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Research paper

Novel polyglycidol-lipid conjugates create a stabilizing hydrogen-bonded layer around cholesterol-containing dipalmitoyl phosphatidylcholine liposomes

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ABSTRACT

Hybrid liposomes resulting from co-assembly of dipalmitoylphosphatidylcholine and polyglycidol-derivatized lipids were prepared. The latter were composed of a lipid-mimetic residue to which a linear polyglycidol chain (degree of polymerization, DP, in the 23–110 range) was conjugated. Formulations with varying copolymer type and content were prepared by film hydration technique followed by extrusion. The hybrid structures were studied by means of dynamic and electrophoretic light scattering, cryogenic transmission electron microscopy, and fluorescence spectroscopy. Cytotoxicity towards OPM-2 (multiple myeloma) and EJ (human urinary bladder carcinoma) cell lines was assessed as well. Predominantly unilamellar liposomes with mean hydrodynamic diameters in the 113–134 nm range and neutral to slightly negative surface potential were prepared. The integrity of liposomes containing copolymers with DP of the polyglycidol chain 23 and 30 was preserved at copolymer contents up to 10 mol %. Bilayer disks were observed at somewhat lower contents of the copolymers of the highest DP of the polyglycidol chain. The hybrid structures were less leaky than the plain liposomes, which was attributed to formation of a strongly hydrogen-bonded polyglycidol layer around the bilayer membrane. They exhibited low toxicological potential, favorable physicochemical characteristics, and ability to act as containers for sustained release.

1. Introduction

Nanotechnology promises new medical therapies, precise, sensitive and rapid diagnostic tools as well as new materials for tissue engineering. It presents potential opportunities to create medicines that would be less harmful towards normal tissues and more effective towards diseased tissues by using advanced drug delivery systems. The latter can be hybrid nanoparticles and chimeric nanosystems [1–3] resulting from co-assembly of, for instance, an amphiphilic copolymer with other substances such as surfactants, lipids, (co)polymers, proteins, oligo- and polynucleotides. It is remarkable that the introduction of an additional entity even in small amounts in the hybrid nanostructures can be manifested in significant alteration of the aggregate morphology and characteristics and can produce new functionality and properties [4]. The sterically stabilized (*Stealth*) liposomes are such hybrid structures that are composed of a self-closed lipid bilayer, enclosing part of the surrounding media into their interior, which is surface modified by polymer-derivatized lipids. The lipid residue is anchored in the bilayer membrane, whereas the polymer (typically poly(ethylene glycol), PEG) creates a repulsive barrier around the liposomes.

The PEG-lipid is introduced in quantities of less than 10 mol% (typically 4–6 mol%), which is sufficient to confer colloidal and *in vivo* stability to the liposomes, to reduce the opsonization (i.e., adsorption of marker macromolecules on the liposomal surface) as well as the uptake of the marked species by the macrophages, which results in prolongation of circulation time [5,6]. The approach to improve the performance and prolong the blood

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circulation time by creating sterically stabilized liposomes is not free of drawbacks and disadvantages. At certain critical content, the PEG-lipids induce transition from bilayers to micellar phase [7–10], which may occur via intermediate structures such as open bilayer disks. Such structures are useless as far as encapsulation and delivery of water-soluble active substances are concerned. Furthermore, the PEG-lipids, even in small amounts, may give rise to rapid leakage of entrapped water-soluble substances due to spontaneous formation and stabilization of transient holes in the bilayer [11]. In addition, many reports have emphasized that repeated administration of PEG-coated liposomes causes an unexpected immunogenic response, known as "accelerated blood clearance (ABC) phenomenon," which results in disappearance of the long-circulation characteristics of the liposomes [12–14]. Furthermore, in the case when PEG is conjugated to phosphatidyl ethanolamine a net negative charge is introduced at the bilayer membrane surface, which has been reported to play a key role in complement activation and anaphylotoxin production [15] in addition to substantial alteration of the properties and performance of the liposomes [16,17].

Although the PEG-lipids, which are nowadays commercially available in diversity of lipid anchors and molar masses of the PEG chain, are the most widely studied and extensively used materials to provide *Stealth* properties to liposomes, other polymer-derivatized lipids may offer important advancement in creation of such hybrid structures. PEG is the polymer of choice to be conjugated to a lipid-mimetic residue, whereas polymers such as poly(N-isopropylacrylamide) and poly(2-alkyl-2-oxazoline) have been occasionally used as PEG substitutes [18–23]. A number of polyglycerol-derivatized phospholipids with degree of polycondensation of the polyglycerol chain below 40 have been synthesized [24,25]. The individual derivatives were incorporated into distearoylphosphatidylcholine/cholesterol liposomes in order to achieve long circulation time *in vivo* [24] and to prevent induction of the ABC phenomenon against long-circulating liposomes upon repeated administration [25].

We have recently reported on the synthesis and self-association of a series of novel non-phospholipid polyglycidol conjugates [26]. These completely non-ionic conjugates are composed of a polyglycidol chain of degrees of polymerization (DP) in the 23–110 range attached to a lipid-mimetic residue, consisting of two C_{12} fully saturated hydrocarbon chains covalently linked to a glycerol skeleton via ether linkages. The structural formula and molecular characteristics of the conjugates are presented in Fig. 1 and Table 1, respectively. Linear polyglycidol is a flexible, hydrophilic, and biocompatible polymer with biological tolerability, comparable to or even better than those of PEG, poly(N-vinylpyrrolidone) and other biocompatible polymers [27]. Polyglycidol is structurally similar to PEG: both consist of a polyether backbone, however, unlike PEG, the polyglycidol chain bears a hydroxymethylene group in each repeating monomer unit (Fig. 1), the presence of which gives platform for further functionalization. In this study we investigate the effects of the novel non-phospholipid polyglycidol conjugates on the physicochemical properties, morphology, and membrane integrity of dipalmitoylphosphatidylcholine/cholesterol liposomes. Cytotoxicity assays against OPM-2 (multiple myeloma) and EJ (human urinary bladder carcinoma) cell lines are performed as well.

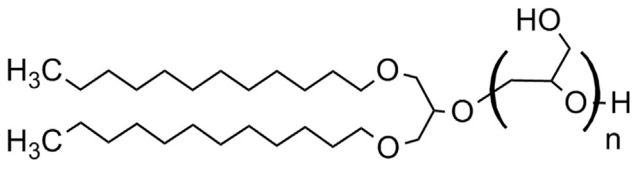

Fig. 1. Chemical structure of the investigated polyglycidol-derivatized lipids, DDP-$(G)_n$. DDP: 1,3-didodecyl/tetradecyloxy-propane-2-ol; G: glycidol monomeric unit; n = 23–110.

Table 1
Composition and nominal molar masses of the investigated polyglycidol-derivatized lipids.

Composition	Nominal molar mass (g mol^{-1})
DDP-$(G)_{23}$	2130
DDP-$(G)_{30}$	2650
DDP-$(G)_{54}$	4420
DDP-$(G)_{110}$	8570

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-phosphocholine (DPPC) (PubChem CID: 452110), cholesterol (CHOL) (PubChem CID: 5997), 5(6)-carboxyfluoresceine (CF), Triton X100 (PubChem CID: 5590), chloroform (PubChem CID: 6212) and methanol (PubChem CID: 887) were purchased from Sigma Aldrich. The synthesis and characterization of the novel non-phospholipid polyglycidol conjugates (hereinafter DDP-$(G)_n$ polymers) are presented in details elsewhere [26]. Fig. 1 and Table 1 present the structural formula and molecular characteristics of DDP-$(G)_n$ polymers, respectively.

2.2. Preparation of liposomes

Chloroform solutions of DPPC and CHOL (2:1 M ratio, 3 mM total lipid concentration) were placed into glass tubes to which a methanol solution of the respective polymer in a defined polymer/lipid molar ratio was added. The solvents were evaporated under a stream of argon and all traces of solvent were removed under vacuum overnight. Phosphate buffer solution (PBS) (pH 7.4) was added to the dry lipid/polymer film and the resulting dispersions were subjected to ten freeze–thaw cycles and then extruded 30 times through polycarbonate filters of pore size 100 nm using a LiposoFast handle type extruder (Avestin Inc., Canada). For the leakage experiments the hydration step was performed with 100 mM buffered solution of CF (pH 7.4). The unentrapped dye was removed by passing through a Sephadex G50 column (Pharmacia, Uppsala, Sweden), equilibrated with PBS buffered saline (pH 7.4).

2.3. Tumor cell lines

The human tumor cell line OPM-2 (multiple myeloma) was supplied by DSMZ GmbH, Germany, whereas EJ cells (human urinary bladder carcinoma) originated from the American Type Cell Culture, USA. The cells were cultured routinely in cell culture flasks with RPMI-1640 liquid medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, housed in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) at 37 °C in 5% CO_2 humidified atmosphere. The cell cultures were maintained in logarithmic growth phase by supplementation with fresh medium twice weekly. EJ cells were used before seventh passage.

2.4. Methods

2.4.1. Dynamic light scattering

The size and size distribution were determined using photon-correlation spectrometer ZetaSizer Nano ZS (Malvern Instruments), equipped with a He–Ne laser (633 nm) and a NIBS