1 Introduction

The recent decade has witnessed various materials potentially used in drug delivery systems (DDS). The interaction between drugs and materials becomes thus a key basic topic. While the influence of drugs on polymeric properties such as glass transition temperature and the influence of polymers on physical properties of drugs such as solubility and diffusivity have been extensively investigated, only a few reports concern the influence of materials on the chemical properties of drugs. The corresponding fundamental studies are thus quite meaningful, especially if the chemical property of a drug could be altered without any reaction between the drug and material. Herein we employed a famous family of amphiphilic block copolymers poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) (PEG–PPG–PEG) called Pluronics as model materials and a well-known family of camptothecins (CPT) as model drugs, and focused upon a physicochemical study of the material cue to influence the equilibrium between the active and inactive chemical forms of a commercialized drug 10-hydrocamptothecin (HCPT) in water.

HCPT is one of the most important drugs of CPT with its chemical structure shown in Fig. 1. HCPT and most of the other CPT analogues have two chemical forms, depending upon the E-ring, as shown in Fig. 1. The lactone form is not due to a kinetic delay of the ring-opening of drugs within micelles, but a thermodynamic shift of the equilibrium between the active and inactive forms driven by drug–material interactions. Further, the equilibrium constant of HCPT within micelles was determined based on analysis of partition between micelles and their medium of both the lactone and carboxylate forms, which by we afforded the so-far only equilibrium constant of the CPT-family drugs within suspended nanoparticles.

10-Hydrocamptothecin (HCPT) exists, like most of the antitumor drugs in the camptothecin (CPT) family, in the form of either lactone or carboxylate, but only the former with a closed E-ring has antitumor efficacy and the lactone fraction at physiological pH is quite low. Herein, we examined the effect of addition of an amphiphilic copolymer Pluronic P123 on HCPT in water. P123 is composed of major poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) and minor diblock copolymers. The lactone fraction of HCPT in the P123 aqueous solution was increased with the copolymer concentration, and the transition P123 concentration was higher than the critical micelle concentration by about two orders of magnitude and lower than critical gelation concentration by 1.5 orders of magnitude. We anticipated that the key event was the co-assembly between drug molecules and “mature micelles” instead of either physical gelation or merely the nascent micelle formation. Such anticipation was confirmed by the fluorescence anisotropy of HCPT at various P123 concentrations. We also explicitly indicated that the enhancement of the lactone fraction was not due to a kinetic delay of the ring-opening of drugs within micelles, but a thermodynamic shift of the equilibrium between the active and inactive forms driven by drug–material interactions. Further, the equilibrium constant of HCPT within micelles was determined based on analysis of partition between micelles and their medium of both the lactone and carboxylate forms, which by we afforded the so-far only equilibrium constant of the CPT-family drugs within suspended nanoparticles.

Tianyuan Ci, Ting Li, Liang Chen, Guangtao Chang, Lin Yuab and Jiandong Ding*

State Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Advanced Materials Laboratory, Fudan University, Shanghai 200433, China. E-mail: jdding1@fudan.edu.cn; Fax: +86-21-65640293; Tel: +86-21-65643506

Key Laboratory of Smart Drug Delivery of Ministry of Education and PLA, School of Pharmacy, Fudan University, Shanghai 201203, China

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3py00118k

Cite this: Polym. Chem., 2013, 4, 3245
2.2 Determination of CMC of P123 copolymers in water

CMC of the block copolymers in PBS solution was determined with a hydrodynamic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Briefly, copolymer solutions of a series of concentrations were prepared with the final DPH concentration 4 μM. The medium pH was measured with a pH-meter, and all the following pH measurements were conducted with the same pH-meter (Aurora Scientific Instruments (Shanghai) Co., Ltd). After adjusting the pH to 7.4 and equilibrating at 37 °C for 12 h, the UV-visible absorption of DPH was detected. The optical densities (ODs) at 337, 356 and 377 nm were increased if DPH was partitioned into the hydrophobic core upon micelle formation. The CMC value was estimated from the transition point of the curves of absorbance difference between 377 and 400 nm versus logarithmic polymeric concentration.

2.3 Dynamic laser scattering (DLS) of micelles

DLS was detected in a light scattering spectrophotometer NanoZS90 (Malvern) with a vertically polarized incident beam at 532 nm. Series of P123 solutions with concentrations ranging from 0.01 wt% to 2.5 wt% were prepared and equilibrated at 37 °C for 12 h. Prior to detection, all the samples were filtered through a 0.22 μm filter and equilibrated for another 10 min. The hydrodynamic radii of P123 micelles were calculated by Stokes–Einstein equation, and data were fitted by GENERAL approach. Each specimen was tested three times.

HCPT-loading P123 micelles were also prepared with 5 μg ml⁻¹ of HCPT and 0.01–2.5 wt% of P123. The sample preparation, detection and data processing were the same as those in dealing with the blank P123 micelle solutions as stated above.

2.4 Transmission electron microscopy (TEM) of micelles

TEM was conducted on a Hitachi electron microscope. The copper grids with carbon films were immeresed into 0.078 wt% and 1.25 wt% P123 micelle solutions. After drying under an infrared lamp, samples were observed at an accelerating voltage 75 kV.

2.5 Phase diagram of concentrated P123 aqueous systems

Concentrated systems of P123 copolymers in water could exhibit a sol–gel transition upon increase of temperature. The sol or gel states of P123 aqueous systems at various concentrations and temperatures were determined by the vial inverting method. P123 solutions of a series of concentrations were prepared first. Then 0.8 ml P123 solutions were added into test vials. All the vials were incubated in a water bath with a temperature increase of 1 °C per 10 min. The sol–gel transition temperature was defined as the starting temperature when no flow of the solution was observed within 15 s.

2.6 Determination of solubility of the lactone-form HCPT in P123 aqueous solutions

P123 solutions of a series of concentrations [0.625 wt%, 1.25 wt%, 2.5 wt%, 5 wt% and 10 wt%] with a superfluous amount of HCPT powder were prepared and adjusted to pH 2 to guarantee
the lactone form of HCPT. After being incubated in a water bath at 37 °C for 7 days with a shaking rate 50 rpm, the suspensions were filtered through a 0.8 μm filter (Millipore) to remove the insoluble HCPT powder. The filtered solutions were detected by HPLC to determine the HCPT concentrations under the detection conditions described in Section 2.7.

2.7 HPLC analysis of the drug

The lactone fractions of HCPT were determined by high performance liquid chromatography (HPLC) with Waters Separation Module e2695 and Waters UV/visible detector 2486. The lactone and carboxylate forms were separated in a reverse phase column of 5 μm C18 (150 mm × 4.6 mm, SunFire™) with column temperature 25 °C and flow rate 1 ml min⁻¹. The mobile phase was 23% methanol, 70% phosphate buffer (pH 6.5, 25 mM), 7% tetrahydrofuran. The detection wavelength was set as 382 nm.

The lactone fraction \( f_{\text{lactone}} \) was calculated by

\[
 f_{\text{lactone}} = \frac{A_{\text{lactone}}}{A_{\text{total}}} \quad \text{Here, } A_{\text{lactone}} \text{ and } A_{\text{total}} \text{ refer to the peak areas of the lactone forms of the samples before and after acidification.}
\]

The latter reflects the total concentration of lactone and carboxylate before acidification.

2.8 Ring-opening or ring-closing kinetics of HCPT upon an abrupt pH change

For the ring-opening process, 5 mg HCPT was first suspended in 1 ml acidic medium, making all of the drug molecules in the lactone form; for the ring-closing process, HCPT was first dissolved in an alkaline medium to ensure all of the drug molecules in the carboxylate form. Then, 10 μl of the stock solution was quickly poured into 10 ml PBS or polymeric PBS solution, followed by adjusting pH to 7.4 and keeping at that pH at 37 °C. Some samples were taken out and detected by HPLC. The change of \( f_{\text{lactone}} \) was fitted exponentially.

2.9 The pH dependence of equilibrium lactone fractions

HCPT-containing PBS or P123 solutions were prepared at pH 4–10. After 12 h at 37 °C, the equilibrium lactone fractions were detected by HPLC. We used the equation

\[
 f(pH) = f_\ast + \frac{f_\ast - f_\ast}{1 + 10^{\varphi (pH - pH_1/2)}}
\]

to fit the pH-dependent lactone fractions, resulting in the transition pH termed as pH₁/₂ by us. The lactone fraction is between the two extremes \( f_\ast \) and \( f_\ast \). The equation employs the form of Henderson–Hasselbalch (HH) previously used in our studies of polymer ionomers and biomacromolecules.⁴⁰-⁴² Both the transition pH and HH index \( n \) could be obtained via fitting.

2.10 Dependence of the lactone fraction upon polymeric concentration

P123 solutions of a series of polymeric concentrations from 10⁻³ wt% to 35 wt% with a fixed amount of HCPT (50 μg ml⁻¹) were adjusted to pH 7.4 ± 0.1 and kept at 37 °C for 12 h. Then HPLC measurements were made to determine the equilibrium lactone fraction.

2.11 Temperature dependence of equilibrium lactone fraction of HCPT in P123 solutions

10 wt% P123 solutions loaded with 50 μg ml⁻¹ HCPT were prepared and adjusted to pH 7.4 ± 0.1. The stock solutions were equilibrated at 4, 10, 15, 25 and 37 °C for 3 days. Then 0.5 ml samples were taken out for HPLC analysis after diluted with the mobile phase.

2.12 Fluorescence anisotropy measurements

P123 solutions containing 5 μg ml⁻¹ HCPT were prepared and adjusted to the predetermined pH (4.0, 7.4 and 10.0). After equilibrating at 37 °C for 12 h, 3 ml samples were taken out. The steady-state fluorescence polarization measurements were conducted in the fluorescence spectrophotometer FLS920 (Edinburgh Instrument) with the excitation and emission wavelengths of 380 and 570 nm, respectively. The intensities of fluorescence of HCPT through polarizer and analyzer with horizontal (H) or vertical (V) orientation were detected. The fluorescence anisotropy was defined as

\[
 \text{Fluorescence anisotropy} = \frac{I_{HH}I_{VV} - I_{HV}I_{VH}}{I_{HH}I_{VV} + 2I_{HV}I_{VH}} \quad (2)
\]

The value was calculated automatically by the software in the spectrophotometer.

3 Results

3.1 Detection of ring-opening and ring-closing kinetics of HCPT

HPLC was used to separate and quantify the lactone and carboxylate forms of HCPT, and some chromatogram profiles are shown in Fig. 2A. Under extreme acidic or alkaline conditions, just the lactone or carboxylate form was observed. The retention times of the lactone form and carboxylate form of HCPT were about 13 min and 4 min, respectively. The peak area is linearly related to the drug concentration, with the standard curve given in ESI Fig. S1. At neutral medium with pH 7.4, both peaks appeared but with a minor lactone peak, and the presence of copolymer P123 led to a significant lactone peak in Fig. 2A.

An abrupt change of medium pH might result in significant ring-opening or ring-closing. Both processes were detected, with the HPLC profiles presented in ESI Fig. S2 and the calculated lactone fractions shown in Fig. 2B. The fractions changed almost exponentially. The equilibrium lactone fraction of HCPT in PBS at pH 7.4 and 37 °C was just about 13%. At the same pH and temperature, the fraction was enhanced to about 45% in the 10 wt% P123 solution.

3.2 The pH dependence of equilibrium lactone fraction

We then detected equilibrium fractions of the lactone form of HCPT under a series of pH values, and obtained complete titration curves, as shown in Fig. 3. A significant increase of the
transition pH was found, resulting in $\mathrm{pH_{1/2}}$ changing from 6.6 in PBS to 7.3 in 10 wt% P123 solution. So, the lactone fraction was influenced to a large extent especially at neutral and weakly acidic conditions.

### 3.3 Dependence of the lactone fraction of HCPT upon copolymer concentrations

The concentration dependence of the lactone fraction of HCPT at pH 7.4 is illustrated in Fig. 4. The condensed states of the polymeric system also changed with concentration of this amphiphilic block copolymer. A transition from single molecules to micelles happened at a low concentration with CMC of P123 about 0.005 wt% in PBS at 37 °C (Fig. 4A); a sol–gel transition occurred at a high P123 concentration, with CGC about 26 wt% (Fig. 4B). The significant enhancement of the lactone fraction happened at P123 concentrations much lower than CGC. So, micelle formation is physically more essential than hydrogel formation as far as the material cue to influence the ring-closing of CPT analogues is concerned.

### 3.4 Polymer concentration dependence of P123 micelles in water

We also used DLS to detect micelle sizes and distributions at copolymer concentrations higher than CMC. The hydrodynamic radii of P123 micelles decreased with the increase of copolymer concentrations in the examined range. Such a trend was observed both in the presence and absence of the drug, as presented in Fig. 5A.

The direct visualization via TEM confirms that P123 micelles formed at a relatively higher polymer concentration were relatively smaller and denser than those formed at a relatively lower concentration (Fig. 5B). The micelles were spherical with a core-corona structure.

### 3.5 Fluorescence analysis of HCPT in P123 solutions

CPT could emit fluorescence when properly excited. We found that its fluorescence emission spectrum was not very sensitive to medium pH in PBS (absence of copolymer), as shown in Fig. 6A; however, the fluorescence anisotropy was significantly affected by the medium pH in the presence of the amphiphilic copolymer. We interpret such pH and concentration sensitivities as an indicator of co-assembly of the lactone form of HCPT into P123 micelles, as schematically presented in Fig. 6B. The lactone form of HCPT at low pH is relatively hydrophobic, and thus ready to be self-assembled into the P123 micelles. The lower mobility of the anisotropic fluorescent dye in the micelles led to higher fluorescence anisotropy.

The fluorescence anisotropy of the HCPT/P123 solutions did not exhibit significant concentration-sensitivity in basic medium, as shown in Fig. 6C. In contrast, a transition took place at 0.6–0.7 wt% in the acidic and neutral media. We define this value as the critical co-assembly concentration $C_{\text{co-assembly}}$. 

---

**Fig. 2** (A) HPLC chromatogram profiles of HCPT under the indicated conditions. The complete lactone specimen was obtained at an acidic medium (pH 1.0), and the complete carboxylate specimen, at an alkaline medium (pH 12). In PBS at pH 7.4, the E-ring of the drug was predominantly opened. The close-ring form was however enhanced after addition of the copolymer P123. (B) Kinetic processes of ring-opening and ring-closing of HCPT in the aqueous systems. The drugs were pre-treated at pH < 4.0 or pH > 9.0 to achieve complete close-ring and open-ring, respectively. Then, the drugs were put into a large amount of neutral PBS solution or the P123 (10 wt%) PBS solution (pH 7.4) to detect the ring-opening or ring-closing kinetics.

**Fig. 3** Equilibrium lactone fraction of HCPT as a function of medium pH in the PBS solution or 10 wt% P123 PBS solution. The data were fitted by eqn (1), resulting in $\mathrm{pH_{1/2}} = 6.6$ (HH index $n = 0.93$) for PBS and 7.3 ($n = 1.03$) for the 10 wt% P123 solution.
3.6 Temperature dependence of the equilibrium lactone fraction of HCPT in P123 solutions

For Pluronics in water, the micellization depends upon not only concentration, but also temperature. For the 10 wt% P123 solution, micelles were formed with increase of temperature, and the critical micelle temperature (CMT) was between 4–10 °C, as reflected in the UV-visible absorption spectra of DPH in 10 wt% P123 solutions at different temperatures, as presented in ESI Fig. S3.† We also examined the lactone fractions of HCPT at 4 °C, 10 °C and higher temperatures. Although the increase of medium temperature exhibited a slight decrease of the equilibrium lactone fraction of HCPT in PBS (data not shown), such a “default” trend was significantly altered among 10–25 °C after addition of the amphiphilic block copolymer P123, as shown in Fig. 7.

3.7 Solubility of the lactone-form HCPT in P123 aqueous solutions

We designed HCPT solubilization experiments mainly in order to indirectly estimate the partition coefficient of HCPT into block copolymer micelles. While the carboxylate form of HCPT is highly soluble in water, the lactone form is relatively hydrophobic with only a limited solubility. The solubility of HCPT in PBS at pH 1.9 at 37 °C was determined by us as 2.56 μg ml⁻¹, which is consistent with the report in the literature.29 The addition of the amphiphilic block copolymer P123 increased the solubility of HCPT to a large extent, as shown in Fig. 8. The further calculation of the partition coefficient of HCPT between

---

Fig. 4 (A) Absorption difference at 377 and 400 nm as a function of P123 concentration with a given amount of hydrophobicity-sensitive dye DPH. The CMC measurement was carried out in PBS at 37 °C. (B) Phase diagram of P123 in the PBS solution. The critical gelation concentration (CGC) is ready to be determined from the diagram. The insets show images of a sol and a gel with the experimental conditions indicated by the asterisks. (C) The lactone fraction of HCPT in the P123 solution (with PBS as medium) at different P123 concentrations.

Fig. 5 (A) DLS data of intensity distributions of hydrodynamic radii of P123 micelles in water $f(R_\text{H})$ at indicated weight concentrations of copolymers with or without HCPT. The drug concentration was 5 μg ml⁻¹. The dashed lines and arrows are just guides of the eyes to indicate the change of peak positions. (B) TEM images of P123 micelles formed at copolymer concentrations of 0.078 and 1.25 wt%. The lower row shows the magnified images of the local regions in the upper row marked by the dashed squares.
4 Discussion

4.1 Kinetic retardation or thermodynamic equilibrium shifting?

In the present study, we employed P123/HCPT as the model system, and illustrated that the equilibrium lactone fraction of HCPT was altered after addition of the block copolymer even at neutral pH. Besides the ring-opening kinetics, ring-closing kinetics were also tested in polymer solution in Fig. 2B, and the equilibrium lactone fraction of HCPT increased from 13% in PBS to about 45% in the 10 wt% P123 solution, indicating a significant thermodynamic equilibrium shifting. Because the lactone fraction upon addition of amphiphilic block copolymers was enhanced due to the equilibrium shifting instead of the kinetic retardation, the low pH in preparation of drug-loading micelles was NOT absolutely necessary, although sometimes helpful if the low pH within drug carriers could be maintained under the physiological conditions.

The equilibrium lactone fractions within a wide range of medium pH were also detected, with the results shown in Fig. 3. Considering that the ring-opening of the E-ring of CPT and its analogues is closely related to ionization of the carboxylate, the hydrolysis process in water actually includes two steps:25

\[
L \rightleftharpoons \frac{K_{\text{open}}}{K_a} C \rightleftharpoons C^- + H^+ \tag{3}
\]

Here, L, C, and C\(^-\) denote the lactone form, nonionized carboxylate form and ionized carboxylate form, respectively; \(K_{\text{open}}\) and \(K_a\) are the equilibrium constants with respect to the first and second steps, respectively. After neglecting the difference between concentration and activity, both equilibrium constants are directly related to the corresponding

Fig. 6 (A) Normalized fluorescence spectra of HCPT in the PBS solution at room temperature with excitation wavelength 380 nm. (B) Schematic presentation of the different co-assembly states between HCPT and P123 at low and high pH. (C) Fluorescence anisotropy of HCPT in P123 solutions at 37 °C as a function of P123 concentrations at indicated medium pH. The excitation and emission wavelengths were 380 and 570 nm, respectively. The fluorescence anisotropy of HCPT was calculated via eqn (2).

Fig. 7 Lactone fractions of HCPT in 10 wt% P123 solutions (PBS as medium) at pH 7.4 and indicated temperatures. Three independent measures were carried out for each group. The p values from Student t-tests are listed in ESI Table S1.† Significant differences were found between any two groups except 4 °C versus 10 °C, and 25 °C versus 37 °C.

Fig. 8 Solubility or solubilization limit of lactone-form HCPT in copolymer suspension \([L]_{\text{susp},s}\) over that in PBS \([L]_{\text{PBS},s}\) as a function of P123 concentration at 37 °C. The lactone form was guaranteed by preparing HCPT solutions at pH 2 (actually 1.9). After shaking in a 37 °C water bath at 50 rpm for 7 days, the solubilities in the saturated solutions were measured following removal of the undissolved HCPT in the acidic solutions through a 0.8 μm filter (Millipore). The data were linearly fitted with a fixed intercept of 1, resulting in a slope of 72 and squared correlation coefficient of 0.99.
concentrations as \( K_{\text{open}} = [C][L] \) and \( K_a = [C^-][H^+][C] \). The global equilibrium constant \( K \) is written as

\[
K = \frac{[C^-][H^+]}{[L]} = K_{\text{open}}K_a
\]

so, we have

\[
pK \equiv -\lg K = pH - \lg([C^-]/[L]) = pK_{\text{open}} + pK_a
\]

Usually [C] is very low, and thus the drug exists mainly in the form of either the lactone form (L) or the ionized carboxylate form (C–).

We define the medium pH with respect to half ring-opening as pH1/2, where equal lactone form and carboxylate form occur. The following relation is, based upon eqn (5), ready to obtain

\[
pH_{1/2} = pK_{\text{open}} + pK_a
\]

Fig. 3 indicates that pH1/2 changed from 6.6 in PBS to 7.3 in 10 wt% P123 solution. Such an increase of pH1/2 led to a significant increase of the equilibrium lactone fraction at neutral and weakly acidic conditions. This pH range is particularly meaningful in medicine. The neutral pH is standard in most in vitro biological investigations and refers to normal tissues; the weakly acidic environment corresponds to the interior of tumor tissues in vivo due to accumulation of acidic metabolism products in the rapid proliferation of tumor cells.11, 44–47

4.2 Theoretical analysis and experimental determination of the equilibrium constant of HCPT within a P123 micelle

Since the equilibrium was shifted due to the formation of micelles, it was natural for us to try determination of the shifted equilibrium constant within micelles. However, we found no present characterization protocol. Unexpectedly, the equilibrium constants of any member of the CPT family within any kind of micelles or other suspended particles have never been reported in the literature; thus we had to set up an approach by ourselves. A candidate detection might come from the straightforward measurement of the lactone and carboxylate forms of CPT-like drugs in micelles. It must be based upon the precise and complete isolation of drug-loading micelles from the corresponding suspension, which is however very hard if not impossible. Another difficulty comes, in our opinion, from that the process of transformation within a micelle is closely related to that out of micelles and the partition of both lactone and carboxylate forms between micelles and medium, as schematically presented in Fig. 9.

We herein suggest to determine \( K_{\text{micelle}} \) indirectly from other constants. The four constants are defined as

\[
K_{\text{micelle}} = \frac{[C^-][H^+]}{[L]}
\]

\[
K_{\text{PBS}} = \frac{[C^-][H^+]}{[L]}
\]

\[
[L]_{\text{in}} = K_L[L]_{\text{out}}
\]

\[
[C^-]_{\text{in}} = K_C[C^-]_{\text{out}}
\]

and for the global equilibrium between the lactone and carboxylate forms in the micelle suspension, we have

\[
K_{\text{susp}} = \frac{[C^-]_{\text{susp}}[H^+]_{\text{susp}}}{[L]_{\text{susp}}}
\]

in the writing of eqn (7), (8) and (11), the difference between concentration and activity has been neglected. Among those five equilibrium constants, \( K_{\text{PBS}}, K_{\text{susp}} \) and \( K_L \) could be determined experimentally; \( K_C \) and \( K_{\text{micelle}} \) should be calculated through other quantities.

4.2.1 Determination of \( K_{\text{out}} \) and \( K_{\text{susp}} \) via \( pH \) titrations of equilibrium lactone fractions without and with the presence of copolymers. The transformation constant outside of micelles \( K_{\text{out}} \) is assumed to be equal to that in PBS without copolymer, namely, \( K_{\text{PBS}} \) (that is why we have replaced \( K_{\text{out}} \) by \( K_{\text{PBS}} \) in Fig. 9 and eqn (8)). This constant could be obtained from the proton concentration \([H^+]\) when \([C^-] = [L] \) in PBS. So, \( K_{\text{PBS}} \) is ready to be obtained from \( pH_{1/2,\text{PBS}} \), which reads 6.6 in Fig. 3.

Similarly, \( K_{\text{susp}} \) was obtained from the \( pH \) titration of lactone fraction as shown in Fig. 3, with \( -\lg K_{\text{susp}} = pH_{1/2,P123} = 7.3 \).

4.2.2 Determination of \( K_L \), the partition coefficient of the lactone form of HCPT between P123 micelles and outer-phase medium indirectly from the solubility measurements. The solubilization limit, namely, saturated concentration of the lactone-form HCPT, \([L]_{\text{susp,s}}\) in a series of P123 micelle suspensions is a function of the lactone concentrations both within micelles \([L]_{\text{in}}\) and in medium \([L]_{\text{out}}\) as well as of the volume fraction of micelles \( \phi \) as

\[
[L]_{\text{susp,s}} = \phi[L]_{\text{in}} + (1 - \phi)[L]_{\text{out}}
\]

Combination of eqn (9) and (12) leads to

\[
\frac{[L]_{\text{susp,s}}}{[L]_{\text{out}}} = (K_L - 1)\phi + 1 = (K_L - 1)sC_{P123} + 1
\]

Here, \( C_{P123} \) is the weight concentration of the block copolymer P123, and \( s \) is the corresponding swelling factor of micelles.
defined as the volume of micelles over the weight of copolymers. The parameter $s$ is in no way strictly characterized so far, yet it could be estimated from CGC in the phase diagram of P123. Physical gelation happened at concentration 26 wt% according to Fig. 4B, in which a percolated micelle network forms. If micelles occupied 100% volume of the suspension in the state of the physical hydrogel, the parameter $s$ might be 100%/26% ~ 3.8. In the following derivation, this value was assumed not significantly changed with polymer concentration, which could be accepted unless $C_{P123} >$ CGC.

While $[L]_{\text{susp}}$ is ready to be measured, $[L]_{\text{out}}$ and $[L]_{\text{in}}$ are not, because it is very difficult to isolate micelles and media with exact quantities. Nevertheless, in the extreme case when HCPT exists only in the lactone form (under the acidic condition) and is saturated, $[L]_{\text{out}} = [L]_{\text{PBS,s}}$. Thus the values of $[L]_{\text{susp, PBS,s}}$ must be linearly related to the weight concentration of P123 with slope $(K_c - 1)s$, as shown in eqn (13), if the equilibrium constant $K_c$ and the swelling factor $s$ do not change with copolymer concentration. Our experimental measurements (Fig. 8) confirm our assumption in the examined concentration range. The value of the slope was 72. Therefore, we determined $K_c = 20$. Since $K_c \gg 1$, the lactone form prefers to be enriched in the micelles significantly.

**4.2.3 Determination of $K_c$, the partition coefficient of the carboxylate form of HCPT between P123 micelles and outer-phase medium indirectly from its relation to $K_{\text{susp}}$ and other available quantities.** The global equilibrium constant $K_{\text{susp}}$ could be expanded as

$$K_{\text{susp}} = \frac{\varphi [C^-]_{\text{in}} + (1 - \varphi) [C^-]_{\text{out}}}{\varphi [I]_{\text{in}} + (1 - \varphi) [I]_{\text{out}}}$$

For $[H^+]_{\text{susp}} = [H^+]_{\text{out}}$, the combination of eqn (8)–(10) and (14) leads to

$$\frac{K_{\text{susp}}}{K_{\text{PBS}}} = \frac{\varphi K_c + (1 - \varphi)}{\varphi K_L + (1 - \varphi)} = \frac{(K_c - 1)sC_{P123} + 1}{(K_L - 1)sC_{P123} + 1}$$

In the 10 wt% P123 solution, $pK_{\text{susp}} = 7.3$, $pK_{\text{PBS}} = 6.6$, $s = 3.8$ and $K_c = 20$. We obtained $K_c = 2.7$. It is interesting that even the carboxylate form of HCPT prefers to be enriched in the micelles since $K_c > 1$. So, the solubility of HCPT could be enhanced after addition of P123 at different pH. Anyway, $K_c \ll K_L$, which is consistent with the higher $f_{\text{lactone}}$ in the presence of amphiphilic block copolymers over the co-assembly concentration.

**4.2.4 Eventual determination of $K_{\text{micelle}}$, the equilibrium constant of transformation of HCPT within P123 micelles.** Now $K_{\text{out}} (K_{\text{PBS}})$, $K_c$ and $K_L$ have been experimentally determined, it is ready to calculate $K_{\text{micelle}}$ ($K_{\text{micelle}}$) of HCPT in micelles at 37 °C as

$$K_{\text{micelle}} = \frac{K_c}{K_L}K_{\text{PBS}} = \frac{K_{\text{PBS}}}{7.4} \approx 3.4 \times 10^{-8}$$

$$pK_{\text{micelle}} = -\lg K_{\text{micelle}} \approx 7.5 = pK_{\text{PBS}} + 0.9$$

The decrease of the equilibrium constant (the increase of $pK_{\text{micelle}}$) leads to a higher equilibrium fraction of the lactone form in presence of the amphiphilic block copolymer.

**4.2.5 Dependence of the global lactone fraction of HCPT in P123 suspensions upon copolymer concentrations: theory versus experiment.** We derived the global lactone fraction $f_{\text{lactone}}$ in P123 suspensions as a function of weight concentration of the copolymer $C_{P123}$ upon given partition coefficients of both lactone and carboxylate forms between micelles and outer-phase medium $K_L$ and $K_c$, swelling factor of micelles $s$, and proton concentration of the medium $[H^+]$ as

$$f_{\text{lactone}}(C_{P123}) = \frac{(K_L - 1)sC_{P123} + 1}{(K_L - 1)sC_{P123} + 1 + [(K_c - 1)sC_{P123} + 1]K_{\text{PBS}}/[H^+]$$

(18)

The derivation process is given in the ESL†. At pH 7.4, the theoretical lactone fractions under different copolymer concentrations are presented in Fig. 10, along with the experimental data. The theory and experiment agree well with each other in Fig. 10. The deviation of the theoretical predictions from the experimental measurements at high polymer concentrations over CGC might be mainly from the inaccuracy in estimation of the swelling factor $s$. Eqn (18) is, in principle, justified only between $C_{\text{co-assembly}}$ and CGC. Nevertheless, consistency between theory and experiment was also found in concentrations between CMC and $C_{\text{co-assembly}}$, and even below CMC. This is because the lactone fractions prior to the co-assembly between drugs and micelles are close to that in PBS in the present experimental system. The transition of the lactone fraction occurs near the co-assembly concentration.

**4.3 The underlying reason for the equilibrium shift of HCPT in polymer solutions.** In the present study, the equilibrium lactone fractions of HCPT were detected in a broad polymer concentration range crossing both CMC and CGC, with results shown in Fig. 10. The transition point of the lactone fraction happened after polymer

---

**Fig. 10** Theoretical and experimental lactone fractions of HCPT in the P123/PBS aqueous system at 37 °C and pH 7.4. The dashed lines indicate CMC for the micellization transition obtained from the UV-visible absorption experiments with the hydrophobic probe DPH in Fig. 4A. CGC for the physical gelling transition obtained by the phase diagram in Fig. 4B, and the concentration of co-assembly between the drug HCPT and the amphiphilic block copolymer P123 obtained from the fluorescence anisotropy in Fig. 6C.
micellization and before physical gelation. Thus the hydrogel state is not necessary, and the micellization effect seems more important. Furthermore according to the temperature dependence of f_lactone (Fig. 7), the lactone fractions of HCPT would increase with temperature, if the environmental temperature was over its critical micelle temperature, which strengthened the importance of the micellization on the equilibrium of HCPT.

A puzzling question arises: why the transition concentration of the lactone fraction is not equal to, but significantly higher than CMC by two orders of magnitude (Fig. 12). We anticipated that the key point may be the co-assembly between drugs and block copolymeric micelles instead of merely micellization. Then we used fluorescence anisotropy, which has been widely used to monitor the interaction between small molecules with proteins etc., to detect the critical concentration of co-assembly between drugs and block copolymers, employing the property of fluorescence emission of this antitumor drug itself. We introduced the parameter C_{co-assembly} (the critical concentration of fluorescence anisotropy) to quantify the polymer concentration where drug molecules and polymer micelles started to co-assemble. The resulting C_{co-assembly} indicates well the transition concentration of lactone fraction of HCPT in Fig. 10.

Our experiments imply that the micelle structure might change with the increase of polymer concentration. No significant shape change was seen from the direct TEM observations (Fig. 5B), but the micelle sizes were surprisingly reduced with polymer concentration in our observation range. We also quantitatively measured the hydrodynamic radii of P123 micelles at different polymer concentrations, which strengthened the decrease of micelle sizes of P123 micelles with increasing polymer concentration. At relative lower P123 concentrations (<0.6 wt%), the micelles shrunken from about 45 nm to nearly 10 nm; at higher P123 concentrations (>0.6 wt%), the micelle size tended to remain constant (Fig. 5A).

A similar phenomenon was reported by Chaibundit et al. The phenomenon could be attributed to the impurity of Pluronic series have been emphasized by some researchers, mainly referring to the diblock copolymer impurity. For instance, Wu et al. noted “the copolymer was purified by six extractions with hexane to remove impurities, including diblock PEO–PPO chains... such a purification process is vitally important for a credible study of this kind of triblock copolymer.”

We herein describe the physical picture as presented in Fig. 11. The Pluronic P123 is a mixture of triblock copolymer and some shorter chains, probably diblock copolymer PEG–PPG. Since PEG–PPG is relatively less hydrophilic than PEG–PPG–PEG, the diblock copolymers form micelles first. The nascent micelles are loose for unknown reasons. With the involvement of the triblock copolymer, the mixture micelles became smaller and denser. The real entrapment of HCPT happened upon formation of mature micelles at P123 concentrations significantly higher than CMC, and then the fraction of the close-ring form was enhanced. The physical hydrogel has a structure of percolated micelle network, and thus its effect on drugs is essentially similar to that of micelles.

Then, why could the co-assembly shift the equilibrium between the two forms of CPT analogues? In the present system of neutral block copolymers, the interaction or driving force might be the hydrophobic interaction between HCPT and P123. The pK_a of the carboxylate form of CPT analogues is theoretically determined by the free energy difference in a dissociation process, and thus written as

$$pK_a = pK_{a,0} + (\Delta H - T\Delta S)/(2.303RT)$$

(19)

Here, R and T are the molar gas constant and the absolute temperature, respectively; \(\Delta H\) and \(\Delta S\) are the change of enthalpy and that of entropy in the process of ionization of the open-ring form. As is known, the hydrophobic interaction always leads to the change of the orientational entropy of water molecules and thus essentially an entropy effect. Under a local environment of amphiphilic polymers, the entropy term in eqn (19) is expressed as

$$\Delta S = S_{C^-/H^+} + S_{C^-/env} - S_{C/env}$$

(20)

Here, \(S_{C^-/H^+}\) is the mixing entropy between C^- and H^+; \(S_{C/env}\) and \(S_{C^-/env}\) are the entropies of the drug molecule interaction with their environment before and after the carboxylate ionization, respectively. In the presence of amphiphilic polymers, the hydrophobic interaction might be predominant over mixing in contribution to \(\Delta S\). Since a non-ionized chemical is more hydrophobic than its ionized counterpart, we reasonably anticipate that \(S_{C/env} > S_{C^-/env}\). So, \(\Delta S < 0\) in an ionization process in our experimental system, and the preferred hydrophobic interaction between the non-ionized carboxylate form and its environment leads to an increase of pK_a. This increase must shift the equilibrium described in eqn (3) to the left, and thus enhances the lactone fraction under a given medium pH. Or, pH_{1/2} must be increased with pK_a according to eqn (6), and the change of pH_{1/2} of HCPT in Fig. 3 turns out to reflect the increase of pK_a of the open-ring form.

Another CPT analogue, topotecan, was encapsulated into the hydrogel of poly[dl-lactic acid-co-glycolic acid]-b-poly(ethylene glycol)-b-poly[dl-lactic acid-co-glycolic acid] by us, and the lactone fraction was also found to be enhanced. We believe that the enhancement of the lactone fraction of CPT analogues by copolymers might be a universal phenomenon, only if some co-assembly could happen due to the hydrophobic interaction between drugs and materials. Both the hydrogel and micelle states were neither necessary nor sufficient conditions. Our findings and interpretations might be extended to more cases, and the influence of materials to the active forms of drugs might be stimulating for DDS design.
Fig. 11 Schematic presentation of self assembly states of some amphiphilic block copolymers presented by P123 (dual-color lines) in water and the corresponding partition of lactone-form CPT drugs represented by HCPT (red dots) at varied copolymer concentrations. At concentrations just above CMC, relatively more hydrophobic diblock copolymer chains are aggregated into clusters. The nascent micelles are not sufficiently dense to entrap HCPT, which is relative less hydrophobic than DPH (the probe to determine CMC). Only after the triblock copolymer micelles formed at relative higher polymer concentrations could the drugs be entrapped. A percolated micelle network forms at higher concentrations over CGC, leading to a physical gel.

5 Conclusions

The E-ring of HCPT preferred to be closed after addition of a sufficient amount of amphiphilic block copolymer Pluronic P123, and the effect is so significant that the lactone fraction was changed from about 13% in PBS at 37 °C and pH 7.4, to about 45% in the presence of 10 wt% P123 at the same pH and temperature. It is due to a real thermodynamic change instead of the kinetic retardation. A comprehensive theoretical deduction and corresponding experiments were further carried out, and the equilibrium constant of HCPT within micelles as well as the partition coefficients of either the lactone or carboxylate form between micelles and outer-phase aqueous medium were successfully determined.

The equilibrium shifting exhibited a strong dependence of copolymer concentration as well as medium pH and temperature. The transition polymer concentration was higher than CMC by about two orders of magnitude and lower than CGC by about 1.5 orders of magnitude for HCPT in P123 suspensions at pH 7.4 and 37 °C. So, the hydrogel was not the essential material state to shift the equilibrium of HCPT, and the micellization is not a sufficient condition to enhance the lactone form. The micelle shrinkage with the P123 concentration above CMC was observed, which might be due to the early micellization of some diblock copolymers as an impurity in the main component triblock copolymer in the commercialized P123. The equilibrium lactone fraction of HCPT was significantly increased in P123 micelle solutions only when the “mature micelles” formed at higher copolymer concentrations. For the enhancement of the active drug form in carriers, the local pH of micelles was not necessarily lowered. The mature micelles afforded a sufficiently strong hydrophobic local environment, which increased pH_{1/2} of HCPT due to the hydrophobic interaction between drugs and polymers and thus enhanced f_{lactone} at a given pH, particularly significant around neutral and weakly acidic conditions.

Acknowledgements

The authors are grateful for the financial supports from NSF of China (grants no. 91127028, no. 21034002 and no. 51273046), and Chinese Ministry of Science and Technology (973 programs no. 2009CB930000 and no. 2011CB606203). A critical reading of the manuscript by Professor Chi Wu is appreciated.

References