PHYSICOCHEMICAL BIOLOGY

Original article

Synthesis of three-dimensional matrices based on collagen–pectin–polyacrylate grafted copolymers using the $RbTe_{1.5}W_{0.5}O_6$ photocatalyst

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Abstract. *The development of new promising materials of three-dimensional structure from available bioresorbable, biointegrable and biocompatible polymers is in demand and relevant in connection with the intensive development of regenerative medicine. In this work, hydrogels of the grafted copolymers of methyl methacrylate/butyl acrylate onto a mixture of collagen and pectin were obtained during photocatalysis in the presence of a complex oxide $RbTe_{1.5}W_{0.5}O_6$. The characteristics of the synthesis products were obtained by gel penetrating chromatography, elemental analysis, electron microscopy, and biological biocidality tests. The collagen content in an amount of less than 40%, the microstructure of the polymer in the form of a fine-mesh, and the biocidity of the sample films were established. The polymer product was identified on the catalyst surface after the separation of the oxide powder from the aqueous dispersion. The main advantage of such materials is the unique combination of properties of their components assembled into a specific structure. The advantages of the obtained material include, among other things, the environmental advantage of the initial components - natural renewable raw materials: collagen was isolated from cod processing waste, pectin from fruit and vegetable processing waste.*

Keywords: *fish collagen, pectin, complex oxide $RbTe_{1.5}W_{0.5}O_6$, photocatalysis, grafted copolymers, three-dimensional matrix*

Funding. *This work was supported by the Ministry of Education and Science of the Russian Federation (assignment FSWR-2023-0024) on the equipment of the Collective Usage Center "New Materials and Resource-saving Technologies" (Lobachevsky State University of Nizhny Novgorod).*

For citation: Semenycheva L.L., Prodaevich V.V., Rumyantseva V.O., Fukina D.G., Koryagin A.V., Valetova N.B., et al. Synthesis of three-dimensional matrices based on collagen–pectin–polyacrylate grafted copolymers using the $RbTe_{1.5}W_{0.5}O_6$ photocatalyst. *Proceedings of Universities. Applied Chemistry and Biotechnology.* 2024;14(4):556-566. DOI: 10.21285/achb.951. EDN: YOETUS.

ФИЗИКО-ХИМИЧЕСКАЯ БИОЛОГИЯ

Научная статья
УДК 547.96:547.458.88:544.526.5
EDN: YOETUS
DOI: 10.21285/achb.951

Синтез трехмерных матриц на основе привитых сополимеров коллаген–пектин–полиакрилат с использованием фотокатализа в присутствии сложного оксида $RbTe_{1,5}W_{0,5}O_6$

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Аннотация. *Разработка новых перспективных материалов трехмерной структуры из доступных биорезорбируемых, биоинтегрируемых и биосовместимых полимеров востребована и актуальна в связи с интенсивным развитием*

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регенеративной медицины. В данной работе гидрогели привитых полимеров метилметакрилата/бутилакрилата на смесь коллагена и пектина были получены в ходе фотокатализа в присутствии сложного оксида $RbTe_{1,5}W_{0,5}O_6$. Характеристики продуктов синтеза были получены с помощью гель-проникающей хроматографии, элементного анализа, электронной микроскопии и биологических тестов на биоцидность. Было установлено содержание коллагена в количестве менее 40%, микроструктура полимера в виде мелкоячеистой сетки и биоцидность образцов пленок. Полимерный продукт был идентифицирован на поверхности катализатора после отделения порошка оксида от водной дисперсии. Основным преимуществом таких материалов является уникальное сочетание свойств их компонентов, собранных в определенную структуру. К преимуществам полученного материала относится в том числе экологическая ценность исходных компонентов – натурального возобновляемого сырья: коллаген был выделен из отходов переработки трески, пектин – из отходов переработки фруктов и овощей.

Ключевые слова: *рыбный коллаген, пектин, комплексный оксид $RbTe_{1,5}W_{0,5}O_6$, фотокатализ, привитой сополимер, трехмерная матрица*

Финансирование. *Данная работа выполнена при поддержке Министерства образования и науки Российской Федерации (задание FSWR-2023-0024) на оборудовании Центра коллективного пользования «Новые материалы и ресурсосберегающие технологии» (Нижегородский государственный университет им. Н.И. Лобачевского).*

Для цитирования: Семенычева Л.Л., Продаевич В.В., Румянцева В.О., Фукина Д.Г., Корягин А.В., Валетова Н.Б. [и др.]. Синтез трехмерных матриц на основе привитых сополимеров коллаген–пектин–полиакрилат с использованием фотокатализа в присутствии сложного оксида $RbTe_{1,5}W_{0,5}O_6$ // Известия вузов. Прикладная химия и биотехнология. 2024. Т. 14. N 4. С. 556–566. DOI: 10.21285/achb.951. EDN: YOETUS.

INTRODACTION

The three-dimensional supramolecular structure is characteristic of biological origin macromolecules, such as proteins, nucleic acids or carbohydrates. It is known that the shape and conformation of macromolecules and the interaction between their components play an important role in their functioning and interaction with each other and determine the active sites where processes occur that allow macromolecules to bind to other molecules, such as enzymes, receptors or drugs [1]. Artificial modeling of three-dimensional structures from available bioresorbable, biointegrable and biocompatible polymers has become one of the most developing areas of scientific research in polymer chemistry. It is due to the practical aspects of using such structures in regenerative medicine, targeted drug delivery, etc. Nowadays, there are several approaches to creating three-dimensional structures based on fibrillar macromolecules of synthetic and natural origin: strategies of double mesh, double crosslinked or interpenetrating nets, chemical modification due to covalent and non-covalent interactions along the bonds involved in these mechanisms [2–4].

Chemical crosslinking is an important step to give satisfactory properties to collagen-based materials. There are several comparative studies on the crosslinking of collagen-based fibrillar gels, which are preferred biomaterials due to properties similar to native tissues with various crosslinking agents [5, 6]. The main advantage of such materials is the unique combination of properties of their components assembled into a specific structure. The advantages of the new material include the environmental advantage of the initial components – natural renewable raw materials: collagen was isolated from cod processing waste, pectin from fruit and vegetable processing waste. The literature provides examples in which pectin can act as a compatible component of collagen in the production of three-dimensional matrices, but the process requires optimization for specific compositions [7, 8]. Various examples can be given: the fibrillar gel was prepared from tilapia collagen and alginate in [9], using tilapia collagen and hyaluronic acid in [10], the invention [11] reveals a method for obtaining and using a recombinant type III collagen gel and sodium hyaluronate with double crosslinking. These examples indicate the prospects of research on the production of three-dimensional structures based on natural polymers of different natures: proteins and polysaccharides. It should be noted that such an approach to obtaining new materials for medicine is dictated by the nature of living organisms: the main substances of the intercellular matrix of any living organism performing structure-forming, protective, and other functions are proteoglycans and glycoproteins, which are structured copolymers of proteins and glycans (polysaccharides) [12–15].

Earlier in our research, we obtained grafted copolymers of some vinyl monomers for cod collagen (CC) [16–20] and pectin [21] synthesized using photocatalysis in the presence of complex oxide $RbTe_{1.5}W_{0.5}O_6$ and known modifying additives (triethylene glycol dimethacrylate (TEDMA), polyethylene glycol (PEG), acrylic acid (AA)). It was found in [21] that the addition of TEDMA in in small quantities and AA in an amount commensurate with the concentration of methyl methacrylate (MMA) to the reaction mixture upon obtaining grafted copolymers of acrylates onto cod collagen, after neutralization of the reaction mixture with 1M NaOH solution at the end of synthesis, it is possible to obtain a mixture of grafted and cross-linked polymer, which is stable in aqueous solution and does not form a coagulate. The addition of PEG to the initial mixture in an amount commensurate with the amount of collagen, after neutralization of the reaction mixture, makes it possible to obtain coagulate of a cross-linked structure. Such a hydrogel easily releases water when dried in vacuum and forms a polymer composite film.

The change in the structure of macromolecules when polysaccharide is added into the initial reaction mixture along with collagen is associated with known interactions such as van der Waals, electrostatic, hydrophobic, and hydrogen bonds. In the presence of active radicals pectin interacts during synthesis, as in the case of collagen, with a hydroxyl radical due to the separation of the hydrogen atom of the hydrocarbon part of the polysaccharide molecule is possible, presumably according to scheme 1. In addition,

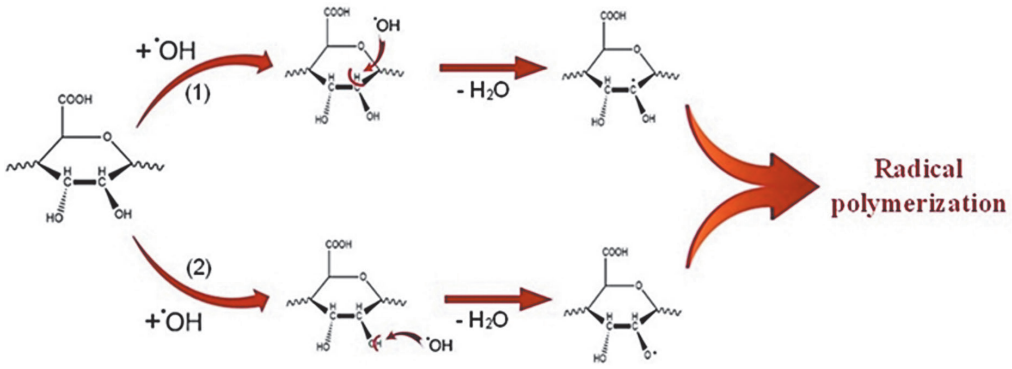

Fig. 1. Scheme of a hydroxyl radical interaction with polysaccharides

Рис. 1. Схема взаимодействия гидроксильного радикала с полисахаридами

this may occur due to the separation of the hydrogen atom from the hydroxyl group of the polysaccharide, presumably according to scheme 2 (Fig. 1), forming radicals in the macromolecule of the polysaccharide [22–24].

These newly formed radicals are active in all radical transformations in the reaction mixture: they interact with the monomer and form a grafted synthetic fragment or participate in a chain transfer reaction, or disproportionate with another active radical and also recombine, i.e. they form additional covalent bonds in the material[1].

In this work, the purpose is to obtain new hydrogels based on grafted copolymers of MMA and butyl acrylate (BA) on fish (cod) collagen synthesized using the $RbTe_{1.5}W_{0.5}O_6$ photocatalyst, with simultaneous addition of polysaccharide – pectin to the composition of the initial reaction mixture (Pect-CC-PMMA and Pect-CC-PBA). Pectin is not only a modifying agent, but also an additional polymer substrate, along with collagen, on which centers of initiation of radical polymerization of acrylate monomers are formed. The obtained new materials should be characterized for a number of important indicators. In this regard, the main task of the study was to introduce into the initial reaction mixture (fish collagen – vinyl monomer (MMA, BA)) as a modifier and a second natural component of pectin in amounts commensurate with collagen, and to control its effect on the composition and properties of the resulting composite in comparison with the MMA/BA copolymer on collagen. It should be especially noted that collagen and pectin are constantly renewable raw materials. Both polymers are isolated from production waste: fish collagen from fish industry waste, pectin from fruit and vegetable processing waste. Currently, it is important if we consider the prospect of using the development in medicine.

EXPERIMENTAL

Materials. Commercial reagents were used without pretreatment: apple pectin (“S.Pudov”), acetic acid, sodium hydroxide, acrylic acid, TEDMA, PEG. MMA and BA were pre-purified from the stabilizer by washing with a solution of sodium hydroxide, then rinsed with cold water to a neutral pH, dried using calcium chloride and distilled in vacuum. The photocatalyst $RbTe_{1.5}W_{0.5}O_6$ was obtained by the method [25].

Isolation of cod collagen. Collagen was isolated by the method [26] by extraction with acetic acid for one day at room temperature. The resulting acetic acid dispersion was dried to a constant weight under vacuum (1.33 Pa) at 50 °C.

Synthesis of graft copolymers under photocatalysis conditions. Pectin and collagen were dissolved separately in a small amount of water at a temperature from 50 to 70 °C with stirring, an emulsion was prepared by mixing the components in the ratio pectin:collagen:monomer:water = 2,5:2,5:5,0:90, a catalyst was added, argon was bubbled for 15 minutes, then stirred (800 rpm) under irradiation with a visible light LED lamp (LED, 30 W, λ = 400–600 nm) in argon current for 5 hours. After polymerization, the reaction mixture was centrifuged at 4000 rpm to separate the catalyst. The catalyst was washed in water 3 times at 50 ° C for 20 minutes and dried to a constant weight.

Analysis of molecular weight characteristics by gel-penetrating chromatography. Molecular mass characteristics were determined by gel-penetrating chromatography (GPC). Aqueous solutions of copolymers isolated from the reaction mixture were analyzed using a high-performance liquid chromatograph manufactured by Shimadzu CTO 20A/20A C (Japan) using LC solutions. Separation was performed using a Tosoh Bioscience TSKgel g3000swxl column with a pore diameter of 5 microns and a low-temperature light scattering detector ELSD-LT II. The eluent was 0.5 M acetic acid solution, the flow rate was 0.8 ml/min, and narrowly dispersed dextran standards with a molecular weight range (MW) of 1–410 kDa (Fluca) were used for calibration.

Scanning electron microscopy. The surface of lyophilically dried copolymer samples was studied using a scanning electron micro-scope JSM-IT 300 (Jeol ltd., Japan) with an electron probe diameter of 5 nm (operating volt-age 20 kV), using detectors of low-energy secondary electrons and backscattering electrons in low vacuum mode to avoid the samples charging.

Elemental analysis of copolymers. The analysis of dried samples was carried out by the CHNS analysis method on the vario EL cube element analyzer for simultaneous determination of CHNS(O). Elemental analysis of the catalyst surface was performed using X-ray microprobe analysis (RMA) with a detector X-MaxN 20 (Oxford Instruments) Kα(O) and Lα(Rb, Te, W).

[1] Handbook of radical polymerization / eds K. Matyjaszewski, T.P. Davis. New York: Wiley Interscience, 2002. 936 p.

Freeze drying. The sponges of the samples were obtained by lyophilic drying. The solvent was distilled under vacuum (1.33 Pa) using deep freezing by liquid nitrogen.

Studies of polymer films for resistance to microscopic fungi. The study of mushroom resistance was carried out according to GOST 9.049-91 "Polymer materials and their components" method 1. The film samples were placed in Petri dishes. Then the samples surface was inoculated with a suspension of spores of micromycetes test cultures, active destructors of polymer materials: Aspergillus oryzae F-2096, Aspergillus niger F-1119, Aspergillus terreus F-1025, Chaetomium globosum F-109, Paecilomyces variotii F-378, Penicillium funiculosum F-1115, Penicillium chrysogenum F-245, Penicillium cyclopium F-245, Trichoderma viride A-1117. Petri dishes with samples were placed in a thermostat for 28 days at a temperature of 29±2 °C and humidity of more than 90%. After the test time, the fungal resistance of the samples was evaluated on a 6-point scale (Table). The area of biofouling and the degree of micromycetes development on the samples were taken into account when assessing fungal resistance. Samples of materials on which the growth of fungi did not exceed 2 points were considered to be fungal resistant.

Assessment of the samples fungal resistance

Оценка устойчивости образцов к грибкам

Score	Characteristic of the score
0	No germination of spores and conidia was detected under the microscope
1	Sprouted spores and a slightly developed mycelium are visible under the microscope
2	A developed mycelium is visible under the microscope, sporulation is possible
3	The mycelium and/or sporulation are barely visible to the naked eye, but are clearly visible under a microscope
4	The development of fungi covering less than 25% of the test surface is clearly visible to the naked eye
5	The development of fungi covering more than 25% of the test surface is clearly visible to the naked eye

RESULTS AND DISCUSSION

The effect of pectin on the structure and properties of grafted copolymers "pectin – CC – polyacrylate" was controlled by physicochemical characteristics. Once again, it should be noted that collagen isolated from any natural substrates, even with the most delicate isolation in the form of a high-molecular polymer with MM ~300 kDa, does not have sufficient mechanical strength. It is due to the fact that the intra- and intermolecular cross-links that arise during the biosynthesis of collagen in vivo and give its fibrils a stable structure, mechanical strength and resistance to the action of enzymes are destroyed during its release into solution. In this regard, an important step in the creation of any collagen materials is the development of crosslinking conditions that ensure mechanical strength and stability during the whole life of the material. At the same time, crosslinking agents are used for collagen fibers of various natures [3, 4].

The process of grafted copolymers synthesis was carried out in dispersion, using aqueous solutions with the inclusion of MMA (Pect-CC-PMMA) and BA (Pect-CC-PBA). After the end of the syntheses and the stopping of mixing, the gel coagulated from the reaction mixture (Fig. 2).

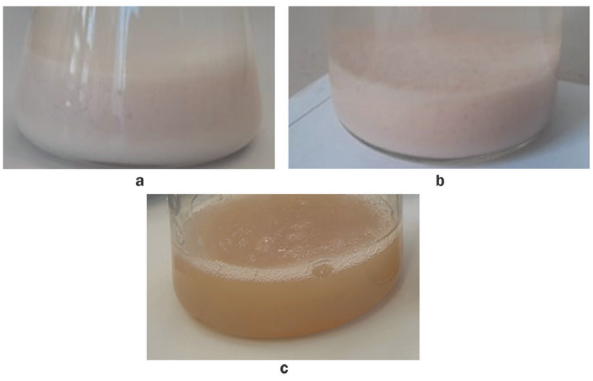

Fig. 2. Images of reaction mixtures Pect-CC-PMMA (a) and Pect-CC-PBA (b) after synthesis, Pect-CC-MMA before synthesis (c)

Рис. 2. Фотографии реакционных смесей Пект-ТК-ПММА (a) и Пект-ТК-ПБА (b) после синтеза, Пект-ТК-ММА до синтеза (c)

Analysis of the nitrogen content in the isolated and dried polymer products showed that they contain collagen and its amount is significantly less than for the sample of the copolymer PMMA-collagen–PEG-AA (CCC-1) (Fig. 3) [20]. It indicates that pectin is more part of the formed copolymers than PEG.

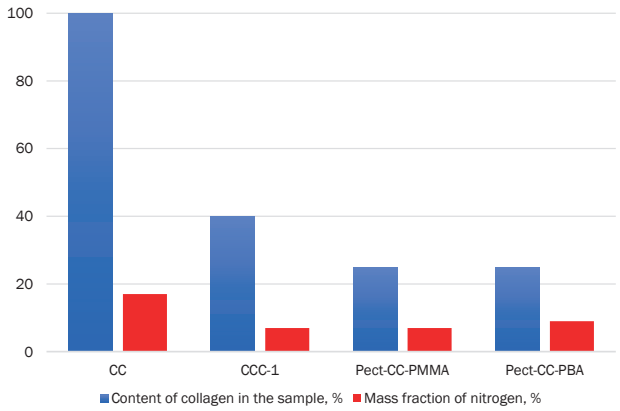

Fig. 3. Nitrogen and collagen content in the samples according to elemental analysis data

Рис. 3. Содержание азота и коллагена в образцах по данным элементного анализа

The SEM images of the new grafted copolymers Pect-CC-PMMA and Pect-CC-PBA (Fig. 4, *a–d*) indicate the cellular structure of the copolymers. Comparing them with the morphology image of previously obtained samples from PEG – sample CCC-1 (Fig. 4, *e*) [20] shows that the morphology of the new materials differs markedly, including the size, shape and dimensional organization of the cellular structure. It can be seen that the pore size in the case of grafted copolymers with pectin for both MMA and BA is significantly smaller than for copolymers obtained under comparable conditions with PEG (samples with the same magnification: *a*, *b* – with pectin, *e* – with PEG). At the same time, collagen fibers are significantly

more compacted in the case of PEG copolymers (see Fig. 4, *e*) than for grafted copolymers with pectin (see Fig. 4, *a*, *b*). That is, the introduction of pectin into the composition of copolymers with both MMA and BA leads to the formation of a small-cell matrix with less pronounced outlines of structural fibrils. In addition, it can be noted that with a greater magnification in grafted copolymers with pectin (see Fig. 4, *c*, *d*), the interpenetration of external and internal pores is clearly visible, which is important in scaffold technologies to characterize the degree of channel interconnection of matrix pores [27, 28].

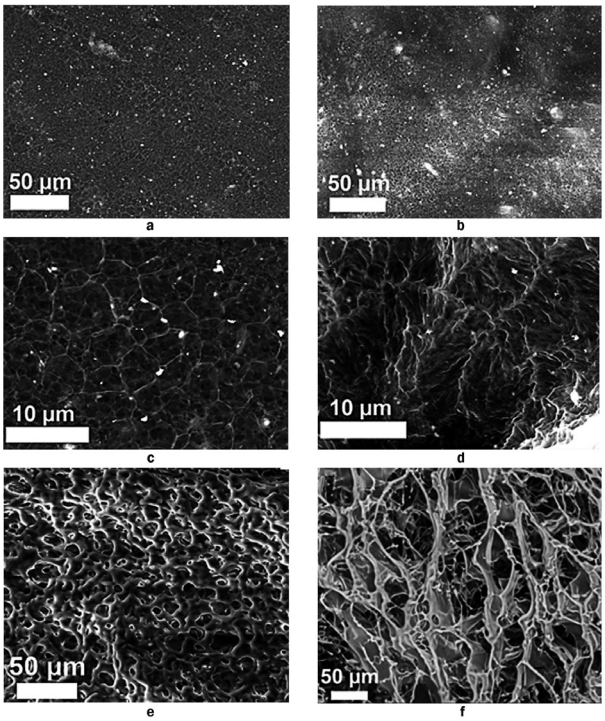

Fig. 4. Scanning electron microscope images of copolymers Pect-CC-PMMA (a, c), Pect-CC-PBA (b, d), CCC-1 (e) [20] and collagen (f) [16]

Рис. 4. Выполненные при помощи сканирующего электронного микроскопа изображения сополимеров Пект-ТК-ПММА (a, c), Пект-ТК-ПБА (b, d), CCC-1 (e) [20] и коллагена (f) [16]

In the aqueous phase, after coagulation of the Pect-CC-PMMA and Pect-CC-PBA copolymers, polymer fractions with MW ~190 kDa, ~11 kDa and ~22 kDa and oligomeric fragments with MW less than 0.5 kDa were found not embedded in the grafted copolymer matrix. Most likely, the high-molecular polymer is collagen, fragments with MW ~11 and ~22 kDa can be attributed to both collagen and pectin, since it is precisely such low-molecular collagen that is formed because of collagen hydrolysis and is determined in pectin [29, 30]. Oligomeric fractions are characteristic of pectin [30]. In general, an important conclusion from the analysis by the GPC method is the statement of the presence of the initial polymers residues that are not embedded in the structure of the Pect-CC-PMMA and Pect-CC-PBA copolymers. This may be primarily due to the insufficient amount of hydroxyl radicals necessary for the formation of grafted and crosslinked polymers. This is due to the adsorption of synthetic monomers and the grafting of synthetic polymer products on the catalyst' surface. Apparently, in this case, the process of interaction of electron-hole pairs with water becomes hysterically difficult, and the formation of a hydroxyl radical practically stops. In addition, increasing the electron migration path along the chain of polymer molecules from the catalyst to the radical center in solution increases the probability of recombination of the electron-hole pair. This leads to a sharp decrease in the number of active particles and formed radicals, and, consequently, to a slowdown and stop of the polymerization reaction in solution [18].

It is important to note that polymer materials based on natural polymers are easily damaged by biological agents under operating conditions, the main among which are microscopic fungi[2] [31]. It was previously noted that the PMMA-CC copolymer has fungal resistance due to the content of $RbTe_{1.5}W_{0.5}O_6$ oxide particles in micro quantities with sizes ≤ 2 nm [32]. In Fig. 4, *a–d*, such particles are clearly visible. It was necessary to find out whether the new materials Pect-CC-PMMA and Pect-CC-PBA have fungal-resistant properties. During the experiments, it was shown that the growth of fungi on Pect-CC-PMMA and Pect-CC-PBA polymer materials films was 0 scores. It suggests that these materials are not capable of being used by fungi as a food source. It is important because the biostability of polymer composites is a critical property of such materials, since it determines the long-term operation function of a particular product in conditions of possible fungal damage.

It should be mentioned that, as in previously published research [16, 21], fragments of polymer macromolecules were found on the catalyst surface (Fig. 5), which are isolated on the surface by homogeneous filaments with a large diameter or plates. Such organic fragments are formed on the surface due to the grafting of acrylate onto the radicals of the catalyst surface, the occurrence of which is associated with the interaction of hydroxyl radicals with hydroxyl groups always present on the surface of metal oxides. It has already been discussed earlier in the works [16, 21].

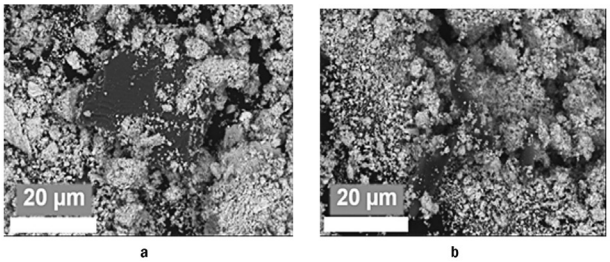

Fig. 5. Scanning electron microscope images of the catalyst surface

Рис. 5. Выполненные при помощи сканирующего электронного микроскопа изображения поверхности катализатора

[2] Ерофеев В.Т., Смирнов В.Ф., Морозов Е.А. Микробиологическое разрушение материалов: учеб. пособие для студентов. М.: Изд-во Ассоциации строительных вузов, 2008. 123 с.

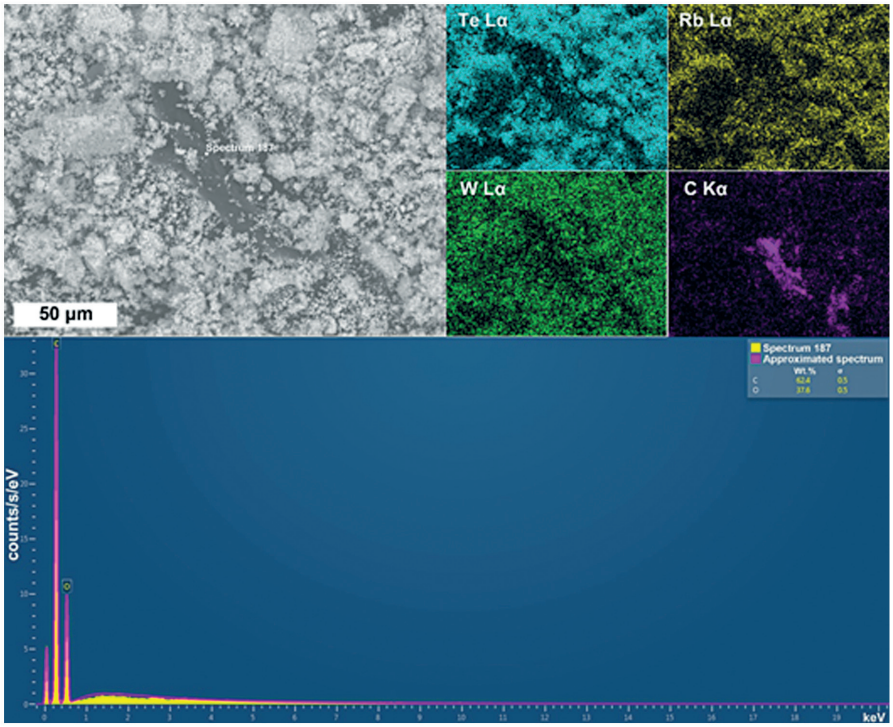

Fig. 6. Scanning electron microscope images of the catalyst surface after synthesis and maps of the elements distribution

Рис. 6. Выполненные при помощи сканирующего электронного микроскопа изображения поверхности катализатора после синтеза и карты распределения элементов

The evidence of the organic nature of such filaments is provided by studies of the elemental composition of the samples, which were carried out using the X-ray microanalysis (Fig. 6). To determine how atoms are distributed in the sample, maps of the elements distribution were obtained (see Fig. 6). In this case, it can be seen that the distribution of the elements Rb, Te, W on the studied surface is homogeneous and corresponds to powder particles of photocatalyst. Fragments of organic filaments are clearly visible on all maps, and the chemical composition of the sample in the selected area (C and O content), shown in the spectrum and in the table in Fig. 6, indicates the organic nature of the selected area.

CONCLUSIONS

As a result of the conducted studies, hydrogels of the grafted copolymers Pect-CC-PMMA and Pect-CC-PBA were obtained using photocatalysis in the presence of a complex oxide $RbTe_{1.5}W_{0.5}O_6$. Polymer products are characterized by methods of elemental analysis and electron microscopy. Dry polymer products, as it turned out, contain collagen in an amount of less than 40%. The SEM images demonstrate the formation of a small-cell matrix for these polymer composites. By the GPC method, it was found that some of the natural polymers of collagen and pectin remained in aqueous dispersion and did not enter the structure of the new material. It was revealed using an X-ray microprobe analyzer that a part of polyacrylates is identified on the catalyst surface after its separation from the aqueous dispersion. The new materials are fungus-resistant, which is important for the long-term operation function of a particular product in conditions of possible fungal damage. The results of the study indicate the expediency of developing research on modified materials based on grafted copolymers of methyl methacrylate, pectin and fish collagen prepared by the $RbTe_{1.5}W_{0.5}O_6$ photocatalysis to obtain a wide range of medical products: colloidal solutions, gels, films.

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Conflict interests

The authors declare no conflict of interests regarding the publication of this article.

The final manuscript has been read and approved by all the co-authors.

Information about the article

Поступила в редакцию 29.05.2024.
Одобрена после рецензирования 22.11.2024.
Принята к публикации 30.11.2024.

Конфликт интересов

Авторы заявляют об отсутствии конфликта интересов.

Все авторы прочитали и одобрили окончательный вариант рукописи.

Информация о статье

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