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Review

Analysis of liposomes

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Abstract

Liposomes are highly versatile structures for research, therapeutic, and analytical applications. In order to assess the quality of liposomes and obtain quantitative measures that allow comparison between different batches of liposomes, various parameters should be monitored. For liposomes used in analytical and bioanalytical applications, the main characteristics include the average diameter and degree of size polydispersity; encapsulation efficiency; the ratio of phospholipids to encapsulant concentration; lamellarity determination. A detailed description of today's most commonly used methods and of novel techniques for the quantification of these aspects is presented in this report citing 182 references. Their advantages and limitations are discussed where appropriate in order to provide the reader with an understanding of the current state of the art assessment of liposome quality.

Keywords: Liposomes; Analysis; Characteristics; Review

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1. Introduction

Liposomes are highly versatile structures for research, therapeutic, and analytical applications. They are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar headgroups of the lipids oriented towards the extravesicular solution and inner cavity. Their structure is similar to that of cells, and thus can be used as a more easily characterized vessel for studying interactions between membrane lipids and biomolecules such as DNA [1] and proteins [2], permeability of ions [3,4] and drugs [5], and elucidating the mechanism of action of pesticides [6] and antibiotics on target organisms [7,8]. Liposomes have been used as models in several recent studies for estimating the partitioning of drugs into cells by surface plasmon resonance [9,10] and chromatography [11–13]. A general overview of liposomes as analytical tools is given by Edwards et al. [14]. In this report, techniques and methods are described that can be used to quantitatively describe liposomes. This provides a means of comparing different batches of liposomes and generates data that assist in the understanding of liposomes and

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their use in a variety of different application areas. Characteristics discussed in this report include liposome lamellarity, diameter and size distribution, lipid composition and concentration determination, the encapsulant concentration and its encapsulation efficiency. Techniques used to determine these properties include ^{31}P NMR, dynamic light scattering, atomic force microscopy (AFM), fluorescence spectroscopy, HPLC and various wet chemistry methods. Other commonly monitored parameters, that are not described in more detail here, include surface charge through zeta potential measurements [15], phase transitions through differential scanning calorimetry [16–18], and quantification of residual solvents through gas chromatography [19]. For a general background and discussion of traditional liposome preparation, analysis, and application the reader is referred to an excellent book that has also been published recently [20].

2. Lamellarity determination

The lamellarity of liposomes made from different lipids or preparation procedures varies widely. This is evidenced by reports showing that the fraction of phospholipid exposed to the external medium has ranged from 5% for large multilamellar vesicle (LMVs) [21] to 70% for SUVs [22]. Liposome lamellarity determination is often accomplished by ^{31}P NMR. In this technique, the addition of Mn^{2+} quenches the ^{31}P NMR signal from phospholipids on the exterior face of the liposomes [23]. Mn^{2+} interacts with the negatively charged phosphate groups of phospholipids and causes a broadening and reduction of the quantifiable signal [24]. The degree of lamellarity is determined from the signal ratio before and after Mn^{2+} addition. While frequently used, this technique has recently been found to be quite sensitive to the Mn^{2+} and buffer concentration and the types of liposomes under analysis [24,30]. Other techniques for lamellarity determination include electron microscopy [25,26], small angle X-ray scattering (SAXS) [27–29], and methods that are based on the change in the visible or fluorescence signal of marker lipids upon the addition of reagents [30]. The latter approach will be reviewed in more detail since it is a relatively simple procedure that can easily be carried out in a standard lab.

Several lipids can be incorporated into liposomes which will exhibit a change in signal upon the addition of certain reagents. These include amino lipids, such as phosphatidylethanolamine (PE) or phosphatidylserine (PS); fluorescently labeled lipids, such as 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-phosphatidylethanolamine; hydroxyl-containing lipids, such as phosphatidylglycerol, phosphatidylinisitol, or glycolipids [30]. All of these methods rely on the comparison of the total signal to the signal achieved from the reaction of the marker lipids with specified reagents. For example, the UV-absorbance at 420 nm of 2,4,6-trinitrobenzenesulfonic acid (TNBS) increases in the mixture as a result of complex formation with primary amines [31]. This property has been used for the detection of aminolipids at 420 nm [32,33]. Under certain conditions, the bilayer permeability of TNBS is minimized such that only the aminolipids on the exterior bilayer contribute to the signal. Lysis of liposomes by a surfactant such as Triton X-100 allows TNBS to interact with interior aminolipids as well and yields the total signal [30]. In another method, the addition of periodate to phosphatidylglycerol results in the oxidation of the diol present in the lipid yielding an aldehyde and release of formaldehyde. The released formaldehyde reacted with chromotropic acid to yield a product, which was subsequently detected at 570 nm [34]. This method has been used for the determination of external reactive groups on liposomes [35]. In another method, the quenching of NBD fluorescence is obtained by sodium dithionite [36,37]. NBD-labeled lipids are highly fluorescent at low concentrations (<1 mol%) in membranes, but undergo self-quenching at increased concentrations [38]. In this approach, the NBD–PE fluorescence initially is from all lipids in the sample. Under appropriate conditions, the addition of sodium dithionite quenches the fluorescence of only the NBD–PE existing on the outer bilayer [36]. The percentage of external lipid is found by dividing the change in fluorescence upon dithionite addition by the total fluorescence, corrected for scattering [30].

These methods assume that the lipid of interest is distributed evenly over all lipid layers, that the rate of inversion between layers is negligible, and the reagents used to elicit the signal change are impermeable to the membrane over the time course of the measurements. A detailed comparison of some of these lamellarity determination methods is available in a recent study by Gruber and Schindler [30].

3. Size determination

Several techniques are available for assessing submicrometer liposome size and size distribution. These include static and dynamic light scattering [22,39,40,57], several types of microscopy techniques [41–43], size-exclusion chromatography (SEC) [44], field-flow fractionation [45–47], and analytical centrifugation [41]. Several variations on electron microscopy (EM) such as transmission EM using negative staining [28,35], freeze-fracture TEM [48,52], and cryo EM [42,49,50], provide valuable information on liposome preparations since they yield a view of morphology and can resolve particles of varying sizes. However, they require complicated sample preparation, remove the liposomes from their native environment, generate artifacts, can induce shrinkage and shape distortion, and are time consuming to obtain a representative size distribution of the population, thus are not amenable to being a routine measurement [51–53]. Some of these problems may be overcome to yield reproducible and accurate results through careful attention to sample preparation, as outlined in a recent review of cryoelectron microscopy of liposomes [42].

Another more recently developed microscopic technique known as atomic force microscopy has been utilized to study liposome morphology, size, and stability [54–57]. This technique relies on the raster scanning of a nanometer sized sharp probe over a sample which has been immobilized onto a carefully selected surface, such as mica or glass, which is mounted onto a piezoelectric scanner [58,59]. The tip is attached to a flexible cantilever. Deflection resulting from passage of the tip over sample attributes is measured by a laser beam. The reflected laser beams are then detected at photodiode array detectors which through a feedback mechanism, maintain the distance of the probe, amplitude of oscillation, or the cantilever deflection constant, depending on the scanning mode [43,62]. The end result is a high resolution three-dimensional profile of the surface under study. Different modes of AFM are available, including contact/repulsive mode (either constant height, constant deflection, or tapping modes) and non-contact/attractive mode [60–62]. In constant height mode, the probe is maintained at a constant distance above the sample while the deflection of the cantilever is monitored [43,60]. In constant force mode, the deflection of the cantilever is maintained constant and the height of the piezoelectric scanner supporting the sample is adjusted [43,63]. In tapping mode, the sharp probe oscillates at high frequency while scanning across the sample. Sample features change the amplitude of oscillation as the probe makes contact on its bottom stroke of oscillation. Tapping mode is often applied for the analysis of soft materials since it minimizes frictional and adhesive forces [55,64]. While the former exerts lateral forces on the sample under study, the latter minimizes these forces but exerts larger point forces [43]. The high resolution ($\sim$1 Å) afforded by AFM has been utilized to examine the effects of cholesterol on the mechanical stability of egg yolk phosphatidylcholine liposomes [55]; the effects of actin encapsulation on liposome shape [65]; the effects of lipid composition on liposome stability in terms of size and shape [56]. The technique permits visualization of liposomes without much alteration of their native form, provided that the requisite surface immobilization does not adversely affect the sample and that the force of the probe itself does not have deleterious effects on the vesicles. While both modes have been used for liposome analysis, the latter effects are minimized when AFM is performed in tapping mode where the probe is not in constant contact with the sample [66]. However, in addition to probe-dependent effects, the native structure of liposomes may be destroyed upon interaction with the surface through the formation of planar bilayers, an outcome which is dependent on lipid compositions, liposome sizes, and buffer compositions [67–69].

HPLC using size-exclusion chromatography can be used to separate and quantify liposome populations according to a time-based resolution of hydrodynamic size. The porous packing material used in this technique excludes large species from the internal pore volume which is more available to smaller species leading to their longer retention on the column. This mechanism leads to separation based on the elution of large particles before smaller particles. Conventional SEC is frequently used for the separation of liposomes from unencapsulated materials as a final purification step, but the use of HPLC-SEC for analysis offers increased resolution of liposome populations, reduced sample size, and better reproducibility [44]. More thorough discussions of HPLC-SEC for size determination of liposomes are available in recent articles by Grabielle-Madelmont et al. [44], Lundahl et al. [13], and Lesieur et al. [70]. One recommended commercially available column is the ethylene glycol-methacrylate gel packed TSK-G6000PW which has a separation range from 20 to >500 nm [70,71]. These columns have also been used in series with G5000PW or G4000PW columns to provide higher resolution of smaller liposomes or low molecular weight species [44,72,73]. An osmotically balanced mobile phase at relatively low pressures (10–15 bars [44]) helps to prevent damage, swelling, or shrinkage of the liposomes [44]. Coupled with refractive index detection, fluorescence detection, or detection of radiolabeled lipids (where applicable), HPLC-SEC can offer a powerful technique for not only size distribution determination, but also stability in terms of aggregation [70] and vesicle permeability [73]. Detection of turbidity in the UV range suffers from light scattering effects, which require correction for particle size and morphology [70,90]. Off-line analyses such as phosphorous or cholesterol determinations (discussed further in Section 4) may also be done to provide an assessment of the lipid quantities present in each separated peak. Other light scattering detectors such as MALS and DLS will be discussed further in this section. Disadvantages of HPLC for size determination of liposomes mainly stem from recovery issues. These include unwanted adsorption of lipids to the column packing and destruction of liposomes which contain lipids that have higher affinity for the column material than the composite lipids, both of which necessitate presaturation of the LC column with lipids prior to analysis [13,44]. In addition, the rigidity of the lipid bilayer, which is a function of the lipid composition, plays a role in the retention and recovery of liposomes. The rigidity of the bilayer dictates whether liposomes of larger diameter can deform to enter pores smaller than their hydrodynamic diameters would otherwise dictate. While the slow diffusion of liposomes limits resolution at high flow rate, low flow rates can result in increased adsorption of liposomes to the column packing materials [13]. Thus, while hydrodynamic size and subsequent molecular weight information can be obtained through this technique, the accuracy of this determination is based on the use of a well-matched (both by shape and chemical composition) set of standards [74]. Potential van der Waals and electrostatic interactions of sample components with the column packing material may affect the elution profile and lead to the misrepresentation of molecular weight [75]. Lastly, while suitable packing materials are available for the resolution of small to moderately sized liposomes, the resolution of large liposomes (>0.8 μm) is not possible using existing commercially available packing materials [13].

Field-flow fractionation (FFF) is a technique which overcomes some of the limitations of HPLC in liposome analysis, and composes a family of techniques, including electrical (El) [76], thermal (Th) [77], sedimentation (Sd) [78–80], and flow (Fl) FFF (symmetrical [81] and asymmetrical [82,83]). These techniques rely on the application of a field which is perpendicular to the direction of flow. Excellent reviews of FFF are available in several recent articles [84–86]. While SdFFF has been used for the analysis of liposomes [87–89], the relative simplicity of the FlFFF technique has increased its application towards liposome analysis in recent years [44,47,107]. The mechanism of FlFFF versus SdFFF for liposome analysis differs in that FlFFF separates vesicles on a hydrodynamic size basis, whereas SdFFF separates them on a weight basis. The former technique yields diameter data for multilamellar vesicles versus unilamellar vesicles, whereas the latter treats similarly sized MLVs as comparatively heavy particles [107]. Subsequent references to FFF in this document will be to the FlFFF form. Rather than a solid phase as is used in HPLC, FFF uses a channel wall which consists of a semipermeable membrane chosen with a MWCO suitable for the particles under study. This membrane allows the carrier fluid to pass, but not the particles of interest. The laminar flow profile causes particles located closer to the walls of the channel to move slower than particles in the center of the channel, while the perpendicular flow propels all particles toward the membrane [53]. Diffusion due to Brownian motion of particles in a size-based manner reduces the accumulation of smaller particles against the membrane wall. Retention times in this technique are proportional to the hydrodynamic diameter of the particles since smaller particles reach an equilibrium position further from the channel walls and hence experience less drag from the perpendicular flow [90]. The equation relating the retention time to the particle's Stoke's radius is shown in Eq. (1), where d_s is the Stoke's diameter, t_r the retention time, k the Boltzman constant, T the temperature, η the carrier viscosity, ω the channel thickness, V the volumetric flow rate of channel flow, and V_r is the cross-flow rate [46].

$$d_s = t_r \frac{2kTV}{\pi\eta\omega^2 V_e} \tag{1}$$

Whereas in HPLC-SEC, large liposomes elute first, in normal mode FFF, small liposomes elute first due to their higher diffusion coefficient and subsequent higher elevation from the accumulation wall [91]. Moon et al. demonstrated baseline separation of five polystyrene latex standards ranging from ~50 to 430 nm diameter within 35 min [46]. Additional mechanisms of separation termed steric or hyperlayer modes have been elucidated for particles greater than 1 μm diameter and result in the elution of larger species prior to small species. This change in elution pattern is due to further protrusion and hydrodynamic lift forces, respectively, of larger particles which place them into faster portions of the carrier stream [85,92,93]. The advantages of the FFF technique include the wide range of particle sizes that can be separated (~1 nm–100 μm) with high resolution; the lack of a packed column which reduces the potential of shear degradation and losses due to adsorption of the samples under study; minimal sample preparation requirements [84,85,94,95]. The disadvantages of FFF include the complexity and expense of the instrumentation; limited commercially available membrane options leading to potential loss of sample through adsorption or permeation; separation mechanism considerations above a vesicle diameter of 1 μm; liposome retention dependence on ionic strength, as outlined in [75,96,107].

Both of these separation techniques rely on calibration standards to correlate observed retention times with molecular weights, unlike dynamic and static light scattering methods. Dynamic light scattering ((DLS) (otherwise known as quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS)) is extensively used in liposome size distribution analysis [40,97,98]. DLS measures the time-dependent fluctuations of light scattered from particles experiencing Brownian motion, which results from collisions between suspended particles and solvent molecules. Light scatter from monodisperse particles yields an intensity correlation function of the form outlined in Eq. (2) [99] where I, t, and τ are the intensity, time, and the shift in time from the previously measured time, respectively.

$$G(\tau) = \int_0^\infty I(t)I(t+\tau)\,\mathrm{d}t \tag{2}$$

$$G(\tau) = B + A\mathrm{e}^{-2q'D\tau} \tag{3}$$

As time $\to \infty$, $G(\tau)$ is normalized to 1 (the baseline). The diffusion coefficient (D) is found through fitting the correlation curve to the single exponential decay form shown in Eq. (3), where B is the baseline, A the amplitude, and q is the scattering vector which depends on the refractive index of the medium, the wavelength of the incident laser, and the angle at which the scattered light is detected [99]. Multimodal distributions exhibit a correlation function which is best fit by a multi-exponential model, whereas that from random noise lacks the exponential decay form. Calculation of the diffusion coefficient (D) yields the hydrodynamic radius (r_h) of the particles under study through the Stokes–Einstein equation, shown in Eq. (4), where k is the Boltzmann constant, T the temperature, and η is the solvent viscosity.

$$r_h = \frac{kT}{6\pi\eta D} \tag{4}$$

The hydrodynamic radius (Stokes radius) of the particle is defined as the apparent size of the hydrated sphere and is calculated from the radius of a sphere that diffuses at the same rate [99]. The diffusion coefficient is a function of the decay rate of the autocorrelation function as well as a scattering vector which is dependent on the refractive index of the medium, the incident wavelength, and the scattering angle. Further discussions on light scattering theory are available in several recent review articles [39,99]. The correlation function contains particle size information for all of the particles measured and requires deconvolution by an autocorrelator for resolu-

tion of particle sizes [99]. The mathematical methods used for deconvolution are beyond the scope of this report, however they typically consist of an intensity weighted, single exponential fit assuming a monomodal distribution (cumulant method [100,101]) and a method which is applicable for multiple exponential fits, and thus can resolve multimodal distributions (CONTIN [39] or non-negative least squares (NNLS) are common examples [99].) The strengths of the technique include the ability to make measurements in native environments; its sensitivity to small quantities of high molecular weight aggregates; ease of commercially available instrument operation; minimal sample volume, concentration, and/or preparation requirements [99,96]. It also covers a large size range of species spanning the low nanometer to low micrometer range. However, DLS is dependent on the algorithms used for deconvolution, and thus care should be taken when comparing the results from one study to another when different instruments are employed [97,172]; resolution and size analysis of multimodal samples can be unreliable, depending on the relative amounts of each species [102]; the technique does not yield particle shape information [46]; it can yield a bias towards reporting larger diameters when small quantities of high molecular weight aggregates or impurities are present in the sample [127].

While DLS relies on detection of light scatter at 90° using time-dependent light scattering fluctuations, static light scattering (SLS) measures the time-average intensity of scatter as a function of the angle [103]. Multi-angle light scattering (MALS) utilizes the angular distribution of scattered light to determine the root mean square radius, the weight-averaged molecular weight, and the geometry of the particles [45]. The expression for the scattering of dilute particles is given in Eq. (5).

$$\frac{KC}{R_\theta} = \left(\frac{1}{M} + 2A_2C\right)\left[1 + \frac{16\pi r^2 R_g^2}{3\lambda^2}\sin^2\left(\frac{\theta}{2}\right)\right] \quad (5)$$

where K is an optical constant based on refractive index and wavelength components, C the particle concentration, R_θ the ratio of scattered light intensity to incident light intensity (Raleigh ratio), M the absolute molecular weight, A_2 the second virial coefficient, R_g the radius of gyration, λ the vacuum wavelength of the incident radiation, and θ is the scattering angle [104]. This technique requires knowledge of analyte concentration and refractive index increment for the calculation of the molar weight and the second virial coefficient, however this is unnecessary for the determination of the root mean square (rms) radius [105]. The latter is defined as the mass weighted average distance from the center of mass. MALS provides an absolute measure of molecular weight without comparison to reference standards, unlike size-exclusion chromatography [106].

Both of these light scattering techniques can benefit from the prior application of a method of particle size separation [53,108]. A recent article highlighted the importance of particle size separation for the accurate resolution of broadly distributed liposomes prior to analysis by DLS [127]. With liposomes of high polydispersity, fractions collected following HPLC or field flow fractionation separation may be analyzed by DLS or MALS for a more accurate resolution of particle size. Coupled with DLS [107] or MALS [46,47,108], FFF has been applied for liposome size distribution analysis in several recent reports. The combination of a size-based separation mechanism requiring calibration standards with an absolute means for detection provides a powerful means for size distribution analysis. In addition, since DLS and MALS signals are dependent on both the molecular mass and the concentration of the species of interest, separation also provides for concentration detection (i.e. refractive index or absorbance).

Several other less conventional techniques have been applied for liposome size distribution analysis that are not discussed in more detail here, including NMR [109], flow cytometry [110–112], right-angle light scattering [37,113,114], capillary zone electrophoresis [115,116], and turbidity [37,113].

4. Quantitative lipid analysis

Several wet chemistry techniques are commonly used for the determination of phospholipid content. Most of these techniques include the use of molybdate-containing reagents to yield a blue-colored product. One such method is the Bartlett assay which relies on the digestion of organic materials in liposome samples by 160 °C sulfuric acid, oxidation to inorganic phosphates by hydrogen peroxide, phosphomolybdate formation upon interaction with ammonium molybdate, followed by reduction through interaction with 1,2,6-aminonaptholsulfonic acid at 100 °C [117]. A blue product forms which can then be read at 830 nm for the quantitative assessment of phospholipids in the preparation. In the ascorbic acid method, ammonium molybdate reacts with orthophosphates formed from acid digestion to yield phosphomolybdic acid. This compound is then reduced with ascorbic acid to yield a blue-colored solution, read at 820 nm [118,119]. Through reaction of molybdophosphoric acid with nonpolar molecules containing quaternary ammonium salts or amines, such as lecithin or phosphatidylcholine, a water insoluble salt is formed which may be extracted into chloroform for the colorimetric determination of nitrogenous phospholipids at 680 nm [120]. This method is specific in that only hydrophobic molecules which contain an electrophilic nitrogen are extracted into chloroform and yield the blue color. Phospholipids can also be analyzed through complex formation with ammonium ferrothiocyanate, extraction into chloroform, and absorbance measurement at 488 nm [121]. This method does not suffer from interference by endogenous inorganic phosphates, but the signal is dependent on the headgroups of the lipids present and shows poor response to phosphatidylglycerol [20]. Fluorescence enhancement of 1,6-diphenyl-1,3,5-hexatriene

in the presence of phospholipids has also been reported [122].

Enzymatic assays for phosphatidylcholine [123] and cholesterol [124] are commercially available and widely used [125–127]. The former method utilizes phospholipase D to hydrolyze phospholipids to release free choline; the free choline is then oxidized to form betaine aldehyde, followed by betaine and hydrogen peroxide, by choline oxidase; the generated hydrogen peroxide causes oxidative coupling of phenol and 4-aminoantipyrine mediated by peroxidase to yield quinoneimine dye which is measured at 505 nm [128,129]. The latter method relies on hydrolysis of cholesterol esters with cholesterol ester hydrolase, followed by oxidation of the cholesterol by cholesterol oxidase and subsequent production of hydrogen peroxide. This product also oxidatively couples 4-aminoantipyrine to phenol in the presence of peroxidase to yield a blue-colored quinoneimine dye, which shows strong absorption at 500 nm [124].

Chromatographic techniques such as HPLC, GC, and thin layer chromatography (TLC) can be used to separate and quantify the lipids composing lipid bilayers [130,131]; can be used to quantify coupling efficiencies [132,133]; to measure lipid hydrolysis or oxidation [134–136]. These approaches eliminate significant restrictions which are otherwise placed on liposomes during preparation when either chemical or enzymatic methods are used. For example, liposomes cannot be prepared using buffers containing phosphates when the standard Bartlett assays are required and the encapsulant must not contain substances which would cause spectral interference with the enzyme assays. These assays are also both time-consuming and labor intensive. Thus, chromatographic approaches are advantageous since they can separate and quantify each lipid in the mixture, including lipids labeled with biorecognition elements [125] or lipids that have become oxidized or hydrolyzed during processing or storage [137].

TLC methods for phospholipid analysis often rely on lipid separation using a mixture of chloroform, methanol, and water [132] or ammonium hydroxide [140]. Detection is frequently accomplished using molybdenum blue in sulfuric acid and ninhydrin stains for the detection of phosphate and primary amino groups, respectively. Alternatively, non-specific detection can be accomplished through charring or incubation with iodine [132]. Following separation by TLC, components can also be assayed by the aforementioned wet chemical methods [138] or with flame ionization detection (FID) [139,140] to determine their phospholipid content. For HPLC analysis, detection of lipids in the UV range is limited to ~200–210 nm due to their lack of chromophores. While feasible, this range restricts the solvents that can be used as mobile phases, hence HPLC detection of lipids is more frequently accomplished by refractive index (RI) [141–143] or evaporative light scattering (ELS) detection [126,144,145]. HPLC analysis conditions have included 100% methanol on a S-5 ODS-1 column for the determination of cholesterol using UV detection [130]; on a YMC Diol-NP column running a gradient method composed of acetone, triethylamine, acetic acid, *n*-hexane, and methanol for the separation of (lyso)phospholipids and phospholipids (including phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine) using ELSD [144]; a gradient method utilizing methanol, chloroform, ammonium hydroxide, and water for the separation of common phospholipids using ELSD on a diol column [145]; a trimethylsilyl column with chloroform, methanol, water for the separation of cholesterol, DPPC, and a carbohydrate-labeled DPPE [125]. GC analysis of lipids typically requires a derivatization step to ensure sufficient volatility of the components, either through trimethyl silylation [135] or methyl esterfication [134,141] prior to detection by flame ionization (FID) [134] or MS [135].

In many cases, pretreatment of liposomes to disrupt the lipid bilayers is completed prior to chromatographic analysis using methods such as dilution of the aqueous liposome suspension with alcohols such as 2-propanol, ethanol, or methanol [130,134]; extraction of lipids into chloroform solvent mixtures [134,146,147]; or surfactant-induced bilayer disruption [148]. The choice of procedure is dependent on the mobile phase employed in the analytical method and the degree of lipid solubility/recovery. However, some methods have been reported that do not require lipid extraction [149].

5. Encapsulant determination

Methods for determining the amount of material encapsulated within liposomes typically rely on destruction of the lipid bilayer using the methods outlined in Section 4 and subsequent quantification of the released material [150,164]. In these measurements, the signal due to intact liposomes is typically monitored prior to bilayer disruption. The techniques used for this quantification depend on the nature of the encapsulant and include spectrophotometry [151,152], fluorescence spectroscopy [153], enzyme-based methods [154], and electrochemical techniques.

If a separation technique such as HPLC or FFF is applied, the percent encapsulation can be expressed as the ratio of the unencapsulated peak area to that of a reference standard of the same initial concentration [45,155]. This method can be applied if the liposomes do not undergo any purification (SEC, dialysis, etc.) following preparation. Either technique serves to separate liposome encapsulated materials from those that remain in the extravesicular solution and hence can also be used to monitor the storage stability in terms of leakage or the effect of various disruptive conditions on the retention of encapsulants. In the latter case, total lysis can be induced by the addition of surfactant [44]. Some authors have combined the size distribution and encapsulation efficiency determination in one assay by using FFF-MALS coupled to a concentration detector suitable for the encapsulant [46].

Since techniques used to separate free from liposome-encapsulated contents can potentially cause leakage of contents and, in some cases, ambiguity in the extent of separa-

tion, research using methods that do not rely on separation are of interest. Reported methods have included ^{1}H NMR where free markers exhibited pH sensitive resonance shifts in the external medium versus encapsulated markers [156]; diffusion ordered 2D NMR which relied on differences in diffusion coefficients of entrapped and free marker molecules [157]; fluorescence methods where the signal from unencapsulated fluorophores was quenched by substances present in the external solution [158]; electron spin resonance (ESR) methods which rely on the signal broadening of unencapsulated markers by the addition of a membrane-impermeable agent [159,160].

The terminology varies widely with respect to the ability of various liposome formulations to encapsulate the target molecules. Many papers express results in terms of 'percent encapsulation' (sometimes referred to as 'incorporation efficiency' [101], 'trapping efficiency [164]', or the encapsulation efficiency (EE) [161,162]) which is typically defined as the total amount of encapsulant found in the liposome solution versus the total initial input of encapsulant solution. This value depends not only on the ability of the liposomes to capture the encapsulant molecules (dependent on lipid/buffer composition, liposome type (small unilamellar vesicle (SUV)/multilamellar vesicle (MLV)/large unilamellar vesicle (LUV)), preparation procedure, etc., as reviewed by Kulkarni et al. [163]), but also on the initial molar amount of encapsulant. When systematic liposome characterizations are undertaken for the purpose of enhancing the degree of entrapment, initial lipid and encapsulant concentrations should be maintained constant for comparison. This representation of the degree of encapsulation is suitable for comparing preparation processes provided that no losses of the encapsulant occur during preparation.

Other authors define the encapsulation efficiency, or encapsulation capacity [163], as the molar amount of marker per mole of lipid [164–166] which is obtained by dividing the concentration of encapsulant by the concentration of lipid. A similar definition is suggested expressing EE on a weight (mg) encapsulant per mM of lipid basis [167]. Another commonly used parameter is the captured (or encapsulation) volume, defined as μL of entrapped volume/μmol of lipid [168]. An excellent review of captured volume obtained from various procedures used to make liposomes is available in Perkins et al. [168]. This number ranges from 0.5 μL/nmol for SUV and MLV preparations to 30 μL/nmol for LUVs [169]. Unlike the 'percent encapsulation' parameter cited previously, these representations require knowledge of the phospholipid concentration through methods outlined in Section 4. Both of these values are dependent on liposome size and lamellarity [170]. While the initial concentration of target in the aqueous phase may affect the process leading to altered encapsulation, the initial value is not factored into the final mol target:mol phospholipid calculation. Moribe et al. have reported that the encapsulation efficacy following preparation (weight encapsulant per weight of lipid) was dependent on the initial weight ratio of encapsulant to phospholipids [171]. Thus, all aspects of the processes under consideration may be compared directly using these latter parameters.

6. Characterization of liposomes with respect to manufacturing

Despite their versatility, difficulties in manufacturing have hindered more widespread applications of liposomes. Poor particle size reproducibility, high cost of manufacture, and questionable stability are contributing factors [169,172]. Liposome size, shape, and lamellarity dictate the amount of material that can be encapsulated within their aqueous cavities. Thus, a good understanding of ideal liposome characteristics for specific applications is required and liposomes need to be quantitatively studied for this purpose. Ideally, liposomes used for commercial purposes should have a reproducible, homogeneous size distribution. Liposome size is dependent on the details of the preparation technique (i.e. sonication times, extrusion pressures, and lipid composition [98,173–175]) and dependent on the lipid composition, liposomes may fuse or aggregate over time [176]. Many techniques are available for size distribution assessment. A concise overview of many of these size determination techniques, including others not covered here, is available in a review article by Provdor [96]. The optimal method for the application is dependent on several factors, including ease of use/interpretation, whether individual vesicles or bulk sampling is desired, and whether observation of liposomes in their native environment is preferable. While it is often assumed that liposomes are spherical in shape, some studies have reported an elongated structure, dependent on process, osmotic, and lipid properties [177,178]. Thus, an analysis method which incorporates both size and morphology information is often desireable. The lipids used in liposome formation can become oxidized or hydrolyzed to form lysophospholipids resulting in bilayer permeability changes. These effects can be minimized by adding antioxidants such as α-tocopherol or BHT; storing the liposome preparation under an atmosphere of nitrogen or argon; ensuring that peroxide forming solvents are completely removed from the preparation prior to storage [170]. The chromatographic methods cited in this review provide the ability to monitor lipid, and subsequent, liposome stability.

The methods outlined in this article provide quality control for many key liposome characteristics, however additional methods specific to the application may be required [179]. These methods include characteristics such pH, osmolality, and surface charge, or may need to address other issues, such as removal of endotoxins and liposome sterilization, which need to be considered when the end liposome product is to be used for medicinal purposes. Residual solvents may be toxic when liposomes are used to encapsulate drugs and can cause liposome destabilization upon storage. Solvent removal poses a more significant issue when the batch size of liposomes is increased [169]. All of these consid-

erations are accentuated when liposomes are to be used as drug delivery vehicles [169]. If liposomes are prepared under well-controlled conditions, and if their characteristics are optimized for their specific application, they can be truly amazing and versatile tools ameliorating the specific application significantly. Several pharmaceutical compounds using liposomes as a drug-delivery system are currently approved by the FDA, including doxorubicin, daunorubicin, amphotericin B, morphine, and cytarabine [180,181]. When used for drug encapsulation, liposomes offer the ability to formulate a drug for sustained release, targeted delivery, and extended longevity of sensitive encapsulated molecules. Another of many positive examples of liposome uses is their application in bioanalytical systems. They have be shown to provide enormously lowered limits of detection in simple biosensor assays while staying functional and stable for more than 12 months of storage at 4 °C [182].

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