Nebulization of Liposomes. II. The Effects of Size and Modeling of Solute Release Profiles

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A series of carboxyfluorescein (CF)-containing multilamellar vesicle (MLV) dispersions was prepared and extruded through polycarbonate membranes ranging in size from 0.2 to 5 μm. Vesicle dispersions were nebulized for 80 min using a Collison nebulizer, and the release of CF was monitored during nebulization. Solute retention was dependent upon the size of the vesicles and leakage ranged from 7.9 ± 0.4% (N = 3) for vesicles extruded through 0.2-μm filters to 76.8 ± 5.9% (N = 3) for liposomes that were not filtered. Solute release profiles obtained over ≥420-min nebulization periods conformed to a two-compartment kinetic model and exhibited a "fast" initial phase (k_1 = 0.052 ± 0.0043) followed by a "slow" terminal phase (k_2 = 0.0034 ± 0.00018). The results show that CF retention can be increased by nebulizing small vesicles and modeling suggests that the rate of CF leakage from the bilayers is faster than from the core of the liposomes.

KEY WORDS: aerosols; carboxyfluorescein; liposomes; nebulizers.

INTRODUCTION

A method of experimentally following the release of solute from nebulized liposomes has previously been described, and the effects of changing the lipid composition of vesicles has been studied (1). In this report the effects of altering the size of liposomes are investigated, as some evidence suggests that liposomes are broken down to smaller sizes as they are nebulized (2). Although it is not known what physically happens to the vesicles as they are nebulized, by producing liposomes which are substantially smaller than the mass median aerodynamic diameter (MMAD) of the droplets produced by the nebulizer, solute leakage might be minimized. In conjunction, mathematical modeling of the (CF) concentration vs time profiles was also performed as an aid to investigate the mechanism of solute release.

THEORY

In an aqueous liposome dispersion the free concentration of solute, in this case purified CF, C_{free}, is related to the total concentration of CF, C_{tot}, by

$$C_{tot} = C_{encaps} + C_{free} \quad (1)$$

where C_{encaps} is the effective concentration of CF which is encapsulated by the liposomes. Specifically, this concentration is the concentration increase which would take place within the total volume of dispersion if the encapsulated CF were completely released from the liposomes.

It has been shown by Mercer *et al.* (3) and noted by others (4–6) that solutions undergoing nebulization become concentrated by a loss of solvent, due to evaporation from reentrained nebulized droplets. Since over 99% of all droplets are reentrained in the Collison nebulizer (4,7), concentration of the solution will take place. This process is described by a power function (3,4) which can be written for the total CF in the liposome dispersion as

$$C_{tot}^{\#} = C_{tot} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} \quad (2)$$

where $C_{tot}^{\#}$ is the total concentration of CF in solution after release from the liposomes at any time during nebulization (μg/ml), V_o is the initial volume of the dispersion (ml), F is the output flow rate of air from the nebulizer (liters/min), S_v is the solvent loss, and S_n is the solution loss from the dispersion (ml/liter/min). Although convenient to use it should be recognized that it is not possible to present the expressions in terms of absolute amounts since the volume of the dispersion during nebulization is unknown and only the concentration of solute in the dispersion can be analyzed directly. If the concentration effect is accounted for in Eq. (1), then

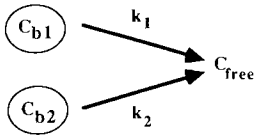
$$C_{tot}^{\#} = C_{free}^{\#} + C_{encaps}^{\#} \quad (3)$$

or

$$C_{tot} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} = C_{free} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} + C_{encaps} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} \quad (4)$$

The free ($C_{free}^{\#}$) and total ($C_{tot}^{\#}$) CF concentrations in dispersions were monitored during nebulization experiments as described elsewhere (1).

Results indicated that CF was being released from the liposomes due to nebulization (1). This increase or accumulation of C_{free} can be described by Scheme I, where b1 and b2 represent two independent compartments within the multilamellar liposomes which release CF according to the ap-



Scheme I

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parent first-order rate constants k_1 and k_2, respectively. The rate of accumulation of C_{free} by definition can therefore be written as

$$\frac{dC_{free}}{dt} = k_1 * C_{b1} + k_2 * C_{b2} \quad (5)$$

By stating that

$$C_{encaps} = C_{b1} + C_{b2} \quad (6)$$

the rate of release of encapsulated CF is given by

$$\frac{dC_{encaps}}{dt} = -k_1 * C_{b1} - k_2 * C_{b2} \quad (7)$$

Integrating Eqs. (5) and (7) with respect to time gives

$$C_{free} = \{C_{fo} + C_{b1o} * [1 - \exp^{(-k_1*t)}] + C_{b2o} * [1 - \exp^{(-k_2*t)}]\} \quad (8)$$

and

$$C_{encaps} = C_{b1o} * e^{-k_1*t} + C_{b2o} * e^{-k_2*t} \quad (9)$$

where C_{fo} is the initial free concentration of CF in the dispersion and C_{b1o} and C_{b2o} are the initial concentrations of CF in the two compartments. From Eqs. (3) and (4) it is readily shown that

$$C_{free}{}^{\#} = C_{free} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} \quad (10)$$

and substituting the right-hand side of Eq. (8) for C_{free} in Eq. (10) gives

$$C_{free}{}^{\#} = \{C_{fo} + C_{b1o} * [1 - \exp^{(-k_1*t)}] + C_{b2o} * [1 - \exp^{(-k_2*t)}]\} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} \quad (11)$$

This general expression can be fit to experimental data obtained for the free concentration of CF. As it stands, however, some seven parameters require estimation. This can be reduced significantly by using Eq. (2) to obtain independent estimates of S_v and S_n by nonlinear regression. Also, from Eq. (6), C_{b1o} can be substituted by $[C_{encaps(0)} - C_{b2o}]$ where $C_{encaps(0)}$ is the initial encapsulated CF concentration. This reduces the nonlinear regression estimates to three for k_1, k_2, and C_{b2o}. Giving the usable expression

$$C_{free}{}^{\#} = \{C_{fo} + [C_{encaps(0)} - C_{b2o}] * [1 - \exp^{(-k_1*t)}] + C_{b2o} * [1 - \exp^{(-k_2*t)}]\} * \left[\frac{V_o}{V_o - ((S_v + S_n) * F * t)}\right]^{\left(\frac{S_v}{S_v + S_n}\right)} \quad (12)$$

MATERIALS AND METHODS

Liposome Size. Liposomes of soy phosphatidylcholine (SPC) (Phospholipon 90; American Lecithin Co., Atlanta, GA) and dipalmitoyl phosphatidylglycerol (DPPG) (Avanti Polar Lipids Inc., Birmingham, AL) were prepared and characterized as described previously (1). In this case the liposomes were extruded three times through polycarbonate membrane filters (Nucleopore Inc., Pleasanton, CA), prior to dialysis, ranging from 0.2 to 5 μm in pore size. In addition one batch was not extruded. A volume of liposome stock dispersion equivalent to 200 μg total CF was placed in a glass Collison flask and diluted to 50 g with phosphate-buffered saline (PBS). Nebulization with a Collison nebulizer (3 inlet/outlet; BGI Inc. Waltham, MA) was carried out for 80 min. Samples were removed periodically from the nebulized dispersions and assayed for their content of free CF and total CF by fluorimetry (Perkin-Elmer LS-3 fluorescence spectrometer; Perkin-Elmer Corp., Norwalk, CT) (1). The experiments were repeated three times for each size and with two independently prepared batches, A and B.

Modeling CF Release. For modeling purposes one batch of 1.0-μm liposomes were nebulized for ≥420 min. Estimates of S_n and S_v were obtained by fitting Eq. (2) to the average data obtained for the total CF during the experiments. Similar experiments were also performed with a solution of 200 μg CF in 50 g PBS. Model fitting was carried out using the NONLIN module of SYSTAT v3.0 for the Apple Macintosh (SYSTAT Inc., Evanston, IL). Using the fitted estimates of S_n and S_v, Eq. (12) was applied to the observed free CF data for the liposome dispersion. Estimates for k_1, k_2, and C_{b2o} were obtained using the same computer program.

RESULTS AND DISCUSSION

Liposome Size. For given batches of liposomes, as the extruded size increased, the release of encapsulated CF increased during nebulization (Fig. 1). The percentage of encapsulated CF released after 80 min of nebulization is shown in Table I and ranged from 7.9 ± 0.4% for 0.2-μm liposomes to 76.8 ± 5.9% for unextruded liposomes. Data are also shown for the percentage release after 10 min of nebulization, which is typically as much as a patient can tolerate at one sitting. Clearly, to minimize the release of solute the size of the liposomes should be small. However, it is well known (8) that as you decrease the size of liposomes, their entrapment efficiency is reduced, and so to deliver a given quantity of drug to the lung, the ratio of lipid to solute will be increased. The quantity of lipid and drug administered to the lung might become greater than a patient can tolerate during a period of nebulization. How small the vesicles will have to be may also be dependent upon the type of nebulizer which is used. The aerosol particle sizes produced by the Collison nebulizer were estimated to have an MMAD of 1.2 μm ± 1.7 σg (N = 10) as measured by an Andersen cascade impactor (1), and this is quite small compared with many of the nebulizers used clinically (9,10). When the percentage release of encapsulated CF after 80 min of nebulization is plotted against the extruded size of the liposomes, there is a cutoff in release at around the MMAD produced by the Collison (Fig. 2). Liposomes exceeding the mean droplet size of the aerosol may be fragmented (11) or severely distorted by nebulization, resulting in leakage of solute. Liposomes which are progressively smaller than the aerosol droplets are likely to be increasingly less exposed to the forces inducing droplet breakup. The observed plateau seen with the larger liposome

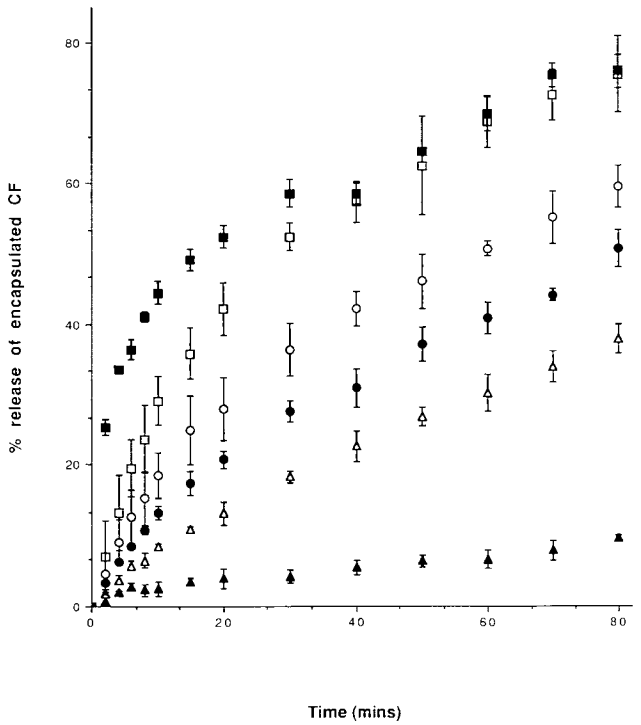

Fig. 1. The percentage release of encapsulated CF as a function of the nebulization time for different sizes of liposomes. Results are shown for liposomes of batch A as unextruded (■), 5.0 μm (□), 1.0 μm (○), 0.8 μm (●), 0.4 μm (△), and 0.2 μm (▲). Results are the averages of three experiments. Error bars are the range. Results for 2.0-μm liposomes are not shown for the purposes of clarity.

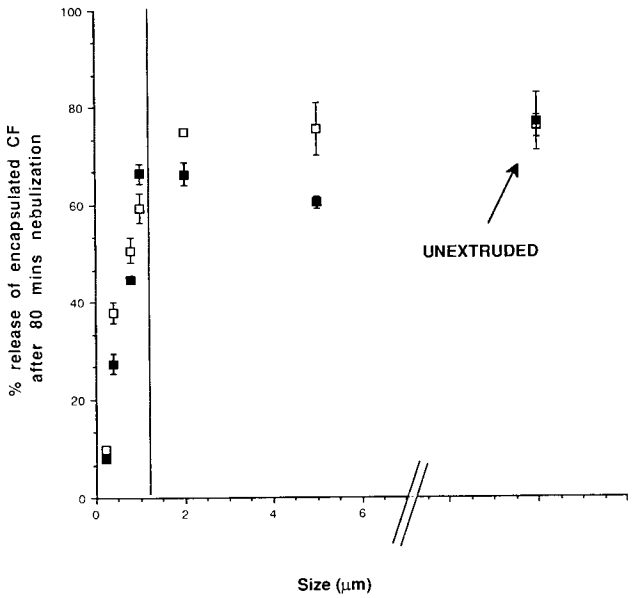

Fig. 2. The percentage release of encapsulated CF after 80 min of nebulization as a function of the extruded size of the liposomes. The line represents the MMAD of the droplets produced by the nebulizer. The averages of three experiments are shown for liposome batches A (□) and B (■). The error bars are the range.

dispersions may be due to the fact that the majority of CF is contained within liposomes which are greater than the droplet size. Similar CF leakage might therefore be expected, as the gas flow and hence the energy input for nebulization were constant. If this is true, then the results imply that, under similar conditions, nebulizers which produce a larger mean droplet diameter may have less impact on the solute retention of the liposomes. In general, if vesicles are nebulized for short periods and prepared significantly smaller the mean size of the aerosol droplets, the majority of solute may be retained within the vesicles and this can be enhanced by modifying the lipid composition of the vesicles as shown previously (1).

Modeling CF Release. The estimates of S_v and S_n are shown in Table II. There was some concern that the concentration vs time profile for the total CF may not just reflect a concentration of CF through evaporation. Additional CF "tightly" bound to the lipids may have been released only through prolonged nebulization and thus overestimate the concentration effect taking place. The model estimates of S_v and S_n for the liposome dispersion and free CF solution are of a similar order of magnitude. In fact, the concentration of solution apparently takes place faster than for the liposomes (Fig. 3). This is not entirely unexpected, as the presence of the lipid might reduce the degree of evaporation. Consequently, the results indicate that the use of Eq. (2) is valid when applied to a liposome dispersion. The estimates for k_1,

Table I. Percentage Release of Carboxyfluorescein (CF) After 10- and 80-min Nebulization of the Liposome Preparations

Liposome size (μm)[a]	% release of CF[b]			
	A		B	
	10 min	80 min	10 min	80 min
0.2	2.6 ± 2.4	9.7 ± 0.5	2.9 ± 0.5	7.9 ± 0.4
0.4	8.6 ± 0.3	37.9 ± 2.1	3.9 ± 0.7	27.3 ± 2.0
0.8	13.3 ± 1.0	50.7 ± 2.6	11.9 ± 0.7	44.8 ± 0.7
1.0	18.6 ± 3.3	59.4 ± 3.0	20.3 ± 0.9	66.4 ± 2.1
2.0	35.1 ± 0.9	74.8 ± 0.6	27.1 ± 0.4	66.3 ± 2.4
5.0	29.1 ± 3.5	75.5 ± 5.4	24.7 ± 0.8	60.4 ± 1.3
unx[c]	44.5 ± 1.6	75.9 ± 2.3	54.3 ± 1.2	76.8 ± 5.9

[a] Size of polycarbonate membrane through which initially prepared liposome dispersions were extruded.
[b] The percentage release of encapsulated CF from two batches (A and B) of liposomes after 10 and 80 min of nebulization. Results are the average of three experiments ± range.
[c] Liposomes which were not extruded.

Table II. Nonlinear Regression Estimates of the Parameters in Eqs. (2) and (12)

Parameter	Estimate[a]	
	Liposomes	Solutions
S_v (ml/L/min)	0.0083 ± 0.0013	0.0094 ± 0.0005
S_n (ml/L/min)	0.0067 ± 0.002	0.0097 ± 0.0017
k_1 (min^{-1})	0.052 ± 0.0043	
k_2 (min^{-1})	0.0034 ± 0.00018	
C_{b2o} (μg/ml)	1.90 ± 0.33	

[a] Values are the average estimates obtained from three experiments ± range of estimate.

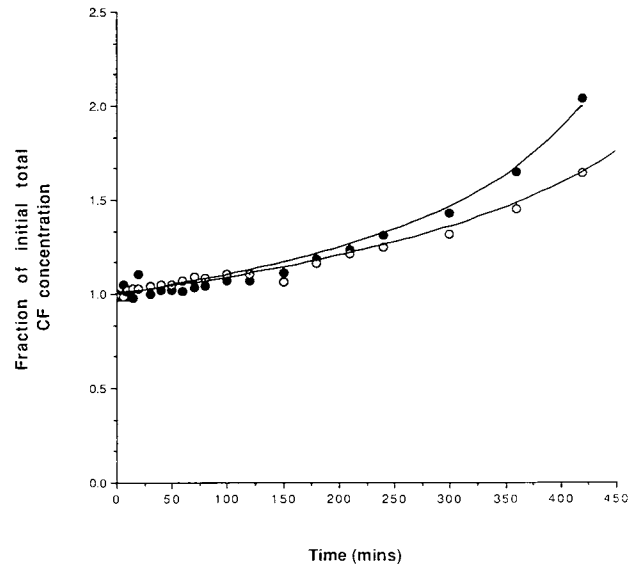

Fig. 3. The effect of nebulization on the concentration of a solution of carboxyfluorescein (●) and a liposome dispersion ($C_{tot}^{\#}$; ○) extruded through 1.0-μm-pore size filters. The results are shown as a fraction of the initial concentration in the nebulizer.

k_2, and C_{b2o} are also given in Table II and the concentration vs time plot is shown in Fig. 4. The good fits indicate that the solute release profiles can be effectively modeled by assuming a two-compartment model and accounting for the solute concentration which takes place during nebulization. The model was also applied to the liposome size data, where k_2 was fixed at 0.0034 min^{-1}, which was the average result

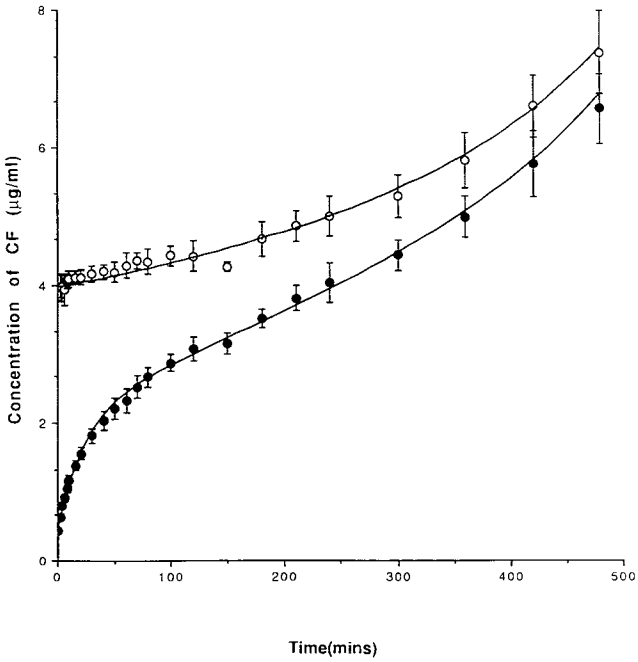

Fig. 4. Plots of the observed concentration data for $C_{tot}^{\#}$ (○) and $C_{free}^{\#}$ (●) obtained using liposomes extruded through 1.0-μm polycarbonate membrane filters. The results are the averages of three experiments. Error bars are the range. The lines associated with the plots represent the best nonlinear regression fits of the average data.

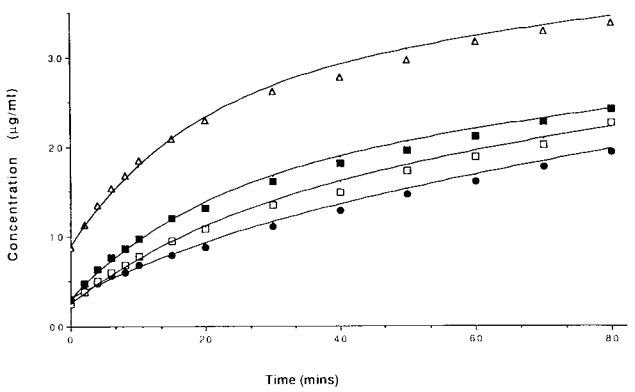

Fig. 5. Concentration vs nebulization time plots showing the release of CF from liposomes (batch A) of 0.4 μm (●), 0.8 μm (□), 1.0 μm (■), and 2.0 μm (△) together with their nonlinear regression fits (—). Insufficient data were available to obtain a computer fit of the 0.2-μm liposome data. The others were left out for the purpose of clarity.

obtained for the extended experiments (Table II). It was assumed that the slow component of the release profile was similar for liposomes of sizes 0.4 μm and greater. Nonlinear regression fits and the average experimental data are shown in Fig. 5 for liposomes of batch A, sized at 0.4, 0.8, 1.0, and 2.0 μm.

An interpretation of the CF release behavior is shown in Fig. 6. The aqueous core may constitute one of the compartments, b2, the other being the solute retained between the bilayers, b1. Presumably diffusion across the individual bilayers occurs at similar rates and hence the overall release rate can be represented by k_1. It is more difficult to speculate why a second and independent release of solute may be occurring from the liposomes. If from the internal core, it may be that the first or first several surrounding lipid bilayers are more "tightly" packed than subsequent ones and present a rate-limiting barrier to the release of the encapsulated solute. It can also be expected that the outer bilayers are more exposed to the rigors of nebulization than the inner ones.

This work has shown that the size of MLVs markedly

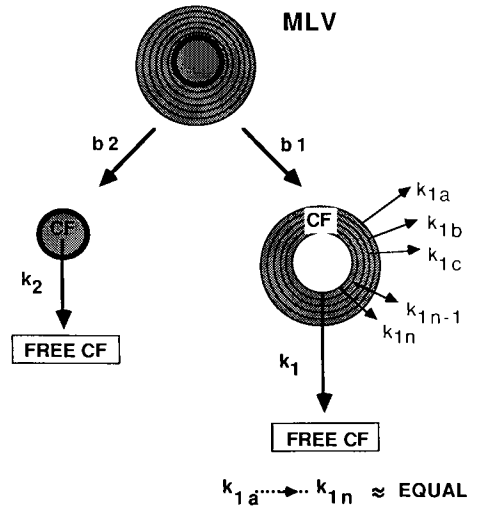

Fig. 6. An interpretation of the kinetic release mechanism of CF from the liposomes.

affects their ability to retain CF while undergoing nebulization, and it can be inferred from the mathematical modeling that the release of CF is biphasic in nature.

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NOMENCLATURE

CF	5,6-Carboxyfluorescein
C_{tot}	Total concentration of CF in a set volume of liposome dispersion as estimated by disruption of vesicles with Triton X-100 (μg/ml)
C_{free}	Free concentration of CF in solution (μg/ml)
C_{encaps}	Effective concentration of CF encapsulated within the liposomes (μg/ml)
$C_{tot}^{\#}$	Total concentration of CF in solution after accounting for evaporation during nebulization (μg/ml)
$C_{free}^{\#}$	Free concentration of CF in solution after accounting for evaporation during nebulization (μg/ml)
$C_{encaps}^{\#}$	Effective concentration of CF encapsulated within the liposomes after accounting for evaporation during nebulization (μg/ml)
V_o	Initial volume of the dispersion/solution to be nebulized (ml)
S_v	Theoretical rate of solvent loss from the nebulizer (ml/liter/min)
S_n	Theoretical rate of solution loss from the nebulizer (ml/liter/min)
F	Output flow rate of air from the nebulizer (liters/min)
b1, b2	Pharmacokinetic compartments containing CF within the liposomes (μg)
C_{b1}, C_{b2}	Effective concentrations of CF within the compartments of the liposomes (μg/ml)
k_1, k_2	Rate constants describing the rate of release of CF from compartments b1 and b2 within the liposomes (min^{-1})
t	Time (min)

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