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Self-Assembly of a 5-Fluorouracil and Camptothecin Dual Drug Dipeptide Conjugate

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Nano-formulated, combinatory therapeutics that control the spatiotemporal aspects of drug release have potential to overcome many of the challenges faced in cancer therapy. Herein, we describe a peptide nanotube functionalized with two anticancer drugs, 5-fluoruracil (5-FU) and camptothecin (CPT). The nanotube was formed via peptide self-assembly, which positioned 5-FU on the surface at the aqueous interface; whereas, CPT was sequestered within the hydrophobic walls. Thus, two different release profiles were observed: rapid release of 5-FU, followed by slower, sustained production of CPT. This profile emerged from the rapid hydrolytic cleavage of 5-FU at the aqueous/nanotube interface, which produced a smaller nanotube comprised of the peptide fragment.

The delivery of combinations of anticancer drugs using nanotechnology-based carriers has emerged as an important strategy to effectively treat cancer.[1,2] Delivery vehicles with nanoscale dimensions also benefit from the enhanced permeability and retention (EPR) effect, which allows the drugs to be delivered selectively into tumor cells.[3] Combination therapy offers the potential to synergize multiple mechanisms of drug action, to reduce systemic toxicity and to suppress drug resistance.[4-6] One strategy to coordinate the cellular uptake of multiple therapeutic agents relies on encapsulation within a single delivery vehicle, such as a liposomal or polymeric nanoparticle.[7-9] However, formulating these systems using drugs with different physiochemical properties, such as size, charge and/or solubility, has been difficult and often produces systems exhibiting unpredictable release rates and noncoordinated biodistribution of the drugs.[10] Covalent conjugation or modification to render the drugs more compatible with the vector have produced carriers with more precise ratiometric control over drug loading.[11-14]

The self-assembly of amphiphilic or peptidic drug derivatives provides another strategy to create delivery vehicles without the need for excipients.[15] Carrier-free, nanoscale systems created by the self-assembly of two or more covalently-linked drugs offer the additional advantage of high loading, improved biocompatibility and the assurance of drug colocalization within the cell.[16-23] The ability to tailor the amphiphilic monomers with hydrophilic and hydrophobic segments allows some of the difficulties of incorporating drugs with different physical properties to be overcome with proper design. These platforms often release each drug at a similar rate because loss of the first drug alters structure of the nanostructure building block, which leads to changes in the stability of the assembly.[24] In this work, we report the self-assembly of a dual drug peptide into a nanotube that hydrolytically releases 5-FU without loss of the nanotube structure, thereby allowing for slower, sustained release of CPT.

Combination therapy using irinotecan, a CPT analog, along with 5-FU has shown efficacy against gastrointestinal cancers.[25-27] To explore the potential of combining two drugs with vastly different solubility into a single, self-assembled nanotube, we functionalized a dilysine peptide, via succinyl linkages, with 5-FU at the *N*-terminus, and with CPT at the ε-amino group of a lysine residue. In contrast to the hydrophobic nature of CPT, which exhibited low water solubility, the structure of 5-FU was hydrophilic and water soluble. We reasoned that the juxtaposition of the two drugs along the peptide backbone of **1** (Fig. 1) would create an amphiphilic structure capable of assembling via β-sheet interactions and hydrophobic sequestration of the CPT segment.[28-30] Nanotube assembly in aqueous media would position the hydrophilic 5-FU group at the aqueous interface with the CPT molecule sequestered within the hydrophobic walls. This arrangement would be expected to permit the 5-FU to undergo hydrolytic release more

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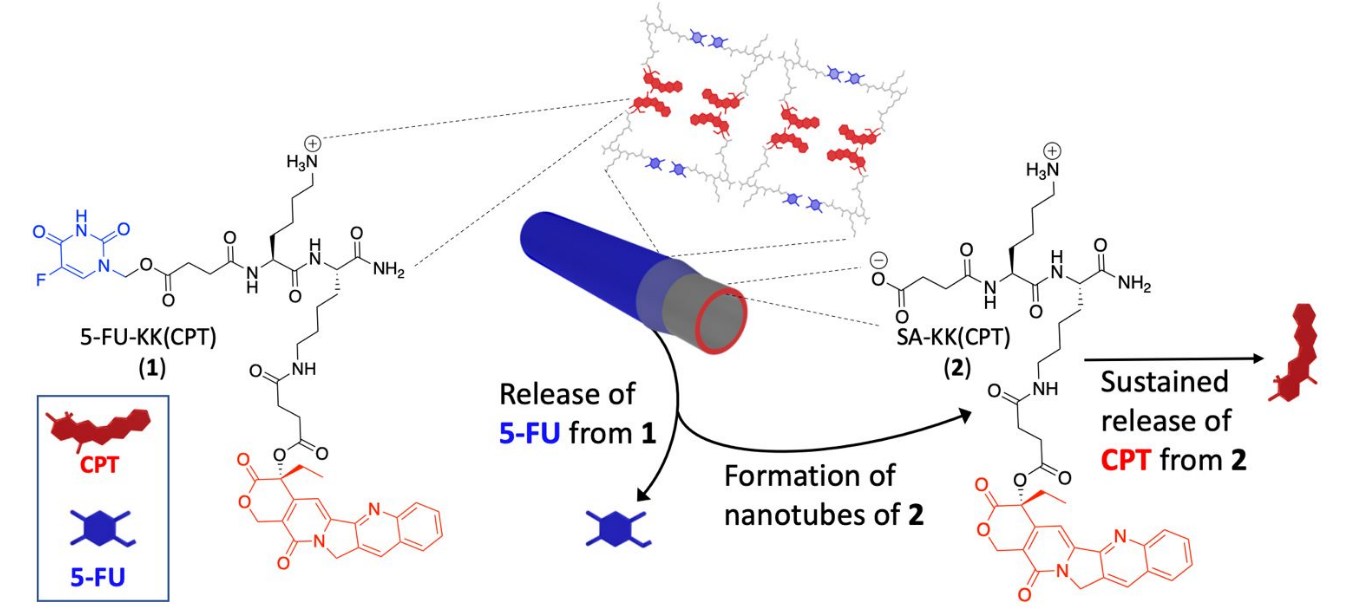

Fig. 1. Stepwise release of CPT and 5-FU from nanotubes, created by the self-assembly of 5-FU-KK(CPT) (**1**). Hydrolytic cleavage of 5-FU from **1** produces nanotubes of SA-KK(CPT) (**2**).

rapidly than CPT. However, the remaining CPT-peptide fragment (**2**) would retain amphiphilicity due to the presence of the negatively charged succinate at the *N*-terminus. On that basis, we hypothesized that the nanotube structure would remain intact following release of the 5-FU, which would permit slower, sustained release of CPT.

To evaluate the assembly characteristics of the peptide carrier prior to and following hydrolytic release of 5-FU, peptides **1** (5-FU-KK(CPT)) and **2** (SA-KK(CPT)) were prepared using standard Fmoc/*t*-Boc solid-phase peptide synthesis (Scheme S1). CPT-peptide **2** would serve as the carrier of CPT upon initial hydrolytic release of 5-FU from **1.** Accordingly, stepwise deprotection of the Fmoc and MTT groups of a dilysine backbone, followed by on-resin amidation allowed for the selective introduction of 5-Fu (**1**)/succinic acid (**2**), and CPT at the *N*-terminal and sidechain positions, respectively. The self-assembly of **1** and **2** was then explored by transmission electron microscopy (TEM) and UV-Vis/CD spectroscopy. Each peptide was incubated at a concentration of 10 (for **2**) or 20 mM (for **1**) in PBS for 72 h to induce self-assembly. After diluting to 1 mM, TEM imaging revealed the formation of an array of nanotubes from both peptides, showing average diameters of 84 ± 11 nm for **1** and 72 ± 11 nm for **2** (Fig. 2). The UV-Vis spectrum of **1** displayed absorption bands at 254 and 368 nm, corresponding to transitions of the 5-FU and CPT chromophores, respectively in PBS (Fig. 3a). The circular dichroism (CD) spectrum of **1** exhibited signals in the 230-280 and 350-400 nm ranges, overlapping with the absorption ranges of both drugs. In contrast, **2** exhibited only weak CD signals in PBS, indicative of comparatively weaker interactions between the CPT chromophores in the assembly. The small amount of red-shifting in CPT absorptions in the UV-Vis spectra, and the relatively weak CD signals of **1** and **2** indicated that the packing of the drugs within the nanotubes emanated primarily from hydrophobic effects, rather than well-defined π–π interactions.[31] Deconvolution of the Fourier-transform infrared (FTIR) spectra in PBS (20 mM, D_2O) revealed amide I bands indicative of 86% (**1**) and 53 % (**2**) β-sheet character of the nanotube secondary structures (Figs S12-13).[32] The critical aggregation concentrations (CAC) of the nanotubes were determined to be 5 μM and 263 nM for **1** and **2**, measured by recording the concentration dependence of the CPT fluorescence (Figs. S14-15).[33,34] These CAC values suggested that the stability of the nanotube increased after release of the 5-FU.

The presence of helically coiled intermediates among the fully formed nanotubes of **1** and **2** in TEM images indicated that assembly proceeded via the coiling and lateral fusion of β-sheet ribbons (Figs. 2a/c, insets). AFM imaging of the nanotubes provided cross-sectional heights of 9-10 (**1**) and 5-6 nm (**2**), reflecting twice the thickness of the walls due to compression of the nanotube by the AFM tip (Figs. S18-19). Thus, the extended dimensions of **1** (2.1 x 2.5 nm, Fig. S9) and **2** (1.4 x 2.5

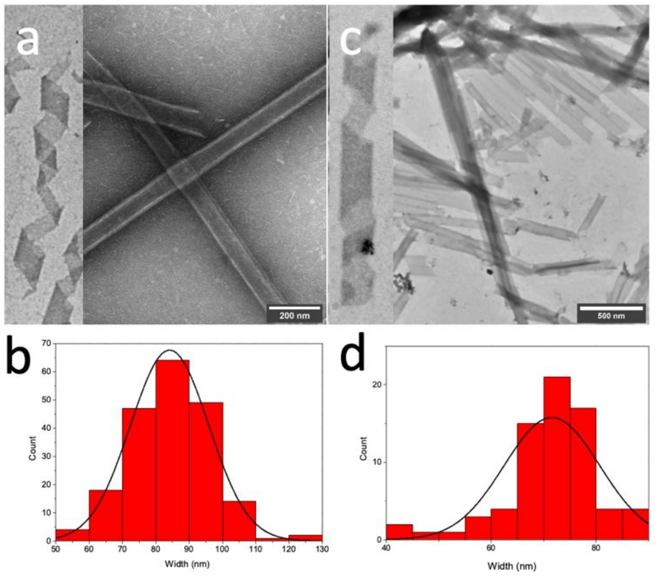

Fig. 2. TEM images and width distribution histograms of the nanotubes formed by 5-FU-KK(CPT) (**1**) (am) and SA-KK(CPT) (**2**) (c,d). The average diameters were 84 ± 11 nm and 72 ± 11 nm for **1** and **2**, respectively. Samples were aged in PBS at 10 (for **2**) or 20 mM (for **1**) for 72 h and imaged on copper coated grids at 1 mM, stained with 2% uranyl acetate. TEM insets highlight helically coiled precursors to the nanotubes.

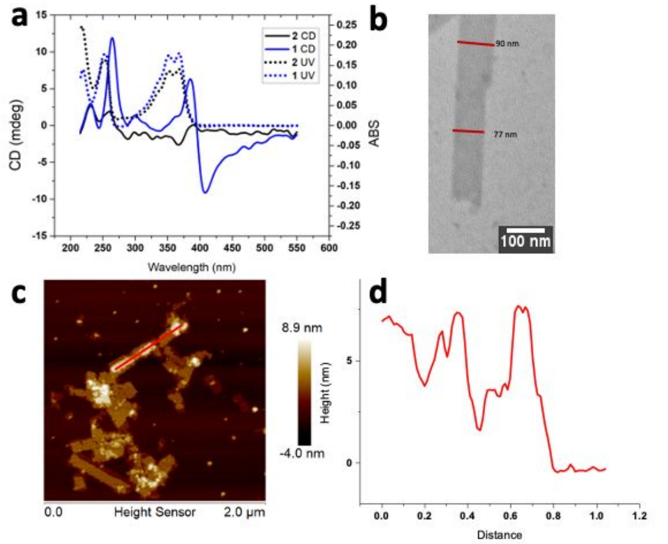

Fig. 3. (a) Co-plot of CD (solid lines) and UV (dotted lines) of **1** (blue) and **2** (black). Samples were aged in PBS (pH 7.4) at 10 mM for 72 h then diluted to 0.1 mM before analysis. (b) TEM images of **1**, assembled in PBS (pH 7.4) for 72 h at 10 mM, then diluted to 0.1 mM and heated to 37°C for 72 h to induce drug release. Image shows regions of the nanotube with smaller diameters, consistent with hydrolytic release of 5- FU and the formation of nanotubes from **2**. (c) AFM images and (d) cross-sectional height analysis of **1** after drug release at 37°C for 24 h at 0.1 mM. Samples were prepared by incubating **1** in PBS (pH 7.4) at 10 mM for 72 h, then diluting to 0.1 mM and heating at 37°C for 24 h. Samples for TEM were stained with 2% uranyl acetate solution. AFM samples were deposited on a freshly cleaved, mica disk and analyzed in tapping mode.

nm, Fig. S10), suggested that the nanotube walls of both structures were comprised of β-sheet bilayers of approximately two molecules, as shown in Fig. 1. Therefore, a structural model of nanotubes formed by **1** would sequester the hydrophobic CPT moieties within the bilayer wall with the hydrophilic 5-FU molecules projected toward the aqueous interface.

Hydrolytic release of 5-FU required initial cleavage of the acyloxymethylene linkage, followed by subsequent collapse of the resultant hemiaminal intermediate, whereas hydrolysis of the 20-*O*-succinyl linkage would release CPT from the nanotube carrier.[35,36] The positioning of 5-FU at the surface of the nanotube exposes the ester linkage to the aqueous phase, and would portend a faster rate of hydrolytic release, compared to CPT. The release of both 5-FU and CPT were recorded by HPLC over 5 days in PBS and human serum (HS) at 37°C at 0.1 mM and 1 mM (Figs. 4, S22-24). It is notable that the rate of 5-FU release was significantly higher than CPT in PBS, but the rate difference decreased at lower concentration. For example, whereas 90% of 5-FU was released after 48 h at 1 mM, only 15% of CPT had been released at the same time point. Although the amount of 5-FU released at 0.1 mM was similar (95%) after 48 h, the proportion of free CPT increased to 31%. Whereas, the release rate 5-FU was similar in human serum, CPT was released at a slightly higher rate compared with PBS. For example, 80% 5-FU and 44% CPT was produced after 40 h at 0.1 mM in HS. (Fig. S24). The difference in the rate of release of 5-FU and CPT emerged from the selective shielding of CPT from the aqueous phase by the nanotube structure. At lower concentrations, a shift in the nanotube-monomer equilibrium toward the monomer state increased the release rate and decreased the rate difference between 5-FU and CPT.

Self-assembled nanocarriers with multiple, covalently bound drugs often release each drug at a similar rate because chemical cleavage of one drug reduces the stability of assembled structure. Although the concentration of **1** decreased rapidly upon exposure to PBS at 37°C, concurrent with the release of free 5-FU and **2**, the amount of **2** remained reasonably stable over 120 h. The stability of **2**, and the slower extended release of CPT over time, emerged from the ability of **2** to remain assembled into nanotubes following 5-FU release, with similar dimensions to those of **1**. Imaging the nanotubes as the drugs were released by TEM over time in PBS at 37°C showed regions with abrupt decreases in diameters from ~90 to 77 nm, consistent with the dimensions measured for nanotubes formed by **1** and **2**, respectively (Fig. 3b, S20). Similar changes in cross-sectional heights could similarly be observed by AFM after 24 h at 37°C (Figs. 3c, S21). These presence of these steps in

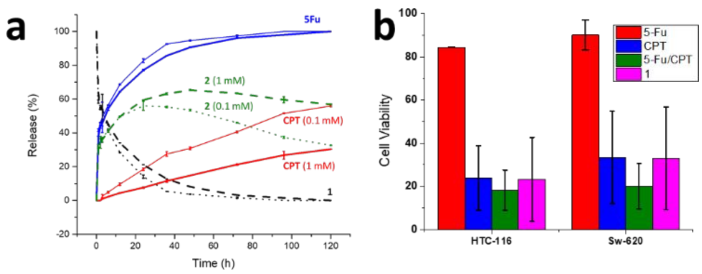

Fig. 4. (a) Release of free CPT (red) and 5-FU (blue), and breakdown/formation of **1** (black) and **2** (green), as monitored by HPLC, from nanotubes of **1** in PBS at 37.5 at 0.1 (thin lines) and 1 mM (thick lines) over 120 h. (b) MTT cytotoxicity assay comparing 5-FU, CPT, 5-FU/CPT and **1** activities against human non-small cell lung cancer cell lines H116 and SW620.

diameter suggested that hydrolytic erosion of the nanotube surface produced another slightly smaller nanotube, derived from **2**, that persisted after initial release of 5-FU

The dual drug nanotube (**1**) was assessed for efficacy against human non-small cell lung cancer (NSCLC) cell lines H116 and SW620 and was compared to CPT, 5-FU and the combined solution of 5-FU and CPT. The cytotoxic activity was assayed using an MTT-assay over the course of a 96 h incubation period to determine cell viability. Peptide **1** exhibited significantly higher cytotoxicity than 5-FU alone, due to the presence of CPT in the conjugate, and similar efficacy as CPT alone or as a combination with 5-FU, against both cell lines. Further studies will be required to evaluate the ability of this dual-drug construct to reduce systemic toxicity and drug resistance in vivo.

Conclusions

We have described a dual-drug, peptide nanotube that sequentially released free 5-FU and CPT in PBS and showed efficacy against two human lung cancer cell lines. The nanotube vehicle was created by the self-assembly of **1** in aqueous media, which positioned the hydrophilic 5-FU molecule at the surface of the nanotube and sequestered CPT within the hydrophobic nanotube walls. This structural feature caused the release profiles of the two drugs to be distinct. Accordingly, a rapid release of 5-FU was followed by the slower, sustained production of CPT. The slower sustained release profile of CPT was maintained by the persistence of nanotubes of peptide **2**, which were produced from **1** upon hydrolytic release of 5-FU. The ability to design self-assembled drug carriers that remain assembled after initial drug releases should facilitate the development of new strategies for delivering combination therapy.

Conflicts of interest

There are no conflicts to declare.

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