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Hydrogen-bonded supramolecular polymer micelles with pH/photothermal-responsive carmofur release and combined chemo-photothermal therapy.

Yanggui Wu,^{a†} Huiying Wang,^{a†} Qianqian Liu,^b Frédéric Lortie,^c Julien Bernard,^c Wolfgang H. Binder,^d Senbin Chen,^{a*} Jintao Zhu^{a*}

We report here on the construction of hydrogen-bonded (H-bonded) supramolecular polymeric micelles loaded with chemotherapy drugs (carmofur) and photothermal agents (IR780) for combined chemo-photothermal therapies (CT/PTT).

Polymeric micelles built from amphiphilic copolymers have been widely exploited as a remarkable platform for pharmaceutical applications,^{1,2} including both therapeutic delivery systems (e.g., drug, protein, gene), and in diagnostic medicine. Although significant progress has been made during the last few decades, several characteristics of polymeric micelles often limit their further in-depth applications, such as inadequate drug loading efficiency, poor micellar stability or uncontrolled drug release kinetic with unsatisfied drug activity and unintended side effects.³

Research at the interface of supramolecular chemistry and biomedical science starts a new era with the development of delivery systems that maximize drug activity and minimize side effects. Promoting additional supramolecular interactions,⁴⁻⁸ such as host-guest interactions,⁹ H-bonds,¹⁰⁻¹² van der Waals or metal-coordination between the polymer chains can stabilize nanocarriers in physiological fluids via non-covalently cross-linking, thus enhance the drug loading content due to molecular recognition/association. Moreover, it paves the way to the design of a stimuli-responsive drug delivery system sensitive to different parameters such as temperature,¹³ pH,¹⁴ or radiation.¹² Therefore, such highly stable polymeric micelles,^{8,15,16} can lead to improved circulation times and superior transport performance, with the ability to trigger drug release at a specific location. Recently, we have constructed a supramolecular polymeric micelles consisting of H-bonded doxorubicin (Dox) via urea/thiourea, along with encapsulated photothermal agent (indocyanine green, ICG), showing enhanced antitumor activity.¹³

Carmofur, (1-hexylcarbonyl-5-fluorouracil, HCFU), a 5-fluorouracil derivative, is an approved hydrophobic antitumor agent which has been used in colorectal cancer therapy since 1980s, also showing clinical benefits towards bladder, gastric, and breast cancers.¹⁷⁻²⁰ A very recent study²¹ also reveals that HCFU can treat COVID-19 with the enhanced inhibitory efficacy, by binding to coronavirus SARS-CoV-2 main protease. Noteworthy, the structure of HCFU ensures the formation of triple H-bonding arrays and selective complexation with heterocomplementary 2,6-diaminopyridine (DAP) derivatives.

Herein, we use the amphiphilic ABA triblock copolymer POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA ($M_n = 12.0$ kDa, $\mathcal{D} = 1.48$, DAPA/TFEA = 2/3, molar ratio),²² with hydrophilic and biocompatible POEGEA (poly(oligo (ethylene glycol) ethyl acrylate), $M_n = 3.8$ kDa, $\mathcal{D} = 1.34$) as the exterior corona, and hydrophobic P(DAPA-*co*-TFEA) (poly(diaminopyridine acrylamide-*co*-2,2,2-trifluoroethyl acrylate)) as the interior core displaying multiple H-bonding DAP moieties. The presence of DAP moiety within the hydrophobic core enhances the loading of HCFU, thanks to the establishment of specific DAP/HCFU triple H-bonding interactions. We investigate how supramolecular interactions between HCFU and P(DAPA-*co*-TFEA) segments located in the inner part of the micelles impact the drug loading efficiency and drug loading content (**Figure 1**). We further show how these specific interactions between DAP moiety and HCFU combined with the use of a photothermal agent (IR-780) can be exploited to precisely control the release

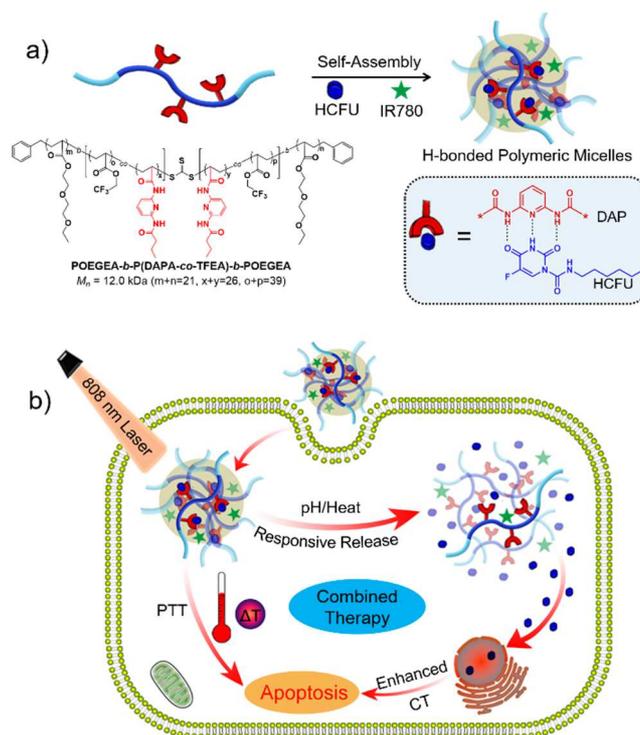


Figure 1: Schematic illustration showing HCFU/IR780 co-loading and self-assembly to afford H-bonded supramolecular polymeric micelles (a), pH/heat-triggered HCFU release and combined CT/PTT (b).

of HCFU upon near-infrared laser (NIR) irradiation under phosphate-buffered saline (PBS), which mimics physiological conditions. IR-780 iodide is a lipophilic cation heptamethine dye with a high fluorescence intensity, which is more stable than ICG. Yet, the hydrophobicity and toxicity of IR780 severely restrict its further clinical applications, therefore IR780 is encapsulated and co-loaded in H-bonded supramolecular polymeric micelles to overcome these disadvantages. At last, we investigate cytotoxicity of HCFU/IR780-co-loaded H-bonding micelles against a model Hela cancer cell line according to different scenarios.

Incorporating chemotherapeutic molecules, e.g. HCFU, through the establishment of triple H-bonds with some parts of the copolymer chains constitutive of the micelles can display the following advantages: firstly the H-bonding linkage between HCFU and the nanocarriers can promote its stability in physiological fluids, it also enhances the loading content of anti-tumor drugs, and ensures the stimuli-responsive dissociation of H-bonds, such as pH and heat, to perform a controlled release of HCFU.

Characterization of H-bonding association between POEGEA-*b*-P(DAPA-co-TFEA)-*b*-POEGEA and HCFU. The synthetic procedures to prepare H-bonding drug delivery vehicle POEGEA-*b*-P(DAPA-co-TFEA)-*b*-POEGEA, and a control polymer devoid of H-bonding moieties, POEGEA-*b*-PTFEA-*b*-POEGEA ($M_n = 14.2$ kDa, $\mathcal{D} = 1.26$) are described in our recent work.²² Our interest in studying the fluorine-containing polymers stems from their promising application fields,²³ such as enhanced

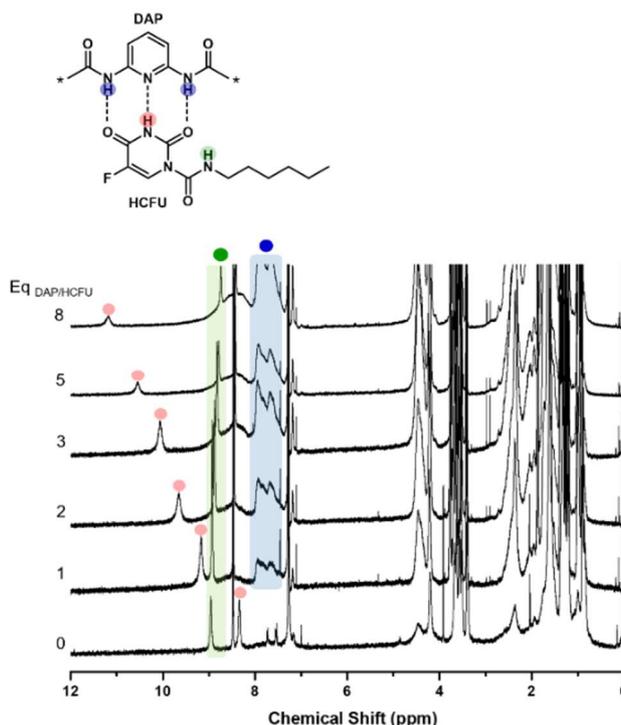


Figure 2: ^1H NMR spectra of HCFU mixed with different amount of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA, recorded in CDCl_3 at 21 $^\circ\text{C}$ ($[\text{HCFU}] = 3 \text{ mM}$, $\text{Eq}_{\text{DAP/HCFU}} = 0 - 8$). Red and green spots: HCFU NH protons; blue spots: DAP NH protons.

chemical/enzyme resistance. Prior to the preparation of supramolecular polymeric micelles, we explore the H-bonding association behavior of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA with HCFU in deuterated chloroform. Using a well-established ^1H NMR titration experiment, DAP/HCFU associations via triple H-bonding forces are evidenced (**Figure 2**). Increasing the concentration of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA and precisely tuning the DAP/HCFU ratio from 0 - 8 results in a gradual downfield shift of HCFU NH proton (from 8.31 to 11.22 ppm, red spots in **Figure 2**).

Using a relevant mathematical model, a binding constant (K_a) of around 100 M^{-1} is determined for DAP/HCFU pair. This value is comparable to that of analogous H-bonding systems.²⁴ It is well established that the strength of H-bonding interactions is strongly impacted by the temperature. Consequently, increasing the solution temperature from 21 to 40 $^\circ\text{C}$ is accompanied by a significant upfield shift of the HCFU NH proton suggesting a substantial weakening of H-bonding interactions upon heating. Note that the NH peak shifts back to its original position when the sample is finally cooled down to 21 $^\circ\text{C}$, highlighting the reversible character of these H-bonding interactions (**Figure S1**).

Drug loading of H-bonded supramolecular polymeric micelles. Aiming at fabricating well-defined micellar particles, the emulsion-solvent evaporation method^{25,26} is used to achieve 3D confined assembly from H-bonded supramolecular polymeric micelles. Morphologies and sizes of the obtained particles are characterized by TEM (transmission electron microscope) and

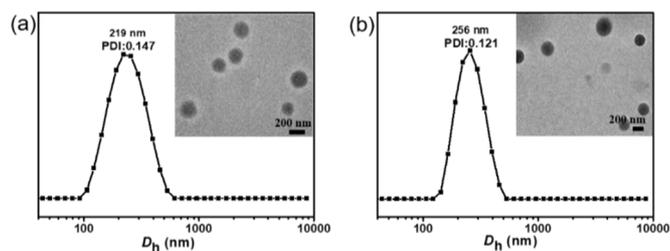


Figure 3: DLS results and TEM images (inserted) of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA without (a) and with (b) HCFU/IR780 loading.

DLS (dynamic light scattering). The hydrodynamic diameters (D_h) of the micelles increase from 219 (Figure 3a) to 256 nm (Figure 3b) after the encapsulation of HCFU and IR780 into POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA. Such increase in dimensions indicates the successful accommodation of drugs in the interior of the micelles. TEM images further demonstrate the micelles diameters (183 ± 19 nm, Figure 3a inserted) from POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA, and (217 ± 24 nm, Figure 3b inserted) from POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780, respectively. These values are slightly smaller than those determined by DLS due to the collapse of the hydrophilic corona after drying. On the other hand, comparable micellar morphologies and sizes are obtained from the control polymer POEGEA-*b*-PTFEA-*b*-POEGEA (Figure S2).

Subsequently, quantitative characterization of the drug loading content (DLC) and drug loading efficiency (DLE) are carried out using UV-Vis absorbance spectroscopy. Compared to the DLC/DLE of HCFU (10.8%/28.0 %, Table S1) in control DAP-free POEGEA-*b*-PTFEA-*b*-POEGEA@HCFU/IR780 micelles, significantly higher drug loading efficiency (15.6%/40.4%) are obtained in H-bonding supramolecular POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles, proving that the presence of the DAP moieties facilitates HCFU trapping within the micelles to achieve a higher drug payload. Regarding DLC/DLE of IR780, comparable values are observed between POEGEA-*b*-PTFEA-*b*-POEGEA@HCFU/IR780 (4.5%/29.6%) and POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 (5.8%/38.7%, Table S1).

Photothermal conversion properties of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780. We introduce IR780 iodide in H-bonded supramolecular polymeric micelles to induce local heating under irradiation and consequently trigger the release of the H-bonded HCFU. Using a NIR laser emitting at 808 nm, we investigate the photothermal conversion properties produced by POEGEA-*b*-P(DAP-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles. As illustrated by Figure S3, we find that the temperature of solutions containing POEGEA-*b*-P(DAP-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles increases rapidly (Figure S3) as a function of the laser irradiation power (from 0.6 to 2 W/cm², Figure S3a) and of micelle concentration (from 0.55 to 1.65 mg/mL, Figure S3b). Note that at 2 W/cm² of laser power density and a micellar concentration of 1.65 mg/mL, the solution temperature increases rapidly and levels off after

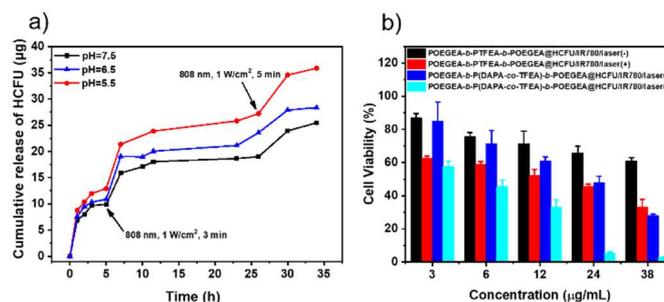


Figure 4: *In vitro* drug release profiles for POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles in PBS buffer at pH 7.5, 6.5 and 5.5 with/without laser irradiation (a). Cell viability from POEGEA-*b*-PTFEA-*b*-POEGEA@HCFU/IR780, POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 with/without laser irradiation (b).

only ~30 and 60 s, respectively, due to the rapid consumption of active IR780.

***In vitro* drug release and chemo-photothermal therapy.** *In vitro* cytotoxicity of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 and POEGEA-*b*-PTFEA-*b*-POEGEA@HCFU/IR780 towards Hela, 3T3 and B16-F10 cells are subsequently evaluated using the CCK8 assay. After incubation at 37 °C for 24 h, both POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 and POEGEA-*b*-PTFEA-*b*-POEGEA@HCFU/IR780 micelles do not exert any significant cytotoxic effects towards all Hela, 3T3 and B16-F10 cells at concentrations as high as 300 µg/mL (Figure S4), indicating that the obtained micelles exhibit extremely low toxicity towards cells.

In vitro drug release profiles of loaded POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA micelles in PBS buffers at different pHs are given in Figure 4. POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles show a very slow release of HCFU in the absence of 808 nm laser irradiation (Figure 4a). A decrease in pH value (pH 6.5 and 5.5) slightly enhances HCFU release rate, with ca. 11-13% of the HCFU out of the micelles after 5 h without 808 nm laser irradiation. All together, these results confirm that H-bonded supramolecular polymeric micelles are relatively stable under such conditions without substantial loss of cargo after several hours. To confirm that the photothermal effect of IR780 provides an effective method to break H-bonds between HCFU and DAP moieties anchored to the carrier and to release HCFU from micelles, 808 nm laser irradiation is applied for 3 or 5 min at defined intervals of time. Exposure of solutions to NIR light results in an instantaneous burst of drug release from POEGEA-*b*-P(DAPA-*co*-TFEA)-

b-POEGEA@HCFU/IR780 micelles (**Figure 4a**), attributed to the cleavage of DAP/HCFU H-bonding complexes at elevated temperatures.

To explore the capacity of H-bonded supramolecular polymeric micelles to induce effective chemo-photothermal therapy, HeLa cells are then incubated with either free HCFU, free IR780, or POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 with/without 808 nm laser irradiation (**Figure 4b**). In the case of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780, comparable therapeutic effect is observed to that of POEGEA-

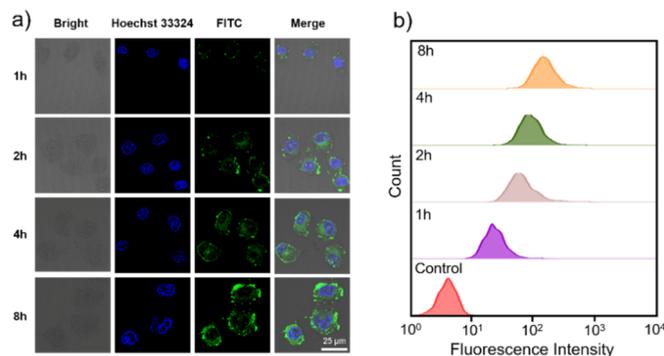


Figure 5: CLSM images of the time-dependent cellular uptake of H-bonded supramolecular polymeric micelles (a), and fluorescence signal intensity extracted from CLSM images (b).

b-PTFEA-*b*-POEGEA@HCFU/IR780 at low concentrations (0.5 and 1 $\mu\text{g}/\text{mL}$), due to the very small amount of active drugs. In contrast, more pronounced therapeutic effect is observed at the concentrations from 2 to 6 $\mu\text{g}/\text{mL}$, which is ascribed to the higher HCFU loading content through the H-bonding association. Additionally, upon 808 nm laser irradiation, POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles induce dramatically enhanced cytotoxicity in comparison with chemotherapy alone under the same experimental conditions, this outcome is undoubtedly resulting from the combined chemo-photothermal therapeutic effect between HCFU and IR780, responsible for a temperature increase and subsequently enhanced HCFU release.

Cellular internalization of H-bonded supramolecular polymeric micelles. Cellular uptake of HeLa cells of the H-bonded supramolecular polymeric micelles is investigated by confocal laser scanning microscope (CLSM, **Figure 5a**). With that aim, HeLa cells nuclei are labeled using blue-fluorescent Hoechst 33324 and green-fluorescent fluorescein isothiocyanate (FITC) is coupled to POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 micelles to monitor the internalization of the micelles. With increasing co-incubation times (from 1 to 8 h, **Figure 5a&b**), higher fluorescence intensities are observed indicating that H-bonded supramolecular polymeric micelles are efficiently internalized by HeLa cells and mostly located in the cytoplasm as suggested by the merged images. Flow cytometry analysis confirmed a gradual enhancement of cellular fluorescence intensity with co-incubation time (**Figure S5**), further proving the effective, yet time-dependent cellular uptake of H-bonded supramolecular polymeric micelles.

Calcein-AM (labels live cells) and propidium iodide (PI, labels dead cells) staining are finally performed to evaluate the chemo-photothermal therapeutic capabilities of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 (**Figure S6**). As expected, the sole application of free HCFU, NIR laser, or non-irradiated free IR780 does not induce considerable cytotoxicity towards HeLa cells. In contrast, the administration of POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 without laser irradiation provokes the death of a non-negligible fraction of HeLa cells. However, cell mortality significantly increases when the POEGEA-*b*-P(DAPA-*co*-TFEA)-*b*-POEGEA@HCFU/IR780 is exposed to laser irradiation (**Figure S6**), further corroborating the superior efficacy of combined chemo-photothermal therapies.

Conclusions

A supramolecular polymeric micelles platform based on H-bonding-driven self-assembly strategy is developed to generate HCFU carriers. It is proven that the delivery of HCFU can be finely controlled upon heating. The hyperthermia generated by IR780 not only induces the dissociation of H-bonds, triggers the responsive HCFU release for CT treatment, but also collectively contributes to the PTT treatment. Such stimuli-responsive H-bonded supramolecular polymeric micelles constitute new tools for effective and smart nano-theranostics strategies.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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