Arch. Pharm. Res. Vol. 16, No. 2, pp. 129-133, 1993

Preparation and Evaluation of Temperature Sensitive Liposomes Containing Adriamycin and Cytarabine

Chong-Kook Kim, Suk-Kyeong Lee and Beom-Jin Lee

College of Pharmacy, Seoul National University, Seoul 151-742, Korea

(Received April 18, 1993)

Temperature sensitive liposomes (TSL) containing adriamycin (ADM) and cytarabine (Ara-C) were prepared. ADM and Ara-C were selected as model compounds of amphiphilic and hydrophilic drug, respectively. Encapsulation efficiency of ADM entrapped into TSL was about twice greater than that of Ara-C. It might be due to different polarity of the drugs. Lipid compositions of TSL had no effect on the encapsulation efficiency of drugs. Thermal behavior of TSL using a differential scanning calorimetry (DSC) was also investigated. Phase transition temperature (T_d) of TSL was dependent on the lipid compositions of TSL. ADM broadened thermogram of TSL but Ara-C did not. However, T_c of TSL was not changed by any drug. Release rate of drugs was highly dependent on temperature. The release profile of ADM was similar to that of Ara-C. The maximum release rate of drugs from TSL was occurred at the near T_c and observed at 39-41℃ for DPPC (Dipalmitoylphosphatidylcholine) only, 52-54℃ for DSPC (Distearoylphosphatidylcholine) only, 41-43℃ for DPPC and DSPC (3 : 1), and 43-45℃ for DPPC and DSPC (1 : 1), respectively. Effect of human serum albumin (HSA) on the release rate of ADM was investigated. HSA had no significant effect on the release of ADM below T_c. However, ADM release from TSL was increased at the near and above T_c. The HSA-induced leakage of drug may result from the interaction of liposomal constituents with HSA structure at the near T_c. From the fact that the release profiles of ADM from freshly prepared TSL and stored TSL for 1 week at 4℃ was not changed, the TSL was considered to be stable for at least 1 week at 4℃. Based on these findings, TSL may be useful to deliver drugs to preheated target sites due to its thermal behaviors.

Key words: Temperature sensitive liposomes, Adriamycin, Cytarabine, Human serum albumin, Encapsulation efficiency, Thermal behavior, Drug release

INTRODUCTION

Liposomes have paid considerable attention as carriers of various substances such as anticancer agents, hormones, proteins and DNA because of their nontoxic, biocompatible and biodegradable properties (Kim and Park, 1987; Kim and Lee, 1987; Kim *et al.*, 1991). In chemotherapy, one exquisite method to deliver anticancer drugs to locally heated tumors in animals employs temperature sensitive liposomes (TSL) (Yatvin *et al.*, 1978; Weinstein *et al.*, 1979; Weinstein *et al.*, 1980; Bassett *et al.*, 1986). TSL must have phase transition temperature (T_d) above the physiological temperature and release drugs at a range of temperature (near 40℃) attainable by mild local hyperthermia. However, little information is available on TSL containing anticancer drugs.

Amphiphilic adriamycin (ADM) and hydrophilic cytarabine (Ara-C) were used as model drugs. The enhanced antitumor activity of Ara-C (Kim and Park, 1987; Rustum *et al.*, 1979; Juliano *et al.*, 1980) and ADM (Olson *et al.*, 1982; Gabizon *et al.*, 1982; Rahman *et al.*, 1980) encapsulated into TSL was previously reported. Polar drugs like methotrexate and cytarabine incorporated into TSL may interact with liposomal bilayer (Weinstein *et al.*, 1979; Margin and Weinstein, 1984). Obvious synergism in the cytotoxicity of ADM at 42-43℃ hyperthermia was reported because ADM go into cell much easily at 43℃ than 37℃ (Zborowski *et al.*, 1977). While it is of interest to deliver anticancer drugs to preheated target sites using TSL, the physicochemical properties of TSL have not been studied intensively.

The purpose of this preliminary study was to investigate physicochemical properties of TSL such as encapsulation efficiency of drugs, thermal behavior of

Correspondence to: Chong-Kook Kim, College of Pharmacy, Seoul National University, San 56-1, Shinrim-Dong, Kwanak-Ku, Seoul 151-742, Korea

TSL and drug release properties from TSL. Effects of human serum albumin (HSA) on the release of ADM from TSL was also investigated.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and human serum albumin (HSA; fraction V powder) were purchased from Sigma Co. Ltd. (St Louis, MO, USA). The purity of lipids was checked by thin layer chromatography. Adriamycin (ADM) and cytarabine (Ara-C) were kindly supplied courtesy of Il-Dong Pharm. Co. (Seoul, Korea) and Choong-Wae Pharm. Co. (Seoul, Korea), respectively. All other chemicals were of reagent grade and used without further purification.

Preparation of TSL

Liposomes were prepared by the modification of reverse phase evaporation technique (Szoka and Papahadjopoulos, 1978). A mixture of DPPC and DSPC was dissolved in an appropriate amount of chloroform and the solvent was evaporated using a rotary evaporator. The dried thin lipid film was then redissolved in a mixture of 2 ml of chloroform and 4 ml of isopropyl ether as an organic phase. Isopropyl ether was freshly washed with 10% sodium bisulfite to remove any peroxides, if present. Drug (ADM or Ara-C) dissolved in 0.9% NaCl solution was added to organic phase. The two phases consisting of 6 ml of organic and 2 ml of aqueous phase were warmed up to 56-65℃ and vortexed for 30 sec followed by 90 sec incubation in a water bath. When a homogeneous emulsion was formed, the solvent was removed on a rotary evaporator at the pressure of 350 mmHg. The newly formed liposomes were allowed to anneal at 50-65℃ in a water bath for 30 min or longer.

Determination of drug content in TSL

The amount of drug in TSL was determined as follows. Liposomes were centrifuged for 10 min at 180,000 g using a ultracentrifuge (MSE Europe 65) at 5-10°C. The pellets were washed 4 times with 0.9% NaCl solution. The resulting supernatant was combined and adjusted to determine the amount of free drug. The free ADM at 480 nm or Ara-C at 272 nm was determined using a UV spectrophotometer (Pye Unicam PU 8800). The amount of drug entrapped is the total amount of drug minus the free drug content in the supernatant.

$$\text{Encapsulation efficiency}(\%) = \frac{(\text{Total drug} - \text{Supernatant drug})}{\text{Total drug}} \times 100$$

Thermal behavior of TSL

Phase transition temperature (T_c) of TSL was measured using a Perkin-Elmer DSC. Liposomal pellets sedimented by ultracentrifugation was scanned from 20 to 70℃ at a rate of 5℃/min against empty reference pan. The sensitivity of DSC is 0.5 mcal/sec. The intersection of straight line to the upward of deflection of the transition curve with the base line of the thermogram was projected to the temperature axis to determine T_c.

Drug release from TSL

The TSLs having different lipid compositions and entrapping ADM or Ara-C were suspended in the 0.9% saline solution to adjust 7 mM lipid concentration. Then, 1 ml aliquot of each TSL was placed in 5 ml centrifuge tube and incubated at a given temperature for 5 min. When the incubation was completed, TSL was rapidly cooled in an ice bath and then ultracentrifuged at 5-10℃. The amount of ADM or Ara-C released in the supernatant was determined according to the method previously mentioned.

The effects of HSA on the release of ADM from TSL consisting of DPPC and DSPC (3 : 1) were also investigated. HSA (1.5 mg/ml) was added to 1 ml aliquot of TSL. After incubation, the amount of ADM released was determined according to the method previously mentioned. The percentage of ADM released was plotted as a function of temperature.

The appropriate volume of TSL consisting of DPPC and DSPC (3 : 1) in 0.9% NaCl solution was stored for 1 week at 4℃ to evaluate physical stability of TSL. One milliliter of TSL was drawn every 24 hours. The amount of ADM released was determined. Release profiles of ADM between freshly prepared TSL and 1 week stored TSL were compared.

RESULTS AND DISCUSSION

Encapsulation efficiency

Encapsulation efficiency of drugs into TSL are shown

Table I. Encapsulation efficiency of drug into TSL

Composition[a]	Encapsulation Efficiency (%)	
(DPPC:DSPC)	ADM	Ara-C
1 : 0	38.7± 5.1[b]	15.2± 3.4
3 : 1	38.2± 5.7	14.9± 2.4
1 : 1	37.9± 6.0	14.5± 3.2
0 : 1	37.8± 4.9	14.7± 3.5

Lipid compositions of TSL were varied. The amount of ADM and Ara-C was invariably 1.8 mg and 2 mg, respectively.
[a] Ratio of DPPC and DSPC by weight
[b] Mean± standard deviation ($n \geq 5$)

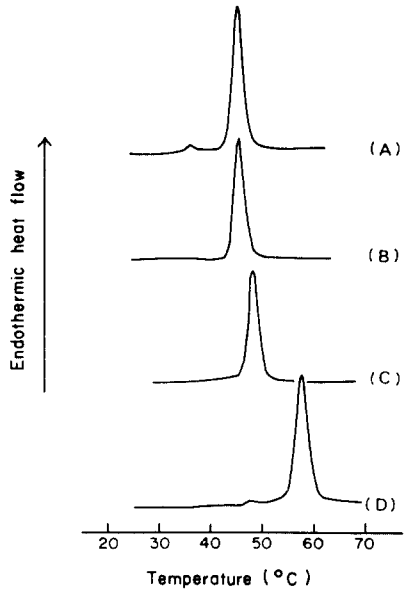

Fig. 1. Thermogram of TSL consisting of various lipid compositions. (A) DPPC only; (B) DPPC:DSPC (3 : 1); (C) DPPC: DSPC (1 : 1); (D) DSPC only.

in Table I. Liposomal lipid compositions had no effect on encapsulation efficiency of drugs. However, encapsulation efficiency was dependent on the type of drugs. Efficiency of ADM entrapped into TSL was about twice greater than that of Ara-C. Hydrophilic Ara-C was less encapsulated compared to amphiphilic ADM. This result suggests that Ara-C with high polarity is entrapped within the aqueous compartment of TSL, while ADM with low polarity is distributed into both aqueous and lipid compartment of liposomal bilayer (Juliano and Stamp, 1976; Kursch *et al.*, 1983).

Thermal behavior of TSL

The thermogram of TSL, varying lipid compositions are shown in Fig. 1. Phase transition temperature (T_c) of TSL was dependent on the lipid compositions of TSL. T_c of TSL was observed at 41℃ with DPPC only, 43℃ with a mixture of DPPC and DSPC (3 : 1), 45℃ with a mixture of DPPC and DSPC (1 : 1), and 54℃ with DSPC only.

The effect of cationic drugs on the thermal behavior of phosphatidylcholine liposomes was previously reported (Juliano and Stamp, 1976). The effect of drug on the thermogram of TSL is given in Fig. 2. Regardless of lipid compositions of TSL, T_c was unchanged by drugs even at a high molar ratio of drug and lipid (1 : 1). However, amphiphilic ADM resulted in peak broadening of thermogram although Ara-C did not change thermogram significantly. It has been reported that amphiphilic molecules alter the packing arrangement of lipid bilayer and T_c at high ratio of drug and lipid (Margin and Weinstein, 1984). It appears

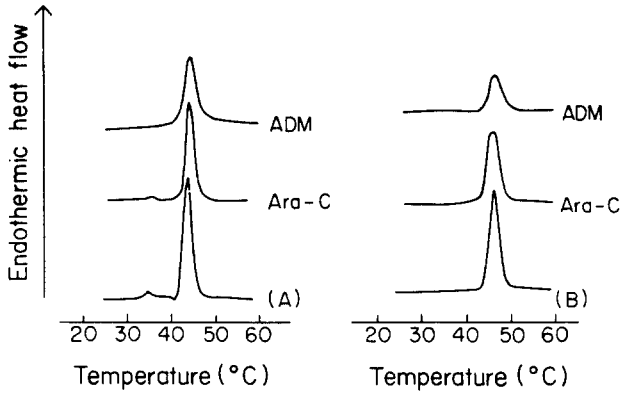

Fig. 2. Effect of ADM and Ara-C on the thermogram of TSL. Lipid compositions of TSL are as follows. (A) DPPC only; (B) DPPC:DSPC (3 : 1).

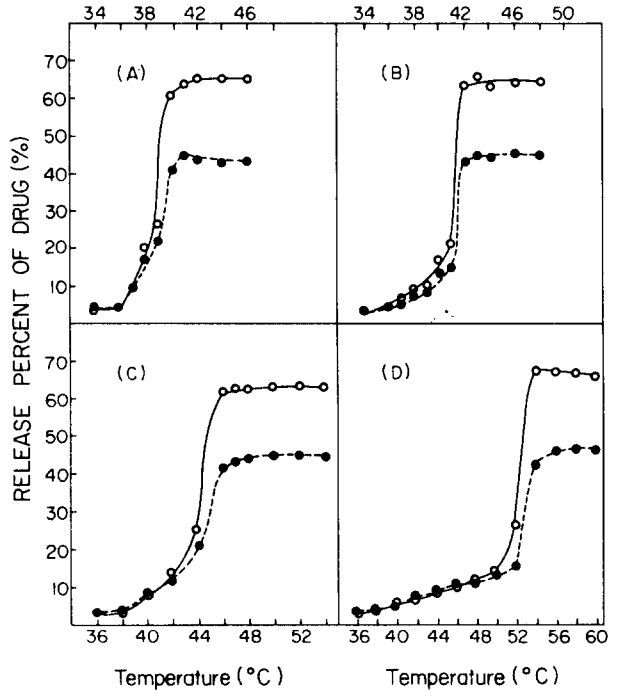

Fig. 3. The release of ADM (●) and Ara-C (○) from TSL as a function of temperature. Lipid compositions of TSL are as follows. (A) DPPC only; (B) DPPC:DSPC (3 : 1); (C) DPPC: DSPC (1 : 1); (D) DSPC only. TSL was incubated and the amount of drug released for 5 min was determined.

that the amount of ADM encapsulated would be too small to associate with the head group and/or acyl chain portion of lipid bilayer in a perturbing manner.

Drug release from TSL

The percentage of drug released from TSL as a function of temperature was shown in Fig. 3. The release profile of ADM was similar to that of Ara-C. Release rate of drug was highly dependent on temperature. The maximum release rate of drugs from

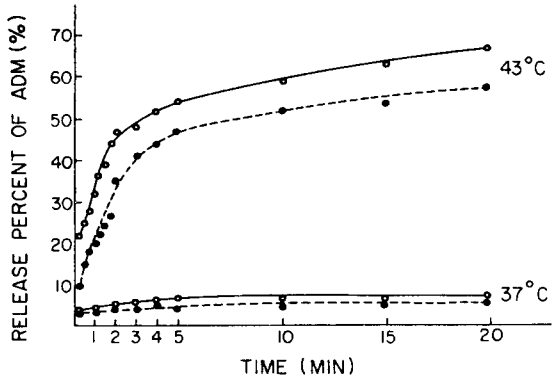

Fig. 4. Effect of HSA on the release of ADM from TSL consisting of DPPC and DSPC (3 : 1) at 37℃. and 43℃. (T_c). Control (●); HSA (1.5 mg/ml) (○). TSL was incubated for 20 min.

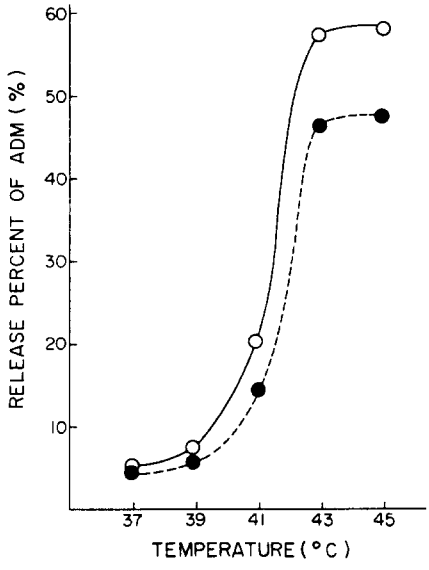

Fig. 5. Effect of HSA on the release of ADM from TSL consisting of DPPC and DSPC (3 : 1) as a function of temperature. Control (●); 1.5 mg/ml HSA (○). TSL was incubated at 37, 39, 43, and 45℃ and the amount of drug released for 5 min was determined.

TSL was occurred at the near T_c and observed at 39-41℃ for DPPC only, 41-43℃ for DPPC and DSPC (3 : 1), 43-45 ℃ for DPPC and DSPC (1 : 1), and 52-54℃ for DSPC only, respectively. At the near T_c, 40-50% of ADM released while 60-70% of Ara-C released for 5 min incubation. Only 5% of drug released below T_c. These results suggest that the structure of TSL may have a discontinuity at the near T_c resulting in changes of TSL fluidity. In view of target specific deliveries, TSL consisting of DPPC and DSPC (3 : 1) may be useful because it remains impermeable at physiological body temperature but reaches maximum drug release when site of disease is heated to 41-43℃.

Effect of HSA on the release of ADM from TSL

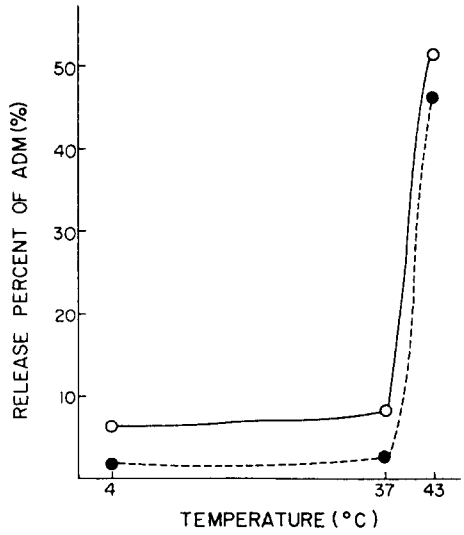

Fig. 6. Comparison of release characteristics of ADM between freshly prepared TSL (●) and TSL stored for 1 week at 4℃ (○). TSL was also incubated at 37 and 43℃ and the amount of drug released for 5 min was determined.

Table II. Percentage of ADM released from TSL consisting of DPPC and DSPC(3 : 1) during 1 week storage at 4℃

Time (day)	1	2	3	4	5	6	7
ADM released (%)	2.3	2.7	3.4	3.9	4.6	5.7	6.4

consisting of DPPC and DSPC (3 : 1) is compared in Fig. 4. At 37℃, HSA had no significant effect on ADM release. At the T_c, more than 40% of ADM was released within 4 min but only less than 5% of ADM was released thereafter. HSA-induced release of ADM from TSL was noted at the T_c. Effect of HSA on the ADM release for 5 min from TSL consisting of DPPC and DSPC (3 : 1) as a function of temperature is compared in Fig. 5. Regardless of HSA, ADM was largely released at near T_c. HSA-induced release of ADM from TSL was more obvious near and above the T_c. It has reported that serum albumin (Kim *et al.*, 1991) and other plasma components interact with liposomal structure and induce leakage of entrapped materials (Zborowski *et al.*, 1977; Tyrrell *et al.*, 1977; Allen and Cleland, 1980). Large induction of ADM release by HSA may result from the defects of lipid bilayer. Although the mechanism of HSA-induced leakage of ADM from TSL is not well-known, TSL may be useful to deliver drugs to preheated target sites due to its thermal behavior.

TSL was stored at 4℃ for 1 week to evaluate the stability of TSL consisting of DPPC and DSPC (3 : 1). The percentage of ADM released from TSL is given in Table II. The amount of ADM released from TSL was relatively small and only about 6.4% of ADM released for 1 week storage. The percentages of ADM

released from freshly prepared TSL and stored TSL for 1 week at 4℃ as a function of temperature are compared in Fig. 6. The profile of ADM released from freshly prepared TSL was similar to that from 1 week stored TSL. These results suggest that TSL remains stable for at least 1 week at 4℃.

CONCLUSIONS

Encapsulation efficiency of amphiphilic ADM in TSL was about twice greater than that of hydrophilic Ara-C possibly due to its low polarity. Lipid compositions of TSL had no effect on the encapsulation efficiency of drugs. Phase transition temperature (T_c) of TSL was dependent on the lipid compositions of TSL. ADM broadened thermogram of TSL but Ara-C did not. However, both drugs did not affect T_c. Release rate of drug was highly dependent on temperature. The maximum release of drug from TSL occurred at the near T_c. HSA had no significant effect on the release of ADM below T_c. However, HSA increased the release of ADM from TSL at the near and above T_c. The HSA-induced leakage of drug from TSL may result from the interaction of liposomal compositions with HSA structure at the near T_c. From the fact that the release profiles of ADM from freshly prepared TSL and 1 week stored TSL at 4℃ was not changed, the TSL was considered to be stable for at least 1 week at 4℃.

ACKNOWLEDGEMENTS

This research work was supported in part by the research grants from the Ministry of Education and from Korea Science and Engineering Foundation.

REFERENCES CITED

Allen, T. M. and Cleland, L. G., Serum induced leakage of liposome contents. *Biochem. Biophys. Acta.*, 597, 418-426 (1980).

Bassett, J. B., Anderson, R. U. and Tacker, J. R., Use of temperature sensitive liposomes in the selective delivery of methotrexate and cis-platinum analogues to murine bladder tumor. *J. Urol.*, 135, 612-615 (1986).

Gabizon, A., Dagan, A., Goren, D., Barenholz, Y. and Fuks, Z., Liposomes as in vivo carriers of adriamycin: Reduced cardiac uptake and preserved antitumor activity in mice. *Cancer Res.*, 42, 4734-4739 (1982).

Juliano, R. L. and McCullough, H .N., Controlled delivery of an antitumor drug: Localized action of liposome encapsulated cytosine arabinoside administered via the respiratory system. *J. Pharmacol. Exp. Ther.*, 214, 381-387 (1980).

Juliano, R. L. and Stamp, D., Lectin mediated attachment of glycoprotein bearing liposomes to cells. *Nature* (London), 261, 235-237 (1976).

Kim, C. K. and Lee, B. J., Effect of salts on the entrapment of calf thymus DNA into liposomes. *Arch. Pharm. Res.*, 10, 110-114 (1987).

Kim, C. K. and Park, D. K., Stability and drug release properties of liposomes containing cytarabine as a drug carrier. *Arch. Pharm. Res.*, 10, 75-79 (1987).

Kim, C. K., Kim, H. S., Lee, B. J. and Han, J. H., Effect of bovine serum albumin on the stability of methotrexate-encapsulated liposomes. *Arch. Pharm. Res.*, 14(4), 336-341 (1991).

Kursch, B., Lullmann, H. and Mour, K., Influence of various cationic amphiphilic drugs on the phase-transition temperature of phosphatidylcholine liposomes. *Biochem. Pharmac.*, 32, 2589-2594 (1983).

Magin, R. L. and Weinstein, J. N., The design and characterization of temperature sensitive liposomes, In Gregoriadis, G. (Eds.), Liposome Technology, Vol. III, CRC Press, Boca Raton, FL, 1984, pp. 137-155.

Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka, F., Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. *Eur. J. Cancer Clin. Oncol.*, 18, 167-176 (1982).

Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P. and Schein, P. S., Liposomal protection of adriamycin-induced cardiotoxicity in mice. *Cancer Res.*, 40, 1532-1537 (1980).

Rustum, Y. M., Dave, C., Mayhew, E. and Papahadjopoulos, D., Role of liposome type and route of administration in the antitumor activity of liposome-entrapped 1-β-D-arabinofuranosylcytosine against mouse L1210 leukemia. *Cancer Res.*, 39, 1390-1395 (1979).

Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large intestinal aqueous space and high capture by the reverse phase evaporation. *Proc. Natl. Acad. Sci.*, 75, 4194-4198 (1978).

Tyrrell, D. A., Richardson, V. J. and Ryman, B. E., The effect of serum protein functions on liposome-cell interaction in cultured cells and the perfused rat liver. *Biochem. Biophs. Acta.*, 497, 469-480 (1977).

Weinstein, J. N., Cysyk, R. L. and Zaharko, D. S., Treatment of solid L1210 murine tumors with local hyperthermia and temperature sensitive liposomes containing methotrexate. *Cancer Res.*, 40, 1388-1395 (1980).

Weinstein, J. N., Magin, R. L., Yatvin, M. B. and Zaharko, D. S., Liposomes and local hyperthermia: Selective delivery of methotrexate to heated tumors. *Science*, 204, 188-191 (1979).

Yatvin, M. B., Weinstein, J. N., Dennis, W. H. and Blumenthal, R., Design of liposomes for enhanced local release of drugs by hyperthermia. *Science*, 202, 1290-1293 (1978).

Zborowski, J., Roerdink, F. and Scherphof, G., Leakage of sucrose from phosphatidylcholine liposomes induced by interaction with serum albumin. *Biochem. Biophys. Acta.*, 497, 183-191 (1977).