International Journal of Pharmaceutics xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Interfacial behavior of PEGylated lipids and their effect on the stability of squalenoyl-drug nanoassemblies

Véronique Rosilio [a,b,*], Fawzia Bekkara-Aounallah [a,b,c], Anshuman Ambike [a,b], Ruxandra Gref [a,b], Patrick Couvreur [a,b]

[a] Univ Paris-Sud, IFR 141, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, Châtenay-Malabry Cedex 92296, France
[b] CNRS, UMR 8612, Institut Galien Paris-Sud, Châtenay-Malabry, France
[c] Université Djillali Liabès de Sidi Bel Abbès, Faculté des Sciences, LCOPM, BP 89, Sidi Bel Abbès 22000, Algeria

ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form 29 April 2014
Accepted 30 April 2014
Available online xxx

Keywords:
Nanoassembly
Squalenoyl-gemcitabine
Squalenoyl-dideoxycytidine
Squalenic acid
Surface tension
Lipopolymer

ABSTRACT

Squalenoyl-gemcitabine (Sq-Gem) and squalenoyl-deoxycytidine (Sq-ddC) are amphiphilic prodrugs that self-assemble in water to form nanoassemblies (NAs) with well-defined structures and size. However, like other drug nanocarriers, these nanoassemblies are rapidly cleared from the blood stream by the reticulo-endothelial system. By adding squalenoyl-PEG (Sq-PEG) or cholesterol-PEG (Chol-PEG) to the squalenoyl prodrugs, composite nanoassemblies (CNAs) were formed, with different sizes and structures. The effect of the PEG-lipids on the formation and stability of these nanoassemblies was assessed by transmission electron microscopy, quasi-elastic light scattering and surface tension measurements in various conditions. The results revealed different stabilities with time for Sq-ddC and Sq-Gem nanoassemblies in aqueous medium, the latter being much less stable than the former. They also demonstrated that the presence of Sq-PEG or Chol-PEG in composite Sq-ddC nanoassemblies contributed to their rapid destabilization. The analysis of the adsorption kinetics of Sq-PEG into a prodrug monolayer below and above its critical aggregation concentration allowed getting a better insight into prodrug-lipopolymer molecular interactions, and their consequences on the formation of composite prodrug nanoassemblies.

1. Introduction

Biocompatible nanostructures formed by self-assembling of amphiphilic prodrugs show remarkable therapeutic potential as they combine high drug loading, improved drug controlled release, and reduced toxicity compared to common nanocarriers. They are particularly appropriate for drugs with critical limitations like low oral bioavailability, rapid metabolism, short circulation half-life, induced resistance and severe side-effects. Many systems have been developed over the last decade, such as long-chain alkyl or cholesteryl conjugates of nucleoside anticancer and antiviral agents forming colloidal dispersions (Jin et al., 2006, 2009, 2012b). In 2006, Couvreur and co-workers have proposed an original strategy to increase the therapeutic index of nucleoside analogues by coupling them to the acyclic isoprenoid chain of squalene (Sq). Squalene is a natural nontoxic lipid, widely distributed in the nature and precursor in cholesterol biosynthesis (Desmaële et al., 2012; Dosio et al., 2013; Reddy and Couvreur, 2009). Remarkably, coupling squalene to nucleosides analogues led to amphiphilic prodrugs, which self-organized in water as nanoassemblies (NAs) of 100–300 nm in diameter, irrespective of the nucleoside analogue used and the location of the covalent linkage. This has been assigned to the highly coiled and compact conformation of the squalene moiety in water (Cattel et al., 1992). This strategy has been first successfully applied to gemcitabine, a pyrimidine nucleoside analogue used in chemotherapy to treat non small-cell lung cancer, pancreatic, bladder, ovarian and breast cancers (Eli Lilly Co., 1997; Sandler and Ettinger, 1999). The squalenoyl gemcitabine nanoassemblies (Sq-Gem) were found to exhibit superior anticancer activity *in vitro* in human cancer cells and in gemcitabine-resistant murine leukemia cells, as well as *in vivo* in experimental leukemia both after intravenous and oral administration (Couvreur et al., 2006; Reddy et al., 2007, 2008a, 2008b). The squalenoylation of other antiretroviral nucleosides (including 2′,3′-dideoxycytidine, ddC) also led to more potent drugs when tested in primary cultures of HIV-infected lymphocytes (Hillaireau et al., 2013). High-resolution synchrotron X-ray diffraction and electron microscopy studies revealed that whereas Sq-Gem molecules were packed in inverse hexagonal phases (Couvreur

* Corresponding author at: Institut Galien Paris-Sud, UMR CNRS 8612, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296, Châtenay-Malabry Cedex, France. Tel.: +33 1 46 83 4518; fax: +33 1 46 83 5312.
E-mail address: veronique.rosilio@u-psud.fr (V. Rosilio).

http://dx.doi.org/10.1016/j.ijpharm.2014.04.072
0378-5173/

et al., 2008), Sq-ddC molecules organized in an inverse bicontinuous cubic phase in equilibrium with excess water (Bekkara-Aounallah et al., 2008).

It has been well established in earlier studies that drug-loaded nanocarriers are rapidly removed from the blood stream by the reticulo-endothelial system, and cannot reach their target (for example a tumor). A preferred strategy to dramatically increase their blood half-lives is the covalent coupling of poly(ethylene glycol) (PEG) chains (PEGylation) at their surface (Gref et al., 1994; Needham and Kim, 2000; Peracchia et al., 1998). For Sq-ddC, PEGylation could seem unnecessary since macrophages are the main HIV reservoirs (Aquaro et al., 2002; Jin et al., 2009). However, it has been shown that HIV reservoirs located in the brain are particularly difficult to eradicate, because it necessitates passage of the nanocarriers across the blood-brain barrier (BBB) (Lafeuillade and Stevenson, 2011; Namanja et al., 2012; Thompson et al., 2011). Crossing BBB was shown to be possible by coating the nanocarriers with PEG shells and appropriate ligands (Huang et al., 2013; Lu et al., 2014,; Zhang et al., 2014). PEGylation may also favor stabilization of nanoparticles intended for oral delivery (Beloqui et al., 2013; Hillaireau et al., 2013; Shen et al., 2013; Yoncheva et al., 2005). Pioneering studies have shown that PEGylation of Sq-ddC and Sq-Gem nanoassemblies was possible. Composite NAs (CNAs) were formed by co-nanoprecipitation using prodrugs mixed with either cholesterol-PEG (Chol-PEG) or squalene-PEG (Sq-PEG), in the absence of any other surfactant. The preparation procedure was successful, as in all cases monodisperse colloidal suspensions of 80 upto 200 nm in diameter CNAs were obtained without aggregates, whatever the weight ratio (from 1:0.002 to 1:0.7) between the squalenoyl nucleoside analogue and the PEG-lipid (Bekkara-Aounallah et al., 2008). The higher the PEG ratio was, the smaller the size of the resulting nanoassemblies. Similarly, Duhem et al. (2014) have recently reported that the addition of $TPGS_{2000}$ to *N*-doxorubicin-α-D-tocopherol succinate significantly reduced the size of prodrug aggregates.

Many questions remain to fully understand the formation of the CNAs and their supramolecular organization, which are crucial for their use in biomedical applications. It is anticipated that the PEG coating, responsible for the increased *in vivo* half-lives, establishes during NAs formation at the interface between the organic droplets and the aqueous phase. However, when preparing CNAs, there might be competition at the interface between Sq-ddC and Chol-PEG or Sq-PEG molecules. Physico-chemical studies at the interfaces would, therefore, be decisive for a better understanding of the CNAs formation. Besides, crucial information could be obtained on the stability of the resulting CNAs.

The present study shows new data on the interfacial behavior of Sq-ddC, Sq-Gem and Chol-PEG or Sq-PEG mixtures, as well as on the stability of composite nanoassemblies. We have first determined the critical aggregation concentration of Chol-PEG and Sq-PEG, studied the interfacial behavior of prodrug nanoassemblies and composite PEG/prodrug nanoassemblies at the free air/water interface. The analysis of the kinetics of films formation, changes in morphology of nanoassemblies upon addition of the PEGylated lipids, and adsorption of a PEG-lipid into a prodrug monolayer, below and above its critical aggregation concentration allowed to get a better insight into the organization and stability of CNAs in the presence of Sq-PEG and Chol-PEG.

2. Material and methods

2.1. Materials

Zalcitabine (2′,3′-dideoxycytidine, ddC, Mw: 211 g mol^{-1}) and squalene were purchased from Sigma–Aldrich Chemical Co., France. Gemcitabine hydrochloride (Sq-Gem, Mw: 299.66 g mol^{-1}) was provided by Sequoia Research Products (UK). The squalenoyl nucleoside analogues Sq-ddC (593.9 g mol^{-1}) and Sq-Gem (645.82 g mol^{-1}) were synthesized by covalent coupling with 1,1′,2-tris-nor-squalenic acid (Sq-Ac) onto the amino group of the nucleosides heterocycle as previously described (Couvreur et al., 2006). Squalene-poly(ethyleneglycol) (Sq-PEG, 2380 g mol^{-1}) was synthesized and characterized as reported earlier (Bekkara-Aounallah et al., 2008). Cholesterol-poly(ethyleneglycol) (Chol-PEG) (1982 g mol^{-1}) was supplied from NOF Europe (Belgium). Ultrapure water (γ = 72.4 mN m^{-1} at 22 °C) was produced by a Millipore Synergy 185 apparatus coupled with a RiOs5, with a resistivity of 18.2 MΩ/cm. Prior to experiments, glassware was cleaned in a TFD4 solution (Franklab) and abundantly rinsed with ultrapure water.

3. Methods

3.1. Preparation of the nanoassemblies

Sq-Gem and Sq-ddC nanoassemblies (1.55 mM and 1.68 mM, respectively) were prepared by nanoprecipitation. Briefly, a prodrug was dissolved in acetone (10 mg mL^{-1}) and added dropwise under stirring (500 rpm) into 2 mL water. Composite nanoassemblies (CNAs) were prepared by co-nanoprecipitation of a prodrug with either Chol-PEG or Sq-PEG. Precipitation of nanoassemblies occurred spontaneously at room temperature. After solvent evaporation under reduced pressure, an aqueous suspension of nanoassemblies was obtained. Different Chol-PEG or Sq-PEG molar ratios were tested, ranging from 0.05% to 21%. Squalenic acid (Sq-Ac) could also self-aggregate and form nanoassemblies, but x-ray diffraction analysis showed none of the structures observed for Sq-Gem or Sq-ddC nanoparticles. Apparently, the Sq-Ac nanoassemblies were not organized enough to produce a diffraction spectrum. They also showed poor stability with time.

For all experiments, the composite nanoassemblies were freshly prepared and used within 24 h (conservation at 4 °C). Their size and polydispersity index (PDI) were measured immediately after preparation, by quasi-elastic light scattering (QELS) (Zetasizer 4, Malvern Instruments Ltd., UK). The selected angle was 90° and the measurement was made using a 50 μg mL^{-1} concentration in MilliQ® water.

The morphology of nanoassemblies and composite nanoassemblies was visualized using transmission electron microscopy (TEM), directly or after freeze-fracture (FF-TEM). For direct TEM observations, 5 μL of aqueous nanoassemblies suspensions were deposited onto a 600 mesh copper grid coated with a very thin carbon film. Grids were washed with 5 μL of aqueous 2% (w/v) uranyl acetate, dried and observed in annular dark-field, in a LEO-Zeiss 902 electron microscope. For FF-TEM observations, 1 mL of nanoassemblies suspension (1 mg mL^{-1}) was incubated with glycerol (30% v/v), used as a cryoprotectant. A drop of each sample was placed on a copper support, immediately frozen in liquid propane, and then kept in liquid nitrogen. Fracturing and shadowing were performed in a Balzers BAF 400 freeze-etching unit. The replicas were washed in THF and distilled water, and placed on copper grids. Observations were made under a TEM JEOL 100SX.

3.2. Surface tension measurements

Equilibrium surface tensions γ of the Chol-PEG and Sq-PEG solutions were measured at 22 ± 1 °C by the Wilhelmy plate method using a digital tensiometer (K10ST, Krüss, Germany). The surface tension of NAs and CNAs was measured with time, without detaching the plate from the interface. The thermostated measurement cells were enclosed in a chamber under saturated vapor pressure to limit water evaporation during the experiments

Please cite this article in press as: Rosilio, V., et al., Interfacial behavior of PEGylated lipids and their effect on the stability of squalenoyl-drug nanoassemblies, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.04.072