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Liposome-Encapsulated Silver Sulfadiazine (SSD) for the Topical Treatment of Infected Burns: Thermodynamics of Drug Encapsulation and Kinetics of Drug Release

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ABSTRACT

Liposomes encapsulating silver sulfadiazine (SSD), the drug of choice for topical treatment of infected burns, are investigated as an improved delivery system that could act as a locally targeted sustained-release drug depot. This communication reports the first stage of the investigation and is focused on (a) the development of spectrophotometric assays for liposome-encapsulated and for free (aqueous soluble) and SSD, (b) on evaluation of the efficiency of encapsulation and kinetics of drug release. DMSO containing 140 mM NH_3 was found to be the best solvent for dissolution of the liposomes and for determination of their SSD content. Peak absorption of liposome-originating SSD in this solvent is at 263 nm with e_m values of 23×10^3–26×10^3. Peak absorption of SSD in aqueous solutions is at 254 nm with e_m magnitudes varying from 2×10^3 to 23×10^3, depending on the electrolytic composition of the system. Kinetic studies of drug release and separations by centrifugation and by gel-exclusion chromatography all indicate that the SSD in the liposomal system is distributed among three states: encapsulated, soluble unencapsulated, and stable (unencapsulated) aggregates that reside in the aqueous phase in which the liposomes are suspended. The liposomal SSD systems were found to meet the essential requirements of high-efficiency encapsulation and sustained drug release. Encapsulation efficiencies of $> 80\%$ at 10 mM lipid, reaching up to 95% at 100 mM lipid, were obtained. The release of encapsulated SSD follows first-order kinetics, with half-life up to 24 hr and with sensitivity to the electrolytes in the system. It is concluded that SSD–liposomal systems are feasible, have potential benefits over treatment with free SSD, and merit further pursuit into providing local targeting.

ABBREVIATIONS

CH, cholesterol; MLV, multilamellar Vesicle; PC, phosphatidyl choline; SSD, silver sulfadiazine; SDMC, solvent dilution microcarrier.

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INTRODUCTION

SSD, the antibacterial drug of choice for the topical treatment of infected burns [1–5], affects a broad spectrum of organisms and the incidence of resistance (or its development) is considerably lower than with most antibiotics. Effective therapy, using the current SSD dosage form which is a 1% cream, requires several daily (usually two–four) applications. This frequency in itself interferes with the healing, whether a therapeutic agent is used at all and (if used) independent of its specific nature, as every change of dressings can be painful to the patient, can cause further trauma to a wound/burn, and exposure afresh to infecting agents. Further trauma and pain are involved in the present case since the SSD cream is not biodegradable and requires removal prior to reapplication. The conceptual approach of the present study is that the frequencies of dosing and of interference with the injury and with the self-healing processes could be significantly lowered (and with it the accompanying problems) were the administered SSD not in a cream form, but encapsulated within bioadhesive liposomes that can act, when applied topically, as site-adherent sustained-release drug-depots [6, 7]. Indeed, encouragement for this approach is found in in vivo studies reported for an SSD dosage form consisting of in situ association of SSD with a lipid-based carrier (SDMC) on a dressing matrix [8].

For SSD-encapsulating liposomes to be effective delivery systems that provide a real improvement over the current situation, the liposomes should encapsulate sufficiently high SSD doses and be capable of releasing it over several days. The present report is the first stage in the development of liposomal–SSD systems, focused on the development of assays for liposomal SSD, on the thermodynamics of SSD encapsulation, and on the kinetics of its release.

SSD is reported to have low solubility in aqueous systems, which is only slightly better in organic systems [1, 9–11]. Thus, it cannot be labeled a "hydrophobic" molecule. Furthermore, it is subject to specific ion effects on its silver component, effects that can lead to dissociation of the molecule into Ag^+ and SD^- and to changes (up or down) in its solubility [1, 9–11]. With this background, care must be taken in selecting the solvent system that will serve as a vehicle in which the SSD–liposome systems will be administered. To that end, the physicochemical studies were conducted in the following potential vehicles: (a) HEPES buffer at pH = 7.2, at a total salt concentration of 150 mM, maintained through mixtures of the buffer components and KNO_3; and (b) KNO_3/KCl solutions, adjusted to the pH range of 6–7, maintaining a total salt concentration of 140 mM.

RESULTS AND DISCUSSION

Assays for Free and Liposome-Encapsulated SSD

Free SSD in aqueous solutions could be determined by its absorbance in a given solvent system. For all aqueous systems, peak absorption was determined to be 254 nm and linearity was observed over the SSD concentration range of 0–55 μM. The molar extinction coefficients determined, listed in Table 1, show reproducibility and differences in sensitivity that seem to be anion-specific. It was verified that the increase in absorbance in the chloride (compared to those

TABLE 1. Molar Extinction Coefficients of SSD in Aqueous and in Liposome Systems

System	Assay Medium	ϵ_M
Aqueous SSD in:		$\lambda = 254$ nm
$HEPES:KNO_3$ 10:140	$HEPES:KNO_3$ 10:140	2000 ± 50
$HEPES:KNO_3$ 20:130	$HEPES:KNO_3$ 20:130	2140 ± 2
$HEPES:KNO_3$ 35:115	$HEPES:KNO_3$ 35:115	2600 ± 15
$HEPES:KNO_3$ 50:100	$HEPES:KNO_3$ 50:100	2800 ± 10
Liposome-encapsulated SSD in:		$\lambda = 263$ nm
$HEPES:KNO_3$ 10:140		
$HEPES:KNO_3$ 20:130	$DMSO\text{-}NH_{3r}$	$22,500 \pm 200$
$HEPES:KNO_3$ 35:115		
$HEPES:KNO_3$ 50:100		
Aqueous SSD in:		$\lambda = 254$ nm
KCl 140	KCl 140	$22,800 \pm 90$
$KCl:KNO_3$ 80:60	$KCl:KNO_3$ 80:60	$20,000 \pm 170$
$KCl:KNO_3$ 60:80	$KCl:KNO_3$ 60:80	$18,070 \pm 30$
KNO_3 140	KNO_3 140	3060 ± 60
Liposome-encapsulated SSD in:		$\lambda = 263$ nm
KCl 140		
$KCl:KNO_3$ 80:60	$DMSO\text{-}NH_{3r}$	$25,500 \pm 200$
$KCl:KNO_3$ 60:80		
KNO_3 140		

in the nitrate)-containing systems, which is expressed in the ϵ_M values, was not due to the formation of insoluble salt aggregates that could scatter the light.

Obviously, determination of a liposome–drug system by absorption spectroscopy requires dissolution or micellization of the liposomes (in order to clarify the solution) together with dissolution of the encapsulated matter. Moreover, as the system can include both encapsulated and unencapsulated drug, the liposome-solvating procedure and materials should allow for the determination of all drug in the system, whether it originated from the encapsulated or from the unencapsulated fraction. For the present case, it was found that DMSO containing 140 mM NH_3 was the best solvent for liposomal–SSD, with capability to clarify liposomal suspensions up to 3 mM lipid, which was sufficient for all systems investigated. It was also verified that empty liposomes as well as the NH_3 did not have absorbance in DMSO at the relevant region, and that additions of water to pure DMSO (water which would come from the liposomes, from the aqueous SSD stock solutions, and from the NH_3 stock solution) did not have any effect on the DMSO spectra.

For all systems tested, independent of the composition of the aqueous media in which the liposomes were suspended prior to their introduction into the $DMSO/NH_3$ media, peak absorption was determined to be 263 nm and linearity was observed over the SSD concentration range of 0–40 μM. The molar extinction coefficients determined, also listed in Table 1, show good reproducibility and are of sufficient magnitude to indicate that this assay can be used with a high level of confidence for SSD concentrations down to the μM range.

Based on these results, SSD was assayed throughout this study using the protocols listed under Methods.

Efficiency of SSD Encapsulation

The efficiency of SSD encapsulation was pursued, for each vehicle (solvent system) explored, at three liposome concentrations. Typical results, determined as detailed under Methods, are listed in Table 2, in the column titled "% encapsulation by centrifugation." As can be seen, regardless of the specific vehicle, the data invariably show high encapsulations, the majority higher than 90%. Furthermore, there is no sensitivity to lipid concentration. To further demonstrate this point, data for a selected system are also plotted on the left-hand side of Figure 1.

The combination of high encapsulation, already achieved at 10 mM lipid, and the insensitivity to liposome concentration are contrary to the theoretical expectations and to all previous experimental support [12].

Through theoretical thermodynamic analysis supported by experimental data, it had been previously established [12] that the efficiency of encapsulation is a product of the drug partition coefficient and the liposome concentration. Thus, for a given drug and liposome species (in terms of type and lipid composition), the liposome concentration is the major factor affecting the efficiency of encapsulation, which is expected to increase with the increase in liposome concentration. It takes a highly lipophilic drug, such as Progesterone [12]

TABLE 2. Efficiencies of SSD Encapsulation in Multilamellar Liposomes

Buffer System	[Lipid] mM	% Encapsulation	
		Centrifugation	Kinetics
KNO_3	10	94 ± 3	67 ± 5
	50	94 ± 1	75 ± 3
	100	96*	85 ± 6
KCl/KNO_3	10	89 ± 3	88 ± 1
20:120	50	89 ± 1	90 ± 1
	100	94*	95 ± 2
KCl/KNO_3	10	80 ± 2	77 ± 6
80:60	50	78 ± 1	94 ± 1
	100	78*	84 ± 10
KCl	10	83 ± 1	64 ± 3
	50	88 ± 1	66 ± 1
	100	70 ± 6	94 ± 3
$HEPES/KNO_3$	10	98*	78 ± 5
10:140	50	97*	71 ± 3
	100	97*	85 ± 6
$HEPES/KNO_3$	10	97*	75 ± 6
20:130	50	97*	75 ± 4
	100		91 ± 2
$HEPES/KNO_3$	10	84 ± 0.8	65 ± 11
35:115	50	97*	86 ± 5
	100	90 ± 2	94 ± 2
$HEPES/KNO_3$	10	96*	64 ± 3
50:100	50	96*	88 ± 9
	100	94 ± 1	78*

* Indicates standard deviations < 0.5.

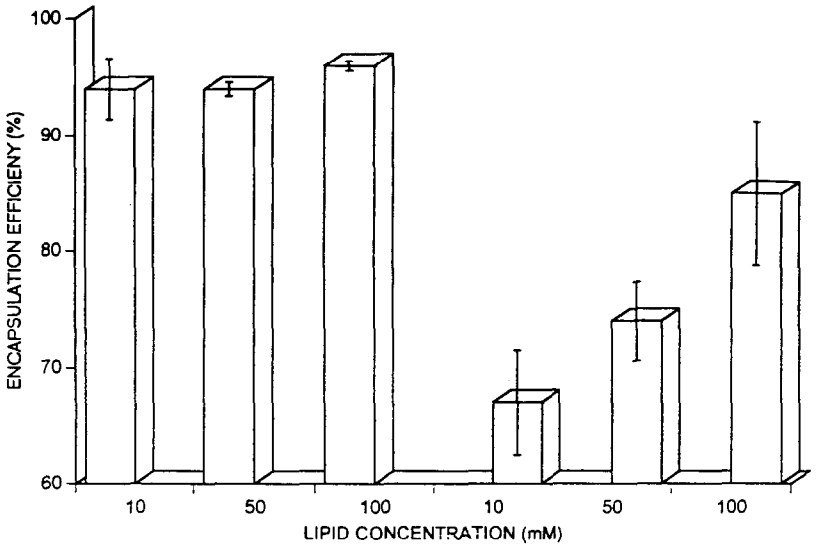

FIGURE 1. The efficiency of SSD encapsulation in MLV as function of liposome concentration (expressed in mM lipid) calculated from separation of aliquots by centrifugation (left-hand side) and from the kinetics of SSD release (right-hand side). Data are for preparations in which the swelling solution as well as the liposome-suspension vehicle was 140 mM KNO_3 adjusted to neutral pH.

Retinoic acid or Cyclosporin A (Linenberg, Mimrod & Margalit, unpublished data) to be encapsulated at efficiencies higher than 90% at liposome concentration as low as 10 mM. SSD is neither a hydrophilic nor a lipophilic molecule, having low solubility in both aqueous and organic solvents [2, 9–11]. Thus, the high encapsulation and the insensitivity to liposome concentration cannot be attributed to high lipophilicity, and accounting for the present findings requires some departure from the conditions under which the thermodynamic theory is valid.

One avenue of departure would arise if not all SSD in the system participated in the equilibrium distribution of the drug between the liposomes and the external aqueous medium in which the liposomes are suspended. Accordingly, it is postulated that the SSD in the liposomal system is distributed among the following three fractions: (i) encapsulated within the liposome, presumably within the lipid bilayer as it does prefer organic to water systems; (ii) unencapsulated but soluble in the external aqueous phase; and (iii) SSD that has exceeded the limits of solubility in the aqueous and/or lipid phases and is therefore "salted out" in the form of highly stable aggregates. If this is the case, then the SSD found in the pellet (postcentrifugation) contains both the truly encapsulated fraction and the SSD aggregates. It follows, then, that for this specific drug, separation of liposomes from the external aqueous phase by centrifugation is not a suitable method for the determination of the efficiency of encapsulation.

If, indeed, the system contains SSD aggregates that are large enough and heavy enough to precipitate during the centrifugation, they could be in the external aqueous phase or inside the liposomes. Additional support for this hypothesis together with localization of the aggregates has been sought through

gel-exclusion chromatography, which is another (and independent from centrifugation) separation method.

Gel-exclusion chromatography of drug–liposome systems usually separates the preparation into two fractions. The dominant components of the first are the liposomes and the drug encapsulated within them. The second fraction is the unencapsulated drug, which usually requires an elution volume distinctly larger than that of the liposomes. As shown in Figure 2, the outcome of such chromatographies for the MLV–SSD systems are somewhat different.

Both expected fractions, i.e., the liposomes (containing the encapsulated drug) and the unencapsulated drug, are obtained. In addition, there is a third drug fraction that constitutes 20% of the total drug in the system and precedes the liposomes. This fraction is attributed to the proposed SSD aggregates, and its location indicates that these aggregates reside at the external aqueous phase in which the liposomes are suspended, rather than inside the liposomes.

The Kinetics of SSD Release

Typical results of the kinetics of SSD release from MLV, displaying the increase in cumulative drug release vs. time, are shown in Figure 3 for three liposome concentrations and for the same solvent system. As can be seen from the data, there is a continuous release of SSD over the 10 hr period measured, with an inverse relationship of rate and liposome concentration. This is similar to the patterns observed with other drugs, and indicates that the previously derived multipool mechanism [6, 7, 12] might fit the present case also. If all drug in the liposomal system is available for release, then the release kinetics can be

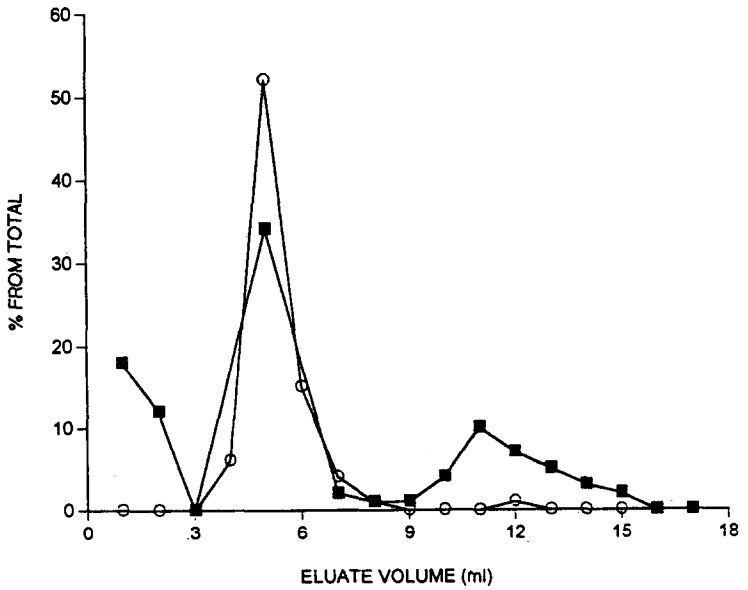

FIGURE 1. Gel-exclusion chromatography of an SSD–liposome system (■: SSD; ○: lipid) on a Sephadex G-50 (1*20) column. Elution was by 140 mM KCl adjusted to pH = 6.

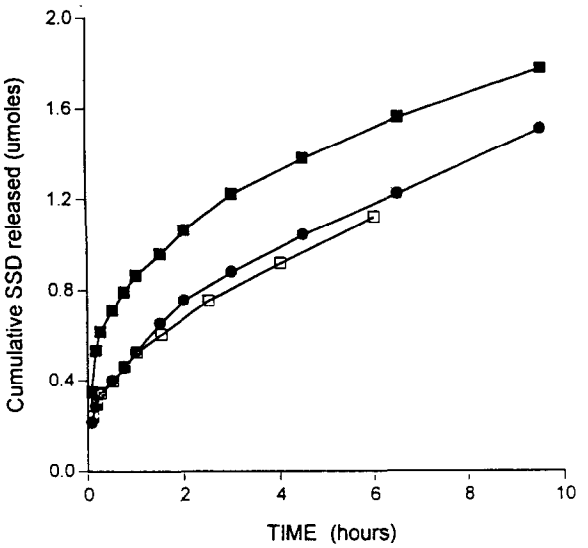

FIGURE 3. Kinetics of SSD release from MLV as function of time and of liposome concentration. Points are the experimental data, and the curves are the theoretical expectations according to equation (3) in the test. ■: 10 mM lipid; ●: 50 mM lipid; ○: 100 mM lipid.

expressed by equation (1):

$$f = \sum_{j=1}^{n} f_j(1 - e^{-k_j t}) \tag{1}$$

where f is the cumulative released drug, normalized to the total drug in the system at time = 0, n is the number of drug pools participating in the release, f_j denotes the fraction of the total drug in the system occupying the jth drug pool at time = 0, and k_j denotes the rate constant of drug release from the jth pool. However, based on the data from the previous section, it can no longer be assumed that all drug in the sac at time = 0 participates in the release process. This requires some rearrangement of equation (1) into equation (2) below:

$$[D] = \sum_{j=1}^{n} [DP_j]_o(1 - e^{-k_j t}) \tag{2}$$

where D denotes the drug released into the dialysate, from all participating pools, at time = t, DP_j denotes the jth drug pool, and $[DP_j]_o$ is the concentration of drug in the jth pool at time = 0. All other parameters are as defined for equation (1) above.

The data were processed according to equation (2) above, and were found to fit a two-pool case, as expressed in equation (3) below:

$$[D] = [DP_u]_o(1 - e^{-k_u t}) + [DP_e]_o(1 - e^{-k_e t}) \tag{3}$$

where the subscripts u and e denote parameters of the unencapsulated (but soluble) and encapsulated drug, respectively, and all other parameters are as defined in equations (1) and (2). Moreover, the sum of $[DP_u]_o$ and $[DP_e]_o$, which represents the quantity of drug in the sac at time = 0 that participated in the kinetic process, was found to be lower than the total quantity of drug in the system at time = 0. Thus, these data constitute another, and independent, support of the proposal postulated (and supported there by data) in the previous section, with respect to the three states of SSD in the liposomal preparation. The data of this and of the previous section clearly indicate that the aggregated fraction is excluded from the encapsulated–unencapsulated equilibrium, and that the expectation for the effects of partition coefficient and liposome concentration on the efficiency of encapsulation should be imposed on the equilibrated SSD alone, as defined in equation (4):

$$\% \text{ ENCAPSULATION} = \left(\frac{[DP_e]_o}{[DP_e]_o + [DP_u]_o} \right) * 100. \quad (4)$$

The % encapsulation, calculated from the results of the kinetic experiments according to equation (4) above, are also listed in Table 2, under the heading "by kinetics" and a representative case is illustrated on the right-hand side of Figure 1. As clearly seen, these data now comply with the theoretical expectation for the increase in encapsulation with the increase in liposome concentrations. They also allow the conclusion that SSD is a well-encapsulated drug, as even at the lower end of liposome concentrations there is appreciable encapsulation and complete encapsulation is approached at the higher end of lipid concentrations.

The rate constants obtained for the release of the encapsulated SSD are presented in Figure 4A and B, for the HEPES:KNO_3 and the KCl:KNO_3 solvent systems, respectively. Based on previous experience, the rate constants were expected to decrease with the increase in liposome concentration due to increases in deviations from ideality (Margalit et al., 1991). This expectation is observed, for the most part, with the HEPES:KNO_3 systems (4a). There were no significant differences in the magnitudes of the rate constants (for similar lipid concentrations) from one solvent system to the other, except for the increase of the rate constant at the highest lipid concentration for the system in which the HEPES-to-KNO_3 molar ratio was 50:100. The situation in the nitrate:chloride system (Figure 4B) is quite different. While the expected decrease is quite clear for the nitrate-only system, with the introduction of chloride into the system and with the increase in its level, the trend reverses and the magnitudes of the rate constants change. This phenomenon implies the existence of a specific ion (chloride) effect that masks that of the general deviations from ideality.

CONCLUSIONS

Liposome–drug systems, indicated for the topical therapy of infected wounds and burns, must meet several physiochemical criteria before their biological activity can be considered at all. Specifically, such systems should show high efficiencies of drug encapsulation and rates of drug release that would conform

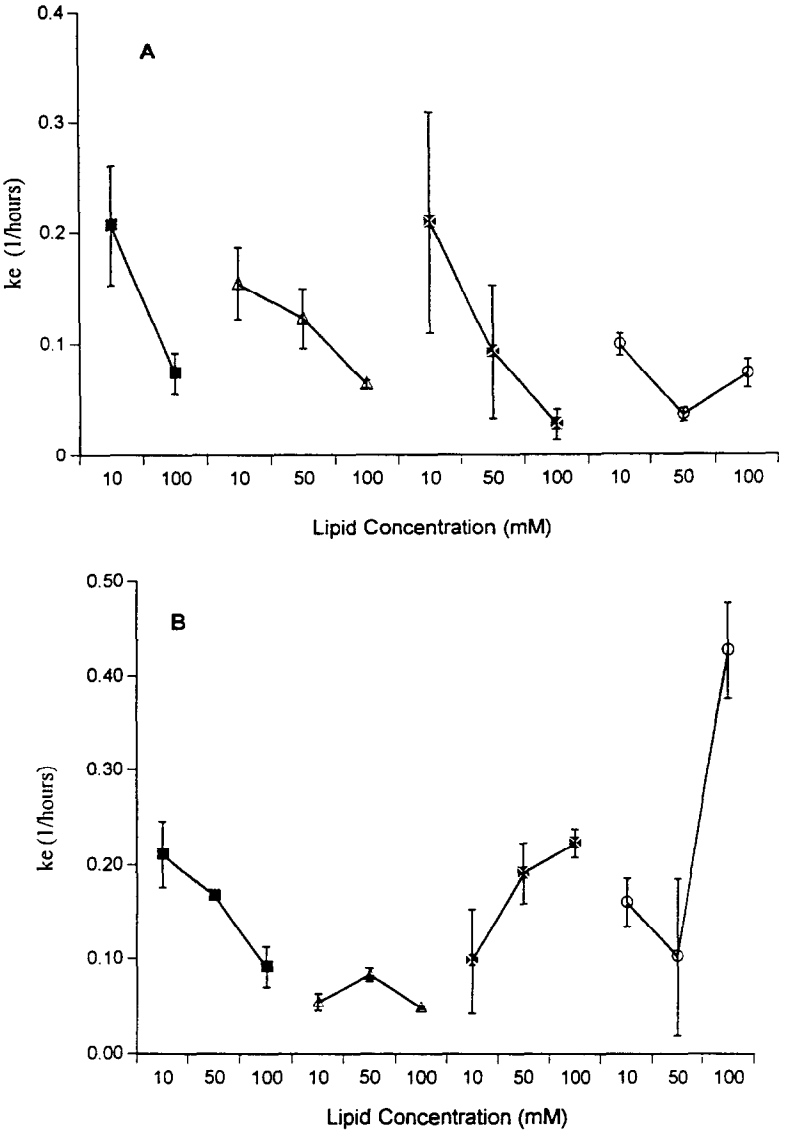

FIGURE 4. Effects of liposome concentration (expressed in lipid concentration) on the rate constant of the release of the encapsulated SSD as function of media composition. The rate constants were obtained from the data processing of experiments of the type shown in Figure 3, using equation (3) in the text. (A) Data for solvent systems composed of HEPES buffer and nitrate ions. ■: HEPES: KNO_3 10/140; △: HEPES: KNO_3 20/130; ⊠: HEPES: KNO_3 35/115; ○: HEPES: KNO_3 50/100 (see "Materials and Methods" for additional details). (B) Data for solvent systems composed of the potassium salts of nitrate and chloride. ■: KNO_3 140; △: KNO_3:KCl 120/20; ⊠: KNO_3:KCl 60/80; ○: KCl 140 (see "Materials and Methods" for additional details).

to behavior of sustained-release depots, and would allow dose reapplications at intervals of a day or more, instead of several times a day. Studying a panel of SSD–liposome preparations that vary in lipid composition and in the nature of the vehicle (especially with respect to electrolyte content), applying physicochemical principles, the following conclusions have been drawn:

(a) Independent of the solvent system used and of the liposome concentration, it is possible to achieve exceptionally high levels of SSD encapsulation.
(b) Among the systems studied, the best with respect to sustained release behavior are those in which HEPES/KNO_3 at molar ratios of 35/115 or 50/100 are the vehicle for liposome concentrations of 50 and 100 mM (lipid). These yield half-life of SSD release in the range of 20–25 hr.
(c) The specific-ion effect on the SSD release, noted for chloride, carries the implication that the rate-limiting step is at a location close enough to the interface between the liposomal membrane and the external aqueous medium to allow access for such ions. Furthermore, it carries the implication that predictions on the rate of release in vivo should be based on in vitro studies in actual or in simulated would fluid.
(d) The complexities of SSD, observed in this study, indicate that strong association of this drug with a microparticulate carrier, be it a microsphere, a liposome, or other lipid-based carriers, cannot be taken as evidence for encapsulation alone. Rather, combination of several independent lines of investigation are required to give insight into the state(s) of the drug in each particular carrier. Such data are required not only for better basic understanding of the system, but also for evaluation of the delivery of the drug from the carrier in vivo and the extent to which targeting attempts via the carrier can be successful.

The results of the present study are encouraging enough to merit progression to the next step, modifying the SSD-encapsulating liposomes into the bioadhesive systems for local targeting [7, 12].

EXPERIMENTAL

Materials

SSD was a kind gift from American International Chemicals, Inc. (MA, U.S.). Cholesterol and Sephadex G-50 were from Sigma Chemical Co. (St. Louis, MO, U.S.). High-purity soybean phosphatidylcholine (PC) was purchased from American Lecithin Company (Atlanta, GA, U.S.). ^{3}H-cholesterol and ^{14}C-cholesterol were purchased from Amersham (Arlington Heights, IL, U.S.) and found to be stable. Spectra/Por4 dialysis tubings (molecular weight cutoff of 12,000–14,000) were purchased from Spectrum Medical Industries (Los Angeles, CA, U.S.). All other reagents were of analytical grade.

Absorbance spectra were measured using Bausch & Lomb 601 and Shimadzu 160-A spectrophotometers. High-speed centrifugation was performed using a Beckman L-8 ultracentrifuge. Liquid scintillation counting was performed with a Kontron Analytical Betamatic I counter.

Methods

Liposome Preparation. MLV, composed of PC:CH at mole ratios of 1:1 with a trace of ^{3}H-CH, were prepared according to our usual procedures [6, 7, 12]. The following systems were used as swelling solutions: (a) HEPES/KNO_3 mixtures at molar ratios of 10/140, 20/130, 35/115, 50/100, and (b) KCl/KNO_3 mixtures at molar ratios of 0/140, 20/120, 80/60, and 140/0.

SSD Encapsulation. SSD was encapsulated in the course of liposome preparation, according to the procedures for drugs that have poor water solubility [6, 7, 12]. Briefly, SSD powder of desired weight (usually at a dose corresponding to 5 μmoles/mL in the final liposome preparation) was added to the weighed lipid mixture and the combined drug–lipid system was dissolved in chloroform. A clear solution was obtained, indicating satisfactory SSD dissolution. The solution was evaporated to dryness in a rotary evaporator under reduced pressure and subjected to the following steps in liposome (MLV) preparation. Namely, swelling with one of the swelling solutions listed above, with extensive vortexing and incubation for 2 hr in a shaker bath, at 37°C and 80 strokes/min. When desired, the liposomes were separated from excess unencapsulated matter by high-speed centrifugation, for 1–2 hr, at a g force of 250,000 and at 4°C.

SSD Determination. SSD was determined by the spectrophotometric assay developed in the course of this study, and discussed in the Results section. Based on the results, the following protocols were adopted and used throughout this study:

(i) Complete SSD–MLV preparations: The solvent system for this assay was DMSO containing 140 mM NH_3. The assay wavelength was 263 nm, and 3 mL of the DMSO/NH_3 solution served as the blank. Aliquots withdrawn from the liposome were added stepwise to 3 mL of the DMSO/NH_3 solution, not exceeding total addition of 15 μL, and the absorbance of each addition was recorded. The concentration of SSD was determined for each step in the titration using the suitable value of ϵ_M as listed in Table 1, and the average of all determinations was calculated. Standard deviations did not exceed 2%.

(ii) Aqueous SSD systems, originating from centrifuge-separated unencapsulated fractions, or from dialysates of drug release studies: (a) As described in (i) above. (b) As described in (i) above, but in the appropriate buffer system instead of DMSO/NH_3. In these cases, the absorbance was recorded at 254 nm and the concentrations were determined using the suitable values of ϵ_M listed in Table 1. As in (i) above, the final determination was the average of all samples, and standard deviations did not exceed 2%.

(iii) SSD–MLV originating from centrifuge-separated liposome pellets: The pellets were resuspended in the original buffer system to a desired volume and assayed according to (i) above.

Efficiency of SSD Encapsulation. Samples of each liposome preparation were subjected to high-speed centrifugation under the conditions listed above, and the quantities of SSD in the supernatants and in the pellets (resuspended in the original buffer system), as well as that of the total preparation prior to centrifugations, were determined using the spectrophotometric assay. In addition, aliquots of each preparation were taken to β counting in order to determine the lipid concentration.

Gel-Exclusion Chromatography. SSD–liposomes were subjected to gel-exclusion chromatography on a Sephadex G 50 (1 * 20) column, with elution by 140 mM KCl adjusted to pH = 6. 1 mL fractions were collected and assayed, each, for lipid (via the radiolabel) and for SSD.

Kinetics of Drug Release. The kinetics of SSD release from the liposomal preparations were studied in a dialysis setup according to previously established methods [6, 7, 12]. Briefly, 0.5 or 1.0 samples were transferred into a dialysis sac, and the sac was immersed into 10 mL of drug-free buffer (i.e., one of the solvent systems) that was constantly stirred. At designated time internals, the sac was transferred into a fresh reservoir of drug-free buffer. The SSD was assayed in the sac before and at the end of the run and in all the dialysates. The experiments were conducted with samples from the original preparations, without any fractionation or centrifugation. Experiments were run for a total of 24 preparations, which correspond to the solvent systems listed above, with three liposome concentrations for each solvent system. For each case, dialysis was against the same solvent system used in the swelling solution.

Unless specifically desired, all SSD-containing systems were continuously protected from light.

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