Nile blue-based nano-sized pH sensors for simultaneous far-red and near-infrared live bioimaging

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1. Table of Contents

1. Table of Contents ........................................................................................................... 1
2. Tables ............................................................................................................................... 2
3. Figures ............................................................................................................................... 2
4. Materials ........................................................................................................................... 3
5. Synthesis of NBM monomer ............................................................................................ 4
6. Synthesis of NBC monomer ............................................................................................. 4
7. Reverse-phase HPLC analysis of Nile Blue monomers ................................................... 5
8. Dye purification ............................................................................................................... 5
9. Synthesis of PMPC macro-CTA via RAFT polymerization ........................................... 5
10. ATRP of PMPC with Nile Blue dye present throughout the polymerization .......... 6
11. ATRP of MPC with Nile Blue dye added at high MPC conversion .............................. 6
12. Synthesis of PMPC-PDPA-NBM with Nile Blue dye present throughout ............... 7
13. Synthesis of PMPC-PDPA-NBX with dye added at high conversion ....................... 7
14. Synthesis of PGMA by RAFT with and without Nile Blue Monomers ..................... 7
15. RAFT synthesis of PMPC_{50-0.2} in ethanol ................................................................ 8
16. Addition of Nile Blue Monomers to PMPC Macro-CTAs ........................................... 8
17. Determination of molar absorption coefficients and integrated absorption coefficients for dyes in ethanol........................................................................................................... 8
18. Spectroscopic determination of polymer dye contents in ethanol ............................ 9
19. Absorption of Polymers in PBS and determination of relative quantum yield ......... 9
20. pH-dependent absorption of Nile Blue-containing PMPC-PDPA diblock copolymers in PBS ......................................................................................................................... 9
21. pH-dependent emission of Nile Blue-containing PMPC homopolymers in PBS .... 10
22. pH-dependent emission of Nile Blue monomers ....................................................... 10
23. pH-dependent emission of Nile Blue-labeled PMPC-PDPA diblock copolymers in PBS 10
24. Cell culture ..................................................................................................................... 11
25. Cell viability: MTT-ESTA assay ................................................................................... 11
26. Cellular uptake of Nile Blue vesicles: Live imaging via confocal fluorescence microscopy. ................................................................. 11
27. 3D multicell tumour spheroid (MCTS) culture and image analysis ................. 11

2. Tables

Table S1: Absorption spectroscopy data obtained for Nile Blue and Nile Blue-based methacrylic monomers in ethanol after purification. Integrated absorption coefficient according to reference S10.
Purity = [Area of monomer peak]/[Area of all peaks] at 254 nm. No apparent impurities by HPLC at this wavelength. ................................................................................................................................. 13

Table S2: Summary of data obtained for all PMPC polymers. GPC studies conducted in a 3:1 CHCl₃:CH₃OH mixed eluent containing 2mM LiBr. Based on absorption coefficient data at the λmax.
Based on integrated absorption coefficient data. In PBS at pH 7.2. In PBS excited at 550 nm. .... 15

Table S3. Summary of data obtained for the three PMPC-PDPA copolymers used in this study .... 15

3. Figures

Figure S1: Normalized monomer spectra (c: 1·10⁻⁵ M to 3·10⁻⁵ M) in ethanol ................................................................. 16

Figure S2. Kinetic plots obtained for the ATRP of MPC in ethanol at 30 °C in the presence of three Nile Blue-based vinyl monomers (NBM and NBMP) and Nile Blue alone. (A) Conversion vs. time curves for 33 % w/v MPC using[MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:2:1; (B) corresponding semi-logarithmic plots; (C) conversion vs. time for 67 % w/v using[MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:3:1.5; (D) corresponding semi-logarithmic plots. ........................................................................................................... 16

Figure S3: Conversion and ln[M]/[M] for the polymerization of glycerol monomethacrylate (DP = 60) with and without NBC. .................................................................................................................... 17

Figure S4: Chemical structures of radical polymerization inhibitors Phenothiazine and Methylene Blue compared with Nile Blue .................................................................................................................. 17

Figure S5: (A) General kinetic equations describing the free radical polymerization of a vinyl monomer in the presence of an inhibitor dye (methylene blue) according to Reference S11. (B) Suggested specific reaction between Nile Blue and an alkyl radical to form labeled polymer chains in 2 steps: 1) Hydrogen abstraction from Nile Blue by a polymeric radical leading to formation of a Nile Blue radical (corresponding to reaction (6) in (A)) and 2) Reaction between a Nile Blue radical and a (different) polymer radical chain (corresponding to reaction (7) in (A)). ................................................................. 18

Figure S6: Normalized absorption spectra of monomers (c: 1-3·10⁻⁵ M) and corresponding polymers (1-4 mg/mL) in ethanol .................................................................................................................. 19

Figure S7: Normalized absorption spectra of polymers (1-4 mg/mL) and Nile Blue (NB, 5.4·10⁻⁵ M) in PBS pH 7.3 .................................................................................................................. 19

Figure S8: pH-dependence of the fluorescence emission spectra recorded for Nile Blue (NB) (~9 µM) and Nile Blue-based PMPC homopolymers in dilute aqueous solution (~1 mg/mL) ................................................................. 20

Figure S9: (A)-(D) Fluorescence emission vs. solution pH recorded for ~ 1 mg/mL aqueous solutions of PMPC-NBC, PMPC-NBMP, PMPC-NB and Nile Blue (~ 9 µM in its ClO₄⁻ salt form). (E)-(H) Fluorescence emission vs. solution pH recorded for aqueous solutions of NBC (~5 µM in its Cl⁻ salt form), NBM (~6 µM in its Cl⁻ salt form) and Nile Blue (~ 9 µM in its ClO₄⁻ salt form) (A), (E) Relative emission at 700 nm and 670 nm (excitation wavelength = 550 nm); (B), (F) Relative emission at 700 nm and 670 nm (excitation wavelength = 650 nm); (C), (G) Integrated emission intensity from 560 nm to 800 nm determined for an excitation wavelength of 550 nm relative to the integrated intensity observed at the lowest pH value; (D), (H) Integrated emission intensity from 660 nm to 800 nm determined relative to the integrated intensity observed at the lowest pH value (excitation wavelength = 650 nm) ..................................................................................................................................... 21

Figure S10: Fluorescence emission spectra vs pH recorded for NBM (~6 µM) and NBC (5 µM) .... 22
Figure S11: Figure S1: Differential uptake of PMPC-PDPA diblock copolymer vesicles and PMPC homopolymer chains by HDF cells. Cells were incubated with (A) rhodamine 6G-labeled PMPC\textsubscript{25}-PDPA\textsubscript{70} diblock copolymer vesicles or (B) rhodamine 6G-labeled PMPC\textsubscript{100} homopolymer at 37 °C in 5% CO\textsubscript{2} for 2 h. After incubation with the (co)polymer, the cells were rinsed three times with PBS and placed in DMEM (1.0 mL) for live cell imaging. DAPI (blue, left) is used to locate the cell nuclei. The PMPC\textsubscript{25}-PDPA\textsubscript{75} diblock copolymer vesicles (red, middle) are readily uptaken by the cells, whereas the PMPC\textsubscript{100} homopolymer is not uptaken at all under the same conditions (no signal, middle). Merged images are shown on the right. Scale bar = 100 µm for all images.

Figure S12: Fluorescence emission vs pH for 1 mg/mL aqueous solutions of various Nile Blue-labeled PMPC-PDPA diblock copolymers. Conditions: excitation slit: 5 nm; emission slit: 5 nm; photomultiplier voltage = 800 V. (A) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{0.12} excited at 550 nm; (B) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{0.12} excited at 650 nm; (C) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{M0.15} excited at 550 nm; (D) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{M0.15} excited at 650 nm; (E) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{C0.08} excited at 550 nm; (F) PhO-PMPC\textsubscript{25}-PDPA\textsubscript{70}-NB\textsubscript{C0.08} excited at 650 nm.

Figure S13: 24 h viability of Human Dermal Fibroblasts exposed to 1 mg/mL Nile Blue labeled PMPC-PDPA diblock copolymer vesicles.

Figure S14: pH-dependent staining of multicell tumour spheroids (MCTS) by Nile blue-labeled PMPC-PDPA copolymers. Bright-field optical images of Nile Blue polymer treated MCTS and controls were recorded using a Leica DMI4000B instrument equipped with a Nuance Multispectral imaging System. Untreated controls were used to subtract the background noise from the images. The image analysis was performed double-blinded. Four independent observers were asked to score the center of the tumor (white star), the edge (red dotted line) and identify the acidic/blue color boundary (black dotted line). Hypoxic (low pH regions) of the spheroids were compared to a positive control marker for hypoxia (Glut-1 immunostaining).

4. Materials

Lithium bromide (LiBr, 99 +%), Nile Blue A (sulfate) were obtained from Acros Organics (Geel, Belgium) and were used as received. Nile Blue A ( perchlorate) (95 %), Sodium hydrogen carbonate (99.7 +%, A.C.S. grade), CuBr (99.999 %), 2,2'-bipyridine (bpy, 99 %), Dimethylaminopyridine (DMAP, ≥99 %) methacrylic anhydride (94 %), deuterated chloroform (CDCl\textsubscript{3}, 99.8 Atom % D) trifluoroacetic acid (TFA, 99+ %), triethylamine (Et\textsubscript{3}N, ≥ 99 %), 2-cyano-2-propyl dithiobenzoate (CPDB), 4,40-azobis(4-cyanopentanoic acid) (ACVA; V-501; 99%), were all purchased from Sigma Aldrich UK (Dorset, UK) and were used as received. HPLC grade acetonitrile, diethyl ether, dichloromethane, methanol, dialysis membranes (Spectra/Por 6, MWCO 1,000) were obtained from Fisher Scientific (Loughborough, UK) and were used as received. Magnesium sulfate (MgSO\textsubscript{4}), sodium chloride (NaCl), triethylamine (Et\textsubscript{3}N) and sodium sulfate (Na\textsubscript{2}SO\textsubscript{4}) were laboratory reagent grade from Fisher Scientific (Loughborough, UK) and were used as received. Deuterated methanol (CD\textsubscript{3}OD, 99.96 atom %), was purchased from Goss Scientific (Nantwich, U.K.).

2-(Methacryloyloxy)ethyl phosphorylcholine (MPC; > 99%) was kindly donated by Biocompatibles Ltd. (Farnham, Surrey, UK). Glycerol monomethacrylate (GMA; 99.8%) was donated by Cognis Performance Chemicals (Hythe, U.K.) 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako Pure Products and 4-cyano-4-(dodecylsulfanylthiocarbonyl)sulfonyl pentanoic acid (DTTC) was synthesized according to a literature protocol.\textsuperscript{1} ATRP initiators, 4-(2-bromoisobutyroyl ethyl)morpholine (MEBr) and 2-Phenoxyethyl 2-Bromoisobutyrate (PhOBr) were prepared according to previously published procedures.\textsuperscript{2,3}
5. Synthesis of NBM monomer

Nile Blue A (sulfate salt form) (5.032 g, 13.7 mmol) was placed in a 500 mL round-bottom flask fitted with a magnetic stir bar. Dichloromethane (250 mL) and triethylamine (6 mL, 4.356 g, 43.0 mmol) were added and the resulting dark red mixture was placed under nitrogen and cooled on an ice-bath. Methacrylic anhydride (3 mL, 3.1 g, 20.1 mmol) was added as a solution in dichloromethane (50 mL), followed by DMAP (0.1072 g, 0.877 mmol), and the mixture was allowed to heat to room temperature (~22 °C). The reaction was monitored by TLC (dichloromethane:methanol 19:1 v/v). After 22 h, further methacrylic anhydride (1.10 mL, 1.14 g, 7.38 mmol) and triethylamine (2.00 mL, 1.45 g, 14.3 mmol) was added and the reaction mixture was stirred for a further 26 h. Solvent was evaporated at 35 °C under reduced pressure. The solid was washed with water (4 x 150 mL) and filtered.

The solid was redispersed in diethyl ether (500 mL). Then, 2 M HCl in diethyl ether (10 mL, 20 mmol HCl) was added, which led to the formation of a dark blue precipitate. The flask was placed at -25 °C. After 15 h, the flask was allowed to heat to room temperature and the resulting crystals were collected on filter paper and washed with diethyl ether (250 mL). The product was dried in a vacuum oven at 25 °C to give 4.3 g (74 %) of product.

The product was further purified on a silica column using a 9:1 dichloromethane:methanol mixture as a mixed eluent. Data below are reported for the non-columned material.

$^1$H NMR (400 MHz, CD$_3$OD) δ 8.95 (d, 1H, J=8.070 Hz), 8.48 (s, 1H), 8.28 (d, 1H, J=8.314 Hz), 8.01 (d, 1H, 9.781 Hz), 7.85 (m, 3H), 7.27 (d, 1H, J=2.690 Hz), 6.11 (s, 1H), 5.8 (s, 1H), 3.98 (m, 4 H), 2.18 (s, 3H), 1.48 (br t, 6H, 7.091 Hz) ppm

$^{13}$C NMR (400 MHz, CD$_3$OD)

δ 168.77, 158.43, 154.26, 151.32, 150.24, 146.07, 141.13, 140.21, 135.11, 130.63, 130.17, 128.32, 124.61, 123.22, 122.89, 122.60, 121.68, 106.76, 96.70, 45.56, 17.53 ppm

ESI-MS, m/z (M+H)$^+$ 386

Accurate Mass (Calculated), (M): 386.1860 (386.1869) corresponding to an elemental composition of C$_{24}$H$_{24}$N$_3$O$_2$ (C$_{24}$H$_{24}$N$_3$O$_2$)

6. Synthesis of NBC monomer

Nile Blue A (sulfate salt form) (1.1141 g, 3.04 mmol) and dichloromethane (25 mL was mixed and placed under nitrogen. Triethylamine (5.0 mL, 3.6 g, 36 mmol) was added and the solution was placed on ice. After 15 min, 2-isocyanatoethyl methacrylate (0.50 mL, 0.55 g, 3.5 mmol) was added dissolved in dichloromethane (10 mL). The reaction mixture was stirred for 20 h. Then methanol (2 mL, 1.6 g, 49 mmol) was added to quench the reaction. After 6 h, the solvent was evaporated and the residue was washed with water (50 mL). The precipitate was dissolved in dichloromethane (10 mL) and diethyl
ether (500 mL). 2 M HCl in ether (2.5 mL) was added, which led to precipitation. The solution was placed at -25 °C for 2 h to precipitate fully. The precipitate was isolated by filtration, washed with diethyl ether (250 mL) and dried under vacuum to yield 1.1 g (71 %) of product.

The product was further purified on a silica column using a 9:1 dichloromethane:methanol mixed eluent. Data below are reported for the non-columned material.

\[ ^1H\text{ NMR (400 MHz, CD}_3\text{OD)} \delta 9.05 (br 1H), 8.72 (br, 1H), 8.35 (d, 1H, J=8.2 Hz), 7.95 (m, 3H), 7.70 (br, 1H), 7.25 (br, 1H), 6.21 (s, 1H), 5.70 (s, 1H), 4.36 (t, 2H, J=5.38 Hz), 3.91 Hz (br q, 4 H), 3.70 (t, 2H, J=5.14 Hz), 1.90 (s, 3H), 1.44 (t, 6H, 7.09 Hz) ppm \]

\[ ^13C\text{ NMR (400 MHz, 3:1 CDCl}_3\text{: CD}_3\text{OD)} \delta 167.32, 161.54, 147.85, 140.79, 136.29, 130.96, 130.30, 128.59, 125.38, 123.45, 122.17, 121.95, 120.67, 115.54, 110.83, 106.33, 105.48, 100.56, 96.28, 92.21, 63.54, 46.42, 38.71, 17.10, 7.90 ppm \]

ESI-MS, m/z (M+H)\(^+\): 460

Accurate Mass (Calculated), (M): 460.2226 (460.2236) corresponding to an elemental composition of C\(_{27}\)H\(_{30}\)N\(_3\)O\(_4\) (C\(_{27}\)H\(_{30}\)N\(_3\)O\(_4\))

7. **Reverse-phase HPLC analysis of Nile Blue monomers**

HPLC chromatograms were acquired using a Shimadzu UFLC XR system consisting of two LC20AD XR pumps, a SIL-20A-XR autosampler, a SPD-M20A diode array detector and a 15 cm x 3.0 mm Genesis C18 120A 4µ HPLC column. Chromatographic conditions: eluent A: 0.1 % v/v TFA in water. eluent B: 0.1 % v/v TFA in acetonitrile. 0-5 min: 60 % A, 40 % B, 5-15 min: 60 % A, 40 % B to 0 % A, 100 % B. 15-20 min: 100 % B. Re-equilibration, 60 % A, 40 % B, 9 min. Detection: diode array detection from 190 nm - 700 nm. Data were collected and analyzed using Shimadzu Labsolutions LCSolution version 1.25 software.

8. **Dye purification**

Due to the relatively low level of dye incorporation the amount of dye which is not incorporated into the polymer becomes significant. Therefore, it is beneficial that the dye is relatively cheap or can be prepared with a minimal amount of purification. For this reason, a technical quality of Nile Blue was used for incorporation into polymers and for preparing NBM and NBC. Similarly, only precipitation was used to purify NBM and NBC used for incorporation into polymers. As shown in Table S1, these dyes each had purities of at least 85 % as measured by analytical HPLC prior to further chromatographic purification.

9. **Synthesis of PMPC macro-CTA via RAFT polymerization**

A mixture of MPC (24.660 g; 83.52 mmol), DTTC (0.749 g; 1.86 mmol) and ethanol (17.040 g; 21.60 mL) was placed in a 50 mL round-bottom flask equipped with a magnetic stirring bar (target degree of
polymerization = 45). After purging with nitrogen for approximately 20 min, VA-044 (150 mg, 0.46 mmol) was added and the mixture was purged for another 5 min. The flask was placed in a pre-heated oil bath at 40°C and the reaction was left to proceed for 20 h. The polymer (PMPC conversion = 97%; aqueous GPC: $M_n = 14900$ g mol$^{-1}$, $M_w = 15900$ g mol$^{-1}$, $M_w/M_n = 1.06$) was purified by dialysis against methanol (5 times) using semi-permeable cellulose tubing (SPECTRA/POR, MW cut-off of 1000), the solvent was removed by a rotary evaporator and the polymer was dried in a vacuum oven. The actual degree of polymerization of this PMPC macroCTA was around 48 as judged by $^1$H NMR spectroscopy.

10. **ATRP of PMPC with Nile Blue dye present throughout the polymerization**

High monomer concentration: A stock solution of MPC (20.1 g, 68.1 mmol, 50 eq.) and MEBr (0.380 g, 1.36 mmol, 1 eq.) in ethanol (30 mL) was prepared. This solution was purged with nitrogen for 30 minutes. A sample of this stock solution was analyzed by $^1$H NMR in CD$_3$OD, the results of which were used as the zero time point in the kinetics measurements.

General protocol for kinetics measurements:

2.0 mL stock solution (1.34 g, 4.54 mmol MPC, 0.0253 g 90 µmol MEBr) was transferred via syringe to a mixture of bpy (0.0423 g, 271 µmol, 3 eq.), CuBr (0.0194 g, 1.5 µmol, 1 eq.) and dye (0.15 eq.-1.5 eq.) in a reaction vessel at 30 °C. 0.1 mL samples were removed at regular intervals and analyzed by $^1$H NMR. A similar blank reaction was performed without the dye.

After the final sample was removed, the reaction mixture was diluted with methanol and dialysed against methanol until the dialyzate was colorless. The solution was then dialysed against water and freeze-dried.

Low monomer concentration: A stock solution of MPC (10.2056 g, 34.6 mmol) and PhOBr (0.1952 g, 680 µmol) in 20.0 mL ethanol.

A catalyst stock solution was prepared by dissolving bpy (0.2157 g, 1.38 mmol) and CuCl (0.0673 g, 680 µmol) in 10.0 mL nitrogen-purged ethanol.

General procedure for kinetics measurements:

Dye (20 µmol, 0.3 eq.) was placed in a reaction vessel and 2.0 mL MPC stock solution was added via syringe (1.0 g, 3.39 mmol MPC, 0.020 g, 68 µmol PhOBr). The solution was purged with nitrogen for further 10 min before it was placed at 30 °C and 1.0 mL catalyst stock solution (0.022 g, 138 µmol bpy and 0.0067 g, 68 µmol CuCl), was added. 0.1 mL samples were removed at regular intervals and analysed by $^1$H NMR. A similar reaction was performed without the dye, this is the blank.

After the final sample was removed, the reaction mixture was diluted with methanol and dialyzed against methanol until the dialyze was colorless. The solution was then dialyzed against water and freeze-dried.

11. **ATRP of MPC with Nile Blue dye added at high MPC conversion**
A stock solution of PhOBr (0.1963 g, 0.68 mmol, 1 eq.) and MPC (10.0468 g, 34.0 mmol, 50 eq.) was dissolved in 20 mL ethanol. This MPC/PhOBr stock solution was purged with nitrogen for 20 minutes. A catalyst stock solution was prepared by dissolving bpy (0.216 g, 1.38 mmol) and CuBr (0.098 g, 0.683 mmol) in 10 mL nitrogen-purged ethanol. 2.0 mL of the MPC/PhOBr stock solution (1.0 g, 3.39 mmol MPC, 0.020 g, 69.6 µmol PhOBr) was placed in a reaction vial fitted with a septum and a magnetic stir bar and purged for 10 minutes before adding 1.0 mL catalyst stock solution (0.022 g bpy, 141 µmol, 0.01 g CuBr, 69.7 µmol). The solution was then placed at 30 °C. After 2 h, 1 eq. of Nile Blue dye was added and the reaction was left for a further 17 h. The viscous solution was then diluted with ethanol and transferred to a dialysis bag (MWCO 1,000 Da) and dialyzed against methanol until the dialyzate was colorless, followed by dialysis against water. Finally the polymer was freeze-dried overnight.

12. Synthesis of PMPC-PDPA-NBM with Nile Blue dye present throughout

MEBr (0.0392 g, 140 µmol, 1 eq.) and MPC (1.0363 g, 3.5 mmol, 25 eq.) was dissolved in 1.5 mL ethanol and the solution was purged with nitrogen for 20 minutes. Then, bpy (0.0445 g, 285 µmol, 2 eq.) and CuBr (0.0274 g, 191 µmol, 1.4 eq.) was added. The dark brown solution was left at 30 °C for 1.5 h. Then a nitrogen-purged solution of DPA (2.0310 g, 9.52 mmol, 68 eq.) and NBM (0.0057 g, 14.7 µmol, 0.1 eq.) in ethanol (3 mL) was added. The reaction mixture was stirred for 18 h, whereupon 1H NMR indicated that all vinylic groups had reacted. Then the viscous solution was exposed to the atmosphere and diluted with ethanol (50 mL). The solution was passed through a silica column using ethanol as eluent to remove spent catalyst. Then the ethanolic solution was transferred to a dialysis bag (MWCO 1,000) and dialyzed against methanol, then methanol and finally water. The opaque solution was freeze-dried to give the polymer as a beige powder.

13. Synthesis of PMPC-PDPA-NBX with dye added at high conversion

PhOBr (0.0761 g, 265 µmol, 1 eq) and MPC (2.071 g, 7.01 mmol, 25 eq.) was dissolved in 3.0 mL ethanol. The solution was purged with nitrogen for 30 minutes. Then, bpy (0.0863 g, 552 µmol, 2 eq.) and CuBr (0.0399 g, 278 µmol, 1 eq.) was added. The dark brown solution was stirred at 30 °C for 1.5 h. Then a nitrogen-purged solution of DPA (4.0440 g, 19 mmol, 68 eq.) in ethanol (6 mL) was added. The reaction mixture was stirred at 30 °C for 16 h. Then Nile Blue or Nile Blue monomer (265 µmol, 1 eq.) was added as a solid and the reaction mixture was stirred for a further 24 h. The viscous solution was exposed to the atmosphere, diluted with methanol (50 mL) and dialyzed (MWCO 1,000) against methanol until the dialyzate was colorless, followed by dialysis against water. Finally the polymer was freeze-dried overnight.

14. Synthesis of PGMA by RAFT with and without Nile Blue Monomers
The general protocol described in the literature was followed: CPDB RAFT agent (1.5 mmol, 0.33 g, 75% purity as judged by \(^1\)H NMR spectroscopy) and GMA monomer (89.6 mmol, 14.35 g) were weighed into a 50 mL round-bottomed flask and purged with nitrogen for 20 min. ACVA (0.2-0.4 molar eq. relative to CPDB), Nile Blue monomer (0.1-0.2 molar eq. relative to CPDB) and anhydrous ethanol (40 w/v %), which had been purged with nitrogen for 30 min, were then added, and the resulting red solution was purged for a further 10 min. The sealed flask was immersed into an oil bath set at 70 °C. Samples were taken periodically and analyzed by \(^1\)H NMR spectroscopy.

15. RAFT synthesis of PMPC\(_{50}\)-co-NBX\(_{0.2}\) in ethanol

MPC (5.0 g, 16.92 mmol, target DP = 50), cyanopropyl dithiobenzoate (CPDB) (0.120 g, 0.34 mmol, 1 eq.) and either NBM (0.029 g 0.068 mmol, 0.2 eq) or NBC (0.034 g, 0.068 mmol, 0.2 eq) were dissolved in ethanol (4.2 mL). After purging with nitrogen for 20 minutes in an ice bath, ACVA (V-501) initiator (0.024 g, 0.009 mmol, 0.25 eq., CPDB/ACVA = 4.0) was added and the mixture was purged for a further 5 minutes. At this point the flask was immersed in an oil bath at 75 °C. Kinetic samples were obtained over the course of the reaction by removing 0.1 mL aliquots and diluting in 1 mL cold CD\(_3\)OD for \(^1\)H NMR analysis.

16. Addition of Nile Blue Monomers to PMPC Macro-CTAs

PMPC\(_{48}\) macro-CTA (0.5 g, approximately 35 µmol CTA), Nile Blue A sulfate (12.3 mg, 34 µmol) or Nile Blue Methacrylamide (NBM, 13.5 mg, 32 µmol) and ACVA (10 mg, 36 µmol) was dissolved in 2 mL ethanol. The solution was purged with nitrogen for 30 min before it was placed at 70 °C for 19 h. After cooling, the solution was transferred to a dialysis bag (MWCO 1,000 Da) and dialyzed against methanol until the dialyzate was colorless, followed by water. Finally the polymer was freeze-dried overnight.

17. Determination of molar absorption coefficients and integrated absorption coefficients for dyes in ethanol

A PC-controlled CARY 50 PROBE UV/Visible spectrophotometer was used to record spectra from 300 nm to 800 nm at a scan rate of 4800 nm min\(^{-1}\). All measurements were performed using disposable UV-grade cuvettes. Solutions for measuring the molar absorption coefficient of Nile Blue and Nile Blue monomers were prepared by weighing approximately 10.0 mg of dye in a 50 mL volumetric flask using a microbalance and filling to the mark with ethanol. Three stock solutions were made for each monomer. Serial dilution of these stock solutions using pipettes and volumetric flasks gave solutions with absorbances ranging between 1.0 and 1.5. Further sequential dilutions allowed evaluation of the molar absorption coefficient after subtraction of a blank (ethanol), which is expressed as the slope of the plot of maximum absorbance versus concentration for four dilutions. In addition, the integrated absorbance was evaluated as described in the literature. Initially, absorbance values from 400 nm to 800 nm of each back-ground corrected spectrum were divided by the square of the wavelength. These
values were then summed and plotted as a function of concentration. The slope of the resulting line gave the integrated absorption coefficient.

18. Spectroscopic determination of polymer dye contents in ethanol

Between 10.0 and 20.0 mg polymer was weighed into a 10 mL volumetric flask. Three solutions were prepared for each polymer. In general, the absorption maximum of these solutions was between 0.2 and 1.2. For all solutions, the maximum absorbance and integrated absorbance (determined as described above) was used to assess the dye content in the solution. Knowledge of the polymer molecular weight from the target degree of polymerization and monomer conversion (as judged by $^1$H NMR) was used to obtain the value given in Table 2, see main text.

19. Absorption of Polymers in PBS and determination of relative quantum yield

Relative quantum yields were determined using the procedure of Fery-Forgues and Lavabre. Between 10.0 and 20.0 mg sample was weighed in a 5 mL or 10 mL volumetric flask on a microbalance (three significant digits) and dissolved PBS. In general the final concentration had a maximum absorbance below 0.5 but above 0.1. The absorption spectrum between 300 nm and 800 nm was obtained and the values at 550 nm and 650 nm were recorded. The solution was then diluted ten times with PBS and the emission spectrum was obtained on excitation at 550 nm and 650 nm. Spectra were integrated using Microsoft Excel software. Nile Blue Perchlorate in water was used as a standard ($\Phi = 0.01$ in water). A PC-controlled CARY 50 PROBE UV/Visible spectrophotometer was used to record spectra from 300 nm to 800 nm at a scan rate of 4800 nm min$^{-1}$. All measurements were performed using disposable UV-grade cuvettes.

A PC-controlled Fluoromax-3 fluorimeter was used for obtaining fluorescence spectra under the following conditions: excitation wavelength = 550 nm or 650 nm, emission scans from 550-900 nm at 1200 nm min$^{-1}$, an excitation slit width of 5 nm, an emission slit width of 5 nm and a PMT voltage of 800 V.

20. pH-dependent absorption of Nile Blue-containing PMPC-PDPA diblock copolymers in PBS

Between 95.0 and 105.0 mg polymer was dissolved in 0.40 mL 1 M HCl and 19.6 mL water to give a solution with a final pH of approximately 2. After measuring the solution pH using a calibrated pH meter (Hanna Instruments) an aliquot was removed and the absorption spectrum between 300 nm and 800 nm was recorded. The solution pH was increased using 0.1 M NaOH and 1 M NaOH and the absorption spectra were recorded at predetermined pH values (Figure 1 in the main text). A PC-controlled CARY 50 PROBE UV/Visible spectrophotometer was used to record spectra from 300 nm to 800 nm at a scan rate of 200 nm min$^{-1}$ and a band width of 1.5 nm. All measurements were performed using disposable UV-grade cuvettes.
21. pH-dependent emission of Nile Blue-containing PMPC homopolymers in PBS

To 10.0 mL of a solution of the homopolymer in PBS used for evaluating quantum yield (see above), was added 0.5 mL 1 M HCl. This lowered the pH to below 2. A 3.0 mL aliquot was removed after measuring the solution pH using a calibrated pH meter (Hanna Instruments) and the emission spectrum was measured, exciting at 550 nm and 650 nm. The aliquot was then recombined with the original solution. The settings of the spectrofluorimeter was adjusted to give a maximum emission of less than 50 % of the maximum range. In general, settings were those used for obtaining the quantum yield (see above) but in some cases it was necessary to adjust the slit widths when the dye content was very low. These settings were maintained throughout the experiment. The pH was then slowly increased using 1 M NaOH added via a syringe pump (World Precision Instruments). At the desired pH, the emission spectrum was obtained by removing 3.0 mL of solution, followed by replacing it as described above.

22. pH-dependent emission of Nile Blue monomers

0.100 mL of the ethanolic monomer solution used for absorption studies was transferred to a 10 mL volumetric flask and filled to the mark with 0.1 M aqueous HCl. The pH was increased using 0.1 M NaOH and 1 M NaOH via an autotitrator (Malvern MPT-2) connected to a flow-cell (Hellma Type No. 176.751-QS, 3 mm light path) placed within a Fluoromax-3 fluorimeter. The fluorescence spectra were recorded at predetermined pH values. The spectrofluorimeter settings were adjusted to give a maximum emission of less than 50 % of the maximum range. In general, settings were those used for obtaining the quantum yield (see above) but in some cases it was necessary to adjust the slit widths when the dye content was very low. These settings were maintained throughout the experiment.

23. pH-dependent emission of Nile Blue-labeled PMPC-PDPA diblock copolymers in PBS

Approximately 10.0 mg labeled polymer (determined to 3 significant figures) was placed in a 10 mL volumetric flask, which was filled up to the mark with 0.1 M HCl. The pH was increased using an autotitrator (Malvern MPT-2) connected to a flow-cell (Hellma Type No. 176.751-QS, 3 mm light path) placed within a Fluoromax-3 fluorimeter. The solution pH was increased using 0.1 M NaOH and 1 M NaOH and the fluorescence spectra were recorded at predetermined pH values. The spectrofluorimeter settings were adjusted to give a maximum emission of less than 50 % of the maximum range. In general, settings were those used for obtaining the quantum yield (see above) but in some cases it was necessary to adjust the slit widths when the dye content was very low. These settings were maintained throughout the experiment.
24. Cell culture

Primary human dermal fibroblasts (HDFs) were obtained from LGC standards (Teddington, UK). Cells were maintained in DMEM (Biosera, UK) supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.625 µg/ml amphotericin B (all from Sigma-Aldrich, UK). Cells were sub-cultured routinely using 0.02 % (w/v) trypsin-EDTA (Sigma-Aldrich, UK) and used for experimentation between passages 4 and 8. MDA-MB-231 human breast cancer cells were a kind gift from Prof. Nicola Brown, Medical School, University of Sheffield. These cells were maintained RPMI-1640 supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin.

25. Cell viability: MTT-ESTA assay

An MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used to measure cellular metabolic potential of treated cells after vesicle treatment. In brief, 3–4 × 10^4 HDF cells were cultured per well in 24-well plates until 70% confluence (typically 48 h). Cells were incubated for 24 h with varying vesicle concentrations. After treatment, cell cultures were thoroughly washed in PBS and then incubated with MTT solution (0.50 mg/ml MTT in PBS, 1.0 ml per well in a 24-well plate) for 45 min at 37°C and in a 95% air / 5% CO₂ environment. Intracellular dehydrogenase activity reduces MTT to a purple-colored formazan salt. After 45 minutes, the solution was aspirated and the insoluble intracellular formazan product was solubilized and released from cells by adding acidified iso-propanol (0.3 ml per well of 24-well plate or 1 ml / cm² cultured tissue) and incubated for 10 min. The optical density at 570 nm was then measured (with a reference filter at 630 nm) using a plate reading spectrophotometer. For statistical analysis (Student’s t-test), experiments were performed in triplicate wells with a total of N = 3 independent experiments.

26. Cellular uptake of Nile Blue vesicles: Live imaging via confocal fluorescence microscopy.

Cells were seeded at a density of 5 × 10^3 cells/well in BD Falcon 96-well imaging plates and grown until 50% confluence. Cells were treated overnight (typically 16 h) with 1.0 mg/ml vesicles. The cells were washed three times with PBS and imaging medium (culture medium without phenol red) was added to each well in preparation for live imaging with a Zeiss LSM510 Meta system (40X). The pH-dependent subcellular localization was monitored using a commercial early endosomes marker for Rab-5 (CellLight® Early Endosomes-GFP, Invitrogen) and a commercial lysosomal marker (Lysotracker®, Invitrogen) following the manufacturer’s instructions.

27. 3D multicell tumour spheroid (MCTS) culture and image analysis
3D MCTS cultures of the MDA-MB-231 breast cancer cell line were produced using ultra-low attachment (ULA) 96-well round bottomed plates, as previously described. For optimal 3D growth and high reproducibility, $1.5 \times 10^4$ cells per well were seeded in 200 µl of cell media (RPMI-1640 supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin). Spheroids were fed on the third day after seeding and thereafter every other day until the sixth day, whereupon they were fed daily thereafter.

MCTS were treated with Nile Blue vesicles (1.0 mg/ml in cell medium) for approximately 30-36 h. Afterwards, live spheroids were washed three times with PBS and imaged with a digital camera (5x, full spheroid per image (Figure 2B) or under an optical microscope (20x magnification, approximately 25 % of a spheroid per image, see Figure S13) in order to identify the boundary for the color change from physiological pH (pink) to hypoxic/low pH (blue). Bright-field optical images were recorded using a Leica DMI4000B equipped with a Nuance Multispectral imaging system. Untreated control spheroids were used to subtract the background noise from the images. The image analysis was performed using a double-blind protocol. Four independent observers were asked to score the center of the tumor (marked with a white star in Figure S13), the edge (red dotted line in Figure S13) and identify the color change, the acidic/blue color margin line (black dotted line in Figure S13). Following this analysis, acidic regions (blue colored) of the spheroids were compared to the full size of the MCTS and the ratio of the hypoxic (acidic) radius to the total radius of the MCTS was calculated over time.

Hypoxic (low pH regions) of the spheroids were compared to a control marker for hypoxia (Glut-1). Briefly, control spheroids were grown under the same conditions as described above and fixed with 4% buffered paraformaldehyde at specific time points. Control spheroids were then incubated for 1 h in a solution of 5% BSA to block unreactive sites. Immunolabeling was performed using rabbit-anti human Glut-1 1:100 (Abcam, UK) for 30 mins followed by goat-anti rabbit alkaline phosphatase conjugated IgG 1:1000 (Abcam, UK) for 30 mins. The spheroids were thoroughly washed and incubated with the chromogenic substrate (SIGMAFAST™ BCIP/NBT) following the manufacturer’s specifications until the signal was visible.

Mean ratios were calculated for N = 3 independent experiments and analysis of differences between the groups was performed using the Student’s paired t-test.

![Scheme S1: Electronic structure of Nile Blue dye dissolved in polar and non-polar solvents.](image-url)
| Abbreviation | Name                                    | $\lambda_{\text{max}}$(Abs) / nm | $\varepsilon_{\text{max}}$ / M$^{-1}$·cm$^{-1}$ | $\varepsilon_f$ / 10$^{19}$M$^{-2}$·cm$^{-1}$ | Purity after chromatography 254 nm$^b$ | Purity before chromatography 254 nm$^b$ |
|-------------|-----------------------------------------|-------------------------------|---------------------------------|-----------------------------------|--------------------------------|----------------------------------|
| NB          | Nile Blue (NB)                          | 629 ± 0.3                     | 39,000 ± 800                    | 1.56 ± 0.03                       | N/A                            | 93 %                             |
| NBM         | Nile Blue Methacrylamide (NBM)          | 544 ± 0.1                     | 37,400 ± 1,300                  | 1.46 ± 0.03                       | 92 %                            | 85 %                             |
| NBC         | Nile Blue (2-methacycloxyethyl carbamate) (NBC) | 534 ± 0.1                     | 41,400 ± 800                    | 1.52 ± 0.03                       | N/D$^c$                         | 92 %                             |

Table S1: Absorption spectroscopy data obtained for Nile Blue and Nile Blue-based methacrylic monomers in ethanol after purification.\textsuperscript{a} Integrated absorption coefficient according to reference S10 \textsuperscript{b} Purity = [Area of monomer peak]/[Area of all peaks] at 254 nm. \textsuperscript{c} No apparent impurities by HPLC at this wavelength.
| Entry | Target Polymer | [MPC] % w/v | Conversion % after 24 h (by $^1$H NMR) | M_n | M_w / M_n | Polymer Composition | Polymer Composition | Relative dye content vs. target (F) / % | $\lambda_{max, Abs}$ nm | $\lambda_{max, Em}$ (550 nm, 650 nm)$^a$ | Quantum yield$^d$ |
|-------|---------------|-------------|----------------------------------------|-----|------------|---------------------|---------------------|---------------------------------|------------------|----------------|----------------|
| 1     | ME-PMPC$_{50}$-NBM$_{0.15}$ | 67          | 99                                     | 31,900 | 1.19       | ME-PMPC$_{50}$-NBM$_{0.01}$ | ME-PMPC$_{50}$-NBM$_{0.02}$ | 13                       | 587              | 654, 699       | 0.03           |
| 2     | ME-PMPC$_{50}$-NBC$_{0.17}$ | 67          | 98                                     | 35,700 | 1.20       | ME-PMPC$_{50}$-NBC$_{0.05}$ | ME-PMPC$_{50}$-NBC$_{0.10}$ | 59                       | 588              | 669, 682       | 0.16           |
| 3     | PhO-PMPC$_{50}$-NBC$_{0.3}$ | 33          | 66                                     | 29,800 | 1.12       | PhO-PMPC$_{33}$-NBC$_{0.06}$ | PhO-PMPC$_{33}$-NBC$_{0.10}$ | 33                       | 590              | 670, 685       | 0.14           |
| 4     | ME-PMPC$_{50}$-NB$_1$       | 67          | 98                                     | 39,200 | 1.22       | ME-PMPC$_{49}$-NB$_{0.14}$ | ME-PMPC$_{49}$-NB$_{0.15}$ | 15                       | 650              | 669, 682       | 0.14           |
| 5     | PhO-PMPC$_{50}$-NBM$_1$     | 33          | 81                                     | 30,200 | 1.13       | PhO-PMPC$_{41}$-NBM$_{0.13}$ | PhO-PMPC$_{41}$-NBM$_{0.16}$ | 16                       | 596              | 677, 705       | 0.02           |
| 6     | PhO-PMPC$_{50}$-NBC$_1$     | 33          | 81                                     | 30,500 | 1.14       | PhO-PMPC$_{41}$-NBC$_{0.10}$ | PhO-PMPC$_{41}$-NBC$_{0.10}$ | 10                       | 579              | 670, 680       | 0.09           |
| 7     | PhO-PMPC$_{50}$-NB$_1$      | 33          | 75                                     | 29,500 | 1.15       | PhO-PMPC$_{28}$-NB$_{0.04}$ | PhO-PMPC$_{28}$-NB$_{0.08}$ | 8                        | 659              | 658, 696       | 0.05           |

Dye added after 2 h to ATRP reaction

Conversion after 2 h

PMPC$_{50}$-Macro-CTA+ACVA+Dye

$^a$ $\lambda_{max, Em}$ (550 nm, 650 nm) is given for comparison. $\lambda_{max, Abs}$ is the absorption maximum in nm.

$^d$ Quantum yield is given for comparison.
Table S2: Summary of data obtained for all PMPC polymers. \(^a\) GPC studies conducted in a 3:1 CHCl\(_3\):CH\(_3\)OH mixed eluent containing 2mM LiBr. \(^b\) Based on absorption coefficient data at the \(\lambda_{max}\). \(^c\) Based on integrated absorption coefficient data. \(^d\) In PBS at pH 7.2. \(^e\) In PBS excited at 550 nm.

| Target Copolymer Composition | Actual Copolymer Composition (by \(^1\)H NMR) | \(M_n\) \(^a\) | \(M_w/ M_n\) \(^a\) | Final Copolymer Composition\(^b\) (including Nile Blue label) |
|-----------------------------|---------------------------------------------|---------------|-----------------|-------------------------------------------------|
| PMPC\(_{25}\)-PDPA\(_{70}\)-NB\(_1\) | PMPC\(_{25}\)-PDPA\(_{61}\) | 61,900 | 1.14 | PMPC\(_{25}\)-PDPA\(_{61}\)-NB\(_{0.13}\) |
| PMPC\(_{25}\)-PDPA\(_{70}\)-NBC\(_1\) | PMPC\(_{25}\)-PDPA\(_{69}\) | 63,700 | 1.15 | PMPC\(_{25}\)-PDPA\(_{69}\)-NBC\(_{0.08}\) |
| PMPC\(_{25}\)-PDPA\(_{70}\)-NBM\(_1\) | PMPC\(_{25}\)-PDPA\(_{62}\) | 63,000 | 1.18 | PMPC\(_{25}\)-PDPA\(_{62}\)-NBM\(_{0.13}\) |

\(^a\) GPC studies were conducted in a 3:1 chloroform/methanol mixed eluent containing 2.0 mM LiBr using a refractive index detector and a series of near-monodisperse poly(methyl methacrylate) calibration standards. \(^b\) Based on integrated absorption coefficient data.

Table S3. Summary of data obtained for the three PMPC-PDPA copolymers used in this study
Figure S2: Normalized monomer spectra (c: 1·10^{-5} M to 3·10^{-5} M) in ethanol

Figure S3. Kinetic plots obtained for the ATRP of MPC in ethanol at 30 °C in the presence of three Nile Blue-based vinyl monomers (NBM and NBM) and Nile Blue alone. (A) Conversion vs. time curves for 33 % w/v MPC using [MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:2:1; (B) corresponding semi-logarithmic plots; (C) conversion vs. time for 67 % w/v using [MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:3:1.5; (D) corresponding semi-logarithmic plots.
Figure S4: Conversion and $\ln[M]/[M]$ for the polymerization of glycerol monomethacrylate (DP = 60) with and without NBC.

Figure S5: Chemical structures of radical polymerization inhibitors Phenothiazine and Methylene Blue compared with Nile Blue.
**A**

| Reaction | Rate | Kinetic Equation |
|----------|------|------------------|
| (1) I $\rightarrow$ 2R$\cdot$ | $k_d[I]$ | Radical decomposition |
| (2) R$\cdot$ + M $\rightarrow$ M$\cdot$ | $2k_d[I]$ | Initiation |
| (3) M$_n^\cdot$ + M $\rightarrow$ M$_{n+1}^\cdot$ | $k_2[M^\cdot][M]$ | Propagation |
| (4) M$_n^\cdot$ + M$_m^\cdot$ $\rightarrow$ M$_{n+m}^\cdot$ | $k_2[M^\cdot][M]$ | Recombination |
| (5) M$_n^\cdot$ + M$_m^\cdot$ $\rightarrow$ M$_n$ + M$_m$ | $k_{2a}[M^\cdot][M]$ | Disproportionation |
| (6) M$_n^\cdot$ + Dye$^+$ $\rightarrow$ M$_n$ + Dye$^\cdot$ + H$^+$ | $k_d[M^\cdot][Dye+]$ | Transfer to dye |
| (7) M$_n^\cdot$ + Dye$^\cdot$ $\rightarrow$ M$_n$-Dye | $k_5[M^\cdot][Dye^\cdot]$ | Dye recombination |
| (8) M$_n^\cdot$ + Dye$^\cdot$ $\rightarrow$ M$_n$ + DyeH | $k_5a[M^\cdot][Dye^\cdot]$ | Dye-polymer disprop. |
| (9) Dye$^\cdot$ + Dye$^\cdot$ $\rightarrow$ Dye$^+$ + DyeH | $k_6[Dye^\cdot]^2$ | Dye dismutation |
| (10) Dye$^+$ + DyeH $\rightarrow$ Dye$^\cdot$ + Dye$^\cdot$ | $k'_6[Dye^\cdot][DyeH]$ | Reverse dye dism. |

**B**

1) $M_n^\cdot$ + $\text{Nile Blue}^{\frac{1}{2}\text{SO}_4^2-}$ $\rightarrow$ RH + $\text{Nile Blue}^{\frac{1}{2}\text{SO}_4^2-}$ + H$^+$

2) $M_n^\cdot$ + $\text{Nile Blue}^{\frac{1}{2}\text{SO}_4^2-}$ $\rightarrow$ $\text{Nile Blue}^{\frac{1}{2}\text{SO}_4^2-}$ + RH

Figure S6: (A) General kinetic equations describing the free radical polymerization of a vinyl monomer in the presence of an inhibitor dye (methylene blue) according to Reference S11. (B) Suggested specific reaction between Nile Blue and an alkyl radical to form labeled polymer chains in 2 steps: 1) Hydrogen abstraction from Nile Blue by a polymeric radical leading to formation of a Nile Blue radical (corresponding to reaction (6) in (A)) and 2) Reaction between a Nile Blue radical and a (different) polymer radical chain (corresponding to reaction (7) in (A)).
Figure S7: Normalized absorption spectra of monomers (c: 1-3×10⁻⁵ M) and corresponding polymers (1-4 mg/mL) in ethanol

Figure S8: Normalized absorption spectra of polymers (1-4 mg/mL) and Nile Blue (NB, 5.4×10⁻⁶ M) in PBS pH 7.3
Figure S9: pH-dependence of the fluorescence emission spectra recorded for Nile Blue (NB) (~9 µM) and Nile Blue-based PMPC homopolymers in dilute aqueous solution (~1 mg/mL)
Figure S10: (A)-(D) Fluorescence emission vs. solution pH recorded for ~ 1 mg/mL aqueous solutions of PMPC-NBC, PMPC-NBM, PMPC-NB and Nile Blue (~ 9 µM in its ClO\textsubscript{4} salt form). (E)-(H) Fluorescence emission vs. solution pH recorded for aqueous solutions of NBC (~5 µM in its Cl\textsuperscript{-} salt form), NBM (~6 µM in its Cl\textsuperscript{-} salt form) and Nile Blue (~ 9 µM in its ClO\textsubscript{4} salt form) (A), (E)

Relative emission at 700 nm and 670 nm (excitation wavelength = 550 nm); (B), (F) Relative emission at 700 nm and 670 nm (excitation wavelength = 650 nm); (C), (G) Integrated emission intensity from 560 nm to 800 nm determined for an excitation wavelength of 550 nm relative to the integrated intensity observed at the lowest pH value; (D), (H) Integrated emission intensity from 660 nm to 800 nm determined relative to the integrated intensity observed at the lowest pH value (excitation wavelength = 650 nm).
Figure S11: Fluorescence emission spectra vs pH recorded for NBM (~6 µM) and NBC (5 µM)

Figure S12: Differential uptake of PMPC-PDPA diblock copolymer vesicles and PMPC homopolymer chains by HDF cells. Cells were incubated with (A) rhodamine 6G-labeled PMPC\textsubscript{25}-PDPA\textsubscript{70} diblock copolymer vesicles or (B) rhodamine 6G-labeled PMPC\textsubscript{100} homopolymer at 37 °C in 5% CO\textsubscript{2} for 2 h. After incubation with the (co)polymer, the cells were rinsed three times with PBS and placed in DMEM (1.0 mL) for live cell imaging. DAPI (blue, left) is used to locate the cell nuclei. The PMPC\textsubscript{25}-PDPA\textsubscript{70} diblock copolymer vesicles (red, middle) are readily uptaken by the cells, whereas the PMPC\textsubscript{100} homopolymer is not uptaken at all under the same conditions (no signal, middle). Merged images are shown on the right. Scale bar = 100 µm for all images.
Figure S13: Fluorescence emission vs pH for 1 mg/mL aqueous solutions of various Nile Blue-labeled PMPC-PDPA diblock copolymers. Conditions: excitation slit: 5 nm; emission slit: 5 nm; photomultiplier voltage = 800 V. (A) PhO-PMPC$_{25}$-PDPA$_{70}$-NB$_{0.12}$ excited at 550 nm; (B) PhO-PMPC$_{25}$-PDPA$_{70}$-NB$_{0.12}$ excited at 650 nm; (C) PhO-PMPC$_{25}$-PDPA$_{70}$-NBM$_{0.15}$ excited at 550 nm; (D) PhO-PMPC$_{25}$-PDPA$_{70}$-NBM$_{0.15}$ excited at 650 nm; (E) PhO-PMPC$_{25}$-PDPA$_{70}$-NBC$_{0.08}$ excited at 550 nm; (F) PhO-PMPC$_{25}$-PDPA$_{70}$-NBC$_{0.08}$ excited at 650 nm.
Figure S14: 24 h viability of Human Dermal Fibroblasts exposed to 1 mg/mL Nile Blue labeled PMPC-PDPA diblock copolymer vesicles.
Figure S15: pH-dependent staining of multicell tumour spheroids (MCTS) by Nile blue-labeled PMPC-PDPA copolymers. Bright-field optical images of Nile Blue polymer treated MCTS and controls were recorded using a Leica DMI4000B instrument equipped with a Nuance Multispectral imaging System. Untreated control spheroids were used to subtract the background noise from the images. The image analysis was performed double-blinded. Four independent observers were asked to score the center of the tumor (white star), the edge (red dotted line) and identify the acidic/blue color boundary (black dotted line). Hypoxic (low pH regions) of the spheroids were compared to a positive control marker for hypoxia (Glut-1 immunostaining).

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