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SCIENTIFIC PAPER

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MASS TRANSFER RANKING OF POLYLYSINE, POLY-ORNITHINE AND POLY-METHYLENE-CO--GUANIDINE MICROCAPSULE MEMBRANES USING A SINGLE LOW MOLECULAR MASS MARKER

On the long way to clinical transplantable hybrid systems, comprising of cells, acting as immuno-protected bioreactors microencapsulated in a polymeric matrix and delivering desired factors (proteins, hormones, enzymes etc.) to the patient's body, an important step is the optimization of the microcapsule. This topic includes the selection of a proper coating membrane which could fulfil, first of all, the mass transfer as well as biocompatibility, stability and durability requirements. Three different membranes from polymerised aminoacids, formed around exactly identical alginate gel cores, were considered, concerning their mass transport properties, as potential candidates in this task. The results of the evaluation of the mass ingress and mass transfer coefficient h for the selected low molecular mass marker, vitamin B_{12}, in poly–L–lysine (HPLL), poly–L–ornithine (HPLO) and poly–methylene–co–guanidine hydrochloride (HPMCG) membrane alginate microcapsules demonstrate the advantage of using the mass transfer approach to a preliminary screening of various microcapsule formulations. Applying a single marker and evaluating mass transfer coefficients can help to quickly rank the investigated membranes and microcapsules according to their permeability. It has been demonstrated that HPLL, HPLO and HPMCG microcapsules differ from each other by a factor of two concerning the rate of low molecular mass marker transport. Interesting differences in mass transfer through the membrane in both directions in–out was also found, which could possibly be related to the membrane asymmetry.

Investigation of the mass transport properties in a microcapsule–marker system *in vitro* is of vital importance for the evaluation of the microencapsulation quality in any final application. To give two examples from a broad range of biotechnological and medical applications, let us consider an enzyme trapped inside a gel capsule, serving as a biocatalyst, and cells supplying a hormone, microencapsulated in a gel and immuno–protected by a semipermeable, poly–aminoacid based membrane layer. A good example for the first kind of application is the microencapsulated enzyme inulase II, entrapped inside a calcium alginate matrix which should permit the ingress of substrate, inulin of MW more than 5 kDa, and the egress of product, di–fructofuranose– dianhydride (DFA III – a possible substitute of sucrose in a functional food) of MW around 300 Da [1]. In the second kind of application, transplanted micro- encapsulated parathyroid cells should be able to release parathyroid hormone of MW more than 9 kDa to the surrounding tissue and should be protected from direct interaction with immune defence factors of the host, immunoglobulines G of MW above 160 kDa. This objective could be accomplished by entrapment of the parathyroid cells in a sodium alginate liquidized gel matrix, coated by poly–lysine membrane and an additional membrane made of alginate gel (APA capsules, e.g. in [2]). The mass transport properties of diverse microcapsule formulations (as illustrated above) are often investigated by determination of the MMCO (Molecular Mass Cut–Off) curve [3], using a variety of marker substances of different molecular masses, sizes and chemical characteristics. The application of many markers with different molecular masses from several hundred Daltons up to a few hundred thousand Daltons (polypeptides, proteins, polysaccharides, polyethylene-glycols, etc.) or using a broad molecular mass standard such as dextran probes (as recommended in [4]) can give an idea about the general permeability of the selected microcapsule formulation and its exclusion limit.

Hydrogels, including calcium alginate gel as a typical example, are generally characterized by a large content (often more than 90%) of free and bound water inside the gel matrix [5]. This fact and their chemical structure influence the enhanced porosity and permeability of these materials. Estimations of the "average pore size" of alginate capsules yield values of several tens of nanometers, while their molecular cut–off values are usually well above 200 kDa, as investigated with dextran markers [6]. A membrane coating is usually applied to improve their biocompatibility after transplantation, to prevent the ingress of large molecules including immunoglobulins and, sometimes, to modulate their mechanical properties [7]. Thus, the coating of an alginate capsule with a poly–lysine membrane can reduce the cut–off values to approximately 150 kDa for dextrans [8], depending on the actual formulation. Clearly, to estimate the cut–off value of a selected microcapsule formulation, large molecular mass markers are needed. For a typical microcapsule, a low molecular mass marker will enter the capsule interior freely and an equidistribution of the marker mass between the solution and capsule phases can be reasonably expected (on the condition that the marker diffusion process will be not influenced by any interactions between charged, polar, apolar and

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hydrophobic groups of the marker molecules, microcapsule material and solution phase as highlighted generally in [5]). Thus, for different microcapsule formulations which are sufficiently permeable, no differences between microcapsules would be observed by measuring the equilibrium mass ingress values for a neutral low molecular mass marker. This will be further illustrated in this work for the selected marker, vitamin B_{12} and three different microcapsule types: poly–L–lysine (HPLL), poly–L–ornithine (HPLO) and poly–methylene–co–guanidine (HPMCG) membrane coated low viscosity guluronic alginate microcapsules.

Concerning the detection of differences between various microcapsules, the investigation of diffusion kinetics and determination of the mass transfer coefficient h can be advantageous. In this case the mass transfer rate i.e. the rate of marker penetration into (ingress) and out of (egress) the microcapsule phase is evaluated not the equilibrium distribution of the marker mass between the solution and microcapsule phases. It is shown below that valuable information (not present in the mass ingress equilibrium data) about the microcapsule properties can be obtained, which enables one to differentiate and classify different microcapsule formulations.

MATERIALS AND METHODS

Materials

The microcapsule samples were obtained according to the following procedure: first, alginate beads from 1.8% low viscosity high guluronic (LVG) Pronova alginate were formed using an electrostatic droplet generator (8 kV, 5.9 ml/h) and gellified in calcium chloride solution. Next, coating membranes were formed by washing in:

– 0.05% poly–L–Lysine for 5 minutes (HPLL microcapsules),

– 0.05% poly–L–ornithine for 5 minutes (HPLO microcapsules),

– 0.5% (v/v) poly–methylene–co–guanidine for 1 minute (HPMCG microcapsules).

After the poly–aminoacid coating step, all the microcapsules were coated with an additional alginate layer by washing in 0.1% Pronova LVG alginate for 5 minutes. The obtained microcapsules were preserved in mannitol solutions with antibiotic/antimitotic agents, carefully washed and incubated for 24 h in 0.9% NaCl before the diffusion experiments. Microscopic images of the microcapsule samples are shown in Figure 1.

A solution of vitamin B_{12} (0.015%) in 0.9% NaCl was used in the kinetic (ingress and egress) experiments for the investigation of marker diffusion in HPLL, HPLO and HPMCG microcapsules.

METHODS

Microcapsule sizes

The microcapsule size distributions and average diameters were determined using an optical microscope, equipped with a movable table (accuracy 10 μm). The microcapsule sizes in 0.9% NaCl were used in further calculations. Photographic documentation of the investigated samples was obtained using a reversed light optical microscope (Olympus CKX 41), equipped with a photo–camera.

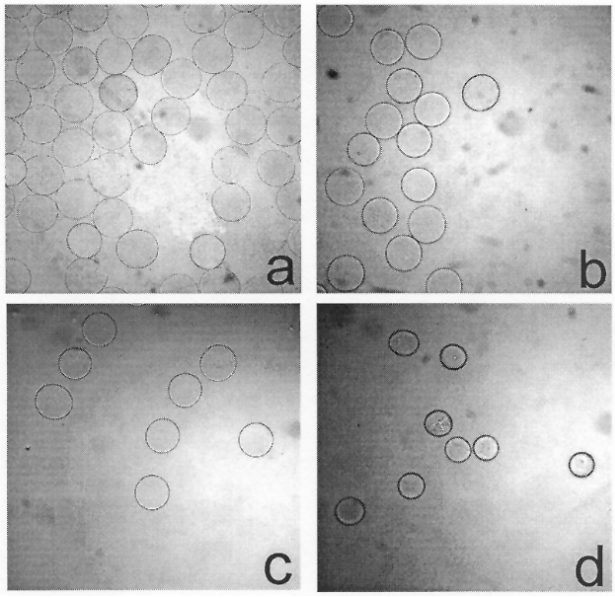

Figure 1. Microscopic images in transmitted light of: a) LVG alginate beads before coating, b) HPLL, c) HPLO, d) HPMCG microcapsules in PBS solution

Kinetic experiments

The diffusion of vitamin B_{12} into the microcapsules (ingress experiment) and, consecutively, from the microcapsules into 0.9% NaCl (egress experiment), was investigated by shaking equal volumes of either solution or saline and microcapsules (2 ml) at 37°C for one and a half hours. Changes in the vitamin B_{12} concentration C in time were measured spectrophotometrically after collecting samples of 50 μl volume at 0, 2, 5, 10, 20, 30, 60 and 90 min during the experiment. The vitamin B_{12} concentrations were determined by measuring the absorbance at 360 nm on a Varian Cary–Bio 50 spectrophotometer. The results were presented as kinetic curves of marker ingress and egress in terms of the relative dimensionless concentrations C/C_0 (where C_0 denotes the initial marker concentration) of the investigated marker as a function of time.

Marker ingress and egress

Mass ingress/egress was defined in an equilibrium state as the mass diffused into/out of the capsules, expressed as the percent of the initial mass in the solution and calculated as $(1–C_{eq}/C_0) \times 100\%$ (ingress) and $(C_{eq_C}/C_0) \times 100\%$ (egress) where C_{eq} and C_{eq_C} denote the respective equilibrium concentrations in the solution in ingress and egress.

For vitamin B_{12} ingress and egress were determined experimentally from kinetic curves

calculating the equilibrium concentration C_{eq} as the average value of the dimensionless concentrations C/C_0 at 30, 60 and 90 minutes of the experiment. The relative experimental error of the ingress determination was about 5%, as assessed in previous investigations [9].

Determination of the mass transfer coefficient h

The mass transfer coefficient values (h) were determined from a general expression [10], describing the transfer of a substance between two phases:

$$V \frac{dC}{dt} = -hS\,(C - C_S) \quad \text{ingress} \tag{1}$$

$$V_S \frac{dC_S}{dt} = -hS\,(C_S - C) \quad \text{egress} \tag{2}$$

where: C – is the concentration of the marker in solution outside the microcapsules, C_S – the concentration of the marker inside the microcapsules close to the interphase boundary, V – the extracapsullar volume of the solution, V_S – the intracapsullar volume of the solution, S – the total external surface of microcapsules being the interphase boundary, h – the mass transfer coefficient.

Special simple solutions of Equations (1) and (2) exist when marker transport has a diffusion character and the marker penetrates freely into the microcapsule volume. This can be checked by the inspection of the ingress and egress kinetic curves (equal distribution of the mass between the solution and microcapsule phases).

Marker ingress

The assumption of free access of the marker to the microcapsule interior (and equality of the intracapsullar and extracapsullar volumes under the conditions of the experiment) yields a simplified solution (3) of the equation (1) [9]:

$$C/C_0 = C_{eq} / C_0 + (1 - C_{eq} / C_0) \exp(-12\,ht/D) \tag{3}$$

where: C_0 –is the initial concentration of the marker, C_{eq} – the equilibrium concentration in the solution outside the microcapsules and D is the microcapsule diameter.

Marker egress

With the same assumptions as above, Equation (2) can be solved, yielding a simplified solution, given by Equation (4):

$$C / C_0 = (C_{eq_C} / C_0)(1 - \exp(-12\,ht/D)) \tag{4}$$

where: C_0 –is the initial concentration of the marker in the ingress experiment, C_{eq_C} – the equilibrium concentration in the solution outside the microcapsules in egress.

As the microcapsule size distribution and the average microcapsule diameter D can be determined by any relevant method, fitting of the theoretical curves given by Equations (3) and (4) to the experimental C/C_o points, determined in both types of experiment, enables one to deteermine the value of the mass transfer coefficient h. Determination of the h value was performed by the computer program Origin 7, using a criterion of minimalization of the sum of the squares of the deviations of the experimental points from the theoretical curves (3) and (4).

RESULTS

The investigated microcapsules in the HPLL, HPLO and HPMCG samples were spherical and uniform, with slightly different sizes in the three samples (Fig.1). The results of the determination of the average diameter D along with its standard deviation SD and size variation coefficient VC (in parentheses, ratio of the SD and the average D expressed in percent) are presented in Table 1.

Table 1. Average diameters of the microcapsules along with the SD, coefficient of variation (in parentheses) and swelling of the microcapsules

	Diameter in mannitol [mm]	Diameter in saline [mm]	Swelling $\Delta V/V_0$ [%]
HPLL	0.55 ± 0.02 (4%)	0.60 ± 0.03 (5%)	30
HPLO	0.65 ± 0.03 (5%)	0.68 ± 0.06 (8%)	14
HPMCG	0.53 ± 0.02 (4%)	0.75 ± 0.04 (6%)	83

All the investigated samples presented a very similar pattern of vitamin B_{12} diffusion in both directions (Figure 2). A slight transient disturbance of the concentration course in time was observed in the first twenty minutes of the ingress process (especially for HPLL and HPMCG samples) and did not occur during the egress process. For HPLL, HPLO and HPMCG microcapsules the final equilibrium marker concentration reached 54, 50 and 55% of the initial value C_0 in ingress and 24, 23, and 24% of the initial value of C_0 in egress. As approximately half of the initial marker mass enters the microcapsules and, consecutively, a half of that amount leaves the microcapsules, this proves the validity of the assumption about free, diffusion marker access. Moreover, the results prove that the investigated microcapsules are practically indistinguishable from the point of view of equilibrium marker ingress and egress.

The differences in vitamin B_{12} transport in HPLL, HPLO and HPMCG microcapsules, especially in the beginning of the process, become already evident during the fitting of the theoretical solutions of mass transport Equations (1) and (2) to the experimental points, illustrated in Figure 3. Transport of the marker in both directions is hindered in HPMCG microcapsules relative to the other two samples. This is confirmed by evaluation of the mass transfer coefficients h in both directions, presented in Figure 5. The mass transfer coefficients for HPMCG microcapsules are significantly lower than the respective values for the HPLL and HPLO microcapsules. For the purpose of comparison, the previous results of the mass transfer determinations [9] for poly–methylene–co– –guanidine coated mixed

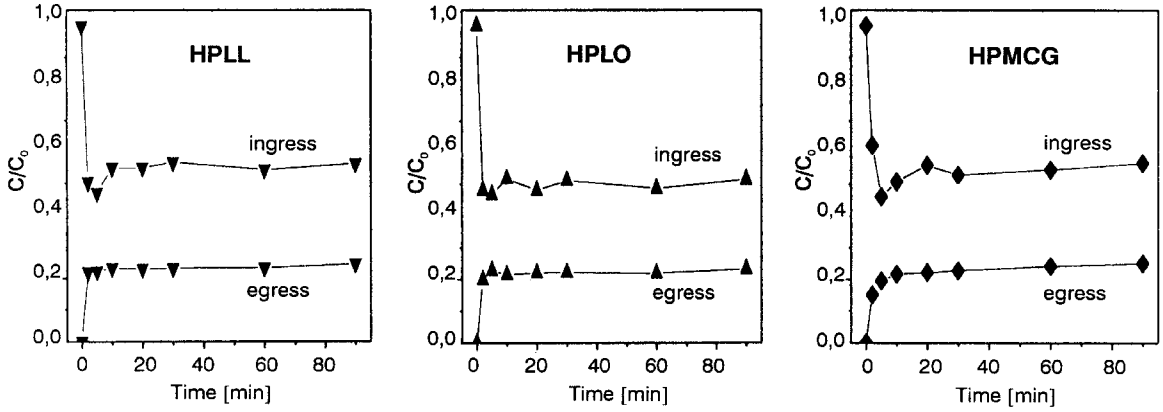

Figure 2. Experimental results of vitamin B_{12} diffusion measurements (kinetic curves) in ingress and egress for HPLL microcapsules (left), HPLO microcapsules (middle) and HPMCG microcapsules (right).

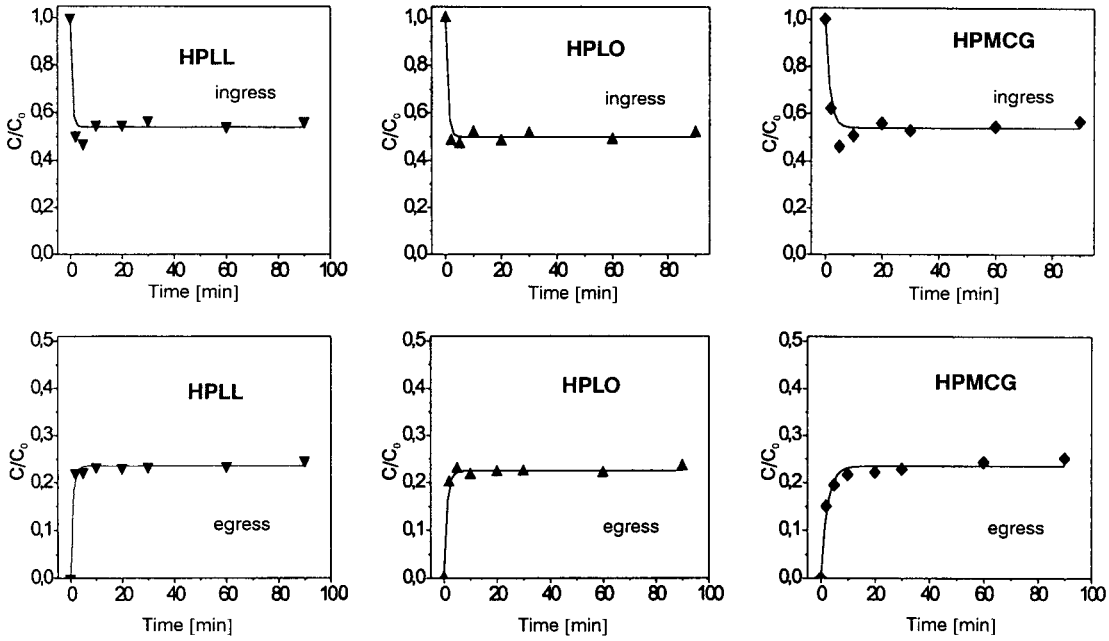

Figure 3. Fitting of experimental points by theoretical solutions of mass transfer equation for ingress (Equation (3) in the upper row) and for egress (Equation (4) in the bottom row) diffusion. From left to right HPLL, HPLO and HPMCG microcapsules, respectively.

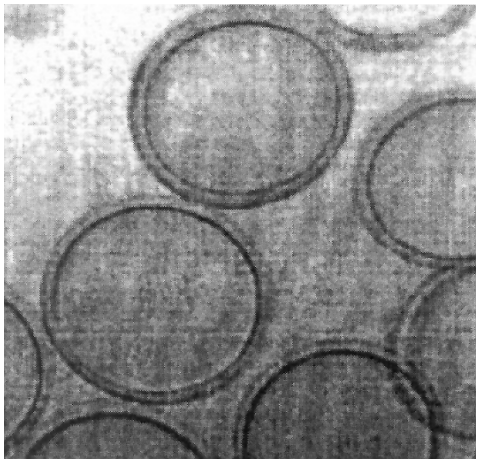

Figure 4. Microscopic image in transmitted light of ACPMG microcapsules in NaCl solution

alginate and cellulose sulphate core microcapsules ACPMG (average diameter D=0.93 mm, average membrane thickness 0.059 mm, sample shown in Figure 4) and for medium viscosity high mannuronic Sigma alginate beads, denoted as Alginate, (average diameter D=0.75 mm) are shown on the same diagram (Figure 5).

To eliminate the possible dependence of the mass transport properties on microcapsule size [4,9] and to compare the investigated samples in a more objective way, the ratio of the determined coefficient h and microcapsule diameter D was calculated as the standardized index of mass transport properties h/D for this low molecular mass marker (Figure 6). This presentation of the microcapsule transport properties for vitamin B_{12} (where the transfer rate is normalized to a unit of the microcapsule diameter) confirms that HPLL and HPLO microcapsules have similar properties,

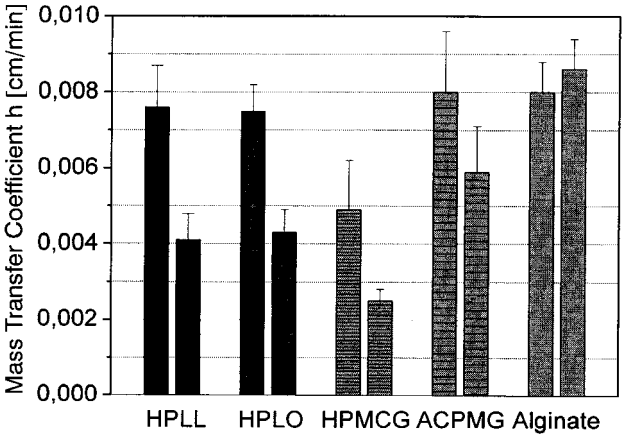

Figure 5. Mass transfer coefficients h, expressed in cm/min, for ingress (left bars) and egress (right bars) diffusion. From left to right HPLL, HPLO and HPMCG microcapsules in comparison with another type of poly-methylene-guanidine microcapsules (ACPMG) and uncoated alginate beads (Alginate).

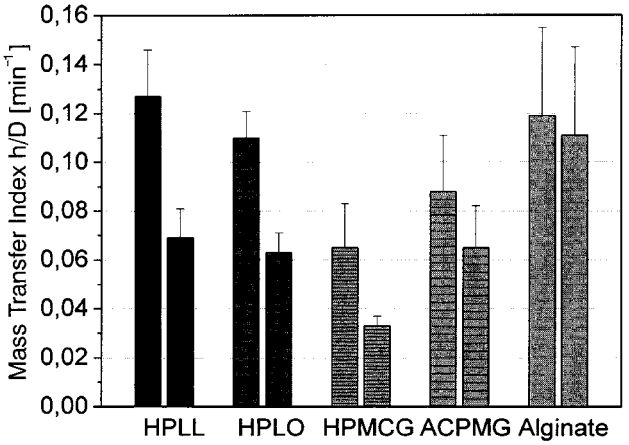

Figure 6. Mass transfer indexes h/D, expressed in min^{-1}, for ingress (left bars) and egress (right bars) diffusion. From left to right HPLL, HPLO and HPMCG microcapsules in comparison with another type of poly-methylene-guanidine microcapsules (ACPMG) and uncoated alginate beads (Alginate).

comparable to typical uncoated alginate beads. The poly–methylene–co–guanidine membrane considerably hinders marker transport in HPMCG microcapsules, which is also evident by comparison with ACPMG microcapsule formulation.

DISCUSSION

The main topic of the paper is the evaluation of the mass transport properties of microcapsules coated with three different poly–aminoacid membranes. They are formed during the processes of the poly–electrolyte interphacial precipitation of poly–cationic (poly–lysine, poly–ornithine and poly–methylene–co–guanidine) polymers onto a poly–anionic alginate polymer network and subsequent precipitation of anionic alginate polymer. A direct conclusion from the inspection of the experimental results (Figure 5 and Figure 6) is that poly–lysine and poly–ornithine membranes are similar and offer much less resistance to vitamin B_{12} transfer than the poly–methylene–co guanidine membrane. Both membranes are fairly permeable and, therefore, HPLL and HPLO microcapsules are similar to uncoated alginate beads in the transport of low molecular mass marker (in the ingress process). It is worthwhile to point out that the observed differences between the microcapsules are revealed only in the mass transfer approach (determination of the mass transfer coefficient h); in the equilibrium mass ingress/egress approach no significant differences were found between the investigated capsule types. This seems to be an advantage of using the mass transfer approach and the analysis of the kinetic curves of marker diffusion. Concerning the differences between HPLL, HPLO and HPMCG microcapsules, observed in the mass transfer investigations, they are most probably related to the different membrane structures (and some extent to the membrane thickness) which makes the difference between the investigated capsules. It is also evident from modern structural investigations that the application of various polymers for membrane formation results in different membrane structures. Recently such different structures were observed e.g. by the inspection of the membrane formation process in poly–lysine–alginate and poly–vinylamine–alginate systems using the analytical centrifuge technique [11].

Another interesting aspect of the mass transport investigations is the observed difference in the ingress and egress mass transfer coefficient values (Figure 5 and Figure 6). For all the investigated microcapsules the ingress process is faster than egress with the $h_{INGRESS}/h_{EGRESS}$ ratio in the range between 1.75 and 2. The same phenomenon was observed for another formulation of the poly–methylene–co–guanidine membrane in ACPMG microcapsules, but to a lesser extent with the $h_{INGRESS}/h_{EGRESS}$ ratio equal to 1.36. On the other hand, the mass transfer rates in both directions are practically equal in alginate beads with no coating membrane ($h_{INGRESS}/h_{EGRESS}$ = 0.93). Recalling that the equality of the marker effective diffusion coefficients D_{eff} in ingress and egress is often assumed to be a proof of the purely diffusional character of the marker transport process [11], this could also concern the mass transfer coefficients. Therefore, the inequality of both mass transfer coefficients for all poly–aminoacid microcapsules would suggest that during ingress diffusion is accompanied by another process (marker adsorption), whereas diffusion predominates during egress. This could also explain the difference in the shape of the ingress and egress kinetic curves (Fig. 2), where a transient disturbance occurs in the first twenty minutes ("tooth" shape) in the ingress process, whereas the egress curves are rather smooth. Interestingly enough, such a "tooth" effect was not observed in the ACPMG kinetic curves (experimental data to [9]). In conclusion it seems that the mass transfer coefficients for ingress in poly–aminoacid microcapsules were overestimated due to phenomena interfering with vitamin B_{12} diffusion in the beginning of the ingress process. Nevertheless, the comparison of the course of mass ingress/egress processes in HPMCG and ACPMG microcapsules shows that the effects of membrane

asymmetry could also contribute to the observed inequality of the mass transfer coefficients in both directions of mass transport. The question of evaluation of membrane asymmetry in mass transport investigations of microcapsules remains to be approached. Inspection of the results for HPMCG and ACPMG microcapsules in relation to their structures illustrates the complexity of the factors which determine the mass transport properties of the microcapsules. Not only the membrane structure and thickness influence the mass transfer rates, but also the microcapsule matrix (gel core) including the chemical structure of the alginates and the presence of other polymers inside the microcapsule core [12].

CONCLUSION

Vitamin B_{12} freely permeates all the investigated microcapsule membranes during a predominately diffusion process. A higher mass transfer rate for this marker was observed for poly–lysine and poly–ornithine based alginate microcapsule formulations than for the poly–methylene–co–guanidine one.

Comparison of the mass transfer rates, represented by the standardized mass transfer coefficients h (i.e. mass transfer index h/D), enables one to obtain valuable information about differences in the microcapsule mass transport properties relative to the results of the mass ingress experiments.

ACKNOWLEDGEMENTS

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IZVOD

KOEFICEJENT PRENOSA MASE, ALGINATNE ČESTICE, POLY–L–LYSINE, POLY–L–ORNITHINE, POLY–METHYLENE–CO–GUANIDINE, IMOBILIZACIJA ĆELIJA

(Naučni rad)

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Rezultati određivanja ukupnog koeficijenta prenosa mase (h) za odabrani molekulski marker (male molekulske mase, vitamin B12) kroz alginatne– poly–L–lysine (HPLL), poly–L–ornithine (HPLO) i poly–methylene–co–guanidine hydrochloride (HPMCG) mikrokapsule pokazala su na osnovu preliminarnih eksperimenata opravdanost korišćenja proračuna za predviđanje daljih istraživanja na osnovu proračuna ukupnog koeficijenta prenosa mase kroz eksperimentalne membrane. Na osnovu eksperimenalnih podataka (traseri male molske mase) verifikovana je metoda proračuna ukupnog koeficijenta prenosa mase kroz alginat– HPLL, HPLO i HPMCG. Takođe je pokazano da nije ista difuzija iz membrana i u membranu što je, verovatno, posledica različite strukture unutrašnje i spoljašnje površine posmatranih membrana.

Ključne reči: Ukupni koeficejent prenosa mase • Alginatne mikrokapsule • Poly–L–Lysine • Poly–L–Ornithine • Poly–Methylene–Co––Guanidine • Imobilizacija ćelija •
Key words: Overall mass transfer coefficient • Alginate microcapsules • Poly–L–lysine • Poly–L–ornithine • Poly–methylene–co––guanidine • Cell encapsulation •