranged from 1.0 to 2.0 mg/L against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* The activity of the gel against these three species was significantly enhanced ($p < 0.05$) compared to 400 mg/L Asimi standard. This optimized silver nanoparticle dosage form demonstrated a high potential for further development for the clinical treatment of bacterial vaginosis.

1. Introduction

Bacterial vaginosis (BV) is the most common vaginal infection in women of child bearing age. In the 2001–2004 National Health and Nutrition Examination Survey, 29% of women aged 14 to 49 years were tested positive for BV with 3.13 times greater prevalence among African Americans than white women (JE and JF, 2007). Bacterial vaginosis is considered an important risk factor for obstetric complications such as preterm birth, low birth weight and post-partum endometritis (Kekki et al. 2004). Bacterial vaginosis infection can also increase a woman's susceptibility to human immunodeficiency virus (HIV) infection if she is exposed to the HIV virus. In addition, BV can increase a woman's susceptibility to other sexually transmitted diseases (STDs), such as herpes simplex, chlamydia, and gonorrhea (Myer et al. 2005; CDC 2008). Therefore, treatment of BV is especially important in pregrant women. Bacterial vaginosis responds to oral or topical antibiotic therapy (Milani et al. 2003) by either oral clindamycin or vaginal metronidazole (Austin et al. 2005). The cure rate is often low and the recurrence rate of BV has been reported to remain high up to 58% after treatment. Therefore, an alternative form of treatment is urgently needed to fully eradicate the infection and limit a patient's susceptibility to STDs.

Silver nanoparticles have shown promise in the development of effective antibacterial, antifungal, anti-viral and anti-inflammatory agents due to their remarkable physical, chemical and biological properties (Vaidyanathan et al. 2009; Pal et al. 2007). Compared with other metals, silver nanoparticles show higher toxicity to microorganisms with lower toxicity to mammalian cells (Zhao and Stevens 1998). Silver nanoparticles have commonly been used on the surfaces of household appliances and used as the cathode in a silver-oxide battery for a number of years since they are relatively free of adverse effects in humans (Shin et al. 2007). To date, the most promising applications have been shown in the medical or pharmacological fields, such as biosensors (Sun et al. 2009) and wound treatment for infection (Lu et al. 2008; Muangman et al. 2006). Silver nanoparticles are available as antimicrobial gel formulations for conventional topical antimicrobial agents, especially for burn treatment (Jain et al. 2009). However, conventional gels and ointments have certain disadvantages in vaginal drug delivery, such as the need for a special application syringe to deliver the gel, which may soften or liquidate unpredictably, and move away from the treatment site rapidly.

Temperature-sensitive hydrogels with unique gelling properties for drug delivery and tissue engineering have recently shown good biocompatibility and biodegradation (Rezwan et al. 2006). Pluronics are thermo-sensitive polymers that have been widely utilized as drug carriers since they exhibit unique sol-gel transition behaviors in response to temperature in aqueous solution (Chung et al. 2008). Pluronic F127 and F68 are di-functional nonionic surfactants/block copolymers that terminate in primary hydroxyl groups, are 100% active, and are relatively nontoxic

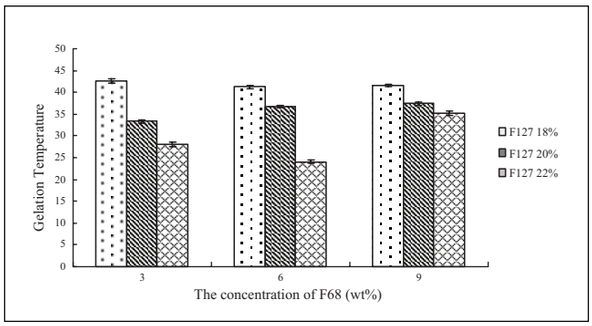


Fig. 1: Gelation temperature of three different concentrations of Pluronic F127 (18%, 20%, 22%) and F68 (3%, 6%, 9%) was performed to evaluate the proportion effects to obtain a suitable gelation at 37 °C. The gelation temperature decreased with an increase in the concentration of F127 over the temperature range of 25–90 °C. Values represent temperature means ± standard deviations

(Zhao et al. 2007). A temperature-sensitive gel in the proper dosage form possesses numerous advantages over conventional gel formulations and ointments (Zhang et al. 2009). For example, it may increase residence time at the absorption site, improve contact between the gel and the absorption site, and provide localization to mucosa to enhance bioavailability. Therefore, we decided to test the feasibility of incorporating silver nanoparticles into Pluronic F127 and F68 to make a temperature-sensitive gel. It can yield a liquid at 25 °C to spray and semi-solid at 37 °C to have better retention and bioavailability characteristics in the vagina.

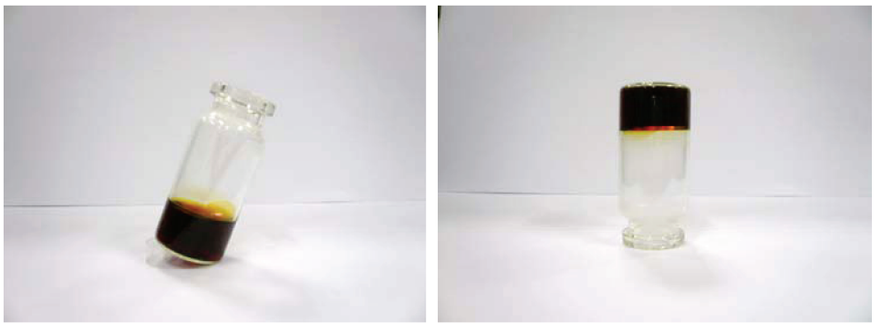
In the present study, a novel gel formulation composed of Pluronic F127 and F68 was prepared and characterized as a function of temperature. Optimum ratios of F127 and F68 were investigated to attain a suitable gelation temperature in an aqueous media at 37 °C. Furthermore, we also studied a novel temperature-sensitive gel containing silver nanoparticles. Antimicrobial activity was investigated using a disk diffusion test, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC).

2. Investigations, results and discussion

2.1. Gelation temperature of ratio F127/F68

Pluronic solutions were composed of two high-performance thermoplastic polymers, hydrophilic poly(ethylene oxide) (PEO) and hydrophobic Poly(p-phenylene oxide) (PPO) blocks. Therefore, the ratio of Pluronic F127/F68 ultimately affected the ratio of PEO/PPO and resulted in different gelation temperatures. Gelation temperature is defined as the temperature at which a liquid phase makes a transition to a gel. Wei et al. (2002) reported that solutions containing less than 15% F127 did not form gels over 25 °C, while a F127 concentration higher than 25% led to difficulty in preparation and administration. The gelling temperature initially increased to a maximum for concentrations containing approximately 10% F68, then decreased with further increases in F68 (Wei et al. 2002). This research indicated that we should be able to alter the proportion and concentration of F127/F68 to obtain a suitable gelation temperature at 37 °C. Therefore, our current study was performed to alter the proportion and concentration of F127/F68 using three different concentrations of Pluronic F127 and F68, which is 18%, 20%, 22% and 3%, 6%, 9%, respectively. Gelation temperature of various ratios of F127/F68 is shown in Fig. 1. During the tested temperature ranges, the gelation temperature decreased with an increase in the concentration of F127. These results are in agreement with those of Wei et al. (2002) showing that although both of the two Pluronics had significant effects on gelation temperature, F127 concentration was more important in modulating gelation temperature. The temperature-sensitive concentration of 20.0 wt% F127 and 6.0 wt% F68 yielded a liquid at 25 °C and semi-solid at 37 °C (Fig. 2). This novel formulation allows for easy application of a liquid at room temperature that becomes semi-solid at body temperature and therefore has better retention and bioavailability characteristics in the vagina.

(a)



(b)

Fig. 2: The temperature-sensitive concentration of 20.0 wt% F127 and 6.0 wt% F68 yielded a liquid at 25 °C (Fig. 2a) and semi-solid at (37 ± 0.5) °C (Fig. 2b)

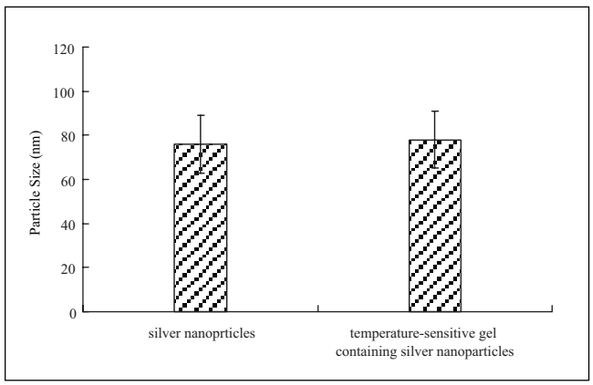


Fig. 3: Particle size distribution of the silver nanoparticle solution yielded a narrow absorption peak from (76 ± 13) nm and particle size distribution of the temperature-sensitive gel containing silver nanoparticles yielded (78 ± 10) nm range

2.2. *Physicochemical properties of the gel*

2.2.1. *pH*

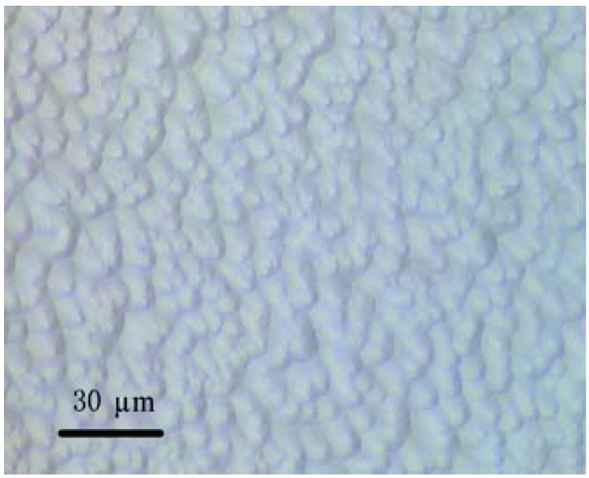
The pH of the temperature-sensitive gel containing silver nanoparticles was 5.3. Since this was within the pH range that commonly occurs during a vaginal infection (pH 5-6) (Amaral et al., 2006), it was not necessary to adjust the pH.

2.2.2. *Particle size determination*

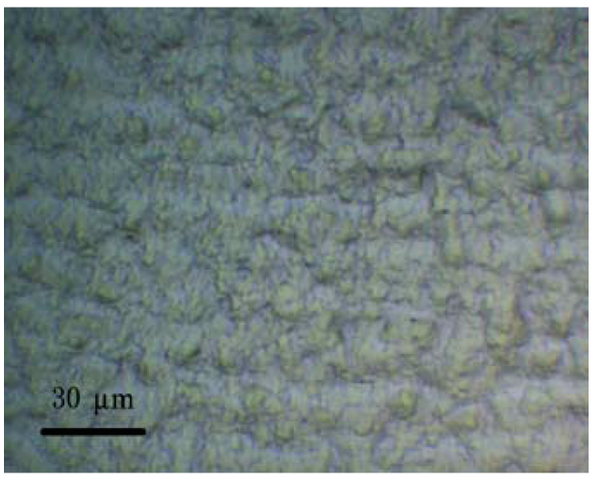
Silver nanoparticles can attach to bacterial cell membranes as well as inside the cells. Thus, the particle size is an important factor which can significantly affect the level of nanoparticles uptaken by the bacterial cell. Xu et al.(2004) showed that silver nanoparticles with sizes of < 80 nm accumulate inside living P. aeruginosa cells, demonstrating that these silver nanoparticles were transported across the outer and inner cell membrane. In this respect, assessment of size distribution of a temperature-sensitive gel containing silver nanoparticles was essential. Fig. 3 shows the particle size distribution of the silver nanoparticles solution and temperature-sensitive gel containing silver nanoparticles. Particle size distribution of the silver nanoparticle solution yielded a narrow absorption peak from 76 ± 13 nm and particle size distribution of the temperature-sensitive gel containing silver nanoparticles yielded 78 ± 10 nm range. Nanoparticle size distribution was not significantly different ($p < 0.05$) when incorporated into the Pluronic F127/F68 gel. We believe that the structure of F127/F68 (PEO-PPO-PEO tri-blocks) does not effect the size distribution of the silver nanoparticles in the temperature-sensitive gel. This implies that silver nanoparticles in the F127/F68 gel matrix should have excellent permeability with a particle size approximately < 100 nm.

2.2.3. *Morphology observation*

Microscopic images of the temperature-sensitive gel with and without silver nanoparticles are shown in Fig. 4. Micographs of the temperature-sensitive gel without silver nanoparticles appeared light in color and finely textured with 4-6 granules/30 μM (Fig. 4a) and temperature-sensitive gel containing silver nanoparticles appeared dark in color and coarsely textured with 2-3 granules/30 μM (Fig. 4b). The microstructure of the gel appeared to be made up of evenly distributed networks but differed in size with the addition of silver nanoparticles. Microscopic observation revealed that the structure of temperature-sensitive gel containing silver nanoparticles are good for usage and may have better adhesive ability due to its coarse texture.



(a)



(b)

Fig. 4: Microscopic images of the temperature-sensitive gel without silver nanoparticles appears light in color, finely textured with 4-6 granules/30 μM (Figure 4a) and temperature-sensitive gel containing silver nanoparticles appears dark in color, coarsely textured with 2-3 granules/30 μM (Figure 4b)

2.3. *Rheological studies*

The viscosity of temperature-sensitive gel containing silver nanoparticles was measured as a function of temperature and presented in Fig. 5. Viscosity data demonstrated minimal changes between 25 °C (94.5 mPa s) and 36.5 ° C (195 mPa s), with a slight inflection point at 37 °C (265.3 mPa s), and rapid increase at 37.2 °C to 1070 mPa s in the temperature-sensitive gel containing silver nanoparticles. There was a maximum viscosity at 38 ° C of 2180 mPa s. This rapid temperature dependent change in liquid/gel physical state may be attributed to the degree of PEO/PPO molecular structure at 37.2 °C where the formation of a multi-block copolymer structure increased its cross-linking density and resulted in the enhanced mechanical strength of the hydrogel network. These results may be promising for the clinical application of the temperature-sensitive gel.

2.4. *Minimal inhibitory concentration(MIC) and Minimal backsicidal concentration(MBC)*

The MIC and MBC results for the temperature-sensitive gel containing silver nanoparticles demonstrated excellent activity against *S. aureus, E. coli,* and *P. aeruginosa* (Table 1). The

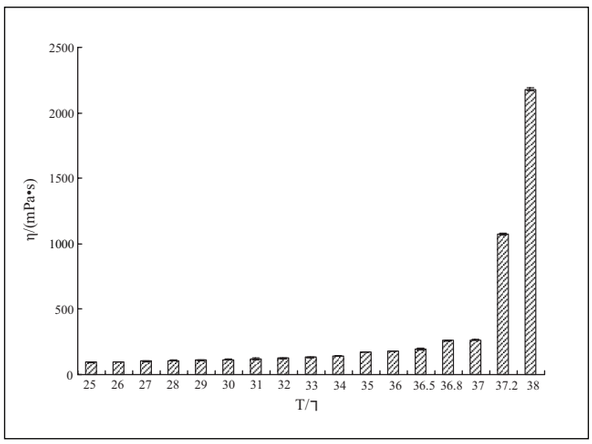


Fig. 5: Viscosity of the temperature-sensitive gel containing silver nanoparticles increased slowly over the temperature range from 25 to 36 °C and showed marked increase above 37 °C

Table 1: Antimicrobial activity of temperature-sensitive gel containing silver nanoparticles (n = 3)

Bacterium	MIC	MBC
S. aureus	1.0 mg/L	1.0 mg/L
E. coli	1.0 mg/L	2.0 mg/L
P. aeruginosa	2.0 mg/L	2.0 mg/L

MIC and MBC of the temperature-sensitive gel containing silver nanoparticles ranged from 1.0 to 2.0 mg/L against the three bacteria. The antimicrobial activity was attributed to the slow release of the silver nanoparticles in the gel.

2.5. Antibacterial effect

The temperature-sensitive gel containing silver nanoparticles was evaluated for its antibacterial effect against *S. aureus, E. coli,* and *P. aeruginosa* using a disk diffusion test determined by the size of visible zone of inhibition. The temperature-sensitive gel containing silver nanoparticles at four different concentrations (50, 100, 200, and 400 mg/L) showed an antibacterial activity. The commercial antibacterial product containing silver particles Asimi was used as an internal standard (Table 2). The antibacterial results demonstrated that the growth of *S. aureus, E. coli,* and *P. aeruginosa* were significantly inhibited in a dose dependant manner by the temperature-sensitive gel. Antibacterial activity against *S. aureus* was demonstrated as a 10.7 mm zone (50 mg/L) and increased to 17.9 mm (400 mg/L), against *E. coli* as a 8.2 mm zone that increased to 11.3 mm, against *P. aeruginosa* as a 9.2 mm zone that increased to 14.8 mm. The most susceptible species was *S. aureus* (17.9 mm inhibition zone) and the most least susceptible was *E. coli* (8.2 mm inhibition zone). Asimi is an antimicrobial gel comprised of silver nanoparticles and carbomer, which is used as a vaginal formulation for women. The activity of the temperature-sensitive gel containing silver nanoparticles against *S. aureus, E. coli,* and *P. aeruginosa* was significantly enhanced ($P < 0.05$) when compared with the commercially available 400 mg/L Asimi standard. Although both the Pluronics and carbomers are non-ionic polymers, it is postulated that the Pluronics co-block structure provides a more stable environment for the silver nanoparticles, resulting in higher antibacterial activity. We know from our on-going antimicrobial studies that antimicrobial zone size is dependent on molecular weight and test compound solubility and diffusion characteristics. We understand the limitations in comparison between compounds tested in various *in vitro* assays. Comparision between Pluronic polymers and carbomer polymer is not exact. However, the carbomer based nanoparticles (Asimi) commonly standard used in clinical treatment is the closest to the Pluronic silver nanopartical gel matrix. The results reported indicate promising antibacterial activity of temperature-sensitive gel containing silver nanoparticles.

2.6. Conclusion

This work developed a novel application of silver nanoparticles as a therapeutic formulation in a temperature-sensitive gel for the effective treatment of bacterial vaginosis. We established the optimum ratio of Pluronic F127/F68 to achieve a suitable gelation temperature at 37 °C for a thermosetting vaginal drug delivery system and successfully improved the antibacterial activity of silver nanoparticles as compared to Asimi. The use of silver nanoparticles in the Pluronics formulation offers a valuable alternative to conventional therapy which is relatively free of adverse effects of many antibiotics, demonstrates low toxicity, and excellent tissue tolerance. The temperature-sensitive gel containing silver nanoparticles described in this study possesses the potential for developing a highly efficient in situ gelling formulation for drug delivery. We believe that gelatinization of the formulation at 37 °C will increase patient compliance and acceptance of this formulation. Better bioavailabilty will be provided for thorough treatment of BV and ultimately a reduction in disease incidence. This novel silver formulation should also allow for the successful treatment of drug-resistant bacterial strains without generating further drug-resistance.

3. Experimental

3.1. Materials

Pluronic® F127 and F68 used for temperature-sensitive gel preparation were obtained from BASF Corp. Silver nanoparticles were purchased from Shanghai Tinaph Nano-Tech Co., Ltd (Shanghai, China), glycerol from Guangzhou-hung Instrument Co., Ltd (Guangzhou, China), and ethylparaben from the Development Center of Tianjin Kemiou Chemreagent (Tianjin, China). Double distilled and deionized water was used throughout the experiment and all other materials used were of analytical or pharmaceutical grade.

Table 2: Antibacterial effect of temperature-sensitive gel containing silver nanoparticles

Organisms	Temperature-sensitive gel containing silver nanoparticles				Asimi®
	50 mg/L	100 mg/L	200 mg/L	400 mg/L	400 mg/L
S. aureus	10.7 ± 0.3	13.3 ± 0.5	15.3 ± 0.7	17.9 ± 0.6	16.7 ± 0.4
E. coli	8.2 ± 0.4	9.5 ± 0.5	10.5 ± 0.4	11.3 ± 0.3	10.5 ± 0.2
P. aeruginosa	9.2 ± 0.2	11.2 ± 0.4	13.2 ± 0.4	14.8 ± 0.5	13.9 ± 0.5

Asimi® used as the standard, is a conventional antimicrobial gel comprised of silver nanoparticles and carbomer, which is used as a vaginal formulation for women. Values represent antibacterial growth inhibition means (mm) ±standard deviations

3.2. Measurement of optimum polymer ratio

Pluronic F127 (18.0-22.0%) and F68 (3.0-9.0%) were used initially to determine the optimum polymer ratio for gelation temperature (the change from liquid to gel) at 37 °C by tube inversion experiments. Combinations of different Pluronic F127 and F68 concentrations (3.0 g dispersed in 10 mm glass tubes) were placed in a water bath over the temperature range of 25-90 °C. The temperature was increased at a rate of 5 °C/h and the change from liquid to gel (or vice-versa). In all cases the gelation temperature was reproducible within 0.1 °C and the gel melted completely within a 0.2-0.3 °C range (Choi et al., 1998).

3.3. Preparation of gel formulation

Glycerol and ethylparaben were dissolved in double distilled and deionized water in the concentration of 1.0% and 0.1%, respectively. Pluronic F127 (12.0 wt%) and F68 (6.0 wt%) were prepared in aqueous glycerol and ethylparaben according to the cold method of El-Kamel (2002). The dispersion was refrigerated to increase the rate of swelling. Silver nanoparticles were added to achieve a final silver concentration of 350 mg/L. If the pH is within the range of pH 5-6, no adjustment will be necessary (Amaral et al. 2006). All formulations were allowed to equilibrate for 24 h at 25 °C before performing experimental studies.

3.4. Physicochemical properties of the gel

A pH meter (Shanghai precision & scientific instrument Co., LTD, China) was used to determine the pH value of the Pluronic F127/F68 gel. Particle size was evaluated using a laser diffraction particle size analyzer (Malvern Zatasize NanoZS90, Malvern Instruments Ltd., UK) and the concentration of 4.2 mg/L. Digital microscopic images of the temperature-sensitive gel containing silver nanoparticles were obtained with a MDJ200 Dia-reflex metaloscope (Guangzhuo Precision Equipment Co. Ltd, China).

3.5. Rheological measurements

The rheological behavior of gel formulations was determined using a Brookfield's Model DV-III$^+$ Viscometer (Brookfield, USA) at a controlled rate mode. Gel consistency and flow measurements were made in a cone-and-plate geometry with a diameter of 20 mm and a cone angle of 2 ° at 25-40 °C and the shear rates ranged from 0.5 to 100 seconds. Formulation sample (15.0 mL) of Pluronic F127/F68 in aqueous glycerol and ethylparaben was applied to the lower plate using a spatula to ensure that formulation shearing and air bubbles did not occur. Formulation sample was at a heating rate of 0.5-1.0 °C/min, a constant frequency (1.0 Hz) within specified temperatures and at the determined polymer concentrations at 37 °C. The temperature sweeps were performed using non-destructive oscillatory measurements for the gels. Mean was calculated from triplicates and experiments were repeated twice.

3.6. Microorganisms and growth conditions

Staphylococcus aureus (ATCC25923), *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were supplied by the Experimental Center for Basic Medical Teaching of Sun Yat-sen University, Guangzhou, China. Bacterial strains were cultivated on Mueller-Hinton (MH) broth medium (Hangzhou Tianhe Microorganism Reagent Co., Ltd, China) and were incubated at 37 °C for 24 h prior to testing. Cell suspensions were adjusted with a sterile saline solution to obtain of concentration of 1.5×10^8 cells/mL by comparison with a 0.5 McFarland turbidity standard.

3.7. Determination of MIC and MBC

Minimum inhibitory concentration (MIC) was determined using a broth micro-dilution method with a 96-well micro-titer plate (NCCLS, 2008). One series of 2-fold dilutions of each sample (ranging from 128 to 0.5 mg/L) for each microbial strain was prepared using MH broth medium. Each series was inoculated with 10 μL of the 1.5×10^8 cells/mL microbial strain and 200 μL of sample. Experiments also included a positive control well containing nutrient media without silver nanoparticles and a negative control well containing silver nanoparticles without bacterium. MIC was determined after incubation for 24 h at 37 °C under 5% CO_2, and in an upright position. All experiments were repeated twice.

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of drug that kills 99.9% of the bacteria (Mims et al. 1993). Bacteria were subcultured using a 10 μL loop from the existing bacterial culture and inoculated on solid Mueller-Hinton Agar (MHA) in order to establish the bactericidal effect. Subcultures were incubated at 37 °C for 24 h under 5% CO_2. The lowest concentration at which there was no bacterial growth on MHA was determined to be MBC.

3.8. Disk diffusion test

Bacterial sensitivity to antibiotics is commonly tested using a disk diffusion test (Rios et al. 1988). Petri plates (150 mm in diameter) containing 20 mL of nutrient agar were inoculated with 100 μL of bacterial culture and were allowed to dry in a sterile chamber. Bacterial suspensions were also the concentration of 1.5×10^8 cells/mL. Filter paper discs (6 mm in diameter) impregnated with 5 μL of temperature-sensitive gel containing silver nanoparticles at four concentrations (400, 200, 100, 50 mg/L) were placed on the inoculated agar surface. The antibacterial agent Asimi (400 mg/L, Shenzhen Tsinghua Yuanxing Pharmaceutical Co., LTD, China) was placed as a control. The plates were incubated at 37 °C for 24 h under 5% CO_2. Antibacterial activity against each test organism was quantified by determining the mean zone of inhibition around the paper discs. All assays were carried out in triplicate.

3.9. Statistical analysis

Means and standard deviations were obtained from three experiments and were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test. Statistically significant differences were identified when $p < 0.05$.

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