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Intracellular and transdermal protein delivery mediated by non-covalent interactions with a synthetic guanidine-rich molecular carrier

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ABSTRACT

The impermeability of the cell plasma membrane is one of the major barriers for protein transduction into mammalian cells, and it also limits the use of proteins as therapeutic agents. Protein transduction has usually been achieved based on certain invasive processes or cell penetrating peptides (CPP). Herein we report our study in which a synthetic guanidine-rich molecular carrier is used as a delivery vector for intracellular and transdermal delivery of proteins. First a sorbitol-based molecular carrier having 8 guanidine units (Sor-G8) was synthesized, and then was simply mixed with a cargo protein of varying sizes to form the non-covalent complex of carrier-cargo proteins. These ionic complexes were shown to have efficient cellular uptake properties. The optimum conditions including the molar ratio between cargo protein and carrier, and the treatment time have been defined. Several protein cargoes were successfully examined with differing sizes and molecular weights: green fluorescent protein (MW 27 kDa), albumin (66 kDa), concanavalin A (102 kDa), and immunoglobulin G (150 kDa). These non-covalent complexes were also found to have excellent transdermal penetration ability into the mouse skin. The skin penetration depth was studied histologically by light microscopy as well as two-photon microscopy thus generating a depth profile. These complexes were largely found in the epidermis and dermis layers, i.e. down to ca. 100 μm depth of the mouse skin. Our synthetic Sor-G8 carrier was found to be substantially more efficient that Arg8 in both the intracellular transduction and the transdermal delivery of proteins. The mechanism of the cellular uptake of the complex was briefly studied, and the results suggested macropinocytosis.

1. Introduction

Delivery of proteins into live cells is often required for a variety of biology and biotechnology works such as probing signal transduction pathways, imaging cytoplasmic organelles, cellular reprogramming, structure-function analyses of cytoplasmic proteins as well as delivery of therapeutic proteins (Kim et al., 2009; Pawson and Nash, 2000; Ramakrishna et al., 2014; Rekha and Sharma, 2013; Zuris et al., 2014). However, transduction of proteins into cells is highly limited due to their larger size, hydrophilic nature, and tight permeability of cellular plasma membrane. Until recently, the entry of proteins into a cell had been achieved mostly by microinjection, electroporation, and lipofectamine-mediated transfection. However, these techniques are labor intensive and invasive to cells, which could result disruption of cell membrane and low cell viability (Bamford et al., 2014; Qin et al., 2015). Transdermal delivery of proteins is much more difficult due to the "skin barrier" of stratum corneum, the outermost epidermal sublayer (Bos and Meinardi, 2000; Madison, 2003; Naik et al., 2000). This permeation barrier is the main hurdle in the transdermal transport of proteins. Despite these disadvantages (e.g. invasive, repeated administration requirement), the

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parenteral administration is still the most-used route for the delivery of protein-based drugs (Herwadkar and Banga, 2012; Kalluri and Banga, 2011).

During the study of the human immunodeficiency virus type 1 (HIV-1) transcription activator, it was found that a functional domain of Tat (transcription activating protein of HIV-1) was essential to cell membrane permeability (Frankel and Pabo, 1988; Green and Loewenstein, 1988). This domain, called protein transduction domains (PTDs) is rich in basic amino acids (lysine and arginine). Starting from this discovery, many kinds of cell-penetrating peptides (CPPs) were discovered or developed: Tat peptide, poly-arginine, penetratin, transportan, MAP, etc. (Mussbach et al., 2011; Oehlke et al., 1998; Torchilin, 2008; Trabulo et al., 2010). For example, green fluorescent protein (GFP) was coupled to the cellular translocating peptide (transportan) and the resulting fusion proteins or covalently linked proteins were shown to penetrate into mammalian cell lines. Subsequent advances in CPPs have enabled transduction of proteins into cells and tissues/organs (Pooga et al., 2001; Schwarze and Dowdy, 2000; Suzuki et al., 2007; Wadia and Dowdy, 2002). A 21-AA amphipathic peptide carrier (Pep-1) was shown to help the cellular uptake of various proteins such as GFP, β-Gal and antibodies without the need for prior chemical covalent coupling with the cargoes (Morris et al., 2001). Arginine-rich intracellular delivery (AID) peptides were also shown to facilitate the delivery of proteins into cells and skin tissues of mouse (Hu et al., 2009). An arginine heptamer was conjugated to cyclosporin A (protein for inhibition of inflammation) through a pH-sensitive linker, and it was efficiently transported into cells in mouse and human skin (Rothbard et al., 2000). In another case, AID-mediated peptides (Tat-PTD, R9, and R9Z) were able to facilitate the delivery of proteins into cells and to penetrate through skin tissues of mouse (Hou et al., 2007). This cellular and transdermal delivery was made without covalent conjugation, probably thanks to the protein–protein interactions between carrier proteins/peptides and cargo proteins (Chang et al., 2007; Liu et al., 2013).

Recently we have designed and prepared a series of novel molecular carriers which are based on carbohydrate scaffolds rather than peptides (Biswas et al., 2008; Chung et al., 2008; Ghosh et al., 2010; Lee et al., 2011, 2009; Maiti et al., 2007, 2006). Multiple guanidine units have been attached to carbohydrate scaffolds such as monosaccharides (glucose, mannose, allose and galactose), disaccharides (sucrose, lactose, and trehalose), inositols and sorbitols, and their delivery properties (plasma membrane penetration, intracellular localization, and mechanism, etc.) have been studied. We have also utilized some of these synthetic molecular carriers for the delivery of various small and large cargo molecules into mammalian tissues and organs (Biswas et al., 2017; Im et al., 2012, 2011a, 2011b, 2009; Jin et al., 2011). A number of the synthetic molecular carriers displayed interesting and unique properties. For example, the sorbitol-based G8 carrier was found to penetrate the cellular membranes so efficiently that it could drag a very large cargo into cells and tissues including the mouse brain. The molecular complex (diameter of ca. 18–20 nm), prepared from a streptavidin conjugated quantum dot and the biotin-conjugated Sor-G8 carrier was found to penetrate into HeLa cells and furthermore into the mouse brain, overcoming the blood-brain barrier (BBB) (Im et al., 2011a). The taxol-conjugated Sor-G8 carrier exhibited good anti-tumor activity in the mouse model of glioblastoma (Jin et al., 2011). Hence it has been envisioned that when the positively charged Sor-G8 carrier is suitably complexed with an anionic cargo (proteins as well as small molecules), the ionic complex might be able to cross the cellular membranes and even possibly the skin barriers, although the carrier-cargo binding is not expected to be strong enough to penetrate into more deep seated tissues/organs. Thus, we have carried out such studies and herein we wish to report the affirmative results.

2. Materials and methods

2.1. General methods of chemical synthesis

All commercial chemicals were used as received except for solvents, which were purified and dried by standard methods prior to use. Analytical TLC was performed on a Merck 60 F254 silica gel plate (0.25 mm thickness), and visualization was done with UV light (254 nm and 365 nm), and/or by spraying with a 5% solution of phosphomolybdic acid or ninhydrin, followed by charring with a heat gun. Column chromatography was performed on Merck 60 silica gel (70–230 or 230–400 mesh). NMR spectra were recorded on a Bruker DRX 500 (^{1}H NMR at 500 MHz; ^{13}C NMR at 125 MHz) spectrometer. Analytical HPLC was performed on Agilent 1220 infinity LC with an analytical column (eclipse XDB-C18, 3.5 μm, 4.6 × 150 mm). High resolution mass spectra were obtained on MALDI TOF mode on a Voyager-DE STR system.

2.2. Synthesis of 1,6-O-bistrityl-D-sorbitol (compound **1**)

To a solution of D-sorbitol (1 g, 5.5 mmol) in pyridine (15 ml), was added trityl chloride (3.37 g, 12.1 mmol), and the temperature was slowly increased to 100 °C and with stirring overnight. After completion of the reaction, pyridine was removed under reduced pressure and the resulting solid was dissolved in CH_2Cl_2 and washed with water and brine. The crude product was purified by column chromatography on 70–230 mesh size silica gel using EtOAc/hexane solvent system, followed by trituration with 5% ether in hexane to give compound **1** (1.4 g, 38%) as off white solid. Characterization data of the compound was in good agreement with the published report (Maiti et al., 2007).

2.3. Synthesis of 1,6-O-bistrityl-2,3,4,5-tetra-O-(N-{bis-[3-(N′, N′-bis-Boc-guanidino)-propyl]}-6-aminohexanoyl)-D-sorbitol (compound **2**)

Compound **1** (0.1 g, 0.15 mmol) in dry CH_2Cl_2 (5 ml) was stored in a vial containing freshly dried 4 Å molecular sieves powder (100 mg). To this solution, compound **4** (0.66 g, 0.9 mmol), EDC (0.2 g, 1.05 mmol), DMAP (0.037 g, 0.3 mmol) in CH_2Cl_2 (2 ml) were added. The reaction mixture was stirred for 3 days at rt under inert atmosphere. The reaction mixture was filtered and the filtrate was washed with CH_2Cl_2, and saturated $NaHCO_3$ solution. Organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum to give the crude product, which was purified by column chromatography (70–230 mesh size silica; 8% MeOH in CH_2Cl_2 as eluent) to give compound **2** (0.34 g, 64%) as a foamy white solid. R_f: 0.5 (8% MeOH in CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 11.5 (s, 8H), 8.5 (s, 8H), 7.44–7.18 (m, 30H), 5.76–5.74 (m, 1H), 5.24–5.02 (m, 3H), 3.45–3.37 (m, 16H), 3.28 (brs, 2H), 3.11–3.01 (m, 2H), 2.46–1.88 (m, 32H), 1.69–1.25 (m, 184H); ^{13}C NMR ($CDCl_3$): δ 172.7, 172.3, 172.2, 171.7, 163.7, 156.2, 153.2, 153.1, 143.6, 143.5, 128.8, 128.7, 127.9, 127.8, 127.2, 127.1, 86.8, 86.7, 83.0, 82.9, 79.2, 79.1, 71.1, 69.3, 68.4, 68.2, 62.3, 61.9, 51.7, 39.7, 34.4, 34.3, 34.0, 33.8, 29.7, 28.5, 28.4, 28.1, 27.4, 27.2, 26.3, 24.9, 24.8, 24.6.

2.4. Synthesis of 2,3,4,5-tetra-O-[N-{bis-(3-guanidinopropyl)}-6-aminohexanoyl]-D-sorbitol·8HCl (compound **3**)

Compound **2** (75 mg, 0.02 mmol) was dissolved in 4 M HCl in dioxane (0.3 ml), and stirred for one day at rt. Reaction mixture was filtered and the residue was washed with EtOAc. The resulting solid was dissolved in deionized water, filtered through a PTGE syringe filter and lyophilized to give crude product. The crude product was