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Promotion of micelle stability *via* a cyclic hydrophilic moiety†

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A novel strategy to promote micelle stability was reported by altering the topological structure of polymer species. Specifically, a cyclic hydrophilic moiety offers greater stability for the self-assembled micelles than a linear analogue. This study thus provides an alternative to enhance micelle stability for drug delivery.

Polymeric micelles self-assembled from amphiphilic block copolymers represent one of the most investigated drug delivery systems[1–4] due to their ability to encapsulate lipophilic drugs in the hydrophobic core for improved bioavailability and to stabilize the core–shell nanoparticles by the hydrophilic corona toward prolonged circulation.[5,6] However, the stability of micelle drug delivery systems in the blood circulation remains a substantial challenge for their practical applications and clinical translations because the drug-loaded micelles must show sufficient stability to survive extreme dilution, sharp change of salt and pH gradients, and interactions with cells and biomolecules available in the blood after injection, which is crucial for minimizing the off-target-associated side effects and for promoting *in vivo* long circulation.[7,8]

Various factors affect the stability of polymeric micelles including polymer composition,[9–12] drug encapsulation[13,14] and environmental conditions.[15–17] Among these factors, the polymer composition is probably regarded as the most fundamental parameter as it is an inherent property of polymers. The groups of Wishart and Leroux reported that the cohesion of the micellar core could be enhanced toward better micelle stability by increasing the chain lengths of hydrophobic segments.[9,10] Okano and Harada enhanced micelle stability by introducing alkyl chains or aromatic moieties to the core-forming blocks of amphiphilic block copolymers due to the stronger hydrophobic–hydrophobic interactions or an additional π–π stacking effect.[11,12] The stability as well as morphology of the resulting self-assemblies have a delicate balance between the hydrophilic and hydrophobic building blocks of amphiphilic block copolymers. Most researchers focused on the promotion of micelle stability from the perspective of the hydrophobic segments to minimize the interfacial free energy, which is the main driving force for micelle formation, whereas, only a few studies concentrated on the hydrophilic moieties and reported the fabrication of tadpole-shaped amphiphilic block copolymers.[18,19]

Besides polymer compositions, the advanced topological structures of polymers, such as cyclic,[20–24] star-like,[25] and hyper-branched[26] architectures, have been reported to exert a significant effect on the self-assembly behaviors and the properties of self-assembled nanostructures. Cyclic polymers, relative to their linear analogues, exhibit some unique properties such as smaller hydrodynamic volume, higher glass transition temperature (T_g), lower intrinsic viscosity and higher critical solution temperature due to their endless chain topology.[24,27,28] Our recent studies revealed that micelles formed by cyclic brush copolymers showed higher stability than the bottlebrush copolymer-based analogues.[22,29,30] Very recently, Kim *et al.* reported the contribution of micelle stability *via* a cyclic monomer-based hydrophobic moiety.[31]

Inspired by the steric hindrance of the cyclic topology, herein we designed an amphiphilic block copolymer with a cyclic hydrophilic moiety and a linear hydrophobic segment, and studied the effect of cyclic topology on the stability of self-assembled micelles. For this purpose, poly(oligo(ethylene glycol) monomethyl ether methacrylate) (POEGMA) was chosen as the hydrophilic block due to its well-documented polymerizable properties by controlled living radical polymerizations.[25,32]

We next synthesized tadpole-like[18,19] amphiphilic block copolymer (*c*-POEGMA)-*b*-poly(ε-caprolactone) (*c*-POEGMA)-*b*-PCL (C), and its linear analogue (*l*-POEGMA)-*b*-PCL (L) (Scheme 1), and further compared the stability of their self-assembled micelles in terms of critical micelle concentrations

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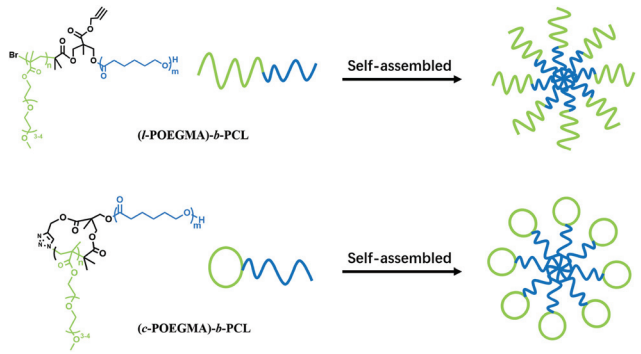
†Electronic supplementary information (ESI) available: Experimental details, characterization and discussion on the synthesis of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL are available in Scheme S1, Fig. S1–S11. See DOI: 10.1039/c8py00299a



Scheme 1 Structural formula and schematic illustration of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL.

(CMCs), micellar size, *in vitro* drug loading and drug release properties, and *in vitro* cytotoxicity.

A triple-head agent, propargyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate (PBH; refer to the ESI† for the synthesis details), was first prepared. The tadpole-like amphiphilic block copolymer (*c*-POEGMA)-*b*-PCL was later prepared in three steps including, (a) preparation of (*l*-POEGMA)-OH by atom transfer radical polymerization (ATRP) of OEGMA using PBH as the initiator, (b) synthesis of (*c*-POEGMA)-OH by Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAc) of (*l*-POEGMA)-OH under highly diluted conditions, and (c) preparation of the target block copolymer by the $Sn(Oct)_2$-catalyst ring-opening polymerization (ROP) of ε-CL using (*c*-POEGMA)-OH as a macroinitiator. The linear amphiphilic block copolymer, (*l*-POEGMA)-*b*-PCL was prepared following similar procedures except for the direct ROP of CL using (*l*-POEGMA)-OH as a macroinitiator (Scheme S1†). The molecular weights (MWs), polydispersity indexes (PDIs) and degrees of polymerization (DPs) of all the synthesized polymers were determined by ^{1}H NMR, size exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) analyses. Notably, the SEC trace of (*c*-POEGMA)-OH showed a clear shift toward a longer retention time (Fig. S5b†) and the FT-IR spectrum showed the absence of the characteristic band of the azide group (~2120 cm^{-1}) after cyclization compared to that of the linear precursor, (*l*-POEGMA)-OH, which supports the successful cyclization of the hydrophilic POEGMA block. All the polymers showed uni-modal and narrowly distributed molecular weights (Fig. S5†), demonstrating well controlled ATRP and ROP processes. The molecular parameters of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL block copolymers are summarized in Table 1.

Table 1 Summary of M_n, PDI, and DP of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL block copolymers

	n^a	m^a	$M_n{}^b$ (kDa)	PDI^b
(*l*-POEGMA$_n$)-*b*-PCL$_m$	18	26	17.5	1.22
(*c*-POEGMA$_n$)-*b*-PCL$_m$	18	24	19.4	1.19

^{a} Determined by ^{1}H NMR. ^{b} Determined by SEC-MALLS.

CMC is defined as the minimal polymer concentration required for micelle formation;[7] therefore it is a fundamental parameter for the characterization of micelle stability. Amphiphilic copolymers self-assemble in an aqueous phase with hydrophobic segments associating to form the inner core domain of micelles and hydrophilic moieties extending to construct the stabilizing outer corona. In other words, the hydrophilic shell shields the hydrophobic core from interactions with aqueous environments, reducing the interfacial free energy of the polymer–water system. Hence, a lower CMC value indicates greater thermodynamic stability of micelles.[33,34] As shown in Fig. 1, a dramatic increase in $I_{393\ nm}$ was recorded clearly, which suggests the formation of micelles. The CMCs of L and C copolymers were determined to be 17.38 and 6.23 μg ml^{-1}, respectively. The much lower CMC of C copolymers relative to that of L copolymers indicates the greater stability of micelles self-assembled from C copolymers over L-based analogues. This is likely due to the topology of the hydrophilic moiety of C micelles (*c*-POEGMA). The cyclic topology enhances the steric hindrance of OEG brushes; therefore it needs fewer polymer chains to reach the equilibrium stage when forming micelles, and as a result, the aggregation number of C micelles is lower. To further validate the stability of the two micelles, we determined the CMCs of three mixed copolymers of C and L at different molar feed ratios of 1 : 3, 1 : 1, and 3 : 1.[34] The CMCs of the three mixed copolymers are 15.35, 10.98 and 7.22 μg ml^{-1}, respectively. The results demonstrate that increasing the relative amount of C copolymers leads to lower CMCs of the mixed copolymers, which apparently confirms the greater stability of C micelles.

The size of polymeric drug carriers is a vital factor affecting their properties and performance. The ideal size (10–100 nm) of polymeric micelles is expected to restrict their uptake by the mononuclear phagocyte system and allows for the passive targeting of cancerous or inflamed tissues through the enhanced permeation and retention (EPR) effect.[3] We next examined the micelle size as indirect evidence to reflect the relationship between polymer topology and stability of self-assembled micelles. The micelle solutions were prepared at a low concentration of 0.25 mg ml^{-1}. Transmission electron microscopy

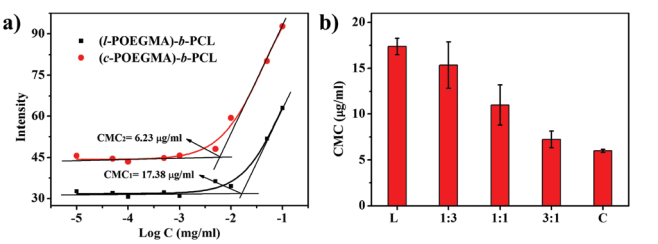


Fig. 1 (a) The intensity of $I_{393\ nm}$ in the emission spectra as a function of the logarithm of the concentrations of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL copolymers, (b) CMC values of L micelles, C micelles and three hybrid micelles. 1 : 3, 1 : 1, and 3 : 1 represent molar ratios (C : L) of 1 : 3, 1 : 1, and 3 : 1 of the (*c*-POEGMA)-*b*-PCL and (*l*-POEGMA)-*b*-PCL copolymers, respectively.

(TEM) observation (Fig. 2a & b) reveals the formation of well-dispersed nanoparticles with regular spherical shapes for both copolymers, and the average size of the micelle nanoparticles formed by L and C copolymers was estimated to be 23.7 nm and 20.5 nm, respectively. The sizes determined by dynamic light scattering (DLS) were 55.8 nm and 29.5 nm in water (Fig. S8a & c†), and 40.2 nm and 30.38 nm in PBS (pH 7.4) (Fig. S8b & d†) for L and C micelles, respectively. The size observed by TEM is smaller than that determined by DLS. Such a discrepancy is reasonable given that the latter is the hydrodynamic diameter of micelles in solution, whereas the former reflects the morphological size of micelles in a dry/dehydrated state.[28] The size of C micelles was statistically significantly smaller than that of L micelles in both water and PBS (Fig. 2c) phases, which is consistent with the lower CMC of C copolymers. The smaller size of C micelles than that of their L analogues could be attributed to the different packing behaviors caused by the hydrophilic POEGMA moiety with different topologies according to the very recent studies of Liu and Cheng.[35,36] They explored the self-assembly behaviors of polystyrene–polyhedral oligomeric silsesquioxane(s) (PS-APOSS) conjugates with different numbers and topologies of hydrophilic APOSS head groups, and revealed that the morphology change between PS-A5 with linear APOSS block and PS-A5b with branched APOSS chain was due to the different packing parameters. Similar to their findings, herein due to the stronger steric hindrance, *c*-POEGMAs have to stay farther away from each other than *l*-POEGMAs, leading to a significantly decreased aggregation number and subsequently more uniform package of (*c*-POEGMA)-*b*-PCL chains for micelle formation. This effect is supported by the remarkably lower CMC value obtained for (*c*-POEGMA)-*b*-PCL as well as the much smaller PDI of C micelles (0.103) than those of their L analogues (0.236) at the same polymer concentration. In addition, the sizes of both micelles in water were similar to those recorded in PBS, indicating that both micelle constructs can maintain the stability in the physiological environment (Fig. 2c). The zeta potential was measured to determine the surface charges of the two formulations (Fig. S9†). The L (−12.90 ± 0.22 mV) and C (−11.23 ± 0.69 mV) micelles show negative potentials with similar values likely due to the hydrophilic P(OEGMA) shell, which is believed to increase the stability of micelles in the physiological environment, and to prolong the circulation time.

In vitro drug loading and drug release study of the two micelles was performed next. The anti-cancer drug, doxorubicin (DOX), was used as the model drug, and encapsulated within the hydrophobic PCL core following the classical dialysis method.[22] The drug-loading content (DLC) and entrapment efficacy (EE) of C micelles are 5.12% and 43.76% which are slightly higher than those of L micelles (3.96% and 39.12%). The greater stability contributes probably to the larger drug-loading capacity of C micelles. *In vitro* DOX release behaviors were evaluated under physiological conditions (PBS, pH 7.4) at 37 °C (Fig. 3a). This pH value represents the typical extracellular pH such as that in blood circulation and normal tissues. Notably, the drug release rate of C micelles was consistently slower than that of L micelles. Incubation at pH 7.4 results in ~58% and 43% DOX release for L micelles and C micelles in 72 h, respectively. The lower release rate of C micelles not only reflects the lower permeability (draining property) of this formulation from the view of micelles, but supports the better protection of the encapsulated drug from the perspective of loaded cargoes. Both effects are attributed to the greater stabi-

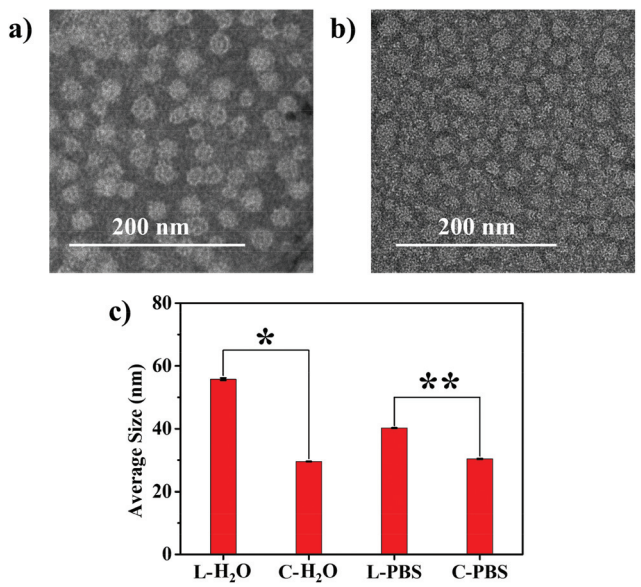


Fig. 2 TEM images of (a) L micelles and (b) C micelles at a polymer concentration of 0.25 mg ml^{-1}. (c) Average size of L and C micelles in H_2O and PBS (pH 7.4) at a polymer concentration of 0.25 mg ml^{-1}. Data are shown as mean ± SD (n = 3; Student's t test, *p < 0.01, **p < 0.02, both p values indicate the statistical significance of the diversity between each group).

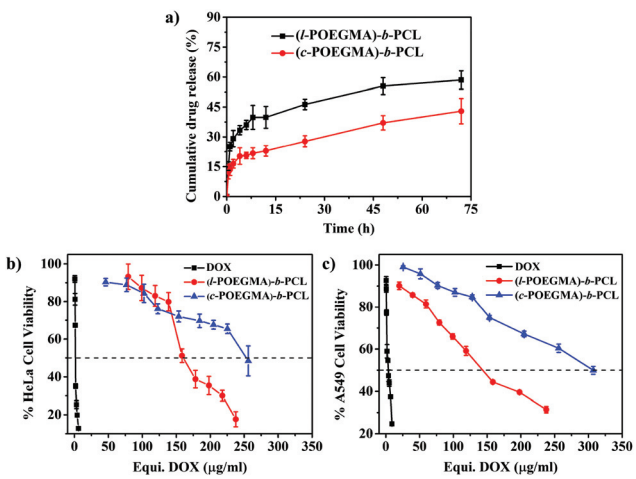


Fig. 3 (a) *In vitro* drug release profiles of DOX-loaded L and C micelles in PBS (pH 7.4). Cell viability of (b) HeLa cells and (c) A549 cells incubated with Dox, Dox-loaded L and C micelles at various concentrations for 24 hours. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells.

lity of C micelles, which is beneficial for minimizing the side effect caused by premature and burst drug release.

Ideally, the zero-order kinetics is highly desirable for drug delivery applications, wherein the release rate remains constant throughout the delivery periods,[37] but actually few reported block copolymer-based systems have realized such constant release profiles. In this study, a cyclic hydrophilic moiety was introduced to produce a tadpole-like amphiphilic block copolymer, and the linear analogue could be regarded as a traditional linear block copolymer. The release profiles of C and L micelles gradually leveled off and approached the "zero-order" release profile after 12 h, demonstrating the great potential of both micelle constructs for sustained drug release. More importantly, C micelles show much slower drug release than their L analogues due to the cyclic hydrophilic moiety-generated greater micelle stability, which constitutes the uniqueness of C micelles developed in this paper.

Finally, the cytotoxicity of L and C micelle constructs to HeLa (cervical cancer cell line), A549 (lung cancer cell line) and L02 (normal liver cell line) cells (all the three cell lines were kindly provided by Stem Cell Bank, Chinese Academy of Sciences) was assessed by MTS cell viability assay, respectively. The blank L and C micelles were non-toxic to all cell lines (with a cell viability above 80%) up to a concentration of 1.6 mg ml^{-1} (Fig. S10†). The half maximal inhibitory concentrations (IC_{50}) of free DOX and DOX-loaded L and C micelles were determined to be 1.68 (1.40, 2.01) μg ml^{-1}, 169.2 (160.2, 178.8) μg ml^{-1}, and 303.8 (242.9, 379.8) μg ml^{-1} in HeLa cells (Fig. 3b) and 3.643 (3.133, 4.236) μg ml^{-1}, 146.5 (136.3, 157.6) μg ml^{-1}, and 314.5 (294.2, 336.2) μg ml^{-1} in A549 cells (Fig. 3c), respectively. All the DOX-loaded micelles exhibit less cytotoxic activity than the free DOX likely due to the slower internalization mechanism (endocytosis *vs.* direct membrane permeation) and release kinetics of the free drug from the micelles. The much higher IC_{50} (less cytotoxicity) of DOX-loaded C micelles, relative to that of L analogues in both cancer cell lines also results from the greater stability of C micelles. The results are in good agreement with the *in vitro* drug release profiles. Note that the cell viability of DOX-loaded L and C micelles is well above 60% in L02 cells at the same tested range of equivalent DOX concentrations for the other two cancer cell lines (Fig. S11†), which implies the lower cytotoxicity of both DOX-loaded micelles to normal cells.

In summary, a cyclic hydrophilic moiety was introduced to an amphiphilic block copolymer to generate a tadpole-like copolymer, (*c*-POEGMA)-*b*-PCL. The self-assembled C micelles showed greater stability than the L analogues in terms of lower CMC, smaller micelle size, slower *in vitro* drug release profile and lower *in vitro* cytotoxicity against HeLa and A549 cells. This work thus reveals that the cyclic hydrophilic moiety can offer extra stability to the self-assembled micelles due to the topology-enhanced steric hindrance and packing behavior, which provides an alternative to fabricate polymeric micelles toward enhanced stability for drug delivery applications. The incorporation of biologically relevant links to the tadpole-like amphiphilic block copolymers is currently underway to provide a solution to the tradeoff between extracellular stability and intracellular high therapeutic efficacy of this delivery system.

Conflicts of interest

There are no conflicts to declare.

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