Polyethylene glycol-stabilized sulphur nanoparticles: an effective antimicrobial agent against multidrug-resistant bacteria

Samrat Roy Choudhury†, Subhasree Roy‡, Arunava Goswami† and Sulagna Basu‡*

1Biological Sciences Division, Indian Statistical Institute, Kolkata-700108, India; 2Division of Bacteriology, National Institute of Cholera and Enteric Diseases, Kolkata-700010, India

*Corresponding author. E-mail: supabasu@yahoo.co.in
†Samrat Roy Choudhury and Subhasree Roy have equally contributed to the work.

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Objectives: To elucidate the antibacterial efficacy of chemically synthesized and custom-made sulphur nanoparticles (SNPs) of two different sizes and surface modifications against a number of multidrug-resistant Gram-negative bacilli (GNB) harbouring the New Delhi metallo-β-lactamase 1 enzyme (NDM-1).

Methods: Antimicrobial susceptibility of the isolates was determined. The strains were evaluated for the presence of carbapenemases, β-lactamases, 16S rRNA methylases and integrons. Chemically synthesized, polyethylene-glycol (PEG)-stabilized SNPs of 10 nm and custom-made non-capped SNPs of 60 nm were physicochemically characterized and evaluated for their antibacterial efficacy against multidrug-resistant GNB using the agar dilution method (ADM) and the broth microdilution method (BMD). The cytotoxicity of the chemically synthesized SNPs was evaluated with a human-derived hepatoma (HepG2) cell line using a WST-1 assay kit.

Results: All isolates were multidrug-resistant and possessed NDM-1 along with other β-lactamases, 16S rRNA methylases and integron 1. Chemically synthesized PEGylated SNPs showed a bactericidal effect against all tested strains at a concentration between 9.41 and 18.82 mg/L using BMD. The ADM data revealed that SNPs had uniform MICs (18.82 mg/L) for all tested strains. On the other hand, custom-made SNPs failed to impart any antibacterial effect at the equivalent concentrations of chemically synthesized SNPs. The WST-1 assay revealed no significant cytotoxicity of the PEGylated SNPs even at the highest concentration (94.08 mg/L).

Conclusions: To the best of our knowledge, this is the first attempted study to show the effectiveness of nanoparticles against multidrug-resistant GNB harbouring NDM-1.

Keywords: PEG-400, multidrug resistance, NDM-1, bactericidal

Introduction

Carbapenems are a class of antibiotics that are being used increasingly as the last resort against serious infections caused by Gram-negative bacilli (GNB). Emergence of carbapenem resistance among GNB is a global healthcare concern.1 Enzymes of the carbapenemase family hydrolyse not only carbapenems but almost all hydrolysable β-lactams, and most are resistant against inhibition by the commercially viable β-lactamase inhibitors. In addition, pathogens carrying such enzymes are typically resistant to many other antibiotic classes. The carbapenemase New Delhi metallo-β-lactamase 1 (NDM-1) has recently been reported from India, Pakistan and the UK4 and within a short span of time human infections with bacteria carrying the NDM-1 enzyme were reported from at least 20 countries.5

The gene encoding NDM-1, i.e. blaNDM-1, is known to coexist with other antibiotic-resistance genes.1 A combination using aztreonam or similar monobactams, generally resistant to hydrolysis by metallo-β-lactamases (MBLs), seems to be inactive against NDM-1-harbouring strains because many of these NDM-1-bearing strains possess AmpC cephalosporinases and/or extended-spectrum β-lactamases (ESBLs) that can hydrolyse aztreonam. Certain NDM-1-harbouring strains may even produce 16S rRNA methylases, making the aminoglycosides ineffective. In general, NDM-1 β-lactamase-producing bacteria show susceptibility to colistin or tigecycline, but probably this phenomenon will also be transient, as colistin-resistant NDM-1-producing strains have already been found.1 Thus, options for treatment are becoming extremely limited.

When antibiotics are reaching their limits of sustainability and viability, nanotechnology-based applications might provide potential novel drugs and therapeutics to address the problems mentioned above. Elemental sulphur (ES) has long been known to be an eco-friendly antimicrobial agent. Nanotechnology offers opportunities to re-explore the antimicrobial efficacy of
ES particles by manipulating their size and surface. The antifungal efficacy of surface-engineered sulphur nanoparticles (SNPs) has already been reported for by our group.\(^4,5\) In this study we report the antibacterial efficacy of chemically synthesized and custom-made SNPs of two different sizes and surface modifications against a number of multidrug-resistant GNB, all harbouring NDM-1. A simultaneous study was carried out to evaluate the biocompatibility of the synthesized SNPs with the human-derived hepatoma (HepG2) cell line.

**Materials and methods**

**Molecular characterization of the isolates**

Carbapenem-resistant GNB were isolated from neonatal specimens (blood or body site cultures) in India and identified with an ID 32 E or ID 32 GN kit (bioMérieux, Marcy l’Etoile, France). Antibiotic susceptibility and MICs were determined using the Etest (bioMérieux) or the broth microdilution method (BMD; only for imipenem).\(^5\) PCR was used to detect the \(\text{bla}_{\text{NDM-1}}\) gene in the strains.\(^6\) One representative isolate of each species (Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Stenotrophomonas maltophilia and Enterobacter aerogenes) possessing NDM-1 was included in the study. Since MBLs are plasmid-mediated and their genetic determinants are often associated with integrons, the approximate size of megaplasmids and the presence of class 1, 2 and 3 integrons were determined.\(^7\) Phenotypic and genotypic detection of other \(\beta\)-lactamases that hydrolyse cephalosporins, such as ESBLs (\(\text{bla}_{\text{SHV-1}, \text{TEM-1}, \text{CTX-M-15}}\)) and AmpC \(\beta\)-lactamases (\(\text{bla}_{\text{OXD-CT, DHA-ACC, EBC, FOX}}\)), carbapenemases other than NDM-1 (\(\text{bla}_{\text{OXA-4, IMI, VIM-1, GES-1, AOM-1, FOX-1}}\)) and 16S rRNA methylases were also undertaken for these strains.\(^7\)

**Characterization of SNPs**

The size, shape, purity and composition, surface modification and thermal decomposition pattern of the synthesized SNPs have been reported earlier.\(^4,5\) Further pulverization of stock sulphur \((\sim 1 \mu\text{m})\) reduced the final size of SNPs \((\sim 10 \text{ nm})\) and this preparation was used throughout for the present study. Custom-made SNPs were purchased from NK Impex, Canada. The X-ray diffraction pattern revealed that the allotropic nature of each of the SNPs in use was orthorhombic (data not shown). A dynamic light scattering (DLS) study was performed to determine the size distribution of SNPs in colloidal medium. High-resolution transmission electron microscopy (HR-TEM) (2010F, JEOL Ltd, Tokyo, Japan) was carried out at 200 kV on a carbon-coated copper grid to determine the size of core SNPs. Fourier transform-infrared (FT-IR) spectroscopic analyses were performed to confirm the surface modification of each of the SNPs in use, employing potassium bromide beads. Evaluation of active ingredients (sulphur) in the stock solution \((1176 \text{ mg/L})\) of this novel formulation (patent file number 1599/DEL/2011) had been determined earlier.\(^6,7\) Each of the concentrations of SNPs required for the present study were fixed and prepared from the above-mentioned SNP stock.

**Antibacterial and cytotoxicity study with SNPs**

The antibacterial effect of the nanoparticles was tested by both the agar dilution method (ADM) and the BMD method. Inocula were adjusted to \(10^5 \text{ cfu per spot for the ADM and } 5 \times 10^7 \text{ cfu per well for the BMD method to determine MICs following the standard protocol.}^{10}\) The strains were treated with serially diluted (2-fold) SNPs (starting from \(75.27 \text{ mg/L}\) and the MICs were determined as the lowest concentration that did not show any visible growth in the test media. The ADM and BMD assays were both performed in triplicate.

The cytotoxicity (biocompatibility) of the PEGylated SNPs was evaluated in terms of cell proliferation using HepG2 cells (ATCC HB8065) using a WST-1 assay kit (Cayman Chemical Company, MI, USA). HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with \(10\%\) heat-inactivated fetal bovine serum, \(1 \text{X}\) glutamine \((0.584 \text{ mg/L})\), \(1 \text{X}\) sodium pyruvate \((110 \text{ mg/L})\) and \(1 \text{X}\) non-essential amino acids under \(5\%\) CO\(_2\) at \(37\) °C. Cells were seeded in a 96-well plate in \(200 \text{ mL}\) of medium per well at a density of \(10000 \text{ cells/well}\) for 24 h. The medium was then replaced with \(200 \text{ mL}\) of medium containing SNPs at different concentrations \((4.74, 11.76, 23.52, 47.04 \text{ and } 94.08 \text{ mg/L})\) and incubated for \(24 \text{ h}\) in triplicate. An aliquot of \(10 \text{ mL}\) of WST-1 reagent was then added to each well and the wells were further incubated for \(4 \text{ h}\) prior to measurement of absorbance at \(450 \text{ nm}\). All media and reagents were purchased from Sigma-Aldrich, MO, USA, unless specified.

**Results and discussion**

All strains were resistant to a range of antibiotics except for minocycline, colistin and tigecycline (Table S1, available as Supplementary data at JAC Online). The MICs for imipenem were \(32 \text{ mg/L}\) for all tested strains except A. baumannii (128 mg/L). Phenotypic and genotypic features of the NDM-1-positive strains are summarized in Table 1. Large plasmids in the range of \(\sim 40 \text{ to } 400 \text{ kb}\) were detected in the strains. All strains possessed integron 1, indicating a probable association of integron 1 with \(\text{bla}_{\text{NDM-1}}\).

**Table 1. Phenotypic and genotypic characterization of SNP-susceptible NDM-1-harbouring isolates**

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Phenotypic presence of (\beta)-lactamases</th>
<th>Genotypic detection of (\beta)-lactamases</th>
<th>Size of megaplasmids (kb) and presence of integron</th>
<th>MIC (mg/L) of PEGylated SNPs by the ADM</th>
<th>MIC (mg/L) of PEGylated SNPs by the BMD</th>
</tr>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>MBL, ESBL and AmpC</td>
<td>NDM-1, CTX-M-15, TEM-1, OXA-1, CMY-59, RmtB</td>
<td>83, 49, integron 1</td>
<td>18.82</td>
<td>18.82</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>MBL</td>
<td>NDM-1, SHV-11, ArmA</td>
<td>385, 158, 43, integron 1</td>
<td>18.82</td>
<td>18.82</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>MBL</td>
<td>NDM-1, OXA-23, OXA-51, ArmA</td>
<td>470, integron 1</td>
<td>18.82</td>
<td>9.41</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>MBL, ESBL and AmpC</td>
<td>NDM-1, CTX-M-15, TEM-1, CMY-42, RmtB</td>
<td>600, 83, integron 1</td>
<td>18.82</td>
<td>9.41</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>MBL and ESBL</td>
<td>NDM-1, CTX-M-15, TEM-1, SHV-1, OXA-1</td>
<td>150, integron 1</td>
<td>18.82</td>
<td>18.82</td>
</tr>
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</table>
DLS measurements revealed average size distributions of SNPs in terms of hydrodynamic diameters in the range of 10–100 nm (data not shown) and 20–50 nm (data not shown) for custom-made and synthesized SNPs, respectively. HR-TEM micrographs revealed that the actual particle size (core) of the synthesized SNPs was around 10 nm (Figure 1c). Actual particle size for the custom-made SNPs was around 60 nm (Figure 1a). Chemically synthesized SNPs were capped with PEG-400, and their surface modifications (Figure 1d) have been explained in an earlier article.\textsuperscript{5} The FT-IR spectrum for the custom-made SNPs (Figure 1b) revealed characteristic peaks similar to those of the FT-IR spectrum of ES (Sigma-Aldrich) (data not shown) and also confirmed that no surfactants were used for the preparation of the commercial batch of SNPs.

From BMD studies it was observed that PEGylated SNPs inhibited bacterial growth at a concentration as low as 9.41 mg/L for \textit{A. baumannii} and \textit{S. maltophilia} and at a concentration of 18.82 mg/L for the other strains [Table 1 and Figure S1 (available as Supplementary data at JAC Online)]. In comparison, the ADM data revealed that PEGylated SNPs had uniform MICs (18.82 mg/L) for all tested strains. Subtle (one dilution error) differences in the susceptibility pattern between the ADM and BMD results for the tested \textit{A. baumannii} and \textit{S. maltophilia} strains may have been due to multiple factors. Interpretation of BMD results following the CLSI (formerly NCCLS) guideline of reading the endpoint as the lowest concentration ‘that completely inhibits growth of the organism, as detected by the unaided eye’ can lead to ignoring subtle or sporadic growth in the well.\textsuperscript{11} Slight growth that cannot be detected by the naked eye may, however, be visible in the ADM. Differences in the ability to form biofilms, the growth pattern and the degree of susceptibility to different antimicrobial agents in solid or liquid media may also be considered crucial factors.\textsuperscript{12} Moreover, the rates of diffusion and bioavailabilities of nanoparticles in different media\textsuperscript{13} (broth and agar) may be different for certain organisms.

PEGylated SNPs inhibited bacterial growth at concentrations lower than the MICs of imipenem (≥32 mg/L). In contrast,
custom-made SNPs failed to impart any antibacterial effect even at the highest concentration tested (75 mg/L). The antibacterial effect, therefore, can be correlated to the size difference and the presence of stabilizing agents on the surface of the SNPs. Irrespective of their allotropic composition (orthorhombic; data not shown), the diminutive size of chemically synthesized SNPs (~10 nm) is able to generate enhanced surface reactivity and translocation potential within the reaction media. Moreover, the PEG-400 coating on the surface of SNPs allows lateral dispersion and surface camouflage that overcomes the resistive forces of bacteria.14

The WST-1 assay revealed no significant cytotoxicity of the PEGylated SNPs even at the highest concentration tested (94.08 mg/L). The results were plotted on a fitted curve and expressed in terms of percentage of viable cells at different concentrations of SNPs (Figure 2). Preferential toxicity to bacterial cells and biocompatibility with mammalian cells (HepG2) suggest the emergence of SNPs as a future putative antibacterial drug.

Work is currently in progress to determine the broad-spectrum antimicrobial efficacy and probable mode of action of SNPs. However, this preliminary investigation shows that SNPs hold immense promise. Bacterial strains producing NDM-1 or other carbapenemases are formidable and demand alternative therapeutics, such as nanoparticles. The small size and novel mode of action of nanoparticles make them effective when antibiotics fail, and bacteria will probably take longer to find ways to resist them.

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Transparency declarations

The SNPs used in the present work were patented at the Institute Technology Management Unit of the Indian Agricultural Research Institute (IARI), India by the researchers of the Indian Statistical Institute, Kolkata and IARI. No company is associated with the product and no individual investors are likely to financially benefit from the product.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References