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IN VITRO SCREENING OF BIOCOMPATIBILITY OF MODIFIED POLYETHERETHERKETONE AS AN IMPLANT MATERIAL: CYTOTOXICITY AND CELL ADHESION

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Introduction. In modern medical practice, bone implants are in demand as an effective solution for restoring bone tissue, including for the rehabilitation of patients with wounds sustained in combat. Polyetheretherketone (PEEK) is considered a promising polymer material for surgical implant production due to a number of properties that closely mimic bone tissue. However, the use of unmodified PEEK in implantology is limited by its biological inertness and poor osseointegration. Modification of this material, including through structural changes to the surface, can increase its biological activity, osteoconductivity, and antibacterial properties.
Objective. To evaluate the cytotoxicity and surface adhesion properties of PEEK modified via various methods.
Materials and methods. PEEK powder was synthesized via polycondensation under nucleophilic substitution conditions by reacting hydroquinone with 4,4'-difluorobenzophenone; for further studies, disk-shaped specimens were fabricated via milling. PEEK surface was modified using one of three methods: sulfonation with concentrated sulfuric acid, treatment with piranha solution followed by functionalization with glycine, or high-temperature fusion with NaCl followed by leaching. The structure of the obtained materials was studied using IR spectrometry and scanning electron microscopy. Cytotoxicity was assessed using the MTT test. The adhesive properties of PEEK materials were evaluated by culturing human dermal fibroblasts on their surface, followed by fluorescence visualization using Calcein AM and Hoechst 33342 fluorochromes. Analysis of adhesion and morphology of attached cells was performed using an inverted Leica DMI 3000 B microscope.
Results. It was shown that PEEK obtained via polycondensation of hydroquinone and 4,4'-difluorobenzophenone does not exhibit a cytotoxic effect on anchorage-dependent cells. Under the influence of the 1-day extract of PEEK and its dilutions, relative cell viability ranged from 96 to 126%, while for the 7-day extract, it varied from 132 to 141% (vs. 100% for the control); stimulation of proliferative activity of cells by PEEK extract was demonstrated. It was found that modification of the specimens does not lead to increased cell adhesion. The porous PEEK specimen obtained by pressing with NaCl followed by its leaching was characterized by uneven adhesion and impaired viability of most cells on the specimen surface. Treatment of PEEK with glycine led to decreased adhesion, impaired viability, and altered morphological characteristics of a large proportion of cells on the specimen surface. On the surface of sulfonated PEEK, the cells remained viable; however, the morphology of the attached cells differed significantly from the typical pattern. Specifically, instead of a subconfluent monolayer of flattened cells characteristic of successful adhesion, cell conglomerates of various sizes, formed by rounded and, less frequently, spindle-shaped cells, were unevenly distributed across the surface.
Conclusions. When modifying PEEK via sulfonation with concentrated H_2SO_4, treatment with glycine, or high-temperature fusion with NaCl, no improvement in the biocompatibility of the material was detected; on the contrary, impairments in the viability and morphology of anchorage-dependent cells adhered to the surface of the modified PEEK were observed. These findings highlight the need to continue searching for PEEK modification methods to overcome the limitations of the material and expand its areas of application in solving urgent interdisciplinary biomedical problems.

Keywords: implant materials; polyetheretherketone, PEEK; surface modification; sulfonation; piranha solution; glycine; porous PEEK; cytotoxicity; MTT assay; cell adhesion; IR spectroscopy; scanning electron microscopy

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СКРИНИНГ *IN VITRO* БИОСОВМЕСТИМОСТИ МОДИФИЦИРОВАННОГО ПОЛИЭФИРЭФИРКЕТОНА КАК МАТЕРИАЛА ДЛЯ ИМПЛАНТАТОВ: ЦИТОТОКСИЧНОСТЬ И КЛЕТОЧНАЯ АДГЕЗИЯ

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Введение. В современной медицинской практике костные имплантаты востребованы в качестве эффективного решения для восстановления костной ткани, в том числе для реабилитации пациентов с ранениями, полученными в боевых условиях. Одним из перспективных полимерных материалов для производства хирургических имплантатов считается полиэфирэфиркетон (ПЭЭК) благодаря ряду свойств, сближающих его с костной тканью. Однако применение в имплантологии немодифицированного ПЭЭК из-за его биологической инертности и низкой остеоинтеграции ограниченно. Повысить биологическую активность, остеокондуктивность и антибактериальные свойства может модификация материала, в том числе путем структурных изменений поверхности.
Цель. Оценка цитотоксичности и адгезионных свойств поверхности полиэфирэфиркетона, модифицированного различными способами.
Материалы и методы. Синтез порошка ПЭЭК осуществляли методом поликонденсации в условиях нуклеофильного замещения взаимодействием гидрохинона с 4,4'-дифторбензофеноном; для дальнейшего исследования образцы формировали путем фрезерования в форме дисков. ПЭЭК модифицировали одним из трех методов: сульфонированием концентрированной серной кислотой, обработкой раствором «Пиранья» с последующей функционализацией глицином, высокотемпературным сплавлением с NaCl с последующим выщелачиванием. Изучение структуры полученных материалов выполняли с применением ИК-спектрометрии и сканирующей электронной микроскопии. Оценку цитотоксичности проводили с помощью МТТ-теста. Адгезивные свойства ПЭЭК-материалов оценивали путем культивирования на их поверхности дермальных фибробластов человека с последующей флуоресцентной визуализацией флуорохромами Calcein AM и Hoechst 33342. Анализ адгезии и морфологии прикрепившихся клеток производили с помощью инвертированного микроскопа Leica DMI 3000 B.
Результаты. Показано, что ПЭЭК, полученный методом поликонденсации взаимодействием гидрохинона и 4,4'-дифторбензофенона, не проявляет цитотоксического действия в отношении поверхностно-зависимых клеток. Под действием 1-суточного экстракта ПЭЭК и его разведений относительная жизнеспособность клеток варьировала от 96 до 126%, под действием 7-суточного — от 132 до 141% (против контроля 100%); показана стимуляция экстрактом ПЭЭК пролиферативной активности клеток. Обнаружено, что модификация образцов не ведет к повышению адгезии клеток. Пористый образец ПЭЭК, полученный методом прессования с NaCl с последующим его выщелачиванием, характеризуется неравномерной адгезией с нарушением жизнеспособности большей части клеток на поверхности образцов. Обработка ПЭЭК глицином приводит к снижению адгезии, нарушению жизнеспособности и изменению морфологических характеристик большой доли клеток, находящихся на поверхности образцов. На поверхности сульфонированного ПЭЭК клетки сохраняли жизнеспособность, однако морфология прикрепившихся клеток существенно отличалась от типичной: вместо субконфлюэнтного монослоя, сформированного клетками распластанной формы, характерного для успешной адгезии, были обнаружены клеточные конгломераты разного размера, неравномерно распределенные по поверхности и сформированные округлыми, реже веретеновидными клетками.
Выводы. При модификации ПЭЭК сульфонированием концентрированной H_2SO_4, обработкой глицином, высокотемпературным сплавлением с NaCl улучшение биосовместимости материала не отмечено; напротив, наблюдались нарушения жизнеспособности и морфологии поверхностно-зависимых клеток, адгезированных на поверхности модифицированного ПЭЭК. Полученные результаты подчеркивают необходимость дальнейшего поиска способов модификации ПЭЭК, что позволит преодолеть ограничения материала и расширить области его применения для решения актуальных междисциплинарных биомедицинских задач.

Ключевые слова: имплантационные материалы; полиэфирэфиркетон, ПЭЭК; модификация поверхности; сульфонирование; раствор «Пиранья»; глицин; пористый ПЭЭК; цитотоксичность; МТТ-тест; клеточная адгезия; ИК-спектроскопия; сканирующая электронная микроскопия

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Соответствие принципам этики. Все доноры дали добровольное информированное согласие на предоставление их утильного биологического материала для выделения культур клеток с целью их последующего использования для проведения научных экспериментов. Проведение исследования одобрено на заседании локального биоэтического комитета ФГБОУ ВО «Приволжский исследовательский медицинский университет» Министерства здравоохранения Российской Федерации (протокол № 9 от 30.06.2023).

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INTRODUCTION

Trauma in peacetime and wartime differs in the nature of injuries and treatment methods. In combat trauma caused by high-velocity projectiles, complex shrapnel wounds and extensive bone destruction predominate, requiring surgical interventions to remove fragments and bone debris along with the restoration of bone tissue — such treatment is necessary for 85–100% of the wounded [1]. Due to the increasing flow of such patients, including those undergoing rehabilitation after injuries, the need for bone implants is currently growing.

Despite the widespread use of titanium in bone grafting [2], there is a growing interest in alternative materials in modern medicine, particularly in polyetheretherketone (PEEK) due to its physical and mechanical properties closely mimicking those of human bone, as well as its biocompatibility, hypoallergenicity, radiolucency, low density, and manufacturability [3–5]. Currently, extensive experience has been accumulated in the use of PEEK implants in both clinical and experimental practice [6–9].

The clinical potential of PEEK and its composites has been in spinal surgery [6, 10–14], as well as in dentistry [15–19], traumatology and orthopedics [20–23], cranioplasty [24, 25], and maxillofacial surgery for facial implants [26]. Information on the use of PEEK in neurosurgery is provided in the review by Muthiah et al. [13]. Having analyzed over 4000 articles, the authors concluded that PEEK offers several advantages as a material for interbody devices, including a low elastic modulus and radiolucency.

However, the use of PEEK is associated with certain limitations. In a study by Khan et al. involving 95 patients with cervical spine pathologies, the reported rates of postoperative complications included transient dysphagia (15.8%), wound hematoma (4.21%), and wound infection (1.05%) [14]. At the same time, excellent outcomes were observed in 79% of cases. The complication rate of cranioplasty using PEEK can reach 15.4% [24]. Due to the hydrophobic nature of PEEK surfaces, osteoblast adhesion to the implant surface is insufficient, thereby hindering osseointegration. Furthermore, during implantation, the formation of a fibrous capsule is often observed, which inhibits the bonding of PEEK to bone. Consequently, PEEK use is associated with a higher risk of pseudarthrosis development and a lower degree of osseointegration compared to titanium [13, 27].

Although the biological inertness of PEEK limits its widespread use in reconstructive surgery [28, 29], its bioactivity can be increased through surface and bulk modification of the polymer [12, 28]. Potential modifiers include titanium oxide, bioglass, calcium silicate, β-tricalcium phosphate, natural amorphous silica fibers, magnesium phosphates, and hydroxyapatite, including fluorine- or strontium-doped hydroxyapatite [30]. PEEK composites generally exhibit greater mechanical strength than the unmodified PEEK; however, these modifications alter the material's biological properties. Therefore, an optimal balance between mechanical strength and biological functionality must be achieved when designing PEEK composites.

Studies demonstrate that PEEK surface modification enhances cell adhesion and proliferation, thereby improving the biocompatibility and osteogenic properties of PEEK implant materials [31]. Current research actively focuses on developing surface modification methods that improve the cellular response of the surface and promote osseointegration [32]. These methods are mainly aimed at changing the surface morphology, creating roughness and porosity [33], as well as increasing polarity through the grafting of functional groups [34]. Several studies report increased osteoblast proliferation at an early stage (48 h) on porous PEEK surface obtained by pressing with NaCl followed by washing. It has been established that cell proliferation improves regardless of pore size; however, over time the cell density becomes lower than on a smooth PEEK surface [35]. One strategy for PEEK bioactivation involves the fabrication of porous PEEK and its composites [36]. The most widely used method for creating a porous structure is sulfonation via treatment with concentrated H_2SO_4 [33]. In this process, PEEK or its composite is immersed in concentrated H_2SO_4 (95–98%), and then residual sulfur compounds are subsequently removed by a hydrothermal reaction at 120 °C. For example, Dos Santos et al. [37, 38] enhanced the biological activity of PEEK surface using H_2SO_4 and piranha solution, resulting in a significant increase in cell viability compared to the unmodified material. The modified surfaces also demonstrated a greater capacity to stimulate cell growth, indicating improved cell adhesion and proliferation.

Surface modification is an effective method for improving the mechanical and biological characteristics of material surfaces while preserving their bulk properties [39]. In their study, Chen et al. proposed a surface modification strategy combining sulfonation and cold pressing (in the presence of NaCl) to form a three-dimensional hierarchical porous structure on the surface of PEEK implants. According to the authors, the technology is straightforward to implement and allows precise regulation of macropore size by adjusting the parameters of the porogen. Sulfonation yielded a finely porous structure with a pore size of 0.5–10 μm, promoting cell adhesion. In contrast, cold pressing with a porogen (NaCl) followed by particle leaching generated large pores (100–200 μm) on the PEEK surface. In addition to facilitating cell ingrowth, this modification method enhanced osteogenic differentiation and mineralization *in vitro* [39].

Functional group grafting is another approach for enhancing the osteogenic potential of PEEK materials. In a study by Buck et al., the PEEK surface was functionalized with two types of groups (-NH_2 and -COOH), which are capable of stimulating pro- and anti-inflammatory responses in macrophages, respectively. Differences in protein adsorption on the PEEK surface due to the distinct functional groups, elicited bidirectional macrophage responses, thereby influencing the osteogenic differentiation of mesenchymal stem cells. The authors

concluded that simultaneous introduction of both functional groups (-NH_2 and -COOH) onto the PEEK surface can improve implant integration with bone [40].

Both approaches — the development of PEEK composites and surface modification — can be applied in reconstructive medicine, depending on the required balance between bioactivity and mechanical strength. However, the available data on the use of PEEK (including modified forms) in reconstructive surgery remain contradictory, highlighting the need for further investigation.

In this study, surface modification was selected to preserve the bulk properties of PEEK, particularly its elasticity, while avoiding the increased brittleness commonly observed in highly filled composite materials. The aim of the study was to evaluate the cytotoxicity and adhesive properties of PEEK surfaces modified by three methods: sulfonation with sulfuric acid, treatment with glycine, and fusion with NaCl followed by leaching.

MATERIALS AND METHODS

Specimen fabrication. Polyetheretherketone (PEEK) was synthesized at the Center for Advanced Materials and Additive Technologies, Kabardino-Balkarian State University, according to the method described in [41]. The key thermal and mechanical characteristics of the synthesized PEEK were as follows: glass transition temperature, 148 °C; melting temperature, 343 °C; melt flow index, 7.8 g/10 min; flexural modulus, 3900 MPa; yield strength, 89 MPa; and elongation at break, 81%.

Specimens for the study were fabricated as disks with a diameter of 9 mm and a thickness of 5 mm. These disks were produced by milling on a Roland MDX-50 machine (Roland, Japan) from injection-molded PEEK bars. The bars were fabricated using an SZS-20 injection molding machine (Ningbo Haitai Machinery, China) with the following parameters: cylinder temperature, 410–420 °C; mold temperature, 150 °C; and clamping pressure, 8 bar.

Sulfonation. Disk-shaped specimens were incubated in 50 mL of 98% H_2SO_4 (analytical grade; Ekos-1 JSC) for 30 s at room temperature. Subsequently, the specimens were washed repeatedly with distilled water (produced using a DE-25M aquadistiller, Electromedoborudovanie Plant) until the pH of the wash water reached a neutral value. Finally, the samples were dried under vacuum at 120 °C for 12 h in a ULAB UT-4630V vacuum drying oven (ULAB, China).

Treatment with piranha solution and glycine. Disk-shaped specimens were immersed in piranha solution (a mixture of 98% H_2SO_4 [analytical grade; Ekos-1 JSC] and 37% H_2O_2 [grade A; Khimprom] at a volume ratio of 2:1) for 90 s. Subsequently, the specimens were washed repeatedly with distilled water until the pH of the wash water reached neutrality (pH ≈ 7). The samples were then incubated in 100 mL of 0.002 M glycine solution (99%; Servicebio Technology, China) at 23 ± 2 °C for 30 min. After washing three times with distilled water, the specimens were dried under vacuum at 80 °C for 12 h in a vacuum drying oven.

Fusion with NaCl. PEEK powder was mixed with NaCl (particle size, 2–4 mm) at a mass ratio of 3:1. The mixture was then fused in an EKPS-10 muffle furnace (Smolensk SKTB SPU OJSC) at 370 °C for 35 min. The resulting bar-shaped products were washed repeatedly with hot distilled water until the residual salt content was removed, and subsequently dried under vacuum at 120 °C for 12 h. The residual salt content in the wash water was monitored by conductometry using a SevenCompact S230 instrument (Mettler Toledo, USA). Washing was considered complete when the conductivity of the wash water stabilized at the level of the original distilled water (≤ 6 μS/cm). Disks were fabricated from the obtained bars by milling. The porosity of the specimens was determined by hydrostatic weighing with a Liboshi MH-300A electronic densimeter (Liboshi, China) and was found to be 18%.

Polymer structure was analyzed using a Spectrum Two™ IR spectrometer (PerkinElmer, USA) in the wavenumber range of 4000–450 cm^{-1}. The measurements were acquired with an attenuated total reflectance (ATR) accessory, a spectral resolution of 4 cm^{-1}, and 4 scans. The surface morphology of the specimens was examined by scanning electron microscopy (SEM) using a Tescan VEGA3LMH microscope (Tescan, Czech Republic). The imaging conditions were as follows: accelerating voltage, 5 kV; SE detector; chamber pressure, 2×10^{-2} Pa; and Resolution scanning mode.

Primary cultures of human dermal fibroblasts at passages 4–6 were used as test cells. The initial cell population was isolated from unaltered waste skin tissue obtained from donors aged over 18 years during elective plastic surgeries. All donors were screened prior to tissue collection for hepatitis B and C, syphilis (via the Wassermann reaction, RW), and HIV. Donors with a history of cancer, tuberculosis, or skin diseases were excluded from the study.

The cell culture protocol is described in detail in [42]. The cultures were confirmed to be sterile and free from mycoplasma and viral contamination. Prior to the experiment, the cells exhibited a homogeneous morphology, with a predominant spindle-shaped appearance and formed a subconfluent monolayer. Cell viability was determined to be 97–98%. The fibroblast phenotype was consistent with that of mesenchymal stem (stromal) cells, as evidenced by the expression profile of a panel of surface markers: $CD90^+$, $CD105^+$, $CD73^+$, $CD10^+$, and the absence of hematopoietic and monocytic markers ($CD45^-$, $CD14^-$, $CD34^-$, $HLA\text{-}DR^-$).

Cytotoxicity study (MTT assay). To prepare the extracts, 0.5 g aliquots of the synthesized PEEK powder were mixed with 5 mL of growth medium. The medium consisted of DMEM/F12 supplemented with 1% penicillin/streptomycin (antibiotics) and 2% fetal bovine serum (FBS). All reagents were supplied by PanEco (Russia). The tubes containing the PEEK aliquots were incubated in a SANYO MCO-18 CO_2 incubator (Sanyo Electric Co., Japan) under controlled conditions: 37 °C, 5% CO_2, and 95% relative humidity. Two time points were used for

extraction: one tube was incubated for 24 h to obtain the 1-day extract, and the second tube for 7 days to obtain the 7-day extract. After the extraction period, the residual fine powder was removed by centrifugation to pellet the particulate matter.

The obtained extracts were sterilized by sequential filtration through 0.8- and 0.22-μm pore-size filters (Corning, USA). Subsequently, the extracts were diluted with growth medium at volume ratios of 1:1, 1:2, 1:4, and 1:8. Throughout the preparation of the extracts and their dilutions, the pH remained stable within the range of 7.2–7.4, as indicated by the colorimetric indicator present in the growth medium.

The extracts and their dilutions were applied to the test cultures seeded in a flat-bottom 96-well plate (Costar, USA), with 8 wells allocated per sample. Prior to exposure, cells were seeded at a density of 5000 cells per well (equivalent to 10^3 cells/cm^2) in 100 μL of growth medium. The cells were pre-cultured for 24 h in a CO_2 incubator under controlled conditions (37 °C, 5% CO_2, and 95% relative humidity) prior to the addition of the test extracts. As a control, the state of the test culture on the untreated culture plastic was assessed; 8 control wells were allocated for each experimental series.

After 72 h of cultivation with the PEEK extracts, the morphological state of the cell culture on the well surface was assessed using an inverted Leica DMI 3000 B microscope equipped with LAS software (version 4.3; Leica Microsystems, Germany). Following fixation and image analysis, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at a concentration of 5 mg/mL was added to each well and incubated for an additional 3 h in a CO_2 incubator under standard conditions (37 °C, 5% CO_2, 95% relative humidity). After the incubation period, the supernatant was carefully removed and replaced with an equal volume of dimethyl sulfoxide (DMSO; PanEco LLC, Russia). The optical density was then measured at 540 nm using an Infinite F50 microplate analyzer (Tecan, Austria).

Cell viability was used as the primary criterion for cytotoxicity assessment. Relative cell viability (RV) was calculated according to Eq. (1). Cytotoxicity was classified using the following grading scale: rank 0 (RV = 100%) and rank 1 (RV = 70–99%), indicating the absence of cytotoxicity; rank 2 (RV = 50–69%), corresponding to mild cytotoxicity; rank 3 (RV = 25–49%), representing moderate cytotoxicity; rank 4–5 (RV = 0–24%), indicating pronounced cytotoxicity.

$$RV(\%) = \frac{\text{average OD in the experimental series}}{\text{average OD in the control}} \times 100, \quad (1)$$

where RV is relative viability, and OD is optical density.

Cell adhesion. For the qualitative assessment of cell adhesion and viability, human dermal fibroblasts were seeded onto the surface of sterile test specimens placed in the wells of a 24-well plate (Costar, USA). The seeding density was standardized at 30 000 cells/cm^2 per specimen, with 3 specimens allocated for each experimental series. Negative controls consisted of cells cultured in 3 wells without PEEK specimens, under identical culture conditions. The plates were incubated in a CO_2 incubator for 24 h under standard conditions (37 °C, 5% CO_2, and 95% relative humidity) in DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). After the incubation period, cells were stained with fluorescent probes from BD Pharmingen™ (USA): Calcein AM, which selectively labels the cytoplasm of viable cells, and Hoechst 33342, a marker highly specific for double-stranded DNA. Fluorescence images of cells on the specimen surfaces were acquired at magnifications of ×40, ×100, and ×200.

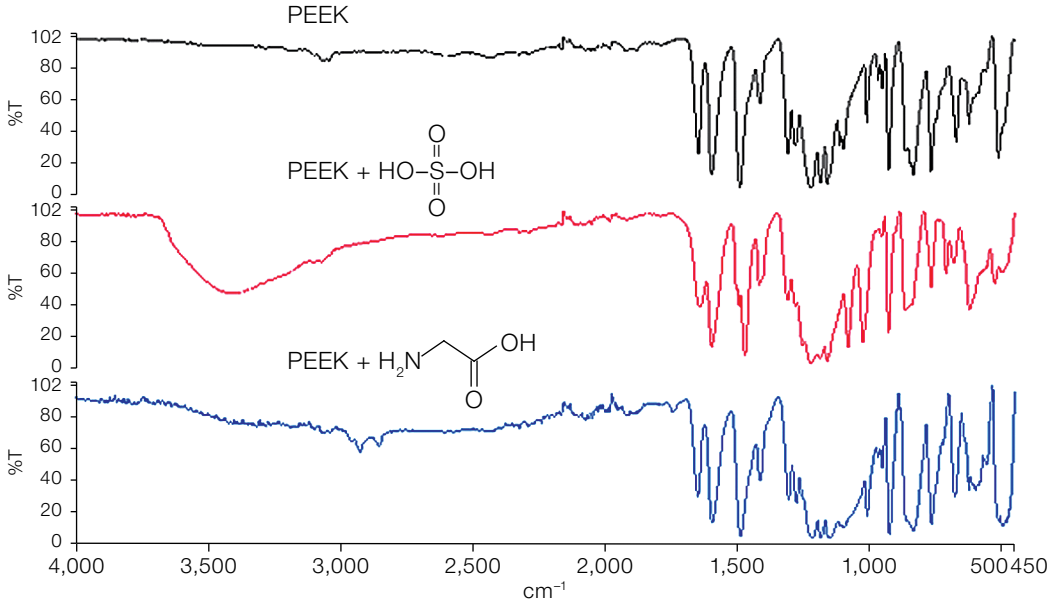


Figure prepared by the authors

Fig. 1. IR spectra of polyetheretherketone (PEEK) samples before modification and after treatment with H_2SO_4 (PEEK•H_2SO_4) and with piranha solution followed by glycine functionalization

Representative images were compared with those obtained from cells cultured on untreated culture plastic (negative controls).

RESULTS AND DISCUSSION

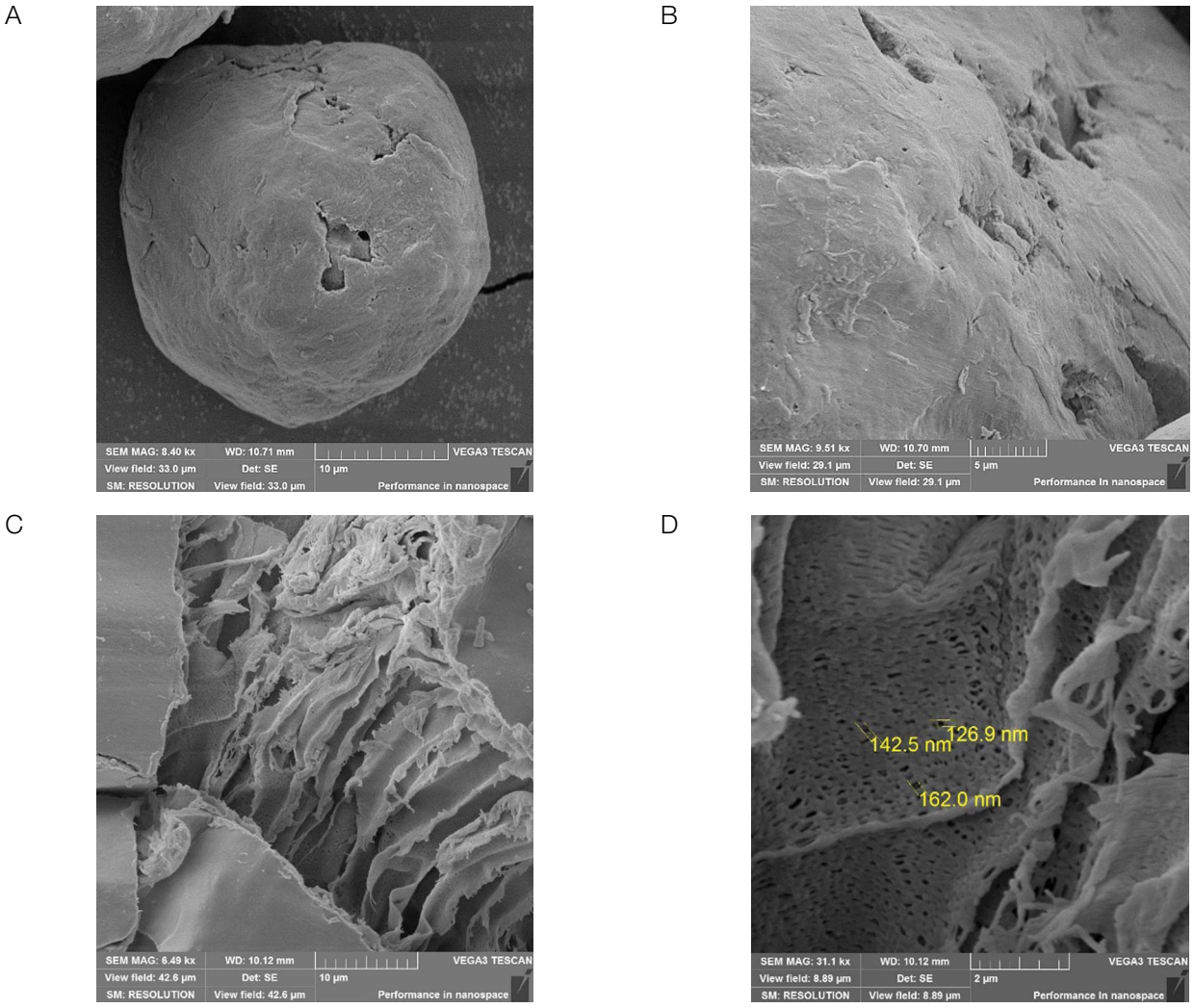
The IR spectrum of untreated PEEK (Fig. 1) exhibits characteristic absorption bands indicative of its molecular structure. The asymmetric stretching vibration of the aromatic ether C–O bond is observed at 1221 cm^{-1}. Absorption bands at 1650, 1490, 926, 1157, and 1185 cm^{-1} are assigned to diphenyl ketone moieties. The carbonyl groups of the ketone bond are represented by bands at 1306 cm^{-1} and 1278 cm^{-1}. Furthermore, the stretching vibrations of the C=O bonds in the benzophenone fragments yield absorption features at 1594 cm^{-1} and 1648 cm^{-1} [41].

Upon sulfonation, a pronounced absorption band appears in the FTIR spectrum of PEEK at approximately 3440 cm^{-1}. This band corresponds to the O–H stretching vibration of the hydroxyl group in the sulfonic acid moiety, confirming the oxidation of PEEK and the resulting increase in surface polarity. Additionally, the band observed at 1050 cm^{-1} is assigned to the symmetric stretching vibration of the S=O bonds [43].

In the spectrum of glycine-modified PEEK, no major structural changes are evident. Only low-intensity peaks are detected in the 2700–2900 cm^{-1} region, which are characteristic of N–H and C–N stretching vibrations associated with glycine. These features indicate successful surface modification with the amino acid.

The surface morphology of the PEEK specimens was evaluated by scanning electron microscopy (SEM; Fig. 2). No significant differences in morphological characteristics were observed between the unmodified PEEK and the glycine-treated PEEK samples; both surfaces exhibited a relatively smooth topography. In contrast, the microstructure of the sulfonated PEEK showed marked alterations, with a notable increase in surface roughness and porosity, as illustrated in Fig. 2C.

During the colorimetric analysis, a slight but consistent stimulation of cell proliferation was observed in the



Photos taken by the authors

Fig. 2. Surface of polyetheretherketone specimens. A — unmodified; B — treated with glycine; C, D — treated with H_2SO_4; pores are indicated by yellow arrows, with pore diameters indicated next to them

Table. Cytotoxicity of polyetheretherketone extracts and their dilutions

Series	Parameter	Extraction duration	
		1 day (24 h)	7 days
Control (*n* = 8)	Optical density	0.277 ± 0.031	0.611 ± 0.024
	Relative viability, %	100	100
	Cytotoxicity level	0	0
Undiluted extract (*n* = 8)	Optical density	0.266 ± 0.031	0.805 ± 0.025*
	Relative viability, %	96	132
	Cytotoxicity level	1	0
Extract 1:1 (*n* = 8)	Optical density	0.330 ± 0.030	0.860 ± 0.017*
	Relative viability, %	119	141
	Cytotoxicity level	0	0
Extract 1:2 (*n* = 8)	Optical density	0.305 ± 0.030	0.834 ± 0.023*
	Relative viability, %	110	136
	Cytotoxicity level	0	0
Extract 1:4 (*n* = 8)	Optical density	0.337 ± 0.022	0.835 ± 0.015*
	Relative viability, %	122	137
	Cytotoxicity level	0	0
Extract 1:8 (*n* = 8)	Optical density	0.350 ± 0.028	0.802 ± 0.031*
	Relative viability, %	126	131
	Cytotoxicity level	0	0

Table prepared by the authors based on their own data

Note. * — $p < 0.05$ compared to the control group, Wilcoxon test; *n* — sample size (number of observations = number of wells).

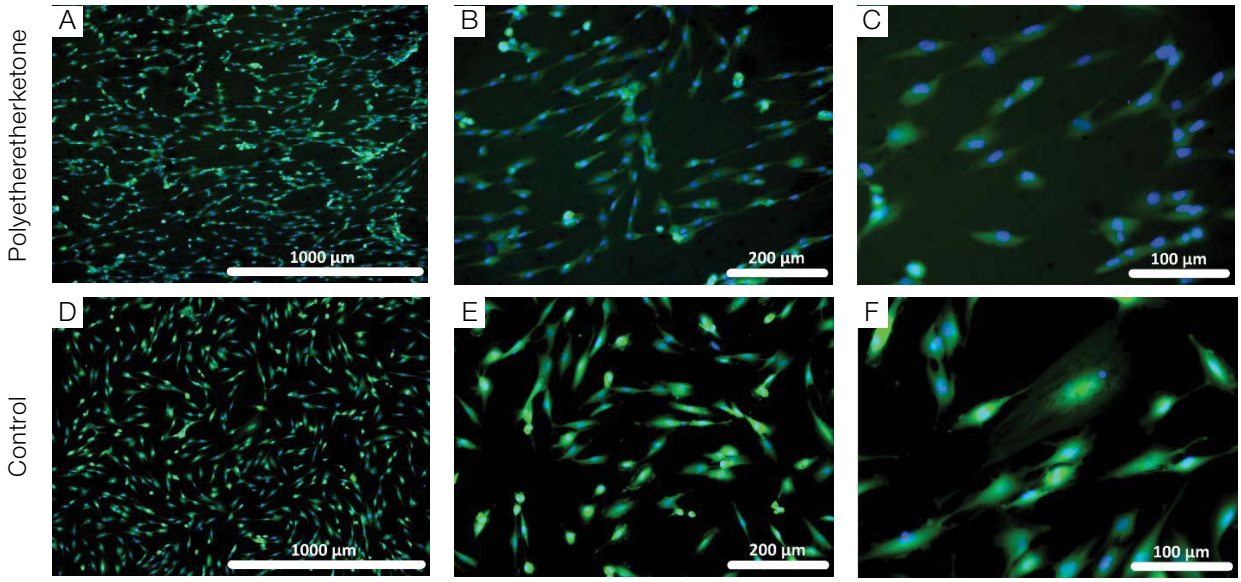


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Fig. 3. Test culture cells on the surface of unmodified polyetheretherketone and inside the control wells. Fluorescence microscopy: green — Calcein AM, blue — Hoechst 33342

presence of diluted extracts, as evidenced by relative cell viability (RV) values exceeding 100%. This effect was more pronounced with the 7-day extract and its dilutions. These observations merit further investigation.

Cell adhesion was evaluated on unmodified and modified PEEK specimens prepared under various treatment conditions. On the surface of unmodified PEEK, spindle-shaped cells were observed with a relatively uniform distribution in the central region (Fig. 3). In contrast, the cell distribution along the specimen edges was heterogeneous, with multiple areas exhibiting a lack of cell attachment. Background fluorescence enabled characterization of the specimen surface as predominantly smooth. Notably, surface irregularities were detected at the edges, appearing externally as film-like fragments.

When studying cell adhesion on the sulfonated PEEK specimens, cells displaying a normal fibroblast-like morphology and a relatively uniform distribution were visualized on the surface of the control wells (Fig. 4A–C). At the same time, no monolayer was observed on the surface of the PEEK•H_2SO_4 specimens; the cells formed conglomerates, whose distribution varied from specimen to specimen (Fig. 4D–L). Specifically, specimen No. 1 exhibited individual conglomerates of rounded cells, sparsely distributed both in the center and along the edges (Fig. 4D–F); specimen No. 2 showed large conglomerates formed by rounded and spindle-shaped cells in contact with each other, with a higher cell density in the center than along the periphery (Fig. 4G–I); specimen No. 3 presented predominantly spindle-shaped cells forming small conglomerates, where the cells

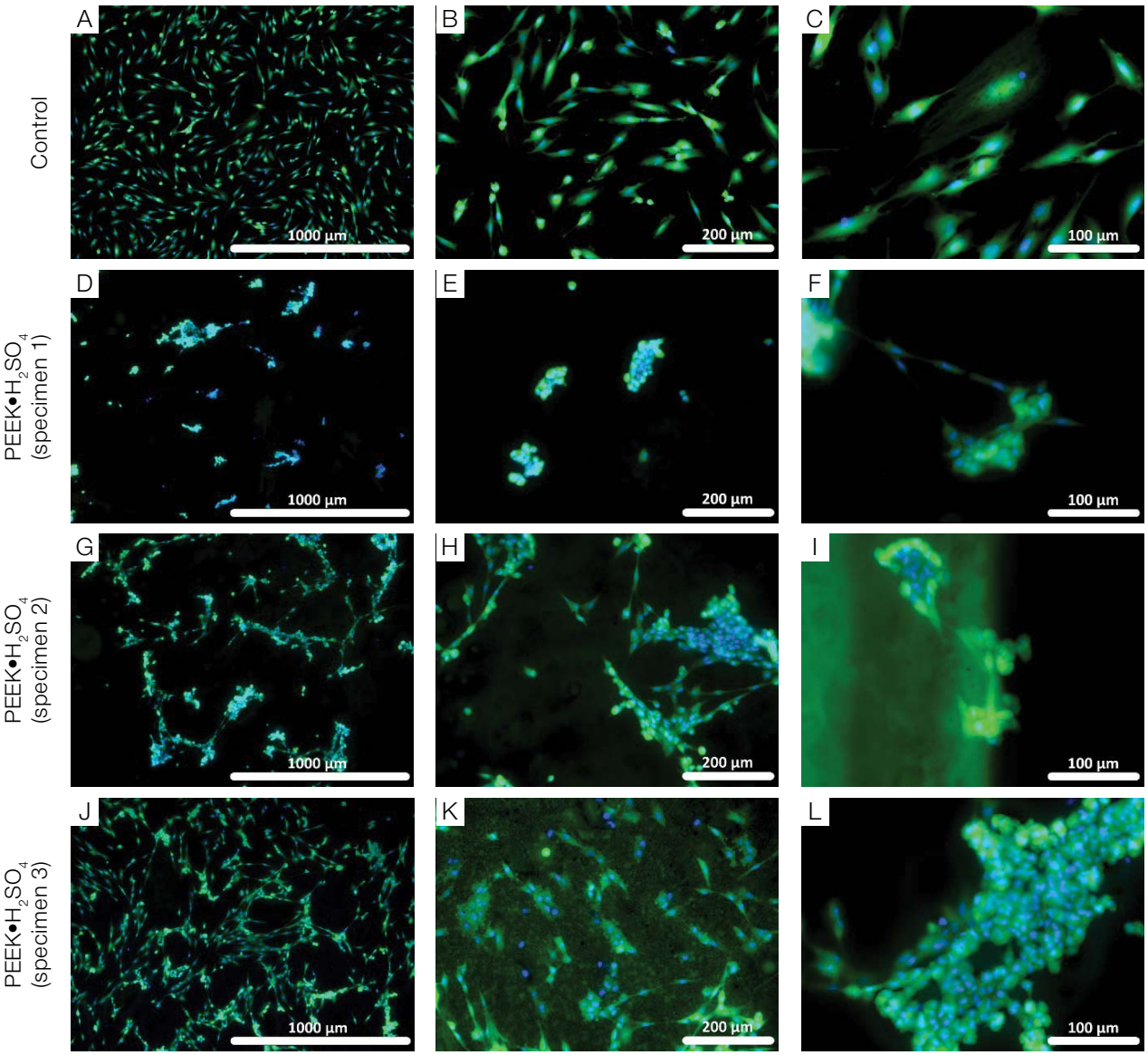

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Fig. 4. Test culture cells on the surface of control wells and polyetheretherketone specimens treated with concentrated H_2SO_4 (PEEK•H_2SO_4). Fluorescence microscopy: green — Calcein AM, blue — Hoechst 33342

created a relatively uniform sparse network in the central region, while remaining unevenly distributed along the edges (Fig. 4K–L).

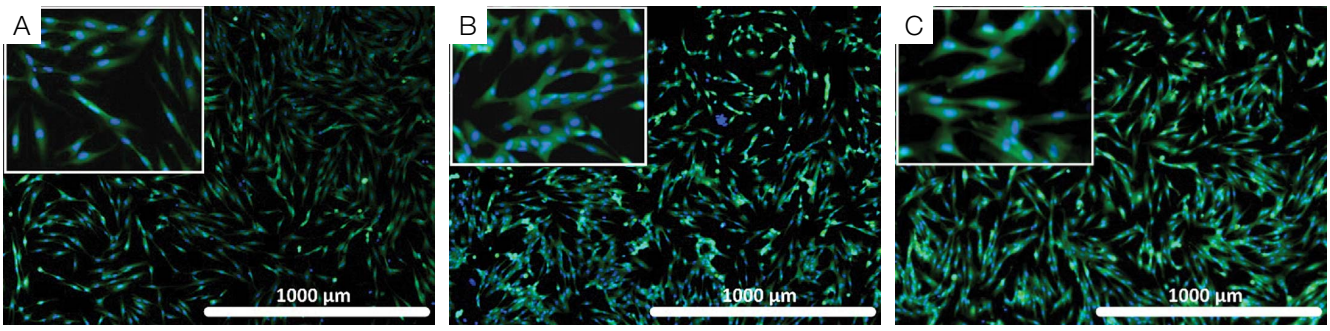
The observed agglomeration of cells on the sulfonated PEEK surface may be attributed to the chemical modifications induced by exposure to 98% H_2SO_4. According to the literature, such treatment leads to the opening of the aromatic ring of the monomer unit, thereby increasing the number of available functional groups. This alteration in surface chemistry could promote cell conglomeration, as previously reported by Dos Santos et al. [38]. It is important to note a key methodological difference between the present study and the work of Dos Santos et al.: in their study, the PEEK surface was pretreated with sandpaper to generate a microrelief, which likely facilitated a more uniform distribution of adherent cells. In contrast, the specimens used in the current investigation were not subjected to mechanical pretreatment, and thus differences in surface roughness may have influenced the pattern of cell adhesion and distribution. Consequently, the discrepancies between the published data and the results obtained in this study following H_2SO_4 treatment may stem from initial differences in the structure and chemical composition of the surface layer. Further investigation is required to confirm this hypothesis.

Notably, a substantial number of viable cells retaining their typical fibroblast-like morphology were observed within the wells after removal of the specimens (Fig. 5). This observation indicates the absence of a cytotoxic effect associated with the H_2SO_4-based surface modification of PEEK. Consequently, the morphological alterations observed in the cells attached to the specimen surface are most likely attributable to the modified surface properties acquired during the treatment process, rather than to systemic cytotoxicity.

On PEEK specimens subjected to piranha solution treatment followed by glycine functionalization (Fig. 6), cell adhesion was reduced relative to the control wells, and the distribution of adherent cells was heterogeneous. Large cell-free areas were observed, with isolated adherent cells predominantly localized along the specimen edges. The lowest number of adhered cells was recorded for Specimen No. 2 (Fig. 6G–I). Concurrently, the majority of the attached cells remained viable, as indicated by the intense green fluorescence of the cytoplasm following Calcein AM staining. In addition to cells displaying a typical fibroblast-like morphology (spindle-shaped with well-developed processes), individual cells with altered morphology were detected, characterized by shortened or absent cellular processes.

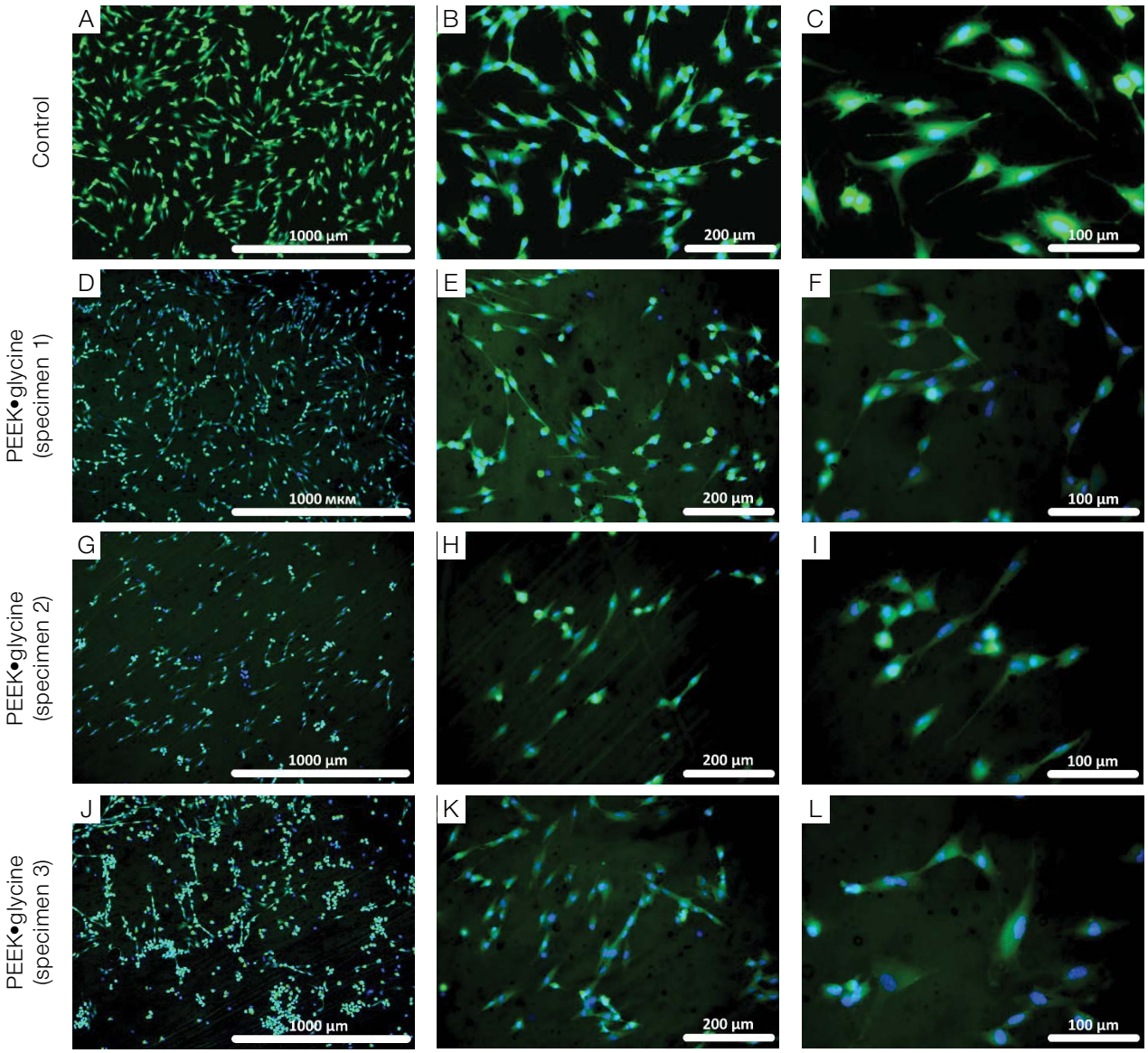
Cell adhesion was evaluated on physically modified porous PEEK specimens prepared by high-temperature fusion with NaCl followed by salt leaching. Compared to the control, a reduction in cell adhesion was observed, although the distribution of adherent cells across the specimen surface was relatively uniform. For example, on specimen No. 1, a large number of adhered cells of round or indeterminate shape were recorded, showing either absent or shortened processes and unstained or weakly green-stained (Calcein AM) cytoplasm (Fig. 7A–C); however, a significant proportion of them were nonviable and deformed. Specimen No. 2 exhibited a fairly large number of adhered cells with high viability (bright green cytoplasm) and preserved typical morphology of dermal fibroblasts (elongated shape with pronounced processes) (Fig. 7G–I). On specimen No. 3, the cells also maintained viability and characteristic morphology, but their number was significantly lower (Fig. 7K–L). These findings indicate that the applied modification protocol does not yield a consistent alteration of surface topography within the specimen series, which is reflected in the variable patterns of cell adhesion observed across individual samples.

The observed reduction in the number of viable cells on porous PEEK specimens fabricated by pressing PEEK with NaCl followed by leaching is likely attributable to residual NaCl within the deep pores located in the bulk of the material. This may explain the discrepancy with the findings reported in [35], where specimens with surface porosity were investigated. In that study, the open architecture of surface pores enabled more efficient removal of the porogen. In contrast, the bulk porous structure of the current specimens limits diffusion, thereby hindering



Photos taken by the authors

Fig. 5. Viable cells within the wells after removal of polyetheretherketone specimens treated with concentrated H_2SO_4 (PEEK•H_2SO_4). Fluorescence microscopy: green — Calcein AM, blue — Hoechst 33342



Photos taken by the authors

Fig. 6. Cell adhesion and distribution on the surface of control wells and polyetheretherketone-glycine specimens. Fluorescence microscopy: green — Calcein AM, blue — Hoechst 33342

the complete elimination of NaCl. Further research is required to optimize the fabrication protocol and develop a method for producing porous PEEK with controlled pore size. Such a material would better support cell penetration and facilitate vascularization.

The present findings do not support the efficacy of the tested surface modification methods — including H_2SO_4 treatment, glycine functionalization, and high-temperature fusion with NaCl — in enhancing the biocompatibility of PEEK. Cell adhesion serves as a critical criterion for evaluating polymers intended for medical applications, particularly in reconstructive surgery, where establishing a favorable interaction between the implant and host bone tissue is essential. A high degree of cell adhesion to PEEK is indicative of biocompatibility and suggests the potential to foster conditions conducive to bone tissue formation around the implant.

The present study demonstrates that surface modification of PEEK can alter its surface morphology and polarity — key parameters that substantially influence the material's biocompatibility. The applied modification protocols not only affected the adhesion of anchorage-dependent cells but also induced changes in their morphology and viability. These findings emphasize that the evaluation of implant materials should extend beyond cytotoxicity testing. Cell adhesion to the specimen surface represents merely the initial screening stage of biocompatibility assessment. To more reliably predict the *in vivo* behavior of implantable materials, a broader characterization is required, including the evaluation of functional cellular activities. A combined qualitative assessment of adhesion and viability enables the rational selection of the most promising candidates at the early development stage.

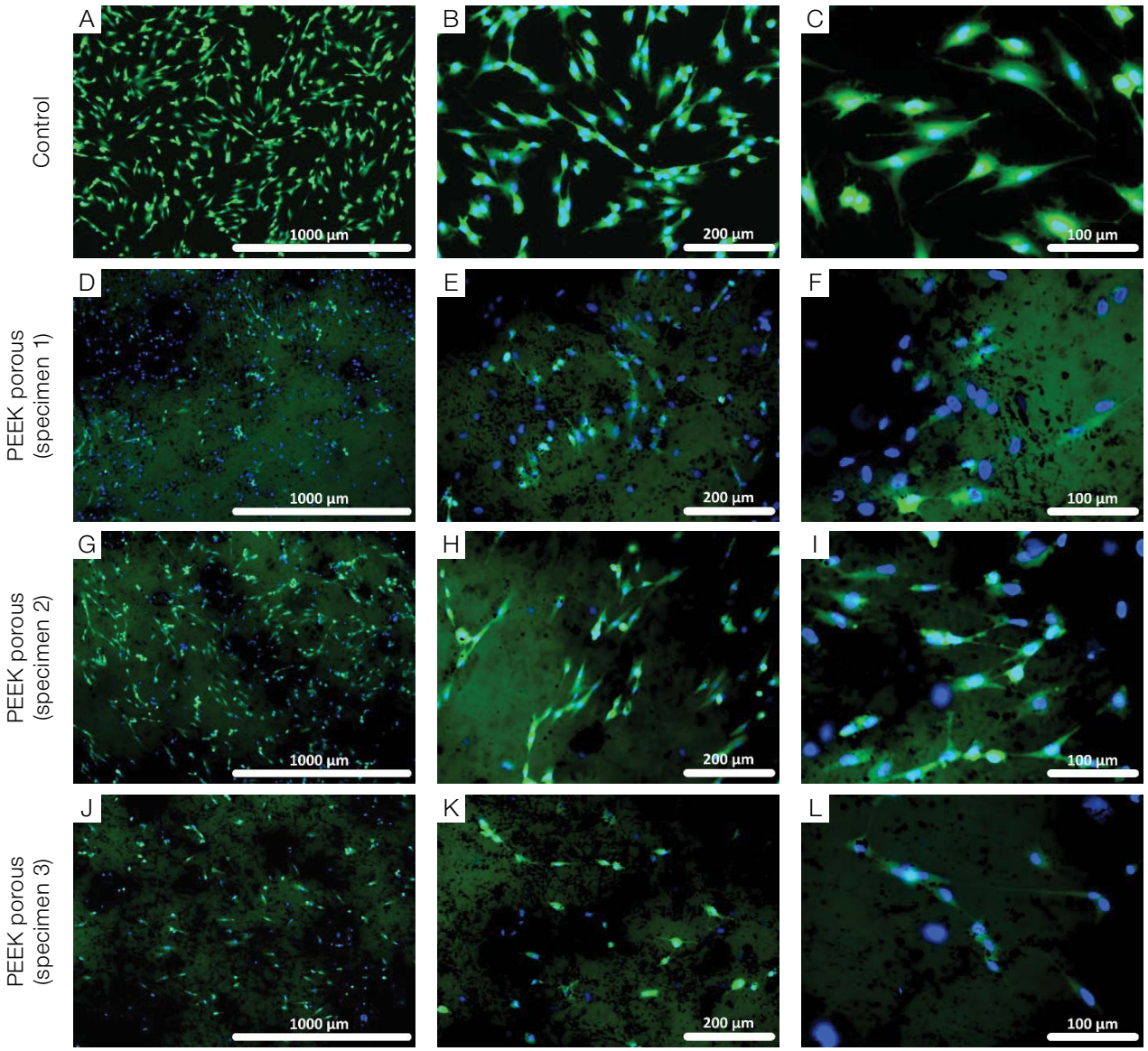


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Fig. 7. Cell adhesion and distribution on control wells and porous PEEK specimens modified by high-temperature fusion with NaCl and subsequent leaching. Fluorescence microscopy: green — Calcein AM, blue — Hoechst 33342

CONCLUSIONS

This study demonstrates that PEEK, synthesized via by polycondensation under nucleophilic substitution conditions by reacting hydroquinone with 4,4'-difluorobenzophenone, exhibited no cytotoxic effect on anchorage-dependent cells, with cell viability remaining at or above 70% *in vitro*. Unmodified PEEK specimens supported the adhesion of anchorage-dependent cells while maintaining their viability and characteristic morphology, showing no significant differences compared to the control. Surface modification of PEEK through H_2SO_4 treatment, piranha solution exposure, glycine functionalization, or high-temperature fusion resulted in variable effects on cell adhesion. The observed reductions in cell viability and alterations in cellular morphology indicate that the tested modification protocols may compromise the material's biocompatibility, thereby limiting its suitability for biomedical applications. These findings underscore the importance of not only optimizing the modification techniques themselves but also performing a comprehensive biological evaluation of the resulting materials at the preclinical stage, using both *in vitro* and *in vivo* models.

Despite the substantial body of experimental research on PEEK composites, current modification technologies remain in the optimization and development phase. Notably, methods reported in the literature as successful do not consistently yield reproducible outcomes under different experimental conditions. This discrepancy is likely attributable to the specific nuances, subtleties, and execution parameters inherent to

each modification technique. Therefore, future research should prioritize the development of more effective and practically feasible modification methods. In addition, long-term preclinical and clinical evaluations of modified PEEK composites are necessary to address existing challenges and facilitate their translation into various biomedical applications.

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