Supporting Information (SI)

Bottlebrush polymer with dual functionality for osteoarthritis treatment: curcumin delivery and lubrication properties

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1. Experimental section

4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPTPA), 2,2′-azobis(2-methylpropionitrile) (AIBN, 98 %), methacryloyl chloride (MAC, 97%), triethylamine (TEA, 99.5%), 4-aminophenylboronic acid pinacol ester (4-APBPE, 97%), and 2,2'-bipyridyl (bpy, 98%) were purchased from Sigma Aldrich and used as received. Methyl methacrylate (MMA, 98%) and 2-hydroxyethyl methacrylate (HEMA, 99%) were obtained from Sigma Aldrich and purified from inhibitors by distillation under reduced pressure. 2-Methacryloyloxyethyl phosphorylcholine (MPC, 97%) was also received from Sigma Aldrich and used without further purification. The monomers including 2-(2-bromoisobutyryloxy)ethyl methacrylate (BIBEMA), and N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide (TMDBPMA) were synthesized according to recent literature [1, 2]. All solvents including, methanol, diethyl ether, acetonitrile, n-hexane, dichloromethane (DCM), and N,N-dimethylformamide (DMF) were purchased from Pure POCH.

β-nicotinamide adenine dinucleotide sodium salt, iodonitrotetrazolium chloride (INT), lithium L-lactate, and Tris base for LDH reagent were purchased from Sigma Aldrich (Saint-Louis, MO, United States). Triton X-100 BioXtra was also received from Sigma Aldrich. Phenylmethylsulfonyl fluoride (PMS) was obtained from BioBasic Canada Inc. (Markham, ON, Canada). MTS reagent was obtained from Abcam. DMEM, with or without phenol red, and fetal bovine serum were purchased from Wisent Inc. (Saint-Jean-Baptiste, QC, Canada). Trypsine-EDTA and Penicillin-Streptomycin-Glutamine were purchased from Invitrogen and Gibco (Carlsbad, CA, United States).

1.1. Instrumentation

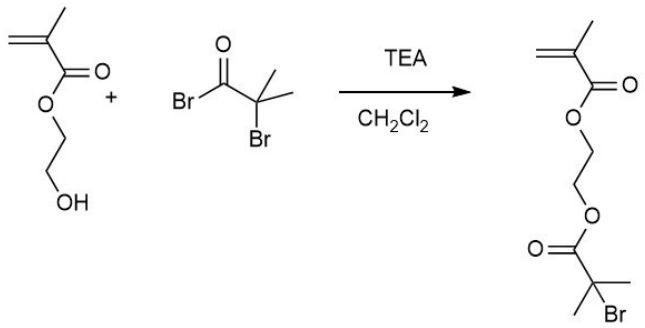
Fourier transform infrared spectroscopy (FTIR) was used for the characterization of the chemical structure and determination of functional groups of the samples. The measurements were performed in the region of 500-4000 cm^{-1} with 32 scans using an FTIR Nicolet 6700 spectrophotometer and OMNIC 3.2 software (Thermo Scientific Products: Riviera Beach, FL, USA). The ATR accessory equipped with a single reflection diamond ATR crystal on ZnSe plate was used for all the analyses. Hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to determine the chemical structure of the synthesized compounds, as well as monomer conversion during the polymerization reactions. The ^{1}H NMR spectra were also used and recorded on a Bruker Advance DPX 700 MHz instrument using chloroform-d[3]

($CDCl_3$), and methanol-d^4 (CD_3OD), as the analysis solvents. The GPC measurements were performed with a Wyatt (Wyatt, Dernbach, Germany) instrument equipped with two Perfect Separation Solutions (PSS) columns and one guard column (GRAM Linear (10 μm, M_n between 800 and 1,000,000 Da)), differential refractometer (RI) and light scattering (LS) detectors. The measurements were performed in DMF as eluent, containing 50 mmol LiBr, at a flow rate of 1 mL/min. Poly(methyl methacrylate) (PMMA) standards (M_n = between 602 and 2,200,000 Da) were used. The average hydrodynamic diameter of the synthesized polymers was measured by dynamic light scattering (DLS) using a Zetasizer NanoZS90 instrument at 25 °C. The dispersions of polymer samples were diluted in Water.

1.2. Synthesis of monomers

1.2.1. Synthesis of 2-(2-bromoisobutyryloxy)ethyl methacrylate (BIBEMA)

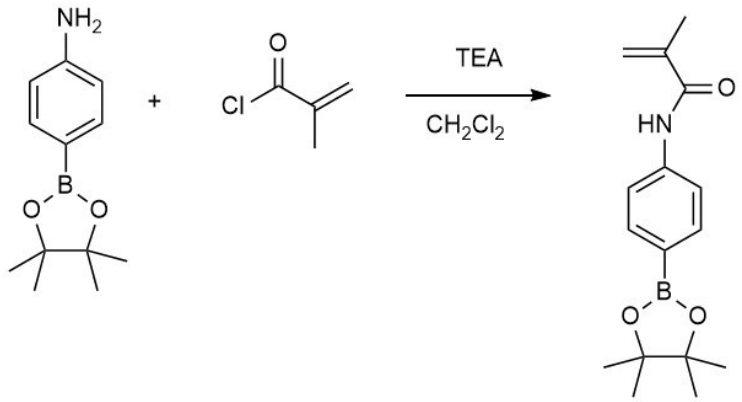
In a dry 100 mL flask, 2-hydroxyethyl methacrylate (HEMA) (5.0 mL, 41.2 mmol), triethylamine (TEA) (6.95 mL, 50 mmol) and dichloromethane (50 mL) were charged under argon atmosphere. The flask was placed in an ice bath for 45 min and a solution of 2-bromoisobutyryl bromide (10.9 g, 47.4 mmol) in 10 mL of dry dichloromethane was added dropwise. The mixture was stirred for 1 h and then at room temperature overnight. The mixture was filtered, and the filtrate was washed with de-ionized water (2 × 50 mL), 0.5 M $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The organic layers were dried with Na_2SO_4, and the solvent was removed by a rotary evaporator under vacuum conditions. The crude product was purified with column chromatography in n-hexan/ethyl acetate (9/1) to yield the product as a slightly yellow liquid [1].



Scheme 1: Synthesis of 2-(2-bromoisobutyryloxy)ethyl methacrylate (BIBEMA)

1.2.2. Synthesis of N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide (TMDBPMA)

In a three-necked round bottom flask, 4-aminophenylboronic acid pinacol ester (20.0 mmol, 4.381 g) was dissolved in dichloromethane (160 mL) and then TEA (5,57 mL, 40.0 mmol) was added to the mixture. The mixture was kept at 0 ^{o}C for 45 min and methacryloyl chloride (2.44 mL, 25.0 mmol) was added dropwise and the resulting solution was stirred at room temperature overnight. Then the reaction mixture was poured into saturated ammonium chloride and the aqueous layer was extracted with dichloromethane (3×15 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2; 20% EtOAc in n-hexane) to obtain monomer [2].



Scheme 2: Synthesis of N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide (TMDBPMA)

1.3. Synthesis of bottlebrush polymer

1.3.1. Preparation of mono-block poly(N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide)-co-poly(methyl methacrylate)-ended chain transfer agent, (PTMDBPMA-co-PMMA)-CTA, by RAFT polymerization

CPBDT (13.28 mg, 0.06 mmol), MMA (0.962 mL, 9.0 mmol), TMDBPMA (2.58 g, 9.0 mmol), and DMF (3.0 mL) were charged in a dry Schlenk flask. AIBN as an initiator (1.48 mg, 0.009 mmol) was added to the flask (taken from a stock solution of DMF). The resulting solution was degassed for 45 min via argon gas and the polymerization reaction was initiated by immersing the flask in a preheated oil bath at 70 °C. The polymerization progress was monitored by ^{1}H NMR, and the reaction mixture was terminated after reaching the desired degree of polymerization by exposing the reaction to air. Finally, the reaction mixture was precipitated three times in cold methanol and re-dissolved in THF to obtain the polymer. The purified polymer, PTMDBPMA-*co*-PMMA-CTA, was dried under vacuum conditions at 30 ºC overnight.

1.3.2. Preparation of di-block poly(N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide)-co-poly(methyl methacrylate)-b-poly(2-(2-bromoisobutyryloxy)ethyl methacrylate)-co-poly(methyl methacrylate), (PTMDBPMA-co-PMMA)-b-(PBIBEMA-co-PMMA), by RAFT polymerization

In a dry Schlenk flask, PTMDBPMA-*co*-PMMA-CTA (0.012 mmol) was dissolved in DMF (4 mL). Then, MMA (0.64 mL, 6 mmol), and BIBEMA (1.28 mL, 6 mmol) were added into the flask. After adding AIBN as an initiator (0.2 mg, 0.6 mmol), taken from a stock solution of DMF into the flask, and the flask was degassed for 45 min via argon gas. Subsequently, the polymerization was initiated by immersing the flask in a preheated oil bath at 70 °C. The obtained polymer was precipitated three times in cold methanol to obtain the block copolymer. The resulting polymer, di-block (PTMDBPMA-*co*-PMMA)-*b*-(PBIBEMA-*co*-PMMA), was dried under vacuum conditions at 30 ºC overnight.

1.3.3. Preparation of poly(N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methacrylamide)-co-poly(methyl methacrylate)-b-((poly(2-(2-bromoisobutyryloxy)ethyl methacrylate)-co-poly(methyl methacrylate))-g-poly(2-methacryloyloxyethyl phosphorylcholine)), (PTMDBPMA-co-PMMA)-b-((PBIBEMA-co-PMMA)-g-PMPC), by ATRP polymerization

A dry 5 mL Schlenk flask was charged with (PTMDBPMA-*co*-PMMA)-*b*-(PBIBEMA-*co*-PMMA) (0.018 mmol), MPC (2.25 mmol), bpy (0.0612 mmol), and copper (II) chloride (0.0036 mmol), methanol (2.5 mL), and acetonitrile (2.5 mL). The solution was degassed by three freeze-pump-thaw cycles. During the final cycle, the flask was filled with nitrogen, and CuCl (2.67 mg, 0.027 mmol) was quickly added to the frozen reaction mixture. The flask was sealed, evacuated, and backfilled with nitrogen five times, and then immersed in an oil bath at 60 °C. The reaction was stopped by exposing it to the air after reaching the desired monomer conversions. The resulting bottlebrush polymers were purified by dialysis (pore size molar mass cut off 50,000 Da) against methanol for 4 days to remove all unreacted monomers and copper catalysts. Finally, to obtain bottlebrush polymers in an aqueous solution, the polymer solution was dialyzed against deionized water for an additional three days. The resulting solution was freeze-dried.

The brush without anchoring side was prepared in an analogous way, using CPBDT as CTA for the synthesis of (PBIBEMA-*co*-PMMA), followed by MPC polymerization as side chains (data not shown).

1.3.4. Preparation poly(4-(methyl)propenamidophenylboronic acid)-co-poly(methyl methacrylate)-b-poly(2-(2-bromoisobutyryloxy)ethyl methacrylate)-g-poly2-Methacryloyloxyethyl phosphorylcholine)-co-poly(methyl methacrylate), (PTMDBPMA-co-PMMA)-b-((PBIBEMA-co-PMMA)-g-PMPC), (PBAPMA-co-PMMA)-b-((PBIBEMA-co-PMMA)-g-PMPC) by hydrolysis of pinacol borate ester under acid condition

In a 10 mL round bottom flask, 50 mg of (PTMDBPMA-*co*-PMMA)-*b*-((PBIBEMA-*co*-PMMA)-*g*-PMPC) was dissolved in 5 mL HCl solution (0.5 M). The mixture was stirred at room temperature overnight. The resulting solution was purified by dialysis (pore size molar mass cut off 50,000 Da) against water for two days. The resulting solution was freeze-dried, and the obtained powders were stored under an inert atmosphere for further *in vitro* studies.

1.4. *In vitro* drug loading study

An appropriate amount of bottlebrush polymer was dissolved in methanol and then curcumin (4 mol/mol boronic acid group) was added to the solution. The mixture was stirred at room temperature under dark conditions for 24 hrs. Then the mixture was dialyzed against methanol for two days to remove unloaded curcumin. Afterward, the mixture was dialyzed against deionized water for two days to exchange methanol with water. The resulting curcumin-loaded aqueous solution was freeze-dried and used for the calculation of curcumin loading efficiency and *in vitro* studies.

To calculate the curcumin loading efficiency (LE), the appropriate amount of curcumin-loaded bottlebrush polymer was dissolved in 0.5 M HCl solution with a polymer concentration of (1 mg/mL) and stirred overnight. The mixture was centrifuged at 1200 rpm for 10 min and the pellet was collected for measuring the loading efficiency. The pellet was dissolved in methanol and the absorbance was measured at a wavelength of 427 nm by UV–Vis spectrophotometer. The absorbance was converted to concentration by interpolating the absorbance of curcumin to their calibration curves. Finally, the concentration was converted to the mass of curcumin. The following formula was used for the calculation of the drug loading efficiency.

$$Drug\ Loading\ Efficiency\% = \frac{mass\ of\ loaded\ curcumin\ in\ the\ bottlebrush}{mass\ of\ bottlebrush} \times 100$$

1.5. *In vitro* drug release study

In vitro drug release profile of curcumin from the bottlebrush polymer was done by direct dispersion method in the releasing buffer (PBS, pH ~ 6.0 and 7.4). The release study was performed for four weeks, and the samples were analyzed at the end of every week. A known quantity of curcumin-loaded bottlebrush polymer was dissolved in an appropriate volume of PBS solution (pH ~ 6.0 and 7.4). The solution was divided into 4 conical tubes. All tubes were incubated in the water bath at 37 °C. At predetermined time intervals (one week, two weeks, three weeks, and four weeks), one tube was taken and centrifuged at 1200 rpm for 10 min. The pellet was dissolved in methanol and the absorbance was analyzed at 427 nm by UV–Vis spectrophotometer. The following formula was used for the calculation of the drug release rate. The release study was performed in triplicate, and the average value was reported.

$$Drug\ Release\% = \frac{mass\ of\ release\ drug}{mass\ of\ loaded\ drug} \times 100$$

1.6. Morphological study

The synthesized bottlebrush polymer was imaged by atomic force microscopy (AFM; Multimode Dimension 3100). The polymer was dissolved at a concentration of 25 μg/mL and deposited on a freshly cleaved mica surface. The polymer was left to adsorb, and the supernatant was rinsed three times to isolate polymer single chains. The surface was dried before AFM measurements. The AFM equipped with a nanoscope VIII controller (Digital Instruments) was set on the peak force QNM mode. The Scanasyst-air tips were used for AFM imaging. Images of the bottlebrushes were analyzed using ImageJ software.

1.7. Tribological study

1.7.1. Friction test equipment

A Surface Forces Apparatus model 2000 (SFA 2000, SurForce, LLC, U.S.A.) was used for the experiment equipped with a spectrometer and digital camera (Andor Technology, U.S.A.), a function/arbitrary waveform generator (Agilent 33250A, Agilent Technologies, Inc., USA) to drive the bimorph slider and a signal conditioning amplifier (Vishay Measurements, 2310B) to recover the signal from the friction sensor.

1.7.2. Experiment preparation

The experiment was based on protocols reported before [3]. Two smooth mica surfaces with the same thickness coated with a 55 nm thick silver coating were glued (silver side down), on cylindrical glass disks with 2 cm curvature using an epoxy glue (Epon 1004F). The disks were

positioned in SFA, and the thickness of the mica surfaces was measured by bringing them into contact in dry air. Next, surfaces were separated and a polymer solution of concentration 40 ug/mL in pure water was injected between them and let to rest for 1 hour of adsorption. To limit the evaporation a small reservoir with water was placed inside SFA chamber.

1.7.3. Friction test

Friction force was measured at different normal loads ranging from 0.2 to 5 mN. At every load at least 3 shearing cycles were applied. The experiments were performed with a frequency of 50 mHz and a 5 V amplitude (corresponding to 10 μm amplitude).

During the measurement, a white light beam was transmitted through the surfaces which allowed the fringes of equal chromatic order to be recorded through a spectrometer equipped with a digital camera [4]. Based on this data, the change in distance between the surfaces during the measurement was continuously recorded.

1.8. Cytotoxicity Assays

Human chondrocytes from our biobank cells (Dre Florina Moldovan, CHU Sainte-Justine, Montreal, QC, Canada, #2252) were isolated from patients undergoing knee surgery [5, 6]. Chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and L-glutamine (PSG). Cells were seeded in culture flasks within three passages at 37°C in a humidified incubator with 5% CO_2 until they reached approximately 70% confluency.

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and lactate dehydrogenase (LDH) colorimetric assays were used to measure the polymers' cytotoxicity. Cells were seeded at the density of 3.3 x 10^4 cells per well into 96-well plates in quadruplicate. The culture media was changed after 24 hours, and cells were incubated with corresponding treatment in DMEM media supplemented with 2.5% FBS and 1% PSG. Cells were treated with 1, 2, 8, 16, 31, 62.5, 125, 250, 500, or 1000 μg/mL of each polymer solubilized in DMEM for 24h, 48h, or 72h. These two viability assays were completed as previously described [7]. Optical densities (OD) were measured at 490 nm and 680 nm using a microplate reader (CLARIOstar Plus, BMG LABTECH GmbH, Ortenberg, Germany). Untreated cells were the negative control, while cells treated with Triton X-100 0.1% (v/v) were the positive control. Three independent experiments were performed.

1.9. Statistical Analysis

The data were analyzed using GraphPad® Prism statistical software (version 9.0.0, GraphPad® Software, San Diego, CA, USA) and are expressed as means and standard deviations (SDs). One-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons tests, was conducted to investigate the MTS assay. Two-way ANOVA, followed by Tukey's multiple comparisons tests, was used to compare paired samples of each polymer over time. To compare the two polymers, a Student's t-test was applied. One-way ANOVA, followed by Dunnett's multiple comparisons tests, was performed to analyze the LDH assay. Values of $p < 0.05$ were considered significant for all comparisons.

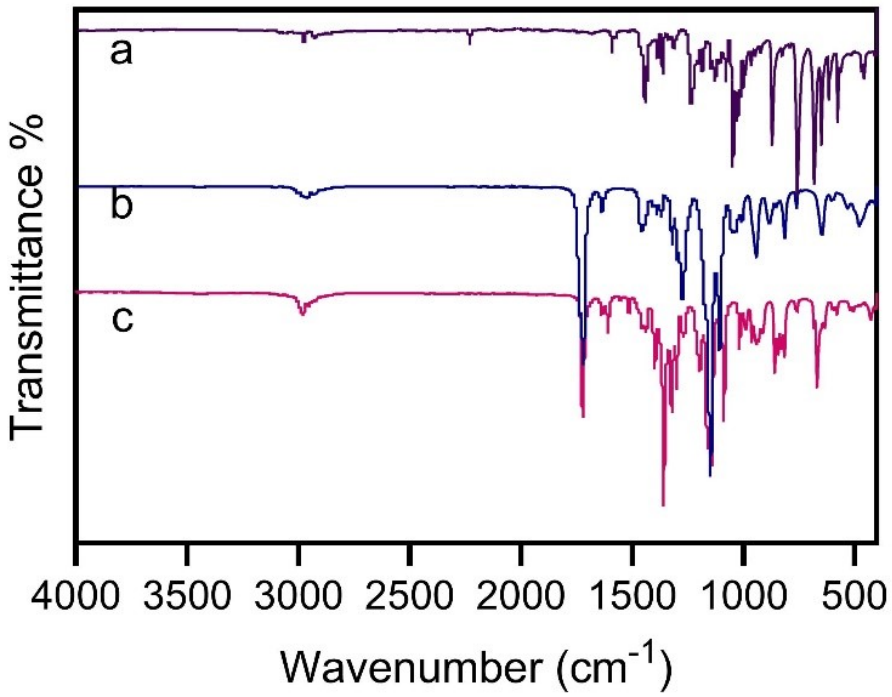


Figure S1: The FTIR spectra of CPBDT (a), BIBEMA (b) and TMDBPMA (c) in the wavenumber ranges of 400–4000 cm^{-1}.

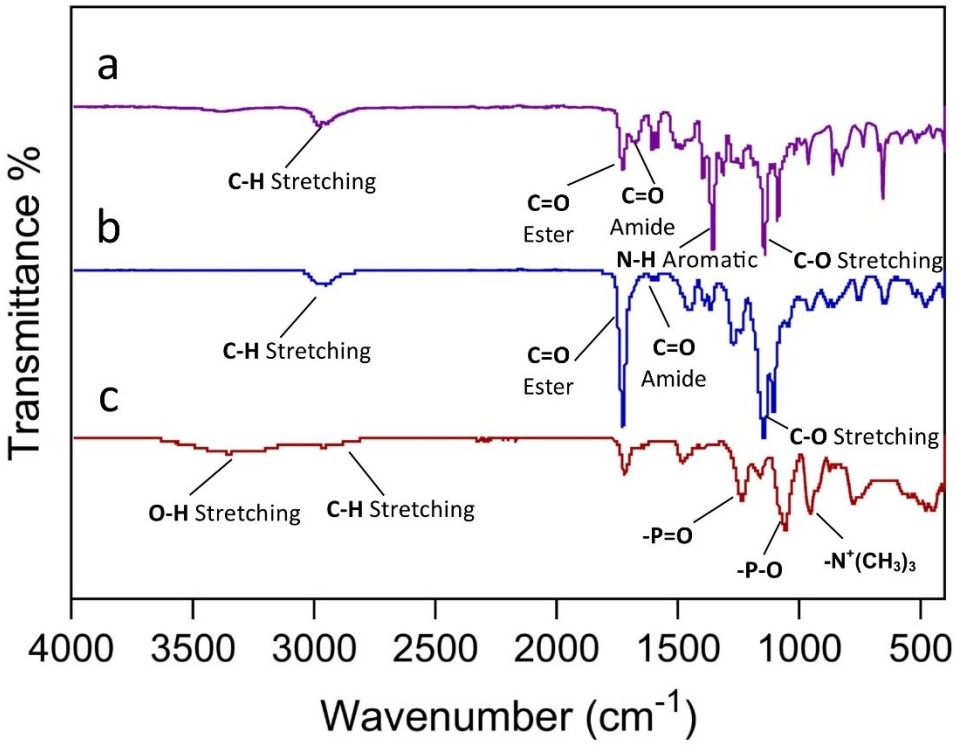


Figure S2: The FTIR spectra of (PTMDBPMA$_{52}$-*co*-MMA$_{56}$), (PTMDBPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*co*-PMMA$_{230}$), and (PBAPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*g*-PMPC$_{50}$-*co*-PMMA$_{230}$) in the wavenumber ranges of 400–4000 cm^{-1}.

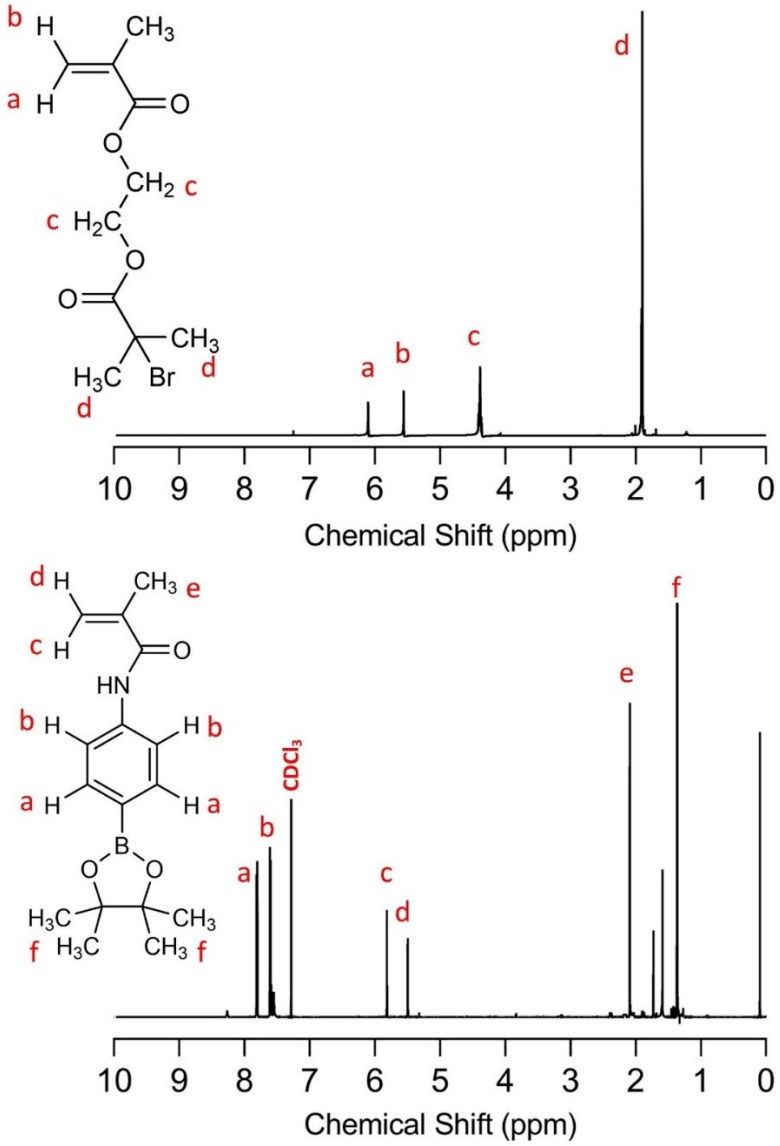


Figure S3: The ^{1}H NMR spectra of the of TMDBPMA and BIBEMA in chloroform-d4

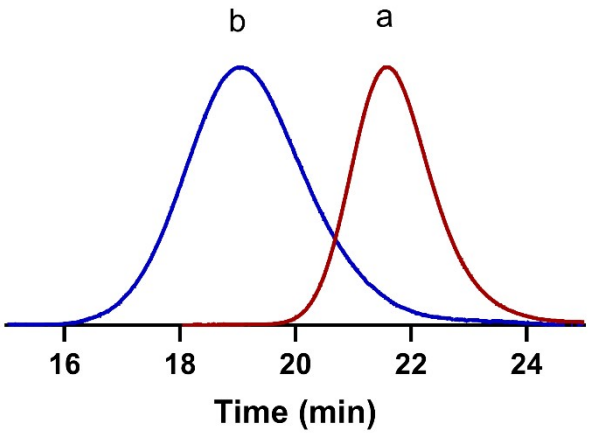


Figure S4: GPC traces of mono-block (PTMDBPMA$_{52}$-*co*-PMMA$_{56}$) copolymer (a) and di-block (PTMDBPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*co*-PMMA$_{230}$) copolymer (b)

Table S1. GPC and ^{1}H NMR characterization of mono-block PTMDBPMA$_{52}$-*co*-PMMA$_{56}$ and di-block (PTMDBPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*co*-PMMA$_{230}$) copolymers

Sample	Mono-block Composition (DP) [a]		Di-block Composition (DP) [a]		$M_{n\ theor}$ [a]	M_n [b]	$Đ$ [b]
	PAPBPEMA [c]	PMMA	PBIBEMA	PMMA			
PTMDBPMA-*co*-PMMA	52	56	-	-	20,500	13,620	1.33
(PTMDBPMA-*co*-PMMA)-*b*-(PBIBEMA-*co*-PMMA)	52	56	230	237	106,000	83,380	1.40

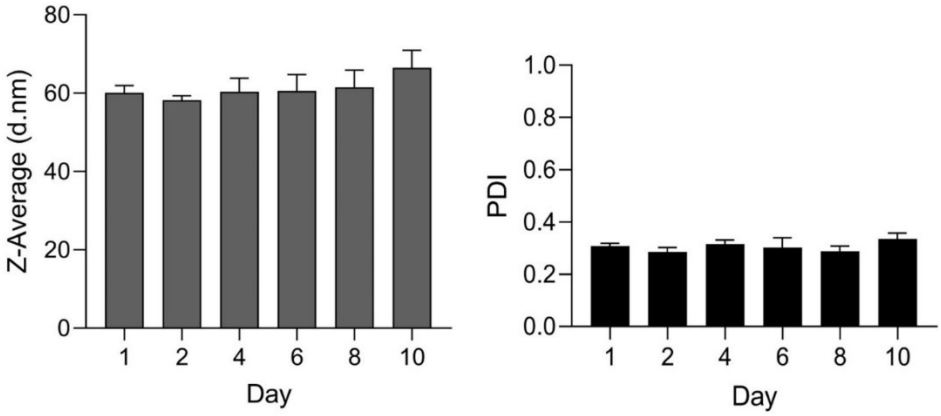


Figure S5: The hydrodynamic diameter (D_h) of (PBAPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*g*-PMPC$_{50}$-*co*-PMMA$_{230}$) determined by dynamic light scattering in water at 25 ºC.

Table 2. The ^{1}H NMR and DLS characterization of bottlebrush polymers with PMPC side chains.

Samples	PMPC DP [a]	$M_{n\ theor.}$ [a] (g/mol)	Diameter, PDI [b]
(PBAPMA$_{52}$-*co*-PMMA$_{56}$)-*b*-(PBIBEMA$_{237}$-*g*-PMPC$_{50}$-*co*-PMMA$_{230}$)	50	3,604,850	60.11± 5.19 nm, 0.302 ± 0.03

[a] Theoretical molecular weight based on monomer conversion calculated from ^{1}H NMR results.

[b] Determined by DLS in water in the concentration of 5 mg/mL right away after solution preparation.

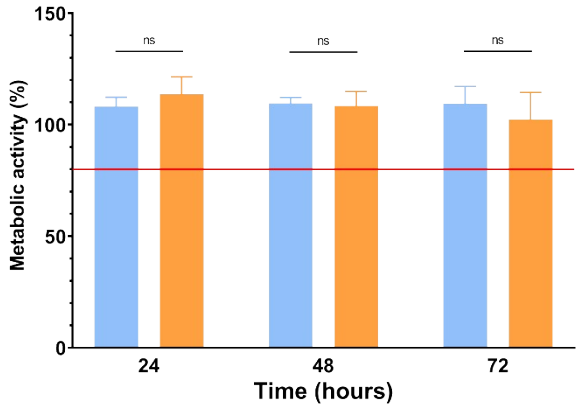


Figure S6. Comparison of metabolic activities of chondrocytes treated with 1 mg/mL of di-block bottlebrush polymer (in blue) or curcumin-loaded bottlebrush polymer (in orange) over time. Metabolic activity was assessed by MTS assay and was calculated as follows: $(OD_{sample}/OD_{negative\ control}) \times 100$. The red line represents the limit of cell biocompatibility (80%). The values presented (mean ± SD) are from three independent experiments (n=3) in quadruplicate (ns: nonsignificant).

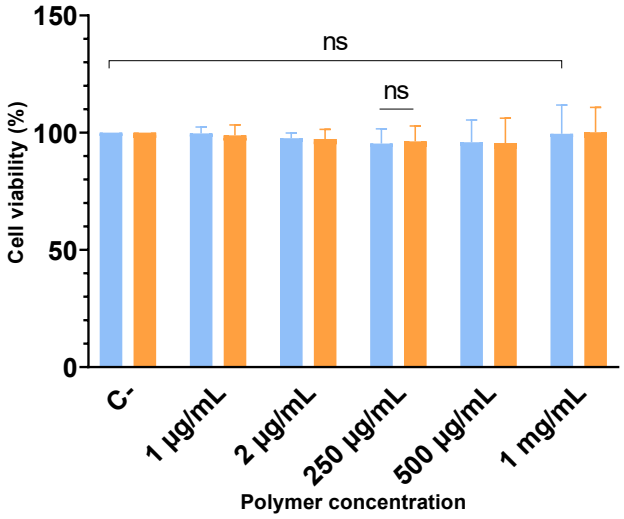


Figure S7. Cell viability of chondrocytes treated with di-block bottlebrush polymer (in blue) and curcumin-loaded bottlebrush polymer (in orange) for 72 hours. Cell viability was assessed by LDH assay and was calculated as follows: $100-((OD_{sample}-OD_{negative\ control})/(OD_{positive\ control}-OD_{negative\ control}) \times 100$. From the formula, the results obtained for chondrocytes without treatment are considered spontaneous LDH activity (0%), and those for chondrocytes treated with Triton X-100 are maximum LDH activity (100%). The values presented (mean ± SD) are from three independent experiments (n=3) in quadruplicate (ns: nonsignificant).

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