Investigations on the impact of the introduction of the *Aloe vera* into the hydrogel matrix on cytotoxic and hydrophilic properties of these systems considered as potential wound dressings

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Abstract

In the paper, synthesis of chitosan-based hydrogels modified with *Aloe vera* juice is presented. The novelty of the research was a combination of hydrogel materials with properties beneficial in viewpoint of their use as modern wound dressings and *Aloe vera* juice supporting the wound healing process. Hydrogels have been received via UV radiation. The impact of the amount of the crosslinking agent as well as the introduction of the *Aloe vera* juice into the hydrogel matrix has been determined. Performed measurements involved analysis of the swelling ability, characteristics of the surface roughness, determining the release profile of *Aloe vera* and the contact angles of hydrogels. Furthermore, the analysis of the dehydration process of the polymer membrane, investigations on the cytotoxicity of hydrogels via MTT reduction assay and the neutral red uptake assay as well as the studies on the pro-inflammatory activity have also been performed. It was proved that the addition of *Aloe vera* juice improves the hydrophilic properties of the materials (e.g. contact angle changed from 82.5° to 73.0°). Next, the use of 25% more of the crosslinker resulted even in the increase of the contact angle by 86%. Modified hydrogels showed higher swelling properties even by 15% than unmodified materials. Furthermore, obtained hydrogels show an ability to release *Aloe vera* – after 5 h approx. 80%

of this additive has been released in an acidic environment. Tested materials do not exhibit cytotoxic properties, the addition of *Aloe vera* results in an improvement of the viability of L929 murine fibroblasts and, importantly, these materials show lower pro-inflammatory activity than the positive control. Performed investigations allow to state that obtained materials show a great application potential.

Keywords: hydrogels; chitosan; *Aloe vera*; MTT reduction assay; water desorption; roughness profiles; wettability

1. Introduction

In recent years, a significant increase in the application of plant extracts for biomedical purposes has been observed wherein particular attention is directed towards *Aloe vera*. This plant has been known and used for centuries due to the therapeutic properties which result from its composition including such chemical compounds as proteins, minerals, polysaccharides, phenolic compounds or enzymes such as e.g. alkaline phosphatase, amylase and superoxide dismutase [1-2].

Hęś et al. performed investigations on the antioxidant properties of the *Aloe vera* extract wherein its antioxidant activity was determined against linoleic acid using the radicals DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]). Next, the phenol content was determined spectrophotometrically with the Folin-Ciocalteu reagent using gallic acid as a standard. Ac a result of the experiments, it was proved that *Aloe vera* extract exhibit antioxidant activity [3]. In turn, the main purpose of studies conducted by Nejatzadeh-Barandozi was to identify and quantify compounds with antioxidant and antibacterial properties contained in *Aloe vera* leaf gel extracts. Such compounds as phenolic acids, polyphenols, indoles or alkaloids, which are responsible for antioxidant properties of *Aloe vera,* have been identified. The oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays were performed and allowed to confirm the mentioned properties [4]. Next, Quispe et al. determined the antioxidant activity of extracts from various parts of the *Aloe vera* (i.e. peel, flowers, gel and roots). The total antioxidant activity of ethanol extracts prepared from various parts of the tested plant was measured via determining the scavenging capacity of DPPH and ABTS radicals, while the iron-reducing antioxidant power (FRAP) was assessed using spectroscopic analyses. The studies showed that the plant skin extracts exhibited the highest antioxidant activity, which is particularly interesting

due to the fact that most of the peel is commonly considered as waste while the most frequently used part of *Aloe vera* is gel obtained from the leaves [5].

Apart from the antioxidant activity, among essential features of *Aloe vera* such may be mentioned as antibacterial [6], anti-inflammatory [7], anti-diabetic [8], immunomodulatory [9] or anti-cancer ones [10]. As a result, *Aloe vera* is more often considered in various investigations on materials designed for biomedical applications [11-13]. For example, Abo-Youssef et al. analyzed the antidiabetic effect of *Aloe vera* leaf extract. In vitro studies were performed on isolated pancreatic islets of adult female albino rats. Additionally, series of experiments, in which diabetes was experimentally induced, has also been conducted. It was proved that *Aloe vera* extract significantly decreased the level of glucose in the tested serum. Moreover, in vitro studies showed an increase in basal and stimulated insulin secretion from isolated pancreatic islets [14]. Next, Shalabi et al. determined the antitumor activity of *Aloe vera* and *Calligonum comosum* extracts. The studies were performed on HepG2 liver cancer cells. Cell viability was assessed via MTT reduction assay, while assessment of apoptosis and DNA damage was performed using the annexin V apoptosis detection kit. The results received indicated that *Aloe vera* and *Calligonum comosum* extracts may induce cytotoxic and genotoxic effects on liver cancer cells (HepG2) by apoptosis modulation [15].

Aloe vera find also the application as material that accelerates the wound healing process [16]. For example, the main purpose of Molazem et al. was to determine the efficiency of the caesarean section wound healing using dressing containing *Aloe vera* gel. Clinical investigations have been performed among 90 women. The patients were randomly divided into two groups of 45. In one group, the wounds were covered with a dressing containing *Aloe vera* gel, while in the other group the conventional dressing was applied. Wound healing was assessed 24 h and 8 days after the cesarean surgery. The results obtained confirmed efficient activity of the mentioned gel as well as the acceleration of the wound healing process caused by the application of this additive compared to the wound treated via traditional dressing [17]. In turn, Panahi et al. proposed the combination of *Aloe vera* extract with olive oil and the use of such formed system for the chronic wound treatment. It was proved that antibacterial and anti-inflammatory properties of proposed combination affected the tissue regeneration and finally the significant improvement of wound healing treated with *Aloe vera* extract/olive oil combination has been observed [18]. Application of *Aloe vera* in the synthesis of materials for wound healing has been also proposed via Aghamohamadi et al. [19] or Anjum et al. [20]. Moreover, *Aloe vera* found also application in preparation of drug delivery systems. For

example, Joshy et al. prepared *Aloe vera* modified solid lipid nanoparticles which were analyzed as carriers of zidovudine – a well known medicine used for HIV treatment [21].

Hydrogel polymers also belong to the group of materials with growing popularity in the application in wound healing processes. This is related to their properties such as biocompatibility [22], non-toxicity [23], elasticity [24] and the big sorption capacity [25]. Particular attention is more often paid to the hydrogels based on chitosan. This compound belong to the group of polysaccharides with antibacterial activity [26]. Many investigations on the development of chitosan-based hydrogels considered as modern wound dressings have been recently presented. For example, Li et al. described hydrogel polymers based on N,O-carboxymethyl chitosan and oxidized chondroitin sulfate. It was proved that such materials showed antibacterial activity, hemostatic properties and, importantly, good adhesion to the biological tissue [27]. Next, Zhang et al. performed studies on hydrogels based on chitosan, lignin and polyvinylpyrrolidone (PVP) and stated that such wound dressings may accelerate wound healing process [28]. Application of chitosan-based hydrogels as dressings for treatment diabetic wounds has been proposed by Lee et al. [29]. Other investigations on the development of chitosan-based hydrogels considered as dressing materials have been also conducted by Deng et al. [30] and Hamdi et al. [31].

In presented paper, investigations on the chitosan-based hydrogels modified additionally with *Aloe vera* juice have been presented. Such materials have been selected for the synthesis of the mentioned polymers due to their widely reported properties desirable in viewpoint of application as dressing materials. Both chitosan and *Aloe vera* have been described in literature as materials which show antimicrobial and anti-inflammatory activity. Moreover, both these components are characterized as materials that positively influence the wound healing process. Therefore the main purpose of the proposed investigations was to develop hydrogel materials based on chitosan and modified with *Aloe vera* extract which have been designed and investigated in viewpoint of their potential application as innovative dressings for chronic wound treatment. The mentioned materials have been obtained via UV radiation and subjected to various studies aimed at determining the impact of *Aloe vera* juice on physicochemical, hydrophilic and biological properties of such modified hydrogels.

2. Materials and methodology of investigations

2.1. Materials

Chitosan (high molecular weight, deacetylation degree 75-85%), diacrylate poly(ethylene glycol) (PEGDA, crosslinking agent, average molecular weight 700 g/mol, d =

1.12 g/mL, contains 100 ppm Mequinol (MEHQ) as inhibitor), and 2-hydroxy-2-methylpropiophenone (Darocur 1173, photoinitiator, 97%, d = 1.077 g/mL) were purchase in Sigma Aldrich (Saint Louis, Missouri, USA).

Aloe vera juice (99.5%) used as a modifier of hydrogels was bought in Herbal Pharmaceuticals (Krakow, Poland). It is a viscous, thick liquid obtained from the flesh of *Aloe vera* leaves it consists of a wide spectrum of substances including vitamins (B12, A, E), enzymes (e.g. amylase, lipase or catalase), saccharides (xylose, fructose, glucose or arabinose), amino acids, fatty acids, minerals or anthraquinones (aloin, emodin) [32-33].

2.2.Synthesis of hydrogels

The procedure of the preparation of hydrogels as well as the investigations on their compositions have been described in our previous paper [34]. In brief, firstly 1% solution of chitosan in 0.05% acetic acid solution has been prepared. Next, adequate amounts of crosslinker (diacrylate (poly(ethylene glycol) with average molecular weight 700 g/mol) and photoinitiator (2-hydroxy-2-methylpropiophenone) have been added to the chitosan solution. The whole mixture has been intensively stirred, poured on a Petri dish and subjected to the UV radiation for 120 s. Photopolymerization process has been performed using EMITA VP-60 lamp (power: 180 W, λ = 320 nm). In Table 1. compositions of hydrogels obtained have been reported.

Table 1. Compositions of hydrogel polymers prepared via UV radiation.

1% chitosan solution in 0.05% acetic acid solution, mL	**Photoinitiator, mL[a)]**	**Crosslinking agent, mL[b)]**	**Sample name**
50.0	0.5	8.0	8-700
		10.0	10-700
		12.0	12-700

[a)] *2-hydroksy-2-metylopropiofenon, Darocur 1173*

[b)] *diacrylate poly(ethylene glycol) with average molecular weight 700 g/mol (PEGDA M_w=700)*

Further step involved preparation of hydrogels modified with *Aloe vera* juice that was conducted analogously to the synthesis of unmodified polymers. The only difference was that adequate amount of the additive was added to the chitosan solution. Next, selected amounts of

crosslinker and photoinitiator were introduced into such formed mixture and the whole was intensively stirred, poured into the Petri dish and treated with UV radiation for 120 s.

The amount of the crosslinking agent has been selected considering the fact that the volume of the reaction mixture was greater than before due to the presence of the additive (*Aloe vera* juice) which may affect the crosslinking process. The use of 8 mL of PEGDA 700 (as in the case of unmodified hydrogel – sample 8-700) did not result in the preparation of properly crosslinked hydrogel structure (i.e. the reaction mixture remained wholly or partially liquid). Consequently, except that 10 mL and 12 mL of the crosslinker has been used, 14 mL of this reagent has also been proposed for the synthesis. Compositions of all mentioned modified hydrogels are presented below in Table 2.

Table 2. Compositions of hydrogels modified with *Aloe vera* juice.

1% chitosan solution in 0.05% acetic acid solution, mL	**Photoinitiator, mL[a)]**	**Crosslinking agent, mL[b)]**	***Aloe vera* juice, mL**	**Sample name**
50.0	0.5	10.0	10.0	10-700-a
		12.0		12-700-a
		14.0		14-700-a

[a)]*2-hydroksy-2-metylopropiofenon, Darocur 1173*

[b)]*diacrylate poly(ethylene glycol) with average molecular weight 700 g/mol (PEGDA M_w=700)*

In Fig. 1. sample images of hydrogel materials obtained are presented.

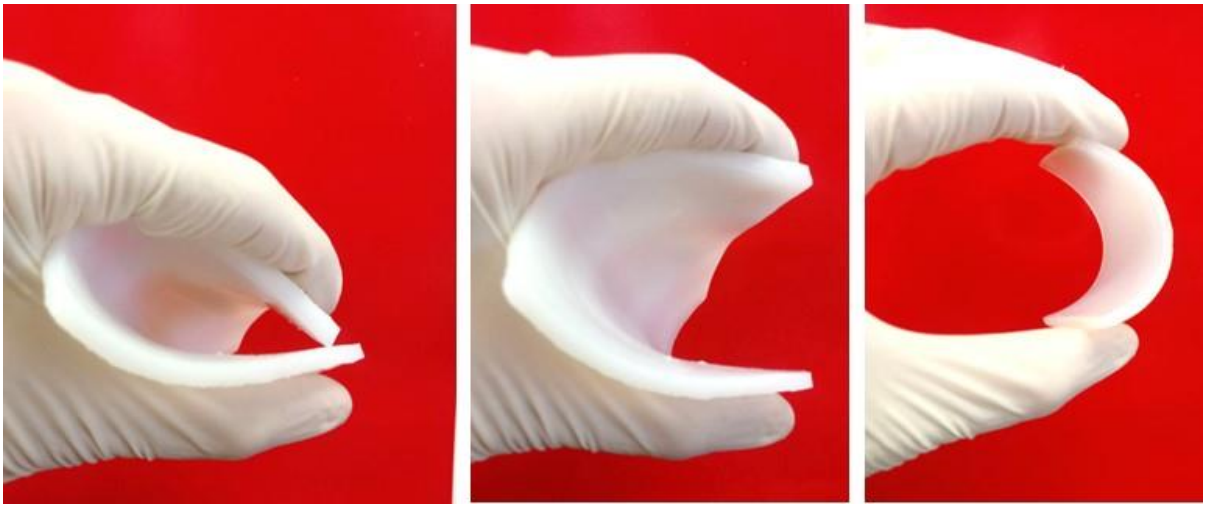

Fig. 1. Images samples of hydrogel materials obtained.

Subsequently, obtained hydrogels have been subjected to the numerous investigations aimed at the determining their physicochemical properties including e.g. surface roughness, behavior in simulated physiological liquids as well as the evaluation of their pro-inflammatory activity and cytotoxicity towards selected cell lines.

2.3.Methodology of measurements

2.3.1. Investigations on the sorption capacity of hydrogels obtained

One of the most characteristic properties of hydrogels are their sorption capabilities. Analysis of these properties is particularly important in viewpoint of their potential application as dressing materials with the ability of the wound exudate absorption. The study consisted of the introduction of 1 g of weighed dry hydrogel sample into the sterile vessel containing 50 mL of SBF (simulated body fluid, isotonic to the plasma of human blood) solution. Next, a tested sample was separated from a solution after a certain period of time (i.e. 1 h, 24 h and 72 h) and weighed in a swollen state. The scheme of the swelling analysis is presented in Supplementary File (Fig. 1S).

The study was performed at room temperature. The sorption capability of hydrogel materials have been determined using swelling ratio α [g/g] and calculated according the following equation (1):

$$\alpha = \frac{(m - m_0)}{m_0}, \text{ where} \qquad (1)$$

α – swelling ratio, g/g
m – mass of a swollen hydrogel, g
m_0 – mass of a dry hydrogel, g.

2.3.2. Studies on the roughness and the geometric structure of hydrogels

Further part of the investigations involved the analysis of the structure geometry of obtained hydrogel materials. The surface structure geometry refers to the set of its all unevenness including such elements as the shape deviations, waviness or roughness wherein the last of the mentioned characteristics is considered as one of the most important parameters of the surface layer of the hydrogel.

The surface structure geometry measurements were performed using the Taylor Form Talysurf Intra 50 profilometer equipped with the measuring head, the sliding table providing the positioning of the measuring tip in the y axis and the vertical column providing the

positioning in the z axis. The analyses of the geometric structure of the surface layer of the hydrogels were performed via the contact method, i.e. the measuring tip was continuously moved over the measured surface. Its vertical movements corresponding to the surface unevenness were recorded and as a result parameters of the surface roughness were determined. A measuring tip with a conical blade (with a tip rounding radius approx. 2 μm) was used for the measurements. During the measurements, pressure of the measuring tip of a value of 1 mN was applied. In order to determine the impact of the applied measuring pressure on the shape of easily-deformable surfaces of the measured samples, microscope images the tested surfaces have also been taken. Measurements were performed on a 1 mm x 1 mm surface of tested materials. Before the analyses, the hydrogels have been dried at room temperature for 24 h. The results obtained were analyzed using the TalyMap Silver software.

2.3.3. Studies on the wettability of hydrogels

In order to determine the hydrophilicity of the hydrogels obtained, the investigations on the contact angle of their surface were performed. In general, the contact angle is determined via the drop shape analysis. The analysis was conducted using the Kruss DSA 100M apparatus and involved the placing of the hydrogel sample on a stationary base and subsequently dropping the measuring liquid (distilled water) using a micropipette on the tested sample. The image of the drop shape formed in contact with analyzed hydrogel was recorded via the optical system with a digital camera.

2.3.4. Analysis of the water desorption from swollen hydrogel samples

Hydrogels obtained were subsequently subjected to the study aimed at the determining their weight loss resulting from the process of water desorption. In this investigation, the measurement of the water loss over time by the hydrogel material was performed using terahertz (THz) sensing. The rationale for this type of measurement over conventional gravimetric analysis is that it can be performed with high sensitivity and without physical contact to the sample of interest [35]. The transmission geometry of the technique differs to reflection geometry [36-37] by allowing water content to be quantified. Fig. 2S (in Supplementary File) shows the THz setup using an IMPATT diode source, operating at 0.1 THz, and a 16x16 THz camera with 1.5 mm pixel size (Terasense Inc., CA, USA). The THz source emits a divergent beam, which is collimated by a plano-convex lens. The collimated beam passes through the sample and is subsequently directed onto the THz camera. For comparison purposes, the sample's weight loss was also simultaneously measured. The samples were prepared by

preparing dry hydrogels into squares ~20 x 20 mm^2 and immersing them in distilled water for 24 h. Prior to measurement, however, excess surface water was removed by means of paper wipes. The samples were placed in a sample holder held in place using binder clips. The entire desorption process was performed under an ambient condition at room temperature. To accelerate the dehydration process, a laboratory fan was used. In Supplementary File the scheme of the system applied is presented (Fig. 2S).

By monitoring the relative THz intensities, weight of water across each sample (EW) can be estimated as a function of drying time using Beer-Lambert Law, under the assumption of constant water density and uniformity [37], as shown by Equation (2),

$$EW = \sum_{pixel=1}^{169} \frac{-(l_{pixel})^2 \rho \, ln\left(\frac{I_{pixel}}{I_{0,pixel}}\right)}{\alpha} \quad (2)$$

where α corresponds to the absorption coefficient of water at 100 GHz and 25°C (11 mm^{-1}), I_{pixel} and $I_{0,pixel}$ correspond to the THz intensities for hydrated and dried membranes, respectively, on each pixel, ρ corresponds to the water density at 25°C (1 mg/mm^3) and l_{pixel} corresponds to the pixel size (1.5 mm). For liquid water weight estimation, a 13x13 (19.5x19.5 mm^2) pixel matrix, which roughly corresponds to the sample size (20x20 mm^2), is used. For the gravimetric studies, the water weight (W_{H2O}) is calculated by Equation (3):

$$W_{H_2O} = W(t) - W_{dry} \quad (3)$$

where W(t) is the sample weight at time t (mg) and W_{dry} is the weight of the dry sample (mg).

2.3.5. Investigations on the behavior of hydrogels in simulated body fluid

In order to determine the behavior of hydrogels in environment simulating conditions occurring in human body incubation studies have been performed. Analysis involved the incubation in simulated body fluid (SBF), hemoglobin (porcine hemoglobin, 2% wt. solution in distilled water) and distilled water. Procedure was as follows: hydrogel samples (with a weight of approx. 1 g) were introduced into the sterile vessels and treated with 50 mL of a given liquid. Such immersed samples were stored in laboratory incubator at 37°C, i.e. at conditions simulating temperature of human body. Study was performed for 12 days during which the pH values were measured.

2.3.6. Studies on the release of the modifying agent from the hydrogel matrices

The main purpose of this study was to determine the release profile of the active substance introduced into the hydrogel matrix. The modification of hydrogel polymers may

give them new properties thus increasing the spectrum of their potential application. Therefore, it is important to determine the release profile of the active substances from their interior as well as determining the conditions in which this process takes place the most effectively.

The study was performed for samples obtained using various amounts of crosslinker and containing *Aloe vera* juice. Analyses were immersed in two different environments, i.e. in an acidic pH environment (2% citric acid solution, pH = 2.0) and in a slightly alkaline pH environment (phosphate buffer, pH = 7.4).

Aloe vera juice is characterized by a very rich composition (saccharides, hormones, enzymes, fatty acids, amino acids etc.) and it was necessary to base on substances which presence may be determined via e.g. UV-Vis spectrophotometry during the release investigations. As substances determining the potential release of *Aloe vera* juice to the tested environments saccharides have been selected. Potassium iodide has been used due to the fact that this compound forms colorful complexes with saccharides which may be determined spectrophotometrically. Firstly, the absorbance of the *Aloe vera* juice used for the preparation of the reaction mixtures of tested hydrogels has been measured and define as 100%. The maximum absorbance was observed at 310 nm. Next, the absorbances determined during the release investigations have been measured and compared to the initial value. UV-Vis spectrophotometric analyses were conducted using UV-Vis Visible Spectrophotometer V-500 apparatus.

In order to perform the measurements, at the beginning the flasks with adequate tested environments (200 mL each) and immersed hydrogel samples were placed in the shaking incubator apparatus (Hanchen ES-60E Temperature Controlled Incubator & Shaker Scientific Incu-Shaker Shaking Incubator) and subjected to the shaking process (rpm = 80). The study was performed at the temperature which imitated the conditions occurring in human body, i.e. at 36.6°C. After a specified period of time (i.e. after 0.5 h, 1 h, 2 h, 3 h, h, 5 h, 6 h, 7 h, 8 h, 9 h and 10 h), 3 mL of each solutions were taken, introduced into the cuvettes, treated with 0.125 mL of KI and remained for 30 min. After this time, the active substance was analyzed spectrophotometrically. The measurement was recorded at room temperature. The flasks were replenished with phosphate buffer or citric acid solution to the original volume after each sampling for UV-Vis analysis.

2.3.7. Evaluation of the cytotoxicity of the hydrogels toward L929 murine fibroblasts

In viewpoint of the potential application of hydrogel materials received for biomedical purposes it was particularly important to perform biological investigations. The evaluation of the *in vitro* cytotoxicity of the hydrogels received allows to assess their usefulness for biomedical uses. The MTT reduction assay constitutes one of the most frequently used analysis for the determining of the cytotoxicity of biomaterials. This test is based on the defining the viability of selected cell lines via determining their metabolic activity. For this purpose, the activity of the mitochondrial dehydrogenase, i.e. the enzyme which is responsible for the conversion of the soluble tetrazole salt – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (known as MTT reagent) into the insoluble formazan, is evaluated. Formazan crystals are dissolved in isopropanol (or DMSO) and the color intensity of such formed solution may be determined spectrophotometrically (within the wavelength range 492 – 570 nm). Next, the measured color intensity of formazan formed as a result of the enzymatic reaction correspond to the amount of the enzyme secreted via the tested cells and thus to their viability. The viability of cells described the cytotoxicity of materials with which the cells were treated.

The study was performed against L929 murine fibroblasts received from American Cell Type Culture Collection (Rockville, USA). Firstly, the mentioned cells were incubated in standard conditions (i.e. 37°C, 5% CO_2, > 90% humidity). The following substrate was used during the incubation: RPMI – 1640 medium supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL) and inactivated bovine serum (10% wt.). Next, a monolayer of cells was formed by placing 100 μL of the cell suspension (concentration: 2 x 10^5 cells/mL)/well in a 96-well plate and their subsequent 24 h incubation in standard conditions.

The next step involved the preparation of the hydrogels. Fir this purpose, the materials were cut into samples (with sizes corresponding to the 1/10 of the well surface), placed in 5 mL of the substrate and incubated for 30 min. such formed samples were subsequently placed in the 96-well plate (1 sample/well). Such prepared plates were incubated in standard conditions for 24 h. after this period of time, the substrate was removed and replenished by the 100 μL of the fresh one. Next, 20 μL of the MTT reagent was introduced into each well (conc.: 5 mg/mL). After 4 h incubation in standard conditions, the plate was centrifuged for 10 min (1200 rpm). The supernatant was removed and the solution obtained via the dissolution of formed formazan crystals in 150 μL of DMSO and subsequently in 25 μL of glycine buffer was incubated at room temperature for 15 min. Finally, 160 μL of this solution was subjected to the UV-Vis spectrophotometric analysis at a wavelength of 570 nm using Spectramax multi mode microplate reader from Thermo Fisher Scientific Inc. company. The scheme of the MTT reduction assay is shown in Supplementary File (Fig. 3S).

Subsequently, in order to support the results obtained via MTT reduction assay, obtained hydrogel materials have also been subjected to the Neutral Red Uptake (NRU) assay. This study involves the measurement of the ability of the tested material to inhibit the neutral red uptake. This dye penetrates the cell membranes and accumulates intracellularly in the lysosomes of living cells. The absorbance intensity (at a wavelength of approx. 540 nm) of the dye which is released from the cells' lysosomes under the influence ethanol/acetic acid solution corresponds to the number of viable cells in the tested culture. Before the investigations, the monolayer of cells was washed using buffered saline. Subsequently, 100 μL of the dye solution is introduced into each plate containing the cells treated with the tested hydrogels (procedure of introducing the hydrogel samples into the 96-well plate is the same as in the case of MTT reduction assay) and the whole is incubated for 3 h in standard conditions. After the incubation, the liquid from the cell monolayers was removed. Next, the cells were washed again with buffered saline, treated with 150 μL of glacial acetic acid/ethanol/H_2O mixture (in 1:50:49 ratio) and incubated for 10 min. Then, 130 μl of liquid from each well of the plate was moved to the other 96-well plate and the absorbance of the liquids obtained was measured at 550 nm via Multiskan EX reader from Thermo Fisher Scientific Inc. company.

2.3.8. Analysis of the pro-inflammatory activity of the hydrogels

Investigations on the pro-inflammatory activity have been performed using human monocytic THP-1XBlueCells™ cell line (Invivogen, San Diego, USA). This cell line is used as an indicator of the activation of the NFκB transcription factor detected indirectly by quantifying the secretion of the embryonic alkaline phosphatase (SEAP) into the cell culture medium. Activation of THP1-XBlue™ cells via one of the Toll receptors (TLRs) induces the nuclear factor (NF) κB followed by the release of SEAP which is detected in cell culture using Quanti-Blue™ reagent (Invivogen, San Diego, USA).

Firstly, tested cells were cultured in RPMI-1640 growth medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM of hydroxyethyl piperazineethanesulfonic acid (HEPES) supplemented with penicillin-streptomycin (100 U/mL - 100 μg/mL) and selection factors, i.e. normocin (100 μg/mL) and blastocidin (10 μg/mL), incubated at 37°C in 5% CO_2.

The viability of the cells was assessed using trypan blue staining. For this purpose, a cell suspension was prepared with the density: 9 x 10^5 cells/mL. Next, 96-well culture plates were incubated with the cell suspension at a working volume of 100 μL/well. Then, tested

hydrogel samples were prepared in cell culture medium on a separate plate, transferred to wells containing THP1-XBlue™ cells and incubated under standard conditions for 24 h. Wells containing cells in cell culture medium were used as negative controls; cultures treated with Escherichia coli lipopolysaccharide (LPS) O55: B5 at a final concentration of 1 µg/mL was used as a positive control of monocyte activation. After incubation, plates were centrifuged (1400 rpm) for 10 min and the supernatants (20 µL) were transferred to the appropriate wells of the plate containing 180 µL/well of Quanti-Blue™ Detection Reagent and incubated for a further 3 h. The absorbance was determined at 620 nm using a Thermo Fisher Scientific multi mode microplate reader.

3. Results and Discussion

3.1. Investigations on the sorption capacity of hydrogels obtained

In Fig. 2. results of the swelling investigations have been presented.

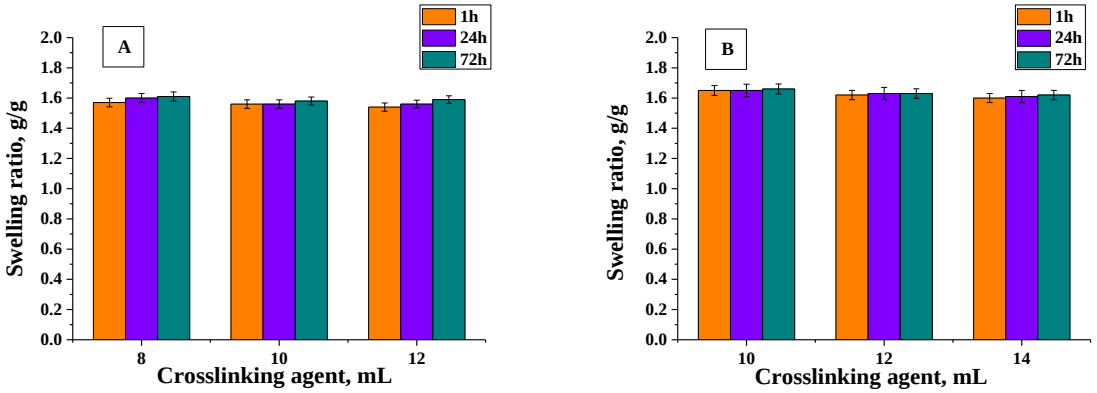

Fig. 2. Swelling in SBF of: A – unmodified hydrogels *(average value* $\overline{SD} = 1.30\%$, *number of repetitions n = 3)*; B – hydrogels modified with 10 mL *Aloe vera (average value* $\overline{SD} = 1.35\%$, *number of repetitions n = 3).*

The investigations on the swelling ability of hydrogels are one of the primary studies because they aimed at the determining the ability of these polymers to absorb various liquids. During the swelling process, the hydration of the functional groups in the polymer chains occurs and as a result an increase in the weight of the tested material is observed (Fig. 2.). Unmodified hydrogels subjected to this investigation are characterized by a swelling ratio of approx. 1.5 g/g (Fig. 2A.), and the materials modified with *Aloe vera* juice exhibit the swelling ratio of approx. 1.7 g/g (Fig. 2B.). Materials containing *Aloe vera* juice show a slightly higher swelling ability which is probably related to the hydrophilic nature of *Aloe vera*. Such a hydrophilic nature of the modifier results in an increase in the swelling ability of the materials which was also

reported in [38]. At the same time, the release of the *Aloe vera* juice from the hydrogel matrix is observed that results in the formation of bigger spaces between polymer chains. Finally, more functional groups may hydrate. Moreover, the crosslinking agent used in the synthesis of the analyzed materials may also have an impact on the swelling ability of these materials, both its amount and type used. The effect of the type of crosslinker (its molecular weight) has been clearly discussed in [34]. Considering the impact of the amount of the crosslinking agent on the swelling properties of hydrogels, it may be observed that its higher amount results in the decrease in the sorption capacity of such formed hydrogel. This is caused by the increase in the crosslinking degree of the hydrogel and therefore lower amount of functional groups may hydrate and consequently the swelling ratio of such crosslinked hydrogel is lower. The selection of the adequate hydrogel material which is characterized by a desirable swelling properties is strictly correlated with the future application of such material. Depending on the use, the hydrogel may be characterized by low or high swelling capacity.

3.2.Studies on the roughness and the geometric structure of hydrogels

In Figs 3-8 results of the geometric analyses (surface roughness, isometric roughness view and layered roughness view) are shown. Additionally, roughness profiles of tested samples are presented in Supplementary File (Figs 4S-9S). Investigations involved analyses of the roughness including also the isometric and layered view of the roughness and the profiles of the analyzed surfaces. Additionally, selected parameters of the hydrogels' surfaces as e.g. arithmetic mean height of the surface or arithmetic mean deviation of the roughness profile.

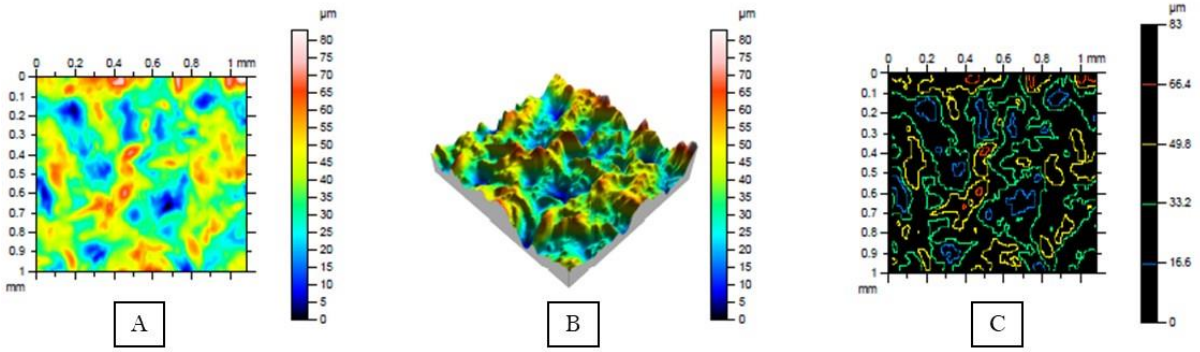

Fig. 3. Surface analysis of hydrogel material with **8 mL PEGDA**: A- surface roughness; B- isometric roughness view; C- layered roughness view.

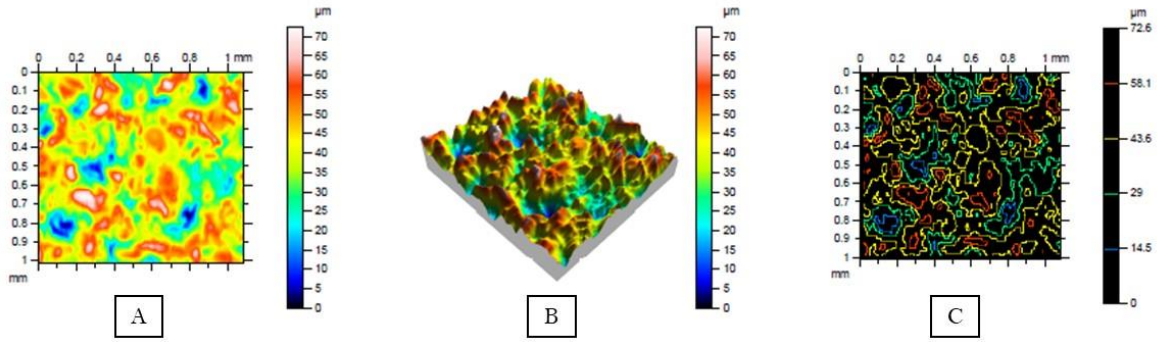

Fig. 4. Surface analysis of hydrogel material with **10 mL PEGDA**: A- surface roughness; B- isometric roughness view; C- layered roughness view.

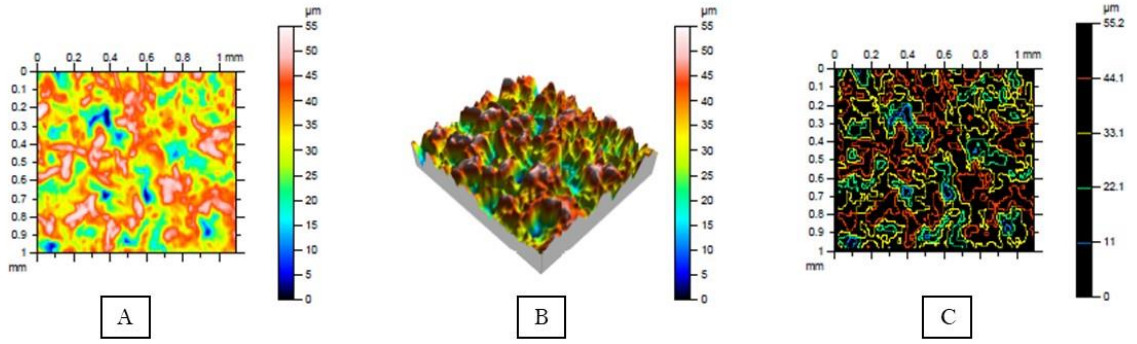

Fig. 5. Surface analysis of hydrogel material with **12 mL PEGDA**: A- surface roughness; B- isometric roughness view; C- layered roughness view.

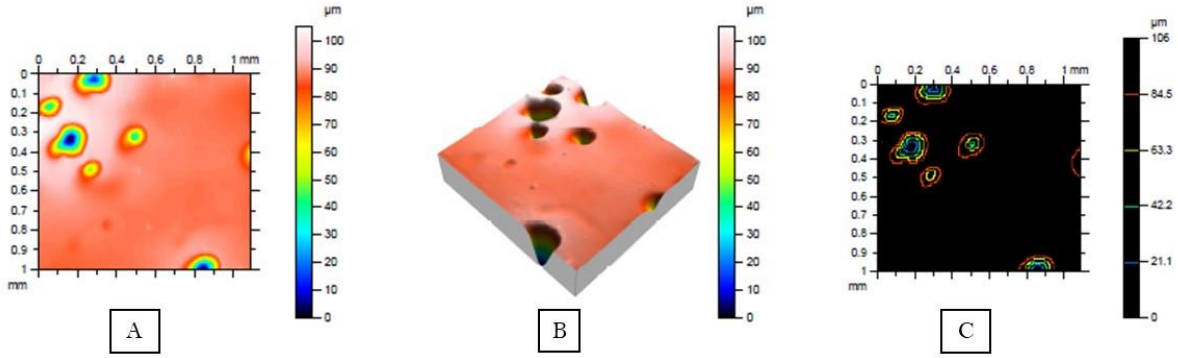

Fig. 6. Surface analysis of hydrogel material with **10 mL PEGDA +10 mL *Aloe vera***: A- surface roughness; B- isometric roughness view; C- layered roughness view.

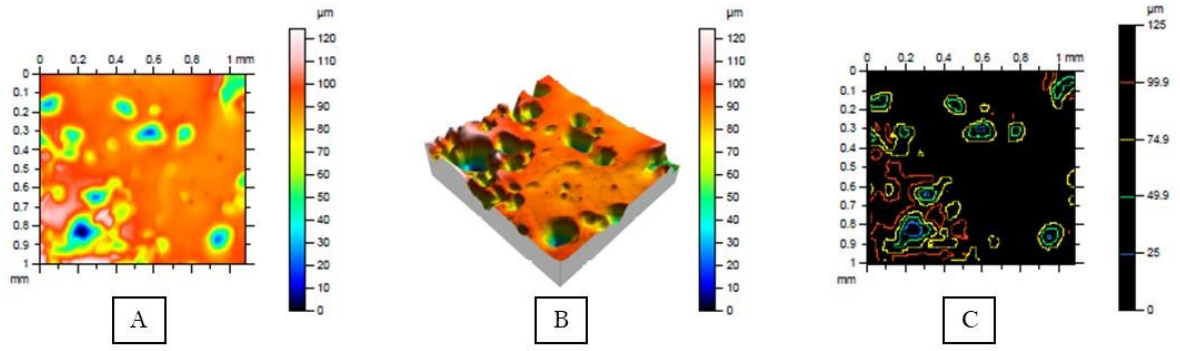

Fig. 7. Surface analysis of hydrogel material with **12 mL PEGDA +10 mL *Aloe vera***: A- surface roughness; B- isometric roughness view; C- layered roughness view.

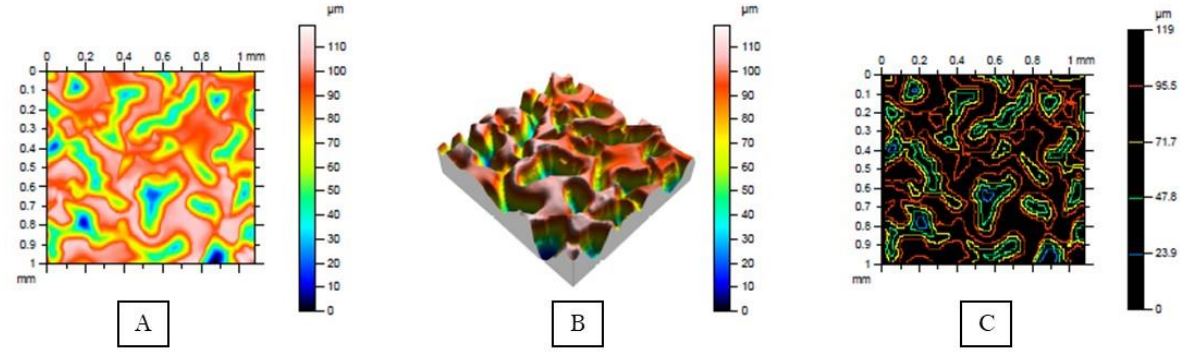

Fig. 8. Surface analysis of hydrogel material with **14 mL PEGDA +10 mL *Aloe vera***: A- surface roughness; B- isometric roughness view; C- layered roughness view.

Table 3. Parameters of the geometric structure of the surface of obtained hydrogel materials.

Parameter	**Value, µm**					
	8 mL PEGDA	**10 mL PEGDA**	**12 mL PEGDA**	**10 mL PEGDA + 10 mL *Aloe vera***	**12 mL PEGDA + 10 mL *Aloe vera***	**14 mL PEGDA + 10 mL *Aloe vera***
S_a	10.10	9.24	7.59	14.20	10.40	18.40
S_z	83.00	72.60	55.20	146.00	125.00	119.0
S_v	46.00	40.90	34.90	83.90	86.30	81.80
R_a	8.15	6.72	7.72	16.80	3.09	15.10
R_z	41.20	35.50	44.00	90.70	26.20	64.80
R_v	19.40	13.70	27.70	51.00	17.30	38.60
S_a (arithmetic mean surface height)				*S_z (maximum surface height)*		
S_v (maximum pit height)				*R_a (roughness average)*		
R_z (average maximum height of the profile)				*R_v (maximum profile valley depth)*		

The surface roughness analysis allow to characterize the outer surface layer of the tested hydrogels and therefore it is possible to determine the differences between the analyzed surfaces from the basic, layered and isometric view of their roughness. Furthermore, the study allows

also to define e.g. the maximum surface heights and the maximum valley depth of the roughness profile of analyzed materials (Tab. 3.). Based on the performed analysis, it is possible to observe clear differences in the determined roughness profiles depending on the amount of the crosslinker used in the synthesis of the tested hydrogels. The higher amount of the crosslinking agent, the stronger the folding of the outer structure of the hydrogel (Fig. 3. – Fig. 5.). This is probably caused by stronger packing of the polymer chains which reduces the swelling ability of such material because the absorbed liquid does not have as much access to the functional groups in the polymer chains as in the case of the material obtained using lower amount of crosslinking agent. Next, hydrogels modified with *Aloe vera* juice are characterized by a much smoother surface. This is probably a result of the presence of the *Aloe vera* juice in the pores of the hydrogel material therefore the surface of such modified material is significantly smoother. Moreover, modified materials (Fig. 6. – Fig. 8) are characterized by significantly higher values of S_z than hydrogels without the additive (Tab. 3.). This is probably related to the presence of the *Aloe vera* juice which, due to its additional volume in the hydrogel matrix, affects the maximum height of the surface. Performed analysis is interesting and in this case the determined roughness profiles allow also to refer to the results of swelling investigations and confirm discussion over the swelling studies presented in this paper and also in other work [38] concerning the impact of the *Aloe vera* juice on the increase in the swelling capacity of materials modified with this additive. This allows to suppose that introduced additive is deposited in the pores of the tested material.

3.3.Studies on the wettability of hydrogels

Fig. 9. presents contact angles determined as a results of the investigation on the wettability of obtained hydrogels.

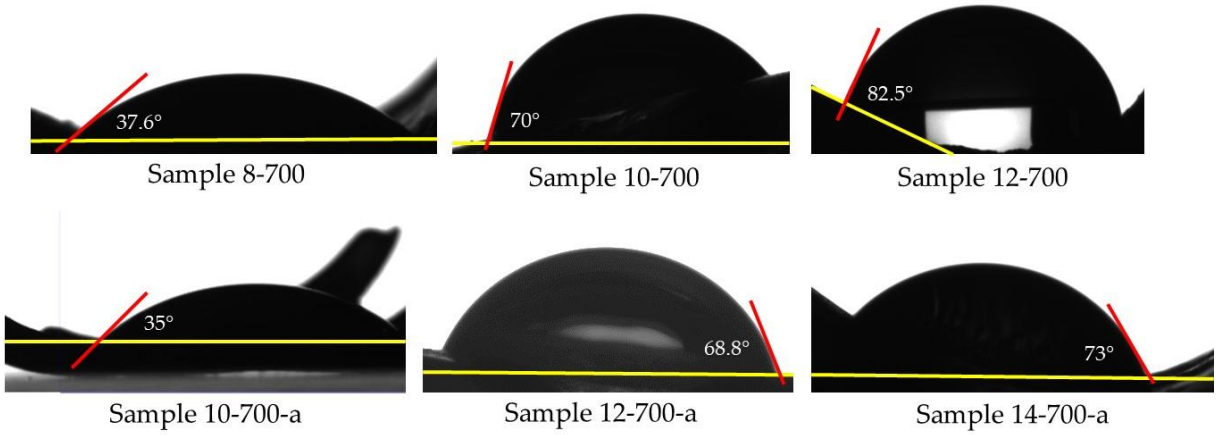

Fig. 9. Contact angles of obtained hydrogel materials.

The investigations on the surface wettability provide information on the behavior of the hydrogel materials (tested in a dry state) when in contact with a drop of water. It is important because in this study the first contact of the material with water is the main research subject and not, as in the case of swelling studies, the behavior of the material after 1 h, 24 h or even 72 h after the immersion in the liquid. The study confirms also the thesis that the increase in the hydrophilicity of the material may be achieved via its modification with appropriate additives which was demonstrated in [39]. In the mentioned article, it was clearly stated that the introduction of adequate hydrophilic additives into the hydrogel matrix results in an increase in its hydrophilicity. Here, an increase of hydrophilicity of hydrogels modified with *Aloe vera* juice compared to the hydrophilicity of unmodified polymers is observed. This investigation also correlates well with the results of the roughness analysis because previously presented structure geometry helps to better understand the wettability characteristics presented in this section of the article. An increasing value of the contact angle of unmodified hydrogels is related to the increase in its crosslinking degree as a result of which the strongly folded structure observed during the roughness analysis prevents the spillage of water drops over the surface that is caused precisely by this corrugated surface. In the case of hydrogels obtained using lower amount of the crosslinker, the contact angle is 37.6°. Next, considering the wettability of hydrogels modified with *Aloe vera* juice, their contact angle is lower which is related probably to the fact that the introduced additive is deposited in the pores of the modified material. Additionally, the modifying agent also exhibits hydrophilic properties. Therefore, the *Aloe vera* juice probably interacts with the water molecules and this is a reason why any rapid spillage of water over the surface of the modified material is not observed even though its smooth surface might indicate such a behavior.

3.4.Analysis of the water desorption from swollen hydrogel samples

In Fig. 10. results of the investigations on the water desorption from the swollen hydrogels are presented.

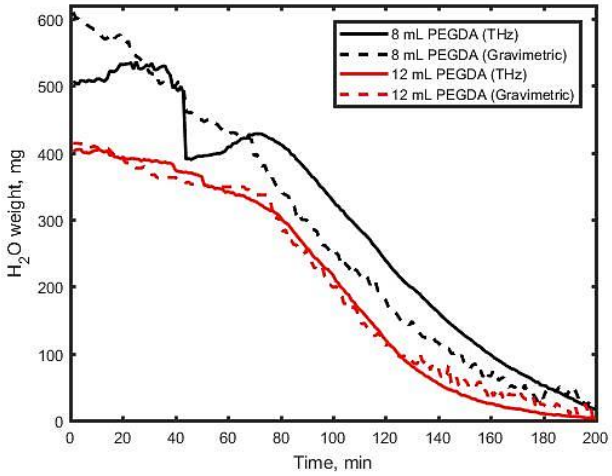

Fig. 10. Water desorption from hydrogel materials with 8 mL (black) and 12 mL (red) of the crosslinker. Straight and dashed lines represent the estimated and measured weight, respectively.

Fig. 10 shows the water desorption profiles of the swollen hydrogels under accelerated drying conditions throughout the 200 min process. It can be observed that the initial amount of water in the hydrogel obtained using 12 mL of the crosslinker is lower than 8 mL because of a reduction in sorption properties. In the case of analysis of hydrogel with lower amount of the crosslinking agent i.e. 8 mL (black line), a significant mass loss is observed from the estimated data from THz measurements after 40 min of the study. This was caused by a physical rupture to the sample thus interfering with the measurement. However, in this instance the ruptured sample was quickly placed back again into the sample holder. Sample rupturing is commonly observed in these specimens because these hydrogels return to its initial state (i.e. before the swelling) upon drying. Fig. 10. also shows a good correlation between the estimated and measured data for both hydrogels. For example, a similar trend is observed characterized by an initial period of relatively constant water weight (0-80 minutes) followed by a steady decrease (80-200 minutes). The latter is consistent with exponential decay behavior also observed in the drying behavior of ionomer-based fuel cell [40], though at a faster rate

3.5.Investigations on the behavior of hydrogels in simulated body fluid

Results of the incubation studies presenting the behavior of obtained hydrogel materials in environments simulating physiological body fluids are shown in Fig. 11.

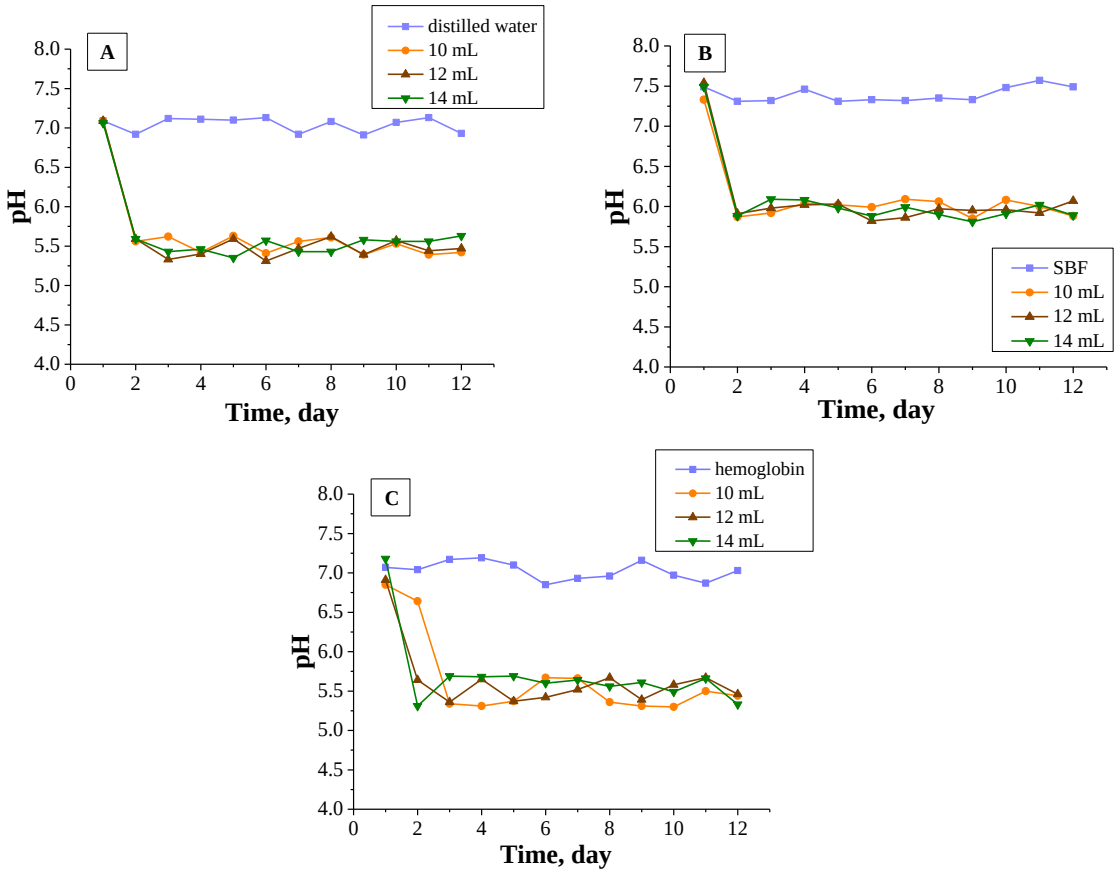

Fig. 11. Changes in pH values determined during incubation of hydrogels modified with *Aloe vera* in: A- distilled water; B- SBF; C- hemoglobin solution.

In above-presented analysis, the impact of the hydrogel materials modified with *Aloe vera* juice on the pH values of distilled water, SBF (simulated body fluid) and hemoglobin has been determined. As it may be observed, during the first days of the study a rapid decrease in pH values is observed. Next, pH of the tested solutions stabilizes and maintains at the similar level. The mentioned rapid change in pH is probably related to the release of the *Aloe vera* juice to the tested environments, i.e. simulated physiological liquids. As a result, the acidification of these liquids occurs which is caused by the fact that the *Aloe vera* juice shows pH = 4.16 [41]. However, this is only a preliminary study to determine the release ability of *Aloe vera* juice from hydrogel materials and this process is presented more specifically in the next section.

3.6.Studies on the release of the modifying agent from the hydrogel matrices

Results of the investigations on the release of active substance from the hydrogel matrices performed in both acidic and alkaline environments are shown in Fig. 12.

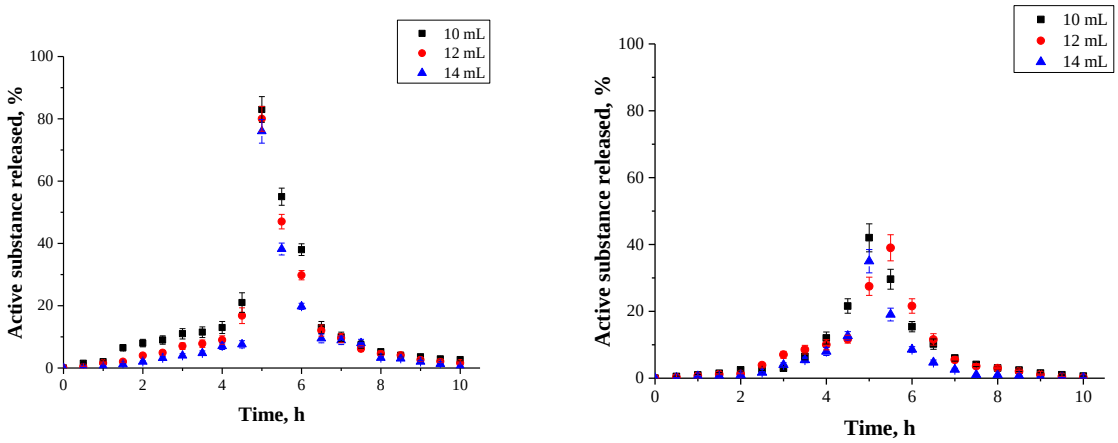

Fig. 12. The release profiles of materials modified with *Aloe vera* determined in: A- 2% citric acid solution; B- phosphate buffer.

During the modification of hydrogel materials, it is important to determine the release ability of the modifying agent from the hydrogel matrix. In this case, the release of *Aloe vera* juice was determined via UV-Vis spectrophotometry. The release analysis was conducted in two environments, i.e. in an acidic and a slightly alkaline one (Fig. 12A). It was proved that in an acidic environment (citric acid solution) the release process proceeds more efficiently compared to the second tested environment (phosphate buffer) (Fig. 12B). In an acidic environment, the NH_2 groups present in the polymer chains of chitosan are protonated, as a result of which NH_3^+ ions forming repel each other and accelerate the loosening of the polymer chains which in dry state form a structure that resembles a tightly coiled thread ball. Larger spaces in the polymer network thus facilitate the release of the *Aloe vera* juice into the tested environment. Such an effect was not observed in the case of the study performed in an alkaline environment (Fig. 12B). In the phosphate buffer solution, only 50% of *Aloe vera* juice compared to the acidic environment is released. The course of the process is the same in both cases but the release intensity is much higher in an acidic environment. Most likely, at the beginning of the study the additive remaining in the pores of the hydrogel is released and in the next step the *Aloe vera* juice present inside the polymer network is released.

In the case of the hydrogels tested in a slightly alkaline environment, such a phenomenon is not as effective as in the case of materials subjected to the study in an acidic environment.

3.7.Evaluation of the cytotoxicity of the hydrogels materials

Evaluation of the cytotoxicity of the hydrogels is important in viewpoint of their potential application for biomedical purposes. Below – in Figs 13-14 – results of the biological analyses, i.e. MTT reduction assays and the investigations on the pro-inflammatory of the hydrogels obtained, are presented. In both assays the negative control ("K1", i.e. cells in the culture medium, untreated with other chemicals) and the positive control ("K2", i.e. cells treated with 1% phenol solution, a substance characterized by a strong cytotoxicity) have also been performed.

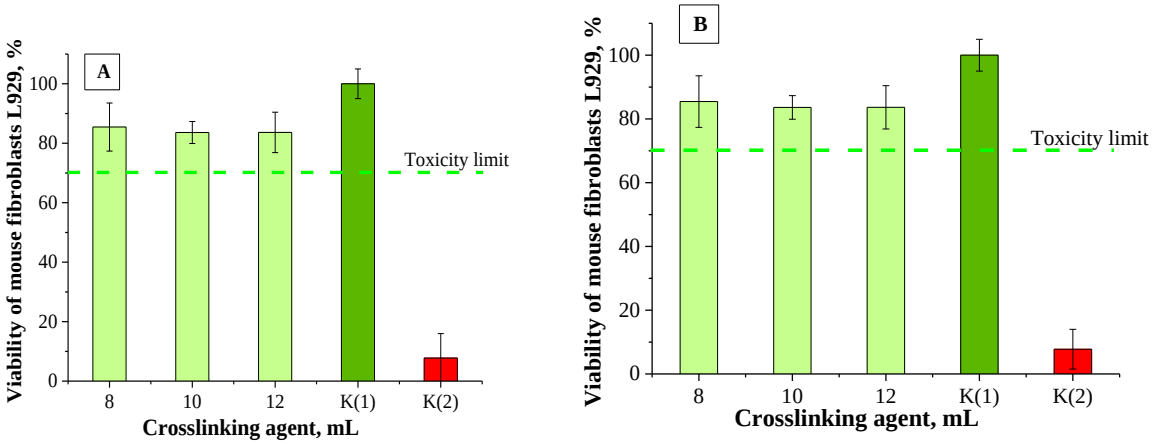

Fig. 13. Cytotoxicity analysis of unmodified hydrogels*:* A- neutral red uptake (NRU) assay; B- MTT reduction assay.

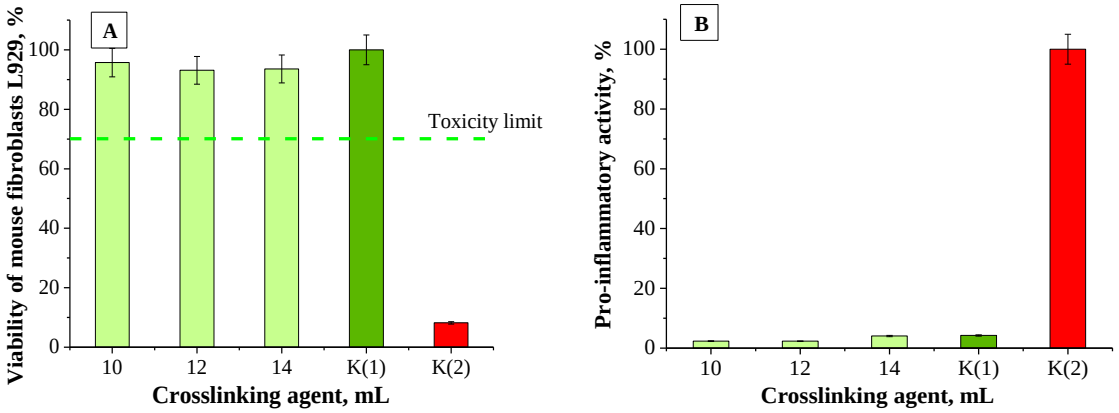

Fig. 14. Cytotoxicity analysis of hydrogel materials modified with *Aloe vera*: A- neutral red uptake (NRU) assay; B- pro-inflammatory activity.

In Fig. 13. results of the cytotoxicity analysis of unmodified hydrogels are presented. The viability of murine fibroblasts (L929 cell lines) was evaluated via neutral red uptake (NRU) assay and MTT reduction assay according to the ISO-10993-5-2009 Standard. According to the

ISO standards, a material is considered as cytotoxic when the viability of cells exposed to this material for 24 h is lower than 70%. In the case of the tested hydrogels, all determined viability values are above the standard level of toxicity (above 70%) therefore tested materials may be defined as non-toxic to murine fibroblasts (L929 cell lines). Moreover, the cytotoxicity analysis was performed also for hydrogels modified with *Aloe vera* juice using MTT reduction assay (Fig. 14A). It was proved that hydrogels containing the mentioned additive do not exhibit cytotoxic properties and, importantly, the viability of L929 murine fibroblasts was greater compared to the viability of cells treated with unmodified polymers. Such conclusions were also reported by Sathiyaseelan et al. who investigated cytotoxic properties of chitosan based composites containing *Aloe vera* juice and silver nanoparticles. They showed that the introduction of *Aloe vera* juice into the composites doe not induce cytotoxic properties and significantly increases the viability of tested cell lines compared to the viability of cells treated with composites without this additive [42]. What is more, apart from the lack of cytotoxicity, these materials are also characterized by a lack of pro-inflammatory activity (Fig. 14B.) and, importantly, the measured pro-inflammatory activity of modified hydrogels is lower than that of the material tested in the negative control (K1).

4. Conclusions

→ The investigations on the swelling capacity of materials received showed that hydrogels containing *Aloe vera* juice exhibited higher swelling properties even by 15% than unmodified materials. Introduction of the additive results in the increase in the swelling ratio of such modified materials due to the hydrophilic nature of this additive.

→ The addition of various amounts of the crosslinker into the hydrogel matrix affects both the crosslinking degree of hydrogel materials and their surface roughness. The greater amount of the crosslinking agent, the lower swelling ability of hydrogels and the higher surface roughness.

→ Studies on the surface roughness showed also that the *Aloe vera* juice added to the hydrogel matrix largely fills the outer pores of the material because the surface of the modified materials is characterized by a much smoother surface and a maximum surface height (S_z) of over 100 μm while unmodified hydrogels exhibited the value of this parameter even at a level of 55 μm.

→ Analysis of the surface wettability showed that the introduction of the *Aloe vera* juice into the hydrogel matrix improves the hydrophilic properties of the materials (e.g. contact angle

changed from 82.5° to 73.0°). Next, the use of 25% more of the crosslinker resulted even in the increase of the contact angle by 86%.

→ The dehydration study confirmed that these materials undergo a process which is opposite to the swelling process, i.e. dehydration. However, this process takes a longer period of time compared to the dehydration of the traditional membranes. This is because the hydrogel materials are characterized by a very high sorption capacity and therefore they are defined as superabsorbents (because they may absorb very large amounts of aqueous solutions). Uniquely, we have shown measurement using THz sensing, benchmarked against simultaneous gravimetric.

→ Results received as a result of the incubation studies conducted in simulated physiological liquids gave preliminary information about the probable release of *Aloe vera* juice to the tested environment because in the 3. day of the study a rapid decrease in pH values of the simulated body fluids to a value of about pH = 5.5-6.0 was observed

→ The preliminary thesis reported after the incubation study was confirmed by the spectroscopic determination of *Aloe vera* juice released from the hydrogel matrix into the acidic and alkaline environment. The ability of the release of *Aloe vera* juice from hydrogel matrix was confirmed wherein this process is much more effective in an acidic environment.

→ The cytotoxicity studies confirmed that the synthesized hydrogels do not show cytotoxic properties against L929 murine fibroblasts and that the addition of the *Aloe vera* affects at the same their viability.

→ Modification of hydrogels with *Aloe vera* resulted in the decrease in their pro-inflammatory activity. Materials with the additive showed lower pro-inflammatory properties than the tested negative control.

→ Results of the performed investigations allow to state that obtained hydrogels are characterized by a great application potential and may find application as innovative dressing materials or as transdermal systems supporting the wound healing process due to the biomedical properties of *Aloe vera* juice.

→ The obtained research results allow us to state that the synthesized materials have a very high application potential and can be used as dressings or transdermal systems supporting wound healing, due to the biomedical properties of *Aloe vera*.

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