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Liposome encapsulated vitamin A compounds exhibit greater stability and diminished toxicity

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Abstract

Absorption and fluorescence studies of retinol (vitamin A alcohol) and retinol palmitate (vitamin A palmitate) intercalated in phosphatidylcholine (PC) liposomes show that these compounds are bound to the lipid bilayer. It is further found that retinol binds liposomes with greater affinity as compared to retinol palmitate. In addition, the delivery of liposome-incorporated retinoids to the blood has also been studied and it is found that these systems reduce blood viscosity and cause less lysis of red blood cells than retinoid compounds not complexed in liposomes.

Keywords: Liposome; Retinol; Retinol palmitate; Absorption; Fluorescence; Binding; Viscosity; Lysis; Delivery

1. Introduction

Inspite of much biological and pharmacological effects, the therapeutic use of retinoids (Fig. 1) is still limited by reasons of many adverse effects that characterize the activity profiles of retinoids, especially when topically employed at higher doses or administered systematically [1–9]. The failure to adopt synthetic retinoids and their parent compounds, such as retinol, retinoic acid and the corresponding esters for clinical use is mainly due to strong toxicity (hypervitaminosis), poor chemical stability, long-lasting tetratogenicity, etc. In addition, the hydrophobic nature of retinoids makes their administration difficult by either intravenous or oral routes, requiring the utilization of oily formulations or surfactant containing aqueous solutions.

In the past, liposomes and immunoliposomes (referred to as biological couriers or biological missiles) have been suggested as possible pharmaceutical delivery systems [10]. Production, characterization and antiproliferative activity on neoplastic cells by liposome-associated retinoids have been reported [11]. Penetration of spin-labelled retinoic acid from liposomal preparation

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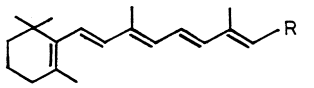

1 : R = $-CH_2OH$
2 : R = $-CH_2OCOC_{17}H_{35}$
3 : R = $-COOH$
4 : R = $-CHO$

Fig. 1. Chemical structures of vitamin A compounds. (1) Retinol (vitamin A alcohol); (2) retinol palmitate (vitamin A palmitate; (3) retinoic acid (vitamin A acid); (4) retinal (vitamin A aldehyde).

into the skin of SKHI hairless mice has been studied by EPR tomography techniques [12]. Earlier we have demonstrated that liposomes can stabilize vitamin A compounds [13,14]. With the aim of finding ways to administer retinoids, possibly overcoming or alleviating the solubility, specificity and toxicity problems associated with retinoids use, we have studied the properties of retinol and retinol palmitate intercalated in phosphatidylcholine (PC) liposomes. Liposomal–vitamin A compounds have been characterized by absorption and fluorescence spectroscopy, and biocompatibility and lysis effects of liposomal vitamin A compounds have been evaluated. It is found that retinol binds more efficiently to liposomes as compared to retinol palmitate. Furthermore, liposome-associated retinoids cause less lysis of RBC membranes, and these are also found to reduce the viscosity and rigidity of RBC solutions.

2. Experimental details

2.1. Materials and general procedures

Retinol, retinol palmitate and retinal were purchased from Fluka. The L-α-phosphatidylcholine was from Sigma. All spectroscopic studies were done in the UV-vis grade solvents procured from M/s Spectrochem, Mumbai. Fresh human blood samples of blood group A+, B+ and O+ were obtained from the Department of Biomedical Engineering, I.I.T, Bombay. All other chemicals were from the Aldrich Chemical Company, USA and used as received. Double distilled and de-ionized water was used for sample preparations. All the retinoids were handled in dim light (red) conditions, and if required were stored in sealed vessel as *n*-hexane solution in a nitrogen atmosphere. Their stereochemical purity was checked by HPLC analysis [15] and only all-*trans* isomers were used in the present studies.

UV-vis measurements were carried out on a Hitachi-U2000 spectrophotometer. Steady state fluorescence studies were performed on a Spex Spectrofluorolog instrument equipped with a 16810.22-m spectrometer as the single-grating monochromator, a 16800.22-m double spectrometer as the double-grating emission monochromator and a 450-W xenon lamp as the light source. Samples were adapted to normal room temperature (25°C) prior to measurements. Fluorescence lifetime measurements were done on an Applied Photophysic SP-70 nanosecond spectrofluorimeter equipped with a 250-W xenon lamp and 200 KHz nanosecond flash lamp as a light source. The lifetime was recorded using the single photon counting technique and data were processed by deconvolution methods. Excitation was done at 325 nm and emission was monitored at 495 nm. A PHM-84 Research pH meter from Radiometer, Copenhagen was used for measuring the pH values. Sonications were carried out with Branson B-12 sonifier (output power 400 W, frequency 20 KHz) equipped with microprobes. Centrifugations were done in Beckman L8-55M Ultracentrifuge using a 45Ti rotor. Viscosity measurements on blood samples were made in Contraves 30 Low Shear Viscometer and by following the literature procedure [16,17]. All operations related to retinoids were carried out in a dim light (red) condition.

2.2. Preparation of retinoids — constituted liposomes

Incorporation of retinoids in liposomes was done as described earlier [14]. In a typical procedure, a chloroform solution of retinoid (20 μl, 1.30×10^{-3} M) was mixed with a *n*-hexane solution of L-α-phosphatidylcholine (0–200 μl, 2.5×10^{-2} M). [A chloroform solution of cholesterol ($2.00–7.00 \times 10^{-5}$ M) was added in case of lipo-

somes containing cholesterol.] The mixture contained in a round-bottomed flask was diluted with 2 ml of chloroform. Most of the organic solvent was removed from the mixture on a rotary evaporator under diminished pressure at 4°C. The mixture was further kept under vacuum (10^{-4} torr) for 3 h to ensure complete removal of organic solvent when a thin shiny film of the residual material is formed in the flask. The desired buffer (2 ml) of particular pH was then added to the flask and the mixture was kept at 4°C for 3 h allowing the phospholipid to swell. The solution was sonicated for 7 min and then centrifuged at 5400 *g* for 20 min at 4°C. Liposome-associated retinoids were further subjected to gel permeation chromatography over Sephadex G-25 with corresponding buffers as eluants. The liposomal preparations of retinoids thus obtained were kept under an argon atmosphere in the dark for further studies.

2.3. Fluorescence studies

From ethanolic stock solutions (5.27×10^{-3} M) of retinol and retinol palmitate, a 4-μl solution was taken and added separately to 2 ml PC solution with varying lipid concentration ($0–2.0 \times 10^{-4}$ M) in different glass vials. The liposomal solutions were incubated at room temperature for 20 min and the fluorescence spectra were recorded. From the values of the change in intensity of emission maximum, the binding constants were calculated according to the literature procedure [18]. For characterizing the microenvironment of retinoids in liposome, quenching experiments were performed by addition of 4 μl of a concentrated ethanolic solution of retinal (2.50×10^{-3} M) to the liposome-associated retinol palmitate. The excitations were done at 325 nm.

2.4. Studies with red blood cells

Fresh human blood samples of blood group A+, B+ and O+, obtained from the Department of Biomedical Engineering, I.I.T, Bombay, were centrifuged at 4000 rev./min for 5 min. The upper plasma and white buffy coats were removed by a syringe and RBCs remained in the lower portion were washed thrice with water and with saline (0.9% NaCl in water). A sample of 1% blood suspension was made by diluting it with phosphate buffered saline [19] (pH, 7.2). The retinoids (3.3×10^{-5} M) were added and incubated at 37°C with gentle shaking. The extents of lysis of the RBC were determined by the increase in absorption of haemoglobin at 540 nm at different time intervals.

Biocompatibility investigations were done by addition of retinol or retinol palmitate to RBC solutions or to whole blood and measuring the viscosity at different time intervals. Control experiments were performed following similar experimental protocols.

3. Results and discussion

3.1. Binding of retinol and retinol palmitate with PC liposomes: absorption and fluorescence studies

The UV-vis absorption characteristics of retinol and retinal palmitate are similar in the ethanol and liposomal matrix (Table 1). The absorption band at approx. 326 nm is due to π,π^* transition of the retinoid's polyene chain. The binding of retinol and retinol palmitate to PC liposomes has been determined by spectrofluorimetric analysis. Excitation of liposome-bound retinoids retinol and retinol palmitate at 325 nm resulted in fluorescence bands with λ_{max} at 490 nm (Fig. 2). The fluorescence intensities of retinol and retinol palmitate increase with the increase in phospholipid concentration with a fixed concentration of retinoids. Typical fluorescence intensity variation with increasing lipid concentrations for retinol are shown in Fig. 2. Similar variations are observed for retinol palmitate also. The binding constants of retinoids (with liposomes) have been de-

Table 1
UV-vis absorption maxima of retinol and retinol palmitate

Compound	λ_{max}, nm (ε)	
	Ethanol	Liposome[a]
Retinol	325 (52,995)	328 (27,657)
Retinol palmitate	327 (51,177)	330 (32,339)

[a] Phosphatidylcholine, 5×10^{-4} M.

Table 2
Binding constant (K, M^{-1}) of retinol and retinol palmitate with liposomes

Compound	K (M^{-1})[a]
Retinol	3.61×10^4
Retinol palmitate	1.03×10^4

[a]An average of three sets of experiments; error ~ ±3%.

termined by a plot of $(\phi/\phi_a - 1)^{-1}$ vs. C_1^{-1}; where ϕ = fluorescence quantum yield of retinoid in liposome, ϕ_a = fluorescence quantum yield of retinoid in aqueous solution, K = binding constant, and C_1 = phospholipid concentration [M]. A typical plot of $(\phi/\phi_a - 1)^{-1}$ vs. C_1^{-1} for retinol is shown in Fig. 3. A similar straight-line plot was obtained for retinol palmitate also. The slope of these straight-line plots gave the K-values (Table 2). The high binding constant values of the order of four indicate efficient binding between retinoids and liposomes. The K-value of retinol is higher as compared to the K-value of retinol palmitate. This is due to the presence of relatively more polar hydroxyl group in retinol which can interact more with the polar head groups of phospholipid than does the relatively less polar ester group of retinol palmitate.

Fluorescence quenching experiments and fluorescence lifetime measurements of retinol palmitate have enabled us to further characterize the binding site of retinoids in a liposomal matrix Table 3. The all-*trans*-retinal quenches the fluorescence of liposomal retinol palmitate. The corresponding Stern–Volmer plot [20] for this quenching experiment is shown in Fig. 4. Retinol palmitate in liposomes shows a fluorescence lifetime of 2.17 ns and the corresponding fluorescence decay profile is presented in Fig. 5. The Stern–Volmer constant K_{sv} and the microviscosity (η) of the localization domain for liposomal retinol palmitate is found to be 6.73×10^4 M^{-1} and 3.22×10^{-13} N s m^{-2}, respectively.

These values indicate that retinol palmitate resides in a rather less viscous and less hydrophobic liposomal domain. This becomes obvious when we compare the K_{sv} and η values of

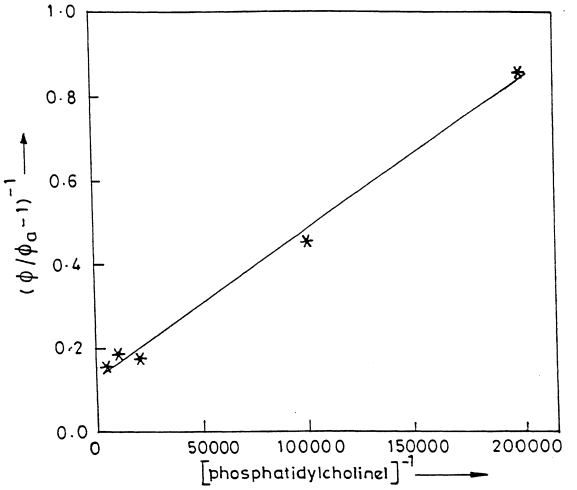

Fig. 3. A plot of $(\phi/\phi_a - 1)^{-1}$ vs. [phosphatidylcholine, PC, M]$^{-1}$, for retinol in PC liposomes.

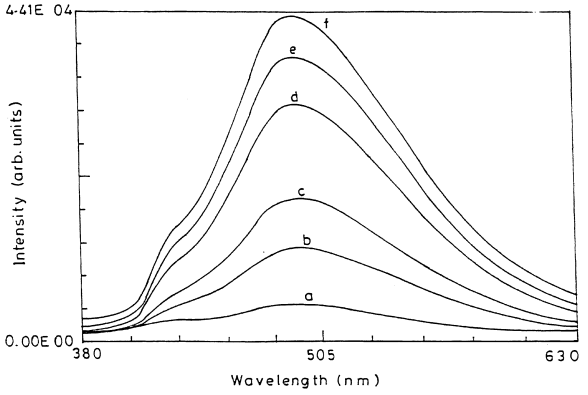

Fig. 2. Fluorescence spectra of retinol in phosphatidylcholine (PC) liposomes. PC concentrations are [a], 0.0 M; [b], 5×10^{-6} M; [c], 1×10^{-5} M; [d], 5×10^{-5} M; [e], 1.0×10^{-4} M; [f], 2×10^{-4} M. Excitation at 325 nm.

Table 3
Fluorescence characterization data for retinol palmitate in the liposome

Parameter	Value
Stern–Volmer constant, K_{sv}	6.73×10^4 M^{-1}
Fluorescence lifetime, τ_0	2.17 ns
Quenching constant, k_q	3.10×10^{13} M^{-1} s^{-1}
Microviscosity, η	3.22×10^{-13} N s m^{-2}

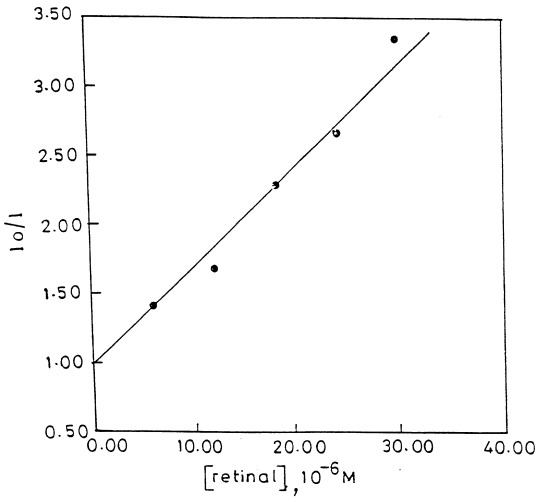

Fig. 4. Stern–Volmer plot for quenching of retinol palmitate fluorescence by retinal. Excitation at 325 nm.

retinal-caused fluorescence quenching of hydrophobic pyrene which is expected to localized in a hydrophobic domain of the liposome.

Liposomes are spherical, self-closed structures composed of curved lipid bilayers which entrap part of the aqueous media in which they freely float into their interior. The thickness of the bilayer is approx. 40 Å [21]. Depending on whether the molecule is hydrophillic or hydrophobic, it could be incorporated in the entrapped aqueous interior or in the lipid bilayer. The hydrophobicity of a molecule could be judged by its polarity. Between retinol and retinol palmitate, the former is more polar than the later. It is observed that in reverse phase HPLC (C_{18}) retinol elutes faster than retinol palmitate [22]. The length of retinoids can be calculated from the energy minimized structures using SYBYL (Tripos, St Louis, MO, USA) and it is found that retinol and retinol palmitate, respectively, have lengths of 15.5 and 35.8 Å, and steric energy of 41.64 and 58.18 kcal mol^{-1}. Thus, retinoids can easily be accommodated in the lipid bilayer as the thickness of the bilayer is approx. 40 Å. Thus, retinoids can be stabilized in the lipid bilayers of liposomes.

3.2. Effect of liposomal and non-liposomal retinol and retinol palmitate on red blood cells (RBC)

Effect of liposomal and non-liposomal retinol and retinol palmitate on RBC membranes has been investigated by monitoring the erythrocyte's absorption band at 540 nm. As seen in Fig. 6, retinol has a drastic lytic effect on human RBCs. In contrast, retinol palmitate causes little or no

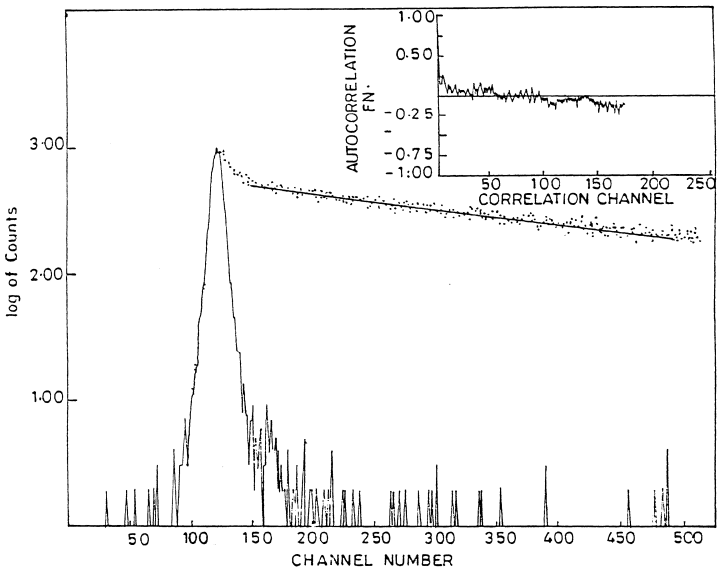

Fig. 5. Fluorescence decay profile of retinol palmitate in PC liposomes.

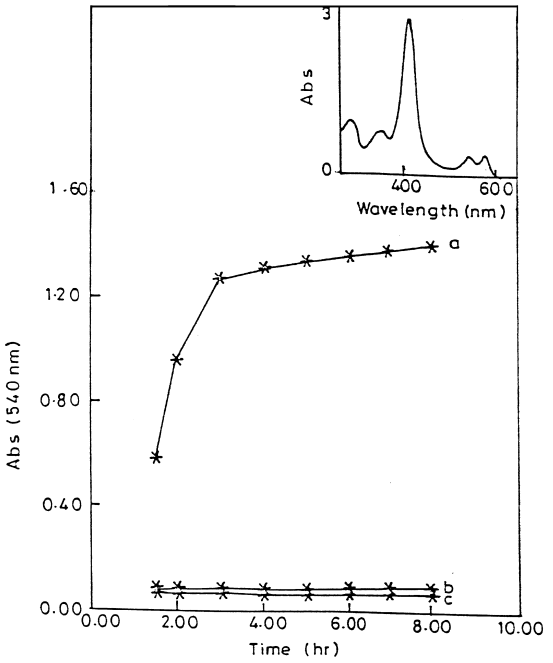

Fig. 6. A plot of change in absorbance at 540 nm with time for incubation of (a) retinol; (b) retinol palmitate; (c) control with erythrocyte in phosphate buffered saline at 37°C. Inset: absorption spectrum of erythrocyte after lysis by retinoids.

lysis at the concentrations used in the present experiments. Liposomal retinol palmitate lysis data are not shown, since the reduction in lysis for liposomal and non-liposomal retinol palmitate is very less as compared to the liposomal and non-liposomal retinol. Control experiments were done without adding any retinoid and it was found that lysis of the cells does not occur. The lytic effects of retinol may be due to the polarity of its functional group. When retinol was incorporated in PC or in PC/cholesterol liposomes [14] and incubated with human RBCs, it caused lysis to a lesser degree (Fig. 7). The lytic effect of the two types of liposomes are, however, comparable.

This study shows that retinol, unlike retinol palmitate, can interact strongly with natural membrane. The initial action of retinol on RBC can be a penetration and expansion of the cell membrane as evidenced by observations from a sequence of changes in the fine structure of the cells during lysis by the vitamin [23]. However, when retinol is incorporated in liposomes, only a

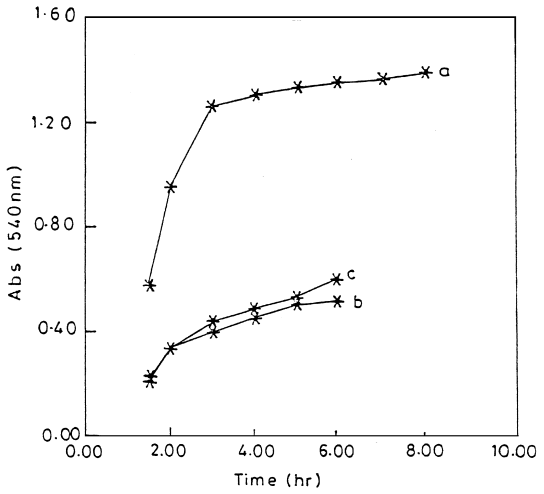

Fig. 7. A plot of change in absorbance at 540 nm with time for incubation of retinol in (a) phosphate buffered-saline; (b) PC liposomes; (c) PC liposomes with erythrocyte at 37°C.

small amount of lysis occurs; presumably the liposomes can sequester the drug and prevent its interaction with erythrocytes.

At present the mechanism underlying the reduced toxicity of liposomal retinoids is not very clear. It is well known, however, that incorporation of drugs into liposomes can affect its behaviour in vivo in a variety of complex ways including alteration of pharmacokinetics, changes in tissue distribution and modification of immune functions.

3.3. Biocompatibility of liposomal retinol palmitate — effect on blood viscosity

Biocompatibility of liposomal retinol palmitate has been investigated by measuring the viscosity changes of RBC and the whole blood. The effects of retinol palmitate and liposome-associated retinol palmitate on erythrocyte's viscosity as a function of shear rate is shown in Fig. 8. In Fig. 9 the effects of liposomal and non-liposomal retinol palmitate on the viscosity of the whole blood at a shear rate of 95.5 s^{-1} are depicted. It is found that the viscosity is reduced in the presence of retinol palmitate and is further reduced in the presence of liposome-associated retinol palmitate. The decrease in viscosity is more at a low shear

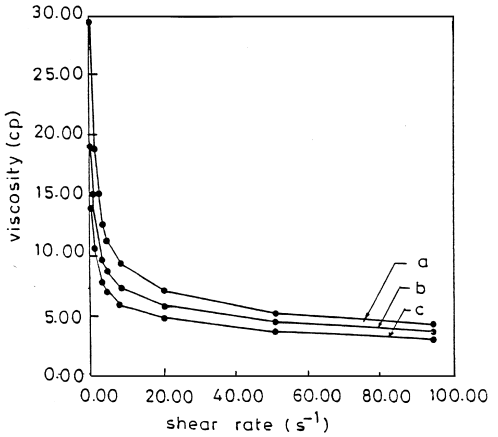

Fig. 8. Change of viscosity with shear rate. (a) Erythrocyte; (b) erythrocyte and retinol palmitate; (c) erythrocyte and PC-liposomal. [Retinol palmitae], 3.3×10^{-5} M; [phospholipid] 4.15×10^{-4} M.

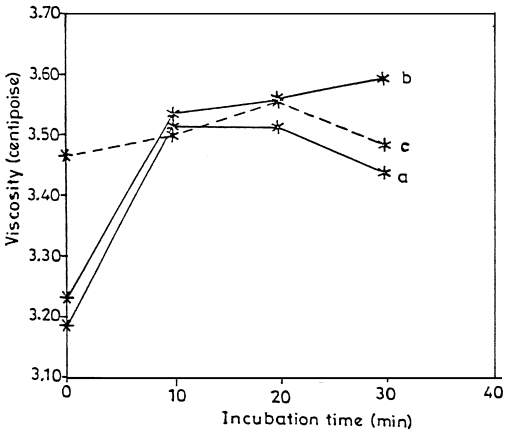

Fig. 9. Change of viscosity with incubation time for whole blood at a shear rate of 94.5 s^{-1} for (a) whole blood; (b) retinol palmitate in whole blood; (c) liposomal (PC)-retinol palmitate in whole blood. Retinol palmitate 3.3×10^{-5} M; phospholipid 8.30×10^{-4} M.

rate. It is possible that in the presence of an external lipid, the lipids of RBC get exchanged with the former, and as a result, the rigidity of the red cells decreases. Consequently, decrease in the viscosity is observed. The red cell membrane bilayer comprise mainly of phospholipids and cholesterol with a small amount of glycolipids and membrane proteins. The length and degree of saturation of fatty acid residues of the phospholipid strongly influences the fluidity of the membrane. With increasing chain length and degree of circulation, the fatty acids become less fluid and the membrane becomes more viscous [24].

4. Conclusions

In conclusion it can be said that liposomes can be used as a retinoid delivery system which can also reduce the toxicity of the retinoids. This approach of intercalating vitamin A compounds in liposomes provides a means of increasing the much desired stability and structural and functional specificity of retinoids and may lead to the further development of other novel systems for use in vitamin A therapeutics. We have not shown how the retinoids could be released from the liposomes. In this context, it may be mentioned that recently the rate of transfer of radioactive retinoids from vesicles to erythrocyte has been studied and it is found that inter-membranous transfer of retinol is much faster than the retinyl palmitate [25]. This is because the long fatty acyl chain in the latter makes it more hydrophobic and since the long chain fatty acyl retinyl esters are not transferable at substantial rates, putative binding proteins for these hydrophobic derivatives would be expected to play a catalytic role in inter-membranous transfers. The targeted release could be achieved by making immuno-liposomes, i.e attaching antibodies with the liposomal retinoids, and further work in this area is highly warranted.

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