

ORIGINAL ARTICLE

Evaluation of the antimicrobial activity of novel composite plastics containing two silver (I) additives, acyl pyrazolonate and acyl pyrazolone

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Summary. *Background:* Public health systems today face the dual challenges of controlling infections and curbing the increase in antimicrobial resistance manifested in drug-resistant microorganisms in hospitals and elsewhere. In the last ten years, research has been conducted to develop new materials with antimicrobial properties to be used in medical devices, increasingly found to harbour critical nosocomial infections. *Methods:* Two next-generation composites using the antimicrobial qualities of silver were tested against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* with the purpose of evaluating their antimicrobial and anti-fungal activity. These tests applied the standardized method according to ISO-2216: Plastics-Measurement of Antibacterial Activity on Plastics Surfaces. Testing was carried out using polyethylene (PE) enriched with AgNO₃ as a positive control and PE as a negative control. *Results:* The antimicrobial activity of the composites proved to be between medium (bacteriostatic) and very good (bactericidal). In particular, PE2 showed the highest scores against all microorganisms, with values ranging from good to very good. Instead, PE1 had lower scores, with a value of medium for *Escherichia coli* and slight for *Candida albicans*. Statistical analysis carried out with the t-test for unpaired data showed a statistically significant difference between the positive control and the other polymers ($p < .0001$). *Conclusions:* Based on our findings, we conclude that the test, conducted to ISO-2216 standards, could be extended to include fungal strains and that the new composites could be used to produce antimicrobial surfaces for medical devices, for example, intubation tubes, urinary catheters, vascular prostheses, and mechanical heart valves. This would reduce the risk of microbial contamination and biofilm formation, ensuring better health outcomes for patients treated with these devices. Further testing should be done to evaluate potential future applications of these composites and the possibility of adding fungal strains to the ISO-2216 standard. (www.actabiomedica.it)

Key words: antimicrobial resistance, silver composites, nosocomial infections, antimicrobial activity

Introduction

Even today, infectious diseases, especially in hospitals, continue to present a major public health problem with socio-economic and cultural consequences (1-5). Even though the standards of health care and

medical technology have risen significantly, the World Health Organization (WHO) declared that infections occur in 15% of patients under medical care in hospital, particularly in Intensive Care Units (ICUs) (2,6).

In 2015, the European Center for Disease Control reported that 8.3% of hospitalized patients in

intensive care for more than two days acquired at least one healthcare associated infection (HAI). Specifically, 6% of patients were affected by pneumonia, 4% by bloodstream infections (BSI), and finally 2% by urinary tract infections (UTIs) (7). Intubation is the cause of infection in 97% of patients, catheters have been associated with 43% of BSIs, and urinary catheters with 97% of UTIs (7). Regarding the most common bacteria, *Pseudomonas aeruginosa* was identified in pneumonia episodes, *Staphylococcus spp* in bloodstream infections, and *Escherichia coli* in urinary catheter infections. In fact, the surfaces of medical devices that remain in place for days provide a substrate for biofilm production and the growth of these microorganisms (4-7). In the last decade, *Candidemia* has also been defined as a severe and often life-threatening infection. *Candida* strains can cause invasive candidiasis (IC) in tertiary care hospitalized patients and also in catheter-associated urinary tract infections (CAUTI) (11,12).

Hospital-acquired infections can be caused by contamination due to incorrect conduct on the part of healthcare workers, for example, failure to wash their hands properly, to store food at the correct temperature, or prepare it in properly hygienic conditions (11-14). This superficial attitude can be attributed to the high levels of stress to which these professional categories are subjected and the speed required for the performance of their duties (15-24).

Another factor in the spread of infections is the public's limited awareness and understanding of the proper use of medicines. Better education about this would improve the effectiveness of treatment and reduce costs for patients and society, thus avoiding risks for health and the waste of resources (25, 26). One of the first steps to take in encouraging the correct use of medicines is to improve the public's health literacy, defined by the WHO as "the cognitive and social skills which determine the motivation and ability of individuals to gain access to, understand and use information in ways which promote and maintain good health" (27,28). One area in particular to be improved is the understanding of the medical terminology commonly used in manuals or package inserts.

Furthermore, in the last decade the over-prescribing of antibiotics or their misuse by patients has increased the risk of hospital infections caused by drug-

resistant microorganisms (29). The WHO has recorded an increase in antimicrobial resistance (AMR) among clinical bacteria and has defined this phenomenon one of the most critical threats for human health, in particular in vulnerable patients (29).

At the same time, the European Center for Disease Control highlighted the very rapid increase of infections caused by bacteria like carbapenemase-producing Enterobacteriaceae (CPE) in patients hospitalized in Italian and in European institutions from 2014 to 2017 (30-32). Therefore, public health services are facing a double challenge: controlling infections and limiting AMR diffusion (33, 34).

As part of the efforts to tackle these problems, researchers have been developing polymers with antimicrobial activity, and in the last decade, a new group of materials including antimicrobial peptides (AMPs), cationic synthetic polymers, and nanoparticles has been created (7, 35). In particular, antimicrobial-bio-compatible polymers have been developed for medical devices in order to prevent the formation of biofilms and the insurgence of nosocomial infections, and as such provide an innovative method to combat these problems that is both gentle and safe. Two types of antimicrobial polymers can be used for the production of medical devices: polymers that already exhibit antimicrobial activity and polymers that are modified to confer antimicrobial properties (10).

In particular, among the wide range of existing antimicrobial plastics, metal-polymer-nano composites and in particular silver-polymers are the subject of increasing interest (36,37). Silver compounds are known to show strong antibacterial activity towards a broad spectrum of bacteria, and the interest in silver derivatives and their potential application as antimicrobial agents has led researchers to investigate different classes of ligands in order to obtain novel silver(I) complexes. The aim of this study was to evaluate possible antibacterial and antifungal activity of new silver-containing polymers (I) synthesized by the Chemistry Department of the University of Camerino (UNICAM), specifically as a potential antibacterial and antifungal agent to embed in plastics. The results obtained against *Escherichia coli* and *Staphylococcus aureus* were compared with the results obtained against *Candida albicans*.

Methods

1. Novel composites containing silver (I) acylpyrazolonate additives

The polyethylene composite materials named PE1 and PE2 were prepared, by the Chemistry Department of UNICAM. PE1 was obtained by mixing, at 150 °C, the complex $[Ag(Q^{fb})]_n$ with polyethylene in a 1:1000 weight ratio, the final product was shaped into 50x50 mm square (10 mm thick). PE2 was similarly obtained by using the complex $[Ag(Q^{cy})]_n$ which differs only for the R substituent (Figure 1). The detailed preparation procedure and the complete characterization have been previously reported (39, 40).

The antimicrobial action of the composite materials PE1 and PE2 has been tested against two microbial strains (*E. coli*, *S. aureus*) and one fungal strain (*C. albicans*).

Molecular structures of silver(I) acylpyrazolonate additives 1 and 2, respectively used in the composite polyethylene materials PE1 and PE2.

2. ISO-2216: Plastics-Measurement of Antibacterial Activity on Plastics Surfaces

The antimicrobial activity by contact exerted by each of the PE composites was measured according to ISO standard-2216 (*ISO-22196: Plastics – Measurement of Antibacterial Activity on Plastics Surfaces*) (38). Square pieces of the composites measuring 50x50 mm (10 mm in thickness) were tested against two bacteria

strains and one fungal strain in triplicate: (Gram-positive) *Staphylococcus aureus* ATCC 25923, (Gram-negative) *Escherichia coli* ATCC 25922 and the fungal strain *Candida albicans* ATCC 24433. Unloaded PE samples were used as negative control; PE loaded with $AgNO_3$ was used as positive control. The appropriate culture medium was inoculated with the test microbes and cultivated for 24 h at $35 \pm 1^\circ C$ under aerobic conditions to achieve the concentration of 10^7 CFU/mL. Bacterial suspensions (0.4 mL) were inoculated onto the test surface (in triplicate) and the inoculums were covered with a piece of polyethylene film (40x40mm), gently pressed down to spread the inoculum to the edges. The Petri dishes containing the inoculated test specimens were incubated at $(35 \pm 1)^\circ C$ with a relative humidity of no less than 90% for 24 ± 1 h. After the incubation time, the inoculum was processed by adding 10 mL SCDLP broth (Soybean casein digest broth with lecithin and polyoxyethylene sorbitan monooleate). From the SCDLP broth, tenfold serial dilutions were made in phosphate-buffered physiological saline (PBS-saline) and aliquots of 1 mL for each dilution were placed in Petri dishes, and 15 mL of plate count agar (PCA) was added to disperse the bacteria. The inverted Petri dishes were incubated at $(35 \pm 1)^\circ C$ for 48 h. After incubation, the number of colonies in the Petri dishes was counted. The number of bacteria surviving on the specimens tested was compared to the number of colonies present on the negative controls. Antimicrobial performance (R) was determined according to the Japanese industrial standards method (*JIS L-1902: 2002, Testing Method for Antibacterial Activity of Tex-*

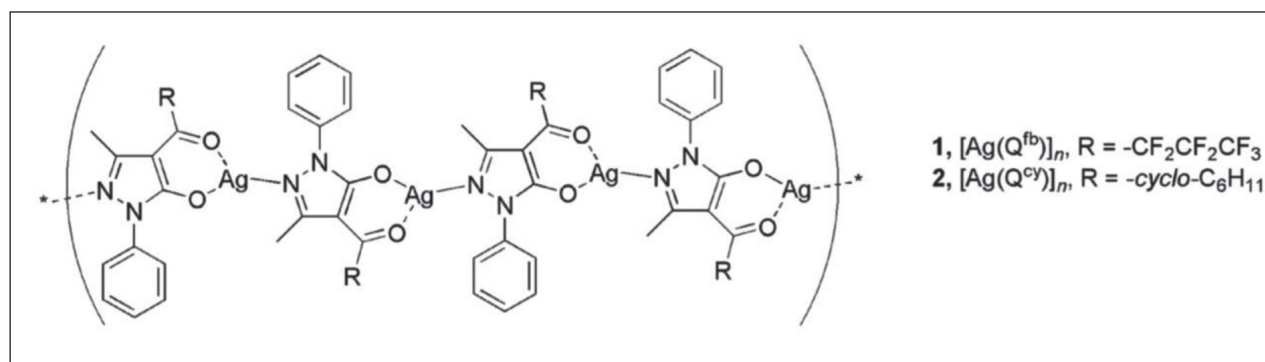


Figure 1.

tiles) (41) and based on the following classification: no antimicrobial activity = ≤ 0.5 log microbial growth reduction (<68.4% reduction); slight antimicrobial activity = 0.5–1 log microbial growth reduction (<68.4% to <90% reduction); medium antimicrobial activity = > 1 to ≤ 2 log microbial growth reduction (90% to <99% reduction); good antimicrobial activity = 2 to <3 log microbial growth reduction (99% to <99.9% reduction); very good antimicrobial activity = > 3 log microbial growth reduction (>99.9% reduction). In addition, the % of reduction was interpreted for bacteria strains in terms of the bactericidal activity (>99.9% of inoculum reduction) or bacteriostatic activity (90 to 99.9% of inoculum reduction) of each composite.

3. Statistical analysis

To verify whether there may be a difference between the resilience and coping values before and after rescue, the unpaired t-test was used and data was processed with the XLstat software (*XLSTAT. Statistical software and data analysis add-on for Excel. Addinsoft (2017)*) (42).

Results

To verify the antimicrobial activity of the loaded composites by contact, the results of the experiments were elaborated according to the JIS L-1902:2002: *Testing Method for Antibacterial Activity of Textiles* (41), applying the standardized method (ISO-2216) (38). After an incubation of 24h, the data obtained relating to microbial charge found on different types of composites was transformed into logarithmic units to calculate antimicrobial performance as Log reduction (R value). The evaluation of bactericidal, bacteriostatic and antifungal activity provided us with much more detailed information about the behavior of the substances tested against two microbial and a fungal species.

In general, an inhibition of microbial growth on the contact surface between the PE composite squares and the inoculums of the microorganisms was revealed.

Our data show that within 24h of exposure, both PE1 and PE2 exhibited antimicrobial activity against

both bacteria, ranging between a medium and very good level, in line with the behavior shown by the positive control (PEAgNO₃). Tested against *C. albicans*, PE1, unlike PE2, showed only slight activity, measuring between 68% and 90%. The positive control (PEAgNO₃) achieved a medium-good antibacterial performance against all bacteria strains.

In detail, tested against *E. coli*, composites PE1 and PE2 show bacteriostatic activity (<99.9%), with PE2 performing better than PE1 (Figure 2). In fact, PE1 achieved an R value from 90% to <99%, corresponding to good antimicrobial performance. The R value of PE2, instead, was within 99% to 99.9%. In addition, compared with the positive control (PEAgNO₃), PE1 achieved only 1.59 log microbial growth reduction, whereas PE1 showed a value of 2.81 against the 2.21 reached by PEA_gNO₃ (Figure 2).

Tested against *S. aureus*, composites PE1 and PE2 achieved very good antimicrobial performance, corresponding to bactericidal activity largely exceeding a log value of 3. Instead, PEA_gNO₃ showed only a medium antimicrobial performance, with a range of antimicrobial performance from 90% to <99% (Figure 3).

Tested against *Candida albicans*, only PE2 achieved good antimicrobial performance. PE1 showed only slight antimicrobial activity. The observation that PE2 and PEA_gNO₃ had similar activity suggests that the ISO standard test can be used to test new polymers against fungi (Figure 4).

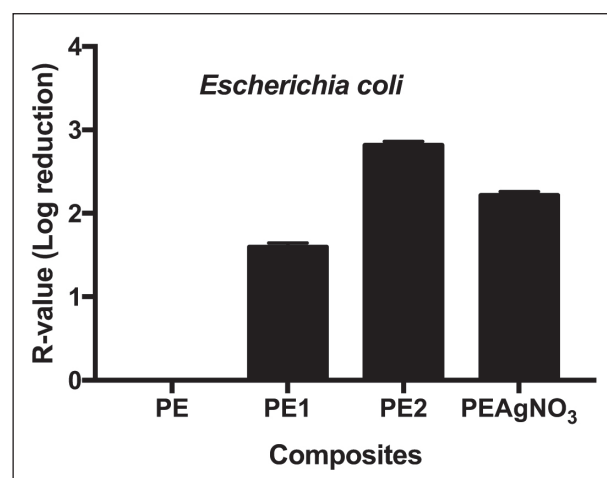


Figure 2. R-values obtained for *Escherichia coli*; unpaired t test of PE1 vs PAgNO₃ was $t=131.5$ with a ***p value < .0001, and of PE2 $t=127.3$ with a ***p value < .0001

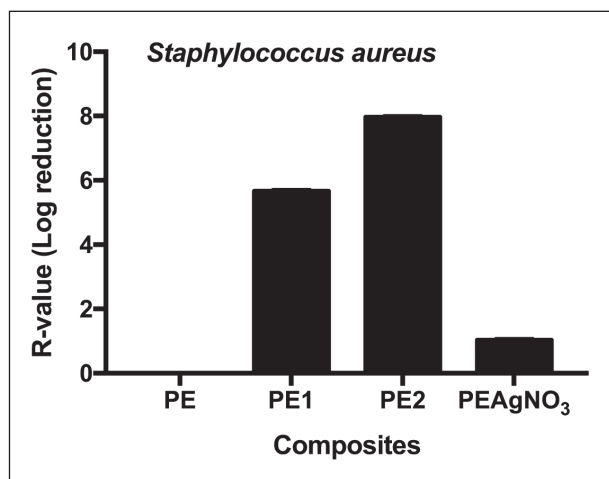


Figure 3. R-values obtained for *Staphylococcus aureus*; Unpaired t test of PE1 vs PAgNO₃ was $t=983.6$ with a ***p value < .0001, and of PE2 $t=1041$ with a ***p value < .0001

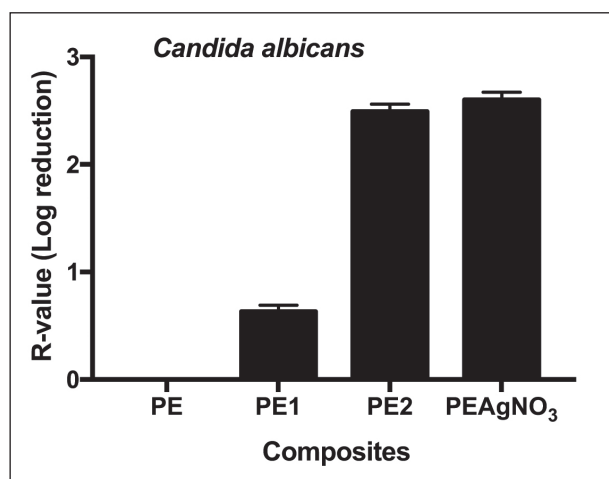


Figure 4. R-values obtained testing PE, PE1, PE2 and PAgNO₃ against *Candida albicans*; unpaired t test of PE1 vs PAgNO₃ was $t=575.29$ with a ***p value < .0001, and of PE2 $t=26.99$ with a ***p value < .0001

Discussion

All experiments performed on the composites against two bacterial and one fungal strain in accordance with the ISO 22196:2007 (38) confirmed the antimicrobial and antifungal activity of all polymers tested. In general, the results showed that the composites embedded in PE maintain the broad-spectrum activity of silver, targeting both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria and the *Candida albicans* fungus.

The positive results of the contact test, [standardized by the ISO 22916:2007 (38)], against two bacteria strains and also against a fungal strain (*Candida albicans*) suggest that these composites can be used against fungal strains as well. In depth, analysis of the results indicates some differences between the antimicrobial activities displayed by the various PE composites. The most important result was recorded for PE1, which showed very good antimicrobial performance measured as bactericidal activity against *Escherichia coli* and *Staphylococcus aureus*, and the highest performance (good) against *Candida albicans*. Even though PE1 showed only medium and slight performance against *Candida albicans* and *Escherichia coli* respectively, it performed very well against *Staphylococcus aureus*.

The higher performance of PE1 may indicate that the mechanism of interaction between this composite and the bacteria cell is stronger than that of the PE2 composite. The silver in the structure of polymer 1 probably exhibits stronger antimicrobial activity. In fact, the hypothesis that silver may act against microorganisms (43) is based on the observation that the oxidized form of silver (Ag^+) can bind strongly to thiols, phosphates, and other electron donating functional groups, in an interaction that disrupts the microbial cell membrane, causing leakage of cellular contents and finally cell death. A consequence of this interaction is the disruption of the cell membrane, followed by the leakage of cellular contents and finally microbial cell death (44).

Our results confirmed the possible application of the novel polymers to control nosocomial infections and AMR. In fact, new polymers characterized by antimicrobial activity are being investigated for use as materials for medical devices (45). In particular, silver has been widely used as an alternative antimicrobial agent in medical devices such as vascular prostheses, urinary catheters, and mechanical heart valves, and the results obtained in the present research confirm this application (46,47).

In addition, this feature is useful in that the various PEs can be used in place of plastic composites of AgNPs, in order to avoid the negative side effects of the latter, such as silver release and harmful environmental impact. "Contact action by polymer/polymer composites," achieved by embedding insoluble silver

(I) coordination polymers in a polymeric matrix offers a new concept in the field of plastics endowed with permanent antimicrobial activity. Thus our composite plastics may prove useful in a number of different situations in which the accumulation of unwanted material is often overlooked.

Conclusions

In the last decade, there has been increased research to develop new antimicrobial polymeric materials free of negative effects on human health. Study of the mechanisms of interaction between such polymers and microorganisms should move forward with the use of standardized protocols. For this reason, we tested the antimicrobial and antifungal activity of two silver (I) coordination polymers, applying the standard methodology reported in ISO-22196: Plastics – Measurement of Antibacterial Activity on Plastics Surfaces. For future clinical applications, it will be important to test the composites against clinically isolated microbes, especially multidrug-resistant strains, and evaluate the *in vitro* and *in vivo* biocompatibility of composites.

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Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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