Novel Pyridinium Dyes That Enable Investigations of Peptoids at the Single-Molecule Level

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Single-molecule microscopy is a powerful tool for investigating various uptake mechanisms of cell-penetrating biomolecules. A particularly interesting class of potential transporter molecules are peptoids. Fluorescence labels for such experiments need to comply with several physical, chemical, and biological requirements. Herein, we report the synthesis and photophysical investigation of new fluorescent pyridinium derived dyes. These fluorescent labels have advantageous structural variations and spacer units in order to avoid undesirable interactions with the labeled molecule and are able to easily functionalize biomolecules. In our case, cell-penetrating peptoids are successfully labeled on solid supports, and in ensemble measurements the photophysical properties of the dyes and the fluorescently labeled peptoids are investigated. Both fluorophores and peptoids are imaged at the single-molecule level in thin polymer gels. With respect to bleaching times and fluorescence lifetimes the dye molecules and the peptoids show only slightly perturbed optical behaviors. These investigations indicate that the new fluorophores fulfill well single-molecule microscopy *and* solid-phase synthesis requirements.

1. Introduction

Peptoids (oligo-*N*-alkylglycines) with positively charged side chains are known for their ability to enable or enhance cellular uptake therewith functioning as molecular transporters.[1] Their cargo can be either covalently linked or bound by supramolecular interactions, whereas the latter ones are engineered in order to deliver DNA or siRNA to cell nuclei in view on nonviral gene therapies.[2] Various studies show the influence of peptoid structures on cellular uptake efficiency or intracellular localization.[3,4] For tailoring the transporters and thus for optimization of the delivery, the exact mechanism of peptoid internalization into cells needs to be understood. Single-molecule imaging is a well-suited method for studying this.

Since the initial experiments on single molecules in a biologically relevant environment,[5] many groups have used single-molecule methods for both in vitro and in vivo investigations.[6] For example, Lee et al. have tracked the motion of peptide transporters on plasma membranes and therefore obtained detailed information about cellular uptake mechanisms.[7]

Fluorescent dyes have to fulfill several physical, chemical, and biological requirements to be attractive for single-biomolecule investigations: (i) They should be brilliant (i.e., display a high molar extinction coefficient related to intense light absorption and a high fluorescence quantum yield); (ii) they need to be particularly photostable; (iii) organic fluorophores usually show fluorescence quenching in aqueous media, so an ideal conjugate for a transporter needs to be highly fluorescent in physiological media; (iv) the molecular mass (or size) should be minimal in order to not burden the transporter; and (v) the fluorophore needs to have a reactive functional group to allow an easy and stable conjugation to biomolecules of interest. To conduct experiments in living cells, it is also desirable that they should absorb and especially fluoresce in the green or red spectral region for diminishing the background due to the cellular autofluorescence. Because of these stringent requirements, the development of new and possibly improved emitters for single-biomolecule detection is presently an actively pursued endeavor.[8–10]

The majority of published single-molecule reports being relevant to biological questions mainly addresses proteins or DNA. Most labeling procedures of these water-soluble biomolecules are carried out in aqueous solution.[7,11,12] On the contrary, smaller molecules such as peptides or peptoids are routinely synthesized and labeled on solid supports using organic solvents.[1,3,4,13] Interestingly, within their synthetic protocols, groups rarely use single-molecule suitable dyes.[14,15] One of the reasons is that the photophysical properties of fluorescent dyes can be negatively affected by solid-phase synthesis (SPS). Especially the cleavage of fluorescently labeled biomolecules from the resin by the commonly used trifluoroacetic acid (TFA) could be a critical step affecting the dye. Since the chemical stability during the peptoid synthesis is a main issue, we here report on fluorophores which satisfy the miscellaneous requirements of single-molecule methods *and* SPS.

We investigated new pyridinium derived dyes (in the following named as Pyr-dyes) as promising candidates. The molecules are synthesized from pyrylium salts which Kostenko et al. used as fluorescent dyes.[16] Pyrylium salts fluoresce slightly and are quite small labels that react under mild conditions with various amino groups of proteins. Thereby they form in only

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one step positively charged *N*-substituted pyridinium derivates which fluoresce strongly. This property explains the use of pyrylium salts for several biochemical applications.[17–19] Furthermore, they are readily obtained with practically any substitution pattern in preparatively useful yields from cheap starting materials.[20] Thereby it is also straightforward to change the Pyr-dyes photophysical properties by structural variations.[21]

Herein, we report the synthesis of new fluorescent Pyr-dyes and their suitability for investigating labeled peptoids by single-molecule fluorescence microscopy. Compared to previous work using pyridinium salts,[17] the present fluorophores have advantageous structural variations and spacer units in order to avoid undesirable interactions with biomolecules which usually lead to fluorescence quenching. We then successfully labeled cell-penetrating peptoids with these dyes on solid support, and the photophysical properties of the Pyr-dyes and the fluorescently labeled peptoids were determined in ensemble and single-molecule measurements. To the best of our knowledge, this represents the first report describing investigations of fluorescently labeled peptoids at a single-molecule level. In thin poly(methyl methacrylate) (PMMA) gels, both Pyr-dyes and peptoids could be successfully imaged at nanomolar concentrations. We analyzed both bleaching times as well as fluorescence lifetimes. The fluorophores and the peptoids showed only slightly perturbed optical behavior which indicates that Pyr-dyes fulfill well the requirements of single-molecule microscopy *and* SPS.

2. Results and Discussion

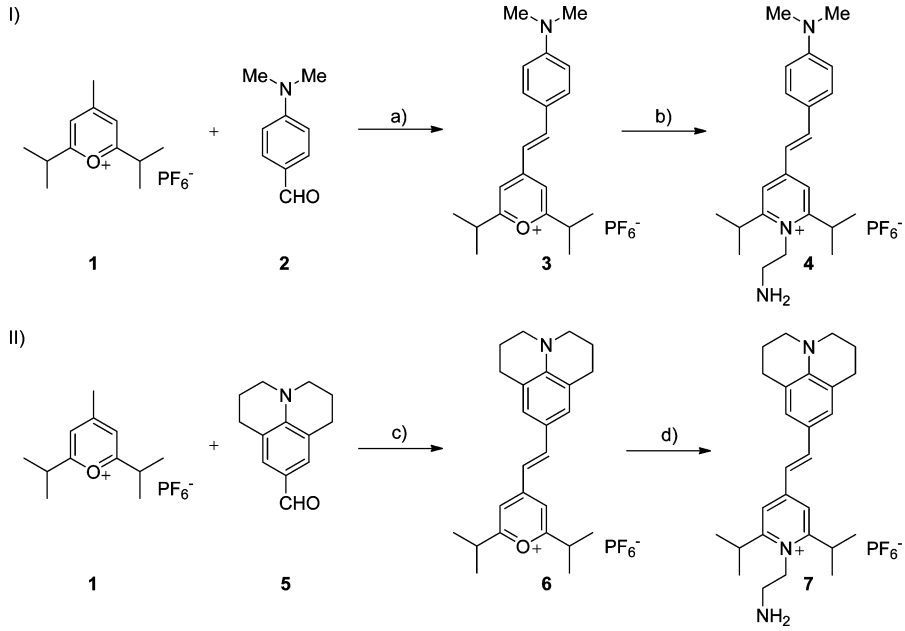
2.1. Synthesis. "Chameleon dyes" derived from styryl pyrylium salts have been reported.[17] In this previous approach, commercial 2,4,6-trimethylpyrylium tetrafluoroborate was reacted with 4-(*N*,*N*-dimethylamino)benzaldehyde to afford the 4-styryl-substituted salts. Subsequently, these compounds were reacted with proteins where ε-amino groups of lysines for instance directly form unspecifically the corresponding pyridinium salts. For our purpose this synthetic strategy is not suitable. First, the condensation with benzaldehyde affords considerable amounts of 2- and/or 6-styryl-substituted pyrylium salts. Second, the pyrylium to pyridinium conversion is not quantitative (80% at best) with anilines being the most prominent secondary products.[22] Inhomogeneous materials could thus be obtained by coupling to peptoids. And importantly, anilines can act as potent fluorescence quenchers as they can undergo facile photoinduced electron transfer. Additionally, the tetrafluoroborate anion is incompatible with most cell lines as its cytotoxic activity has been proven especially when associated with ionic liquids.[23]

To exploit the advantages of solid-phase synthesis,[24] an alternative synthetic strategy was developed. First the two methyl groups in position 2 and 6 within the pyrylium salt were replaced by two 2-propyl groups. This simple modification, without increasing significantly the mass of the molecule, has three evident advantages: (i) The two isopropyl groups do not react with benzaldehydes. Thus the latter can selectively attack the 4-methyl group forming the corresponding 4-styrylpyrylium salt in high yields; (ii) albeit being bulkier than methyl groups the two isopropyl groups react in excellent yields with primary amines to afford the corresponding pyridinium salts due to their Janus-type behavior (the Janus facet is based on what an incoming reagent experiences: either a small methyl group or a bulky *tert*-butyl group depending on which conformation of the isopropyl group is preferred);[25] (iii) the two isopropyl groups impart an increased solubility in organic solvents used for the SPS.

Two synthetic methods using low-cost starting materials such as either *tert*-butanol, isobutyric anhydride, and hexafluorophosphoric acid, or *tert*-butyl chloride, aluminum chloride, and isobutyryl chloride have been described recently for the multigram synthesis of the starting pyrylium salt **1**.[26] This was reacted further with either 4-*N*,*N*-dimethylaminobenzaldehyde **2** or the benzaldehyde derived from julolidine **5** to give the pyrylium salts **3** and **6**, respectively.

In a second synthetic step, these pyrylium salts were converted to the corresponding *N*-(2-aminoethyl)pyridinium salts **4** and **7** in good yields and multigram quantities. The 2-ami-

SCHEME 1: Synthesis of Pyr-Dyes 4 and 7[a]



[a] (I) Reagents, conditions and yields: (a) ethanol, reflux, 15 min, 74%; (b) 2.7 equiv of ethylenediamine, ethanol, reflux, 2 h, 58%. (II) Reagents, conditions and yields: (c) ethanol, reflux, 30 min, 82%; (d) 1.5 equiv of ethylendiamine, ethanol, reflux, 2 h, 94%.

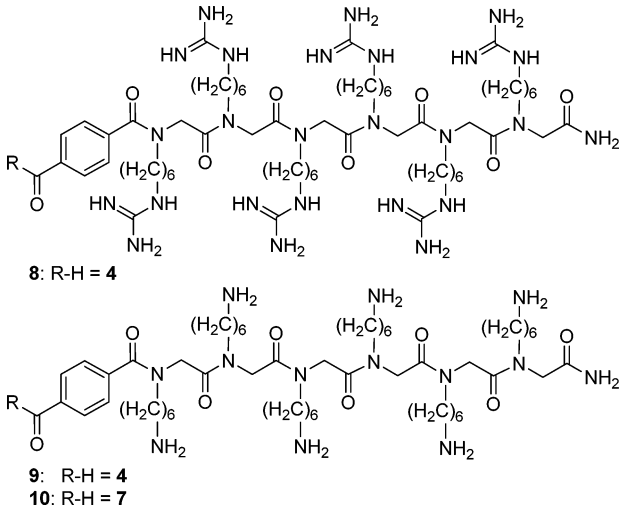


Figure 1. Fluorescently labeled peptoids after deprotection and cleavage from the solid support.

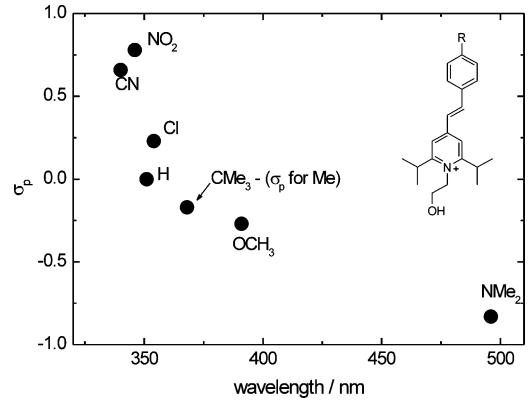


Figure 2. Hammett plot showing the absorption maxima in dry dichloromethane for *N*-(2-hydroxyethyl)-4-styryl pyridinium salts having various 4′-R-substituents. σ_p represents the Hammett substituent constant for the para-position.

noethyl handle assures the reactive linking group of the fluorophore onto peptoids being compatible with SPS protocols and acts as short spacer. Scheme 1 details the syntheses, reaction conditions and yields. Furthermore, the introduction of other anchoring groups such as maleimides or ethanolamine was possible as well.[27] Hence, Pyr-dyes could label various biomolecules also by thio or ester group linkages.

The synthesis and the labeling procedure as well as purification of fluorescently labeled peptoids are based on our previously described protocols.[3,4] All steps were performed on solid supports. Peptoids (Figure 1) were obtained in satisfying yields and excellent purities.

2.2. Photophysical Properties in Ensemble Measurements. We further investigated if the Pyr-dyes are suitable for studies at the single-molecule level of biological probes, namely, cell-penetrating peptoids. In our standard protocols we incubate cells with these biomolecules in aqueous solution.[3,4] Therefore we initially determined the bulk photophysical properties of free Pyr-dyes as well as of fluorescently labeled peptoids in water. Additionally, the stationary absorption and fluorescence spectra of the fluorophores were measured in dichloromethane. Table 1 summarizes the photophysical data.

First of all, it was evident that the auxochromic groups on the styryl moiety of the dyes strongly influenced their spectral properties (Figure 2). Independent of the solvent, the absorption as well as the emission bands of Pyr-dye **4** shifted toward the blue region (35−50 nm) in comparison to Pyr-dye **7**. The same result was observed after coupling to the peptoid structures due to the effect of the rigid julolidine rings in Pyr-dye **7** compared to the *N*,*N*-dimethylaniline part in Pyr-dye **4**.[28] This makes especially Pyr-dye **7** a favorable candidate for measurements in cells. Excitation above 500 nm is effectively possible, where autofluorescence problems in a cell environment are suppressed. Furthermore, the spectral adjustability of the Pyr-dyes allows a choice of the right fluorescent molecule for a specific application and microscope setup.

Second, local environments influenced the absorption and fluorescence maxima of the Pyr-dyes. Changing the dielectric environments from water toward the less polar solvent dichloromethane, both dyes, **4** and **7**, showed significant bathochromic shifts (54 and 48 nm, respectively) in absorption and hypsochromic shifts (14 and 8 nm, respectively) in fluorescence. Hence, the Stokes shifts in water are higher than in dichloromethane and above all remarkable (164 nm for **4**, 143 nm for **7**). Thereby an effective separation of fluorescence signals from the excitation light in imaging experiments is guaranteed, promoting thus Pyr-dyes as easily available and interesting candidates for single-molecule experiments.

In addition to the spectral shifts a drastic increase in quantum yield occurred in dichloromethane compared to water. This effect can be due to inhibition of unspecific aggregation. Such aggregation phenomena drastically reduce the fluorescence, because of concentration quenching. It is the main reason why "sticky" organic, hydrophobic fluorophores, which are extremely brilliant in their monomeric form, only dully fluoresce in water. Additionally, for related structures (e.g., 9-(2-carboxy-2-cyanovinyl)julolidine) a twisted intramolecular charge-transfer (TICT) by rotation around the C═C bond is known as a nonradiative relaxation pathway.[29,30] Moreover, TICT as well as planar ICT (PICT) processes involving the *N*-stilbenyl bond are discussed.[31,32] We assume that similar excited-state events are occurring for the Pyr-dyes: After excitation, the molecule can relax to an ICT state. This state is characterized by an increased charge separation and is especially well-stabilized in

TABLE 1: Summary of the Photophysical Properties of Chromophores in Free Form and of Fluorescently Labeled Peptoids

molecule	solvent	$\lambda_{max,abs}$[a] (nm)	ε_{max}[b] ($M^{-1}cm^{-1}$)	$\lambda_{max,em}$[c] (nm)	Φ_F	τ (ns)
4	CH_2Cl_2	498	47000 ± 1000	594	0.5 ± 0.01	0.235 ± 0.007
	H_2O	444	12600 ± 300	608	0.0094 ± 0.0004	0.611 ± 0.005
8	H_2O	460	12000 ± 400	606	0.054 ± 0.005	1.03 ± 0.06
9	H_2O	453	8600 ± 100	604	0.045 ± 0.006	0.985 ± 0.008
7	CH_2Cl_2	545	50000 ± 3000	632	0.08 ± 0.007	0.29 ± 0.02
	H_2O	497	23000 ± 3000	640	0.0045 ± 0.0004	0.66 ± 0.02
10	H_2O	501	2290 ± 50	641	0.022 ± 0.002	0.776 ± 0.007

[a] Absorption maxima have a ±1 nm imprecision. [b] For the peptoids the calculation is based on the molar mass of the free amines without a counter ion. [c] Fluorescence maxima are reproducible within a ±2 nm range.

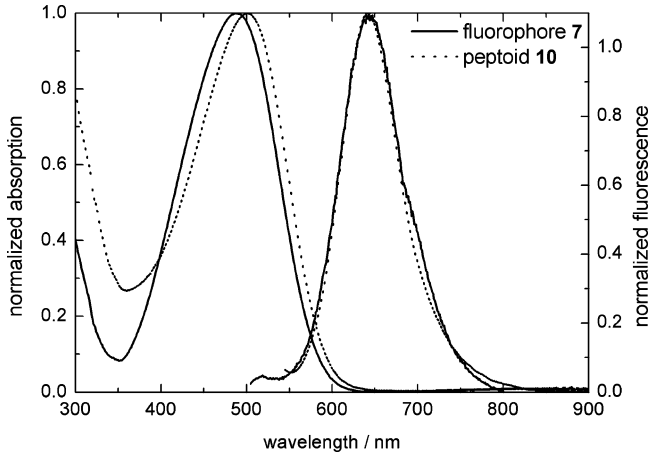


Figure 3. Normalized absorption and fluorescence spectra of Pyr-dye **7** and peptoid **10** in water.

polar solvents such as water. On the contrary, in less polar solvents the ITC stage is less favorable. Thus, the nonradiative relaxation pathway is reduced and the quantum yield is increased. Such solvent/aggregation dependency can be a very useful feature for spatially and spectrally resolved imaging of biological probes with different polarities.

Figure 3 shows normalized absorption and fluorescence spectra of Pyr-dye **7** and peptoid **10** in water. Slight shifts in absorption maxima to higher wavelengths occurred after coupling Pyr-dyes to peptoids. Nevertheless, in comparison with the above-discussed shifts, these differences are negligible. Noticeable is that the emission curves were almost identical. Thus, the spectral properties of the Pyr-dyes were only slightly perturbed, even after the coupling steps on solid supports.

Molar extinction coefficients in water decreased for Pyr-dyes after coupling to peptoids. In the case of Pyr-dye **4** the effect was moderate and values were still in the range of other single-molecule dyes such as perylenes.[12] Worth mentioning is that the quantum yields increased by almost 1 order of magnitude but were still lower than the quantum yields of perylenes (Φ_F in water = 0.39−0.66).[12] Nevertheless, other emitters, suitable for single-molecule imaging in cells, have comparable values in water. For example Lord et al. present dicyanomethylene-dihydrofuran fluorophores which show quantum yields < 0.003 in water that were sufficient for single-molecule imaging within cell membranes.[9] Thus, the low quantum yields of Pyr-dyes and their derived labeled peptoids are not necessarily an obstacle for cellular imaging. Fluorescence lifetime curves for ensemble measurements showed monoexponential decays (data not shown). The fitted values were around 0.6 ns for the fluorophores and between 0.7 and 1 ns for the peptoids and thus similar to related structures.[18]

2.3. Single-Molecule Properties. For future single-molecule tracking of fluorescently labeled peptoids in cells we investigated Pyr-dye **7** and peptoid **10** more extensively. These molecules fluoresce at higher wavelengths and are more advantageous for biological experiments.

First of all, a fluorescent dye is applicable in single-molecule microscopy experiments if imaging is possible. Initially, judging from their extinction coefficients and quantum yields at the ensemble level, it was not obvious that Pyr-dyes are suitable for such experiments. Nevertheless, our setup allowed us to obtain single-molecule images of immobilized Pyr-dye **7** molecules as well as fluorescently labeled peptoid **10** (Figure 4A) at nanomolar concentrations (5−10 nM) in thin films of PMMA. Fluorescence intensity traces give an indication of single-molecule observation[33] (Figure 4B). Additionally, the resistance to enter long-lived dark states is visible in the exemplary trace. Both the free Pyr-dye **7** and the peptoid **10** exhibited little or no blinking on the time scale of our experiments (2−100 ms).

We recorded 166 traces of Pyr-dye **7** molecules and also of 127 molecules of peptoid **10**. By fitting the histogram for the duration of transients (Figure 5), it was possible to obtain the mean survival times. For the free dye it was 1.87 ± 0.15 s and for the peptoid 1.9 ± 0.3 s, respectively, the values being thus identical within the experimental error. The observed survival times were not as high as those reported for other fluorescent dyes.[8] However, a comparison is not unambiguously possible as experimental setups and measurement conditions in diverse

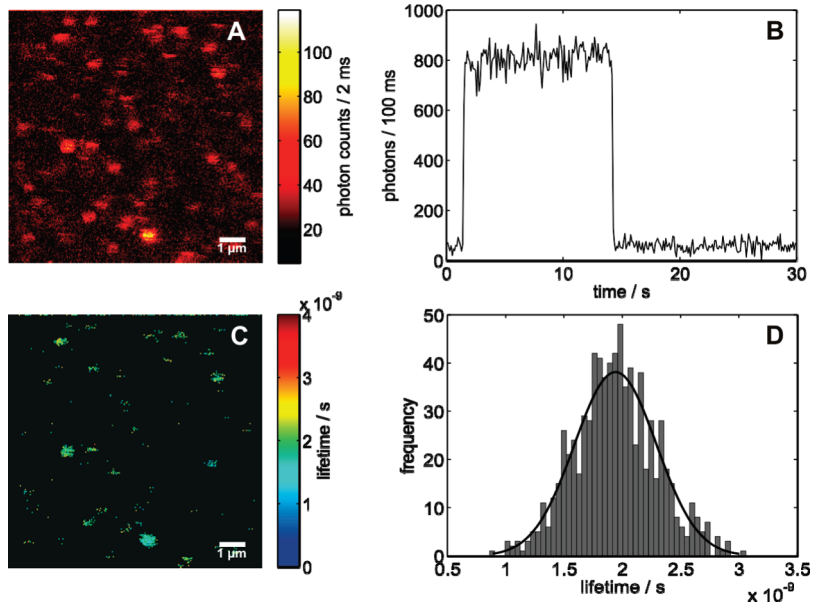


Figure 4. (A) Typical fluorescence image of single copies of peptoid **10** in PMMA (c = 10 nM). (B) Fluorescence intensity as a function of time for a single molecule. (C) Fluorescence lifetime image of the same area as in A. (D) Distribution of lifetimes from the fluorescence lifetime image with a maximum around 2.1 ns.

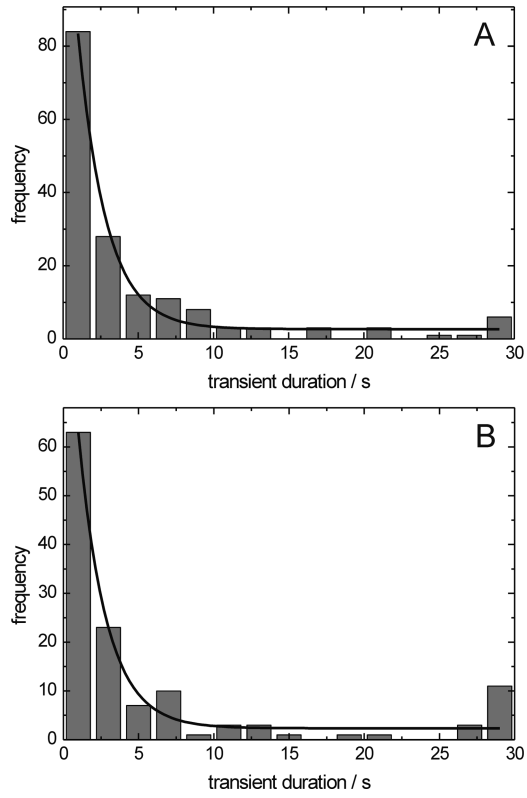


Figure 5. Distribution of the duration of transients for Pyr-dye **7** (A) and peptoid **10** (B) molecules. Data were fitted as a monoexponential function.

reports differ. Fluorophores, such as, e.g., the fluorescent protein DsRed, show different survival times in different polymer gels.[34,35] Furthermore, the measured survival times of the Pyr-dye and the labeled peptoid were on similar or even longer time scales as residence times and dynamic events of cell-penetrating molecules on membranes. For example Lee et al. analyzed oligoarginine transporter conjugates on the plasma membrane of cells which all showed residence times lower than 1.6 s.[7] Ciobanasu et al. successfully tracked the cell-penetrating peptide HIV TAT1 within biological model membranes. In this case, analyzing the binding and mobility was possible out of movie frames no longer than 500 ms in total.[36] For this reason we assume that the photobleaching time of peptoid **10** should be sufficient and adequate for cellular imaging. Peptoids labeled with Pyr-dyes could in fact already successfully be imaged in cells in higher concentrations ($c = 10-50\ \mu$M, data not shown). We also expect that upcoming single-molecule experiments on living cells will give us further insight into internalization mechanisms of peptoids.

As pulsed laser light excited the samples, fluorescence lifetime images could also be obtained. Figure 4C shows the same area as Figure 4A, but with lifetime information. Values were plotted against their frequency and fitted by a Gaussian (Figure 4D). The measured fluorescence lifetime was 2.1 ± 0.1 ns for Pyr-dye **7** and 2.0 ± 0.1 ns for peptoid **10**, again identical values within the error margins. This and the identical values for photobleaching statistics show an important fact: in single-molecule experiments, the fluorescence behavior of the dyes is almost independent of the conjugated peptoid. Additionally, the Pyr-dyes were stable enough to overcome SPS procedures.

Fluorescence lifetimes for both, Pyr-dye **7** and peptoid **10** were longer than those in solution, what might be due to the change in viscosity. Such sensitivity is known for chemical structures related to Pyr-dyes[9] as well as other emitters such as dicyanomethylenedihydrofuran fluorophores[29,30] and can be once again explained with reduced nonradiative relaxation pathways. Because a C=C bond twist to the TICT state is less favorable in rigid environments, the fluorescence quantum yield and thus the fluorescence lifetime (which is in general proportional to the quantum yield) could increase. Besides, fluorophores chemically related to Pyr-dyes show such strong changes in fluorescence lifetime (from 1.0 up to 2.4 ns) upon conjugation to bulky proteins, a fact which was successfully used in an affinity assay.[18] This sensitivity to local environment is a useful tool for, e.g., fluorescence lifetime imaging as well as to follow dynamic changes even on the single-molecule level.

3. Conclusions

We presented the synthesis and spectral investigation of new functionalized fluorescent dyes and fluorescently labeled peptoids. Fluorophores based on new styrylpyridinium salt structures have a major advantage: the possibility to modify their spectral properties by varying the *p*-auxochromic groups on the benzaldehyde synthon. Furthermore, their remarkably high Stokes shifts in comparison to other commonly used fluorophores allow an effective separation from excitation sources and represent an advantage especially for single-molecule experiments. Because of optimized synthetic protocols, the fluorescent dyes were available in large quantities and high purity. In reaction with anchor molecules, they formed highly fluorescent pyridinium salts. These were chemically compatible with functional groups in biomolecules, such as peptoids. Rapid and efficient bioconjugation was possible by optimized solid-phase techniques. As notably small fluorophores, they minimally burden the conjugate, thus enabling efficient cargo trafficking. Due to these facts, Pyr-dyes have a great potential as labels for peptoids and for many other molecules, e.g., peptides, proteins, and sugars.

We presented the first single-molecule investigation of fluorescently labeled peptoids. In PMMA gels, both pyridinium derived dyes and peptoids were imaged at the single-molecule level and showed similar photophysical properties with respect to bleaching times and fluorescence lifetimes. Consequently, Pyr-dyes were stable enough for labeling on solid supports. Both free dyes and peptoid molecules undergo minimal blinking on the single-molecule level—an advantage for tracking experiments. Moreover, the sensitivity to the viscosity of the environment makes them promising candidates for further analytical investigations.

We thus assume that the new fluorophores are promising candidates for upcoming single-molecule tracking experiments. Future work will address the single-molecule analysis of peptoids in aqueous polymers and the investigation of hydrodynamic radii for Pyr-dyes and fluorescently labeled peptoids. Single-molecule experiments will give further insight into internalization mechanisms of peptoids into cells and will allow one to fine-tune their cargo delivering ability.

4. Experimental Methods

4.1. Synthetic Procedures. All reagents were purchased from commercial sources and used without further purification. Solvents of technical quality were distilled before using; solvents with the quality per analysis (p.a.) were purchased from commercial sources. Dichloromethane was dried over $CaCl_2$, refluxed over CaH_2, and distilled. For microwave assisted syntheses the single-mode CEM Discover microwave was used.

Melting points were recorded with Stuart melting point apparatus (Bibby Sterilin). Routine NMR spectra were measured

with a Bruker DPX 300 Avance spectrometer at 300 MHz and a Bruker AM 400 spectrometer at 400 MHz. All spectra are referenced to the respective solvent signals (δ = 0 ppm): chloroform-d_1 with 7.26 ppm for ^{1}H NMR and 77.0 ppm for ^{13}C NMR, acetone-d_6 with 2.05 ppm for ^{1}H NMR ppm and 206.0 ppm for ^{13}C NMR. Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, sept = septet, and m = multiplet. Coupling constants (J) are given in hertz. FT-IR spectra were measured on IFS 88 spectrometer (Bruker). Intensities of transmission (T) are described as follows: vs, very strong (0−10% T); s, strong (10−40% T); m, mean (40−70% T); w, weak (70−90% T); vw, very weak (90−100% T). Matrix assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF-MS) of the Pyr-dyes were recorded with PerSeptive Biosystems Voyager-DE Pro with 100 laser pulses and an acceleration voltage of 20 kV in isovanillin (3-hydroxy-4-methoxybenzaldehyde) as matrix. For the peptoids we used Bruker Biflex IV with a nitrogen laser (λ = 337 nm) and FlexControl version 1.1 and XMASS-XTOF version 5.1.1 as software. Each sample was shot 100−300 times with a repetition rate of 1−3 Hz. We used Bruker standard targets of aluminum 386 "spots" and 2,5-dihydroxybenzoic acid (DHB) 50% acetonitrile and 0.1% TFA in water as matrix. Fast atom bombardment mass spectra (FAB-MS) were measured with MAT 95 (Finnigan). Elemental analyses were recorded on the CHN rapid instrument from Elementar.

4.2. Synthesis of the 2,6-Diisopropylpyridinium Derived Dye. ***2,6-Diisopropyl(pyrylium-4-yl)vinyl-N,N-dimethylbenzamine Hexafluorophosphate (3).***[25] *N*,*N*-Dimethylaminobenzaldehyde (**2**; 0.69 g, 4.63 mmol, 1.50 equiv) and 2,6-diisopropyl-4-methylpyrylium hexafluorophosphate (**1**; 1.00 g, 3.09 mmol, 1.00 equiv) were dissolved in ethanol (70 mL) and stirred for 15 min under reflux. The blue mixture was then allowed to cool to room temperature. The precipitate was filtered and dried in vacuo. The product was recrystallized from 2-propanol (1.04 g, 74% yield). Mp 190−193 °C. ^{1}H NMR (300 MHz, chloroform-d_1): δ 8.12 (d, J = 15.2 Hz, 1 H, 13-CH), 7.73 (d, J = 8.8 Hz, 2 H, 16-, 20-CH), 7.31 (s, 2 H, 3-, 5-CH), 6.91 (d, J = 15.2 Hz, 1 H, 14-CH), 6.60 (d, J = 9.1 Hz, 2 H, 17-, 19-CH), 3.11 (s, 6 H, 22-, 23-CH_3, 1.35 (d, J = 6.9 Hz, 12 H, 8-, 9-, 11-, 12-CH_3). ^{13}C NMR (300 MHz, chloroform-d_1): δ 177.0 (2-, 6-C), 160.8 (4-C), 154.6 (18-C), 152.8 (13-, 14-C), 134.5 (16-, 20-C), 122.9 (15-C), 112.3 (17-, 19-C), 111.3 (3-, 5-C), 39.9 (22-, 23-C), 33.3 (7-, 10-C), 19.8 (8-, 9-, 11-, 12-C). IR (KBr): ν = 2977 (w), 2939 (w), 1655 (m), 1617 (m), 1533 (m), 1374 (m), 1334 (m), 1274 (m), 1172 (m), 937 (m), 839 cm^{-1} (m). UV/vis (CH_2Cl_2): λ_{max} (log ε_{max}) = 603 (3.12), 297 nm (2.63). FAB-MS (*m*/*z*): 455.3 [$M^+ + PF_6^-$], 310.4 [M^+] (100), 191.5 [$M^+ + H - C_8H_{10}N^\bullet$]. HRMS [ESI]: [$M + H$]$^+$ calcd for $C_{21}H_{28}NO$, 310.2213; found, 310.2220.

***[1-Ethylamino(2,6-diisopropylpyridinium-4-yl)]vinyl-N,N-dimethylbenzamine Hexafluorophosphate* (4).** Pyrylium salt (**3**; 0.50 g, 1.10 mmol, 1.00 equiv) was dissolved in ethanol (15 mL) under nitrogen atmosphere and heated to reflux. Then ethylendiamine (180 mg, 0.20 mL, 3.00 mmol, 2.7 equiv) was added fastly, whereas the blue solution changed its color to red. The mixture was stirred for 2 h under reflux. After cooling to room temperature the solvent was removed under reduced pressure. The precipitate was taken up in a small amount of dichloromethane and added to a mixture of *n*-hexane and diethylether (2:1). The product precipitated as a red solid, it was filtered, washed with a small amount of *n*-hexane, and dried under reduced pressure. It was recrystallized from ethanol and the product was obtained as an orange powder (320 mg, 58% yield). Mp 215 − 217 °C. ^{1}H NMR (300 MHz, acetone-d_6): δ 7.94 (s, 2 H, 3-, 5-CH), 7.60 (d, J = 8.8 Hz, 2 H, 19-, 23-CH), 7.16 (d, J = 16.2 Hz, 2 H, 16-, 17-CH), 6.78 (d, J = 8.8 Hz, 1 H, 20-, 22-CH), 4.97 (t, J = 5.9 Hz, 2 H, 7-CH_2), 4.04 (sept, J = 6.7 Hz, 2 H, 10-, 13-CH), 3.84 (t, J = 5.8 Hz, 2 H, 8-CH_2), 3.05 (s, 6 H, 25-, 26-CH_3), 2.80 (s, 2H, 9-NH_2), 1.46 (d, J = 6.8 Hz, 12 H, 11-, 12-, 14-, 15-CH_3). ^{13}C NMR (300 MHz, acetone-d_6): δ 165.7 (2-, 6-C), 155.6 (21-C), 154.0 (4-C), 143.4 (17-C), 131.3 (19-, 23-C), 124.7 (16-C), 120.3 (3-, 5-C), 119.0 (18-C), 113.7 (20-, 22-C), 52.7 (7-C), 51.9 (8-C), 41.0 (25-, 26-C), 32.8 (10-, 13-C), 23.7 (11-, 12-, 14-, 15-C); IR (KBr): ν = 3404 (vw), 2977 (w), 2944 (w), 2807 (w), 1588 (m), 1530 (m), 1369 (m), 1192 (m), 1162 (m), 843 (s), 558 cm^{-1} (m). UV/vis (CH_2Cl_2): λ_{max} (log ε_{max}) = 498 (4.67), 286 (2.43), 254 nm (2.36). FAB-MS (*m*/*z*): 369.2 (100) [Matrix], 352.4 [M^+], 309.4 [$M^+ + H - C_2H_6N^\bullet$]. HRMS [ESI]: [$M + H$]$^+$ calcd for $C_{23}H_{34}N_3$, 352.2753; found, 352.2748.

4.3. Synthesis of the Julolidine Derived Dye. Samples of 2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinoline (julolidine) and 2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinoline-9-carbaldehyde (**5**) were synthesized according to literature.[37]

***2,6-Diisopropyl(pyrylium-4-yl)vinyl[2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline] Hexafluorophosphate* (6).** 2,6-Diisopropyl-4-methylpyrylium hexafluorophosphate (**1**, 2.00 g, 6.17 mmol, 1.00 equiv) was dissolved in ethanol (100 mL) and treated in an ultrasonic bath. A saturated solution of julolidinecarbaldehyde **5** (1.24 g, 6.17 mmol, 1.00 equiv) in ethanol (approximately 60 mL) was added, and the color changed from green to blue. The mixture was stirred for 30 min under reflux. After cooling to room temperature the precipiate was filtered off, washed with a little amount of diethyl ether, and dried in vacuo. The product was recrystallized from ethanol to give a blue powder (1.16 g, 82% yield). Mp 208−210 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 8.26 (d, J = 14.9 Hz, 1 H, 15-CH), 7.25−7.58 (m, 11-, 13-, 17-, 21-CH), 7.04 (d, J = 14.9 Hz, 1 H, 14-CH), 3.55 (t, 4 H, 2-, 10-CH_2), 3.15 (sept, J = 6.9 Hz, 2 H, 22-, 25-CH), 2.79 (t, J = 6.2 Hz, 4 H, 4-, 8-CH_2, 2.02−1.96 (m, 4 H, 3-, 9-CH_2), 1.38 (d, J = 6.9 Hz, 12 H, 23-, 24-, 26-, 27-CH_3). ^{13}C NMR (400 MHz, acetone-d_6): δ 177.5 (18-, 20-C), 160.6 (16-C), 153.6 (15-C), 152.6 (6-C), 134.4 (11-, 13-, 17-, 21-C), 133.8 (12-C), 124.9 (5-, 7-C), 115.8 (14-C), 52.5 (2-, 10-C), 35.2 (22-, 25-C), 29.0 (4-, 8-C), 22.5 (3-, 9-C), 21.3 (23-, 24-, 26-, 27-C). IR (KBr): ν = 3428 (vw), 2975 (m), 2941 (m), 2874 (w), 1650 (m), 1557 (s), 1522 (s), 1435 (m), 1276 (s), 1252 (s), 1166 (s), 1079 (m), 936 (m), 840 (s), 558 cm^{-1} (m). UV/vis (CH_2Cl_2): λ_{max} (log ε_{max}) = 646 (3.18), 299 nm (2.21). FAB-MS (*m*/*z*): 508.3 [$M^+ + H^+ + PF_6^-$], 507.3 [$M^+ + PF_6^-$], 362.3 [M^+] (100). HRMS [ESI]: [$M + H$]$^+$ calcd for $C_{25}H_{32}NO$, 362.2526; found, 362.2524.

[1-Ethylamine(2,6-disopropyl(pyridinium-4-yl)]vinyl[2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline] Hexafluorophosphate (7). Pyrylium salt **6** (1.00 g, 1.97 mmol, 1.00 equiv) was dissolved in ethanol (80 mL) under nitrogen atmosphere and was heated to reflux. Then ethylendiamine (0.18 g, 0.20 mL, 2.99 mmol, 1.50 equiv) was added quickly, and the blue solution changed its color to red. The mixture was stirred for 2 h under reflux, and after cooling to room temperature the solvent was removed under reduced pressure. The precipitate was taken up in a small amount of dichloromethane and added to a mixture of *n*-hexane and diethyl ether (2:1). The product precipitated as a red solid and was recrystallized from ethanol and dried in vacuo (1.02 g, 94% yield). Mp 158 − 160 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.88 (s, 2 H, 17-, 21-CH), 7.83 (d, J = 16.0 Hz, 1 H, 15-CH), 7.13 (s, 2 H, 11-, 13-CH), 7.04 (d, J =

16.0 Hz, 1 H, 14-CH), 4.95 (t, $J = 5.9$ Hz, 2 H, 22-CH_2), 3.90 (sept, $J = 6.6$ Hz, 2 H, 25-, 28-CH), 3.74 (t, $J = 5.9$ Hz, 2 H, 23-CH_2), 3.28 (t, $J = 6.2$ Hz, 4 H, 2-, 10-CH_2), 2.72 (t, $J = 6.3$ Hz, 4 H, 4-, 8-CH), 1.90−1.97 (m, 4 H, 3-, 9-CH_2), 1.45 (d, $J = 6.6$ Hz, 12 H, 26-, 27-, 29-, 30-CH_3), 0.94 (t, 2 H, NH_2). ^{13}C NMR (300 MHz, acetone-d_6): δ 165.3 (18-, 20-C), 155.7 (16-C), 147.1 (6-C), 143.9 (14-C), 129.6 (11-, 13-C), 123.7 (12-C), 122.8 (14-C), 119.8 (17-, 21-C), 117.6 (5-, 7-C), 52.8 (22-C), 51.7 (2-, 10-C), 37.5 (23-C), 32.7 (4-, 8-C), 29.1 (25-, 28-C), 23.7 (26-, 27-, 29-, 30-C), 23.0 (3-, 9-C). IR (KBr): $\nu = 3591$ (w), 2945 (w), 2843 (w), 1582 (m), 1524 (w), 1315 (m), 1259 (w), 1158 (w), 842 (m), 559 (w), 1257 (m), 1155 (m), 1126 (m), 1096 (m), 977 (m), 840 (s), 558 cm^{-1} (m). UV/vis (CH_2Cl_2): λ_{max} (log ε_{max}) = 544 nm (4.70). FAB-MS (*m*/*z*): 549.3 [$M^+ + PF_6$], 518.4 (100) [$M^+ + H^+ + PF_6 - CH_4N$], 458.4 (100), 404.3 [M^+], 360.3 [$M^+ - C_2H_6N$]. HRMS [ESI]: [M + H]$^+$ calcd for $C_{27}H_{38}N_3$, 404.3107; found, 404.3112.

4.4. Synthesis of the Peptoid Conjugates. ***Construction of the Peptoids:***[38] The amino-functionalized Rink amide resin (200 mg, 128 μmol, 1.00 equiv) was covered with 5 mL of *N*,*N*-dimethylformamide (DMF) and swollen for 60 min at room temperature. The resin was washed with DMF and treated with 20% piperidine in DMF (3 × 5 min with 3 mL each), and thoroughly washed with DMF. A solution of *N*-(8-(*tert*-butoxycarbonylamino)hexyl)-*N*-(9*H*-fluorene-9-ylmethoxycarbonyl)amino acetic acid (191 mg, 384 μmol, 3.00 equiv, synthesized according to published methods with slight modifications[3]) in DMF (3.80 mL), 1-hydroxybenzotriazole hydrate (HOBt·H_2O; 58.9 mg, 384 μmol, 3.00 equiv), and *N*,*N*′-diisopropylcarbodiimide (DIC; 97.8 μL, 79.7 mg, 384 μmol, 3.00 equiv) was added to the preswollen resin (128 μmol, 1.00 equiv). The reaction vessel was subjected to microwave irradiation to keep the temperature at 60 °C for 30 min while being stirred. The reaction solution was filtered and the resin treated a second time with freshly prepared reaction solution using the above-mentioned conditions (double coupling). The resin was washed with DMF (3 × 5 mL each). This procedure was repeated five times to obtain the yellowish resin bound hexamer. To a solution of terephthalic acid (63.8 mg, 384 μmol, 3.00 equiv) in DMF (3.80 mL) were added HOBt·H_2O (118 mg, 768 μmol, 6.00 equiv) and DIC (100 μL, 81.5 mg, 588 μmol, 4.59 equiv) before being added to the resin (128 μmol, 1.00 equiv). The reaction vessel was subjected to microwave irradiation to keep the constant temperature at 60 °C for 30 min while being stirred. The reaction solution was filtered off and the resin treated a second time with freshly prepared reaction solution using the above-mentioned conditions (double coupling). The resin was washed with DMF (3 × 5 mL each). The resin was split into four (128 μmol/4 = 32 μmol) portions.

Labeling of the Peptoids. ***Peptoid* 9.** A solution of **4** (47.8 mg, 96.0 μmol, 3.00 equiv) in DMF (1.00 mL), HOBt·H_2O (14.7 mg, 96.0 μmol, 3.00 equiv), and DIC (24.5 μL, 20.0 mg, 96.0 μmol, 3.00 equiv) was added to the resin synthesized as described above (32.0 μmol, 1.00 equiv). The reaction vessel was subjected to microwave irradiation to keep the temperature at 60 °C for 30 min while being stirred. The reaction solution was filtered off and the resin treated a second time with freshly prepared reaction solution using the above-mentioned conditions (double coupling). The resin was washed with DMF (3 × 5 mL each). To cleave the peptoid from solid support, a solution of 1 mL of TFA in dichloromethane (50:50) was added to the resin and left for 2 h at room temperature. The resin was rinsed two times with 0.5 mL of methanol. This procedure of adding cleavage solution and rinsing with methanol was repeated until the cleavage solution was colorless. Water was added, and the mixture was lyophilized to give the crude peptoid **9**. After HPLC purification (method 1, $R_t = 22.6$ min) 1.44 mg of the product was obtained with a purity of 93%. MALDI-TOF-MS [*m*/*z*]: [M]$^+$ calcd for $C_{79}H_{135}N_{16}O_8^+$, 1437.02 (average mass); found, 1437.1.

***Peptoid* 10.** A solution of **7** (52.8 mg, 96.0 μmol, 3.00 equiv) in DMF (1.00 mL), HOBt·H_2O (14.7 mg, 96.0 μmol, 3.00 equiv), and DIC (24.5 μL, 20.0 mg, 96.0 μmol, 3.00 equiv) was added to the resin synthesized as described above (32.0 μmol, 1.00 equiv). From here on the same procedure was used as reported for peptoid **9** to give the crude peptoid **10.** After HPLC purification (method 2, $R_t = 33.2$ min) 2.39 mg of the product was obtained with a purity of 91%. MALDI-TOF-MS [*m*/*z*]: [M + H]$^+$ calcd for $C_{83}H_{139}N_{16}O_8^+$, 1489.09 (average mass); found, 1490.3.

***Peptoid* 8.** A solution of **4** (47.8 mg, 96.0 μmol, 3.00 equiv) in DMF (1.00 mL), HOBt·H_2O (14.7 mg, 96.0 μmol, 3.00 equiv), and DIC (24.5 μL, 20.0 mg, 96.0 μmol, 3.00 equiv) was added to the resin synthesized as described above (32.0 μmol, 1.00 equiv). From here on the same procedure was used as reported above to give again the crude peptoid **9**. A solution of 1*H*-pyrazole-1-carboxamidine hydrochloride (128 mg, 870 μmol, 20.0 equiv) in *N*,*N*-dimethylacetamide (DMA; 3.00 mL) and *N*,*N*-diisopropyl-*N*-ethylamine (DIPEA; 303 μL, 225 mg, 1.74 mmol, 40.0 equiv) was added to the peptoid **9**. The reaction vessel was subjected to microwave irradiation to keep the constant temperature at 60 °C for 120 min while being stirred. Water was added and the mixture was lyophilized to give peptoid **8**. After HPLC purification (method 3, $R_t = 19.1$ min) 3.19 mg of the product was obtained with a purity of 99%. MALDI-TOF-MS [*m*/*z*]: [M]$^+$ calcd for $C_{85}H_{147}N_{28}O_8^+$, 1689.26 (average mass); found, 1688.6.

HPLC Purification. Reverse-phase analytical HPLC was performed using Agilent Series 1100, employing a C18 column PerfectSil Target (MZ-Analytik, 3−5 μm, 4.0 × 250 mm). Reverse-phase semipreparative HPLC was performed using Agilent Series 1200, using a C8 Zorbax 300SB-C8 column (Agilent, 5 μm, 9.4 × 250 mm). Flow rate: 1.5 mL; A, 0.1% TFA (trifluoroacetic acid) in H_2O; B, 0.1% TFA in acetonitrile.

Method 1 (peptoid **9**): 25% B to 55% B over 30 min at 15 °C, $R_t = 22.6$ min.

Method 2 (peptoid **10**): 5% B to 77% B over 38 min at 50 °C, $R_t = 33.2$ min.

Method 3 (peptoid **8**): 25% B to 65% B over 20 min at 60 °C, $R_t = 19.1$ min.

Separation of the peptoids was monitored with UV-detection in the range of 200−650 nm, and UV spectra along with MALDI-TOF-mass spectrometry were used to identify the product peaks. Manually collected fractions of the semipreparative runs were freeze-dried and immediately used in the assays. Prior to lyophilization, fraction aliquots were directly reinjected onto the analytical column to quantify purity, which was determined by integration of the respective single peak area from the chromatograms at 218 nm.

4.5. Bulk Experiments. All bulk solution UV/vis spectra were recorded with a Cary 500 scan (Varian) or Lambda 5 UV/vis spectrometer in 1 cm cuvettes. All bulk solution fluorescence spectra were obtained using quartz cuvettes in a Cary Eclipse fluorescence spectrometer (Varian). Quantum yields were measured at room temperature against a perylene (*N*-(2,6-diisopropylphenyl)-*N*′-[3-(*N*-succinimidyl)carboxyethyl]-1,6,7,12-tetra(4-sulfophenoxy)perylene-3,4:9,10-tetracarboxydiimide) with known quantum yield.[12] Fluorescence lifetimes were measured on samples prepared

by dropping 10 μL of the concentrated solutions (20−150 μM) on clean glass coverslips (170 μm thickness). Samples were studied using a microscope (Axiovert, Zeiss) in an epifluorescence configuration. For illumination, a white light laser source (SuperK Versa, 80 MHz, Koheras) was used. The laser light passed bandpass filters to selectively illuminate the samples with light in the range of 510−560 nm (Filter set 14, Zeiss). The emission light passed an objective (40×, 0.6 NA, Zeiss), a dichroic mirror, and a filter to remove scattered excitation light. It was detected by single-photon avalanche photodiodes (APD; SPCM-AQRH, PerkinElmer). Acquisition was done using a time correlated single-photon counting (TCSPC) PC card (SPC 830, Becker & Hickl GmbH). Signals were recorded in first in, first out (FIFO) mode, where time intervals of each photon with respect to the excitation pulse as well as the time lag with respect to the previously detected photon were recorded. The decay curves were fitted exponentially.

4.6. Single-Molecule Experiments. ***Sample Preparation.*** Glass coverslips (170 μm thickness) were cleaned to remove sources of fluorescence. They were baked for 8 h at 510 °C followed by a cleaning procedure: five parts deionized water and one part 27% ammonia solution (Carl Roth) were heated to 70 ± 5 °C. The solution was removed from the hot plate, and one part 30% hydrogen peroxide (Carl Roth) was added. Coverslips were soaked in the solution for 15 min. Glass slides were washed several times with deionized water and dried with nitrogen. Solutions for spectroscopy measurements were prepared in pure solvents. Samples in poly(methyl methacrylate) (PMMA; AR-P649.04, MW = 200000 g/mol, 4% (w/w) in ethyl acetate, Allresist) were prepared as follows: solutions of PMMA were doped with a small volume of nanomolar concentrated dye solution for single-molecule samples. Solutions were spin-coated onto clean cover slides (900 rpm for 10 s, 4000 rpm for 90 s) and dried.

Measurement Procedure. Samples were studied using the same setup as described for the lifetime measurements of bulk solutions but with an oil immersion objective (100×, 1.46 NA, Zeiss). 2D-images were recorded by point-by-point measurements of the samples with a raster-scanning *x*,*y*−piezo stage (JPK Instruments, TAO-stage). Signals were recorded in the FIFO mode and synchronized to each pixel. Therefore, also fluorescence lifetime images were generated and analyzed. Single molecules were examined by targetting the laser focus on individual fluorescent spots, and fluorescence intensity traces (transients) were recorded. Data were analyzed using homemade Matlab programs and OriginPro 8G.

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