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Supramolecular nanoparticles of calcitonin and dipeptide for long-term controlled release

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ABSTRACT

Salmon calcitonin (sCT) is a therapeutic polypeptide drug widely used to treat bone diseases such as osteoporosis (more than 200 million patients all over the world). The half-life of sCT is very short (~1 h), thus various delivery systems have been developed for sCT in order to avoid frequent injections. However, most delivery systems use polymeric materials, which may limit their applications in clinic formulations due to the biocompatibility issue. We observed that a very simple dipeptide (Asp-Phe, DF) was co-assembled with sCT into supramolecular nanoparticles. These nanoparticles can significantly prolong the acting time of sCT to beyond one month after just a single subcutaneous injection. The assembling and releasing mechanisms were thoroughly investigated by both *in vitro* and *in vivo* methods, as well as by molecular dynamics simulations. This work provides an alternative strategy of designing protein/peptide drug delivery systems with long-lasting therapeutic effects.

1. Introduction

The clinic applications of protein/peptide drugs have been restricted by various limitations such as physical and chemical instability, low oral bioavailability, susceptibility to enzymatic degradation, and short *in vivo* half-lives [1,2]. For example, calcitonin (CT) is a polypeptide hormone drug containing 32 amino acid residues. It is capable of inhibiting the bone resorption of calcium through the interaction with osteoclasts, and thus has been applied as a therapeutic agent for bone diseases for more than 30 years [3]. Such bone diseases include osteoarthritis (OA) [4], osteoporosis, Paget's disease, hypercalcemia and bone associated pain conditions [5,6]. Among all the CT species, salmon calcitonin (sCT) is by far the most widely used one in clinical practice because it offers the highest intrinsic potency (40–50 times higher than human calcitonin) and its greater analgesic effect [3,7]. Although oral delivery is the favoured administration method due to its convenience and patient compliance [8–10], so far there is no commercial oral sCT product because of its poor absorption and rapid proteolytic degradation [11–15]. Although intranasal spray sCT is commercially available, the pulmonary barrier, the possibility of mucociliary clearance and the digestion of pulmonary enzymes significantly influence the absorption of sCT and deduce its bioavailability [16–18]. Currently, subcutaneous or intramuscular injection is the preferred sCT administration method, but is associated with poor patient compliance due to the need for frequent injections (the half-life is around 1 h). Therefore, many researchers have attempted to develop long-term controlled release systems that are based on polymeric materials for sCT, so as to extend its therapeutic efficacy after a single injection [19–23]. Such systems have shown remarkable prospects for the controlled delivery of sCT, but the design of suitable polymers remains a challenge and their approval for clinical trials may be hard and time-consuming.

Supramolecular assemblies of protein/peptide play important roles in living organisms. Inspired by these natural structures, many protein/peptide supramolecular assemblies have been constructed to offer new functionalities. Among these, short peptides are excellent building

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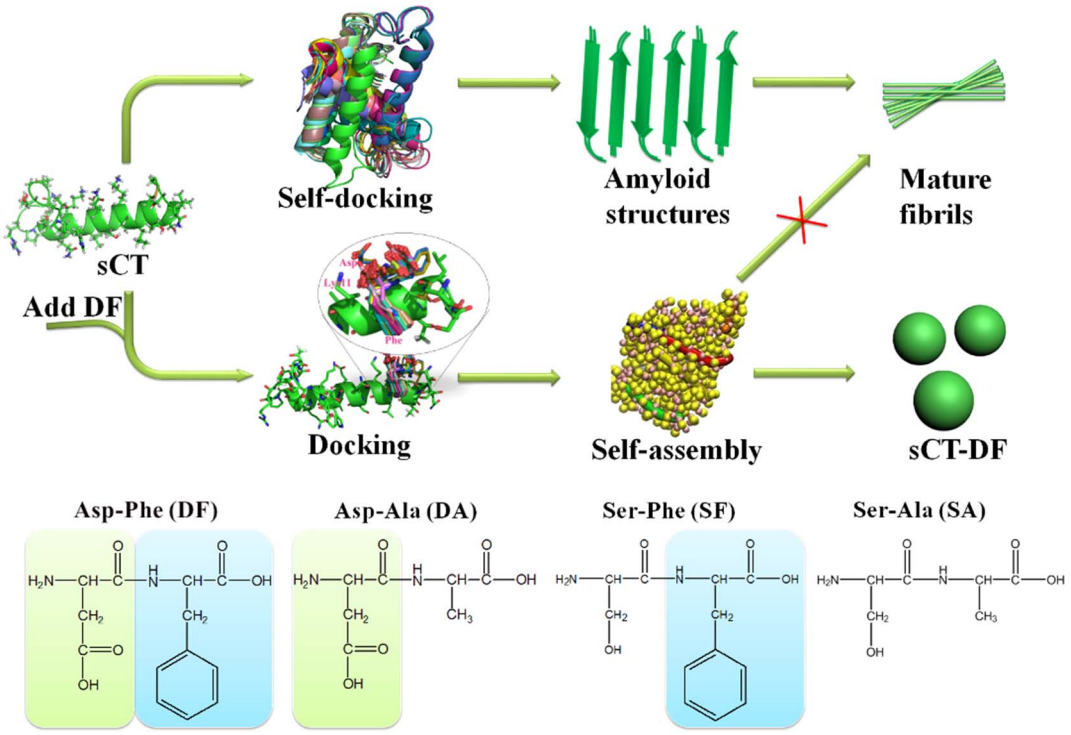

Scheme 1. Schematic representation of the self-assembly of the supramolecular nanoparticles. sCT easily forms fibrilous aggregates, as shown by self-docking studies of sCT, leading to the formation of amyloid structures that self-assemble into mature fibrils. However, with the addition of Asp-Phe (DF) into fresh salmon calcitonin solution, DF interacts with sCT through both hydrophobic and electrostatic interactions (docking models of sCT-DF), leading to the self-assembly of supramolecular nanoparticles rather than mature fibrils.

blocks of various supramolecular assemblies [24,25] owing to their low cost of production, ease of running molecular dynamics simulations and many promising properties such as energy transfer/photoluminescence [26,27], tuneable mechanical properties [28], and biocompatibility/bioactivity [29]. For example, Yang et al. presented an elegant example of core-shell nanoparticles assembled from an amphiphilic cationic peptide. These particles exhibited strong antimicrobial properties, with a higher therapeutic index against infection than that of their un-assembled peptide counterparts [30]. Recently, Hecht et al. also demonstrated the feasibility of incorporating dipeptides and their analogues into proteins by ribosome-mediated process [31]. From the viewpoints of both biocompatibility and feasibility, peptides (especially short ones) can be good candidates for forming supramolecular assemblies with protein/peptide drugs and can potentially improve their therapeutic efficiency.

In this research, we rationally designed short dipeptides that could assist in the assembly of sCT into supramolecular nanoparticles (Scheme 1). The isoelectric point (pI) of sCT is 9.3, thus it is positively charged at neutral pH (7.4). We designed a short dipeptide (Asp-Phe, DF) that contains a negatively charged residue (Asp) and a hydrophobic residue (Phe). Docking studies showed a strong electrostatic interaction between the Lys11 of sCT and the Asp of the dipeptide. The benzene ring of Phe in the dipeptide was located outside of the alpha-helix groove of sCT, which indicated the presence of strong hydrophobic interactions between the dipeptide and sCT. Thus, the dipeptide interacted with sCT through both electrostatic and hydrophobic interactions, and then co-assembled into supramolecular nanoparticles. The supramolecular dipeptide-calcitonin nanoparticles (SDCT-NPs) retained the bioactivity of sCT. Thus, they could act as a drug depot after subcutaneous injection and prolong acting time of sCT *in vivo*.

2. Materials and methods

2.1. Materials

Salmon calcitonin (sCT) and dipeptides were obtained from GL Biochem (Shanghai, China). Heparin sodium, thioflavin T (ThT), the kits for serum calcium and salmon calcitonin and other materials were purchased from Baoxin Biotechnology Co. Ltd. (Chengdu, China). Commercially available reagents were used without further purification. Ultrapure water was used in all experiments.

2.2. General methods

Samples for atomic force microscopy (AFM) measurement were diluted 20-fold with water and then spin-coated on freshly cleaved mica using a spin processor, then left to dry overnight. All AFM images were captured in tapping mode with a spring constant of 40 N/m and a resonant frequency of 300 KHz, using a Nanoscope Multimode (Veeco Instruments, USA). Transmission electron microscopy (TEM) samples were vortexed and diluted 100-fold. They were then immediately absorbed onto 300 mesh copper grids, incubated in 2% uranyl acetate for 2–3 min, and dried under infrared light to be examined by negative staining. The grids were visualized at 200 kV by Transmission Electron Microscope (FEI Tecnai G2 F20 S-TWIN, USA).

2.3. Preparation and ThT study of SDCT-NPs

Salmon calcitonin (0.1 mM) and dipeptides (1.0 mM) were dissolved in 5 mM phosphate buffer (PB, pH 7.4), and then incubated at 25 °C for predetermined time periods. Then the SDCT-NPs obtained after 3 h of incubation were lyophilized for the following tests (except for the ThT test). The self-assembly process of SDCT-NPs was monitored by ThT fluorescence from the very beginning as follows: 20 μL of SDCT-NPs samples was added to 980 μL of 3 μM ThT (in 5 mM PB, pH 7.4), and then incubated at 25 °C for 15 min. Fluorescence intensity was