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Antibacterial activity of copper-containing clinoptilolite/PVC composites toward clinical isolate of *Acinetobacter baumannii*

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Abstract: The multidrug-resistant bacteria *Acinetobacter baumannii* cause serious hospital infections. Commercial poly(vinyl chloride) (PVC) used for endotracheal tubes was modified in order to obtain a composite with an antibacterial effect towards a clinical isolate of *A. baumannii* ST145. The composites were prepared by addition of different amounts of copper-containing zeolite tuff (CuZ) and by successive impregnation with D-tyrosine (D-Tyr) solution. The composites that were obtained by addition of CuZ (CuZ–PVC) only did not exhibit an antibacterial effect. The impregnation of the CuZ–PVC by D-Tyr resulted in an antibacterial effect which was explained by a synergistic effect of CuZ and D-Tyr. Rheological tests confirmed that the modification of PVC by CuZ does not affect its processability and reformability.

Keywords: natural zeolite; multidrug resistance; polymers; poly(vinyl chloride); amino acids.

INTRODUCTION

Worldwide, there has been a growing demand for the design of novel antimicrobial materials for biomedical applications. Most of the reported materials exerting antimicrobial activity were based on a suitable matrix in which metals, metal oxides, or novel engineered nanoparticles were incorporated.[1–3] These antimicrobial agents interact with microbial cells through variety of mechanisms. The interactions include protein dysfunction and loss of enzyme activity, production of reactive oxygen species, influence on membrane function, interfere with nutrient transport or exert genotoxicity.[4]

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Recently, naturally occurring minerals, such as clays and zeolites, have attracted great attention considering their possible use as antimicrobial agents in a variety of applications.[5–8] The negatively charged aluminosilicate lattice of zeolites has a unique possibility to host different metal cations, allowing their controlled release into the environment and, accordingly, preventing the metal ions from causing a concentration-dependent toxicity in the environment.[9]

Bacterial infections in hospitals are frequently caused by pathogenic bacteria species hosted in the ventilation systems and on the surfaces of medical devices.[10] *Acinetobacter baumannii* usually cause nosocomial infections in intensive care units.[11,12] These Gram-negative bacteria show resistance to most common antimicrobial agents and survive for a long period in the environment.[13,14] *Acinetobacter* spp. colonizes medical devices for mechanical ventilation (endotracheal tubes)[15] commonly produced from thermoplastic polymers. Accordingly, great effort has been expended in the modification of polymers to inhibit bacterial attachment. Composites based on different polymers were prepared mainly with Ag particles,[16] but also with various metal oxides.[3,17] It should be noted that although the antimicrobial effect of Ag is the most applicable and efficient, the main advantages of metals such as copper or zinc over silver is in their physicochemical stability and cost benefits. Recently, the antimicrobial effects of copper and zinc were investigated and both the metals and their oxides were found to be promising candidates for disinfectants. The metal ions and their oxides exhibited antibacterial efficacy towards *Escherichia coli* and *Staphylococcus aureus*.[6,18]

Furthermore, it was recently reported that some amino acids, in particular D-tyrosine (D-Tyr), can prevent the formation of the biofilm formed by some pathogenic bacteria.[5,19]

Considering these facts, two types of poly(vinyl chloride) (PVC) composites were prepared and their antibacterial activity towards a clinical isolate of *A. baumannii* ST145 investigated. The first composite, denoted as CuZ–PVC, was prepared by adding different amounts of copper-containing zeolite tuff (CuZ) into the PVC matrix and the second type, denoted as D-Tyr–CuZ–PVC, was obtained by impregnation of the CuZ–PVC composite with D-Tyr. Dynamic and mechanical analyses were performed on the CuZ–PVC composite in order to test the reformability of the PVC matrix.

EXPERIMENTAL

Preparation of the composites

Natural zeolite tuff (Z) obtained from the Zlatokop Mine, Vranjska Banja deposit, containing 73 wt. % of clinoptilolite, was enriched by copper according to a previously reported procedure.[6] The copper-loaded zeolite (containing 1.54 wt. % Cu) was added into a solution containing 1 g of PVC in 20 cm^3 tetrahydrofuran (THF). CuZ was added in different amounts (5–15 wt. %). The suspensions were firstly homogenized with magnetic shaker (RTC

standard, IKA for 2 h at 500 rpm) and then intensively by an Ultra Turrax mixer (IKA T18 Basic at 8000 rpm for 10 minutes). The composites CuZ5–PVC, CuZ10–PVC and CuZ15––PVC (number denotes CuZ percentage in the composite) were obtained by drying for 72 h at 25°C, than in vacuum oven (0.4 kPa, 25 °C) for 6 h and finally cut into small squares (1 cm^2, thickness around 350 μm). The composites were submerged in 70 wt. % ethanol for 10 min and dried at 37 °C until the ethanol had evaporated completely.

Impregnation of the composites with D-Tyr was performed by immersing the CuZ–PVC into a solution of D-Tyr (100 mg dm^{-3}), following the incubation in a temperature programmed water bath at 37 °C for 16 h with shaking at 150 rpm. The composites D-Tyr–CuZ5––PVC, D-Tyr–CuZ10–PVC and D-Tyr–CuZ15–PVC were washed with phosphate buffer solution (PBS, pH 7.2). The sterility of both types of composites was checked by placing the composites into nutrient agar followed by incubation at 37 °C for 24 h. No microbial contamination of the composites was observed.

Antibacterial activity test

The antibacterial activity was examined toward a clinical isolate of *A. baumannii* ST145 deposited at the University Hospital Centre, Split, Croatia. *A. baumannii* was first pre-grown on a blood agar for 16 h at 37.0±0.1 °C to obtain the culture in the log phase of growth. The bacterial biomass was then suspended in PBS (initial concentration of the suspension was around 10^8 CFU cm^{-3} (colony forming units per cm^3)). The tubes were left to incubate in the dark for 24 h at 37.0±0.1 °C without stirring. The number of planktonic bacteria was determined at beginning of the experiment and after 24 h of contact. 0.1 cm^3 of the original sample or serially diluted sample (10^{-1}–10^{-8}) was inoculated (by the spread plate method) onto the nutrient agar. The plates were incubated for 24 h at 37.0±0.1 °C. The number of immobilized *A. baumannii* cells was determined after 24 h of contact. The immobilized cells were separated from the composites by vortex treatment (45 Hz/5 min). The number of bacteria was determined and presented as CFU cm^{-3} or CFU cm^{-2}.

In order to check antibacterial activity of D-Tyr itself, an experiment with D-Tyr at a concentration of 100 mg dm^{-3} was performed. The statistical analysis was realised using Statistica Software 10.0 (StatSoft, Tulsa, OK, USA). The number of planktonic and immobilized cells was logarithmically presented in order to equalize the variances of the measured parameters. The comparisons between composites were performed using one-way analysis of variance (ANOVA) and subsequently the *post hoc* Duncan test was performed for the calculation of pair-wise comparisons. Statistical decisions were made at a significance level of $p < 0.05$. Leaching of copper ions from CuZ15–PVC in PBS solution was tested after 24 h of the experiment by measuring the Cu concentration in filtrate by atomic absorption spectroscopy (SpectrAA 55B, Varian).

Mechanical and rheological testing

Dynamic rheological data, *i.e.*, the dynamic storage modulus (G'), the loss modulus (G''), the phase angle (tan δ) and the complex dynamic viscosity (η^*) of PVC and the CuZ–PVC samples were obtained using a Discovery Hybrid rheometer HR2 (TA Instruments, New Castle, DE, USA). The rheological properties of molten samples were analysed on pastilles in the dynamic shear mode between two parallel plates (diameter 25 mm; gap 1.5 mm) at three different temperatures: 180, 190 and 200 °C. Frequency sweep scans were conducted from 0.1 to 100 rad s^{-1} and at a strain of 0.5 %. The complex dynamic viscosity of the sample melts obtained at different temperatures were used to calculate the activation energy of flow (E_a),

which was calculated using the relation between viscosity and temperature (Arrhenius equation).

RESULTS AND DISCUSSION

The antibacterial activities of the CuZ–PVC composites are given in Fig. 1. The data on the activity of the composites with Z and D-Tyr–Z are not shown here. In a previous work,[5] it was found that these materials do not exhibit antibacterial activity towards *A. baumannii* ST145.

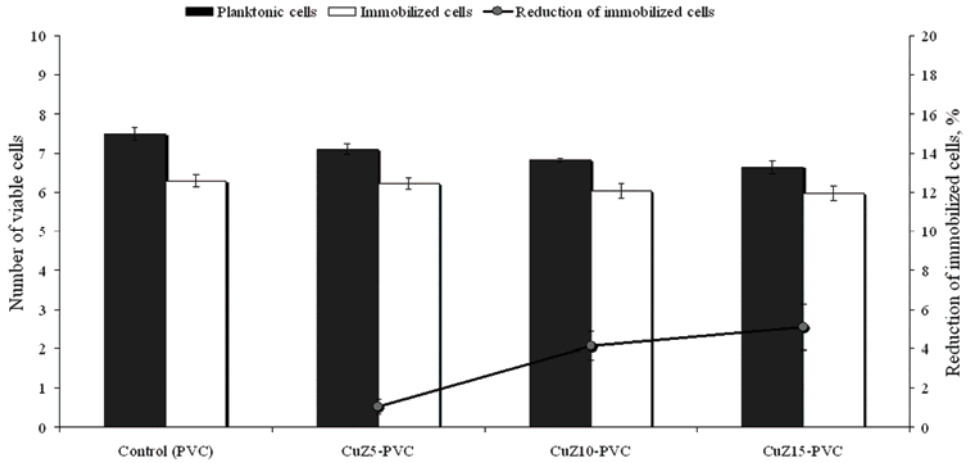

Fig. 1. Antibacterial activity of the control material (PVC) and CuZ–PVC composites after 24 h contact (number of viable cells is expressed as log (*CFU* cm^{-3}) or log (*CFU* cm^{-2}) for planktonic and immobilized cells, respectively). t_0 *A. baumannii* (log *CFU* cm^{-3}) = 7.79±0.20.

The composites containing CuZ in all examined amounts did not exhibit antibacterial activity towards either immobilized or planktonic cells (antibacterial activity was only 5 % reduction of immobilized cells for the CuZ15–PVC composite). This could be explained by the fact that the *A. baumannii* produces a high amount of biofilm which protect the cells of *A. baumannii* from antibacterial agents. The explanation is supported by the antibacterial effect found for D-Tyr––CuZ–PVC (Fig. 2).

The reduction of planktonic cells was not significant, but the reduction of immobilized cells on the D-Tyr–CuZ–PVC composites was evident. There was a slight increasing trend with increasing amount of CuZ in the composites. The reduction of the immobilized cells varies from 10 % (D-Tyr–CuZ5–PVC) to 14 % (D-Tyr–CuZ15–PVC). A one log CFU reduction in the immobilised *A. baumannii* cells on D-Tyr–CuZ15–PVC was achieved in comparison to the control (a commercial endotracheal tube).

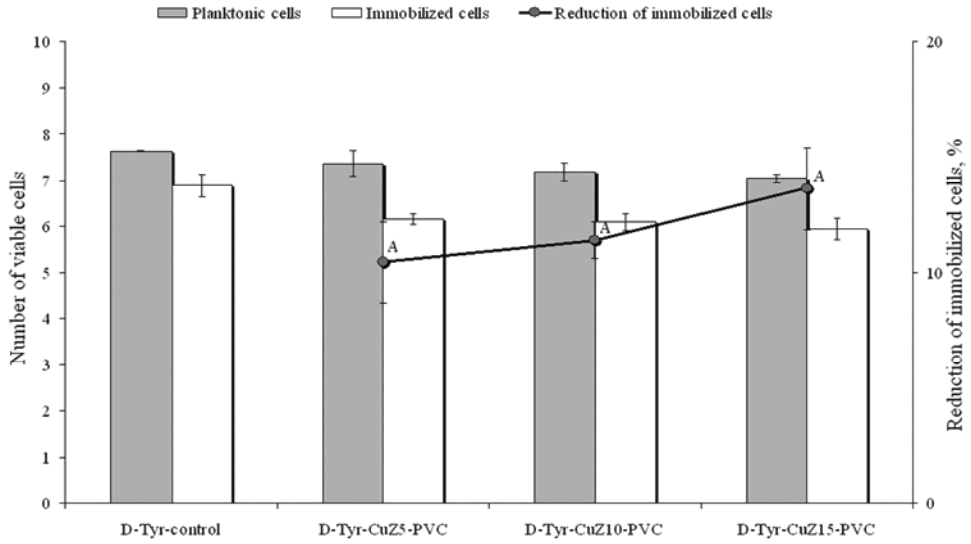

Fig. 2. Antibacterial activity of D-Tyr–PVC (control) and D-Tyr–CuZ–PVC composites after 24 h of contact (number of viable cells is expressed as log (*CFU* cm^{-3}) or log (*CFU* cm^{-2}) for planktonic and immobilized cells, respectively) t_0 *A. baumannii* (log *CFU* cm^{-3}) = 7.72±0.13; A – significant reduction as compared to the control.

D-Tyr showed a repellent activity towards microbial aggregates, including *Amaricoccus macauensis* strain, *Leifsonia* sp., *Microbacterium* sp., *Mesorhizobium* sp., *Burkholderia cepacia*, *Alicycliphilus* sp. and *Acidovorax* sp.,[19] *Staphylococcus aureus*[20] and *Pseudomonas aeruginosa*.[21] In this study, it was found that the antibacterial activity of CuZ15–PVC towards *A. baumannii* develops only in a presence of D-Tyr. Moreover, pH value did not change significantly during the experiments. It increased from 6.70 to 7.20, which is the optimal pH range for growth of *Acinetobacter* spp.[22] This supports the fact that antibacterial effect cannot be ascribed to differences in the pH.

Since antibacterial activity of various metal-containing antibacterial materials has been attributed to leaching of metal ions,[23] the possibility of Cu leaching from the composite with the highest amount of CuZ (CuZ15–PVC) was examined. It was found that less than 1 % of the total amount of Cu present in the CuZ had leached into PBS after 24 h of contact. This indicates that free Cu ions are not responsible for the antibacterial activity. In addition, antibacterial effect of a D-Tyr solution at the concentration used for the impregnation of the composite was examined. No activity of the D-Tyr towards *A. baumannii* ST145 was found. The results obtained in this study show that the mechanism of the antibacterial activity of the prepared composites towards *A. baumannii* includes a synergetic activity of CuZ and D-Tyr. Further work will be directed towards detailed investigations on the mechanism itself.

Finally, in order to check whether the addition of CuZ influences the dynamic mechanical properties of the PVC matrix, the dynamic mechanical properties of the CuZ15–PVC composite and neat PVC in the molten and solid states were compared (Fig. 3).

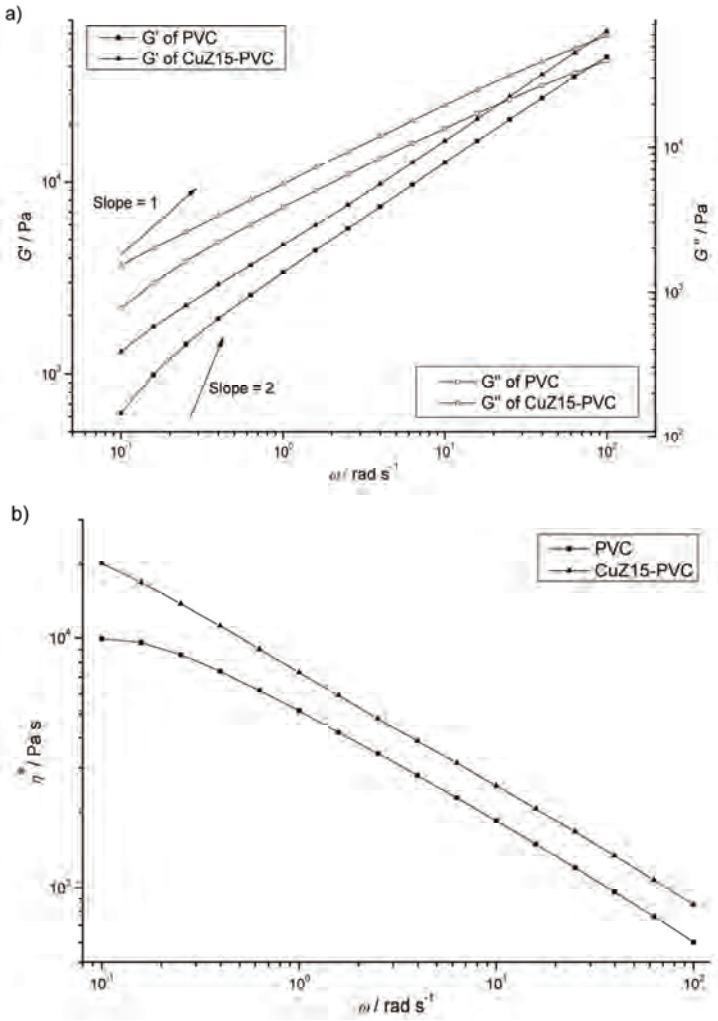

Fig. 3. a) Frequency dependences of the storage and loss modulus and b) the complex viscosity of PVC (control) and CuZ15–PVC at 200 °C.

Considering the higher values of rheological parameters, activation energy of flow (E_a) and reduced slopes of G', G'' and η^* at 0.1 rad s^{-1} ($\eta^*_{0.1}$) and the frequency dependences (ω) at low frequencies (Table I), it could be concluded that the addition of CuZ into PVC improved its rheological behaviour.

TABLE I. Results of dynamic–mechanical analysis

Sample	$\eta^*_{0.1}$ / kPa s	Melts				Solid	
		$\eta^* \approx \omega^p$	$G' \approx \omega^m$	$G'' \approx \omega^n$	E_a kJ mol^{-1}	G' at 25 °C MPa	G' at 100 °C MPa
PVC	9.9	–0.077	0.889	0.808	38.5	24	4.14
CuZ15–PVC	20.1	–0.415	0.603	0.575	75.5	75	8.4

The addition of CuZ improved G' in the rubbery state (T > 60 °C), as well as at 25 °C, and enhanced the elastic behaviour of PVC. The improved rheological and mechanical properties of the PVC composite indicated that CuZ could play the role of physical crosslinker due to the possibility of the formation dipole––dipole interactions between the electronegative chlorine atoms on the PVC chains and the polar zeolite particles.[24]

CONCLUSIONS

The study showed that commercial PVC could be modified by a simple procedure to achieve an antibacterial effect towards the multidrug resistant *A. baumannii*. The rheological results showed that the addition of natural zeolite (15 wt. %) did not affect the processability and formability of the PVC. On the contrary, the addition of the zeolite to PVC improved its rheological behaviour.

Preliminary results indicated that D-Tyr and copper-containing zeolite are promising candidates for the modification of thermoplastic polymers that are used in biomedical devices. Further research will be directed towards a study of the antibacterial mechanism as well as to optimisation of the modification process.

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ИЗВОД

АНТИБАКТЕРИЈСКА АКТИВНОСТ КОМПОЗИТА НА БАЗИ БАКРОМ ОБОГАЋЕНОГ ЗЕОЛИТА/ПОЛИ(ВИНИЛ-ХЛОРИДА) ПРЕМА КЛИНИЧКОМ ИЗОЛАТУ *Acinetobacter baumannii*

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Мултирезистентна бактерија *Acinetobacter baumannii* изазива озбиљне болничке инфекције. У овом раду комерцијални поли(винил-хлорид), који се користи за производњу ендотрахијалних тубуса, модификован је бакром обогаћеним зеолитом у циљу добијања композита који показују антибактеријску активност према клиничком изолату

Acinetobacter baumannii ST145. Композити су припремани додавањем различите количине бакром обогаћеног зеолитног туфа (CuZ), а затим су импрегнисани раствором D-тирозина (D-Tyr). Композити са CuZ (CuZ–PVC) нису показали антибактеријску активност. Композити CuZ–PVC који су импрегнисани раствором D-Tyr показали су антибактеријску активност. Активност је објашњена синергистичким деловањем CuZ и D-Tyr. Реолошким мерењима показано је да модификација поли(винил-хлорида) бакром обогаћеним зеолитом не утиче на процесибилност овог полимера као ни на његову поновну обраду.

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