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Contact Mechanism of the Ag-doped Trimolybdate Nanowire as An Antimicrobial Agent

Yujie Jiang[1], Jian Gang[1], Sheng-Yong Xu[1,*]

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Abstract: Antibacterial Ag-agents are intensively applied as broad spectrum, high-stability, high-efficiency and high-safety inorganic antibacterial agents. We have developed a new kind of antibacterial Ag-agent, namely $Ag_{2-x}(NH_4)_xMo_3O_{10}\cdot 3H_2O$ nanowires (NWs). Carrying Ag atoms in the lattice and Ag-rich nanoparticles on the surface, the Ag-doped NWs show strong antibacterial effects for a variety of bacteria including *E. coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. By performing systematic comparison experiments, we have proven that the main antibacterial effects are neither resulted from the tiny amount of Ag^+ ions released from the Ag-doped NWs in aqueous solutions, nor resulted from Ag-rich nanoparticles of fragments of the NWs when they are slowly dissolved in the Martin broth. Instead, the effects are mainly resulted from a contact mechanism, under which, the Ag-doped NWs need to be physically in contact with the bacteria to be eliminated. This is a novel phenomenon observed in the interactions between nanomaterials and live cells, which is worthy of further investigation at the molecular scale. As the Ag-doped NWs are not dissolved in pure water or weak acids, one may find practical antibacterial applications in textile industry and food storage industry for these unique nanomaterials.

Keywords: Antibacterial agent; Antibacterial Ag-agent; Silver ions; Silver-rich nanoparticles; Silver-doped trimolybdate nanowire; Bio-safety of nanomaterials; Contact mechanism

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Introduction

Antibacterial agents are important materials that are intensively applied in hospitals, clinics, biological laboratories, medical and food industries, as well as in military practices and daily lives. There are natural antibacterial agents, organic antibacterial agents and inorganic antibacterial agents. Among inorganic antibacterial agents, the antibacterial Ag-agents that consist of element Ag have been widely investigated, and they are extensively applied in many cases as the first choice, because these Ag-agents usually inhibit low cost, broad spectrum, high stability, high efficiency, and above all, high safety [1-10].

To date two main effective parts have been recognized for the strong antibacterial properties of the Ag-agents: Ag^+ ions, and Ag-rich nanoparticles. For the antibacterial effects of Ag^+ ions, two kinds of mechanism have been reported. First, it is resulted from the toxic effects of the heavy element Ag^+ released from the agent. Second, it is attributed to the strong oxidization effects of reactive oxygen species (ROS) produced by Ag^+, which consequently causes permanent damages on macromolecules or membrane of the bacterium [11-21]. On the other hand, the exact antibacterial mechanism of Ag-rich nanoparticles is not clear. However, at the molecular level one major difference has been reported: In the cells under test the Ag-rich

[1]Key Laboratory for the Physics and Chemistry of Nanodevices and Department of Electronics, Peking University, Beijing 100871, China
[1]These authors contributed equally to the paper.
*Corresponding author. E-mail: xusy@pku.edu.cn

nanoparticles do not cause a dense DNA phenomenon, but it is always observed in Ag^+ treated cells [22-31].

We have reported that Ag-doped trimolybdate NWs, with a chemical formula of $Ag_{2-x}(NH_4)_xMo_3O_{10}\cdot 3H_2O$, have strong antibacterial effects. These NWs carry a certain amount of Ag atoms in the lattice and Ag-rich nanoparticles on the surface, and they can be synthesized at one-atmosphere from aqueous solutions. A 2-15 ppm concentration of the Ag-doped NWs in water could sufficiently eliminate the growth of *E. coli* and *Staphylococcus aureus*, while still being safe to certain kind of human cells [32]. The crystalline structure of the Ag-doped NWs is similar to the alkali family of trimolybdate NWs, namely $\Theta_m(NH_4)_{2-m}Mo_3O_{10}\cdot nH_2O$ (Θ=Li, Na, K, Rb; m=1, 2). The Ag-doped NWs are hard to be dissolved in solutions with pH values of 2-9, but they can be dissolved in Martin broth [33].

However, it is not clear whether the antibacterial mechanism of the Ag-doped NWs is caused by the Ag^+ ions, or by the Ag-rich particles released from the NWs, or it is due to other unknown mechanism. In this work, we have performed systematic experiments and revealed a contact mechanism for the antibacterial effects of the Ag-doped trimolybdate NWs.

Experimental details

We have followed the method reported previously to synthesize the Ag-doped trimolybdate NWs [32, 33]. Basically, the Ag-doped NWs were synthesized from a mixed aqueous solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (purity 99.999%) and $AgNO_3$ (purity 99.999%) that were separately dissolved in de-ionized water. For each run, the masses of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and $AgNO_3$ were typically 2 g and 0.25 g, respectively, and the reaction temperature was set at 60±5℃. After continuously stirring of the mixed solution for a few minutes, yellowish-white precipitates started to occur in the solution. The precipitates were collected, centrifuged and rinsed in de-ionized water for 6 cycles to get the pure NWs. The final solid samples were dried in air. The morphology and crystalline structures of the solid samples, recognized as Ag-doped trimolybdate NWs, were characterized by means of a field-emission scanning electron microscope (SEM, Tecnai XL30F) operated at 3-15 kV and a transmission electron microscope (TEM, TecnaiG20) operated at 200 kV. The density of bacteria in the solution was determined with optical density measurement on a UV-2100 spectrophotometer (UNIC Optoelectronics Technology), operated at the wave length of 600 nm. The measured data, referred as OD_{600} values, linearly indicated the colony density of bacteria about 1×10^9 cfu/ml at OD_{600}=1. The inductive coupled plasma (ICP) spectrum was performed on a PROFILE SPEC (Leeman) atomic emission spectroscopy system. The culturing of various bacteria was performed following a standard biological procedure. A dozen groups of different experiments were done to figure out the antibacterial mechanism of the Ag-doped trimolybdate NWs.

In the first sets of experiments, we examined whether the antimicrobial effect of the Ag-doped NWs was resulted from the Ag^+ ions in the solution released from the NWs. To determine the concentration of Ag^+ ions in an aqueous solution of Ag-doped NWs, a suspension of 1 wt% Ag-doped NWs was prepared by adding 500 mg NWs into 50 ml de-ionized water, stirred thoroughly and kept for 24 hrs at room temperature. After that, an acrodisc syringe filter with average pore diameter of 0.45 μm (Article No. PN 4614, Pall Co.) was applied to filtrate the suspension. Then 10 ml filtered solution was measured with an ICP atomic emission spectroscopy system. For comparison, we prepared 5 aqueous solutions of $AgNO_3$ with Ag^+ ion concentrations of 44.7 ppm, 18.6 ppm, 7.76 ppm, 3.66 ppm and 0.7 ppm, respectively. Then 20 ml of each of the six kinds of solutions were individually mixed with Martin medium powder, at a ratio of 1000 ml to 28.5 g. Each mixed solution was further mixed with 1 ml of yeast suspension (namely *Saccharomyces cerevisiae*, showing an OD_{600} value of 0.4), and the final samples were cultured at 37℃ for 24 hr.

Next, control experiments, namely Groups A, B and C, were performed for culturing the same yeast sample. For Group A, we prepared mixture of Martin medium powders with 20 ml de-ionized water. For Group B, a mixture of Martin medium powder was prepared with 20 ml filtrate of the suspension of 1 wt% Ag-doped NWs in de-ionized water. For Group C, the Martin medium powder was mixed with a suspension of 100 ppm Ag-NWs in 20 ml de-ionized water. The ratio of powder to solution for Groups A, B and C was all kept at 28.5 g to 1000 ml. Then 1 ml yeast suspension (showing OD_{600} of 0.4) was added into each sample of Groups A, B and C. All these samples were cultured at 37℃ for varied time up to 20 hrs, and their OD_{600} values were measured at different stages of the cultured samples. We have repeated this set of experiments for 3 times.

The OD_{600} method was applied to measure the dissolvability in time of the Ag-doped NWs in the Martin broth. Then 5 sets of samples were prepared to determine the solvent(s) for the Ag-doped NWs. In every 28.5 g of Martin powder, there were 5 g of tryptone, 2 g of yeast extract, 20 g of glucose, 1 g of K_2HPO_4 and 0.5 g of $MgSO_4$. For each set of testing samples, 6 mg Ag-doped NWs suspended in 20 ml de-ionized water was mixed with one of the 5 ingredients of the Martin medium. Their OD_{600} values were measured at the time when the mixtures were just prepared, and at the time after they were treated in a shaker for 8 hrs.

Finally, we examined whether the antimicrobial effect was resulted from Ag-rich nanoparticles released from the Ag-doped NWs, or the productions when the Ag-doped NWs were dissolved in Martin broth. The Martin broth was prepared at a ratio of 1000 ml de-ionized water to 28.5 g of Martin medium powder. First, the density change of the a 100 ppm Ag-doped NWs suspension (i.e., 2 mg Ag-doped NWs in 20 ml Martin broth) was continuously measured over a period of 24 hrs by means of the OD_{600} method. Next, 4 groups of samples, namely Groups D, E, F and G, were prepared. The samples of Group D contained 20 ml pure Martin broth. For Group E samples, the prepared suspension, where 2 mg Ag-doped NWs were totally dissolved in 20 ml Martin broth, was filtered with the acrodisc syringe filter. Group F samples contained originally 100 ppm solid Ag-doped NWs mixed with 20 ml of Martin broth, and no filtering treatment was done. For samples in Group G, 20 ml of pure Martin broth was filtered with the acrodisc syringe filter. Each sample was added 1 ml of the same yeast (*Saccharomyces cerevisiae*, with original OD_{600} value of 0.4) suspension and was cultured at 37℃. The samples were taken out at different culturing time from 0 to 10 hrs for OD_{600} measurements. These sets of experiments were repeated for 3 times.

In addition, we conducted preliminary experiments to check the safety of applying the Ag-doped NWs on the skin of live animals. The skin sensitivity tests were performed with 4 rabbits weighing 2.3-2.5 kg for 14 days. The temperature and humidity of the rabbit hutch were kept at 18-26℃ and 40-61%, respectively. Hairs on both sides of spine of each rabbit were totally sheared away, leaving two pieces of clean skin, each about 3 square centimeters. On one piece of the clean skin, 0.5 ml of 1000 ppm Ag-doped NWs in de-ionized water was smeared uniformly, while the other piece was left untreated for contrast. The same experiment was repeated for continuous 14 days.

Results and discussions

Measured in SEM, the as-fabricated Ag-doped NWs typically had a high length-to-diameter aspect ratio of 10^2-10^4 with smooth surfaces. Their lengths were ranged from a few to a hundred micros, and their diameters usually ranged in 10-100 nm, as shown in Fig. 1(a). When examined in a TEM, however, although each of these Ag-doped NWs did have a good uniformity in diameter and a smooth surface, the

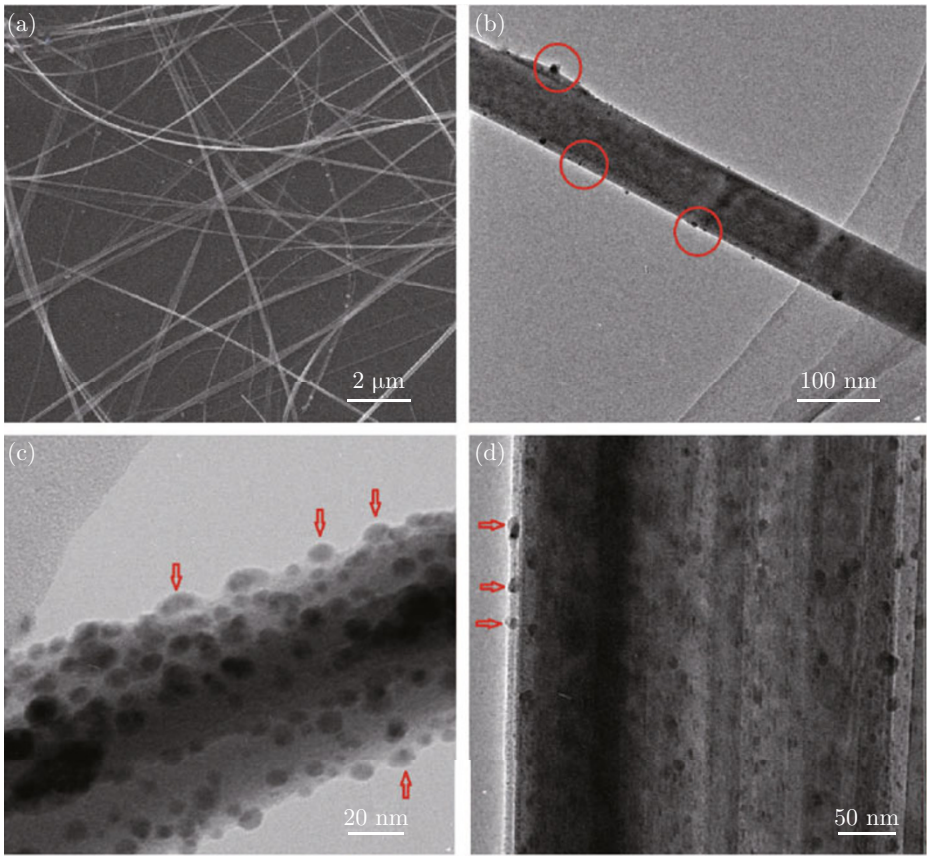

Fig. 1 (a) A SEM micrograph of the Ag-doped, $Ag_{2-x}(NH_4)_xMo_3O_{10}\cdot 3H_2O$ NWs. (b)-(d), TEM micrographs of individual Ag-doped NWs at different magnifications, where Ag-rich nanoparticles, appearing as dark dots in the images, are highlighted with red circles in (b) and with red arrows in (c) and (d).

surface was not clean. As shown in Fig. 1(b) and 1(c), many dark nanoparticles were observed on the surface. These nanoparticles were characterized to be Ag-rich, probably being Ag_2O nanoparticles [32]. As highlighted with red circles and arrows in Fig. 1(c) and 1(d), many Ag-rich nanoparticles were observed at the side of individual NWs, indicating these particles were located within the surface layer of the NS. We have confirmed this point by focusing a high-intensity electron beam of the TEM on individual Ag-doped NWs. After irradiation treatment of the high-intensity electron beam, the Ag-doped NW kept its shape, but many Ag-rich particles shifted away from their original positions.

As most Ag-doped NWs were much longer than 10 μm, we expected that after filtrated with the acrodisc syringe filters, whose average pore diameter was 0.45 μm, the Ag-doped NWs in the suspension were all removed from the remaining broth. That is, in the filtered solution, there were no Ag-doped NWs in their full lengths. If the NWs were partially of totally dissolved in the solvent, i.e., the Martin broth, however, besides molecules and molecule-clusters, there could be fragments of the dissolved NWs.

To examine whether the antimicrobial effect of the Ag-doped NWs was resulted from the Ag^+ ions in the solution released from the NWs, we first measured the Ag^+ concentration in the suspension of 1 wt% Ag-doped NWs in de-ionized water. The suspension was stirred thoroughly, kept for 24 hrs and filtered with a 0.45 μm-pore filter. In the suspension the concentration of Ag-doped NWs (1 wt%) was much higher than its minimum inhibitory concentration observed previously for antibacterial effect, which was 2-15 ppm [32, 33]. This setup ensured that, if Ag^+ ions could be released from the Ag-doped NWs, in the filtered solution one should found enough Ag^+ ions, and the solution should have obvious antibacterial effect. But the measured results were all negative. No antibacterial effects were observed by using the filtered solution. Indeed the concentration of Ag^+ ions in the filtered solution was only 1.7±0.3 ppm, as measured in ICP tests.

The low concentration of Ag^+ ions in the suspension of Ag-doped NW in water was confirmed by the control experiments, where antibacterial effects of aqueous $AgNO_3$ solutions with Ag^+ ion concentrations from 0.7 ppm to 44.7 ppm were systematically examined. The results showed that, 44.7 ppm and 18.6 ppm solutions of Ag^+ strongly eliminated the growth of yeasts, while in the rest 3 samples, with Ag^+ concentration of 7.76 ppm, 3.66 ppm and 0.7 ppm, respectively, the antibacterial effects were weak. It showed that, under the conditions of our experiments, the minimum concentration of Ag^+ ion concentration for antibacterial application was somewhere between 18 ppm and 7 ppm, a value much higher than the 1.7±0.3 ppm measured from the suspensions of Ag-doped NWs.

Therefore, the results have shown that the tiny amount of Ag^+ ion released in aqueous solutions from the solid Ag-doped NWs is not the major antibacterial factor observed in our experiments [32, 33]. The aqueous solution of Ag^+ ions is known as a strong antibacterial agent. When the Ag^+ ions are in a Martin broth, the measured minimum concentration of Ag^+ for antibacterial effect in our experiments, around 10 ppm, was consistent with previous reports [36]. But under other conditions, the minimum concentration of Ag^+ for showing moderate antimicrobial effects could be as low as 0.1 ppm [34, 35]. The main reason could be the appearance of K_2HPO_4 in the Martin medium powder, which seems weakening the bactericidal effect of Ag^+.

The experimental results samples of Group A, Group B and Group C are shown in Fig. 2, where roughly, the OD_{600} values of 0.4, 1, 3 and 6 are calibrated as corresponding to the yeast densities of around 4×10^8, 10^9, 3×10^9 and 6×10^9 cfu/ml, respectively. No difference is seen between the results of Group A and Group B. As the Group A samples are the standard, original materials for culturing bacteria, the results clearly indicate that the materials prepared for Group B do not have any antibacterial effect for yeast. In other words, the amount of Ag^+ ions in the sample, if there is any, is not enough to eliminate the growth of yeasts. In sharp contrast, in Group C samples the measured yeast density is obviously lower than those in Groups A and B. And, this low density looks not changing with the culturing time, indicating an excellent elimination of the growth of yeasts at the right beginning of the experiment. This confirmed the strong antibacterial effect of the Ag-doped NWs themselves. We repeated the

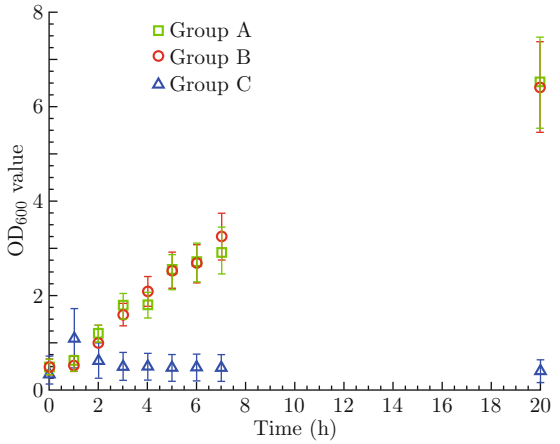

Fig. 2 OD_{600} measurement results for density of bacterium samples of Groups A, B and C. For Group A, pure Martin broth is used. For Group B, the powder is mixed with 20 ml filtrate of the suspension of wt.1% Ag-doped NWs in de-ionized water. For Group C, the powder is mixed with 100 ppm Ag-NWs in 20 ml de-ionized water. No evidence for antibacterial effect is observed in Groups A and B, but that for Group C shows a strong antibacterial effect. All experiments are repeated for 3 times.

experiments three times, and obtained the consistent results.

A detail is noted in Fig. 2. In the first two hours, the yeast density in the Group C slightly increases and then it decreases to a value close to the original one. It may indicate that the NWs need some time to be effective for elimination of the growth of yeasts.

Figure 3 shows the solubility of the Ag-doped NWs in the aqueous solution of Martin broth powder. One sees that the OD_{600} value decreases rapidly with time. In 10-16 hrs the NWs are almost totally dissolved. Figure 4 shows the results that determine which of the five ingredients of the aqueous solution of Martin broth powder (tryptone, yeast extract, glucose, K_2HPO_4 and $MgSO_4$) plays the key role for dissolving the Ag-doped NWs. The initial OD_{600} values of 6mg in the test solutions are all about 0.6. The OD_{600} values for Ag-NWs

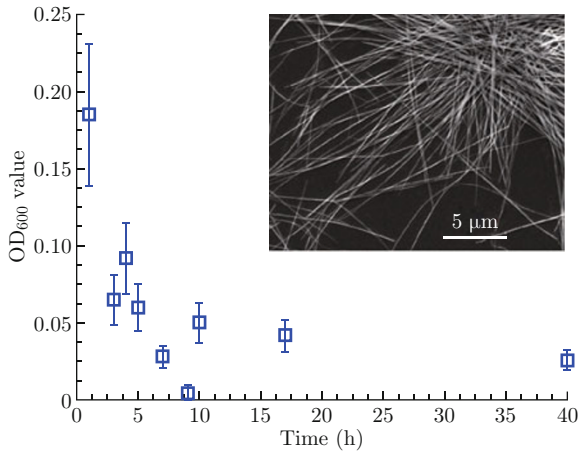

Fig. 3 The OD_{600} measurements results for 2mg Ag-doped NWs in 20 ml Martin broth, showing the density of NWs remained in the solution decreases rapidly in time. After 10-16 hrs the NWs are almost totally dissolved. The inset shows a SEM micrograph of the Ag-doped NWs.

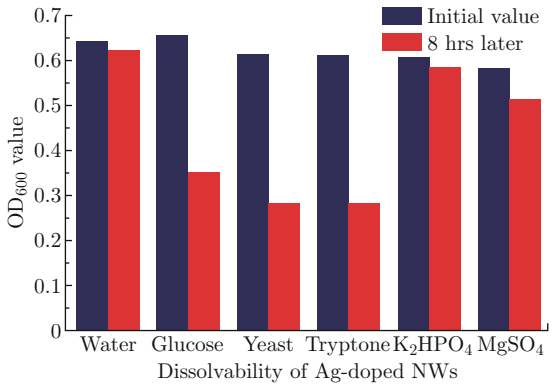

Fig. 4 A plot of the measurement results, showing which ingredient of a Martin broth being the solvent for dissolving Ag-doped NWs. Originally their OD_{600} values are the same. After 8 hrs, the OD_{600} values for Ag-NWs mixed in tryptone, yeast extract and glucose decrease to around 0.3, while the rest do not change much.

mixed in tryptone, yeast extract and glucose decreased to around 0.3 after the samples had been shaken for 8 hrs in the solutions, indicating the NWs were partially dissolved in these three solvents. For samples mixed with K_2HPO_4 and $MgSO_4$, the OD_{600} values did not change. So we conclude that the tryptone, yeast extract, glucose are the solvents for dissolving the Ag-doped NWs. As there are nanoparticles on surface of the NWs and they may not be firmly attached to the NWs, there could be free Ag-rich nanoparticles in the solution after the NWs are dissolved. The following results reveal whether the possible free Ag-rich nanoparticles, fragments or any other productions in the solution, where the Ag-doped NWs have been dissolved in Martin broth, cause the antimicrobial effects.

Figure 5 plots the OD_{600} data showing yeast density in samples of Groups D, E, F and G. Group D samples are the blank control samples with the pure Martin broth, so the data indicate the normal growth trend of the yeasts over time. Within the measurement error, the samples of Groups D, E and G give the same yeast density at each time point, implying the materials prepared in Groups E and G have no antibacterial effects. But in Group F, where 2 mg NWs were added for an original concentration of 100 ppm Ag-NWs, strong antibacterial effect is seen. The main difference in these repeated experiments is that, in samples of Group F, there are solid Ag-doped NWs of original concentration of 100 ppm, while the samples of the rest groups originally contain no solid Ag-doped NWs.

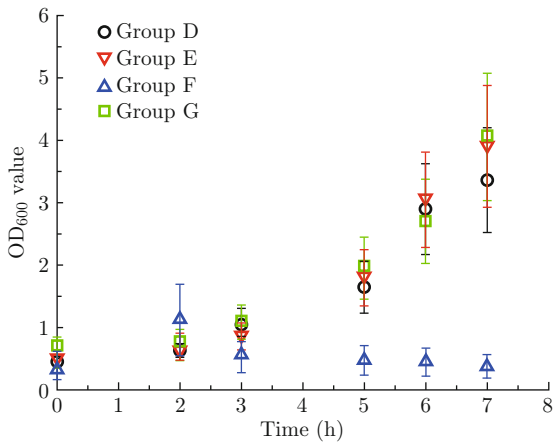

Fig. 5 The OD_{600} data measured from samples of Groups D, E, F and G. Clearly, yeasts grow well in samples of Groups D, E, and G, but not in samples of Group F. The main difference is, in samples of Group F, there are solid Ag-doped NWs of original concentration of 100 ppm, while the samples of the rest groups originally contain no solid Ag-doped NWs. All experiments are repeated for 3 times.

The data for Group F are quite similar to that for Group C shown in Fig. 2. Once again, the common point is that the materials prepared for Group C and Group F both contain a large amount of solid Ag-doped

NWs. Also in a similar way, in the Group F the yeast density slightly increases in the first two hours then decreases to a value close to the original. These results have shown that the antimicrobial effect is neither caused by the nanoparticles, nor by the dissolving productions of the Ag-doped NWs in the Martin broth, but rather by the un-dissolved, solid Ag-doped NWs. It needs further investigation at the molecular level to understand how the solid Ag-doped NWs interplay with the bacteria under test.

Finally, the skin sensitivity tests showed that, after applying 0.5 ml of 1000 ppm Ag-doped NWs in de-ionized water on the rabbit skin for continuous 14 days, the skin stayed in good condition free of erythema or dropsy. This preliminary result implies a potential application for the solid, water-proof Ag-doped trimolybdate NWs as high-performance antibacterial agent in textile industry, e.g., as the surface agent of underwares and socks. They may also be used as the coating materials for the inner surface of food containers, e.g., a refrigerator.

Conclusions

In short, we have synthesized $Ag_{2-x}(NH_4)_xMo_3O_{10}\cdot 3H_2O$ NWs with the one-atmosphere, aqueous solution technique, and systematically studied the antimicrobial mechanism of these Ag-doped NWs for bacteria *E. coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. The Ag-doped NWs are not dissolvable in water, but can be slowly dissolved in the Martin broth, an agent for culture of bacteria. When wt.1% of the Ag-doped NWs are mixed in de-ionized water and kept for 24 hrs, the Ag^+ ion concentration in the solution is around 1.7±0.3 ppm. We have confirmed that, this tiny amount of Ag^+ ions do not have obvious antibacterial effects under the setup of our experiments. We also confirmed that dissolved fragments of the Ag-doped NWs in the Martin broth do not show obvious antibacterial effects. We show unambiguously that the antibacterial effects of the Ag-doped NWs are mainly resulted from a contact mechanism of the nanomaterials with the bacteria under test. This contact mechanism has been utilized to sense the occurrence of Ag-doped NWs in a diffusing process passing through porous ager medium [37]. In addition, this new phenomenon observed in the interactions between nanomaterials and live cells may have a potential for practical antibacterial applications in textile industry and food storage industry [38-40].

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