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ORIGINAL RESEARCH

Triclosan resistance reversion by encapsulation in chitosan-coated-nanocapsule containing α-bisabolol as core: development of wound dressing

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Abstract: The use of nanoparticles may be particularly advantageous in treating bacterial infections due to their multiple simultaneous mechanisms of action. Nanoencapsulation is particularly useful for lipophilic drugs. In this scenario, triclosan is considered a good candidate due to its lipophilicity, broad-spectrum activity, and safety. In the present study, we have developed and characterized an antimicrobial suspension of triclosan and α-bisabolol against pathogenic strains that are resistant (*Pseudomonas aeruginosa*) and susceptible (*Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*) to triclosan. We also aimed to determine the minimum inhibitory concentration, using serial microdilution adapted from a CLSI methodology (Clinical and Laboratory Standards Institute). Challenge test was used to confirm the antimicrobial effectiveness of the nanocapsule formulation, as well as after its incorporation into a commercial wound dressing (Veloderm®). The zeta potential of *P. aeruginosa* before and after contact with cationic nanocapsules and the ratio between the number of nanocapsules per colony forming unit (CFU) were determined to evaluate a possible interaction between nanocapsules and bacteria. The results showed that nanoencapsulation has improved the antimicrobial activity when tested with two different methodologies. The number of nanocapsules per CFU was high even in great dilutions and the zeta potential was reverted after being in contact with the cationic nanocapsules. The nanocapsules were able to improve the activity of triclosan, even when tested within 28 days and when dried in the wound dressing.

Keywords: antimicrobial effect, triclosan, α-bisabolol, chitosan, nanocapsules

Introduction

Nanoparticles have been studied for their antimicrobial properties[1–3] and as carriers for antimicrobial drugs, which have shown promising results.[4,5] Even when tested against resistant microorganisms, nanoemulsion,[6] liposomes,[7,8] and nanoparticles[9,10] could reverse drug resistance. The many different modes of action of nanocarries make the occurrence of multiple concurrent mutations unlikely to develop resistance to nanoparticles.[11]

Lipophilic drugs are suitable candidates for encapsulation in organic nanoparticles for presenting positive logarithm values of drug distribution (Log D), which determines their mechanism of encapsulation in polymeric nanocapsules.[12] Log D is the lipophilicity of molecules estimated by calculating the logarithm of the octanol–water distribution coefficient of a molecule, considering the pH value of the medium that affects the proportion of unionized and ionized species and their distributions in the organic and aqueous phases.[12]

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Triclosan is a lipophilic antimicrobial drug that has been used for over 30 years in the treatment of infections. It is the most potent and widely used bisphenol with a favorable safety and nontoxic profile.[13] Triclosan has a broad spectrum of antimicrobial activity against a variety of microorganisms.[14] However, bacteria, such as *Pseudomonas aeruginosa*, present resistance to triclosan due to efflux pump mechanisms.[15] Some characteristics, such as high log D (5.17), thermal stability, and broad spectrum of activity, make triclosan a good candidate for encapsulation into organic nanoparticles, such as nanocapsules. Polymeric nanocapsules contain two domains, ie, an oily core and a polymeric wall, that are dispersed in water with the use of surfactants.

In order to properly carry the drug, the oily core of the nanocapsules needs to be able to disperse triclosan. Keeping that in mind, α-bisabolol, which is a monocyclic sesquiterpene alcohol, was considerd to be a good candidate because of its characteristics. Bisabolol is a viscous oil known to have anti-inflammatory, antimicrobial, and wound-healing properties,[16,17] and it is also a lipophilic antibiotic activity enhancer.[18] The log D of α-bisabolol is 5.07, demonstrating its lipophilic character, which can guarantee the encapsulation of poorly water-soluble drugs, such as triclosan.

Studies of nanoparticles have demonstrated the importance of the positive charge, which improves interaction with microorganisms.[19,20] To take advantage of positively charged nanoparticles, many studies have been reported on the use of chitosan as coating.[21,22] In addition, the use of this polycationic biopolymer in an antimicrobial formulation is interesting due to its biodegradability and antimicrobial activity.[1,23]

An innovative strategy for antimicrobial nanoparticle formulations is to incorporate them into medical products,[24] mainly for wound dressings impregnated with silver nanoparticles[25,26] to inhibit bacterial growth (BG). A good candidate for this application is Veloderm®, which is a biological wound dressing with good healing properties.[27,28] The use of biological wound dressing alone had few problems reported, such as infections, and consequently the interruption of treatment due to its lack of antimicrobial effect.[29] This problem could be solved by incorporating an antimicrobial nanocapsule formulation into this occlusive dressing.

Therefore, the aims of this study were to develop and characterize an antimicrobial nanocapsule formulation containing triclosan and α-bisabolol, to evaluate its effect against pathogenic strains that are resistant (*Pseudomonas aeruginosa*) and susceptible (*Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*) to triclosan, and to verify its suitability for incorporation into wound dressings.

Materials and methods

Materials

Poly(epsilon-caprolactone) (PCL, Mn 80 kDa), chitosan of low molecular weight (50–190 kDa) and 75%–85% deacetylation degree, Mueller-Hinton broth 2, RPMI-MOPS, and MTT bromite of 3-(4,5-dimetiltiazol-2-il)-2, 5-dipheniltetrazolium) were obtained from Sigma-Aldrich (St Louis, MO, USA). Medium-chain triglyceride (MCT) was purchased from Delaware (Porto Alegre, RS, Brazil), α-bisabolol and triclosan were acquired from Fragon (São Paulo, SP, Brazil), isopropanol and acetone were obtained from Vetec (São Paulo, SP, Brazil), and acetonitrile and ethanol HPLC standard were purchased from Tedia (São Paulo, SP, Brazil). Glacial acetic acid, Lipoid S75® (soybean lecithin), and polysorbate 80 were acquired from Fmaia (Belo Horizonte, MG, Brazil), Lipoid (Ludwigshafen, RP, Germany), and Henrifarma (São Paulo, SP, Brazil), respectively.

Production of nanocapsules and controls

Interfacial deposition of preformed polymers was the method used to produce the nanocapsules.[30] α-bisabolol was used as oil core and PCL as polymeric wall. The nanocapsules were coated with soybean lecithin, polysorbate 80, and chitosan. An ethanol solution of lecithin was added to an organic phase composed of PCL, α-bisabolol, and triclosan in acetone. The organic phase was injected into an aqueous phase containing polysorbate 80, as stabilizer. Polysorbate 80 and lecithin were used in the same proportion. Reduced volume of aqueous phase (20 mL) compared to the organic phase (25 mL) was used as previously proposed.[31] The nanocapsules were coated with the cationic biopolymer using 0.7% of chitosan in 1% acetic acid aqueous solution, based on an adapted technique from Mayer et al.[32] Triclosan or α-bisabolol were dispersed in polysorbate 80 aqueous solutions in the same proportion used to obtain the nanocapsule formulations. Blank nanocapsule formulation (NC_{BL}) was prepared using MCT in the place of α-bisabolol. All formulations were adjusted to the same concentration with a final volume of 10 mL, as described in Table 1.

Physicochemical characterization of nanocapsules

The characterization of nanocapsules were carried out to determine the size distribution profiles, mean diameters, polydispersity, zeta potential, pH, drug content, encapsulation efficiency, release profile, transmission electronic microscopy, and particle number density. Laser diffraction analysis (from 40 nm to 2 mm) was performed

Table 1 Final compositions of nanocapsules and controls (mg/mL)

Formulation/components	T_{P80}	α_{P80}	NC_{BL}	NC_{BC}	NC_{AC}
Triclosan	0.9	–	–	0.9	0.9
α-bisabolol	–	14.4	–	14.4	14.4
Chitosan	–	–	0.7	–	0.7
MCT	–	–	14.4	–	–
Polysorbate 80	6.9	6.9	6.9	6.9	6.9
Lecithin	–	–	6.9	6.9	6.9
PCL	–	–	9	9	9

Abbreviations: MCT, medium-chain trigyceride; NC_{AC}, chitosan-lecithin-polysorbate 80-coated nanocapsules; NC_{BC}, lecithin-polysorbate 80-coated nanocapsules; NC_{BL}, blank nanocapsules; PCL, poly(epsilon-caprolactone); T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.

in a Mastersizer 2000 equipment (Malvern Instruments, Malvern, UK). Each sample was inserted in the wet unit containing distilled water, without any previous treatment, for a laser obscuration of 2%. Refraction indexes of 1.590 (before coating with chitosan) and 1.345 (after coating with chitosan) were used to calculate the volume-weight mean diameters (D[4,3]), the polydispersity (SPAN), and the median diameter by number of particles [$d(0.5)_n$]. Dynamic light scattering analysis (from 0.6 to 1,000 nm) was carried out in a Zetasizer Nano ZS instrument (Malvern Instruments). Each sample was diluted (500 times) in pre-filtered (Millipore®, 0.45 μm) ultrapure water. The particle diameter profiles were determined to calculate the hydrodynamic mean diameter (D_h) and the polydispersity index (PDI) for each batch of formulation. Zeta potential values were determined by electrophoretic mobility using the same instrument (Zetasizer Nano ZS) after diluting each sample (500 times) in 10 mmol/L NaCl aqueous solution. The pH values were determined by direct measurement using potentiometer (B474; Micronal, São Paulo, Brazil) calibrated at 4.00 and 7.00 with phosphate buffer.

Encapsulation efficiency (EE%) was analyzed by ultrafiltration-centrifugation method[33] using a ultrafiltration-centrifugation unit (Millipore; Amicon® Ultra, cut-off 10 kDa), centrifuged at 1,844× *g* (RCF) for 5 minutes. The same methodology was applied in diluted formulations in ultrapure water (1:100 and 1:1,000, v/v) in order to determine the ability of the drug to remain encapsulated, allowing the serial microdilutions to perform the microbiological experiments. All triclosan quantifications were performed by high performance liquid chromatography (HPLC) (HPLC model LC 20A, Shimadzu Co., Tokyo, Japan) using a previously validated method,[31] regarding accuracy, linearity, precision, and specificity parameters.[34] Nova-Pax RP-18 column, Waters® (Milford, CT, USA), was used as a stationary phase. A mixture of acetonitrile:H_2O (60:40, v/v) with apparent pH of 4.5 corrected with acetic acid was used as a mobile phase. The drug was detected at λ=280 nm. The particle number density (d_{NP}) was determined by turbidimetry[35] in a spectrophotometer Cary 50 UV-Vis (Varian, Palo Alto, USA) at λ=395 nm.

The drug release profile was determined using cellulose acetate dialysis bags Sigma-Aldrich with a cut-off of 14 kDa. The release medium was sampled in 10 points of time interval (10, 30, and 60 minutes and 3, 6, 12, 18, 24, 36, and 48 hours).[36] A mixture of ethanol:water (1:1, v/v) (150 mL) was used as release medium to keep the sink condition.[14] The drug release data were modeled to determine the best release profile, according to the monoexponential and biexponential models to define the best adjustment by mathematical modeling.[35] MicroMath Scientist® was used to analyze the profiles, and the model was determined according to the best correlation coefficient, the best model selection criteria, and the best graphic adjustment. The morphology of the nanocapsules (NC_{BC} and NC_{AC}) were evaluated by transmission electronic microscopy[37] using a JEM 1200 Exll, operated at 80 kV and stained with uranyl acetate solution (2% w/v).

Minimum inhibitory concentration (MIC) in bacteria

Bacterial MICs were determined in liquid growth media Mueller Hinton against *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923), using serial microdilution, in 96-well plates.[38] In order to avoid natural turbidity caused by the nanocapsules, which could interfere in visual inspection, a spectrophotometer was employed to measure the turbidimetry. To determine BG, the measures were taken before (T_0) and after 24 hours of incubation (T_{24}). Therefore, absorbance results of T_0 were subtracted from T_{24}, according to Equation 1. MICs were obtained considering the lowest concentration which had no statistically significant difference between T_{24} and T_0 ($p>0.05$). Inoculums and serial microdilution were prepared according to CLSI 2003[38] in microdilution plates (96 U-shaped wells), and the wavelength used to measure the turbidity was 625 nm, with T_0 as initial value.

$$BG = T_{24} - T_0 \quad (1)$$

MIC in yeast

The MIC determination of *Candida albicans* (ATCC 24433) for different formulations was made in liquid growth media RPMI-MOPS, using serial microdilution, according to CLSI 2008.[39] MTT assay was used to determine viability detection,[40] which had an absorbance in the two wavelengths

evaluated (570 and 690 nm). Breakpoints were determined as the minor concentration, which reached 80% of cellular damage. Inoculums and microdilution were prepared following CLSI 2008[39] and the time of incubation was 48 hours. To verify any possible interference due to redox reaction, and consequently, formazan precipitation, all samples were incubated in growth media for 24 hours without the presence of inoculums.[40]

Zeta potential of *P. aeruginosa* before and after contact with nanocapsules

Zeta potential values were evaluated for pure inoculum of *P. aeruginosa* and for inoculum in contact with nanocapsules in the same proportion (1:1) to simulate the same conditions as in the first microdilution well. The inoculum with nanocapsules was homogenized and left in contact for 15 minutes to ensure a proper interaction. Dilutions in 10 mmol/L NaCl aqueous solutions were made for the nanocapsule analysis, as described earlier. Results were expressed as a mean of 3 independent measurements,[41] and bacterial concentration was fixed at 5×10^5 CFU/mL to reproduce the first well of the serial microdilution testing condition.

Number of nanocapsules per colony forming unit (CFU)

To determine the number of nanocapsules that could interact with each CFU, a theoretical ratio calculation (R), Equation 2, was preformed using the particle number density (nanocapsules per milliliter, d_{NP}), as described earlier. The number of CFU was established during inoculum preparation (~5×10^5 CFU/mL).

$$R = d_{NP}/\text{CFU} \quad (2)$$

Nanocapsules incorporation into wound dressing

To verify the feasibility of incorporating the NC_{AC} into the hemicellulose wound dressing (Veloderm®), a spraying method was applied. Briefly, the wound dressing was cut into small rectangles (2×6 cm^2), weighted before and after spraying the nanocapsule formulation, and dried (24 hours protected from the wind). Spraying process was carried out two times with a commercial spray (Brand New®) at 15 cm distance. The product was named WD-NC_{AC}, and triclosan from incorporated NC_{AC} was extracted from dried wound dressing with pure acetonitrile (3 mL) and quantified by HPLC (λ=280 nm) with a validated method.

Challenge test

To verify the maintenance of the antimicrobial effect, the challenge test was performed. The strains tested were *Escherichia coli* ATCC 8739 (in MacConkey agar), *Staphylococcus aureus* ATCC 25923 (Baird-Parker agar), *Pseudomonas aeruginosa* ATCC 27853 (cetrimide agar), and *Candida albicans* ATCC 10231 (potato glycosylated agar). The procedure was adapted from a previously reported methodology,[42] by dilution in buffered sodium chloride-peptone solution, adjusted to pH 6.0–8.0, in the same proportion (1:9). Samples were taken at time intervals (24 hours and 7, 14, 21, and 28 days) after incubation and cultivated for 24 hours; visual quantifications were made. This experiment was performed for NC_{AC} suspension and NC_{AC} incorporated into a wound dressing.

Statistical analysis

Significant differences between measurements were detected by two-way ANOVA, followed by Bonferroni's multiple comparison test. Differences between comparisons were considered to be significant at $p<0.05$. All analyzes were performed using GraphPad Prism 5.0® software (GraphPad Software, Inc., San Diego, CA, USA).

Results and discussion

Development of nanocapsules

The nanocapsule formulations containing triclosan (NC_{BC} and NC_{AC}) showed narrow size distribution profiles by laser diffraction with similar D[4,3] and polydispersity (Table 2). NC_{AC} showed a calculated median diameter by number of particles [$d(0.5)_n$] of 130±2 nm. NC_{BC} and NC_{AC} analyzed

Table 2 Size and zeta potential characterization results of NC_{BC} and NC_{AC} formulations

Formulations	Laser diffraction[a]		Dynamic light scattering[a]		Electrophoretic mobility
	D[4,3] (nm)	Span	D_h (nm)	PDI	Zeta potential (mV)
NC_{BL}	136±1	1.05±0.13	142±1	0.14±0.01	+13.6±0.4
NC_{BC}	133±2	1.02±0.12	141±1	0.07±0.00	−15.6±2.2
NC_{AC}	137±2	0.98±0.03	144±2	0.13±0.02	+13.7±0.4

Notes: Values were expressed as mean ± standard deviation; n=3. [a]Calculated from the size distribution curves by volume of particles.
Abbreviations: D[4,3], volume-weight mean diameters; D_h, hydrodynamic mean diameter; NC_{AC}, chitosan-lecithin-polysorbate 80-coated nanocapsules; NC_{BC}, lecithin-polysorbate 80-coated nanocapsules; NC_{BL}, blank nanocapsules; PDI, polydispersity index.

by dynamic light scattering (DLS) (Table 2) had similar D_h with narrow size distributions, since PDIs were below 0.15. Zeta potential was negative before chitosan coating (NC_{BC}) and it was reverted to a positive value after the interfacial reaction (NC_{AC}) (Table 2), corroborating a previous study.[22] Potentiometry analyses showed that pH was neutral for NC_{BC} formulation (7.04±0.17); however, after adding the chitosan solution, the pH values decreased to 4.09±0.1 (NC_{AC}). The acidity increased due to the presence of acetic acid used to disperse chitosan in water, as previously reported.[22] Values of pH between 4 and 5 can be suitable for a topical application, since skin surface has slight acidity.[43] Nanocapsules prepared without triclosan and with MCT in the place of α-bisabolol (NC_{BL}) showed similar physicochemical attributes than those presented by NC_{AC}.

After preparation, NC_{AC} had an experimental triclosan content of 0.86±0.02 mg/mL, which was close (95.5%) to the theoretical concentration (0.9 mg/mL). After 30 days, this formulation showed a similar ($p>0.05$) triclosan content (0.82±0.01 mg/mL). Particle number density for NC_{AC} was (7.88±0.96) $\times 10^{13}$ nanoparticles per milliliter. This value is ~10 times higher than the one observed for other nanocapsule formulation.[35] The difference is based on the use of ethanol to prepare the former nanocapsules compared to the ethanol-free process generally employed for the latter, which particles have mean size >100 nm.

No triclosan was detected by HPLC in the ultrafiltrate for NC_{AC} irrespective of whether samples were undiluted or diluted, indicating an EE% of 100%. NC_{AC} are polymeric nanocapsules prepared with other nucleous than the lipid-core nanocapsules. However, this result corroborated our previous study,[12] in which we proposed the use of log D as the main parameter to estimate the mechanism of drug encapsulation in polysorbate 80-coated lipid-core nanocapsules with a core composed of MCT and sorbitan monostearate. For polysorbate 80-lipid-core nanocapsules, drugs with log D >4 are concentrated in the nanocapsule core. Triclosan (log D 5.17) is likely to concentrate within the core of NC_{AC}, which is composed of α-bisabolol.

Transmission electron microscopy was used to show the morphological characteristics of the nanoparticles (Figure 1). NC_{BC} are spheroids with a higher pigment density at the corona (Figure 1A). This characteristic was previously observed for lecithin-polysorbate 80-lipid-core nanocapsules,[44] with a corona formed by spherical and cylindrical micellar structures. NC_{AC} are also spheroids having a corona pigmentation of lower intensity with a characteristic fringe (Figure 1B) due to the presence of chitosan in the formulation.

Regarding the release experiment, the triclosan dialyzed from the polysorbate 80 dispersion formulation (T_{P80}) was ~80%, in 24 hours. In contrast, for NC_{AC}, the triclosan released in 24 hours was 64%, showing a controlled profile, which reached a plateau in 36 hours (Figure 2). The results fitted to a monoexponential first-order model for both formulations T_{P80} (k= (3.2±1.5) $\times 10^{-3}$/min and $t_{1/2}$=4.9±2.6 hours) and NC_{AC} (k= (9.2±3.3) $\times 10^{-4}$/min and $t_{1/2}$=14.1±4.2 hours). These results showed that nanoencapsulated formulations were able to control the drug releasing rate (k), 3 times slower, and half-life ($t_{1/2}$), 3 times higher, when compared to T_{P80}.

MIC

MIC results are described based on the concentrations of triclosan (MIC_T), α-bisabolol (MIC_α), and chitosan (MIC_{CHI}) (Table 3 and Figures S1–S4). The strains were chosen for being the most common pathogens found in infected wounds.

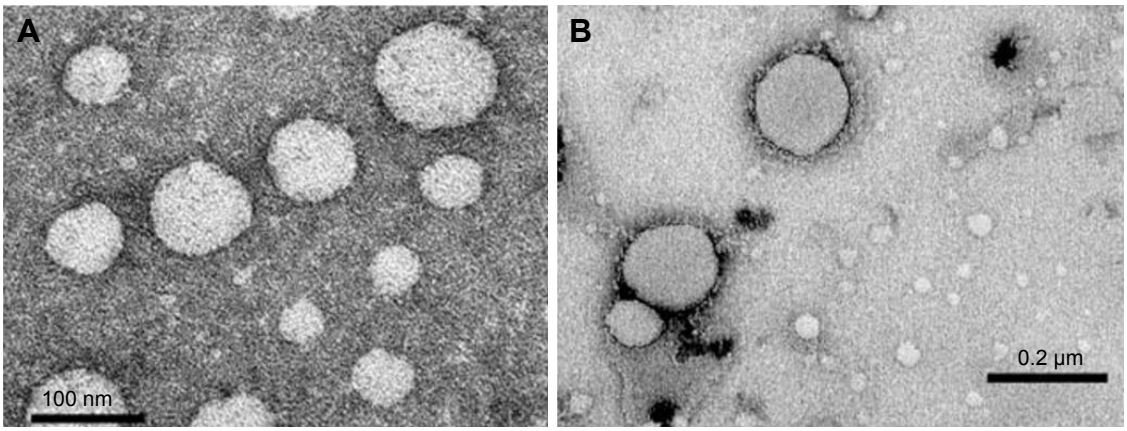

Figure 1 Transmission electronic microscopy images of (**A**) nanocapsules before being coated by chitosan (NC_{BC}) and (**B**) nanocapsules after being coated by chitosan (NC_{AC}).
Abbreviations: NC_{AC}, chitosan-lecithin-polysorbate 80-coated nanocapsules; NC_{BC}, lecithin-polysorbate 80-coated nanocapsules.

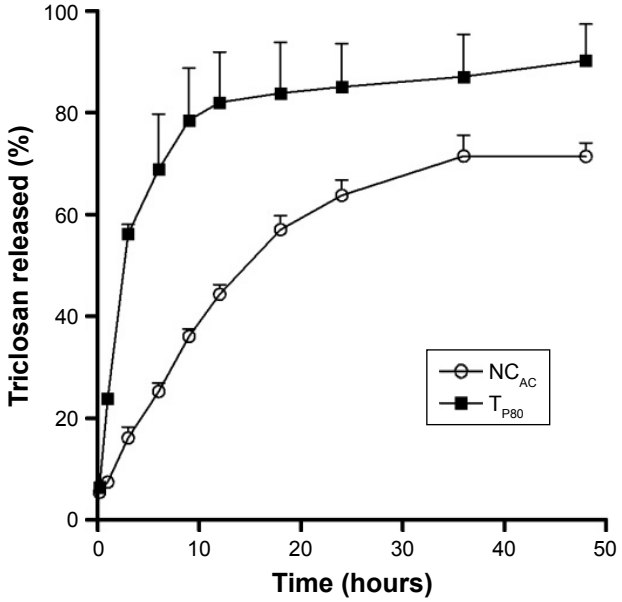

Figure 2 T_{P80} and NC_{AC} releasing profile in ethanol:H_2O (1:1) receptor media.
Note: Mean and error bars are represented at each point, n=3.
Abbreviations: NC_{AC}, chitosan-lecithin-polysorbate 80-coated nanocapsules; T_{P80}, triclosan dispersed in polysorbate 80.

S. aureus colonization may occur within the first 48 hours and *P. aeruginosa* and *E. coli* within the first 72 hours, while *Candida sp*. is the most common cause of fungal infections.[44] For *S. aureus* and considering triclosan, NC_{BC} showed a slight reduction in sensitivity compared to the free drug dispersed in polysorbate 80 (T_{P80}). Conversely, after coating the nanocapsules with chitosan, NC_{AC} showed a sensitivity increase close to 4-folds compared to T_{P80}. Considering α-bisabolol, both nanocapsule formulations (NC_{BC} and NC_{AC}) were more effective than the free drug dispersed in polysorbate 80 (α_{P80}) (917-folds and 7,333-folds, respectively). It is worth noting the difference between the formulations containing or not containing chitosan as the coating material. The presence of chitosan was a determinant to increase the sensitivity of *S. aureus* against triclosan and α-bisabolol. Our previous study[45] showed a better antimicrobial activity of chitosan-lecithin-polysorbate 80-coated lipid-core nanocapsules against *S. aureus*, which was attributed to the antimicrobial activity of this polysaccharide against Gram-positive and Gram-negative bacteria.

For *E. coli*, the nanoencapsulation of triclosan in NC_{BC} or NC_{AC} has slightly reduced the strain sensitivity compared to T_{P80} (Table 3), while the nanoencapsulation of α-bisabolol in NC_{BC} and in NC_{AC} increased the strain sensitivity compared to a_{P80} (113-folds and 449-folds, respectively). The results suggest that a formulation containing only α-bisabolol, as an anti-inflammatory drug, is very promising for further studies.

For *P. aeruginosa*, T_{P80} did not show any activity as previously reported for triclosan (free drug).[46,47] In fact, *P. aeruginosa* is reported[13] to be highly resistant to triclosan, reaching values >1 mg/mL, due to its efflux pump in the outer membrane.[15,48,49] In contrast, the nanoencapsulation of triclosan showed MIC_T of 220 mg/mL (NC_{BC}) and 56 mg/mL (NC_{AC}) (Table 3). NC_{BC} demonstrated a reduction in strain sensitivity compared to a_{P80}, while NC_{AC} showed an increase of 1.8-folds compared to the free drug dispersed in polysorbate 80. Once again, the effect of chitosan as a coating is an important parameter to obtain results. Thereby, the main explanation relies on the cationic character of the particles, once lipopolysaccharide-mediated resistance of Gram-negative bacteria to neutral and anionic detergents were overcome by cationic nanoemulsions.[6] A study conducted with Al_2O_3-cationic nanoparticles suggested that those structures may have an easier interaction with negative cell membrane, enhancing their penetration into the cell,[3] a hypothesis that can be taken into consideration.

Table 3 Minimum inhibitory concentration (μg/mL) determined for all formulations tested against bacteria and yeast calculated using triclosan, α-bisabolol, and chitosan concentrations (MIC_T, MIC_α, and MIC_{CHI}, respectively)

MIC (μg/mL)												
Formulations	***S. aureus* (ATCC 25923)**			***E. coli* (ATCC 25922)**			***P. aeruginosa* (ATCC 27853)**			***C. albicans* (ATCC 24433)**		
	MIC_T	MIC_α	MIC_{CHI}	MIC_T	MIC_α	MIC_{CHI}	MIC_T	MIC_α	MIC_{CHI}	MIC_T	MIC_α	MIC_{CHI}
T_{P80}	0.0034	–	–	0.014	–	–	>450	–	–	450	–	–
α_{P80}	–	110	–	–	220	–	–	1,800	–	–	900	–
NC_{BC}	0.0068	0.12	–	0.11	1.95	–	220	4,000	–	>450	>8,000	–
NC_{AC}	0.00086	0.015	0.00067	0.027	0.49	0.021	56	1,000	44	28	500	22
NC_{BL}	–	–	87.5	–	–	87.5	–	–	44	–	–	87.5

Abbreviations: NC_{AC}, nanocapsule after coating; NC_{BC}, nanocapsule before coating; NC_{BL}, blank-nanocapsules; nd, not determined; T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.

For *C. albicans*, NC_{BC} did not show any inhibitory effect in relation to both T_{P80} and a_{P80} (Table 3). The slight negative zeta potential could be a barrier to prevent NC_{BC} interaction with the bacterial membrane, which also has negative zeta potential.[41] On the other hand, NC_{AC} promoted an increase of 1.6-folds and 1.8-folds in comparison to T_{P80} and a_{P80}, respectively. In a study performed with positive PCL nanocapsules containing chlorhexidine (free base), it was suggested that the cationic nanoparticles were able to interact with bacteria, due to their opposite charges, diffusing the drug from the core of the nanocapsules to the bacterial cell membrane.[4]

For all strains, α_{P80} showed high MIC_a values (Table 3). Actually, this drug is more remarkably known as a potentiator of antibiotic activity by disarranging the cell membrane structure[18] than by having an antimicrobial activity.[50] Nevertheless, MIC_a values for NC_{AC} were reduced compared to α_{P80}, remarkably in the case of *S. aureus* and *E. coli*.

The MICs of chitosan (MIC_{CHI}) against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* were determined using chitosan-coated blank-nanocapsule formulation (NC_{BL}) (Table 3). The most relevant result was the antimicrobial effect against *P. aeruginosa*, considering the comparison between NC_{BL} and NC_{AC} (MIC_{CHI} 44 µg/mL for both). The inhibitory effect of NC_{AC} observed for NC_{AC} was exclusive from the presence of chitosan at the nanocapsule surface suggesting a possible reduction in triclosan and α-bisabolol doses in the formulation. Previously, studies in the literature reported the antimicrobial effect of chitosan.[45,51–53] Regarding *S. aureus*, *E. coli*, and *C. albicans*, the inhibitory effect observed for NC_{BL} was lower than that observed for NC_{AC}.

The increased susceptibility reversal of triclosan resistance by the nanoencapsulation should be highlighted as an important achievement, since *P. aeruginosa* is among the most common burn infectious agents, possessing many intrinsic and acquired resistance mechanisms, which makes burn wounds infected by these bacteria difficult to treat.[54] Therefore, it was selected as the activation strain model to test the hypothesis of electrostatic interaction.

Zeta potential of *P. aeruginosa* before and after contact with the nanocapsules

This experiment was performed to analyze the zeta potential alterations of pure inoculum and inoculum after contact with NC_{AC}. In general, Gram-negative bacteria, such as *P. aeruginosa*, possess negative zeta potential due to the presence of lipopolysaccharides, phospholipids, and membrane proteins.[55] The zeta potential determined for the *P. aeruginosa* inoculum was −4.18±1.6 mV (pure inoculum). Negative potential enhanced the interaction with positive ions,[41] such as cationic-coated nanocapsules. The zeta potential of *P. aeruginosa* inoculum shifted to +3.19±0.27 mV after contact with NC_{AC} (Figure 3). The positive surface potential enhabled NC_{AC} to bind to the *P. aeruginosa* surface.

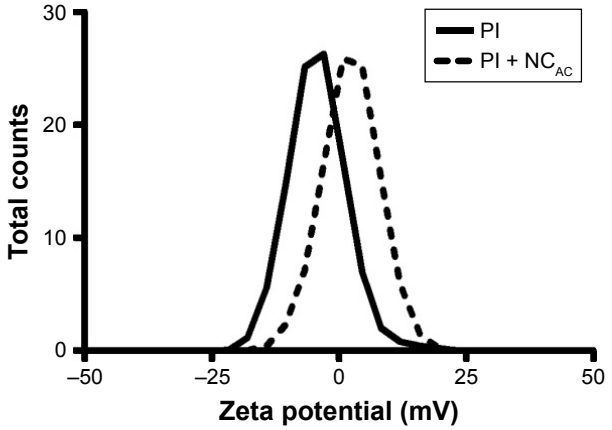

Figure 3 Zeta potential distributions: *Pseudomonas aeruginosa* inoculum (solid line) and *P. aeruginosa* inoculum added with NC_{AC} (1:1) (dashed line).
Note: Both results are expressed as the mean of three independent measurements (n=3).
Abbreviations: NC_{AC}, chitosan-lecithin-polysorbate 80-coated nanocapsules; PI + NC_{AC}, *Pseudomonas aeruginosa inoculum* after contact with NC_{AC}; PI, *Pseudomonas aeruginosa* inoculum

Number of nanocapsules per CFU

The results of the ratio between NC_{AC} and CFUs are shown in Table 4. These results may be applied to any bacterial strain, whereas the number of CFU is the same for all of them. In experimental conditions, the nanocapsules have displayed, even in small concentration (initial dose 0.39%), high magnificence (10^6 nanocapsules/CFU). The high number of cationic nanocapsules interacting with the bacterial surface might destabilize or affect the membrane leading to the leakage of intracellular components and, consequently, to cell death.[1] The adsorption of bacteria by the nanocapsules may prevent the electrostatic interaction between the bacteria and the surface, disabling bacterial fixation.[16] Another possibility would be a great number of nanocapsules diffusing their antimicrobial drug from the core directly to the cytoplasm of microorganisms.[4]

Incorporation of NC_{AC} into wound dressing and challenge test

NC_{AC} was incorporated into the wound dressing (WD-NC_{AC}) showing a drug recovery of 93.28%±7.27%, corresponding to 23.41±2.54 µg/unit. The challenge test was performed in

Table 4 Ratio between the number of nanocapsules (NC_{AC}) and the number of colony forming units (CFUs) in each dilution

	50%	25%	12.5%	6.75%	3.12%	1.56%	0.78%	0.39%
d_{NP}	7.8×10^{12}	3.9×10^{12}	1.95×10^{12}	9.75×10^{11}	4.9×10^{11}	2.44×10^{11}	1.22×10^{11}	6.1×10^{10}
CFU	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000
Ratio	7.8×10^{8}	3.9×10^{8}	1.95×10^{8}	9.75×10^{7}	4.9×10^{7}	2.44×10^{7}	1.22×10^{7}	6.1×10^{6}

Abbreviation: d_{NP}, density of NC_{AC} in the well (particles/mL).

order to verify the antimicrobial effect of WD-NC_{AC} after production and after 28 days of storage. Results shown in Table 5 confirm that after dilution (1:9), in experimental condition, the triclosan concentration (90 μg/mL) was able to inhibit microorganism growth within 28 days. This result was expected once all MIC values obtained from serial microdilution were <90 μg/mL. However, when we take into account the triclosan concentration in WD-NC_{AC} after dilution (2.34 μg/mL), we observed that MIC obtained for *P. aeruginosa* (56.25 μg/mL) was ~24 times higher and *C. albicans* had its growth totally inhibited after 21 days.

Due to the importance of protecting the wounds toward contamination, Veloderm® with the NC_{AC} incorporated could act in two ways. On the first contact, the nanocapsules would be able to kill microrganisms and inhibit growth of bacteria for at least 28 days. While a single application of wound dressing seals the wound bed, avoiding exposure and contact with new infectious agents, making a suitable environment for proper healing. It is important to note that both systems are complementary.

Conclusion

The present study described the development of a cationic nanostructured system, presenting highly homogeneous size of nanoparticles, without any micrometric contaminants, with acceptable pH for cutaneous use, and with the ability to control the release of triclosan. In terms of MIC results, the nanocapsules after chitosan coating (NC_{AC}) presented the best results when compared to all controls. The wound dressing containing those nanocapsules maintained antimicrobial activity. The results also included species with high resistance to free triclosan, such as *P. aeruginosa*, which became susceptible to a dose nearly 8-folds smaller. In order to understand the mechanism of action of NC_{AC}, physicochemical tests were performed revealing a large number of nanocapsules per CFU with an inversion of zeta potential after adding the formulation into the bacterial inoculum. Considering the state of the art, the results give us a light over a possible mechanism of action of NC_{AC} and the promising use of those nanocapsules as a platform to develop novel drug delivery systems intended to increase microorganism susceptibility.

Table 5 Results obtained from the challenge test for NC_{AC} and NC_{AC} incorporated into a wound dressing (WD-NC_{AC})

	E. coli (ATCC 8739)	*S. aureus* (ATCC 25923)	*P. aeruginosa* (ATCC 27853)	*C. albicans* (ATCC 10231)
Inoculum (T=0)	6.2×10^{5} CFU/g	2.4×10^{5} CFU/g	6.2×10^{4} CFU/g	4.5×10^{4} CFU/g
24 hours				
NC_{AC}	–	–	–	–
WD-NC_{AC}	–	–	–	2.0×10^{4} CFU/g
7 days				
NC_{AC}	–	–	–	–
WD-NC_{AC}	–	–	–	2.0×10^{4} CFU/g
14 days				
NC_{AC}	–	–	–	–
WD-NC_{AC}	–	–	–	8.6×10^{3} CFU/g
21 days				
NC_{AC}	–	–	–	–
WD-NC_{AC}	–	–	–	–
28 days				
NC_{AC}	–	–	–	–
WD-NC_{AC}	–	–	–	–

Abbreviations: (–), absence of growth; CFU, colony forming units; NCAC, nanocapsules after chitosan coating.

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Disclosure

The authors report no conflict of interest in this work.

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Supplementary materials

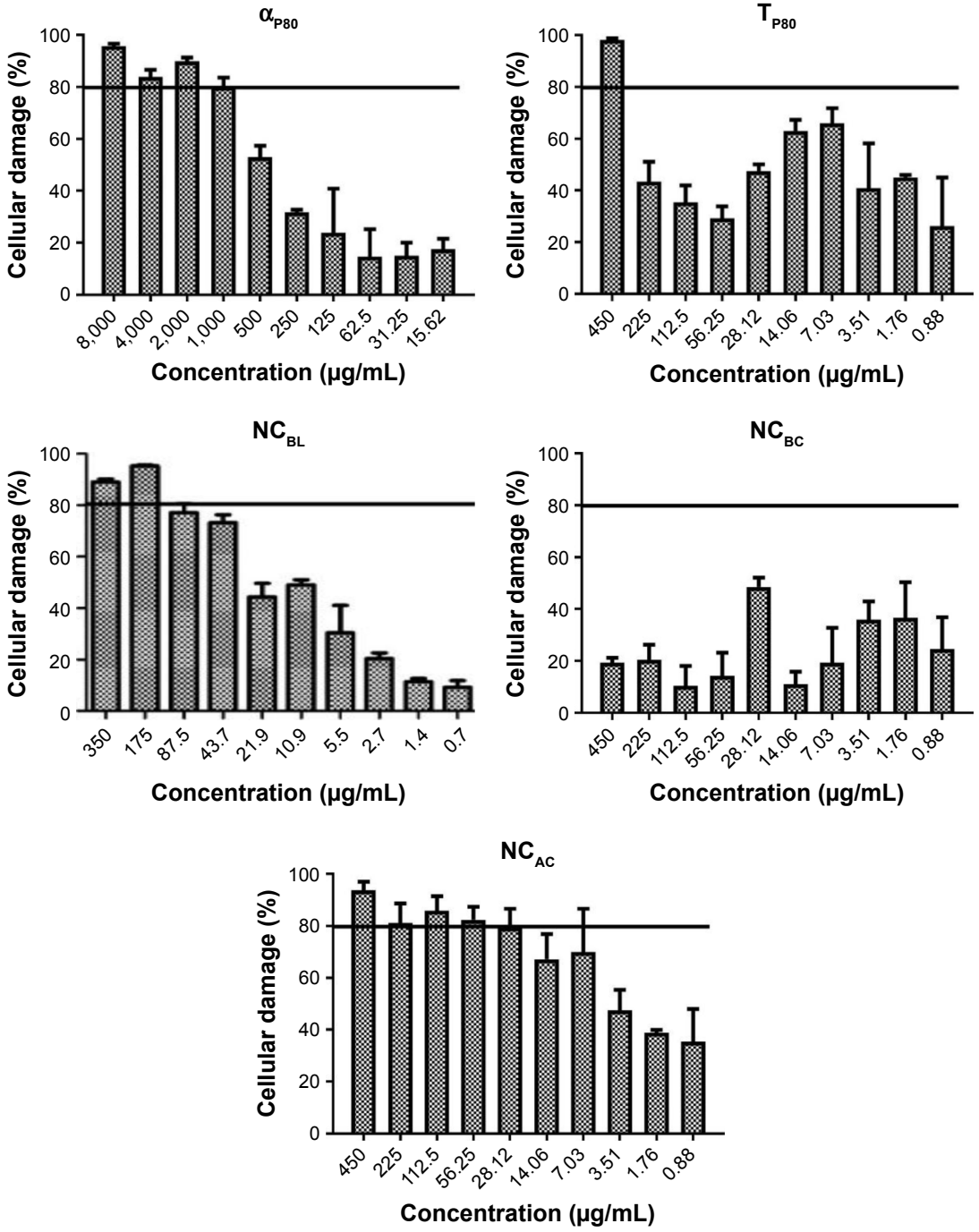

Figure S1 MIC_{80} results from MTT assays for *C. albicans* facing four different formulations.
Note: For T_{P80}, NC_{BC} and NC_{AC} concentration refers to triclosan, for NC_{BL} to chitosan, and for α_{P80} to α-bisabolol.
Abbreviations: MIC, minimum inhibitory concentration; NC_{AC}, α-bisabolol and triclosan-coated nanocapsule suspension; NC_{BC}, α-bisabolol and triclosan-uncoated nanocapsule suspension; NC_{BL}, blank nanocapsules; T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.

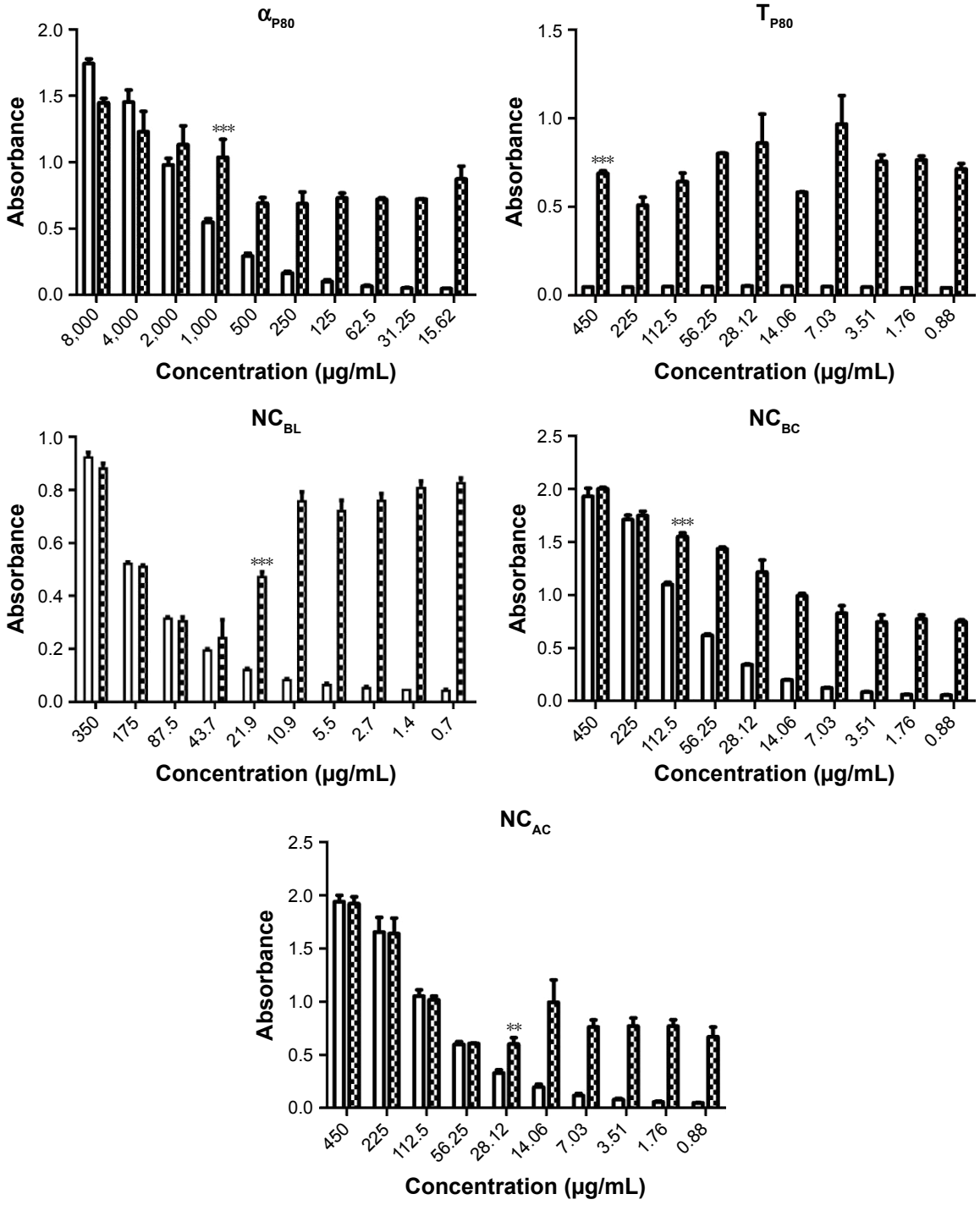

Figure S2 MIC results from for *P. aeruginosas* facing five different formulations.

Notes: ***$p<0.001$, **$p<0.01$. For T_{P80}, NC_{BC} and NC_{AC} concentration refers to triclosan, for NC_{BL} to chitosan, and for α_{P80} to α-bisabolol. Y-axis refers to absorbance read at 625 nm.

Abbreviations: MIC, minimum inhibitory concentration; NC_{AC}, α-bisabolol and triclosan-coated nanocapsule suspension; NC_{BC}, α-bisabolol and triclosan-uncoated nanocapsule suspension; NC_{BL}, blank nanocapsules; T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.

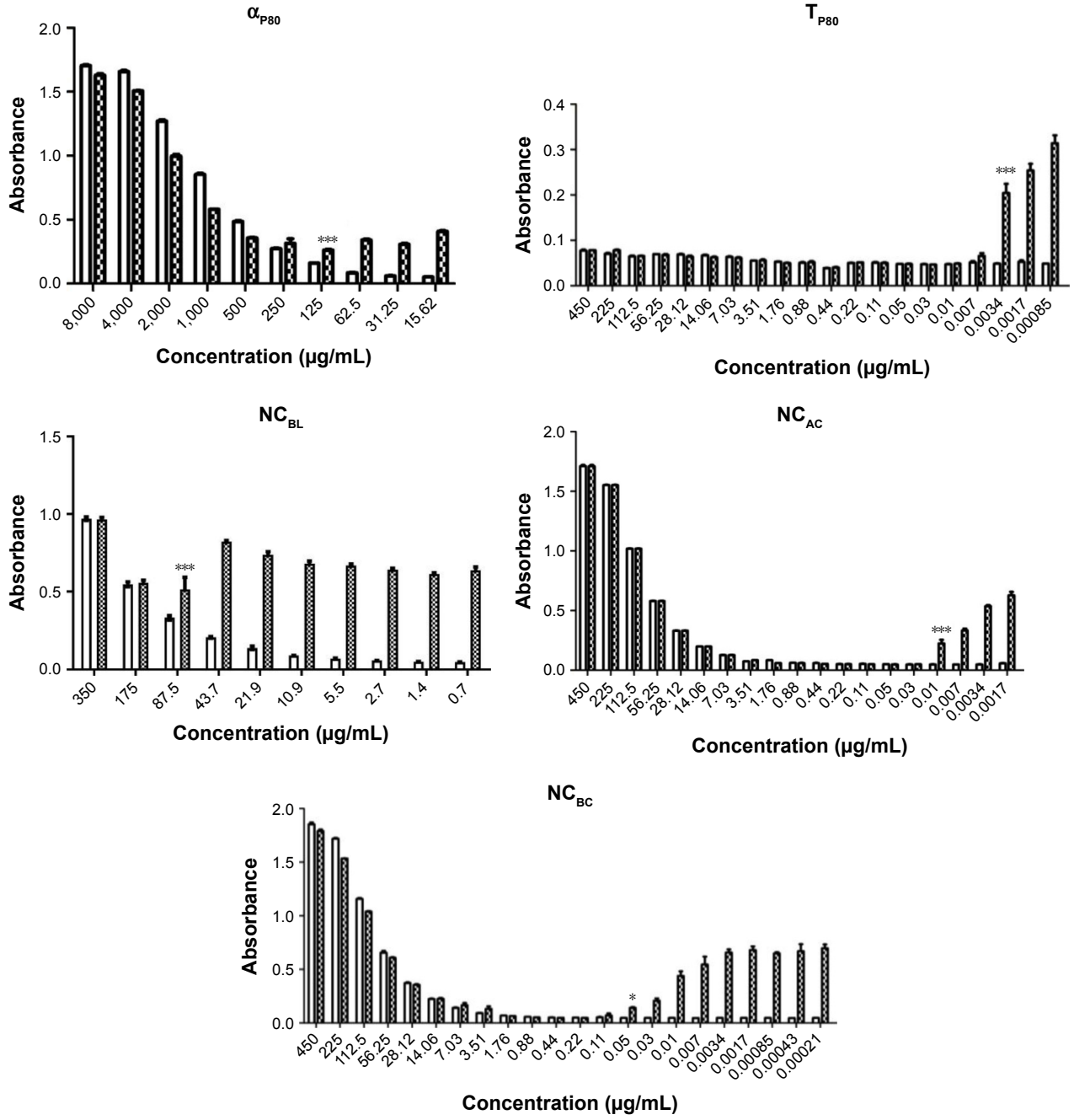

Figure S3 MIC results from for *E. coli* facing five different formulations.

Notes: ***$p<0.001$ and *$p<0.05$. For T_{P80}, NC_{BC} and NC_{AC} concentration refers to triclosan, for NC_{BL} to chitosan, and for α_{P80} to α-bisabolol. Y-axis refers to absorbance read at 625 nm.

Abbreviations: MIC, minimum inhibitory concentration; NC_{AC}, α-bisabolol and triclosan-coated nanocapsule suspension; NC_{BC}, α-bisabolol and triclosan-uncoated nanocapsule suspension; NC_{BL}, blank nanocapsules; T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.

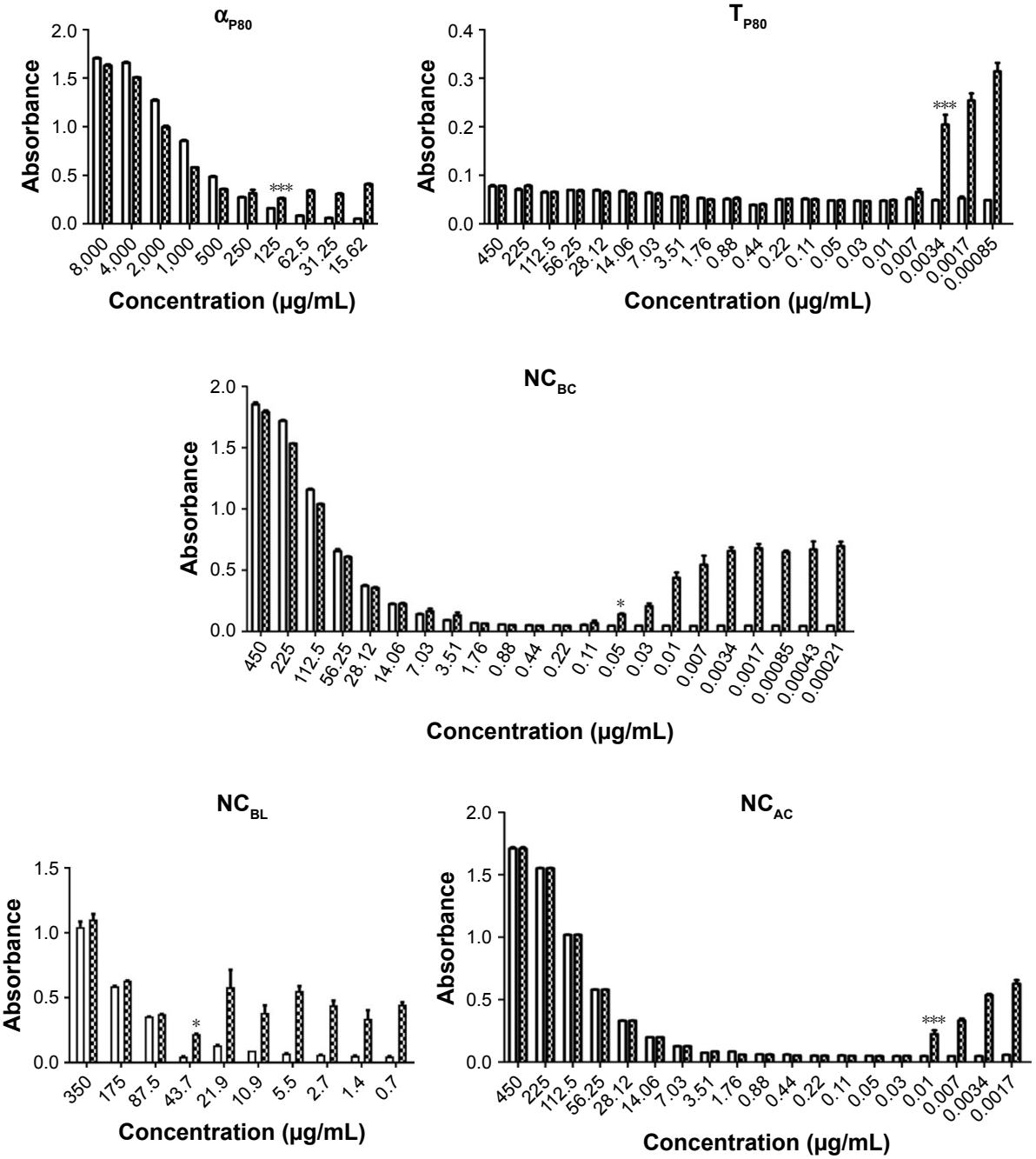

Figure S4 MIC results from for *S. aureus* facing five different formulations.

Notes: ***$p<0.001$ and *$p<0.05$. For T_{P80}, NC_{BC} and NC_{AC} concentration refers to triclosan, for NC_{BL} to chitosan, and for α_{P80} to α-bisabolol. Y-axis refers to absorbance read at 625 nm.

Abbreviations: MIC, minimum inhibitory concentration; NC_{AC}, α-bisabolol and triclosan-coated nanocapsule suspension; NC_{BC}, α-bisabolol and triclosan-uncoated nanocapsule suspension; NC_{BL}, blank nanocapsules; T_{P80}, triclosan dispersed in polysorbate 80; α_{P80}, α-bisabolol dispersed in polysorbate 80.