Polyolefin Matrixes with Permanent Antibacterial Activity: Preparation, Antibacterial Activity, and Action Mode of the Active Species

Sandrine Lenoir,[†] Christophe Pagnoulle,[‡] Moreno Galleni,[§] Philippe Compère,[||] Robert Jérôme,*[,†] and Christophe Detrembleur[†]

Center for Education and Research on Macromolecules (CERM), University of Liège, Sart-Tilman, B6, 4000 Liège, Belgium, Physiol S.A., Parc Scientifique du Sart-Tilman, allée des Noisetiers 4, 4031 Liège, Belgium, Département des Sciences de la Vie, Macromolécules Biologiques, Centre d'Ingénierie des Protéines, University of Liège, Sart-Tilman, B6, 4000 Liège, Belgium, and Département des Sciences et Gestion de l'Environnement, University of Liège, Sart-Tilman, B6, 4000 Liège, Belgium

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Poly[2-(*tert*-butylamino)ethyl methacrylate] (PTBAEMA) belongs to a novel class of water-insoluble biocides. Dispersion of a poly(ethylene-*co*-butylene)-*b*-poly[2-(*tert*-butylamino)ethyl methacrylate] diblock copolymer (PEB-*b*-PTBAEMA) within low-density polyethylene (LDPE) imparts antimicrobial properties to the polyolefin as assessed by the viable cell counting method against *Escherichia coli* (*E. coli*). This diblock copolymer has been synthesized by atom transfer radical polymerization (ATRP) with a poly(ethylene-*co*-butylene) (PEB) oligomer end-capped by an activated bromide as a macroinitiator for the polymerization of 2-(*tert*-butylamino)ethyl methacrylate (TBAEMA). Morphological changes of *E. coli* bacteria in contact with modified LDPE have been observed by transmission and scanning electron microscopy and indicate that the diblock copolymer is bactericide rather than bacteriostatic. Finally, the action mode of the PEB-*b*-PTBAEMA copolymer more likely relies on the displacement of the Ca^{2+} and/or Mg^{2+} ions of the outer membrane of the bacteria, which is disorganized and finally disrupted.

Introduction

The use of biocidal compounds in synthetic polymers is steadily increasing as an answer to the need for safety and hygiene. Any compound that inhibits the growth of microorganisms is referred to as a biocide. Biocides are nowadays additives for polymers used in food packaging, textiles, shower curtains, garden furniture, soles of trainer shoes, and so forth. There are two categories of materials depending on whether the biocide is temporarily trapped within the polymer or permanently attached to the chains. In the first category, a low molecular weight biocide is released from a traditional binder, in which it is physically entrapped or to which it is attached through a hydrolytically unstable bond. 2,4,4′-Trichoro-2-hydroxydiphenyl ether (Triclosan from CIBA) is the biocide most frequently used in this strategy.[1−4] The uncontrolled migration and release of the biocide is the major drawback of this approach. Most often, the release is fast, and an initially high concentration of the active compound is observed in the environment, which results in the time limitation of the biocidal activity. In the other extreme, the biocide is so strongly immobilized within the polymer that the release is extremely low, and the activity is negligible. In the second category of materials, the active compound is covalently attached to the polymer chains, and the biocidal action is based on the contact between the biocide and the microorganisms, with no release of the active molecule. Typical examples are polymers substituted by quaternary ammonium salts,[5−11] phosphonium salts,[12−14] and pyridinium groups,[15,16] respectively. As a rule, these cationic biocides are intended to target the negatively charged membrane of bacteria, which is accordingly disrupted and disintegrated.

The aim of this paper consists of loading traditional polymers, mainly polyolefins, with biocidal polymers bearing amino groups. The purpose is to combine sustainable activity and low toxicity. Poly[2-(*tert*-butylamino)ethyl methacrylate] (PTBAEMA) is a typical representative water-insoluble biocide that can be used for this purpose. According to the patent literature, PTBAEMA exhibits inherent biocidal properties and has potential in antifouling paints and coatings.[17−22] This activity results from the pendant bulky secondary amine of the methacrylate backbone, without the need of quaternization, as is the case for other amine-containing polymers.[23] It must be noted that, although PTBAEMA has high antimicrobial activity and low toxicity, the parent monomer is completely inactive, which suggests that the antimicrobial action is closely related to the molecular structure and conformation of the chains.[23] The strategy envisioned in this work is to incorporate PTBAEMA within low-density polyethylene (LDPE) by direct mixing within conventional converting equipment (extruder). Because polyethylene consists of carbon and hydrogen atoms, the permanency of the antibacterial activity can rely only on the physical anchoring of the active polymer within LDPE. For this purpose, a block copolymer that combines a short polyolefin block miscible with LDPE (anchoring block) and a PTBAEMA block has been synthesized by controlled atom transfer radical polymerization (ATRP) of TBAEMA initiated by a poly-

* rjerome@ulg.ac.be.
[†] Center for Education and Research on Macromolecules (CERM), University of Liège.
[‡] Parc Scientifique du Sart-Tilman.
[§] Centre d'Ingénierie des Protéines, University of Liège.
[||] Département des Sciences et Gestion de l'Environnement, University of Liège.

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(ethylene-*co*-butylene) (PEB) polymer end-capped by an activated bromide. The binary blends have been compression-molded as flasks, and the surface has been analyzed by attenuated total reflection spectroscopy (ATR). Finally, the antimicrobial activity of the flasks has been assessed in the presence of *Escherichia coli* by using the vial cell counting method.[24] Morphological changes of the *E. coli* cells in contact with modified LDPE have been observed by scanning electron microscopy and transmission electron microscopy, in order to understand the mechanism of action of PTBAEMA.

Experimental Section

Materials. CuCl (Aldrich, 98%) was dispersed within glacial acetic acid under stirring for a few hours, filtered, washed with ethanol, dried under reduced pressure at 80 °C, and stored under nitrogen. 2-Bromoisobutyryl bromide (Aldrich, 98%), 1,4,7,10,10′-hexamethyltriethylenetetramine (HMTETA) (Aldrich, 97%), hydroxyl end-capped poly(ethylene-*co*-butylene) (PEB−OH; $M_n = 3900$ and $M_w/M_n = 1.1$) from Shell Chemicals (Kraton, L-1203), and LDPE 4023 from Exxon Chemicals were used as received. 2-(*tert*-Butylamino)ethyl methacrylate (TBAEMA) (Aldrich, 97%) was dried over CaH_2, distilled under reduced pressure, and stored under N_2 at −20 °C. Methylene chloride (CH_2Cl_2) was refluxed over CaH_2 and degassed by nitrogen bubbling for 20 min. Toluene was refluxed over Na/benzophenone complex, distilled, and degassed by nitrogen bubbling for 20 min.

Preparation of the Macroinitiator (PEB−Br). 150 mL of dry and degassed methylene chloride and 1.54 g (15.3 mmol) of triethylamine were added to a 500 mL two-neck round-bottom flask equipped with a dropping funnel and a magnetic stirrer, closed by a three-way stopcock, and placed under nitrogen. After cooling to 0 °C, 1.9 mL of 2-bromoisobutyryl bromide (15.5 mmol) was added, followed by the dropwise addition of 30 g (7.1 mmol) of PEB−OH in 300 mL of dry, degassed CH_2Cl_2 for 4 h. The temperature was allowed to increase up to room temperature, and the reaction took place under stirring overnight. Half of the solvent was distilled off, and an equal volume of toluene was added. The solution was filtered, and the PEB−Br macroinitiator was precipitated in methanol. After two additional precipitation steps from toluene to MeOH, the macroinitiator was collected and dried in vacuo. ^{1}H NMR ($CDCl_3$), δ: 1.80−1.90 (s, 6H), 4.15−4.25 (m, 2H), 1.24 (m, 473H), 0.8 (t, 111H) ppm.

Synthesis of PEB-*b*-PTBAEMA. In a typical experiment, the macroinitiator (PEB−Br), CuCl, HMTETA, toluene, and a magnetic stirrer were introduced in a glass flask that was closed by a three-way stopcock. This solution was degassed by bubbling of nitrogen for 20 min. The monomer was added with a syringe, and the reactor was heated in an oil bath thermostated at 70 °C. Progress of polymerization was followed by picking up samples that were treated in the Sartorius MA50 analyzer (infrared heater; "standard desiccation" at 150 °C) for the gravimetric determination of the polymer. The collected polymer was analyzed by size exclusion chromatography (SEC). Before SEC analysis, the catalyst was extracted by elution of a copolymer solution in THF through an Al_2O_3 filled column. When the conversion was complete, the reaction mixture was diluted with THF and eluted through Al_2O_3. After solvent elimination, the polymer was dried in vacuo (for one night, at 40 °C).

Preparation of the Blends. Melt blending was carried out in a ZSk twin screw extruder at 100 rpm. LDPE was melt-blended with 10 wt % of the PEB-*b*-PTBAEMA (4100−14 000) at 140 °C. Blends were compression-molded as flasks by Optim Test Center S.A. (Liège, Belgium) at 170 °C.

Antimicrobial Assessment (Shake Flask Method). A freeze-dried ampule of *Escherichia Coli* (DH5α) was opened, and the culture was picked up with a micropipet and placed in 2 mL of nutrient broth (composition for 1 L of nutrient broth (Luria Bertani): 10 g bactotryptone, 5 g of extract of yeast, sodium chloride) and incubated (incubator shaker model G25; New Bruswick Scientific Co., Inc.; Edison, NJ) at 37 °C overnight. Then, 200 μL of the culture was placed in 100 mL of nutrient broth; the bacterial culture was incubated at 37 °C for 4 h. At this stage, the culture of *Escherichia Coli* contained ca. 10^8 cells/mL (absorbance at 600 nm = 0.6) and was used for the antibacterial test. 0.5 cm × 0.5 cm pieces of LDPE melt-blended with 10 wt % of PEB-*b*-PTBAEMA were dispersed in a sterile saline water mixture (prepared by dissolving 8.5 g of sodium chloride in 1 L of MilliQ water in a "Schott" bottle followed by sterilization at 121 °C for 20 min). This dispersion was diluted with sterile distilled saline water until the desired concentration (0.2, 0.3, and 0.4 g/mL) was reached when 9 mL of this biocide solution preequilibrated at 37 °C was combined with 1.0 mL of the bacterial culture that contained $\pm 10^8$ cells/mL. In parallel, 1.0 mL of the same culture was added to 9 mL of sterile saline water that contained 0.4 g/mL of LDPE (control). At regular time intervals, 100 μL samples were removed, and decimal serial dilutions (until 10^5) were carried out by mixing 100 μL with 900 μL of sterile saline water. From these dilutions, the surviving bacteria were counted by the spread plate method (100 μL of decimal dilutions were spread on a Petri dish that contained LB agar). The Petri dishes were incubated at 37 °C overnight. After incubation, the colonies were counted.

Characterization. Size exclusion chromatography (SEC) was performed in THF containing 5 vol % of triethylamine at 40 °C, with a Waters 600 liquid chromatograph equipped with a Waters 410 refractive index detector and Styragel HR columns effective in the 100−5000 (HR 1), 500−20 000 (HR 2), and 5000−600 000 (HR 4) molecular weight ranges. The styragel columns were calibrated with PMMA standards.

^{1}H NMR spectra were recorded with a Brucker AN 400 (400 MHz) in $CDCl_3$ at 25 °C.

ATR spectra were recorded with an attenued transmission reflection infrared spectrometer (ATR-FTIR, Thermo Nicolet Continuμm, Ge 800−1800 nm).

A JEOL JSM-840A scanning electron microsope (SEM) operating at an accelerating voltage of 20 kV was used to observe the cells. Fixation and dehydration of samples were carried out by conventional technique.

Morphology of the cells was observed with a Philips CM100 transmission electron microscope after fixation and dehydration of samples by conventional technique. A Reichter-Jung ultracryomicrotome equipped with a diamond knife was used to prepare ultrathin samples at −78 °C.

Table 1. M_n Data for the PEB−Br Macroinitiator

$M_{n,th}$[a]	$M_{n,exp}$[b]	$M_{n,exp}$[c]	PDI[c]
3900	4100	7900	1.05

[a] Specified by the supplier (PEB−OH). [b] Determined by ^{1}H NMR. [c] Determined by PMMA-calibrated SEC.

Results and Discussion

Preparation of the Macroinitiator.[25] The hydroxyl endgroup of a commercially available poly(ethylene-*co*-butylene) oligomer (PEB−OH) has been bromoacetylated by 2-bromoisobutyryl bromide, so making a macroinitiator, PEB−Br, available to ATRP of TBAEMA. Success of the reaction has been confirmed by ^{1}H NMR. Indeed, the $-CH_2OH$ multiplet of PEB−OH at 3.66 ppm has disappeared upon bromoacetylation, and two new signals have been observed, i.e., a singlet at 1.80−1.90 ppm for the $[(CH_3)_2C(Br)-]$ dimethyl groups and a multiplet at 4.15−4.25 ppm for the $[-CH_2-OC(O)]$ methylene groups. The PEB−Br macroinitiator shows two characteristic ^{1}H NMR peaks that can be assigned to the terminal group ($\delta = 4.2$ ppm) and the repeat units ($\delta = 1.2$, 0.8 ppm), respectively. M_n of PEB−Br has been calculated from the integrals of these two peaks as reported in Table 1, together with M_n SEC (PMMA calibration).

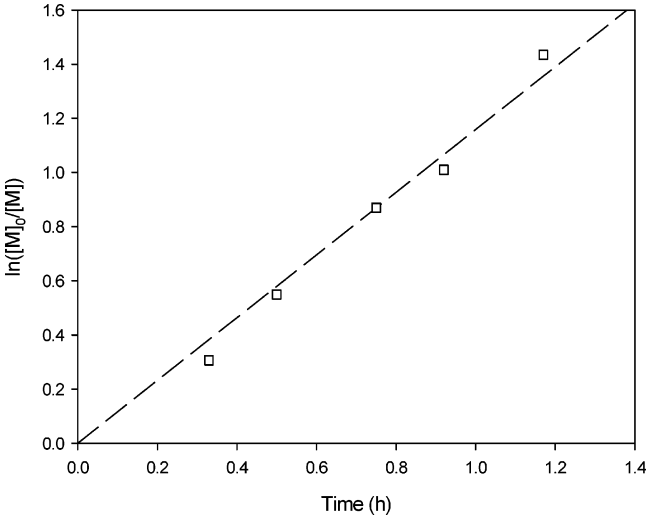

Figure 1. Time dependence of ln([M]$_0$/[M]) for the ATRP of TBAEMA initiated by PEB−Br catalyzed by CuCl/HMTETA at 70 °C in toluene: [TBAEMA]$_0$ = 4 M; [PEB−Br]$_0$ = [HMTETA]$_0$ = [CuCl]$_0$ = 50 mM.

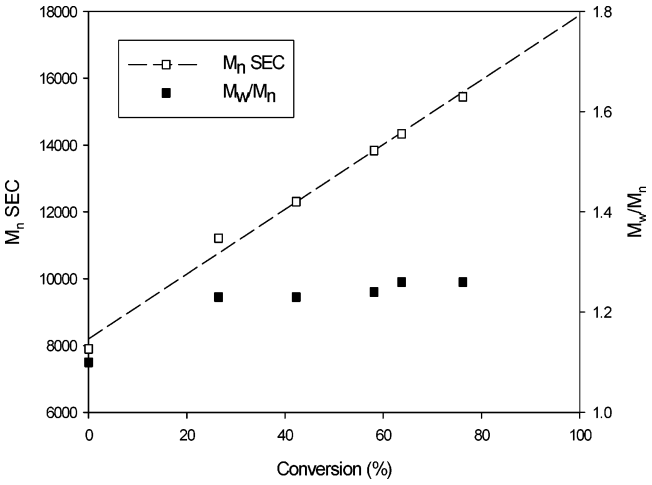

Figure 2. Conversion dependence of M_n and M_w/M_n for the ATRP of TBAEMA initiated by PEB−Br and catalyzed by CuCl/HMTETA at 70 °C in toluene: □ M_n SEC, ■ M_w/M_n. [TBAEMA]$_0$ = 4 M; [PEB]$_0$ = [lHMTETA]$_0$ = [CuBr]$_0$ = 50 mM.

Synthesis of PEB-*b*-PTBAEMA. Polymerization of TBAEMA has been initiated by the PEB−Br macroinitiator (M_n = 4100), with CuCl as a catalyst ligated by HMTETA in toluene at 70 °C. Figures 1 and 2 show the time dependence of ln([M]$_0$/[M]) and how the molecular weight and polydispersity depend on the monomer conversion. Concentration of the active species is constant, as supported by the linearity of ln([M]$_0$/[M]) vs time. The molecular weight increases linearly with time, and polydispersity remains narrow (M_w/M_n = 1.2). These observations are consistent with a controlled process. The SEC chromatograms reported at different monomer conversions are monomodal all along the polymerization (Figure 3), in agreement with a fast consumption of the macroinitiator compared to chain propagation. Moreover, the PEB-*b*-PTBAEMA block copolymer is formed within a very short reaction time (76% monomer conversion after 70 min). Figure 4 shows the ^{1}H NMR spectrum and the assignment of the signals for the PEB-*b*-PTBAEMA. M_n of the PTBAEMA block (14 000) has been determined from the relative intensity of the ^{1}H NMR signals for the methacrylate units [−CH_2−OC(O)] at 4.1 ppm and for PEB at 0.8 ppm [−CH_2−CH(CH_2−CH_3)−$(CH_2)_4$−].

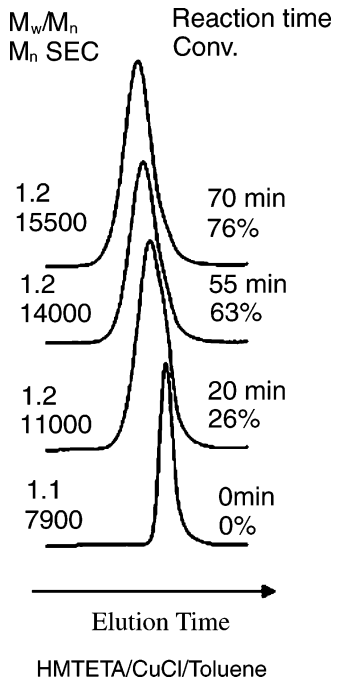

Figure 3. Evolution of the SEC chromatograms with TBAEMA conversion in the presence of CuCl ligated by HMTETA.

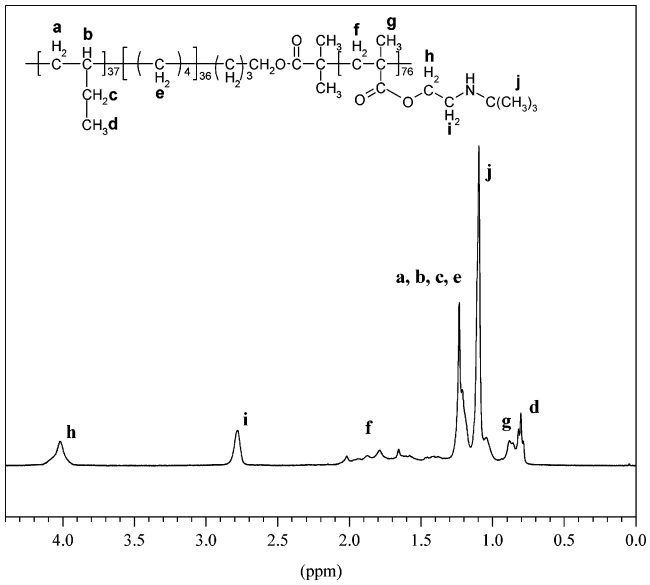

Figure 4. ^{1}H NMR spectrum for PEB-*b*-PTBAEMA.

Preparation and Molding of LDPE/PEB-*b*-PTBAEMA Blends. 10 wt % of PEB-*b*-PTBAEMA (4100-*b*-14 000) have been incorporated into LDPE by melt-blending at 140 °C using a ZSk twin screw extruder, and flasks (8.5 cm × 6 cm (L × D)) have been compression-molded at 170 °C. ATR spectroscopy of the surface and bulk of the flasks has been conducted in order to localize the PEB-*b*-PTBAEMA, at the surface and/or in the bulk. Indeed, because the PEB-*b*-PTBAEMA copolymer is antimicrobial by contact, the permanent location of the active compound at the LDPE surface is very crucial for the final product to exhibit significant and sustainable antibacterial properties. Figure 5 shows the ATR spectra for LDPE (1), surface and bulk of the flasks (10 wt % of PEB-*b*-PTBAEMA within LDPE) (2) and (3), and PTBAEMA (4). PTBAEMA shows an absorption peak at 1735 cm^{-1} characteristic of the ester bond (CO), thus in a range in which LDPE does not absorb.

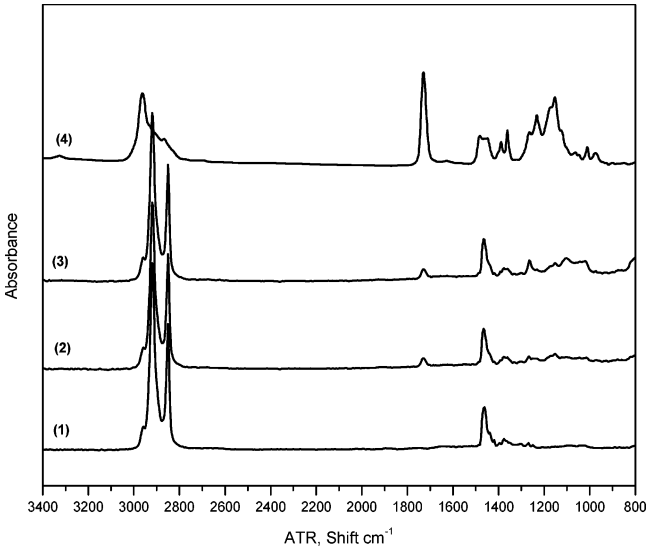

Figure 5. ATR spectra for LDPE (1), LDPE containing 10 wt % PEB-*b*-PTBAEMA (surface, 2; bulk, 3), and PTBAEMA (4).

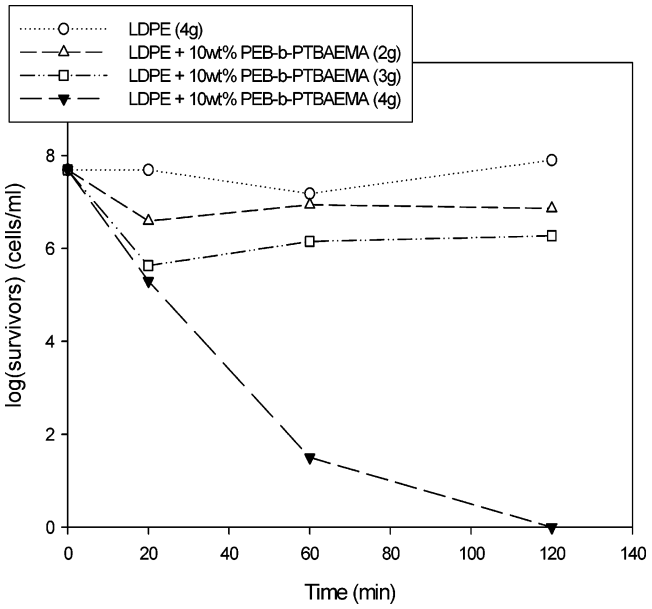

Figure 6. Plots of log(survivors) versus exposure time for LDPE and LDPE containing 10 wt % of PEB-*b*-PTBAEMA against *E. coli*. ○, 4 g of LDPE; ▽, □, and ▲, 2 g, 3 g, and 4 g of modified LDPE, respectively.

The common region for each compound at 2800−3000 cm^{-1} corresponds to the elongation of the alkyl groups (C−H of CH_2 and C−H of CH_3). Analysis by ATR spectroscopy clearly shows that the block copolymer is homogeneously distributed within the flask, i.e., at the surface and in the bulk. Only the PEB-*b*-PTBAEMA copolymer located at the surface can impart an antimicrobial activity to the LDPE flasks.

Antimicrobial Activity. The dynamic shake flask method has been used to assess the ability of the LDPE surface to kill bacteria. For these experiments, 0.5 cm × 0.5 cm pieces (2 g, 3 g, 4 g) of modified LDPE and LDPE (control, 4 g) were shaken with 10 mL of a bacterial suspension (10^8 cells/mL) for 20, 60, and 120 min at 37 °C. The number of viable cells in the suspension was counted after incubation by dilution of the samples, followed by overnight incubation on agar plates. The permanency of the antimicrobial compound (PEB-*b*-PTBAEMA) in LDPE has been assessed by placing a 2 cm × 2 cm piece of modified LDPE and LDPE, respectively, on the surface

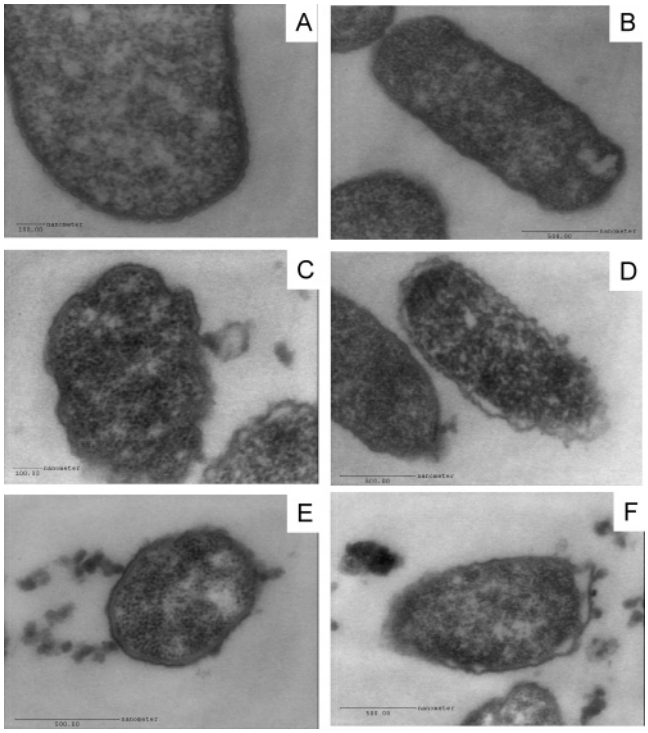

Figure 7. TEM micrographs of *E. coli* in contact with neat LDPE after 60 min (A,B) and modified LDPE (C,D) and (E,F), after 30 min and 60 min of contact, respectively.

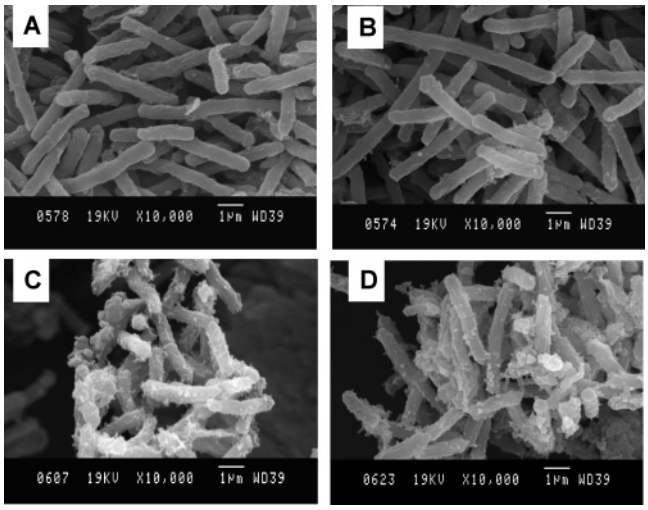

Figure 8. SEM micrgraphs of *E. coli* in contact with neat LDPE (A) and modified LDPE (B−D) after 15 (B), 30 (C), and 60 min (A,D) of contact time.

of an agar plate that was previously inoculated with approximately 1 × 10^6 *E. coli*. The lack of growth inhibition just around these pieces indicates that no active compound is diffusing out of the modified LDPE. Moreover, modified LDPE was incubated for one week in water in the absence of bacteria. After separation, the solution was inoculated, and growth of bacteria was observed consistent with lack of diffusion of antimicrobial molecules out of the matrix. Figure 6 shows a plot of the log(number of survivors) against the exposure time when the same amount of *E. coli* (10^8 cells/mL of bacteria) is exposed to 2 g, 3 g, and 4 g of LDPE containing 10 wt % of PEB-*b*-PTBAEMA (0.5 cm × 0.5 cm pieces) and 4 g of LDPE (control), respectively. In all the cases, the polymer was used as a collection of 0.5 cm × 0.5 cm pieces. The total amount of PTBAEMA (active compound) available both at the surface and

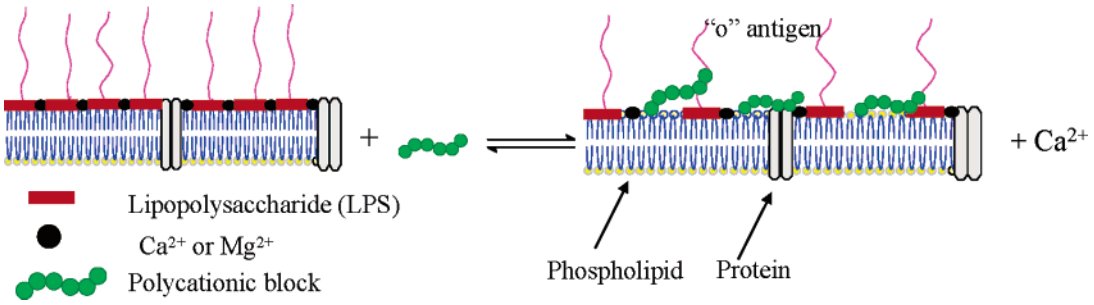

Figure 9. Equilibrium between the outer membrane of bacteria and cationic PTBAEMA.

in the bulk is 1.67 mmol (4 g of blend), 1.25 mmol (3 g of blend), and 0.83 mmol (2 g of blend), respectively. LDPE with 10 wt % of PEB-*b*-PTBAEMA is able to kill all the bacteria cells within 120 min when 0.4 g of modified LDPE is used per milliliter of bacterial suspension (0.167 mmol PTBAEMA/mL suspension). When less modified LDPE is used (0.3 g/mL and 0.2 g/mL), the concentration of bacteria decreases for 20 min and then levels off at approximately 10^6 and 10^7 cells/mL for 0.3 and 0.2 g/mL, respectively. Therefore, a PEB-*b*-PTBAEMA block copolymer has the ability to provide LDPE with an antimicrobial activity merely by contact.

Morphological Changes of Bacterial Cells. For these experiments, 0.5 cm × 0.5 cm pieces (1.3 g) of modified LDPE and LDPE (control, 1.3 g), respectively, were shaken with 3.33 mL of an *E. coli* suspension (10^8 cells/mL) for 15, 30, and 60 min at 37 °C. Then, 900 μL of the culture medium were picked out, fixed, dehydrated by conventional technique,[26] and analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Transmission electron microscopy has shown striking structural alterations for *E. coli* exposed to modified LDPE. The observations reported in Figure 7 are representative of the bacteria exposed to neat LDPE (A and B) and modified LDPE for 30 min (C and D) and 60 min (E and F), respectively. The double membrane is clearly seen in untreated bacteria, which is no longer the case when bacteria have been in contact with 0.4 g/mL of modified LDPE. Retraction of cytoplasm from the outer membrane is also a typical consequence of the treatment of bacteria by PEB-*b*-PTBAEMA containing LDPE. An additional characteristic feature is the release of fibrous and granular material, presumably cell content, through damaged membranes. This phenomenon, which is pronounced after 60 min of contact, is straight evidence for the destruction of the bacteria membrane.

E. coli treated with modified LDPE have also been observed by scanning electron microscopy. The surface morphology of the bacterial cells remains unchanged in the presence of neat LDPE (Figure 8A) even after 60 min of contact. When treated by modified LDPE for increasing periods of time (up to 60 min), the surface of *E. coli* shows steadily more pronounced wrinkles and blebs (Figure 8B−D). The membrane of many cells is disintegrated, and fibrous and cellular material accumulates as result of the leakage and lyses of the cells.

Importantly, the surface of the blends has been observed after 15, 30, and 60 min of contact by scanning electron microscopy (SEM). No bacteria has been observed sticking on the active surface, which means that this surface is not bacteriostatic and that bacteria are killed by contact and released in the aqueous environment. Therefore, the activity of the surface should not be inhibited.

Action Mode of PEB-*b*-PTBAEMA. This work has confirmed that PTBAEMA is an antimicrobial polymer as result

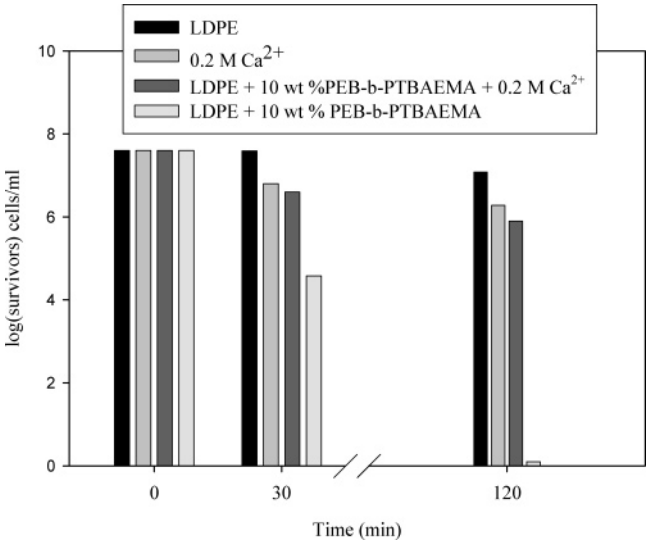

Figure 10. Plots of log(survivors) versus exposure time for LDPE (0.4 g/mL), $CaCl_2$ (0.2 M), and modified LDPE (0.4 g/mL) with and without $CaCl_2$ (0.2 M) against *E. coli*.

of its ability to disorganize the bacterial membrane. Sosna et al.[23] suggested that the antimicrobial activity results from the pendant bulky secondary amine attached along the helical methacrylate backbone and oriented face outward. The outer membrane of Gram-negative bacteria, such as *E. coli*, is composed of phospholipids, proteins, and lipopolysaccharides (LPS). Its major role is to take a variety of environmental molecules away from the cell and to allow for selective uptake of other molecules. The divalent cations, i.e., Ca^{2+} and Mg^{2+}, that cross-bridge adjacent negatively charged LPS molecules in the membrane are key players.[27] Polymyxin, which is a polycationic amphipathic antibiotic, binds strongly to LPS. The divalent cations at the binding sites are then replaced by polymyxin molecules and released from *E. coli* cells, which results in the disruption of the outer membrane.[28,29] According to the pK_a of TBAEMA (9.12), most of the tertbutyl amino groups of PTBAEMA are charged in water, which make this polymer similarly to polymyxin. In qualitative agreement with TEM and SEM of *E. coli* in contact with LDPE loaded by PEB-*b*-PTBAEMA, the charged amino groups of the PTBAEMA block have the potential to replace the divalent cations of the outer membrane as illustrated in Figure 9, followed by membrane disorganization and lyses of the cells. To confirm this hypothesis, an excess of Ca^{2+} (0.2 M) compared to the secondary amino groups (0.167 M) has been added to the culture medium. Figure 10 shows the plot of the log(number of *E. coli* survivors) versus the exposure time for 4 g of LDPE (control, 0.5 cm × 0.5 cm pieces), with 0.2 M of $CaCl_2$ (control calcium) and for 4 g of modified LDPE (0.5 cm × 0.5 cm pieces) with and without 0.2 M of $CaCl_2$. Clearly, the antibacterial activity

of LDPE is no longer observed, which strongly suggests that the equilibrium shown in Figure 9 is displaced to the left by the excess of calcium ions in the medium, thus in favor of free amino groups.

Conclusions

Melt-blending of LDPE with 10 wt % of a diblock copolymer (PEB-*b*-PTBAEMA) synthesized by controlled atom transfer radical polymerization is an easy way to prepare a novel antimicrobial material active by contact with bacteria. This polyblend has been molded with a flask shape, and the antimicrobial activity has been assessed against *E. coli*. Effective antimicrobial activity has been observed for a content of 0.4 g of blend/mL. The morphological changes exhibited by the bacterial cells in contact with modified LDPE have been studied by TEM and SEM. The inner membrane is hardly distinguished anymore, and fibrous and granular material, presumably cell content, is released through the damaged membrane. A reasonable explanation for this effect is that the secondary amines bound to the PTBAEMA block of the PEB-*b*-PTBAEMA diblock copolymer are protonated in water and displace by exchange the Ca^{2+} or/and Mg^{2+} cations of the outer membrane. Indeed, these cations are responsible for the cohesion and integrity of the lipopolysaccharides that make up the membrane. Once they are displaced by an external agent, the outer membrane is disorganized, and the lysis of cells occurs, which results in death of the bacteria.

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