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In vitro and in vivo study of the biocompatibility and adjacent soft tissue response to synthetic polyvinyl formal hydrogel implant

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Background: *Given a rising tendency in the rate of ocular trauma, there is an increasing need in surgeries for orbital, ocular adnexal and periorbital reconstruction. The susceptibility to resorption is a common drawback of the biological tissues used as a plastic material in contemporary orbital surgery. Non-biological hollow porous polyvinyl formal (PVF) hydrogel implants capable of integration with adjacent biological tissues offer a new opportunity for reconstructive surgery.*

Purpose: *To examine the in vivo and in vitro biocompatibility of a synthetic PVF-based hydrogel implant material.*

Material and Methods: *Scanning electron microscopy (SEM) analysis; in vitro evaluation of the biocompatibility of the PVF hydrogel implant material in terms of its cytotoxicity, genotoxicity and effects on marker enzymatic activities of L929 fibroblasts; and in vitro study on implant incorporation.*

Results: *A cross-linked polyvinyl formal hydrogel for orbital endoprosthesis surgery was synthesized. Our in vitro cytotoxicity and genotoxicity tests of the hydrogel and tests of its effects on marker enzymatic activities of L929 fibroblasts demonstrated safety. All hydrogel implant samples, after being sealed into polypropylene bags and autoclaved for 15 min at 121 °C and 1.04 atm steam pressure, were found to be sterile. In all rats in which the hybrid hydrogel implant was placed subcutaneously in the back, there was prompt relief of edema at the site of postoperative suture. The inflammatory reactions seen in the tissues adjacent to the implant included mostly mild diffuse lymphocytic infiltration close to the implant surface, with no inflammatory reactions seen at the sites more remote from the implant.*

Conclusion: *Our in vivo and in vitro study of the PVF hydrogel demonstrated its high biocompatibility in terms of its cytotoxicity, genotoxicity and effects on marker enzymatic activities of fibroblast cells, with no post-implantation complications, but a step-by-step improvement in the relationship between the implant and adjacent tissues. The findings of this study allow us to recommend the developed PVF hydrogel for further research on the potential for using it as an implant for filling postoperative cavities and endoprosthesis in orbital and periorbital reconstruction.*

Keywords:
hydrogels, orbit, orbital and periorbital area, biocompatibility, implantation

Introduction

In recent decades, there has been a tendency to increasing prevalence of craniofacial injuries in Ukraine. This was mostly caused by anthropogenic and criminal-related ocular and orbital injuries. Most orbital injuries are attributed to the violation of orbital wall integrity. In addition, there has been a significant increase in the number of patients who suffered from combat actions in Ukraine. Combat injuries due to mine blast are characterized by substantial damage to ocular tissues and socket and multiple fragmentation wounds, which are frequently concomitant with injuries to the face and other body parts [1]. Given a rising tendency in the rate of ocular trauma, there is an increasing need in surgeries for orbital, ocular adnexal and periorbital reconstruction.

The ocular surgeon has to use implant materials to replace soft tissue and bone structures during restorative and reconstructive surgeries. Presently, such biological tissues as autotissue (autocartilage, autofat, dip fascia of thigh and allogenic tissue) [2, 3] are employed as implant materials in orbital reconstruction. Legal requirements and limitations for donor material harvesting in Ukraine and over the world are being made stronger year by year. Therefore, it is important to develop advanced synthetic materials for orbital and facial bone reconstruction and filling postoperative cavities.

Non-biological hollow porous implants capable of integration with adjacent tissues offer a new opportunity for reconstructive surgery. We believe that a polyvinyl formal (PVF) porous hydrogel may be a promising material for these applications. We have demonstrated previously [4] that PVF porous hydrogels have a high antimicrobial activity and are capable of immobilization and prolonged release of a variety of therapeutic agents [5, 6].

The purpose of the study was to examine the in vivo and in vitro biocompatibility of a synthetic PVF hydrogel implant material as well as the response of soft tissues adjacent to the implant surface.

Material and Methods

The reagents used for hydrogel synthesis were as follows: linear polyvinyl alcohol (PVA; 98%; Appli Chem GmbH, Darmstadt, Germany; 72 kDa); formaldehyde (37%; LAB-SCAN); concentrated sulfuric acid, H2SO4 (Merck, chemical grade, 98%); and TritonX-100 (Polyethyleneglycol tert-octylphenylether C14H22O(C2H4O)n) (Appli Chem GmbH). Bi-distilled water was used as an eluent in all experiments.

Synthesis of spongy polymer matrix based on cross-linked PVF. PVA was condensed with formaldehyde in the presence of strong acid to perform PVA acetylation [4-6]. The percentages of PVA and formaldehyde in the reaction mixture were 6.6% and 7.5%, respectively. Fig. 1 shows a spongy polymer matrix based on cross-linked PVF.

Scanning electron microscopy (SEM) images were obtained with a TESCAN MIRA 3 LMU device with energy dispersive spectroscopy (EDS, Oxford Max-80) and a precision etching coating system (PECS, Gatan 682) to obtain data on the morphology and structure of pores of PVF sponges.

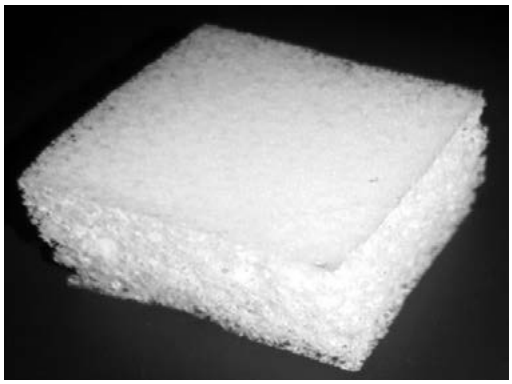

Fig. 1. Photograph of cross-linked polyvinyl formal hydrogel.

Thermogravimetric analysis (TGA) of the synthesized hydrogels was carried out by a TGA Q50 device, and differential thermal analysis, by a MOM Q-1500 derivatograph.

The biocompatibility of the synthesized PVF hydrogel was measured as per International Standard Organization (ISO) guidelines [7]. The cytotoxicity was assessed by three hydrogel exposure methods. In the first method, hydrogel sample was placed on a monolayer of test cells. In the second method, the hydrogel sample-conditioned medium was employed. In the third method, some cell suspension and hydrogel were deposited into a plate well together. The eukaryotic L929 fibroblast cell line was obtained from the Cell Culture Bank of the Zabolotnyi Institute of Microbiology and Virusology, National Academy of Sciences of Ukraine, and used in cytotoxicity tests. Cells were monolayer-cultured in flasks (75 cm2) in DMEM/F12 medium supplemented with 10% inactivated fetal calf serum (FCS) and Antibiotic-Antimycotic mix at 37ºC with 5% CO2. The cell response to potential toxic exposure was determined after 24 h of exposure of the formed L929 cell monolayer to the pre-conditioned test sample. Crystal violet test and MTT assay allow to characterize the key viability indices (the total number of attached cells and the mitochondrial metabolic activity) of the cells and were used to determine the state of the cells after their exposure to the synthesized PVF hydrogel. A metabolic activity index (MAI) was defined as a ratio of the percentage of metabolically active cells and the percentage of total attached cells and was employed for generalization and analysis of results.

The in vitro biocompatibility of the synthesized PVF hydrogel (in terms of genotoxicity) was evaluated by alkaline comet assay on L929 fibroblast cells [8]. The cells were grown and incubated in the medium preconditioned with the synthesized PVF hydrogel. Non-treated test cells were used as negative control cells (no genotoxic effect), and the test cells treated with 1 mM of N-nitrosomethyl urea were used as positive control cells.

The in vitro biocompatibility of the synthesized PVF hydrogel (in terms of effects on marker enzymatic activities, adenosine triphosphate (ATP) activity and lactate dehydrogenase activity) was evaluated with the use of the membranous and cytosolic fractions obtained by fractionation of test cell culture of L929 fibroblasts. Protein concentrations of the obtained fractions were determined by the Lowry method [9]. The total ATP activity of the membranous fraction was determined as previously reported [10, 11]. The lactate dehydrogenase activity of the cytosolic fraction was assessed spectrophotometrically by the dihydroniconinamide adenine dinucleotide (NADH) oxidation at 340 nm [10].

The marker enzymatic activities of the membranous and cytosolic fractions of naive cells were used as negative controls. The enzymatic activities of the fibroblasts exposed to the hydrogel sample extract with the established cytotoxic effect were used as positive controls (the marker of cytotoxic effect).

Sabouraud broth was used concurrently with thioglycollate medium for sterility testing of hydrogel samples. Cells were cultured in two parallel tubes for each medium, in a volume sufficient for complete submersion of the test sample. The cells in the thioglycollate medium were incubated at 32 ± 1°C, and the cells in the Sabouraud broth, at 22 ± 1°C, for 8 days. The material was declared sterile if there was no growth in any culture.

A study of soft-tissue response to the hybrid hydrogel implant included in-vivo experiments on 6 rats in the vivarium of SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine".

All animal experiments were performed in compliance with the Law of Ukraine on Protection of Animals from Cruel Treatment No. 3447-IV dated 21.02.2006 and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from the European Treaty Series (Strasbourg, 1986), and approved by a local Bioethics Committee of the Filatov Institute (Minutes No. 2 dated 25.06.2020).

Prior to surgical procedure, animals were anesthetized with thiopental sodium 0.1% (1.0 mL/kg body weight, intramuscularly). A hybrid hydrogel implant sized 10.0 x 5.0 x 2.5 mm was placed subcutaneously in the back of each animal, and the wound margins were sutured with interrupted 6-0 silk sutures. At days 2, 5 and 10 after implantation surgery, important clinical signs, such as tissue edema, state of sutures, and discharge, if any, were noted. Animals were euthanized by air embolism immediately after removal of implants with adjacent tissues under anesthesia at days 10 and 30.

A histological study to evaluate the effects of implantation of the examined hydrogel was conducted at the Pathomorphology and Electron Microscopy Laboratory, SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine" (Laboratory Competence Certificate No.PT-397/23 dated 31.10. 2023).

Results

SEM was used to examine the microstructure (morphology) of synthesized hydrogels after hydrogel samples swelled to the equilibrium water content state and were lyophilized. Respective microscope photographs with different magnifications are presented in Fig. 2. The synthesized hydrogels belong to porous materials with a system of connected and equally distributed pores and heterogenous multi-level porous architecture. PVF hydrogels have a cellular structure with regularly shaped pores sized 10.0–22.0 ± 1.6 μm. The small pores of the lowest level formed the substructure of the walls of large pores and had a diameter of 3–7± 1.2 μm (Fig. 2).

TGA findings (Fig. 3) included a loss of moisture (4.7%) up to 121° C, an inflection point around 186.82° C, three weight loss maxima at 319.9° C, 417.4° C and 518.9° C, weight stabilization at 622.6° C, post-stabilization residual weight percentage of 0.8%, and a total weight loss percentage of 99.2%. Therefore, there was TGA evidence of a wide range of thermostability of the synthesized medical hydrogel materials, with this range exceeding their specified operating and processing temperature range. It was demonstrated that steam sterilization does not affect the physical and chemical properties of hydrogels.

The in vitro biocompatibility of the PVF-based hydrogel implant material was evaluated in terms of its cytotoxicity, genotoxicity and effects on biochemical markers.

Cytotoxicity findings for the hydrogel are reviewed in Table 1. The toxicity of the hydrogel-conditioned medium was taken into account in the analysis because this characteristic is a reflection of the prolonged toxicity of the hydrogel sample.

The results of the analysis of PVF hydrogel biocompatibility in terms of in vitro genotoxicity are presented in Table 2. The PVF-based hydrogel implant material was found to biocompatible in terms of in vitro genotoxicity (Table 2).

The results of the tests of PVF hydrogel biocompatibility in terms of the effect of the hydrogel on marker enzymatic activities are presented in Table 3.

The exposure to the hydrogel sample known to be toxic (positive control) resulted in 70% inhibition of ATP activity of the membranous fraction and a more than two times stimulation of lactate dehydrogenase activity of the cytosolic fraction of L929 fibroblasts compared to negative control (naive cells) (Table 3). In contrast, the PVF hydrogel was characterized as non-toxic and biocompatible in terms of its effect on marker enzymatic activities of L929 fibroblasts.

All hydrogel implant samples, after being sealed into polypropylene bags and autoclaved for 15 min at 121 °C and 1.04 atm steam pressure, were found to be sterile.

In all rats in which the hybrid hydrogel implant was placed subcutaneously in the back, there was edema at

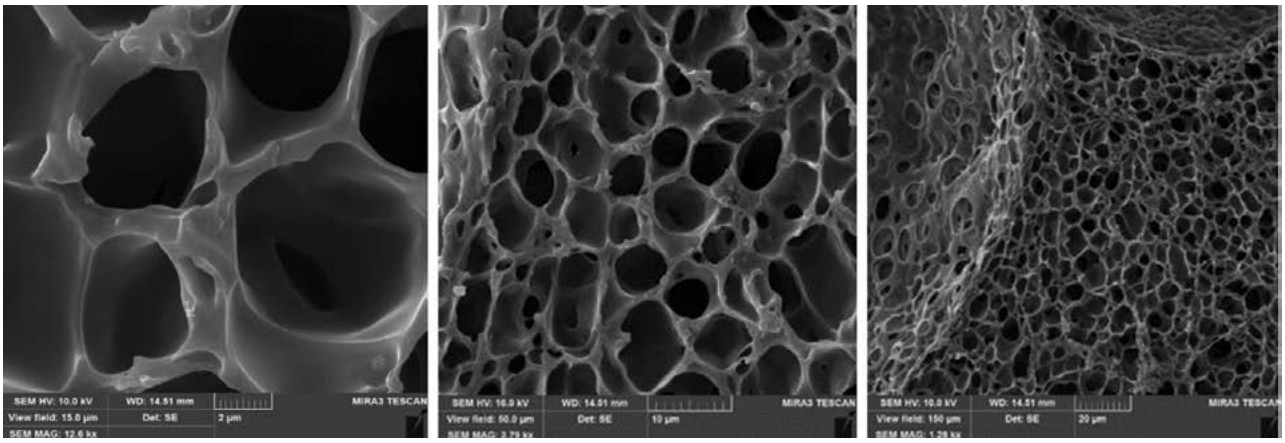

Fig. 2. Scanning electron microscopy (SEM) of the cross-linked polyvinyl formal hydrogel

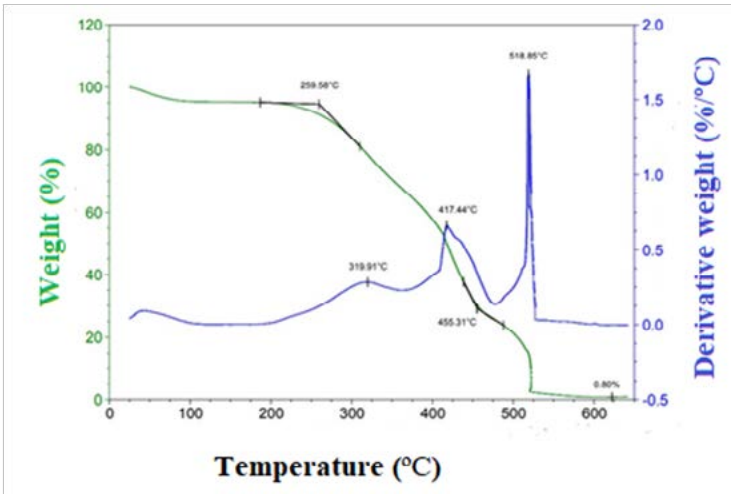

Fig. 3. Thermogravimetric analysis (TGA) curves for the cross-linked polyvinyl formal hydrogel

Table 1. Cytotoxicity of the polyvinyl formal (PVF) hydrogel as assessed based on metabolic activity index (MAI) for L929 fibroblasts

Sample type	Hydrogel sample placed on a monolayer of fibroblast cells	Fibroblasts deposited on the surface of the hydrogel sample	Hydrogel sample-conditioned medium employed
Control	1.0	1.0	1.0
PVF hydrogel	0.8	1.6	0.9

Table 2. In vitro evaluation of polyvinyl formal (PVF) hydrogel biocompatibility in terms of genotoxicity by culturing L929 fibroblasts

Control/Sample	Comet assay index	Conclusion on toxicity
Negative control	0,042±0,002	Non genotoxic
Positive control	2,051±0,001	Genotoxic
PVF hydrogel	0,046±0,001	Non genotoxic

Table 3. In vitro evaluation of polyvinyl formal (PVF) hydrogel biocompatibility in terms of effects on marker enzymatic activities of L929 fibroblasts, adenosine triphosphate (ATP) activity and lactate dehydrogenase (LDH) activity

Control (Ao)/ Substance (Ae)	ATP activity of the membranous fraction			LDH activity of the cytosolic fraction		
	ATP activity (A), nMol Pi/mg protein per h	Ae/ Ao,%	Conclusion on substance toxicity	LDH activity (A), μmol NADH/ mg protein per min	Ae/Ao, %	Conclusion on substance toxicity
Negative control (naïve cells)	6978	100	Control	10.33	100	Control
Positive control (cells exposed to cytotoxic hydrogel sample)	1938	28	Toxic	24.82	240	Toxic
PVF hydrogel	6757	97	Non-toxic	3.04	9	Non-toxic

the site of postoperative suture in the first five days after implantation. The edema began to decrease thereafter and disappeared on day 8 to day 10. On examination of post-surgical skin wound at day 1 and the following days after implantation of the hybrid hydrogen implant, post-surgical wound healing occurred by primary intention.

After the response of adjacent soft tissues to the hybrid hydrogel implantation was clinically found to be satisfactory, we assessed the cell response to the implant, the presence of any adjacent tissue growth into implant structure, and resorption susceptibility of the implant. Thus, at day 10 after implantation, in animals with a subcutaneously placed implant, there was histological evidence of slight to moderate infiltration in the soft tissue structures adjacent to it, with the infiltrate containing not only lymphocytes but also a certain number of eosinophilic and macrophage-histiocytic cells similar in type to tissue basophil cells.

At day 30 after implantation, there was histological evidence of inflammatory response in the structures adjacent to the implant and profound fibroblast proliferation in vessels (Fig. 3). In addition, we noted massive fibroblast growth with the formation of fibrous tissue and numerous vessels in the implant. At some sites, the implant adhered to the epidermis, and no distinct fibrous capsule was seen except at the subepidermal stroma (Fig. 4).

We noted severe fibroblast ingrowth with the formation of fibrous tissue. The implant appeared surrounded by a thin fibrous capsule, which was not sufficiently distinct from the rest of stroma, with mild diffuse lymphocytic infiltration in the adjacent stroma. At some sites, a thin connective tissue layer appeared to be between the implant and adjacent skeletal muscle (Fig. 5).

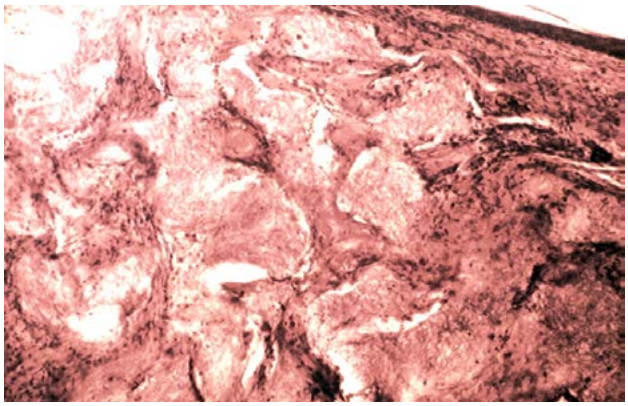

Fig. 4. The implant adhered to the epidermis (can be partially seen at the top right-hand side of the image), and there was no distinct fibrous capsule. Magnification, 100x.

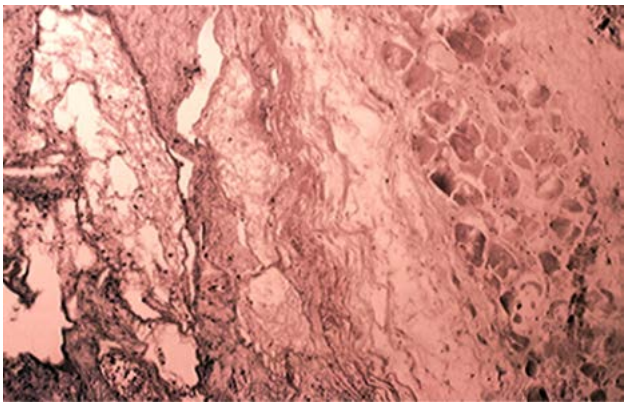

Fig. 5. Some fibrous tissue was seen inside the implant. A thin connective tissue layer appeared to be between the implant and adjacent skeletal muscle. Magnification, 100x.

Discussion

In Ukraine, any developer of implantable medical products (e.g., the hybrid hydrogels examined in the current study) must comply with the national "Technical regulation on active implantable medical products" which was approved by the Order № 755 of the Cabinet of Ministers of Ukraine of 2 October, 2013, and is presently valid as amended on 30 November, 2022 [12]. Of note that the technical regulation was developed based on the European Council Directive 90/385/EEC of 20 June 1990 on the approximation of the laws of the Member States relating to active implantable medical devices. The acceptance of these implantable medical devices in clinical practice must be preceded by conducting well-designed randomized preclinical and clinical trials with adequate sample size. Studies on the biocompatibility and safety of the synthesized hybrid hydrogels as implants are an essential component of a preclinical trial and a prerequisite for further clinical studies and the introduction of these materials into clinical practice. Our in vitro cytotoxicity and genotoxicity tests of the hydrogel and tests of its effects on marker enzymatic activities of L929 fibroblasts demonstrated safety.

The regulations require an implantable medical device to be sterile. Our microbiological studies demonstrated that the used sterilization regimen (15 min at 121 °C and 1.04 atm steam pressure) allows sterility of hydrogel implants. This indicated the adequacy of the selected sterilization regimen and the conformance to the regulations on hygiene for implants [12].

In our in vivo study of post-implantation effects of PVF hydrogels, we observed some manifestations of inflammatory responses to implantation. However, the inflammatory reactions seen in the tissues adjacent to the implant included mostly mild diffuse lymphocytic infiltration close to the implant surface, with no inflammatory reactions seen at the sites more remote from the implant. Most commonly, the inflammatory reactions were seen on day 10, and tended to decrease by day 30. In most histological observations, there was fibrous tissue proliferation at the implant periphery. This in itself does not facilitate capsule formation, because this leads to no clear distinction between the implant and adjacent tissues. Therefore, the relationship between the implant and adjacent tissues indicate better implant integration compared to the implants that are distinct from adjacent tissues.

In conclusion, our in vivo and in vitro study of PVF hydrogel demonstrated its high biocompatibility in terms of its cytotoxicity, genotoxicity and effects on marker enzymatic activities of fibroblast cells, with no post-implantation complications, but a step-by-step improvement in the relationship between the implant and adjacent tissues, thus indicating the conformance to the implantable medical device regulations. The findings of this study allow us to recommend the developed PVF hydrogel for further research on the potential for using it as an implant for filling postoperative cavities and endoprosthesis in orbital and periorbital reconstruction.

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Disclosures

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Author Contributions: *YuMS: Conceptualization and Design of the Study, Literature Data Collection and Analysis, Writing-original draft, Writing-review & editing. APM: Conceptualization and Design of the Study. SMD: Conceptualization and Design of the Study, Biocompatibility Evaluation. TGG, LSR and VIP: Biocompatibility Evaluation. NMZh: Fibroblast Cell Culturing, Cytotoxicity Evaluation. LOK: Hydrogel Synthesis and Characterization, Writing-original draft, Writing-review & editing. NOP and TPP: Hydrogel Synthesis and Characterization. IIeM: Literature Data Collection and Analysis. IIV: Literature Data Collection and Analysis, Writing-original draft, Writing-review & editing. OVA: Histological studies. NMB: Literature Data Collection and Analysis. All authors reviewed the results and approved the final version of the manuscript.*

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Conflict of interest: *The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or material discussed in this manuscript.*

Abbreviations: *MAI, metabolic activity index; PVA, polyvinyl alcohol; PVF, polyvinyl formal; SEM, scanning electron microscopy*