Materials Science and Engineering C 67 (2016) 214–220

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Antimicrobial surface functionalization of PVC by a guanidine based antimicrobial polymer

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ARTICLE INFO

Article history:
Received 16 February 2016
Received in revised form 5 May 2016
Accepted 12 May 2016
Available online 13 May 2016

Keywords:
Antimicrobial coating
PVC
Poly hexamethylenediamine guanidine hydrochloride
Non-leaching coating

ABSTRACT

Antimicrobial polyvinyl chloride (PVC) plastic was obtained by covalent bonding of poly hexamethylenediamine guanidine hydrochloride (PHMG), a guanidine based antimicrobial polymer. This was achieved by grafting mercaptopropyltrimethoxysilane onto PVC, followed by aminopropyltriethoxysilane. Glutaraldehyde is a bifunctional crosslinker that was bonded to the free amine groups found in the treated PVC on one side and PHMG on the other. The treated PVC samples were characterized by FT-IR and XPS, showing that the PVC samples were successfully modified. Energy Dispersive X-ray spectroscopy showed the spatial distribution of the elements Si and S, indicating that the coatings were homogeneous. The resulting PVC samples showed high antimicrobial activity against Gram-positive and Gram-negative bacteria. Furthermore, the biofilm formation was negligible in comparison with the untreated material. The coating elusion assay indicated that its antimicrobial ability was achieved via direct contact rather than a controlled release mechanism.

1. Introduction

The polymer polyvinyl chloride (PVC) is one of the most used plastic materials in the world and its versatility allows it to be used in biomedical devices, tubes, bottles, different packagings, coatings, plastisols, films, etc. It is a low cost and durable plastic, with good mechanical properties, and easy high-temperature processability. PVC itself is hard and rigid but the addition of plasticizers makes it soft and pliable [1].

Upon contact with biological fluids, as other plastic materials, PVC is prone to suffer microbial colonization, [2–5] probably due to its hydrophobic characteristics [6]. Typically, they do not have inherent antibacterial properties, an important feature in preventing infections in humans or material deterioration by microorganisms present in the living environment [7]. For this reason, in order to obtain an antimicrobial material, PVC modification is currently being studied because it would diminish the risk of infections and cross contamination in industrial processes [8–13]. One possible antibacterial strategy is based on the use of biocides agents, either bound to the surface or to be released to the surroundings [14–20]. Although some antibiotics such as nisin or triclosan had been incorporated to PVC products, [21,22] the most used antibacterial agent is silver [10,23,24].

When the antibacterial agent leaching is not desirable, one alternative is to achieve the PVC derivatization by making use of a nucleophilic substitution reaction. It is well known that thiol compounds can be used for this purpose due to the high nucleophilicity and low basicity of the sulfur [25]. Although it has been demonstrated that no modification of the polymer takes place when only water is used as solvent, a good solvent for PVC can be added to the water suspension to yield highly modified polymers [26].

Many polymers with antimicrobial activity have been covalently bonded to different substrates [27], such as guanidine-based cationic polymers, which show excellent antibacterial, antifungal and antiviral activities [28] Poly hexamethylenediamine guanidine hydrochloride (PHMG) is a guanidine-based polymer. Its mechanism of antimicrobial action has been clearly determined: PHMG possesses multiple positive charges within a single molecule that are able to disturb the negative charges present on the cell walls of microorganisms. Therefore, PHMG diffuses through the cellular membrane and binds to the cytoplasmic membrane forming a complex with the phospholipid molecules of the lipid bilayer, destabilizes the osmotic equilibrium and destroys cytoplasmic membrane, causing leakage of the cell content [14]. As an advantage, PHMG has a great potential in the development of covalently bound antimicrobial materials [29,30–32].

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http://dx.doi.org/10.1016/j.msec.2016.05.052
0928-4931/

The aim of this paper was to develop a PVC antimicrobial coating by immobilizing the polymer PHMG to the PVC surface. This was achieved by grafting mercaptopropyltrimethoxysilane (MPTMS) onto PVC, followed by aminopropyltriethoxysilane (APTES). Glutaraldehyde (Glut), a bifunctional crosslinker, was bonded to the free amine group from the APTES. Finally, the PHMG molecules were bonded to the free aldehyde groups from the Glut. The coated PVC was characterized by Infrared Spectroscopy (FT-IR), X-ray photoelectron spectroscopy (XPS) and Energy Dispersive X-Ray Spectroscopy (EDS). The antimicrobial properties of the pure and modified PVC were studied against *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Bacillus subtilis*. The biofilm formation was assessed against *P. aeruginosa*.

2. Materials and methods

2.1. Materials

Medical grade PVC urinary catheters were purchased from Barcat (Argentina) and cut in order to get samples of 1 cm^2. 3-Mercaptopropyltrimethoxysilane (MPTMS, 98%) and 3-aminopropyltriethoxysilane (APTES, 99%) were acquired from Sigma (St Louis, MO, USA). Glutaraldehyde 25% in water was purchased from J.T Baker (New Jersey, USA). Poly (hexamethylenediamine guanidinium chloride) 20% in ethanol solution (PHMG) was acquired from Diransa (Buenos Aires, Argentina). All other reagents were of analytical grade.

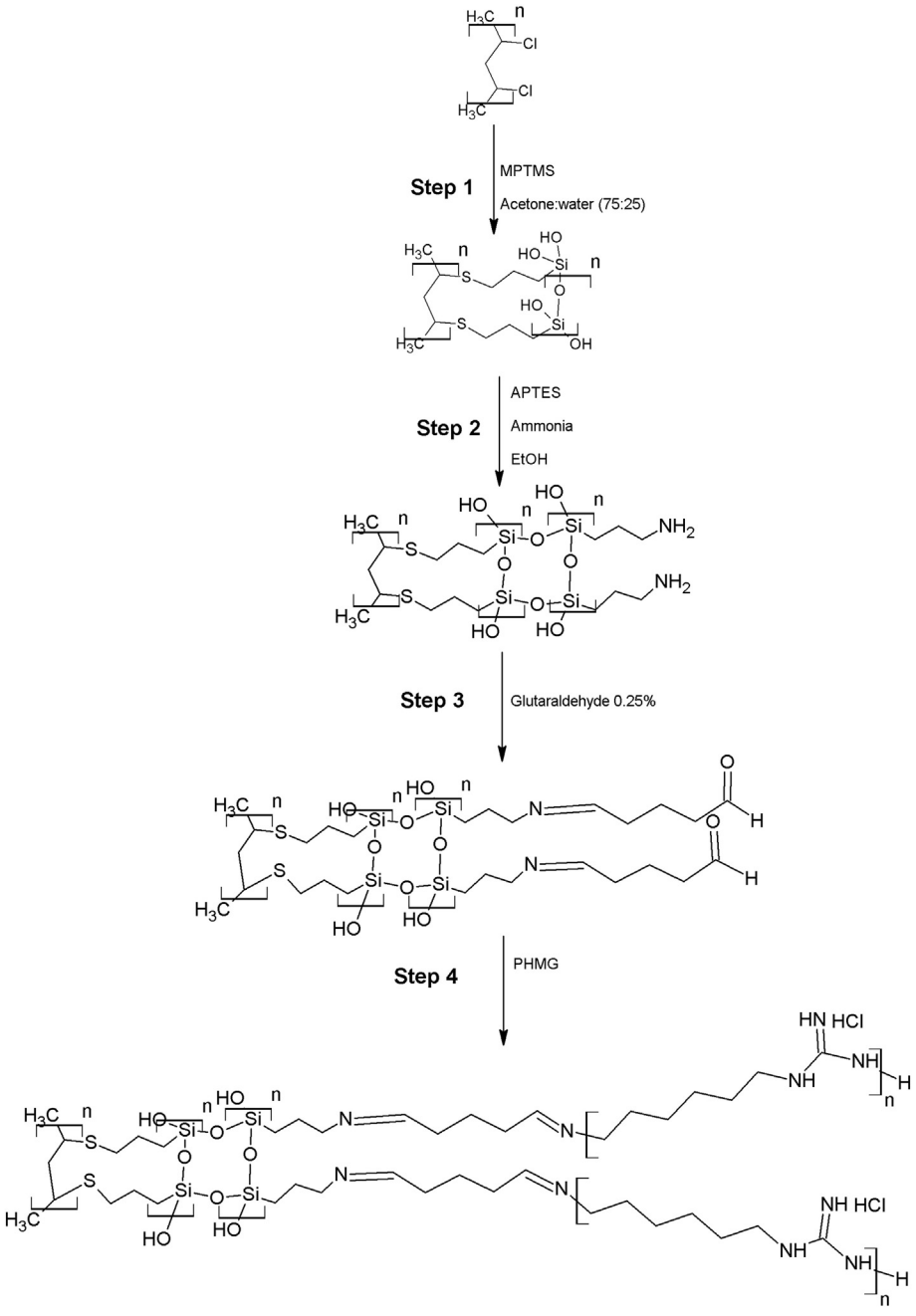

Fig. 1. Schematic representation of the coatings synthesis.

P. aeruginosa ATCC 27853, *S. aureus* ATCC 29213 were gently provided by the Microbial Culture Collection of Facultad de Farmacia y Bioquímica (CCM 29), University of Buenos Aires and *A. baumannii* wild type and *B. subtilis* wild type were isolated from a hospital environment. All microorganisms were grown at 35 °C for 24 h on Luria Bertani (LB) medium (Britania, BA, Argentina).

2.2. Solvent - non solvent MPTMS modification and APTES funcionalization

These treatments were performed according to a previous work [33]. Briefly, MPTMS substituted PVC (M-PVC) was prepared, using a MPTMS solution in acetone:water (75:25) at room temperature for 30 min. When PVC is exposed to a nucleophile such as a thiol group, a substitution reaction occurs (Step 1 in Fig. 1) [34].

After that, the M-PVC samples were incubated with a 3.76 mM APTES solution comprising APTES, ethanol (2.5 mL), aqueous ammonia (0.5 mL) and deionized water at room temperature with gentle shaking for 18 h. Afterwards, they were rinsed with deionized water and air dried. These samples were named A-PVC (Step 2 in Fig. 1).

2.3. Glutaraldehyde and PHMG derivatization

The A-PVC samples were left in a Glutaraldehyde 0.25% solution in phosphate buffer 0.2 M pH 7.5 for 30 min. Later, they were rinsed with deionized water and air dried. These samples were named G-PVC (Step 3 in Fig. 1).

G-PVC samples were incubated in the PHMG solution as received for 18 h. After that time, they were rinsed with deionized water and air dried. These samples were named P-PVC (Step 4 in Fig. 1).

2.4. Characterization

2.4.1. Infrared spectroscopy, energy dispersive X-ray analysis (EDX) and X-ray photoelectron spectroscopy (XPS)

ATR–FTIR transmission spectra were acquired in the range of 4000–650 cm^{-1}, using a Fourier transform infrared spectrometer (FT-IR) with a diamond attenuated total reflectance (ATR) system (Perkin Elmer, Spectrum One). All slides were previously dried for 24 h at 60 °C to avoid interference from water related bands. Elemental analyses were carried out using an EDX analyzer (OXFORD instrument) coupled to a Zeiss Supra 40 Scanning Electron Microscope. The XPS measurements were carried out with a spectrometer (model Physical Electronics 5700), using a Mg-Kα source (1253.6 eV) (model 04-548 Dual Anode X-rays Source). The X-ray source was run at a power of 300 W (10 keV and 30 mA). The pressure inside the vacuum chamber was 5×10^{-8} Torr. A hemispherical analyzer was employed (10-360 Precision Energy Analyzer) with a multi-channel detector (16 channels, which uses a chevron pair of multi-channel plates with 16 discretes anodes and 16 channels of amplification, discrimination and counting electronics). The lens system (Omni Focus IV Lens) was used to scan the spectrum and to define the size of the analysis area. All spectra were obtained using a 720 μm diameter analysis area.

2.4.2. Guanidine density

Guanidine density was quantified by using the method developed by Martin F. Chaplin [35], based on the formation of a colourless adduct between ninhydrin and guanidine groups. Briefly, a ninhydrin solution (113 μM) was allowed to react with the guanidine groups present in the P-PVC samples in a 0.1 M pH 9.0 NaH_2PO_4/Na_2HPO_4 buffer at 37 °C for 18 h. The guanidine groups were determined by the loss in absorbance at 232 nm. A PVC control was also performed.

2.4.3. Plasticizer loss

The plasticizer loss was measured according to ASTM D1203-1992 (Test Method A, 70 °C, 24 h, using activated carbon method) [36,37]. Briefly, the PVC samples were weighted individually on an analytical balance and designated this weight as W_1. Weights of individual specimens were 0.1 g within a tolerance of 10%. Activated carbon was spread on the bottom of a container. One specimen was placed on top of the activated carbon and covered with activated carbon. A second specimen was placed on top of the first and covered carbon, followed by a third specimen and then more of activated carbon. A cover was placed on the container in such a manner that the container was vented. The containers were placed in an oven at 70 ± 1 °C for 24 h. At the end of the 24 h period, the container was removed from the oven. Then, the specimens were removed from the container and brushed free of carbon.

After reconditioning, the specimens were reweighed and this weight was designated as W_2.

The volatile loss was calculated as Eq. (1):

$$\text{weight loss}\% = [(W_1 - W_2)/W_1] \times 100 \quad (1)$$

where:

W_1 = initial weight of test specimen, and

W_2 = final weight of test specimen.

2.5. Test for antimicrobial activity and efficacy

The antibacterial activity of the coatings against *P. aeruginosa* was carried out according to a modified assay from Japanese Industrial Standards (JIS) Z 2801 [38] using microorganisms grown in LB medium for 24 h. The preparation of the inoculum was performed diluting LB broth with sterile physiological solution to a 500-fold volume. Then the inoculum was prepared diluting the original inoculum until the microorganisms concentration was $2.6 \cdot 10^6$ cfu/mL.

Each PVC sample was immersed in 1 mL of 70% ethanol for the disinfection, and used for the antimicrobial efficacy assay after washing three times with 1 mL of sterilized water. Then they were placed in sterilized conic vessels and 0.020 mL of the previously described bacterial suspension was added, maintaining the vessel in horizontal position. The interaction between the samples and the bacterial suspension was performed at 35 °C for 24 h without agitation. After that time, 0.5 mL of sterilized saline solution was added to each vessel and the surviving bacteria were counted by the spread plate method. Decimal dilutions were spread on a Petri dish that contained LB agar and were incubated at 35 °C overnight. After incubation, the colonies were counted [39]. *A. baumannii*, *S. aureus*, and *B. subtilis* bioburden reduction was also studied in the P-PVC samples following the protocol described above. The results were presented in terms of value of antimicrobial activity

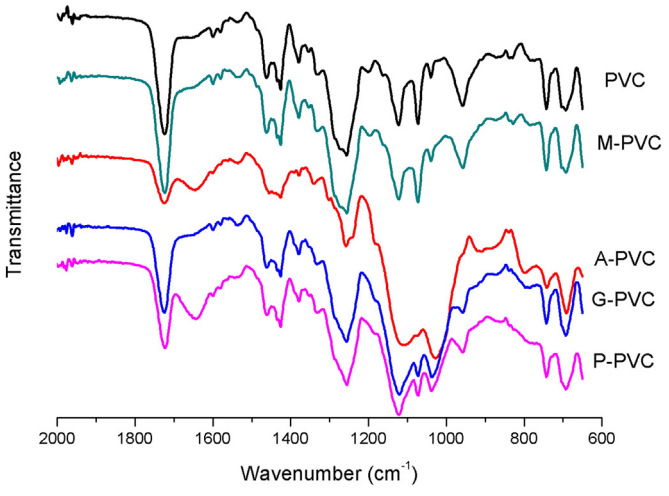

Fig. 2. FT-IR of untreated and treated samples (PVC: untreated sample; M-PVC: PVC + MPTMS; A-PVC: M-PVC + APTES; G-PVC: A-PVC + Glut; P-PVC: G-PVC + PHM; G).

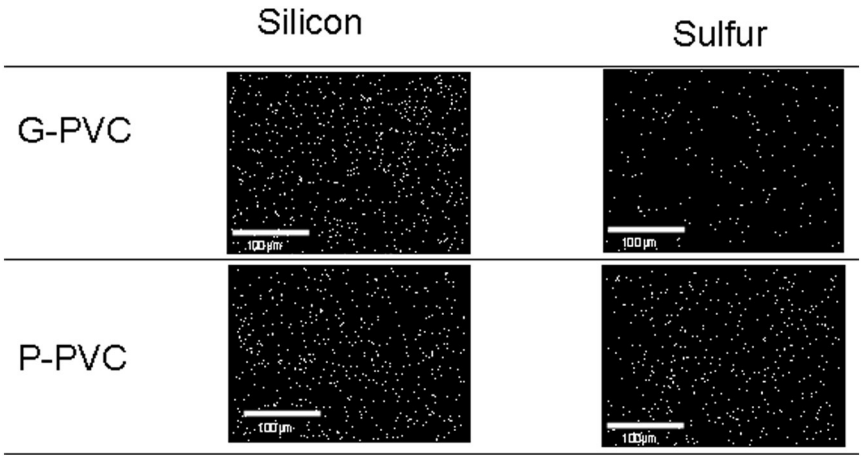

Fig. 3. EDX mapping of treated samples.

(R(log)) and % bacterial reduction (D %) in Eqs. (2) and (3):

$$R(\log) = [\log(A) - \log(B)] \quad (2)$$

$$D\% = (A-B)/B \times 100 \quad (3)$$

where A is the average of the number of viable cells of bacteria on the untreated PVC samples after 24 h and B is the average of the number of viable cells of bacteria on the treated piece after 24 h.

The antimicrobial activity of G-PVC and P-PVC was studied at different times after their synthesis (t_1: 1 day after synthesis, t_2: 7 days after synthesis, t_3: 30 days after synthesis and t_4: 60 days after synthesis) to evaluate the antimicrobial activity stability.

2.6. Antimicrobial elution assay

In order to assess if the antimicrobial was released during the antimicrobial assay, P-PVC samples were left in sterile saline solution during 24 h. Supernatant antimicrobial activity was measured in a diffusion agar test.

2.7. Biofilm formation test

Biofilm formation of PVC and P-PVC samples was evaluated. This assay was carried out using *P. aeruginosa* (10^8 cfu/mL) grown in LB medium for 24 h. Each sample was sterilized with ethanol 70% and then washed with sterilized water three times. Then, they were incubated by total immersion in the previously described bacteria suspensions at 37 °C for 48 h. After that time, the samples were rinsed with sterilized water three times, put in a conical vessel with physiological solution and sonicated for 5 min at 35 kHz [40]. The bacteria present on the supernatant were counted by the spread plate method as described above. This method was chosen in order to evaluate killing of biofilm cells.

2.8. Statistics

All quantitative results were obtained from triplicate samples. Data were expressed as means ± SD. Statistical analysis was carried out using a One-way ANOVA test and a Bonferroni post test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussions

3.1. Infrared spectrum

The FT-IR spectra of the uncoated and coated PVC samples (PVC, M-PVC, A-PVC, G-PVC and P-PVC) were analyzed (Fig. 2 and Supplementary data 1). Each step of the PVC grafting was assessed by the appearance of new functional groups corresponding to each new molecular link. In the PVC spectrum, characteristic absorption bands could be found at 695 cm^{-1}, due to C—Cl stretching vibration, at 732, 943, 1066 and 1114 cm^{-1}, attributable to PVC chain stretch, at 1247 cm^{-1}, corresponding to C—H bend, at 1342 and 1413 cm^{-1}, due to C—H_2 bend, at 2850 and 2908 cm^{-1}, owing to C—H_2 stretching and at 2933 cm^{-1}, corresponding to C—H stretching, neighboring CH—Cl groups [41,42]. Since diethyl hexyl phthalate (DEHP) is the plasticizer used in this PVC sample, absorption bands due to its presence could also be found in the PVC spectrum. Absorption bands at 742 and 1720 cm^{-1} corresponded to C—H from the aromatic compound and the carbonyl group from the plasticizer, respectively. Furthermore, an absorption band due to the alkane C—H bond from PVC and DEHP was found around 1250 cm^{-1} [40].

The M-PVC sample showed a typical silicon oxide broad band at 815 cm^{-1} corresponding to symmetric Si–OH stretching. Asymmetric Si—O—Si bond stretching band at 1021 cm^{-1} was found in A-PVC, G-PVC and P-PVC spectra, probably due to Si—OH condensation between MPTMS and APTES molecules [43–46]. In P-PVC and A-PVC spectrum a band corresponding to N—H stretching at 1655 cm^{-1} was found, showing that APTES and PHMG functionalization was successfully performed.

Table 1
Atomic concentration (%) of the surface obtained by XPS.

	C	O	Cl	N	Si	S
PVC	80.43 ± 1.23	6.10 ± 0.03	13.18 ± 1.86	ND	ND	ND
M-PVC	58.10 ± 1.89	20.46 ± 0.56	<0.31	ND	11.80 ± 0.95	9.36 ± 0.23
A-PVC	61.47 ± 1.26	18.36 ± 0.24	<0.29	3.79 ± 0.17	8.55 ± 0.8	7.56 ± 0.18
G-PVC	60.83 ± 4.69	20.31 ± 0.76	0.53 ± 0.15	0.69 ± 0.33	11.30 ± 0.67	6.28 ± 5.15
P-PVC	84.36 ± 0.92	6.82 ± 0	3.57 ± 1.16	2.46 ± 0.47	2.00 ± 0.17	0.79 ± 0.06

3.2. Energy dispersive analysis and guanidine density

In order to assess the coating homogeneity, EDX elemental analysis and mapping were performed by means of Si and S spatial distribution in the two last steps of the coating. The Si came from the organosilanes used in the coating and the S from the MPTMS. As it was demonstrated in our previous work, the organosilane grafting steps can achieve homogeneous coatings [33]. EDX mapping of the G-PVC and P-PVC samples showed that the Si and S distribution was not altered by random local concentrations of the glutaraldehyde or PHMG in the final steps and the homogeneity was maintained (Fig. 3). Otherwise, the elemental ratio would vary among the surface.

Due to the low sensitivity of the N atoms for the EDX method, the quantification of the guanidine groups density present in the surface was performed by a spectrophotometric assay. The guanidine density was 2.67 μmol/cm^2. This result also showed evidence that the PHMG was successfully grafted to the surface.

3.3. X-ray photoelectron spectroscopy

XPS analysis was performed in order to characterize the functional groups present at the surface, The XPS profiles were shown in Supplementary data 3 and 4. Results exposed in Table 1 indicated that the atomic concentration (%) of C and Cl diminished in M-PVC, A-PVC and G-PVC due to the rise of the atomic concentration of other elements that are not present in PVC. In P-PVC an increment was observed due to the significant presence of C and Cl in the polymer.

Regarding O and Si atomic concentration, an increase was observed in *M*-PVC, A-PVC and G-PVC. These results were in accordance to the incorporation of the organosilanes (O and Si) and Glut (O). The binding energy at 532.0 eV was characteristic of O in the Si—OH group and 533.0 eV corresponded to an O in the Si—O—Si group. The binding energy at 530.0 eV could be assigned to the presence of a carboxylic group in the plasticizer DEHP and other PVC additives or due to the carbonyl group from the Glut. Concerning Si, in all derivatized samples the binding energy at 102.3 eV was found, owing to the presence of Si(IV) from the silanol group. In P-PVC the O and Si atomic concentration decreased in comparison with the other treated PVC samples, because of the absence of these elements in the polymer.

The photoemissions corresponding to N are not present until the incorporation of APTES. In P-PVC the atomic concentration increases respecting G-PVC due to the presence of this element in the PHMG. The binding energy at 400.1 eV found in A-PVC indicated the presence of N in amine groups that are at the end of an aliphatic chain. This result reinforced the idea that the reaction between M-PVC and APTES occurred because of the reaction between the APTES silanols groups and the Si—OH present in the M-PVC. In P-PVC the photoemission at 399.6 eV corresponded to a —N═ group.

Finally, the presence of S was observed first after the coating with MPTMS and in the following grafting steps. The S atomic concentration decreased along with the rest of the treatments. The binding energies of 163.2 eV (signal S $2p_{3/2}$) and 164.4 eV (signal S $2p_{1/2}$) eV are typical of a thioether group [47].

3.4. Plasticizer loss

As it was mentioned before, the PVC plasticizer present in the samples was DEHP, which could be released from the bulk of the solid to the human fluid in contact [48]. Therefore, any treatment to PVC should not increase plasticizer loss. The volatile loss weight found in P-PVC (3.15 ± 0.31%) was not significantly different from the one in PVC (3.25 ± 0.65%; $p > 0.05$). These results showed that none of the coatings induced DEHP loss.

Table 2
Antimicrobial efficacy assay against *P. aeruginosa* of untreated, PHMG treated samples and all the intermediate (t_1: 1 day after synthesis).

	cfu/mL	D %	R(log)
PVC	2.7×10^8		
M-PVC	1.6×10^8	33.338	0.1
A-PVC	1.3×10^8	51.838	0.4
G-PVC t_1	2.9×10^3	99.989	4.4
P-PVC t_1	50	99.999	6.4

3.5. Test for antibacterial activity and efficacy

In order to evaluate the antibacterial activity of the different coating stages, the treated and control samples were first tested against *P. aeruginosa*. The results (Table 2) showed that in PVC, M-PVC and A-PVC samples, the bacterial growth was not significantly different ($p > 0.05$) among all treatments indicating that neither MPTMS nor APTES treatments contributed to the antibacterial activity.

In G-PVC and P-PVC samples an excellent antimicrobial activity was found, being P-PVC much more active than G-PVC. In Fig. 4 the R(log) of *P. aeruginosa* against G-PVC and P-PVC after different coating storage times after their synthesis is shown. In G-PVC, a quick drop of the antimicrobial activity against time could be found. This may be due to Glut degradation, which is known to be unstable. In P-PVC, although the activity decreased mildly, the effectiveness of the coating lasted at least for two months. This effect might have been due to the presence of Glut residues which had not reacted with PHMG. As soon as time passed, Glut degradation took place and the overall antimicrobial activity in P-PVC diminished.

In order to assess the antibacterial spectrum of the coating, the antibacterial efficacy test was also performed in P-PVC samples against *A. baumannii*, *S.aureus* and *B. subtilis*. Results describing the R(log) and D (%) against the previously mentioned species are shown in Table 3. The antibacterial reduction was above 3.5 in all of them, which is above the R(log) > 2 required by the JIS standard. This confirms that this coating may be useful to prevent infections caused by Gram negative and Gram positive microorganisms.

3.6. Antimicrobial elusion assay

This assay was made by incubating P-PVC in saline solution in order to assess if the coating was released during the antibacterial assay. As could be seen in Supplementary data 2, supernatant antibacterial activity was undetectable. This confirms that the antibacterial activity of the coating is achieved by contact of bacteria with the modified PVC surface and not by the leaching of the antimicrobial to the surrounding media.

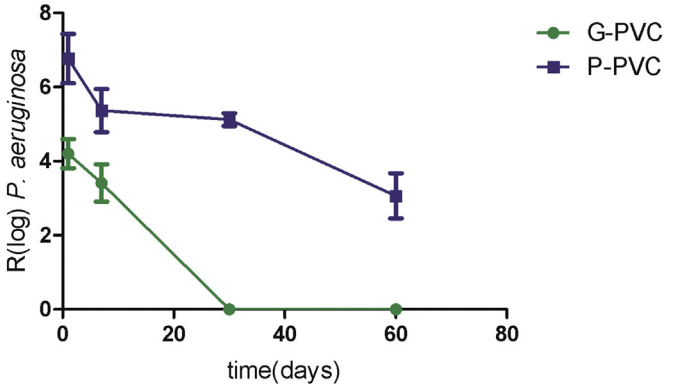

Fig. 4. R(log) of *P. aeruginosa* against G-PVC and P-PVC 1, 7, 30 and 60 days after their synthesis.

Table 3
Evaluation of the antimicrobial efficacy spectrum of P-PVC samples against Gram positive and Gram negative bacteria.

Strain	D %	R(log)
P. aeruginosa	99.999	6.4
S. aureus	99.999	4.9
A. baumannii	99.956	3.7
B. subtilis	99.999	5.4

3.7. Biofilm formation test

The biofilm formation test showed that the bacterial adhesion in treated samples was 78.8% lower than in the control showing that the coating could prevent biofilm formation and its consequences. Biofilm formation is usually promoted by rich environments, high bacterial number and long exposure times. By exposing the coatings to a 1×10^8 cfu/mL in LB medium for 48 h the coatings where challenged in a worst case scenario. Even in these conditions the coatings showed that the bacterial adhesion was much lower in the treated samples than in the control.

Surface roughness may influence bacterial attachment [49,50], in fact, in a specific study.

PVC has been nanotextured in order to diminish the bacterial colonization [51]. When comparing PVC and P-PVC surface topography no differences could be found in the SEM images (Supplementary data 5). Thus, it can be concluded that the bactericidal effect of the PHMG residues was the main responsible for the biofilm formation decreasement and roughness parameter was considered negligible.

4. Conclusion

According to the reported results in this paper, it was concluded that medical grade PVC samples could be modified for the obtaining of an antibacterial plastic with high activity against *P. aeruginosa*, *S. aureus*, *A. baumannii* and *B. subtilis* when they were derivatized with the polycationic polymer PHMG.

The treated PVC samples were characterized by FT-IR and XPS, proving that the PVC samples were successfully modified. EDX mapping showed the distributions of the elements Si and S, indicating that the coatings were homogeneous.

P-PVC samples showed an excellent antibacterial activity against Gram positive and Gram negative microorganisms. Besides, the treated samples showed a lower bacterial attachment, which made them suitable for preventing biofilm formation. Also, this development presents an improvement in the antibacterial activity compared to similar antimicrobial coatings for PVC [33]. Moreover, the antimicrobial activity has proved to be maintained at least for 60 days.

The antimicrobial elution assay indicated that its antimicrobial ability was via direct contact rather than a controlled release mechanism.

Acknowledgments

M.E.V. is grateful for her posdoctoral fellowship granted by Consejo Nacional de Investigaciones Científicas y Técnicas. J.A.G. is grateful for his doctoral fellowship granted by Consejo Nacional de Investigaciones Científicas y Técnicas. This work was supported with grants from Universidad de Buenos Aires (UBACYT 20020130100780BA), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220120100657CO) and project of Junta de Andalucía P12-RNM-1565 (Spain) and FEDER funds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2016.05.052.

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