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LIPOSOMES WITH α-TOCOPHEROL MEMBRANE

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Liposomes, closed spherical structures formed by phospholipid bilayers, have been recognized as a controlled drug delivery system. We have studied the preparation and biocompatibility of liposomes based on soya phospholipids encapsulated with α-tocopherol. The binding efficiency of α-tocopherol was analysed by comparing two methods for liposome preparation: a) dry film (DFM) and b) solvent infusion method (SIM). The degree of encapsulation achieved (88-93%) suggested a high affinity of α-tocopherol for liposome membrane binding. The initial concentration of α-tocopherol had a significant effect on the degree of encapsulation, while the effect of method used was less pronounced. In general, a higher degree of encapsulation was achieved with smaller liposome size fractions. Based on an experimentally obtained size distribution function, it can be concluded that if smaller liposomes are used, SIM seems to be more efficient due to the higher content of smaller vesicles. The in vivo application of the liposomes to CBA mice confirmed the biocompatibility and nontoxicity of such preparations. The analysis of hematological parameters in peripheral blood (determination of mature blood cells with differential count) revealed that liposomes did not express cytotoxic effects on any of the parameters tested.

Key words: liposomes, α-tocopherol, encapsulation, blood cells

INTRODUCTION

Liposomes, vesicles formed by phospholipid bilayers dispersed in aqueous media, originally were introduced by Bangham in the early 1960s (Bangham et al., 1965) and since then are considered as an excellent model in basic research on the properties of biological membranes (Paphadjopoulos and Miller, 1967; Bangham et al, 1974; Michalet et al., 1994). These closed spherical structures that have encapsulated part of the liquid medium in their interior, are currently employed in many other applications, from biomedicine to cosmetics (Brooks

and McManus, 1990; Sato and Sunamoto, 1992; Gabizon, 1994; Gregoriadis, 1995; Lasić, 1994; Kedar et al, 1994.).

Due to their physico-chemical properties, which can be well controlled during preparation, as well as biocompatibility, biodegradability and nontoxicity, liposomes have raised considerable interest as carriers for diagnostic agents and pharmaceuticals, adjuvants in vaccination, transfection vectors in genetic engineering, etc. New possibilities and perspectives for liposome use are still arising, and many specific problems are yet unsolved. Currently, it is very attractive for researchers to develop specific liposome systems with a precisely defined size range, type and composition of lipid membrane.

In this work, we have studied liposomes based on soya phospholipids encapsulated with α-tocopherol, since it is well known that α-tocopherol could contribute to higher stability of liposomes. Liposomes were prepared by two methods: dry film evaporation (DFM) and solvent infusion (SIM), which produce different types of liposomes. Using dry film evaporation, multilamellar vesicles are obtained, while the solvent infusion method produces large unilamellar vesicles. In this paper, the effect of the method used on liposome size, and on degree of encapsulation was investigated. Liposome preparations consist of a heterogeneous dispersion of vesicles where the size distribution is dependent on the method used. We have also studied the size distribution of the prepared liposomes and the degree of encapsulation of the particular size fractions. The biocompatibility and toxicity of the liposomes were tested in CBA mice and hematological parameters in peripheral blood were analysed up to 72 h after *in vivo* administration of liposome suspensions.

MATERIALS AND METHODS

Deoiled soya lecithin for liposome production was obtained from INR Uljarica (Belgrade). Deoiled lecithin was enriched with phosphatidylcholine (PC) fraction according to the ethanol extration procedure and a 70% PC enriched fraction was used for liposomes preparations.

dl-α-Tocopherol oil of min. 97% purity was purchased from Fischer Chemicals AG (Zurich, Switzerland).

All other reagents were of p. a. grade and were used without further purification.

Dry film evaporation method (DFM): Liposomes were prepared initially by the technique of Bangham et al. (1965). The soya lecithin fraction with 70% of PC (0.5 g) was dissolved in chloroform (20 ml) and various amounts of α-tocopherol (0.03 g, 0.05 g, 0.15 g, 0.20 g and 0.25 g) were added to the lipid-solvent mixture. The lipid fraction was placed on the sides of a round-bottomed flask by removing the organic solvent in a rotary evaporator. The residual traces of solvent were removed under a stream of nitrogen. Dried lipid was hydrated with distilled water (10 ml) and the thin film was then removed from the surface of the flask by hand shaking followed by 10 min sonication in an ultrasonic bath (USH 28). Using this

method, multilamellar liposomes, detected by optical light microscopy (Olympus, Japan) were obtained.

Solvent infusion method (SIM): Using this method, liposomes were prepared according to the procedure described by Deamer and Bangham (1976). The soya lecithin fraction with 70% PC (0.5 g) was dissolved in petroleum ether (5 ml) and various amounts of α-tocopherol (0.03 g, 0.05 g, 0.15 g, 0.20 g and 0.25 g) were added. The mixture was injected into the flasks with an aqueous solution (10 ml) at a temperature below 60^{0}C. The rate of injection was 0.25 ml/min. After the end of the injection the flasks were gently hand shaken. Upon removing the petroleum ether by evaporation at 60^{0}C, large unilamellar liposomes were obtained.

Determination of the average diameter of liposomes: The average diameter of liposomes was measured according to the procedure described by Ohsawa et al. (1985a; 1985b). The method is based on the determination of the turbidity of the diluted liposome suspension (0.025%) by measuring the absorbance at 600 nm at 25^{0}C in a 1 cm^3 cuvette using a spectrophotometer (Specol, Carl Zeis, Jena).

Determination of the liposome size distribution: The size distribution of the obtained liposomes was determined by a sieve screening method. The liposome solution was passed through a series of Millipore filters (3.0 μm, 1.2 μm, 0.45 μm and 0.2 μm). As a result, five diameter fractions were obtained: I) D>3.0μm; II) 3.0μm>D>1.2μm; III) 1.2μm>D>0.45μm; IV) 0.45μm>D>0.2μm and V) D<0.2 μm. Quantitative distribution of the above fractions was determined by measuring the concentration of phosphorus. The method was based on the proportional relationship between the size of liposome fractions and the presence of phosphorus. A similar approach was adopted by Brendzel and Miller (1980a; 1980b). The concentration of phosphorus was determined spectrophotometrically using ammonium-molybdate as the colour developing agent.

Determination of the encapsulation percent: The percent of encapsulated α-tocopherol was calculated on the basis of α-tocopherol concentrations found in filtrates after sieve screening. α-Tocopherol concentration was determined according to the Emmerie-Engel analytical procedure. The extinction was measured at 520 nm on a spectrophotometer (Specol, Carl Zeis, Jena).

Assessment of hematological parameters: Normal male CBA mice, weighing 20-22 g, were injected intravenously with 0.2 ml of liposome suspensions prepared by both methods and extruded to uniform diameter by passage through 0.2 μm filters. Blood samples were collected 0,24, 48 and 72 h after treatment. Groups of 6 mice were used for each time point. In each animal the following parameters were estimated in peripheral blood: the total number of white blood cells (WBC), platelets (PLT) and erythrocytes (ER), as well as hematocrit. The differential counts of peripheral nucleated cells were made on 100 cells on blood smears stained by the May-Grunwald-Giemsa procedure, and the cells were divided into the following comparments: metamyelocytes (META), mature granulocytes (GRAN), monocytes (MONO) and lymphocytes (LYMPHO). The significance of differences between the groups was calculated by Student's t-test.

RESULTS

The effect of different initial α-tocopherol concentrations on the degree of encapsulation into the liposomes obtained using two different methods (DFM and SIM) are presented in Figure 1A. Initial α-tocopherol concentrations were varied

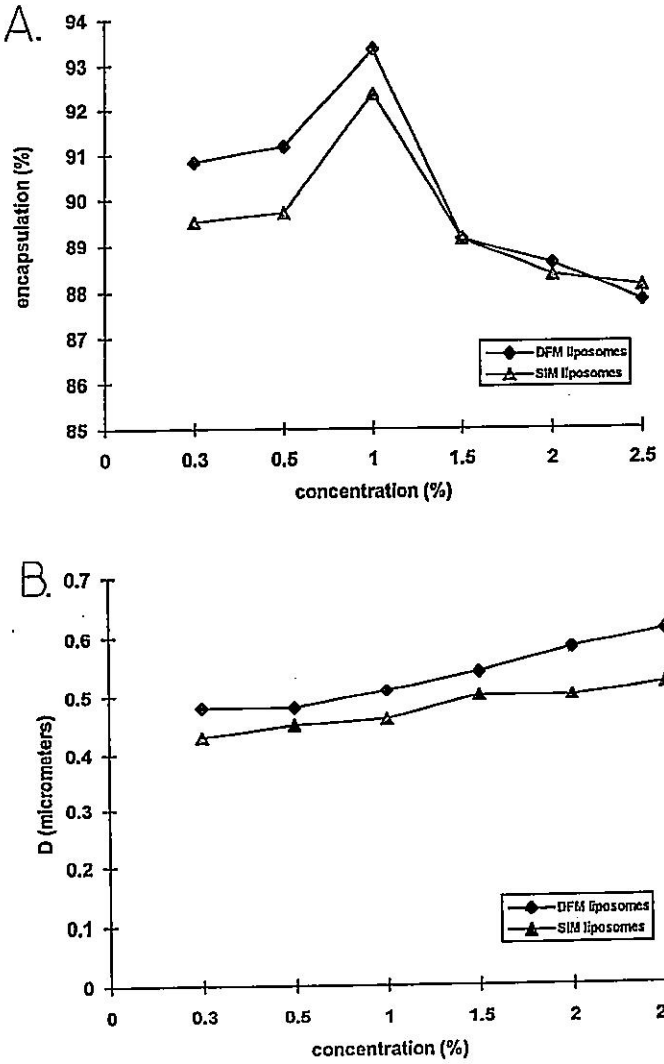

Figure 1. A. Effect of α-tocopherol concentration on its encapsulation in liposomes obtained by DFM and SIM;
B. Effect of α-tocopherol concentration on the average diameter of liposomes obtained by DEM and SIM.

from 0.3% to 2.5% and the percent of the achieved encapsulation for both methods was rather high and ranged from 88% to 93%. These results suggested a high binding affinity of α-tocopherol to liposomes. The highest percent of encapsulation in both methods was achieved with a very low initial concentration of α-tocopherol (1%). In general, as shown in Figure 1A, a slightly higher encapsulation efficiency was reached with liposomes prepared by DFM.

The effect of initial concentrations of α-tocopherol on the average diameter of liposomes obtained by DFM and SIM is shown in Figure 1B. By increasing the initial concentration of α-tocopherol, the average diameter of liposomes obtained by both methods increased. In general, the liposomes prepared by DFM had a higher average diameter. The average diameter of liposomes obtained by SIM ranged from 0.43 to 0.52 μm, while for liposomes obtained by DFM it ranged from 0.48 to 0.65 μm.

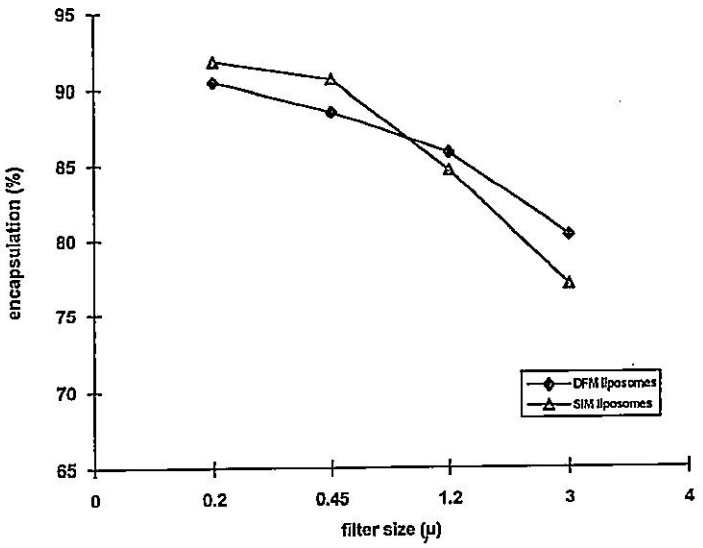

Figure 2. Effect of the diameter of α-tocopherol encapsulated liposomes (1% concentration of α-tocopherol) on the percent of encapsulation.

In Figure 2 the percentage of encapsulation of different liposome size fractions obtained by sieve screening is presented. It may be seen that the smaller size fractions of liposomes obtained by both methods showed a higher degree of encapsulation and over 90% of α-tocopherol was encapsulated in liposomes sieve screened through 0.2 μm pores.

The size distribution of liposomes obtained by DFM and SIM is presented in Figure 3.

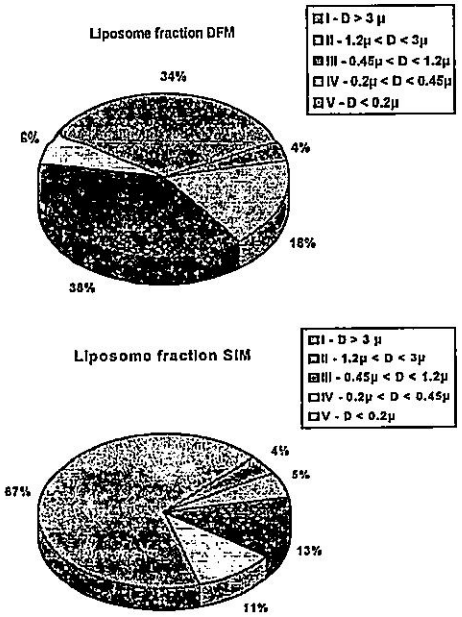

Figure 3. The size distribution of liposomes obtained by DFM and SIM.

Liposome size distribution showed some differences between the methods examined in our study. The dominant liposome fraction in SIM prepared liposomes was the smallest fraction V ($D<0.2\mu m$), that included 67% of the total liposomes. Participation of fractions with a higher diameter was in the range of 4% to 13%. DFM produced a significantly larger and non uniform size distribution of liposome fractions. By DFM, the dominant size fraction was fraction III ($0.45\ \mu m<D<1.2\mu m$), which included 38% of the total liposomes. The participation of larger and smaller fractions ranged from 4% to 34%.

Normal CBA mice were injected intravenously with 0.2 ml of liposome suspensions prepared by both methods. The analysis of hematological parameters in peripheral blood determined 24, 48 and 72 h following treatment with DFM-prepared liposomes is presented in Table 1. During the observed period liposome-treated animals exhibited no overall changes in the numbers of erythrocytes and platelets, as well as hematocrit (data not shown), as compared to the control group of non-treated mice (0 h). Some transient decrease in the number of total white blood cells (WBC) was seen 24 h after the application of liposomes. The differential count of nucleated cells indicated that this decrease was caused by a decline in the number of granulocytes and lymphocytes. During the next two time points (48 and 72 h) the number of total WBC, including granulocytes and lymphocytes, normalized and the values obtained were at the level of the controls. However, compared to the pre-treatment levels, significant increases in the number of monocytes were observed 48 and 72 h after liposome injection. When SIM-prepared liposomes were applied, the same changes in the

hematological parameters occurred as in the case of DFM-obtained liposomes (data not shown).

Table 1. Peripheral blood cell counts with differential counts before (0 h) and 24, 48 and 72 h after treatment with DFM-prepared liposomes

Hours	ER x 10^{12}/l	PLT x 10^9/l	x 10^9/l					
			Total WBC	META	GRAN	Total GRAN	MONO	LYMPO
0	7.1 ± 0.5	0.87 ± 0.12	5.150 ± 1.63	0.080 ± 0.08	1.013 ± 0.30	1.093 ± 0.3	0.095 ± 0.07	3.962 ± 1.29
24	7.2 ± 0.6	0.78 ± 0.2	2.510* ± 0.53	0.042 ± 0.03	0.517 ± 0.15	0.559* ± 0.18	0.073 ± 0.05	1.878* ± 0.53
48	7.0 ± 0.1	0.94 ± 0.1	5.816 ± 2.63	0.088 ± 0.05	1.098 ± 0.56	1.186 ± 0.58	0.247 ± 0.18	4.383 ± 1.93
72	6.8 ± 0.7	0.88 ± 0.1	5.670 ± 0.92	0.054 ± 0.06	1.289 ± 0.72	1.343 ± 0.69	0.314* ± 0.06	4.013 ± 1.35

Abbreviations are given in Materials and Methods.
Values are expressed as mean numbers ± SD from 6 mice tested individually.
Significant difference from control (0 h) by t-test:* $p<0.01$.

DISCUSSION

α-Tocopherol is a hydrophobic molecule that becomes incorporated into the liposome membrane contributing to the higher stability of the vesicles. Our study on the encapsulation of α-tocopherol in liposomes obtained by two methods, DFM and SIM, demonstrated generally very high affinity of α-tocopherol for binding to liposome membranes. The percent of encapsulation ranged from 88% to 93%. These results are in agreement with the findings (Juliano, 1981) that hydrophobic substances have high encapsulation efficiencies, even in the case of small unilamellar vesicles and that the stability of the association between liposome and drug is greater for hydrophobic drugs than hydrophilic ones (VanBloois et al., 1987). The obtained data demonstrated that the initial concentration of α-tocopherol had a significant effect on the degree of encapsulation. The effect of the method used was less pronounced and for both methods the maximum percentage of encapsulation was achieved with 1% of α-tocopherol.

The average diameter of encapsulated liposomes was also influenced by the initial α-tocopherol concentration and increased with increasing concentrations of α-tocopherol. DFM gave larger liposomes with average diameters in the range of 0.48-0.65 μm, while SIM produced liposomes of average diameter within the range of 0.43-0.52 μm. However, for both methods, α-tocopherol encapsulation was higher in liposome fractions with smaller diameter. The liposome size distribution obtained by these two methods showed some differences. The dominant liposome fraction in SIM prepared liposomes was the fraction with $D<0.2$ μm, which included 67% of the total liposomes, while DFM produced liposomes with the dominant size fraction 0.45 μm$<D<$1.2 μm (38%). For intravenous administration of liposomes, small vesicles, preferably about or less

than 0.2 μm are usually required to obtain extended circulation lifetimes and optimal effect (Djordjević and Ivankovich, 1988; Bally et al. 1994; Gabizon, 1994). Thus, the extrusion of liposomes through 0.2 μm filters is often the best way to ensure a suitable size as well as their sterility for *in vivo* application (Djordjević and Ivankovich, 1988).

The *in vivo* application of liposomes with α-tocopherol membranes, obtained by both methods, to CBA mice, confirmed the biocompatibility and nontoxicity of such preparations. According to our results it is evident that these liposome suspensions are nontoxic, since they did not alter the numbers of erythrocytes and platelets. The temporary decrease in the number of total WBC, as well as in the numbers of granulocytes and lymphocytes, observed 24 h after treatment, could not be attributed to the cytotoxic effects of liposomes. It is known that liposomes are recovered from blood after i. v. administration but a substantial amount is distributed over several organs, particularly in the liver and spleen (Mellissen et al., 1994). Thus, the observed decrease in the number of total WBC may be the consequence of migration of these cells from the circulation to main target organs and tissues. An increased number of monocytes with time could be expected, since liposomes are avidly ingested and metabolized by circulating monocytes after i. v. injection. This property, i. e. the affinity of liposomes for the mononuclear phagocyte system provides a highly effective system for selective delivery of drugs or immunomodulators (cytokines) to monocytes/macrophages (Kirsh and Poste, 1984). Furthermore, phagocytic cells circulating in blood play an important role in the transport and accumulation of intravenously administered liposome-encapsulated drugs to inflammatory sites *in vivo* (Bally et al., 1994; Mehta et al., 1994). In addition, these liposomes with α-tocopherol membranes could be useful as potential drug carriers with a controlled release mechanism for anticancer therapies, since it was shown that α-tocopherol (vitamin E) could protect against carcinogenesis in tumor growth and also could reduce the toxicity of several anticancer therapies (Das, 1994).

In summary, liposomes with α-tocopherol as a membrane constituent have been found to be suitable for *in vivo* application. However, some alterations in peripheral white blood cells were detected. In order to elucidate the changes observed in peripheral blood cells induced by liposome administration, further investigations of the involvement of the immunohematopoietic system are in progress.

REFERENCES

1. *Bally, M. B., Masin, D., Nayar, R., Cullis, P. R. and Mayer, L. D.* 1994. Transfer of liposomal drug carriers from blood to the peritoneal cavity of normal and ascitic tumor-bearing mice. *Cancer Chemother. Pharmacol. 34*, 137-146.

2. *Bangham, A. D., Standish, M. M. and Watkins, J. C.* 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol. 13,* 238-252.

3. *Bangham, A. D., Hill, A. W. and Miller, N. G. A.* 1974. Preparation and use of liposomes as models of biological membranes. In: Bangham, A. D. (ed.) *Methods in Membrane Biology, Vol. I, Plenum Press, New York,* pp. 1-68.

4. *Brendzel, A. M. and Miller, I. F.* 1980a. Thermotropic characterization of liposome filtration. *Biochim. Biophys. Acta 596,* 129-136.

5. *Brendzel, A. M. and Miller, I. F.* 1980b. Effect of lipid-soluble substances on the thermotropic properties of liposome filtration. *Biochim. Biophys. Acta 601,* 260-270.

6. *Brooks, G. J. and McManus, R. C.* 1990. Finding new uses for liposomes in cosmetics. Inform. 1, 891-897.

7. *Das, S.* 1994. Vitamin E in the genesis and prevention of cancer. A review. *Acta Oncologica 33,* 615-619.

8. *Deamer, D. and Bangham, A. D.* 1976. Large volume liposomes by an ether vaporization method. Biochim. *Biophys. Acta 443,* 629-634.

9. *Djordjević, Lj. and Ivankovich, A. D.* 1988. Liposomes as carries of hemoglobin. *In: Gregoriadis, G. (ed.) Liposomes ad Drug Carriers. John Wiley & Sons, New York, pp.* 551-567.

10. *Gabizon, A. A.* 1994. Liposomal anthracyclines. *Hematol. Oncol. Clin. N. 8,* 431-450.

11. *Gregoriadis, G.* 1995. Liposomes as immunological adjuvants. In: Stewart-Tull D. E. S. (ed.) *The Theory and Practical Application of Adjuvants. Jonh Wiley & Sons, New York, pp.* 145-169.

12. *Juliano, R. L.* 1981. Pharmacokinetics of liposome-encapsulated drugs. In: Knight, C. G. (ed). Liposomes: From Physical Structure to Therapeutic *Applications. Elsevier, Amsterdam, pp.* 391-407.

13. *Kedar, E., Braun, E., Rutkowski, Y., Emanuel, N. and Barenholz, Y.* 1994. Delivery of cytokines by liposomes. II. Interleukin-2 encapsulated in long-circulating sterically stabilized liposomes: immunomodulatory and anti-tumor activity in mice. *J. Immunother. 16,* 115-124.

14. *Kirsh, R. and Poste, G.* 1984. Liposome encapsulation of biological response modifiers: activation of tumoricidal macrophages. Gregoriadis, G. (ed.) *Liposome Technology, Vol. II. CRC Press, Boca Raton, Florida, pp.* 33-53.

15. *Lasić, D. D.* 1994. Sterically stabilized vesicles. *Angew. Chem. Int. Ed. Engl. 33,* 1685-1698.

16. *Mehte, R. T., McQueen, T. J., Keyhani, A. and Lopez-Berestein, G.* 1994. Phagocyte transport as mechanism for enhanced therapeutic activity of liposomal amphotericin B. *Chemotherapy 40,* 256-264.

17. *Mellissen, P.M. B., vanVianen, W., Leenen, P. J. M. and Bakker-Woundenberg, I. A. J. M.* 1994. Tissue distribution and cellular distribution of liposomes encapsulating muramyltripeptide phosphatidyl ethanolamine. *Biotherapy 7,* 71-78.

18. *Michalet, X., Julicher, F., Fourcade, B., Seifert, U. and Bensimon, D.* 1994. La physique des liposomes. *Le Recherche 269,* 1012-1018.

19. *Ohsawa, T., Miura, H. and Harada, K.* 1985a. Evaluation of a new liposome preparation technique, the freeze-thawing method, using 1-asparaginase as a model drug. *Chem. Pharm. Bull. 33,* 2916-2923.

20. *Ohsawa, T., Miura, H. and Harada, K.* 1985b. Studies on the effect of watersoluble additives and on the encapsulation mechanism in liposome preparation by the freeze-thawing method. *Chem. Pharm. Bull. 33,* 5474-5483.

21. *Paphadjopoulos, D. and Miller, N.* 1967. Phospholipid Model Membranes. I. Structural characteristics of hydrated liquid crystals. *Biochem. Biophys. Acta 135,* 624-638.

22. *Sato, T. and Sunamoto, J.* 1992. Recent aspects in the use of liposomes in biotechnology and medicine. *Prog. Lipid Res. 31,*345-372.

23. *Van Bloois, L., Dekker, D. D. and Crommelin, D. J. A.* 1987. Solubilization of lipophilic drugs by amphiphiles. *Acta Pharm. Technol. 33,* 136-141.

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SADRŽAJ

INKAPSULACIJA α-TOKOFEROLA U LIPOZOME

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Lipozomi, mikrosfere izgrađene od fosfolipidnih dvoslojnih membrana, su danas vrlo atraktivni kao potencijalni nosači dijagnostičkih agenasa i lekova. U ovom radu ispitivana je inkapsulacija α-tokoferola u lipozome na bazi fosfolipida soje dobijene pomoću dve metode: metode tankog filma (MTF) i metode injektiranja rastvarača (MIR). Praćen je uticaj izabranih metoda na efekte inkapsulacije, uticaj polazne koncentracije α-tokoferola na procenat inkapsulacije i srednju veličinu čestica, a ispitivan je i stepen inkapsulacije α-tokoferola u frakcijama lipozoma određenog raspona veličine prečnika. U lipozomima proizvedenim pomoću obe metode konstatovan je visok stepen inkapsulacije α-tokoferola i to 88%-93%, što ukazuje na izrazit afinitet α-tokoferola za vezivanje sa lipozomima. Neznatno bolja inkapsulacija je utvrđena u lipozomima dobijenim MTF. Najbolji efekti inkapsulacije su ostvareni sa koncentracijom α-tokoferola od 1%. Sa povećanjem koncentracije α-tokoferola u suspenziji lipozoma povećavao se srednji prečnik lipozoma i to u opsegu od 0.43 μm do 0.52 μm za lipozome dobijene MIR i od 0.48 μm do 0.65 μm za lipozome dobijene MTF. Sekvencijalnim filtriranjem lipozoma kroz filtre različitih veličina pora lipozomi su frakcionisani u pet frakcija različite veličine, a procentualni udeo ovih frakcija utvrđen je merenjem raspodele sadržaja fosfora. Rezultati merenja procenta inkapsulacije α-tokoferola u pojedinim frakcijama su pokazali da se on efikasnije inkapsulira u manje lipozome. Ukoliko su za određene svrhe potrebni lipozomi manjih dimenzija (n. pr. za medicinske ili farmaceutske svrhe) smatra se da prednost ima MIR u odnosu na MTF, jer sadrži 67% lipozoma manjih od 0.2 μm. *In vivo* primena ovih lipozoma kod CBA miševa potvrdila je biokompatibilnost i netoksičnost ovih čestica. Određivanje broja zrelih krvnih elemenata sa diferencijalnim sastavom ćelija pokazalo je da lipozomi ne uzrokuju citotoksične efekte na ove ćelije.