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Published on Web 09/02/2004

Stimuli-Responsive Supramolecular Assemblies of Linear-Dendritic Copolymers

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Abstract: With the goal of developing a pH-responsive micelle system, linear-dendritic block copolymers comprising poly(ethylene oxide) and either a polylysine or polyester dendron were prepared and hydrophobic groups were attached to the dendrimer periphery by highly acid-sensitive cyclic acetals. These copolymers were designed to form stable micelles in aqueous solution at neutral pH but to disintegrate into unimers at mildly acidic pH following loss of the hydrophobic groups upon acetal hydrolysis. Micelle formation was demonstrated by encapsulation of the fluorescent probe Nile Red, and the micelle sizes were determined by dynamic light scattering. The structure of the dendrimer block, its generation, and the synthetic method for linking the acetal groups to its periphery all had an influence on the critical micelle concentration and the micelle size. The rate of hydrolysis of the acetals at the micelle core was measured for each system at pH 7.4 and pH 5, and it was found that all systems were stable at neutral pH but underwent significant hydrolysis at pH 5 over several hours. The rate of hydrolysis at pH 5 was dependent on the structure of the copolymer, most notably the hydrophobicity of the core-forming block. To demonstrate the potential of these systems for controlled release, the release of Nile Red as a "model payload" was examined. At pH 7.4, the fluorescence of micelle-encapsulated Nile Red was relatively constant, indicating it was retained in the micelle, while at pH 5, the fluorescence decreased, consistent with its release into the aqueous environment. The rate of release was strongly correlated with the rate of acetal hydrolysis and was therefore controlled by the chemical structure of the copolymer. The mechanism of Nile Red release was investigated by monitoring the change in size of the micelles over time at acidic pH. Dynamic light scattering measurement showed a size decrease over time, eventually reaching the size of a unimer, thus providing evidence for the proposed micelle disintegration.

Introduction

The development of self-assembling polymeric systems is of interest for a wide range of applications, including catalysis,[1] nanoscience,[2] and drug delivery.[3] The ability to control the polymeric assembly in such systems has the potential to significantly enhance their performance by tuning the catalyst microenvironment, turning on and off the activity of nanoscale devices, or triggering payload release selectively at the target. Thus far, a number of copolymer micelle systems have been developed that are responsive to stimuli such as ultrasound[4] or changes in temperature[5] or pH.[6] Changing the pH is particularly attractive for applications involving biological systems because of the numerous pH gradients that exist in both normal and pathophysiological states. For example, the extracellular pH of tumors[7] as well as the endosomal and lysosomal compartments of cells are slightly more acidic than blood and normal tissues.[8]

Current approaches toward the development of pH- responsive micelles involve either the incorporation of "titratable" groups including amines[9] or carboxylic acids into the copolymer[10] or the use of linkages such as hydrazones[11] or ortho esters that

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10.1021/ja0463738 CCC: $27.50

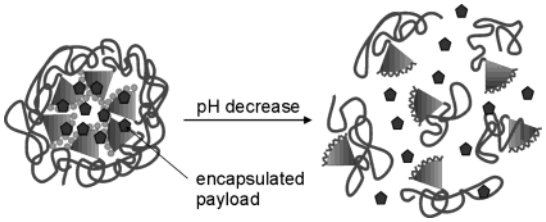

Figure 1. Schematic for drug release from a pH-sensitive micelle.

degrade under acidic conditions.[12] However, the number of systems that are responsive within the physiologically accessible pH range of approximately 4.5−7.4 is quite limited. Here we describe the development of a new and general approach to stimuli-responsive micelles using PEO−dendrimer hybrids as the copolymer backbone. This approach involves the attachment of hydrophobic groups to the surface of the core-forming dendrimer block by highly sensitive acetal linkages.[13] The system is designed such that upon hydrolysis of the acetals, the hydrophobic dendrimer periphery becomes hydrophilic, thus removing the driving force for self-assembly and destabilizing the micelle. This destabilization should enable release of the contents of the micelle from its encapsulating compartment as illustrated in Figure 1.

The stepwise synthesis and plurivalency of linear-dendrimer block copolymers make them ideal copolymer scaffolds for the development of these pH-responsive systems. The length of the linear poly(ethylene oxide) block and the dendrimer generation can be easily tuned to afford micelles with a range of sizes and stabilities. In addition, subtle changes in the copolymer structure affect the microenvironment at the micelle core and can significantly affect the rate of acetal hydrolysis, enabling the control of the rate of micelle degradation. Because these micelle systems are designed to be very stable at neutral or physiological pH (7.4), but degrade under mildly acidic conditions, they are potentially applicable as pH-responsive drug delivery systems capable of selectively releasing drug molecules in tumor tissue or within tumor cells.

Results and Discussion

Design. Acetals formed from the 1,3-diols and 2,4,6-trimethoxybenzaldehyde were chosen as the pH-sensitive linkages such that polar diol moieties are revealed on the core-forming block upon hydrolysis. While cyclic acetals are known to hydrolyze relatively slowly in comparison to their noncyclic analogues,[14] the electron-donating methoxy groups in the ortho and para positions accelerate the rate of hydrolysis, providing a half-life of 1 h at pH 5.0 and 37 °C as previously measured for low molecular weight model compound.

The amine of a diol such as serinol or the third hydroxyl of 1,1,1-tris(hydroxymethyl)ethane can provide a functional handle for attachment of the acetals to the polymer backbone. Although in initial studies the amine of serinol could be conjugated directly to a copolymer backbone having pendant carboxylic acids, the yields for this conjugation were not quantitative and titratable carboxylic acid groups remained along the copolymer backbone.[13] Since then, we have determined that it is possible to quantitatively convert pendant amine groups along a copolymer backbone to carbamates by reaction with 4-nitrophenyl carbonates. Methods for the high-yielding functionalization of hydroxyl groups on a polymeric backbone have also been previously developed in our group.[15] With these issues in mind, the target copolymers for this work are designed to contain blocks with pendant amines or hydroxyl groups for functionalization.

Although linear block copolymers of PEO with polylysine and polyserine have the desired pendant amine and hydroxyl groups, respectively, a drawback to this approach is that the polymerization of amino acid *N*-carboxyanhydrides initiated from PEO−NH_2[16] in our hands was not well controlled, leading to relatively high polydispersities and homopolymer impurities that were difficult to remove. Linear-dendrimer hybrid copolymers are attractive alternatives because they can be prepared by a stepwise sequence leading to well-defined structures with a controlled number of peripheral functional groups on the dendrimer for subsequent functionalization. The self-assembly of various linear-dendrimer hybrids in water has been investigated by several groups, who have reported the formation of aggregates ranging from unimolecular micelles to multimolecular spherical micelles and even vesicles.[17] In pioneering work, Chapman and co-workers have prepared PEO−dendritic polylysine copolymers having amine or BOC-protected amine groups on the dendrimer periphery, with the BOC-functionalized copolymers showing micellar properties in aqueous solution.[18] We therefore hypothesized that it should be possible to attach acid-sensitive hydrophobic acetal groups to the peripheral amine groups in an efficient coupling reaction to afford acid-sensitive micelles. PEO−dendritic polyester block copolymers have previously been synthesized in our group, but their micellization properties have not yet been investigated.[15a] These copolymers have peripheral hydroxyl groups on the dendrimers that can serve as functional handles for attachment of the acetals. Using both of these well-defined systems, it should be possible to tune the micelle properties such as size, critical micelle concentration (CMC), and drug release rates by varying the length of the PEO chain, the dendrimer generation, and the linkage between the acetals and dendrimer backbone.

Synthesis. To prepare the target acetal with a single remaining reactive group available for subsequent coupling to the amine functionalized copolymers, 2,4,6-trimethoxybenzaldehyde (**1**) was reacted with 1,1,1-tris(hydroxymethyl)ethane in dry THF in the presence of molecular sieves and catalytic *p*-toluenesulfonic acid to give the acetal **2**. The remaining hydroxyl group was then activated by reaction with 4-nitrophenyl chloroformate, providing the carbonate **3** as shown in Scheme 1. The third-generation PEO−dendritic polylysine copolymers **4a** (PEO-5K-

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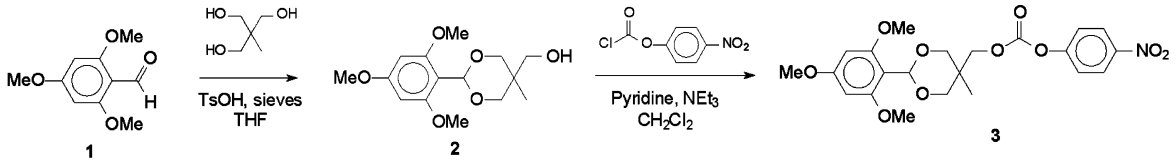
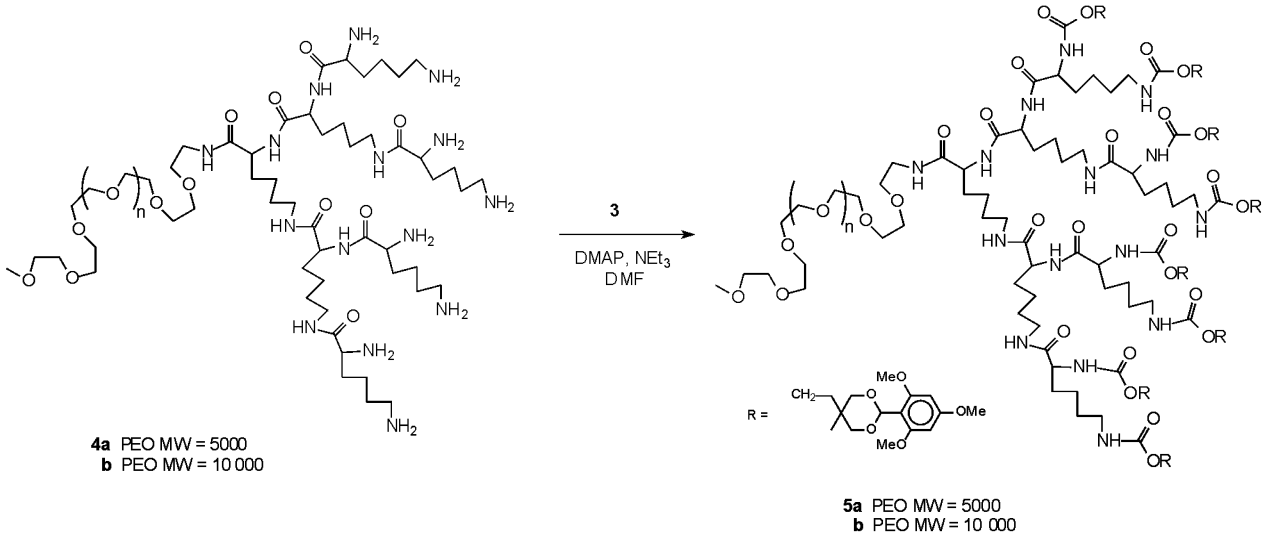
Scheme 1



Scheme 2



[G-3]-polylysine-NH_2) and **4b** (PEO-10K-[G-3]-polylysine-NH_2) with eight peripheral primary amine groups were prepared using the previously reported divergent approach starting from *O*-(2-aminoethyl)-*O*′-methylpoly(ethylene glycol) (PEO−NH_2) with molecular weights (MW) 5000 and 10 000 g/mol, respectively.[18] The peripheral amine groups could then be reacted efficiently with excess of **3** in the presence of 4-(dimethylamino)pyridine (DMAP) to provide the target pH-sensitive amphiphilic copolymers **5a** (PEO-5K-[G-3]-polylysine-acetal) and **5b** (PEO-10K-[G-3]-polylysin-acetal) as shown in Scheme 2, followed by purification by dialysis to remove low MW material. In the case of the 10 000 MW PEO−NH_2 "initiator", the PEO−dendritic polylysine copolymer was extended to the fourth generation with 16 peripheral amine groups and was coupled with **3** to afford the target **6** (PEO-10K-[G-4]-polylysine-acetal). ^{1}H NMR spectroscopy was used to confirm that at least 85−90% of the peripheral amine groups had been successfully converted to carbamates and that no residual primary amine groups were detected by ninhydrin staining.

The PEO−dendritic polyester copolymers **7a** (PEO-5K-[G-3]-polyester-OH) and **7b** (PEO-10K-[G-3]-polyester-OH) with eight peripheral hydroxyl groups were prepared by the previously reported divergent method starting from poly(ethylene glycol) monomethyl ether of MWs 5000 and 10 000 g/mol, respectively.[15a] In one acetal attachment strategy, the dendrimer hydroxyl groups could be activated as 4-nitrophenyl carbonates by reaction with 4-nitrophenyl chloroformate to provide copolymers **8a** (PEO-5K-[G-3]-polyester-PNPC) and **8b** (PEO-10K-[G-3]-polyester-PNPC). These activated carbonates could then be efficiently coupled to our previously reported amine-functionalized acetal **9** in the presence of DMAP to provide the target copolymers **10a** (PEO-5K-[G-3]-polyester-carbamate-acetal) and **10b** (PEO-10K-[G-3]-polyester-carbamate-acetal), where the acetals are attached to the dendrimer periphery by a carbamate linkage (Scheme 3). This approach was also carried out on a PEO−dendritic polyester copolymer (PEO MW 10 000 g/mol) with a fourth-generation dendrimer having 16 hydroxyl groups to afford the 4-nitrophenyl carbonate-functionalized copolymer **11** (PEO-10K-[G-4]-polyester-PNPC) and finally copolymer **12** (PEO-10K-[G-4]-polyester-carbamate-acetal) with 16 acetal groups on the dendrimer periphery. According to ^{1}H NMR spectroscopy, both the activation of the hydroxyl groups and the carbamate formation were essentially quantitative reactions leading to near complete functionalization of the dendrimer periphery in each case.

Because the method for attaching the acetal to the dendrimer periphery influences the hydrophobicity of the dendrimer block and thus the core environment of the resulting micelle, we were interested in exploring other attachment strategies involving esters in addition to carbamates for conjugating the acetals to the peripheral hydroxyl groups. In contrast to the carbamate, an ester lacks the hydrogen bond donor ability, and thus it is expected that a more hydrophobic micelle core will be obtained with ester linkages. An acetal having a carboxylic acid functional handle can be conveniently prepared as shown in Scheme 4 from the monomer 2,2-bis(hydroxymethyl)propionic acid **13**, which constitutes the dendrimer repeat unit. The resulting acetal must be isolated and used as its triethylamine salt **14** because of the incompatibility of a carboxylic acid group with the highly acid-sensitive acetal. Coupling of **14** to the peripheral hydroxyl groups of copolymer **7a** was achieved using 1,3-dicyclohexylcarbodiimide (DCC) in the presence of DMAP and 4-(dimethylamino)pyridinium *p*-toluenesulfonate (DPTS)[19] to provide the target **15** (PEO-5K-[G-3]-polyester-ester-acetal).

In contrast to the ester linkage that was expected to make the micelle core more hydrophobic, we were also interested in

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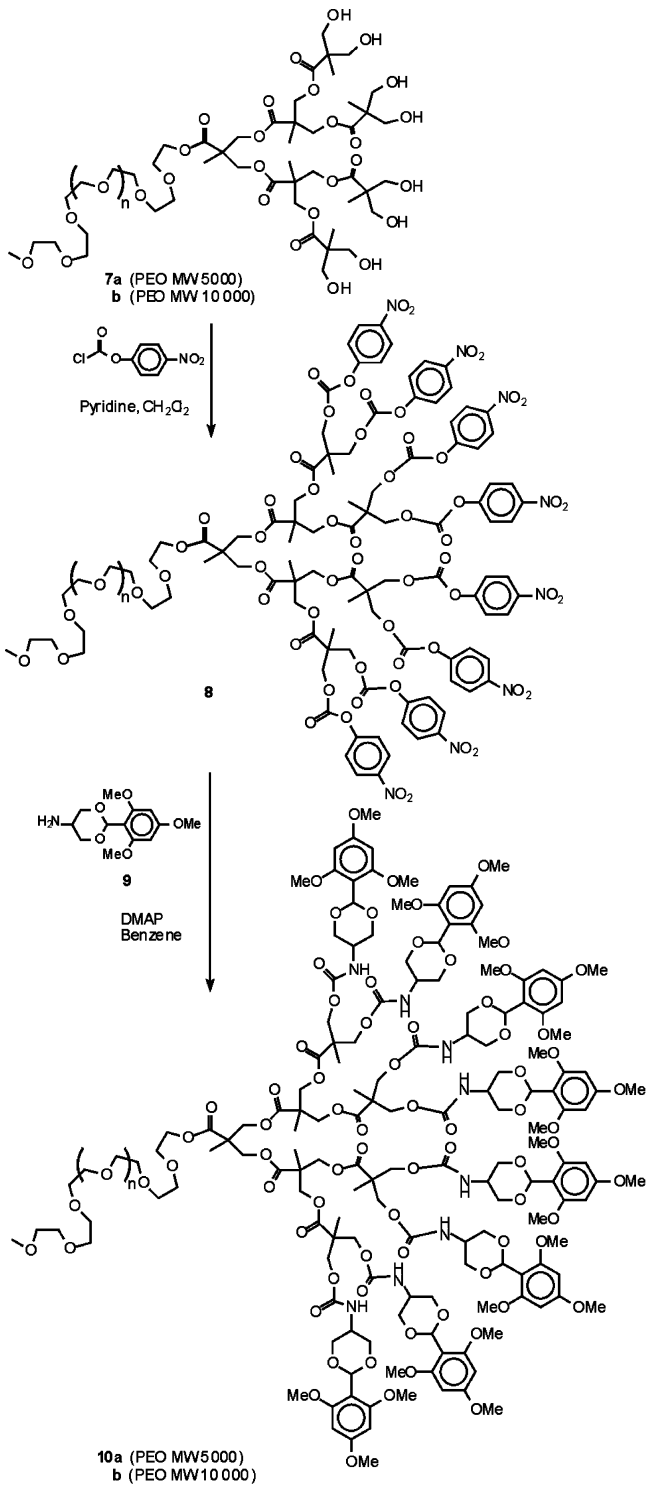
Scheme 3



incorporating a more hydrophilic spacer to determine its effect on the micelle properties. Therefore, 2-aminoethoxyethanol (**16**)- was chosen as the starting material for the linker synthesis, and the amine group was protected as a trifluoroacetamide by reaction with ethyl trifluoroacetate to provide **17** as shown in Scheme 5. The hydroxyl group was then oxidized to a carboxylic acid under Jones conditions, giving **18**, which could be coupled to the amine **9** using DCC to afford the acetal with a protected linker (**19**). Amine **20** was then obtained by removal of the trifluoroacetamide group under basic conditions and was coupled to copolymer **8a** to afford the target **21** (PEO-5K-[G-3]-polyester-linker-acetal).

Characterization. Critical micelle concentrations for the different copolymers were determined at pH 7.4 using the fluorescent probe technique. Nile Red was chosen as the hydrophobic dye constituting the micellar payload because its fluorescence is negligible in aqueous solution but is known to increase substantially in the hydrophobic environments found in some membranes or micelles.[20] Furthermore, its absorption λ_{max} is at 553 nm, far from interfering absorbances of the aromatic groups of the copolymer. The results are summarized in Table 1, showing the effect of the dendrimer backbone, dendrimer generation, linear PEO length, and nature of the linkage between the acetal and the dendrimer. The CMCs for the copolymers having the polylysine dendrimer block rather than the polyester dendrimer are typically higher. This can be attributed to the increased polarity and hydrogen bonding ability of the polylysine backbone. The effect of increasing the length of the PEO block on the CMC is quite minimal, while increasing the generation of the dendrimer block, thus doubling the number of hydrophobic groups attached to the periphery, lowers the CMC significantly for the polylysine copolymer **6**. This is probably because of the large increase in hydrophobicity of the core-forming block that strongly favors its self-assembly in water. In the case of the polyester system, the effect of the attachment strategy for linking the acetals to the dendrimer periphery on the CMC is quite significant. For copolymer **15** with the ester linkage, the CMC is lowered almost 3-fold compared to that of copolymer **10a** with the carbamate linkage. The CMC for copolymer **21** with the hydrophilic spacer is increased 3-fold, consistent with the hydrophobicity argument.

For biological applications, micelle size is an important factor. To avoid clearance by the liver and spleen, while at the same time facilitating uptake by nonphagocytic cells such as tumor cells, a size of less than 100 nm is ideal.[21] The micelle size and polydispersity was determined for each copolymer system by dynamic light scattering. A comparison of the volume average sizes determined in 10 mM pH 7.4 buffer at 37 °C is shown in Table 1. The micelles were in the ideal size range, with larger aggregates of 100−200 nm being observed only for copolymers **5a** and **21**. As expected, doubling the length of the PEO chain from 5000 to 10 000 MW somewhat increases the size of the micelles, while the size is increased even more dramatically by increasing the dendrimer generation to [G-4]. This may be due either to the increased size of the micelle core itself or to a higher aggregation number due to its greater hydrophobicity. An example of a size distribution profile is shown for copolymer **5b** in Figure 2.

The hydrolysis rates of the acetal groups located at the micelle cores were determined for each copolymer system at pH 7.4 and pH 5.0 at 37 °C. The kinetics could be conveniently monitored by UV−visible spectroscopy because the 2,4,6-trimethoxybenzaldehyde that is released in the hydrolysis absorbs strongly at 290 nm. The results for the lysine-based copolymers are shown in Figure 3a. The rates of hydrolysis for

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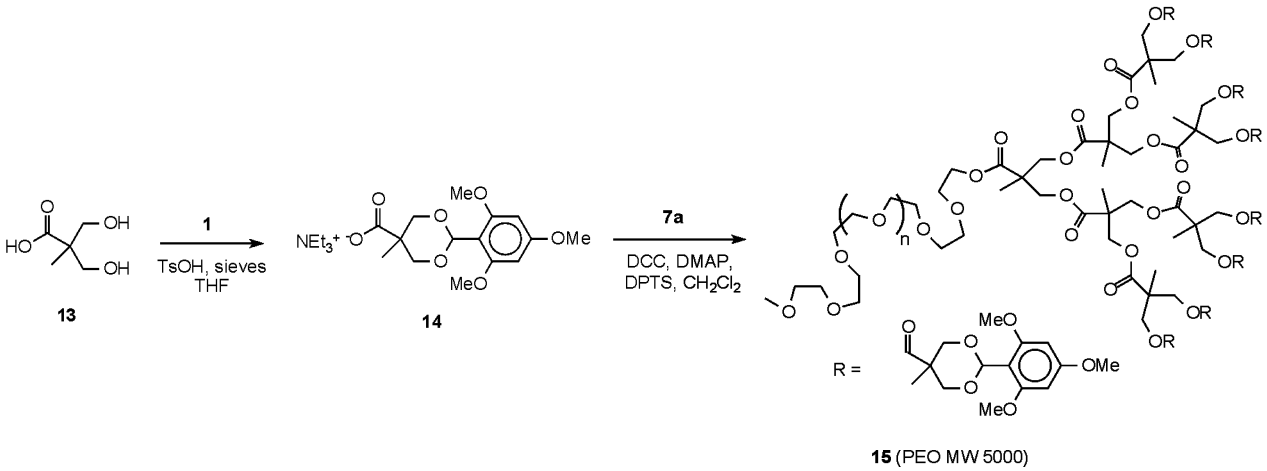
Scheme 4



Scheme 5

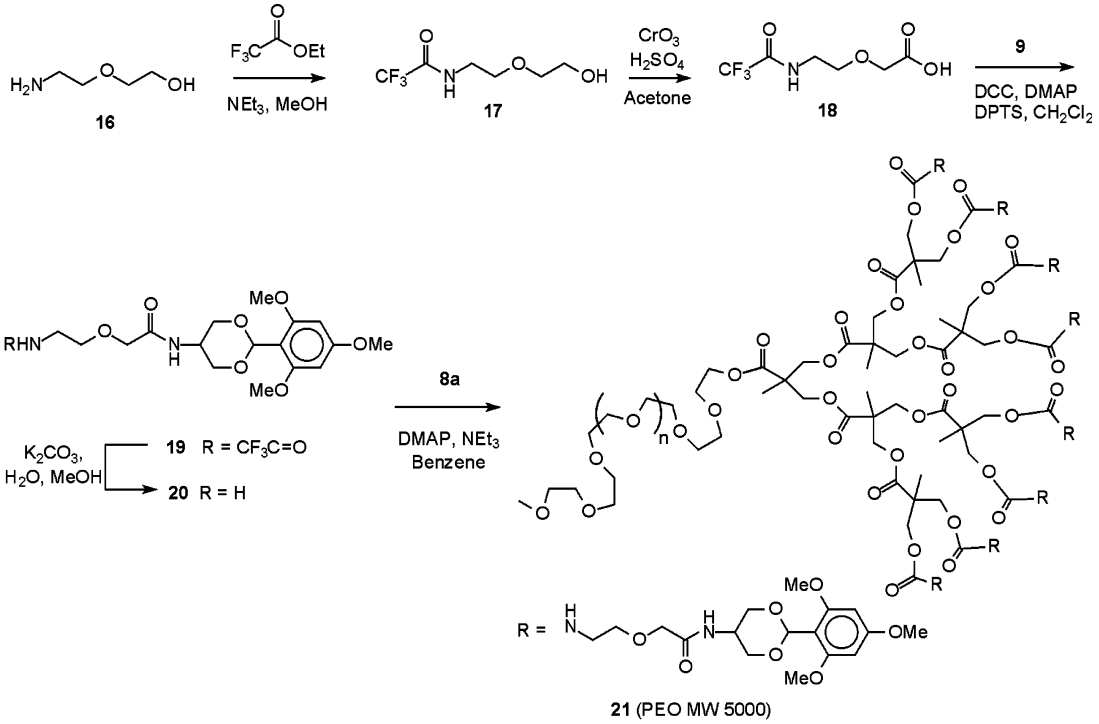


Table 1. Critical Micelle Concentrations and Diameters of Micelles Formed from the PEO−Dendritic Copolymers

copolymer	CMC (mg/L)	volume average size (nm)	polydispersity
5a (PEO-5K-[G-3]-polylysine-acetal)	120	20[a]	0.49
5b (PEO-10K-[G-3]-polylysine-acetal)	110	26	0.24
6 (PEO-10K-[G-4]-polylysine-acetal)	40	34	0.02
10a (PEO-5K-[G-3]-polyester-carbamate-acetal)	55	22	0.19
10b (PEO-10K-[G-3]-polyester-carbamate-acetal)	40	27	0.30
12 (PEO-10K-[G-4]-polyester-carbamate-acetal)	32	50	0.19
15 (PEO-5K-[G-3]-polyester-ester-acetal)	20	22	0.21
21 (PEO-5K-[G-3]-polyester-linker-acetal)	150	17[a]	0.41

[a] Larger aggregates of 100−200 nm were also formed.

copolymers **5a** and **5b** having [G-3] dendrimer blocks are very similar, with 50% of the acetals hydrolyzed after approximately 2.5 h at pH 5.0. Interestingly, this is somewhat slower than the rate of hydrolysis for the same acetal in our previously reported system and for a low MW model compound, both of which have half-lives of 1 h at pH 5.0.[13] The decrease in reaction rate is presumably due to the increased hydrophobicity of the nanoenvironment at the micelle core in these systems when compared to the previous system. Both the decreased local concentration of water and the destabilization of polar and charged transition states that are involved in the acetal hydrolysis mechanism likely contribute to the rate decrease. This effect of the nanoenvironment on the reaction rate is similar to that observed in enzymatic catalysis and has previously been

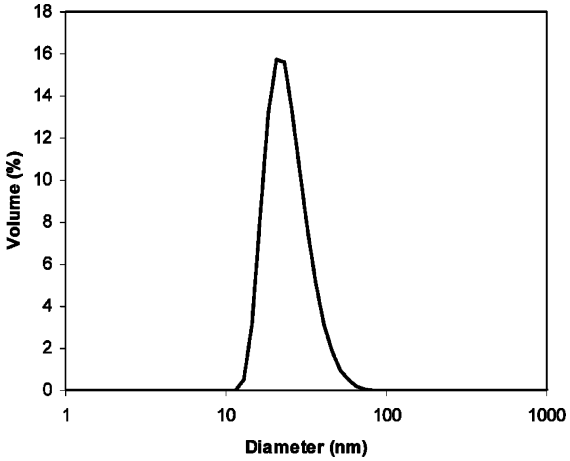


Figure 2. Size distribution profile for micelles of copolymer **5b**, measured by dynamic light scattering.

exploited in micellar systems to accelerate reactions.[22] In the case of our system, the environment is constantly changing throughout the hydrolysis reaction as hydrophobic groups are released to reveal more hydrophilic hydroxyl groups. Therefore, the rate of the reaction does not follow simple pseudo-first-order kinetics, and the measurement of a true half-life for hydrolysis is not straightforward. As predicted, the rate of hydrolysis of copolymer **6** is even slower because of the presence of its [G-4] dendron with its increased hydrophobicity, and 7 h are required to reach 50% hydrolysis at pH 5.0. All of the systems showed only negligible amounts of hydrolysis at pH 7.4 over the time period of the experiment.

The hydrolysis rates for the acetals in the polyester systems are shown in Figure 3b. In general, the acetals in the polyester system hydrolyze somewhat more slowly than those in the polylysine system. Micelles of copolymers **10a** and **10b** with a [G-3] polyester dendron and a carbamate linkage reach 50% hydrolysis after approximately 4 h at pH 5.0, while **12** with the [G-4] dendron requires 11 h. Because of their increased hydrophobicity, micelles of copolymer **15** with the ester linkage also hydrolyze quite slowly, with 50% hydrolysis after about 8 h at pH 5.0. In the case of **21** with its hydrophilic spacer, 50% hydrolysis is reached after 1.5 h only. Therefore, it is clear that the hydrophobicity of the dendron constituting the micellar core has a strong influence on the rate of acetal hydrolysis.

The ability of the micelles to release encapsulated molecules in response to acetal hydrolysis at acidic pH was also investigated using Nile Red. Release from the micelle environment to aqueous solution is easily monitored by fluorescence spectroscopy since the intensity of emission from Nile Red is decreased dramatically in aqueous solution relative to in the micellar environment.[20] Therefore, the fluorescence of Nile Red encapsulated in micelles was monitored over time at pH 7.4 and pH 5.0 at 37 °C. The micelles were first equilibrated with Nile Red overnight in 10 mM pH 7.4 buffer, and then the pH was adjusted to 5.0 by addition of a small volume of concentrated pH 5.0 acetate buffer. A sample at pH 7.4 was adjusted to the same salt concentration (100 mM), confirming that the micelles are stable to this 10-fold increase in salt concentration and that the fluorescence intensity of Nile Red was not affected. As shown in Figure 4a for the PEO-[G-3]-polylysine systems **5a** and **5b**, the fluorescence of Nile Red decreases at pH 5.0 over a time scale similar to that of the acetal hydrolysis, while the fluorescence of the sample at pH 7.4 remained relatively constant over this time period. These observations are consistent with release of Nile Red from the micelle, and the time dependence of the fluorescence change strongly suggests that it is indeed triggered by hydrolysis of the acetals. In the case of the [G-4] system **6**, the fluorescence does not begin to significantly decrease until after about 10 h, when 80% hydrolysis of the acetals has been hydrolyzed. This indicates that the micelles likely remain stable until a critical number of hydrophobic groups have been lost.

A similar trend is observed with the polyester systems as shown in Figure 4b, where the slower hydrolysis rates correspond to a slower release of Nile Red from the micelle. Again in the case of the [G-4] polymer **12**, a significant amount of acetal hydrolysis must occur before release of Nile Red can occur. The expected trend is observed for copolymers having a different type of connection to the acetal groups, with the more hydrophobic system **15** releasing Nile Red most slowly and the

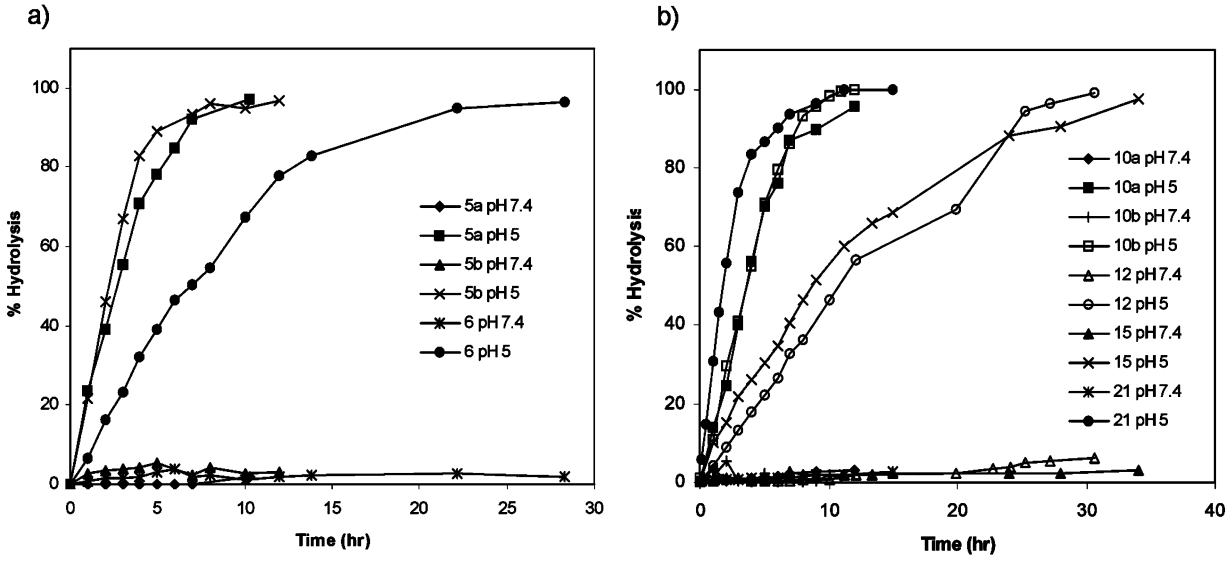


Figure 3. Acetal hydrolysis rates at pH 7.4 and pH 5.0 for (a) polylysine copolymers **5a**,**b** and **6** and (b) polyester copolymers **10a**,**b**, **12**, **15**, and **21**.

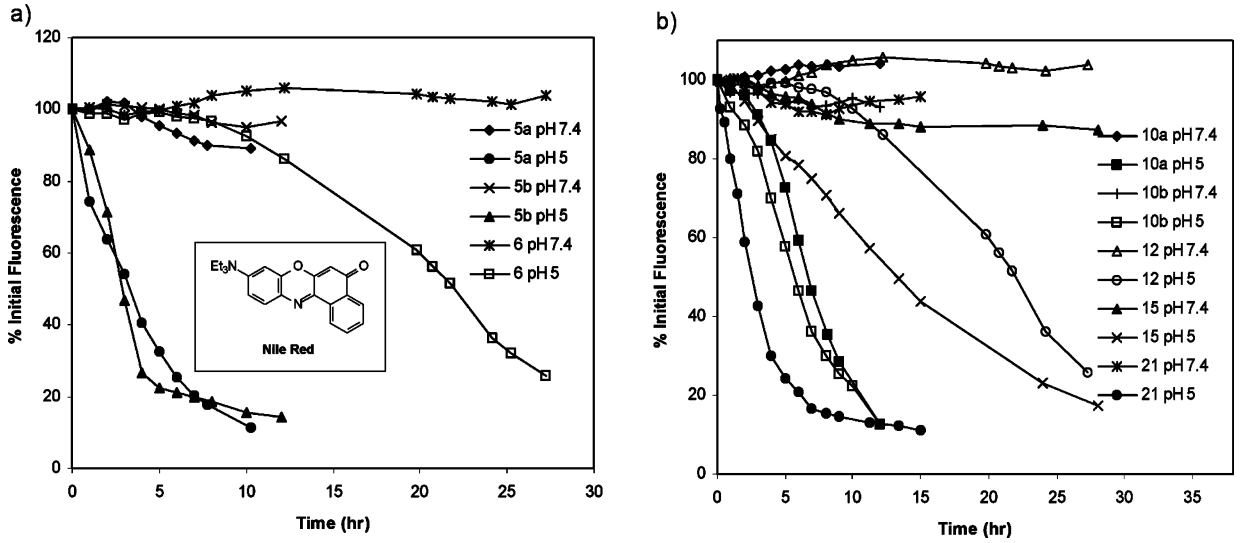


Figure 4. pH dependence of Nile Red fluorescence in micelles of (a) polylysine copolymers **5a**,**b** and **6** and (b) polyester copolymers **10a**,**b**, **12**, **15**, and **21**.

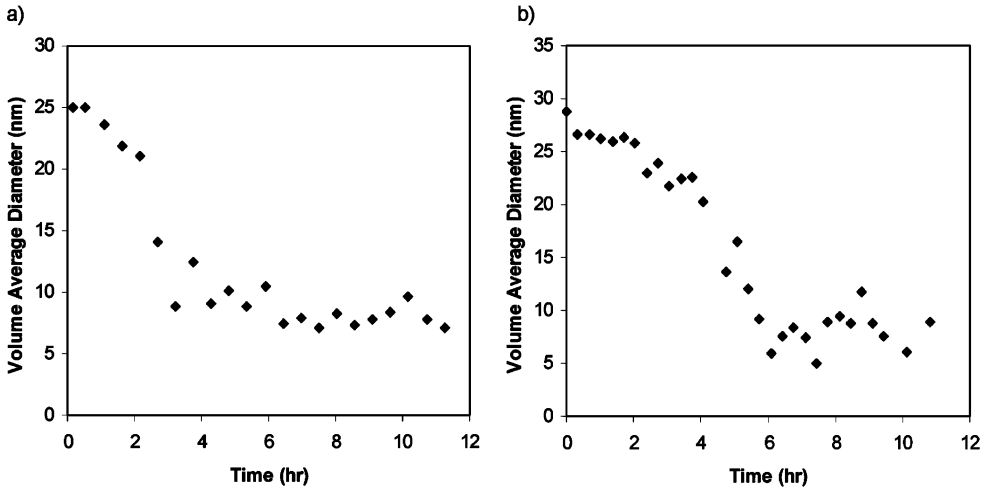


Figure 5. Time dependence of micelle size (volume average diameter) for copolymer (a) **5b** and (b) **10b** at pH 5.0 and 37 °C monitored by dynamic light scattering.

hydrophilic system **21** most rapidly. Overall, these results confirm that there is a strong correlation between the hydrolysis rate of the acetals within the micelle and the disruption of the micelle, triggering Nile Red release. This indicates that it should be possible to control the rate of payload release from these triggerable carriers by tuning the structure of the dendrimer block of the copolymer.

Because the proposed mechanism of release from these carriers involves disintegration of the micelle structure as the hydrophobicity of the core-forming block is lost through hydrolysis, we also explored changes in the size of the micelle over time at acidic pH. The size of the micelles was therefore monitored by dynamic light scattering at pH 5.0 and 37 °C for a representative polylysine system **5b** and the polyester system **10b**. As shown in Figure 5, parts a and b, the volume average micelle size decreases over time for both the polylysine and polyester systems, with the decrease somewhat slower for the polyester system, consistent with the hydrolysis and Nile Red release rates. After several hours, both systems approach a size of 5−10 nm. In the size distribution profiles for each system, rather than observing a bimodal distribution corresponding to intact micelles and released polymer chains, the volume average size of one micelle population was observed to gradually decrease, consistent with a decreasing aggregation number of the micelles as the acetals were hydrolyzed. The degree of scatter in the data points is likely due to the dynamic nature of the disassembly process. For comparison, the size we determined by light scattering for the linear PEO block alone with a MW of 10 000 g/mol is about 5 nm. Therefore, 5−10 nm is a reasonable size estimate for the resulting block copolymer. Therefore, these results provide convincing evidence that the proposed disintegration of the micelles into unimers in solution does in fact take place following hydrolysis of the acetal groups.

Conclusion

A number of linear-dendrimer hybrids consisting of PEO and polylysine or polyester dendrimers having hydrophobic groups attached to the dendrimer periphery by acid-sensitive linkages have been prepared and used to assemble pH-responsive micelles. The micelles were all in the desired size range for

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drug delivery applications, with their CMCs controlled by the structure of the dendrimer backbone, its generation, and the specific functionalization strategy. Most importantly, it was found that these structural features could be used to tune the pH sensitivity of the systems and, thus, the rate of release of encapsulated payload. Furthermore, it was confirmed that the proposed method of release via disintegration of the micelle into unimers at acidic pH was valid. This new approach is therefore promising for the development of micelle systems capable of responding to subtle pH changes in the physiological environment. The well-defined nature of the system and the ability to tune its properties in predictable ways should facilitate its development in a range of drug delivery applications. Some of these applications are currently being explored.

Acknowledgment. The Center for New Directions in Organic Synthesis is supported by Bristol-Myers Squibb as a sponsoring member and Novartis Pharma as supporting member. We thank the National Institutes of Health (GM 65361 and EB 002047) and the U.S. Department of Energy (DE-AC03-765F00098) for support of this research.

Supporting Information Available: Full experimental details and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0463738