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## Biofouling: The Journal of Bioadhesion and Biofilm Research

Publication details, including instructions for authors and subscription information:

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Published online: 17 Sep 2014.

To cite this article: Jelena Milenkovic, Jasna Hrenovic, Ivana Goic-Barisic, Milos Tomic, Jasna Djonlagic & Nevenka Rajic (2014) Synergistic anti-biofouling effect of Ag-exchanged zeolite and D-Tyrosine on PVC composite against the clinical isolate of *Acinetobacter baumannii*, *Biofouling: The Journal of Bioadhesion and Biofilm Research*, 30:8, 965-973

To link to this article: <http://dx.doi.org/10.1080/08927014.2014.959941>

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## Synergistic anti-biofouling effect of Ag-exchanged zeolite and D-Tyrosine on PVC composite against the clinical isolate of *Acinetobacter baumannii*

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(Received 22 July 2014; accepted 26 August 2014)

Due to their susceptibility to bacterial biofilm formation, commercial tubes for medical use are one of the main sources of hospital infections with *Acinetobacter baumannii*. The anti-biofouling activity of novel composites against the clinical isolate of the multi-drug resistant *A. baumannii* is reported here. The composites were prepared by addition of micronised silver-exchanged natural zeolite (Ag-NZ) into poly(vinyl chloride) (PVC), followed by coating of the composites with D-Tyrosine (D-Tyr). The Ag-NZ composites (containing 1–15 wt% of Ag-NZ) coated with D-Tyr (Ag-NZ-Tyr) showed a bactericidal effect (100% or a 6.9 log CFU reduction) towards immobilised bacterial cells. The uncoated Ag-NZ composites showed a reduction of up to 70% (4.4 log CFU) of immobilised bacteria in comparison with the original PVC. Rheological testing of the composites revealed that the addition of Ag-NZ slightly affected processability and formability of the PVC and increased the elasticity of the polymer matrix.

**Keywords:** antibacterial activity; bacteria; biofilm; endotracheal tube; public health

### Introduction

The use of biomedical devices is frequently connected with a risk of nosocomial infection. A high rate of infection of biomaterials is particularly encountered when such materials are brought into contact with human bodies. Thus, endotracheal tubes and catheters usually act as reservoirs for infection since various microorganisms tend to become attached to surfaces and form biofilms. In the last decade the bacterium *Acinetobacter baumannii* has been intensively studied since it causes various nosocomial infections, particularly in intensive care units. Infections attributed to *A. baumannii* are extremely difficult to control (Gordon & Wareham 2010; Chiang et al. 2011) and the Infectious Diseases Society of America (IDSA) placed *A. baumannii* on a list of the six top-priority dangerous drug-resistant microbes among pathogens. Moreover, IDSA targeted *A. baumannii* in its call to develop new antibiotics by 2020 (Gilbert et al. 2010).

Endotracheal tubes and catheters are commonly produced from thermoplastic polymers. Accordingly, a great deal of research has been directed towards the development of novel materials based on polymers with antimicrobial properties. Generally, there are two approaches to enhancing the antimicrobial properties of polymeric materials. The first is based on incorporation of metals or metal compounds into polymer matrices such as poly-

ethylene, polyamide, poly(propylene), poly(vinyl chloride) (PVC) or polyurethane (Yeo et al. 2003; Balazs et al. 2004; Jeong et al. 2005; Kumar & Munstedt 2005). The second approach is based on coating the surface with different antibacterial agents using different techniques such as vapour or electrochemical deposition, sputtering or ion beam deposition. Silver (Ag) is most frequently used as a broad-spectrum antimicrobial agent. Thus, nano-Ag coatings have been reported to exhibit superior antimicrobial properties (Dowling et al. 2001; Samuel & Guggenbichler 2004; Jain & Pradeep 2005; Afessa et al. 2010). However, most surface coating methods suffer from poor adhesion onto the surface or from a lack of coating uniformity (Boschetto et al. 2012). Nano-Ag coatings have been reported to release a high concentration of Ag, which might be toxic to both human and animal cells (Hadrup & Lam 2014).

Incorporation of Ag compounds into molten polymers has recently been considered as a promising method for the preparation of antimicrobial polymer composites (Kumar & Munstedt 2005; Damm & Munstedt 2008; Zampino et al. 2011; Loo et al. 2014). Zeolites (both synthetic and natural) have been considered to be promising inorganic reservoirs for hosting Ag ions. Open-framework zeolite lattices control the rate of Ag release and thus exert antibacterial activity (Ferreira et al. 2012). Different D-amino acids were found to

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possess a repellent activity for biofilm formation. Among them D-Tyrosine (D-Tyr) has been reported as the most effective (Kolodkin-Gal et al. 2010; Hochbaum et al. 2011; Xu & Lui 2011; Si et al. 2014). PVC is one of the most frequently used polymers with various medical equipment applications. Many studies have been directed toward improvements in the mechanical as well as other properties of PVC polymer by adding inorganic filler phases such as clays, titania, silica or calcium carbonate (Abu-Abdeen 2012). Accordingly, the study of the mechanical and rheological properties of polymer composites is crucial for their processing and for the design of novel materials.

Taking these facts into account, the aim of the present study was to prepare a new anti-biofouling PVC composite that would show activity against the clinical isolate of *A. baumannii*. The Ag-exchanged natural zeolite (clinoptilolite) incorporated in PVC was used as a source of antibacterial Ag ions, and D-Tyr was used as a repellent coating. A dynamic-mechanical analysis of the PVC composites obtained was carried out in order to examine how the addition of zeolite affected PVC processability and its mechanical properties.

## Materials and methods

### Materials

Natural zeolitised tuff was obtained from a sedimentary deposit in the Zlatokop mine (Serbia). Previous detailed mineral analysis (Rajic et al. 2010; Stojakovic et al. 2011) showed that the tuff contains about 73 wt% of clinoptilolite, 14 wt% of feldspar plagioclase and 13 wt% of quartz. Silver nitrate used for the ion exchange of the tuff was purchased from Merck (Darmstadt, Germany) and D-Tyr was supplied by Sigma Aldrich (Hamburg, Germany). Commercial endotracheal tubes were purchased from Rusch (Teleflex Medical, Keren, Germany). Isolation of *A. baumannii* was performed on blood agar (Bio-Rad, Hercules, CA, USA), whereas nutrient agar (Biolife, Milan, Italy) was used for antibacterial assays.

### Preparation of composites

Ag-exchanged zeolite was prepared by an ion-exchange procedure described in detail in previous work (Hrenovic et al. 2013). A 6 mM aqueous solution of AgNO<sub>3</sub> was used for the ion exchange. The Ag-exchanged zeolite contained 43.50 mg Ag g<sup>-1</sup> of the dry zeolite. Both the natural zeolite and the Ag-exchanged zeolite were finely ground for 1 h at 250 rpm (Pulverisette 6, Fritsch, Idar-Oberstein, Germany) to obtain a particle size of 0.5–1.5 µm and the micronized products were denoted as NZ and Ag-NZ, respectively.

1.0 g of PVC (obtained by cutting the tubes) was first dissolved in 20 ml of tetrahydrofuran (THF, Sigma Aldrich, Germany) and then NZ or Ag-NZ was added in the weight percentage of 0 (control material), 1, 3 (only Ag-NZ), 5, 10 and 15 wt%. The mixtures prepared were stirred using a magnetic stirrer for 2 h at 500 rpm (RCT standard, IKA, Staufen, Germany), and then using an Ultra Turrax (IKA T18 basic at 8,000 rpm) for 10 min. They were then placed in Petri dishes to air dry for 72 h followed by 6 h in a vacuum dryer (Duo-vac oven, Labline Instruments, Kochi, India). The processed discs (~350 µm thick) were separated from the dishes and cut into 1 cm<sup>2</sup> square plates. The composite samples were denoted as NZ0 (control material), NZ5, NZ10, NZ15, and Ag-NZ1, Ag-NZ3, Ag-NZ5, Ag-NZ10, Ag-NZ15, where the number indicates the weight percentage of zeolite in the composite.

Original composites were sterilised in 70 vol. % ethanol (Carlo Erba, Rodano, Italy) for 10 min and then dried at 37°C. The sterilised composites were coated with D-Tyr by immersion in a solution of D-Tyr (100 mg l<sup>-1</sup>) and incubated for 16 h at 37 ± 0.1°C with stirring at 150 rpm, and then washed with a phosphate buffer solution (PBS) (0.203 g KCl, 0.611 g Na<sub>2</sub>HPO<sub>4</sub>, 0.25 g KH<sub>2</sub>PO<sub>4</sub>, 7.946 g NaCl, distilled water up to 1 l, pH=7.2). The sterility of the prepared composites was checked by placing them on nutrient agar, followed by incubation at 37°C for 24 h. No microbial contamination of the coated composites was observed.

### Bacterial isolate

130 clinical isolates of *A. baumannii*, collected during a 10-year surveillance period at the University Hospital Centre Split, Croatia, were assayed for the production of biofilm. The ability for biofilm production was determined in microtitre plates using a standard procedure (Espinal et al. 2012). Of all the isolates examined, *A. baumannii* ST145 exhibited the best biofilm formation ability, and therefore it was chosen for the study. This isolate originated from a tracheal aspirate of a patient hospitalised in the intensive care unit of the University Hospital Centre Split in 2009. The isolate displayed the following multiresistant antibiotic profile: resistant to ceftazidime, cefepime, amikacin, gentamicin, ciprofloxacin, imipenem and meropenem; reduced susceptibility to ampicillin/sulbactam; and fully susceptible only to colistin. Genotyping, using pulsed-field gel electrophoresis (PFGE) followed by macrorestriction of genomic DNA with *ApaI* (New England Biolabs, Hitchin, UK) as the restriction enzyme, revealed that this isolate belonged to the international clonal lineage 2 (Goic-Barisic et al. 2011). The international clonal lineage 2 has been the leading clone implicated in *A. baumannii* hospital infections in Croatia, especially in intensive care units (Goic-Barisic & Kaliterna 2011).

### Antibacterial activity test

Original composites and composites coated with D-Tyr were tested for antibacterial activity. The *A. baumannii* isolate ST145 was first pre-grown on blood agar for 16 h at  $37 \pm 0.1^\circ\text{C}$  to obtain a culture at a log phase of growth. The bacterial biomass was then suspended in PBS. The prepared composites were added to 5 ml of bacterial suspension. Tubes with the control material (PVC without NZ) served as a positive control. The tubes were left to incubate in the dark for 24 h at  $37 \pm 0.1^\circ\text{C}$  without stirring. To confirm the antibacterial activity of Ag and D-Tyr, experiments with solutions of  $\text{AgNO}_3$  (with a metal concentration similar to Ag-NZ15) and D-Tyr ( $100 \text{ mg l}^{-1}$ ) in PBS were set up in the same manner as the composite experiments, where the suspension of bacterial cells in PBS served as a positive control.

The number of planktonic cells was determined at the beginning of the experiment and after contact for 24 h. 0.5 ml of the original sample or a 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  $37 \pm 0.1^\circ\text{C}$ . After incubation the bacterial colonies were counted (Stuart Colony Counter SC6) and the number of colony forming units ( $\text{CFU ml}^{-1}$ ) was determined. The number of cells immobilised on the composites was determined after contact for 24 h. In preliminary experiments, several different methods to recover immobilised cells from the composites were tested. The composites were transferred to fresh PBS solution and the immobilised cells were separated by employing either vortex only (45 Hz/5 min, Kartell TK3S, Milan, Italy) or ultrasound (Sonopuls HD 2070, probe KE 76, Bandelin, Berlin, Germany) and then vortexing. A third method consisted of composites transferred into a solution of D-Tyr ( $100 \text{ mg l}^{-1}$ ) and left for 2 h at  $37^\circ\text{C}$  with stirring at 200 rpm. Immobilised cells were separated either directly from the solution of D-Tyr or after employing a vortex step. For the vortex-only experiments, the combination of ultrasound and vortex experiments, and the combination of D-Tyr solution and vortex experiments the number of recovered CFU was similar (Table 1). The number of recovered immobilised

cells was significantly lower for the treatment only with D-Tyr in comparison with the other methods. As a result, vortex treatment was chosen for the recovery of immobilised cells in all experiments. The number of immobilised bacteria that were recovered in PBS was determined on nutrient agar as described above and reported as  $\text{CFU cm}^{-2}$ . All experiments were performed in quadruplicate.

Statistical analyses were carried out using Statistica Software 10.0 (StatSoft, Tulsa, USA). The CFU counts were logarithmically transformed. The antibacterial activity of composites was expressed as the reduction of log CFU ( $\log \text{CFU}_{\text{control}} - \log \text{CFU}_{\text{composite}}$ ) and as the percentage reduction of log CFU as compared with the positive control ( $100 * [1 - \{\log \text{CFU}_{\text{composite}} / \log \text{CFU}_{\text{control}}\}]$ ). The comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the *post hoc* Duncan test was performed for calculations of pair-wise comparisons. Statistical decisions were made at a significance level of  $p < 0.05$ . The biofilm on the surface of the composites was examined by scanning electron microscopy (SEM). The composites were rinsed with PBS, fixed, dehydrated and prepared for the SEM analysis (using JSM-6610LV SEM, Jeol, Peabody, MA, USA) by a procedure consisting of ethanol treatment, drying, and coating with gold.

### Release of silver from composites

The leaching of Ag ions from Ag-NZ15 was examined during a 24 h period in order to investigate the kinetics of Ag release from composite material. The experiment with 12 replicates was set up as described above for the antibacterial activity test. The Ag concentration in solution was measured by atomic absorption spectrophotometer (SpectrAA 55B, Varian, Palo Alto, CA, USA).

### Rheological and mechanical measurements

Dynamic rheological data, ie the dynamic storage modulus ( $G'$ ), the loss modulus ( $G''$ ), the phase angle ( $\tan\delta$ ) and the complex dynamic viscosity ( $\eta^*$ ) of PVC and PVC/NZ composites, were obtained by 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  $200^\circ\text{C}$ . Frequency sweep scans were conducted from 0.1 to  $100 \text{ rad s}^{-1}$  and at a strain of 0.5%. In addition, the dynamic mechanical properties of the rectangular samples were analysed in a torsion rectangular mode in the temperature range of 25 to  $120^\circ\text{C}$  at a fixed strain of 0.5% and an angular frequency of 1 Hz. pastilles (25 x 1.5 mm). Rectangular test specimens (50 x 12 x 1 mm)

Table 1. Recovery of immobilised *A. baumannii* cells from the control material after contact for 24 h by employing different methods.

Method	Immobilised cells ( $\log \text{CFU cm}^{-2}$ )
Vortex only	$6.43 \pm 0.06$
Ultrasound and vortex	$6.42 \pm 0.06$
D-Tyr only	$5.91 \pm 0.11^*$
D-Tyr and vortex	$6.47 \pm 0.07$

Note:  $t_0$  *A. baumannii* ( $\log \text{CFU ml}^{-1}$ ) =  $8.26 \pm 0.06$ ; \*significantly lower as compared with vortex only.



were prepared by melting PVC and the PVC/NZ thin film in a three-part metal mould at 180°C for 1 h, followed by compression at 1.5 MPa for 30 min at room temperature. The complex dynamic viscosity of the sample melts, obtained at different temperatures, was used to calculate the activation energy of flow ( $E_a$ ). The activation energy of flow ( $E_a$ ) is calculated based on the relation between viscosity and temperature (Arrhenius equation):

$$\eta^*(T) = A e^{E_a/RT} \quad (1)$$

where  $\eta^*$  is the complex dynamic viscosity of the sample melts,  $T$  is the temperature,  $R$  is the universal gas constant and  $A$  is a constant related to the polymer.

## Results

### Antibacterial activity of composites

The antibacterial activity of the control material and composites with different weight percentages of NZ or Ag-NZ after contact for 24 h is shown in Figure 1. The composites containing NZ did not exhibit antibacterial activity either towards planktonic or immobilised bacteria. The Ag-NZ composites exhibited a significant reduction in the planktonic and immobilised cells in comparison with the control material. Antibacterial activity slightly increased with increasing Ag-NZ concentrations in the composites, and ranged for planktonic cells from 4.7–5.5 log CFU reduction (54–74%), for Ag-NZ1

and Ag-NZ15, respectively, while for immobilised cells, it ranged from 4.1–4.4 log CFU reduction (65–70%) for Ag-NZ1 and Ag-NZ15, respectively.

Figure 2 shows the antibacterial activity of the composites coated with D-Tyr after contact for 24 h. Coating with D-Tyr had no effect on the antibacterial activity of the NZ composites (NZ-Tyr). Coating of the Ag-NZ composites with D-Tyr did not significantly affect antibacterial activity toward planktonic cells (4.6–5.3 log CFU reduction or 60–69% for Ag-NZ1-Tyr and Ag-NZ15-Tyr, respectively). However, coating with D-Tyr resulted in a bactericidal effect (100% reduction corresponding to 6.9 log CFU reduction) against the immobilised cells for all the Ag-NZ-Tyr composites tested. In order to check whether the antibacterial activity could be ascribed to the Ag ions or to D-Tyr itself, the antibacterial activities of Ag solution (at a concentration equivalent to the amount of Ag in Ag-NZ15 composites) and D-Tyr solution (100 mg l<sup>-1</sup>) were tested. The Ag ions displayed bactericidal activity in comparison with the control (7.77 ± 0.08 log CFU reduction), while D-Tyr itself showed no antibacterial activity (0.04 ± 0.01 log CFU reduction, data not shown).

SEM analysis of the composites after contact with bacteria for 24 h confirmed the presence of a few immobilised cells on the Ag-NZ composite and the absence of immobilised *A. baumannii* cells on the Ag-NZ-Tyr composite (Figure 3). Differences in the pH between the control and experimental systems after contact for 24 h were

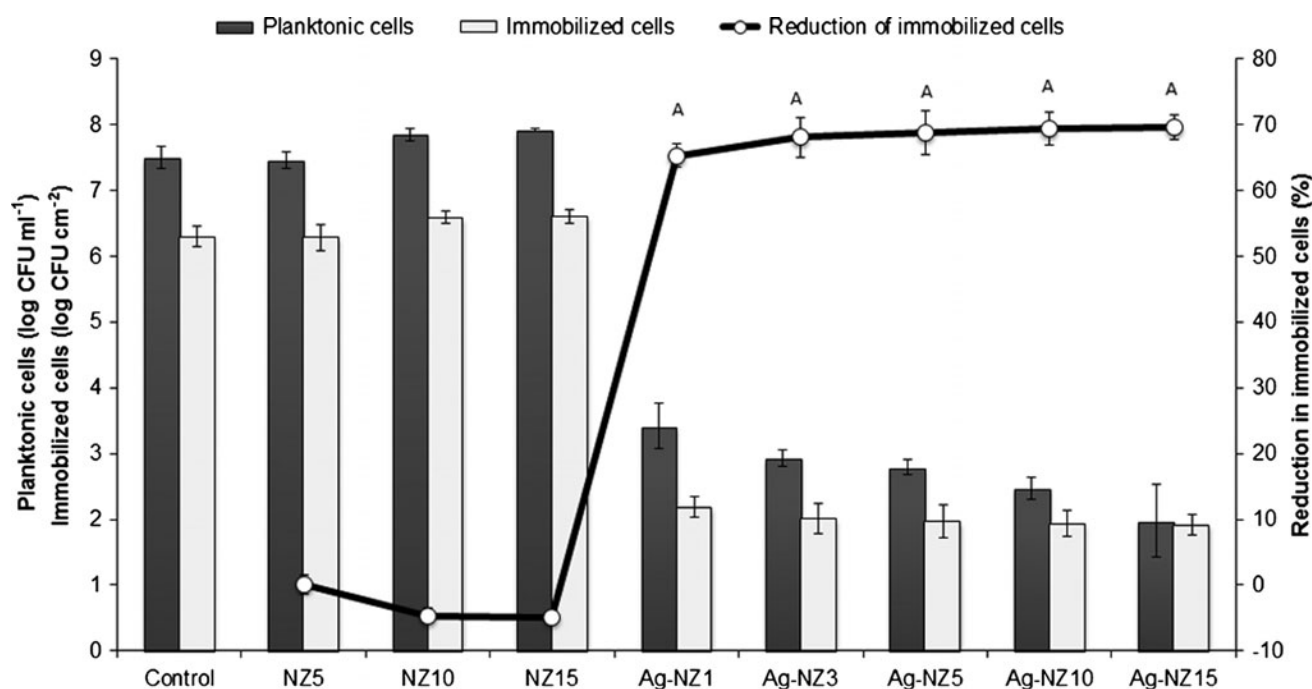


Figure 1. Antibacterial activity of the control material and of the NZ and Ag-NZ composites after contact for 24 h.  $t_0$  *A. baumannii* (log CFU ml<sup>-1</sup>) = 7.79 ± 0.20; <sup>A</sup> = a significant reduction compared with the control material.

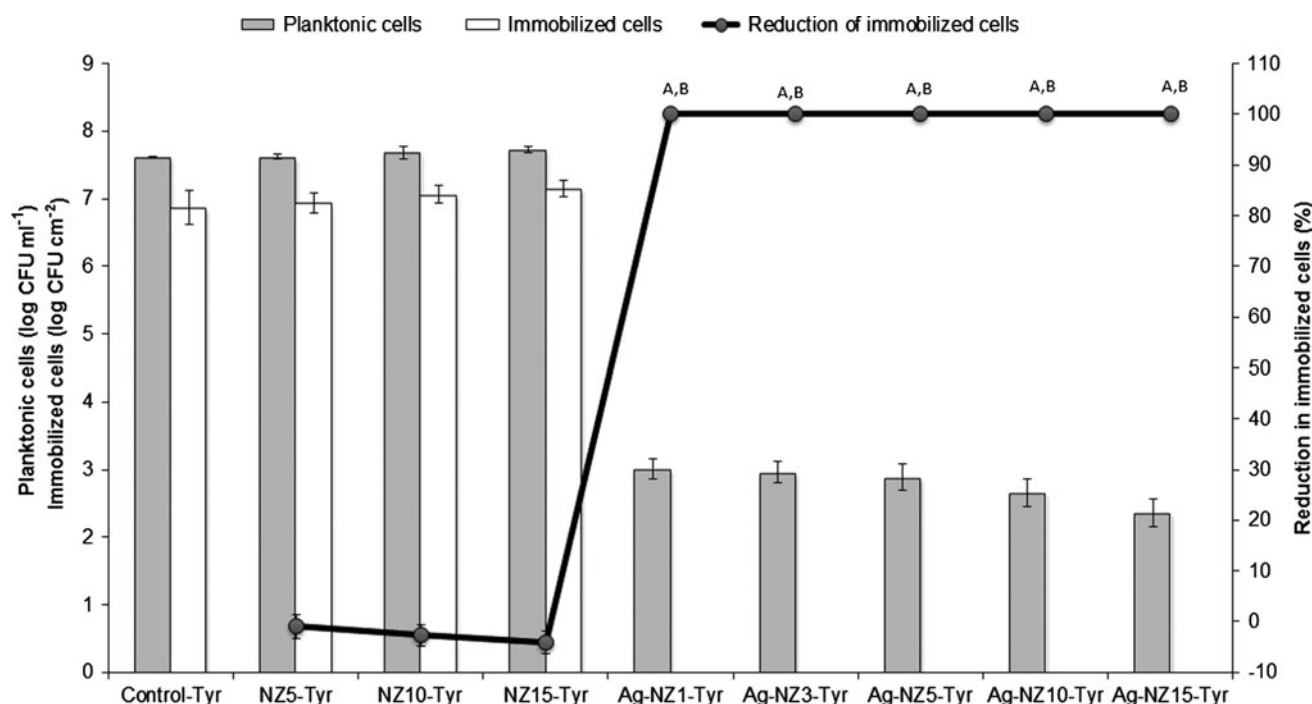


Figure 2. Antibacterial activity of the control material and of the NZ-Tyr and Ag-NZ-Tyr composites after contact for 24 h.  $t_0$  *A. baumannii* (log CFU ml<sup>-1</sup>) =  $7.72 \pm 0.13$ ; <sup>A</sup> = a significant reduction compared with the control material coated with D-Tyr; <sup>B</sup> = a significant reduction compared with the corresponding uncoated material; the limit of detection of immobilised cells was 1 CFU cm<sup>-2</sup>.

not greater than 0.5 (data not shown). The lowest and highest measured pH values were 6.8 and 7.5, respectively. Since *Acinetobacter* sp. grow in the pH range 5.0 to 8.0 (Garritty et al. 2005), antibacterial activity cannot be ascribed to the differences in pH.

### Release of silver from composites

In order to examine whether the antibacterial activity could be ascribed to the Ag ions released from the composites and to predict the durability of the composites, a leaching test was performed for the Ag-NZ15 composite which contained the highest wt% of Ag-NZ. The concentration of Ag released from Ag-NZ15 was about 1% of its initial concentration in the composite after contact for 24 h (Figure 4). Consistent results for Ag leaching in replicated measurements suggest a homogenous distribution of Ag-NZ in the composites. Analysis of Ag release with time showed that the release kinetics are in a good agreement ( $r^2 > 0.99$ ) with the Korsmayer–Peppas mathematical model (Costa & Sousa Lobo 2001) as shown by Equation 2:

$$F = k \cdot t^n \quad (2)$$

where  $F$  is the fractional release of Ag from the surface of the composite,  $k$  is the kinetic release constant,  $t$  is

the elapsed time and  $n$  is the release exponent. The value for  $n$  was found to be 0.36, indicating that diffusion is the predominant mechanism in Ag release from Ag-NZ15 (Costa & Sousa Lobo 2001).

### Rheological and mechanical behaviour of the composites

In order to check whether the addition of zeolite influenced the mechanical behaviour and processability of PVC, the dynamic mechanical properties of the composites were examined in the molten state since they are important parameters for the processing of PVC. The dynamic rheological behaviour of the PVC matrix (NZ0), and of the NZ15 and Ag-NZ15 composites was analysed by measuring the storage shear modulus ( $G'$ ), the loss shear modulus ( $G''$ ) and the complex shear viscosity ( $\eta^*$ ) in the frequency range of 0.1–100 rad s<sup>-1</sup>. The storage and loss shear modulus reflects the ability of a material to store energy (elastic behaviour) and to disperse energy (viscous behaviour) under applied deformation, respectively. Figure 5 shows the frequency dependence of  $G'$ ,  $G''$  and  $\eta^*$  for PVC (NZ0) and the composites in a molten state at 200°C. The  $G'$  and  $G''$  of NZ0 and of the composites (NZ15 and Ag-NZ15) increased with the increase in frequency. The addition of NZ enhanced the  $G'$  of PVC, while the presence of

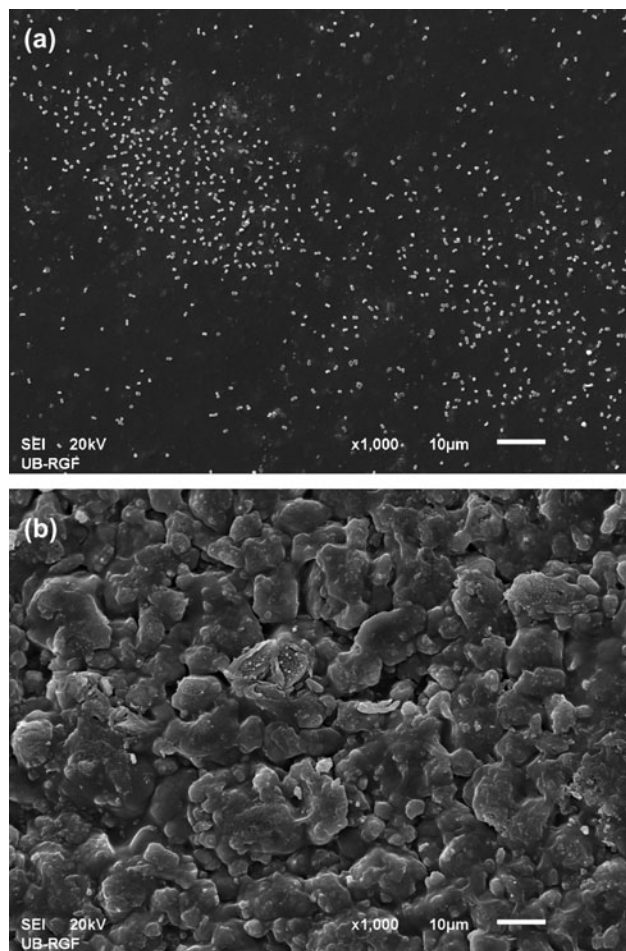


Figure 3. SEM images after contact for 24 h of (a) immobilised cells of *A. baumannii* without any sign of biofilm formation on the surface of the Ag-NZ15 composite, and (b) the absence of bacterial cells on the surface of the Ag-NZ15-Tyr composite. The side of one surface of the composites was somewhat coarse due to the addition of NZ (imaged for Ag-NZ15-Tyr), while the side of the other surface was shiny (imaged for Ag-NZ15).

Ag-NZ15 affected  $G'$  only at high frequencies. On the other hand,  $G''$  for PVC was enhanced by the addition of NZ15 or Ag-NZ15 regardless of frequency. The composites exhibited non-Newtonian behaviour as the complex viscosity decreased with increasing frequency (shear thinning).

Table 2 shows that the complex viscosity of composites was higher than NZ0 in all frequency ranges and that the NZ15 composite had the highest complex viscosity. The same trend was observed for the frequency dependence of  $G'$  and  $G''$ . For NZ0 a zero shear or Newtonian viscosity was present at low frequencies, while the addition of either NZ15 or Ag-NZ15 induced non-Newtonian behaviour in the low-frequency region. Moreover, the presence of NZ led to a reduction in the

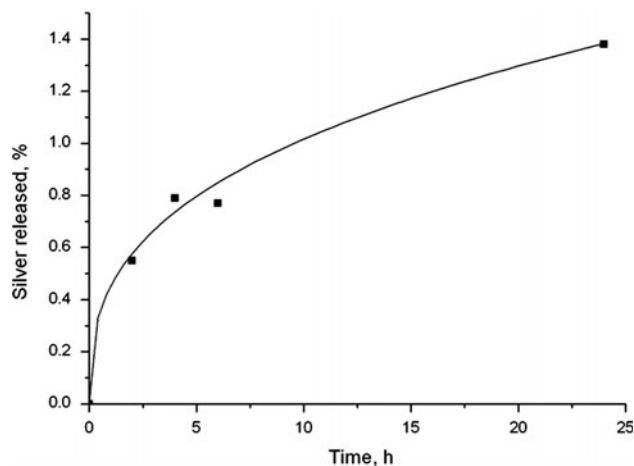


Figure 4. Percentage of Ag ion released from the Ag-NZ15 composite over 24 h.

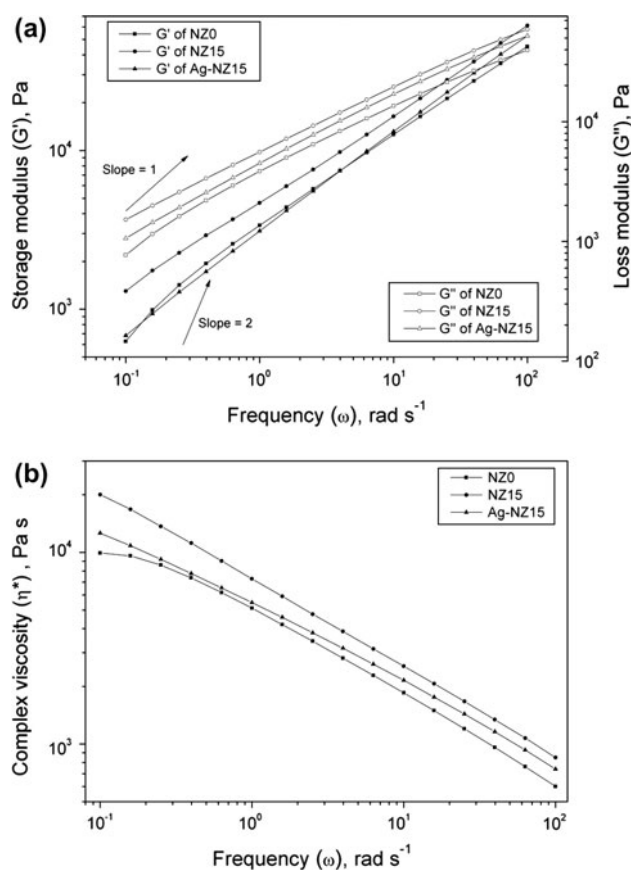


Figure 5. Frequency dependences of (a) the storage and loss modulus, and (b) the complex viscosity of the control material (NZ0) and NZ15 and Ag-NZ15.

slope values, indicating possible interfacial interactions between the PVC and the NZ particles. The slopes of  $G'$  (0.89) and  $G''$  (0.81) obtained for NZ0 were lower than

Table 2. Rheology results for PVC (NZ0) and composites (NZ15 and Ag-NZ15).

	Solid sample		$\eta^*$ , kPa s, at 0.1 rad $s^{-1}$	Melts			Activation energy of flow, $\text{kJ mol}^{-1}$
	$G'$ at 25°C, MPa	$G'$ at 100°C, MPa		$\eta^* \sim \omega^p$	$G' \sim \omega^m$	$G'' \sim \omega^n$	
NZ0	24.0	4.14	9.9	-0.077	0.889	0.808	38.5
NZ15	75.0	8.40	20.1	-0.415	0.603	0.575	75.5
Ag-NZ15	58.0	7.40	12.6	-0.342	0.685	0.647	82.0

2 and 1, respectively, which could be ascribed to the interaction of the plasticiser with the non-plasticised PVC (Persico et al. 2009). Moreover,  $E_a$  was higher for the composites than for NZ0, indicating that the flowability of the PVC melts is disrupted by the addition of zeolite.

The dynamic mechanical properties of the NZ0, NZ15 and Ag-NZ15 composites were investigated on solid rectangular samples in a temperature range of 25 to 120°C (data not shown). The temperature dependence of the storage modulus ( $G'$ ) and of  $\tan\delta$  indicated that the samples exhibited characteristics for  $\alpha$ -relaxation or the transition from glassy to rubbery state ( $T > 60^\circ\text{C}$ ). The glass transition temperature ( $T_g$ ), defined as the inflection point of the temperature dependence of  $G'$  and as a maximum of the temperature dependence of  $\tan\delta$ , was not evident, which could be attributed to the influence of the plasticiser. Therefore, only the storage modulus ( $G'$ ) at 25 and 100°C is given in Table 2. The addition of either NZ or Ag-NZ improves  $G'$  in the rubbery state ( $T > 60^\circ\text{C}$ ), as well as at 25°C, and enhances the elastic behaviour of PVC. A greater improvement in  $G'$  in the rubbery state was noticed for NZ15 (103% in the rubbery state) than for the Ag-NZ15 composite (79%).

## Discussion

Bacterial biofilms can develop on endotracheal tubes after < 24 h post-intubation of patients (Augustyn 2007). Attachment of *Pseudomonas aeruginosa* and *Staphylococcus aureus* onto polyvinyl alcohol hydrogels was evident after contact for 6 h (Loo et al. 2014). Therefore, it seems necessary to develop endotracheal tubes that will prevent the formation of bacterial biofilm during the first 24 h post-intubation, retain their anti-biofouling activity until the end of intubation, and at the same time release no antibacterial compound at concentrations which may be toxic to humans. Although biofilm formation by *A. baumannii* has been widely studied as a bacterial virulence factor, this nosocomial pathogen has not been used in studies on the anti-biofouling activity of novel materials. The prepared PVC composites were effective in the

prevention of biofilm formation by the clinical multi-drug resistant *A. baumannii* isolate during 24 h of contact. The Ag-NZ composites were able to eradicate up to 4.4 log CFU (see Figure 1) and the Ag-NZ composites coated with D-Tyr eradicated 6.9 log CFU of immobilised *A. baumannii* cells compared with untreated controls (see Figure 2). For comparison, polyvinyl alcohol hydrogels containing Ag-nanoparticles reduced colonisation of *P. aeruginosa* by 2 logs CFU and < 1 log CFU of *S. aureus* after contact for 24 h (Loo et al. 2014). An Ag coating on polyurethane resulted in a 2.5–3.8 log CFU reduction of immobilised cells of *Staphylococcus epidermidis* after contact for 24 h (Dowling et al. 2001). The PVC composite containing Ag-exchanged synthetic zeolite reduced *Escherichia coli* and *S. epidermidis* by 4–6 log CFU after contact for 24 h (Zampino et al. 2011).

Silver is a widely known bactericidal agent. Bactericidal activity of Ag-exchanged natural zeolite against the clinical isolate of *A. baumannii*, which belongs to the international clonal lineage 2, has been shown to be due to the activity of Ag ions loaded onto zeolite and not to the zeolite used as the support material (Hrenovic et al. 2013). This was confirmed in this study, since the NZ composites showed no antibacterial activity while the Ag-NZ composites were antibacterial (see Figure 1). In a previous study, Ag solutions at concentrations of  $1.6 \text{ mg l}^{-1}$  and  $0.13 \text{ mg l}^{-1}$  of Ag leached from Ag-exchanged natural zeolite showed bactericidal activity and reduced *A. baumannii* by 7 log CFU (Hrenovic et al. 2013). As confirmed in a separate experiment with silver nitrate, the Ag content in the Ag-NZ15 composites was sufficient to inactivate all the *A. baumannii* (7.77 log CFU reduction). However, in this study, 1% of the leached Ag from the Ag-NZ15 composites (see Figure 4) inactivated 5.5 log CFU of planktonic bacteria and 4.4 log CFU of immobilised bacteria (see Figure 1). It should be mentioned here that in the experiments on the antibacterial activity of composites, the immobilised bacteria presented a minority of the total bacterial population in the system. In the experiment with the Ag-NZ15-Tyr composites (see Figure 2) the antibacterial activity against planktonic bacteria was similar (5.3 log CFU



reduction) to that for the Ag-NZ15 composites. This suggests that the antibacterial activity of composites containing AgNZ is a function of the release of Ag ions from the composites. Only 1% of Ag was leached from the composites after exposure for 24 h, suggesting that the anti-biofouling activity could be retained for a longer period of time. Leaching of the Ag ions from a PVC composite containing the commercial Ag-exchanged synthetic zeolite was highest during the first 24 h of exposure, and antibacterial activity was retained for up to 20 days (Zampino et al. 2011). The rate of Ag diffusion out of the composite depends on the type of zeolite structure serving as a source of Ag ions. The small size and the shape of pore openings in the zeolite lattice imposes constraints on the release of large Ag ions and thus influences not only the diffusion rate but also the concentration of Ag ions on the surface of the composite. Accordingly, the use of a zeolite, whose lattice imposes a slow release of Ag ions, could be a promising alternative to Ag salts or Ag nanoparticles usually used for Ag incorporation into a polymer matrix. Ag nanoparticles have been reported to release high amounts of Ag ions (Hadrup & Lam 2014).

The antibacterial activity of the Ag-NZ composites against immobilised bacterial cells was significantly enhanced (for 2.5–2.8 log CFU) by coating the composites with D-Tyr. Many studies have indicated that D-amino acids possess a repellent activity towards biofilm formation, with D-Tyr being the most effective. D-Tyr is incorporated in the peptidoglycan layer of the cell wall where it replaces D-alanine and disrupts the connection between the extracellular matrix and the cells (Kolodkin-Gal et al. 2010; Xu & Lui 2011; Si et al. 2014). D-Tyr prevents biofilm development at the stage of initial attachment of bacteria at concentrations of only 3  $\mu$ M (Kolodkin-Gal et al. 2010). The anti-biofouling effect of D-Tyr was a result of a controlled diffusive release of D-amino acid from the impregnated polymer surface (Hochbaum et al. 2011). This suggests that the anti-biofouling property of the Ag-NZ-Tyr composites may be due to the synergistic toxic effect of Ag ions and the repellent activity of D-Tyr. The absence of antibacterial activity of the D-Tyr solution against *A. baumannii* is consistent with other observations that D-Tyr did not inhibit growth of the bacterial cells (Kolodkin-Gal et al. 2010; Hochbaum et al. 2011).

The prepared anti-biofouling PVC composites have comparable rheological and mechanical properties to commercial endotracheal tubes. These results are consistent with the report of Zampino et al. (2011) that the addition of up to 20 wt% of the Ag-exchanged synthetic zeolite did not affect the processability and formability of the PVC composites. Addition of either NZ15 or Ag-NZ15 slightly affected the rheological behaviour of PVC. The improved rheological parameters and the

reduced slope values (non-Newtonian rheological behaviour) at low frequencies could be attributed to a dipole–dipole interaction between the PVC chains and the zeolite particles (Wan et al. 2003) since highly electronegative chlorine atoms of the PVC chains provide a strong polarity to PVC. Zeolite has a negatively charged aluminosilicate lattice, making the zeolite surface also polar. This suggests that zeolite could act as a physical cross-linker with PVC (Sodeifian et al. 2012). The intensity of interfacial interactions was lower with Ag-NZ than with NZ, indicating that the presence of Ag ions in the zeolite lattice affects the interactions. It seems likely that the ion exchange of a smaller  $\text{Na}^+$  (116 pm) by a larger  $\text{Ag}^+$  ion (129 pm) changes the polarity of the zeolite surface, which affects the interaction of zeolite with PVC.

## Conclusions

Novel PVC composites containing the Ag-exchanged natural zeolite and coated with D-Tyr showed anti-biofouling activity against a clinical isolate, multi-drug resistant *A. baumannii*. A 6.9 log CFU reduction in the immobilised *A. baumannii* cells on Ag-NZ-Tyr composites was achieved as compared with commercial endotracheal tubes. Moreover, the PVC composites reduced the number of unattached planktonic bacteria. The anti-biofouling activity of the Ag-NZ-Tyr composites originated from both the repellent action of D-Tyr and the release of Ag ions from the composites. The zeolite Ag ion lattice slowly released the ions, which diffused out of the composite and then exhibited antibacterial activity. A modification of PVC by the addition of only 1 wt% of the Ag-exchanged natural zeolite and subsequent coating of the composite with D-Tyr is a promising method for obtaining new anti-biofouling materials for biomedical applications.

## Acknowledgements

This work was supported by the Serbian Ministry of Science (project nos. 172018 and 172062) and the Ministry of Science, Education and Sports of the Republic of Croatia (project no. 1191155-1203). The authors are grateful to Prof. Juraj Sipusic, Faculty of Chemical Engineering and Technology, University of Zagreb, for micronization of the zeolite.

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