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Complementary hydrogen bonding interaction triggered co-assembly of amphiphilic peptide and anti-tumor drug

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We reported a new tumor-targeting amphiphilic peptide that can form complementary hydrogen bonds with anti-tumor drug methotrexate (MTX), leading to a reversible self-assembled morphology transition from loose micelles to densely packed nanorods or nanofibers. The MTX loaded nanorods can target tumor cells and show more than 2-fold higher cytotoxicity (IC_{50} = 0.38 mg/L) than that of normal cells (IC_{50} = 0.89 mg/L).

In the past few decades, peptide-based materials have been a class of extremely important biomaterials that have found wide applications in drug delivery, tissue engineering and gene therapy. In particular, because bioactive peptides such as tumor-targeting, membrane penetration and nuclear localization signal peptides could efficiently overcome various extra- and intracellular barriers, their self-assembly has been widely used to deliver therapeutic drugs for disease treatment. To realize drug loading and delivery, the general strategy is to mix drugs with peptide-based materials and employ the subsequent self-assembly to load drug into the self-assembled nanostructures. However, their clinical translation is significantly hindered by many barriers, including unsatisfied drug loading, batch-to-batch variations of drug loading and undesirable drug leakage during sample storage. In an attempt to address these issues, peptide-based prodrugs have been considered as an effective strategy in recent years.

Through conjugating therapeutic drugs to the peptides by stimuli-responsive linkages, the drugs could be easily delivered and rapidly released after the breakage of the stimuli-responsive linkages. Xu and co-workers reported the first example of taxol-conjugated peptide-based prodrug. Yang and co-workers recently demonstrated that conjugation of two complementary therapeutic drugs could confer the resulted peptide-based prodrug with improved therapeutic efficacy. These pioneering researches prove the feasibility of peptide-based prodrugs for disease treatment, however, ingenious design and synthesis and technical purification are necessary.

In this communication, we reported a new and convenient strategy to construct drug-loaded platform by using complementary hydrogen bonding interaction. It is known that cyanuric acid and melamine are a classic hydrogen bonded pair with the capacity to form complementary hydrogen bonds (Fig. 1A), which has been widely employed to construct self-assembled materials such as multilayer films. As a clinical anti-tumor drug, methotrexate (MTX), has the similar structure to melamine (Fig. 1A), which shows a great potential to form complementary hydrogen bonding interaction with cyanuric acid. Along this principle, we designed and synthesized a new tumor-targeting amphiphilic peptide with cyanuric acid conjugated to the hydrophobic tail (CA-C11-GGGRGDS). As shown in Fig. 1B, when dissolving this amphiphilic peptide in aqueous solution, the hydrophobic interaction among the hydrophobic tail facilitates the molecular aggregation to form spherical micelles. If mixing this amphiphilic peptide with hydrophobic MTX, the complementary hydrogen bonding interaction between MTX and the terminal cyanuric acid group leads to an increase in the overall hydrophobicity of the self-assembling system, resulting in the formation of MTX loaded nanorods (low MTX concentration) or nanofibers (high MTX concentration). As the MTX release, the hydrophilic-hydrophobic balance is disturbed, inducing the recovery of micelles from nanorods or nanofibers.

Fig. 1. (A) Molecular structures of the hydrogen bonded pair (cyanuric acid and melamine) and the anti-tumor drug MTX; (B) Molecular structure of the amphiphilic peptide and the illustration of the co-assembly of this amphiphilic peptide with MTX.

Starting from the commercial N-fluorenyl-9-methoxycarbonyl (FMOC)-protected amino acids, the amphiphilic peptide was...
prepared by FMOC standard solid-phase peptide synthesis (SPPS). We first investigated the self-assembly behavior of this amphiphilic peptide. As shown in Fig. 2A, after dissolving the amphiphilic peptide in aqueous solution (4 mg/mL, pH 7.0) and placing at room temperature for 30 min, it can self-assemble into spherical micelles with an average diameter of ~20 nm, which is relatively smaller than the result of dynamic light scattering (DLS) analysis (~49.9 nm, Fig. S5, ESI†). By using pyrene as a fluorescent probe (Fig. S6, ESI†), the critical micelle concentration (CMC) is determined as ~109.6 mg/L (Fig. 2B).

The self-assembly of the amphiphilic peptide was characterized by circular dichroism (CD) and fourier transform infrared spectroscopy (FT-IR). The negative band at ~216 nm in CD spectrum (Fig. S8A, ESI†) and the absorbance of amide I band at ~1649 cm⁻¹ in FT-IR spectrum (Fig. S9A, ESI†) indicate the random-coil conformation adopted by the amphiphilic peptide.¹⁶

Prior to investigating the co-assembly of the amphiphilic peptide and MTX, we examined the possibility of the hydrogen bond formation between MTX and the cyanuric acid. From the ¹H NMR spectra (Fig. S3, ESI†), the signals at ~6.7 and 7.5 ppm correspond to the protons of the amine groups of MTX. After mixing with cyanuric acid, these two peaks respectively shift to ~7.3 and 7.9 ppm, demonstrating the formation hydrogen bonds between MTX and cyanuric acid.¹⁷ By using the classic Benesi-Hildebrand equation,¹⁸ the hydrogen bond association constant ($K_b$) between the amphiphilic peptide and MTX is determined as ~664 M⁻¹ (Fig. S4, ESI†). To investigate the co-assembly of the amphiphilic peptide and MTX via the complementary hydrogen bonding interaction, the amphiphilic peptide and MTX were mixed in methanol with an equal molar ratio, followed by sonication and evaporation. After dissolution-sonication-evaporation thrice, distilled water was added and the solution pH was adjusted to a value of 7.0. After placing at room temperature for 30 min, TEM was employed to observe the morphology of the self-assembled amphiphilic peptide/MTX complex. As shown in Fig. 2C, at a concentration of 1 mg/mL, the amphiphilic peptide/MTX complex could self-assemble into nanorods with a length of 400-500 nm, which are the aggregations of numerous thin and short nanofibers (Fig. 2D). If increasing the complex concentration to 2 mg/mL, besides the aggregation of short nanofibers, the majority of the amphiphilic peptide molecules self-assemble into long nanofibers (Fig. 2E). Further increasing the concentration to 4 mg/mL, all the building blocks self-assemble into long nanofibers (Fig. 2F), which is different with the spherical morphology self-assembly from the amphiphilic peptide without MTX at the same conditions (Fig. 2A). CD and FT-IR were also employed to examine the conformation of the peptide backbone in the self-assembled nanofibers. The negative band at ~216 nm in CD spectrum (Fig. S8B, ESI†) and the absorbance of amide I band at ~1630 cm⁻¹ in FT-IR spectrum (Fig. S9B, ESI†) indicate that the backbone of the amphiphilic peptide forms β-sheet like superstructure in the nanofibers.¹⁹ In comparison with the self-assembly behavior of the amphiphilic peptide without MTX, the morphology change and conformation transition upon MTX addition strongly demonstrate the amphiphilic peptide prepared in this study has a good ability to load MTX. Herein, the MTX encapsulation efficiency for nanorods or nanofibers is higher than 80% (Table S1, ESI†).

For most amphiphilic peptides, there is nearly no change in the self-assembled morphology or peptide conformation after drug loading.⁷,¹⁰ However, in current work, due to the hydrogen bonding interaction between MTX and the amphiphilic peptide, the MTX loading leads to obvious morphology and conformation changes. As shown in Fig. 1B, there is a hydrophilic cyanuric acid group conjugated to the hydrophobic tail of the amphiphilic peptide. The overall hydrophobicity of this building block is weaker than that of the traditional amphiphilic peptides such as the well-studied peptide amphiphiles (PAs).³,⁹ As a result, it is apt to aggregate into loose structure such as micelle. With the addition of hydrophobic MTX, the terminal cyanuric acid group could form complementary hydrogen bonds with MTX to improve the overall hydrophobicity (~24.5 mg/L for the critical aggregation concentration, Fig. S7, ESI†), resulting in the formation of densely packed nanorod. Like many other reported peptide self-assembly with concentration dependence,¹⁶,²⁰ if increasing the concentration of the amphiphilic peptide/MTX complex, the hydrophobic aggregation degree will be strengthened and thus induce the formation of long nanofiber.

The release behavior of MTX loaded nanofibers was investigated at physiological temperature (37 °C). As shown in Fig. 3A, the nanofibers present a sustained drug release behavior at pH 7.4. Around 43.5% of loaded MTX is released from the nanofibers within 4 h and the cumulative release within 36 h reaches to ~66.9%. If changing the external pH to an acidic (pH 5.0) or basic (pH 9.0) environment, the MTX release rate increases and more than 85% of loaded MTX can be released within 36 h. This increased release rate is mainly attributed to the ionization of MTX. Both amine and carboxylic acid groups are contained in MTX (Fig. 1A), ionization will thus appear when increasing or decreasing external pH, resulting in a transition of MTX from hydrophobicity to hydrophilicity. Consequently, the hydrophilic-hydrophobic balance of the self-assembled system is disturbed, leading to a rapid MTX release. To verify the aforementioned statement, we used TEM to observe the morphology change of the nanofibers during MTX release at pH 7.4. After placing at 37 °C for 4 h, nanofibers could be observed although part of the loaded MTX has been released (Fig. 3B).

Further incubating the nanofibers for 36 h, the MTX release...
induces the decrease in the length and amount of nanofibers (Fig. 3C). In addition, numerous spherical micelles are formed in the self-assembled system. After 7 days incubation, almost all the nanofibers change into spherical micelles (Fig. 3D). All these results strongly demonstrated that the release of MTX could disturb the hydrophilic-hydrophobic balance of the self-assembled system, leading to a morphology transition from nanofibers to spherical micelles. Herein, although there is a morphological change as incubation time increase, it is not conflicting with our goal since the loaded MTX could be sustained released to exert the following therapeutic effect.

![Figure 3](image-url)

**Fig. 3.** (A) Cumulative release of MTX from the MTX loaded nanofibers at physiological temperature; (B-D) TEM images of the MTX loaded nanofibers incubated at physiological temperature for 4 h (B), 36 h (C) and 7 days (D), respectively; (E, F) CLSM images of HeLa (E) and COS7 (F) cells incubated with the MTX loaded nanorods for 4 h; (G, H) Flow cytometry (G) and MFI (H) of HeLa and COS7 cells incubated with the MTX loaded nanorods for 4 h; (I) Cytotoxicity of free MTX and MTX loaded nanorods against HeLa and COS7 cells.

We then evaluated the anti-tumor effect of the MTX loaded nanostructures. Herein, the MTX loaded nanorods was chosen to evaluate the corresponding cytotoxicity against tumor cells. Before evaluating the cytotoxicity, the intracellular uptake of the MTX loaded nanorods was observed using confocal laser scanning microscope (CLSM). Noting that, the self-assembled amphiphilic peptide/MTX still maintains the nanorod morphology even dispersion in cell culture medium (Fig. S10, ESI†). As shown in Fig. 3E, due to the presence of tumor-targeting RGD sequence in the amphiphilic peptide, HeLa cells with over-expressed intergrins on the membrane show a higher uptake than that of COS7 cells (Fig. 3F). The result of flow cytometry quantitative analysis (Fig. 3G) is consistent with that of CLSM observation. The mean fluorescence intensity (MFI) of HeLa cells incubated with the nanorods (Fig. 3H) is more than 3-folder higher than that of COS7 cells. The cytotoxicity of the MTX loaded nanorods against HeLa and COS7 cells is shown in Fig. 3J. The free MTX shows the similar cytotoxicity against HeLa and COS7 cells. In contrast, due to the relatively high intracellular uptake, the cytotoxicity of the nanorods against HeLa cells is much stronger than that of COS7 cells. The corresponding half maximal inhibitory concentration (IC50) for HeLa cells is ~0.38 mg/L, which is more than 2-fold lower than that of COS7 cells (~0.89 mg/L). In case of the single amphiphilic peptide, there is no apparent cytotoxicity within the concentration range from 0~10 mg/L (Fig. S11, ESI†), suggesting a great potential of this amphiphilic peptide for drug delivery.

In conclusion, we have designed and prepared a new tumor-targeting amphiphilic peptide with a terminal cyanuric acid group. This new building block presents a satisfied ability to load and release anti-tumor drug of MTX, leading to a reversible self-assembled morphology transition from spherical micelles to nanofibers.

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### Notes and references

A new tumor-targeting amphiphilic peptide with terminal cyanuric acid group was designed and prepared. This new building block presents an excellent capacity to load and release the anti-tumor drug methotrexate (MTX), leading to a reversible self-assembled morphology transition from spherical micelles to nanofibers.