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(57) Abstract

Liposomes having covalently bound PEG moieties on the external surface have improved serum half-life following intravenous administration.

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LIPOSOMES

The present invention relates to liposomes bearing polyethylene glycol (PEG) moieties covalently linked to the external surface.

Many ways have been sought to prolong the half life of liposomes in the circulation. Methods have included incorporation of gangliosides in the lipid bilayer, as described by Allen, T.M. et al. Biochim. Biophys. Acta 818: 205-210, and coating the liposomal surface with molecules such as glycosides, as described by Gosh, P. and Bachawat, B.K. Biochim. Biophys. Acta. 632: 562-572, and poloxamers, as described by Senior J. CRC Critical Reviews in Therapeutic Drug Carriers 3: 123-193 (1987).

There is however, a need for a technique which increases the surface hydrophilicity of liposomes (whether these are small unilamellar vesicles or multilamellar vesicles or large unilamellar vesicles of defined size) while quantitatively retaining aqueous solutes, without crosslinking the vesicles and without conferring on the vesicle a net charge.

A particular problem arises in the use of liposomes to modify the circulation lifetime characteristics of magnetic resonance imaging agents such as Gd-DTPA described by Unger et al., Radiology, 171 81-85 (1989) and Tilcock et al., Radiology, 171: 77-80 (1989). For use as a perfusion agent it would be desirable to increase the circulation lifetimme of liposomal Gd-DTPA.

Once administered i.v., the liposomes are subject to numerous interactions with plasma proteins (eg. HDL) and the Reticulo-endothelial system (RES) which result in destabilisation and clearance of the vesicles from the circulation. Methods that have been employed to date to improve vesicle stability in the circulation have been to

incorporate sterols such as cholesterol or glycolipids within the lipid composition of the vesicles. The drawback to both approaches is that it has been shown that the sterol or other high phase transition lipid decreases the permeability of the vesicle membrane to water and so results in a decreased relaxivity for the entrapped Gd-DTPA, thereby decreasing its effectiveness as a contrast agent.

We have surprisingly discovered that the covalent linkage of PEG to the external surface of liposomes can extend the circulation life-time of the liposomes without disrupting the lipid bi-layer.

The present invention therefore provides liposomes having covalently bound PEG moieties on the external surface.

Preferably the PEG moieties are linked to amino groups in the head group of at least one phospholipid species forming the liposome. Suitable phosholipids having amino groups in the head group include phosphatidylethanolamine (PE) and phosphatidyl serine (PS).

The liposomes may be formed of any suitable phospholipid or phospholipid mixture, of which a great many are already known in the literature, provided that at least one of the phospholipid species has a suitable head group for binding PEG. The space within the liposomes may contain any conventional aqueous phase and the liposomes may be presented as an aqueous suspensions or as any other conventional formulation, for instance as pharmaceutical formulations also comprising a pharmaceutically acceptable carrier or diluent, for instance as formulations for intravenous administration. Preferred carriers include sterile water for injection with optional accessory ingredients such as buffers, preservatives, antioxidants and isotonic salts.

Preferably the liposomes are large unilamellar vesicles prepared by extrusion (LUVettes), more preferably lipid bilayers consist of a 7:3 to 5:5 molar ratio of

dioeylphosphatidyl choline and dioleylphosphatidyl ethanolamine and most preferably the liposomes contain aqueous Gd-DTPA.

The invention further provides a process comprising treating liposomes with a reactive derivative of polyethylene glycol, preferably 2,2,2-trifluoroethanesulphonyl (tresyl) monomethoxy PEG. Tresyl monomethoxy PEG (TMPEG) and its production is described in our co-pending British application no. 8824591.5.

Preferably the reaction between the reactive PEG derivative and the liposomes is conducted in aqueous solution at ambient or physiological temperatures. The reaction occurs at near neutral pH, for instance in physiological buffer but is faster and more extensive at pH9-10. By controlling the ratio of reactive PEG derivative to liposomes, the number of PEG moieties linked to the liposomes may be controlled.

Poly(ethylene glycol) (PEG) is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal hydroxyl groups:

$$HO(CH_2CH_2O)_nCH_2CH_2OH$$

PEG's are classified by their molecular weights, thus PEG 6000, for example, has a molecular weight of about 6000 and n is approximately 135.

PEG's can be covalently linked to proteins by a variety of chemical methods. We have used tresyl chloride (2,2,2-trifluoroethane sulphonyl chloride) to activate the single free hydroxyl group of monomethoxy PEG 5000 (MPEG) but other tresyl halides and other reactive derivatives of MPEG may be used. By having the other hydroxyl group of PEG "blocked" as the unreactive methyl ether, the possibility of producing PEG activated at both ends, which would give rise to cross-linked lipids in the coupling stage, is avoided.

The phospholipids phosphatidylethanolamine (PE) and

phosphatidyl serine (PS) have a free amino group in the polar head group. In aqueous solutions phospholipids show lyotropic mesomorphism; most phospholipids adopt closed vesicle structures comprising lipid bilayers (liposomes). PE on its own adopts the H_{II} phase, but in mixtures with phosphatidylcholine (PC) adopts bilayer organisations. We have prepared liposomes from PE/PC mixtures to provide lipid vesicles with the amino groups of PE exposed at both the outer and inner surface. Only the outer PE molecules are accessible to the tresyl-PEG, so the modification is asymmetric.

The amount of PEG linked to the liposome surface can be controlled by varying the lipid composition, the ratio of the reactive derivative of polyethylglycol to the phospholipid having an amino group-containing head group, the duration of the reaction and the pH. The production process may be optimised by systematic studies using, for instance, release of entrapped dye as a marker for disruption of the integrity of the lipid bilayer and by monitoring half-life of treated liposomes in, for instance, the blood stream of mice following intravenous administration.

The major fate of untreated liposomes injected in to the circulation, regardless of size, is uptake by the Kupfer cells of the liver and by fixed macrophages in the spleen. Such uptake by the reticulo-endothelial system (RES) limits the applicability of liposomes in applications such as the formation of reservoirs for the slow release of biologically active molecules and for treatment of tissues other than RES tissues. Treatment of the liposomes according to the present invention, in order to introduce PEG moieties on the external surface surprisingly reduces the interaction between serum and the liposome and surprisingly increases the circulation life-time following intravenous administration.

A particularly preferred use of the PEG-bearing

liposomes of the present invention is in the delivery of MR imaging agents such as Gd:diethylenetriaminepentacedic acid chelate.

The invention further provides the use of liposomes having PEG moieties bound to their external surfaces in therapeutic and diagnostic methods practised on the human or animal body, for instance as delivery means for drugs and for contrast agents for Magnetic resonance (MR) imaging. The invention provides a therapeutic or diagnostic process comprising intravenous administration of an effective, non toxic amount of a PEG-bearing liposomes as hereinbefore described containing a diagnostic or therapeutic agent to a human or non-human animal in need thereof.

The invention will now be illustrated by the figures of the accompanying drawings which:

Fig. 1. shows a comparison of the clearance of PEGylated SUVs and unPEGylated SUVs from the circulation in mice.

Fig. 1A: SUVs of composition DSPC:PE:Cholesterol (molar ratio 0.4:0.1.5) either PEGylated ( ) or untreated ( ) were injected iv into mice (0.4mg/25g mouse). Blood levels of CF (dose ± se, 5 animals) are shown; ^{3}H phospholipid clearance was similar (not shown).

Fig. 1B: and 1C: Identical conditions to Fig. 1A except that the SUV preparation had been centrifuged to 100,000g for 1 hr to remove larger vesicles and the injected dose was 0.8mg/25g mouse. Both CF Fig. 1B clearance and ^{3}H phospholipid clearance Fig. 1c are shown for PEGylated () and unPEGylated () vesicles.

The invention will now be illustrated by the following Examples:

EXAMPLES 1-10

PREPARATION OF PEGYLATED LIPID VESICLES

A. Preparation of activated Tresyl-MPEG

Tresylated monomethoxy PEG (TMPEG) was obtained by treating dry monomethoxy PEG 5000, which is available from Union Carbide, in dichloromethane, with tresyl chloride (2,2,2-trifluoroethane-sulphonyl chloride) which is available from Fluka, at room temperature, using pyridine as a base catalyst. Dichloromethane was removed under reduced pressure and the solid obtained dissolved in methanol-HCl mixture (0.3ml conc HCl per 1000ml) and reprecipitated at between -20 and 0^0. The solid was isolated by centrifugation, the process repeated until the sample was free of pyridine (detected at 255nm), and then the solid was reprecipitated from methanol until acid free.

B. PEGylation of lipid vesicle surfaces

The resulting TMPEG was reacted with lipid vesicles at room temperature in buffered solutions (see below). The MPEG covalent attachment of the MPEG to the outer surface of the vesicles was demonstrated by the alteration in the partitioning behaviour of the vesicles in aqueous two-phase systems of PEG and dextran, by a method similar to that of Tilcock et al., Biochim. Biophys. Acta 979:208-214 (1989). The composition of the phase system was adjusted so that the vesicles showed a low partition in the top PEG-rich phase; vesicles were at the interface or in the MPEG bottom dextran-rich phase. Attachment of MPEG to the vesicle surface makes them more "PEG-like" (increases their wetting by the PEG-rich phase) and they partition to the top phase.

EXAMPLE 1

PEGylation of MLVs (multilamellar vesicles)

Multilamellar vesicles containing 20% (w/w) egg phosphatidylethanolamine (EPE) and 80% (w/w) egg phosphatidylcholine (EPE) and ^{14}C EPC were prepared in 0.125M

NaCl containing 0.05M sodium phosphate buffer, pH 7.5 (PBS) at 10 mg total lipid/ml. 0.1ml samples of vesicles were incubated with solutions of TMPEG prepared in PBS (final concentrations 0-170 mg/ml) for 2 hours at room temperature. Samples were partitioned by adding samples (0.05 ml) to a biphasic system (1 ml of top phase and 1 ml of bottom phase of a phase system of 5% (w/w) PEG 6000 and 5% (w/w) Dextran T500 in 0.15M Nacl containing 0.01M sodium phosphate, pH 6.8, mixing the systems and measuring the radioactivity in samples taken from the mixture immediately after mixing (total) and from the top and bottom phases after phase separation was completed (20 min).

The results in Table 1 show that exposure of the liposome to TMPEG increases their partition into the PEG-rich top phase. This indicates that PEG has become attached to the liposome, presumably by the covalent attachment to the amino group of the EPE.

TABLE 1

The effect of TMPEG on the partitioning behaviour of multilamellar vesicles of EPE/EPC (2:8).

FINAL TMPEG	PARTITION (%)			n
(mg/ml)	Top Phase	Interface	Bottom Phase	
0.0	9.1±4.7	84.5± 4.1	6.4± 2.4	9
2.0	14.5±5.4	80.2± 4.2	5.3± 1.6	3
8.0	44.9±6.3	50.8± 6.5	4.3± 0.4	3
12.5	74.7±9.5	20.1±10.5	5.2± 1.4	3
25.0	96.3±7.8	3.1± 3.6	4.6± 0.8	4
50.0	89.3	6.5	4.5	1
100.0	88.8	5.1	6.1	1
170.00	89.3	6.5	4.2	1

The presence of PE in the vesicle is required for TMPEG to have any effect. When MLVs of 100% EPC were treated with TMPEG for two hours and then partitioned in a 5%/5% PEG 6000-Dextran T500 systems in 0.15M NaCl buffered with 0.01M sodium phosphate, pH 6.8 there was no difference compared to MLVs treated with buffer (Table 2).

TABLE 2

Effect of TMPEG on eggPC Multilamellar vesicles.

FINAL TMPEG	PARTITION (%)			n
(mg/ml)	Top Phase	Interface	Bottom Phase	
0	22.5 ± 13.0	71.6 ± 12.0	5.9 ± 1.0	5
25	25.8 ± 13.0	67.8 ± 14.0	6.4 ± 1.0	5

The activity of TMPEG declines on storage. Samples that had lost their ability to PEGylate proteins were found to have no effect on the partitioning of liposomes containing EPE. This observation, taken together with the inablity of TMPEG to effect non-PE containing vesicles supports the conclusion that TMPEG attaches to PE specifically, and that altered partitioning does not arise from adsorption of TMPEG to vesicle surfaces.

EXAMPLE 2

PEGylation of SUVs (Small Unilamellar Vesicles)

SUVs composed of distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylethanolamine (DPPE) and cholesterol in molar ratio 0.8:0.2:1 were prepared by the method of Senior et al., Biochim. Biophys. Acta, 839: 1-8 (1985), with tracer ^{3}H-DPPC (6 x 10^{6} dpm per 30 mg phospholipid): 25 mg DSPC, 5.5 mg DPPE and 15 mg cholesterol were hydrated in 2 ml PBS (0.125M NaCl buffered with 0.05M

Naphosphate buffer, pH 8.5). To measure liposomal retention of water-soluble molecules during the coupling reaction and subsequent procedures, Carboxyfluorescein was partially purified and entrapped at 0.15M as described by Senior et al., Biochim. Biophys. Acta 839: 1-8 (1985). 0.5ml SUV were incubated with an equal volume of TMPEG, prepared in PBS (0.125M NaCl buffered with 0.05M Naphosphate buffer, pH 8.5) at 125 mg/ml. for 2 hours at room temperature (Ratio of TMPEG to total DPPE is 6.25). The vesicles were then separated from unreacted TMPEG by gel filtration on Sepharose 4B-CL and partitioned as in Example 1 in a phase system of 5% PEG 8000 (Union Carbide) and 5% Dextran T500 (Pharmacia) in 0.15M NaCl containing 0.01M sodium phosphate, pH 6.8. The results in Table 3 show that exposure of the liposomes to TMPEG increases their partition into the PEG-rich top phase compared with vesicles treated only with buffer (control). This suggests that PEF has been covalently linked to the amino group of the DPPE. PEGylation proceded without the loss of the entrapped CF.

Table 3

Phase Partitioning of PEGylated and unPEGylated SUVs.

VESICLES	PARTITION[1] (%)		
Phase	Top Phase	Interface	Bottom
Untreated	1.4 ± 0.2	36.0 ± 5.0	62.5 ± 5.1
TMPEG-treated	96.5 ± 1.0	1.4 ± 1.1	2.1 ± 0.4

[1] mean ± n = 6

EXAMPLE 3

The SUVs, as used in Example 2, were treated with TMPEG (125 mg/ml) and their partitioning compared with SUVs treated with MPEG (125mg/ml) or buffer: the TMPEG treated vesicles were completely (100%) partitioned into the top phase, whereas the MPEG-treated vesicles and buffer-treated vesicles showed no top phase partitioning, and similar even distributions between the interface and the bottom phase. This provides additional support for the suggestion that TMPEG acts by covalent attachment to the vesicle surface, and not by adsorption.

EXAMPLE 4

PEGylation of LUVettes (Large Unilamellar Vesicles prepared by Extrusion) of defined size.

LUVettes were prepared as described by Tilcock et al., Biochim. Biophys. Acta 979:208-214 (1989).

LUVettes of 100nm diameter were prepared at a final concentration of 10mg/ml. Mixtures of dioleyl-phosphatidylcholine (DOPC) and dioleylphosphatidyl ethanolamine (DOPE) in chloroform at various molar ratios (total 20mmoles) were combined with 2uC of ^{3}3H DPPC and the solvent removed by evaporation under reduced pressure (<0.1mm Hg) for 2 hours. The lipid was dispersed by vortex mixing at room temperature in 1.55ml of 50 mM Hepes, 100mM NaCl pH 7-9 to give a final lipid concentration of 10mg/ml. Large unilamellar vesicles were then produced by repeated extrusion (10 times) of the lipid dispersion MLVs through two stacked 100nm polycarbonate filters using the Extruder device (Lipex Biomembranes, Canada) by the method of Hope et al., Biochim. Biophys. Acta 812: 55-65 (1985). Diameters determined by QEL using a Nicomp model 270 particle analyzer.

The vesicles were PEGylated by incubation with 40 ul of buffer containing TMPEG at room temperature. At intervals

20 ul samples were removed and partitioned in a phase system of 1.5ml top phase and 1.5ml bottom phase of a 5% PEG 8000 (Union Carbide) and 5% Dextran T500 (Pharmacia) system prepared 0.15M NaCl buffered with sodium phosphate pH 6.8 at room temperature. Samples of top and bottom phase were removed for counting 20 min after the phase had been mixed and allowed to separate. This phase system was selected so that the partitioning of the untreated vesicles into the top phase was extremely low (>5%); the majority of the vesicles were approximately equally divided between the bottom phase and the bulk interface.

EXAMPLE 5

The time course and pH dependency of the PEGylation reaction using a two-fold excess of TMPEG to the DOPE present at the outer surface of LUVettes are used in Example 4. At pH 8-9 incubation with TMPEG rapidly caused a time dependant transfer of vesicles to the top phase. At pH 7.5 the reaction was considerably slower and at pH 7.0 there was virtually no transfer to the top phase. In a separate experiment in which the bottom phase and interface partitioning was also measured it is seen that at pH 7.2, although top phase partitioning does not alter there was decrease in bottom phase partitioning with an increase in interface partitioning, indicating that PEGylation proceed at pH 7.2 albeit more slowly than at higher pHs. At pH 8 the partitioning moves from the bottom phase to the interface and then to the top phase; at pH 9 and 10 vesicles are moved rapidly from the interface and bottom phase to the top phase. Thus the PEGylation reaction is very sensitive to pH and appropriate choice of conditions of time and pH can determine the degree of PEGylation. The extent of PEGylation can also be controlled by the amount of TMPEG used. Treating 100nm Luvettes of DOPE/DOPC (0.2:0.8) at pH 9.0 with varying molar ratios of TMPEG increased partitioning into the top phase

consistent with increasing PEGylation. There was a marked increase in top phase partitioning between the molar ratios 1.0 and 1.3 from 20% to 90%. When the partitioning in the bottom phase and at the interface is also measured (Table 4) it can be seen that PEGylation at the lower ratios of TMPEG:outerDOPE molar ratio causes a progressive change in the partition from the bottom phase to the interface and subsequently to the top phase demonstrating gradations in the degree of PEGylation.

It is clear from the time course of the partitioning that reaction at pH 9 is virtually complete by 1 hour. Thus defined
degrees of PEGylation are obtained by control of the TMPEG:DOPE ratio.

TABLE 4

Molar ratio TMPEG: DOPE at outer surface	Partitioning (%)		
	Bottom	Interface	Top
0	50	40	10
0.2	56	41	3
1.0	28	58	13
1.3	1	9	89

Measurement of the fraction of amino groups (from PE) exposed at the outer surface of the LUVettes, made by the method of Hope, M.J. and Cullis. P.R. J. Biol. Chem. 262: 4360-4366 (1987) in 0.05M TNBS in borate buffer at pH 8.5, gave values of 47% for DOPC:DOPE vesicles (8:2), close to the theoretical value of 50% for equal distribution of the PE between the inner and outer surfaces. PEGylation caused a decrease in the PE content detectable by this assay, suggesting covalent attachment of the MPEG to the free NH_2 group of PE. For example, when a 3-fold mole excess of TMPEG to outer PE was added to DOPC:DOPE vesicles of 7:3 molar ratio for 1 hour, the percentage of outer PE PEGylated was 36%; when a 6-fold molar excess was added, this percentage PEGylation increased to 45%.

EXAMPLE 6

Stability of lipid vesicles to PEGylation.

The stability of lipid vesicles was measured by the extent of efflux of 6CF (6-carboxyfluorescein) as described by Senior and Gregoriadis in "Liposome Technology" (G Gregoriadis ed) vol 3, p.263 (1984) CRC Press. LUVettes of 100nm composed of DOPC:DOPE were prepared with entrapped 50mM 6CF (6-carboxyfluorescein) in 100mM NaCl at pH 8.5, external 6CF was removed by column chromatography on Sephadex G-25 using 50mM Hepes, 100mM NaCl, pH 8.5 as eluant. Samples for latency measurement were added to 4ml of buffer (100mM NaCl, 50 mM HEPES pH 9) and fluorescence measured (dye released), and to 4ml of buffer containing 25mM octylglucoside, incubated for 30 mon at 37° to ensure complete disruption of the vesicles and fluorescence measured (total dye). Fluorescence was measured at 490 nm excitation and 520 nm emission.

LUVettes of 100nm were PEGylated with TMPEG without any loss of latency. Vesicles of DOPC: DOPE 8:2 were treated with a 3 fold molar ratio of TMPEG to DOPE present in the outer vesicle surface at pH 8.5 to ensure extensive PEGylation (demonstrated by phase partitioning). There was no leakage of 6CF out of the vesicles over a period of 2 hours demonstrating that PEGylation occurs without disruption of the lipid bilayer.

EXAMPLE 7

Interaction of SUVs with serum.

0.1 ml of SUVs of composition DSPC:PE:Cholesterol (molar ratio 0.4:0.1:0.5), with or without coupled PEG (see above) were incubated at 376° with 0.5 ml of fresh plasma (mouse) or buffer. Samples were removed at intervals and partitioned as in Example 2 above. SUVs partitioned about

20% top phase, 60% interface and 20% bottom phase. Treatment with serum caused an immediate (within 1 min) alteration in the vesicle surface properties indicated by their partit ion: 0% top phase, 40% interface and 60% bottom phase. The plasma proteins alone partitioned mainly to the bottom phase (68% bottom, 32% top; Partition coefficient $=0.47 \pm 0.02$, $n=4$). Thus it appears that the SUVs are immediately coated with serum proteins which then cause the vesicles to partition with similar characteristics to the proteins. PEGylation of the SUVs increased their partition into the top phase (almost 100%); on exposure to serum there was a change in their partition towards the interface and the bottom phase, but importantly this process was very slow compared with the virtually instantaneous effect of serum on unPEGylated SUVs. Since the partitioning behaviour relates to the sum of the forces imposed by the PEGylation and serum binding, and with the former is not a linear function, it is not simple to determine whether the effect of serum on partition is equal for the PEGylated and for the unPEGylated liposomes. This could, however, be determined with a detailed dose response analysis of the effect of PEGylation on the partition coefficient so that the influence of serum could be determined at various parts of the dose response curve in "PEG-equivalents". This would establish whether serum had different effects on the PEGylated and unPEGylated liposomes. The order of magnitude differences in partition behaviour suggests that PEGylation slows down the adsorption of serum components onto the vesicles.

Separation of the SUVs exposed to serum by gel chromatography gave vesicles which showed partitioning behaviour close to that of the vesicles before exposure. Thus the interaction between vesicles and serum is reversed by reisolation of the vesicles.

These experiments also demonstrate that the altered

surface properties of the SUVs imposed by PEGylation are not substantially reversed by serum protein adsorption.

EXAMPLE 8

Stability of LUVettes to serum is increased by PEGylation

To determine the stability of LUVettes to serum vesicles containing entrapped 6CF (50ul) were incubated at 37° with 0.5 ml serum (freshly hydrated lyophilised human serum, Monitrol-ES, Dade Diagnostics) to provide a final lipid concentration of approx 1mg/ml, a concentration corresponding to the maximum in vivo serum concentrations expected on the basis of the imaging experiments of Unger et al Radiology 171: 81-85. Samples were removed at intervals and the 6CF released was measured fluorimetrically. Vesicles were PEGYlated with a 3-fold excess of TMPEG to outer surface DOPE overnight at room temperature, after which time there had been loss of latency.

50 nm vesicles of DOPC:DOPE at 8:2 molar ratio showed considerable loss of latency in the presence of serum (eg only 10% latency remained after 2hrs) which PeGylation did not decrease; 100nm vesicles showed a latency of 35% after 2hrs which was unaffected by PEGylation; 200 nm vesicles showed a smaller loss of latency (eg 65% latency remained after 2hrs), which also was not inhibited by PEGylation. However, for 100nm vesicles of 7:3 molar ratio DOPC:DOPE, PEGylation decreased serum induced loss of latency by a factor of 2. Increasing the DOPE content to 40 mole% and 50 mole % increased the stability of the vesicles to serum; nevertheless PEGylation produced additional stabilisation. Table 5 summarises these data.

Table 5

Stabilisation of 100nm LUVette latency to serum (2hr, 37°) by PEGylation

DOPC:DOPE molar ratio	Latency (%)	
	UnPEGYLATED	PEGylated
8:2	35	35
7:3	55	83
6:4	90	95
5:5	92	99

EXAMPLE 9

PEGylation does not alter the relaxivity of encapsulated Gd-DTPA

Gd-DTPA was encapsulated in LUVettes composed of DOPC:DOPE 7:3 by the method of Tilcock et al Radiology 171: 77-80 (1989).

Half of the sample was PEGylated with TMPEG (molar ratio of TMPEG: PE on outer surface of 3:1). Both control and PEGylated samples were diluted in saline buffer (139 mM NaCl, 10 m Hepes, 6mM KCl, pH 8.5) to give four samples with effective Gd concentrations of 2, 1, 0.5, and 0.25 mM (calculated as described by Tilcock et al., Radiology 171: 77-80 (1989) given the known trap volume of the vesicles, the lipid concentration and assuming the concentration of entrapped Gd-DTPA was 0.67M.) Samples of 10-12 ml were imaged with a Toshiba 0.5T MRT-50A whole body scanner. Relaxivites are obtained from linear regressions of 1/T1 (spin lattice relaxation time constant) against the effective Gd-DTPA concentration. These were unaffected by PEGylation of the vesicles.

EXAMPLE 10

PEGylation of SUVs decreases their in vivo clearance

SUVs of composition DSPC:PE:Cholesterol (molar ratio 0.4:0.1:0.4) (0.2ml containing 0.4mg phosphpholipid) were

injected intravenously into the tail vein of male TO mice (5 in each group). Clearance of PEGylated and unPEGylated vesicles was assessed from entrapped CF and ^{3}H-radiolabelled phospholipid measured in blood samples (25ul) withdrawn at intervals in the method of Senior and Gregoriadis in "Liposome Technology" vol 3 pp263-282 (1984), CRC Press. In another experiment an 0.8 mg dose of phospholipid was given as the supernatant from ultracentrifugation at 100,000g for 1 hour, which contains small vesicles of 20-100nm (average 50 nm) as described by Senior et al Biochim Biophys Acta 839: 1-8 (1985).

Fig 1A shows the clearance of SUVs after intravenous administration of a sonicated, uncentrifuged preparation. This preparation contains, presumably, some larger vesicles which are cleared rapidly, in both the PEGylated and unPEGylated samples. However the slower clearance phase corresponds to about 50-60% of the lipid dose and showed a marked difference in the half life of the PEGylated sample (10hr) compared with the unPEGylated preparation (7hr). In the preparation in which the larger vesicle had been removed (FIG 1B and FIG 1C) the PEGylated vesicles had half life of 14 hr compared with untreated vesicles of 12 hr.

CLAIMS

1. Liposomes having covalently bound PEG moieties on the external surface.

2. Liposomes according to claim 1 wherein the lipid bilayers consist of a mixture of dioleylphosphatidylcholine (DOPC) and dioleylphosphatidylethanolamine (DOPE).

3. Liposomes according to claim 2 wherein the lipid bilayers consist of a 7:3 to 5:5 molar ratio of DOPC to DOPE.

4. Liposomes according to any one of claims 1 to 3 comprising an aqueous phase containing Gd:diethylenetriaminepentaacetic acid (Gd-DTPA).

5. A pharmaceutical composition comprising an aqueous suspension of liposomes according to any one of claims 1 to 4 and a pharmaceutically acceptable carrier or diluent.

6. Liposomes according to any one of claims 1 to 4 for use in a method for the treatment of the human or animal body or in a diagnostic method practised on the human or animal body.

7. Use of liposomes according to any one of claims 1 to 4 in the preparation of a medicament for use in a method of treatment of the human or animal body or a method of diagnosis practised on the human or animal body.

8. Use according to claim 8 of liposomes comprising an aqueous phase containing a contrast agent in a diagnostic method comprising magnetic resonance imaging of the human or animal body.

9. A process for producing a liposome according to any one of claims 1 to 4 comprising treating liposomes with a reactive derivative of polyethylene glycol.

10. A process according claim 9 wherein the reactive derivative is 2,2,2-trifluoroethane sulphonyl-monomethoxy-polyethylene glycol.

11. A therapeutic or diagnostic method comprising

intravenous administration of an effective, non-toxic amount of liposomes according to claim 1 comprising an aqueous phase containing a diagnostic or therapeutic agent to a human or non-human animal in need thereof.

Fig. 1A.

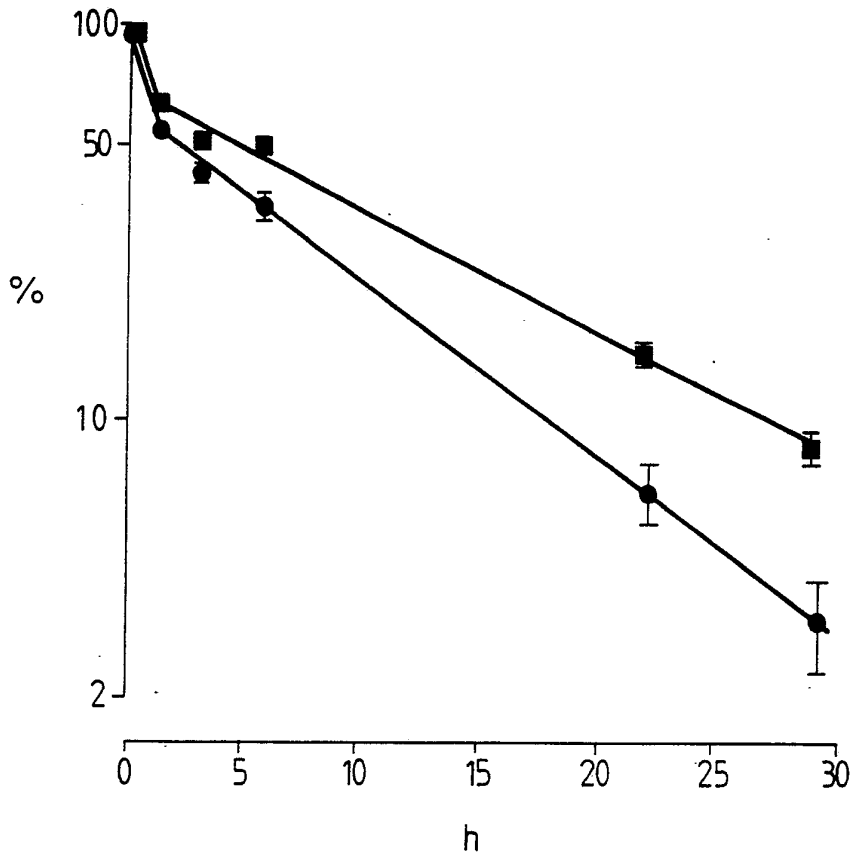


Fig. 1B.

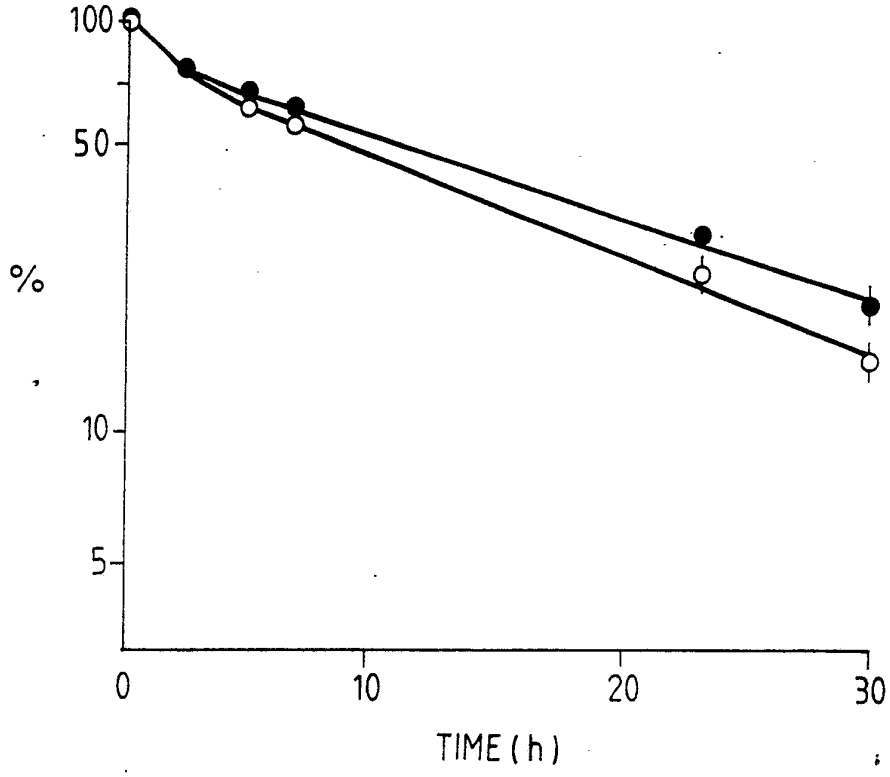
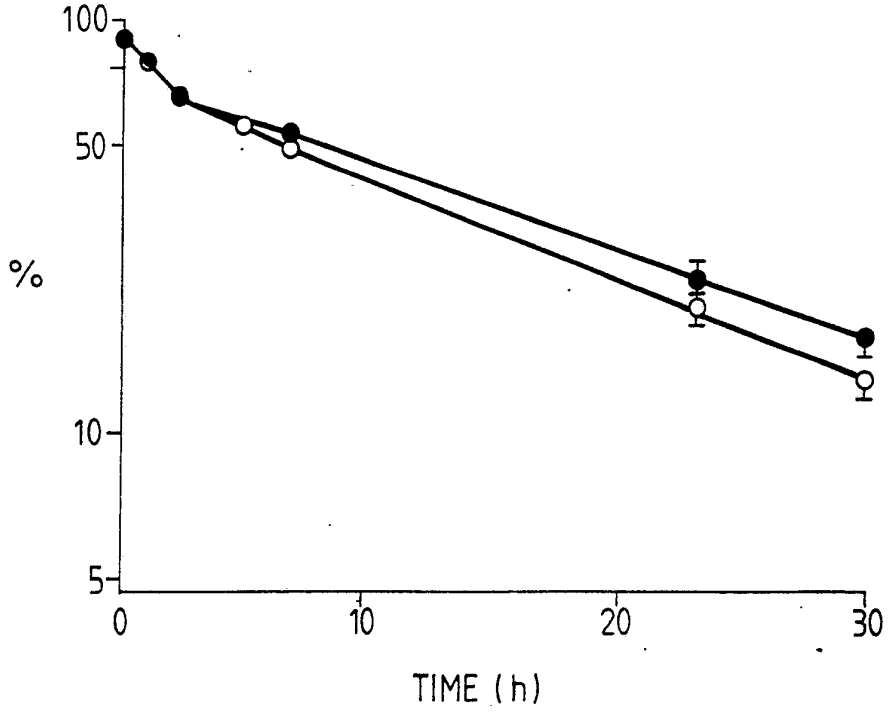


Fig. 1C



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01262

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 9/127, A 61 K 49/00, A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0171946 (TECHNICON INSTR. CORP.) 19 February 1986 see page 4, lines 13-25; page 7, lines 30-35; page 8, lines 22-31; page 9, lines 25-34; claims	1
Y	--	1-10
Y	GB, A, 2185397 (COSMAS-DAMIAN LTD) 22 July 1987 see page 1, lines 37-42; page 6, lines 41-53	1-10
Y	-- STN File Server, (Karlsruhe), Chemical Abstracts, volume 109, no. 3, 1988, (Columbus, Ohio, US), C. Hofmann et al.: "Transfer of functional insulin receptors to receptor-deficient target cells", see abstract no. 17441m, & Endocrinology (Baltimore), 122(6), 2865-72	1-10
	-- ./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th February 1990	27. 03. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Radiology, volume 171, no. 1, April 1989, E.C. Unger et al.: "Hepatic metastases: liposomal Gd-DTPA-enhanced MR imaging", pages 81-85 see page 81, abstract cited in the application	1-10

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 11, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv):
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [ ] The additional search fees were accompanied by applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8901262 SA 31851

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/03/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0171946	19-02-86	US-A-	4707441	17-11-87
		AU-B-	550838	10-04-86
		AU-A-	4555185	20-02-86
		CA-A-	1258627	22-08-89
		JP-A-	61053568	17-03-86
GB-A- 2185397	22-07-87	DE-A-	3700911	23-07-87