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# Antimicrobial and antioxidant amphiphilic random copolymers to address medical device-centered infections

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ABSTRACT

Microbial biofilms are known to support a number of human infections, including those related to medical devices. This work is focused on the development of novel dual-function amphiphilic random copolymers to be employed as coatings for medical devices. Particularly, copolymers were obtained by polymerization of an antimicrobial cationic monomer (bearing tertiary amine) and an antioxidant and antimicrobial hydrophobic monomer (containing hydroxytyrosol, HTy). To obtain copolymers with various amphiphilic balance, different molar ratios of the two monomers were used. $^{1}$H NMR and DSC analyses evidenced that HTy aromatic rings are able to interact with each other leading to a supramacromolecular re-arrangement and decrease the copolymer size in water. All copolymers showed good antioxidant activity and $Fe^{2+}$ chelating ability. Cytotoxicity and hemolytic tests evidenced that the amphiphilic balance, cationic charge density and polymer size in solution are key determinants for polymer biocompatibility. As for the antimicrobial properties, the lowest minimal inhibitory concentration (MIC = 40 μg/mL) against *Staphylococcus epidermidis* was shown by the water-soluble copolymer having the highest HTy molar content (0.3). This copolymer layered onto catheter surfaces was also able to prevent staphylococcal adhesion. This approach permits not only prevention of biofilm infections but also reduction of the risk of emergence of drug-resistant bacteria. Indeed, the combination of two active compounds in the same polymer can provide a synergistic action against biofilms and suppress reactive species oxygen (ROS), known to promote the occurrence of antibiotic resistance.
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## 1. Introduction

Implantable medical devices are associated with a high risk of bacterial and fungal infections. Staphylococci account for the majority of these infections due to their strong ability to adhere to different kinds of materials thus promoting biofilm formation. Antibiotic treatments often fail in eradicating biofilm-based infections due to the inherent high antibiotic resistance of biofilm-growing microorganisms [1,2].

In the last three decades, to prevent these infections, several antimicrobial agents, such as rifampicin, clindamycin, chlorhexidine and silver, have been loaded onto the surface of different medical devices [3–5], obtaining variable degrees of success depending on the type of medical device, used drugs and site of device implantation. In general, the main limitation of these drug-loaded devices is the rapid drug release, the short antibacterial action and the risk for selecting antimicrobial resistant strains. Therefore, there is a need for a continuous update in the strategies for biofilm prevention and killing.

Intrinsically antimicrobial polymers (also known as biocidal polymers) have recently emerged as interesting candidates for the prevention of microbial adhesion and biofilm formation [6–8]. Indeed, biocidal polymers do not release antimicrobial substances but exert their killing action when microorganisms contact the surface. They can be obtained by introducing phosphonium salts or quaternary amine compounds in the polymer backbone or side-chain, functionalities exerting antimicrobial activity [8,9].

Different kinds of polymers bearing cationic quaternary ammonium salts have been recently developed [7,10,11]. The antibacterial activity of these polymers is known to be related to their ability to establish electrostatic interactions with the negatively charged bacterial cell membrane causing cell wall and/or membrane disruption, leakage of cytoplasmic material and cell death [12,13]. Although the positive charge is fundamental for the antimicrobial

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activity of cationic polymers, a too high charge density can decrease their selectivity toward bacteria, with consequent toxic effects on human cells [14,15].

To increase selectivity versus bacterial cells, different studies [16,17] have demonstrated that spatial arrangement [20] and amphiphilicity [16,21,22], together with positive charges [18] and low molecular weight [19], are key parameters to regulate antimicrobial and hemolytic activities of polymers.

Particularly, to obtain amphiphilic cationic polymers, three different strategies have been mainly employed: (i) introduction of neutral hydrophilic moieties, such as PEG, into hydrophobic polymers [23,24], (ii) modification of the nature of the hydrophobic groups [16] and (iii) synthesis of random copolymers with a proper ratio of the cationic and the hydrophobic groups in the side chain [25]. In all these strategies, usually the hydrophobic fraction did not have any antimicrobial activity but just provided the polymer with a suitable amphiphilic balance.

In this work, novel dual-function amphiphilic random copolymers, endowed with both antimicrobial and antioxidant properties, by copolymerization of an antimicrobial cationic monomer (containing tertiary amine groups [26]) and an antioxidant and antimicrobial hydrophobic monomer (containing hydroxytyrosol).

The rationale of the work relies on the knowledge that the accumulation in the biofilm of reactive oxygen species (ROS) is one of the factors causing the high antibiotic resistance of biofilm-growing bacteria. Indeed, ROS increase the natural mutation frequency promoting the selection of antibiotic resistant strains [27,28]. In addition, ROS seem to be involved in signaling pathways in both bacteria and fungi playing a role in the regulation of biofilm formation [29].

Therefore, the development of a polymer provided with intrinsic antimicrobial and antioxidant activities seems to be a novel and promising approach to reduce the emergence of drug resistant bacteria in biofilm and combat biofilm infections.

In addition, since the antioxidant hydroxytyrosol (HTy) chosen in this study (a polyphenolic compound present in virgin olive oil and in olive oil wastewaters) also possesses antimicrobial activity against *Staphylococcus epidermidis* and *Staphylococcus aureus* [30,31], its presence in the polymer together with cationic charges could further contribute to the success of the polymer itself, providing a potential synergistic action against microbial biofilms.

Since HTy is poorly available and expensive, Tyrosol (Ty), an abundant phenolic compound also present in olive oil, was used as a precursor molecule. Particularly, an acrylic monomer containing Ty was synthesized and used for copolymerization. Later, the catecholic function was inserted by a patented chemo- and regio-selective aromatic hydroxylation [32].

To obtain copolymers with various amphiphilic balance, different molar ratios between the two monomers were used. After physical characterization, cytotoxicity of the copolymers was assessed by using the FMC 74 cell line while their hemolytic activity was monitored by determining the hemoglobin release from human blood cells (RBCs). The antioxidant activity of the copolymers was evaluated by both direct reaction with free radicals (2,2-Diphenyl-1-picrylhydrazyl as anionic radical) and chelation of transition metal (ferrous ions). Finally, the antimicrobial and antifouling properties of the copolymers were assessed against a strain of *S. epidermidis*, that is one of the most involved pathogens in medical device-related infections.

## 2. Materials and methods

### 2.1. Materials

Sodium metabisulphite ($Na_2S_2O_5$) and potassium monobasic phosphate ($K_2HPO_4$) were purchased from Carlo Erba. Acryloyl chloride (Ac) 96% and N, N-diethylethylenediamine (DED) were supplied from FLUKA. Tyrosol (2-(4-Hydroxyphenyl)ethanol, Ty), acrylic acid (AA), hydroquinone, potassium persulfate ($K_2S_2O_8$), *p*-toluenesulfonic acid (pTSOH), sodium dithionite ($Na_2S_2O_4$), Ferrozine, Ferrous sulfate ($Fe_2SO_4$), and Ferrous chloride ($FeCl_2$), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich as were all other solvents and reagents. DMSO $D_6$ 100% were supplied from CIL (Cambridge Isotope Laboratories, Inc). All chemicals were of analytical grade and used as received. 2-iodoxybenzoic acid (IBX) was prepared in the laboratory as described in the literature [33].

### 2.2. Synthesis of AcDED and pAcDED

Syntheses of the acrylate monomer based on N,N-diethylethylendiamine (AcDED) and of the respective homopolymer have been described elsewhere [26]. Briefly, AcDED was synthesized by adding N,N-diethylethylendiamine (0.029 mol) into a solution of acryloyl chloride (0.038 mol) in dimethylcarbonate (DMC, 75 ml, Sigma Aldrich) containing $K_2HPO_4$ (0.08 mol) used as a base [34]. The reaction was carried out for 4 h at room temperature. Then, the solution was filtered to remove the inorganic and organic salt and the monomer was recovered by solvent evaporation (yield ranging from 85 to 90%).

The homopolymer synthesis was carried out at 25 °C for 24 h by using a 1.0 M water solution of AcDED (5 ml) and $K_2S_2O_8$ ($2.8 \times 10^{-4}$ mmol) plus $FeSO_4$ ($2.4 \times 10^{-4}$ mmol) as radical initiators. The resulting polyacrylamide was called pAcDED ($pK_b = 8.61$).

In the previous work [26], several homopolymers of different chain length were obtained by adding sodium metabisulfite ($Na_2S_2O_5$, Carlo Erba) as a chain transfer agent (T) to the synthesis. In this work, the [T]/[AcDED] = 0.1 M ratio was employed obtaining a polymer, herein called pAcDED, with a molecular weight of $94 \times 10^3$ g/mol and a polydispersity index of 1.32.

### 2.3. Synthesis of AcTy

The tyrosol-based acrylic monomer (AcTy) was synthesized by dissolving 0.1 mol of Ty in 75 mL of toluene in the presence of 20 mg of hydroquinone and 750 mg of *p*-toluenesulfonic acid (pTSOH). Then, 0.12 mol of acrylic acid was added and the mixture and the solution were refluxed for 1.5 h in a flask equipped with a Dean–Stark apparatus. At the end of the reaction, the mixture was cooled and treated with sodium bicarbonate (6 g) and water (1.5 mL), until carbon dioxide evolution ended. The mixture was then dried with anhydrous sodium sulfate and filtered. The solvent was removed under vacuum. The reaction yield was assessed by $^1$H NMR.

### 2.4. Synthesis of p(AcDED-co-AcHTy) copolymers

To obtain copolymers possessing different contents of tertiary amines and phenolic groups in the side chain, the two monomers AcDED and AcTy were reacted in different AcDED/AcTy molar ratios (90:10, 80:20, 70:30, 60:40 and 50:50). Particularly, AcDED (in variable quantity according to the chosen ratio between monomers) was dissolved in 3 mL of water and added to an acetone solution (3 mL) containing AcTy. The mixture was kept under continuous stirring until homogeneity. Later, the initiator (potassium persulfate) and the chain transfer agent (sodium metabisulfite) were added and the solution was kept at 80 °C for 2 h to distillate acetone and then at room temperature for 6 h. At the end of the reaction, the copolymer solution (named p(AcDED-co-AcTy), Scheme 1) was dialyzed in water by using a membrane of regenerated cellulose (Spectr/por membrane BIOTECH) with a cut off of

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