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Cathepsin nanofiber substrates as potential agents for targeted drug delivery

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ABSTRACT

The development of reactive drug carriers that could actively respond to biological signals is a challenging task. Different peptides can self-assemble into biocompatible nanostructures of various functionalities, including drugs carriers. Minimal building blocks, such as diphenylalanine, readily form ordered nanostructures. Here we present the development of self-assembled tetra-peptides that include the diphenylalanine motif, serving as substrates of the cathepsin proteases. This is of great clinical importance as cathepsins, whose activity and expression are highly elevated in cancer and other pathologies, have been shown to serve as efficient enzymes for therapeutic release. Based on the cathepsins affinity around the active site, we generated a library of Phe-Phe-Lys-Phe (FFKF) tetra-peptide substrates (TPSs). We inserted various N-termini capping groups with different chemical properties to investigate the effect on protease affinity and self-assembly. All nine TPSs were cleaved by their targets, cathepsins B and L. However, solvent switching led to nanofibers self-assembly of only seven of them. Due to its rapid self-assembly and complete degradation by cathepsin B, we focused on TPS4, Cbz-FFKF-OH. Degradation of TPS4 nanofibers by cathepsin B led to the release of 91.8 ± 0.3% of the incorporated anti-cancerous drug Doxorubicin from the nanofibers within 8 h while only 55 ± 0.2% was released without enzyme treatment. Finally, we demonstrated that tumor lysates fully degraded TPS4 nanofibers. Collectively, these results suggest that tetra-peptide substrates that form nanostructures could serve as a promising platform for targeted drug delivery to pathologies in which protease activity is highly elevated.

1. Introduction

To date, several drug delivery systems have been developed and applied overcoming both pharmacological limitations such as solubility, elimination half-life, stability and absorption, and pharmacodynamic difficulties such as specificity and low therapeutic index. Passive targeting of various nanoparticles like micelles and liposomes loaded with drugs have demonstrated great promise as drug-carriers utilizing the enhanced permeability retention (EPR) effect for delivery (for reviews, see [1–3]). In parallel, numerous groups have applied active targeting by decorating particles with targeting moieties such as receptor ligands [4], sugars [5], antibodies [6,7] and small molecule inhibitors [8].

The release of targeted drugs is often achieved by attaching the drug load to a carrier through specific peptide sequences which are cleaved by proteases that are highly expressed at the desired location. The peptide-drug conjugate can be created using hydrophobic or other non-covalent interactions, covalent modifications or as a part of a polymeric chain [9,10]. For example, drug-loaded liposomes and mesopores with short peptide targeting sequences have been introduced to enable drug release upon enzyme recognition and cleavage [11,12]. The cathepsin proteases thus often serve as the enzymes that release the drugs from their carrier.

Lysosomal cathepsins are a subfamily of cysteine proteases known to be involved in several processes of cancer progression such as tumor growth, invasion and metastasis [13,14]. Overexpression and activity of cathepsins B have been found in cancer as well as other pathologies, making them exceptional targets for precision therapeutics [14]. Tumor cell cathepsins are often secreted and translocate from their lysosomal location to the tumor microenvironment, providing an additional opportunity for targeting non-cell permeable therapeutics [8,15]. In particular,

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cathepsin B has been found to be highly overexpressed and active in tumor associated macrophages (TAMs) making them a potential target for cancer immunotherapy [16]. An essential component of substrate specificity of cathepsins, as in other proteases, is recognition of the sequence around the scissile (cleaved) bond within the substrate. Many studies have revealed cathepsin proteases affinity for positively charged amino acids at the P1 site and bulky hydrophobic amino acids at the P2 and P3 sites [17–19]. A recent example of a cathepsin substrate that meets the above criteria is the Cbz-Phe-Lys that has been used by several groups for targeting of therapeutics and imaging reagents [18,20–25].

The use of short peptide sequences for nanotechnology and nanomedicine has recently gained popularity due to their biodegradability, simple preparation, reproducibility, and ability to spontaneously form 3D structures [26–28]. The most prominent are hydrophobic peptide building blocks that can self-assemble into well-organized nanostructures [29–32]. The process of self-assembly can be induced by different triggers such as enzymatic catalysis [33], light induction [34], or changes in temperature [35], pH [36,37] or solvent [38,39]. The simplest and most widely studied of these building blocks are the Phe-Phe (FF) variants whose self-assembly is mostly governed by hydrophobic and aromatic π-π stacking interactions [40,41]. Due to their lack of toxicity, FF nanostructures are highly biocompatible and therefore used for biological applications. Recently, FF nanostructures have been reported in several biological systems for applications in 3D cell culture [42,43], bio-imaging [44], biosensors [32,45] and drug delivery, where FF nanostructures, known to undergo endocytosis, were applied for transporting oligonucleotides and small molecules to intracellular destinations [46,47].

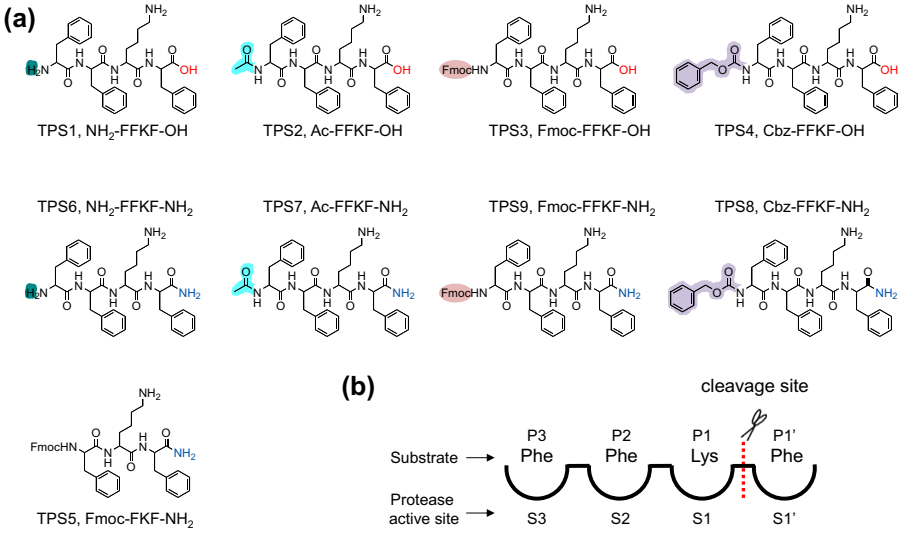
The popularity of the FF motif for biological applications together with the known cathepsin affinity for hydrophobic amino acids encouraged us to design a self-assembly cathepsin tetra-peptide substrate (TPS) for therapeutic delivery. We reasoned that a short peptide sequence that contains di-aromatic and a positively charged amino acid could serve as a potential cathepsin substrate and simultaneously allow for self-assembly. Therefore, we explored a variety of Phe-Phe-Lys-Phe (FFKF) analogs as self-assembled cathepsin substrates to be used as carriers with specific drug release properties.

2. Results and discussion

To target the delivery of therapeutics to cancerous tissues with elevated cathepsin activity, we designed a series of TPSs based on the FFKF scaffold. To optimize the TPSs self-assembly our peptide designs contain unique N-termini chemical groups and charge variation at the C-termini (Scheme 1a). Aromatic chemical groups were introduced at the N- and C-termini to ensure that the addition of the Lys to the FF variant will not impair the self-assembly process. Following synthesis of these peptides (Scheme S1), we studied their ability to be recognized and cleaved by cathepsin proteases. The peptides were incubated with either cathepsin B or cathepsin L and their cleavage products were analyzed using mass spectroscopy (MS). A tri-peptide fragment FFK was the expected cleavage product since the target site for cleavage by these cathepsins is

postulated to be the amide bond after the P1 Lys, recognized by the protease S1 pocket (Scheme 1b) [17–19,48]. Results obtained from MS analysis revealed that all TPSs were recognized and cleaved by both enzymes. In addition to the expected tri-peptide cleavage product, di-peptides and/or single amino acids were detected (Fig. 1), as accounted for by the known highly promiscuous nature of the cathepsin proteases [49]. It is widely accepted that the sequence determines the substrate specificity to a protease, in some cases we found correlations between the cleavage pattern and the peptide N-termini capping group. In cathepsin B for example, a charged amine group led to removal of a single amino acid while bulky hydrophobic groups (Fmoc or Cbz) led to removal of two or three amino acids.

Next, we investigated the ability of the different TPSs to self-assemble into ordered nanostructures using a solvent-mediated approach to trigger the assembly [50,51]. Nanostructures formation was verified by transmission electron microscopy (TEM), revealing that all TPSs self-assembled into ordered structures with morphology of elongated nanofibers network, excluding TPS6 and 7 (Fig. 2 and Supplementary Fig. S1). In most cases the addition of a charged amino acid, Lys, to the fundamental FF structure did not prohibit structure formation, most likely because of the many aromatic rings within the TPSs. We observed variance in the nanofibers diameters of the different TPSs (Table S1) that we attribute to the chemical modifications at the N- and C-termini. Aromatic moieties (such as Fmoc and Cbz) and carbonyl/amide groups (Scheme 1) can contribute to the total π-π and hydrogen bond interactions, respectively. These additional interactions may enable stronger stacking forces yielding “well-packed” nanostructures with relatively smaller diameter [52]. Interestingly, upon assembly initiation TPS4 stood out since it instantly formed fibers, while most other TPSs took over 2 h to assemble, as was confirmed by microscopic examination (data not shown).



Scheme 1. Structures of TPSs. (a) Pairs of TPSs were designed based on the FFKF scaffold, each pair carrying a unique N-termini chemical group and a variation in the C-termini charge. Carboxyl group is marked in red and amide group in blue. (b) Protease-substrate standard notation, protease binding pockets (S) and corresponding substrate amino acid (P). The cleavage site is the scissile bond between the *P*1 (N-termini to the scissile bond) and *P*1′ (C-termini to the scissile bond) residues. Adopted from Schechter and Berger [47].