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Fabrication of self-assembling nanofibers with optimal cell uptake and therapeutic delivery efficacy

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ABSTRACT

Effective strategies to fabricate finite organic nanoparticles and understanding their structure-dependent cell interaction is highly important for the development of long circulating nanocarriers in cancer therapy. In this contribution, we will capitalize on our recent development of finite supramolecular nanofibers based on the self-assembly of modularly designed cationic multidomain peptides (MDPs) and use them as a model system to investigate structure-dependent cell penetrating activity. MDPs self-assembled into nanofibers with high density of cationic charges at the fiber-solvent interface to interact with the cell membrane. However, despite the multivalent charge presentation, not all fibers led to high levels of membrane activity and cellular uptake. The flexibility of the cationic charge domains on self-assembled nanofibers plays a key role in effective membrane perturbation. Nanofibers were found to sacrifice their dimension, thermodynamic and kinetic stability for a more flexible charge domain in order to achieve effective membrane interaction. The increased membrane activity led to improved cell uptake of membrane-impermeable chemotherapeutics through membrane pore formation. *In vitro* cytotoxicity study showed co-administering of water-soluble doxorubicin with membrane-active peptide nanofibers dramatically reduced the IC50 by eight folds compared to drug alone. Through these detailed structure and activity studies, the acquired knowledge will provide important guidelines for the design of a variety of supramolecular cell penetrating nanomaterials not limited to peptide assembly which can be used to probe various complex biological processes.

1. Introduction

Supramolecular assembly of peptides has been widely used as a bottom-up approach to generate functional nanomaterials [1–5]. These materials exhibit well-defined molecular structure, internal ordering and nanostructure, which were found to be important factors to manipulate their interactions with cells and tissues [6–11]. Fundamental understanding the relationship between the molecular/supramolecular nanostructure and bioactivity of these assemblies is crucial to develop self-assembled peptides with optimized biological properties. In the last two decades, structure-activity correlation has been primarily focused on peptide nanofibers of infinite dimension for tissue engineering application [12–17]. It came to realization that the impact of finite peptide nanostructures could also be far-reaching particularly for the development of systemic therapeutic delivery vehicles where the length scale of the assembly plays important roles for cell uptake and tissue penetration as dictated by the enhanced permeation retention (EPR) effect. There have been numerous studies on the design of inorganic [18–21], polymeric [22–25], and protein-based rod-like nanoparticles [26–30] as long-circulating anisotropic nanocarriers. However, limited research was reported on finite anisotropic nanomaterials based on rationally designed and engineered peptide assembly. The lack of related research is partly due to the difficulty of fabricating peptide nanofibers with precisely controlled morphology, optimally below 100 nm that can potentially be used as long circulating nanocarriers. Notably, supramolecular peptides may also overcome some of the intrinsic limitations associated with single chain peptides, e.g. stability to greatly expand their biomedical utility [31–34].

We have been dedicating to the development of water-soluble

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finite supramolecular peptide nanostructures with built-in biological functions and understanding their sequence-structure-activity correlation on both the molecular and supramolecular level [31–35]. Self-assembling Antimicrobial Nanofibers (SAANs) [32] and Filamentous Cell Penetrating Peptides (FCPPs) [31,34] are two families of supramolecular peptides that we developed to mimic natural antimicrobial peptides and cell penetrating peptides respectively with dramatically improved stability, bioactivity and cytocompatibility. In particular, FCPPs were designed and fabricated as a highly effective gene delivery system based on the self-assembly of *de novo* designed cationic β-sheet forming multi-domain peptides (MDPs) [31]. In our previous study, we compared the cell penetrating activity of nanofiber forming peptides with their monomeric analogue [34], showing the important role of nanofiber formation in increasing peptides' membrane activity. In the current work, we seek to understand the structure-activity relationship (SAR) of these peptide nanofibers and identify critical structural features governing the cell penetrating activity of these assemblies. Despite the multivalent charge presentation, not all fibers led to high levels of membrane activity and cellular uptake. The interaction between peptides and the cell membrane is governed by combined chemical and physical parameters and the flexibility of the cationic charge domains on self-assembled nanofiber is critically important for effective membrane perturbation. Nanofibers were found to sacrifice their dimension, thermodynamic and kinetic stability for a more flexible charge domain in order to achieve effective membrane interaction and therapeutic delivery efficacy. We believe rational design of peptide building blocks to form FCPPs and detailed understanding of their molecular and supramolecular nanostructure and their effect on biological activity is crucial for the development of highly effective supramolecular cell penetrating peptides. The fundamental knowledge showed here can also be applied to the design of other types of protein/polymeric cell penetrating nanomaterials which can be used to probe various complex biological processes.

2. Materials and methods

2.1. Materials

MBHA rink amide resin, Fmoc-protected amino acids, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem. Piperidine and diisopropylethylamine (DIPEA) was purchased from Sigma-Aldrich. All other reagents and solvents for peptide synthesis and purification were purchased from Fisher Scientific and used as received. Desalting column VariPure IPE was ordered Agilent Technologies (Apple Valley, MN). Dulbecco's modified Eagle medium (DMEM) culture medium, hoechst 33342 and LysoTracker Red DND-99 was purchased from Life Technologies. Fetal Bovine Serum (FBS) was ordered from VWR (Radnor, PA). CCK8 assay kit was ordered from Dojindo Molecular Technologies (Rockville, MD). Fluorescence measurements were performed on Varian Cary Eclipse fluorescence spectrophotometer. Reversed-phase HPLC was carried out using HITACHI L-7100 pump. UV absorbance was measured on a micro-plate reader (Vitor2 1420 Multilabel Counter, PerkinElmer) for cell toxicity experiment.

2.2. Peptide synthesis and purification

The synthesis of MDPs followed standard Fmoc-solid phase peptide synthesis method. Briefly, Fmoc group was removed with 20% piperidine/DMF (V/V) for 5 min and the deprotection reaction was repeated once. Fmoc-protected amino acids (5 eq), coupling reagent, HBTU (5 eq) and diethylpropylamine (10 eq) were added to the solid resin and the coupling reaction run for 45 min. Upon completion of the synthesis, the N-terminus of the peptide was capped with acetic anhydride in the presence of DIPEA in DMF for 1 h and the completion of acetylation reaction was confirmed by Kaiser test. Cleavage cocktail including trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O (95/2.5/2.5 by volume) was added to the resin and mixed for 3 h. Cleavage solution was collected and the resin was rinsed with neat TFA for two times. Excessive TFA was evaporated by air blow and residual peptide-TFA mixture was triturated with cold diethyl ether. Precipitates were isolated by centrifugation and washed with cold diethyl ether for three times. Peptide powder was dried under vacuum overnight before HPLC purification. A linear gradient of a binary water/acetonitrile solvent containing 0.05% TFA was used for HPLC purification on a preparative reverse phase C18 column. HPLC fraction was collected, combined and desalted to remove residual TFA salts. The desalted peptide solution was frozen in liquid nitrogen and lyophilized for 3 days. Mass was confirmed by MALDI-TOF. Expected mass for K10: 3225.80, Experimental result: 3226.10. Expected mass for K6: 2713.40, Experimental result: 2712.34. Expected mass for D-K10: 3225.80, Experimental result: 3228.99.

2.3. Critical aggregation concentration (CAC) measurement

CACs were determined using a previous protocol based on the fluorescence intensity change of tryptophan [35,36]. Fluorescence measurements were performed at room temperature by monitoring the emission spectrum of peptides between 295 nm and 440 nm using an excitation wavelength at 280 nm. Peptide stock solution (160 μM) was added in 200 μL Tris buffer (20 mM, pH = 7.5) with an increment of 2 μL each time. Fluorescence intensity at 350 nm was plotted as a function of peptide concentration. The CAC was determined at the concentration in which onset of nonlinearity was observed.

2.4. Fluorescence recovery experiment for kinetic stability measurement

Both FITC-labeled and non-labeled peptides were dissolved in Tris buffer (20 mM, pH = 7.5) separately and incubated overnight before further use. FITC-Labeled peptides were prepared at a concentration of 15 μM and the non-labeled peptides were prepared at a concentration of 3 mM. The two solutions were mixed at a molar ratio of 1: 40. Time-dependent fluorescence intensity was recorded every 30 s for 24 h with the excitation wavelength at 497 nm and emission at 527 nm. The excitation slit was set to 2.5 nm and emission slit was set to 2.5 nm. The subunit exchange rate was estimated by fitting the experimental data into two-rate first order kinetic equation.

2.5. Patch clamp electrophysiology

For patch clamp electrophysiology experiments, HEK293 cells were seeded onto glass coverslips, and transferred to a bath positioned on the stage of an inverted Olympus IX51 microscope. Cells were continuously perfused with a divalent-free extracellular solution containing 140 mM NaCl, 10 mM glucose, 10 mM HEPES, (pH adjusted to 7.4 with NaOH). Peptide solution was diluted in divalent-free extracellular solution to reach a final concentration of 16 μM for K10 and 26 μM for K6. Single cell current recordings were made in the broken patch whole cell voltage clamp configuration according to conventional methods [37] using low resistance (0.5–3 MΩ) borosilicate glass electrodes. Membrane potential was held at −40 mV for the duration of the experiment. Current

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