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Design of novel cell penetrating peptides for the delivery of trehalose into mammalian cells

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ABSTRACT

Stabilization of cells in a desiccated state can significantly simplify the storage and transportation and save expenses for clinical applications. Introduction of the impermeable disaccharide, trehalose, into cells is an important step to improve the desiccation tolerance of cells. In this study, a novel cell penetrating peptide, KRKRWHW, was developed based on molecular simulations. The peptide exhibited little cytotoxicity and high penetrating efficiency into mammalian cells. The cell viability of mouse embryonic fibroblasts (MEFs) after the incubation with various concentrations of KRKRWHW from 0.01 mM to 5 mM at 37 °C for 4 h was maintained at around 100%. The peptide was able to penetrate into MEFs within 1 h at 37 °C with an efficiency of around 90% at 0.1 mM. Trehalose, as a cargo coupled with the peptide of KRKRWHW through hydrogen bond and π–π bond, was successfully loaded into the MEFs. This novel peptide provides a novel approach for the delivery of trehalose into mammalian cells.

1. Introduction

Stabilization of cell-based products has become a critical barrier against their successful commercialization in tissue engineering and cell therapy etc. [1]. Lyophilization and cryopreservation have been widely used to stabilize cells and tissues to ensure the off-the-shelf availability during long-term storage [2]. Due to constraints in the storage and transportation at cryogenic temperature in cryopreservation [3], storage of cells in the desiccated state would greatly simplify the storage and transportation and facilitate their applications in clinical therapies.

Trehalose as a non-reducing disaccharide having a high glass transition temperature and a dihydrate crystalline structure has been demonstrated to be able to increase the stability of dried liposomes [4], bacteria [5], yeast and retrovirus [6] and platelets [7]. The stabilization mechanism provided by trehalose is not fully elaborated. It has been suggested that trehalose may replace the water shell around macromolecules to prevent protein aggregation and denaturation [2], depress the phase transition temperature of membranes to remain membrane integrity during drying [8,9], and form stable glasses during freeze-drying [10].

Introduction of trehalose into cells is an important step to provide protection against cell damage during lyophilization [11]. In recent years numerous approaches have been developed to load trehalose into mammalian cells to improve desiccation tolerance, including passive trehalose influx by microinjection [12], genetically engineered pores [11–13], thermal shock and osmotic shock [14], transient membrane leakage at the phase transition temperature [15], and fluid-phase endocytosis [16]. The major drawback with these methods is that the processes of intracellular delivery are not trehalose specific, which may further result in the loss of intracellular components through the open pores. In addition, gene expression biosynthesis of trehalose using a recombinant adenovirus vector has been applied to introduce trehalose into human primary fibroblasts [17]. However, this may raise concerns on the risk during clinical applications. Hence, it is necessary to develop a novel method to specifically deliver trehalose into cells without causing damage to the cells.

Cell penetrating peptides (CPPs), rich in basic amino acids, are short peptides which can translocate through cell membranes and can be conjugated with a cargo, such as oligonucleotides [18], plasmids [19], proteins [20], and enter living cells [21]. The mechanism behind the translocation is still unclear; presumably endocytosis plays an important role in the translocation especially when cargo molecules are conjugated [22]. Since CPPs can deliver the specific cargo into the cells and no intracellular components escape from the cells, CPPs have been widely used to deliver a variety of cargos into cells without entrapment in lysosome [23,24]. However, due to the cytotoxicity of CPPs and the

Abbreviations: CPP, Cell penetrating peptide; DAPI, 4′,6-diamidino-2-phenylindole; DIEA, N,N-Diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's medium; DMF, N,N-Dimethylformamide; EDT, 1,2-eth-anedithiol; FITC, fluorescein isothiocyanate; HOBt, 1-Hydroxybenzotriazole; MEF, mouse embryonic fibroblast; MTT, dimethyl thiazolyl diphenyl tetrazolium salt; PI, propidium iodide; RMSD, root mean square deviation; TBTU, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoro-borate; TFA, trifluoroacetic acid; TIS, triisopropylsilane

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difficulty in efficient release of the covalent conjugated target molecules, an alternative CPP offering low cytotoxicity and efficient intracellular delivery of target molecules needs to be developed.

In this study, a CPP was designed based on the molecular docking and dynamics simulations and synthesized in order to provide a more effective and specific technique for intracellular delivery of trehalose. The novel CPP is rich in basic amino acid and contains several amino acids having the ability of coupling with trehalose through non-covalent bonding. We evaluated the cytotoxicity of the novel CPP and further examined the efficiency of the CPP entering into living MEFs. The capacity of intracellular trehalose loading using the CPP was also determined. Our results reveal that the designed CPP exhibits little cytotoxicity and a high efficiency of intracellular trehalose delivery.

2. Materials and methods

2.1. Materials

Peptide synthesis reagent O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoro-borate (TBTU), N,N-Dimethylformamide (DMF), 1-Hydroxybenzotriazole (HOBt), N,N-Diisopropylethylamine (DIEA), isopropanol, trifluoroacetic acid (TFA), phenol, thioanisole, 1,2-eth-anedithiol (EDT), piperidine were purchased from DaTianFengTuo Chem (Beijing, China). Amino acids and Wang resins used in peptide synthesis were purchased from GL Biochem (Shanghai, China). Trehalose was purchased from Yuanye Bio-Technology (Beijing, China), fluorescein isothiocyanate (FITC) from Merck (USA) and dimethyl thiazolyl diphenyl tetrazolium salt (MTT) from Amresco (USA). Fetal bovine serum (FBS), trypsin, Dulbecco's Modified Eagle's medium (DMEM), phosphate buffered saline (PBS), L-glutamine and nonessential amino acid were purchased from Hyclone (USA). Paraformaldehyde and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (USA). Calcein-AM and propidium iodide (PI) were purchased from Fanbo Biochemicals (Beijing, China).

2.2. Design of CPPs

The structures of trehalose (TRE) and heparin sulfate (1HPN) were downloaded from the RCSB protein data bank (PDB). The structure of polypeptides utilized in the molecular simulations was sketched using the Discovery Studio Visualizer (DSV) 3.0. Topology and parameters of force field for the trehalose and heparin sulfate were generated using the PRODRG2 server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg_beta) [25]. Molecular docking using the AutoDock 4.0 with Lamarckian Genetic Algorithm (LGA) was carried out to investigate the interactions between the designed peptides and trehalose and heparin sulfate. Except for a maximum number of 2,500,000 energy evaluations and 100 runs in $60 \times 60 \times 60$ grid box with spacing at 0.375 Å, all the other parameters were set as the default.

Molecular dynamics (MD) simulations were carried out using the GROMACS 4.5.4 and the standard GROMOS96 force field. Long-range electrostatics interactions were treated by the Particle Mesh Ewald (PME) [26]. The peptide was first immersed in a box containing the Simple Point Charge (Extended) water model, and then the trehalose and heparin were subsequently added into the system. Each system for the simulations was neutralized by adding Na^+ or Cl^-. Before each system was equilibrated for 20 ps, an energy minimization was employed. For each system, the simulation was performed at 300 K, physiological pH and 1 atm for a total of 10 ns to assess the stability of the tested systems. The system stability was verified by analyzing the energy components and the root mean square deviation (RMSD) of the structures in the trajectory compared to the starting conformation. The simulation results were visualized using the Visual Molecular Dynamics (VMD) and DSV. All computing was performed on the Lenovo ThinkStation E30 (Lenovo, China).

2.3. Synthesis of CPPs

Peptides were synthesized manually on a Wang resin using the standard Fmoc solid-phase synthesis strategy [27]. Fmoc was removed by DMF containing 20% (v/v) piperidine for up to 30 min, followed by three washes in isopropanol and DMF at room temperature, respectively. Stepwise coupling of Fmoc-amino acids to the growing peptide chain on the resin was performed in the reaction mixture of TBTU 0.91 g, DMF 10 ml, HOBt 0.45 g and 0.52 ml DIEA at room temperature for 2 h. After each amino acid was added, the step of deprotection of Fmoc group was performed. After the last amino acid was added to the peptide chain, the cleavage of peptide from the resin was carried out in the mixture of TFA, triisopropylsilane (TIS), H_2O and EDT (94:1:2.5:2.5, v/v) at 0 °C ice-water while being stirred at 3000 rpm. All crude peptides were purified using a reverse-phase high performance liquid chromatography (RP-HPLC, LC-8A, SHIMADZU, Japan) on a C18 column to purity >95%. The identity of the purified peptides was determined using a MALDI-TOF mass spectrometer (Autoflex III LRF200-CID, Bruker, Germany). To visualize the penetration of CPPs, the C-terminal of the CPP was conjugated with FITC, a fluorescence tag, at the R group of Lys when synthesized.

2.4. Cell culture

MEF cells were cultured and maintained in complete culture medium, DMEM supplemented with 10% FBS, 1% nonessential amino acids and 1% L-glutamine. All cells were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

2.5. Cytotoxicity and cell viability assays

Cytotoxicity was assessed using the MTT assay. The MEF cells were seeded at 8000 cells/well in 96-well plates and cultured for 24 h before treatment. The cells were incubated in the serum-free culture medium with various concentrations of peptides in the range from 0.01 mM to 5 mM for 4 h in a 5% CO_2 humidified atmosphere at 37 °C. To investigate the effect of trehalose loaded by the designed CPPs on the cell metabolic rate, the cells were incubated with the CPP and trehalose together for 2 h in a 5% CO_2 humidified atmosphere at 37 °C. The concentration of the CPP was 1 mM, and the ratio of the CPP to trehalose was set at 1:8. The cytotoxicity was determined immediately after the incubation and after subsequent 24 h of culture by the MTT assay. For the MTT assay, MTT was added to the tested wells at the final concentration of 1 mg/ml and incubated for 4 h at 37 °C and then 100 μl of DMSO was added to the wells after the medium was removed. The absorbance was determined using a microplate reader (Infinite F20, Tecan, Switzerland) at 490 nm. The cells cultured with the normal medium were used as the negative control.

Cell viability of adherent MEFs before and after the incubation with the CPP was evaluated using the Calcein-AM and PI staining method. Briefly, the cells were seeded at the density of 4×10^4 cells/well onto 15 mm of coverslips in 24-well plates and incubated with the medium containing the CPP only or the CPP together with trehalose for 24 h. The cells immediately after the incubation with the CPP or the CPP together with trehalose and after subsequent 24 h culture with normal medium were stained with 1 μM Calcein-AM and 2 μM PI for 10–15 min in the dark, and then washed with PBS for three times. The green and red fluorescence in the stained cells were visualized using a fluorescence microscope (Nikon, Japan) excited at 488 nm and 535 nm, respectively, connected to an imaging capture system (Tucsen, China). The cell morphology was visualized using a microscope.

2.6. The cellular uptake assay of CPPs

The MEF cells were seeded onto the coverslips with a diameter of 15 mm in 24-well plates at the density of 4×10^4 cells/well and cultured for 24 h. After the incubation with the serum-free culture medium