Supporting Information

Fructose-coated Nanoparticles: a Promising Drug Nanocarrier for Triple-negative Breast Cancer Therapy

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1. Synthesis

Chemicals. D-fructose (99%, Aldrich), dichloromethane (DCM; anhydrous, >99.8%, Aldrich), methacrylic anhydride (94%, Aldrich), 4-Dimethylaminopyridine (DMAP; 94%, Aldrich), sulfuric acid (95%-98%, Ajax Finechem), acetone (HPLC grade, Ajax Finechem), fluorescein *O*-methacrylate (97%, Aldrich) and *N*,*N*-dimethylformamide (DMF; 99%, Ajax Finechem) were used as received. 1,4-dioxane (99%, Ajax Finechem) and pyridine (99%, Ajax Finechem) were purified by reduced-pressure distillation. Butyl acrylate (>99%, Aldrich) was passed over basic aluminum oxide to remove the inhibitor. 2,2-azobis(isobutyronitrile) (AIBN; 98%, Fluka) was recrystallized

from methanol for purification. The RAFT agent 4-cyanopentanoic acid dithiobenzoate (CPADB) was synthesized according to a literature procedure.[1]

Synthesis of 1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose and 2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose. These two compounds were synthesized according to the previously reported procedure.[2]

Synthesis of 3-*O*-methacryloyl-1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (3-*O*-MAipFru). Into a solution of 1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (3 g, 11.5 mmol), 4-dimethylaminopyridine (1 g) and pyridine (6 mL) in anhydrous dichloromethane (165 mL) at 0 ℃ methacrylic anhydride (2.2 mL) was added. After it stirring for 48 h at room temperature, the mixture was poured into ice-cold saturated $NaHCO_3$ solution (165 mL), and the organic layer was separated. The aqueous layer was extracted with dichloromethane, and combined organic layers were dried using $MgSO_4$ and concentrated under reduced pressure. The product was further purified by flash column chromatography with ethyl acetate : hexane (1:1) as eluent. (yield: 85%). ^{1}H NMR (300 MHz, chloroform-*d*) δ 6.24 (dq, *J* = 1.9, 1.0 Hz, 1H), 5.66 (p, *J* = 1.6 Hz, 1H), 5.22 (d, *J* = 8.0 Hz, 1H), 4.38 (dd, *J* = 8.0, 5.3 Hz, 1H), 4.27 (ddd, *J* = 5.3, 2.4, 1.0 Hz, 1H), 4.25 – 4.06 (m, 2H), 3.99 (d, *J* = 9.3 Hz, 1H), 3.84 (d, *J* = 9.3 Hz, 1H), 2.00 (dd, *J* = 1.6, 1.0 Hz, 3H), 1.61 (s, 3H), 1.52 (s, 3H), 1.41 (s, 3H), 1.39 (s, 3H) ppm. ^{13}C NMR (75 MHz, chloroform-*d*) δ 166.7, 135.7, 126.7, 112.1, 109. 7, 103.8, 75.0, 73.8, 71.7, 70.4, 60.4, 27.8, 26.4, 26.4, 26.1, 18.3 ppm.

Synthesis of 1-*O*-methacryloyl-2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (1-*O*-MAipFru). The synthesis of 1-*O*-methacryloyl-2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose followed the same procedure as that of 3-*O*-methacryloyl-1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose. (yield: 88%). ^{1}H NMR (300 MHz, chloroform-*d*) δ 6.17 (dq, *J* = 1.8, 0.9 Hz, 1H), 5.62 (dd, *J* = 1.9, 1.3 Hz, 1H), 4.64 (dd, *J* = 7.9, 2.7 Hz, 1H), 4.50 (dd, *J* = 11.9, 0.6 Hz, 1H), 4.40 (dd, *J* = 2.7, 0.6 Hz, 1H), 4.27 (ddd, *J* = 7.9, 1.9, 0.8 Hz, 1H), 4.17 (dd, *J* = 11.9, 0.7 Hz, 1H), 3.96 (dd, *J* = 13.0, 1.9 Hz, 1H), 3.79 (dt, *J* = 13.0, 0.8 Hz, 1H), 1.99 (dt, *J* = 1.6, 0.8 Hz, 3H), 1.56 (s, 3H), 1.50 (s, 3H), 1.41 (s, 3H), 1.37 (s, 3H) ppm. ^{13}C NMR (75 MHz, chloroform-*d*) δ 166.7, 136.0, 126.0, 109.1, 108.8, 101.6, 70.8, 70.3, 70.1, 65.0, 61.3, 26.5, 25.9, 25.4, 24.0, 18.5 ppm.

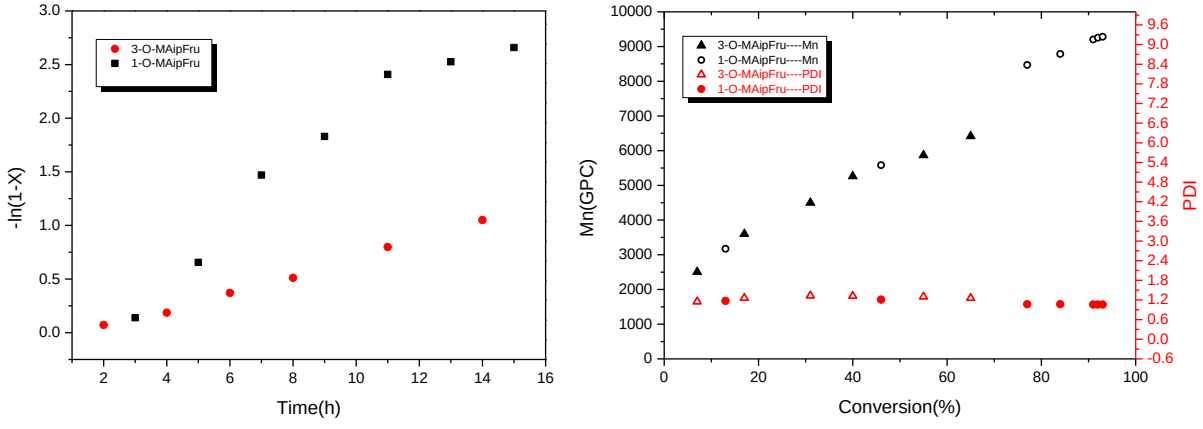
Synthesis of Macro RAFT Agent based on 3-*O*-MAipFru. In a Schlenk tube, 3-*O*-methacryloyl-1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (1 g, 1.83 mmol), AIBN (0.8 mg, 4.8×10^{-3} mmol)

and CPADB (10 mg, 0.037 mmol) were dissolved in 1,4-dioxane (4.3 mL). Then the tube was degassed by three freeze-pump-thaw cycles. The polymerization was carried out at 70 ℃ and stopped after 15 h by cooling the solution in iced water. The polymer solution was poured into a large excess of diethyl ether for precipitation. The viscous polymer was dried under vacuum for 24 h.

Synthesis of Macro RAFT Agent based on 1-*O*-MAipFru. In a Schlenk tube, 1-*O*-methacryloyl-2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (1 g, 1.83 mmol), AIBN (0.8 mg, 4.8×10^{-3} mmol) and CPADB (10 mg, 0.037 mmol) were dissolved in 1,4-dioxane (4.3 mL). Then the tube was degassed by three freeze-pump-thaw cycles. The polymerization was carried out at 70 ℃ and stopped at 7 h by cooling the solution in ice water. The polymer solution was poured into a large excess of diethyl ether for precipitation. The viscous polymer was dried under vacuum for 24 h.

Polymerization Kinetics of Two Glycomonomers

To evaluate the suitability of the RAFT agent CPADB, kinetics studies were carried out using AIBN as initiator in 1,4-dioxane at 70℃ under the reaction condition of $[\text{Monomer}]_0$ / $[\text{CPADB}]_0$ / $[\text{AIBN}]_0$ = 50 : 1 : 0.13 (molar ratio). The conversion versus molecular weight and pseudo first-order kinetics are shown in **Figure S1**, the linearity of –ln(1-x) versus time plot indicates that polymerization of both two glycomonomer follow first-order kinetics.



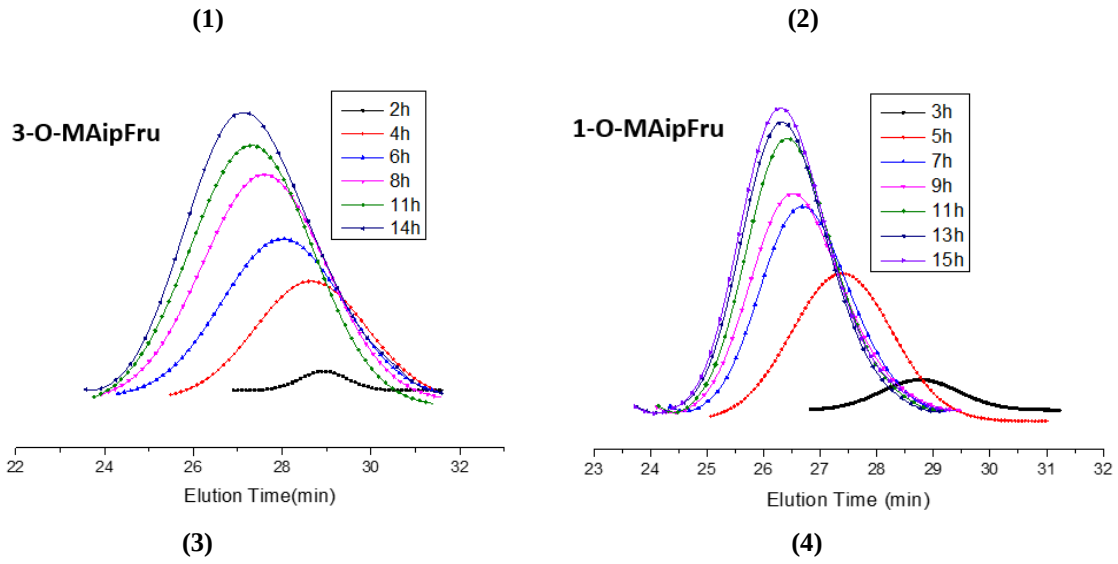


Figure S1 (1) Pseudo first-order kinetic plots for the RAFT polymerization of 3-O-MAipFru and 1-*O*-MAiPrFru; (2) Number-average molecular weight and PDI of 3-*O*-MAiPrFru and 1-O-MAipFru as a function of conversion; (3) SEC traces of Poly(3-*O*-MAiPrFru) evolution over the kinetic course; (4) SEC traces of Poly(1-*O*-MAiPrFru) evolution over the kinetic course.

The SEC traces of two glycopolymers samples taken at increasing reaction time show continuous increasing of molecular weight. The narrowing of the molecular weight during the polymerization course, which is characterized by polydispersity index, indicates the occurrence of a successful controlled radical polymerization. The kinetics study also indicates that two glycomonomers have different reactivity. The polymerization reaction rate of 1-*O*-MAiPrFru was much faster than that of 3-*O*-MAiPrFru due to the difference of steric hindrance between two monomers. Compared to 3-O-MAipFru, 1-*O*-MAiPrFru has further distance between double bond and six-membered ring. The steric hindrance affects the polydispersity of glycomonomers as well. The PDI of Poly(1-*O*-MAiPrFru) is slightly higher than that of Poly(3-*O*-MAiPrFru).

Chain Extension of Macro RAFT Agents with *n*-Butyl Acrylate. A typical procedure was described as follows: macro RAFT agent (100 mg, 9×10^{-3} mmol), *n*-butyl acrylate (175 mg, 1.37 mmol), AIBN (9×10^{-4} mmol) were dissolved in 1,4-dioxane (1.1 mL). The tube was degassed by three freeze-vacuum-thaw cycles. The polymerization was carried out at 70 ℃ and stopped at 14 h by cooling the solution in ice water. The polymer solution was poured into a large excess of *n*-hexane for precipitation. The viscous polymer was dried under vacuum for 24 h.

Deprotection of Block Copolymers. The deprotection of the block copolymers was carried out under acidic conditions. The polymer (80 mg) was added into 1.59 mL of TFA/H_2O (9:1 v/v) in a vial with stirring at room temperature for 30 minutes. After reaction, the polymer solution was dialyzed against deionized water for two days (MWCO 3500). The deprotected polymer was then lyophilized.

Self-assembly of Block Copolymers. The deprotected polymer (8 mg) was dissolved in DMF (0.4 mL) at first. 3.6 mL MQ water was added to the polymer solution using a syringe pump at a rate of 0.2 mL/h. Then the solution was dialysed against deionized water to remove the DMF.

2. Characterization Techniques

Nuclear Magnetic Resonance (NMR) Spectrometry. All NMR spectra were obtained using a Bruker Avance III 300 spectrometer (300 MHz). All chemical shifts are recorded in ppm (δ) relative to tetramethylsilane (δ = 0 ppm), referenced to the chemical shifts of residual solvent resonances (^{1}H and ^{13}C). The multiplicities were explained using the following abbreviations: s for singlet, d for doublet, t for triplet, m for multiplet and bs for broad signal.

Size Exclusion Chromatography (SEC). The molecular weight and polydispersity of synthesized polymers were analyzed via size exclusion chromatography (SEC). A Shimadzu modular system comprising a SIL-10AD auto-injector, DGU-12A degasser, LC-10AT pump, CTO-10A column oven and a RID-10A refractive index detector was used. A 5.0-lm bead-size guard column (50 × 7.8 mm) followed by four 300 × 7.8 mm linear columns (500, 10^3, 10^4, and 10^5 Å pore size, 5 μm particle size) were employed for analysis. *N*,*N*-Dimethylacetamide (DMAc; HPLC grade, 0.05% w/v 2,6-di-butyl-4-methylphenol (BHT) and 0.03% w/v LiBr) with a flow rate of 1 mL/min at 50 ℃ was used as mobile phase. 50 μL of polymer solution with a concentration of 2 mg/mL in DMAc was used for every injection. The calibration was performed using commercially available narrow-polydispersity polystyrene standards (0.5-1000 kDa, Polymer Laboratories).

Dynamic Light Scattering (DLS). Particle sizes (the average diameters and size distributions) were determined using a Malvern Zataplus particle size analyser (laser, 35mW, λ = 632 nm, angle = 90°) at a polymer concentration of 1 mg/mL. Samples were prepared in deionized water and purified from dust using a micro filter (0.45 μm) prior to the measurements.

Transmission Electron Microscopy (TEM). The TEM micrographs were obtained using a JEOL1400 transmission electron microscope comprising of a dispersive X-ray analyser and a Gatan CCD facilitating the acquisition of digital images. The measurement was conducted at an accelerating voltage of 80 kV. The samples were prepared by casting the micellar solution (1 mg/mL) onto a copper grid. The grids were dried by air and then negatively stained with uranyl acetate.

Micelle Cytotoxicity Analysis (SRB assay). Human breast cancer MCF-7 cells were seeded in 96-well plates (4000 cells per well) with culture medium Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.2 g/L $NaHCO_3$, 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in the incubator (5% CO_2/ 95% air atmosphere at 37 ℃) for 24 h. After incubation, cells were treated with trichloroacetic acid 10% w/v (TCA) and incubated at 4 ℃ for 40 min, and then washed five times with Milli-Q water to get rid of the TCA solution. TCA- fixed cells were stained for 30 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. SRB outside the cells was removed by washing the plates with 1% acetic acid. The plates were left to air-dry overnight followed by the addition of 100 μL of 10 mM Tris buffer per well to dissolve the dye in the cells. The absorbance at 570 nm of each well was measured using a microtiter plate reader scanning spectrophotometer to calculate cell viability [cell viability (%) = (test - blank) / (control - blank) × 100].

Flow Cytometry. Cellular uptake was measured using a flow cytometer (BD FACSort). Cells (CHO cells, RAW264.7 cells, MCF-7 cells and MDA-MB-231 cells) were seeded in 6-well tissue culture polystyrene plates at a density of 5×10^5 cells per well in 3 mL culture medium and 50 μg/mL micelles. Cells were measured after 24 h by analysing the fluorescence intensity along with the number of cells. The medium was then replaced with fresh medium (200 μL) containing gradient concentrations of micelles and incubated in the same conditions for 24 h. Non-treated cells were used as controls.

Fluorescence Intensity. The fluorescence intensities of micelles were measured using a Cary Eclipse Fluorescence Spectrophotometer (Aglient Technologies). The excitation and emission wavelengths were 490 and 512 nm, respectively.

Laser Scanning Confocal Microscopy. Cells (CHO cells and MDA-MB-231 cells) were seeded in 35 mm Fluorodishes (World Precision Instruments) at a density of 5×10^5 cells per well in 3 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.2 g/L $NaHCO_3$, 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Micelles were loaded to the cells at a working concentration of 100 μg/mL and incubated in a humidified incubator (5% CO_2/ 95% air atmosphere at 37 ℃) for 24 h. After incubation, the cells were washed thrice with PBS, and then stained with 2.0 μg/mL Hoechst 33342 (Invitrogen) for 5 min. Finally, 100 nM Lyso Tracker Red DND-99 (Invitrogen) for 1 min. A laser scanning confocal microscope system (Zeiss LSM 780) consisted of a Diode 405-30 laser, an argon laser and a DPSS 56110 laser (excitation and absorbance wavelengths: 405, 448 and 561 nm) connected to a Zeiss Axio Observer.Z1 inverted microscope (oil immersion ×100/1.4 NA objective) was used for observation. The Zen2011 imaging software (Zeiss) was used for imaging acquisition and processing.

3. Characterization of Block copolymers.

The polymerization conditions were summarized in **Table S1**. The SEC traces of obtained glycopolymers are shown in **Figure S2**.

Table S1. Synthesis of Glycopolymers via RAFT Polymerization at 70 ℃ in 1,4-Dioxane.

Glycopolymers	[monomer]:[CTA / macro-CTA]:[AIBN]	PDI
Poly(3-*O*-MAipFru)$_{33}$ [a]	50 : 1 : 0.13	1.28
Poly(3-*O*-MAipFru)$_{33}$-*b*-(BA)$_{100}$ [b]	150 : 1 : 0.13	1.18
Poly(3-*O*-MAipFru)$_{33}$-*b*-(BA)$_{202}$ [b]	300 : 1 : 0.13	1.28
Poly(3-*O*-MAipFru)$_{33}$-*b*-(BA)$_{300}$ [b]	600 : 1 : 0.13	1.35
Poly(1-*O*-MAipFru)$_{35}$ [c]	50 : 1 : 0.13	1.11
Poly(1-*O*-MAipFru)$_{35}$-*b*-(BA)$_{74}$ [d]	150 : 1 : 0.13	1.17
Poly(1-*O*-MAipFru)$_{35}$-*b*-(BA)$_{198}$ [d]	300 : 1 : 0.13	1.24
Poly(1-*O*-MAipFru)$_{35}$-*b*-(BA)$_{348}$ [d]	600 : 1 : 0.13	1.41

[a] CPADB was used as CTA, 3-*O*-MAipFru was used as monomer; [b] Poly(3-*O*-MAipFru)$_{33}$ was used as macro-CTA, butyl acrylate was used as monomer; [c] CPADB was used as CTA, 1-*O*-MAipFru was used as monomer;

[d] Poly(1-*O*-MAipFru)$_{35}$ was used as macro-CTA, butyl acrylate was used as monomer. Every reaction system has 1% fluorescein *O*-methacrylate as fluorescein monomer.

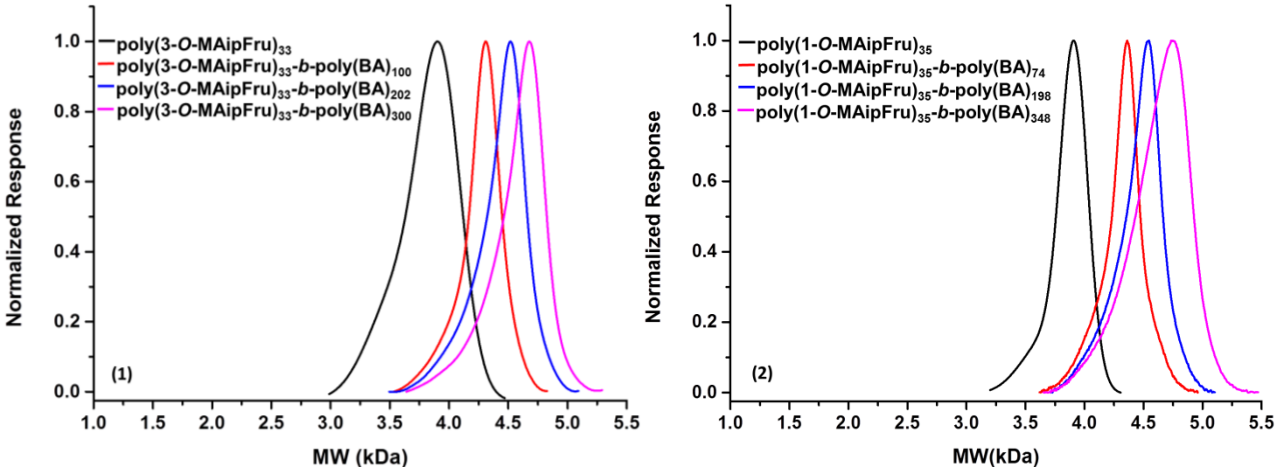


Figure S2. SEC Traces of Glycopolymers Based on 3-*O*-MAipFru and 1-*O*-MAipFru.

4. Deprotection of block copolymers

The isopropylidene protons (δ 1.2-1.6 ppm, ^{1}H NMR; 30-35 ppm, ^{13}C NMR) completely disappear after hydrolysis **(**Figure S3). The characteristic signals corresponding to butyl acrylate block are still existing, indicating the ester groups remain intact under this hydrolysis condition.

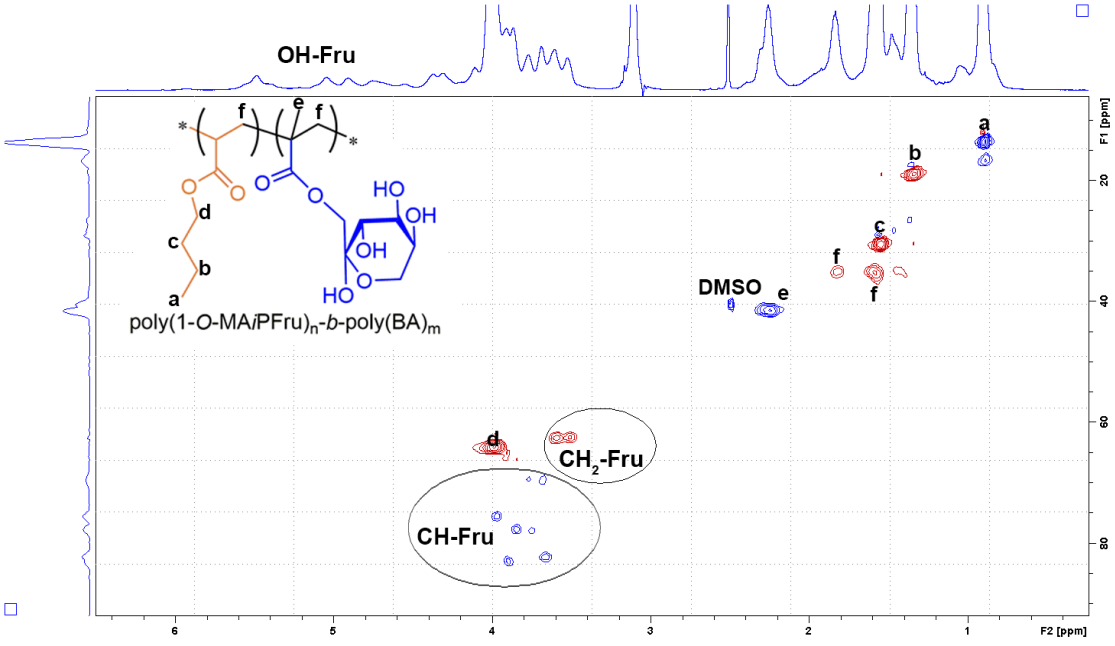


Figure S3. HSQC NMR Spectra (600 MHz, 72 °C, DMSO-d_6). The spectra were obtained at elevated temperature in order to average the signals, hence decrease the broadness of the peaks, resulting from different forms of deprotected fructose (open chain, pyranose and furanose)

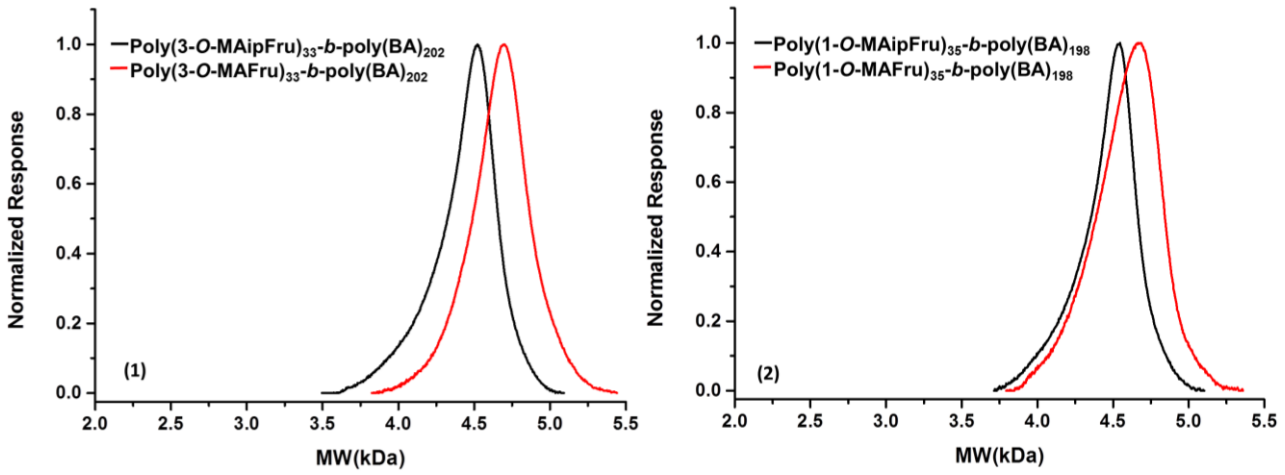


Figure S4. SEC Traces of Block Glycopolymers Before (Black Curves) and After (Red Curves) Deprotection. (1) poly(3-*O*-MAipFru)$_{33}$-*b*-poly(BA)$_{202}$ / poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{202}$; (2) poly(1-*O*-MAipFru)$_{35}$-*b*-poly(BA)$_{198}$ / poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{198}$.

The deprotection of isopropylidenyl groups is also qualitatively confirmed by SEC (Figure S4). SEC curves of deprotected glycopolymers show a shift to higher molecular weight due to a large increase of hydrodynamic volume after deprotection. The well-defined and unimodal molecular weight distributions of block glycopolymers after hydrolysis indicate that pendant fructose residues still exist after hydrolysis.

5. Investigation in the stability of micelles

Briefly, a stock solution of pyrene was made by dissolving pyrene (1 mg, 5 µmol) in acetone (200 mL) to form a 2.5 x 10^{-5} M solution. The pyrene solution (48 µL) was dropped into empty vials, and the acetone was evaporated overnight under reduced pressure. A stock solution with micelles and cross-linked micelles was serially diluted with deionized water starting with the concentration of 0 to 100 µg mL^{-1}. Each polymer solution (2 mL) was transferred to a vial containing pyrene and stirred overnight. The final concentration of pyrene in the polymer solutions was 6 x 10^{-7} M (which is less than the pyrene saturation concentration in water).[45] Fluorescence measurements were carried out using an excitation wavelength of λ= 237.96 nm, using a 2.5 nm slit width for excitation and a 2.5 nm slit width for emission. Emission wavelengths were scanned from 350 to 450 nm. The intensities of I_1 (372 nm) to I_3 (383 nm) vibronic bands were evaluated for each sample, and the ratio of these were plotted against the log of the concentration of each polymeric sample.[46] The

critical micelles concentration (CMC) was taken as the intersection of two regression lines calculated from the linear portions of the graphs.

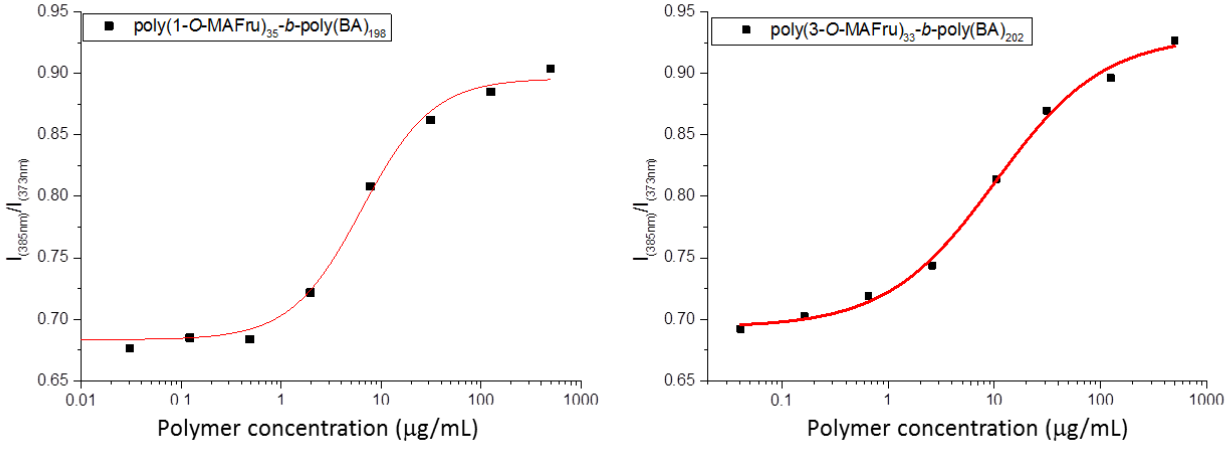


Figure S5. Ratio of fluorescence intensity of pyrene at λ=385 nm and λ= 373 nm in presence of increasing amounts of block copolymer

6. Cytotoxicity of Micelles

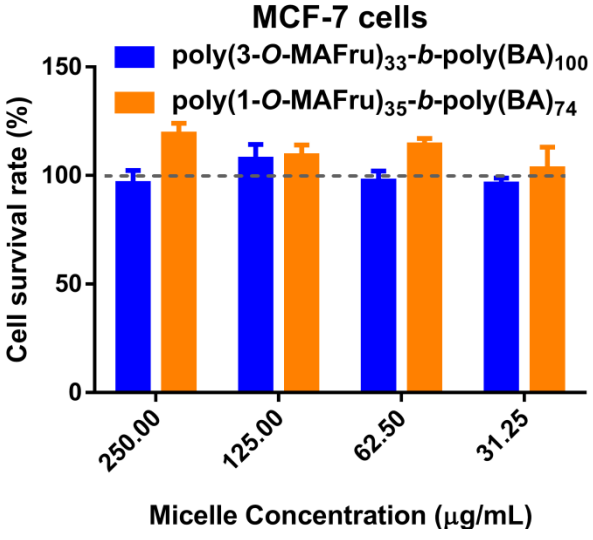


Figure S6. Cell viability of MCF-7 cells exposed to micelles with different concentrations.

7. Stability against surface fouling

The solutions used to measure DLS were mixed with BSA to yield a solution of 0.5 wt% BSA and 5wt% BSA. The particle size distributions were recorded after 1 h and 10 h.

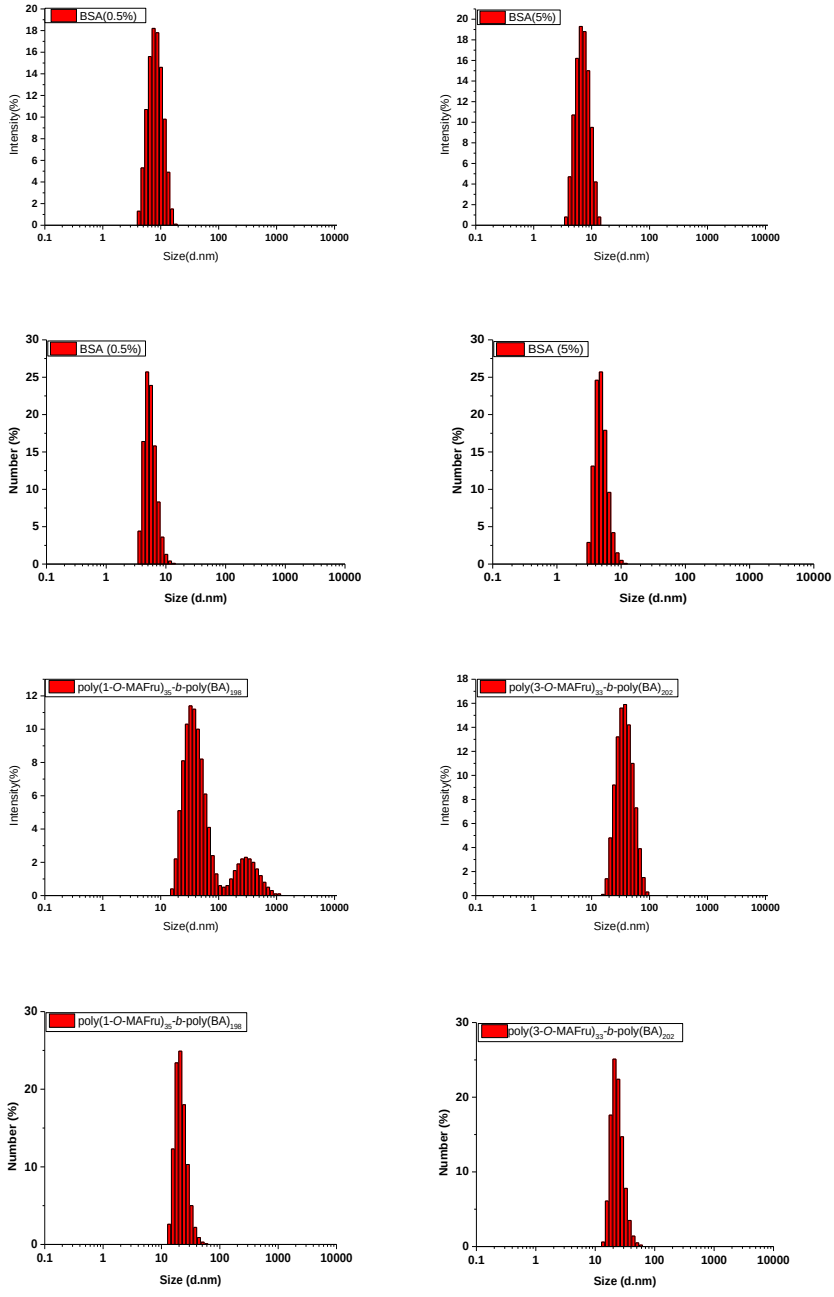


Figure S7. Hydrodynamic diameter (intensity and number distribution) measured by DLS of BSA and block copolymer only as control

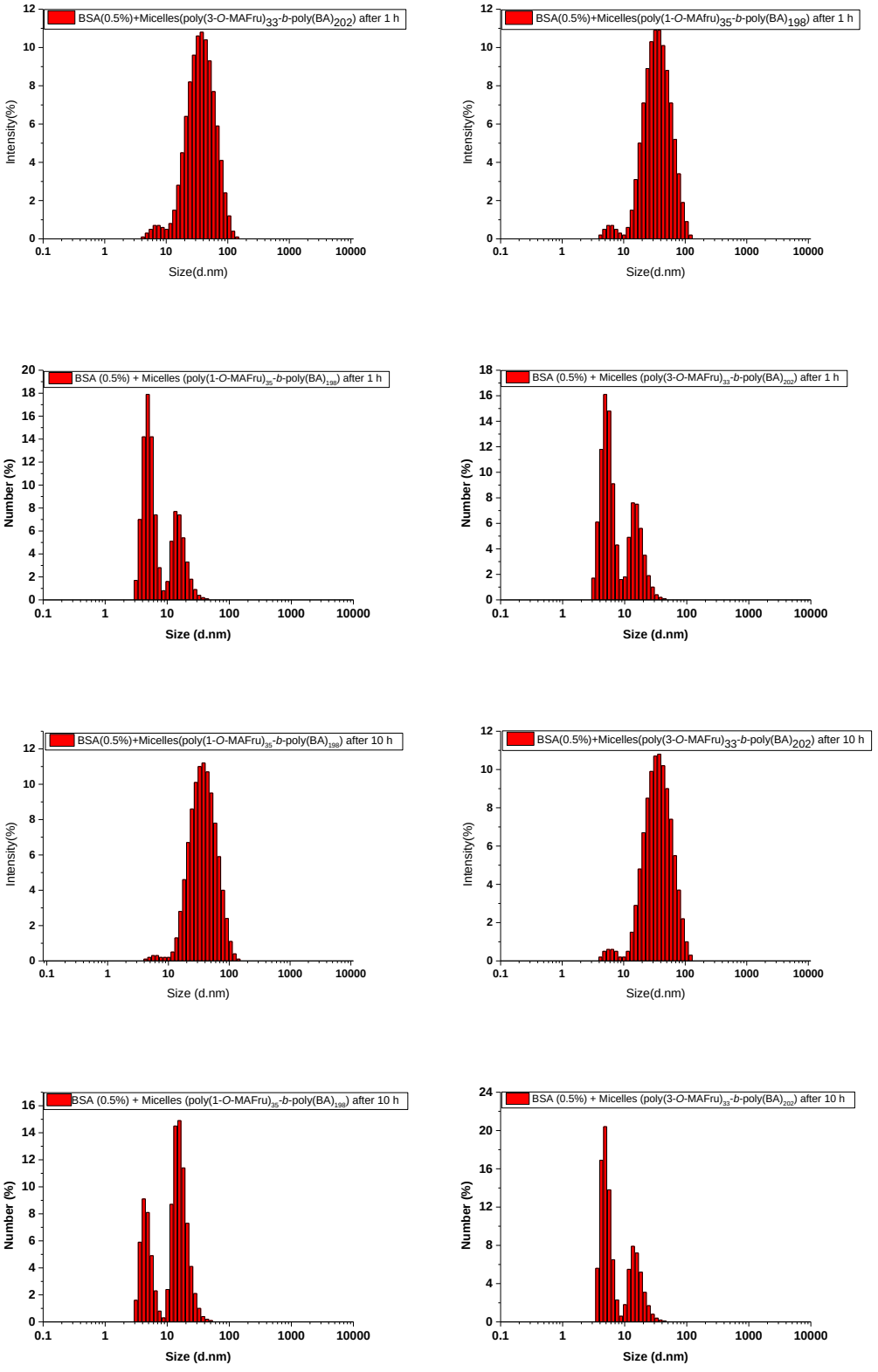


Figure S8. Hydrodynamic diameter (intensity and number distribution) measured by block copolymer in 0.5wt% BSA after 1 and 10 h.

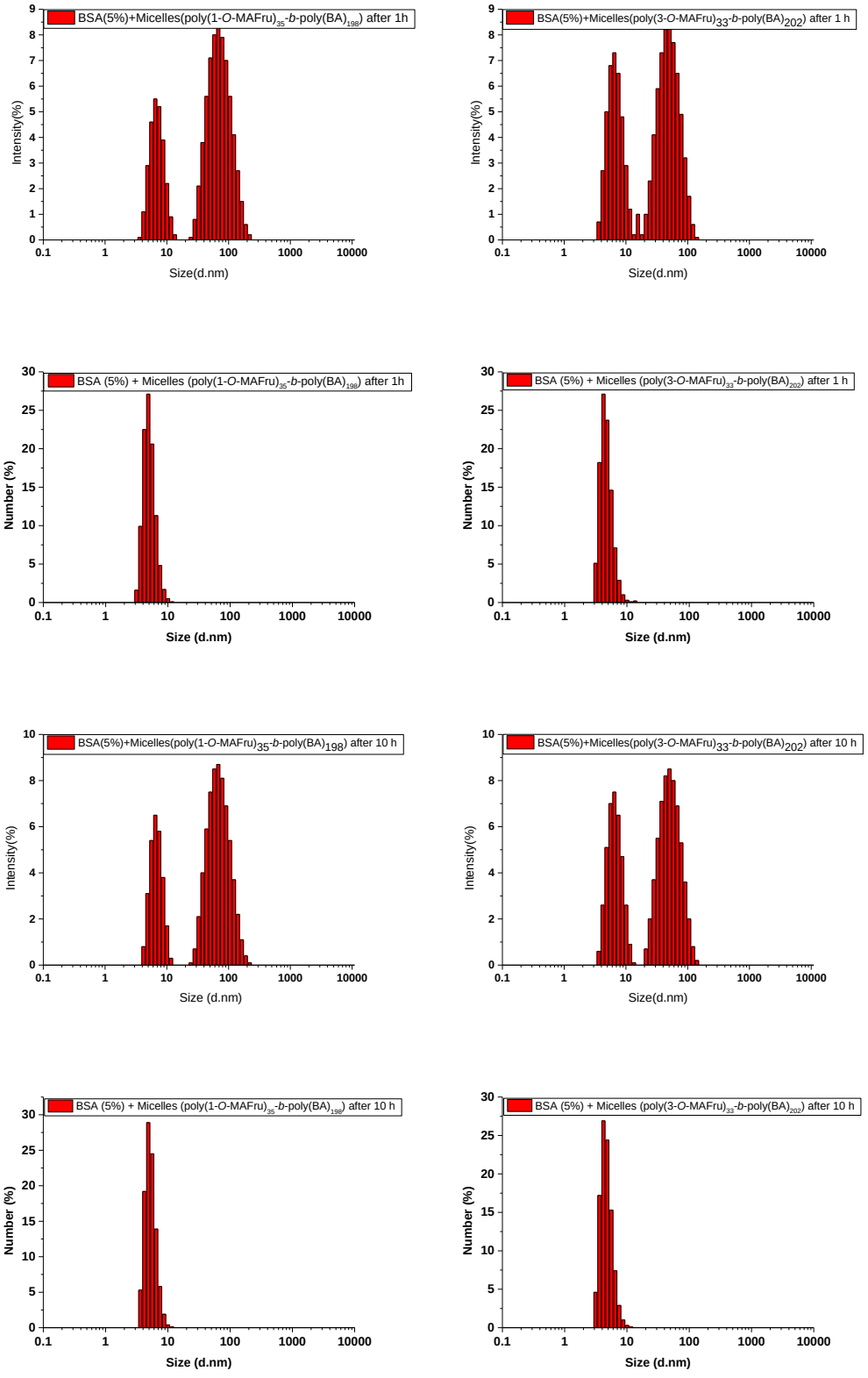


Figure S9. Hydrodynamic diameter (intensity and number distribution) measured by block copolymer in 5wt% BSA after 1 and 10 h.

8. Flow cytometry

Table S2. Fluorescence Intensity of Different Micelles Used for Flow Cytometry.

Micelle	Polymer	Fluorescence Intensity[a] (a.u.)	Normalization factor
A	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{100}$	344	0.746
B	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{202}$	461	1.000
C	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{300}$	459	0.996
D	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{74}$	421	0.913
E	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{198}$	393	0.852
F	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{348}$	436	0.946

[a] The fluorescence intensity was measured at 512 nm with excitation wavelength of 490 nm (a.u. – arbitrary units).

Table S3. Mean Fluorescence Intensity of Different Cells with Internalized Micelles Measured by Flow Cytometry.

Micelle	Polymer	Normalized Mean Fluorescence Intensity[a]			
		CHO	RAW264.7	MCF-7	MDA-MB-231
A	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{100}$	300	194	556	409
B	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{202}$	313	199	610	483
C	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{300}$	326	196	683	520
D	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{74}$	313	177	605	441
E	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{198}$	311	188	605	474
F	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{348}$	314	186	675	495
-	control	297	146	432	319

[a] The MFI values were normalized by the factors given in Table S2 after subtraction of the autofluorescence from control (blank samples)

Table S2 indicates that micelles have different fluorescence intensity due to different level of fluorescence in every glycopolymer. In order to make the flow cytometry data comparable, normalized mean fluorescence intensity (MFI) was calculated using equation 1.

$$\text{Normalized MFI} = \frac{MFI(Sample) - MFI(control)}{Normalized\ FI\ (micelles)} \quad (1)$$

7. References

(1) Mitsukami, Y.; Donovan, M. S.; Lowe, A. B.; McCormick, C. L. *Macromolecules* **2001**, *34*, 2248.

(2) Brady Jr, R. F. *Carbohydrate Research* **1970**, *15*, 35.