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Bioimaging with Macromolecular Probes Incorporating Multiple BODIPY Fluorophores

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Abstract

Seven macromolecular constructs incorporating multiple borondipyrromethene (BODIPY) fluorophores along a common poly(methacrylate) backbone with decyl and oligo-(ethylene glycol) side chains were synthesized. The hydrophilic oligo(ethylene glycol) components impose solubility in aqueous environment on the overall assembly. The hydrophobic decyl chains effectively insulate the fluorophores from each other to prevent detrimental interchromophoric interactions and preserve their photophysical properties. As a result, the brightness of these multicomponent assemblies is approximately three times greater than that of a model BODIPY monomer. Such a high brightness level is maintained even after injection of the macromolecular probes in living nematodes, allowing their visualization with a significant improvement in signal-to-noise ratio, relative to the model monomer, and no cytotoxic or behavioral effects. The covalent scaffold of these macromolecular constructs also permits their subsequent conjugation to secondary antibodies. The covalent attachment of polymer and biomolecule does not hinder the targeting ability of the latter and the resulting bioconjugates can be exploited to stain the tubulin structure of model cells to enable their visualization with optimal signal-to-noise ratios. These results demonstrate that this particular structural design for the incorporation of multiple chromophores within the same covalent construct is a viable one to preserve the photophysical properties of the emissive species and enable the assembly of bioimaging probes with enhanced brightness.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjugchem.7b00166.

Synthesis of **6** and **8**; Normalized absorption and emission spectra of **10**; Critical concentration of **1e**; Normalized absorption and emission spectra of **10** and **1e**; Fluorescence decays of **10** and **1e**; Absorption and emission spectra of the model Alexa-conjugated antibody.(PDF)

Video of *Caenorhabditis elegans* injected with blank PBS (AVI)

Video of *Caenorhabditis elegans* injected with **10** (AVI)

Video of *Caenorhabditis elegans* injected with **1e** (AVI)

The authors declare no competing financial interest.

Graphical abstract

INTRODUCTION

Fluorescence measurements¹ permit the detection of cells in biological fluids² and the visualization of tissues in living organisms.³ Fast response, inherent sensitivity, and noninvasive character are the main reasons behind their widespread application in cytometry and imaging. Their experimental implementation, however, requires first the labeling of a given biotarget with appropriate fluorescent probes.⁴ Generally, a fluorescent chromophore is attached to a targeting agent, in the form of an antibody, an aptamer, a peptide, or even a relatively small ligand, capable of selectively directing the signaling unit to the target of interest. The supramolecular association of the complementary components then immobilizes the fluorophore on the target and allows the detection of the latter after collecting the emission of the former. Under these conditions, the brightness of the fluorophore ultimately dictates the intensity of the detected signal and the sensitivity of the overall protocol.^{2,3} This particular photophysical parameter is equal to the product of the molar absorption coefficient (ϵ) of the fluorophore at the excitation wavelength and its fluorescence quantum yield (ϕ).¹ For most organic fluorophores, ϵ is lower than $10^2 \text{ mM}^{-1} \text{ cm}^{-1}$, and therefore, their brightness ($\epsilon \times \phi$) barely approaches $10^2 \text{ mM}^{-1} \text{ cm}^{-1}$, even when ϕ is close to unity. For example, indocyanine green, which is one of the most common synthetic dyes for fluorescence measurements *in vivo* and is approved for use in humans, has a brightness of only $11 \text{ mM}^{-1} \text{ cm}^{-1}$.⁵

In principle, more than one fluorescent chromophore can be attached to a single targeting agent, in order to increase the values of ϵ and, hence, the brightness associated with each binding event. However, the clustering of independent fluorophores within a restricted volume generally leads to significant interactions in the excited state that inhibit radiative deactivation.¹ Therefore, the increase in ϵ with the assembly of multiple chromophores into a single molecular, macromolecular, or supramolecular construct is often counteracted by a pronounced decrease, or even complete suppression, of ϕ . As a consequence of this fundamental limitation, inherent to the excitation dynamics of organic chromophores, the identification of viable designs to integrate multiple fluorophores within relatively compact synthetic structures to enhance brightness remains far from trivial.^{6–15}

Hundreds of identical chromophores can be encapsulated within the same polymer particle to generate fluorescent assemblies with a brightness approaching $10^4 \text{ mM}^{-1} \text{ cm}^{-1}$.^{16–20} Such brightness levels are several orders of magnitude greater than those accessible with individual organic fluorophores and far exceed even those of semiconductor quantum dots.^{21,22} Nonetheless, the encapsulation of the fluorescent guests within the polymer particles and, often, also the structural integrity of the host matrix itself are a result of noncovalent interactions. The reversible nature of these supramolecular contacts can lead to the leakage of the guests out of the particles, as well as to the disassembly of the hosts, under the extreme dilution that can occur in biological fluids. A possible solution to this problem can be the covalent connection, rather than the noncovalent encapsulation, of many emissive chromophores to a common polymer chain^{11,15} or cross-linked scaffold.¹² For example, polymers with BODIPY fluorophores in their side chains can be assembled with relatively simple synthetic procedures to efficiently generate covalent constructs with a brightness that can also approach $10^4 \text{ mM}^{-1} \text{ cm}^{-1}$.^{23,24} Nonetheless, the hydrodynamic diameter (d_H) of these fluorescent constructs, as well as those of their noncovalent counterparts, exceeds 50 nm in most instances.^{16–20} Such physical dimensions are more than 2 orders of magnitude greater than those of a single chromophore and can have detrimental effects on the supramolecular association of the targeting agent, as well as complicate administration in vivo and clearance from the organism. Thus, viable structural designs to engineer fluorescent probes with bright emission together with long-term stability, compact dimensions, targeting compatibility, and lack of toxicity are still very much needed. The availability of such materials would be particularly valuable in the biomedical laboratory and could ultimately facilitate the implementation of clinical and surgical applications based on convenient fluorescence measurements.

Our laboratories developed a series of amphiphilic polymers with multiple decyl and oligo(ethylene glycol) side chains along a common poly(methacrylate) backbone.^{25–28} In aqueous environments, these macromolecules assemble spontaneously into particles with d_H close to 20 nm, hydrophilic surface, and hydrophobic interior. In the process of assembling, they can capture multiple hydrophobic fluorophores in their interior and transfer these, otherwise insoluble, chromophores into aqueous solutions. The supramolecular shell encapsulating the fluorescent components protects them from the aqueous environment and preserves their photophysical properties. Furthermore, the hydrophobic side chains of the macromolecular components isolate the entrapped fluorophores from each other, preventing interchromophoric interactions in both the ground as well as excited states. As a result, a negligible influence on ϕ is generally observed upon encapsulation of multiple fluorophores within these supramolecular containers, offering the possibility of constructing supramolecular assemblies with relatively large brightness. Nonetheless, noncovalent contacts are solely responsible for holding fluorophores and polymers together. In fact, these supramolecular nanocarriers can discharge their fluorescent cargo into the many lipophilic domains found in biological preparations.^{28,29} These considerations suggested the design of similar amphiphilic macromolecular constructs with multiple fluorophores covalently connected to the polymer backbone, instead of being noncovalently encapsulated into the corresponding supramolecular nanocarriers.²⁸ This article reports the synthesis and characterization of two families of macromolecular probes engineered around this structural

design, together with the spectroscopic investigation of their photophysical properties and the assessment of their performance in model biological preparations.

RESULTS AND DISCUSSION

Design, Synthesis, and Structural Characterization

Two structural designs for the covalent integration of BODIPY chromophores within amphiphilic macromolecular constructs were envisaged. In one, the chromophoric units are directly attached to a poly(methacrylate) backbone together with decyl and oligo(ethylene) side chains (**1** in Figure 1). In the other, the chromophores are connected to the ends of the hydrophobic side chains (**2** in Figure 2). Both were prepared from the random polymerization of the corresponding methacrylate monomers in tetrahydrofuran (THF), under the assistance of azobis(*i*-butyronitrile) (AIBN). In the first instance, the stoichiometry of the three monomers was varied systematically to produce five macromolecules (**1a–e** in Table 1) differing in the number (*N* in Table 1) of chromophores per polymer chain and in the ratio (χ in Table 1) between the hydrophobic and hydrophilic side chains. The second design was exploited to assemble macromolecules without and with (**2a** and **2b** in Figure 2) a third side chain terminated by a carboxylic acid. The latter was treated with *N*-hydroxysuccinimide (NHS), *N,N*-dicyclohexylcarbodiimide (DCC), and 4-*N,N*-dimethylaminopyridine (DMAP) in dichloromethane and then coupled to a secondary antibody (**9** in Figure 2) in *N,N*-dimethylformamide (DMF) and bicarbonate buffer (BCB). The resulting conjugate (**2c** in Figure 2) was isolated from any unreacted starting materials by size-exclusion chromatography.

Gel permeation chromatography (GPC) of **1a–e** and **2a,b** indicated their number-average molecular weight ($\bar{M}_n$ in Table 1) to range from 14.0 to 24.9 kDa with a dispersity index (in Table 1) varying from 1.42 to 2.36. Integration of the resonances associated with protons of the BODIPY chromophores, hydrophobic chains, and hydrophilic tails in the corresponding ^1H NMR spectra provided estimates of *N* and χ (Table 1). For example, the ^1H NMR spectrum (Figure 3) of **1e** shows a multiplet at 7.30 ppm for the two pairs of homotopic protons on the phenylene ring of the BODIPY chromophores, a singlet at 3.36 ppm for the methoxy protons at termini of the hydrophilic chains, and a multiplet at 0.88 ppm for the methyl protons at the ends of the hydrophobic chains. These three sets of resonances integrate for 4, 5, and 18 protons, respectively. These values correspond to *N* of 5.3 and χ of 3.6. Similarly, the ^1H NMR spectrum (Figure 3) of **2b** shows a pair of doublets at 8.16 and 7.39 ppm for the phenylene protons of the BODIPY chromophores and a singlet at 3.36 ppm for the methoxy protons of the hydrophilic chains. These resonances integrate for 16 and 40 protons, respectively, and correspond to *N* and χ of 6.4 and 0.3, respectively. Additionally, the spectrum shows also a multiplet at 2.64 ppm for the methylene protons adjacent to the carboxylic acid, integrating for 4 protons. This value suggest that **2b** incorporates an average of 1.6 chains with terminal carboxylic acids within its macromolecular backbone.

Absorption and Emission Spectroscopies

The absorption and emission spectra (Figure S3) of a model monomer (**10** in Table 2) dissolved in THF show the characteristic bands of the BODIPY chromophore with maxima at wavelengths (λ_{Ab} and λ_{Em} in Table 2) of 527 and 541 nm. The very same bands are also observed for all polymers (Figure 4) with minimal shifts in λ_{Ab} and λ_{Em} . The presence of multiple BODIPY chromophores, however, enhances the molar absorption coefficient (ϵ in Table 2) at λ_{Ab} from 58.0 mM⁻¹ cm⁻¹ for monomer **10** to up to 429.2 mM⁻¹ cm⁻¹ for polymer **2a**, but has negligible influence on the fluorescence quantum yield (ϕ in Table 2). Specifically, ϕ is 0.63 for **10** and ranges from 0.45 to 0.71 for the polymers. As a result, the brightness ($\epsilon \times \phi$ in Table 2) of all polymers, with exception of **1c**, is greater than that of the monomer and approaches 206.0 mM⁻¹ cm⁻¹ for polymer **1e**, while it is only 36.4 mM⁻¹ cm⁻¹ for **10**. Furthermore, this photophysical parameter increases with N for both series of polymers. The only outlier on this trend is **1d**, which has more chromophores per polymer chain, but lower brightness, than **1e**. Presumably, this apparent contradiction is a consequence of the high relative amount (χ in Table 1) of hydrophobic components in **1e**, which ensure the effective insulation of the chromophores from each other. These observations suggest that the brightness of these macromolecules can be optimized even further with the elongation of their polymer backbone, and hence an increase of their $\bar{M}_n$, as long as a significant excess of hydrophobic components, relative to their hydrophilic counterparts, is maintained within the overall amphiphilic construct.

The transition from THF to phosphate buffer saline (PBS) has negligible influence on the photophysical parameters of the monomer (Table 2 and Figure S3). By contrast, it shifts bathochromically λ_{Em} and depresses ϕ for all polymers (Table 2 and Figure 4). These observations suggest that the environment around the BODIPY chromophores changes drastically on going from organic to aqueous solution only when they are attached to a macromolecular backbone. Presumably, the amphiphilic polymers alter their geometry significantly to avoid direct exposure of their hydrophobic domains to water molecules and encourage the nonradiative deactivation of the excited BODIPY chromophores. In spite of these effects, the brightness of some of the polymers remains greater than that of the monomer, which is only 22.6 mM⁻¹ cm⁻¹ under these conditions. Specifically, **1e** maintains the largest brightness, out of all the macromolecular constructs tested, to approach 61.2 mM⁻¹ cm⁻¹.

The photophysical parameters of **1a–e** and **2a,b** were determined at a concentration of 30 μ g mL⁻¹. This value is lower than the corresponding critical micellar concentration (CMC in Table 1), which ranges from 43.4 to 104.1 μ g mL⁻¹, and was selected to avoid the aggregation of the amphiphilic polymers into supramolecular assemblies in aqueous environment. In turn, the CMC of all macromolecules was determined with the aid of a hydrophobic BODIPY chromophore (**14** in Figure S4) with extended electronic conjugation. This compound is essentially insoluble in PBS and its fluorescence can be detected only in the presence of sufficient amounts of any one of the seven amphiphilic polymers. Indeed, plots (Figure S4) of the emission intensity of **14** against the polymer concentration all show a sudden fluorescence increase above a given concentration threshold, which is the corresponding CMC value. These observations suggest that at concentrations greater than

the threshold values the amphiphilic macromolecules assemble into supramolecular nanocarriers capable of capturing **14** in their hydrophobic interior, transferring it into the aqueous phase, and allowing the detection of its fluorescence. Consistently, dynamic light scattering (DLS) measurements performed at concentrations greater than CMC confirm the formation of nanoscaled aggregates with hydrodynamic diameters (d_H in Table 1) ranging from 8.0 to 21.0 nm.

The methyl groups in positions 1 and 7 of the BODIPY chromophores of all polymers, as well as of the model monomer, are expected to hinder the rotation of the heterocyclic platform about the [C–C] bond connecting it to the adjacent phenylene ring and preserve ϕ . Indeed, literature data^{30–35} demonstrate that the conformational freedom of unsubstituted BODIPY derivatives facilitates instead the nonradiative deactivation of their excited state and translates into a significant dependence of their ϕ and fluorescence lifetime (τ) on the viscosity of the surrounding environment. By contrast, the normalized absorption and emission spectra (Figure S5) of **10** in THF and glycerol are essentially identical and ϕ is 0.63 in one solvent and 0.66 in the other, respectively. In both environments, the fluorescence decays monoexponentially with similar kinetics and τ is 4.67 ns in THF and 5.99 ns in glycerol. These observations confirm that the steric hindrance engineered into the BODIPY of **10** restricts rotation, in agreement with the logic behind the structural design of these chromophores. The very same constraints are also designed into the BODIPY components of the polymers. Nonetheless, their photophysical properties appear to be affected by the viscosity of the solvent. Specifically, comparison of the normalized emission spectra (Figure S5) of **1e** in THF and glycerol shows a bathochromic shift of 11 nm and considerable broadening with a viscosity increase, while the absorption spectra in both solvents are almost identical. Additionally, ϕ decreases from 0.67 in THF to only 0.14 in glycerol and τ shortens from 4.66 ns in one solvent to 1.41 ns in the other (Figure S6). These trends are in contrast to the negligible changes observed for the model monomer and the enhancements in ϕ and τ with viscosity reported for unsubstituted BODIPY derivatives.^{30–35} Such an apparent contradiction indicates that the influence of viscosity on the photophysical properties of **1e** must be a result of the restricted conformational freedom on the amphiphilic scaffold, rather than the actual chromophoric components. Presumably, the inability of the amphiphilic polymer to reorganize in a viscous medium, upon excitation of the chromophores, is responsible for promoting the nonradiative deactivation of the BODIPY components and causing the observed decrease in ϕ and τ .

Fluorescence Imaging

Images of live *Caenorhabditis elegans*, microinjected with either blank PBS (**a** in Figure 5) or a PBS solution of **10** (**b** in Figure 5) in the gonadal region, show the fluorescent probes to accumulate in the fat droplets in the intestinal tract of the nematode. Under identical experimental conditions, injection of **1e** (**c** in Figure 5) resulted in bright, diffuse fluorescence throughout the rest of the nematode with the exception of the eggs. Presumably, the different distributions are a consequence of the amphiphilic character of **1e** and hydrophilic nature of **10**. The amphiphilic polymer can diffuse much more easily than the hydrophilic model into additional tissues of the worm, possibly via trafficking through the coelomocytes.

The emission intensity detected within the living organism for the macromolecular probe is significantly greater than that measured for the model monomer. Fluorescence profiles (*e* and *f* in Figure 5), collected along lines drawn across the imaged nematodes, reveal a 4-fold enhancement in emission for the polymer, even although the concentration (50 μM) of the probes was the same in both injected solutions. These observations are fully consistent with the brightness measured spectroscopically for the two systems in PBS, which is $61.2 \text{ mM}^{-1} \text{ cm}^{-1}$ for **1e** but only $22.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for **10** (Table 2), and demonstrate that the macromolecular probe retains its photophysical properties within the living organism. Furthermore, the injected polymer does not appear to have any effect on the nematode. Sequences of images (Videos S1–S3), recorded to monitor the movements of the worms in real time, do not reveal any significant difference in the behavior of three injected nematodes over the course of 60 min.

The ability of **2c** to immunolabel biological preparations can be assessed with the aid of Alexa Fluor 647-conjugated affiniPure goat anti-mouse IgG (H+L). This model bioconjugate incorporates the very same secondary antibody of **2c** and is known to associate with *anti- α -tubulin* to allow the imaging of tubulin filaments in a variety of cell lines.⁴ Furthermore, the absorption and emission bands of the BODIPY fluorophores of **2c** and those of the Alexa dyes (Figure S7) of the model system are sufficiently resolved across the visible region of the electromagnetic spectrum to permit the imaging of both in separate detection channels. As a result, cells can be labeled with the two conjugates simultaneously and their localization probed independently. Specifically, images of HeLa cells treated with both bioconjugates show the BODIPY fluorescence in one channel (*a* in Figure 6) and Alexa emission in the other (*b* in Figure 6). Both images reveal the characteristic shape of the tubulin filaments and an overlay (*c* in Figure 6) of the two clearly shows the colocalization of the two sets of probes. These observations demonstrate that the conjugation of the macromolecular probe to the antibody does not affect the ability of the biomolecule to associate selectively with its target and that such antibody–polymer construct can, indeed, be employed to label and visualize biological preparations.

CONCLUSIONS

Multiple BODIPY chromophores can be appended to a common poly(methacrylate) backbone together with decyl and oligo(ethylene glycol) side chains. The hydrophobic decyl components isolate the fluorophores effectively from each other, preventing interchromophoric interactions. As a result, the fluorescence quantum yield of these macromolecular constructs remains relatively high and is comparable to that of a model BODIPY monomer. In turn, the hydrophilic components ensure solubility in aqueous environment, where the photophysical properties of the emissive species are, once again, preserved by the effective insulation of the hydrophobic side chains. The presence of multiple chromophoric units, however, enhances the molar absorption coefficient significantly and translates into a 3-fold increase in brightness, relative to the model BODIPY. Such macromolecular probes can be microinjected into living nematodes, where they retain their characteristic brightness levels and allow the visualization of the organisms with signal-to-noise ratios greater than those accessible with the model monomer. Furthermore, the fluorescent polymers do not have any significant effects on the behavior of

the living organisms. The architecture of these macromolecular assemblies can be modified to permit the subsequent conjugation of antibodies and enable the immunolabeling of biological preparations. Specifically, carboxylic acids can be appended to the termini of some of the hydrophilic chains and then connected to the primary amino groups of a secondary antibody. Comparison of the resulting antibody conjugates to model systems demonstrate that the biomolecules retain their ability to bind their complementary targets and allow the visualization of the tubulin filaments of model cells with optimal signal-to-noise ratios. In summary, these particular structural designs for the covalent integration of multiple fluorescent chromophores into the same construct (1) provide brightness levels greater than those accessible with conventional fluorophores, (2) allow the imaging of living organisms, and (3) enable the immunolabeling of model biological preparations. Thus, a general strategy for the assembly of macromolecular probes with optimal photophysical properties and targeting capabilities for bioimaging applications can ultimately evolve from these investigations.

EXPERIMENTAL PROCEDURES

Materials and Methods

Chemicals were purchased from commercial sources and used as received with the exception of THF, which was distilled over Na and benzophenone, and H₂O, which was purified with a Barnstead International NANOpure Diamond Analytical system. Compounds **3–5**, **10**, **12**, and **14** were prepared according to literature procedures.^{29,36–38} The synthesis of **6** and **8** are illustrated in Figures S1 and S2. GPC was performed with a Phenomenex Phenogel 5- μ m MXM column (7.8 $\times$ 300 mm) operated with a Shimadzu Nexera X2 system in THF at a flow rate of 1.0 mL min⁻¹. Monodisperse polystyrene standards (2.7–200.0 kDa) were employed to determine the $\bar{M}_n$ and $\bar{M}_w$ of the polymers from the GPC traces, following a literature protocol.³⁹ EISMS was performed with a Bruker micrOTO-Q II spectrometer. NMR spectra were recorded with a Bruker Avance 500 spectrometer. DLS measurements were performed with a Malvern ZEN1600 apparatus. The values listed for d_H in Table 1 are averaged over ten independent experiments of ten runs of 10 s each. Absorption spectra were recorded with a Varian Cary 100 Bio spectrometer, using quartz cells with a path length of 1.0 cm. Emission spectra were recorded with a Varian Cary Eclipse spectrometer in aerated solutions. Fluorescence quantum yields were determined with a fluorescein standard, following a literature protocol.⁴⁰ Time-correlated single-photon counting measurements were performed with an Edinburgh Analytical Instruments nF920 spectrometer. Samples were excited at 405 $\pm$ 10 nm, using a light-emitting diode with a pulse width of 1 ns, and the emission intensity was recorded at 540 nm.

General Procedure for the Synthesis of 1a–e

A solution of **3** (23.2 mg, 0.05 mmol), **4** (40.9 mg, 0.18 mmol for **a**; 56.8 mg, 0.25 mmol for **b**; 40.9 mg, 0.18 mmol for **c**; 18.2 mg, 0.08 mmol for **d**; 68.1 mg, 0.3 mmol for **e**), **5** (540 mg, 0.27 mmol for **a**; 540 mg, 0.27 mmol for **b**; 340 mg, 0.17 mmol for **c**; 280 mg, 0.14 mmol for **d**; 300 mg, 0.15 mmol for **e**), and AIBN (4.9 mg, 0.03 mmol) in degassed THF (8 mL) was heated for 72 h at 75 °C under Ar in a sealed vial. After cooling down to ambient temperature, the reaction mixture was transferred to a centrifuge tube and diluted with THF

to a total volume of 10 mL. Hexane was added in portions of 1 mL and the tube was shaken vigorously, after each addition, until the formation of a precipitate was clearly observed. After centrifugation, the oily layer at the bottom of the tube was separated from the supernatant and dissolved in THF (10 mL). The treatment with hexane, followed by centrifugation, was repeated 3 times and the final oily residue was dried under reduced pressure to give **1** (90 mg for *a*; 100 mg for *b*; 65 mg for *c*; 39 mg for *d*; 48 mg for *e*) as a red solid.

1a. GPC— $\bar{M}_n = 14.0$ kDa, $\eta_{inh} = 1.42$; $^1\text{H NMR}$ (CD_2Cl_2): $\delta = 0.82\text{--}0.95$ (19H, m), 0.96–1.18 (17H, m), 1.20–1.46 (58H, m), 1.54–1.74 (15H, m), 1.76–2.06 (11H, m), 2.09–2.16 (4H, m), 2.33 (4H, bs), 2.44–2.56 (6H, m), 3.36 (15H, s), 3.39–3.82 (702H, m), 3.86–4.25 (16H, m), 7.18–7.42 (4H, m).

1b. GPC— $\bar{M}_n = 21.8$ kDa, $\eta_{inh} = 1.90$; $^1\text{H NMR}$ (CD_2Cl_2): $\delta = 0.81\text{--}0.96$ (13H, m), 0.96–1.19 (15H, m), 1.21–1.48 (45H, m), 1.54–2.22 (34H, m), 2.34 (3H, s), 2.50 (4H, s), 3.36 (12H, s), 3.41–3.86 (551H, m), 3.88–4.35 (17H, m), 7.18–7.44 (4H, m).

1c. GPC— $\bar{M}_n = 17.9$ kDa, $\eta_{inh} = 1.69$; $^1\text{H NMR}$ (CDCl_3): $\delta = 0.75\text{--}0.90$ (28H, m), 0.94–1.02 (20H, m), 1.18–1.38 (106H, m), 1.55–1.66 (16H, m), 1.68–1.92 (20H, m), 2.29 (8H, bs), 2.45–2.57 (14H, m), 3.37 (23H, s), 3.52–3.74 (1024H, m), 3.89–4.20 (23H, m), 7.16–7.24 (4H, m).

1d. GPC— $\bar{M}_n = 16.4$ kDa, $\eta_{inh} = 1.53$; $^1\text{H NMR}$ (CD_2Cl_2): $\delta = 0.81\text{--}0.96$ (6H, m), 0.97–1.05 (4H, m), 1.19–1.46 (23H, m), 1.65 (4H, bs), 2.14 (2H, s), 2.20–2.40 (4H, m), 2.44–2.56 (6H, m), 3.36 (3H, s), 3.41–3.82 (187H, m), 3.90–4.22 (64H, m), 7.19–7.41 (4H, m).

1e. GPC— $\bar{M}_n = 24.9$ kDa, $\eta_{inh} = 2.34$; $^1\text{H NMR}$ (CD_2Cl_2): $\delta = 0.81\text{--}0.95$ (18H, m), 0.96–1.09 (11H, m), 1.19–1.48 (63H, m), 1.65 (9H, bs), 1.72–1.86 (6H, m), 2.23–2.42 (5H, m), 2.51 (5H, bs), 3.36 (5H, s), 3.43–3.82 (254H, m), 3.86–4.24 (11H, m), 7.22–7.38 (4H, m).

General Procedure for the Synthesis of **2a** and **2b**

A solution of **6** (100 mg, 0.15 mmol), **7** (225 mg, 0.45 mmol for *a*; 205 mg, 0.41 mmol for *b*) without (for *a*) or with **8** (29 mg, 0.03 mmol for *b*), and AIBN (5 mg, 0.03 mmol) in degassed THF (8 mL) was heated for 72 h at 75 °C under Ar in a sealed vial. After cooling down to ambient temperature, the reaction mixture was transferred to a centrifuge tube and diluted with THF to a total volume of 10 mL. Hexane was added in portions of 1 mL and the tube was shaken vigorously, after each addition, until the formation of a precipitate was clearly observed. After centrifugation, the oily layer at the bottom of the tube was separated from the supernatant and dissolved in THF (10 mL). The treatment with hexane, followed by centrifugation, was repeated 3 times and the final oily residue was dried under reduced pressure to give **2** (100 mg for *a*; 110 mg for *d*) respectively as a red solid.

2a. GPC— $\bar{M}_n = 20.1$ kDa, $\eta_{inh} = 2.26$; $^1\text{H NMR}$ (CDCl_3): $\delta = 0.80\text{--}0.91$ (25H, m), 0.95–1.02 (11H, m), 1.25–1.30 (19H, m), 1.32–1.40 (15H, m), 1.58–1.65 (4H, m), 1.70–1.78 (4H,

m), 1.80–1.90 (8H, m), 2.50–2.56 (6H, s), 3.38 (15H, s), 3.51–3.72 (158H, m), 3.85–3.96 (3H, m), 4.04–4.14 (10H, m), 4.32–4.40 (2H, m), 7.41 (2H, d, 8 Hz), 8.18 (2H, d, 8 Hz).

2b. GPC— $\bar{M}_n$ = 15.8 kDa, $\bar{M}_w/\bar{M}_n$ = 2.36; ^1H NMR (CDCl_3): δ = 0.75–0.90 (7H, m), 0.92–1.05 (11H, m), 1.25 (7H, s), 1.28–1.38 (12H, m), 1.40–1.1.48 (3H, m), 1.55–1.62 (3H, m), 1.75–1.90 (14H, m), 2.25–2.35 (4H, m), 2.58 (6H, s), 2.60–2.67 (1H, m), 3.36 (10H, s), 3.55–3.70 (113H, m), 3.78–3.82 (1H, m), 3.85–3.95 (2H, m), 4.08 (7H, bs), 4.30–4.37 (2H, m), 7.39 (2H, d, 8 Hz), 8.16 (2H, d, 8 Hz).

Synthesis of 2c

A solution of DCC (19.3 mg, 0.09 mmol) in CH_2Cl_2 (2 mL) was added dropwise, over the course of 20 min, to a solution of **2b** (150 mg, 0.009 mmol), DMAP (2.2 mg, 0.1 mmol) and NHS (12.9 mg, 0.11 mmol) in CH_2Cl_2 (6 mL) maintained at 0 °C under Ar. The reaction mixture was allowed to warm up to ambient temperature and was stirred for 24 h under these conditions. The resulting precipitate was filtered off and the solvent was distilled off under reduced pressure to afford a red oil (120 mg). ^1H NMR (CDCl_3): δ = 0.78–0.90 (7H, m), 0.92–1.05 (10H, m), 1.22–1.28 (10H, m), 1.30–1.40 (12H, m), 1.42–1.1.50 (3H, m), 1.55–1.65 (3H, m), 1.75–1.90 (13H, m), 2.23–2.34 (4H, m), 2.52 (6H, bs), 2.80–2.86 (2H, m), 3.36 (10H, s), 3.55–3.70 (109H, m), 3.78–3.82 (1H, m), 3.85–3.95 (2H, m), 4.04–4.15 (8H, m), 4.30–4.37 (2H, m), 7.39 (2H, d, 8 Hz), 8.16 (2H, d, 8 Hz). An aliquot (1.5 mg) of the residue was dissolved in PBS (100 μL) and combined with an aqueous solution of **9** (Jackson ImmunoResearch Inc. 115–005–003; 1 mg, 435 μL), whose pH was adjusted to 8.8 with BCB (1 M, 75 μL). The mixture was stirred for 1 h at ambient temperature and purified by column chromatography (Sephadex G25, PBS). The collected fractions (0.4 mL each) were analyzed by absorption spectroscopy and those showing the characteristic absorption of the BODIPY chromophores at 528 nm were used for further spectroscopic and imaging experiments.

6—A solution of DCC (106.6 mg, 0.52 mmol) in CH_2Cl_2 (2 mL) was added dropwise, over the course of 20 min, to a solution of **11** (114 mg, 0.48 mmol), DMAP (12 mg, 0.1 mmol), and **12** (200 mg, 0.48 mmol) in CH_2Cl_2 (5 mL) maintained at 0 °C under Ar. The reaction mixture was allowed to warm up to ambient temperature and was stirred for 24 h under these conditions. The resulting precipitate was filtered off and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO_2 : hexane/ EtOAc (4:1, v/v)] to afford **6** (120 mg, 39%) as a red oil. ESIMS: m/z = 671.3783 [$\text{M} + \text{Na}$] $^+$ (m/z calcd. for $\text{C}_{38}\text{H}_{51}\text{BF}_2\text{N}_2\text{O}_4\text{Na}$ = 671.3808); ^1H NMR (CDCl_3): δ = 0.97 (6H, t, 8 Hz), 1.27 (6H, s), 1.30–1.43 (10H, m), 1.45–1.52 (2H, m), 1.65–1.72 (2H, m), 1.78–1.85 (2H, m), 1.95 (3H, s), 2.28–2.234 (4H, q, 8 Hz), 2.55 (6H, s), 4.13–4.17 (2H, t, 8 Hz), 4.36–4.39 (2H, t, 8 Hz), 5.55 (1H, s), 6.10 (1H, s), 7.40–7.42 (2H, d, 8 Hz), 8.17–8.19 (2H, d, 8 Hz); ^{13}C NMR (CDCl_3): δ 11.9, 12.5, 14.5, 14.7, 17.1, 17.8, 18.3, 18.9, 26.0, 28.6, 29.3, 29.4, 64.8, 65.5, 125.2, 128.6, 128.7, 130.1, 130.3, 130.9, 133.1, 136.5, 138.1, 138.7, 140.6, 154.2, 166.2, 167.6.

8—A solution of succinic anhydride (2.28 g, 22.8 mmol) and **13** (4.0 g, 7.6 mmol) in pyridine (21 mL) was stirred for 48 h at ambient temperature. The solvent was distilled off

under reduced pressure and the residue was purified by column chromatography [SiO₂:hexane/EtOAc (3:2, v/v)] to afford **8** (3.2 g, 67%) as a colorless oil. ESIMS: m/z = 649.3031 [M + Na]⁺ (m/z calcd. for C₂₈H₅₀O₁₅Na = 649.3047); ¹H NMR (CDCl₃): δ = 1.92 (3H, s), 2.62 (6H, bs), 3.55–3.72 (56H, m), 4.20–4.30 (5H, m), 5.55 (1H, s), 6.10 (1H, s).

11—A solution of DCC (1.3 g, 6.3 mmol) in CH₂Cl₂ (5 mL) was added dropwise, over the course of 20 min, to a solution of 1,10-decanediol (1.0 g, 5.7 mmol), DMAP (140 mg, 1.1 mmol), and methacrylic acid (494 mg, 5.7 mmol) in CH₂Cl₂ (5 mL) maintained at 0 °C under Ar. The reaction mixture was allowed to warm up to ambient temperature and stirred for 24 h under these conditions. The resulting precipitate was filtered off and the solvent was distilled off under reduced pressure. The residue was purified by column chromatography [SiO₂:hexane/EtOAc (4:1, v/v)] to afford **11** (700 mg, 50%) as a colorless oil. ESIMS: m/z = 265.1780 [M + Na]⁺ (m/z calcd. for C₁₄H₂₆O₃Na = 265.1780); ¹H NMR (CDCl₃): 1.24–1.40 (12H m), 1.50–1.70 (6H, m), 1.92 (3H, s), 3.59–3.63 (2H, t, 8 Hz), 4.09–4.13 (2H, t, 8 Hz), 5.52 (1H, s), 6.07 (1H, s); ¹³C NMR (CDCl₃): δ = 17.8, 18.3, 18.9, 25.7, 25.9, 28.6, 29.2, 29.4, 29.5, 32.8, 63.0, 64.8, 125.1, 136.5, 141.8, 154.0, 167.6, 173.8.

Nematode Imaging

Caenorhabditis elegans strain KG1188 *lite-1(ce314)* strain⁴¹ was used for fluorescence imaging. Microinjections were done using a Zeiss Axio Observer inverted microscope in age-matched adult hermaphrodites ~24 h past the L4 stage as described.⁴² Borosilicate glass needles were loaded with PBS or PBS solutions of **1e** (1.10 mg mL⁻¹) or **10** (0.12 mg mL⁻¹) by capillary action and injections (~200 pL each) were performed into worm gonads. Worms were recovered in M9 buffer, mounted onto Nematode Growth Media agar chunks, and overlaid with a glass coverslip for imaging using a HC Plan-Apo 20× (0.7 NA) objective Leica SP5 laser-scanning confocal microscope, as described.^{43,44}

Cell Imaging

HeLa cells were cultured in Dulbecco's modified Eagle's media supplemented with fetal bovine serum (10% v/v), penicillin (100 U mL⁻¹), and streptomycin (0.01% v/v) at 37 °C. Cells were fixed in MeOH (0.2 mL) at -20 °C for 15 min, washed with PBS (0.1 mL) three times, and maintained in a PBS solution of bovine serum albumin (BSA, 10 mg mL⁻¹, 0.1 mL) for 30 min at ambient temperature. Then, PBS solutions of *anti- α -tubulin* antibody (mouse monoclonal antibody, DM1A, Sigma; 0.8 μ g mL⁻¹, 0.1 mL) and BSA (10 mg mL⁻¹, 0.1 mL) were added to the extracellular matrix and the resulting sample was maintained for 1 h at ambient temperature. Cells were washed with a PBS solution of BSA (10 mg mL⁻¹, 0.1 mL) three times, incubated in a PBS solution (0.1 mL of 1% BSA) of **2c** (20 μ L, 2 μ g mL⁻¹) and Alexa Fluor 647-conjugated affipure g anti-mouse IgG (H+L) (100 μ L, 3 μ g mL⁻¹) for 1 h at ambient temperature, washed with a PBS solution of BSA (10 mg mL⁻¹, 0.1 mL) a further three times, and imaged with a Leica SP5 confocal laser-scanning microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Synthesis of amphoteric polymers **1a–e**.

Figure 2.
Synthesis of amphiphilic polymers **2a** and **2b** and antibody conjugation of the latter to produce **2c**.

Figure 3. ^1H NMR spectra (500 MHz) of **1e** and **2b** in CD_2Cl_2 and CDCl_3 , respectively, at 25 °C.

Figure 4. Normalized absorption and emission spectra ($30 \mu\text{g mL}^{-1}$, $\lambda_{\text{Ex}} = 470 \text{ nm}$) of **1e** and **2b** in THF and PBS at 25°C .

Figure 5.

Overlaid fluorescence ($\lambda_{\text{Ex}} = 514 \text{ nm}$, $\lambda_{\text{Em}} = 530\text{--}650 \text{ nm}$) and transmittance images (scale bar = $100 \mu\text{m}$) of *Caenorhabditis elegans* injected with PBS (a), a PBS solution of **10** (b), or a PBS solution of **1e** (c). Plots of the emission intensity measured along lines drawn across the nematodes injected with PBS (d), a PBS solution of **10** (e), or a PBS solution of **1e** (f) in the rectangular areas indicated in the corresponding image. Each plot is the average of five lines parallel to the short axis of the rectangle and equally spaced along the long axis.

Figure 6.

Fluorescence images (scale bar = 100 μm) of HeLa cells, immunolabeled with **2c**, and Alexa Fluor 647-conjugated affipure goat anti-mouse IgG (H+L), recorded with either a λ_{Ex} of 514 nm and a detection window of 530–650 nm (**a**) or a λ_{Ex} of 633 nm and a detection window of 650–750 nm (**b**) together with an overlay (**c**) of the two frames.

Table 1Structural Parameters of 1a–e and 2a,b^a

	M_n (kDa)	N	χ	CMC ($\mu\text{g mL}^{-1}$)	d_H (nm)	
1a	14.0	1.42	1.2	1.3	57.1	13.4
1b	24.9	2.34	2.7	1.1	53.7	8.7
1c	17.9	1.69	1.0	1.2	61.5	13.4
1d	16.4	1.53	5.6	2.0	104.1	21.0
1e	21.8	1.90	5.3	3.6	98.3	10.0
2a	20.2	2.26	7.4	0.2	43.4	9.0
2b	15.8	2.36	6.4	0.3	41.7	8.0

^aThe number-average molecular weight (M_n) and dispersity index (χ) were determined by GPC in THF, against monodisperse polystyrene standards (2.7–200.0 kDa). The number (N) of chromophore per polymer chain and the ratio (χ) between the hydrophobic and hydrophilic segments of each macromolecule were determined by ¹H NMR spectroscopy in CD₂Cl₂ or CDCl₃. The critical micellar concentration (CMC) was determined by emission spectroscopy in PBS. The hydrodynamic diameter (d_H) were measured by DLS in PBS at a concentration greater than the corresponding CMC.

Table 2

Absorption (λ_{Ab}) and Emission (λ_{Em}) Wavelengths, Molar Absorption Coefficient (ϵ), Fluorescence Quantum Yield (ϕ), and Brightness ($\epsilon \times \phi$) of **1a–e**, **2a,b**, and **10** at 25 °C^a

	λ_{Ab} (nm)		λ_{Em} (nm)		ϵ (mM ⁻¹ cm ⁻¹)		ϕ		$\epsilon \times \phi$ (mM ⁻¹ cm ⁻¹)	
	THF	PBS	THF	PBS	THF	PBS	THF	PBS	THF	PBS
1a	526	528	538	546	69.6	53.28	0.71	0.38	49.4	20.2
1b	526	527	537	541	156.6	119.88	0.54	0.18	84.6	21.6
1c	526	528	540	545	58.0	44.4	0.46	0.17	26.7	7.5
1d	526	529	541	550	324.8	248.64	0.55	0.10	178.6	24.9
1e	526	529	541	546	307.4	235.32	0.67	0.26	206.0	61.2
2a	526	529	542	550	429.2	328.56	0.45	0.18	193.1	59.1
2b	526	529	542	553	359.6	275.28	0.50	0.18	179.8	49.6
10	527	528	541	541	58.0	44.4	0.63	0.51	36.4	22.6

^aThe photophysical parameters of **1a–e** and **2a,b** were determined at a concentration (30 μ g mL⁻¹) that is lower than the corresponding CMC. The values of ϵ listed for **1a–3** and **2a,b** are the products of that of **10** and the number (*N* in Table 1) of chromophores per polymer chain. The values of ϕ were determined in aerated solutions against a fluorescein standard.