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The use of biodegradable polymers in design of cellular scaffolds*

Zastosowanie biodegradowalnych polimerów w projektowaniu rusztowań komórkowych

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

The objective of this work was to demonstrate the usage of biodegradable polymers, made of calcium alginate and dibutyrylchitin, in the design of cellular scaffolds having broad application in reconstructive therapy (dentistry, orthopedics).
To visualize cells seeded on calcium alginate and dibutyrylchitin polymers DAPI staining of fibroblasts nuclei was used. The cytotoxicity of the materials and microscopic evaluation of the viability of seeded cells was tested with a PKH 67 fluorescent dye. To assess the cellular toxicity the proliferation of fibroblasts adjacent to the tested polymers was examined. The vitability of cells seeded on polymers was also evaluated by measuring the fluorescence intensity of calcein which binds only to live cells.
The conducted experiments (DAPI and PKH 67 staining) show that the tested materials have a positive influence on cell adhesion crucial for wound healing – fibroblasts. The self-made dibutyrylchitin dressing do not cause the reduction of viability of cells seeded on them.
The *in vitro* study illustrated the interactions between the tested materials, constructed of calcium alginate or dibutyrylchitin and mouse fibroblasts and proved their usefulness in the design of cellular scaffolds. Examined polymers turned out to be of great interest and promise for cellular scaffolds design.

Key words: **cellular scaffolds • tissue engineering • biopolymers**

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Introduction

Tissue engineering is an interdisciplinary field combining the biological, chemical and medical sciences giving a chance of cure for patients suffering from tissue or organs damage.

In 1985, Dr. Fung from the University of California, proposed at the meeting of the National Science Foundation of the United States to isolate "Tissue Engineering" as a discipline of science. At the time the current definition was proposed: "Tissue engineering - an interdisciplinary field that uses the base engineering and life sciences to obtain biological substitutes that restore, maintain or improve the functioning of individual tissues or organs"[1].

In 1987, group of scientists at the European Society of Biomaterials Conference defined the word biomaterial as „non viable material used in medical device, intended to interact with biological systems"[24].

In 1993, two American scientists, R. Langer and J. Vacanti, described new tissue culture method using the three major "components": cells, cell scaffolds and appropriate signal substances such as growth factors, hormones, and a number of substances with a paracrine effect [2].

Cellular scaffolds play a crucial role of physiological extracellular matrix in tissues, performing the function of retaining the cells and the skeleton together [21]. ECM (ang. *extracellular matrix*) is a complex of various macromolecules, including collagen and non-collagenous proteins, suspended in aqueous polysaccharide gel. The extracellular matrix is a dynamic structure, being a vast reservoir of a series of cytokines, signaling molecules enabling contact between cells. The ECM is responsible for the proliferation and differentiation of cells in tissues [10,14].

As a scaffolds for cells to replace the ECM various materials were proposed, however researchers mostly focused their attention on the polymeric materials currently derived either from natural sources or obtained by synthesis [15,17,24]. Biomaterials useful for designing scaffolds should possess a suitable properties which allow their implantation- biocompatibility, biostability, and biodegradability [9].

Cellular scaffolds for tissue engineering must be characterized by high porosity which allows correct cell proliferation and angiogenesis and also enables the exchange of nutrients and growth factors. Scientists have made a number of efforts to develop new technologies of production of porous cellular scaffolds. Seeding therapeutic cells in pre-made porous scaffolds made of biodegradable biomaterials has become the most commonly used scaffolding approach. Technologies used for production of cellular scaffold can be classified into three categories: (1) processes using porogens in biomaterials, (2) solid free-form or rapid prototyping technologies and (3) techniques using woven or non-woven fibers [3].

In this study, we tested two polymers - calcium alginate and dibutyrylchitin as potential cell scaffolds.

Alginic acid derivatives are biocompatible and biodegradable materials useful for medical and pharmaceutical applications, including tissue engineering and drug delivery. Alginates have been used extensively in the culturing of numerous types of cells [4,11].

Calcium alginate, a common and easy-to-handle biocompatible polymer, has a polysaccharide structure constructed from residues of α-L-guluronic (G) and β-D-mannuronic (M) acids and is obtained from brown algae *(Phaeophyceae)*, or produced by bacteria *Azotobacter vinelandii* and *Peudomonas sp*.[22].

Under the influence of the exchange of calcium ion, associated with subunits of guluronic acid on sodium ion derived from exudate, scaffold made from calcium alginate takes the form of a gel by binding excess exudate around the fibers. The remains of the polymer are biodegradable and do not cause tissue irritation [19].

In the present study a commercially available fibre non-woven calcium alginate dressing was used.

Second tested biomaterial was an ester derivative of chitin – dibutyrylchitin (DBC). The unique method of receiving dibutyrylchitin has been developed at the Technical University of Łódź [20].

Chitin itself is extremely interesting polymer for tissue engeneering applications, but the low solubility has limited its use [16]. Dibutyrylchitin is a polysaccharide structure material obtained by semi-synthesis, it is characterized by good solubility in most organic solvents such as ethanol or acetone. Chitin and its derivatives, due to their properties such as biocompatibility, biodegradability and non-toxicity, are also increasingly used in tissue engineering [5,6,12,25].

The main techniques of transforming the dibutyrylchitin solutions in ethyl alcohol into cellular scaffolds, suitable for tissue engeeneering are electrospinning and spraying of polymer solution [7,8,18,23].

In the present study scaffolds made of dibutyrylchitin using as a porogen sodium chloride was tested.

The aim of this study was to determine the suitability of a commercially available wound dressing constructed from calcium alginate and in-house made dibutyrylchitin dressing as cellular scaffolds that may replace the extracellular matrix (ECM) prior to tissue regeneration, thus ensuring the optimal environment for cell adhesion and proliferation.

Materials and methods

Dressings

– Dressing constructed from calcium alginate - Sorbalgon ® producer: Paul Hartmann AG., Heidenheim Germany, no. series: 200337111, d 09.2017 in.
– Dibutyrylchitin (sample No.13 IPS, degree of esterification ˃ 98%) Dibutyrylchitin polymer was purchased at the Institute of Leather Industry in Lodz in the Department of Experimental Application. Dibutyrylchitin was synthesized using methanesulphonic method. The purchased 3% polymer solution (w/v) was prepared in 95.6% ethanol and poured onto a Petri dish. After the initial evaporation of ethanol, the NaCl crystals were applied to the surface of the polymer and dried at 37 ° C for 48 hours. NaCl crystals were then rinsed with the PBS. Prepared films were then sterilized by autoclaving (121 ° C, 15 min) and used as a material for further research.

Equipment

- Microscope Evosfl ® AMG
- Microscope JuLi Smart®
- Air flow laminar compartment BioSpherix ®

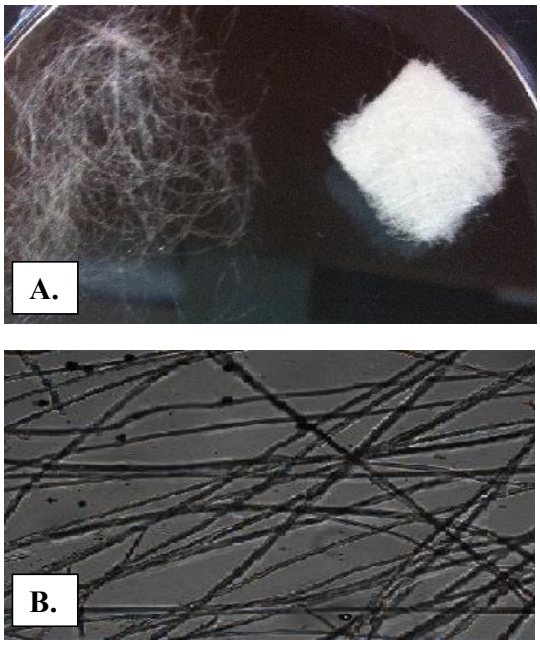


Fig. 1. Commercially available biomaterial (Sorbalgon ®) built from calcium alginate; A - Macroscopic image, B - Image from the microscope JuLI Smart ®, 10x

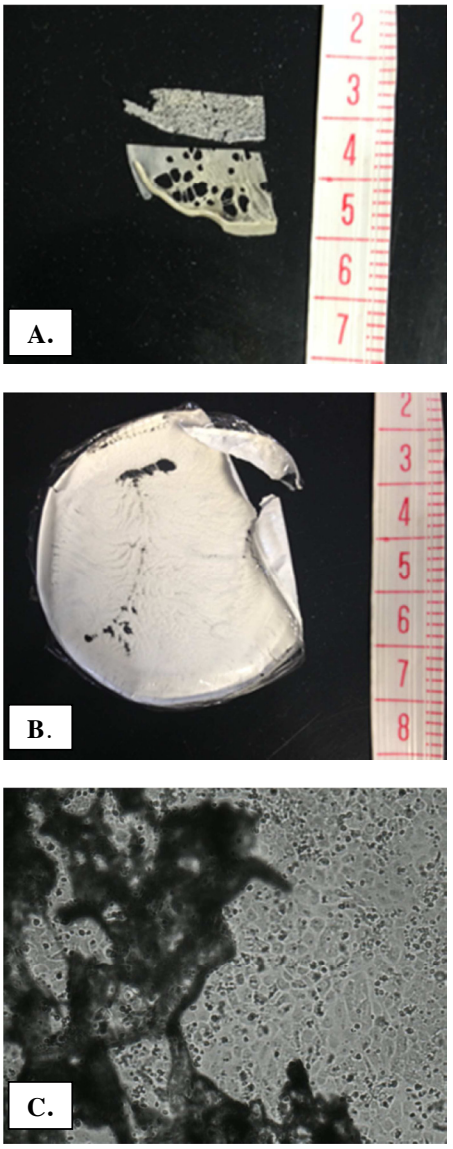


Fig. 2. Synthesized biomaterial built from dibutyrylchitin.
A.,B. Macroscopic images,
C. Image from the microscope JuLI Smart ®, 10x

- Centrifuge (Sartorius Sigma 6-16K)
- ADAM NanoEntec ®
- Multi-Mode Microplate Reader Synergy 2, BioTek®

Cell cultures

Commercially avaliable murine fibroblast cell line from ATCC® CCL-163 (Balb/c 3T3, *Mus musculus, embryo*) was used in the experiment.

Reagents

– Dulbecco's Modified Eagle's Medium without phenol red (DMEM), ATCC®

– Dulbecco's Modified Eagle's Medium with phenol red,(DMEM), ATCC®
– Foetal Calf Serum, (FCS), ATCC®
– Penicyllin/Streptomycin Solution, ATCC®
– Mounting Medium with DAPI, VECTASHIELD®
– PKH 67 Cell Linker KIT, Sigma- Aldrich®
– PBS (Phosphate buffered saline), Gibco®
– 0,25% trypsin/EDTA Solution, Sigma- Aldrich®
– Calcein AM; LIVE/DEAD ® Viability/Cytotoxicity Kit for mammalian cells, Molecular Probes®

DAPI staining of fibroblasts nuclei

The interaction between cells and ECM are very important in the wound healing context. Our experiment allowed to determine the *in vitro* effect between the tested dressings, performing the role temporary ECM and the seeded cells. The study also assessed the number of cells associated directly with the tested materials.

At the 4-well plates the fragments of tested materials were placed and then fibroblast line Balb/3T3 CCL163 were seeded. After four days, the culture medium was removed and the wells were rinsed with PBS solution followed by application of antifade containing DAPI. DAPI fluorescent dye strongly binds to double-stranded DNA, allowing nuclei staining.Cells nuclei were visualised in ultraviolet light using a Evosfl ® AMG microscope.

Analysis of cytotoxicity of examinated materials

Biocompatibility test was carried out for a period of 7 days by incubating cell cultures (murine fibroblast line ATCC® CCL163) with the biopolymers at 37 ° C in an atmosphere containing 5% CO_2.

The experiment was conducted on 6-well plates. To each well the piece of tested material (1cm x 1cm) was applied and the specified amout of cells were plated (1,79 x 10^5 cells to each well). The cells were previously counted using ADAM NanoEntec ®. At 3rd, 5th and 7th day of incubation, sterile dressing fragments were removed from appropriate wells. The wells were rinsed with sterile PBS solution containing Ca^{2+} and Mg^{2+} ions and to each was poured into 1 mL a solution of 0.25% trypsin/EDTA. The test plate was transferred to the incubator for several minutes allowing the cells to detach from the cell culture vessel.

After about 10 minutes, trypsin was inactivated by addition of approximately 3 mL of complete medium containing serum. The cell suspension was transferred to a sterile Falcon tube and centrifuged at 900 rpm for 10 minutes.

The supernatant was removed and the pellet was resuspended in the appropriate cultured medium.

Cells were counted using ADAM cel counter.

As a blank cells seeded on 6-well plates without tested materials in the medium containing: DMEM + 10% FCS + 1% P/S was used. The cell culture medium every three days was changed.

Cell staining with PKH 67 linker kit

The experiment was performed on 6-well plates.

The cells (mouse fibroblast Balb/3T3,ATCC® CCL163) were seeded on the tested dressings.

After 72 hours of incubation at 37 ° C, the staining was performed. The cell cultures were rinsed with sterile PBS. 8 µL of PKH 67 solution was mixed with 4 mL Diluent C reagent (available in PKH 67 staining kit) and prepared solution was poured into each well. Incubation was carried out for 5 minutes in a laminar chamber in darkeness. The PKH 67 dye binds to the cell membranę and stains live cells in green.

Observations were carried out using fluorescence microscope Evosfl ® AMG.

Calcein AM staining of live cells

The experiment was performed on 12- well plates.

The cells before seeding were counted using ADAM NanoEntec ® (7 x 10^5 cells/mL). To the appropriate wells the cell suspension was poured (200µL cell suspension per well). After 24 hours of incubation at 37 ° C, the pieces of tested materials (1cm x 1cm) were applied to the wells. After 24 hours of incubation with polymers Calcein AM staining was performed. Acetoxymethyl ester of calcein (Calcein AM) is a lipid-soluble diester fluorogenic esterase substrate that passively crosses the cell membrane and is frequently used to stain viable cells. Inside the cells, it is converted by intracellular esterases into a polar, lipid-insoluble fluorescent calcein that is retained by cells with intact membranes but is released from the damaged ones

The cell cultures, after incubation for 24 hours with tested polymers, were rinsed with sterile PBS and to each 1 mLof DMEM without phenol red containing 0,5µL Calcein AM was poured into. After 25 minutes of incubation at room temperaturę, 2mL of DMEM without phenol red was added to each well.

Intensity of calcein fluorescence in live cells was assessed hourly for 24 hours using Multi-Mode Microplate Reader Synergy 2, Biotek®.

The fluorescence of calcein was tested on excitation/emission wavelengths of 485 nm and 528 nm respectively.

As a control Calcein AM stained cells seeded on appropriate wells in 12-well plates without tested materials was used. Background signal was subtracted from the results of the experiment.

Results

DAPI staining of fibroblasts nuclei

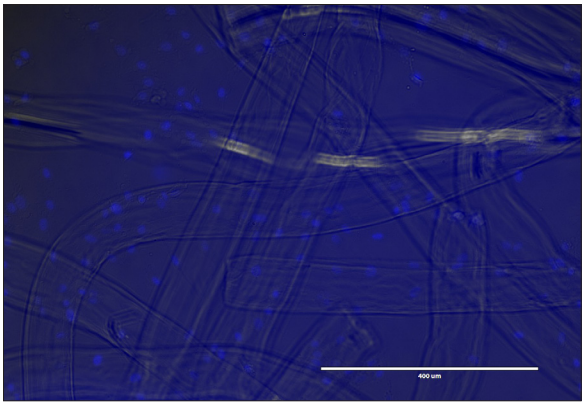


Fig3. The photo shows the outline of dressing fibres consisting of calcium alginate (Sorbalgon ®). Between the fibres the nuclei stained with DAPI dye are visible. Seeded cells are incorporated between fibres of polymer.

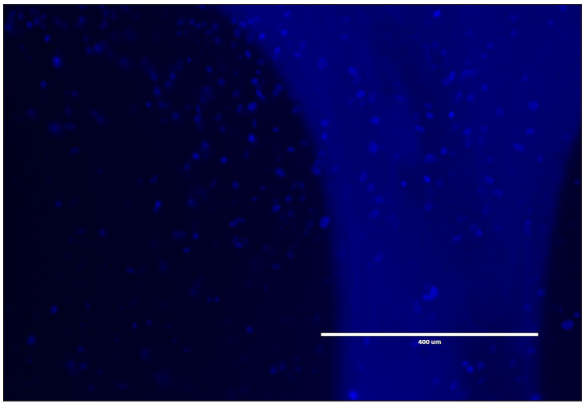


Fig.4 The photo shows the outline of the material consisting of dibutyrylchitin. Between the pores of the material nuclei stained with DAPI dye are visible. Cells seeded onto the polymer are incorporated in the pores

Analysis of cytotoxicity of examinated materials

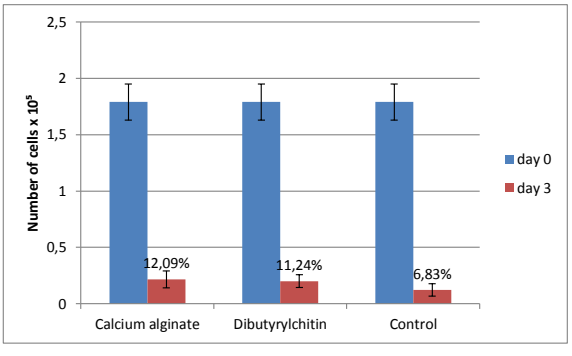


Fig. 5. Comparison of the ability of mouse fibroblast proliferation on the tested dressings relative to a control (3rd day of incubation)

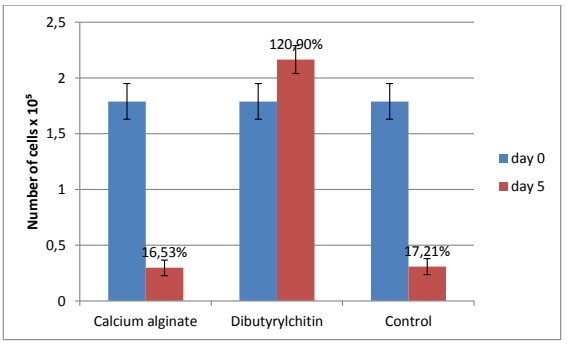


Fig. 6. Comparison of the ability of mouse fibroblast proliferation on the tested dressings relative to a control (5th day of incubation)

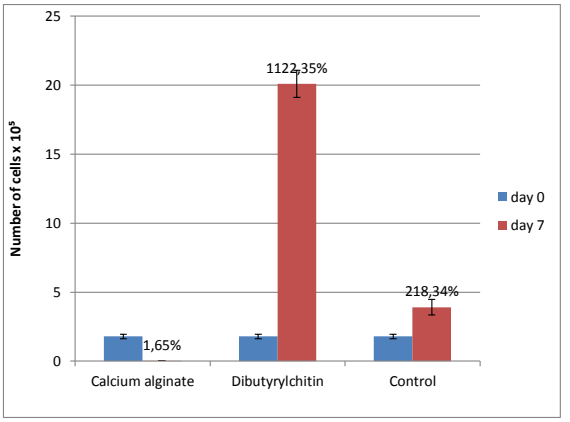


Fig. 7. Comparison of the ability of mouse fibroblast proliferation on the tested dressings relative to a control (7th day of incubation)

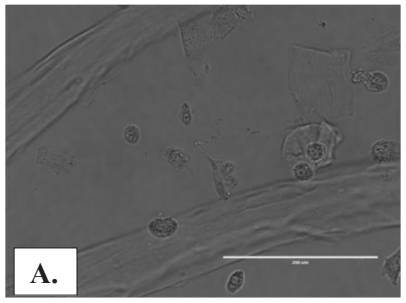
Results and Discussion

Performed experiments illustrate the interaction between the commercially available dressing composed of calcium alginate and three-dimensional scaffold composed of dibutyrylchitin and the cells seeded on them (murine fibroblast cell line). Fibroblasts seeded on polymers exhibit "positive tropism" to them (Fig.3, 4, 8, 9).

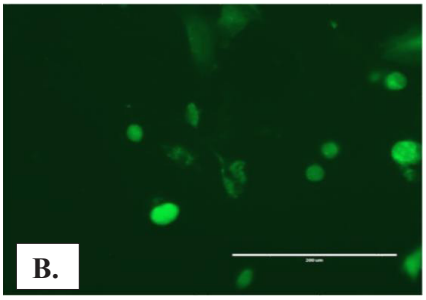
Commercially available Sorbalgon® Hartmann dressing inhibited the proliferation of the murine cell line fibroblasts in the 3rd, 5th and 7th day of observation (Figure 5, Figure 6, Figure 7). Dibutyrylchitin showed no cellular toxicity. Interesting results were obtained by staining cells plated on calcium alginate and dibutyrylchityn using Calcein AM. 24 hours viability profile of fibroblasts incubated with the polymers was significantly higher than the control profile. Presence of dibutyrylchitin and calcium alginate significantly stimulated cell proliferation (Fig. 10).

The results indicate that the material constructed of dibutyrylchitin accelerated cell division of mouse fibroblasts

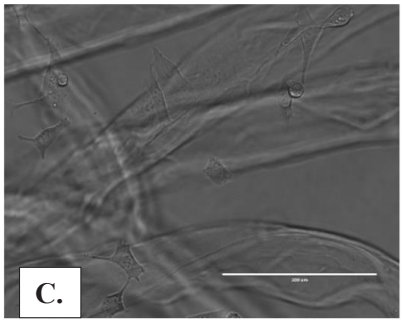
Cell staining with PKH67 linker kit Calcium Alginate:



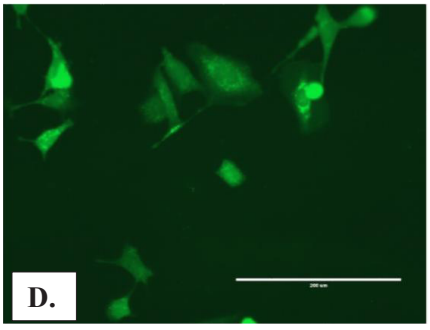
Picture A. Microscopic image (visible light)



Picture B. Microscopic image (UV light), (pictures A and B - the same setting)



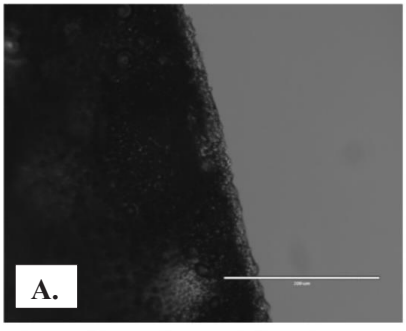
Picture C. Microscopic image (visible light)



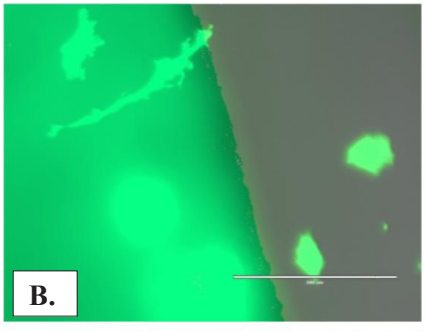
Picture D. Microscopic image (UV light), (pictures C and D - the same setting)

Figure 8. Live cells stained with dye PKH 67. Presence of calcium alginate does not cause cell death.

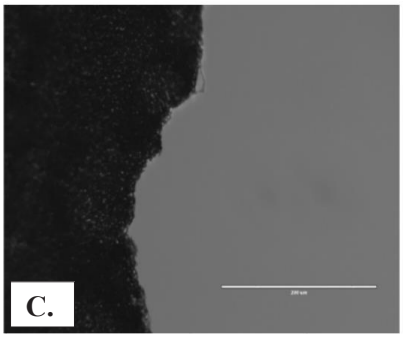
Dibutyrylchitin:



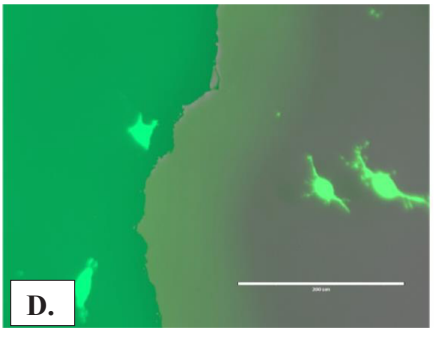
Picture A. Microscopic image, (visible light)



Picture B. Overlay of microscopic image in UV light with Picture A



Picture C. Microscopic image, (visible light)



Picture D. Overlay of microscopic image in UV light with Picture C

Figure 9. Live cells stained with dye PKH 67. Presence of dibutyrylchitin does not cause cell death.

Calcein AM staining of live cells

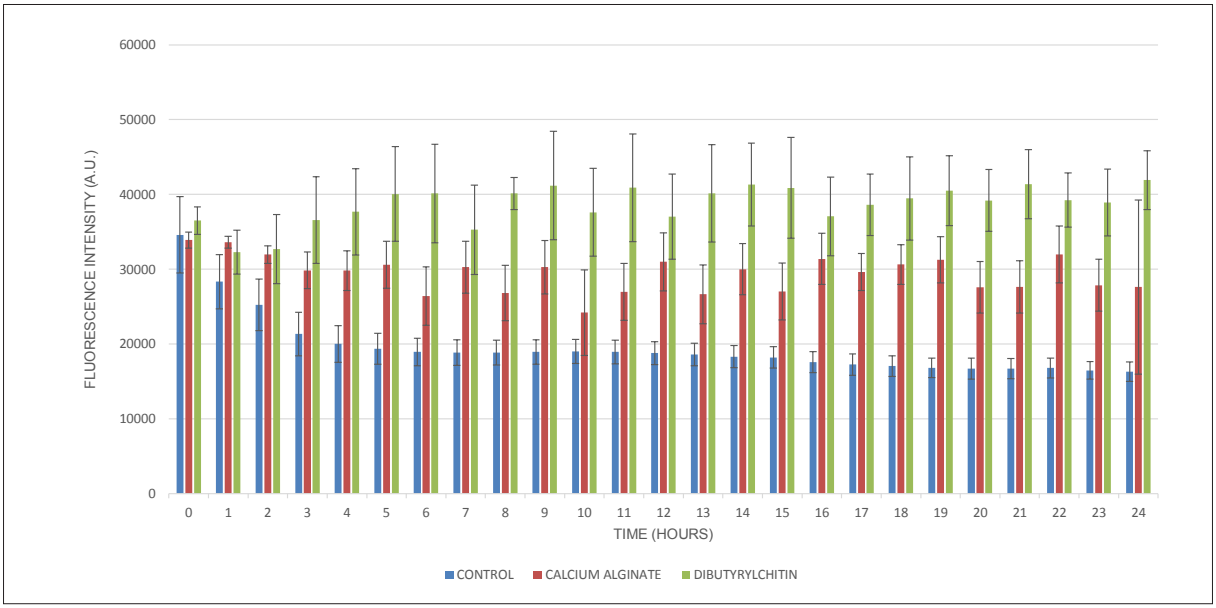


Fig. 10. Comparison of the fluorescence intensity of calcein associated with live cells under the influence of calcium alginate and dibutyrylchitin. As a control calcein AM stained cells seeded on appropriate wells in 12-well plates without tested materials were used

both in the short-term study (Fig. 10) as well as long--term study (Fig. 5, 6, 7).

Dressing built of dibutyrylchitin do not cause the reduction of viability of cells seeded on them (Figure 4, Figure 9). After 72 hours of incubation the microscopic image did not show an increased number of dead cells separating from the bottom of the cell culture 6-well plate.

Cells colonize between the fibers, in the case of commercial dressing and in the pores of the material built with dibutyrylchitin (Fig.3, 4, 8, 9).

Role of regenerative medicine is to implant scaffolding materials for regenerating tissue based on the recruitment of native cells into the scaffold, and subsequent deposition of extracellular matrix (ECM). Scaffolds play a crucial role - they act as an artificial ECM to provide a temporary environment to support the cell to adhere, proliferate and differentiate [10].

Cellular scaffold should provide optimal mechanical support for the cells. In order to enable new tissue organization, it should have an optimal porosity that maintain adequate space for cell growth and tissue development [15].

Polymers useful as cellular scaffolds for tissue engineering should be characterized, notably by the biocompatibility and biodegrability and lack of cytotoxicity. Degradation products of scaffolds should also be non-toxic.

During our experiments we used two types of cell scaffolds. The first type of scaffold was obtained from dibutyrylchitin using porogen sodium chloride. The second type of scaffold is commercially available dressing constructed of non-woven calcium alginate fibers.

The conducted experiments confimed the lower biocompatibility and cytotoxicity in the long-term study of calcium alginate, however cells seeded on alginate scaffolds showed positive tropism relative to the polymer, by setting the spaces between the fibers.

Scaffold made of dibutyrylchitin positively fulfilled the requirements for tissue engineering. Dibutyrylchityn stimulate cell division proving its biocompatibility. Pores made with a porogen were settled by inoculated cells, enabling the exchange of nutrients and signaling factors.

The conducted experiments show that the tested material built of dibutyrylchitin has a positive influence on cell adhesion and proliferation (Figure 4, Figure 9) which suggests that the applied material may play the role of a temporary ECM [13].

Dibutyrychitin is valuable and interesting polymer in terms of their potential applications in tissue engineering. Tested biomaterial is promising cellular platform for regenerative therapy.The unique combination of polymer properties such as biocompatibility, biodegradability, and ease of processing indicates a valuable and highly interesting material for further *in vitro* and *in vivo* studies.

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The authors have no potential conflicts of interest to declare.