Nanocapsules: lipid-coated aggregates of cisplatin with high cytotoxicity

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Cisplatin is one of the most widely used agents in the treatment of solid tumors, but its clinical utility is limited by toxicity. The development of less toxic, liposomal formulations of cisplatin has been hampered by the low water solubility and low lipophilicity of cisplatin, resulting in very low encapsulation efficiencies. We describe a novel method allowing the efficient encapsulation of cisplatin in a lipid formulation; it is based on repeated freezing and thawing of a concentrated solution of cisplatin in the presence of negatively charged phospholipids. The method is unique in that it generates nanocapsules, which are small aggregates of cisplatin covered by a single lipid bilayer. The nanocapsules have an unprecedented drug-to-lipid ratio and an *in vitro* cytotoxicity up to 1000-fold higher than the free drug. Analysis of the mechanism of nanocapsule formation suggests that the method may be generalized to other drugs showing low water solubility and lipophilicity.

The clinical use of *cis*-diamminedichloroplatinum(II) (cisplatin) and many of its analogs faces three major problems: 1) serious dose-limiting toxicities in particular nephrotoxicity and neurotoxicity; 2) rapid inactivation of the drug as a result of complexation to plasma and tissue proteins; and 3) the frequent occurrence of platinum resistance[1–5]. In general, these problems can be reduced by shielding of a drug from the extracellular environment by means of a lipid coating. However, in many cases this approach fails because of inefficient encapsulation of the drug in lipid formulations resulting in low drug uptake by the tumor[6]. This is particularly true for cisplatin: the low water solubility and low lipophilicity of cisplatin result in lipid formulations with a very low drug-to-lipid ratio[7–9]. One approach is to synthesize lipophilic derivatives of cisplatin that can be efficiently encapsulated in large multilamellar liposomes[10]. Here, we describe a new method to efficiently encapsulate native, non-derivitized cisplatin in a lipid formulation.

Nanocapsules of cisplatin

Our method involves hydration of a dry lipid film composed of equimolar amounts of dioleoyl-phosphatidylserine (PS) and dioleoyl-phosphatidylcholine (PC), with a buffered solution (pH 7.4) of 5 mM cisplatin followed by 10 freeze-thaw (FT) cycles, and removal of free (extravesicular) cisplatin by centrifugation. The cisplatin-containing lipid suspension (cisPt-PS/PC) was extremely cytotoxic (Fig. 1*a*) with a typical IC_{50} (the drug concentration at which cell growth is inhibited by 50%) of approximately 2 nM as compared with 0.5 μM for the free drug (conventional cisplatin). A lipid suspension not loaded with cisplatin (blank) was not cytotoxic, and mixing conventional cisplatin with the blank lipid suspension did not increase the cytotoxicity of cisplatin.

Omitting the freeze-thaw step, or leaving out the negatively charged PS in the lipid mixture, resulted in a dramatic decrease in cytotoxicity (Fig. 1*b*). This decrease in cytotoxicity was paralleled by a similar decrease in the encapsulation efficiency suggesting a direct relation between drug-to-lipid ratio and cytotoxicity. Omitting freeze-thawing or leaving out PS typically decreased the drug-to-lipid molar ratio 10-fold and 4-fold, respectively. The cisplatin-to-lipid molar ratio was 0.5 ± 0.1 (n = 17), which translates into a theoretical intravesicular concentration of cisplatin in excess of 30 mM (based on an encapsulated volume of 15 l/mol phospholipid[11]). This value by far exceeded the solubility limit of cisplatin (~8 mM in chloride-free media[12]), and suggested that the lipid formulations contained aggregates of cisplatin. Indeed, cryo-electron microscopy (cryo-EM) examination (Fig. 2*a*) and elemental microanalysis (EDX (energy-dispersive X-ray analysis); data not shown) revealed the presence of nanocapsules of cisplatin—small, electron-dense particles containing platinum and coated by a thin lipid layer. Much higher drug-to-lipid ratios of 2.5 ± 0.1 (n = 4) were obtained when, during the preparation of the cisplatin formulation, a solution of cisplatin in H_2O instead of buffer was used. Under these conditions, nanocapsules were much more abundant and could be isolated using sucrose density centrifugation (Fig. 2*b*). EM analysis

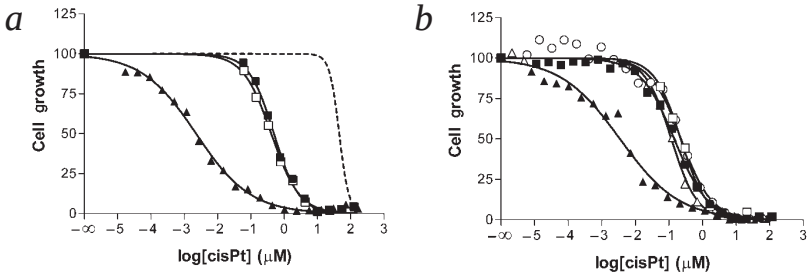

Fig. 1 Cytotoxicity towards human ovarian carcinoma cells. ***a***, Lipid suspension of cisplatin (cisPt-PS/PC; ▲), conventional cisplatin (■), conventional cisplatin mixed with a blank (cisplatin-free) lipid suspension (□; same lipid concentration as in ▲), blank lipid suspension (dashed line; same lipid concentration as in ▲). ***b***, Variations on the standard protocol, omitting FT (△), omitting PS (○). The cytotoxicity of conventional cisplatin (■) is not influenced by FT (□).

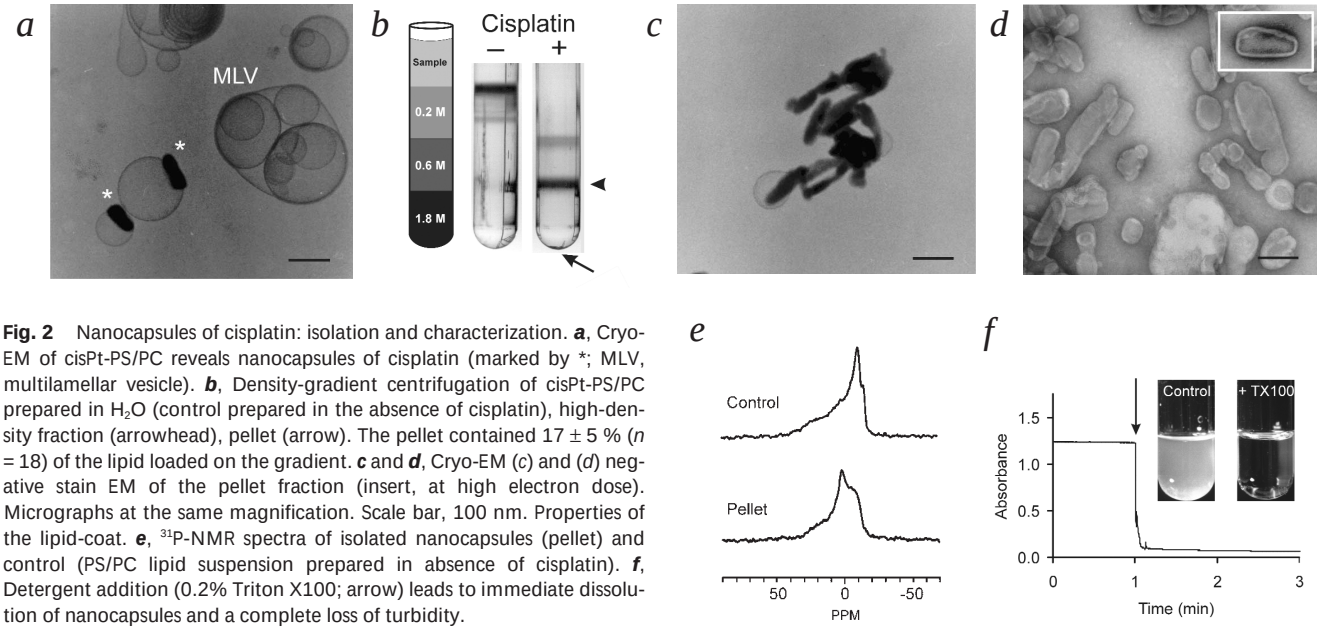

Fig. 2 Nanocapsules of cisplatin: isolation and characterization. ***a***, Cryo-EM of cisPt-PS/PC reveals nanocapsules of cisplatin (marked by *; MLV, multilamellar vesicle). ***b***, Density-gradient centrifugation of cisPt-PS/PC prepared in H_2O (control prepared in the absence of cisplatin), high-density fraction (arrowhead), pellet (arrow). The pellet contained 17 ± 5 % (*n* = 18) of the lipid loaded on the gradient. ***c*** and ***d***, Cryo-EM (*c*) and (*d*) negative stain EM of the pellet fraction (insert, at high electron dose). Micrographs at the same magnification. Scale bar, 100 nm. Properties of the lipid-coat. ***e***, ^{31}P-NMR spectra of isolated nanocapsules (pellet) and control (PS/PC lipid suspension prepared in absence of cisplatin). ***f***, Detergent addition (0.2% Triton X100; arrow) leads to immediate dissolution of nanocapsules and a complete loss of turbidity.

of the gradient fractions showed that the pellet fraction existed of nanocapsules (Fig. 2*c*) and that lipid vesicles were virtually absent (data not shown). Most of the particles were bean-shaped, measuring 46 ± 16 nm by 86 ± 32 nm (inclusive of lipid coat). Negative stain EM showed electron-dense particles surrounded by a bright layer not accessible to the stain (Fig. 2*d*). This bright layer corresponds to the hydrated lipid coating and its thickness, 5–9 nm for approximately 80% of the particles, indicated that most nanocapsules of cisplatin carry a single lipid bilayer (a coat of two bilayers should be at least 10.4-nm thick[13]). The presence of a bilayer coat was supported by ^{31}P nuclear magnetic resonance (NMR) spectroscopy (Fig. 2*e*), which showed a bilayer-type of spectrum typical for membranous particles of this shape and size[14], as well as by freeze-fracture analysis (data not shown). An occasional cross-fracture through a nanocapsule revealed a multi-layered structure for the enclosed cisplatin, suggesting a quasi-crystalline organization. Disruption of the lipid coat leads to the immediate and complete dissolution of the nanocapsules; resuspension of the pellet resulted in a colloidal solution with a milky appearance, which immediately turned transparent upon addition of detergent (Fig. 2*f*). The drug-to-lipid molar ratio of the pellet fraction was exceptionally high, 11 ± 2 (*n* = 18), which translates into approximately 3.3 mg cisplatin per µmol phospholipid. The cytotoxicity of the pellet fraction was much greater than that of conventional cisplatin, and could be further enhanced by filtering the pellet fraction (Fig. 3*a*). A narrow size distribution with an average apparent size of 127 nm was observed (Fig. 3*b*). The cytotoxicity was the highest measured so far, with a typical IC_{50} of 0.3 nM, that is, about 1,000 times more cytotoxic than conventional cisplatin tested in parallel (Fig. 3*a*).

Mechanism of formation and mode of action

We propose the following mechanism for nanocapsule formation (Fig. 4*a*). A nearly saturated solution of cisplatin in H_2O, in the absence of added chloride, contains a mixture of the neutral dichloride- and dihydroxo-species of cisplatin with a low solubility in water, and positively charged aquo-species of cisplatin with a much higher solubility[1,4,12,15]. During freezing, solutes are excluded from the expanding ice phase[16] and cisplatin is progressively concentrated in the residual fluid. The

Table 1 Factors determining cisplatin nanocapsule formation

	Experimental conditions			Nanocapsule formation:
Lipid	Cisplatin	Medium (H_2O)	FT	Pellet[a]
PS/PC (1/1) Control	+	+	+	+ (100%)
None	+	+	+	-
PS/PC (1/1)	-	+	+	-[b]
PS/PC (1/1)	+	+	-	-
PC	+	+	+	-
PS/PC (1/1)[c]	+	+	+	+
PA/PC (1/1)	+	+	+	+
PG/PC (1/1)	+	+	+	+
PS/PE (1/1)	+	+	+	+
PS/SM (1/1)	+	+	+	+
PS/SM/Chol (3/3/4)	+	+	+	+
PS/PC (1/1)	+	+150 mM NaCl	+	-
PS/PC (1/1)	+	+NaOH (pH 8.0)[d]	+	-
PS/PC (1/1)	+	+50 mM NaCl	+	+/- (15%)
PS/PC (1/1)	+(2% diaquo)	+50 mM NaCl	+	+/- (50%)
PS/PC (1/1)	+(10% diaquo)	+50 mM NaCl	+	+
PS/PC (1/1)	+	+150 mM $NaNO_3$	+	+/- (35%)

[a], Nanocapsule formation was determined by sucrose density centrifugation as the amount of cisplatin in the pellet fraction relative to that in the control pellet (PS/PC, 5 mM cisplatin in H_2O, FT): >70% (+), 15–50% (+/-), <10% (-). [b]Visual inspection.[c], Cisplatin added to preformed liposomes (unilamellar or multilamellar). [d], 5 mM cisplatin in H_2O has pH 5.5 and contains ~10% diaquated (aquo/hydroxo) species of cisplatin. PA: dioleoyl-phosphatidic acid, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, SM: sphingomyelin, Chol: cholesterol.

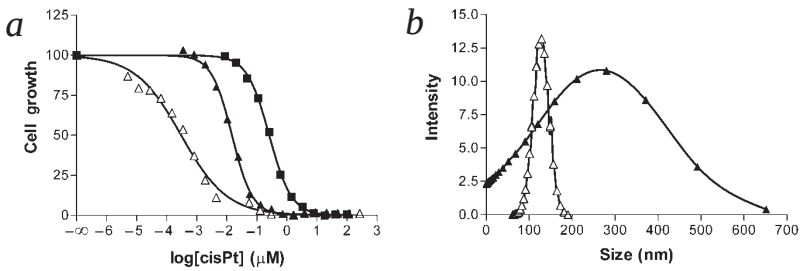

Fig. 3 Cytotoxicity and sizing of nanocapsules of cisplatin. ***a***, Cytotoxicity of conventional cisplatin (■), and of isolated nanocapsules before (▲) and after (△) sizing. ***b***, Dynamic light scattering analysis before (▲) and after (△) sizing.

solubility limit of the neutral species of cisplatin is exceeded first and small aggregates form, which are subsequently covered by positively charged aquo-species of cisplatin. The highly positively charged cisplatin aggregates interact with the negatively charged lipid vesicles, and membranes reorganize to cover the surface of the aggregates resulting in nanocapsules—small lipid-coated aggregates of cisplatin. Only those aggregates of cisplatin that are completely covered by lipid do not redissolve upon thawing. This model is in close agreement with the experimental results indicating that aggregation of cisplatin depends on freezing and lipid coating on electrostatic interactions between cisplatin and lipids (Table 1). First, nanocapsule formation required the presence of both negatively charged lipids and cisplatin, and critically depended on freeze-thawing. The choice of anionic and zwitterionic lipids and the stage at which cisplatin was added were not critical, PS could be replaced by phosphatidic acid (PA), and the same results were obtained adding cisplatin to a dry lipid film or to preformed liposomes. Second, high chloride concentrations and alkaline pH, conditions that inhibit the formation of positively charged 'aquated' species of cisplatin[4,5], prevented nanocapsule formation. At a chloride concentration of 50 mM, nanocapsule formation could be restored completely by adding 10% of preformed diaquated cisplatin just before freeze-thawing. Finally, high concentrations of salts such as $NaNO_3$, which prevent phase separation of ice and solutes[16] and thus aggregation of cisplatin during freezing, strongly inhibited nanocapsule formation. Note that our method results in a colloidal solution of extremely small (lipid-coated) particles, and is clearly different from simple precipitation of cisplatin from an oversaturated solution, which generally results in irreversible inactivation of the drug.

The most likely reason for the higher cytotoxicity of the nanocapsules is a reduced drug inactivation. The lipid coat prevents the reaction of cisplatin with substrates present in the extracellular environment. After cell-surface binding or endocytic uptake (Fig. 4*a*), the coat is destabilized, and after membrane passage, cisplatin ultimately interacts with nuclear DNA triggering cell death. The possibility of endocytic uptake of nanocapsules is supported by EM analysis (Fig. 4*b*).

Medical and pharmaceutical implications

The nanocapsules of cisplatin have an unprecedented drug-to-lipid ratio, 2–3 orders of magnitude higher than published previously[7–9], and an unprecedented *in vitro* cytotoxicity, up to 1000-fold higher than that of the free drug. The nanocapsules can be stored after freeze-drying, and are stable in water at 4 °C with less than 10% release in 8 days (a slow release of cisplatin is found at 25 °C and 37 °C, with halftimes of 29.5 h and 6.5 h, respectively; data not shown). Lipid composition, size distribution, and surface charge can be manipulated and the anti-cancer formulation optimized for local or systemic use. We have previously shown that under appropriate conditions, cisplatin reacts with the head group of PS forming a neutral coordination complex[17]. This reaction can be used to reduce membrane surface charge: incubating a suspension of nanocapsules with 1 mM cisplatin in water for 24 hours at 37 °C changed the ζ (zeta) potential, a direct measure of surface charge, from –44 ± 1 mV to a value identical to that of PC liposomes (data not shown). Reduction of membrane surface charge is an important tool to improve the stability of drug–lipid formulations in

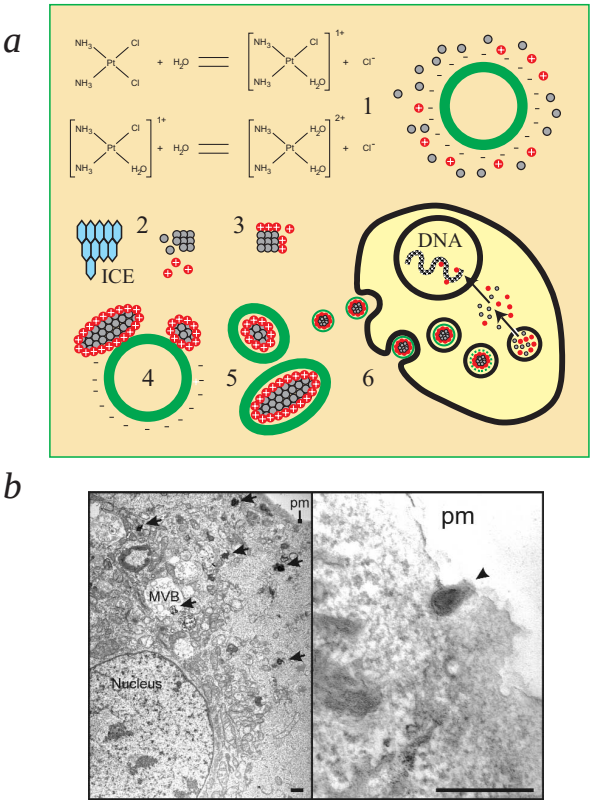

Fig. 4 Mechanism of nanocapsule formation and cell interaction. ***a***, A model: partial hydrolysis of cisplatin in water yields positively charged weak acids. Neutral and positively charged species of cisplatin are present in a suspension of negatively charged liposomes before freeze-thawing (1). Freezing results in ice-crystallization and aggregation of the neutral species (2), co-aggregation with positively charged species (3), interaction of positively charged cisplatin aggregates with negatively charged liposomes (4), lipid-coated aggregates (nanocapsules) of cisplatin (5). Endocytic uptake of nanocapsules, destabilization and intracellular release of cisplatin (6). ***b***, EM analysis of nanocapsule–cell interactions. Clusters of cisplatin nanocapsules (arrows) after internalization at 37 °C (30 min). Occasionally, an individual nanocapsule is observed presumably at an early stage of endocytosis (arrowhead in right panel). pm: plasma membrane, MVB: multivesicular body. Scale bars, 1 μm.

Methods

Lipid formulations. Cisplatin (Sigma) was dissolved in Pipes-EGTA (10 mM Pipes-NaOH, 1 mM EGTA, pH 7.4) or MilliQ water and incubated in the dark overnight at 37 °C. Lipid dispersions (1.2 mM) were prepared by adding 5 mM cisplatin to a dry film of phospholipids (Avanti Polar Lipids, Birmingham, Alabama), incubating at 37 °C for 15 min, followed by ten freeze-thaw (FT) cycles using ethanol/dry-ice (–70 °C) and a water bath (37 °C). Free (extravesicular) cisplatin was removed by centrifugation (3 times, 10 min, 20 °C, 70,000g_{max}), resuspending the membrane pellet in Pipes-EGTA. Alternatively, the dispersion (1 ml) was loaded on top of a step gradient consisting of 1 ml each of 1.8 M, 0.6 M and 0.2 M sucrose in Pipes-EGTA, centrifuged (30 min, 4 °C, 400,000g_{max}), and the pellet resuspended in MilliQ water. Filtered pellet fractions were obtained by high-pressure extrusion[11] (200-nm pore size), and re-isolation of the pellet.

Encapsulation efficiency and cytotoxicity. The phospholipid content was determined[2], and cisplatin was quantified by atomic absorption spectroscopy[2]. Human-derived ovarian tumor cells (IGROV-1) were grown on plastic in RPMI (ref. 2). Formulations were diluted in RPMI without FCS to a cisplatin concentration of 233 μM. Tumor cell growth inhibition was determined using 96-well plates and the sulforhodamine-B assay[19]. Approximately 1,000 cells were seeded per well, the cisplatin formulations were added after 48 h (20 concentrations, in triplicate), and the cells further incubated for 120 h at 37 °C. Data were fitted to a sigmoidal dose-response curve (variable slope) using GraphPad Prism (GraphPad Software, San Diego, California). To study nanocapsule–cell interactions, IGROV-1 cells (6-well plates, 80% confluency) were incubated with cisplatin–lipid suspensions (1 mM cisplatin in RPMI with FCS) for 30 min at 37 °C, washed with ice-cold PBS, and processed for electron microscopy.

Electron microscopy. Dispersions were visualized by cryo-electron microscopy (cryo-EM) at low dose[20]. Negative-stain EM was performed using 4% uranyl acetate. For thin-section EM, cells were fixed in 2% glutaraldehyde, postfixed with OsO_4 and $K_3Fe(CN)_6$, dehydrated in the presence of 1% p-phenylene-diamine, and embedded in epon.

Miscellaneous procedures. Diaquated cisplatin was prepared using $AgNO_3$ (ref. 15). Large unilamellar vesicles were prepared by extrusion[11]. Particle size and surface charge were determined on a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Absorbance at 400 nm was used as a measure of turbidity. ^{31}P-NMR spectra of 1-ml samples (7 mM phospholipid) were recorded on a Bruker MSL 300 (Bruker Analytik, Rheinstetten, Germany), acquiring 60,000 scans at 30 °C.

the blood stream and decrease uptake by the reticulo-endothelial system[6].

A major problem of the lipid formulations of cisplatin used so far, appears to be the low drug-to-lipid ratio, which limits the bioavailability of cisplatin in the tumor and which may result in low cytotoxicity[18] and in regrowth of platinum-resistant tumors[9]. Our method is expected to overcome these problems. Thus, two major goals may be achieved, reduction of systemic toxicities and, in combination with dose-escalation, prevention and counteraction of platinum resistance. Apart from likely benefits in intravenous therapy, the nanocapsules of cisplatin have a high potential in intracavitary therapy and for delayed/sustained release.

The mechanism underlying the efficient encapsulation of cisplatin in nanocapsules suggests that the same methodology may also prove successful in the encapsulation of other compounds, which, like cisplatin, are notoriously difficult to enclose in liposomes because of their low water solubility and low lipophilicity. In line with this suggestion, we recently succeeded in producing nanocapsules containing lanthanum chloride (R.W.H.M.S., unpublished observation).

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