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Original Paper

Interaction of parylene C with biological objects

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The aim of the present work was to examine the interactions of parylene C with such selected biological objects as: blood plasma proteins, platelets, endothelial cells, and bacterial biofilm produced by *E. coli* cells. The results obtained strongly support the thesis that parylene C is a material worth considering for biomedical use. Parylene C coating on polished medical steel significantly reduces platelet adhesion to this surface. On the other hand, in the case of the surface of machined medical steel coated with parylene C, the number of adhered platelets is significantly higher. This also means that surface texture of substrate material is very well reproduced by parylene C coating and is an important factor facilitating the platelet adhesion. Adsorption of plasma proteins at parylene C surface is very effective, and this finding confirms a notion that cell interaction with surfaces is mediated by the adsorbed proteins. In the light of the above, a high susceptibility of parylene C surface to bacterial colonization is easy to explain. The results showing reduced proliferation and changes in endothelial cell gene expression should also be seriously analysed when parylene C is considered for the use in contact with blood vessels.

Key words: *blood–material interaction, cell adhesion, endothelial cells, plasma protein, platelets, polymer, protein adsorption, thrombogenicity*

1. Introduction

Parylene is a generic name of a class of unusual polymers, the principal member of which is poly(para-xylylene) presented in figure 1.

The outstanding importance of xylylene polymers arises from the fact that they constitute the only class of polymeric hydrocarbon materials that are commercially produced by a chemical vapour deposition (CVD) technique. The parylene process was developed in the sixties of the twentieth century [1] and put on the market by the Union Carbide Corporation [2].

A precursor compound for xylylene polymers is paracyclophane, a cyclic dimer, whose formula is shown in figure 2.

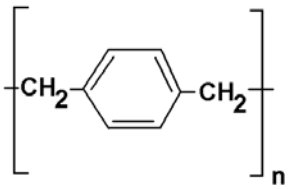

Fig. 1. Structural formula of poly(para-xylelene)

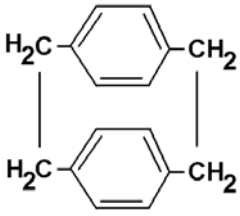

Fig. 2. Structural formula of paracyclophane

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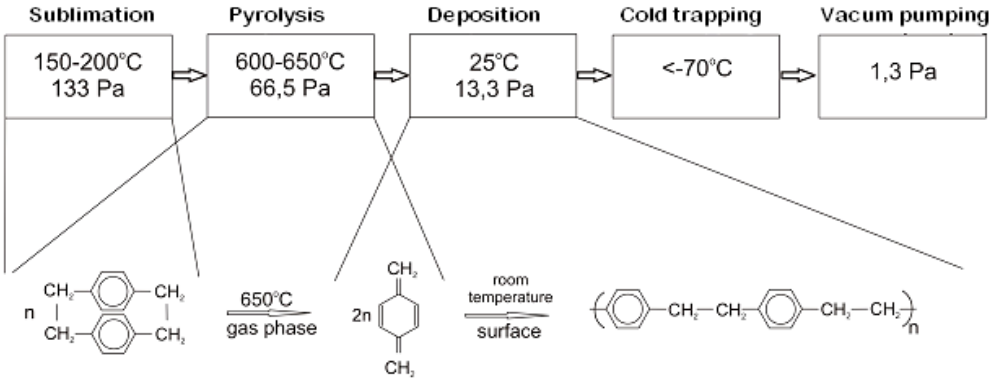

Fig. 3. Schematic representation of the parylene technology and basic chemistry of the process

The dimer molecule consists of two benzene rings connected with one another in para positions with ethylene bridges. Such a structure is subjected to a large molecular strain. Because of the strain energy stored in its molecule, paracyclophane possesses a number of unusual chemical properties, of which it is a relative ease of thermal cleavage of ethylene bonds that makes it so useful in the thin-film technology. Thermal decomposition of the dimer takes place at 650 °C and results in a quantitative formation of para-xylylene, an extremely reactive monomer species, which forms polymer films immediately upon its condensation on any surfaces. A schematic representation of the parylene process, together with the respective chemical reactions, is presented in figure 3.

Xylylene polymers have a number of very useful properties, ranging from high mechanical strength and low friction coefficient, through superb dielectric and insulation characteristics to excellent barrier behaviour and extraordinary chemical resistance. Since the process is also relatively simple to handle, easy to integrate with other vacuum technologies, with the resulting coatings being characterized by uniform thickness and extraordinary penetration abilities, it is widely applied in various areas of life and technology. There are a number of review articles, published as entries to several encyclopedia [3]–[5], that discuss these applications in detail.

Among many potential applications of various xylylene polymers, a chlorinated parylene, known as Parylene C, is a promising candidate for metallic implant coatings separating an implant body from the surrounding tissues. Unfortunately, little is known about its biocompatibility, although some papers suggest its good hemo- or thrombocompatibility [6], [7].

Any implant introduced into the human body will rapidly interact with body fluids, triggering the first and very important response. Proteins present in these fluids adsorb on the surface of the implant and, depending on its hydrophobic properties, a very thin protein film is created. This initial contact is responsible for the further history of the implant interaction with the surrounding tissues. Implants designed for tissue integration should exhibit a high affinity to proteins of body fluids, since these proteins mediate further cell–surface interactions. Those materials that are intended for a contact with blood should exhibit quite different properties. On one hand, adhesion of platelets to the implant surface should be excluded because of the possibility of thrombosis. On the other one, however, both adhesion and growth of endothelial cells, lining blood vessels, are welcome. This situation makes it difficult to design and to produce an appropriate implant surface.

The above difficulties are additionally deepened by the susceptibility of artificial surfaces to bacterial colonization, also mediated by the adsorbed proteins. Once a microbial biofilm is formed, it is extremely difficult to be overcome, which very often causes health problems. Tissue–surface interactions on a molecular level and a possible risk resulting from implant presence in the human body are described by WALKOWIAK [8].

The aim of the present work was to examine the interactions of parylene C, coating medical steel substrates, with such selected biological objects as: blood plasma proteins, platelets, endothelial cells and *E. coli* cells.

2. Materials and methods

The samples for study were prepared as follows: round bar (8 mm in diameter) of commercially available stainless steel (AISI 316 L) was cut into 3-mm thick discs. The samples obtained by means of machining or polishing procedures were then coated with parylene. The coating procedures were carried out in

a self-designed parylene deposition reactor [9] based on the process presented schematically in figure 3. Before each coating procedure, substrates were put in the reactor's deposition chamber and a carefully weighed amount of the dimer was placed in the sublimer. For one μm of the coating thickness we need approximately 1 g of the dimer. After the evacuation of the reactor down to the pressure of 1 Pa, the pyrolysis oven was heated up to 650 °C. When the oven reached the desired temperature, another heater, heated up to 150 °C, was inserted onto the sublimer. This initiated the deposition process, which was later carried out up to a complete expenditure of the dimer. Medical steel was coated with parylene powder particles (ca. 1-mm mean diameter) which was carried out in a tumbler rotating reactor.

Hydrophobicity of the surfaces studied was estimated by the measurement of a contact angle of a drop of deionized water. The values of the contact angle were determined with the use of commonly available software Image J.

For testing the plasma protein adsorption under flow conditions, parylene C was deposited onto commercially available glass plates of the sensor precoated with gold (SIA kit Au, BIACore AB, Uppsala, Sweden). For the proper operation of the employed BiaCore X instrument, the thickness of parylene layer must not exceed 20 nm.

Adsorption of blood plasma proteins at parylene C surface, under flow conditions, was measured with BiaCore X system (BIACore AB, Uppsala, Sweden). The prepared sensor was subjected to a routine test of sensitivity [10]. Next, the sensor was brought to a contact with flowing diluted blood plasma proteins. Changes in the mass of adsorbed proteins were proportional to the surface plasmon resonance (SPR) signal. For experiments we used human blood plasma diluted 1000 times, and the flow of plasma proteins was changed in the range between 10 and 100 μl/min. As a reference surface, pure gold film was used.

Interaction of parylene C surface with platelets was studied by a standard method developed in our laboratory [11]. Blood used for experiments, accepted by the Bioethical Committee of the Medical University of Łódź, was collected from healthy volunteers. The donors have not been treated with any antiplatelet drugs for at least two weeks prior to the examination. The investigated surfaces were immersed in the whole citrated blood at 37 °C for one hour. Blood was constantly kept in motion by end-to-end mixing. Thereafter, the samples were rinsed twice in 0.1 M phosphate buffer, pH 7.4. The fixing procedure was carried out with glutaraldehyde and sample dehydration was achieved with ethanol used in an increasing concentration. Finally, the surface was sputtered with a thin layer of gold. Quantitative analysis of SEM images, obtained from thirty randomly selected areas, was carried out for every sample.

Endothelial immortalized cell line EA.hy 926 was used for the experiment [12]. Cells were grown, in the presence of medical steel powder particles both coated with parylene or not coated, to 80% of confluence. For a control, cell culture without any powder particles was employed. Total RNA was then isolated from the cells. The purity and quality of the RNA obtained were evaluated by electrophoresis in 1.5% agarose gel. As the next step, cRNA was obtained in PCR reaction, and then it was amplified and labelled with biotin-16-dUTP. The synthesized molecular probes (biotin-cRNA) were used for hybridization (12 hours) with oliogonucleotide fragments immobilized on commercially available arrays (SuperArray). Chemiluminescent reaction was triggered by adding CDP-Star (1,2-dioxetane) and recorded by means of autoradiography. Gene expression was determined by the spot darkness analysis on X-ray film (Kodak) by using Image ScannerII and ImageMaster 2D software (Amersham Biotech.). For proliferation experiment the cells were grown on the parylene C surface coating the disc of medical steel substrate. Proliferation and viability of the cells were tested with bis-benzimide (live cells) and propidium iodide (dead cells) fluorescence probes [13].

E. coli cells were cultured on the surface of parylene C coating a medical steel substrate. The samples were incubated for 24 h in a medium containing *E. coli* cells (DH5α strain) at 37 °C under stationary or flow conditions. An electromagnetic stirrer set at 150 or 350 rpm forced the rotational flow. After incubation, sample surfaces were extensively washed with deionised water and labelled by immersion in 10 ml of a fluorescent dyes solution. The solution contained two fluorescent dyes, bis-benzimide and propidium iodide, which made the visualization of both live and dead cells possible [14].

Both *F*-Snedecor's test and unpaired Student's *t*-test or alternatively nonparametric ANOVA test were used for statistical analysis of the results. The values of $p < 0.05$ were considered as significant.

3. Results

Independently of the way of preparing medical steel samples, parylene C coatings increase their surface hydrophobicity (figure 4).

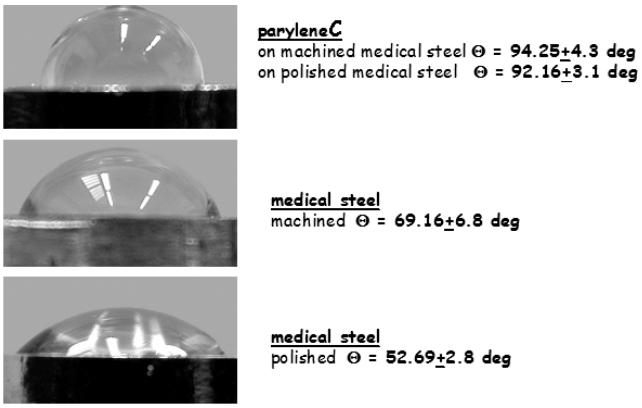

Fig. 4. Photographs of the drop of deionised water (20 µl) on the surfaces examined. The measured values of contact angle are presented next to the photographs

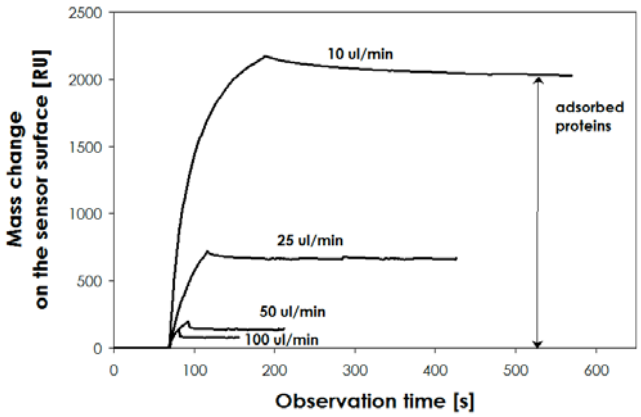

Fig. 5. Example of sensograms obtained for blood plasma proteins flowing by parylene C surface

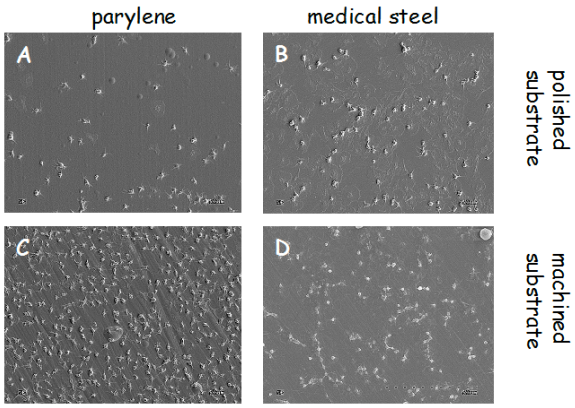

Fig. 6. Platelets adhered to the surfaces studied. A and C – parylene C coating samples of polished and machined medical steel, respectively, B and D – polished and machined medical steel, respectively

Protein adsorption at parylene C surface was assessed at different flow rates, i.e. 10, 25, 50, and 100 µl/min. It is evident that an increase in shear stress, with increasing flow rate, caused a decrease in the amount of adsorbed proteins (figure 5).

Table 1 summarizes the results of measurements of blood plasma proteins adsorption on parylene C and on control gold surfaces.

One-hour contact of the samples tested with citrated whole blood resulted in adhesion of numerous platelets to the surfaces. Only single platelet adhesion, without forming any aggregates, and all three forms of platelet activation were observed for all the samples (figure 6).

The highest and the lowest number of adhered platelets were found respectively on the machined

Table 1. Comparison of mass change on the sensor surfaces as a function of the flow rate. Shear stress was calculated for regular cuboid channel, and amount of adsorbed proteins was estimated from the following approximation: 1 R.U. ~ 1 pg/mm^2

	Gold	Parylene C	Gold	Parylene C	Gold	Parylene C	Gold	Parylene C
Flow (µl/min)	10		25		50		100	
Shear stress (Pa)	0.74		1.92		3.83		7.39	
Mass change (R.U.)	335.9 ±60.8	1186.5 ±62.9	181.3 ±22.5	342.8 ±32.1	139.8 ±19.9	259.4 ±10.3	110.3 ±12.2	204.9 ±56.7
Amount of proteins (ng/mm^2)	0.34	1.19	0.18	0.34	0.14	0.26	0.11	0.20
Significance	S ($p < 0.003$)		S ($p < 0.03$)		S ($p < 0.02$)		NS	

Table 2. Mean number of adhered platelets per surface unit

Surface	Number of adhered platelets per surface unit	Significance
Polished medical steel	1.82±0.18	S ($p < 0.05$)
Polished medical steel coated with parylene C	0.89 ± 0.09	
Machined medical steel	2.45±0.23	NS
Machined medical steel coated with parylene C	3.07±0.24	

medical steel and on the polished medical steel surfaces coated with parylene C. A detailed analysis of the data collected indicates that parylene C deposited onto polished medical steel significantly reduces the platelet adhesion (table 2).

The presence of parylene C powder particles caused any measurable changes neither in endothelial cells proliferation nor in their viability, but the culture of the cells on the parylene C surface significantly reduced cells proliferation and increased their mortality (figure 7 and table 3).

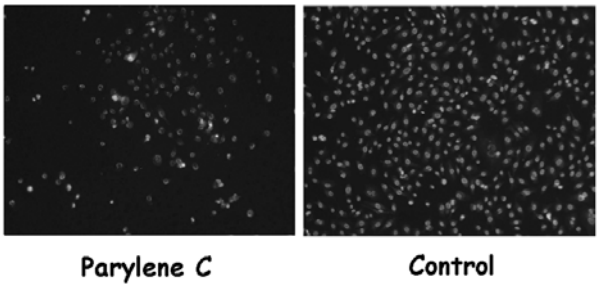

Fig. 7. EA.hy 926 cells grown on the surfaces studied were labelled with with bis-benzimide and propidium iodide

Table 3. Endothelial cells proliferation and mortality assessed for cells cultured on parylene surface. As the control surface a standard COSTAR multi-well cell culture plate was employed. Each sample was subjected to fluorescence microscopy examination, and six randomly selected areas were documented and processed

Surface	Number of independent readings	Mean number of living cells	Mean number of dead cells
Control	6	682 ± 38	5 ± 1
Parylene C	6	77 ± 52	33 ± 5
Significance		S ($p < 0.0001$)	S ($p < 0.0001$)

The culture of endothelial cells in the presence of parylene C coating medical steel substrate resulted in a high level of changes in expression of genes responsible for cell cycle and apoptosis path. It mainly concerns cyclins, cyclin-dependent kinases and genes responsible for apoptosis. Changes occur also in genes, which are responsible for cell proliferation and growth. The gene expression changes were detected for both materials, i.e. medical steel and parylene C, but parylene C caused more changes and they were more profound (see figures 8 and 9 and table 4).

Table 4. Summary of changes in gene expression observed with cell cycle and apoptosis microarrays. Position of the particular gene on the microarray is presented in parentheses

Gene type	Polished medical steel	Parylene coating polished medical steel
OligoGEArray® Human Cell Cycle microarray		
	(gene position on the cell cycle microarray)	
S phase and DNA replication	overexpression (89) suppression (109, 113)	overexpression (89, 113) suppression (85, 109)
G2 phase and G2/M transition	overexpression (5, 26) suppression (4)	overexpression (5, 12, 26, 81) suppression (4)
G1 phase and G1/S transition	suppression (36)	overexpression (36) suppression (23)
Cell cycle checkpoint and cell cycle arrest	overexpression (16, 55, 56) suppression (8, 36, 50, 57, 62, 82, 83, 112)	overexpression (16, 36, 50, 55, 56, 57) suppression (8, 27, 62, 63, 82, 83, 96, 112)
Regulation of the cell cycle	overexpression (17, 33, 35, 42, 60, 67) suppression (2, 22, 24, 28, 41, 48, 51, 69, 75, 90, 94, 95)	overexpression (17, 33, 35, 41, 42, 60, 67, 75, 94, 95) suppression (2, 22, 24, 28, 48, 51, 66, 70, 90)
Negative regulation of the cell cycle	overexpression (13) suppression (112)	overexpression (13) suppression (112)
M phase		suppression (98)
OligoGEArray® Human Apoptosis microarray		
	(gene position on the apoptosis microarray)	
p53 and DNA damage response	overexpression (3, 67)	overexpression (3, 67)
TNF receptor family	overexpression (84) suppression (80, 87)	overexpression (84) suppression (80, 87)
Death domain family	overexpression (66, 108)	overexpression (66, 108)
Bcl-2 family	overexpression (34, 73) suppression (13)	overexpression (73) suppression (13)
IAP family	overexpression (28) suppression (25)	suppression (25)
Caspase family	suppression (50)	suppression (50)
Anti-apoptosis	suppression (70)	suppression (70)

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