JPP
Journal of
Pharmacy and Pharmacology

JPP 2005, 57: 527–531
© 2005 The Authors
Received September 23, 2004
Accepted December 22, 2004
DOI 10.1211/0022357055830
ISSN 0022-3573

# Development of simple thiol-reactive liposome formulations, one-step analysis and physicochemical characterization

E. Kaourma, S. Hatziantoniou, A. Georgopoulos, A. Kolocouris and C. Demetzos

## Abstract

The aim of this study was to synthesize simple thiol-reactive conjugates from maleimide and lipoamines (stearylamine or oleylamine) and to develop a simple, fast and low-cost method for the preparation of lyophilized general-purpose thiol-reactive liposomes. A formulation of egg phosphatidylcholine–dipalmitoylphoshatidylglycerol (9:0.1 molar ratio) was developed and characterized. Freeze-drying methodology was established to produce a stock of liposomes and the physicochemical characteristics of the reconstituted liposomes were compared with those of the initial preparation. The physicochemical properties (size and $\zeta$-potential) of the new liposomal formulations were studied. High-performance thin-layer chromatography coupled to a flame ionization detector was applied for one-step analysis of the liposomal components and for determining the maleimide–lipoamine conjugates phospholipid molar ratio. The differences concerning the incorporation efficiency of the synthetic conjugates into liposomes were discussed on the basis of their conformational properties. The small difference in structure between the two thiol-reactive conjugates (i.e., the C18 alkyl chain double bond) causes a considerable difference in phospholipids packing of the resulting lipidic bilayers of the liposomes; the conformational bending of conjugate maleimide-oleylamine may contribute to the final architecture of liposomes.

Department of Pharmaceutical Technology, School of Pharmacy, Panepistimiopolis-Zografou, 157 71, Athens, Greece

E. Kaourma, S. Hatziantoniou, A. Georgopoulos, C. Demetzos

Department of Pharmaceutical Chemistry, School of Pharmacy, Panepistimiopolis-Zografou, 157 71, Athens, Greece

A. Kolocouris

**Correspondence:** C. Demetzos, Department of Pharmaceutical Technology, School of Pharmacy, Panepistimiopolis-Zografou, 157 71, Athens, Greece. E-mail: demetzos@pharm.uoa.gr

**Acknowledgement:** This study was funded by grants from the Special Account of Research (E.L.K.E) of the National and Kapodistrian University of Athens.

**Note:** This paper is dedicated to the memory of Professor Demetrios Papahadjopoulos.

## Introduction

Liposomes are effective and non-toxic drug carriers composed mainly of phospholipids, which form bilayers after hydration. Some liposomal drugs, such as Caelyx/Doxil, the liposomal formulation of doxorubicin, are currently in clinical use (Woodle 1995; Allen & Cullis 2004). The cytotoxicity and specificity of drugs to target cells have been shown to be improved via the active targeting process (Iden & Allen 2001; Allen & Cullis 2004) after their encapsulation in immunoliposomes compared with conventional non-targeting liposomes. Immunoliposomes using an antibody targeting ligand have attracted much attention in recent years and have been proven as efficient carriers of anti-cancer drugs to the target tissues, dramatically reducing their toxicity, while improving therapeutic efficiency. Today the preparation of liposomal drugs with high specificity to target cells is an attractive scientific field, and efforts have been focused on the preparation of ligand-targeted liposomes (LTL) and immunoliposomes, which meet the established criteria for ideal drug carriers (Martin & Papahadjopoulos 1982; Papahadjopoulos et al 1991; Kirpotin et al 1997).

To prepare immunoliposomes, an antibody fragment must be chemically coupled to suitable groups at the liposome. Maleimide (Mal)-terminated lipids are thiol-reactive conjugates; they have been used widely as the substrates for the chemical coupling between an antibody peptide fragment and the surface of liposomes via a Michael reaction. Then the drug should be encapsulated within this LTL to produce the immunoliposomal drug. Various techniques have been described in the literature for preparing LTLs (Hansen et al 1995) and the requirements for introducing them to clinical trials should include biocompatibility, stability and selective delivery of drugs (Kirpotin et al 1997; Iden & Allen 2001; Allen & Cullis 2004).

For many thiol-reactive liposomes the Mal-terminated lipid is Mal–PEG (Papahadjopoulos et al 1991; Kirpotin et al 1997; Sapra & Allen 2003), an expensive commercial product, while various conjugates of Mal–lipids have been reported (Boeckler et al 1998). Thus, to develop general purpose liposomes, the simplicity, stability and low cost of the liposome formulation substrate, before the insertion of the desired drug and its linkage with the antibody, must be successfully realized. The preparation process must be simple, fast and reproducible; furthermore, for introduction to clinical trials the LTL must selectively deliver the drug to the target cell and should be biocompatible and non-toxic to normal cells (Sapra & Allen 2003; Allen & Cullis 2004).

The concept of this study was to synthesize simple thiol-reactive conjugates from Mal and lipoamines (stearylamine (SA) or oleylamine (OA)) and to develop a simple, fast and low cost method for the development of lyophilized general-purpose thiol-reactive liposomes. The overall procedure that we followed was simple and well documented from the literature in the case of the synthesis of Mal–lipoamines and for the preparation of liposomes. Furthermore, we applied, for the first time, an HPTLC/FID (high-performance thin-layer chromatography coupled to a flame ionization detector) methodology for the determination of all liposomal components in a one-step analysis. This procedure can simplify the manufacturing and offer an efficient methodology for preparing LTLs.

## Materials and Methods

### Materials

Stearylamine (SA), oleylamine (OA), *N*-succinimidyl-3-maleimidopropionate (NSMP) and sucrose were purchased from Sigma (St Louis, MO). Egg phosphatidylcholine (EggPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Lipoid (Ludwigshafen, Germany) and from Avanti Polar Lipids Inc. (Alabaster, AL), respectively; the organic solvents used were of spectroscopic grade. Sephadex G-75 was purchased from Fluka Biochemica.

### Synthesis of Mal–lipoamine conjugates 3 and 4

To a solution of SA (**1**, 231.8 mg, 0.86 mmol) or OA (**2**, 230 mg, 0.86 mmol) in chloroform (3 mL), a solution of NSMP (266.2 mg, 1 mmol) in chloroform (3 mL) was added. After stirring for 24 h at room temperature, the chloroform solution was washed with water and brine and dried ($Na_2SO_4$). After solvent evaporation, conjugate **3** or **4** was obtained. The full $^1H$ and $^{13}C$ spectra assignment of compounds **3** and **4** was accomplished using the DEPT, COSY and HMQC NMR experiments on Bruker 400 and 200 MHz spectrometers (detailed data on NMR will be supplied by the authors upon request). After some experimentation, an optimum mixing time of 200 ms was used for the 2D NOESY spectrum; the relaxation delay used was 1.5 s.

### Molecular calculations

Molecular mechanics calculations were performed using the MM+ force field provided by the software Hyperchem on a Pentium IV platform. An initial structure was constructed and minimized using conjugate gradient and Newton-Raphson algorithms and an energy gradient tolerance of 0.01 kcal mol$^{-1}$ Å$^{-1}$. This structure was then manipulated using Hyperchem software modules to produce the target structures (Spellmeyer et al 1997).

### Liposome preparation and physicochemical characterization

The lipid film was prepared by dissolving EggPC (50 $\mu$mol) and DPPG (0.55 $\mu$mol) in a chloroform solution of **3** or **4** (1.0 $\mu$mol). The solvent was slowly evaporated in a flash evaporator (Buchi Waterbath B-480) and the film was dried under vacuum for at least 12 h. Multilamellar vesicles (MLVs) were prepared by hydrating the lipid film with HPLC-grade water in a water bath above the gel to liquid phase transition of the lipid mixture (41°C) and stirring for 1 h. The MLV suspension was subjected to 10 freeze–thaw cycles (i.e. successive immersion of the samples in a dry ice/*n*-butanol bath and then in a 40°C water bath). Large unilamellar vesicles (LUVs, size > 100 nm) or Small unilamellar vesicles (SUVs, size < 100 nm) were prepared from the resultant liposomal suspension, which was subjected to sonication for two 5-min periods interrupted by a 5-min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0,7 – UP 200S; Dr Hielsher GmbH, Berlin, Germany). The resultant vesicles were left to stand for 30 min to anneal any structural defects. The unentrapped lipoamine conjugate **3** or **4** was removed at room temperature by gel filtration chromatography through a saturated Sephadex (G-75) column equilibrated with HPLC-grade water (pH 5.6). Then the liposomal suspension was frozen ($CO_2$ + propanol) and freeze-dried in volumes of 300 $\mu$L for 24 h. To prepare liposomes that can be reconstituted after lyophilization, LUVs, including the conjugate **3** or **4**, were prepared by adding 150 mM sucrose, instead of water, to the lipid film. The sucrose-to-lipid weight ratio was 4.5:1 for **3** and **4**. The physicochemical properties (i.e. size and $\zeta$-potential) of the liposomes were studied using Zetasizer 3000 $HS_A$ (Malvern Instruments, Malvern, UK) and the physical stability (t = 4°C, HPLC-grade water pH 5.6) was monitored during a period of 35 days.

### Determination of phospholipids and Mal conjugates by HPTLC/FID

In this work high-performance thin-layer chromatography coupled to a flame ionization detector (HPTLC/FID) has been applied to analyse the liposomal formulation. The overall preparative procedure can be tracked to identify possible losses of raw material and errors in the liposomes' preparation, resulting in the amelioration of the method (Hatziantoniou & Demetzos 2003; Goniotaki et al 2004). HPTLC/FID was performed on an Iatroscan MK-5$^{new}$ instrument (Iatron Lab. Inc., Tokyo, Japan) with the following chromatographic conditions: hydrogen flow rate,

160 mL min$^{-1}$; airflow rate, 1900 mL min$^{-1}$; scan speed, 30 s/scan; Chromorods–SII (Iatron Lab. Inc) in set of 10 rods were used as a stationary phase; two mobile phases were used to separate the liposomal components – the phospholipids were separated using $CHCl_3$–$CH_3OH$–$H_2O$ (45:25:5 v/v) and subsequently the liposomal components **3** or **4** were separated using $CHCl_3$–$Et_2O$ (80:20 v/v) and $Et_2O$ 100%, respectively (De Schrijver et al 1991). The quantification of the constituents of the liposomal preparations was achieved by using calibration curves of the components.

### Statistical analysis

Statistical analysis of the effect of the liposome type (Table 1) on the size, PI (polydispersity index) and ζ-potential was performed using one-way analysis of variance followed by a post-hoc Tukey's HSD test (SPSS for Windows release 11). All the results were from four ($n = 4$) independent experiments. The differences in the two liposomal formulations (Table 2) regarding incorporation efficiency were also analysed using unpaired two-tailed *t*-test (SPSS for Windows release 11). All the results were from six ($n = 6$) independent experiments.

## Results and Discussion

### Synthesis of Mal–lipoamine conjugates 3 and 4 and conformational properties

The conjugates Mal–SA (**3**) and Mal–OA (**4**) were synthesized using the active ester methodology, by the reaction of SA (**1**) or OA (**2**) with NSMP. The 2D NOESY spectra gave information on the conformational properties of the two Mal–lipoamine conjugates **3** and **4**. For the unsaturated molecule **4**, a strong dipolar correlation between $(CH_2)X$–$CH_2N(CO)_2$ was observed, indicating that a clustering between the lipophilic chain and the polar head is favoured. This is in agreement with molecular mechanics calculations showing that a bending conformation is stabilized for **4**.

However, the absence of similar nOe correlations for conjugate **3** suggests that this molecule adopts an *all-trans* or a loose conformation of the lipophilic chain. In contrast, in molecule **4** the *cis*-double bond acts as a constraint, allowing the molecule to bend its lipophilic part (Figure 1) (Mason et al 1991).

### Characterization of the new liposome formulations incorporating maleimide–lipoamine conjugates 3 and 4

The application of a liposome substrate in biological tests, after drug encapsulation and antibody linkage, is the main matter of concern in the current literature (Sapra & Allen 2004). Nevertheless, when developing a new liposome formulation substrate it is important to measure the physicochemical properties of the new vesicles and to determine qualitatively and quantitatively all the constituents, using a simple and fast analytical method. Both the above properties are important, especially from a manufacturing point of view.

SUVs composed of EggPC–DPPG (9:0.1 mol/mol) were prepared (Gabizon & Papahadjopoulos 1998). The

**Table 1** Size (nm) and ζ-potential (mV) values for the liposomal formulations (25°C) on the day of preparation and after reconstitution

| Liposome composition (mol/mol) | EggPC–DPPG (9:0.1) | 5 (EggPC–DPPG–3 9:0.1:0.18) | 6 (EggPC–DPPG–4 9:0.1:0.18) | 5–sucrose[c] (9:0.1:0.18) | 6–sucrose (9: 0.1:0.18) |
|---|---|---|---|---|---|
| Size[a] (nm) | 49.0 ± 4.7 | 296.7 ± 3.2 | 256.0 ± 4.1 | 123.8 ± 2.1 (114.1 ± 1.8)[d] | 88.1 ± 1.2 (84.4 ± 1.8)[d] |
| PI[b] | 0.50 ± 0.02 | 0.51 ± 0.03 | 0.62 ± 0.02 | 0.53 ± 0.01 (0.62 ± 0.03)[d] | 0.51 ± 0.02 (0.54 ± 0.02)[d] |
| ζ-Potential (mV) | −16.9 ± 7.4 | −39.7 ± 4.5 | −47.1 ± 2.5 | −23.3 ± 1.7 (−32.7 ± 2.4)[d] | −45.5 ± 1.4 (−41.9 ± 5.2)[d] |

[a]z-average mean diameter; [b]polydispersity index; [c]sucrose was added as lyoprotectant (= protection against damage by dehydration); [d]values for reconstituted liposomal formulations.

**Table 2** One-step HPTLC/FID analysis of liposome constituents of the new thiol-reactive liposomes **5** and **6**

| Liposome composition | Incorporation efficiency (%) | Initial molar ratio of conjugate to phospholipids | Experimental molar ratio of conjugate to phospholipids | Phospholipid recovery (%) | Calculated no. of molecules of 3 or 4 incorporated per liposome (outer surface) |
|---|---|---|---|---|---|
| EggPC–DPPG–**3** (9:0.1:0.18) | 11.8 ± 0.8 | 1:50.6 | 1:416.8 (2.4 $10^{-3}$) | 97.4 ± 2.5 | 1124 |
| EggPC–DPPG–**4** (9:0.1:0.18) | 28.2 ± 1.3 | 1:50.6 | 1:116 (8.6 $10^{-3}$) | 92.4 ± 3.2 | 2825 |

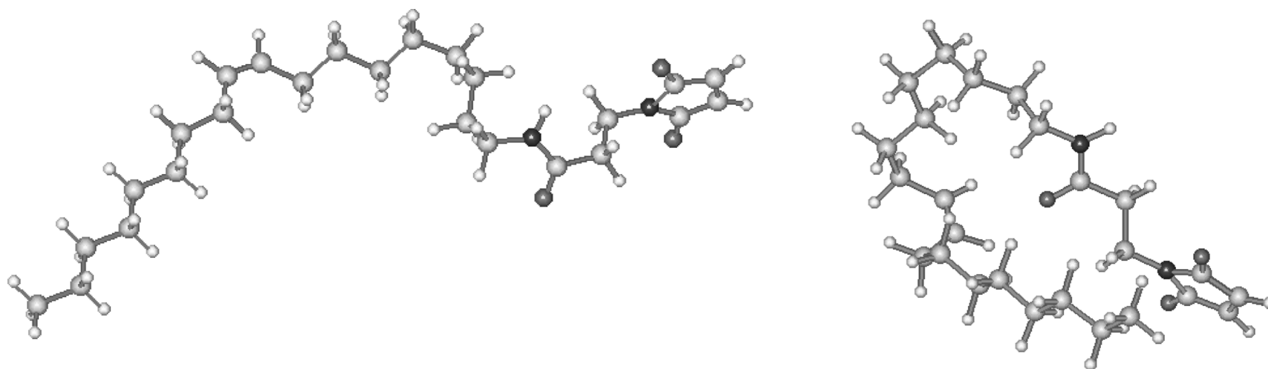

**Figure 1** Conformational snapshots for Mal–OA (**4**) consistent with nOe data. A bending conformer (right hand part) can be populated because of the bending induced by the *cis* double bond.

physical stability of the liposomes was studied through monitoring their size and ζ-potential. These liposomes were found to retain their stability with particle sizes of $49.0 \pm 4.7$ nm, while the ζ-potential remained stable ($-16.9 \pm 7.4$) over a 35-day period. The incorporation of **3** and **4** led to statistically significant differences ($P < 0.05$) between EggPC–DPPG (9:0.1 mol/mol) and liposomal formulation **5** and **6** regarding size and ζ-potential, while PI values were found to be statistically significant only in the case of **6**. Comparison of the physicochemical properties of the resulting liposome formulations **5** and **6** led to some interesting observations. Particle size was increased by 40.7 nm ($P < 0.05$), while ζ-potential and PI were decreased by 7.4 mV (differences were not statistically significant) and 0.09 ($P < 0.05$) for liposomes **5** compared with liposomes **6** (Table 1).

A stock of freeze-dried liposomes (**5** and **6**; Table 1, last two columns) incorporating the conjugates Mal–SA (**3**) and Mal–OA (**4**) was prepared. The freeze-dried liposomes **5** and **6** were reconstituted to the original volume by adding HPLC-grade water. The physicochemical properties of the reconstituted liposomes were statistically significant ($P < 0.05$) for PI and ζ-potential only for the liposomal formulation **5**/sucrose (Table 1).

It is known that delivery systems such as LTLs and immunoliposomes can deliver drug molecules to the target tissues using only a few dozen active macromolecules or antibody molecules on the liposome surface, respectively (Park et al 1997). The current practice of liposomal analysis focuses on the determination of only the main liposomal constituents: the active component (encapsulated drug) using HPLC and GC and the total phospholipid fraction through measuring the inorganic phosphorus content. These methods are time consuming and often lack accuracy (Ratz et al 2001). In addition, apart from the main components, a liposome may contain additives that regulate its physicochemical characteristics, such as cholesterol or lipids that are used for providing charge on the surface of liposomes, and regulate their stability (i.e. DPPG).

The coupling efficiency of the new thiol-reactive liposomes **5** and **6** to antibody fragments depends on the number of Mal groups on the vesicle surface that can bind peptide-SH targeting moieties. HPTLC/FID analysis of all liposomal components showed that the incorporation efficiency of the Mal–SA or Mal–OA conjugate **3** or **4** into lipid bilayers was different ($P < 0.05$), while the difference in recovery of phospholipids was not statistically significant (Table 2). According to Table 2, for encapsulating 1 mol of **3** or **4** into the liposomal formulation used (EggPC–DPPG 9:0.1), 416.8 or 116 moles of lipids were needed respectively.

Assuming that liposomes are spherical with a bilayer thickness of 4 nm and a surface area per phospholipid molecule of 0.6 $nm^2$, a theoretical calculation of the number of linker molecules per liposome surface was undertaken (Vance & Vance 1996). The number of conjugates on the outer surface of liposomes was found to be 1124 and 2825 for liposome formulation **5** (EggPC–DPPG–**3** 9:0.1:0.18) and **6** (EggPC–DPPG–**4** 9:0.1:0.18), respectively (Table 2).

It seems that the different C18 lipophilic chain structure of the two Mal–lipoamine conjugates **3** and **4** affect the phospholipid packing, resulting in different incorporation efficiency into lipid bilayers and different size between the two liposomal formulations **5** and **6**. According to our results, the number of Mal–SA **3** molecules incorporated per liposome **5** is ~ 2.5-fold smaller and the size of the corresponding liposome is ~ 1.3-fold bigger compared with liposomes **6**, including Mal–OA **4**.

These observations suggest a better packing of Mal–OA **4** with EggPC–DPPG lipids, in relation to Mal–SA **3**, which can be explained in terms of: firstly, the structural similarity between the alkyl chains of Mal–OA **4** and EggPC lipids, bearing in mind that EggPC consists mainly of unsaturated phospholipids; and, secondly, the different conformational properties of the two conjugates **3** and **4** in that Mal–OA **4** adopts a more compact conformation, while Mal–SA **3** adopts a loose conformation, which can no longer be packed well between EggPC–DPPG lipid chains, resulting in bigger liposomes **5** ($P < 0.05$) and more effective fluidization of the lipid bilayer of liposomes. Thus, the number of Mal residues anchored in the liposome surface that can react with ligands is smaller for liposomes **5**, while their surface is bigger.

Future work will involve investigation of the effect of the Mal–lipoamine conjugate structure on liposome physicochemical properties using new synthetic conjugates and the pharmacokinetic properties of immunoliposomes bearing encapsulated drugs.

### Conclusion

In this work we have prepared low-cost general-purpose lyophilized thiol-reactive liposomes. The new liposomes **5** and **6** were composed from EggPC–DPPG bilayers and the synthetic Mal–SA or Mal–OA conjugates **3** or **4**, respectively. Their constituents were analysed in a one-step method using HPTLC/FID, a general, simple, fast and accurate analytical method. We focused on the physicochemical characterization and correlation of properties with structure, rather than the biological applications of the new liposomes **5** and **6**, which are substrates for immunoliposomes; liposomes **5** and **6** have an active surface composed by Michael acceptor Mal groups anchored in their surface, which can bind various ligands to produce LTLs, like immunoliposomes. The small difference in structure between the two thiol-reactive conjugates **3** and **4**, representing only 2% of the total lipids, which is the C18 alkyl chain double bond, caused a considerable difference in phospholipid packing of the resulting liposome vesicles **5** and **6**; the conformational bending of conjugate **4** may contribute to the final architecture. Thus, the number of Mal–SA **3** molecules anchored in the surface of liposome **5** is $\sim$ 2.5-fold smaller and the size of the corresponding liposome is $\sim$ 1.3-fold bigger with regards to liposomes **6**, which include Mal–OA **4**. The results obtained from this study could be useful as a tool for drawing liposomal formulation and may have future applications for incorporation and delivery of biologically active molecules.

## References

Allen, T. M., Cullis, P. R. (2004) Drug delivery systems: entering the mainstream. *Science* **303**: 1818–1822

Boeckler, C., Frisch, B., Schuber, F. (1998) Design and synthesis of thiol reactive lipopeptides. *Bioorg. Med. Chem. Lett.* **8**: 2055–2058

De Schrijver, R., Vermeulen, D. (1991) Separation and quantification of phospholipids in animal tissues Iatroscan TLC/FID. *Lipids* **26**: 74–76

Gabizon, A., Papahadjopoulos, D. (1998) Liposomes formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl Acad. Sci. USA* **85**: 6949–6953

Goniotaki, M., Hatziantoniou, S., Dimas, K., Wagner, M., Demetzos, C. (2004) Encapsulation of naturally occurring flavonoids into liposomes: physicochemical properties and biological activity against human cancer cell lines. *J. Pharm. Pharmacol.* **56**: 1217–1224

Hansen, B. G., Kao, G. Y., Moase, E. H., Zalipsky, S., Allen, T. M. (1995) Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta* **1239**: 133–144

Hatziantoniou, S., Demetzos, C. (2003) Abstracts of Papers, Sixth International Conference on Liposome Advances. London, December 15–19

Iden, D. L., Allen, T. M. (2001) In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim. Biophys. Acta* **1513**: 207–216

Kirpotin, D., Park, J. W., Hong, K., Zalipsky, S., Li, W.-L., Carter, P., Benz. C. C., Papahadjopoulos, D. (1997) Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36**: 66–75

Martin, F. J., Papahadjopoulos, D. (1982) Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J. Biol. Chem.* **257**: 286–288

Mason, R. P., Rhodes, D. G., Herbette, L. (1991) Reevaluating equilibrium and kinetic binding parameters for the lipophilic drugs based on structural model for drug interaction with biological membranes. *J. Med. Chem.* **34**: 869–877

Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K., Woodle, M. C., Lasic, D. D., Redemann, C., Martin, F. J. (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl Acad. Sci. USA* **88**: 11460–11464

Park, J. W., Hong, K., Kiprotin, D. B., Meyer, O., Papahadjopoulos, D., Benz, C. C. (1997) Anti-HER2 immunoliposomes for targeted therapy of human tumors. *Cancer Lett.* **118**: 153–160

Ratz, H., Schnell, H., Rischer, M. (2001) Separation and quantitation of alkylphosphocholines and analogues of different liposome formulations by HPLC. *J. AOAC Int.* **84**: 1277–1282

Sapra, P., Allen, T. M. (2003) Ligand-targeted liposomal anticancer drugs. *Prog. Lipid Res.* **42**: 439–462

Sapra, P., Allen, T. M. (2004) Improved outcome when B-cells lymphoma is treated with combinations of immunoliposomal anticancer drugs targeted to both CD19 and CD20 epitopes. *Clin. Cancer Res.* **10**: 2530–2537

Spellmeyer, D. C., Wong, A. K., Bower, M. J., Blaney, J. M. (1997) Conformational analysis using distance geometry methods. *J. Mol. Graph. Model.* **15**: 18–36

Vance, D. E., Vance, J. E. (eds) (1996) *Biochemistry of lipids, lipoproteins and membranes*. Elsevier, Amsterdam

Woodle, M. C. (1995) Sterically stabilized liposomes therapeutics. *Adv. Drug. Del. Rev.* **16**: 249–265