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Hyaluronan is a key component in cryoprotection and formulation of targeted unilamellar liposomes

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Abstract

Lyophilized unilamellar liposomes (ULV), the dosage form of choice for shelf-life, revert upon reconstitution to the larger multilamellar liposomes (MLV), which is detrimental to the many carrier-mediated therapies that require small particles. High doses of sugars such as trehalose, sucrose and others, included in the original formulations for cryoprotection, were shown to prevent the conversion to MLV. In this study we set out to test whether hyaluronan (HA), the surface-bound ligand in our previously developed targeted bioadhesive liposomes (BAL), can also act as a cryoprotectant. The studies included structural and physicochemical characterization of original and reconstituted hyaluronan-ULV (HA-ULV). For each HA-ULV, similar regular ULV (RL-ULV) served as controls. Four properties were tested: particle size, zeta potential, encapsulation efficiency and half-life of drug release ($\tau_{1/2}$), for three drugs—chloramphenicol (CAM), vinblastine (VIN) and mitomycin C (MMC). Encapsulation efficiencies of the original systems were quite alike for similar RL-ULV and HA-ULV ranging from 25% to 70%. All systems acted as sustained-release drug depots, $\tau_{1/2}$ ranging from 1.3 to 5.3 days. Drug species and lipid composition were the major determinants of encapsulation and release magnitudes. By all tests, as anticipated, lyophilization generated significant changes in the reconstituted RL-ULV: 17-fold increase in diameter; tripling of zeta potential; 25–60% drop in encapsulation efficiencies; 25–30% decrease in $\tau_{1/2}$. In contrast, the reconstituted HA-ULV retained the same dimensions, zeta potentials, encapsulation efficiencies and $\tau_{1/2}$ of the original systems. These data clearly show HA to be a cryoprotectant, adding another clinically relevant advantage to HA-BAL. We propose that, like the sugars, HA cryoprotects by providing substitute structure-stabilizing H-bonds.

Keywords: Unilamellar liposome; Hyaluronan; Bioadhesive; Lyophilization; Cryoprotection

1. Introduction

Lyophilization (freeze-drying) is the method of choice for long-term storage of biological materials, including free and drug-encapsulating liposomes [1–3]. In the process, most of the water molecules are excluded from the specimen and the aqueous suspension becomes a powder (lyophilizate) that can be stored at selected, even ambient, temperatures. Prior to use, reconstitution of the particulate system is achieved by rehydration of the dry powder [4]. Lyophilization of multilamellar liposomes (MLV) and subsequent recovery of the very same liposome type—MLV—is simple and straight forward [2,3]. With unilamellar liposomes (ULV) the situation is more complex. Unless specific precautions are taken, the small original ULV revert, upon lyophilization and reconstitution, to the much larger MLV [2,5,6].

It is well recognized that small particle size (on the nanoscale) is critical for carrier-mediated treatment of pathologies that require systemic administration and long-term circulation [7–12]. Relapse of the small-sized ULV into the much larger MLV is obviously detrimental to such treatments. A solution to the problem that allows recovery of ULV from lyophilized ULV powders was found to be the inclusion of relatively high concentrations (~ 30%) of cryoprotectants, such as trehalose, sucrose, mannose or glucose, in the original ULV preparation [5,6,13]. The role attributed to these cryoprotectants is replacement of structure-stabilizing water-based hydrogen bonds at the liposo-

Abbreviations: BAL, bioadhesive liposomes; CAM, chloramphenicol; CH, cholesterol; DOC, deoxycholate; EDC, ethyl-dimethyl-aminopropyl-carbodiimide; HA, hyaluronan, hyaluronic acid; MLV, multilamellar vesicles; MMC, mitomycin C; PBS, phosphate-buffered saline; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PEG, polyethylene glycol; RL, regular liposomes; ULV, unilamellar vesicles; VIN, vinblastine

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mal surface, which are lost in the process of drying [5,6,13]. The downside of this approach is that these sugars are also present in the formulation administered to the patient.

In this report, we offer another approach to cryoprotection, which fits bioadhesive liposomes (BAL) and does not require the inclusion of additives, such as the sugars listed above, in the formulation.

BAL are regular liposomes (RL), modified by the covalent anchoring of ligands such as collagen, gelatin, EGF or hyaluronan (HA) to their surface [14–23]. We have developed and investigated these liposomes originally for topical and regional, and lately also for systemic, applications [14–21]. We have found that the surface-bound HA provides liposomes with the advantages of long circulation and of high affinity to recognition sites that are overexpressed in tumors [22,23]. Focusing here on HA-BAL, the abundance of hydroxyl residues on the liposomal surface has lead us to hypothesize that this HA coat may also act as a cryoprotectant for lyophilized ULV. This would eliminate the need to include sugar residues in the formulation and be especially beneficial to some patients for which the sugar residues (particularly at the doses used) may trigger unacceptable adverse effects.

To pursue this hypothesis, we prepared several systems of RL and of HA bioadhesive ULV in which we encapsulated vinblastine (VIN), mitomycin C (MMC) or chloramphenicol (CAM). Each original preparation, regular and bioadhesive, was characterized by structural and by physicochemical properties, focusing on EM, particle size, zeta potential, efficiency of drug encapsulation and kinetics of drug efflux. The systems were then lyophilized, reconstituted by rehydration, and the structural and physicochemical properties determined anew. We also tested the influence of two liposome parameters on the lyophilization/reconstitution properties: lipid composition, tested for both the regular and the bioadhesive systems, and (for the BAL) the density of the HA coat at the liposomal surface.

As will be shown, the results confirmed the working hypothesis. The present results reveal another advantage for the HA-BAL—the ability to act as a cryoprotectant for unilamellar liposomes.

2. Materials and methods

2.1. Materials

High-purity (Phospholipon 100) soybean phosphatidylcholine (PC) was a kind gift from Nattermann phospholipid GMBH (Germany). All other high-purity lipids, ethyldimethyl-aminopropyl-carbodiimide (EDC) and CAM were purchased from Sigma Chemical Company, St. Louis, MO. [^{3}H]Vinblastine (VIN) and [^{3}H]cholesterol were purchased from Amersham (Buckinghamshire, England) and found to be stable. MMC was a kind gift from Dexon Ltd., Israel. HA (hyaluronic acid) from bovine trachea was a kind gift from Hyal Pharmaceutical Corporation, Canada. Dialysis tubing (molecular weight cutoff of 12 000–14 000) was from Spectrum Medical Industries (Los Angeles, CA, USA). Polycarbonate membranes were from Nucleopore (Pleasanton, CA, USA). All other reagents were of analytical grade.

Liposome extrusion was performed with the Lipex extrusion device (Vancouver, Canada). Centrifugation was performed using a Beckman Optima TLX, tabletop ultracentrifuge. Lyophilization was performed with an Alpha 1-4 freeze drier (CHRIST, Germany). Absorbance spectra were measured using a Cary UV–Visible spectrophotometer and a Thermomax microplate reader. Liquid scintillation counting was performed with a Kontron Analytical Betamatic. ALV-NIBS (Berlin, Germany) was used for particle sizing and the Malvern Zetasizer IV (MA, USA) was used to determine the zeta potential. Scanning electron microscopy (SEM) was done with a JSM-840A, Joel Microscopy (Japan) after coating the lyophilized powder with gold using the SEM coating unit E5100 (England).

3. Methods

3.1. Liposome preparation and drug encapsulation

Regular MLV, the ‘‘raw material’’ for RL-ULV, were prepared essentially as previously described [14,18,20]. Four liposome formulations, differing in their lipid composition, were prepared for each encapsulated drug as follows: Formulation A—phosphatidyl ethanolamine (PE)/phosphatidyl choline (PC) (5:95), Formulation B—PE/PC (10:90), formulation C—PE/PC (20:80) and formulation D PE/CH/PC (20:20:60). The numbers in parenthesis are the mole ratios. All formulations were prepared at the same liposome concentration of 50 mg lipid/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.

ULV were obtained by extrusion of the MLV through the Lipex device, operating the extrusion device at room temperature and under nitrogen pressures of 200 to 500 psi. The extrusion was carried out in stages using progressively smaller pore-size membranes, with several cycles per pore-size [21].

3.2. Liposome modification

The modification was performed on the ULV, according to our previously reported process [14,15,20]. Briefly, HA was dissolved in water and pre-activated 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, and this reaction mixture was incubated for 24 h at 37 °C. At the end of incubation, the liposomes were separated from excess reagents and by-products by centrifugation ($1.3 \times 10^5 g$, 4 °C and 40 min) 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. HA concentrations in the reaction mixture were 0.5, 2.0 or 5.0 mg/ml, and the resultant HA-BAL were denoted $BAL_{0.5}$, $BAL_{2.0}$ and$BAL_{5.0}$.

3.3. Drug diffusion

The kinetics of drug diffusion was studied as previously described [18,20,21]. 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 (PBS at pH 7.2). The buffer volume in the receiver vessel was 10- to 16-fold higher than that of the liposome sample in the dialysis sac. 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 multi-pool kinetic model [18,21]. Eq. (1) expresses the relationship between free and dependent variables for this model.

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

Where t denotes time, $f(t)$ is the cumulative drug released into the dialysate at time t, normalized to the total drug in the system at time = 0, f_j is the fraction of the total drug in the system occupying the jth pool at time = 0, and k_j is the rate constant for drug diffusion from the jth pool.

3.4. Encapsulation efficiency

Defined as the ratio of entrapped drug to the total drug in the system, encapsulation efficiency was determined by two independent methods: (1) 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 was calculated, and conservation of matter was verified. (2) Efflux kinetics. As discussed above, data analysis yields the parameter f_j. When the efflux experiment was performed on samples from the complete liposome preparation, the magnitude of f_j for the pool of encapsulated drug was also the efficiency of encapsulation.

3.5. Lyophilization and reconstitution

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.

3.6. Quantitative determinations

Cholesterol (CH) and vinblastine were assayed using traces of [^{3}H]cholesterol and [^{3}H]vinblastine, respectively. MMC was assayed by its absorbency at 365 nm, which was linear in the range of 0–100 μg/ml, with extinction coefficients of 0.0127 and 0.0153 ml/μg cm in buffer and in 5% deoxycholate (DOC), respectively. CAM was assayed by its absorbency at 280 nm, found to be linear in the concentration range of 0–100 μg/ml, with extinction coefficients of 0.0315 and 0.0354 ml/μg cm in buffer and in 5% DOC, respectively.

Statistics was done using Student's t test.

4. Results

4.1. Structural properties

4.1.1. Particle size

Particle sizing, pre- and post-lyophilization, was performed using the ALV-NIBS device as detailed under Section 2. Typical results, presented in Table 1, demonstrate the effects of the HA coat on retention of liposome size. The original liposomes, irrespective of whether they are regular or bioadhesive, were unilamellar (as expected) with diameters in the range of 100 nm. Upon lyophilization and reconstitution, the RL were found to undergo a substantial increase in diameter size—17-fold higher than the original particles. This high level of increase, yielding liposomes with an average diameter of 2400 nm, is indicative of the expected relapse of ULV to MLV, in the absence of cryoprotectants. In contrast, the three formulations of bioadhesive ULV that were

Table 1
The effects of lyophilization on particle size of RL and HA-BAL

Liposome specification	Liposome diameter (nm)	
	Pre-lyophilization	Post-lyophilization
RL[a]	138 ± 43[b]	2330 ± 735
$BAL_{0.5}$	150 ± 40	224 ± 100
$BAL_{2.0}$	172 ± 90	240 ± 150
$BAL_{5.0}$	195 ± 65	300 ± 180

[a] RL denotes regular liposomes; $BAL_{0.5}$, $BAL_{2.0}$ and $BAL_{5.0}$ denote the hyaluronan bioadhesive liposomes, prepared at different hyaluronan coating densities (see Section 2 for details).

[b] Each value is an average of seven determinations.

surface-coated with HA showed good retention of the ULV size range, post-lyophilization and reconstitution.

4.1.2. Zeta potentials

The zeta potentials measured pre- and post-lyophilization are listed in Table 2. The zeta potentials of the RL-ULV, pre- and post-lyophilization, are close to zero, but were tripled post-lyophilization and reconstitution. The zeta potentials of the original (i.e., pre-lyophilization) bioadhesive ULV are negative, increasing in magnitude with the increase in the density of the HA coat. Lyophilization and reconstitution generated small differences in the respective potentials. The surface modification is done on pre-formed liposomes (see methods), hence all of the HA anchored covalently to the lipid are originally on the outermost leaflet of the lipid bilayer membrane. Were the lyophilized powders composed of individual lipid molecules and lipid–HA conjugates, it is conceivable that in the course of reconstitution by rehydration, some of the lipid-attached HA would reside in the interior of the liposomes. This, in turn, would make the zeta potential of a reconstituted liposome less negative than the respective original system. The observation that this did not occur is taken as an indication that structural liposomal elements were preserved in the lyophilized BAL. It also implies that the HA remained at the surface, in a position to replace the lost water-originating hydrogen bonds.

4.2. SEM

The results obtained from SEM of the lyophilized liposome powders are illustrated in Fig. 1A for RL-ULV and in Fig. 1B for the bioadhesive ULV. The lyophilizate of the RL-ULV (1A) is seen to be composed of large particles, ranging from 1000 to 4000 nm. In contrast, the dominant particles in the lyophilizate of the bioadhesive ULV (1B) are much smaller, at the edge of detection under the magnification used. These findings fit well with the above presented results of liposome dimensions (Table 1) and zeta potentials (Table 2).

4.3. Efficiency of encapsulation

Typical magnitudes obtained for the efficiency of encapsulation for each of the tested drugs in all four liposome formulations (see methods for details), in both regular and coated vesicles, pre- and post-lyophilization, are shown in the three parts of Fig. 2.

Starting with the data for CAM, shown in the top part of Fig. 2, several findings stand out: increasing the PE concentration in the liposome formulation, from 5 to 10 to 20 mol% (formulations A, B and C) resulted in a substantial increase in the efficiency of encapsulation for both regular and coated ULV. For both types of liposomes, replacing 20 mol% of the PC by CH (formulation D) resulted in a further increase of encapsulation efficiency. For the coated liposomes in all tested formulations, the lyophilization and reconstitution did not generate any change in the encapsulation efficiency. In contrast, in all RL formulations lyophilization and reconstitution resulted in decreased encapsulation efficiencies. Similar trends were found for the MMC and for the VIN systems (shown in the middle and bottom parts of Fig. 2, respectively). For the latter, encapsulation efficiencies of the

Table 2
ζ potential of HA-coated liposomes pre- and post-lyophilization

Liposome specification	ζ potential (mV)	
	Pre-lyophilization	Post-lyophilization
RL	0.012 ± 0.003[a]	0.031 ± 0.002
$BAL_{0.5}$	− 6.21 ± 0.45	− 8.4 ± 1.9
$BAL_{2.0}$	− 13.0 ± 0.90	− 15.4 ± 4.5
$BAL_{5.0}$	− 15.4 ± 1.10	− 18.6 ± 3.7

[a] Each values is an average of seven determinations.

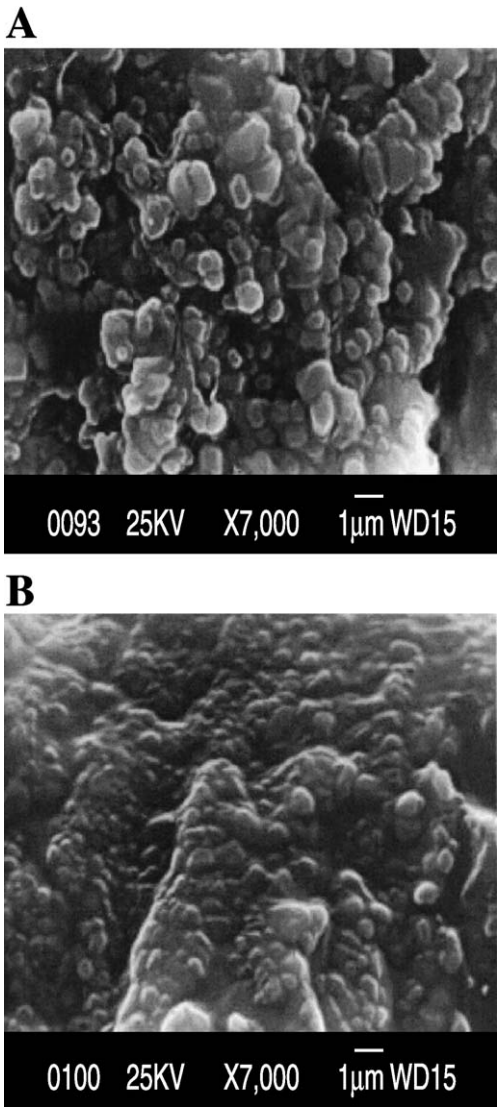

Fig. 1. SEM of lyophilized unilamellar liposomes. (A) RL. (B) HA-BAL. Both micrographs were taken under the same magnification as indicated in the figure.

coated liposomes actually increased from the original to the reconstituted systems.

4.4. Kinetics of drug efflux

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

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

where indices 1 and 2 are for the unencapsulated and encapsulated drugs, respectively.

Intrinsic drug properties were found, as expected, to be the dominant factor dictating the efflux rate constant of the encapsulated matter, and its sensitivity to the different liposomal systems (RL and BAL, each type pre- and post-lyophilization). Unlike the case of encapsulation efficiency, sensitivity to lipid composition—for each drug within a given liposomal system—was quite low. Increase in mol% PE either made no change or slightly increased the efflux, the addition of CH (for the same % mole PE) induced a slight decrease in the efflux, but none of these differences

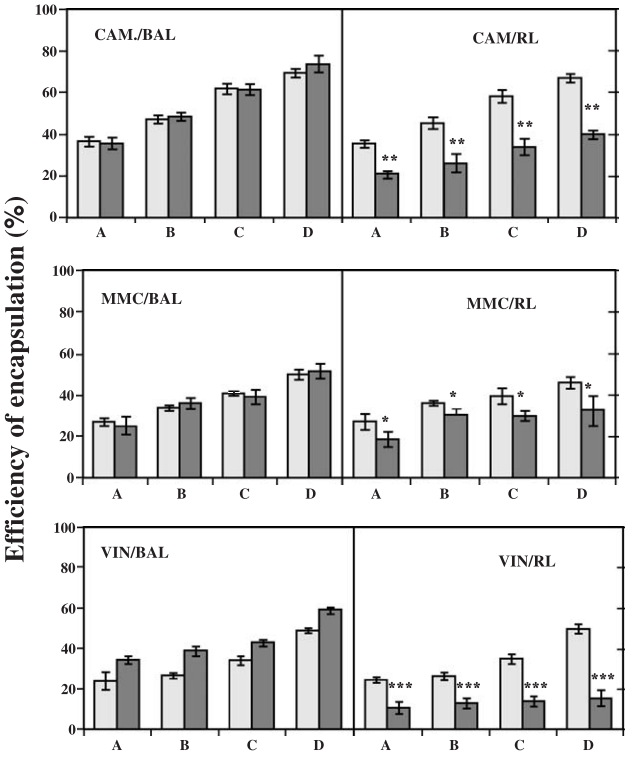

Fig. 2. The effects of lyophilization and reconstitution of regular and of hyaluronan bioadhesive unilamellar liposomes on the efficiency of drug encapsulation. Top part is the chloramphenicol (CAM) data, middle part is the mitomycin C (MMC) data and bottom part is the vinblastine (VIN) data. Encapsulation of each drug, pre- and post-lyophilization, is reported for four liposome formulations: (A) PE/PC (5:95), (B) PE/PC (10:90), (C) PE/PC (20:80) and (D) PE/CH/PC (20:20:60). Light-shaded and dark-shaded bars are for pre- and post-lyophilization, respectively. Each bar is an average of three determinations and the error bars represent the standard deviations. *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table 3
Efflux of encapsulated drugs from RL and from BAL, pre- and post-lyophilization

Drug	Efflux half-life (days)			
	RL		BAL	
	Pre	Post	Pre	Post
Chloramphenicol	5.26 ± 0.53	3.61 ± 0.05	4.00 ± 0.54	4.33 ± 0.45
Mitomycin C	1.73 ± 0.27	1.28 ± 0.10	1.99 ± 0.23	2.35 ± 0.47
Vinblastine	1.68 ± 0.22	1.32 ± 0.24	3.20 ± 0.27	4.72 ± 0.53

were statistically significant (data not shown). Therefore, for each drug in a given liposomal system, the rate constants for the efflux of the encapsulated drug (i.e., k_2) were transformed into the corresponding half-life values, averaged over all lipid compositions tested, and listed in Table 3.

All systems performed as sustained-release drug depots, half-lives ranging from 1.3 to 5.3 days. Among the three drugs tested, CAM had the slowest diffusion. The data (Table 3, first row) show that in the pre-lyophilized state, CAM diffusion was slower from the regular than from the BAL. Lyophilization, however, did not change the efflux from the BAL, while it made a significant change—increasing the efflux—for the regular ones. These data fit with the findings reported in previous sections of this communication that the lyophilized and reconstituted unilamellar BAL remain quite similar to the original ones, whereas the RL undergo drastic changes. For MMC (Table 3, second row) the efflux from RL and BAL is quite similar in the original state. Lyophilization and reconstitution generated slight and opposite changes in MMC efflux from the two types of liposomes—increase in the case of the regular and decrease in the case of the bioadhesive, systems. Similar trends, with the obvious drug-specific quantitative differences, are seen for the efflux of VIN (Table 3, third row).

5. Discussion

Lyophilization and reconstitution of a pharmaceutical product are usually viewed as technical procedures per se that do not change the nature of the original product and therefore do not require molecular investigative efforts [1]. This situation is changed when it comes to unilamellar liposomes [2,5,6,13], where pursuit of the effects of lyophilization becomes a necessity, in particular for therapies in which a small particle size is critical [7–12].

The well-recognized phenomena discussed in the introduction of this report, that lyophilized small (unilamellar) liposomes revert to the much larger MLV [2,5,6,13], was reaffirmed by findings of this study. In the absence of any cryoprotectant in the system, lyophilization and reconstitution had a substantial effect on the size of regular unilamellar liposomes. The quantitative evidence provided through particle size analysis shows a 17-fold increase in diameter size, from the original diameter range of 140 nm,

to that of 2300 nm for the reconstituted systems (Table 1). The EM results (Fig. 1A) add qualitative support to this radical increase in size.

The findings of this study clearly validate the working hypothesis—that HA, anchored to the surface of BAL, acts as a cryoprotectant of lyophilized ULV. Unlike the RL-ULV (discussed above), lyophilization and reconstitution generated a relatively small increase (<1-fold) in the original size of HA-ULV. This is seen (Table 1) for three different HA-ULV formulations, which vary in their bioadhesive coat. The original diameters, in the range of 150, 170 and 200 nm for the three formulations, increased to respective diameters in the range of 220, 240 and 300 nm. Furthermore, as clearly seen from the standard deviation in Table 1, the size distribution broadened from the original to the reconstituted systems. The original bioadhesive systems (as any preparation in which ligands are attached to the liposomal surface) probably contain some regular (or poorly bioadhesive) liposomes. These, upon lyophilization and reconstitution, would obviously revert to the larger MLV. If the share of the RL, within the bioadhesive preparation, is not enough for resolution into two distinct populations, their conversion to larger particles could account for the slight increase seen (for the whole system) in diameter size and for the broader distribution. Consequently, it might be that in terms of particle size, the protection offered by the HA coat is even better than what the data show. As in the case of the RL, the EM data concur with the quantitative particle size analysis: the HA-coated liposomes remained small (Fig. 1B), where the RL increased in size (Fig. 1A).

Another demonstration that the BAL retained their structural features throughout the drying and the reconstitution comes from the zeta potentials (Table 2). For each bioadhesive liposome formulation, the zeta potential remains quite the same for the original and for the lyophilized and reconstituted systems. Were the dry state an amorphous powder of lipid molecules, some of the HA originally localized at the liposomal surface should have ended inside the liposome. Were this to happen, the reconstituted liposomes would have had less negative zeta potentials. The data suggest that this did not happen, indicating structural elements were conserved in the dry state, the HA remaining through the drying and rehydration at the surface of the liposome.

Encapsulation efficiency and efflux kinetics are two key physicochemical parameters that influence drug delivery performance of particulate carriers. Both parameters were tested for three different drugs to evaluate how lyophilization and reconstitution affect them. Three distinct features of this probing, shown in Fig. 2, clearly stand out: In all cases, replacing PC by increasing levels of PE, from 5 to 20 mol% (systems A to C), and a further replacement of PC by 20 mol% CH (system D), resulted in an increase of encapsulation efficiencies. For a given type of liposome, increase in liposome concentration and decrease in the standard electrochemical potential of the drug in the liposome [$(\mu_D^o)_{liposome}$] were identified long ago as two independent parameters that generate increase in encapsulation efficiency [21]. Since the lipid concentration was held constant in all systems tested, the increase in encapsulation efficiency (within each liposome type, i.e., ULV or MLV) is attributed to the changes in lipid composition. The observation that the same trend was seen for different drugs is further support that this is a liposome-related phenomena, not restricted to a specific drug or liposome type.

As also seen in Fig. 2, for each of the drugs in all four lipid-different formulations, encapsulation efficiencies in the RL dropped from the original to the reconstituted systems. This is another indication that the lyophilization and reconstitution change regular, unprotected, ULV. This drop is also consistent with the conversion from ULV to MLV, since for the same lipid concentration and composition, the liposome (i.e. particle) concentration decreases from ULV to MLV. Lowering liposome concentration, as already indicated above, tends to reduce encapsulation efficiency [21].

For CAM and MMC, encapsulation efficiency in the BAL remained unchanged from the original to the reconstituted systems; another indication that the HA offers effective cryoprotection. For vinblastine in the BAL, encapsulation efficiency is seen to increase from the original to the reconstituted systems. Taking into account that, unlike the other two drugs, vinblastine is quite lipophilic, it may be that in the course of lyophilization and reconstitution, vinblastine relocalized within the liposome, gaining a state of lower $(\mu_D^o)_{liposome}$. We have yet to pursue the molecular origins of this phenomena, yet regardless of the origins, for the task of drug delivery it is a change in a positive direction.

The effects of lyophilization and reconstitution on the half-life of drug release—a direct measure of carrier performance as a sustained-release drug depot—are listed in Table 3. Since drug specifications are the major factor dictating the kinetics, this tends to reduce the impact of lyophilization and reconstitution. Yet, some common trends are observed. For all three drugs, efflux from the RL increased after lyophilization. In contrast, lyophilization and reconstitution of the BAL either made no change (CAM) or slowed down the efflux (MMC and VIN). This indicates that for BAL, the process of lyophilization and reconstitution either retains or improves the performance as sustained-release drug depots.

Lyophilization of liposome suspensions obviously removes water molecules, including those at the surface of the liposomes that are involved in structure-stabilizing hydrogen bonding [5,6,13]. It is the loss of these hydrogen bonds that apparently destabilizes the unilamellar structure to the point that MLV are recovered upon rehydration of the dry lipid powder [5,6,13]. The cryoprotection mechanism attributed to the sugar molecules stems from their ability to engage in hydrogen bonding that substitutes for those lost water molecules [5,6,13].

The mechanism we suggest for liposomal cryoprotection by HA also involves retention of structure-stabilizing hydrogen bonding at the liposomal surface. HA could perform this function in several ways: HA is known to engage extensively in hydrogen bonding—to itself (intra- and intermolecular) and to other molecules, including water [24–28]. In addition, HA is known for its extensive ability to adsorb water molecules, as well as for its reluctance to relinquish all of them upon drying [28]. On the basis of these properties we offer the following scenario: The original BAL could already benefit from structure stabilization that stems from the hydrogen bonding abilities of HA and from its water-holding capacity. These liposomes may be less vulnerable, from the beginning, to structural destabilization brought upon by removal of water molecules. As the drying process progresses, the HA can substitute—just as the sugar residues do—hydrogen bonding instead of the lost water molecules, further preventing structural destabilization of the dried systems.

In conclusion, on the basis of the data reported in this study (coming from several independent lines of investigation), HA anchored at the surface of unilamellar BAL clearly acts as a built-in cryoprotectant. This HA ability is not restricted to a specific drug, specific liposome formulation or ULV size range—similar cryoprotection was effective for HA-ULV of 60–70 nm diameter prepared for systemic administration [22,23]. There is an obvious need for further probes into the mechanism(s) involved, yet the benefits for drug delivery are already evident. The dried BAL can be taken "off the shelf", reconstituted and administered to the patient without the risks of change in particle size (that would undermine the therapy) and without the need to include high levels of foreign matter in the formulation that could cause adverse effects.

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