IN SITU-FORMED BACTERIAL EXOPOLYSACCHARIDE (EPS) AS A POTENTIAL CARRIER FOR ANCHORAGE-DEPENDENT CELL CULTURES

Piotr Komorowski[1,2*], Agnieszka Kołodziejczyk[1], Krzysztof Makowski[1], Sylwia Kotarba[1], Bogdan Walkowiak[1,2]

[1] Bionanopark Ltd,
Dubois 114/116, 93-465 Lodz, Poland
[2] Department of Biophysics,
Institute of Materials Science and Engineering,
Lodz University of Technology,
Stefanowskiego 1/15, 90-924 Lodz, Poland
*e-mail: p.komorowski@bionanopark.pl

Abstract

The study involved the use of a bacterial strain isolated from environmental samples which produce the biopolymer in the form of pellets in the submerged culture. This material (bacterial exopolysaccharide) is produced by bacteria of the Komogateibacter xylinus which are prevalent in the environment. The aim of this study was to characterize bacterial exopolysaccharides and commercial dextran-based "microcarriers" in terms of their roughness and cell culture effects, including the morphology and viability of the human hybridoma vascular endothelial cell line EA.hy926. The pellets were characterized using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The resulting structures were used for cell culture of adherent cells (anchorage-dependent cells). At the same time, the cultures with commercial, dextran-based "microcarriers" were carried out for comparative purposes. After completion of the cell culture (24 hours of culture), the cellulose and commercial "carriers" were analyzed using SEM and AFM. Finally, the obtained cell densities (fluorescence labelling) and their morphological characteristics (SEM) were compared. The obtained results strongly support the applicability of bacterial exopolysaccharide (EPS) in tissue engineering to build innovative 3D scaffolds for cell culture, the more so that it is technologically possible to produce EPS as spatially complex structures.

Keywords: *bacterial exopolysaccharides, dextran-based "microcarriers", scanning electron microscopy, atomic force microscopy, roughness parameters, three-dimensional cell culture*

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Introduction

Bacterial exopolysaccharides (EPSs) have various environmental functions and different physicochemical properties, depending on their structure. One of the first microbial exopolysaccharides to be characterized was microbial cellulose, which was discovered over 100 years ago [1]. This material is produced by *Komogateibacter xylinus* bacteria that are prevalent in the environment [2]. EPSs protect microbial cells in their natural environments against unfavorable conditions, such as drying, osmotic stress, antibiotics, toxic compounds, phagocytosis, etc. [3,4]. In addition to this, the EPS layer protects *Komogateibacter xylinus* bacteria environment against the competitive colonization by other microbial species. Furthermore, the presence of a gelled polysaccharide layer around a cell may have a predominant effect on its diffusion properties both into and out of the cell [5]. Among other functions, the polymer allows cells to remain on the surfaces of different liquids, where they have access to oxygen and nutrients. Despite many years of research, work on the potential applications of EPSs is still underway in many research centers around the world, most of which is focused on its use as a film on the surface of culture medium in static cultures [6]. However, only a small number of literature reports have discussed the possibilities offered by using this biopolymer formed as pellets. In such a form, EPSs could be used as blood flow improving agents in medicine, as microcarriers in tissue cultures and also as an immobilization matrix [7]. Many of the biological applications of EPSs have been previously discussed [8-12], mostly in relation to their structure, composition, and molecular weight. Additionally, bacterial EPSs have been explored as agents that could be useful in creating a more environmentally friendly, metal nanoparticle production process. This is due to the fact that they can reduce metal ions to form nanoparticles and by acting as capping agents can also stabilize them [13]. Of all known EPSs, bacterial cellulose (BC) has been studied most thoroughly as a material for dental implants, wound dressings, and as a temporary skin substitute. This research has resulted in the commercialization of several biomedical products [14-17]. Another very important aspect is the structural and spatial arrangement of EPSs, which could constitute a scaffold for eukaryotic cells and their potential use in bone regeneration [18].

In recent years, three-dimensional (3D) cultures of various cell types have become very popular [19-21]. The transition from 2D to 3D cell culture makes it possible to simulate conditions that are closer to the physiological processes observed in the human body [22]. The question is whether EPSs are suitable as matrices of micro scaffolds used for the cell culture and how their physicochemical properties will affect the cell culture. The aim of this study was to characterize bacterial exopolysaccharides and commercial dextran-based "microcarriers" in terms of their roughness and effects on the cell culture, regarding the morphology and viability of the human endothelial cell line EA.hy926. Endothelial cells line the inner surface of blood vessels and are one type of barrier cells responsible for the control of processes involved in transporting substances from barrier tissues to blood and from blood to deeper tissues [23,24]. The study was performed using atomic force microscopy and scanning electron microscopy working in an environmental mode, whereas the endothelial cell viability test was performed by labelling nuclei after the 24 h cell culture.

Materials and Methods

The EPS pellet production by *Komogateibacter xylinus* bacteria was developed in the framework of this work. Two commercial samples of dextran-based microcarrier beads, i.e. Cytodex™ 1 (17-0448-03) and Cytodex™ 3 (17-0485-01) were purchased from GE Healthcare Ltd, UK, and were used as reference samples. The particle size of Cytodex™ 1 and 3 averages 190 and 175 μm when swelled in a liquid solution, respectively. Cytodex™ 3 reference sample contains a layer of acid-denatured porcine collagen on the surface of the microporous matrix. This data refer to the specification sheets provided by the manufacturer. Reference samples were processed according to the manufacturer's manual.

Atomic Force Microscopy and Scanning Electron Microscopy

The substrate topography was measured using NTEGRA scanning probe microscope (NT-MDT, Russia) with NSG-01 cantilevers in the air (with resonant frequency from 87 to 230kHz, NT-MDT Probes, Russia) in a semi-contact mode. To obtain the precise visualization, at least 5 scans were performed of 5, 15, and 50 μm scan size. Additionally, the average roughness parameters (Sq – root mean square, Sa – average roughness, St – area peak-to-valley height, S10z – ten-point height, Sdr – surface area ratio, Sds – density of summits of the surface, Ssc – mean summit curvature) from 15 μm x 15 μm AFM images of the bacterial EPS surface, EPS surface etched with 0.1M NaOH and reference commercial samples were calculated. The statistically significant differences between the samples are marked as (*) for $p < 0.05$ and (**) for $p < 0.01$ (ANOVA).

The scanning electron microscopy (SEM) evaluation was performed using a Quanta FEG 250 microscope (FEI, The Netherlands) equipped with a large-field detector (LFD). The microscope was working in a "wet" environmental mode of scanning electron microscopy (ESEM) at an accelerating voltage of 5 kV, the sample temperature of -15°C, humidity 100%, and pressure 194 Pa. The SEM images were collected in five randomly selected areas.

Cell culture

The human hybridoma vascular endothelium cell line EA.hy926 (ATCC CRL-2922) was grown in culture medium Dulbecco's Modified Eagle Medium (DMEM, ATCC, USA) with 10% of Fetal Bovine Serum (Gibco, USA) and Penicillin/streptomycin cocktail (ATCC, USA) solution at 37°C in a humidified atmosphere of 5% CO_2. All the cell culture reagents were sterile. The cell culture protocol is consistent with our previous work [25]. The cells were passaged every 2-3 days when reached 90-100% confluence on the cell culture dishes. The cells were detached from the culture dishes with Trypsin-EDTA (ATCC, USA) applied for 3 min, and then neutralized in the 10-fold volume of complete DMEM medium. Next, the cells were centrifuged for 5 min at 800 rpm and seeded at the desired density on specified substrates (EPS-etched samples and cytodex microcarries). The sterile substrates were placed into a 96-well plate prior to seeding the cells. All types of the samples were processed in 5 replicates.

Cell morphology

A scanning electron microscope was used to observe differences in cell morphology on the surface of the tested samples. After 24 h of incubation with the suspension of cells, the culture medium was removed and the samples were rinsed twice with phosphate-buffered saline (PBS) and fixed for 1 h at 4°C with 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer.

Fluorescence labelling

The evaluation of the proliferation rate of EA.hy926 cells on the tested substrates was performed by measuring the blue fluorescence of the cells nuclei stained with Hoechst 33342 dye (Life Technologies, USA), which intercalates into DNA. The fluorescence was measured by Multifunctional microplate reader Victor X4 (Perkin-Elmer, USA). Each experiment was repeated in triplicate, and five independent procedures were performed for each experiment.

Results and Discussions

Characterization of EPS pellets by AFM

In FIG. 1, the surface topography of EPS pellets obtained for the selected growing time periods of 6 h (FIG. 1A, D), 10 h (FIG. 1B, E), and 24 h (FIG. 1C, F) is presented. Due to the larger scanning range of the surface (upper images), *Komogateibacter xylinus* bacteria can be observed, as well as the global pattern of EPS. To accurately compare the cellulose fibers themselves, AFM was performed for a smaller surface scan range of about 5 μm in the areas between the bacteria. The AFM images show a random microfiber network of cellulose chains, in which the length and arrangement of the fibers change with the growth time (FIG. 1D, E, F). FIG. 2 shows a comparison of the roughness parameters for bacterial cellulose in the form of pellets obtained for different growth times. The highest roughness values were obtained for 24 h, therefore this EPS growth time was used for further experimental purposes.

In the next step, etching with NaOH was carried out in order to remove bacteria. The surface topography of etched EPS pellets was compared with commercially available "microcarrier" beads (FIG. 3). Both reference samples present a microporous matrix, but with different packing densities and pore sizes. TABLE 1 shows the mean values of the roughness parameters for commercial samples and for the etched EPS. Based on the obtained results, one can conclude that the highest roughness values are observed for the etched EPS samples. Substrates with higher roughness values are most often characterized by better eukaryotic cell adhesion [26]. Nano/micro-topography stimulates behavioral changes in cells and plays a critical role in modifying their proliferation and vitality, as well as the strength of adhesion to substrates [27].

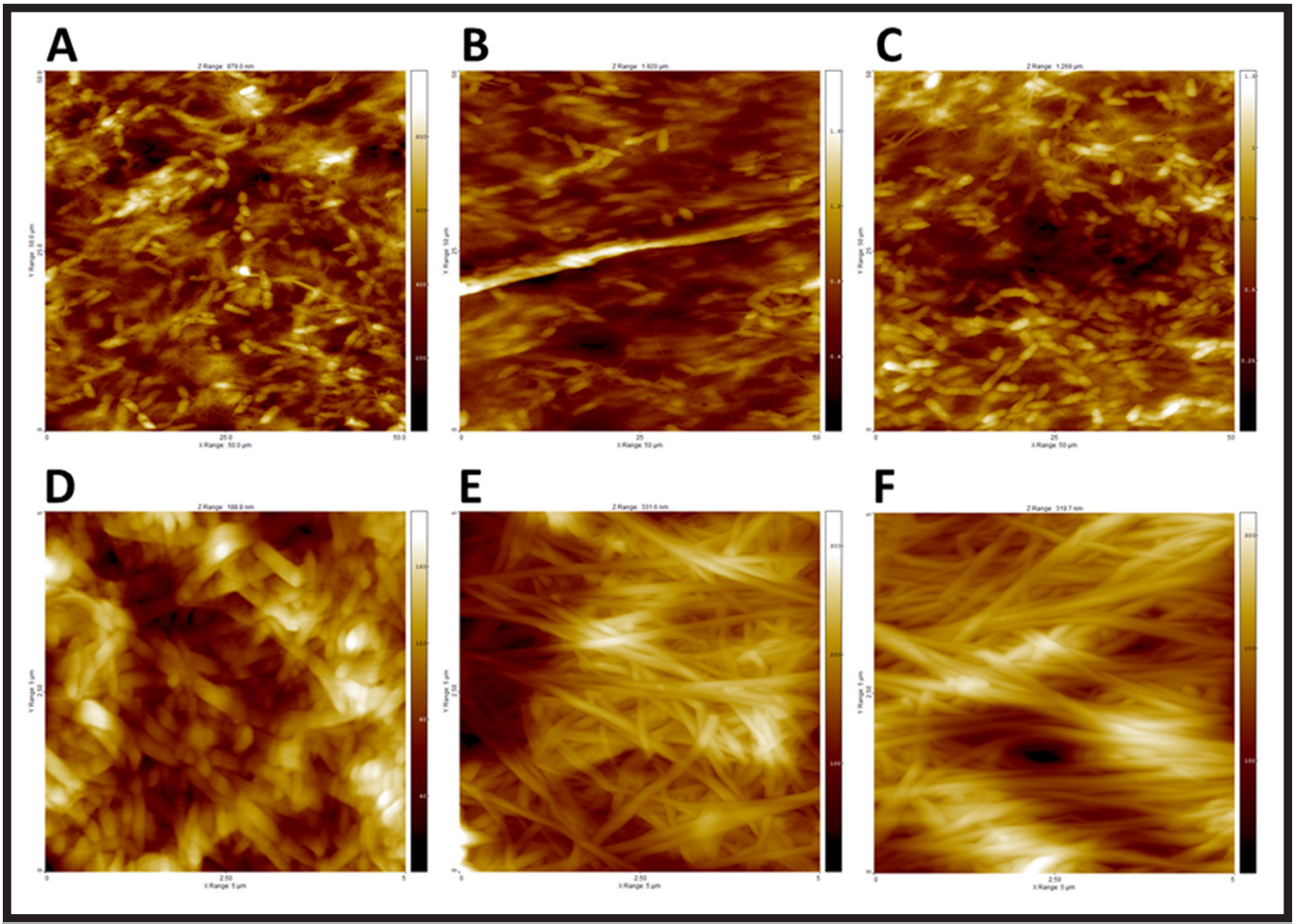

FIG. 1. AFM images of EPS surface for selected growing time periods, scan sizes 50 µm x 50 µm (A-C) and 5 µm x 5 µm (D-F).

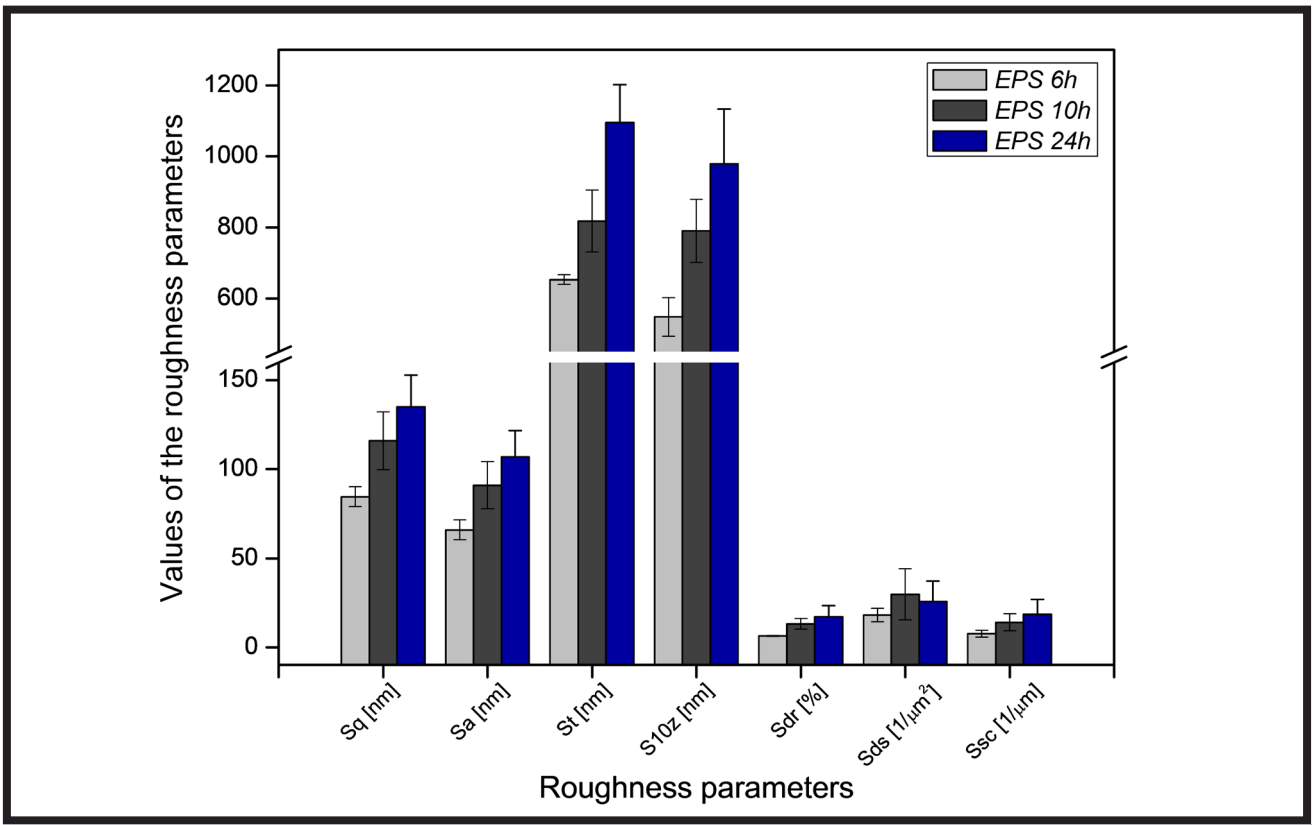

FIG. 2. Values of selected roughness parameters for EPS samples.

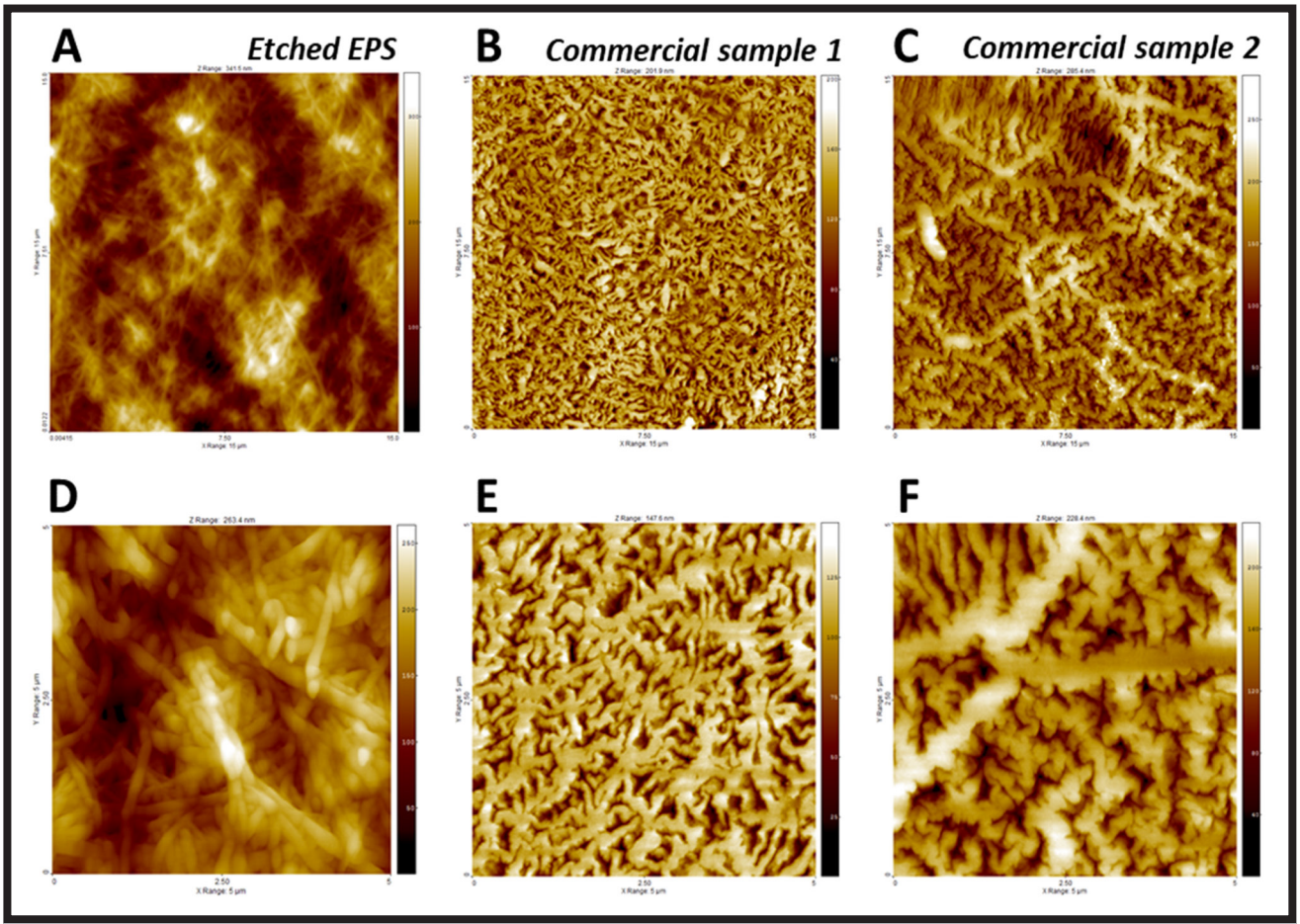

FIG. 3. AFM images of etched EPS surface and reference samples, scan sizes 15 µm x 15 µm (A-C) and 5 µm x 5 µm (D-F).

TABLE 1. Average values of roughness parameters for commercial samples and etched EPS.

Roughness parameters	Commercial sample 1	Commercial sample 2	Etched EPS (0.1M NaOH)
Sq [nm]	25.69 ± 1.21	37.77 ± 1.04	63.89 ± 15.03
Sa [nm]	19.96 ± 1.37	29.42 ± 0.31	51.65 ± 12.32
St [nm]	281.40 ± 123.17	359.63 ± 62.88	434.69 ± 76.28
S10z [nm]	247.74 ± 79.95	324.90 ± 71.80	394.56 ± 75.48
Sdr [%]	13.77 ± 5.31	9.47 ± 0.10	2.55 ± 1.29
Sds [1/um²]	68.64 ± 16.58	56.75 ± 3.29	18.49 ± 3.59
Ssc [1/um]	13.66 ± 1.64	13.88 ± 0.52	6.17 ± 1.58

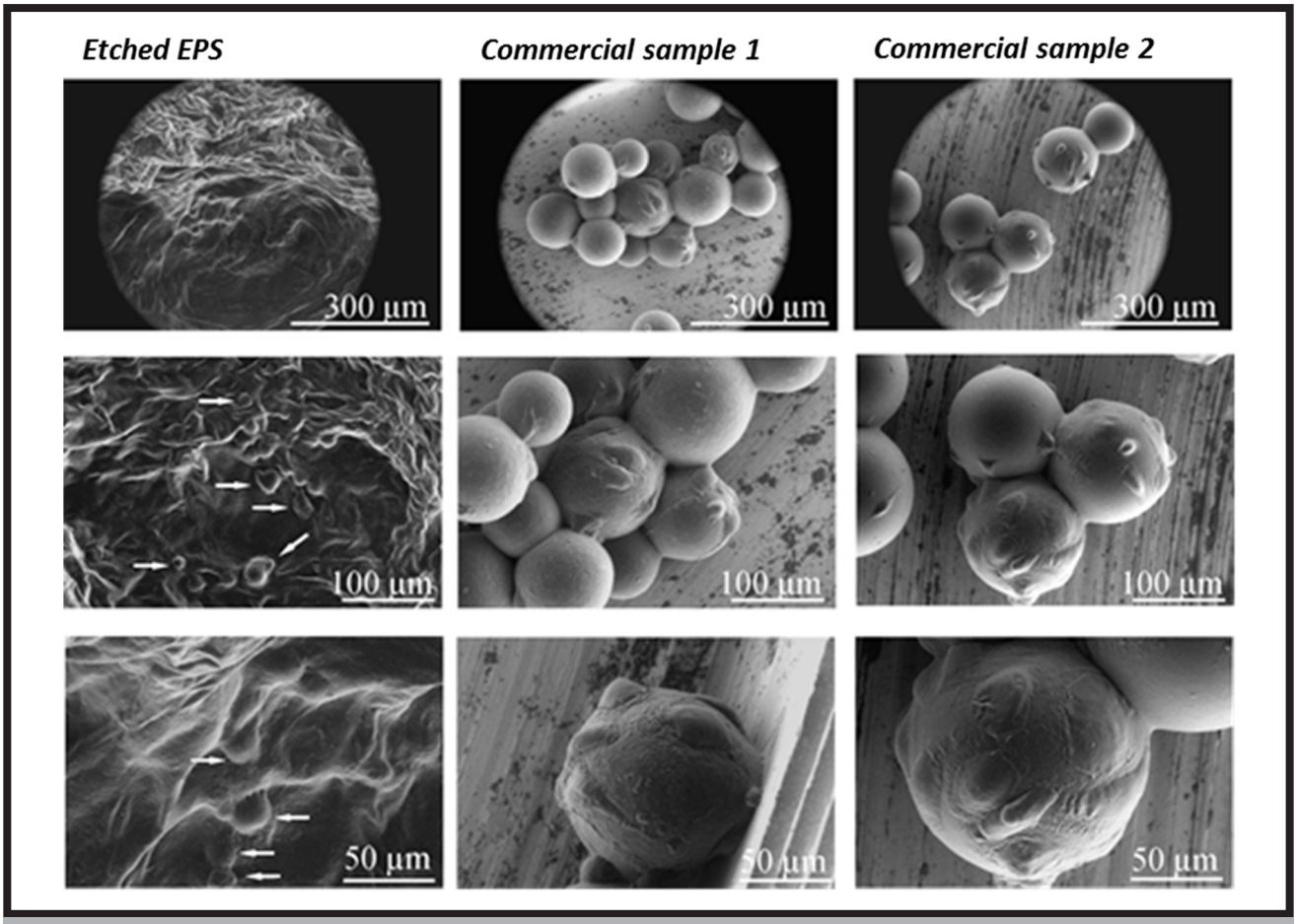

FIG. 4. SEM images of etched EPS surface and reference samples after 24 h of endothelial cell culture. White arrows indicate the EA.hy926 cells on the etched EPS surface. SEM images were collected for different magnifications, the scales are marked on the images.

Cells morphology visualized with SEM and fluorescence labelling

The well-flattened endothelial cells adherent to the selected substrates (etched EPS strains and commercial samples 1 and 2) can be observed in the SEM images (FIG. 4). On the etched-EPS pellets after the cell culture, the central parts of the endothelial cells can be distinguished (the areas with the cell nucleus marked with the white arrows). It can be concluded that EA.hy926 cells adhering to the etched-EPS pellets conform to their undulating surface. In the case of the reference samples, we found both single cells and whole microcarrier beads covered by endothelial cells. The highest cell proliferation values were obtained for the endothelial cells cultured on an etched EPS sample (statistically significant difference, $p < 0.05$). For commercial sample 1 and commercial sample 2 there were no differences comparing to the control sample, as shown in FIG. 5. This may indicate the good properties of the EPS substrate, produced by *Komogateibacter xylinus* bacteria, for the adhesion and proliferation of endothelial cells. The adhesion of cells to the surface of the biomaterial is very important for proper integration with tissues and should be taken into account when designing new biomaterials [28]. Many proteins from the extracellular matrix (including fibronectin, collagen, laminin, etc.) as well as cytoskeleton proteins (e.g. actin, vinculin) and membrane receptors (integrins) are involved in the cells adhesion processes to the 2 or 3D substrates. Mainly the interactions between these proteins and their specific receptors induce signal transduction, which in turn influences cell growth and differentiation [29].

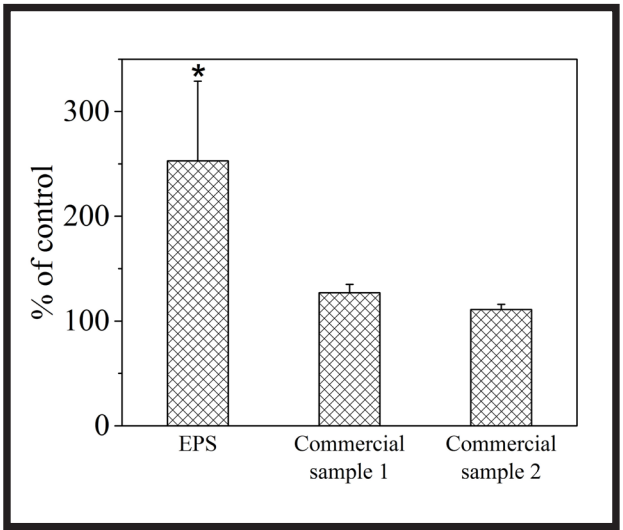

FIG. 5. Proliferation of endothelial cells (EA.hy926) on different surfaces after 24 h of cell culture.

Conclusions

In the present study, an innovative model based on bacterial exopolysaccharide was developed for potential three-dimensional cell cultures. It can be concluded that extending the time of bacterial cellulose synthesis results in higher parameters of its surface roughness. Moreover, the roughness parameters (Sq and Sa) for the etched EPS surface are much higher, as compared to the commercial reference samples, which may suggest better adhesion of the cells grown on the EPS surface. This paper also shows the importance of fully characterizing the substrate topography and measuring roughness parameters. Nano/micro-topography has been recognized as fundamental in the design of bio-inspired materials. The obtained results strongly support the idea of using bacterial EPS in tissue engineering to build innovative 3D scaffolds for cell culture, more so that it is technologically possible to produce EPS in the form of spatially complex structures.

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ORCID iDs

P. Komorowski: https://orcid.org/0000-0002-4035-7501
A. Kołodziejczyk: https://orcid.org/0000-0003-3184-4831
K. Makowski: https://orcid.org/0000-0002-7885-3420
S. Kotarba: https://orcid.org/0000-0003-1490-0627
B. Walkowiak: https://orcid.org/0000-0003-4867-5456

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