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Original paper

Surface characterization, collagen adsorption and cell behaviour on poly(L-lactide-*co*-glycolide)

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Poly(L-lactide-*co*-glycolide) (PLG) was modified through the adsorption of collagen to improve the behaviour of fibroblasts and osteoblasts. As reference materials cell-resistant polystyrene (PS) and cell-conductive tissue-culture polystyrene (TCPS) were also evaluated. The physicochemical surface properties of the materials were studied by X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and water contact angle measurements. The morphology of cells was examined using optical microscopy, while their growth was evaluated by both crystal violet and MTT tests. Nitric oxide level and protein concentration were tested in cell supernatants. The results showed that the adsorbed amount and the organization of the adsorbed collagen were influenced by surface hydrophobicity. Cell culture experiments on native substrates revealed that cell attachment, spreading and growth enhanced, depending on the substrate, in the following order: PS<PLG<TCPS. Coating the substrates with collagen led to distinct changes in the cell behaviour: the cells were more numerous, better spread and more homogeneously distributed on the surface compared to the bare polymers. Improvements in cell growth and protein secretion were also observed. The results obtained show that surface modification of PLG by simple adsorption of collagen promotes the distribution and proliferation of fibroblasts and osteoblasts.

Key words: *collagen, poly(L-lactide-co-glycolide), AFM, XPS, cell growth*

1. Introduction

Adsorption of proteins at solid surfaces is an important process in a wide range of applications, including in vitro cell growth and implant biocompatibility [1], [2]. The organization of protein layers is affected by specific properties of individual proteins, as well as by particular properties of the surfaces [3]. The adsorbed proteins influence subsequent cell reactions [3], [4]. Thus, the biomaterial surface can be designed in such a way as to specifically adsorb proteins, which would enable the cell/material interactions to be controlled [5]. Depending on the requirements, it is possible to design protein-repulsive surfaces which prevent cell adhesion (e.g., for blood-contacting devices), and substrates which promote cell adhesion and differentiation (e.g., for skin and bone substitutes) [6].

Collagen is an extracellular matrix (ECM) protein, abundantly found in living organisms (in the animal kingdom, collagen constitutes 20–40% of all proteins). Type I collagen occurs in skin, bones and tendons [7]. The type I collagen molecule (molar mass of 300 000 g/mol, length of 300 nm, diameter of 1.4 nm) is a semi-flexible triple helix, made of three polypeptide chains wrapped together. Collagen is involved in tissue structuring and cell-recognition processes [8]. Through an influence on the interactions between the

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cells and ECM, it controls the processes of growth, differentiation, metabolism and gene expression. Therefore, modification of the surface properties using collagen being able to provide cell-recognition sequences can improve cell alignment, adhesion and proliferation [9]–[11].

PLG is an amorphous copolymer of lactide and glycolide. The ester linkages in the copolymer backbone allow its gradual hydrolytic degradation. PLG has been used as degradable sutures and elements for osteosynthesis [12]. More recently it has been often developed as drug delivery systems and scaffolds for tissue engineering applications [13]. PLG possesses attractive bulk properties, such as degradability, resorbability and relatively high tensile strength. On the other hand, the PLG surface is not most advantageous for cell adhesion. Our preliminary studies show that initial adhesion of fibroblasts (4 h of culture) can be improved when the PLG surface is modified through adsorption of type I collagen [14].

The first aim of the present study was to find a relationship between surface properties of PLG and collagen adsorption process. The second aim was to examine the effect of the adsorbed collagen on PLG surface on fibroblasts' and osteoblasts' behaviour. Two model substrates: polystyrene (PS) and tissue-culture polystyrene (TCPS) were also analysed as reference materials. PS is regarded as a cell-resistant material, while TCPS is known for good cell attachment and spreading on its surface [14], [15]. This research is mainly concerned with the modification of a resorbable biomaterial surface (PLG) through collagen adsorption with the aim to provide adhesion sequences for cell receptors. In particular, the effect of adsorbed collagen on cell morphology, growth and secretion was examined.

2. Materials and methods

2.1. Samples' preparation

PLG with the molar ratio of the comonomers being 85:15, M_n = 51 kDa, d = 2.2 (from the Centre of Polymer and Carbon Materials, PAN, Zabrze, Poland) was synthesized with an initiator – zirconium(IV) acetylacetonate, $Zr(acac)_4$. The synthesis of PLG was described in detail elsewhere [16]. The foils were slip-casted from 10% (w/v) polymer solution in dichloromethane (POCh, Gliwice, Poland) on glass Petri dishes. After air drying for 24 h and vacuum drying for the next 72 h, the polymer films were detached from the Petri dishes. PS (M_n = 140 kDa, d = 1.8; from Sigma-Aldrich, Germany, the foils obtained as above) and TCPS, originating from 24-well multidishes (Nunclon, Denmark), were used as reference materials.

Type I collagen (from calf skin; catalog no. C8919, Sigma, Germany) was received as an aqueous solution in citric acid (1 mg/ml, pH 3). Dilution to 40 μg/ml was performed in phosphate buffered saline (PBS) (137 mM NaCl, 6.44 mM KH_2PO_4, 2.7 mM KCl, 8.0 mM Na_2HPO_4, all chemicals from POCh, Gliwice, Poland, or Merck, Darmstadt, Germany). The PLG and PS samples were placed in cell culture wells and 2 ml of the collagen solution at 37 °C were added. The collagen solution was also poured into empty wells (TCPS). The adsorption was performed for 2 h at 37 °C. The samples were rinsed 10 times with ultra-high quality water (UHQ-water, produced in the UHQ-PS apparatus from Elga, UK or in the Milli-Qplus apparatus, Millipore, France) without a complete removal of the solution, by pumping out 1 ml of the liquid and adding 1 ml of the UHQ-water. Then, the samples were dried by flushing with a gentle nitrogen flow (fast drying) for about 10 s and stored in a desiccator prior to use [17].

2.2. Surface characterization

2.2.1. Atomic force microscopy

The topography measurements of the polymer substrates were carried out with an Explorer atomic force microscope (Thermomicroscopes, Vecco, USA). Contact-mode topographic images were recorded in air using Si_3N_4 tips with a spring constant of 0.05 N/m and with a nominal radius of curvature of 20 nm (Veeco NanoProbeTM Tips, model MLCT-EXTM-A). The images were recorded with the scan area of 5 μm × 5 μm for three randomly chosen places (300 × 300 data points) and at a scan rate of 3 lines/s. All the images were flattened using the third-order polynominal algorithm provided with the instrument. Based on the software SPMLab602, the root-mean-square roughness (R_{RMS}) was calculated.

2.2.2. X-ray photoelectron spectroscopy (XPS)

The XPS spectra were recorded using a Kratos Axis Ultra spectrometer (Kratos Analytical-UK), equipped with an aluminium anode and a quartz monochromator. The angles between the normal to the sample and the

direction of photoelectron collection were 0° or 60°. The analyzed area was approximately 700 μm × 300 μm. The pressure in the analysis chamber was about 10^{-7} Pa. Charge stabilization was achieved by using the Kratos Axis device. The pass energy of the hemispherical analyzer was set at 160 eV for the survey spectra and at 40 eV for narrow scans. In the latter conditions, the full width at half maximum (FWHM) of the Ag $3d_{5/2}$ peak of a standard silver sample was about 0.9 eV. The following sequence of spectra was recorded: survey, C_{1s}, O_{1s}, N_{1s}, Si_{2p} and Zr_{3d}, and C_{1s} again to check the charge stability as a function of time and the absence of sample degradation during the analysis.

The data treatment was performed with the CasaXPS software (Casa Software Ltd, UK). The binding energy scale was fixed by setting the component due to carbon only bound to carbon and/or hydrogen (C-(C,H)) at 284.8 eV. A linear background subtraction was used. Intensity ratios were converted into molar concentration ratios by using the sensitivity and transmission factors provided by the manufacturer. Peak decomposition was realized by the addition of several components (mixed Gaussian/Lorentzian product function in proportion 70:30) and by obtaining the best fit. Some FWHM and peak positions were fixed [18]. The theoretically expected concentrations of carbon and oxygen were determined for PLG and PS on the basis their chemical structure.

2.2.3. Water contact angle measurement (θ)

The water contact angles of the substrates were measured by the sessile-drop method at ambient temperature and humidity, before and after collagen adsorption with the drop-shape analysis system (DSA 10 Mk2, Krüss, Germany). The UHQ-water droplet volume was 0.2 μl. The final results were obtained by averaging 10 measurements of individual droplets.

2.3. Cell culture

For the cell culture studies, the polymers were sterilized using UV radiation (1 h on each side) and placed in 24-well dishes (Nuclon, Denmark). L929 fibroblasts or MG63 osteoblasts (European Collection of Cell Cultures, Salisbury, UK) were seeded on both raw polymeric materials and polymers after collagen adsorption, at the initial density of 3×10^4 cells per well in 1 ml of RPMI 1640 culture medium (Sigma, Germany), supplemented with 10% fetal bovine serum (ICN, USA), 1% *L*-glutamine (Sigma, Germany) and antibiotics: penicillin (100 μg/ml) and streptomycin (100 μg/ml) (Sigma, Germany). The cells were cultivated for 1, 3 and 7 days at 37 °C.

2.3.1. Cell morphology and growth

The mass of adhered cells was measured by means the crystal violet test (CV), according to the method described previously [6]. In brief, the samples were rinsed with PBS, fixed in 2% formalin for 1 h at 21 °C and stained with crystal violet (0.5% in 20% methanol, Sigma, Germany) for 5 min. After washing with tap water and drying in air, the cells were examined under an optical microscope. Later, the absorbed stain was extracted using 100% methanol (POCh, Gliwice, Poland). Finally, the optical density (O. D.) was measured at 570 nm with an Expert Plus spectrophotometer (Asys Hitach, Austria). The activity of mitochondrial enzymes of the cells in culture (MTT test) was measured by the reduction of the tetrazolium salt MTT (3-[4,5--dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan. The MTT solution (5 mg/ml) was added to the wells with cells. After 1 h, the reaction was stopped using isopropanol/HCl (0.04 M) and the optical density of the blue dye was determined at 570 nm [6].

2.3.2. Nitric oxide and proteins

The activities of fibroblasts and osteoblasts were evaluated by the Griess method that allows measurement of nitric oxide (NO) concentration in the supernatants collected from cell cultures. Firstly, 100 μl of the supernatant were added to wells of a 96-well plate and then 100 μl of a mixture of the Griess reagent A and B (1:1) were added (A: 1% sulfanilamide in 5% H_3PO_4, B: 0.1% naphtylenediamine in H_2O; Sigma--Aldrich, Germany). After incubation for 5 min, the optical density was measured at 540 nm [6].

Protein concentration in the supernatants collected from the cell cultures was measured by the colorimetric BCA method. A mixture of copper(II) sulfate solution (CS, Sigma, Germany) and bicinchoninic acid solution (BCA, Sigma, Germany) in the ratio of 1:50 was prepared. Later, 10 μl of each sample tested were transferred to wells of a 96-well plate and then 200 μl of the CS/BCA mixture were added. The plates were incubated for 30 min in the dark, and the optical density was measured at 570 nm [19].

2.3.3. Statistics

The data are presented as means ±SEM (standard error of the mean) of three similar experiments carried

out in triplicate. *T*-Tukey test was used for statistical analysis; $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Properties of the polymers before and after collagen adsorption

Table 1 presents the chemical composition obtained by XPS, the measured water contact angles, and the surface roughness deduced from AFM pictures for the polymer substrates before and after collagen adsorption.

The XPS results show that the highest molar percentage of oxygen was detected on raw PLG, and the lowest on PS with an intermediate value on TCPS (table 1). Nitrogen was found in a very small amount on PLG and TCPS. The sample of PLG contained traces of zirconium, being the remnants of the zirconium acetylacetonate used as an initiator in the ring-opening polymerization of this material. The analysis of the surface revealed also the presence of silicon, which is a common contaminant, often detected on materials analyzed by XPS [20].

The XPS analysis was conducted with two angles of photoelectron collection (0° and 60°) (table 2). The depth of analysis is related to the cosine of that angle in such a way that for higher angles of analysis, the signal originates from a layer of smaller thickness [21], [22]. The expected molar percentages of carbon and oxygen functionalities in PLG and PS are presented in table 2 for comparison. It was found that there were significant differences between theoretical and measured amounts of carbon and oxygen on the surface of PLG. The influence of the depth of analysis on the compositions obtained was as follows: the smaller the thickness of the layer analyzed, the higher the contribution of C–(C, H) and the smaller the contribution of C–O and COO. The concentration of Zr did not depend on the angle of photoelectron collection, while the amount of Si was higher for the angle of 0°. The measured surface composition of PS was similar to the expected one, the concentration of

Table 1. Surface chemical composition (except hydrogen) measured by XPS, water contact angle (θ), root-mean square roughness (R_{RMS}) of the polymer substrates before and after collagen adsorption. The average and standard deviations are calculated for $n = 10$ (θ) or $n = 3$ (R_{RMS}, %C, %O, %N). The angle of photoelectrons collection in the XPS analysis was 0°

	Chemical composition (mole %)			Other elements < 1%	θ (°)	R_{RMS} (nm)
	C	O	N			
PLG	67.5 (2.9)	30.9 (3.5)	1.0 (0.0)	Zr, Si	76.9 (1.3)	7.0 (2.2)
PLG + coll	67.8 (0.1)	26.1 (0.2)	5.2 (0.1)	Zr	73.0 (1.8)	6.4 (4.6)
PS	99.1 (0.5)	0.9 (0.5)	bdl	Si[a]	93.7 (2.2)	16.9 (6.6)
PS + coll	78.5 (0.4)	13.4 (0.3)	7.6 (0.2)	Si, Cl[a]	77.8 (1.0)	14.9 (9.4)
TCPS	90.8 (1.5)	8.7 (1.5)	0.4 (0.0)	Si[b]	64.8 (6.1)	7.3 (1.3)
TCPS + coll	78.7 (2.4)	12.7 (1.8)	8.2 (0.8)	S[a], F[a]	38.1 (2.2)	7.1 (1.4)

bdl – below detection limit; [a] – detected on one sample; [b] – detected on two samples.

Table 2. Molar percentage of carbon, oxygen and silicon determined by XPS at the surface of PLG, PS and TCPS, depending on the angle of photoelectron collection (0° and 60°). Expected molar percentages of carbon and oxygen functionalities in PLG and PS are given for comparison

		C_{1s}			O_{1s}	Si_{2p}	Zr_{3d}	N_{1s}
		C–(C,H)	C–O	COO				
		284.8 eV	286.9 eV	288.9 eV	532.2–533.6 eV	103.0 eV	182.7 eV	400.5 eV
PLG	expected	18.0	20.5	20.5	41.0	0	0	0
	0°	23.5	20.5	20.2	34.9	0.8	0.1	bdl
	60°	28.1	17.6	18.3	34.6	1.3	0.1	bdl
PS	expected	100	0	0	0	0	0	0
	0°	99.7[a]	bdl	bdl	0.3	bdl	bdl	bdl
	60°	99.8[a]	bdl	bdl	0.2	bdl	bdl	bdl
TCPS	0°	80.8	6.8	3.6	8.3	bdl	bdl	0.5
	60°	74.6	7.8	4.8	12.0	0.1	bdl	0.7

bdl – below detection limit; [a] – including C–(C,H) ring, C–(C,H) aliphatic and shake-up.

oxygen being negligible. The amount of oxygen on TCPS increased from 8.3% for the angle of 0° to 12.0% for the angle of 60°.

The lowest and the highest water contact angles among raw materials were measured on TCPS and on PS, respectively (table 1). Representative AFM images of the polymer surfaces, before and after collagen adsorption, are presented in figure 1. The PLG surface was quite smooth, with some scratches but without pores (figure 1A), the surface of PS was porous (figure 1C), while on TCPS many scratches were visible (figure 1E). The surface roughness was similar for both PLG and TCPS, but twice higher for PS (table 1, last column).

After incubation in collagen solution the water contact angles on all the materials examined have been decreased, while their R_{RMS} roughness values practically have not changed (table 1). A protein layer with a fine granular structure was created on the PLG surface (figure 1B). On PS, the collagen formed discontinuous patches (figure 1D), while no features attributable to adsorbed collagen were visualized in the topographic images of TCPS (figure 1F). After conditioning in collagen solution, nitrogen was found in a larger amount on all the samples (table 1). The nitrogen fraction varied from 8.2% for TCPS to 5.2% on PLG.

Figure 2 presents the C_{1s} spectra recorded on all the polymer materials, before and after collagen adsorption, and of freeze-dried collagen powder. The main C_{1s} component in all substrates, at 284.8 eV,

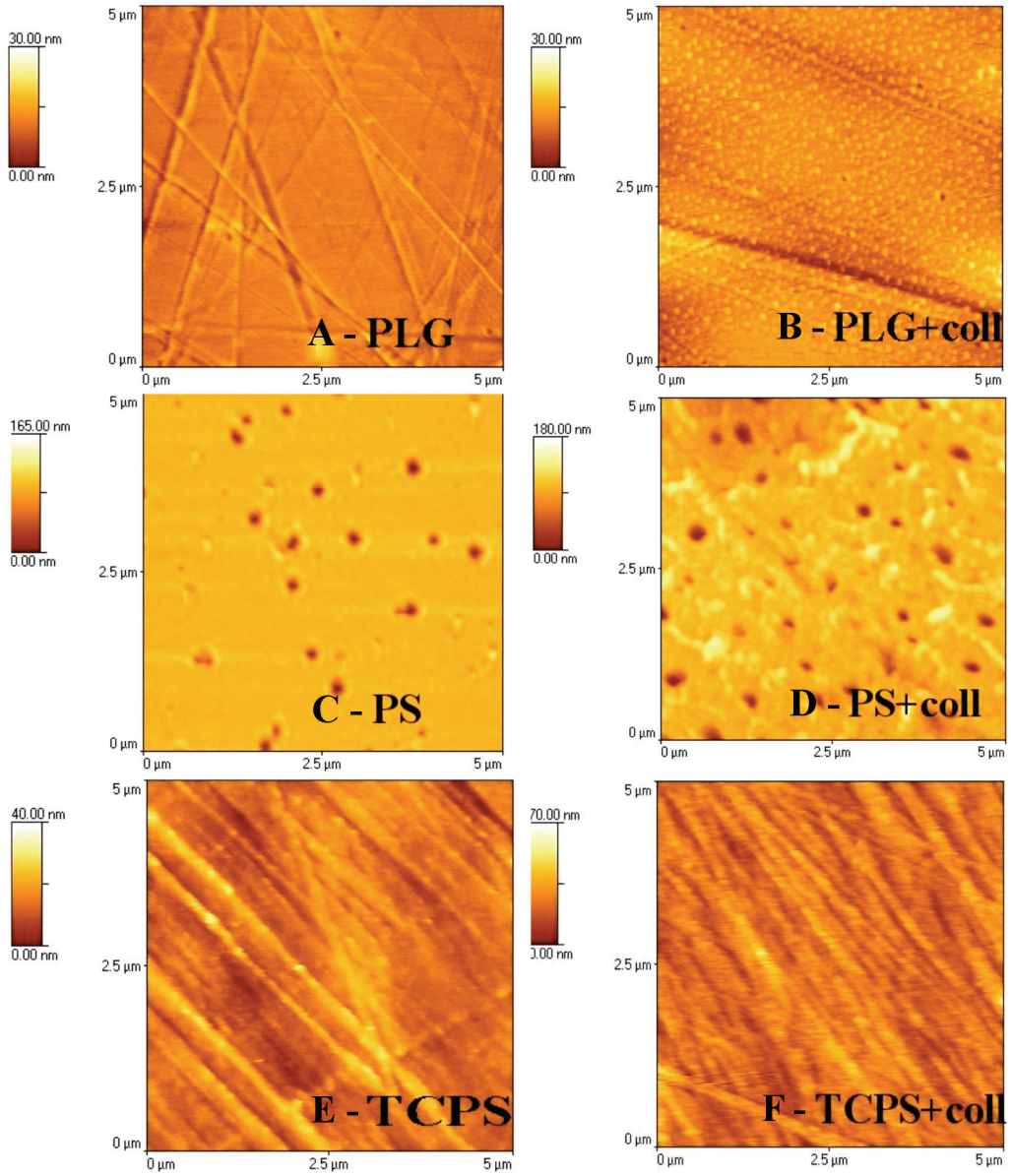

Fig. 1. AFM topographic images (5 µm × 5 µm, topographical height z = 30, 40, 70, 165 or 180 nm) of the polymer surfaces before and after collagen adsorption: A – PLG, B – PLG + coll, C – PS, D – PS + coll, E – TCPS, F – TCPS + coll

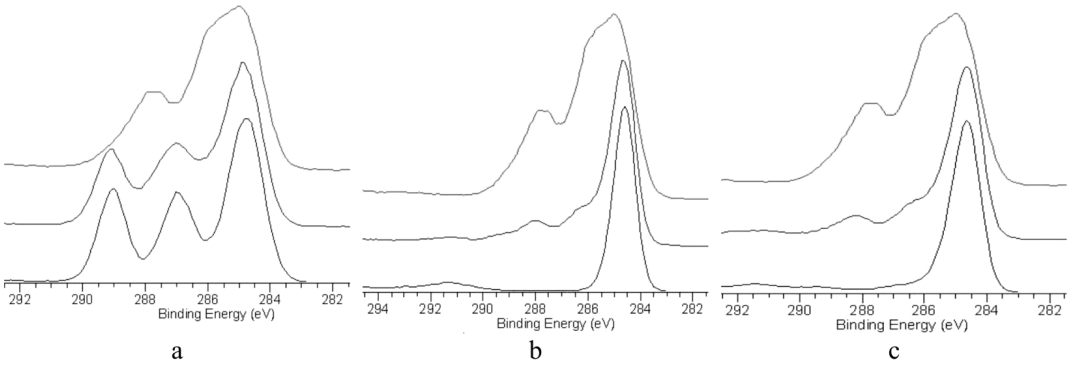

Fig. 2. C_{1s} spectra of PLG (a), PS (b) and TCPS (c) prior to the (bottom) and after (middle) collagen adsorption. The spectrum for a freeze-dried collagen powder (top) is also shown for comparison. The ordinate scale was adjusted to obtain the maximum of the peak at the same height. The angle of photoelectron collection in the XPS analysis was 0°

was, due to carbon, singly bound to carbon and hydrogen C–(C,H). In the spectra of PLG, components at about 286.9 eV and 288.9 eV, attributed to C–O and COO functionalities, respectively, were detected. The component at 291.3 eV present on PS was due to the energy loss attributed to the transition of the aromatic ring (shake-up) [23]. Differences in the C_{1s} spectra of PS and TCPS occurring at binding energies higher than 284.8 eV were due to oxidized carbon functions created by plasma treatment on the surface of TCPS [23]. The spectrum of pure collagen is characterized by three main components, due to carbon singly bound to carbon or hydrogen (C–(C,H)) at 284.8 eV, due to carbon singly bound to oxygen or nitrogen (C–(O,N)) at about 286.3 eV, and due to carbon involved in the peptidic link (amide bond, O=C–N) at about 287.8 eV. After collagen adsorption on the polymers, a relative increase of these two latter components was observed, confirming the presence of collagen.

3.2. Results of cell culture

The morphology of fibroblasts and osteoblasts examined under optical microscope is presented in figures 3 and 4, respectively. On the first day of culture, the fibroblasts on raw PLG were numerous and some of them already spread over the surface (figure 3). After three days, the number of cells increased; also, their spreading area was larger. The seven-day culture had flattened cells, although the whole surface was not covered with the cells. On PS the cells were sparse and round. After 3 and 7 days, the cells were proliferating, but still they stayed grouped together to form aggregates. On TCPS, the cells were spindle-shaped and polygonal. After 7 days, the cells covered the entire surface and formed a uniform layer. Former incubation in collagen solution had improved cell growth. It was particularly visible in the case of PS and PLG, where the number of cells increased significantly and the cells were distributed more homogenously. The fibroblasts were also much better spread than before. On TCPS, no such serious changes were observed.

The osteoblasts cultured on raw PLG were well flattened, although they were grouped together and non-homogenously distributed all over the surface (figure 4). Similar cell morphology was also observed on bare TCPS surface. On PS, the cells were poorly spread, spindle-shaped and formed aggregates. After collagen adsorption, the number and distribution of cells were considerably improved on all the samples. Also, cell spreading area increased, especially in the case of PS+coll.

The results of CV and MTT tests estimating cell growth confirmed the microscopic examinations (figure 5). The number of fibroblasts on the raw polymers increased in the following order: PS < PLG < TCPS (figure 5a, c). Incubation of the substrates in collagen solution led to a significant increase in the cell proliferation on all the materials examined. This tendency was also revealed in the case of PLG (figure 5c), although in this particular sample the difference was not statistically different. The number of osteoblasts on the raw PS was significantly lower than on both TCPS and PLG (figure 5b, d); after collagen adsorption it significantly increased (figure 5b).

Figure 6 presents the amounts of nitric oxide (NO) and proteins released by the cells. Fibroblasts and osteoblasts cultured on all raw polymeric samples released similar levels of NO and proteins. After collagen adsorption the release of NO by fibroblasts was significantly higher in the case of TCPS and PLG (figure 6a). The level of proteins secreted by both fibroblasts and osteoblasts cultivated on the substrates after collagen adsorption showed the tendency to

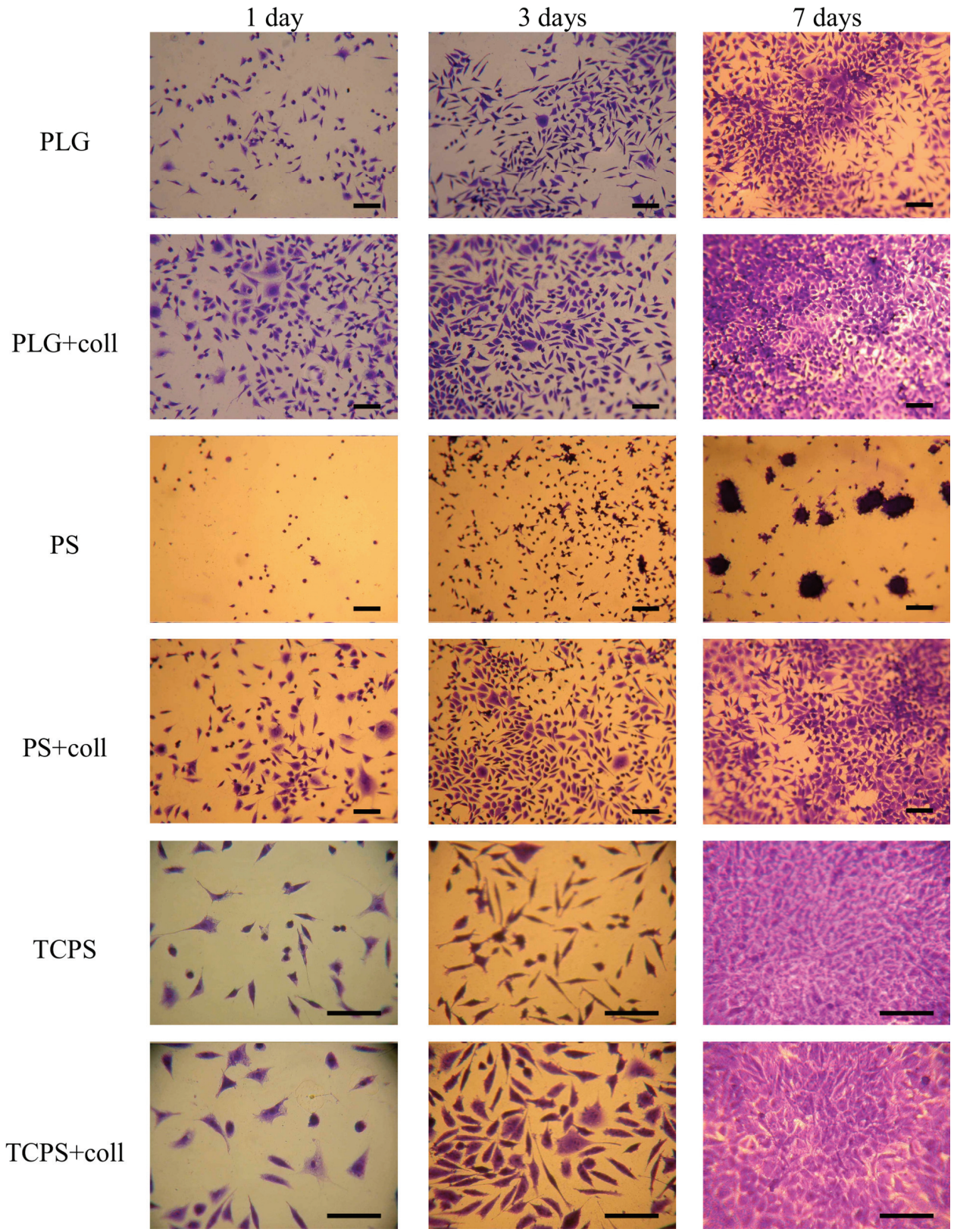

Fig. 3. Morphology of L929 fibroblasts on PLG, PS and TCPS before and after collagen adsorption. Cell culture time: 1 day (panel 1), 3 days (panel 2) and 7 days (panel 3). Morphology of cells was examined under optical microscope with obj. 10× (cells on PLG and PS) or using inverted microscope, obj. 20× (cells on TCPS) after staining with crystal violet. Bar 100 μm

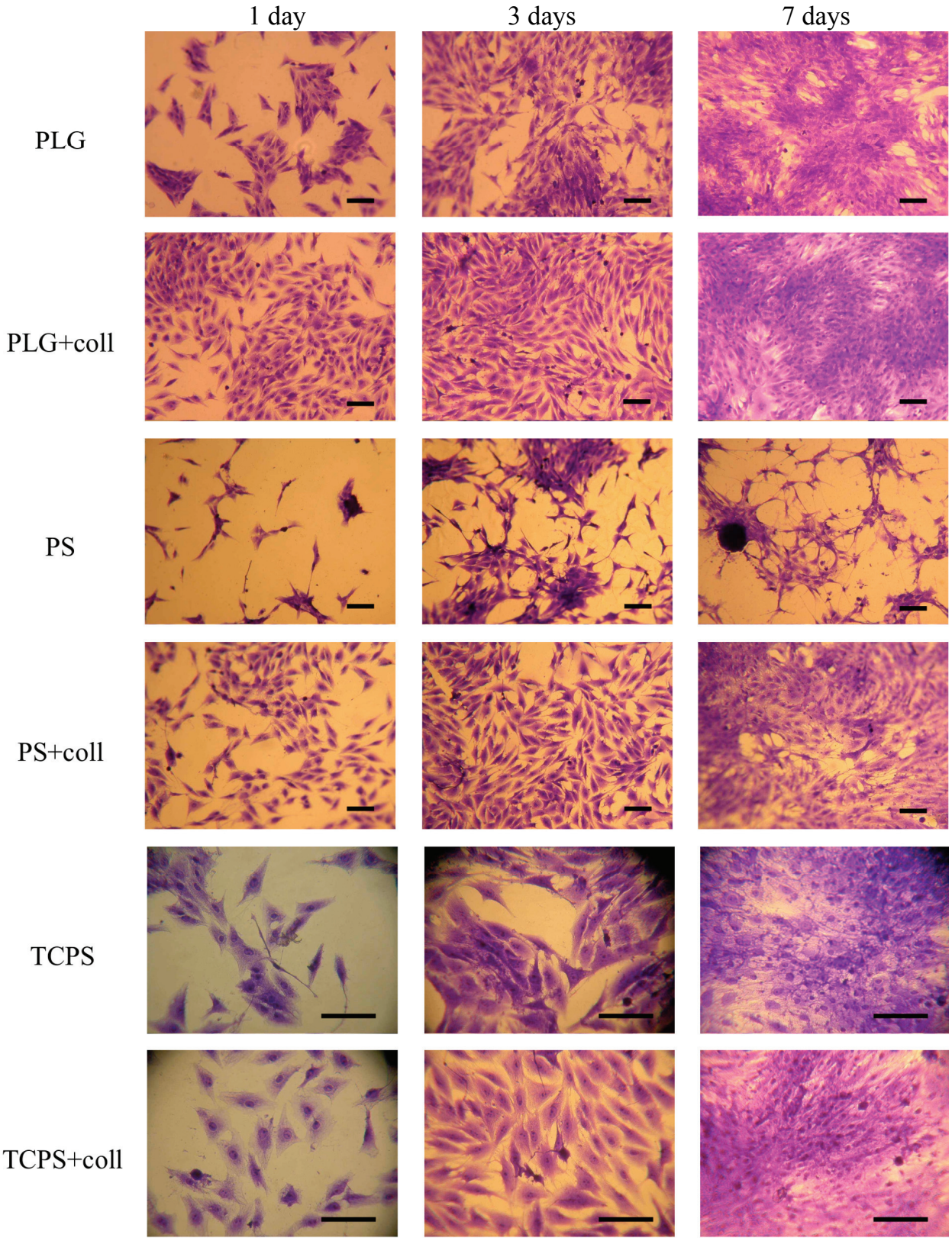

Fig. 4. Morphology of MG63 osteoblasts on PLG, PS and TCPS before and after collagen adsorption. Cell culture time: 1 day (panel 1), 3 days (panel 2) and 7 days (panel 3). Morphology of cells was examined under optical microscope with obj. 10× (cells on PLG and PS) or using inverted microscope, obj. 20× (cells on TCPS) after staining with crystal violet. Bar 100 μm

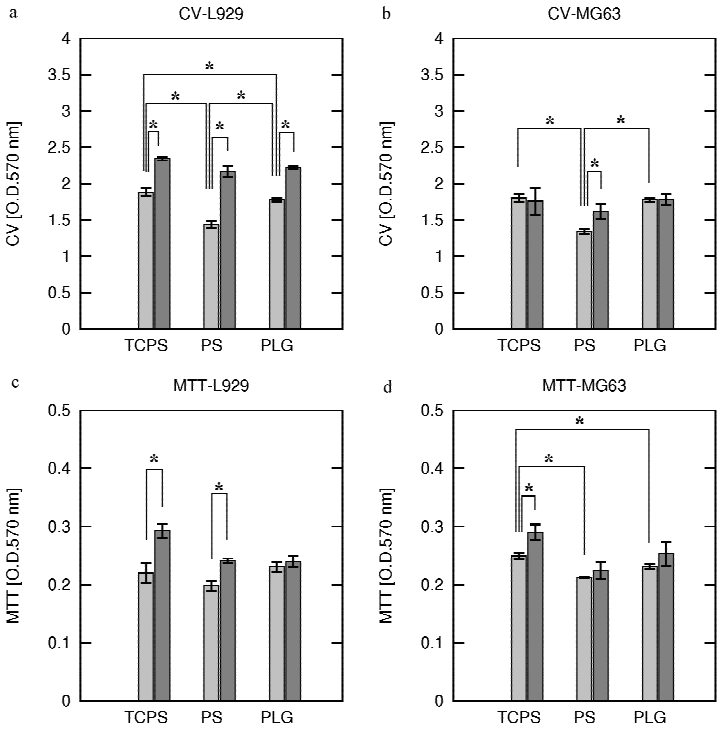

Fig. 5. Crystal violet – CV test (a, b) and MTT test (c, d) of L929 fibroblasts (a, c) and MG63 osteoblasts (b, d) on TCPS, PS, PLG before (light grey) and after (dark grey) collagen adsorption. The cells were cultured for 7 days. The data are expressed as the mean ± SEM of the three similar experiments carried out in triplicate. Asterisks indicate a statistical significance: $*p < 0.05$

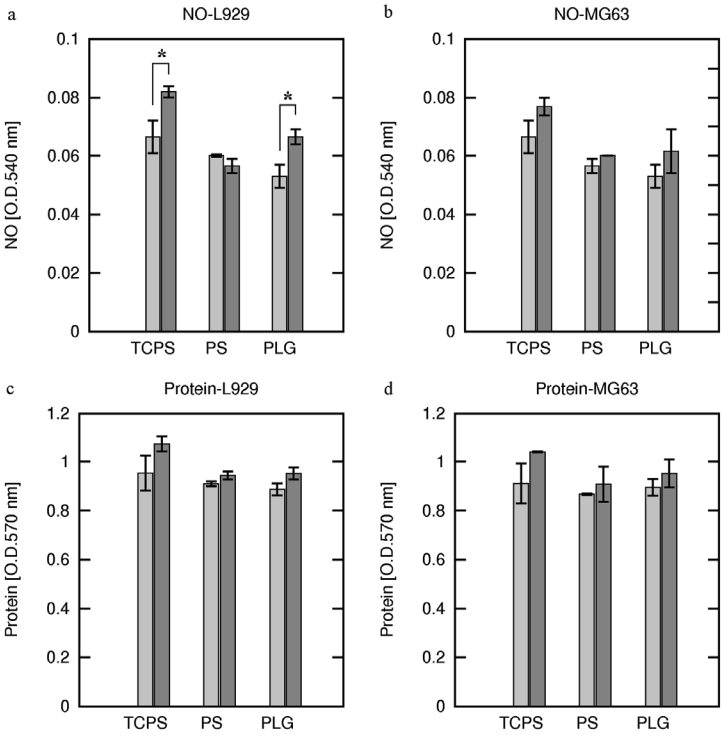

Fig. 6. Nitric oxide – NO (a, b) and protein concentration (c, d) in the supernatants of L929 fibroblasts (a, c) and MG63 osteoblasts (b, d) cultured on TCPS, PS, PLG before (light grey) and after (dark grey) collagen adsorption. The cells were cultured for 7 days. The data are expressed as the mean ± SEM of the three similar experiments carried out in triplicate. Asterisks indicate a statistical significance: $*p < 0.05$

increase on all the samples, but those results were not statistically significant (figure 6c, d).

4. Discussion

4.1. Properties of the raw polymers

XPS analysis of the polymeric surfaces revealed that the molar percentage of oxygen increased in the following order: PS < TCPS < PLG. The highest contact angle (~ 94°) was measured on PS, while PLG had a lower contact angle (~ 77°). TCPS, in spite of its moderate amount of oxygen (significantly less than on PLG), had the lowest contact angle (~ 65°) among the polymer materials. However, it should be kept in mind that XPS provides a mean analysis of a thin surface layer between 1 and 10 nm [21]. Therefore, the electrons are expelled not only from the atoms of the very top layer of the surface, but also from several atomic layers below. For PLG and PS the chemical structure is expected to be identical in bulk and on the surface, provided that no contaminants are present. In the case of TCPS, only the extreme surface has been modified by oxygen plasma treatment. This means that the XPS signal may be collected also from subsurface layers containing less (or any) oxygen, which results in the reduction of the mean oxygen content. This hypothesis was confirmed by the results obtained using the XPS analysis at different angles. The higher the angle of analysis and the lower the probed depth, the higher the content of oxygen on the TCPS surface (table 2).

XPS analysis performed at different angles also proved that the differences between the expected and actual molar percentages of carbon and oxygen at the surface of PLG may be associated with surface contamination. Adsorption of atmospheric hydrocarbon-containing molecules onto surfaces prepared in laboratory environment before introduction into the ultra-high vacuum conditions may result in an increase of the C–(C, H) peak intensity. Typically, one to several monolayers of contaminants could be present. After quantification of the data by the standard methods, this could result in an increased carbon concentration of up to a few tens of molar percentage [24]. Indeed, when the analysis is carried out for a thinner layer at the sample surface (i.e., at 60°), the C–(C, H) component of the C_{1s} peak increases, while it comes closer to the value expected for PLG when analysis is performed at 0°.

4.2. Properties of the polymers after collagen adsorption

It has already been reported that the organization of the collagen adsorbed on polymer substrates is, among other things, a function of the properties of these substrates, e.g., their chemical nature and roughness. As a general trend, rather smooth layers are found on more hydrophilic substrates, while patterned structures attributed to the association of a few collagen molecules are formed on more hydrophobic substrates [17]. From this point of view, the results obtained agree with expectations. The foils of PLG, more hydrophilic than PS, were covered with a granular layer of proteins, while collagen formed elongated structures on PS. It is more difficult to estimate the influence of the surface chemical nature of TCPS on the organization of collagen, because no collagen features were visualized using AFM. This is presumably due to the small size of the collagen molecules, compared to the high substratum roughness, as previously reported by others [25]. However, it must be kept in mind that the presence of the collagen adsorbed on all the samples, including TCPS, was confirmed by water contact angle measurements and by the detection of nitrogen and associated components in the C_{1s} peak by XPS.

The molar percentage of nitrogen present at the surface of the samples increased according to the order: PLG < PS < TCPS, while the contact angle increased according to the order: TCPS < PLG < PS. The relationship between the amount of nitrogen on the surface and the substrate hydrophobicity is not straightforward. It is well known that hydrophobic surfaces tend to bind more proteins than the hydrophilic ones. However, it has been reported that collagen bound on hydrophobic surfaces has also a lower mechanical resistance when is displaced by AFM tip [17]. Moreover, rather smooth layers of adsorbed collagen are found on hydrophilic surfaces, while on hydrophobic ones, patterned structures are formed [9], [26]. It should be stressed that XPS is sensitive to a combination of the amount of adsorbed protein molecules and their distribution within the adsorbed layers [26]. Therefore, a direct relationship between the measured nitrogen surface concentration and the actual adsorbed amount of collagen is not necessary. A good example is the comparison of the collagen organization on PLG and PS. The amount of nitrogen measured on PLG should be related to the adsorbed amount because of the collagen organization (rather continuous and thin granular layer). On PS, the colla-

gen has formed discontinuous patterned structures, where places without proteins still can be found. If the height of the collagen features exceeds the depth analyzed, the screening of the nitrogen signal will occur, and the adsorbed amount will be underestimated.

The effect of the surface roughness on the organization of the adsorbed collagen is very complex. In general, a greater roughness creates more area for potential interactions with proteins [4]. At the same time, collagen can form structures attributed to supramolecular assemblies on smooth substrates. If the topographic variations of the substrate surface are greater than the diameter of the collagen molecules, they can hinder collagen mobility along the surface. Therefore, these supramolecular structures will not be observed on rough materials [9]. As a matter of fact, such collagen assemblies were found on the surface of PS, characterized by a high roughness. However, the surface of PS can be regarded as quite smooth if the holes are excluded. Collagen assemblies were not found on TCPS, which revealed many topographical features, whose size was higher than the thickness of collagen molecules or their assemblies (see figure 1).

4.3. Biological properties of polymer substrates

In vitro studies showed that on TCPS, the most hydrophilic material, both types of cells were most numerous, well spread and most homogenously distributed on all over the surface. On PLG the fibroblasts have similar morphology to that on TCPS (figure 3), although their number was lower (figure 5a). The number of osteoblasts on PLG was similar to that on TCPS (figure 5b) and they have similar morphology to that on TCPS (figure 4). The excellent cell-conductive properties of TCPS can be explained in terms of its ability to passively adsorb adhesive ECM proteins (e.g., fibronectin, vitronectin, collagen) from serum, with sufficient conformational accessibility, and sufficient tractability among more plentiful adsorbed non-ECM proteins to permit cell receptor access, engagement, and mechanical tension on the surface [15]. For these reasons, TCPS is probably the best available material to culture cells in vitro.

PS, the most hydrophobic among the polymers tested, is regarded as a cell-resistant material, passively adsorbing non-ECM proteins (e.g., albumin, globulins, lipoproteins) from serum, thus resulting in surface densities of ECM components insufficient to support cell adhesion [27]. And indeed in this study, the morphology of both fibroblasts and osteoblasts was significantly different: the cells were round, less spread than on the other polymers, and tended to aggregate (figures 3 and 4). Also, a relatively small area of the cells was in contact with the surface. Compared to TCPS and PLG, the number of fibroblasts and osteoblasts on PS was significantly reduced (figure 5).

After incubating the substrates in collagen solution, the cells on PS were better spread and flattened; no big aggregates were formed anymore. Collagen positively affected proliferation of both types of cells. This means that the surface modification of the polymers under study with adsorbed collagen ensures good accessibility of recognition sequences for cell receptors, as already reported for endothelial cells (HUVECs) cultured on polystyrene and plasma-oxidised polystyrene [10].

The fibroblasts cultured on PLG and TCPS with adsorbed collagen were more numerous, better spread and their number was higher. Collagen adsorbed on all substrates considerably influenced the morphology and distribution of osteoblasts. Contrary to the raw polymers, the cells were homogenously distributed on the entire surfaces. This phenomenon is especially important in cell cultures for tissue engineering applications, where materials supporting homogenous cell cultures are particularly advantageous.

The obtained results of cell activity measurements suggest that the cells cultured on all substrates (except fibroblasts on TCPS + coll and PLG + coll) exhibited similar production of nitric oxide (figure 6a, b). However, it should be kept in mind that the cells cultured on TCPS and PLG were also most numerous. The nitric oxide level on PS was comparable to those on TCPS and PLG (figure 6a), while the number of cells cultured on PS was significantly lower (figure 5). This means that NO production per cell was the highest on PS. Nitric oxide is one of the active nitrogen compounds, thus an increase in its production/release might have a cytotoxic effect on the surrounding cells and tissues which manifested itself in slowing down the growth of the cells [28].

The culture of fibroblasts and osteoblasts on different polymeric materials has a minor effect on the level of protein synthesis and release. The concentration of proteins released into the supernatants tended to be higher when the cells were cultured on collagen-coated polymers, though these data were not statistically significant. The proteins released by fibroblasts in physiological conditions consist of growth factors (e.g., FGF, fibroblast growth factor) necessary for normal metabolic processes and mitosis of the cells [29]. Therefore, an increased protein release by fibroblasts incubated with collagen-modified materials may lead to an increased viability of those cells.

5. Conclusion

Fibroblasts and osteoblasts were cultured on raw PLG and two reference materials: PS and TCPS. As expected, on cell-conductive TCPS the cells were flattened and their growth was the best, while on cell-resistant PS the cells were round, weakly spread, formed aggregates and their growth was inhibited. On PLG the morphology of the cells was similar to that of the cells on TCPS; although their growth was worse than on TCPS, but better than on PS. This phenomenon can be explained in terms of surface hydrophobicity of PLG compared to PS. The presence of higher amount of oxygenated polar functional groups on PLG enhances adsorption of ECM proteins from culture medium supplemented with serum, thus providing stable ligands for cell receptors and enabling cell adhesion and proliferation, which is not the case for PS.

The cells were also cultured on collagen-conditioned substrates with the aim of enhancing their adhesion and growth. It was found that the amount and organization of adsorbed collagen are different and depend on surface chemistry and hydrophobicity. On hydrophilic TCPS and PLG collagen forms continuous layers, while on more hydrophobic PS the patterned structures are observed. It is interesting that irrespectively of the collagen morphology, the cells grow better and are more homogeneously distributed on all these collagen-modified substrates. The results show that surface modification of PLG by simple adsorption of this particular protein promotes the distribution and growth of osteoblasts and fibroblasts. All these findings are interesting from the point of view of tissue engineering. This approach can be further explored by culturing cells on PLG in serum free-media, where simple methods supporting growth of cells are of a key importance.

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