Liposomes in topical drug delivery

Helene E. Schaeffer and David L. Krohn

The possible use of liposomes as topical drug delivery vehicles for both water- and lipid-soluble drugs has been investigated. Data for two characteristic drugs, penicillin G and indoxole, are presented. Liposome uptake by the cornea is greatest for positively charged liposomes, less for negatively charged liposomes, and least for neutral liposomes, suggesting that the initial interaction between the corneal surface and liposomes is electrostatic adsorption. Positively charged unilamellar liposomes enhanced transcorneal flux of penicillin G across isolated rabbit cornea more than fourfold. Liposomal entrapment of drug is prerequisite to enhanced transport; corneal penetration was not enhanced when liposomes that were preformed in the absence of drug were mixed with penicillin G immediately before application to the cornea. Although penicillin G is water-soluble, the findings indicate that it secondarily associates with liposome membranes, possibly by insertion of its hydrophobic end into the lipid bilayer. Indoxole, however, was incorporated directly into the membranes of pure phosphatidyl choline liposomes. Liposome-mediated drug flux efficiency after topical instillation in rats was significantly greater than that obtained with equivalent concentration of drug delivered in polysorbate 80. Ten times more drug in polysorbate 80 was required to equal liposome-mediated flux efficiency. The findings suggest that liposomes enhance corneal penetration of drug by adsorbing to the corneal surface, with direct transfer of drug from liposomal to epithelial cell membranes.
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Key words: liposome, topical drug delivery vehicle, rabbit cornea, penicillin G, indoxole, rat

Liposomes were first described by Bangham,[1] who demonstrated that when phospholipids are suspended in excess aqueous solution they spontaneously form multilamellar concentric bilayer vesicles. The term liposome now refers to any of a variety of unilamellar or multilamellar phospholipid vesicles, which may vary with respect to size and method of production. Liposomes have been used extensively as model biological membranes for study of various membrane-related phenomena.[2–6] The potential application of liposomes as carrier vehicles with which to introduce poorly permeable biologically active molecules and drugs into cells has more recently been investigated[7–12] and reviewed.[13]

Many currently employed ophthalmic drugs and others of potential use penetrate the cornea poorly, particularly in the case of polar water-soluble drugs. The greatest barrier to corneal penetration appears to be the surface epithelial cell layer.[14, 15] We have explored the use of liposomes to enhance intracorneal and transcorneal penetration of both water- and lipid-soluble drugs. In this article, data

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for two characteristic drugs, penicillin G and indoxole, are presented.

Materials and methods

Materials. Purified phospholipids (>99% purity) were obtained from P-L Biochemicals, Inc., Milwaukee, Wisc., and radiochemicals were obtained from Amersham Corp., Arlington Heights, Ill.: ^{14}C-phosphatidyl choline (60 mCi/mmol), ^{3}H-cholesterol (9.5 Ci/mmol), and ^{14}C-benzyl penicillin potassium (59.5 mCi/mmol). Indoxole was supplied as a courtesy by the Upjohn Co., Kalamazoo, Mich.

Liposome preparation. Multilamellar liposomes were prepared by the method of Bangham.[1] Briefly, phospholipids dissolved in chloroform were dried under nitrogen to a thin lipid film. The lipid was resuspended in phosphate-buffered saline (PBS) by vigorous vortexing for 5 min and was allowed to swell for 2 hr at room temperature before use. Unilamellar liposomes were produced from multilamellar vesicles by sonication until clarification under humidified nitrogen in a bath-type sonicator (Heat Systems, 80W). Clarification of the previously turbid suspension indicates the conversion of a large portion of multilamellar vesicles to unilamellar liposomes.[16]

For experiments with penicillin G, liposomes were prepared in PBS containing 3.0×10^{-5}M ^{14}C-benzyl penicillin potassium. Several liposome types were studied: (1) neutral liposomes—phosphatidyl choline and cholesterol (molar ratios, 9:1); (2) positively charged liposomes—phosphatidyl choline, stearylamine, and cholesterol (7:2:1); (3) negatively charged liposomes—phosphatidyl choline, dicetyl phosphate, and cholesterol (7:2:1). Both unilamellar and multilamellar forms of each liposome type were prepared.

Indoxole was incorporated directly into the membranes of pure phosphatidyl choline (PC) vesicles (1 mg indoxole/15 μM lipid) by its inclusion in the organic solvent, along with phospholipids, during vesicle preparation. The liposomes were then suspended in PBS.

Phospholipid concentration in all liposome preparations was 15 μM/ml aqueous solution. Fresh preparations were made for each experiment.

Liposome-corneal interactions. The uptake of phosphatidyl choline, the major liposomal constituent, has been shown to be a reliable index of liposome uptake.[17] Therefore liposomes of various charges labeled with ^{14}C-phosphatidyl choline were prepared in PBS without drug. Corneas from freshly killed (intracardiac sodium pentobarbital) female New Zealand white rabbits (1.5 to 2.0 kg) were mounted in transport chambers,[18] and the epithelial surface of each cornea was exposed to 200 μl of liposome preparation. After 1 hr corneas were drained, blotted dry, digested, and assayed for ^{14}C-phosphatidyl choline uptake by liquid scintillation counting (LSC).

Table I. In vitro corneal button uptake of ^{14}C-phosphatidyl choline (rabbit cornea)

Liposome	*No. of experiments*	*Mean (nmole)*	*S.E.*
Neutral multilamellar	3	7.358	0.800
Neutral unilamellar	3	7.575	0.517
Negative multilamellar	4	14.43	3.13
Negative unilamellar	3	11.45	0.926
Positive multilamellar	3	34.80	5.23
Positive unilamellar	4	24.77	1.42

Corneal penetration of drug

Penicillin G. Corneas were mounted in transport chambers[18] and exposed for 1 hr to 200 μl of liposome suspension prepared in 3.0×10^{-5}M penicillin G. Two additional groups of corneas were exposed to equivalent doses of (1) free drug or (2) free drug mixed with liposomes (positive unilamellar) preformed in the absence of drug. Corneas were then washed, digested, and assayed for ^{14}C-penicillin G uptake by LSC. Penicillin G flux across the cornea was also measured; the fluid-filled chamber on the endothelial side of the cornea was drained and its contents were assayed for ^{14}C-penicillin G.

Indoxole. Male Sprague-Dawley white rats (250 to 300 gm) were anesthetized by intraperitoneal sodium pentobarbital. Ten microliters of one of the following indoxole preparations were delivered to the lower conjunctival sac with a Hamilton syringe: 1.0 mg/ml PC liposome suspension, 1.0 mg/ml in polysorbate 80, or 10 mg/ml in polysorbate 80. One hour after instillation the eye was irrigated with 0.2 ml proparicaine, and 10 μl of aqueous fluid was removed with a Hamilton syringe fitted with a 26-gauge needle. The aqueous tap was flushed into 2.0 ml absolute ethanol and was assayed for indoxole fluorometrically.[19]

To determine whether liposomal delivery induced corneal damage, rat eyes were treated twice daily for 8 days with the above liposome-indoxole preparation. Rats were then killed by intracardiac pentobarbital, the enucleated eyes were fixed immediately in buffered formalin, and the corneas were processed for histologic observation.

Liposome-drug interaction. During formation,

Table II. In vitro ^{14}C-penicillin G flux in 1 hr (rabbit cornea)

Preparation	*No. of experiments*	*Mean (nmole)*	*S.E.*	*Mean (% dose)*	*S.E.*
Free drug	12	0.027	0.0027	0.46	0.045
Drug + preformed unilamellar positive liposomes	10	0.022	0.0016	0.37	0.026
Drug + multilamellar neutral liposomes	10	0.027	0.0025	0.48	0.045
Drug + unilamellar neutral liposomes	12	0.042	0.0022	0.72	0.036
Drug + multilamellar positive liposomes	7	0.064	0.0050	1.08	0.087
Drug + unilamellar positive liposomes	10	0.122	0.0091	2.11	0.16
Drug + multilamellar negative liposomes	17	0.073	0.0041	1.30	0.075
Drug + unilamellar negative liposomes	12	0.055	0.0030	1.01	0.049

Table III. In vitro corneal button uptake of ^{14}C-penicillin G (rabbit cornea)

Preparation	*No. of experiments*	*Mean (nmole)*	*S.E.*	*Mean (% dose)*	*S.E.*
Free drug	11	0.051	0.0051	0.866	0.0875
Drug + preformed unilamellar positive liposomes	9	0.043	0.0056	0.722	0.0948
Drug + multilamellar neutral liposomes	10	0.039	0.0031	0.680	0.053
Drug + unilamellar neutral liposomes	12	0.077	0.0056	1.33	0.0926
Drug + multilamellar positive liposomes	7	0.049	0.0038	0.82	0.061
Drug + unilamellar positive liposomes	10	0.115	0.0081	2.01	0.153
Drug + multilamellar negative liposomes	17	0.066	0.0042	1.19	0.075
Drug + unilamellar negative liposomes	12	0.081	0.0051	1.51	0.0872

Table IV. Rat aqueous humor indoxole concentration in 1 hr

Indoxole preparation	*No. of experiments*	*Nanograms per 10 µl aqueous humor*	*S.E.*
1.0 mg/ml polysorbate	10	74	12
10.0 mg/ml polysorbate	10	200	59
1.0 mg/ml liposome suspension	10	180	43

liposomes spontaneously entrap at least a portion of available drug by (1) direct incorporation of lipid-soluble drug into liposome membranes, (2) entrapment within aqueous compartments of polar solutes present in aqueous solution, or (3) entrapment of amphiphilic moieties partially into both aqueous and lipid phases.[20]

Penicillin G. To determine percent entrapment of 3.0×10^{-5}M ^{14}C-penicillin G by liposomes of various charge and size, ^{3}H-cholesterol–labeled liposome preparations containing both entrapped and unentrapped drug were chromatographed on Sephadex G-200; fractions were analyzed by double-label LSC. Liposomal lipid, together with entrapped drug, elutes first in the void volume; unentrapped drug is included in the gel and elutes later.

To determine whether entrapped penicillin is partially membrane associated, the lipid peak (together with entrapped drug) obtained from positively charged unilamellar liposomes was rechromatographed on Sephadex G-200 after disruption of liposomes with (1) Triton X-100, (2) sonication for 1 hr at pH 6.0, (3) sonication for 1 hr at pH 8.0, or (4) no further treatment.

Indoxole. A known aliquot of 1.0 mg/ml indoxole in ^{14}C-labeled PC vesicles was chromatographed on Sephadex G-200. Fractions were analyzed for lipid (LSC) and for indoxole (spectrophotofluorometry).[19]

Statistical analysis. Comparisons were based on analysis of variance, and where appropriate, a modified t test for differences between means.

Results

Liposome-corneal interactions. After 1 hr, uptake of liposomal ^{14}C-phosphatidyl choline by the cornea was greatest for positively charged liposomes, less for negatively charged liposomes, and least for neutral liposomes (Table I).

Corneal penetration of drug

Penicillin G

FLUX. Positively charged unilamellar liposomes prepared in 3.0×10^{-5}M penicillin G

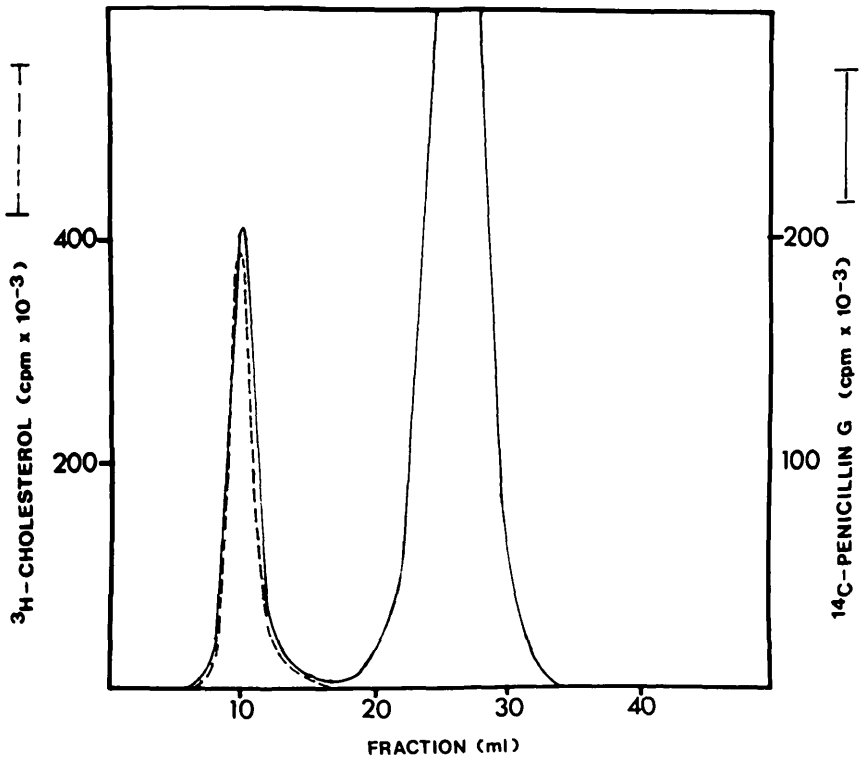

Fig. 1. Representative elution curve of liposomes prepared in 3.0 × 10^{-5}M penicillin G, chromatographed on Sephadex G-200. Values of percent entrapment for each liposome type are as follows: multilamellar neutral, 2.0; unilamellar neutral, 16.4; multilamellar negative, 2.2; unilamellar negative, 4.3; multilamellar positive, 6.5; unilamellar positive, 17.5, 18.8 (duplicate values are comparable).

enhanced transcorneal drug flux more than fourfold, as compared with an equivalent concentration of free drug (Table II). In contrast, when preformed positively charged unilamellar liposomes were mixed with free drug immediately before application to the cornea, flux was not increased ($p > 0.05$). Therefore liposomes alone do not enhance transport if no portion of available drug is liposome entrapped. Flux was increased by almost threefold by negatively charged multilamellar liposomes and greater than twofold by negatively charged unilamellar and positively charged multilamellar liposomes, with an insignificant difference between them ($p > 0.25$). A smaller but still significant increase was also found with neutral unilamellar liposomes ($p < 0.001$) but not with neutral multilamellar liposomes ($p > 0.10$).

CORNEAL UPTAKE. The largest increase in corneal uptake of penicillin G over free drug was mediated by positively charged unilamellar liposomes prepared in 3.0 × 10^{-5}M penicillin G (Table III). A significant increase was also obtained with negatively charged unilamellar, neutral unilamellar, and negatively charged multilamellar liposomes ($p < 0.0005$). Drug uptake was not altered by neutral multilamellar liposomes ($0.10 > p > 0.05$) or by positively charged multilamellar liposomes ($p > 0.25$). As was the case for transcorneal flux, no increase in drug uptake was obtained with preformed positively charged unilamellar liposomes ($0.10 > p > 0.05$).

Indoxole. A significantly greater aqueous humor indoxole concentration was found 1 hr after topical instillation of 1.0 mg indoxole/ml liposome suspension, compared with that found after instillation of 1.0 mg indoxole/ml

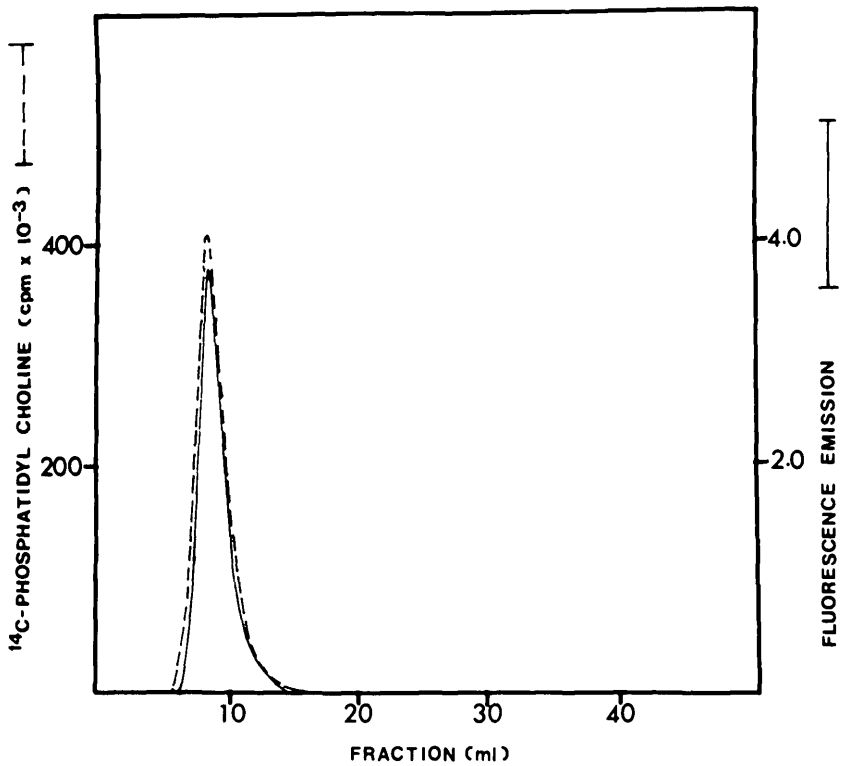

Fig. 2. Chromatography on Sephadex G-200, showing association of indoxole with sonicated pure phosphatidyl choline liposomes; indoxole was incorporated directly into liposome membranes (15 mg indoxole/15 μM lipid). Fluorescence intensity is expressed in arbitrary units.

Table V. Rechromatography of penicillin-entrapped liposome peak (positive unilamellar) on Sephadex G-200

Treatment of liposomes prior to rechromatography	*Lipid-associated penicillin after rechromatography (%)*
Disruption of liposomes with Triton X-100	76.0
Sonication for 1 hr at pH 6.0	72.0
Sonication for 1 hr at pH 8.0	71.0
None	73.0

polysorbate 80 ($p < 0.05$) (Table IV). There was no significant difference ($p > 0.25$) after instillation of 10 mg indoxole/ml polysorbate 80 or 1.0 mg indoxole/ml of liposome suspension. Therefore, liposome-mediated indoxole delivery is considerably more efficient than delivery in polysorbate 80. In addition, histologic observation of corneas exposed to indoxole in liposomes over an 8 day period did not show the corneal toxicity (stromal hyalinization) found to have been associated with polysorbate 80 delivery (unpublished data).

Liposome-drug interation

Penicillin G. Liposome entrapment of penicillin G is shown in Fig. 1. Although penicillin G is water-soluble, entrapment appears to be membrane associated, since (1) both positively charged and neutral unilamellar liposomes (with more available surface area for solute binding) entrap several times more penicillin than do their multilamellar forms and (2) after exposure of penicillin G–entrapped liposomes to liposome-disrupting treatments, more than 70% of the drug remains associated with liposomal lipid (Table V). The remaining 25% to 30% was lost mainly through efflux. This is consistent with the duration of the experiments and with the finding that upon dialysis of penicillin G–entrapped positively charged unilamellar liposomes against PBS, penicillin G effluxes from these

liposomes at the rate of 2% to 3% per hour (unpublished data).

Indoxole. Chromatography on Sephadex G-200 of indoxole-entrapped phosphatidyl choline liposomes indicated that all of the entrapped indoxole was associated with membrane lipid (Fig. 2).

Discussion

Liposomes are a promising new topical drug delivery vehicle for both water- and lipid-soluble drugs. They offer the advantage over most ophthalmic preparations of being completely biodegradable and relatively nontoxic. Histologic preparations of corneas treated with indoxole-entrapped PC liposomes showed no evidence of damage on light microscopy. In addition, uptake of large numbers of vesicles by 3T3 cells in culture has been reported to result in no apparent cytotoxicity.[8] However, a dose-dependent cytotoxicity associated with stearylamine-containing liposomes has been reported.[21–24] This may result from destabilization of lysosomal membranes by stearylamine, with subsequent release of lysosomal hydrolases into the cytoplasm.[24] Unilamellar stearylamine-containing liposomes produced the greatest increase in flux and corneal uptake of penicillin G. This increase was not likely a result of corneal epithelial cell damage, since a mixture of free drug and unilamellar stearylamine-containing liposomes does not increase flux.

Corneal uptake of liposome-associated radioactivity was found to be greatest for positively charged liposomes. Similar findings have been reported for other cell types.[22, 25] Because at physiologic pH the cell surface bears a net negative charge,[26] the initial interaction between liposomes and the corneal surface may be electrostatic adsorption.[22, 23] This is suggested by the relative affinities of liposomes for the corneal surface based on charge alone.

Liposome-drug interactions were examined for possible insight into mechanisms of enhanced corneal drug penetration. Indoxole is completely lipid soluble and was therefore entrapped entirely within the lipid phase. Although water soluble, penicillin G entrapment is not limited to the aqueous phase, since entrapped drug was refractory to release by membrane rupturing treatments; polar drugs found only in the internal aqueous compartments of liposomes are readily released when the membranes are ruptured. Therefore penicillin G behaves as an amphiphilic drug, which secondarily intercalates into liposome membranes, probably by insertion of its hydrophobic end.[27] We speculate then that liposomes, adsorbed to the corneal surface, transfer their membrane-associated drug directly to corneal epithelial cell membranes, thereby facilitating drug transport across the cornea. The results of this study are consistent with this interpretation in that liposome entrapment of drug is prerequisite to enhanced translocation of drug into ocular tissues, and all liposome types studied bind to the cornea, as shown by corneal uptake of liposome-associated radioactivity. Direct membrane-(liposome) to-membrane (cell) transfer of membrane-associated moieties (e.g., cholesterol) has been described previously.[17, 28] Whether other mechanisms such as endocytosis of liposomes or fusion of liposome membranes with the plasmalemma[29] are also involved in enhanced transport remains to be determined.

Penicillin G flux was enhanced best by positively charged unilamellar liposomes, which may reflect their greater binding capacity for the corneal surface. In contrast, neutral liposomes bind least and have a correspondingly small effect on transcorneal flux. Despite the better binding of positively charged multilamellar liposomes, penicillin G flux was found to be greatest with positively charged unilamellar liposomes. This discrepancy might be explained by the small size of unilamellar liposomes, about 25 nm,[20] allowing closer apposition of liposome to cell membrane, thereby facilitating more efficient drug transfer.

Difficult to reconcile with the above scheme are the findings that negatively charged multilamellar liposomes are significantly better than negatively charged unilamellar liposomes ($p < 0.025$) in their ability to enhance transcorneal penicillin G flux and that nega-

tively charged unilamellar and positively charged multilamellar liposomes enhance drug flux to the same extent ($p > 0.25$). In addition, with the exception of the highest and lowest extremes (mediated by positively charged unilamellar and neutral multilamellar liposomes, respectively), there is a general lack of correspondence between transcorneal penicillin G flux and corneal uptake of drug. However, other factors, including the effects of different liposome types on corneal surface ultrastructure, may account for these inconsistencies.

Indoxole, a potentially powerful but poorly soluble anti-inflammatory agent, was previously found to penetrate the cornea maximally when solubilized in polysorbate 80.[30] However, corneal toxicity of the latter is limiting. Liposome-mediated indoxole delivery is considerably more efficient than delivery in polysorbate 80 and appears to be noncytotoxic. Therefore the use of liposomes as an indoxole delivery vehicle may renew the potential usefulness of this drug.

Liposomes enhance corneal permeability of penicillin G in vitro. However, if attachment of liposomes to the cornea is through electrostatic binding, methods to enhance retention of liposomes under physiologic conditions may be desirable. Covalent attachment to the liposome surface of suitable ligands that have a strong affinity for the corneal epithelium is under investigation.

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