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# Dual antibacterial effect of immobilized quaternary ammonium and aliphatic groups on PVC †

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A coating comprising quaternary ammonium salts (QAS) and aliphatic moieties was performed in Poly(vinyl chloride) (PVC) surface in order to confer antibacterial activity. This was achieved by grafting mercaptopropyltrimetoxysilane onto PVC, followed by aminopropyltriethoxysilane. Betaine and Dodecyl Succinic Anhydride (DDSA) were bonded to free amine groups. The modified PVC samples were characterized by FT-IR, showing that the PVC surface was successfully coated. Energy Dispersive X-ray spectroscopy showed the spatial distributions of the elements Si and S, indicating that the coatings were homogeneous. Betaine and DDSA coated PVC showed a better antibacterial performance than the controls. This antibacterial effect was extremely reinforced in Betaine-DDSA modified PVC showing greater antibacterial activity than both treatments separately applied. Antibacterial activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis* was studied in the treated samples showing that the coating was effective against Gram positive and Gram negative species.

## 1 Introduction

The polymer polyvinyl chloride (PVC) is a low cost and durable plastic used in pipes, food packaging, biomedical devices, etc. As other plastic materials, PVC is prone to suffer microbial colonization.<sup>1-5</sup> However, it does not have inherent antibacterial properties, an important feature in preventing infection in humans by microorganisms present in the living environment.<sup>6</sup> Bacterial colonization in PVC products can be disadvantageous in various settings. Hospital environment is especially susceptible to pathogenic bacteria contamination, which is especially hard to deal when it is found in biofilms.<sup>7,8</sup> The rise in nosocomial infections is a direct consequence of this. For example, infections resulting from microbial adhesion to biomaterials have been observed on nearly all medical devices, such as catheters and orthopaedic implants, leading to severe economic and medical consequences.<sup>9-11</sup> Respecting industry related environments, the contamination caused by microorganisms during the manufacture, processing, and packaging of food and medicines is of considerable importance to public health and consequently it is a major issue for industry.<sup>12</sup> For this reason, PVC derivatization in order to obtain an antibacterial material would diminish the risk of infections.<sup>13-16</sup> One possible antibacterial strategy is based on the use of biocides agents, either bound to the surface or to be released to the surroundings.<sup>8,17-21</sup> Although some organic antibiotics such as nisin or triclosan had been incorporated to PVC products,<sup>22-24</sup> the most used antibacterial agent is silver.<sup>15,25,26</sup>

One alternative to achieve the PVC derivatization is 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 sulphur.<sup>27</sup>

Quaternary ammonium salts (QAS) have been known to be active moieties against microorganisms by contact with the cell membrane.<sup>28</sup> Therefore, QAS-containing polymers have been prepared and were the subject of several investigations.<sup>29-31</sup> Betaine esters,<sup>32,33</sup> methylbenzethonium chloride,<sup>34</sup> cetalkonium chloride<sup>35</sup> and cetylpyridinium chloride<sup>36</sup> are some of the QAS known for their antimicrobial activity. Betaine derivatives are harmless for human usage since the products obtained from the hydrolysis are normal metabolites of the host.<sup>32</sup> Most QAS have a lipophilic moiety in their structures. This moiety is involved in the interaction of the molecule with microbial membranes enhancing penetration of the antibacterial,<sup>32</sup> for example in cetylpyridinium chloride, cetalkonium chloride, benzalkonium chloride.

The aim of this study was to develop a PVC antibacterial coating by separately immobilizing onto the surface the quaternary amine and the lipophilic moieties of a QAS. In this work we present the preparation of an antibacterial dual coating containing betaine and a 12 carbon chain, covalently bonded to the PVC surface. This was achieved by grafting mercaptopropyltrimetoxysilane (MPTMS) onto PVC, followed by aminopropyltriethoxysilane (APTES) where betaine and Dodecyl Succinic Anhydride (DDSA) were bonded to free amine groups. The coated PVC was characterised by Infrared Spectroscopy (FT-IR) and Energy Dispersive X-Ray Spectroscopy (EDS). The -NH<sub>2</sub> moieties were quantified by the picric acid method and the quaternary ammonium moieties were qualitatively detected through iodine reaction. The antibacterial properties of the pure and modified PVC were studied against *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Bacillus subtilis*.

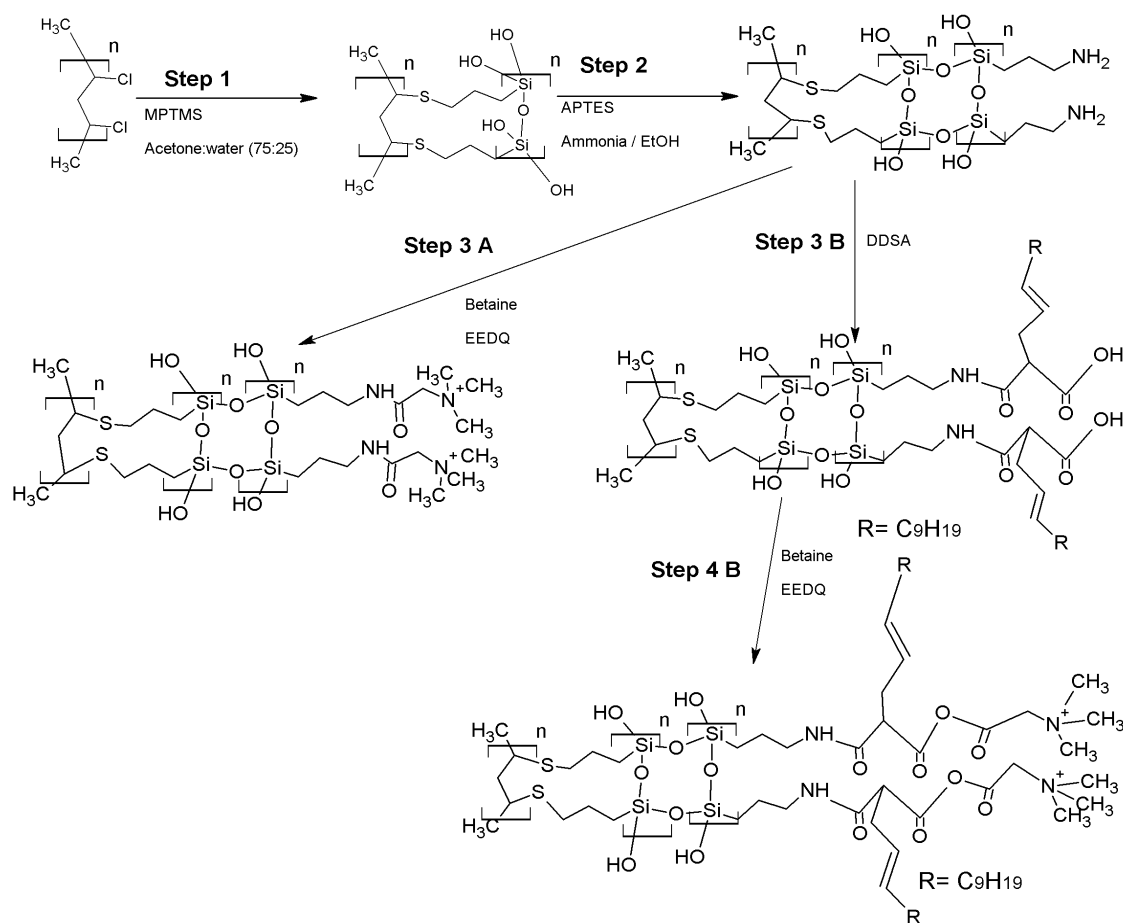


Figure 1. Schematic representation of the coatings synthesis.

## 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<sup>2</sup>. 3-mercaptopropyltrimethoxysilane 95% (MPTMS), 3-aminopropyltriethoxysilane 99% (APTES), 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline 99% (EEDQ) and Betaine hydrochloride 99% were acquired from Sigma (St Louis, MO, USA). Dodecyl Succinic Anhydride (DDSA) was purchased from Fullam Inc. (New York, USA). All other reagents were of analytical grade.

*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

MPTMS substituted PVC (M-PVC) was prepared, using a 5% 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 reactions

occur ( Step 1 in Figure 1).<sup>37</sup>

### 2.3 APTES coating

In order to optimize the number of amine groups per cm<sup>2</sup> over the surface, three different solutions had been prepared mixing APTES, absolute ethanol ( 2.5 mL), ammonia 35% (0.5 mL) and deionized water. The APTES concentration of each solution was 3.76 mM, 8.53 mM and 17.0 mM, respectively. The M-PVC samples were incubated with the previously mentioned APTES solutions at room temperature with gentle shaking for 18 h. After that, they were rinsed with deionized water and air dried. These samples were named A-PVC and those derivatized with 3.76 mM solution were chosen for the rest of the assays (Step 2 in Figure 1).

### 2.4 Betaine linking

The betaine linking solution had been prepared mixing 100 mg of solid EEDQ with a 1% w/v betaine hydrochloride solution. The A-PVC samples were left in the previously described solution for 18 h. Later, they were rinsed with deionized water and air dried. These samples were named B-PVC. EEDQ was used to couple the amine group with the carboxylic acid. The activation of carboxylic acids by EEDQ involves the transient formation of a mixed carbonic anhydride which cannot be isolated presumably because of a

rapid breakdown.<sup>38–40</sup>

## 2.5 DDSA-betaine derivatization

A 12 carbon chain moiety was added to the A-PVC samples by incubating them in a 50 % v/v DDSA solution in ethanol at 60 °C for 2 h. After that, they were rinsed with ethanol, deionized water and finally air dried. These samples were named D-PVC. Such samples were left in a solution prepared by mixing 100 mg of solid EEDQ with a 1% betaine hydrochloride solution for 18 h. Afterwards, they were rinsed with ethanol, and then deionized water and air dried. These samples were named BD-PVC.

## 2.6 Characterisation

### 2.6.1 Infrared spectrum and Energy dispersive X-ray analysis

ATR–FTIR transmission spectra were acquired in the range of 4000–650 cm<sup>-1</sup>, using a Fourier transform infrared spectrometer (FT-IR) with ZnSe flat-plate attenuated total reflectance (ATR) (Nicolet). All slides were previously dried for 24 h at 60 °C to avoid interference from water related bands. Elemental analyses were carried out in freeze-dried and gold coated samples using an Energy Dispersive analyser (EDX) analyser (OXFORD instrument) coupled to a Zeiss Supra 40 Scanning Electron Microscope.

### 2.6.2 Amine groups density in A-PVC

In order to determine the amine groups density of A-PVC samples, the picric acid method was followed. Briefly, three A-PVC samples were neutralized with 0.3 M NaOH for 6 min, and then washed with deionized water for 2 min. The samples were treated with 0.1 M picric acid for 10 min and washed five times with deionized water for 2 min each time. The picrate was eluted with the NaOH solution for 6 min. The solution absorption at 354 nm was measured spectrophotometrically. The concentration of the amine groups in the solution was obtained by comparing the absorption at 354 nm of the unknown solution with that of a standard picrate solution.<sup>41,42</sup>

### 2.6.3 Determination of Quaternary Ammonium Compounds

This qualitative assay was performed as follows: 1 ml of reagent (10 g of resublimed iodine + 12.4 g of KI in 1 L of water) was added to the PVC samples. Brown colour indicated the presence of quaternary ammonium salts.

### 2.6.4 Plasticizer loss

The plasticizer loss was measured according to ASTM D1203-1992 (Test Method A, 70°C, 24 h, using activated carbon method).<sup>43,44</sup> Briefly, the PVC samples were individually weighted using an analytical balance and designated this weight as W<sub>1</sub>. 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 containers were removed from the oven. Then, the specimens were removed from the containers and brushed free of carbon.

After reconditioning, the specimens reweighed and this weight was designated as W<sub>2</sub>.

The volatile loss was calculated as Eq (1):

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

where:

W<sub>1</sub> = initial weight of test specimen, and

W<sub>2</sub> = final weight of test specimen.

## 2.7 Antibacterial activity and efficacy test

The antibacterial activity of the coatings against *P. aeruginosa* was performed according to a modified assay from Japanese Industrial Standards (JIS) Z 2801.<sup>45</sup> For this test microorganisms were grown in LB medium for 24 h. The challenge inoculum was prepared diluting the grown bacteria with a culture medium (LB medium diluted 500-fold in physiological sterile solution) until the microorganism concentration was 1.9 10<sup>6</sup> cfu/ml.

Treated and untreated PVC samples were immersed in 1 mL of 70 % ethanol for the disinfection, and used for the antibacterial efficacy assay after washing three times with 1 mL of sterilized water. Then, they were incubated in 0.020 mL of the previously described bacterial suspensions.

After 24 h, the surviving bacteria on the supernatant 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 24 h. After incubation, the colonies were counted.<sup>46</sup>

The results were presented in terms of value of antibacterial activity (R(log); Eq (2)) and % bacterial reduction (D%; Eq (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. In order to assess the antimicrobial spectrum Figure 2. FT-IR of untreated and treated samples.

*A. baumannii*, *S. aureus*, and *B. subtilis* bioburden reduction was also studied in the BD-PVC samples following the protocol described above.

All experiments were conducted in triplicate, each time utilizing a fresh cell suspension.

## 2.8 Determination of coating release.

In order to assess if the coating was released during the antibacterial assay, BD-PVC samples were left in 1 mL of sterile saline solution during 24 h. Supernatant antibacterial activity was measured in a diffusion agar test.

## 2.9 Biofilm formation test

Biofilm formation of *P. aeruginosa* in PVC and BD-PVC samples was evaluated. For this test microorganisms were grown in LB medium for 24 h ( $10^8$  cfu/mL) and then used as the challenge inoculum. On the other hand, each PVC sample was sterilized with 70 % ethanol and then washed with sterilized water. After that, they were incubated in the previously described bacteria suspensions for 48 h. After that period, the PVC samples were rinsed with sterilized water, put in a tube with sterilized physiological solution and sonicated for 5 min.<sup>47</sup> The bacteria present in the supernatant were counted by the spread plate method as described above. All experiments were conducted in triplicate, each time utilizing a fresh cell suspension.

## 2.10 Statistics

All quantitative results were obtained from triplicate samples. Data were expressed as means  $\pm$  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 discussion

As it was described before, a PVC surface derivatization was performed in order to confer antibacterial activity. This was achieved by grafting MPTMS onto PVC, followed by APTES. Betaine and Dodecyl Succinic Anhydride (DDSA) were bonded to APTES free amine groups in order to obtain a coating comprising QAS and aliphatic moieties. This samples (BD-PVC: A-PVC + Betaine + DDSA) were characterized and tested against bacteria as well as all the intermediates: M-PVC (PVC + MPTMS), A-PVC (M-PVC + APTES), B-PVC (A-PVC + Betaine) and D-PVC (A-PVC + DDSA).

### 3.1 Surface characterisation

The FT-IR spectra of the uncoated and coated PVC samples (PVC, M-PVC, A-PVC, B-PVC, D-PVC and BD-PVC) were characterised (ESI † 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  $695\text{ cm}^{-1}$ , due to C-Cl stretching vibration, at  $732$ ,  $943$ ,  $1066$  and  $1114\text{ cm}^{-1}$ , attributable to PVC chain stretch, at  $1247\text{ cm}^{-1}$ , corresponding to C-H bend, at  $1342$  and  $1413\text{ cm}^{-1}$ , due to C-H<sub>2</sub> bend, at  $2850$  and  $2908\text{ cm}^{-1}$ , owing to C-H<sub>2</sub> stretching and at  $2933\text{ cm}^{-1}$ , corresponding to C-H stretching, neighbouring CH-Cl groups.<sup>48</sup> 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  $742$  and  $1720\text{ cm}^{-1}$  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  $1250\text{ cm}^{-1}$ .<sup>47</sup>

The M-PVC sample showed a typical silicon oxide broad band at  $815\text{ cm}^{-1}$  corresponding to symmetric Si-OH stretching. Asymmetric Si-O-Si bond stretching band at  $1021\text{ cm}^{-1}$  was found in A-PVC, B-PVC, D-PVC and BD-PVC spectra.<sup>20,49,50</sup>

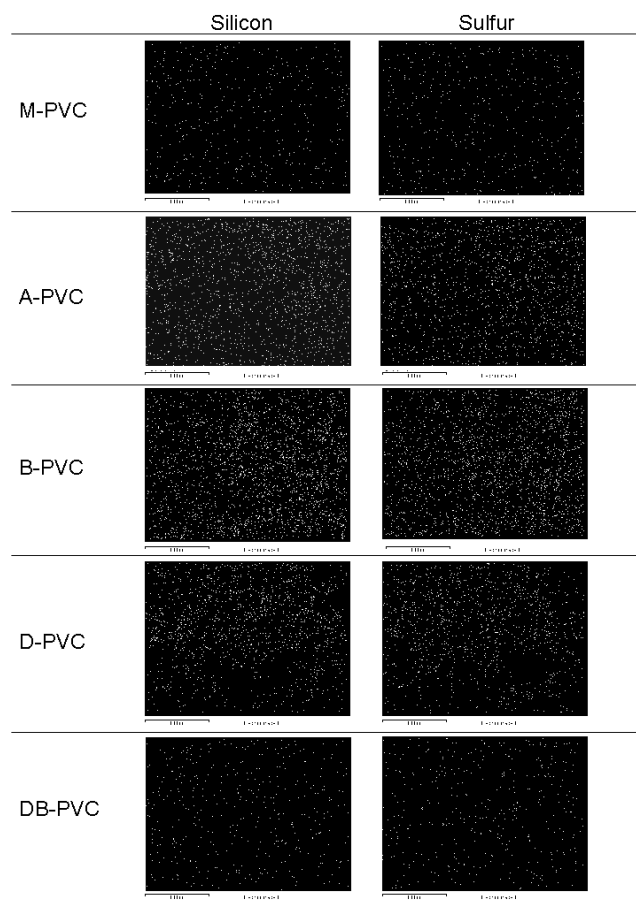


Figure 2. EDX mapping of untreated and treated samples. Coating homogeneity was evaluated by Silicon and Sulfur surface distribution.

This band accounted for the presence of a polymerized silicon oxide network, which became significant after APTES addition. This would be in relation to the coupling between. This band accounted for the presence of a polymerized silicon oxide network, which became significant after APTES addition. This would be in relation to the coupling between MPTMS and APTES silanol groups.

In the D-PVC and BD-PVC spectra, the broadening of the band at  $1720\text{--}1710\text{ cm}^{-1}$  accounted for the presence of new carbonyl groups from DDSA. The adsorption band due to the alkane C-H bond from DDSA carbon chain was found around  $2950\text{ cm}^{-1}$  in D-PVC and BD-PVC as well. The intensity of the bands was higher in the former.

### 3.2 Coating homogeneity

In order to assess the coating homogeneity EDX elemental analysis and mapping were performed. In the PVC samples the presence of Cl, C and O was observed (ESI † 2). In the coated samples the Si and the S were also present. The Si came from the organosilanes used in the coating and the S from the MPTMS. The elemental mapping showed that upon addition of MPTMS, homogeneous distribution of Si and S has been achieved in the coated surface (Figure 2). Moreover, with the addition of the subsequent link molecules the

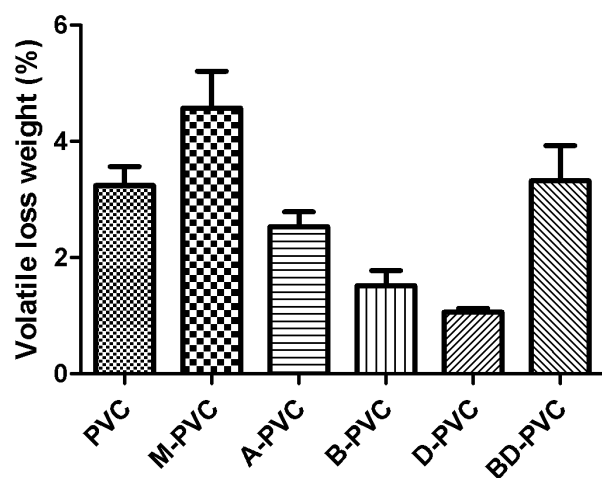


Figure 3. Volatile loss weight of untreated, betaine-DDSA treated samples and all the intermediates. The effect of the treatments in the plasticizer loss is evaluated.

homogeneous distribution of Si and S is maintained. This would indicate that next coating stages would also be homogeneous. Otherwise, the elemental ratio would vary among the surface.

### 3.3 Amine groups density in A-PVC and presence of Quaternary Ammonium Compounds

The amine group density of samples coated with 3.76 mM, 8.53 mM and 17.0 mM APTES solutions was  $3.5 \pm 0.6 \mu\text{mol}/\text{cm}^2$ ,  $3.3 \pm 0.8 \mu\text{mol}/\text{cm}^2$  and  $4.4 \pm 0.7 \mu\text{mol}/\text{cm}^2$  respectively. Since the densities found in the three concentrations tested were not significantly different ( $p > 0.05$ ), the A-PVC samples chosen for the following assays were those exposed to the lower APTES concentration (3.76 mM). As shown in ESI † 3, those PVC slides treated with betaine turned brown after periodate exposure. This confirmed that the grafting with quaternary ammonium groups was successful.

### 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. Therefore, any treatment to PVC should not increase plasticizer loss. Although significant difference could be found among the samples, none of the treated samples was significantly different from PVC ( $p > 0.05$ ). (Figure 3). These results showed that none of the coatings induced DEHP loss.

### 3.6 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 1) showed that in PVC, M-PVC and A-PVC samples the bacterial growth was

Table 1. Antimicrobial efficacy assay against *P. aeruginosa* of untreated, betaine-DDSA treated samples and all the intermediates

	ufc/mL	R%	R(log)
PVC	$2.7 \times 10^8$		
M-PVC	$1.6 \times 10^8$	33.338	0.1
A-PVC	$1.3 \times 10^8$	51.838	0.4
B-PVC	$2.3 \times 10^7$	86.633	1.3
D-PVC	$1.3 \times 10^6$	99.227	2.3
BD-PVC	$1.4 \times 10^5$	99.913	3.5

Table 2. Evaluation of the antimicrobial efficacy spectrum of BD-PVC samples against Gram positive and Gram negative bacteria.

	cfu/mL in PVC	cfu/mL in BD-PVC	R%	R(log)
<i>S. aureus</i>	$4.0 \times 10^7$	$1.1 \times 10^5$	99.7	2.9
<i>A. baumannii</i>	$1.9 \times 10^8$	$1.7 \times 10^6$	99.0	2.2
<i>B. subtilis</i>	$2.8 \times 10^6$	$2.4 \times 10^4$	99.2	2.4

not significantly different ( $p > 0.05$ ) in all of them indicating that neither MPTMS nor APTES treatment contributed to antibacterial activity.

As it was described before, the antibacterial moiety chosen was the quaternary ammonium present in betaine. It is generally accepted that lipid bilayer structures of cell membranes are principal targets for this class of compounds. A possible mechanism that can explain their activity is that a highly charged surface (like those obtained in this research) can induce what is essentially an ion exchange between the positive charges on the surface and structurally critical mobile cations within the membrane. Upon approaching a cationic surface, the structurally essential divalent cations of the membrane are relieved of their role in charge neutralization of the membrane components and are thus free to diffuse out of the membrane. The loss of these structural cations results in a loss of membrane integrity.<sup>51-53</sup> In the process of binding, the presence of a hydrocarbon chain would enhance antibacterial activity by intercalating into the hydrophobic interior of the microbial membrane, and the cationic polar head group would participate in charge interactions with neighbouring surface structures.<sup>54</sup>

As could be seen in Table 1, in B-PVC, D-PVC and BD-PVC the cfu/mL was significantly lower than in untreated PVC. We found the highest bactericidal activity for the BD-PVC ( $R(\log)=3.5$ ). D-PVC and B-PVC samples also showed antibacterial activity ( $R(\log)=2.3$  and  $R(\log)=1.3$  respectively). Accordingly, since the betaine portion and its quaternary ammonium moiety were identical in BD-PVC and B-PVC, the differences in antibacterial activities between them were dependent on the presence of the lipophilic moiety.<sup>32</sup> This effect suggested that the membrane disruption

caused by the hydrophobic chain is of relevance in the mechanism of action.

In order to assess the antibacterial spectrum of the coating, the antibacterial efficacy test was also performed in BD-PVC samples against *Acinetobacter baumannii*, *Salmonella choleraesuis* and *Bacillus subtilis*. Results describing the R(log) and D (%) against the previously mentioned species are shown in Table 2. The antibacterial reduction was important in all of them, confirming that this coating may be useful to prevent infections caused by Gram negative and Gram positive microorganisms.

### 3.7 Coating release.

This assay was made by incubating BD-PVC in saline solution in order to assess if the coating was released during the antibacterial assay. As could be seen in ESI † 4, 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, being the leaching of the coating negligible by means of its antibacterial effect.

### 3.8 Biofilm formation

The biofilm formation test showed that the bacterial adhesion was 82 % lower in treated samples 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  $1 \times 10^8$  cfu/ml in LB medium for 48hs the coatings were challenged in a worst case scenario. Even in this conditions the coatings showed that the bacterial adhesion was much lower in the treated samples than in the control.

## 4. Conclusions

According to the reported results in this paper, it was concluded that medical grade PVC samples could be transformed into antibacterial plastics with a high antibacterial activity against *P. aeruginosa*, *S. aureus*, *A. baumannii* and *B. subtilis* when they were derivatized with a 12 carbon aliphatic chain and a QAS moiety.

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

BD-PVC samples showed an excellent antibacterial activity against *P. aeruginosa*, *S. aureus*, *A. baumannii* and *B. subtilis*, which implies antibacterial activity for both Gram positive and Gram negative microorganisms. Besides, the treated samples showed a lower bacterial attachment, which makes them suitable for preventing biofilm formation. The antibacterial activity of the coated PVC is related to the interaction between cationic and aliphatic moieties and microbial cells. Hydrophobic chains are of main importance in biocidal activity.

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## Notes and references

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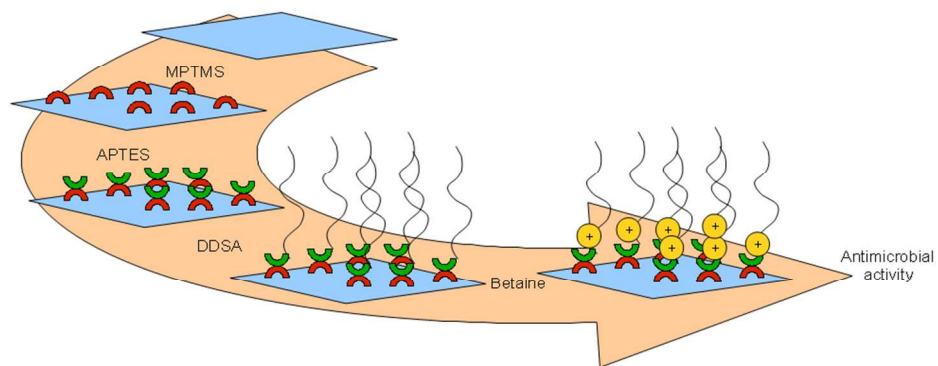
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Quaternary ammonium salts and lipophilic moieties were separately immobilized onto PVC to obtain a broad spectrum antimicrobial coating.