Polyethyleneimine and polyethyleneimine-based nanoparticles: novel bacterial and yeast biofilm inhibitors

M. M. Azevedo,1,2,3 P. Ramalho,1 A. P. Silva,1,3 R. Teixeira-Santos, 1 C. Pina-Vaz1,3,4 and A. G. Rodrigues1,3,5

Correspondence
M. M. Azevedo
maria.manuel.azevedo2011@googlemail.com

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INTRODUCTION

Biofilms are commonly involved in medical device-related infections. The purpose of this study was to determine the antimicrobial and anti-biofilm activity of polyethyleneimine (PEI) and PEI-based nanoparticles (nanoPEI) against Staphylococcus aureus, Staphylococcus epidermidis, Acinetobacter baumannii and Candida albicans (clinical and ATCC strains), and to evaluate their effect upon biofilm formation on polyurethane (PUR)-like catheters. MICs and minimal lethal concentrations of PEI and nanoPEI were determined according to CLSI microdilution reference protocols. For PEI, the MIC value was 195.31 mg l$^{-1}$ for all the bacteria and 48.83 mg l$^{-1}$ for the yeast strains. For nanoPEI, the MIC value was 1250 mg l$^{-1}$ for all the strains except A. baumannii, for which it was 2500 mg l$^{-1}$. Biofilm formation was assessed with PUR-like catheter segments and biofilm metabolic activity was quantified by colorimetry with a tetrazolium reduction assay. Plasma membrane integrity and membrane potential were assessed by flow cytometry after staining microbial cells with a membrane-impermeable dye, propidium iodide, and a membrane-potential marker, DiBAC$_4$(3). PEI inhibited growth of all microbial species; higher concentrations of nanoPEI were needed to inhibit growth of all species. Biofilm formation in the presence of antibacterial PEI activity was dose-dependent (except for S. epidermidis) and species-related. NanoPEI at 0.5×MIC and MIC significantly reduced the metabolic activity of biofilms of S. aureus, S. epidermidis and A. baumannii, whereas 2×MIC was required in order to inhibit biofilm metabolic activity.

Abbreviations: CRBSI, catheter-related bloodstream infection; DiBAC$_4$(3), (bis-1,3-dibutylbarbituric acid) trimethine oxonol; MLC, minimal lethal concentration; nanoPEI, polyethyleneimine-based nanoparticles; PEI, polyethyleneimine; PI, propidium iodide; PUR, polyurethane.

INTRODUCTION

Biofilms are communities of micro-organisms attached to a surface. These structured communities can cause serious medical problems, such as indwelling device-related infections. Recent estimates show that over 65% of all hospital infections originate from biofilms (Mah & O’Toole, 2001). Very frequently, colonization of medical indwelling devices through microbial adhesion and subsequent biofilm formation may precede bacteremia/fungaemia and sepsis (Tamura et al., 2007; Rupp, 2014). Central venous catheters are a particularly high-risk category of devices (Randolph et al., 2005). Catheter-related bloodstream infections (CRBSIs) result in increased healthcare costs (Orsi et al., 2002), longer hospital stays (Polderman & Girbes, 2002) and increased mortality (Sitges-Serra & Girvent, 1999). In the USA more than 200,000 healthcare-related bloodstream infections occur each year, most of them associated with intravascular devices (Mermel et al., 2001). The preponderance of such infections among Intensive Care units is also frequently associated with such medical devices.

Several approaches have been suggested to diminish the incidence of CRBSIs, such as the coating of catheters with antibiotics or antisepsics (Raad et al., 2007). Catheters impregnated with chlorhexidine–silver sulfadiazine appear to be effective in reducing colonization and infection. However, hypersensitivity reactions have been documented (Oda et al., 1997), silver-impregnated catheters are not...
associated with a lower rate of colonization (Kalfon et al., 2007) and, despite catheters coated with minocycline and rifampicin significantly decreasing the incidence of CRBSIs, some concern still exists regarding the development of antimicrobial resistance (Gilbert & Harden, 2008). The current challenge is to discover new compounds, non-cytotoxic to human tissues, exhibiting a potent and broad range of antimicrobial activity.

Polyethyleneimine (PEI) is a synthetic polymer, weakly basic, aliphatic and nontoxic, and also polycationic due to the presence of primary, secondary and tertiary amino groups. This compound is obtained by cationic polymerization of aziridine (Yudovin-Farber et al., 2010). PEI can be used for several purposes, for example as a drug (Weber et al., 2012) and in the development of catalyst supports due to effective neutralization of excess anionic colloidal charge, especially under acidic and neutral conditions (Honraet et al., 2005), and is a common microbialcid ingredient in a variety of formulations ranging from washing agents to packaging materials (Helander et al., 1997).

Polycations exhibit antibacterial properties, since they can interact with and disrupt bacterial cell membranes (Kawabata & Nishiguchi, 1988). According to a previous study, PEI has a strong permeabilizing effect; however, no bactericidal effect was observed upon Gram-negative bacteria (Helander et al., 1997). Nevertheless, other observations showed that PEI enhances the bactericidal efficacy of hydrophilic and hydrophobic antibiotics (Khalil et al., 2008), and more recently (Hyun et al., 2011) the cytotoxic effects of PEI and PEI silver nanoparticles upon bacteria were confirmed.

Considering that biofilms formed by Staphylococcus aureus, Staphylococcus epidermidis, Acinetobacter baumannii and Candida albicans potentially cause indwelling catheter infections, the aim of this work was: (1) to investigate the antimicrobial activity of PEI and PEI-based nanoparticles (nanoPEI) upon such species; (2) to test the efficacy of PEI and nanoPEI as regards anti-biofilm formation on polyurethane (PUR)-like catheters; and (3) to assess the induction of cell membrane lesions by such compounds.

METHODS

Chemicals. PEI (branched polyethyleneimine) solution (mean $M_r$ ~60,000 by gel permeation chromatography, mean $M_r$ ~750,000 by light scattering, 50% in $H_2O$) was purchased from Sigma-Aldrich (ref.181978).

Synthesis of PEI-based nanoparticles (a kind gift from Professor Ascenção Lopes, Engineering Faculty, University of Porto) was performed via reductive amination as follows.

PEI (10 g, 0.23 mol monomer units) was dissolved in 100 ml absolute ethanol. Glutaraldehyde solution (230 µl) was added at a 1:0.01 molar ratio (primary amine of PEI monomer units/glutaraldehyde). The cross-linking reaction was carried out at room temperature for 6 h. N-Alkylation was conducted by adding octanal at a 1:1 molar ratio (primary amine of PEI monomer units/octanal). The alkylation step was carried out at room temperature for 24 h. The resulting yellow suspension was reduced with sodium cyanoborohydride, added at a 1:0.33 molar ratio (octanal/NaCNBH$_3$) for 24 h at room temperature. Methylation was carried out with 43 ml methyl iodide (0.69 mol), which was added at a 1:3 molar ratio (monomer units of PEI/methyl iodide) at 42 °C for 48 h. During the methylation step, a yellow precipitate was formed. An equivalent amount of sodium bicarbonate (0.23 mol, 19 g) was added to release HI during the methylation step. Neutralization was continued under the same conditions for an additional 24 h. The crude precipitate was allowed to reach room temperature; it was afterwards washed with hexane and double-deionized water to remove traces of unreacted octanal, methyl iodide, sodium bicarbonate and sodium iodide and was then freeze-dried. The purification step was repeated with additional amounts of hexane and double-deionized water, and then freeze-dried. The mean yield was 75 mol%.

Microbial strains. One type strain from the American Type Culture Collection (ATCC) and one clinical isolate of each of four microbial species were used; type strains tested were S. aureus ATCC 29213, S. epidermidis ATCC 155, A. baumannii ATCC 19606 and C. albicans ATCC 90028. The clinical strains S. aureus Sa1, S. epidermidis Se1, A. baumannii Ab1 and C. albicans Ca1 had been previously recovered from central venous catheters removed from critical care patients (Cobrado et al., 2012). All clinical isolates had been identified by the Vitek2 system (bioMérieux).

Antimicrobial activity. MICs and minimal lethal concentrations (MLC) of PEI and nanoPEI were determined according to CLSI microdilution reference protocol M07-A9 for bacteria (CLSI, 2012a) and M27-A3 and M27-S4 for yeasts (CLSI, 2008, 2012b); test concentrations ranged from 0.763 to 12500 mg l$^{-1}$ and 39.06 to 5000 mg l$^{-1}$, respectively. MIC end point was defined as the lowest concentration that inhibited visually at least 80% of the growth of micro-organisms after overnight incubation.

After MIC had been read, to determine the minimal lethal concentration (MLC), 20 µl from each microplate well was plated on Luria–Bertani agar (bacteria) or Sabouraud agar (yeasts); plates were incubated at 37 °C for 24 h (bacteria) or 35 °C for 48 h (yeasts) with subsequent enumeration of c.f.u. MLC was defined as the lowest concentration killing at least 99.9% of the final inoculum.

Biofilm formation on PUR-like catheters. Strains were grown overnight in LB broth (bacteria) or Sabouraud broth (yeasts) at 37 °C and 180 r.p.m.; cells were harvested by centrifugation, washed with PBS, and adjusted to MacFarland standard no. 0.5, which corresponds to $1–2 \times 10^8$ cells ml$^{-1}$ in brain–heart broth for bacteria and $1–5 \times 10^6$ cells ml$^{-1}$ in Sabouraud broth for yeasts.

The anti-biofilm effect was evaluated in the presence of four concentrations of PEI: 2×MIC, MIC, 0.5×MIC for bacteria and 4×MIC, 2×MIC, MIC, 0.5×MIC for yeast. (The MIC value for yeast strains is similar to the MLC value.)

The effect of nanoPEI on biofilms was evaluated in the presence of three concentrations: 2×MIC, MIC and 0.5×MIC. (The MIC value for bacterial strains is similar to the MLC value.)

A single fragment of PUR-like intravenous catheter (BD Vialon16G, 1.7 × 45 mm), 1 cm in length, was placed in each well of 12-well plates, containing 1 ml standardized microbical suspension.

After incubation with shaking (24 h for bacteria and yeasts) at 37 °C, 150 r.p.m., each catheter fragment was removed, gently washed with PBS and placed in a new plate well to assess biofilm metabolic activity with 2,3-bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay (Chandra et al., 2001). All the assays were performed in triplicate. The study was conducted using three biological replicates in different days. Mean and standard deviation values were calculated.
Mechanism of action: effects on cytoplasmic membrane.

Plasma membrane integrity and membrane potential were assessed by flow cytometry after staining microbial cells with a membrane-impermeable dye, propidium iodide (PI), and a membrane-potential marker, DiBAC4(3) [(bis-1,3-dibutylbarbituric acid) trimethine oxonol], respectively. Fifty milliliters of a cell suspension adjusted to 1 x 10^6 cells ml^-1 of S. aureus ATCC 29213, S. epidermidis ATCC 155, A. baumannii ATCC 19606 or C. albicans ATCC 90028 was prepared in sterile saline solution.

In order to assess the effect of PEI, cell suspensions of each ATCC strain were incubated with concentrations corresponding to 4 x MLC and MLC for 15, 30, 60 and 120 min. To assess the effect of nanoPEI, bacterial suspensions were incubated with concentrations corresponding to 2 x MLC and MLC, and yeast suspensions were incubated with concentrations corresponding to 4 x MIC and 2 x MIC for 15, 30, 60 and 120 min. Afterwards, cells were centrifuged and the pellet was stained with PI or DiBAC4(3) at a final concentration of 1 μg ml^-1 or 0.5 μg ml^-1, respectively, and incubated for 15 min in the dark, at room temperature. Suspensions of untreated and of dead cells (treated with 99 % 2-propanol) were stained using similar conditions and used as controls of viable and dead cells (cell membrane lesion).

Cell suspensions were analysed in a FACSCalibur flow cytometer (BD Biosciences). Cell fluorescence was determined at FL1 (515 nm) for PI and at FL3 (620 nm) for PI. The intensity of fluorescence shown by cells treated with PEI or with nanoPEI was determined and compared with non-treated cells and with dead cells.

Viability assessment. To confirm the results obtained by flow cytometry analysis, 100 μl of each cell suspension was plated on brain–heart agar (for bacteria) or Sabouraud agar (for yeast). The plates were incubated at 35 °C for 24 h (bacteria) or 24–48 h (yeasts), with subsequent enumeration of c.f.u.

Data analysis. Biofilm metabolic activity in the presence of PEI and nanoPEI was expressed as a percentage relative to the control. Values were divided by 1000 and arcsine square root transformed to achieve a normal distribution and homoscedasticity. For each compound, the other concentrations tested, corresponding to MIC and 0.5 x MIC, did not impair biofilm metabolic activity.

NanoPEI at concentrations corresponding to 0.5 x MIC and MIC significantly reduced biofilm metabolic activity of all S. aureus, S. epidermidis and A. baumannii (P<0.05) strains (Fig 2a, b). In respect of C. albicans, only the highest concentration tested, 2500 mg l^-1 (corresponding to 2 x MIC), inhibited biofilm metabolic activity of both clinical and ATCC strains. For the clinical yeast strain the biofilm inhibition was 34 % and for the ATCC strain it was 57 %. The other concentrations tested, corresponding to MIC and 0.5 x MIC, did not inhibit biofilm (data not shown).

Mechanisms of action

A cytometric assay aiming to characterize the effect of PEI and nanoPEI in bacteria and yeast strains was implemented, based upon the use of two fluorescent markers: PI, a marker of cell death (only penetrates cells with severe membrane lesions), and DiBAC4(3), which detects depolarized cells. DiBAC4(3) has a high degree of voltage sensitivity (Bräuner et al., 1984) and enters depolarized cells, binding to lipid-rich intracellular components.

Untreated cells stained with PI or DiBAC4(3) showed a very low intensity of fluorescence. However, 2-propanol-treated cells (dead cell control) showed higher intensity fluorescence. S. aureus and S. epidermidis treated with PEI stained with PI after 30 min (43 % of S. aureus cells and 24 % of S. epidermidis cells were dead) (Table 1). A. baumannii treated with PEI did not stain with PI. As regards DiBAC4(3), all the bacterial species exhibited a high percentage of cells depolarized, higher in S. aureus than in S. epidermidis. PI was unable to stain C. albicans, although a significant increase in membrane depolarization was detected after 2 h (Table 1).

NanoPEI resulted in a higher percentage of dead S. aureus and S. epidermidis cells than did PEI (56 and 32 % respectively); additionally, increased depolarization was evident (Table 1). For A. baumannii, the effect of nanoPEI was similar to that of PEI (Table 1). For C. albicans, the effect of PEI on the cell membrane permeability was more pronounced than the effect of nanoPEI, unlike what happens to membrane depolarization, for which both PEI and nanoPEI concentrations tested showed similar results.

Viability assessment

During cytometric assays, cells that had been exposed to different PEI and nanoPEI concentrations were plated on

RESULTS

Antimicrobial activity

PEI MICs were 195.31 and 48.83 mg l^-1 respectively for bacteria and yeast strains. MLCs corresponded to 32 x MIC for S. aureus (Sa1), S. aureus ATCC and A. baumannii (Ab1). For S. epidermidis (Se1), S. epidermidis ATCC and A. baumannii ATCC, the MLC value corresponded to 8 x MIC. For C. albicans, the MIC and MLC value was 48.83 mg l^-1.

NanoPEI MICs were 1250 mg l^-1 for S. aureus (Sa1), S. aureus ATCC, S. epidermidis (Se1), S. epidermidis ATCC, C. albicans (Ca1), C. albicans ATCC 90028, and 2500 mg l^-1 for A. baumannii (Ab1) and A. baumannii ATCC.

Biofilm formation on PUR-like catheters

PEI at concentrations corresponding to MLC, 2 x MIC, MIC and 0.5 x MIC significantly reduced biofilm metabolic activity of both clinical and ATCC strains of S. aureus and of A. baumannii (P<0.05) in a dose-dependent way (Fig. 1a, b). For S. epidermidis, although all the concentrations tested significantly inhibited the biofilm metabolic activity (Fig. 1a), such inhibition was not dose-dependent (P>0.05). For yeasts, concentrations ≥48.83 mg l^-1 (4 x MIC, 2 x MIC and MIC) significantly inhibited biofilm metabolic activity (P<0.05) (Fig. 1c); only the lowest concentration used, 24.42 mg l^-1 (0.5 x MIC), did not impair biofilm metabolic activity.

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Fig. 1. Effect of polyethyleneimine (PEI) on biofilm formation (as a percentage of the control) by: (a) Gram-positive bacteria *S. aureus* and *S. epidermidis*, ATCC and clinical strains; (b) Gram-negative bacterium *A. baumannii*, ATCC and clinical strains; and (c) *Candida albicans*, ATCC and clinical strains. A decrease in biofilm metabolic activity is observed for all strains.

Fig. 2. Effect of nanoPEI on biofilm formation (as a percentage of the control) by: (a) Gram-positive bacteria *S. aureus* and *S. epidermidis*, ATCC and clinical strains; and (b) Gram-negative bacterium *A. baumannii*, ATCC and clinical strains. A decrease in biofilm metabolic activity is observed for all strains.
agar medium and c.f.u. were counted after 24 and/or 48 h. Independent of the concentration tested and the exposure time, no microbial cells were recovered, showing a lethal effect of PEI and nanoPEI.

**DISCUSSION**

Healthcare-related infections involving medical indwelling devices represent a serious clinical challenge. Consequently, it is imperative to find new antimicrobial agents that are non-cytotoxic to humans.

In this study, we evaluated the antimicrobial activity of PEI and nanoPEI against selected Gram-negative and -positive bacteria and yeast, as well as their effect on biofilm formation on PUR-like catheters. Our results demonstrate the bacteriostatic and fungicidal effects of PEI and nanoPEI.

Concerning bacterial growth inhibition by PEI, our results were similar for all the strains studied. Interestingly, we found that a lower concentration of PEI is able to inhibit yeast growth compared with bacteria strains.

In order to confirm the mechanism of action of PEI, we tested its effect upon plasma membrane integrity and membrane potential by flow cytometry. Two fluorescent dyes were used, namely PI and DiBAC<sub>4</sub>(3). Exposure to PEI resulted in cell depolarization and disruption of cell membrane integrity. This latter effect was not observed with *A. baumannii*. NanoPEI was less active against *A. baumannii*, and at the tested concentration no cell membrane permeability was seen with PI. Several explanations can be put forward. Both PEI and nanoPEI seem to be less active against *A. baumannii* than against *Staphylococcus* spp. Positive PI-staining means that cells are dead and exhibiting evident membrane lesion; cells could be dead but negative to PI. A higher concentration of the treatment is probably needed to permeabilize the cell membrane to PI in such a case. The Gram-negative cell membrane is also more complex, preventing staining. In the case of *C. albicans*, only cell depolarization was detected. It is generally agreed that the mechanism of the bactericidal action of polycationic biocides involves destructive interaction with the cell wall and/or cytoplasmic membrane (Kawabata & Nishiguchi, 1988). The molecular mechanism by which PEI increases the permeability of the outer membrane has not yet been determined (Helander et al., 1998). The enhanced antibacterial activity is probably due to a redistribution of phospholipids from the inner to the outer layer of the outer membrane caused by PEI. PEI is able to disorganize the outer membrane not only transiently but irreversibly (Helander et al., 1997) and is known to bind to and precipitate various cellular materials such as lipids and nucleic acids (Salt et al., 1995).

The work by Helander et al. (1997) showed that PEI is a potent permeabilizer of the outer membrane of Gram-negative bacteria like *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, and additionally showed
that PEI increased the susceptibility of the tested species to the hydrophobic antibiotics clindamycin, erythromycin, fucidin, novobiocin and rifampicin, without being directly bactericidal. According to our results, PEI at subinhibitory concentrations resulted in a significant reduction of biofilm metabolic activity in S. aureus, S. epidermidis and A. baumannii. Thus, PEI is more active against Gram-positive than Gram-negative bacterial biofilms. Interestingly, inhibition of C. albicans biofilm metabolic activity was only detected at concentrations above the MIC value.

NanoPEI gave similar MIC and MLC values for S. aureus and S. epidermidis, with A. baumannii strains once again being more resistant. Accordingly to the literature, nanoPEI exhibit a strong antibacterial effect (Beyth et al., 2006, 2008). The high activity of polycationic agents reflects the adsorption of positively charged polymers onto negatively charged bacterial surfaces (Beyth et al., 2008). This process is thought to be responsible for the increase in cell permeability and disruption of the cell membrane. As described by Beyth et al. (2006), PEI nanoparticles exhibited a strong antibacterial effect against Streptococcus mutans, and the same study demonstrated that composite resin materials incorporating PEI nanoparticles maintained antibacterial properties for over 1 month. Moreover, quaternary ammonium-PEI (QA-PEI) particles embedded in restorative composite resin resulted in complete inhibition of growth of Streptococcus mutans. It has been demonstrated that QA-PEI nanoparticles completely inhibited growth of Gram-positive (Staphylococcus aureus) and Gram-negative (E. coli) bacteria. Such a study confirmed that polycations bearing quaternary ammonium moieties inhibit bacterial growth in vitro and may have potential uses as additives in medical devices (Yudovin-Farber et al., 2010). The study by Beyth et al. (2008) also supports a differential bacterial sensitivity to nanoPEI.

A key feature to have in mind is the biocompatibility issues of these compounds. QA-PEI-based nanoparticles in dental composite resin at 1 % (w/w) was considered biocompatible (Yudovin-Farber et al., 2008; Beyth et al., 2008). A recent work also shows that incorporation of QPEI nanoparticles in bone cements has a long-lasting antibacterial effect without compromising the cement’s biocompatibility (Beyth et al., 2013).

In our study, similarly to PEI, nanoPEI treatment resulted in the disruption of cell membrane integrity and in cell depolarization (except for A. baumannii strains). In the case of C. albicans, only cell depolarization was detected.

Concerning biofilm inhibition, a recent study demonstrated that covalently bound quaternized polyDMAEMA (polydimethylaminoethylmethacrylate) and PEI reduced biofilm growth of C. albicans by up to 92 % (De Prijck et al., 2010). Alkylated PEI attached to flat macroscopic surfaces and to surfaces of nanoparticles made these materials highly bactericidal for Gram-positive and Gram-negative bacteria. According to our results, the anti-biofilm effect of nanoPEI at subinhibitory concentrations was prominent for all bacteria tested; however, inhibition of C. albicans biofilm metabolic activity was only observed at 2 MIC. In this specific case, the MLC value was not determined.

According to our results, the inhibition pattern of biofilm formation by PEI and nanoPEI appears to be promising and may justify its future evaluation for the development of indwelling catheters and other indwelling medical devices that are less prone to bacterial and yeast biofilm formation. In vitro testing of nanoparticle systems represents a key aspect of current investigations envisaging future clinical applications of nanomaterials. The in vivo effectiveness of PEI and nanoPEI can only be speculated, as it will require animal model testing. Our results regarding their activity against biofilms of clinically relevant bacterial and fungal pathogens on the surface of PUR-like catheters stress the need to investigate their possible use in medical devices.

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