BACTERIA DEACTIVATION AND REMOVAL FROM WASTEWATER AND POLLUTED AIR

Dusan KIMMER\(^a\), Ivo VINCENT\(^a\), Jan DUDAK\(^a\), Eva BERGEROVA\(^a\), David PETRAS\(^a\),
Jaroslav LEV\(^a\), Marek HOLBA\(^b,d\), Libor KALHOTKA\(^c\), Premysl MIKULA\(^d\), Radka KORINKOVA\(^e\)
and Lubomir KUBAC\(^e\)

\(^a\)SPUR a.s., Zlin, Czech Republic, EU, dusan.kimmer@spur.cz
\(^b\)ASIO spol. s r.o., Brno, Czech Republic, EU
\(^c\)Mendel University of Brno, Brno, Czech Republic, EU
\(^d\)Institute of Botany of the ASCR, Brno, Czech Republic, EU
\(^e\)COC s.r.o., Rybitvi, Czech Republic, EU

Abstract

Procedures permitting to prepare homogeneous functionalized nanofibre structures based on polyurethanes modified by phthalocyanines (PCs) by employing a suitable combination of variables during the electrospinning process are presented. Compared are filtration and bacteria deactivation properties of nanostructures without PCs, modified in bulk and with PCs embedded into polyurethane chain by a covalent bond protecting the release of active organic compound during the filtration process. Finding that the functionalized nanofibre structures have an effect on bacterial growth was confirmed by microbiological, physico-chemical and molecular biological analyses, such as the inoculation in a nutrient agar culture medium, flow cytometry and real-time polymerase chain reaction.

Keywords: Nanofibre, Nanostructure, Electrospinning, Filtration efficiency, Phthalocyanines, Bacteria deactivation

1. INTRODUCTION

At present, the requirements on elimination of ultrafine particles by microfiltration (i.e. removal of particles having a size ranging from 100 nm to 15 μm) and ultrafiltration (removal of particles ranging from 5 to 100 nm in size) are increased by a demand for materials capable of not only capturing but also deactivating bacteria and viruses from air and water \([1,2]\). This requirement is very relevant primarily in case of airconditioning equipment, in which bacteria multiply when the equipment is not used for a long time and after the equipment is restarted and if sufficiently efficient filters are not used, the bacteria penetrate the environment in increased concentrations. The filtration materials capable of deactivating bacteria during filtration process will always offer an advantage as regards elimination of microorganisms in comparison with the materials permitting a barrier type of microorganism capture only.

A correct assessment of antibacterial properties of filtration nanomaterials requires an assessment accuracy and comparison of the results obtained using preferably several specific analyses. We have chosen the microbiological one (cultivation on a selective medium), qualitative as well as quantitative molecular biological analysis (polymerase chain reaction technique - real-time PCR), physical-chemical (flow cytometry) and also fluorescent microscopy. By combining results obtained by the above methods not only filtration efficiency of nanostructures but also bacteria deactivation efficiency can be evaluated.
2. EXPERIMENTAL

2.1 Materials
A modified polyurethane (PU) solution in dimethyl formamide for electrostatic spinning was prepared from diisocyanate, polyesterdiol, 1,4 butane diol (BD) and phthalocyanine (PC) containing amino or hydroxyl group in at the temperature of 90°C for a time period of 6 hours. The per partes method of synthesis was used. The solution prepared in this way was diluted with DMF to a viscosity of 1 - 1.5 Pa.s and its conductivity was increased to 50 - 150 µS/cm.

Phthalocyanines (tetrabenzo-fused 5,10,15,20-tetraazaporphyrins) belong to the group of photosensitizers capable of generating singlet oxygen and other reactive oxygen species upon interaction with light of suitable wavelength in the presence of oxygen. Singlet oxygen and other reactive oxygen species have strong biocidal effect on algae, bacteria, fungi and yeasts.

2.2 Filter Sample Preparation by Electrospinning Process
Nanofibre layers were prepared from polymeric solutions with a commercially available NanoSpider™ machine (Elmarco s.r.o. Liberec, Czech Republic, http://www.elmarco.com/) equipped with a patented rotating electrode with 3 cotton cords spinning elements (PCT/CZ2010/000042) and/or SpinLine 120 equipment (SPUR a.s., Zlín, Czech republic, http://www.spur-nanotechnologies.cz) using nanofibres forming jets. The experimental conditions were as follows: relative humidity: 25 - 36%, temperature: 23°C, electric voltage applied to PU solution: 35 through to 125 kV, distance between electrodes: 170 - 210 mm, speed of supporting textile collecting nanofibres: 0.16 - 1.20 m.min⁻¹. Nanofibres were collected on polypropylene (PP), polyester or viscose nonwoven textiles.

2.3 Proving embedding of PC in PU chains
Thin films were cast from samples of aqueous extracts of modified nanostructures with embedded and unembedded PC on a silica substrate and subsequently FTIR spectra were measured with the aid of Nicolet ISS spectrometer using a wave number ranging from 800 to 4000 cm⁻¹.

2.4 Filter Sample Characterization
Nanofibre-based filter prepared by the electrospinning process was characterized by a scanning electron microscope (SEM, Vega 3, Tescan, Czech Republic). In some cases the SEM images obtained were consequently used to determine fibre diameter, nanofibre layer thickness and fibre diameter/pore size distribution by using the recently proposed digital image analysis technique [3-7].

2.5 Laboratory water-filtration equipment
For some experiments the filter was lumminated by LED diode (wave length 660 nm) placed in a distance of 3 cm from the filter.
2.6 Laboratory air-filtration equipment

Three bubblers for capturing bacteria (passed through the filter) in distilled water by sorption were placed between the filtering equipment and the final filter. As a light source providing visible light necessary for initiation of deactivation processes, a 11W Narva LD 11W/Aquarium Color fluorescent tube emitting light suitable for PC initiation was used. Escherichia coli (E. coli) bacteria of the CCM 2024 non-pathogenic strain in model samples were detected and enumerated in a VRBL agar (Biokar Diagnostics, France) at 37°C for 24 hours.

2.7 Characterization of antibacterial properties

Microbiological analysis - a method used to determine number of microorganisms by cultivation on a nutrient medium is a standard technique prescribed by standards (namely EN ISO 6222:1999 or EN ISO 9308-1:2000). Employing this method it is possible to cultivate the sort of microbiological organism
Flow cytometry (FCM) represents one of the most prospective analytical methods in aquatic microbiology, ecology and ecotoxicology. FCM enables not only fast and accurate counting of cells, but also a determination of a number of physiological, genetical and other parameters of cell populations contained in sample suspensions based on their optical characteristics. In our study, bacteria were enumerated using CyFlow ML flow cytometer (Partec, Muenster, Germany) equipped with blue excitation laser (λ = 488 nm) and four optical parameters (forward- and side scatters as well as two fluorescent channels). Before the measurement started, samples were stained with SYBR Green I fluorescent stain and incubated for 15 minutes in the dark [9]. SYBR Green enters the cells of all bacteria regardless their physiological state and binds to cellular nucleic acids. After staining, green fluorescent bacteria are easily distinguishable on flow cytometric diagrams from the background, which enables determination of bacterial counts (or rather concentration) in analyzed sample. In addition to that, dual staining of samples by SYBR Green I and propidiumiodide (PI) (with 15 minutes of incubation in the dark) was performed to measure bacterial membrane integrity [10]. Unlike SYBR Green I, PI may enter the cell only in a case of cell membrane damage. Since PI possess red fluorescence, on flow cytometric histograms, cells with damaged cell membrane can be discriminated from intact cells based on their high red and low green fluorescence (on the contrary, intact cells have high green and negligible red fluorescence). Cells with damaged cell membrane can be considered as “dead”, as they are not able to reproduce or even face the adverse insults from their environment, which, sooner or later, leads to their lysis.

Real-time polymerase chain reaction (PCR) analysis. At present, the real-time PCR analysis is already a commonly used technique and belongs to relatively reliable and accurate methods intended for detection of any living organism. The basic principle of the polymerase chain reaction is generating millions of copies of the selected DNA sequence, which permits to detect and quantify the DNA sample tested. The reaction proceeds in several steps and cycles in a special equipment called cycler. For the PCR analyses the cycler GeneAmpR PCR System 9700 (Applied Biosystems, Foster City, USA) and iQ5 cycler (Bio-Rad, Sergate, Italy) were used.

2.8 Analysis of decomposition products of deactivating action of PC

 UV-VIS spektrophotometry was used to determine protein components and concentration of DNA, i.e. possible products of deactivating action of PC on bacteria (proteins, toxins) during filtration of air. The analyses were performed behind the filtration material (in bubblers and on final High-Efficiency Particulate Air /HEPA/ filters) on SmartSPec Plus Spectrofotometer (Bio-Rad, Sergate, Italy).

At the wavelength of 280 nm no measurable values of the protein components were obtained. This means that the bacteria deactivation does not lead to a complete decomposition of the bacteria. Bacteria form a filtration cake on the surface of and inside the nanostructures. Values of DNA concentrations at 260 nm were comparable with the concentrations of the bacteria detected with other analytical methods.

3. RESULTS AND DISCUSSION

All currently marketed biocide-modified polymer materials are bulk or surface treated. Compatibility with the binder is provided mostly by Van der Wals forces only or is not provided at all. Therefore, the biocides can be removed from the polymers by the stream of the media filtered or by extraction into water [1,2].

In order to eliminate the above shortcomings a procedure can be used based on nanostructures formed by nanofibres having a diameter from 50 nm to 500 nm and a pore size (a diameter of inscribed spheres) ranging from 200 nm to 1500 nm, which are highly efficient in capturing microorganisms and bacteria, in filtering air as well as in capture of viruses. The biocidal substances are embedded in the polymer being spun (primarily polyurethane) directly with a covalent bond so that they could not escape spontaneously.
from the filtration materials. The covalent bond between the molecule of the biocide and that of the polyurethane (PU) is formed during the polyadduct synthesis proper and results from reaction of the isocyanate group located at the end of the PU chain with acidic hydrogen of the amino group (equation 1 - during which urea bonds are formed), from reaction of the hydroxy group (equation 2 - during which urethane bonds are formed), or from reaction of carboxy group (during which amides are formed - equation 3). The amino, hydroxy and/or carboxy groups being a part of the biocidal molecule.

\[
\text{PU} \quad \text{NCO} + \text{H}_2\text{N} \quad \rightarrow \quad \text{PU} \quad \text{NHCONH} \quad \text{PC} \quad \quad (1)
\]

\[
\text{PU} \quad \text{NCO} + \text{HO} \quad \rightarrow \quad \text{PU} \quad \text{NHCOO} \quad \text{PC} \quad \quad (2)
\]

\[
\text{PU} \quad \text{NCO} + \text{HOOC} \quad \rightarrow \quad \text{PU} \quad \text{NHCOOOC} \quad \text{PC} \quad \quad (3)
\]

3.1 Embedding of PC into nanostructures

The highest efficiencies of PC embedding into PU were achieved in case of PC containing a free amino group. The FTIR spectra in Fig. 3 compare PU modified in bulk with unembedded sulphonated PC and PU with embedded cationic PC with substituent containing amino group. The absorption peaks at wave numbers of 1723 cm\(^{-1}\) (C=O from urethane bond) and 1589 cm\(^{-1}\) (N-H group originating from the urea bond) show formation of an embedding into the PU chain [8].

![Fig. 3 FTIR spectra of PC embedded (red) and unembedded (blue) into PU chains](image)

3.2 Model filtration of water

Tab. 1 presents results of cultivation and flow cytometry of the most efficient PC number 5 (see Tab. 2) examined (1 mass percent in PU nanofibres) for filtrations of model suspensions of E. coli bacteria in water.
The starting E.coli suspension contained 8.33E+06 colony forming unis (CFU)/ml determined by the cultivation method or 6.89E+06 cell number/ml determined by flow cytometry.

**Tab. 1 - Filtration**{1) of a model sample of E. coli bacteria in water through PC-modified PU nanostructures.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass per square area/ filtration time/ volume filtered</th>
<th>Filter efficiency ² for E. coli (%)</th>
<th>Analyzed place</th>
<th>Cultivation</th>
<th>Flow cytometry</th>
<th>Substance balance (CFU in front of / CFU on and behind filter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bacteria content (CFU)</td>
<td>Bacteria content (cell number)</td>
<td>Deactivated³ (%)</td>
</tr>
<tr>
<td>1 exposed to light-illuminated</td>
<td>3.0 g/m² 2 min 13 s 1000 ml</td>
<td>44.1</td>
<td>filter - shake out</td>
<td>1.52E+07</td>
<td>9.40E+08</td>
<td>52.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>filtrate</td>
<td>3.34E+07</td>
<td>3.85E+09</td>
<td>65.31</td>
</tr>
<tr>
<td>2 not exposed to light-unilluminated</td>
<td>3.0 g/m² 10 min 780 ml</td>
<td>98.8</td>
<td>filter - shake out</td>
<td>1.39E+09</td>
<td>1.39E+09</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>filtrate</td>
<td>2.36E+08</td>
<td>6.21E+07</td>
<td>5.98</td>
</tr>
</tbody>
</table>

1) At the pressure of 1 bar
2) Counted from the bacteria content before filtration and in the filtrate determined by FCM, the calculation from the shake-out could be distorted by shake-out quality (also by gluing of deactivated bacteria into the nanostructure – see the Fig. 6).
3) Membrane integrity (viability) measured after 24 hours of storing the samples in dark

The content of live bacteria itself in both the shake-out as well as the filtrate of sample 1 determined by the cultivation method suggests that their deactivation in comparison with the initial quantity could occur. The deactivation efficiency both in the shake-out and the filtrate in case of sample 1 is relatively high, with the filtration of 1000 ml of suspension and hence also the illumination (exposure to light) lasting 2 min 13 s only. Comparison with sample 2 shows a positive effect of the exposure to light.

The samples 1 and 2 differ also by the space arrangement of the nanostructures as can be concluded from the filtration efficiencies achieved at identical mass per square area. Open structure (Fig. 4) in comparison with the planar one (Fig. 5) can assure a more perfect contact of bacteria with nanofibres and thus increase also the deactivation capacities during passage of the bacteria through the nanostructure (Fig. 6). The space structure will be more passable and will let pass through more dead but also live bacteria. A filtration cake is formed quickly on a filter with a uniform distribution of nanofibres (Fig. 7). The filtration cake formed prevents the required contact of filtered bacteria with the active surface of the nanostructure and hinders also its illumination.
Fig. 4 Open nanostructure of sample 1 with a wide distribution of nanofibre diameters, magnification 5000×

Fig. 5 Planar nanostructure of sample 2, magnification 5000×

Fig. 6 Sample 1 with captured bacteria inside the nanostructure, magnification 5000×

Fig. 7 Filtration cake from E. coli bacteria of sample 2, magnification 5000×
3.3 Model filtration of air

Tab. 2 summarises bacteria captures and deactivation action of PC on a filter, Petri dish with agar, in bubblers and on final filter at a model filtration of air. PU nanostructures with PC embedded in chains (samples 3 to 7) as well as those modified in bulk only (samples 8 and 9) are presented. In all cases the PC concentration used was 1 mass percent, with the exception of sample 9 where the PC concentration in PU was 3 mass percent.

Tab. 2 PCR analysis of PC nanostructures in a model filtration of air

<table>
<thead>
<tr>
<th>Sample Modifying PC</th>
<th>Filter Mass per square area</th>
<th>Captured (CFU)</th>
<th>Deactivation efficiency (^1) (%)</th>
<th>Cultivation on a Petri dish (CFU)</th>
<th>Water sorption Captured (CFU)</th>
<th>Final filter Captured (CFU)</th>
<th>Substance balance Captured (CFU in front of / CFU on and behind filter)</th>
<th>Filtration efficiency (^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Zn, urea derivative 3.2 g/m(^2)</td>
<td>3.15E+02</td>
<td>99.60</td>
<td>2</td>
<td>1.26E+03</td>
<td>3.76E+04</td>
<td>7.52E+06 / 3.92E+04</td>
<td>99.5</td>
</tr>
<tr>
<td>4</td>
<td>Al, urea derivative 3.0 g/m(^2)</td>
<td>9.81E+05</td>
<td>99.994</td>
<td>nd.</td>
<td>3.38E+04</td>
<td>3.20E+02</td>
<td>4.29E+06 / 1.02E+06</td>
<td>99.2</td>
</tr>
<tr>
<td>5</td>
<td>Al, aminopyridine derivative 3.0 g/m(^2)</td>
<td>5.04E+06</td>
<td>99.998</td>
<td>nd.</td>
<td>7.40E+04</td>
<td>5.72E+02</td>
<td>4.29E+06 / 5.11E+06</td>
<td>98.3</td>
</tr>
<tr>
<td>6</td>
<td>Zn, amino derivative 2.2 g/m(^2)</td>
<td>7.83E+05</td>
<td>99.993</td>
<td>nd.</td>
<td>7.29E+04</td>
<td>4.50E+01</td>
<td>4.29E+06 / 8.56E+05</td>
<td>98.3</td>
</tr>
<tr>
<td>7</td>
<td>Zn, sulphamidic derivative 2.2 g/m(^2)</td>
<td>1.46E+04</td>
<td>99.991</td>
<td>1</td>
<td>2.94E+03</td>
<td>3.10E+03</td>
<td>7.52E+06 / 2.06E+04</td>
<td>99.9</td>
</tr>
<tr>
<td>8</td>
<td>Al, pyridine derivative 2.6 g/m(^2)</td>
<td>7.29E+03</td>
<td>99.983</td>
<td>5</td>
<td>3.53E+03</td>
<td>1.76E+03</td>
<td>7.52E+06 / 1.26E+04</td>
<td>99.9</td>
</tr>
<tr>
<td>9</td>
<td>Al, sulphonated 2.4 g/m(^2)</td>
<td>1.013E+07</td>
<td>99.999</td>
<td>1</td>
<td>3.63E+05</td>
<td>9.80E+03</td>
<td>1.51E+07 / 1.05E+07</td>
<td>97.5</td>
</tr>
<tr>
<td>No filter</td>
<td>-</td>
<td>-</td>
<td>1.01E+06</td>
<td>7.85E+03</td>
<td>1.26E+04</td>
<td>1.51E+07 / 1.03E+06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) Deactivation determined from the total PCR of the content analyzed and live bacteria determined by the cultivation method.

2) The overall filtration efficiency was determined from the content of bacteria before and after filtration, it was not calculated from the capture on the filter due to difficult shaking out of bacteria from morphologically voluminous nanostructures.

In the materials compared, the difference between embedded (samples 3 to 7) and unembedded (samples 8 and 9) PC will not show under the given experimental conditions. The reference filtration carried out without any filter did not prove presence of a significant quantity of damaged cells during sample injection.

When filtering a suspension of Escherichia coli bacteria (CM2024 non-pathogenic strain) in air, the quantities filtered are approximately by 3 orders lower and due to a sterically unhindered contact of the bacteria with the nanostructure the deactivation efficiencies are markedly higher than in case of filtering water suspensions (compare Figs. 8 and 9 with Fig. 7). The deactivation efficiencies are high in cases of both higher and lower captures of bacteria on filter. Also, duration of the light exposure (30 minutes) affects positively deactivation of E. coli bacteria.
4. CONCLUSIONS
The work presents experimental procedures permitting preparation of biocidal nanostructures containing PC molecules firmly attached to PU chains by a covalent bond. A system of microbiological analyses was worked out allowing a demonstration of inhibition effects of PC on microorganisms. The amount of the bacteria filtered, exposure of the filter to visible light and morphology of biocidal nanostructures have been found to be able to affect favourably the deactivation action of PC.

ACKNOWLEDGEMENTS
This work has been supported by the grants of Czech Ministry of Industry and Trade No. FR-TI1/053 and Technology Agency of the Czech Republic No. TA01010356.

LITERATURE
[2] CZ 303243