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POLY(ALKYLIMIDE) AND POLY(VINYL ALCOHOL) MEDICAL HYDROGELS – TESTING WITH U937 CELL LINE

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Abstract. Direct interactions with a cell line of poly(vinyl alcohol) and poly(alkylimide) hydrogels were tested using optical microscopy. After the interaction neither necrotic cells nor changes in cell activity and morphology were noticed although poly(vinyl alcohol) showed better compatibility. IR spectroscopy and differential scanning calorymetry were used giving characteristics of hydrogels studied.

Key words: hydrogels, medical fillers, poly(vinyl alcohol), poly(alkylimide), U937 cell line, biocomatibilty.

1. Introduction

Polymer hydrogels are a new group of materials used in plastic and reconstructive surgery as an injectable prosthesis and in tissue engineering as scaffolds or matrices not only for correction but also for improving some aesthetic imperfections or reconstructing some serious defects. They represent a new group of biomaterials used in medicine for non-invasive therapeutic treatment in opposition to popular well known biomaterials demanding surgical procedures [1]. In the last decade, there has been a great development in the field of polymer-based hydrogels. Those water-swellable polymeric materials are a class of biomaterials very similar to a soft tissue because of their high water content, mechanical properties, low invasiveness, softness, oxygen permeability and high biocompatibility. Polymer hydrogels can be either chemically stable and remain unchanged in host organism or degradable and dissolve *in vivo* [2, 3].

Some examples of polymer-based hydrogels are represented by poly(vinyl alcohol) (PVA), poly(alkylimide) (PAI) [4], polyethylenoglycol (PEG), poly(2-hydroxyethyl methacrylate) (polyHEMA) [5] and polyacrylamide (PAA) [6]. The fundamental aspect of their proper use is the knowledge about the potential cytotoxicity and interaction with the surrounding human tissue. There are many tests for estimating chemical, physical and microbiological properties of biomaterials, especially of solid polymers. There are also many devices to be used in testing, such as atomic force microscopy analysis (AFM), light microscopy, cryogenic transmission electron microscopy (cryo-TEM), differential scanning calorymetry (DSC), solid-state nuclear magnetic resonance or X-ray scattering [4, 7, 8, 9].

In this paper poly(vinyl alcohol) and poly(alkylimide), commercially available synthetic hydrogels, are the subject of interest. They both are used as reconstructive surgery injectable prosthesis. There is a great need to enrich methods of testing hydrogels. In the paper the new test is proposed to be used for polymer hydrogels examination. It is the test with human malignant cell line U937 usually performed for evaluating the influence of pharmacological substances such as retinoic acid, myristate acetate [10] or lipid based reagents [11] on tissue. That simple *in vitro* test was innovatory used for estimating the behavior of hydrogels *in vivo* and host response to their presence.

U937 cells are characterized by round and smooth surface; they grow attached to the matrix and to each other by extending pseudopodia and developing 3-dimensional aggregates upon exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) [12-14]. All the presented experiments are focused on comparing the morphological changes in U937 cells after the interaction with synthetic hydrogels containing alkylimide groups or alcohol vinyl groups. In this way we want to receive the answer if the same procedure of testing used for pharmacological substances can be accomplished for polymer hydrogels. As there is no information in the medical leaflet or patent on the details of chemical composition of both hydrogels studied the characterization by IR and DSC analysis is also undertaken in this paper to be useful for future comparison of those hydrogels with other ones of that type.

2. Experimental

2.1. Materials

Two kinds of hydrogels were studied, *i.e.* permanent poly(alkylimide) (PAI) and biodegradable poly(vinyl alcohol) (PVA). PAI hydrogel was supplied by Polymekon, Italy (commercially available as Bio-Alcamid®). It is supplied as a solution of polyakrylimide in pyrogenic water in closed and sterile synergies (4 % PAI and 96 % of pyrogen free water).

PVA hydrogel was supplied by Polmax, Czestochowa, PL; (commercially available as Bioinblue®). From the literature it is known, that PVA hydrogel is prepared in a series of freeze and thawing cycles [15-17]. It is also supplied as a solution of PVA in pyrogenic water in closed and sterile synergies (4 % PVA and 96 % of pyrogen free water).

As it was mentioned in the introduction the supplier delivered no details about the chemical structure. That is why certain physico-chemical characteristics of the studied materials are determined as well by infrared spectroscopy (IR) and differential scanning calorymetry (DSC).

2.2. Methods

2.2.1. Infrared spectroscopy(IR)

The hydrogel samples were analyzed by IR (spectrophotometer FTIR Nicolet 8700- Thermo Electro Corporation) in a wet and dry state (drying at room temperature in the air to a constant weight. The IR examination of water was included as a comparison. As a result of IR analysis the proper absorption spectra were obtained. All spectral peaks were interpreted in accordance with the literature data [18].

2.2.2. Differential scanning calorymetry (DSC)

DSC thermograms (Perkin Elmer DSC 7) were performed on hydrogel samples in a dry state (dried in the same way as for IR analysis). The DSC program consisted of heating, cooling and additional heating steps. The first heating step for PVA proceeded from 298.15 to 488.15 K at the constant heating rate of 30°/min. Then the sample was kept for one minute at 488.15 K and cooled down to 293.15 K at the rate of 30°/min. After reaching 293.15 K the sample was kept for one minute at that temperature and again heated from 293.15 to 523.15 K. Thus, sets of thermograms representing three runs (heating, cooling and heating) were obtained. The first heating step for PAI was within the temperature range from 298.15 to 523.15 K.

The distinct transitions were analyzed by the Pyris™ 7 Software- PerkinElmer®.

2.2.3. Interaction test

The U937 cell line was cultured in fibronectin Phosphate Buffer Saline (PBS), at concentration of 1 mg of fibronectine in 10 ml of PBS, for 18 h at 277.15 K. U937 cells were treated with PAI and PVA hydrogels in Phosphate Buffer Saline (PBS); one dish was left as a control one and treated with all the same substances but the polymer. The interactions of hydrogels with U937 cells were studied using two kinds of samples, *i.e.* cells in suspension and cells attached to the slides. In the case of attached cells, small slides were placed inside each sample, so the cells could attach to them. Later, the cells were differentiated with solution of bovine serum albumine (BSA) in PBS at concentration of 1g per 100 ml. Afterwards the BSA-containing solution was removed and a solution of TPA at 50 ng per 1 ml of medium was added. All samples were incubated for 72 h at 310 K in 5% CO_2. In the next step, cells were fixed in 4% formalin and placed onto the microscope slides. Cells on slides were treated with hematoxyline and stained with activated eosine (0.5 %) to color cytoplasm pink color. After staining, samples were dehydrated in ethanol (dehydration series) and air-dried for 24 h.

Stained slides were evaluated and cell counts were performed using the light microscopy at proper magnification (40, 60 and 100X).

The cells were counted by two methods. For the samples containing a large number of live cells (*i.e.* control and sample after the interaction with PVA), ten randomly chosen fields on the slide were counted. For samples with only a few surviving cells (*i.e.* sample after interaction with PAI), the whole slide area was assessed.

For the cells in suspension, two sub-samples (marked I and II) of the same sample were placed onto the two ends of one slide (Fig. 1). Counting was performed for both areas of the slide, and an average from the sub-samples I and II was calculated. The fixation and staining method for the suspended cells was identical to that used for the attached cells.

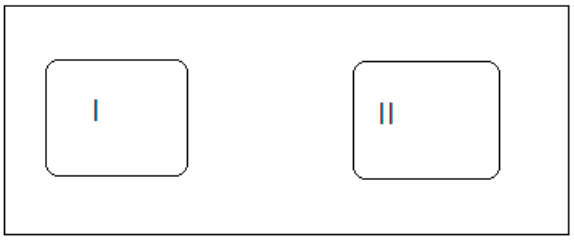


Fig. 1. Schematic arrangement of samples on microscopic slide

3. Results and Discussion

As a result of IR analysis, a set of absorption spectra for water (Fig. 2), PAI (Fig. 3) and PVA (Fig.4) was obtained.

IR spectra presented in Figs. 2, 3a and 4a are very similar. Broad absorption bands are visible in the range of 3500–3400 cm^{-1} and a narrow one at 1630–1615 cm^{-1}.

They are typical for hydroxyl groups of –OH water band, and are due to valence vibrations. It means that no signal coming from the polymer is observed in Figs. 3a and 4a. This can be explained by the very low concentration of polymers (4 %) in the analyzed hydrogels. That is why we analyzed the hydrogels in dry states as well.

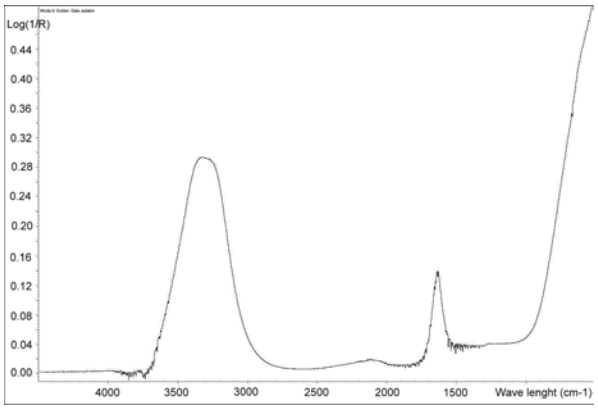


Fig. 2. IR absorption spectrum for water

In Fig. 3b (PAI in a dry state) the absorption bands characteristic for imide, amide and hydroxyl groups (as a result of water bound to hydrogel despite of drying) are found at 3400–3200, 3500–3300 and 3500–3490 cm^{-1}, respectively. In the range of 3500–3200 cm^{-1}, the peaks resulting from valence vibrations of different molecules are very close to each other; a very broad enhanced pick can be observed. In the range of 1305–1200 cm^{-1}, the pick due to deformation vibrations in amides is present. From those results we can state that studied PAI hydrogel has alkylimide-amide groups.

Fig. 4b shows the results of IR analysis for the dry PVA hydrogel. The absorption bands characteristic for water are visible in the range of 3500–3490 cm^{-1}; those for –OH groups in alcohol (valence vibration) at 3356 cm^{-1}; bands due to deformation vibration in –OH groups in alcohols at 1400–1340 cm^{-1}; bands for –C–OH bond which is typical for alcohols (visible due to drying) at 1125–1085 cm^{-1}; and those for primary alcohol at 1075–1000 cm^{-1} [7]. Thus studied PVA posses primary and secondary alcohol groups.

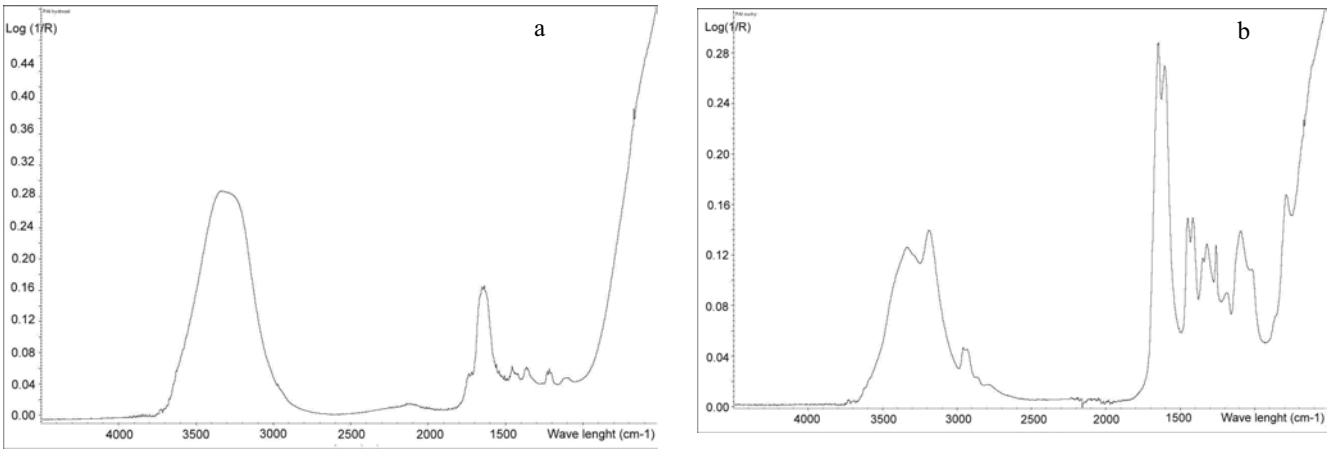


Fig. 3. IR absorption spectra for PAI analyzed in wet (a) and dried (b) states

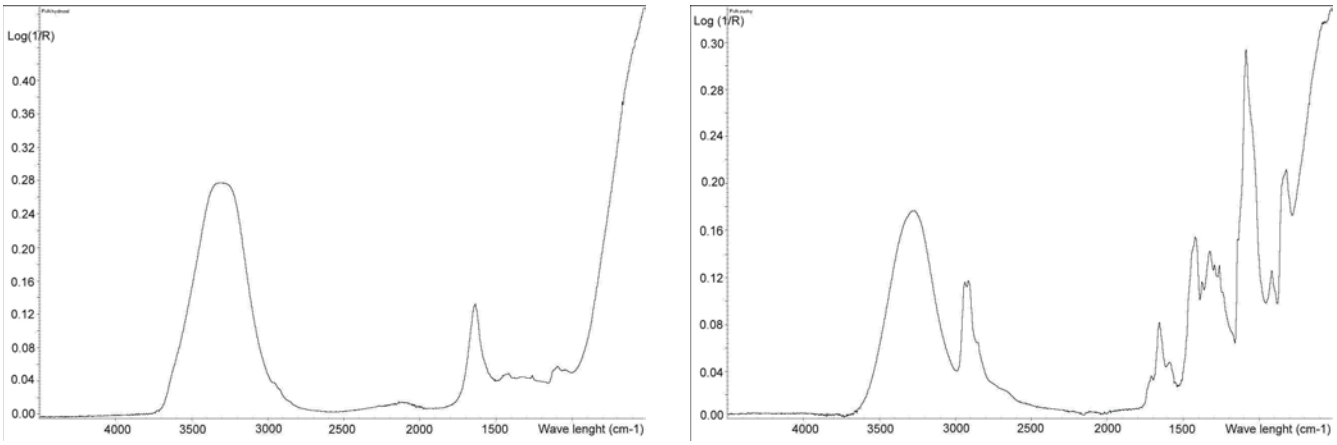


Fig. 4. IR absorption spectra for PVA hydrogel analyzed in wet (a) and dried (b) states

In Table 1 all visible transitions have been summarized. The glass transitions (T_g) are well visible in the heating runs. In the first heating run T_g equals to 398.55 K (T_{g1}) while in the second heating run it is 351.75 K (T_{g2}). Brundrup and Immergut, who studied PVA, have reported about T_g value of around 458 K, and two melting points [14]. One melting point was found by them at 485 K for isotactic PVA and another one at 505–540 K for syndiotactic PVA. In our study, the melting point in the first run was 475 K (T_{m1}) (Fig. 5d), which is close to isotactic PVA. The crystallization process was observed during the cooling procedure in the temperature range of 413–453 K (Fig. 5c). Assuming the enthalpy (ΔH^0) for PVA in its completely crystalline state to be 152 J/g [8], the crystallinity was estimated at 0.42 %. After heating to 488.15 K and quick cooling, the crystallinity of PVA increased to 10.57 %. T_g determined during the first heating run was higher by about 40 degrees than that for the second heating run. These results indicate that after heating the hydrogel to 488 K (which is close to the melting point and the degradation range) the crosslinked network is destroyed and chains become more flexible. Thus it can be concluded that under specific conditions the analyzed PVA is a crystallizable polymer. The crystallization process has been reported in the literature as responsible for weathering of some hydrogels after many cycles of cooling and heating [7]. Therefore it is worth to consider this fact while planning future applications of the investigated PVA. The authors will devote a separate paper to this issue.

DSC analysis did not reveal any thermal transitions in PAI hydrogel that had been subjected to the similar procedure as the one used for PVA.

Table 1

The set of results for DSC analysis of PVA

Run	1st heating				Cooling			2nd heating		
Parameters	T_{g1}	ΔCp_1	T_{m1}	ΔH_{m1}	T_c	ΔH_c	T_{g2}	ΔCp_2	ΔH_{m2}	T_{m2}
Unit	K	J/g·K	K	J/g	K	J/g	K	J/g·K	J/g	K
Value	398.55	0.221	375.25	0.64	438.27	-20.3	351.28	0.25	16.07	479.7

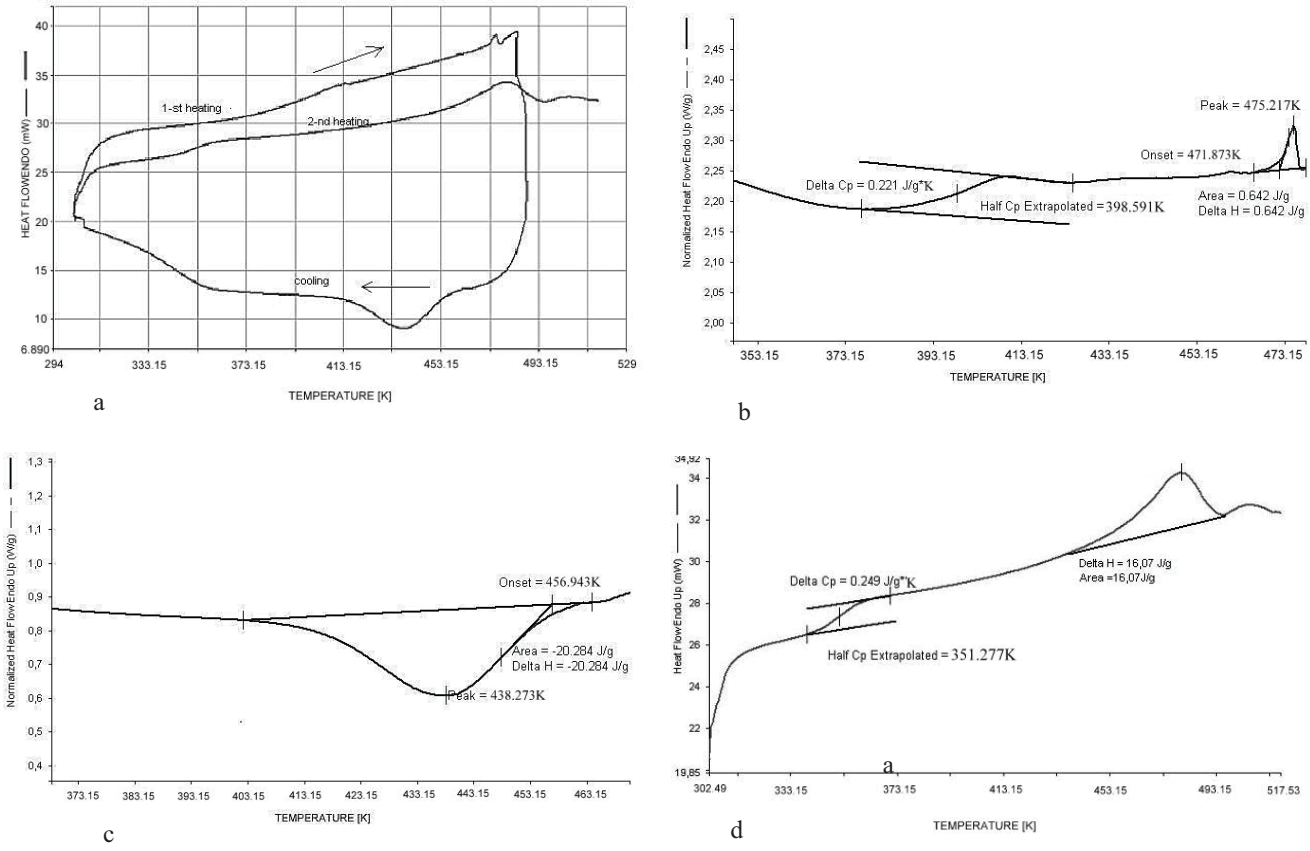


Fig. 5. The general view of DSC thermograms for PVA in a dried state (all three runs) and examples of a computer calculation (a); calculation of glass transition and melting point in the first run (1st heating) (b); calculation of DH for crystallization in the second run (cooling) (c) and calculations of the second glass transition (2nd heating) (d)

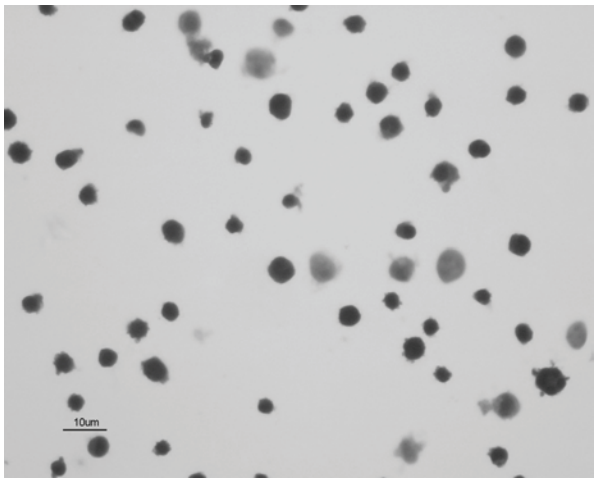
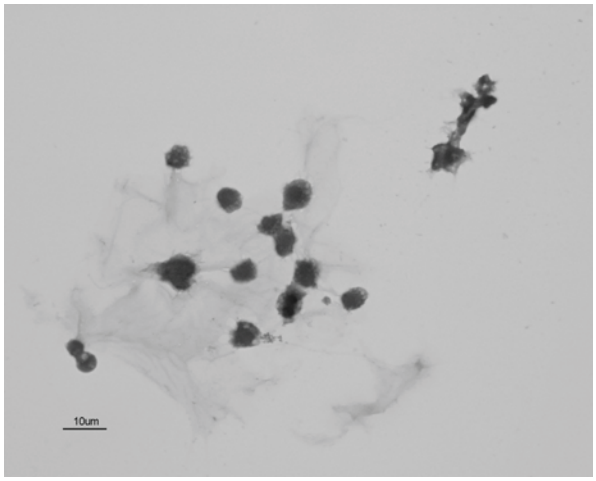
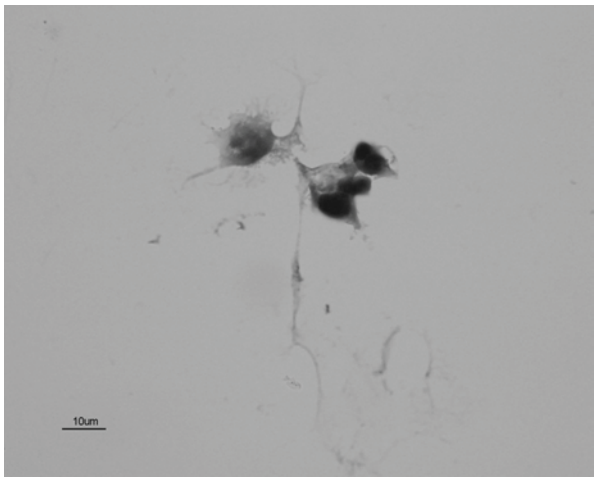


Fig. 6. The example of the control sample with cells in suspension observed at magnification of 60x



a



b

Fig. 7. The example of the sample with cells after interaction with PAI: in suspension at magnification of 40x (a) and cells attached at magnification of 60x (b)

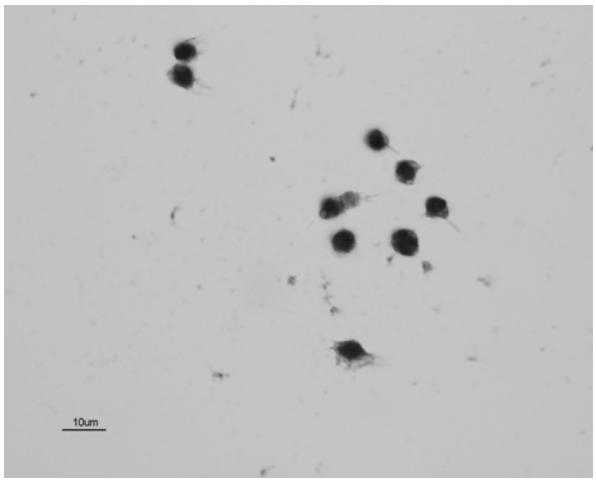


Fig. 8. Sample with cells attached after interaction with PVA at magnification 60x

Figs. 6-8 show the results of microscopic observation of the U937 cells before (control sample) and after the interaction with PAI and PVA hydrogels. Normal and apoptotic cells were present, while necrotic cells were not observed. Also, no important changes in morphology were visible.

The results on counted cells are presented in Table 2 as the average number of cells after interaction and compared to the average number of cells in control samples.

Table 2

Comparison of the average cells number: in the control sample (ctr) and after interaction with PAI and PVA

Sample	Attached cells	Cells in suspension	
		I	**II**
Ctr	2566	1340	1319
		Average: **1329.5**	
PVA	2474	919	739
		Average: **829**	
PAI	413	250	78
		Average: **164**	

The number of cells after direct contact with PVA is comparable to the control sample, while the number of vivid cells after interaction with PAI is significantly lower- 6 times (attached cells) or even 8 times (cells in suspension) lower than in control cells and 5-6 times lower than in case of interaction with PVA hydrogel.

4. Conclusions

IR spectra of the investigated PVA hydrogels show that some OH-ended dangling chains were present in the

sample as well as the primary alcohol groups. The absorption bands characteristics for imide, amide and hydroxyl groups were identified in dry PAI hydrogel.

The PVA hydrogel used in the Bioinblue product is hardly crystalline after drying at room temperature. The crystallinity was estimated to be 0.42 %. Its T_g (determined during the first heating process) is 398.55 K and melting point is 475 K (close to isotactic PVA).

Any thermal transitions were observed in PAI Bioalcamid hydrogel in the range of 298.15 to 523.15 K. The above data are the subject of interest in the future estimating of polymer hydrogel stability after longer contact with human tissue.

According to authors' experiments testing of PVA and PAI hydrogels with the cell line U937 is appropriate polymer hydrogels and can be used in future for other solid biomaterials. After the interaction of U937 cells with both hydrogels no cell necrosis was observed, whereas apoptosis was visible in many cases. There were no visible changes in morphology of the cells. U937 cells after the interaction with PVA showed higher viability as compared to those exposed to PAI. The trial to use the important test so far used for pharmaceutical substances appeared to be done for polymer hydrogels and can be used for comparing different hydrogels.

Acknowledgements

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ТЕСТУВАННЯ ПОЛІАЛКІЛІМІДНИХ ТА ПОЛІВІНІЛСПИРТОВИХ МЕДИЧНИХ ГІДРОГЕЛІВ ЗА ДОПОМОГОЮ ЛІНІЇ ГЕНЕТИЧНО ОДНОРІДНИХ КЛІТИН U937

***Анотація**. З використанням оптичної мікроскопії проведено тести безпосередньої взаємодії лінії генетично однорідних клітин полівініл-спиртових та поліалкілімідних гідрогелів. Встановлено, що після взаємодії не спостерігається ні наявності некротичних клітин, ні змін в активності та морфології клітин, незважаючи на те, що полівініловий спирт виявив кращу сумісність. Для характеристики гідрогелів використано ІЧ-спектроскопію та диференційну скануючу калориметрію.*

***Ключові слова:** гідрогелі, медичні наповнювачі, полівініловий спирт, поліалкілімід, лінія генетично однорідних клітин U937, біосумісність.*