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Binding of bilirubin with albumin-coupled liposomes: implications in the treatment of jaundice

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Abstract

In the present study, we demonstrated the suitability of liposomes as a method of removing plasma bilirubin in hyperbilirubinemic rats. The liposomes have innate tendency to bind with bilirubin through hydrophobic interaction. Among different types of liposomes, the positively charged liposomes were found to have maximum affinity to free bilirubin. However, the entrapment or coupling of serum albumin on the surface of egg phosphatidylcholine liposomes can render a several-fold increase in their bilirubin binding capacity. The proteoliposomes were able to preferentially bind with bilirubin even in the presence of erythrocytes. Interestingly, these liposomes were found to displace bilirubin bound on the surface of erythrocytes as well. The results of the present study further demonstrate that albumin-bearing liposomes were equally effective in removing plasma bilirubin in experimental jaundiced animals. These observations indicate that liposome-mediated selective homing of excess plasma bilirubin to the liver cells (cf. hepatocytes) may help in the development of safer strategy for the treatment of hyperbilirubinemic conditions in the model animals.

Keywords: Bilirubin; Liposome; Jaundice; Albumin; Kernicterus; Reticuloendothelial system

1. Introduction

Bilirubin, a potentially toxic catabolic product of haemoglobin, is normally cleared from plasma after conjugation with glucuronic acid in the hepatocytes and finally excreted into the bile [1,2]. Under normal physiological conditions, plasma albumin interacts with bilirubin stoichiometrically and helps in its transportation to the liver [3,4]. However, in certain metabolic disorders such as jaundice or in newborn infants with genetic deficiency or low levels of bilirubin glucuronosyltransferase, the amount of unconjugated bilirubin (UB) in blood increases [2–4]. Besides, the plasma UB level can also rise due to the overproduction of the pigment in conditions such as hemolytic anemia [5]. The elevated bilirubin level interferes with the normal functioning of the cellular machinery and eventually manifests systemic toxicity [4,6]. Moreover, the high serum level of the bilirubin when crossed the threshold level can contribute to number of sequels specifically impairing cellular function by blocking oxidative phosphorylation, inhibiting respiration and finally resulting in fatal Kernicterus [1,4,7].

The interaction of bilirubin with lipid vesicles as well as basolateral membranes has been studied extensively [8]. The liposomes are believed to bind with bilirubin involving both ionic as well as hydrophobic interactions [8,9]. Further, the liposomes can help in targeting of the bilirubin to the liver and spleen, the two main components of the reticuloendothelial system (RES), for its final removal from the systemic circulation [10]. Keeping in view the high affinity of bilirubin to lipids, we attempted to exploit the liposomes (biodegradable lipid vesicles) for the removal of bilirubin from systemic circulation of the jaundiced animals.

Abbreviations: RES, reticuloendothelial system; ULVs, unilamellar lipid vesicles; MLVs, multilamellar lipid vesicles; egg PC, egg phosphatidylcholine; PS, phosphatidylserine; SA, stearylamine; HSA, human serum albumin; RSA, rat serum albumin; PSA, porcine serum albumin; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase

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Incidentally, bilirubin has high affinity for albumin as well, thus entrapment or coupling of albumin onto the surface of liposomes should result in several-fold increase in their binding with bilirubin [11]. To test our hypothesis, we evaluated various types of liposomes for their binding with free as well as plasma membrane-bound bilirubin. Finally, we determined whether such liposomes would facilitate hepatic uptake and vectorial transport of bilirubin. The in vivo studies demonstrate that liposome-based bilirubin lowering formulations will not only help in the formation of liposome–bilirubin complex but also facilitate the clearance of bilirubin from systemic circulation in the model animals.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC) was isolated from egg yolk and purified as described earlier [12]. Cholesterol (Chol) was purchased from Centron Research laboratory, Mumbai, India, and was used after crystallizing it three times with methanol. Stearylamine (SA), phophatidylserine (PS), gangliosides, sodium cyanoborohydride, fatty acid free albumin (various animal sources) and bilirubin were bought from Sigma. Sodium metaperiodate and ethylenediaminetetraacetic acid (EDTA) were obtained from Sisco Research Laboratory, Mumbai, India. Sephadex G-75 and Sepharose 6B were procured from Pharmacia Fine chemicals.

2.2. Methods

2.2.1. Preparation of bilirubin solution

Keeping into account the fact that contaminating impurities present in the commercially supplied bilirubin might interfere in liposome–bilirubin binding studies, it was purified following the published procedure [13]. The recrystallized bilirubin (about 5 mg) was dissolved in 1 ml of 38 mM sodium carbonate solution containing 1 mM EDTA (pH 11.0). This solution was centrifuged at 5000 rpm for 10 min to remove insoluble bilirubin. The pH of the bilirubin solution was adjusted to 8.0 with Tris buffer saline (10 mM Tris, 150 mM sodium chloride, pH 7.6). The concentration of bilirubin was determined spectrophotometrically using molar extinction coefficient 6.2×10^4 M^{-1} cm^{-1} at 453 nm. Bilirubin solution was protected from light to prevent photodegradation and used within 30 min of its preparation. All experiments were conducted with exclusion of daylight, using light source covered with a double layer of orange cellophane, transmitting very less illumination at 440 nm.

2.2.2. Preparation of liposomes

In the present study, the bilirubin was allowed to interact with liposomes of different physicochemical properties. The compositions of various types of the liposomes were as follows:

Neutral liposomes: egg PC and Chol (7:3 molar ratio)
Negatively charged liposomes: egg PC, Chol and PS (7:2:1 molar ratio)
Positively charged liposomes: egg PC, Chol and SA (7:2:1 molar ratio)
Albumin coupled [human serum albumin (HSA)/OUT] liposomes: egg PC, Chol and gangliosides
Albumin coupled/entrapped (HSA IN/OUT) liposomes: egg PC, Chol and gangliosides (20:20:4 molar ratio)

Unilamellar lipid vesicles (ULVs) were prepared by sonication method following published procedure as modified in our lab [14]. Briefly, the desired composition of the lipids was dissolved in a chloroform–methanol mixture in a thick glass tube, and the solvents were removed under a slow jet of N_2 to get a thin lipid film on the wall of the tube. The traces of the solvents were removed by leaving the tube overnight in vacuo. The film was dispersed in 0.8 ml of the borate-buffered saline (10 mM borate, 60 mM NaCl, pH 8.4). The lipid dispersion was sonicated with probe sonicator for a total of 30 min (pulsed mode, 3 s pulse 2 s rest) at 4 °C under N_2 atmosphere. The sonicated preparation was centrifuged in RC5C cooling centrifuge at 10,000 rpm for 1 h at 4 °C to effect the removal of the titanium particles as well as poorly dispersed lipid. Only the top two-thirds of the supernatant was used in the further studies. The mean diameter of these liposomes, as determined by negative staining electron microscopy, was about 60 ± 10 nm.

2.2.3. Covalent coupling of serum albumin with liposomes

The entrapment of serum albumin in the liposomes was effected by freeze–thaw method followed by coupling of albumin onto the surface of liposomes [15]. The serum albumins from various sources were covalently coupled to the liposome surface as described earlier [16]. Briefly, the liposomes were subjected to periodate oxidation for generating aldehyde group from gangliosides present on the surface of liposomes. Subsequently, the vesicles were separated from the periodate on a 1.5×15-cm Sephadex G-75 column equilibrated in 20 mM borate, 120 mM NaCl (pH 8.4). The liposome peak was concentrated on an Amicon centriflo cone (1500 rpm, 4 °C) using RC 3B cooling centrifuge. For conjugation, oxidized liposomes (10 μmol lipid in 1 ml) were mixed with 10 mg of given serum albumin in 0.5 ml of 20 mM borate, 120 mM saline (pH 8.4). To the above mixture was added 15 μl of $NaBH_3CN$ (2 M) and the reaction mixture was left for 14 h at room temperature. The uncoupled albumin was removed by passing this mixture through 1.6×90 cm sepharose 6B column, equilibrated with sucrose-supplemented Tris saline (10 mM Tris, 44 mM sucrose, 120 mM NaCl and 5 mM EDTA, pH 7.4) and albumin-coupled liposomes were eluted in void volume. The peak corresponding to vesicles was

concentrated using centriflo cone as described above. The protein-to-lipid ratio in HSA/OUT liposomes was about 80 μg/μmol of lipid Pi, while it was 96 μg/μmol of lipid Pi for HSA IN/OUT liposomes, as estimated by BCA reagent method [17].

2.2.4. In vitro bilirubin binding to liposomes

The kinetics of bilirubin binding with liposomes was determined by incubating various liposomal preparations with bilirubin solution at 37 °C. The interaction was studied in concentration- as well as time-dependent manner. Briefly, liposomes (62.5–500 nmol lipid Pi/500 μl) were incubated with 500 μl of 2:1 bilirubin/HSA mixture (total bilirubin 150 μmol) for 1 h at 37 °C in Tris buffer (10 mM Tris, 150 mM saline, pH 8.0). The unbound bilirubin was separated on a 1.5 × 30-cm Sephadex G-75 column equilibrated in 10 mM Tris, 150 mM NaCl (pH 8.0). The liposomal peak was concentrated on an Amicon centriflo cone (1500 rpm, 4 °C) using RC 3B cooling centrifuge. We also determined bilirubin binding with liposomes in the presence of isolated erythrocytes as well as whole blood. Briefly, liposomes (250 nmol lipid Pi) were incubated with 2:1 bilirubin/HSA mixture (total bilirubin 150 μmol) in the presence of human erythrocytes (1×10^8 cells) for 1 h at 37 °C, keeping final volume of the reaction mixture 1 ml with Tris buffer saline. The relative interaction of bilirubin with liposomes versus erythrocyte surface was studied by incubating liposomes with erythrocytes that were already saturated with bilirubin. Briefly, bilirubin-saturated erythrocytes (1×10^8 cells) were incubated with various liposomal preparations (250 nmol lipid Pi) for 1 h at 37 °C, keeping final volume 1 ml with Tris buffer. The liposomes were separated by centrifugation at 2000 rpm for 10 min at 4 °C and liposome-bound bilirubin was estimated as described earlier [18]. Similarly, the potential of erythrocytes to displace liposome-bound bilirubin was studied by allowing them to interact with bilirubin-saturated liposomes. Briefly, 250 nmol lipid Pi was allowed to interact with erythrocytes (1×10^8 cells) at 37 °C for 1 h. The erythrocytes and liposomes were separated by centrifugation for 30 min at 2000 rpm at 4 °C using RC 3B centrifuge. Erythrocytes were further washed with TBS by centrifugation at 2000 rpm for 10 min at 4 °C (× 3). Both liposomes as well as erythrocytes were analyzed for their bilirubin content following the published procedure [19].

2.2.5. In vitro cytotoxicity assay of liposomes

The toxicity of different liposomal preparations was measured by using the MTT—micro culture tetrazolium assay using published procedure [20]. Briefly, human embryonic kidney (HEK) cells at the exponential growth phase were harvested and centrifuged at 2000 × *g* for 5 min, resuspended in complete medium. The cells were dispensed in a 96-well plate (2×10^4 cells in 180 μl DMEM/well) and incubated in a 5% humidified CO_2 incubator at 37 °C. After 24 h, 20 μl liposomal solution was added in six replicates to give final concentration of 0–2000 nM of liposomes (lipid Pi) per milliliter. After 48 h of incubation at 37 °C, 20 μl/well of MTT (stock solution 5 mg/ml PBS) was added and the plate was again incubated for 4 h after which the medium was removed by aspiration. DMSO (150 μl) was added to each well and the formazan was dissolved by gentle shaking. The plate was read immediately in a micro plate reader (Ceres uv900c, Bio-Tech Instruments, USA) operating at 540 nm. Wells with complete medium, liposome and MTT, but without cells, were used as blanks. Vincristine sulphate (40 nM) was able to induce 100% killing of the cells and was used as positive control.

2.2.6. In vivo interaction of liposomes with plasma bilirubin in jaundiced rats

Male albino Wistar rats were exposed with bilirubin/rat serum albumin (RSA) (2:1 molar ratio) solution for three alternate days to develop hyperbilirubinemic condition. Each animal received 1 ml of bilirubin/RSA solution corresponding to 75 μM bilirubin per dose; the first two doses were administered by subcutaneous route, followed by last intravenous injection of the same amount of bilirubin (75 μM in 1 ml isotonic buffer). The animals were divided in six different groups; each consists of 10 animals.

1. Normal rats given no treatment
2. Hyperbilirubinemic rats given no liposomal treatment
3. Hyperbilirubinemic rats given free RSA treatment
4. Hyperbilirubinemic rats given PC-liposomes treatment

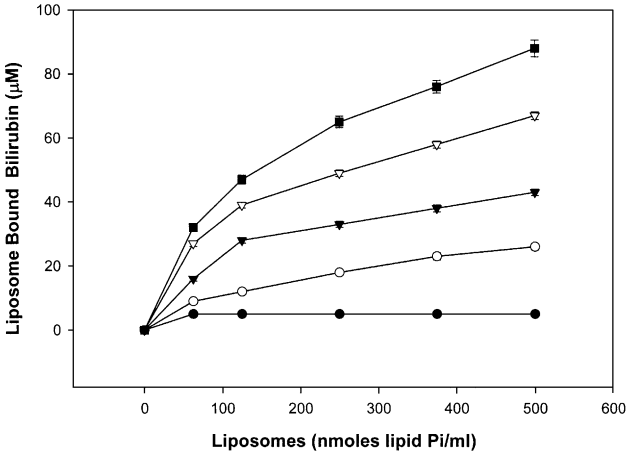

Fig. 1. Concentration-dependent binding of liposomes with bilirubin. The effect of increasing amount (62.5–500 nmol lipid Pi) of liposomes on binding with bilirubin was determined by incubating them with 2:1 HSA and bilirubin (total bilirubin 150 μM) for 1 h at 37 °C, keeping final volume 1 ml in Tris buffer saline. Liposome-bound bilirubin was estimated following published procedures as described in the Materials and methods section. (○), Egg PC liposomes; (●), negatively charged liposomes; (▼), positively charged liposomes; (▽), HSA (OUT) egg PC liposomes; (■), HSA (IN/OUT) egg PC liposomes. Each value is the mean of three different experiments ± S.D.

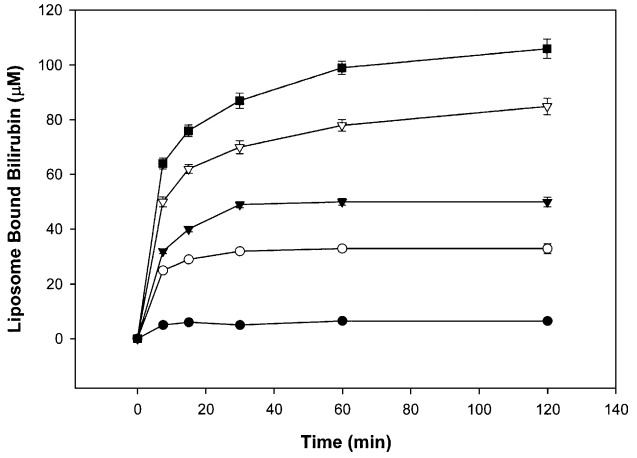

Fig. 2. Time-dependent binding of bilirubin to various liposomal preparations. Unilamellar liposomes (625 nmol lipid Pi) were incubated with 2:1 HSA and bilirubin (total bilirubin 150 μM) for different time intervals at 37 °C in TBS solution. The liposome-bound bilirubin was estimated following published procedures as described in the Materials and methods section. (○), Egg PC liposomes; (●), negatively charged liposomes; (▼), positively charged liposomes; (▽), HSA (OUT) egg PC liposomes; (■), HSA (IN/OUT) egg PC liposomes. Each value is the mean of three separate experiments ± S.D.

5. Hyperbilirubinemic rats given RSA/OUT liposome treatment
6. Hyperbilirubinemic rats given RSA IN/OUT liposome treatment

The treatment of the hyperbilirubinemic rats with various liposomal preparations was started the next day to the last dose (i.v. injection) of bilirubin. Each animal was treated with 250 nmol lipid Pi (0.4 ml buffer) corresponding to various liposomal preparations daily through intravenous route for four consecutive days. The serum level of bilirubin as well as other liver functioning parameters were determined following published procedure [21]. Briefly, the blood was collected by retro-orbital puncture on day 0 (6 h after the last dose of liposome treatment), day 3 and day 9 post-liposomal treatment and analyzed for its bilirubin as well as other enzyme contents.

Table 1
Relative affinity of liposomised serum albumin with erythrocyte-bound bilirubin

Liposomes	Percentage eluted bilirubin
Rat serum albumin	42.66 ± 1.38
Human serum albumin	89.33 ± 0.94
Pig serum albumin	97.33 ± 1.4

Human erythrocyte (1×10^8) cells were incubated with 150 μM bilirubin/HSA solution in TBS as described earlier for 1 h at 37 °C. The unbound bilirubin was removed by washing erythrocytes extensively with TBS. The bilirubin-bound erythrocytes were allowed to incubate with albumin-bearing liposomes for 1 h at 37 °C. The bilirubin transferred to liposomes was determined as described earlier. The spontaneous leaching of bilirubin from untreated erythrocytes was less than 1%. The values shown are mean of three different experiments ± S.D.

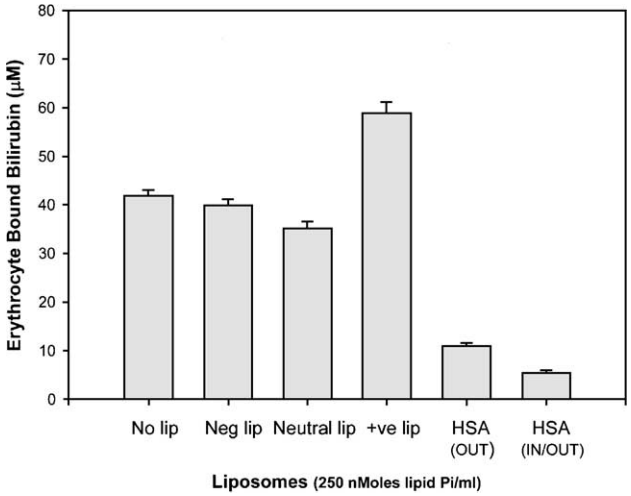

Fig. 3. Binding of bilirubin with erythrocytes in the presence of lipid vesicles. Human erythrocytes (1×10^8 cells) were incubated with 2:1 HSA and bilirubin (total bilirubin 150 μM) in the presence of different liposomal preparations (250 nmol lipid Pi) for 1 h at 37 °C. The mixture was centrifuged to separate erythrocytes from liposomes and unbound bilirubin. The erythrocyte-bound bilirubin was estimated following published procedures as described in the Materials and methods section. The values shown represent mean of five different experiments ± S.D.

2.2.7. Statistical analysis

The effect of proteoliposome treatment on the plasma bilirubin level of hyperbilirubinemic animals was tested by the Student's *t*-test. A P value of less than <0.05 was considered statistically significant.

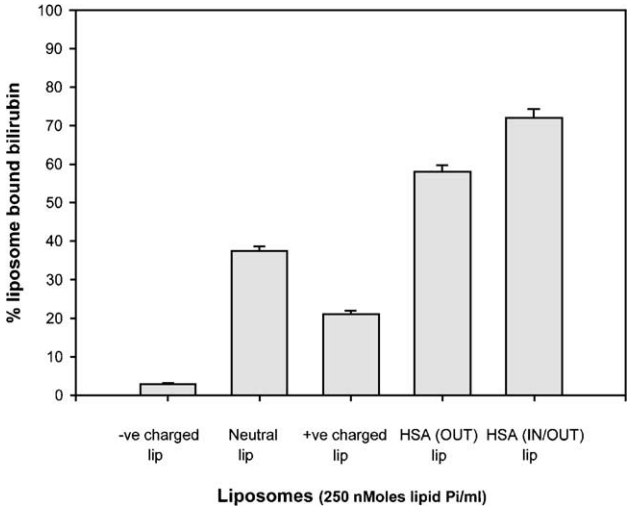

Fig. 4. Relative affinity of bilirubin with erythrocyte versus liposomes. Human erythrocytes (1×10^8 cells) were incubated with 2:1 HSA and bilirubin (total bilirubin 150 μM) for 1 h at 37 °C. The unbound bilirubin was removed by extensive washing with TBS. The erythrocytes were then incubated with different liposomal formulations for 1 h at 37 °C. The mixture was centrifuged at 2000 rpm to separate erythrocytes. Liposome-bound bilirubin was estimated by analyzing supernatant for its bilirubin content following published procedures as described in the Materials and methods section. The spontaneous leaching of bilirubin from erythrocytes (no liposome treatment) was less than 1%. The data shown express mean of five separate experiments ± S.D.

3. Results

The bilirubin was found to interact with various liposomal preparations in concentration- as well as time-dependent manner (Figs. 1 and 2). The composition of the liposomes seems to play an important role in such interactions. Among various types of liposomes, the positively charged liposomes were shown to have higher bilirubin binding as compared to the neutral liposomes. Negatively charged liposomes did not interact substantially with bilirubin at any concentration. However, the bilirubin binding capacity of the neutral liposomes increased significantly when serum albumin (HSA) was either entrapped inside or covalently coupled onto the surface of liposomes (Figs. 1 and 2). The binding of the bilirubin with albumin-bearing liposomes depends on the source of the albumin and differs from species to species. Among different kinds of albumins, the rat albumin was found to bind the least while porcine albumin has the maximum bilirubin binding capacity (Table 1).

We have also demonstrated that the presence of erythrocytes did not affect the bilirubin binding capacity of albumin-coupled liposomes (Fig. 3). The results of the present study suggest that bilirubin, when present in excess, can interact with both erythrocytes as well as neutral liposomes with equal propensity, that is, the presence of liposomes or erythrocyte in the same reaction mixture did not affect their binding with bilirubin substantially. On the other hand, the presence of negatively charged liposomes did not affect the binding of bilirubin with erythrocytes (Fig. 3). The positively charged liposomes behaved differently; they were adsorbed onto the surface of erythrocyte (personal observation) resulting in overall increase in binding of bilirubin with erythrocyte (Figs. 3 and 4). Further, we also studied the relative binding of bilirubin to erythrocytes in the presence of liposomes. Our results show that erythrocyte-bound bilirubin can be easily knocked out by both HSA/OUT as well as HSA IN/OUT liposomes (Fig. 4). On the contrary, erythrocytes were not effective in the displacement of proteoliposome-bound bilirubin (Fig. 5). In other words, proteoliposomes bind bilirubin much better as compared to erythrocytes under these conditions.

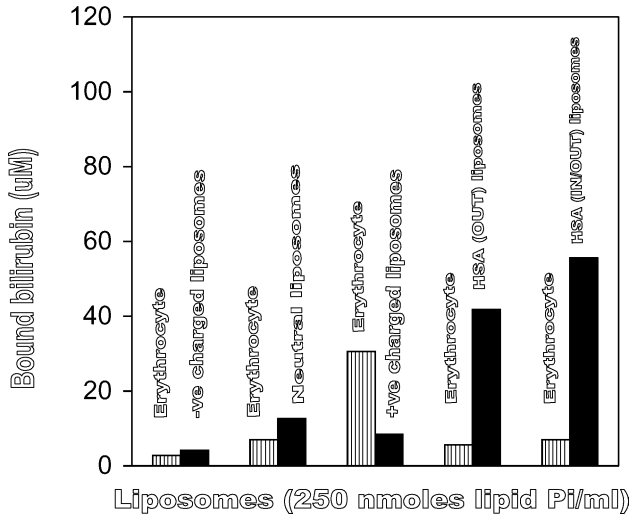

Fig. 5. Transfer of liposome-bound bilirubin to human erythrocytes. Liposomes (250 nmol lipid Pi) were preincubated with 2:1 HSA and bilirubin (total bilirubin 150 μM) for 1 h at 37 °C, keeping final volume 1 ml with TBS. The unbound bilirubin was removed and bilirubin-containing liposomes were then incubated with erythrocytes for 1 h at 37 °C. After a stipulated time period, the erythrocytes were separated from the above mixture by centrifugation. The bilirubin transferred to the erythrocytes/retained by liposomes was estimated following published procedure as described in the Materials and methods section. The data shown in the figure are representative of five different experiments.

Table 2
Cytotoxic effect of different liposomal preparations on HEK cells

Dose (nmol Pi/ml)	Percentage cell survival			
	Neutral liposomes	Negatively charged liposomes	Positively charged liposomes	HSA/OUT liposomes
0	98.5 ± 0.8	98.0 ± 1.0	99.2 ± 1.11	99.6 ± 0.6
125	98.8 ± 0.6	98.4 ± 0.6	97.2 ± 0.6	98.4 ± 1.2
250	97.2 ± 0.5	97.2 ± 0.8	96.2 ± 0.4	97.8 ± 0.8
500	96.2 ± 1.1	96.2 ± 1.2	95.5 ± 0.9	96.2 ± 0.4
1000	96.0 ± 1.2	96.4 ± 0.6	93.0 ± 0.8	96.6 ± 0.6
2000	96.4 ± 0.5	96.4 ± 0.8	92.2 ± 0.8	96.0 ± 0.2

The HEK cells were dispensed in a 96-well plate as described in Materials and methods section. The cells were treated with increasing doses (in six replicates) of 0–2000 nmol of liposomes (lipid Pi) per milliliter. After 48 h of incubation at 37 °C, MTT was added and formazan was dissolved by gentle shaking. The plate was read immediately in a micro plate reader (Ceres uv900c) operating at 540 nm. Wells with complete medium, liposome and MTT, but without cells, were used as blanks. Vincristine sulphate (40 nM) was able to induce 100% killing of the cells and was used as positive control. The percentage toxicity was determined by calculating cells surviving in the test wells as compared to the liposome-untreated HEK cells. The data represent means of six different observations ± S.D.

The toxicity of different liposomal preparations was ruled out by incubating cells with liposomes for different time periods. The killing of the cells was considered as parameter for assessing the safety of liposomes. Results shown in Table 2 clearly demonstrate that liposomes are safe at the dose of 2 μM Pi/ml.

Finally, various liposomal preparations were evaluated for their potential to eliminate plasma bilirubin from systemic circulation in model animals. Our preliminary experiments demonstrated that the single dose therapy (250 nmol lipid Pi) was not much effective in the elimination of plasma bilirubin from systemic circulation of the model animals. For example, the RSA IN/OUT liposomes were able to remove only 35–40% of the excess bilirubin present in the hyperbilirubinemic rats (data not shown). Moreover, it was followed by recurrence in the plasma bilirubin level and suggested that single dose therapy would not be successful in the treatment of hyperbilirubinemic conditions. We tried several dosage regimens and found that the schedule followed in the present study was most successful in normalizing the plasma bilirubin level in the hyperbilirubinemic rats.

Table 3
Liver functioning test of the bilirubin-exposed rats after treatment with different liposomal preparations

Group	Determination of liver functioning test	Alkaline phosphatase (IU/l)	SGOT (IU/l)	SGPT (IU/l)	Bilirubin (mg/dl)
Normal	Day 0	8.14 ± 0.74	15.5 ± 0.41	9.5 ± 0.41	0.53 ± 0.05
	Day 3	8.26 ± 0.90	16.0 ± 0.90	9.8 ± 0.85	0.56 ± 0.05
	Day 9	8.10 ± 0.40	16.2 ± 0.78	9.7 ± 0.80	0.51 ± 0.04
Untreated	Day 0	28.2 ± 0.85	34.13 ± 0.70	20.2 ± 0.58	1.84 ± 0.08
	Day 3	29.4 ± 0.60	35.0 ± 1.10	19.8 ± 0.90	1.85 ± 0.08
	Day 9	28.8 ± 0.52	35.2 ± 1.1	20.0 ± 0.80	1.89 ± 0.09 *
Egg PC liposomes	Day 0	20.8 ± 0.19	20.4 ± 1.1	15.5 ± 0.41	1.30 ± 0.047
	Day 3	20.0 ± 0.08	17.4 ± 0.80	15.2 ± 0.84	1.20 ± 0.09
	Day 9	19.8 ± 0.07	17.0 ± 0.60	15.0 ± 0.60	1.13 ± 0.09 *
RSA/OUT liposomes	Day 0	11.2 ± 0.49	16.8 ± 0.71	10.8 ± 0.24	0.84 ± 0.09
	Day 3	10.6 ± 0.38	16.0 ± 0.28	10.3 ± 0.24	0.80 ± 0.07
	Day 9	10.0 ± 0.34	15.8 ± 0.46	10.0 ± 0.30	0.76 ± 0.05 *
RSA IN/OUT liposomes	Day 0	9.4 ± 0.80	15.1 ± 0.18	9.9 ± 0.12	0.56 ± 0.04
	Day 3	9.2 ± 0.60	15.3 ± 0.28	9.8 ± 0.20	0.54 ± 0.02
	Day 9	8.4 ± 0.40	15.4 ± 0.45	9.6 ± 0.80	0.53 ± 0.02 *

The male albino Wistar rats ($n = 10$ for each group) were injected with bilirubin and RSA (2:1 ratio, total bilirubin 75 μM) in TBS as described in Materials and methods section. The hyperbilirubinemic animals were then treated with various liposomal preparations. The bilirubin level was determined after different time interval post-liposome treatment. The table shows level of different liver functioning parameters in 10 animals from each group. The animals were randomly bled before the treatment and the LFT was determined following published procedures. The data shown are mean of the values from 10 different animals ± S.E.
* $P < 0.001$.

Among different liposomal formulations tested on day 0, the egg PC liposomes eliminated around ~28% of the excess bilirubin, while RSA/OUT liposomes were effective in removal of the ~55% of the excess bilirubin when compared with the untreated animals. The level of plasma bilirubin was finally reduced to 1.13 and 0.76 mg/dl in egg PC liposome ($P < 0.001$) and RSA OUT ($P < 0.001$) liposome-treated animals, respectively, when determined on day 9 post-treatment. The RSA IN/OUT liposomes were found to be most effective as these successfully normalized the plasma bilirubin concentrations within 6 h (day 0) post-treatment ($P < 0.001$). Interestingly, there was no recurrence in plasma bilirubin level in RSA IN/OUT treated animals even on day 9 post-treatment. Besides, these liposomes were also successful in normalizing the other liver functioning enzyme levels as well (Table 3). There was no substantial reduction in plasma bilirubin level of the animals (control group) treated with free albumin even on day 9 post-treatment (data not shown).

4. Discussion

We have recently demonstrated that bilirubin has domain-specific binding sites on serum albumin [22,23]. In fact, the normal serum level of bilirubin is maintained by its specific binding with albumin that poses significant physiological repercussions in terms of bilirubin toxicity [1,4,6]. The bilirubin–albumin complex, when reaches to the liver, is specifically recognized by certain factors, the putative albumin receptors of the hepatocytes, which induce conformational changes in the albumin molecules to facilitate the release of bilirubin for subsequent degradation in the liver cells [24]. However, the bilirubin detoxifying function of the liver cells cannot cope well under conditions when plasma bilirubin exceeds its normal level [1,3,4]. The excess bilirubin remains in the systemic circulation for longer duration and exerts its cytotoxic effect to different tissues including brain cells [1,4,7].

It is widely believed that UB, a lipophilic compound, has an intrinsic affinity for lipids (cf. phospholipid) of biological membranes and other lipid structures such as vesicles and micelles [8,9]. In concordance with the earlier reports, the results of the present study confirm that liposome can interact with free bilirubin in time- and concentration-dependent manner (Figs. 1 and 2). Moreover, we observed that the size as well as lamellarity of the liposomes was not contributing much in binding with bilirubin (data not shown). However, the surface charge of the liposomes was crucial in bilirubin–liposomes interaction. The higher bilirubin binding to the positively charged liposomes indicated the involvement of electrostatic interactions in the binding process. In other words, it appears that among the three species of bilirubin, both bilirubin di-anion as well as mono-anion have preferences over bilirubin acid to bind with liposomes. The higher binding of the bilirubin with positively charged liposomes could be explained on the premise that apart from hydrophobic interactions, the anionic species of bilirubin should have an extra edge in binding with positively charged liposomes through ionic interactions as well.

Our study that involves interaction of bilirubin with liposomes in the presence of blood components implicates that bilirubin-bound positively charged liposomes have a tendency to undergo adsorption onto the surface of the erythrocytes (personal observation). This interaction can be attributed to the respective charges present on two

interacting moieties. Thus, the use of positively charged liposomes may have detrimental effects on erythrocytes, and might not be the right strategy for its future use in the treatment of hyperbilirubinemia. The observed binding behavior of positively charged liposomes with bilirubin in the presence of erythrocytes or whole blood led us to tailor the neutral egg PC liposomes for increasing their bilirubin binding capacity.

Keeping into consideration the higher affinity of albumin with bilirubin, we speculated that its entrapment in the aqueous core compartment, or coupling onto the outer surface of liposomes would enhance its binding with bilirubin several folds to that of the free albumin. The results of the present study clearly show the increased binding capacity of albumin-bearing liposomes with bilirubin (Figs. 1 and 2). The bilirubin–proteoliposome interaction was not affected by the presence of erythrocytes (Fig. 3). These liposomes not only possess higher binding to the free bilirubin but also have the potential to dislodge plasma membrane-bound bilirubin from the living cells as well (Fig. 4). It seems that such liposomes have the ability to bind with the free bilirubin through lipid moiety as well as albumin residues present on the surface.

For most of the in vitro experiments, we used HSA as model albumin with the idea that results of such studies can have direct correlation with their possible future use in the human subjects. However, we preferred to use RSA-bearing liposomes for in vivo studies performed in the Wistar rats. Nevertheless, RSA more closely mimics the physical properties of human albumin, at least with regard to bilirubin binding properties among different serum albumins [25]. Moreover, use of HSA was likely to exert some allogenic immunological responses in rats.

Our in vivo results suggest that proteoliposomes can offer an alternate approach to treat the hyperbilirubinemic conditions (Table 3). The liposome-based therapy should certainly find wide application, especially in the situations when hyperbilirubinemic conditions are induced by different pathological conditions or reduced albumin binding in sick or premature infants [26]. Similarly, jaundice induced by glucose-6-phosphate dehydrogenase deficiency with concurrent infection could also be successfully treated with albumin-bearing liposomes [27]. However, this approach would find limited scope in conditions when hyperbilirubinemic condition is inflicted by impairment of bilirubin glucuronosyl transferase, an enzyme responsible for glucuronidation of bilirubin before its final excretion in the bile [2–4].

Since liposomes are avidly taken up by the RES (liver and spleen), one can argue that liposome-based treatment can redirect bilirubin to its site of origin. This can be explained on the premise that after reaching the liver, the liposomes will have equal access to both kupffer cells as well as hepatocytes [28]. Besides, the presence of albumin receptors as well as bilirubin carrier molecules (bilitranslocase) on the surface of hepatocytes can facilitate the uptake of bilirubin-bearing liposomes [4,23,27]. Further, the proteoliposome can provide temporary sink to the bilirubin thus minimizing the risk of bilirubin toxicity. In fact, liposome-mediated uptake of bilirubin could be more advantageous in the sense that single molecule of the bilirubin upon interaction with its carrier present in the plasma membrane of the hepatocyte can carry several other molecules present in the single liposome.

The earlier methods for removing bilirubin from the plasma include use of resins or charcoals [29,30]. The resin columns are likely to induce hypokalemia/hypocalcemia-like situations by binding to the essential ions of the plasma [31], while charcoal has limitation in being minimally effective, as well as suffer with other blood compatibilities such as generation of particulate emboli [32]. Besides, most of these methods are prone to extracorporeal blood clotting and need use of excessive anticoagulants for hemoperfusion [33]. Similarly, phototherapy used for the treatment of jaundiced infants has its own limitations as the optimal dose of light is not yet clear and shorter wavelength light (350–450 nm) may be mutagenic [34,35].

Since higher plasma bilirubin level usually results from the liver disorders mainly [1–3,36], the liposomes can be proved of multipurpose importance as we can load them with the drugs having liver-correcting functions. Finally, we conclude that liposome-based therapy can have advantage over other cumbersome methods such as hemoperfusion or photooxidation where hospitalization as well as presence of medical staff become essential for the treatment and special arrangements have to be made for passing the blood through columns containing bilirubin-binding resins.

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References

[1] W.B. Karp, Pediatrics 64 (1979) 361–366.
[2] F. Vanstapel, N. Blanckaert, N. Tavoloni, P.D. Berk, Hepatic transport and bile secretion, Physiology and Pathophysiology, Raven Press, New York, 1993, pp. 477–488.
[3] S.C. Hauser, J.C. Ziurys, J.L. Gollan, Gastroenterology 91 (1986) 287–296.
[4] G.R. Gourley, Adv. Pediatr. 44 (1997) 173–229.
[5] W.H. Schwesinger, W.E. Kurtin, J. Surg. Res. 35 (1983) 520–524.
[6] S. Schenker, A.M. Hoyumpa, D.W. McCandless, Bilirubin toxicity to brain (kernicterus) and other tissues, in: J.D. Ostrow (Ed.), Bile Pigments and Jaundice, Marcel Dekker, New York, 1986, pp. 395–415.
[7] T.W.R. Hansen, D. Bratlid, Acta Paediatr. Scand. 75 (1986) 513–522.
[8] E.F. Erikson, D. Henning, R. Broderson, J. Biol. Chem. 256 (1981) 4269–4274.

[9] M.G. Mustafa, T.E. King, J. Biol. Chem. 245 (1970) 1084–1089.
[10] M.J. Hsu, R.L. Juliano, Biochim. Biophys. Acta 720 (1982) 411–419.
[11] R. Broderson, J. Biol. Chem. 254 (1979) 2364–2369.
[12] W.S. Singleton, M.S. Gray, M.L. Brown, J. Am. Oil Chem. Soc. 42 (1965) 53–56.
[13] A.F. McDonagh, F. Assisi, Biochem. J. 129 (1972) 797–800.
[14] M. Owais, C.M. Gupta, Eur. J. Biochem. 267 (2000) 3946–3956.
[15] M. Owais, G.C. Varshney, A. Chaudhury, S. Chandra, C.M. Gupta, Antimicrob. Agents Chemother. 39 (1995) 180–184.
[16] T.D. Heath, B.A. Macher, D. Papahadjopoulos, Biochim. Biophys. Acta 640 (1981) 66–81.
[17] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Oslon, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[18] J. Fog, Scand. J. Clin. Lab. Invest. 10 (1958) 241–245.
[19] S. Tayyab, M.K. Ali, J. Biochem. Biophys. Methods 39 (1999) 39–45.
[20] D.A. Scudiero, R.H. Shoemaker, K.D. Paull, A. Monks, S. Tierney, H. Nofziger, M.J. Currens, D. Seniff, M.R. Boyd, Cancer Res. 48 (1988) 4827–4833.
[21] M.A. Jafri, J.M. Subhani, K. Javed, S. Singh, J. Ethnopharmacol. 66 (1999) 355–361.
[22] M.M. Khan, S. Tayyab, Biochim. Biophys. Acta 36356 (2000) 1–15.
[23] S. Tayyab, N. Sharma, M.M. Khan, Biochem. Biophys. Res. Commun. 277 (2000) 83–88.
[24] R.G. Reed, C.M. Burrington, J. Biol. Chem. 264 (17) (1989) 9867–9872.
[25] S.D. Zucker, W. Goessling, J.L. Gollan, J. Biol. Chem. 270 (1995) 1074–1081.
[26] H.D. Arkans, G. Cassady, J. Paediatr. 92 (1978) 1001–1005.
[27] E. Beutler, Blood 84 (1994) 3613–3636.
[28] D.K. Meijer, P. Vander Sluijs, Pharm. Res. 6 (2) (1989) 105–108.
[29] S. Sideman, L. Mor, J.M. Brandes, Trans. Am. Soc. Artif. Intern. Organs 25 (1979) 497–500.
[30] T.S. Lie, W.I. Kim, A. Holst, U. Grunn, K. Rommenshein, Acta Hepato-Gastroenterol. 23 (1976) 262.
[31] J.L. Rosenbaum, G. Onesti, C. Heider, J. Am. Med. Assoc. 180 (1962) 762–764.
[32] T.M.S. Chang, A. Gonda, J.F. Dirks, T. Lee-Burns, Trans. Am. Soc. Artif. Intern. Organs 18 (1972) 465–467.
[33] Y. Idezuki, M. Hamaguchi, S. Hamabe, H. Moriya, T. Nagashima, H. Watanabe, T. Sonoda, K. Teramoto, T. Kikuchi, H. Tanzawa, Trans. Am. Soc. Artif. Intern. Organs 27 (1981) 428–430.
[34] J.F. Ennever, Clin. Perinatol. 17 (1990) 467–481.
[35] S. Onishi, S. Itoh, K. Isobe, Biochem. J. 236 (1986) 23–25.
[36] J. Gollon, R. Schmid, R. Wright, G.H. Millward-Sadler, K.G.M.M. Albertii, S. Karran (Eds.), Liver and Biliary Disease, 2nd edn., Baillere-Tindall, London, 1985, pp. 301–357.