ARTICLE IN PRESS

BBA - Biomembranes xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Biomembranes

journal homepage: www.elsevier.com/locate/bbamem

Cerium oxide nanoparticles as potential antibiotic adjuvant. Effects of CeO_2 nanoparticles on bacterial outer membrane permeability

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ARTICLE INFO

Keywords:
CeO_2 nanoparticles
Drug synergism
Antimicrobial resistance
Bliss independence theory
KPC beta-lactamase
K. pneumoniae

ABSTRACT

Background: Therapeutic options against Multi Drug Resistant (MDR) pathogens are limited and the overall strategy would be the development of adjuvants able to enhance the activity of therapeutically available antibiotics. Non-specific outer membrane permeabilizer, like metal-oxide nanoparticles, can be used to increase the activity of antibiotics in drug-resistant pathogens. The study aims to investigate the effect of cerium oxide nanoparticles (CeO_2 NPs) on bacterial outer membrane permeability and their application in increasing the antibacterial activity of antibiotics against MDR pathogens.

Methods: The ability of CeO_2 NPs to permeabilize Gram-negative bacterial outer membrane was investigated by calcein-loaded liposomes. The extent of the damage was evaluated using lipid vesicles loaded with FITC-dextran probes. The effect on bacterial outer membrane was evaluated by measuring the coefficient of permeability at increasing concentrations of CeO_2 NPs. The interaction between CeO_2 NPs and beta-lactams was evaluated by chequerboard assay against a *Klebsiella pneumoniae* clinical isolate expressing high levels of resistance against those antibiotics.

Results: Calcein leakage increases as NPs concentrations increase while no leakage was observed in FITC-dextran loaded liposomes. In *Escherichia coli* the outer membrane permeability coefficient increases in presence of CeO_2 NPs. The antibacterial activity of beta-lactam antibiotics against *K. pneumoniae* was enhanced when combined with NPs.

Conclusions: CeO_2 NPs increases the effectiveness of antimicrobials which activity is compromised by drug resistance mechanisms. The synergistic effect is the result of the interaction of NPs with the bacterial outer membrane. The low toxicity of CeO_2 NPs makes them attractive as antibiotic adjuvants against MDR pathogens.

1. Introduction

The phenomenon of antimicrobial resistance has emerged among pathogenic bacteria since the beginning of the antibiotic era as consequence of the selective pressure generated by the use, abuse and misuse of antibiotics in human and veterinary medicine. Nowadays, pathogenic organisms expressing Multi Drug Resistance (MDR) phenotype are among the most important cause of infections in nosocomial and community settings. Therapeutic options for infections sustained by MDR pathogens are limited and often ineffective, and new drugs are urgently needed [1]. To overcome antibiotic-mediated resistance, the overall strategy would be, so far, the use of combinations of drugs or the development of adjuvants that, acting in concert with licensed agents, can enhance their antimicrobial activity even against resistant strains. Substances able to increase susceptibility to currently licensed agents, would be very attractive and useful [2–4].

The development of antibiotic adjuvants does not necessarily imply the discovery of new targets in bacterial cells, on the contrary, most of the known cell targets can be exploited (*e.g.* beta-lactamase inhibitors), or even non-specific compounds like outer membrane permeabilizer can be used to increase the activity of antibiotics. Outer membrane in Gram-negative bacteria is a semipermeable barrier that confers inherent resistance to most antibiotics [5,6]. The entrance of hydrophilic antibiotics such as beta-lactams, is allowed trough channel-forming proteins called porins which represent < 1% of the surface area. Moreover, the rate of uptake into the cell is often reduced by the

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https://doi.org/10.1016/j.bbamem.2018.07.002
Received 19 March 2018; Received in revised form 29 June 2018; Accepted 9 July 2018
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Please cite this article as: Bellio, P., BBA - Biomembranes (2018), https://doi.org/10.1016/j.bbamem.2018.07.002

presence of efflux pumps, making actually the accessible area, ungenerous. The passage of hydrophobic compounds is also prevented by the presence in the outer layer of the outer membrane, of the polyanionic lipopolysaccharide (LPS) which is stabilised by divalent cations. The displacement of the stabilising cations operated by polycations such as polymyxins, aminoglycosides or cationic peptides [6] makes outer membrane increasingly permeable to other compounds [7]. Following this logic, positively-charged metal oxide nanoparticles (MeO-NPs) have been demonstrated firmly bind bacterial cell membrane [8].

In the last years the interest in MeO-NPs is enormously increased as potential antibacterial agents against drug-resistant pathogens [8]. The nanometer size of metal oxide NPs as the physical and chemical properties, are strictly related to their antimicrobial activity [9,10]. Specifically, ceria nanoparticles (NPs) have been extensively studied for a variety of potential applications in several fields, including nanomedicine [11–18]. Their ability to act as antioxidant has been well established [16,19–21], but investigations about their antimicrobial potentiality are still undergoing. Recently has been demonstrated that coated ceria NPs are able to inhibit up to 50% the growth of *Pseudomonas aeruginosa* bacterial strain [22]. Thill et al. [23] have demonstrated the cytotoxicity of CeO_2 NPs against the enterobacterial *Escherichia coli* and Shah et al. [12] demonstrated that dextran coated cerium oxide nanoparticles are able to induce toxicity against *Escherichia coli* [12,23]. Pelletier et al. [24] showed as cerium oxide NPs exert a moderate bactericidal activity against *E. coli* and *Bacillus subtilis*. Moreover, several studies demonstrated that morphology, size and composition of CeO_2 NPs surface, characterize their antibacterial properties [25–27].

Has been postulated that CeO_2 NPs adsorb *via* electrostatic attraction to the bacterial surface but do not penetrate them [23,24,28]. The electrostatic interaction between NPs and the membrane seems to be so strong that they might stick at the surface for a very long time [23] and Ce^{4+} atoms close to the membrane surface are reduced to Ce^{3+}, resulting in oxidative stress on the major components of the membrane such as lipids and/or proteins [23]. The oxidation of the bacterial cell would create mesosoma like structures, therefore several elementary and essential functions as DNA replication, cell division are changed and consequently the surface area of bacterial cell membrane is increased [29] properly because the formation of membrane invaginations. However, despite the attempts to demonstrate and ameliorate the toxic activity of ceria NPs against pathogenic bacteria [12,22–24,30], they still cannot be defined as pure and efficient antibacterial agents.

2. Materials and methods

2.1. Antibiotics and reagents

All tested antibiotics, cefotaxime (CTX), imipenem (IMP), amoxicillin (AMX) and clavulanate (CLV) were from Sigma-Aldrich (Milan, Italy). All reagents used for the preparation of ceria nanoparticles were from Sigma-Aldrich (Milan, Italy). Nitrocefin was kindly provided by professor Shahriar Mobashery laboratories (Notre Dame University, US-IN). *Escherichia coli* total lipid extract for calcein-entrapping liposomes preparation were from Avanti Polar Lipids (Alabaster, US-AL) and calcein was from Sigma-Aldrich (Milan, Italy). Fluorescein isothiocyanate dextran D-4, D-20 D-70 were from Sigma-Aldrich (Milan, Italy).

2.2. Organisms

Escherichia coli HB101, host cells (genotype, F- *mcrB mrr hsdS20(rB-mB-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) glnV44* λ-) from Promega (Madison, US-WI) were used for permeability assays.

Klebsiella pneumoniae carbapenemase (KPC)-producing, named KP1/11, was used for drug interaction assays [31]. The *K. pneumoniae* KP1/11 strain shows the simultaneous presence of the resistance genetic determinants bla_{KPC-3} and bla_{VIM-2} encoding for a serine-beta-lactamase and a metallo-beta-lactamase, respectively. Moreover, the presence of *aac*A29b and *aac*(6′)-Ib genes, confers resistance to aminoglycosides.

2.3. CeO_2 nanoparticles synthesis

Cerium oxide nanopowders were synthesised using a modified precipitation method with cerium nitrate hexahydrate as precursor [32]. The process of chemical synthesis, the structural and electronic properties of the nanoparticles of cerium oxide used in this study, were previously reported [33]. Specifically, CeO_2 NPs used in this work, were calcined in an air furnace at temperature T = 500 °C for 8 h.

2.4. Calcein loaded liposomes

Calcein-entrapping liposomes of different lipid compositions were prepared as previously described [34] with some modifications. Briefly, 1 mL of a solution of calcein 60 mM in sodium phosphate buffer 50 mM, pH 7.4, containing EDTA 0.1 mM, was cosonicated with 1 mL of *E. coli* total lipid extract dissolved in chloroform. After that, lipid vesicles were prepared by reverse phase evaporation method [35]. Untrapped calcein was removed by gel filtration (Sephadex G-50, 1.5 × 15 cm column, equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA). The lipid concentration in the separated vesicular fractions was determined by the method of Stewart [36].

Lipid vesicles preloaded with calcein were treated with cerium oxide NPs at concentrations ranging from 50 μg/mL to 600 μg/mL in sodium phosphate buffer 50 mM, pH 7.4 and EDTA 0.1 mM. The release of calcein from liposomes was monitored fluorimetrically on a Perkin-Elmer LS 50 B spectrofluorimeter; excitation and emission wavelengths were 490 nm and 517 nm, respectively. The maximum fluorescence intensity was determined by the addition of 20 μL of a water solution of Triton X-100 (10%, v/v) to the sample containing calcein loaded liposomes.

The percentage of calcein released was calculated according to the equation [37]:

$$Leakage\ (\%) = 100 \times (F - F_0)/(F_t - F_0)$$

where F and F_t are the intensity of fluorescence before and after the addition of the detergent and F_0 is the fluorescence of intact vesicles.

2.5. Dextran loaded liposomes

Dextran loaded vesicles containing the FITC-D of choice (FITC-D 4, 20 or 70) were prepared as reported elsewhere [38]. The release of dextran from loaded vesicles upon interaction with cerium oxide NPs, at the same concentrations used for calcein loaded liposomes, was examined fluorimetrically; excitation and emission wavelengths were 494 nm and 520 nm, respectively. In a typical experiment, an aliquot of a solution of cerium NPs in potassium phosphate buffer 50 mM, pH 7.4 and EDTA 0.1 mM, was incubated 10 min with a suspension of dextran-loaded vesicles, with a final lipid concentration of 50 μM. The mixture was gently stirred for 10 min in the dark and then centrifuged at 22500*g* for 30 min. The supernatant was recovered, and its fluorescence intensity recorded. The maximum fluorescence intensity was determined by the addition of 20 μL of 10% (v/v) Triton X-100 water solution to the vesicle suspension. The apparent percentage leakage value was calculated as previously done for calcein. All experiments were carried out at room temperature and in triplicate.

2.6. Outer membrane permeability assay

Outer membrane permeability was determined as previously described by Zimmermann and Rosselet [39]. *E. coli* HB101 was transformed with the expression vector pBR322 containing the gene bla_{TEM-}

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