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Curcumin-polymer conjugates with dynamic boronic acid ester linkage for selective killing of cancer cells

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Experiment Section

1. Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMA, M_n ~ 950 g mol^{-1}, Sigma Aldrich), 2-(acetoacetoxy) ethyl methacrylate (AEMA, Aladdin, 95%), 4-formylphenyl boronic acid (Ark Pharm, 98%), benzaldehyde (Macklin, 98%), ammonium acetate (Sinopharm Chemical Reagent Co., Ltd, 99%), dimedone (Aladdin, 99%), glycine (Sinopharm Chemical, 99%), 2,2'-azobis(2,4-dimethyl) valeronitrile (ABVN, Energy Chemical, 98%), ethyl acetoacetate (Aladdin, 99%), curcumin (Heowns, 97%), apigenin (Adamas, 97%), phosphate buffered saline (PBS, pH ~ 7.2-7.4, 0.01 M, Solarbio), fetal bovine serum (FBS, Gibco), 1640 RPMI culture medium (Gibco), DMEM culture medium (HyClone), penicillin-streptomycin solution (Gibco), immunol staining fix solution (Beyotime), trypsin-EDTA (Gibco, 0.25%), cell counting kit-8 solution (Beyotime),

fluorescein diacetate (FDA, Sigma), propidium iodide (PI, Sigma, 94%) and lectin (MACKLIN) were used as purchased. Solvents such as acetonitrile, ethanol and diethyl ether were purchased from Sinopharm Chemical Reagent and used directly without further purification. The small 1,4-dihydropyridine (DHP) molecule, similar to the P1 (P2) side group, was synthesized as previously report.[1]

2. Instrumental Analysis

The ^{1}H NMR spectra of all samples were obtained using a JEOL JNM-ECA400 spectrometer. Gel permeation chromatography (GPC) system included a Shimadzu LC-20AD pump 45 system consisting of an auto injector, a MZ-Gel SDplus 10.0 μm guard column (50 × 8.0 mm, 102Å), a MZ-Gel SDplus 5.0 μm bead-size column (50 – 106Å, linear) and a Shimadzu RID-10A refractive index detector. Narrow molecular weight distribution polystyrene standards ranging from 200 to 106 g mol^{-1} were calibrated for this system, and N, N-dimethyl formamide (DMF) were used as the eluent. The fluorescence measurements for binding constants experiments were obtained on Shimadzu RF-6000 spectrofluorophotometer. The size of nanoparticles was measured by dynamic light scattering (DLS) on a Brookhaven zeta potential analyzer. The detailed morphology of nanoparticles was recorded by transmission electron microscopy (TEM) on Hitachi H-7650B with accelerating voltage of 80 kV. UV analyses were carried out by a PerkinElmer Lambda 750 UV/Vis/NIR spectrophotometry. The fluorescent intensity in cells (485/535 nm) were collected by VICTORTM X3 PerkinElmer 2030 Multilabel Plate Reader. The orbital shaker (ZD-9566, HLD laboratory equipment Co., China) and a peristaltic pump (BT/101S, Baoding Lead Fluid Technology Co., Ltd., China) were used for the dynamic release study. Confocal images were collected by a Zeiss LSM-710 confocal microscopic system.

3. Methods

3.1 Preparation of M1

AEMA (8.58 g, 40 mmol), dimedone (5.60 g, 40 mmol), 4-formylphenylboronic acid (6.00 g, 40 mmol), ammonium acetate (4.64 g, 60 mmol), glycine (0.30 g, 4 mmol) were dissolved in 20 mL of acetonitrile. The mixture was kept in a 70 ℃ oil bath for 4 hours. The monomer (**M1**) was obtained by precipitation in diethyl ether and deionized water twice as a pale-yellow powder (20.0 g, 96.2% yield).

^{1}H NMR (400 MHz, d_6-DMSO, δ/ppm): 9.05 (s, 1H, NH), 7.54 (m, 2H, C_6H_4), 7.11 (m, 2H, C_6H_4), 4.77 (s, 1H, CH), 4.23 (m, 4H, $C\underline{H}_2C\underline{H}_2OC{=}O$), 4.22 (m, 2H, $B(OH)_2$), 5.92 (s, 1H, $C{=}CH_2$), 5.61 (s, 1H, $C{=}CH_2$), 2.27 (m, 2H, $CH_2C{=}O$), 1.77 (s, 3H, $C\underline{H}_3CNH$), 2.31 (s, 3H, $C\underline{H}_3C{=}CH_2$), 2.11 (m, 1H, $C\underline{H}_2CNH$), 1.96 (m, 1H, $C\underline{H}_2CNH$), 0.92 (s, 3H, CH_3CCH_2), 0.47 (s, 3H, CH3CCH2).

^{13}C NMR (100 MHz, methanol-D4, δ/ppm): 196.9, 167.8 (167.6), 149.4 (149.0), 145.1, 138.1, 133.2 (133.0), 128.3 (128.0), 126.1, 111.3, 105.1, 102.7, 63.1 (62.9), 50.6, 39.8, 37.6, 32.0, 28.1, 26.2, 17.9, 15.1.

IR (ν/cm^{-1}): 3205, 2961, 1723, 1697, 1607, 1485, 1411, 1368, 1281, 1217, 1165, 1138, 1111, 1043, 938, 845.

ESI-MS: observed (expected): 468.2775 (468.2775) $[M+H]^+$.

M2 were similarly synthesized using different reactants (18.3 g, 95.8% yield).

^{1}H NMR (400 MHz, d_6-DMSO, δ/ppm): 9.05 (s, 1H, NH), 7.15 (m, 5H, C_6H_5), 4.77 (s, 1H, CH), 4.27 (m, 4H, $C\underline{H}_2C\underline{H}_2OC{=}O$), 5.92 (s, 1H, $C{=}CH_2$), 5.63 (s, 1H, $C{=}CH_2$), 2.27 (m, 2H, $CH_2C{=}O$),

1.77 (s, 3H, $C\underline{H}_3CNH$), 2.31 (s, 3H, $C\underline{H}_3C{=}CH_2$), 2.11 (m, 1H, $C\underline{H}_2CNH$), 1.96 (m, 1H, $C\underline{H}_2CNH$), 0.92 (s, 3H, CH_3CCH_2), 0.47 (s, 3H, CH3CCH2).

^{13}C NMR (100 MHz, methanol-D4, δ/ppm): 196.9, 167.8 (167.6), 149.4 (149.0), 145.1, 138.1, 130.6 (130.1), 127.3 (127.0), 126.5, 126.1, 111.3, 102.7, 63.1 (62.9), 50.6, 39.8, 37.6, 32.0, 28.1, 26.2, 17.9, 15.1.

IR (ν/cm^{-1}):3281, 2961, 1721, 1702, 1640, 1605, 1483, 1450, 1380, 1274, 1210, 1160, 1140, 1106, 1046, 997, 885, 761.

ESI-MS: observed (expected): 424.3116 (424.3116) $[M+H]^+$.

3.2 Preparation of P1

The phenylboronic acid contained polymers (**P1**) were easily prepared using **M1** by convenient radical polymerization.[1] **M1** (1.84 g, 4 mmol), PEGMA (7.60 g, 8 mmol), ABVN (0.06 g, 0.24 mmol) were dissolved in 10 mL of N,N-dimethylformamide. The mixture was purged with nitrogen flow to remove oxygen followed by keeping in a 70 ℃ oil bath for 8 hours. The polymerization was quenched in an ice-water bath, samples were taken out for ^1H NMR and GPC analyses. The final polymer **P1** was obtained by precipitation in diethyl ether three times as a white powder.

The control polymer (**P2**) only containing phenyl group was similarly synthesized.

3.3 Measurement of binding constants

Binding studies between natural drug molecules and **MA** were carried out as descried in the previous essay.[1,2]

Curcumin exhibits a significant change in fluorescent intensity upon bonded boronic acid. Briefly, a buffer solution of curcumin (solution A) was freshly prepared (9.0×10^{-6} M) in sodium phosphate buffer/ethanol mixture (ethanol: buffer = 1: 1 (v/v), pH ~7.4, 0.10 M). Solution B was prepared by adding **MA** to solution A to achieve a final concentration of 9.0×10^{-6} M curcumin and 1.8×10^{-3} M **MA**. Solution B was then titrated into solution A to obtain a series of mixtures with constant curcumin concentration and a range of **MA** concentrations. Each mixture was equilibrated for 10 min prior to measurements. Fluorescence intensity was recorded at 520 nm with an excitation wavelength at 480 nm in microplate readers. All experiments were ran thrice and binding constants were calculated using the Benesi-Hildebrand method[2].

Binding constants of curcumin (CUR) - MA were determined based on the following equilibria:

$$CUR + MA \xleftrightarrow{K_{CUR\text{-}MA}} CUR\text{-}MA$$

CUR-MA represent the curcumin and MA complexes, with binding constants $K_{CUR\text{-}MA}$.

$K_{CUR\text{-}MA}$ is determined according to:

$$\frac{1}{\triangle FI} = \left(\frac{1}{\triangle Kp_0 I_0 K_{CUR/MA}}\right)\frac{1}{[CUR]} + \left(\frac{1}{\triangle Kp_0 I_0}\right)$$

From the intercept and slope of the plot ΔFI^{-1} vs. $[CUR]^{-1}$. ΔK, p_0 and I_0 are parameters of the spectrophotometer.

$K'_{CUR\text{-}MA}$ (pH~5.5) was calculated by dropping acetic acid to adjust the pH of solutions.

$K_{API\text{-}MA}$ were similarly measured using apigenin instead of curcumin.

3.4 Stability study of the curcumin-polymer nanoparticles

The curcumin and polymers were dispersed in sodium phosphate buffer/ethanol mixture (ethanol: buffer = 5: 95 (v/v), pH ~7.4, 0.10 M), and the suspensions (curcumin: PBA in polymer = 1: 3 (M/M), curcumin 1 mg/mL, 2 mL) were treated by ultrasonic dispersion for 30 minutes for complete binding. The suspensions were diluted with buffer/ethanol mixture (ethanol: buffer = 5: 95 (v/v), pH ~7.4, 0.10 M) then measured the sizes and size distribution indices of the nanoparticles by dynamic light scattering (DLS).

3.5 Curcumin release study

The curcumin was released by a circulation mode[3]. The nanoparticles suspension (ethanol: buffer = 5: 95 (v/v), pH ~7.4, curcumin: PBA in polymer = 1: 3 (M/M), curcumin 1 mg/mL, 100 mL) was added to a dialysis bag (MWCO: ~ 3.5 K), and connected to a peristaltic pump by inserting I/O ducts. The dialysis bag was put into a beaker (1 L) containing PBS/ethanol solution (700 mL, ethanol: buffer = 5: 95 (v/v), pH ~7.4, 0.10 M) on an orbital shaker (90 rpm). The rotation rate of the peristaltic pump was set at 108 rpm (9.5 mL/sec). Samples (2 mL) were taken from the outside PBS solution at different time points to measure released curcumin (UV: 359 nm), then put back for continue dialysis.

Curcumin release at different pH was similarly studied.

3.6 Cell experiments

Cell culture was maintained in a 37 °C incubator with 5% CO_2, culture medium was changed every one or two days for maintaining the exponential growth of the cells. HeLa cells (derived from cervical cancer cells) and A375 cells (derived from malignant melanoma cells) cultured in DMEM medium (high glucose) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin and streptomycin. L929 cells (derived from mice) cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin.

3.6.1 Cell viability assay. The cell viability assays of P1 to L929, HeLa and A375 cells were evaluated using cell count kit-8 (CCK-8)[4]. Briefly, cells (~5×10^4 cells/mL) were seeded in a 96-well plate in 100 μL of culture medium. After attachment, cells were washed twice by PBS and cultured with different concentrations of P1 for 24 h, respectively. Then, cells were washed three times by PBS and cultured in 100 μL of culture medium containing 10% CCK-8 solution for 2 h prior to observation. The CCK-8 dye absorbance was measured under 450 nm and the reduction of CCK-8 dye was compared to positive control (cells in pure culture medium, 100% CCK-8 dye reduction) and negative control (no cells in plate, 0% CCK-8 dye reduction), the absorbance ratio to the positive control and negative control reflects the cell viability. The results were presented as mean ± standard deviation (SD).

P1-curcumin conjugates were similarly tested.

3.6.2 Endocytosis analysis. Cells (~10^5 cells/mL) were incubated in culture media containing different concentrations of P1 (10 mg/mL, 8 mg/mL, 6 mg/mL, 4 mg/mL, 2mg/mL, 1mg/mL) for 4 h, respectively. The cells were washed three times with PBS (pH ~ 7.4) then kept in PBS (pH ~ 7.4) for observation. The fluorescent images of cells were recorded by a confocal microscopy (Zeiss LSM-710) under UV (405 nm) and were processed by a Zeiss software.

Supporting Data

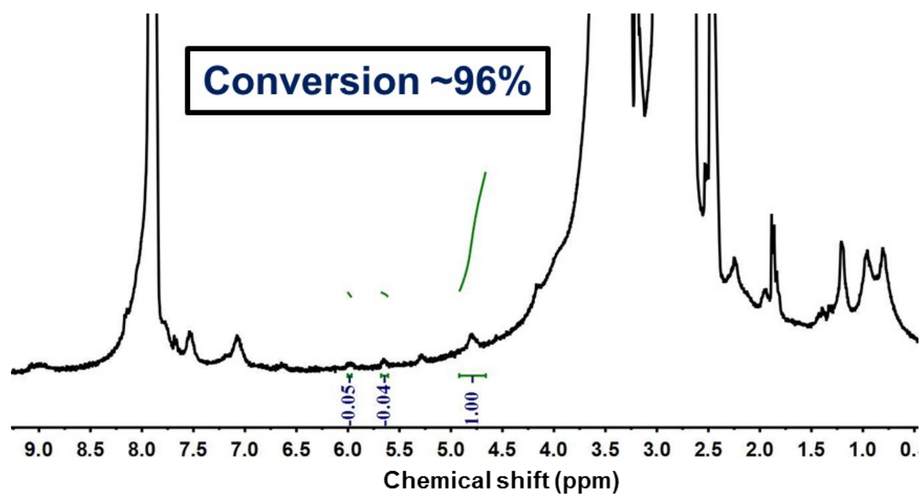


Figure S1. ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of crude **P1** after radical polymerization for calculating conversion.

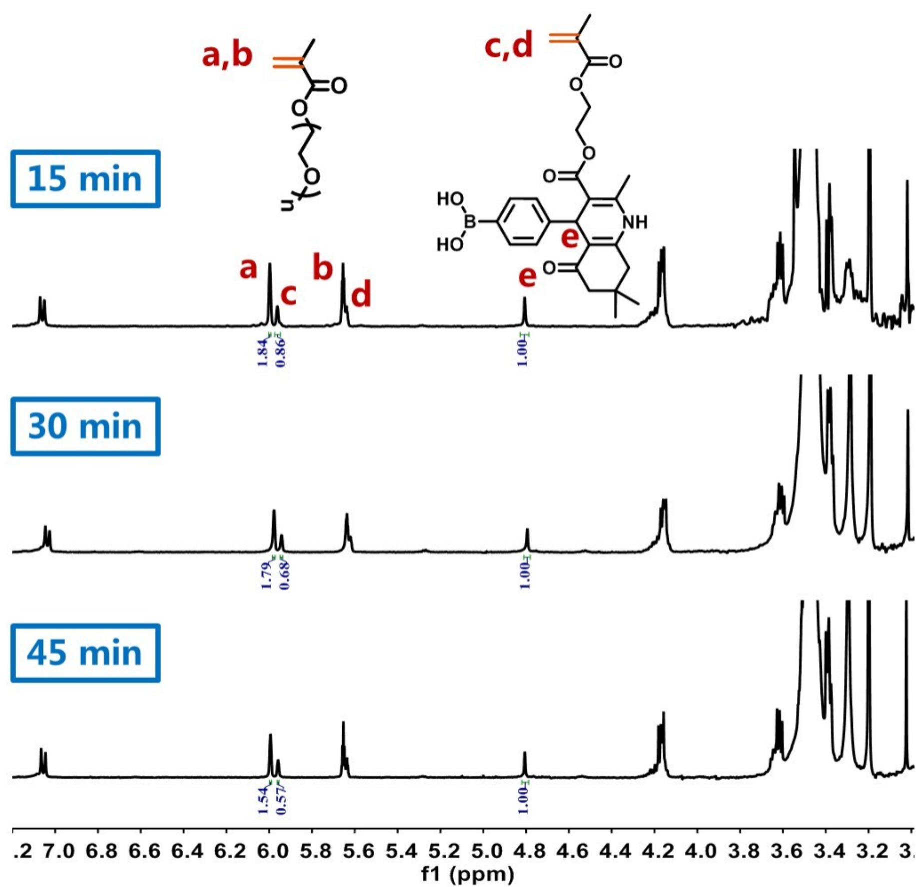


Figure S2. ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of crude **P1** after 15/30/45 minutes radical polymerization for calculating conversion.

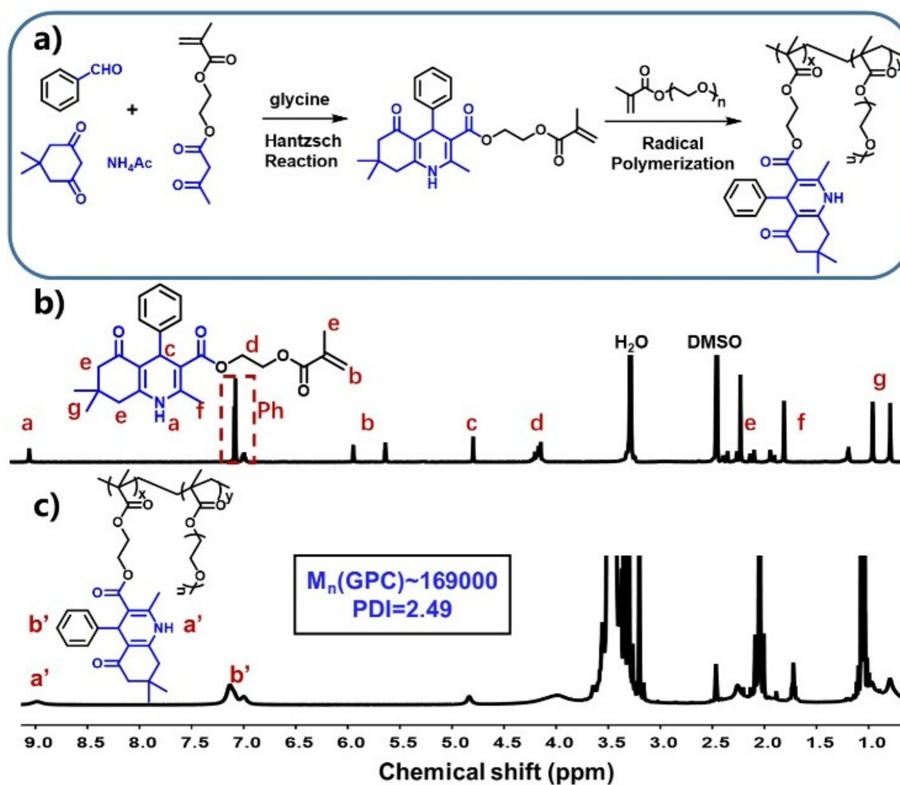


Figure S3. a) Preparation of **P2** by Hantzsch-radical polymerization, [AEMA]/[dimedone]/[benzaldehyde]/[ammonium acetate]/[glycine] = 100/100/100/150/10, [AEMA] = 2.0 M in 1.0 mL of CH_3CN, 70 °C, 4 h; [**M2**]/[PEGMA]/[ABVN] = 100/200/6, [**M2**] = 0.4 M in 1.0 mL of DMF, 70 °C, 8 h; b) ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of the monomer; c) ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **P2**.

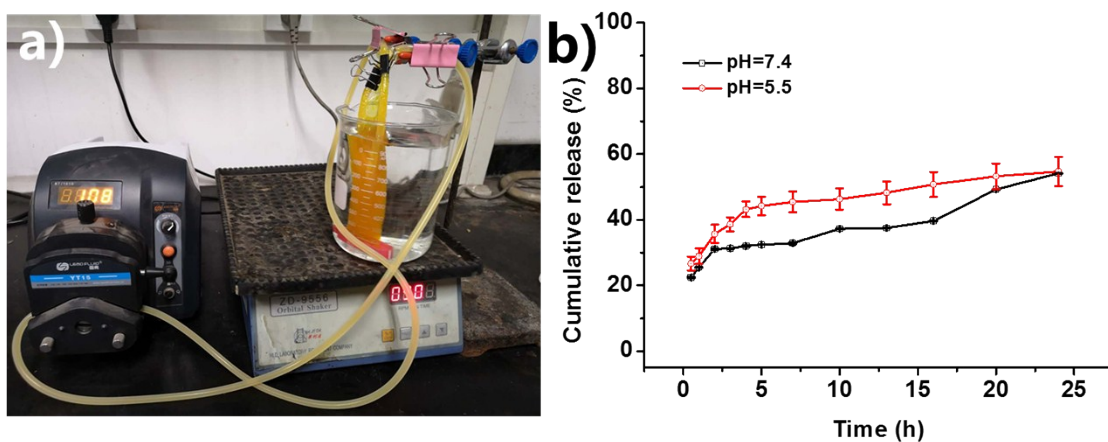


Figure S4. a) Experiment setup of the dynamic release model. b) Cumulative released curcumin versus time at pH 7.4 (black) and pH 5.5 (red).

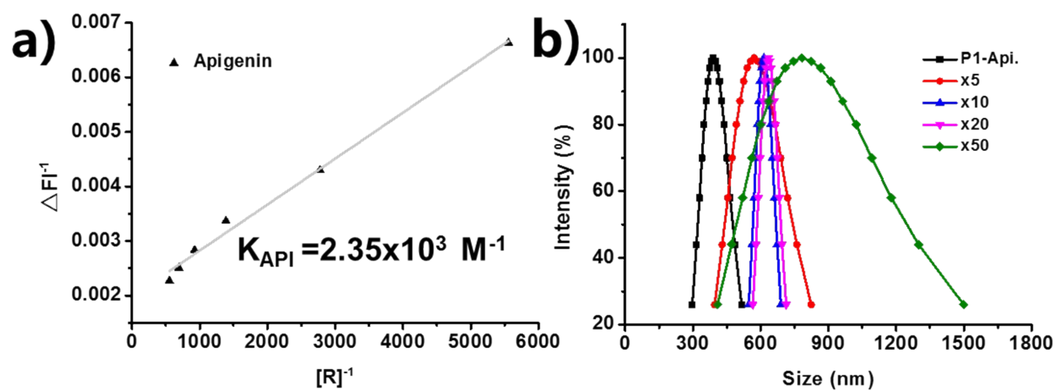
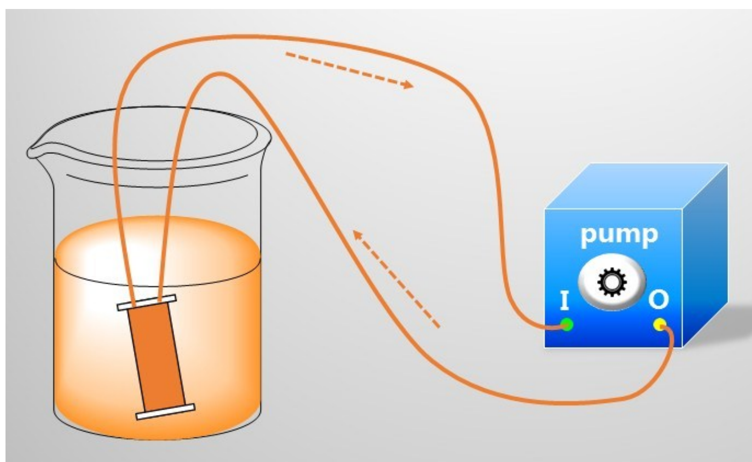


Figure S5. a) ΔFI^{-1} vs $[R]^{-1}$ plot to calculate the binding constants between **MA** and apigenin under neutral pH (~7.4); b) DLS results of the multiple dilution experiments of **P1**-apigenin.



Scheme S1. The dynamic release system for polymer-curcumin conjugate.

Table S1 Properties of the as-synthesized copolymers.

Copolymer	**Conversion (%)**[a]	**Ratio of Monomer : PEGMA**[b]	$\mathbf{M_n}$**(GPC)**[c]	**PDI**[d]
P1	96.0	1.95:1	156000	2.07
P2	93.0	1.91:1	169000	2.49

a. Polymerization conversion of monomers, calculated by ^{1}H-NMR (DMSO-d_6, 400 MHz).

b. Polydispersity index, measured by GPC using dimethylformamide (DMF) as eluent (1 mL/min).

Table S2 Binding constants of MA-cur. and P1-cur..

Ratio of Borate: Curcumin	△FI MA-Cur.	△FI P1-Cur.
20:1	3295.7	3482.4
40:1	3598.2	3811.6
80:1	3744.9	3930.5
120:1	3844.3	4081.2
160:1	3891.6	4100.5
200:1	3926.7	4139.1
K[a]	**2.64×10^4**	**2.68×10^4**

a. Binding constants of MA-cur. and P1-cur..

References

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