Capsule Permeability via Polymer and Protein Ingress/Egress

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ABSTRACT: Static incubation tests, where microcapsules and beads are contacted with polymer and protein solutions, have been developed for the characterization of permselective materials applied for bioartificial organs and drug delivery. A combination of polymer ingress, detected by size-exclusion chromatography, and protein ingress/egress, assessed by gel electrophoresis, provides information regarding the diffusion kinetics, molar mass cutoff (MMCO) and permeability. This represents an improvement over existing permeability measurements that are based on the diffusion of a single type of solute. Specifically, the permeability of capsules based on alginate, cellulose sulfate, polymethylene-*co*-guanidine were characterized as a function of membrane thickness. Solid alginate beads were also evaluated. The MMCO of these capsules was estimated to be between 80 and 90 kDa using polymers, and between 116–150 kDa with proteins. Apparently, the globular shape of the proteins (radius of gyration (R_g) of 4.2–4.6 nm) facilitates their passage through the membrane, comparatively to the polysaccharide coil conformation (R_g of 6.5–8.3 nm). An increase of the capsule membrane thickness reduced these values. The MMCO of the beads, which do not have a membrane limiting their permselective properties, was higher, between 110 and 200 kDa with dextrans, and between 150 and 220 kDa with proteins. Therefore, although the permeability estimated with biologically relevant molecules is generally higher due to their lower radius of gyration, both the MMCO of synthetic and natural water-soluble polymers correlate well, and can be used as *in vitro* metrics for the immune protection ability of microcapsules and microbeads. This article shows, to the authors' knowledge, the first reported concordance between permeability measures based on model natural and biological macromolecules. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 75: 1165–1175, 2000

Key words: bioartificial organs; electrophoresis; encapsulation; permeability; gel permeation chromatography

INTRODUCTION

Encapsulation has been developed as a tool for the immunoisolation of cells to be transplanted into the human organism. The therapeutic potential of encapsulated cells is promising for treating patients who suffer from tissue loss, neurodegenerative disorders, diabetes, liver failure, and other diseases caused by specific vital cellular dysfunctions. To be efficient for biomedical applications, capsules must be able to not only protect the cells from attack by the host immune system but also maintain viable cell functions by allow-

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ing the passage of oxygen and nutrients such as, hormones, proteins as well as the egress of the cellular products.[1] Consequently, it is necessary to control, and hence to measure, the permeability of capsules.

There are several analytical methods that have been proposed to characterize solute diffusion through capsule membrane. Klein et al.[2] applied inverse size-exclusion chromatography (ISEC) to calcium/alginate beads to estimate their pore size. They packed beads in column and applied various dextrans of known size as solutes. Stewart et al.[3] performed similar measurements with proteins in the place of dextrans. These authors emphasized that, for the determination of the pore size, dextrans would not provide a measure as accurate as proteins themselves would. Prokop et al.[4] reported the use of polyethylene oxide standards to evaluate the permeability of multicomponent microencapsulation systems with ISEC. However, they considered that this method could not be used for a massive screening of capsule permeability due to its significant time requirements.

Although alginate-polylysine microcapsules are often applied as bioartificial organs, permitting the ingress of peptides and proteins, Coromoli et al.[5] used dextran polymers to analyze the intra/extracapsular changes in concentration. Briefly, they added dextran solutions to a defined volume of capsules. Intracapsular dextran concentration was obtained by destruction of the capsules, filtration, and analysis of the dextran present in the washing water, whereas extracapsular dextran concentration was determined by analysis of the dextran present in the supernatant. Both analyses were carried out by size-exclusion chromatography (SEC). To evaluate alginate/poly(L-lysine) capsule permeability, Brissova et al.[6] compared ISEC measurements for the detection of the dextran standards through alginate/poly(L-lysine) capsules with static SEC measurements of dextran diffusion. The results were in good agreement, but SEC measurements of dextran diffusion exhibited the advantage to be more rapid as well as avoiding the irreproducibility in column packing and the problems of capsule damages due to the mechanical strength exerted by the flow rates and inlet pressures.

Numerous methods have also been developed that involved radioactive components. For example, magnetic monodisperse polymer particles (Dynabeads) coated with antibodies against selected proteins were encapsulated in alginate/poly(L-lysine) microcapsules, and the capsule membrane permeability was estimated by measuring the binding of ^{125}I-labeled proteins to the encapsulated antibody-coated Dynabeads.[7] To complement the SEC experiments, Brissova et al.[8] measured the influx/efflux of labeled IgG and Interleukin-1b using a protein A sepharose–antibody complex. Alternatively, Prokop et al.[4] proposed a so-called efflux method that consisted to equilibrate capsules with a [^{3}H] tracer protein solution and to detect the progress of tracer to equilibrium in a buffer solution.

For a systematic evaluation of capsule permeability for bioartificial organs, a realistic approach would be to mimic cellular encapsulation conditions, with reciprocal exchanges as can occur *in vivo,* between encapsulated cells and environmental medium. In the experiments discussed herein, proteins with an extended range of molecular weights have been chosen as representative encapsulated diffusible molecules. To support results on the diffusion of proteins, natural polymers such as dextrans have also been applied. The permeability characteristics of the capsules were then compared to that of alginate beads, which consist of a water-swollen network of alginate polymer, and are characterized by the absence of a membrane. Finally, the influence of the capsule membrane thickness has also been evaluated.

EXPERIMENTAL

Materials

Sodium alginate (Keltone HV) was purchased from Staerkle & Nagler AG (Zürich, Switzerland), cellulose sulfate (Acros 17781-500) from Chemie Brunschwig AG (Basel, Switzerland), and poly(methylene-*co*-guanidine) (36.5 wt % aqueous solution) from Scientific Polymer Products Inc. (New York, NY). Dextrans were obtained from Polfa, Kutno Pharmaceutical Co. (Kutno, Poland), and pullulan and polyethylene oxide standards from Polymer Standard Services (Mainz, Germany). High-range molecular weight protein standards were purchased from Bio-Rad Laboratories AG (Glattbrugg, Switzerland), with yeast alcohol dehydrogenase obtained from Sigma (Buchs, Switzerland). The characteristics of proteins utilized are presented in the Table I.

Polyacrylamides were synthesized according to the method described in ref. 9. Salts and buffer solutions were commercially available as analyt-

Table I Characteristics of Proteins Utilized for Permeability Estimation

Protein	Molar Mass[a] (kDa)	Radius of Gyration[b] (nm)	Isoelectric Point	Ionic Form at pH 7[c]	Structure
Hen egg white ovalbumin	45.0	2.9	4.6	++	Globular shape
Bovine serum albumin	66.2	3.4	4.9	++	Globular shape
Rabbit muscle phosphorylase b	97.4	3.9	5.5	+	Globular shape
E. coli β-galactosidase	116.25	4.2	4.3–7.1	+	Globular shape
Yeast alcohol dehydrogenase	150.0	4.6	5.4	+	Globular shape
Rabbit skeletal muscle myosin	200.0	—	5–7.4	+/−	Long fiber (length 150 nm)

[a] Molar masses were supplied by the respective producers.
[b] Polymer dimensions were estimated using eq. (1).
[c] ++: highly positive, +: positive, +/−: ampholytic.

ical reagents or laboratory grade materials from Fluka Chemie AG and Sigma (Buchs, Switzerland). Products were used as received, and all the polymer and salt solutions were filtered on a 0.2-μm filter before use.

Capsule and Bead Preparation

The capsules were prepared from five active components: sodium alginate (Alg), cellulose sulfate (CS), poly(methylene-*co*-guanidine) hydrochloride (PMCG), calcium chloride, and sodium chloride.

Capsules have been obtained by dropping of a 0.6 wt % Alg/CS solution in PBS generated from a syringe with a needle (27 $G^{3/4}$) to a polycation solution. This solution was composed of 1.2 wt % PMCG, 1 wt % $CaCl_2$ in 0.9 wt % NaCl at pH 7–7.4. The standard reaction time was 1.5 min, but this time was varied to modify the thickness of the capsule membrane. After several washings with 0.9 wt % NaCl solution, capsules were immersed for 7 min in a large volume of 50 mM sodium citrate solution buffered with 0.47 wt % NaCl and 20 mM D-fructose at pH 7–7.4. Capsules were washed and stored in 0.9 wt % NaCl solution. Capsule sizes and membrane thicknesses were measured using optical microscope.

Beads of alginate were prepared by dropping 0.6 wt % Alg solution into a 1.1 wt % $CaCl_2$ solution buffered with 10 mM HEPES, 20 mM D-fructose. The time of reaction between the alginate and calcium ions was 2 min. Beads were washed in PBS solution.

Permeability Measurements

Two methods were employed to assess the permeability of the polymeric multicomponent capsules. The apparent diffusion of the substances through the membrane was detected by SEC and by SDS-polyacrylamide gel electrophoresis. These techniques utilized polymer and protein standards respectively.

For the SEC method, 2 mL of empty capsules or beads were placed into a small recipient bath. Two milliliters of 0.1 wt % solution of polymer standards or 0.05 wt % solution of alcohol dehydrogenase in 0.9 wt % NaCl were added under agitation. Aliquots of the medium were withdrawn at various times and injected into a liquid chromatograph.

The liquid chromatograph was comprised of a L6000 isocratic pump (Merck Hitachi), equipped with Knauer DRI detector. A Protein KW-804 HQ column and a Protein KW-G guard column (Shodex) were employed as stationary phases. The eluent was 0.9 wt % NaCl applied at a flow rate of 0.5 mL/min, with a resulting pressure between 1.6–1.8 MPa. The polymer or protein concentrations were linearly proportional to the maximum height of the detected chromatographic peaks (Beers' Law) with an extinction coefficient independent of the polymer concentration. According to the respective beads, or capsules, to medium volume ratios, the permeability was maximal when the polymer or the protein concentration in the medium decreased from 100 to 66.6%.

The efflux of alcohol dehydrogenase from the capsules and beads has been measured using SEC. One hundred milliliters of 0.25 wt % alcohol dehydrogenase in 0.9 wt % NaCl were mixed with 500 mL of 0.6 wt % Alg/CS or Alg solution in PBS for the preparation of, respectively, capsules or beads. Capsules or beads were immersed in 400 mL of 0.9 wt % NaCl solution. Aliquots of the

medium were collected at different times, and the concentration of alcohol dehydrogenase in the medium was determined. In respect to beads, or capsule, to medium volume ratios, the equilibrium is reached when the protein concentration in the medium increased from 0 to 40%. At this point, the permeability was maximized.

The gel electrophoresis method involved protein standard separation to determine capsule and bead molar mass cutoffs (MMCO). Proteins were immobilized in capsules or beads prepared in mixing 50 mL of protein solution at the concentration of 2 mg/mL each with 500 mL of 0.6 wt % Alg/CS or Alg solution in PBS, respectively. Capsules or beads were then formed as previously reported. They were immersed in 400 mL of 0.9 wt % NaCl solution. Fifty milliliters of the medium were collected at different times and heated in 1× SDS gel loading buffer. Samples were loaded onto 8% SDS polyacrylamide gel and electrophoresis was performed in Tris-glycine buffer at 80–100 V for 2 h. Gels were stained with Coomassie Brilliant Blue R-250.

The radius of gyration R_g is the root-mean-square distance of the macromolecule segments from the center of mass. The value of the radii of gyration of globular proteins and polymers used as permeants was calculated according to the following formulae with the molar masses (MM) used as supplied by the producers:

$$R_g = 0.051\ \mathrm{MM}^{0.378} \text{ for globular proteins}^{6} \quad (1)$$

$$R_g = 0.026\ \mathrm{MM}^{0.495} \text{ for dextrans}^{6} \quad (2)$$

$$R_g = 0.015\ \mathrm{MM}^{0.550} \text{ for pullulans}^{6} \quad (3)$$

$$R_g = 0.016\ \mathrm{MM}^{0.59} \text{ for polyethylene oxides}^{10} \quad (4)$$

$$R_g = 0.0255\ \mathrm{MM}^{0.56} \text{ for polyacrylamides}^{11} \quad (5)$$

RESULTS

Multicomponent Polymeric Capsule Preparation

Capsule preparation was carried out in two steps. First, gelled beads containing a polyelectrolyte complex membrane were formed. Calcium chloride permitted the gelification of anionic Alg/CS beads and the ingress of the cationic PMCG formed the external membrane via a reaction, predominantly with cellulose sulfate. The cellulose sulfate imparts the capsule network structure, whereas alginate influences the droplet rheology.[4] The second step involved the liquefaction of the core of the capsule with sodium citrate. Sodium chloride was used to modulate the properties of capsules in the initial stage of the complex formation. In this report, the size of the prepared capsules was within the range of 2–3 mm. The standard membrane thickness was approximately 90 μm, corresponding to a reaction time between Alg/CS and $CaCl_2$/PMCG of 1.5 min.

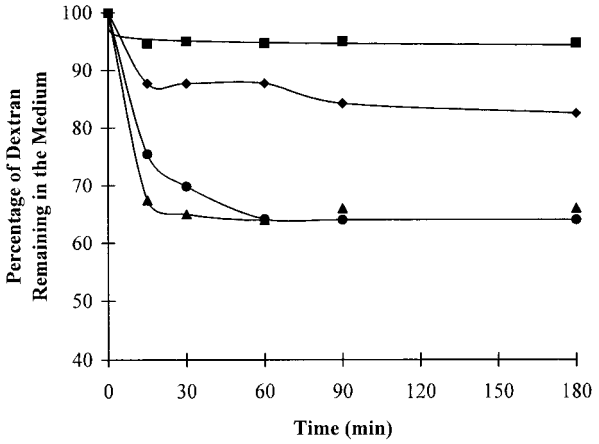
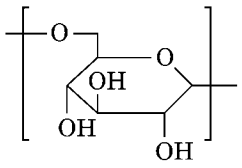
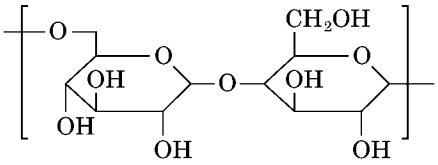


Figure 1 Alginate/cellulose sulfate/polymethylene-*co*-guanidine/$CaCl_2$/NaCl capsule ingress permeability as measured by the diffusion of dextran polymers into capsules. Detection was by SEC. The ratio between dextran solute concentration in the medium and dextran solute concentration in the medium, at $t = 0$, is plotted vs. time of diffusion. ■ Dextran 110 kDa, R_g = 8.3 nm, ◆ dextran 70 kDa, R_g = 6.5 nm, ● dextran 15 kDa, R_g = 1.6 nm, ▲ dextran 4 kDa, R_g = 1.6 nm.

Multicomponent Polymeric Capsule Permeability

Characterization of the Alg/CS/PMCG/$CaCl_2$/NaCl capsule permeability was carried out with SEC using dextran standards with molar masses of 4, 15, 70, and 110 kDa, corresponding to radii of gyration of 1.6, 3, 6.5, and 8.3 nm, respectively (Fig. 1). For standard capsules as described above, the concentration of 4, 15, and 70 kDa dextrans in the supernatant decreased until it reached, after 3 h, 66, 66, and 82%, respectively (Fig. 1). The maximal permeability at the equilibrium (66%) is reached in about 1 h for dextrans of 4 and 15 kDa, whereas the diminution of the concentration of 110 kDa dextran in the medium was negligible even after 3 h. This implies that

Table II Permeability of the Alginate/Cellulose Sulfate/Polymethylene-*co*-Guanidine/$CaCl_2$/NaCl Capsule as Measured by Polymer Ingress

Polymer	Repeat Unit	Molar Mass[a] (kDa)	Radius of Gyration[b] (nm)	Percentage of Polymers in the Medium after 3 h[c]
Dextran		15	3.0	72
		70	6.5	76
		110	8.3	100
Pullulan		22.9	3.7	70
		95.3	8.2	88
Polyethylene oxide	$-(CH_2-CH_2-O)-$	25	6.3	86
		92	13.6	96
Polyacrylamide	$-(CH_2-CH(CO-NH_2))-$	21.9	6.9	66
		79	14.0	79

the membrane was permeable to the 4, 15, and 70 kDa dextrans though the kinetics were slower for the 70 kDa dextran. The capsules completely excluded larger dextran molecules with molar mass of 110 kDa and higher. In summary, the capsule MMCO was determined to be between 70 and 110 kDa.

Additional SEC experiments were conducted that used different polymer standards. The diffusion of pullulans, polyethylene oxides, and polyacrylamides in the same range of molar masses has complemented measurements with dextrans (Table II). These polymers of similar molar mass have different coil sizes as expressed by their radii of gyration, varying from 3 to 14 nm. Polyethylene oxides and polyacrylamides possess a greater chain expansion in aqueous solutions than dextrans and pullulans. The results from Table II indicate that the percentage diffusion into capsules was roughly the same for all polymers tested. This suggests that the membrane exhibits the same permeability characteristics independent of the chemistry of the macromolecular solute. This implies that the SEC method is accurate. In summary, the MMCO determined with synthetic polymers can be approximately estimated between 80 and 90 kDa.

Alginate Bead Permeability

When a sodium alginate solution is added dropwise to a solution containing calcium ions, it forms transparent, spherical, and homogeneous gel beads. These gels are the result of the chelation between the carboxylate groups of alginate guluronate and the calcium ions. The bead diameter was approximately 2–3 mm. Figure 2 shows the evolution of the dextran concentration in the medium in presence of beads. After 3 h of diffusion, 68, 67, and 93% of dextrans, with molar masses of 70, 110, and 200 kDa, respectively, remained in the medium. Equilibrium was reached for 70 and 110 kDa dextrans after, respectively, 60 and 90 min, whereas 200 kDa dextran did not diffuse into the beads. The permeability of the beads was higher than that of the capsules, as dextrans with molar masses of 70 and 110 kDa could penetrate. The MMCO of the beads was, therefore, estimated to be between 110 and 200 kDa.

Permeability via Gel Electrophoresis

To mimic the exchange capability that may exist between encapsulated biological material and the *in vivo* environment, capsule and bead permeabilities were also assessed in evaluating the dif-

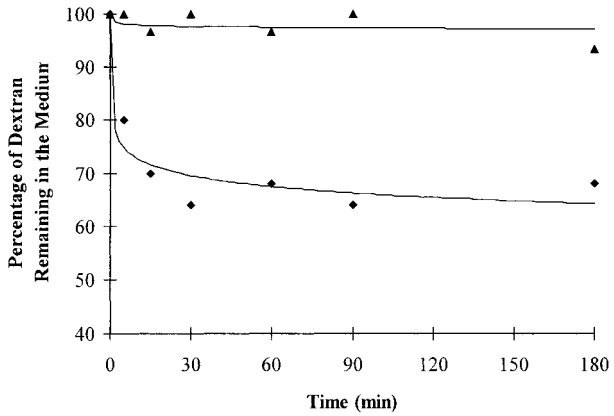


Figure 2 Alginate/$CaCl_2$ bead ingress permeability as measured by the diffusion of dextran polymers into beads. Detection was by SEC. The ratio between dextran solute concentration in the medium and dextran solute concentration in the medium, at $t = 0$, is plotted vs. time of diffusion. ▲ Dextran 200 kDa, R_g = 10.9 nm, ◆ dextran 70 kDa, R_g = 6.5 nm.

fusion of encapsulated proteins by gel electrophoresis with Coomassie Blue staining. The protein standard applied was comprised of ovalbumin (45.0 kDa, R_g = 2.9 nm), bovine serum albumin (66.2 kDa, R_g = 3.4 nm), rabbit muscle phosphorylase b (97.4 kDa, R_g = 3.9 nm), β-galactosidase (116.25 kDa, R_g = 4.2 nm), and rabbit muscle myosin (200 kDa, R_g = 5.1 nm) (Table I). The results presented in Figure 3 shows that the capsules and beads were permeable to the ovalbumin, bovine albumin, phosphorylase b, β-galactosidase. They were not, however, permeable to the myosin after 4 h of diffusion, as the band corresponding to the myosin has not been detected in the medium extract. Furthermore, the diffusion rate was faster for the beads than for the capsules with regard to protein efflux. In addition to that, the band corresponding to the phosphorylase b is barely detected in the capsule experiments compared to that with beads. As each protein is approximately in the same concentration, this could be explained either by a slight interaction between phosphorylase b and one or several components of the capsular membrane, either by a unfolded conformation of the protein in pH conditions used. Nevertheless, the capsule MMCO measured in this experiment with proteins was slightly higher than that observed by SEC with dextrans, and its value can be estimated between 116 and 220 kDa.

Diffusion of High Molar Mass Permeant

To refine the permeability measures, a permeant with a molar mass in the range of 110–200 kDa was required. Unfortunately, few were commercially available in this range. We have used alcohol dehydrogenase, which is a globular protein extracted from yeast, with a molar mass of 150 kDa and a radius of gyration of 4.6 nm. Upon analysis by gel electrophoresis, the protein is broken down into four subunits due to the denaturing conditions of electrophoresis. These subunits were detected by Coomassie Blue staining in the sample reference, which was the protein alone. However, it has not been possible to observe the encapsulated alcohol dehydrogenase release from the capsules by Coomassie Blue staining. SEC measurements were subsequently carried out with columns preventing the protein adsorption.

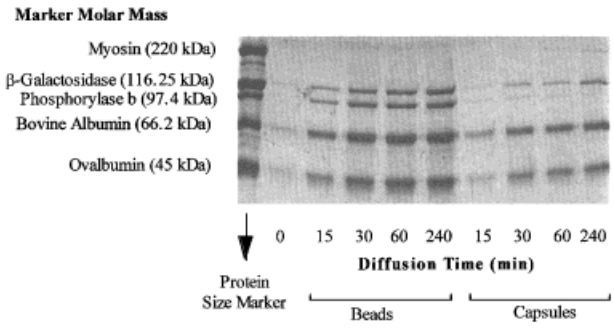


Figure 3 Alginate/cellulose sulfate/polymethylene-*co*-guanidine/$CaCl_2$/NaCl capsule and Alg/$CaCl_2$ bead permeability as measured by the exo-diffusion of protein standards from capsules and beads. Detection was by gel electrophoresis and Coomassie Blue staining.

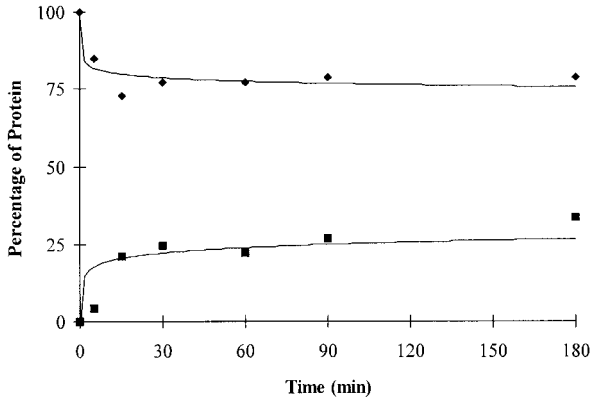


Figure 4 Diffusion of the alcool dehydrogenase into alginate/$CaCl_2$ beads (◆) and egress from beads (□). Detection was by SEC.

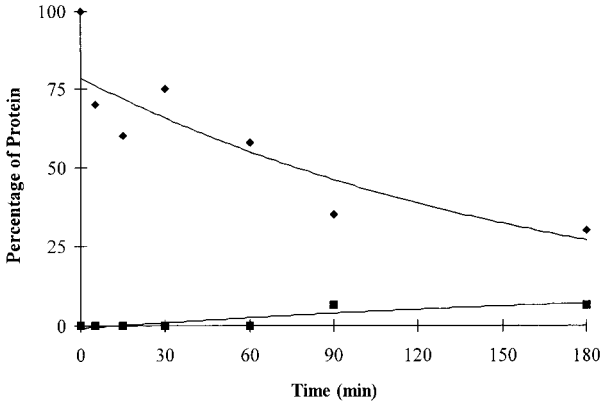


Figure 5 Diffusion of the alcool dehydrogenase into alginate/cellulose sulfate/polymethylene-*co*-guanidine/$CaCl_2$/NaCl capsules (◆) and egress from capsules (□). Detection by SEC.

Therefore, the diffusion of the alcohol dehydrogenase (ingress and egress) to the capsules and beads has been quantitatively analyzed.

Figure 4 shows the ingress and the egress of the alcohol dehydrogenase in the beads. The percentages of protein in the medium were 79% after 3 h of diffusion inside the beads, and 33% after 3 h of diffusion outside the beads. In this case, there was good agreement between the experimental and calculated percentages of protein present in the medium at the maximum permeability. These values indicated that the protein does not adsorb at the surface of the beads. In considering all the experiments, the MMCO of the beads was, therefore, estimated between 150 and 200 kDa.

Figure 5 illustrates the diffusion of the alcohol dehydrogenase into and from the capsule. The ingress of the protein into the capsule started after 30 min of contact but seemed maximal after 3 h. In the majority of experiments, as shown in Figure 5, the value obtained for 3 h reached 30%, which was not consistent with the calculated value of maximal permeability at the equilibrium (66%). Surprisingly, the encapsulated protein did not seem to diffuse outside the capsules, as the percentage of alcohol dehydrogenase in the medium did not exceed 5% after 3 h of capsule immersion in saline solution. One hypothesis to explain these results was that the protein did not diffuse at all in or from the capsules. When the protein was present in the medium, it adsorbed at the capsule surface and did not go through the membrane inside the capsule. Therefore, the percentage of adsorbed protein contributed to decrease the theoretical percentage of maximal permeability. To validate this hypothesis, capsules were soaked in alcohol dehydrogenase solution for 3 h. After several washings, they were scratched and intracapsular content was retrieved. Both washing solutions and intracapsular medium were loaded on gel electrophoresis. The protein was only detected in washing solutions. No protein was found in the analysis of the intracapsular medium. This could confirm that the protein was adsorbed at the capsule surface and, consequently, did not pass through the membrane. All protein diffusion experiments considered, the MMCO of the capsules was, therefore, estimated between 116 and 150 kDa.

Membrane Thickness as a Permeability Parameter

In Figure 6, the capsule membrane thickness was plotted as a function of the time of reaction between the Alg/CS drops and the $CaCl_2$/PMCG solution. As expected, the thickness increased with the reaction time. After 15 s of reaction, a thin membrane already appeared, with a size of 45 μm. A thickness of 90 μm was obtained in 1.5 min, which correspond to the standard conditions used above. A thickness of 720 μm was obtained after 15 min of reaction. The upper limit of the membrane thickness was the capsule size itself, as the membrane was formed by diffusion and complexation of the PMCG inside the capsule.

The permeability of capsules having different membrane thicknesses (60, 130, 180, and 250 μm) was measured by gel electrophoresis in following the diffusion of encapsulated protein standard from the capsules. The gel obtained after the Coomassie Blue staining is shown in the Figure 7. Capsule membranes with a size of 60 and 130 μm were permeable to β-galactosidase (116 kDa),

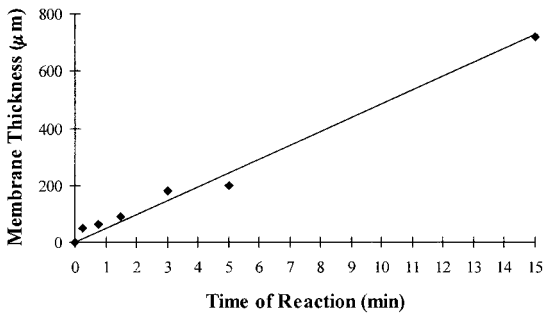


Figure 6 Variation of the capsule membrane thickness as a function of time for the reaction between alginate/cellulose sulfate and polymethylene-*co*-guanidine/$CaCl_2$.

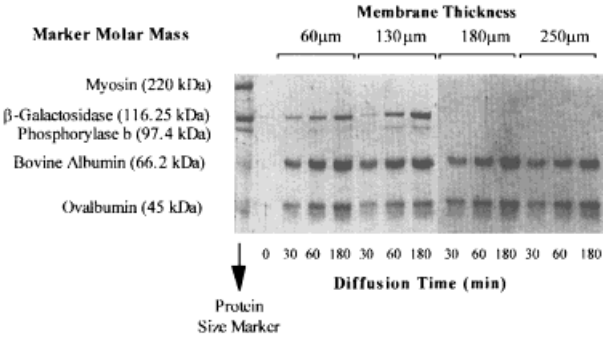


Figure 7 Alginate/cellulose sulfate/polymethylene-*co*-guanidine/$CaCl_2$/NaCl capsule permeability as measured by the exo-diffusion of protein standards from various capsules. Detection was by gel electrophoresis and Coomassie Blue staining. The membrane thickness is shown at the top of the photo.

whereas capsule membranes with a size of 180 and 250 μm were not, as no band corresponding to this proteins was detected. It is difficult to conclude with phosphorylase b as the band corresponding is poorly seen or not visible. The β-galactosidase (116 kDa) is, therefore, chosen as upper limit. The MMCO of capsules, with a membrane size of 60 and 130 μm, was evaluated between 116 and 150 kDa, and that of 180 and 250 μm membrane capsules between 66 and 116 kDa. This implies that the thickness of the capsule membranes altered their permeability towards proteins.

DISCUSSION

Multicomponent polymer capsules were prepared from five active components. These capsules exhibit a liquid core, surrounded by a membrane obtained by polyelectrolyte complexation reaction. By the process employed, the encapsulation system retained the biological materials within a semipermeable membrane, and the encapsulated materials, such as cells, can grow and function.[12] This system is usually known for its ability to create a complex membrane with adaptable permeability and is a promising candidate for the immunoisolation of cells.[4,12,13] By modification of component concentration, a series of capsules having a range of permeabilities between 40 to 230 kDa has been developed.[12] Thus, by varying the capsule permeability, the degree of immunoprotection of the encapsulated cells vis-à-vis the host immune system can be adjusted. This can explain the good performance of these capsules in islet encapsulation, perifusion, and implantation studies.[14] Specifically, these capsules have been shown to reverse diabetes in NOD mice (xenograph islets from rats) for period exceeding 120 days.[4]

Polysaccharide Ingress

Dextran is a linear polysaccharide bearing a low density of charged groups. Thus, noncovalent interactions with the capsule membrane and SEC column are expected. The dextran diffusion should be controlled only by its molecular size. This should also be the case for polymers such as pullulans and polyethylene oxides that carry hydroxyl and ether functional groups. Polyacrylamides possess amide functions, which can create hydrogen bonds, as can proteins in aqueous media. SEC measurements with dextrans have permitted the determination of a MMCO range between 70 and 110 kDa for capsules with a membrane thickness of 90 μm. The use of different polymer standards has narrowed this range to 80–90 kDa. These polymers carried various chemical groups and their macromolecular chains exhibited a different radius of gyration for equivalent molar masses (Table II). However, the ingress of polymers into the capsules seemed to be comparable for all chemical structures, therefore validating, the generalization of the method.

Protein Ingress and Egress

Proteins can be classified as complex ampholytes. They are generally charged and can electrostatically interact with charged components of the capsule. Thus, in addition to steric hindrance, electrostatic interactions can also influence permeability measurements with proteins. All the used proteins, with the exception of the myosin, have a globular shape. Their radius of gyration varies from 2.9 to 4.6 nm. All proteins possess an isoelectric point (defined as the pH at which the net charge averaged over all the protein is zero) in the range between 4.6 and 7.4 (Table I). Indeed, a large statistical study has shown that 70% of the proteins have pI values below 7.[15,16] At the physiological pH used for the permeability experiments (pH 7–7.4) the proteins are in an anionic form (Table I). Following the recommendation of Stewart et al.,[3] the eluent pH should be above the pI of protein permeants to minimize ionic adsorption to the negatively charged polymers such as

Table III Calculated Equivalent Molar Mass of Globular Proteins and Dextrans vs. the Radius of Gyration

Polymer	Molar Mass (kDa)[a]	Radius of Gyration (nm)[b]	Equivalent Molar Mass of Globular Proteins (kDa)[b]	Proteins	Molar Mass (kDa)[a]	Radius of Gyration (nm)[b]	Equivalent Molar Mass of Dextrans (kDa)[b]
Dextran	4	1.6	9.1	Ovalbumin	45.0	2.9	13.7
Dextran	15	3	48	Albumin	66.2	3.4	19
Dextran	70	6.5	370	Phosphorylase b	97.4	3.9	25
Dextran	110	8.3	700	β-Galactosidase	116.25	4.2	29
—	—	—	—	Alcohol dehydrogenase	150.0	4.6	35

[a] Molar masses were supplied by the respective producers.
[b] Protein and dextran dimensions were estimated using eqs. (1) and (2), respectively.

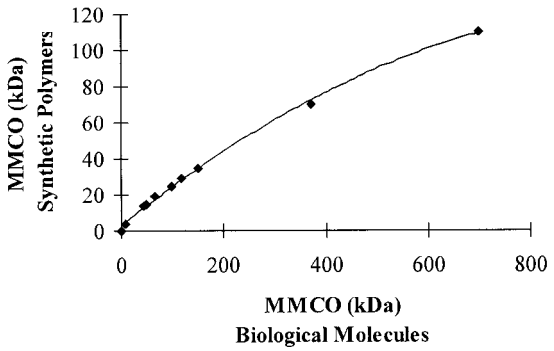
alginate. Thus, in comparison of results obtained with protein diffusion, it is expected that the influence of charge density carried by the protein will be negligible. The characterization of the capsule permeability with proteins has mainly been carried out using gel electrophoresis followed by Coomassie Blue staining. This technique does not require either the handling of radioactive compounds or radiolabeled proteins and/or an antibody against the protein of interest. Furthermore, it allows a large screening of protein detection with a reasonable gel preparation number. Exodiffusion of proteins from the capsules has been so measured. Proteins were encapsulated and capsules were soaked in physiological sodium chloride solution. By this method, the value of capsule MMCO can be evaluated between 116 and 220 kDa after 4 h of capsule immersion. Refinement of the measurement, by complementary protein diffusion tests and SEC detection, has led to estimate the MMCO between 116 and 150 kDa. These values are higher than that obtained with dextrans and other polymers. In Table III, it has been reported the calculated equivalent molar mass of globular proteins and of dextrans vs., respectively, the radius of gyration of used dextrans and globular proteins. For example, in considering the size of proteins, the β-galactosidase (116 kDa) should theoretically diffuse in the same manner as a dextran with a molar mass of 29 kDa, and the alcohol dehydrogenase (150 kDa) as a dextran with a molar mass of 35 kDa. However, the results have shown that the capsules were permeable to dextrans with a molar mass inferior to at least 70 kDa, and proteins with a molar mass inferior to at least 116 kDa. Thus, relative to the dextrans, the globular shape or hard sphere conformation of proteins certainly facilitated their passage through the capsule membrane, compared to the flexible coil conformation of polymers such as dextrans.

Equivalence of Methods

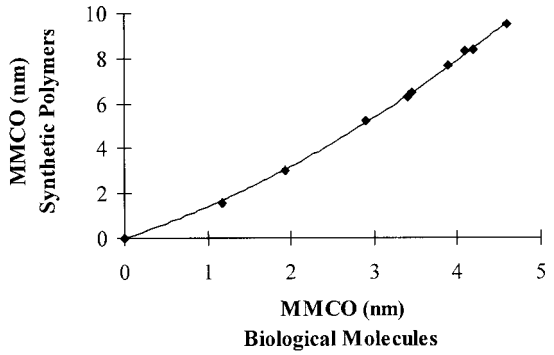
Molar mass cutoff, as an indicator of permeability, is a relative measure related to the permeate size in solution, and is dependent on the chemistry of the macromolecule and the solvent quality. Therefore, one would not expect the MMCOs to be numerically equivalent if estimated using polysaccharide and protein based probes. Nonetheless, one would anticipate having a qualitative agreement between the two metrics. Figure 8 shows a very good correlation between MMCO evaluated with dextrans and proteins for both capsules and beads. The following section will demonstrate that these metrics can be used to assess the function of a permselective barrier for various structural conditions, such as membrane thicknesses, providing a valuable tool in the optimization of bioartificial organs.

Effect of Membrane Thickness

Different capsular membrane thicknesses have been obtained in varying the reaction time between the Alg/CS drops and the $CaCl_2$/PMCG solution. Prokop et al.[4] have shown that a longer reaction time led to more extensive ionotropic gelation, and could therefore regulate the permselectivity of the membrane. The authors considered that the capsules having a thick walls of



(a)



(b)

Figure 8 Comparison of the MMCO in kDa (a) and nm (b) estimated using synthetic polymers with GPC detection and natural polymers with electrophoretic detection. A clear correspondence is observed.

30–100 μm were the most suitable for bioartificial pancreas application. Our experiments provide a measurement of the corresponding permeability. The MMCO of capsules with a membrane size of 60 and 130 μm was evaluated between 116 and 150 kDa, whereas that of 180 and 250 μm membrane capsules between 66 and 116 kDa. The permeability of the capsules decreases, therefore, with an increase in membrane thickness.

Bead Permeants

The various measurements of alginate bead permeability have shown that their MMCO was higher than that of capsules, between 110 and 200 kDa, with dextrans and between 150 and 220 kDa with proteins. Indeed, beads consist of a water-swollen network of alginate polymer and are characterized by the absence of a membrane. Therefore, the permeability is regulated by the crosslinking between the alginate chains and calcium, conferring to the beads less permselective properties. This system is not adequate for long-term application, as the presence of monovalent cations generally leads to bond breakage between the calcium and the alginate chains. Beads are more appropriate, therefore, for the development of drug delivery applications where the permeability performance requirements are less stringent than for bioartificial organs.[17]

Unresolved Questions

A number of questions are still unresolved concerning the notion of permeability and particularly the optimal molecular weight cutoff.[18] For example, it is not clear what molecules should be excluded or allowed to pass through the membrane. Calafiore et al.[19] recommended a MMCO of range from 50 to 70 kDa for alginate/polyaminoacids microcapsules of pancreatic islet graft immunoisolation. Other authors[6] believe that the exclusion limit of the membrane must correspond to the size of immunoglobulin molecules, with a molar mass of approximately 150 kDa. Furthermore, to optimize the permselectivity of the capsules and so to control their immunoisolation properties, it has been initially suggested that, with an appropriate choice of a cutoff pore size, the polymer membrane can as act as a barrier. In a recent paper, Wang[1] considered that the notion of membrane barrier is limited, as the capsule pore size homogeneity and the pore surface distribution can influence the permeability. Thus, if the pore size and their distribution are not homogeneous, the host immune molecules can penetrate in the membrane but the smaller pores present in the membrane should retain the immunoglobulins. Therefore, the membrane should serve more as an entrapment zone than as a barrier. Generally, the methods employed for permeability assessment permit evaluation of an apparent pore size regardless of the disparity distribution and size. Further characterizations, principally at a microscopic level, are necessary to take into account the concept of pore heterogeneity and distribution.

Another questions concern the ability of some proteins to be adsorbed on the inner or outer surface of the capsules. Further studies should be aimed to evaluate this adsorption. Moreover, understanding the molecular mechanisms of the interactions between the proteins and the capsular

components may allow to develop new capsule systems in which these interactions could be reduce or absent. This system should be ideal for cell encapsulation to reduce necrotic fibrose due to immunoglobulins penetration in the capsular membrane, but also to permit a total secretion of cellular nutrients which are toxic when they accumulate.

CONCLUSIONS

In this study, two methods, which did not involve radioactive compounds, were employed to assess the permeability of polymeric multicomponent capsules. These capsules, based on five active compounds, Alg/CS/PMCG/$CaCl_2$/NaCl, exhibited a 90-μm membrane thickness for a standard reaction time of 1.5 min. Their MMCO was estimated between 80 and 90 kDa using polymers such as dextrans, pullulans, polyethylene oxides, and polyacrylamides, and between 116 and 150 kDa using proteins. Apparently, the globular shape of the proteins facilitated their passage through the capsule membrane, comparatively to the polysaccharide coil conformation. The membrane size influenced the MMCO, as the thickness increase leads to a reduction of its MMCO. Alginate beads were more permeable with a MMCO estimated in the range of 110 and 200 kDa with dextrans and in the range of 150 and 220 kDa with proteins.

These results illustrate that the permeability can be estimated by using both polymer and biologically relevant molecules influx and efflux. This evaluation differs from prior approaches, which have defined a MMCO based on the permeability of a single solute. This article shows, to our knowledge, the first reported concordance between permeability measures based on model natural and biological macromolecules. We speculate that both the chromatography and gel electrophoresis methods can provide reasonable metrics allowing to approach *in vivo* conditions, and can be adopted for a complete evaluation assessment of capsule and bead permeability. The ability to measure and control the permeability may lead to the improvement of existing encapsulation procedures and contribute to extensive development opportunity in cell microencapsulation, bioencapsulation, drug delivery systems and bioartificial organs.

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