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E. coli as a model for the description of the antimicrobial mechanism of a cationic polymer surface: cellular target and bacterial contrast response

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ABSTRACT

In this study we use *E. coli* as a model to investigate the antimicrobial mechanism of a film made of a copolymer based on mPEG, MMA and DMAEMA and whose surface is active towards Gram-negative and Gram-positive bacteria. The polymer contains not quaternized amino groups able to generate a charged surface by protonation when in contact with water. For the purpose, we adopted a dual strategy based on the analysis of cell damage caused by contact with the polymer surface, and on the evaluation of the cell response to the surface toxic action. The lithic effect on the protoplasts of *E. coli* showed that polymer surface can affect the structure of cytoplasmic membranes while assays of calcein leakage from LUVs at different phospholipids composition indicated that action on membranes does not need a functionally active cell. On the other hand, the significant increase in sensitivity to actinomycin D demonstrates that polymer interferes also with the structure of the outer membrane, modifying its permeability. The study on gene expression, based on the analysis of the transcripts in a temporal window where the contact with the polymer is not lethal and the damage is reversible, showed that some key genes of the synthesis and maintenance of OM structure (*fab*R, *fad*R, *fab*A, *waa*A, *waa*C, *kds*A, *pld*A, *pag*P), as well as regulators of cellular response to oxidative stress (*sox*S), are more expressed when bacteria are exposed to polymer surface.

All together these results identified the outer membrane as the main cellular target of antimicrobial surface and indicated a specific cellular response to damage, providing more information on the antimicrobial mechanism. In perspective, data here reported could play a pivotal role in a microbial growth control strategy based not only on the structural improvements of the materials, but also on

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the possibility of intervening on the cellular pathways involved in the contrast reaction to these and other polymers with similar mechanisms.

Nanomaterials are attracting enormous interest owing to their potential in specific applications such as electronics, information technology, medicine, food safety etc. In this framework, synthetic polymers can represent an excellent opportunity of developing materials with e.g. inherent antimicrobial and/or biocidal activity. This can represent an answer to the increasing demand for antimicrobial active food packaging, medical devices or water purification systems. In contrast, most is still unknown on the biological mechanism of action of such materials, a fundamental requirement for designing polymers with higher antimicrobial efficiency and possibly very low toxicity.[1]

So far, biologists and material scientists identified a few features of bacterial cells that can be strategically exploited to make polymer inherently antimicrobial. In particular, it is well known that the external envelope of bacteria is characterized by the presence of a net negative charge generally stabilized by divalent cations such as Ca^{2+} and Mg^{2+}. The negative charge comes from the teichoic or lipothecoic acids of the Gram-positive cell wall (CW) or from lipopolysaccharides and phospholipids of the Gram-negative bacteria outer membrane (OM). Being composed of phospholipids and embedded proteins, cytoplasmic membrane (CM) is also negatively charged. As a consequence, synthetic antimicrobial polymers soluble in water present cationic groups; however, they must also possess some degree of hydrophobicity to allow initial polymers adhesion to negatively charged cell surface and subsequent membranes (CM and OM) penetration and permeabilization.

As support to such mechanistic hypothesis, Ikeda et al.[2] reported that the lysis of protoplast of *Bacillus subtilis* showed that polymers containing quaternary ammonium groups (QA) were able to damage the CM of cells with release of cytoplasmic constituents such as K^+, DNA and RNA. These results were confirmed by more recent studies carried out with techniques aimed to monitor loss of cells constituents and demonstrating that QA of synthetic polymers provoked cell lysis and consequently cells death by damaging the outer envelope as well as the CM.[3-9]

Polymeric surfaces have probably a similar mechanism of action with respect to the soluble chains, even if biocidal charged groups are tethered to the surface and are often unable to completely penetrate the envelope reaching the CM due to their short length. In this case, a mechanism of action via contact between cell and charged surface is often invoked. However, cell lysis, deriving from disruption of cellular membranes, is probably still working as evidenced by the presence of

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cells constituents in solution when coated materials[10-12] or polymer microbeads[13] containing QA were mixed with a suspension of bacteria. In this contest, it is also worth noting the necessity of reaching a minimum surface charge-density for an optimum biocidal efficiency of cationic surfaces,[14-16] either to foster adhesion of bacteria cells on the surfaces or cause leaching of Ca^{2+} and Mg^{2+}. We found that the loss of stabilizing ions is a key ingredient of surfaces in killing bacteria, as emerged from the fact that films made of copolymers containing crown ether pedants, the latter introduced with the aim of sequestering alkali-earth ions, were biocidal versus *E. coli*.[17] Such a result indirectly confirmed the presence of a mechanism of action that starts by leaching or complexing the envelope stabilizing cations.

Although a destabilizing action on cellular membranes is recognized for few cationic polymers, no experimental evidence of the cellular response resulting from the contact with surfaces is reported in literature as far as we knows. Therefore, possible regulators mediating the response to the damaging action are not yet known; similarly, it is not clear whether the antimicrobial action is a "passive" event, which is mediated only by polymer properties, or requires a cellular response as induction of a death signal. The answer to these questions is essential to understand antimicrobial mechanism of these materials and to provide more targeted and efficient growth control strategies by the synthesis of new materials and the use of specific substances (activators and inhibitors) able to control involved cellular pathways.

In this study we use *E. coli* as a model to investigate the mechanism of action of a bactericidal polymer surface active towards several Gram-negative cells and Gram-positive *S. aureus*.[16,18] The film is made of copolymers constituted by a block of monomethylether poly(ethylene glycol) (mPEG) covalently linked to two gradient-copolymeric chains based on methyl methacrylate (MMA) and 2-dimethyl(aminoethyl) methacrylate (DMAEMA) (mPEG-(MMA-ran-$DMAEMA)_2$).[18,19] The amino groups of DMAEMA (34% in mol) were not quaternized but able to generate a charged surface by protonation when in contact with water (Figure 1).

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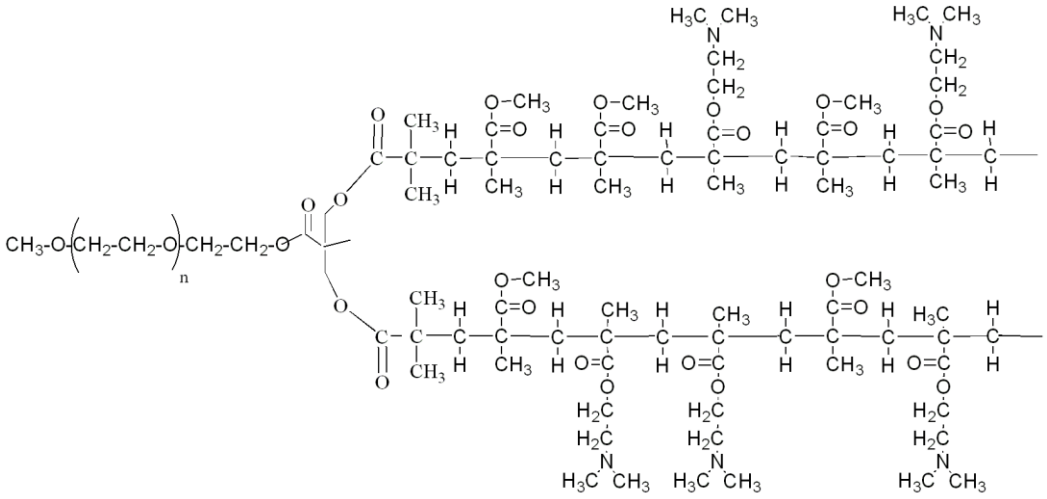

Figure 1. Structure of mPEG-(MMA-ran-DMAEMA)$_2$ copolymer.

Specifically, the morphological and structural damages induced by the contact with cationic surfaces and the genetic effects on the expression of key genes of specific cellular processes were investigated.

RESULTS AND DISCUSSION

Structural and morphological alterations. As first aspect of the study, we investigated the morphological and structural effects induced by polymer films on *E. coli*. For the purpose we used both fluorescence and transmission electronic microscopy (TEM) and compared intact cells (control sample) with those subjected to the action of the film. Fluorescence analysis were conducted in accordance with Mangoni method (2004),[20] which involves the simultaneous use of three fluorochromes: a DNA binding fluorochrome with the aim of highlighting total cells, both viable and dead,[21] FITC probe, which labels cells with alterations in envelope permeability[20,22] and finally CTC that evidences vital cells.[23] As shown in Figure 2, in the control population the cells were labeled both by the Hoechst probe and CTC (blue and orange fluorescence, respectively), indicating a high density of living bacteria. Population treated with film was marked both by the Hoechst and FITC probe (blue and green fluorescence, respectively), while mostly of cells resulted negative to the CTC, which means altered permeability of the envelope and presence of non-viable cells. The results of the cells viability were in accordance with kinetic data of the antimicrobial action previously reported for this polymer surface, which showed a high mortality rate (> 95%) after a treatment time close to that used for fluorescence assays (4-5h).[16,18]

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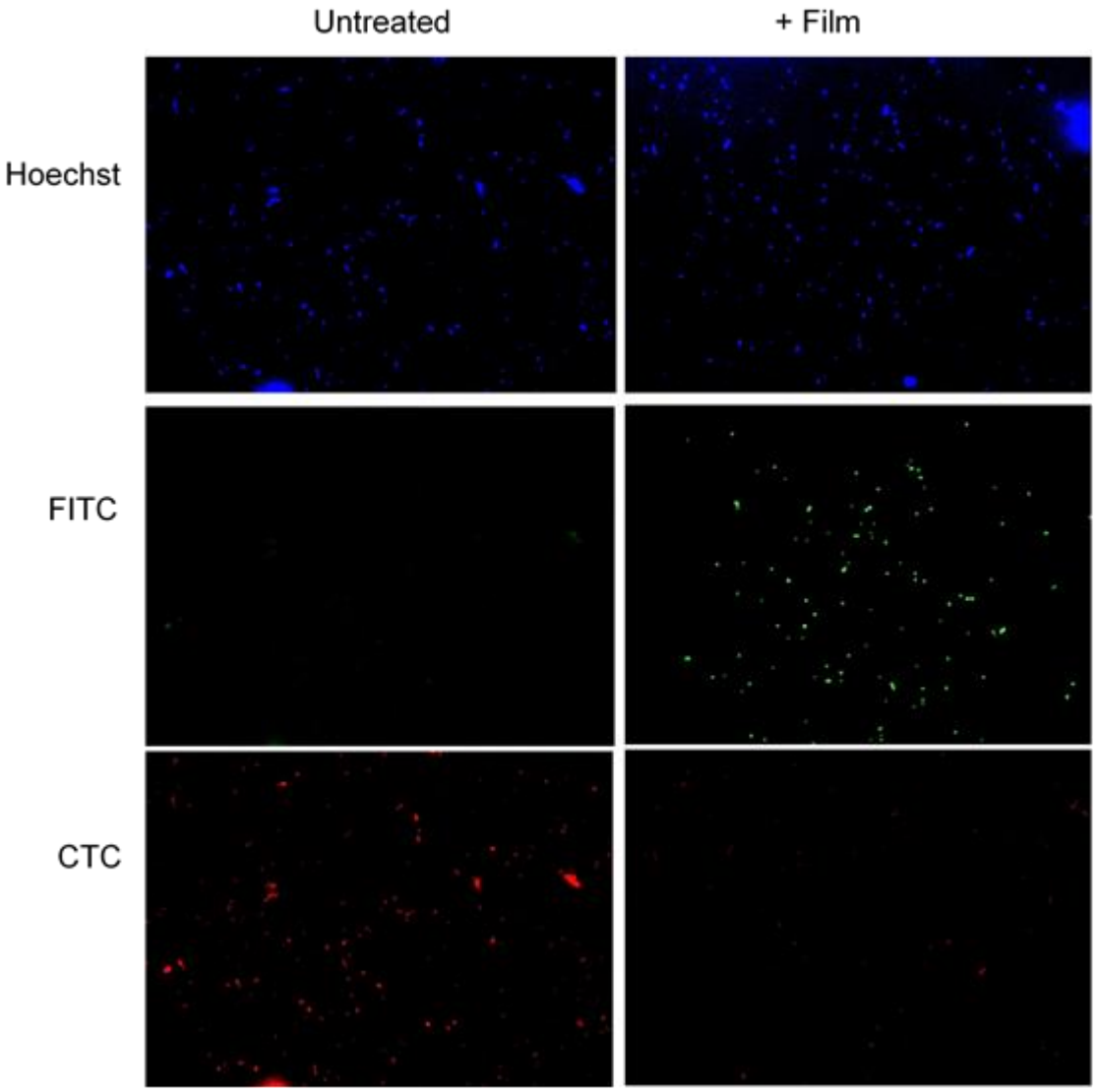

Figure 2. Triple staining with fluorescent probes. *E. coli* cells examined after incubation in presence (+Film) or in absence (Untreated) of antimicrobial film. The blue fluorescence is emitted by the total bacteria, regardless of their viability and envelope permeability. Specific cellular conditions are evidenced by CTC and FITC: untreated cells, positives to red fluorocrome CTC (viable cells) and negative to green FITC (non-permeabilized cells); treated cells, negatives to red CTC probe (not viable cells) and positive to green FITC (permeabilized cells).

TEM images evidenced a few important morphological and structural cellular alterations, as reported in Figure 3. To have a greater vitality rate and therefore better highlight cellular alterations, we shortened polymer treatment times (1.5h) compared to the ones used for fluorescence microscopy. After treatment, the number of cells with rounded shape significantly increased with respect to the ones with rod-like shape, the typical cells morphology of *E. coli*, compared to control population. In the altered bacteria, envelope membranes appeared to be less structured and the surface more wrinkled. Furthermore, the cytoplasmic compartment showed various alterations such as a greater presence of clear areas, typically associated to the loss of cytoplasmic material, other

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than precipitates, associated to phenomena of molecular agglutination. Cellular ghosts were also detected.

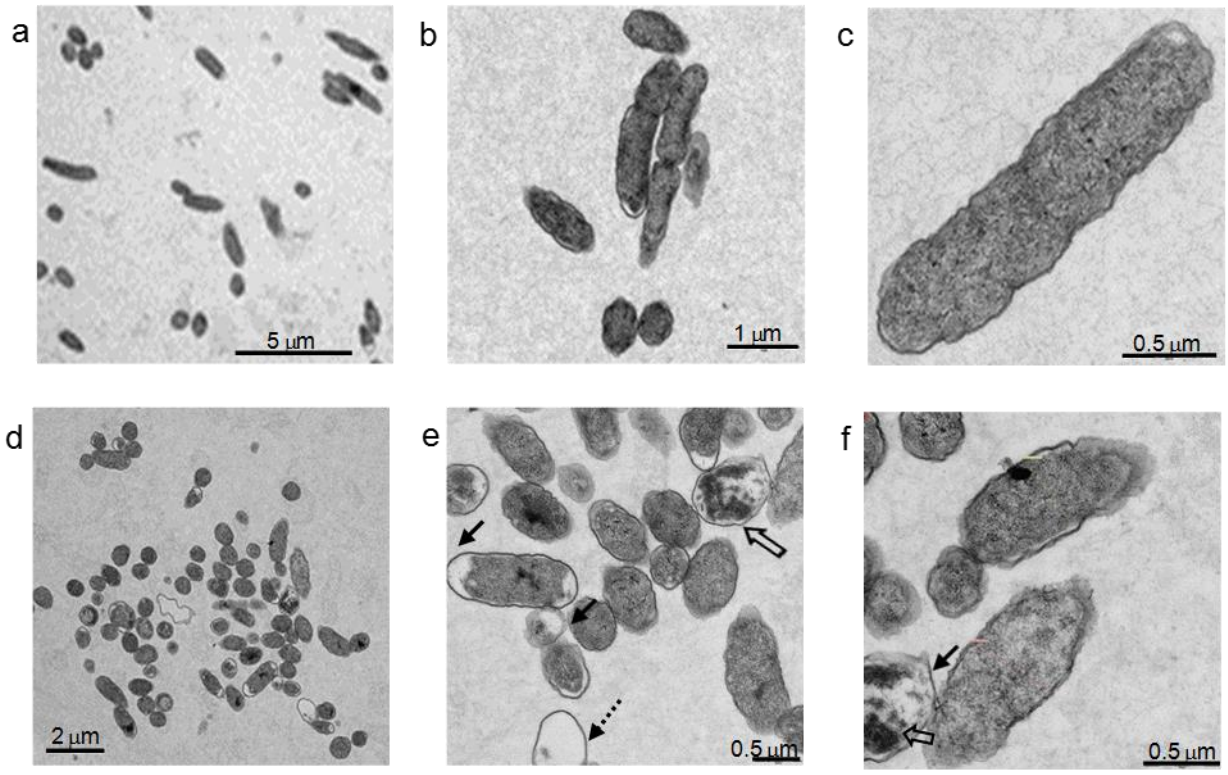

Figure 3. Transmission electronic microscopy (TEM). *E. coli* cells examined after incubation in absence (*a, b, c*) and in presence (*d, e, f*) of antimicrobial film. The treatment results in an increased number of cells with a rounded shape (*d, e, f* versus *a, b, c*). The membranes of the envelope appear less structured and the surface is more wrinkled (*e, f*). In *e* and *f* are indicated few cytoplasmic alterations: clear areas, due to loss of material (full arrows), agglutination areas (empty arrows). A cell ghost is present in *e* (dashed arrows). For each image the scale is indicated by the bar size.

Envelope alterations. To confirm the results of microscopy and to better define the action of the polymer film on the bacterial envelope, we evaluated the release of cytoplasmic materials and the sensitivity to osmotic shock of treated and untreated cells.

Release of cytoplasmic material was measured by variation of the electrical conductivity in the surrounding medium over more than 5h of contact of bacteria suspension with the film.[24,25] The conductivity of such a suspension reached 12µS/cm with a polyphasic trend over the time (Figure 4).

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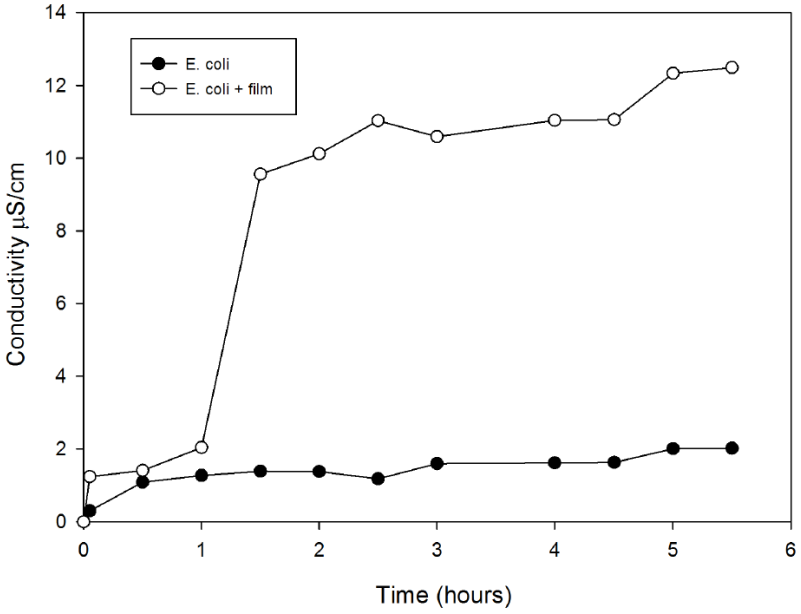

Figure 4. Effect of film on cellular leakage. Cellular leakage from *E. coli* measured by electrical conductivity in the incubation medium up to 5.5 hours. (Treated bacteria: empty circles; untreated cells: full circle). Values are the average of three independent experiments.

In fact, the conductivity was low (about 1.5-2.0 µS/cm) throughout the first hour, rapidly reached the value of 9 µS/cm (>70% of the maximum value) after 30 min (at 1.5 h), and gradually increased to its maximum over the next 3.5 h. For the untreated population, the conductivity remained constantly low (between 1.5-2.0 µS/cm). Cytoplasmic release data were further confirmed by quantization of nucleic acids by measuring absorbance at 260 nm in the medium over the time (data not shown).

Osmotic shock tests are generally used to highlight and estimate damage to the envelope membranes, which reduces cellular resistance to lysis induced by environments with low osmotic pressure. In our experimental conditions, bacteria populations were pretreated with the film at different times (30, 60 and 90 min); the resistance to osmotic shock was subsequentely assessed by determining cellular survival up to 48 h. The pretreatment times were chosen basising on kinetic data for specific antimicrobial activity[16] and in order to obtain increasing levels of alteration without a high mortality percentage that would compromise the reliability of the results. As shown in Figure 5, pretreatment lasting 1h did not produce a significant reduction in population vitality as compared to original population; conversely, vitality was reduced to about 60% after 90 min. A damage of the bacterial envelope was however evident in all three cases: after removing the polymer film, a fraction of the viable microbial population underwent osmotic lysis. As expected, the amount of damage to the envelope was proportional to the duration of pretreatment; in fact, the damaged cells fraction and lysis rate increased upon increasing pretreatment time (30%, 60% and > 70% at 30, 60

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and 90 min, respectively). Besides, kinetic data of the osmotic shock were consistent with those of the cytoplasmic release, the drop in cell viability due to the pretreatment (time 0 in Figure 5) and the conductivity values up to 1.5 h (Figure 4) being superimposable.

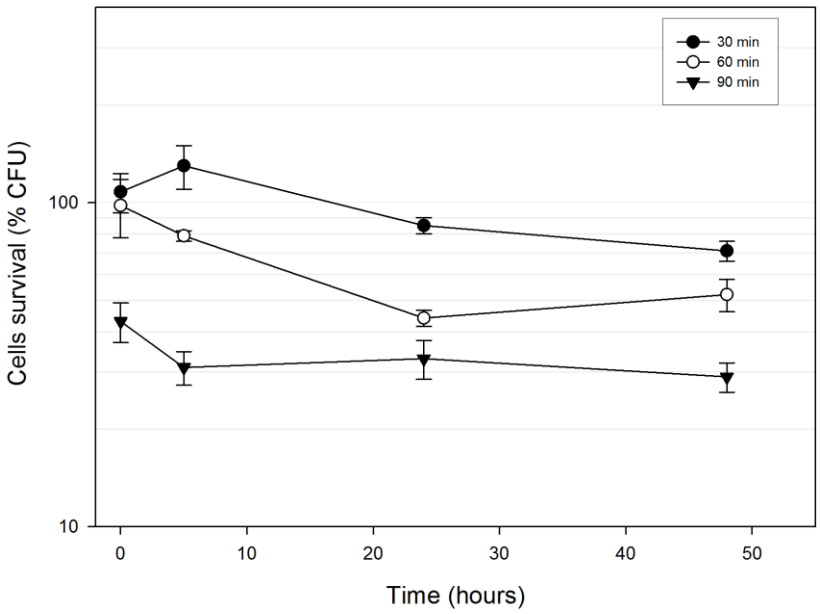

Figure 5. Assays of shock osmotic as evidence of membranes destabilization. Bacteria cells were pre-treated with films for 30, 60 and 90 minutes, then the density of each population was measured at 0, 5, 24 and 48 h by CFUs determination, in absence of polymer and in an hypotonic environment. For each indicated time, the cell survival of the treated population is reported as a percentage ratio with the relative untreated control. The values are the mean ± standard deviation of three independent analysis. The y-axis is in logarithmic scale.

Thus, all data discussed in this section confirmed the results obtained by microscopy, highlighting an important role of the active polymer surface in altering the integrity of the external barrier.

Action of film surface on cytoplasmic membrane. Envelope constitutes the external barrier of permeability of bacteria and consists of the CM membrane and the cell wall. In Gram-negative bacteria such as *E. coli*, the main element of the cell wall is the OM, a double asymmetric lipid layer consisting of proteins, lipopolysaccharides (LPS) in the outer sheet and phospholipids in the inner sheet. To better direct our study on the mechanism, it was investigated which of the two membranes is the main target of the polymer surface.

To evaluate the effects on CM, we used two approaches: one based on protoplasts, viable cells without cell wall, and another on liposomes, an acellular system of vesicles surrounded by a synthetic lipid bilayer.

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Protoplasts retain most of the biochemical and molecular characteristics of intact cells, but are extremely sensitive to osmotic lysis and to be maintained viable require media with high osmolarity. However, agents that damage the membrane, further increase their sensitivity.

Protoplasts of *E. coli* were produced with lysozyme as described by Dathe (2002)[26] and their sensitivity to polymer surface was evaluated by incubating with the film and measuring the variation of population density up to 24 h. Two control populations were inserted, consisting of protoplasts and intact cell of *E. coli,* respectively. The first control was introduced to check viability of the protoplasts in absence of antimicrobial material and verify the specific action of the surface. The second one was used to highlight a possible role of cell wall in mediating the toxic action of film. As evidenced in Figure 6, the contact with polymer significantly reduced the number of the protoplasts. At 4 h, surviving cells were 50% of the initial population and about 3% at 24h, while control protoplasts did not experience significant effects. On the other hand, intact cell population was protected by antimicrobial action of film in sucrose solution, showing only a slight reduction (20%).

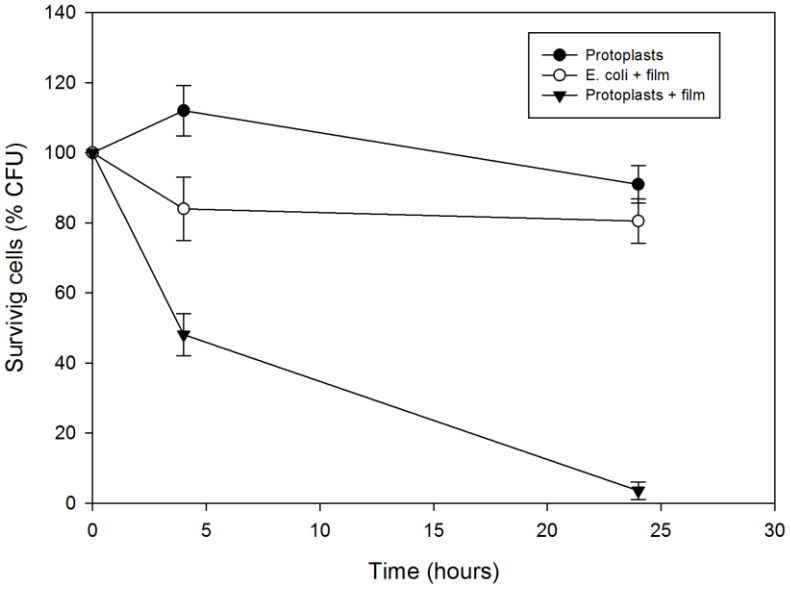

Figure 6. Sensitivity of protoplast to lysis induced by film. Cell survival is shown during 24 h treatment with the film in an osmotically protected environment (10% sucrose medium). (Full circles: untreated protoplasts; full triangle: treated protoplast; empty circles: treated intact *E. coli* cells). For each, population survival over the time is determined as a percentage ratio of the number of CFUs compared to the time 0. The values are the mean ± standard deviation of at least three independent analysis.

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We evaluated the direct action on the CM by studying the effects of polymer on synthetic membranes mimicking the general composition of bacterial ones.[27,28] Specifically, we produced unilamellar liposomes (LUVs) composed of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) in the ratio of 4:1 for Gram-negative-like vesicles[29] and PG and cardiolipin (CL) in the ratio of 12:1 for Grams-positive-like.[30] Both LUVs were loaded with calcein dye.

The action on the membranes was evaluated by measuring the fluorescence emitted by calcein released into the medium as a result of LUVs lysis. Two further populations were also included in these studies. The first one was not treated with film to check the effects of experimental conditions on the integrity of vesicles. The second population was subjected to the action of a polymer surface lacking of the charged component ($A(B_2)$) responsible of the antimicrobial action on intact cells.[18] Such a population allowed evaluating the sensitivity of liposomes to other polymeric elements and a different antimicrobial mechanism of film (see Figure 7).

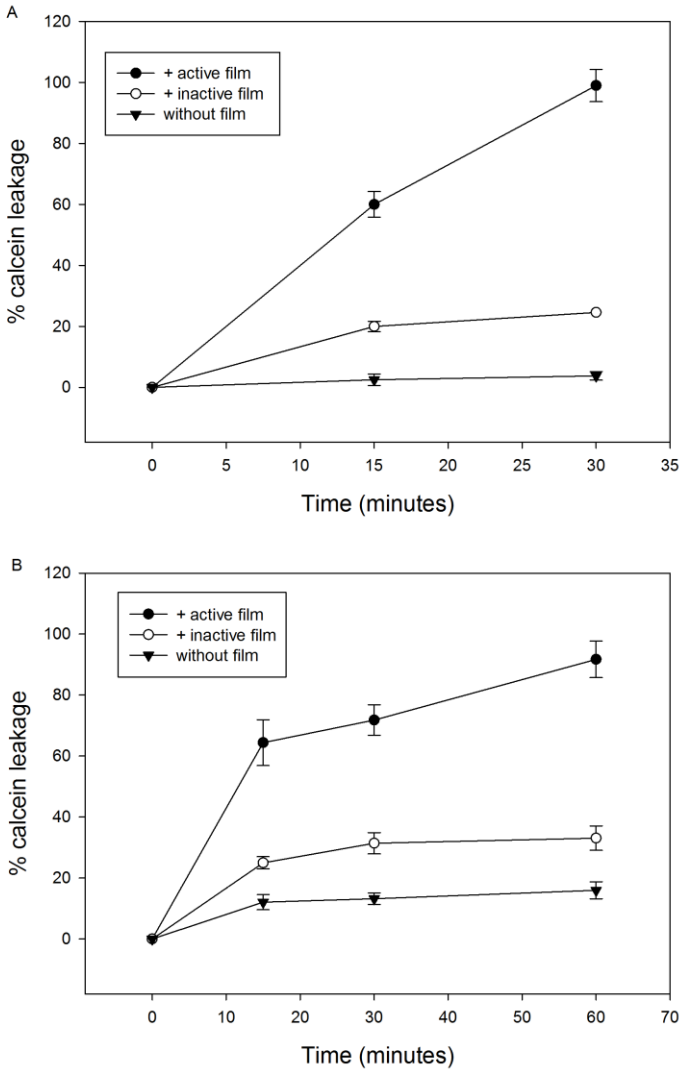

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Figure 7. Film-induced calcein leakage from LUVs. A. LUVs mimicking the cytoplasmic membranes of Gram-negative (DOPE:DOPG = 4:1). B. LUVs mimicking the cytoplasmic membranes of Gram-positive (DOPG:CL = 12:1). LUVs have been incubated with antimicrobial active film, inactive film (A(B_2)) and in the absence of film. Lysis has been measured quantifying the leakage of fluorescent calcein by fluorometry and the values reported as percentage of leakage with respect to the maximum leakage obtained by treatment of intact LUVs with Triton X. The active film induces complete lysis (A and B), with efficiency dependent on lipid composition. On the contray (A (B_2)) has a low lysis capacity (25-30% loss of calcein) and an action less dependent on lipid composition. Values are the mean ± standard deviation of more than three independent experiments.

The presence of active film caused the release of more than half of the total incorporated calcein, meaning 60% and 64% for the vesicles mimicking the Gram-negative and Gram-positive membranes respectively, after 15 min. The complete release (due to a total lysis) required different times, meaning 30 min for the Gram negative (99% of leakage) and over 60 min for the Gram positive-like membranes (92% at 60 minutes). In the presence of non-active film, the lysis was strongly reduced (25-30% of calcein release) and the kinetic of release was not dependent on the lipid composition, confirming the toxicity of the cationic groups for the phospholipid membranes and their role in antimicrobial effect.

All together, these data indicated that polymer can directly damage CM, leading to cell lysis, but the presence of cell wall hinders this direct action on membrane to the point that cell remains vital in an osmotically protected environment. In addition, the lytic effect on liposome evidenced that toxic action does not require metabolically active cells and any cellular response, although this does not exclude that it may be modulated by the cell.

Action of film surface on the OM. To detect the effects of polymer films on the OM, we evaluated the variation of sensitivity to actinomycin D. *E. coli* and many other Gram-negative bacteria are resistant to actinomycin D due to the presence of the OM, and it has been shown that treatments with agents that increase the permeability of the OM also increase bacterial sensitivity to antibiotic.[31,32] For the purpose, bacterial cells were first preincubated for different times with the polymer film, after they were grown for 16h in rich medium without film and in the presence of 25, 50, and 70 μg/ml of actinomycin D, finally the effects on growth were determined. To optimize experimental conditions and to not alter results, pretreatment times (30 and 60 min) were chosen to avoid decimating the population before putting it in contact with antibiotic and to have the maximum number of damaged but viable cells in the population (see Figure 4). Two populations were used as controls: the first (CntAD) was subjected to the same treatments as the previous

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population, but without the use of polymer film to evaluate the specificity of the surface effects, while the second one (CntGr) was obtained as for CntAD, but it was not treated with actinomicyn D and it was used as a reference for the growth. Experimental results are reported in Table 1.

Table 1. Film modification of the sensitivity of *E. coli* to actinomicyn D

Samples ratio	Pretreatment time (min.)	Relative Growth[d]		
		Actinomycin D μg/ml		
		25	50	75
CntAD[a]/CntGr[b]	30	1.10±0.11	0.97±0.04	0.71±0.02
	60	0.98±0.03	1.00±0,05	0.70±0,01
+Film[c]/CntGr	30	1.08±0.09	0.72±0.03	0.61±0.01
	60	1.11±0.12	0.74±0.01	0.41±0.02

[a]Population of *E. coli* not pretreated with film and grown without actinomycin D;
[b]Population of *E. coli* not pretreated with film and grown with actinomycin D;
[c]Population of *E. coli* pretreated with film and grown in the presence of actinomycin D.
[d]Ratio of optical densities at 600nm. The sensitivity increases with the reduction of the values.

While *E. coli* strain was not sensitive to actinomycin D up to 50 μg/ml, there was a 30% reduction of population at 75 μg/ml (CntAD/CntGr ratio equal to 1 and 0.7, respectively). Pretreatment with films induced a significant increase in sensitivity with a reduction of population density of almost 30% at 50 μg/ml, following 30 or 60 min of pretreatment. The growth inhibition was higher at 75 μg/ml and was dependent on the pretreatment time. In fact, cell density reduction was 40% and 60%, respectively after 30 and 60 minutes. Since we did not find the growth inhibitory dose (MIC) in our conditions, to estimate the sensitivity we considered the MIC_{50}, the antibiotic concentration which reduced the population by 50% compared to that grown in the absence of antimicrobial compound. As deduced by table 1, MIC_{50} for untreated *E. coli* was higher than 75 μg/ml, while after 60 min of incubation with film MIC_{50} was between 50 and 75 μg/ml.

In conclusion, these data indicate that film surface acts by destabilizing bacterial lipid membranes. In addition to the effects on the cytoplasmic ones, film also influences the barrier function of cell wall by increasing the permeability of the OM.

Molecular study of antimicrobial mechanism. The antimicrobial action of the film is closely related to the positive charges of cationic pendants. It has been estimated that the length of the latter is lower than the thickness of *E. coli* cell wall,[16,18] and they can not reach the CM. Furthermore, the experiments with protoplasts confirm that the presence of the wall prevents direct damage to the

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plasma membrane (Figure 6). We hypothesized that the damage to the OM was the main cause of the antimicrobial action and that cell lysis was due to destabilization of the wall and to consequent reduced protection of the protoplast from osmotic pressure. For this reason, we focused on analyzing the alterations induced by film on the OM using a genetic approach to detect variations in the expression of key genes involved in the synthesis and maintenance of the OM structure , Genes of LPS synthesis (*kds*A, *waa*A, *waa*C),[33-35] of maintenance of membrane asymmetry and stability (*pld*A, *pag*P),[36-40] and of lipid metabolism (*fad*R, *fab*R, *fab*A)[41-47] were specifically investigated (Table 2, S4). Unlike the latter, the first two groups are specific to processes related to the OM, so their variations are associated to the changes of this lipid layer. To get more information on the mechanism, we have also considered genes associated with other cellular functions, such as *rec*A,[48-50] which is involved in DNA repair, and *sox*S,[51-54] involved in the response to oxidative stress (S4).

Expression analysis of OM related genes

The expression variations were evaluated by analysis of transcripts of considered genes by Real-Time-PCR technique (RT-PCR); specifically, we applied a method based on comparison with the expression of a less variable internal gene (internal standard), in our case the *rrs*G encoding for ribosomal rRNA 16S. The expression analysis was focused on two different growth conditions. *E. coli* was first incubated with film (treatment phase, T phase) up to obtain the largest fraction of damaged cells without induction of a significant bacteria mortality in the population; successively, it was immediately incubated in rich medium without film to allow cells repairing the damages of OM (recovery phase, R phase). In this last phase, cells treated with the film have extended by 20 min the latency phase to repair damages up to the recovery of all functions and resistance to osmotic stress. Subsequently, they enter the exponential phase, showing the same growth characteristics of non-pretreated cells (Figure S2, S3). Transcripts analysis was conducted right after 1h of treatment, in accordance with osmotic shock results on cell viability (Figure 5), and 12 min during the recovery phase. Reference population (control) was similarly processed in absence of the polymer. For each phase total RNA was extracted and retrotranscripted for RT-PCR analysis with the primers reported in Table 2.

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Table 2. Sequences characteristics of oligonucleotide primers used in this study and target genes description.

Primer name[a]	Target gene[b]	Primer sequence	Annealing position[c]	Gene description
fadR -F1 fadR-R2	*fadR*	5'-GATAATTTGCTGTCGGTGCG-3' 5'-GCAAAGGCATCGGCGTGATC-3'	295-314 406-425	Transcriptional regulator of fatty acid metabolism
fabR-F1 fabR-R2	*fab*R	5'-CCACATTTATGGAGTTCATCG-3' 5'-CCAGATAGTCCGCAAGTTCCG-3'	278-298 398-418	Transcriptional repressor of fatty acid biosynthetic genes *fab*A and *fab*B
fabA-F1 fabA-R2	*fab*A	5'-CGGATCTGTGGTTCTTCGGATG-3' 5'-GTGAATTTCACTTCGCCAAC-3'	188-209 325-344	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase
waaA-F1 waaA-R2	*waa*A	5'-GCGTCATCGTTATCCTGATTTACC-3' 5'-GTCGCGCGTTAGCGATCACCAGCGG-3'	212-237 439-464	3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase)
waaC-F1 waaC-R2	*waa*C	5'-GG TTCGCACAGATTCCTTCCTG-3' 5'-GGCTGGCTAAAGGTTCGCGAGC-3'	119-141 355-377	ADP-heptose:LPS heptosyl transferase I
kdsA-F1 kdsA-R2	*kds*A	5'-GCTATGACAACCTGGTTGTCGATATGC-3' 5'-GGACCATCACATTTCGCATGTTCCG-3'	518-545 721-746	3-deoxy-D-manno-octulosonate 8-phosphate synthase
pldA-F1 pldA-R2	*pld*A	5'-GCGATCTGAATAAAGAAGCGATTGC-3' 5'-GCGA TCTGAATAAA GAAGCGATTG-3'	197-222 407-430	Outer membrane phospholipase A
pagP-F1 pagP-R2	*pag*P	5' -GCTAACGCAGATGAGTGGATGACAAC-3' 5'-CACGAGTCCTTAAATGCCATGG-3'	73-99 287-309	Phospholipid:lipid A palmitoyltransferase
soxS-F1 soxS-R2	*sox*S	5'-GGTACTTGCAACGAATGTTCC-3' 5'-GTCTGCTGCGAGACATAACC-3'	107-127 241-260	Superoxide response regulon transcriptional activator
recA-F1 recA-R2	*rec*A	5'-GTAACCTGAAGCAGTCCAACAC-3' 5'-GACGGATGTCGAGACGAACAG-3'	542-563 662-682	DNA recombination and repair protein
rrsG-F1 rrsG-R2	*rrs*G	5'-GGTTCCCGAAGGCACATTCT-3' 5'-CGGCCGCAAGGTTAAAACTC-3'	505-524 631-650	16S ribosomal RNA of rrnG operon

[a]F and R: Forward and Reverse primers, respectively.
[b]The sequences of the genes used for the construction of the primers were those of the *E. coli* strain K-12 (substrain MG165), all present in the database of the National Center for Biotechnological Information (NCBI) to the access number: NC_000913
[c] Numbering is relative to the sequences of *Escherichia coli* strain K12

Results in Table 3 show that in the treatment phase, the film induced RNA expression of genes involved in each of the three processes relative to structural maintenance of OM. The specific genes of LPS synthesis, *waa*A and *waa*C, and of enzymes of the outer leaflet, *pld*A and *pag*P, were 1.5, 2.4, 2.0 and 1.8 times, respectively, more expressed than in the control population. The genes involved in biosynthesis of fat acids, *fad*R and *fab*A, were also 2.0 times more expressed. In addition to these, contact with the antimicrobial film activated the *sox*S gene 1.5 times, while the expression of *rec*A was not significantly different between the two populations. Differently, all the genes considered were from 2 at 4.5 times more expressed in untreated control cells respect to treated one in the recovery phase. These changes in the expression trend were in accordance with

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the shorter latency phase of the control population after incubation in rich medium (Figure S3). Therefore, the early activation of the genes involved in bacterial growth, such as those regulating the biosynthetic pathways, canceled up to reverse the differences of expression observed in the treatment phase.

Table 3. Transcript changes for *E. coli* treated with film *versus* not treated control strain.

Genes	Expression Fold (EF)[a]		Relate functions
	T phase-1h	*R phase-12 min*	
*kds*A	0.98 ± 0.13	[b]0.46 ± 0.13	LPS biosynthesis
*waa*A	[b]1.52 ± 0,18	[b]0.22 ± 0.04	
*waa*C	[b]2.36±0.28	[b]0.33 ± 0.16	
*pag*P	[b]1.77 ± 0.32	0.38 ± 0.10	Maintenance of the OM asymmetry and permeability functions
*pld*A	[b]1.97 ± 0.24	0.52 ± 0.11	
*fad*R	[b]2.11 ± 0.37	[b]0.40 ± 0.12	Fatty acid metabolism
*fab*R	1.32 ± 0.43	[b]0.32 ± 0.11	
*fab*A	[b]2.13 ± 0.11	[b]0.44 ± 0.11	
*sox*S	[b]1.51 ± 0.43	[b]0.25 ± 0.11	Cellular response to oxidative stress
*rec*A	1.31 ± 0.21	[b]0.43 ± 0.04	DNA repair

[a]Mean for three indipendent experiments ± standard deviations. T: treatment. R: recovery
[b]Significant variations *versus* control strain calculated with Student's t test ($p<0.05$)

CONCLUSION

In this study we investigated the antimicrobial mechanism of a cationic film surface made of copolymers containing mPEG, MMA and DMAEMA in a branched architecture that increases the degree of ionization of the DMAEMA amino group, the latter responsible of the bactericidal activity of surface.[16] For the purpose, an *E. coli* strain was used as a model for Gram-negative bacteria. In a first phase, microscopy, the measure of cytoplasmic release and osmotic lysis assays have shown that film surfaces induces an alteration of the permeability of the envelope, followed by bacterial lysis. The nature of the damage has been further investigated by specifically evaluating the action on the two individual components that give to the envelope its characteristic of permeability barrier, CM and the OM. The use of protoplasts, very sensitive to lysis induced by agents that alter structure and permeability of cellular membranes, and of phospholipidic liposomes confirmed the ability of the film to damage the CM. Furthermore, the results on liposomes, very simple membranous systems lacking biochemical activity, have shown that the toxic activity is not strictly dependent on the induction of a cellular death pathway. The OM, due to its particular composition,

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plays an important role in counteracting the passage of small liposoluble molecules, making Gram-negative bacteria resistant to several harmful substances (fatty acids, bile salts, antibiotics, detergents).[55-57] We found that pretreatment of *E. coli* with polymer film for a time sufficiently short not to alter the density of the living population induces a reduction of MIC_{50} for the liposoluble antibiotic actinomycin D, thus highlighting its ability to modify also the OM permeability. Basing on the specific architecture of the Gram-negative envelope, the short length of the cationic polymeric pedants[18] and the protective action of cell wall towards direct action on the CM, we decided to focus subsequent genetic analyses only on the OM.

From osmotic lysis assays and growth kinetic studies, we identified a temporal window during which interrupting the treatment with the films leaves the entire population still vital, albeit damaged to an extent depending on the duration of treatment. If placed under appropriate growth conditions in the absence of the film, treated populations undergo a phase of damage recovery. It also emerged that during T phase film induces an increased expression of *pld*A, *pag*P, *waa*A and *waa*C genes, indicating that OM undergoes serious structural damages and confirming the results on the altered permeability. A major expression of *pld*A and *pag*A genes indicate an increase of the amount of phospholipids in the outer leaflet and an imbalance in the concentrations of divalent ions that stabilize LPS.[36-38] The latter result is in accordance with previous data reporting the bactericidal activity of surfaces made of polymer containing crown ethers pendants as chelating agents of divalent cations.[17] The cations imbalance also induced a loss of LPS.[39,55] This last hypothesis is supported by the increase in the expression of the biosynthetic genes of the LPS core, *waa*A and *waa*C.[33] The expression of bifunctional regulator gene of fatty acids metabolism, *fad*R, also resulted increased in T phase with respect to untreated bacteria, as well as the expression of *fab*A gene, positively regulated by FadR (S4). Differently, there are no significant differences in the expression of the *fab*R repressor of the biosynthetic genes. This aspect agree with the expression variation evidenced for genes *pld*A, *pag*P, *waa*A and *waa*C. In fact, a loss of fatty acids by the outer leaflet of OM as an effect of both degradation of phospholipids and loss of LPS, would require an increase of the synthesis of fatty acids. However, *kds*A gene, coding for the enzyme involved in the synthesis of 3-deoxy-D-manno-octulosonic acid (Kdo) of the LPS oligosaccharide core (OS)[34,35] was apparently in contrast with the above gene expression profile, as its transcription level was not significantly altered compared to untreated cells. Despite this, no conflict is suggested with the synthesis of the LPS induced by the film, as the control of Kdo availability does not strictly correlated with that of the *kds*A gene transcription as evidenced in several bacterial strains. In *E. coli* K-12 a half-life of enzyme KdsA higher than that of the relative transcript was described.[58] *Salmonella typhimurium* was able to synthesize LPS even in absence of Kdo synthesis, probably

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using a pool of cellular components.[59] One may therefore speculate that during treatment phase the greater stability of KdsA enzyme and/or the use of a reserve of cellular elements would be sufficient to support, at least for a short time, a more modest increase in the synthesis of LPS than required during the growth phase in rich medium.

The analysis of expression of *rec*A gene did differ from the case of untreated population, indicating that films do not induce DNA damages in pretreatment phase, at least those inducing *recA* system.[48-51] On the contrary, *sox*S mRNA level resulted significantly higher than in control population. It is worth nothing that we did not use redox-active compounds, while the superoxide anion is the only activator recognized as able to regulate the expression of the *sox*S gene.[52-54,60,61] However, it is possible that stress conditions induced by films could elevate cellular formation rates of reactive oxygen species (ROS) or alternatively that other unknown non-oxidative regulators come to be involved as already described for *E. coli*.[62] It has been reported that stress conditions due to free fatty acids over-production result in compromised membrane permeability and induce the expression of MarA/Rob/SoxS regulon by increasing mRNA levels of the global regulators MarA, Rob and SoxS. Overall, genes of the regulon are implicated in multiple resistance to antibiotics, resistance to solvents and other small toxic molecules and oxidative stress.[63,64] The control of the regulon SoxRS (S4) by factors affecting the permeability barrier of bacteria still remains unsolved, but the functions of some genes of this regulon are consistent with the response to injury. In fact, SoxS is able to promote *micF* transcription *in vivo*[65-68] and *lpx*C.[68,69] MicF acts as an antisense repressor of the *omp*F gene transcription, encoding for the OmpF porin, a protein channel that regulates the permeability of the outer membrane in a coordinated fashion with the smaller OmpC channel. The relative proportion of the two proteins is controlled by environmental conditions (osmolarity, temperature, toxic ingredients), so that microorganisms obtain a reduction or an increase of membrane permeability by reducing OmpF and increasing OmpC levels and vice versa, respectively.[70-72] The gene *lpx*C encode UDP-3-Oacyl-N-acetylglucosamine deacetylase, enzyme that catalyzes the lipid A biosynthesis of LPS.[68] Thus, it could be hypothesized that in our conditions the control of these genes could play a role in counteracting the increased permeability and the effects on the structure produced by membrane damage.

In conclusion, we highlight an action on the membranes in some respects similar to that described for some cationic soluble polymers, but for the first time the mechanism is investigated also at genetic level. We identify some key genes involved in the cellular response to the toxic action and provide a starting point also for other antimicrobial surfaces with similar functionality. The control of these genes could improve the antimicrobial efficacy and have an implication also in countering the occurrence of resistance mechanisms. Further work is needed to deeper understand their role, by

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studying more in detail the regulation and function of related pathways and the structural and molecular changes of cell membranes.

MATERIALS AND METHODS

Materials. Escherichia coli, strain JM109 was purchased at Promega (http: //www.promega.com/products; cat. No P9751). Fluorochromes [CTC(5-cyano-2,3-di-(P-tolyl)tetrazolium), Hoechst 33342, FITC (fluorescin isothiocyanate dextran)], Poly (L-lysine) and Calcein were purchased from Sigma-Aldrich and used without further purification. Phospholipids 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE), cardiolipin (CL), and the mini extrusion kit-extruder were purchased at Avanti Polar Lipids. Dehydrated compounds for the growth medium of *E. coli* were purchased from Oxoid.

Poly(ethylene glycol) monomethylether (m-PEG) (M_n = 2000 Da), CuBr, 2,2'-bipyridine (bpy), chloroform and Al_2O_3 were purchased from Aldrich and used without any further purification.
All manipulations involving air-sensitive compounds were carried out under nitrogen atmosphere using Schlenk or drybox techniques. Toluene (Aldrich) was dried over sodium and distilled before use. CH_2Cl_2 (Carlo Erba), methylmethacrylate (MMA) and 2-(dimethylamino)ethyl methacrylate (DMAEMA) (Aldrich) were dried over CaH_2, then distilled, the latter under a reduced pressure of nitrogen.

Synthesis of branched m-PEG-(PMMA-PDMAEMA)$_2$ *copolymer.*[18] Synthesis was carried out in toluene at 90°C, in a 50 ml glass flask charged, under nitrogen atmosphere, with 0.1 g of m-PEG-Br_2 macroinitiator[18] in 15 ml of dry toluene. After the dissolution of the macroinitiator, 0.03 g of CuBr, 0.05 g of bpy, 5 ml of MMA and 2.5 ml of DMAEMA were added. The mixture, thermostated at 90°C, was magnetically stirred for 18h then the reaction was stopped with *n*-hexane. The copolymer was recovered, dissolved in the minimum amount of chloroform and passed over a column of activated Al_2O_3 to remove the catalyst. The solution was dried in vacuum, the polymer was recovered, washed with cold methanol and then dried.
NMR Analysis. Spectrum was recorded on a Bruker Avance 400 MHz spectrometer at 25°C with D1 = 10 sec. The sample was prepared by introducing 20 mg of copolymer in 0.5 mL of $CDCl_3$ into a tube (0.5 mm outer diameter). TMS was used as internal reference.

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^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.83-1.10(C*H*$_3$ main chain), 1.79-1.87 (C*H*$_2$ main chain), 2.27 (-N(C*H*$_3$)$_2$), 2.56 (-O-CH$_2$-C*H*$_2$-N(CH$_3$)$_2$), 3.57 (-OC*H*$_3$), 3.61(-OC*H*$_2$C*H*$_2$-), 4.06 (-O-C*H*$_2$-CH$_2$-N(CH$_3$)$_2$). ^{13}C-NMR (400 MHz, $CDCl_3$): δ 16.6-18.9 (*C*H$_3$ main chain), 44.9 (quaternary carbon in the main chain), 46.0 (-N(*C*H$_3$)$_2$), 52.0 (-O*C*H$_3$, MMA), 54.5 (*C*H$_2$ main chain), 57.2 (-O-CH$_2$-*C*H$_2$-N(CH$_3$)$_2$), 63.2 (-O-*C*H$_2$-CH$_2$-N(CH$_3$)$_2$), 70.7 (-O*C*H$_2$*C*H$_2$-), 176.3-178.2 (-*C*=O).
The chemical composition in mol of the copolymer was determined by ^{13}C-NMR and resulted to be: mPEG = 8%, MMA = 58%, DMAEMA = 34%. The molar mass was determined by the degree of polymerization, as evaluated from ^{13}C-NMR, by the molar mass of monomers and by the signal intensities[18] and resulted to be 82 kDa.

Preparation of polymer film by casting.[16,18] Film of ~ 400 *μm* of thickness was prepared by dissolving 200 mg of copolymer in 50 ml of CH_2Cl_2 at 25°C. The solution was cast in a teflon petri dish (diameter 6 cm) and the solvent evaporated at room temperature. The film was removed from the petri dish and stored in a vacuum oven at 30°C for three days.

*Fluorescent analysis***.** Fuorescent analysis was conducted by the triple staining method,[20] using the following fluorochromes: the blue fluorescent Hoechst (that bind to double stranded DNA), to stain viable and non viable bacteria;[21] the red CTC (for detection of viable cells);[23] and the green FITC, which is unable to traverse the cytoplasmic membrane of cells unless permeabilized.[20,22] CTC, Hoechst and FITC were made up as stock solutions at a concentration of 6mg/ml in water, 10mg/ml in water and 10 mg/ml in acetone, respectively. For analysis cells of *E. coli* in exponential growth in Lauria-Bertani medium (5.0 g/L yeast extract, 10 g/L peptone, 10g/L NaCl, pH 7.0) were resuspended at a density of 5 x 10^7 CFU (colony forming units)/ml (0.05 OD at 600nm) in sterile distilled water and incubated 4 hours in the presence or absence (control population) of the film (1 cm^2/ml), at 37 °C and with constant agitation at 50rpm by vertical rotator Multi-bio RS-24 (Bioscientifica Srl, Italy). Subsequently, aliquots of 50 μl of bacterial suspension were incubated with 900 μl of CTC [(1.5mg/ml in phosphate buffered saline (PBS)] for 90 minutes at 37 °C with constant agitation (150 rpm). After, cells were poured on to glass slides previously coated with poly(L-lysine) and incubated 45 min at 30 °C to allow the adhesion of microorganisms to the support. The glass slides were washed with sodium phosphate buffer, treated with Hoechst solution (10μg/ml in PBS) and incubated 30 min at 30 °C. After were rinsed with PBS and treated with FITC solution (6μg/ml in PBS) for 30 min at 30 °C. Finally the glass were washed as above with PBS and examined by phase-contrast and fluorescence microscopy. For all treatment slide were placed in the wells of a polystyrene plate (Corning Italy). Phase-contrast images as well as images

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under fluorescence light were recorded using a Leica DM4000-6000B equipped with an oil-immersion objective (x 40). Acquisition and processing were performed using LAS AF software.

Transmission electron microscopy analysis (TEM). E. coli cells were prepared as for fluorescence analysis except for the incubation time with the film which was 1.5 h. After removing the polymer the cells were harvested by centrifugation at 3500g for 10 min. The pellet was resuspended in water and treated for TEM analysis as described by Park et al. (2012).[73] The prepared specimens were examined by Zeiss LEO 912AB EFTEM.

*Electrical conductivity***.** The cellular leakage from bacterial population was measured by the detection of electrolyte leakage into the incubation medium with a conductivity meter (DDS-307, Precision & Scientific Instrument Co. Ltd., Shanghai, China) in accordance with the method of Lee et al. (1998).[24] For the analysis, suspensions of 5 x 10^7 CFU/ml *E. coli* cells both in the presence or absence of the film (control population) were prepared as indicated above and kept under stirring at 37 °C for 5.5 h. The measurement of electrical conductivity was made at regular intervals for both populations. For both samples the conductivity variations were reported with respect to the background value of the control sample at time 0.

*Osmotic shock***.** The state of integrity of the cellular permeability barrier was evaluated with osmotic shock assays, following the method below. *E. coli* cells were grown as above in rich medium (LB) up to middle of the exponential phase, collected by centrifugation at 3500g for 15 min and rinsed once with sterile saline (0.9% NaCl). Then, the cell suspension was centrifuged (3500g, 15 min) to remove the saline solution and the pellet resuspended in distilled sterile water (mother suspension) at an optical density of 0.01 at 600nm (10^7 CFU/ml). Immediately, aliquots of cell suspension were incubated with the polymer (1 cm^2/ml) for 30, 60 and 90 minutes at 37 °C, under constant agitation at 50 rpm by vertical rotator. From the mother suspension, also were produced control populations for each treatment time, subjected to the same conditions, but in the absence of the film. At the end of each fixed time (named time 0) the polymers were removed from the bacterial suspensions and cell survival was determined at 0, 5h, 24h and 48h with count plate method, by spreading appropriate sample dilutions on LB agar dishes (LB, 15 g/l agar) and calculating colony forming units (CFUs) after incubation at 37 °C for 24 h. The values of cell survival were graphically reported as a percent of the ratio between the number of CFU of treated populations and that of the relative controls and represent the mean ± standard deviation of three independent analyses.

*Protoplasts of E. coli***.** Protoplasts were prepared as described by Dathe (2002).[26] Briefly, an aliquot of an overnight grown culture of *E. coli* was further cultivated in LB up to an OD600 = 0.8 at 37 °C with gentle shaking at 120 rpm. Then, 200 μl cell suspension was centrifuged at 4 °C for 10 min at

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3000g. The pellet was resuspended in 2.5ml ice cold sucrose solution (10% sucrose in 50mM Tris, 10mM $MgCl_2$, pH 8.0) and mixed with 0.5 ml lysozyme (5mg/ml in 50mM Tris, pH 8.0) for 5 min at 4 °C. Subsequently, 1 ml of EDTA (25 mM in 50 mM Tris, pH 8.0) was added and stirred gently for 5 minutes at 4 °C before adding 1 ml of Tris buffer (50 mM, pH 8.0) and mix it gently for further 15 min at 37 °C. Then, 2 ml 10% sucrose solution was added and the suspension was centrifuged for 20 min at 2000g. The cellular pellet was resuspended in 10% sucrose solution for further use. To evaluate the sensitivity to polymer 300µl of protoplasts suspension were incubated with 0.5 cm^2 of polymer at 37 °C and with constant agitation as described for intact cell. Untreated protoplasts and intact cells of *E. coli* were introduced as control samples. The cellular density of the three populations was measured up to 24 hours (0, 5h, 24h) with the plate counting method, distributing 100 µl of appropriate sample dilutions on LB agarized plates (10% of sucrose) and counting the CFUs after 24 hours of growth at 37 °C. For each populations the number of CFUs at different times was compared to that present at t0 and the value expressed as percentage. The graphic of cell survival was obtained by reporting the mean ± standard deviation of three independent analyses.

Large unilamellar vesicles (LUVs) production.[27-30] Phospholipids utilized for liposomes preparation were 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1',3'-bis[1,2-dipalmitoyl-sn-glycero-3-phospho]-glycerol (cardiolipin, CL), all as a solution of 25 mg/ml in chloroform. Phospholipids were purchased from Avanti Polar lipids (Alabaster, AL). LUVs mimiking Gram-negative membranes were prepared by utilizing DOPE:DOPG = 4:1, those Gram-positive, with DOPG:CL =12:1 as follow. The appropriate lipid solution was transferred in glass tubes and chloroform was evaporated by gentle nitrogen stream at RT. The produced lipid film was hydrated with PBS (pH 7.4) in the presence of 50 mM of calcein for 1 h in constant shaking at RT. Then, it was subjected to 5 cycles of freezing and thawing and extruded 10 times through a 400 nm pore size polycarbonate membrane on lipid extruder (Avanti Mini-Extruder from Avant Polar Lipids, Alabaster, AL). Unencapsuled calcein was removed by gel filtration chromatography (Sephadex G-50) and the final lipid concentration was determined as total phosphorus.[74] For liposome assay, LUVs were diluted in PBS (pH 7.4) to 50 µM and 300µl of suspension were incubated in the presence or absence (control) of antimicrobial film (0.5 cm^2) at 37 °C, with constant agitation by vertical rotator at 50 rpm. A further control was included, incubating LUVs with the polymer without the charged component $[A(B_2)]^{18}$ responsible for the antimicrobial action on intact cells. The lysis of vesicles was monitored over the time by exciting the suspension at 490 nm (λ_{ex}= 450 nm) and recording fluorescence emission from released calcein at 510nm (λ_{em}= 510 nm). The maximum fluorescence emission was determined by the

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addition to untreated samples of 20µl Triton X-100 (in 20% Dimethyl sulfoxide) to disrupt vesicles. A baseline was derived from untreated vesicles at time 0, to subtract the background emission from all measurements. Release was reported as a percentage of the maximum fluorescence emission and the values were expressed as the average from experiments performed in triplicate.

Evaluation of sensibility to actinomycin D. *Escherichia coli* cells were prepared as for osmotic shock assays and pellet resuspended in distillate sterile water at an OD600 of 0.01 (10^7 CFU/ml). Before evaluating the sensitivity to antibiotic, aliquots of cell suspension were incubated (pretreatment phase) for 30 min and 60 min respectively, with films (500µl/cm^2 of film) at 37 °C with constant agitation at 50 rpm by vertical rotator. Similar aliquots were incubated for the same times in the absence of the film, for the experimental controls CntAD and CntGr (see below). After the pretreatment, sensibility to actinomycin D was tested, according to the CLSI (Clinical and Laboratory Standards Institute, formerly the NCCLS) guidelines for determination of minimum inhibitory concentration (MIC)[75] as follows. Cell suspensions pretreated and not with the film (for the control CntAD) were diluted in fresh LB medium at $5x10^{-5}$ OD600 (that mean about 5 x 10^5 CFU/ml) and incubated with 25, 50 and 75 µg/ml of antibiotic. Another control (CntGr) was introduced to evaluate the growth of *E. coli* in the experimental conditions in the absence of actinomycin D. All samples were incubated 16 hours at 37 °C with constant agitation at 250 rpm. Then, the entity of the growth was evaluated for each suspension by measuring optical density at 600nm. To estimate the change in sensitivity to antibiotic the OD600 measurements were expressed in relation to the CntGr. The values were reported as the average of three independent experiments, each conducted in triplicate.

Molecular analysis

Specifics of primers and of the target genes. Primers and genes for expression analysis are indicated in Table 2. The gene sequences for primers construction were derived from the chromosomal sequence of *E. coli* strain K-12 (substrain MG165), present in the database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov), accession number: NC_000913. The studied genes are localized at following regions of the chromosome: *fad*R, bases 1234938-1235657; *fab*R, bases 4161067-4161771; *fab*A, bases 1015952-1016470; *waa*A, bases 3808540-3809817; *waa*C, bases 3795979-3796938; *kds*A, bases 1268165-1269019; *pld*A, bases 4004862-4005731; *pag*P, bases 656557-657117; *sox*S, bases 4277060-4277383; *rec*A, bases 2822708-2823769; *rrs*G, bases 2729616-2731157. The primers were synthesized as a service of Eurofins Genomics (https://eurofinsgenomics.com). Before proceeding with the expression analysis, the primers were tested for their functionality and specificity by the polymerase chain

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reaction (PCR) technique, using the *E. coli* genomic DNA as a template, and electrophoresis on agarose gel. For PCR, GoTaq® Green Master Mix (M712) system (Promega) was used. All reactions were performed in a final volume of 25 μl, according to the manufacturer's instructions, with 50 ng of genomic DNA and 500 nM of each primer. The amplification conditions were as follows: 5 min of initial denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec. The final extension was set at 72 °C for 10 min. The electrophoresis of the products was performed on 1.5% agarose gel.

RNA extraction and cDNA synthesis. The total RNA was isolated from *E. coli* using RNeasy Mini Kit (Qiagen) associated to RNA Protect Bacteria Reagent (Qiagen), that provides immediate stabilization of RNA prior to RNA isolation procedures, ensuring reliable gene expression and gene-profiling date. For gene expression analysis *E. coli* cells were grown in LB and resuspended in sterile distilled water as described for the osmotic shock assays, but at a higher density, $5x10^8$ CFUs in a final volume of 10ml (OD600=0.5) for each RNA extraction. Bacterial suspensions were incubated 1h (treatment phase, T phase) in the presence or absence of the film (1 cm^2/ml), at 37 °C with constant agitation. Both for the cells treated with the film and for the untreated ones, the expression analysis was carried out at the end of the treatment phase and after 12 min of incubation in a LB rich medium (recovery phase, R phase). For this latter, after the time of treatment, the cell suspensions were diluted in 1 vol of LB twice more concentrated and incubated for a further 12 min at 37 °C and constant shaking. For RNA extraction all suspensions were mixed with 2 vol of Protect Bacteria Reagent and RNA extracted, according to the manufacturer's instructions of RNeasy Mini Kit. The RNAs were treated with DNase I (RQ1 RNase-Free DNase, Promega) to clear genomic DNA. For cDNA synthesis 500 ng of total RNAs were reverse transcribed using GoScript reverse Trascription System (Promega) in a volume of 40 μl, using 500 ng of random hexamer primers, according to the manufacturer's instructions. To verify the absence of DNA template contamination a parallel no reverse transcriptase reaction (containing all components, including template RNA, except for the reverse transcriptase), was carried out for each reverse transcriptase reaction and used as a template for subsequent PCR negative control.

Semi-quantitative Real-Time PCR analysis (RT-PCR). For real-time PCR analysis, QuantiTect SYBR Green PCR kit (Qiagen) and IQ5 Thermocycler (Bio-Rad) were used. As internal standard was used gene *rrs*G, codifying for 16S ribosomal RNA. All PCR reactions were carried out in a final volume of 20 μl, using 10 μl of 2X SYBR green master mix, 1 μl of reverse-transcription reaction (approximately 2-5ng of total cDNA) and 500nM of each primers (Table 2). For the more expressed 16S rRNA, amplification was carried out with 1 μl of 500 fold diluted reverse-

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transcription reaction (approximately 4-10 pg of total cDNA). Negative controls were included for each specific PCR reaction, consisting of the amplification mixture without the cDNA. The amplification conditions were as follows: 1 cycle at 95°C for 3 min and 45 cycles at 95°C for 10 s, 57°C for 15 s and 70 °C for 15 s. To ensure the specificity of the PCR products, melting curve analysis was performed by heating products to 95 °C for 15 s, followed by cooling to 60°C and slowly heating to 95°C while monitoring fluorescence (data not shown). Data collection and analysis was carried out by use of IQ5 Optical System software (version 2.1, Bio-Rad). Data were normalized to levels of *rrs*G and analyzed by use of the comparative critical threshold (CT) method for calculation of the ΔΔCt and Expression Fold (EF $=2^{-\Delta\Delta Ct}$) between the treated and untreated samples. The values of EF were reported as the average ± standard deviations of three independent experiments, each conducted in triplicate. Statistical significance was calculated with Student's *t* test and a *p* value <0.05 was considered significant.

ASSOCIATED CONTENT

Supporting information

13C-NMR copolymer characterization; data supporting bacterial damage reversibility; short summary describing the characteristics and functions of the considered genes.

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