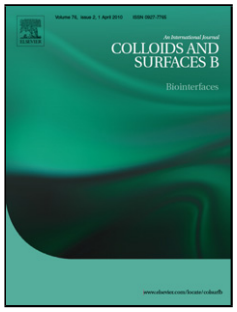


Title: Transforming an inert nanopolymer into broad-spectrum bactericidal by superstructure tuning

Authors: Natalia A. Scilletta, Magdalena Pezzoni, Martín F. Desimone, Galo J.A.A. Soler-Illia, Paolo N. Catalano, Martín G. Bellino

PII: S0927-7765(19)30138-9
DOI: https://doi.org/10.1016/j.colsurfb.2019.02.056
Reference: COLSUB 10047

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 16 January 2019
Revised date: 26 February 2019
Accepted date: 27 February 2019

Please cite this article as: Scilletta NA, Pezzoni M, Desimone MF, Soler-Illia GJAA, Catalano PN, Bellino MG, Transforming an inert nanopolymer into broad-spectrum bactericidal by superstructure tuning, *Colloids and Surfaces B: Biointerfaces* (2019), https://doi.org/10.1016/j.colsurfb.2019.02.056

Transforming an inert nanopolymer into broad-spectrum bactericidal by superstructure tuning

Natalia A. Scilletta[1], Magdalena Pezzoni[2], Martín F. Desimone[3], Galo J. A. A. Soler-Illia[4], Paolo N. Catalano[1] *, Martín G. Bellino[1] *.

[1] Departamento de Micro y Nanotecnología, Instituto de Nanociencia y Nanotecnología - Comisión Nacional de Energía Atómica y Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. General Paz 1499, B1650KNA San Martín, Argentina.

[2] Departamento de Radiobiología, Comisión Nacional de Energía Atómica, Av. General Paz 1499, B1650KNA San Martín, Argentina.

[3] Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Facultad de Farmacia y Bioquímica. Junín 956, Piso 3° (1113), Buenos Aires, Argentina.

[4] Instituto de Nanosistemas, Universidad Nacional de San Martín, Av. 25 de Mayo 1021, 1650 San Martín, Argentina.

*** Corresponding Authors**

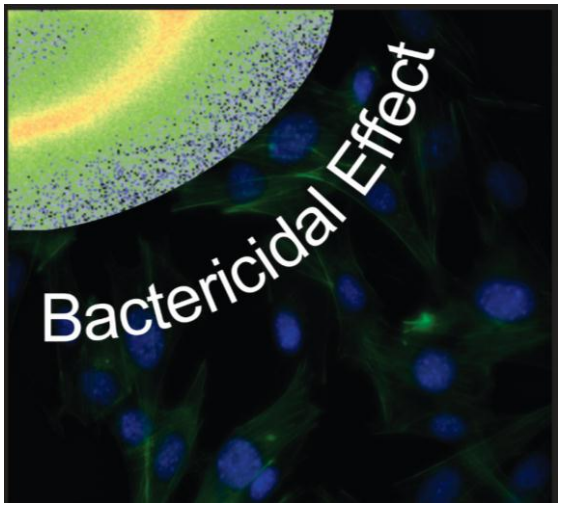
catalano@cnea.gov.ar (P. N. Catalano); mbellino@cnea.gov.ar (M. G. Bellino).

Statistical Summary

Nº of words: 4208 (excluding figure legends, table title and references)

Nº of tables / figures: 1 / 6

Graphical abstract



Highlights

Nano-engineered hybrid Pluronic-titania films show bactericidal activity

Superstructuration can turn inert self-assembled polymer into highly bactericidal

Maximum bactericidal efficiency relies on appropriate superstructured configuration

A hybrid biocompatible coating with broad-spectrum antimicrobial activity is shown

ABSTRACT

Poloxamer block copolymers (also known as Pluronic) are particularly useful for drug delivery and self-assembly techniques. These nanopolymers are generally considered to be biologically inert and they were used to generate only bacteria repellent surfaces but keeps bacteria alive and as a latent threat. However, the inherent capabilities of these nanopolymers to kill bacteria have been largely overlooked. Here, we report that Pluronic shaped as

superstructures (self-organized array of micelles) in fact possess a broad-spectrum bactericidal activity (capability of killing bacteria) similar to that shown for some antibiotics. This further represents the first report that shows that appropriate control of superstructured mesophase architecture is a key parameter for bactericidal efficacy. Based on this finding, we have developed a highly bactericidal coating (>99.9 % kill) against all tested Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Salmonella typhimurium LT2*, *Escherichia coli K12* and *Pseudomonas aeruginosa PAO1*) bacteria which moreover allows the adhesion and proliferation of mammalian cells. The inexpensiveness and ease of production make these versatile nanopolymer structures a powerful tool for the development of a new generation of highly effective antimicrobial coatings.

Keywords: bactericidal; broad-spectrum antibacterial; nano-engineered superstructures; pluronic nanopolymers; functional nano-coatings

INTRODUCTION

There is currently huge interest in the application of polymer-based nanotechnology for a wide range of material designs and biomedical applications.[1,2] Among available polymer nanomaterials, poloxamer block copolymers – hydrophilic poly (ethylene oxide) [PEO)] and hydrophobic poly (propylene oxide) [PPO] blocks arranged in a tri-block structure: PEO-PPO-PEO – which were introduced in 1950s as additives in food and pharmaceutical preparations, are of particular interest for applications in drug delivery and self-assembling techniques.[3,4] Owing to their amphiphilic properties, poloxamers (also named as Pluronic) are known to form micelles in aqueous solution above a critical polymer concentration called critical micelle concentration (CMC). Further increase in surfactant concentration result in the self-organization of micelles into superstructured mesophases.[5] For instance, Pluronic structures have been used

for drug delivery and gene targeting, for imaging molecules, as nano-containers and as templating agents for more complex structures.[6-11]

In the attempt to meet the increasing demand of generating surfaces with the ability to create antibacterial environments, Pluronic coatings have shown to offer either an anti-adhesive function by modifying the hydrophilicity of the coated surface or the possibility of being impregnated or conjugated with antibiotic agents. These approaches combine the improved drug pharmacokinetics and biodistribution power of the Pluronic polymer delivery with the antibacterial activity of the incorporated antibiotic.[12-14] For example, Pluronic-lysozyme conjugates have been used as anti-adhesive and antibacterial coating[15], and a poloxamer composed hydrogel functionalized with Cateslytin allowed prevention of medical implant infection[16]. These dual-functional coatings are based on two components: the Pluronic polymer providing an anti-adhesive, structural and cohesion function, and the incorporated antibiotic agent offering the antibacterial effect. In fact, although a modest number of Pluronic-based bacteriostatic nanomaterials (capable of inhibiting the growth or reproduction of bacteria) have been reported in the literature, [17,18] none of them demonstrated a bactericidal effect (capable of killing bacteria) as was described in this study. All these former Pluronic systems have been typically based on superficial monolayer functionalization or on polymer molecules in solution or gels, showing at most a slight bacteriostatic activity. In particular, lack of bactericidal effect of Pluronic nanopolymers was reported.[19,20] Moreover, poloxamer hydrogels were used to mimic biofilm environment for assessment of confined bacteria susceptibility towards antimicrobial treatments, thus remarking its harmlessness in these experimental conditions.[21-23]

In this work, in contrast, we described that Pluronic nanopolymers possess a broad-spectrum bactericidal activity, and we illustrated the dramatic influence of nanopolymer superstructuration in the bactericidal performance. The synthesis and assessment of mesostructured titania thin films synthesized by the sol-gel method combined with evaporation-induced self-assembly of Pluronic micelles was described. Titania was,

indeed, selected as a matrix owing to its high biocompatibility and low dissolution rate in physiological media.[24] The observed bactericidal and biological properties spotlight its application as a smart coating with highly efficient bactericidal activity (>99.9 %) against all tested Gram-negative (*Salmonella typhimurium LT2*, *Escherichia coli K12* and *Pseudomonas aeruginosa PAO1*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). Moreover, the developed coating promotes the adhesion and proliferation of mammalian cells as assessed by culturing osteoblastic cells over the coated surfaces.

From a biochemical point of view, Pluronic architectures resemble structural motifs of the protective-wall constituents of pathogens, making these nanopolymers capable of interfering in this cell wall synthesis. However, the fact that Pluronics have been conjugated to antibiotic agents to offer an antibacterial activity, including delivery systems and coatings, demonstrates that the presence of this activity has so far been ignored. This work is the first to report that nanopolymer systems based on PEO-PPO-PEO have a bactericidal activity and that it can selectively affect bacterial function while remaining innocuous to mammalian cells. We further showed that appropriate control of the self-assembled nanopolymer superstructure is key to bactericidal efficacy. The fact that these tuned nanopolymer architectures can exhibit an antibacterial activity gives them the potential to become novel smart biomaterial platforms for the development of alternative antimicrobial therapeutics.

MATERIALS AND METHODS

Synthesis and deposition of Pluronic F127-based mesostructured titania thin film coatings

Standard microscope glass slides pieces of 26 mm long and 20 mm wide (5.2 cm^2) or 20 mm-diameter cover glasses (3.14 cm^2) (Marienfeld, Lauda-Königshofen, Germany) were thoroughly washed with neutral detergent (Extran®, Merck Millipore, Darmstadt, Germany), then sequentially rinsed with miliQ water, acetone and isopropanol, and

finally dried under nitrogen flow. The titania mesostructured thin film coatings were obtained from a $TiCl_4$/ethanol solution to which the organic surfactant Pluronic F127 or Pluronic P123 (Sigma-Aldrich, Saint Louis, MO) and water were added. The final composition of the precursor solution was $TiCl_4$:ethanol:H_2O:Pluronic equal to 1:40:10:*S* mol ratios where *S* was varied between 0.0025 and 0.02. Pluronic-free titania thin film coatings were prepared following the same protocol but without the addition of Pluronic surfactant. The coatings were deposited on the glass substrates by spin-coating (2500 rpm) at 35 ºC and at a relative humidity (RH) of 15%. After deposition, the samples were placed in a 50% RH chamber for 24 h and subjected to a consolidation thermal treatment consisting of three successive stages of 1 h at 60ºC, 130 °C and 180ºC.

Film coating characterization

Electron micrographs were recorded using a field-emission scanning electron microscope (FE-SEM; SUPRA 40, Carl Zeiss AG) in the secondary-electron mode, using an in-lens detector to improve resolution. Film mesostructure was analyzed using Small-angle X-ray scattering (XEUSS 1.0 from XENOCS equipment, France; λ = 0.15419 nm that is the weighted average of X-ray wavelength of the *Cu-*$K_{\alpha 12}$ emission lines) at glancing angle (0.3°) incidence and a sample to detector distance of 1377.11 mm. This setup is equipped with a 2D photon counting pixel X-ray detector Pilatus 100k (DECTRIS, Switzerland).

Determination of antibacterial activity of film coatings

The clinically significant pathogens used in this study were: the Gram-negative bacteria *Salmonella typhimurium* (LT2), *Escherichia coli* K12 and *Pseudomonas aeruginosa* (PAO1), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213) and *Bacillus subtilis* (laboratory collection). These strains were routinely grown at 37ºC in Luria–Bertani (LB) broth (yeast extract, 5 g L^{-1}; NaCl, 10 g L^{-1} and triptone, 10 g L^{-1}); 15

g L^{-1} agar was added when solid medium was used. The antibacterial activity of Pluronic-based mesostructured film coatings deposited on glass slide pieces of 5.2 cm^2, was tested in the darkness on bacterial strains following the standard methodology to characterize antibacterial coatings.[25] Sample surfaces were exposed to 300 µL-inoculums with 1 x 10^6 CFU (colony forming units) mL^{-1}. Inoculums were prepared by dilution in sterile saline solution of overnight cultures and were then spread on the surface of each sample in triplicate. Inoculated samples were incubated at 37ºC for 4, 8, 16 or 24 h in a humidified chamber to avoid evaporation. Aliquots of bacterial suspensions were taken before (0 h) and after exposure to film coatings; the neat suspension and serial dilutions were plated onto LB solid medium. The plates were incubated at 37ºC for 24 h and the colonies were counted to assess cell viability. Control samples were Pluronic-free titania-coated glass slides. The bactericidal efficiency (%) was expressed in reference to CFU at 0 h. The survival fraction was calculated as CFU after incubation with film coatings over CFU at 0 h.

Cell membrane integrity was determined by staining with a mixture of SYTO 9 and propidium iodide fluorescent dyes. The recovered cells were washed with distilled water and immersed in a solution containing 3.5 µM SYTO 9 and 20µM propidium iodide in distilled water, then incubated in the dark for 15 min at room temperature. SYTO 9 is a fluorescing green dye that permeates both intact and damaged membranes of the cells. On the other hand, propidium iodide is a fluorescing red dye that enter only cells with significant membrane damage and binds with higher affinity to nucleic acids than SYTO 9. Images were visualized with an Epifluorescence Microscope Olympus BX51.

Bacterial DNA was quantified in the supernatants recovered from the coated slides previously centrifuged in order to remove the cells, by evaluating the absorbance at 260/280 nm using a Nanodrop 2000 (Thermo Scientific NanoDrop®).

The antibacterial activity was calculated using two different parameters: percent reduction (*D%*) and value of antimicrobial activity (*R*). *D%* was calculated from

Pluronic-free titania coatings – Pluronic-based mesostructured coatings microbial count percentage ratio.

R was calculated according to JIS Z 2801:2000: [25]

R = log (B/C)

where B is the average number of viable bacteria on the Pluronic-free titania coatings after 24 hs and C is the average number of viable bacteria on the Pluronic-based mesostructured coatings after 24 h.

Osteoblastic cell culture over film coatings: adhesion, proliferation and morphology assays

MC3T3E1 osteoblastic mouse calvaria-derived cells (ATCC® CRL-2593™) were grown in 100 mm culture dishes at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 80 mg L^{-1} gentamycin and 10% (v/v) fetal bovine serum (FBS). When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin-1 mM EDTA in Ca^{2+}–Mg^{2+} free phosphate buffered saline PBS. For experiments, 20 mm-diameter cover glasses coated with Pluronic-based mesostructured titania films or Pluronic-free titania films (control samples) were placed in 12-well plates. Cells were seeded on film coatings at a density of 2 x 10^4 cells per well. A 250 L volume containing the cells was dropped onto the samples for 4 h to allow cells adhesion. Afterwards, 2 mL of medium were added to each well. Control cells were seeded directly on culture plate wells.

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay immediately after the cell adhesion allowing step (day 0) and after 1, 3 and 7 days of culture. After incubation periods, a 0.4 mg mL^{-1} solution of MTT was added to each well and plates were incubated (37ºC, 5% CO_2) for 4 h. Afterwards, the MTT solution was removed, the wells were washed with PBS and the blue formazan crystals were dissolved using DMSO. The purple solution was read at 540 nm. The percentage of cell metabolic activity was expressed

relative to control group at day 0. The results are from triplicate samples in three independent cultures.

F-actin and nucleus of cells were identified by fluorescent Alexa Fluor® 488-phalloidin (Thermo Fisher Scientific, Waltham, MA) and Hoechst 33258 (Sigma–Aldrich), respectively. After 2 days in culture, the samples were fixed with 3.7% paraformaldehyde for 20 min and permeabilized/blocked with 0.1% Triton X-100, 1% BSA in PBS for 30 min, respectively. Then, the samples were stained with Alexa Fluor® 488-phalloidin (1:40 dilution) and Hoechst 33258 (10 g mL^{-1}) for 30 min. The samples were then imaged using a fluorescence microscope (Nikon Eclipse TS100). The staining was analyzed in triplicates.

Statistics

All quantitative results were obtained from triplicate samples. Data were expressed as mean ± SEM. Statistical analysis was carried out using T-test or two-way ANOVA. A value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

In this study, the antibacterial activity of Pluronic-loaded oxide thin film coatings presenting different lyotropic mesophases was assessed. For this purpose, crack-free hybrid titania-Pluronic mesostructured thin films (film thickness ranges between 250 and 290 nm – see Figure 1A) were obtained via a sol−gel method combined with supramolecular self-assembly, following protocols described in the literature.[26,27] Briefly, these hybrid films were produced by spin–coating of glass substrates in water/alcohol acidic solutions containing the inorganic precursor ($TiCl_4$) and the Pluronic templates. The use of Pluronic F127 and Pluronic P123 surfactants allowed to modulate the mesophase structure where we have systematically changed the Ti:Pluronic mol ratio. Pluronic P123 ($PEO_{19}PPO_{69}PEO_{19}$) indeed presents roughly half the molecular weight of Pluronic F127 ($PEO_{100}PPO_{65}PEO_{100}$) polymer. SAXS patterns

collected in grazing incidence to the substrates showed well-defined diffraction rings, indicating locally ordered domains at the mesoscale for the samples used in this study. Figure 1B-H show these typical SAXS patterns that illustrate the periodic nanostructures obtained displaying different average interdistances by adjusting the relevant synthesis parameters such as the nanopolymer size and/or amount. The observed SAXS signal distortion (not distributed along a circle but along an ellipse) is caused by an anisotropic contraction that takes place from the adhesion of the films to the substrate. By analyzing the SAXS patterns, the Pluronic templated mesophases appeared elliptical, distributed homogeneously and were of expected nanometric interdistances (average distances parallel and perpendicular to the film surface that show the different hybrid mesophases obtained are inset in Figure 1B-H). As also expected, in the Pluronic-free titania films no SAXS signal was observed. Raman analyses confirmed the successful syntheses of these hybrid titania-nanopolymer coatings (Supplementary Fig. S1 in Supplementary Information). Optical profilometry characterizations further support the existence of a typical nano-roughness in the hybrid films (see Fig S2 in SI).

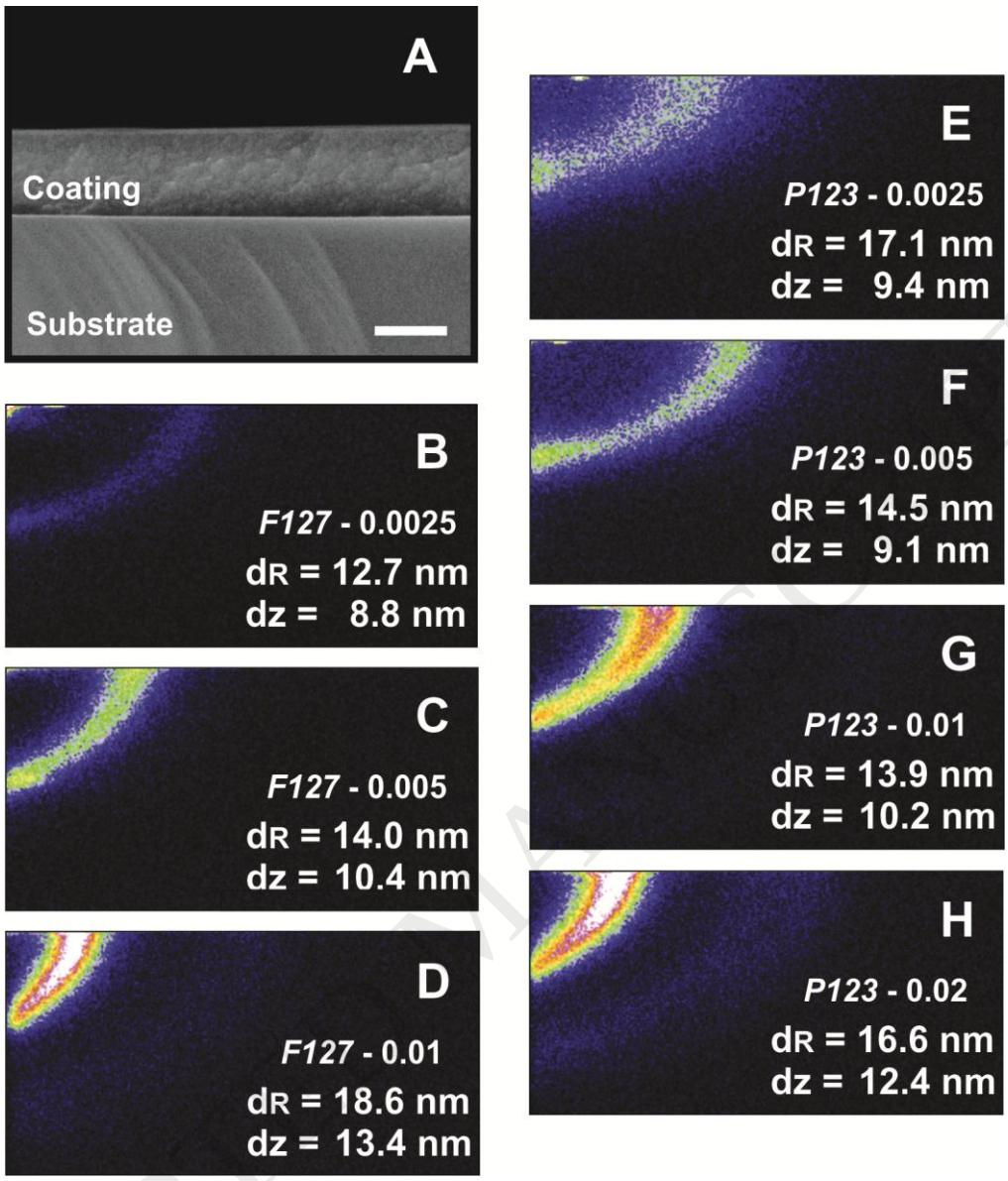


Figure 1. Structural characterization of Pluronic mesostructured thin films: A) Typical FE-SEM cross-section view of the Ti:F127 = 0.005 loaded film (the scale bar represents 200 nm); B-H) representative 2D SAXS patterns of the samples with variable concentrations of surfactant used in this study, depicting the local order arrangement. Average distances parallel (d_R) and perpendicular (d_Z) to the film surface measured by SAXS patterns according to Ti:Pluronic molar ratios are shown for each sample, in the lower right corner of each graph.

To evaluate the bactericidal properties of the Pluronic-loaded titania films, bacterial inoculums were incubated in the darkness on the coating surfaces and the bactericidal efficiency was monitored using a standard viability plate-count technique. Figure 2 shows the results from an assay in which the films loaded with Pluronic F127 and P123 prepared with varying amounts of surfactants were challenged with 1 x 10^6 CFU mL^{-1} of *Salmonella typhimurium* bacterium. Bacterial viability was assessed 24h after inoculating the surfaces. These hybrid coatings possess bactericidal activity as shown in Figure 2, whereas the titania control films (i.e., without Pluronic F127) showed no antibacterial activity. Although it is known that titania-based systems can present bactericidal activity under UV-visible light irradiation,[28,29] as expected since our experiments were performed in the dark, no antibacterial activity has been observed with the Pluronic-free titania films. The data moreover showed that to one tuned amount of template in the formulation, the antibacterial activity of the surface increases for both Pluronic F127 and P123 surfactant. Indeed, at the extremes of Ti:Pluronic ratio the surfaces are moderately active. However, if the weight percent is at an appropriately modulated value the surfaces become extremely active. The unlike activities are, indeed, traced back to their dissimilar textures. More interestingly, both Pluronic F127 and P123 templated surfaces showed the highest performance at coincident topographic interdistances parallel to film surface closer to 14 nm (see Figure 1C and 1G). These results demonstrate that a straightforward Pluronic-loaded surface with the appropriate mesoarchitecture is able to kill bacteria with high efficiency. This illustrates that structural characteristics of the mesostructured titania system provide distinctive features that presents an extraordinary antibacterial efficiency. In what follows, the attention shall be centered on the activity of Pluronic F127-loaded coating with the 14 nm characteristic parallel distance (highest antibacterial activity film - hereafter labelled TF14). These TF14 films, in addition, showed a fast antibacterial response, killing up to 65 % of bacteria in the first 4 h (Figure 3). The bactericidal efficacy is worthy of mentions, because these Pluronic-

based mesostructures has killing rates of 136,000 bacteria cm^{-2} area over the first 4 h.

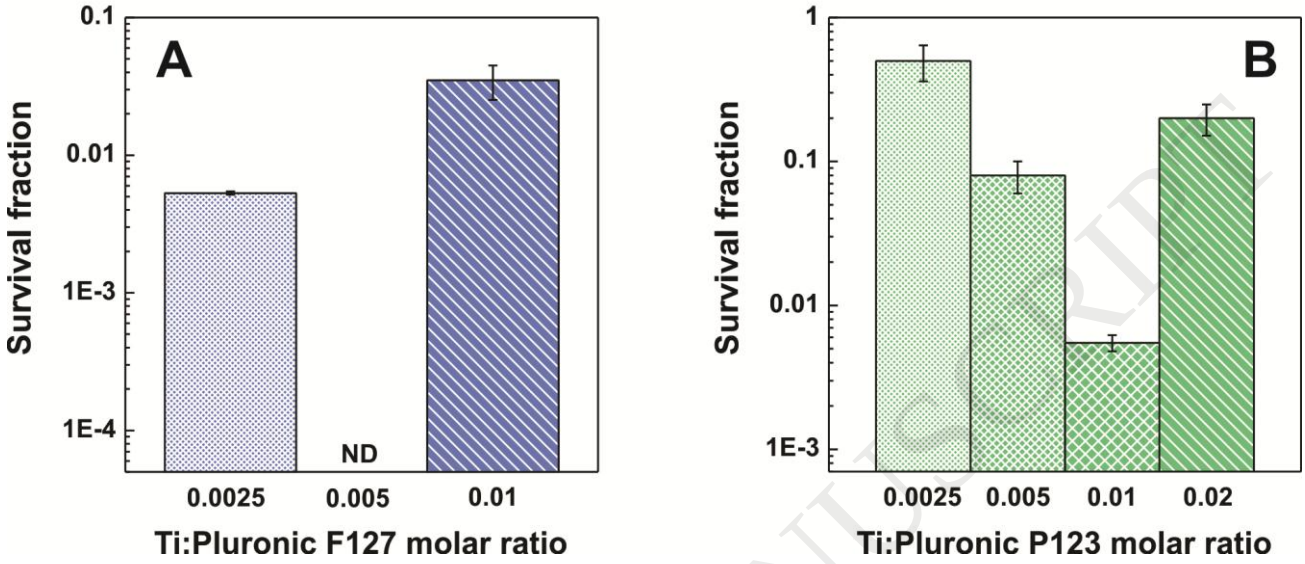


Figure 2. Bactericidal activity of the mesostructured surfaces: Pluronic F127 (A) and Pluronic P123 (B) loaded films with variable concentrations of surfactant were challenged with 10^6 CFU ml^{-1} of *Salmonella typhimurium*. Survival fraction of bacteria after 24 h on the various Pluronic-loaded films is reported. ND: Non-detectable.

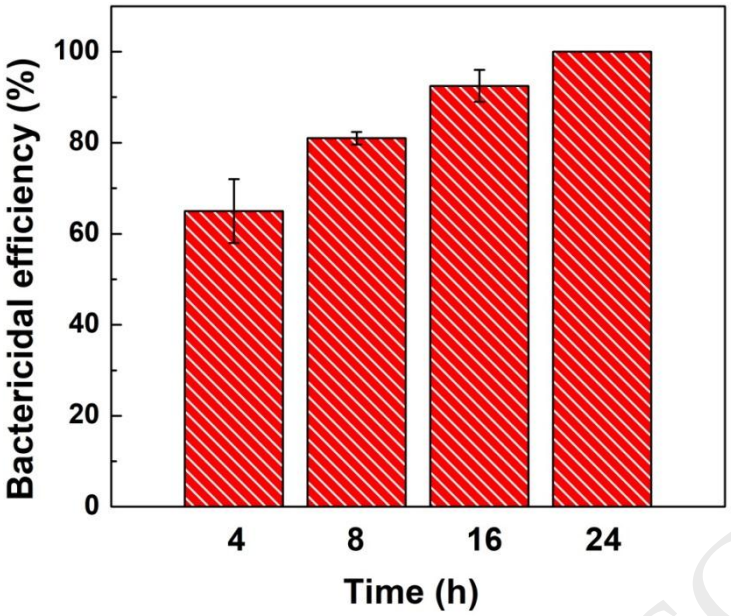


Figure 3. Bactericidal response over time: progressive % kill of *Salmonella typhimurium* when incubated with TF14 films for up to 24 h.

To assess Pluronic F127-loaded coating effect on bacteria viability, the suspension recovered from the surfaces were analyzed using the fluorescent dye propidium iodide which penetrates all ruptured bacterium, causing red fluorescence to indicate the dead bacteria that have damaged membranes, whereas bacterial cells that look green are live cells with intact membranes. This bacterial viability assay showed that bacteria wall integrity was significantly disrupted and bacteria dead after incubation on the F127-loaded coatings but remained mainly intact after incubation on the Pluronic-free titania films (Figure 4A-B). DNA present in the bacteria suspension after inoculating the Pluronic-based films confirms the massive cell wall disruption induced by these mesostructured surfaces (Figure 4C). The effect of coatings on bacterial morphology was also carried out using electron microscopy after 24 h incubation (Figure 4). Lesions and holes were evident in the bacteria cell wall of the F127-loaded films treated bacteria (Figure 4E), while the morphology of bacteria treated with F127-free coatings was observed with no nicks, tears or membrane disintegration (Figure 4D). The results indicated that the disrupting action exerted by mesostructured Pluronic-

loaded coatings on the bacterial wall is substantial, and confirmed the significant potential for Pluronic superstructures to be used in leading to bactericidal tools.

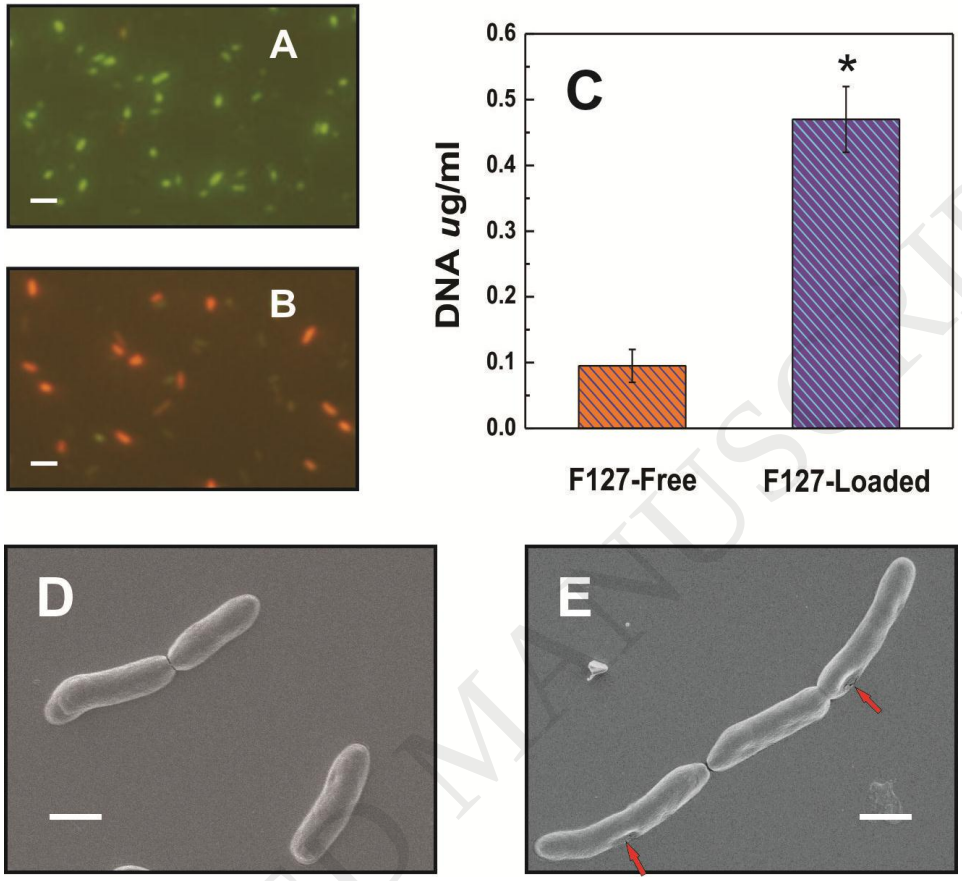


Figure 4. Cell membrane integrity: Fluorescence microscopy images of the bacteria suspension after 24 h inoculating F127-free films (A) and TF14 films (B). Bacteria that have damaged membranes are labelled in red and cell with intact membranes in green. Scale bars represent 10 μm. (C) The amount of DNA present in the bacteria suspension after incubation on the surfaces. T-test, $p<0.005$, * significantly different from F127-free coatings. Morphology of *Salmonella typhimurium* after contact with F127-free coatings (D) and TF14 films (E) (scale bars = 1 μm). Red arrows indicate some of the lesions and holes observed on the bacteria cell wall after incubation with F127-loaded coatings.

In order to analyze the bactericidal activity spectrum of the Pluronic-mesostructured coatings, five species of bacteria were incubated on the TF14 surfaces for periods of 24h and the bacterial viability was monitored. The selected bacterial strains, representing significant prokaryotic taxa towards models in biofilm and bacterial suspension studies, included Gram-negative and Gram-positive important opportunistic pathogens in humans. In this sense, prototypical bacterial strains were selected: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and the aforementioned *Salmonella typhimurium*. Figure 5 shows the results from the assay in which the TF14 coatings were challenged with 1×10^6 CFU mL^{-1} to emulate a high contaminated scenario. These hybrid coatings were extremely active against all tested bacterial strains, unlike Pluronic F127-free titania films that showed no antibacterial activity. In fact, Pluronic-loaded coatings demonstrated an inherently broad-spectrum antibacterial activity with a killing efficiency >99.999, except for *Bacillus subtilis* for which, despite being a sporulating bacteria, the antibacterial efficiency was >99.9% (Table 1). Moreover, the value of antimicrobial activity (*R*) for these coatings, obtained by the JIS Z 2801:2000 testing method, was significantly higher than the minimum established by the standard norm to recognize the surface antimicrobial efficacy of a product (Table 1).[30]

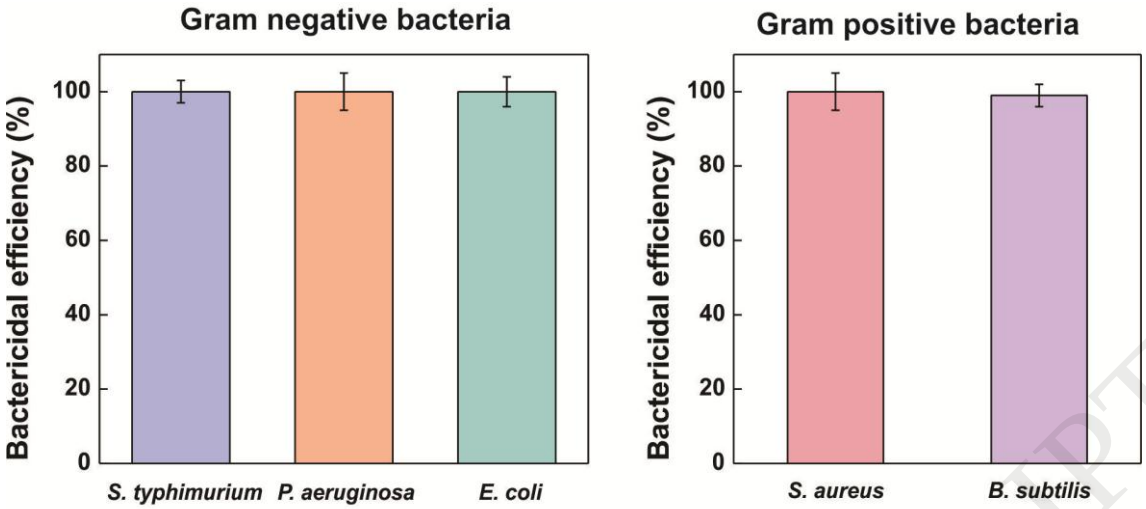


Figure 5. Broad spectrum bactericidal activity: Antimicrobial activity of Pluronic F127-loaded titania coatings against five clinically significant bacterial strains after 24 h (challenged with 1 x 10^6 CFU mL^{-1}).

Table 1. Pluronic F127-loaded titania coating antibacterial activity test results for five clinically significant bacterial strains.

Strains	CFU[a] 0 h	F127-loaded coatings		
		CFU 24 h	D%[b]	R[c]
S. typhimurium	2,28 x 10^6	<10	>99,999	>6,43
P. aeruginosa	4,2 x 10^6	<10	>99,999	>6
E. coli	6,2 x 10^6	<10	>99,999	>5,69
S. aureus	3,4 x 10^6	<10	>99,999	>3,74
B. subtilis	2,5 x 10^6	1 x 10^3	99,933	2,67

[a] CFU was calculated from the mean of 3 CFU counts.

[b] Percent reduction.

[c] Value of antimicrobial activity.

The high yield of bactericidal activity raised the question of whether the bacteria were simply attached to the surface during incubation. The fact that, after incubation, only a very few bacteria were found on the surface through optical microscopy analysis, confirmed that the Pluronic-containing surface killed the bacteria. These results highlight the successful antimicrobial activity of the Pluronic-loaded titania coatings. It is important to rule out the possibility that the observed activity is caused by leaching of nanopolymer into bacterial dispersion. To test this, we incubated the Pluronic-loaded films in the standard bacterial medium for 24 h (the time taken to observe bacteria killing) and then removed the medium from the surface. This leaching solution had no antibacterial activity, showing that the observed activity cannot be attributed to the leaching of the nanopolymer into solution, but occurs on the surface of the Pluronic-loaded titania films. In addition, to discard a possible effect of Pluronic solutions, a wide range of nanopolymer concentrations were tested showing absence of antibacterial activity, in accordance with previous reports.[19,20,31,32] Considering these results, we evaluated bactericidal properties of the films after extracting Pluronic by calcination. These nanostructured titania films show no capacity of killing bacteria, reinforcing the hypothesis that both features of the system (topographical arrangement and nanopolymer chemistry) are necessary to achieve antibacterial activity.

In order to assess the potential biocompatibility of these antibacterial films, MC3T3E1 osteoblastic cells were seeded and grown on them over 7 days and cell adhesion and proliferation was assessed by the Methyl tetrazolium (MTT) assay. Figure 6A shows that the cell metabolic activity increases with culture time, indicating cell viability and proliferation on the Pluronic F127-loaded coatings. No differences in osteoblastic cell adhesion (time 0) and proliferation (1, 3 and 7 days) on these nanopolymer-loaded surfaces were, indeed found compared with Pluronic-free films or even culture plate well surfaces. As nanopolymer-contaning surface did not affect osteoblastic cell viability and growth, we performed additional studies to assess cell appearance. To investigate the morphology and the cytoskeletal organization of

osteoblasts, cells were double-stained with Alexa Fluor® 488−phalloidin and Hoechst 33258 (Figure 6B). Cells grown 48 h over the Pluronic F127-loaded coatings exhibited well-formed cytoskeletal structure, similar to what was observed in Pluronic F127-free titania films and in conventional culture plates. These results together, show that the nanopolymer-loaded coatings are capable of avidly killing bacteria while sparing mammalian cells.

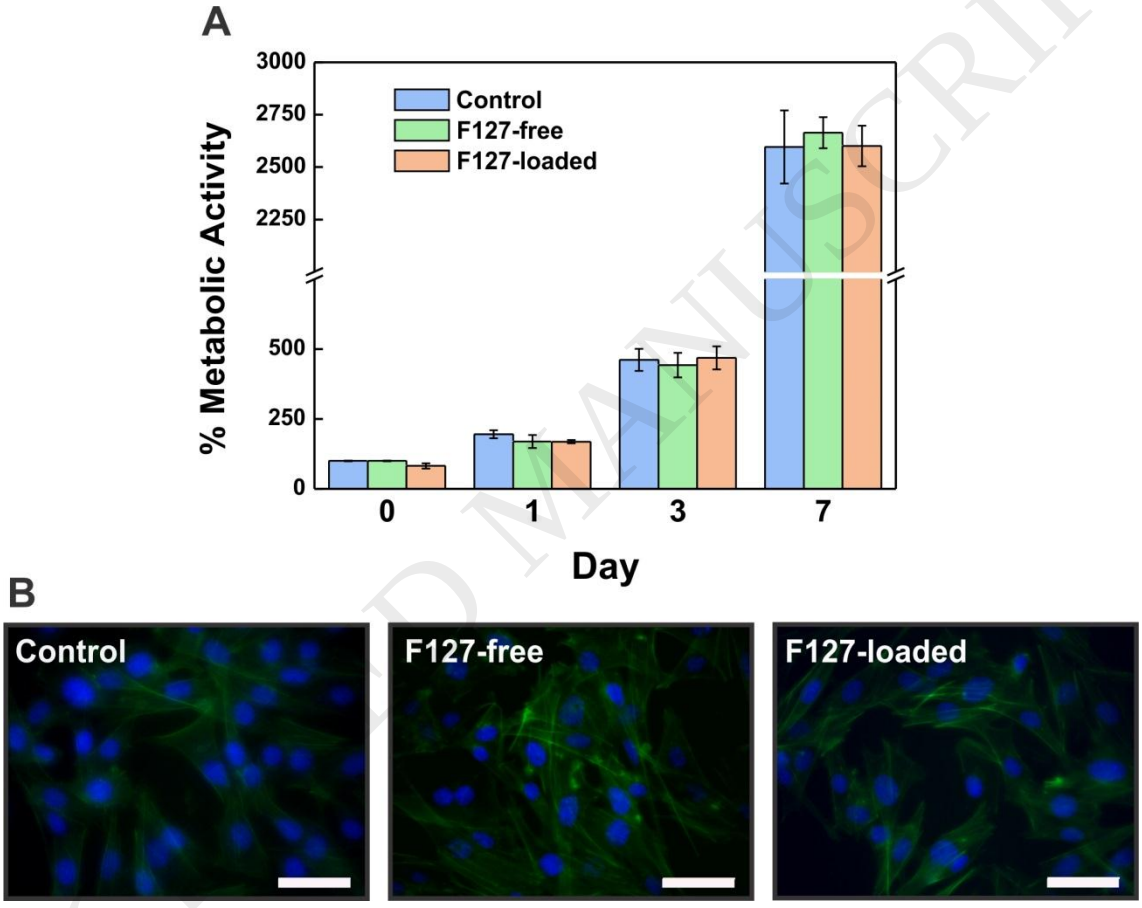


Figure 6. Biocompatibility studies: A) Percentage of metabolic activity of osteoblasts (MTT assay) grown on traditional culture plates (Control), Pluronic F127-free titania coatings (F127-free) and Pluronic F127-loaded titania coatings (F127-loaded), where Control group at day 0 were normalized to 100% at each time point; two-way ANOVA: ns between groups; B) Fluorescence micrographs of osteoblasts on the same samples after 48 h of incubation. The magnification is 400X. The scale bars represent 50 μm.

The strategy in the selection of the mesostructured Pluronic system to eventually kill the microbes as well as offer good biocompatibility was based on five observations: (1) the strategy of micro and nano-structuring surfaces to achieve remarkable antibacterial effects was observed in nature[33-35], (2) unlike previously applied configurations, the hybrid coatings reported here present a superstructured mesophase, (3) a broad family of antibiotics act by interfering the continuous renovation of the protective outer wall of bacteria, weakening the peptidoglycan scaffold within the bacterial wall and leading to osmotic lysis and causing bacteria death, (4) there is evidence of similarities between PEO-PPO-PEO chemical domains and sugars found in the lipopolysacharide and peptidoglycan structures present the bacterial membrane and wall[36] and (5) the family of Pluronic block copolymers are classically considered among the least toxic of known surface-active agents. From the above arguments, we postulate that mesostructured Pluronic interfaces to which bacteria interact offer particular topo-chemical tags that can hamper peptidoglycan assembly machinery during the synthesis and renovation of the bacterial cell wall, compromising the structural bacteria wall conformation to effectively damage bacteria integrity. Since mammalian cells lack a peptidoglycan wall structure, this action selectively targets bacteria with no negative effect on mammalian cells, as done by antibiotics. In fact, osteoblastic cells normally attached to the surface coatings and proliferated as done by cells seeded on standard culture plates, showing a normal structural integrity. This is an important finding, as previous coatings based on Pluronic F127 monolayer functionalization for antiadhesive applications suppress cell integration. Furthermore, the physico-chemical characteristic of this antibacterial mechanism is also attractive in terms of the potentially low probability of bacterial resistance development.

CONCLUSIONS

We here provide the first report that mesostructured systems based on PEO-PPO-PEO nanopolymers possess a broad spectrum bactericidal activity. The appropriate

tuning of the self-assembled nanopolymer superstructure determines the overall bactericidal efficiency of the hybrid coatings. We have exploited Pluronic-mesostructured titania films synthetized by means of a simple process to create a highly bactericidal coating against the five clinically significant pathogens tested with an effectivity above 99.9%, including Gram-negative and Gram-positive bacteria. We postulate that both the topographical arrangement and nanopolymer chemistry of mesostructured Pluronic interfaces to which bacteria interact, compromise bacterial cell wall integrity and are responsible for the high bacteria killing. The osteoblastic cell culture results showed that the coatings have no detrimental effect on mammalian cells which could attach and proliferate on the film surface, opening the way to novel biomaterial coatings to generate self-sterilized implants and prosthesis. It is worth mentioning that these nano-coatings are inexpensive, modular and scalable; and the production and processing steps involved are compatible with the techniques and substrates used in the biomaterial industry. A straightforward application of this coating type in route to advanced biotechnology and medicine can therefore be anticipated. Furthermore, the proficient ability of incorporating selected drugs in the templated mesophases within the oxide matrix to provide more benefits suggests the future development of antibacterial biomaterial coatings with additional functionalities. Our findings pave the way for a wide range of new potential applications of Pluronic nanopolymer based systems in the field of biomaterials and in other biomedical developments.

ACKNOWLEDGMENTS

The authors thank Ms. Mariel Atia for her assistance with cell culture preparations. N.A.S. is grateful for her doctoral fellowship granted by CONICET. M.P. is member of CNEA and CONICET. M.F.D., G.J.A.A.S-I, P.N.C. and M.G.B. are members of CONICET. This work was supported by CONICET (PIP 561) and ANPCYT (PICT-2016-0647 and PICT- 2016-1781), Argentina.

AUTHOR CONTRIBUTIONS

P.N.C. and M.G.B. conceived and designed the experiments. N.A.S. and M.P. carried out the preparation of the samples and performed the experiments. M.F.D. participated in the design of biocompatibility assays. P.N.C. and M.G.B. wrote the paper and are equally responsible for the work. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS

The authors declare no competing financial interests.

MATERIALS & CORRESPONDENCE

Correspondence and requests for materials should be addressed to P.N.C. (catalano@cnea.gov.ar) or to M.G.B. (email: mbellino@cnea.gov.ar).

REFERENCES

1 Kesharwani, S. S., Kaur, S., Tummala, H. & Sangamwar, A. T. Multifunctional approaches utilizing polymeric micelles to circumvent multidrug resistant tumors. *Colloids and Surfaces B: Biointerfaces* 173, 581-590, doi:https://doi.org/10.1016/j.colsurfb.2018.10.022 (2019).

2 Sun, L., Wu, Q., Peng, F., Liu, L. & Gong, C. Strategies of polymeric nanoparticles for enhanced internalization in cancer therapy. *Colloids and Surfaces B: Biointerfaces* 135, 56-72, doi:https://doi.org/10.1016/j.colsurfb.2015.07.013 (2015).

3 Lundsted, L. & Schmolka, I. The applications of block copolymer polyol surfactants. *Block and graft copolymerization. John Wiley & Sons, New York*, 113-255 (1976).

4 Nace, V. *Nonionic surfactants: polyoxyalkylene block copolymers*. Vol. 60 (CRC Press, 1996).

5 Brinker, C. J., Lu, Y., Sellinger, A. & Fan, H. Evaporation-induced self-assembly: nanostructures made easy. *Advanced materials* 11, 579-585 (1999).

6 Zhang, Y. *et al.* Therapeutic surfactant-stripped frozen micelles. *Nature Communications* 7, 11649 (2016).

7 Alakhov, V. *et al.* Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. *Colloids and Surfaces B: Biointerfaces* 16, 113-134 (1999).

8 Valle, J. *et al.* A phase II, window study of SP1049C as first-line therapy in inoperable metastatic adenocarcinoma of the oesophagus. *Journal of Clinical Oncology* 22, 4195-4195 (2004).

9 Li, S. D. & Huang, L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. *Gene Therapy* 13, 1313, doi:10.1038/sj.gt.3302838 (2006).

10 Batrakova, E. V. & Kabanov, A. V. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. *Journal of Controlled Release* 130, 98-106 (2008).

11 de Freitas, C. F. *et al.* PEG-coated vesicles from Pluronic/lipid mixtures for the carrying of photoactive erythrosine derivatives. *Colloids and Surfaces B: Biointerfaces* 175, 530-544, doi:https://doi.org/10.1016/j.colsurfb.2018.12.031 (2019).

12 Veyries, M. *et al.* Controlled release of vancomycin from poloxamer 407 gels. *International journal of pharmaceutics* 192, 183-193 (1999).

13 Muszanska, A. K. *et al.* Antiadhesive polymer brush coating functionalized with antimicrobial and RGD peptides to reduce biofilm formation and enhance tissue integration. *Biomacromolecules* 15, 2019-2026 (2014).

14 Wang, R., Chua, K. L. & Neoh, K. G. Bifunctional coating with sustained release of 4-amide-piperidine-C12 for long-term prevention of bacterial colonization on silicone. *ACS Biomaterials Science & Engineering* 1, 405-415 (2015).

15 Muszanska, A. K., Busscher, H. J., Herrmann, A., van der Mei, H. C. & Norde, W. Pluronic–lysozyme conjugates as anti-adhesive and antibacterial bifunctional polymers for surface coating. *Biomaterials* 32, 6333-6341 (2011).

16 Mateescu, M. *et al.* Antibacterial peptide-based gel for prevention of medical implanted-device infection. *PLoS ONE* 10, e0145143 (2015).

17 Jagannath, C., Allaudeen, H. & Hunter, R. L. Activities of poloxamer CRL8131 against Mycobacterium tuberculosis in vitro and in vivo. *Antimicrobial agents and chemotherapy* 39, 1349-1354 (1995).

18 Hunter, R. L., Jagannath, C., Tinkley, A., Behling, C. A. & Nolte, F. Enhancement of antibiotic susceptibility and suppression of Mycobacterium avium complex growth by poloxamer 331. *Antimicrobial agents and chemotherapy* 39, 435-439 (1995).

19 Veyries, M.-L., Faurisson, F., Joly-Guillou, M.-L. & Rouveix, B. Control of staphylococcal adhesion to polymethylmethacrylate and enhancement of susceptibility to antibiotics by poloxamer 407. *Antimicrobial agents and chemotherapy* 44, 1093-1096 (2000).

20 Trott, A. T. in *Wounds and Lacerations (Fourth Edition)* (ed Alexander T. Trott) 73-81 (W.B. Saunders, 2012).

21 Clutterbuck, A. L., Cochrane, C. A., Dolman, J. & Percival, S. L. Evaluating antibiotics for use in medicine using a poloxamer biofilm model. *Annals of Clinical Microbiology and Antimicrobials* 6, 2 (2007).

22 Yamada, H., Koike, N., Ehara, T. & Matsumoto, T. Measuring antimicrobial susceptibility of Pseudomonas aeruginosa using Poloxamer 407 gel. *Journal of Infection and Chemotherapy* 17, 195-199 (2011).

23 Gilbert, P. *et al.* The use of poloxamer hydrogels for the assessment of biofilm susceptibility towards biocide treatments. *Journal of applied microbiology* 85, 985-990 (1998).

24 Schmidt, J. & Vogelsberger, W. Aqueous long-term solubility of titania nanoparticles and titanium (IV) hydrolysis in a sodium chloride system studied by adsorptive stripping voltammetry. *Journal of solution chemistry* 38, 1267-1282 (2009).

25 Association, J. S. Antimicrobial products-Test for antimicrobial activity and efficacy, Japanese Industrial Standard JIS Z 2801, Reference number. *JIS* (2000).

26 Crepaldi, E. L. *et al.* Controlled formation of highly organized mesoporous titania thin films: from mesostructured hybrids to mesoporous nanoanatase TiO2. *J Am Chem Soc* 125, 9770-9786 (2003).

27 Soler-Illia, G. *et al.* Mesoporous hybrid and nanocomposite thin films. A sol–gel toolbox to create nanoconfined systems with localized chemical properties. *Journal of sol-gel science and technology* 57, 299-312 (2011).

28 Dalrymple, O. K., Stefanakos, E., Trotz, M. A. & Goswami, D. Y. A review of the mechanisms and modeling of photocatalytic disinfection. *Applied Catalysis B: Environmental* 98, 27-38 (2010).

29 Joanne, G. & Zisheng, Z. Applications of Photocatalytic Disinfection. *International Journal of Photoenergy* 2010 (2010).

30 Catalano, P. N. *et al.* Optically transparent silver-loaded mesoporous thin film coating with long-lasting antibacterial activity. *Microporous and Mesoporous Materials* 236, 158-166 (2016).

31 Rodeheaver, G. T., Smith, S. L., Thacker, J. G., Edgerton, M. T. & Edlich, R. F. Mechanical cleansing of contaminated wounds with a surfactant. *The American Journal of Surgery* 129, 241-245 (1975).

32 Demirci, S. *et al.* Boron and poloxamer (F68 and F127) containing hydrogel formulation for burn wound healing. *Biological trace element research* 168, 169-180 (2015).

33 Ivanova, E. P. *et al.* Natural Bactericidal Surfaces: Mechanical Rupture of Pseudomonas aeruginosa Cells by Cicada Wings. *Small* 8, 2489-2494, doi:doi:10.1002/smll.201200528 (2012).

34 Rigo, S. *et al.* Nanoscience-Based Strategies to Engineer Antimicrobial Surfaces. *Advanced Science* 5, 1700892, doi:doi:10.1002/advs.201700892 (2018).

35 Minoura, K. *et al.* Antibacterial effects of the artificial surface of nanoimprinted moth-eye film. *PLoS ONE* 12, e0185366, doi:10.1371/journal.pone.0185366 (2017).

36 Moghimi, S. M., Wibroe, P. P., Wu, L. & Farhangrazi, Z. S. Insidious pathogen-mimicking properties of nanoparticles in triggering the lectin pathway of the complement system. *European Journal of Nanomedicine* 7, 263-268 (2015).