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Preparation and stability of lipid-coated nanocapsules of cisplatin: anionic phospholipid specificity

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Abstract

Cisplatin nanocapsules represent a novel lipid formulation of the anti-cancer drug *cis*-diamminedichloroplatinum(II) (cisplatin), in which nanoprecipitates of cisplatin are coated by a phospholipid bilayer consisting of a 1:1 mixture of zwitterionic phosphatidylcholine (PC) and negatively charged phosphatidylserine (PS). Cisplatin nanocapsules are characterized by an unprecedented cisplatin-to-lipid ratio and exhibit increased in vitro cytotoxicity compared to the free drug [Nat. Med. 8, (2002) 81]. In the present study, the stability of the cisplatin nanocapsules was optimized by varying the lipid composition of the bilayer coat and monitoring in vitro cytotoxicity and the release of contents during incubations in water and in mouse serum. The release of cisplatin from the PC/PS (1:1) nanocapsules in water increased with increasing temperature with a $t_{1/2}$ of 6.5 h at 37 °C. At 4 °C, cisplatin was retained in the nanocapsules for well over 8 days. Replacement of PS by either phosphatidylglycerol or phosphatidic acid revealed that nanocapsules prepared of PS were more stable, which was found to be due to the ability of PS to form a stable cisplatin–PS coordination complex. Mouse serum had a strong destabilizing effect on the cisplatin nanocapsules. The PC/PS formulation lost over 80% of cisplatin within minutes after resuspension in serum. Incorporation of poly(ethylene glycol 2000) (PEG)-derivatized phosphatidylethanolamine and cholesterol in the bilayer coat extended the lifetime of the cisplatin nanocapsules in mouse serum to almost an hour. The results demonstrate that specificity in the interaction of cisplatin with anionic phospholipids is an important criterium for the formation and stability of cisplatin nanocapsules.

Keywords: Cisplatin; Nanocapsule; Lipid formulation; Anionic phospholipid; Stability; Drug delivery

1. Introduction

The anticancer drug *cis*-diamminedichloroplatinum II (cisplatin) is commonly used in the treatment of a variety of malignancies including genitourinary, head and neck, and non-small-cell lung cancers [1–3]. There is strong evidence that the nuclear DNA is the most important target of cisplatin [4]. The DNA–cisplatin adducts formed interfere with transcription and/or replication, eventually leading to cell death. The clinical use of cisplatin is impeded by toxic side effects such as nephrotoxicity, peripheral neuropathy, and ototoxicity [5,6]. Failure of treatment is often caused by the development of resistance to cisplatin [7]. Another drawback of cisplatin is its rapid inactivation due to complexation with plasma and tissue proteins [8].

One approach to reduce the toxic side effects and to increase the therapeutic index of cisplatin is encapsulation in liposomes. Several liposomal formulations of cisplatin have been developed [9–11], the most recent being SPI-077, in which cisplatin is enclosed in pegylated (“stealth”) liposomes consisting of hydrogenated soy phosphatidylcholine (PC), cholesterol, and polyethylene glycol (PEG)-derivatized distearoylphosphatidylethanolamine [12,13]. SPI-077 liposomes were reported to be stable in plasma, to have a prolonged circulation time, and to exhibit increased efficacy against tumors and reduced toxicity compared to free cisplatin [12,14]. However, in the clinical phase I–II studies carried out so far, no antitumor efficacy of SPI-077 was observed (see, e.g. Refs. [15,16]). It

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appears that the bioavailability of cisplatin in the tumor is the limiting factor of conventional liposomal cisplatin formulations [17]. The low solubility of cisplatin in water, resulting in low encapsulation efficiencies in liposomes, has so far hampered the development of an effective lipid formulation of cisplatin.

Recently, we reported a new method for the encapsulation of cisplatin in a lipid formulation [18]. The method takes advantage of the limited solubility of cisplatin in water and produces cisplatin nanocapsules, bean-shaped nanoprecipitates of cisplatin coated by a lipid bilayer. The nanocapsules represent a novel lipid formulation of cisplatin characterized by an unprecedented cisplatin-to-lipid ratio and exhibiting strongly improved cytotoxicity against tumor cells in vitro as compared to the free drug. The formation of the nanocapsules critically depends on the presence of negatively charged phospholipids and positively charged aqua-species of cisplatin [18].

The present study is focused on the stability of cisplatin nanocapsules and its dependence on the lipid composition of the bilayer coat. The release of cisplatin from cisplatin nanocapsules composed of several different lipid compositions was followed during incubations in vitro in water and in serum. Furthermore, the influence of the nature of the anionic phospholipid head group and of the acyl chain composition on the formation, the cisplatin-to-lipid ratio and the in vitro cytotoxicity of cisplatin nanocapsules are reported. For the purpose of long-term storage, the effect on the nanocapsules of lyophilization and rehydration was examined. Coating of the surface of liposomes with PEG is a popular strategy to increase the stability and circulation time of liposomes [19,20]. Following that strategy, the effect of PEG on the stability of the cisplatin nanocapsules was studied by incorporating PEG conjugated to phosphatidylethanolamine (DSPE-PEG2000).

2. Materials and methods

2.1. Materials

Cisplatin, K_2PtCl_6, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly (ethylene glycol)2000] (DSPE-PEG2000) were obtained from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoserine (DSPS), phosphatidylinositol (PI) from soy bean, egg sphingomyelin, and cholesterol were all purchased from Avanti Polar Lipids Inc., (Birmingham, AL). Mouse serum was purchased from Harlan (Horst, The Netherlands). All other chemicals were analytical grade.

2.2. Preparation of cisplatin nanocapsules

Cisplatin was dissolved in water and subsequently incubated overnight in the dark at 37 °C to ensure full equilibration. Cisplatin nanocapsules were prepared by hydrating dry lipid films (1.2 μmol) consisting of mixtures of lipids in molar ratios as indicated, with 1.2-ml 5 mM cisplatin in water at 37 °C for 30 min, followed by 10 freeze-thaw cycles using ethanol/dry-ice (− 70 °C) and a water bath at 37 °C [18]. The resulting suspension was centrifuged at low speed (4 min at 2100 rpm) in an Eppendorf centrifuge to collect the nanocapsules in the pellet and remove multilamellar liposomes (MLV) and free cisplatin. Subsequently the nanocapsules were washed by resuspension in 1 ml of water and centrifugation as above. Using a lipid mixture of DOPC/DOPS 1:1, typically 30% of cisplatin and 15% of phospholipid phosphorus were recovered in the nanocapsule pellet.

2.3. Retention of cisplatin in nanocapsules

In order to characterize the stability of nanocapsules with different phospholipid compositions, the nanocapsules were resuspended at a phospholipid phosphorus concentration of 0.25 mM in water or in mouse serum. Aliquots (150 μl) of the suspension were incubated at 37 °C unless indicated otherwise. At appropriate time points the aliquots were centrifuged at 20,000 × *g* for 4 min at 20 °C, the supernatant was analysed for Pt-content by non-flame atomic absorption spectroscopy (NFAAS), and this was related to the total Pt-content of the suspension (100%). Some of the preparations were lyophilized, stored and rehydrated after different periods of time. After centrifugation at 20,000 × *g* for 4 min at 20 °C, supernatants of the rehydrated nanocapsules were analysed for Pt-content.

2.4. Electron microscopy (EM)

Cisplatin nanocapsules were visualised by negative staining. A suspension of nanocapsules was transferred to a carbon-coated grid, dried and stained with 4% (w/v) of uranyl acetate for 45 s [21]. The specimens were observed with a Philips CM10 electron microscope at an accelerating voltage of 80 kV.

2.5. In vitro cytotoxicity assay

Cytotoxicity was assayed as described [18]. Briefly, human IGROV-1 ovarian tumor cells [22] were grown on plastic dishes (Costar, Cambridge, MA) in RPMI (Gibco, Glasgow, UK) supplemented with 25 mM HEPES, 10% (v/v) fetal calf serum (FCS; Gibco) and 100 units/ml each of penicillin and streptomycin (Gibco). Approximately 1000 tumor cells were seeded per well in a 96-well microtiter plate in 200-μl growth medium. After 48 h the cisplatin formulations were added at the concentrations indicated (in

triplicate or quadruplicate) and the cells were further incubated for 5 days at 37 °C. Prior to administration to the cells, the lipid formulations of cisplatin in water had been diluted in RPMI without FCS to a Pt concentration of 233 μM. Tumor cell growth inhibition induced by cisplatin was determined using the sulforhodamine B (SRB) assay [23]. The data were fitted to a sigmoidal dose response curve (variable slope) using GraphPad Prism (GraphPad Software, San Diego, CA).

2.6. Other methods

Pt-contents were quantified by non-flame atomic absorption spectroscopy (NFAAS) on a SpectrAA-400 Zeeman spectrometer (Varian, Palo Alto, CA), using a modifier solution of 0.5% Triton X-100 in water, and K_2PtCl_6 as a standard [24]. Phospholipid contents were determined by phosphate (P_i) analysis after destruction with perchloric acid [25]. Complex formation of cisplatin with DOPS was analyzed as described [26]. The half-life of the nanocapsules was calculated from the apparent first order rate constant of the cisplatin release (k), which was obtained by fitting the time dependent release of cisplatin to:

$$R(t) = R(0) + R(\max)(1 - e^{-kt}) \quad (1)$$

with $R(t)$ the release of cisplatin at time t, $R(0)$ the release at time zero, $R(\max)$ the maximal release obtained, and t the time of the incubation.

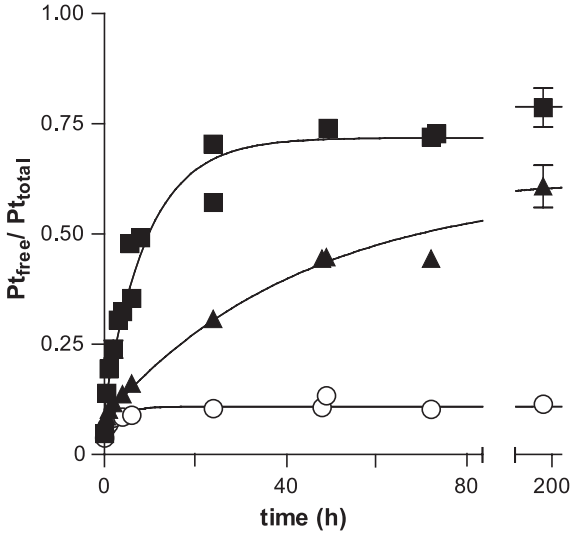

Fig. 1. Temperature dependence of the kinetics of release of cisplatin from nanocapsules prepared of DOPC/DOPS (1:1) upon incubation in water at 4 °C (○), 25 °C (▲), and 37 °C (■). After centrifugation the Pt-content of the supernatant was determined and normalized to the Pt-content of the suspension of cisplatin nanocapsules before centrifugation. Data from three (4, 25 °C) or four (37 °C) independent experiments were averaged with the standard deviation never exceeding 0.05.

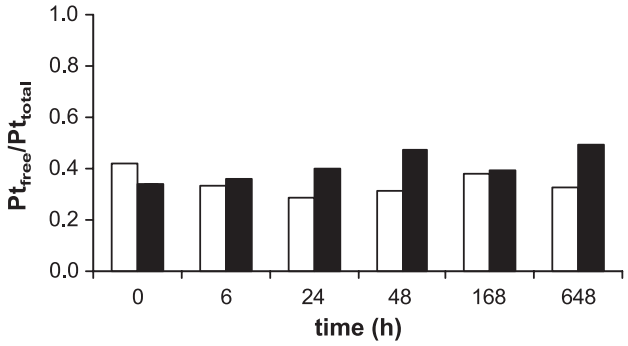

Fig. 2. Release of cisplatin from lyophilized cisplatin nanocapsules (DOPC/DOPS 1:1) upon rehydration. Lyophilized nanocapsules were stored in the dark for the times indicated at 4 °C (white columns) and at room temperature (black columns). After rehydration, nanocapsules were centrifuged and the Pt-content of the supernatant was determined and normalized to the total Pt-content of the suspension.

3. Results

3.1. Stability in water

We previously showed that hydration of a dry lipid film composed of equimolar amounts of DOPC and DOPS with an aqueous solution of 5 mM cisplatin followed by 10 freeze-thaw cycles yields cisplatin nanocapsules that can be isolated by sucrose gradient centrifugation [18]. Here, we applied a less laborious version of the protocol, in which the nanocapsules are collected by low speed centrifugation rather than by gradient centrifugation. The preparation of nanocapsules obtained at the optimal centrifugation rate (see Section 2) is characterized by a cisplatin-to-lipid (Pt/P_i) ratio of 11 ± 2 ($n = 9$), which translates into 3.3 mg cisplatin/μmol phospholipid, and is comparable to that obtained with the original protocol [18].

The release of cisplatin from DOPC/DOPS (1:1) nanocapsules was examined upon incubation in water at different temperatures (Fig. 1). At 4 °C, there is hardly any release and 90% of the cisplatin initially present in the nanocapsules is retained for at least 200 h. Increasing the incubation temperature leads to the release of ~ 75% of cisplatin from the nanocapsules, with the rate of release depending on the temperature. By fitting the kinetics of release to a mono-exponential, half-life values for cisplatin retention of 6.5 h at 37 °C and 28 h at 25 °C were calculated.

We employed lyophilization of nanocapsules followed by hydration in order to evaluate the possibility to store the formulation in the freeze-dried state, which could be useful for future clinical use. Upon rehydration of lyophilized DOPC/DOPS (1:1) cisplatin nanocapsules, some 60% of the originally encapsulated cisplatin is recovered in the low speed pellet, irrespective of the time and temperature of storage (Fig. 2). The cytotoxicity of the rehydrated lyophilized formulation is similar to that of the standard preparation (data not shown). The nanocapsules before and after lyophilization and rehydration were compared by electron

microscopy. Negative staining revealed a heterogeneous population of elongate, bean-shaped particles with an average width of 45 ± 15 nm and a length in the range of 50–150 nm (Fig. 3A), as described previously [18]. The thickness of the bright layer surrounding the dark area was estimated to be between 5 and 6 nm, which corresponds to the size of a single bilayer. The rehydrated lyophilized samples revealed particles with shapes and sizes similar to those seen before freeze-drying (compare Fig. 3A and B).

3.2. Lipid specificity of nanocapsule stability

Negatively charged lipids were found to be essential for the formation of cisplatin nanocapsules [18]. Cisplatin nanocapsules prepared of equimolar mixtures of DOPC and several anionic phospholipids were tested for encapsulation efficiency, stability in water and cytotoxicity. Replacement of DOPS by either DOPA or DOPG produced nanocapsules in similar yields (not shown) and with similar Pt/P_i ratios (Table 1). However, hydration in 5 mM cisplatin of mixtures of DOPC/PI in various ratios (from 1:1 to 9:1), followed by freezing and thawing, did not yield nanocapsules, demonstrating specificity for the nature of the anionic lipid in the nanocapsule formation process. The stability in water of nanocapsules prepared from DOPC/DOPA and from DOPC/DOPS does not significantly differ (Fig. 4). In contrast,

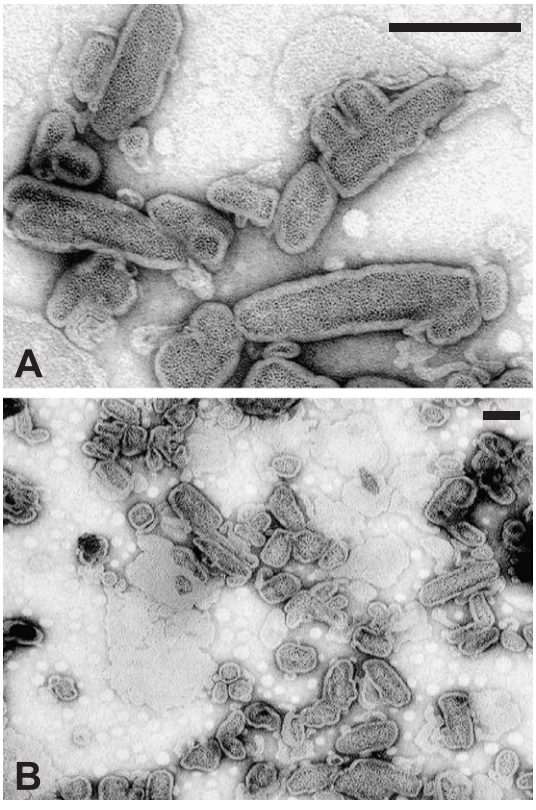

Fig. 3. Negative stain electron micrographs of cisplatin nanocapsules prepared of DOPC/DOPS 1:1 before (A) and after lyophilization and rehydration (B). The scale bars reflect 100 nm.

Table 1
Lipid dependence of the formation of cisplatin nanocapsules

Lipid composition (molar ratio)	Formation of nanocapsules	Pt/P_i ratio[a] ± S.D.
DOPC/DOPS, (1:1)	+	11 ± 2 (n=9)
DOPC/DOPG, (1:1)	+	16 ± 2 (n=4)
DOPC/DOPA, (1:1)	+	12 ± 4 (n=4)
DOPC/PI, (1:1)	–	
DSPC/DSPS, (1:1)	–	
DOPC/DSPS, (1:1)	–	
DSPC/DOPS, (1:1)	±	1.2 ± 0.1 (n=3)
DSPC/DOPC/DOPS, (1:1:2)	+	3.5 ± 0.7 (n=3)
DOPC/DOPS/chol/DSPE-PEG, (27:27:40:6)	+	12 ± 3 (n=7)

[a] The Pt/P_i molar ratio of the nanocapsule pellet is presented as averaged value from n experiments (± S.D.).

replacing DOPS or DOPA by DOPG leads to a dramatic decrease of the half-life value for cisplatin retention from 6.5 h to 20 min, again pointing to specificity in the role played by the anionic phospholipid. Within 3 h, all cisplatin is released from DOPC/DOPG nanocapsules (Fig. 4). Fig. 5 shows the growth inhibition of human ovarian IGROV-1 tumor cells induced by cisplatin nanocapsules prepared from different negatively charged phospholipids. The DOPC/DOPG and DOPC/DOPA formulations display approximately similar cytotoxic activities as free cisplatin with IC50 values around 0.4 μM, suggesting that cisplatin rapidly leaks from these formulations in cell culture medium. The DOPS-containing formulation exhibits increased cytotoxic activity, with an IC50 value 1 order of magnitude lower than that of free cisplatin (cf. Ref. [18]).

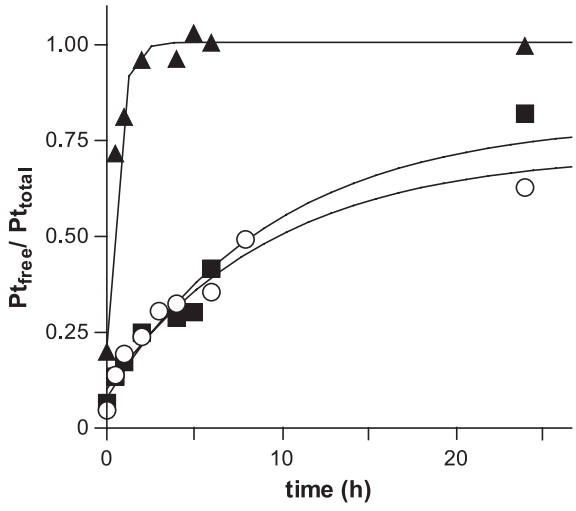

Fig. 4. Time course of release of cisplatin from cisplatin nanocapsules prepared from DOPC/DOPG 1:1 (▲), DOPC/DOPA 1:1 (■) and DOPC/DOPS 1:1 (○) upon incubation in water at 37 °C. After centrifugation, the Pt-content of the supernatant was determined and normalized to the Pt-content of the suspension of cisplatin nanocapsules before centrifugation. Data from four independent experiments were averaged with the standard deviation never exceeding 0.05.

The dramatic difference in stability between DOPC/DOPS and DOPC/DOPG nanocapsules may be due to the ability of PS but not PG to form a stable coordination complex with cisplatin [26]. To investigate whether the presence of the PS–cisplatin complex affects the release of cisplatin from nanocapsules, the content of PS–cisplatin complex of the nanocapsules at the start of the release experiment was varied. This was accomplished by varying the time of hydration of the lipid film in 5 mM cisplatin, prior to the freeze-thaw cycles. Analysis by thin layer chromatography (data not shown) revealed that the content of PS–cisplatin complex present at the start of the release experiment (expressed as percentage of total phospholipids) increased from 10% after 0-min hydration via 20% after 30-min hydration to 45% after 24 h of hydration in the presence of 5 mM cisplatin, in agreement with our published results [26]. As shown in Fig. 6, the retention of cisplatin is prolonged as the pre-incubation with cisplatin is extended from 0 min up to 24 h. We conclude that the nanocapsules are stabilized by the presence of the PS–cisplatin coordination complex in the bilayer coat.

In exploring other ways to improve the stability, cisplatin nanocapsules were prepared of lipid mixtures containing lipids with saturated acyl chains, which have been commonly used in liposomal formulations of cisplatin [12]. Application of the standard procedure to equimolar mixtures of DSPC and DSPS or DOPC and DSPS failed to generate nanocapsules. A 1:1 mixture of DSPC and DOPS did yield a low speed pellet, albeit with an apparently tenfold lower cisplatin-to-lipid ratio, compared to DOPC/DOPS (1:1) nanocapsules (Table 1), consistent with co-pelleting of "empty" liposomes. The rate of leakage of cisplatin from the DSPC/DOPS (1:1) formulation was strongly reduced compared to that of DOPC/DOPS nanocapsules (half-life values of ~25 versus 6.5 h). The cisplatin-to-lipid ratio (Table 1) and the stability in water of a DSPC/DOPC/DOPS (1:1:2) formulation were found to be intermediate between the DOPC/DOPS and DSPC/DOPS formulations. The introduction of DSPC in the nanocapsule formulation did not improve the efficacy of the nanocapsules in vitro, in the cytotoxicity assay (data not shown).

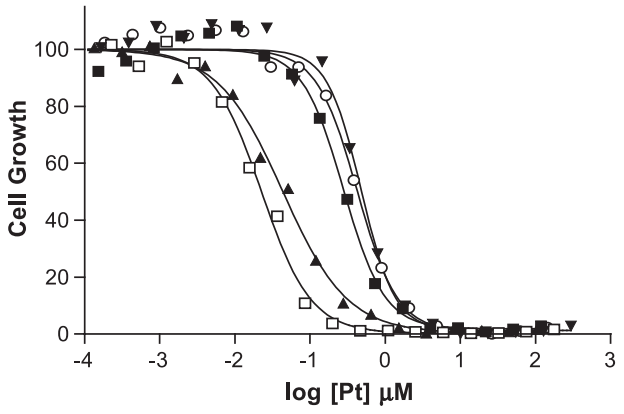

Fig. 5. Cytotoxicity of several cisplatin nanocapsule formulations towards IGROV-1 human ovarian carcinoma cells. The growth inhibition in response to increasing concentrations of cisplatin is shown for cisplatin nanocapsules prepared from DOPC/DOPS 1:1 (▲), DOPC/DOPA 1:1 (■), DOPC/DOPG 1:1 (▼), DOPC/DOPS/cholesterol/DSPE-PEG 27:27:6:40 (□), and compared to that in response to conventional cisplatin (○). For experimental conditions, see Section 2.

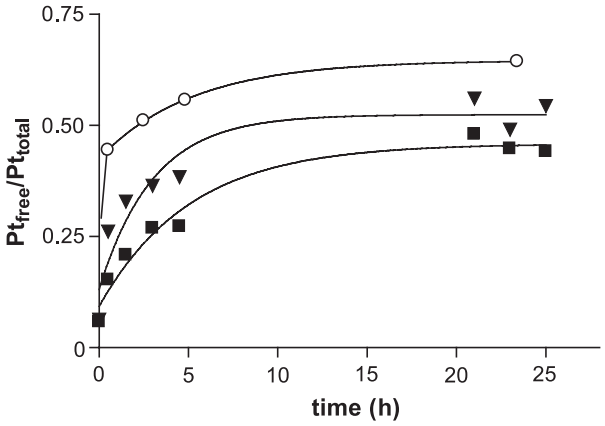

Fig. 6. Time course of release of cisplatin from cisplatin nanocapsules with varying initial contents of PS–cisplatin complex, obtained by pre-incubating the DOPC/DOPS (1:1) lipid film in 5 mM cisplatin for 0 min (○), 30 min (▼), and 24 h (■) prior to the freeze-thaw cycles. A pre-incubation time of 0 min was obtained by hydrating the lipid film in 0.6-ml water, and adding 0.6-ml 10 mM cisplatin just before the freeze-thaw cycles. Immediately after the freeze-thaw cycles and the wash step, nanocapsules were incubated in water at 37 °C. After centrifugation at the time points indicated, the Pt-content of the supernatant was determined and normalized to the Pt-content of the suspension of cisplatin nanocapsules before centrifugation.

3.3. Stability in mouse serum

Fig. 7 shows the destabilizing effect of mouse serum on the nanocapsules. Resuspension of the standard DOPC/DOPS (1:1) nanocapsules in mouse serum leads to the almost instantaneous release of virtually all cisplatin. Centrifugation of such samples failed to produce a pellet. The ability of cholesterol to reduce the membrane permeability is well established [27,28]. For this reason, most liposomes used for drug delivery contain cholesterol at a concentration around 40 mol% (reviewed in Ref. [29]). Further stabilization can be achieved by the incorporation of both cholesterol and sphingomyelin, which form intermolecular hydrogen bonds [30]. However, the incorporation of cholesterol at 40% (mol/mol) with respect to total lipid, with or without the concomitant substitution of DOPC by sphingomyelin (not shown), did not affect the half-life value of cisplatin retention of DOPC/DOPS (1:1) nanocapsules in water (data not shown), nor in mouse serum (Fig. 7).

Conjugation of PEG polymer to the membrane surface of liposomes is known to provide a steric barrier against protein binding, thus stabilizing and extending the circulation time of liposomes [19,29]. Incorporation of 6 mol% DSPE-PEG into DOPC/DOPS/cholesterol (3:3:4) nanocapsules significantly improves the stability in mouse serum,

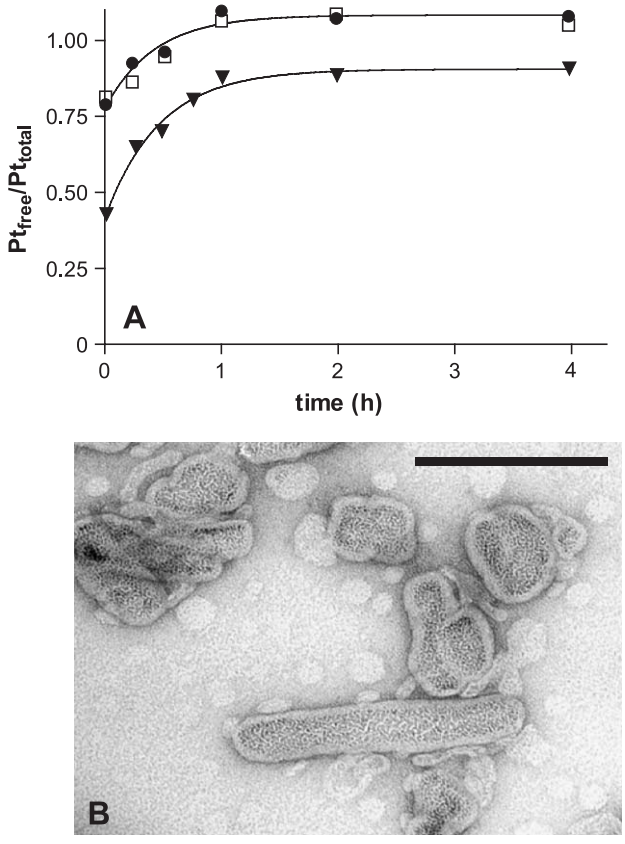

Fig. 7. (A) Time course of release of cisplatin from cisplatin nanocapsules prepared from DOPC/DOPS 1:1 (●), DOPC/DOPS/cholesterol 3:3:4 (□), and DOPC/DOPS/cholesterol/DSPE-PEG 27:27:40:6 (▼) upon incubation in mouse serum at 37 °C. After centrifugation the Pt-content of the supernatant was determined and normalized to the Pt-content of the suspension before centrifugation. Data from four independent experiments were averaged with the standard deviation never exceeding 0.05. (B) Negative stain electron micrograph of cisplatin nanocapsules composed of DOPC/DOPS/cholesterol/DSPE-PEG 27:27:40:6. The scale bar reflects 100 nm.

although still considerable leakage of cisplatin occurred. In the first few minutes, 40% of the cisplatin leaked out and after 75 min a loss of 85% was found (Fig. 7). Increasing the DSPE-PEG2000 content of the nanocapsules from 6 to 10 mol% of total lipids, did not further delay the release of contents. When the content of DSPE-PEG2000 is further increased, the nanocapsules become less stable (data not shown). Increasing the amount of the cisplatin–PS complex present at the start of the incubation in serum up to 45% (leaving 5% PS) did not significantly improve stability either (not shown). Cisplatin nanocapsules prepared from DOPC/DOPS/cholesterol/DSPE-PEG2000 exhibit the characteristic Pt/P_i ratio of around 11 (Table 1), and are morphologically indistinguishable from their DOPC/DOPS counterparts, as judged by negative stain EM (compare Figs. 3A and 7B). The incorporation of DSPE-PEG2000 and cholesterol in the formulation increased the in vitro cytotoxicity of the nanocapsules, compared to that of the DOPC/DOPS nanocapsules (Fig. 5).

4. Discussion

Cisplatin nanocapsules constitute a new lipid formulation of the anticancer drug cisplatin, in which a nanoprecipitate of the drug is covered by a single phospholipid bilayer consisting of an equimolar mixture of DOPC and DOPS [18]. In this study the stability of the cisplatin nanocapsules upon incubation in water and in serum was investigated, in particular its dependence on the lipid composition of the bilayer coat. For this purpose we simplified the preparation protocol (see Section 3).

The formation of nanocapsules critically depends on the presence of negatively charged phospholipids that have been proposed to electrostatically interact with the positively charged aqua-species of cisplatin [18]. When negatively charged DOPS in the original formulation was replaced by DOPG or DOPA, nanocapsules were obtained in similar yields and with similar encapsulation efficiencies. Substitution of DOPS by (soy bean) PI did not yield nanocapsules, even though the affinities of binding of positively charged cisplatin species to large unilamellar vesicles composed of DOPS and PI are similar [31]. We conclude that electrostatic interaction by itself is not sufficient for nanocapsule formation, and speculate that the inositol headgroup of PI interferes with the interaction between cisplatin and negatively charged phospholipids required for the formation of nanocapsules.

The DOPC/DOPS cisplatin nanocapsules released their contents in a temperature-dependent way upon resuspension in water. While stable at 4 °C, they lost about 75% of their contents with a half-time of 6.5 h at 37 °C. In contrast, the DOPC/DOPG formulation rapidly released all of its contents at 37 °C. Since PS can form a stable coordination complex with cisplatin whereas PG cannot [26], these results pointed to a role for the cisplatin–PS complex in nanocapsule stability. By varying the content of cisplatin–PS complex at the start of the release experiment, we were indeed able to show that the complex reduces the rate of release of contents (Fig. 6). Conceivably, the initially electrostatic interaction of the solid core of the nanocapsules with the bilayer is stabilized as the cisplatin–PS coordination complex forms. In addition, complex formation may influence the permeability properties of the bilayer coat by affecting the acyl chain order.

Interestingly, the kinetics of cisplatin release in water of the DOPC/DOPA formulation were similar to those of DOPC/DOPS, suggesting that complex formation may also occur between cisplatin and PA. Analysis by thin layer chromatography of lipid extracts of DOPC/DOPA nanocapsules indeed showed the appearance of new spots originating from the cisplatin–PA interaction (our unpublished results), in agreement with this supposition. Unlike the DOPC/DOPS formulation, the DOPC/DOPA formulation did not exhibit increased cytotoxicity towards IGROV-1 cells compared to free cisplatin. This would indicate that the stabilizing effect provided by the putative cisplatin–PA complex in water does not withstand cell culture medium.

How can the remarkable anionic lipid specificity in nanocapsule formation and stability with PI < PG < PA = PS be understood? The stabilizing role of complex formation between PS and cisplatin and PA and cisplatin has already been alluded to. It is tempting to speculate that the difference between PI and PG in nanocapsule formation originates from the different number of hydroxyl groups present in the headgroup. In this scenario, the strongly hydrated hydroxyl groups would influence the interaction between the positively charged cisplatin aqua-species and the negatively charged phosphate group of PI such that the formation of nanocapsules is abolished.

Based on the literature on stabilization of liposomal formulations of cisplatin and other drugs [12,29], a number of lipid compositions were tested for their ability to improve the stability of the cisplatin nanocapsules. A (partial) replacement of DOPC by saturated DSPC, which is commonly used in liposomal formulations of drugs, reduced the rate of leakage of cisplatin from the nanocapsules in water. However, it did not improve the in vitro cytotoxicity properties and it reduced the yield of nanocapsules. The latter indicates that the fluidity of the bilayer coat is another constraint for nanocapsule formation. The introduction of cholesterol up to 40 mol% is known to increase the stability of liposomes carrying drugs and to prolong their circulation time upon intravenous administration, with sphingomyelin having an added effect [19,29]. However, cholesterol with or without sphingomyelin did not improve the stability of the cisplatin nanocapsules, neither in water nor in serum.

The positive effect of the cisplatin–PS coordination complex on nanocapsule stability on the one hand, and the deleterious effect of gel state phospholipids on their formation on the other, together indicate that it is crucial that the surrounding bilayer has the flexibility to tightly cover the irregular surface of the cisplatin nanoprecipitate, a condition not met by saturated phospholipids. Using solid state NMR, the molecular order of the bilayer coat of cisplatin nanocapsules is being addressed (V. Chupin et al., manuscript in preparation).

Given the special architecture of the nanocapsules and the constraints imposed on the composition of the lipid coat, it is remarkable that suspensions of cisplatin nanocapsules can be lyophilized and rehydrated to yield a preparation of nanocapsules still containing 60% of the amount of cisplatin initially enclosed, and without loss of efficacy in the in vitro cytotoxicity assay. Although the loss of 40% of the contents is undesirable from the point of view of future clinical applications, these results do illustrate the stability in water of the active nanocapsules.

The stability of the nanocapsules in serum was investigated in view of a possible future clinical application. Upon resuspension in mouse serum, the DOPC/DOPS nanocapsules with or without cholesterol immediately collapsed. The presence of the negatively charged PS, a prerequisite for the formation of nanocapsules, is most likely the cause for the instability in serum. Although part of the PS reacts with cisplatin to form the neutral cisplatin–PS coordination complex, the remaining negative surface charge [18] leads to high affinity binding of serum proteins to the bilayer coat, destabilizing the nanocapsules. PEG conjugated to PE has been successfully used in liposomes as a steric barrier against plasma protein binding and clearance from the circulation [20]. Whereas incorporation of 5 mol% DSPE-PEG effectively shields neutral liposomes composed of PC and cholesterol, including the liposomal cisplatin formulation SPI-077 [12], liposomes containing PS are more rapidly cleared from the circulation [32], and require elevated levels of DSPE-PEG2000 [33]. A recent report shows that as much as 15 mol% DSPE-PEG2000 is required for protecting liposomes containing 10 mol% PS in the blood circulation [34].

Upon incorporation of 6 mol% DSPE-PEG2000 into DOPC/DOPS/cholesterol (3:3:4), cisplatin nanocapsules were obtained with a yield, Pt/P_i ratio, and morphology indistinguishable from the original DOPC/DOPS nanocapsules. The stability of the cisplatin nanocapsules in serum was drastically improved by incorporating 6 mol% of DSPE-PEG2000. A further increase of the DSPE-PEG content did not reduce but slightly enhanced the leakage of contents, possibly by impairing the interaction between the cisplatin core and the bilayer coat. The observed increased cytotoxicity in vitro of the pegylated nanocapsules compared to the DOPC/DOPS nanocapsules is most likely due to the protection provided by DSPE-PEG against leakage induced by the 10% FCS present in the culture medium. The lifetime of the pegylated nanocapsules in mouse serum was not prolonged upon decreasing the negative surface charge by increasing the content of the neutral PS–cisplatin complex at the expense of negatively charged PS [18], suggesting that the surface charge is not the limiting factor for the stability in serum.

In conclusion, the release of contents of cisplatin nanocapsules depends on the temperature, the surrounding medium, and the lipid composition of the bilayer coat. Cisplatin nanocapsules containing 6 mol% DSPE-PEG will serve as the starting formulation for in vivo studies addressing the anti-tumor efficacy of cisplatin nanocapsules in tumor-bearing mice.

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References

[1] M. Rosencweig, D.D. von Hoff, M. Slavik, F.M. Muggia, Cis-diamminedichloroplatinum (II). A new anticancer drug, Ann. Intern. Med. 86 (1977) 803–812.

[2] P.J. Loehrer, L.H. Einhorn, Drugs five years later. Cisplatin, Ann. Intern. Med. 100 (1984) 704–713.
[3] B. Lippert (Ed.), Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug, Wiley-VCH, New York, 1999.
[4] S.M. Cohen, S.J. Lippard, Cisplatin: from DNA damage to cancer chemotherapy, Prog. Nucleic Acid Res. Mol. Biol. 67 (2001) 93–130.
[5] J.M. Hill, R.J. Speer, Organo-platinum complexes as antitumor agents (review), Anticancer Res. 2 (1982) 173–186.
[6] H. Calvert, I. Judson, W.J. van der Vijgh, Platinum complexes in cancer medicine: pharmacokinetics and pharmacodynamics in relation to toxicity and therapeutic activity, Cancer Surv. 17 (1993) 189–217.
[7] G. Giaccone, Clinical perspectives on platinum resistance, Drugs 59 (Suppl. 4) (2000) 9–17.
[8] D.F. Long, A.J. Repta, Cisplatin: chemistry, distribution and biotransformation, Biopharm. Drug Dispos. 2 (1981) 1–16.
[9] R. Reszka, I. Fichtner, E. Nissen, D. Arndt, A.M. Ladhoff, Preparation, characterization, therapeutic efficacy and toxicity of liposomes, containing the anti-tumour drug *cis*-dichlorodiamineplatinum(II), J. Microencapsul. 4 (1987) 201–212.
[10] P.A. Steerenberg, G. Storm, G. de Groot, J.J. Bergers, A. Claessen, W.H. de Jong, Liposomes as a drug carrier system for *cis*-diammine-dichloroplatinum(II): I. Binding capacity, stability and tumor cell growth inhibition in vitro, Int. J. Pharm. 40 (1987) 51–62.
[11] J.A. Gondal, H.G. Preuss, R. Swartz, A. Rahman, Comparative pharmacological, toxicological and antitumoral evaluation of free and liposome-encapsulated cisplatin in rodents, Eur. J. Cancer 29A (1993) 1536–1542.
[12] M.S. Newman, G.T. Colbern, P.K. Working, C. Engbers, M.A. Amantea, Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long–circulating, pegylated liposomes (SPI-077) in tumor-bearing mice, Cancer Chemother. Pharmacol. 43 (1999) 1–7.
[13] T. Peleg-Shulman, D. Gibson, R. Cohen, R. Abra, Y. Barenholz, Characterization of sterically stabilized cisplatin liposomes by nuclear magnetic resonance, Biochim. Biophys. Acta 1510 (2001) 278–291.
[14] J. Vaage, D. Donovan, E. Wipff, R. Abra, G. Colbern, P. Uster, P. Working, Therapy of a xenografted human colonic carcinoma using cisplatin or doxorubicin encapsulated in long-circulating pegylated stealth liposomes, Int. J. Cancer 80 (1999) 134–137.
[15] K.J. Harrington, C.R. Lewanski, A.D. Northcote, J. Whittaker, H. Wellbank, R.G. Vile, A.M. Peters, J.S. Stewart, Phase I-II study of pegylated liposomal cisplatin (SPI-077) in patients with inoperable head and neck cancer, Ann. Oncol. 12 (2001) 493–496.
[16] E.S. Kim, C. Lu, F.R. Khuri, M. Tonda, B.S. Glisson, D. Liu, M. Jung, W.K. Hong, R.S. Herbst, A phase II study of STEALTH cisplatin (SPI-77) in patients with advanced non-small cell lung cancer, Lung Cancer 34 (2001) 427–432.
[17] J.M. Meerum Terwogt, G. Groenewegen, D. Pluim, M. Maliepaard, M.M. Tibben, A. Huisman, W.W. ten Bokkel Huinink, M. Schot, H. Welbank, E.E. Voest, J.H. Beijnen, J.M. Schellens, Phase I and pharmacokinetic study of SPI-77, a liposomal encapsulated dosage form of cisplatin, Cancer Chemother. Pharmacol. 49 (2002) 201–210.
[18] K.N.J. Burger, R.W.H.M. Staffhorst, H.C. de Vijlder, M.J. Velinova, P.H. Bomans, P.M. Frederik, B. de Kruijff, Nanocapsules: lipid-coated aggregates of cisplatin with high cytotoxicity, Nat. Med. 8 (2002) 81–84.
[19] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Yong, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, Biochim. Biophys. Acta 1066 (1991) 29–36.
[20] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Mathhay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J. Martin, Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11460–11464.
[21] A. Bremer, M. Häner, U. Aebi, in: J.E. Celis (Ed.), Negative Staining in Cell Biology, A Laboratory Handbook, vol. 3, Academic Press, San Diego, 1998, pp. 277–284.
[22] J. Bénard, J. da Silva, M.C. de Blois, P. Boyer, P. Duvillard, E. Chiric, G. Riou, Characterization of a human ovarian adenocarcinoma line, IGROV 1, in tissue culture and in nude mice, Cancer Res. 45 (1985) 4970–4979.
[23] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (1990) 1107–1112.
[24] K.N.J. Burger, R.W.H.M. Staffhorst, B. de Kruijff, Interaction of the anti-cancer drug cisplatin with phosphatidylserine in intact and semi-intact cells, Biochim. Biophys. Acta 1419 (1999) 43–54.
[25] G. Rouser, S. Fleischer, A. Yamamoto, Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[26] G. Speelmans, R.W.H.M. Staffhorst, K. Versluis, J. Reedijk, B. de Kruijff, Cisplatin complexes with phosphatidylserine in membranes, Biochemistry 36 (1997) 10545–10550.
[27] R.A. Demel, S.C. Kinsky, C.B. Kinsky, L.L.M. van Deenen, Effects of temperature and cholesterol on the glucose permeability of liposomes prepared with natural and synthetic lecithins, Biochim. Biophys. Acta 150 (1968) 655–665.
[28] R. Fettiplace, D.A. Haydon, Water permeability of lipid membranes, Physiol. Rev. 60 (1980) 510–550.
[29] D.C. Drummond, O. Meyer, K. Hong, D.B. Kirpotin, D. Papahadjopoulos, Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors, Pharmacol. Rev. 51 (1999) 691–743.
[30] M.B. Sankaram, T.E. Thompson, Interaction of cholesterol with various glycerophospholipids and sphingomyelin, Biochemistry 29 (1990) 10670–10675.
[31] G. Speelmans, W.H.H.M. Sips, R.J.H. Grisel, R.W.H.M. Staffhorst, A.M.J. Fichtinger-Schepman, J. Reedijk, B. de Kruijff, The interaction of the anti-cancer drug cisplatin with phospholipids is specific for negatively charged phospholipids and takes place at low chloride concentration, Biochim. Biophys. Acta 1283 (1996) 60–66.
[32] H.H. Spanjer, M. van Galen, F.H. Roerdink, J. Regts, G.L. Scherphof, Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition, Biochim. Biophys. Acta 863 (1986) 224–230.
[33] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target, Biochim. Biophys. Acta 1062 (1991) 142–148.
[34] G.N.C. Chiu, M.B. Bally, L.D. Mayer, Selective protein interactions with phosphatidylserine containing liposomes alter the steric stabilization properties of poly(ethylene glycol), Biochim. Biophys. Acta 1510 (2001) 56–69.