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CHARACTERIZATION OF LOADED LIPOSOMES

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ABSTRACT

The examination of liposomes using traditional (SEC) and high performance (HPSEC) size discharge chromatography is the main topic of this paper. Regarding the field of liposome applications, the effectiveness of both procedures is evaluated. The use of an HPLC system coupled to gel columns with a size selectivity range permitting liposome characterisation in addition to particle separation significantly increases the potentiality of conventional SEC. Experimental aspects of size exclusion chromatography are reviewed, and a method based on HPSEC coupled to multidetection modes for on-line analysis of liposomes via label or substance encapsulation is offered. Applications of conventional SEC and HPSEC that deal with polydispersity, size and encapsulation stability, bilayer permeabilization, liposome production and reconstitution, and integration of amphiphilic compounds are presented. A straightforward and effective method for examining the encapsulation, insertion, and interaction of compounds from.

Abbreviations: Ca, calcium; C16G2, diglycerol hexadecyl ether; Chol, cholesterol; CholHS-PEG2000, cholesterylhemisuccinate monomethoxypolyethylene glycol; Con A, concanavalin A; DCP, dicetyl phosphate; Dil, 1,1V-dioctadecyl-3,3,3V-tetramethylindocarbocyanine amide perchlorate; DG, dodecyl-h-D-maltoside; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; EPA, egg phosphatidic acid; EPC, egg phosphatidylcholine; SEC, size exclusion chromatography; FITC-dextran, fluorescein-isothiocyanate dextran; Hepes, N-[hydroxyethyl]piperazine-NV-[2-ethanesulfonic acid]; HPSEC, SEC performed with a high performance chromatography (HP) equipment; LUV, large unilamellar liposomes; M-PEG-cholesterol, monomethoxypoly (ethylene glycol) cholesteryl carbonate; MLV, multilamellar liposomes; NSV, sonicated non-ionic monoalkyl

surfactant-cholesterol liposomes; OD, optical density; OG, octylglucoside (n-octyl-h-D-glucopyranoside); PA, phosphatidic acid; PEG-Chol, poly(ethylene glycol) cholesteryl ether; SUV, small unilamellar liposomes; SPC, soy phosphatidylcholine; SPG, soy phosphatidylglycerol; Sph, sphingomyelin; 99mTc, technetium.

KEYWORDS: High performance gel exclusion chromatography; Gel filtration; Liposome; Liposome reconstitution; Encapsulation; Permeability.

INTRODUCTION

Liposome are small article vesical of spherical shape created when phospholipids self-build in water. One or more curved lipid bilayers make up their membrane, which also contains some liquid medium which they are floting.[1–4] Liposomes can encapsulate in water heating compounds in the aqueous interior cavity or hydrophobic molecules in the bilayer membrane in addition to amphiphilic substances because of the phospholipids' amphiphilic nature and their organisation in closed structures (Fig. 1, Parts 1 –6). These unique qualities have led to several uses for lipid liposomes as in vivo drug delivery systems and models for biological membranes. Depending on the solubility and polarity properties of the drug, liposomes and drugs can interact in a variety of ways. They can be intercalated in the polar head group region, adsorbed on the membrane surface, anchored by a hydrophobic tail, or trapped in the inner aqueous compartment in addition to being inserted in the lipid chain bilayer region (Fig. 1, Parts 7– 8). The development of techniques enabling for liposome preparation control, including particle size, stability, and encapsulation rates, as well as the determination of the encapsulated compounds' leakage engertic, is a precondition for the application of drug loading liposomes in vivo. The lipids' makeup and the processes used to create liposomes determine their chemical and physical properties. For their usage as model systems and drug carriers, it is crucial to determine their structural properties, including the structure of the lipidic bilayer, the average number of lamellae per liposome, the average size and size distribution, and the internal volume. The characterization of liposomes has been carried out using small-angle X-ray diffraction and scattering, static and dynamic light scattering, freeze fracture electron microscopy, analytical ultracentrifugation, sedimentation field flow fractionation, size (or gel) exclusion chromatography, and internal volume measurements. High performance size exclusion chromatography (HPSEC)[11-14] and conventional size exclusion chromatography (SEC)[5-10] are two methods that are particularly intriguing because they allow for the physical separation of liposomes from small solutes as well as the division

of liposomes into a number of subpopulations. This makes it possible to determine size and size distribution precisely. Furthermore, since this method does not harm the materials, fractions after collection can be tested for a variety of additional assays. However, HPSEC outperforms traditional SEC in terms of both analysis reproducibility and separation effectiveness.[11-15] It has been demonstrated that HPSEC is a very effective approach for liposome size distribution analysis, size stability investigation, liposome fusion and aggregation analysis, among other applications.[14]

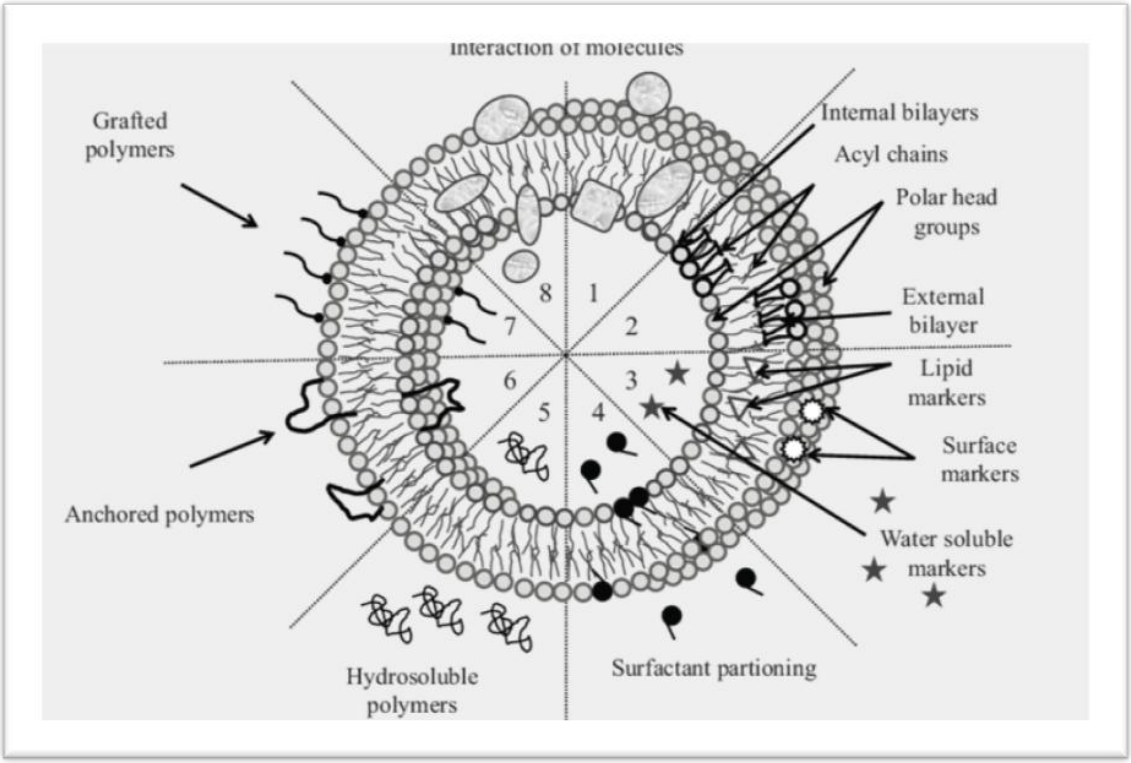

Fig.1: Illustration of unilamellar liposomes in a schematic representation.

Fig. 1. Schematic representation of unilamellar liposomes (1) and filled substance locations (2 – 8). Water-soluble markers are introduced in the internal and exterior aqueous media, amphipatic labels are put in the polar head groups, and hydrophobic markers are primarily found inside the acyl chains of the bilayer. (2). hydrophilic polar moiety is divide between the liposome bilayer and the aqueous media. (3). Entrapment of hydrosoluble polymers with different average molecular weights is possible. (4). Covalently coupled (5) and anchored liposomes may be used to create stealth liposomes. (6) polymers. Drugs, proteins, and biological macromolecules all have different interaction sites (7, 8). Transmenbrane proteins connect the lipid bilayer with locations exposed to the aqueous phase, amphiphilic molecules orient to create bilayers, and water-soluble molecules can be loaded into the core of the

liposome or bound to the bilayer surface. Numerous components and uses of traditional SEC and HPSEC have already been explored in relation to liposomes and proteoliposomes.[13,14,16] We focus on the SEC and HPSEC analyses of loaded liposomes in this paper. We go through some useful aspects of size exclusion chromatography and show how a method based on HPSEC coupled to multiple detection modes may be used to investigate the on-line stability and label encapsulation of liposomes. Only a few examples of applications are provided, such as polydispersity, size and encapsulation stability, bilayer permeabilization, liposome synthesis and reconstitution, and incorporation of amphiphilic chemicals. They emphasise the significance of the information that this method offers.

2. Conventional SEC and HPSEC

The terminology now used to describe the separation of solutes or aggregates by their molecular size includes Molecular Sieve Chromatography, Gel Filtration, Gel Permeation, Size Exclusion Chromatography, and others. As stated by Hagel[17], The legal name of the method's descriptive phrase, SEC, stands for the separation principle. Gel filtration is the process of using SEC in aqueous solutions. For the sake of clarity, the following will make use of both conventional SEC and HPSEC.

2.1. Principle

The same fundamental idea underlies both conventional SEC and HPSEC, namely the size-dependent penetration of solubilized molecules or dispersed molecular aggregates across the relevant gel. Both separation and dispersion processes are included in the conclusion.[18] The first category includes any secondary retention technique that isn't a size exclusion strategy. The latter expands the chromatograms since it depends on molecular diffusion and column packing. The premise behind a real size exclusion approach is that interactive processes like partition and adsorption won't have an impact on retention behaviour. Liposome retention is greatly influenced by liposome size and morphology. But the stiffness or flexibility of the bilayer may matter depending on the lipid composition of the liposomes. The encapsulated substance, the lipid bilayer, and the gel's chemical make-up may all interact with the gel matrix in some way, with the existence of charged species making this more likely to happen. These interactions may modify the properties and content of the studied liposomes by affecting sample recovery from the column gel.

2.2. Available gels

For the traditional SEC of liposomes, various soft gels, such as crosslinked dextrans and large-pore Agarose gels (Bio-gel A150, Sepharose 2B or CL-2B, and Sepharose 4B or CL-4B), have been utilised (Sephadex). These gels are widely used to remove minute solutes that liposomes are unable to retain. The correct gel can be employed to create a separation in which the liposomes are excluded and the solute is fully integrated. These gels' upper exclusion limit prevents them from being divided into a wide range of sizes (e.g., 60-nm liposomes for Sepharose 4B[19], 100-nm liposomes for Sepharose 2B[7,15,19-22], and 150-nm liposomes for Agarose-150-m[23]; see also Table 1 in Ref.[16]). It is possible to size and separate liposomes up to 200-300 nm using a copolymer of allyl-dextran and methylene bisacrylamide known as Sephacryl S-1000.[8-10,24,25] For both HPSEC and conventional SEC chromatography, this gel is suitable. It displays good mechanical stability for separations at low pressures and flow rates (0.3 to 0.5 ml/min).[11] Despite the fact that these standards, solid polymer spheres, are extremely different from the liposome structure, which is based on an aqueous core separated by a lipid shell, polystyrene monodisperse latex was utilised to calibrate the column for liposome sizing using this gel.[9,26] A more relevant selectivity curve for Sephacryl S-1000 has been performed with unilamellar liposomes with a diameter range of 17 to 181 nm.[11] It has been demonstrated that when the column is presaturated with lipid, the liposomes recover well and keep their integrity (small unilamellar liposomes, SUV). A Sephacryl S-1000 column may be used with low exclusion limit sepharyl gels, such as S-500 Hr, S-400 Hr, and S-300 Hr (from 100 to 60 nm).[26–28] Numerous prepacked HPSEC gel columns have been used in the field of liposomes.

By sequentially extruding through 0.4, 0.2, 0.1, and 0.05 Am filters, egg phosphatidylcholine (EPC)/egg phosphatidic acid (EPA) liposomes of different sizes were created. The efficacy of TSK Type gels based on semi-rigid hydroxylated polyether (GDNA PW, G6000 PWXL, G6000 PW, and G5000 PW, Toyo Soda, Tokyo, Japan) was examined using nuclepore membranes. The TSK-G6000 PW gel was demonstrated to be the most effective for determining average size and size distribution[14], lipid recovery[11,13,14], and size range (20 to over 500 nm).[11] When separating smaller diameter liposomes from other substances including those with low molecular weights, the G6000 PW column can be utilised in conjunction with the G5000 PW and/or G4000 PW.[13,29] Superose 6, a semi-rigid cross-linked polysaccharide, ranges in size from 1 to 1.5 nm micellar aggregates to 25 nm liposomes and has a narrow size range with excellent lipid recovery.[30]

2.3 Technical consideration

The methodology of chromatographic analysis is described in details in Refs.[13,14,16] Here, we briefly discussed a few key elements that are crucial for using this technology in a practical and effective manner, including the selection of the gel and column, material recovery, the type of detection, and column calibration.

The size range of the liposomal suspension and the kind of separation or analysis to be carried out dictate the choice of column and gel bed. Before analysis, the gel must be saturated with lipids to prevent material loss due to lipid adsorption on the gel. The elution buffer, which is often where the liposomes are suspended, is used as the preferred column pre-treatment method because the liposomes' small sizes ensure effective lipid penetration within the gel pores. When liposomes are injected at constant sample loadings, they achieve presaturation when they elute at the same elution volumes and produce peaks with the same regions.

The usual rule is that the eluent should be isoosmolar with both the internal and external aqueous media of the liposomes to prevent osmotic shocks, which could injure or cause the liposomes to shrink. The eluent should be in equilibrium with the loaded liposomes when water-soluble chemicals are present. The eluent should correspond to the effective continuous medium in equilibrium with the mixed aggregates, which can partition between the liposome bilayer and the watery continuum, in order to maintain the system's true properties throughout the analysis while using amphiphilic substances like surfactants (see Section 3.3.4). The deformability of the gel beads and/or packing down with soft gels may have an effect on size separationIt is necessary to control the flow rate in particular to avoid compression of the gel beads. The pressure should be between 10 and 15 bars for semi-rigid gels and should not exceed 1 bar for soft gels. Depending on the scale, either the preparative scale or the analytical scale, the gel volume and sample loading are customised. The void and total volumes of columns can be determined using nonextruded multilamellar liposomes (MLV) and incredibly small solutes (NaCl, sodium azide, and glucose), respectively.

Online detection should be chosen whenever possible since it guarantees a precise description of the elution profiles. Otherwise, fraction collections for later analyses must be gathered. Liposomes can be found directly through the measurement of light scattering, refractive index, total lipid concentration, or indirectly through the use of fluorescent or radiolabel markers placed either inside the liposomes' bilayers or their interior aqueous spaces. Light

scattering can be used to determine the size and shape of liposomes, but it cannot be used to determine how much lipid is actually within because there is a nonlinear relationship between the intensity of the scattered light and liposome size. The ability to quantify the amount of lipid in the liposomes is made possible by the use of refractive index detection[13,14], phosphorus analysis, radiolabeled lipids (14C-DPPC, 3 H-DPPC), or fluorescent lipids (diphenylhexatriene).[31] It is feasible to evaluate the efficiency of encapsulation, the content of the release, and the kinetics of leakage using water-soluble radioactive, fluorescent, or other chromophore labels.

To construct calibration curves for applications using liposomes loaded with water-soluble fluorescent labels, the loaded chemicals can be eluted in relation to their concentration (such liposome leakage analysis).

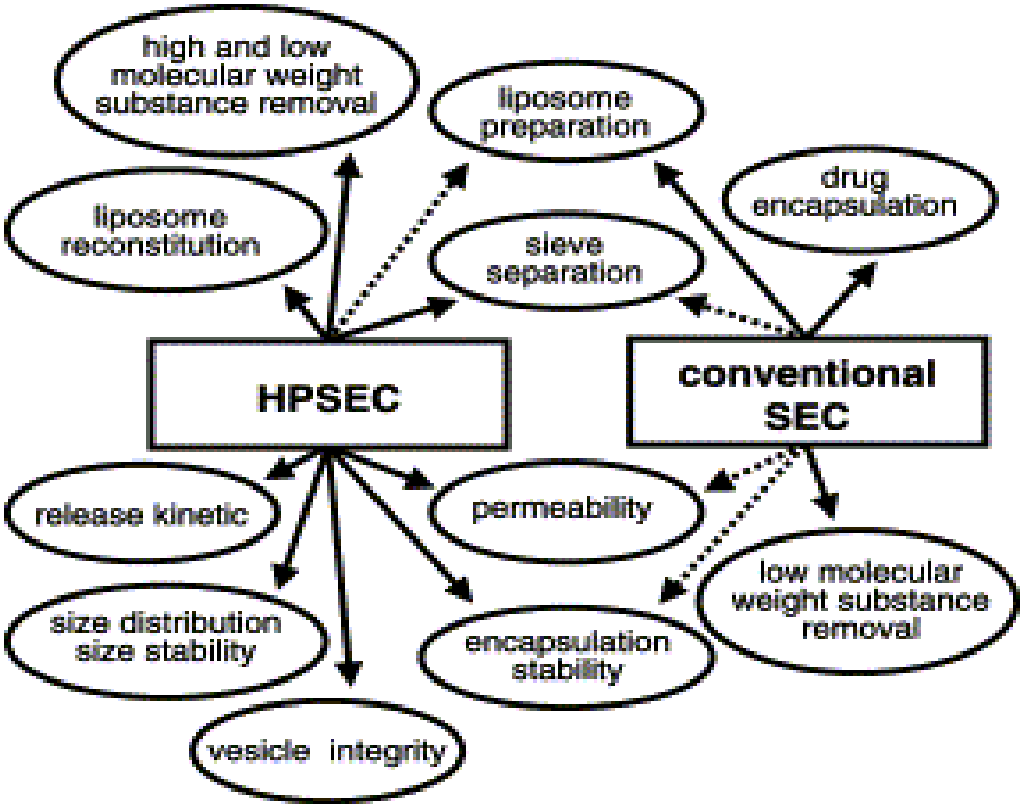

Fig. 2: To construct calibration curves for applications using liposomes loaded with water-soluble fluorescent labels, the loaded chemicals can be eluted in relation to their concentration (such liposome leakage analysis).

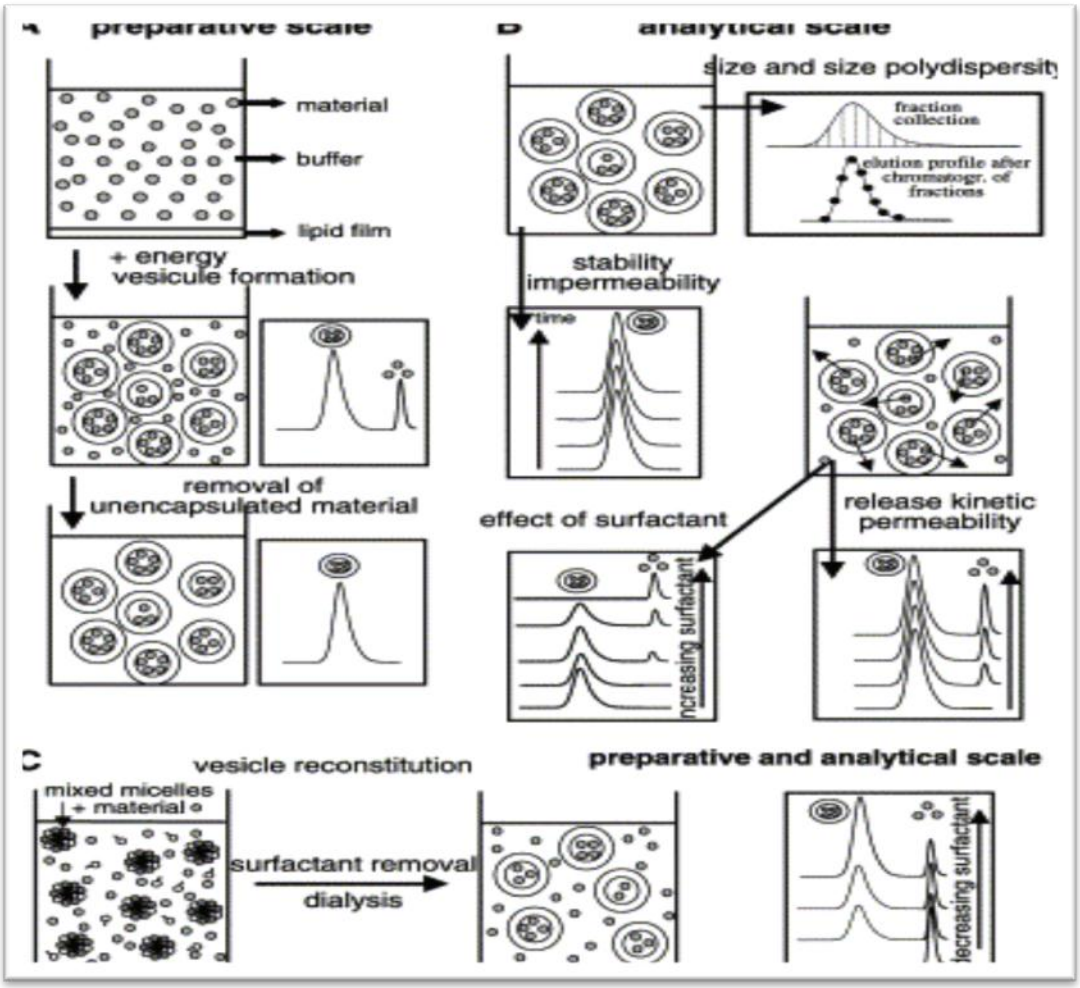

Fig. 3. The various chromatogram sequences that were obtained based on the results of the liposome study are represented schematically. The creation of loaded liposomes and elimination of unencapsulated material, the measurement of liposome size, size polydispersity, size stability, and impermeability, the observation of liposome solubilization by surfactant molecules, and studies of release kinetics are all examples of liposome-related processes; (C) reconstruction of liposomes. On a preliminary level, traditional SEC is quite useful, but additional research necessitates an HPSEC system. both intensity and concentration. Liposomes and fluorescent molecules are separated by elution on the columns, so it is not necessary to encapsulate a lot of labels due to the high sensitivity of the fluorescence detection. By doing this, the issue of quenching or inner filter effects is avoided[32], which result in a divergence from the linear connection between fluorescence intensity and label concentration. After the non-entrapped molecules have been eliminated, the total number of molecules encapsulated is determined by lysing the liposomes with a very small volume of a concentrated

surfactant solution for negligible dilution. Because of how effectively the surfactant diffuses and dissolves within the lipid bilayer, it was chosen.

2.4 Conventional SCE and HPSCE performance

The unique characteristics of the available columns connected with the usage of an HPLC system are the reason why HPSEC has benefits over traditional SEC. Faster run times, smaller sample sizes, much higher peak resolution, analysis reproducibility, and separation efficiency are all made possible by the penultimate step.[11-13,15] According to the separation and analysis efficiencies of both forms of chromatography, the application fields for each are reported in Fig. 2. the HPSEC the following is advised for liposome characterization: size distribution, size stability, integrity, removal of high molecular weight compounds, time-resolved studies (release kinetic, permeability, and encapsulation stability), and liposome reconstitution. For example, by removing surfactants, conventional SEC is well suited to produce liposomes, encapsulate drugs, and remove compounds with low molecular weight. Both methods can conduct liposome subpopulation separation, although the standard SEC gel columns only accommodate a limited range of liposome sizes.

On a preparative level, traditional SEC provides the opportunity to match the column size to the separation to be performed and so reduce the dilution factor, which could be an issue with prepacked HPLC columns. The ability to recover liposomes at the gel's empty volume also makes it possible to gather concentrated fractions. The inexpensive cost of conventional gels, which do not require regeneration in the event of contamination, is another important consideration. Analytically, HPSEC is by far the more effective. It offers accurate data in a range of research. The sequences of chromatograms associated with the process under study are shown in Fig. 3, which provides an overview of the many analysis types that can be carried out.

APLLICATON

3.1 Liposome loading

Numerous methods can be used to create multilamellar and unilamellar liposomal systems.[33] Materials in liposomes are encapsulated using the same techniques.[34] Here is a summary of a few of the steps.

By dissolving the lipid(s) in an organic solvent and then allowing the solvent to evaporate, embrane markers or lipophilic compounds are introduced into the lipid(s). In the aqueous

buffer used for the liposomal preparation, compounds that are soluble in water are dissolved. Their affinities to the various sections of the liposome are related to their location in relation to the liposome bilayer[35] fig.1. Multilamellar liposomes, tiny unilamellar liposomes (Sonicated Liposomes, SUV), or extruded liposomes made by sequential extrusion down through polycarbonate membranes can all be made, depending on the application. Another method of encapsulation entails the formation of loaded liposomes through the removal of surfactant molecules from a mixture of mixed lipid-surfactant micelles. Either this mixed micellar solution or the buffer used to make the liposomes without the use of surfactants can be utilised to administer the medication. By using SEC, dialysis, or dilution with pure buffer, detergent can be removed. Preformed liposomes can also be used for encapsulation; in this case, the encapsulant is added to the liposome suspension's external medium. To enhance the trapping, several cycles of freezing and thawing are frequently used. Whatever the method utilised to manufacture the liposomes, only a small portion of the solute molecules are trapped while the majority are still in free solution. The removal of unentrapped material is frequently carried out by centrifugation, dialysis, or traditional SEC.

In general, the removal technique employed should not harm the liposomes, as this could result in a partial loss of the contained material. With the use of centrifugation, this might be the case. Because different research used centrifugation settings that ranged from 700 g to 150,000g[36], At high g, small liposomes can be lost in the supernatant while the loaded solute can be removed from the liposomes, allowing for the first possibility—a diversity of liposome sizes—to be produced. According to research by Schubert et al.[37], liposome separation from released inulin on a Sepharose CL-4B was much less effective than ultracentrifugation (140,000 g) in releasing entrapped inulin from liposomes. The cutting off of the dialysis membrane limits the amount of chemicals that can be removed by dialysis. This is true of large compounds like dextrans.

Because the liposomes are excluded and the solute is fully incorporated in the available gels (Sephadex, Sepharose), the separation of tiny molecules by traditional SEC is typically effective.[36] The difficulty comes from effectively separating high molecular mass solutes from the liposomes. In fact, if the distance between the liposomes' and unentrapped molecules' respective elution volumes becomes too small, both elution peaks may overlap. utilising Sephacryl S1000 columns as well as additional HPSEC using TSK-G6000, G5000,

or G4000 PW HPLC columns in series[13,29] combined with a low elution rate significantly improves the separation.

Regardless of the method employed to remove the unencapsulated drug, management of its effectiveness is crucial since a residual concentration of the free substance in the liposomes' external medium might obstruct future analyses of stability, release kinetics, or permeability.

3.2 HPSEC methodology for analysing loaded liposome.

Here, we present a method created in our lab.[29] Two TSK-PW columns (30 * 0.75 cm, Toyo Soda, Japan) are used in the HPSEC experimental setup (Fig. 4), one of which is effective in size liposomes (G6000 PW), and the other at sizing smaller particles (G4000 PW). A Hitachi pump (Model L-6000) and a precision injection valve are included with the HPLC system (Rheodyne). Online elution monitoring is done with a three Window circulating quartz cell (ref 176.353-QS, Hellma, France) installed in X-format spectrofluorimeter Fluorolog SPEX (FL1T11) under computer control and thermostated at 25 jC. If necessary, fraction collection is used.

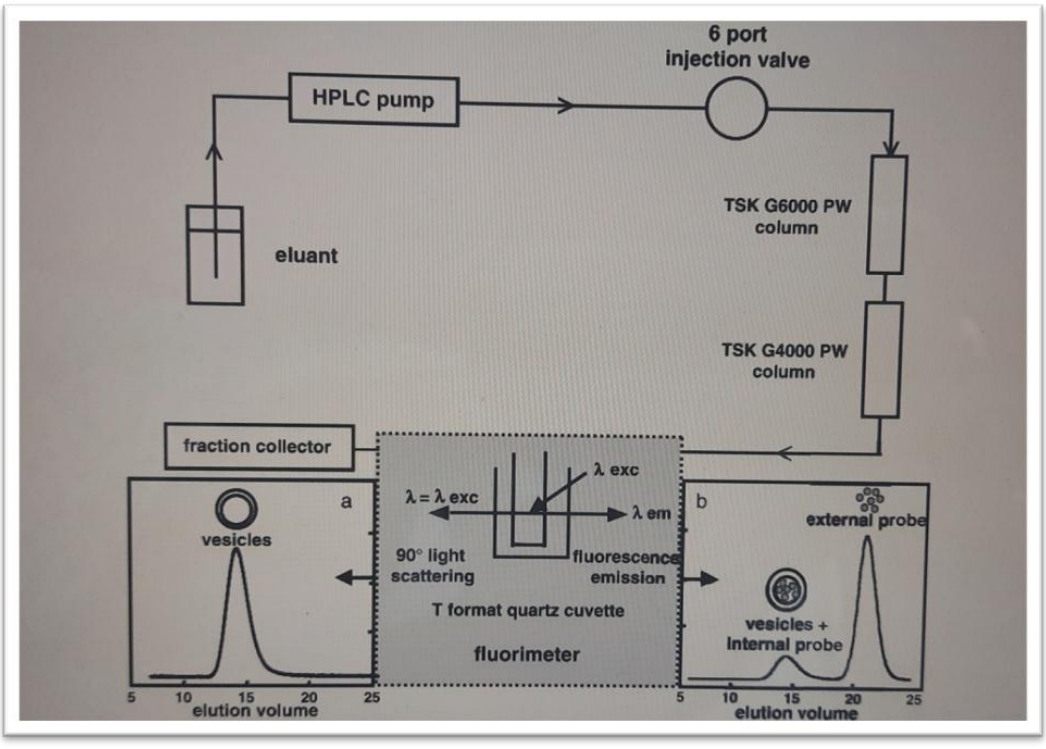

Fig. 4: HPSEC experimental set-up coupled with fluorescence (a) and 90j light scattering (b) on-line detections: (a) liposome characterization, (b) separation of unencapsulated probe from the loaded liposomes.

Extruded EPC/EPA liposomes containing water-soluble fluorescein-isothiocyanate dextrans (FITC-dextrans) with molecular weights ranging from 4400 to 147,800 were used to investigate the separation effectiveness of the HPSEC setup.[29,38] For all the polymers investigated, complete separation of the FITC-dextrans that had not been captured from the FITC-loaded liposomes was achieved, with the entrapping effectiveness sharply declining as the polymer chain length increased.[39]

The FITC-dextran 4400 is used as an example in Fig. 5 to show how the technique works. The simultaneous assessment of the marker concentration and the turbidity of the liposomes is made possible by the twofold on-line detection by fluorescence (excitation wavelength = 493, emission wavelength = 514 nm) and 90j light scattering at 493 nm.

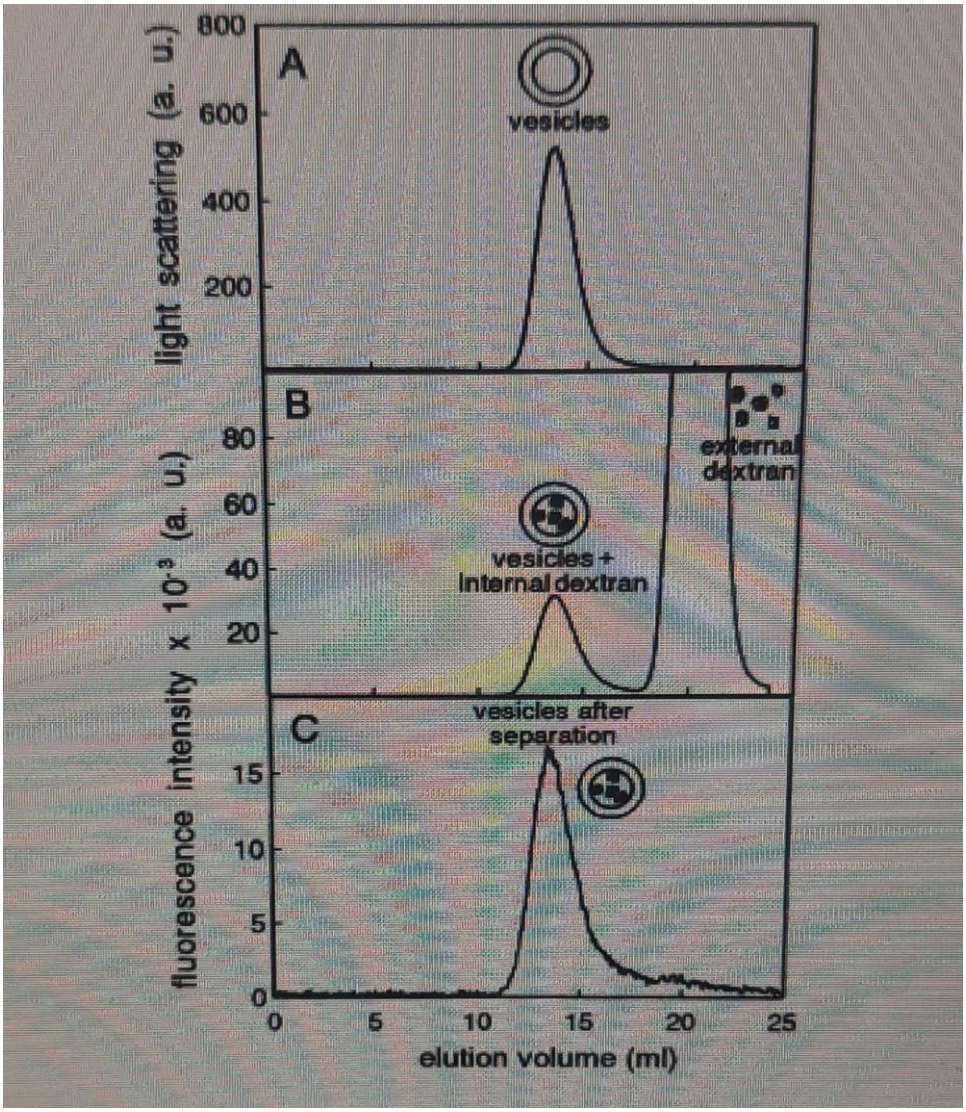

Fig. 5: Analysis of loaded liposomes using HPSEC. Elution profiles of extruded EPC/EPA (9:1, w/w) liposomes on TSK-G6000 and -G4000 PW columns connected in

series. Without dextran encapsulation, (A), produced in an aqueous solution containing 1 mg/ml of FITC-dextran 4400, (B), and (C), FITC-4400 liposomes after the unencapsulated marker has been separated. Aqueous buffer, eluent (145 mM NaCl, 10 mM Hepes, pH 7.4). 200 Al of sample loaded; 1.0 ml/min of flow. Online detections included the following: fluorescence (excitation and emission wavelengths were 493 and 514 nm, respectively); 90j light scattering at 493 nm (A) (B and C). from Ref.[29]

While the fluorescence intensity measured during the elution profile centred at Ve = 13.45 ml depicts the fluorescent dextran contained inside the liposomes, the peak observed at Ve roughly 20 ml is related to the dextran in the outer medium of the liposomes. As shown by the liposomes' post-separation elution curve[fig. 5C], the percentage of eluted liposomes that is not contaminated by the marker can be determined using online detection. This procedure also provides information about the loaded liposomes' prepared features. The fact that the elution parameters (Ve and halfheight Ve) of the dextran-loaded liposomes are the same as those of the unloaded liposomes produced using the same procedure demonstrates that dextran encapsulation does not alter the size or polydispersity of the liposomes. By injecting the liposome solutions containing the probe at different times, it is possible to control the stability of the encapsulation as well as the stability of the liposome size vs time.

3.3 Application field of conventional SEC and HPSEC

3.3.1 Liposome polydispersity

A crucial aspect of liposomes' physical makeup is their polydispersity. In order to evaluate the size and size distribution of liposome populations utilising HPSEC on a TSK-G6000 PW column, we thoroughly analysed a number of methods.[14] They can be employed with different kinds of column gels as long as the liposomes are not eluted at the column's void volume and are instead recorded preferentially by online detection for accurate drawing of the elution profile. However, precise measurements can only be made if the volume of the eluted liposomes sits on the plateau region of the selectivity curve. The selectivity curve of the column can be utilised to quantify the size of liposomes. This last stage also needs to be established using liposomes with various mean diameters that match the separation range of the column. It is recommended to first fractionate the liposomes across the column by gathering small fractions at the peak of the elution peak. This fraction should then be rechromatographically analysed, with the mean diameter being determined independently by quasi-elastic light scattering. The selectivity curve's accuracy will increase as a result.

Rechromatography of narrow fractions acquired during the elution profile makes it straightforward to determine the size polydispersity of a liposome preparation (for details, see Ref.[14]). Procedures that involve labelling the lipid bilayers together with the internal aqueous volume can be used to calculate the size distribution. Small, unilamellar liposomes composed of dipalmitoylphosphatidylcholine (DPPC) (Table 1, line 1). were labelled with 3 H-DPPC and 14C-mannitol. The exterior labelling was eliminated using conventional SEC. The liposomes were evaluated by HPSEC using a two-column system with a TSK-G6000 and a TSK-G5000 column in series.[14] Turbidity was discovered online, and liposome fractions were collected. The relevant effective liposome radius, computed from the 14C/3 H ratio, and the lipid concentration of each fraction, estimated from 3 H counts, were used to construct the size distribution. A limited size distribution was discovered through study. A heterogeneous liposomal DPPC suspension that was tagged with 14C-DPPC and contains self-quenched calcein was analysed using the same procedure (Table 1, line 2). The dequenching calcein fluorescence/14C ratio was used to compute the effective liposome radius of each collected fraction, and the lipid content was used to determine the liposome size distribution. To ascertain whether there is a linear relationship between the calcein concentration and the fluorescence intensity, it is crucial to keep in mind that the quantitative measurement of calcein after liposome lysis should be performed in a dilution regime for which the inner filter effect and self-quenching are completely suppressed.[32]

3.3.2 Liposome stability

The following paradox should be resolved in the area of drug delivery via liposomes: the same substance should be released at a controlled pace after administration, despite being entrapped for as long and completely as feasible during storage. To imitate the stability of drug encapsulation, SUV, large unilamellar liposome (LUV), and MLV liposomes with various lipid compositions were used to encapsulate glucose, the sodium salt of quinoline yellow, and the lipophilic colour oil scarlet(Table 1, line 3).[40]

Table 1: Synopsis of conventional SEC and HPSEC studies: size parameters and stability of liposomes.

No.	Lipids	Membrane marker	Aqueous marker	Column gel	Applications	Aim of analysis	References
1	DPPC SUV	^{3}H-DPPC	^{14}C-mannitol	Sephadex G25M satured with DPPC SUV TSK-G6000+	Free marker removal Vesicle	Size distribution	14

				G5000PW	fractionation		
2	DPPC polydisperse vesicles	^{14}C-DPPC	80mM calcein	TSK-G6000	Vesicle fractionation	Size distribution	14
3	Soja lecithin , hydrogenated soja lecithin, chol, MLV LUV SUV	Oil scarlet	Na quinoline yellow glucose	Sephadex G50	Vesicle/free probe separation	Liposome stability	40
4	EPC/DCP/Chol (70/20/10) MLV LUV SUV	^{3}H-EPC	^{14}C-inuline horseradish peroxidase	Sephadex G200 Sepharose 2B	Vesicle/free probe separation	Liposome stability kinetic of release	41
5	EPC/Chol/PA (7/2/1), EPC/Chol/PA (7/7/1), 200-nm SUV 70-nm SUV	^{14}C-DPPC, ^{99m}Tc			Vesical/free probe separation	Liposome stability	15
6	$C_{16}G_2$/Chol/DCP (47.5/47.5/5 wt.%) Sonicated NSV			Scpharosc 4B, TSK-G5000 PW TSK-G6000 PW	On-line size stability	Vesical stability	42,43

On Sephadex G-50, conventional SEC was used to analyse the kinetics of the substance release as a function of the average liposome size, lipid composition, storage temperature, and addition of adjuvants. Chromatography of the liposome suspensions was performed at after lysis, the released marker was examined spectrophotometrically and at various time points those liposomes. A polydispersity index and the typical particle size were separately calculated assessed using QELS.

For applications in vivo, liposome stability in biological fluids should also be managed since serum or plasma components can harm the bilayer structure. One method to improve the carrier capacity of liposomes is to modify their phospholipid content. Large, tiny, and multilamellar unilamellar liposomes (Table 1, Line 4) were marked with 14C-inulin, 3 H-phosphatidylcholine, and horseradish peroxidase.[41] The unentrapped marker was removed by conventional SEC, and the aqueous label release from the filled liposomes following incubation in serum or plasma was examined. The breakdown of phospholipid bilayers is thought to be the cause of the leakage. Conventional SEC has demonstrated how leakage varies with incubation conditions and how high cholesterol (Chol) content has a stabilising effect. Sphingomyelin (Sph) incorporation improves the liposomes' structural stability.

The role of cholesterol as a protective agent against serum harmful activity was also studied using negatively charged liposomes that were both cholesterol-poor and cholesterol-rich and loaded with technetium (99mTc), a liposome surface ligand [table 1, line 5].[15] Their stability was evaluated for both in vitro (in saline and in serum) and in vivo (living) circumstances using conventional SEC on Sepharose 4B and HPSEC on a TSK-G5000 PW column. The 200- and 70-nm liposomes required to be pre-processed by sonication in order for them to elute at the void volumes of the SEC and HPSEC columns, respectively. Their stabilities were assessed by co-eluting the radiolabels at the void volume of both columns. Both labels entered the gels across a wide molecular mass range when instability occurred after being at least partially transported from the empty volume on both columns. In this case, differences in the labels' elution profiles on Sepharose 4B and G5000 PW columns were observed, which were attributed to the different void volumes and sizes of the two liposome preparations in the two columns. The results showed that high ratio cholesterol liposomes reduced phospholipid loss when exposed to serum or in vivo, but cholesterol-poor liposomes were destroyed in serum and drastically altered in vivo. One of the advantages of using an HPSEC system has been noted, and the TSK-G5000 PW is good for monitoring the transfer of phospholipids between liposomes and serum proteins and for spotting variations in liposome sizes. The size stability of non-ionic surfactant liposomes (NSV) containing cholesterol was investigated using HPSEC [Table 1, line 6].[42,43] The two criteria that were looked at were the evolution of the liposome mean diameter as determined by the selectivity curve of the column[12] and their size distribution as deduced from the pattern of the elution profile. However, because unilamellar larger NSV (z 170 nm) are completely retained by the gel column, the study can only be done with NSV that have a small mean diameter (120 nm) when using the TSKG6000 PW column. This behaviour demonstrates how the above-mentioned elution method is affected by the liposomes' bilayer stiffness.

3.3.3 PEG-coted liposome

A second way to defend liposomes against solubilizing agents and immune system protein adsorption is to glue hydrophilic polymer chains to the liposome surface. Important factors to take into account are the coating's efficacy and the amount of polymer connected to the liposomes. These data were gathered using traditional SEC methods.[44-46] The stability of PEG-Chol liposomes (EPC/PEG-Chol and/or Chol) and their incorporation into liposomes (PEG-Chol) were examined [Table1, line 2].[44] In order to monitor PEGChol by fluorescence, the liposome membrane was tagged with 1,1V-dioctadecyl-3,3,3V-

tetramethylindocarbocyanine amide perchlorate (Dil), and the phospholipid concentration was determined using a conventional approach. PEG-coated liposomes and free PEG-Chol molecules were isolated using chromatography. Profiles obtained after gel filtering were used to calculate the levels of PEG-Chol incorporation in liposomes. According to the results, PEG-Chol cannot be completely assimilated at large molar ratios, and concentration levels vary depending on the length of the PEG chain. Traditional SEC on Sephacryl 400 was used to study the incorporation of cholesterylhemisuccinate monomethoxypolyethylene glycol (CholHS-PEG2000) into the membrane of contrast-carrying liposomes.[45]

Table 2: Synopsis of conventional SEC and HPSEC studies: polymer loading.

No	Lipids	Polymers	Membrane markers	Aqueous markers	Column gel	Applications	Aim of analysis	References
1	EPC/PEG-Chol and/or Chol extruded vesicles	PEG2200-Chol, PEG4400-Chol, PEG8800-Chol	^{3}H-DPPC	Dil	Sephorus CL-4B	coated vesicle/ free polymer	PEG incorporation liposome stability	44
2	SPC/Chol/SPG (6/3/1), SPC/Chol (7/3)	CholHS-PEG$_{2000}$		iopromide Gd-DTPA	SephacrylS-400	coated vesicle/ free polymer	CholHS-PEG2000 incorporation	45
3	DL-a-DPPC/Chol (3/1 mol) extruded vesicles	Hydrophobized polysaccharides		FITC-Polysaccharide	Sepharose -4B	coated vesicle/ free polymer separation	Efficiency of anchoring	46
4	DMPC, DMPC/Chol (7/3) extruded vesicles	PNIPAM-Py, b-PNIPAM-Py			Sephacryl S-1000 XK column	Coated vesicle/ free polymer separation	efficiency of anchoring	48
5	C16G2/Chol(50/50)/ M-PEG-Chol (50/50 –x/x) mol% SUV	M-PEG1000-Chol, M-PEG2000-Chol		calcine	TKS G-6000+ PW TSK G-6000+ G4000 PW	On-line analysis free calcein removal calcein release	PEG-Chol vesicle characterization Stability	49 50

These liposomes (Table 2, line 2) were created by passing the water-soluble radiographic contrast agent iopromide through polycarbonate membranes with progressively smaller pore sizes in conjunction with freeze-thaw cycles.[47] Iopromide-liposome: soy phosphatidylcholine (SPC)/cholesterol/soy phosphatidylglycerol (SPG), 6:3:1. It was examined how the surface modification of the liposomes influenced their properties using conventional SEC and other methods. CholHSPEG2000 has been successfully incorporated into liposomal bilayers via SEC, as shown in Fig. 6. A variety of hydrophobized polysaccharides were used to make polysaccharide-coated liposomes (Table 2, line 3).[46] The amount of polymer bonded to the liposome surface was measured using SEC on Sepharose 4B, and the polysaccharide-coated liposomes were distinguished from free polysaccharide. The polymers are FITC-labeled. This sum, which was extracted from liposomes, was defined in relation to the first added sum by the proportion of the polymer.

The coating effectiveness was examined in relation to different polysaccharides, including pullulan, dextran, and mannan, as well as the hydrophobicity of the anchors (cholesteryl, hexadecyl, and a,aV-dodecyldiglyceryl diether groups). The coating effectiveness of liposomal surfaces was shown by conventional SEC to be closely correlated with the hydrophobicity of the anchors, with the cholesteryl moiety being the best. A modest link between pullulan and liposomes was also revealed by the chromatographic examination. Pullulan was thus removed from the liposomes during gel filtration even though pullulan binding to liposomes was identified by fluorescence because this binding was too weak. Similar outcomes were attained for the binding of poly-(N-isopropylamides) modified by the same hydrophobic substituent, either at random locations along the macromolecular chain or specifically at one chain end, to liposomes containing dimyristoylphosphatidylcholine (DMPC) (Fig. 7).[48] The end labelled polymer is strongly anchored into the liposomes as they coelute, according to conventional SEC (Table 2, line 4) (Fig. 7A and B). While eluting through the column, the randomly labelled polymer liposomes totally disintegrated and no polymer was found in the lipid fraction (Fig. 7C and D). The increased hydrophobicity of the end-labeled polymer was thought to be the cause of the variation in elution behaviour.

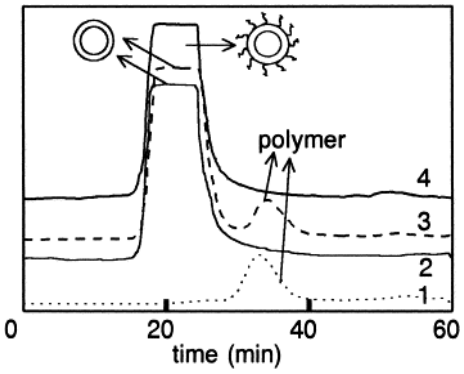

Fig. 6. Polymer integration into liposome bilayers. Liposomes with 5 mol% of CholHS-PEG (curve 4) and conventional SEC on Sephacryl S400 of CholHS-PEG polymer (curve 1), SPC/Chol/SPG liposomes (curve 2), liposome/polymer combinations (curve 3), and conventional SEC on Sephacryl S400 of CholHS-PEG polymer (curve 1). (see text). Based on Reference.[45]

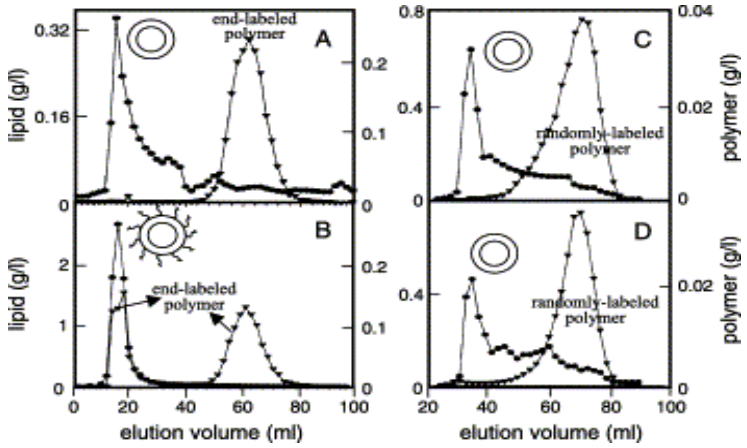

Fig. 7: Polymer architecture's impact on binding to liposome bilayers Conventional SEC on Sephacryl S1000 of (A) DMPC liposomes (.), (B) DMPC liposomes (.) with b-PNIPAM-Py (z) end-labeled polymer indicating polymer anchoring into liposomes, and (C) of DMPC/Chol liposomes (.) with PNIPAM-Py (z) randomly labelled polymer indicating no polymer/liposome from Ref.[48]

By providing them with a steric structure, monomethoxypoly(ethylene glycol) cholesteryl carbonates (M-PEG-cholesterol) were able to stabilise non-ionic surfactant liposomes.[49] Two alternative chain lengths were examined along with M-PEG-cholesterol (MPEG1000-cholesterol and M-PEG2000-cholesterol). NSV was produced by sonicating M-PEG-cholesterol with hydrated mixed films of C16G2, cholesterol, and NSV. Using a TSK-G6000 PW column with turbidity on-line detection, HPSEC performed a sizing investigation that revealed that M-PEG-cholesterol was entirely incorporated into the C16G2/cholesterol

membrane (Table 2, line 5). (Fig. 8A). However, the M-PEG-cholesterol1000 and M-PEG-cholesterol2000NSV could only be kept intact by a small amount of polymer (10-20 mol percent for MPEG1000-cholesterol and 5-10 mol percent for M-PEG2000-cholesterol). The growth of disk-shaped bilayer portions caused the elution profiles to trend toward smaller particles above this point, according to findings from cryo-transmission electron microscopy. The stability and impermeability properties of conventional NSV (cholesterol/C16G2/dicetylphosphate) and M-PEG-cholesterol-NSV containing small amounts of M-PEG-cholesterol were examined using calcein encapsulation within the internal aqueous volume of the liposomes.[50] The TSKG6000 PW and -G4000 PW two-column systems were used for the analysis together with the aforementioned on-line fluorescence and 90j light scattering detections (Table 2, line 5).[32] The partial release of calcineurin by NSV and M-PEG-cholesterol-NSV. In spite of the fact that calcium diffusion through the lipidic membrane was three times higher in the presence of cholesterol grafted onto polymer chains, regardless of the length of the polymer chains, over the course of two weeks of storage at room temperature, the efflux from the liposomes followed a first order law.

(Fig. 8B). The M-PEG-cholesterol-physical NSV's stability was evaluated using both 90j light scattering and fluorescence detections.

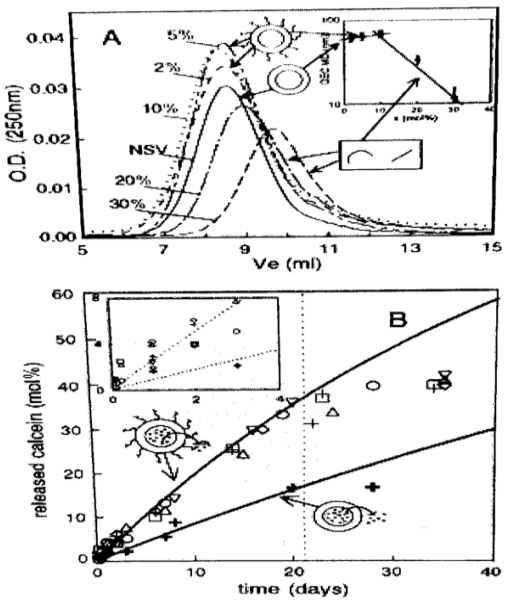

Fig. 8: Non-ionic surfactant liposomes with PEG coating. (A) HPSEC was performed on a TSK-G6000 PW column using NSV (C16G2/Chol/DCP, 48.0/48.0/3.6 mol percent) and (C16G2/Chol/M-PEG2000, 50/50 -x/x mol percent) mixed aggregates created by

ultrasonication. Polymer molar ratios x are used to express quantities. The growth of the mean hydrodynamic diameter in response to the amount of polymer, as determined by the chromatograms, is shown in the inset. The integrity of PEG-coated liposomes is maintained up to 10 M-PEG2000 mol%, although smaller particles start to form after that. From Reference[49], modified. (B) HPSEC employing the TSK-G6000 and -G4000 PW connected in a sequence of NSV and M-PEG-Chol-NSV loaded with calcein.

For NSV (thick +) and M-PEG-Chol-NSV comprising 2 (D), 5 (o), 7.5 (w), and 10 (q) mol percent M-PEG1000-Chol or 2 ((5), 5 (+) mol percent M-PEG2000-Chol, the percentage of calcein produced from liposomes is plotted against time. 1 curves (NSV) calcein released versus time is represented by and 2 (data of the two M-PEG-Chol-NSV systems) by a firstorder kinetic law. Enlarged image of the first four days in the inset. from Ref.[50]

3.3.4 Liposome permeability characterised by surfactant

Since they control the effectiveness of drug retention, the distribution of trapped molecules, and the physical stability of the liposomes under various biological environments, the permeability properties of liposomes are crucial for their employment as drug carriers Knowing how liposomes behave in relation to their lipid-solubilizing capabilities the removal of detergent from surfactants has ramifications for liposome drug loading . The fate of liposomes in living organisms, a mixed micellar solution, and membrane protein reconstitution. The lipids in the liposome dissolve into lipid-surfactant mixed micelles as more surfactant molecules are added, and vice versa (via a sequence of aggregation states), liposome synthesis by surfactant removal from lipid-surfactant micelles occurs.[51–53] Among other techniques, conventional SEC and HPSEC have been used to study the threshold of the liposome closure in the liposome formation process[54], liposome reconstitution[57–59], and size evolution of the vesicular surfactant–lipid intermediates (light scattering, fluorescence, small-angle X-ray scattering).

The cholate-induced membrane solubilization of EPC, EPC/Sph, and EPC/cholesterol liposomes was seen using [14c] DPPC as a lipid marker (Table 3, line 1). To check for their resistance to sodium cholate, liposomes that had been holding 3 H-inulin were released. Traditional SEC of liposome/cholate combinations was performed using Sepharose 4B-Cl for 70 nm liposomes and Sepharose 2B-Cl for 220-nm liposomes. On 220-nm liposomes, but not on 70-nm liposomes, the size of mixed lipid/cholate liposomes shrank when cholate was added. The chromatographic results show that the gradual release of the trapped carbohydrate

observed at low cholate concentrations is not caused by liposome solubilization but may instead be brought on by transient intramembrane holes.[37] In the presence of calcium, cholate induces liposome formation via a fusion mechanism.[55] Researchers discovered this method by observing the preservation of FITC-dextran (MW: 70,000) in 30-nm EPC liposomes in a Ca2 + (10 mM CaCl2)-containing solution following cholate addition (Table 3, line 2). Before and after the addition of cholate, aliquots were eluted through small (8 8 mm) concanavalin A (Con A) - sepharose columns, which kept the fluorescent dextran that wasn't entrapped but not the portion that was in the liposomes. Eluent functioned as the Ca2 + buffer that was used to make liposomes. The amount of dextran that remained in the liposomes after being solubilized by too much cholate was measured using fluorescence techniques.

Calculated from the selectivity curve of the TSK G6000 PW column [12].

No.	Lipids	Vesicles nm	Markers	Surfactant	Column gel	Eluent	Applications	Information from chromatography	References
1	EPC, EPC/Sph. 7/3, EPC/Chol 7/3	70, 200	^{14}C-DPPC, ^{3}H-inulin	No Cholet	Sepharose CL-4B, Sepharose CL-2B	10 mM phosphate, 150 mM NaCl, pH 7.35	vesicle/free probe separation size analysis	vesicle size growth probe release via transient pores	[37]
2	extruded EPC vesicles	30	FITC-dextran 70,000	No Cholet	Con A Sepharose	135 mM NaCl, 10 mM CaCl2, pH 7.4	vesicle/free probe separation, free probe removal	probe release via vesicle fusion	[55]
3	EPC SUV	23		OG	TSK-G6000 PW Sephacryl S1000 TSK-G6000 PW	145 mM NaCl, 10 mM Hepes, pH 7.4 145 mM NaCl, 10 mM Hepes,	On-line size analysis on-line size analysis	Vesical Size	[54]
4	EPC SUV	34	FITC-dextran 70,000	OG	Con A Sepharose	135 mM NaCl, pH 7.4	vesicle/free probe separation, free probe removal	Probe release via a lipid transfer mechanism	[56]
5	C16G2/Chol/DCP (47.5/47.5/5 wt.%) sonicated NSV	72(66)[a]		10 mM OG	TSK-G6000 PW TSK-G6000 PW	145 mM NaCl, 10 mM Hepes, pH 7.4 same buffer with 10 mM OG	On-line size analysis	Probe release via a lipid transfer mechanism vesicle impermeability	[56] [43]
6	EPC/EPA 9/1 extruded vesicles	136-147	FITC-dextrans	OG NaTC	TSK-G6000 PW + G4000	Vesicle medium, pH 7.4	on-line analysis:	release kinetic mechanism via	[29,38]

			4400 to 148,000		PW		encapsulated probe, probe release, size analysis	transient pores (OG) and permanent pores (NaTC)	
7	EPC/OG mixed micelles		^{14}C-glucose, ^{3}H-inulin		Sephadex G25 (PD10)	145 mM NaCl, 10 mM Hepes, 14C-glucose, pH 7.4	OG Removal	vesicle closure from micelle dilution	[54]
8	enzymatically formed DPPC vesicles DPPC/DG vesicles		calcein 1 mM	Sephacryl S1000, TSK-G6000 PW + G4000 PW	145 mM NaCl, 10 mM Hepes, pH 7.4	145 mM NaCl, 10 mM Hepes, pH 7.4	On-line size analysis	Vesical formulation	[58,59]

The size of the liposomes increases when subsolubilizing octylglucoside (n-octyl-h-D-glucopyranoside) concentrations are added to EPC SUV.[54] Liposomes were chromatographed with or without 10 mM OG using a gel exclusion column (TSK-G6000 PW) that was equilibrated with or without 10 mM OG, respectively (Table 3, line 3). EPC SUV with a 23 nm starting diameter eluted at a wavelength that corresponded to 25.6 nm in OG. When liposomes were exposed to 10 mM OG and quickly returned to low surfactant concentration, they were eluted on a Sephacryl S1000 gel column at the same position as the initial SUV (23 nm diameter), demonstrating that this liposome swelling caused by OG insertion in the lipid bilayer was reversible. The EPC-OG aggregates' elution quantities in 20 mM OG from the TSK-G6000 column matched smaller structures (20 nm). . However, when SUV was exposed to 20 mM OG or more and then rapidly diluted to a low OG concentration, the liposomes produced were noticeably larger than SUV (61 nm in diameter). Almog et al.[56] hypothesised a method of liposome expansion via lipid transfer above a ratio of OG to phospholipid in the liposome bilayer of 1, based on FITC-dextran (MW: 70,000) retention studies and chromatography on Con A -Sepharose column (Table 3, line 4). Large OG stabilised holes would become visible if 3 H-inulin were to leak, according to Ollivon et al.[54] Cholesterol significantly slows down the rate of OG entry into membranes OG cannot pass through NSV, which are synthesised with 50% cholesterol, up to a surfactant concentration of 10 mM. According to HPSEC (Table 3, line 5), pure sonicated NSV will elute over a TSK-G6000 PW column at the same rate with either OG-free buffer or buffer containing 10 mM OG.[43] As with the EPC-OG system, liposome development is seen after OG is able to permeate the bilayers. Using a TSK-G6000 PW column that had been pre-saturated with a buffer containing 10 mM OG, the elution patterns of pure NSV and mixed aggregates at two stages of the NSV solubilization (E and D, Fig. 9 inset) were examined. Surfactant partitioning between the lipidic membrane and the aqueous media causes the liposomes to gradually become permeable without damaging their bilayers.After the membrane has been saturated by surfactant molecules, solubilization takes place.[51,53] Extruded EPC/EPA (90/10 mol/mol) liposomes encasing FITC-dextrans of increasing average molecular weights have been used to study the permeabilization processes [29,38]. With the use of the HPSEC system previously described (see Section 3.2, Fig. 4), the effect of the leakage of the markers caused by OG and sodium taurocholate surfactants was examined. Figure 10 shows an illustration of the release kinetics obtained with FITC-dextran 4400 loaded liposomes and OG (Table 3, line 6). Using a syringe pump, a 100 mM OG solution is continually added while being stirred into the liposomes at a steady rate. By

sequentially injecting the surfactant-treated solutions onto the TSK-G6000 and -G4000 columns, the dextran leakage was examined. The continuous media that the mixed OG-liposomes were in equilibrium with was the eluent. In fact, it is crucial to keep the medium in equilibrium with the mixed aggregates formed at the end of OG addition throughout the elution process. Elution with free buffer would inevitably remove surfactant from the liposome membrane by dilution of the surfactant in the exterior aqueous phase of the mixed aggregates. A calibration curve (FITC fluorescence intensity vs polymer concentration; see Fig. 10 inset) was created by eluting solutions of FITC-dextran 4400 at the same circumstances as the permeability tests, and it was utilised to determine the release content quantitatively. The 90j light scattering makes it possible to monitor changes in the liposomes' size as well as their structural integrity. The HPSEC data demonstrates that below a specified OG concentration, no release of FITC-4400 from the liposomes is detectable, and that the number of surfactant molecules in the liposome membrane subsequently controls the leakage. Independent of the average molecular weights of the FITC-dextrans, two distinct mechanisms of efflux of FITC-dextrans from the loaded liposomes have been demonstrated. The release of the entrapped markers occurs via the formation of transient intramembrane pores with OG and permanent ones with sodium taurocholate.[38]

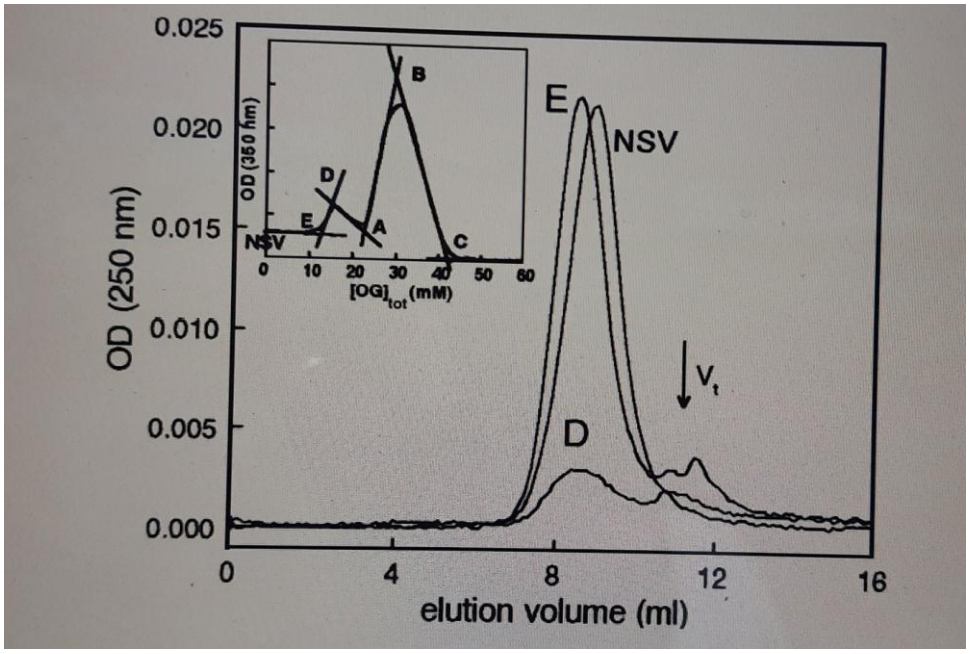

Fig. 9: Size growth of liposomes induced by surfactant during the liposome-micelle transition. HPSEC was performed on a TSKG6000 PW column using sonicated NSV (mean diameter MD = 66 nm, initial lipid concentration [lip]init = 2.5 mM, elution profile NSV), as well as mixed aggregates at points E and D of the solubilization curve

shown in the inset (MD = 131 nm, [lip]init = 2.3 mM, elution profile E and D, respectively). This curve was created by adding OG continuously at a rate of 6.94 10-5 mmol/min. The buffer, which served as both an eluent and pre-equilibrator for the column, contained 10 mM OG. 1 ml/min flow rate; 50 Al sample loading From Ref.[43]

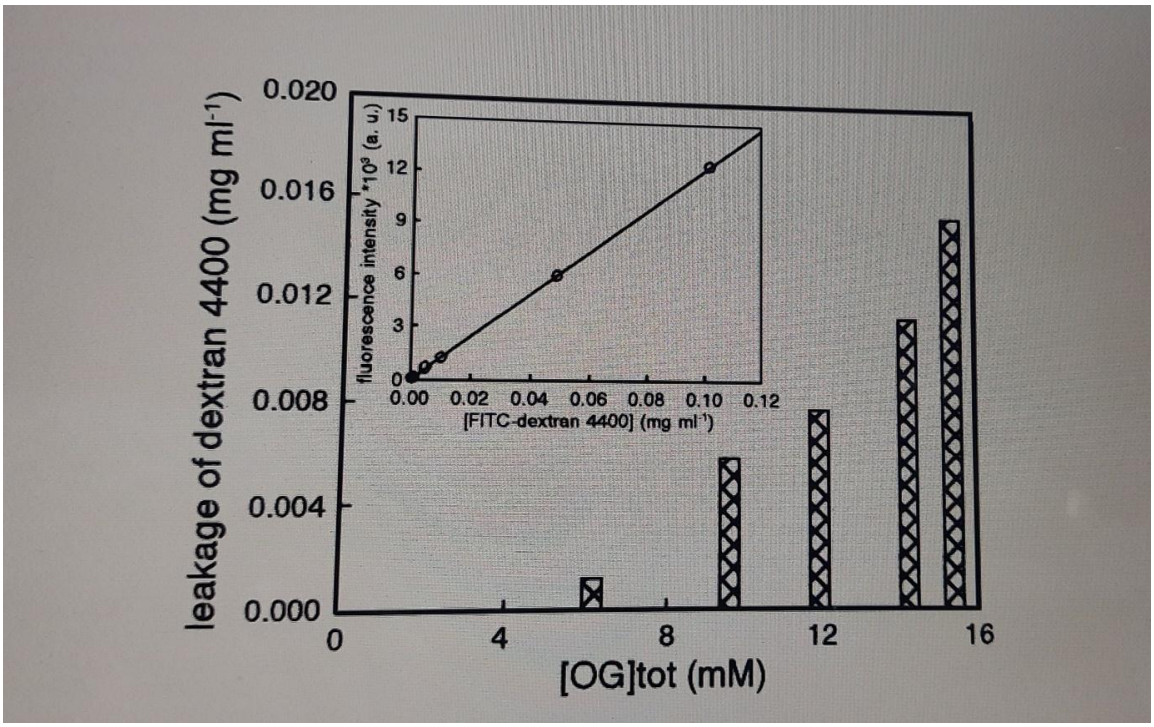

Fig. 10: Liposome permeability to surfactant. The extruded EPC/EPA (9/1, w/w) liposomes loaded with FITC-dextran 4400's permeability to OG. The HPSEC system used series-connected TSK-G6000 and -G4000 PW columns to track the release's kinetics. The calibration curve shown in the inset was used to calculate the amount of the marker released from the liposomes as a function of the OG concentration in the sample ([OG]tot). Fluorescence intensity at the peak of the dextran elution peak in buffer solution plotted against FITC-dextran 4400 concentration. The HPSEC circumstances shown in Fig. 5. Adapted from Reference.[29]

3.3.5 Liposome formation and constituent

Surfactant removal from mixed micelles is the foundation of a traditional method for liposome reconstitution.[53] The most effective method to encapsulate labile pharmaceuticals or biological molecules like proteins, peptides, or nucleic acids is to use the right surfactant nucleic acids while keeping their biological properties intact. Surfactant elimination can accomplished by dilution[54,55,57,60], SEC[10,61,62], dialysis[8,19,23], or adsorption onto Sephadex, Biobeads SM2, and other hydrophobic polymer beads.[63–66] SEC provides the important

forming liposomes and removing the unencapsulated material in one process is advantageous. When buffer free of surfactants is eluted through gel beads, liposomes form as a result of the surfactant being removed from the mixed micelles by a dilution process that is sped up by the gel sieving between the monomers and mixed micelles that can pass through the gel pores and the liposomes that are excluded from them.[53] The mixed micelles' surfactant depletion during the reconstitution of the liposomes causes bilayer curvature, which leads to liposome closure. Large molecules that were entrapped at the time of closure can be used to estimate this specific point. For the purpose of converting EPC-OG mixed micelles into liposomes through surfactant dilution, inulin was used[54] (Line 7 in Table 3). OG-free buffer with similar concentrations of EPC (12.8 mM), OG (40 mM), 3 H-inulin, and 14C-glucose was used to dilute the combination by 11 times amounts of unlabeled inulin and 14C-glucose. The OG, EPC, and inulin are diluted. Until the liposomes seal, while following closure, the inulin inside the liposomes is not diluted even more. The remaining OG was eliminated at each dilution phase by a passage of the sample successively on a Sephadex G150 column to remove any leftover OG, then a Sephadex G25 column prewashed with buffer containing 14Cglucose and EPC liposomes the outside glucose and inulin. Liquid scintillation counting was performed using aliquots of the initial mixture and washed liposome fractions. The ratio of inulin to the entrapped volume marker, 14C-glucose, and the concentration of OG at closure-inulin were calculated. A method for transitioning from a micelle to a liposome using an enzyme has been created. In the presence of 1 mM calcein, dodecyl h-D-maltose (DM)-DPPC mixed micelles were converted into DPPC liposomes by two sequential enzymatic processes involving the enzymes amyl glucosidase and h-D-glucosidase, respectively.[58] A marker for bilayer closure was calcein. A useful method to evaluate the formation of close formations is conventional SEC, which can distinguish between calcein molecules free in the external aqueous medium and those contained in the aqueous cavity of aggregates. Thus, chromatography of the generated aggregates using a Sephacryl S1000 in conjunction with on-line fluorescence was used to demonstrate the first instance of DPPC liposome production.[58] Then, using HPSEC with the configuration shown in Fig. 4 (Table 3, line 8), it was possible to compare the elution profile detected by 90j light scattering (Fig. 11B, curves a to c) for the aggregate turbidity to the elution profile observed by fluorescence for the fraction of the encapsulated calcian.[59] Parallel to this, using the same HPSEC method, the viability of producing DPPC -dodecyl-h-Dmaltoside (DG) liposomes by sonication from a DPPC -DG mixed film hydrated with isotonic buffer containing 1 mM calcein was examined.[67] (Table 3, line 8). As mentioned before, the presence of closed liposomes is indicated by the trapping

of calcein (Fig. 11C and D). The purpose of utilising a gel column in the size range of the liposomes to be studied, as already noted, is to learn more about their physical features. As can be seen, the elution behaviour of the enzyme-formed liposomes is comparable to that of the reference sonicated unilamellar DPPC liposomes (Fig. 11, curve d).

3.3.6 Interaction and Insertion of biological amphiphilic molecule

Size exclusion chromatography has been investigated by Lundahl et al. in the analysis of drug-entrapped liposomes and protein-liposome interaction.[16] Traditional SEC or HPSEC are efficient investigational techniques that have provided information on the conformational behaviour of gramicidin, the glyceride solubility limit (3.8%) in EPC liposomes, and the distribution of drugs and cytokines between the liposome membrane and the internal and external aqueous compartments. Melittin's pore formation and sizing in liposomes[71,72], the distribution of drug and cytokine protein between the liposome membrane and Liposome-polyelectrolyte interactions[75,76], the activity of biotin peroxidase in targeted streptavidin-liposomes[77], the interaction of charge isomers of myelin basis protein with negatively charged liposomes[78], formation of proteoliposomes and characterization of the size distribution .

CONCLUSION

The statistics presented here demonstrate that conventional SEC and HPSEC provide a range of information in various liposome fields. This method's capacity to fractionate heterogeneous suspensions into monodisperse subpopulations and to separate liposomes from solutes is also of considerable interest. The analysis essentially poses no stress for the liposome structure if the elution buffer is isoosmotic with the aqueous medium of the liposomes. With little SEC equipment as well as fully automated and advanced HPSEC gear, it can be utilised as a straightforward routine approach or as a significantly more complex one. Both analytical and preparative scales can be used to scale conventional SEC and HPSEC. On an analytical scale, it is advised to test the preparative separation-appropriate conditions first.

Studies using traditional SEC demonstrate that the liposomes systematically elute at the void volume of the columns because of the commonly utilised gel bed. This favours the separation gap between the liposomes and the unloaded solute but has a significant disadvantage in that the liposomes cannot be separated and their size and size distribution are not quantified. This is further confirmed by employing low porosity HPLC exclusion gels, which elute both

liposomes and the void volume. Therefore, two columns must be connected in series, one covering the size range of liposomes (for example, TSKG6000 PW or Sephacryl S1000 columns), and the other that of smaller particles, in order to evaluate loaded liposomes. While multi-mode ones offer simultaneous information gathered from the same sample under the same conditions, precise description of the elution profiles necessitates on-line detection. Additionally, this saves time by avoiding the laborious process of fraction collection and subsequent analysis.

Since the quick run time allows for serial time sampling and time resolved analysis in addition to reproducibility, separation efficiency, and quantitative determination, HPSEC with TSK PW series columns has a number of obvious advantages, especially for kinetic analysis (stability, material release, interaction/insertion of exogenous substances, etc.). When utilising fluorescent markers, which are widely used in permeability studies, it is sufficient to encapsulate a little amount because they can be easily separated from unencapsulated labels on the gel column by the liposomes. This eliminates the requirement for a quenching effect. Furthermore, when the encapsulated marker does not show fluorescence attenuation, liposome leakage can only be detected by separation by chromatography.

Regardless of the nature of the foreign substances—charged or uncharged, water-soluble, amphiphilic or hydrophobic—the SEC technique offers the benefit of being a modular tool that is extremely relevant for the control and understanding of any loaded liposome system.

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