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ENCAPSULATION OF CHONDROCYTES IN HYDROGEL SYSTEMS EFFECT OF CHITOSAN VISCOSITY AND MICROCAPSULE SHAPE

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Alginate – chitosan – alginate multilayer hydrogel encapsulation systems were investigated for encapsulation of chondrocytes. Hydrogel is crosslinked due to ionic interaction between cationic chitosan and anionic alginate, and additionally by calcium ions. Two types of chitosan with molecular weight were investigated. Cells were encapsulated in two shape microcapsules, microbeads with diameter size 300 – 400 and 500 - 600 µm and fibres with diameter 500 - 600 µm. The work provides a detailed examination of the impact of the microencapsulation process on the growth of cells. The viability of chondrocytes can be influenced by the size of produced microcapsules, while the shape of microcapsules has no important significance on cell viability. The applied encapsulation methods do not contain harmful stages and create conducive conditions for cell growth. A possible application area of the developed system is dressing and regeneration of damaged joint cartilage.

Keywords: microencapsulation; ACA microcapsule; chondrocytes

1. INTRODUCTION

Due to the ageing of societies damage to the articular cartilage caused by the wear, overload, misalignment and accidents became a common and also difficult therapeutic problem. Unfortunately, natural cartilage, probably due to a very low amount of blood capillaries, practically does not heal. Despite the development of many techniques, a selection of the appropriate and effective method for damage treatment is still not a trivial matter. Traditional methods of treatment include the loss of full-thickness debridement of adamaged joint, the stimulation of bone marrow cells through abrasion, drilling and microfracture, the transplantation of bone chondromyxoid pulp and chondromyxoid transplantation - bone in the plasticity of a mosaic. The application of these techniques, results in cartilage substitution of a damaged place by the fibrous cartilage which is not the desired type of cartilage. The resulting improvement is usually short-lived. That is why microencapsulated autologus chondrocytes or differentiated stem cells implantation could be an effective clinical procedure for cartilage repair. To perform an effective local tissue regeneration cells should be kept in place and a proper environment for cells should be also assured. To achieve that microencapsulation of cells in a the proper biomaterial is applied. This creates conditions for the reconstruction of the more valuable, specialised tissue, and also reduces the risk of complications. Three-dimensional scaffold of a suitable shape directs the development of tissue and allows for convenient introduction and adhesion of cells to patients' joints. Proper encapsulation protects cells from immunological reactions and mechanical stress, providing diffusion of oxygen, nutrients, and metabolic products (Gombotza and Wee, 1999). There are several challenges in the development of encapsulating membranes that have to be solved.

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The most important problem is reaching an optimum balance between the mechanical strength of the microcapsule and mass transport properties of a membrane. In addition, most of the developed membranes lack appropriate vascularisation, an obstacle to ensuring continuous nutrients and oxygen diffusion to enclosed cells (Fig. 1). The encapsulating material must allow for cell survival and differentiation while maintaining its physio-mechanical properties throughout the required implantation period (Nafea et al., 2011).

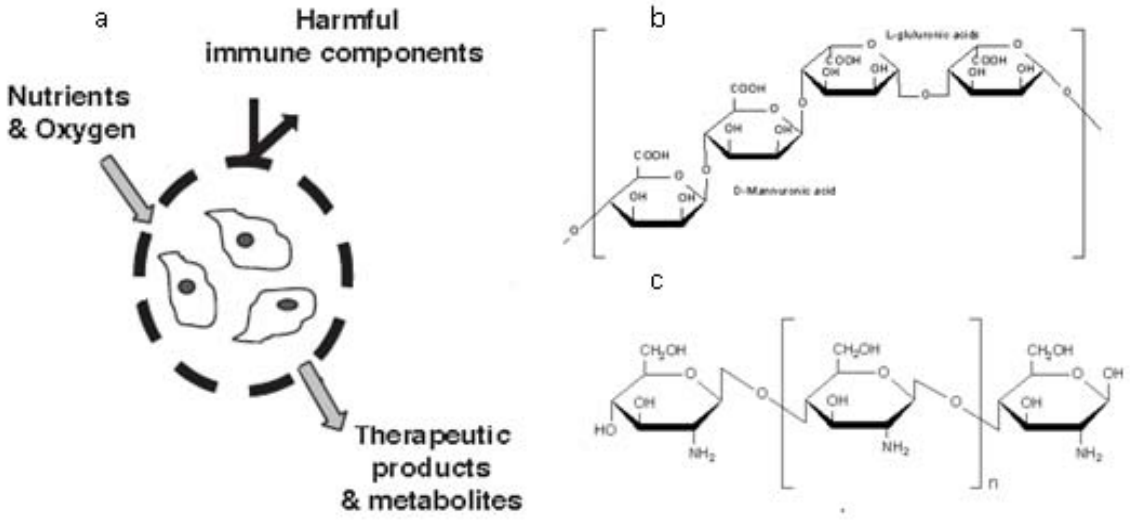

Fig. 1. (a) Idea of a semi-permeable membrane for immunoisolation of encapsulated cells; (b) representative chain portion of alginate. Alginates are linear copolymers consisting of blocks of continuous mannuronate residues, guluronate residues or alternating guluronate/mannuronate residues; (c) representative chemical structure of chitosan. Chitosan is produced commercially by deacetylation of chitin

In the conventional methods microcapsules are prepared by dispersing sodium alginate solution into water solution of cross-linking divalent (Ca^{2+}, Ba^{2+}, Sr^{2+}) or trivalent cations (Al^{3+}, Fe^{3+}) to form a jelly, and then coated by a cationic polyelectrolyte (Gombotza and Wee, 1999). The coating slows down the swelling and degradation of microcapsules, but it may also cause immunological reactions and fibrotic growth (De and Robinson 2003; Gaserod et al., 1999a; 1999b). To reduce these undesirable effects microcapsules are coated one more time by alginate which is more biocompatible. Alginate is a copolymer of L-guluronic acid. It is bioadhesive, biocompatible and provides an inert aqueous environment within the matrix; it polymerises in a mild room temperature, encapsulation process is free of organic solvents, it has a high gel porosity which allows macromolecules diffusion. Moreover, it has the ability to control this porosity with simple coating procedures, as well as process of dissolution and biodegradation of athe system under normal physiological conditions (Puppi et al., 2010). The main advantage is the fact that chondrocytes do not dedifferentiate in alginate and produce high levels of GAG (glicosylaminoglycans) and collagen type II (Chia et al., 2005; Domm et al., 2002; Sittinger et al., 1997; Wang et al 2003). After the first study of Lim and Sun (Lim and Sun, 1980) alginate – poly-lysine- alginate (APA) microcapsules became the most widely used method for cell entrapment. Still, due to the high cost of poly-lysine and its unsuitability for long-term transplantation different polycation methods are worth investigating. The alternative is provided by alginate - chitosan- alginate microcapsules. Chitosan is less immunological, more stable in vivo and has better properties for cryopreservation than lysine (Haque et al., 2005). Chitosan (-1,4-linked N-glucosamine) is a biodegradable cationic aminopolysaccharide derived from naturally occurring acetylated chitin. Chitosan is a homopolymer which has a hydrophilic surface promoting cell adhesion, proliferation and differentiation. Moreover, it is endowed with antibacterial activity and good biocompatibility with acceptable host response (Puppi et al., 2010)].

Here, alginate - chitosan - alagiante microcapsules were studied for their potential use as a scaffolding material for cartilage repair and regeneration. Two types of microcapsules were obtained: micro-beads and micro-fiber. The aim of the presented study was to investigate the cell number and the viability of chicken articular chondrocytes, which were chosen as a model, when cultured in alginate - chitosan - alginate microcapsules. Obtained results were compared with non - encapsulated chondrocytes culture. Because of slow proliferation of chondrocytes a long culture period - 30 days, was chosen. In the paper influence of geometrical diffusing properties of different shape of microcapsules on chondrocytes growth were considered. Micro-beads have a bigger surface interface contact than fibre, which inducted a better diffusing ratio of nutrients and oxygen through microcapsule membrane. Also the influence of chitosan molecular weight on the cultured chondrocytes was evaluated.

2. MATERIALS AND METHODS

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), Nutrient mixture F-12 (Ham F-12), chitosan, collagenase type I, fetal chicken serum (FCS), penicillin G, streptomycin, tryptinase, trypan blue were obtained from Sigma – Aldrich. Two types of chitosan with medium viscosity 200 - 800 cP (1 wt. % in 1% acetic acid, 25°C, Brookfield) (M) and hight viscosity >1000 cPa (H) were evaluated. Alginate made from brown seaweeds reach in guluronic residues was purchased from Fluka, while Ascorbate acid from Carlo ERBA Reagents. Chemicals were have been used as received, without further purification.

2.2. Chondrocytes isolation

Cartilage was harvested from chicken joints, then it was cut into small pieces and subjected to an enzymatic digestion (tryptinase, collagenase) in Ham F-12 followed by digestion in DMEM (supplemented with 50µm/ml gentamicin and 100µm/ml streptomycin) with 0.06% collagenase over night in 37^{o}C, in a humid atmosphere of 5% CO_2 incubator. After digestion the solution was filtered (filter porosity 70 µm), washed and centrifuged in order to isolate chondrocytes. Cells were resuspended in 20ml of distilled and aseptic water, counted and their viability was quantified by 0.4% trypan blue in distilled water.

2.3. Microencapsulation and culture conditions

Preparation of ACA beads was performed in aseptic conditions. The cell suspension was adjusted to 5.0x10^{6} chondrocytes/ml of sterile 1.5 % alginate solution in 0.9% sodium chloride. Beads were formed by extrusion of the solution in the air 4 cm above the 0.1 M calcium chloride solution. High voltage generator connected to the needle was applied to facilitate liquid jet breakup into small droplets, which is the process of electrostatic micro dripping (Ciach, 2007). Different diameters of microbeads were received by using two potential values (4.5 kV and 6.1 kV), at a constant flow rate (25.5 ml/h) and needle size (27-gauge). Fibres where prepared by forcing out the cell solution in alginate through 30- gauge needle at a constant flow rate 350ml/h to 0.1M calcium chloride. The tip of the needle was immersed below the gelling bath surface. The beads and fibres were hardened for 30 min. Afterwards, both were placed for 30 min in 0.5% (w/v) chitosan solution in 0.1 mol/l sodium acetate – acetic buffer, *pH* 4.5. In order to investigate the effects of chitosan molecular weight on cell growth two types of chitosan were exanimate. After that period the microcapsules where rinsed with physiological saline to remove excess chitosan and followed by treatment for 10 min in 0,15% alginate solution to contract

charges on the membrane. Before culturing in full culture media, the microcapsules were rinsed in physiological saline.

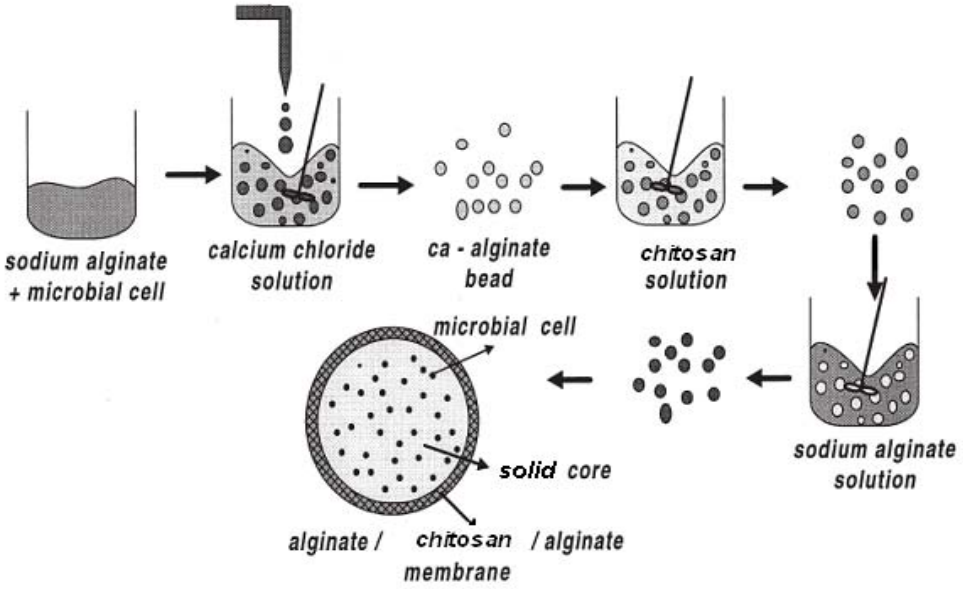

Fig. 2. Schematic of microencapsulation process

Cell cultured medium was composed of 1:1 DMEM : Ham F-12 and 10% FCS supplemented white 25 µg/ml ascorbate acid, 100 µm /ml streptomycin and 50µg/ ml gentamycin and changed two times a week. Chondrocytes were cultured for 30 days in 24-well plates. Cultures were done at 37°C in a humidified atmosphere of 97% air and 5% CO_2. In order to successfully compare the results, a monolayer culture of chondrocytes was carried out in the same condition. To estimate the size and morphology of capsules a sample of 15 microbeads and microfibers was taken from each type and was measured using a microscope. The average diameter was calculated. A monolayer culture of chondrocytes was cared under this same condition and was used as the control. The structure of microencapsules was investigated using a SEM (Phenom). Samples were lyophilized in a freeze dryer. Samples were not sputtered before examination, to enable observation of the monolayer of chitosan and alginate.

2.4. Cell concentration and viability

Every 84h microscopic observations were made (Nikon eclipse 80i). At the same time 0.5g samples of microbead and microfiber were taken. These samples were dissolved in 0.05M sodium citrate in shacked flask for dissolution of alginate followed by adding an acetic acid for dissolution of chitosan. Cell viability was assessed using a trypan blue staining technique. Trypan blue 0.4% was used to distinguish viable cells from non-viable cells. After 2 min of incubation with dye, viable cells appeared round and clear while non-viable were asymmetrical and adsorbed the dye, and their colour turned blue there for appearing blue. Total chondrocytes cell concentration was estimated using a Thoma counting chamber. Cell viability percentage was determined by the following equation. Mean viability and associated standard deviations (n =5) are reported.

$$\frac{number\ of\ alive\ cells}{number\ of\ all\ counted\ cells\ (dead + alive)} \times 100\% \qquad (1)$$

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2.5. Degradation of ACA microcapsules

In order to determined the degradation of different microcapsules with two chitosan viscosity values, weight changes were analysed during the culture time period. Samples of each type of microcapsules and microfibers were prepared and were put in PBS saline with *pH* 7.4. Every 84 h 3 samples of 0.5g (*Ws*) were taken. Microcapsule dry weight was determined by drying the microcapsules at 70°C for 14h (*Wd*). The percentage of water content was calculated using the formula. Mean water content and associated standard deviations (n =3) are reported.

$$\frac{Ws - Wd}{Ws} \times 100\% \tag{2}$$

3. RESULTS AND DISCUSSION

Based on the literature data and results from previous experiments we decided that the most adequate material for microencapsulation is alginate with high guluronic residues and concentration 1.5%(w/v) (Constantinidis et al., 1999; Moresi and Bruno, 2007; Stabler et al., 1999). This concentration makes microcapsules mechanical strong enough and provides good exchange of substrates and metabolic products. For this reasons the lower concentration 0.1 M of calcium chloride was selected. When the alginate solution was pumped at a rate 25.5 ml/h through a 27 guage needle with a voltage of 4.5 kV, microbeads with a diameter of about 540 ± 40 µm were formed , in a later paragraphs were named as microcapsules from the range of 500 - 600 µm. Smaller microbaeds with diameter between 300 – 400 µm were formed at a higher voltage 6.1 kV and had about 360 ± 50 µm . Only one diameter (560 ± 40 µm) of microfibers was chosen for a culture of chondrocytes, because early research shows that the micro-fibres are easily breakable. Microscopic observation of microcapsules and micro-fibres showed a highly porous structure (Fig. 3a, b). SEM showed that scaffold fibre generally was covered by alginate in one direction. An application of subsequent layers smoothed the surface of microbeads. Taken images also showed, that the imposition of alginate on last layer was not complete, as white spots were visible on the chitosan layer in SEM images. After preparation and cultured for 24h, ACA microcapsules were observed every 84h with optical microscopy. Fig. 3c, d present representative microscope images of the prepared and cultured microcapsules after 24h. It can be seen that ACA microbeads were spherical and intact with smooth surface. The dark line represents the outer layer of microcapsules.

There were no differences in microcapsule properties for 28 days of culture. Regardless of molecular weight of chitosan, water content of microcapsules was found to show similar patterns. Water content initially decreased over the first 5 days and then slowly returned to the initial values (Fig. 4). These results are similar to those obtained by Wang and al. The initially decreases were probably caused by molecular rearrangement, while swelling was the result of osmosis caused by unbound carboxyl groups which can cause disintegration of microcapsules. During culturing manipulation and microscopic observations no changes in the appearance of microcapsules or their tendency to disintegrate were noticed.The microfibers confirmed their tendency to tear, which can result in mechanical release of cells to growth medium while the activities associated with culturing.

Cell appearance and shape did not change a lot during culture time, they were slightly reduced in size and slightly shrank. During microscopic observation a the secretion of extracellular matrix was observed – which means that cells started to produce cartilage components (Fig. 5). Chondrocytes were encapsulated at 5.0×10^6 cells/ml, after the immobilisation, the concentration of cells was reduced to about 4.5×10^6cell/ml (Table 1).

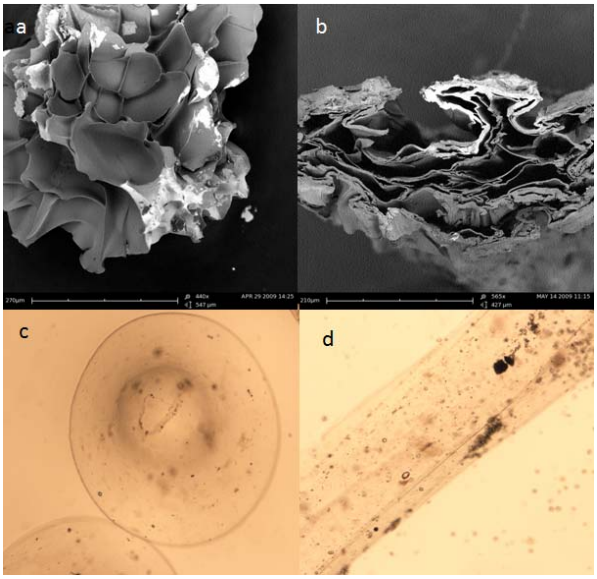

Fig. 3. Scanning electron microscopic images of (a) microcapsules, (b) micro-fibres. Imagination of chondrocytes culture of (c) microcapsule, (d) micro-fibres after 24h

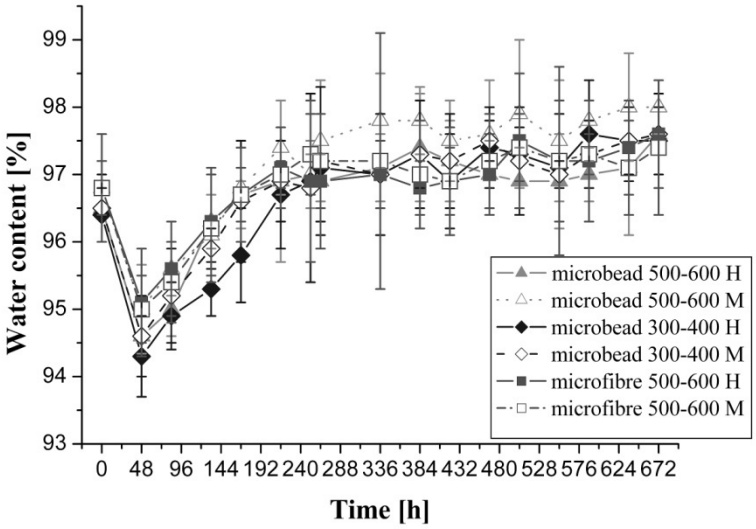

Fig. 4. Percentage of water content in microbead and microfiber coated by high (H) and medium (M) viscosity chitosan (*MV*±*SD*, *n* =3)

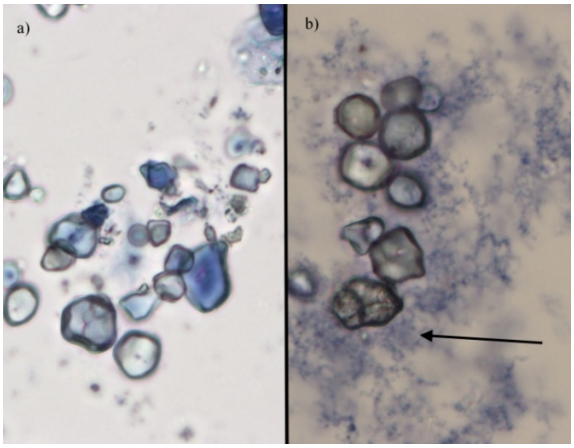

Fig. 5. Microscopic observation of chondrocytes a) after microencapsulation, b) after 24 days of culture, extracellular matrix is shown by the arrow (*MV*±*SD*, *n* =3)

Table 1. Changes in chondrocyte concentrations before and after microencapsulation in microcapsules and fibres coated by high (H) and medium (M) viscosity chitosan (*MV*±*SD*, *n* =5)

	Before [10^6 cell/ml]	After [10^6 cell/ml]
microcapsules 300-400µm M	5	4.6 ± 0.09
microcapsules 300-400µm M	5	4.6 ± 0.05
microcapsules 500-600µm H	5	4.5 ± 0.06
microcapsules 500 -600 µm H	5	4.45 ± 0.05
fibres H	5	4.48 ± 0.08
fibres M	5	4.5± 0.06

A reduction in cell concentration was mainly caused by cell release during encapsulation and destruction of them, while they were pumped through a drain and needle. The concentration of the cells subtly varied (Fig. 6).

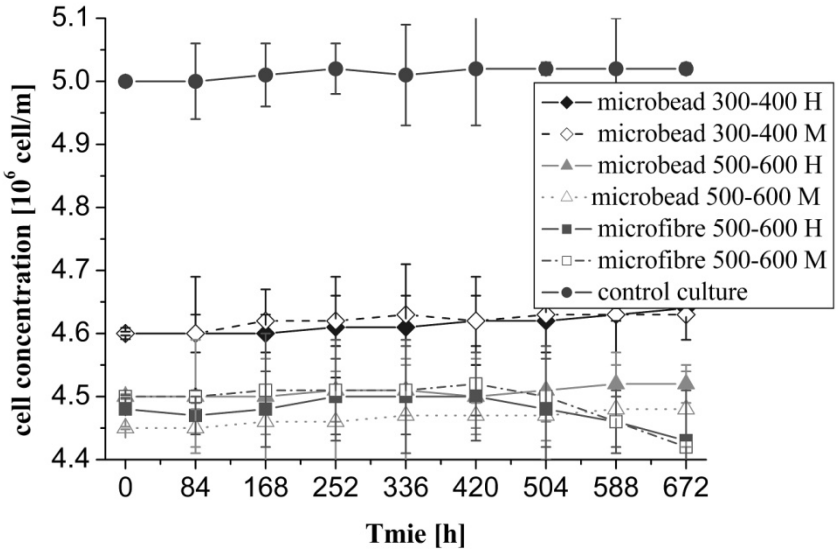

Fig. 6. Changes in cell concentration during culture in microcapsules and fibres coated by high (H) and medium (M) viscosity chitosan (*MV*±*SD*, *n* =5)

The reduction in cell concentration is autonomous of the type of chitosan applied, but it is dependent on the geometry of microcapsules. There is a clear decrease of cell concentration in the microfibers which is the result of the tearing the fibres and cell release to culture medium. For the microcapsules of diameter 300 - 400 µm a high increase in concentration from $4.6x10^6$ to $4.64x10^6$ cell/ml was observed. For the capsules of diameter 500 - 600 µm concentration increased only from $4.5x10^6$ to $4.52x10^6$ cell/ml. The lower concentration of cell in diameter range 500 - 600 µm was effected by beater substrate and metabolism diffusion. For microcapsules 300 - 400 µm cell concentration increased similarly to the control culture ($5.0x10^6$ - $5.05x10^6$cell/ml). The increase of concentration and shrinking of cell suggest that cells were dividing.

A comparison of cell viability before and after immobilisation showed that there are no harmful stages in the encapsulation procedure. The encapsulation stage, which was critical for the process and the most harmful for cells, was the stage of microcapsules coating with chitosan which solution *pH* was 4.5. The previous immobilisation of cells in alginate protected them from destructive environment. Cell

viability was reduced while conducting culture and it depended on the type of chitosan (Fig. 7). In the control culture viability was decreasing constantly while there were two stages in immobilised cultures. There was no difference in the viability of chondrocytes encapsulated in micro-fibre and micro–beads. Previous studies on model organism Saccharomyces cerevisiae showed an influence of microcapsule shape on cell viability and proliferation. This result could be explained by a difference in the diffusion of substrates inside and metabolites outside microcapsules. Chondocytes are slowly proliferating cells so microcapsule shape has no influence on the examined values. The shape of microcapsules can have an effect in the case of a higher density of cells or in the situation when cells start forming aggregates.

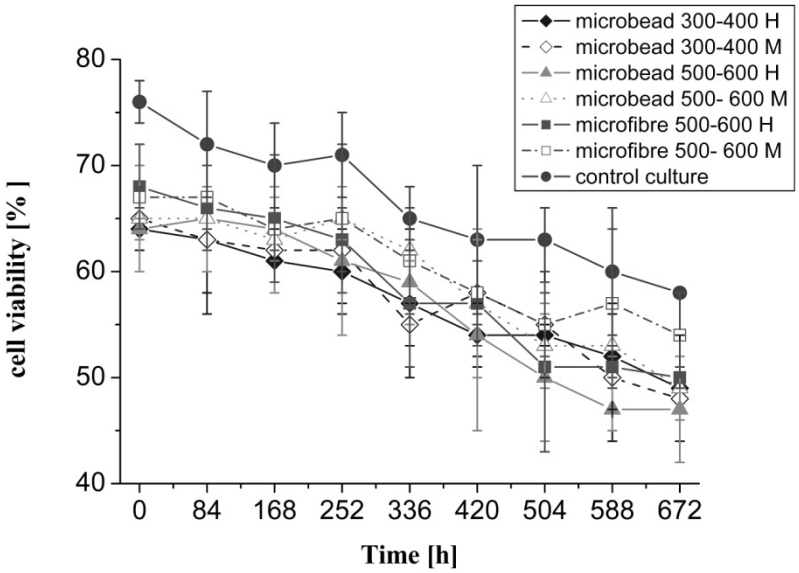

Fig. 7. Change in chondrocyte viability in microcapsules and fibres coated by high (H) and medium (M) viscosity chitosan (*MV*±*SD*, *n* =5)

In the first 252 h cell viability was stable, but after that it was starting to decrease. At the end of the culture cell viability in microcapsules and microfibers, which were both coated by medium viscosity chitosan was 5% higher than that of those coated by high viscosity chitosan (Table 2).

Table 2. Chondrocyte viability at the end of the culture (*MV*±*SD*, *n* =5)

	high viscosity chitosan	medium viscosity chitosan
microcapsules 300-400 µm	57% ± 8	62% ± 3
microcapsules 500-600 µm	56% ± 4	60% ±5
Fibres	60% ±2	64% ±6

This fact can be explained by differences in chitosan layer thickness (Gaserod et al.,1999b). High chitosan viscosity makes the layer on a alginate thicker, and because of that metabolite diffusion is lower. Finally, cell viability was reduced by about 20% compared to control culture. This small difference shows that immobilisation of chondrocytes in ACA system did not limit their growth.

4. CONCLUSIONS

A method of alginate – chitosan – alginate multilayer hydrogel encapsulation system for chondrocytes was investigated. The presented method allows to encapsulate chondrocytes in microbeads and fibres, retaining their viability and promoting extracellular matrix production. Alginate or chitosan were

prepared from a solution of neutral *pH* which offered an additional advantage of allowing protein or drug to be uniformly incorporated in its matrix structure with no or minimal denaturisation. Microcapsule coatings make microcapsule mechanically resistant and did not loseng the integrity of their structure within 30 day culture. There is no significant influence of microcapsule shape on slowly proliferating cells. Thanks to all these advantages ACA system appears to be a promising method for the reconstruction of damaged cartilage. To confirm the use of ACA system for cartilage reconstruction further studies on the release of glucosaminoglycans and collagen in long term cultures followed by animal experiments are needed.

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