Biocybernetics and Biomedical Engineering
2010, Volume 30, Number 3, pp. 65–76

Three Dimensional Polyethersulphone Scaffold for Chondrocytes Cultivation – the Future Supportive Material for Articular Cartilage Regeneration

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The this paper method of obtaining broad-pore membranes for application as scaffolds for chondrocyte cultivation is described. These membranes were obtained from polyethersulfone. They are characterized by the presence of both macropores of relatively large diameter, as well as micropores. These membranes are intended first of all for use in cultivation of the cartilage cells (chondrocytes). The membranes are obtained by the phase inversion method followed by dissolving cellulose present in the membrane. Cellulose is a macropore precursor. Cellulose is dissolved by means of a copper hydroxide ammonia complex. The membranes obtained are not cyto-toxic. The culture of chondrocytes derived from White New Zeeland breed rabbits developed very well on these membranes. The cell cultures were studied by observation under an optical microscope and scanning electron microscope. The protein mass increase on the membrane was determined by flame analysis. The results of these experiments did not show any negative effects of the membranes on the cell culture. Just the opposite, the cartilage cells development on the membranes proceeded very well. The results obtained show that the membrane developed is a very good scaffold for cell cultivation.

K e y w o r d s: chondrocytes cultivation, polyethersulfone scaffolds, polyethersulfone membrane

1. Introduction

The articulation joint cartilage must continuously carry considerable loads resulting from the normal work of the articulation joint. This concerns especially the

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Received 17 November 2009; accepted 22 March 2010

knee articulation joint. The cartilage shows limited possibilities of self-regeneration. If slight damages occur then the articulation joint functions are not impaired. In case of considerable damages, often blocking of the articulation joint occurs due to the pain felt by the patient. Damages of dimensions larger than 2–4 mm very seldom heal spontaneously by themselves. This type of damages usually occurs in sports, especially competitive ones and cannot be easily cured [1, 2]. In the very advanced stage of illness it may be necessary to implant an articulation joint prosthesis [3, 4]. Works leading to improvement of articulation joints and articulation joint cartilage conditions are also carried out [5]. To avoid implantation prosthetic joints attempts are made to regenerate the damaged articulation joint [6, 7]. This is performed by implantation of cartilage to the damaged articulation joint in such a way that after implantation the cartilage cells spontaneously regenerated the damaged articulation joint by themselves [8, 9]. The implantation of deposited on dishes free cells is applied. The cultivation on a dish proceeded on the surface – under these conditions the cultivation in three dimensions was not observed [10, 11]. Better effects are provided by cartilage cells cultivation under 3D conditions. It is then much easier to apply them for implantation [12, 13]. In this case the effect depends on the scaffold used [14–16]. Polyurethane – polycaprolactone scaffolds are an example of this [17]. Chondrocytes develop on such a synthetic scaffold and it is possible to implant them together with the scaffold. Contrary to the hydrogel of fibrin, this polymer is much more durable. Due to this, the implant stays in the damaged place long enough for the tissue to regenerate. Although the fibrin scaffolds better affect the chondrocytes development, the stability has been determined for 2 weeks, which is too short time to apply such scaffolds in medical practice [18]. Good results have been obtained on scaffolds of polyglycolic acid. Correct development of the cartilage cells was observed. Biodegradability is an advantage of such scaffold [19]. Scaffolds of satisfying mechanical resistance for chondrocyte cultivation can also be obtained from alginate gel [20]. Gels such as collagen, agarose, synthetic peptide PuraMatrix are also used [21].

The purpose of our work was obtaining of new porous scaffolds, on which the cartilage cells development would be possible. We tried to obtain membranes of spatially highly developed internal pore structure and possibly the greatest scaffold thickness. We attempted to obtain such scaffold for the chondrocytes to rise also into it and develop within all the scaffold's volume. Due to this we hoped to obtain the largest possible cell mass per membrane volume unit. Besides this, contrary to the cartilage cells cultivated on a surface, in the 3D cultivation no unfavorable differentiation of the chondrocytes occurs. Such type of material could find application in treatment of damaged articulation joints cartilage [22]. Moreover, the scaffold should isolate the newly growing cartilage from the enzymes liberated from the inside of bones. They can have a very unfavorable effect on the development of the new cartilage.

2. Experimental Part

2.1. Materials and Methods

Polyethersulphone Ultrason E2020P of BASF was the membrane-forming polymer. Prior to application it was dried at 130–150°C for 8 h. The dry polymer was stored in tightly closed containers containing a drying agent (molecular sieves 4A). Auxiliary porophore, poly(vinyl pyrrolidone) 10 000 (PVP), Sigma, was dried prior to use at 100–110°C for 8 h. The dried porophore was also stored in tightly closed containers with a drying agent (molecular sieves 4A). Chromatographic paper Whatmann no. 3 was used as the main porophore. 1:1 mixture of N,N-dimethylformamide, (DMF), of POCh and 1-methyl-2-pyrrolidone, (NMP), of Fluka was used as the solvent. N,N-dimethylformamide was distilled before use through a high-performance rectification column (the fraction boiling at 153 °C was collected) and stored in a closed flask with a drying agent (molecular sieves 4A). 1-methyl-2-pyrrolidone did not require distillation. It was kept in a closed flask with a drying agent (molecular sieves 4A). Deionized water of 18.2 MΩcm resistance was used for gelation. Copper sulfate (analar), sodium hydroxide (analar), ammonia water (analar), ethanol 96% and 99.8%, were of POCh production.

Chondrocytes were obtained from a 16-week-old White New Zealand rabbit. (Research protocol of this experiment with rabbit was reviewed and approved by the ethical committee of Medical University of Warsaw). Collagenase from *Clostridium histolyticum*, and deoxyrybonuclease I were produced by Sigma. DMEM/F-12 with Glutamax™-1, penicillin/streptomycin and bovine serum were produced by (Invitrogen Company, Gibco). Nylon mesh for cell isolation was produced by BD Falcon Cell Strainers.

2.2. Preparation of the Copper Hydroxide Ammonia Solution $[Cu(NH_3)_4](OH)_2$

Solution of copper hydroxide ammonia complex was obtained as follows: from the 10% $CuSO_4$ aqueous solution $Cu(OH)_2$ was precipitated by means of 5% aqueous NaOH, the precipitate was filtered off and washed with water until SO_4^{2-} ions disappeared. The washed precipitate was dissolved in 25% ammonia aqueous solution.

2.3. Preparation of Membrane-forming Mixture

In 100 g of DMF and NMP mixture (1:1 ratio by weight), 25 g of Ultrason E2020P and 2.5 g of PVP 10 000 were dissolved, while stirring in an anhydrous atmosphere (argon) at 42–44°C for 40 h. The solution was used directly after preparation.

2.4. Preparation of a Scaffold with a Double-sided Skin Layer

The Whatmann no. 3 chromatographic paper carefully dried at 105°C was placed in a membrane-forming solution and left for 30 minutes. Then, the excess of polymer

was removed by scooping with a knife so only a thin layer remained on the surface of the chromatographic paper. The solution was then gelled with deionized water for 4 h. The ready membrane was transferred to a copper hydroxide ammonia solution in which it stayed until cellulose dissolved. After complete dissolution of cellulose the remaining copper compounds were dissolved in 1% HNO_3 solution. The ready membrane was washed with deionized water until achieving neutral reaction and removal of NO_3^- ions. The membranes were stored in ethanol in sealed polyethylene bags.

2.5. Preparation of a Scaffold without Skin Layers

The membrane was prepared analogously as described in chapter 2.4. The difference consisted in removal of the skin layer. After saturating the cellulose chromatographic paper with the membrane-forming solution, the future membrane was placed between pieces of thin chromatographic paper saturated with DMF. The membrane was kept between the chromatographic paper sheets for 3 minutes, followed by placing together with them in deionized water. After gelation the individual layers were not separated from each other, as they were strongly tied with each other and such an attempt would cause mechanical damage of the membrane. Further the process proceeded as in chapter 2.4. In this way membranes lacking of skin layers and having an open pore structure were obtained.

2.6. Preparation of a Scaffold with One Dense and One Perforated Skin Layer

The Whatmann no. 3 chromatographic paper carefully dried at 105°C was placed in a membrane-forming solution and left for 30 minutes. Then, the excess of polymer was removed by scooping with a knife so on the surface of the chromatographic paper only a thin layer remained. Then the chromatographic paper with the membrane-forming mixture was placed on the chromatographic paper saturated with DMF and left in air for 15 minutes. The membrane was then gelled with deionized water. Further procedure was analogous as in chapter 2.4.

2.7. Isolation of Chondrocytes

16-week-old White New Zealand rabbits were used. Fresh articular cartilage slices were taken from the knee joints of the rabbits, transferred to a Petri dish, washed 2–3 times with phosphate-buffered saline (PBS) and cut into approximately 1-mm-thick slices. The chondrocytes were isolated by treatment with Collagenase from *Clostridium histolyticum* in culture medium with addition of deoxyrybonuclease I for 4 hours at 37 °C in a 50 ml centrifuge tube on a roller. The medium was DMEM /F-12 with Glutamax™-1 supplemented with 10% fetal bovine serum and penicillin/streptomycin. The isolated cells were centrifuged, washed by culture me-

dium and filtered through a sterile 70-μm nylon mesh. After removing of undigested cartilage the chondrocytes were resuspended in the culture medium and finally plated in 24-well plates.
Research protocol of this experiment with rabbit was reviewed and approved by the ethical committee of Medical University of Warsaw

2.8. Membrane Preparation for Cell Culture

Membranes of $d = 5$ mm diameter were cut under sterile conditions. Then they were placed in 75% ethyl alcohol for 30 minutes followed by washing fragments of the membranes twice with sterile water and buffered saline (salt).

2.9. Chondrocyte Cell Culture

The prepared fragments of the membranes were placed in 24 well-plates. A suspension of cells was deposited onto the porous side of the membrane. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2-3 days. The chondrocytes were kept in culture from 2 weeks to 6 weeks in order to reach at least one million cells.

2.10. SEM images of Membranes

The membranes obtained were analyzed by means of a Hitachi TM-1000 electron microscope. When covering of the samples with a layer of gold was necessary, an Emitech K550X sputter coater was used. The layer of gold deposited was of ca. 7 nm thickness.

2.11. Elemental Analysis

Elemental analysis was carried out by means of a CHNS analysis of Elementar model Vario EL III. Two analyses were performed for each sample.

3. Results and Discussion

As a result of the work carried out, three types of the broad-pore semi-permeable membranes of 0.8–1.0 mm thickness for application as scaffolds for the chondrocyte cultivation were obtained. The interior of the membranes obtained by us contained two types of pores. The first one consisted in macropores formed after washing out cellulose fibers. The surfaces of these pores are the real scaffold for cell cultivation. The pore walls have a micropore structure. The micropores assure possibility of easy diffusion transport of nutritious products and oxygen to cells and metabolism

products outside when the entire volume of macropores fills up. The first obtained and studied membrane was that with two skin layers. This membrane showed good mechanical properties. Unfortunately, as a cultivation scaffold it did not fulfill requirement of rapid and efficient penetration inside the membrane. The second type of membrane completely lacking of skin layers appeared to be mechanically too weak and practical its use by surgeons was extremely difficult. However, this membrane perfectly performed as a cultivation scaffold. The seeded cells very well grew inside the macropores. However, lack of the bottom barrier which is the skin layer caused some losses of the chondrocytes which migrated to the plate. The above reservations caused that we concentrated on development of a membrane which would not have the faults of both previous types.

The membrane comprising one solid layer and the second skin layer with perforation (Fig. 1) appeared to be such a membrane. The perforated skin layer did not constitute a barrier for the chondrocytes. The chondrocytes penetrated inside the membrane where they freely developed. The second skin layer constituted, however, a non-permeable barrier for the cells. Since this barrier has a cut-off point at about 20 kD, it is also non-permeable for enzymes and proteins. It permeates all low-molecular weight nutritious products. Due to maintaining of both skin layers, the resistance of such membrane is sufficiently high to easily work with.

Figure 1 presents a cross-section of such membrane. The whole membrane together with the solid skin layer (at the top of the picture) and the layer of great porosity constituting a border on the opposite side (at the bottom of the picture) are visible. A sponge type structure is visible between these layers. The empty spaces result from dissolution of the cellulose fibers from the raw membrane.

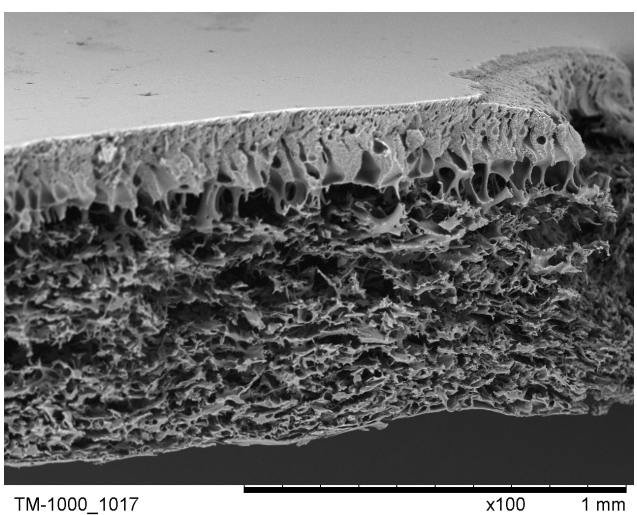

Fig. 1. Cross-section through the membrane. Solid skin layer at the top and perforated skin layer at the bottom are visible. The interior of the membrane is filled with a sponge structure. Magnification 100×

The thickness of the skin layer is considerable and ranges between 0.10–1.15 mm. It is practically a separate membrane integrally connected with the remaining structure. In Figure 2 it is seen that the broad-pore membrane skin layer consists of several layers.

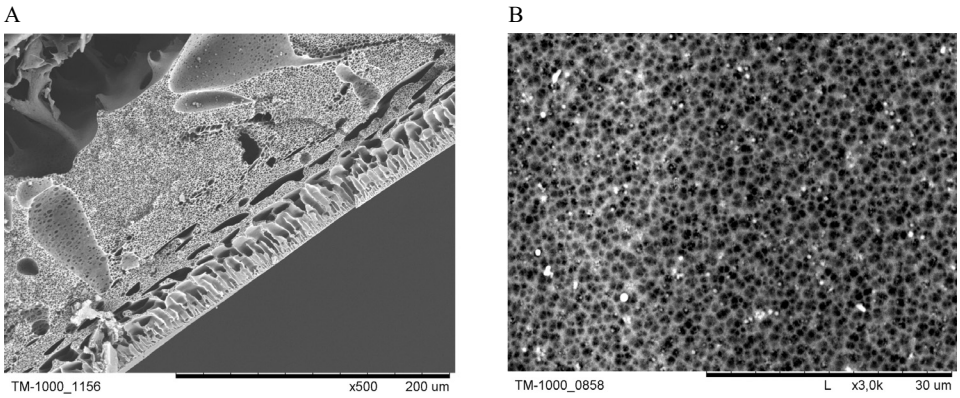

Fig. 2. A – Cross-section through the membrane skin layer. The broad-pore membrane skin layer structure is visible. Magnification 500×. B – Surface of the skin layer. Magnification 3000×

In Figure 3 the perforated skin layer is presented. The very large non-continuities of the skin layer are visible. The size of perforation causes that the chondrocytes can penetrate without any problems into the interior of the membrane. The internal macropores enable migration and growth of the cells within the entire membrane volume.

The width of the perforation gaps is quite differentiated and ranges from10 μm to 50 μm. The length often exceeds 1 mm. Besides this, on the surface other regions lacking of skin layer are present. One is visible in the right upper corner in Fig. 3A,

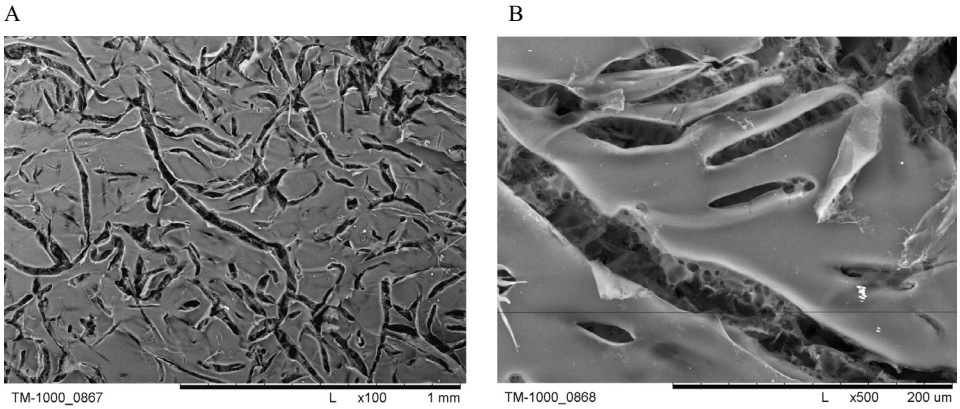

Fig. 3. Photomicrograph of perforated skin layer. A – magnification 100× B – magnification 500×

and its dimensions are 150 × 80 μm. Despite such considerable perforation the skin layer maintained its mechanical resistance. Due to this the entire membrane is sufficiently mechanically resistive. The polyethersulfone membranes obtained according to our method have been not cytotoxic and were earlier successfully used for hepatocytes cultivation [23–25]. In this work we took up cultivation of the chondrocytes for the regeneration of losses in the knee articulation joint cartilages in the White New Zeeland rabbits. The chondrocytes were isolated from the cartilage taken from healthy 16-week old rabbits of the same breed. Then the isolated cells were seeded on ring slices cut out of the above described polyethersulfone membranes.

The cells growing on the edge of the membrane were monitored by means of an optical microscope. Pictures were taken directly of the plate under sterile conditions without taking out the membrane from the cultivation well.

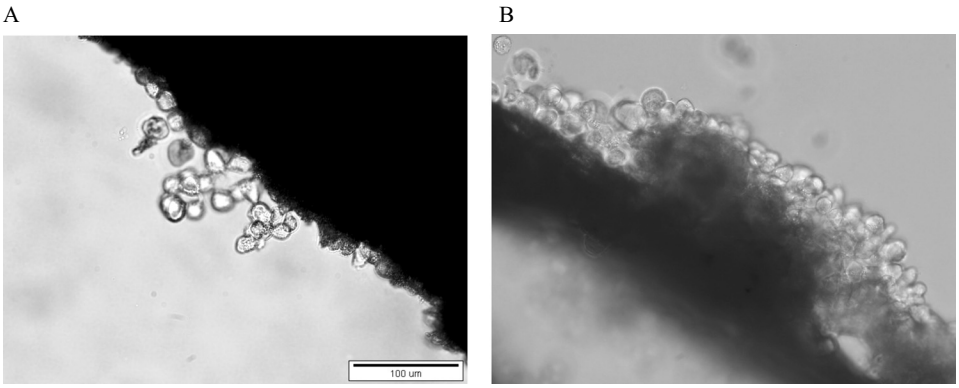

Fig. 4. Photomicrograph from the optical microscope of chondrocytes isolated and seeded onto the polysulfonic membrane. A – after 5 days; B – after 13 days. The images show chondrocytes on the edge of the membrane

In Figure 4 the difference in the number of cells at the edge of the membrane in the 5 and 13 day of cultivation is clearly visible. The cells could be found both on the membrane and the cultivation plate. However, the cells on the membrane grew faster than those on the plate alone. During the consecutive cultivations no essential problems in the growth of chondrocytes on the membrane were found.

In the SEM pictures taken of lyophilized samples from the cell culture it is clearly visible that the chondrocytes develop both on the membrane surface (Fig. 5) as well as in its interior (Fig. 6).

Figure 6 shows the image from half of the membrane thickness. This is proof that the cells penetrate to the interior of the membrane where they freely develop and proliferate. For comparison, in Figs. 5 and 6 on the right side is presented an identical membrane without chondrocytes (at the same magnification).

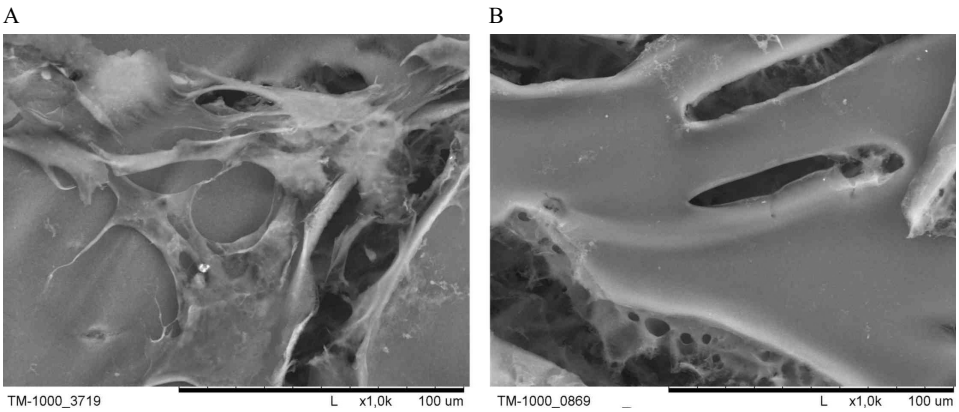

Fig. 5. Photomicrograph A – perforated skin layer with chondrocytes, B – perforated skin layer. Magnifications 1000×

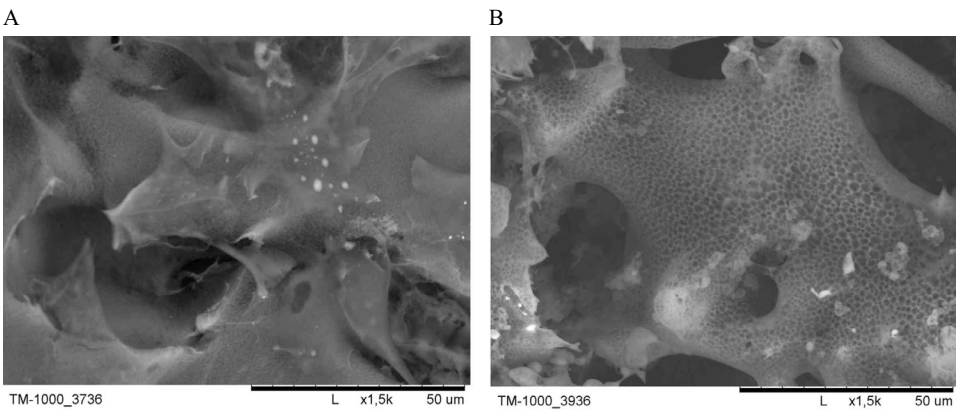

Fig. 6. Photomicrographs: A – membrane interior with chondrocyte cells, B – interior of the membrane alone. Magnifications 1500×

To estimate exactly how in time the mass of chondrocytes on our scaffold increases an elemental analysis was carried out. Samples for the elemental analysis were collected every 7 days starting from the second week of cultivation. After collection the samples were immediately frozen to –82°C, and stored at that temperature. A membrane with the culture frozen without cultivation was used as a reference sample. The protein content was calculated on the basis of the nitrogen content in the samples. The average nitrogen value in proteins of 6.25% was assumed for the calculations. The defrosted samples were dehydrated with 99.8% ethanol and then dried at 105°C. The results of the elemental analysis are presented in Fig. 7. Those are average values of burning four different membranes with chondrocytes. The results show a systematic, considerable increase in the protein mass on the membrane and

inside the membrane. Between the 2 and 6 week of cultivation a double increase in the cell mass and protein mass generated by them was observed. This is a considerable increase as for 4 weeks of cultivation. It indicates that the chondrocytes have good conditions for development.

The method of obtaining of broad-pore membranes is relatively easy. The size of macropores is defined by the size of the cellulose fibers. Since for production of the chromatographic papers such as the one used by us cotton cellulose is used, then it is decisive of the size of pores. Due to this the pores have an ideally repeatable size, and hence the entire process of membrane production is reproducible. The membranes of required size can be obtained. The only limitations are the size of sheets or rolls of the chromatographic paper used.

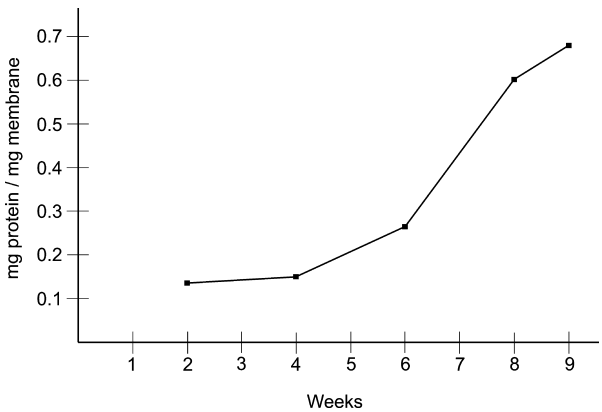

Fig. 7. Results of elemental analysis of samples of chondrocytes cultivated on a broad-pore polyethesulfone scaffold

Moreover, obtaining of the membranes in the sterile form is relatively easy. The membranes can be sterilized by radiation. The use of bioconforming polymers causes that the membrane in the final form will also be bioconforming. The membrane obtained is stable in time. For at least two years it did not change its form and properties.

4. Conclusion

The developed broad-pore membrane is a non-cytotoxic membrane. The method of its production is reproducible. The membrane has sufficient stability. Sterilization is also simple. 3D spatial structure of the unique type inside the membrane allows to achieve considerable cell and protein masses per volume unit. This is especially important in cultivation of the chondrocytes predicted for regeneration implants.

The sufficient mechanical resistance causes that this membrane can be convenient for cell cultivation without fear of its damage.

All these properties of the developed membrane permit to assume that it will be a good and convenient scaffold for cultivation of the chondrocytes. It can also find application in medicine in the regeneration of cartilage in joints.

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