Electronic Supporting Information to the paper: **Superior proapoptotic activity of curcumin-loaded mixed block copolymer micelles with mitochondrial targeting properties**

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***Materials***

Poly(ε-caprolactone) diol (HO-$PCL_{70}$-OH, CAPA® 2803, molar mass 8000 $g.mol^{-1}$, kindly donated by Solvay Chemicals) and methoxy poly(ethylene glycol) ($PEO_{113}$-OH, molar mass 5000 $g.mol^{-1}$, Fluka) were precipitated in cold methanol (-40 $^oC$), filtered and dried under vacuum at 40 $^oC$ overnight. 2-(Dimethylamino)ethyl methacrylate (DMAEMA, Sigma-Aldrich) was stirred overnight over calcium hydride (Merck) and distilled under reduced pressure. α-Bromoisobutyryl bromide (BIB, Sigma-Aldrich), triethylamine ($Et_3N$, Fluka), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA; Sigma-Aldrich), 4-pentynoic acid (Acros), copper (I) bromide (CuBr, Sigma-Aldrich), N,N,N′,N″,N″ pentamethyldiethylene-triamine (PMDETA, Sigma-Aldrich), pyridinium p-toluenesulfonate (Sigma-Aldrich), sodium azide (Fluka), 4-dimethylaminopyridine (DMAP, Sigma-Aldrich), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Merck), 4-bromobutyltriphenylphosphonium bromide (Sigma-Aldrich),curcumin (Sigma-Aldrich), methanol (Merck), tetrahydrofuran (Merck), 1,4-dioxane (Merck), hexane (Fisher Chemicals), acetone (Fisher Chemicals), toluene (Merck), isopropanol (Fisher Chemicals), silica gel (70-230 mesh, Merck) and activated neutral aluminum oxide (Fluka) were used as received.Dichloromethane (Sigma-Aldrich) and chloroform (Sigma-Aldrich) were stirred overnight over calcium hydride and distilled.N,N-dimethylformamide (Sigma-Aldrich) were dried over diphosphoruspentaoxide and distilled.

***Synthesis of block copolymers***

*Br-PCL$_{70}$-Br macroinitiator:* HO-PCL$_{70}$-OH (30.0 g, 3.75 mmol) was dissolved in 100 mL of toluene and dried by azeotropic distillation. Then, the dry polymer was dissolved in 150 mL of freshly distilled THF and TEA (6.276 mL, 45.0 mmol) was added via syringe. The solution was cooled down to 0°C by dint of an ice bath and α-bromoisobutyryl bromide (2.78 mL, 22.49 mmol) was added dropwise. The reaction mixture was stirred at 750 rpm for 168 h at 20$^{o}$C. Thereafter, the reaction mixture was filtered off to remove the insoluble white precipitate of HBr salt. The residual solution of a brominated product in THF was concentrated by rotary evaporation prior to precipitation into a 10-fold excess of cold methanol (-40 $^{o}$C) and recovered by filtration. $^{1}$H-NMR ($CDCl_3$, δ ppm): 4.2-3.9 (m,-C$\underline{H}_2$-CO(O)-), 2.35-2.15 (m, -C$\underline{H}_2$-O-CO-), 1.93 (s, -C-C$\underline{H}_3$ end groups), 1.7-1.55 (m, -C$\underline{H}_2$-CH$_2$-C(O)-), 1.45-1.25 (m, -CH$_2$-C$\underline{H}_2$-CH$_2$-).

*PDMAEMA$_{20}$-b-PCL$_{70}$-b-PDMAEMA$_{20}$ triblock copolymer:* A single-neck round-bottom flask equipped with a stir bar was charged with Br-PCL$_{70}$-Br (1.0683 g, 0.133 mmol, 1 equiv), Cu(I)Br (0.0179 g, 0.125 mmol, 1 equiv ), DMAEMA (1.07 ml, 6.818 mmol, 50 equiv) and freshly distilled THF (10.7 mL). After that, the flask was rapidly sealed off with a septum, and the reaction mixture was left stirring at ambient temperature under argon flow for 20 minutes. After the degassing with argon, HMTETA (0.05759 g, 0.025 mmol, 2 equiv) was added via syringe. The flask was immersed in an oil bath preheated to 60°C, and the reaction was allowed to proceed for 16 h. Then, the reaction mixture was allowed to cool down to ambient temperature under exposure to air. THF was added additionally to dissolve the polymer formed. Purification was achieved by dialysis against distilled water (cellulose membrane, cut-off = 2 000 g.mol$^{-1}$) for 7 days. Finally, the copolymer was recovered by freeze drying. $^{1}$H-NMR ($CDCl_3$, δ ppm): 4.2-3.9 (m,-C$\underline{H}_2$-CO(O)- of PCL and -C$\underline{H}_2$-O-CO- of PDMAEMA), 2.65-2.47 (m,-CH$_2$-C$\underline{H}_2$–N= of PDMAEMA), 2.35-2.15 (m, -C$\underline{H}_2$-O-CO- of

PCL and -N-(C<u>H</u>$_3$)$_2$ of PDMAEMA), 1.95-1.75 (m,-C-C<u>H</u>$_2$- of PDMAEMA), 1.67-1.55 (m, -C<u>H</u>$_2$-CH$_2$-C(O)- of PCL), 1.45-1.25 (m, -CH$_2$-C<u>H</u>$_2$-CH$_2$- of PCL), 1.15-0.8 (m,-C-C<u>H</u>$_3$ of PDMAEMA).

*PDMAEMA$_{20}^{(TPP+)}$-b-PCL$_{70}$-b-PDMAEMA$_{20}^{(TPP+)}$ triblock copolymer:* PDMAEMA$_{20}$-b-PCL$_{70}$-b-PDMAEMA$_{20}$ (500 mg, 1 eq) was dissolved in dry $CH_2Cl_2$ (10 ml) under inert atmosphere in 50 ml round-bottom flask. 4-Bromobutyl triphenyl phosphonium bromide (0.25 eq. to PDMAEMA groups (40 units per chain)) was added to the above solution, and the reaction mixture was heated and stirred for 48 h at 35 °C. Finally, the product was dialysed against MeOH. $^1$H-NMR ($CDCl_3$, δ ppm): 7.9-7.65 (m, Ar signals of 4-bromobutyl triphenyl phosphonium moiety), 4.1-4.01 (m,-C<u>H</u>$_2$-CO(O)- of PCL and -C<u>H</u>$_2$-O-CO- of PDMAEMA), 2.65-2.45 (m,-CH$_2$-C<u>H</u>$_2$–N= of PDMAEMA), 2.30-2.27 (m, -C<u>H</u>$_2$-O-CO- of PCL and -N-(C<u>H</u>$_3$)$_2$ of PDMAEMA), 1.95-1.75 (m,-C-C<u>H</u>$_2$ of PDMAEMA), 1.7-1.55 (m, -C<u>H</u>$_2$-CH$_2$-C(O)- of PCL), 1.4-1.3 (m, -CH$_2$-C<u>H</u>$_2$-CH$_2$- of PCL), 1.15-0.8 (m,-C-C<u>H</u>$_3$ of PDMAEMA).

*PEO$_{113}$ macroreagent with cleavable acetal and clickable azide terminal groups (PEO-N$_3$):* PEO monomethyl ether (2.5 g, 0.5 mmol) and pyridinium*p*-toluenesulfonate (12.6 mg, 0.05 mmol) were added in a flask under $N_2$ atmosphere. After three azeotropic distillations by toluene, dry $CH_2Cl_2$ (14 mL) was added and the solution was cooled down to 0°C. 2-Chloroethyl vinyl ether (254 μL, 2.5 mmol) was added dropwise. The reaction medium was stirred at 25°C for 1 h under $N_2$ atmosphere. The obtained reaction mixture was quenched by adding 5 wt% of basic $Na_2CO_3$ aqueous solution (~ 60 mL), diluted with another 75 mL of $CH_2Cl_2$; washed with 75 mL of water and 2x75 mL portions of brine. All aqueous layers were further extracted with 50 mL of $CH_2Cl_2$ and the combined $CH_2Cl_2$ solutions were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The product was redissolved in 10 mL of a $CH_2Cl_2$/THF (1/1 v/v) mixture and precipitated into 100 mL of a cold mixture of hexane and diethyl ether (1/1 v/v) to give PEO$_{113}$-Cl; $^1$H-NMR ($CDCl_3$, δ ppm): 4.82 (q, H$_3$C-

C<u>H</u>(OR)(OR') – acetal group), 3.85-3.75 (m, Cl-C<u>H</u>$_2$CH$_2$O-), 3.75-3.5 (m, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-), 3.37 (s, C<u>H</u>$_3$-O- of PEO), 1.33 (d, <u>H</u>$_3$C-CH(OR)(OR') – acetal group).

The as-obtained $PEO_{113}$-Cl (2.25 g, 0.45 mmol) and $NaN_3$ (292.5 mg, 4.5 mmol) were then dissolved in 14 ml of anhydrous N,N-dimetylformamide under inert atmosphere. The temperature was increased and reaction was carried out at 120 °C for 3 h. The solution was concentrated under reduced pressure on rotary evaporator, diluted with 75 mL of $CH_2Cl_2$, washed with 75 mL of water and 2x75 mL portions of brine. All aqueous layers were further extracted with 50 mL of $CH_2Cl_2$ and the combined $CH_2Cl_2$ solutions were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The product was redissolved in 10 mL of a mixture of $CH_2Cl_2$ and THF (1/1 v/v) and precipitated into 100 mL of a cold hexane/diethyl ether mixture (1/1 v/v). The product was further filtered and washed well with $Et_2O$ to give PEO carrying a cleavable acetal and clickable azide terminal group. $^1$H-NMR ($CDCl_3$, δ ppm): 4.82 (q, $H_3$C-C<u>H</u>(OR)(OR') – acetal group), 3.75-3.5 (m, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-), 3.40-3.35 (m, $N_3$-C<u>H</u>$_2$CH$_2$O-), 3.37 (s, C<u>H</u>$_3$-O of PEO), 1.33 (d, <u>H</u>$_3$C-CH(OR)(OR') – acetal group).

*Alkyne terminated $PCL_{70}$ macroreagent ($HC\equiv C$-$PCL_{70}$-$C\equiv CH$):* PCL-diol (1.25 g, 0.16 mmol, 1 eq.) was dissolved in dry $CH_2Cl_2$ (20 ml) under inert atmosphere in 50 ml round-bottom flask. Pentynoic acid (125.6 mg, 1.28 mmol, 8 eq), EDC (122.7 mg, 0.64 mmol, 4 eq) and DMAP (39.8 mg, 0.32 mmol, 2 eq) were added consequently to the solution at room temperature. The reaction was carried out for 24 h, followed by dilution with 60 ml of $CH_2Cl_2$ and extraction with 2x75 ml of distilled $H_2O$. The organic phase was dried over $MgSO_4$, filtered and the solvent was evaporated. The product was re-dissolved in 10 ml of $CH_2Cl_2$ and precipitated in 100 ml chilled MeOH. Finally, the precipitated product was filtered and washed well with hexane. $^1$H-NMR ($CDCl_3$, δ ppm): 4.05 (t, -C<u>H</u>$_2$-CO(O)- of PCL), 2.56-2.52 (m, -C<u>H</u>$_2$-CH$_2$–C≡CH group), 2.52-2.47 (m, -CH$_2$-C<u>H</u>$_2$–C≡CH group), 2.3 (t, -C<u>H</u>$_2$-O-CO- of

PCL), 1.98 (t, -$CH_2$-$CH_2$-C≡C<u>H</u> group), 1.7-1.55 (m, -C<u>$H_2$</u>-$CH_2$-C(O)- of PCL), 1.42-1.33 (m, -$CH_2$-C<u>$H_2$</u>-$CH_2$- of PCL).

*$PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ triblock copolymer:* HC≡C-$PCL_{70}$-C≡CH (1 g, 0.125 mmol, 1 eq), $PEO_{113}$-Ac-$N_3$ (1.875 g, 0.375 mmol, 3 eq), and CuBr (0.359 g, 2.5 mmol, 20 eq) were added in a flask under nitrogen atmosphere. After three freeze-pump-thaws degassing cycles, anhydrous DMF (12 ml) was added via a syringe and the solution was purged with nitrogen and stirred vigorously for 20 min. PMDETA (626.0 µl, 3.0 mmol, 24 eq) was added via a syringe and the solution was degassed by nitrogen for additional 20 min. The reaction mixture was heated up to 30°C and stirred for 24 h. After addition of 30 ml THF, the solution was passed through a neutral $Al_2O_3$ column to remove the main part of copper salts. The solution was evaporated on a rotary evaporator and the dissolved in water product was finely purified by dialysis in water. Selective removal of the excess of pristine PEO was achieved by ultrafiltration (membrane MW cut off 10 000 $g.mol^{-1}$). $^1$H-NMR ($CD_2Cl_2$, δ ppm): 7.48 (s, -$N_3$-<u>H</u>$C_2$- triazole ring), 4.7 (q, $H_3$C-C<u>H</u>(OR)(OR') – acetal group), 4.47 (t, N-C<u>$H_2$</u>-$CH_2$-O-), 4.03 (t, -C<u>$H_2$</u>-CO(O)- of PCL), 3.72-3.45 (m, -O-C<u>$H_2$</u>-C<u>$H_2$</u>O- of PEO), 3.33 (s, C<u>$H_3$</u>-O- of PEO), 2.98 (t, OC(O)-$CH_2$-C<u>$H_2$</u>-N), 2.68 (t, OC(O)-C<u>$H_2$</u>-C<u>$H_2$</u>-N), 2.29 (t, -C<u>$H_2$</u>-O-CO- of PCL), 1.77-1.53 (m, -C<u>$H_2$</u>-$CH_2$-C(O)- of PCL), 1.4-1.3 (m, -$CH_2$-C<u>$H_2$</u>-$CH_2$- of PCL), 1.25 (d, <u>$H_3$</u>C-CH(OR)(OR') – acetal group).

***Measurements***

$^1$H-NMR spectra were recorded on Bruker Avance- DRX 250 and II+600 apparatuses at room temperature in $CDCl_3$ and $CD_2Cl_2$. Size exclusion chromatography (SEC) of PDMAEMA-b-PCL-b-PDMAEMA triblock copolymer was performed on a Agilent Technologies 1200 chromatograph equipped with a degasser, an isocratic HPLC pump, an autosampler, a refractive index (RI) detector and three columns: a guard column 5 µm PL gel and two

columns 5 µm PL gel mixed-D. Tetrahydrofuran (THF) at 35°C was used as the eluent at a flow rate 1 $mL.min^{-1}$, sample concentration was 1 $mg.mL^{-1}$ and SEC was calibrated with polystyrene standards. SEC of PEO-b-PCL-b-PEO was performed on Shimadzu Nexera HPLC chromatograph equipped with a degasser, a pump, an autosampler, a RI detector and three columns: 10 µm PL gel mixed-B, 5 µm PL gel 500Å and 50Å. THF was used as the eluent at flow rate 1.0 $mL.min^{-1}$ and temperature 40 $^{o}C$. Sample concentration was 1 $mg.mL^{-1}$ and SEC was calibrated with polystyrene standards.

*Critical micelle concentration (CMC).* CMC was determined by using the hydrophobic dye 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. 20 ml of DPH dissolved in methanol (0.4 mM) was added to 2 ml aqueous dispersions of micelles (8 different concentrations ranging from 0.001 to 1 $g.L^{-1}$) and the samples were incubated in the dark for 16 h at room temperature. UV–vis absorption spectra of DPH were recorded in the 250–600 nm range at 20 $^{o}C$. The CMC value of each system was determined as an inflection point in the absorbance intensity *vs.* concentration curve.

***Table S1.*** *Equieffective concentrations ($IC_{50}$) of free curcumin, curcumin-loaded mixed $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$/$PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ micelles and curcumin-loaded single $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ micelles on a panel of human cell lines.*

| **Compound** / **Cells** | **$IC_{50}$ (µg/ml)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | HEK-293 | Huvec | HL-60 | HL-60/ DOX | PC-3 | HeLa | MCF-7 |
| Free Curcumin | 10 | 12.15 | 8.0 | 11.8 | 12.67 | 8.77 | 16.68 |
| Curcumin-loaded mixed micelles | 7.5 | 5.84 | 5.7 | 5. 9 | 6.83 | 3.25 | 5.93 |
| Curcumin-loaded single PEO-b-PCL-b-PEO micelles | 9.8 | 8.93 | 6.1 | 7.7 | Not tested | Not tested | Not tested |

Figures:

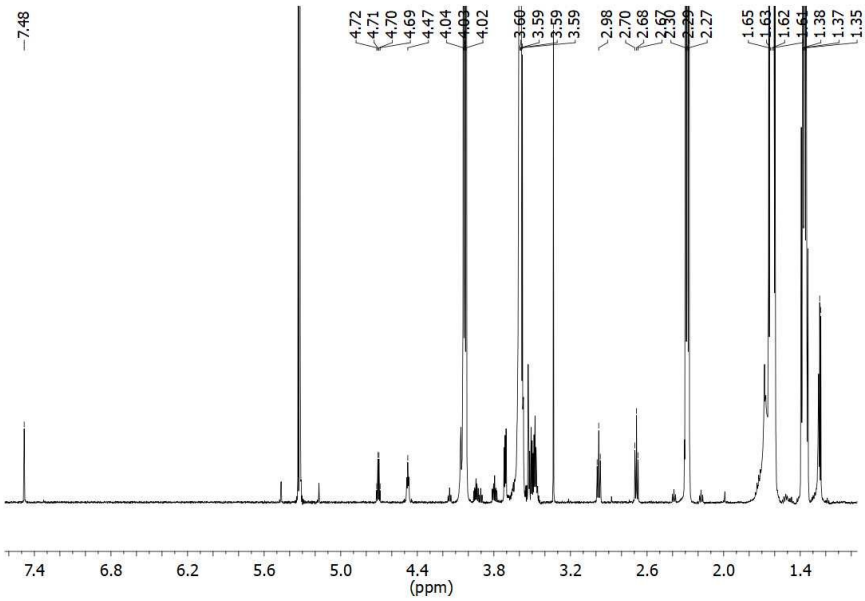


***Figure S1.****[1]H-NMR spectrum of $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ triblock copolymer in $CD_2Cl_2$.*

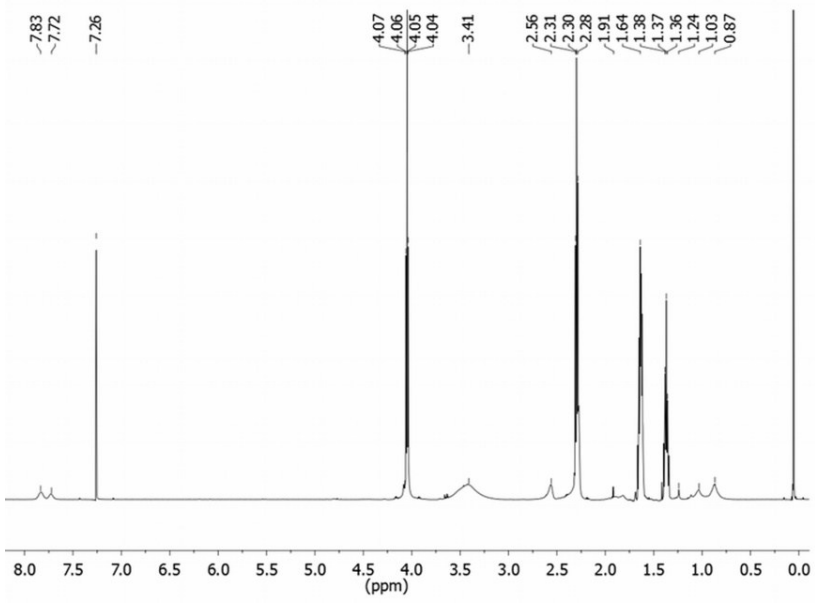


***Figure S2.****[1]H-NMR spectrum of $PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ triblock copolymer in $CDCl_3$.*

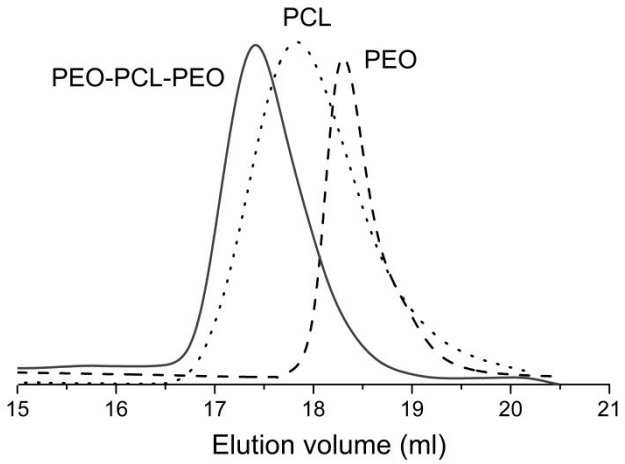


***Figure S3.*** *SEC chromatograms of $PEO_{113}$-OH and OH-$PCL_{70}$-OH precursors, and $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ triblock copolymer.*

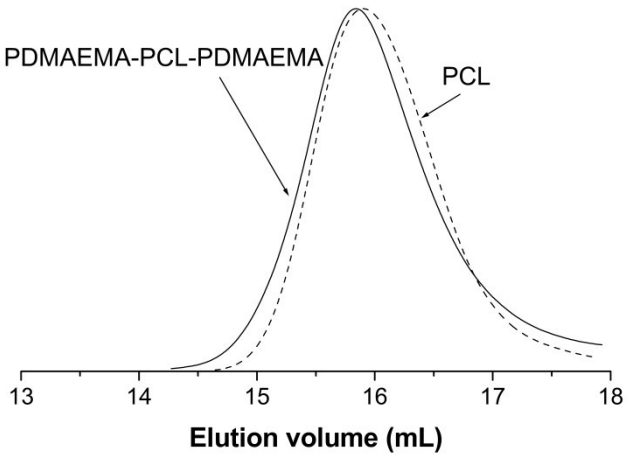


***Figure S4.*** *SEC chromatograms of Br-$PCL_{70}$-Br precursor and $PDMAEMA_{20}$-b-$PCL_{70}$-b-$PDMAEMA_{20}$ triblock copolymer.*

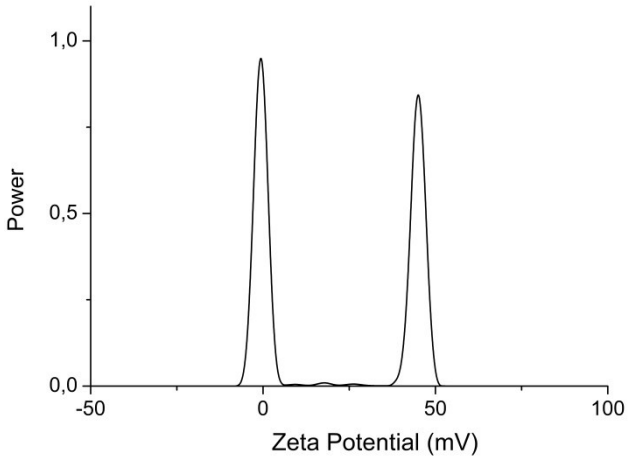


***Figure S5.*** *Zeta potential of a mixture of preformed single $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ and $PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ micelles.*

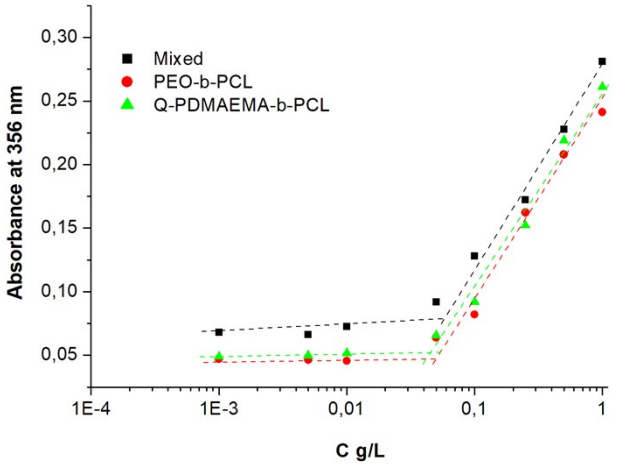


***Figure S6****. Determination of the critical micellar concentration of single $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ and $PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ micelles and mixed $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$ /$PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ micelles by the DPH method.*

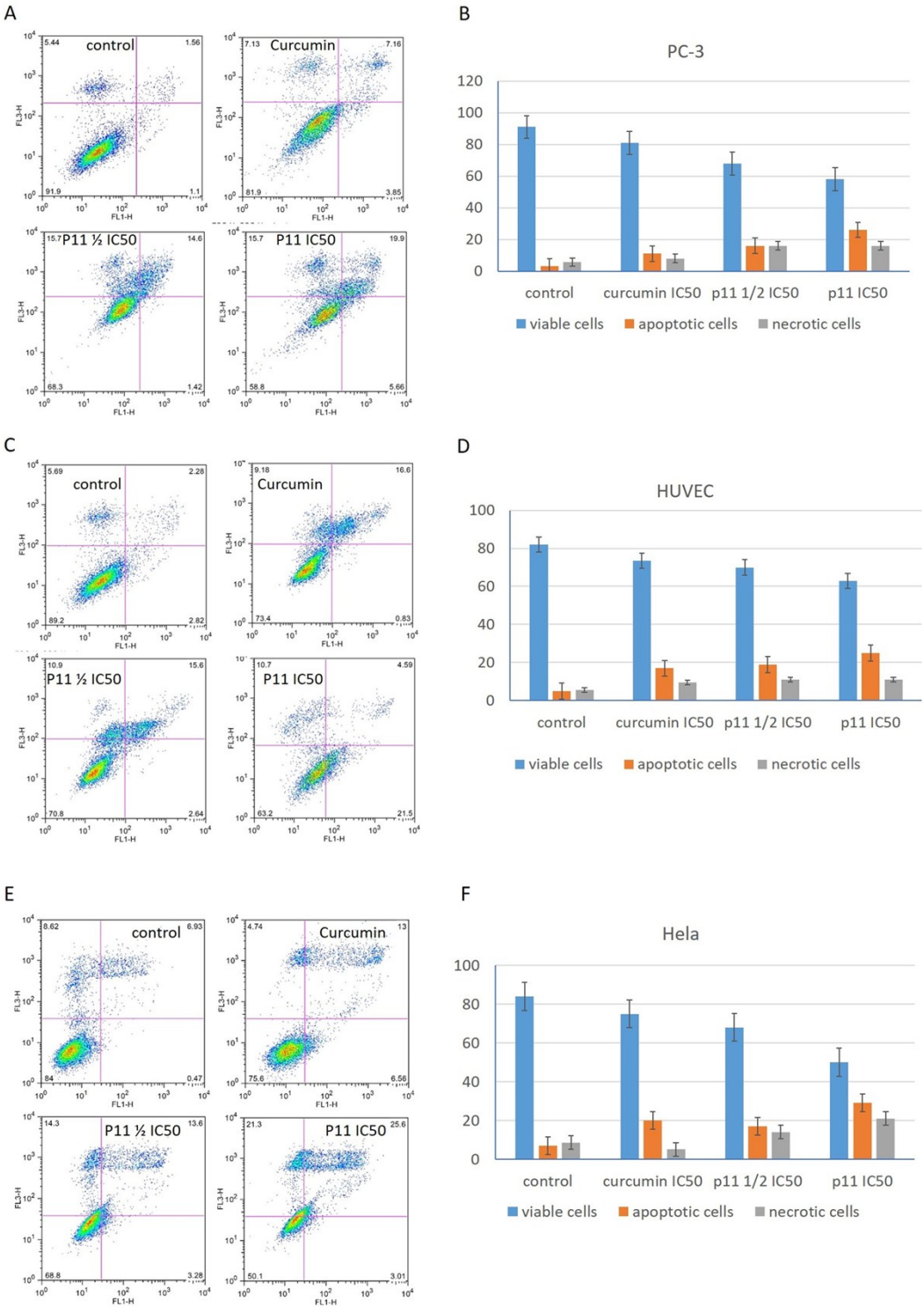


***Figure S7****. Flow cytometry analysis of free curcumin at $IC_{50}$ and curcumin-loaded mixed $PEO_{113}$-b-$PCL_{70}$-b-$PEO_{113}$/$PDMAEMA_{20}^{(TPP+)}$-b-$PCL_{70}$-b-$PDMAEMA_{20}^{(TPP+)}$ micelles (P11) at ½ $IC_{50}$ and $IC_{50}$ as determined by Annexin V-PI staining. The dot plots (left) and the percentage (%) of cell distribution (right) of PC-3(A,B), HUVEC(C,D) and Hela (E,F) after cells treatment with different concentrations of the formulations for 24 hours are presented. The data is the average value of three independent experiments.*