COMPARATIVE STUDY OF BIOLOGICAL RESPONSE OF Ag AND Pd NANOWIRE ARRAYS SUPPORTED ON POLYETHYLENE NAPHTHALATE FOR MEDICAL APPLICATIONS

POLÍVKOVÁ Markéta, SIEGEL Jakub, STASZEK Marek, VALOVÁ Michaela, PIŠLOVÁ Markéta, ŠVORČÍK Václav

Department of Solid State Engineering, University of Chemistry and Technology Prague, Czech Republic, EU, polivkoa@vscht.cz

Abstract

In recent days, advancements in nanotechnology ensure everyday applications of nanostructured noble metals, especially silver. Particularly in the medicinal applications, where they profit from their unique antibacterial effects, a serious risk of cytotoxic side-effects may occur. In this work, we studied biological (antibacterial and cytotoxic) response of commonly used silver nanostructures (nanowires, NWs), and compared them with Pd ones, as their possibly safer alternative. Both metal nanostructures were supported on biocompatible polymer - polyethylene naphthalate. The surface of metal/polymer composites was thoroughly characterized by XPS, FIB-SEM and AFM. Thereafter, biological testing of the samples was accomplished. Antibacterial properties of both, Ag and Pd NWs were examined and mutually compared by using Gram-negative bacteria *Escherichia coli* and Gram-positive *Staphylococcus epidermidis*, which commonly cause hospital-acquired infections. Finally, the comparative cytotoxic study of both types of composites was carried out using mouse embryonic fibroblasts. Such approach was chosen to determine, whether Pd nanostructures might compete with Ag ones in their medical applications.

Keywords: Silver, palladium, nanowires, medical applications, antibacterial effects, cytotoxicity

1. INTRODUCTION

Nowadays, the exploitation of nanotechnology in the development of antimicrobial polymeric materials gains an increasing interest, predominately utilizing silver. Nanostructured silver is highly effective material able to fight broad spectrum of nosocomial pathogens, whose main sources are the skin of the patients and/or clinicians during a surgical intervention [1]. For this reason, medical applications of this metal in its various nanostructured forms are widely spread, e.g., antimicrobial coating of medical devices [2] (surgical instruments, catheters, endotracheal tubes etc.), without taking into account its potential human cytotoxicity.

The mechanism of antimicrobial action of nanostructured silver, particularly clarified for silver nanoparticles, is based on partial oxidation, which results in the release of Ag^+ ions. Thereafter, silver ions act as the major bactericidal agent. They are able to interact with four main components of bacterial cell: cell wall, plasma membrane, bacterial DNA and specific enzymes in vital cellular processes. Silver ions cause the degradation of cell wall, which results in cell lysis. Subsequent penetration of Ag^+ into cell interior is followed by binding to DNA bases and causes DNA condensation and chromosomal aberrations. DNA loses its replication ability. Another Ag^+ exposition sites are mitochondria and ribosomes, where silver ions cause mitochondrial dysfunction, ribosome denaturation, and subsequent inhibition of protein synthesis, leading to degradation of plasma membrane [3].

In recent days, silver being used more and more in matters of general consumption, however, complex studies warning about its toxicity goes hand in hand. Contaminated wastewater was reported to negatively affect fish, and, in particular, direct use of antimicrobially treated medical devices causes serious complications to human [3]. Available studies reported that the most cytotoxic are silver nanowires [3-5]. For this reason, we prepared, characterized, and investigated biological response of silver nanowires supported on laser-treated polyethylene naphthalate (PEN), and palladium NWs/PEN composites, and compared them between

themselves. Since PEN is commonly used material in medical applications (e.g., in the preparation of very thin and strong balloon catheters), it was chosen as a model biocompatible polymer.

2. EXPERIMENTAL

2.1. Materials, apparatus and procedures

Polyethylene naphthalate foil (50 μm, Goodfellow Ltd., UK) was used as a substrate. The preparation of periodic ripples on the samples' surface was performed by KrF excimer laser (COMPexPro 50F, Coherent, Inc., wavelength 248 nm, pulse duration 20-40 ns, repetition rate 10 Hz) using linearly polarized laser light with UV-grade fused silica prism (model PBSO - 248-100) on an aperture with the area of 5×10 mm^2. The samples were irradiated by 6,000 laser pulses using laser fluence of 10 $mJ \cdot cm^{-2}$ (sample area of 2×2 cm^2).

The deposition of metals (Pd and Ag pellets of 3.18×3.18 mm^2, purity 99.99 %, Safina, a.s., CZ) onto patterned PEN surface was carried out by vacuum evaporation (LEYBOLD-Heraeus, Univex 450 device) to produce metal nanowire arrays (20 nm thick). Deposition conditions: room temperature, pressure of $3 \cdot 10^{-4}$ Pa, deposition rate of 0.33 $nm \cdot s^{-1}$, and glancing angle of φ = 70. Quartz crystal microbalance was used for *in situ* monitoring of metal thickness (Δf_{Ag} = 640 Hz, Δf_{Pd} = 220 Hz), and AFM scratch method (AFM VEECO CP II device) measured metal effective thickness (glass substrate) [6]. Thickness variations did not exceed 5 %.

2.2. Analytical methods

The atomic concentrations of C(1s), O(1s) Ag (3d) and Pd(3d), on the samples´ surface were measured by X-ray photoelectron spectroscopy (XPS) using Omicron Nanotechnology ESCAProbeP spectrometer. The X-ray source was monochromated at 1486.7 eV. The step size was 0.05 eV. The electron take-off angle was set to - 81 and 81° (with respect to surface normal) [7]. Spectra evaluation was done by CasaXPS software.

Focused Ion Beam Scanning Electron Microscope (FIB-SEM, LYRA3 GMU, Tescan, CZ) was used for visualization of NWs. FIB cuts were made by a Gallium ion beam. The analyzed surface was cleaned and flattened by polishing procedure. The images were taken under the angle of 54.81° and voltage of 5 kV.

Surface morphology of the samples was determined by atomic force microscopy (AFM) using AFM VEECO CP II device. Tapping mode minimized potential damage of the samples. A Veeco oxide-sharpened P-doped silicon probe RTESPA-CP attached to a flexible micro-cantilever working near its resonant frequency of 300 kHz was used. The scans were acquired at the line scanning rate of 0.5 Hz. Surface roughness (R_a), periodicity (Λ) and height (h) were then evaluated [7]. Variations of these parameters did not exceed 5 %.

2.3. Biological tests

Antibacterial response was investigated by the drop plate method [7,8] using Gram-negative (G^-) *Escherichia coli* (DBM 3138) and Gram-positive (G^+) *Staphylococcus epidermidis* (DBM 3179). Tested and control samples (triplicates) were immersed in 2 ml of physiological solution (PS), and inoculated with 1.1×10^4 of colony forming units (CFU) per 1 ml of *E. coli* and 2.2×10^4 of CFU per 1 ml of *S. epidermidis*. The samples were incubated under both, static and dynamic (shaking at 130 rpm) conditions at 24 °C (*E. coli*) and 37 °C (*S. epidermidis*) for 3 and 24 h. Afterwards, the aliquots of 25 μl from each sample were placed on agar plates (LB for *E. coli*, PCA for *S. epidermidis*) in 10-fold repetitions. After overnight incubation, CFUs were counted by Lucia Image 4.8 software. The experiments were accomplished under sterile conditions.

Cytotoxic response was examined using WST-1 assay (Sigma, USA) [7] using mouse embryonic fibroblasts L929. The samples were sterilized by UV irradiation, inserted into 12-well plates (VWR, USA, Ø 2.14 cm), and then seeded with 30,000 cells per well in triplicates in 1 ml of Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS). The cells were incubated for 24, 48 and 72 h (cultivation conditions: 37 °C, 5% CO_2, and 95% humidity). The culture medium was replaced with fresh phenol red free FluoroBrite

DMEM (480 µl) supplemented with 20 µl of WST-1 reagent per well. After 2 h of incubation, the medium of individual wells was aliquoted into 96-microtiter plates. The absorbance was measured at 450 nm (reference wavelength of 630 nm) using UV-Vis spectrometer (BioRad). Cells cultivated on standard tissue culture polystyrene (TCPS) and on pristine and rippled PEN served as controls.

3. RESULTS AND DISCUSSION

3.1. Surface characterization

The combination of laser modification of polymer and vacuum evaporation of metals was used for the preparation of self-organized, fully separated Ag and Pd nanowire arrays (thickness of 20 nm). Due to the experimental set-up of vacuum evaporation process (glancing angle of 70°) resulting in the shadow effect [9], this two-step preparation process produce metal NWs preferably from one side of rippled PEN (for graphical representation see ref. [7]). This phenomenon was confirmed by XPS (see **Table 1**) and FIB-SEM (**Figure 1**).

The determination of atomic concentrations of elements (at. %) by XPS was carried out from both sides of the samples; left and right (for graphical representation see ref. [7]). The analytical information was provided from 1-2 atomic layers. Concentrations of C and O are given by PEN stoichiometry, and were extended by hydrocarbon impurities adsorbed from air [10]. The presence of Ag and Pd originates from vacuum evaporation process. Tůma *et al.* [11] found that after two-step deposition of Ag, the concentration of Ag is independent on the detection angle. Contrary to our one-step metal deposition (shown in **Table 1**), in which higher values for both, Ag and Pd were found from the left side (Ag - left 24.8 %, right 8.6 %, Pd - left 25.9 %, right 9.0 %, respectively). Thus, the difference between the results originates from symmetric and asymmetric metal deposition, respectively. Significant differences in concentrations of both metals (Ag and Pd samples) confirmed the formation of NWs from one (in this case left) side of the ripples, caused by above-mentioned shadow effect [9]. The results for pristine and rippled PEN revealed no noticeable differences in element concentration from the left and right side. Slight concentration discrepancy, found by comparing the samples of pristine and rippled PEN, was given by the compositional change of the samples during laser patterning, as well as by associated change of their surface morphology, evident from AFM images (**Figure 2**).

Table 1 Atomic concentrations of C(1s), O(1s), Ag(3d) and Pd (3d) measured by XPS under the electron take-off angle of - 81 and 81° (left and right side of the samples)

side	sample	atomic concentrations of elements (at. %)			
		C	O	Ag	Pd
Left (-81°)	pristine PEN	73.0	27.0	-	-
	rippled PEN	79.3	20.7	-	-
	Ag NWs/PEN	63.1	12.1	24.8	-
	Pd NWs/PEN	62.1	12.0	-	25.9
Right (81°)	pristine PEN	73.1	26.9	-	-
	rippled PEN	79.5	20.5	-	-
	Ag NWs/PEN	75.9	15.5	8.6	-
	Pd NWs/PEN	74.4	16.6	-	9.0

Visual representation of metal NWs formed from ripples´ one side shows **Figure 1**. These results corresponds to AFM ones (see below, **Figure 2**), however, FIB-SEM analysis is more informative; it might measure various parameters, such as the distribution and shape of NWs. In particular, AFM scans cannot provide the imaging of fully separated metal NWs [12]. In **Figure 2**, one can observe the interface between a polymeric substrate (PEN) and metal (**Figure 1a**) Ag, and b) Pd NWs).

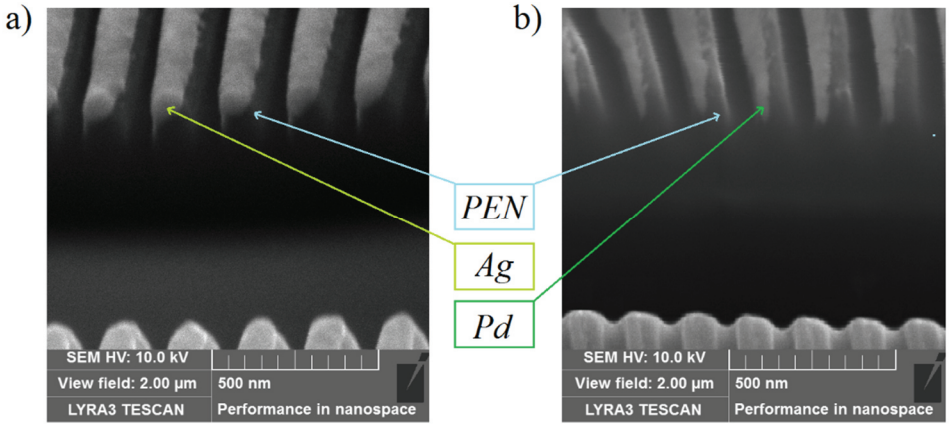

Figure 1 FIB-SEM images of a) silver, and b) palladium nanowire arrays supported on laser-patterned PEN

Surface modification of polymer, such as laser patterning, enables a direct control over the material biocompatibility; might significantly increase the adhesion and proliferation of human cells [13]. For this reason, AFM was used to evaluate the surface morphology before and after laser patterning of the samples. One of the most important parameter related to material biocompatibility is its surface roughness, which also plays an important role in its resulting antibacterial effects [13]. It follows that the enhancement of those two types of biological responses by the combination of laser patterning and metal deposition gives a promising combination of materials properties for their medical applications. **Figure 2** shows periodic ripples homogenously distributed over the PEN surface (sample of rippled PEN). The value of surface roughness (R_a) was considerably increased after laser patterning (PEN 4.4 nm, rippled PEN 9.9 nm). No significant differences were observable after metal deposition (Ag NWs/PEN 9.2 nm, Pd ones 9.9, respectively). The values of periodicities (Λ) and heights (h) (Λ_{Ag} = 215.3 nm, h_{Ag} = 36.9 nm, and Λ_{Pd} = 217.8 nm, h_{Pd} = 34.8 nm for Ag and Pd NWs/PEN, respectively) were mildly decreased compared to rippled PEN sample (224.0 nm, 37.4 nm), because of partial filling of the ripples by metal during vacuum evaporation process.

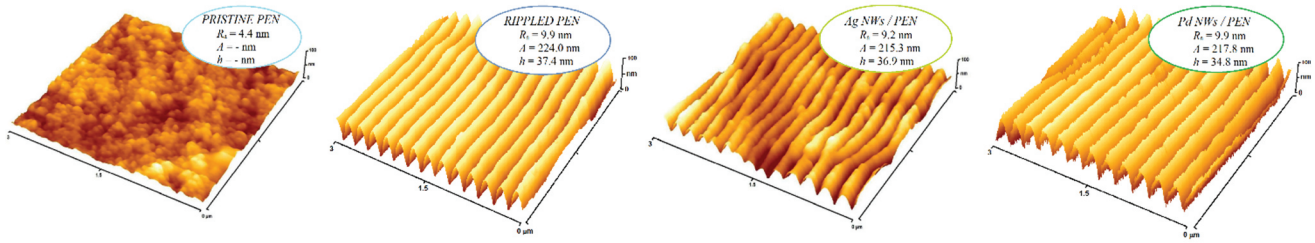

Figure 2 AFM images together with corresponding surface roughness (R_a), periodicity (Λ) and height (h)

3.2. Biological tests

The results of antibacterial testing with two environmental bacterial strains of G^- *E. coli* and G^+ *S. epidermidis* are shown in **Figure 3a, b**, respectively. The samples were incubated in both, static and dynamic mode for 3 and 24 h. In **Figure 3**, one can see that pristine and rippled PEN were not antibacterially active in all conditions, with the exception of rippled PEN in dynamic mode after 24 h of cultivation of *S. epidermidis* (**Figure 3b**). Mild antibacterial effect (about 30 % of control sample) might be a consequence of higher sensitivity of G^+ bacteria to surface properties of a materials with which they are in a contact, such as surface morphology and roughness [14] (see **Figure 2**). Antibacterial effects of both Ag and Pd/PEN composites, increased with incubation time in all conditions. After both, 3 and 24 h of incubation of *S. epidermidis* in dynamic mode (**Figure 3b**), no countable CFUs were detected, unlike *E. coli* (**Figure 3a**). The different results for *E. coli* and *S. epidermidis* were a consequence of a different composition of their cell walls; positively charged noble

metals easily penetrate through the cell walls of G^+ bacteria (*S. epidermidis*) [15]. The samples of Ag and Pd NWs showed comparable antibacterial effects in all conditions, which enables medical applications of Pd NWs as an alternative of Ag ones.

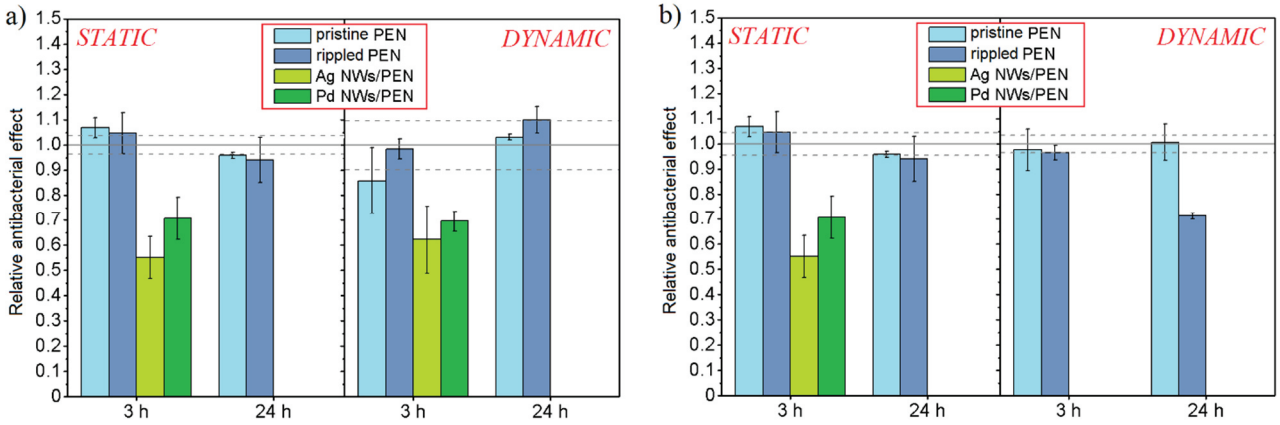

Figure 3 Relative antibacterial effect (CFU of sample / CFU of control) against a) *E. coli* and b) *S. epidermidis*, in static and dynamic mode. Gray line is a reference level together with its uncertainty

For safer applications of metal NWs/polymer composites, primarily medical ones, relative viability of L929 cell line was studied for cultivation times of 24, 48 and 72 h. One can see the results in **Figure 4**. Pristine PEN was not cytotoxic in all conditions. However, rippled one showed mild cytotoxicity after 24 and 48 h of cultivation (ca 15 % of TCPS), which disappeared after 72 h. Observed cytotoxic effects were probably caused by more difficult adaptation and fixation of cells onto rippled surface, since L929 line is characterized by high sensitivity to rougher surfaces [16]. Our cytotoxicity tests confirmed significant cytotoxicity of Ag NWs reported earlier in ref. [3-5], which was more than 50 % of TCPS and increased with cultivation time under conditions. Contrary to that, Pd NWs were not found to be cytotoxic, which represents safer alternative to Ag ones.

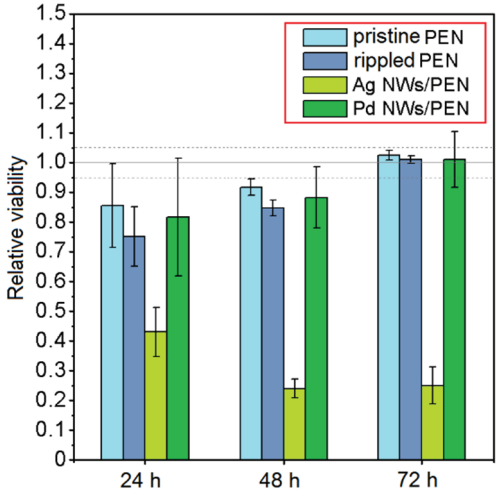

Figure 4 Relative viability (absorbance of sample / absorbance of control) of L929 cell line. Gray line represents a reference level together with its uncertainty (dash line)

4. CONCLUSION

We present a comprehensive study of nanostructured, antibacterial, metal-based coatings of polymeric medical devices (metal NWs). The most commonly used nanostructured metal (silver) was confronted with Pd one. It was found that the increase of surface roughness did not lead to enhanced biocompatibility, L929 cell line was highly sensitive to rougher surfaces. From the results of biological testing, it turned out that both, Ag

and Pd NWs/PEN composites have the appropriate antibacterial properties, however, only Pd ones fulfil the condition of cells´ safety, which making them suitable candidates for medical applications. Due to relatively high cytotoxicity, the applications of Ag NWs in medicine are limited and their contact with living tissues, e.g., in the treatment of medical devices, should be minimized.

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