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Octreotide labeled aggregates containing platinum complexes as nanovectors for drug delivery‡

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The synthesis, formulation and a complete physico-chemical characterization, by dynamic light scattering and small angle neutron scattering techniques, of new liposomal aggregates obtained by co-assembling an amphiphilic molecule containing a platinum complex, Peg_{1500}-Lys(Pt-aminoEtGly)-Lys$(C18)_2$, (abbreviated as $(C18)_2$-PKAG-Pt), with a second amphiphilic monomer, $(C_{18}H_{37})_2NCO(CH_2)_2CO(AdOO)_5$-Oct ($(C18)_2L_5$-Oct), containing the octreotide bioactive peptide, is reported. Liposomes of $(C18)_2$-PKAG-Pt present a radius of 48 nm, whereas the mixed aggregates $(C18)_2$-PKAG-Pt/$(C18)_2L_5$-Oct at 90/10 M ratio give larger liposomes with a radius of 84 nm. In both cases, the bilayer thickness is ~5.3 nm. Encapsulation of doxorubicin in mixed liposomes is also obtained by using the pH gradient method. The obtained liposomes could represent a new target selective cargo system for delivery of cisplatin based drugs and/or doxorubicin on cells overexpressing the sstr2 and sstr5 somatostatin receptors. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: supramolecular aggregates; platinum complexes; drug delivery; octreotide; SANS; doxorubicin

Introduction

(SP-4-2)-diamminedichloridoplatinum(II) (cisplatin) and many of its analogs are currently used in the treatment of a large number of solid tumors [1]. Despite the large use, the clinical treatments based on platinum drugs find three major problems: (i) serious dose-limiting toxicities in particular nephrotoxicity, neurotoxicity and ototoxicity; (ii) rapid inactivation of the drug as a result of complexation to plasma and tissue proteins; and (iii) frequent occurrence of platinum resistance [2–5]. One strategy to reduce these side effects and save their therapeutic efficacy lies in shielding the drugs from the extracellular environment by means of a lipid coating. The ability of liposomes to prevent organ specific toxicities such as cardiotoxicity is well known [6]. These preparations exhibit an extended circulation time, increased anti-tumor efficacy and reduced toxicity compared with the free drug. Moreover, a liposomal formulation may overcome platinum resistance by delivering a high dose of the administered drug at the tumor site. Another advantage could lie in different mechanism of the liposome to cross the cell membrane barrier.

Anyway, most of platinum drugs have low hydrophilicity and at the same time a very low affinity for the hydrophobic compartment. For this reason, a very low drug-to-lipid ratio is found in liposomal preparation [7–9]. Two strategies have been pursued to increase the amount of platinum complexes in liposomes or in other supramolecular aggregates. One approach is to synthesize lipophilic derivatives of cisplatin that can be efficiently encapsulated in large multilamellar liposomes [10]. The lipophilic Pt complex NDDP is an example of such a complex, and its liposomal formulation L-NDDP (aroplatin) has entered Phase II clinical trials [10–13]. Furthermore, in order to improve the liposolubility of NDDP, highly branched aliphatic carboxylate groups were used, greatly increasing the molecular weight and making passive diffusion through the cell membrane difficult. Recently, salicylate derivatives of platinum complexes have been efficiently encapsulated showing greater antitumor activity and less toxicity than carboplatin and oxaliplatin [14]. The second approach is based on improving the loading methods by using highly water soluble platinum complexes, for example, *cis*-diamminedinitratoplatinum (II), as drug [15].

Several liposomal formulations of cisplatin have been already proposed; anyway, some drawbacks have been found. For

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List of abbreviations: *AdOO, 8-amino-3,6-dioxaoctanoic acid; DIPEA, N,N-diisopropylethylamine; DLS, dynamic light scattering; DMF, dimethylformammide; DPPG, dipalmitoyl phosphatidyl glycerol; ESI MS, electrospray ionization-mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, (1-hydroxybenzotriazole); HPLC, high performance liquid chromatography; mPEG DSPE, 1,2-distearoyl-phosphatidyl ethanolamine-methyl-polyethyleneglycol; MBHA resin, 4-methylbenzhydrylamine resin; NDDP, (cis-bis-neodecanoato-trans-R, R-1,2-diaminocyclohexane platinum(II)); PEG, polyethylene glycol; SANS, small angle neutron scattering; SPPS, solid-phase-peptide-synthesis; sstr2 and sstr5, somatostatin receptor 2 and 5; TFA, trifluoracetic acid; TIS, trisisoprpylsilane; USANS, ultra small angle neutron scattering.*

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example, negatively charged-lipid coated cisplatin nanocapsules [16] fail in their clinical applications because of inefficient encapsulation of the drug in lipid formulations limiting the bioavailability and resulting in low drug uptake by the tumor [17–19], and in the poor serum stability [20]. In liposome-coated cisplatin SPI-077, high levels of platinum were detected at tumor sites [7,21]. Despite a prolonged circulation time and enhanced tumor uptake, this formulation showed little activity when evaluated clinically [22]. In fact, they showed very low release kinetics of cisplatin from the liposomes, resulting in poor therapeutic efficacy [18,23,24]. Another liposomal cisplatin formulation (Lipoplatin), composed of DPPG, soy phosphatidyl choline, cholesterol and mPEG-DSPE, is currently undergoing a number of Phase II and Phase III clinical trials in combination with other cytotoxic agents, demonstrating substantially reduced renal toxicity, peripheral neuropathy, ototoxicity and myelotoxicity [25,26].

An improvement in the use of liposomes or other supramolecolar aggregates as tools for drug delivery is based on the possibility to decorate the external surface of the supramolecular carrier with bioactive molecules to improve drug accumulation at tumor site. For example, liposomes encapsulating As and Pt based drugs were decorated by folate in order to target cells overexpressing specific folate receptors [27].

In the last years, we developed new mixed aggregates modified with a bioactive peptide on their external surface and able to deliver contrast agents and/or drugs on tumor cells overexpressing the appropriate receptors for the chosen peptide [28]. These aggregates have been obtained by co-aggregation of two monomers: the first monomer containing a chelating agent able to coordinate a radioactive or paramagnetic metal ion and having a lipophilic moiety and the second monomer containing a bioactive peptide linked to a similar lipophilic moiety [29,30], or by using a single monomer containing in the same molecule the bioactive peptide, the chelating agent and the lipophilic moiety [31,32].

In this paper, we report on the synthesis, formulation and physico-chemical characterization of new aggregates obtained by co-assembling an amphiphilic molecule containing a platinum complex with the second amphiphilic monomer containing the octreotide bioactive peptide. The obtained liposomes could represent a new target selective cargo system for delivery of cisplatin based drugs on cells overexpressing the sstr2 and sstr5 somatostatin receptors. Moreover, the encapsulation of the second drug (doxorubicin) is explored in order to develop target selective delivery systems with two drugs.

Results

Synthesis and Formulation

The schematic structure of the two monomers used to assemble supramolecular aggregates is reported in Figure 1, both monomers contain a hydrophobic moiety and a more hydrophilic region. The hydrophobic region of the two monomers is based on two aliphatic chains with 18 carbon atoms each. This hydrophobic moiety was chosen for its similarity to the components of membrane phospholipidic bilayer, thus avoiding toxic and haemolytic effects on the cells [33]. Moreover, the 18 carbon atom chains provide sufficient Van der Waals interactions to obtain stable aggregates in water, also when diluted in the blood for *in vivo* applications.

The monomer $(C_{18}H_{37})_2NCO(CH_2)_2CO(AdOO)_5$-Oct ($(C18)_2L_5$-Oct) was previously designed and already used by us, in order to vehicle contrast agents to tumor cells overexpressing somatostatin receptors [34]. It contains the cyclic peptide octreotide, a well known somatostatin analog, characterized by long *in vivo* stability and able to bind with nanomolar affinity, the somatostatin receptors sstr2 and sstr5. The bioactive peptide is spaced from the hydrophobic moiety by five units of 8-amino-3,6-dioxaoctanoic acid (AdOO). The five AdOO linkers, that constitute a PEG fragment, were chosen both to increase the hydrophilicity of the head without changing the monomer charge and to reduce aggregate clearance through the reticulo-endothelial system [35].

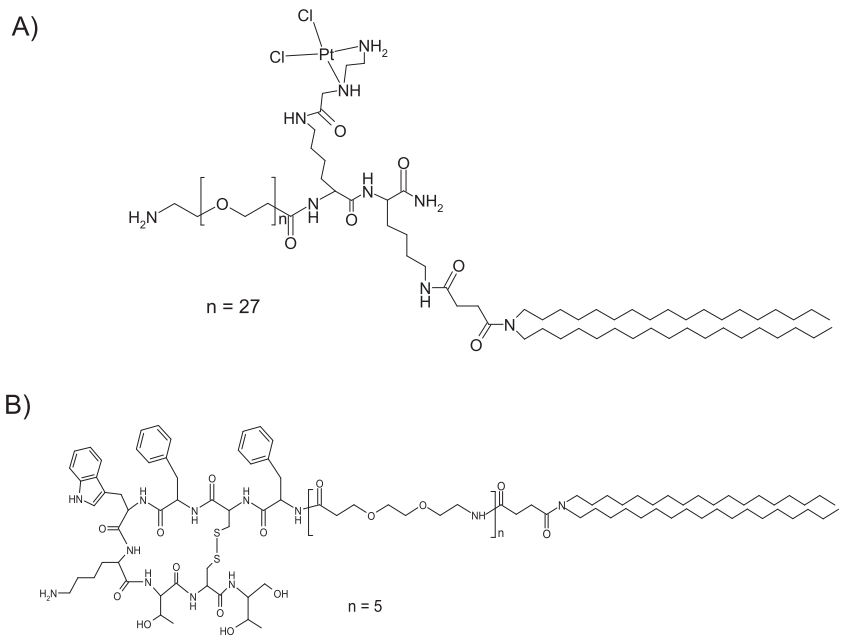


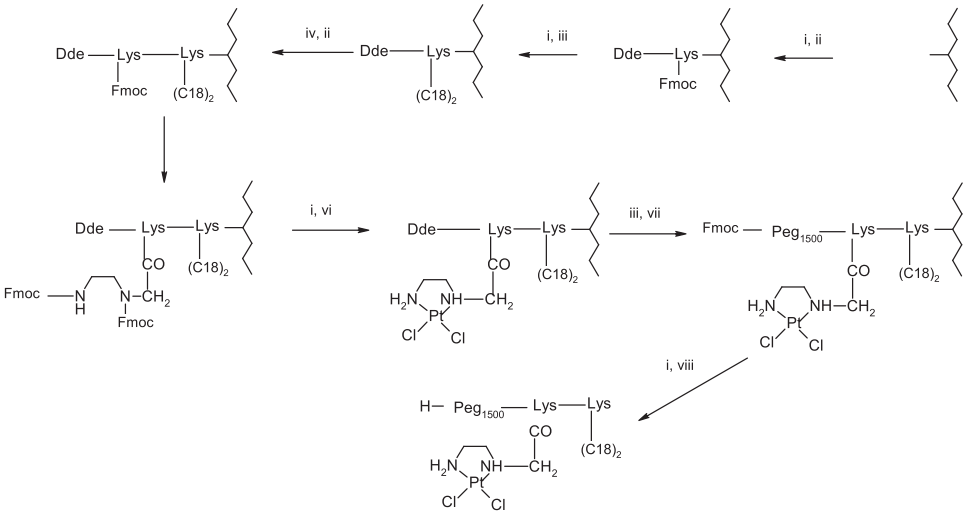
Figure 1. Schematic representation of the two amphiphilic monomers: (a) Peg_{1500}-Lys(Pt-aminoEtGly)-Lys$(C18)_2$ ($(C18)_2$-PKAG-Pt) and (b) $(C18)_2L_5$-Oct.

The platinum containing amphiphilic monomer, Peg_{1500}-Lys (Pt-aminoEtGly)-Lys$(C18)_2$, ($(C18)_2$-PKAG-Pt) was designed to follow two requirements: (i) the platinum complex has to retain a cytotoxic efficacy also when it is bound to the entire monomer and/or to the reconstituted liposome, and (ii) the hydrophilic head has to contain a PEG chain to give hydrophilicity to the head and to allow a long *in vivo* circulation time of the reconstituted liposomes. The monomer is based on a Lys–Lys moiety, in which the first Lys residue is derivatized with a Peg1500 moiety on its α-amino function, and with *N*-2-aminoethylglycine chelating the $PtCl_2$ fragment, on the epsilon amino function; the second Lys residue is derivatized on the side-chain with the hydrophobic moiety containing two 18 carbon atom chains. Both monomers were synthesized in solid phase modifying the well assessed procedure of SPPS with Fmoc strategy. The synthetic pathway used to obtain $(C18)_2$-PKAG-Pt is reported in Figure 2. Following the synthetic procedure proposed by Reedjiak [36], the platinum ion was added by reacting a stoichiometric amount of K_2PtCl_4, dissolved in N-methyl-2-pyrrolidone (NMP)/water mixture, with the free amino functions of *N*-2-aminoethylglycine moiety present on the epsilon amino group of the Lys–Lys fragment bound to the Rink amide resin. The monomer was cleaved from the resin adding a TFA/H_2O mixture. The TIS scavenger, usually used in cleavage peptide procedure from resin, was avoided. This molecule could favor the formation of platinum at elementary state, through oxidative addition followed by a rapid reductive elimination. The platinum monomer was purified by crystallization steps by adding diethylether dropwise to TFA cleavage solution. The product identity was confirmed by ESI MS and by ^1H NMR spectroscopy. The presence of Pt(II) was found 40% of that expected by ICP-MS measurements. Peaks found in ESI MS spectrum correspond to the platinum complex containing chloride ligands and platinum complexes in which chloride atoms are substituted, during HPLC, with water molecules because of the *trans* effect of the amino nitrogen atoms. The ESI spectrum also shows the platinum complex without chloride ligands and the full monomer lacking of the platinum center.

Self assembling aggregates containing the platinum monomer $(C18)_2$-PKAG-Pt and mixed aggregates between platinum monomer $(C18)_2$-PKAG-Pt and peptide monomer $(C18)_2L_5$-Oct, at 90/10 M ratio, were prepared by well-assessed sonication and extrusion procedures in 0.1 M phosphate buffer at pH 7.4 and physiological ionic strength conditions (NaCl 0.9 wt%). Larger amount of peptide monomer in the final composition of mixed aggregates were avoided in order to prevent inter-chain peptide interactions, and to preserve the right peptide conformation for receptor binding. Peptide concentration in mixed aggregates was determined by UV spectroscopy. The exposure of the bioactive octreotide moiety of the monomer containing peptides on aggregates surface was evaluated by monitoring the fluorescence emission produced by the indole moiety of the tryptophan residue. Usually, this fluorophore shows an emission peak centered at 350 nm in polar solvents, whereas in hydrophobic solvents the maximum shifts to 330 nm [37]. A fluorescence emission centered at 345 nm was observed confirming the exposure of the most of the indole groups on the hydrophilic surface of supramolecular aggregates. By considering that the Trp-Lys fragment in a β-turn conformation is responsible for octreotide and somatostatin binding to membrane receptors, the found emission wavelength value allows to predict binding properties for the entire aggregate. ICP-MS measurement confirmed the quantitative presence of platinum both in self-assembling and in mixed supramolecular aggregates, thus indicating the complex stability also during supramolecular aggregate preparation.

Dynamic Light Scattering and Small Angle Neutron Scattering

In Figure 3, an example of the hydrodynamic radius distribution function has been reported for the binary system $(C18)_2$-PKAG-Pt/water and for the ternary system $(C18)_2$-PKAG-Pt/$(C18)_2L_5$-Oct/water at 90/10 M ratio among the two synthetic components.



i) Piperidine 30% in DMF; ii) Dde-Lys(Fmoc)-OH, HBTU/HOBt/DIPEA in DMF; iii) N,N-dioctadecylsuccinamic acid, HBTU/HOBt/DIPEA in DMF; iv) 2% NH_2NH_2 in DMF; v) N-Fmoc[N'-Fmoc-2-aminoethyl]Gly-OH, HBTU/HOBt/DIPEA in DMF; vi) K_2PtCl_4 1 equiv. in NMP/H_2O, vii) Fmoc-Peg_{1500}-OH, HBTU/HOBt/DIPEA in DMF; viii) TFA/H_2O (95/5).

Figure 2. Scheme of synthesis, by solid phase methods, of $(C18)_2$-PKAG-Pt monomer.

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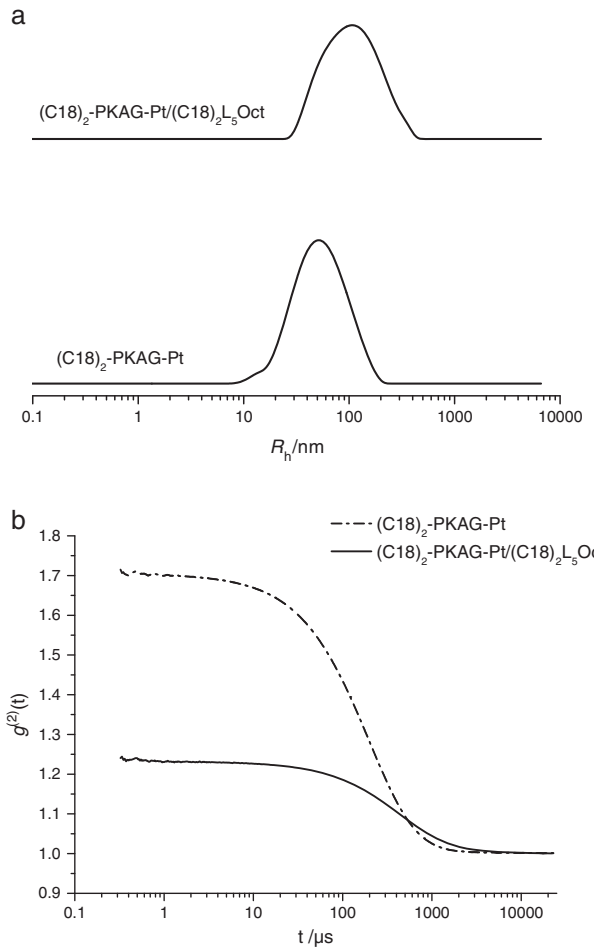


Figure 3. DLS measurements: (a) Hydrodynamic radius distribution functions obtained at 25 °C for $(C18)_2$-PKAG-Pt and $(C18)_2$-PKAG-Pt/$(C18)_2$L5-Oct (90/10 M ratio) aqueous dispersions at pH 7.4; (b) Intensity correlation functions at θ = 90° for 1 mM liposomal solution.

In both cases, it is possible to observe that the distribution is clearly mono-modal, i.e. there is a presence of a single mode. Hydrodynamic radius R_h distribution function is strictly connected to the diffusion coefficient D distribution function through the Stokes-Einstein

$$R_H = \frac{k_B T}{6\pi\eta_0 D} \quad (1)$$

where k_B is the Boltzmann constant, T is the absolute temperature and η is the medium viscosity. Rigorously speaking, Eqn (1) holds at infinite dilution. However, because of high dilution, it can be reasonably used to estimate the hydrodynamic radius of the aggregates [38]. Extrapolation of diffusion data at zero angle, i.e. zero q values, allows establishing the presence of aggregates with diffusion coefficients of $(5.1 \pm 0.5) \cdot 10^{-8}\,cm^2\,s^{-1}$ and $(2.9 \pm 0.3) \cdot 10^{-8}\,cm^2\,s^{-1}$ for binary and ternary systems, respectively. These values correspond to a mean radius of (48 ± 4) nm and (84 ± 6) nm, for binary and ternary systems, respectively. The found values fall in the typical range of vesicular or liposomal aggregates and would be in agreement to the preparation method of the aggregates.

In order to have information on the morphology of the aggregates formed in the systems, SANS measurements have been carried out and reported in Figure 4. Inspection of the figure allows

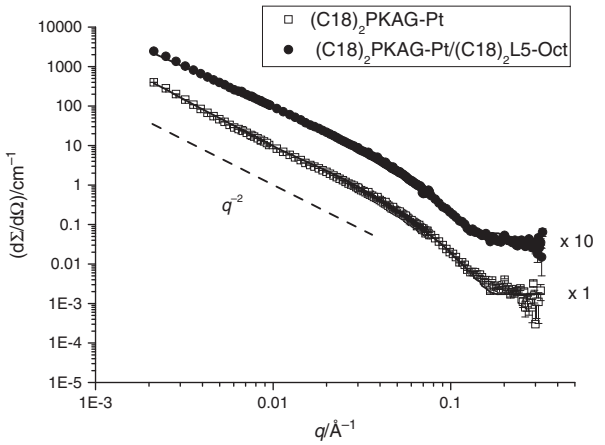


Figure 4. Scattering cross-sections obtained at 25 °C for (□) $(C18)_2$-PKAG-Pt and (○) $(C18)_2$-PKAG-Pt/$(C18)_2L_5$-Oct (90/10 M ratio) aqueous dispersions at pH 7.4. Solid lines represent fitting curves of the experimental data. To allow for a better visualization, data have been multiplied for a scale factor, as indicated in the plot.

observing the scattering cross-sections for both the systems scale with a power law $(d\Sigma/d\Omega) \propto q^{-2}$ typical of bi-dimensional structures such as sheets. Actually, the presence of large objects such as unilamellar liposomes with radii of tens of nanometers does not allow for a full characterization of these supramolecular structures, because their Guinier regime falls in the USANS domain ($q < 10^{-4}$ nm). Consequently, liposomes can be regarded as a collection of randomly oriented planar sheets, whose cross-section dependence on q is given by [39]:

$$\frac{d\Sigma}{d\Omega} = 2\pi(\rho_c - \rho_0)^2 S d^2 \frac{1}{q^2} \frac{\sin^2\left(\frac{qd}{2}\right)}{\left(\frac{qd}{2}\right)^2} + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh} \quad (2)$$

where d is the plane thickness, and S is the plain surface per unit volume, and $(d\Sigma/d\Omega)_{incoh}$ represents the incoherent contribution to the total scattering cross-sections, mainly because of the presence of hydrogenated molecules in the systems. Fitting of Eqn (2) to the experimental data, also reported in Figure 4, allows obtaining an estimation of the lateral thickness of the liposome double layer, which results to be (5.3 ± 0.2) nm for ternary and binary systems.

Doxorubicin Loading

Doxorubicin was loaded in mixed liposomes by using the pH gradient method [40]. Liposomes were prepared at pH 4.0, and then the pH outside the liposomes was brought to 7.4 by adding NaOH dropwise. Doxorubicin loading content (DLC) was calculated by UV–vis measurements for subtraction of the amount of free doxorubicin, eluted by gel filtration, from the total amount of initial doxorubicin. Several loading assays were carried out to optimize the DLC. Four different percentage of drug (wt)/lipid (wt) from 0.025 to 0.1 were used. An encapsulation >95% was observed at 0.025 (drug (wt)/lipid (wt)). This ratio is lower with respect to the other liposome such as commercially available liposomes (Myocet and Doxil). However, because of higher molecular weight of monomers used in this formulation respect to widespread phospholipids, the molar ratio is comparable with that used for Myocet and Doxil. In the final formulation, the amount of platinum complex was $2 \cdot 10^{-5}$ mol, whereas the amount of doxorubicin is tenfold lower ($2 \cdot 10^{-6}$ mol). DLS measurements

indicate that liposome structure and size do not change after doxorubicin loading (data not shown).

Discussion

In last year, liposomal carriers for drug delivery have been easily obtained by using natural or synthetic phospholipids; these molecules are commercially available, also in GMP grade, and guarantee high *in vivo* stability and low toxicity of the supramolecular aggregates they form in water solution. Generally, the size of the liposomes is appropriate for *in vivo* use and is not affected by drug encapsulation process. As a consequence, about one-tenth of liposomal drugs have been introduced in the market and others are currently in development or already used in clinical studies.

More recently, liposomes have also been obtained employing synthetic amphiphilic monomers opportunely designed to introduce special functions on the final aggregate such as targeting molecules or metal complexes acting as contrast agents in MRI or in nuclear medicine [28–34].

Now, we have obtained new supramolecular aggregates by self-assembling, in water solution, a synthetic amphiphilic monomer containing a platinum complex, a long PEG chain, and a hydrophobic moiety with two C18 alkyl chains. The liposome structure has been deeply characterized by dynamic light scattering and SANS measurements, and it is schematized in Figure 5. The PEG moiety in the monomer structure is essential to give hydrophilicity to the monomer head allowing liposome formation: a similar monomer lacking of the PEG chain on the lysine α-amino function results completely insoluble in water solution and does not aggregate in supramolecular structures (data not shown). Moreover, the advantage of pegylated liposomes respect to non-pegylated aggregates has been well assessed for drug delivery purpose: in fact, liposome coating with PEG moieties allows a longer *in vivo* circulating time and confers stealth properties to the aggregates [41]. ICP measurements obtained on the single monomer $(C18)_2$-PKAG-Pt indicate that only 40% of the chelating agent *N*-2-aminoethylglycine in the single monomers complex the platinum(II) ion; anyway, the platinum(II) ion remains stable complexed to the two amino functions, as confirmed by comparing ICP data, on the single amphiphilic monomer and on the aggregate formulations. The other two ligands of the platinum(II) center, in its square-planar structure, are chloride ions or water molecules according to the solution composition, as in the case of cisplatin $[Cl_2Pt(NH_3)_2]$, in which the chloride ions are substituted by water molecules in the cell environment characterized by low chloride concentration. The biological activity of platinum(II) ions complexed by *N*-2-aminoethyl-glycine bound to peptide sequences in monomeric peptide derivatives has been already studied by Reedijk [36] and by Mancini [42]. They found a cytotoxic activity lower than cisplatin; in the aggregate formulation based on Peg_{1500}-Lys(Pt-aminoEtGly)-Lys$(C18)_2$, the presence of a large amount of platinum complexes, even if with reduced cytotoxic activity of each platinum center, should give a potential high cytotoxic efficacy to the full liposome.

Figure 5 also reported a schematic structure, and the geometrical parameters of mixed liposomes in which 10% of the amphiphilic monomers are constituted by the $(C18)_2L_5$-Oct peptide containing monomer. An increase of about 70% of the hydrodynamic radius of the mixed aggregates respect to self-assembling liposomes is observed. This increase could be probably because of the presence of the octreotide peptide on the liposome external surface. The exposition of the octreotide peptide, confirmed by fluoresce data, could assure targeting properties of the platinum containing aggregate to cancer cells overexpressing the sstr2 and/or sstr5 somatostatin receptors. Moreover, it seems reasonable to expect that the platinum containing liposomes could be efficiently modulated by inserting in the final composition other $(C18)_2L_5$-peptide amphiphilic monomers containing bioactive peptides to target other biologically relevant receptors for selective drug delivery.

Finally, we have investigated the possibility to obtain target selective multi-drug delivery systems by coupling the platinum based drug and the well known cytotoxic doxorubicin in the same liposomal cargo. Loading data confirm that the proposed procedure allows obtaining doxorubicin encapsulation in the inner liposome compartment without structural modification of the cargo system, as confirmed by DLS data (Figure 5). The combined use of doxorubicin and platinum based drugs is a very

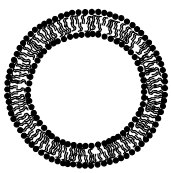
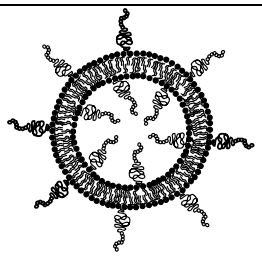
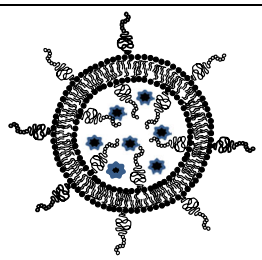
LIPOSOMES	$(C18)_2$PKAG-Pt	$(C18)_2$PKAG-Pt / $(C18)_2L_5$-Oct	$(C18)_2$PKAG-Pt / $(C18)_2L_5$-Oct/Doxo
$(C18)_2$PKAG-Pt $(C18)_2L_5$-Oct Doxo			
D $x10^{-8}$/ cm^2s^{-1}	5.1 ± 0.5	2.9 ± 0.3	2.9 ± 0.3
R_h / nm	48 ± 4	84 ± 6	84 ± 6
Thickness / Å	53 ± 2	53 ± 2	

Figure 5. Schematic representation and physic-chemical parameters of $(C18)_2$-PKAG-Pt; $(C18)_2$-PKAG-Pt/$(C18)_2L_5$-Oct (90/10 M ratio); and $(C18)_2$-PKAG-Pt/$(C18)_2L_5$-Oct/doxorubicin liposomes.

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important approach in several tumor therapeutic purposes. The results obtained in A Multi-National, Randomized, Phase III, GCIG Intergroup Study Comparing Pegylated Liposomal Doxorubicin (CAELYX®) and Carboplatin vs. Paclitaxel and Carboplatin (CAPYPSO trials) demonstrate a superior therapeutic index respect to carboplatinum in relapsed/recurrent ovarian cancer [43].

Materials and Methods

General Experimental Procedures

Protected N^{α}-Fmoc-amino acid derivatives, coupling reagents and Rink amide MBHA resin were purchased from Calbiochem–Novabiochem (Laufelfingen, Switzerland). Fmoc-AdOO-OH was purchased from Neosystem (Strasbourg, France). Fmoc-Peg_{1500}-OH was purchased by Iris Biotech (Marktredwitz, Germany). *N*,*N*-dioctadecylsuccinamic acid was prepared according to the experimental procedure reported in literature [44]; *N*-Fmoc[*N*′-Fmoc 2-aminoethyl]Gly-OH was obtained by Fmoc protection of the commercially available [*N*′-Fmoc-2-aminoethyl]Gly-OH (Bachem, Switzerland). K_2PtCl_4 and all other chemicals were commercially available by Sigma-Aldrich (St. Louis MO USA), Fluka (Buchs, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. All solutions were prepared by weight using double distilled water. Samples to be measured by SANS techniques were prepared using heavy water (Sigma-Aldrich, purity > 99.8%). The pH of all solutions was kept constant at 7.4.

Synthesis of peptide conjugate was carried out as previously describe in solid phase under standard conditions using Fmoc strategy [45]. Experimental conditions for purification and identification data have been reported elsewhere [34].

Synthesis of Peg_{1500}-Lys(Pt-aminoEtGly)-Lys$(C18)_2$ ($(C18)_2$-PKAG-Pt)

Synthesis of platinum containing amphiphilic monomer was carried out in solid phase under standard conditions using Fmoc/tBu strategy. Rink amide MBHA resin (0.55 mmol g^{-1}, 0.1 mmol scale, 0.180 g) was used as polymeric support. The N^{α}-protecting group on the resin was removed by DMF/piperidine (70/30) mixture. Dde-Lys(Fmoc)-OH residue (0.186 g, 0.2 mmol) was activated by 1 equivalent of HBTU and HOBt, and 2 equivalents of DIPEA in DMF and coupled on the resin stirring the slurry suspension for 1 h. The coupling was repeated twice. Then, the N^{ε}-Fmoc protecting group of the lysine residue was removed and 0.124 g (0.2 mmol) of *N*,*N*-dioctadecylsuccinamic acid was condensed twice for 1 h in DMF/DCM (1/1) mixture. The lipophilic moiety was activated *in situ* by the standard HOBt/HBTU/DIPEA procedure. The coupling was monitored by the qualitative Kaiser test. After the Dde protecting group removal by a solution of 2% hydrazine solution in DMF, a Dde-Lys(Fmoc)-OH residue was coupled to H-Lys(Fmoc). Then, the N^{ε}-Fmoc protecting group of the lysine residue was removed, and *N*-Fmoc[*N*′-Fmoc-2-aminoethyl]Gly-OH was linked to the ε-amino group. The coupling was performed in DMF using 2 equivalents of *N*-Fmoc [*N*′-Fmoc-2-aminoethyl]Gly-OH (0.117 g) activated with HBTU and HOBt. After the assembly was complete, the Fmoc groups were removed and K_2PtCl_4 0.05 M in NMP/H_2O (9 : 1 v/v, 10 ml) was added and leaved under stirring for 24 h. The resin was filtered and washed with H_2O and NMP. Upon cleavage of the Dde protecting group, 308 mg Fmoc-Peg_{1500}-OH (2 equivalents) was added and activated by HOBt and HBTU in DMF. After 1 h, the resin was washed with DMF, DCM and treated with TFA/H_2O (95 : 5 v/v, 2.5 ml) for 1.5 h. After filtration, the resin was washed with TFA, and the combined TFA filtrates was precipitated with Et_2O in a cold bath at 0 °C. The product was recollected in good yield and used without further purification.

ESI MS (positive ions) peaks: $[M + 3H]^{3+}/3 = 843$ a.m.u.. (calculated 2529 a.m.u.), $[M - 2Cl]^{2+}/2 = 1230$ a.m.u. (calculated 2460 a.m.u.), $[M - 2Cl + 2H_2O]^{2+}/2 = 1248$ a.m.u. (calculated 2496 a.m.u.), $[M - Pt - 2Cl + 2H]^{2+}/2 = 1132$ a.m.u. (calculated 2260 a.m.u.).

^1H NMR (CD_3OD) δ: 3.78 (m, 2H, α Lys). 3.2, 3.31 (m, 4H, ε Lys), 3.00 (s, 27H, OCH_3), 2.75 (m, 2H, CH_2N), 2.53 (m, 2H, CH_2N), 2.49 (m, 2H, CH_2NH_2), 2.39 (m, 4H, $COCH_2$), 1.62 (m, 2H, δ Lys), 1.53 (m, 4H, γ Lys), 1.44 (m, 4H, δ Lys), 1.34 (m, 4H, β LH_2 Lys), 1.30 (m, 64H,) 0.90 (t, 6H)

ICP measurements: ICP (calc): 1555 ppb (3900 ppb). Yield: 40%.

Liposome Preparation

Self assembling and mixed aggregates at 90/10 M ratio were prepared by dissolving the components in a small volume (2.0 ml) of 50/50 methanol/chloroform mixture. After removing solvent under N_2 flow, the lipidic film was hydrated in 0.1 M phosphate buffer at pH 7.4 and physiological ionic strength conditions (NaCl 0.9 wt%), sonicated for 30 min and extruded 10 times through a 0.1 μm carbonate membrane. Water volume used allows obtaining $2{\cdot}10^{-3}$ M liposome solutions. Peptide concentration in mixed aggregates was determined by absorbance on a UV–vis Jasco V-5505 spectrophotometer (Easton, MD, USA) equipped with a Jasco ETC-505T Peltier temperature controller with a 1-cm quartz cuvette using a molar absorptivity (ε_{280}) of 5630 M^{-1} cm^{-1} for octreotide, because of the contribution of tryptophan residue present in the primary octreotide structure.

Fluorescence Measurements

Emission spectrum was recorded using a Jasco Model FP-750 spectrofluorometer (Jasco Easton, MD, USA) equipped with a Peltier temperature controller in 1.0 cm path length quartz cell at 25 °C. Equal excitation and emission bandwidths (5 nm) were used throughout experiments, with a recording speed of 125 nm min^{-1} and automatic selection of the time constant. The tryptophan emission spectrum in 290–450 nm range was obtained exciting at 280 nm liposome solution.

DLS Measurements

DLS investigations were performed at (25.00 ± 0.05) °C with a home assembled instrument composed by a Photocor compact goniometer, a 50 mW light source operating at 532.5 nm and a PMT correlator purchased from *Correlator.com*. Temperature was kept constant through the use of a thermostat bath.

In all the experiments, the intensity autocorrelation function has been measured and, through a variation of CONTIN algorithm, the diffusion coefficient distribution function has been obtained at various angles, from 30 to 90°. Finally, the *z*-average of the diffusion coefficient *D* has been obtained from extrapolation of diffusion data [46]:

$$D = \lim_{q \to 0} D(q) \tag{3}$$

where $q = 4\pi n_0/\lambda \sin(\theta/2)$ is the modulus of the scattering vector, n_0 is the refractive index of the solution, λ is the incident wavelength and θ represents the scattering angle.

SANS Measurements

SANS measurements have been performed at 25 °C with the KWS2 instrument located at the Heinz Maier-Leibnitz neutron source of the Jülich Centre for Neutron Science (Garching, Germany). Neutrons with an average wavelength of 0.7 and 1,9 nm and a wavelength spread $\Delta\lambda/\lambda \leq 0.2$ were used. A two-dimensional 128×128 array scintillation detector at two different collimation (C)/sample-to-detector(D) distances (C_8D_8 and C_8D_2, with all the lengths expressed in m) measured neutrons scattered from the samples. These configurations allowed collecting data in a range of the scattering vector modulus $q = 4\pi/\lambda \sin(\theta/2)$ located between 0.0002 and 0.025 nm^{-1}. The obtained raw data were then corrected for background and empty cell scattering. Detector efficiency corrections, radial average and transformation to absolute scattering cross-sections were made with a secondary plexiglas standard [47,48].

Doxorubicin Loading

$(C18)_2$-PKAG-Pt/$(C18)_2$-L5Oct/Doxo liposomal formulation was prepared by remote loading using the pH gradient method. Briefly, $2 \cdot 10^{-3}$ M liposomal solution was prepared as reported earlier at pH 4.0 by using 0.1 M citrate-phosphate buffer. Then, pH was adjusted from 4.0 to pH 7.4 adding dropwise a stock solution of 1.0 M NaOH, and 100 µl of $2.1 \cdot 10^{-3}$ M doxorubicin solution was added to 1.0 ml of liposomal solution. This suspension was stirred for 1 h at 60 °C. Doxorubicin concentration, in all experiments, was determined by spectroscopic measurements (UV–vis) using calibration curves obtained by measuring absorbance at 480 nm. Subsequently, unloaded doxorubicin was removed by using a Sephadex G50 (Amersham Pharmacia Biotech Inc (Piscataway, NJ USA)) column pre-equilibrated 0.1 M phosphate buffer at pH 7.4. The DLC (defined as the weight ratio of the encapsulated doxorubicin vs the amount of the amphiphilic moieties) was quantified for subtraction of the amount of doxorubicin removed from the total amount of doxorubicin loaded.

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