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Simple synthesis, self-assembly, and cytotoxicity of novel dimeric cholesterol derivatives

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ABSTRACT

A simple and economic methodology to synthesize three types of novel dimeric cholesterol derivatives (DCDs) was developed. Results obtained from dynamic light scattering and transmission electron microscopy show that spherical and/or angular nano-structural aggregates of DCDs are formed by self-assembly in aqueous solution. The size and morphology of DCD dispersions depend on the spatial arrangement of the substituents and polarity of the head group in the DCD structures. The cytoxicity of DCD dispersions to human keratinocytes (HaCaT) and squamous cell carcinomas (SCC25) cells was also evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The present novel DCD dispersions were not toxic to HaCaT and SCC25 cells at appropriate tested concentrations.

1. Introduction

Molecular self-assembly is an incredibly powerful phenomenon in modern molecular science. Small or macro-molecules can aggregate into new or special structures with various functions under specific conditions through molecular self-assembly [1]. Over the past decade, molecular self-assembly has drawn significant attentions for its potential applications in many areas because of the possibility of forming micro- [2,3] or nano-scale [3–6] aggregates with special well-organized structures [6], morphology [7], and functions [8]. Self-assembly is driven mainly by molecular interactions, such as electrostatic interactions, van der Waals' force, dipole interactions, hydrogen bonding, hydrophobic interactions, and π–π interactions [9]. Among them, hydrophobic interaction is the strongest driving force of the self-organization of amphiphilic molecules in water [9–11]. Molecular self-assemblies form various morphological transformations, which are determined by the structural conformation of aggregated molecules and the environmental conditions [9–11].

Cholesterol is one of the main constituents of cell membranes and has been considered to govern the membrane fluidity and permeability of the hydrophilic drug [11,12]. It was found that the stability of liposomes, an artificial vesicle composed of a lipid bilayer, could be enhanced by the incorporation of cholesterol into the lipid bilayer [13]. A growing number of experimental results demonstrated that cholesterol and its relevant derivatives have potential applications in biomedical science owing to their excellent properties such as biocompatibility, biodegradability, and low toxicity [4,14–23]. Therefore, it would be expected that an amphiphile formed by attachment of a cholesterol moiety to a polar head group exhibits sound biocompatibility.

The steroid backbone of cholesterol is a suitable hydrophobic domain for the self-assembly [23–27]. Various amphiphilic cholesterol derivatives with the unique molecular characteristics have been developed and extensively studied [23,27–34]. The feasibility of amphiphilic self-aggregated nanoparticles as hydrophobic drug carriers has been demonstrated [28]. Yang et al. [29] synthesized a novel water-soluble amphiphilic sodium alginate derivative on which was grafted three types of cholesteryl group per 100 hexuronic acid residues. It can self-assemble into stable and compact nano-aggregates under the influence of the intra- and intermolecular hydrophobic interactions between cholesteryl moieties in NaCl solution. Wang et al. [31] synthesized a series of cholesterol-modified *O*-carboxymethyl chitosan conjugates, which formed the monodispersed self-aggregated nanoparticles upon sonication in water. Dimeric cholesterol derivatives (DCDs) have recent emerged as one of the most promising platforms for drug delivery due to their rigid structure, strong hydrophobic force

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[25,33,35–40], and ability to aggregate into responsive vesicles [40], nanoparticles [25,31,39], organogels [8,24,40,41] and liquid crystals [42]. Some DCDs are important to the pharmaceutical chemistry and exhibit such characteristics as antibacterial activity, anticancer potential [43], antimalarial activity [44], and serum cholesterol-lowering activity [45]. Therefore, cholesterol segments are some of the best candidates for providing strong hydrophobic interactions to self-assembly. In this study, cholesteryl chloroformate was used to synthesize novel cholesterol-based dimeric derivatives.

Compared to relevant analyses for DCDs, most of which are synthesized through complex synthetic steps [25,35,39,46,47], those for biocompatibility and efficacies of DCDs are still very limited. For example, Bajaj et al. [46] synthesized a series of unique DCDs from the precursor cholest-5-en-3β-oxyethan- *N,N′*-dimethylamine by reacting it with α,ω- dibromoalkanes. This complicated processes had high cost with low yield. Hou et al. [47] used three benzene rings, two amide structures, and two carbamate groups to synthesize novel DCDs with low yields. In this study, we propose a simple and economical means to synthesize three types of novel DCDs with high yields and low toxicity. The molecular structures of these DCDs were verified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and mass spectroscopy (MS). The physicochemical properties of DCD dispersions were investigated using dynamic light scattering (DLS) and transmission electron microscopy (TEM). If DCD aggregates are applied to the transdermal delivery system, they will come into contacting with the keratinocytes in the epidermis. It was demonstrated that the human keratinocytes (HaCaT) cells are premalignant and have a non-tumorigenic characteristic [48]. Squamous cell carcinomas (SCC25) cells derived from a cancer of the oral cavity, are used as a tumorigenic contrast to HaCaT [49]. To determine the influence of DCD dispersions on cells with non-tumorigenic and tumorigenic characteristics, respectively, HaCaT and SCC25 cells were used to interact with DCD dispersions at various concentrations. The effects of DCD dispersions on cell viability and the cytotoxicity mechanism for HaCaT and SCC25 cells were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the observation of cell morphology.

2. Materials and methods

2.1. Materials

Cholesteryl chloroformate (Sigma-Aldrich), 1,8-diaminonaphthalene, 1,5-diaminonaphthalene, and 4-nitro-*o*-phenylenediamine (Alfa Aesar) were dried by molecular sieve. All solvents (ECHO Chemical Co., Ltd) were purified, dried, or freshly distilled as required. The pure water with a resistivity of 18.2 MΩ cm obtained from a Milli-Q plus purification system (Millipore) was used in all experiments.

2.2. Synthesis of DCDs

Diamine in dry dichloromethane (20 mL) was added slowly into cholesteryl chloroformate (1.1 equiv) in dichloromethane (10 mL), and the resultant solution was admixed with vigorous stirring for 30 min at room temperature under nitrogen atmosphere. The mixed solution was filtered and followed by concentration in vacuo. The residue was subsequently purified by recrystallization to form solid DCDs. Note that the DCDs formed at room temperature were very stable and no observable change occured after several months storage in a closed container.

2.2.1. Preparation of bis((3S,8S,9S,10R,13R,17R)-10,13-dimethyl-17-((R)-5-methylhexan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-3-yl) naphthalene-1,5-diyldicarbamate, DCD-1

1,5-Diaminonaphthalene (0.35 mL, 2.23 mmol) was reacted with cholesteryl chloroformate for 12 h, and then purified by recrystallization twice with ethyl acetate/hexane = 1/1 to obtain a purple solid (3.18 mg, yield = 75%, purity > 97%). IR (KBr pellet, cm^{-1}): $\nu = 3316\ cm^{-1}$ (stretching of –NH), 2962–2867 cm^{-1} (stretching of $–CH_3$ and $–CH_2–$), 1708 cm^{-1} (stretching of –C=O), 1534 cm^{-1} (stretching of –NH bending), 1095, 1020 cm^{-1} (stretching of C–O–C), ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm), 7.97 (s, 2*H*, CONH), 7.68 (d, *J* = 8.4 Hz, 2*H*, benzene), 7.51 (t, *J* = 7.8 Hz, 2*H*, benzene), 6.96 (s, 2*H*, benzene), 5.42 (s, 2*H*, alkenyl), 4.67 (m, 2*H*, oxycyclohexyl), 0.69–2.49 (m, 86*H*, cholesteryl protons). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm), 11.843, 18.689, 19.341, 21.020, 22.533, 22.820, 23.795, 22.820, 23.795, 27.999, 28.068, 28.221, 31.826, 31.891, 35.779, 36.144, 36.560, 36.941, 38.421, 39.481, 39.684, 42.277, 49.948, 56.066, 56.641, 75.260, 122.789, 126.089, 133.361, 139.563. MS (ESI): *m/z* calcd for $C_{64}H_{94}N_2O_4$ [(M + Na)$^+$]: 954.72, found: 977.00.

2.2.2. Preparation of bis((3S,8S,9S,10R,13R,17R)-10,13-dimethyl-17-((R)-5-methylhexan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-3-yl) naphthalene-1,8-diyldicarbamate, DCD-2

1,8-Diaminonaphthalene (0.35 mL, 2.23 mmol) was reacted with cholesteryl chloroformate for 8 h, and subsequently purified by recrystallization twice with ethyl acetate/hexane = 1/1 to gain purple solid (3.27 mg, yield = 77%, purity > 97%). IR (KBr pellet, cm^{-1}): $\nu = 3466\ cm^{-1}$ (stretching of –NH), 2946–2862 cm^{-1} (stretching of $–CH_3$ and $–CH_2–$), 1738 cm^{-1} (stretching of –C=O), 1536 cm^{-1} (stretching of –NH bending), 1199 cm^{-1} (stretching of C–O–C), ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm), 7.71 (s, 2*H*, CONH), 7.68 (d, *J* = 7.8 Hz, 4*H*, benzene), 7.42 (t, *J* = 7.8 Hz, 2*H*, benzene), 5.39 (s, 2*H*, alkenyl), 4.63 (m, 2*H*, oxycyclohexyl), 0.69–2.47 (m, 86*H*, cholesteryl protons). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm), 11.859, 18.689, 19.308, 21.020, 22.553, 22.820, 23.807, 24.264, 27.999, 28.116, 28.217, 31.806, 31.875, 35.783, 36.139, 36.520, 36.933, 38.454, 39.481, 39.684, 42.277, 49.940, 56.070, 56.653, 75.297, 122.776, 125.673, 126.758, 132.208, 135.987, 139.503, 154.412. MS (ESI): *m/z* calcd for $C_{64}H_{94}N_2O_4$ [(M + Na)$^+$]: 954.72, found: 977.00.

2.2.3. Preparation of bis((3S,8S,9S,10R,13R,17R)-10,13-dimethyl-17-((R)-5-methylhexan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-3-yl) (4-nitro-1,2-phenylene)dicarbamate, DCD-3

4-Nitro-*o*-phenylenediamine (0.17 mL, 1.12 mmol) was reacted with cholesteryl chloroformate for 8 h, and sequently purified by recrystallization twice with ethyl acetate/hexane = 1/1 to obtain a yellow solid (1.69 mg, yield = 80%, purity > 97%). IR (KBr pellet, cm^{-1}): $\nu = 3412\ cm^{-1}$ (stretching of –NH), 2945 cm^{-1} (stretching of $–CH_3$ and $–CH_2–$), 1714 cm^{-1} (stretching of –C=O), 1536 cm^{-1} stretching of $–NO_2$ bending), 1511 cm^{-1} (stretching of –NH bending), 1042 cm^{-1} (stretching of C–O–C), ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm), 9.62 (s, 1*H*, benzene), 8.51 (d, *J* = 9.6 Hz, 1*H*, benzene), 8.35 (s, 1*H*, CONH), 7.59 (d, *J* = 6.6 Hz, 1*H*, benzene), 6.63 (s, 1*H*, CONH), 5.42 (s, *J* = 4.8 Hz, 2*H*, alkyl), 4.62 (m, 2*H*, oxycyclohexyl), 0.68–2.43 (m, 86*H*, cholesteryl protons). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm), 11.835, 19.308, 21.007, 22.549, 22.820, 23.795, 24.260, 27.886, 27.995, 28.213, 31.806, 31.867, 35.775, 36.139, 36.524, 36.552, 36.876, 36.900, 38.239, 38.328, 39.481, 39.660, 42.269, 49.916, 49.957, 56.062, 56.616, 56.653, 75.762, 121.502,122.890, 122.995, 130.990, 132.713, 136.003, 139.300, 139.442, 152.721. MS (ESI): *m/z* calcd for $C_{60}H_{91}N_3O_6$ [(M + Na)$^+$]: 950.38, found: 976.80.