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ARTICLE

Tuning the molecular weight of polymeric amphiphiles as a tool to access micelles with a wide range of enzymatic degradation rates

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Enyzme-responsive polymeric assemblies hold great potential for biomedical applications due to the over-expression of disease-associated enzymes, which can be utilized to activate such systems only in afflicted tissue. Herein we demonstrate that the overall molecular weight of polymeric amphiphiles, which have the same hydrophilic/hydrophobic ratio, can be utilized to create polymeric micelles with an extreme range of degradation rates. This approach expands the available set of molecular parameters that can be adjusted to tune the degradation rate of polymeric assemblies, paving new possibilities for rational design of polymeric systems with controlled degradation rates.

Introduction

Stimuli-responsive polymeric micelles have emerged as promising candidates to serve as drug delivery systems, due to their ability to encapsulate or covalently bind hydrophobic drugs in their hydrophobic core and release them upon specific cue.[1–6] Among the various types of stimuli, enzymes are very appealing for triggering drug release from nano, micro, and macro delivery systems due to their high specificity and overexpression of disease-associated enzymes in afflicted tissues.[7–9] Despite great potential, there are still significant challenges to overcome in order to achieve efficient utilization of enzymes as triggers, as it is often challenging to tune the enzymatic degradation rates and many enzyme-responsive assemblies show very limited degradability. These limited responses are due to the lack of direct access of the activating enzyme to its substrates, which are usually concealed inside the hydrophobic core of the assembly. As suggested in previous reports by Heise,[10,11] Thayumanavan,[12–14] and our group,[15] it is reasonable to assume the interaction between the enzyme and its hydrophobic substrate occurs with non-assembled amphiphiles that are present due to the monomer-assembly equilibrium. This equilibrium-based mechanism gives rise to non-linear dependency of the enzymatic degradation rates on the hydrophilic/hydrophobic ratio, which governs the thermodynamic and kinetic stability of the assemblies. By utilizing the high molecular precision of PEG-dendron hybrids,[16] which were first introduced by Frechet, Gitsov, Hawker, and Wooley,[17,18] we were able to recently show that small changes in the amphiphilic ratio can lead to large differences in the enzymatic degradation rates of these hybrid amphiphiles. Tuning either the length of the PEG[15] or the hydrophobicity of the dendron, by changing the number[19,20] or type of end-groups,[21] allowed us to prepare well-defined polymeric amphiphiles with diverse degradation rates ranging from rapidly degraded amphiphiles to highly stable assemblies that show no apparent degradation or disassembly even after long incubation periods with the activating enzyme.

Herein, taking advantage of the high molecular precision of PEG-dendron amphiphiles, we studied the effect of the overall molecular weight of these amphiphiles on their self-assembly,

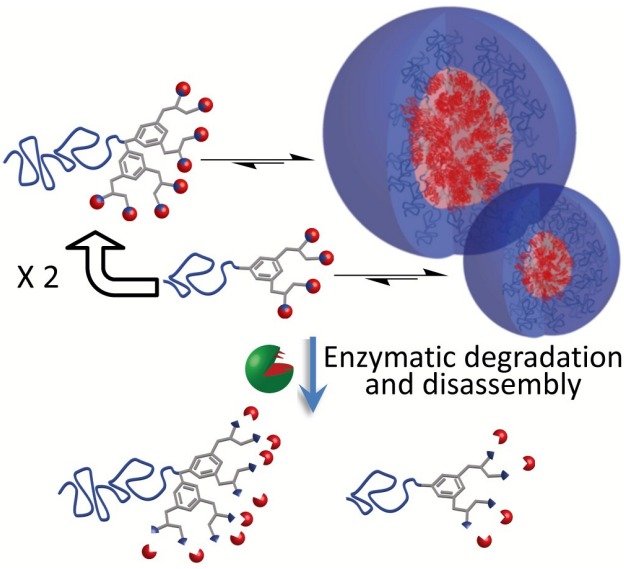

Figure 1: Illustration of two types of enzyme-responsive PEG-dendron hybrids with similar hydrophilic/hydrophobic ratio and two fold difference in total molecular weight (hydrophilic = blue; hydrophobic = red)

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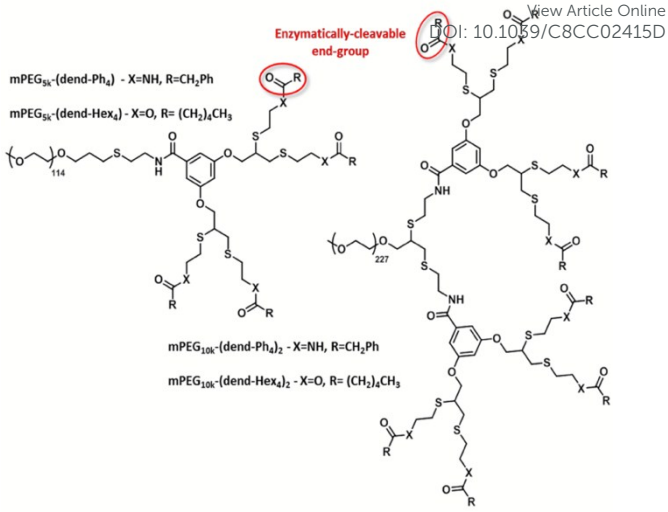
loading capacity, and enzymatic responsiveness of polymeric amphiphiles with the same hydrophilic/hydrophobic ratios. Toward this goal, we designed and synthesized two amphiphilic hybrids based on 10-kDa monomethyl PEG as the hydrophilic block and dendrons bearing eight amidase- or esterase-cleavable end-groups. These new hybrids were designed to have the same hydrophilic/hydrophobic ratio and twice the molecular weight of our previously reported PEG-dendron amphiphiles, which were based on 5-kDa PEG. As illustrated in Scheme 1, the dendritic blocks of the higher molecular weight amphiphiles contain exactly two copies of the dendrons that were used to construct the smaller 5-kDa-based amphiphiles.

Results and discussion

The synthesis of the amphiphiles starts from mono-amine or diamine PEG, $mPEG_{5k}$-NH_2, or $mPEG_{10k}$-$(NH_2)_2$ and required only two high yielding synthetic steps to reach the final hybrids: In the first step, the amine or amines of the mPEG were conjugated with branching units that contained two propargyl moieties. In the second step, the acetylenes were further branched by using thiol-yne click reaction with a pre-made thiol that contained the enzyme-responsive hydrophobic end-group (Figures S12-S14, S17). The two larger amphiphiles have eight phenylacetamide ($mPEG_{10k}$-$(dend\text{-}Ph_4)_2$) or hexanoate ester ($mPEG_{10k}$-$(dend\text{-}Hex_4)_2$) end-groups, which can be cleaved by penicillin G amidase (PGA) or porcine liver esterase (PLE), respectively. The two smaller amphiphiles bear four amidase or esterase-cleavable end-groups, respectively. The amphiphilic hybrids were expected to self-assemble into micelles in aqueous solutions. Upon enzymatic cleavage of the hydrophobic end-groups, the original amphiphilic nature of the hybrid will be altered, leading to fully soluble polymer that will no longer form micelles (Figure 1).

All hybrids were synthesized through the new accelerated two-step methodology in quantitative overall yield (>95%) and were characterized by ^1H and ^{13}C NMR, HPLC, GPC, IR, and MALDI-TOF. The experimental and expected theoretical data were in good agreement (see supporting information).

After completion of the synthesis, we studied the self-assembly of the four hybrids in aqueous media (phosphate buffer, pH 7.4). First the CMC values of the amphiphiles were determined using the Nile Red method;[22] the values are shown in Table 1. Although each of the two pairs of our hybrids has the same hydrophilic/hydrophobic ratio, we expected that the larger amphiphiles, $mPEG_{10k}$-$(dend\text{-}Ph_4)_2$ and $mPEG_{10k}$-$(dend\text{-}Hex_4)_2$, would have lower CMC values than the smaller ones, $mPEG_{5k}$-$dend\text{-}Ph_4$ and $mPEG_{5k}$-$dend\text{-}Hex_4$, respectively. The obtained results indeed showed the expected trend, which correlates well with previous publications showing that increasing the molecular weight of amphiphilic diblock copolymers while keeping the hydrophilic/hydrophobic ratio the same led to some decrease in the CMC values.[23] Next, the micellar diameters were determined using dynamic light scattering (DLS), which indicated the formation of structures with sizes of 18-34 nm (Figure 3 solid lines and table 1). Transmission electron microscopy (TEM) images confirmed the formation of spherical structures (Figure 2) and sizes were in agreement with



Scheme 1: Chemical structures of amphiphilic PEG-dendron hybrids based on 5- or 10-kDa PEG bearing one or two identical dendrons, respectively.

DLS. Based on the molecular weights of the amphiphiles, it is not surprising that both the DLS and TEM data showed that the two 10-kDa-based hybrids formed micelles with larger diameters than those of the 5-kDa-based ones.

Considering the differences in the diameters of the micelles and the fact that at equal weight percent concentration of the 5- and 10-kDa-based hybrids the absolute amount of PEG and dendrons in each solution is equal, we were interested to evaluate the encapsulation capacity of hydrophobic cargo in the four types of micelles. We chose camptothecin (CPT), an anti-cancer drug, which has very low solubility in water, as a model hydrophobic cargo.[24] The encapsulation experiments were conducted in aqueous solutions with similar weight percent of the amphiphiles and commercial 5- and 10-kDa mPEG-OH were used as control. Remarkably, solutions containing the micelles of the larger amphiphiles encapsulated roughly two-fold higher amounts of CPT than the solutions containing the smaller micelles, although the total weight concentration of the amphiphiles was kept constant (Figure 4). The higher loading capacity can be attributed to the larger size of the dendrons, resulting in significant increases in the polymer/CPT ratios for the larger amphiphiles (Table 1).

Finally, we were interested to see how the molecular weight of the amphiphiles influenced enzymatic degradability. Based on the differences in the CMC values between the larger and smaller amphiphiles, we were expecting the 10-kDa-based hybrids to be somewhat more stable and to show longer degradation times. The enzymatic degradation of the hybrids was directly monitored using HPLC, and the analysis of the results is presented in Figure 5. In the case of PGA-responsive hybrids, when the kinetic experiments were conducted at low

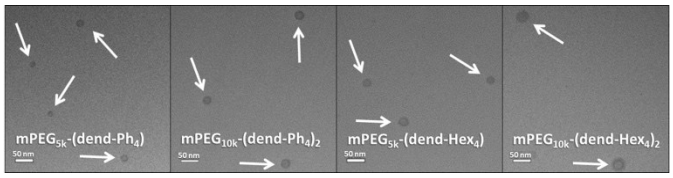


Figure 2: TEM images of micellar solutions of the four amphiphiles. Arrows indicate micellar structures. Scale bars, 50 nm.

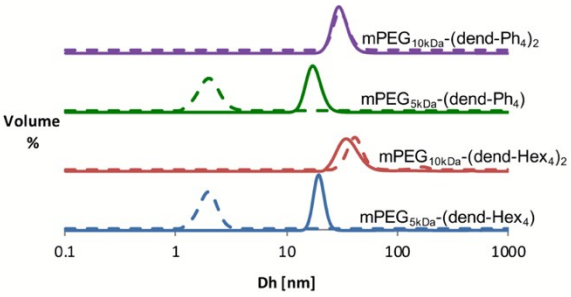


Figure 3: DLS of micellar solutions before (solid lines) and after (dashed) 24 hours incubation with the activating enzymes ([PLE] = 14nM, [PGA] = 5 nM).

concentration of the enzyme (5 nM), we saw that $mPEG_{5k}$-dend-Ph_4 was fully degraded in approximately 16 hours. Although we expected slower degradation of $mPEG_{10k}$-(dend-Ph_4)$_2$, we were surprised to see almost no degradation of the larger amphiphile under these conditions. In order to evaluate the extent of the stability difference, we significantly increased the PGA concentration by 100 fold to 500 nM. Under this relatively high concentration of activating enzyme, the smaller amphiphile $mPEG_{5k}$-dend-Ph_4 was fully degraded in less than 30 minutes, and we also observed substantial degradation the larger amidase-responsive amphiphile, $mPEG_{10k}$-(dend-Ph_4)$_2$, although this occurred significantly slower with nearly 75% degraded at 24 hours. As mentioned before, the inverse correlation of higher stability with lower CMC values for amphiphilic di-block copolymers with same hydrophilic/hydrophobic ratio and larger molecular weights was previously reported.[23] However, our results demonstrate that the effect of the total molecular weight on enzyme-responsiveness seems to be much more substantial than the difference in the CMC values for the two amidase-responsive amphiphiles. Intrigued by this extreme stability difference, we conducted enzymatic degradation experiments on PLE-responsive $mPEG_{10k}$-(dend-Hex_4)$_2$ and $mPEG_{5k}$-dend-Hex_4. By now, we were not surprised to find that at low PLE concentration (14 nM) the 5-kDa-based hybrid was degraded completely in 16 hours and the 10-kDa-based amphiphile showed nearly no degradation at this time point. For these esterase-responsive amphiphiles, the stability difference was so dramatic that even after 100-fold increase in PLE concentration only 25% of $mPEG_{10k}$-(dend-Hex_4)$_2$ was degraded after 24 hours.

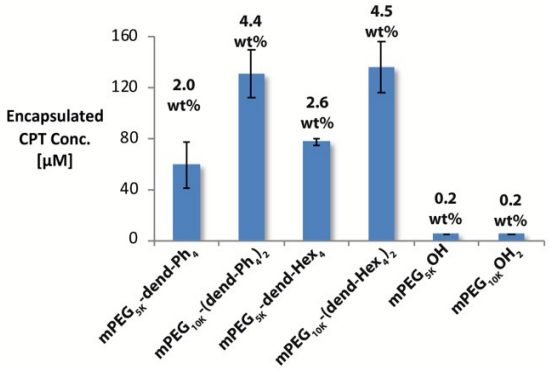


Figure 4: Encapsulation capacity of CPT (in μM and wt%) in micelles formed by the different amphiphiles ([hybrid] = 1 mg/ ml). PEG 5 kDa and 10 kDa were used as controls.

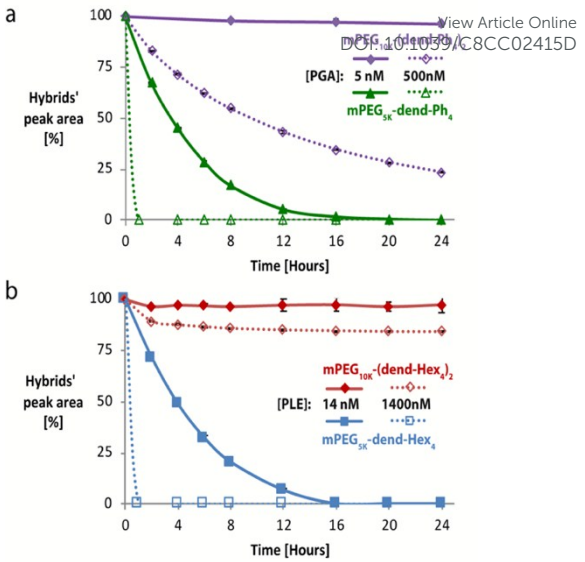


Figure 5: HPLC-based enzymatic degradation profiles of (a) amidase-responsive amphiphiles at enzyme concentration of 5 and 500 nM (solid and dotted, respectively) and (b) esterase-responsive amphiphiles at enzyme concentration of 14 and 1400 nM (solid and dotted lines, respectively) ([hybrid] = 1 mg/ ml).

In addition to directly monitoring the degradation of the hybrids by HPLC, we also used DLS to monitor the presence of self-assembled or disassembled structures in the solution (Figure 2, dashed lines). As expected, the two 5-kDa-based amphiphiles showed no traces of the assembled structures after the time at which degradation was confirmed by HPLC, and new peaks appeared indicative of smaller structures that corresponded to the degraded hybrids. On the other hand, the 10-kDa-based hybrids showed structures of similar sizes to the ones measured before the addition of the enzyme. When the concentrations of the enzymes were increased, we still observed micelles and also smaller structures that corresponded to both the non-assembled hydrolyzed hybrids that have higher hydrophilic/hydrophobic ratios and the activating enzymes, which were used at relatively high concentrations in order to try and push the degradation of the larger amphiphiles.

It is important to note that although the differences in CMC values and sizes of the micelles were anticipated based on previous reports in the literature,[23] the significant effect on the loading capacity and the exceptionally large differences in degradation rates, illustrate the importance that overall molecular weight has in determining the stability and enzymatic degradability of polymeric assemblies. The increased stability may also be attributed to steric hindrance due to the longer PEG chains and larger dendrons. The results of this work deepen the understanding of the different structural parameters that govern enzymatic degradability of polymeric amphiphiles. Furthermore, the higher loading capacity and enhanced micellar stability of the larger amphiphiles make them highly promising for the construction of stable delivery systems with adjustable release rates. Cell studies (internalization and toxicity) of these micellar systems are currently under further investigation.

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Hybrid	PEG Mn [kDa]	Dend. Mn [Da]	Hydrophilic/hydrophobic [%][a]	Theoretical Mn [kDa]	Mn [kDa][b]	Mw/ Mn[b]	Mn [kDa][c]	Micellar diameter [nm][d]	CMC [μM / ng/ml]	polymer /CPT mol. ratio
$mPEG_{5k}$-(dend-Ph_4)	5.0	1112	82/18	6.1	6.2	1.06	6.2	18 ± 2	24 ± 1 / 145 ± 6	2.7 ± 0.8
$mPEG_{10k}$-(dend-Ph_4)$_2$	10.0	2180	82/18	12.2	12.8	1.18	11.5	31 ± 6	5 ± 2 / 61 ± 24	0.6 ± 0.1
$mPEG_{5k}$-(dend-Hex_4)	5.0	1036	83/17	6.0	6.2	1.06	6.1	20 ± 2	10 ± 2 / 60 ± 12	2.1 ± 0.1
$mPEG_{10k}$ (dend-Hex_4)$_2$	10.0	2028	83/17	12.0	12.5	1.13	11.9	34 ± 6	3 ± 1 / 36 ± 12	0.6 ± 0.1

Table 1: Characterization table of the two series of PEG-dendron hybrids. [a]weight ratio of PEG/dendron. [b]measured by GPC. [c]measured by MALDI-TOF [d]measured by DLS.

Conclusions

To summarize, we prepared two pairs of enzyme-responsive PEG-dendron hybrids with nearly identical hydrophilic/hydrophobic ratios that differ in their total molecular weights. Each of the pairs of amphiphiles was designed to respond to a different enzyme: an amidase and an esterase, based on the type of cleavable end-groups. The amphiphiles were synthesized through high yielding step-efficient synthesis by a combination of amidation and thiol-yne reactions. As expected, the 10-kDa-based hybrids had lower CMC values and formed micelles of larger diameters than the smaller hybrids. Interestingly, although we kept the overall polymer concentration (weight %) similar, the larger micelles could encapsulate approximately twice the amount of CPT, which was used as model hydrophobic cargo, in comparison with the smaller micelles. Most importantly, there was a tremendous difference between the enzymatic responsiveness and degradation rates for the larger and smaller hybrids for both enzymes (amidase and esterase) that were tested. The observed kinetic trends indicate that the absolute molecular weight of the hydrophobic block strongly affects the enzymatic responsiveness of the amphiphiles and their assembled structures. Our results demonstrate the potential utilization of the overall molecular weight of amphiphiles as a modular tool for tuning their enzymatic degradation rates, extending the molecular tool box beyond the more frequently used approach of adjusting the hydrophilic/hydrophobic ratio.

Conflicts of interest

There are no conflicts to declare.

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