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SIBS TRIBLOCK COPOLYMERS IN CARDIAC SURGERY: *IN VITRO* AND *IN VIVO* STUDIES IN COMPARISON WITH ePTFE

M.A. Rezvova[1], E.A. Ovcharenko[1], P.A. Nikishev[2, 3], S.V. Kostyuk[2, 3, 4], L.V. Antonova[1], T.N. Akent'eva[1], T.V. Glushkova[1], Y.G. Velikanova[1], D.K. Shishkova[1], E.O. Krivkina[1], K.Yu. Klyshnikov[1], Yu.A. Kudryavtseva[1], L.S. Barbarash[1]

[1] Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russian Federation
[2] Research Institute for Physical Chemical Problems, Belarusian State University, Minsk, Republic of Belarus
[3] Faculty of Chemistry, Belarusian State University, Minsk, Republic of Belarus
[4] Institute of Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russian Federation

Implantation of polymeric heart valves can solve the problems of existing valve substitutes – mechanical and biological. **Objective:** to comprehensively assess the hemocompatibility of styrene-isobutylene-styrene (SIBS) triblock copolymer, synthesized by controlled cationic polymerization in comparison with expanded polytetrafluoroethylene (ePTFE) used in clinical practice. **Materials and methods.** SIBS-based films were made by polymer solution casting method; *in vitro* biocompatibility assessment was performed using cell cultures, determining cell viability, cell adhesion and proliferation; tendency of materials to calcify was determined through *in vitro* accelerated calcification; *in vivo* biocompatibility assessment was performed by subcutaneous implantation of rat samples; hemocompatibility was determined *ex vivo* by assessing the degree of hemolysis, aggregation, and platelet adhesion. **Results.** The molecular weight of synthesized polymer was 33,000 g/mol with a polydispersity index of 1.3. When studying cell adhesion, no significant differences ($p = 0.20$) between the properties of the SIBS polymer (588 cells/mm^2) and the properties of culture plastics (732 cells/mm^2) were discovered. Cell adhesion for the ePTFE material was 212 cells/mm^2. Percentage of dead cells on SIBS and ePTFE samples was 4.40 and 4.72% ($p = 0.93$), respectively, for culture plastic – 1.16% ($p < 0.05$). Cell proliferation on the ePTFE surface (0.10%) was significantly lower ($p < 0.05$) than for the same parameters for SIBS and culture plastic (62.04 and 44.00%). Implantation results (60 days) showed the formation of fibrous capsules with average thicknesses of 42 μm (ePTFE) and 58 μm (SIBS). Calcium content in the explanted samples was 0.39 mg/g (SIBS), 1.25 mg/g (ePTFE) and 93.79 mg/g (GA-xenopericardium) ($p < 0.05$). Hemolysis level of red blood cells after contact with SIBS was 0.35%, ePTFE – 0.40%, which is below positive control ($p < 0.05$). Maximum platelet aggregation of intact platelet-rich blood plasma was 8.60%, in contact with SIBS polymer – 18.11%, with ePTFE – 22.74%. **Conclusion.** In terms of hemocompatibility properties, the investigated SIBS polymer is not inferior to ePTFE and can be used as a basis for development of polymeric prosthetic heart valves.

Keywords: polymeric heart valve prostheses, poly(styrene-b-isobutylene-b-styrene), polytetrafluoroethylene, hemocompatibility.

INTRODUCTION

Prosthetic heart valves are currently the preferred choice in correcting valvular defects. Functioning efficiency, being durable without the use of anticoagulant therapy and ability to be implanted in patients of various age groups are the key requirements for this type of valves [1, 2]. Prosthetic heart valves can be mechanical (with a rigid flap) or biological (based on xenomaterials), which have been used in clinical practice for several decades. Both types of prostheses have setbacks: the mechanical one has high thrombogenicity, while the biological one has limited durability as a result of xenotissue degradation [1, 2]. However, with higher number of younger patients and longer life expectancy of the population, there is need for a paradigm shift in the technology of valve structures and related materials today. Leaflet flap apparatus based on elastomeric polymers has prospects for successful use in clinical practice and can address the problem of bioprosthesis durability (reoperation). It can eliminate the need for permanent use of anticoagulants required for implantation of mechanical valve devices and provide hemodynamics that is comparable to physiological hemodynamics [3]. The ability to design a leaflet device with a given structure and properties is another advantage of synthetic products [4, 5]. In addition, biostable polymer matrices are attractive for use in heart valve tissue engineering [6].

Corresponding author: Rezvova Maria Alexandrovna. Address: 6, Sosnoviy blvd, 650002, Kemerovo, Russian Federation. Tel. (913) 079-61-40. E-mail: rezvovamaria@mail.ru

Despite the considerable number of researches geared towards finding suitable materials, polymer valves are still used in clinical practice due to high degree of instability of thermoplastic elastomers [7] and the thrombosis and calcification processes triggered by their use [8]. That is why the search for an effective combination of biostability and hemocompatibility properties is a pressing issue standing on the way of identifying polymer valve with clinical reality. Beginning in the 1950s, there were attempts to use a number of polymers as the basis for leaflet apparatus: polyurethanes [9], silicones [10], 3D cross-linked polyvinyl alcohol [11], polytetrafluoroethylene (PTFE and ePTFE) [12, 5], POSS–PCU nanocomposite [13], styrene-isobutylene-styrene (SIBS) triblock copolymer [14], etc. However, no ideal solution has yet been found.

SIBS block copolymers are of particular interest due to several factors: high hemocompatibility and biostability [15]; their properties can be controlled by varying the length and structure of polymer units; processing them by extrusion or injection molding is relatively easy; there is considerable experience in using them in medicine [16]. So, SIBS30 (30% styrene) manufactured by Innovia is the basis of modern cardiosurgical stents. It demonstrates high biocompatibility [17]. However, because pure SIBS30 has low physical and mechanical characteristics and given available experimental data on thrombosis [18], there was the need to look for new triblock copolymer modifications that could be used to develop a leaflet prosthetic heart valve, in particular, surface modification and creation of composites with reinforcing networks made of stronger polymers [19, 20].

This paper describes the synthesis of SIBS model polymer by controlled cationic polymerization. It also looks at the study of *in vitro* and *in vivo* hemocompatibility properties. The work uses, for the first time, an integrated approach to assessing hemocompatibility properties of the presented SIBS-based polymeric materials. This approach is especially important for adequately predicting the potential use of the polymers in valve structures.

MATERIALS AND METHODS

Synthesis of SIBS polymers

Stabilized styrene (Sigma-Aldrich, USA, >99%) was treated with 10% KOH solution, then washed with distilled water until neutral reaction, then dried with $CaCl_2$ and twice distilled over CaH_2 under reduced pressure. Methylene chloride and n-hexane (Ekos-1, Russia, reagent grade) were treated with concentrated H_2SO_4, a solution of soda, distilled with water until neutral reaction, then dried with $CaCl_2$, boiled twice and distilled over CaH_2. Titanium tetrachloride (Sigma-Aldrich, USA, 99.9%) was distilled over copper chips under reduced pressure. Isobutylene (Sigma-Aldrich, USA, >99%) was dried by passing it through a column filled with calcium chloride. 2,6-di-tert-butylpyridine (Sigma-Aldrich, USA, 97%) and dicumyl alcohol (Aldrich, USA, 97%) were used without prior purification.

Polymerization was carried out in a three-necked flask, which was first vacuumized and filled with argon. An initiator – freshly prepared dicumyl chloride (0.095 mmol) – was added to the flask through Kaszas technique [21]. Then it was dissolved in a "hexane: methylene chloride = 3 : 2" mixture and 0.20 mmol of 2,6-di-tert-butylpyridine was added, cooled to –60 °C in an alcohol bath, and isobutylene (34 mmol) cooled to –60 °C was added. Monomer concentration was 1.0 M. Next, the temperature was lowered to –80 °C and 1.9 mmol of titanium tetrachloride (20-fold excess) was added for commencement of polymerization.

After 25 minutes from start of polymerization, 9.2 mmol of pre-chilled styrene (2.0 M solution in a "hexane: methylene chloride = 3 : 2" mixture) was added. After 50 minutes from the start, the reaction was stopped by adding 2 mL of ice-cold methanol. The resulting sample was precipitated twice in a 10-fold excess of ice-cold ethanol. The precipitated sample was separated by centrifugation, washed with small amount of ethanol, and dried in vacuum at 55–60 °C/2 mm Hg until change in mass stopped.

Gel permeation chromatography

Molecular mass characteristics of the obtained block copolymers and efficiency of block formation were determined by gel permeation chromatography on an UltiMate 3000 instrument equipped with a PLgel precolumn (7.5×50 mm, 5 μm particle size), PLgel MIXED-C column (7.5×300 mm, 5 μm particle size) with refractometric and UV detectors (Thermo Fisher Scientific, Germany). Tetrahydrofuran was used as solvent; elution rate was 1 mL/min at a column temperature of 30 °C. Average molecular weight (M_n) and polydispersity (M_w/M_n) of polymers were calculated using the Chromeleon 7.0 software package (Thermo Scientific Dionex, Germany) using elution curves based on calibration dependences obtained applying polystyrene standards (Agilent EasiCal) with $M_w/M_n \leq 1.05$.

Polymer film production

Polymer films were obtained by irrigation from a polymer solution in chloroform (ratio of 1.6 g of substance to 8 mL of solvent) on a glass surface. The area of the obtained samples was 21 cm^2. The films were formed at 25 °C temperature for one hour and then heated to 35 °C for three hours at atmospheric pressure and 50% air humidity. Additionally, the samples were dried using a Labconco FreeZone 2.5 Benchtop vacuum dryer (USA) at –40 °C temperature and <0.133 mbar pressure.

In vitro assessment of cytotoxicity using cell cultures

The experiment was performed using the EA.hy926 cell line – hybridoma of human umbilical vein endothelial cells (HUVEC) and human lung carcinoma cells (cells provided by Dr. Cora-Jean S. Edgell, University of North Carolina, USA), which allows the cell line to reproduce the main phenotypic and functional characteristics of human microvessel endothelial cells. GORE-TEX® ePTFE vascular grafts (Gore & Associates, Inc, USA) were used as the comparison group, while culture plastic was used as the control group.

Sterile samples (n = 3 for each group; ethylene oxide sterilization) using a 0.6% agarose solution (Helicon, Russia) were fixed to the bottom of sterile 24-well culture plates. 2.0×10^5 cells were introduced into the polymer samples and cultured for 5 days in DME/F12 medium (Sigma Aldrich, USA) containing 1% HEPES buffer (Hyclone, USA), 10% fetal bovine serum (Sigma Aldrich, USA), 1% L-glutamine, 100 u/mL penicillin, 0.1 μg/mL streptomycin, 0.1 μg/mL amphotericin B, Hypoxanthine-Aminopterin-Thymidine (HAT) (Sigma Aldrich, USA) at 37 °C temperature and 5% CO_2. The medium was changed once every 2 days. Absolute cell count per 1 mm^2 of the surface and relative content of dead cells were evaluated using fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Germany). For this purpose, 2 μg/mL of Hoechst 33342 nuclear fluorescent dyes (Molecular Probes, USA) and 0.03 mg/mL of ethidium bromide (AppliChem, Spain) were added 30 minutes before microscopic examination (orange staining of dead cell nuclei). To prepare the samples for microscopy, they were separated from agarose and transferred to a sterile 24-well plate. Cell count was determined in 5 different fields of view at ×200 magnification, followed by conversion to 1 mm^2 of the studied surface. The relative number of dead cells was determined from the ratio of the absolute number of dead cells per 1 mm^2 of the surface to the absolute number of cells per 1 mm^2.

Cell proliferation was evaluated on sterile samples (n = 3 for each group) using the Click-iT Plus EdU cell proliferation imaging kits (Molecular probes, USA): the nuclei of all cells were stained blue with nuclear DAPI dye, while the nuclei of proliferating cells were stained green with Alexa Fluor 488 fluorescent dye. When DNA synthesis takes place, thymidine integrates into DNA and, having an affinity for Alexa Fluor 488, selectively detects synthesized DNA, which is expressed in green fluorescent nuclei. Fluorescence microscopy was performed on an LSM 700 laser scanning microscope (Carl Zeiss, Germany). The relative number of proliferating cells was determined from the ratio of the absolute number of proliferating cells per 1 mm^2 of the surface to the absolute number of cells per 1 mm^2 of the surface.

Determination of calcification *in vitro*

Calcification resistance was determined through accelerated *in vitro* calcification. Samples 5×5 mm in size (n = 5 for each group) were placed in 2 mL of a DMEM culture medium solution (Sigma, USA) and albumin serum (FBS, Sigma, USA) containing $CaCl_2$ and Na_2HPO_4, and kept in a CO_2 incubator at a 37 °C temperature and 5% CO_2. Degree of calcification was evaluated after the third and sixth week of incubation. GORE-TEX® ePTFE vascular prosthetic material (Gore & Associates, Inc, USA) was used as the comparison group; bovine xenopericardium preserved by standard method with glutaraldehyde (GA) (NeoCor JSC, Russia) was used as positive control. Cryosections of the biomaterial and polymer samples were stained for calcium with alizarin red S (Reachim, Russia) and analyzed using an Axio Imager A1 microscope (Carl Zeiss, Germany).

In vivo biocompatibility assessment

Tissue response

In vivo inflammatory response and calcification was examined by subcutaneous implantation of 5×5 mm samples of male Wistar rats (weight 55–70 g) for a 2-week (tissue response assessment, n = 5) and 2-month (tissue response assessment, n = 5, degree of calcification, n = 5) follow-up period. After the experiment, some of the film samples were biopsied with surrounding tissues, fixed in a 4% solution of neutral formalin (MiniMed, Russia), and then restricted in paraffin (Biovitrum, Russia). Sections were stained with hematoxylin and eosin (Biovitrum, Russia) according to Van Gieson with a mixture of acid fuchsin and picric acid (Biovitrum, Russia) and alizarin red S (Reakhim, Russia). They were examined using an Axio Imager A1 microscope (Carl Zeiss, Germany).

Inflammatory response was analyzed according to the ISO 10993-6:2016 standard using a semi-quantitative evaluation system, for which the number of neovascularization foci in five fields of view was calculated for each animal at ×400 magnification. The response was also evaluated based on the thickness of the fibrous capsule (average of 10 equidistributed measurements of the width of the capsules taken over the entire dense tissue closest to the implant), fatty infiltrate and number of lymph nodes.

In vivo calcification

Another part of samples was hydrolyzed to evaluate quantitative calcium content. For this purpose, part of calcium was placed in 0.5 mL of 50% perchloric acid and kept at 150 °C until a clear solution was obtained (ePTFE was not subjected to hydrolysis, calcificates that were formed during the experiment passed into the solution). The cooled samples were diluted with distilled water. Calcium content was evaluated by inductively coupled

plasma optical emission spectrometry on an iCAP 6500 DUO spectrometer (Thermo Scientific, USA).

GORE-TEX® ePTFE vascular prosthetic material (Gore & Associates, Inc, USA) was used as the comparison groups, while bovine xenopericardium preserved by standard method with glutaraldehyde (GA) (NeoCor JSC, Russia) was used as positive control.

In vitro hemocompatibility assessment

Degree of hemolysis

The study was performed in accordance with the ISO 10993-4:2017 standard. To estimate the level of red blood cell (RBC) hemolysis, fresh donor blood was used with addition of 3.8% sodium citrate solution in a 1:9 (citrate:blood) ratio. The test samples 25 cm^2 in size (n = 5 for each type of material) were placed in containers, 10 mL of physiological saline was added and placed in a thermostat at 37 °C for 120 minutes. Saline and distilled water were used as negative and positive controls, respectively, to assess the degree of erythrocyte hemolysis. After that, 200 µl of citrated blood was added to each box, mixed and again kept in an incubator at 37 °C for 60 minutes. After incubation, the solution was taken from the boxes into tubes, followed by 10-minute centrifugation at 2800 rpm to precipitate RBCs. Optical density of the resulting solutions was measured at 545 nm wavelength on a GENESYS 6™ spectrophotometer (Thermo Scientific, USA).

The degree of hemolysis (H) in % was determined by the formula:

$$H = \frac{D_t - D_{ne}}{D_{pe} - D_{ne}} \times 100\%,$$

where D_t is the optical density of sample incubated with the studied polymer; D_{ne} is the optical density of positive control; D_{pe} is the optical density of the sample after 100% hemolysis.

For complete absence of hemolysis, the arithmetic mean value of the optical density index when measuring the positive control (saline solution with citrated blood) was taken; for 100% hemolysis, the arithmetic mean value of the optical density of the device when measuring distilled water with citrated blood was taken. Comparison groups were GORE-TEX® ePTFE (Gore & Associates, Inc, USA) and Polyethylene (LDPE) ERM – EC590 (Merck, Germany).

Platelet aggregation

The study was performed in accordance with the ISO 10993-4:2017 standard. The study used fresh donor blood with addition of 3.8% sodium citrate, in a 1:9 (citrate: blood) ratio. To obtain platelet-rich plasma (PRP), citrate blood was centrifuged at 25 °C for 10 minutes at 1000 rpm. Platelet-poor plasma (PPP) was obtained by centrifuging citrated blood for 20 minutes at room temperature and at 4000 rpm rotation speed. This speed was used to calibrate the device. An intact PRP was used as a control. ERM-EC590 polyethylene (LDPE) (Merck, Germany) was used as a comparison group for high blood platelet aggregation rates.

Measurements were taken without aggregation inducers in spontaneous mode. In the present study, a semi-automatic 4-channel platelet aggregation analyzer (photometer) "ARAST 4004" (LABiTec, Germany) was used. To initiate spontaneous aggregation in citrated blood, the level of Ca^{2+} ions was restored, and the prepared $CaCl_2$ solution with a 0.25 M molar concentration was used as reagent. The sample-to-reagent ratio was 250 µl PRP + 25 µl $CaCl_2$. The duration of contact between the studied samples and PRP was 3 minutes.

Platelet adhesion

PRP obtained from fresh citrated donor blood via 10-minute centrifugation at 1000 rpm was used for the study. Comparison groups were GORE-TEX® ePTFE (Gore & Associates, Inc, USA) and Polyethylene (LDPE) ERM – EC590 (Merck, Germany).

Samples 1 cm^2 in size were incubated with 500 µl PRP at 37 °C for 2 hours, then carefully washed with phosphate-buffered saline (PBS) to remove non-adsorbed plasma components. Then, the samples were fixed in a 2% glutaraldehyde solution prepared on 0.1 M phosphate buffer for 6 hours. Next, the samples were dehydrated in a series of alcohols of ascending concentration (50, 75, 95 and 100%) for 15 minutes each. Platelet adhesion was assessed using a S3400N scanning electron microscope (Hitachi, Japan). The adhesive capacity of the surface of the materials was evaluated in randomly selected 8 fields of view, as the platelet activation index, which was calculated by the formula:

Activation index = (number of level I platelets × 1 + number of level II platelets × 2 + number of level III platelets × 3 + number of level IV platelets × 4 + number of level V platelets × 5) / total number of platelets (Table 1).

Table 1

Platelet activation levels

Level	Characteristic
I	Disc-shaped platelet, without activation
II	Platelet is increased in size with rudiments of pseudopodia in the form of protrusions (initial stage of activation)
III	Platelet is significantly increased in size, irregular in shape, with pronounced pseudopodia, platelets accumulate
IV	Platelet spreading, cytoplasm spreads between pseudopodia
V	Platelet in the form of a spot with granules, pseudopodia cannot be identified due to cytoplasm spread

Statistical processing

Quantitative data were processed by generally accepted statistical techniques using the Statistica 6.0 application package (StatSoft, Inc., USA) for processing medical and biological information. The nature of distribution in the samples was evaluated using the Kolmogorov–Smirnov test. Non-normal distribution ($p < 0.01$) was observed in the groups, and therefore all data were presented as medians (M) and quartiles (25 and 75%). Statistical significance of differences between two independent groups was evaluated using the nonparametric Mann–Whitney U test. Differences were considered significant at a $p < 0.05$ significance level.

RESULTS

SIBS polymer synthesis

Poly(styrene-block-isobutylene-block-styrene) obtained through successive addition of monomers in controlled cationic polymerization is characterized by a number average molecular weight of 33,000 g/mol and a polydispersity index of 1.3. The polymer structure is confirmed by gel permeation chromatography.

In vitro cytotoxicity assessment using cell cultures

In studying cell adhesion (Fig. 1), there were no significant differences ($p = 0.20$) found between SIBS

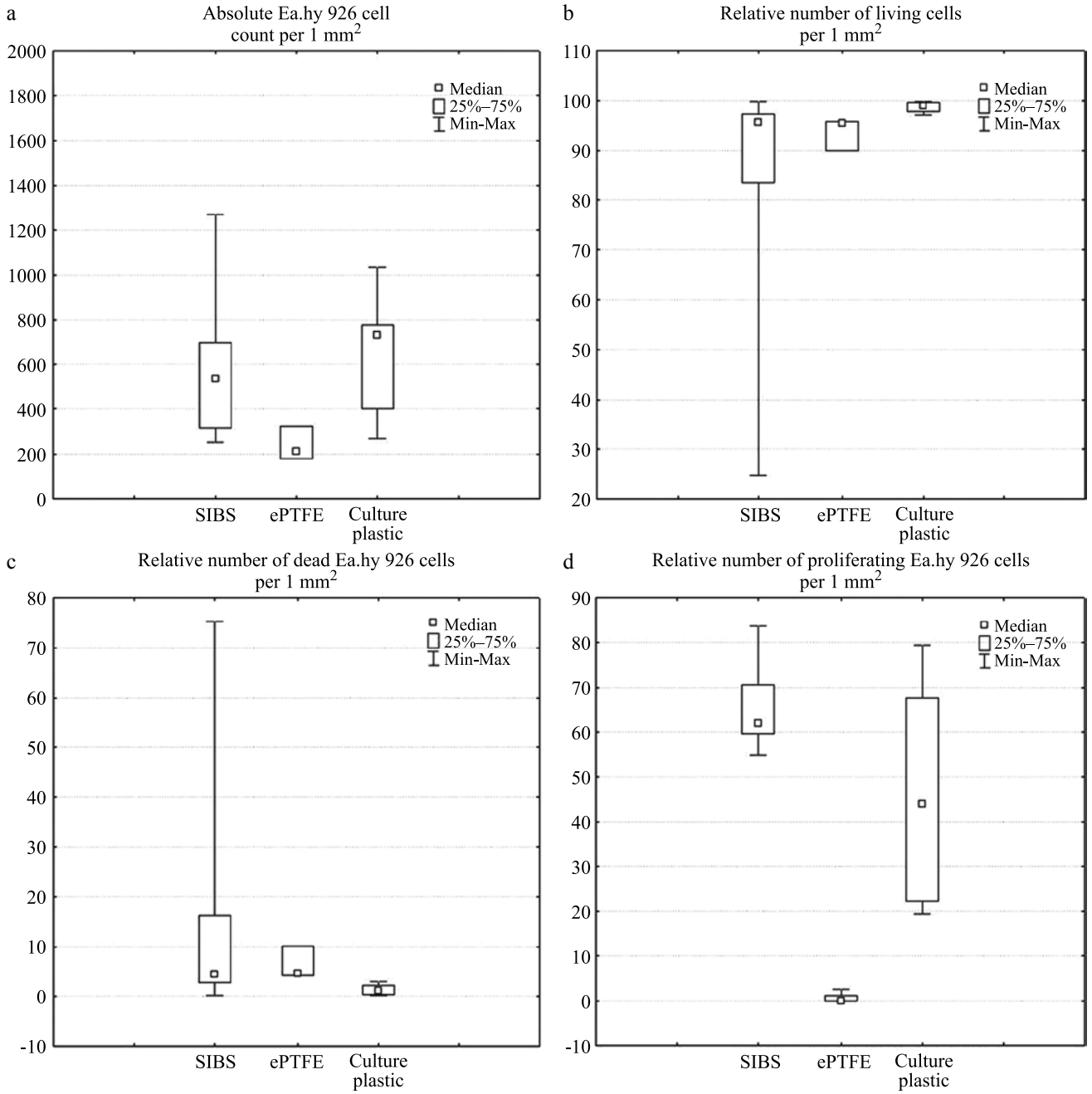

Fig. 1. Absolute number of cells (a), relative number of living cells (b), relative number of dead cells (c), relative number of proliferating cells (d). Ea.hy 926 on various polymer surfaces 5 days after cultivation

polymer properties (588 cells/mm^2) and culture plastic properties (732 cells/mm^2) that is considered the gold standard for *in vitro* cell adhesion. The absolute quantity of adherent cells for the ePTFE material (212 cells/mm^2) was 3 times less than for the SIBS polymer ($p < 0.001$).

The relative number of viable EA.hy926 cells in different polymer samples (Fig. 1, b) did not significantly differ ($p = 0.56$). Accordingly, the same situation was the case with the relative number of dead cells. In the studied polymer samples (SIBS, ePTFE), the number of dead cells did not differ among themselves ($p = 0.93$) – 4.40 and 4.72%, respectively. At the same time, this parameter had a value of 1.16% for culture plastic which turned out to be four times lower (Fig. 1, c) than on the SIBS and ePTFE surfaces ($p < 0.05$).

However, extremely low cell proliferation on the ePTFE surface (0.10%) made cell adhesion and proliferation on this material (Fig. 1, d) impossible. As can be seen from Fig. 2, maximum proliferative activity of EA.hy926 cells was detected on the SIBS polymer surface (62.04%), which was 1.4 times higher than on culture plastic (44.00%), ($p < 0.05$).

Determination of calcification *in vitro*

Polymeric materials SIBS, ePTFE (Fig. 3, b, c) were found to be resistant to calcification in an *in vitro* test, in contrast to biological tissue samples, which had already started mineralizing by the 3rd week of incubation and became slightly more pronounced by the 6th week (Fig. 3, a).

In vivo biocompatibility assessment

Tissue response

After 14 days following implantation, there was a moderate macrophage tissue response to implanted ma-

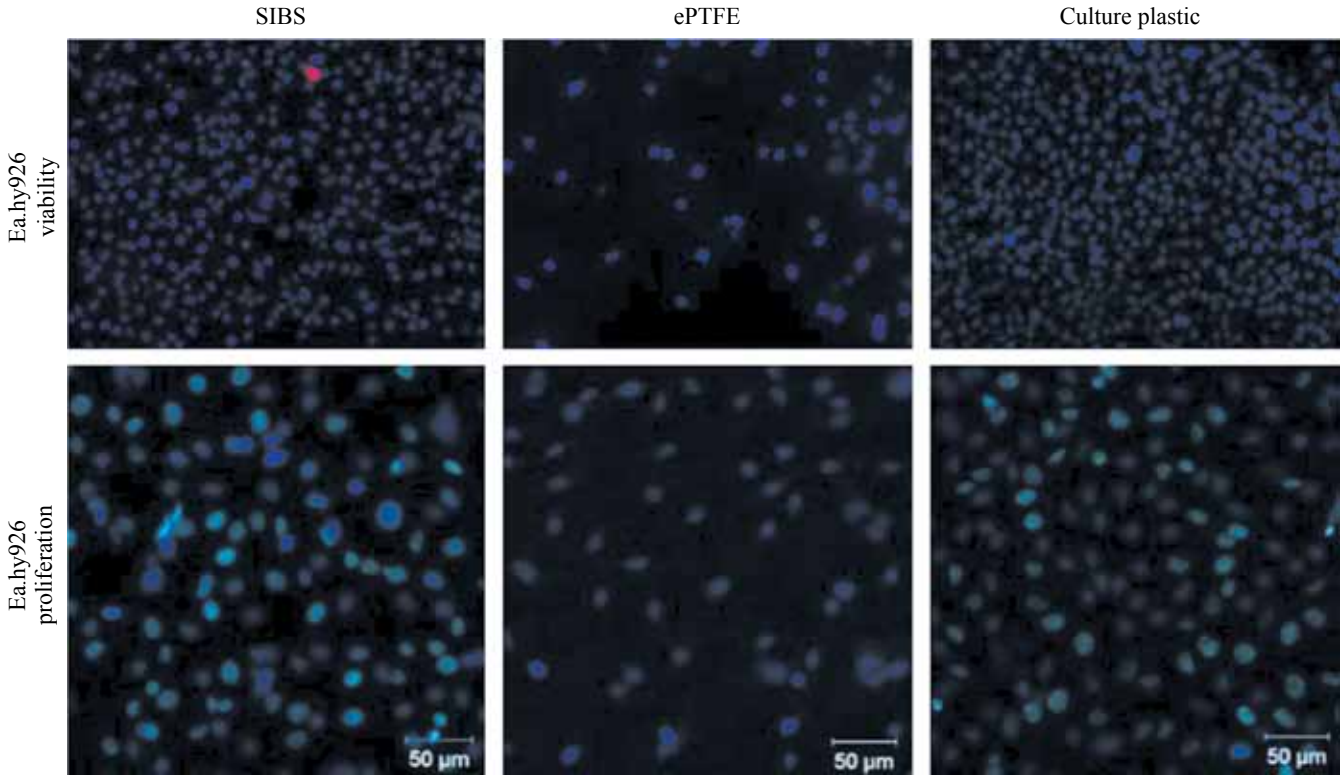

Fig. 2. Fluorescence microscopy of matrices with cells. ×200

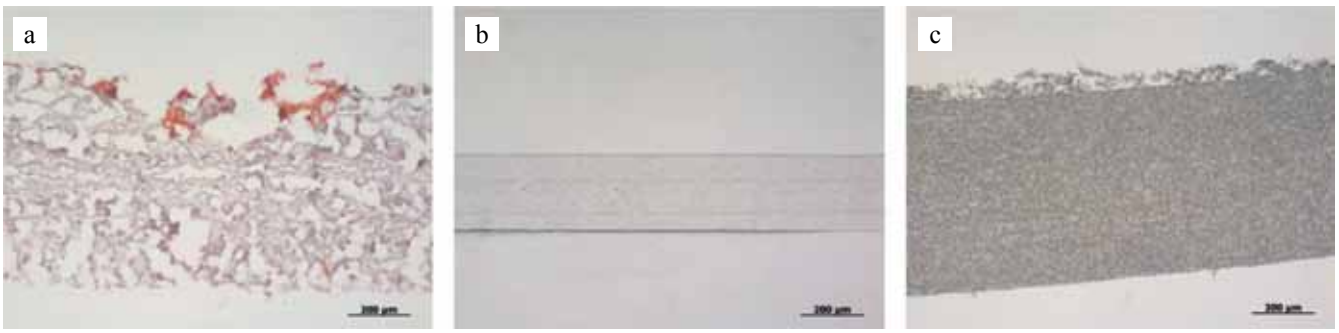

Fig. 3. Light microscopy, *in vitro* calcification test, 6 weeks incubation; samples of materials: GA-preserved bovine pericardium (a), SIBS (b), ePTFE (c). ×100

terials and no giant cells. This suggests good biocompatibility of the polymers (Fig. 4, a–c). For all types of polymers, lymph nodes were found in the tissue surrounding the implants. This indicates inflammatory processes, while lymph nodes were found in minimal number for the SIBS sample. Histological examination showed formation of loose fibrous tissue preceding the formation of a connective tissue capsule covering polymer samples with an average thickness of 69 μm (ePTFE), 50 μm (GA-xenopericardium) and 98 μm (SIBS). Fatty infiltrate associated with collagen fibers was observed in the case of biological samples. The degree of neovascularization was comparable for all types of materials – minimal capillary proliferation, 1–3 neovascularization foci with fibroblast structures in the field of view (×400 magnification). In the case of biological samples and ePTFE (due to its porous structure), cell infiltration was noted, especially active for GA-xenopericardium.

Long-term implantation (60 days) led to formation of dense connective tissue capsule in the form of collagen fibers, with an average thickness of 42 μm for ePTFE and 58 μm for SIBS (Fig. 4, e, f). In the case of GA-xenopericardium, the capsule was not clearly visualized due to active inflammatory process, which was caused by significant calcification. The presence of foreign-body giant cells two months after implantation was observed for a polymer sample of ePTFE and GA-xenopericardium; in the case of SIBS, foreign-body giant cells were not detected.

From the standpoint of a semi-quantitative assessment (ISO 10993-6:2016) based on analysis of tissue response indicators (neovascularization, fibrosis, fatty infiltrate), SIBS polymer can be classified as biocompatible relative to comparison samples (ePTFE, preserved xenopericardium, which are used today in the clinical practice of heart valve replacement).

In vivo calcification

Another biocompatibility criterion is the degree of calcification of material in contact with the body environment. Histological examinations showed active calcification of GA-xenopericardium (Fig. 4, h). The presence of calcifications in the case of ePTFE was detected on the surface of the polymer sample (Fig. 4, i).

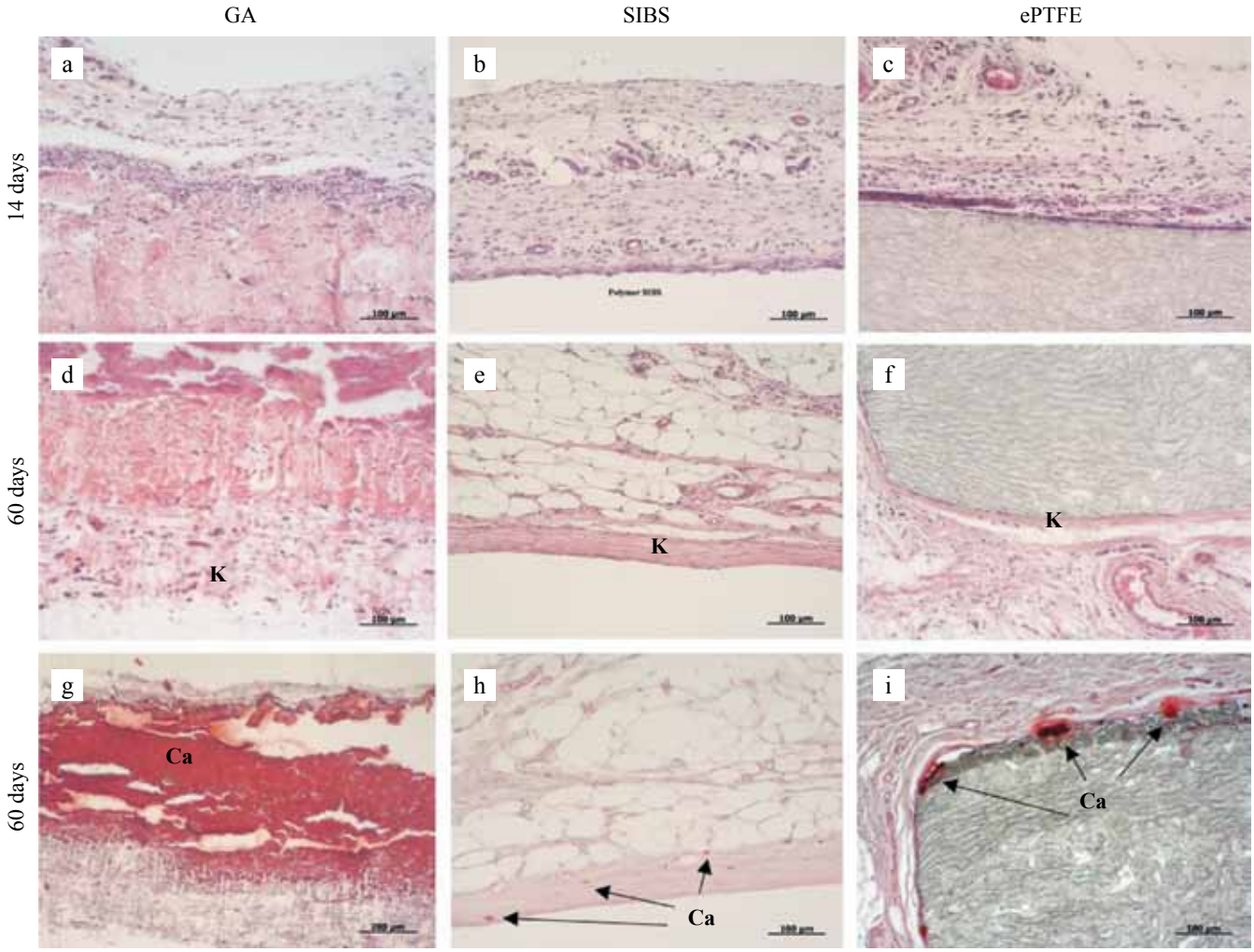

Fig. 4. Histological sections of GA-xenopericardium polymer matrices (a, d, h), SIBS (b, e, i), ePTFE (c, f, i), after implantation in rats for a period of 2 weeks (a, b, c) and 2 months (d, e, i). Stained with hematoxylin-eosin (a–f), alizarin red S (g–i). ×200

Crystalline calcium dot sites are noted in the thickness of the connective tissue capsule formed around SIBS.

Quantitative results showed statistically significant differences in the calcium content of SIBS and ePTFE samples ($p < 0.05$). Significant increase in calcium was also found in GA-xenopericardium samples ($p < 0.05$). A full description of the results is presented in Table 2.

Table 2

Calcium content in the samples (mg/g)

	Min	25%	Me	75%	Max
SIBS	0.23	0.23	**0.39**	0.51	0.55
ePTFE	0.49	0.53	**1.25**	2.70	2.95
GA-xenopericardium	1.77	2.57	**93.79**	155.30	159.80

In vitro hemocompatibility assessment

Level of hemolysis

The study showed no negative effect of SIBS polymer on the RBC membrane. Hemolysis level of RBCs after contact with SIBS was 0.35% (min: 0.03; max: 0.60; 25%: 0.11; 75%: 0.40), which is statistically less than that of negative control ($p < 0.05$).

When assessing the degree of RBC lysis after contact with ePTFE polymer, hemolysis level was 0.40% (min: 0.11; max: 2.40; 25%: 0.31; 75%: 0.67), which is also statistically significantly lower than that of the positive control ($p < 0.05$). Hemolysis level of RBCs after contact with polyethylene was 1.82% (min: 1.16; max: 2.30; 25%: 1.41; 75%: 2.10). Comparison of two polymer groups (SIBS and ePTFE) with each other found no significant differences in severity of hemolysis ($p = 0.57$). Comparison of two polymer groups (SIBS and ePTFE) with polyethylene found significant differences ($p < 0.05$).

Platelet aggregation

Maximum platelet aggregation of intact platelet-rich plasma was 8.60% (min: 7.79%; max: 15.91%; 25%: 8.02; 75%: 10.12%). Moreover, platelet-rich plasma samples in contact with polyethylene showed 73.40% platelet aggregation level (min: 67.73%; max: 82.74%; 25%: 72.35; 75%: 78.99%) Assessment of the degree of platelet aggregation after contact with SIBS and ePTFE polymers showed the following results: maximum aggregation after contact with SIBS polymer was 18.11% (min: 16.40%; max: 23.78%; 25%: 16.66; 75%: 20.42%); maximum platelet aggregation after contact with ePTFE was 22.74% (min: 18.6%; max: 28.56%; 25%: 22.45; 75%: 24.52%), which is statistically significantly lower than the maximum polyethylene-induced aggregation ($p < 0.05$).

Platelet adhesion

Platelets adherent to the surface were detected for all studied materials (Fig. 5). It is interesting to note that on one of the SIBS samples, there were individual areas with uncharacteristic accumulation of type III platelets located in the central part of the sample in a targeted manner.

In calculating the activation index of platelets adherent to polymer surface, the following indicators were obtained: activation index of platelets adherent to polyethylene surface was 3.61 (min: 2.0; max: 4.25; 25%: 2.82; 75%: 4.0). For polyethylene, level IV platelets were more characteristic – 61.29% of the total count of ad-

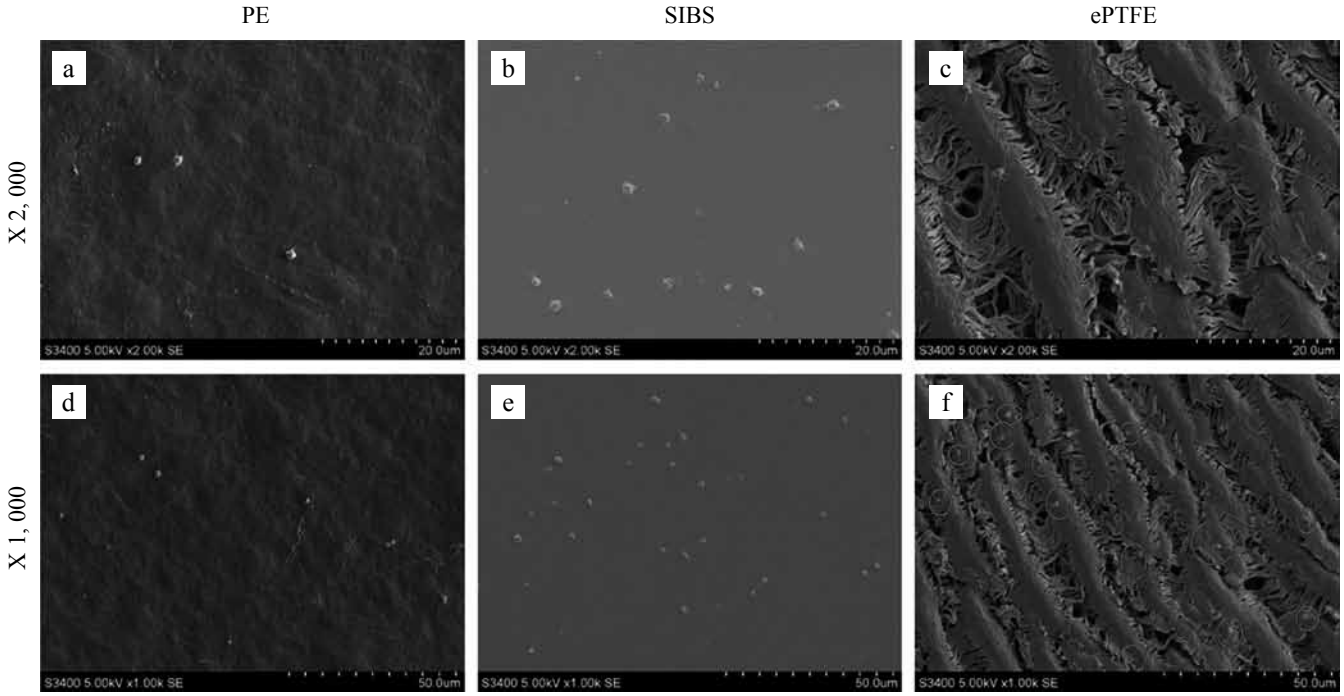

Fig. 5. SEM images of platelet adhesion on the surface of polyethylene polymer matrix (a, d), SIBS (b, e) and ePTFE (c, f)

herent platelets. Activation index of platelets adherent to SIBS polymer surface was 3.25 (min: 2.1; max: 4.0; 25%: 3.06; 75%: 3.69). Level II and III platelets were predominantly found on SIBS surface, which is 61.63% and 8.4%, respectively, as a percentage of the total number of adherent platelets. Activation index of platelets adherent to ePTFE surface was 3.76 (min: 3.0; max: 4.09; 25%: 3.29; 75%: 3.96). Level III and IV platelets were more characteristic for ePTFE surface, which is 41.54 and 26.76%, respectively.

Comparison of activation indices of platelets adherent to SIBS and ePTFE polymer surfaces with polyethylene showed no statistically significant differences ($p = 0.54$ and $p = 0.72$, respectively).

DISCUSSION

Copolymers and composites are widely used in medical practice today due to their unique properties – ability to combine structural fragments of various functionality. In particular, styrene-isobutylene-styrene block copolymers have thermoplasticity and high strength. They are resistant to hydrolytic, oxidative, and enzymatic effects due to the linear nature of carbon chain (with alternating secondary and quaternary carbon atoms) and biological inertness of side groups (Fig. 6) [15].

Assessment of the cytotoxicity of SIBS films synthesized by controlled cationic polymerization showed that they have high hemocompatibility. Proliferative potential can complement the picture of functional activity of cells in the case of short-term experiments where full dynamics of changes in cell viability does not always manage to reach maximum values. Experiments showed that the surface of the ePTFE material has low ability to cell adhesion. This is consistent with the results of other studies [22]. Ability to cell adhesion and proliferation is important in tissue engineering of heart valves [6]. Considering the results obtained during experiment, it can be concluded that SIBS polymers has a greater ability for spontaneous endothelization and greater potential in development of tissue-engineered constructs than ePTFE.

Implantation of any foreign material is inevitably accompanied by a response from the body, while formation of a stable connective tissue (fibrous) capsule indicates completion of the inflammatory process. Thickness of the fibrous capsule characterizes the degree of biocompatibility of the studied material. For the SIBS sample, the degree, after 60 days of experiment, turned out to be slightly higher than that of ePTFE sample. This correlates with the results of other experiments [17]. Formation of connective tissue on the leaflet apparatus of the implantable prosthesis creates a protective barrier, separating the synthetic material of the implant from the body environment. At the same time, this process is necessary for formation of a tight connection with the aortic root in order to prevent paravalvular regurgitation and ensure strong fixation of the implant. Foreign-body giant cells observed for explanted samples of ePTFE and GA-xenopericardium are capable of secreting reactive oxygen species and other chemical agents, potentially leading to oxidative damage and destruction of implanted devices [23]. No cells of this type were found for SIBS material after 60 days of experiment. Most likely, SIBS biocompatibility is related to the fact that the outer part of the polymer (10 nm) is a polyisobutylene block, while polystyrene blocks are located inside the polymer sample and are not in direct contact with biological tissues [24].

Calcification is the main cause of native aortic valve stenosis and bioprosthetic valve stenosis. It involves initiation and growth of calcium phosphate crystals, required for bone tissue, in atypical locations. Unlike xenomaterials, polymers do not contain phosphorus-rich cell deposits and destroyed collagen capable of promoting mineralization. They are therefore more resistant to this process. Based on results obtained from *in vitro* experiment, it can be concluded that the studied polymers do not trigger formation of crystal forms of calcium. Moreover, active calcification of GA-xenopericardium confirms the data obtained in previous studies [25].

Tendency of the test materials to calcify was also evaluated in an *in vivo* model during implantation in rats. Indicators of quantitative calcium content for the GA-xenopericardium that was selected as a positive control are consistent with published reports [26] and significantly exceed same values for the studied polymers.

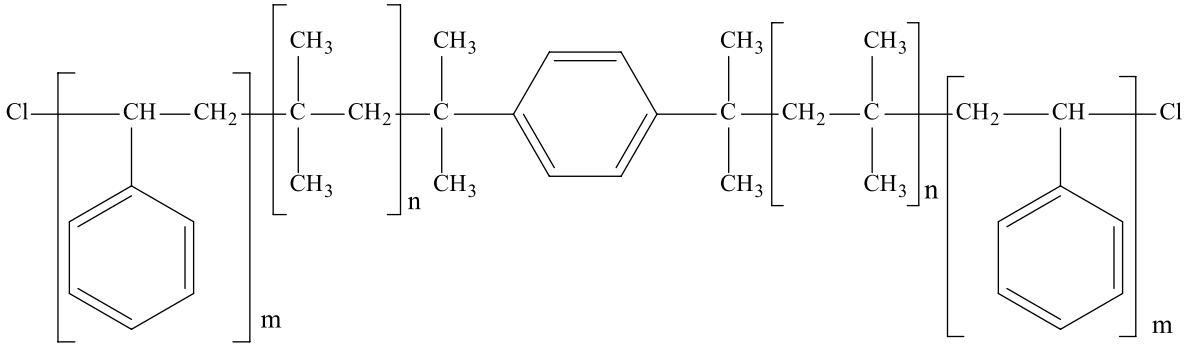

Fig. 6. Structural formula of poly(styrene-block-isobutylene-block-styrene)

There are two mineralization mechanisms arising as a result of contact between the synthetic material and the body's environment: nucleation of calcium phosphate crystals on the surface or at the interface between the connective tissue capsule and the implant; and calcification of biological tissue formed around the implant [27]. For ePTFE, the first mineralization mechanism was observed – calcifications were located on the surface of the sample; while for SIBS material, calcium was found in the fibrous capsule. According to quantitative assessment, SIBS polymer had significantly higher calcification resistance than ePTFE. This suggests that SIBS can be used as the basis for synthetic prosthetic heart valve. Literature sources also show that ePTFE materials have the tendency to mineralization in clinical experiments [8]. The high tendency of ePTFE to calcify may be due to the porous structure of its surface, resulting in accumulation of cellular elements and newly formed extracellular matrix.

In evaluating the degree of erythrocyte hemolysis induced by SIBS polymer surface, it was revealed that polymer has no toxic effect on formed elements (red blood cells). Also, ePTFE polymer, which was used as a comparison group, did not cause destruction of the cell membrane of red blood cells. At the same time, hemolysis level of RBCs after contact with polyethylene was 1.82%, which indicates that polyethylene is susceptible to thrombosis. Assessment of RBC hemolysis showed that the studied SIBS and ePTFE polymers can be considered hemocompatible, since the hemolysis level of RBCs after contact with SIBS and ePTFE polymer surfaces did not exceed 0.4% with permissible hemolysis degree of up to 2% [28]. Our findings are consistent with those of foreign authors [29].

Platelet adhesion occurs as a result of different charges existing between platelet surfaces and the surface of materials in contact with blood. Platelet adhesion is the initial stage of blood clot formation. However, adhesion alone is not enough to trigger thrombogenic reactions. Only activated platelets can release substances into the blood, which can lead to irreversible aggregation. Adhesion of platelets with regular round shape that have not yet been deformed does not carry a thrombogenic hazard, since adsorption of level I platelets is reversible and can be easily returned to the bloodstream [30]. Our results on assessment of adhesion properties suggest that platelets adhere to all polymers, regardless of type. Perhaps this is due to the potential difference between the surface of polymers and platelets [31].

A study of the effect of polymer surface on the level of platelet aggregation found that SIBS and ePTFE polymers do not increase platelet aggregation, unlike polyethylene. Going by obtained data suggesting high degree of blood aggregation after contact with polyethylene, it can be assumed that this polymer contains low molecular weight impurities that are irritating to blood platelets. This in turn leads to platelet activation and release of aggregation inducers. SIBS and ePTFE do not contain such impurities.

CONCLUSION

In terms of hemocompatibility properties, SIBS synthetic model polymer is not inferior to ePTFE used in clinical practice as a material for heart valve replacement. Moreover, experiments to determine the degree of calcification, cell adhesion and proliferation demonstrated that the studied SIBS polymer is superior. It can be concluded, therefore, that SIBS material has potential in development of new-generation polymeric prosthetic heart valves. Additional modification of the surface of the polymeric material in order to increase hydrophilicity would reduce its adhesive properties. The hemocompatibility of the model polymer can also be increased by modifying it with various antithrombotic agents, particularly heparin.

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The authors declare no conflict of interest.

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