Nanotransformation of vancomycin overcomes the intrinsic resistance of Gram-negative bacteria

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Abstract

The increased emergence of antibiotic resistant bacteria is a growing public health concern, and although new drugs are constantly being sought, the pace of development is slow compared to the evolution and spread of multidrug resistant species. In this study, we developed a novel broad-spectrum antimicrobial agent by simply transforming vancomycin into nano-form using sonochemistry. Vancomycin is a glycopeptide antibiotic largely used for the treatment of infections caused by Gram-positive bacteria, but inefficient against Gram-negative species. The nanospherization extended its effect towards the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, making these bacteria up to 10 and 100 times, respectively, more sensitive to the antibiotic. The spheres were able to disrupt the outer membranes of these bacteria, overcoming their intrinsic resistance towards glycopeptides. The penetration of nanospheres into a Langmuir monolayer of bacterial membrane phospholipids confirmed the interaction of the nano-antibiotic with the membrane of *Escherichia coli* cells, affecting their physical integrity as further visualized by scanning electron microscopy. Such mechanism of antibacterial action is unlikely to induce mutations in the evolutionary conserved bacterial membrane therefore reducing the possibility of acquiring resistance. Our results indicated that the nanotransformation of vancomycin could overcome the inherent resistance of Gram-negative bacteria towards this antibiotic, and disrupt mature biofilms at antibacterial-effective concentrations.

Keywords: Vancomycin, sonochemistry, antibiotic nanospheres, antibiotic resistance, biofilms

Introduction

Bacterial infections caused by antibiotic resistant strains are one of the world's public health challenges,[1] thus the development of novel therapeutic approaches have been extensively researched. Special focus has been given to Gram-negative bacteria, which outer and inner membranes, porins and efflux pumps form a complex barrier system impermeable to many clinically relevant antibiotics. These inherent structural characteristics impart Gram-negative bacteria a first line of defense against bactericides.[2,3]

Vancomycin, a member of a class of several clinically used antibiotics including teicoplanin, dalbavancin, oritavancin, and telavancin, is a glycopeptide that is structurally unrelated to any available antibiotic. It inhibits bacterial cell wall synthesis through a five-hydrogen bond interaction between the heptapeptide backbone of the antibiotic and the C-terminal L-Lys-D-alanyl-D-alanine dipeptide, a component of bacterial peptidoglycan, extending from the cell wall.[4] This peptide is located in the periplasmic space, which is directly accessible to antibiotics in Gram-positive bacteria. By contrast, vancomycin is inefficient against Gram-negative bacteria due to its large molecular size and inability to penetrate the outer bacterial membrane, which makes the bacteria intrinsically resistant to vancomycin.

Nanotechnology has been considered as a novel tool for overcoming microbial resistance development. Liposomes, nanocapsules and particles are commonly investigated as drug carriers for delivering antimicrobial agents to the infection site, enhancing their clinical effectiveness.[5–7] However, the transformation of the antimicrobial agents themselves into nano-sized entities has been only sparsely reported.[8,9] The assemblies of the molecules at nanometer dimensions have unique properties, differing from those of the free molecules and the bulk materials with the same composition.[5] Nanotransformations of antimicrobial agents into nanospheres (NSs) have been carried out in our group using the ultrasonic emulsification method.[8,10,11] This approach drastically changes the physical properties of the sonochemically

processed compounds allowing for novel modes of action towards bacteria. Antibacterials processed into nano-sized particles are claimed to be more efficient than their non-processed counterparts.[5,6,8]

The exact mechanisms by which nanomaterials interact with bacteria are not fully understood, but it is thought that the nanoparticles cause an increase in the microbial cell membrane permeability, osmotic damage, and flow of cytoplasmic components out of the cell, eventually leading to cell death.[6] This mode of action reduces the possibility of developing new resistant strains, because bacterial membrane is highly evolutionarily conserved and therefore unlikely to be changed by a single gene mutation.[12] In addition, nanoparticulate systems are also able to tackle multiple biological pathways and many concurrent mutations should have to occur in bacteria in order to develop resistance.[5]

Besides avoiding the occurrence of antibiotic resistance, nanotechnology has been considered an efficient tool to control biofilm-associated infections.[13–16] In fact, the biofilm mode of growth is by itself a resistance mechanism that bacteria use to survive in harsh environments. Bacteria encased in biofilms are over 1000 times more resistant to antibiotics and host immune system than in culture.[17,18] Infections due to biofilms are very difficult to treat and account for the increased morbidity and mortality in hospitals.[19]

This work aims at developing vancomycin NSs with antibacterial efficiency against Gram-negative species. We hypothesized that sonochemical nano-spherization of vancomycin would extend its activity to Gram-negative bacteria, relying on bacterial membrane disturbance effect. We additionally expected to improve the activity of vancomycin towards bacteria embedded in biofilms, due to enhanced penetration of the NSs into the biofilm matrix. The single step nanotransformation process will save on time- and resource-consuming screening for new drug entities. Since the chemical structure and the target of the drug will not be altered by adding a new mechanism of activity, such structural engineering

rather than synthetic approach would shorten the validation process in terms of novel antibiotic development. The fact that only two new classes of antibiotics with limited activity against Gram-negative bacteria have been introduced into the clinic over the last two decades, supports the timeliness of this study.[20,21]

Results and discussion

Vancomycin exerts its antimicrobial activity against Gram-positive bacteria binding to a L-Lys-D-alanyl-D-alanine dipeptide present in the bacterial peptidoglycan layer and inhibiting the cell wall synthesis, which leads to bacterial lysis and death. However, the double membrane of the Gram-negative bacteria is impermeable to the large glycopeptide molecules of vancomycin, restricting their access to the targeted dipeptide and therefore neutralizing their antibacterial efficiency. Examples of vancomycin-insensitive bacteria are the *Escherichia coli (E. coli)* in urinary tract infections, and the *Pseudomonas aeruginosa* (*P. aeruginosa*) in bloodstream infections and pneumonia.[22] These clinically relevant Gram-negative species together with the Gram-positive *S. aureus* were first tested for susceptibility to the non-processed vancomycin. The Gram-negative bacteria showed to be insensitive even to high vancomycin concentrations, while *S. aureus* was fully eradicated at antibiotic dosage above 15 μg/mL ($O.D._{600} \approx 0$ in Fig. 1). Aiming at extending the activity spectrum of vancomycin also to Gram-negative bacteria, the free drug was sonochemically processed into NSs.

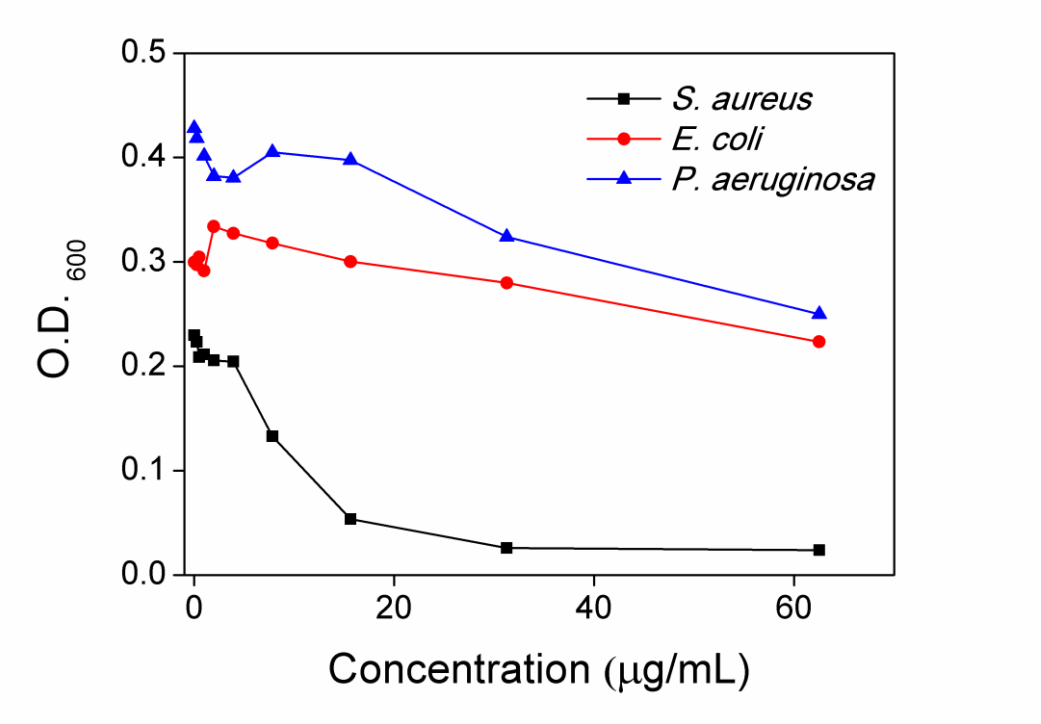

Figure 1. *P. aeruginosa*, *E. coli* and *S. aureus* growth assessed at optical density at 600 nm (O.D. $_{600}$) after 24 h incubation with vancomycin in solution (0 – 62.5 µg/mL).

We previously applied this nano-formulation approach to boost the antibacterial activity of biopolymers such as aminocellulose and thiolated chitosan as well as other antibiotics such as penicillin.[8,11,23,24] Nanoparticle tracking analysis (NTA) of the sonochemically generated control NSs (without antibiotic) and vancomycin NSs revealed average particle sizes of 115 ± 2 nm and 147 ± 5 nm, respectively (Fig. S2). The nanosized vancomycin spheres were further observed by transmission electron microscopy (TEM) (Fig. 2). Zeta potential measurements confirmed a stable suspension of negatively charged spheres (- 50 ± 0.5 mV), owing to the use of a non-ionic surfactant (poloxamer 407) in the process of spheres generation. This was further evidenced by the long-term physical stability of the vancomycin NSs, which did not aggregate in solution for more than 6 months stored at 4 ºC.

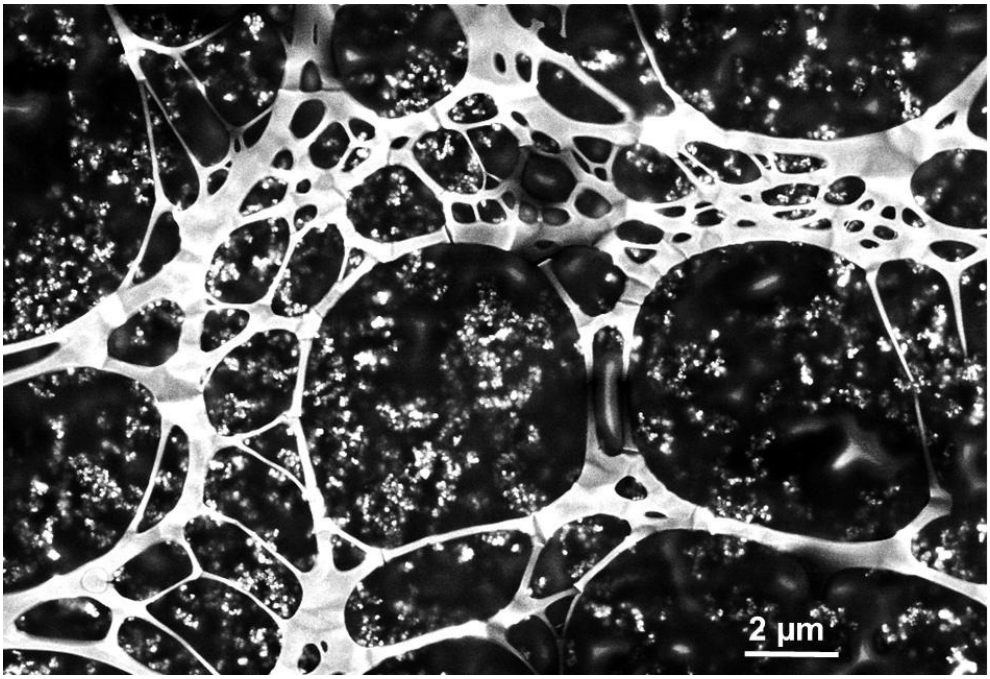

Figure 2. TEM image of vancomycin NSs generated in presence of 0.1 % poloxamer 407.

In order to assess the amount of the encapsulated vancomycin, the NSs were separated from the bulk solution by ultracentrifugation, and both supernatant and NSs were analyzed by HPLC (Fig. S1). The difference between the antibiotic concentration in the supernatant and the solution prior to the sonication revealed that only 15 % of vancomycin was transformed into NSs (Table S1). However, the amount of vancomycin quantified after NSs demulsification (disassembling with ethanol) was 2-fold lower (7.5 ± 0.8 %) probably because of the affinity partitioning of the antibiotic into the hydrophobic vegetable oil phase of the biphasic system.[25] Most likely, part of vancomycin was retained in the NS shell readily acting on bacteria, while the remaining antibiotic formed the sphere core, being inaccessible to bacteria at first, but gradually released over time.

We further investigated whether the conversion of vancomycin into NSs was accompanied by any structural or chemical changes. FTIR spectra were acquired for both vancomycin and vancomycin NSs, as well as for the oil and surfactant used to form the NSs (Fig. 3). The

spectra did not revealed any changes in the chemical structure of vancomycin upon spherization. The new peak appearing at 1750 cm^{-1} corresponds to the presence of oil (inset in Fig. 3), whereas the increase of the broad band at 3500 cm^{-1} was assigned to hydrogen bonding effect due to the stretching of the hydroxyl groups in vancomycin. Therefore, we may conclude that the sonochemically-induced formation of vancomycin NSs led to the creation of a hydrogen bond network contributing to the stabilization of the spheres. Similar effect has been observed processing sonochemically penicillin NSs and tetracycline nanoparticles. [9,11]

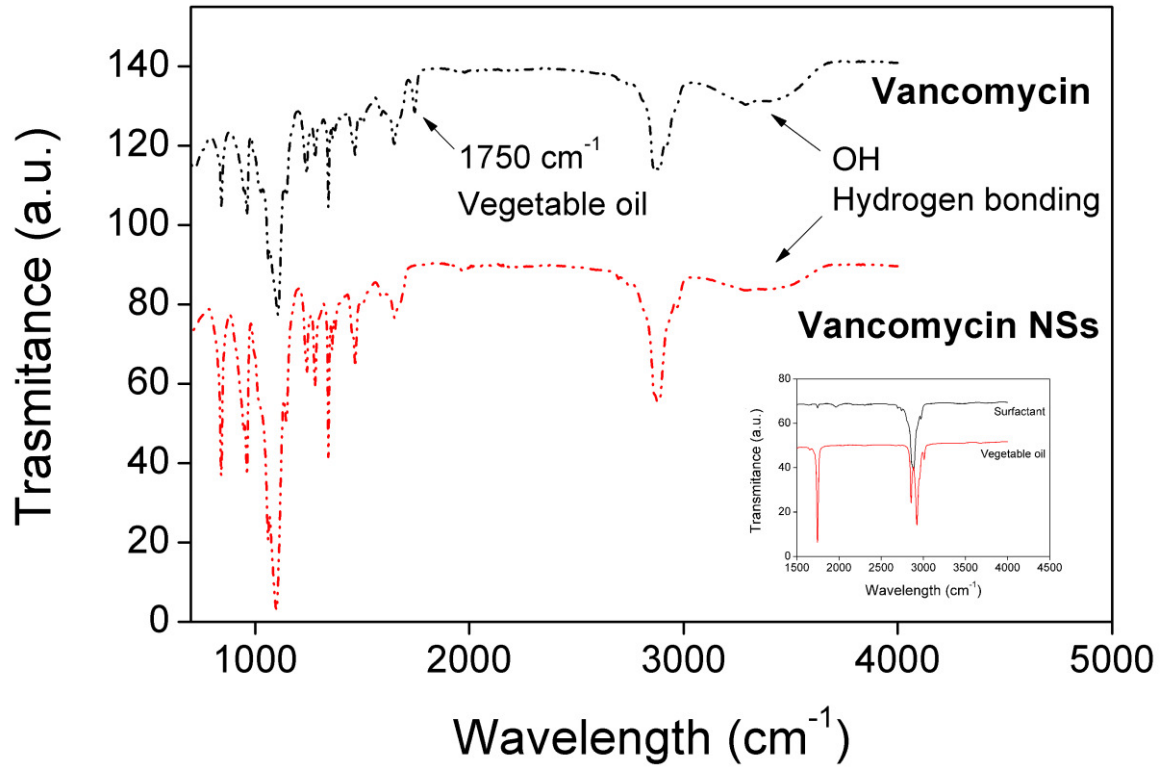

Figure 3. FTIR spectra of lyophilized vancomycin, vancomycin NSs, surfactant (poloxamer 407) and vegetable oil.

Cationic nanoparticles are reported to interact with negatively charged microbial membranes through electrostatic interactions causing membrane disruption and cell leakage, an effect that

we have observed for other positively charged NSs,[8] while negatively charged particles penetrate the cells through nonspecific binding and clustering on the cationic sites of the membrane followed by internalization via endocytosis.[26] The interaction of our negatively charged spheres with a membrane model built with phosphatidylethanolamine (PE), a major constituent phospholipid from *E. coli,*[27] was studied by Langmuir monolayer technique. The Langmuir technique is widely used for building monolayers mimicking natural membranes and provides information about the interactions between structural lipids and different molecules or nanoparticulate systems, without considering other constituents of the cell membrane.[8,28]

The isotherms of PE using as a subphase water or vancomycin solution presented a lift-off area at ca. 100 $Å^2$/molecule, whereas the presence of NSs (both control and vancomycin NSs) increased this molecular area to around 110 $Å^2$/molecule for the control NSs and 165 $Å^2$/molecule for the vancomycin NSs. This behavior indicates that PE forms stable monolayers, and the presence of vancomycin in solution does not induce any disturbing effect on the bacterial mimetic membrane. By contrast, the NSs in the subphase significantly expanded the PE monolayers, indicating their strong membrane disturbing effect, especially when vancomycin was included in the NSs (Fig. 4 a).

After compression, at surface pressure higher than ca. 45 mN/m, the PE isotherms with control and vancomycin NSs in the subphase nearly overlap with the isotherm with only water in the subphase. Although the slope of these isotherms was still different, this behavior indicates that, at high surface pressures, part of the NSs are expelled from the monolayer down to the solution.[29] However, at the characteristic surface pressure of natural bacterial cell membranes (33 mN/m),[30] the PE monolayer with a subphase containing vancomycin NSs was still expanded when compared to the PE isotherm with vancomycin in solution, control NSs and water in the subphase (Fig. 4 a), suggesting that these NSs induce a strong disturbing

effect on the membrane that may result from their protruding in between the phopholipid molecules at the air-water interface.[8,11]

To corroborate these results, control NSs, vancomycin NSs and vancomycin in solution were injected into the subphase beneath the PE monolayer, previously formed at 33 mN/m, and the increase on the surface pressure was recorded with time (Fig. 4 c). The increase of the surface pressure as a function of time demonstrated the membrane disrupting effect of both vancomycin NSs and control NSs while no effect was observed when the subphase contained vancomycin in solution. The vancomycin NSs, however, induced a higher surface pressure increase when compared to the control NSs, accounting for their membrane disturbing effect.

Analyzing the compressibility modulus (C_s^{-1}) calculated from the slope of the π-A isotherm in Fig. 4 a, we intended to obtain information about the phase transitions occurring in the monolayer in presence of NSs (Fig. 4 b). The isotherms reached a liquid condensed state (LC) when the subphase contained either water or vancomycin in solution. On the contrary, the NSs (control and vancomycin NSs) induced two physical states: liquid expanded (LE) and LC, being LC less compact than the one observed with water and vancomycin in solution. Therefore, this indicated that NSs avoided the packing of the phospholipid molecules in the membrane, reducing the C_s^{-1} maximum value and avoiding the formation of highly compact states, thereby conferring fluidity to the membrane (Fig. 4 b).[29] These results confirmed one more time the capacity of vancomycin NSs to disturb the bacterial membrane model.

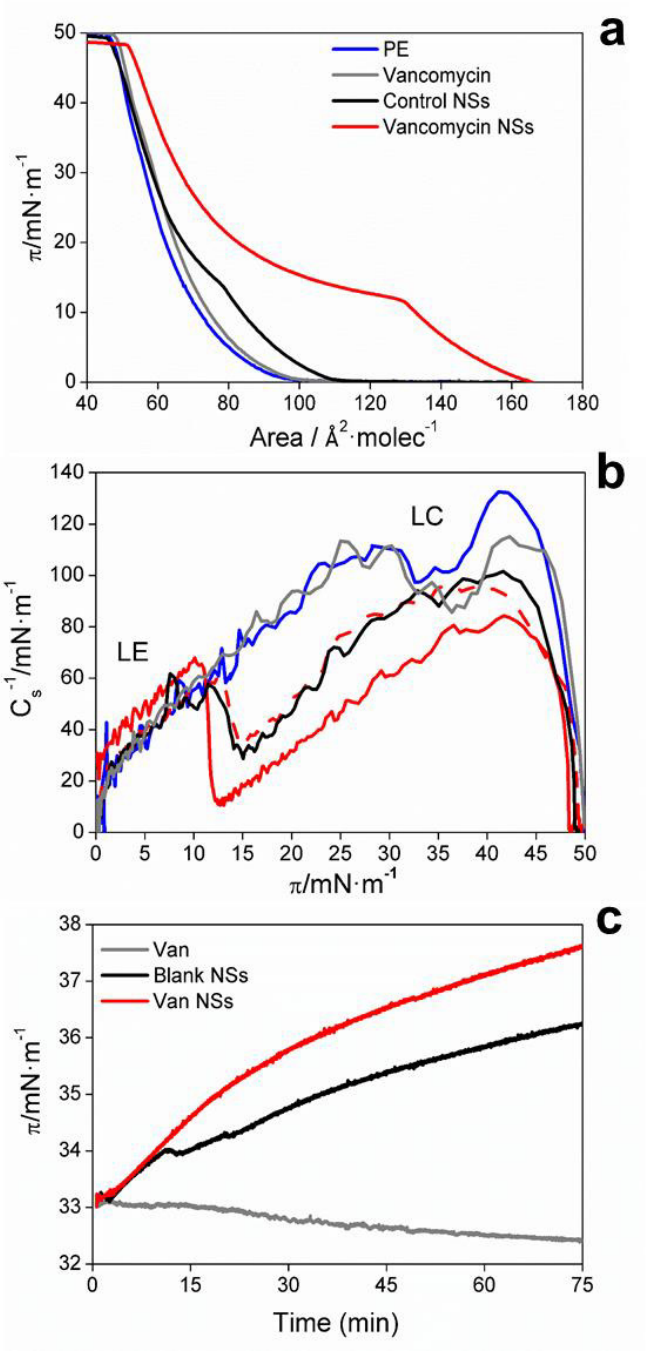

Figure 4. Surface pressure-area isotherms of a PE monolayer (the area per molecule in X-axe refers to *E. coli* PE) in water with vancomycin (2.5 µg/mL), vancomycin NSs and control NSs (2.1 ± 0.1 x10^8 NSs/mL) (a), and respective compression modulus (b). (c) Kinetic adsorption process resulting from the incorporation of free vancomycin, vancomycin NSs and control NSs into the air/water interface of the PE monolayer at a surface pressure of 33 mN/m.

In order to validate the disturbing effect of the vancomycin NSs on the PE membrane model, the Langmuir-Blodgett (LB) films were transferred to mica and the interaction between the NSs and the membrane model was verified using atomic force microscopy (AFM) (Fig. 5 a). AFM maps the profile/height of the monolayer surface, which is altered by the presence of NSs underneath. The light and dark shades in the AFM images correspond to different heights of the monolayer surface. At low surface pressures, the PE with water in the subphase formed a uniform and compact monolayer with no relevant structures observed (Fig. 5 a, left image), while PE with vancomycin NSs in the subphase produced bright round patches (Fig. 5 a, right image), which indicates that the integrity of the membrane was affected by the spheres. The round patches mean that the PE monolayer is in a LC state, whereas the rest of the lipid molecules are in a more fluid (LE) state, corresponding to the dark shade in the AFM image. Thus, the surface profile of the PE monolayer confirmed its disturbance by the vancomycin NSs.

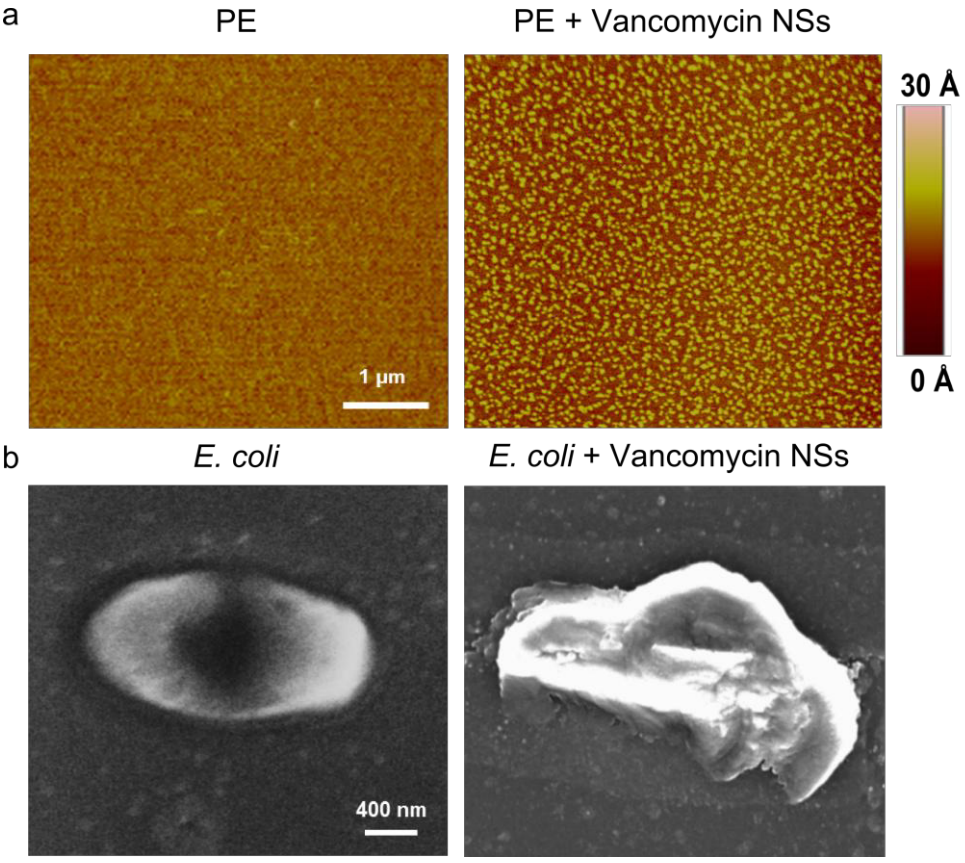

Figure 5. Effect of vancomycin NSs on a mimetic membrane and *E. coli* bacterial cells. a) AFM topographic images of LB film before (left) and after treatment with vancomycin NSs (right). The LB monolayers were transferred on mica at 23 ºC at π = 5 mN/m. b) SEM images of *E. coli* bacterium in absence (left) and presence of vancomycin NSs (right).

To further corroborate the membrane disturbing effect of the NSs demonstrated in Fig. 4 and 5a the vancomycin NSs were incubated with *E. coli* and the changes in cell morphology were visualized using scanning electron microscopy (SEM) (Fig 5 b). The non-treated *E. coli* cells were intact with a typical rod like shape, smooth surface and size about 0.8 μm in width by 1.5 μm in length (Fig. 5 b, left image), while significant morphological changes were observed after incubation with vancomycin NSs (Fig. 5 b, right image). The altered cell morphology (Fig. 5 b) indicates cell damage, which eventually led to leakage of cytoplasmic contents and cellular death.

Proven the fact that the NSs of vancomycin were able to disturb the biomimetic membrane and alter the morphology of bacterial cells, we further analyzed the antimicrobial and antibiofilm activities of the NSs towards two Gram-negative species. The vancomycin NSs induced a 2.8 log reduction of *P. aeruginosa* (**$P \leq 0.01$) and 1.9 log reduction of *E. coli* (*$P < 0.05$) growth, while vancomycin in solution (using the same dilution factor) caused less than 0.8 log reduction of both microorganisms (Fig. 6). A 4- and 3-fold improvement, respectively, was attained in the inhibition of these planktonic Gram-negative bacteria. It is worth mentioning that the nanotransformation of vancomycin plays a key role in the inhibition of bacterial growth, considering the low concentration of antibiotic in the spheres.

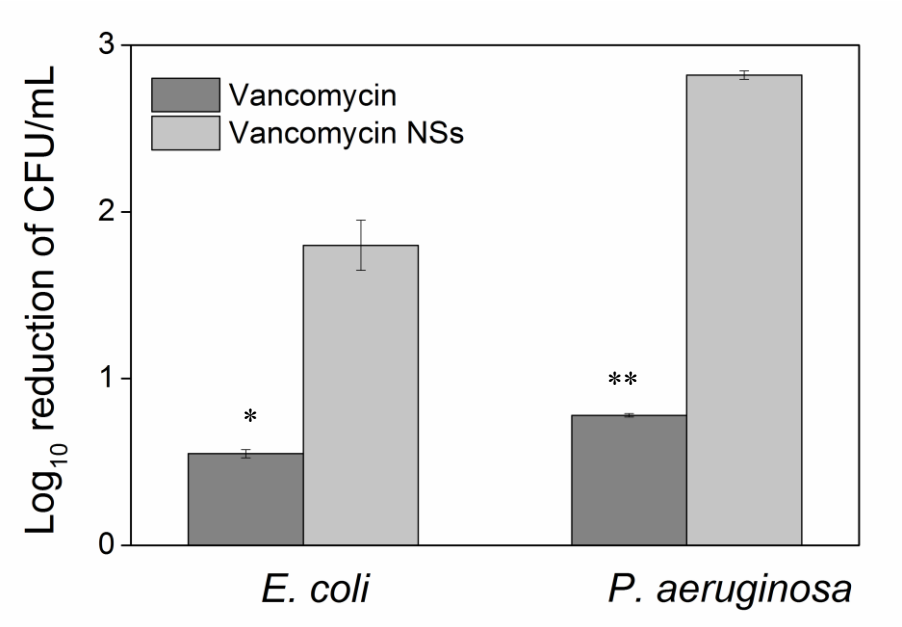

Figure 6. Antimicrobial activity of vancomycin in solution (125 µg/mL) and in the form of NSs (≈ 2.1 ± 0.1 x 10^{13} NSs/mL) on *E. coli* and *P. aeruginosa* after 24 h of contact. The error bars indicate the standard deviation of three independent experiments and statistical significance was calculated using two-tailed Student's t Test. *P < 0.05 and **P ≤ 0.01 vancomycin vs vancomycin NSs.

In general, the antibacterial effect of nanostructures depends on various parameters including their size, shape, surface charge and the method of synthesis. The electrostatic attraction between positively charged molecules and negatively charged bacterial cell membrane is thought to predominate over the other parameters in the antibacterial efficacy of the particles. Carboxyl, amide, phosphate and hydroxyl groups, and carbohydrate moieties present in the cell wall of bacteria provide sites for interactions with cationic NSs.[31] Similarly, the positively charged PE, present in the outer membrane of Gram-negative bacteria cells is the component providing reactive sites for interaction with the negatively charged vancomycin NSs. Bacterial cell lysis may be induced by clustering on the cationic sites of the membrane followed by internalization via endocytosis or membrane disruption, due to an increased antibiotic

concentration at the site of bacteria. Such effect has been reported when a combination of nanoparticles and antibiotics was applied.[26] Recent studies have also shown that combining nanoparticles with antibiotics not only reduced the toxicity of both agents towards human cells by decreasing the requirement for high dosages, but also enhanced their bactericidal properties. The nanoparticle ability to destroy antibiotic-resistant bacteria has also been shown.[26] In our study, the enhanced killing effect was due to the nanotransformation of the antibiotic itself, instead of its encapsulation, thereby combining both bactericidal and nanoscale effects in a single construct. Therefore, we believe that nonspecific binding and diffusion as well as membrane damage, govern the antibacterial efficiency of the vancomycin NSs against Gram-negative bacteria.

In a next step, we evaluated the effect of the nanoformulated vancomycin on biofilms of Gram-negative bacteria. Vancomycin either in solution (125 μg/mL) or in the form of NSs (≈ $1.05 \pm 0.06 \times 10^{13}$ NSs/mL) was applied on mature biofilms and the effect on the cells viability was assessed by fluorescence microscopy after Live/Dead staining (Fig. 7). The biofilm disruption was more pronounced upon treatment with the antibiotic NSs, and in the case of *P. aeruginosa,* a large number of red stained cells, i.e. dead bacteria, was observed. The control NSs without antibiotic neither decreased the biomass nor killed the *E. coli* confirming that the nanoparticulate form alone is not able to eradicate the biofilm form of the bacterium. On the other hand, the red stained cells detected in the *P. aeruginosa* biofilm, treated with control and vancomycin NSs, may result either from antibacterial effect due to membrane disturbance, or from staining of the extracellular DNA component of *P. aeruginosa* biofilm by the propidium iodide dye used for cells viability assessment.[32]

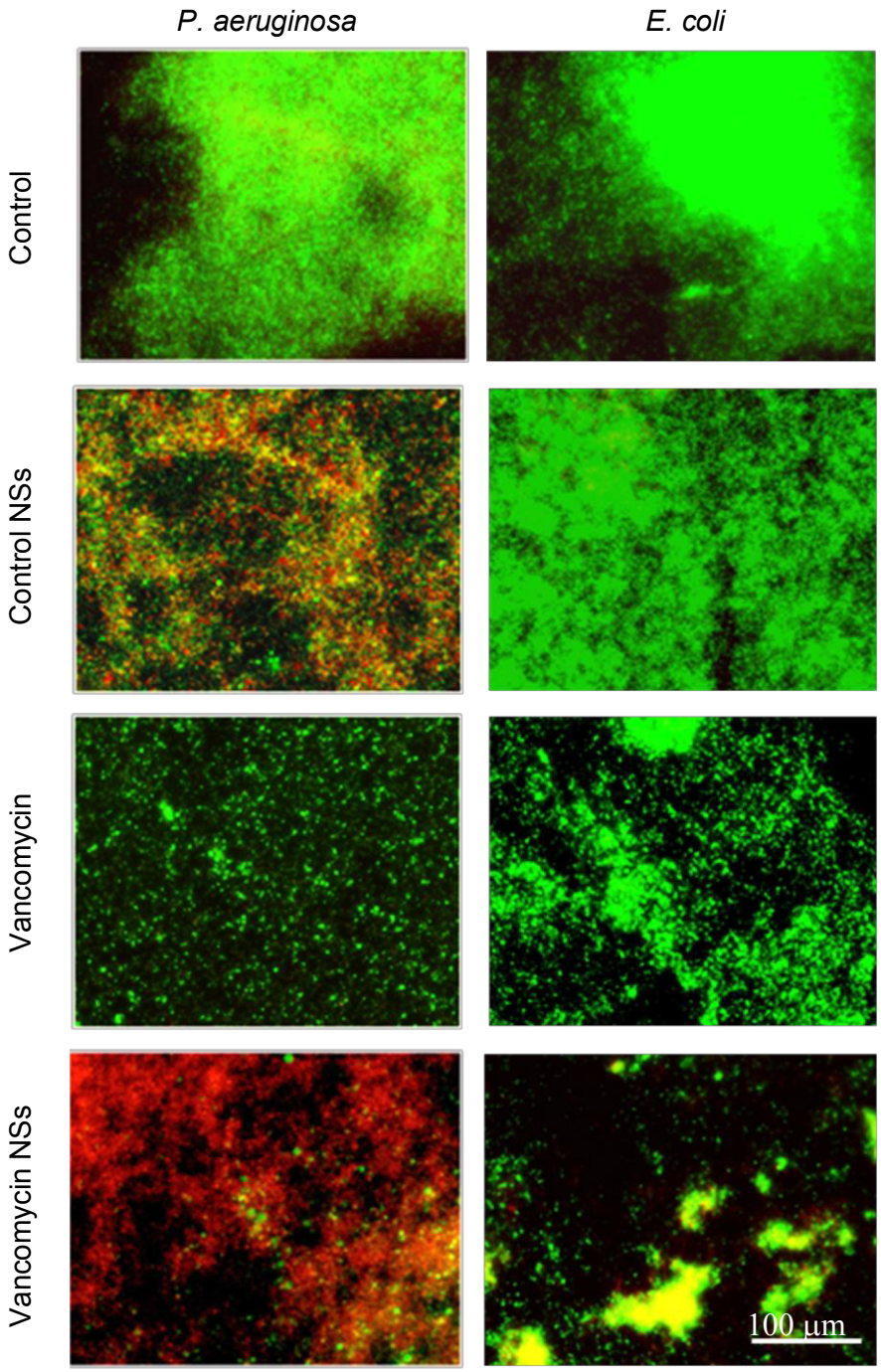

Figure 7. Fluorescence microscopy images of *P. aeruginosa* and *E. coli* biofilms after 24 h treatment with vancomycin (125 µg/mL), vancomycin NSs and control NSs (≈ 2.1 ± 0.1 x 10^{13} NSs/mL). Live cells are represented in green and dead cells in red.

It was, therefore, necessary to quantify the susceptibility of bacteria within biofilms through enumeration of the viable microorganisms using a CBD-MBEC™ assay, and confirm the

ability of vancomycin NSs to kill biofilm growing *P. aeruginosa*, as visualized by fluorescence microscopy. CBD-MBEC™ technique allows microorganisms to grow and form biofilms on 96 identical pegs protruding down from a plastic lid. Placing the grown biofilm in contact with the antimicrobial compounds and further plating the cells on specific agar makes possible to quantitatively evaluate the reduction of the biofilm-encased bacteria. The vancomycin NSs significantly decreased *P. aeruginosa* (**P < 0.01) and *E. coli* (*P < 0.05) cells within the biofilm when compared to vancomycin in its bulk form. *P. aeruginosa* was at least 10 times (ca. 1 log reduction) more susceptible to the antibiotic NSs than *E. coli* (Fig. 8). In contrast, we did not observe any decrease of the live bacterial cells in the biofilm upon the application of vancomycin solution, corroborating the results found by fluorescence microscopy and antibacterial assays (Fig. 6 and 7).

Expectedly, the activity of vancomycin NSs on the sessile bacteria was lower than on the planktonic cells because of the increased resistance provided by the build-up of the biofilm matrix. Besides the conventional resistance mechanisms, the biofilm-grown bacterial cells express properties distinct from the planktonic cells that play important role in their resistance to the most common antimicrobials.[18] Our NSs do not possess hydrolytic activity to degrade the biofilm matrix components, e.g. extracellular polysaccharides, but they were able to eradicate *P. aeruginosa* and *E. coli* cells in the mature biofilms. This effect could be attributed to the nano-form of the antibiotic and its enhanced penetration through the biofilm to reach the bacteria embedded therein. The interaction between the biofilms and NSs is a complex interplay of particles characteristics, such as charge and size, and biofilm features. It is possible that the negatively charged vancomycin NSs interact electrostatically with oppositely charged matrix molecules and attach to the biofilm structure.[33] Once NSs are adsorbed on the biofilm they may diffuse deeper into it or accumulate within the matrix as a consequence of the active outgrowth of the biofilm, forming new layers above the NSs.

The obtained antibiotic NSs could be further used in either free form or engineered as coatings for controlling bacterial infections and the emergence of drug resistance. They could be exploited in a wide range of biomedical applications, such as antibacterial and anti-biofilm indwelling medical devices and implants, hospital textiles, skin care products for treatment of skin infections, and wound dressings.

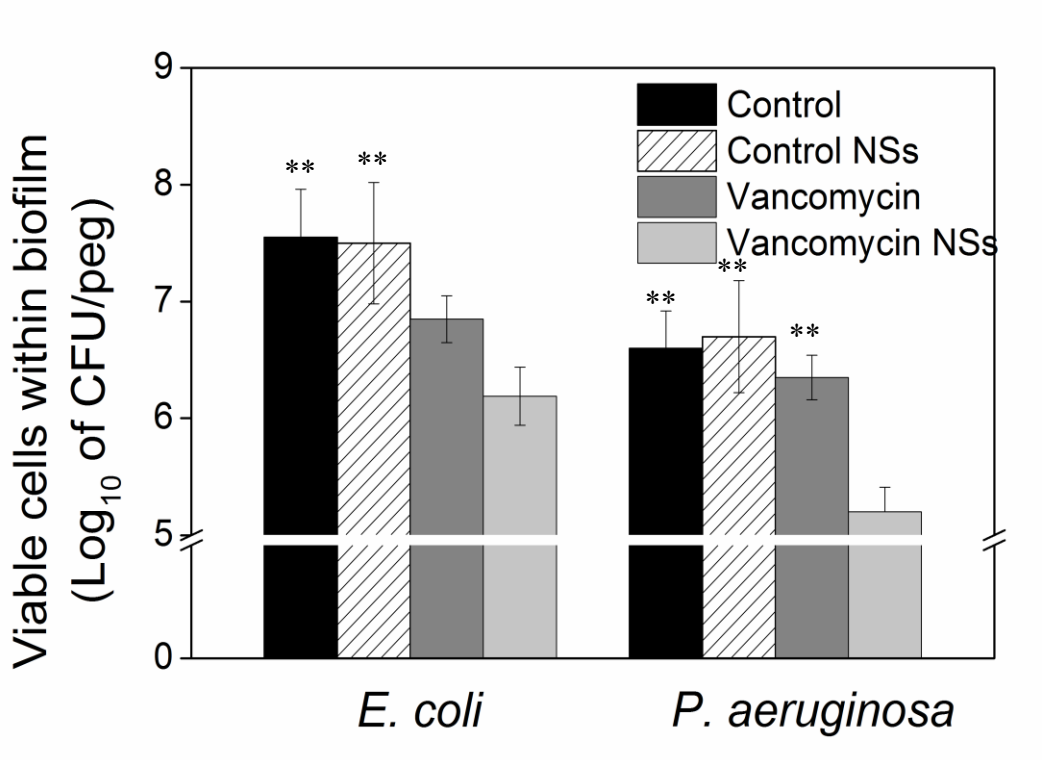

Figure 8. Viable cells within *P. aeruginosa* and *E. coli* biofilms after applying of vancomycin in solution (125 μg/mL) vancomycin NSs and control NSs. *E. coli* and *P. aeruginosa* control samples (black bars) correspond to the growth of bacteria in biofilm form (i.e. viable cells) in absence of vancomycin and vancomycin/control NSs. The error bars indicate the standard deviation of three independent experiments and statistical significance was calculated using one-way ANOVA. **P < 0.01 when compared to vancomycin NSs.

Conclusions

The accelerated emergence of drug resistant bacterial strains and their ability to form biofilms

are one of the most serious problems of modern medicine and the difficulties in finding alternatives make it even more challenging. The advent of nanotechnology has allowed for significant advancement in this field. The current work demonstrated that the nanospherization could boost the activity of vancomycin towards Gram-negative bacteria. These species became up to 100 times more sensitive to the drug in planktonic culture, and 10 times more susceptible in the form of biofilm. The proposed antibiotic engineering approach allowed extending the antibacterial spectrum of vancomycin towards Gram-negative species relying on a mechanism of action that comprises mechanical interaction of the NSs with the bacterial membrane in addition to the specific antibiotic activity. Such mechanism of membrane disturbance is thought to reduce the possibility of resistance emergence in bacterial pathogens according to the literature evidences. The outcomes of our study could be further valorized, having in mind that the main reason for bacterial resistance is the overuse of antibiotics, while the NSs carry only 15 % from the tested vancomycin in solution. Low antibiotic dosage is posing less evolutionary stress on bacteria. Thus, the concept of nanoscale formulation of clinically validated antibiotics could constitute an important, yet simple tool for treatment of infections caused by antibiotic-resistant pathogens.

Materials and methods

Materials

The bacterial strains: *Staphylococcus aureus* (*S. aureus* ATCC 25923), *Escherichia coli* (*E. coli,* ATCC 25922) and *Pseudomonas aeruginosa* (*P. aeruginosa,* ATCC 10145) were obtained from American Type Culture Collection (ATCC LGC Standards, Spain). L-α-phosphatidylethanolamine (PE) from *E. coli*, was purchased from Avanti Polar Lipids, Inc.

(Alabama, USA). Vancomycin hydrochloride hydrate (Sigma reference: 861987) was purchase from Sigma-Aldrich (Spain) as well as all other reagents, and used without further purification. Ultrapure MilliQ water with a resistivity of 18.2 MΩ cm was used in the experiments.

Sonochemical preparation of vancomycin nanospheres

Vancomycin NSs were prepared by an adaptation of the Suslick method.[34] Briefly, a two-phase solution containing 70 % of 0.25 g/L antibiotic and 0.1 % (w/v) poloxamer 407 (aqueous phase), and 30 % of commercial sun-flower oil (organic phase) was prepared and placed into a thermostated sonicator cell (8 ºC ± 1 ºC). The same biphasic system was used for the preparation of control poloxamer NSs, comprised solely of surfactant and oil, but without vancomycin, in order to determine the effect of the surfactant and spherical shape on the interaction with the membrane model and bacteria. The NSs were synthesized with a high-intensity Vibra-Cell VCX 750 ultrasonic processor (Sonics & Materials, Inc., USA) using 20 kHz Ti horn at 35% amplitude. The bottom of the probe was positioned at the aqueous-organic interface, employing an acoustic power of ~ 0.5 W/cm^3 for 3 min using an ice-cooling bath. The resulted NSs dispersion was purified by three consecutive centrifugations at 1500 rpm for 15 min, for the removal of the organic phase, followed by an ultracentrifugation at 14 000 rpm for 45 min and re-suspension in water for separation of the NSs from the solution.

Nanospheres characterization

NTA was used to determine the size of the NSs and their number per mL. NTA was performed in flow mode with a NanoSight LM10 instrument (Malvern Instruments Inc., UK) equipped with LM10 Laser 405 nm nanoparticle viewing unit and sCMOS camera. NTA Software NTA 3.0 was used to capture several frames of the NSs suspension and correlate the

particle size with the sample concentration. Results are displayed as distribution plots of concentration (NSs/mL) vs. particle size. The reported average particle sizes represent the mean values ± standard deviations of five measurements per sample. The determination of zeta potential was carried out using a Zetasizer Nano Z (Malvern Instruments Inc., UK) after appropriate dilution of NSs using MilliQ water. The shape and morphology of NSs was visualized with Zeiss Neon FIB microscope (Carl Zeiss, Germany) in STEM mode operating at 30 kV acceleration voltage. The samples for characterization were drop-casted on a TEM holey carbon grid. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) using a Spectrum 100 FT-IR spectrometer (Perkin Elmer, USA) was used to determine the structural changes in vancomycin before and after sonication. Drops of vancomycin NSs, vancomycin in solution as well as the control NSs, oil or surfactant (powder) were placed in contact with FTIR sampling accessory. The spectra were obtained in the range of 4000–625 cm^{-1} performing 64 scans at 4 cm^{-1} resolution and the data were analyzed using essential eFTIR-3.00.019 software.

Vancomycin concentration in the nanospheres

The amount of vancomycin transformed into NSs was determined by high-performance liquid chromatography (HPLC) as previously described.[35] The analysis was performed using an Agilent Technologies 1200 HPLC system, consisting of a degasser, a quaternary pump, and an automatic sample injector. The system also includes a diode array detector for recording the absorption spectra of the samples. A Microsorb-MV C-18 reserved-phase column (150 x 4.6 mm, 5 µm particle size) was used to perform the chromatographic separation. The flow rate was 1 mL/min and the vancomycin was detected at 214 nm. Gradient elution was carried out using solvent A (0.1 % v/v trifluoroacetic acid (TFA) and a solvent B containing acetonitrile and 0.1 % TFA (95 : 5 v/v). During the analysis, the solvent gradient was

programmed from 10 % to 100 % B in A in 30 min. With column cleaning and equilibrium steps, the total run time for each sample was 40 min. Agilent ChemStation software was used for data acquisition and processing. Calibration curves were obtained by plotting the peak area of vancomycin against its concentration. No interfering peaks were found in the chromatographic separation at the retention time. To determine the vancomycin concentration, the NSs were separated from the suspension by ultracentrifugation (14000 rpm for 45 min). Then, the supernatant (containing non-encapsulated vancomycin and small size NSs) was diluted in MilliQ water and introduced in the HPLC system. The vancomycin content in the NSs was also evaluated. The NSs were demulsified with known volumes of absolute ethanol (EtOH). After EtOH evaporation, the solutions were centrifuged again and the aqueous phase containing the antibiotic was subjected to HPLC analysis.

Antibacterial and antibiofilm properties

Susceptibility to antibiotics. The susceptibility of bacteria to antibiotics in bulk form was determined by the broth microdilution method, according to the NCCLS 2003 standard.[36] At first, 190 µL of antibiotics solution prepared at different concentrations (0 - 80 µg/mL) in nutrient broth (NB) medium (Sharlab, Spain) were placed in 96-well plate. Then, 10 µL inoculum of *P. aeruginosa*, *E. coli* and *S. aureus* (concentration of $\approx 5 \times 10^5$ CFU/mL) were added and the samples were incubated at 37 °C overnight with shaking. At the end of incubation, the optical density (O.D.) at 600 nm was recorded and the susceptibility of the bacterial strains evaluated. All the experiments were performed in triplicate.

Planktonic growth inhibition. The inhibition of *P. aeruginosa* and *E. coli* growth by vancomycin in both bulk and nanoform was further evaluated. Bacteria were grown overnight and then diluted to an O.D. = 0.01 at 600 nm (concentration of $\approx 5 \times 10^5$ CFU/mL) in NB medium. Thereafter, 200 µL of the antimicrobial agents, vancomycin at 125 µg/mL or

vancomycin NSs at 2.1 ± 0.1 x 10^{13} NSs/mL (corresponding to the same dilution factor applied to the vancomycin in solution), and 200 µL of bacterial inoculum of *P. aeruginosa* or *E. coli* were mixed in test tubes. The tubes were further incubated at 37 ºC and 230 rpm for 24 h. After 24 h the suspensions were serially diluted in sterile buffer solution (0.3 mM KH_2PO_4), plated on appropriate plate count agar and further incubated at 37 ºC for 24 h to determine the number of viable bacterial cells. The bacterial reduction (log_{10} reduction) was calculated using the following formula:

Bacterial log_{10} reduction = log_{10} (CFU/mL control sample) – log_{10} (remaining CFU/mL after exposure to specific antimicrobial agent). All antibacterial data represent mean values ± SD (n=3).

Biofilm disruption. The viability of *P. aeruginosa* and *E. coli* within the biofilms after treatment with vancomycin solution, and vancomycin and control NSs was assessed by fluorescence microscopy using Live/Dead BacLight® bacterial viability kit (Invitrogen, Molecular probes L7012) and by Calgary Biofilm Device (CBD) using a MBEC™ High-throughput assay (Biofilm Technologies, Calgary, Alberta).

Live/dead kit: *P. aeruginosa* and *E. coli* biofilms were grown on tissue culture-treated polystyrene 24-well plates for 24 h at 37 °C as previously described.[37,38] Then, the biofilm was washed with tryptic soy broth (TSB) under aseptic conditions to eliminate non-adhered bacteria and 1 mL of the samples diluted 2 times in TSB to obtain a concentration of 125 µg/mL of vancomycin and 2.1 ± 0.1 x 10^{13} NSs/mL of vancomycin NSs and control NSs, was added to each well. After exposure to vancomycin solution, and vancomycin NSs and control NSs for 24 h at 37 °C the solutions were discarded and biofilm washed with 0.9 % sodium chloride (NaCl) pH 6.5. The biofilm disruption was analyzed using Live/Dead BacLight kit according to the instructions of the manufacturer. The Live/Dead kit contains two nucleic acid stains – green-fluorescent Syto 9 that labels all viable bacteria in the population in green and

red-fluorescent propidium iodide that labels dead bacteria in red. After washing with 1 mL 0.9 % NaCl pH 6.5 the biofilms were stained for 15 min in the dark with a mixture of both stains (1 : 1) and observed using fluorescence microscopy at 480/500 nm for Syto 9 and at 490/635 nm for propidium iodide. Biofilms non-treated with antibiotic were used as a negative control (lack of biofilm eradication).

Calgary Biofilm Device: Biofilms of *P. aeruginosa* and *E. coli* were grown on a device comprised of a microtiter plate lid with 96 pegs, which provide the surface for cells to adhere, colonize and form a uniform biofilm. The pegs fit precisely into the wells of a standard 96-well microtiter plate. The lid was used in conjunction with special troughs for growing of bacteria, washing, and incubating. The trough was inoculated with bacteria and placed in contact with the pegged lid and further incubated in a Plate Shaker-Thermostat for 24 h at 37 º C, 100 rpm. Afterwards, the pegs with the developed biofilm were washed twice with 0.9 % NaCl pH 6.5 and then placed in contact with the 96-well plate containing different concentrations of the test compounds including vancomycin solution, and vancomycin and control NSs at 37 °C for 24 h. The pegged lids were then transferred in 96-well plate containing 200 µl of recovery medium (Mueller-Hinton broth) supplemented with 20 g/L saponin, 10 g/L Tween-80 and 2.5 % (v/v) neutralizing agent containing L-histidine (50 g/L), L-cysteine (50 g/L) and reduced glutathione (100 g/L) and placed in a ultrasonic bath for 10 min. The viable bacterial cells after treatment with antibacterial agents were count after appropriate dilution in sterile buffer and plating on specific agar.

Interaction with *E. coli* cells

The effect of vancomycin NSs on the *E. coli* cultures was also evaluated with scanning electron microscopy (SEM). Briefly, vancomycin NSs (2.1 ± 0.13 x 10^{13} NSs/mL) were mixed with *E. coli* inoculum in NB (O.D. = 0.01 at 600 nm) and the samples were incubated

for 6 h at 37 ºC with shaking. Immediately after the treatment the samples were centrifuged, washed twice with sterile phosphate-buffered saline (PBS) and re-suspended in 50 µL PBS. Afterwards, 15 µL of the suspension were spread on glass slide and allowed to air dry. The cells were fixed in 4 % (v/v) formaldehyde for 30 min, washed with sterile PBS and sequentially treated with 25, 50, 75, and 96 % EtOH for 10 min each. The effect of vancomycin NSs on *E.coli* was studied using a Zeiss Neon FIB microscope (Carl Zeiss, Germany) operating in SEM mode.

Interaction with cell membrane models

The interaction of vancomycin, vancomycin NSs and control NSs with cell membrane models was assessed using Langmuir and Langmuir-Blodgett (LB) techniques. The experiments were carried out in a Langmuir trough equipped with two mobile barriers (KSV NIMA Langmuir-Blodgett Deposition Troughs, model KN2002, Finland) with a total area of 273 cm^2 and total volume 120 cm^3 mounted on an antivibration table and housed in an insulation box at a temperature of 23 ± 1 ºC. The surface pressure was measured using paper Whatman 1 held by a Wilhelmy balance connected to a microelectronic system registering the surface pressure (π).

Langmuir monolayer compression measurements: Vancomycin solution and vancomycin and control NSs were diluted in MilliQ water to a concentration of 2.5 µg/mL and 2.1 ± 0.1 x 10^8 NSs/mL, respectively, and their interaction with *E. coli* PE monolayers was evaluated. The Langmuir trough was cleaned with chloroform and water several times. Afterwards, the subphase (MilliQ water containing vancomycin, control or vancomycin NSs) was poured into the through and 35 µL of 0.5 mg/mL PE solution in chloroform were spread dropwise with a gas-tight syringe. The isotherm recording was performed after the complete spreading and

evaporation of the chloroform (10 minutes). All the experiments were carried out at least three times with barrier closing rates at 25 cm^2/min.

The interaction of the vancomycin NSs with the PE monolayers was also studied by AFM. The LB film transfer was conducted dipping the freshly cleaved mica through the air liquid interface on the subphase before adding the solution, waiting five minutes for monolayer stabilization after pressure set point (5 mN/m) was achieved, obtaining transfer ratios > 80 %. AFM topographic images of LB films were acquired in an air tapping mode using a Multimode AFM controlled by Nanoscope IV electronics (Veeco, Santa Barbara, CA) under ambient conditions. Triangular AFM probes with silicon nitride cantilevers and silicon tips were used (SNL-10, Bruker) (nominal spring constant of 0.35 N/m and a resonant frequency of 50 kHz). Images were acquired at 1.5 Hz line frequency and at minimum vertical force to reduce sample damage.

NSs insertion into phospholipid monolayer: The kinetics of insertion of vancomycin NSs and control NSs into monolayers was measured using the *E. coli* PE monolayer built at π = 33 mN/m, corresponding to the pressure of natural cell membranes.[30] Briefly, 35 µL of 0.5 mg/mL of PE was spread in MilliQ water and the monolayer was compressed to 33 mN/m. After the PE monolayer stabilization, 300 µL of the vancomycin solution and NSs to a final concentration of 2.5 µg/mL and 2.1 ± 0.13 x 10^8 NSs/mL, respectively were injected in the subphase. The changes in the π were monitored over the time.

Data analysis

Replicate samples were used for each measurement, and all the values are presented as mean values ± the respective standard deviations (SD). Results were analysed using graph pad prism version 7.0c (Graph Pad Software, San Diego, CA, U.S.A.). Statistical significances were determined using a one-way ANOVA followed by the Dunnett post-hoc test or by the

unpaired two-tailed Student's t-test method. P values ≤ 0.05 were considered statistically significant.

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Supporting information: Description of the methods for the determination of yield of vancomycin NSs formation; HPLC-UV-Vis chromatogram of vancomycin standard solution and supernatant (diluted) and pellet fractions from vancomycin NSs; Size distribution of Vancomycin NSs and Control NSs.

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