The influence of cationic dendrimers on antibacterial activity of phage endolysin against *P. aeruginosa* cells

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ABSTRACT

Nowadays, the researchers make a big effort to find new alternatives to overcome bacterial drug resistance. One option is the application of bacteriophage endolysins enable to degrade peptidoglycan (PG) what in consequence leads to bacterial cell lysis. In this study we examine phage KP27 endolysin mixed with poly(propyleneimine) dendrimers to evaluate an antimicrobial effect against *Pseudomonas aeruginosa*. Polycationic compounds destabilize bacterial outer membrane (OM) helping endolysins to gain access to PG. We found out that not only bacterial lipopolysaccharide (LPS) is the main hindrance for highly charged cationic dendrimers to disrupt OM and make endolysin reaching the target but also the dendrimer surface modification. The reduction of a positive charge and concentration in maltose poly(propyleneimine) dendrimers significantly increased an antibacterial effect of endolysin. The application of recombinant lysins against Gram-negative bacteria is one of the future therapy options, thus OM permeabilizers such as cationic dendrimers may be of high interest to be combined with PG-degrading enzymes.

1. Introduction

In 21th century bacterial resistance to antibiotic is gradually increasing, in particular in Gram-negative bacteria. The vast majority of pathogens belonging to multidrug resistant ESKAPE group (*Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas, Enterobacter*) are Gram-negatives. ESKAPE members are considered to be particularly dangerous, because of a high adaptability to colonize various niches ranging from water and soil to plants and animal tissues. Most of them have a broad spectrum of virulence factors which cause a serious damage to host tissues and also compromises the immune system [1]. Since the multidrug resistant (MDR) bacteria have expanded, therapeutic and prevention options have become limited.

Bacterial viruses (phages) are especially attractive as a potential tool for MDR strains eradication and recently the interest in lytic phages is constantly growing [2]. Some studies showed that bacteriophage-derived proteins especially peptidoglycan (PG)-degrading enzymes (lysins) as efficient antimicrobials exhibit an economical potential for food industry and agriculture [2,3].

Phages produce two major types of muralytic enzymes: virion-associated lysins (VALs) and endolysins. VALs as a part of virion particle degrade PG at the beginning of phage infection allowing to inject phage DNA into the host cell. Endolysins are used in the end of replication cycle to degrade host PG from within causing cell lysis and phage progeny release [3–5].

The specificity and host range of lysins depend mostly on the protein origin, such as phages infecting Gram-positive or -negative bacteria [3,5,6].

The PG degrading effect provided by lysins can be seen in seconds as an osmotic lysis of targeted cell, making these enzymes a desired and efficient antibacterial agent. Lysins present a high effectiveness against Gram-positive pathogens, including *Streptococcus pneumoniae* and *Staphylococcus aureus,* even methicillin resistant strains (MRSA). In contrast, PG-degrading enzymes have limited effect on Gram-negatives, when applied exogenously, because of a hampered penetration through the outer membrane (OM) layer. There are only few endolysins described as external agents (SPN9CC, PlyF307, CfP1gp153) which are able to overcome OM and gain access to PG [7–9].

There are in fact some options to improve an antibacterial activity of lysins towards Gram-negative bacteria. The simplest way is to
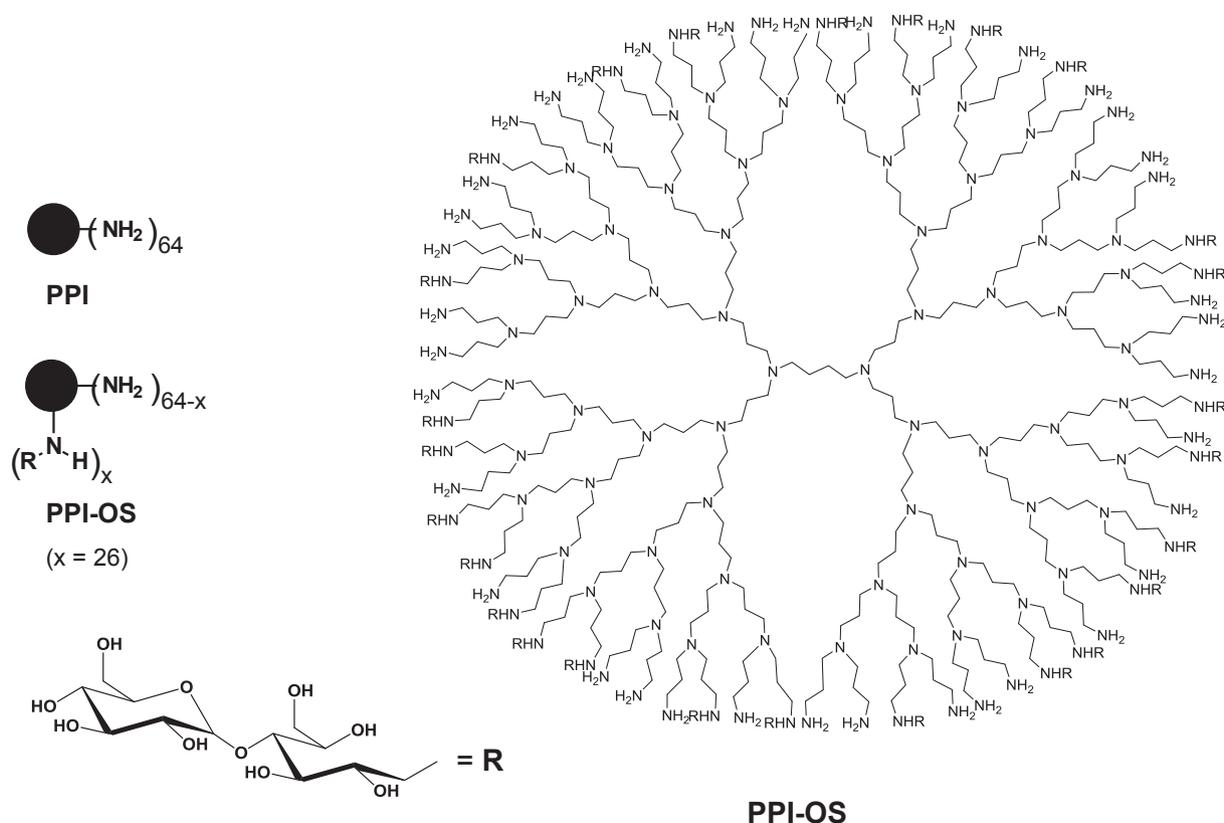
Fig. 1. Structures of dendrimers used in the study. The parental fourth generation poly(propyleneimine) dendrimer (PPI) with terminal amino groups and PPI glycodendrimer modified with maltose (PPI-OS), meaning about 20% maltose attachment on 4th generation PPI dendrimer PPI-OS.

combine the enzyme with OM destabilizing agents such as EDTA, weak organic acids (citric acid) or polycationic agents [10]. There is also a possibility to genetically modify those enzymes by the addition of polycationic, hydrophobic or amphipathic peptides causing OM disruption [11,12]. Several endolysins (Ply511, PlyA, CD27L, OBPgpLYS) have been already modified and patented as Artilysins® [13]. There is also a possibility to use membrane-interacting/destabilizing dendrimers as an additive to phage endolysin to improve OM penetration and antibacterial activity.

The dendrimers properties such as high degree of molecular uniformity, narrow molecular weight distribution, tunable size and surface charges let them be a potential antimicrobial agent themselves. The synthesis and further modification of polycationic dendrimers allow for an effective internalization in Gram-negative bacteria [14]. The capacity of dendrimers for the disruption of biological membrane is strictly connected to their supramolecular structure and charge distribution. The cationic charge is a main driver in attraction and permeabilization of bacterial membrane. However, a higher positive charge may also increase cytotoxic activity against other biological environment, for example, inducing hemolytic effects [15]. Most polycationic dendrimers (poly(propyleneimine) (PPI) and poly(aminoamide) (PAMAM)), metallodendrimers and glycodendrimers have antibacterial properties against Gram-negative bacteria, but at concentrations above 10 μM [16,17]. Moreover, a hemolytic effect is very often visible at the same concentration. Therefore, it is necessary to modify dendrimers making them more selective towards bacterial membrane. The modification of dendrimers surface with sugar units (in about 20%) increases their hydrophilicity, but still preserves the cationic charge necessary to interact with lipid membrane of bacteria. There are many biological applications of glycodendrimers. For instance PPI glycodendrimers are very promising candidates for an effective anti-HIV DC-based immunotherapy [18]. GlycoPAMAM dendrimers can mimic *T*-antigen markers on breast cancer cells [19]. The addition of maltose to

dendrimers structure decreases the hemolytic effect and cytotoxicity [20]. Moreover, glycodendrimers can behave like glycoproteins on the cell surface interacting with bacterial lectin. Therefore, these compounds can also be used as anti-adhesive macromolecules [21].

The combination of polycationic dendrimers as bacterial membrane permeabilization agents with an antibiotic or mesoporous silica nanoparticles gave promising results [22,23]. For instance, PPI dendrimers with amoxicillin show an increased antimicrobial activity compared to antibiotic alone. Very effective was also the covalent grafting of PPI dendrimers to mesoporous silica nanoparticles (loaded with levofloxacin) that allowed for an internalization into Gram-negative bacteria.

In this study we present the first attempt to combine the cationic dendrimer and/or glycodendrimers macromolecules with phage-borne enzyme (KP27 endolysin) to enhance an antibacterial efficacy against Gram-negative bacteria. The parental PPI dendrimers with highly dense cationic charges and maltose modified glycodendrimers with open shell (PPI-OS) with reduced positive charge on the surface (due to maltose presence) were chosen as permeabilization agents in this study. The idea of this work was to examine a possible synergistic effect of these two agents: First, cationic dendrimers at low concentration would permeabilize OM enabling endolysin to get access to its target (PG). We investigated a panel of first properties (surface modification and charge) of cationic dendrimers to find out the best synergic composition against Gram-negative bacteria. Moreover, the endolysin combined with dendrimers was tested in terms of its stability and activity.

2. Materials and methods

2.1. Dendrimers

Poly(propyleneimine) dendrimer of the 5th generation (MW 7167.97 g/mol) with 64 terminal amino groups was obtained from

SyMO-Chem (Eindhoven, The Netherlands) and specified as 4th generation (PPI) following the uniform nomenclature of polyamine dendrimers for poly(propyleneimine) and poly(amidoamine) macromolecules [24]. The maltose-modified dendrimers (Fig. 1) were synthesized in Leibniz Institute of Polymer Research Dresden, Germany. Parental PPI dendrimer of the 4th generation (PPI) with primary surface amino groups was partially modified with maltose (PPI-OS, molecular weight (MW) 15053 g/mol) (so-called "open shell" = OS) glycodendrimer; Fig. 1) which was prepared and characterized as described previously [24,25]. For the reductive amination of PPI dendrimer a molar ratio of 1:0.25 between primary amino groups and maltose was used in sodium borate buffer [24]. Degree of maltosylation on PPI dendrimer surface was provided by MALDI-TOF MS as indicated and described in Ref. [24]. Thus, 26 maltose units (MW 15053 g/mol determined by MALDI-TOF MS) have been coupled to PPI dendrimer which indicated an average modification of 20% (Fig. 1), when, finally, each amino group of 64 amino groups in PPI dendrimer can theoretically bear 2 coupled maltose units (100% modification addresses 128 maltose units for dense maltose shell) [24].

2.2. Bacterial strains

Antibacterial activity of KP27 endolysin combined with dendrimers was tested using *P. aeruginosa* PAO1 wild-type and its knock-out mutants deficient in biosynthesis of LPS A-band and B-band O-antigen (Table 1).

2.3. Recombinant endolysin preparation

The recombinant phage-borne endolysin was prepared according to the method described previously by Maciejewska et al. [26]. Briefly, the coding sequence of *Klebsiella* phage KP27 endopeptidase was amplified using Pfu polymerase (Thermo Fisher Scientific Waltham, MA, USA) and cloned into the commercially available pEXP-5-CT/TOPO® TA expression vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer recommendations. The expression was conducted for 18 h at 20 °C using *E. coli* BL21 (DE3) pLysS (Agilent Technologies, Santa Clara, CA, USA) and isopropyl-β-D-1-thiogalacto-pyranoside (IPTG; the final concentration of 0.5 mM) as an inductor of the expression. The recombinant protein was purified from the filtered supernatant by affinity chromatography using NGC Medium Pressure Chromatography Systems (Bio-Rad, Hercules, CA, USA) combined with 5-ml nickel columns: Bio-Scale Mini Profinity IMAC Cartridges (Bio-Rad, Hercules, CA, USA) and dialyzed against PBS buffer. The concentration of purified recombinant enzyme was then determined fluorimetrically (Qubit® Protein Assay Kit, Molecular Probes, Thermo Fischer Scientific, Waltham, MA, USA).

2.4. Thermal stability of endolysin in the presence of dendrimers measured by circular dichroism

Circular dichroism (CD) was measured in the far-UV region using a J-815CD spectrometer (Jasco, Japan). The experiments were done in 10 mM sodium phosphate buffer (pH 7.4). The concentration of proteins was 0.5 µM. First CD spectra of protein alone and in the presence of dendrimers were obtained in the range between 260 and 195 nm and changes in mean residual ellipticity (θ/θ_0) in characteristic minimum at 220 nm were measured at temperature range from 20 to 80 °C, where θ_0 is a mean residual ellipticity at 20 °C. Quartz 0.5 cm path length cells (Hellma, Germany) were used for all CD experiments. The mean residue ellipticity, θ expressed as $cm^2\ dmol^{-1}$, was calculated. The CD spectra were taken for pure protein and after adding of dendrimers at a molar ratio of 1:4 (protein:dendrimer).

2.5. Enzymatic activity of endolysin in the presence of dendrimers

The enzymatic activity of endolysin was tested by measuring peptidoglycan (PG) degradation. PG was isolated from *E. coli* ATCC 8739 cells. Briefly, *E. coli* was cultivated under aerobic conditions in a fermenter (BioStat A, Sartorius Stedim Biotech) in nutrient broth (BTL, Poland) under controlled conditions (37 °C, pH 7.2–7.4, pO_2 70–86%). Cells were harvested at the end of the logarithmic growth phase, centrifuged (5000 g, 30 min), washed with distilled water, and lyophilized. PG was isolated in accordance with the method described by Bera et al. [26]. The rate of degradation was evaluated spectrophotometrically in the presence of dendrimers: PPI (unmodified polypropylene imine dendrimers) and PPI-OS (PPI dendrimers modified with 20% of maltose on the surface). Endolysin (10 µM) was preincubated with both types of dendrimers at 1:1 and 1:4 M ratio (protein:dendrimers) for 15 min and then the scans were recorded at different temperature from 20 °C to 37 °C. The 0.25 mg/ml of PG was added to the sample and the kinetics of its degradation was measured at 560 nm using Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland).

2.6. Antimicrobial activity of dendrimers in the presence of endolysin

The antibacterial activity of dendrimers and endolysin was measured by using the spectrometric method at 600 nm. Antimicrobial properties of endolysin (12 µM) alone, dendrimers (12 and 50 µM) alone and dendrimers in presence of endolysin (12 µM) were expressed as the percentage of *P. aeruginosa* culture growth (optical density OD_{600}) compared to the non-treated bacterial culture (control) using Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland).

3. Results and discussion

3.1. Thermal stability of endolysin in the presence of dendrimers

The aim of the first experiment was to check whether dendrimers chosen for combination with endolysin do not affect the secondary structure of enzyme disturbing the activity of its catalytic centre. Fig. 2 shows the dependence of secondary structure changes in molar ellipticity (θ/θ_0) of endolysin in the function of temperature. The experiments were performed in the presence or absence of dendrimers PPI and PPI-OS. The increase of molar ellipticity for endolysin was observed after heating up to 50 °C for both combinations. In the presence of PPI dendrimers an earlier increase of molar ellipticity of endolysin was observed after heating to 40 °C compared to protein in the presence of PPI-OS. Moreover, the changes in mean residue ellipticity slope (at $\lambda = 220$ nm) in presence of PPI dendrimers were much steeper. In contrast, PPI-OS dendrimers did not affect the stability of KP27

Table 1
P. aeruginosa PAO1 wild-type and mutants used in this study.

| Bacterial strains	Phenotype
P. aeruginosa PAO1 (ATCC 15692)	Wild type
Δ*rmd* (A−, B+)*	Deficiency in D-rhamnose biosynthesis; lack of A-band LPS
Δ*waaL* (A−, B−)*	Lack of WaaL ligating O-polymer to core-lipid A; LPS is devoid of A-band and B-band, semirough (SR-LPS, or core-plus-one O-antigen)
Δ*wbpL* (A−, B−)*	Lack of glucosyltransferase WbpL essential for initiation of both A-band and B-band synthesis
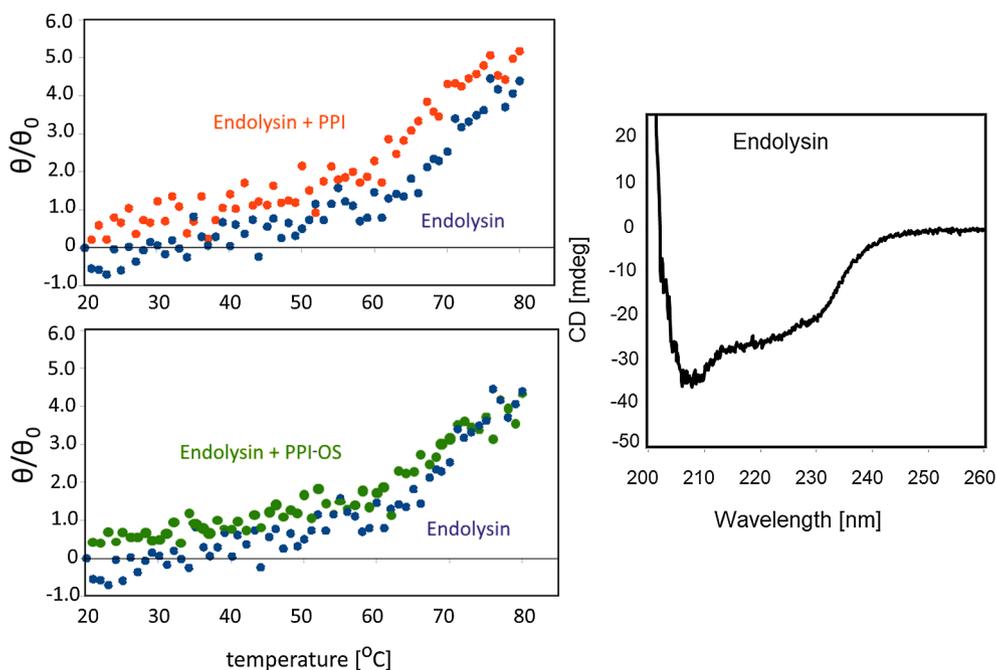
Fig. 2. Changes in the mean residual ellipticity (θ/θ_0) of KP27 endolysin at 220 nm in the function of temperature. Endolysin incubated alone and with unmodified PPI dendrimer (upper scan) or endolysin incubated alone and with maltose modified dendrimer PPI-OS (lower scan). On the right, the CD spectrum of endolysin is presented.

endolysin. The differences in the interaction of PPI and PPI-OS dendrimers with phage enzyme are caused by the disparity of quantity of positively charged amine groups present on the macromolecule surface. PPI dendrimers possess more cationic groups, while PPI-OS surface is partially neutralized by maltose moieties. Our data correspond to the previous studies describing the interaction of poly(propyleneimine) dendrimers with other proteins that sugar modification of PPI dendrimers minimizes the interaction strength with cationic and anionic proteins [27].

3.2. Enzymatic activity of endolysin in the presence of dendrimers

The enzymatic activity of endolysin in the presence of dendrimers was measured by peptidoglycan (PG) degradation at different temperatures and different protein:dendrimer molar ratios (1:1 and 1:4) (Table 2). The highest endolysin activity was observed at 37 °C with complete degradation of PG (0.25 mg/ml) after 10–15 min of incubation. This process slowed down along with the temperature. From circular dichroism measurements small changes in the secondary structure of endolysin were observed in the presence of both dendrimers. The endolysin was inactive at 20 °C without or with dendrimers. After heating to 26 °C or higher temperatures, the activity raised up to 100% and PG degradation was seen. At 26 °C and 32 °C the activity of endolysin in presence of PPI and PPI-OS dendrimers was lower, but was increasing to 100% at 37 °C. It is worth noticing that both dendrimers strongly inhibit endolysin activity at the molar ratio of 1:1 compared to 1:4. However, the results show that higher activity is observed at lower temperature with excess of dendrimers in solution. It seems that, PPI

dendrimer has much lower inhibitory effects on the enzyme activity as found in case of PPI-OS. 1:1 molar ratio between endolysin and PPI-OS shows highest inhibitory effects due to tight complex formation process or blocking the enzymatic center for the degradation of PG. This effect is temperature dependence. The inhibitory effect disappears after heated to 37 °C. These differences in inhibition of enzyme can be due to existence of ionic and hydrogen bonds interaction, where H-bonds of maltose units play a major role in comparison to PPI alone. The ionic interactions could be destroyed at higher concentration (excess PPI only), while 1:1 with PPI shows similar effects as shown for PPI-OS. It is worth to notice that also small changes in the thermal stability of endolysin in the presence of dendrimers seem not to be correlated with the enzyme activity, but only with the properties of used dendrimers.

3.3. Antimicrobial properties of endolysin in presence of dendrimers

The optical density of bacterial culture was measured at 600 nm to check the influence of endolysin and/or dendrimers on *P. aeruginosa* PAO1 wild-type and its three different LPS knock-out mutants (Fig. 3). Bacterial strains varied in the length and type of LPS O-chain. PPI and PPI-OS dendrimers at concentrations of 12 μM and 50 μM were used in this study. The PPI dendrimers at 12 μM and PPI OS dendrimers modified by maltose at 50 μM have been already confirmed as nontoxic against eukaryotic cells [20].

The decrease of optical density in the presence of PPI dendrimers was observed for all tested bacterial strains, especially for *ΔwaaL* and *ΔwbpL* mutants. The maltose-modified dendrimers PPI OS decreased the optical density only at 50 μM for *ΔwaaL* mutant. It indicates that

Table 2

Enzymatic activity of endolysin in the presence of dendrimers at different protein:dendrimers molar ratio at different temperatures after 15 min of incubation with PG. The percentage of activity means the percentage of peptidoglycan degradation by endolysin after this time.

PG degradation [%]

Temperature [°C]	Endolysin	Endolysin + PPI [MR 1:1]	Endolysin + PPI [MR 1:4]	Endolysin + PPI-OS [MR 1:1]	Endolysin + PPI-OS [MR 1:4]
20	0	0	0	0	0
26	100	25	60	8	45
32	100	38	90	10	40
37	100	100	100	100	100

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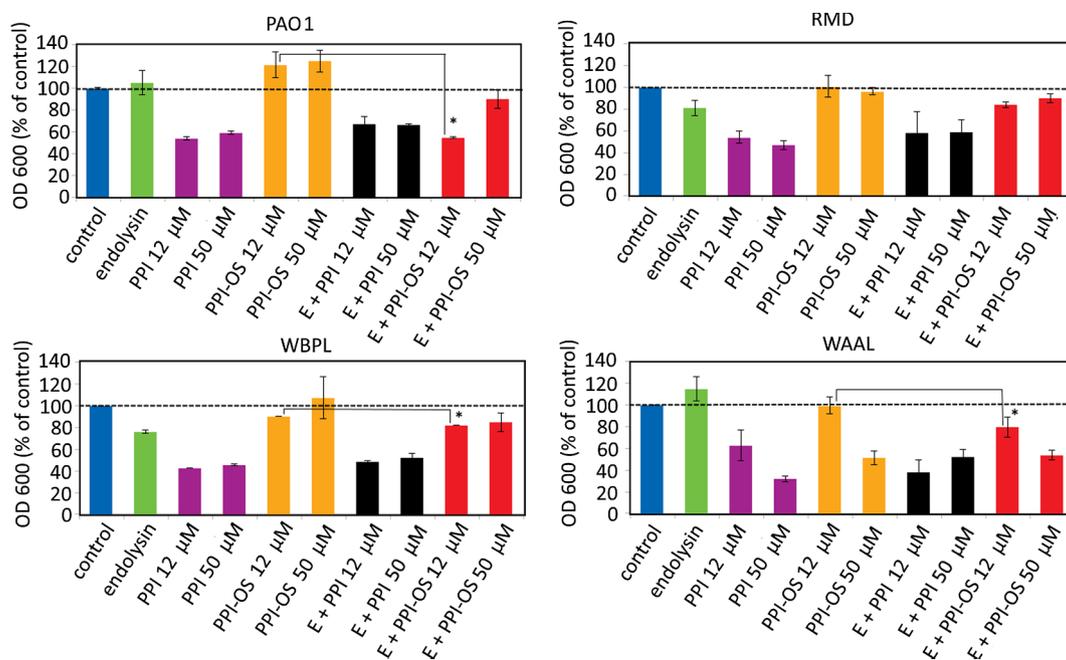**Fig. 3.** The optical density of PAO1 wild-type and its mutants (*Δrmd, ΔwbpL and ΔwaaL*) measured at 600 nm in the presence of endolysin (12 μM), dendrimers and combined at different concentrations and molar ratio (1:1; 1:4). The results are expressed as the percentage, where 100% is a non-treated bacterial culture (control). PPI (poly(propyleneimine) dendrimer), PPI-OS poly(propyleneimine) dendrimer with maltose – 20% on the surface), E-PPI and E-PPI OS (dendrimers mixed with endolysin). Culture growth for 24 h in 37 ˚C.

antibacterial properties of cationic dendrimers depend on the modification of dendrimers surface by maltose which might promote an electrostatic interaction with outer membrane of bacterial cells, which is negatively charged and possess lectins at the surface.

There was no visible additional antibacterial effect of endolysin in combination with PPI dendrimers. In opposite a significant improvement of enzyme activity was only noted in the presence of PPI-OS at 12 μM for PAO1, *ΔwaaL* and *ΔwbpL* mutants (p < 0.05). It could be due the possible aggregation effect of PPI OS dendrimers at 50 μM. It seems that the higher number of cationic groups presented on PPI dendrimers surface can be a hindrance for proper antibacterial activity of endolysin. It is worth mentioning, that at 12 μM PPI OS dendrimers are nontoxic to human cells [20].

4. Conclusions

The aim of the study was to check the additional antibacterial effect of endolysin in presence of poly(propyleneimine) dendrimers. Effective antibacterial properties of poly(propyleneimine) dendrimers against Gram-negative bacteria are present. This is triggered by the surface composition of PPI dendrimers and their used dendrimer concentration. The poly(propyleneimine) dendrimers with maltose mixed with recombinant phage protein enhance antibacterial activity of KP27 endolysin against PAO1 wild-type and mutants with reduced antigen O in LPS structure, in contrast to both components alone. Maltose dendrimers possess less cationic charges, which promote the binding with LPS and/or lectins present on the *P. aeruginosa* cell wall. This effect is mediated by dendrimers at low non-cytotoxic concentration (12 μM). A high dendrimer concentration (50 μM) leads to loss of dendrimer-protein interactions. We can conclude that PPI-OS dendrimers may help disrupt the outer bacteria membrane and help endolysin reach peptidoglycan, what in consequence may lead to overcome bacteria resistance. Obtained results are promising to start further study on the development of new antimicrobial complexes composed of glycodendrimers and phage-borne PG-degrading enzymes.

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