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MICROWAVE-ASSISTED SYNTHESIS AND CHARACTERIZATION OF NOVEL CHITOSAN-BASED BIOMATERIALS FOR PELVIC ORGAN PROLAPSE TREATMENT

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Pelvic organ disorders affect up to one in four women in the United States. The prevalence of pelvic organ prolapse (POP) is increasing with each year, particularly in the setting of prolonged life expectancy and an aging population. Current treatment approaches, including polypropylene monofilaments are associated with numerous painful and worrisome side-effects. Therefore, scientists are looking for new solutions. A promising alternative to the current treatment is tissue engineering, which can be utilized to re-create support to the vagina and pelvic organs. Tissue engineering requires the use of three-dimensional scaffolds, derived from biocompatible materials. Chitosan is a natural polymer, obtained from shellfish exoskeletons. It is known for its biodegradability, lack of cytotoxicity and non-pyrogenicity. Due to the presence of free hydroxyl and amino groups, it may undergo various modifications. In this paper, we describe a new type of chitosan-based biomaterials, which can be used as a new alternative scaffold that may provide support to prolapse organs. The chitosan scaffold was obtained under microwave radiation using multifunctional amino and organic acids. We discuss the scaffold's characteristics, with an emphasis on its chemical structure and morphology. Fourier transform infrared spectroscopy (FT-IR) analysis confirmed cross-linking processes with preservation of free amino groups. Moreover, mechanical durability, the stability and swelling ability of the scaffolds in a simulated body fluid were investigated. All of the prepared scaffolds demonstrated very good antioxidant activity and biodegradability. Importantly, the biocompatibility of chitosan scaffolds was examined on human vaginal VK2/E6E7 cell line. No evidence of toxicity was documented, and the cells maintained their presence on the studied materials. These results allude to the lack of toxicity of the scaffolds, and indicate that chitosan-based scaffold should be further investigated in *in vivo* studies as they may be a promising alternative treatment to pelvic organ prolapse.

Key words: *chitosan, pelvic organ prolapse, mesh erosion, vaginal mucosa cells, toxicity, free radical species, biodegradability, biocompatibility*

INTRODUCTION

Despite numerous advances in modern medicine, securing implantable tissues for repair procedures remains an ongoing challenge (1-4). Acute, chronic or congenital injuries often require tissue or organ replacement. In addition, reconstructive surgeries often rely on extensive amount of tissue, and the patient is usually the sole source of the autologous material (3). Tissue engineering evolved from the field of biomaterials development and describes the process whereby cells, engineering and biochemical factors are combined to create living and functional tissues which can be used for therapeutic purposes. Specifically, these engineered tissues can help with the restoration and regeneration of defective native tissues (2, 4, 5). Engineered tissues contain cells, scaffolds and growth-stimulating signals, commonly referred to as the tissue engineering triad (5).

In the human body, cells are the building blocks of tissues, which in turn are organized into organs. Generally, groups of cells make and secrete their own support structure, called extracellular matrix (ECM) (5-7). The ECM acts as a scaffold, providing structural cellular support and also acting as a relay station for cellular signalling molecules (5-7).

In this study we describe the possibility of utilizing chitosan, a chitin derived polysaccharide, as a potential biomaterial that can be used in scaffolds formation. A chitosan-based cross-linked scaffold mesh was created using microwave-radiation. The application of microwaves enabled the elimination of toxic solvents and allowed chemical cross-linking using with carboxylic acids (COOH) and amino acids (NH2). This cross-linking is not seen in conventional heating methods which use photoionators or neurotoxic glutaldehyde, thus microwave radiation was used.

Table 1. Chitosan scaffolds synthesis parameters.

Sample	Chitosan, [g]	Crosslinking acids	Crosslinkers [g]	Power [W]	Time [min]	Temp. [°C]	Freezing temp. [ºC]
1	0.50	aspartic; glycolic	0.33; 0.13	200	8	140	–20
2	0.50	aspartic; glycolic	0.33; 0.13	200	8	140	–80
3	0.50	aspartic; glycolic	0.33; 0.13	200	10	140	–20
4	0.50	aspartic; glycolic	0.33; 0.13	200	10	140	–80
5	0.50	adipic; levulinic	0.50; 0.50	200	8	140	–20
6	0.50	adipic; levulinic	0.50; 0.50	200	8	140	–80
7	0.50	adipic; levulinic	0.50; 0.50	200	10	140	–20
8	0.50	adipic; levulinic	0.50; 0.50	200	10	140	–80

The main purpose of the designed scaffold was to provide an alternative treatment solution to pelvic organ prolapse (POP). Apart from bones and muscle which provide gross structural support, the vaginal integrity is maintained by facia and ligaments. Defects or injuries to these supportive structures in the pelvic floor lead to POP. The primarily injury in this process is sustained by the surrounding connective tissue and ECM. Recent gynecological applications of polypropylene monofilaments as well as synthetic polymers such as poly(lactic acid), poly(caprolactone) or poly(vinyl alcohol) in the treatment of POP resulted in complications associated with material degradation and undesired cellular response (8-15). As such, there is a dire need for an alternative material that can help re-establish support to the surroundings without causing additional harm. Developing a non-toxic scaffold that can help with the restoration and regeneration of damaged vaginal tissue can ultimately improve the quality of life of patients and be a promising alternative to current management.

Our hypothesis was that chitosan scaffolds can provide an appropriate environment for new, viable vaginal tissue formation with minimal cytotoxic effect on its surroundings. This is a novel alternative to POP treatment and if deemed successful, the current reliance on polypropylene mesh applications will decline.

To decrease the risk of cytotoxicity, the scaffolds were prepared using only biocompatible reagents. Additionally, our goal was to maintain some favorable features of the chitosan like biodegradability and antioxidant activity which would positively affect tissue regeneration by decreasing the risk of cells apoptosis due to reactive oxygen species presence or some external factors.

In order to best characterize the chitosan scaffolds, a series of experiments were designed. The chemical structure of the scaffolds was determined using Fourier-transform infrared spectroscopy (FT-IR) and X-ray fluorescence (XRF). Next, their morphology and porosity were determined. The stability and swelling ability of the chitosan scaffolds were also investigated, using a simulated body fluid. Their mechanical properties and biodegradability were also evaluated. Antioxidant activity was investigated using conventional 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Finally, the cytotoxic effect on human vaginal epithelial VK2/E6E7 cell line was evaluated to confirm the compatibility of the scaffolds and their potential applicability in gynaecological surgeries and tissue engineering.

MATERIALS AND METHODS

Materials

Chitosan (300 000 g/mol, degrees of deacetylation (DD = 80%) was purchased from Vanson, USA. The DD was determined by the NMR method by the supplier. Acetic acid, glycolic acid, aspartic acid, adipic acid, levulinic acid, 1,2-propanodiol, methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and isopropanol were purchased from Sigma Aldrich, Poland. Components used for simulated body fluid (SBF) preparation included: NaCl, KCl, HCl, $CaCl_2$, $NaHCO_3$, $NaNO_3$, KCl, Na_2SO_4, $KH_2PO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$. All were purchased from POCH, Poland. The VK2/E6E7 cell line, Keratinocyte-SFM (1X) serum free medium (Gibco) with gentamicin (Lonza) were purchased from ATCC.

Chitosan scaffolds synthesis

To obtain the chitosan scaffolds, 1.0 g of the biopolymer with 80% DD was dissolved in an aqueous acetic acid solution (4%) on a magnetic stirrer, and left until homogenous mixture was obtained (1 hour). In the next step, an appropriate amount of cross-linking agent (glycolic acid, aspartic acid, adipic acid and levulinic acid) as well as 6 ml of propylene glycol were added to each sample. Then, reacting mixtures were placed in Prolabo Synthewave 402 microwave reactor. All syntheses were carried out in the field of microwave radiation in various synthesis conditions (*Table 1*). The obtained chitosan hydrogels were

washed out from cross-linking agents residues using distilled water. In the last step, hydrogels were frozen at two different temperatures and lyophilized.

For the following experiments, samples number 1, 2, 7 and 8 were used.

Fourier transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FT-IR) analysis was performed using IR Nicolet 6700 spectrometer, USA. The range was between 400 and 4000 cm^{-1} with 32 scans and 4 cm^{-1} resolution. Dried samples were placed in KBr pellets. For each analysis, 5 mg of analyzed sample was mixed with 200 mg of KBr.

Scanning electron microscope analysis and X-ray microanalysis

Scanning electron microscope (SEM) analysis was performed using FEI QUANTA 650 FEG. To perform the analysis, the lyophilized samples were cut into small fragments and fixed on the carbon tape. Microphotographs were taken under pressure of 50 Pa and HV of 20.00 kV. X-ray microanalysis of the materials was performed by an energy dispersive spectroscopy method using FEI QUANTA 650 FEG microscope equipped with EDS detector.

Porosity and density

The density and porosity of the obtained chitosan materials were determined by isopropanol displacement, since it does not wet the sample. Investigated biomaterials were placed into the previously measured volume of isopropanol. After a fixed amount of time (5 min) the change in volume of the alcohol-impregnated aerogel was measured. Then studied chitosan scaffold was removed from the isopropanol. In the last step, the difference in isopropanol volume was measured. Basing on the obtained data, the density (equation 1) and porosity (equation 2) of the material were calculated using the following equations:

$$d = \frac{w}{v_1 - v_2} \times 100\% \quad (1)$$

$$p = \frac{v_1 - v_3}{v_2 - v_3} \quad (2)$$

where: d - density, g/cm^3; p - porosity, %; W - weight of the investigated sample, g; V_1 - initial volume of isopropanol, cm^3; V_2 - volume of isopropanol with immersed sample, cm^3; V_3 - volume of isopropanol after sample removal, cm^3.

Swelling properties and incubation study

To determine swelling properties of the obtained biomaterials, samples were weighed and placed in distilled water. Then, the scaffolds were left for 24 hours. Next, samples were weighed again and their swelling degree (%) was calculated. The stability of the scaffolds in a simulated body fluid (SBF) was determined by pH changes measurements for 96 hours. Hydromet ERH-11 (Poland) pH electrode was used for the pH measurements. The experiments were repeated 3 times.

Mechanical properties study

To determine mechanical properties of the prepared biomaterials, the scaffolds were cut into shapes with dimensions 50 mm × 10 mm then placed between cardboard grips. The average thickness of the scaffolds was determined applying SEM method. Next, the nanocomposites were conditioned in a saturated sodium nitrate solution at 21ºC temperature and 30% relative humidity for 48 hours. After conditioning process, the scaffolds were stretched with 100 N load cell at cross-head speed of 5 mm per min and an initial grip separation of 20 mm. Parameters such as tensile strength, elongation at break and elastic modulus were calculated with the obtained force deformation data using the following equations: deformation data using the following equations:

$$\text{TS (MPa)} = \frac{F}{A} \quad (3)$$

where: TS - tensile strength; F - peak force at failure, N; Scaffold crosssectional area (0.50 mm);

$$E_b = \frac{l_b}{l_0} \times 1000 \quad (4)$$

where: l_b - elongation at break, mm; l_0 - the original sample length (20 mm);

$$\text{EM (MPa)} = \frac{\frac{\Delta F}{A}}{\frac{\Delta l}{l_0}} \quad (5)$$

where: ΔF - the change in the force; Δl - corresponding change in the sample length during initial linear deformation.

The experiments were repeated 3 times.

Biodegradation study

In vitro biodegradation study was conducted for 7 days using human lysozyme - enzyme which naturally occurs in human tears and in serum at concentration of 7 – 13 mg/l. Lysozyme is an enzyme hydrolyzing glycosidic bonds. For the study, weighed chitosan aerogels where immersed in SBF solution with lysozyme concentration = 10 mg/l at 37ºC to imitate human body conditions. The samples were taken out, washed with distilled water, dried and weighted at fixed time intervals. The percentage of degradation and biodegradation was calculated using equation 6:

$$\text{BD} = \frac{W_o - W_t}{W_o} \times 100\% \quad (6)$$

where: BD - biodegradation degree, %; W_o - initial weight of the analyzed sample, g; W_t - sample weight after time = t, min.

All experiments were repeated 3 times.

Antioxidant study

Antioxidant properties of the prepared chitosan scaffolds were investigated by a standard DPPH method. For this purpose, a solution of DPPH in methanol was prepared, such that the solution absorbance was 1.0 at 517 nm using Aligent 8453 spectrophotometer. To determine the ability of free radicals scavenging, 0.10 g of each sample was placed in 5 ml of DPPH solution and left in darkness for 1 hour with constant shaking. Then, the absorbance of each solution was measured at 517 nm. The percent of the free radicals removed was calculated using the equation 7:

$$\%S = \frac{A_S - A_C}{A_C} \quad (7)$$

where: %S - the % of the free radicals which were neutralized; A_c - the absorbance of the DPPH solution without the sample; A_s - the absorbance of the DPPH solution containing sample.

Cell culture study

For the cell culture experiments, the VK2/E6E7 cell line was used. The culture was carried out using Keratinocyte-SFM (1X) serum free medium (Gibco) with gentamicin (Lonza). The

cells were cultured at 37ºC, 95 CO_2% concentration and 95% air humidity. For the cytotoxicity study, chitosan scaffolds were divided into small fragments and conditioned in PBS supplemented with penicillin/streptomycin solution for 24 hours. Then, scaffolds were conditioned in pure PBS to wash out the excess of antibiotics. Next, scaffolds were placed on Petri dishes (Ø35 mm) and culture medium was added. After 24 hours, VK2/E6E7 cells were seeded on each scaffold/dish (100,000 cells per dish). The studies lasted 8 days. Cell culture carried out at standard conditions were used as a control (Petri dish and T75 bottle).

RESULTS

Fourier transform infrared spectroscopy study

As a result of microwave-assisted cross-linking, four three-dimensional (3D) scaffolds were obtained. Although all of the proposed synthesis parameters enabled cross-linking process to occur, only four samples maintained 3D structure after lyophilization (samples number 1, 2, 7 and 8).

Fig. 1 presents FT-IR spectra of the pure chitosan and the aforementioned samples. It can be noticed that the spectrum of

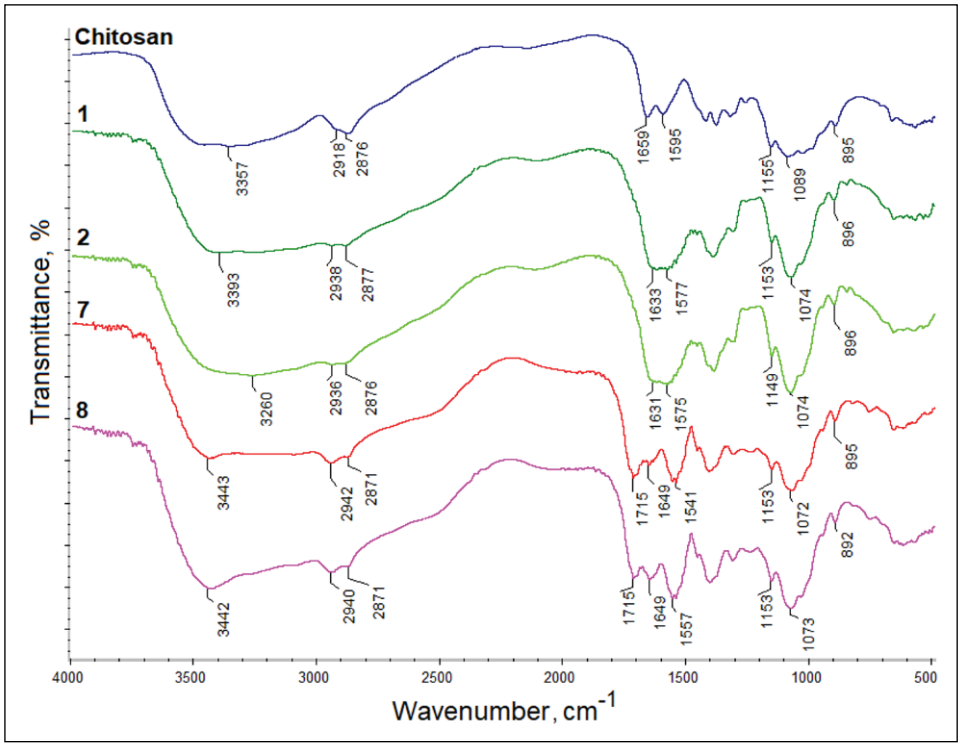

Fig. 1. FT-IR spectrum of the pure chitosan, sample 1, 2, 7 and 8.

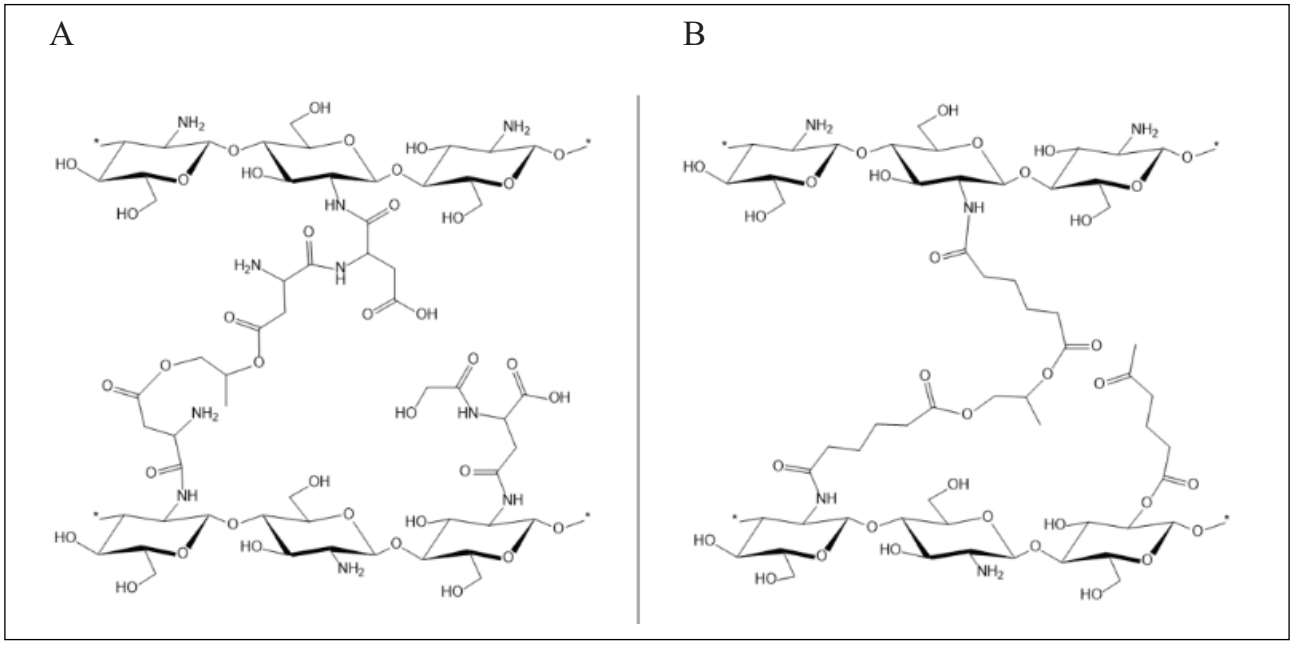

Fig. 2. (A) Chemical structure proposed for the scaffolds prepared from aspartic and glycolic acid; (B) chemical structure proposed for the scaffolds prepared from adipic and levulinic acid.

the raw polymer presents a band typical for free hydroxyl groups (–OH) at 3357 cm^{-1} as well as bands characteristic for aliphatic groups at 2918 cm^{-1} and 2876 cm^{-1}. Additionally a band typical for amide bonds coming from N-acetylaminoglucose mers is visible with a peak at 1659 cm^{-1}. Bands with a peak at 1595 cm^{-1} and 1155 cm^{-1} can be assigned to free amino groups (NH_2) present in amino glucose mers (deacetylated units). Moreover, bands characteristic for glycosidic bonds and glucopiranose ring are present at 1089 cm^{-1} and 895 cm^{-1} respectively.

FT-IR spectra of the samples cross-linked with glycolic and aspartic acid show bands at 3393 cm^{-1} (sample 1) and 3260 cm^{-1} (sample 2) which can be assigned to hydroxyl and carboxylic groups. It suggests that microwave radiation caused small surface degradation of the polymer. At the same time bands typical for –CH– and $–CH_2–$ groups are still present at the spectrum of sample 1 (2938 cm^{-1} and 2877 cm^{-1}) as well as at the spectrum of sample 2 (2936 cm^{-1} and 2876 cm^{-1}). Of note, the intensity of bands typical for amide bonds (for sample 1 1633 cm^{-1} and for sample 2 1163 cm^{-1}) is visibly greater relative to the pure chitosan sample, which confirms that the cross-linking process has occurred between the carboxylic acid groups from the acids and the free amino groups from the deacetylated mers (10, 12). Such results suggest that amino groups in the microwave assisted conditions are the first to react with carboxyl ones. Additionally, the bands coming from free amino groups are visible at 1557 cm^{-1} 1153 cm^{-1} for sample 1 and 1575 cm^{-1} and 1149 cm^{-1} for sample 2. This suggests that although amino groups took part in the cross-linking process, aspartic acid, which also contains these groups in its chemical structure, enabled the compensation of the loss of free NH_2 groups in chitosan (not sure what this means). This fact is very important from a biological point of view, since amino groups in the biopolymer determine its favourable properties. The presence of bands typical for glycosidic

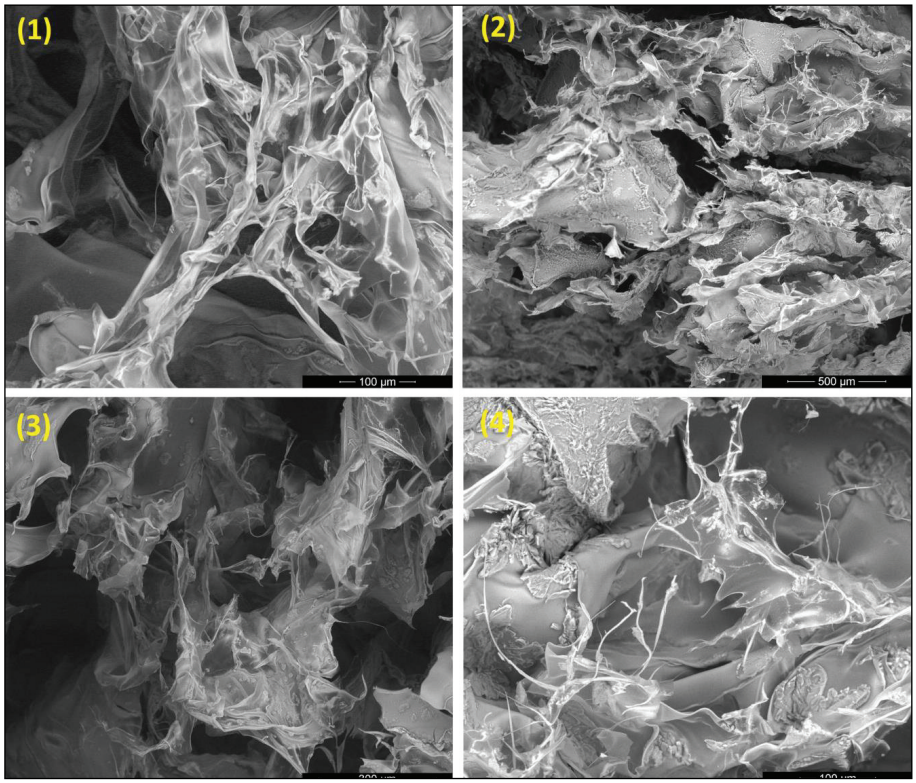

Fig. 3. SEM microphotographs of the prepared scaffolds; (1) sample 1, (2) sample 2, (3) sample 7, (4) sample 8.

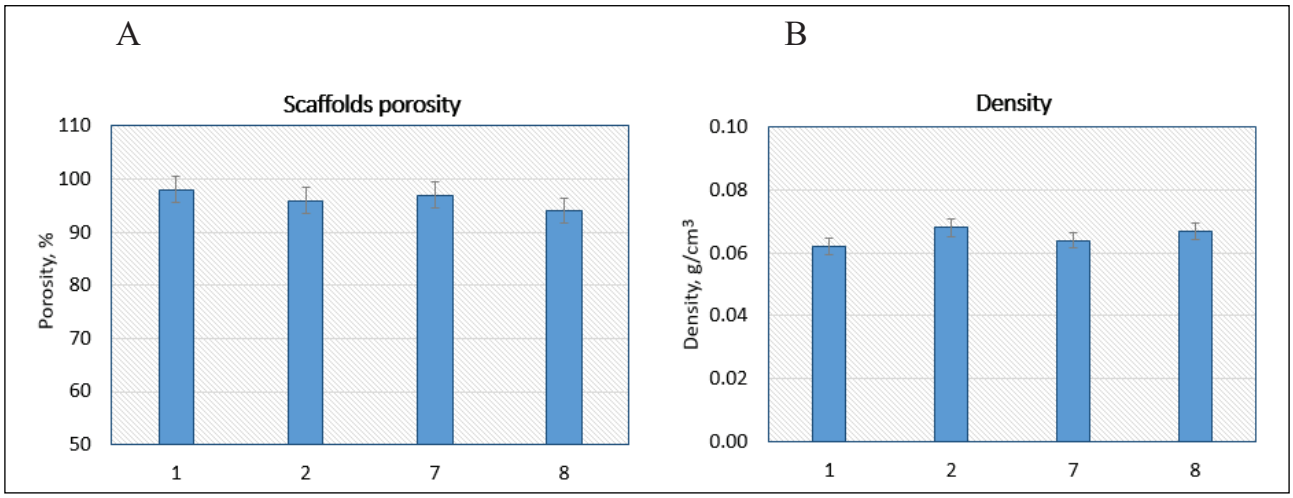

Fig. 4. (A) Scaffolds porosity, (B) scaffolds density.

bonds at 1074 cm^{-1} as well as at 1896 cm^{-1} for both samples proves that there was no significant polymer chain degradation (12, 13).

The FT-IR spectra of samples 7 and 8 are similar to these described above. Again, bands typical for free hydroxyl and carboxylic groups are visible at peaks at 3443 cm^{-1} and 3442 cm^{-1}. However, in this case it can be noticed that the amount of free carboxyl groups is significantly higher due to the application of adipic acid, which contains two carboxylic acid groups. This suggests that not all functional groups participated in the reaction. As previously, bands coming from aliphatic groups are present at 2936 cm^{-1} and 2876 cm^{-1} (sample 7) as well as 2940 cm^{-1} and 2287 cm^{-1} (sample 8). In contrast to sample 1 and sample 2, a new band coming from carbonyl group is visible at 1175 cm^{-1} (sample 7) and (sample 8) due to the application of levulinic acid as a crosslinker. Again, amide bonds are visible at 1649 cm^{-1} for both samples and the intensity is greater due to cross linking. Bands coming from free NH_2 groups also can be noticed at 1154 cm^{-1} (sample 7) and 1557 cm^{-1} (sample 8) as well as at 1153 cm^{-1}. Again, bands coming from glycosidic bonds at 1072 cm^{-1} (sample 7) and 1073 cm^{-1} (sample 8) are present as well as bands typical for pyranose ring of the chitosan mers are present at 895 cm^{-1} (sample 7) and 892 cm^{-1} (sample 8), which confirms maintenance of the polymeric structure (12-15).

Basing on the data coming from performed FT-IR analysis, the chemical structure of the prepared scaffolds is given in *Fig. 2*.

Scaffolds morphology study

Biomaterials dedicated to tissue engineering must be characterized by high porosity. Porous structure is essential to provide an appropriate environment for cells growth. Pores of appropriate shape and size should enable cell anchorage as well as cellular proliferation, as cultured cells must be able to migrate inside the three-dimensional structure of the scaffold.

Additionally, porosity is crucial for the delivery of nutrients, gases and important macromolecules such as growth factors (15, 16) and pores are also important as they enable the removal of metabolites and carbon dioxide. *Fig. 3* shows scanning electron microscope (SEM) microphotographs of the prepared samples. Notably, all of the evaluated biomaterials demonstrate a porous structure, and the pore sizes are above 100 µm. Another significant finding is that the scaffold preparation method (freezing temperature) can affect the size and shape of the pores. Evidently, samples frozen at –20ºC have bigger pore diameters than samples frozen at –80ºC. Also, one can observe that samples prepared at higher temperature have more delicate shape. Such results are due to the differences in the speed at which water molecules form crystals. At lower temperatures (–80ºC), there is more nucleation of crystallization than at –20ºC, therefore more crystals are formed and they are of a smaller size.

Porosity and density studies

Fig. 4 presents results of the porosity and density studies. The results confirm that obtained biomaterials are characterized by very high porosity and very low density. It can be also noticed, that density and porosity are strongly correlated with the freezing temperature, as it was visible on the SEM microphotographs.

X-ray fluorescence analysis

Although the prepared scaffolds are designed for a three-dimensional cell culture, the interactions between the biomaterial surface and the cells is very important. The first stage of the epithelial cells culture is adhesion. Therefore, it is crucial to locate appropriate functional groups which can interact with cellular membranes. Performed FT-IR analysis showed that the scaffolds contain numerous free amino groups. Positively charged amino groups attract the negatively charged carboxyl groups present on the cell surface (15). *Fig. 5* presents XRF analysis of the samples surface. Both biomaterials contain atoms typical for chitosan elemental composition including carbon, oxygen and nitrogen. Additional trace amounts of chloride and calcium may be observed, which can attributed to residues from the raw material used to prepare the chitosan (shrimps exosceletons). Nevertheless, their presence is irrelevant.

Swelling capability and stability study

Biomaterials which are dedicated to cell culture must provide an environment that is conducive for cellular growth, such as high humidity and accessible oxygen and nutrients.

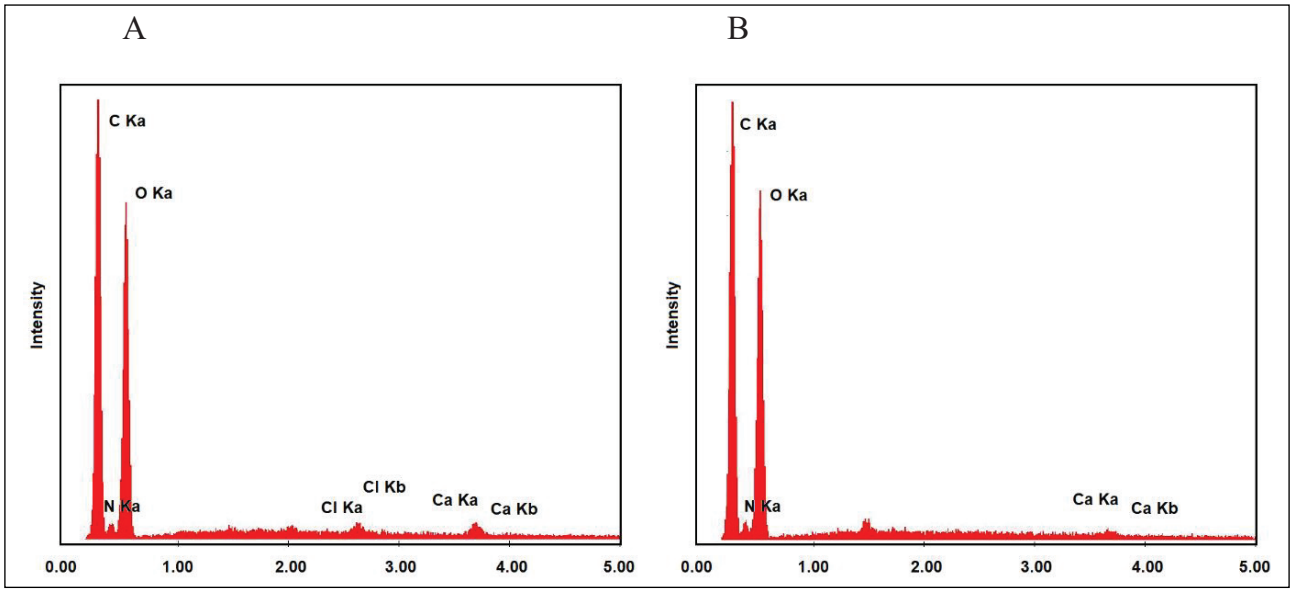

Fig. 5. XRF analysis of the prepared scaffolds; (A) elemental composition of the scaffolds prepared from aspartic and glycolic acid (B) elemental composition of the scaffolds prepared from adipic and levulinic acid.

Tissue engineering enables cell culture in three-dimensions. Therefore, the gases as well as components of a culture medium must be delivered inside the scaffold. This is possible when interconnecting pores are present. The ability to deliver nutrients to cells is determined by biomaterials swelling properties (*Fig. 6A*). In general, sorption of the aquatic solutions is determined by the presence of hydrophilic functional groups as well as the porosity.

Mechanical properties study

Biomaterial dedicated to damaged tissue regeneration applications must be characterized by good durability. To support proliferating, cells should maintain porous structure so to provide oxygen and nutrients deliver as well as enable neo-vascularization. Scaffolds prepared from the raw chitosan exhibit quite low mechanical durability, especially in aquatic media. Therefore, their

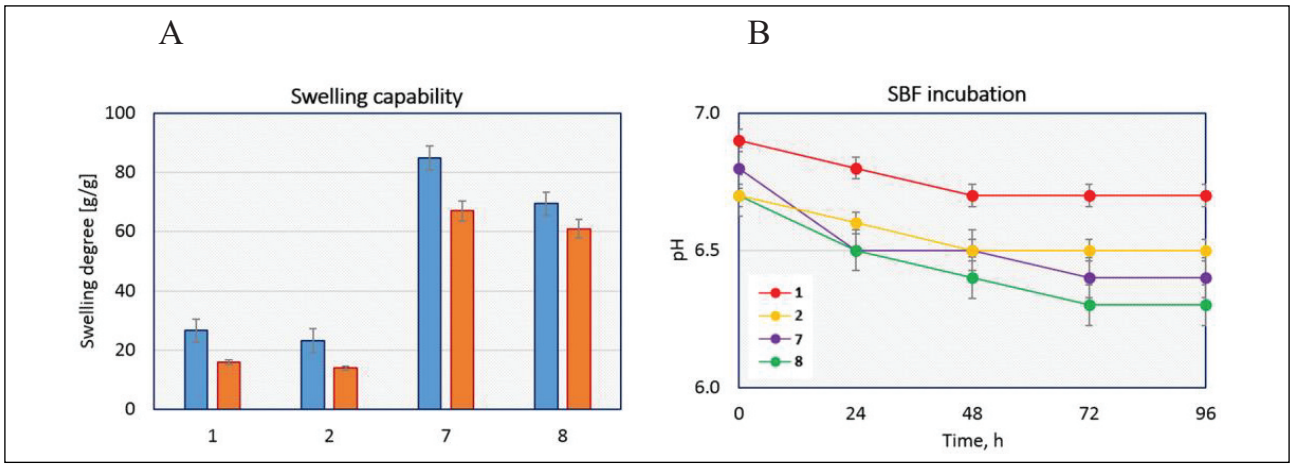

Fig. 6. (A) Scaffolds swelling capability, (B) Scaffolds stability in SBF.

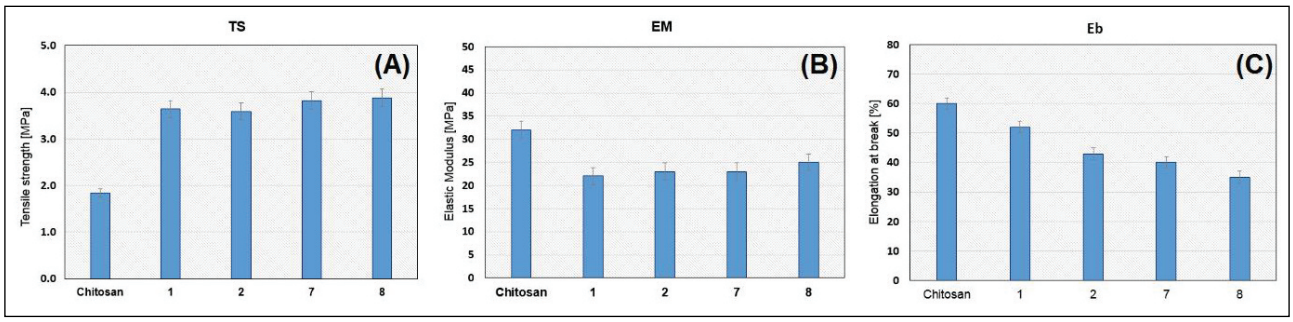

Fig. 7. Mechanical properties of the biomaterials; (A) TS (B) Eb 7 (C) EM.

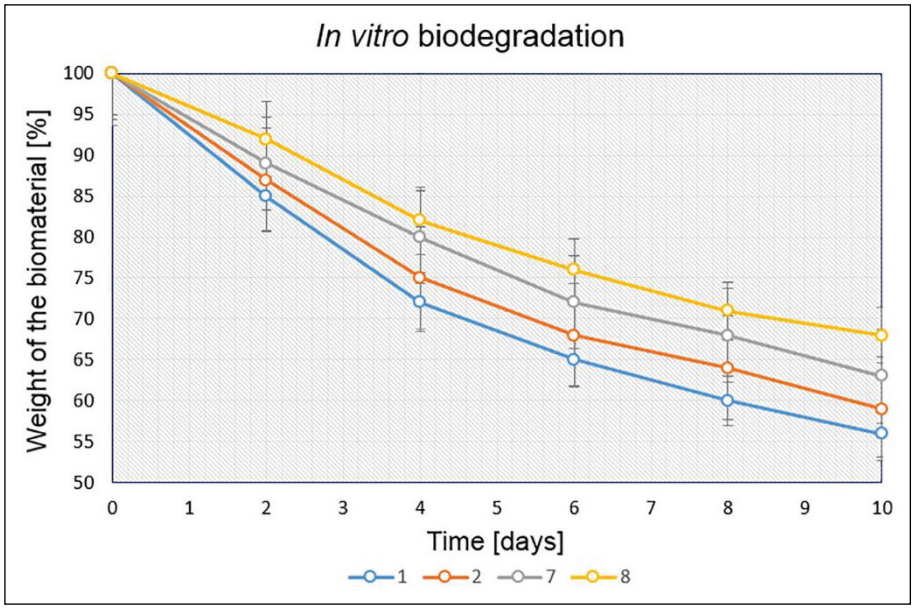

Fig. 8. Scaffolds *in vitro* biodegradation.

application in regenerative medicine is limited. It is known, that chemical cross-linking results in a denser, highly branched structure, which significantly improves mechanical properties (15). *Fig. 7A* presents results of the tensile strength of the raw chitosan scaffold and biomaterials prepared from the modified polymer. It can be noticed that proposed cross-linking strategy resulted in more durable materials. The highest increase is seen in sample 8 (almost two times more durable than pure chitosan). Such phenomenon can be explained by the introduction of C5 and C6 chains between chitosan molecules while maintaining hydrogen bonds.

Biodegradation study

An important feature of biomaterials that enables their application in regenerative medicine is their biodegradability (8, 15). Scaffolds should be resorbed by the body after tissue regeneration so to prevent reoperations. Although certain polymers such as poly(lactic acid) can be applied both *in vitro* and *in vivo*, once degraded, these polymers release lactic acid which leads to local pH decrease. This may cause undesired internal reactions in the surrounding tissues. On the other hand, chitosan is known to be biodegradable by local, naturally occurring lysozyme which hydrolyze glycosidic bonds without an adverse effect on the surrounding tissue (15). *Fig. 8* presents the results of a biodegradation study carried out for ten days. It can be noticed, that the weight loss of the material occurs due to the breaking of bonds between chitosan mers. The process is constant and it can be assumed that it will continue in time. Results given in the *Fig. 6* illustrate that during incubation with sterile SBF, no significant pH decrease was noticed and the same observation was obtain during biodegradation under human like conditions.

Antioxidant properties study

Free radicals, especially in the form of reactive oxygen species (ROS) very often have a toxic effect on cells, leading to their apoptosis. Additionally, oxidative stress my cause random enzyme activation and damage to cellular systems (17, 18). Therefore, during tissue regeneration, it is important to prevent cells from exposure to superoxide anions, hydroxyl radicals as well as hydrogen peroxide. Chitosan in known of its antioxidant activity. However, this property is strongly correlated with its deacetylation degree as well as its origin. *Fig. 9* demonstrated the ability of the prepared scaffold to neutralize free radicals. Evidently, both pure chitosan as well as prepared scaffolds have a very good antioxidant activity. However, one can notice that the ability of free radical removal is greatest in samples 7 and 8. These results were surprising, since antioxidant properties are strongly correlated with the amount of free OH and NH_2 groups, as they are able to neutralize free radicals (17).

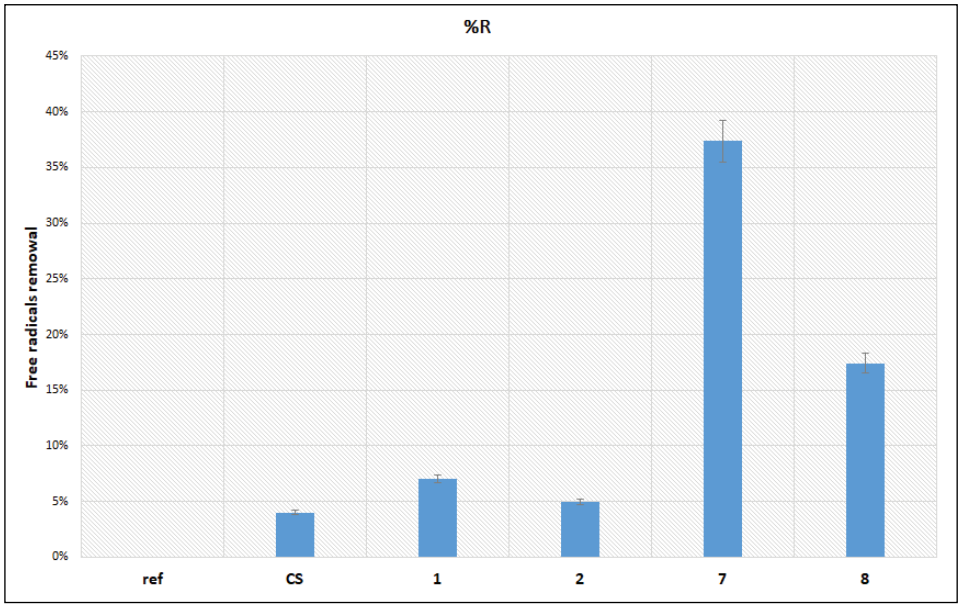

Fig. 9. Antioxidant properties study by DPPH method.

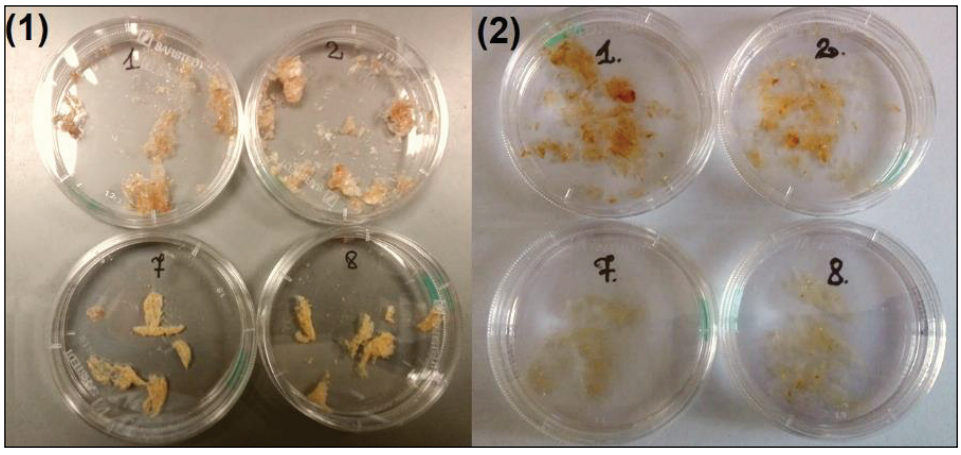

Fig. 10. (1) Scaffolds prepared for the VK2/E6E7 cell line culture (2) Scaffolds immersed in culture medium after 24 hours.

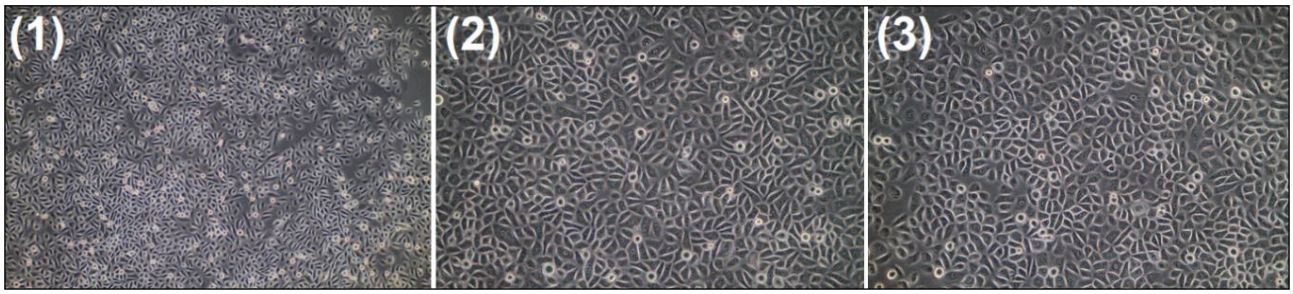

Fig. 11. (1) VK2/E6E7 cultured on T75 bottle after 4 passage, 8 day (2) VK2/E6E7 cultured on T75 bottle after 4 passage, 8 day (3) VK2/E6E7 cultured on Petri dish after 5 passage, 7 day.

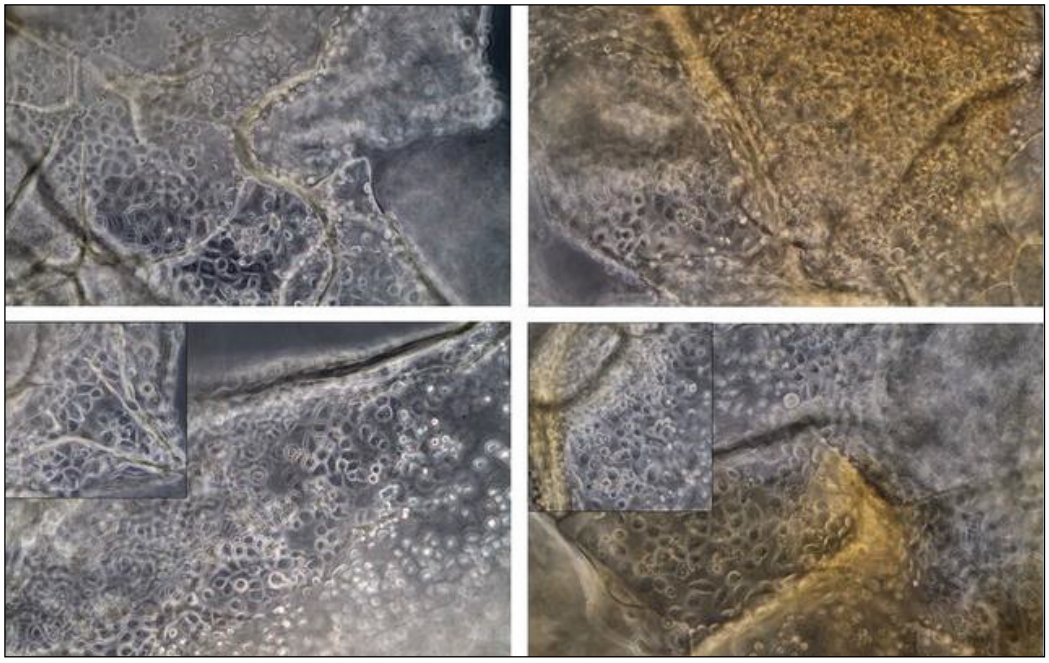

Fig. 12. VK2/E6E7 cells cultured on chitosan scaffolds - samples prepared using aspartic and glycolic acid.

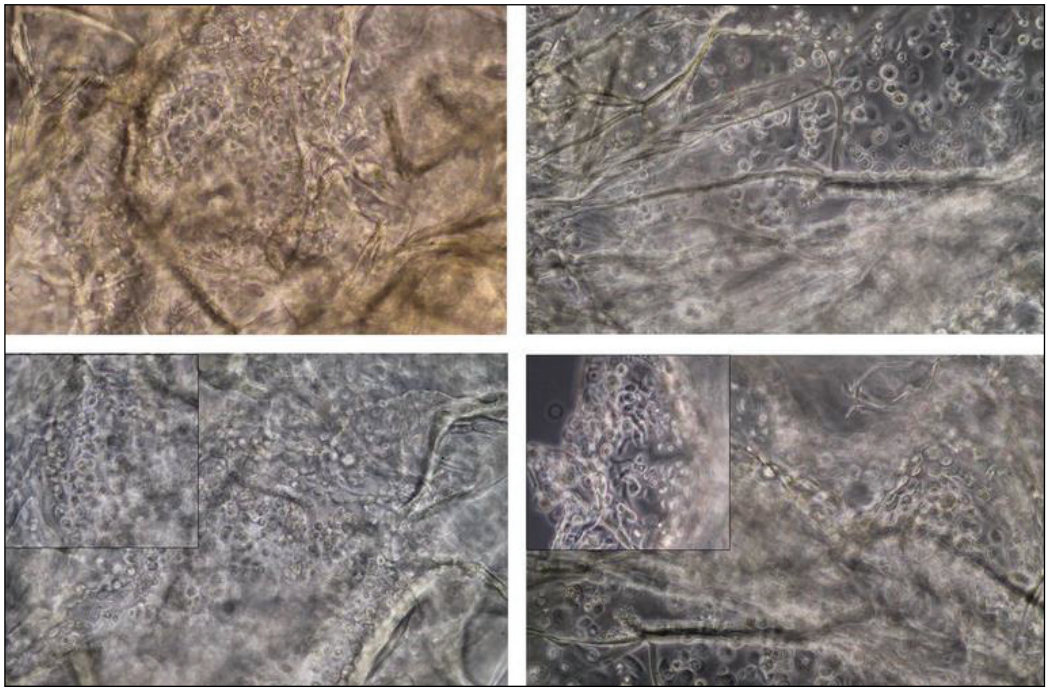

Fig. 13. VK2/E6E7 cells cultured on chitosan scaffolds - samples prepared using adipic and levulinic acid.

Cytotoxicity study

The prepared biomaterials should serve as scaffolds for tissue engineering in the field of urogynecology. Therefore their cytotoxicity was investigated on human vaginal epithelial VK2/E6E7 cell line. For the experiments, scaffolds were placed in DMEM medium. *Fig. 8* presents chitosan scaffolds after its contact with culture medium. It can be observed that samples 1 and 2 were very fragile. When dividing into smaller fragments, the cells crumbles and formed "pellets" that floated in the medium. On the other hand, sample 7 and 8 remained more compact, "sticky" and resilient. They can be seen slagging on the bottom after flooding with the medium. The results suggest that sample 7 and 8 will be more suitable for the *in vivo* tests on the large animal model since probably they will remain their primary structure after suturing into pelvis. The culture medium after 24 h has not changed its color, indicating that the pH has not decreased in a significant amount (*Fig. 10*).

Fig. 11 presents standard VK2/E6E7 cell culture on T75 bottles and Petri dish. All of the cultures show standard cells morphology.

Figs. 12 and *13* shows the results of the cell culture on the prepared scaffolds. It can be noticed that none of the biomaterials were toxic to the cells. One may observe that VK2/E6E7 cells cover the bottom of the plates around the scaffolds and do not differ morphologically from the cells normally grown on the bottle or slides. The cells also grow on the scaffolds. It can be observed, that a greater number of viable cells are seen on samples 1 and 2. The cells that grew on the scaffold were able to retain their morphology, although some cells with irregular edges and granular cytoplasm are present.

DISCUSSION

The FT-IR analysis (*Fig. 1*) shows that the modified chitosan is rich in free amino groups, which determine its ability to support cell adhesion (15-18). Additionally, the structure is rich in amide and glycosidic bonds, which are known to be biodegradable in the human body. Their presence alludes to the biodegradability of the material. Moreover, the presence of carboxylic, amino and hydroxyl groups determines the hydrophilic character of the biomaterial as well as its swelling capability (12).

The SEM analysis confirmed the porous morphology suggests that cultured cells will not only be present at the surface but will also have a possibility to migrate inside the scaffold. If the structure is not porous, cells do not move inside the biomaterial, and there is a serious risk that the structure may collapse due to the accumulated load on its surface. Additionally, cells present at the edges of the scaffold may block pores, and thus deeper situated cells will die due to lack of oxygen and nutrients. The presented chitosan structure is porous, which will enable cells to proliferate and reorganize in order to engage in angiogenesis processes that are essential for further tissue regeneration. The appropriately sized pores and interconnected channels will enable the formation of blood vessels, which provide nutrient delivery after being implemented inside a patient's body (15).

The porosity and density study which are shown in the *Fig. 4* indicate that lower freezing temperature provides the structures with a lower porosity and a higher density (sample 2 and 8). Nevertheless, the differences in the aforementioned values are negligible. Importantly, in all cases, the porosity is above 90%, indicating that the prepared samples meet the requirements of tissue engineering and will be suitable for cell culture (15, 16). Very low density of the prepared biomaterials, which is less than 0.07 g/cm^3, suggests that they may be defined as aerogels. The low density is correlated with the high porosity.

Importantly, the XRF analysis (*Fig. 5*) confirms the absence of heavy metal atoms which can potentially have a cytotoxic effect on the VK2/E6E7 vaginal cells. Therefore, the scaffold is deemed safe in this *in vitro* setting. Furthermore, the presence of nitrogen atoms confirms the presence of functional groups which may engage in electrostatic interactions with the cultured cells, leading to cell proliferation and migration (15).

As shown in *Fig. 3*, the scaffolds have spread porosity, which enables water molecules migration. Notably, all of the prepared biomaterials contain carboxylic acid, amino and hydroxyl groups. The FT-IR and XRF analysis show that samples 1, 2 and 7, 8 have a very similar composition. However, it is evident that materials prepared using adipic and levulinic acid have much better swelling ability (more than 80 g per g). This may be caused by the fact that adipic acid (C6) contains long aliphatic chain ending with two carboxylic groups, whereas levulinic (C5) acid contains carboxyl and carbonyl group. Therefore, the hydrophilic groups are perhaps more available for the water molecules relative to the samples prepared from aspartic acid (C4); one amino and one carboxyl group and glycolic acid (C2) one carboxylic and one hydroxyl. The other factor that contributes to swelling ability is the porosity of the biomaterial. Samples with bigger pores (sample 1 and 7) have better sorption properties which can be explained by better accessibility of the biomaterials channels inside the scaffold (12-15).

Fig. 6 showed that the biomaterials have great swelling properties. In general, sorption of the aquatic solutions is determined by the presence of hydrophilic functional groups as well as the porosity. The studies on swelling abilities carried out using SBF as a medium demonstrated that the sorption properties remain appropriate and the same correlations may be observed. A small decrease in the swelling capability can be noticed due to the presence of various ions in the SBF which may interact with the functional amino, carboxyl and hydroxyl groups of the biomaterials. This may hamper the penetration of water molecules inside the polymeric matrix, however, the effect is negligible. Overall, it is clear that the prepared samples meet the requirements of tissue engineering and will provide appropriate conditions for cultured epithelial cells.

Fig. 7 presents the results of mechanical properties study. The tissue swelling of almost four MPa obtained for all samples suggests that they have good durability. It is known, that biomaterials for urigynecology should be characterized by very good flexibility (19-22). *Fig. 7B* and *7C* show the results of elongation at break and elastic modulus study results. It is evident that although crosslinking process enhanced biomaterials stiffness, it had a negative effect on its elasticity (23).

The proposed chitosan biomaterials are characterized by satisfactory mechanical properties, although other polymers including polypropylene, poly(lactic acid) or poly(caprolactone) may demonstrate higher resistance. The native tissue is composed of muscles and extracellular matrix, and exhibit flexibility and very high durability. Thus, the biomaterials should not replace the damaged area by themselves alone only provide appropriate conditions for tissue regeneration (19-22). The results suggest that the best option for the scaffolds application in pelvis organ prolapse treatment will be to use biomaterials seeded with the autologous vaginal cells which will form a new tissue under *in vivo* conditions.

Fig. 8 shows that the samples are biodegradable. Since the acids used for the cross-linking were weak and biocompatible, it can be assumed that their release would not cause any pyrogenic *in vivo* reactions. The biodegradability of the material is caused by the presence of hydrolyzable amide and ester bonds formed

during chemical modification as well as glycosidic bonds present in the native chitosan (12-15). It can be noticed that both the porosity and the type of cross-linking agent can impact biodegradability. The results given in *Fig. 8* show that the most susceptible to biodegradation are biomaterials prepared using L-aspartic an glycolic acids as the cross-linking agents containing higher number of pores. The samples prepared from the acids of a slightly longer aliphatic chain (C5-6) exhibited lower weight loss after ten days which is caused by the fact that C-C bonding is significantly more durable than others due to its covalent nature. The results suggest that after *in vivo* implantation, the biomaterials will be bioresorbed and will not require an additional surgery for their removal. In the further research carried out on the large animal model, the biodegradability will be once more investigated to evaluate the effect of other enzymes present in the female human body on the scaffolds stability. Also, the local pH value decrease after biomaterial degradation will be evaluated as well as the pyrogenic states of the surrounding tissues.

Fig. 9 proves antioxidant properties of the scaffolds. Previously discussed FT-IR results (*Fig. 1*) showed that sample 1 and 2 contain more free amino groups due to the type of the cross-linkers applied. Interestingly, in the case of sample 1 and 2 the antioxidant activity is only slightly higher when comparing to pure chitosan, whereas samples 7 and 8 removed a significantly greater number of free radicals than raw polymer. The antioxidant properties can be correlated with the accessibility of the hydroxyl and amino groups to free radicals due to better sorption properties. Thus, the obtained results suggest that the prepared samples may play a protective role during cell culture by preventing reactive oxygen species from degrading DNA, protein and lipids (12, 17).

The cytotoxicity study showed that all samples are biocompatible (*Figs. 11-13*). The results suggests that the components of the biomaterials did not affect cell cycles such as glycolysis, perhaps since they do not permeate cell membrane (15). However, human vaginal cells mechanism of proliferation is complex, and further research on this issue should be performed since there is not enough data regarding these cells (24). Moreover, studies show that the VK2/E6E7 cell line may differ in the multiplication pathway then primary vaginal cells (24). It seems that the proposed chitosan chemical modification did not alter its biocompability, since it was able to maintain its free amino groups. These functional groups interact with cell membrane, enabling cells adhesion and attachment (15, 25-30). Additionally, it may be assumed that the antioxidant activity of the scaffolds could prevent VK2/E6E7 cells from reactive oxygen species which could be present due to oxidative stress associated with cell culture (12, 17, 18). Chitosan is similar in its chemical structure to the extracellular matrix components such as chondroitin sulphate (31, 32). Therefore the biopolymer does not cause any undesired reactions to the surrounding cells. Although modification of the polymer may result in the deterioration of its favourable biological properties, well-chosen cross-linking agents will render it safe. *Fig. 9* shows the scaffold prepared from chitosan using aspartic acid as the one of cross-linkers. It suggests that vaginal epithelial cell receptors can recognize regions similar to the natural ECM, resulting in a satisfactory level of proliferation. Therefore these cells may be used as scaffolds for primary cells culture derived from patients (19, 21, 33). Ultimately, the performed studies demonstrated that all obtained scaffolds are not cytotoxic and may be utilized in tissue engineering treatment for urogynecology disorders. For their commercialization further investigation is required. Thus, the upcoming studies will be focused on the biocompability evaluation on the large animal model. It is well-known, that animal models are suitable for testing novel treatment methods and discovering natural pathologies. Nevertheless, the right choice of the animal species for the study may be a challenging issue since human are known of their unique pelvic orientation. In the future, for the biological properties study sheep will be used due to the high resemblance in the anatomy of gynecological organs to humans. Female pelvis provides support due to the presence of tendons and muscles (34). Therefore, for the study, previously sterilized scaffolds will be sutured into pelvis muscles and their potentially cytotoxic effect will be investigated. Further trials will include placement of the scaffolds seeded with autologous vaginal cells and validation of the tissue recovery progress (*Figs. 12* and *13*).

The prevalence of pelvic organ prolapse and disorders of the female reproductive organs has been increasing in the past decade, particularly in the setting of prolonged life expectancy. Current treatments that utilize propylene monofilaments are associated with undesired cell response. As such, the aim of our study was to design a new kind of scaffold mesh which may be used in pelvic organ prolapse treatment and help recreate support to the vaginal and pelvic organs. The scaffold was made of chitosan - a chitin derived polysaccharide. In addition to being biocompatible, chitosan is known for its mucoadhesive, antibacterial, antioxidant as well as anti-pyrogenic properties (25-30). It may also create physical and chemical hydrogels which may promote the proliferation of surrounding embryonic and adult epithelial cells (19, 21, 33, 34-38).

The chitosan scaffolds were prepared under microwave radiation, which promoted cross-linking and removed toxic solvents. Next, the properties of the prepared scaffolds were examined extensively, with a focus on their chemical composition and morphology. The FT-IR and XRF studies demonstrated that the scaffolds are free of toxic metals, are able to resist degradation, and contain nitrogen groups that can enhance their cross-linking. SEM studied of the scaffolds illustrated high porosity which provides a favourable environment for cells growth, cell anchorage, proliferation, as well as delivery of nutrients and growth factors. Furthermore, the porosity also enables blood vessel formation, which helps with nutrient delivery post implantation.

In addition, the chitosan-based materials were stable in simulated body fluid and demonstrated excellent swelling properties. The mechanical properties investigation showed that the biomaterials are durable. The studies also showed that all samples are biodegradable in human-like conditions. The antioxidant study of the scaffolds suggest that the cells may provide a protective role in cell culture by preventing DNA, protein and lipid degradation caused by reactive oxygen species. This can be attributed to the presence of hydroxyl and amino groups in chitosan and their ability to access free radicals. Lastly, cell culture studies illustrated that the vaginal epithelial VK2/E6E7 vaginal cells remain intact while growing on the scaffold, which proves that the scaffold lacks *in vitro* cytotoxic qualities.

In summary, our data suggests that chitosan-based scaffolds have a great potential in future applications in treating female pelvic organ prolapse. Future studies may investigate *in vivo* applications of the scaffolds and the ability of the surrounding tissues to regenerate and grow. Next stage of the research will be focused on the evaluation of the biomaterials biocompability using large animal model (sheep) which has a high resemblance in the anatomy of pelvis (39).

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