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Crosslinked dendronized polyols as a general approach to brighter and more stable fluorophores†

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Fluorescent, aqueous-soluble, crosslinked dendronized polyols (CDPs) are obtained through a sequential process involving ring-opening metathesis polymerization (ROMP), intra-chain ring-closing metathesis (RCM), and hydrolysis. This general strategy improves the quantum yields (QYs), brightness, and photostability of five common fluorophores whose emission wavelengths cover 150 nm. Additionally, the availability of the polymeric fluorophore to the cytosomes opens the door to applications in bioimaging and intracellular delivery.

Fluorescent probes have been widely used in bioimaging, especially for monitoring supramolecular and biological processes at the molecular level.[1] To best enable these applications, aqueous-soluble, bright, photostable, and biocompatible fluorophores are needed. To achieve aqueous solubility, the fluorescent dyes may be linked to charged functional groups[2] or aqueous soluble macromolecules.[3] Efforts toward the other desirable properties listed above have come from different perspectives, such as designing new fluorophore structures,[4] adding anti-fading agents,[5] attaching functional species,[6] and introducing protective sheaths[7] and scaffolds.[8] However, it remains difficult to achieve all of these desirable features simultaneously.

Polyglycerol dendrimers (PGDs) are globular macromolecules with multiple periphery hydroxyl groups.[9] PGDs have attracted considerable interest because of their biocompatibility and charge-free character across a wide pH range.[10] They have been reported to enhance the aqueous solubility and brightness,[3a,b] as well as to reduce the blinking of hydrophobic fluorescent dyes.[3d] However, the incorporation of fluorophores onto the polyglycerol scaffold is synthetically non-trivial, and is generally limited to either a single copy at the core, or multiple copies at the periphery. Introducing multiple copies of a specific moiety inside the dendritic structure can be quite challenging.[11]

A less explored macromolecular architecture for solubilizing and stabilizing fluorophores is linear dendronized polymer. These polymers can be prepared by grafting dendrons onto the repeating units of a linear polymeric backbone or by the direct polymerization of dendronized monomers.[12] As dendron generation increases, direct polymerization can be increasingly demanding because of growing steric hindrince. In this regard, ROMP has been successfully applied to the synthesis of this challenging macromolecular structure because of its rapid rate and the high functional group tolerance of the catalyst.[13] Dendronized polymers can be further diversified by co-polymerizing different monomers while maintaining the high density of functional groups along the backbone.[14]

To our knowledge, although intramolecular cross-linking has been widely used to synthesize polymeric nanoparticles,[15] little research has been directed toward high-generation ($g \geq 3$) dendronized polymers.[16] Herein, we report the synthesis and study of crosslinked dendronized polyols (CDPs) as a general platform for fluorescent dye encapsulation and protection using a sequential ROMP and intra-chain RCM process. A similar ROMP–RCM strategy was recently reported to prepare polymeric organic nanoparticles (ONPs) for fluorescein protection.[17] However, a dihydroxylation step with potassium osmate[18] was required to provide aqueous solubility preventing generalization of the method to more reactive dyes such as BODIPY and coumarin.

To demonstrate the generality of the new CDP approach for dye encapsulation and protection, we selected five representative, commonly used dyes with emission wavelengths ranging from 450 nm to 590 nm, thus covering most of the visible spectrum. The five dyes are 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (BODIPY or **B**), coumarin (**C**), fluorescein (**F**), perylene diimide (PDI or **P**) and tetramethylrhodamine (TAMRA or **R**). All dyes are readily available from straightforward synthesis and are conveniently linked to an *exo*-norbornenyl moiety. Indeed, these ROMP monomers were prepared on gram scale in just two or three steps as detailed in the ESI.† The phenol groups of

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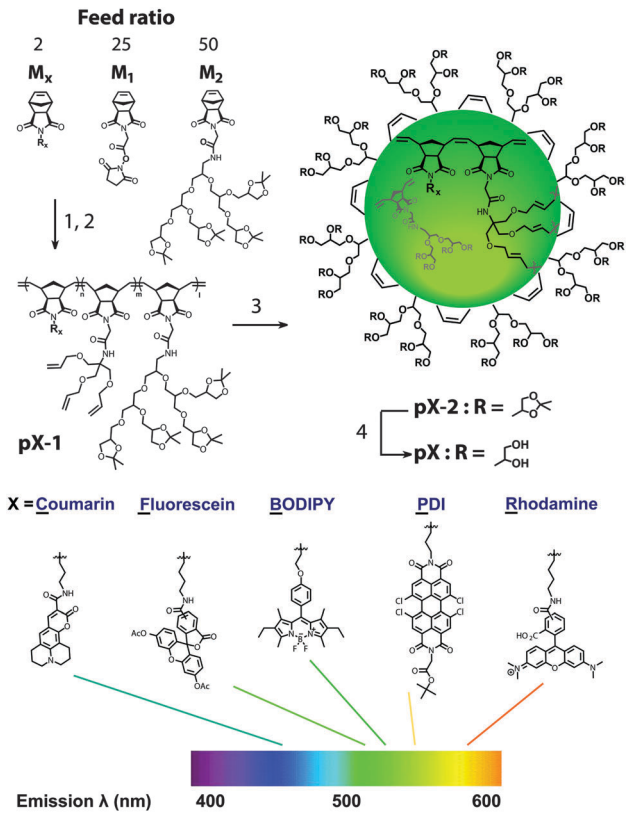
F were protected by esterification with acetic anhydride using a reported procedure.[17] All the other fluorophores (**C**, **B**, **P**, and **R**) are inherently compatible with the operation of the alkene metathesis catalysts, *i.e.*, Grubbs 1st and 3rd generation catalysts, because no isolated alkenes are present and all the amino groups are alkylated.[19]

For the preparation of CDPs, M_1 (an active ester) and M_2 (a protected polyglycerol dendron) were used in all of the polymerizations along with one of the five different M_x's, where X = **C**, **B**, **F**, **P** and **R** as described above. With the appropriate monomers (M_1, M_2, M_x) in hand, the synthesis of fluorescent CDPs required four steps as outlined in Scheme 1. First, a linear, random copolymer was synthesized by ROMP using a 25 : 50 : 2 feed ratio of M_1, M_2, and M_x. Post-functionalization with tris(allyloxymethyl)-aminomethane (tri-*O*-allyl tris), intra-chain RCM, and hydrolysis afforded aqueous-soluble, fluorescent, dendronized polymers with a semi-rigid and compact macromolecular structure.

The successful synthesis of the dye-conjugated, dendronized polymers was confirmed by ^{1}H NMR, dynamic light scattering (DLS), gel permeation chromatography (GPC) coupled with a multi-angle laser light scattering (MALLS) detector. Comparing ^{1}H NMR spectra



Scheme 1 Synthesis of the crosslinked dendronized polymer **pX**. (1) Grubbs 3rd generation catalyst, DCM; (2) tri-*O*-allyl tris, nitrobenzene, DCM, 40 °C; (3) Grubbs 1st generation catalyst, DCM, high dilution conditions (crotyl groups drawn to indicate cross-linking *via* ring closing metathesis); (4) TFA, DCM, acetone, H_2O, 40 °C. **X** represents the specific dye incorporated. The corresponding emission wavelengths of different dyes are displayed on the visible spectrum.

of **pF-1** to **pF-2** (Fig. S3, ESI†), the new proton signals centered at *ca.* δ 5.24 and 5.16 ppm corresponding to olefin groups indicated the successful incorporation of the tri-*O*-allyl tris. Intra-chain cross-linking *via* RCM under high dilution condition was verified by the almost complete disappearance of the terminal alkene proton signals and the narrow polymer GPC traces with slightly longer retention times (Fig. S4, ESI†). MALLS analysis of **pF-1** and **pF-2** showed a molecular weight of 84 kDa (PDI = 1.08) and 79 kDa (PDI = 1.12), respectively. The molecular weight of the other fluorescent dendronized polymers could not be determined in the same way because their fluorescence interferes with the MALLS detector. However, their GPC retention times were comparable to **pF-1** and **pF-2**, suggesting similar molecular weights (Fig. S5, ESI†).

The previously prepared ONPs showed a much larger increase in retention time upon RCM (*e.g.*, >1 min).[17] Both polymeric nanostructures are fully crosslinked according to NMR, so the different extent of contraction observed with the ONPs and CDPs (Fig. S4, ESI†) can be attributed to a more compact structure already present in the pre-crosslinked dendronized polymers. Indeed, because of the densely packed dendrons along the polymer backbone, a linear, dendronized polymer tends to adopt a semi-rod-like structure.[13b,c,20] As a result, the CDPs might contain primarily short-range cross-links, whereas the more flexible ONPs could favor the long-range RCM commonly observed in dendrimers.[21]

With the completion of the RCM, attention was turned to the deprotection of the dendrons. Different acids, including aqueous TFA, HCl, H_2SO_4 and Dowex resin, were screened and dilute aqueous TFA solution was found to remove the acetal groups efficiently while maintaining the fluorophore integrity. DLS analysis of **pF** indicated a hydrodynamic size of *ca.* 6 nm and the other CDPs were assumed to have similar sizes (Fig. S6, ESI†). This size is close to that of a recently developed quantum dot and in the appropriate size range for biomolecule labeling applications.[22]

The quantum yields (QYs) of **pX**s and free dyes were determined using fluorescein as the standard (QY = 0.95).[23] The brightness for each CDP was calculated as one-thousandth of the product of QY and molar extinction coefficient (Table 1). The fluorescein and TAMRA units exhibited similar QYs in the CDPs (*i.e.*, **pF** and **pR**) and free solutions, whereas **pB** and **pP** were dramatically brighter than free BODIPY and PDI, respectively. The quantum yield of coumarin decreased by more than half once incorporated in the CDP, which might stem from the sensitivity of coumarin's spectral properties towards the microenvironment.[24] An enhancement in brightness was observed for all five chromophores, including coumarin, because each CDP contains two fluorophores on average. However, the results for **pB** and **pP** far exceeded a factor of two, suggesting that the polymer scaffolds not only provided aqueous solubility but also inhibited dye–dye quenching.[25] For **pP**, additional evidence for the scaffold inhibiting PDI π–π stacking comes from its UV-vis spectrum (Fig. S7, ESI†).

The relative photostability of the **pX**s and their respective free dyes were compared by irradiating the **pX** or fluorophore (*ca.* 100 nM) in phosphate buffer (PB, pH 7.4) with an LED light

Table 1 QY and brightness of free dyes and polymers

	Free fluorophores		pX	
Fluorophore	QY (%)	Brightness[b] ($M^{-1}cm^{-1}$)	QY (%)	Brightness[b] ($M^{-1}cm^{-1}$)
Coumarin	96	9.9	34	13
BODIPY[a]	1.3	0.056	46	22
Fluorescein	95[14]	71	71	112
PDI[a]	3.3	0.97	4.6	3.6
Rhodamine	12	11	11	20

[a] Water-insoluble fluorophores (BODIPY and PDI) were first dissolved in dioxane and then diluted with 0.1 M phosphate buffer (pH = 7.4). [b] Brightness = QY·molar extinction coefficient/1000.

source (470 nm). Water-insoluble BODIPY and PDI were dissolved in dioxane first, and then diluted with PB so that their photobleaching behaviours could be studied in aqueous solutions. Plots of fluorescence intensity over time are shown in Fig. 1. Significantly improved photostability was observed in the case of **pC**, **pF** and **pP**. After 4 h of irradiation, **pC** and **pP** maintained about 55% of their initial fluorescence intensity whereas the free coumarin and PDI's intensity quickly dropped to the background level. Similarly, the final fluorescent intensity of **pF** was about 14 times higher than that of free fluorescein. TAMRA is known as a very robust fluorophore, and its stability was well preserved when incorporated to the CDPs. However, it was difficult to compare the photostability of **pB** and free BODIPY because the resulting BODIPY solutions had very low quantum yield (<1%). PEG-conjugated BODIPY and PDI were also prepared and studied. In the case of BODIPY, the photostability was significantly reduced upon PEG conjugation with apparent QY improvement,

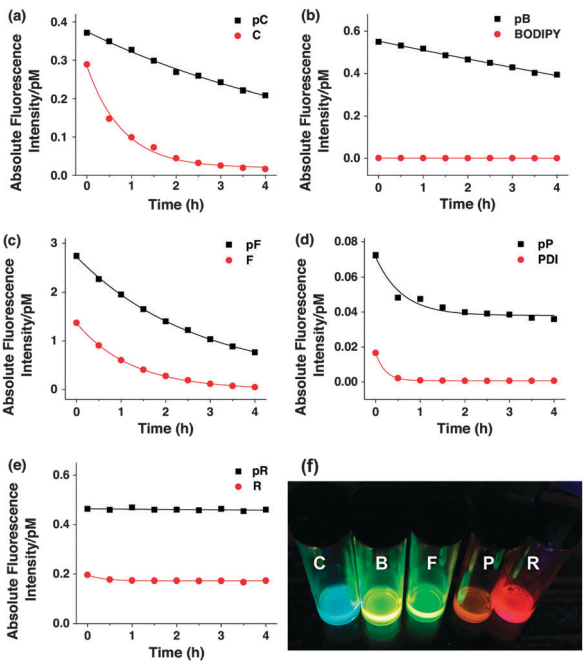


Fig. 1 Absolute Fluorescence intensity over time during the photobleaching study: (a) **C** and **pC**; (b) **B** and **pB**; (c) **F** and **pF**; (d) **PDI** and **pP**; (e) **R** and **pR**; (f) pictures of CDP solutions (0.5 mg mL^{-1}, illuminated with TLC UV lamp).

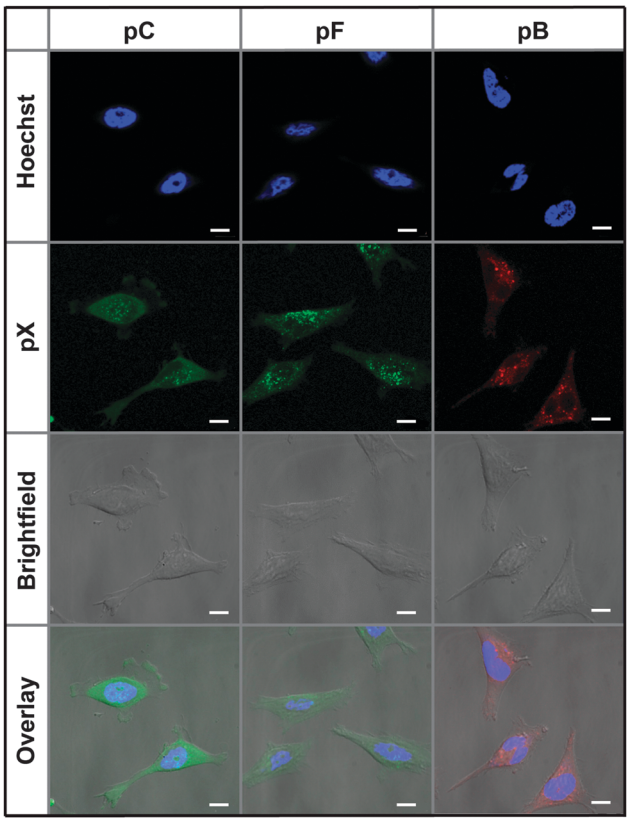


Fig. 2 Confocal microscopy images of live HeLa cells treated with **pX**. First row: blue channel images showing nucleus staining with Hoechst; second row: fluorescence images from **pX**; third row: bright-field images; fourth row: overlay of nucleus, fluorescence from **pX** and bright-field images. (X = **C**, **F** and **B**); scale: 10 μm.

whereas PEG-functionalized PDI had even lower QY (Fig. S8, ESI†). These PEG conjugation studies indicated that linear PEG cannot protect BODIPY nor prevent PDI from dye–dye quenching.

Polyvinyl alcohols such as Mowiol 4-88 are known to act as anti-fading agents for fluorescence microscopy.[26] This type of polymer carries multiple hydroxyl groups analogous to the CDPs, although the mechanism of the stabilization is not known. To test whether the free fluorescent dyes can be stabilized with CDPs as additives, control studies were performed by preparing a CDP without M_x and mixing it with free dyes (1 : 2 molar ratio) at the same concentration used for **pX**s. No photostabilization was observed, indicating the importance of the covalent encapsulation of the dyes in the CDPs.

To explore the potential of these fluorescent CDPs in applications such as bioimaging and cellular delivery, cytotoxicity and cell uptake studies were performed (ESI†). The toxicity of all the CDPs was measured in HeLa cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell viability assay. All five polymers exerted minimal cytotoxicity at a concentration as high as 100 μg mL^{-1} (Fig. S9, ESI†). Furthermore, similar to non-dendronized ONPs, live-cell confocal microscopy showed that all five CDPs readily entered the cells (Fig. 2 and Fig. S10 and S11, ESI†). Interestingly, although the ONPs were largely localized in endosomes,[17] the fluorescence of the CDPs was

observed throughout the cytoplasm (Fig. S12, ESI†). An in-depth study of the internalization and intracellular trafficking mechanism is beyond the scope of the current study, but this is an intriguing result.

In conclusion, we have developed a new approach to construct brighter and more stable polymeric fluorophores. The incorporation of dendronized polyglycerol monomers affords high aqueous solubility as well as enhanced photo-stability. By avoiding the use of potassium osmate, the current approach has allowed a much broader range of fluorophores to be used. Multiple copies of dyes can be introduced into the CDPs, which increases the brightness. The availability of these polymeric nanoparticles to cytoplasm opens the door to more applications in the future, and the different cellular localization patterns of ONPs and CDPs may be used to tailor cellular delivery vehicles for different purposes.

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