International Journal of Pharmaceutics 528 (2017) 723–731

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Self-assembly nanostructure controlled sustained release, activity and stability of peptide drugs

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ARTICLE INFO

Article history:
Received 27 February 2017
Received in revised form 26 May 2017
Accepted 15 June 2017
Available online 16 June 2017

Keywords:
Self-assembly
Nanostructure
Anticancer peptide
Stability
Drug delivery

ABSTRACT

Peptides are considered as a new generation of drugs due to their high structural and functional diversity. However, the development of peptide drugs is always limited by their poor stability and short circulation time. Carriers are applied for peptide drug delivery, but there may be problems like poor loading efficiency and undesired xenobiotic toxicity. Peptide self-assembly is an effective approach to improve the stability and control the release of peptide drugs. In this study, two self-assembling anticancer peptides are designed by appending a pair of glutamic acid and asparagine to either the N-terminus or the C-terminus of a lytic peptide. This simple, yet rational sequence modification was made to change the amphiphilic pattern and secondary structural content of the parent peptide, thereby modulating its self-assembly process. It was found that the N-terminus modified peptide favors the formation of nanofibrils and the peptide with C-terminal modification formed micelles. Although both nanostructures showed prolonged action profiles and improved serum stability compared to the parent peptide, the morphology of the nanostructures is highly critical to manipulate the release profile of the free peptide from the assembly and regulate their bioactivity. We believe the self-assembly approach demonstrated in this study can be applied to a variety of therapeutic peptide drugs to improve their stability and therapeutic activity for the development of carrier-free drug delivery system.

1. Introduction

Peptide drugs are considered as a new generation of drugs due to their high specificity and diversity, especially in targeted therapy (Dissanayake et al., 2017; Raucher and Ryu, 2015; Chen et al., 2015; Sigg et al., 2016). However, their application is greatly limited by their short circulation time in body, which is mainly caused by the clearance of reticuloendothelial system (RES) and enzymatic degradation. Therefore, carriers are usually designed for peptide delivery (Jallouk et al., 2015; Saffran et al., 1986; Kim et al., 2008). Nevertheless, problems such as complicate preparation, poor loading efficiency, and potential xenobiotic toxicity are sometimes found (Angelova et al., 2011; Du and Stenzel, 2014). Regarding this point, novel delivery system for peptide drugs are necessary in order to promote their clinical application.

Over the past decades, numerous advances have been made in the field of molecular self-assembly, which was commonly used to direct the design and synthesis of biomaterials with unique properties (Whitesides and Grzybowski, 2002; Zhang, 2003; Gazit, 2015). Due to their design modularity, biocompatibility and ease of synthesis and modification, peptides have been proven easy yet effective tools for the fabrication of biomaterials with various nanostructures, such as nanotube (Hamley, 2014), nanotape (Castelletto et al., 2012; Miravet et al., 2013) and nanofibril (Gazit, 2007; Mandal et al., 2014) *etc*. Distinct from other synthetic molecules, peptides exhibit a wide range of biological activity and specificity, which make them highly attractive as an important class of molecular building blocks.

Anticancer peptides (ACPs) are mainly derived from antimicrobial peptides (AMPs), which have inherent amphiphilic structures (Zasloff, 2002). Self-assembling peptides based on ACPs have been demonstrated through modification of their primary sequences to induce supramolecular assembly. For instance, Chen et al. designed a peptide CL-1 (FLGALFRALSRLL), which was derived from a well-studied ACP PTP-7 (FLGALFKALSKLL) by replacing both lysine

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http://dx.doi.org/10.1016/j.ijpharm.2017.06.051
0378-5173/

residues with arginine residues. The resulting peptide CL-1 formed nanofibrils which showed much higher serum stability and cell selectivity (Chen and Liang, 2013). Such a strategy takes the advantage of the inherent bioactivity of the peptides and well-defined hierarchical nanostructures, providing a new solution to the delivery of peptide drug with dramatically improved stability and activity. Up to date, although the general principles governing peptide assembly have been well studied, reports on new design methods and rules to direct the self-assembly of bioactive peptides are quite limited. The impact of the study of functional peptide assembly would be enormous given the implication of peptides in various biological processes and biomedical applications (Bai et al., 2016; Goktas et al., 2015; Xu et al., 2014; Chen et al., 2016).

In previous studies, we constructed a self-assembling peptide PTP-7S (EENFLGALFKALSKLL) by adding two negatively charged glutamic acids and one asparagine to the N-terminus of the peptide PTP-7. Due to charge neutralization and its amphipathic feature, PTP-7S self-assembled into nanofibrils showing improved stability and prolonged anticancer effects (Tu et al., 2007). Based on the understanding about the self-assembling mechanism of PTP-7S, two new self-assembling peptides EN (ENFLGALFKALSKLL) and NE (FLGALFKALSKLLNE) were designed to maintain the helical secondary structure, but varying in their amphiphilic pattern. The two peptide analogues self-assemble into two nanostructures, namely nanofibrils and micelles as dictated by the sequence specific intermolecular interactions between peptide chains. As is presented in this study, both the nanofibrils and micelles displayed improved serum stability and time-dependent anticancer activity. Interestingly, the fibril-forming peptide EN showed much higher cytotoxicity than the micelle-forming peptide NE, but relatively lower serum stability. We expect that the approach of molecular self-assembly of bioactive peptides with fine-tuned amphiphilic pattern could be applicable to a wide range of functional peptides, not limited to the ACP demonstrated in this work, for the development of new types of peptide therapeutics.

2. Experimental section

2.1. Materials

All Fmoc-protected resins and amino acids, piperidine (>99.5%), *N*, *N*-Dimethylformamide (DMF, >99.5%), dichloromethane (DCM, >99.5%) were purchased from Beijing Bo Mai Jie Technology Co., Ltd. (Beijing, China). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluo-rophosphate (HBTU, >99%), N, *N*-Diisopropylethylamine (DIEA, >99%) and 1-hydroxybenzot-riazole (HOBt, >99%) were purchased from GL Biochem Ltd. (Shanghai, China). Trifluoroacetic acid (TFA, HPLC grade), acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Dikma Technologies Inc. (Beijing, China). 1, 2-Dipalmitoyl-sn-*glycero*-3-phosphocholine (DPPC), and 1, 2-dihexadecanoyl-sn-*glycero*-3-phospho-L-serine (DPPS, sodium salt) were purchased from Corden Pharma LLC. (Liestal, Switzerland). A LIVE/DEAD bacteria staining kit was purchased from Invitrogen Life Technologies (CA, USA). All other chemicals were purchased from Sigma-Aldrich Co. (MO, USA).

2.2. Peptide synthesis and purification

All peptides were synthesized via a solid-phase method using standard Fmoc chemistry. Reverse-phase HPLC equipped with a semi-preparative C18 column (Shimadzu LC-6AD, Cosmosil C18 peptide/protein column) was employed to purify the crude peptides. HPLC solvents consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 8:2 acetonitrile/water) and a linear gradient from 10% to 90% solvent B was carried out for 101 min. The peptide purities were verified by analytical HPLC (Shimadzu LC-6AD) and ESI–MS (Thermo LTQ Orbitrap XL equipped with an electrospray ionization source). The results showed that they were synthesized correctly and the purity was >95% (Fig. S1–S3).

2.3. Circular dichroism (CD)

Circular dichroism (CD) spectra of the peptides were collected using a Jasco J-810 spectropolarimeter. All the peptide samples were diluted from pre-dissolved stock solution (10 mM in 10% v/v $DMSO/H_2O$) with various solutions and then examined on the CD spectrometer. All the data were collected from 250 nm to 190 nm at room temperature (RT) and averaged from 4 scans with each scan at a rate of 50 nm/min and a bandwidth of 1 nm. Molar residual ellipticity (MRE) was calculated using ellipticity in millidegrees (θ), path length in cm (l), molecular weight in g/mol (M), peptide concentration in mg/mL (c), and number of residues (nr). $[\theta] = (\theta \times M)/(10 \times c \times l \times nr)$. The helical contents of the peptides were calculated from ratio of the experimentally observed $[\theta]_{222}$ to the predicted molar ellipticity $\times$ 100%. The ratio of the molar ellipticities at 222 nm and 208 nm ($[\theta]_{222}/[\theta]_{208}$) was calculated to estimate the formation of coiled-coil structures (Choy et al., 2003; Bruch and Hoyt, 1992).

2.4. Detection of peptide aggregates

The processes of peptide self-association were monitored using a fluorescence probe 1-anilinonaphthalene-8-sulfonic acid (1, 8-ANS). Various concentrations of peptide solutions were prepared from a stock solution (10 mM in 10% DMSO/water), and then incubated with 1, 8-ANS (20 μM) for 10 min. The increase of 1, 8-ANS fluorescence emission spectrum induced by peptide aggregation was recorded on a fluorescence spectrophotometer (Hitachi F-4600, Hitachi Ltd., Tokyo, Japan) by setting excitation wavelength at 369 nm.

2.5. Determination of critical micelle concentration (CMC)

Pyrene fluorescence spectroscopy was employed to determine the critical micelle concentration of the peptides, which could be used as an indicator of the self-assembling ability of peptides. Briefly, peptide stock solution (10 mM in 10% DMSO/water) was diluted with 10 mM PBS (pH = 7.4) to various concentrations (1.56–800 μM for EN, 0.19–400 μM for NE). A known amount of pyrene in acetone (0.035 mg/mL, 20 μL) was added to 1.5 mL vials and dried under a stream of nitrogen. Then, 1 mL of peptide solution at various concentrations was added to each vial, and incubated overnight (~16 h) to equilibrate the pyrene and the peptide aggregate. The final concentration of pyrene was 3.46 μM. Fluorescent spectra of peptide samples were measured by a fluorescence spectrophotometer (Hitachi F-4600, Hitachi Ltd., Tokyo, Japan) with a slit width of 5 nm. The emission wavelength was set at 395 nm, and the excitation wavelength range was set between 300 and 380 nm. The CMCs were determined by plotting I_{339}/I_{333} *vs.* peptide concentrations.

2.6. Transmission electron microscope

Diluted peptide samples were deposited onto carbon grids for TEM imaging. A total of 20 μL of peptide solution was pipetted onto a carbon mesh copper grid and allowed to sit for 1 min. After that, excess peptide solution was carefully removed by filter paper. Then the sample was dried for 1 min, followed by the addition of 10 μL of 2 wt% phosphotungstic acid aqueous solution (pH was adjusted to 7.0) for negative staining. After 90 s, excess staining solution was removed and the TEM samples were allowed to air-dry before