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Journal of Controlled Release 116 (2006) 353–359
journal of controlled release
www.elsevier.com/locate/jconrel

Intracellular protein delivery with a dimerizable amphiphile for improved complex stability and prolonged protein release in the cytoplasm of adherent cell lines

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Received 27 June 2006; accepted 2 October 2006
Available online 13 November 2006

Abstract

Direct delivery of functionally active proteins into cells represents an emerging strategy for laboratory investigation and therapeutic applications. For this purpose, we developed a novel amphiphile (CholCSper) consisting of cholesterol linked to carboxy-spermine by a cysteine. This amphiphile is dimerizable upon mild oxidation of the thiol to disulfide and it was used in formulation with DOPE to prepare an intracellular protein delivery system. The stabilization of the CholCSper assemblies by chemical conversion of CholCSper into its gemini amphiphile afforded the production of homogeneous assemblies with proteins whose sizes are easier to control. Furthermore, the cholesterol moiety has an effect on the density of the complexes formed with proteins and leads to a prolonged protein release in the cytoplasm of cells exposed to the protein carrier assemblies.

Keywords: Delivery vehicle; Lipoplexes; Protein transduction; Nanoparticle; Supramolecular chemistry

1. Introduction

Anionic proteins are amenable to cytoplasmic delivery by complex formation with cationic lipids [1] or amphiphilic cationic peptides [2]. We previously showed that protein/cationic lipid complexes [3] follow the same pathway as DNA/lipid or DNA/polymer complexes to enter adherent cells [4,5] and that the lipid micelles protect the proteins from degradation certainly by steric hindrance. The efficient cellular entry is based on the formation of positively charged assemblies, which, in turn, anchor to the external face of cell plasma membranes through electrostatic interactions with heparane sulfate proteoglycans [6]. Among these negatively charged receptors for cationic particles, pharmacological results indicate that the transmembrane syndecans are responsible for particle engulfing into intracellular compartments aimed for degradation [7]. At this stage, cationic lipids with bilayer-disrupting properties in combination with fusogenic lipids [8,9] may lead to various degrees of macromolecule escape into the cytosol and to the subsequent biological effect. However gene delivery experiments indicate that the efficiency of delivery relies not only on the vector [10] but also on the preparation of suitable macromolecule/vector complexes, ranging from 100 to 1000 nm in diameter [11–14]. However, compared to nucleic acid polymers, proteins are highly variable in terms of composition, shape, charge density and aggregating properties. This variability impacts significantly their encapsulation in nanomaterials [15–18] and the formation of complexes between a protein and the cationic vectors. Although some general rules and protocols were proposed for generic protein delivery using cationic lipids, delivery optimization of a given protein with a given lipid has to be carried on empirically [1,3]. In particular, it appears that the size of the protein/lipid complexes is difficult to control and reproduce with accuracy. This behavior may be partially explained by the irreversible self-association of lipids into polydisperse and large objects (micelles) upon hydration. Subsequent association of proteins to lipid surfaces will thus lead to even less controlled assemblies.

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doi:10.1016/j.jconrel.2006.10.005

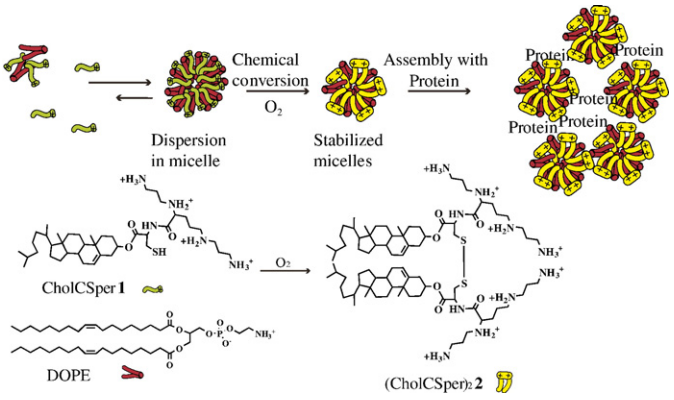

Fig. 1. Schematic representation of complex formation between proteins and micelles of $(CholCSper)_2$ ***2***.

Reversible self-association of cationic surfactants drives the assembly thermodynamically towards homogeneous aggregates with a low aggregating number. Unluckily, the other consequence of reversible associations is that these assemblies do not withstand dilution or competition with plasma membrane anionic receptors, hence cannot be used for delivery. As proposed earlier for gene compaction, *in situ* stabilization of the surfactant assemblies by chemical conversion of surfactants into dimers [19] or polymers [20] may produce a more homogeneous assembly, which could be easily modified for specific cell targeting [21] (Fig. 1). In this context, we prepared a cysteine-based cationic amphiphile capable of dimerization by oxidation of the thiol into disulfide using the air naturally dissolved in aqueous solutions and investigated its properties in comparison with a cationic lipid, DOGS. In particular, we report the synthesis of a cationic surfactant consisting of cholesterol and carboxyspermine connected by a cysteine (CholCSper) ***1*** and the protein delivering properties of its corresponding dimer $(CholCSper)_2$ ***2*** formulated with dioleoylphosphatidylethanolamine (DOPE).

2. Materials and methods

2.1. Materials

4-Methoxytrityl-chloride resin (1.73 mmol/g), benzotriazole-1-yl oxytrispyrrolidino-phosphonium hexaphosphate (PyBOP) were purchased from Novabiochem (Meudon, France). L-Cysteine-bis-allyl ester bis(toluene-4-sulfonate), cholesterol, *N*,*N*′ dimethyl-aminopyridine (DMAP), *N*-(9-fluorenylmethoxycarbonyloxy)succinamide, *N*,*N*′-diisopropylethyleneamine (DIEA), piperidine, trifluoroacetic acid (TFA), *N*-methylmorpholine (NMP) were from Fluka (St Quentin Fallavier, France). Dichloromethane (DCM), *N*,*N*′-dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH), chloroform ($CHCl_3$) were from Carlo Erba (Val de Reuil, France). We used a glass column ended with a joint connection and a glass frit (porosity n°2) on the upper and lower ends, respectively, as a reaction vessel for solid-phase reactions. The column was connected to a rotary evaporator for stirring and the reaction was carried out at room temperature. *R*-phycoerythrin was from Molecular Probes (Eugene, OR, USA; P-801). Dioleoylphosphatidylethanolamine (DOPE), cell culture media and supplements were purchased from Sigma (St Quentin Fallavier, France). The Syrian hamster kidney cell line (BHK-21, ATCC number CCL-10) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics. Cell lines were grown according to ATCC guidelines and were seeded at about 60 000 cells/well in 24-well tissue culture plates 1 day before the experiments.

2.2. Amphiphile synthesis

The synthesis of the amphiphile was performed according to a published procedure starting from 4-methoxy chloride resin (200 mg scale) except for the acid-grafted resin esterification with cholesterol [22]. This esterification was done by addition of a solution of cholesterol (0.254 g, 0.66 mmol), dicyclohexylcarbodiimide (0.150 g, 0.726 mmol) and *N*,*N*-dimethylaminopyridine (88 mg, 0.726 mmol) in $CHCl_3$ (5 mL) to the resin. After gently stirring at room temperature, the vessel was drained and washed with $CHCl_3$ (3 × 15 mL), MeOH (3 × 15 mL) and dichloromethane (3 × 15 mL). The reaction process was repeated 3 times to ensure complete reaction and the amphiphile was then cleaved of the resin and deprotected by addition of 20% TFA in CH_2Cl_2 (5 mL) for 1 h at room temperature. The filtrate was evaporated under reduced pressure, washed with cold diethylether and dried under reduced pressure to afford the CholCSper ***1*** as a yellowish solid in 65% overall yield.

^{1}H NMR (EtOH-d6): δppm 0.62–0.85 (m, 5*H*), 1.13–1.32 (m, 8*H*) 1.41–2.26 (m, 26*H*), 2.61–3.28 (m, 25*H*), 3.80–4.13 (m, 3*H*), 4.51–4.73 (m, 2*H*), 6.90 (m, 1*H*), HRFABMS (m/z): calcd for $C_{41}H_{76}N_5O_3S$ (MH^+): 718.5668, found: 718.5650.

2.3. Formation of protein/amphiphile complexes

For all experiments, the CholCSper ***1*** and DOPE were solubilized in ethanol (stock solution) at a final concentration of 3.33 mM and 1.16 mM for ***1*** and DOPE respectively. The stock solution was stored in aliquots at −80 °C to avoid ethanol evaporation and thiol oxidation.

Method A. 3 μL of the CholCSper/DOPE ethanolic solution was added to 50 μL of 20 mM Hepes buffer, pH 7.4. After mixing, the amphiphile in solution was immediately added to 50 μL of 20 mM Hepes buffer, pH 7.4 containing 2 μg of the phycoerythrin. The assembly medium was then gently mixed for 20 to 30 s and the mixture was left at room temperature during 48 h for complete oxidation of the thiol to disulfide.

Method B. 30 μL of the ***1***/DOPE ethanolic solution was added to 500 μL of 20 mM Hepes buffer, pH 7.4. After mixing, the CholCSper/DOPE micelles were incubated for 48 h for micelles stabilization by full conversion of the free thiol into disulfide using the air dissolved into the aqueous phase. 50 μL of the oxidized micelles (200 μM in ***1***, 70 μM DOPE) was then added to a 50 μL solution of the phycoerythrin (2 μg, 8.3 pmol) in 20 mM Hepes buffer pH 7.4. After gently mixing for 20 s by pipetting up and down, the complexes were incubated for 20 min at room temperature and added onto cells.