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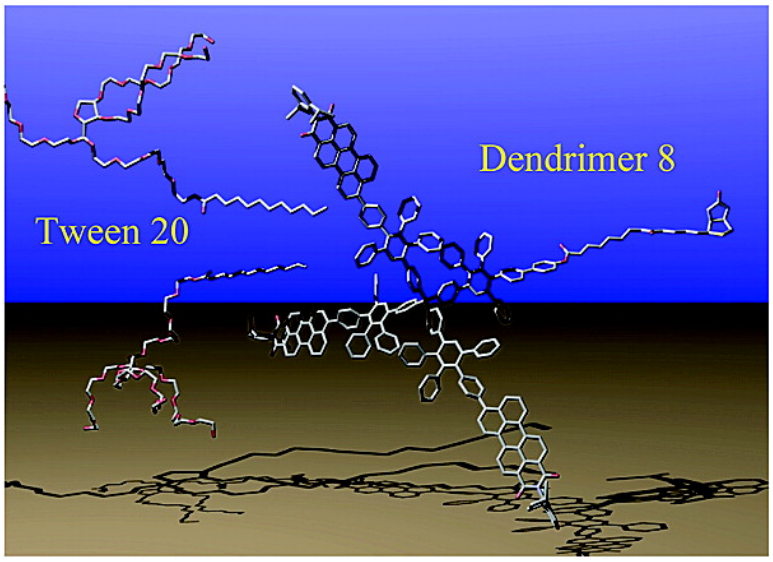
J. Am. Chem. Soc., **2003**, 125 (19), 5832-5838• DOI: 10.1021/ja0285058 • Publication Date (Web): 16 April 2003



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A Polyphenylene Dendrimer−Detergent Complex as a Highly Fluorescent Probe for Bioassays

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Abstract: The synthesis of a polyphenylene dendrimer carrying three perylenemonoimide dyes as well as one biotin group is presented. Due to the hydrophobic polyphenylene scaffold, this dendrimer is insoluble in water thus preventing investigations in aqueous media. However, the use of an appropriate detergent results in the formation of well-defined supramolecular dendrimer−detergent complexes being soluble in aqueous media. The dendrimer−detergent complexes have a constant hydrodynamic radius of 7.1 nm measured by light scattering and fluorescence correlation spectroscopy and exhibit a high stability in the presence of blood serum proteins. The specific binding of the dendrimer−detergent complexes carrying a single biotin group to the protein streptavidin is demonstrated using a magnetic bead assay.

Introduction

In recent years, there has been an increasing interest in the development of new materials for diagnostics and sensoring technology.[1] Especially, the rapid progress in molecular biology and herein in the field of genomics[2,3] and proteomics[4] increases the demand for a technique where very low concentrations of an analyte down to the recognition of a single molecule can be detected. In this context, assays based on fluorescence probes are frequently used, since this approach provides low detection limits with the setup being relatively inexpensive.[5] So far, a single fluorescent dye can be recognized down to nanomolar concentrations. Since the sensitivity of a fluorescence assay often directly correlates with the number of chromophores, multichromophoric arrangements such as fluorescent bioconjugates[6] have been investigated in view of a reduction of the detection limit. Although these systems have shown a higher fluorescence intensity and thus an increased capacity in detecting lower analyte concentrations than their monochromophoric analogues, the unknown composition, for example, the number of chromophores of such aggregates, often prevents a calibration thus making it impossible to quantify the concentration of the analyte. Therefore, an "ideal" fluorescent probe should bear a large but also distinct number of chromophores as well as a single biologically active group.

With regard to their defined size as well as the presence of large amounts of substituents at their periphery, cascade molecules such as dendrimers[7−9] are interesting candidates for applications in medicine and diagnostics.[8,10] Recently, the detection of proteins by using radiolabeled dendrimers[11] or the visualization of tumor cells with dendrimers bearing gadolinium ions[12,13] has successfully been demonstrated by using appropriately functionalized dendrimers. Up to now, only dendrimers carrying one type of functionality at the periphery have been investigated for biological applications. Therefore, especially dendrimers bearing signaling sites, for example, chromophores or radioactive groups as well as one biologically active recognition site represent attractive candidates as highly sensitive and selective probes for bioassays.

In this article, we will present the synthesis of a polyphenylene dendrimer bearing three chromophores as well as a single biotin group (vitamin H) at the periphery. The synthesis of unsymmetrically substituted polyphenylene dendrimers is based on a synthetic procedure which has been described previously.[14] We chose biotin as a bioactive group, since it can be easily attached to a dendrimer and it interacts with the protein streptavidin with a high affinity constant ($K_a \approx 10^{13}$ M^{-1}).[15,16]

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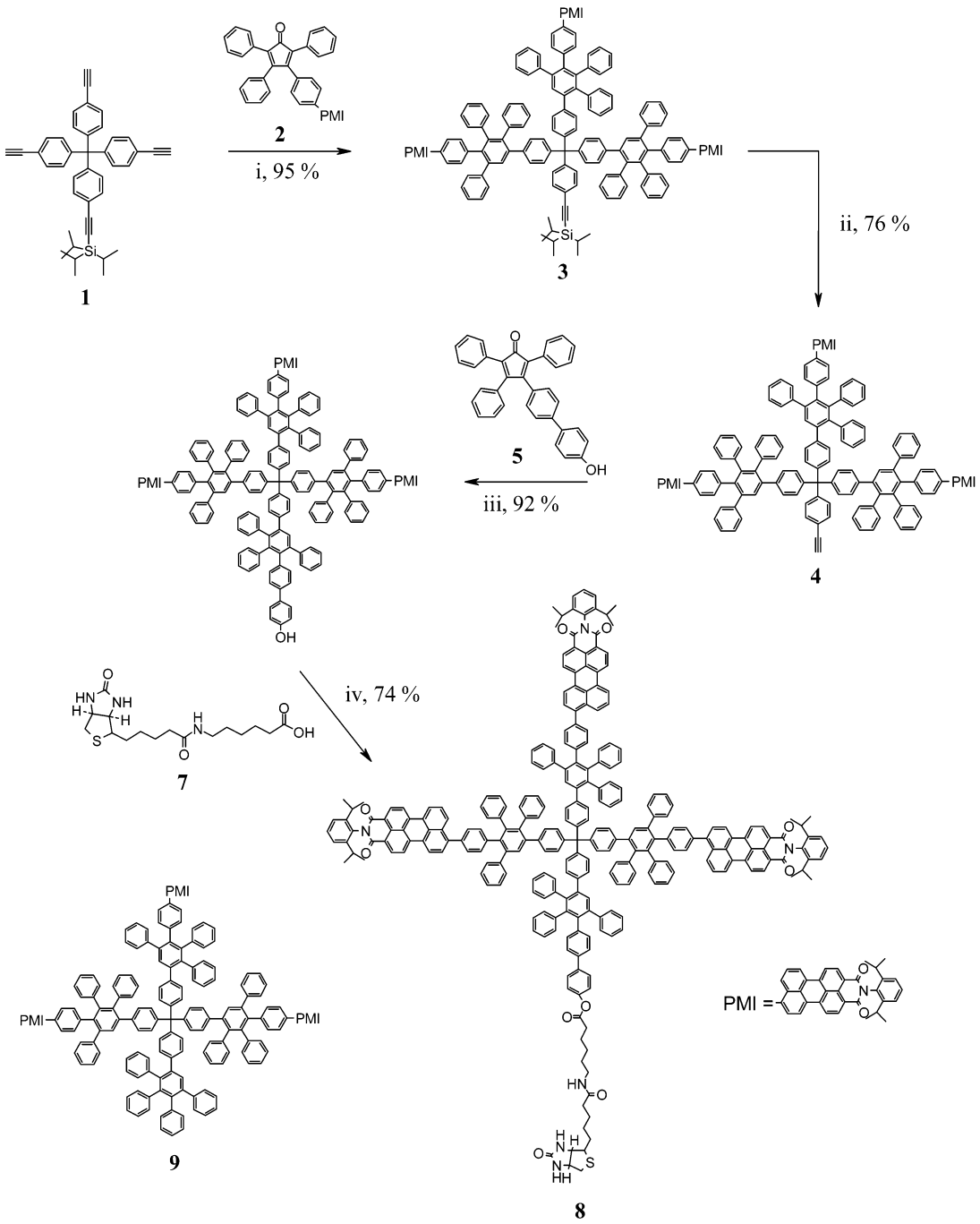
10.1021/ja0285058 CCC: $25.00

Scheme 1[a]



[a] (i) 3.75 equiv of **2**, *o*-xylene, 12 hr; (ii) 2 equiv of NH_4F, Bu_4NF (catalytic), THF, 2 hr; (iii) 1.25 equiv of **5**, *o*-xylene, 170 °C, 12 hr; (iv) 3.3 equiv of biotin-ACS **7**, 3.5 equiv of EDC, DMAP (catalytic), DCM/DMF (3:1), rt, 7 days.

As chromophores, we chose highly fluorescent and extremely photostable perylenedicarboximide chromophores[17] (PMI, Scheme 1) which already have been applied successfully as fluorescent probes in single molecule spectroscopy.[18,19] Therefore, their use should in principle facilitate the realization of bioassays even at the single molecule level. However, PMI chromophores as well as polyphenylene dendrimers are both highly hydrophobic molecules and, therefore, not soluble in aqueous media. Since the solubility in water is a key concern to accomplish bioassays, we focused on applying an appropriate detergent to solubilize the dendrimer in water. We strongly believe that dendrimers combining multifunctionality as well as bioactivity and water solubility have a high potential as sensitive probes for investigations in a biological environment including those down to the single molecule level.

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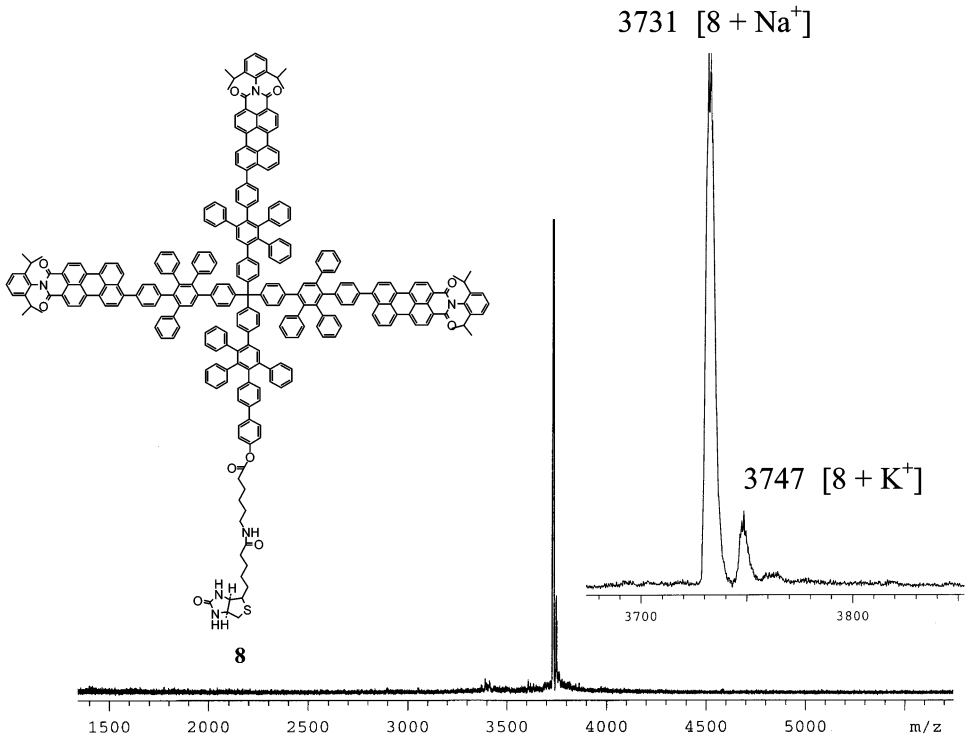

Figure 1. MALDI-TOF mass spectrum of **8** (calculated molecular weight, MW = 3708 g/mol; matrix, dithranol).

Results and Discussion

Synthesis and Characterization of a Fluorescent, Biologically Active Polyphenylene Dendrimer. The synthesis of asymmetrically substituted polyphenylene dendrimers is based on a convergent growth approach[14] and requires a partially protected core molecule which has already been described in the literature.[14] The *Diels−Alder* cycloaddition of a tetraphenylmethane core **1** bearing three free ethynyl groups as well as one tri*iso*propylsilyl-protected ethynyl group with 4.5 equiv of a cyclopentadienone carrying one perylenedicarboximide chromophore (PMI, Scheme 1) gave the asymmetrically grown polyphenylene dendrimer **3**. After column chromatography, **3** was obtained in nearly quantitative yield. Subsequently, the remaining tri*iso*propylsilyl group was cleaved by using 2 equiv of ammoniumfluoride and catalytic amounts of tetrabutylammoniumfluoride in tetrahydrofuran to give **4** in 76% yield. A further cycloaddition of **4** and 1.5 equivalents of cyclopentadienone **5** yielded a first generation polyphenylene dendrimer **6** bearing three PMI chromophores as well as one single hydroxyl group at the surface. Since a longer spacer between biotin and a given substrate improves the binding constant of biotin and the protein avidin,[20] the commercially available biotinyl-6-aminocapronic acid (**7**) was attached to dendrimer **6**. This reaction proceeded by applying *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and catalytic amounts of *N*,*N*-dimethylaminopyridine (DMAP) in dimethylformamide and methylenchloride (DCM, 3:1). After 7 days, the reaction mixture was washed several times with water and purified in dimethylformamide (DMF) via dialysis. Via this reaction sequence, dendrimer **8** carrying three chromophores as well as one biotin group was obtained as a bright red solid in 74% yield. All molecules reported herein were characterized by applying NMR spectroscopy and mass spectrometry confirming the structural perfection and monodispersity of each molecule (Experimental Section).

To exclude unspecific binding of compound **8** to streptavidin, the synthesis of a dendritic model compound bearing three PMI chromophores but no biotin unit is crucial. Compound **9** in Scheme 1 represents such a model compound whose synthesis and characterization have already been published.[14]

Figure 1 reveals a MALDI-TOF mass spectrum of dendrimer **8** in the presence of a sodium salt. Only one main peak is detected which corresponds to an [**8** + Na^+] cluster. The second peak at 3747 g/mol can be attributed to an [**8** + K^+] cluster being formed due to the presence of small amounts of K^+ during the sample preparation.

Solubilization of the Dendrimer in Water. Dendrimer **8** is well soluble in organic solvents such as dichloromethane (DCM) and tetrahydrofuran (THF) but it shows only a limited solubility in methanol and no solubility in water. For the solubilization of dendrimer **8** in aqueous media, several commercially available detergents have been investigated. In general, an appropriate detergent should solubilize dendrimer **8** in a way that no aggregates are formed. Since it is often difficult to distinguish a homogeneous solution from a solution bearing small aggregates, we used laser scanning microscopy (LSM) and fluorescence correlation spectroscopy (FCS) to evaluate the solubilization behavior of each detergent. Table 1 in the Supporting Information gives an overview over different detergents as well as their *critical micellar concentration* (CMC)[21,22] and the aggregation behavior of dendrimer **8**. Nonionic detergents such as *Tween 20* and *Tween 60* showed the most promising solubilization behavior. *Tween 20* (poly-

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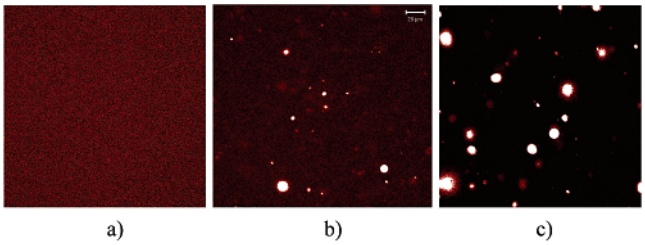

Figure 2. Fluorescence micrographs of dendrimer **8** solutions in the presence of decreasing amounts of detergent (concentration of the dendrimer, $c = 50$ nM) with a molar ratio *Tween 20*/dendrimer **8** of (a) 11600, (b) 2910, and (c) 580.

oxyethylenesorbitan monolaureate) gave the best results leading to the formation of a homogeneous dendrimer solution which might be explained by its flexible hydrophobic tail having an optimal length to fit into the hydrophobic pockets of the dendrimer.

Since it is our concern to study the suitability and stability of dendrimers incorporated in micelles for their application in a biotin-based assay, we applied three buffer solutions (composition see Supporting Information) typically used in biotin-streptavidin technology. All three buffers in combination with the detergent *Tween 20* could be applied to homogeneously solubilized dendrimer **8**. A detailed solubilization procedure is given in the Supporting Information.

To investigate the CMC[21,22] of *Tween 20*, a titration experiment was realized by using a constant concentration of dendrimer **8** (50 nM) and a varying concentration of the *Tween 20* detergent from 1.45×10^{-5} to 1.8×10^{-3} M. Figure 2 shows three fluorescence images of the solubilized dendrimer **8** in buffer B. At a detergent concentration of 5.8×10^{-4} M, no aggregates were detected (Figure 2a). At this point, the concentration of *Tween 20* was higher than its *CMC* indicating that "empty" micelles of *Tween 20* as well as dendrimers surrounded by *Tween 20* coexist in solution. A further decrease in the concentration of *Tween 20* from 1.45×10^{-4} to 1.45×10^{-5} M rapidly increased the amount and the size of the aggregates (Figure 2b,c).

Since the dendrimer carries highly fluorescent chromophores, fluorescence correlation spectroscopy (FCS) is a straightforward method which was used to study the size of the fluorescent probes.[23,24] Figure 3a depicts the experimentally determined correlation function $G(\tau)$ as a function of the diffusion time of the particles in the observed volume.

The data evaluation, as described in the Supporting Information, reveals that the solution exhibits two distinct populations consisting of a fast and a slow diffusive component. Figure 3b shows that the fast component exhibits a constant diffusion time of 168 μs over the entire concentration range, which corresponds to a hydrodynamic radius of 7.1 nm. Above concentrations of 1.7×10^{-4} M of *Tween 20*, the slow component reveals a diffusion time of 2470 μs indicating a hydrodynamic radius of 80 nm. The influence of both components on the correlation function is dependent on the emission intensity of the particles,[25] which is approximately proportional to the volume of the particles. Therefore, we conclude from the data evaluation that

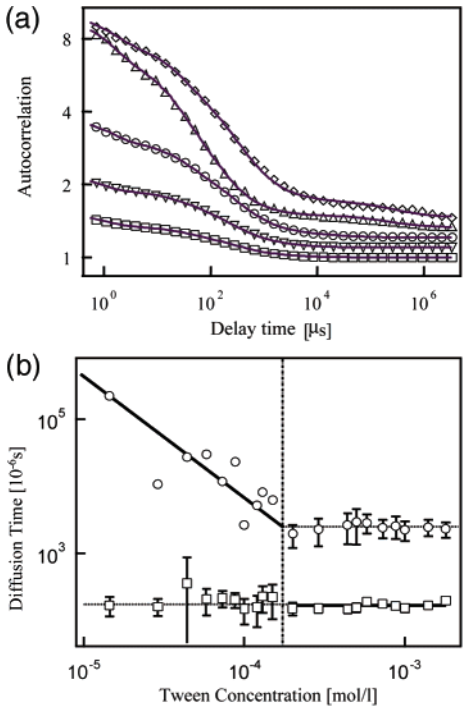

Figure 3. (a) Fluorescence time autocorrelation $G(\tau)$ of particles at different *Tween 20* concentrations: (△) 2.9×10^{-6}M, (◇) 1.45×10^{-5}M, (○) 8.75×10^{-5}M, (▽) 8.8×10^{-4}M, (×ff□) 1.8×10^{-3}M. The solid lines correspond to fits with two diffusion modes. For presentation reasons, each curve has an offset of 10%. (b) Diffusion times in relation with the concentration of *Tween 20*: (○) slow mode, (×ff□) fast mode. Above 1.7×10^{-4} M, the faster mode with an average diffusion time of $\tau_D = 168$ μs dominates the autocorrelation function with nearly 100%. Below this transition concentration, the slower population is more significant.

the ratio between the slow and fast component, at this concentration range, is below 6×10^{-7}. At detergent concentrations below 1.7×10^{-4} M, a divergence of the slow component diffusion time is observed corresponding to aggregates with a hydrodynamic radius of 80 nm and higher.

In this context, the fast diffusion time corresponds to a nonaggregated dendrimer−detergent complex, while the slow diffusion time corresponds to aggregated complexes. These experimental results can be explained by a transition from aggregated toward nonaggregated complexes. This transition occurs at detergent concentrations of 1.7×10^{-4} M, thus leading to a molar ratio (detergent/dendrimer) of 3400. At lower *Tween 20* concentrations, the detergent molecules were unable to completely solubilize the dendrimer in aqueous solution leading to the formation of aggregates.

To understand the aggregation behavior of the dendrimer, it is further important to investigate the dependence of the concentration of aggregates on different concentrations of dendrimer **8**. For this experiment, an initial solution of the detergent (2.9×10^{-4} M) has been used. This solution has then been diluted by using buffer C to adjust different dendrimer concentrations. Figure 4 reveals the relation of the number density of particles and the concentration of dendrimer **8** in buffer C.

One can clearly see a region in the range between 20 and 1000 nM where the number density of particles increases linearly with the concentration of the dendrimer. This observation can be explained by the fact that only one dendrimer is isolated inside the micelle under given conditions.

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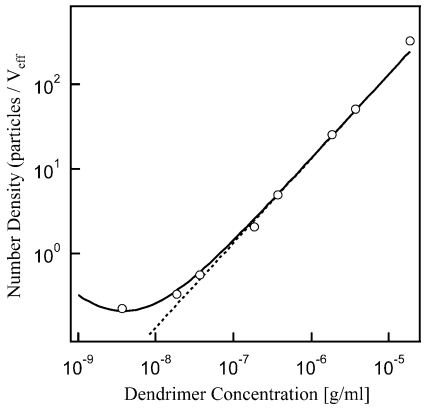

Figure 4. Number density of particles (N/V_{eff}) versus the concentration of dendrimer **8** in buffer C measured by FCS. The dashed line correspondence to slope 1 indicates the proportionality of weighted dendrimer concentration and measured number density.

Size of the Dendrimer−Detergent Complex. The size of the dendrimer−detergent complex has been calculated by applying different techniques such as dynamic light scattering (DLS), fluorescence correlation spectroscopy, and molecular modeling. In each experiment, a concentration of the dendrimer of 50 nM in different buffers under standard conditions (see Supporting Information) has been used. According to light scattering measurements (Zetasizer, Malvern), the dendrimer−detergent complex has an average radius of 7.35 nm. By applying fluorescence correlation spectroscopy, we are able to calculate the hydrodynamic radius of the dendrimer−detergent complex in an indirect way from the specific diffusion constant and the diffusion time according to eq 3 given in the Supporting Information. According to FCS measurements, a radius of approximately 7.1 nm is obtained. Furthermore, the radius of the complex remains constant at concentrations of the detergent above 1.7×10^{-4} M (Figure 3) indicating that the micelles have a distinct and over a long-range constant size.

For a visualization of the arrangement of the detergent around the dendrimer, molecular mechanics calculations have been applied.[26] The calculated radius of the dendrimer is 2.9 nm being measured from the center of the dendrimer up to the top of the imide structure of the PMI chromophore. *Tween 20* detergent molecules have an approximate chain length of about 3.9 nm. Therefore, the radius of the dendrimer−detergent complex lies in the range of about 7 nm, which is in a good agreement with the experimentally obtained values (DLS, FCS). This clearly indicates that the supramolecular dendrimer−detergent complex should contain only one dendrimer in the interior. In Figure 5a and b, a visualization of such a dendritic micelle is depicted which reveals a possible arrangement of the molecules.

Optical Characterization. The PMI chromophores of the dendrimer can be regarded as a sensitive probe which reflects changes in the polarity of the environment as well as the formation of PMI aggregates via $\pi\pi$-interactions.[27,28] Therefore, optical measurements provide important information in view of the spatial isolation of the dendrimer inside the micelle as well as the formation of aggregates between different dendritic molecules. Absorption and emission spectra have been recorded in aqueous solution prepared according to the standard protocol described previously. Furthermore, the influence of the buffer solutions A, B, and C on the optical properties has been investigated. Structured UV spectra as well as sharp fluorescence spectra were obtained which strongly resemble the spectra of nonaggregated PMI chromophores in organic solvents such as toluene.[14,29] In contrast to a water-soluble PMI chromophore which shows only one absorption maximum,[28] the dendrimer−detergent complex displays two absorption maxima. In the emission spectrum of solubilized dendrimer **8**, only a small bathochromic shift is detected. Since the emission spectrum of aggregated PMI displays a strong shift of about 50 nm toward a longer wavelength, it is highly evident that only one dendrimer is located inside the micelle and that no aggregation takes place. Table 1 gives an overview over the spectroscopic data of dendrimer **8** in different solvents. The fluorescence quantum yields have been calculated by using dendrimer **9** as a reference whose quantum yield has already been reported in the literature ($\phi_F = 0.98$ in toluene).[29] Obviously, the dendrimer−detergent complexes reveal comparatively high quantum yields in the range of 57 up to 65% for each PMI chromophore thus indicating that the chromophores are effectively shielded from the aqueous environment and that no aggregation of the PMI chromophores occurs. Since a single dendrimer contains three PMI chromophores, the fluorescence intensity of the whole dendrimer is very high (the quantum yields given in Table 1 refer to one single PMI chromophore of the dendrimer). In this way, the fluorescence intensity of dendrimer **8** solubilized in aqueous solution is around 2.5 times higher in comparison to one rhodamine 6G chromophore ($\phi_F = 0.89$ in aqueous solution).

Stability of the Complex toward Protein Solutions. We have shown that dendrimer **8** could be dissolved in water by using the detergent *Tween 20* and that highly fluorescent micelles were obtained. However, the application of such micelles as fluorescent probes in a biological environment requires a sufficient stability in the presence of proteins or antibodies. Since detergents are often used to solubilize hydrophobic proteins, there exists a strong affinity between these molecules which might have an influence on the equilibrium of the dendrimer−detergent complex. Therefore, the optical spectra of the dendrimer complex in the presence of complexation agents such as EDTA, bovine serum albumin (BSA), immunoglobulin G, fibronectin, and human serum (male) were investigated. For these experiments, a standard solution (given in the Supporting Information) of the solubilized dendrimer was diluted with a solution consisting of 0.1 mg/mL of the aforementioned components. Thereafter, absorption and emission spectra of the solutions were recorded and compared with the spectra of a sample consisting of the dendrimer−detergent complex in the absence of protein. One can clearly see that the absorption and emission intensity only slightly decreases in the presence of the proteins (Figure 6). However, a distinct

(26) Polyphenylene dendrimer **8** carrying three PMI chromophores has been minimized by applying the MM+ force field implemented in the program package HyperChem 6.0 of Hypercube Inc. The minimization protocol of this type of molecules has already been described in the literature. Then, the detergent *Tween 20* has been optimized separately by using the same method.
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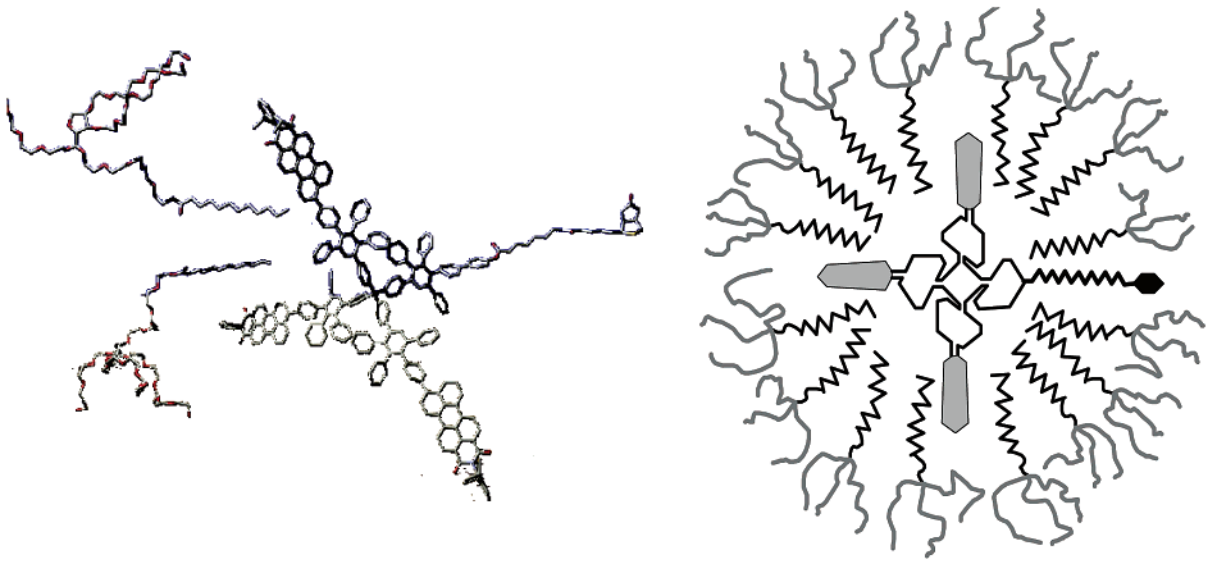

Figure 5. (a) Visualization of the dendrimer−detergent complex obtained via force field calculations. (b) Scheme of the dendrimer−detergent complex.

Table 1. Optical Data of Dendrimer **8** in Aqueous Solution (λ_{exc} = 488 nm)

	absorption maximum [nm]	emission maximum [nm]	fluorescence quantum yield [%]
8−Tween 20 in buffer A	501, 523	602	57 ± 0.1
8−Tween 20 in buffer B	500, 522	601	62 ± 0.1
8−Tween 20 in buffer C	500, 522	602	65 ± 0.1
8, DMF	517	580	90 ± 0.1
8, toluene	495, 520	560, 595	98 ± 0.1

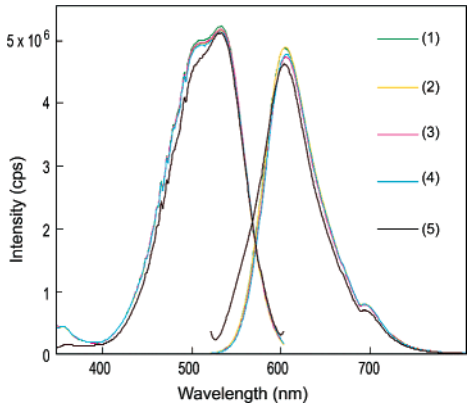

Figure 6. Fluorescence spectra of dendrimer−detergent complexes in buffer C (1) are almost unaffected in the presence of 0.1 mg/mL BSA (2), 0.1 mg/mL immunoglobulin G (3), and 0.1 mg/mL fibronectin (4). Small deviations are observable in case of human serum (5).

fluorescence signal is observed even after 24 h incubation in the presence of all of the herein investigated proteins which finally enables the use of such dendritic micelles in biological media.

Biological Activity: Binding of the Complex to Streptavidin. The stability of the micelles in the presence of proteins now permits us to investigate their binding to the protein streptavidin. For an interaction with the protein streptavidin, it is highly crucial that the biotin linker is situated at the outer sphere of the micelle, since the presence of the *Tween 20* layer could in principle prevent an interaction of the supramolecular complex with the protein. To investigate the binding capacity of the dendrimer−detergent complex, magnetic particles coated with streptavidin (Roche, Mannheim) have been used. These beads are nonfluorescent and have a size of about 1 μm. Their magnetic properties facilitate their isolation as well as their characterization. After the rinsing of 0.5 mg of the magnetic particles with buffer C, 50 μL of a solution of dendrimer−detergent complex at 22.4 μM have been introduced and stirred for various times (1, 4, or 24 h). In parallel, a control experiment has been performed by using dendrimer **9** carrying no biotin group.[14]

Thereafter, the particles were washed with buffer C, diluted, and analyzed by FCS and LSM. After incubation of dendrimer **9** bearing no biologically active group, only a very weak fluorescence is observed (Figure 7c) which can be due to only a weak tendency of the dendrimer to absorb on the surface of the magnetic bead. This phenomenon can be attributed to a non specific adsorption probably due to the detergent layer. However, after incubation of dendrimer **8** under the same conditions, fluorescent particles with a size of around 5 μm were obtained (Figure 7a,b). The observation of globular, fluorescent particles only in the case of dendrimer **8** proves that the biotin ligand is available for an interaction with the protein streptavidin.

After separation of the magnetic beads, FCS measurements on the dendrimer−detergent complex solution have been recorded. A decrease in fluorescence intensity of the supernatant solution has been observed which is in direct relation to the amount of molecules which have already reacted with streptavidin. This finding permits us to determine the approximate coupling yield according to Figure 4, where the plot *N* versus *C* is in the linear regime. In Table 2, the relative coupling yields as a function of the contact time between the dendrimer−detergent complex and the beads are given. Obviously, already after a 1 h reaction time, nearly every dendrimer−detergent complex has reacted with the particles.

As a control, three competition experiments were performed to investigate the binding of the supramolecular complex in the presence of free biotin. A detailed description of these experiments is given in the Supporting Information. After incubation of the magnetic particles first with biotin and then with the dendrimer−detergent complex, only a weak fluorescence of the particles was detected (Figure 7d). This observation indicates

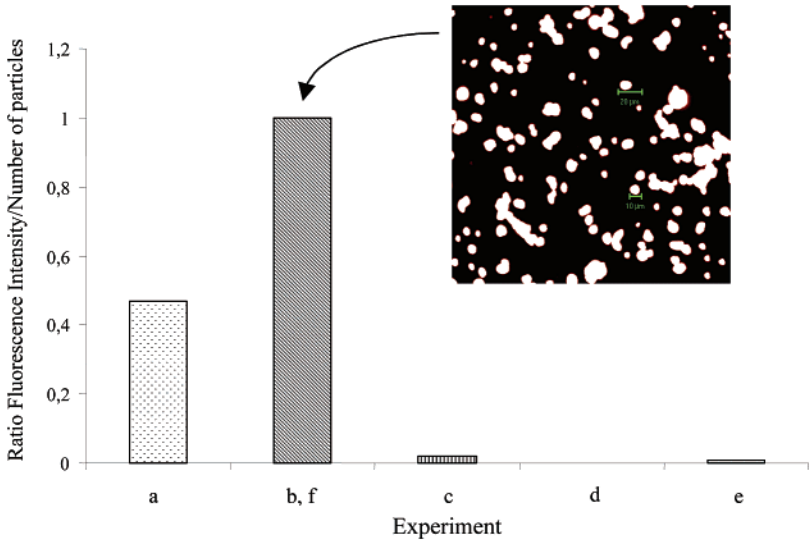

Figure 7. Magnetic bead binding assay. The plot shows the ratio of fluorescence intensity of coupled dendrimers per bead. (a) After a 1 h incubation time with **8**. (b) After a 24 h incubation time with **8**. (c) After a 24 h incubation time with **9**. (d) Incubation with free biotin and then with **8**. (e) Incubation with free biotin and **8**. (f) Incubation with **8** and then with free biotin. The inset shows a fluorescent image of streptavidin beads coated with bound dendrimers.

Table 2. Approximate Coupling Yield of the Dendrimer−Detergent Complex as a Function of the Contact Time

contact time (h)	coupling yield (%)
1	73 ± 0.1
4	76 ± 0.1
24	83 ± 0.1

that all binding sites of streptavidin were blocked by biotin due to its high affinity for this protein and the weak fluorescence was due to small amounts of the dendrimer being adsorbed on the surface of the particles.

In a second competition experiment, the magnetic beads were stirred with the same concentration of both free biotin and the dendrimer−detergent complex. In this case a weak fluorescence of the particles was detected (Figure 7e) which could be attributed to the faster interaction of biotin with streptavidin. Obviously, the affinity of the supramolecular complex for streptavidin is lower in comparison to free biotin which might be due to the larger size of the complex.

Incubation of the magnetic particles first with the dendrimer−detergent complex and then with biotin results in highly fluorescent particles (Figure7f), though free biotin is not able to replace the dendrimer−detergent complex which has already been coupled to streptavidin. This observation further indicates that the affinity of the dendrimer−detergent complex for the protein streptavidin is comparatively high which enables its application in biological assays.

Conclusion

In this article, we have presented the first synthesis of a dendrimer bearing chromophores as well as one biologically active group at the periphery. However, the use of this dendrimer for biological applications normally requires a sufficient solubility in aqueous media. This challenge has been achieved by using the detergent *Tween 20* which forms a supramolecular complex with the dendrimer. The size of this complex lies in the range of 7 nm, as determined independently by FCS and DLS, respectively. Based on the experimentally obtained value, a visualization of dendrimer micelles with the detergent molecules partially located inside the hydrophobic pockets of the dendrimer emerges. The optical spectra of the supramolecular complex in water reveal that the dendrimer is spatially isolated inside the micelle and its fluorescence quantum yield is independent of buffer conditions in particular the content of blood proteins. The highly fluorescent particles are stable in the presence of proteins and strong complexation agents which is a prerequisite for the realization of assays in biological media. In a first attempt, the biological activity of the supramolecular complex has been demonstrated by using magnetic particles coated with streptavidin which were coupled to the supramolecular complex via the biotin group of the dendrimer.

The use of detergents for the solubilization of hydrophobic polyphenylene dendrimers in water loaded with chromophores and a biologically active group is straightforward. Dendrimer−detergent complexes require less synthetic reaction steps in comparison to dendrimers carrying covalently attached substituents in order to provide water solubility. It remains to be shown that with the help of this novel polychromophore dendrimer−detergent complex an improved sensitivity and therefore a decrease in the detection limit of substrates such as hormones or antibodies will be achieved. It is a special feature of perylenedicarboximide chromophores that even investigations at the single molecule level are feasible which are currently underway.

Acknowledgment. The authors thank the European Associated Laboratory (LEA) and the BMBF nano-center for financial support. Last but not least, we would like to thank Cornelia Beer for her synthetic support.

Supporting Information Available: Experimental data for compounds **3**, **4**, **6**, and **8** and buffer compositions. Procedure for solubilization of the dendrimer **8** in water and table comparing solubilities of **8** in the presence of different detergents and solvents. Procedures for determining the stability of the complex toward protein solutions and biological activity. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0285058