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PAPER

Synthesis and properties of fluorescent dyes conjugated to hyperbranched polyglycerols†‡

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Convergent syntheses of polyglycerol hyperbranched polymers containing fluorescent labels (fluorescein or perylene diimide (PDI)) at their core are presented. The hyperbranched polyglycerol (HPG) precursors were synthesized using a one step polymerization reaction wherein the initiator leaves a single reactive group for dye functionalization. For further site isolation, allylated HPG was synthesized allowing cross-linking *via* ring closing metathesis and subsequent dihydroxylation to produce water-soluble, fluorescent nanoparticles. The dyes produced showed improvements in photostability, water solubility, or quantum yield, depending on both the dye used and cross-linking. These fluorescent nanoparticles outperformed similar dyes that incorporated linear polyethylene glycol (PEG) polymers.

Introduction

Organic fluorescent dyes are a ubiquitous part of current biomedical and cellular biology research, finding use in applications ranging from fluorescence cell sorting,[1] cellular[2] and biomedical imaging,[3] to the measurement of biomolecule dynamics.[4] Enabling these technologies are the broad range of commercially available reactive fluorescent dyes.[5] Despite their long history and versatile use, there are still features that can be improved in these dyes such as enhanced brightness and improved photostability.[6] Outside the development of new synthetic dyes, researchers have shown that many of the traditional dyes such as the xanthine and cyanine dyes can be improved through molecular encapsulation.[7] For instance, Nau and coworkers showed that the encapsulation of rhodamine 6G in cucurbit[7]uril leads to reduced aggregation and reduced photobleaching.[8] Anderson and coworkers synthesized a rotaxane incorporating a cyanine dye threaded by α-cyclodextrin which led to reduced photobleaching and increased fluorescence in organic media but reduced fluorescence in aqueous solutions.[9]

One of the drawbacks to molecular encapsulation is the inherent reversibility of the binding event, which may lead to loss of dye over time or in the presence of competitive binding agents. Typically encapsulation is host/guest specific making a general method for improving dye properties difficult through host/guest encapsulation. An alternative approach is the covalent attachment of the fluorophore directly to the encapsulating host giving rise to site isolated dyes with solubility properties similar to the host and not the dye. Indeed, a number of groups have used this strategy to encapsulate hydrophobic dyes, such as Rubpy,[10] phthalocyanines,[11] and PDI, using dendritic molecules.[12] In each case, dendrimer conjugation led to increased water solubility, and generation dependent increases in fluorescence quantum yield (Φ_{fl}).

Recently Zimmerman,[13] and Haag,[14] have demonstrated independently, convenient synthetic methods for the rapid synthesis of both dendritic polyglycerol (PG) and hyperbranched polyglycerols (HPG) containing single site reactive species for dye conjugation. These non-ionic water soluble polymers are ideal for applications involving cellular and biomedical imaging because of their low toxicity and minimal nonspecific interactions.[15] Furthermore, the highly branched structure of these dendritic polymers may provide the steric bulk necessary to minimize dye–dye aggregation. Herein we describe the synthesis of several dye HPG conjugates, incorporating PDI and fluorescein. These conjugates were synthesized using a clickable HPG and further refined through metathesis based cross-linking. Preliminary studies to determine the extent of site isolation of the dyes within these polymer structures is reported.

Results and discussion

Polyglycerol synthesis

The most straight forward approach to the synthesis of dye functionalized HPG would be a direct anionic polymerization from a dye core. However the harsh reaction conditions used for the anionic polymerization of the HPG prevents this approach from being a general one, especially for more reactive dyes. For this reason a polymer first approach was employed.

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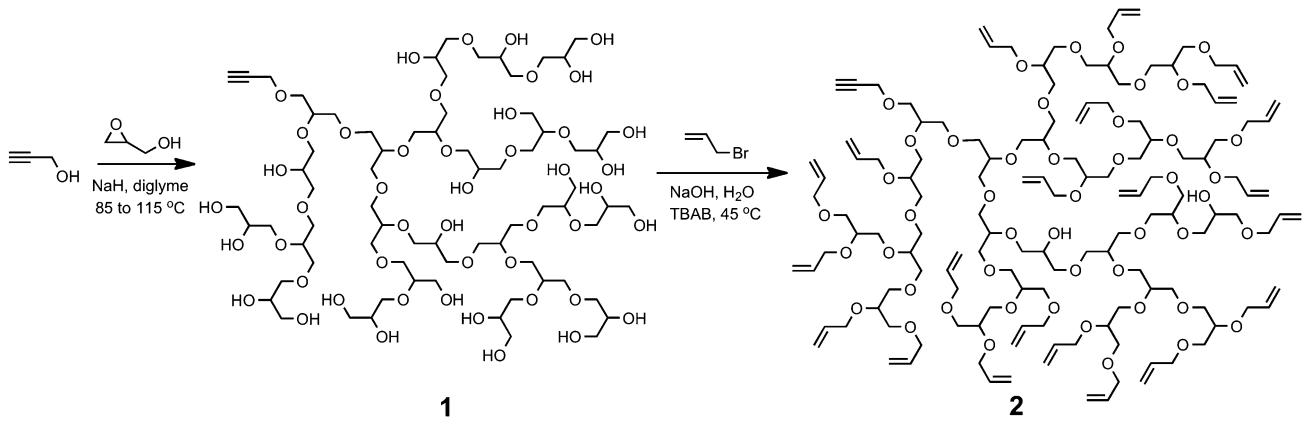


Scheme 1 Synthesis of HPG polymers used in this study. HPG structures are representative.

The general route to alkyne core HPG is outlined in Scheme 1 and follows the previously reported method of polymerizing glycidol with a propargyl alcohol initiator.[13b] The crude polymer was purified by methanol/acetone fractionation to afford **1**. Each polymer contained a single alkyne group for subsequent azide alkyne cycloaddition. These polymers were further allylated to form **2** to allow for ring-closing metathesis-mediated cross-linking.[16] In addition, an alkyne modified polyethylene glycol (PEG), **3** (structure not shown), was also synthesized from propargyl bromide and M_n = 2000 PEG monomethylether.

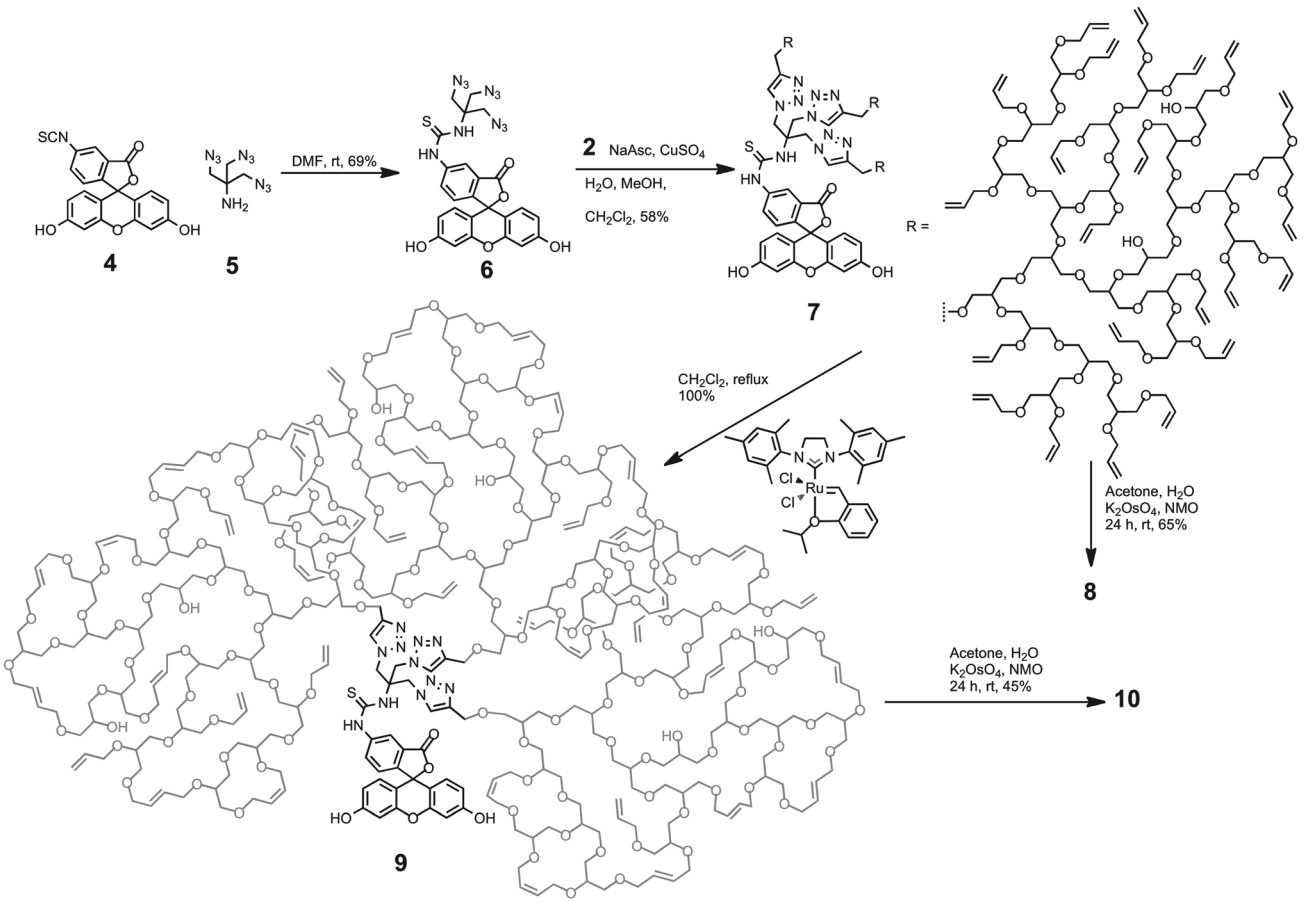
Fluorescein dye conjugates

Fluorescein is a well-known commercial dye used for fluorescence microscopy and other applications[17] and its functional derivatives are readily available. In particular, the commercially available fluorescein derivative fluorescein isothiocyanate (FITC, **4**) is a work horse for protein tagging and fluorescence imaging. Despite its widespread use, fluorescein still suffers from several drawbacks, including pH dependent fluorescence and a tendency to photobleach under illumination. The photobleaching process is thought to originate with the long lived triplet state and proceed through several routes including photoxidation, or dye to dye interaction.[18] Although oxygen removal and oxygen scavenging reagents have been used to reduce photobleaching,[19] these methods are not generally applicable, especially in live cell imaging. Alternatively the use of highly branched and cross-linked polymer may act as a barrier to reduce dye–oxygen and dye–dye interactions and yield highly stable fluorescent dyes.

Many commercially available, reactive dyes like FITC contain an amine reactive group. Thus, a general synthesis of PG-dye conjugates would start by converting the amine reactive group to an azide to act as a click partner for alkyne **1** or **2**.[20] To illustrate this approach with FITC, triazide amine linker **5** was synthesized following a procedure by Diaz[21] and coupled with FITC to yield fluorescein **6** carrying three azide groups (see Scheme 2). Triazide **6** was clicked to an allylated HPG polymer **2** (M_n = 2000) with sodium ascorbate and copper sulfate using a variation of the conditions described by Kim and coworkers.[22] Size exclusion chromatography (SEC) was performed to remove unreacted alkyne polymer and provided **7** in 81% yield. SEC analysis of crude material indicated a 3 fold increase in molecular weight, M_p = 4800 to M_p = 12 500, and IR analysis of the product showed almost no sign of the strong azide peak at 2100 cm^{-1}. Because of its large size ^1H NMR characterization was dominated by the PG backbone and terminal allyl groups. Peaks corresponding to the core dye were significantly broadened making assignment of specific peaks or an estimation of MW difficult. The polymer was made water soluble following a simple osmium catalyzed dihydroxylation in aqueous acetone to produce uncross-linked dye **8**. The allylated HPG was also intramolecularly cross-linked using Hoveyda-Grubbs catalyst prior to osmium catalyzed dihydroxylation to produce cross-linked dye **10**. A third water soluble fluorescein derivative, **11**, was synthesized which incorporated PEG (M_n = 2000) chains in place of the HPG. A summary of these dyes is shown in Table 1.

Site isolation of fluorescein in the non-cross-linked and cross-linked HPG derivatives **8** and **10**, respectively was studied using Stern–Volmer analysis, Fig. 1a. Thus, NaI has been shown to be an effective quenching agent for fluorescein with a small static component.[23] Both the cross-linked and uncross-linked polymers proved effective in protecting the dye from iodide quenching with "apparent" K_Q values four times lower than a simple fluorescein derivative prepared by reacting FITC with 6-azidohexan-1-amine (FITC-C6-Azide). A comparison of the cross-linked and uncross-linked PG showed a negligible difference indicating that the cross-linking process did not aid in the effective site isolation of the dye. The exact reason for this is not clear, but could be a result of imperfect encapsulation in the cross-linked network, leaving the dye exposed to the environment.

A similar trend is observed in the photobleaching of the two dyes. Simple experiments were performed using a 470 nm LED on bulk samples which were monitored for fluorescence periodically. Typical photobleaching curves are shown in Fig. 1b. The dye polymer conjugates **8** (not shown) and **10** were both more stable than fluorescein with a half life 3.5 times greater than fluorescein alone. However, these two dyes again performed almost identically, indicating that the cross-linking process did not localize the polymer around the dye. Both cross-linked and uncross-linked HPG outperformed PEG in photobleaching studies, indicating that the HPG is superior in protecting the dye from O_2 and from dye–dye photobleaching pathways. However, only dyes **8** and **11** maintained a high initial Φ_{fl} typical of fluorescein in aqueous solutions.



Scheme 2 Synthesis of cross-linked and uncross-linked fluorescein HPG conjugates.

Table 1 Comparison of fluorescein based dyes synthesized in this study

Dye	Polymer	M_n[a]	M_w/M_n[a]	% Cross-linking	λ_{max} H_2O (nm)	$Fluor_{max}$ H_2O (nm)	Φ_{fl} H_2O
8	HPG	6900[b]	NA	0	498.5	519	0.83
10	HPG	15 400	1.54	90%	499.5	520	0.45
11	PEG	13 800	1.14	NA	494	515	0.71

[a] Determined by SEC based on polystyrene standards. [b] Determined by MALDI-MS.

One explanation for the reduced fluorescence of **10** compared to dyes **8** and **11** is that it is unable to fully ionize in the hydrophobic environment created by the collapsed cross-linked polymer shell. However, a comparison of the pH dependent fluorescent curves of **10** and fluorescein show that the two dyes respond almost identically to solution pH, see the ESI.‡ Another more likely explanation would be the presence of residual Ru metal in the polymer shell. Heavy metals are known to quench dye excited states[24] and the ruthenium from Grubbs catalysts are notoriously difficult to remove.[25] This makes the simple two step approach to water soluble dye conjugation as described for **8**, or the one step approach described below even more useful.

Perylene diimides

PDIs represent a highly fluorescent and stable class of dyes. However, they are highly insoluble, especially in aqueous solutions where the tendency to aggregate results in reduced fluorescence.[26] Recently several groups have overcome these limitations. For instance Müllen and coworkers[27] used the bay substituted PDIs to incorporate multiple charged species to the dye producing several highly fluorescent ionic dyes which could be further modified at their periphery allowing incorporation of biotin functionality or protein conjugation. Haag and coworkers[12b] incorporated perfectly dendritic PG at the PDI termini to produce some of the most fluorescent PDI dyes observed in aqueous solution. However, these dyes lacked further functionality for tagging proteins or conjugation to small molecules (*e.g.*, biotin). Thus a route to PDIs with HPG at the bay position could provide increases to Φ_{fl} while allowing further functionalization at the imide position.

For this reason we started with the bay substituted tetrachloro PDI as described by Müllen in his synthesis of tetrasulfated PDIs.[27] Initial attempts to effect a direct conversion of the chloride groups to azide groups through treatment with sodium azide resulted in dye decomposition as observed by a red to dark blue color change and dye precipitation. This was not surprising as successful substitution at this position has only been described for phenol derivatives.[28] We thus began our synthesis from the tetrahydroxy PDI **12** (Scheme 3).[29] The four hydroxy groups were easily converted into tosyl groups, producing intermediate **13** (not shown), which in turn was treated with sodium azide to produce tetraazide **14**. Compound **14** served as a core reacting with HPG **1** (M_n = 3000) using standard click chemistry conditions (CuI and DMF) to produce polymer **15**.

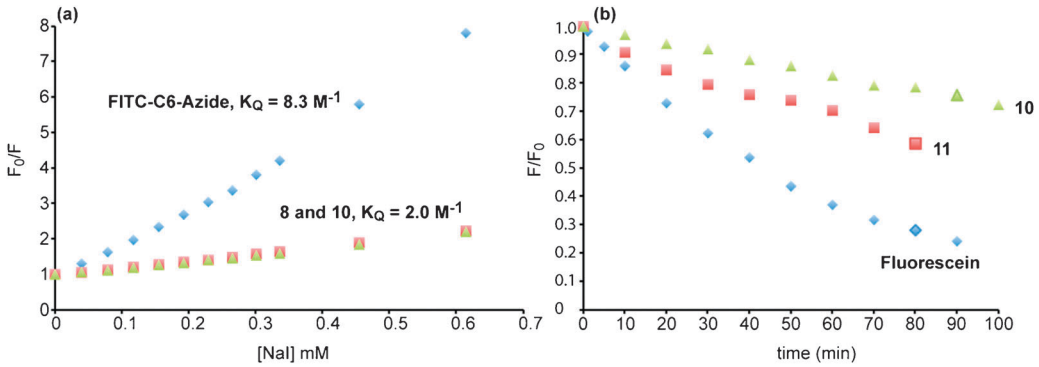


Fig. 1 (a) Stern–Volmer plots of fluorescein derivatives FITC-C6-Azide, **8**, and **10** obtained from fluorescence quenching using NaI as a quenching agent. Values reported are "apparent" K_Q values as fluorescence lifetime measurements were not obtained. (b) Photobleaching curves for cross-linked HPG fluorescein conjugate **10**, PEG fluorescein conjugate **11**, and free fluorescein. Samples were exposed to 470 nm LED for 10 min time intervals and their fluorescence peak relative to their initial fluorescence peak is reported.

A visual inspection of the dye solutions indicated that the addition of the HPG on dye **15** was sufficient to dissolve it in methanol solutions producing a red fluorescent solution. In aqueous solutions this dye became light purple and its fluorescence was reduced indicating some aggregation was occurring. The red fluorescence was recovered after addition of a small amount of SDS. By UV-Vis a 24 nm red shift was observed in aqueous solutions compared to DMF which is the opposite of the results described by Webber and coworkers when charged PDI were examined with surfactants.[30] The Φ_{fl} of **15** was also determined and compared to a PEG derivative, **16**. Compound **16** was prepared with alkyne functionalized polyethylene glycol (PEG) (M_n = 2000) using the same reaction conditions as above. Although the HPG derivative showed reduced Φ_{fl} in aqueous solutions compared to DMF, it was not nearly as dramatic as the loss of fluorescence observed for the PEG derivative **16** (Table 2) indicating that it has a strong tendency toward aggregation whereas aggregation is hindered by the branched nature of the HPG chains on **15**.

To further protect the dye from the aqueous environment and reduce aggregation and solvent interactions a cross-linked HPG shell was attached to the dye. This was accomplished using allyl HPG **2** (M_n = 4700) and click chemistry to produce **17**. Cross-linking under high dilution yielded an intermediate **18**, which was dihydroxylated to yield water soluble **19**. Again a reduction in Φ_{fl} was observed on going from DMF to an aqueous environment. However the reduction was not as dramatic as in the case for compound **15**. This is seen in Fig. 2 which shows the decrease in relative brightness of dyes **15** and **19** on going from a DMF solution to an aqueous solution.

Conclusion

We have demonstrated several simple synthetic routes to dendritic dye conjugates that impart enhanced photophysical properties to the dye molecules. Because of the use of click chemistry to attach the polymers to the dyes there was no sign of intermolecular cross-linking during the dye polymer conjugation. In the case of fluorescein, the dendronized dyes showed improvements in photostability over the free dye and a comparable fluorescein PEG conjugate. Conjugation of the hyperbranched polymer to PDI produced water soluble dyes with a Φ_{fl} four times greater than a similar PEG conjugated dye. These polymers could be cross-linked to reduce their size, however the cross-linking did not provide additional photostability and caused a decrease in Φ_{fl}. Because of the simplicity of the chemistry demonstrated, these techniques offer an excellent method to explore further dye encapsulation and to prepare stable and biocompatible fluorophores for use in biological and photophysical studies.[31]

Experimental

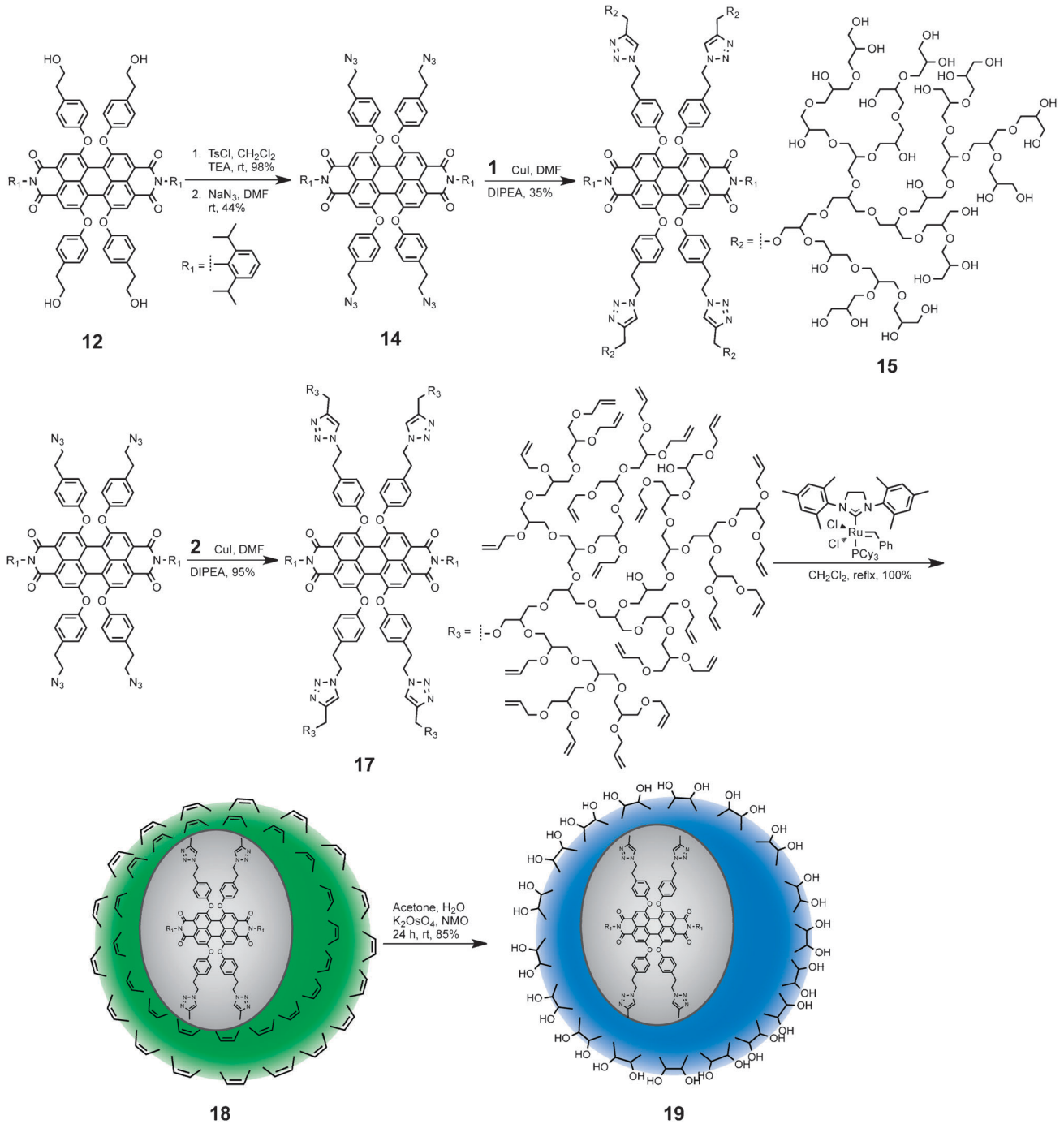
Synthetic procedures

General methods. All reactions were performed under an atmosphere of N_2 unless otherwise stated. Chemicals were used as received unless otherwise stated The synthesis of compounds **1**,[13b] **2**,[16f] **5**,[21] and **12**[32] were synthesized as described previously.

Preparative aqueous SEC was performed using Bio-Rad Bio-Gel® p-10 Gel medium mesh eluting in 20% methanol in water. Analytical SEC was performed using a Viscotek Viscogel I series LMW cutoff 2×, and HMW cutoff 1× columns in series with 0.05% (w/w) LiBr in DMF as the eluent with a column temperature of 50 °C and a flow rate of 1.0 mL min^{-1} or a Waters Ultrahydrogel™ 120 1× and Ultrahydrogel™ 250 1× columns in series with 0.01% (w/w) NaN_3 in water as the eluent with a flow rate of 1.0 mL min^{-1}. SEC-derived molecular weights were based on calibration with linear polystyrene.

^{1}H NMR and ^{13}C NMR data were obtained on a 500 MHz Varian U500 instrument. ^{1}H NMR spectra obtained in $CDCl_3$ were referenced to residual $CHCl_3$ at 7.26 ppm, CD_3OD spectra were referenced to residual CD_2HOD at 3.31 ppm, and D_2O were referenced to residual HDO at 4.79 ppm. ^{13}C NMR spectra obtained in $CDCl_3$ were referenced to 77.0 ppm and CD_3OD spectra were referenced to 49.0 ppm. ^{1}H NMR end group analysis based on methylene peak at 4.18 ppm and polymer backbone 3.9–3.4 ppm assuming 74.01 g mol^{-1} per 5 H.

Propargyl PEG monomethyl ether (3). To 2.5 g (M_n = 2000 g mol^{-1}, 1.25 mmol, 1 equiv.) PEG monomethyl ether



Scheme 3 Synthesis and cross-linking of PDI based dye polymer conjugates.

Table 2 Comparison of PDI based dyes synthesized in this study

Dye	Polymer	M_n[a]	M_w/M_n[a]	% Cross-linking	λ_{max} DMF(nm)	λ_{max} H_2O (nm)	$Fluor_{max}$ DMF (nm) ND[b]	$Fluor_{max}$ H_2O (nm) ND[b]	Φ_{fl} DMF	Φ_{fl} H_2O
15	HPG	26 300	1.14	0	579	593	609	627	0.49	0.14
16	PEG	11 400	1.20	NA	578	588			0.41	0.04
19	HPG	10 600	1.58	75	578	588	607	618	0.29	0.16

[a] Determined by SEC based on polystyrene standards. [b] ND = not determined.

dissolved in 50 mL THF was added 200 mg (5 mmol, 4 equiv.) NaH. This was stirred under nitrogen until bubbling ceased. The solution was cooled on an ice bath and 1.5 g (12.5 mmol, 1.5 equiv.) propargyl bromide was slowly added. The reaction was allowed to cool to room temperature and stirred 30 h. The solution was gravity filtered using CH_2Cl_2 to wash product

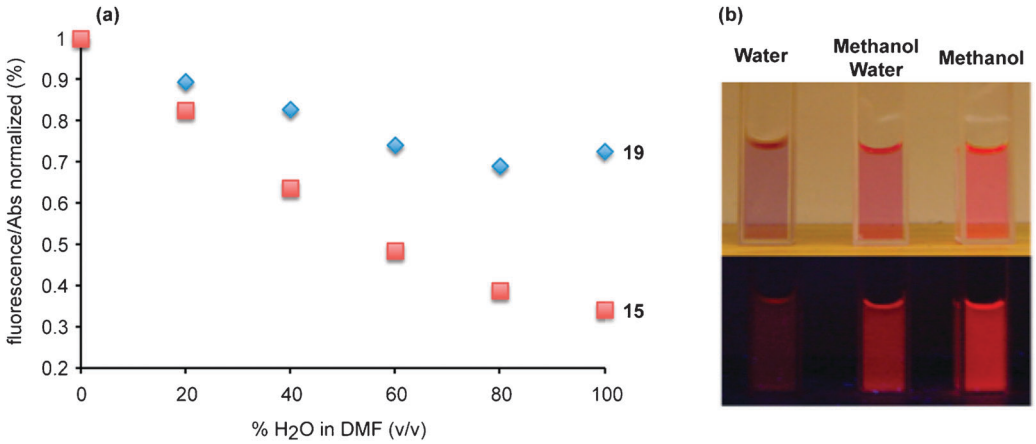


Fig. 2 (a) Change in Φ_{fl} with change in solvent for cross-linked **19** and uncross-linked **15** PDI HPG conjugates. (b) Images of uncross-linked PDI **15** in water and methanol. Top image in white light, bottom image, fluorescence produced from 327 nm UV lamp.

through paper. The filtrates were treated with 25 mL H_2O and the aqueous solution was extracted three times with 25 mL portions of CH_2Cl_2. The organic layer was concentrated to roughly 10 mL under vacuum, then precipitated with 150 mL ether and the mixture was stored overnight at −20 °C. The yellowish solid was isolated by vacuum filtration and the solid was dissolved in CH_2Cl_2 and precipitated again. Thorough drying yielded 2.9 g (100%) of an off white powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.21 (d, J = 2 Hz, 2H), 3.75–3.65 (bm, 239H), 3.39 (s, 3H), 2.45 (t, J = 2 Hz, 1H).). SEC (DMF) M_n = 4870 g mol^{-1}, M_w/M_n = 1.06. MS (MALDI) m/z 2163.43, (M + H$^+$), theoretical 2163.55, peak separation 44.1 g/mol.

1-(1,3-Diazido-2-(azidomethyl)propan-2-yl)-3-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thiourea (6). To 100 mg FITC (0.231 mmol, 1 equiv.) in 1 mL dry DMF was added 125 mg (0.64 mmol, 2.8 equiv.) of **5** and 1 mL of DMF. The reaction was monitored by TLC (1 : 1 ethyl acetate : hexanes 1% acetic acid R_f 0.15) and when no further reaction was observed, 5 mL of methanol was added and the solution was dry loaded onto silica and eluted using a gradient (40% ethyl acetate in hexanes to 40% ethyl acetate 2.5% acetic acid in hexanes). The combined fractions yielded 93 mg (69%) of **6** as an orange solid. ^{1}H NMR (500 MHz, CD_3OD) δ 8.14 (dd, J = 2 Hz, 0.5 Hz, 1H), 7.75 (dd, J = 8.5 Hz, 2 Hz, 1H), 7.55 (dd, J = 8 Hz, 0.5 Hz, 1H), 6.682 (m, 4H), 6.54 (dd, J = 8.5 Hz, 2.5 Hz, 2H), 4.05 (s, 6H). MS (HRES ESI) m/z 586.1348 (M+), theoretical 586.1370.

Fluorescein-thiourea allyl-HPG conjugate (7). A solution of 88 mg (0.44 mmol, 20 equiv.) of sodium ascorbate and 55 mg (0.22 mmol, 10 equiv.) of $CuSO_4$ was dissolved in 2 mL of water. This solution was added to 2 mL of a 1 : 1 methanol CH_2Cl_2 solution containing 13 mg (0.022 mmol, 1 equiv.) of **6** and 181 mg (0.089 mmol, 4 equiv.) of **1** ($M_{n(nmr)}$ = 2000, $M_{n(sec)}$ = 3850, M_w/M_n = 1.69). The biphasic solution was sealed and stirred vigorously for 14 h. The solution was filtered and dried under vacuum. The residue was dissolved in toluene and dried under vacuum to afford a pasty red residue. The residue was dissolved in a minimum of ethyl acetate and passed through a plug of silica using ethyl acetate as eluting solvent. This solution was treated with $MgSO_4$, filtered, and dried to afford a red viscous oil. The residue was dissolved in a minimum of toluene and passed through a preparative SEC column collecting the product as a dark red band. Solvent removal afforded 142 mg of a red oil that was 80% polymer and 20% toluene by mass based on ^{1}H NMR, totaling 116 mg (80%) product. Note thorough drying of the product leads to polymer cross-linking. The material was not suitable for long-term storage. ^{1}H NMR (500 MHz, CD_3OD) δ 5.8–6 (b, 1H), 5.1–5.3 (b, 3 peaks, 2H), 3.9–4.1 (b, 2 peaks, 2H), 3.4–3.8 (b, 2 peaks, 5H). SEC (DMF) M_n = 12 100 g mol^{-1}, M_w/M_n = 1.39.

Osmium catalyzed oxidation of uncross-linked fluorescein allyl-HPG conjugate (8). To 25 mg (0.22 mmol of allyl groups, 1 equiv.) of **7** in 1 mL of acetone was added 105 μL 50% (w/w) solution (0.44 mmol, 2 equiv.) of NMO in H_2O followed by 2 mg (0.0054 mmol, 0.025 equiv.) of $K_2OsO_4{\cdot}2H_2O$. The solution was sealed and stirred for 30 min and a precipitate formed. The solution was diluted with 2 mL of water to produce a transparent solution and stirred for 1 h. The solution was diluted with 4 mL of water to maintain a homogeneous mixture. The solution was stirred 24 h, and 20 mg of Smopex type 105 metal scavenger resin was added. This mixture was stirred 24 h and filtered. The filtered solution was dialyzed against water (MWCO = 1400 Daltons) and dried to afford 21.5 mg (65%) of **8** as a red oil. ^{1}H NMR (500 MHz, D_2O) δ 3.4–3.9 (b). MS (LRES-MALDI) m/z = 6900 at peak.

Metathesis cross-linking of fluorescein allyl-HPG conjugated (9). To 50 mg (0.439 mmol of allyl groups, 1 equiv.) of **7** dissolved in 250 mL of CH_2Cl_2 was added 5.5 mg (0.0088 mmol, 0.02 equiv.) of Hoveyda-Grubbs catalyst. The reaction was monitored by removal of aliquots for ^{1}H NMR analysis. The mixture was stirred and heated to reflux for 4 h and 5 mg (0.008 mmol, 0.018 equiv.) of Hoveyda-Grubbs catalyst was added. The mixture was kept at reflux for 50 h and 5 mL of ethyl vinyl ether and 100 mg of Ph_3P was added. The mixture was stirred 12 h. To the mixture was added 10 mL of toluene to prevent complete solvent removal during evaporation. The mixture was dried under vacuum to approximately 3 mL

and was passed through a preparative toluene SEC column collecting the fastest moving red band. Solvent removal revealed 68 mg of a red oil that was 46 mg (100%) product **9** and 22 mg toluene based on ^{1}H NMR. (Thorough drying of the product leads to polymer cross-linking. The material was not suitable for long-term storage) ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.6–6 (b, 1H), 5.1–5.3 (b, 2 peaks, 0.2H), 3.8–4.4 (b, 2 peaks, 2H), 3.1-3.8 (b, 5H). SEC (DMF) M_n = 6800 g mol^{-1}, M_w/M_n = 1.85.

Osmium catalyzed oxidation of cross-linked fluorescein allyl-HPG conjugate (10). To a vial containing 1 mL of acetone was added 22 μL of a 9.3 mg mL^{-1} solution (20 mg, 0.1 mmol allyl, 1 equiv.) of **9** followed by 100 μL of a 50% (w/w) solution (48 mg, 0.2 mmol, 2.5 equiv.) of NMO in H_2O. To this was added 1 mg (0.0027 mmol, 0.027 equiv.) of $K_2OsO_4{\cdot}2H_2O$. The vial was covered in aluminum and stirred at room temperature. To the solution was added 1 mL of H_2O every 2–4 h for 12 h to maintain homogeneity. The solution was stirred 12 h. The solution was filtered, diluted with 10 mL H_2O, and washed with three 5 mL portions of CH_2Cl_2. The aqueous layer was reduced in volume and dialyzed against H_2O. The dialyzed product was dried under vacuum to reveal 11 mg (48%) of **10** as a thin film. ^{1}H NMR (500 MHz, D_2O) δ 3–4 (b). SEC (DMF) M_n = 15 400 g mol^{-1}, M_w/M_n = 1.51.

Fluorescein-thiourea PEG (M_n 2000) conjugate (11). To a 10 mL scintillation vial was added 205 mg (0.10 mmol, 3.1 equiv.) of **9**, 1 mL of DMF, and 20 mg (0.034 mmol, 1 equiv.) of **6**. The mixture was stirred until it became homogeneous and 6.5 mg (0.034 mmol, 1 equiv.) of CuI, and 48 μL (0.272 mmol, 8 equiv.) of DIPEA were added. The reaction was monitored by SEC and stirred for 36 h. The reaction was diluted with water and dialyzed (MWCO = 2000) against water. Solvent removal afforded 132 mg (60%) of **11** as an orange flaky solid. SEC (DMF) M_n = 13 800 g mol^{-1}, M_w/M_n = 1.14.

5-(3-(6-Azidohexyl)thioureido)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (FITC-C6-Azide). To a screw cap scintillation vial containing 100 mg (0.26 mmol, 1 equiv.) of fluorescein-5-isothiocianate and 54 mg (0.38 mmol, 1.4 equiv.) of 6-azido-hexan-1-amine[33] was added 1 mL of DMF. The mixture was stirred for 48 h at room temperature. The reaction was dry loaded onto a silica column and purified by column chromatography using silica gel (2 : 1 ethyl acetate:hexanes). Solvent removal afforded 98 mg (72%) of the product as a dark red solid. ^{1}H NMR (500 MHz, CD_3OD) δ 8.20 (s, 1H), 7.81, (d, J = 7.3 Hz, 1H), 7.19, (d, J = 8 Hz, 1H), 6.83 (d, J = 9 Hz, 2H), 6.78, (d, J = 2 Hz, 2H), 6.65, (dd, J = 8.5, 2 Hz, 2H), 3.63 (br s, 2H), 1.75–1.60 (m, 4H), 1.5–1.4 (m, 4H). MS (HRES) m/z 532.1655 (M$^+$), theoretical 532.1655.

(((2,9-Bis(2,6-Diisopropylphenyl)-1,3,8,10-tetraoxo-1,2,3,8,9,10-hexahydroanthra[2,1,9-def:6,5,10-d′e′f′]diisoquinoline-5,6,12,13-tetrayl)tetrakis(oxy))tetrakis(benzene-4,1-diyl))tetrakis(ethane-2,1-diyl) tetrakis(4-methylbenzenesulfonate) (13). A round bottom flask containing 315 mg (0.251 mmol, 1 equiv.) of **12** and 1.0 g (3.02 mmol, 20 equiv.) of 4-toluenesulfonyl chloride was cooled to 0 °C in an ice bath. The solids were dissolved by slowly adding a 50 mL solution of CH_2Cl_2 containing 350 μL (2.5 mmol, 10 equiv.) of triethylamine. The reaction mixture was taken out of the ice bath and warmed to room temperature, stirred for 36 h, and monitored by TLC. An additional 300 mg (1.58 mmol, 6.3 equiv.) of 4-toluenesulfonyl chloride and 150 μL (1.1 mmol, 4.3 equiv.) of triethylamine were added. The solution was stirred overnight at which point TLC analysis yielded a single spot (CH_2Cl_2, R_f 0.17). The solution was reduced in volume by rotary evaporation, and an equal volume of ethyl acetate was added. The solution was filtered and the eluent was dried under vacuum. The residue obtained was purified on a silica column (CH_2Cl_2 to 10% acetone in CH_2Cl_2). Solvent removal afforded 458 mg (97%) of **13** as a red flaky solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (s, J = 4H), 7.76 (dd, J = 6.5 Hz, 2 Hz, 8H), 7.44 (t, J = 7.7 Hz, 2H), 7.28 (d, J = 8.5 Hz), 7.28 (d, J = 7.5 Hz, 4H), 7.07 (d, J = 8.5 Hz, 8H), 6.87 (m, 8H), 4.18 (t, J = 7 Hz, 8H), 2.94 (t, J = 7 Hz, 8H), 2.69 (sep, J = 7 Hz, 4H), 2.39 (s, 12H), 1.12 (d, J = 7 Hz, 24H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.2, 155.9, 154.2, 145.7, 145.0, 133.3, 133.0, 132.8, 130.7, 130.1, 129.6, 128.0, 124.0, 123.0, 120.8, 120.5, 120.3, 120.1, 70.5, 34.7, 29.2, 24.1, 21.7. MS (HRES ESI) m/z 1871.5732 (M + H$^+$), theoretical 1871.5674.

5,6,12,13-Tetrakis(4-(2-Azidoethyl)phenoxy)-2,9-bis(2,6-diisopropylphenyl)anthra[2,1,9-def:6,5,10-d′e′f′]diisoquinoline-1,3,8,10-(2*H*,9H)-tetraone (14). To 1 mL of dry DMF was added 63 mg (0.0267 mmol, 1 equiv.) of **13** and 50 mg (0.534 mmol, 16 equiv.) of NaN_3. The heterogeneous mixture was stirred at room temperature for 16 h and monitored by TLC (solvent CH_2Cl_2, R_f 0.75). The reaction mixture was transferred to a separatory funnel using 15 mL of toluene and the solution was washed four times with 10 mL portions of H_2O. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The concentrated solution was purified on silica gel (CH_2Cl_2 to 5% acetone in CH_2Cl_2) to afford 42 mg (93%) of **14** as a reddish brown solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.25 (s, 4H), 7.434 (t, J = 8 Hz, 2H), 7.28 (d, J = 7.5 Hz, 4H), 7.14 (dt, J = 9 Hz, 2.5 Hz, 8H), 6.93 (dt, J = 9 Hz, 2.5 Hz, 8H), 3.47 (t, J = 7 Hz, 8H), 2.85 (t, J = 7.5 Hz, 8H), 2.69 (sep, J = 7 Hz, 4H), 1.12 (d, J = 6.5 Hz, 24H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.5, 156.2, 154.4, 145.9, 134.8, 133.5, 130.9, 130.7, 129.8, 124.3, 123.3, 121.1, 120.7, 120.6, 120.4, 52.7, 35.0, 29.4, 24.4. MS (HRES ESI) m/z 1355.5570 (M + H$^+$), theoretical 1355.5579.

Tetrasubstituted perylene diimide HPG conjugate (15). To a screw cap vial containing 1 mL of DMF was added 560 mg, (0.18 mmol, 5 equiv.) of **1** (M_n = 3000 based on ^{1}H NMR) and 50 mg (0.147 mmol N_3, 4 equiv.) of **14**. An additional 1 mL DMF was used to transfer **14** into the reaction vessel. To the solution was added 17 mg (0.089 mmol, 0.61 equiv.) of CuI. The solution was stirred 1 h and 0.1 mL DIPEA was added. The solution immediately turned black. The reaction was monitored by SEC for 10 h and the solution was reduced in volume and the oily residue was dissolved in water. The product was purified using a preparatory SEC column, (MWCO = 6000), and dialyzed (MWCO = 1400) against water. The dialyzed product was dried under vacuum to afford 180 mg (35%) of **15** as a red oil. ^{1}H NMR (500 MHz, D_2O)

δ 3.2-4 (b, 910H), 1.03 (b, 24H). SEC (DMF) M_n = 22 600 g mol^{-1}, M_w/M_n = 1.14.

Tetrasubstituted perylene diimide PEG conjugate (16). In a 10 mL scintillation vial was added 90 mg (0.045 mmol, 4.1 equiv.) of propargyl PEG monomethyl ether, 0.5 mL of DMF, and 15 mg (0.011 mmol, 1 equiv.) of **14**. The mixture was stirred until it was homogeneous and 2.0 mg (0.011 mmol, 1 equiv.) of CuI, and 100 μL (0.57 mmol, 51 equiv.) of DIPEA were added. The solution was stirred for 48 h and the reaction was monitored by SEC. The solution was diluted with H_2O and dialyzed (MWCO = 2000) against H_2O. Solvent removal yielded 82 mg (78%) of **16** as a dark red fluffy solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (b, 4H), 7.39 (b, 4H), 7.24 (b, 2H), 6.86–7.04 (b, 14H), 4.97 (b, 6H), 3.4–3.8 (716 H), 2.64 (b, 6H), 1.08 (bs, 24H). SEC (DMF) M_n = 19 800 g mol^{-1}, M_w/M_n = 1.25

Tetrasubstituted perylene diimide allyl-HPG conjugate (17). To a screw cap vial containing 0.5 mL DMF was added 625 mg (0.13 mmol, 6 equiv.) of **2** (M_n = 4700 by ^{1}H NMR) and 30 mg (0.088 mmol N_3, 4 equiv.) of **14** in 0.5 mL of DMF. To the solution was added 14 mg (0.074 mmol, 0.84 equiv.) of CuI. The solution was stirred 1 h and 0.1 mL DIPEA was added. The solution immediately turned black. The reaction was monitored by IR by following the disappearance of the azide peak at 2100 cm^{-1}. The mixture was stirred for 18 h and 10 mg (0.052 mmol, 0.6 equiv.) of CuI was added. The mixture was heated to 50 °C for 8 h. The mixture was filtered and washed with 20 mL of 1N HCl and washed four times with 20 mL portions of H_2O. The organic layer was dried with $MgSO_4$ and passed through a silica plug with ethyl acetate. The solution was concentrated and the product was purified on a toluene SEC column. Solvent removal afforded 150 mg (95%) of **17** as a red oil. The product was not pumped completely dry to avoid intermolecular cross-linking. The mass obtained is based on ^{1}H NMR integrations of product peak and toluene solvent peak. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.9–6 (b, 120H), 5.10–5.25 (b, 3 peaks, 240H), 3.9–4.2, (b, 2 peaks, 240H), 3.4–3.8 (b, 2 peaks, 600H), 1.1 (bs, 24H). SEC (DMF) M_n = 11,400 g mol^{-1}, M_w/M_n = 1.20.

Metathesis cross-linking of perylene diimide allyl-HPG conjugate (18). To 500 mL of CH_2Cl_2 was added 50 mg (0.438 mmol allyl, 1 equiv.) of **17** and 4.5 mg (0.0053 mmol, 0.12 equiv.) of Grubbs 2nd generation catalyst. The solution was refluxed under N_2 and monitored by ^{1}H NMR for 18 h at which point the polymer was approximately 60–70% cross-linked. The solution was treated with 66 mg of Smopex # 105 MTD metal scavenger resin and 3 mL of ethyl vinyl ether and refluxed for 2 h. The solution was filtered and reduced in volume and purified on a toluene SEC column (MWCO = 6000). The red fraction was collected off the column, stirred with 25 mg Smopex #105 scavenger resin and filtered. Solvent removal afforded 85 mg of a crude product which was 53% **18** by mass based on ^{1}H NMR, 45 mg (100%), remaining mass is toluene. Product not dried to avoid intramolecular cross-linking. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.6–6 (b, 1H), 5.1–5.3 (b, 3 peaks, 0.6H), 3.9–4.3, (b, 2 peaks, 2H), 3.2–3.8 (b, 5H). SEC (DMF) M_n = 8500 g mol^{-1}, M_w/M_n = 1.32.

Oxidation of cross-linked perylene diimide allyl-HPG conjugate (19). To a 2 mL solution of acetone containing 45 mg (0.281 mmol alkene, 1 equiv.) of **18** was added 66 mg of citric acid, 1 mg (0.0027 mmol, 0.01 equiv.) of $K_2OsO_4 \cdot 2H_2O$, and 530 mL of a 50% (w/w) solution (2.25 mmol, 8 equiv.) of NMO. The acetone solution was slowly diluted with water over 12 h to maintain homogeneity. The solution was treated with 38 mg Smopex-105 metal scavenger resin for 2 h and filtered twice. The filtrates were dialyzed against pure water. Solvent removal afforded 49 mg (75%) of **19** as a dark red oil. ^{1}H NMR (500 MHz, D_2O) δ 3.2–3.9 (b, 1244H), 1.06 (b, 24H). SEC (DMF) M_n = 10 600 g mol^{-1}, M_w/M_n = 1.58.

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