ELSEVIER

Biochimica et Biophysica Acta 1285 (1996) 237–245

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Effect of sterical stabilization on macrophage uptake in vitro and on thickness of the fixed aqueous layer of liposomes made from alkylphosphocholines

Reiner Zeisig [a,*], Kazuhiko Shimada [b], Sadao Hirota [b], Dieter Arndt [a]

[a] *AG Phospholipids, Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin, Germany*
[b] *School of Pharmaceutical Sciences, University of Shizuoka, Yada, Shizuoka 422, Japan*

Received 18 June 1996; revised 12 August 1996; accepted 13 August 1996

Abstract

A serious problem using liposomes for therapeutic purposes is the fast removal from blood circulation by components of the reticuloendothelial system (RES) most likely after opsonization of the vesicles. This study was performed to quantify the reduction in macrophage uptake in vitro of sterically stabilized liposomes (PEG-liposomes) prepared from hexadecylphosphocholine, cholesterol and poly(ethylene glycol$_{2000}$) distearoylphosphoethanolamine (PEG$_{2000}$DSPE) for the first time. The uptake was determined using HPC-liposomes of different defined size (125, 250 and 1000 nm) without and with sterical stabilization by incorporating 5 mol% of PEG$_{2000}$DSPE. HPTS was used as fluorescence marker allowing the discrimination between general uptake and the part of liposomes internalized into the low pH-compartment (Daleke, L.D., Hong, K. and Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366). Liposomal uptake by J774 mouse macrophage-like cells was time-dependent. Both the uptake and internalization were clearly reduced for PEG-liposomes compared to plain liposomes. Sterical stabilization reduced the general uptake of liposomes in vitro by more than 50% and the internalization by about 50–60%. PEG-liposomes additionally showed a delay in internalization into the macrophages during the first 6 h. Size of used liposomes had only a minor influence on liposomal uptake but highest concentration of lipid was found for large multilammelar vesicles (MLV). The fixed aqueous layer thickness (FALT) was determined by zeta potential measurements of plain and sterically stabilised HPC-liposomes (100 nm) in solutions of different ion concentrations. The calculation of the thickness was based on the linear correlation between ln ζ (zeta-potential) and κ (Debye Hückel-Parameter). FALT was calculated and found to be for plain HPC-liposomes 0.83 ± 0.17 nm and for PEG-HPC-liposomes 3.57 ± 0.17 nm. Exchange of the HPC by an alkylphospholipid with different head group has no or only minor effect (PEG-OPP-liposomes 3.44 ± 0.31 nm). Thus the reduced uptake of HPC-LUVET correlates with an increased thickness of

Abbreviations: APL, alkylphospholipids; CH, cholesterol; DCP, dicetylphosphate; DSPE, distearoylglycerophosphoethanolamine; DMG, dimyristoylglycerol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; FALT, fixed aqueous layer thickness; FCS, heat-inactivated fetal calf serum; HPC, hexadecylphosphocholine; HPTS, 8-amino-naphthalene-1,2,3-trisulfonate; LUVET, large unilamellar vesicles by extrusion technique; MLV, multilamellar vesicles; MPS, monocyte phagocytic system; NO, nitroxide; OPP, octadecyl (1,1'-dimethyl-piperidino-4-yl)phosphate; PBS, phosphate-buffered saline solution; PC, phosphocholine; PEG, poly(ethylene glycol); PEG$_{2000}$DSPE, *N*-(methoxy-poly(ethylene-glycol))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; PI, polydispersity index; PS, phosphoserine; SUV, small unilamellar vesicles; TNF, tumor necrosis factor

* Corresponding author. Fax: +49 30 94063686; E-mail: rzeisig@mdc-berlin.de

PII S0005-2736(96)00167-8

the fixed aqueous layer around these liposomes and could support the hypothesis that the thickness is an important property responsible for preventing opsonization and resulting finally in a reduced macrophage uptake.

Keywords: Hexadecylphosphocholine; Macrophage; Phagocytosis; Sterical stabilization; Poly(ethylene glycol); Liposome; Mononuclear phagocytic system; Zeta potential; Large unilamellar vesicle

1. Introduction

The use of liposomal preparations of anticancer drugs offers several advantages in comparison to the application of the free drug. Side effects are prevented and targeting by conjugation of antibodies is easily possible. In contrast to classical cancerostatica, which are encapsulated into vesicles prepared from natural lipids, antineoplastic alkylphospholipids (APL) are amphiphilic compounds with the ability to form stable liposomes in combination with cholesterol (CH) and a charged lipid (for example dicetylphosphate, DCP) by themselves [1,2].

The most serious problem in using liposomes is that they are quickly removed from blood circulation by components of the reticuloendothelial system (RES), most likely after opsonization of the vesicles [3–5].

Several attempts have been made to circumvent this disadvantage. Besides variation of liposome composition [6–12], size [6,7], amount of cholesterol [8,9], surface charge [10], addition of phosphatidylserine [7] or gangliosides [10], best results have been obtained using sterical stabilization by incorporation of phosphoethanolamine derivatives of poly(ethylene glycol). These PEG-liposomes (or Stealth® liposomes) exhibited an enhanced time of circulation in vasculature [11–14] (for review see Refs. [15,16]) and subsequently an increase was found in the drug targeted by liposomes to tumor tissue [17,18].

First experiments demonstrated that it is possible to prepare sterically stabilized HPC-liposomes without any problems by incorporation of up to 10% poly(ethylene glycol) derivatives of phosphatidylserine [19]. Indirect evidence was found for a reduced interaction with mouse peritoneal macrophages or permanent cell lines comparing the NO- and TNF-release from cells coincubated with PEG-liposomes or with plain liposomes [19,20] in vitro. Additional carbon ink assay in vivo indicated an inhibition of immune system response [20].

A great number of data are already available for changed biodistribution in vivo [15,16,21]. Several experiments have been carried out to investigate the influence of different factors on macrophage uptake in vitro [6–8,22,23]. On the other hand, only few results have been given for a direct comparison of PEG- and plain liposome uptake quantified in vitro. [24].

This study was performed to quantify in vitro the macrophage uptake of sterically stabilized liposomes prepared from alkylphosphocholines for the first time.

Several reasons for the reduction of macrophage uptake are now under discussion. It is most likely that either the sterical barrier of hydrophilic chains directly or induction of a fixed aqueous layer at the liposomal surface indirectly prevents the opsonization of the vesicles.

The fixed aqueous layer thickness can be determined by zeta-potential measurements. First results using doxorubicine encapsulating liposomes prepared from DMPC demonstrated a clear difference in the thickness of this layer for plain- and PEG-liposomes depending on the concentration of the PEG-lipid [25].

FALT was determined for APL for the first time and allows the comparison of the biological property uptake and the physical property thickness of the fixed aqueous layer.

2. Materials and methods

2.1. Materials

Na-salt of *N*-(*O*-methylpoly(ethylene glycol))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PEG_{2000}-DSPE), with about 45 repetitive ethoxy-units was purchased from Sygena LTD. Liestal, Switzerland. HPC and OPP was a gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany) and was stored in a desiccator at room temperature. Dicetylphosphate (DCP), cholesterol (CH) and calcein was purchased from Serva (Heidelberg, Germany). CH was recrystallized from acetone prior to

use. All other chemicals were commercial products of reagent grade.

2.2. Preparation and characterization of liposomes

Vesicles were prepared from APL, CH and DCP in a molar ratio of 1:1:0.2 (lipid/CH/DCP) by the lipid layer method as reported recently [1,20]. For the preparation of PEG-liposomes additional PEG-lipid (10 mol%) was added as indicated in the tables and figures. The lipid film was hydrated with phosphate-buffered saline solution (PBS, pH 7.4) for MLV preparation.

LUVET were prepared by the extrusion technique according to MacDonald et al. [26]. Liposomes for uptake experiments were obtained by repeated extrusion (19 times) of 10 mM MLV-suspensions (30 mM HPTS)) through polycarbon membranes of 100 nm pore size using a LiposoFa. .™ Basic system (Avestin, Ottawa, Canada). Liposomes for FALT determinations were produced from MLV in lactate buffer (10 mM, pH 4) by a single extrusion through filter with decreasing pore sizes and finally five times through filter of 100 nm pore size at 50°C using the Extruder® (LIPEX, Vancouver, Canada).

HPTS liposomes were separated from HPTS by centrifugation at 19 500 × *g* (MLV) or by chromatography on Sephadex G75.

The lipid content and liposomal composition of the preparations were checked by high performance thin-layer chromatography (Automated Multiple Development System and Linomat IV; CAMAG, Muttenz, Switzerland).

Vesicle size determination was performed by dynamic light scattering measurement at an angle of 90 with a Coulter Counter N4 MD model and the Accu-Comp® System (Coulter Electronics, Hialeah, USA). Mean particle size was calculated by SDP analysis. Size distribution (based on solid sphere weight results) is given as polydispersity index (PI) — varying from 0 (entirely monodisperse) to 1 (completely polydisperse suspension).

2.3. Determination of cellular uptake

These experiments were done according to Daleke et al. [22]. Therefore, $1 \cdot 10^6$ J744 mouse macrophage like cells were seated in a 6-well plate (Costar). The cells were incubated after adherence with HPTS liposomes diluted in RPMI medium without fetal calf serum to a final concentration of 100 μM for the indicated times at 36°C.

Control cells were incubated with the same liposome-media solution at 4°C for 30 min to determine binding under conditions excluding phagocytosis. Fluorescence measurements of marker in low pH compartment of macrophages were done after coincubation of cells with a solution of HPTS in medium (100 mM) for at least 24 h.

2.3.1. Fluorescence measurements

After incubation the cells were harvested by scraping off and counted. Fluorescence of HPTS of cell suspensions were determined using a FARRAD spectrofluorometer MK1 (FARRAD Optical, New York, USA). Excitation wavelength used was 415 and 450 nm and emission wavelength was 510 nm.

Differentiation between general uptake and phagocytosed marker was done using the following equation

$$m = (R_{max} - R_{exp})/(R_{max} - R_{min})$$

where m: amount of HPTS in liposomes associated with cells, given in percent, R_{max}: fluorescence ratio (450:415 nm) in cells after HPTS uptake, R_{min}: fluorescence ratio (450:415 nm) after liposome association at 4°C, R_{exp}: fluorescence ratio (450:415 nm) found for liposomal HPTS in the experiment.

Absolute quantification of HPTS was done using a standard curve, based on fluorescence at excitation wavelength 415 nm.

The amount of associated/internalized HPC was calculated from the HPTS-amount after determination of the lipid content of each liposomal preparation and was finally normalized to the uptake of 10^6 cells.

All experiments were done in duplicate and the results represent the mean of at least 4 separate experiments. Statistic evaluation was done using Student's *t*-test.

2.4. FALT determination

Falt determination was based on measurement of the electrophoretic mobility of liposomes in sample solutions prepared by appropriate dilution with isotonic solution of 10 mM lactate buffer (pH 4) with

various concentrations of NaCl and sucrose (for details see tables and figures) as reported recently [25]. Zeta potential of these suspensions (calculated by applying the Smoluchowski equation) were determined using the electrophoretic light scattering apparatus ELS 800 (Otsuka Electronics, Osaka, Japan) at 25°C.

The ln zeta potential ζ (V) was plotted versus Debye Hückel parameter (k). κ represents $3.3\sqrt{C}$, where C is the concentration of electrolytes in the solution. The slope of the obtained plots indicates the thickness of the fixed aqueous layer in nm.

3. Results

3.1. Cellular uptake

The uptake of alkylphosphocholine liposomes with regard to size, time and sterical stabilization was determined. Liposomes were prepared from MLV (size > 1000 nm, polydispersity index about 0.65) by repeated extrusion through polycarbonate filter with pore size of 100 nm (LUVET 100) or 400 nm (LUVET 400) and obtained with diameters of about 120 ± 10 nm (PI 0.06) and about 250 ± 15 nm (PI 0.28) respectively (Table 1).

Starting with a 30 mM solution the amount of encapsulated hydrophilic fluorescence marker HPTS varied and was found to be between 37.5 (PEG-LUVET 100) and 157.8 pmol/nmol HPC (PEG-MLV).

The cellular uptake was determined based on HPTS encapsulated in the HPC liposomes. The content was estimated using 415 nm excitation wave length and a standard curve in the appropriate concentration range. The total amount of liposomes associated with cells (indicated as uptake and including cell membrane bound vesicles and liposomes already internalized) was determined in a similar way after harvesting the cells. The concentration of HPTS was obtained from the standard curve, normalized to 1 million cells and the amount of HPC calculated based on the amount of encapsulated HPTS.

Determinations were carried out at various time points of up to 18 h during which the uptake of liposomes increased with time (Figs. 1 and 3A). Most liposomal HPC was associated with macrophages coincubated with MLV. The lowest amount of HPC was found at cells treated with small liposomes (Fig. 1). The difference in HPC-uptake with regard to size was significantly different between macrophages co-incubated with MLV and cells incubated with the smaller vesicles after 6 and 18 h ($P < 0.05$).

Sterical stabilization of HPC liposomes resulted in a clear reduction in liposomal uptake for liposomes of all sizes by at least 47% (MLV) after 18 h of incubation (Fig. 2A and Fig. 3A). We already found differences for shorter incubation times but they were not significant ($P > 0.05$). For example, the comparison of plain and sterically stabilized liposomes is given for the 2 h incubation period in Fig. 2B. A complete data set for LUVET 100 is plotted in Fig. 3A.

The internalization of liposomal HPC into the low pH compartment was estimated at the pH-dependent excitation wavelength 450 nm. The changes of the fluorescence ratio 450:415 allow the calculation of

Table 1
Characterization of liposomes used for determination of macrophage uptake

Liposomes	Size (nm)	PI	HPTS (pmol/nmol HPC)
LUVET 100	122.7 ± 10.3	0.062 ± 0.009	41.2 ± 22.1
PEG-LUVET 100	119.4 ± 10.2	0.050 ± 0.037	37.5 ± 10.6
LUVET 400	255.4 ± 11.4	0.265 ± 0.08	84.3 ± 38.8
PEG-LUVET 400	238.4 ± 17.5	0.287 ± 0.085	83.4 ± 33.0
MLV	1917.3 ± 1641.8	0.653 ± 0.302	119.7 ± 57.9
PEG-MLV	1257.2 ± 1030.8	0.696 ± 0.345	157.8 ± 22.4

Liposomes were prepared as described in Section 2. Size was determined by photon correlation spectroscopy using a Coulter M4 particle sizer at an angle of 90°. Data represent the mean ± S.D. for four different liposomal preparations. Diameter and size distribution were based on unimodal results. Content of HPTS was determined by fluorescence spectroscopy at 510 nm emission and 415 nm excitation wavelengths. Concentration of HPC was obtained by HPTLC.

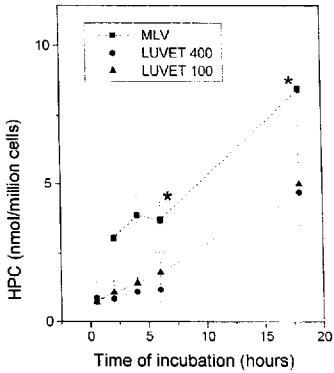

Fig. 1. The amount of HPC (mean ± S.D., $n \geq 3$) calculated for total cellular uptake by J774 cells (about $5 \cdot 10^5$ cells) after coincubation with indicated HPTS-liposomes (100 μM) for different times as described in Section 2 are given. Concentration of HPTS was determined based on a standard curve by fluorescence measurements (F_{EM}: 513 nm/F_{EX}: 415 nm). Significant different to 100 nm LUVET.

the percentage of internalized liposomes. Therefore, control experiments were carried out. The fluorescence ratio for HPTS in liposomes only associated with the cell membrane was obtained after incubation of cells with HPTS-liposomes at 4°C. The fluorescence ratio for HPTS in the low pH compartment after pinocytosis was estimated after macrophage coincubation with molecular HPTS solutions.

The results of internalization are similar to the data already found for the total uptake. The amount of liposomes found within the macrophages were also directly dependent on the size of vesicles. PEG-liposomes were internalized much lower than plain liposomes of the same size. The sterically stabilization seems to hinders the internalization of these vesicles into the cells in contrast to the binding of liposomes which was not delayed. Only after longer incubation times the effect of sterical stabilization was overcome. Here the amount of internalized HPC increased from control level to 75% (LUVET 100), 72% (LUVET 400) and 48% (MLV) of that calculated for total uptake (Fig. 3A,B).

3.2. Zeta-potential measurements

The thickness of the aqueous layer around liposomes was calculated for 3 selected liposomal preparations to investigate the influence of the sterical stabilization (comparison of plain HPC-LUVET and PEG-HPC-LUVET) and to compare the influence of the lipid used as the main 'brick' for liposomal preparation (comparison between PEG-HPC-LUVET and PEG-OPP-LUVET). The liposomes for these experiments were of a average diameter of 106.8 ± 3.8, 127.4 ± 1.4 and 113.1 ± 1.7 nm for HPC-LUVET, PEG-HPC-LUVET and PEG-OPP-LUVET respectively with a polydispersity index lower than 0.08. The zeta-potentials estimated for these liposomes in isotonic lactate buffer with sucrose and sodium chloride in concentrations between 5 and 100 mM are given in Table 2. The pH of the buffer was fixed at 4

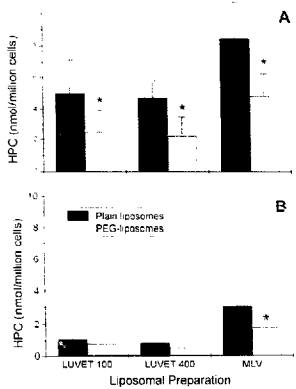

Fig. 2. Comparison of plain and sterically stabilized HPC-liposomes (PEG-liposomes) for total uptake after 18 h (A) or 2 h (B) of coincubation of J774 cells with 100 μM liposomes. Given are the concentration of HPC (mean ± S.D., $n \geq 3$) calculated as described in Section 2 based on fluorescence measurements of HPTS (F_{EM}: 513 nm/F_{EX}: 415 nm). * Significantly different to plain liposomes.

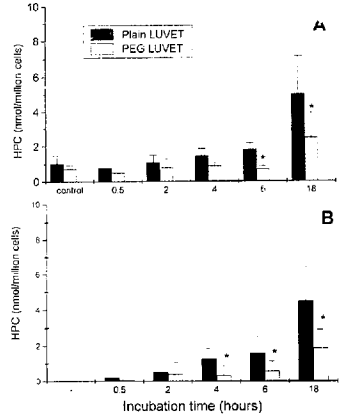

Fig. 3. Total uptake (A) and internalization (B) of HPC- (plain LUVET) and sterically stabilized HPC-liposomes (PEG-LUVET), both extruded through 100 nm pore size, were followed for 18 h. J774 cells were coincubated with 100 μM of liposomes. After indicated times the concentration of the marker HPTS was determined by fluorescence measurements (F_{EM}: 513 nm/F_{EX}: 415 nm, A and F_{EM}: 513 nm/F_{EX}: 450 nm, B). Given are the concentration of HPC (mean ± S.D., $n \geq 3$) calculated as described in Section 2. * Significantly different to plain liposomes.

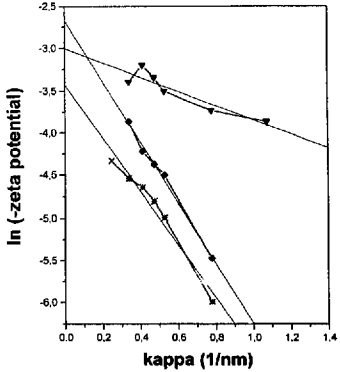

Fig. 4. Electrophoretic mobility of liposomes were measured in isotonic solution of 10 mM lactate buffer (pH 4) with various concentrations of NaCl and sucrose (for details see Section 2 and Table 2). Natural logarithm of the absolute value of zeta potential ζ (V) was plotted versus Debye Hückel parameter (κ). κ represents $3.3\sqrt{C}$, where C is the concentration in mol of electrolytes in the solution. ▼, plain HPC-liposome; HPC/CH/DCP (10:10:2 molar ratio); ♦, PEG-HPC-LUVET, HPC/CH/DCP/PEG_{2000}DSPE (10:10:2:1 molar ratio); *, PEG-OPP-LUVET, OPP/CH/DCP/PEG_{2000}DSPE (10:10:2:1 molar ratio).

to obtain similar conditions compared to previous measurements using liposomes made from dimyristoyl phosphocholine liposomes with and without doxorubicin [25]. The absolute value of the potential decreased with increasing concentration of salt. The decrease was at its smallest with plain liposomes.

Table 2
Dependence of zeta potential on electrolyte concentration

NaCl and lactate (mM)	Plain HPC-LUVET	PEG-HPC-LUVET	PEG-OPP-LUVET
5.6	−31.82 ± 1.73	−15.40 ± 0.51	−13.16 ± 0.35
10.6	−33.86 ± 1.66	−20.98 ± 1.62	−10.66 ± 1.27
15.6	−40.94 ± 6.39	−14.66 ± 2.32	− 9.67 ± 0.55
20.6	−35.96 ± 2.18	−11.08 ± 1.02	− 6.78 ± 0.33
50.6	−23.94 ± 1.39	− 4.19 ± 0.52	− 2.51 ± 1.52
100.6	−20.99 ± 1.04	nd	nd

Zeta potential measurement were done with liposomes composed of APL/CH/DCP (10:10:2) or APL/CH/DCP/PEG_{2000}DSPE (10:10:2:1 molar ratio), diluted in isotonic lactate buffer containing different concentrations of NaCl and sucrose. Results are expressed as means ± S.D. ($n \geq 3$).

Table 3
Thickness of the fixed aqueous layer around APL-liposomes

Liposomal preparation	Thickness (nm)	A [a]	R^2 [a]
Plain HPC-LUVET [a]	0.83 ± 0.17	-3.00	0.922
PEG-HPC-LUVET [b]	3.57 ± 0.17	-2.67	0.996
PEG-OPP-LUVET [b]	3.44 ± 0.31	-3.25	0.988
PEG-DMPC-LUVET [c]	3.2	-3.20	0.980

FALT results were estimated from Fig. 4.
[a] Liposomal composition: APL/CH/DCP (10:10:2);
[b] APL/CH/DCP/PEG_{5000}DSPE (10:10:2:1),
[c] DMPC/CH/DMPG/PEG_{5000}DMG (10:10:6.5:4 molar ratio); data taken from Ref. [25].
[a] Characteristics for linear regression, based on $Y = A + BX$, ($n \geq 5$).

Deviations from the exponential slope were found at a very low salt concentration, but the plot of ln(-zeta) against the Debye-Hückel parameter κ fits best to a linear function (Fig. 4). Here κ represents $3.3\sqrt{c}$, where c gives the concentration of electrolytes including NaCl and the dissociated amount of lactate in the buffer system (for details see [25]). The slope of these plots indicates the thickness of the fixed layer in nm. The results are given in Table 3. Both sterically stabilized liposome preparation have a similar thickness of the aqueous layer of about 3.5 nm. The thickness of this layer was clearly reduced for plain liposomes and calculated to be only about one fourth of the former.

4. Discussion

The main problem to obtain a high efficiency of liposomal drugs on targets different from the RES is the rapid clearance from the blood circulation. The method mostly used to circumvent uptake by macrophages as the main components of the RES is the sterical stabilization by poly(ethylene glycol) derivatives of PE incorporated into the liposomal membrane [11–16].

In a previous study we could demonstrate that it is possible to prepare liposomes of a defined size with and without sterical stabilization using alkylphosphocholines as liposomal and active cancerostatic lipid within one vesicle preparation [1,20].

First experiments gave indirect evidence for a reduced interaction of these PEG-HPC-LUVET with phagocytic cells [20]. The activation of macrophages to release cytotoxic mediators like NO or TNF in vitro induced by APL-liposomes [19,27] was decreased to the level of control cells if PEG-liposomes were used for activation. Additional data demonstrated that the uptake of particles like carbon ink was not affected in vivo by the application of PEG-liposomes prior to ink injection in contrast to classical liposomes where a dramatic change of particle clearance was induced [20].

To continue investigations of sterically stabilized liposomes prepared from alkylphospholipids, the study was directed at two aims. First, we wanted to quantify the uptake of PEG-HPC-liposomes by macrophages in vitro. In a direct way this should prove whether sterical stabilization works with alkylphosphocholine liposomes in the same way as it was found for liposomes made from natural lipids. Secondly, we wanted to characterize the liposomes with regard to thickness of the fixed aqueous layer around the outer membrane which is made responsible for the variation in immune response.

Different methods have been used for the determination of cellular liposomal uptake mostly based on measurements of encapsulated fluorescent [22,23,28–30] or radioactive marker [6,8]. But uptake of liposomes is a process consisting of association of liposomes to the cellular membrane and transport of liposomes into the intracellular compartment by endocytosis. It is possible to differentiate between both liposomal quantities in a classical way by doing two separate experiments, one for general uptake and the other for conditions necessary for binding without endocytosis.

Another possibility for the characterization of macrophage uptake allows the encapsulation of the fluorophore HPTS [22,31]. The amount of marker is determined in this simple and suitable assay using the pH independent excitation wavelength 415 nm for total uptake. The part of internalized liposomes was obtained from fluorescence at excitation wavelength 450 nm, at which fluorescence is strongly dependent on pH. Both data sets were obtained for the same cell population and are highly comparable. Therefore we selected this assay for our experiments.

Until now sterically stabilized liposomes only prepared from natural lipids with a classical glycerol backbone structure were characterized with regard to

immune response in vivo [32–35]. On the other hand, only a few studies in vitro have been reported quantifying the macrophage uptake of PEG-liposomes [6,24,36].

In our experiments we were interested in using an optimal liposome preparation with regard to minimal macrophage uptake. It was demonstrated in an extensive study on liposomal uptake in vitro that the amount of PEG-lipid should be between 5–10 mol% [6]. Theoretical estimation based on calculation of the protective square radius of PEG lipid resulted in 1000 molecules necessary for the complete protection by a polymer of molecular weight 2000 Da [37,38]. This corresponds to 4.7 mol%. Therefore we prepared liposomes containing 4.3 mol% PEG_{2000}DSPE based on complete molar liposomal compounds (or 10 mol% related to HPC).

The data obtained in our study demonstrated that it is possible already in vitro to characterize the influence of liposomal composition on interaction with macrophages. This is in a good agreement with data obtained in comparable studies using liposomes prepared from egg-PC [6,24,36].

The preparation of liposomes using alkylphosphocholines with their simple mono chain structure as main liposomal 'brick' is possible without any problem, if an equimolar amount of cholesterol is used [1,20]. We found no interference of HPC to properties of sterical stabilization. All the liposomes investigated were significantly higher bound to macrophages than the sterically stabilized ones. The liposomes were taken up by J774 macrophages in a time dependent manner.

Another important factor influencing the cellular uptake is the vesicular size. Although there is a generalisation that smaller liposomes were taken up much slower from the blood circulation [21,39] the uptake in vitro was found to be negatively correlated to size [6,40]. In our study uptake was found to be positively correlated to vesicular size irrespective of whether the liposomes were sterically stabilized or not. The highest amount of HPC was determined for coincubation with MLV with a mean diameter larger then 1 μm, whereas the uptake of smaller liposomes was nearly similar.

Taking into account that the transport of vesicles into the cell takes about 30 min [38], the internalization was clearly inhibited up to 6 h for the PEG liposomes compared to plain liposomes. Further, no difference was found comparing the ratio between total uptake and internalization of liposomal preparations with and without sterical stabilization after 18 h of coculture. This indicates that sterical stabilization is mainly influencing the binding process to the cellular surface and only to a lesser degree the transport into the cell [10]. This agrees with the finding that J774 cells have higher binding sites and higher binding constants for PS/PC/CH liposomes than that for PC/CH liposomes, but the rate constants for endocytosis was similar for both liposome preparations [29].

The mechanism(s) of sterical stabilization for prevention of cellular uptake has not yet been completely understood. A marking by the incorporation of serum complements like proteins into the liposomal membrane (opsonization) seems to be an essential process for the recognition of such particles by macrophages [3–5,22,39]. If this opsonization is missing, the liposomal uptake by macrophages is inhibited.

It was demonstrated that the incorporation of proteins into the bilayer or on the liposomal membrane is dramatically reduced after sterical stabilization [40]. There are two different explanations for this phenomenon. First, the high hydrophilic polyethylene chain could form a dense conformational cloud over the outer liposomal surface resulting in a physical sterical barrier which inhibits the interactions of liposomal components with molecules in the solution or prevents the incorporation of proteins [37]. Secondly, the hydrophilic chain results in a formation or increase of a fixed aqueous layer around the outer liposomal surface, which should complicate the opsonization by proteins from serum.

In our understanding there is no real discrepancy between both explanations. It is likely that the hydrophilic chain of the PEG lipids form a steric barrier but with a very dynamic character [37]. This should allow the incorporation of water molecules between the polyethylene chains and thus the forming of the fixed aqueous layer just around the liposomal bilayer. Both could explain the prevention of charge to charge interaction between liposomal membrane and proteins.

Our results demonstrate that even sterically stabilized liposomes made from unusual lipids show simi-

lar protection for macrophage uptake, which could be further enhanced by the careful selection of liposomal size.

Acknowledgements

This work was supported by the grants from the Deutsche Forschungsgemeinschaft (24911D) and by the Japanese Society for Promotion of Science (RC3/96703). We especially thank Ms. Anneliese Schütt for her expert technical assistance.

References

[1] Zeisig, R., Fichtner, I., Arndt, D. and Jungmann, S. (1991) Anti-Cancer Drugs 2, 411–417.
[2] Kaufmann-Kolle P., Unger C. and Eibl H. (1992) in: Progress in Experimental Tumor Research (Eibl, H., Hilgard, P. and Unger, C., eds.), Vol. 34, New Drugs in Cancer Therapy, pp. 12–24, S. Karger, Basel.
[3] Senior, J., Crawley, J.C. and Gregoriadis, G. (1985) Biochim. Biophys. Acta 839, 1–8.
[4] Devine, D.V., Wong, K., Serrano, K., Chonn, A. and Cullies, P.R., (1994) Biochim. Biophys. Acta 1191, 43–51.
[5] Blume, G. and Cevc, G. (1993) Biochim. Biophys. Acta 1146, 157–168
[6] Allen, T.M., Austin, G.A., Chonn, A., Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
[7] Lee, K.D., Nir, S., and Papahadjopoulos, D. (1993) Biochemistry 32, 889–899.
[8] Roerdink, F.H., Regts, J., Handel, T., Sullivan, S.M., Baldeschwieler, J.D. and Scherphof, G.L. (1989) Biochim. Biophys. Acta 980, 234–240.
[9] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591–598.
[10] Lee, K-D., Hong, K. and Paphadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 185–197.
[11] Allen, T.M. and Hansen, C. (1991) Biochim. Biophys. Acta 1068, 133–141.
[12] Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
[13] Papahadjopoulos, D., Allen, T.M., Gabizon, A. Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
[14] Blume, G., Cevc, G., Crommelin, M.D.J.A., Bakker-Woudenberg, I.A.J.M., Kluft, C. and Storm, G. (1993) Biochim. Biophys. Acta 1149, 180–184.
[15] Woodle, M.C. and Lasic, D. (1992) Biochim. Biophys. Acta 1113, 171–199.
[16] Allen, T.M., Hansen, B.C., De Menezes, D.E.L. (1995) Adv. Drug Deliv. Rev. 16, 267–284
[17] Yuan, F., Dellian, M. Fukumura, D., Leunig, M., Berk, D.A., Torchilin, V.P. and Jain, R.K. (1995) Cancer Res. 55, 3752–3756.
[18] Wu, N., Da, D. Rudoll, T. Needham, D., Whorton, R. and Dewhurst, M. (1993) Cancer Res. 53, 3765–3770.
[19] Eue, I., Zeisig, R. and Arndt, D. (1995) J. Cancer Res. Clin. Oncol. 121, 350–356.
[20] Zeisig, R., Eue, I. Kosch, M., Fichtner, I. and Arndt, D. (1996) Biochim. Biophys. Acta 1283, 177–184.
[21] Maruyama, K., Yuda, T. Okamoto A., Kojima, S., Suginaka, A. and Iwatsuru, M. (1992) Biochim. Biophys. Acta 1128, 44–49.
[22] Dalcke, L.D., Hong, K., Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366.
[23] Kronberg, B., Dahlmann, A., Carlfors, J. and Karlsson J. (1990) J. Pharmaceut. Sci. 79, 667–671.
[24] Vertut-Doi, A., Ishiwata H. and Miyajima, K. (1996) Biochim. Biophys. Acta 1278, 19–28.
[25] Shimada, K., Miyagishima, A., Sadzuka, Y., Nozawa, Y., Mochizuki, Y., Oshima, H. and Hirota, S. (1995) J. Drug Targeting 3, 283–289.
[26] MacDonald, R.I., Menco, B.P.M., Takeshita, K., Subbarao, N.K. and Hu, L. (1991) Biochim. Biophys. Acta 1061, 297–303.
[27] Zeisig, R., Rudolf, M. Eue, I. and Arndt, D. (1995) J Cancer Res. Clin. Oncol. 121, 69–75.
[28] Perry, D.G. and Martin, W.J. (1995) J. Immunol. Methods 181, 269–285.
[29] Lee, K.D., Nir, S. and Papahadjopoulos, D. (1993) Biochemistry 32, 889–899.
[30] Stevenson, M., Baillie, A.J. and Richards, R.M.E. (1984) J Pharm. Pharmacol. 36, 824–830.
[31] Straubinger, R.M., Papahadjopoulos, D. and Hong, K. (1990) Biochemistry 29, 4929–4939.
[32] Boerman, O.C., Storm, G., Oyen, W.J., Van Bloois, L., Van der Meer, J.W., Claessens, R.A., Crommelin, D.J. and Corstens, F.H. (1995) J. Nucl. Med. 36, 1639–1644.
[33] Allen, T.M., Newman M.S., Woodle, M.C. Mayhew, E. and Uster, P.S. (1995) Int. J. Cancer 62, 199–204.
[34] Zheng, S., Zheng, Y. and Beissinger, R. (1994) Artif. Cells Blood Substit. Immunobil Biotechnol. 22, 487–501.
[35] Huang, S.K., Stauffer, P.R., Hong, K., Guo, J.W., Phillips, T.L., Huang, A. and Papahadjopoulos, D. (1994) Cancer Res. 54, 2186–91.
[36] Lee, K.D., Hong, K. and Papahjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 185–197.
[37] Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov, A.A., Trubetskoy, V.S., Herron, J.N. and Gentry, C.A. (1994) Biochim. Biophys. Acta. 1195, 11–20.
[38] Yosimura, T. Shono, M. Imai, K. and Hong, K. (1995) J. Biochem. Japan 117, 34–41.
[39] Devine, D.V., Wong, K., Serrano, K., Chonn, A. and Cullies, P. (1994) Biochim. Biophys. Acta 1191, 43–91.
[40] Woodle, M.C., Newman, M.S. and Cohen, J.A. (1994) J. Drug Targeting 2, 397–403.