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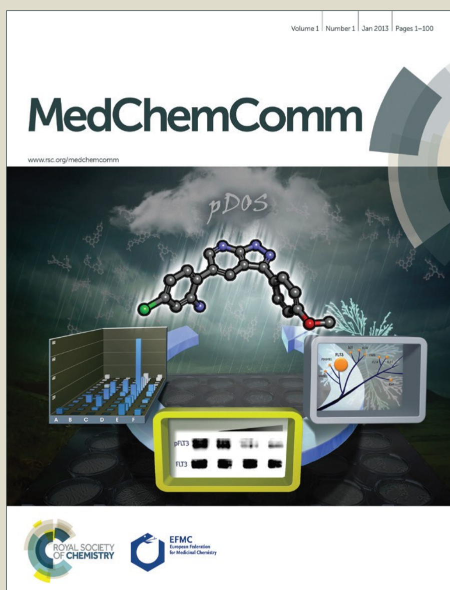

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ARTICLE

Supramolecular, Prodrug-based Micelles with Enzyme Regulated Release Behavior for Controlled Drug Delivery

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Supramolecular polymer micelles (SMPMs) engineered by host-guest interaction of α-cyclodextrin (α-CD) and poly(ε-capralactone) (PCL) homopolymer have been recently reported as robust drug delivery system. The incorporation of supramolecular chemistry affords significantly obvious advantages of convenience in fabrication and controllability in property, in contrast to conventional micelles. This work aims to design and develop novel SMPMs assembled by anticancer drug camptothecin carrying PCL (CPT-PCL) and α-CD. The prodrug strategy developed can not only introduce the steric effect to facilitate the supramolecular nano-assembly, but also provides the capability to prevent the premature release as well as to regulate the drug release by enzyme. CPT drug and PCL polymer were chemically bonded through ester bond linkage hydrolyzable in the present of lipase. The resultant SMPMs were spherical with hydrodynamic size around 220 nm with a fixed drug ratio dependent on molecular weight of PCL employed. The enzyme-induced drug release behavior and cytotoxicity of CPT carrying SMPMs were further evaluated, and the results revealed the promising application of these micelles for controlled drug delivery.

Introduction

Rapid growth of supramolecular chemistry and macromolecular self-assembly has led to the development of many novel structures and systems.[1-6] Non-covalent interactions such as ionic and dipolar interactions, hydrogen bonding, π-π interactions, Van der Waals interactions, and hydrophobic effect are frequently employed to drive the formation of these assemblies. Among those basic non-covalent interactions, “host-guest” interaction, normally involves two components, “host” and “guest” molecules, is highly promising for its specificity as well as convenience. The binding capacity of host and guest molecules such as cyclodextrins (CDs) and poly (ε-caprolactone) (PCL) is attributed to hydrophobic interaction as well as their structures’ complementary characters in size and shape.[7] Intensive works have been done to develop various supramolecular structures for basic science as well as for various applications including drug carrier,[8-10] gene carrier,[11-13] multiple identification and targeting, etc.[14-17] For some examples, one can refer to the publications of the active group led by Harada A in this area.[6, 18] Based on PCL and α-CD, we have developed several novel drug delivery micellar-systems with significant superiority over traditional micelles.[19-21]

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Polymeric micelles have played an important role in drug delivery and cancer therapy research. Their unique core–shell structure provides a robust reservoir to hold the drug in the structure enabling effective protection, solubility improvement, controlled drug release, and even passive and active targeted delivery.[22-26] Previously, we successfully developed a novel strategy to engineer micelles via host-guest interaction of CDs and PCL. In comparison with conventional method to obtain micelles, this method is highly promising for high convenience and controllability. The key technical point is to suppress the crystallization of the formed polyrotaxane, usually observed during the procedure of these assemblies formation, by incorporating hydrogen bond destroyer as well as careful choice of molecular weight of PCL. These unique supramolecular polymer micelles have been found to be very effective platform for drug delivery.[19-21]

Despite of the above, burst release of the drug from these supramolecular polymer micelles remains an issue, similar with most of micellar drug delivery systems. This premature drug release would pose significant challenge in application since it is the major cause for side effect as well as to reduce the bioavailability of the drug. For this consideration, we propose herein a supramolecular “pro-drug” strategy to address this issue, with the aim to optimize release kinetics. Beyond this benefit, prodrug strategy will be able to suppress the crystallization behavior in cyclodextrin based inclusion complexes by steric effect. The release of the drug can be further controlled by lipase taking advantage of the enzyme biodegradability of PCL. The design and synthesis of this novel enzyme sensitive drug delivery system was illustrated in

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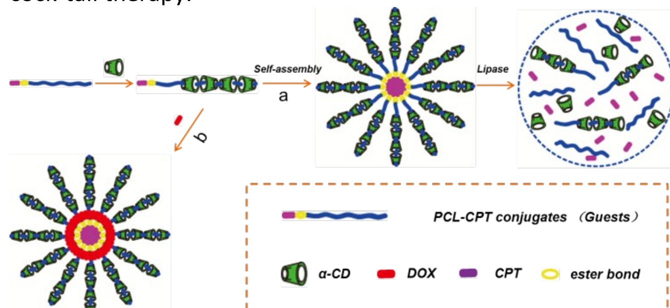
Scheme 1. CPT was attached to PCL via degradable ester linkages (PCL-CPT) and formulated into supramolecular micelles with α-CD, after which lipase was used to regulate the drug release behavior. In addition, this novel supramolecular prodrug micelles can be also used as drug carrier for encapsulation another drug doxorubicin (DOX) for potential cock-tail therapy.



Scheme 1. Schematic diagram showing the "host-guest" interaction of PCL-CPT and α-CD and subsequent hydrophilic - hydrophobic driven assembly of PCL-CPT/α-CD prodrug SMPMs as well as their enzyme sensitive drug release behavior (a), and formation of DOX-loaded PCL-CPT/α-CD prodrug SMPMs (b).

Results and discussion

Fabrication of α-CD/PCL-CPT Prodrug SMPMs

In this study, α-CD and PCL are chosen as the building blocks of SMPMs while CPT is chosen as the drug segment. There are several factors need to be considered for choosing the building blocks. One is the molecular weight of PCL. As we have proven in our previous work,[21] the molecular weight of PCL may have a significant influence on the physiochemical property of SMPMs, as well as on drug conjugation efficiency. Lower molecular weight of PCL will result in higher drug conjugation efficiency but may compromise the stability of SMPMs for crystallization. Therefore, we conducted three parallel experiments, herein, by different molecular-weight of PCL for optimization. Another important consideration is on the molecular size of chosen drug. In the proposed design, the drug will act not only a therapeutic effect but also provide a steric effect for α-CD threading at the drug terminal of the polymer chain. The molecular size of the drug should be therefore large enough to prevent α-CD hosts from slipping out from both ends of the PCL chains, so as to keep the sufficient number of threaded α-CDs required for micellar self-assembly.

As described above, α-CD/PCL-CPT prodrug SMPMs will be obtained via macrocyclic host–guest complexation, which has found to be convenient and controllable in comparison with conventional micelle assembly. In this study, PCL-CPT acts as the guest while α-CD as the host. Before the assembly, PCL-CPT polymer guests need to be chemically synthesized.

Polymer guest PCL-CPT conjugate was fabricated between PCL and CPT bridged by ester bonds which have suitable biodegradability *in vivo*. Typically, a four-step procedure was used to prepare PCL-CPT guest, as illustrated in Fig 1. Firstly, a typical ring-opening polymerization was carried out between ε-CL and L-phenylalanine to prepare PCL [27]. In this study, PCL with three different molecular weight was fabricated by controlling the molar ratio of ε-CL to amino acid ([ε-CL]/[L-phenylalanine]). Secondly, PCL synthesized in the first step was processed by acetic anhydride to inactivate the hydroxyl group. Thirdly, the processed PCL above was reacted with thionyl chloride to turn the terminal carboxyl group at another end into more active acyl chloride group. Finally, the PCL-CPT conjugates were obtained through reaction between PCL acyl chloride and CPT.

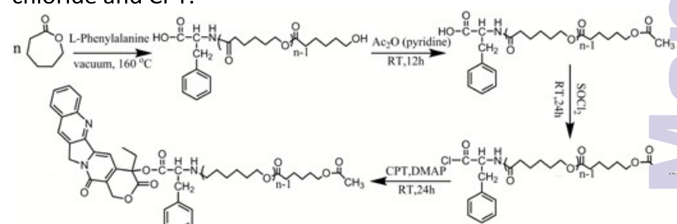


Fig. 1 Synthesis procedure of PCL-CPT polymer conjugates via ester bond linked.

^{1}H NMR spectroscopy and gel permeation chromatography (GPC) were used to characterize the structure as well as measure the molecular weight of PCL guests synthesized above. As a representative sample, the detailed structure of PCL detected by ^{1}H NMR is shown in Fig. 2, which is in consistent with major features of PCL. Furthermore, the peaks at δ= 3.1 and 7.18 ppm are attributed to the protons of methylene and phenyl in the $PhCH_2$- end group. The above characteristic peaks are clear evidences of L-Phe incorporation into the polymer chain, supporting the successful synthesis of PCL.

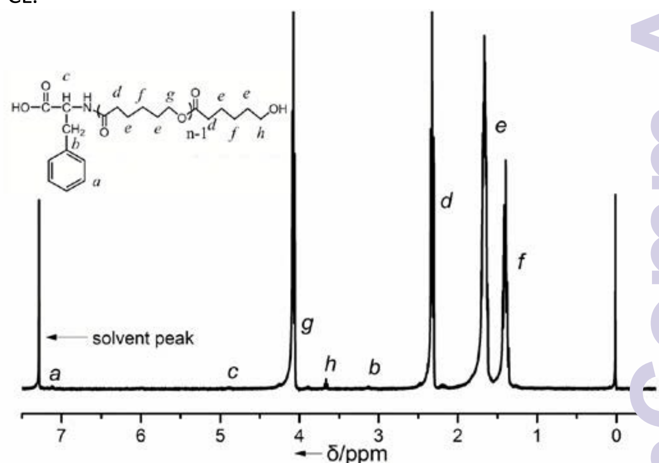


Fig. 2 ^{1}H NMR spectra of L-phenylalanine terminated PCL in $CDCl_3$.

The molecular weight of PCL was estimated according to the ^{1}HNMR spectrum by comparing the proton signal's integration of methylene group in PCL main chain (g) and in terminal L-Phe group (h). The calculated results are summarized in Table 1. GPC was also performed to characterize PCL molecular weight, with $CHCl_3$ used as the eluent. As shown in Fig. 3, the three parallel PCLs synthesized in this study exhibited a retention time of 14 min, 16 min and 17.5 min respectively, corresponding to a mean M_n of 5.5K, 7.5K and 11K. As shown in Table 1, GPC results are nearly in consistence with the

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results obtained by ^{1}HNMR calculation, revealing a fine tuning of molecular weight on PCL precursor by ring-opening polymerization technique.

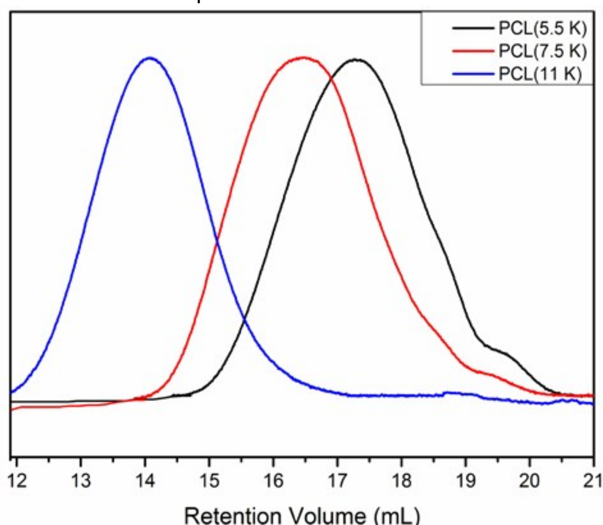

Fig. 3 GPC traces of three PCLs synthesized by controlling the molar ratio of ε-CL to amino acid during ROP.

Table 1 Molecular weight and distribution indexes of three PCLs.

Sample	M_n,NMR[a]	M_n,GPC[b]	M_w^b/M_n^b
PCL(5K)	5300	5500	1.295
PCL(7K)	7000	7500	1.422
PCL(10K)	10000	11000	1.280

[a]Determined by ^{1}H NMR spectroscopy of PCL. [b]Determined by GPC analysis with polystyrene standards. $CHCl_3$ was used as eluent.

The ^{1}H NMR spectroscopy was further used to monitor the synthetic intermediates and product of PCL-CPT guests (Fig. 4). The peak at δ= 2.1 of PCL after acetic treatment assigned to methyl group in acetic anhydride suggests the successful inactivation of hydroxyl group (Fig. 4b). The spectrum of PCL–CPT conjugate contains all the characteristic peaks of PCL and CPT (Fig. 4c), including the $–CH_2–$ of PEG at δ= 3.53 ppm and the $–CH_3$ of CPT at δ= 0.91 ppm, respectively. All of these results demonstrate the successful synthesis of PCL-CPT conjugates. In addition, CPT loading efficiencies of the three different molecular weight of PCL-CPT were calculated by UV/Vis spectroscopy, which are 8%, 6%, 4%, respectively.

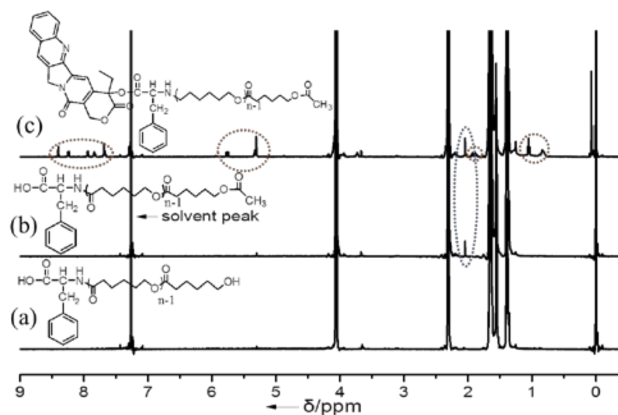

Fig. 4 ^{1}H NMR spectra of PCL (a), esterificated PCL (b), PCL-CPT conjugate (c) in $CDCl_3$.

The α-CD/PCL-CPT prodrug SMPMs were prepared by a method according to our previous work[21]. Herein, α-CD/PCL-CPT prodrug SMPMs were fabricated in parallel with three synthesized PCL-CPT guests with different molecular weight (5.5K, 7.5K, 11K) at two feed ratio (1:1, 10:1) of $m_{α\text{-}CD}$ to $m_{PCL\text{-}CPT}$. In the assembly process of three parallel micelles, a visually observed change from transparent to opaque was found for the solutions, suggesting the formation of micelles. These micelles were purified by exhausted dialysis to remove residual hosts, guests and organic solvent, followed by freeze drying process to collect the product. Yields of micelles were calculated as shown in Table 2. It can be seen that the yields of α-CD/PCL-CPT prodrug SMPMs increases as the M_n of the guest part PCL-CPT increases.

Table 2 Yields of α-CD/PCL-CPT prodrug SMPMs prepared from different molecular weight of PCL prodrug with 2 mg/mL α-CD at different mass ratio of α-CD to PCL-CPT. The yields of SMPMs (not shown here) is lower than 15% by using 20 mg/mL α-CD.

$m_{α\text{-}CD}$: $m_{PCL\text{-}CPT}$ / yeild of SMPMs / PCL with different M_n	1:1	10:1
PCL5.5K	26.7	Little for weighting
PCL7.5K	29.6	40
PCL11K	50.4	55

The yield was calculated based on the equation: weight of obtained SMPMs/weight of added PCL-CPT×100%

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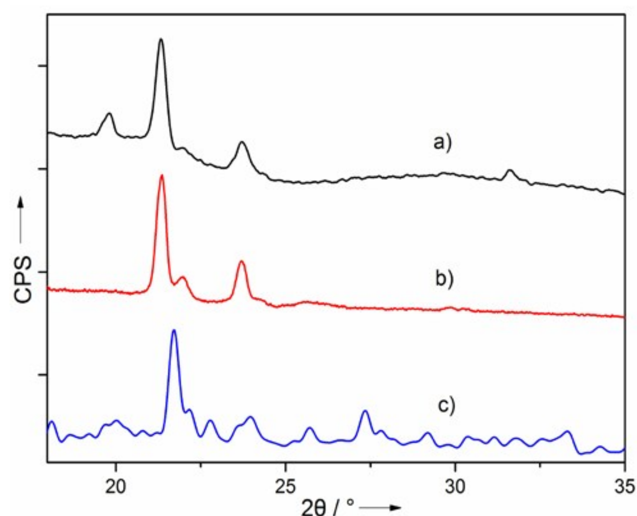

Fig. 5 X-ray diffraction patterns of a) freeze-dried PCL-CPT/α-CD SMPMs; b) PCL-CPT; c) α-CD.

To demonstrate the structure of SMPMs, X-ray diffraction of freeze-dried α-CD/PCL-CPT prodrug SMPMs was conducted (Fig. 5). The pattern of SMPMs is partially different from those of pure PCL-CPT and α-CD, which demonstrates the complexation between α-CD and PCL-CPT. The most intense diffraction peak at 2θ=19.8° in the freeze-dried SMPMs is a fingerprint for the channel structure of α-CD included complexation, which means the α-CDs are stacked on top of each other[28]. The peaks at 2θ=21.3° and 23.6°of PCL-CPT are also present in the spectrum of the freeze-dried SMPMs, indicating the existence of unthreaded PCL-CPT segment within the SMPMs. This confirms the co-existence of threaded PCL-CPT segments and free PCL-CPT segments in α-CD/PCL-CPT prodrug SMPMs, which also indicates the amphiphilic structure formation. The α-CD threaded PCL with the hydrophilic nature would be more possibly act as the micellar shell while unthreaded PCL as the hydrophobic segment acts as the core.

Physiochemical Properties Study of α-CD/PCL-CPT Prodrug SMPMs

The formation of polymeric assemblies mediated by the host–guest interactions between hydrophilic α-CD hosts and hydrophobic PCL-CPT guests was proved by fluorescence spectroscopy using pyrene as a probe which can preferentially partition into hydrophobic micro-domains with a concurrent change in photophysical properties. Taking advantage of this phenomenon, an apparent critical micelle concentration (CMC) of 52 μgmL^{-1} was estimated as shown in Fig. 6. Hydrodynamic diameter of α-CD/PCL-CPT SMPMs from dynamic laser light scattering (DLS) is around 220 nm with very narrow polydispersity index (PDI=0.077) (Fig. 7a). The morphology from transmission electron miscroscopy (TEM) clearly shows well dispersed micelles with average size of about 40 nm, which is smaller than that from DLS test due to dehydration effect during TEM observation (Fig. 7b).

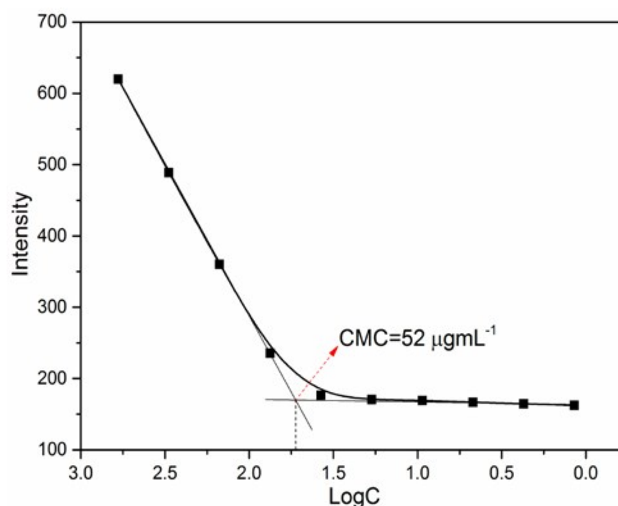

Fig. 6 The fluorescence emission intensity of pyrene at 398 nm as a function of the logarithm of polymer concentration. λex=310 nm; [pyrene]=6×10^{-7} M.

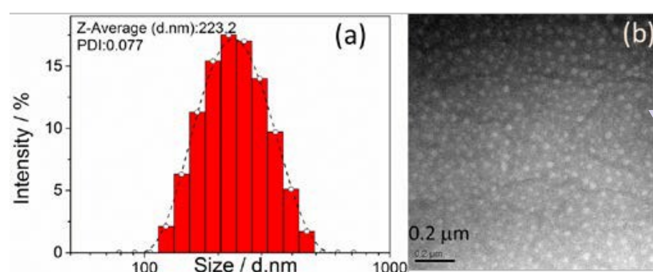

Fig. 7 Size distribution (a) and TEM image (b) of PCL-CPT/α-CD prodrug SMPMs.

Time dependent size alterations of the resultant SMPMs monitored by DLS were further investigated to assess the stability of micelles. The monitoring time lasted for 20 days (Fig. 8). Three micelles formed by different M_n of PCL have no obvious size change even at 5th day indicating the relatively stable property of SMPMs. As time extended, stability of SMPMs formed by α-CD/PCL (11K) was found to be obviously higher (Fig. 8a), while the three groups exhibited nearly initial particle size around 220 nm. The yield and stability results reveal a relatively larger PCL molecular weight may facilitate the assembly process to afford more compact micelles. Therefore, the prodrug SMPMs formed from α-CD/PCL-CPT (11K) was chosen as a representative sample for further study because of its relatively higher stability and yield, except for otherwise indicated.

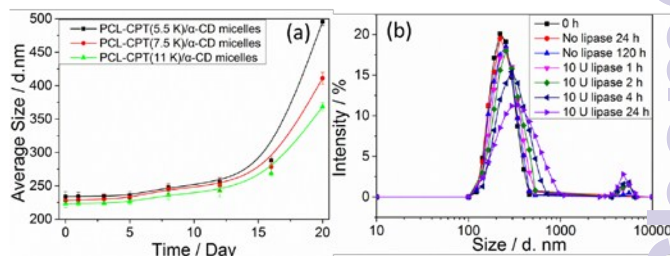

Fig. 8 (a) Size change of PCL-CPT/α-CD prodrug SMPMs over time (b) Size distribution of PCL-CPT/α-CD prodrug SMPMs over time in response to lipase in PBS determined by DLS measurement

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The behavior of enzyme-induced structural disassembly of the formed α-CD/PCL-CPT prodrug SMPMs in PBS was monitored by DLS. The time-dependent changes in size of SMPMs in the presence and absence of 10 U lipase was shown in Fig 8b. The control group without lipase revealed insignificant change in average size of SMPMs. The phenomenon demonstrates the high stability of α-CD/PCL-CPT prodrug SMPMs which is in accordance with the previous stability results. In contrast, the other groups in the presence of 10 U lipase exhibited obvious change in size during 24 h interval, demonstrating the structural change of SMPMs. The increase of size is most probably due to the break of ester bonds, which results in the aggregation of CPT and formation of aggregated micelles.

In Vitro Drug Release Study

In order to investigate the cumulative release of CPT during the disassembling process induced by lipase, the CPT release experiments were conducted under different lipase concentrations. Specifically, three individual experiments were carried out with the concentration of lipase be 0U, 5U and 10U respectively. It can be seen from Fig. 9 that compared to the group without lipase, the two groups with lipase exhibit faster release rate and higher drug release amount. Without lipase, the drug release behavior shows slow and steady release of drug with less than 30% CPT released out over 120 h, moreover, without burst immature release initially. While about 50% CPT was released out for the one with 5U lipase and 70% was released out for that with 10U lipase within 10 h. The lipase contained groups reveal a much faster release rate than the control group, which mainly due to the break of the ester bonds triggered by lipase.

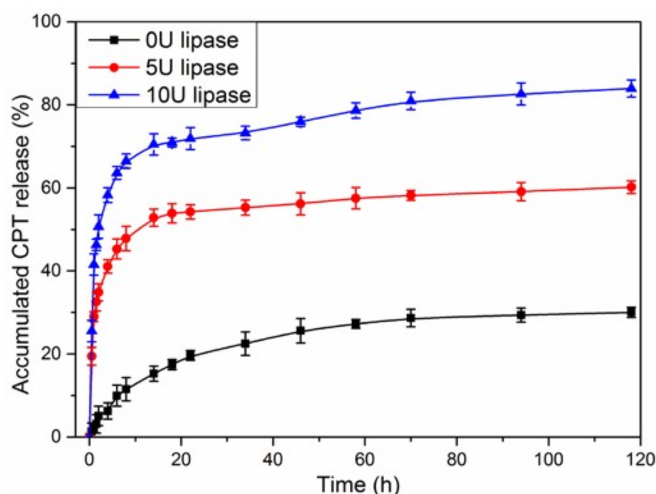

Fig. 9 In vitro release kinetics of CPT from PCL-CPT/ α-CD prodrug SMPMs in the absence and presence of lipase in PBS at 37 ^{o}C .

To evaluate the drug encapsulation capacity of prodrug formed SMPMs, doxorubicin, another hydrophobic anticancer drug was used as a model. DOX was loaded by dialysis method with a drug entrapment efficiency of 43.7% and drug loading efficiency of 17.3% by calculation. The release behavior of physically loaded DOX is compared with chemically conjugated CPT (Fig.10). Apparently, DOX release is much faster than CPT, which is mainly due to physical loading of DOX different from chemical conjugation of CPT. Physically entrapped DOX mainly release through diffusion while chemically conjugated CPT release through hydrolysis of ester bond.

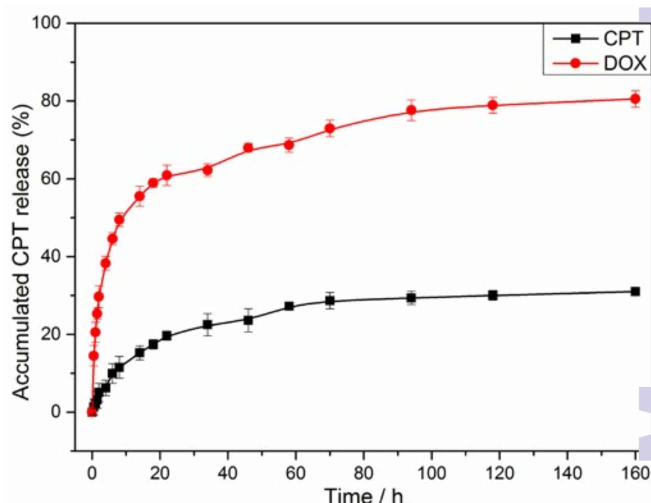

Fig. 10 In vitro release kinetics of CPT and DOX from DOX loaded PCL-CPT/ α-CD prodrug SMPMs in PBS at 37 ^{o}C.

Cytotoxicity Study of α-CD/PCL-CPT Prodrug SMPMs at Cell Level

HepG2 cells were used as model cell line to study the cytotoxicity of SMPMs by MTT assay. Cells were incubated with α-CD/PCL-CPT SMPMs in the absence and presence of lipase as well as free CPT (Equi. dose) for 24 h. The results are summarized in Fig. 11. Except for DOX loaded α-CD/PCL-CPT SMPMs without lipase, α-CD/PCL-CPT SMPMs with 10 U lipase have the highest cytotoxicity of the four while the group without lipase has the minimal inhibition effect. The group of α-CD/PCL-CPT SMPMs with 10 U lipase even has an increased cytotoxicity over free CPT group, which can be explained by the enhanced internalization ability of the nanoscaled micelles and lipase-induced hydrolysis of ester bonds in SMPMs. The group of α-CD/PCL-CPT prodrug SMPMs without lipase has a decreased cytotoxicity over free CPT group, presumably for low hydrolysis extent of ester bonds. Similarly, the cytotoxicity of DOX-loaded α-CD/PCL-CPT SMPMs was evaluated following the same protocol. The cytotoxicity of these two groups, DOX loaded α-CD/PCL-CPT SMPMs and free CPT group respectively show remarkable difference. The enhanced cytotoxicity may be ascribed to the combined effect of DOX released from physical entrapment and the CPT cleaved from the conjugates.

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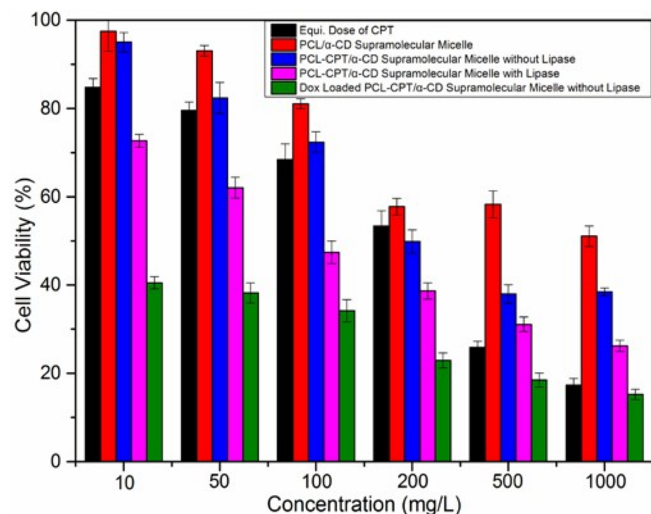

Fig. 11 Cell proliferation of HepG2 cancer cells after 24 h incubation with PCL-CPT/α-CD prodrug SMPMs in presence or absence of lipase, DOX loaded SMPMs, with pure CPT as the control.

Conclusions

In conclusion, a novel α-CD/PCL-CPT prodrug SMPMs was fabricated through host-guest interaction engineered with an enzyme-sensitive drug release mechanism. A prodrug strategy was employed such that CPT can be protected by chemically bioconjugation into the hydrophobic core to avoid the immature release. The assembly based on supramolecular chemistry affords the significant priority of SMPMs over conventional micelles in terms of convenience in fabrication and controllability in micelles structure. In addition, the other drugs such as DOX can be physically encapsulated into the micelles for combination drug delivery. Under tumor-relevant enzyme contained conditions, cleavage of the ester linker initiates micellar rearrangement associated with the rapid release of the therapeutic payload, resulting in enhanced pharmacological efficacy.

Experimental

Materials and Characterizations

Materials: α-CD was purchased from Acros and was used as received. ε-caprolactone (ε-CL, 99%) was purchased from Alfa Aesar and was dried before use. Doxorubicin hydrochloride (Dox·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd (China) and was desalinated before use. Camptothecin (CPT) was obtained from Shanghai Longxiang Co., Ltd. and was used as received. L-phenylalanine, pyridine, acetic anhydride, thionycl chloride, 4-dimethyl-aminopyridine (DMAP) and lipase were purchased from Aladdin Chemistry Co. Ltd.. Chloroform, dichloroform (DCM), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetone, chloroform and tetrahydrofuran (THF) were purchased from Shanghai Chemical Reagent Co. Ltd.. DMF, THF, chloroform and DCM were dried by refluxing over CaH_2 and distilled before use. Dulbecco's modified Eagle's medium (DMEM), trypsin, Dubelcco's phosphate buffered saline (DPBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT), fetal bovine serum (FBS), penicillin-streptomycin were obtained from Gibco Invitrogen Corp. The dialysis bags were obtained from Spectrum Laboratories Inc.

Characterization Methods: ^{1}H NMR spectra were acquired using an Avance 500 MHz spectrometer (Bruker BioSpin Switzerland). Samples were dissolved in $CDCl_3$, and TMS was used as standard. Gel permeation chromatography (GPC) measurements were conducted on a gel permeation chromatographic system, equipped with a Waters 150C separations module and a Waters differential refractometer. The molecular weight and molecular weight distributions were calibrated against polystyrene standards, with $CHCl_3$ as the eluent at a flow rate of 1 mL min^{-1}. UV/Vis detection and fluorescence spectra were obtained with an ultraviolet–visible spectrophotometer (Varian Ltd., Hong Kong) and a Hitachi F2500 luminescence spectrometer (Hitachi Ltd., Hong Kong), respectively. TEM was carried out on a Hitachi H7100 transmission electron microscope (Hitachi Ltd., Hong Kong). Briefly, a drop of sample suspension was placed on a copper grid fitted with a Formvar film and dried before measurement. DLS studies of SMPMs were determined by NanoZS 90 Nanosizer (Malvern Instruments Ltd., Worcestershire, UK) with the scattering angle fixed at 90°. The crystallographic data of the samples were characterized by X-217 ray diffraction (XRD D/MAX-IIIC, Japan). The XRD patterns were obtained from 10 to 90° (2θ) based on Cu Kα radiation at a predetermined scanning rate.

Fabrication of α-CD/PCL-CPT Prodrug SMPMs

Synthesis of PCL–COOH: The guest molecular PCL-COOH was synthesized through ring-opening polymerization of ε-CL initiated by L-Phe (L-phenylalanine). In order to acquire guest molecular with expected molecular weight, the raw material should be mixed with given molar ratio ([ε-CL]/[L-Phe]). Herein, guest molecular with three different M_n were synthesized. Specifically, ε-CL (2.901 g), accompanied with different weight of L-Phe (0.099 g, 0.071 g, 0.050 g respectively) was added into a vacuum-sealed ampule and the mixture was stirred at 160 °C for 24 h in a vacuum-sealed ampule. The residue was purified by dissolving it in THF and then being precipitated with a mixture of CH_3OH and H_2O (v/v=4:1) at RT. The precipitate (OH-PCL-COOH) was finally filtrated and dried in a vacuum at 30 °C for 24 h.

Hyrdroxyl inactivation of HO-PCL-COOH: The HO-PCL-COOH synthesized need to undertake a "hydroxyl group sheilding" process so as not to affect the following reactions. Typically, HO-PCL-COOH (0.5 g) was dissolved into 5mL pyridine with excess acetic anhydride (50 μL) added. The mixture was stirred at room temperature (r.t.) for 24 h. Excess pyridine was removed under reduced pressure, and the residue was redissolved in THF. The processed product $CH_3COOPCL$-COOH was then being precipitated with a mixture of CH_3OH and H_2O (v/v=4:1) at r.t. and was finally filtrated and dried in a vacuum at 30 °C for 24 h.

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Synthesis of PCL-CPT: $CH_3COOPCL-COOH$ (0.3g, 0.06 mmol) was dissolved into 5mL DCM and thionyl chloride (30 μL) was added. The mixture was stirred at room temperature under N_2 atmosphere for 24 h. The product of $CH_3COOPCL-COCl$ was then obtained after removing the excess thionyl chloride and DCM under reduced pressure. CPT (0.02g, 0.06mmol) was suspended in 10 mL of anhydrous chloroform and cooled to 0°C. Then the chloroform solution of $CH_3COOPCL-COCl$ and DMAP (0.0025g, 0.02mmol) were added. The mixture was stirred at room temperature for 24 h. the mixture solution was condensed and cold down to 0 °C, the free CPT crystal was removed by centrifugation, the supernatant was subjected to precipitation by CH_3OH and H_2O (v/v=4:1). The obtained product PCL-CPT was further dried under vacuum at 30 °C for 24 h.

Formation of α-CD/PCL-CPT SMPMs: A solution of α-CD (40 mg) and urea (40 mg) dispersed in deionized water (10 mL) was added dropwise to a stirred solution of PCL with different mass in THF (10 mL) at 60°C, after which the mixture was stirred continuously for 24 h. THF, urea, and the excess α-CD in the mixture were removed through dialysis method against water. Small amounts of precipitate were removed by centrifugation after cooling the mixture to room temperature. The finally obtained solution was freeze-dried before characterization.

Assembly and Disassembly Study of α-CD/PCL-CPT Prodrug SMPMs

Critical micelle formation concentration (CMC) determination: CMC of α-CD/PCL-CPT SMPMs was determined by fluorescence spectroscopy method using pyrene as hydrophobic florescence probe following a previous protocol[29]. Morphology of SMPMs was observed by TEM.

Lipase-induced stability and hydrolysis study of α-CD/PCL-CPT SMPMs: α-CD/PCL-CPT SMPMs (0.5 mg/mL) prepared in 100 mL PBS （0.01 M, pH 7.4） were incubated with 10 U of lipase at 37 °C. The above solution was placed in a shacking bed at 37°C with a rotation speed of 200 rpm. Time-dependent changes in nanomicelle size distribution was monitored for 24 h at different time intervals by DLS. Control group without lipase was performed in parallel.

In Vitro Drug Release Study

Lipase-induced CPT release from α-CD/PCL-CPT SMPMs: Release kinetics of CPT from α-CD/PCL-CPT SMPMs was measured at 37 °C following a modified protocol by Koo et. al[30]. Briefly, 5 mL α-CD/PCL-CPT SMPMs (0.5mg/mL) suspension prepared in PBS (0.01 M, pH 7.4) were filled into a dialysis bag (MWCO = 3.5 kDa) in the presence and absence of lipase (0U, 5U, 10U respectively). Dialysis was performed against PBS (100 mL) under constant stirring (150 rpm). During a period of 120 h, 2 mL aliquots of the dialysis medium was withdrawn with an equal volume of fresh media replenished at predefined time points and CPT content was measured by UV/Vis spectroscopy method (λ= 370 nm). The measurement was performed three times in parallel, and the result is reported as average ± SD.

DOX's encapsulation into α-CD/PCL-CPT SMPMs: DOX was loaded into SMPMs by dialysis method. Typically, a solution of α-CD/PCL-CPT SMPMs (20 mg) and DOX (8.8 mg) in DMF was subjected to dialysis against deionized water for 24 h at 37°C. The dialysis medium was changed five times to remove the free DOX and DMF. The whole procedure was performed in the dark. The drug loaded SMPMs was obtained by freeze drying. For determination of drug loading content, DOX-loaded SMPMs were dissolved in DMF and analyzed with UV spectroscopy with DOX's absorption wavelength at 485nm. The calibration curve was obtained with DOX in DMF solutions under different concentrations. Drug loading (DL) and entrapment efficiency (EE) were calculated according to the following formula:

$$DL(wt.\%) = \frac{\text{mass of drug loaded in micelles}}{\text{mass of drug loaded micelles}} \times 100\%$$

$$EE(wt.\%) = \frac{\text{mass of drug loaded in micelles}}{\text{mass of drug fed initially}} \times 100\%$$

Dual release of CPT and DOX from α-CD/PCL-CPT SMPMs: The release profiles of CPT and DOX from DOX-loaded SMPMs were explored in PBS (0.01 M, pH 7.4) at 37°C following a modified protocol by Koo etal[30]. Typically, 5 mL DOX-loaded α-CD/PCL-CPT SMPMs (0.5mg/mL) suspension prepared in PBS (0.01 M, pH 7.4) were filled into a dialysis bag (MWCO = 3.5 kDa). Dialysis was performed against PBS (100 mL) under constant stirring (150 rpm). During a period of 120 h, 2 mL aliquots of the dialysis medium was withdrawn with an equal volume of fresh media replenished at predefined time points. The content of CPT and DOX was measured by UV/Vis spectroscopy method (370 nm, 485nm respectively).

Study of α-CD/PCL-CPT Prodrug SMPMs at Cell Level

Cell lines: The human liver hepatocellular carcinoma (HepG2) cells were supplied by Cell Center of Tumor Hospital of Fudan University (Shanghai, China). Cells were propagated in T-75 flasks under an atmosphere of 5% CO_2 at 37 °C and grown in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) penicillin/streptomycin.

In vitro cytotoxicity assay: The cytotoxicity of α-CD/PCL SMPMs, α-CD/PCL-PCL prodrug SMPMs and DOX loaded α-CD/PCL-PCL prodrug SMPMs against HepG2 cells were investigated by measuring the cell viabilities through the conventional MTT assay. Typically, HeLa cells were seeded in 96-well plates (5000 cells/well) using DMEM (200 μ L) and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 for 24 h. The medium in each well was then replaced with culture medium (150 μL) containing treatments of free CPT, α-CD/PCL SMPMs, α-CD/PCL-PCL prodrug SMPMs, or DOX loaded α-CD/PCL-PCL prodrug SMPMs. The concentration of α-CD/PCL SMPMs, α-CD/PCL-PCL prodrug SMPMs (in the presence and absence of 10U lipase) and DOX loaded α-CD/PCL-PCL prodrug SMPMs was diluted with culture medium to obtain a concentration range of 1×10^{-3} to 1 mg mL^{-1}. After incubation for 24 h, the cell culture medium in each well was replaced with 100 μL of fresh medium and the cells were incubated for a further 4 h with 20 μL of sterile filtered MTT (5 mg/mL) in warm PBS allowing viable cells to reduce the MTT into purple formazan crystals

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After the removal of the culture medium, 100 μL DMSO was replenished into each well and the plate was placed on a shaking platform for 10 min at 25°C.The absorbance of the resulting solution was measured at 570 nm using a Multiscan MK3 plate reader. Cell viability was calculated according to the following equation: Cell viability = $(OD_{treated}/OD_{control}) \times 100\%$, where $OD_{treated}$ was obtained by comparing the OD with that of control wells containing only cell culture medium. Data are presented as average ± SD.

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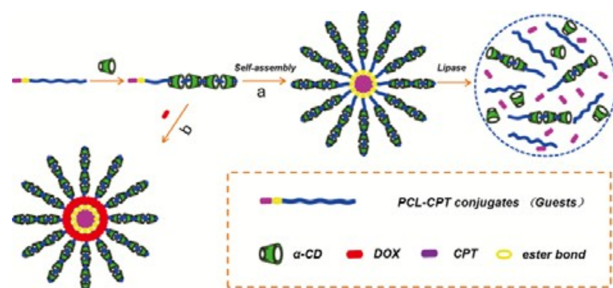
Graphic Abstract:

Supramolecular, prodrug-based micelles (SMPMs) with enzyme-induced drug release behavior were engineered via host-guest interaction of camptothecin carrying PCL and α-cyclodextrin.



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