Electrostatic Microencapsulation of Living Cells

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Microencapsulation of different biologically active material for diverse applications have received increasing interest over the last 20 years. Microencapsulation of living cells seems to be a very promising and prospective technology, especially useful in biotechnology and medical applications. One of the most convenient and precise method for this purpose is an electrostatic technique. Electrostatic droplet generation could be performed using single- or multi-nozzle devices, significantly improving efficiency of the process. The usage of an impulse voltage generator allows to manufacture spherical and uniform microbeads with sizes from 0.2 to 3.0 mm of very narrow size distribution. Proposed two-liquid droplet electrostatic formation technique provides preparation of core/shell microbeads, where all cells are immobilized deeply inside a matrix and surrounded with cell-free polysaccharide layer. Such a solutiuon prevents from cell protrusion out of the capsule. Applied electrostatic field is safe for encapsulated living cells and does not cause any cell dysfunction.

K e y w o r d s: microencapsulation of cells, alginate beads, electrostatic droplet generator

1. Introduction

Microencapsulation is the procedure by which biologically active material is enclosed within microspherical, semipermeable containers of a diameter between 0.2 to 3.0 mm [1–3].

Materials for microencapsulation could be: synthetic and natural medicines, enzymes, hormones, other peptides, genes, cells or their aggregates, pieces of tissues, bacterium (especially genetically modified), fungus, other microorganisms or even whole seeds [4–9]. Nowadays microencapsulation has been widely applied in many modern industries: from the environment protection, through agriculture, food, cosmetics and pharmaceutical industry to medicine [10–15].

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There are two basic areas of medical applications of the microencapsulated cells: the first one, as a very sophisticated drug delivery systems with continuous and controlled release of therapeutic agents. In this case the microcapsules could be implanted into body directly at the target site and could be especially effective for treatment of cancer and neurological diseases (e.g. Alzheimer's, Parkinson's and Huntington's disease) [16–21]. The second field of medical applications of the microencapsulated cells is a replacement or support of organ functions. In this case the microcapsules could be implanted into patient's body, for example as a hybrid pancreas [22–24] or could work as a part of extracorporeal devices, for instance as a bioreactor with hepatic cells for liver support [25–28]. The microcapsule, which contains living cells is also well-known as, so called "an artificial cell". Its principle of action is schematically shown in Fig. 1.
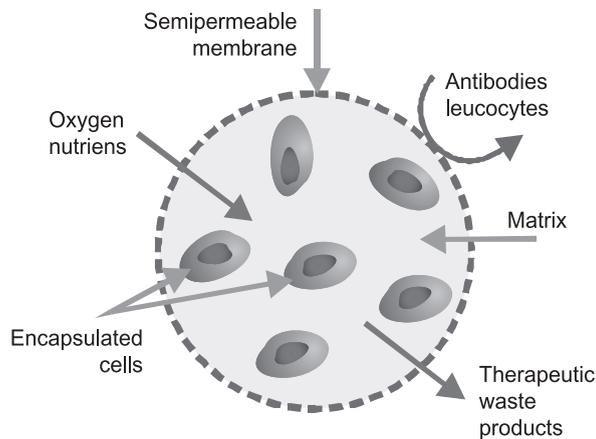

Fig. 1. The principle of action of an artificial cell

Living cells are immobilized inside a spherical matrix, which is surrounded by a semipermeable membrane. The main task of the matrix is a creation of the best possible living conditions for the encapsulated cells. The main task of the membrane is immunoisolation of the encapsulated cells from the destruction by the immunological system of the host. So the membrane have to protect the capsule interior from the penetration of antibodies and leukocytes [2, 3, 8]. The microcapsule have to ensure free oxygen and nutrients transport from the environment to the capsule interior and a reverse transport of metabolites (waste products) and therapeutic substances, produced by the encapsulated cells. The matrix and the membrane have to be highly biocompatible.

All these requirements seriously restrict number of chemical substances which can be used for the microencapsulation of living cells. The best materials for the preparation of the matrix are natural polysaccharides. Among them the most commonly used is sodium alginate, which has an ability to form flexible hydrogels in

the presence of divalent cations at room temperature [29, 30]. For the preparation of the membranes certain synthetic poly-aminoacids like: poly-L-lysine, poly-ornithine and poly-methylene-co-guanidine have been selected [31–35].

2. Preparation of Microcapsules

The microcapsules are usually produced by two-step procedure: in the first step the cells are immobilized within hydrogel microbeads and then the microbeads are covered with semipermeable membrane to obtain a microcapsule [22, 36]. The membrane is formed by the electrostatic interaction between a negatively-charged matrix and positively-charged membrane materials. The second step consists of immersion of the microbeads in the membrane-forming polymer solutions. The membrane thickness and permeability can be modified by selection of the proper polymer concentration and optimization of the immersion time. As the biocompatibility of the poly-aminoacids is not satisfactory, the last covering is usually performed with an alginate solution. This procedure could reduce the alginate gel cut-off from about 230 kDa up to 70–60 kDa.

3. Preparation of Microbeads

All methods of the microbead preparations base on simple dropping of an alginate water solution into a gellifying water bath containing calcium cations – Ca^{2+} (Fig. 2) [37, 38]. Unfortunately, microbeads obtained by this method are too big to ensure a good mass transfer properties. Their minimal diameter is about 1 mm.
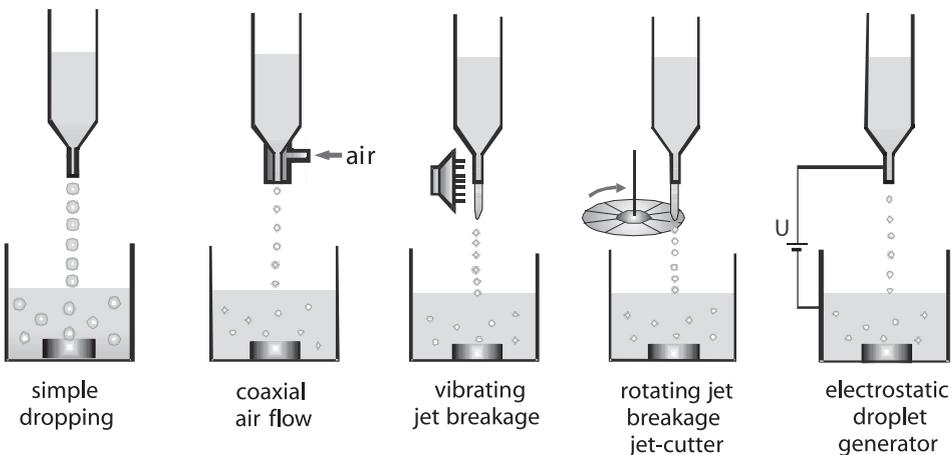

Fig. 2. Methods of the microbeads' production

Therefore different technical modifications have been applied to reduce the size of droplets. The most popular are: coaxial air flow, where an air jet, surrounding a nozzle increases the force available to break a nascent drop; vibrating jet breakage, where a liquid jet is being broken up into droplets due to a nozzle vibrations. Jet-cutter technology, where a jet is cut by a series of rotating knifes and the electrostatic droplet generation. In the last method the reduction of droplet size is caused by applying a high static electric potential between the nozzle and the gellifying bath.

4. Electrostatic Microbead Generation

4.1. Theoretical Principle

The principle of electrostatic droplet generation has been elaborated by prof. Poncelet [39, 40]. "Surface tension γ is the main force maintaining the pendant droplet on the needle. By applying the electric potential, the migration of charged molecules to the surface of the droplet is promoted. These molecules will repulse each other, causing the surface tension to decrease. According to Lippman's theory of the electrocapillary the equilibrium surface tension γ of a charged liquid surface decreases with increasing electrical potential U, as it is expressed by the equation (1)

$$d\gamma = -\sigma \, dU \tag{1}$$

where σ is the electric surface charge. Assuming the pending droplet as a sphere, submitted to the electric potential U, the mean surface density of the electric charge σ my be expressed by the equation (2) where q_0 is a surface charge, d is a droplet diameter, ε_0 is the air dielectric constant, d_c is a inner diameter of a needle.

$$\sigma \approx \frac{q_0}{\pi d^2} = 2\varepsilon_0 \frac{U}{d_c} \, . \tag{2}$$

Combining both equations and integrating over the electric potential U gives an expression of the surface tension in function of the electrical potential U (equation 3).

$$\gamma = \gamma_0 \left(1 - \frac{\varepsilon_0 U^2}{d_c \gamma_0} \right) = \gamma_0 \left(1 - \frac{U^2}{U_c^2} \right). \tag{3}$$

where γ_0 is the surface tension of a liquid at U equal to zero and U_c is the critical electrostatic potential as defined below (equation 4).

$$U_c \approx \sqrt{\frac{d_c \gamma_0}{\varepsilon_0}} \, . \tag{4}$$

Near this value the surface tension becomes negligible and the size of the droplets becomes quasi independent of the applied electric potential" [40].

Increasing of the electrical potential U causes changes in the jet shape, which becomes more longer and narrow and, in consequence the diameter of obtained droplets decreases.

4.2. Electrostatic Microbead Preparation Using an Impulse Voltage Generator

This method has been used in our Institute for over eight years. The experimental set-up (Fig. 3) consists of: a tank for polymer solution, equipped with a steal nozzle with a steel plate to increase the uniformity of the static electric field between the nozzle tip and the gellifying bath. The nozzle is connected with the positive end of the high voltage generator, supplied with a frequency modulator, whereas the gellifying bath is grounded. This set-up enable us to apply pulsed electric voltage of regulated parameters: the electric voltage – U in the range from 0 to 25 kV, the frequency of electrical impulses – f from 1 to 100 Hz and the duration time of the impulses, – τ from 1 to 9 ms. During the process, cell suspension in an alginate sol is forced, under regulated gas pressure P, to flow through the nozzle. Droplets are formed at the nozzle tip and fall into the gellifying bath below. Other regulated parameters are: the gas pressure P, the diameter of the nozzle and the distance between the nozzle tip and the gellifying bath – L.
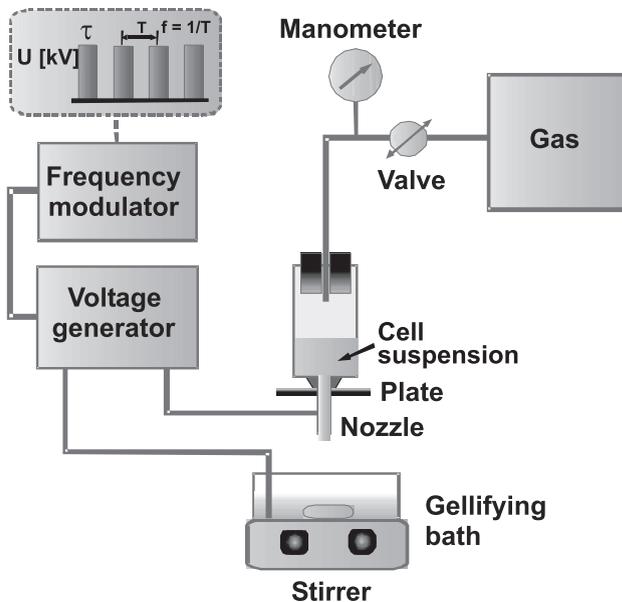

Fig. 3. A schematic view of the experimental set-up

The electrical parameters have a crucial influence on the microbead diameter and size distribution [41]. The graph on the left (Fig. 4a) shows changes of the microbead average diameter D in function of increasing voltage value U.
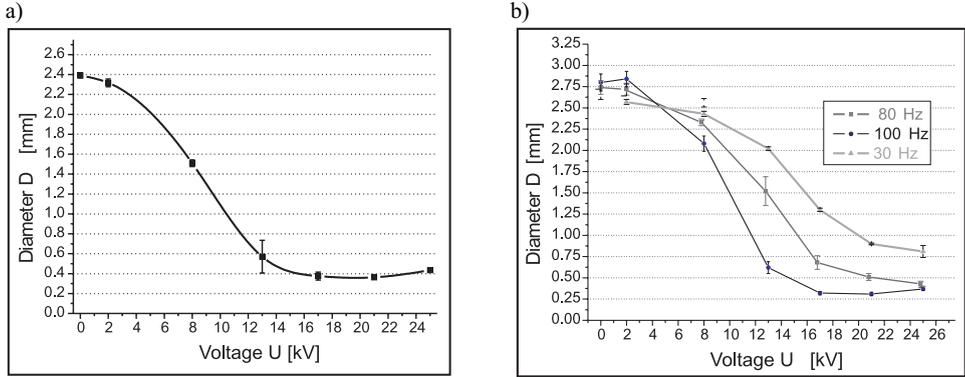
a)

b)

Fig. 4. The influence of electric voltage U (a) and impulse frequency f (b) on the average microbead diameter D

The diameter gradually decreases from about 2.4 mm to 0.4 mm with increasing electric potential from 0 up to 15 kV. Further increase in electric potential U has no impact on the microbead diameter D. Figure 4b shows the same dependence, but experiments have been performed applying three different frequency values. The upper curve has been obtained at the frequency of 30 Hz and the lowest one at the frequency of 100 Hz. So, the diameter of the microbeads, obtained at the same electric potential value U can be smaller or bigger depending on frequency values.One of the most serious problem of all dropping technologies is the size uniformity of the microcapsules. Very often two microcapsule fractions of drastically different sizes (even more than one order of magnitude) have been obtained (Fig. 5a).
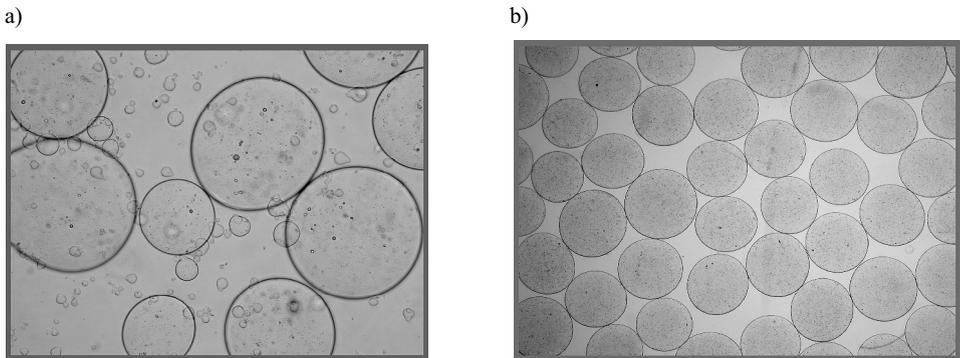
a)

b)

Fig. 5. Alginate microcapsules: with (a) and without (b) the satellite fraction of the very small microbeads

As it is shown in Fig. 6, applying the proper values of impulse duration time τ enables one to avoid this problem.
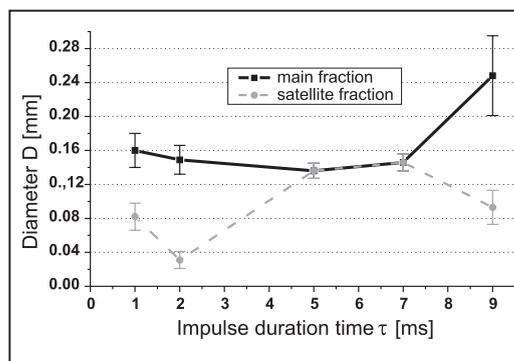

Fig. 6. Absence and presence of the satellite fraction in the bead samples in dependence on impulse duration time τ

The main fraction of the bigger microcapsules is denoted as the solid curve and the satellite fraction as the dashed one. In this experiment, at τ between 5 to 7 ms the satellite fraction disappeared and only the main fraction of the bigger microbeads was obtained.

The electrostatic droplet formation enables one to manufacture spherical beads of desirable diameter between 0.15 to 3.0 mm of a very narrow size distribution – variation coefficient of diameter $VC = (SD/D)100\%$ is usually below 10%, without satellite fraction, where SD is the standard deviation of bead diameter.

4.3. Comparison of Different Bead Production Technologies

Technical limitations of different bead production technologies were investigated within the European project COST action 840 *"Bioencapsulation. Innovations and Technologies"* four years ago [42]. The aim of the study was to obtain microbeads with a resulting diameter of 800 ± 100 µm using diverse technologies by gelation of different alginate solutions, to compare technical limitations of all the applied methods. Seven laboratories from different European countries took part in this study (Table 1). Five different microbead production technologies like: a coaxial air-flow dropping, an electrostatic dropping working in a continuous and a pulse mode, a vibration and a Jet-Cutter method were applied.

The alginate solutions contained from 0.5 to 4.0 percentage of alginate and their viscosity varied from 24 to about 11 000 mPas (Table 2).

Only three of the applied methods were able to obtain desirable mirobeads from all of the five solutions: the coaxial air-flow; the electrostatic method working in a pulse mode and the Jet-Cutter working in soft-landing mode.

Table 1. The bead production technologies used in different labs

Lab	Bead production
Slovak Academy of Sciences, Bratislava	Coaxial air-flow dropping
University of Belgrade, Belgrade	Electrostatic dropping (continuous mode)
Polish Academy of Sciences, Warsawa	Electrostatic dropping (impulse mode)
Institute Meurice, Brussels	Vibration, JetCutter (normal mode)
University of Perugia, Perugia	Vibration
ENITIIA, Nantes	Vibration
FAL and geniaLab GmbH, Braunschweig	JetCutter (soft-landing mode)

Table 2. Dynamic viscosity η of solutions with different sodium alginate contents

Na-alginate content %	η in mPa·s
0.5	24
1.0	92
2.0	667
3.0	2 008
4.0	10 560

Alginate solutions of concentration above 2% were too viscous for the vibration technique and the electrostatic working in continuous mode. The coaxial-air flow method seems to be also improper for very viscous solution, because the shape of obtained microbeads is not spherical. On the other hand the jet-cuter technique working in normal mode is useless for solutions of very low viscosity.

This problem has been successfully solved by the application of the Jet-Cutter method working in a soft-landing mode.

In the soft-landing mode the nozzle is placed about 2 m below the collecting bath. The droplets are pushes high up from the nozzle and then fall into the collecting bath. This way the velocity of droplets is reduced and obtaining of microbeads from low viscous solutions becomes possible.

4.4. Multi-nozzle Device

The electrostatic droplet generation could also be performed using multi-nozzle device. Designed by us device presented in figure 7a consists of a small plastic tank with a steel plate at the bottom. There are six nozzles 13 mm long of inner diameter 0.3 and outer diameter 0.5 mm. The nozzles are evenly distributed around the outer rim of the plate.

The photo on the right (Fig. 7b) shows the nozzle during the microbead production process. The cone-shaped solution jets, which repulse each other are visible. This

way clumping of microdrops is eliminated. The microbead diameter depends on the voltage value in the same way like for a single-nozzle device and is also dependent on the applied frequency (Fig. 8).
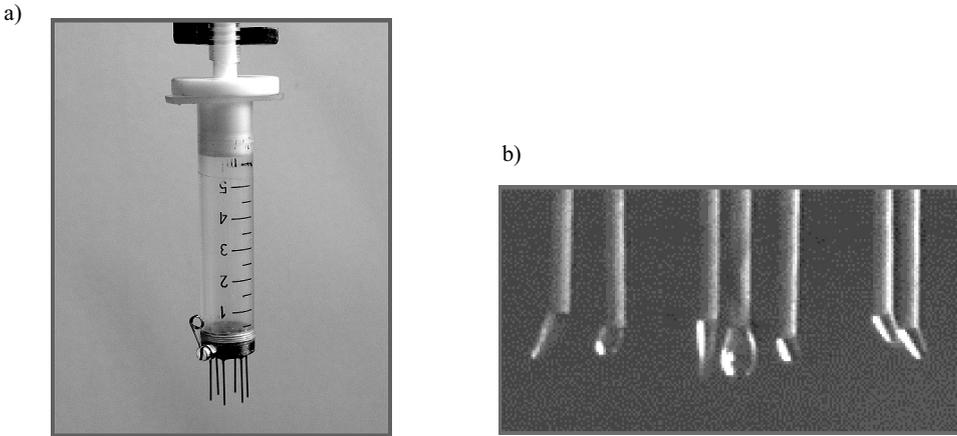
a)

b)

Fig. 7. The multi-nozzle device: (a) the view of the whole nozzle, (b) the nozzle during the microbead production process
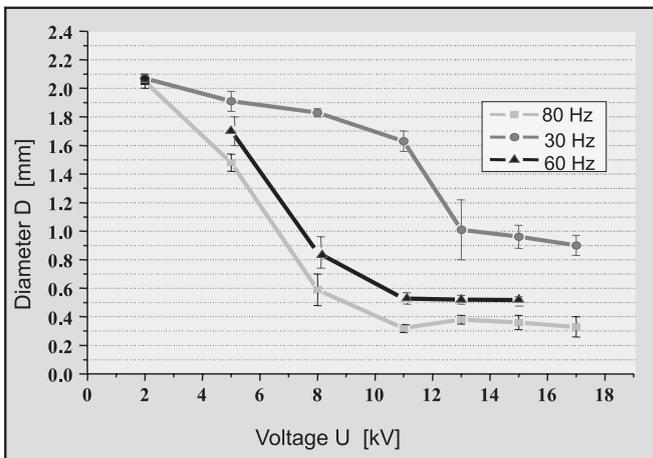
Fig. 8. Dependence of the bead size on the voltage at different frequency values

This device enabled us to obtain spherical microbeads of diameter from 0.32 to more then 2 mm with low size distribution – the variation coefficient was below 10%. Efficiency of this device is much higher than efficiency of the single-nozzle apparatus and could reach 700 ml/h in comparison with about 60 ml/h for the single-nozzle device.

4.5. Electrostatic Microencapsulation of Hepatocytes

The electrostatic microbead generation can be successfully applied for microencapsulation of living cells. Last year we have used it for a sterile encapsulation of hepatocytes.

The aim of the study was to select the proper values of process parameters to encapsulate hepatocytes in the alginate matrixes for a long-term cultivation in a bioreactor. To select an optimal process condition the values of the different process parameters were changed to obtain the microbeads of diameter about 0.80 mm and a narrow size distribution.

The experimental data are summarized in the Table 3.

Table 3. The influence of parameter values on the microbead size and distribution

No of Ex.	U kV	f Hz	τ ms	L mm	P atm.	D mm	VC %
1	11	50	5	20	0.10	0.45	3
2	11	30	5	20	0.10	0.44	8
3	11	30	5	30	0.15	0.51	2
4	8	50	5	30	0.15	0.83	4
5	8	60	5	30	0.25	0.81	1
6	**8**	**50**	**5**	**25**	**0.20**	**0.81**	**1**
7	8	50	5	20	0.20	0.73	7
8	8	50	8	20	0.20	0.59	14
9	8	50	5	23	0.20	0.59	13
10	8	50	5	25	0.23	0.70	3

The values of parameters applied in the sixth experiment were chosen for further investigation. The obtained microbeads are shown in photo a) (Fig. 9). This sample is free of satellite fraction contrary to the sample shown in photo b), where a very small satellite fraction is present.
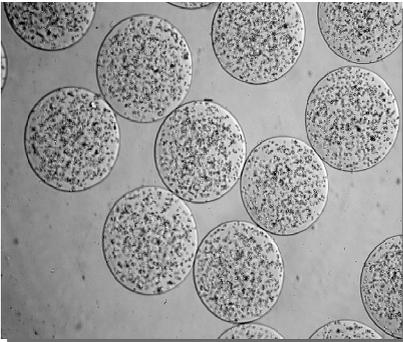
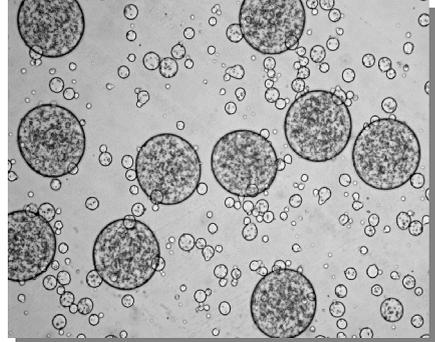(a)   (b)

Fig. 9. The effect of proper and improper encapsulation parameters value: absence (a) and presence (b) of the satellite fraction

The suspensions of two hepatocyte cell lines HepG2 and C3A of concentration 4×10^6 cells/ml in 1.5% sodium alginate in 0.9% NaCl were used. The microbeads were gellified in 1.67% calcium chloride in saline solution with addition of 0.26% of HEPES. The assessment of cell viability was performed using the fluorescent staining method. Four sterile encapsulation of both types of hepatocyte cells were made. The cell viability was very high (about 90%) for both types of cells, proving the safety of the electrostatic method. After 2 weeks of cultivation in DMEM medium high multiplication of hepatocytes was observed (Fig. 10).
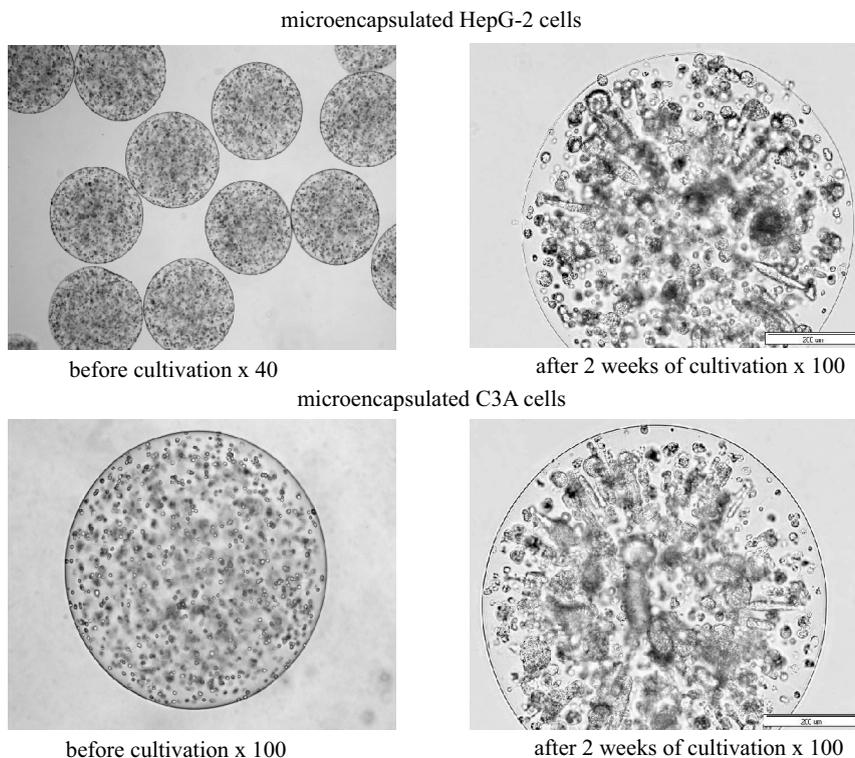
microencapsulated HepG-2 cells

before cultivation x 40

after 2 weeks of cultivation x 100

microencapsulated C3A cells

before cultivation x 100

after 2 weeks of cultivation x 100

Fig. 10. The hepatic cells encapsulated in the alginate microbeads before and after the cultivation

The electrostatic technique using an impulse voltage droplet generator seems to be a simple and very useful method of hepatocyte encapsulation for bioreactor applications [43].

4.6. Two-liquid Droplet Electrostatic Formation Technique for Microencapsulation of Living Cells

Cell protrusion out of the capsules is a significant problem in the therapeutic transplantation of the microencapsulated cells. Incomplete coating of biomaterial by matrix

causes problems with preparation of well adhered membrane and is very often the main reason for direct activation the immunological system of the host and the rejection of transplanted biohybrid organs. Proposed two-liquid droplet electrostatic formation technique (Fig. 11) could solve this problem effectively [44].
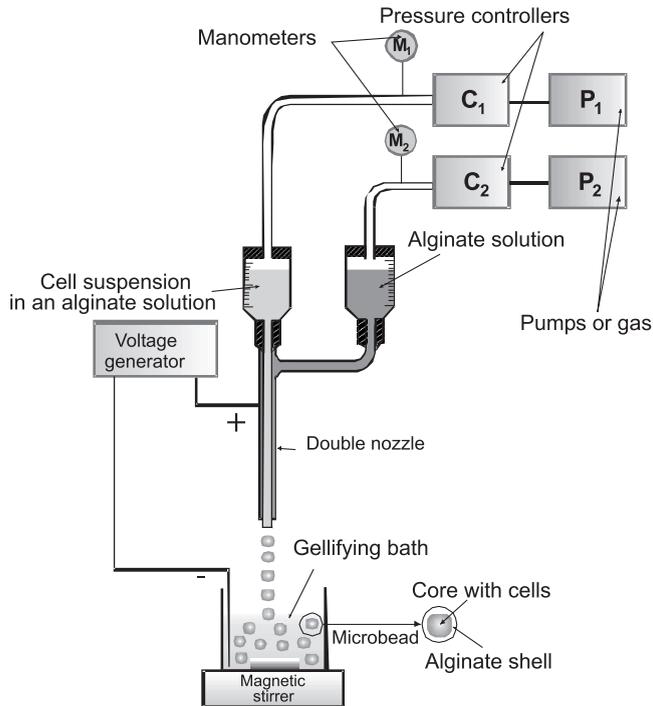

Fig. 11. The experimental set-up

In this method two liquids – internal (which is a cell suspension) and external (alginate solution) are simultaneously pumped through the double nozzle under controlled fluid flow conditions forming "double droplets". After the gellification, a double microbead, comprised of the core (with cells) and the coating layer (cells-free alginate gel) is formed (Fig. 12).

All the cells are enclosed within microsphere of diameter 0.36 mm, which is surrounded by the alginate layer about 90 microns thick. For comparison, the capsule made by the single-nozzle method is shown on the right (Fig. 12) where the protrusion of some cells is clearly visible, especially at the bottom. To test if this method is safe for the encapsulated cells, yeast cells have been encapsulated and then cultivated for 48 h. A view of the microbeads just after encapsulation, 24 hours after incubations and 48 hours is shown in Fig. 13.
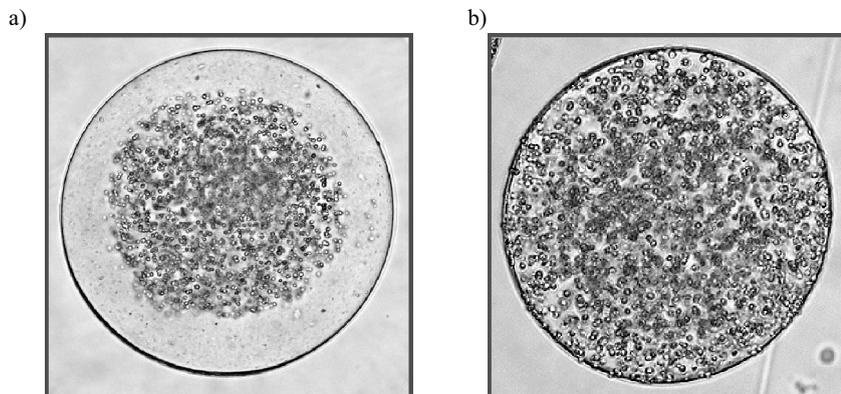
a)   b)

Fig. 12. The microbeads obtained by the double-nozzle (a) and by the single-nozzle (b) method
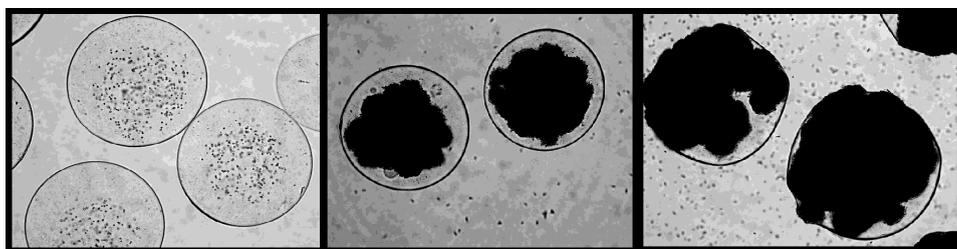

Fig. 13. The encapsulated yeast cells: just after manufacturing (on the left) after 24 h of incubation (in the middle), and after 48 h of incubation (on the right)

After 24 hours of incubation the number of the yeast cells increased rapidly as a result of their multiplication. After 48 h the cells filled almost whole microbeads volume and started to stick out of the gel matrix. The diameter of the microbeads manufactured by two-liquid droplet electrostatic formation technique can vary in the wide range of dimensions and strongly depends on the selected voltage value. As it is shown in the graph (Fig. 14) the diameter of the microbead – (the upper curve) varies from 2.40 to 0.59 mm. The diameter of the microbead core – with the cells (the middle curve) changes from 1.50 to 0.40 mm. The thickness of cell-free alginate layer (the lowest curve) decreases from 442 to 104 μm and can be easily modified by the selection of proper flow rate values of both liquids.

4.7. Conclusion

The electrostatic droplet formation is one of the most precise method, which enables one to manufacture spherical and uniform bead fractions with sizes from 3.0 mm down to 0.2 mm. The usage of the impulse voltage generator enables one to control and regulate process parameters like: the electric voltage U, the impulse frequency
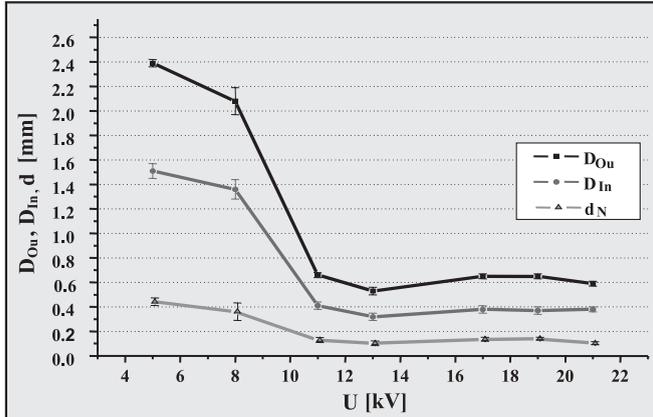
Fig. 14. The influence of voltage value U on the microbead average outer diameter D_{ou}, inner diameter D_{in} and the thickness of cell-free layer d. $f = 50$ Hz; $\tau = 5$ ms; cells concentration 1.6×10^7 cells/ml

f and the impulse duration time τ, which plays a crucial role in the droplet formation. The proper choice of these parameter values allows to avoid the formation of the undesirable satellite fraction of the very small microbeads. Low efficiency of electrostatic process can be significantly improved by multiplication of the nozzles, without any loss of the microbeads quality. The electrostatic technique using an impulse voltage droplet generator seems to be a simple and very useful method of living cells encapsulation.

The two-liquid droplet electrostatic formation technique provides preparation of core/shell microbeads, where all the cells are immobilized deeply inside the matrix and no cell protrusion out of the capsule occurs. Applied electric field is safe for the encapsulated living cells and does not cause any cell dysfunctions.

It is easy to use, fully controlled and reproducible technique.

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