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Report

Adenosine Triphosphate Liposomes: Encapsulation and Distribution Studies

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Four methods for encapsulating adenosine triphosphate (ATP) in liposomes were evaluated. Optimum entrapment required emulsifying ATP with the lipids used to form the liposome membrane in a high-speed homogenizer followed by evaporating the organic solvent with vigorous stirring. Under these optimum conditions ATP entrapment was 38.9%; i.e., the dosage form contained 38.9 g of ATP per 100 g of lipid. The distribution of positively charged liposomes loaded with ATP was studied in dogs with experimentally induced myocardial infarction. Intravenous injection of positively charged ATP liposomes caused accumulation of ATP in myocardial infarct tissue. Myocardial infarct tissue has reduced blood flow; since positively charged liposomes accumulated in infarct tissue, liposomes may be a drug delivery system for this disease state.

KEY WORDS: drug targeting; liposomes; adenosine triphosphate; myocardial infarction.

INTRODUCTION

Drug entrapment in liposomes, expressed as percentage (w/w), is usually low, and this low drug entrapment limits the use of liposomes as a drug delivery system. Numerous methods to entrap water-soluble drugs in liposomes are available (1), but entrapment values are typically less than 10% (w/w). From a manufacturing viewpoint, high drug entrapment is necessary to make the dosage form pharmaceutically feasible. In the present studies adenosine triphosphate (ATP)[4] was used as a model compound and four methods of liposome formation were evaluated in order to optimize ATP entrapment.

Different results were reported in the literature about the accumulation of ATP in myocardial infarct tissue after iv injection of positively charged ATP liposomes. Therefore tritiated labeled ATP was used to evaluate the distribution of liposome encapsulated ATP in dogs with experimentally induced myocardial infarction. We demonstrate accumulation of positively charged liposomes in myocardial infarct tissues, giving direct evidence that liposomes can function as a drug delivery system for this disease state.

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[4] Abbreviations used: ATP, adenosine triphosphate; IEV, improved emulsification vesicles; REV, reverse-phase evaporation vesicles; DEV, double-emulsification vesicles; TFV, thin-film vesicles; LAD, left anterior descending coronary artery; ^{51}Cr-LFTRBC, ^{51}Cr-labeled formalized toad red blood cells; RMBF, relative myocardial blood flow.

MATERIALS AND METHODS

Liposome Preparation Characterization and Sterilization

Thin film-formed vesicles (TFV) were prepared according to the method of Bangham *et al.* (2). Reverse-phase evaporation vesicles (REV) were formed according to the method of Szoka *et al.* (3). Double emulsification vesicles (DEV) were prepared according to the method of Matsumoto *et al.* (4). Improved emulsification vesicles (IEV) were prepared by homogenizing aqueous ATP solutions with an organic lipid solutions in a high-speed homogenizer followed by evaporating the organic solvent (5). All liposomes were prepared having the same composition. The lipid phase was composed of egg lecithin/cholesterol/stearylamine (8:1:1), which was dissolved in chloroform–isopropyl ether solution (1:2). ATP was dissolved in distilled water to make an 8% solution and adjusted with dilute sodium hydroxide solution to pH 7.5. The buffer was pH 7 phosphate-buffered saline (PBS) (50 m*M*).

For entrapment ratio calculations, liposomes were separated from unentrapped ATP by ultrafiltration (6). For stability studies, liposomes were separated from unentrapped ATP by Sephadex G-50 column chromatography (7). ATP concentrations were measured by the method according to (8). Addition of 4% Triton X-100 was necessary to measure total ATP associated with the final liposome population (9). Entrapment ratio is usually calculated as entrapped volume per volume of lipid (10); however, from a manufacturing point of view, we calculate encapsulation ratio as the weight percentage of entrapped drug in the final liposome population. Entrapment ratio was calculated by

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$$\text{entrapment ratio (\%)} = \frac{W_{ATP}}{W_{lipid}} \times 100$$

where W_{ATP} and W_{lipid} are the weights of entrapped ATP and lipid in the final liposome population, respectively.

A Coulter counter (Model TAII) was used to estimate the size distribution of liposomes prepared by the above techniques. Liposomes were diluted in 2% sodium chloride for this measurement. Electron micrographs of ATP liposomes were obtained using a H-600 electron microscope after staining the liposomes with a 3% phosphotungstic acid solution (11). Sterilization of liposomes was conducted by irradiation under nitrogen using a ^{60}Co source with a radiation dose of 1×10^6 rad.

Distribution Studies

Liposome distribution studies used positively charged IEV liposomes composed of egg lecithin/cholesterol/stearylamine (5:5:1). ^3H-ATP was used as an aqueous space marker to follow vesicle distribution. A stock solution of ^3H-ATP (2 mCi/l ml) was obtained from the atomic institution of Shanghai at University Academy Science of China. Coulter counter measurements and electron micrographs indicated that vesicle diameter ranged between 2 μm and 25 nm. ^3H-ATP liposomes contained approximately 50 μCi of ^3H-ATP per ml of liposome.

Experimental animals for tissue distribution and blood flow measurement were divided into two groups (seven dogs/group): the ^3H-ATP group and the ^3H-ATP liposomes group. The dogs were mongrels, eight males and six females; they weighed 8–14 kg, were disease free, and were not used previously for any experimentation. Each dog was anesthesized with 30 mg/kg pentothal sodium intravenously and artificially ventilated with oxygen, and an electrocardiogram recorded during each experiment. A cannulated femoral artery was used for obtaining blood samples and monitoring arterial pressure. A femoral venous catheter was inserted for injection of lipsomes and ^3H-ATP and infusions. A left thoracotomy exposed the heart for catheter insertion into the left atrium. For regional blood flow measurements, ^{51}Cr-labeled formalized toad red blood cells (^{51}Cr-LFTRBC) were infused through this atrial catheter after 2 mg/kg of heparin was given (12). For distribution studies in animals with myocardial infarction, the left anterior descending coronary artery (LAD) was ligated just distal to its first diagonal branch after surgery was completed. After 30 min of ligation, 100 μCi ^3H-ATP or ^3H-ATP liposomes containing ATP 20 mg and lipid 52 mg was injected iv. The injection rate was 2 ml per min for 30 min.

Regional myocardial blood flow was measured with ^{51}Cr-LFTRBC after tissue distribution studies, using radioactive microspheres according to a technique previously described (12,13). Approximately 10^7 ^{51}Cr-LFTRBC suspended in 2 ml of saline (sp act, ~80 μCi) were injected into the left atrial cannula during a period of 20 sec. Starting 5 sec before the injection of ^{51}Cr-LFTRBC and continuing for 120 sec, a reference blood sample was withdrawn through the arterial catheter at a rate of 10 ml/min. Five minutes after ^{51}Cr-LFTRBC injection, the animals were sacrificed, and hearts were immediately removed. After removing the endocardium, vessels, and fat, the heart was cut into pieces of 0.2–0.4 g, weighed to the nearest milligram, and placed in plastic tubes. The reference blood and the heart samples were counted for 1 min in a GP-I-type gamma-scintillation counter.

Table I. ATP Entrapment in Liposomes

Liposome formation method	% entrapped (w/w)[a]
TFV[b]	9.2 ± 1.26
REV	3.47 ± 0.18
DEV	6.90 ± 1.09
IEV	22.56 ± 1.16

[a] Values are the means of three determination.
[b] Liposomes composed of egg lecithin/cholesterol/stearylamine (8:1:1).

Myocardial blood flow was determined using the formula: $Q_m = Q_r \times C_t/C_r$, in which Q_m = myocardial blood flow (ml/min/g); Q_r = reference blood flow (ml/min); C_t = counts per gram in myocardium (cpm/g); and C_r = total counts in the reference blood (cpm).

Measurement of ^3H-ATP in Myocardium Tissue. After gamma-scintillation, myocardium tissue was cut in to pieces of about 100 mg and placed in liquid scintillation bottles. Myocardium samples were digested with acid: 0.1 ml of perchloric acid and 0.2 ml of 3% hydrogen peroxide were added, and samples were incubated at 80°C for 2 hr. After samples equilibrated to room temperature, 15 ml of scintillant was added. Scintillant contained 6 g of 2-(4-biphenyl)-5-(*p*-t-butylphenyl)-1,3,4-oxadiazole and 1 liter of solvent, which was a mixture of xylene and monomethyl ether (5.5:4.5, v/v). The samples were counted for ^3H on a YSI-76 Model liquid scintillation counter. ^3H-ATP was measured by the external standard ratio method. After deducting the interference derived from ^{51}Cr isotope, the radioactivity of ^3H-ATP in myocardium was obtained.

Statistical Analysis. Data are expressed as the mean ±SD. After measuring regional blood flow and radioactivity of ^3H-ATP in myocardium tissue, the relationship between relative myocardial blood flow and relative activity of ^3H-ATP was studied using linear regression and correlation analysis. Relative blood flow in the infarcted area was calculated as the ratio of blood flow supplied by the ligated left anterior descending arteries divided by blood supplied by the left circumflex artery, i.e., infarct-B/normal-B. The relative radioactivity of ^3H-ATP of the infarcted area was the ratio

Table II. ATP Entrapment in Neutral and Charged Liposomes Prepared by IEV

Lipid composition	% entrapped (w/w)[a]
Egg lecithin/cholesterol/stearylamine (5:5:1) (positive)	38.9
Egg lecithin/cholesterol (5:5) (neutral)	27.5
Egg lecithin/cholesterol/phosphatidic acid (5:5:1) (negative)	7.8

[a] Values are the means of two determination.

Table III. Size Distribution of Four ATP Liposomes

Sample	Medium diameter (μm)	σ
TFV	2.26	0.431
REV	5.56	0.206
DEV	1.89	0.221
IEV	1.95	0.235

Table IV. Regional Myocardial Blood Flow of Two Groups (ml/min · g)

	^{3}H-ATP	^{3}H-ATP liposomes
Normal	1.622 ± 0.201	1.552 ± 0.223
	(t = 0.572)	(P > 0.05)
Infarct	0.405 ± 0.141	0.307 ± 0.139
	(t = 0.007)	(P > 0.05)

Fig. 1. Electron micrograph of IEV. ×300,000.

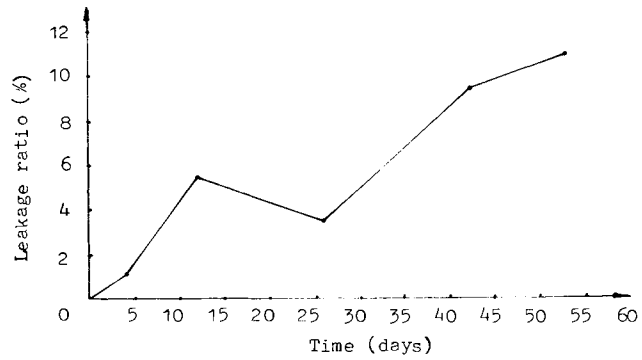

Fig. 2. Leakage curve of ATP liposome. Liposome was prepared with egg lecithin/cholesterol/stearylamine (5:5:1) containing 38.34% ATP, pH 7, and stored at room temperature (20°C). Values are the means of two determination. Leakage ratio (%) = $[(W_t - W_o)/W_i] \times 100\%$, where W_t is the free ATP amount at the beginning of storage, W_o is the free ATP amount during storage, and W_i is the total ATP amount entrapped in the liposome.

between the radioactivity supplied by the left anterior descending vessel divided by that supplied by the circumflex artery, i.e., infarct-A/normal-A.

Paired Student's t test was used for differences of both groups. In addition, corrected coefficient of blood flow activity of ^{3}H-ATP, relative activity of ^{3}H-ATP divided by relative blood flow, was studied, i.e., (infarct-A/normal-A) ÷ (infarct-B/normal-B).

RESULTS

The entrapment ratios of ATP in liposomes prepared by TFV, REV, DEV, and IEV methods are shown in Table I. Optimum entrapment was found for the IEV method, and therefore, the IEV method was used to encapsulate ATP in charged liposomes. Table II shows that positively charged liposomes result in optimum ATP entrapment. The lipid mixtures composed of egg lecithin/cholesterol/stearylamine (5:5:1), and the buffer was pH 7 PBS (50 mM). Liposome size varied with method of liposome formation. Table III shows that REV liposomes elicited the highest vesicle diameter (5.56 μm). Liposomes prepared by the IEV and DEV technique were similar in diameter (~1.9 μm). Electron micrographs of ATP-liposomes were obtained by all techniques (data not shown). Figure 1 is an electron micrograph of ATP liposomes prepared by the IEV method and repeating bilayers are apparent. While electron micrographs of liposomes provide an ambiguous measure of core size, Fig. 1 suggests that IEV liposomes contain a large core. Large cores are necessary for high entrapment of liposomes (10) and the IEV method elicited the highest entrapment of the methods tested.

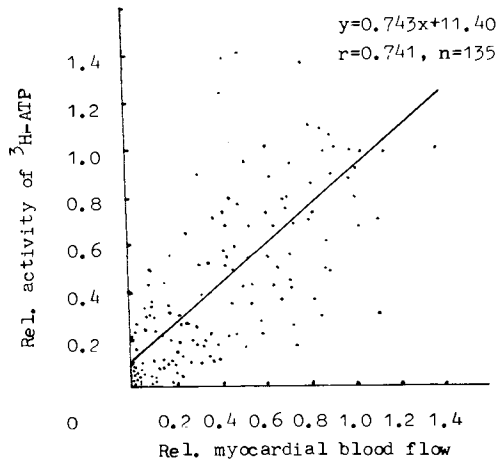

Fig. 3. Relationship of the RMBF and its relative activity of ^{3}H-ATP in the infarct myocardium in the ^{3}H-ATP group.

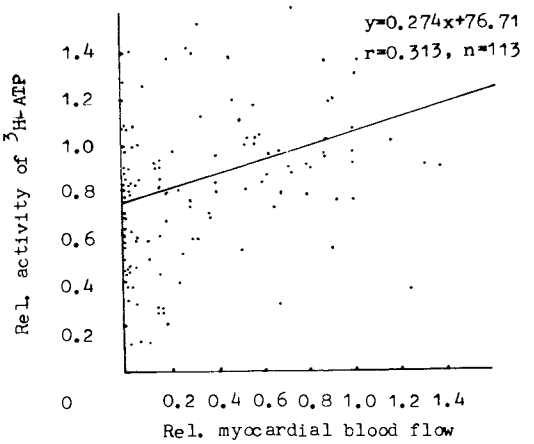

Fig. 4. Relationship of the RMBF and its relative activity of ^{3}H-ATP in the infarct myocardium in the ^{3}H-ATP liposome group.

The stability of IEV liposomes was evaluated as the percentage of ATP released over time during storage. Figure 2 demonstrates that after 54 days of storage at room temperature, approximately 11% of ATP leaked from the IEV liposomes. Radiation sterilization of IEV liposomes caused little effect on liposome size or release of ATP and also little effect on the UV spectrum of ATP liposomes. Microorganism growth was negative after sterilization.

Distribution Studies

Table IV shows that regional blood flow 60 min after ligation is substantially reduced compared to normal myocardial blood flow. No significant difference in tissue near the circumflex artery or left anterior tissue in both groups was apparent. Figures 3 and 4 show the relationship between relative myocardial blood flow and distribution in myocardial tissue 30 min after injecting unencapsulated ^{3}H-ATP (Fig. 3) or IEV-encapsulated ^{3}H-ATP (Fig. 4). A positive correlation between blood flow and tissue was obtained in both figures. However, the correlation coefficient obtained in Fig. 4 is so low (0.313) that no relationship between relative ^{3}H-ATP activity and blood flow is apparent. Although no relationship is apparent, much higher levels of ATP are found in myocardial tissues regardless of blood flow when liposomes are used to deliver ATP (compare Fig. 3 and Fig. 4). Thus, based on Fig. 3, we suggest that myocardial uptake of free ^{3}H-ATP depends on blood flow supplied by the left anterior descending artery, whereas based on Fig. 4, liposomal delivery of ATP depends partially on myocardial blood flow. However, under low-blood flow conditions, liposome delivered ATP causes substantially higher tissue concentrations of ATP (Fig. 4). Table V shows that relative blood flow in both groups of animals was similar. The corrected coefficients of blood flow–activity of ^{3}H-ATP of two groups were significantly different ($t = 4.74$, $P < 0.001$). Therefore there was a negative correlation between the relative blood flow and the relative activity of ^{3}H-ATP in ^{3}H-ATP liposomes group. It appeared that positively charged ^{3}H-ATP liposomes accumulate in ischemic myocardium.

DISCUSSION

Numerous methods have been reported for preparing liposomes, but their entrapment ratio was low. We have examined thin-film formed vesicles, ethanol injection, ether injection, ultrasonic waves, emulsification injection, reverse-phase evaporation vesicles, double emulsification vesicles, and multiemulsification, with similar results. In order to raise the entrapment ratio, we compared four methods and found that the entrapment ratio of IEV is the highest, being 2.5 times higher than that of TFV, 6.5 times higher than that of REV and 3.3 times higher than that of DEV. The positively charged liposomes result in the highest entrapment ratio; since ATP is negatively charged, the electrostatic attraction between ATP and positively charged stearylamine appears to enhance the ATP entrapment.

In this article we define the entrapment ratio as the percentage of drug weight entrapped in liposome to the weight of lipids (including the phosphatid and other adjuvants) (14).

Gel filtration (7,15), dialysis (16), and ultracentrifugation (17) are commonly used to separate the liposome from free drug, but an ultrafiltration technique was chosen in this study, as the filtrated free drug can be recovered for reuse.

Acute myocardial infarction is a severe complication of coronary heart disease. The mortality rates during hospitalization and within 1 year following infarction are 15 and 10%, respectively (18). Myocardial infarction induced by coronary occlusion could lead to metabolic functional and ultrastructural changes of injured myocardium. The cellular content of ATP decreased with injured time. When ATP depleted to less than 30% of control, cellular injury was irreversible (19). In recent years the effect of liposomes in cardiovascular diseases was studied (20–24). Caride and Zaret (20) reported that the liposome concentration in ischemic myocardium was significantly higher than that in normal myocardium in dogs which received positively charged or neutral liposomes 24 hr following coronary anterior occlusion. Maroko *et al.* (23) prepared positive liposomes with ^{3}H-ATP as the liquid phase marker. The dogs whose left anterior descending was ligated received the liposomes intravenously. Their results indicate that the content of ^{3}H-ATP liposomes in the ischemic myocardium is higher than that of ^{3}H-ATP. By electron microscopy, liposomes were identified mainly in the cytoplasm of ischemic myocytes but also in endothelial cells,

Table V. Comparison of RMBF and Its Relative Activity of ^{3}H-ATP in Two Groups

	Group	Mean ± SD	t and P
RMBF	^{3}H-ATP	0.261 ± 0.117	$t = 1.113$
(Infarct-B)/(normal-B)	^{3}H-ATP liposomes	0.198 ± 0.097	$P > 0.05$
Relative activity of ^{3}H-ATP	^{3}H-ATP	0.340 ± 0.198	$t = 4.086$
(Infarct-A)/(normal-A)	^{3}H-ATP liposomes	0.853 ± 0.235	$P < 0.01$

interstitium, and vascular lumens. But Cole *et al.* (24) reported that no evidence was found that liposomes are taken up preferentially by ischemic myocardium. Our data revealed that the content of positive ^{3}H-ATP liposomes in ischemic areas is rather high. We suggested that liposomes may serve as a carrier for drug delivery to infarct zones of low flow.

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