Protocol 6.13

Nanocapsules: a new vehicle for intracellular delivery of drugs

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Introduction

Liposomes, aqueous compartments surrounded by lipid bilayers, have been widely used as transport vectors to deliver (impermeant) substances into the cytoplasm of cells. Examples include the delivery of chemotherapeutic agents, DNA for transfection, and fluorescent probe molecules (for reviews see refs 1 and 2). The molecular mechanism of the liposome–cell interaction leading to uptake of the liposome contents depends on the lipid composition of the liposome membrane and the cell type involved. Liposome–plasma membrane fusion has been reported in a number of cases (e.g. [3]). Endocytosis is considered the major route of entry for liposomes into cells (reviewed in ref. 4).

The efficiency of encapsulation of the compound of interest in the liposomes is an important determinant of the efficiency of uptake by the cell. The structural properties of liposomes allow for highly efficient encapsulation of hydrophilic compounds and lipophilic compounds in the aqueous interior and in the lipid bilayer of the liposome, respectively [1]. Compounds that do not meet either of these criteria were so far not amenable to efficient encapsulation in a lipid bilayer coat.

One such compound is the poorly water-soluble anti-cancer drug cisplatin, *cis*-diamminedichloroplatinum (II), which is commonly used in the treatment of a variety of solid tumours, including genito-urinary, head and neck, and lung tumours [5]. Encapsulation of this drug into liposomes has many advantages including the reduction of premature inactivation of this highly reactive molecule upon entry in the blood and the reduction of deleterious side-effects such as nephro-, oto- and neurotoxicity [6].

The liposomal formulations of cisplatin developed so far (see e.g. ref 7) suffer from a limited bioavailability of the drug in the tumour [8]. A key factor is likely the low water solubility (7 mM at 37 °C) and low lipophilicity of cisplatin, leading to liposomal formulations with low drug-to-lipid molar ratios (of the order of 0.02). Serendipitously, an alternative method was recently discovered, enabling the encapsulation of cisplatin in a lipid formulation with superior efficiency [9]. Our method takes advantage of the limited solubility of the drug in water, and produces cisplatin nanocapsules, nanoprecipitates of cisplatin surrounded by a single lipid bilayer, which exhibit an unprecedented drug-to-lipid ratio and an unprecedented *in vitro* cytotoxicity. The cisplatin nanocapsules may turn out to be the paradigm for encapsulating compounds

with limited solubility in water and with low lipophilicity in a lipid bilayer coat.

Technical principle

Repeated freezing and thawing of a concentrated aqueous solution of cisplatin in the presence of anionic phospholipids result in the formation of cisplatin nanocapsules. The preparation of cisplatin nanocapsules requires the presence of negatively charged phospholipids and of positively charged aqua-species of cisplatin, pointing to an essential role for electrostatic interactions in the mechanism of formation. A solution of cisplatin in water, in the absence of added chloride, contains a mixture of the neutral dichloride- and dihydroxo-species of cisplatin with low solubility in water, and positively charged aqua-species of cisplatin with a much higher solubility [5]. In the model proposed for the mechanism of nanocapsule formation [9], cisplatin is concentrated in the residual fluid during freezing, forming small aggregates when the solubility limit of the dichloro-species is exceeded. As freezing proceeds, the nanoprecipitates of the dichloro-species of cisplatin become covered by the positively charged aqua-species, which have a higher solubility limit. The negatively charged membranes interact with the positively charged cisplatin aggregates and then reorganize to wrap the aggregates in a phospholipid bilayer coat. The resulting nanocapsules do not redissolve upon thawing.

Procedure

1. Cisplatin is dissolved in MilliQ water to a concentration of 5 mM, which is facilitated by incubating at 55 °C for 30 min. The solution is incubated overnight in the dark at 37 °C to ensure full equilibration.
2. Stock solutions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) are prepared in chloroform (concentration ~5 mM). The precise concentrations are determined by phosphate analysis [10].
3. Aliquots corresponding to 0.6 micromole of each phospholipid are mixed, the solvent is removed by rotatory evaporation, and the lipid film is further dried under vacuum overnight.
4. The dry lipid film is hydrated by adding 1.2 ml of the 5 mM cisplatin solution in water and incubating for 15 min at 37 °C.
5. After brief homogenization on a vortex mixer, the dispersion is transferred to a glass tube and subjected to 10 freeze–thaw cycles using ethanol/dry-ice (−70 °C) and a water bath (37 °C).
6. The resulting colloidal solution is transferred to microfuge tubes and centrifuged for 4 min at 470*g* (2100 rpm in an Eppendorf centrifuge) to collect the nanocapsules.
7. After removal of the supernatant, the fluffy white layer on top of the yellow pellet, corresponding to large liposomes, is removed by a micropipette. The yellow pellet containing the cisplatin nanocapsules, is resuspended in 1 ml water and centrifuged as above in order to wash away non-encapsulated cisplatin.
8. Upon resuspending the final pellet in 0.5 ml water, the nanocapsules are stored at 4 °C until use.

Alternatively, the nanocapsules are separated from contaminating liposomes by density gradient centrifugation. Briefly, the dispersion obtained after the freeze–thaw cycles is loaded on top of a step gradient consisting of 1 ml of each 1.8, 0.6 and 0.2 M sucrose in 10 mM Pipes-NaOH, 1 mM EGTA, pH 7.4. After centrifugation

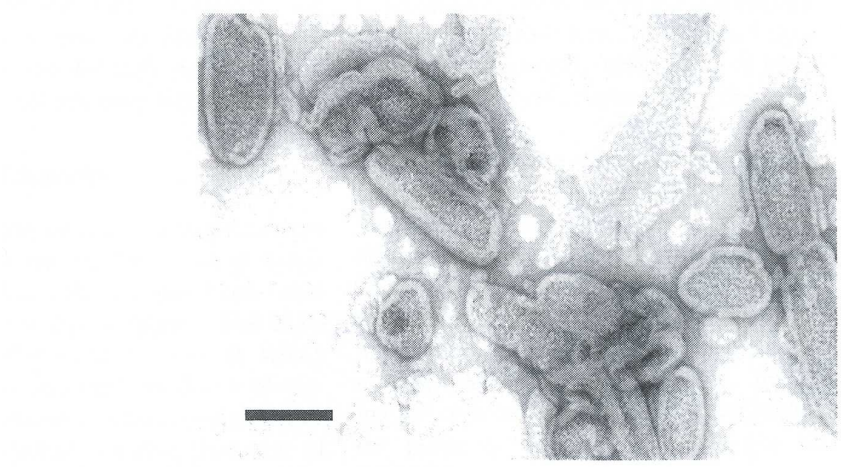

Figure 6.9 Electron micrograph of cisplatin nanocapsules visualised by negative staining. A dilute suspension of nanocapsules was transferred to a carbon-formvar coated grid, and stained with 4% (w/v) uranyl acetate for 45s. Scale bar, 100 nm

at 4 °C for 30 min at 400 000*g*, the pellet fraction corresponding to the nanocapsules is collected and washed as above.

Instead of DOPS, other anionic phospholipids like dioleoyl-phoshatidylglycerol (DOPG) and dioleoyl-phosphatidic acid (DOPA) can be used to prepare cisplatin nanocapsules. DOPC can be replaced by dioleoyl-phosphatidylethanolamine (DOPE) or sphingomyelin. The method is sensitive to high chloride concentrations and alkaline pH, because these conditions prevent the formation of the positively charged aqua-species. Instead of hydrating a lipid film with 5 mM cisplatin, it is also possible to add the cisplatin solution to preformed DOPC/DOPS liposomes and then start the freeze–thaw cycles. The cisplatin nanocapsules can be stored after lyophilization, and retain cisplatin upon rehydration.

Characterization

1. Encapsulation efficiency

The phospholipid content of the nanocapsules is determined by phosphate analysis [10]. The cisplatin content of the nanocapsules is assessed by flameless atomic absorption spectrometry (NFAAS) using K_2PtCl_2 as a standard [11]. Analysis of the cisplatin nanocapsules prepared according to the above protocol, typically yields a Pt/phosphate molar ratio of 11 ± 1. Based on the size of the nanocapsules (see below), this number is estimated to correspond to an internal cisplatin concentration exceeding 0.5 M, which is far beyond the solubility limit of cisplatin and consistent with the quasi-crystalline structure of the encapsulated cisplatin [9]. The method allows encapsulation of cisplatin with an efficiency of approximately 30%.

2. Shape and size

Analysis by negative stain electron microscopy reveals bean-shaped particles consisting of an electron-dense core surrounded by a bright layer (excluding stain), corresponding to the bilayer coat (Figure 6.9). The nanocapsules have a heterogeneous size distribution, with 75% of the population having a length between 50 and 250 nm and a width of around 50 nm. Size analysis by dynamic light scattering yields consistent results. Nanocapsules

of smaller size and with a narrower size distribution have been obtained by high-pressure extrusion through polycarbonate filters with a 200 nm pore size [9].

3. Delivery of contents

The cytotoxicity of the cisplatin nanocapsules towards the human ovarian carcinoma IGROV-1 cell line has been compared to that of free cisplatin. The IC50 value (the drug concentration at which cell growth is inhibited by 50%) of cisplatin administered as nanocapsules is two orders of magnitude smaller than that of the free drug [9]. The higher cytotoxicity is explained by the reduced inactivation of the drug, due to the lipid coat sequestering it from reaction with substrates in the extracellular environment. Upon binding to the cell surface or endocytic uptake of the nanocapsules, the coat is destabilized, and after membrane passage, cisplatin can exert its cytotoxic effect.

Comments

Cisplatin nanocapsules represent a new lipid formulation of cisplatin, distinct from conventional liposomal formulations in that the drug is present as a nanoprecipitate surrounded by a bilayer. This results in a drug-to-lipid ratio that exceeds that of liposomal formulations by two to three orders of magnitude and probably accounts for the typical bean-like shape of the nanocapsules. The high encapsulation efficiency of cisplatin in nanocapsules is expected to increase the bioavailability of the drug and thus improve the therapeutic index as compared to liposomal formulations of cisplatin. Data obtained so far indicate that the cisplatin nanocapsules can gain access to the cell interior via endocytosis [9]. The method for preparing cisplatin nanocapsules may be applicable to a variety of other compounds with limited solubility in water and low lipophilicity that are not efficiently encapsulated in conventional liposomes. Alternatively, compounds of interest may be co-encapsulated together with cisplatin.

Like the surface of conventional liposomes, the membrane surface of nanocapsules can be engineered to include poly (ethyleneglycol)-conjugated lipids (reviewed in ref. 2). This results in more stable nanocapsules without affecting the cytotoxicity [12]. It is expected that the technologies developed for liposomes, including the attachment of ligands or antibodies for purposes of targeting, can also be applied to nanocapsules.

Acknowledgement

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Note added in proof: The molecular architecture of the cisplatin nanocapsules was recently solved (Chupin, V., de Kroon, A. I. P. M., and de Kruijff, B. (2004) *J. Am. Chem. Soc.*, **126**, 13816–13821.)