Colloids and Surfaces B: Biointerfaces 116 (2014) 32–40

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Cholesterol-based cationic lipids for gene delivery: Contribution of molecular structure factors to physico-chemical and biological properties

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ARTICLE INFO

Article history:
Received 19 August 2013
Received in revised form 5 December 2013
Accepted 18 December 2013
Available online 30 December 2013

Keywords:
Cholesterol cationic lipids
Structure–activity relationship
Intracellular uptake
Gene delivery

ABSTRACT

In this work, we prepared a series of cholesterol-based cationic (**Cho-cat**) lipids bearing cholesterol hydrophobe, natural amino acid headgroups (lysine/histidine) and linkage (carbonate ester/ether) bonds. In which, the natural amino acid headgroups made dominant contribution to their physico-chemical and biological properties. Among the lipids, the L-lysine headgroup bearing lipids (Cho-es/et-Lys) showed higher pDNA binding affinity and were able to form larger sized and higher surface charged lipoplexes than that of L-histidine headgroup bearing lipids (Cho-es/et-His), they also demonstrated higher transfection efficacy and higher membrane disruption capacities than that of their L-histidine headgroup bearing counterparts. However, compared to the contributions of the headgroups, the (carbonate ester/ether) linkage bonds showed much less affects. Besides, it could be noted that, Cho-es/et-Lys lipids exhibited very high luciferase gene transfection efficiency that almost reached the transfection level of "gold standard" bPEI-25k, made them potential transfection reagents for practical application. Moreover, the results facilitated the understanding for the structure–activity relationship of the cholesterol-based cationic lipids, and also paved a simple and efficient way for achieving high transfection efficiency by modification of suitable headgroups on lipid gene carriers.

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1. Introduction

During the past decades, transfer of therapeutic gene into disordered cells by using non-viral gene carriers, had been achieved great attention in the field of biomedical and material sciences [1]. To date, a number of cationic lipids [2–7], cationic polymers [8–11] and nanoparticles [12,13] were developed as new non-viral gene carriers for gene delivery. For safe and efficient gene delivery, developing new non-viral gene carriers with the merit of low cytotoxicity and high gene transfection performance is regarded as a major task. Recent researches had revealed that the cytotoxicity [14], gene transfection efficiency [15] and intracellular trafficking [16] of non-viral gene carriers strongly depended on the molecular architectures [17]. Thus, selecting appropriate molecular building blocks to construct low toxic and high efficient gene carriers was considered as an essential problem in gene therapy. To give insights on the affect of molecular architectures on gene delivery, illustrating the structure–activity relationships of synthetic gene carriers is of high demand.

Previous studies revealed that molecular factors such as backbone, cationic headgroup, linker/linkage and topology played vital roles in gene delivery behaviors. As for headgroups [18], Kataoka et al. [19] found the aminoethylene headgroups in the side chain of polyaspartamides could influence the gene transfection capacity. In earlier works, we prepared some [20] polyamine headgroup-modified cationic poly(L-lysine)-*b*-poly(L-lactic acid)-*b*-dendritic poly (L-lysine) block copolymers, found the introducing of polyamine headgroups could largely enhance the gene transfection efficiency. Recently, the utilization of natural amino acids as cationic headgroups to construct biocompatible gene carriers had attracted increasing interests [21–23]. Among the amino acids, L-lysine (L-2,6-diamino-hexanoic acid) demonstrates good cell membrane fusion capability [24,25] and L-histidine (L-2-amino-3-(1*H*-imidazol-4-yl)-propanoic acid) possesses endosomal disrupting ability [26], and they were often utilized as cationic headgroup moieties for the modification of gene vectors. On the other hand, many chemical linkages including biodegradable (e.g. carbonate ester, phosphate ester, etc.) [27] and non-biodegradable linkage (e.g. ether, etc.) were used as connectors in gene carriers,

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0927-7765/$ – see front matter © 2014 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.colsurfb.2013.12.039

which were able to influence the gene transfection capacity. e.g. Han et al. [28] prepared an ether linkage bearing cationic lipid, which showed higher SiRNA delivery efficiencies than ester linkage bearing lipid. So far, although the effects of backbone, headgroup, linker and topology of some non-viral gene carriers have been investigated, nevertheless, to rational control the physico-chemical and biological properties by mean of molecular engineering is still a challenge. Therefore, continuing exploration of the structure-transgenetic activity relationships of non-viral gene carriers is of great importance. Compared to the undefined molecular structures and polydispersity of polymeric gene carriers, cationic lipids possessed well-defined and controllable molecular structures, which made them good models for the structure–activity relationships study.

Cholesterol, as one of the essential natural-based lipids which often participates in many important biological processes including membrane formation, lipid transportation, metabolism and so on, has been developed as a hydrophobic building block for the construction of functional cationic lipids [29]. Prior researches revealed that cholesterol derived cationic lipids could improve gene transfection efficacy. For instance, Bhattacharya et al. developed a series of ether linkage bearing cholesterol lipids [30], Gemini-cholesterol lipids [3,31] and cholesterol-PEI conjugates [32] with good transfection capacities; Chaudhuri et al. [33] prepared some histidine-conjugated cationic cholesterol lipids, which showed pH-responsive affect and enhanced transfection efficiency; Zenkova et al. [34] revealed that pyridine/imidazole-modified cholesterol cationic lipids have low cytotoxicity and high transfection capability. Besides, high efficient gene transfection was also achieved in some cholesterol-macromolecule conjugates or cholesterol-hybridized materials. e.g. Smith et al. [35] revealed that the synergistic effect of cholesterol dendritic polyamine played important roles in pDNA binding and delivery; Yang et al. [36] found that two cholesterol-conjugated oligopeptides HR15-Chol and HR20-Chol could lead to high efficient gene transfection; Rana et al. [37] synthesized some cholesterol-hybridized cationic lipids with enhanced RNAi transfection efficiencies and lower toxic effects. Recently, we developed a series of disulfide linker bearing CHOSS lipids [38] with the merit of low cytotoxicity and high transfection efficiency, notably, they demonstrated interesting "perinucleic localization" effects in COS-7 cells. Although several types of cholesterol derived cationic lipids were synthesized, however, so far, the structure–activity relationships of many cholesterol-based cationic lipids is still not very clear, and the rational design of low toxic and high efficient cholesterol-based lipid gene carriers through molecular engineering is still a big challenge. Additionally, the intracellular fate such as cellular uptake capability, endocytosis pathway and intracellular localization effect of the cholesterol-based lipids are needed to be deeply understood.

In order to investigate the contribution of structure factors (headgroups and linkages) on the physico-chemical and biological behaviors of cholesterol cationic lipids, in this work, we synthesized and characterized a series of cholesterol-based cationic (**Cho-cat**) lipids with well-defined structures, in which hydrophobic cholesterol block was incorporated with natural L-lysine/histidine headgroups through a flexible linker bearing biodegradable ester or non-biodegradable ether linkages. The phase transition properties and dissociation capacities of the lipids were evaluated by DSC analysis and acid–base titration, respectively. The plasmid DNA binding affinities were examined by agarose-gel retardant assay. The average particle size, surface potential and the morphology of their pDNA lipoplexes were analyzed by dynamic laser light scattering instrument (DLS) and atomic force microscopy (AFM), respectively. Furthermore, the cytotoxicity and gene transfection efficacy of the **Cho-cat** lipids were evaluated in COS-7 and HeLa cell lines. Finally, the intracellular uptake and localization of the **Cho-cat** lipids/Cy3-labeled pDNA lipoplexes was investigated by fluorescent microscopy.

2. Materials and methods

2.1. Materials

Cholesteryl chloroformate and 4-toluene sulfonyl chloride were purchased from *Acros Organics*. cholesterol, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 1,6-hexanediol and trifluoroacetic acid (TFA) were purchased from *Shanghai Sinopharm Chemical Reagent Co. Ltd*. Nα,Nim-di(*tert*-butoxycarbonyl)-L-2-amino-3-(1*H*-imidazol-4-yl)-propanoic acid dicyclohexylamine salt (Nα,Nim-Boc-L-His(Boc)-OH·DCHA) was purchased from *GL Biochemical Co. Ltd., Shanghai*. L-2,6-bis((*tert*-butoxycarbonyl)amino) hexanoic acid (Nα,Nε-bis-Boc-L-lysine) was prepared according to literature [25].

Agarose was purchased from *Gene Tech, Shanghai*. Ethidium bromide and branched poly (ethylene imine) (bPEI-25k, $M_w = 25{,}000$) were purchased from *Sigma & Aldrich*. Lipofectamine 2000 was received from *Invitrogene Life Technology, USA*. Phosphate buffer solution (1 × PBS), Dulbecco's Modified Eagle Media (DMEM) and RPMI1640 media were purchased from *Hangzhou Genom Co. Ltd*. Fetal bovine serum (FBS) was purchased from *GIBCO, USA*. Thiazoyl blue tetrazolium bromide (MTT) was purchased from *Biobasic Inc., Markham, Canada*. LDH-cytotoxicity assay kit was purchased from *Abcam Corporation*, *UK*. Luciferase assay kit was purchased from *Promega, USA*. Bicinchoninic acid (BCA) protein quantization kit was purchased from *Applygen technologies Inc., Beijing*. 96-well and 24-well cultivation plates and 50 mL cell cultivation flasks were purchased from *Corning Co. Ltd., USA*. pCMV-Luc plasmid DNA and COS-7/HeLa cell line were kindly provided by the lab of Prof. Yuhong Xu, School of Pharmacy, Shanghai Jiaotong University. pEGFP-N1 plasmids was provided by Dr. Bo Wan (State Key Laboratory of Genetic Engineering, Fudan University, China). *Label* IT® Tracker™ intracellular nucleic acid localization kits, including *Trans* IT®-LT1 transfection reagent was purchased from *Mirus Bio LLC*, *USA*. Lysotracker Green DND-26 was received from *Invitrogene Life Technology, USA*. DAPI was received from *Roche, USA*. In this work, all the other reagents and solvents were analytical grade and were utilized without further purification.

2.2. Methods

2.2.1. Synthesis of cholesterol cationic (Cho-cat) lipids

The synthesis procedures and ^{1}H NMR/MS measurements of the cholesterol cationic (**Cho-cat**) lipids were described in detail in supporting information (SI-1, supporting information).

2.2.2. DSC analysis of Cho-cat lipids

4–8 mg of the synthesized **Cho-cat** lipids were weighed in closed aluminum pans (Standard pan with lid, TA). Scanning runs were performed by differential scanning colorimeter (DSC Q200, TA) under a nitrogen gas flow of 50 mL/min conditions within a heating range of −60–105 °C at a heating rate of 10 °C/min. The glass transition temperature (T_g) and phase transition entropy (ΔH_m) were recorded by TA Q200 analyzer.

2.2.3. Acid titration of Cho-cat lipids

Cho-cat lipids (12–20 mg) were dissolved in 20 mL distilled water to prepare sample solutions, which were adjusted with 0.1 N HCl to pH value lower than 3.0, then each solution was separately titrated with 0.01 N NaOH to pH about 11.0 and the pH values were recorded by a TitroLine easy pH/mV Titrator (SI analytics). The p*K*a

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