Supplementary Information

Cyto-friendly polymerization at cell surface modulates cell fate by clustering cell-surface receptor

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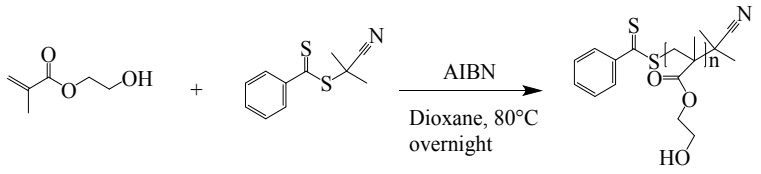
S1 General information

2-Hydroxyethyl methacrylate (HEMA), 2-cyano-2-propyl benzodithioate (CTA), Roswell Park Memorial Institute-1640 (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (USA). Azodiisobutyronitrile (AIBN), meth acryloyl chloride, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), ferrocenecarboxylic acid and β-Cyclodextrin (β-CD) were provided by Aladdin (Shanghai, China). Mal-PEG_3-Mal was obtained from ToYang Bio Tech. Inc. (Shanghai, China). TE buffer was purchased from Solarbio (Beijing, China). Cell Counting Kit-8, Annexin V-FITC/PI Apoptosis Detection Kit, Cell Lysis Buffer, DiI, Horseradish Peroxidase-labeled Goat Anti-Rabbit IgG, Fluo-4 AM, PP2, EGTA, JC-1 and Caspase 3 Activity Assay Kit were purchased from Beyotime (Shanghai, China). The anti-CD20 aptamer (sequence: 5'-TGC GTG TGT AGT GTG TCT GTT TTT TAT CTT CTT TTA TCT ACT CTT AGG GAT TTG GGC GG-3') was synthesized at TSINGKE Biological Technology (Hangzhou, China). Anti-CD20 and β-actin antibodies were brought from Abcam (UK). All solvents used in the research were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

S2 Synthesis and characterization of macromers

Synthesis and characterization of pHEMA 1



pHEMA 1

pHEMA was synthesized by RAFT polymerization of hydroxyethyl methacrylate (HEMA) initiated by AIBN. Firstly, HEMA was passed through a chromatography column filled with neutral alumina to remove the stabilizer (MEHQ). In a typical experiment, a mixture of AIBN (0.0189g, 0.115mmol), CTA (0.127g, 0.575mmol), HEMA (2.85mL, 23mmol) and 7.7mL of dioxane were charged into a 25-mL three-necked flask equipped with a magnetic stirrer. Nitrogen was pumped in and out for 30min to remove the oxygen from the system. Then the flask containing the reaction mixture was immersed into an oil bath which had been preheated to 80°C and allowed to polymerize overnight under the protection of nitrogen. After that, the polymerization was quenched by immersing the flask into liquid nitrogen. Subsequently, a 10-fold excess volume of pre-cooled ether was added into the reaction mixture. The precipitate was collected via centrifuge (4000rpm, 10min) and washed thrice with ether. The resulting product was dried in a vacuum oven at 50°C for 24h to give the final product, pHEMA 1. pHEMA 1 was dissolved in DMSO-*d6* at a concentration of 20mg/mL and analyzed by an NMR spectrometer (AC-80, BrukerBioSpin, Germany). The SEC trace of pHEMA 1 was determined with DMSO as an eluent. PLgel MIXED-C column (particle size: 5μm; dimensions: 7.5mm×300mm) was employed with a differential refractive index detector. The flow rate was at 0.6mL/min.

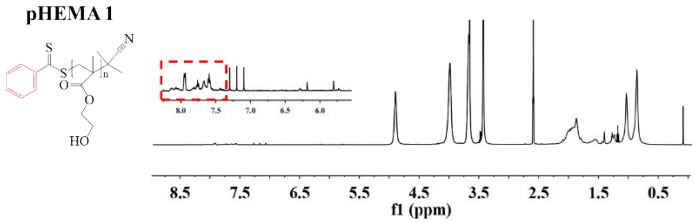
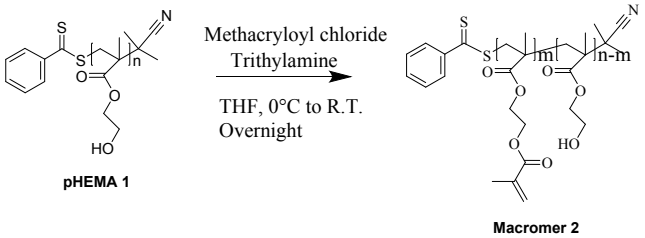


Figure S1 The proton spectrum of pHEMA 1.

Synthesis and characterization of Macromer 2



The methacryloyl chloride was used to react with the hydroxyl groups in pHEMA 1 to obtain the macromer 2. The molar ratio of pHEMA 1: meth acryloyl chloride was at 1:6. Briefly, 400mg of pHEMA 1 was dissolved in 24mL of distilled THF. The solution was transferred into a 100-mL dry three-necked flask equipped with a magnetic stirrer, followed by the addition of trimethylamine (48.4mg). Subsequently, 50mg of methacryloyl chloride dissolved in 2 mL of distilled THF was added dropwise under an ice-bath. One hour later, the ice-bath was removed, and the resulting solution was stirred overnight at room temperature followed by dialysis against deionized water (MWCO: 3.5kDa) for 48h, to give macromer 2a. Macromer 2b and 2c were obtained by adjusting the feed ratio of methacryloyl chloride to 1:20 and 1:30. The proton NMR was performed following the identical procedures as described in the section of pHEMA 1, to verify the structure, molecular weight as well as the content of double-band.

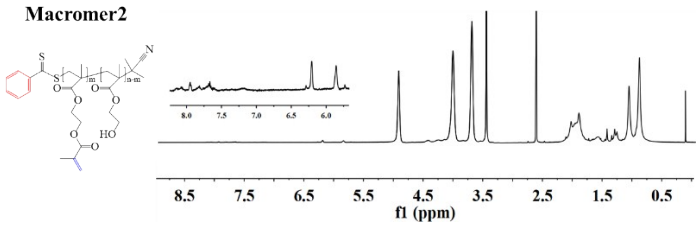


Figure S2 The proton spectrum of macromer 2.

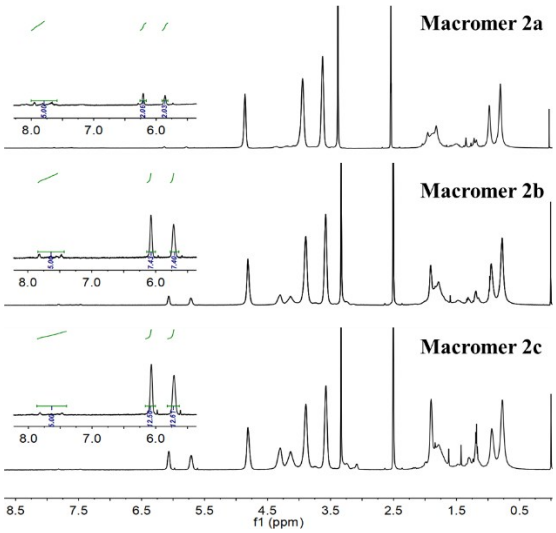
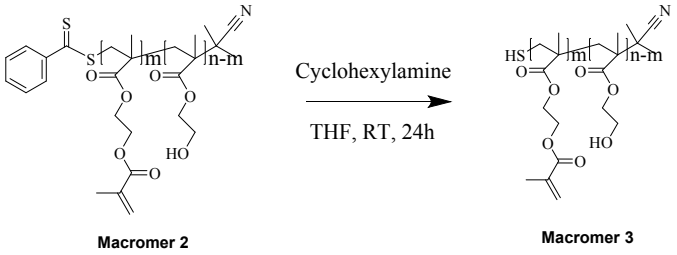


Figure S3 The proton spectra of macromer 2 with various double-bond contents.

Synthesis and characterization of macromer 3



Briefly, 20mg of macromer 2 was weighted into a 10-mL flask equipped with a magnetic stirrer and dissolved in 2mL of distilled THF. 23μL of cyclohexylamine (50-fold excess relative to the thioester bond) was added and the resulting mixture was stirred for 24h under nitrogen protection at room temperature. After that, the product was precipitated in 10-fold excess volume of pre-cooled n-hexane. The precipitate was then washed thrice with n-hexane and dried in a vacuum oven at room temperature for 24h to obtain macromer 3, which was preserved at -20℃ for future usage. The proton NMR was performed following the identical procedures as described in the section of pHEMA 1, to verify the structure.

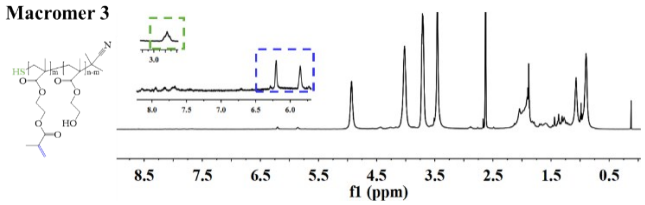
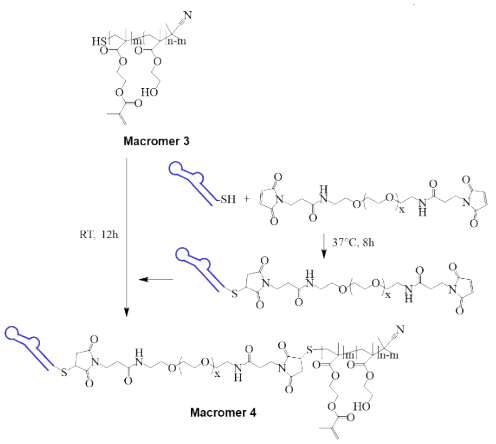


Figure S4 The proton spectra of macromer 3.

Synthesis and characterization of macromer 4



0.02μmol of thiol-ended aptamer dissolved in 1mL of TE buffer was slowly added into 1-mL preheated TE buffer containing 0.1μmol of Mal-PEG_3-Mal at 37℃. Then TCEP (at a final concentration of 1mM) was added to prevent the oxidation of thiol groups. The resulting mixture was stirred for 8 h at 37 ℃ followed by dialysis against deionized water for 24 h to remove any unreacted regents (MWCO: 3.5kDa). Subsequently, the obtained maleamide-functionalized aptamer aqueous solution was mixed with 0.02mmol of macromer 3. The resulting mixture was stirred at room temperature for 12h to afford macromer 4. The size-exclusion chromatography was performed to verify the quantitative reaction, following the identical procedures as describe in section of pHEMA 1. To further demonstrate the successful modification of aptamer to macromer 3, polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. Macromer 3 was labelled by FITC firstly. 250μg of macromer 3 was dissolved in 1mL of deionized water and 39μg of FITC in 1mL of ethanol was added dropwise. The mixture solution was stirred for 14h at room temperature and dialyzed against deionized water for 24h (MWCO: 3.5kDa), followed by lyophilization to obtain FITC-labelled macromer 3- which was subsequently reacted with maleamide-functionalized aptamer to afford FITC-labelled macromer 4. Macromer 3, mixture of aptamer with macromer 3 and macromer 4 were subjected to 20% SDS-PAGE, then electrophoresis was conducted at 100V for 4h under an ice-bath. The resulting gel was observed by Gel Imager (Gel Logic 200, Kodak, USA)

S3 Macromers binding onto the cell surface

Cell culture

Raji and Jurkat cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), streptomycin (0.1mg/mL) and penicillin (100units/mL) at 37°C in a humidified 5%-CO_2 atmosphere. For Bel-7402, A549 and SGC cells, the conditions were the same, except DMEM instead of RPMI-1640 medium was used.

Expression levels of CD20 receptor on tumor cells

CD20-expression levels on Raji, Jurkat, Bel-7402, A549, and SGC tumor cells were determined using western blotting. 5×10^4 tumor cells were lysed in cell lysis buffer.

The obtained cell-lysates were subjected to SDS-PAGE and immunoblotted with anti-CD20 antibody, following the manufactory's instruction. β-actin was used as the reference. The blots were washed and incubated with the Horseradish Peroxidase-labeled Goat Anti-Rabbit IgG for 2h at room temperature, followed by imaging (Chemiluminescence imager, Bio-Rad ChemiDoc XRS+, USA).

Evaluation of binding efficiency of macromer 4 onto Raji cell surface

The selectivity of macromer 4 for Raji cells in a mixed cell population was demonstrated by confocal fluorescent microscope. Raji cells were stained by the cell-membrane dye, DiI, following the manufacture's instruction, to obtain the DiI^+ Raji cells. Then DiI^+ Raji cells were mixed with Jurkat cells in an equal number (5×10^4) in 100μL of FBS-free 1640 medium and seeded in 1.5-mL sterile Eppendorf tubes. FITC-labelled macromer 4 was subsequently added at a final concentration of 0.2μM. After co-incubating for 1h at 37℃ in dark, the cells were collected, washed twice with PBS and fixed by paraformaldehyde. The nucleus was stained by DAPI, followed by an observation *via* Confocal Fluorescent Microscope (Nikon A1R, Japan).

For FACS analysis, Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then 3'-FAM-aptamers or FITC-labelled macromer 4 were added at a final concentration of 0, 0.01, 0.2, 0.5, 1, 2 and 3μM, respectively. After a 1-h-co-incubation at 37℃ in the dark, the cells were harvested and washed twice with PBS. The mean fluorescence intensity (MFI) was measured by FACS (Cytomic FC 500MCL, Beckman, USA). Each treatment had been tested in triplicate. Jurkat cells were taking as the control.

The impact of incubation-time on binding was investigated by confocal fluorescent microscope. Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then 3'-FAM-aptamers or FITC-labelled macromer 4 were added at a final concentration of 0.2μM, respectively. After co-incubating for 1h, 2h, 4h and 8h at 37℃ in the dark, the cells were collected, washed twice with PBS, and stained by a cell-membrane dye, DiI, following the manufacture's instruction. After that, the cells were fixed by paraformaldehyde and observed by Confocal Fluorescent Microscope (Nikon A1R, Japan).

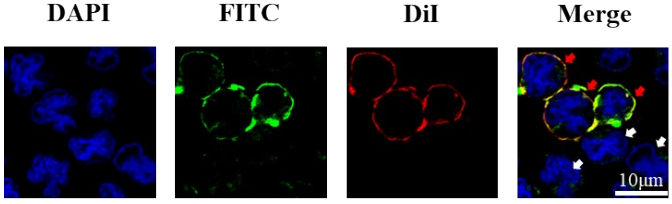


Figure S5. Fluorescent images of the mixed cell population of DiI^+ Raji cells and DiI^- Jurkat cells exposed to FITC-labelled macromer 4 for 1h. Red arrows indicate Raji cells and white arrows indicate Jurkat cells.

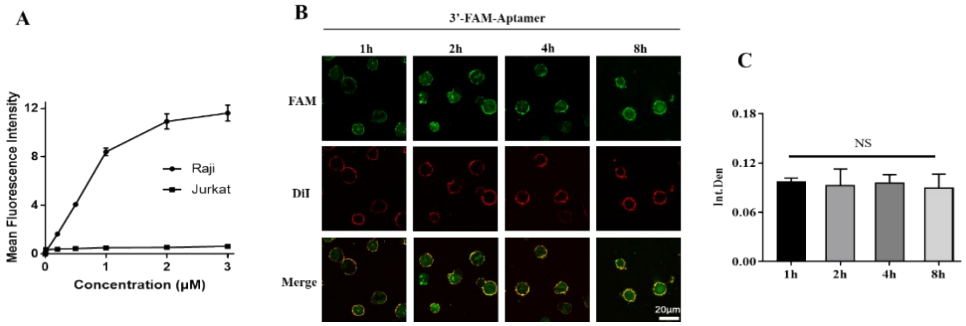


Figure S6 The anti-CD20 aptamer binding onto Raji cells. A) FACS analysis of Raji cells incubated with 3'-FAM-Aptamer at different concentrations; Fluorescent images (B) and semi-quantitative results (C) of Raji cells exposed to 3'-FAM-Aptamer for various time periods, the cell membrane was labeled by DiI.

Quantitation of macromer 4 bound onto Raji cell surface

Ferrocene-tagged macromer 4 was initially synthesized. 450μg of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 270μg of N-Hydroxysuccinimide (NHS) were added into 0.5mL of DMSO containing 92μg of ferrocenecarboxylic acid. The resulting mixture 1 was stirred at room temperature for 1h to activate the carboxyl group of ferrocenecarboxylic acid. Subsequently, 17nmol of macromer 4 was added (the molar ratio of ferrocenecarboxylic acid: macromer 4 was at 20:1) to give mixture 2. The mixture 2 was stirred for an extra 24h, followed by dialysis against deionized water (MWCO: 3.5 kDa). The product ferrocene-tagged macromer 4 was obtained by lyophilization and the tagging efficiency was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (NexION300X, PerkinElmer, USA).

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 500μL per tube. The cell density was fixed at 1×10^6/ml. Then ferrocene-tagged macromer 4 were added at a final concentration of 0, 0.3, 0.5, 1, 2 and 3μM, respectively. After a 1-h- incubation at 37℃ in the dark, the cells were collected, washed twice with PBS, and analyzed by ICP-MS. Each treatment had been tested in triplicate.

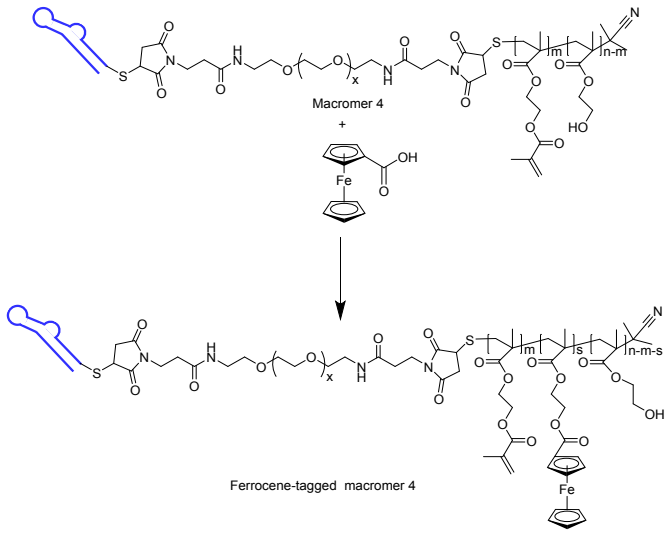


Figure S7 Synthetic scheme of ferrocene-tagged macromer 4.

The polymerized crosslinked polymer was extracted from the cellular milieu

Biotin-tagged macromer 4 was synthesized. First, the macromer 2 was modified by biotin. 35mg of EDC and 23mg of 4-dimethylaminopyridine (DMAP) were added into 4mL of anhydrous DMSO containing 9mg of biotin and the resulting mixture 1 was stirred at room temperature for 4h to activate the carboxyl group of biotin. Subsequently, 20mg of macromer 2 was added (the molar ratio of biotin: macromer 2 was at 10:1) to give mixture 2. The mixture 2 was stirred for an extra 24 h, followed by dialysis against deionized water (MWCO: 3.5kDa). The product biotin-tagged macromer 2 was obtained by lyophilization and the structure was confirmed by proton NMR following the identical procedures as described in the section of pHEMA 1. Then, referring to the synthesis protocols of macromer 3 and 4 mentioned above, the biotin-tagged macromer 2 was reacted with cyclohexylamine to afford biotin-tagged macromer 3, followed by reacting with maleimide-functionalized aptamer to give biotin-tagged macromer 4.

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 500μL per tube. The cell density was fixed at 1×10^6/ml. Then biotin-tagged macromer 4 were added at a final concentration of 2μM. After a 1-h- incubation at 37℃, the cells were collected, washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and lysed using RIPA cell lysis buffer. The polymer was extracted from the cell lysate with streptomycin magnetic beads (M2420, Solarbio, China) according to the manufacturer's protocol. Briefly, the magnetic beads were added to the cell lysate, rotated and mixed for 30min at room temperature to capture the polymers. After that the beads were separated with supernatant by a magnet, the obtained beads were washed three times with water. Then, the beads were resuspended in water and incubated at 40℃ for 10min, the supernatant was collected and repeated this step again. The supernatant was combined and lyophilized. These steps were repeated at

60℃, 80℃ and 97℃ respectively. The supernatant was combined and lyophilized, resuspended in water and analyzed by dynamic light scan.

2μM of biotin-tagged macromer 4 was added to 500μL of water and 5mM of APS was added. After incubating at 37℃ for 30min, the obtained solution was analyzed by dynamic light scan.

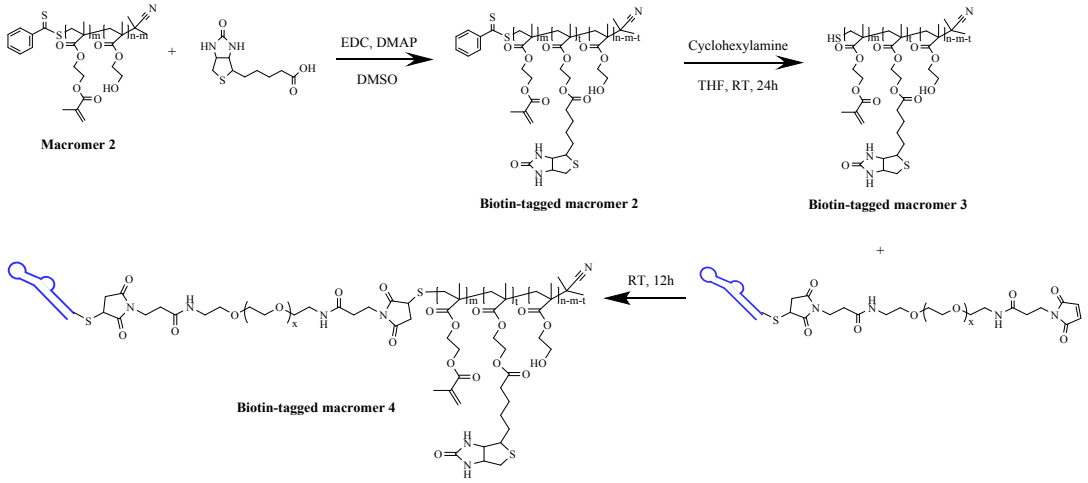


Figure S8 Synthetic scheme of biotin-tagged macromer 4.

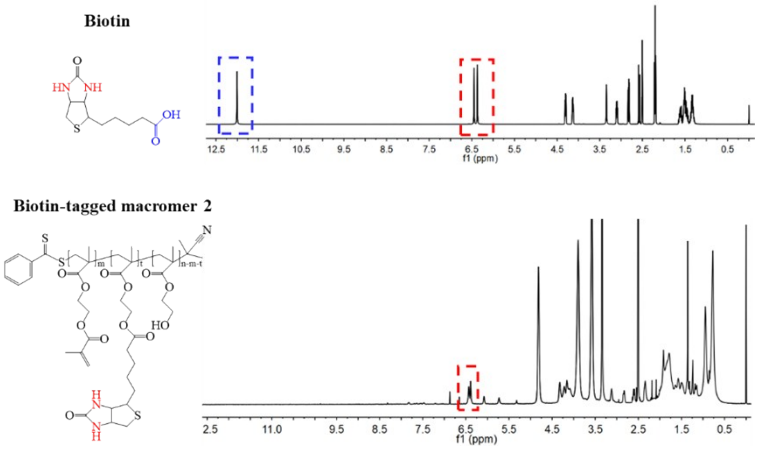


Figure S9 The proton spectra of biotin and biotin-tagged macromer 2.

S4 Polymerization at cell surface

Cytotoxicity of macromer 4

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then macromer 4 was added at a final concentration of 0, 0.5, 1, 2, 3μM, respectively. After a 1h-co-incubation for 1h at 37℃, the cells were washed twice with PBS and seeded in a 96-

well plate. After a 48-h culture, the cell viabilities were measured by CCK-8 assay following the manufacture's instruction. Each treatment was tested in triplicate.

Cytotoxicity of APS

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then APS was added at a final concentration of 0.5, 1.5, 2.5, 3, 5mM, respectively. After a 10-min-co-incubation at 37℃, the cells were washed twice with PBS and seeded in a 96-well plate. After a 48-h culture, the cell viabilities were measured by CCK-8 assay following the manufacture's instruction. Each treatment was tested in triplicate. For the time-course experiment, the protocols were almost identical, except the concentration of APS was fixed at 5mM and the co-incubation time varied from 0 to 30min.

Scanning electron microscope (SEM) observation of Raji cells with and without polymerization

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 500μL per tube and a cell density of 1×10^6/ml. Then macromer 4 was added at a final concentration of 2□M. After a 1-h-co-incubation at 37℃, the cells were washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and collected for a SEM observation. The treatment without the addition of APS was taken as the control.

Transmission electron microscope (TEM) observation of Raji cells with and without polymerization

2μM of ferrocene-tagged macromer 4 in deionized water was polymerized by addition of 5mM of APS at 37℃ for 30min, then the resulted structure was verified by TEM.

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 500μL per well and a cell density of 1×10^6/ml. Then macromer 4 was added at a final concentration of 2□M. After a 1-h-co-incubation at 37℃, the cells were washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and collected for a TEM observation. The treatment without the addition of APS was taken as the control.

S5 Polymerization-induced CD20-receptors clustering

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then macromer 4 was added at a final concentration of 0.5, 1 and 2□M, respectively. After a 1-h-co-incubation at 37℃, the cells were washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and

seeded in a 96-well plate. After a 48-h culture, the cell viabilities were measured by CCK-8 assay following the manufacture's instruction. Each treatment was tested in triplicate. The treatment without the addition of APS was taken as the control. For the study of crosslinking-degree dependence, the procedures were almost the identical, except macromer 4 was used with various contents of double-bond and a fixed final concentration, i.e. 2□M.

Maleamide-functionalized macromer 3 was synthesized from Mal-PEG_3-Mal and macromer 3. Briefly, 45μg of Mal-PEG_3-Mal was dissolved in 1mL of deionized water and transferred into a vial bottle, to which 1mL of deionized water containing 310μg of macromer 3 was added dropwise. The resulting mixture was stirred for 12h at room temperature, followed by dialysis against deionized water for 24h to afford maleamide-functionalized macromer 3. FITC-labelled, maleamide-functionalized macromer 3 was obtained following the identical procedures, except as-synthesized FITC-labelled macromer 3 instead of macromer 3 was used.

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then FITC-labelled maleamide-functionalized macromer 3 was added at a final concentration of 2μM. After a 30-min-co-incubation at 4℃ in the dark, the cells were collected, washed twice with PBS, and stained by a cell-membrane dye, DiI, following the manufacture's instruction. After that, the cells were fixed by paraformaldehyde and observed by Confocal Fluorescent Microscope (Nikon A1R, Japan).

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube. The cell density was set at 1×10^6/ml. Then the maleamide-functionalized macromer 3 was added at a final concentration of 0.5, 1 and 2μM, respectively. After a 30-min-co-incubation at 4℃, cells were washed with FBS-free 1640 medium to remove the free macromer. Then, the cells treated with 5mM of APS for 30 min at 37℃. After that, the cells were washed, resuspended and seeded in a 96-well plate. After a 48-h incubation, the cell viabilities were detected by CCK-8 assay. Each treatment was tested in triplicates.

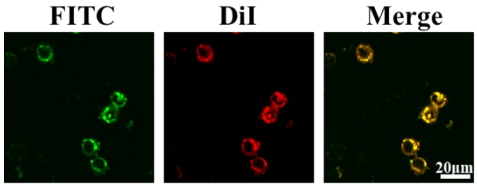


Figure S10 Maleamide-functionalized macromer 3 chemically conjugated onto the cell surface. The macromer was labelled by FITC and the cell membrane was stained by DiI.

Apoptosis detection

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube, at a cell density of 1×10^6/ml. Then macromer 4 was added

at a final concentration of 0.5, 1 and 2μM, respectively. After a 1-h-co-incubation at 37℃, the cells were washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and seeded in a 24-well plate. After a 24-h culture, the cells were collected and stained by Annexin V-FITC/PI according to the manufacturer's instructions, followed by FACS analyzation. Cells treated with 5mM of APS, 2μM of macromer 4 and 2μM of maleamide-functionalized macromer 3 plus 5mM of APS were served as control groups.

S6 Signal events induced by CD20-receptors clustering

Raji cells in FBS-free 1640 medium were seeded in 1.5-mL sterile Eppendorf tubes with a volume of 100μL per tube, at a cell density of 1×10^6/ml. Then macromer 4 was added at a final concentration of 2□M. After a 1-h-co-incubation at 37℃, the cells were washed twice with PBS and exposed to 5mM of APS in FBS-free 1640 medium. Following a 30-min exposure, the cells were washed and seeded in a 24-well plate. After a 24-h culture, the cells were collected and examined for calcium influx, mitochondrial membrane potential, caspase 3 activation, and apoptosis ratio, respectively.

For the calcium influx investigation, cells were loaded with Fluo-4 AM according to the manufacturer's instructions, followed by Confocal Fluorescent Microscope (Nikon A1R, Japan) observed. For the mitochondrial membrane potential study, cells were stained by JC-1 according to the product description and observed by Fluorescence Microscope (Zeiss, Axio Observer A1, Germany). For the detection of caspase 3 activity, a caspase 3 activity assay kit was applied and cells were analyzed following the manufacturer's protocol. For the measurment of apoptosis ratio, cells were stained by Annexin V-FITC/PI according to the manufacturer's instructions, followed by FACS analyzation.

To deplete cholesterol of the rapid rafts, cells were preincubated 1h at 37 °C in the presence of β-CD (2%). To inhibit the activation of Src-family of protein tyrosine kinases (Src-PTKs), the Src-selective inhibitor, PP2, was applied to preincubate with cells for 30min at a concentration of 25μM, and it was still involved in the futher incubation. To deplete the extracellular calcium in the medium, cells were preincubated with 10mM of EGTA for 30min, and it was still involved in the futher incubation.

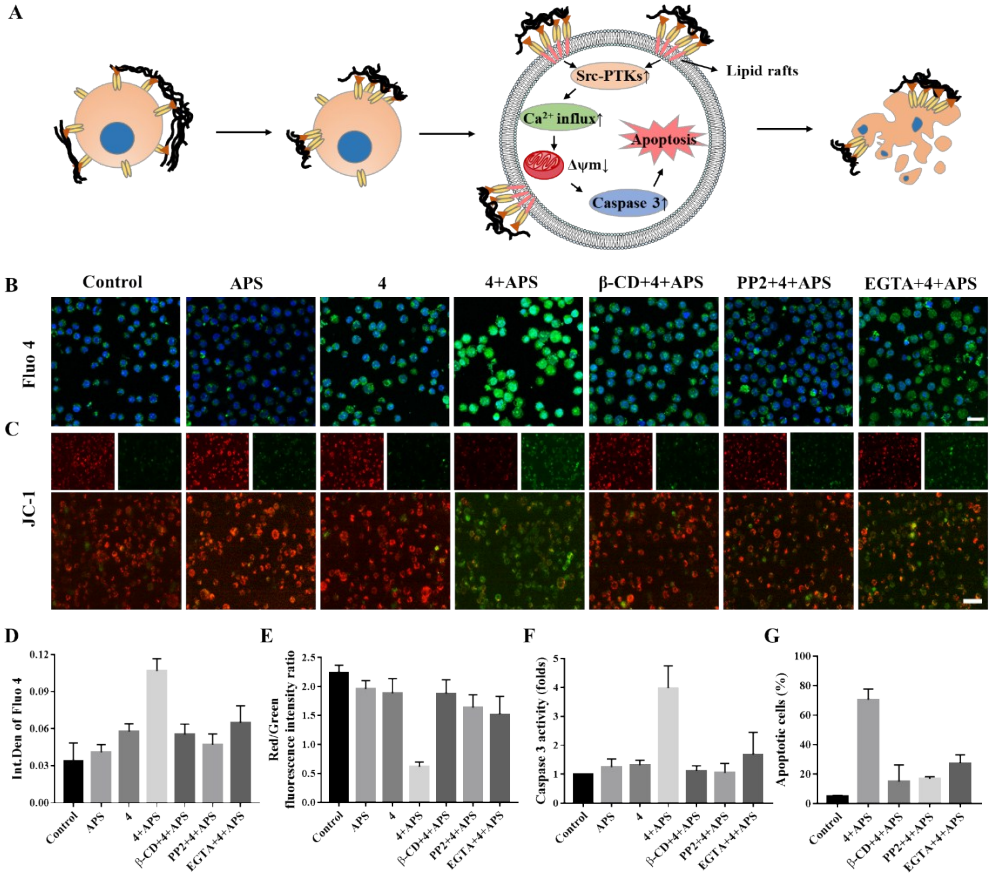


Figure S11 Apoptosis signals triggered by CD20-receptor clustering. A) Illustration of the signal events involved in the apoptosis process. B) Confocal microscopy visualization of Raji cells stained with Fluo-4 AM (green fluorescence) and DAPI (blue fluorescence) after different treatments. Scale Bar=20μm. C) Fluorescence microscope images of Raji cells stained with JC-1 after different treatments. Scale Bar=50μm. D) Semi-quantification analysis of (B) using Image J. E) The red/green fluorescence intensity ratio of JC-1 was calculated based on (C). F) Caspase 3 activity was evaluated by caspase 3 activity assay kit after different treatments. G) Apoptosis proportion was calculated by FACS after different treatments.

S7 Statistical analysis

Statistical calculations were performed using Prism 7 software (GraphPad). Data were expressed as means and SD. Differences were statistically evaluated by one-way analysis of Student's *t* test. The differences were considered to be statistically significant for a p value of <0.05.