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Antimicrobial activity of polystyrene particles coated by photo-crosslinked block copolymers containing a biocidal polymethacrylate block

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Abstract: A commercially available poly(ethylene-*co*-butylene) copolymer, end-capped by a short polyisoprene block and a hydroxyl group (PI-*b*-PEB-OH), has been derivatized into a macroinitiator for atom transfer radical polymerization (ATRP) by esterification of the hydroxyl end-group by an activated bromide-containing acyl bromide. Two types of triblock copolymers, PI-*b*-PEB-*b*-poly-(dimethylaminoethyl methacrylate) (PDMAEMA) and PI-*b*-PEB-*b*-poly[2-(*tert*-butyl-amino)ethyl methacrylate] (PTBAEMA), have been synthesized and used to coat polystyrene particles. These coatings have been permanently immobilized by UV cross-linking of the isoprene units. They exhibit a biocidal activity against Gram-negative bacteria either intrinsically in case of the PTBAEMA block or upon quater-nization of the PDMAEMA block by octyl bromide. The antimicrobial activity is directly related to the concentration of coated PS particles in the medium.

Introduction

Growth of microorganisms must be inhibited at the surface of polymeric materials used in a variety of applications, such as food packaging, antifouling paints [2,3] and hospital furniture. Nowadays, antimicrobials in plastics are a growing sector of the speciality biocides industry. The effectiveness of an antimicrobial material is related to its ability to limit contact with the microorganisms and to inhibit their growth. Bactericidal and fungicidal properties can be granted to a coating whenever a pre-incorporated biocide is slowly released, which is illustrated by antifouling paints used to protect submerged structures in sea water [3,4]. Bioactive molecules can be leached from the coating by different mechanisms, including erosion of the binder and by hydrolysis of a chemical bonding. A drawback of the antifouling paints is found, however, in the toxicity of the active molecule and the short-lived protection. An effective way to tackle this problem consists in developing materials with an intrinsic and thus permanent antimicrobial activity. Nowadays, fibres with inherent antimicrobial activity are available on the market place [5] and used to protect wears,

socks, filters and package materials. Polymer chains with pendant quaternary ammonium salts (QAS) bonded through non-hydrolysable covalent bonds exhibit bactericidal and fungicidal activities in water and in the solid state, as well [6]. As a rule, polycationic biocides with high positive charge density deserve interest in hygiene and biomedical applications. This paper aims at reporting on the synthesis of a novel class of polymeric biocides active by contact. They are block copolymers that consist of a central poly(ethylene-*co*-butylene) block flanked by, respectively, a short polyisoprene block, and a block with biocidal activity, either a quaternized poly-(dimethylaminoethyl methacrylate) block (PDMAEMA) or a poly[2-(*tert*-butylamino)-ethyl methacrylate] (PTBAEMA). These copolymers are intended to coat polyolefins, mainly low-density polyethylene (LDPE) and polypropylene (PP), in order to impart them antimicrobial properties without leaching any compound to the environment. The role of the poly(ethylene-*co*-butylene) block is to anchor the copolymer to poly-olefin surfaces. Because it is cross-linkable upon UV exposure, the polyisoprene block will contribute to the permanency of the coating. Concerning the third block, PDMAEMA must be quaternized by octyl bromide for exhibiting antimicrobial activity [7], in contrast to PTBAEMA that is intrinsically active probably because of the bulki-ness of the pendant secondary amines. Short poly[isoprene-*b*-(ethylene-*co*-butylene)] copolymers are commercially available, with a hydroxyl end-group attached to the polyolefin block. This hydroxyl group has been derivatized into an activated bromide, so making a macroinitiator available to the ATRP polymerization of DMAEMA and TBAEMA. Polystyrene (PS) particles have been coated by the copolymers that were cross-linked upon UV exposure. The antimicrobial activity of the particles has been assessed in the presence of *E. coli* and by using the vial cell counting method [8].

Results and discussion

Preparation of the macroinitiator [9]

The hydroxyl end-group of the commercially available poly[isoprene-*block*-(ethylene-*co*-butylene)] oligomer (PI-*b*-PEB-OH) has been reacted with 2-bromoisobutyryl bromide, in order to make a macroinitiator, PI-*b*-PE-Br, available to ATRP of DMAEMA, TBAEMA and MMA, respectively. Moreover, the short polyisoprene block of this macroinitiator (*c*. 10 double bonds/chain) makes the amphiphilic copolymer cross-linkable upon UV exposure. Success of the reaction has been confirmed by ^{1}H NMR (Fig. 1). Indeed, the -$\underline{CH_2}$OH multiplet of PI-*b*-PEB-OH at 3.66 ppm disap-peared upon bromoacetylation, and a new signal has been observed, viz., a multiplet at 4.15 - 4.25 ppm for the [-$\underline{CH_2}$-OC(O)] methylene group. The PI-*b*-PEB-Br macroini-tiator shows some characteristic ^{1}H NMR peaks, which can be assigned to the iso-prene units (δ = 5.5 - 5 ppm), to the terminal group (δ = 4.2 ppm) and the repeating units (δ = 2.2 - 0.8 ppm), respectively (Fig. 1). Molecular characteristics of PI-*b*-PEB-Br are reported in Tab. 1.

Tab. 1. Molecular weight of the PI-*b*-PEB-Br macroinitiator

$M_{n,th}$ [a]	M_n [a] (PEB)	M_n [a] (PI)	$M_{n,exp}$ [b]	M_n [b] (PEB)	M_n [b] (PI)	$M_{n,exp}$ [c]	PDI [b]
6000	5410	590	5900	5320	544	13 600	1.05

[a] Specified by the supplier. [b] Determined by ^{1}H NMR. [c] Determined by PMMA-cali-brated SEC.

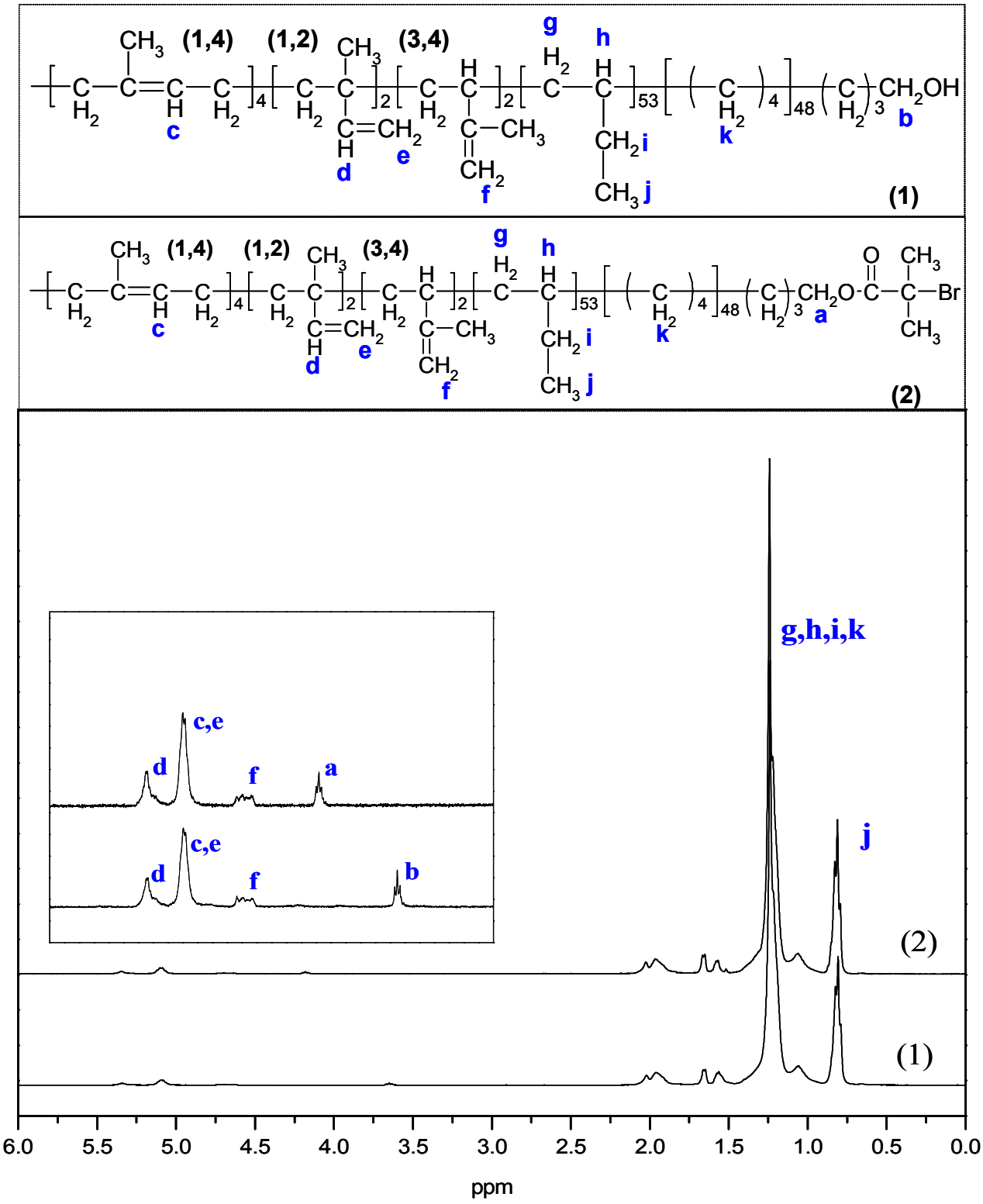

Fig. 1. ^{1}H NMR spectra of PI-*b*-PEB-OH (1) and PI-*b*-PEB-Br (2)

Synthesis of PI-b-PEB-b-PDMAEMA copolymers

ATRP of DMAEMA has been initiated by the PI-*b*-PEB-Br macroinitiator in toluene and catalyzed by a mixture of CuCl and $CuCl_2$ ([CuCl]/[$CuCl_2$] = 1/0.1) ligated by HMTETA. M_n of the PDMAEMA block has been determined from the relative intensity of the ^{1}H NMR signals for the methacrylate units [-$\underline{CH_2}$-OC(O)] at 4.1 ppm and for

PI-*b*-PEB at 5.2 - 5.3 ppm, respectively. M_n of PDMAEMA (6000) is expectedly close to that of PI-*b*-PEB (6000), and the polydispersity is low (1.2).

The PDMAEMA block has been quaternized by octyl bromide, because Kanazawa et al. [10,11] reported that the antimicrobial activity is strongly dependent on the charge density and length of the alkyl chain, which should ideally contain 8 carbon atoms. Indeed, the antibacterial activity decreases whenever the chain length exceeds 8 carbon atoms. There is thus an optimal balance between the hydrophobicity of the cationic disinfectant and the targeted cytoplasmic membrane of bacteria [12-13]. The main constitutive components of the cytoplasmic membrane are proteins and phosphoglycerides with a dual structure consisting of a hydrophilic phosphate (including polar residues) and two long-chain fatty acid tails with 12 - 20 carbon atoms [14]. The higher activity of cationic biocides with an octyl [7] chain would thus result from the stronger interaction with the cytoplasmic membranes of bacteria.

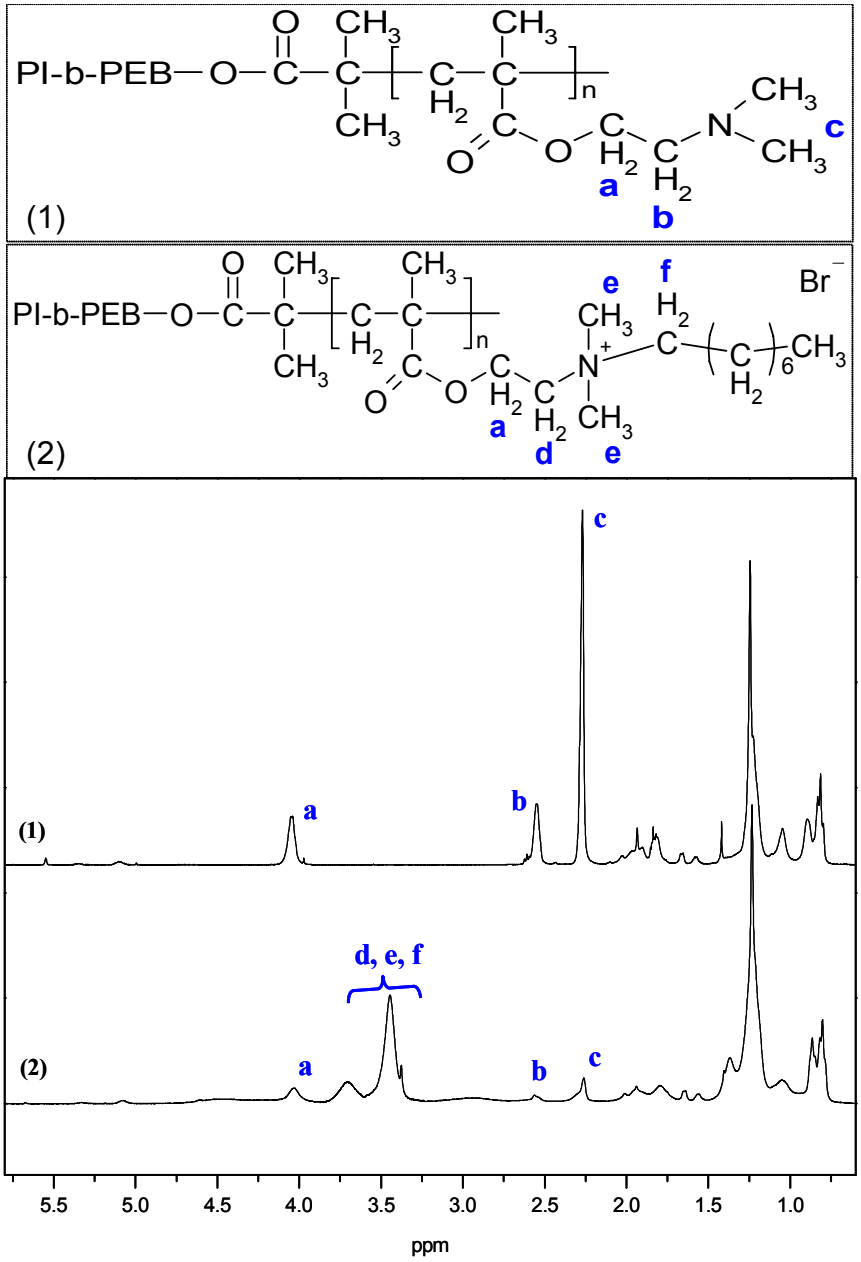

Fig. 2. ^{1}H NMR spectra for (1) PI-*b*-PEB-*b*-PDMAEMA and (2) PI-*b*-PEB-*b*-PDMAEMA quaternized by octyl bromide (86%)

The PDMAEMA block of the diblock copolymer has been quaternized by 0.9 eq of octyl bromide in tetrahydrofuran (THF), at 50°C, overnight. ^{1}H NMR analysis before and after quaternization (Fig. 2) confirmed that 86% of the pendant amines have been quaternized, based on the $\underline{CH_2}$-N(CH_3)$_2$ multiplet and the CH_2-N($\underline{CH_3}$)$_2$ multiplet of poly(DMAEMA) at 2.6 and 2.2 ppm, respectively, that decreased at the benefit of two new signals: a singlet at 3.5 ppm for the methyl groups -N^+-($\underline{CH_3}$)$_2$ and a multiplet at 3.7 ppm for the methylene protons –$\underline{CH_2}$-N^+-$C\underline{H_2}$ of the quaternized units.

Synthesis of PI-b-PEB-b-PTBAEMA copolymers

A PI-*b*-PEB-*b*-PTABEMA diblock has also been prepared by ATRP of TBAEMA initiated by PI-*b*-PEB-Br in the presence of CuBr, HMTETA and toluene, at 70°C. M_n of the polymethacrylate block (8000) has been determined from the relative intensity of the ^{1}H NMR signals for the [-$\underline{CH_2}$-OC(O)] protons of the PTBAEMA block at 4.1 ppm and for the PI-*b*-PEB block at 5.2 - 5.3 ppm, respectively (Fig. 3). The molecular weight distribution is monomodal and symmetric with M_w/M_n = 1.3.

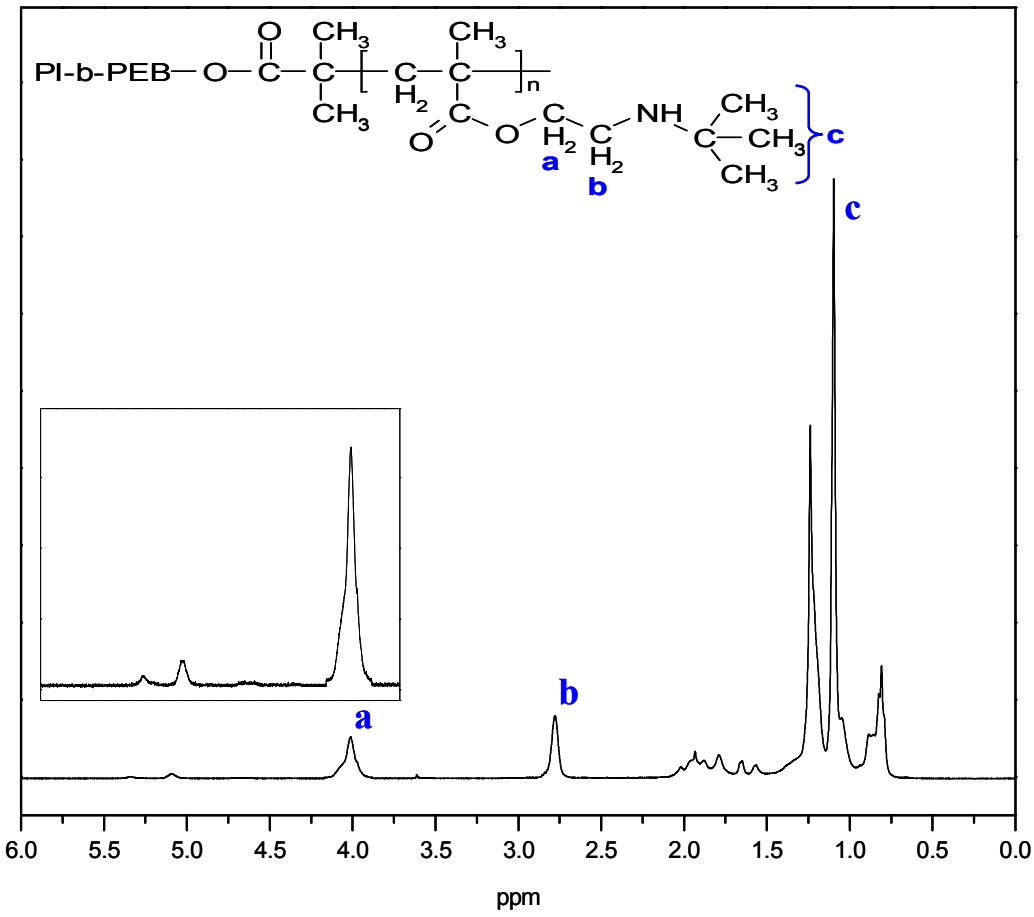

Fig. 3. ^{1}H NMR spectrum of PI-*b*-PEB-*b*-PTBAEMA

Antibacterial activity of PS particles coated by cross-linked PI-b-PEB-b-PDMAEMA (quaternized by octyl bromide) and PI-b-PEB-b-PTBAEMA copolymers

Polystyrene particles (100 - 200 mesh) have been coated by the PI-*b*-PEB-*b*-PDMAEMA (6000-6000) triblock copolymer quaternized by octyl bromide and by the PI-*b*-PEB-*b*-PTBAEMA (6000-8000) copolymer, respectively. Afterwards, the

isoprene units of the diblock copolymer have been cross-linked upon UV irradiation in the presence of benzophenone. Indeed, preliminary tests have shown the beneficial effect of benzophenone on cross-linking. Actually, benzophenone is a known photo-initiator, which generates free radicals under UV and, accordingly, initiates the cross-linking of unsaturated compounds.

After cross-linking of the PI-*b*-PEB block upon UV exposure, the PS particles have been assessed against naturally occurring microbial contaminants. Microbial growth inhibition has been measured by monitoring the microbial viability of *Escherichia coli* in the presence of the surface-coated particles by using the viable cell counting method in solution (shake flask method). Although the diblock copolymers were purified by passing a solution of them through an Al_2O_3 column in order to remove the copper catalyst, a small part of this catalyst persists as assessed by a light green colour. Because copper is known for antimicrobial properties, a poly(isoprene-*b*-(ethylene-*co*-butylene))-*b*-poly(methyl methacrylate) (PI-*b*-PEB-*b*-PMMA) block copolymer which doesn't bear ammonium groups and thus, with no intrinsic biocidal activity, has been synthesized by ATRP of MMA initiated by PI-*b*-PEB-Br. The catalyst has not been eliminated after synthesis of this PI-*b*-PEB-*b*-PMMA in order to assess whether the residual copper catalyst has an antimicrobial activity or not. The PI-*b*-PEB-*b*-PMMA diblock has been deposited at the surface of the PS particles (100 - 200 mesh) and cross-linked by UV.

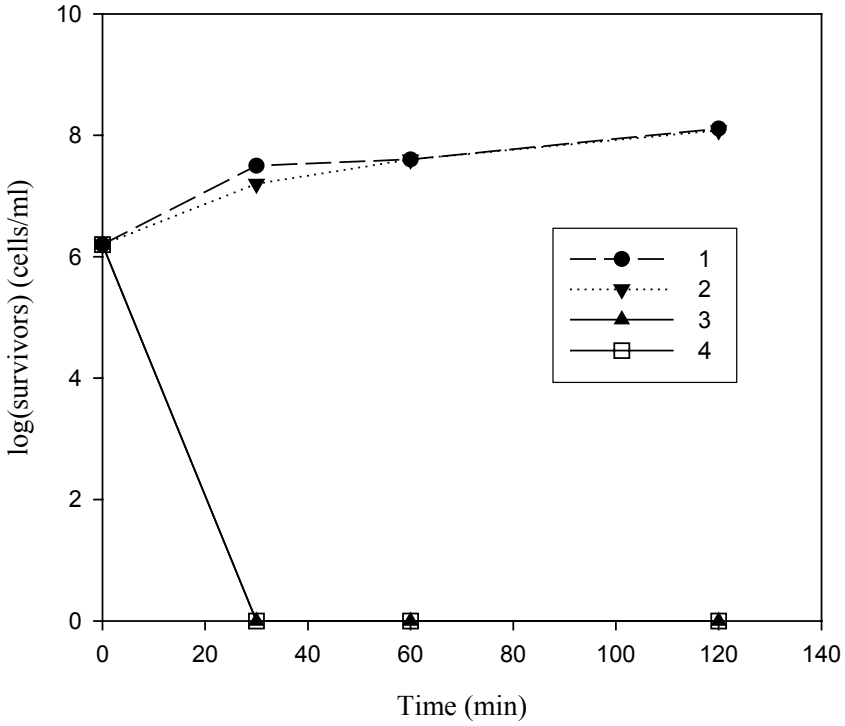

Fig. 4. Plot of log(survivors) vs. exposure time for samples 1 - 4: 1. PS particles (25 mg/mL). 2. PS particles coated by cross-linked PI-*b*-PEB-*b*-PMMA (6000-9000), 25 mg/mL. 3. PS particles coated by cross-linked PI-*b*-PEB-*b*-PDMAEMA (6000-6000) quaternized by octyl bromide, 25 mg/mL. 4. PS particles coated by cross-linked PI-*b*-PEB-*b*-PTBAEMA (6000-8000), 25 mg/mL

Fig. 4 shows how the number of survivors depends on the contact time with the PS particles (25 mg/mL) coated by 10 wt.-% of the PI-*b*-PEB-*b*-PDMAEMA (6000-6000)

copolymer after quaternization (86%) by octyl bromide, the PI-*b*-PEB-*b*-PTBAEMA (6000-8000) copolymer and PI-*b*-PEB-*b*-PMMA (6000-9000), respectively. For the sake of comparison, uncoated PS particles have been used as control at a concentration of 25 mg/mL. The PS particles (25 mg/mL) coated by non-quaternized PI-*b*-PEB-*b*-PTBAEMA and quaternized PI-*b*-PEB-*b*-PDMAEMA kill all the bacteria within 30 min of contact. Quite importantly, PI-*b*-PEB-*b*-PMMA is inactive when used at the same content of 25 mg/mL of coated particles, which indicates that the residual ATRP catalyst doesn't contribute to the antimicrobial properties of the copolymers.

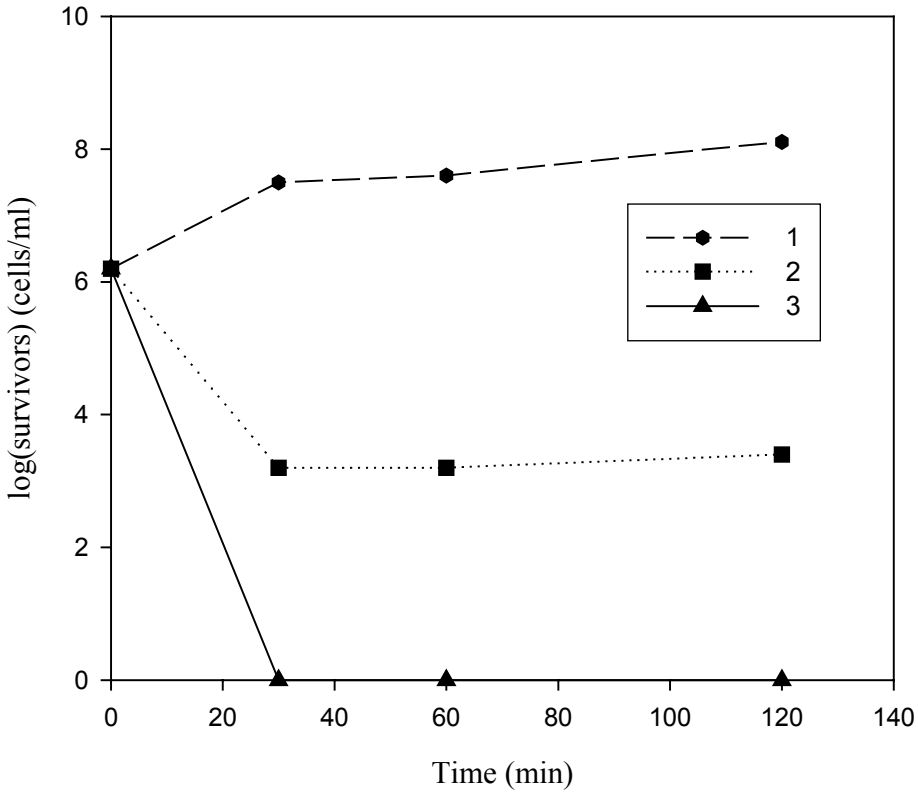

Fig. 5. Plot of log(survivors) vs. exposure time for samples 1 - 3: 1. PS particles (25 mg/mL). 2. PS particles coated by cross-linked PI-*b*-PEB-*b*-PDMAEMA (6000-6000) quaternized by octyl bromide, 1 mg/mL. 3. PS particles coated by cross-linked PI-*b*-PEB-*b*-PDMAEMA (6000-6000) quaternized by octyl bromide, 25 mg/mL

Effect of concentration of PS particles coated by cross-linked PI-b-PEB-b-PDMAEMA (quaternized by octyl bromide) on the antimicrobial activity

For the sake of comparison, the content of PS particles coated by the PI-*b*-PEB-*b*-PDMAEMA block copolymer quaternized by octyl bromide (C) and cross-linked has been changed in the bacterial suspension. At a constant time, Fig. 5 shows that log(survivors) expectedly decreases when the content of antimicrobial PS particles is increased from 1 to 25 mg/mL. Moreover, for 1 mg/mL of the antimicrobial particles, all the bacteria cannot be killed within 2 h of contact, as is the case when the concentration is 25 mg/mL. Actually, the concentration of bacteria decreases for 30 min and then levels off at approximately $2 \cdot 10^3$ cells/mL. When no biocidal agent is used (uncoated PS particles), log(survivors) increases with time. Thus, enough biocide must be made available for killing the bacteria faster than they are formed.

Experimental part

Materials

CuBr (Aldrich, 98%) and CuCl (Aldrich, 99+%) were treated with glacial acetic acid under stirring for a few hours, filtered, washed with ethanol, dried under reduced pressure at 80°C and stored under nitrogen. $CuCl_2$ (Aldrich, 97%), octyl bromide (Aldrich, 99%), 2-bromoisobutyryl bromide (Aldrich, 98%), 1,4,7,10,10-hexamethyl-triethylenetetramine (HMTETA) (Aldrich, 97%), polystyrene (PS) cross-linked with 1% divinylbenzene (100 - 200 mesh from Aldrich), a hydroxyl end-capped poly[isoprene-*b*-(ethylene-*co*-butylene)] oligomer (PI-*b*-PEB-OH; M_n = 6000 and M_w/M_n = 1.05) from Shell Chemicals (Kraton, L-1302) were used as received. 2-Dimethylaminoethyl methacrylate (DMAEMA), 2-(*tert*-butylamino)ethyl methacrylate (TBAEMA) and methyl methacrylate (MMA), from Aldrich, were dried over CaH_2, distilled under reduced pressure and stored under N_2 at -20°C. Methylene dichloride (CH_2Cl_2) was refluxed over CaH_2 and degassed by bubbling of nitrogen for 20 min. Toluene was refluxed over Na/benzophenone complex, distilled and degassed by bubbling of nitrogen for 20 min.

Polymerization

Preparation of the macroinitiator (PI-*b*-PEB-Br)

1.54 g (15.3 mmol) of triethylamine in 150 mL of dry and degassed CH_2Cl_2 was transferred into 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 50 g (7.9 mmol) of PI-*b*-PEB-OH in 300 mL of dry and degassed CH_2Cl_2 for 4 h. The temperature was let to increase up to room temperature, and the reaction took place under stirring overnight. Half the solvent was distilled off and an equal volume of toluene was added. The solution was filtered, and the PI-*b*-PEB-Br macroinitiator was precipitated in methanol (MeOH). After two additional precipitations from toluene to MeOH, the macroinitiator was collected and dried in vacuum.

Synthesis of PI-*b*-PEB-*b*-PDMAEMA copolymer

In a typical experiment, PI-*b*-PEB-Br (3.5 g, 0.6 mmol), CuCl ($5.5 \cdot 10^{-2}$ g, 0.6 mmol), $CuCl_2$ ($8 \cdot 10^{-3}$ g; 0.06 mmol), HMTETA (0.14 g, 0.6 mmol), toluene (8 mL) and a magnetic stirrer were introduced into a glass flask that was closed by a three-way stopcock. This solution was degassed by bubbling of nitrogen for 20 min. DMAEMA (6 mL, 35.7 mmol) was added with a syringe, and the reactor was heated in an oil bath thermostated at 50°C. The polymerization progress was monitored by picking up samples that were treated in the Sartorius® MA50 analyzer (infrared heater; 'standard desiccation' at 150°C) followed by the gravimetric determination of the formed co-polymer. Before analysis of this copolymer by size exclusion chromatography (SEC), 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 vacuum (for one night, at 40°C).

Quaternization of PI-*b*-PEB-*b*-PDMAEMA by octyl bromide

5 g of PI-*b*-PEB-*b*-PDMAEMA (15.3 mmol of DMAEMA units) and 100 mL of tetrahydrofuran (THF) were introduced in a one-neck round-bottom flask and stirred with a magnetic stirrer. Then, 2.7 g of octyl bromide (13.75 mmol) (0.9 eq with respect to the tertiary amine groups) were added to the solution. The glass reactor was thermostated in an oil bath at 50°C and maintained under stirring overnight. The solvent was removed in vacuum, and the quaternized diblock was dried in vacuum at 40°C for one night.

Synthesis of PI-*b*-PEB-*b*-PTBAEMA copolymer

In a typical experiment, the initiator PI-*b*-PEB-Br (3.4 g, 0.5 mmol), CuBr (0.08 g, 0.5 mmol), HMTETA (0.12 g, 0.5 mmol), toluene (8 mL) and a magnetic stirrer were introduced into a glass flask that was closed by a three-way stopcock. This solution was degassed by bubbling of nitrogen for 20 min. TBAEMA (6 mL, 29.7 mmol) was added with a syringe, and the reactor was heated in an oil bath thermostated at 70°C. Collection and analysis (SEC) of the copolymer were carried out as in case of the PI-*b*-PEB-*b*-PDMAEMA counterparts (*vide supra*).

Synthesis of PI-*b*-PEB-*b*-PMMA copolymer

In a typical experiment, the initiator PI-*b*-PEB-Br (3.4 g, 0.5 mmol), CuBr (0.08 g, 0.5 mmol), HMTETA (0.12 g, 0.5 mmol), toluene (8 mL) and a magnetic stirrer were introduced into a glass flask that was closed by a three-way stopcock. This solution was degassed by bubbling of nitrogen for 20 min. MMA (6 mL, 56 mmol) was added with a syringe, and the reactor was heated in an oil bath thermostated at 80°C. The copolymer was collected and analyzed by SEC as reported when DMAEMA was the monomer rather than MMA (*vide supra*).

Coating of PS particles by PI-b-PEB-b-PDMAEMA quaternized by octyl bromide, PI-b-PEB-b-PTBAEMA and PI-b-PEB-b-PMMA

PS particles (0.5 g) were dispersed into a solution of 10 wt.-% copolymer added with 5 wt.-% of benzophenone in a THF/MeOH (25/75 v/v) mixture, followed by evaporation of the solvent at room temperature. The coated PS particles were exposed to UV with a conveyor belt moving at a constant rate of 0.1 m/s. The total irradiation time (50 s/cycle) was fixed by the number of radiation cycles (15). The main benefit of an intermittent exposure compared to a continuous process is to allow the sample to cool down between two UV cycles and to restrict the risk of thermal degradation of the cationic units. The coated PS particles were washed with MeOH/THF to remove the non cross-linked polymer and dried in vacuum.

Characterization

Size exclusion chromatography (SEC) was performed in THF added with 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 (HR 1: M_w = 100 - 5000; HR 2: 500 - 20 000; HR 4: 5000 - 600 000). The columns were calibrated with PMMA standards.

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

Antimicrobial assessment (shake flask method)

A freeze-dried ampoule of *Escherichia coli* (DH5α) was opened and the culture was picked up with a micropipette and placed in 2 mL of nutrient broth (composition for one litre of nutrient broth (Luria Bertani): 10 g bactotryptone, 5 g of extract of yeast, sodium chloride), which was incubated (Incubator shaker model G25; New Brunswick Scientific Co., Inc.; Edison, New Jersey, USA) 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 *E. coli* contained *c*. 10^8 cells/mL (absorbance at 600 nm = 0.6) and was used for the antibacterial test. Diblock-coated PS particles were dispersed in a sterile saline water mixture (prepared by dissolving 8.5 g of NaCl in 1 L of miliQ 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 (1 or 25 mg/mL) was reached when 18 mL of this biocide solution pre-equilibrated at 37°C was combined with 2.0 mL of the bacterial culture that contained *c*. 10^8 cells/mL. In parallel, 2.0 mL of the same culture were added to 18 mL of sterile saline water that contained uncoated PS particles (control). At regular time intervals, 1.0 mL samples were picked out; 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.

Conclusions

A novel class of macromolecular biocides has been synthesized, which consist of a biocidal polymethacrylate block chemically bonded to a short poly[isoprene-*block*-(ethylene-*co*-butylene)] diblock copolymer. This diblock has been designed for making the final copolymer easily anchored to polyolefinic substrates and easily cross-linked upon UV exposure, which provides films/coatings with stability/permanency. In this work, the biocidal block is either poly(dimethylaminoethyl methacrylate) (PDMAEMA) block quaternized by octyl bromide, or poly(2-(*tert*-butylamino)ethyl methacrylate) (PTBAEMA) that does not require quaternization for being active. Radical polymerization of DMAEMA and TBAEMA by ATRP initiated by a macroinitiator directly derived from the polyolefin block is a very effective way to prepare the envisioned biocidal triblock copolymers. Polystyrene beads have been coated by these antimicrobial copolymers, followed by UV cross-linking of the isoprene units. These particles, which are easily handled and recovered, are endowed with a high biocidal activity as assessed by antibacterial testing (shake flask method). Extension of this strategy to polyolefin (LDPE, PP) surfaces is under current investigation.

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