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Mechanistic insight of lysozyme transport through the outer bacteria membrane with dendronized silver nanoparticles for peptidoglycan degradation

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Abstract

The problem of drug resistance has become a global problem, prompting the entire scientific world to look for alternative methods of dealing with resistant pathogens. Among the many alternatives to antibiotics, two appear to be the most promising; membrane permeabilizers and enzymes that destroy bacterial cell walls. Therefore, in this study we provide an insight into the mechanism of two antimicrobial protein (lysozyme) transport strategies using two types of carbosilane dendronized silver nanoparticles (AgNPs): non-Pegylated (Dend-AgNPs) and PEGylated (PEG-Dend-AgNPs), for outer membrane permeabilization and peptidoglycan degradation. Remarkably, studies have shown that Dend-AgNPs can build up on the surface of a bacterial cell, destroying the outer membrane, thereby allowing lysozyme to penetrate inside the bacteria and destroying the cell wall. PEG-Dend-AgNPs, on the other hand, have a

completely different mechanism of action. PEG chains containing complexed lysozyme lead to bacterial aggregation and an increase in the local concentration of the enzyme near the bacterial membrane, thereby inhibiting bacterial growth. This is due to the accumulation of the enzyme in one place on the surface of the bacteria and penetration into it through slight damage of the membrane due to weak but existing interactions of nanoparticles with the membrane. Our multidisciplinary research presents two new approaches, understanding of which could help to invent new, more effective antimicrobial protein nanocarriers to overcome bacterial multidrug resistance.

Keywords: Outer bacteria membrane, permeabilization, lysozyme, silver nanoparticles, carbosilane dendrimer.

1. INTRODUCTION

The advent of antibiotics at the beginning of the 20th century revolutionized medicine and made them an indispensable tool used in the treatment and prevention of bacterial infections. However, it has only been a few years since penicillin was invented when penicillin-resistant bacteria appeared [1–4]. Nowadays, antibiotic resistance has become a very serious global problem and commercially available anti-bacterial measures have become ineffective. Multidrug-resistant (MDR) pathogens are an established and growing worldwide public health problem. Among others, the ESKAPE group (Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas, and Enterobacter) seems to be the most crucial and particularly dangerous [5]. These bacteria have developed many ways to make them resistant to antimicrobials. These include, but are not limited to, enzyme inactivation, decreased cell permeability, target protection, altered target site and increased efflux due to overexpression of cell efflux pumps [6]. This problem mainly affects Gram-negative bacteria due to the presence of an extra outer membrane in these bacteria. The outer bacterial membrane prevents

the penetration of antibacterial substances, including antibiotics, which in turn prevents the elimination of infections in the human body caused by the presence of these pathogens [7]. The constantly deteriorating situation and the emergence of multi-drug resistant strains are forcing scientists around the world to look for alternative ways to fight this problem [8,9]. Currently, there are several alternative methods of controlling drug-resistant strains, they are: bacteriophages[10], antimicrobial proteins (e.g. lysins) [11], immune system stimulators and membrane permeabilizers –antimicrobial peptide [12–14] and nanoparticles [15–18]. However, none of these methods is 100% effective, hence the need to refine existing methods or look for other, more effective.

It has been proven that metal-based compounds, especially silver, are a possible alternative to classic organic drugs. One of the best-studied antibacterial systems are metal nanoparticles (NPs), in particular silver nanoparticles (AgNPs). Nanoparticles have several features that make them of great interest to scientists for their ability to fight pathogens. These include improved drug solubility and stability, ease of synthesis, their biocompatibility with target agents, and proven ability to permeabilize the outer bacterial membrane. Their characteristic drug delivery functionality is achieved thanks to their very small size and enormous surface-to-volume ratio [19–25]. These substances are potentially safe and their surface can be further modified (e.g. dendrons, PEG chains) in order to increase the antimicrobial effectiveness [22,26,27]. In addition to modifying their surface, their antibacterial effectiveness can also be enhanced by combining their action with other antibacterial substances, such as enzymes (lysozyme or phage derived-endolysin) [21,28,29]. Antimicrobial enzymes such as lysozyme break down peptidoglycan and, as a result, kill the bacterial cell [30]. Lysozyme exerts an antimicrobial effect by destroying the bacterial cell wall. Lysozyme works by breaking the glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) molecules. Gram-negative (G-) bacteria are more resistant to lysozyme due to the presence of

an outer cell membrane. Bacteria are protected by a lipopolysaccharide layer, which act as a physical barrier and prevents muramidase from accessing the peptidoglycan found in the cell wall - the target of this enzyme molecule. Therefore, for such an enzyme to work, the outer bacterial membrane must first be damaged, which the enzyme itself cannot damage enough to lead to cell death. Next, when the pores in the membrane are formed, the enzyme is able to enter inside and kill the cell. Among the many studies on the antimicrobial properties of AgNPs conjugated with lysozyme [31–34], it is not easy to find the exact mechanism of interaction with the membrane, transport and the role of lysozyme in this case.

In order to create an effective tool for the transport of antibacterial proteins through the outer bacterial membrane, which will eventually lead to the death of the pathogen, it is first necessary to thoroughly understand the mechanism of this transport. Therefore, in this study, we examined the dual action mechanism of lysozyme combined with AgNP, modified with carbosilane (CBS) dendrons (Dend-AgNPs) and with CBS dendrons and PEG chains (PEG-Dend-AgNPs), to see if membrane permeabilization by these cationic AgNPs could form a pathway for lysozyme to reach and degrade peptidoglycan. This knowledge will aid in the development of a new drug delivery system using antimicrobial proteins.

2. MATERIALS AND METHODS

2.1 Materials

Dend-AgNPs and PEG-Dend-AgNPs were prepared following the methodology previously described [22,35]. The scheme of synthesis and AgNP structure is presented below (Figure 1A) Characteristics of NPs-lysozyme complex in supporting information (Figure S1 and S2). The lysozyme was ordered from Sigma Aldrich, Germany. Lysozyme concentrations were 7 and 37 μg / mL, which corresponds to the maximum capacity of adsorbed lysozyme on the surface of NPs; PEG-dendAgNPs and DendAgNPs respectively[27]. Strategy of transport of

lysozyme by NPs is presented in figure 1B and is further described in Results and discussion section.

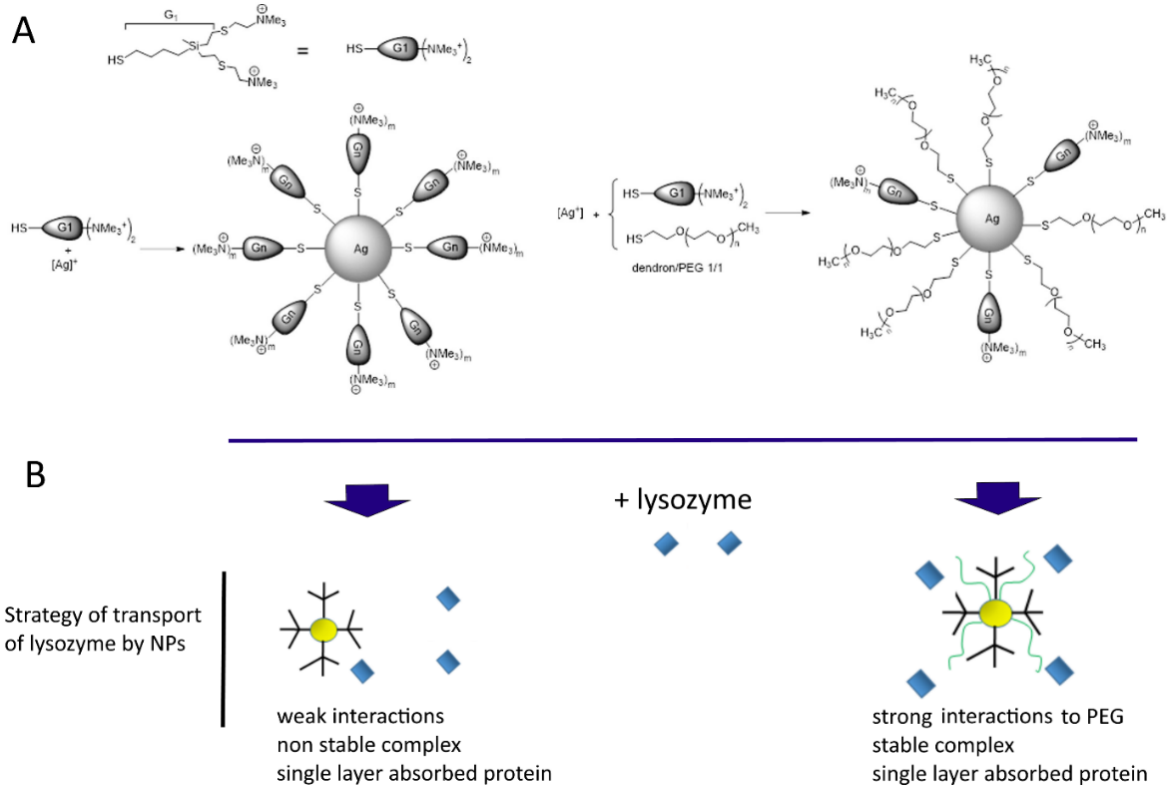

Figure 1. Drawing of first generation cationic CBS dendron and reaction schemes of AgNP synthesis (A); (left, DendAgNP; right, PEG-DendAgNP). (B) Strategy of transport of lysozyme by NPs to peptidoglycan degradation.

2.2 Antimicrobial activity assay

The antimicrobial activity of the peptidoglycan (PG) degrading enzyme in combination with dendrimers was tested using wild-type *P. aeruginosa* PAO1 and its wbpL mutant deficient in A-band biosynthesis and O-band antigen B, provided by Andrew M. Kropiński of the Laboratory of Foodborne Zoones, Guelph, Canada. The antimicrobial activity of dendronized AgNPs and lysozyme was measured on an exponentially growing culture of *P. aeruginosa* (OD 600) by spectrometry at a wavelength of 600 nm. Dendronized AgNPs alone (48 µg / ml,

chosen based on previously carried experiment[21]) and dendronized AgNPs (the same concentration) in combination with lysozyme (7 μg / ml and 37 μg / ml) were added and the effect was expressed as percentage growth of *P. aeruginosa* culture (OD600 optical density) compared to untreated bacterial culture (control) using a TECAN Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland). Experiments were repeated twice.

2.3 Permeabilization of outer bacterial membrane

The experiment was performed using the N-phenyl-1-naphthylamine (NPN) probe uptake assay. The bacteria for the experiment were grown at 37 ° C to obtain the appropriate optical density (OD = 0.5). The bacteria were then centrifuged to obtain pellets. The bacterial pellet was dissolved in Hepes buffer (correspondingly 1 ml of the buffer per 0.5 ml of bacteria before centrifugation) to obtain OD = 0.1. The measurement was performed in a sterile 96-well plate. The NPN fluorescent dye was added to the bacterial suspension on the plate (NPN concentration in the well 10 μM). Then, appropriate concentrations of two types of NPs and lysozyme were added to the mixture (final concentrations of NPs; 48 μg / ml, concentration of lysozyme; 7 μg / ml and 37 μg / ml). NPN fluorescence (emission = 420 nm, excitation = 350 nm) was immediately recorded on a TECAN Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland).

2.4 Determination of Reactive Oxygen Species (ROS)

For this assay, one milliliter of bacteria (10^6 cells/mL) obtained in the exponential growth phase was washed with PBS and resuspended in 1 mL of 10 mM phosphate buffer. The mixtures were treated or not treated with DendAgNPs, DendAgNPs with lysozyme 7 μg/mL, DendAgNPs with lysozyme 37 μg/mL, PEG-dendAgNPs, PEG-dendAgNPs with lysozyme 7 μg/mL, PEG-dendAgNPs with lysozyme 37 μg/mL, H_2O_2 (0.5 mM), lysozyme 7 μg/mL and 37 μg/mL for 15 min at room temperature. After that, CM-H2DCFDA (1 μM) was added. Subsequently, 100 μL of the bacteria were transferred to black 96-well microplates, and the

fluorescence was measured every 5 min using a TECAN Spark Fluorometer with 485/535 nm excitation/emission wavelengths.

2.5 Interaction of NPs with the membrane model (liposomes).

2.5.1 Liposomes preparation. The liposomes used are prepared with 65% DOPE, 23% DOPG and 12% cardiolipin. DOPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, 90% regular lipid, 10% cyanin 5 labeled lipid, Cy5) are neutral phospholipids, DOPG (1,2-dipalmitoyl-sn-glycero-3 [phospho-rac- (1-glycerol)) are negative modulators of phospholipids and cardiolipins outer membrane charge. The appropriate amounts of both lipids and cardiolipin (DOPE [labeled] - 48 mg, DOPG - 18 mg, CL- 0.8 mg) were weighed and added to the flask and then made up with solvent (CH_3Cl/CH_3OH = 70/30-1 ml . Then the solvent was evaporated on a vacuum evaporator so that only the lipid film on the sides of the flask was left. The lipids were dissolved by adding 1ml PBS (Phosphate-Buffered Saline) to the flask. An extruder was used to produce liposomes (diameter of obtained liposomes – 100 nm). After the liposomes were obtained, they were dialyzed.

2.5.2 Fluorescence quenching of liposomes. The experiment was performed on a TECAN Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland), at the wavelengths; emission 640 nm, excitation 670 nm. The liposome fluorescence quenching experiment was performed on a 96-well microplate. Two types of nanoparticles (PEG-dendAgNPs, Dend-AgNPs) and liposomes at concentration 10 mg/mL were used. The measurements were made of the use of different temperatures to determine the thermodynamic constants of the reactions (temperatures used: 298 K, 301 K, 304 K, 307 K 309 K). Interference between fluorescent signals from NPs and from used dyes was not observed.

2.6 Interaction of NPs with the membrane model (liposomes) in presence of lysozyme.

Liposomes were prepared as described previously, but without Cy-5 labeled lipid. Before experiments, dendronized AgNPs were encapsulated with carboxyfluoresceine (CF) and

dialyzed overnight. The appropriate amount of powdered carboxyfluorescein (16.4 mg) was weighed, then supplemented with 1 ml of PBS and dissolved. The fluorescence intensity was measured before and after the addition of the appropriate amount of AgNPs. The final concentration of CF was 4 mM. Next, appropriate concentration of lysozyme was added and fluorescence intensity was measured. The experiment was performed on a TECAN Spark microplate reader (Tecan Group Ltd., Switzerland), at the wavelengths; emission 497 nm, excitation 517 nm. Interference between fluorescent signals from NPs and from used dyes was not observed.

2.7 TEM (Transmission Electron Microscopy**) microscopy. Morphology of bacteria in presence of NPs**

The potential influence of tested nanoparticles carried lyzosyme on *Pseudomonas* cells morphology was verified by transmission electron microscopy (TEM) (Tecnai G2 Spirit, FEI Company). The concentration of AgNPs and lysozyme was the same as was described previously. The resulting mixture was placed on a carbon-coated 200-mesh copper grid (Ted Pella, Inc). Samples were negatively stained with 2% (w/v) uranyl acetate. Magnification of 4000 was used to examine bacteria morphology.

2.8 Fluorescence microscopy. Labeling peptidoglycan with HADA.

In order to verify the interaction of AgNPs and lysozyme with the outer bacterial membrane and the penetration into these substances, fluorescence microscopy and labeled peptidoglycan of the tested bacteria were examined. The bacteria *P. aeruginosa* (PAO1), PEG-dendAgNPs, Dend-AgNPs and lysozyme were used for the experiment. To prepare the bacteria for the experiment, the PAO1 strain was grown overnight on a shaker (37° C) to aerate the culture. The overnight culture was then diluted with TSB medium and grown until an OD600 of 0.5 was reached, then diluted to OD = 0.1. The HADA tag (propyl (2E) -2-cyano-3- (3,4-dihydroxy-5-nitrophenyl) prop-2-enoate) was then prepared following the product guidelines.

Powdered HADA was dissolved in DMSO (dimethyl sulfoxide). The resulting HADA concentration was 100 mM. The marker was added to the bacterial culture (in the case of control, the marker was added to the bacteria: 0.3 ml of culture + 3 µl HADA, in the case of samples containing AgNPs, the elements were added in the following order: 0.3 ml bacteria + 1 µl NPs + 3 µl HADA, in for samples containing lysozyme: 0.3 ml of bacteria + 1 µl of NPs + 1 µl of + 3 µl of HADA, then all samples were placed on a shaker (37° C) for 5 minutes. In the next step, 0.7 ml of ice-cold ethanol was added to the samples (to enhance the permeabilization effect in the case of NPs samples and as a control to allow the marker to penetrate the peptidoglycan) and left on ice for 1 hour. After the specified time has elapsed, the bacteria were centrifuged and the supernatant removed. 1 ml of frozen PBS (Phosphate Buffer Saline) was added to the pellet and pipette, centrifuged again and supernatant was removed again to wash away residual ethanol and unbound HADA. At the end, frozen PBS (200 µl) were added to the pellet to resuspended. The resulting peptidoglycan-labeled bacterial solution was dripped onto a glass slide and covered with a coverslip. The experiment was carried out in 6 variants: control (bacteria only), bacteria + lysozyme, bacteria + PEG-dendAgNPs, bacteria + DendAgNPs, bacteria + PEG-dendAgNPs + lysozyme, bacteria + DendAgNPs + lysozyme. The visualization was performed using a fluorescence microscope (Zeiss Axio Scope.A1) and the ZEN microscopic image analysis program. O filtrach I dlugosciach fali

3. RESULTS AND DISCUSION

3.1 Antimicrobial activity of DendAgNPs and PEG-dendAgNPs carried lysozyme

We first assessed the antibacterial activity of dendAgNPs and PEG-dendAgNPs complexed with lysozyme (at concentration of 37 µg/mL, which correspond to minimal concentration of adsorbed protein onto AgNPs surface, see supporting information and [27]) against the Gram-

negative bacteria *P. aeruginosa*. Two strains of bacteria were used; wild type PAO1 and WBPL mutant deficient in biosynthesis of A-band and B-band O-antigens. Remarkably, both cationic dendronized AgNPs exhibited antibacterial activity against both strains bacteria with MIC values above 96 µg/mL, what suggest moderate antibacterial activity. However, adding lysozyme to both tested AgNPs shifted these values below 96 µg/mL. One can observed significant decrease of OD 600 when lysozyme is complexed to dendronized AgNPs at concentration 15, 24 and 48 µg/mL in case of dendAgNPs and 48 µg/mL in case of PEG-dendAgNPs (Supporting information, figure S3 and S4). Noteworthy, adding lysozyme reduced concentration of AgNPs from 4 to 2 times when compared with non complexed AgNPs.

Our finding of the different antibacterial activities shown by the AgNPs could be ascribed to the different surface charge and surface modification, different possibilities of absorption and transport of lysozyme and thereby distinct interactions with bacteria (Figure 2A). We hypothesize that cationic dendronized AgNPs are able to damage the outer bacterial membrane and thus create a path for an antimicrobial protein (e.g. lysozyme) to degrade peptidoglycan, thereby killing multi-drug resistant bacteria.

To analyze the mechanism of antibacterial properties of these AgNPs carrying lysozyme we chose concentration of both AgNPs; 48 µg/mL where additional lysozyme significantly increase antibacterial activity. To be sure that even lower concentration of lysozyme can be efficiently transported to increase antibacterial activity, we decreased also concentration of lysozyme more than 4 times, to 7 µg/mL (Figure 2B). With this lower concentration of lysozyme, lower activity of dendAgNPs against PAO1 strain was detected. Interestingly, in the case of PEG-denAgNPs, the addition of lower concentration of protein showed higher antibacterial effect (decrease of OD values) when compared to higher concentration of lysozime. PEG-dendAgNPs bearing not only cationic terminals and but also PEG chains offer

more place for negative charged lysozyme to be absorbed onto surface or attaching to PEG chains than non PEGylated AgNPs. This discovery could highlight different properties of surface-modified AgNPs that will be further exploited for the development of new antimicrobial agents that transport antimicrobial proteins. Very similar observation can be found for second used strain WBPL, what make suggest that Lipopolysaccharide as a hindrance has to play less role (supporting information, figure S3 and S4).

It has been known that cationic nanoparticles may permeabilize outer bacteria membrane, based on electrostatic interaction between positively charged NPs and negatively charged membrane [36–39]. Moreover, in the case of metal nanoparticles additional effect of induction of reactive oxygen species (ROS) due to metal ions play crucial role in antibacterial effectiveness [40–42]. Therefore next we check the level of ROS induction in time in presence of AgNPs alone and AgNPs/lysozyme complexes in tested bacteria. Here, we saw higher increase of ROS in time in PAO1 strain (Figure 2C) for dendAgNPs and for PEG-dendAgNPs compared to untreated control. The higher level of ROS was observed for non Pegylated one (dendAgNPs). It is worthy to report that even higher induction of ROSs was visible in case of AgNPs transporting lysozyme at higher concentration of protein and little lower for lower concentration of protein. This may suggest that possible permeabilization may increase number of lysozyme which reach peptidoglycan, degrade it and as result make more AgNPs penetrate into bacteria and induce more ROSs. Similar results were obtained in case of mutant strain (Supporting information, figure S5).

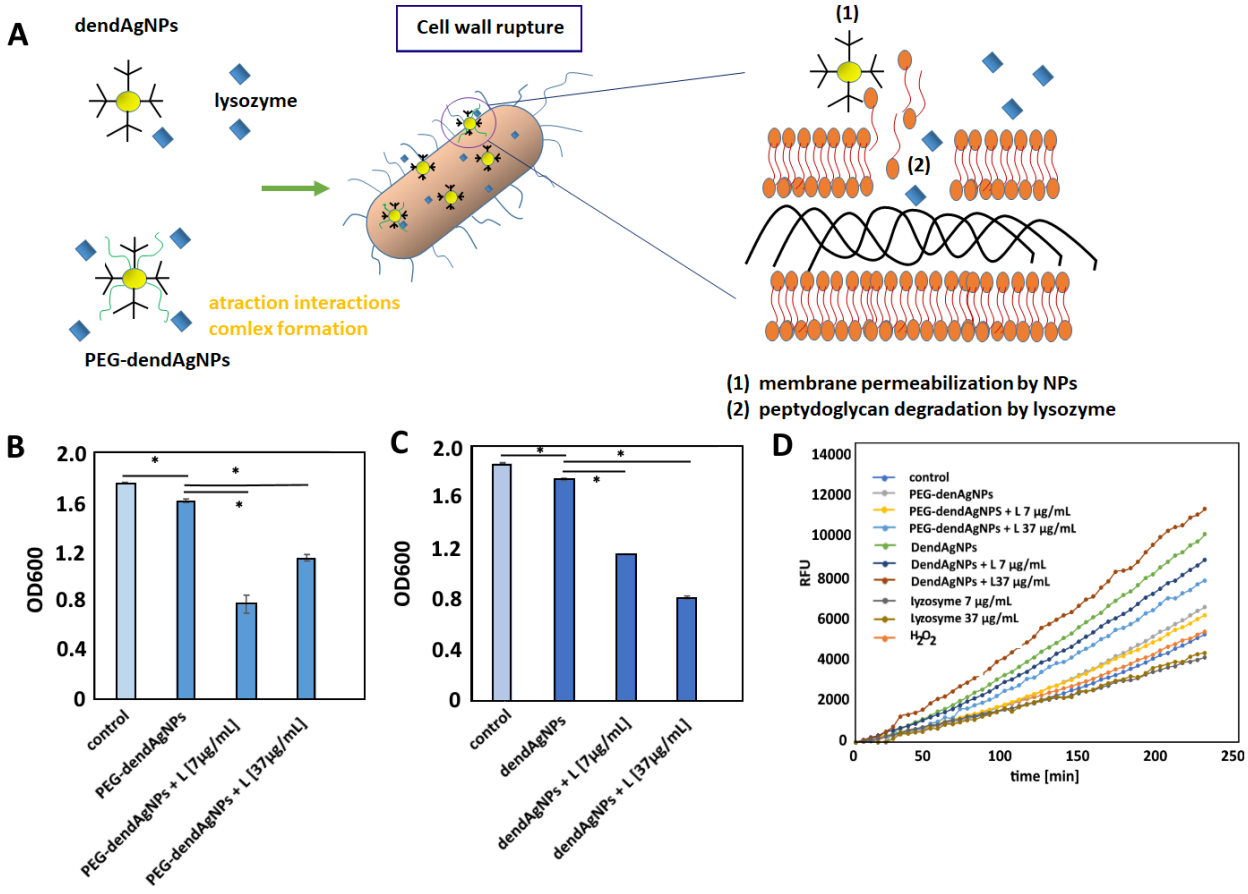

Figure 2. (A) Possible mechanism of permeabilization and peptidoglycan degradation by dendronized AgNPs complexed with lysozyme. (B and C) The optical density of PAO1 wild-type and its mutant (1wbpL) measured at 600 nm without and in the presence of dendronized AgNPs (concentration 45µg/mL) and AgNPs (the same concentrations) combined with lysozyme at two indicated concentrations. DendAgNPs means unmodified nanoparticles with PEG. Culture growth for 24 h at 37°C. (D) Influence of DendAgNPs and PEG-dendAgNPs in absence or presence of lysozyme on intracellular ROS generation. The presence of ROS in PAO1 strain (10^6 CFU/mL) was studied by measuring the fluorescence of CM-H2DCFDA after 15 min of treatment. RFU = Relative Fluorescence Units.

3.2 Permeabilization of outer bacteria membrane (*P. aeruginosa*)

Encourage by significantly better antibacterial activity of AgNPs/lysozyme complexes, we next check the mechanism of action, therefore we further evaluated the permeabilization

properties of tested dendronized AgNPs and lysozyme. 1-N-phenylalanine, a hydrophobic probe, is normally excluded from interaction with an intact G- bacterial membrane (signal is small). Increased uptake of 1-N-phenylalanine fluorescence occurs in samples of bacterial suspensions in which the membrane is damaged and does not function properly. Based on this knowledge, the uptake of 1-N-phenylalanine in samples treated with NPs and lysozyme at various concentrations (7 µg / ml and 37 µg / ml) was investigated for two *P. aeruginosa* strains; PAO1 (Figure 3) and WbpL (supporting information, figure S6). The entire experiment was run for 30 minutes and measurements were taken every minute.

The PAO1 strain treated with dendronized AgNPs without and with lysozyme demonstrated higher fluorescence values compare to the control. The best permeabilization effect was obtained for the sample containing only AgNPs. The results in the samples containing lysozyme showed lower values of fluorescence, respectively, together with the addition of a lower concentration of lysozyme (7µg /mL) we see a decrease in the value of fluorescence compared to the sample without enzyme, while in the sample with the highest concentration of enzyme (37µg /mL) the fluorescence values are the lowest. The explanation of this phenomena is that excess lysozyme may block AgNPs from accessing the surface of the bacteria. The noticeable decrease in the obtained fluorescence values obtained in our results can also be explained by the type of the surface charge of the enzyme. Lysozyme is positively charged, so electrostatic interactions may also occur between the enzyme particles and the bacterial membrane. Hypothetically, the more enzyme particles in the tested sample, the more they prevent AgNPs, also positively charged, from accessing the surface of the bacterial membrane, hence the decreasing fluorescence values along with the increasing concentration of lysozyme in the tested samples.

In the case of assays involving PEGylated AgNPs, Peg-dend-AgNPs, the results are quite different. All fluorescence values of the NPs/enzyme complexes assays turned out to be below

the control value. However, this does not necessarily mean that AgNPs do not affect and do not interact with bacteria membrane. Nazeer et al.[43] report that bacteria in the presence of extracellular polymers, i.e. polyethylene glycol (PEG), can aggregate, clump together in as a result of the osmotic imbalance caused by the PEG chains getting between the bacterial cells. Such a situation could result in the impossibility of the label (NPN) to penetrate into bacteria and disturbed fluorescence measurement, which would be observed as a result of the lack of permeabilization. Similar results were obtained for Wbpl strain (Supporting information, figure S6)

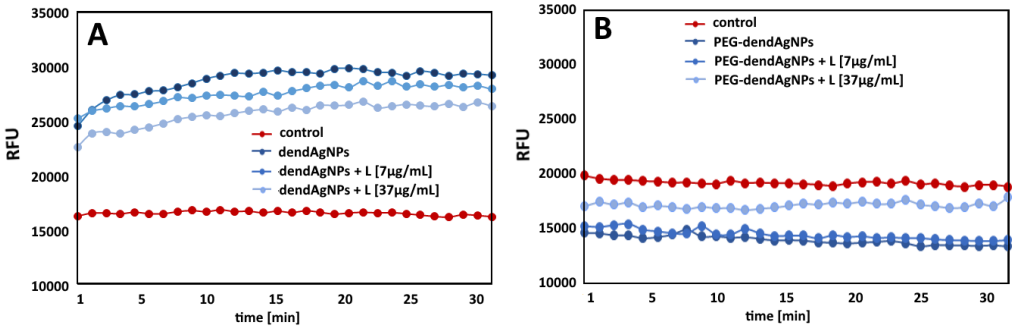

Figure 3. Assessing the permeability of the bacterial outer membrane. Permeability of the bacterial outer membrane in PAO1 strain (A) as evaluated using the N-phenyl-1-naphthylamine (NPN) dye after treatment with NPs without and with lysozyme.

3.3 Interaction of AgNPs and AgNPs/lysozyme complexes with the membrane model (liposomes).

It was previously reported that polycationic systems, e.g. dendrimers, may disrupt bacterial membrane by inducing pore formation via cooperative electrostatic interaction between the cationic dendrimer terminals and the anionic bacterial membrane surface[44]. Therefore, next step was to check whether there is a difference in interaction of tested AgNPs containing cationic CBS dendrons with different charge (zeta potential of DendAgNPs is 20 mV and 4 mV for PEG-dendAgNPs) with bacteria model membrane. This type of interaction between

NPs and model membrane was analyzed thermodynamically.The table 1 and table 2 present the thermodynamic parameters obtained from Stern-Volmer and Van't Hoff analysis (see also supporting information, Figure S7). The type of interaction between AgNPs and fluorescently labeled liposomes can be analyzed by Stern-Volmer analysis, where increase or decrease Stern-Volmer constant (K_{sv}) obtained at different temperatures indicate static or dynamic type of interactions (the fluorescent quenching analysis is presented in supporting information).

In the case of DendAgNPs, it was observed a decrease of K_{sv} with increasing temperature, which suggests static interaction and formation of a stable complex between AgNPs and membrane. Decrease of binding constant K_b, negative enthalpy ΔH^0, the negative values of ΔG^0, and negative entropy ΔS, indicate an exothermic reaction between DendAgNPs and liposomes, which is spontaneous at low temperature. In the case of PEGylated PEG-dendAgNPs, is easy visible increase of K_{sv} what indicate a dynamic type of interaction (less stable complex). Increase of binding constant K_b, positive enthalpy ΔH^0 ,the negative values of ΔG^0, and positive entropy ΔS. Here, this indicate an endothermic reaction, which is spontaneous at higher temperature.

All presented thermodynamic parameters clearly indicate a higher affinity of non PEGylated DendAgNPs to membrane compare with PEGylated PEG-dendAgNPs. The obtained results may confirm that DendAgNPs, due to higher affinity to membrane, has more possibility to damage bacteria membrane (permeabilization and ROS induction) and then transport lysozyme through membrane more effectively.

Next, we tested whether complexed lysozyme may affect the interaction of NPs with membrane. Due to high influence and interference of lysozyme with signal of Cy-5 phospholipids, we decide to use unlabeled liposomes in this experiment. First, AgNPs were encapsulated with Carboxyfluorescein (CF). It is known that CF may be encapsulated into hydrophobic cavities as those presented in CBS dendrons and thus decrease of fluorescence of

CF[45] (Figure 4). While AgNPs attached to membrane the some numbers of CF released from cavities what lead to increase of fluorescence of CF (Figure 4A in case of DendAgNPs and figure 4C in case of PEG-dendAgNPs). Adding DendAgNPs/lysozyme complex (encapsulated with dye) to liposomes showed increase of fluorescence (release of CF from NP's cavities). Surpassingly, adding PEGylated PEG-dendAgNPs/lysozyme complexes showed no visible increase of fluorescence, that is, no release of CF was produced. The explanation for this could be that lysozyme was attached to PEG covered AgNPs surface avoiding the interaction with membrane. This result is in agreement with those obtained from permeabilization and ROS induction experiments. However, it has to be highlighted that even if the interaction of this complex is weak and problematic with the membrane, PEGylated AgNPs/lysozyme complexes are still effective against bacteria comparably to non PEGylated ones, although mechanism of action is completely different.

Table 1.

Stern-Volmer binding parameters of interactions between dendronized AgNPs (Dend-AgNPs, PEG-Dend-AgNPs) and liposomes.

T (K)	DendAgNPs + Liposomes	PEG-DendAgNPs + Liposomes
	K_{sv} (10^6 mol/L)	
298	2.28	3.36
301	1.99	3.37
304	1.81	3.86
307	2.03	4.01
309	1.42	4.27

Table 2.

Thermodynamic parameters of liposomes-AgNPs interactions at different temperatures obtained from Van't Hoff analysis .

T (K)	K_b (L/mol)	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)	ΔS (J/mol)
DendAgNPs + Liposomes				
298	3.65	-56.57	-2549	-181.29

301	1.6		-2005	
304	1.2		-1461	
307	2.5		-917	
309	0.99		-373	
PEG-DendAgNPs + Liposomes				
298	5.39	22.49	-4126	89.32
301	5.60		-4394	
304	6.41		-4662	
307	6.85		-4930	
309	7.57		-5198	

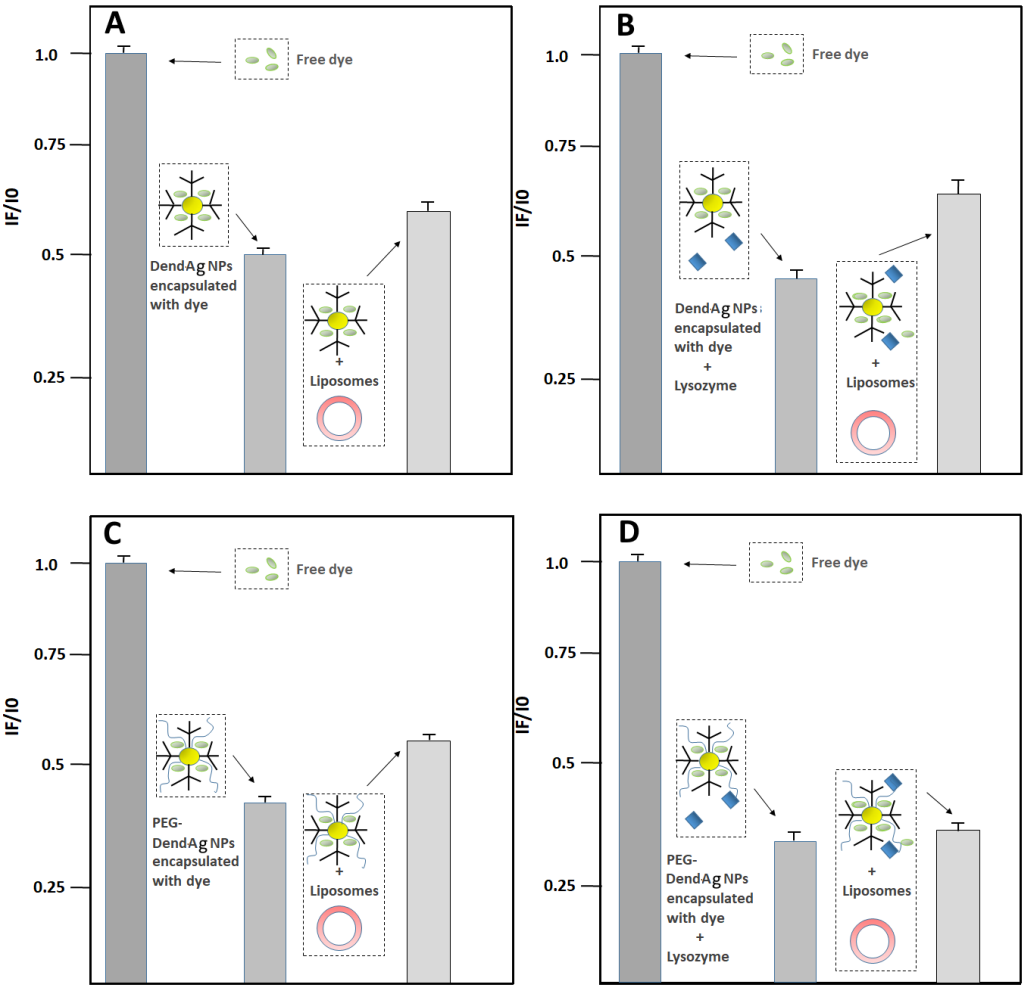

Figure 4. The interaction of CF encapsulated AgNPs with liposomes, complexed with lysozyme (B and D) or without it (A and C). The initial fluorescence come from uncapsulated CF in solution. Encapsulation of CF into hydrophobic cavities inside CBS dendrons attached to AgNPs surface lead to decrease of fluorescence.

3.4 Interaction of NPs and NPs carried lysozyme with the bacteria. Damage of outer membrane and peptidoglycan.

To find clues about the possible effects in bacteria; damage of bacteria membrane and peptidoglycan degradation, of AgNPs and lysozyme, TEM and an experiment with labeled peptidoglycan (HADA tag) were performed on *P. aeruginosa* (PAO1) strain exposed to various research variants of PEGylated and Dendronized AgNPs and lysozyme. First, TEM imaging was performed to check bacteria morphology in presence of AgNPs and lysozyme (Figure 5). In the case of the untreated control (Figure 5A), the bacterial cells showed normal morphological structure, smoothness and a compact surface, without the release of intracellular components. No noticeable pores or cracks. When the bacteria were treated with lysozyme only (Figure 5B), no significant changes in the morphology of the bacterial cell were observed. The next pictures show bacterial cells treated with DendAgNPs (Figure 5C), DendAgNPs/lysozyme complexes (Figure 5D), PEG-dendAgNPs (Figure 5E) or PEG-dendAgNPs/lysozyme complexes (Figure 5F). On the surface of the bacteria, we can observe small black points, these corresponds with AgNPs accumulation on the outer surface of the bacterial membrane. Numerous leakages of cellular contents, ruptures of membranes and cell disintegration can be seen on the surface of the bacteria in presence of DendAgNPs complexed with lysozyme. The weaker effect, but still the same behavior was visible in case of PEG-dendAgNPs complexed with lysozyme. Cells treated with AgNPs and the enzyme demonstrated destruction of the bacterial membrane and leakage of cellular components. In the case of samples treated with PEGylated AgNPs (NPs only and in the presence of lysozyme), less damage to the cellular structures is noticeable, but bacterial cells are sticking together. This could explain the results obtained in the permeabilization experiment with PEGylated AgNPs, which showed that there was no ongoing permeabilization process by these AgNPs due to aggregation of bacterial cells. They may result from the depleted

interactions caused by extracellular polymers (in our case PEG chains) involved in bacterial aggregation[46].

To support the hypothesis of damage to the bacterial outer membrane by AgNPs and penetration by lysozyme of the bacterial cell wall, fluorescence microscopy imaging of bacteria with labeled peptidoglycan (HADA) was performed. The control sample (Figure 5G) shows morphologically normal cells with an undisturbed surface. There are no cracks or unevenness in it. After treating the bacteria with DendAgNPs (Figure 5H), one can see unevenness, pits, and cell ruptures. A lot of fragmented cells can be noticed in the preparation, which proves the complete disintegration of peptydoglycan. After adding lysozyme (Figure 5I) to the system, we also see numerous cavities and irregularities on the surface of the bacteria as well as torn cells and their fragments (the bacteria peptidoglycan was not visible in blue filter, the left bacteria were visible when green filter was applied). Due to the weak effect of permeabilization of outer membrane by PEG-dendAgNPs, we show only results with non PEGylated AgNPs.

TEM analysis and experiment with peptidoglycan-labeled *P. aeruginosa* bacteria treated with AgNPs and lysozyme showed penetration of the bacteria by NPs and their attachment to the bacterial surface eventually leading to morphological changes such as pore formation and cell breakage. The tests containing dendronized NPs and the same NPs with lysozyme showed the best bacterial killing activity. With PEGylated NPs, the effect is weaker, which may be due to the aggregation of bacterial cells in the presence of PEG chains.

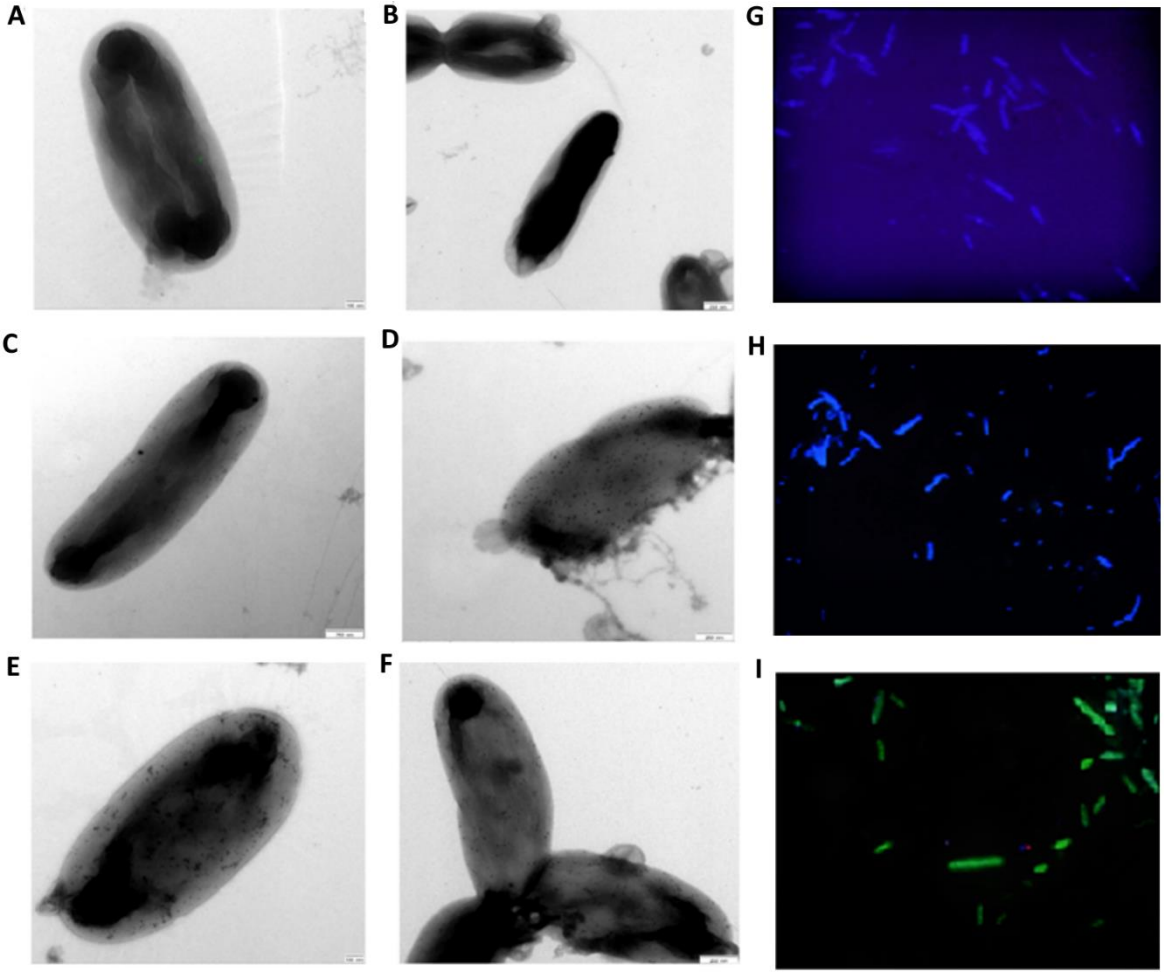

Figure 5. Transmission electron microscopy (TEM) images of P. aeruginosa after treatment with AgNPs and lysozyme: (A) control, (B) Bacteria + lysozyme (7 µg/mL), (C) bacteria + DendAgNPs, (D) Bacteria + DendAgNPs + lysozyme (7 µg/mL), (E) Bacteria + PEG-DendAgNPs, (F) Bacteria + PEG-DendAgNPs + lysozyme (7 µg/mL), and photos taken fluorescence microscope of bacteria labeled with peptidoglycan (HADA); (G) Control, (H) Bacteria + DendAgNPs, (I) Bacteria + DendAgNPs + lysozyme (7 µg/ml).

4. CONCLUSION

The problem of drug resistance is an increasingly serious concern in the international arena. In the particular case of gram-negative bacteria, their outer membrane is extremely difficult to be overcome by commercially available agents. This is forcing scientists around the world to look for alternatives to this problem. The alternative presented in our research may be the

combined action of dendronized AgNPs with antimicrobial proteins such as lysozyme. In this article, we present two strategies for the transport of antimicrobial proteins carried by dendritic nanoparticles through the outer bacterial membrane of Pseudomonas aeruginosa bacteria in order to allow the proteins to access the peptidoglycan and, consequently, to degrade it (Figure 6). We tested the transport of lysozyme via two types of silver nanoparticles; Dend-AgNPs and PEG-Dend-AgNPs.

Studies have shown that in both variants we are dealing with a completely different type of interaction, and consequently with different interactions with bacteria. In the first case, the combination of Dend-AgNPs with the peptidoglycan-degrading enzyme resulted in a strong membrane permeabilization, which allowed lysozyme to penetrate inside the bacterial cell and destroy it. AgNPs deposits on the surface of the bacteria create pores in the bacterial membrane and, consequently, allow the enzyme to enter inside the bacteria, resulting in its death. It appears that Dend-AgNPs have a greater affinity for the bacterial membrane than PEG-Dend-AgNPs, which results in better permeabilization of the bacterial membrane for the first type of AgNPs. In most of our experiments, the addition of lysozyme to AgNPs improved the antimicrobial activity. However, it may happen that too much lysozyme in the sample provoke a decrease in antibacterial activity. This is due to the fact that the enzyme, like AgNPs, are positively charged, and the negatively charged membrane is attractive to the enzyme, which means that it blocks access to the AgNPs membrane, which prevents its damage. When PEGylated PEG-Dend-AgNPs were used as carrier for the enzyme, we obtained different results. PEG-Dend-AgNPs caused bacterial cells to stick together and aggregate, making the permeabilization effect very weak. We obtained greater antimicrobial activity with lower or no lysozyme content in the sample. This may be due to the surface charges of these substances. Positively charged lysozyme can bind to negatively charged PEG chains, thus forming a complex that may prevent NPs from properly interacting with the

bacterial membrane. However, a strong inhibition of the growth of bacterial cells treated with PEG-Dend-AgNPs was noted. This may be due to the local accumulation of lysozyme at the surface of the bacteria. AgNPs disrupt the bacterial membrane, thanks to which the enzyme is able to penetrate its interior. Understanding the exact mechanisms of our strategies is crucial to thoroughly understanding the antimicrobial principle of the systems we propose. In the future, they can be used to create new, more effective methods of fighting drug resistance, such as better drug carriers.

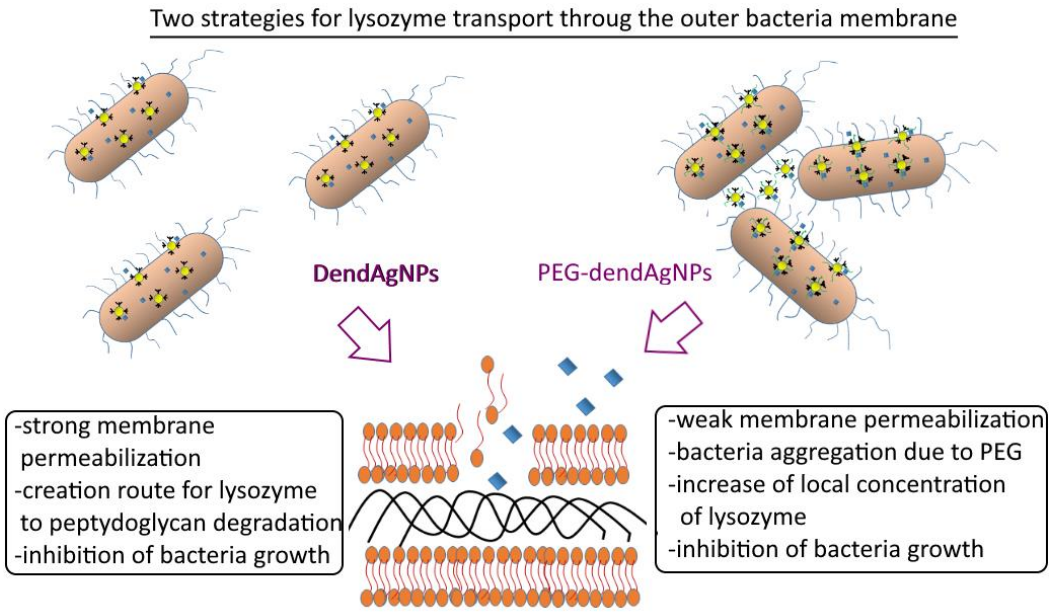

Figure 6. Cartoon illustration of the antibacterial activity shown by dendronized AgNPs complexed with lysozyme via membrane adsorption and disruption and peptidoglycan degradation by lysozyme.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Kinga Skrzyniarz; Conceptualization; Data curation; Formal analysis; Methodology; Visualization; Roles/Writing - original draft,

Javier Sanchez- Nieves; Methodology; Writing - review & editing; **F. Javier de la Mata**; Writing - review & editing, **Małgorzata Łysek-Gładysinka**; Methodology; Software; Visualization, **Karol Ciepluch**; Conceptualization; Funding acquisition; Investigation; Project administration; Writing - review & editing

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Appendix A. Supplementary data

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