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Food storage material silver nanoparticles interfere with DNA replication fidelity and bind with DNA

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Abstract
Nanosilver is increasingly used in the food industry and biomedical applications. A lot of studies have been done to investigate the potential toxicity of nanosilver. But information on whether or how nanosilver particles bring changes in genetic materials remains scant. In this study, the replication fidelity of the rpsL gene was quantified when nanosilver particles were present in polymerase chain reactions (PCRs) or cell cultures of *E. coli* transformed with the wild-type rpsL gene. Three types of nanosilver (silver nanopowder, SN; silver–copper nanopowder, SCN; and colloidal silver, CS) were tested. The results showed that the replication fidelity of the rpsL gene was differentially compromised by all three kinds of nanosilver particle compared with that without nanosilver. This assay could be expanded and applied to any other materials to preliminarily assess their potential long-term toxicity as a food additive or biomedical reagent. Moreover, we found that nanosilver materials bind with genomic DNA under atomic force microscopy, and this might be an explanation for the compromised DNA replication fidelity.

1. Introduction

The application of nanotechnology in the food industry will have a profound impact on a number of products. As a powerful bactericide, silver is used in food storage, packaging and processing, such as storage containers. Although silver in various chemical forms has toxicity to microorganisms [1–3], silver nanoparticles (Ag NPs) have gained increasing interests due to their strong antimicrobial activities [4, 5]. It was found that the silver nanoparticles have antibacterial effects at low concentrations, and the antibacterial properties were associated with the particle surface area [6]. Aymonier and co-workers demonstrated that hybrids of silver nanoparticles with macromolecules exhibited excellent biocidal action against bacteria [7].

Despite the wide application of nanosilver and many related studies on cytotoxicity to bacteria, there is still a serious lack of information concerning their long-term impact on human health and the environment. It has been suggested that DNA loses its replication ability once the bacteria are treated with silver ions [8]. DNA synthesis is required to faithfully replicate genomes with high fidelity. The fidelity of DNA synthesis is beneficial for avoiding harmful mutations that can initiate and promote human diseases such as cancer and neurodegenerative diseases. Brindha and co-workers provided some evidence that the DNA polymerase-induced errors were an important part of cancer-causing gene mutations [9]. However, little is known about the potential risk of whether the use of silver nanomaterials will lead to DNA replication errors that are related to long-term safety issues.

In this study, we started with the fidelity of DNA amplification/replication through a polymerase chain reaction (PCR) with a nanosilver aqueous suspension. The PCR has been proven to be a very useful tool and a basic laboratory procedure for DNA replication *in vitro* [10]. The nucleotide mis-incorporation errors in PCR products can be determined by

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Table 1. The silver nanoparticles and concentrations used in this study.

	Silver nanopowder, SN	Silver–copper nanopowder, SCN	Colloidal silver, CS
Source	Sigma: 576832-5G	Sigma: 576824-25G	Huzhen (Shanghai)
Particle size (nm)	30–50	<100	$\leqslant 1$
Stock solution (μg ml^{-1})	240	40	100
In vitro (PCR) final concentration (μg ml^{-1})	10	1.6	4
In vivo final concentration (μg ml^{-1})	0.6	0.1	0.25

mutation assays. The rpsL forward mutation assay is a direct, time-resolved measurement for PCR fidelity [11]. Besides, bacterial strains transformed with the wild-type rpsL gene can work as a model to detect the effects of nanomaterials on rpsL replication fidelity *in vivo* if the bacterial strains are incubated directly with nanomaterials. In this study, the effect of silver nanoparticles was explored for the first time on the DNA replication fidelity *in vitro* and *in vivo*, and the direct interaction between nanosilver and DNA was observed by atomic force microscopy (AFM).

One important issue in this report is the toxicity assessment methodology of nanoparticles. Approaches for toxicity assessment of nanoparticles are not necessarily different from those used for general chemicals [29], though specific approaches for nanoparticles are expected to develop. Several lines of investigations have been reported to measure the safety parameters of nanomaterials [17, 24–26], and the approaches used in those studies will be all suitable for general chemicals. In this study, perturbed DNA replication fidelity resulting from nanomaterials was employed for the potential long-term toxicity assessment. This is a novel convenient method to calculate the relative capacities of different nanoparticles to introduce DNA replication errors in the rpsL gene-based assays [10–12] both *in vitro* (polymerase chain reaction, PCR) and *in vivo*. The rpsL-based assay used in this study has not been reported in the evaluation of the genotoxicity of chemical, but it is expected to play useful roles for long-term toxicity assessment for many chemicals in future.

Another important issue in this report is the functional difference between silver nanoparticles and silver ions in the context of antibacteria activity and compromising the DNA replication fidelity. Already, lots of studies have been undertaken on antibacteria mechanisms and related biomedical implications with silver nanoparticles and silver ions. Jung *et al* found that silver ions may cause *S. aureus* and *E. coli* bacteria to reach an ABNC (active but nonculturable) state and eventually die [19]. A recent study compared the inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth [20]. Using autotrophic nitrifying organisms from a well-controlled continuously operated bioreactor, Ag NPs, Ag(+) ions ($AgNO_3$), and AgCl colloids, all at 1 mg l^{-1} Ag, inhibited respiration by 86%, 42%, and 46%, respectively. Based on a prolonged microtiter assay at about 0.5 mg l^{-1} Ag, the inhibitions on the growth of *Escherichia coli* PHL628-gfp by Ag NPs, Ag(+) ions, and AgCl colloids were 55%, 100%, and 66%, respectively. Cell membrane integrity was not compromised under the treatment of test Ag species. However, electron micrographs demonstrated that Ag NPs attached to the microbial cells. The above report, together with many other reports [21, 22], provided evidence that silver nanoparticles and silver ions inhibit bacterial growth and other cellular activities under different mechanisms. In this study, silver nanoparticles and silver ions displayed distinct effects as additives for PCRs, and the potential relationship between antibacteria activity and compromised DNA replication fidelity is discussed.

2. Materials and methods

2.1. Preparation of aqueous suspensions of silver nanoparticles

The nanosilver materials and their concentrations in PCRs and cell cultures are presented in table 1. Colloidal silver solution was used directly. Silver nanopowder was made as a water suspension. First, it was necessary that the silver nanopowder was exposed to UV light for about 30 min to be free of DNase and RNase. Then 10 mg silver nanopowder with 0.99 ml sterilized double-distilled H_2O (ddH_2O) was placed in a prepared 1.5 ml sterilized microcentrifuge tube and sonicated in an ultrasonic bath for 1–2 h. Then the 10 mg ml^{-1} silver nanopowder was diluted with ddH_2O to the required concentrations as shown in table 1. The *in vitro* final concentrations of nanosilver materials were those for PCRs optimized for best amplification efficiency in our laboratory. The concentrations used for fidelity measurement were 4–10 μg ml^{-1} for *in vitro* PCR (figure 2) and 0.1–0.6 μg ml^{-1} for *in vivo* bacterial growth (table 1). At 0.1–0.6 μg ml^{-1}, both silver nanoparticles and silver ions displayed partial inhibitory effects on cell growth and were not considered as toxic doses (see section 4 and figure 3).

2.2. Nanosilver materials affect DNA replication in vitro—PCR fidelity assay

The fidelity of DNA replication during the PCR was measured using the rpsL mutation assay, which is based on streptomycin resistance of an rpsL mutant [11]. The rpsL gene encodes the small ribosomal protein S12 to which streptomycin

binds. Some mutations in this gene make cells resistant to streptomycin. Since the streptomycin resistance (Sm^r) is recessive to the wild-type (Sm^s), Sm^r cells with the wild-type rpsL gene exhibit the Sm^s phenotype. Once a mutation inactivates the rpsL function, the cells become resistant to streptomycin. So an rpsL-based system to detect a very small fraction of mutant plasmids among a large number of wild-type plasmids was devised [23]. Briefly, the full length (4.0 kb) of the plasmid pMOL21 containing the ampicillin resistance (Ap^r) and rpsL genes was digested with Sca I as a template. Then the linearized pMOL21 was amplified by a PCR with biotinylated primers (P1 5′-biotin-AAAA AAAA AAAC GCGT CACC AGTC ACAG AAAA GCAT CTTAC-3′ and P2 5′-AAAA AAAA AAAC GCGT CAAC CAAG TCAT TCTG AGAA TAGT-3′). PCR amplifications were performed in 50 μl reaction volumes with 2.5 U Taq DNA polymerase, 200 μM each dNTP, and 0.2 μM each primer, on a T-Gradient Thermal Block (Biometre) and/or a SpeedCycler (Analytik Jena AG). PCRs began with a denaturation step at 94 °C for 2 min, and 25 cycles of amplification were performed using the following conditions: 15 s at 94 °C; 30 s at 58 °C; 5 min at 72 °C. The amplified fragments were purified by Streptavidin beads (Promega) which were attached at the end of primer P1 and then digested by MluI at the primer regions. After ligation by T4 DNA ligase the DNA products were used to transform Sm^r host bacterial cells MF101 [11]. Bacterial colonies formed on LB (Luria–Bertani medium) plates containing 100 μg ml^{-1} ampicillin were counted as total colonies. The number of colonies that formed on LB plates containing both ampicillin and 80 μg ml^{-1} streptomycin were counted as mutant colonies in which the rpsL gene had accumulated some replication (amplification) errors during the PCR. All enzymes used in this study were purchased from New England Biolabs.

2.3. Nanosilver materials affect DNA replication in vivo

In this experiment, bacterial *E. coli* cells MF101 transformed with the plasmid of pMOL21 were cultivated with nanosilver directly. This MF101/pMOL21 strain was cultivated in the LB medium with nanosilver materials for 24 h. The amount of nanosilver materials was 10 μl of the previously mentioned concentrations (table 1) for 4 ml LB medium. After 24 h of cultivation, a portion of the bacteria cultivated in the LB medium with or without nanosilver materials was diluted and plated on ampicillin and streptomycin plates to determine the total number of rpsL mutants, and another portion of diluted bacterial culture was plated on ampicillin plates to determine the total number of cells.

2.4. Atomic force microscopy (AFM) imaging

A total of 20 μl of a suspension of the DNA samples with or without nanosilver was dropped onto a newly cleaved mica surface (0.8 × 0.8 cm^2) by a pipette [13]. Then the samples were blown dry by clean air and were ready for AFM observation. AFM experiments were performed using a Multimode microscope (Nanoscope IIIa, USA) equipped with silicon cantilevers (NSC-11, MikroMasch) with a spring constant of 48 N m^{-1}. A typical resonant frequency of 340 kHz was used. All operations were carried out in air at room temperature under a relative humidity of 25%–40%.

Table 2. Comparison of PCR fidelity with or without silver nanoparticles.

Nanomaterial	Expt.	Total colonies	Mutate colonies	Mutation frequency (%)	Error rate ($\times 10^{-6}$)
Blank control	1	23 088	159	0.69	5.30
	2	777	6	0.77	5.92
	3	2 183	13	0.60	4.61
	Total	26 048	178	**0.68**	**5.23**
SN	1	2 072	20	0.97	7.46
	2	57 128	899	1.57	12.07
	3	9 213	200	2.17	16.69
	Total	68 413	1119	**1.63**	**12.54**
SCN	1	6 845	106	1.55	11.92
	2	2 220	25	1.13	8.69
	3	16 243	259	1.59	12.23
	Total	25 308	390	**1.54**	**11.85**
CS	1	1 073	37	3.45	26.53
	2	851	27	3.17	24.38
	3	3 441	52	1.51	11.62
	4	814	17	2.09	16.07
	Total	6 179	133	**2.15**	**16.54**

3. Results

3.1. The effect of silver nanoparticles on PCR fidelity

The rpsL replication fidelity assay was used to measure the error frequency of PCR products (table 2). Except for the water suspension of silver nanomaterials, all PCR parameters remained constant, including the dNTP, primer and template concentrations, the PCR cycling parameters, and the number of PCR cycles performed. The mutation frequency of Taq DNA polymerase alone (blank control) was 0.68% and the error frequency was calculated as 5.23×10^{-6}. The mutation frequencies in PCR became higher after nanosilver was added, showing a negative influence of silver nanoparticles for rpsL replication fidelity. When the silver nanoparticles present in the reaction, the mutation frequencies were calculated as 1.63% (SN), 1.54% (SCN), and 2.15% (CS), respectively.

In the experiments above, the mutation frequency was determined by dividing the total number of mutant colonies on the ampicillin and streptomycin plates by the total number of colonies on the ampicillin plates. The error rate was calculated using the equation error rate = mutation frequency/(bp × d), where bp is the base pair number of potential mutation sites that cause phenotypic changes in the rpsL gene (here bp is 130 [11]) and d is the number of template doublings. Template doublings (d) were determined using the equation 2^d = (amount of PCR product)/(amount of starting target DNA). Because the error rates do not appear to be significantly influenced by the number of template doublings, the PCR cycling number in this experiment was 30, so we used 10 uniformly as the template doublings (d) [10, 11]. The error rate of adding no nanomaterials calculated by the method described above was 5×10^{-6}; this result was in agreement with the error rate measured by Cline *et al* [10] (the error rate was 8.0×10^{-6}).

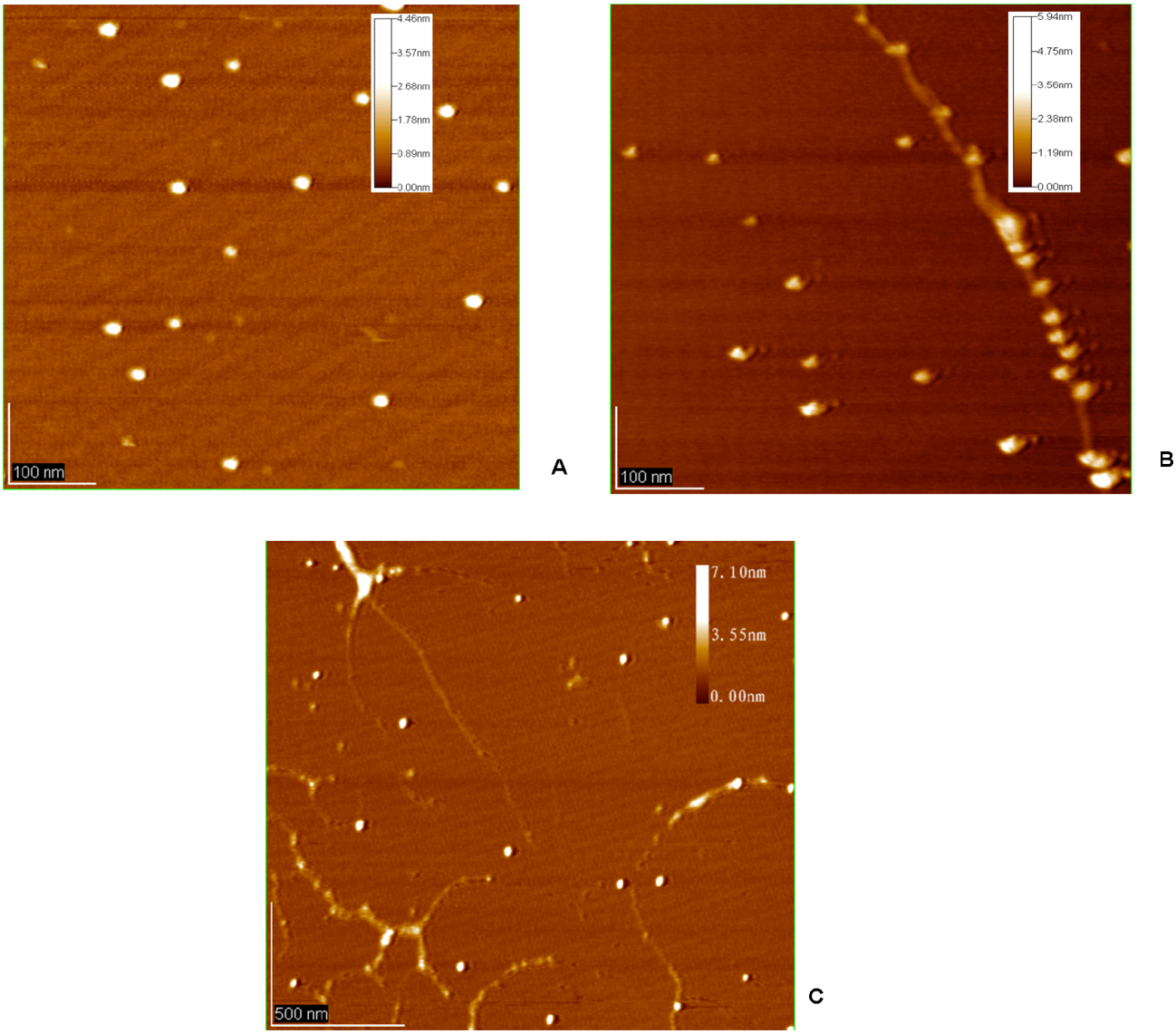


Figure 1. Observed binding between silver nanoparticles and DNA molecule by AFM. Only the results of colloidal silver (CS) are presented. (A) Observation of CS control, with AFM height image of pure silver nanoparticles. Scan size: 600 nm × 600 nm. (B) Part of an image of DNA–CS interaction. Size: 600 nm × 600 nm. (C) The genomic DNA of *E. coli* cultivated with CS. Scan size: 2.00 μm × 2.00 μm.
(This figure is in colour only in the electronic version)

3.2. The effect of silver nanoparticles on DNA replication fidelity in vivo

The mutation frequencies and replication error rates were calculated similarly as in the *in vitro* experiments, but the generation time of *E. coli* is about 20 min, which means that three generations of new cells will be created in 1 h. In our experimental system the cells were almost saturated after 6–7 h. In 6 h there would be 18–21 generations of cells. So we simply estimated d as 20 in the *in vivo* experiments. Comparing tables 2 and 3, the *in vivo* mutation frequencies and error rates were apparently four orders of magnitude lower than those *in vitro*. But remember that the *in vivo* mutation frequencies and replication error rates measured in this study were actually reflecting the replication fidelity of the whole genome plus transformed plasmids, not the single rpsL gene, whereas the *in vitro* mutation frequencies and replication error rates measured in this study were basically reflecting the replication fidelity of the rpsL gene alone. Considering that the length of the *E. coli* genome (about 4.5 × 10^6bp) is 10^4-fold longer than that of the rpsL gene (about 414bp), the mutation frequency induced by silver nanoparticles *in vitro* may be very similar to that *in vivo*, further suggesting that the nanomaterials tested in this study might be able to freely translocate and make contact with the genetic materials in the cell.

3.3. AFM imaging of the potential binding between silver nanoparticles and DNA

The potential binding between silver nanoparticles and double-stranded DNA (48 kb λ DNA) was investigated by atomic force microscopy. Without DNA, the silver nanoparticles dispersed at the surface of mica (figure 1(A)). When binding with DNA, the local heights of a DNA fragment along the surface of mica

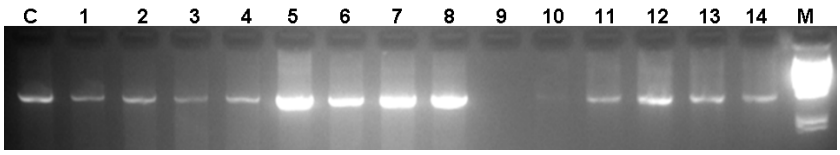
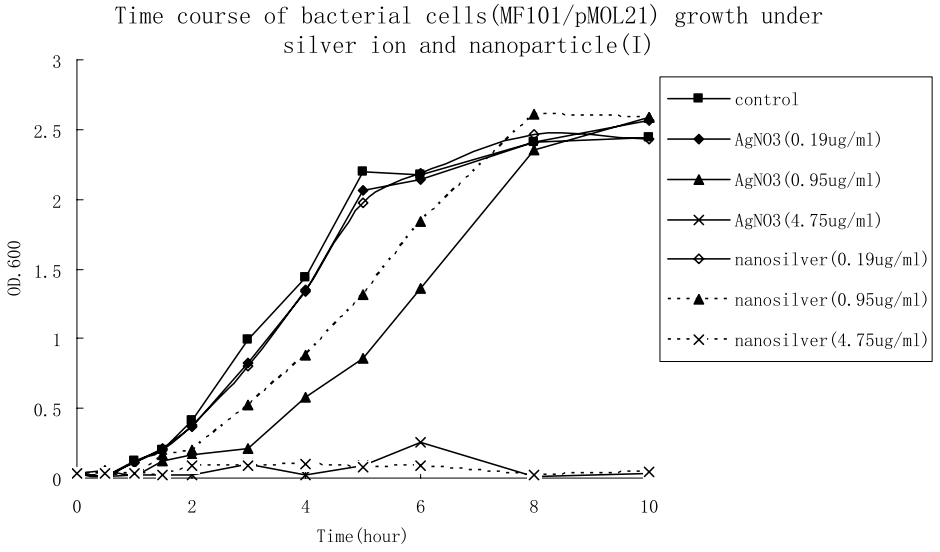


Figure 2. Amplification of linear pMOL21 (4 kb) with silver nanopowder (SN) and silver ion. From lane 1 to lane 14, the PCR additives and their final concentrations were SN (lane 1, 48 μg ml^{-1}), SN (lane 2, 9.6 μg ml^{-1}), SN (lane 3, 4.8 μg ml^{-1}), SN (lane 4, 0.96 μg ml^{-1}), SN (lane 5, 0.48 μg ml^{-1}), SN (lane 6, 0.096 μg ml^{-1}), SN (lane 7, 0.048 μg ml^{-1}), SN (lane 8, 0.0096 μg ml^{-1}), $AgNO_3$ (lane 9, 7.6 μg ml^{-1}), $AgNO_3$ (lane 10, 1.52 μg ml^{-1}), $AgNO_3$ (lane 11, 0.76 μg ml^{-1}), $AgNO_3$ (lane 12, 0.152 μg ml^{-1}), $AgNO_3$ (lane 13, 0.076 μg ml^{-1}), $AgNO_3$ (lane 14, 0.0152 μg ml^{-1}), respectively. M: λDNA/HindIII molecular marker. Note: 0.24 mg ml^{-1} SN and 0.38 mg ml^{-1} $AgNO_3$ had the same molar concentration for silver or silver ion. C: the control without SN or $AgNO_3$.



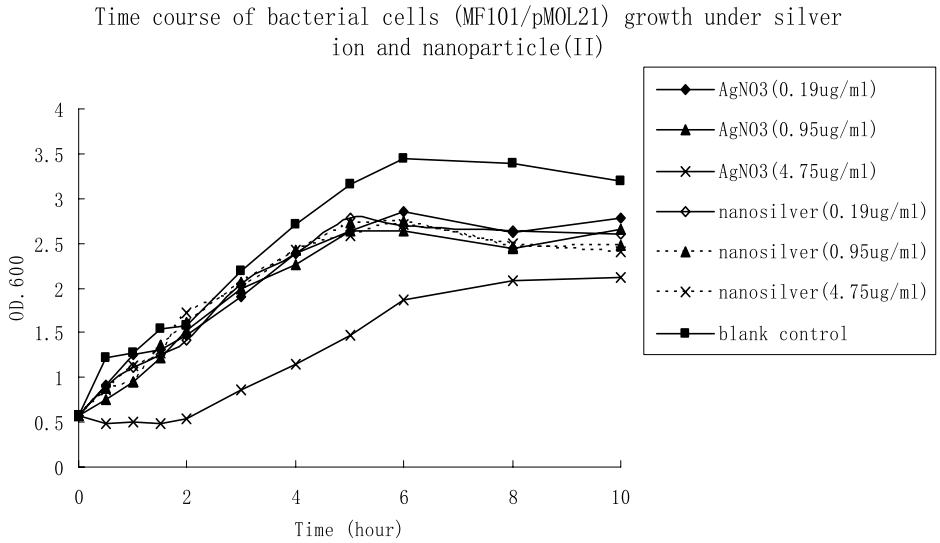


Figure 3. Effects on the growth of bacterial *E. coli* MF101/pMOL21 by silver nanoparticles and silver ions. The starting cell densities in (I) and (II) were 0.5 and 0.013 of OD600, respectively. Data were averaged from three tests.

apparently varied (figure 1(B)). The lining pattern of about 16 silver nanoparticles along the selected DNA fragment strongly suggested that there existed apparent binding between the DNA molecule and silver nanoparticles.

We also tried to observe the potential binding between nanosilver materials with *E. coli* genomic DNA. After being cultured together with nanomaterials, *E. coli* cells were harvested and subjected to genomic DNA isolation. In this stage, nanomaterials often contaminated the final genomic DNA samples, and it was often very hard to get clear AFM pictures for genomic DNA from cells treated with the three nanosilver materials. However, one of the three nanosilver

Table 3. Comparison mutation with or without silver nanoparticles *in vivo*.

Nanomaterials	Expt.	Total colonies ($\times 10^8$)	Mutant colonies ($\times 10^2$)	Mutation frequency ($\times 10^{-6}$)	Error rate ($\times 10^{-10}$)
Blank control	1	88	71	0.81	3.11
	2	28	42	1.50	5.77
	3	24	19	0.79	3.04
	4	20	41	2.05	7.88
	Total	160	173	**1.08**	**4.15**
SN	1	20	39	1.95	7.50
	2	18	27	1.50	5.77
	3	18	87	4.83	18.58
	4	20	86	4.30	16.54
	Total	76	239	**3.14**	**12.08**
SCN	1	16	27	1.69	6.50
	2	18	36	2.00	7.69
	3	27	68	2.52	9.69
	Total	61	131	**2.14**	**8.23**
CS	1	28	70	2.50	9.62
	2	26	36	1.38	5.31
	3	20	55	2.75	10.58
	4	45	109	2.42	9.31
	Total	91	200	**2.19**	**8.42**

materials used in this study, CS, was assumed to easily get cleaned from the cell pellets because the size of CS is very small (<1 nm). After repeated washing and filtration with a 0.45 μm filtering membrane, the purified cells were broken for genomic DNA isolation and AFM observation. Figure 1(C) suggests that the nanosilver CS was able to bind with the genomic DNA *in vivo*.

4. Discussion

Silver is well known for a broad spectrum of antimicrobial activities. Unlike other heavy metals, silver is believed to be safe for the human organism and it has been used as a nontoxic inorganic antibacterial agent for a long time. It is in the last few years that effective antimicrobial activity was found in the form of silver nanoparticles and this has resulted in their widespread use in consumer and industrial products, especially food storage material. Recently, the discussion of the potential risks of nanoparticles on life and environment are beginning to emerge again [14–17]. Despite the continuing increase in the population exposed to silver nanoparticles, the effects of silver nanoparticles have not been thoroughly studied.

This study is likely the first one that has reported an apparent increase of mutation frequency caused by silver nanoparticles during DNA replication *in vitro* and *in vivo*. In a typical PCR system, the error rate of Taq DNA polymerase was calculated as 5.23×10^{-6}. Using a lacI PCR mutation assay, Janice *et al* reported that the error rate of Taq and Pfu was 8.0×10^{-6} and 1.3×10^{-6}, respectively, and these data coincided with the results of an rpsL assay [11, 18]. Furthermore, the rpsL forward mutation assay is significantly faster and simpler than the lacI assay. However, when three different types of silver nanoparticle were added, the fidelity of Taq DNA polymerase decreased with an average error rate of 12.54×10^{-6}, 11.85×10^{-6} and 16.54×10^{-6} for SN, SCN, and CG, respectively. The error rate of Taq DNA polymerase increased by nearly three times after silver nanoparticles were put in the PCR reactions, and the *in vivo* experiment data demonstrated similar results. These results seem to be strong enough to call for a review of the long-term biohazard issues of silver nanoparticles.

One thing needs to be discussed: whether the experiments in this study demonstrated the antibacterial activity of silver nanoparticles by a mechanism interfering with DNA replication. Though silver nanoparticles were able to induce gene mutations, the resulting DNA replication error rates were still very low (though higher than that from Taq DNA polymerase) and would not likely be able to demonstrate the antibacterial activity as quick as silver ions. Antibacterial activity can be presented in several hours, but the influence of DNA mutations resulting from silver nanoparticles (or silver ions within) may not be able to display so quickly as in several hours. So we can say that the antibacterial activity was not caused by a mechanism interfering with DNA replication, or at least not solely by such a mechanism.

From figure 3 it is clear that silver nanoparticles (SN in table 1) and silver ions with similar concentrations displayed similar inhibitory effects on bacterial cell growth. High concentrations of both $AgNO_3$ and silver nanoparticles significantly suppress the bacterial growth from both low and high cell densities. The lower panel of figure 3 indicates that $AgNO_3$ was stronger than silver nanoparticles in inhibiting the growth of bacterial cells at the concentration of 4.75 μg ml^{-1}.

On the other hand, silver nanoparticles and silver ions displayed different behaviors when used for PCR additives. $AgNO_3$ began to eliminate the PCR yield at a concentration about 1 μg ml^{-1}, while silver nanoparticles at even 48 μg ml^{-1} still displayed only partial inhibition (figure 2). So silver ions were more 'toxic' to the PCR than silver nanoparticles. Besides, the purchased silver nanoparticles were very pure (30–50 nm, 576832-5G, purity 99.5%). Supposing that the remaining material $(100 - 99.5 = 0.5)\%$ was all silver ions, then the 48 μg ml^{-1} silver nanoparticle solution will include 0.24 μg ml^{-1} silver ions, which would not be enough to suppress the PCR reaction (figure 2). So which component, silver nanoparticles or silver ions, induced the compromised DNA replication fidelity, needs further investigation, though figure 1 provides evidence that silver nanoparticles can directly bind with DNA. Moreover, there remains the question whether the compromised DNA replication fidelity and cellular toxicity posed by silver nanoparticles and silver ions were two independent processes.

More evidence may help to tackle the above question. Morones *et al* found that the silver nanoparticles stay not only at the surface of cell membrane, but also inside the cell [27]. Besides, Hatchett reported that silver nanoparticles tend to have a high affinity to react with phosphorus- and sulfur-containing compounds such as DNA [28]. So one cannot exclude the possibility that the silver nanoparticles interact with the genetic DNA inside the cell and might also cause the higher mutation.

The genetic information was mostly stored in the form of DNA. It has been reported that silver ions enter into the cells

and turn DNA into a condensed form that loses replication ability [8]. In this study we found that silver nanoparticles bound with double-stranded DNA and, possibly in this way, resulted in the compromised DNA replication fidelity both *in vitro* and *in vivo*. It is not clear whether silver nanoparticles directly interact with DNA polymerases. DNA polymerases have *in vitro* replication error rates that could account for a significant fraction of *in vivo* mutations [9], while the cellular uptake of nanoparticles would likely further raise the error rates. It is necessary that more comprehensive tests including other *in vivo* models (such as animal models) be employed to investigate the potential DNA mutation frequencies posed by both silver ions and different types of silver nanoparticle.

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