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Regular paper

Physicochemical properties and cytotoxicity of hydrogels based on Beetosan® containing sage and bee pollen

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Currently, increasing attention is being paid to issues related to environmental protection, waste management, as well as to the development of polymers with useful properties. The research presented here involved preparation of hydrogels based on Beetosan® – a chitosan derived from the multi-stage processing of dead bees. Moreover, hydrogels were additionally modified with natural substances – i.e. bee pollen and extract of *Salvia officinalis* (sage) that are well known for the presence of many compounds with beneficial properties from a medical point of view. Materials have been first obtained by photopolymerization. Then, their surface morphology, wettability and cytotoxicity to selected cell lines have been determined. It can be stated that such combination of Beetosan® hydrogel matrix and the mentioned additives resulted in a preparation of polymers characterized by negative impact on cancer cells. Impact of hydrogels with sage is slightly more intense due to the presence of substances such as ursalic or rosmaric acid that are characterized to have anticancer activity. Such negative impact has not been observed in case of studies using fibroblasts. Furthermore, addition of natural substances into hydrogels resulted in a more homogeneous surface and in the decrease of wettability angle of the tested polymers. It can be concluded that the use of natural-derived reagents and synthesis of polymers using these reagents (as a result of environmentally friendly photopolymerization) yields materials with interesting properties for medical purposes, with particular emphasis on antitumor activity, and without significant negative impact on fibroblasts.

Key words: hydrogels; cytotoxicity; fibroblasts; chitosan; sage; wettability

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Abbreviations: SEM, Scanning Electron Microscopy; MTT reagent, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoline bromide

INTRODUCTION

Nowadays, particular attention is paid to the healing properties of materials of natural origin. Such materials undoubtedly gain a great popularity and can compete with traditional pharmaceuticals. A great deal of substances that are characterized by features that are desirable from medical point of view can be found in plants or substances resulting from the plants' processing, as well as in substances of animal origin, including bee pollen. Despite the fact that the applied drugs derived from chemical synthesis are widely used in medicine and in pharmacy, a certain part of them can cause undesirable effects. That is the reason for looking for new solutions in the surrounding environment. Special role can be played by plants, such as sage and chamomile, or their byproducts, as bee pollen. The mentioned materials, due to their composition and positive effect on human organisms, constitute interesting reagents for synthesis or modification of materials that potentially can be applied in medicine. Sage or bee pollen contain numerous nutrients, both macro- and microelements, and this is a reason why they can significantly increase the potential use of the mentioned hydrogel polymers (Hasanein *et al.*, 2016; Cabrera de Oliveira *et al.*, 2016).

The main component of synthesized hydrogel matrices is Beetosan®. It is a type of well-known polysaccharide – a chitosan – obtained as a result of multistage chemical treatment of bees. The mentioned insects are individuals that do not survive difficult winter conditions and constitute a waste. Therefore, the proposed methodology is also interesting from the point of view of the waste management (Nemtsev *et al.*, 2004; Draczyński, 2008; Mucha, 2010; Alemdaroglu *et al.*, 2006; Ma *et al.*, 2007). Moreover, preparation of Beetosan can affect the environmental protection. The applied reagents are not toxic and no toxic organic solvent is used. Additionally, preparation of the mentioned substance does not generate any waste and the process is not long-lasting. Therefore it can be concluded that the conducted reaction does not have any negative impact on the environment and, which is also important, natural waste, such as bees that had died of natural causes, are converted into a product that can be further used for preparation of materials for biomedical purposes.

It should be also emphasized that the research presented here is a continuation of previously presented studies (Tyliszczak *et al.*, 2017; Tyliszczak *et al.*, 2016).

MATERIALS AND METHODS

Materials. Beetosan® acting as a hydrogel matrix was prepared at the Cracow University of Technology during a multistage chemical treatment. Diacrylate poly(ethylene glycol) (average Mw 700 g/mol; density: 1.120 g/ml) and 2-hydroxy-2-methylpropiophenone (97%; density: 1.077 g/ml) were bought from Sigma Aldrich S.A. Sage and bee pollen were supplied by Herbapol Lublin Sp. z o.o. In case of gelatin – this reagent was ordered from the Avantor Performance Materials (formerly POCH SA).

Synthesis of hydrogels. The first step involved preparation of materials constituting the hydrogel matrix.

Table 1. Compositions of the synthesized hydrogels.

No.	Base solution [ml]	Bee pollen solution [ml]	Sage infusion [ml]	Crosslinking agent [ml]	Photoinitiator [ml]
1.	50	-	-	8	0.25
2.	50	1	-	8	0.25
3.	50	3	-	8	0.25
4.	50	5	-	8	0.25
5.	50	-	1	8	0.25
6.	50	-	3	8	0.25
7.	50	-	5	8	0.25

For this purpose, gelatin and Beetosan® were dissolved in 0.05% aqueous solution of acetic acid and such a mixture was treated as the base solution. The concentrations used were as follows: gelatin solution 2%; Beetosan® solution 3%. Then, the aqueous suspension of bee pollen (substance in a solid form, concentration: 1.0 wt%) and aqueous extract of sage (0.5 wt%; prepared by treatment of 1 g of ground leaves with water at 70°C) were prepared. Next, suitable amounts of the base solution, additives' solutions, crosslinking agent (diacrylate poly(ethylene glycol), M_w=700 g/mol) and photoinitiator (2-hydroxy-2-methylpropiophenone) were mixed and exposed to UV radiation for 1–2 min. Quartz lamp – Emita VP 60 – was used (power – 120 W; wavelength λ=320 nm) as a source of the mentioned radiation. In Table 1, compositions of Beetosan® based modified hydrogels are presented.

SEM Analysis. The prepared hydrogels have been tested using Scanning Electron Microscopy. Such analysis provides information about the external topography of the tested sample, as well as about the internal structure with consideration of a porosity. The analysis has been conducted by means of Helios NanoLab H50HP FEI. SEM microphotographs with detector set at voltage of 5 kV.

Studies on wettability of hydrogels including determination of contact angles. Synthesized materials have been subjected to the surface wetting. The measurements were conducted by using the Kruss DSA 100 M device. The dried polymer sample was subjected to the wettability angle test with the presence of a drop of distilled water. The surface contact angles for some hydrogel samples were determined. Behavior of the tested material after contact with a drop of distilled water was also photographed.

Studies on cell lines. In order to check cytotoxicity of the synthesized materials, studies on selected cell lines were carried out. Jurkat cells (cancer origin) and WEHI 164 (fibroblasts) have been selected for this analysis. The culture medium of the tested cells was as follows: RPMI1640 (containing such compounds as biotin, vitamin B12, and PABA). However, this type of medium does not contain any proteins or lipids, therefore it should be supplemented with 10% FBS (Fetal Bovine Serum) or NCS and 1% antibiotics. It also uses a sodium bicarbonate buffer system (2.0 g/l), and therefore requires a 5–10% CO_2 environment to maintain physiological pH. Determination of cytotoxicity has been conducted using the MTT test. This assay is based on a reaction of reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoline bromide (known as the MTT reagent) that is accompanied by formation of azure formazan in the living cells. Such reaction makes possible to define enzymatic activity of succinate dehydrogenase in order to define the cellular vitality. In the next step, the formed formazan (whose amount is related to the number of living cells) is measured by a colorimetric analysis.

In order to check cytotoxicity, the samples of modified hydrogels were introduced into vessels containing selected cell cultures. Subsequently, the test was performed by introduction of a small amount of MTT reagent directly to the cell culture, incubating for previously selected periods of time and finally measurement of absorbance using a multifunctional plate reader. Measurement for every sample in the MTT test was conducted six times and the average values are presented.

RESULTS AND DISCUSSION

SEM Analysis

Microphotographs of the obtained modified hydrogels are presented in Fig. 1.

On the basis of the studies conducted, it can be stated that addition of natural substances has a significant impact on the surface of hydrogel materials. Surface of unmodified hydrogels is more heterogeneous in comparison to the one of with additives. This is probably caused by the fact that substances introduced in a form of solu-

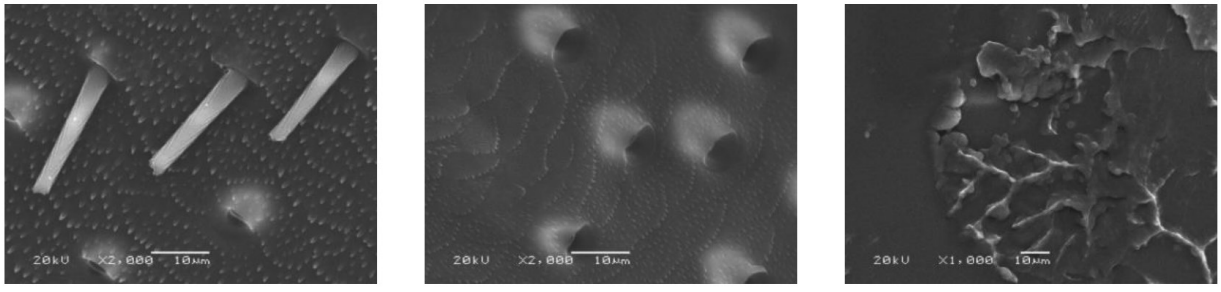

Figure 1. SEM microphotographs of hydrogels modified with: (**a**) sage; (**b**) bee pollen and (**c**) unmodified hydrogel

Table 2. Values of contact angles.

	Contact angle [deg]		
	Unmodified sample	Sample with sage	Sample with bee pollen
I	55.0	37.0	31.0
II	48.0	35.0	33.0
III	47.0	35.0	32.0
Average value	50.0	35.6	32.0

tions fill in some surface inhomogeneities. Moreover, the introduced additives are probably located in areas between polymer chains and therefore the material becomes more homogeneous. Such phenomenon can have an impact on sorption capacity of the tested materials due to the fact that the absorbed liquid penetrates the pores of the material, and hydrogel with a more homogeneous surface will be characterized by less swelling ability. Such information is important from a point of view of future application of these materials. In other words, it is possible to modify a sorption capacity of hydrogel samples by changing the amount of the additives' solutions.

Table 3. Results of cytotoxicity of hydrogels in relation to the Jurkat cells (average values of six measurements).

	3rd Day of cell culture			5th Day of cell culture			7th Day of cell culture		
	All cells [%]	Alive cells [%]	Nonviable cells [%]	All cells [%]	Alive cells [%]	Nonviable cells [%]	All cells [%]	Alive cells [%]	Nonviable cells [%]
Control sample	93.6	96.1	3.4	83.2	89.8	8.8	80.4	99.1	0.5
Sample with sage	76.0	13.3	83.5	65.9	3.6	92.3	61.4	6.4	92.7
Sample with bee pollen	78.2	26.5	70.3	50.5	6.4	87.7	35.2	11.3	87.5

Studies on surface wettability

Results of the conducted wettability studies are presented in Table 2.

Based on the results of wettability studies, it is possible to conclude that all of the tested materials are characterized by hydrophilicity that can be indicated by values of contact angles which are lower than 90 degrees. Hydrogels containing sage and bee pollen have a significantly lower contact angle in comparison to the unmodified sample. This is probably caused by lower porosity, as well as by lower heterogeneity of the modified samples, resulting from the partial closure of the pores of the material by the additives introduced in the form of solutions. Thus, water has a limited ability to penetrate to the interior of the tested hydrogel. It is worth noting that results of wettability studies are compatible with the results of surface morphology analysis.

Studies on cell lines

Results of cytotoxicity of the prepared hydrogel polymers are presented in Table 3–4.

Results of cytotoxicity clearly define the impact of prepared hydrogels on selected cells. In case of cancer cells, the most promising behavior has been observed for hydrogels modified with the sage solution. Almost all cancer cells have been classified as nonviable after 7-days of contact with the hydrogel containing sage. It can be noticed that samples containing sage exhibit greater anticancer activity when compared to those modified with bee pollen. It is probably due to the presence of terpenes or terpenoids, such as caryophyllene and α-humulene, in the sage extract. Additionally, it has been stated that compounds present in the sage extract, such as ursolic acid or rosmarinic acid, have been also defined as substances having negative impact on cancer growth. Their anticancer activity has been noticed by Ghorbani *et al.*, 2017. Similar effect has been observed in case of the hydrogel with bee pollen, however its negative impact on cancer cells is slightly less intense. Maybe it is caused by the fact that the above mentioned compounds are not present in its composition.

Additionally, any impact of the culture medium on the results of the MTT test has not been determined.

Based on these observations, it can be stated that this type of materials can be subjected to more advanced research in order to determine their potential application in the anticancer therapy. In case of hydrogels with bee pollen, a negative impact on cancer cells has been also noted, but to a much lesser extent. Interesting conclusions can be also drawn on the basis of studies with fibroblasts. Only 0.5% cells treated with the hydrogel containing bee pollen were classified as nonviable which indicates that these materials do not have any negative impact on human fibroblasts. Certain negative impact has been observed for hydrogel with addition of bee pollen. These results may be due to the presence of some unreacted reagents in the hydrogel network, such as the crosslinking agent or photoinitiator that can adversely affect the cells.

CONCLUSIONS

A series of Beetosan® based hydrogel materials containing substances of natural origin, such as sage or bee pollen, have been prepared. A type of chitosan – Bee-

Table 4. Cytotoxity of prepared materials in relation to the WEHI 164 cells (fibroblasts) (average values of six measurements).

	WEHI cells (fibroblasts)		
	3rd day of culture		
	Type of cells [%]		
	all	alive	nonviable
Control sample	80.0	96.1	3.9
Sample with sage	43.4	99.5	0.5
Sample with bee pollen	30.6	79.1	20.9

tosan® – obtained from dead honeybees has been used as the major component of the polymer matrix. Moreover, hydrogels have been modified with natural substances characterized by favorable properties from a medical point of view. Hydrogels containing sage and bee pollen have heterogeneous (although more homogeneous in comparison to the unmodified sample) and porous surface, therefore water can penetrate their interior. What is especially important, on the basis of the cytotoxicity studies it can be concluded that hydrogels containing sage and bee pollen have a significantly negative impact on cancer cells. It is an essential information due to the potential application of such materials in medicine, including cancer treatment. It is also worth noting that the prepared materials do not have a significant negative effect on human fibroblasts. The synthesized materials are of great importance from the point of view of such realms as chemical technology and medicine, but also waste management, due to the use of such waste as bodies of honeybees as the major component of the hydrogel matrix.

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