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Self-assembly of a 5-fluorouracil-dipeptide hydrogel†

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The self-assembly of 5-fluorouracil dilysine conjugates into self-supporting hydrogels, comprised of entangled nanofibers or rigid nanotubes with diameters of 10 and 16 nm, respectively, is reported. The rate of release of 5-Fu from the conjugates was highly dependent on concentration in solution, whereas, release from the fully formed hydrogels was significantly slower. The 5-Fu conjugate also exhibited promising *in vitro* cytotoxicity against human tumor cell lines A549, H460 and H23.

Nanotechnology enhanced drug delivery promises a capability to favorably control the pharmaco-kinetics, biodistribution and efficacy of anticancer therapeutics.[1,2] Peptide-based gelators[3–5] are receiving significant interest for biomedical applications such as drug delivery,[6–17] tissue engineering,[18–21] biomacromolecule immobilization,[22–24] and regenerative medicine[25–28] due to their biocompatibility, injectability, and controllable formation/degradation rates. Compared with conventional polymeric hydrogels, peptide gelators are formed primarily through non-covalent interactions such as hydrophobic interactions, π–π stacking, and hydrogen bonding. The non-covalent structures of these hydrogels enables them to be formed, biodegraded and excreted *in vivo*, making them ideal as biomedical delivery vehicles.[29–31] The active drug can often be physically entrapped within the hydrogel matrix, but this strategy often suffers low or variable drug loading/encapsulation levels and uncontrollable release kinetics.[6,7,31,32] In this work, we report a self-assembled, low molecular weight hydrogel composed entirely of a 5-fluorouracil drug conjugate organized into nanotube assemblies.[10,33–35] The hydrogels maintain high drug loading levels, and exhibit slow drug release profiles controlled by the nanotube and hydrogel structures.

5-Fluorouracil (5-Fu) is an antimetabolite drug whose mechanism of action involves the irreversible inhibition of thymidylate synthase *via* competitive binding.[36–38] It has been used as an anticancer agent against various tumors such as anal, breast, colorectal, and skin cancers—as well as for treating actinic or solar keratosis.[39–41] Injectable hydrogel formulations offer localized delivery and sustained release profiles,[27,42,43] and have potential for the treatment of colorectal cancer.[44] Nanoscale carriers have also been reported to reduce the occurrence of acquired drug resistance (ADR),[2,45,46] which often emerges from the high doses of 5-Fu necessary in many therapeutic applications. In dermatological treatment protocols, 5-Fu is generally administered intravenously or as a ~5 wt% topical cream or ointment.[47,48] A low molecular weight hydrogel comprised of 5-Fu organized into nanotubes, has potential to enhance the clinical utility of 5-fluorouracil.

Dipeptides **A** and **B** were prepared using standard Fmoc/*t*-Bu solid-phase peptide synthesis, wherein the 5-Fu moieties were introduced by on-resin modification of the supported peptide (Scheme S5, ESI†). The design was based on previously described, dilysine peptide motifs that effectively assembled into various nanostructures, such as nanotubes, nanobelts, and nanofibers in water.[49–54] In this design, β-sheet self-assembly is driven by hydrophobic π–π association of both the uracil and Fmoc chromophores in aqueous media. The tendency toward infinite β-sheet assembly into insoluble amyloid-type aggregates is opposed by the electrostatic repulsions of adjacent protonated lysines within the assembly, which also promote water solubility. The 5-Fu moiety was appended at N-1 to the ε-amine of the C-terminal lysine residue *via* either a hydrolytic, self-immolative succinate (**A**) or a stable, acetamide linkage (**B**).[55] Based on the structures of **A** and **B**, the calculated drug loadings were 17.6 and 19.6%, respectively (Fig. 1). Accordingly, hydrogels **A** (formed at 20 mM) and **B** (formed at 10 mM) contain 0.26 wt% and 0.13 wt% 5-Fu, respectively.

The hydrogelation of dipeptide conjugates **A** and **B** was studied in phosphate buffered saline (PBS, pH = 7.4). Conjugates **A** and **B**

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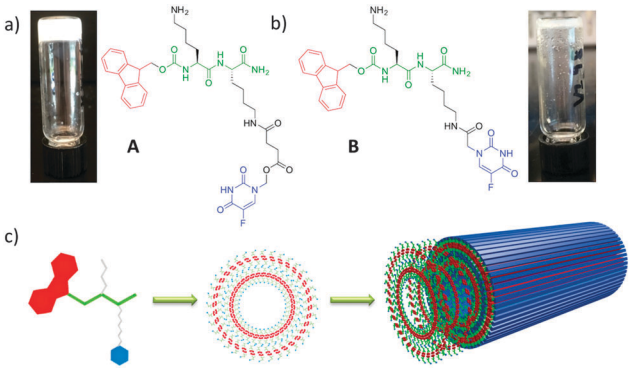

Fig. 1 Structural design and self-assembly of compounds **A** and **B**. (a) Dipeptide **A** and the hydrogel formed after aging at 20 mM in PBS for 3 d; (b) dipeptide **B** and the hydrogel formed after aging at 10 mM in PBS for 3 d; (c) self-assembly of **A** into bilayer rings, which further stack into 1D nanotubes.

both formed clear, self-supporting hydrogels[56] after briefly sonicating (~1 min) at low concentrations (**A**, 20 mM; **B**, 10 mM) in PBS then aging for 72 h. The structure of the hydrogels formed by **A** and **B** were further studied using transmission and scanning electron microscopy (TEM and SEM) (Fig. 2). The hydrogel formed by **A** consisted of a network of one-dimensional nanotubes with diameters of ~16 nm, wall thicknesses of ~4 nm and lengths of several micrometers (Fig. 2a). The dimensions of the nanotube walls are consistent with a bilayer structure comprised of two molecules of **A**. Additionally, the occasional presence of intermediate ring structures with identical dimensions as the nanotubes indicated a progressive assembly process whereby an initially formed bilayer ring subsequently stacked into the nanotube (Fig. 2a, inset).[53] The hydrogel formed by **B** displayed a network of nanofibers

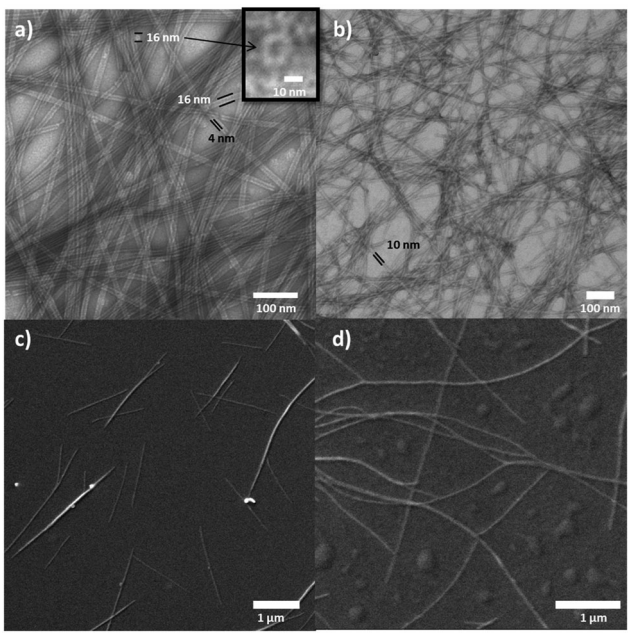

Fig. 2 TEM (top) and SEM (bottom) images of compound **A** (a and c) and **B** (b and d) in PBS samples were prepared by aging compound **A** (20 mM) or **B** (10 mM) in PBS for 3 days at pH 7.4, then diluting to 1 mM.

with diameters of ~10 nm (Fig. 2b). The critical aggregation concentrations (CACs), as measured using the solvochromatic dye Nile Red,[57] of freshly dissolved solutions of **A** and **B** in PBS, prepared without preincubation, were 1.50 and 0.78 mM, respectively (Fig. S1, ESI†). It is noteworthy that a 0.25 mM sample of dipeptide **A** did not undergo gelation, only exhibiting a small degree of non-specific aggregation by TEM, incapable of encapsulating Nile Red (Fig. S5, ESI†). It is likely that these nonspecific aggregates precede the formation of the intermediate ring structures en route to the final nanotube assemblies. However, the unstable nature of the rings, relative to the nanotubes, makes it difficult to assess the concentration at which these form in the assembly process.

The broad bands exhibited by **A** and **B** in PBS at 264 nm in the ultraviolet (UV-Vis) spectra were slightly red-shifted (~2 nm), compared with the spectra in 2,2,2-trifluoroethanol (TFE), in which aggregation was minimal (Fig. 3a). The red shifting of the absorption at 264 nm that occurs upon solvent-induced assembly was indicative of J-type aggregation of the Fmoc chromophore within the assemblies.[58] Deconvoluted Fourier-transform infrared (FT-IR) spectra of samples, prepared by lyophilizing the hydrogels formed in PBS then redissolving in D_2O (20 mM), revealed predominant bands at 1639 cm^{-1} (for **A**) and 1634 cm^{-1} (for **B**), due to the presence of β-sheet secondary structure (Fig. S2, ESI†).

Release of 5-Fu from the nanotubes takes place following hydrolytic cleavage of the acyloxymethylene linkage of **A** and subsequent collapse of the resultant hemiaminal intermediate.[55,59] The release of active drug, 5-Fu, from **A** and **B** was measured by analytical RP-HPLC over time as a function of concentration in PBS (Fig. 3b). Whereas **A** readily released 5-Fu in PBS (pH 7.4) at 37.5 °C, **B** was stable under these conditions, as a consequence of the more stable acetamide linkage between the peptide and 5-Fu (Fig. S6, ESI†). At concentrations above the CAC of **A**, the release of 5-Fu from the nanotube was considerably slower than at concentrations below the CAC. For example, 12.6% of 5-Fu was released after 7 days when aging at 10 mM in PBS, whereas near complete release of 5-Fu was achieved after 7 days at 1 or 0.1 mM. At concentration ranges below the CAC, the rate of release was also inversely dependent on concentration, but to a lesser extent (*e.g.*, 50% free

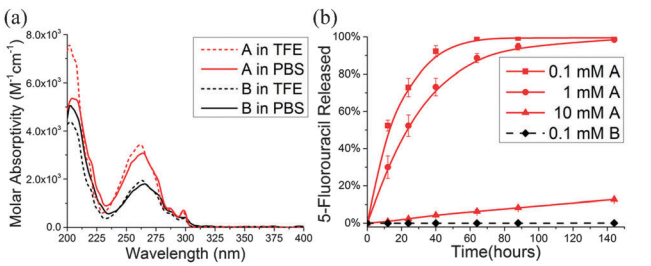

Fig. 3 (a) UV-Vis spectra of **A** and **B** in PBS and TFE. **A** was prepared at 20 mM in PBS or TFE, while **B** was prepared at 10 mM in PBS or TFE. After aging for 3 d, each sample was briefly sonicated then diluted to 0.25 mM. (b) Release of 5-Fu in PBS (pH = 7.4) at 37 °C from solutions of **A** and **B** as a function of concentration. The percentage of 5-Fu released was monitored by analytical reverse-phase HPLC.

5-Fu released after 1 day at 1 mM, compared to 73% at 0.1 mM). As we observed previously,[49] self-assembly slows the hydrolytic release of 5-Fu from **A** by sequestering the ester linkage within the hydrophobic regions of the nanotubes. This observation demonstrates that self-assembly is an effective strategy to slow down the release of free drug by protecting the hydrolyzable bond from exposure to the aqueous media.[49]

Next, the release of 5-Fu from hydrogel **A** was also determined by analytical RP-HPLC over one month. The hydrogel of **A** was formed in PBS (20 mM) in a cylindrical vial (15 × 45 mm), and allowed to equilibrate for 3 days, during which time 5-Fu release was insignificant. A solution of PBS (1 mL) was added on top of the hydrogel (~1 mL) without disturbing the gel. A 10 μL aliquot was then collected, replaced and analyzed for the release of 5-Fu at each time point. Compared with non-gel solutions at 10 mM, the release of 5-Fu from hydrogels was significantly slower due to the extensive self-assembly and slow diffusion within the hydrogel structure. For example, after 7 days, hydrogel **A** released 2.5% of 5-Fu into the top solution, while 12.6% of 5-Fu was released when aged at 10 mM without pre-gelation (Fig. 4a). Hydrogel **A** was stable for over one month, with only 6% of 5-Fu released. This result suggests hydrogel **A** may be used in formulations for sustained local application.

Dipeptides **A** and **B** and the parental drug 5-Fu, were assessed for their cytotoxicity against human non-small cell lung cancer cell lines A549, NCI-H460, and NCI-H23. The cytotoxic activity was assayed using the colorimetric MTT assay after a 96 hours incubation period. The IC_{50} values were 66.1 μM, 96 μM, 114.6 μM for compound **A**, respectively, and 43.4 μM, 47 μM, and 90.6 μM for 5-Fu, respectively (Fig. 4b). Dipeptide **B** exhibited no cytotoxicity against any of the three cell lines, as a consequence of the inability of **B** to release free 5-Fu. Notably, the cytotoxicity of the by-product of hydrolytic release of CPT from **A**, Fmoc-KK(succinic acid), was also assessed and found to have negligible impact cancer cell growth (Fig. S7, ESI†), proving that the cytotoxicity of **A** is from the release of 5-Fu. Overall, 5-Fu exhibits roughly 1.5× greater potency than compound **A** in all cell lines, likely reflecting the slower release of 5-Fu from **A**.

Finally, the mechanical properties of hydrogels **A** and **B** were investigated by rotational shear rheometry.[60] The hydrogels possess distinctive mechanical behavior resulting from their respective linkages to 5-Fu. Stress sweep testing (25 °C, 1.0 Hz) revealed significant differences in the linear viscoelastic regions

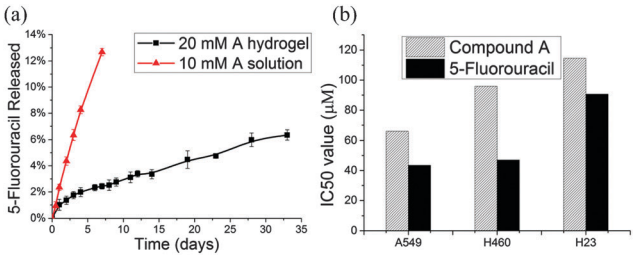

Fig. 4 (a) Release of 5-Fu from hydrogel **A** compared with solution; (b) IC_{50} values of compound **A** and 5-Fu on non-small cell lung cancer cell lines A549, H460 and H23.

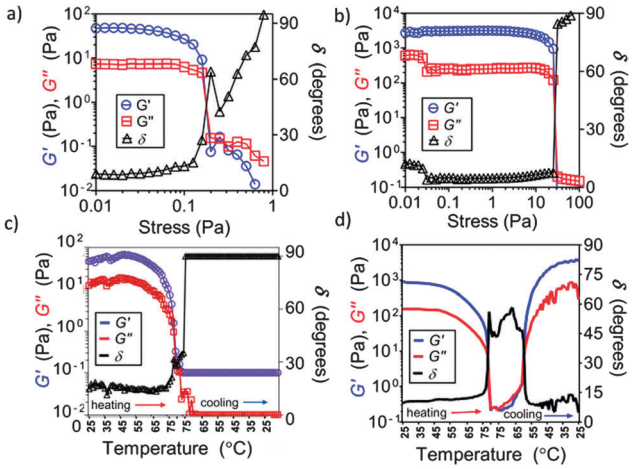

Fig. 5 (a) Oscillatory stress sweep of hydrogel **A** at 20 mM and (b) hydrogel **B** at 10 mM, 25 °C, 1.0 Hz; (c) temperature sweep (heating and cooling) of hydrogel **A** at 20 mM and (d) hydrogel **B** at 10 mM, 1.0 Hz, and 0.1 Pa applied oscillatory shear stress; (G' = storage modulus, G'' = loss modulus, δ = phase angle).

(LVR) between the two hydrogels (hydrogel **A**: LVR < 0.1 Pa; hydrogel **B**: LVR < 10 Pa). Hydrogel **A**, which contains a hydrolyzable succinimidyl ester linkage to 5-Fu, exhibited two log lower storage (G') and loss (G'') moduli than hydrogel **B** (Fig. 5a and b, respectively), which lacks this flexible linker to 5-Fu and instead contains an *N*-acetamide linkage. Temperature sweep testing—performed by ramping the temperature from 25 °C to 80 °C, and back to 25 °C at a rate of ±1 °C min^{-1}—showed that these hydrogels possess similar melting points (determined from the crossover of G' and G'') of ~75–80 °C (Fig. 5b and c), despite their differences in mechanical strength.[61] Hydrogels **A** and **B** behave differently upon heating and cooling. Whereas hydrogel **B** recovers its original mechanical strength upon cooling to <60 °C, hydrogel **A** is not thermally reversible. We suspect that the ester linkage present in hydrogel **A** is hydrolyzed under these conditions, which is supported by 5-Fu release studies on **A** that show this linkage is labile.

In summary, the 5-Fu dilysine conjugates **A** and **B** formed hydrogels *via* the self-assembly of a network of nanotubes or nanofibers in PBS with diameters of 16 and 10 nm, respectively. The nanotube structures from dipeptide **A** provided a protective environment for 5-Fu, thereby affording a slow release of active 5-Fu depending on concentration. Furthermore, the hydrogel structure significantly reduced the release rate of 5-Fu, offering the potential for sustained local drug delivery. The cytotoxicity of **A**, as determined in three cancer cell lines, arose from the release of free 5-Fu, while dipeptide **B**, with a hydrolytically stable, *N*-acetamide linkage, exhibited no cytotoxicity. Additionally, oscillatory shear testing shows that the choice of linkage to the 5-Fu moiety significant impacted the strength, stability, and reversibility of the resulting hydrogels. In summary, a simple strategy to create nanostructured hydrogels for the delivery of 5-Fu based on the self-assembly of dipeptide-5-Fu conjugates has been described.

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