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Journal of Chromatography B, 706 (1998) 141–147

JOURNAL OF CHROMATOGRAPHY B

# Methodology for vesicle permeability study by high-performance gel exclusion chromatography

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**Abstract**

A methodology based on high-performance gel exclusion chromatography (HPLC-GEC) has been developed to perform permeability studies of vesicles. Encapsulation of two marker isothiocyanate fluorescein (FITC) dextrans of 4400 and 40 500 molecular mass was used as a model system. Combination of two TSK-PW columns, one efficient in vesicle sizing (G6000 PW), the other in that of dextrans (G4000 PW), was required to achieve complete particle separation and to remove entirely the unentrapped dextran after encapsulation into vesicles. Coupling fluorescence and light scattering detection allowed to control the efficiency of the separation, to quantify the vesicle leakage and to follow both the integrity of the vesicles and changes in their size. This methodology can be applied to other fields such as encapsulation of water soluble compounds and drug delivery systems. © 1998 Elsevier Science B.V.

*Keywords:* Vesicles; Liposomes; Encapsulation

## 1. Introduction

Vesicles or liposomes are attractive models for biological membrane studies or drug delivery systems because of their molecular organization, consisting of one or more lipid bilayers enclosing aqueous compartments. They can encapsulate either hydrophilic compounds in their aqueous internal cavity or hydrophobic molecules in their bilayer membranes. The nature of the lipids and the methods of preparing liposomes define the chemical and physical characteristics of the vesicles. Their permeability properties are of primordial importance as they govern the efficiency of drug retention, the liberation of entrapped molecules and the stability of the vesicles.

Permeability properties are also indicative of structural changes in the bilayer architecture caused by insertion of foreign molecules. This is, among amphiphilic molecules, the case of solubilizing surfactants usually named detergents. The molecules of a detergent solution, added to a suspension of vesicles, partition between the lipid bilayer and the aqueous medium. A series of lipid–detergent mixed aggregates are formed in equilibrium with a specific detergent concentration in the aqueous medium. The lipid–surfactant interactions lead to a progressive solubilization of the vesicles into lipid–detergent mixed micelles [1–5]. The first step of this solubilization process is characterized by an increase in the permeability of the lipid membrane [3,6–8]. Although different hypotheses have been proposed, such as the formation of transient holes [6,8] in the membrane or the existence of open vesicles stabilized by detergent molecules [2], the mechanism is not yet fully understood. For this reason, we have

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0378-4347/98/$19.00 
*PII* S0378-4347(97)00517-3

chosen to encapsulate isothiocyanate fluorescein dextrans with increasing molecular mass in very stable and well-defined vesicles, and to study the leakage of these probes induced by the addition of a non-ionic detergent. The aim of this investigation was to evaluate the size of the membrane defects created by the insertion of detergent molecules.

In this paper, we present a methodology based on high-performance gel exclusion chromatography (HPLC-GEC) [9–12] specifically developed to perform permeability studies. It includes characterization of the FITC-dextrans, separation of free dextran from vesicles, and analysis of the different parameters which can be simultaneously measured thanks to a double on-line detection by fluorescence and light scattering.

## 2. Experimental

### 2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Avanti (Alabaster, AL, USA). Egg phosphatidic acid (EPA), fluorescein-isothiocyanate dextrans (FITC-dextrans), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes), sodium chloride and *n*-octyl-β-D-glucopyranoside (OG) were obtained from Sigma (St. Louis, MO, USA). Polysaccharide standards of 738, 5800, 12 200, 23 700, 48 000, 100 000, 186 000, 380 000 and 1660 000 molecular mass, were manufactured by Polymer Laboratories (Amherst, MA, USA) and supplied by Touzart et Matignon (Paris, France).

### 2.2. Vesicle preparation

Vesicles (REV) of EPC–EPA (90:10, w/w) without dextran (REVo) and with encapsulated dextrans (4400-REV with FITC-dextran 4400 and 40500-REV with FITC-dextran 40500) were formed using the reverse-phase evaporation technique described by Szoka and Papahadjopoulos [13] and sequentially extruded (Extruder Lipex Biomembranes, Vancouver, Canada) down through polycarbonate membranes (Nuclepore) of 0.8, 0.4, 0.2, 0.1 and 0.05 μm diameter [10]. REVo were prepared in an aqueous buffer (10 m*M* Hepes, 145 m*M* NaCl, pH 7.4), 4400-REV1 and 40500-REV1 in the same buffer containing 1 mg $ml^{-1}$ of the corresponding FITC-dextran and 4400-REV88 in an isotonic buffer (10 m*M* Hepes, 172 m*M* NaCl, pH 7.4) containing 88 mg $ml^{-1}$ of FITC-dextran 4400.

### 2.3. Quasi-elastic light scattering

Hydrodynamic mean diameters (MD) of vesicles were determined by quasi-elastic light scattering (QELS) by using an N4 Coultronics. Calculations were made according to the Stokes–Einstein equation assuming the particles to be independent and spherical. The MD values correspond to the average of three measurements with a standard deviation lower than 5%.

### 2.4. High-performance gel exclusion chromatography (HPLC-GEC)

TSK-G6000 and G4000 PW columns (30×0.75 cm I.D.) were supplied by Toyo Soda (Tokyo, Japan). The HPLC apparatus was equipped with a Hitachi pump (Model L-6000) and a precision injection valve (Rheodyne) [10,12].

For the characterization of dextrans, a single TSK-G4000 PW column was used. Samples of 50 μl were injected. The eluent was water and the flow-rate was 1.0 ml $min^{-1}$. A refractive index (RI) detector was used for sample detection. Aqueous solutions of standard polysaccharides (0.5 mg $ml^{-1}$) and FITC-dextrans (0.3 mg $ml^{-1}$) were prepared according to the procedure provided by Polymer Laboratories. After adding distilled water to the polymer, the solution was allowed to stand for 24 h to ensure complete swelling.

The TSK-G4000 PW column was calibrated with polysaccharide standards ($\overline{M_w}/\overline{M_n} \leq 1.1$). The void ($V_o = 5.17$ ml) and total ($V_t = 9.92$ ml) volumes were determined from elution of a polysaccharide standard of 1 660 000 molecular mass and glucose, respectively. The column parameter, $K_d$, was calculated from the relation:

$$K_d = (V_e - V_o)/(V_t - V_o) \quad (1)$$

where $V_e$ is the sample elution volume.

$V_o$ was taken at the intercept of the baseline with the half-height tangent to the left side of the peak

corresponding to the elution of the excluded polysaccharide standard. Elution volumes ($V_e$, $V_t$) corresponding to the maximum of chromatograms were determined by the intercept of the half-height tangents to the symmetrical HPLC peaks.

In the procedure elaborated for permeability measurement, a TSK-G6000 and G4000 PW columns were used in series. Sample loading was 200 μl, the eluent was aqueous buffer for separation of the unencapsulated markers from the vesicles and the flow-rate was 1.0 ml $min^{-1}$. Detection was performed by fluorescence (excitation wavelength = 493 nm, emission wavelength = 514 nm) and 90° light scattering at 493 nm with a spectrofluorimeter Fluorolog Spex FL1T11, connected to a computer and equipped with a specific quartz cell (Hellma 176.353-QS) thermostated at 25°C. A fraction collector was used to recover the fractions corresponding to the elution peak of vesicles.

### 2.5. Permeability measurement

Permeability experiments were performed on 4400-REV1. A 100 m*M* OG solution in buffer was continuously added to the vesicles under stirring, at a rate of $1.39\times10^{-3}$ ml $min^{-1}$, until the desired OG concentration in the samples was reached according to the procedure previously described [2,4]. The dextran leakage was analysed by injecting the surfactant-treated suspensions on the TSK-G6000 and G4000 PW columns in series (see Section 2.4). The eluent was the continuous medium in equilibrium with the mixed OG–vesicles [2]. The release content was determined from a calibration curve obtained by elution of solutions of FITC-dextran 4400 in the conditions used for the permeability experiments, with aqueous buffer as eluent. The dextran solutions in the $5\times10^{-4}$ to 0.5 mg $ml^{-1}$ concentration range were prepared in buffer according to the protocol described in Section 2.4. The fluorescence intensity was linearly related to the dextran concentration up to $1\times10^{-1}$ mg $ml^{-1}$ (slope, 122 975 cps $mg^{-1}$ ml; correlation coefficient, 0.9999). Concentrations of FITC-dextran as low as $5\times10^{-4}$ mg $ml^{-1}$ could be detected. The limit of quantitation was estimated to be $5\times10^{-3}$ mg $ml^{-1}$ with a standard deviation of 5% for the concentration value. The reproducibility over 24 h was better than 1%.

## 3. Results and discussion

### 3.1. Characterization of the dextrans

The selectivity curve of the TSK-G4000 PW column expressed as molecular mass ($\overline{M}_w$) of the polysaccharide standards versus $K_d$ is shown Fig. 1. By fitting the experimental points to a polynomial of the third degree, the following equation (correlation coefficient, 0.989) was obtained:

$$\log(\overline{M}_w) = 6.17 - 7.11(K_d) + 12.32(K_d^2) - 9.34(K_d^3) \quad (2)$$

The fluorescent dextran characteristics given by the commercial firm ($\overline{M}_w$), polydispersity indices ($\overline{M}_w$)/($\overline{M}_n$) and number of moles of FITC/mol glucose) are summarized in Table 1. The $K_d$ values obtained for the FITC-dextrans (Fig. 1, Table 1) indicate an elution behavior through the G4000 PW column distinct from that of the standard polysaccharides. The apparent molecular masses calculated from Eq. (2) are always lower than the values given by the commercial firm (Table 1). This discrepancy may be due to the use of different methods to measure the molecular mass of the fluorescent dextrans and standard polysaccharides. The results suggest also that the interaction of the FITC-dextrans with the G4000 gel may differ from that of the polysaccharide standards. This could arise from the hydrophobicity of the FITC groups covalently linked to some

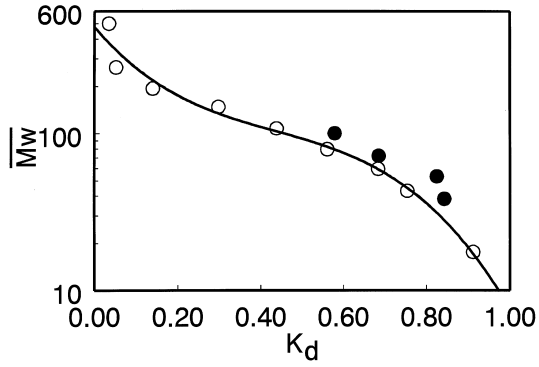


Fig. 1. Selectivity curve for the TSK-G4000 PW column: log ($\overline{M}_w$) versus $K_d$: polysaccharide standards (open circles), FITC-dextrans (full circles). The experimental points (open circles) were fitted by a polynomial of the third degree (full line) (see text).

Table 1
FITC-dextran characteristics, elution parameter and ($\overline{M_w}$) determined by HPLC-GEC

| | | | | |
|---|---|---|---|---|
| Commercial ($\overline{M_w}$)[a] | 4400 | 9400 | 18 900 | 40 500 |
| ($\overline{M_w}/\overline{M_n}$)[a] | <1.5 | <1.25 | <1.25 | <1.25 |
| Mol FITC/mol glucose[a] | 0.004 | 0.008 | 0.008 | 0.003 |
| $K_d$ | 0.843 | 0.825 | 0.685 | 0.579 |
| Calculated ($\overline{M_w}$)[b] | 2158 | 2774 | 11 982 | 23 537 |

[a]Provided by the commercial supplier.
[b]$\overline{M}$ calculated from Eq. (2), see text.

glucose units of the dextrans which could delay their elution through the column, although their number is very low (Table 1). Moreover, because of this very low number, the global conformation of the FITC-dextrans should not be modified. To remove these uncertainties, it would be necessary to measure the FITC-dextran molecular mass by a universal method. However, as the purpose pursued by using dextrans with increasing molecular mass is to evaluate the size of the defects in the lipidic membrane responsible of the vesicle leakage, it seems preferable to undertake the measurement of the gyration radius of the entrapped polymers rather than their hydrodynamic size.

### *3.2. HPLC-GEC methodology for permeability measurement*

Permeability measurements of vesicles require different criteria to be representative of the system studied. First, the vesicles used as a model membrane, must be unilamellar and monodisperse since internal volume, average size and size distribution as well as lamellaricity are factors affecting the permeability properties. EPC/EPA REVs have been demonstrated by HPLC-GEC to fulfil these characteristics and moreover to be stable versus time [10,12].

The procedure used for the permeability measurements is summarized in Fig. 2. The vesicles are prepared in a buffer containing the probe. The resulting vesicles contain the probe both in the internal and external medium. Then the unencapsulated probe has to be completely removed. Control of the efficiency of this elimination is important as the residual concentration of the probe in the external medium of the vesicles would interfere in the further analysis of the leakage. Subsequently, the leakage of the vesicles, induced for instance by addition of detergent, can be analysed as a function of its concentration.

Gel permeation is a commonly used method for the removal of unentrapped solutes. For small molecules the separation is generally easy since with available gels (Sephadex, Sepharose), the vesicles are excluded and the solute totally included [14]. In the case of solutes of high molecular mass like dextrans, the problem arises of their effective separation from the vesicles. Indeed, the gap between the respective elution volumes of the vesicles and free

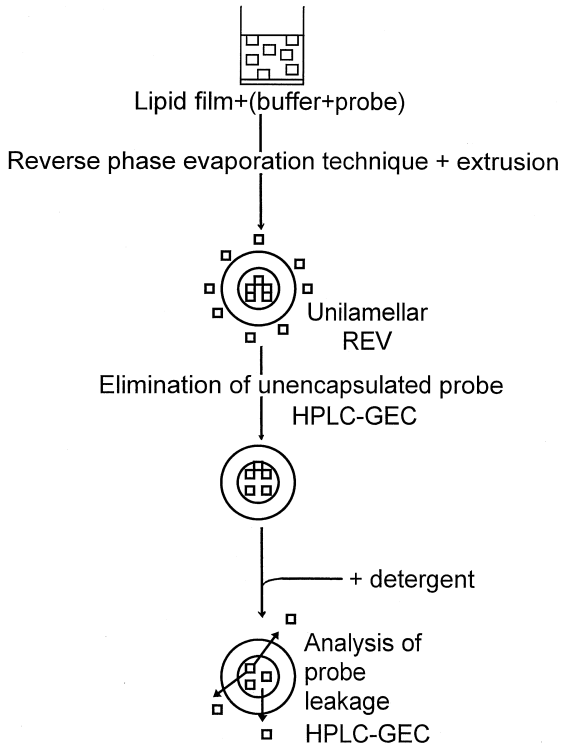


Fig. 2. Procedure used for permeability measurements.

dextrans becomes more and more reduced as the molecular mass of the dextran increases. Moreover, without on-line detection of the elution profile, it is very difficult to estimate the efficiency of the separation and/or to select the fraction of the eluted vesicles which is not contaminated by the marker.

From a general point of view, in permeability studies of vesicles using encapsulation of dextrans [5,7,15–21], conventional GEC was only used to remove the unencapsulated molecules without allowing the size analysis of the vesicles.

HPLC-GEC developed in our laboratory [9–12] has been proved to be a powerful method for sizing and physical characterization of liposomes. The G6000 PW column offers the best performance in terms of selectivity, lipid recovery and separation domain of vesicles. This paper shows that G4000 PW column, which is efficient in the separation of particles smaller than vesicles, corresponds to the size fractionation range of the polysaccharides.

The HPLC-GEC experimental set-up is derived from that used previously for vesicle characterization [12]. For the present study, it was necessary to connect the G6000 and G4000 PW columns in series in order to optimize the separation of the particles. A double on-line detection by fluorescence and 90° light scattering was chosen, which enables simultaneous measurement of the marker concentration and the turbidity of vesicles.

Light scattering (90°) allows detection of the vesicles either containing or not containing dextran. Fig. 3A shows, as an example, the elution profile recorded for the REVo vesicles. The chromatograms obtained by elution of the 4400-REV1 and 4400-REV88 vesicles are presented in Fig. 3B,C, and that of the 40500-REV1 vesicles in Fig. 4A. The first peak centered at $V_e = 13.45$ ml (Table 2) corresponds to the elution volume of the vesicles. The fluorescence intensity measured along the elution peak depicts the fluorescent dextran encapsulated inside the vesicles. The second peak which is observed at a higher elution volume (Table 2) corresponds to the free unencapsulated dextran, i.e. the dextran in the external medium of the vesicles.

The chromatograms clearly indicate that the combination of the two columns allows to achieve a complete separation of the unencapsulated dextran from the vesicles, for dextrans of molecular mass

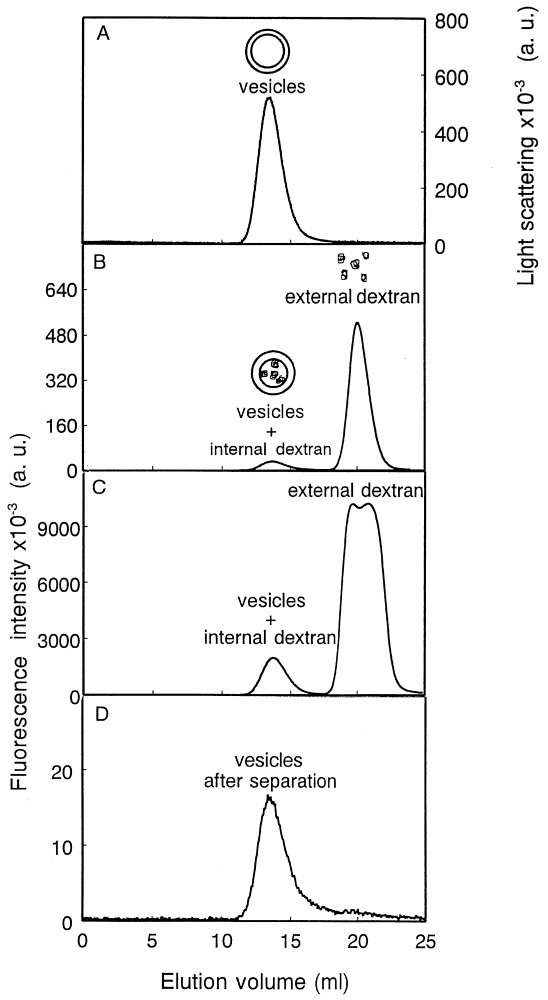


Fig. 3. Chromatograms of EPC–EPA REVs (9:1, w/w) eluted from TSK-G6000 and -G4000 PW columns connected in series. (A) REVo without dextran encapsulated; (B) 4400-REV1 prepared in buffer containing 1 mg $ml^{-1}$ (0.23 m*M*) of FITC-dextran 4400; (C) 4400-REV88 prepared in buffer containing 88 mg $ml^{-1}$ (20 m*M*) of the same dextran (see Section 2); (D) 4400-REV1 after separation of the unencapsulated marker. Eluent: aqueous buffer (145 m*M* NaCl, 10 m*M* Hepes, pH 7.4). Sample loading, 200 μl; flow-rate, 1.0 ml $min^{-1}$. Detection: 90° light scattering at 493 nm for REVo (A); fluorescence (the excitation and emission wavelengths were 493 and 514 nm, respectively) for 4400-REV1 and 4400-REV88 (B–D).

varying from 4400 to 40 500. Moreover, the separation is efficient even for a high amount of the probe. The fraction corresponding to the elution peak of the vesicles was collected and chromatographed again under the same conditions (Fig. 3D, Fig. 4B).

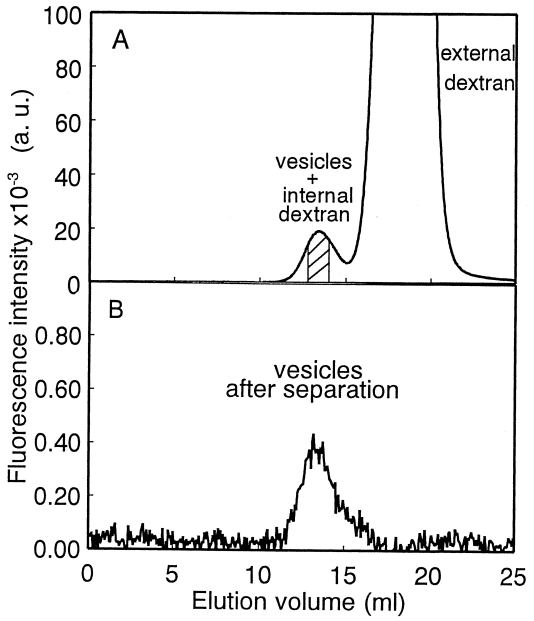


Fig. 4. Separation of 40500-REV1 prepared in buffer containing 1 mg $ml^{-1}$ of FITC-dextran 40500 from unencapsulated marker (A). The elution conditions are the same as in Fig. 3. The shaded area corresponds to the fraction of vesicles collected and chromatographed again under the same conditions (B). Detection: fluorescence (the excitation and emission wavelengths were 493 and 514 nm, respectively).

Only the peak corresponding to the elution of the vesicles is detected, indicating the efficiency of the separation. The elution parameters related to the vesicle peak (Table 2) also demonstrate that encapsulation of dextran does not alter the characteristics of the vesicles. The mean diameters (MD) of the collected vesicles (136–147 nm) measured independently by QELS were found to be similar to that of REVo (141 nm), which confirms the HPLC-GEC results. By injecting the REV suspensions containing the probe at different times, the stability of the

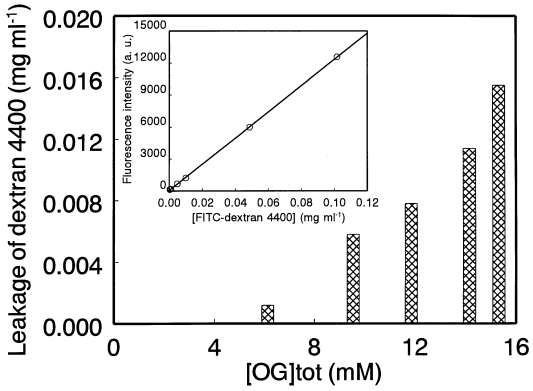


Fig. 5. Evolution of the leakage of FITC-dextran 4400 encapsulated in 4400-REV1 as a function of the OG concentration in the sample $[OG]_{tot}$. The leakage is expressed as mg $ml^{-1}$ of the released marker determined from the calibration curve reported in the inset. Inset: fluorescence intensity vs. concentration of FITC-dextran 4400. Solutions of dextran in aqueous buffer were analysed by HPLC-GEC as described in Fig. 3. The intensity of fluorescence corresponds to the maximum of the elution peak.

vesicle size versus time can be therefore controlled in parallel with stability of encapsulation.

The applicability of the HPLC-GEC method to the permeability studies was tested with 4400-REV1 after complete removal of the unencapsulated marker (Fig. 3D), by using OG as leakage agent. Fig. 5 shows the evolution of the dextran release versus the OG concentration in the samples. Insertion of OG in the lipidic membrane of the vesicles creates passageways [2,6,8] which lead to a progressive leakage of the vesicle content in a manner that depends on both the size of the entrapped marker and the amount of detergent in the vesicle membrane. If these pathways are very small, no release of dextran is observed. The leakage of the probe was quantitatively determined by means of the calibration curve (Fig. 5, inset). Detection by fluorescence proves to be very sensi-

Table 2
Elution parameters of REVo, dextran-loaded REVs and unencapsulated dextran

| Vesicles | Vesicle peak | | Unencapsulated dextran | |
|---|---|---|---|---|
| | $V_e$ (ml) | Half-height $V_e$ values (ml) | $V_e$ (ml) | Half-height $V_e$ values (ml) |
| REVo | 13.38 | 12.68–14.64 | — | — |
| 4400-REV1 | 13.45 | 12.57–14.72 | 19.95 | 19.29–20.94 |
| 4400-REV88 | 13.55 | 12.67–14.74 | 20.37 | 18.74–22.93 |
| 40500-REV1 | 13.43 | 12.51–14.72 | 18.29 | 17.23–19.50 |

tive, since concentrations as low as $5\times10^{-4}$ mg $ml^{-1}$ of FITC-dextran can be detected. The sensitivity of the detection shows that only a small amount of the probe needs to be encapsulated. Likewise, leakage of fluorescent dextrans through the vesicle membrane, when exposed to any other stress conditions such as time, temperature, addition of foreign molecules, etc., can be analysed in the same way.

## 4. Conclusion

The HPLC-GEC system presented in this paper offers major advantages for permeability measurements regarding efficiency in particle separation, control of the integrity of vesicles, quantification and kinetic evaluation of the leakage. The ability of measuring simultaneously different parameters, such as the marker concentration and particle turbidity, is of particular interest.

This methodology can be used with different types of columns and detectors and applied to other encapsulated water-soluble compounds or vesicle incubation in aqueous media. This represents a powerful tool in the fields of drug delivery and liposome technology.

## Acknowledgements

We thank Dr Yoshio Kato (Toyo Soda, Japan) for the gift of the TSK columns. K.A. is supported by I.F.S.B.M.

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