ELSEVIER

International Journal of Pharmaceutics 232 (2002) 157–162

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Effect of dendrimer on entrapment and release of bioactive from liposomes

Ajay J. Khopade [b], Frank Caruso [a], Pushpendra Tripathi [a], Surekha Nagaich [a], Narendra K. Jain [a,*]

[a] *Dr Harisingh Gour Vishwavidyalaya, Sagar-470 003, (M.P.), India*
[b] *Max-Planck Institute of Colloids and Interfaces, D-14424 Potsdam, Germany*

Received 15 March 2001; received in revised form 15 August 2001; accepted 5 October 2001

Abstract

An active encapsulation method to obtain high entrapment in liposomes is described. The method harnesses the ability of dendrimer to interact with oppositely charged phospholipid and solubilize acidic drugs in their interior. The high drug entrapment in liposomes is due to the enhanced entrapment of dendrimer, which creates sink in the liposomal aqueous compartment where the methotrexate (MTX) molecules are fluxed in. The encapsulation increases with dendrimer generation. The release of bioactive was also decreased by this method. The method may be useful to entrap drugs with relatively high therapeutic dose.

Keywords: Liposomes; Dendrimers; Transmembrane gradient; High-entrapment; Methotrexate

Liposomes, the most extensively studied systems for drug delivery, have been commercialized as the formulations of doxorubicin, amphotericin B and cytarabine are in market now and many are in clinical phases (Forssen and Willis, 1998). While stability remains the major issue in its therapeutically successful applicability, the achievement of desired encapsulation in small unilamellar vesicles is yet another constraint. Various tricks can improve encapsulation of hydrophilic molecules like filling the molecules into liposomes by some interior force such as transmembrane pH gradient and chemical potential gradient (Lasic, 1996). Membrane permeable drugs however, quickly leak out of the liposomes after dilution or application therefore, internal immobilization like, complexation; gelation; precipitation; membrane binding is beneficial (Zhu et al., 1996). The use of dendrimers to increase entrapment of drug in the liposomes and modulate release seems promising.

Polyamidoamine (PAMAM) dendrimers consist of central core, the radially extending intermediate repeating units, and the terminal functional groups. Core and the repeating units determine the microenvironment in the interior and the terminal functional groups determine their solubility, physical and chemical interaction in the immediate surrounding environment (Zeng and Zimmer-

* Corrosponding author. Tel./fax: +91-7582-23712.
E-mail address: jnarendr@bom8.vsnl.net.in (N.K. Jain).

0378-5173/02/$ - see front matter
PII: S0378-5173(01)00901-2

man, 1997). The host–guest properties of dendrimers based on physical entrapment, hydrophobic interactions and ionic interactions are thoroughly explored (Bosman et al., 1999). PAMAM dendrimers act as solubility enhancers for acidic drugs through ionic interactions (Milhem et al., 2000). Investigations have shown that the amine terminated PAMAM dendrimers interact with negatively charged surfactants including phospholipids forming an envelope around the dendrimer molecules (Malik, 1998). Dendrimer and phospholipid composites, dendrosomes, are reported for effective gene delivery (Sarbolouki et al., 2000). Purohit et al. (2001) recently studied the interaction of amphipathic cationic partial dendrimers with charged and neutral liposomes.

In this communication, we are reporting the use of amine terminated PAMAM dendrimers for increasing encapsulation of a model acidic drug, methotrexate (MTX), in liposomes. MTX is an anticancer drug usually encapsulated in liposomes by passive method in a dissolved form (Lasic, 1996). The encapsulation of MTX was increased by the use of positively charged lipid stearylamine in liposomes exploiting ion-pair interaction (Kim et al., 1994), lipid-conjugation (Williams et al., 1996) and proliposome preparation (Park et al., 1994). The encapsulation efficiency and loading in these methods is not satisfactory to deliver drugs with relatively high therapeutic dose. PAMAM dendrimers are few nanometers in size in diameter, almost equivalent to the thickness of aqueous space between two liposomal bilayers, and therefore may be encapsulated in aqueous space of liposomes (Fig. 1). Their high positive charge is expected to interact with oppositely charged lipid in liposomes. Hence, an attempt was made to study the effect of dendrimer on liposome formation, encapsulation and release of acidic drug.

Liposomal formulations, different in lipid composition, aqueous phase composition or quantity of MTX in feed, were prepared by film hydration method encoded and summarized in Table 1. The lipids (1.8 mmoles) consist of purified hydrogenated soyphosphatidylcholine (Lipoid GmbH, Germany), cholesterol and dicetylphosphate (Sigma, USA) in 1.5:1:1 molar ratio, with or without (0.011 mmoles) PAMAM dendrimer (Aldrich, UK) was dissolved in chloroform in a round-bottomed flask. The solvent was evaporated to form a film that was hydrated (primary hydration) with 2 ml aqueous phase as shown in Table 1 at 60 °C. The liposomes were sonicated at 60 W for 2 min with a probe sonicator (Imeco sonifiers, India) and filtered through 0.22 μm membrane filter (Millipore, USA). MTX solution (4 mg ml^{-1} as sodium salt) was then added to the dispersion (secondary hydration) and allowed to stand in a 60° bath for 6 h with mild stirring with pH adjustment to 7.4 with 0.1 N oxalic acid solution. The non-encapsulated drug was removed by dialysis against water (pH adjusted to 7.4) for 24 h at 4 °C with three intermittent exchanges. To the liposomal dispersion a drop of 1% w/v of triton X-100 (Sigma, USA) solution was added and suitably diluted with water. The encapsulated drug was analyzed spectrophotometrically (British Pharmacopoeia, 1993). The size of liposomes was determined by dynamic light scattering (Horiba, Japan) and observed under transmission electron microscope (TEM; Philips CMl2). The shift in absorption maximum of copper sulfate was noted for detecting the presence of dendrimer in external phase in blank experiments (Diallo et al., 1999). The release of MTX was studied by placing formulations equivalent to 10 mg of drug in pre-treated dialysis tube (molecular weight cut off 12 000–14 000, Sigma). The tube was then kept in the basket of USP dissolution apparatus. The dissolution medium was 500 ml phosphate buffer of pH 7.4 maintained at 37 °C with constant stirring. The drug released after 8 h in dissolution medium was analyzed spectrophotometrically.

There was a difference in physical behavior of dendrimer formulations compared with tromethamine formulations during primary hydration step due to their ability to electrostatically interact with oppositely charged lipids (Paulo et al., 1998; Iolanda et al., 1997) and induce phase change. The light microscopic (Zeiss, Germany equipped with fluorescence lamp and cross polarizers) observations showed worm-like gel structures, which tend to elongate and break or fuse on application of shear on glass slide and were bire-

fringent under polarized light. The size of freshly filtered liposomes was 860 ± 25 nm but under TEM the size of the final liposome formulation ranged from less than 100 nm to few μm in size due to aggregation and swelling of liposomes (unpublished observations).

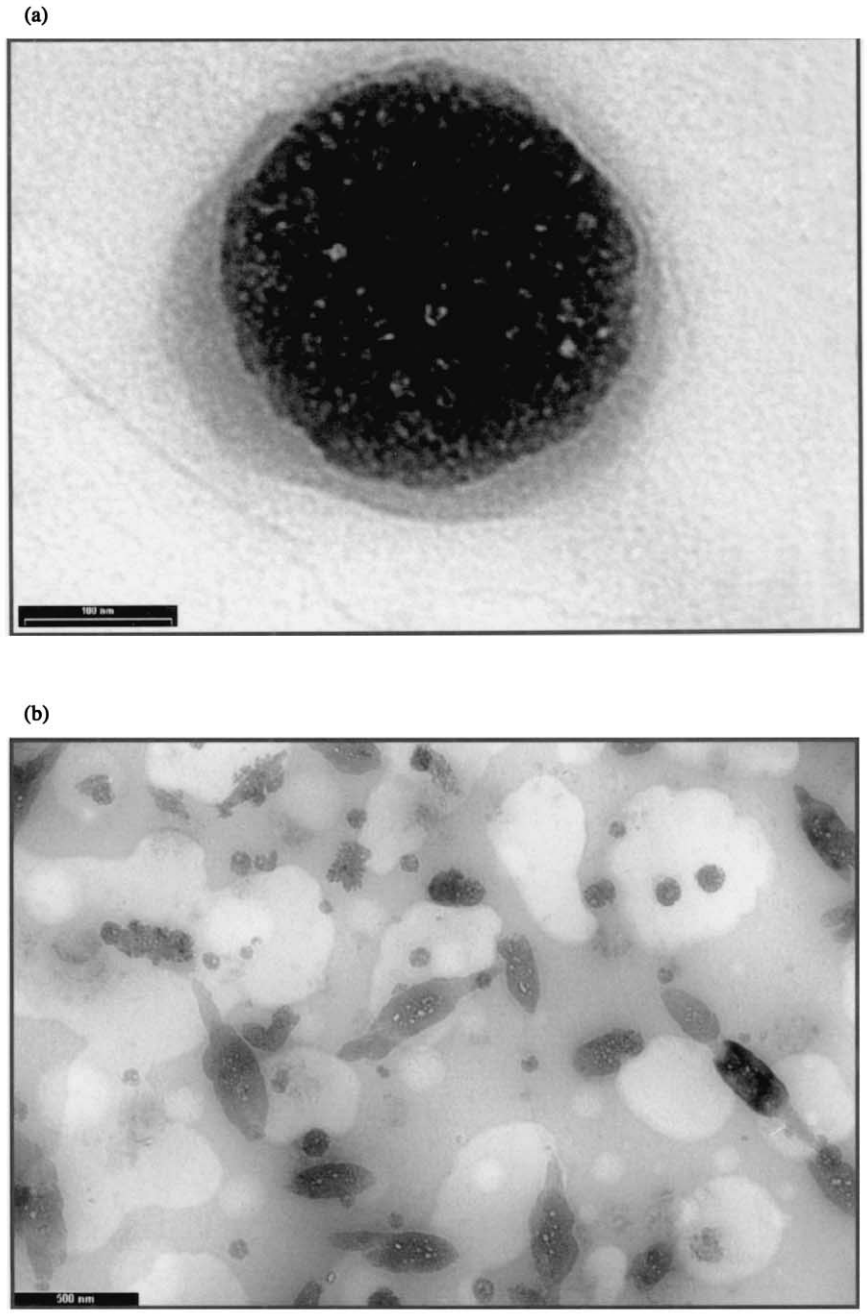

Fig. 1. (a) TEM image of dendrimer containing liposomal formulation. (b) A single lipid particle showing encapsulated dendrimer grains.

Table 1
Codes for liposomal formulations differing in lipid composition and aqueous phase composition used for hydration and their physicochemical characteristics

Formulation code	Lipid film composition	Aqueous phase		Drug loading (mole percent of lipid)	Drug release in 8 h (%)
		Primary hydration	Secondary hydration		
DL-1	HSPC+CH	DCP+4GD	MTX solution	7.21 ± 0.28	–
DL-2	HSPC+CH +DCP+4GD	Distilled water	MTX solution	7.11 ± 0.53	24.5 ± 1.2
DL-3	HSPC+CH +4GD	Distilled water	MTX solution	5.58 ± 0.43	25.7 ± 3.3
3GD-DL-2	HSPC+CH +DCP+3GD	Distilled water	MTX solution	3.92 ± 0.26	34.9 ± 2.8
2GD-DL-F	HSPC+CH +DCP+2GD	Distilled water	MTX solution	2.05 ± 0.70	46.2 ± 3.2
TL-1	HSPC+CH +DCP	Tromethamine solution	MTX solution	1.14 ± 0.07	67.8 ± 2.6
TL-2	HSPC+CH	Tromethamine solution	MTX solution	0.99 ± 0.21	65.4 ± 3.5

HSPC, hydrogenated soy phosphatidylcholine; CH, cholesterol; DCP, dicetylphosphate; MTX, methotrexate; 4GD, fourth generation dendrimer; 3GD, third generation dendrimer; 2GD, second generation dendrimer.

The encapsulation of MTX was increased in the presence of dendrimer (Table 2). The basic interior of the liposome due to dendrimer creates pH gradient that may be the reason of increased influx of MTX. However, the encapsulation was significantly greater than batch where the interior of liposome was made basic with tromethamine (0.15 mmoles) solution (TL-1) equivalent to that of dendrimer pH. This indicates that the increase in encapsulation may be due to interactions between amino groups in the core of dendrimers and MTX in the same way as reported by Kojiyama et al. (2000) in polyethylene glycol grafted PAMAM dendrimer. The encapsulation was less as compared with that report, perhaps due to the low partitioning of MTX across lipid layer or incomplete encapsulation of dendrimer. No shift in peak of $CuSO_4$ solution was however observed by addition of dialysate from blank DL-1 and DL-2 batches indicating strong dendrimer–liposome association.

The negatively charged lipid in liposomes (DL-2) increased drug encapsulation, because of effective electrostatic entrapment of dendrimers. The improved drug encapsulation in neutral liposomes (DL-3) is due to pH dependent interaction of phosphate groups with dendrimer leading to their entrapment. The encapsulated dendrimer seems to act as sink, where the MTX molecules are fluxed in. The drug encapsulation in DL-3 was however, less than DL-1 and DL-2. The drug encapsulation for negatively charged liposomes (TL-1) and neutral liposomes (TL-2) in case of tromethamine formulations was statistically insignificant. The release was lowered from approximately 65–25% for dendrimeric liposomal formulations in 8 h (Table 1). The charge of liposomal lipid was not found to affect the release of drug. The release of dendrimer decreased with increasing dendrimer generation due to their open structure. The study shows that it is possible to modulate the release of drug from the dendrimeric liposomal formulations.

Table 2
Effect of MTX concentration in hydration solution and type of dendrimer in lipid phase on encapsulation efficiency of liposomes

MTX concentration (mg ml^{-1})	Loading (mole percent lipid)		
	Fourth generation	Third generation	Second generation
1	2.10	2.18	1.83
2	4.10	3.86	2.24
3	5.82	4.21	–
4	7.11	3.92	2.05
5	7.21	–	–

The entrapment of drug was proportional to the generation (G) of dendrimer (Table 2). The liposomes containing 4 G dendrimer were able to encapsulate drug approximately two and four times greater than 3 and 2 G dendrimer containing liposomes, respectively, which is due to less solubility of MTX in 3 and 2 G dendrimer. Another possibility may be the proportion of these dendrimers attached with the surface phosphate groups was unable to encapsulate drug due to their open structure. The loading values for dendrimer containing liposomes include the proportion of drug that remained associated with surface attached dendrimer. The loading increased and reached a constant maximum value as the amount of MTX supplied in the secondary hydration phase was increased from 1, 2, 3, 4 and 5 mg ml^{-1} (Table 2). This shows that the influx is controlled by the presence of dendrimer in liposomes, because, the encapsulation is greater at lower MTX supplements compared with TL-1.

In conclusion, we have reported use of dendrimer to entrap higher quantities of acidic drugs in liposomes. This is due to the entrapment of dendrimer by charge interaction that creates pH and solubility gradient across the bilayer and lead to an influx of acidic molecules like MTX. Perhaps, due to same reason the release of drug back into the medium is also slowed down. The studies on the structure, physicochemical, pharmacokinetic and pharmacodynamic properties of these novel liposomal formulations shall be reported in future.

Acknowledgements

The authors are indebted to Sun Pharma Advanced research Center for providing drug sample and some of the facilities. Surekha Nagaich is thankful to University Grants Commission, New Delhi for the grant of fellowship. Dr A.J. Khopade is grateful to Alexander von Humboldt foundation, Germany for the award of post-doctoral fellowship.

References

Bosman, A.W., Janssen, H.M., Meijer, E.W., 1999. About dendrimers: structure, physical properties, and applications. Chem. Rev. 99, 1665–1688.

British Pharmacopoeia, 1993. Int. Ed., vol. 1, London, pp. 419.

Diallo, M.S., Balogh, L., Shafagati, A., Johnson, J.H. Jr, Goddard, W.A. III, Tomalia, D.A., 1999. Poly(amidoamine) dendrimers: a new class of high capacity chelating agents for Cu(II) ions. Environ. Sci. Technol. 33, 820–824.

Forssen, E., Willis, M., 1998. Ligand-targeted liposomes. Adv. Drug Deliv. Rev. 29, 249–271.

Iolanda, P., Rosa, G., Clara, G., Agustín, C., Concepción, A., 1997. Macromolecules in ordered media: 7. Influence of ionic strength and bilayer composition on the association of polyelectrolytes to mixed liposomes. Polymer 38, 5107–5113.

Kim, C.K., Lee, M.K., Han, J.H., Lee, B.J., 1994. Pharmacokinetics and tissue distribution of methotrexate after intravenous injection of differently charged liposome-entrapped methotrexate to rats. Int. J. Pharm. 108, 21–29.

Kojiyama, C., Kono, K., Maruyama, K., Takogishi, T., 2000. Synthesis of polyamidoamine dendrimer having poly(ethylene glycol) grafts and their ability to encapsulte anticancer drugs. Bioconj. Chem. 11, 910–917.

Lasic, D.D., 1996. Liposomes in drug delivery. In: Rosoff, M. (Ed.), Vesicles. Marcel Dekker, New York/Basel, pp. 452–453.

Malik, N., 1998. Internally supported lipid vesicle systems, patent WO9856353.

Milhem, O.M., Myles, C., McKeown, N.B., Attwood, D., D'Emanuele, A., 2000. Polyamidoamine Starburst® dendrimers as solubility enhancers. Int. J. Pharm. 197, 239–241.

Paulo, S.K., Yan, L., Marcia, C.B., 1998. Complex formation between poly electrolytes and ionic surfactants. Chem. Phys. Lett. 298, 51–56.

Park, J.M., Ann, B., Yoon, E.J., Lee, M.G., Shim, C.K., Kim, C.K., 1994. The pharmacokinetics of methotrexate after intravenous administration of methotrexateloaded proliposomes to rats. Biopharm. Drug Disp. 15, 391–407.

Purohit, G., Sakthivel, T., Florence, A.T., 2001. Interaction of cationic partial dendrimers with charged and neutral liposomes. Int. J. Pharm. 214, 71–76.

Sarbolouki, M.N., Sadeghizadeh, M., Yaghoobi, M.M., Karami, A., Lehrasbi, T., 2000. Dendrosomes: a novel family of vehicles for transfection and therapy. J. Chem. Tech. Biotech. 75, 919–922.

Williams, A.S., Camilleri, J.P., Goodfellow, R.M., Williams, B.D., 1996. A single intra-articular injection of liposomally conjugated methotrexate suppresses joint inflammation in rat antigen-induced arthritis. Br. J. Rheumatol. 35, 719–724.

Zeng, F., Zimmerman, S.C., 1997. Dendrimers in supramolecular chemistry: from molecular recognition to self-assembly. Chem. Rev. 97, 1681–1712.

Zhu, G., Oto, E., Vaage, J., Quinn, Y., Newman, M., Engbers, C., Uster, P., 1996. The effect of vincristine–polyanion complexes in stealth liposomes on pharmacokinetics, toxicity and anti tumor activity. Cancer Chemother. Pharmacol. 39, 138–142.