J Incl Phenom Macrocycl Chem (2007) 57:567–572
DOI 10.1007/s10847-006-9250-7

ORIGINAL ARTICLE

A versatile liposome/cyclodextrin supramolecular carrier for drug delivery through the blood-brain barrier

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Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 19 January 2007

Abstract For many commercial drugs, reaching the central nervous system in large amount without damaging the blood-brain-barrier (BBB) remains a challenging task. We present here a supramolecular strategy aiming at using a well-defined cyclodextrin-coated liposomes as drug carrier and adamantoyl saccharides as BBB-interacting ligands. In this study, the liposome is constituted of *n*-alkyldimethylammoniumcyclodextrins incorporated in the lipid bilayer of a 3/7 cholesterol/dipalmitoylphosphatidylcholine mixture and the ligand is constituted of an adamantoyl-glucose molecule whose adamantoyl moiety can be included in the CD cavity. The whole supramolecular assembly has been characterized by light-scattering and ^{31}P NMR measurements. Toxicity and permeability studies on an in vitro model of the BBB clearly demonstrated a 5-fold improved ability of the modified liposome to enter the BBB-endothelial cells compared to the non-coated liposome. Fluorescence labelling of these liposomes is also displayed with DiI as a fluorescent probe.

Keywords Blood-brain-barrier · Cyclodextrin · Drug delivery · In vitro model · Liposome · Saccharide · Supramolecular chemistry

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Abbreviations

DPPC	Dipalmitoylphosphatidylcholine
BBB	Blood-brain barrier
DMA-C_n-CD	Mono–(*N*-*n*-alkyl,*N*,*N*-dimethylamino)-*β*-cyclodextrin
DiI	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

Introduction

Increasing the delivery of drugs through the blood-brain barrier (BBB) currently constitutes the only effective solution to the problem of neurodegenerative diseases (Alzheimer, Parkinson...). However, this strategy comes up against a number of difficulties. Actually, the BBB is made-up of endothelial cells tightly joined by numerous transmembrane and intracytoplasmic proteins such as occludin and actin which prevents the brain uptake of >98% of all potential neurotherapeutics [1]. Moreover, contrary to what was usually observed for the endothelium of other cells, the number of transport vesicles is quite low in the cerebral capillaries. Their scarceness and the absence of fenestration also contribute to restrict the passage of molecules through the cerebral endothelium. In addition to these structural properties, other metabolic characteristics participating in the exchanges between the blood and the brain also need to be considered. As an example, the presence of Multi-Drug-Resistance-type proteins results in enhanced efflux of exogenous molecules. Among these proteins, the P-glycoprotein (P-gp) has been clearly identified as an

efflux pump, hunting out the cytoplasm many anticancer drugs back to the blood stream [2, 3]. However, the brain endothelial cells are fortunately not totally impermeable, allowing nutritious elements, such as amino-acids and glucose, or larger proteins, like transferrin or insulin, to reach the central nervous system.

Knowing that the passage of molecules was low but possible, methods have been developed to favour the transport of drugs through the BBB. Physical methods consisted in injecting directly the drug in the brain but they may cause adverse reactions and harm. Moreover, the highly invasive character of these treatments requires the patient to be followed through the healthcare system. The methods consisting in injecting hypertonic solutions of mannitol in the carotid artery are even more invasive and trigger the fragilisation of the BBB [4] Consequently, in addition to the therapeutic drugs, blood borne molecules that are dangerous to the nervous system may also enter the brain. In this context, soft pharmacological or physiological methods have been developed to improve therapeutic outcomes and reduce adverse consequences. These techniques are based on the use of the natural transport mode to reach the brain, that is to say the blood. By this way, small lipophilic molecules with low molecular weight (400–600 Da) have proved to cross the cerebral endothelium by passive diffusion, even though the percentage remains relatively low. By contrast, lipophilic molecules with high molecular weight are unable to reach the BBB due to their insolubility in the blood and their weak affinity for the cellular sheet of the cerebral endothelium. In order to help them crossing the BBB, molecular shuttles have been designed. These synthetic vectors are nanoparticles or liposomes composed of a hydrophilic shell under which the drug is encapsulated in large amount [5, 6]. To be specifically recognised by the endothelial cell receptors, the shell is often modified by specific ligands covalently grafted on the nanoparticles surface or included in the liposome lipid bilayer [7]. Nevertheless, the fast screening of the efficacy of various ligands on the recognition process and their invagination through the cells is not possible up to now, since nanoparticles or liposomes have to be fully characterised (size, stability, permeability...) when modified by a new ligand.

We recently develop a supramolecular strategy to evaluate the impact of saccharidic ligands on the transport of cyclodextrin-modified liposomes through the BBB. More precisely, the aim is to elaborate a supramolecular edifice constituted of a dipalmitoylphosphatidylcholine (DPPC)-cholesterol liposome coated by amphiphilic cyclodextrins (CDs) incorporated in the liposome bilayer. Once the object well characterized, adamantoylated saccharides may be included in the CD cavity without changing the physicochemical properties of the inner core of the liposomes. The recognition process between the adamantoylated saccharide and the CDs is rendered possible by the good affinity between the adamantoyl moiety and the CD cavity. The strategy is summarised on Scheme 1.

The elaboration of the building blocks of the above supramolecular edifice has been the subject of previous papers. First, we synthesised adamantoyl-saccharide compounds to evaluate their strength of interaction with native and modified CDs [8]. Then, we developed the synthesis of long-alkyl chain ammonium-β-CDs (DMA-C_n-CD with $2 < n < 16$) and showed that the chain was included in the CD cavity. We studied their behaviour in the presence of adamantoylated derivatives and we observed that the adamantoyl group expelled the alkyl chain from the cavity [9]. Recently, using an in vitro model of the BBB, we demonstrated that the longer the DMA-C_n-CD alkyl chain, the lower the toxicity on the BBB [10]. Indeed, we showed that once the CD cavity occupied by the long dodecyl chain, DMA-C_{12}-CD were unable to extract phospholipids and/or cholesterol from the lipid bilayer of the endothelial cells and the integrity of the endothelial cellular sheet was preserved. This paper deals with the last part of our study on coated liposomes since we describe here the elaboration and characterisation of the whole supramolecular structure. The ability of the obtained shuttle to pass through the BBB is also commented.

Experimental

Chemicals and antibodies

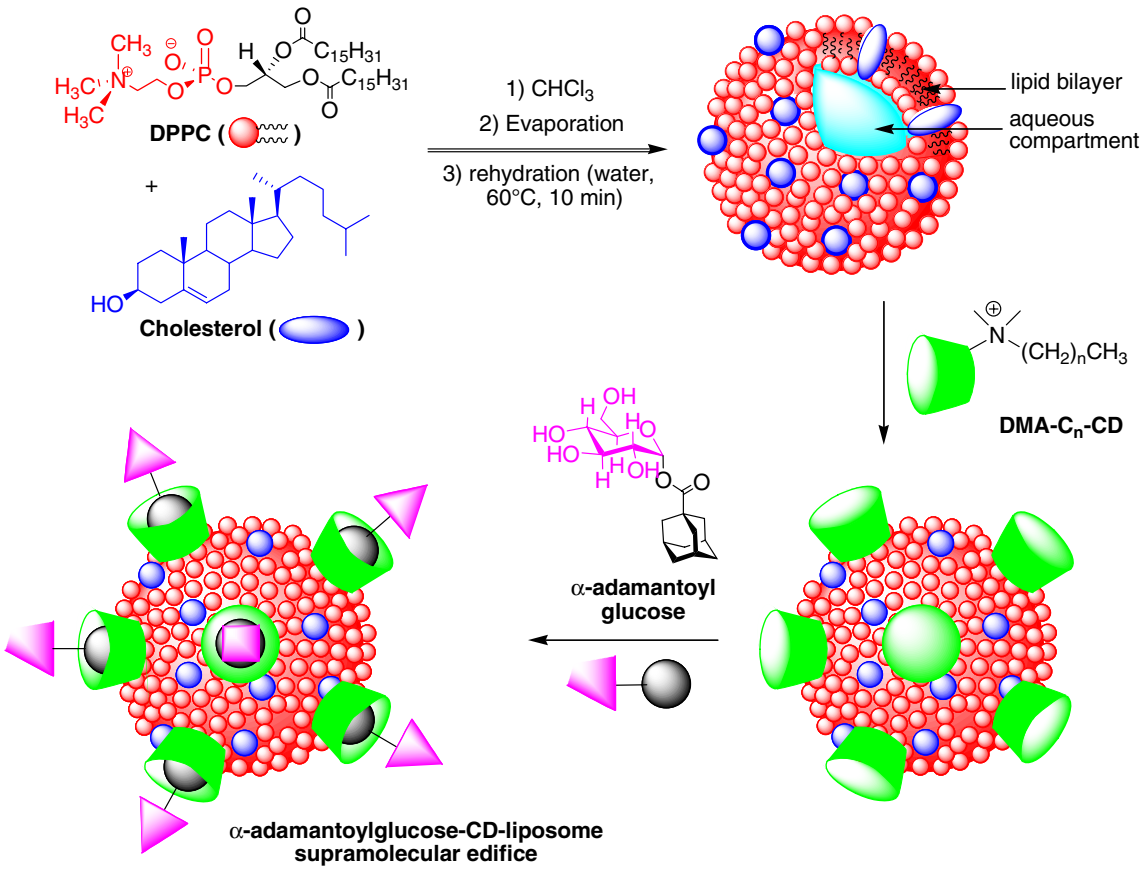
DPPC was purchased from Lipoid (Ludwigshafen, Germany). Cholesterol and DiI were purchased from Sigma-Aldrich and the Hoescht reagent from MP Biomedical. [^{14}C]saccharose (58 mCi/mmol) were obtained from Amersham Biosciences Inc. (Piscataway, NJ).

Elaboration of the DPPC/cholesterol liposome

The liposomes have been elaborated according to a procedure of the literature [11].

NMR

^{31}P NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer at 298 K.



Scheme 1 Elaboration of the supramolecular liposome/DMA-C_n-CD/α-adamantoylglucose assembly

Light scattering measurements

The size distribution of the liposomes has been established with a Coulter® N4 Plus nanosizer (Beckman Coulter).

In vitro model

The in vitro model used for this study has already been widely described previously [12].

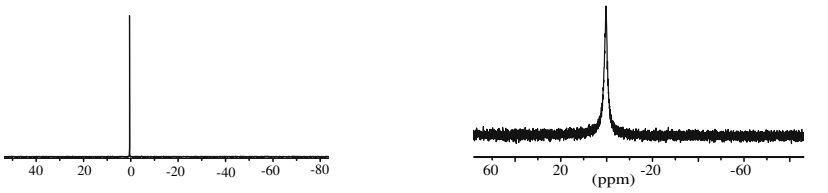
Fluorescence microscopy

To visualize DiI, BCECs were fixed as described previously [13] and photographed with a Leica fluorescence microscope (Leica, Wetzlar, Germany).

Results and discussion

Liposomes have been first described by Bangham et al. in 1965 [11]. Since then, many procedures have been developed to obtain specifically Multi Lamellar Vesicles (MLV), Large Unilamellar Vesicles (LUV), Small Unilamellar Vesiles (SUV) or others [14]. As it has previously been shown that nanoparticles with a diameter of about 100 nm were required to cross the BBB, we have considered SUV as model structures. Indeed, their diameter could be easily controlled using the following procedure: once the lipids (cholesterol and DPPC) dissolved in chloroform, the solvent was removed by evaporation and the remaining lipidic film was rehydrated with water at 60 °C (over the transition temperature of DPPC (40 °C)). The MLV-containing solution was then sonicated several times and extruded on a 0.1 μm filter until SUV with the appropriate diameter were obtained. Light scattering measurements proved the sample to be rather homogeneous in size since the liposome diameter was between 45 and 115 nm with the average population at 70 nm. The broadening of the ^{31}P NMR signal of DPPC was characteristic of its incorporation in the liposome bilayer (Fig. 1).

In a second step, DMA-C_n-CD (n = 14 or 16) have been added to the DPPC/cholesterol liposome solution. For DMA-C_n-CD/lipids molar ratios of 0.4 and 0.8, no modification of the ^{31}P NMR signal of DPPC were observed, indicating that the integrity of the



Free DPPC

Non-coated liposomes ([lipids]=15mM) in ultra pure water (T=25°C)

Fig. 1 ^{31}P NMR signal of the DPPC phosphorus atom depending on its chemical environment

liposome bilayer was maintained in these conditions. Moreover, an increase in the size and polydispersity of the liposomes was measured by light scattering. These observations were consistent with an incorporation of DMA-C_n-CD in the lipid bilayer. As a matter of fact, the disappearance of the DMA-C_n-CD aggregates was accompanied by an increase in the size of the liposomes as clearly shown in Fig. 2.

Indeed, when DMA-C_n-CD was not incorporated in the liposome, the alkyl chain remained included in the CD cavity, preventing the interaction of the cavity with lipids [10]. On the contrary, in the presence of liposomes, DMA-C_n-CD deployed its alkyl chain in the hydrophobic shell of the liposome.

Third, a α-adamantylglucose solution in a stoechiometric ratio in respect to DMA-C_n-CD was poured on the CD-coated liposome solution. As previously demonstrated in our previous paper [8], the inclusion of the adamantoyl moiety in the CD cavity was very strong and supramolecular DMA-C_n-CD/α-adamantoylglucose complexes were thus formed at the surface of the liposome. As observed above for DMA-C_n-CD alone, no modification of the ^{31}P NMR resonance of the phosphorus atom of DPPC was observed. Actually, a broad symmetric peak was detected. Light scattering measurements clearly established the decrease in the liposome population at 75 nm and the increase in the population at 115 and 150 nm (Fig. 2) because of the formation of the supramolecular complexes on the liposomes. No new nanoparticules were detected as logically confirmed with a pure solution of α-adamantoylglucose.

In the last part of the study, the behaviour of the liposomes coated by DMA-C_n-CD/α-adamantoylglucose complexes was evaluated on an in vitro model of the BBB. In particular, a special attention has been devoted to the variation of toxicity and permeability through the BBB induced by the glucose-coated liposomes. The in vitro model of the BBB consisted on two compartments separated by a filter coated on the upper side with brain capillary endothelial cells at a concentration of 4×10^5 cells/mL (Scheme 2) [12]. The liposomes solution was poured on the upper compartment. In order to detect the liposomes in the cell or in the lower compartment of the model, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) has been used as a fluorescent probe and was incorporated in the lipid bilayer of the liposome (1mol% in respect to the lipids) [15]. Four liposomes solutions have been prepared at two different concentrations of 100 and 200 μg/mL with or without 1:1 DMA-C_{16}-CD/α-adamantoylglucose complexes. Whatever the solutions, the liposomes remained stable in physiologic salt concentrations as checked by fluorescence measurements. These solutions have been poured on the upper compartment of the BBB model and the toxicity of the modified liposomes on the endothelial cells has been measured. The toxicity of the liposome solution was evaluated as follows: the integrity of the brain endothelial cell monolayer during exposure to CDs was checked by determination of the endothelial permeability coefficient (P_e) of [^{14}C] saccharose across the BBB [16]. A P_e higher than 1×10^{-3} cm min^{-1} was indicative of a leaky BBB. Whatever the concentration (100 or 200 μg/mL), the liposomes remained non-toxic towards the BBB. Actually, the

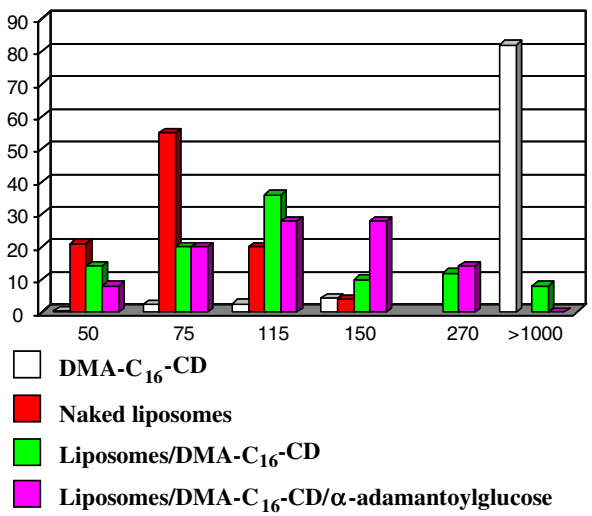


Fig. 2 Distribution of DMA-C_{16}-CD, naked and coated liposomes

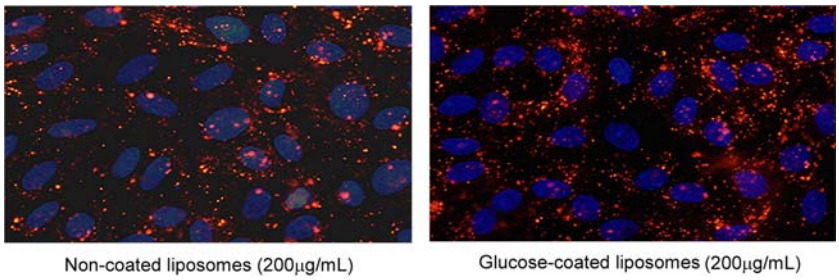
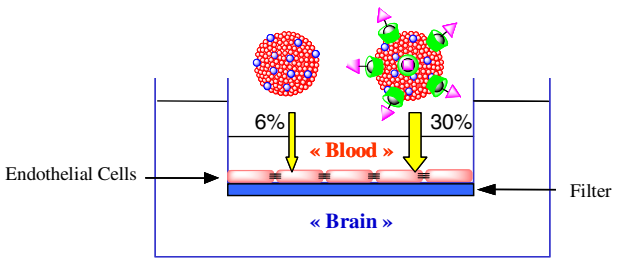


Fig. 3 Fluorometry microscopy of endothelial cells after 4 h incubation in the presence of naked liposomes (on the left) and liposomes/DMA-C_{16}-CD/α-adamantoylglucose (on the right)



Scheme 2 Percentage of incorporation of naked (6%) and coated liposomes (30%) in the endothelial cells of the in vitro of the BBB

P_e was below the toxicity threshold (0.70×10^{-3} cm/min or the 100 μg/mL solution and 0.63×10^{-3} cm/min for the 200 mg/μL solution). The BBB integrity was preserved in these conditions. Knowing that the liposomes solutions were not toxic, we then evaluated their permeability through the BBB. After 2 and 4 h of incubation, the lower compartment has been analysed by fluorometry. No fluorescence was detected suggesting that the liposomes were unable to cross the BBB. However, the analysis of the upper compartment for the four liposomes solutions revealed that the fluorescences after 4 h were below those measured at the beginning of the experiments. For the non-coated liposomes-containing solutions, the fluorescence were similar to those initially measured (94% for the 100 or 200 μg/mL liposomes solutions), showing that the absence of ligand on the liposome surface was prejudicial to the recognition process of the liposome by the BBB receptors, as already demonstrated by others [17]. On the contrary, when the liposomes were coated with DMA-C_n-CD/α-adamantoylglucose complexes, the results were quite different since a decrease in the fluorescence was measured (70% and 76% for the 100 or 200 μg/mL liposomes solutions respectively), revealing a better interaction of the saccharide-coated liposomes with the endothelial cells.

To determine the fluorescence distribution, the cells have been analysed after the experiments by fluorescence microscopy. In addition to DiI, another fluorescent probe (Hoescht reagent) has been used to identify the position of the cells nuclei. The two photos of Fig. 3 are relative to endothelial cells after 4 h incubation with naked liposomes (200 μg/mL) (on the left) and with liposomes/DMA-C_n-CD/α-adamantoylglucose supramolecular edifices (200 μg/mL) (on the right). In both cases, DiI was detected around the nuclei. This observation is characteristic of a degradation way (via lysosomes) and thus indicates that the liposomes, coated or not, take a non-specific path to reach the cytoplasm.

Conclusion

The proof of concept of the liposome/CD/adamantoylsaccharide supramolecular strategy for the crossing of the BBB has been clearly established since an increase in fluorescence inside the cytoplasm of the endothelial cells was measured with the supramolecularly saccharide-coated liposomes (30% vs. 6% for the naked liposome). The main advantage of the system we developed lies in its versatility to study the mechanism of transport through the BBB. Actually, once characterised, the DPPC/cholesterol liposomes could be easily modified on their surface by various ligands (not only saccharides) without altering the stability, the size or permeability of the liposomes. A systematic study is currently under investigation to determine the efficacy of the recognition process with other saccharide- and oligosaccharide-adamantoylated derivatives and the ability of the obtained coated liposomes to cross the BBB.

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