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International Journal of Pharmaceutics 132 (1996) 9–21

international journal of pharmaceutics

Surface modification of continuously extruded contrast-carrying liposomes: effect on their physical properties

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Received 19 May 1995; accepted 26 June 1995

Abstract

Surface-modified, contrast-carrying liposomes were generated by incorporation of amphipathic polymers into the membranes of continuously extruded vesicles. Besides the well described distearoylphosphatidylethanolamine monomethoxypolyethyleneglycol (DSPE-PEG), a new substance, cholesterylhemisuccinate monomethoxypolyethyleneglycol (CholHS-PEG) was tested for the first time. Using the water-soluble radiographic contrast agent iopromide as well as the nuclear magnetic contrast agent Gd-DTPA, the impact of surface modification (SM) on liposome properties like vesicle size distribution, encapsulation efficiency, zeta potential and storage as well as plasma stability was investigated. In the course of the studies, the molar amount of amphipathic polymer employed as well as the time point of SM during the production process were varied. Incorporation of both, DSPE-PEG and CholHS-PEG into the lipid films formed before continuous extrusion resulted in a concentration-dependent decline of encapsulation efficiencies. When SM was carried out after vesicle formation, the observed effect diminished and even disappeared, as soon as PEG-coating was carried out after the last extrusion step. However, when using the latter procedure with DSPE-PEG, mean vesicle diameters showed a strong increase in the course of the pegylation process. The extent of bilayer modification was studied by zeta potential measurements of liposomes containing the negatively charged phospholipid SPG. In the presence of PEG-derivatives the high zeta potentials of unmodified vesicles were significantly reduced, irrespective of whether SM was carried out before, during or after extrusion. This result indicated a successful association of the PEG-derivatives with liposomal bilayers for all procedures. For CholHS-PEG complete incorporation into liposomes after extrusion could be demonstrated using gel filtration. Stability testing revealed an unchanged macroscopic appearance, encapsulation efficiency and vesicle size distribution of unmodified and CholHS-bearing liposomes after 4 months' storage at 2–8°C. In contrast to this, DSPE-PEG-containing vesicles displayed a pronounced size increase when SM was carried out during extrusion. Another important effect of DSPE-PEG incorporation was found during plasma stability experiments. Whereas CholHS-PEG-carrying and unmodified liposomes had similar leakage rates in human plasma, DSPE-PEG caused a concentration-dependent decrease in plasma stability, but only when SM had been carried out before extrusion. Altogether, from a merely

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SSDI 0378-5173(95)04247-8

technological point of view, CholHS-PEG revealed superior properties over DSPE-PEG for SM of continuously extruded contrast-carrying liposomes.

Keywords: Liposome; Contrast agent; Continuous extrusion; Surface modification; Amphipathic polymer; DSPE-PEG; CHOLHS-PEG

1. Introduction

Water-soluble contrast agents which are frequently employed for enhancement of contrast in computed tomography (CT) and magnetic resonance imaging (MRI) usually show similar biodistribution patterns. After intravenous injection they extravasate within minutes and are rapidly cleared by the kidneys (Weinmann et al., 1984; Speck, 1987).

A common way to alter the in vivo behaviour of these molecules is their entrapment in liposomes, small lipid vesicles of a size between 30 nm and several microns, which consist of one or several concentric bilayers, similar to cell membranes. Once introduced to the circulation liposomes like other particulate species are preferentially taken up by cells of the mononuclear phagocyte system (MPS) and thus accumulate to a great extent in organs like liver and spleen (Senior, 1987; Allen, 1988). In the past, this characteristic feature has been used for liver- and spleen-specific targeting of contrast-carrying liposomes (Havron et al., 1981; Ryan et al., 1983; Kabalka et al., 1987). As a result, improved detection of liver metastases in experiments conducted in mice and rabbits has been accomplished by several groups (Henze et al., 1989; Sachse et al., 1993).

Since targeting to non-MPS tissues would require vesicles with longer blood circulation times, researchers eventually concentrated on strategies to avoid liposomal MPS uptake. In the late 1980s, substances like the monosialoganglioside GM1 as well as amphipathic polyethyleneglycol-derivatives (PEG-derivatives), which can modify the surface properties of phospholipid vesicles, were introduced. With these novel lipids, phagocytic uptake was greatly reduced and half-lives of liposomes increased dramatically (Allen and Chonn, 1987; Blume and Cevc, 1990; Klibanov et al., 1990). A reduced interaction between blood components (proteins, lipoproteins and cells) and the vesicular carrier, due to steric hindrance, created by hydrophilic, bulky head-groups was identified as the molecular mechanism of MPS-avoidance (Klibanov et al., 1991; Lasic et al., 1991). Thus, at least as far as PEG-derivatives are concerned, it was shown that opsonisation, which usually precedes phagocytic uptake, is prevented to a great extent after incorporation of amphipathic polymers into the vesicular membrane (Senior et al., 1991; Chonn and Cullis, 1992). As a consequence, accumulation of encapsulated material in tumors, following extravasation through leaky tumor vessels (Needham et al., 1992; Wu et al., 1993), as well as specific targeting of immunoliposomes to desired tissues (Torchilin et al., 1992) became possible.

Since improvement in therapeutic efficacy of drugs, e.g., doxorubicin, could be successfully demonstrated with long-circulating liposomes (Papahadjopoulos et al., 1991), the idea of using such vesicles also for targeting of water-soluble contrast agents to tissues other than liver and spleen evolved. It is expected that MPS-avoiding liposomes might improve detection of tumors and other lesions in CT or NMR and, moreover, enable the use of diagnostics as blood pool agents.

Lately, we have described a new method for the large-scale production of contrast-carrying liposomes of defined size, using a continuous high pressure extrusion apparatus (Schneider et al., 1994; Schneider et al., 1995). The new device permits the effective encapsulation of the radiographic contrast agent iopromide as well as the nuclear magnetic resonance contrast agent Gd-DTPA into liposomes with mean diameters in the range between 90 and 210 nm. Since MPS-avoiding vesicles display longest blood half-lives when their diameters are within this range (Allen, 1992; Allen, 1994), continuous extrusion appeared to be suitable for the generation of long-circulating liposomal contrast agents.

In this study, we report about surface modification (SM) of continuously extruded iopromide and Gd-DTPA-containing vesicles with two PEG-derivatives. Besides the well described distearoylphosphatidylethanolamine monomethoxypolyethyleneglycol (DSPE-PEG), we also employed a new substance, cholesterylhemisuccinate monomethoxypolyethyleneglycol (CholHS-PEG). While other researchers in the past concentrated on the in vivo behavior of surface-modified liposomes, we focussed on the technological aspects of PEG-incorporation into liposomal bilayers. Thus, the impact of different modes of SM on the physical properties of contrast-carrying liposomes like encapsulation efficiency, vesicle size distribution, zeta potential as well as storage and plasma stability were examined and differences between DSPE-PEG and CholHS-PEG were revealed. In these experiments, stable, PEG-coated liposomes with high encapsulation efficiencies for potential in vivo testing were obtained.

2. Materials and methods

2.1. Lipids and contrast agents

Soy phosphatidylcholine (Lipoid S100, SPC) and soy phosphatidylglycerol (Lipoid SPG, SPG) were from Lipoid KG, Ludwigshafen, Germany. Cholesterol (Chol) was from Solvay Duphar B.V., Veenendal, The Netherlands. The amphipathic polymer DSPE-PEG_{2000} was obtained from Genzyme, Haverhill, Suffolk, UK, CholHS-PEG_{2000} from CTS, Berlin, Germany. All lipids were used without further purification.

Ultravist® 370, containing the non-ionic, water-soluble X-ray contrast agent iopromide (Mol. wt. 791.14 g) was obtained from Schering AG, Berlin, Germany, as was Magnevist®, which contains the ionic, water-soluble paramagnetic gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) as its dimeglumine salt (Mol. wt. 938.01 g). Contrast agent solutions were diluted to the desired concentration with either 20 mM tromethamine buffer, pH 7.5 (Ultravist®), or bidistilled water (Magnevist®).

2.2. Liposome preparation

Liposomes, composed of either SPC:Chol:SPG in a molar ratio of 6:3:1 for iopromide or SPC:Chol in a molar ratio of 7:3 for Gd-DTPA, were prepared as described elsewhere (Schneider et al., 1995). Briefly, multilamellar vesicles (MLV) were generated by suspending lipid films, which had been obtained from rotary evaporation of ethanolic solutions (96% (v/v), reagent grade, E. Merck, Darmstadt, Germany), at room temperature with contrast agent solutions. Subsequently, MLV were extruded through polycarbonate membranes (Nuclepore GmbH, Tübingen, Germany) with decreasing pore sizes of 5.0, 1.0, 0.4, 0.2 and 0.1 μm, five passages each, using the continuous high pressure extrusion device Maximator® Model HPE 10.0-250 (Schmidt, Kranz & Co, Zorge, Germany). After passage through membranes with 0.4 μm and before continuing further extrusion, three freeze-thaw cycles in glass vials using methanol/CO_2 (-70°C) and a water bath (70°C) were carried out. For storage, the liposomes were filtered through microbe retentive filters (cellulose acetate, pore size 0.22 μm, Sartorius, Göttingen, Germany) and placed in sterile glass vials under aseptic conditions.

2.3. Surface modification (SM)

SM was carried out at different times during the production process, either before, during or after extrusion. For SM before extrusion, the amphipathic polymer was dissolved together with the other lipids in ethanol at elevated temperatures (60°C) in order to form a lipid film after rotary evaporation. Thus, DSPE-PEG and CholHS-PEG were already incorporated in the vesicular membrane during MLV-formation. For SM during extrusion, PEG-derivatives were dispersed in extruded liposomal preparations after the performance of three freeze-thaw cycles and before continuing further extrusion (16 h at room temperature on a magnetic stirrer). SM after extrusion was performed in the same way, but not until extrusion had been completed.

2.4. Liposome size

Liposome size was determined by photon correlation spectroscopy (PCS) using a Submicron Particle-Sizer Autodilute®, Model 370, Nicomp Instr. Corp., Santa Barbara, CA, USA, as described elsewhere (Schneider et al., 1995).

2.5. Encapsulation efficiency

Encapsulation efficiency (amount of encapsulated contrast agent in the preparation expressed as percent of total recovered), also referred to as entrapment value or trapping efficiency, was determined by equilibrium dialysis, using a Dianorm system, Dianorm, Munich, Germany. For experimental details refer to Schneider et al., 1995.

2.6. Zeta potential

The zeta potential of surface-modified vesicles was calculated from the results of electrophoretic mobility measurements (20 s at 25°C) on a Zetasizer IIc, Malvern Ltd, Malvern, UK.

2.7. Gel filtration

For gel filtration, which was performed to test whether CholHS-PEG was successfully incorporated into the liposomal membrane of void vesicles by SM after extrusion, we employed a system composed of a C-Column 16/70 with two Adaptors A16, Pharmacia LKB Biotechnology AB, Upsala, Sweden, in combination with a peristaltic pump Miniplus 3, Gilson Medical Electronics S.A., Villiers Le Bel, France and a photometer LKB 2238 Uvicord® S II, LKB-Produkter AB, Bromma, Sweden. Tromethamine buffer (20 mM, pH 7.5) was used as the mobile phase, Sephacryl S 400 high resolution, Pharmacia LKB Biotechnology AB, Upsala, Sweden, column length 56 cm, as the stationary phase. Flow rate was 2.4 ml/min and the detection wavelength was set to 206 nm.

2.8. Cryo-electron microscopy

After cryofixation on carbon-coated copper grids in ethane using a cryofixation unit and transfer with a cryo-transfer system, lipid vesicles were observed under a transmission electron microscope CEM 902 (all devices from Carl Zeiss, Oberkochem, Germany) without employment of any fracturing step or staining procedure.

2.9. Plasma stability

Leakage of encapsulated iopromide from liposomes in human plasma (DRK-Blutspendedienst, Berlin, Germany) was investigated at 37°C by equilibrium dialysis using the Dianorm system described above. Each sample was diluted to an iodine concentration of 5 mg/ml with plasma. Encapsulation was determined after 1, 2 and 4 h of dialysis and related to the encapsulation efficiency determined before plasma stability testing (relative encapsulation). Iodine concentration was measured by X-ray fluorescence excitation analysis (FEA) (Kaufmann et al., 1976).

3. Results

Most experiments were carried out with iopromide, since determination of the Gd-DTPA concentration in liposomes is very tedious. The latter was employed for a few experiments only, in order to investigate whether effects observed with iopromide could be verified with other water-soluble molecules as well.

3.1. Increasing amounts of PEG-derivatives: influence on encapsulation and vesicle size

The impact of SM before extrusion on the encapsulation efficiency and vesicle size distribution of iopromide-carrying liposomes was investigated with increasing molar portions of CholHS-PEG and DSPE-PEG between 1 and 10%. As can be seen in Fig. 1, encapsulation efficiencies fell sharply with increasing amounts of either of the two amphipathic polymers, whereas the mean vesicle diameters with values between 90

and 110 nm remained unaffected. In the case of CholHS-PEG entrapment dropped from an average of $24.5 \pm 2.3\%$ for unmodified liposomes to $15.1 \pm 1.3\%$ at 5 mol% and $12.5 \pm 0.8\%$ at 10 mol%. Using DSPE-PEG, trapping efficiencies of $18.4 \pm 0.7\%$ (5 mol%) and $13.5 \pm 0.6\%$ (10 mol%) were achieved.

Similar results were obtained with Gd-DTPA-carrying liposomes when SM was performed before extrusion. Encapsulation of the MRI-contrast agent fell from $53.1 \pm 1.1\%$ for unmodified vesicles to $44.2 \pm 2.3\%$ with 5% CholHS-PEG and $44.8 \pm 2.3\%$ with 5% DSPE-PEG. Again, vesicle size determination revealed no significant changes, when SM was applied. Mean diameters ranged from 130 to 150 nm (lipid concentration, 150 mg/g; Gd concentration, 180 μmol/g; final pore size, 0.2 μm; $n = 3$).

3.2. SM before, during and after extrusion: impact on encapsulation and vesicle size

In order to find possible ways of preventing the above deterioration of encapsulation efficiency after incorporation of amphipathic polymers, SM of iopromide-carrying liposomes was carried out at different times during the production process. Thus, CholHS-PEG and DSPE-PEG were also added to vesicle preparations between two extrusion steps (SM during extrusion) as well as after extrusion had been completed (SM after extrusion).

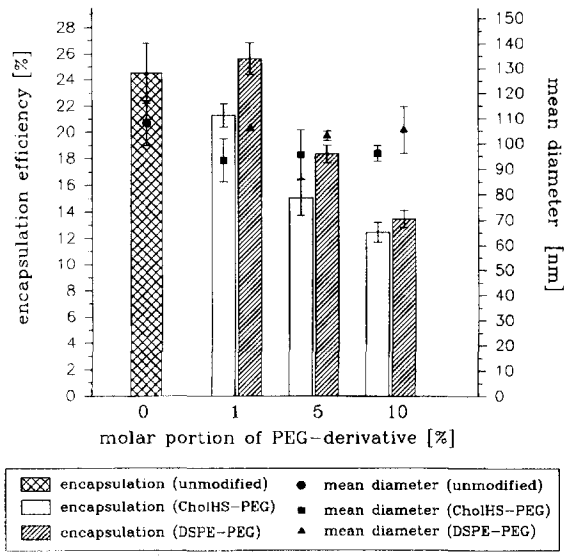

Fig. 1. Increasing amounts of PEG-derivatives during film formation: influence on encapsulation efficiency and mean diameter of iopromide-carrying liposomes. Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size:0.1 μm. Error bars denote SDs, $n = 3$.

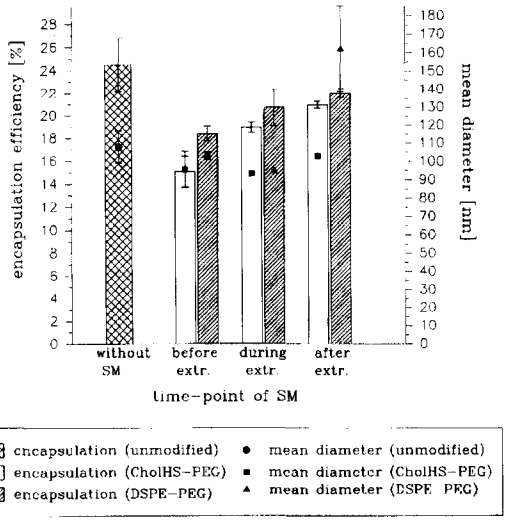

Fig. 2. SM before, during and after extrusion: influence on encapsulation efficiency and mean diameter of iopromide-carrying liposomes: 5 mol% PEG-derivative. Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size: 0.1 μm. Error bars denote SDs, $n = 3$.

SM during extrusion caused smaller declines in encapsulation efficiencies of iopromide than SM before extrusion, whereas SM after extrusion hardly affected the entrapment at all (Fig. 2). With 5 mol% CholHS-PEG, the average trapping efficiency fell from $24.5 \pm 2.3\%$ for unmodified liposomes to $15.1 \pm 1.3\%$ (SM before extrusion), $18.8 \pm 0.5\%$ (SM during extrusion) and $21.0 \pm 0.3\%$ (SM after extrusion). With 5 mol% DSPE-PEG average values of $18.4 \pm 0.7\%$ (SM before extrusion), $20.7 \pm 1.6\%$ (SM during extrusion) and $22.0 \pm 0.4\%$ (SM after extrusion) were obtained. With one exception, mean vesicle diameters remained unaffected by SM and ranged between 90 and 110 nm. However, when DSPE-PEG was incorporated into liposomal preparations after extrusion, mean vesicle diameters

Table 1
Surface-modified liposomes with high encapsulation efficiencies of iopromide and Gd-DTPA

SM	Contrast agent	Encaps. effic. (%)	Mean diam./CV (nm/%)
Unmodified	iopromide	42.3 ± 3.4	131/30.3
5% CholHS-PEG	iopromide	45.2 ± 2.6	154/33.2
5% DSPE-PEG	iopromide	44.0 ± 3.4	177/37.8
unmodified	Gd-DTPA	53.1 ± 1.1	143/33.9
5% CholHS-PEG	Gd-DTPA	51.7 ± 1.9	157/ 30.7
5% DSPE-PEG	Gd-DTPA	58.0 ± 0.8	153/33.3

Lipid concentration: 120 mg/g for iopromide, 150 mg/g for Gd-DTPA. Iodine concentration: 60 mg/g. Gd concentration: 180 μmol/g. SM after extrusion. Final pore size: 0.2 μm ($n = 3$).

showed a marked increase from 108 ± 8.9 to 162 ± 23.1 nm.

3.3. PEG-coated liposomes with high encapsulation efficiencies

An improvement in encapsulation efficiencies of iopromide-carrying, surface-modified liposomes was achieved by decreasing the initial iodine to lipid ratio from 1 to 0.5 as well as increasing the pore size of the final extrusion step from 0.1 to 0.2 μm. For even further improvement, SM was carried out after extrusion. As a result of these changes in production conditions, entrapment values for iopromide as high as 42.3 ± 3.4% were achieved without SM, compared to 45.2 ± 2.6% with 5% CholHS-PEG and 44.0 ± 3.4% with 5% DSPE-PEG (Table 1). Similar preparations containing Gd-DTPA are also shown in Table 1. As can be seen 53.1 ± 1.1% of the total amount of MRI-contrast agent could be encapsulated in unmodified liposomes, compared to 51.7 ± 1.9% in the presence of 5% CholHS-PEG and 58.0 ± 0.8% in the presence of 5% DSPE-PEG.

A strong increase in mean vesicle diameter from about 130 to almost 180 nm was observed again, when SM of iopromide-containing liposomes was performed after extrusion with 5 mol% DSPE-PEG. With Gd-DTPA, however, the increase after SM was considerably smaller.

Fig. 3 shows an example of a cryo-electron micrograph of iopromide-carrying liposomes coated with 5 mol% CholHS-PEG (SM after extrusion). Apparently, all vesicles remained intact after SM. They displayed mostly uni- or bilamellar character, with the greatest portion being composed of only one bilayer.

3.4. Zeta potential measurements

Iopromide-carrying liposomes containing 10 mol% of the negatively charged SPG were employed in order to investigate the influence of SM on the zeta potential of phospholipid vesicles. In the course of this experiment the molar content of PEG-derivatives as well as the time of SM during the production process were varied.

As can be seen in Fig. 4, zeta potentials decreased sharply with increasing molar portion of the PEG-derivatives. While unmodified vesicles had an average zeta potential of − 39.3 ± 2.9 mV, lowest values of − 4.3 ± 1.2 mV with CholHS-PEG were already measured at 5 mol%. With the same amount of DSPE-PEG slightly higher values of − 8.2 ± 1.3 mV were obtained, which could be further reduced to − 2.0 ± 0.7 mV at 10 mol%. Moreover, Fig. 5 shows that this strong decrease in zeta potential could also be achieved when SM with 5 mol% of either CholHS-PEG or DSPE-PEG was carried out during or after extrusion, indicating a successful bilayer association of amphipathic polymers, irrespective of the time of SM. Thus, very small zeta potentials of − 5.4 ± 0.3 mV (CholHS-PEG) and − 4.9 ± 0.9 mV (DSPE-PEG) were measured even when SM was performed after extrusion.

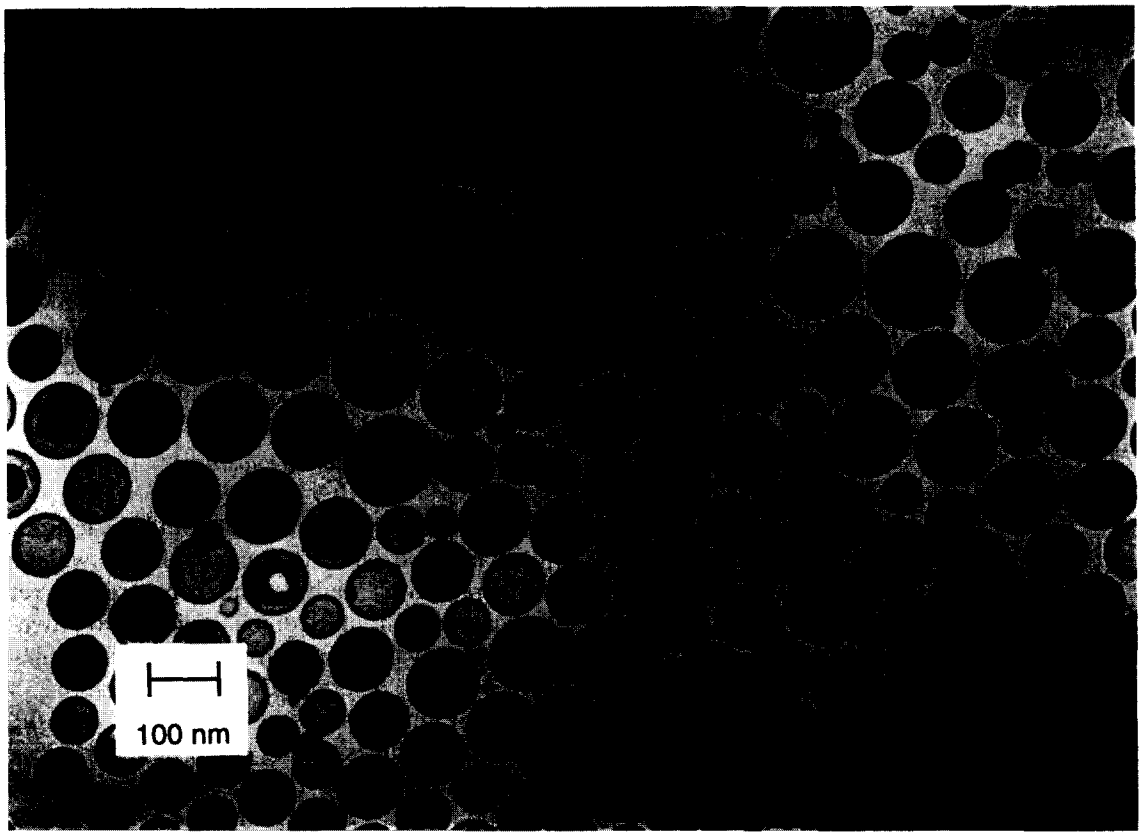

Fig. 3. Cryo-electron micrograph of iopromide-carrying liposomes coated with CholHS-PEG (SM after extrusion): 5 mol% CholHS-PEG. Lipid concentration, 120 mg/g; iodine concentration, 60 mg/g; final pore size, 0.2 μm.

3.5. Gel filtration experiments

In this study, zeta potential measurements were used to examine the effect of pegylation on surface properties of SPG-containing liposomes. The results suggested a successful association of amphipathic polymers with the liposomal bilayer, irrespective of the time of SM. In order to confirm these observations, the bilayer association of CholHS-PEG was also examined by gel filtration.

Experiments were performed with plain CholHS-PEG solutions, plain void liposome suspensions, mixtures of both made just before injection as well as void liposomes treated with 5 mol% of CholHS-PEG for 16 h, after extrusion had been completed. With Sephacryl S 400 columns, sufficient differences in retention times between vesicles (21 min) and the amphipathic polymer (33.5 min) were achieved. While the mixture of both species showed two peaks that were easily distinguishable, surface-modified liposomes displayed only one peak at the retention time of void vesicles (Fig. 6). Since the detection limit of CholHS-PEG in this experiment was 10% of the initial amount, at least 90% of the employed polymer were successfully incorporated by SM after extrusion.

3.6. Storage stability at 2–8°C

For stability testing, surface modified, iopromide-carrying liposomes were stored at 2–8°C without prior removal of the free contrast agent. After 4 months, none of the preparations showed visible inhomogeneities (sedimentation). Encapsulation was almost unaffected by storage, only a

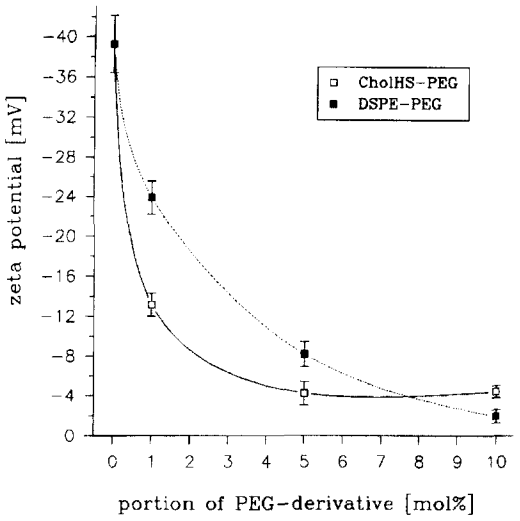

Fig. 4. Zeta potential of iopromide-carrying liposomes depending on the molar concentration of PEG-derivatives (SM before extrusion). Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size, 0.1 μm. Error bars denote SDs, $n = 3$)

small insignificant increase was observed (Table 2). Differences occurred, however, regarding vesicle size distributions of stored liposomes. While

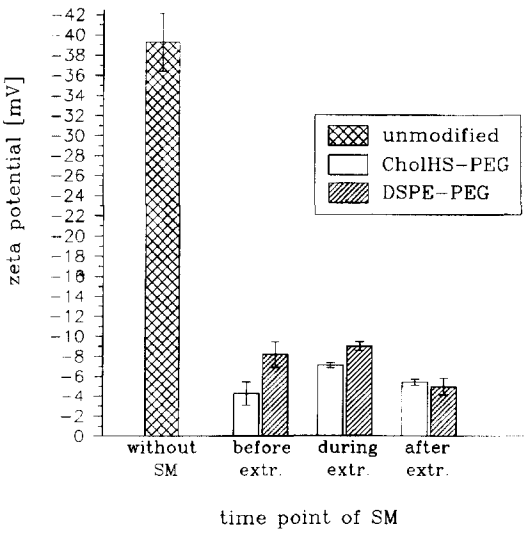

Fig. 5. SM before, during and after extrusion: impact on zeta potential of iopromide-carrying liposomes: 5 mol% PEG-derivative. Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size, 0.1 μm. Error bars denote SDs, $n = 3$.

mean diameters of unmodified and CholHS-PEG-carrying liposomes remained unchanged irrespective of the amount of PEG-derivative or the time of SM all preparations containing DSPE-PEG showed a significant increase in vesicle size. When SM was carried out before extrusion, mean diameters of DSPE-PEG-liposomes rose only moderately from 103 to 124 nm, whereas SM during extrusion resulted in a strong increase from 95 to 195 nm. Even preparations undergoing SM after extrusion, which already had mean diameters of 163 nm before storage, displayed a further increase to 192 nm.

3.7. Stability in human plasma

The stability of surface-modified, iopromide-carrying liposomes in human plasma was tested by equilibrium dialysis and compared to results obtained with unmodified vesicles. Relative entrapment values at various plasma incubation times were determined for preparations with different amounts of PEG-derivatives and for SM at different times during the production process.

As can be seen in Fig. 7, increasing amounts of CholHS-PEG up to 10 mol% did not severely affect the plasma stability when SM was carried out before extrusion. In all cases, between 48 and 58% of the initially encapsulated material still remained within the liposomal compartment after 2 h and between 43 and 51% after 4 h. By contrast, the plasma stability of DSPE-PEG-liposomes showed a concentration-dependent decrease (Fig. 8). With 1 and 5 mol% DSPE-PEG only 41 and 45% of the initially entrapped material, respectively, remained encapsulated after 2 h of plasma incubation compared to 58% for unmodified vesicles. DSPE-PEG (10 mol%) caused a further decrease to 33%. After 4 h, differences became even more pronounced: relative entrapment values of 32% (1 mol% DSPE-PEG), 34% (5 mol% DSPE-PEG) and as low as 16% (10 mol% DSPE-PEG) were obtained, compared to 49% without SM.

Fig. 9 reveals, however, that the observed deterioration in plasma stability with DSPE-PEG only occurred when SM was carried out before extrusion. As soon as the amphipathic polymer was

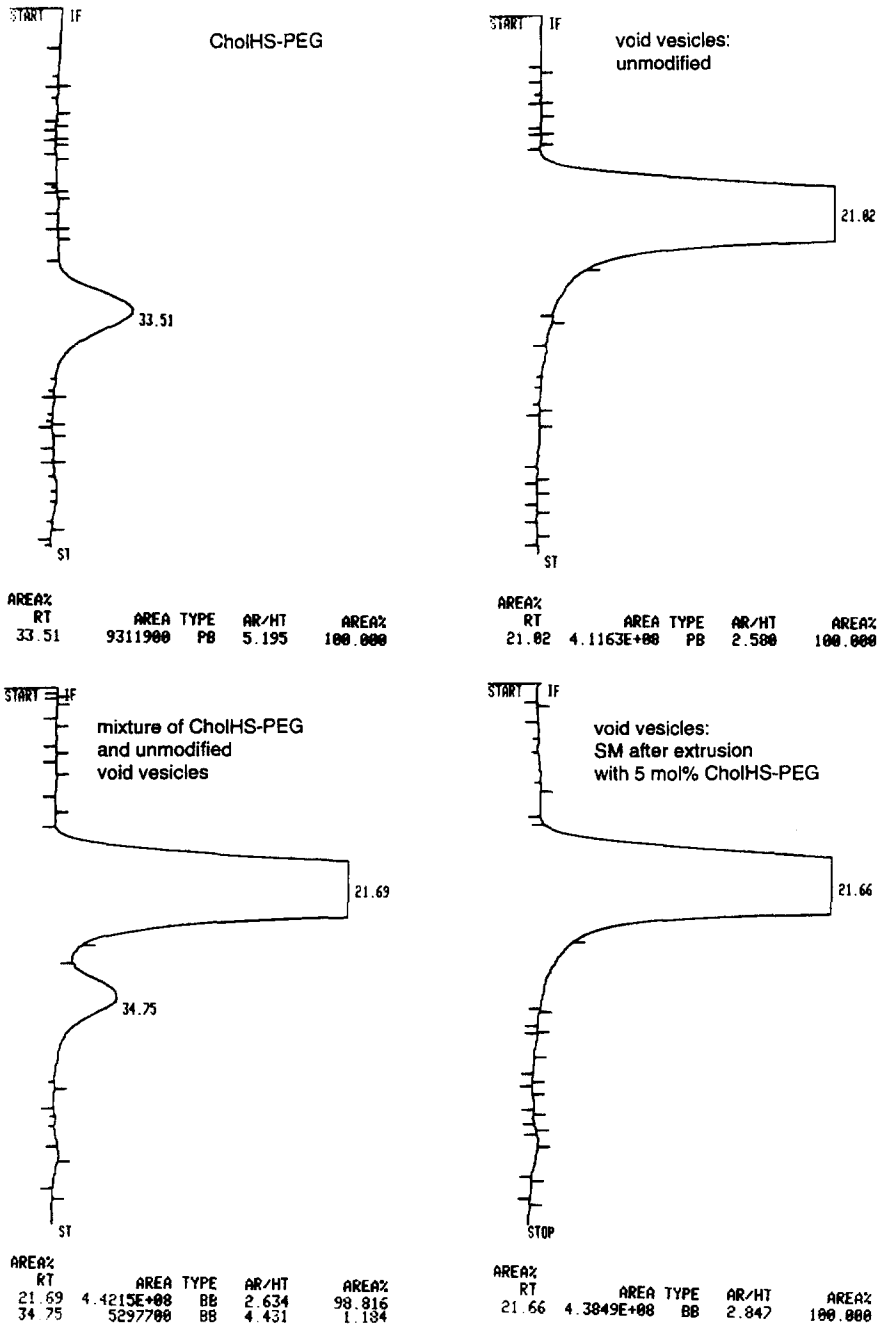

Fig. 6. Gel filtration experiments demonstrating successful incorporation of CholHS-PEG into liposomal bilayers (SM after extrusion). Lipid composition: SPC:Chol:SPG either with or without 5 mol% CholHS-PEG. Lipid concentration, 100 mg/g; final pore size, 0.1 μm; gel, Sephacryl S 400.

Table 2
Long-term stability of surface modified, iopromide-carrying liposomes (storage at 2–8°C for 4 months)

SM	Encaps. eff. after preparation (%)	Encaps. eff. after 4 months (%)	Mean diam./CV after preparation (nm/%)	Mean diam./CV after 4 months (nm/%)
Without SM	24.5 ± 2.3	25.3 ± 3.0	108/24.0	104/25.2
5% CholHS-PEG SM before extrusion	15.1 ± 1.3	16.7 ± 1.5	96/30.1	94/32.5
5% CholHS-PEG SM during extrusion	18.8 ± 0.5	20.2 ± 0.9	93/27.2	94/27.1
5% CholHS-PEG SM after extrusion	21.0 ± 0.3	22.7 ± 1.2	103/27.1	101/23.4
5% DSPE-PEG SM before extrusion	18.4 ± 0.6	20.2 ± 0.8	103/28.9	124/36.7
5% DSPE-PEG SM during extrusion	20.7 ± 1.6	22.5 ± 0.9	95/28.9	195/45.3
5% DSPE-PEG SM after extrusion	22.0 ± 0.4	23.0 ± 0.5	162/42.6	192/49.3

Lipid concentration: 100 mg/g. Iodine concentration: 100 mg/g. Final pore size: 0.1 μm ($n = 3$).

added during or after extrusion, plasma stability became similar to that of unmodified preparations. Use of CholHS-PEG did not considerably alter the plasma stability of iopromide-carrying liposomes, irrespective of whether SM was performed before, during or after extrusion (Fig. 10).

4. Discussion

In this study, the new amphipathic polymer, CholHS-PEG, was introduced for SM of vesicular systems and compared to the well described phosphatidylethanolamine-derivative, DSPE-PEG. The major difference between the two substances is their respective lipid anchor, a sterol in the case

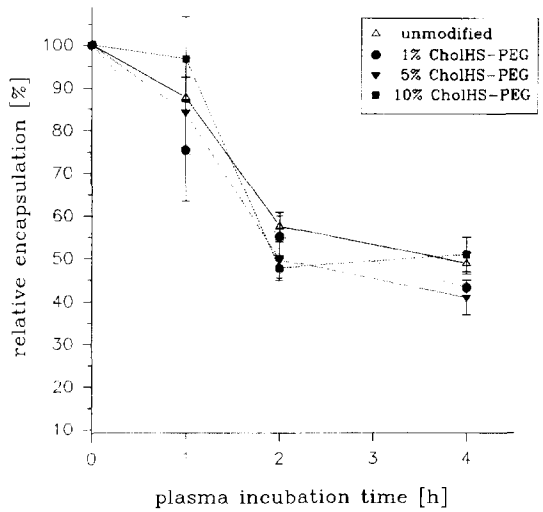

Fig. 7. Plasma stability of iopromide-carrying liposomes with increasing amounts of CholHS-PEG (SM before extrusion). Lipid concentration, 100 mg/g; iodine concentration: 100 mg/g; final pore size, 0.1 μm ($n = 3$).

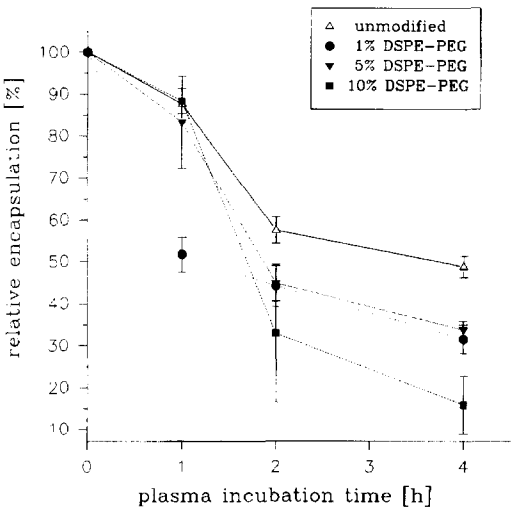

Fig. 8. Plasma stability of iopromide-carrying liposomes with increasing amounts of DSPE-PEG (SM before extrusion). Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size, 0.1 μm ($n = 3$).

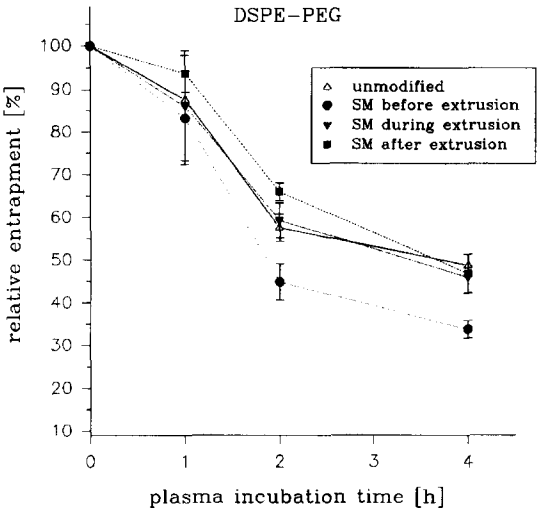

Fig. 9. SM with DSPE-PEG before, during and after extrusion: impact on plasma stability of iopromide-carrying liposomes: 5 mol% DSPE-PEG. Lipid concentration, 100 mg/g; iodine concentration, 100 mg/g; final pore size, 0.1 μm ($n = 3$).

of CholHS-PEG and a phospholipid with long saturated fatty acid chains in the case of DSPE-PEG. Although experiments with these polymers revealed some interesting differences concerning

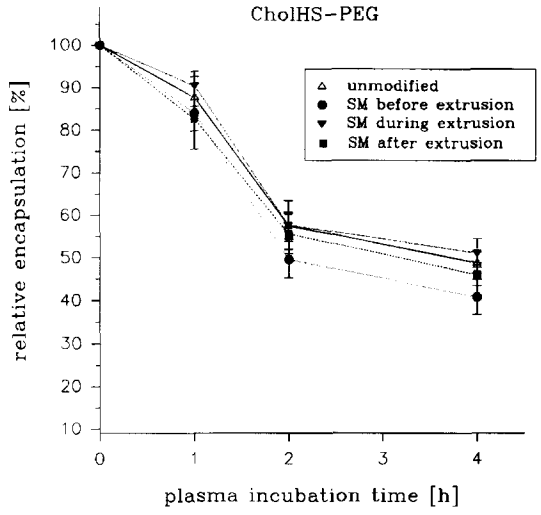

Fig. 10. SM with CholHS-PEG before, during and after extrusion: impact on plasma stability of iopromide-carrying liposomes: 5 mol% CholHS-PEG. Lipid concentration, 100 mg/g; iodine concentration; 100 mg/g; final pore size, 0.1 μm ($n = 3$).

their influence on physical properties of liposomes, they had one important characteristic feature in common: after incorporation before vesicle-formation, they caused a sharp, concentration-dependent diminution in encapsulation efficiencies of water-soluble contrast agents. This observation can be explained by a reduction in internal vesicular volume due to bulky PEG-chains covering not only the outer, but also the inner monolayer of surface-modified liposomes. As could be demonstrated by others (Needham et al., 1992), PEG-chains of e.g., 4 mol% DSPE-PEG_{1900} extend from the surface to a depth of about 5 nm, when incorporated into bilayer membranes. For a respective vesicle with 100 nm inner diameter before SM this would translate into a reduction to 90 nm after PEG-coating, resulting in a theoretical 27.1% decline in internal volume. Taking this into account, the observed decline of 38% with 5 mol% CholHS-PEG and 25% with the same amount DSPE-PEG after SM becomes comprehensible.

The fact that the addition of amphipathic polymers at later times during the production process reduced the overall decrease in encapsulation efficiency further supports this theory. Apparently, SM during extrusion already led to the integration of smaller portions of the PEG-derivative into the inner monolayer than SM before extrusion, whereas with SM after extrusion, hardly any polymer reached the inner monolayer. Polymer-coating after extrusion thus represents an elegant way of changing liposomal surface properties without reducing trapping efficiencies. Unfortunately, however, addition of DSPE-PEG to completely extruded liposomes caused a significant increase in mean diameters, so that small DSPE-PEG-containing vesicles could not be obtained using this method.

Encapsulation efficiencies of both contrast agents, iopromide and Gd-DTPA, reached maximum values of around 45 and over 50%, respectively, when optimum production parameters were employed. The results seem to be sufficient for subsequent in vivo testing since, to our knowledge, they greatly exceed the highest values published so far for entrapment of water-soluble molecules or peptides in PEG-coated vesicles (e.g.,

4–10% carboxyfluorescein-encapsulation (Blume and Cevc, 1990) and 22% vasopressin-encapsulation (Woodle et al., 1992)).

In earlier publications, the extent of SM with amphipathic polymers in vitro was often observed using methods like phase partitioning (Delgado et al., 1990; Senior et al., 1991) or biotin-avidin-agglutination assays (Klibanov et al., 1991). In this study, a new experimental design permitted an examination of the pegylation process by means of zeta potential measurement. For this purpose, the reduction in zeta potential, originally caused by SPG, was determined. The simple experiment revealed a concentration-dependent decrease in zeta potential after PEG-coating, apparently caused by a strong shielding of SPG's negative charge by the hydrophilic headgroups of CholHS-PEG and DSPE-PEG. Similar results were obtained when SM was carried out during or after extrusion, indicating a successful bilayer association irrespective of the time during the extrusion process. Gel filtration experiments employing preparations which were pegylated with the CholHS-derivative after extrusion confirmed these results. Both analytical methods clearly suggest that the addition of amphipathic polymers to existing vesicular systems results in effective bilayer incorporation. The final proof for this assumption has, however, still to be adduced.

During stability testing of surface-modified, iopromide-carrying liposomes, all preparations containing CholHS-PEG remained stable as regards macroscopic appearance, encapsulation and vesicle size, whereas DSPE-PEG caused a significant increase in mean diameter leading to values close to 200 nm when SM was carried out during or after extrusion. With SM before extrusion, the increase was considerably smaller, however, as already mentioned, this method led to the strongest decline in encapsulation efficiency.

Reduced stability of DSPE-PEG-carrying liposomes was also seen in human plasma. While CholHS-PEG had no effect, the former caused a concentration-dependent leakage increase, when SM was carried out before extrusion. Addition of DSPE-PEG at later times during the production process did not have this effect, however. Altogether, the plasma stability of the examined preparations was not satisfactory. After 4 h of incubation, even the most stable systems had lost around 50% of their content. These observations can be explained by the fact that the respective preparations contained mostly unilamellar vesicles, which are less stable than oligo- or multilamellar vesicles.

From a technological point of view, the new amphipathic polymer CholHS-PEG proved to be better suited to PEG-coating of the examined liposomal systems than the well described DSPE-PEG. Compared to unmodified species, pegylated liposomes with similar properties as regards encapsulation efficiency, vesicle size distribution as well as storage and plasma stability could only be obtained using the sterol derivative. The in vivo properties of DSPE-PEG versus CholHS-PEG liposomes still have to be examined in subsequent studies, however.

Acknowledgements

The help and advice of B. Overbeck during zeta potential determination as well as the assistance of U. Joswig (both Schering AG, Berlin) during Gd analysis, are gratefully acknowledged. We would also like to thank K. Karge and R. Kähne (Schering AG, Berlin) for their expert technical assistance during plasma stability experiments.

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