

Luminescence-Tunable Polynorbornenes for Simultaneous Multicolor Imaging in Subcellular Organelles

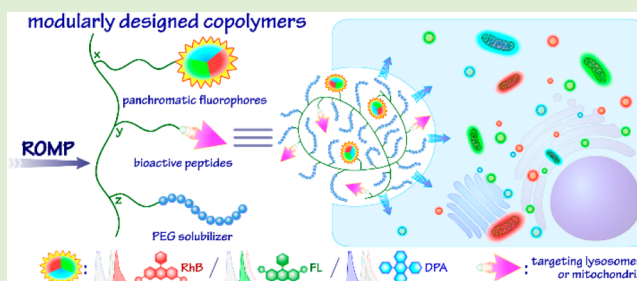
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Supporting Information

ABSTRACT: Through modular ROMP (ring-opening metathesis polymerization), biofunctional polynorbornenes are designed and fabricated from panchromatic fluorophores, bioactive peptides, and polyethylene glycol solubilizer for organelle-specific multicolor imaging. Attributed to the free permutation and combination of highly fluorescent red rhodamine B, green dichlorofluorescein and blue 9,10-diphenylanthracene fluorophores as well as signaling peptide sequences of F_xRF_xK and TAT, we successfully realize simultaneous multicolor imaging toward lysosomes and mitochondria in living cells first utilizing polymeric scaffolds. If more biofunctions could be incorporated, modularly designed copolymer would provide a promising opportunity to facilitate multitasking application to monitoring intracellular alterations and elucidating complex biological processes.



INTRODUCTION

Subcellular imaging in living cells is essential for deciphering biological events and interactions because of its spatiotemporal patterning and specific visualization among different organelles.^{1,2} To support the unmet needs for this purpose, a diverse array of luminescent probes, such as fluorescent proteins,^{3–5} monoclonal antibodies,^{6,7} organic chromophores,^{8–11} and semiconductor nanocrystals,^{12,13} have been involved in subcellular imaging by selectively localizing in targeting organelles and visualizing biological species or physiological markers in a microenvironment. However, the multitasking purpose of integrating a couple of functional groups into one complex system for biological applications makes the exquisite design of luminescent probes to avoid synthetic limits and multistep, intricate preparation rather challenging.

Biofunctional polymer is a promising candidate for luminescent probe that can be conveniently incorporated with imaging labels, targeting ligands, therapeutic agents, and other designable elements, thus implementing multiple tasks with high adaptability and satisfying the demands of diversity, bio-orthogonality, and complexity in biological applications.^{14–21} Ring opening metathesis polymerization (ROMP) as a selectable method for the preparation of functional polymers has a number of advantages like simple protocols, widely functional group tolerance, and polymerizations at room temperature as well as the capacity for living and controlled polymerizations.²² Recently, we have developed a modular strategy to fabricate biofunctional polymers from norbornenyl monomers by a one-pot multicomponent ROMP,^{23,24} which offers a simple and effective toolbox for synthesis of well-defined copolymers with a variety of functional

motifs that could bear chromophores,^{25–28} oligopeptides,^{29–37} DNA or protein analogues,^{38–41} hydrophilic and hydrophobic building blocks,^{42–47} hydrogen-bond interfaces,^{48–50} with controllable molecular weight and narrow PDIs (polydispersity indices).⁵¹ Attributed to the repeating functional motifs on polymer chain, the multivalent effect and the enhanced permeation and retention (EPR) effect are capable of increasing the signal-to-noise ratio in bioimaging, enhancing target-binding specificity and improving the bioavailability or theranostic efficacy.^{52,53}

To study the morphological features and dynamic relations among different organelles, simultaneous multicolor imaging in living cells could be employed as a practical toolkit but that strongly depends on the substantial development of functional fluorophores. To the elaborate design of a molecular system, the multitasking combination of active chromophore requires customized chemistry for covalent tethering of organic dyes with targeting motif individually. Moreover, simultaneous targeting among different organelles often result in biocompatible conflicts in their native setting under multichannel imaging conditions. While utilizing modular ROMP, we could fabricate luminescent polymers with bioactive peptide motifs and tunable emission wavelengths, which also provides a universal toolbox for organelle-specific multicolor imaging with designable polynorbornenes in living cells.^{23,24}

For a polynorbornenyl scaffold, functional monomers could be facilely derived from reactive precursors like carboxyl, bromo-,

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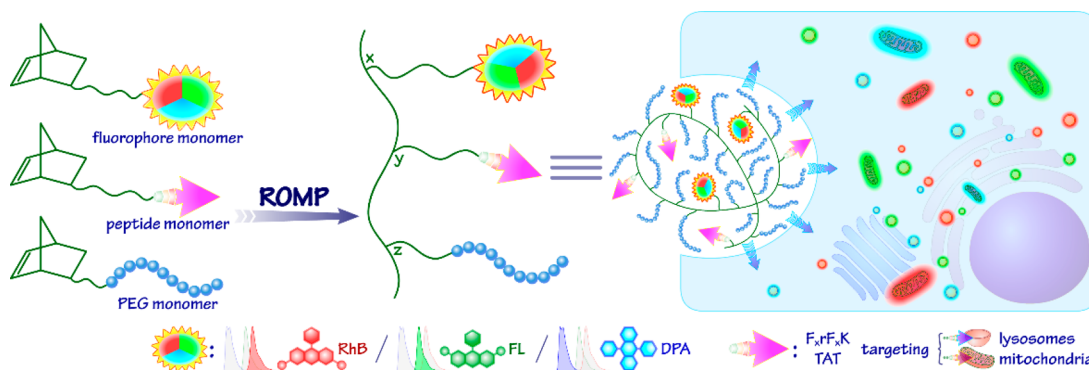


Figure 1. Fabrication of luminescent polynorbornenes and their organelle-specific multicolor imaging in living cells.

amino-, and hydroxyl norbornenes with chemical derivatizations through esterification, amidation, etherization, and so on.^{23,24,29–33} On the basis of the above design rationale, we carefully selected highly fluorescent rhodamine B (RhB), dichlorofluorescein (FL), and 9,10-diphenylanthracene (DPA) from our monomeric libraries as red, green, and blue imaging modules, respectively. These fluorophores possess a set of distinguished properties of bio-orthogonal imaging, panchromatic emission, good photostability, high extinction coefficients and fluorescence quantum yields, which are crucial advantages for sensitive long-term cell tracing. Other functional monomers such as peptide and PEG norbornenes could also be easily obtained from chemical modification and act as targeting or solubilizer module on polynorbornenyl scaffold. In particular, peptide norbornene monomers can be synthesized by a modified SPPS (solid phase peptide synthesis) method, which utilizes *exo*-norbornenyl carboxylic acid as the monomeric synthon like other amino acids and affords full protected peptide monomers with designable sequences to facilitate organelle-targeting capability. Here, we choose cell penetrating peptide TAT (YGRKKRRQRRR) sequence, which had been proven not only to enhance cell permeability, but also to target to lysosomes due to its positive charge,^{54,55} and mitochondria penetrating peptide Fx (F_xR_xF_xK) sequence for subcellular localization of lysosomes and mitochondria, respectively (Figure 1).²⁴ After freely permuting and combining luminescent fluorophores and signaling peptides modules by modular ROMP, the polynorbornenes allow for tunable fluorescence and simultaneous multicolor imaging of specific organelles in living cells.

EXPERIMENTAL SECTION

General. All reactions and manipulations with air- or moisture-sensitive materials were performed utilizing the standard Schlenk techniques under an argon atmosphere. ¹H and ¹³C NMR spectra were recorded with a Bruker Advance DPX 400 MHz spectrometer and were internally referenced to residual protio solvent signals. High-resolution mass spectrometry experiments were performed with a Waters GCT Premier (for EI), or a Bruker Daltonics Apex IV spectrometer (for ESI). A Carlo Erba 1106 elementary analyzer were used for elemental analysis. Polymer molecular weight and dispersity were determined by gel-permeation chromatography (GPC) using a Shimadzu LC-10A pump equipped with both of a multiangle light scattering detector (100 mW 658 nm GaAs laser, Dawn Heleos II, Wyatt, USA) and an Optilab rEX refractive index detector (Wyatt, USA). Elutions were carried out at 0.5 mL/min on a MZ-Gel SDplus 10 μm bead-size column using THF or DMF containing 25 mM LiBr as the eluting solvent at 313 K. Zeta potential (ζ) was measured in 10 mM phosphate-buffered saline (PBS) solution at 298 K on a Malvern Zetasizer Nano ZS90. UV–vis absorption spectra were recorded on either a Hitachi U-3900 or a Shimadzu 1601PC spectrophotometer. Photoluminescent spectra and

lifetime-decay profiles were obtained using TCSPC (time-correlated single-photon counting) techniques on an Edinburgh Analytical Instruments F-900.

Materials and Synthesis. *Exo*-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid and 11-bromoundecyl-*exo*-bicyclo[2.2.1]-hept-5-ene-2-carboxylate were prepared according to published literatures.^{56–59} NB-RhB monomer (5-Rhodamine-B-formyloxypentyl-*exo*-bicyclo[2.2.1]-hept-5-ene-2-carboxylate), organelle-specific peptide monomers (NB-pFx and NB-pTAT monomer), and NB-PEG monomer (methoxypolyethylene-glycol-550-*exo*-bicyclo[2.2.1]-hept-5-ene-2-carboxylate) were synthesized according to our previous report.^{24,60} MitoTracker Red, MitoTracker Green, LysoTracker Green, LysoTracker Red, and Hoechst 33342 were all purchased from KeyGEN Biotech (China). TubulinTracker Green was purchased from Invitrogen Life Technologies (USA). High-glucose Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (USA). Plastic cell culture dishes and plates were purchased from Corning Incorporation (USA). Other materials were obtained from commercial supplies and used as received.

11-(2-(2,7-Dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoyloxy)undecyl-exobicyclo[2.2.1]-hept-5-ene-2-carboxylate (NB-FL Monomer). A mixture of 2,7-dichlorofluorescein (800 mg, 1.99 mmol), 11-bromoundecyl-*exo*-bicyclo[2.2.1]-hept-5-ene-2-carboxylate (740 mg, 1.99 mmol), K₂CO₃ (1.20 g, 8.68 mmol), and DMF (30 mL) was stirred at 80 °C for 4 h under inert atmosphere. After removal of the solvent, the crude product was purified by silica gel column chromatography to give the NB-FL monomer as red solid (1.25 g, 1.81 mmol, yield 91%). TLC, R_f = 0.35 (methylene chloride/ethyl acetate = 1:1). HR-ESI MS: *m/z* = calcd. 689.2073, found 689.2081 for [M – H][–], C₃₉H₃₉Cl₂O₇[–]. ¹H NMR (400 MHz, CDCl₃, ppm) δ: 8.27 (m, 1H), 7.72 (m, 2H), 7.33 (m, 1H), 6.91 (m, 4H), 6.10 (m, 2H), 4.06 (m, 2H), 3.96 (m, 2H), 3.02 (m, 1H), 2.90 (m, 1H), 2.20 (m, 1H), 1.90 (m, 1H), 1.61 (m, 2H), 1.49 (m, 1H), 1.14 (m, 18H). ¹³C NMR (100 MHz, CDCl₃, ppm) δ: 176.50, 169.30, 165.18, 153.79, 151.93, 137.97, 135.72, 133.55, 132.62, 131.34, 130.57, 130.40, 130.07, 128.26, 121.53, 115.82, 110.12, 65.91, 64.62, 46.56, 46.32, 43.21, 41.58, 30.28, 29.40, 29.35, 29.28, 29.13, 29.09, 28.60, 28.24, 25.83 (2C). Anal. calcd. for C₃₉H₄₀Cl₂O₇: C, 67.73; H, 5.83. Found: C, 67.98; H, 5.61.

4-(10-Phenylanthracen-9-yl)phenol. The compound was synthesized via a modified method as light yellow solid according to the literature.⁶¹ After the reaction solvent was changed from EtOH to THF, the yield was improved to 90% in a scale of 5.0 mmol. TLC, R_f = 0.20 (petroleum ether/ethyl acetate = 5:1). ¹H NMR (400 MHz, CDCl₃, ppm) δ: 7.76 (m, 2H), 7.70 (m, 2H), 7.61 (m, 3H), 7.50 (m, 2H), 7.35 (m, 6H), 7.09 (m, 2H), 5.02 (s, 1H).

11-(4-(10-Phenylanthracen-9-yl)phenoxy)undecyl-exobicyclo[2.2.1]-hept-5-ene-2-carboxylate (NB-DPA Monomer). A mixture of 4-(10-phenylanthracen-9-yl)phenol (700 mg, 2.02 mmol), 11-bromoundecyl-*exo*-bicyclo[2.2.1]-hept-5-ene-2-carboxylate (740 mg, 1.99 mmol), K₂CO₃ (1.20 g, 8.68 mmol), and DMF (30 mL) was stirred at 80 °C for 24 h under inert atmosphere. After removal of the solvent, the crude product was purified by silica gel column chromatography to give the NB-DPA monomer as light yellow solid (1.24 g, 1.79 mmol, yield 90%).

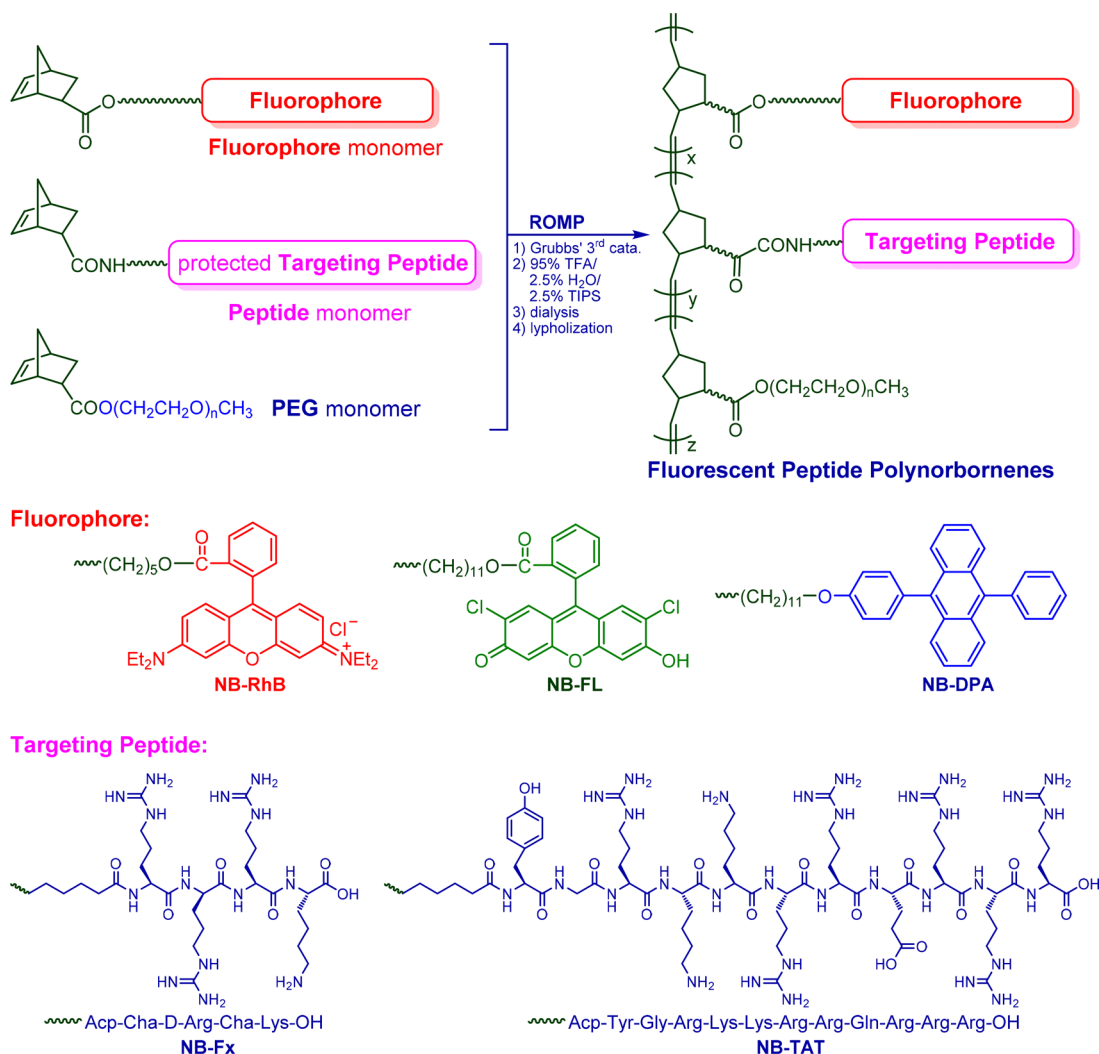


Figure 2. Preparation of luminescent polynorbornene probes.

TLC, $R_f = 0.35$ (petroleum ether/methylene chloride = 2:1). HR-EI MS: $m/z = \text{calcd. } 636.3603$, found 636.3602 for $[M]^+$, $C_{45}H_{48}O_3$. $^1\text{H NMR}$ (400 MHz, CDCl_3 , ppm) δ : 7.78 (m, 2H), 7.70 (m, 2H), 7.61 (m, 3H), 7.50 (m, 2H), 7.39 (d, $J = 8.4$ Hz, 2H), 7.35 (m, 4H), 7.14 (d, $J = 8.4$ Hz, 2H), 6.14 (m, 2H), 4.12 (m, 4H), 3.07 (m, 1H), 2.94 (m, 1H), 2.24 (m, 1H), 1.92 (m, 3H), 1.67 (m, 2H), 1.57 (m, 3H), 1.21 (m, 14H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3 , ppm) δ : 176.27, 158.62, 139.15, 138.01, 137.02, 136.86, 135.77, 132.33, 131.32, 130.85, 130.22, 129.91, 128.36, 127.39, 127.06, 126.91, 124.92, 124.83, 114.39, 68.11, 64.56, 46.61, 46.36, 43.22, 41.63, 30.30, 29.57, 29.50 (2C), 29.44, 29.42, 29.24, 28.71, 26.16, 25.95. Anal. calcd. for $C_{45}H_{48}O_3$: C, 84.87; H, 7.60. Found: C, 85.11; H, 7.52.

Polymer Synthesis General Procedure. Random copolymers were synthesized through modular ROMP with Grubbs' third-generation initiator⁵¹ (~0.01 M) in CHCl_3 at a monomer-to-initiator ratios of 100. For copolymerization, the proportion for fluorophore monomer (NB-RhB, NB-FL, or NB-DPA), protected peptide monomer (NB-pFx or NB-pTAT), and PEG monomer (NB-PEG) were held at 5, 15, and 80, respectively. The polymerization reactions were conducted at room temperature for 15 min, then quenched with ethyl vinyl ether and stirred for an additional 15 min, during which the complete conversion of monomers could be monitored by $^1\text{H NMR}$. The protected copolymers were dried under high vacuum at room temperature and cleaved with a mixture solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and H_2O (95:2.5:2.5). After precipitation into cold ethyl ether, the deprotected polymers were collected by centrifugation and purified by dialysis against water using

Spectra/Por 3 membrane (MWCO 3500) for 5 days in refrigerator at 4 °C to remove all small molecular residues, final lyophilization afforded polymeric products as solids.

Polymer PNB-FL. $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$, ppm) δ : 11.55 (br, 1H), 8.15 (m, 1H), 7.78 (m, 1H), 7.69 (m, 1H), 7.39 (m, 1H), 6.78 (m, 4H), 5.12 (m, 2H), 3.92 (m, 2H), 3.85 (m, 2H), 2.96 (m, 1H), 2.44 (m, 2H), 1.92 (m, 2H), 1.49 (m, 2H), 1.17 (m, 18H).

Polymer PNB-DPA. $^1\text{H NMR}$ (400 MHz, CDCl_3 , ppm) δ : 7.74 (m, 2H), 7.67 (m, 2H), 7.58 (m, 3H), 7.46 (m, 2H), 7.36 (m, 2H), 7.30 (m, 4H), 7.11 (m, 2H), 5.24 (m, 2H), 4.07 (m, 4H), 3.12 (m, 1H), 2.71 (m, 1H), 2.52 (m, 1H), 2.09 (m, 1H), 1.87 (m, 2H), 1.61 (m, 4H), 1.33 (m, 14H), 1.14 (m, 1H).

Polymer PNB-RhB₅-co-pFx₁₅-co-PEG₈₀. $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$, ppm) δ : 12.33 (br, 0.15H), 7.99 (m, 0.95H), 6.97 (m, 0.9H), 5.21 (m, 2H), 4.11 (m, 2.4H), 3.51 (s, 35.44H), 3.42 (m, 1.6H), 3.24 (s, 2.4H), 2.95 (m, 2.35H), 2.48 (m, 2.75H), 1.91 (m, 2.45H), 1.60 (m, 3.25H), 1.36 (m, 8.5H).

Polymer PNB-RhB₅-co-Fx₁₅-co-PEG₈₀. $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$, ppm) δ : 12.09 (br, 0.15H), 7.96 (m, 0.95H), 6.97 (m, 1.2H), 5.20 (m, 2H), 4.11 (m, 2.4H), 3.50 (s, 35.44H), 3.42 (m, 1.6H), 3.23 (s, 2.4H), 3.01 (m, 2.05H), 2.50 (m, 1.85H), 1.94 (m, 2H), 1.62 (m, 3.25H), 1.20 (m, 6.25H).

Polymer PNB-FL₅-co-pFx₁₅-co-PEG₈₀. $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$, ppm) δ : 12.01 (br, 0.2H), 7.84 (m, 0.95H), 6.81 (m, 0.8H), 5.22 (m, 2H), 4.12 (m, 2.4H), 3.51 (s, 35.04H), 3.43 (m, 1.6H), 3.23 (s, 2.4H), 2.95 (m, 2.35H), 2.48 (m, 2.75H), 1.91 (m, 2.45H), 1.61 (m, 3.3H), 1.37 (m, 8.45H).

Table 1. GPC Data, ζ Potentials, and Photophysical Parameters for Luminescent Polynorbornenes

polymer	M_n^a kDa	M_w^a kDa	PDI ^a	ζ^b , mV	λ_{abs}^c nm ($\epsilon/10^3$ L mol ⁻¹ cm ⁻¹)	λ_{em}^c nm	τ_{em}^c ns	Φ_{em}^c
PNB-RhB ₅ -co-TAT ₁₅ -co-PEG ₈₀	99.8	76.3	1.31	+16.5 ± 2.7	561 (111), 525 (39.8), 403 (4.22), 308 (15.8), 285 (23.2)	592, 637 (sh)	1.69	0.50 ± 0.03
PNB-FL ₅ -co-TAT ₁₅ -co-PEG ₈₀	102.2	75.5	1.35	+11.4 ± 2.1	520 (93.7), 490 (46.2), 327 (7.88), 299 (19.0)	548, 592 (sh)	5.42	0.89 ± 0.04
PNB-DPA ₅ -co-TAT ₁₅ -co-PEG ₈₀	89.1	71.8	1.24	+15.3 ± 2.9	398 (15.8), 378 (17.0), 358 (11.1), 341 (6.02)	423, 442, 469 (sh)	8.69	1.00 ± 0.04
PNB-RhB ₅ -co-Fx ₁₅ -co-PEG ₈₀	81.0	68.3	1.19	+11.6 ± 2.4	561 (114), 524 (41.0), 403 (3.53), 307 (14.7), 283 (21.7)	592, 637 (sh)	1.68	0.51 ± 0.03
PNB-FL ₅ -co-Fx ₁₅ -co-PEG ₈₀	92.4	72.1	1.28	+9.3 ± 1.5	520 (96.8), 491 (49.9), 326 (8.15), 298 (19.3)	548, 591 (sh)	5.36	0.88 ± 0.02
PNB-DPA ₅ -co-Fx ₁₅ -co-PEG ₈₀	90.2	74.9	1.20	+11.9 ± 1.8	398 (15.6), 378 (17.1), 359 (11.7), 342 (6.24)	423, 442, 470 (sh)	8.72	1.00 ± 0.05

^aMeasured at 313 K in 25 mM LiBr DMF solution. ^bMeasured in 10 mM PBS at 298 K with polymer concentration of 1.0 mg/mL. ^cMeasured in 10 mM PBS, pH 7.4.

Polymer PNB-FL₅-co-Fx₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 11.97 (br, 0.2H), 7.91 (m, 0.95H), 6.88 (m, 1.1H), 5.21 (m, 2H), 4.11 (m, 2.4H), 3.51 (s, 35.04H), 3.42 (m, 1.6H), 3.24 (s, 2.4H), 3.00 (m, 2.05H), 2.50 (m, 1.85H), 1.96 (m, 2H), 1.65 (m, 3.3H), 1.24 (m, 6.2H).

Polymer PNB-DPA₅-co-Fx₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.14 (br, 0.15H), 7.91 (m, 1.5H), 7.19 (m, 0.7H), 5.22 (m, 2H), 4.12 (m, 2.4H), 3.51 (s, 35.04H), 3.43 (m, 1.6H), 3.24 (s, 2.4H), 2.95 (m, 2.35H), 2.48 (m, 2.75H), 1.95 (m, 2.5H), 1.64 (m, 3.4H), 1.38 (m, 8.35H).

Polymer PNB-DPA₅-co-TAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 11.96 (br, 0.15H), 7.92 (m, 1.5H), 7.18 (m, 1H), 5.21 (m, 2H), 4.12 (m, 2.4H), 3.50 (s, 35.44H), 3.42 (m, 1.6H), 3.23 (s, 2.4H), 2.97 (m, 2.05H), 2.50 (m, 1.85H), 1.90 (m, 2.05H), 1.61 (m, 3.4H), 1.25 (m, 6.1H).

Polymer PNB-RhB₅-co-pTAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.25 (br, 0.15H), 7.91 (m, 2H), 7.15 (m, 2.25H), 6.41 (m, 4.05H), 5.21 (m, 2H), 4.11 (m, 3.6H), 3.51 (s, 35.44H), 3.42 (m, 1.6H), 3.24 (s, 2.4H), 2.94 (m, 6.25H), 2.47 (m, 7.1H), 1.99 (m, 4.85H), 1.63 (m, 0.85H), 1.39 (m, 18.4H).

Polymer PNB-RhB₅-co-TAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 11.93 (br, 0.45H), 7.90 (m, 1.85H), 6.62 (m, 5.25H), 5.20 (m, 2H), 4.10 (m, 3.6H), 3.50 (s, 35.44H), 3.41 (m, 1.6H), 3.23 (s, 2.4H), 3.03 (m, 4.45H), 2.50 (m, 1.7H), 1.93 (m, 2.15H), 1.52 (m, 0.85H), 1.20 (m, 8.95H).

Polymer PNB-FL₅-co-pTAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.18 (br, 0.2H), 7.93 (m, 2H), 7.14 (m, 2.25H), 6.39 (m, 3.95H), 5.22 (m, 2H), 4.12 (m, 3.6H), 3.50 (s, 35.04H), 3.43 (m, 1.6H), 3.22 (s, 2.4H), 2.95 (m, 6.25H), 2.47 (m, 7.1H), 1.98 (m, 4.85H), 1.63 (m, 0.9H), 1.37 (m, 18.35H).

Polymer PNB-FL₅-co-TAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.12 (br, 0.5H), 7.92 (m, 1.85H), 6.58 (m, 5.15H), 5.21 (m, 2H), 4.12 (m, 3.6H), 3.50 (s, 35.04H), 3.42 (m, 1.6H), 3.22 (s, 2.4H), 2.99 (m, 4.45H), 2.50 (m, 1.7H), 1.93 (m, 2.15H), 1.54 (m, 0.9H), 1.24 (m, 8.9H).

Polymer PNB-DPA₅-co-pTAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.15 (br, 0.15H), 7.95 (m, 2.55H), 7.21 (m, 2.25H), 6.43 (m, 3.85H), 5.22 (m, 2H), 4.11 (m, 3.6H), 3.51 (s, 35.44H), 3.42 (m, 1.6H), 3.23 (s, 2.4H), 2.95 (m, 6.25H), 2.47 (m, 7.1H), 1.99 (m, 4.9H), 1.64 (m, 1H), 1.37 (m, 18.25H).

Polymer PNB-DPA₅-co-TAT₁₅-co-PEG₈₀. ¹H NMR (400 MHz, DMSO-*d*₆, ppm) δ : 12.08 (br, 0.45H), 7.88 (m, 2.4H), 6.64 (m, 5.05H), 5.21 (m, 2H), 4.13 (m, 3.6H), 3.51 (s, 35.44H), 3.42 (m, 1.6H), 3.23 (s, 2.4H), 3.00 (m, 4.45H), 2.50 (m, 1.7H), 1.92 (m, 2.2H), 1.53 (m, 1H), 1.22 (m, 8.8H).

Cell Culture. The cancer cells, human hepatocellular carcinoma Bel-7402 and human cervical carcinoma HeLa, were cultured in DMEM (10% FBS, 100 U mL⁻¹ penicillin, 100 mg L⁻¹ streptomycin). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Cell Viability Assay. Cell viability was evaluated by reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to

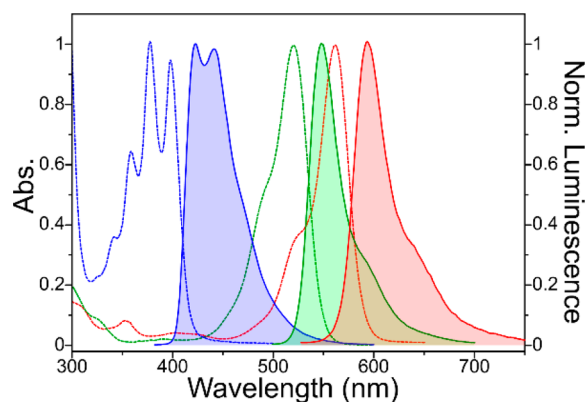


Figure 3. UV-vis absorption and normalized fluorescence spectra of PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ (red), PNB-FL₅-co-Fx₁₅-co-PEG₈₀ (green), and PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ (blue).

formazan crystals using mitochondrial dehydrogenases. Cells were seeded in 96-well plates at density of 5×10^3 /well. After 24 h of incubation, the medium was replaced by the solution of polymeric probes and corresponding commercial organelle trackers at various concentrations, and the cells were then incubated for 24 h, 48 h and 72 h, respectively. After the designated time intervals, the cells were washed three times with PBS and then incubated in 25 μ L of 5 mg/mL MTT PBS solution and 100 μ L of fresh DMEM. After incubation for additional 4 h, the MTT solution was carefully removed and the formed formazan crystals in live cells were dissolved using 200 μ L of dimethyl sulfoxide. The plates were shaken for 10 min, and the absorbance value was recorded at 570 nm on a microplate reader (Thermo Multiskan MK3). Results are expressed as a percentage of cell viability by taking untreated cultures as controls.

Cellular Uptake and Endocytosis Pathway. Carcinoma cells were seeded in six-well plates with a density of 1×10^6 /well and incubated at 37 °C overnight. After being rinsed with PBS, the cells were incubated with polymer probes at given concentration for 4 h at 4 or 37 °C. Thereafter, the cells were washed with PBS three times and harvested by trypsinization. After centrifugation, the cells were suspended in PBS buffer and analyzed immediately on an LSRFortessa flow cytometry (BD Biosciences). To elucidate potential endocytosis pathway of the polymers, cells were preincubated for 30 min with different inhibitors of macropinocytosis (50 nM wortmannin), clathrin-mediated endocytosis (10 μ g/mL chlorpromazine), or caveolae-mediated endocytosis (200 μ g/mL genistein or 5 μ g/mL cytochalasin D), and then the same inhibitors were also present during subsequent incubation with the copolymers.

Colocalization Imaging. Cells were seeded with a density of 1×10^5 /well in 35 mm glass bottom culture dishes. After incubation with the polymeric probes for 4 h, the cells were stained with corresponding organelle trackers, MitoTracker Red or MitoTracker Green,

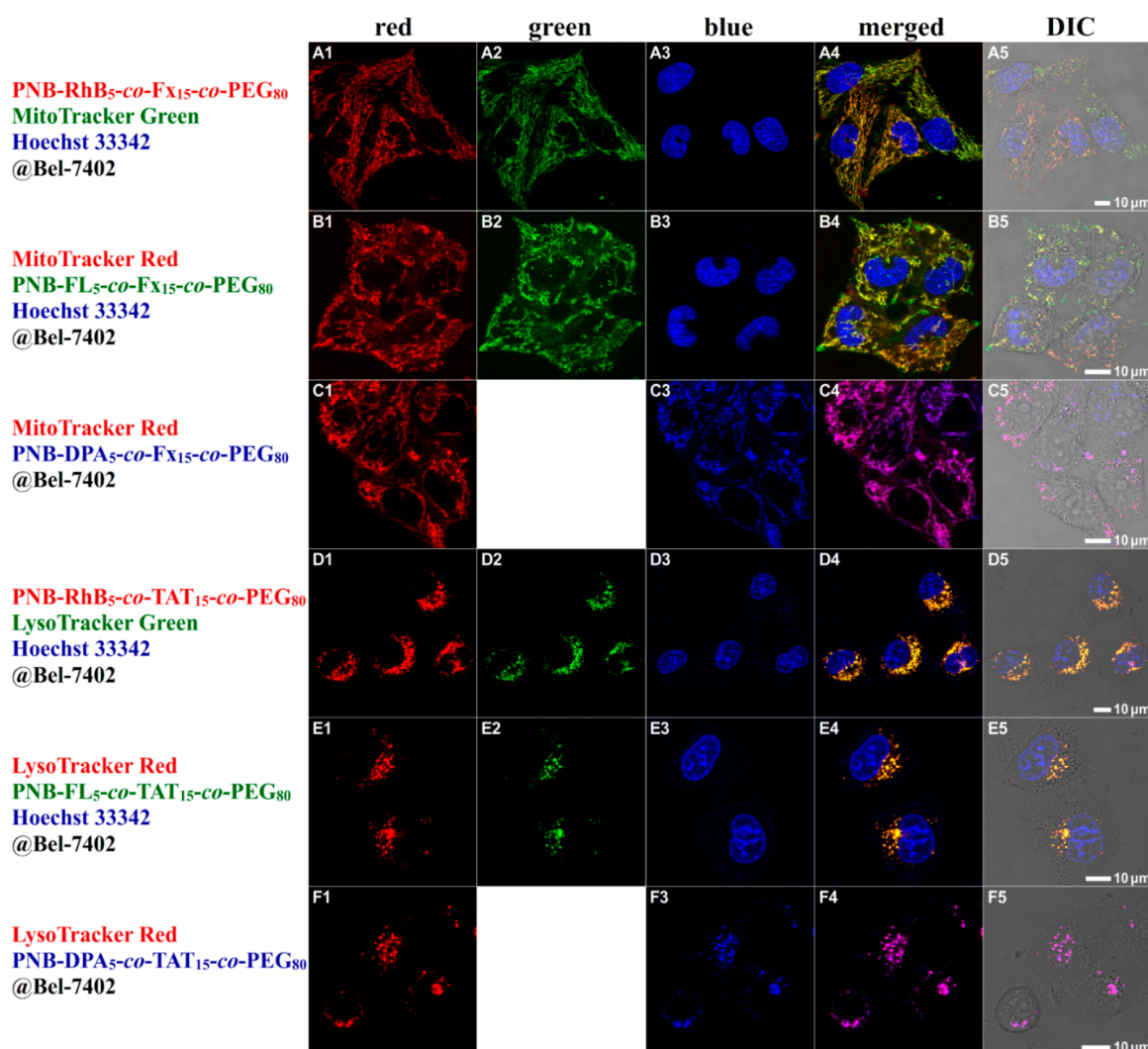


Figure 4. CLSM images for Bel-7402 cells incubated with polymeric probes and corresponding commercial organelle trackers. (A) Colocalization of PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ with MitoTracker Green, and counterstained with Hoechst 33342, Pearson's $r = 0.92 \pm 0.05$, $n = 10$. (B) Colocalization of PNB-FL₅-co-Fx₁₅-co-PEG₈₀ with MitoTracker Red, and counterstained with Hoechst 33342, Pearson's $r = 0.88 \pm 0.08$, $n = 10$. (C) Colocalization of PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ with MitoTracker Red, Pearson's $r = 0.90 \pm 0.06$, $n = 10$. (D) Colocalization of PNB-RhB₅-co-TAT₁₅-co-PEG₈₀ with LysoTracker Green, and counterstained with Hoechst 33342, Pearson's $r = 0.71 \pm 0.13$, $n = 10$. (E) Colocalization of PNB-FL₅-co-TAT₁₅-co-PEG₈₀ with LysoTracker Red, and counterstained with Hoechst 33342, Pearson's $r = 0.59 \pm 0.15$, $n = 10$. (F) Colocalization of PNB-DPA₅-co-TAT₁₅-co-PEG₈₀ with LysoTracker Red, Pearson's $r = 0.77 \pm 0.09$, $n = 10$.

LysoTracker Red or LysoTracker Green for 30 min, and the cell nuclei visualized with Hoechst 33342. For the prolonged incubation period, the cells were costained with the Fx containing copolymers and MitoTracker dyes for 24 h. Then the cells were rinsed thoroughly, placed in fresh serum-free medium, and visualized directly by confocal laser scanning microscopy (CLSM, Leica TCS-SP8). The excitation and emission settings were as follows: PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, PNB-RhB₅-co-TAT₁₅-co-PEG₈₀, MitoTracker Red, and LysoTracker Red (ex, 530 nm; em, 610/30 nm); PNB-FL₅-co-Fx₁₅-co-PEG₈₀, PNB-FL₅-co-TAT₁₅-co-PEG₈₀, MitoTracker Green, and LysoTracker Green (ex, 488 nm; em, 530/30 nm); PNB-DPA₅-co-Fx₁₅-co-PEG₈₀, PNB-DPA₅-co-TAT₁₅-co-PEG₈₀, and Hoechst 33342 (ex, 405 nm; em, 480/30 nm).

Multicolor Imaging. Cells were prepared in dishes as described in the colocalization experiment and simultaneously incubated with four pairs of polymeric probes: PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ and PNB-DPA₅-co-TAT₁₅-co-PEG₈₀, PNB-RhB₅-co-TAT₁₅-co-PEG₈₀ and PNB-DPA₅-co-Fx₁₅-co-PEG₈₀, PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ and PNB-FL₅-co-TAT₁₅-co-PEG₈₀, PNB-RhB₅-co-TAT₁₅-co-PEG₈₀ and PNB-FL₅-co-Fx₁₅-co-mPEG₈₀, which localize in mitochondria and lysosome, respectively, and counterstained with TubulinTracker Green or Hoechst 33342. After rinsing in PBS, the cells were subjected to imaging analysis by using

CLSM with the same light sources and filter settings as the colocalization experiment.

Statistical Analysis. ANOVA test was applied to statistical analysis, and corresponding results were expressed as mean $\pm$ SD. A p value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

According to permutation and combination, all six norbornene copolymers were fabricated from fluorophore monomers (NB-RhB, NB-FL, and NB-DPA) as multicolor imaging module, peptide monomers (NB-pTAT and NB-pFx) as targeting module, and biocompatible PEG monomer (NB-PEG) as hydrophilic solubilizer using modular ROMP as a versatile toolbox. By initiation with Grubbs' third-generation catalyst in CHCl₃, the peptide functionalized fluorescent copolymers can be obtained freely from the combination of the above monomeric function norbornenes (Figure 2). A fixed DP (degree of polymerization) value of 100 was applied to all polynorbornenes to guarantee the molar ratio of total feeding amounts of norbornenyl monomers

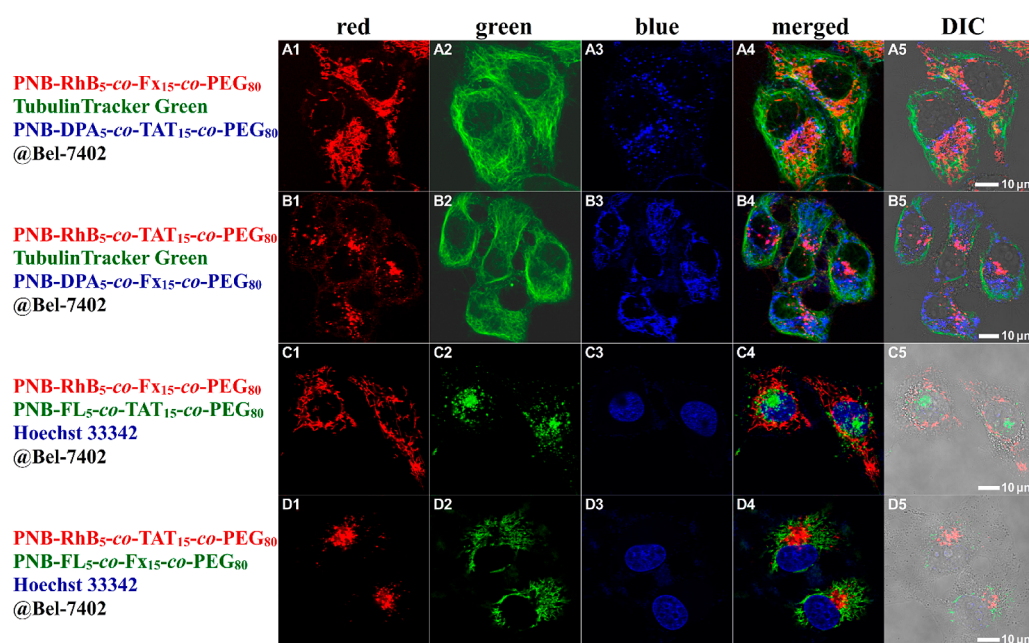


Figure 5. CLSM images for Bel-7402 cells incubated with (A) PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ and PNB-DPA₅-co-TAT₁₅-co-PEG₈₀, (B) PNB-RhB₅-co-TAT₁₅-co-PEG₈₀ and PNB-DPA₅-co-Fx₁₅-co-PEG₈₀, and counterstained with TubulinTracker Green; or internalized with (C) PNB-RhB₅-co-Fx₁₅-co-PEG₈₀ and PNB-FL₅-co-TAT₁₅-co-PEG₈₀, (D) PNB-RhB₅-co-TAT₁₅-co-PEG₈₀ and PNB-FL₅-co-Fx₁₅-co-PEG₈₀, and counterstained with Hoechst 33342.

to Grubbs' initiator. The fluorophore monomer (NB-RhB, NB-FL, and NB-DPA) proportion x , the peptide monomer proportion y and the PEG monomer proportion z are empirically held at 5, 15, and 80, respectively, this doping ratio was optimized with comprehensive consideration of molecule weight, polymer distribution, polymerization dynamics, fluorescent intensity, targeting capability as well as aqueous solubility. After TFA-mediated cleavage and dialysis against water, we obtained the unprotected and bioactivated copolymers with well-controlled molecular weights and PDIs, all these results were reasonably confirmed by GPC and ¹H NMR characterizations (Table 1 and Figures S28–S39).

In agreement with our preliminary research, functional peptides and hydrophilic PEG solubilizer are orthogonal toward fluorophore modules after copolymerization.^{23,24} As we expected, the photophysical properties of red fluorescent RhB, green fluorescent FL and blue fluorescent DPA are interfered little with the combination of either TAT and Fx peptide motifs or hydrophilic PEG solubilizer. As listed in Table 1, polymeric Fx series PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, PNB-FL₅-co-Fx₁₅-co-PEG₈₀, and PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ exhibit typical red, green, and blue emissions at 580–640, 520–570, and 420–470 nm, respectively, and correlated excitations and luminescence match well with CLSM setups and configurations. In addition, RhB, FL, and DPA copolymers present excellent solubility in an aqueous media and exhibit almost identical UV–vis absorption and fluorescence spectra in PBS buffer as compared with their polymeric or monomeric analogues (Figure 3 and Figures S7–S9). Corresponding lifetimes (τ) and fluorescence quantum yields (Φ) are also comparable in similar solvents (Table 1 and Table S2). It is worth noting that both of the blue fluorescent DPA copolymers PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ and PNB-DPA₅-co-TAT₁₅-co-PEG₈₀ possess a fluorescence quantum yield up to 100%, which would be a meaningful exemplar to the scarceness of highly fluorescent blue probes in living cells.

An ideal multicolor imaging probe for practical application should minimally perturb living organism at the concentrations

employed. Hence, the cytotoxicities of these copolymers against two different types of cancer cells, human cervical carcinoma HeLa cells and human hepatocellular carcinoma Bel-7402 cells, were accessed using an MTT assay. The commercial organelle imaging probes MitoTracker (red and green) and LysoTracker (red and green) were coincubated as controls. Figures S1 and S2 display the cell viability percent for cancer cells after treatment with a series of concentrations of PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, PNB-FL₅-co-Fx₁₅-co-PEG₈₀, and PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ polymeric probes for 24 h. At any given concentrations of copolymers, no significant cytotoxicities (cell viability >95%) were observed in all of the tested cells, indicating excellent biocompatibility of these polynorbornenes. In comparison, the commercial MitoTracker dyes showed obvious cytotoxicity over the same time course at comparable concentrations. Moreover, the cells still remain adherent and viable in cultures as exposed to the probing copolymers during a sufficiently long observation period of up to 72 h, this feature offers practical advantage of polynorbornene scaffolds for long-term subcellular monitoring on physiological processes in living cells. In addition, the quantitative analysis (Figure S3) in flow cytometry indicated that the fluorescent polynorbornenes internalized into cells through caveolae rather than clathrin-mediated endocytosis or macropinocytosis pathways, which is consistent with our previous studies.²⁴

Importantly, we intend to emphatically demonstrate the simultaneous multicolor imaging capability of these polymeric probes in distinct cellular compartments while freely permuting and combining luminescent fluorophores (red RhB, green FL, and blue DPA) as well as signaling peptides (Fx and TAT). Colocalization study on organelle trackers reveals that polymeric Fx (or TAT) series with amendable fluorescence of red PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, green PNB-FL₅-co-Fx₁₅-co-PEG₈₀, and blue PNB-DPA₅-co-Fx₁₅-co-PEG₈₀ present excellent targeting to mitochondria (or lysosomes) and exhibit a perfect overlap with MitoTrackers (or LysoTrackers) (Figure 4, Figure S4). Moreover, these polymeric probes did not show any significant

changes in CLSM imaging and morphology of the cells after additional 24 h of incubation; on the contrary, only weak fluorescence were detectable in the MitoTrackers-stained cells after the same incubation period (Figure S5). By coinubation with a triple combination (i.e., counterstaining with two fluorescent polynorbornenes and one commercial dye) in Bel-7402 cells, we found PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, TubulinTracker Green, and PNB-DPA₅-co-TAT₁₅-co-PEG₈₀ will simultaneously occupy the red, green, and blue imaging channels in CLSM measurements. In compliance with their subcellular targeting attribution, red Fx polymer PNB-RhB₅-co-Fx₁₅-co-PEG₈₀, green TubulinTracker, and blue TAT polymer PNB-DPA₅-co-TAT₁₅-co-PEG₈₀ make characteristic patterning toward mitochondria, endoplasmic reticulum, and lysosomes, respectively (Figure 5A). Other triple combinations also carry out perfect visualizations among different organelles such as mitochondria, lysosomes, microtubules, and nucleus, and exhibit excellent bioorthogonality during simultaneous multicolor imaging (Figure 5B–D). This result implies that if more biofunctions could be incorporated, modularly designed polymer scaffold would provide a promising opportunity to facilitate multitasking application to monitoring intracellular alterations and elucidating complex biological processes.

CONCLUSIONS

In summary, we designed and fabricated biofunctional polynorbornenes directly from panchromatic fluorophores, bioactive peptides, and PEG solubilizer for organelle-specific multicolor imaging utilizing a modular ROMP strategy. Attributed to the free permutation and combination of highly fluorescent red RhB, green FL, and blue DPA fluorophores as well as signaling peptide sequences of Fx and TAT, we successfully realize simultaneous multicolor imaging toward lysosomes and mitochondria in living cells first utilizing polymeric scaffolds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.8b00338.

Cytotoxicity of polymers and commercial dyes, multicolor imaging of polymers in HeLa cells, colocalization of polymers with commercial dyes; UV–vis absorption and fluorescence spectra, decay profiles of fluorescence, NMR and mass spectra for monomers and polymers (PDF)

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Notes

The authors declare no competing financial interest.

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