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Guest-Release Control in Enzyme-Sensitive, Amphiphilic-Dendrimer-Based Nanoparticles through Photochemical Crosslinking

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Abstract

Stimuli sensitive, facially amphiphilic dendrimers have been synthesized and their enzyme-responsive nature has been determined with dual fluorescence responses of both covalently conjugated and non-covalently bound reporter units. These dual responses are correlated to ascertain the effect of enzymatic action on micellar aggregates and the consequential guest release. The release of the guest molecule is conveniently tuned by stabilizing the micellar aggregates through photochemical crosslinking of hydrophobic coumarin units. This photo-crosslinking is also utilized as a tool to investigate the mode of enzyme-substrate interaction in the context of aggregate-monomer equilibrium.

Keywords

coumarin; dendrimers; enzymes; micelles; photochemistry

Introduction

For supramolecular assemblies to have a broad impact, it is essential that they not only sequester guest molecules, but also release these bound guest molecules in response to a specific trigger. Among supramolecular assemblies, micelles have attracted great interest due to their ability to sequester lipophilic guest molecules in an aqueous environment.^[1] This ability, combined with the fact that micellar assemblies are often nanoscopic in size, renders these assemblies of interest in a variety of applications, including drug delivery.^[2] To this end, there have been several reports on stimuli-responsive micellar assemblies that respond to variations in pH, redox conditions, light, and temperature.^[3] Assemblies that respond to variations in enzymatic activity, though not as prominent in the literature,^[4] are also of great interest, because aberrations in enzymatic activity or protein concentration are the primary biological imbalances associated with many diseases.^[5]

Amphiphilic assemblies generated from macromolecules, such as polymers and dendrimers, exhibit added advantages as stimuli-responsive systems, since these assemblies exhibit low critical aggregate concentrations (CAC) and high inherent stabilities.^[6] Dendrimers are particularly interesting, allowing for precise control over molecular weights, thus providing a unique opportunity for developing fundamental structure–property correlations.^[7] Enzyme-responsive dendrimers have been reported, wherein an enzyme-induced cleavage of

a bond triggers a cascade of events that results in the covalent disassembly of the dendritic molecules.^[4b,8] In these cases, the molecules, to be released in response to the enzymatic reaction, are covalently attached to the dendrimer. It is interesting to develop strategies in which there is no necessity for covalently modifying the guest molecules to be released, as they allow for using a broad range of lipophilic guest molecules. Our research group has recently reported on such a possibility using our facially amphiphilic dendrimers as the host scaffold.^[9] In this system, the guest molecules are non-covalently sequestered within the dendrimer assembly, and are then released in response to an enzymatic reaction, because of the change in the hydrophilic–lipophilic balance (HLB) caused by the cleavage of the substrate functionality. In that preliminary communication, we have demonstrated some degree of control over the guest release that can be obtained by varying the dendron generation. However, the tunability in the release rate was relatively limited. Thus, we have been interested in developing an approach where systematic control over guest-molecule release can be conveniently achieved. Herein, with the aid of photochemical reactions, we demonstrate that the tunability in the guest-molecule release can be achieved by controlling the availability of the substrate functionalities in the dendrimers to the enzymes. We arrived at this strategy by seeking a correlation between the enzymatic reaction that causes the amphiphilic dendrimer to lose its hydrophilic-lipophilic balance and the release of non-covalently encapsulated guest molecules. We detail our findings on both of these aspects in this manuscript.

Results and Discussion

Molecular design and synthesis

To understand the correlation between the extent of enzyme-induced bond cleavage and the guest-molecule release, it is important that we introduce a reporter element in our dendrons, in which the cleavage reaction is reliably analyzed along with the release of the non-covalently encapsulated guest molecules (Figure 1). Fluorescence is a convenient and sensitive technique for this purpose. In our molecular design, it is essential that the enzyme-induced cleavage reaction disconnects the lipophilic component of the amphiphilic dendrimer thus resulting in a more hydrophilic functionality at the dendron end. This change forms the basis for the stimulus-induced change in HLB, which will result in the release of the guest molecules. Therefore, we searched for a lipophilic fluorophore that is rendered non-fluorescent when attached to the dendron, but becomes fluorescent upon liberation from the dendritic backbone due to the enzymatic reaction. The hydroxycoumarin derivative, 4-methylumbelliferone (MUF), is highly fluorescent; however, the ester derivative of this molecule is not fluorescent.^[10] Therefore we sought to utilize the esterase-induced cleavage of the ester functionality in molecule **A** (Figure 2), as the fluorescence reporting event. However, we found the hydrolytic stability of the phenolic ester in **A** to be poor even in the absence of the enzyme. Since it has been reported that the alkoxy coumarins are also non-fluorescent, we utilized the acetal-modified coumarin ester **B**. In this case, the esterase-induced cleavage of the ester would produce a hemiacetal, which is expected to decompose in situ to afford the fluorescent molecule MUF. When **B** was subjected to an enzymatic cleavage reaction, the solution indeed turned highly fluorescent with time, indicating the formation of MUF.

We envisaged the use of the 1,3-dipolar Huisgen cycloaddition reaction, popularly referred to as click reaction, to attach the fluorophore precursor to the dendritic backbone. Accordingly, our target dendron structure is shown as structure **1** (Scheme 1). In this structure, the pentaethyleneglycol unit forms the hydrophilic functionality of the dendritic molecule, while the coumarin derivative constitutes the lipophilic component. We anticipated that the enzymatic cleavage of the ester bonds in dendron **1** by porcine liver esterase (PLE), should afford the carboxylic acid based dendron product **2** that contains

hydrophilic functionalities at either face of the dendron and thus would lose its ability to non-covalently sequester hydrophobic guest molecules (Scheme 1).

Synthesis of molecule **1** was approached in a modular fashion. The dendritic precursor to target molecule **1** is represented by molecule **5** in Scheme 2. Molecule **5** was assembled from the biaryl building block unit **3** and peripheral unit **4**. We have previously reported the synthesis of the biaryl building block **3** containing the pentaethyleneglycol and propargyl functionalities.^[9] The reaction between **3** and **4** was carried out under potassium carbonate conditions to obtain **5** which was then treated with the acetal-functionalized coumarin **6** containing an azide moiety to obtain the targeted dendron **1** in 70% yield under click chemistry conditions.^[11] Compound **6** was obtained from a reaction between MUF, dibromomethane, and 6-azidohexanoic acid in 36% yield (Scheme 2).

Characterization of the assembly

We were first interested in confirming the micelle-type assemblies formed by G1 dendron **1**. We have previously reported that the most drastic change in the critical aggregation concentration (CAC) occurred from the monomer to G1-dendron; the CAC gain from the G1 to G2 dendron was relatively small.^[4f,9,12] Therefore, we focused on the simpler G1 dendron for the studies outlined here. It is essential, however, that we confirm that the CAC for the G1 dendron **1** synthesized here is indeed in the micromolar range. We decided to determine the CAC of **1** by using the same lipophilic molecule that will be used for observing the non-covalent guest release. To concurrently analyze the release of the non-covalently sequestered guest molecule, it is essential that the photophysical features of the guest dye molecule are very different from that of the coumarin derivatives. Accordingly, we chose 1,1'-dioctadecyl-3,3,3',3'-tetramethylindolyl carbocyanine perchlorate (DiI) as the guest molecule, which absorbs at 530 nm and emits at 573 nm. DiI is a hydrophobic molecule and is therefore not soluble in water by itself. However, when it is embedded in the hydrophobic interior of the micelles in aqueous solution, a finite concentration of this dye molecule would be present in the aqueous solution (Figure 3a).

The CAC for the assembly was determined from the plot of fluorescence response of DiI as a function of the dendron concentration.^[3c] A sudden change in the emission intensity of DiI was observed around 9.40 μM concentration of G1; this can be attributed to the onset of micelle formation (Figure 3b). To confirm the formation of the micellar assemblies, dynamic light scattering (DLS) experiments were conducted at 10 μM concentration of G1 dendron **1**. The excellent correlation function obtained in these measurements suggests that these micellar aggregates have an average hydrodynamic radius of 120 nm and a narrow polydispersity (Figure 3c). Note that this is a rather large size for a micellar assembly from these dendrimers. We believe that these are micelle-type aggregates, rather than a classical micelle. The reason for suggesting that these are indeed micelle-type aggregates is that these sequester lipophilic guest molecules and the lipophilicity of their interiors is similar to that observed in classical micellar assemblies (when tested using fluorescent probes).^[3h,4f,9] The studies, outlined below, were all conducted at 25 μM concentration of G1 dendron **1** (well above its CAC) in 50 mM HEPES buffer at pH 7.2. At this concentration, the dendrons assemble to form the micelle-type aggregates of similar size even in the buffer, as confirmed by DLS. The formation of the assemblies was also confirmed in the dry state by TEM (Figure 3d).

Enzyme sensitive behavior of dendrimer assemblies

One of the main objectives of this study is to understand the correlation between the guest-molecule release and the extent of enzymatic cleavage. To realize this objective, it is essential that we first understand the rate of enzymatic cleavage alone, before we test its

correlation with the rate of guest-molecule release. To accomplish this, we have designed our dendrimer such that the enzymatic cleavage of the substrate will result in liberation of the fluorescent MUF. As a proof of concept, the enzyme sensitive nature of these dendrimers was tested by treating 25 μM dendrimer with 0.02 μM of the enzyme porcine liver esterase. The extent of enzymatic cleavage was then measured by monitoring the fluorescence of the enzyme-cleaved MUF by exciting it at 365 nm. The fluorescence intensity of MUF increased constantly with time and reached a saturation point at about 400 min (Figure 4a). The fluorescence turn-on from this reaction was evident even upon visual inspection of the solution (Figure 4 b). This result confirms that this dendrimer is indeed sensitive to the enzyme and also that the extent of enzymatic cleavage can be monitored through the fluorescence turn-on of MUF.

The MUF fluorescence not only provides a firsthand report of the enzymatic event, but also indicates the extent of imbalance brought into the micellar aggregate. This is because when the enzyme cleaves the hydrophobic substrate, the lipophilic termini are converted to carboxylic acid functionalities, thereby affecting the HLB. This resultant change in HLB can be observed by conveniently monitoring the release of the pre-encapsulated (non-covalently sequestered) guest molecules. To test this hypothesis, guest molecule DiI was non-covalently encapsulated in a 25 μM dendrimer solution in 50 mM HEPES Buffer. This dendrimer solution was treated with increasing concentrations (0.005 μM , 0.01 μM , and 0.02 μM) of PLE and the release of MUF and DiI were monitored by exciting at 365 nm and 530 nm, respectively. It can be noted from Figure 5 that the percentage release of both MUF and DiI increased proportionally with the enzyme concentration. This indicates that the change in HLB (indicated by DiI release) is indeed directly proportional to the extent of enzymatic cleavage (indicated by the MUF release), thereby suggesting a clear correlation between these two processes. Also, though enzymatic cleavage affects the HLB and causes the release of non-covalent guest molecule (DiI), it should be noted that there exists a residual assembly from the product dendron, as observed by DLS. Albeit with much lesser efficiency, due to the altered HLB, these residual aggregates are capable of retaining some of the guest molecules, as seen from the incomplete release of the DiI.

Photo-crosslinking of the dendritic micelles

While the observed difference in release rate can be attributed to differences in the enzymatic activity, it is desirable that we exert control over the extent of enzymatic reaction and the ensuing guest-molecule release, based on the inherent molecular characteristics of the supramolecular assembly. The difference in dendron generation has been shown to provide some control over release kinetics,^[9] but it does not exhibit the level of systematic control desired. To develop such a possibility, we first examined the two limiting mechanistic possibilities for the enzymatic reaction upon the substrate containing dendrons: 1) the enzyme transiently penetrates into the micellar interior to access the lipophilic substrate functionality or 2) the enzyme gains access to the substrate through the monomer–aggregate equilibrium, in which the monomeric state of the dendrons provides clear access to the substrate functionalities. We conceived that it is unlikely that the enzyme will access the lipophilic interior of a rather large micellar aggregate since the energetic penalty for such a step would be high. Therefore, scenario 2 (i.e., presentation of monomeric dendron through monomer–aggregate equilibrium) seems to be a more reasonable pathway for the observed enzyme-based guest release. With this assumption, we hypothesized that limiting the availability of the monomeric aggregates in solution should significantly affect the enzymatic reaction and thus the release kinetics.

One possible way of limiting the availability of the dendron in its monomeric state in solution would involve crosslinking the micellar interior in its aggregated state. As the degree of crosslinking increases, the availability of the dendrons in their monomeric state

should decrease. We show three different scenarios in Figure 6: 1) no crosslinking, 2) low crosslinking density, 3) high crosslinking density. We decided to utilize the coumarin functionality to controllably crosslink the aggregates formed by the dendron. Photodimerization of coumarin functionalities by [2+2] cycloaddition reaction is well known^[31,13,14] (Figure 7 a). For effective crosslinking of these aggregates, we need at least three coumarin functionalities per molecule involved in the assembly. Molecule **1** is the simplest dendritic structure that presents three units. It is also interesting to note that the presence of only three units presents the opportunity to more precisely correlate the extent of the reaction with dendrons available in the monomer–aggregate equilibrium. In the case of dendrons with a higher number of coumarin units, a small percentage of reaction can cause extensive crosslinking of the overall structure. This is another reason for our choice of a simple G1 dendron for these studies (in addition to the observation of the best CAC gain upon going from G0 to G1, rather than from G1 to G2).

Crosslinking was achieved by irradiating a solution of **1** at 365 nm. It is known that hydrophobic environments greatly enhance the rate of coumarin dimerization.^[14] Since this is the case with our system, we anticipated the crosslinking reaction to be efficient. We observed that the required irradiation times for crosslinking are rather short and that the extent of crosslinking can also be tuned by simply varying the irradiation time. As shown in Figure 7b, the absorption peak at 320 nm, corresponding to the coumarin units in **1**, decreases with increasing irradiation time and reaches saturation at approximately 30 min of irradiation. The extent of crosslinking at different irradiation times was estimated using the initial absorbance at 320 nm (that is, at $t=0$ with no irradiation) as 0% crosslinked and the absorbance at saturation as 100% crosslinked (that is, at $t=30$ min of irradiation; see Figure S1 in the Supporting Information).

To test if the observed photochemical transformation had resulted in crosslinking of the micellar aggregates, the stability of these nanostructures was tested. It is known from our previous studies that these facially amphiphilic dendrimers tend to lose their ability to aggregate, when subjected to a non-aqueous polar medium such as dimethylformamide (DMF).^[15] Thus, the stability of the dendritic aggregates formed from the non-crosslinked dendron **1** and crosslinked dendron solutions with varying percentages of DMF were investigated using DLS. As expected, the non-crosslinked dendron solutions exhibited very poor correlation function in DLS and a broad PDI with just 20% of DMF, confirming the instability of the amphiphilic aggregate in this medium (Figure S2 in the Supporting Information). On the other hand, the crosslinked dendritic solutions (all solutions had a final concentration of 25 μM of the dendron **1** with varying DMF/H₂O ratio) did not lose their aggregation behavior even at 60% DMF. In addition, the DLS also showed excellent correlation function and a narrow PDI, independent of the percentage of DMF. These observations confirm that the photochemical irradiation indeed crosslinks the dendritic micellar interiors. As would be expected of a crosslinked nanostructure, these crosslinked particles did show a linear increase in size with increasing percentage of DMF (Figure 7 c) which might be due to swelling.

Upon validation of the variation in crosslinking density based on the irradiation time, we were interested in testing whether there would be a differential availability of the dendrons for enzymatic reaction. Accordingly, we subjected a 25 μM solution of the dendrons to photochemical irradiation (365 nm) for different times to obtain the nanoaggregates with 0, 20, 52, and 87% crosslinks (Figure 8). First, we tested the release of MUF in response to the enzyme and observed a clear correlation between the extent of crosslinking and MUF release from these dendritic assemblies. For instance, only a small percent release of MUF was observed with 87% crosslinked nanoaggregates even after 6 h, while 80% of MUF was released with uncrosslinked nanoaggregates. This observation has an important caveat; note

that if the enzyme molecules were to access the interior of the dendron and cleave the ester bonds of the dimerized coumarin, then the fluorescence from MUF would not be observed, since the hydroxy version of the dimerized coumarin does not fluoresce at this wavelength. This observation simply suggests that the extent of MUF release is controlled by different irradiation times. However, if we concurrently examine the release of the noncovalently sequestered guest molecule, DiI, further insights can be gained.

Note that if the enzyme were able to access the interior of the micelle and cleave the ester bonds, this cleavage reaction would simultaneously uncrosslink the dendritic aggregates and thus cause changes in HLB, independent of the crosslinking density. These changes in HLB should then cause the amphiphilic aggregates to release the non-covalently sequestered guest molecules. On the other hand, if the enzymes do not have access to the interior of these particles, there should be a clear correlation between the previously observed MUF release and the release of the non-covalently sequestered DiI molecules. To differentiate these possibilities, we examined the release of DiI molecules from these assemblies by subjecting the 25 μM solutions of **1** to PLE. Here also, a very small percent release of DiI was observed in the case of the 87% crosslinked structure in 6 h, compared to about 83% release from the uncrosslinked structure over the same time period. The structures with intermediate crosslink densities exhibit intermediate release profiles. These observations support our assertion that the enzyme does not have access to the interior of these assemblies. The utilization of the monomer–aggregate equilibrium to execute the enzymatic reaction and thus affect the HLB of the dendrons in solution seems to be the most reasonable alternate mechanism.

This observation can be further augmented by reversibly enriching the monomer to aggregate concentration through the decrosslinking of crosslinked aggregates. This should then allow more accessible dendrons (in monomeric form) to the enzyme, and thereby show enhanced guest release. The decrosslinking of crosslinked aggregates was achieved through photochemical irradiation of the crosslinked aggregates at 250 nm wavelength. However it is known from the literature^[16a] that irradiation at 254 nm not only causes photocleavage of dimerized coumarin but also the photodimerization and finally reaches the equilibrium (photostationary state). This limits the recovery of the uncrosslinked dendrons to a maximum of about 50%.^[16] The extent of decrosslinking at (250 nm) was calculated by considering the absorbance at 320 nm before and after irradiation (365 nm) as 100 and 0%, respectively (see Figure S3 in the Supporting Information).

As the decrosslinking efficiency was limited to approximately 50%, it is important to note that significant number of crosslinkers are still intact in the crosslinked aggregate and these should be sufficient for retaining the majority of trapped guest molecules. To test this scope, dye-release experiments were conducted on three sets of 25 μM G1 dendrimer solutions: 1) no crosslinking, 2) crosslinked, 3) decrosslinked following initial crosslinking. The absorption spectra depicting these three cases are shown in Figure 9a; the re-emergence of the peak at 320 nm wavelength corresponds to the decrosslinking of the dimerized (crosslinked) MUF. These solutions were then subjected to the enzyme PLE and the dye release was monitored. In the case of covalently attached MUF, the non-crosslinked and crosslinked samples showed 90% and 5% release respectively, whereas about 20% release was observed in the case of decrosslinked sample (Figure 9b). A similar trend was observed for the non-covalently encapsulated DiI (Figure 9c). This observation therefore further validates that the enzyme does access the substrate in the monomeric form, provided through the monomer aggregate equilibrium. However, it is also important to note another possibility in which an individual monomer unit (present in the aggregate) transiently presents the lipophilic units at the surface of the aggregate, during which the enzyme can

accesses its substrate functionality. At this time, we are unable to distinguish this possibility with the one based on the monomer–aggregate equilibrium.

Conclusion

We have designed and synthesized a dendritic molecule containing lipophilic fluorescent precursor functionality, which can self-assemble in aqueous solution to form nanoscopic micelle-like aggregates. Subjecting these dendrons to an enzymatic reaction releases a fluorophore, which has been utilized to monitor the release rate of the lipophilic fluorophore from the dendritic backbone. Since this enzymatic reaction also causes a change in the hydrophilic–lipophilic balance in the dendrons, the amphiphilic supramolecular nanostructures lose their micellar nature in response to the enzyme stimulus. Monitoring the release of non-covalently sequestered guest molecules during the deformation of the assembly suggests that there is a clear correlation between covalent bond cleavage and guest-molecule release. This observation led us to test the possibility of utilizing these dendrons for controlling the release of the guest molecules by limiting the extent of dendron availability in a monomer–aggregate equilibrium. We utilized the photochemical dimerization of coumarin to test this possibility. Indeed, we observed that the extent of guest-molecule release can be precisely controlled by manipulating the extent of crosslinking in these nanoassemblies. These observations also allowed us to rule out the possibility of enzymes accessing the interior of the micellar aggregates to execute the enzymatic reaction. We believe that the molecular-design strategies that emerge from these observations can impact a variety of areas, particularly those involving controlled molecular release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of enzyme induced release of covalently attached reporter units and non-covalently encapsulated guest molecules.

Figure 2.
Coumarin-based substrates for enzymatic cleavage.

Figure 3.

Micelle-type assembly. a) Emission spectrum of an aqueous solution of DiI in the presence and absence of G1 dendron **1** (λ_{ex} =530 nm), b) CAC calculation for **1** by plotting fluorescence intensity of DiI vs. concentration of G1 dendrimer (λ_{ex} =530 nm; λ_{em} =573 nm), c) size of the assembly determined by DLS at 10 μ M concentration of **1**, d) TEM image of G1 dendron **1** confirming formation of assemblies.

Figure 4. Turn-on fluorescence of coumarin (MUF) following the enzymatic action represented by a) emission spectra ($\lambda_{\text{ex}}=365$ nm), b) photograph showing a visual evidence of the fluorescence turn-on upon substrate cleavage.

Figure 5.
Enzyme concentration dependent release of a) MUF, and b) DiI.

Figure 6. Schematic representation, showing the effect of aggregate–monomer equilibrium on enzymatic action.

Figure 7.

a) Reversible photodimerization of coumarin units (dimerized structure inferred from decrease in absorbance at 320 nm^[16a], b) UV absorption spectra indicating the increase in crosslinking density with increasing irradiation (365 nm) time, c) plot showing the swelling behavior of the crosslinked assemblies at 25 μM dendrimer concentration by varying the percentage of DMF.

Figure 8. Temporal release of a) MUF and b) DiI (normalized), at different crosslinking densities.

Figure 9. Decrosslinking and the dye-release studies. a) Absorption spectra showing partial recovery of non-crosslinked coumarin, b) time dependent MUF release, and c) DiI release.

Scheme 1.
Enzymatic action on target dendron 1.

Scheme 2.
Synthesis of target dendron **1**.