

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

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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 (CDCl₃, 99.8 Atom % D) trifluoroacetic acid (TFA, 99+ %), triethylamine (Et₃N, ≥ 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 (MgSO₄), sodium chloride (NaCl), triethylamine (Et₃N) and sodium sulfate (Na₂SO₄) were laboratory reagent grade from Fisher Scientific (Loughborough, UK) and were used as received. Deuterated methanol (CD₃OD, 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)sulfanyl pentanoic acid (DTTC) was synthesized according to a literature protocol.¹ ATRP initiators, 4-(2-bromoisobutyryl ethyl)morpholine (MEBr) and 2-Phenoxyethyl 2-Bromoisobutyrate (PhOBr) were prepared according to previously published procedures.^{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.

¹H NMR (400 MHz, CD₃OD) 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

¹³C NMR (400 MHz, CD₃OD)

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, 11.54 ppm

ESI-MS, m/z (M+H)⁺ 386

Accurate Mass (Calculated), (M): 386.1860 (386.1869) corresponding to an elemental composition of C₂₄H₂₄N₃O₂ (C₂₄H₂₄N₃O₂)

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 NMR (400 MHz, CD₃OD) δ 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 NMR (400 MHz, 3:1 CDCl₃: CD₃OD) δ 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 C₂₇H₃₀N₃O₄ (C₂₇H₃₀N₃O₄)

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: $M_n = 14900 \text{ g mol}^{-1}$, $M_w = 15900 \text{ g mol}^{-1}$, $M_w/M_n = 1.06$) 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 ^1H 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 ^1H NMR in CD_3OD , 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 ^1H 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 dialyzed 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 ^1H 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 dialyzate 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 μ mol 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 μ mol, 0.01 g CuBr, 69.7 μ mol). 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 μ mol, 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 μ mol, 2 eq.) and CuBr (0.0274 g, 191 μ mol, 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 μ mol, 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 ethanol, 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 μ mol, 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 μ mol, 2 eq.) and CuBr (0.0399 g, 278 μ mol, 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 μ mol, 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 literature⁴ was followed: CPDB RAFT agent (1.5 mmol, 0.33 g, 75% purity as judged by ¹H 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 ¹H NMR spectroscopy.

15. RAFT synthesis of PMPC₅₀-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 CD₃OD for ¹H NMR analysis.

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

PMPC₄₈ 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.⁵ 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 $^1\text{H NMR}$) 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.^{6,7} 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 in 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).⁸ 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, $3-4 \times 10^4$ 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.

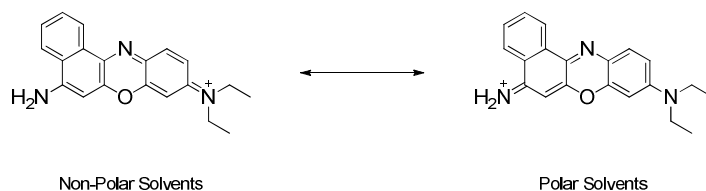
27. 3D multicell tumour spheroid (MCTS) culture and image analysis

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.⁹ For optimal 3D growth and high reproducibility, 1.5×10^4 cells per well were seeded in 200 μ l 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.

Abbreviation	Name	$\lambda_{\text{max}}(\text{Abs})$ / nm	$\epsilon_{\text{max}} /$ $\text{M}^{-1} \cdot \text{cm}^{-1}$	$\epsilon_{\text{F}} / 10^4$ $19 \text{ m}^{-2} \text{ a}$	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- methacryloyloxyethyl 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.^a Integrated absorption coefficient according to reference S10^b Purity = [Area of monomer peak]/[Area of all peaks] at 254 nm. ^cNo apparent impurities by HPLC at this wavelength.

Entry	Target Polymer	[MPC] % w/v	Conversion % after 24 h (by ¹ H NMR)	M _n ^a	M _w / M _n ^a	Polymer Composition A _{Max} ^b	Polymer Composition F ^c	Relative dye content vs. target (F) / %	λ _{max, Abs} nm	λ _{max, Em} nm (λ _{Ex} 550 nm, 650 nm) ^d	Quantum yield ^e
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
Dye added after 2 h to ATRP reaction			Conversion after 2 h								
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 ₃₈ -NB _{0.04}	PhO-PMPC ₃₈ -NB _{0.08}	8	659	658, 696	0.05
PMPC ₅₀ -Macro- CTA+ACVA+Dye											

8	PMPC ₄₈ -NB ₁	25	N / A	23,700	1.13	PMPC ₄₈ -NB _{0.03}	PMPC ₄₈ -NB _{0.05}	5	660	660, 694	0.03
9	PMPC ₄₈ -NBM ₁	25	N / A	21,700	1.14	PMPC ₄₈ -NBM _{0.02}	PMPC ₄₈ -NBM _{0.04}	4	600	656, 660	0.03

Table S2: Summary of data obtained for all PMPC polymers. ^a GPC studies conducted in a 3:1 CHCl₃:CH₃OH mixed eluent containing 2mM LiBr. ^bBased on absorption coefficient data at the λ_{\max} . ^cBased on integrated absorption coefficient data. ^dIn PBS at pH 7.2. ^eIn 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 ₅₉	63,700	1.15	PMPC ₂₅ -PDPA ₅₉ -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

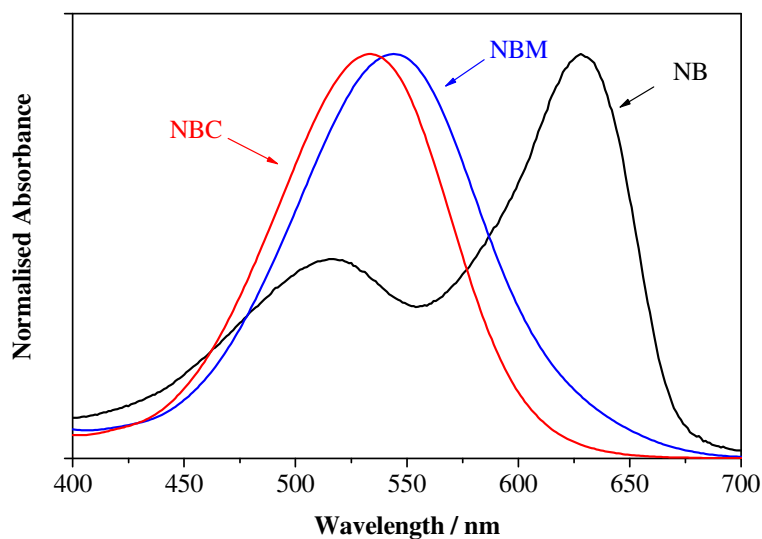


Figure S2: Normalized monomer spectra ($c: 1 \cdot 10^{-5} \text{ M}$ to $3 \cdot 10^{-5} \text{ M}$) in ethanol

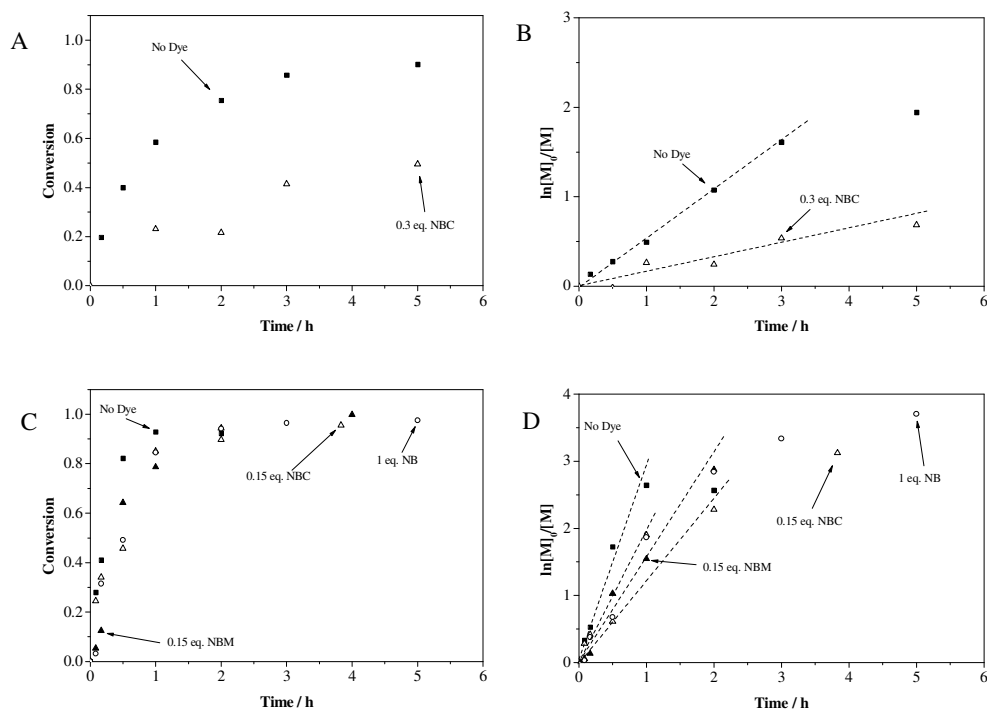


Figure S3. Kinetic plots obtained for the ATRP of MPC in ethanol at 30 °C in the presence of three Nile Blue-based vinyl monomers (NBM and NBM) and Nile Blue alone. (A) Conversion vs. time curves for 33 % w/v MPC using $[MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:2:1$; (B) corresponding semi-logarithmic plots; (C) conversion vs. time for 67 % w/v using $[MPC]:[MEBr]:[bpy]:[CuBr] = 50:1:3:1.5$; (D) corresponding semi-logarithmic plots.

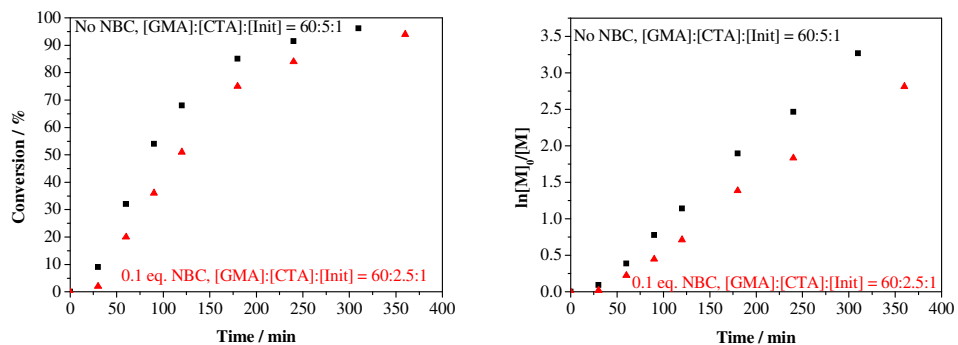


Figure S4: Conversion and $\ln[M]_0/[M]$ for the polymerization of glycerol monomethacrylate (DP = 60) with and without NBC.

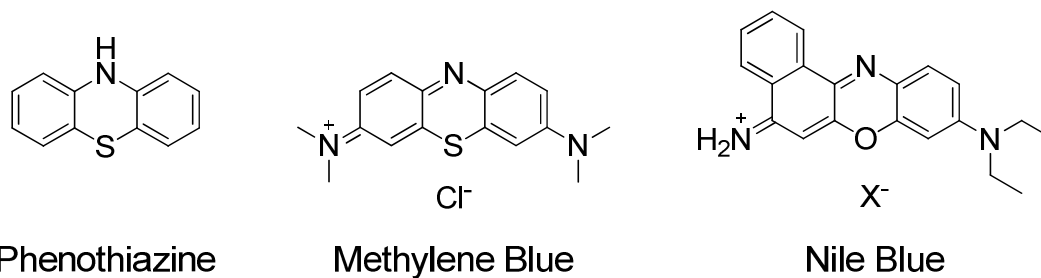


Figure S5: Chemical structures of radical polymerization inhibitors Phenothiazine and Methylene Blue compared with Nile Blue

A		Rate	
(1) I	$\rightarrow 2R\cdot$	$k_d[I]$	Radical decomposition
(2) R \cdot + M	$\rightarrow M\cdot$	$2fk_d[I]$	Initiation
(3) M $_n\cdot$ + M	$\rightarrow M\cdot_{n+1}$	$k_2[M\cdot][M]$	Propagation
(4) M $_n\cdot$ + M $_m\cdot$	$\rightarrow M_{n+m}$	$k_2[M\cdot][M]$	Recombination
(5) M $_n\cdot$ + M $_m\cdot$	$\rightarrow M_n + M_m$	$k_{2a}[M\cdot][M]$	Disproportionation
(6) M $_n\cdot$ + Dye $^+$	$\rightarrow M_n + \text{Dye}\cdot + H^+$	$k_4[M\cdot][\text{Dye}^+]$	Transfer to dye
(7) M $_n\cdot$ + Dye \cdot	$\rightarrow M_n\text{-Dye}$	$k_5[M\cdot][\text{Dye}\cdot]$	Dye recombination
(8) M $_n\cdot$ + Dye \cdot	$\rightarrow M_n + \text{DyeH}$	$k_{5a}[M\cdot][\text{Dye}\cdot]$	Dye-polymer disprop.
(9) Dye \cdot + Dye \cdot	$\rightarrow \text{Dye}^+ + \text{DyeH}$	$k_6[\text{Dye}\cdot]^2$	Dye dismutation
(10) Dye $^+$ + DyeH	$\rightarrow \text{Dye}\cdot + \text{Dye}\cdot$	$k'_6[\text{Dye}^+][\text{DyeH}]$	Reverse dye dism.

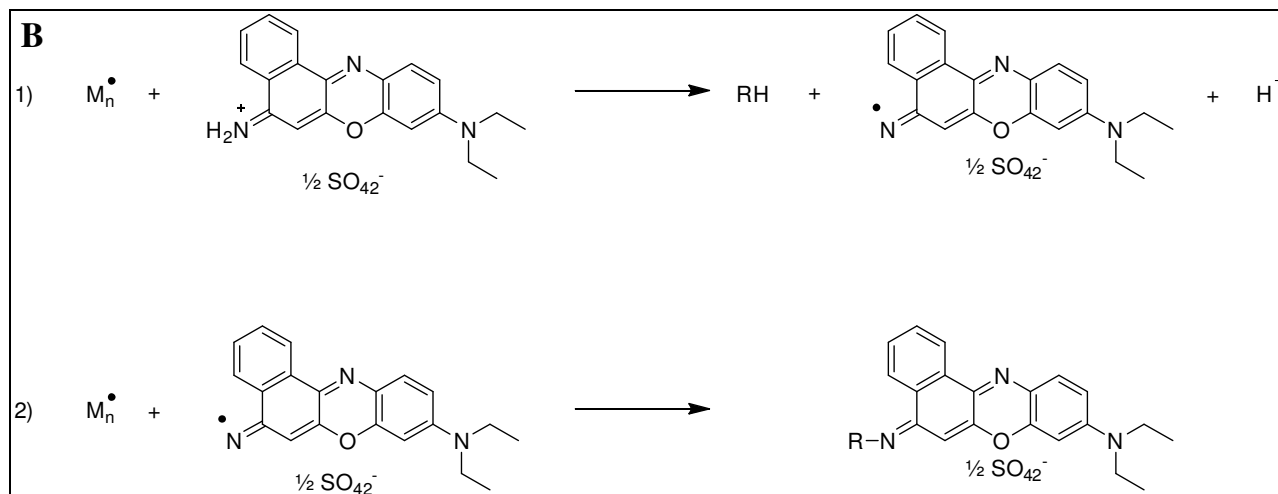


Figure S6: (A) General kinetic equations describing the free radical polymerization of a vinyl monomer in the presence of an inhibitor dye (methylene blue) according to Reference S11. (B) Suggested specific reaction between Nile Blue and an alkyl radical to form labeled polymer chains in 2 steps: 1) 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))

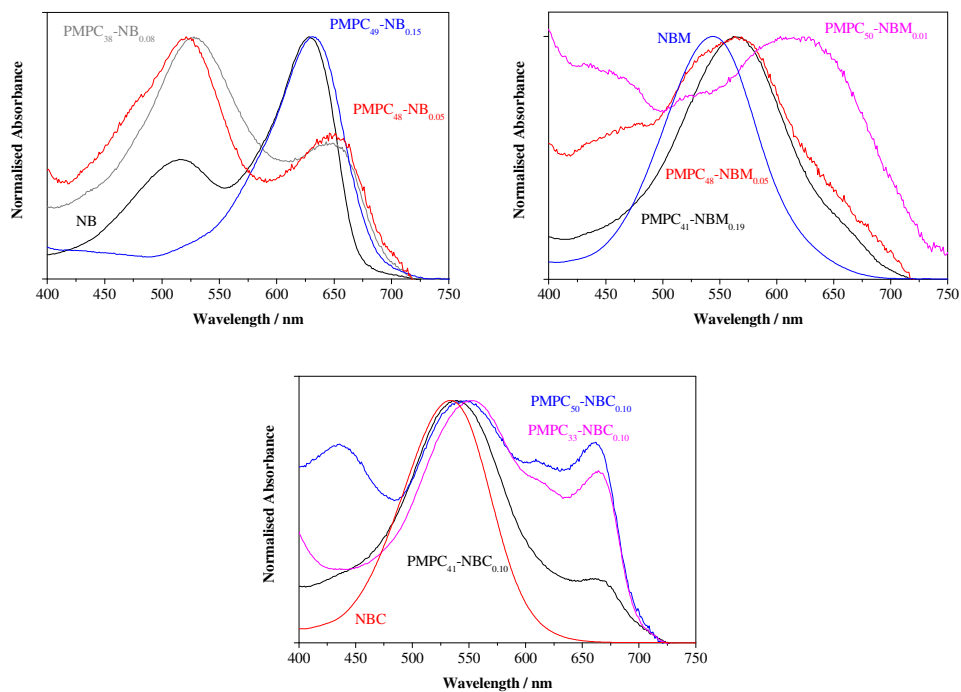


Figure S7: Normalized absorption spectra of monomers ($c: 1\text{-}3\cdot 10^{-5}$ M) and corresponding polymers (1-4 mg/mL) in ethanol

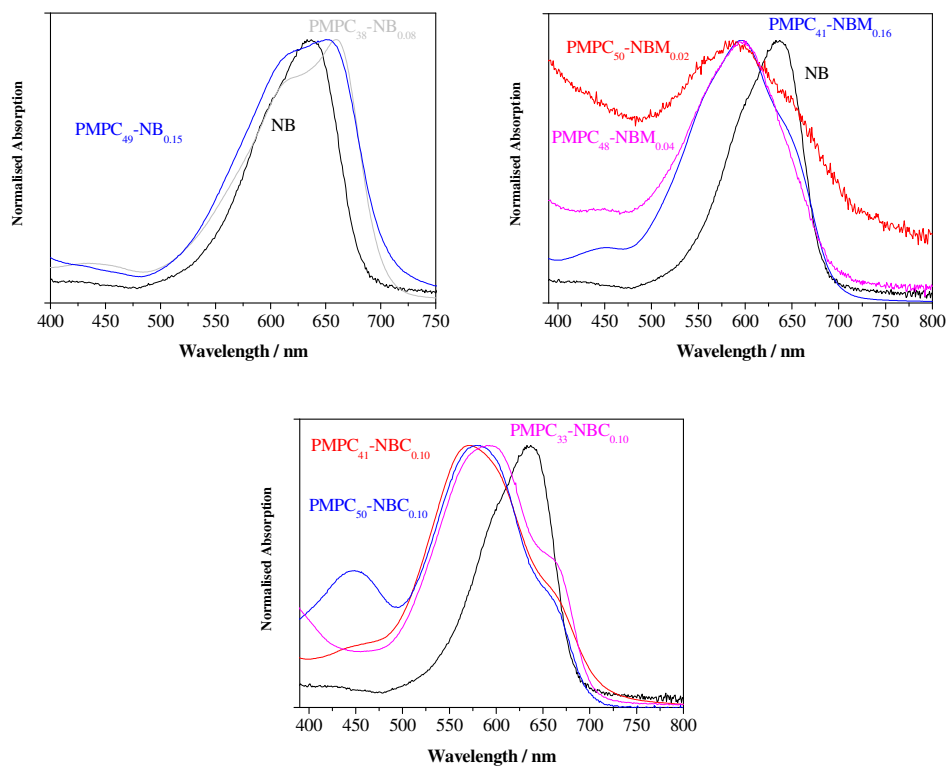


Figure S8: Normalized absorption spectra of polymers (1-4 mg/mL) and Nile Blue (NB, $5.4\cdot 10^{-6}$ M) in PBS pH 7.3

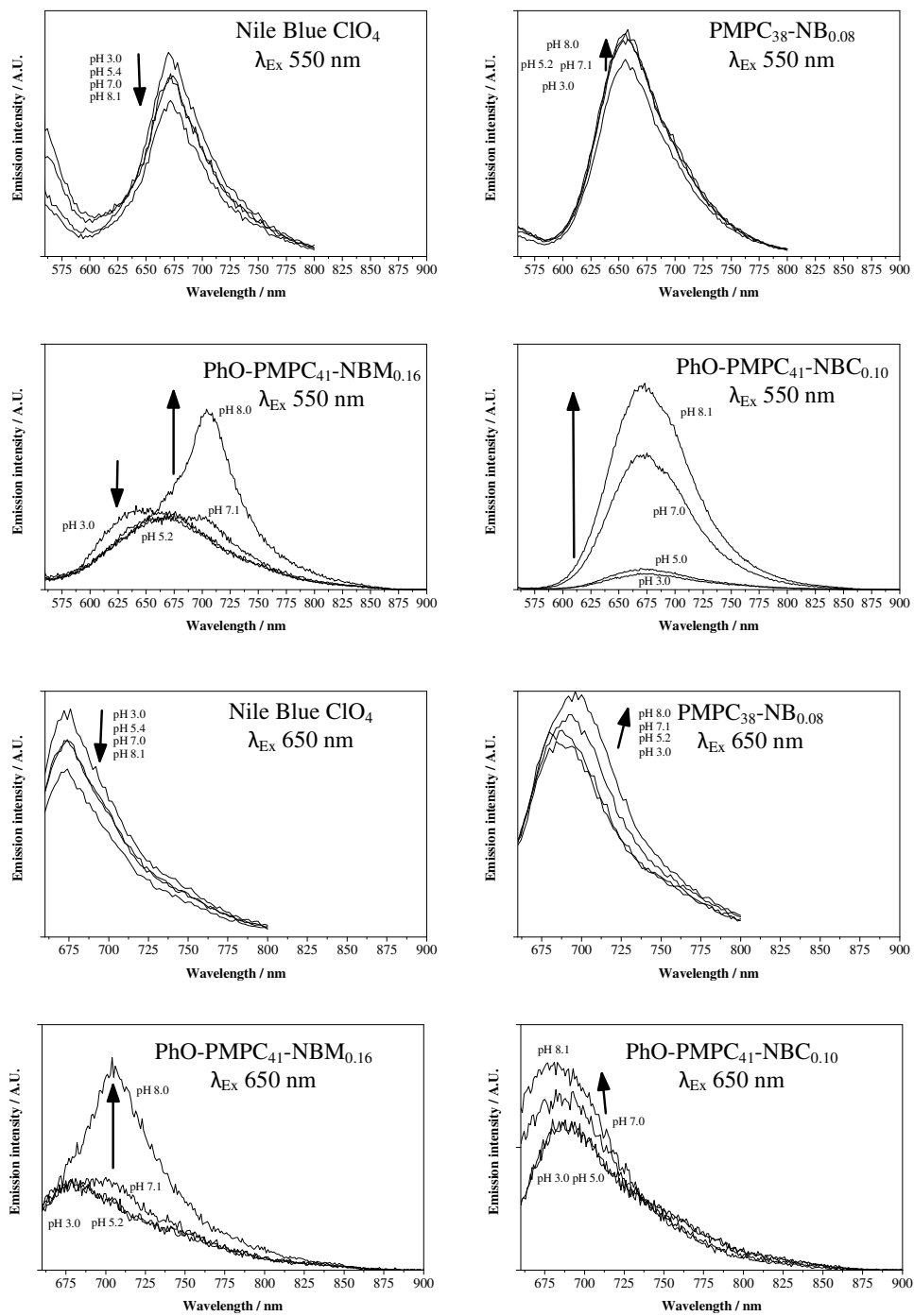


Figure S9: pH-dependence of the fluorescence emission spectra recorded for Nile Blue (NB) (~9 μM) and Nile Blue-based PMPC homopolymers in dilute aqueous solution (~1 mg/mL)

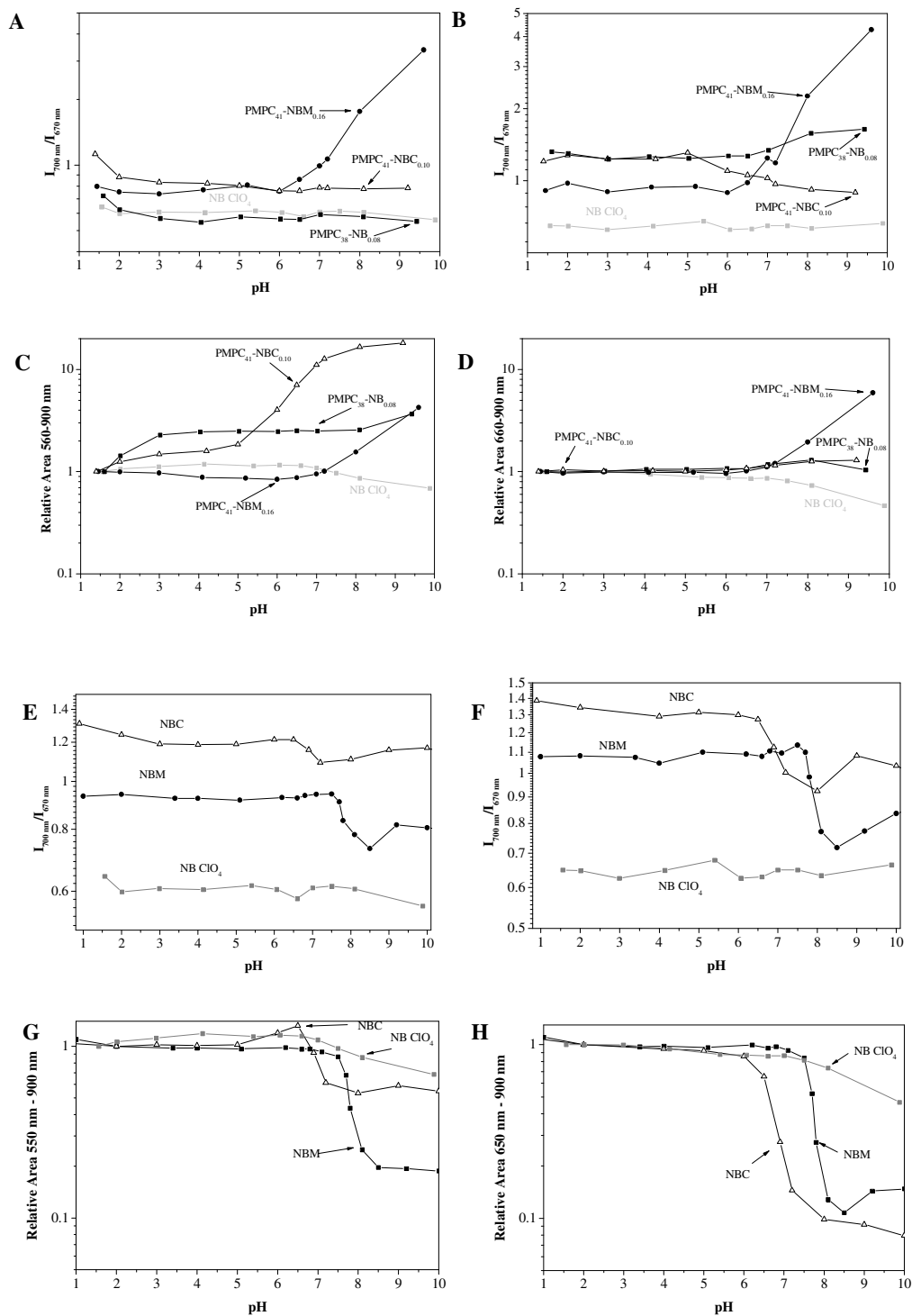


Figure S10: (A)-(D) Fluorescence emission vs. solution pH recorded for ~ 1 mg/mL aqueous solutions of PMPC-NBC, PMPC-NBM, PMPC-NB and Nile Blue (~ 9 μ M in its ClO₄ salt form). (E)-(H) Fluorescence emission vs. solution pH recorded for aqueous solutions of NBC (~5 μ M in its Cl⁻ salt form), NBM (~6 μ M in its Cl⁻ salt form) and Nile Blue (~ 9 μ M 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).

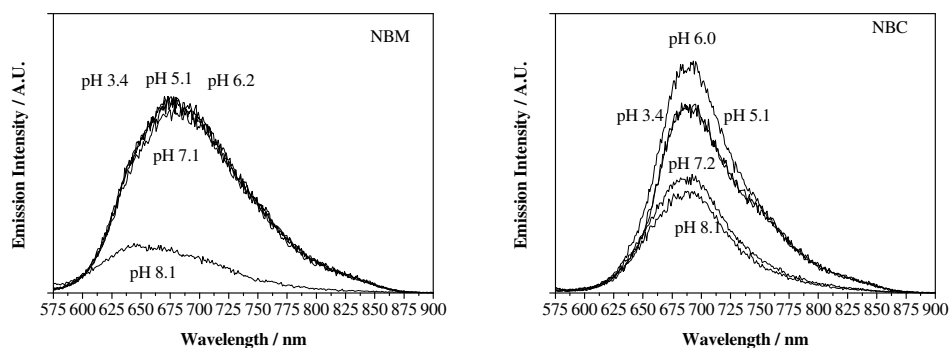


Figure S11: Fluorescence emission spectra vs pH recorded for NBM (~6 μ M) and NBC (5 μ M)

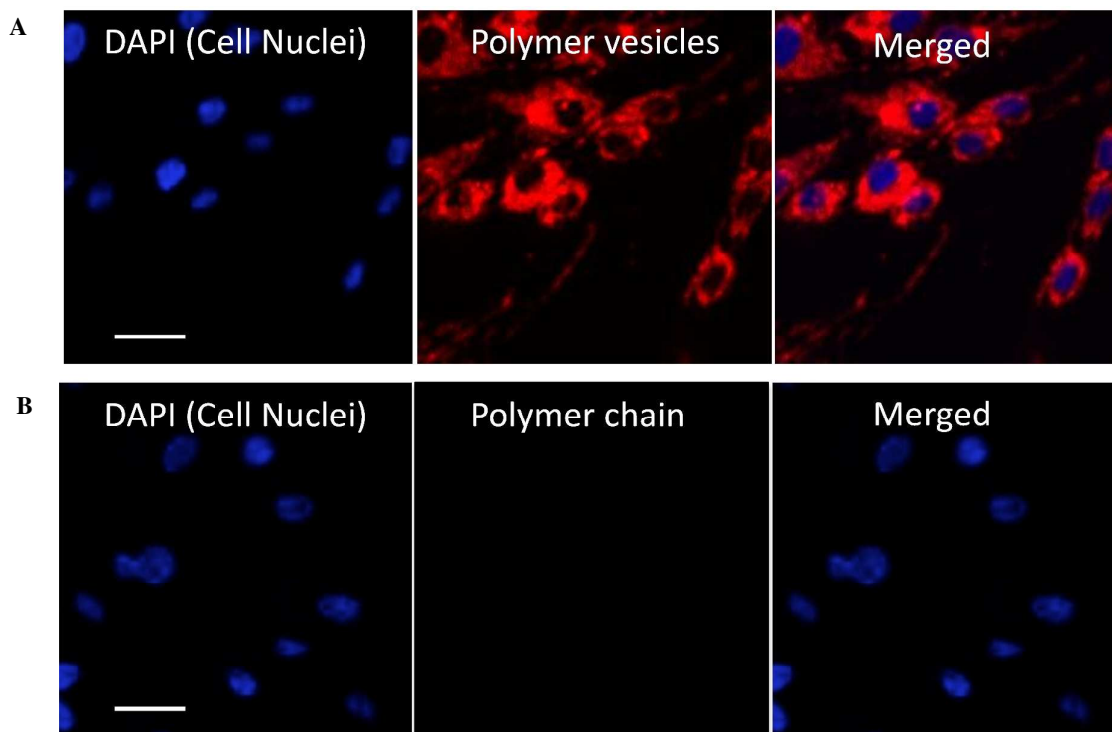


Figure S12: Differential uptake of PMPC-PDPA diblock copolymer vesicles and PMPC homopolymer chains by HDF cells. Cells were incubated with (A) rhodamine 6G-labeled PMPC₂₅-PDPA₇₀ diblock copolymer vesicles or (B) rhodamine 6G-labeled PMPC₁₀₀ homopolymer at 37 °C in 5% CO₂ for 2 h. After incubation with 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 PMPC₂₅-PDPA₇₅ diblock copolymer vesicles (red, middle) are readily uptaken by the cells, whereas the PMPC₁₀₀ homopolymer is not uptaken at all under the same conditions (no signal, middle). Merged images are shown on the right. Scale bar = 100 μ m for all images.

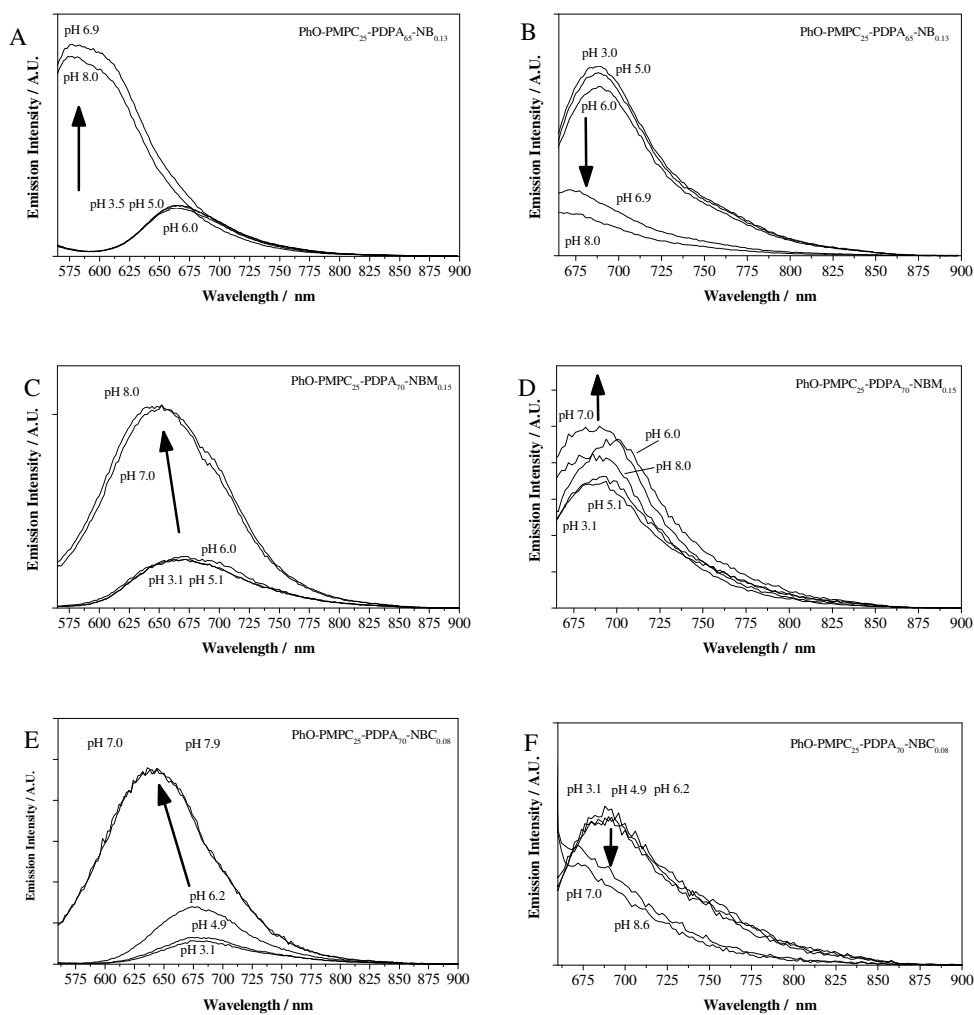


Figure S13: Fluorescence emission vs pH for 1 mg/mL aqueous solutions of various Nile Blue-labeled PMPC-PDPA diblock copolymers. Conditions: excitation slit: 5 nm; emission slit: 5 nm; photomultiplier voltage = 800 V. (A) PhO-PMPC₂₅-PDPA₇₀-NB_{0.12} excited at 550 nm; (B) PhO-PMPC₂₅-PDPA₇₀-NB_{0.12} excited at 650 nm; (C) PhO-PMPC₂₅-PDPA₇₀-NBM_{0.15} excited at 550 nm; (D) PhO-PMPC₂₅-PDPA₇₀-NBM_{0.15} excited at 650 nm; (E) PhO-PMPC₂₅-PDPA₇₀-NBC_{0.08} excited at 550 nm; (F) PhO-PMPC₂₅-PDPA₇₀-NBC_{0.08} excited at 650 nm.

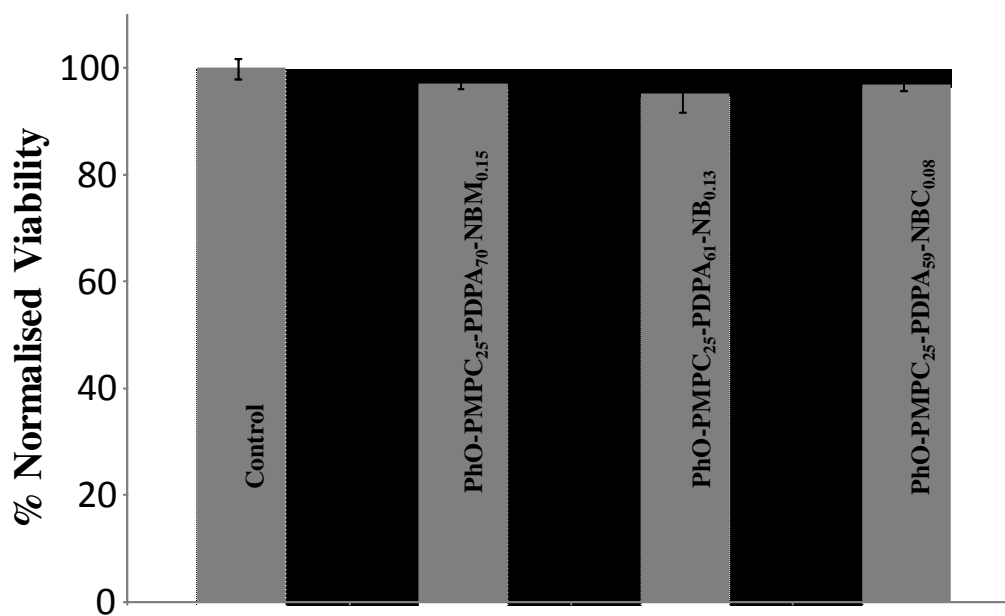


Figure S14: 24 h viability of Human Dermal Fibroblasts exposed to 1 mg/mL Nile Blue labeled PMPC-PDPA diblock copolymer vesicles

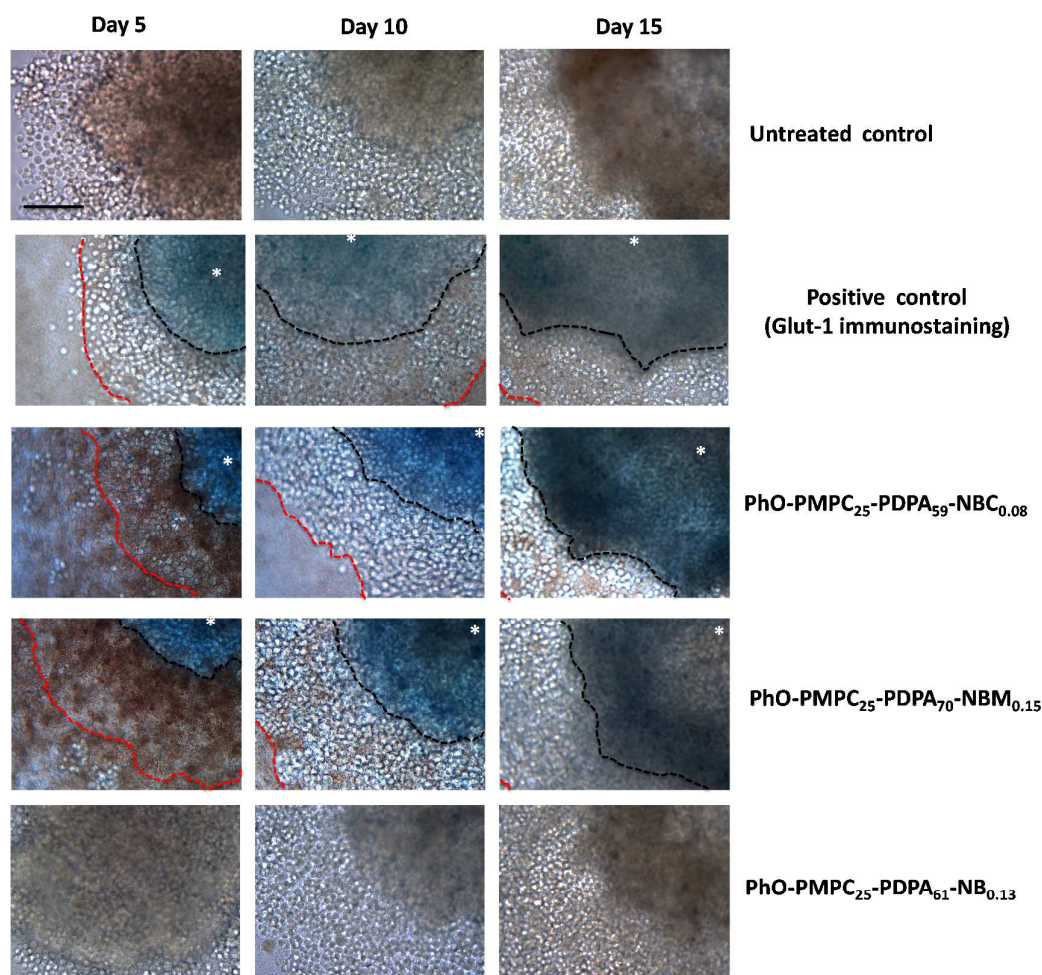


Figure S15: pH-dependent staining of multicell tumour spheroids (MCTS) by Nile blue-labeled PMPC-PDPA copolymers. Bright-field optical images of Nile Blue polymer treated MCTS and controls were recorded using a Leica DMI4000B instrument equipped with a Nuance Multispectral imaging System. Untreated control 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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