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Novel Antimicrobial Polymer Films Active Against Bacteria and Fungi

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We report on the synthesis and the characterization of an azobenzene-based compound. The molecule shows high antimicrobial activity against *Staphilococcus aureus* and *Candida albicans*, as demonstrated by in vitro microbiological test in solution. Two antimicrobial polymeric systems were realized introducing this antimicrobial molecule in two polyolefin matrices. The polymeric materials containing different percentages of azo-compound were mold-casted and the obtained films were tested in vitro against the same Gram-positive bacterium and fungus. The films show biocide activity at low percentage of azo dye (up to 0.01%), holding promise for the fabrication of bacteria-resistant polymer films by means of simple melt processing. POLYM. COMPOS., 00:000–000, 2013. © 2013 Society of Plastics Engineers

INTRODUCTION

Searching for novel antimicrobial drugs is crucial nowadays because an increasing number of microorganisms are developing resistance to the most common antibiotic classes, causing infections that result in prolonged illness and greater risk of death.

Antimicrobial peptides (AMPs) represent archetype host defense effector molecules found within organisms across the phylogenetic continuum. Since the discovery in 1987 by Zasloff [1] of short peptides having antibacterial activities, these AMPs drove an enormous interest. The conventional assumption that AMPs act exclusively as membrane detergents has been shown to be an overly narrow perspective; many of these molecules exert mechanisms of action that appear unique and highly complex. The AMPs' capability to interact with lipid membranes limits the emergence of bacterial resistance, but it is a severe risk of cytotoxicity [2, 3]; moreover, the development of antibiotics based entirely on peptides is limited by their cost and their stability. Because peptides are usually prepared by fermentation, their production cost is very high. The stability issue is a consequence of the instability of polyamide bonds that can be easily cleavaged by numerous enzymes. These two complications limited AMPs to few, mostly topical-use, applications. Therefore, a popular strategy for developing novel antibiotics was to synthesize molecules capable to mimic the AMP self-assembly on bacterial membranes [4]. These materials are more stable in vivo and can have a lower production cost. Our strategy consists in inserting molecules with antibiotic activity in polymer matrices, in order to produce composites having low cost of production, processability, antimicrobial potential, and low cytotoxicity. The final material can be used in all those applications where an intrinsic antimicrobial ability of the material is required, like biomedical tools, antibacterial surfaces, and films for food packaging.

Recently we developed and tested novel antimicrobial agents [5, 6], which exhibit low toxicity and very high activity against Gram-positive bacteria and some fungi. Here, we considered the insertion of one of these molecules in polymeric matrix, addressing chemical issues, microbiocidal and toxicity activities, stability, and processability of the final material. In recent years, several antimicrobial agents have been introduced into polymers [7]; the most common antimicrobial agents used in polymer films are triclosan, chlorhexidine, tetracycline and derivatives, benzophenon, and rifampicin. Most of the work to obtain antimicrobial polymers was done on polystyrene, polyvinylchloride, poly lactic acid or poly (lactide-co-glycolic acid), chitosan, and cellulose. Occasionally, cyclodextrin inclusion compounds were added [7] to stabilize the antimicrobial agents at high polymer-processing temperature. To the best of our knowledge, this is the first time in which a novel azobenzene-based antimicrobial compound was added into polymer films. The choice of the system azo-compound/polyolefin

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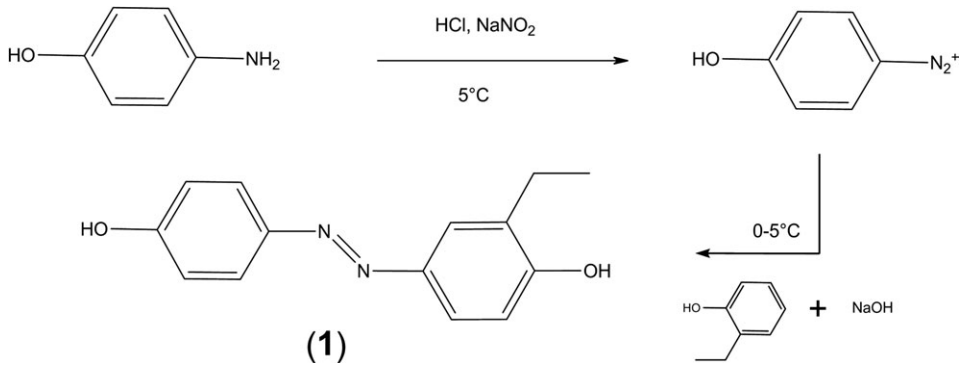

Scheme 1. Synthesis of AZOA1.

permitted the preparation of novel materials by a simple mold-casting technique. Synthesis of polymeric antimicrobial materials was realized by introducing the new azo-molecule with antimicrobial activity in two polymer matrices of polypropylene (PP) and linear low-density polyethylene (LLDPE). The antimicrobial activity of the resulting materials against two demonstrative organisms, a Gram positive (*Staphilococcus aureus*) and a fungus (*Candida albicans*), can be exploited in biomedical field and in the field of antimicrobial active packaging.

EXPERIMENTAL PART

Materials

All reagents and solvents were purchased from Sigma Aldrich and Carlo Erba. Reagents and solvents were used without further purification.

The selected polymer matrices were a PP Moplen RP241H and a m-LLDPE Luflexen 18 TFA, both supplied by LyondellBasell Industries. These polymers have similar densities (PP = 900 kg/m^3 and m-LLDPE = 918 kg/m^3) but different melt flow indexes (MFI of PP at 230°C and 2.16 kg = 1.8 g/10 min; MFI of m-LLDPE at 190°C and 2.16 kg = 3.5 g/10 min).

Synthesis of Azo-Compound

A new compound, named AZOA1, was synthesized according to the classic scheme of diazotization–copulation reaction, as illustrated in Scheme 1.

The procedure for the synthesis of 4'-hydroxy-(4-hydroxy-3-ethyl)-azobenzene (AZOA1) was the following: 2.00 g of p-aminophenol (0.0183 mol) was suspended in a solution containing 16 mL of water and 4 mL of HCl 37% (w/w) and cooled at 0–5°C. Under stirring, a solution of 1.39 g of sodium nitrite (0.0202 mol) in 4 mL of water was added slowly. And stirring was continued at 0–5°C for 20 min. Separately, 20 mL of a basic solution (NaOH 10% (w/v) in water) with 0.0183 mol of 2-ethyl phenol was prepared and added to the first acid solution, under stirring at 12°C. The system was left reacting for 20 min more. Then the final solution was added slowly to 40 mL of a solution of acetic acid 10% (v/v) in water and then stirred for 30 min at 15°C. A dark red precipitate of the azo-compound formed. The crude precipitate was filtered and dried under vacuum at 50°C. The final yield was 40%. The crude product was extracted, crystallized from boiling *n*-octane (500 mL) and dried under vacuum at 70°C. Final crystallization from water/ethanol (10:1) gave pure AZOA1 as gold yellow crystalline material.

Preparation of Polymer Films

To realize flexible and thin active films with antimicrobial activity, we selected two different polymeric matrices of PP (Moplen RP241H) and LLDPE (Luflexen 18 TFA). This PP is commonly used for sheet extrusion, blow molding, and thermoforming processes, whereas the m-LLDPE is involved in cast film processing. The selection was made choosing polymers that can be processed at temperature lower than the temperature of degradation of the azo-compound synthesized and that can be addressed to both rigid and flexible packaging.

Different percentage of AZOA1 molecule (from 0.01 to 5%) were added in bulk to the polymer matrices by mixing at 180°C by a Brabender Do-Corder E 330 for 5 min at 50 rpm. The blended materials were then mold-casted under pressure at 180°C to obtain thin films.

Chemical Characterization

Thermal measurements were performed by a DSC-7 Perkin Elmer calorimeter under nitrogen flow at 10°C/min rate. Thermogravimetric analysis was performed with a TA Instruments SDT 2960 apparatus, in air at 20°C/min. ^{1}H NMR spectra were recorded with a Brucker DRX/400 Spectrometer. Chemical shifts are reported relative to the residual solvent peak. UV-visible (UV-vis) absorption spectrum of AZOA1 was obtained using a Perkin Elmer Lambda 900 Spectrophotometer.

Antimicrobial Test: Biofilm Formation and Quantification

Candida albicans cells were resuspended in 200 μL RPMI Medium 1640 at 10^6 cells/mL, seeded in a flat-bottom

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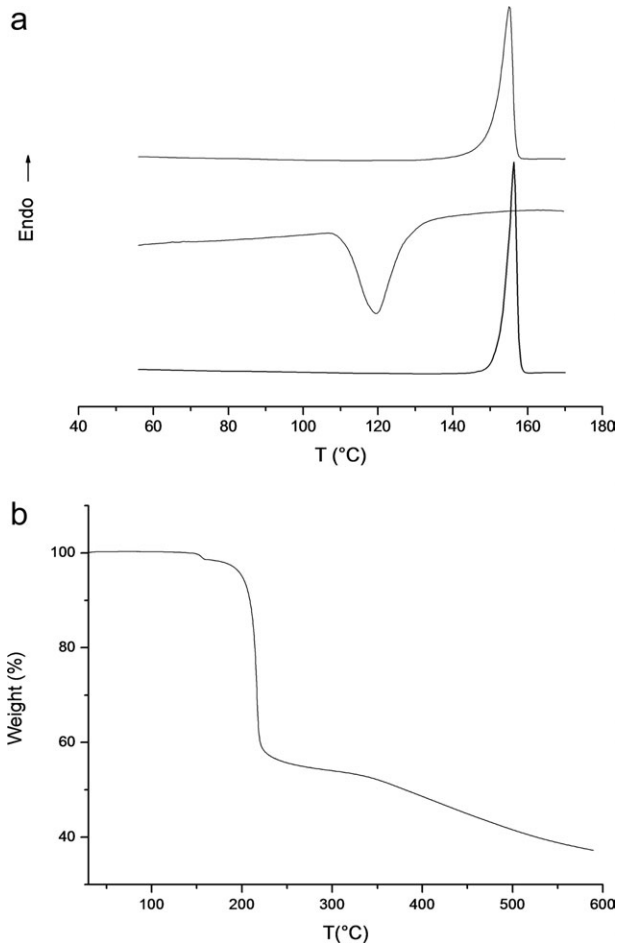

FIG. 1. (a) DSC analysis on AZOA1. From bottom to top: first heating run, cooling run, second heating run. (b) TGA curve of AZOA1.

96-well plate for 12 h at 37°C. After *Candida* biofilm formation, serial dilutions (8, 10, 15, 20, and 30 μg/mL) of AZOA1 were added to each well and incubated 8 h at 37°C. The biofilm degradation was visualized using an XTT assay, which characterizes living biomass of AZOA1 compound. Nonadherent cells were removed by washing the biofilms three times with 200 μL of sterile phosphate-buffered saline (PBS). Biofilm was quantified by the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay [8]. Briefly, XTT (Sigma-Aldrich, Milan, Italy) was dissolved in PBS at a final concentration of 1 mg/mL. The solution was filter-sterilized using a 0.22-μm-pore-size filter and stored at -70°C until required. Menadione (Sigma-Aldrich) solution (0.4 mM) was also prepared and filtered. Before each assay, XTT solution was thawed and mixed with menadione solution at a volume ratio of 20:1. The XTT–menadione solution (250 μL) was then added to each well. The microtiter plates were then incubated in the dark for 1 h at 37°C. Following incubation, 250 μL of the XTT–menadione solution was recovered and centrifuged (to eliminate interference of cells with colorimetric readings); 100 μL of the solution was transferred to new wells; and the color change resulting from XTT reduction was measured at 490 nm with a microtiter plate reader (LAB system multiscan EX). Each assay was performed three times.

The same protocol was used for the *S. aureus* biofilm degradation. *S. aureus* cells ($\sim 10^4$–10^5 CFU/mL) were grown in a 96-well microtiter plate at 35°C. After biofilm formation, serial dilutions (8, 10, 15, 20, and 30 μg/mL) of AZOA1 were added to *Staphylococcus* biofilms and incubated 8 h at 37°C.

For crystal violet staining, the wells were rinsed with water to remove loosely adherent cells and then stained for 1 min with 200 μL of Gram's crystal violet. The wells were then rinsed with water and dried. The amount of biofilm biomass was quantified by destaining the wells with 200 μL of 33% acetic acid and then measuring the absorbance of the crystal violet solution in a microplate spectrophotometer set at 595 nm.

The same conditions were used for the formation of *Candida* or *Staphylococcus* biofilms on PP/AZOA1 films, which were washed to remove nonadherent cells and afterward stained with crystal violet solution 0.3% (w/v). In particular, for *S. aureus*, films were washed, dried, and the weight compared to the pure matrix.

RESULTS AND DISCUSSION

Chemical Characterization

Compound AZOA1 was characterized using thermal analysis, UV-vis absorption spectroscopy, and ^{1}H NMR measurements. The proton resonance data are in agreement with the expected values: (DMSO-d_6), δ (ppm) = 7.77 (d, 2H); 7.61 (s,1H); 7.58 (m, 1H); 6.95 (d, 2H); 6.93 (m, 1H); 2.64 (m, 2H); 1.20 (t, 3H).

DSC traces show a melting temperature of 156.3°C (ΔH_m = 145 J/g) in the first heating run, a broad crystallization peak around 120°C in the cooling run, and a

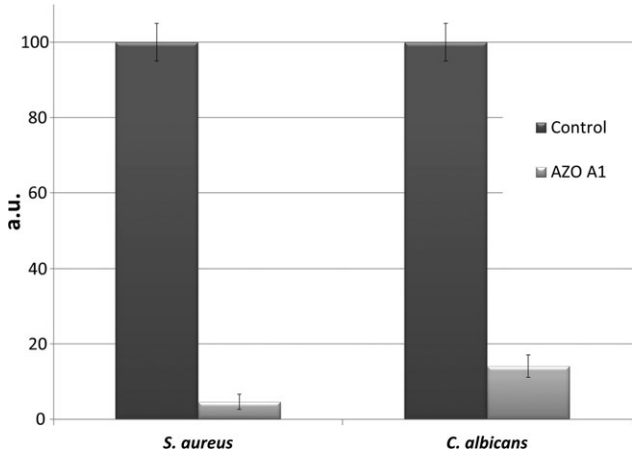

FIG. 2. Antimicrobial activity of AZOA1 (20 μg/mL) against preformed biofilms of *S. aureus* and *C. albicans*. The *y*-axis represents the optical densities (OD) after normalization (considering the OD from the biofilms treated with only vehicle as 100%), hence in arbitrary units (a.u.).

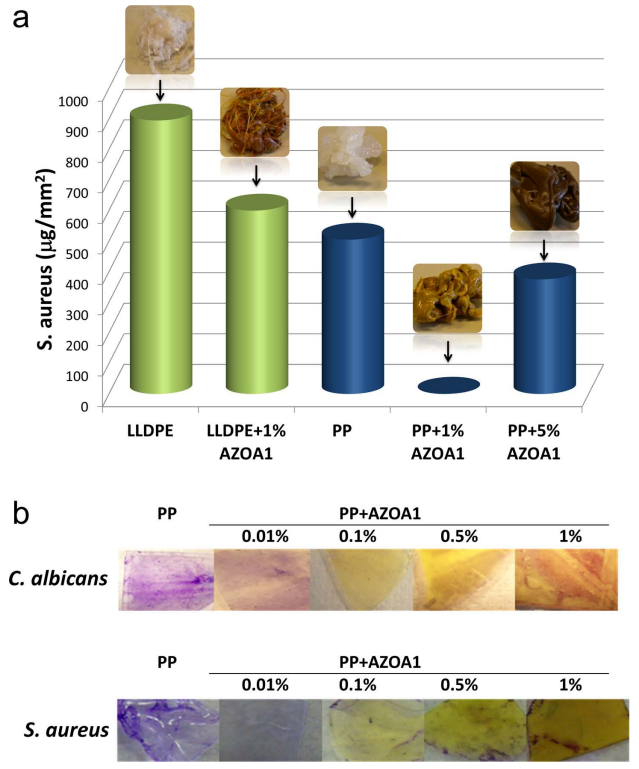

FIG. 3. (a) Antimicrobial activity of PP/AZOA1 and LLDPE/AZOA1 films against *S. aureus*; (b) biofilms formation on PP/AZOA1 films. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

second melt at 155.0°C (ΔH_m = 134 J/g). The broad second melting peak can be attributed to the different degree of crystallization obtained from the melt (Fig. 1a).

The thermogravimetric analysis highlights a first 5% weight loss in the range 150–200°C (Fig. 1b). To avoid the degradation of the molecule, the operating temperature in the mold-casting process must be kept below 200°C.

UV-vis absorption spectrum was recorded at 25°C in $CHCl_3$ solution. The spectral region 650–240 nm was investigated using a cell path length of 1 cm. Chromophore AZOA1 concentration of 4.6×10^{-5} mol/L was used. The UV-vis absorption spectrum showed in the 240–650 nm region a broadband centered around 370 nm (ε_{max} = 29,500 L mol^{-1} cm^{-1}), related to the $\pi \rightarrow \pi^*$ electronic transition of the azobenzene chromophore in *trans* configuration.

Microbiological Characterization

Microbiological tests were performed on AZOA1 to study its antimicrobial activity. In Fig. 2, we evaluated the ability of AZOA1 (20 µg/mL) to destroy preformed biofilms of *S. aureus* (a Gram-positive bacterium) and *C. albicans*, a dimorphic fungus, causal agent of opportunistic oral and genital infections in humans. At this concentration, AZOA1 was able to destroy about 85% of preformed *Candida* biofilm and more than 95% of preformed *Staphylococcus* biofilm, compared with the same biofilms treated with only vehicle (dimethyl sulfoxide).

Antimicrobial activity of the films was tested against *S. aureus* (Fig. 3a). These preliminary tests showed that in the case of PP films at 1% of AZOA1, the formation of *S. aureus* colonies on the polymer surface is completely inhibited. At 5% of AZOA1, the active system starts to decompose, as was evident from the dark color of the produced films (see Fig. 3). Further analysis is underway, aimed at gaining a better understanding of the system behavior. For LLDPE matrix the films surface was quite rough, which could explain the different behavior of this material during the microbiological tests. Similar to the PP system, the active LLDPE film at 5% of AZOA1 also shows high degradation; therefore, the microbiological results were not reported in this work.

We also analyzed the degradation of preformed biofilms of *Staphylococcus* and *Candida* on the active PP-based films, by staining with crystal violet. The destruction of biofilms is indicated by the absence of violet coloration. Figure 3b shows that at concentrations of AZOA1 higher than 0.01% in PP films there is a complete destruction of biofilms, whereas for the film AZOA1/PP 0.01% (w/w) both biofilms are only partially destroyed.

CONCLUSION

This work presents some innovative antimicrobial systems, consisting of an antibiotic azo-dye blended in two different polymer matrices. The films, prepared via mold-casting, exhibit antimicrobial activities and the capability to degrade preformed biofilms of *S. aureus* and *C. albicans* at concentration lower than 0.1% (w/w). Concentration of 0.01% (w/w) permits the preparation of uncolored and transparent polymers with potential applications for food packaging. Remarkably, the concentration described here is lower than typical concentrations reported in literature [7]. The results presented show a viable synthetic route and protocol for the preparation of films with high antimicrobial activity against two typical models of bacteria and fungi.

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