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Toxicity of gamma irradiated liposomes. 1. In vitro interactions with blood components

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Abstract

Gamma irradiation is a potential technique for sterilisation of liposome suspensions. Unfortunately, gamma irradiation may result in chemical degradation of the phospholipids and the toxicological aspects have to be considered. The effects of liposome composition and gamma irradiation on the interactions of the liposomes with the hemostatic mechanisms (hemolysis, aggregation and coagulation) were studied. Non-irradiated liposome suspensions showed no hemolysis of erythrocytes. After irradiation, up to 3.1% hemolysis was measured. Least hemolysis was observed with irradiated liposomes composed of unsaturated or charged phospholipids. The negatively charged DSPG-liposomes (both non-irradiated and irradiated) induced aggregation of platelets as observed by the spectrophotometric method. However, no aggregates were seen in the microscope or measured by the aggregometer. Negatively charged liposomes also affected the coagulation cascade where prolonged coagulation times were measured. Irradiation of the liposome suspensions resulted in even longer coagulation times. The prolonged coagulation times correlated to some extent with the measured binding and depletion of calcium from plasma by the negatively charged liposomes.

Keywords: Liposomes; Gamma irradiation; Toxicity; Blood components

1. Introduction

Gamma irradiation is an approved sterilisation method for some pharmaceuticals (Woods and Pikaev, 1994; Reid, 1995) and might be an alternative sterilisation method for liposomes. Based

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on the radiation response of the radiation resistant spores of *Bacillus pumilus*, a dose of 25 kGy has generally been accepted as a suitable sterilisation dose.

During gamma irradiation, the liposomes may be affected directly by irradiation or through radicals formed by radiolysis of water. Because of the unlimited access for these radicals to attack the liposomal membrane, the indirect effect of irradiation is considered to be most important (Stark, 1991).

A review of the literature on the effects of gamma irradiation on the liposomes has been presented by Albertini and Rustichelli (1993). Unfortunately, degradation of the phospholipids takes place during gamma irradiation (Tinsley and Maerker, 1993; Stensrud et al., 1996; Zuidam et al., 1996a). The physical stability is affected to a lesser extent (Zuidam et al., 1996a; Stensrud et al., 1997). In addition to peroxidation of unsaturated phospholipids (Zuidam et al., 1995), lysophospholipids, free fatty acids, phosphatidic acid and different hydrocarbons have been identified as irradiation induced degradation products (Tinsley and Maerker, 1993; Zuidam et al., 1996a). Zuidam and co-workers have also recently published a review of possible sterilisation methods for liposomes (Zuidam et al., 1996b). They concluded that more studies are necessary in order to evaluate and optimise gamma irradiation as a convenient and safe sterilisation method for liposomes.

Liposomes are composed mainly of phospholipids, and phosphatidyl choline (PC) is usually the major component. These natural occurring compounds have proved to be safe for pharmaceutical use. Prior to acceptance of a liposomal preparation for clinical use, however, possible side effects of the carrier need to be investigated. Adverse effects like impairment of the mononuclear phagocyte system (MPS), blood clotting disturbances, capillary blocking and immunogenicity associated with in vivo administration of liposomes have been reported (Allen, 1993; Parnham and Wetzig, 1993; Storm et al., 1993).

When anticipating possible adverse effects of liposomes by parenteral administration, the interaction of liposomes with hemostatic mechanisms must be considered. Clot formation is a response to blood vessel wall rupture. The clot formation is due to a series of cellular alterations in blood platelets leading to aggregation and the formation of a primary hemostatic plug. The conversion of coagulation factors present in the circulating blood to active enzymes lead to the formation of a fibrin network. Both mechanisms are closely related during the hemostatic process (Zwaal, 1978). Lipids play an important role in the blood coagulation process. Phospholipid vehicles guide and accelerate clot formation in vitro by providing a suitable lipid/water interface for interacting coagulation factors. Phospholipids are crucial both in the intrinsic and the extrinsic coagulation cascade in vivo, where they participate in at least three different complex formations followed by enzyme activation (Williams, 1983). The nature of the phospholipids is important for the coagulation activity. Zwaal (1978) has summarised the specific properties of the lipid surface required to achieve optimal coagulation activity. The factors includes optimal negative surface charge at the lipid/water interface, homogenous charge distribution, presence of additional stabilising nucleophilic groups, size and membrane fluidity.

Liposomes composed of charged phospholipids seem to give more toxic effects than the neutral ones. Negatively charged liposomes stimulates the plasmatic contact activating system as demonstrated by the acceleration of whole blood clotting time and induces reversible platelet aggregation (Zbinden et al., 1989). Charged liposomes may inhibit the response of platelets to physiological stimuli for aggregation (adenosine diphosphate, thrombin) (Juliano et al., 1983; Bonté et al., 1987) and to collagen-mediated platelet aggregation (Zakrevsky et al., 1994). Increased clotting time as measured by activated partial thromboplastin time (APTT) and prothrombin time (PT) was reported for polymerizable phosphatidyl choline vesicles. This was explained as binding and depletion of clotting factor V from plasma (Bonté et al., 1987). The same retarding effects were also observed for negatively charged liposomes by Miller et al. (1992), but in this case the coagulation disturbances were explained by adsorption of Ca^{2+} to the liposomes and the replacement of free Ca^{2+} partially reversed this effect.

Phospholipid degradation products such as lysolecithin (LPC) and peroxidised lipids are known to induce hemolysis of erythrocytes (Weltzien, 1979; Kobayashi et al., 1985). Lysolecithin is a surfactant which forms micelles instead of bilayers and causes destabilisation of the liposomal membrane (Inoue and Kitagawa, 1974). The general steps in the phenomenon of hemolysis induced by lysophophospholipids and short-chain phospholipids are explained as adsorption of the lysophosphatide to the membrane and penetration into the matrix with subsequent induction of changes in the molecular organisation. This results in a change in the permeability of ions through the membrane and a disturbance of the osmotic equilibrium. Finally, haemoglobin leaks out (Reman et al., 1969; Weltzien, 1979; Tanaka et al., 1983). Important parameters controlling the lytic activity of lysophospholipids are temperature, type of erythrocytes employed and the chemical structure of the lysolipid molecule (Weltzien, 1979).

Degradation of the phospholipids takes place during gamma irradiation and the presence of degradation products may enhance the toxic potential of the liposomes. In this study, the possible toxic effects of different kind of gamma irradiated liposomes were evaluated. Interactions of liposomes with the platelets, the coagulation cascade and the erythrocytes were investigated.

2. Materials and methods

2.1. Materials

Dimyristoyl phosphatidyl choline (DMPC), distearoyl phosphatidyl choline (DSPC), distearoyl phosphatidyl glycerol (sodium salt) (DSPG) and soya phosphatidyl choline (soya PC) were kindly provided by Nattermann Phospholipid, Köln, Germany. Egg L-α-phosphatidyl choline (egg PC), Myristic acid (MA), Drabkin's reagent, 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), adenosine diphosphate (ADP) and Triton-X 100 were obtained from Sigma Chemical (St. Louis, USA). Myristoyl lysophosphatidyl choline (LPC-C_{14}), palmitoyl lysophosphatidyl choline (LPC-C_{16}) and egg lysophosphatidyl choline (egg-LPC) were purchased from Avanti Polar-Lipids (Pelham, AL, USA). Cephotest™ (APTT reagent, activated partial thromboplastin time) and the prothrombin reagents Thrombotest™ (bovine thromboplastin) and Normotest™ (rabbit thromboplastin) were purchased from Nycomed Pharma (Oslo, Norway). Cobas® Integra Calcium (CA) was purchased from Roche. All other chemicals were of analytical grade.

2.2. Preparation and characterisation of liposomes

The phospholipids were dissolved in chloroform/methanol (2:1) and evaporated to dryness in a 250 ml round bottom flask. The thin film obtained was further dried under reduced pressure for at least 15 min. The phospholipid film was then hydrated above the phase transition temperature with phosphate-buffered saline (PBS) pH 7.4 (Ph. Eur.) and gently shaken for 15 min. The mixture was allowed to swell for 2 h above the phase transition temperature (N_2 atmosphere). When mannitol was used as a radical scavenger, it was dissolved in the hydration medium (44 mg/ml). The final concentration of the liposome suspension was 10 mg/ml. The liposome suspension was extruded three times through a 2-stacked 0.6 mm polycarbonate membrane (Nucleopore®, Costar, Cambridge, USA) followed by ten passages through a 2-stacked 0.1 mm polycarbonate membrane above the phase transition temperature with a Lipex extruder (Lipex Biomembranes, Vancouver, Canada). LPC-micelle solutions for the hemolysis experiments were prepared in the same way as the liposomes. The liposomes were filled in 5 or 10 ml vials under N_2 atmosphere and stored in the refrigerator (2–8°C).

The liposome suspensions were irradiated the following day at ambient temperature with a ^{60}Co source. The irradiation doses were 10, 25 and 50 kGy with a dose rate of 15 kGy/h ($\pm$ 1 kGy). The exposed dose was measured by a dosimeter (type 4034, Harwell. Before carrying out the experiments, the size of the liposome suspension was measured again and the liposome suspension was finally extruded once more to ensure a small size/size distribution.

The lipid content of the liposomes was eventually measured using the phosphorus assay described by Bartlett (New, 1990).

The mean diameter of the liposomes was measured at a 90° angle at 25°C by photon correlation spectroscopy (PCS, Coulter N4 MD). Samples were diluted in PBS and measurements were performed in triplicate using the unimodal model for size distribution. The refractive index and viscosity of pure water were used in the calculations.

The zeta potential of the liposome suspension was measured with a Doppler-Elecrophoretic Light Scattering Analyser (Coulter DELSA 440®). The liposome suspension was diluted in the hydration medium and analysed at 25°C. The zeta potential (ξ) were calculated from the mobility (U) by means of the Smoluchowsky equation: $\xi = 4\pi\eta U/\epsilon$, where the viscosity of the suspending medium (η) and the dielectric constant (ϵ) were 0.0089 poise and 78.36, respectively.

For the cryo-electron micrographs, the liposome dispersions were vitrified in thin films on 200 mesh copper grids coated with a perforated carbon film. Liquid ethane was used as cryogen. A further description of this method is given by Dubochet et al. (1988). The preparation of the samples was carried out in a flow of humid air, in order to reduce the evaporation and drying effects (Cyrklaff et al., 1990). The samples were observed at −170°C in a Philips CM12 transmission electron microscope using a Gatan 626 cryo-transfer system. Micrographs were recorded under low dose condition.

2.3. Hemolysis experiments

The method was a modification of a method earlier described by Kristensen et al. (1994). A total of 2 ml of EDTA-treated human blood was centrifuged for 15 min at 1500 rpm ($470 \times g$) (KUBOTA, KS-8000, Japan) and the plasma removed. The RBC were washed three times with 5 ml PBS pH 7.4 (Ph. Eur) and centrifuged for 10 min at 2000 rpm ($840 \times g$) between each washing procedure. The RBC were further resuspended in 5 ml PBS. Hematocrit measurements were performed using Microhematocrit Centrifuge (Hematocrit 4, LIC Lars Ljungberg, Sweden) by rotation for 3 min and the concentration (% v/v) was determined. The washed blood cells were stored in the refrigerator and used within 48 h. The same donor blood was used in all experiments.

Samples of 1% (v/v) RBC, 5.5 mM glucose and liposomes (normally 1 mg/ml, ≈ 1.35 μM) were prepared in PBS. Samples without liposomes and samples without RBC were used as references. The samples were incubated in a shaking water bath (Haake SWB 20, Denmark) for 30 min at 37°C. The samples were centrifuged for 10 min at 2000 rpm ($840 \times g$) to remove intact RBC and cell debris. The supernatant was removed and mixed with Drabkin's reagent (1:4). The absorbance of the samples was measured at 540 nm (Shimadzu UV-Vis, UV-160 A spectrophotometer, Japan).

Multiple samples were used ($n = 2$–4), where each sample was prepared in duplicate. Intra-batch relative standard deviation was 6.1% for 3.0% hemolysis ($n = 10$). The lower detection limit was set to 0.5% hemolysis.

The percentage hemolysis was calculated as follows: ABS (hemolysis) = ABS (sample) − ABS (RBC-free reference) − ABS (liposome-free reference).

A standard curve was calculated to convert the absorbance of the sample to percentage hemolysis. The standard curve was prepared by dilution of a 100% hemolysed RBC-suspension (liposome-free reference) assuming that erythrocytes in the sample (1% v/v) were 100% hemolysed after addition of 2 μl/ml Triton-X 100. The standards were prepared and measured in the same way as the samples. A new standard curve was calculated after every 14 days.

2.4. Platelet aggregation experiments

The spectrophotometric method is a modification of a method previously described by Lüthje and Ogilvie (1987) for platelet aggregation measurements in whole blood. The platelet-count in the supernatant is an indirect measure of the extent of platelet aggregation. Citrated platelet-rich plasma (PRP) (200 000 platelets/ml) were obtained from fresh human blood by centrifugation. The plasma was mixed with the liposome suspen-

sion or equal volume of PBS (blank sample, 2.7 mg/ml) and incubated at 37°C. 125 μl probes were withdrawn every third minute and immediately fixed in 0.8 ml EDTA/formalin solution containing EDTA (12 mM), formalin (1%, w/w), KCl (2.68 mM), KH_2PO_4 (1.47 mM), NaCl (136.9 mM) and Na_2HPO_4 (6.46 mM) pH 7.4. After centrifugation for $130 \times g$ for 7 min, 200 μl of the supernatant was dispensed to 96-well plates and measured at 595 nm. Equal concentration of the liposome suspension in PBS was centrifuged and used as reference. The ability of the platelets to aggregate was controlled by addition of 1 μM ADP. The ability of the liposome suspension to inhibit ADP-induced aggregation was performed in the same way. After 10 min incubation at 37°C, 8 μM ADP was added and samples were withdrawn every 20 s. All the experiments were performed in triplicate.

The samples were examined for aggregates in the microscope. PRP was incubated with the equal volume of liposome suspension and incubated for 30 min at 37°C. The sample was mixed with EDTA-formaline fixative and examined.

The ability of the liposome suspensions to induce platelet aggregation was further controlled with the use of an aggregometer. This method is in routinely clinical use. Shortly, the liposome suspension was diluted with PRP and albumin buffer and transfered into a Payton dual channel aggrometer and stirred. Ristocetin was added as aggregating agent (1 mg/ml).

2.5. Coagulation experiments

Immediately before the experiments the fresh human citrated blood was centrifuged for 15 min at 3400 rpm ($2000 \times g$) at 4°C and the plasma was removed. The plasma was incubated with the liposomes (0.2–2.5 mg/ml) or equal volumes of PBS for 30 min at 37°C. APTT and prothrombin time (Thrombotest™ and Normotest™) were then assayed. The former assay is used to evaluate the effects on the intrinsic coagulation system whereas the other two tests evaluates the effects on the extrinsic system (Williams, 1983). The time required to form fibrin was measured using an automated coagulometer (ACL Futura, Instrumentation Laboratory, Milan, Italy). The experiments were performed in triplicate. The same donor was used in all the experiments.

The free and complexed calcium was measured as follows; after incubation of the liposome suspension or PBS with plasma (1:4) for 30 min at 37°C, the mixture were ultrasentrifuged ($100\,000 \times g$ for 1 h). The Ca^{2+} concentration in the supernatant (free and complexed) was measured using an in vitro diagnostic reagent system (Cobas® Integra (CA), Roche) intended for use on an automated instrument (Cobas® Integra). Calcium ions react with *o*-cresolphthalein complexone (p-CPC) under alkaline conditions to form a violet coloured complex. The colour intensity formed is directly proportional to the calcium concentration and is measured as increase in absorbance at 552 nm (Schwarzenbach, 1955). The experiments were performed in triplicate and each sample was analysed three times.

The measurement of free calcium was performed after incubation of the samples with plasma (1:4) for 30 min at 37°C with a calcium ion-selective electrode (model 92–20, Orion, Boston, USA) connected to a pH/ISE meter (model 720 A, Orion, Boston, USA). The experiments were performed in triplicate and each sample was analysed two times.

2.6. Statistical analysis

The observed effects were tested for significance by analysis of variance or linear regression and considered significant when $P < 0.05$.

3. Results

The different liposomal compositions used and the corresponding zeta potentials are shown in Table 1. DSPC, DMPC, soya PC and egg PC are originally slightly negatively charged whereas DSPG is negatively charged. Irradiation of the liposomes increased the negative surface charge.

Table 1
Zeta potential of different liposome suspensions (PBS, pH 7.4) before and after irradiation (10, 25 and 50 kGy)[a]

	Zeta potential (mV)							
	0 kGy		10 kGy		25 kGy		50 kGy	
DSPC	−1.9	(1.1–2.5)	−4.7	(4.0–5.5)	−6.9	(4.8–9.7)	−11.9	(8.6–15.5)
DMPC	−1.6	(1.4–1.9)	−3.6	(3.2–3.8)	−5.4	(5.2–5.5)	−5.2	(2.4–8.1)
DSPG	−30.6	(29.6–31.0)	−30.2	(29.2–31.9)	−29.0	(27.1–30.6)	−31.8	(31.0–32.7)
20% DSPG/DSPC	−13.4	(12.0–15.2)	−16.8	(15.5–17.5)	−15.5	(14.6–16.2)	−25.3	(24.8–25.6)
Soya PC (O)	−1.0	(0.8–1.1)	−4.3	(3.3–6.1)	−6.8	(4.8–8.7)	−7.8	(7.5–8.3)
Soya PC (N)	−1.0	(0.2–1.5)	−4.4	(3.7–5.3)	−6.6	(4.8–7.6)	−5.9	(4.4–7.4)
Egg PC (N)	−2.0	(1.8–2.2)	−2.1	(1.9–2.1)	−4.3	(4.2–4.5)	−5.2	(4.9–5.3)

[a] O denotes oxygen atmosphere and N nitrogen atmosphere. Mean, min and max values for three different batches are reported.

The average diameter and the width of the size distribution (S.D.) of the different extruded liposome suspensions varied from 88 to 104 nm and from 23 to 35 nm, respectively. The same size was measured for both the non-irradiated and the irradiated liposomes. Non-irradiated DSPC-liposomes tended to form aggregates, but de-aggregated easily after heating above the phase transition temperature (T_m).

In Fig. 1, the cryo-electron micrographs of DSPG-liposomes before and after irradiation are shown. The liposomes are in both cases mainly unilamellar and faceted. The size and the shape of the liposomes seem to remain unchanged after the irradiation. We have earlier shown cryo-electron micrographs of neutral liposomes composed of egg PC and DPPC where the size and shape of individual liposomes also remained unchanged after irradiation (Stensrud et al., 1997).

3.1. Platelet aggregation experiments

In vitro aggregation of platelets was observed by incubation with negatively charged DSPG-liposomes by the spectrophotometric technique. The irradiation did not affect the state of aggregation. The liposome suspensions showed no influence on the ADP-induced platelet aggregation. On the other hand, no aggregation of these liposomes was observed when the liposomes were added to plasma and stirred in the aggregometer. No platelet aggregation was observed in the microscope with any of the liposome suspensions studied.

3.2. Hemolysis experiments

No hemolysis of the RBC was detected when incubation with the non-irradiated liposome suspensions. Irradiated liposome suspensions resulted in hemolysis and the hemolysis increased with higher irradiation doses (Fig. 2). Most often this relationship was non-linear. This means that the increase in hemolysis was most pronounced at lower irradiation doses. An adequate fit of the model could be obtained by neglecting the reference point (0% hemolysis) or by using a quadratic term in the model. The significance of the effects was evaluated in accordance to the optimised model. The regression was significant ($P < 0.01$).

Negatively charged liposomes showed less hemolysis after irradiation compared to the neutral ones. The degree of hemolysis caused by irradiated liposomes was as follows: 20% DSPG/DSPC < DSPG < DSPC. DMPC-liposomes (C_{14}-chain length) caused significant more hemolysis compared to DSPC-liposomes (C_{16}-chain length) after irradiation. Significant differences between liposomes composed of unsaturated phospholipids compared to saturated ones were observed as the degree of hemolysis was as follows; soya PC < egg PC < DSPC.

The hemolysis caused by the irradiated liposomes depended upon the liposome concentration. The hemolysis was negligible at concentrations below 0.1 mg/ml (Fig. 3).

The inclusion of mannitol as a radical scavenger in the liposome formulation decreased the

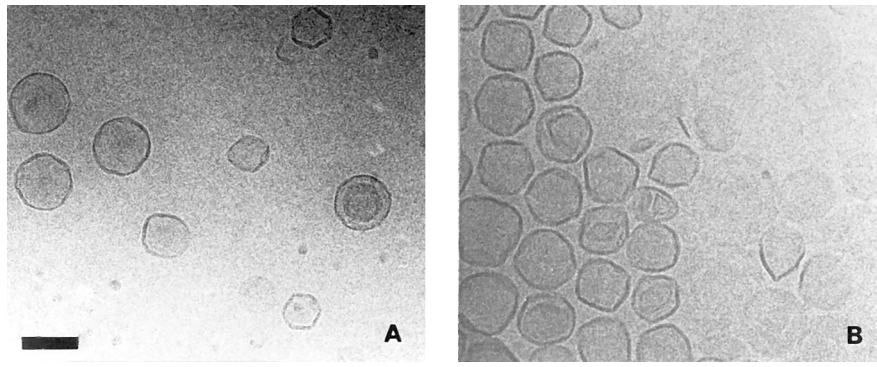

Fig. 1. Cryo-electron micrographs of of non-irradiated (A) and irradiated (B) DSPG-liposomes. Bar represents 100 nm.

irradiated DSPC-liposome induced hemolysis up to 62%. The protective effect was most pronounced at lower irradiation doses (10 and 25 kGy).

An increase in hemolysis was observed when up to 20 mol% LPC-C_{14} was incorporated in the DMPC liposomes (Fig. 4).

The hemolysis induced by increased LPC-micelle concentration was characterised by a sigmoidal lysis curve. Observed L_{50} values (concentration of LPC needed for 50% hemolysis) were 21.7 μM for egg-LPC, 37.7 μM for LPC-C_{14} and 18.7 μM for LPC-C_{16}.

Autoclaving of the DSPC-liposomes (121°C, 20 min) resulted in more hemolysis (4.3%) than liposomes sterilised by irradiation (2.5% with 25 kGy).

3.3. Coagulation experiments

Incubation of plasma with negatively charged liposomes (20% DSPG/DSPC and DSPG) resulted in a significant prolongation of the APTT (Fig. 5). Irradiation induced a further and dose-dependent prolongation of the APTT (Fig. 6). Also liposomes composed of soya PC showed to some extent an extended APTT after irradiation. For the other liposome suspensions no effect was seen. With lower lipid concentrations, the APTT values dropped significantly and approached the normal range (Fig. 7).

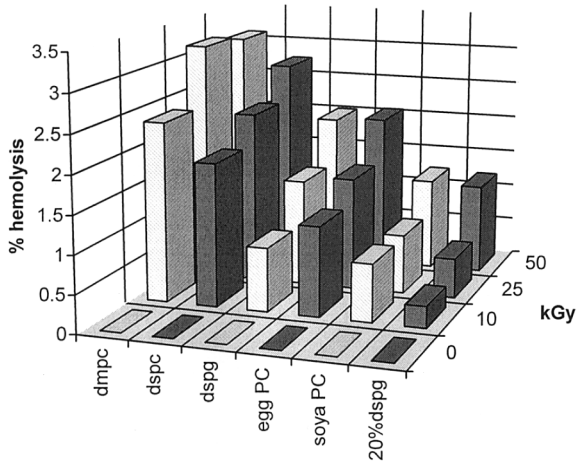

Fig. 2. The percentage hemolysis caused by different liposome-suspensions (1 mg/ml) after irradiation (10, 25 and 50 kGy). Non-irradiated samples showed no hemolysis ($n = 2–4$).

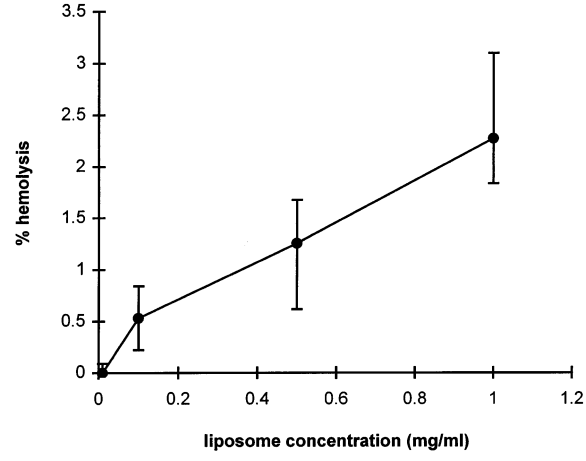

Fig. 3. The percentage hemolysis caused by different concentrations of DSPC-liposomes irradiated 25 kGy. Error bars denote min and max values ($n = 4$).

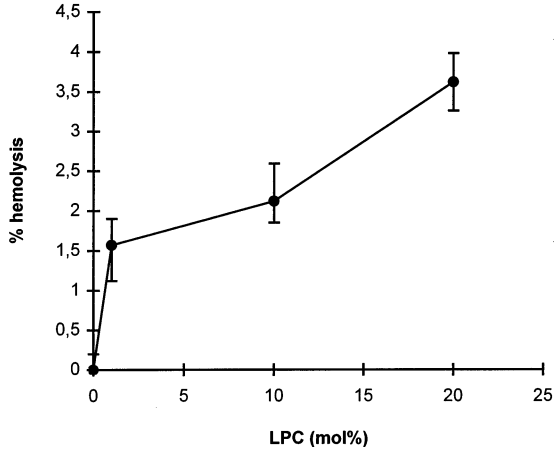

Fig. 4. The percentage hemolysis induced by different amounts of LPC-C_{14} incorporated in DMPC-liposomes (1 mg/ml). Error bars denote min and max values ($n = 3$–4).

The coagulation activity of the extrinsic coagulation system assayed by the use of bovine thromboplastin was only affected by liposomes composed of DSPG. Before irradiation, DSPG-liposomes caused a slight, but non-significant shortening of the prothrombin time (PT). With increasing irradiation doses, the PT's were progressively prolonged (Fig. 8). The effects of DSPG-liposomes on PT was dependent on the concentration with shorter PTs at low concentrations (Fig. 9). The DSPG-liposomes showed even shorter PT than PBS (reference).

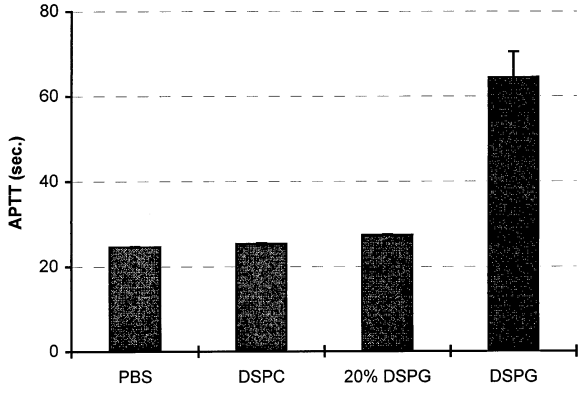

Fig. 5. Effect of charge incorporation in the liposomes (2.5 mg/ml) on the intrinsic coagulation system (APTT, activated partial thromboplastin time). Error bars denote min and max values ($n = 3$).

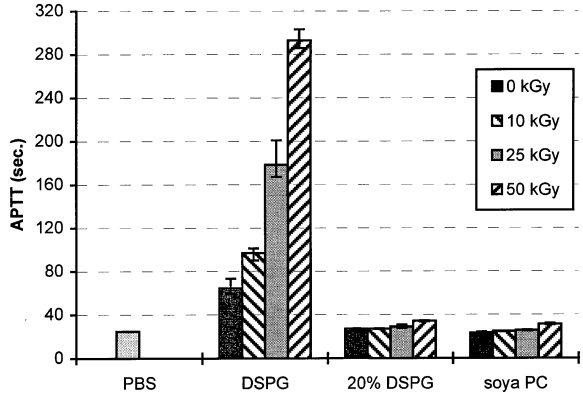

Fig. 6. Effect of non-irradiated and irradiated liposomes (2.5 mg/ml) on the intrinsic coagulation system (APTT, activated partial thromboplastin time). The liposomes were irradiated 0 (non-irradiated), 10, 25 and 50 kGy. Error bars denote min and max values ($n = 3$).

The same effect on the extrinsic coagulation system was also measured by the use of rabbit thromboplastin (Normotest™). Non-irradiated DSPG-liposomes showed a slight, but significantly shorter coagulation time compared with PBS, but after irradiation longer coagulation time was measured (Fig. 10).

Incubation with DSPG-liposomes in plasma showed depletion of Ca^{2+} from plasma, especially when irradiated liposomes were used (Table 2). The neutrally charged DSPC-liposomes (both

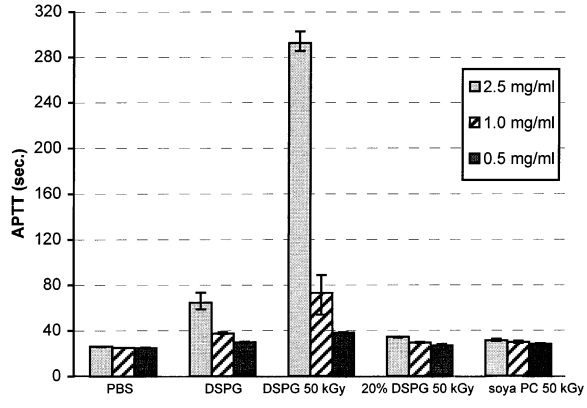

Fig. 7. Effect of various concentrations of liposomes on the intrinsic coagulation system (APTT, activated partial thromboplastin time). The phospholipid concentrations are: 2.5; 1.0; and 0.5 mg/ml. Error bars denote min and max values ($n = 3$).

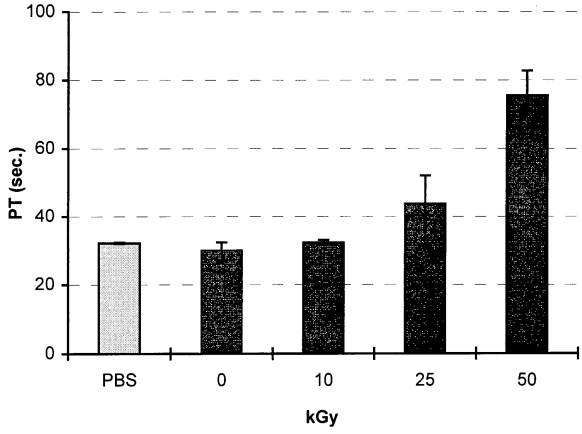

Fig. 8. Effect of non-irradiated and irradiated DSPG-liposomes (2.5 mg/ml) on the extrinsic coagulation system assayed by the use of bovine thromboplastin (PT, prothrombin time). The liposomes were irradiated 0 (non-irradiated), 10, 25 and 50 kGy. Error bars denote min and max values ($n = 3$).

non-irradiated and irradiated) had no influence on the Ca^{2+} concentration.

By addition of calcium to the plasma incubated with liposomes, the effects on blood coagulation due to the negatively charged liposomes were reduced. Differences in the coagulation times were observed when calcium was added before or after the incubation of plasma with liposomes (Fig. 11).

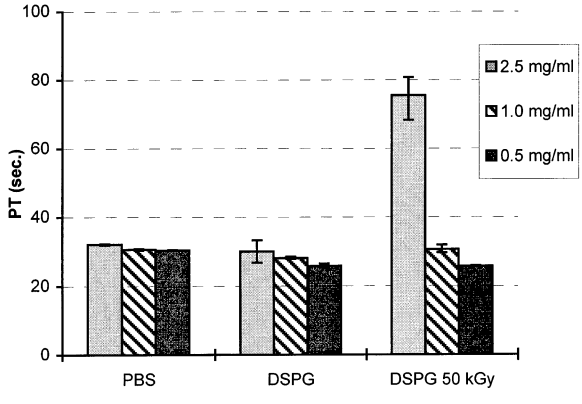

Fig. 9. Effect of various concentrations of DSPG-liposomes (non-irradiated and irradiated 50 kGy) on the extrinsic coagulation system assayed by the use of bovine thromboplastin (PT, prothrombin time). The phospholipid concentrations are 2.5; 1.0; and 0.5 mg/ml. Error bars denote min and max values ($n = 3$).

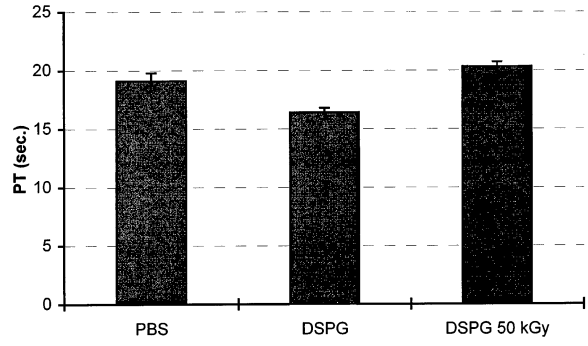

Fig. 10. Effect of negatively charged DSPG-liposomes (non-irradiated and irradiated 50 kGy) on the extrinsic coagulation system measured with the Normotest™ assay (rabbit thromboplastin) (PT, prothrombin time). Error bars denote min and max values ($n = 3$).

4. Discussion

4.1. Platelet aggregation experiments

Notable liposome-induced platelet aggregation was observed only with the negatively charged DSPG-liposomes (spectrophotometric method) in this study, which is in agreement with previously reported studies (Zbinden et al., 1989). Because no aggregation was observed using an aggre-

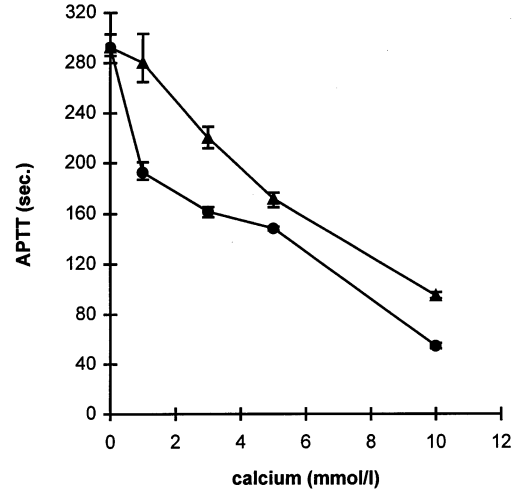

Fig. 11. Effect of addition of calcium to the plasma before (▲) or after (●) incubation with the liposomes on the intrinsic coagulation system (APTT). Error bars denote min and max values ($n = 3$).

Table 2
The concentration of Ca^{2+} in plasma after incubation with different liposome suspensions[a]

	Ca^{2+} (mmol/l)			
	Free and complexed		Free	
Reference (PBS)	1.40	(1.39–1.41)	0.52	(0.49–0.57)
DSPC	1.42	(1.41–1.44)	0.55	(0.50–0.61)
DSPC 50 kGy	1.40	(1.39–1.40)	0.52	(0.51–0.54)
DSPG	1.28	(1.27–1.28)	0.40	(0.37–0.42)
DSPG 50 kGy	1.02	(1.02–1.03)	0.30	(0.27–0.31)

[a] The mean values for three different samples are reported together with min and max values.

gometer of the type normally used for clinical assay of platelet function, these aggregates had to be of the reversible type. Reversible aggregates are usually not retained in the capillary bed and do not therefore represent a potential hazard. In our study, the microscopy method also failed to prove any aggregation. The aggregates were most probably very small which rendered the detection in the microscope difficult.

Zbinden et al. (1989) observed platelet aggregation caused by negatively charged liposomes (PC:PA 8:1) down to a lipid concentration of 0.19 mg/ml by counting the platelets in a counting chamber with a microscope. In addition, they observed platelet aggregation induced by the same liposomes in an in vivo study with guinea pigs. They proved that the aggregates formed are small and of the reversible type. Therefore, the aggregation was not detected with the use of an aggrometer, which is in accordance with our results and with results obtained by other groups using this method (Juliano et al., 1983; Bonté et al., 1987). The observed aggregation was explained by Zbinden et al. (1989) as an interaction of the negatively charged liposomes with the positively charged amino groups (2.5×10^5), which are present in addition to the negatively charged group (20.5×10^5), on the platelets. The liposomes act as bridging agents and keep the platelets sufficiently apart to avoid contact between the platelets.

4.2. Hemolysis experiments

In these hemolysis experiments an approximately logarithmic relationship was observed for the degree of hemolysis caused by the liposomes versus irradiation dose of the liposomes. This means that liposomes irradiated with low doses caused comparatively more hemolysis than liposomes irradiated with higher doses. This observation might be explained by the irradiation induced degradation kinetic of the phospholipids. The slope of phospholipid degradation versus irradiation dose is also logarithmic (Stensrud et al., 1996; Zuidam et al., 1996a). This means that comparatively more degradation take place at lower irradiation doses. In addition, water soluble degradation products were found only after exposure to higher irradiation doses (25 and 50 kGy, Stensrud et al., 1996). These products are formed from degradation of LPC and leads to a decrease in the amount of LPC. Irradiation also resulted in the enhancement of net additionally negative charge of the liposomes (Table 1). The washed red blood cells in our experiments were negatively charged (-16 mV) and liposomes with a negative charge might be repulsed.

Liposomes composed of phospholipids with different headgroups show different susceptibility to gamma irradiation. Phospholipids with glycerol (PG) as the headgroup are more easily degraded than phospholipids with choline (PC) as the headgroup, due to the more direct contact of the more soluble PG salt with the reactive water radiolysis products (Tinsley and Maerker, 1993; Stensrud et al., 1996; Zuidam et al., 1996a). The decreased contact between the negatively charged erythrocytes and the negatively charged liposomes is most probably the explanation for the lower

degree of hemolysis observed for the negatively charged phospholipids, although more chemical degradation is observed for these phospholipids. In addition, irradiation induced degradation of PG might, compared to PC, result in greater amount of other degradation products (non-hematolytic) than the hematolytic active compound LPC (Tinsley and Maerker, 1993). Negatively charged DSPG-liposomes showed more hemolysis after irradiation than negatively charged 20% DSPG/DSPC-liposomes due to the higher content of the easily degradable PG.

Optimal lytic capacity is reported for aliphatic lysocompounds with chains of 16 or 18 carbons due to optimal capability for these compounds to bind to the cells (Reman et al., 1969; Weltzien et al., 1977). We observed, however, that DMPC-liposomes (C_{14}-chain) caused more hemolysis than DSPC-liposomes (C_{18}-chain) after irradiation. This observation can be explained by the different phase transition behaviour of the liposomes since it has been shown that the hemolysis increases above the phase transition temperature (Martin and MacDonald, 1976). DMPC-liposomes are in the fluid state at 37°C ($T_m = 23.5$°C) whereas DSPC-liposomes are in the gel state ($T_m = 55.5$°C). Also Yoshihara and Nakae (1986) reported lower hemolytic activity of the phospholipids in the gel state. In addition, as the chain length of neutral liposomes decreases, the rate of hydrolysis tends to increase (more LPC formed, Grit and Crommelin, 1993). No experiments have so far been carried out in order to determine if the same is true for gamma irradiation induced degradation.

An increase in the number of double bonds lead to a decrease of hemotolytic activity (Gottfried and Rapport, 1963; Reman et al., 1969). We observed less hemolysis for the liposomes composed of unsaturated phospholipids (soya PC and egg PC) than for the saturated ones (DSPC). Soya PC contains more double bonds than egg PC and showed also considerably less hemolysis. This low activity of the unsaturated phospholipids may result from the greater bending and bulkiness of the aliphatic chain and consequently reduced contact with and penetration into the erythrocytes (Gottfried and Rapport, 1963). The liposomes were stored and irradiated under nitrogen atmosphere. This condition seemed to prevent or decrease the oxidation of the unsaturated phospholipids and thereby the possible induction of membrane damage of the erythrocytes by peroxidised liposomes (Kobayashi et al., 1985).

By increasing the liposome concentration, more degradation products are present which again are able to react with the erythrocytes and induce hemolysis.

Mannitol has proved to be an effective hydroxyl radical scavenger by decreasing the irradiation induced degradation of the phospholipids (Stensrud et al., 1996). In this study the inclusion of mannitol in the liposomal formulation significantly reduced the irradiation induced hemolysis.

The hemolysis obtained by incorporation of LPC-C_{14} in the DMPC-liposomes was in the same range as the hemolysis obtained from the irradiated liposomes. For DPPC-liposomes, an irradiation dose of 9.66 kGy resulted in the disappearance of 2.4 mol% of the phospholipids (Tinsley and Maerker, 1993). Of this amount 0.59 mol% was identified to be LPC. An approximately equal amount of LPC is most probably present in our sample after the same irradiation dose and subsequent more LPC at higher doses.

The experiments with the different LPC-compounds support earlier results (Weltzien et al., 1977), that LPC with an aliphatic chain with 16 carbons causes more hemolysis than LPC with 14 carbons. The relatively high L_{50} value obtained for the egg-LPC shows that unsaturated aliphatic chains result in a lower degree of hemolysis compared to saturated chains.

Autoclaving is a well established method to sterilise emulsions containing phospholipids for intravenous administration and is also possible to use for liposomes under proper condition (Zuidam et al., 1993). However, hydrolysis of the phospholipids with the formation of lysophospholipids might be a problem. In our study, the irradiated liposomes caused less hemolysis than the autoclaved liposomes. In regard to hemolysis it seems that irradiation of

liposomes is favourable and that the observed hemolysis do not represent a potential hazard.

4.3. Coagulation experiments

The prolonged activated partial thromboplastin time (APTT) measured with negatively charged liposomes composed of DSPC/20% DSPG and especially with DSPG might be connected to the Ca^{2+}-concentration in plasma. It is reported that negatively charged phospholipids are able to bind calcium (Papahadjopoulos et al., 1976). Calcium is involved in several steps in the coagulation cascade (Williams, 1983). Whereas incubation with DSPC-liposomes showed no reduction in plasma Ca^{2+}-concentration, lower plasma Ca^{2+}-concentration after incubation with liposomes composed of DSPG indicates depletion of calcium from the plasma. This behaviour correlates well with the unaffected and the extented coagulation times measured for the DSPC-liposomes and for the DSPG-liposomes, respectively. The further extended coagulation time measured after incubation with irradiated DSPG-liposomes is closely related to the simultaneous further decreased plasma Ca^{2+}-concentration (28% decrease). Practically no changes were observed in the zeta-potential or in the size and shape of the DSPG-liposomes after irradiation (measured in PBS). This further extended coagulation time is therefore most probably due to the presence of distearoyl phosphatidic acid (DSPA) which is a degradation product formed after irradiation of DSPG (Stensrud et al., 1996; Zuidam et al., 1996a). With two negatively charged groups at physiological pH, the ability to bind Ca^{2+} increases.

The relation between plasma calcium concentration and coagulation times was further supported by adding calcium to the plasma with the irradiated DSPG-liposomes. Shorter coagulation times were measured with higher calcium concentrations. Shorter coagulation times were found when calcium was added just before the analysis instead of addition before the incubation with the liposomes, indicating a time dependent binding of calcium to the liposomes.

The measured levels of calcium (free, free and complexed) in the reference (PBS) showed a small difference compared to normal plasma when the dilution effect was taken into account (Endres and Rude, 1989). The use of citrate as an anticoagulant and the addition of phosphate (PBS) which both form complexes with calcium results in lower free calcium concentrations. A possible disturbance of the binding equilibrium (protein–calcium) or not complete removal of protein bounded calcium during ultra-sentrifugation are other explanations for these discrepancies.

Similar blood coagulation disturbances were also observed by Miller et al. (1992) with incubation of negatively charged liposomes composed of egg lecithin, cholesterol and dicetylphosphate or phosphatidic acid. They calculated that the negatively charged phospholipid could bind up to 0.5 mol Ca^{2+} per mole phospholipid.

However, two other considerations have to be mentioned in this regard. The Cephotest™ used contains an optimal concentration of phosphatidyl ethanolamin (cephalin) as a source for phospholipids. DSPG and especially the formed DSPA might compete with this phosphatidyl ethanolamin in the coagulation reactions. If the activity of DSPG and DSPA is lower this will lead to prolonged coagulation times. In addition, excess lipids reduces the contact between the coagulation factors in the complex formation (tenase complex, prothrombinase complex) because of a too large catalytic surface and this will also contribute to the longer coagulation times observed (Zwaal, 1978).

The extrinsic coagulation activity showed the same prolonged coagulation times for the irradiated DSPG-liposomes. The explanations for this is probably the binding and depletion of calcium from plasma. Thrombotest™ contains adsorbed plasma as a source for factor V and fibrinogen. It is therefore not likely that binding and depletion of factor V from plasma as reported by Bonté et al. is the explanation for these effects (Bonté et al., 1987). In addition, similar results were also obtained when Normotest™ which

utilises rabbit brain thromboplastin was used. However, the somewhat shorter coagulation times observed with non-irradiated DSPG-liposomes indicate activation of the pathway.

5. Conclusion

Non-toxic, sterile, liposome suspensions can be produsedby gamma irradiation after careful selection of the phospholipids used. The platelet aggregates observed by incubation with non-irradiated and irradiated DSPG-liposomes were small and the aggregation reversible. Liposomes composed of negatively charged phospholipids (DSPG) showed toxic effects observed as prolonged plasma coagulation times. After gamma irradiation, the toxicity increased for these preparations. After irradiation, hemolysis of erythrocytes occurred for all the liposomes suspensions tested. The degree of hemolysis depended on the content of unsaturated and charged phospholipids in the liposome suspension. However, the degree of hemolysis was low and is most probably without any clinical relevance.

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