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Polymeric Quaternary Ammonium-Containing Coatings with Potential Dual Contact-Based and Release-Based Antimicrobial Activity

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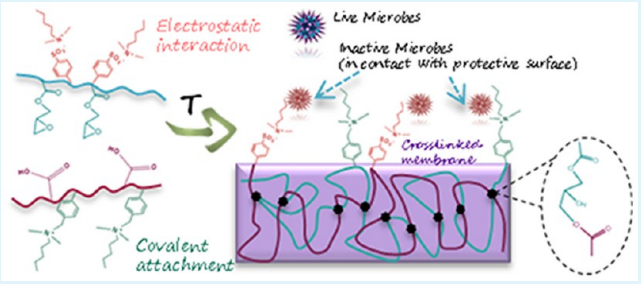
Supporting Information

ABSTRACT: In the present work, reactive blending of copolymers with complementary functional groups was applied to control their antimicrobial activity and antifouling action in real conditions. For this purpose, two series of copolymers, poly(4-vinylbenzyl chloride-*co*-acrylic acid), P(VBC-*co*-AAx), and poly(sodium 4-styrenesulfonate-*co*-glycidyl methacrylate), P(SSNa-*co*-GMAx), were synthesized via free radical copolymerization and further modified by the incorporation of biocidal units either covalently (4-vinyl benzyl dimethylhexadecylammonium chloride, VBCHAM) or electrostatically bound (cetyltrimethylammonium 4-styrenesulfonate, $SSAmC_{16}$). The cross-linking reaction of the carboxylic group of acrylic acid (AA) with the epoxide group of glycidyl methacrylate (GMA) of these two series of reactive antimicrobial copolymers was explored in blends obtained through solution casting after curing at various temperatures. The combined results from the ATR-FTIR characterization of the membranes, solubility tests, turbidimetry, and TEM suggest that the reaction occurs already at 80 °C, leading mostly to graft samples, while at higher curing temperatures (120 or 150 °C) insoluble cross-linked samples are usually obtained. Controlled release experiments of selected membranes were performed in pure water and aqueous 1 M NaCl solutions for a period of two months. The released material was followed through gravimetry and TOC/TN measurements, while the evolution of the integrity and the morphology of the membranes were followed visually and through SEM, respectively. Antimicrobial tests also revealed that the cross-linked membranes presented strong antimicrobial activity against *S. aureus* and *P. aeruginosa*. Finally, a specific blend combination was applied on aquaculture nets and cured at 80 °C. The modified nets, emerged in the sea for 15 and 35 days, exhibited high antifouling action as compared to blank nets.



KEYWORDS: *quaternary compound, glycidyl methacrylate, acrylic acid, cross-linking, membrane, antimicrobial activity, antifouling*

1. INTRODUCTION

The presence of reactive groups in the polymeric chain, either as internal functionality such as ester and amide groups in polyesters and polyamides, respectively, or as additional functionality in the case of reactive copolymers, has been widely explored for the creation of new polymeric materials with controlled properties through reactive extrusion in solid state or stabilization through cross-linking. The idea of transesterification during melting was first demonstrated many years ago.[1] Since then, many efforts have been devoted to the creation of materials with new properties during processing,[2−8] either in the solid state or in solution. Such reactions were also used to synthesize block copolymers composed of chain growth and step growth blocks[9] or to induce reactions at polymer−polymer interfaces,[10] while they are still used for compatibilization of immiscible blends.[11,12] An interesting reactive unit is the epoxide group, because it reacts with a variety of nucleophiles, such as alcohols, phenols, and carboxylic acids. This reaction is applied in epoxy resins[13] in reactive blending,[14−21] in extrusion technologies,[22−25] and in the case of curable coatings,[26] as well as for the development of healing formulations.[27]

The need for development of clean surfaces and articles is well established in our society. Depending on the mechanism involved in bacteria killing, bactericidal surfaces can be divided into release-based or contact-based ones.[28] The use of

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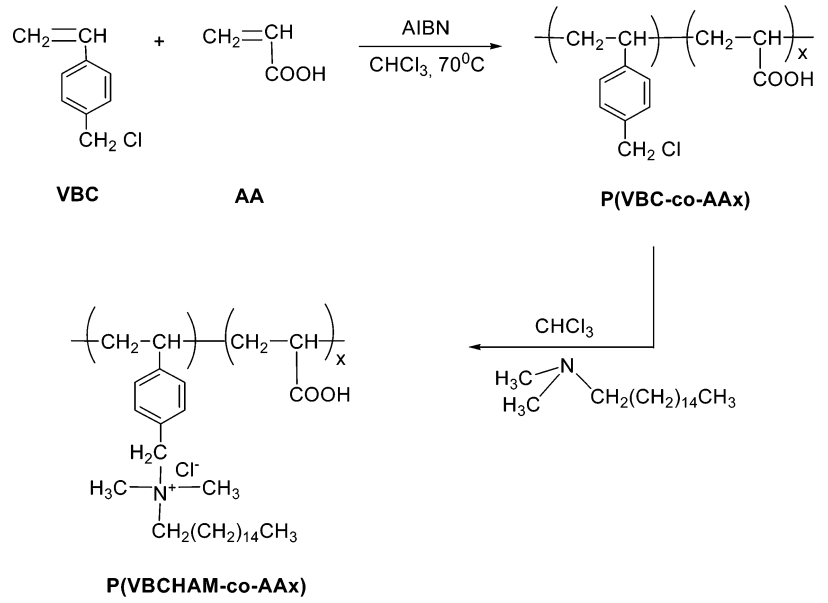
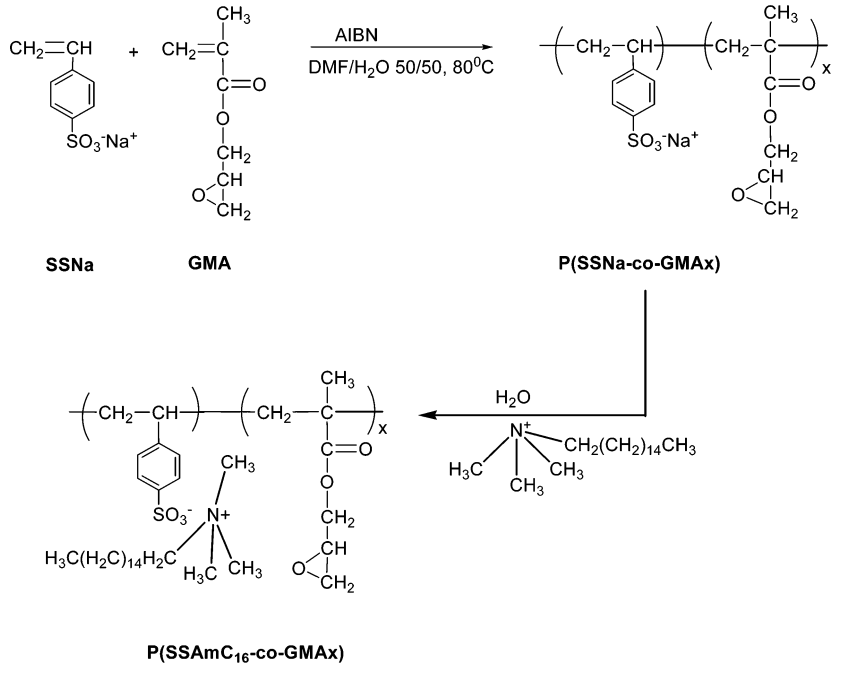
Scheme 1. Reaction Steps for the Synthesis of P(VBCHAM-*co*-AAx) Copolymers



Scheme 2. Reaction Steps for the Synthesis of P($SSAmC_{16}$-*co*-GMAx) Copolymers



polymeric antimicrobial agents is an attractive response to the aforementioned need, especially toward conventional antimicrobial agents. Their advantages, such as no volatility, chemical stability, no toxicity, the difficulty in permeation through the human or animal skin, and the prolonged lifetime have increased the interest of both academic and industrial point of view. Their use is of great importance in various fields especially in hospital surfaces, surgery equipment, dental restoration, water purification, soil sterilization, drugs, textiles, food packaging, and antifouling paints.[29−32] Various attempts have been described in the literature for the creation of antimicrobial polymers and copolymers.[33−49] An important class of polymeric biocides is based on quaternary ammonium compounds. Such compounds are offered for contact-based or release-based applications because the quaternary groups may be either covalently attached on the polymer chain or counterions of a polyanion (electrostatic binding).

A crucial problem in the design of polymeric biocides is to control the critical balance between activity and duration, because the electrostatically bound release-based biocidal groups can be leached out (reducing thus the duration of the action), while the covalently attached contact-based biocidal groups can alone show limited activity. Recently it was reported by our group that covalently attached and electrostatically bound tertiary ammonium groups can act as efficient biocides if they are combined with proper comonomers in either random or block structure.[42,45] While the random copolymers[42] are easily synthesized and used as additives in certain polymeric matrixes such as aromatic polysulfones, the block copolymers show limitations due to the synthetic restrictions on using

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Table 1. Characterization Results for the P(VBCHAM-*co*-AAx) and P($SSAmC_{16}$-*co*-GMAx) Antimicrobial Copolymers

precursors	feed composition % (mol AA or GMA)	^{1}H NMR composition % (mol AA or GMA)	M_w	PDI	antimicrobial copolymers	titration composition % (mol AA)
P(VBC-*co*-AA1.5)	3	1.5	23400	3.0	P(VBCHAM-*co*-AA1.5)	3.3
P(VBC-*co*-AA5.5)	5	5.5	30200	2.5	P(VBCHAM-*co*-AA5.5)	3.9
P(VBC-*co*-AA12)	10	12	34000	2.8	P(VBCHAM-*co*-AA12)	8.5
P(VBC-*co*-AA20)	20	20	28200	2.7	P(VBCHAM-*co*-AA20)	20
P(SSNa-*co*-GMA2)	2	2	28600	2.3	P($SSAmC_{16}$-*co*-GMA2)	-
P(SSNa-*co*-GMA6)	5	6	27800	1.9	P($SSAmC_{16}$-*co*-GMA6)	-
P(SSNa-*co*-GMA20)	15	20	12200	1.8	P($SSAmC_{16}$-*co*-GMA20)	-

charged monomers for their controlled polymerization processes.[50−53] On the other hand, the block copolymeric structure can expand the use of such antimicrobial materials in mixtures or blends, taking advantage of their organizational ability and morphology control that is necessary for the long-term activity of the final materials. Concerning the cytotoxicity of these ammonium compounds, it has been recently demonstrated[54] that carbon nanotubes (CNTs) modified with such electrostatically bound or covalently attached polymeric biocides show reduced cytotoxicity as compared to pristine CNTs, indicating that such polymeric biocides are probably characterized by low cytotoxicity profiles.

Apart from the design of clean surfaces, biocidal species have been historically used as antifouling agents to prevent biofouling, for instance in marine applications.[55] For example, the most effective antifouling coatings contained biocides such as tributyltin and tributyltin oxide (TBTO).[56] However, since the ban of organotin compounds in related applications by the International Maritime Organization in 2008 for environmental reasons,[57] research is now focused on developing new generations of antifouling coatings.[58,59] The use of environmentally acceptable biocidal materials as those based on quaternary nitrogen compounds represents one research direction,[60] among the several strategies investigated toward such applications. In fact, the use of polymeric biocides based on electrostatically bound quaternary nitrogen or phosphonium species is under investigation by our group.[37,61]

Having in mind all possibilities available from the reactive blending, applying this concept for the design and creation of new antimicrobial copolymers bearing covalently attached and electrostatically bound biocidal units was an attractive challenge. To the best of our knowledge, this approach has not been yet investigated. Nevertheless, this would allow the fine-tuning of the amount of each class of biocidal units (covalently attached and electrostatically bound onto the polymeric chains), aiming at structures with the optimum loading, to control activity and durability of the antimicrobial action. Moreover, the fine-tuning of the amount of complementary reactive units, in combination with the extent of the reaction, may lead to varying copolymer architectures, ranging from slightly grafted soluble samples to cross-linked insoluble networks.

Motivated by the aforementioned possibilities, we designed new random copolymers, where rather small fractions of reactive units such as carboxyl groups or epoxides are copolymerized with quaternary ammonium biocidal units, covalently attached or electrostatically bound onto the polymeric chains, respectively (Schemes 1 and 2). The reaction in solid state results in a large number of copolymers with a grafted or cross-linked architecture (depending on composition, stoichiometry, and reaction conditions), comprising blocks of the two classes of biocidal units. Here we focused mostly on the demonstration of the applicability of the reactive blending concept in the case of antibacterial polymers, and we investigated the limitations while we optimized the reaction conditions to obtain cross-linked architectures. Such architectures can be applied as coatings on different polymeric matrixes and articles, offering the advantage of fine-tuned antimicrobial activity. Moreover, selected pairs of complementary reactive copolymers were used for the coating of aquaculture nets which were subsequently emerged in the sea environment, and the antifouling properties were examined for more than a month.

2. EXPERIMENTAL SECTION

2.1. Materials. The monomers glycidyl methacrylate (GMA), sodium 4-styrenesulfonate (SSNa), acrylic acid (AA), and 4-vinylbenzyl chloride (VBC), the homopolymers poly(sodium 4-styrenesulfonate) (PSSNa) and poly(acrylic acid) (PAA), the initiator azobis(isobutyronitrile) (AIBN), the surfactant cetyltrimethylammonium bromide (CTAB), the amine *N*,*N*-dimethylhexadecylamine (HAM), as well as deuterium oxide (D_2O) and deuterated chloroform ($CDCl_3$) were purchased from Aldrich and used as received. The solvents *N*,*N*-dimethylformamide (DMF), chloroform ($CHCl_3$), acetone, and hexane were purchased from Fischer and used as received. Ultrapure water was obtained by means of an SG apparatus water purification unit.

2.2. Synthesis of Precursors. The copolymers poly(4-vinylbenzyl chloride-*co*-acrylic acid) and poly(sodium 4-styrenesulfonate-*co*-glycidyl methacrylate) were synthesized through free radical copolymerization in $CHCl_3$ and DMF/H_2O 3D (50/50), respectively, using AIBN as initiator (Supporting Information). These copolymers (Table 1) are denoted as P(VBC-*co*-AAx) and P(SSNa-*co*-GMAx), where *x* is the mole fraction of AA and GMA units in the copolymer, as determined by the ^{1}H NMR characterization in $CDCl_3$ and D_2O, respectively. The experimental procedures are described in detail elsewhere.[42,54,62]

2.3. Introduction of Quaternized Units. *2.3.1. Covalently Attached Biocidal Species.* The quaternization process of P(VBC-*co*-AAx) copolymers has been described previously.[42] Briefly, the copolymers were dissolved in $CHCl_3$ and quaternized with an excess of *N*,*N*-dimethylhexadecylamine (HAM) at 60 °C for 48 h. The quaternized products, denoted P(VBCHAM-*co*-AAx), were recovered by precipitation in acetone, thoroughly washed with hexane and dried in a vacuum oven at 60 °C for 24 h.

2.3.2. Electrostatically Bound Biocidal Species. For the introduction of electrostatically bound quaternary ammonium cations on the P(SSNa-*co*-GMAx) copolymers, an ion exchange reaction in aqueous solution between the sodium ions of SSNa units with an excess of quaternary cetyltrimethylammonium cations (AmC_{16}) of CTAB was carried out. The final precipitated products, denoted P($SSAmC_{16}$-*co*-GMAx), were obtained through filtration, washed thoroughly with ultrapure H_2O, and dried in a vacuum oven at 60 °C for 24 h.

2.4. Preparation of Antibacterial Membranes. Initially, mother solutions of the copolymers P(VBCHAM-*co*-AAx) and P($SSAmC_{16}$-*co*-GMAx) were prepared in $CHCl_3$ at a 5% (w/v) concentration and left overnight at room temperature under mild stirring. Subsequently, the mother solutions of complementary copolymers were mixed at various

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Table 2. Solubility Tests of Membranes of Complementary Copolymer Blends after the Heat Treatment at Various Temperatures

complementary copolymers		composition % (w/w)	r	curing temperature and time of curing	solubility tests in $CHCl_3$ (1% w/v) visual inspection	solubility tests in $CHCl_3$ (1% w/v) soluble fraction (%)	EtOH-soluble fraction (%)	membrane
P(SSAmC$_{16}$-*co*-GMA2)	P(VBCHAM-*co*-AA1.5)	15/85	1−5	80 °C (1 d)	soluble	100	71	S1-80
				120 °C (4 h)	soluble	100	nd[a]	S1-120
P(SSAmC$_{16}$-*co*-GMA2)	P(VBCHAM-*co*-AA5.5)	40/60	1−5	80 °C (1 d)	soluble	100	34	S2-80
				120 °C (4 h)	insoluble (soft)	nd[a]	nd[a]	S2-120
				150 °C (30 min)	insoluble (soft)	87	nd[a]	S2-150a
				150 °C (5 h)	insoluble (soft)	24	nd[a]	S2-150b
P(SSAmC$_{16}$-*co*-GMA6)	P(VBCHAM-*co*-AA5.5)	20/80	1−5	RT[b] (1 d)	soluble	100	55	S3-RT
				80 °C (1 d)	soluble	100	67	S3-80
				120 °C (4 h)	insoluble	50	nd[a]	S3-120
P(SSAmC$_{16}$-*co*-GMA2)	P(VBCHAM-*co*-AA1.5)	50/50	1−1	RT[b] (1 d)	gel	nd[a]	42	S4-RT
				80 °C (1 d)	gel	nd[a]	41	S4-80
				120 °C (4 h)	gel	90	nd[a]	S4-120
				150 °C (1 h)	insoluble (soft)	28	nd[a]	S4-150a
				150 °C (5 h)	insoluble (soft)	22	31	S4-150b
P(SSAmC$_{16}$-*co*-GMA6)	P(VBCHAM-*co*-AA5.5)	50/50	1−1	RT[b] (1 d)	soluble	100	40	S5-RT
				80 °C (1 d)	soluble	100	51	S5-80
				120 °C (4 h)	insoluble (soft)	63	nd[a]	S5-120
				150 °C (30 min)	insoluble (soft)	80	nd[a]	S5-150a
				150 °C (1 h)	insoluble (soft)	36	nd[a]	S5-150b
P(SSAmC$_{16}$-*co*-GMA2)	P(VBCHAM-*co*-AA1.5)	80/20	5−1	RT[b] (1 d)	soluble	100	33	S6-RT
				80 °C (1 d)	soluble	100	36	S6-80
				120 °C (4 h)	soluble	100	nd[a]	S6-120
				150 °C (1 h)	soluble	100	nd[a]	S6-150
				150 °C (5 h)	soluble	100	52	S6-150
P(SSAmC$_{16}$-*co*-GMA6)	P(VBCHAM-*co*-AA1.5)	60/40	5−1	80 °C (1 d)	soluble	100	53	S7-80

[a]nd: Not determined, due to the nature of the membrane (gel, very soft, or very fragile). [b]RT: Room temperature.

compositions. In these mixtures, the epoxy/carboxyl group molar ratio r was set at the desired values (5:1, 1:1, and 1:5). The mixtures were poured into a Petri dish and left at room temperature for 24 h until complete solvent evaporation. The obtained homogeneous membranes were subsequently cured at room temperature, 80 °C, 120 °C, or 150 °C. All the prepared membranes had a uniform thickness of 100 μm.

2.5. CharacterizationTechniques. *2.5.1. Proton Nuclear Magnetic Resonance (^{1}H NMR).* The samples for ^{1}H NMR characterization were prepared by dissolving the P(VBC-*co*-AAx) copolymers in $CDCl_3$, containing TMS internal standard, and the P(SSNa-*co*-GMAx) copolymers in D_2O. ^{1}H NMR spectra were obtained at 400 MHz at 300 K on a Bruker AVANCE DPX 400 spectrometer. The ^{1}H NMR spectra were used to determine the chemical composition of the copolymers.

2.5.2. Potentiometric Titration. The content of carboxyl groups in the P(VBCHAM-*co*-AAx) copolymers was determined by acid−base titration with standard aqueous 0.1 N NaOH solutions, in the presence of an excess of HCl. The variation of pH was monitored with a SevenEasy pH-meter purchased from Mettler Toledo.

2.5.3. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). The ATR-FTIR spectra of the copolymers or the dried membranes before and after curing were recorded using a Bruker Platinum ATR-FTIR spectrometer.

2.5.4. Size Exclusion Chromatography (SEC). For the organo-soluble precursors P(VBC-*co*-AAx), chloroform ($CHCl_3$) was the mobile phase. SEC was carried out at 25 °C by using a Marathon II HPLC pump, a Fasma 500 UV/vis detector, and two PLgel 5 μm Mixed columns C. Polystyrene standards were used for calibration, and the software Clarity v.3.0.07.662 was used for the spectra analysis. For the water-soluble precursors P(SSNa-*co*-GMAx), a Millipore Waters 501 HPLC chromatographer at 25 °C was used, equipped with two Shodex B-804, B-805 linear columns (8 mm × 500 mm), a differential refractometer (R401) detector, poly(ethylene oxide) standards, and 0.1 M $LiNO_3$ as eluent. The operating flow rate was set at 1 mL/min for both SEC systems.

2.5.5. Scanning Electron Microscopy (SEM) Examination. Scanning electron microscopy (SEM, Zeiss SUPRA 35VP instrument equipped with an energy-dispersive X-ray spectroscopy, EDS, detector) was performed to investigate the membranes' surface morphologies.

2.5.6. Total Organic Carbon (TOC) and Total Nitrogen (TN) Measurements. Simultaneous analyses of TOC and TN were carried out using a Schimadzu TOC analyzer (TOC-VCSH) coupled to a chemiluminescence detector (TNM-1 TN unit).

2.6. Physicochemical Characterization. *2.6.1. Turbidimetry.* The optical density at 500 nm was measured using a Hitachi U-1800 UV−Vis spectrophotometer equipped with a circulating water bath, set at 20 °C. The copolymers or membranes were dissolved/dispersed in $CHCl_3$ under vigorous stirring. The concentration was fixed at 0.01 g/mL. All measurements were performed in triplicate.

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2.6.2. Solubility Studies. Small pieces of each membrane were dipped in glass vials containing the desired solvent and left at room temperature for 24 h. The appearance of the sample/solution was checked visually. In addition, for the insoluble samples, the soluble fraction was determined when possible through gravimetry.

2.6.3. Release Studies. The membranes S2-120 and S5-120 (Table 2) after curing at 120 °C were selected for the release studies. More specifically, small pieces of the membranes were immersed in pure water or aqueous 1 M NaCl solutions and left for a time period ranging from hours up to 2 months. The membranes were taken out at the specific time period, washed (in the case of NaCl solution), and dried. The soluble fraction of the two membranes in pure water or aqueous 1 M NaCl solution was then evaluated gravimetrically. The TOC/TN content of the final solutions was determined, while the membranes were examined through SEM.

2.7. Antibacterial Activity. For the measurement of the antimicrobial effect of the prepared membranes, the bacterial strains *S. aureus* NCTC 6571 and *P. aeruginosa* NCTC 10662 were used. The bacterial culture preparation and the method used for the determination of the antimicrobial activity are described in details elsewhere.[42,63,64] Briefly, pieces of 18 × 18 mm were cut from each membrane and a 20 μL aliquot of an overnight culture of each bacterium was placed on every piece of polymer membrane. Incubation followed at 22 °C for 24 h. After four serial decimal dilutions using phosphate-buffered saline (PBS), nutrient agar plates were inoculated with 100 μL of each decimal dilution and the plates were incubated at 37 ± 1 °C for 18−24 h. Enumerations of the colonies were finally recorded. For each dilution, two plates were inoculated and the result was expressed as the mean of the two numbers. The experimental procedure was repeated at least three times on different days with different bacterial cell suspensions. Within each experiment, three dilutions were made and the mean was calculated from a minimum of three data points.

2.8. Application of Antimicrobial Polymers on Aquaculture Nets. The copolymers P(VBCHAM-*co*-AA20) and P(SSAmC$_{16}$-*co*-GMA20) were dissolved in $CHCl_3$ at 5% (w/v) concentration as previously described. The composition of the complementary copolymers was adjusted to simulate the blends prepared at lab scale. After blend preparation, preweighed nets, 20 × 25 cm, were immersed in the solution and left for 1 h. The polymer uptake of the nets was 15−20% (w/w). The nets were left to dry at RT and were subsequently cured at 80 °C. Then the coated nets were hooked on an aquaculture cage and immersed in an aquaculture unit in the Saronic Bay of Greece for 15 and 35 days during summer time. For comparison reasons, blank nets were also immersed in the sea. All the nets were placed in the same depth to obtain constant lighting and ventilation conditions.

3. RESULTS AND DISCUSSION

As discussed in Introduction, the main goal of the present work was the preparation of grafted/cross-linked materials with a dual contact-based and release-based antimicrobial action, i.e. containing both immobilized and released biocidal units. These materials will be further discussed in terms of their antimicrobial activity and their antifouling action in real conditions. Thus, the first step was the synthesis of complementary reactive copolymers, P(VBCHAM-*co*-AAx) and P(SSAmC$_{16}$-*co*-GMAx), where *x* is the molar content of reactive units. For the preparation of blends and the optimization of the cross-linked membranes, *x* varied between 1.5 and 12% mol AA or GMA. However, for the application of blends on aquaculture nets and their immersion in the sea environment, we preferred to synthesize copolymers with *x* = 20%, to ensure efficient cross-linking while maintaining the optimum copolymers composition in terms of cross-linking reaction and antimicrobial efficiency.

3.1. Synthesis and Characterization of P(VBCHAM-*co*-AAx) and P(SSAmC$_{16}$-*co*-GMAx) Copolymers. Two series of copolymers were prepared. The first series was the P(VBCHAM-*co*-AAx) copolymers, containing acrylic acid (AA) as reactive units and 4-vinylbenzyl dimethylhexadecylammonium chloride (VBCHAM) as immobilized biocidal species, because they are covalently attached onto the polymer chain. The synthesis of these copolymers (Scheme 1) involves the preparation of the P(VBC-*co*-AAx) precursors through copolymerization of 4-vinylbenzyl chloride (VBC) with acrylic acid (AA) followed by modification with *N*,*N*-dimethylhexadecylamine (HAM) to afford the final reactive contact-based antimicrobial materials P(VBCHAM-*co*-AAx). The aqueous solutions of these copolymers are weakly acidic, as a result of the presence of AA units in their structure.

The second series is the P(SSAmC$_{16}$-*co*-GMAx) copolymers, containing glycidyl methacrylate (GMA) as reactive units and cetyltrimethylammonium cations (AmC$_{16}$) as electrostatically bound biocidal species, because they are the counterions of styrenesulfonate units. The synthesis of these copolymers (Scheme 2) involves the preparation of the P(SSNa-*co*-GMAx) precursors through copolymerization of sodium 4-styrenesulfonate (SSNa) with GMA as a first step, followed by ion exchange of sodium ions by AmC$_{16}$ cations as the second step to afford the final reactive release-based antimicrobial materials P(SSAmC$_{16}$-*co*-GMAx).

The chemical structure of the synthesized copolymers was verified through ATR-FTIR spectroscopy (Figure S1) and ^{1}H NMR spectroscopy (Figure S2). The monomer feed composition along with the characterization results from ^{1}H NMR spectroscopy of the synthesized precursors and the final P(VBCHAM-*co*-AAx) and P(SSAmC$_{16}$-*co*-GMAx) antimicrobial copolymers are quoted in Table 1. Moreover, in the case of P(VBC-*co*-AAx) copolymers, we took advantage of the weakly acidic nature of AA unit and we attempted to determine the AA content of these copolymers through acid−base titration. As seen in Table 1 the results from both characterization methods are rather well comparable and, in general, they follow the feed composition used for the synthesis of the precursors.

The molecular weight distributions of the P(VBC-*co*-AAx) and P(SSNa-*co*-GMAx) precursors were determined through size exclusion chromatography in chloroform or aqueous 0.1 M $LiNO_3$ solution, using polystyrene or poly(ethylene oxide) standards, respectively. The weight-average molecular weights, M_w, and polydispersity indices, PDI, are summarized in Table 1. As seen, molecular weights are in the range 10000−30000, indicating that the mean polymerization degrees of the samples are rather low. This, in fact, suggests that the fraction of polymer chains with just one functional unit is quite significant for the samples with the lowest AA or GMA contents, namely P(VBC-*co*-AA1.5) and P(SSNa-*co*-GMA2).

As a consequence, the grafting density of the respective biocidal materials will probably be quite low leading to grafted or slightly cross-linked structures. On the other hand, the reactive sites along the polymer chains for the biocidal materials with higher contents of functional units *x* will be enough to allow efficient cross-linking, depending on curing conditions.

3.2. Optimization of Reaction Process. Cross-linking between the pairs of these complementary copolymers took place through the reaction in solid state of the carboxyl group of AA unit of P(VBCHAM-*co*-AAx) copolymers with the epoxy group of GMA unit of P(SSAmC$_{16}$-*co*-GMAx) copolymers, as shown in Scheme 3. In most cases, curing at higher

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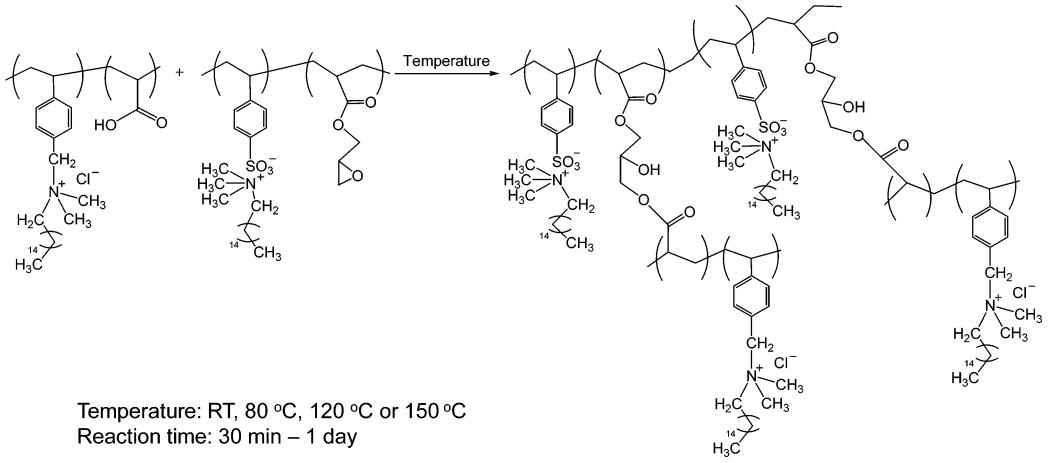
Scheme 3. Reaction between P(VBCHAM-*co*-AAx) and P(SSAmC$_{16}$-*co*-GMAx) Copolymers



temperatures was needed for the successful epoxide ring-opening of GMA.

The different attempts performed to optimize the reaction process are summarized in Table 2. Several pairs of complementary copolymers were investigated, while the composition of the copolymer mixtures varied to cover several mixing ratios r of functional units ($r = n_{GMA}/n_{AA}$), where n_{GMA} and n_{AA} are the equivalents of GMA and AA units, respectively), ranging from r = 1/5 (AA-rich mixtures) up to r = 5/1 (GMA-rich mixtures). In addition, the complementary copolymers were adequately chosen, to obtain SSAmC$_{16}$-rich mixtures (sample S6), VBCHAM-rich mixtures (samples S1 and S3), and mixtures with comparable SSAmC$_{16}$ and VBCHAM weight contents (samples S2, S4, S5, and S7). These mixtures were dissolved in $CHCl_3$, and the membranes were obtained through solvent casting at room temperature. The grafting reaction was then allowed to proceed in the solid state at the desired temperature (room temperature, 80 °C, 120 °C, or 150 °C). The heat treatment was carried out for a period of 30 min up to 24 h, depending on the curing temperature.

First evidence on the progress of the reaction was obtained from the ATR-FTIR characterization of the membranes after the thermal treatment. For this purpose, the absorption peak of epoxy groups at 909 cm^{-1} was mainly examined. As representative examples, the ATR-FTIR spectra of two membranes heat treated at 80 °C for 24 h are shown in Figure 1. As observed, the peak at 909 cm^{-1} completely disappears (Figure 1a) or significantly decreases (Figure 1b). These changes indicate that the ring-opening of GMA indeed took place to a large extent, because GMA is deficient in these membranes (r = 1/5). A similar behavior was observed for the peak at 770 cm^{-1}, which diminished after heat treatment. This, in fact, indicates that the extent of the reaction is rather low and/or that ATR-FTIR characterization cannot lead to definitive conclusions, especially for the blends with high mixing ratios r (GMA-rich blends).

Because the reaction is expected to affect the solubility of the final membranes, solubility studies were carried out by immersing the cured membranes for 24 h in $CHCl_3$ and 95% ethanol. In addition, the soluble fractions of the membranes after their immersion in $CHCl_3$ and 95% ethanol were determined in some cases. These results, giving supporting evidence on the success of the reaction, are also shown in Table 2.

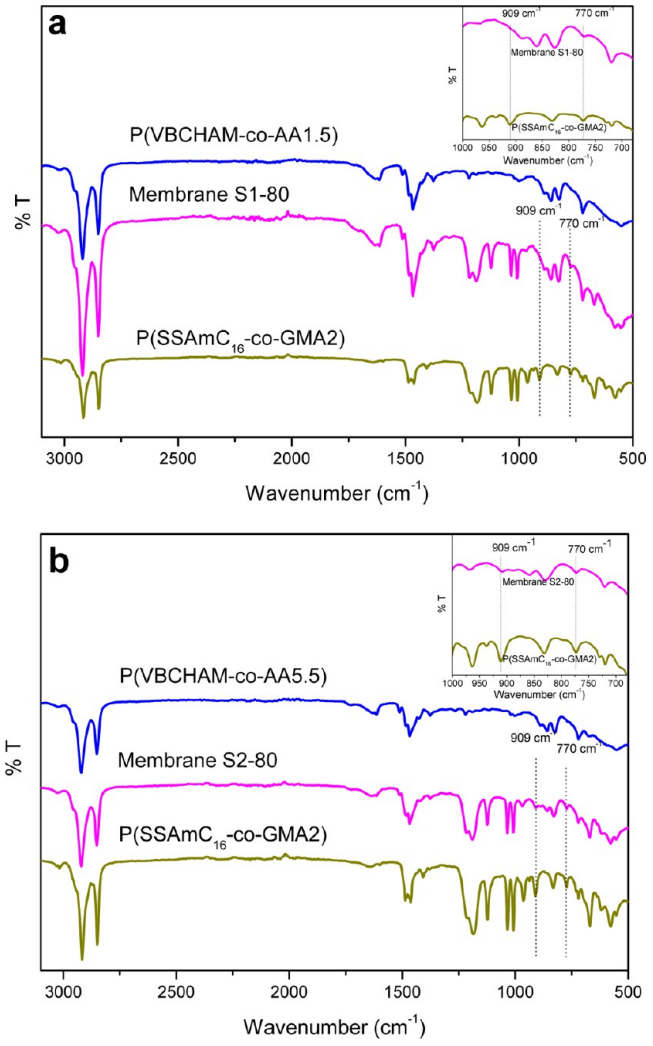


Figure 1. ATR-FTIR spectra of the membranes (a) S1-80 consisting of P(SSAmC$_{16}$-*co*-GMA2) and P(VBCHAM-*co*-AA1.5) with a composition 15/85% (w/w) and (b) S2-80, consisting of P(SSAmC$_{16}$-*co*-GMA2) and P(VBCHAM-*co*-AA5.5) with a composition 40/60% (w/w).

The copolymers P(SSAmC$_{16}$-*co*-GMAx) are partially dissolved in 95% ethanol, whereas the copolymers P(VBCHAM-*co*-AAx) are completely soluble in this solvent. As seen in Table

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2, the cured membranes appear macroscopically self-standing after treatment with 95% ethanol, though a soluble fraction ranging from ~30% (w/w) up to ~70% (w/w) was determined in most cases. These observations could be the result of the cross-linking reaction, in combination with the marginal quality of 95% ethanol as solvent for the P(SSAmC$_{16}$-*co*-GMAx) copolymers.

In contrast to 95% ethanol, all the initial copolymers are completely soluble in $CHCl_3$. In this case, a richer behavior is observed, also giving evidence about the trends of the cross-linking reaction with the curing temperature. Thus, in many cases (for example, samples S1-80, S1-120, S2-80, S3-80, S5-80, S6-RT, S6-80, S6-120, S6-150, and S7-80) the membranes are dissolved in $CHCl_3$ or easily dispersed in this solvent, leading to turbid solutions. In some cases $CHCl_3$-swollen samples are obtained, adsorbing almost the whole solvent quantity (samples S4-RT, S4-80, and S4-120). Finally, some samples (S2-120, S2-150, S4-150a, S4-150b, S5-120 S5-150a, and S5-150b), especially those cured at higher temperatures, do not lose their original membrane shape, but they become soft, because they apparently adsorb some solvent, that acts thus as plasticizer. In addition, these soft samples can be dispersed in $CHCl_3$ under vigorous stirring. Therefore, the turbidity of these solutions/dispersions as a function of curing temperature can be used to derive additional evidence on the trends of the cross-linking reaction (Figure 2). For example, it is seen that the GMA-rich membrane S6 leads to homogeneous transparent solutions, regardless of curing temperature. Transparent solutions are also obtained for all the membranes when curing is carried out at room temperature or at 80 °C. These results suggest that either the extent of the ring opening reaction is low or/and it does not really lead to cross-linked but rather to grafted samples. In contrast, when the curing temperature is raised at 120 °C, turbid solutions are obtained for all samples (with the exception of S6), indicating that now the material is sufficiently cross-linked.

The soluble fraction of the materials in $CHCl_3$, whenever possible to be determined, also reveals some interesting trends. For example, the membrane S5 is soluble in $CHCl_3$ when it is cured at room temperature or at 80 °C and turns to a soft material with a soluble fraction of ~60% (w/w) when it is cured at 120 °C (S5-120) for 4 h. The soluble fraction decreases significantly and becomes just 36% (w/w) when curing is carried out at 150 °C for 1 h (S5-150b). In addition, the time of curing is an important parameter. This is clear for the samples cured at 150 °C in our studies. For example, the soluble fraction of the membrane S5 is as high as 80% (w/w) when the curing at 150 °C takes place for 30 min (S5-150a), as compared to the much lower soluble fraction determined when curing takes place for 1 h (S5-150b). However, this temperature is at the limits of the thermal stability of our materials. For this reason, membranes cured at lower temperatures are used for the following studies.

3.3. Release Studies in Aqueous Environment. Because the main function of the materials developed in the present work is antimicrobial activity, the major potential applications of such materials require direct contact with natural or seawater. For this reason, the evolution of the soluble fraction of selected membranes in pure water or aqueous 1 M NaCl solutions was followed for a period of about two months.

For this investigation, the membranes S2-120 and S5-120, cured at 120 °C, were selected. It should be mentioned that the P(VBCHAM-*co*-AAx) copolymers are marginally soluble in pure water and practically insoluble in salt solution, whereas the P(SSAmC$_{16}$-*co*-GMAx) copolymers show a limited solubility in pure water and they are completely soluble in salt solution. However, both membranes retain their shape and appear macroscopically intact after immersion in pure water or salt solution, as a consequence of the cross-linking reaction. As an example, the appearance of the membrane S5-120 is shown in Figure 3 at various stages of the releasing study. As seen, just after curing, the membrane is homogeneous and slightly turbid but keeps its integrity. When immersed in water, the membrane becomes more flexible. However, it does not appreciably swell. Finally, when the membrane is dried after the release procedure, it still retains the initial characteristics, as it concerns integrity and appearance.

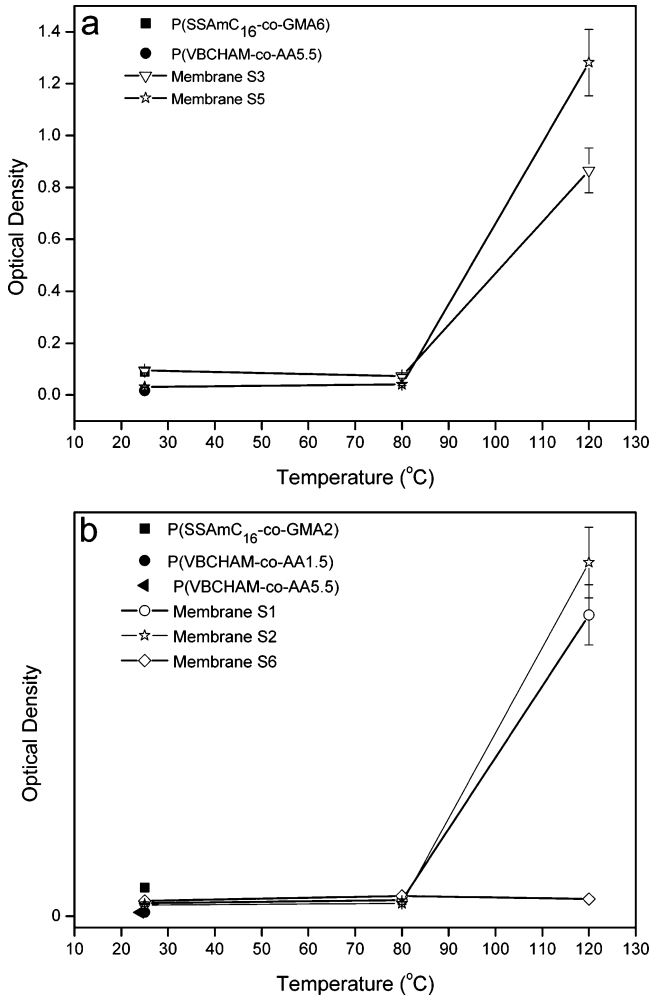

Figure 2. Turbidity studies of the membranes (a) S3 and S5, (b) S1, S2, and S6 versus curing temperature. The turbidity of solutions of the respective complementary copolymers is also shown. The copolymers/membranes were dissolved/dispersed in $CHCl_3$ at 1% (w/v) concentration.

The evolution of the soluble fraction of the two membranes in pure water or aqueous 1 M NaCl solution is shown in Figure 4a.The release levels of membrane S5-120 are higher than membrane S2-120, in agreement with the higher SSAmC$_{16}$ content of the first membrane. In pure water, both membranes exhibit an initial burst release within the first few hours, while a slow release of material is observed for longer time. In contrast, in salt solution much lower release rates and release levels are observed. This observation is, in fact, intriguing, because one

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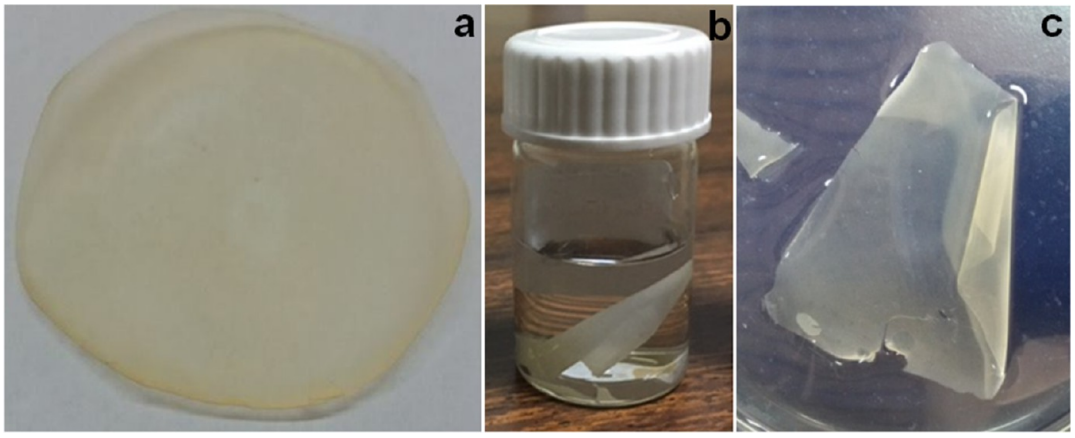

Figure 3. Appearance of (a) the membrane S5-120 after curing, (b) a piece of the membrane immersed in pure water for 30 days, and (c) a dried piece of the membrane after the release study.

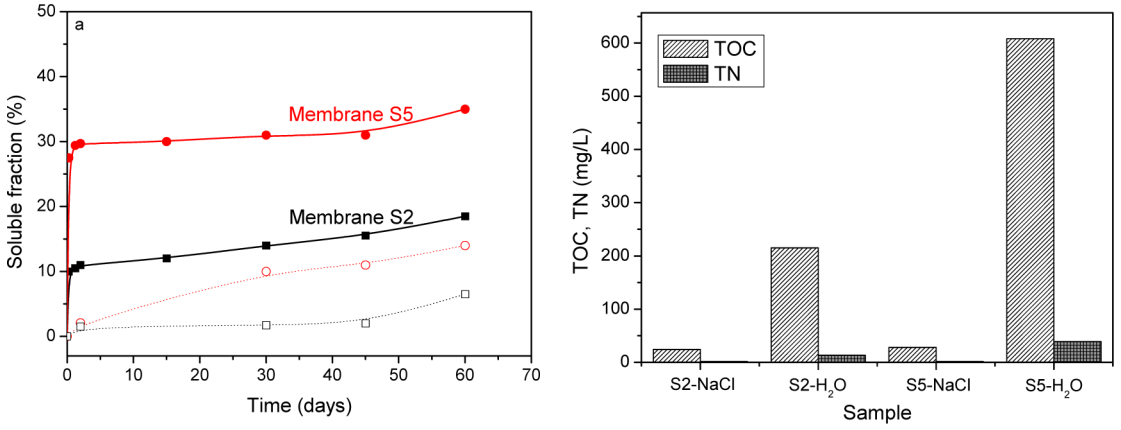

Figure 4. (a) Evolution of the soluble fraction of membranes S2-120 and S5-120 immersed in pure water (solid curves) and in an aqueous 1 M NaCl solution (dashed curves). (b) TOC and TN results of the aqueous media used for the release study of the two membranes for 60 days.

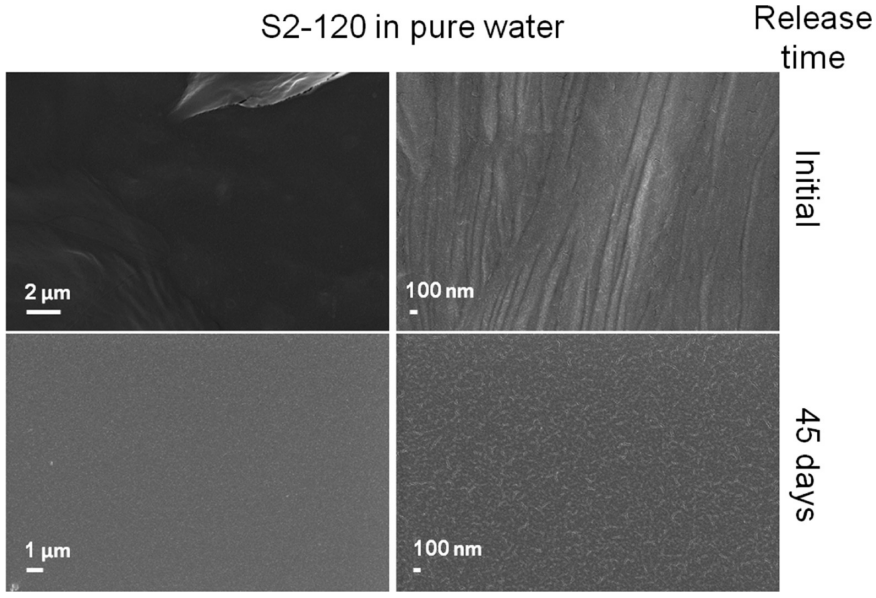

Figure 5. SEM of membrane S2-120 before and after immersion in water for 45 days.

should expect a much easier ion exchange of AmC_{16} cations of $SSAmC_{16}$ units with sodium cations (in agreement with the high solubility of the P($SSAmC_{16}$-*co*-GMAx) copolymers in aqueous 1 M NaCl solutions), leading to faster and easier release of biocidal species in the case of the salt solution.

The aforementioned trends were also verified through the determination of the total organic content (TOC) and total

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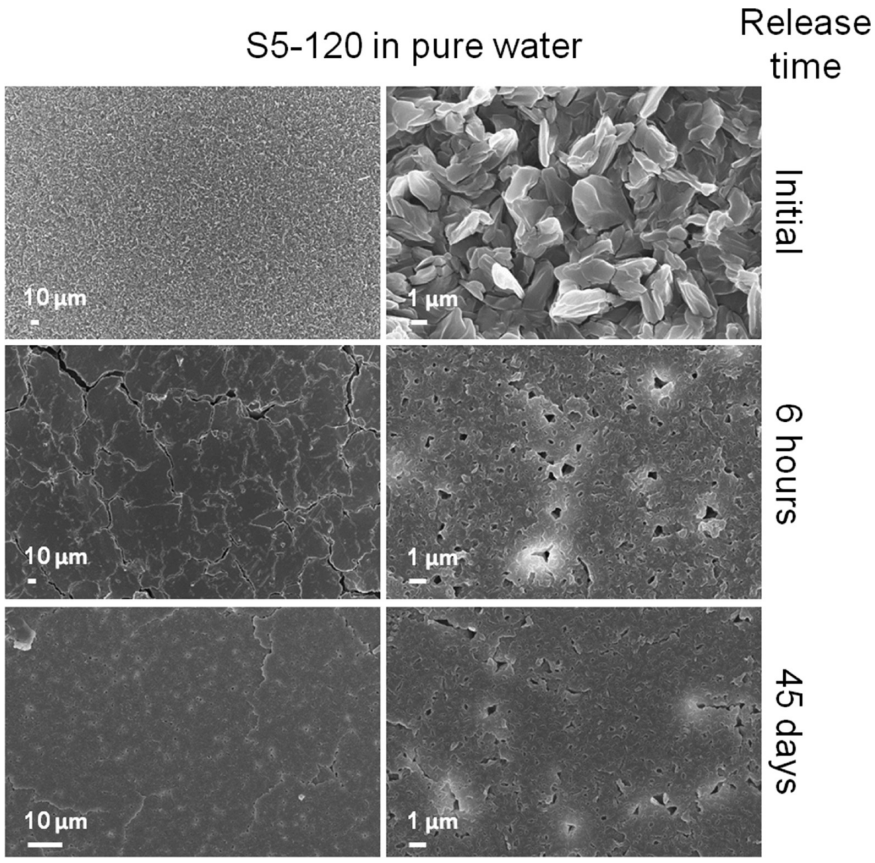

Figure 6. SEM of membrane S5-120 before and after immersion in pure water for 6 h and 45 days.

nitrogen (TN) of the aqueous media used for the samples studied at 60 days (Figure 4b). Though the soluble fraction results are not directly comparable to the TOC or TN results, it is clear that the TOC/TN values for the membranes in pure water are much higher than those studied in salt solution. Moreover, the TOC/TN values for membrane S5-120 are clearly higher than the respective values for membrane S2-120. An additional interesting point is that the mean value for the four samples of the molar C/N ratio is around 19/1 (Table S1), namely the theoretical values of AmC_{16}. This is a first indication that both in pure water or in salt solution the released species is the counterion AmC_{16} and not the polymeric chains containing $SSAmC_{16}$ or VBCHAM segments (the theoretical molar C/N value is 27/1 for both cases). The release of VBCHAM segments is indeed not expected, because the P(VBCHAM-*co*-AAx) copolymers are marginally soluble or insoluble in pure water or aqueous 1 M NaCl solutions, respectively. Moreover, the release of only AmC_{16} cations could be expected in salt solution, as a result of the ion exchange mechanism, provided that all P($SSAmC_{16}$-*co*-GMAx) copolymers are cross-linked with the P(VBCHAM-*co*-AAx) copolymers and the final product (note that $SSAmC_{16}$ units are turned into hydrophilic SSNa form) remains insoluble in the aqueous environment. In contrast, the origin of the release of AmC_{16} cations also in the case of pure water is not clear and merits further investigation. Because, in this case, no small cations are available in the solvent to exchange with AmC_{16} cations, a possible exchange mechanism could involve VBCHAM cations incorporated in the polymer chains.

The weight contents of membrane S5 and membrane S2 in AmC_{16} cations are 30% and 24%, respectively. Considering that these cations are released in salt solution, we can conclude from Figure 4a that the released material from the membranes within two months is somewhat less than the one-third of the releasable material in 1 M NaCl solution.

Microscopic examination using SEM has shown that in the case of S2 membrane in water (Figure 5) the smooth appearance of the surface before and after immersion in water for 45 days does not change, in agreement with the release experiment findings where a very minimum amount of the active species is released.

In the case of membrane S5 (Figure 6) that shows a rough surface before immersion, a drastic change was observed after immersion to water for 6 h, showing a collapse of the structure probably because of some plasticization. This structure, observed after 6 h, remained more or less unchanged for the 45 day period. This behavior is in agreement with the release experiments where a drastic change was observed in the very early stage and remained at the same level for the following period.

3.4. Antimicrobial Activity. At a next step, the antimicrobial activity of selected membranes cross-linked at 120 °C was evaluated against Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria. The results for the cross-linked membranes S2-120 (P($SSAmC_{16}$-*co*-GMA2)/P-(VBCHAM-*co*-AA5.5), weight composition: 40/60) and S3-120 (P($SSAmC_{16}$-*co*-GMA6)/P(VBCHAM-*co*-AA5.5), weight composition: 20/80) are shown in Figure 7. The antimicrobial effect was measured in terms of log reduction of the bacterial cells after 24 h contact with each material at 22 °C.

We have tested in previous studies the antimicrobial activity of a wide range of random and block copolymers at various

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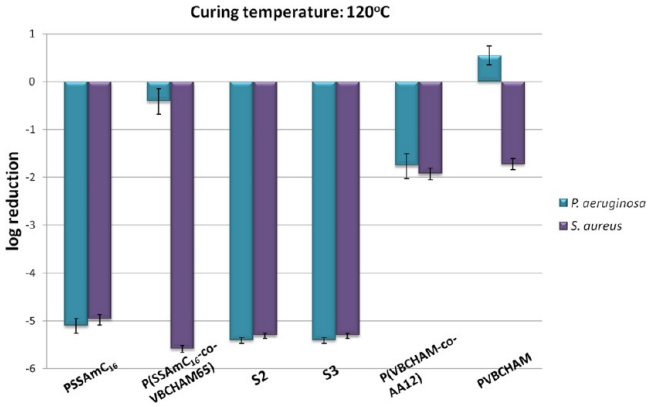

Figure 7. Antimicrobial effect of the membranes S2 and S3 after cross-linking at 120 °C, the copolymers P(SSAmC$_{16}$-*co*-VBCHAM65) and P(VBCHAM-*co*-AA12), and the homopolymers PSSAmC$_{16}$ and PVBCHAM. The experiments were conducted after 24 h of contact with *S. aureus* and *P. aeruginosa* at 22 °C. Each bar represents the log reduction from three independent experiments done in duplicates (mean ± standard deviation).

monomeric ratios[42,45] containing electrostatically bound and covalently attached quaternary ammonium groups. In fact, the antimicrobial activity of random copolymers against *S. aureus* and *P. aeruginosa* depends on the composition. For example, as shown in Figure 7, the random copolymer P(SSAmC$_{16}$-*co*-VBCHAM65) with a rather low SSAmC$_{16}$ content (35% mol), is very effective against *S. aureus*, while it presents a negligible antimicrobial activity against *P. aeruginosa*. On the other hand, the antimicrobial activity of the homopolymer PSSAmC$_{16}$ is pronounced against both microorganisms, whereas that of the homopolymer PVBCHAM is marginal. In the latter case, the antimicrobial effect seems to be slightly enhanced through the incorporation of a low content of AA units, as illustrated in Figure 7, for the copolymer P(VBCHAM-*co*-AA12). This means that the antimicrobial activity of the specific copolymers depends not only on the high content of the unit bearing covalently attached quaternary ammonium groups but also on the combination of this unit with the hydrophilic AA moiety. Thus, in the present work the AA moieties in the copolymer were maintained at low levels to preserve a significant antimicrobial activity on the one hand and to achieve the optimum ratio between AA and GMA functional groups for a successful grafting reaction on the other hand. As a consequence, the polymer films prepared in the present work, even with limited SSAmC$_{16}$ content (20% for S3 and 40% for S2), presented very high antimicrobial activity against both microorganisms. More specifically, a log reduction of ∼5.4 is observed against *P. aeruginosa* and ∼5.3 against *S. aureus*; namely, the membranes show improved antimicrobial properties as compared to the random copolymer P(SSAmC$_{16}$-*co*-VBCHAM65), probably arising from the cross-linked structure of the material. The determined log reductions are considered

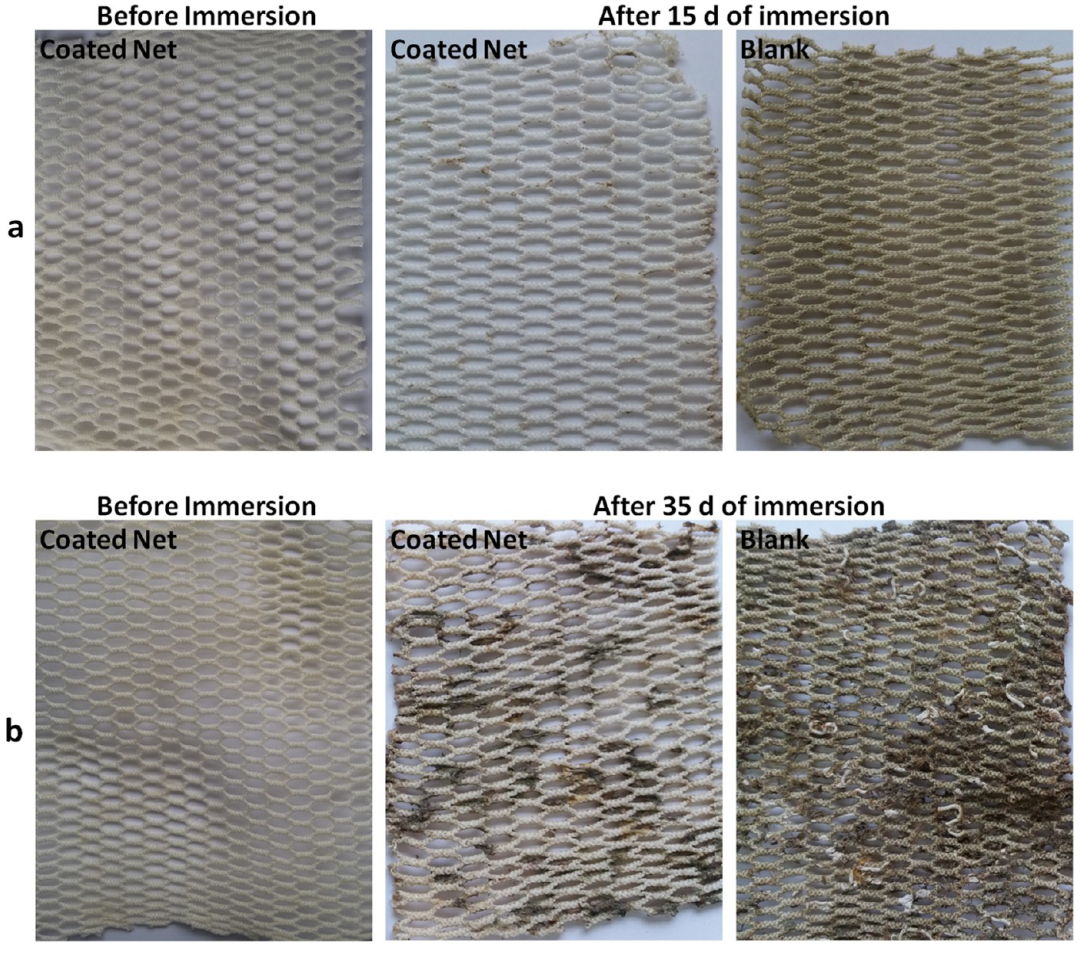

Figure 8. Photographs of the coated and uncoated nets (blank) after immersion in the sea environment for 15 and 35 days.

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sufficient to ensure the antimicrobial activity of the final materials, and they are well comparable with cationic biocidal polymers reported in the literature.

3.5. Antifouling Properties in Real Conditions. To test the antifouling action of the above-mentioned blends, a copolymer mixture was prepared as described previously. The composition of the complementary copolymers (P(SSAmC$_{16}$-*co*-GMA20) and P(VBCHAM-*co*-AA20)) used for the coating of nets was adjusted to the desired level to achieve the best cross-linking behavior and high antimicrobial efficiency.

The coated nets together with the blank nets were mounted onto an aquaculture cage and immersed in the sea for 15 and 35 days. Photographs of the immersed nets are presented in Figure 8. As observed through optical observation, the uncoated nets had the highest fouling compared to coated nets. More specifically, after 15 d of immersion the coated net remained almost intact whereas the color of the blank net had already darkened. A remarkable difference was observed between the coated and uncoated nets after 35 days of immersion, where a large number of fouling organisms was observed at the uncoated net, mainly attached at the nodes. Note that the net immersion in the aquaculture unit was conducted during summer when the daylight periods were longer, the light was more intense, and the temperatures were warmer, enhancing the reproduction and growth of fouling organisms. Thus, the low fouling concentration observed at the coated nets after 35 days is of high importance, as it renders the coating material very promising for further studies with longer periods of immersion in the sea environment.

4. CONCLUSIONS

In the present work, reactive blending of copolymers with complementary reactive groups, namely AA and GMA, was applied to control the antimicrobial activity of copolymers bearing active tertiary amine biocidal groups, either covalently (VBCHAM) or electrostatically (SSAmC$_{16}$) attached. On the basis of previous experience, the type of biocidal group along with the copolymer composition determines to a certain extent the final antimicrobial activity as well as the duration of the action. For this purpose, two copolymer series, namely P(VBCHAM-*co*-AAx) and P(SSAmC$_{16}$-*co*-GMAx), were synthesized through free radical copolymerization in organic solvent and characterized with ^{1}H NMR and ATR-FTIR spectroscopy.

The main goal of the present work was the optimization of the reaction between the two copolymers to control their activity and longevity. Overall, from the combined solubility test/turbidity results, the ATR-FTIR investigation, and the soluble fraction of the membranes in $CHCl_3$ after curing, it can be concluded that the ring-opening of the epoxide unit of GMA by the carboxylic acid unit of AA is favored by increasing the curing temperature. Though the details of the macroscopic behavior are also controlled by the ratio of the complementary reactive units, the reaction already takes place to a significant extent at 80 °C, leading to grafted samples rather than cross-linked ones. Insoluble cross-linked samples may be obtained by a further increase in curing temperature at 120 or 150 °C. The study of the release of active species in pure water or aqueous NaCl solution has revealed that, depending on the curing temperature and coating composition, very low release rates were obtained especially in the salt solution.

The antimicrobial activity of selected membranes cross-linked at 120 °C was pronounced, as they presented a log reduction ∼5.4 against *P. aeruginosa* and ∼5.3 against *S. aureus*. Finally, aquaculture nets that were treated with the blend composition presented the higher antimicrobial activity and cured at 80 °C. The immersion of the coated nets in the sea environment for 15 and 35 days during the summer period indicated significant antifouling action compared to the uncoated nets. This performance of the coating materials, as well as their strong antimicrobial activity and their behavior when they are in contact with an aqueous environment, suggests that such materials are promising for antifouling applications. In fact, the concept presented here offers an practical alternative for the preparation of coatings with a dual contact-based and release-based antimicrobial action. The strength and duration of this action can be fine-tuned by the relative contents of the two biodidal species, the extent of the cross-linking reaction, and the density of cross-links.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b14463.

Synthesis of precursors, ATR-FTIR characterization of the developed copolymers, ^{1}H NMR characterization of the developed copolymers, TEM images of selected copolymers and membranes, and TOC/TN results for the released study of selected membranes (PDF)

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Notes

The authors declare no competing financial interest.

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