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Physicochemical Evaluation of a Stability-Driven Approach to Drug Entrapment in Regular and in Surface-Modified Liposomes

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The traditional mode of encapsulating drugs in liposomes poses risks to drug stability, especially when recognition agents are attached to the liposomal surface to obtain targeted liposomes. To reduce such risks, we devised a simple, novel method to entrap drugs in liposomes, consisting of (i) preparation and lyophilization of drug-free regular and surface-modified liposomes and (ii) drug encapsulation in the course of liposome reconstitution through rehydration in an aqueous solution of the drug. In this paper, we report physicochemical studies in which we compared regular and surface-modified liposomes made by this novel approach (denoted N-liposomes) to respective liposomes made by the traditional mode (denoted T-liposomes). The studies were performed with fluorescein, sucrose, histidine, mitomycin C (MMC), and chloramphenicol (CAM) encapsulated (each) in regular and in bioadhesive liposomes, the latter having hyaluronic acid as the surface-bound ligand. Our major findings are as follows: (1) The drug-specific encapsulation efficiencies spanning the range of 10–90% were, excepting sucrose, either similar in the N- and T-liposomes or better in the N- than in the T-liposomes, for both regular and bioadhesive liposomes. (2) For all liposome types and methods of preparation, fluorescein, histidine, and MMC did not adsorb to the liposomal surface. Sucrose and MMC did adsorb to the liposomal surface irrespective of the liposome preparation mode, sucrose favoring bioadhesive over regular liposomes and MMC having the opposite trend. (3) For both regular and bioadhesive liposomes, the mechanism of drug efflux from the N-liposomes was found to be governed by a single rate constant, as previously found for the T-liposomes. The magnitudes obtained, ranging from 3.5(±0.2) × 10^{-3} to 400(±17) × 10^{-3} h^{-1}, were always drug specific and occasionally also liposome type (i.e., regular or bioadhesive) specific. For MMC and CAM, the novel approach rendered liposomes with improved sustained release. The results reported here attest, overall, to the potential of this novel approach, meriting further investigations. Studies currently underway with MMC indicate N-liposomes also have functional advantages.

Key Words: **liposomes; hyaluronic acid; modified liposomes; lyophilization; bioadhesion; drug encapsulation; drug release.**

Covalent attachment of recognition agents to the surface of liposomes is one of the major strategies in the efforts to endow liposomes with targeting ability. Among the agents investigated for the task are receptor-specific molecules such as EGF,[2] transferrin and folate (1–3), antibodies, lectins (4, 5), and bioadhesive molecules such as collagen and hyaluronic acid (6–8).

Drug integrity and drug activity, especially for drugs of biological origin, may be at risk in the course of the chemical procedures employed to position the targeting agent at the liposomal surface. In addition, the unavoidable steps of cleaning the modified system from excess reagents and from by-products may cause loss of encapsulated drug and attempts to reduce such losses by bolstering the wash buffers with free drug may be insufficient and rather wasteful.

This situation prompted us to pursue other ways to prepare drug-encapsulating regular and surface-modi-

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[2] Abbreviations used: BAL, bioadhesive liposomes; CAM, chloramphenicol; CH, cholesterol; DOC, deoxycholate; EGF, epidermal growth factor; HA, hyaluronic acid; MLV, multilamellar vesicles; MMC, mitomycin C; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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fied liposomes that would be free of the limitations discussed above. The concept we developed, which is rather simple and straightforward, stems from the practice of lyophilizing drug-encapsulating liposomes for long-term stability and shelf life (9–12). When needed, liposomes are reconstituted from these freeze-dried powders by rehydration with pure, electrolyte-free, water.

Our approach is a two-step process: first, preparation of drug-free regular and surface-modified "empty liposomes" (i.e., encapsulating buffer alone) followed by their lyophilization; second, reconstitution of liposomes from the dried powder through rehydration in an aqueous solution of the desired drug. This approach offers several distinct advantages over the traditional mode. It avoids processes that may compromise the drug, as all the modification steps are done in the absence of drug. This absence also means the risk of losing encapsulated drug is nonexistent, and there is no need to waste drug by its inclusion in wash buffers. The new approach not only retains the same level of long-term stability and shelf life achieved with lyophilized drug-encapsulating drugs but may actually surpass it, as liposomes and drug can be stored, separately, in powder form. A single liposome preparation (be it regular or surface modified) could be reconstituted with different drugs, to make several different liposome–drug formulations.

To initiate testing of this simple concept, we compared side by side—for the same drug—physicochemical properties of regular and of surface-modified liposomes prepared by this novel mode and by the traditional mode. In the following, we will refer to the novel and the traditional formulations as N-liposomes and T-liposomes, respectively. The tests were performed on five pairs of liposome formulations, differing in the encapsulated matter, each pair consisting of regular and surface-modified liposomes. Sucrose, histidine, fluorescein, mitomycin C (MMC), and chloramphenicol (CAM) were the five encapsulated entities. As a representative of surface-modified liposomes, we selected from our bioadhesive liposomes the species in which hyaluronic acid (HA) is the surface-anchored ligand (7, 8). HA is a large naturally occurring glycosaminoglycan containing repeating disaccharide units of *N*-acetylglucosamine and glucuronic acid. HA is found in living systems in both free (for example, in synovial fluid) and attached (for example, as an extracellular matrix component) forms (13, 14).

Bioadhesive liposomes (7, 8) are regular liposomes that have undergone surface modification through the covalent anchoring of specific adhesive ligands to the liposomal surface. To date, there are four species of bioadhesive liposomes, differing by the surface-bound ligands, selected from collagen, gelatin, EGF, and HA (7). By becoming an integral part of the liposome, these surface-anchored ligands endow the liposome with the ability to bind with high affinity to selective sites such as membrane-embedded receptors and components of the extracellular matrix (5–7). Having chosen, as detailed above, for the present study the bioadhesive liposome species in which HA is the surface-bound bioadhesive ligand, we will refer to them as HA-BAL.

The physicochemical properties measured were efficiency of drug encapsulation, drug adsorbance to the liposomal surface, and kinetics of drug efflux.

MATERIALS AND METHODS

Materials

High-purity (Phospholipon 100) soybean phosphatidylcholine (PC) was a kind gift from Nattermann Phospholipid GmbH (Germany). All other high-purity lipids, ethyl((dimethylamino)propyl)carbodiimide (EDC), fluorescein, L-histidine, and chloramphenicol, were purchased from Sigma Chemical Co. (St. Louis, MO). The following radiolabels were purchased from Amersham (Buckinghamshire, England) and found to be stable: [^{3}H]cholesterol, [^{14}C]sucrose, and [^{3}H]histidine. Mitomycin C (MMC) was a kind gift from Dexon Ltd. (Israel). Hyaluronic acid (from bovine trachea) was a kind gift from Hyal Pharmaceutical Corp. (Canada). All other reagents were of analytical grade. Analytical grade sucrose was purchased from BDH (England). Spectra/Por dialysis tubing (MWCO 12,000–14,000) was purchased from Spectrum (Gardena, CA).

Centrifugation was performed using a Beckman Optima TLX tabletop ultracentrifuge. Absorption spectra were measured with a Cary UV–visible spectrophotometer and a Thermomax microplate reader. Liquid scintillation counting was performed with a Kontron Analytical Betamatic. Lyophilization was performed with an Alpha 1-4 freeze-drier.

Methods

Liposome preparation and drug encapsulation. Multilamellar vesicles (MLV) were prepared essentially as previously described (1, 6, 7, 15). For histidine, sucrose, and MMC, we used the lipid composition of PC:PE:CH at mole ratios of 3:1:1 and a lipid concentration of 100 mg/ml. The same lipid composition was used for fluorescein, but at a lipid concentration of 50 mg/ml. For CAM, the liposomes were composed of PC:PE at a mole ratio of 19:1 and a lipid concentration of 50 mg/ml. For drug-free liposomes, the swelling solution was phosphate-buffered saline (PBS) at a pH of 7.2. The same buffer was used for the drug-encapsulating liposomes and the drugs were introduced through the swelling solution.

Liposome modification. The modification was performed according to our previously reported process (6–8). Briefly, HA was dissolved in water and preactivated by incubation with EDC at pH 4 (controlled by titration with HCl) for 2 h at 37°C. At the end of this step, the activated HA was added to a suspension of PE-containing liposomes in 0.1 M borate buffer to a final pH of 8.6. Incubation with the liposomes was for 24 h at 37°C and, upon termination, the liposomes were separated from excess reagents and by-products by centrifugation (as described above) and repeated washings. When modifying drug-encapsulating liposomes, all steps were carried out in the presence of drug in the external medium, in order to minimize drug loss during the process. The final ratio of ligand to lipid was 57 μg of HA/μmol of lipid.

Drug diffusion. The kinetics of drug diffusion were studied as previously described (1, 6, 15). Briefly, a suspension of liposomes (0.5–1.0 ml) was placed in a dialysis sac and the sac was immersed in a continuously stirred receiver vessel containing drug-free buffer

(phosphate-buffered saline at pH 7.2). Receiver-to-liposome sample volume ratios were 10–16. At designated periods, the dialysis sac was transferred from one receiver vessel to another containing fresh (i.e., drug-free) buffer. Drug concentration was assayed in each dialysate and in the sac (at the beginning and end of each experiment).

In order to obtain a quantitative evaluation of drug release, experimental data were analyzed according to a previously derived multipool kinetic model (1, 15). For this model, the relationship between time (the free variable) and the dependent variable $f(t)$—the cumulative drug released into the dialysate at time t normalized to the total drug in the system at $t = 0$—is expressed in Eq. [1]

$$f(t) = \sum_{j=1}^{n} f_j(1 - e^{-k_j t}), \qquad [1]$$

where f_j is the fraction of the total drug in the system occupying the jth pool at $t = 0$ and k_j is the rate constant for drug diffusion from the jth pool.

Encapsulation efficiency. Defined as the ratio of entrapped drug to the total drug in the system, encapsulation efficiency can be determined by two independent methods: (1) By centrifugation. Samples of complete liposome preparation (i.e., containing both encapsulated and unencapsulated drug) were centrifuged as described above. The supernatant, containing the unencapsulated drug, was removed and the pellet, containing the liposomes with encapsulated drug, was resuspended in drug-free buffer. Drug was assayed in the supernatant and in the pellet, as well as in the complete preparation, from which the encapsulation efficiency and conservation of matter can be calculated. (2) From data analysis of efflux kinetics. As discussed above, data analysis yields the parameter f_j. When the efflux experiment is performed on samples from the complete liposome preparation, the magnitude of f_j for the pool of encapsulated drug is also the efficiency of encapsulation.

Lyophilization. Lyophilization of liposome suspensions was performed on 1.0-ml aliquots. Samples were frozen for 2–4 h at −80°C and lyophilized for 48 h. Reconstitution was to original volume using distilled water (with/without drug).

Quantitative determinations. Cholesterol, histidine, and sucrose were assayed using a trace of radioisotope: [^{3}H]cholesterol, [^{3}H]histidine, and [^{14}C]sucrose. Fluorescein was assayed by its absorbency at 493 nm, which was linear in the concentration range 0–15 μM, with molar extinction coefficients of 69,700 and 61,500 M^{-1} in buffer and in 5% DOC, respectively. MMC was assayed by its absorbency at 391 nm, which was linear in the range 0–100 μg/ml, with extinction coefficients of 0.0127 and 0.01526 μg/ml in buffer and in 5% DOC, respectively. CAM was assayed by its absorbency at 280 nm, found to be linear in the concentration range 0–100 μg/ml, with extinction coefficients of 0.0315 and 0.0354 μg/ml in buffer and in 5% DOC, respectively.

Statistics. Statistical significance was evaluated by Student's t test with $P < 0.01$.

RESULTS AND DISCUSSION

I. Efficiency of Encapsulation

Typical magnitudes obtained for the efficiency of encapsulation for each of the tested drugs in both regular and surface-modified liposomes by the two liposome production methods are shown in Fig. 1. The plotted data are those obtained by the efflux kinetics method. Data analysis of such kinetic results provides a more accurate measure of encapsulation efficiency as it is free of overestimation that can occur with the centrifugation results, especially in the case of drugs that have appreciable adsorption to the liposomal surface (see section II).

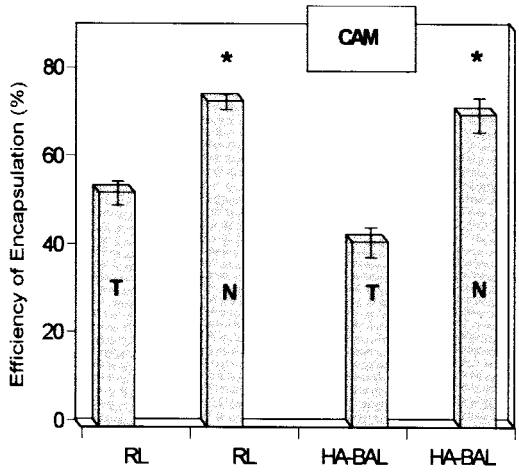
Screening, first, the data shown in Fig. 1 for the bioadhesive liposomes, it becomes clear that with the exception of sucrose, replacing the traditional by the novel method of preparation generated no significant loss, and on occasion a gain, in encapsulation efficiencies. Similar trends, for the most, are also seen for the regular liposomes: encapsulation of CAM, fluorescein, and histidine in liposomes made by the novel mode is similar to, or better than, that in liposomes made by the traditional mode. The novel approach continues to disfavor sucrose, significantly. For MMC, encapsulation within regular liposomes made by the traditional mode is slightly better than in the case of the novel mode.

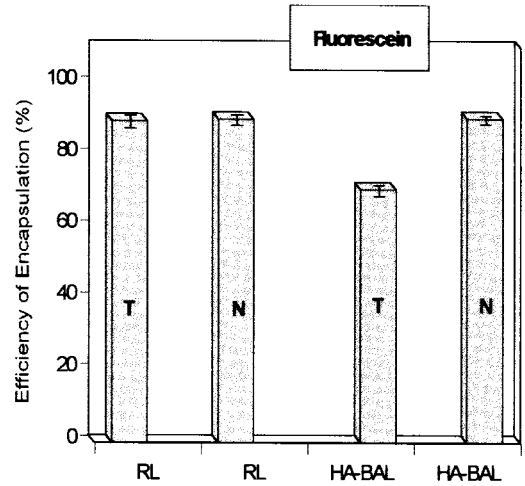
II. Drug Adsorption to the Liposomal Surface

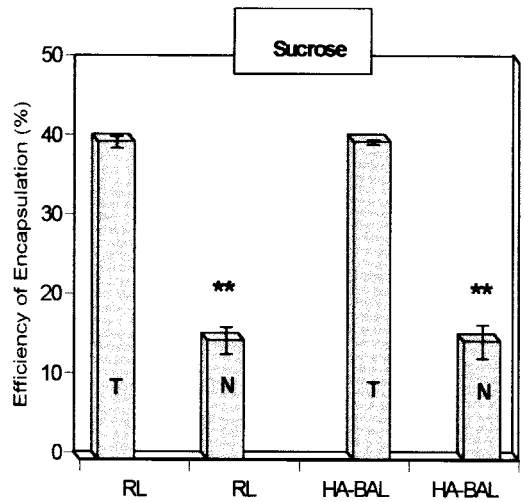
As detailed under Methods, efficiency of drug encapsulation for each liposome–drug system was determined by two independent methods—centrifugation and efflux. For a given system, the ratio of encapsulation efficiencies determined by both methods (denoted here the centrifugation/efflux ratio) should have a value of one, provided the drug does not adsorb to the liposomal surface. In contrast, for drugs with significant adsorption to the liposomal surface, encapsulation efficiency determined by centrifugation is expected to be higher than by efflux, yielding a centrifugation/efflux ratio larger than one.

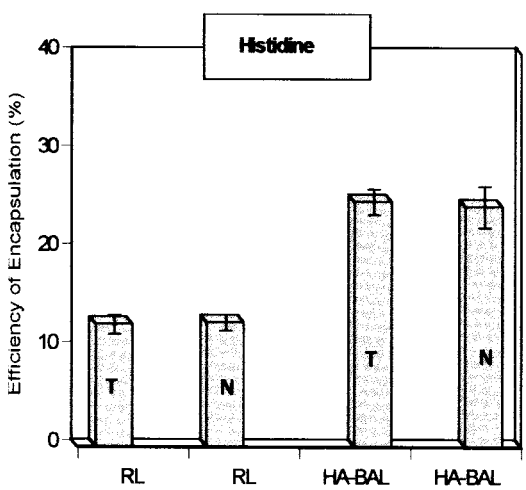
For histidine, sucrose, and fluorescein, encapsulated (each) in either regular or bioadhesive liposomes, produced by the traditional or the novel mode, we consistently found a centrifugation/efflux ratio of one (data not shown). The case of MMC was fairly similar (see Fig. 2, left-hand side), with a slight, although not statistically significant, elevation in regular liposomes made by the traditional mode. The exception was CAM (see Fig. 2, right-hand side), for which the ratio is higher than one for all systems, whether produced by the traditional or by the novel method, with the latter slightly higher.

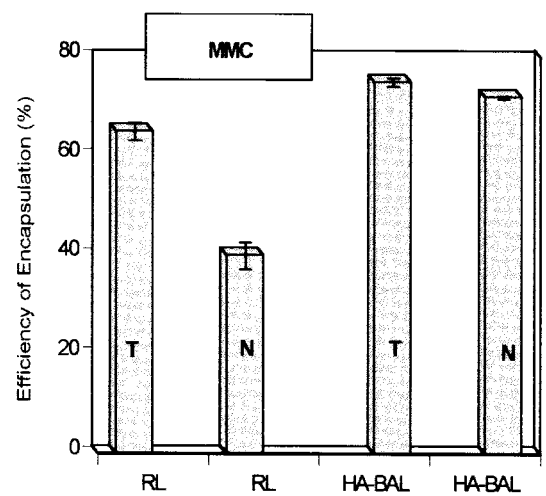
Overall, on the issue of drug adsorption, the novel method seems to be quite comparable to the traditional one. Aside from the current objectives, we would like to point out that a comparison of the type done here—between two independent methods of determination—has other uses. For example, significant drug adsorption to the liposomal surface may impact performance as a drug delivery system, and the present type of comparison can provide insight into the existence and extent of such adsorption.


CAM
Efficiency of Encapsulation (%)
0
20
40
60
80
*
*
T
N
T
N
RL
RL
HA-BAL
HA-BAL


Fluorescein
Efficiency of Encapsulation (%)
0
20
40
60
80
100
T
N
T
N
RL
RL
HA-BAL
HA-BAL


Sucrose
Efficiency of Encapsulation (%)
0
10
20
30
40
50
**
**
T
N
T
N
RL
RL
HA-BAL
HA-BAL


Histidine
Efficiency of Encapsulation (%)
0
10
20
30
40
T
N
T
N
RL
RL
HA-BAL
HA-BAL


MMC
Efficiency of Encapsulation (%)
0
20
40
60
80
T
N
T
N
RL
RL
HA-BAL
HA-BAL

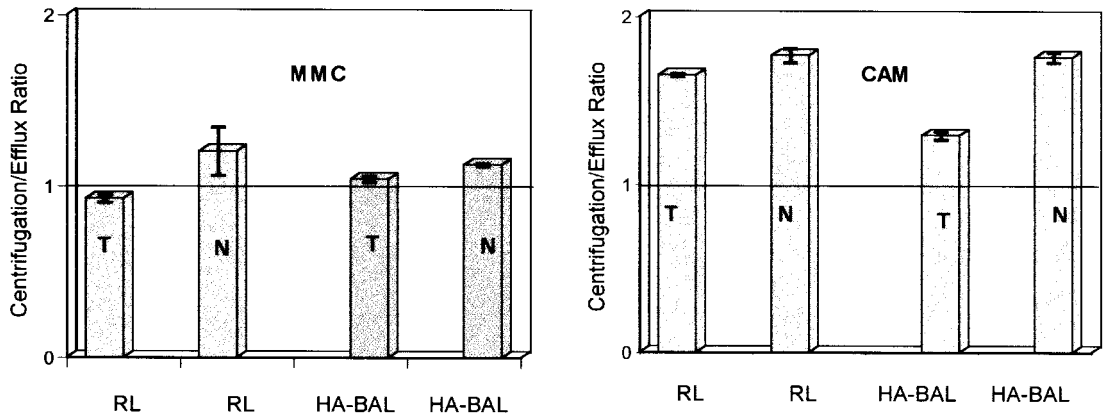


FIG. 2. Drug adsorption to the liposomal surface calculated from the ratio between efficiencies of encapsulation determined for the same system by two independent methods (centrifugation and efflux kinetics), hence denoted the centrifugation/efflux ratio (see text for further details). RL and HA-BAL represent regular and bioadhesive liposomes, and T and N indicate liposomes made by the traditional and novel methods. Left-hand side: liposomes encapsulating mitomycin C. Right-hand side: liposomes encapsulating chloramphenicol. The error bars represent the standard deviation among the batches.

III. Kinetics of Drug Efflux

For all systems studied, we found the data to fit a two-term kinetic equation (see Eq. [1), corresponding to

$$f(t) = f_1(1 - e^{-k_1 t}) + f_2(1 - e^{-k_2 t}),$$

where the indices 1 and 2 are for the unencapsulated and encapsulated drug, respectively. Typical results of k_2, the rate constant for the efflux of the encapsulated drug, are listed in Table I.

The data in Table I make it clear that, as expected, the properties of the encapsulated drug are the major factor determining the diffusion constant. Some drug specificity also carries into the question of whether drug diffusion is affected by the liposome production method. For sucrose, k_2 is similar for both regular and bioadhesive liposomes within the traditional liposomes and, separately, within the novel liposomes, yet in the latter, efflux is 8- to 9-fold faster. For histidine in regular liposomes and for fluorescein in regular and in bioadhesive liposomes, the novel method makes either no change or a very minor one. For CAM and MMC in both regular and bioadhesive systems as well as for histidine in bioadhesive liposomes, efflux from the novel liposomes is slowed down significantly compared to the corresponding traditional liposomes.

In the context of liposome performance as sustained-release drug depots, in 17 of the 20 different systems for which data are shown in Table I, we propose that the effects of the novel method on drug efflux are beneficial, as k_2 is either unchanged or decreased. Although we do not know the molecular origins of the decrease in k_2, the phenomenon is of particular value for CAM and MMC, the two actual drugs among the five test molecules. The slowdown observed in the novel liposomes corresponds to significant increases in the half-life of drug release. For CAM, the half-life of drug release increases from 13 and 12 h for regular and for bioadhesive liposomes made by the traditional mode to 20 and 28 h for the corresponding systems made by the novel mode. For MMC, it is more dramatic, from 1.7 and 8.5 h (regular and bioadhesive liposomes, respectively, traditional mode) to corresponding values of 35 and 50 h in the novel mode.

IV. Conclusions

On the basis of the results of the three physicochemical tests performed on two different liposome types and five different drugs, we conclude that the novel

FIG. 1. Comparison of encapsulation efficiencies for each of the five tested drugs, in regular and in surface-modified liposomes, each prepared by the traditional and novel modes. All liposomes were MLV. Liposomes encapsulating histidine, sucrose, and MMC were composed of PC:CH:PE at a mole ratio of 3:1:1 at a lipid concentration of 100 mg/ml. Liposomes encapsulating fluorescein had a similar liposome composition but were at a lipid concentration of 50 mg/ml, and liposomes encapsulating CAM were composed of PC:PE at a mole ratio of 19:1 and a lipid concentration of 50 mg/ml. RL and HA-BAL represent regular and bioadhesive liposomes, respectively. T and N represent traditional and novel modes of liposome preparation, respectively. Each bar represents an average of several independent liposome batches (three for fluorescein, histidine, and MMC, and two for sucrose and CAM), and the error bars represent the standard deviation among the batches. Intrabatch scatter was smaller than interbatch scatter. $P < 0.01$ and $P < 0.001$ are indicated by * and **, respectively.

TABLE I

Rate Constants for Drug Efflux from Regular (RL) and Bioadhesive (HA-BAL) Liposomes

Drug of interest	Liposome type	k_2 (h) × 1000 Traditional method	k_2 (h) × 1000 Novel method
Fluorescein	RL	17 ± 0.8	19 ± 0.5
	HA-BAL	18 ± 0.8	22 ± 0.6*
Sucrose	RL	4.0 ± 1.0	30 ± 1.7**
	HA-BAL	3.5 ± 0.2	31 ± 0.8**
Histidine	RL	18 ± 1.2	17 ± 1.3
	HA-BAL	7 ± 0.3	4 ± 0.1**
MMC	RL	403 ± 17	20 ± 0.7**
	HA-BAL	82 ± 5	14 ± 0.6**
CAM	RL	52 ± 6	34 ± 5**
	HA-BAL	54 ± 6	25 ± 7**

Note. Statistically significant differences between the traditional and novel methods; $P < 0.01$ (*), $P < 0.001$ (**).

method of liposome production is at least as good as the traditional method, and in some cases, better.

- By the encapsulation efficiency test, excepting sucrose, the same levels are retained and, in the case of the bioadhesive liposomes, occasionally increased.
- By the adsorption test, both methods are quite similar.
- By the kinetics of drug efflux test, excepting sucrose, the novel method is similar to or better than (for sustained-release depots) the traditional one.

Taking these experimental results together with the intrinsic advantages of the novel method detailed in the introduction, we find this novel approach to surpass the traditional mode. This is especially noticeable for the two actual drugs studied, MMC and CAM, in the surface-modified liposomes. Further studies testing biological activities of drugs in liposomes prepared by this novel method are under way and preliminary results with MMC (to be reported elsewhere) also attest to the benefits of the novel approach. Yet a word of caution should be interjected: in adopting the novel method for a drug of choice, an investigator should first compare both methods as there is a measure of drug specificity. Chances are, though, that the novel method would serve well.

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