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# Temperature Sensitivity Trends and Multi-Stimuli Sensitive Behavior in Amphiphilic Oligomers

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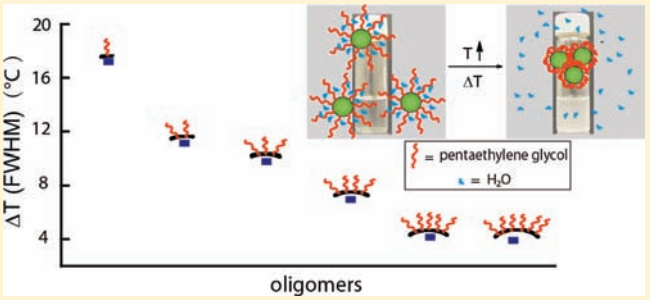
Supporting Information

**ABSTRACT:** A series of oligomers, containing oligo(ethylene glycol) (OEG) moieties, with the same composition of amphiphilic functionalities has been designed, synthesized, and characterized on the basis of their temperature-sensitive behavior. The non-covalent amphiphilic aggregates, formed from these molecules, influence their temperature sensitivity. Covalent tethering of the amphiphilic units also has a significant influence on their temperature sensitivity. The lower critical solution temperatures of these oligomers show increasingly sharp transitions with increasing numbers of OEG functional groups, indicating enhanced cooperativity in dehydration of the OEG moieties when they are covalently tethered. These molecules were also engineered to be concurrently sensitive to enzymatic reaction and pH. This possibility was investigated using porcine liver esterase as the enzyme; we show that enzymatic action on the pentamer lowers its temperature sensitivity. The product moiety from the enzymatic reaction also gives the amphiphilic oligomer a pH-dependent temperature sensitivity.




## INTRODUCTION

Stimuli-sensitive systems have generated interest in a variety of areas, including controlled drug delivery vehicles,[1] sensing,[2] tissue engineering,[3] coatings,[4] catalysis,[5] and separations.[6] The stimuli employed for these so-called smart materials can be broadly divided into two categories: physical and chemical. Among the physical stimuli, materials that respond to temperature variations have garnered particular interest due to implications in biomedical applications such as drug delivery and tissue engineering.[7,8] Thermo-responsive materials have also found utility in areas such as thermal affinity separation, enzyme recycling, and protein chromatography.[9]

Thermal sensitivity is endowed into a material, especially polymeric ones, by engineering the molecular structures so that the polymer undergoes a coil-to-globule transition when the temperature changes.[9d,10] When this transition results in solubility differences, the material is thought to exhibit lower critical solution temperature (LCST) behavior. For experimental convenience, the onset temperature at which the polymer-containing solution becomes turbid is commonly probed to extract relationships between structure and LCST properties. Among the temperature-sensitive polymers, poly(*N*-isopropylacrylamide) (p-NIPAM) has attracted a great deal of interest.[11] Another class of materials that has attracted substantial interest in temperature-sensitive materials, due to their biocompatibility and anti-fouling features, comprises ethylene glycol-based polymers. Both poly(ethylene glycol) (PEG) and oligo(ethylene glycol) (OEG) have been used as components of polymeric scaffolds that exhibit temperature-sensitive behavior.[12] At higher temperatures, hydrogen bonds between the ethylene glycol units and the water molecules break, rendering them lipophilic. This is thought to be the reason for the thermal sensitivity of these functional groups.

It is interesting that incorporating a single OEG unit, such as a penta(ethylene glycol) moiety, by itself or as part of a small molecule does not lead to any noticeable thermal sensitivity. However, when penta(ethylene glycol) is attached onto a scaffold that presents these units in a multimeric form due to self-assembly, the system exhibits significant temperature sensitivity. We have observed this phenomenon while comparing amphiphilic dendrimers with the corresponding amphiphilic small molecules.[13] We were interested in identifying the underlying reasons why a polymer would be more sensitive than the small molecule. We approach this by studying well-defined oligomers and comparing them to the corresponding monomers. This article outlines our findings, where we have designed, synthesized, and characterized the temperature-sensitive behavior of a set of amphiphilic oligomers containing penta(ethylene glycol) as the hydrophilic OEG moiety.

Additionally, systems that are concurrently sensitive to more than one stimulus have attracted significant interest in recent years, because they provide unique opportunities to fine-tune their response to each stimulus independently, as well as to precisely regulate the release profile under the combined effect of multiple stimuli.[14] Our molecular design provides a great opportunity for testing the sensitivity of these oligomers to multiple

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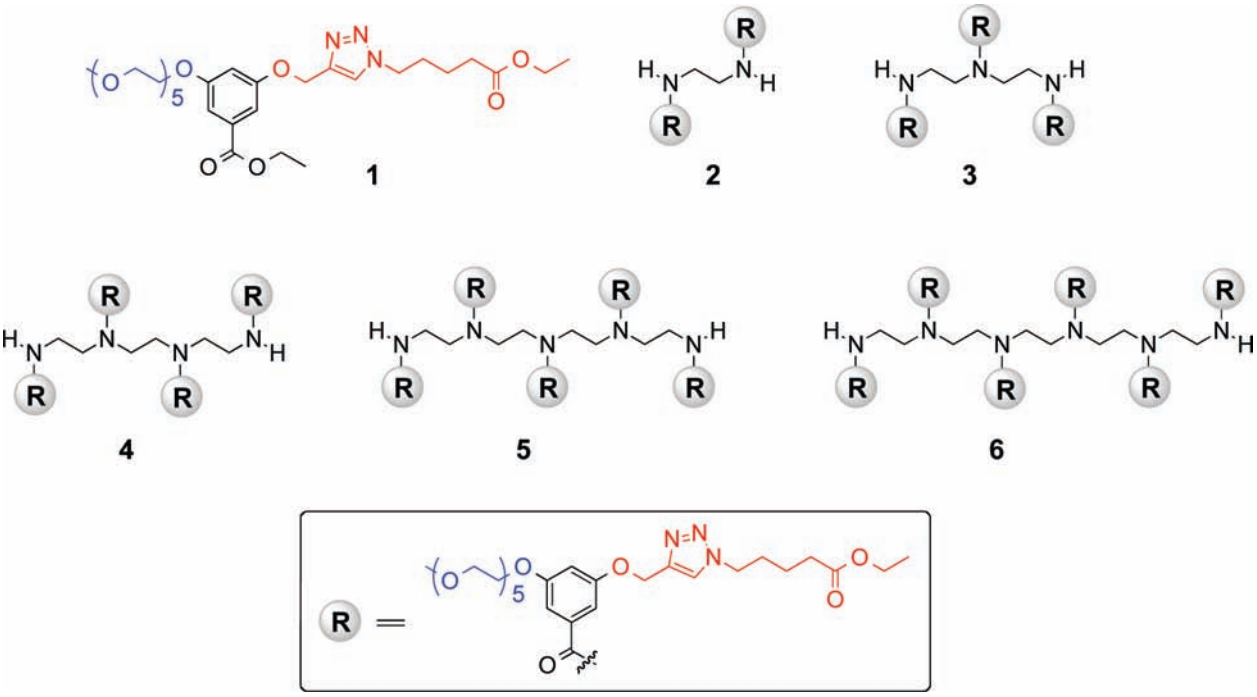
**Chart 1. Structures of the Amphiphilic Oligomers Used in This Study**




stimuli, i.e., temperature and enzyme. We take advantage of this opportunity and test our oligomers for concurrent sensitivity to these stimuli. We particularly focus on esterase-based enzymes because (a) they are convenient model systems to study enzyme sensitivity and (b) they are ubiquitous in biology and thus relevant to temperature-sensitive systems that are of interest for several biomedical applications.

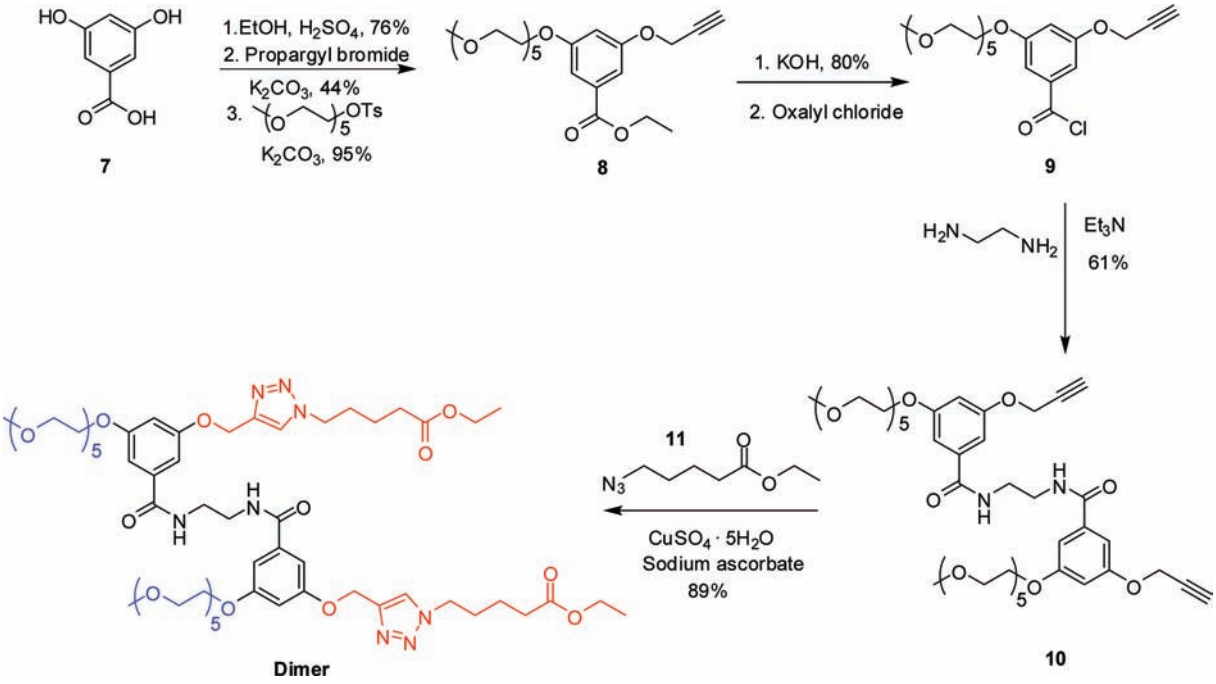
## RESULTS AND DISCUSSION

**Molecular Design and Synthesis.** The effects of compositional variations of OEG-based monomer and another monomer on the temperature-sensitive behavior of polymers have been previously investigated.[15] However, since our interest lies in developing an understanding of the cooperative effect of OEG units when they are covalently tethered together, our self-imposed design criterion is that the hydrophilic−lipophilic balance (HLB) in all oligomers should be the same. In our study, we have focused on variations ranging from monomeric to hexameric amphiphiles. Penta(ethylene glycol) and alkyl moieties were attached to the *meta*-positions of a benzoyl building block as the hydrophilic and lipophilic moieties, respectively. This basic building block was then converted to an oligomer using the corresponding commercially available oligoamine scaffolds (Chart 1). Note that the lipophilic alkyl moiety is terminated with an ester functionality. This ester functionality provides the substrate handle for esterases; the reaction between them affords the corresponding carboxylic acid. Conversion of the ester moiety to the corresponding carboxylic acid functionality, in the presence of the enzyme, alters the HLB of the amphiphilic molecule and thus the LCST of the oligomers. Additionally, it is easy to imagine that the carboxylic acid moiety, thus generated, would provide an avenue for pH-sensitivity.

In all these cases, the commercially available oligoamines were treated with the benzoyl chloride molecule **9** (Scheme 1). To synthesize **9**, 3,5-dihydroxybenzoic acid (**7**) was first converted to its ethyl ester, followed by the installation of a propargyl functionality on one of the phenolic groups through an alkylation reaction in the presence of potassium carbonate, as shown in Scheme 1. Treatment of the mono-substituted product with penta(ethylene glycol) tosylate under similar alkylation conditions provided the precursor **8**. Hydrolysis of the ethyl ester, followed by treatment with oxalyl chloride, afforded the targeted aryloyl chloride molecule **9**. Treatment of this molecule with oligoamines in the presence of a base afforded the targeted oligomers. This is exemplified by the synthesis of dimer **2** in Scheme 1. Note that the molecule **10** does not contain the ester-based lipophilic functionality installed in the molecule yet. This functionality was attached to the oligomers in the last steps of the synthesis using the Huisgen 1,3-dipolar cycloaddition reaction, the so-called "click" chemistry. This was necessary because we had to generate the acid chloride species in order to generate that targeted benzamide molecules. The azide counterpart in the Huisgen reaction is ethyl 5-azidovalerate (**11**), which was synthesized from the corresponding alkyl bromide by treating it with sodium azide. Higher oligomers were synthesized through a similar route using the corresponding oligoamine; the details of these syntheses are shown in the Supporting Information. All products were characterized using $^{1}$H NMR, $^{13}$C NMR, and mass spectrometry (see Supporting Information for details).

Since these are amphiphilic oligomers, we also anticipated that these molecules would aggregate in aqueous media above a certain concentration, the critical aggregation concentration (CAC). We were specifically interested in this concentration, since this feature could further influence the temperature-sensitive behavior of these molecules. It is reasonable to anticipate that the assemblies obtained in the aqueous phase from these amphiphilic molecules will contain a hydrophobic interior, which can potentially sequester lipophilic guest molecules. The lipophilic guest

**Scheme 1. Synthetic Route for the Amphiphilic Oligomers, Exemplified with the Dimer 2**



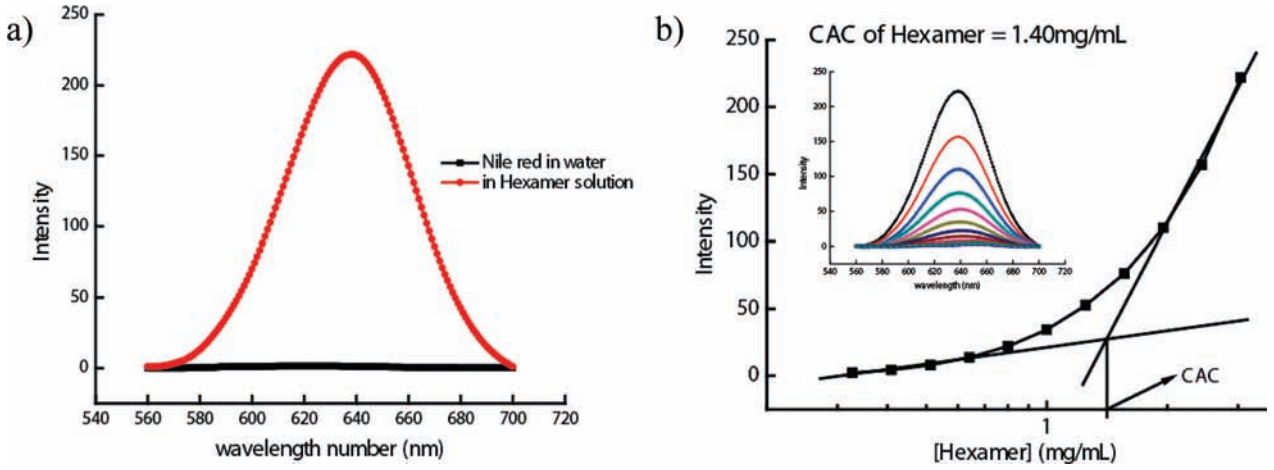




**Figure 1.** (a) Fluorescence of Nile red in water and hexamer **6** solution. (b) CAC calculation for hexamer.

**Table 1. CAC and DLS Size of Oligomers**

| oligomer | CAC, mg/mL | CAC, mM | size, nm (PDI) |
|---|---|---|---|
| monomer (**1**) | 3.84 | 6.14 | NA |
| dimer (**2**) | 1.55 | 1.27 | 18 (0.24) |
| trimer (**3**) | 1.75 | 0.95 | 23 (0.26) |
| tetramer (**4**) | 1.66 | 0.67 | 25 (0.24) |
| pentamer (**5**) | 1.65 | 0.54 | 30 (0.24) |
| hexamer (**6**) | 1.40 | 0.38 | 60 (0.19) |

sequestration event is typically used as the pathway for identifying the CAC. We have used Nile red as the lipophilic guest molecule for this purpose; note that Nile red by itself is not soluble in water (Figure 1a). A plot of concentration of the surfactant molecules against the Nile red fluorescence provides a sigmoidal curve. The point at which there is a significant change in the slope of the curve is taken to be the CAC. This is exemplified in Figure 1b, and the CACs of molecules **1**−**6** are shown in Table 1. CAC values are usually reported as concentrations. However, since the amphiphilic repeat unit in each of these molecules is the same, it is more relevant to assess the relative CACs in terms of mg/mL, and these values are also tabulated. We found that while there is a significant gain in CAC between the monomer and the dimeric amphiphile, there is no real difference in CACs among other oligomers.

The sizes of our amphiphilic aggregates, estimated using dynamic light scattering (DLS) measurements, are shown in Table 1 for comparison. We used 3 mM concentration of the molecules,

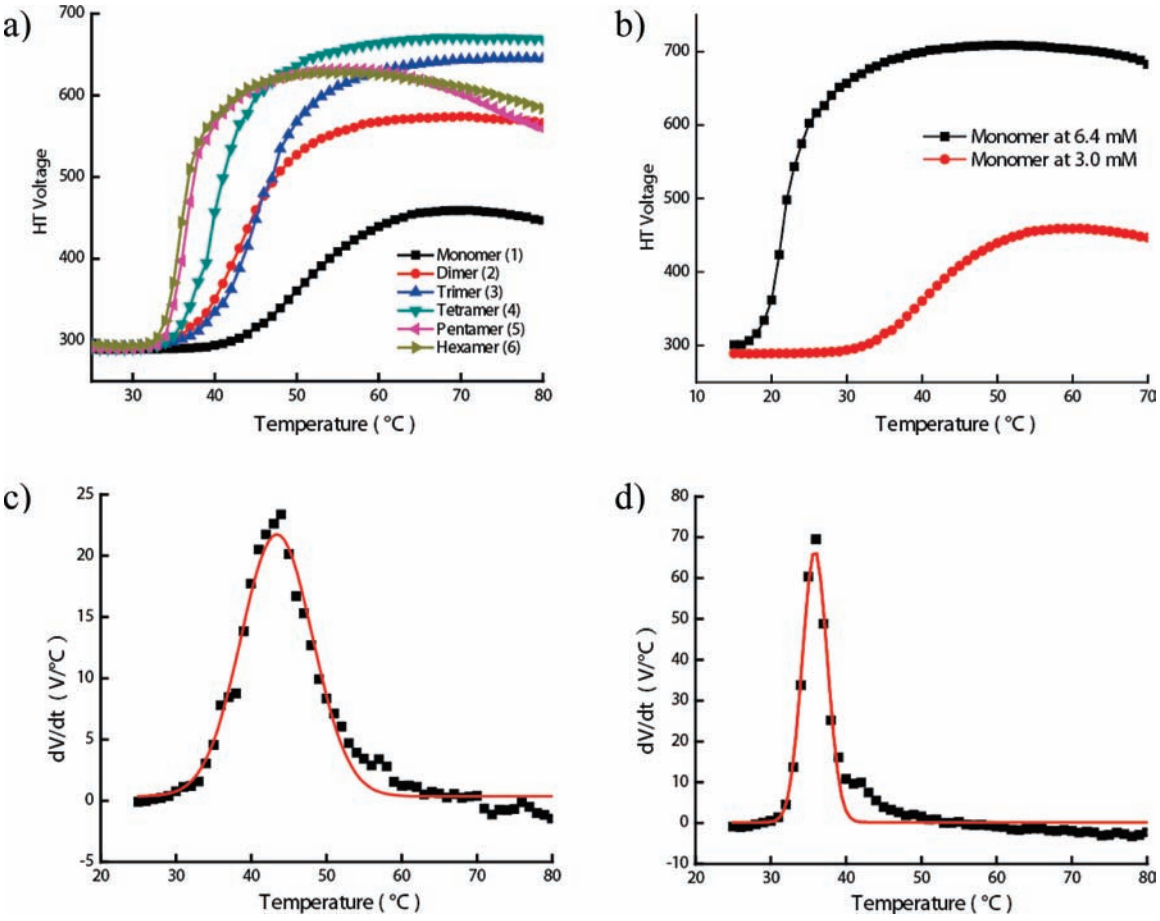



**Figure 2.** (a) Plot of HT voltage vs temperature for molecules **1**−**6** in water. (b) LCST of Momomer **1** at 6.4 and 3.0 mM. (c) First differential plot for dimer **2**. (d) First differential plot for hexamer **6**.

**Table 2. Transition Temperature and Full Width at Half-Maximum of Oligomers**

| oligomer | $T_t$ (°C) | FWHM |
|---|---|---|
| monomer (**1**) | 51.3 | 17.2 |
| dimer (**2**) | 43.4 | 11.2 |
| trimer (**3**) | 45.3 | 9.9 |
| tetramer (**4**) | 40.1 | 6.9 |
| pentamer (**5**) | 36.4 | 4.1 |
| hexamer (**6**) | 35.8 | 3.9 |

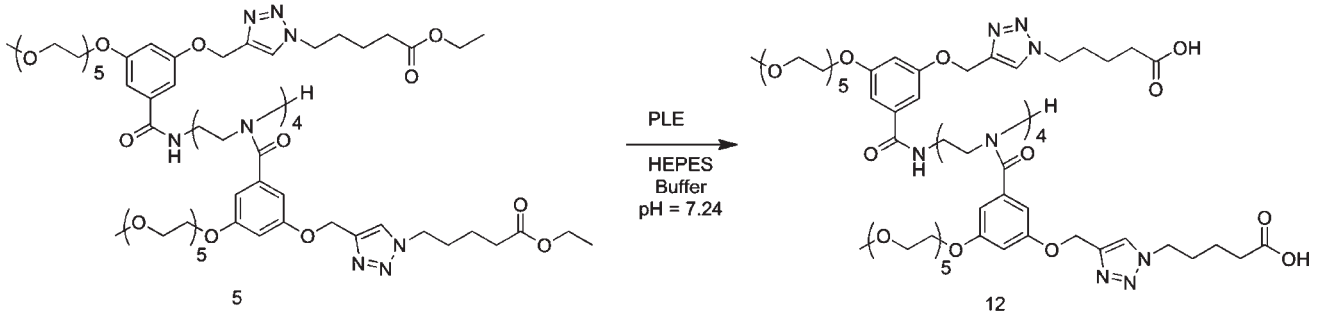
and the sizes seem to increase with increasing oligomeric length. It should be noted that the sizes of our assemblies are somewhat larger than those of small-molecule-based surfactant micelles. Thus, we believe that our assemblies are higher order aggregates with micelle-like lipophilic interiors. The rather shallow transition in the CAC estimation in Figure 1 further suggests that these are higher order aggregates.

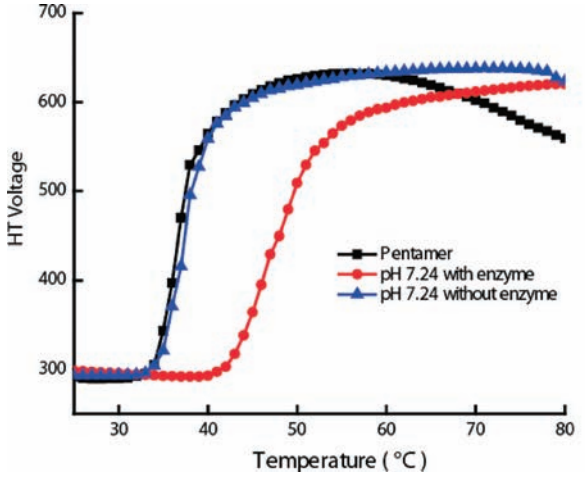
**Temperature-Sensitive Behavior.** The temperature-sensitive behavior of the oligomers was studied using turbidity measurements by measuring the high-tension (HT) voltage response of the photomultiplier on a circular dichroism (CD) spectrometer.[13,16] The CD spectrometer was used simply because of the conveniently available temperature control in this equipment in our laboratories. Aqueous solutions of molecules **1**−**6** were monitored for solution turbidity with increasing temperature at 650 nm. The temperature increase was done at a rate of 1 °C/min.

In all these studies, we kept the concentration of the OEG units in solution constant, as this allows for an understanding of the possible cooperativity due to covalent tethering of the OEG units. The concentrations of the oligomer solutions were consistently kept at 3 mM with respect to the OEG unit; i.e., since the monomer **1** contains only one OEG unit, a 3 mM solution of molecule **1** was used. However, we used a 1.5 mM concentration of **2** when the dimer was used, as this solution provided an overall OEG concentration of 3 mM. Note that, with the exception of **1**, all these molecules are well above their CACs, and therefore we study the behavior of the aggregates in all these cases.

Figure 2a shows the temperature sensitivity plots for the amphiphilic molecules **1**−**6**. Two observations can be immediately made from these plots: (i) There is a systematic change in the temperature sensitivity of the oligomeric amphiphiles. Monomer **1** is less sensitive to temperature; i.e., it exhibits a much higher transition temperature compared to the higher oligomers. (ii) The transition itself seems sharper in higher oligomers. Both of these features can be more clearly visualized and better quantitative data can be acquired if we analyze the first differential of these plots. Figure 2c,d shows the first differential of the plots for dimer **2** and hexamer **6**, respectively. It is readily seen that the transition in the hexamer is indeed sharper than that in the dimer.

**Scheme 2. Change in HLB in Pentamer 5 Due to the Enzymatic Reaction**




| Oligomers | $T_t$ (°C) | FWHM |
|---|---|---|
| Pentamer (**5**) | 36.4 | 4.1 |
| with enzyme | 47.3 | 8.9 |
| without enzyme | 37.2 | 4.6 |

**Figure 3.** LCST profile of pentamer **5** after enzymatic reaction.

A Gaussian function was applied to fit the nonlinear graph, leading to the temperature at the sharpest transition point, which is taken to be the transition temperature ($T_t$) (Table 2). A full width at half-maximum (FWHM) can also be extracted from these curves, which provides an important insight into the rate of the transition, as this is a measure of the steepness of the transition (Table 2). This rate can be correlated with the cooperativity among the OEG units, because the concentrations of OEG units in all these solutions are the same.

Note that the onset temperatures in all oligomeric molecules **2**−**6** are very similar, while that of **1** is significantly different. To explain this observation, we need to first examine the underlying reasons behind the OEG units exhibiting a temperature-sensitive coil-to-globule transition. It is generally accepted that the driving force involves the breaking of hydrogen bonds between OEG units and water molecules with increasing temperature.[12] This is thought to occur because of the entropy gained due to the shedding of the ordered water molecules around OEG units. Note that, in our experiments, the concentrations of OEG units are the same in all cases. However, the onset temperature is different for the monomer **1** compared to other oligomers. We attribute this to the difference in LCST of OEG-based aggregates in **2**−**6** vs monomeric OEG-based molecule **1**. Note that the oligomers **2**−**6** are present in solution above its CAC, while the monomer **1** is below its CAC. Thus, a high percentage of the oligomer is present as non-covalent nanoscale assemblies, where a number of OEG units are presented on their surfaces, a feature that is not present in **1**. To test this further, we measured LCST behavior of **1** at a concentration above its CAC (6.4 mM). Indeed, the $T_t$ decreased to 21.6 °C, with a corresponding sharpening of the transition (see Figure 2b). These could also be attributed to the inherent increase in the concentration of OEG units in the solution. We could not distinguish these possibilities. However, when put together with other results, this result is consistent with our hypothesis that the non-covalent nanoscale assembly formation influences the onset temperature.

Although there are very small differences, if any, in the onset temperatures among the oligomers, the FWHM's of the oligomers exhibit a systematic difference in these molecules. As mentioned above, FWHM is related to the rate of the coil-to-globule transition. Since the OEG concentration in all oligomers is identical, the difference in rate of the transition can be attributed to the difference in cooperativity in the OEG units shedding the water in response to the temperature variation. This, in turn, suggests that a greater cooperativity is observed in higher oligomers compared to the lower oligomers (for example, the FWHM of the dimer is 11.2, while that of the hexamer is only 3.9). Overall, these results suggest that formation of a non-covalent assembly provides a pathway for providing a critical change in the onset temperature in the coil-to-globule transition of the OEG units. In addition, the rate of transition in these non-covalent assemblies can be tuned by covalently tethering amphiphilic units, as this provides a useful vehicle for cooperative dehydration of the OEG units.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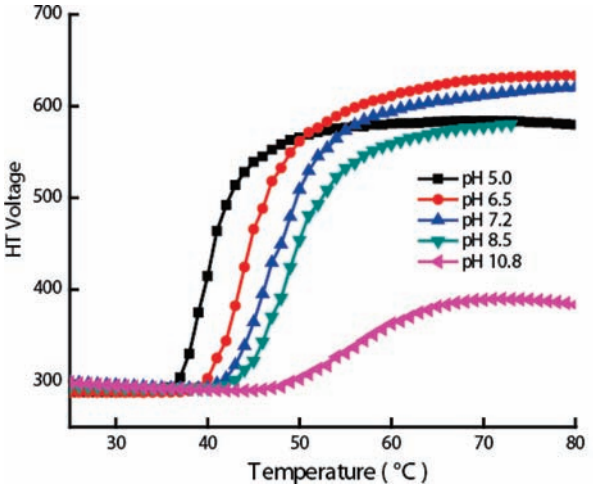
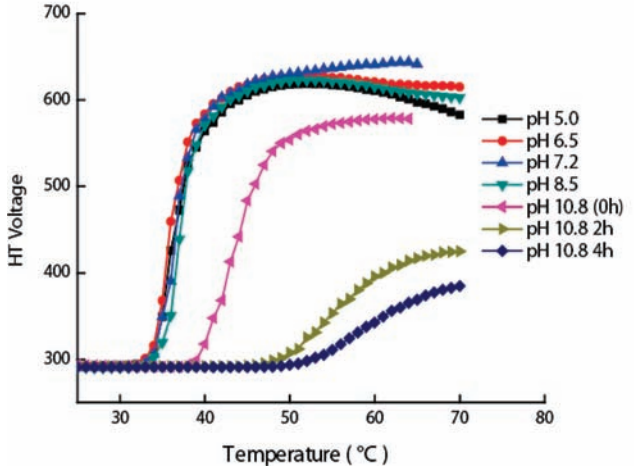



| pH | Tt (°C) | FWHM |
|---|---|---|
| pH 5.0 | 39.9 | 5.3 |
| pH 6.5 | 44.1 | 7.0 |
| pH 7.2 | 47.3 | 8.9 |
| pH 8.5 | 48.8 | 8.3 |
| pH 10.8 | 56.2 | 16.5 |

**Figure 4.** LCST profile of pentamer **5** after enzymatic reaction at different pH's.




| pH | Tt (°C) | FWHM |
|---|---|---|
| pH 5.0 | 36.2 | 4.3 |
| pH 6.5 | 35.8 | 3.8 |
| pH 7.2 | 36.5 | 3.8 |
| pH 8.5 | 37.0 | 3.3 |
| pH 10.8 (0h) | 43.4 | 7.0 |
| pH 10.8 2h | 55.4 | 12.8 |
| pH 10.8 4h | 59.4 | 14.6 |

**Figure 5.** LCST profile of pentamer **5** control at different pH's and at different times for pH 10.8.

**Multi-Stimuli Sensitivities.** Next, we were interested in testing whether esterase activity will modulate the LCST behavior of these amphiphilic molecules. The appeal of this is in the fact that esterase-based degradation is often the basis for degradation of biomaterials. Therefore, it is interesting to examine how the temperature sensitivity of a molecule can be modulated after an enzymatic reaction. Note that we have engineered the molecule in a fashion such that an esterase reaction would alter the HLB in the amphiphilic oligomers, which is expected to alter the LCST behavior. This is anticipated because esterases cleave esters to produce carboxylic acid; since the aliphatic esters are more lipophilic than the corresponding carboxylic acids, the products of the enzymatic reaction would possess markedly different HLB values as compared to the reactants (Scheme 2). In our case, the molecule is expected to become more hydrophilic, resulting in a significant change in its LCST behavior.

We examined this possibility with the pentamer **5** using porcine liver esterase (PLE). Accordingly, we subjected a 3 mM solution of **5** in HEPES buffer at pH 7.24 to reaction with PLE for 20 h, after which we examined the temperature-dependent turbidity generation in the solution. Indeed, we noted that the onset temperature increased by ~10 °C in the enzyme-treated solution (Figure 3). As a control experiment, the LCST behavior of the same pentamer solution in HEPES buffer at pH 7.24 without enzyme was examined after 20 h. No change in the temperature sensitivity was observed, suggesting that the observed change in LCST is indeed due to an enzymatic reaction rather than any adventitious hydrolysis over time in the aqueous phase. Also, partial hydrolysis of the ester functionalities in the mixture due to the esterase reaction was confirmed by $^1$H NMR (see Supporting Information).

Note that the molecular design is also engineered to exhibit a pH-sensitive behavior, since the carboxylic acid formed after the enzymatic reaction can present different hydrophilicities, depending on whether the carboxylic acid is protonated or deprotonated. Thus, the pH of the solution can significantly alter the LCST behavior of the molecule. We tested this possibility by monitoring the LCST of the enzymatic product at different pH values. After the enzymatic reaction, the pH of the solution was adjusted to 5.0, 6.5, 8.5, and 10.8 by addition of acid or base to the

solution. The LCST behaviors were examined at these different pH values (Figure 4). Deprotonation of the carboxylic acid at basic pH increases the hydrophilicity due to an increase in the number of negatively charged carboxylate anions. Similarly, protonation of the carboxylates at lower pH should decrease the hydrophilicity. Indeed, at pH 5.0 and 6.5, we found that $T_t$ decreases to 39.9 and 44.4 and the FWHM decreases to 7.0 and 5.3, respectively, compared to a $T_t$ of 47.3 and FWHM of 8.9 at neutral pH, 7.2. Similarly, at pH 8.5 and 10.8, $T_t$ increased compared to that at neutral pH, while the FWHM did not significantly change at pH 8.5 (in fact, it decreased slightly) and increased greatly at pH 10.8. These observations are indeed consistent with our hypothesis.

Although consistent, there is a possibility that the esters might also be hydrolyzed at different pH's, and this could cause further changes in the HLB. We were concerned about this, especially because of the rather significant change noted at pH 10.8. Accordingly, we incubated the pentamer **5** at different pH's (Figure 5). When analyzed from pH 5 to 8.5, the free pentamer **5** did not exhibit any difference. However, at pH 10.8, the pentamer **5** did exhibit a significant change that indicates hydrolysis of the ester. To further test this, we analyzed the pentamer **5** solution at different time intervals, and indeed the LCST systematically evolved with time, further supporting the observation that hydrolysis of the ester over time affects the observed LCST behavior (Figure 5). Under these conditions at lower pH, however, there is no observable hydrolysis of the ester. Therefore, it is clear that there is indeed a pH-dependent temperature sensitivity of the enzymatic product. However, these observations are not reliable at high pH (>8.5), where an independent base-catalyzed hydrolysis of the ester occurs.

## SUMMARY

We have designed, synthesized, and characterized a series of amphiphilic oligomers containing penta(ethylene glycol) functionalities as the hydrophilic segment and esters as the hydrophobic moiety. By systematically comparing the oligomers, we note that (i) non-covalent organization of the OEG units through aggregation causes a significant increase in temperature sensitivity; (ii) cooperativity is further enhanced when these OEG units are covalently tethered in the oligomers, as evidenced by the increase in transition kinetics with increasing oligomerization; (iii) when an enzyme-sensitive functionality is incorporated onto the lipophilic segment of the amphiphile, these molecules can be rendered sensitive to both enzyme and temperature; and (iv) since the product of the enzymatic reaction provides a pH-sensitive functionality, the amphiphilic assembly is rendered responsive to three different stimuli. Overall, our studies here provide insights into the need for multimeric presentation of oligoethylene glycol units based on either non-covalent assemblies and/or covalent tethering. Our report also outlines a strategy to design a molecule that can be sequentially sensitive to three different stimuli. We believe that this work will have implications in designing molecular scaffolds for applications such as drug delivery and tissue engineering, where stimuli-sensitive materials are used.

## ASSOCIATED CONTENT

**Supporting Information.** Synthesis and other experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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