Original Article

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The effect of different positively charged silver nanoparticles against bacteria, fungi and mammalian cell line

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Abstract

The bactericidal efficiency of various positively charged silver nanoparticles has been extensively evaluated in literature, but there is no report on efficacy of various positive charged silver nanoparticles. The goal of this study is to evaluate the role of different positive electrical charge at the surface of silver nanoparticles on antibacterial activity against a panel of microorganisms and their biofilm activities and their cytotoxicity. Four different silver nanoparticles were synthesized by different methods, providing four different electrical surface charges (two ionic liquids (imidazolium and pyridinium) with 12 and 18 alkyl chain length) namely C12Im, C12Py, C18Im and C18Py, respectively. The antibacterial activity of these nanoparticles was tested against gram-positive (i.e., *Staphylococcus aureus*, *Bacillus subtilis*), gram-negative (i.e., *Escherichia coli* and *Salmonella typhi*) bacteria and *Candida albicans* as fungi. Disc diffusion and micro-dilution tests were used to evaluate the bactericidal activity of the nanoparticles according to CLSI methods. Also primary cytotoxicity assay of nanosilvers was assessed by MTT test. According to the obtained results, C12Py showed the highest bactericidal activity against all microorganisms tested. C18Im had the least and the C12Im had intermediate antibacterial activity. The most resistant bacteria were *Escherichia coli*. Different positive surface charge of silver nanoparticles was a significant factor affecting their bactericidal activity. Although the nanoparticles capped with pyridinium and 12 alkyl chains showed the highest level of effectiveness against the organisms tested, the silver nanoparticles capped with imidazolium and 12 alkyl chains were also potent against most bacterial species. Cytotoxicity of the silver nanoparticles was negligible.

Keywords: Alkyl chains, Antimicrobial activity, Imidazolium, Pyridinium, Silver nanoparticles, Surface charge,

1. Introduction

Due to the outbreak of the infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, pharmaceutical companies and researchers was stimulated to seek new class of antibacterial agents. In an interesting scenario, nanoscale metallic materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties (1). Silver nanoparticles are most promising as they have proved to be most effective against bacteria, viruses and other eukaryotic microorganisms (2). Actually, silver has had his-

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torical background as an antimicrobial agent (3, 4). In modern medicine, silver has been used in the medical field for antimicrobial applications such as elimination of microorganisms on textile fabrics (5, 6), disinfection in water treatment (7), prevention of bacteria colonization on catheters (8), etc. It has also been found to prevent HIV from binding to host cells (9).

Reducing the particle size of metals is also an efficient and reliable tool for improving their biocompatibility, which facilitates their applications in different fields such as bioscience and medicine. As silver works as a bulk material, the use of nano-size silver has also been appealing (10). Many studies have established the bactericidal effect of nanosilver (AgNPs) against Gram negative and Gram positive bacteria (11, 12). Depending on the method of synthesis, AgNPs can exhibit a broad size and shape distribution, different surface charges and varying levels of bioactivity. In this respect, the capping agents such as ionic liquids, which are normally used to change the surface charges of the nanoparticles, can also influence the bioactivity of the AgNPs (13-15). The superiority of the positively charged AgNPs over the negatively charged particles, in terms of the antibacterial activity, was demonstrated (11) but there is no study to evaluate the biological effects of different positively charged AgNPs. In this study, different capping agents were used to provide four different positively charge nano-silvers, and the effect of surface charge on their antibacterial and antifungal activity was investigated by two standard method including disc diffusion and micro dilution broth. Also, because of global concern about toxicity of new nanoparticles, the primary cell cytotoxicity of synthetized nanosilver is assessed by MTT test.

2. Materials and methods

2.1. 2.1.Synthesis of the AgNPs

The ionic liquid-protected silver nanoparticles were prepared according to the procedure described by Samari and Dorostkar (16). In brief; all glassware was placed in 1:3 HCl/HNO_3 solution, rinsed with triple distilled water three times; then, 1.0 mL of 0.01 mol/L $AgNO_3$ aqueous solution was added to 20 mL of 6.2 mmol/L appropriate ionic liquid, and the solution was stirred vigorously. The ionic liquids that were used in this study included: 1-dodecyl-3-methylimidazolium chloride [C12I], 1-dodecyl-3-methylpyridinium chloride [C12P], 1-octadecyl-3-methylimidazolium chloride [C18I], 1-octadecyl-3-methylpyridinium chloride [C18P]. The freshly prepared 0.4 mol/L $NaBH_4$ aqueous solution was then added to the stirred solutions dropwise until the colour of the solution became golden. Subsequently, the colloidal solutions were centrifuged for 20 min to remove excess amount of ionic liquids. The resultant golden-coloured solution was stored at room temperature. The Final synthetized nanoparticles were 1-dodecyl-3-methylimidazolium chloride protected silver nanoparticles (C12IAgNP), 1-dodecyl-3-methylpyridinium chloride protected silver nanoparticles (C12PAgNP), 1-octadecyl-3-methylimidazolium chloride protected silver nanoparticles (C18IAgNP), 1-octadecyl-3-methylpyridinium chloride protected silver nanoparticles (C18PAgNP), respectively.

2.2. Preparation of the experimental solutions

In this study 4 experimental aqueous solutions were prepared C12IAgNP, C12PAgNP, C18IAgNP and C18PAgNP with concentration of 6.2 mM.

2.3. Evaluation of Antibacterial Activity

A combination of gram-negative and gram-positive bacteria and a fungus was selected. The gram-positive microorganisms used in this study were *Staphylococcus aureus* (ATCC29737), *Enterococcus faecalis* (PTCC1394) and *Bacillus subtilis* (PTCC1720) and the gram-negative ones were *Escherichia coli* (ATCC15224) and *Salmonella typhi* (PTCC1609) and the fungi was *Candida albicans* (PTCC5027).

The antibacterial assessment was performed using two methods

2.3.1. Disk diffusion

The standard disk diffusion test was performed on each microorganism using Mueller-Hinton agar (MHA) and standard sterile paper disks. At first, MHA was prepared from the dehydrated medium according to the manufacturer's

Table 1. Radius of inhibition zone in disk diffusion test (mm).

	C12P	C12I	C18P	C18I	Ampicillin
Staphylococcus aureus	25.17±3.6	23.83±4.22	9±1.1	7.17±3.6	20.17±1.6
Bacillus subtilis	24.33±1.51	25±1.1	11±5.19	9.67±5.12	10.83±1.33
Escherichia coli	16.33±1.97	16.33±3.44	0	4.4±2.83	0
Salmonella typhi	18.67±115	17.33±2.52	0	0	0
Candida albicans	20.25±0.5	21.5±2.38	10.25±1.2	9.25±0.96	16.71±3.

instructions. Then, each microbial suspension with optical density of about 0.13 was prepared and 200 µl of this suspension was transferred to MHA plate. The disks contains 10 µl of nanoparticles were placed on the Plates. The plates were incubated For 24 hour in 37 °C and then the zone of halo diameters were measured in the usual manner. The test was conducted six times.

2.3.2. Micro Dilution Broth

MIC and MBC determination of AgNPs against Bacteria was conducted triplicate by a standard microdilution method with Mueller-Hinton broth (Himedia, Mumbai, India). All procedures were performed according to the guidelines presented by the Clinical and Laboratory Standards Institute (CLSI) (Wikler 2010). The MIC_{90} were defined as the lowest concentration, which inhibited 90% of the growth when compared with growth control. The concentration of nanosilvers in these tests were 6.2, 3.1, 1.55, 0.775, 0.388 and 0.194 mM.

2.4. MTT assay for Cytotoxicity of Nanoparticles

To evaluate the cytotoxicity of nanoparticles, MTT assays were employed as previously described by Gholami *et al.* (17). Briefly, HepG2 cells were seeded at 1×10^4 cells per well and incubated for 24 h at 37 °C, 5% Co_2 incubator. Then the cells were exposure by different concentration of nanoparticles. After 24 h, the test medium was discarded, and cells were

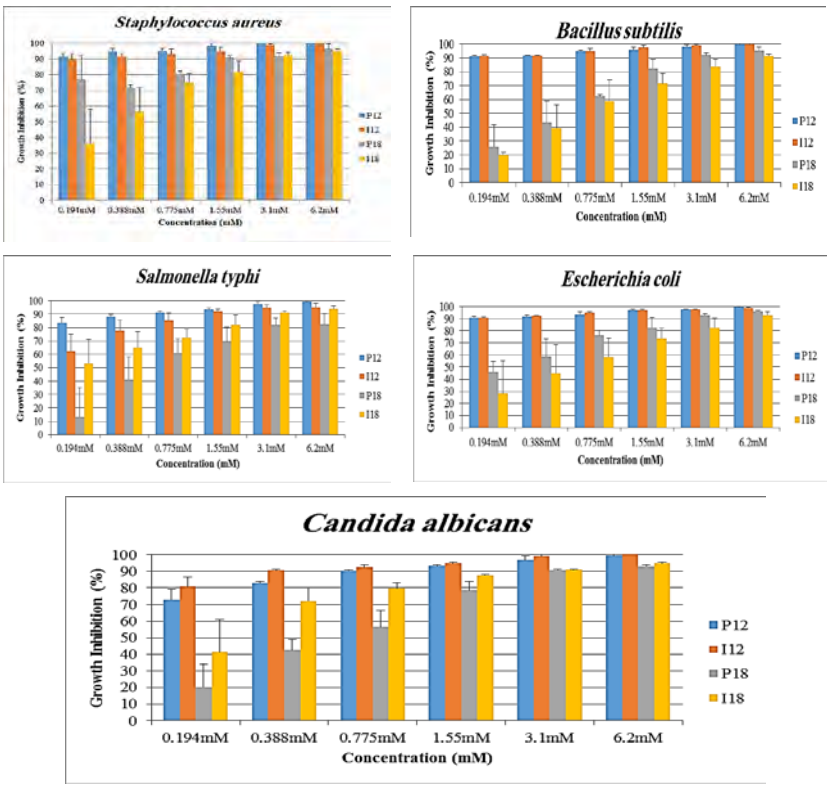

Figure 1. The effect of concentration of different nanosilvers on antibacterial activities against different bacteria's and a fungi.

Table 2. Micro dilusion broth results.

	Candida albicans		Salmonella typhi		Escherichia coli		Bacillus subtilis		Staphylococcus aureus	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
C12P	0.194	0.194	0.194	0.194	0.194	0.194	0.775	0.775	0.775	0.775
C12I	0.194	0.194	0.194	0.194	0.194	0.194	1.55	1.55	0.388	0.388
C18P	1.55	1.55	3.1	3.1	3.1	3.1	>6.2	>6.2	3.1	3.1
C18I	3.1	3.1	6.2	6.2	6.2	6.2	3.1	3.1	3.1	3.1

incubated with 100 µl of MTT solution for 3 h at 37 °C. Subsequently, the MTT solution was discarded and 20 µl of DMSO was added to each well. Optical density (OD) was read by a microplate reader at 540 nm, with a reference at 655 nm (EL800, Bio-Tek Instruments, Inc.). Cell viability for each nanoparticle was calculated as the ratio of the mean OD of replicated wells relative to that of the control (only cell culture medium added).

3. Results

3.1. Disk diffusion

Disk diffusion tests provided diameter of inhibition zone of each AgNPs against bacteria and fungi. The measured halo diameters were shown in table.1. As a general norm, these results showed that C12s have better antibacterial effects than C18s.

3.2. Micro dilution broth

Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and minimum biofilm inhibitory concentration (MBIC) were calculated by Serial dilution test. The effect of AgNPs on the bacterial growth was demonstrated in Figure 1. As seen in the figure, C12 AgNPs in the lower concentrations than C18 had antimicrobial effect on all tested strains. Table 2 was summarized MIC and MBC values of Ag-NPs against tested microbes.

Also, table.2 is shown that the results of MIC and MBC are the same.

3.3. Cytotoxicity

As illustrated in Figure 2, cytotoxicity of the all tested silver nanoparticles on HepG2 cell line in concentration used was negligible and their cytotoxicity was depends on concentration. The maximum cytotoxicity is for C12P in 6.2 mM with viability of 75%.

4. Discussion

Previous works were shown that surface charge of silver nanoparticles has significant effect on their antibacterial activities (11, 14). In this study, four types of silver nanoparticles were synthesized with positive surface charges to assess their antimicrobial activity against gram positive, gram negative bacteria and fungi. For this phase of study, disc diffusion and micro dilution broth tests were done. In the second phase, their cytotoxic effects were evaluated by MTT assay.

The disk diffusion results indicated C12P and C12I have acceptable effects on gram positive,

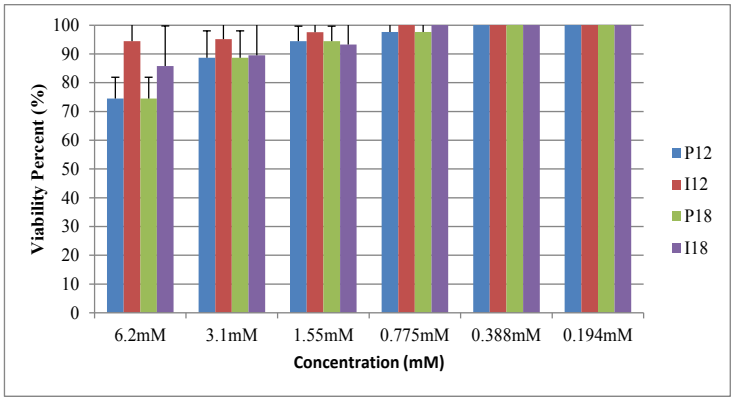

Figure 2. The effect of concentration and type of silver nanoparticles on HepG2 cell line.

Table 3. Average size and surface charge of AgNPs and their ratio.

	Ratio of surface potential to nanoparticle sizes	Surface potential (mV)	Size of nanoparticle (nm)
C12I	9	+50	5.56
C18I	33	+58.2	1.76
C12P	7	+25	3.57
C18P	29	+57.6	1.99

gram negative and fungi microorganisms. These effects were better on gram positive and fungi than gram negative bacteria. C18P and C18I were shown 'weak to average' antibacterial effects. For evaluating of the effective concentration of nanoparticles, micro dilution broth test were used. There are many studies that used micro dilution broth test for assessing antimicrobial effects of nanoparticles. Patil *et al.* demonstrated that silver nanoparticles covered by 1-(dodecyl)-2-amino-pyridinium bromide, had powerful antibacterial effects (18). Lara *et al.* were showed that MIC value of silver nanoparticles against *Escherichia coli* was 83.8 mM (19). Abbaszadegan *et al.* were assessed antibacterial effects of silver nanoparticles with different surface charge (11). Their reported MIC for *Escherichia coli* and *Staphylococcus aureus* was 5.7×10^{-12} molar, for *Streptococcus mutans* was 5.7×10^{-11} molar, for *Protus vulgaris* was 5.7×10^{-8} molar. The MIC results of this study showed that silver nanoparticles with positive surface charge were active in very low concentrations. As the concentration increased, the antimicrobial effects increased. The MIC measured for C12I and C12P on *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* was 0.194 mM. These silver nanoparticles with 12 alkyl chain length have better antibacterial effects than 18 alkyl chain length and the best antibacterial effect was attained by C12P and c12I. Table 2 showed the zeta potentials and average sizes of all tested nanoparticles. As shown in the table, the more the ratio of surface potential to nanoparticle sizes, the bigger the antimicrobial effect. It seems that positively charged-nanoparticles more likely to attack the negatively-charged cell membranes of the microorganisms to disrupt cellular enzymes and destroy tight-controlled cellular permeability; subsequently, cell lysis and cell death was happened (20, 21). However, this rule was not predominant in high surface potentials. The effects of C12P and C12I were near, while their surface potential was too different.

The results of MBC illustrated that all of the nanoparticles had the same MBC and MIC. These results showed that the concentration of nanoparticles for bacterial growth inhibition and killing the bacteria are the same. This also helps to supposed that the antibacterial mechanism of Ag-NPs may be bactericidal. Three common mechanisms proposed for this bactericidal effect are: (a) destruction of replication of DNA and ATP synthesis by uptake silver ions (22) (b) production of reactive oxygen species due to interference of silver ions and AgNPs themselves (23, 24), and (c) loss of cell membrane integrity directly caused by nanoparticles (25). Another aspect of this study was evaluation cytotoxicity of these silver nanoparticles by MTT assay. This primary cytotoxicity study proposed that al form of AgNPs had acceptable cytocompatibility and their moderate toxicity behavior was concentration dependent. As concentration increased, cytotoxicity of silver nanoparticles increased. C12P had the most cytotoxicity in comparison with other nanoparticles. It seems that capping molecules that bind to the entire surface of nanoparticles and control their self-aggregation can also change their cytotoxic behavior. The ionic liquids such as pyridinium and imidazolium salt which was applied in the synthesis of AgNPs is well-known as a green solvent for organic synthesis (26) and help the nanoparticles to have little cytotoxicity (27, 28). In our work, the results showed that ionic liquid capped AgNPs had little cytotoxicity to HepG2 cells whilst they were very active against bacterial and fungal strains. Although AgNPs can cross both prokaryotic and eukaryotic cell membranes, but intracellular antioxidant settings in mammalian cells may keep them from possible oxidative stresses (29). On the

other hand, the difference between the amounts of harvested nanoparticle by different cell types also has an impact on their different activity.

4. Conclusion

Generally, without considering that which ionic liquids (imidazolium and pyridinium) was used, silver nanoparticles with 12 alkyl chain length had better antibacterial effect than 18 alkyl chain length against gram positive and gram negative bacteria. Silver nanoparticles in pyridinium ionic liquid had better antibacterial activities than imidazolium. Also, the results illustrated that silver nanoparticles in pyridinium ionic liquid with 12 alkyl chain length had the most cytotoxicity than others.

Conflict of Interest

None declared.

5. References

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