Electronic supplementary information for

A thin hydrogel barrier linked onto cell surface sialic acids through covalent bonds induces cancer cell death *in vivo*

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Materials

D-Mannosamine hydrochloride, 2-azidoacetic acid, acetic anhydride, pyridine, alginic acid sodium salt (M_w: 100,000) and azide fluor 545 were purchased from Sigma-Aldrich. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM) was purchased from Wako Pure Chemical. WST-1 was purchased from Dojindo. DBCO-carboxyrhodamine 110 and dibenzylcyclooctyne-PEG_4-amine (DBCO-PEG_4-amine) were purchased from Click Chemistry Tools. Other reagents and solvents available in extra-pure grade were obtained commercially and used without further purification.

Synthesis of Ac_4ManNAz

D-Mannosamine hydrochloride (500 mg, 2.3 mmol) was added to an aqueous solution of azidoacetic acid (200 μL, 2.4 mmol). DMT-MM (664 mg, 2.4 mmol) was added to this solution and the reaction mixture was stirred at 45 ºC for 2 days. The solvent was removed by evaporation to give a solid state crude reaction mixture and the *N*-azidoacetyl D-mannosamine (ManNAz) product was extracted from the crude mixture by washing three times with methanol, then purified by silica gel chromatography (eluting solution: methanol/chloroform = 2/1, v/v). Acetic anhydride (380 μL, 4.0 mmol) was added to a solution of ManNAz (170 mg, 0.66 mmol) in anhydrous pyridine (5 mL, 62 mmol) and the reaction mixture was stirred overnight at room temperature under a nitrogen atmosphere. The

solution was concentrated, resuspended in dichloromethane, and washed with 1 M hydrogen chloride, saturated sodium hydrogen carbonate, and then saturated sodium chloride. The organic phase was dried using magnesium sulfate, filtered, and evaporated to give solid tetraacetylated *N*-azidoacetyl mannosamine (Ac_4ManNAz). ^{1}H-NMR (500 MHz, $CDCl_3$): 1.98–2.22 (–OCOC*H*$_3$, 12H), 3.81 (C2HN*H*CO–, 1H), 3.92 (–COC*H*$_2$N$_3$, 2H), 4.05–4.13 (C3*H*, C4*H*, and C5HC*H*$_2$, 4H), 4.27 (C5*H*, 1H), 4.62 (C2*H*, 1H), 5.18 (C1*H*, 1H). ESI-MS, calc. 430.4; found 429.08.

Cell culture

MCF-7 cells (human breast adenocarcinoma cell line), PC-3 (human prostate cancer cell line), and THP-1 cells (human acute monocytic leukemia cell line) were purchased from ATCC. MCF-7 and PC-3 cells were cultured in DMEM supplemented with 10% FBS, 2.0 mM L-glutamine, and antibiotic solution containing penicillin (100 units mL^{-1}) and streptomycin (100 µg mL^{-1}) at 37 ºC in a humidified atmosphere containing 5.0% CO_2. THP-1 cells were cultured in RPMI 1640 medium supplemented with 5% FBS, 2.0 mM L-glutamine, and antibiotic solution containing penicillin (100 units mL^{-1}) and streptomycin (100 µg mL^{-1}) at 37 ºC in a humidified atmosphere containing 5.0% CO_2.

Preparation of azide-modified cells

MCF-7 cells (1.0×10^5) were seeded on a 3 cm glass bottom dish, then 2 mL of DMEM containing Ac_4ManNAz (100 μM) was added and the dish was incubated at 37 ºC for 3 days. The supernatant was removed and then 2 mL of DMEM was freshly added to the dish. DBCO-carboxyrhodamine 110 (final concentration: 5 μM) was added to the dish and incubated at 37 ºC for 1 hour. Cells attached on the dish were washed twice with phosphate buffered saline (PBS), 1 mL of Live Cell Imaging Solution (Life Technologies) was added, and then the cells were observed by confocal laser scanning microscopy (CLSM, ZEISS LSM700). The azide-modification of PC-3 and THP-1 cells was performed similarly.

Synthesis of alkyne-modified alginate

DBCO-PEG_4-amine (76 mg, 112.6 μmol) and DMT-MM (50 mg, 180.6 μmol) dissolved in 5 mL of pure water was added to 15 mL of Alg (500 mg, 5 μmol) solution and stirred at room temperature for 6 days. The reaction mixture was dialyzed (MWCO: 14,000) against pure water for 2 days, then the resultant solution was freeze-dried to give a white powder of DBCO-modified alginate (Alg-DBCO). The molecular structure of Alg-DBCO was determined by ^{1}H-NMR analysis (D_2O, 85 ºC).

Bioorthogonal click reaction between azide-modified cells and Alg-DBCO

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 3 cm glass bottom dish were washed

twice with PBS, then FITC-labeled Alg-DBCO (0.5 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the cells were washed with PBS twice, then 1 mL of Live Cell Imaging Solution was added and the cells were observed by CLSM. The bioorthogonal click reaction was conducted similarly with PC-3 and THP-1 cells.

Formation and characterization of the thin hydrogel on cell surface glycans

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 3 cm glass bottom dish were washed twice with PBS, then FITC-labeled Alg-DBCO (0.5 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the supernatant was completely removed and the cells were rinsed with PBS twice, then DMEM was freshly added. Aqueous calcium chloride solution (4.5 mM) was added and the cells were incubated at 37 ºC for 1 hour to allow physical gelation of the alginate covalently bound to the cell surface glycans. The treated MCF-7 cells were observed by CLSM. SEM (JSM-7001FA, JEOL) observation was conducted by fixing the treated MCF-7 cells with 10% neutral buffered formalin for 10 min, then subjecting the cells to sequential dehydration in graded ethanol (50, 70, 80, 90, 95, 99, and 100%), followed by lyophilization.

Adhesion assay of cells with a surface thin hydrogel

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 6 well plate were washed twice with PBS, then Alg-DBCO (0.5 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the supernatant was completely removed and cells were rinsed with PBS twice, then DMEM was freshly added. Aqueous calcium chloride solution (4.5 mM) was added and the cells were incubated at 37 ºC for 1 hour to allow physical gelation of the alginate covalently bound to the cell surface glycans. The cells were treated with 0.25% trypsin, harvested, then seeded (1.0×10^5) in a collagen-coated 6 well plate and incubated for 24 and 48 hours at 37 ºC in a humidified atmosphere containing 5.0% CO_2. The cells were stained with crystal violet (0.1%) and the adhered cells were observed by microscopy (KEYENCE, BIOREVO BZ-9000). The adhesion of control cells on collagen-coated dishes was also observed. The number of adhered cells per 1 mm^2 was counted. Values represent means ± S.D (n = 3).

Migration assay of cells with a surface thin hydrogel

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 6 well plate were washed twice with PBS, then Alg-DBCO (0.5 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the supernatant was completely removed and the cells were rinsed with PBS twice, then DMEM

was freshly added. Aqueous calcium chloride solution (4.5 mM) was added and the cells were incubated at 37 ºC for 1 hour to allow physical gelation of the alginate covalently bound to the cell surface glycans. The cells were treated with 0.25% trypsin, harvested, then seeded (8.0×10^5) in a collagen-coated 6 well plate and incubated for 24 hours at 37 ºC in a humidified atmosphere containing 5.0% CO_2. A linear scratch was made on the cell monolayer surface using a sterile pipette tip, then after 48 hours the scratch was visualized by ultra violet staining and photographed under a microscope (KEYENCE, BIOREVO BZ-9000). The wound recovered area was analyzed using Image J software and normalized to the time 0 wound. The assay was similarly performed for control cells. Values represent means ± S.D (n = 3).

Invasion assay of cells with a surface thin hydrogel

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 6 well plate were washed twice with PBS, then Alg-DBCO (0.5 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the supernatant was completely removed and the cells were rinsed with PBS twice, then DMEM was freshly added. Aqueous calcium chloride solution (4.5 mM) was added and the cells were incubated at 37 ºC for 1 hour to allow physical gelation of the alginate covalently bound to the cell surface glycans. The cells were treated with 0.25% trypsin, harvested, then plated ($3.0 \times$

10^4) in collagen-coated transwell inserts (8 μm pore size) and incubated in DMEM at 37 ºC in a humidified atmosphere containing 5.0% CO_2. After 72 hours, cells that had migrated through the inserts were stained with crystal violet and imaged by microscopy (KEYENCE, BIOREVO BZ-9000). The number of adhered cells per 1.0×10^5 μm^2 was counted. The assay was similarly performed for control cells. Values represent means ± S.D (n = 3).

Proliferation assay of cells with a surface thin hydrogel

Azide-modified MCF-7 cells (7.0×10^4) cultured on a 6 well plate were washed twice with PBS, then Alg-DBCO (0.5 mg/mL or 2.0 mg/mL) dissolved in DMEM was added and the cells were incubated at 37 ºC for 24 hours. Following the resulting bioorthogonal click reaction, the supernatant was completely removed and the cells were rinsed with PBS twice, then DMEM was freshly added. Aqueous calcium chloride solution (4.5 mM) was added and the cells were incubated at 37 ºC for 1 hour to allow physical gelation of the alginate covalently bound to the cell surface glycans. The cells were treated with 0.25% trypsin, harvested, then plated (8.0×10^3) in a 96 well plate and incubated at 37 ºC in a humidified atmosphere containing 5.0% CO_2. After a predetermined time, the supernatant was removed and 100 μL of fresh DMEM and 5 μL of WST-1 solution was added to each well and the plate was incubated at 37 ºC for 2 hours. Absorbance at 450 nm and 620 nm was measured by a microplate reader. The assay was similarly performed for control MCF-7 cells and for THP-

1 cells. Values represent means ± S.D (n = 3). The viability of MCF-7 cells 24 hours after formation of the surface thin hydrogel was investigated using the Live/Dead assay. Calcein-AM (DOJINDO) and propidium iodide (PI, DOJINDO) were added in the medium and reacted for 1 hour at 37 ºC, then CLSM observation was carried out in 1 mL of Live Cell Imaging Solution.

In vivo distribution of Alg-DBCO in azide-modified tumor-bearing mice

All animal experiments and all experimental protocols were approved by Konan University (protocol No.: K-18-06) and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Female nude mice (ICR-nu/nu) 5 weeks of age were purchased from Charles River. For surgery, the animals were anesthetized using isoflurane and the surgical area was cleaned. PC-3 cells (1.2×10^6 cells) in 150 μL of Matrigel (BD Bioscience) were injected subcutaneously into the back bilaterally using a disposable syringe and a 26-gauge needle. When the tumor size reached 100 mm^3, the animals were randomly divided into 4 groups (FITC-Alg-DBCO, FITC-Alg-DBCO/Ca^{2+}, Ac_4ManNAz/FITC-Alg-DBCO, and Ac_4ManNAz/FITC-Alg-DBCO/Ca^{2+}). AC_4ManNAz (3 mg) was dissolved in 100 μL of PBS/DMSO (42/58, v/v). The mice were anesthetized using isoflurane, then the AC_4ManNAz solution was administrated by local injection into the tumor tissue. This administration was carried out for 4 consecutive days. After 24 hours, aqueous

FITC-Alg-DBCO solution (0.5 mg/100 μL) was administrated by local injection into the tumor tissue and this administration was carried out for 2 consecutive days. After 24 hours, 50 μL of aqueous calcium chloride solution (4.5 mM) was administrated by local injection into the tumor tissue, then the mice were sacrificed after a further 48 hours and the tumor tissues and main organs (brain, heart, lung, liver, spleen, and kidney) were excised carefully. The tissues and organs were rinsed with PBS twice, the cell nuclei was stained with Hoechst33342, then the tissues and organs were sliced and observed by CLSM observation. The excised tissues and organs were then homogenized thoroughly and incubated in 1 mL of PBS at 4°C for 12 hours, centrifuged at 4000 rpm for 10 min, and each supernatant was transferred to a quartz cell. The fluorescence intensity at 513 nm was measured to calculate the amount of FITC-Alg-DBCO distributed into each tissue/organ. The fluorescence intensity is the average of the triplicate experiments using different three mice (n = 3) and is expressed as mean ± S.D.

In vivo behavior of cancer cells with a surface thin hydrogel

Female nude mice (ICR-nu/nu) 5 weeks of age were purchased from Charles River. For surgery, the animals were anesthetized using isoflurane and the surgical area was cleaned. PC-3 cells (1.2×10^6 cells) in 150 μL of Matrigel (BD Bioscience) were injected subcutaneously into the back bilaterally using a disposable syringe and a 26-gauge needle. When the tumor size reached 100 mm^3, the animals were randomly divided into 4 groups (PBS only, Alg-

DBCO/Ca^{2+}, Ac_4ManNAz/Alg-DBCO, and Ac_4ManNAz/Alg-DBCO/Ca^{2+}). AC_4ManNAz (3 mg) was dissolved in 100 μL of PBS/DMSO (42/58, v/v). The mice were anesthetized using isoflurane, then the AC_4ManNAz solution was administrated by local injection into the tumor tissue. This administration was carried out for 4 consecutive days. After 24 hours, an aqueous FITC-Alg-DBCO solution (0.5 mg/100 μL) was administrated by local injection into the tumor tissue and this administration was carried out for 2 consecutive days. After 24 hours, 50 μL of aqueous calcium chloride solution (4.5 mM) was administrated by local injection into the tumor tissue. The tumor size was measured each day using a caliper and the volume was calculated according to the formula: tumor volume = (shorter diameter)^2 × (longer diameter)/2. The body weights of the mice were recorded each day. The tumor size and the body weight are the average of the triplicate experiments using different three mice (n = 3) and is expressed as mean ± S.D.

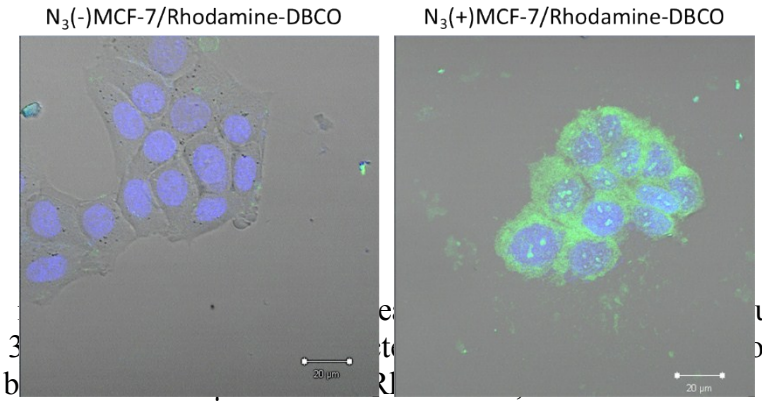


Fig. S1. CLSM [illegible] ut (left) 100 μM Ac_4ManNAz for 3 [illegible] oxyrhodamine 110 for 1 hour. Scale b [illegible]

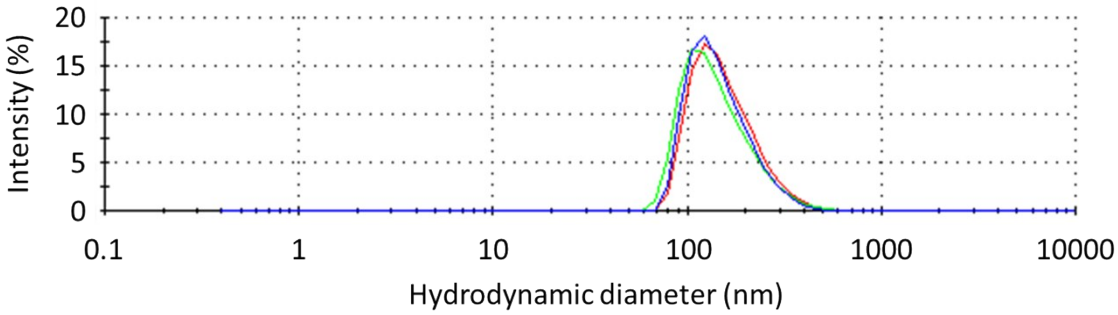


Fig. S2. Hydrodynamic diameter of Alg-DBCOs in HEPES and their distribution analyzed by DLS.

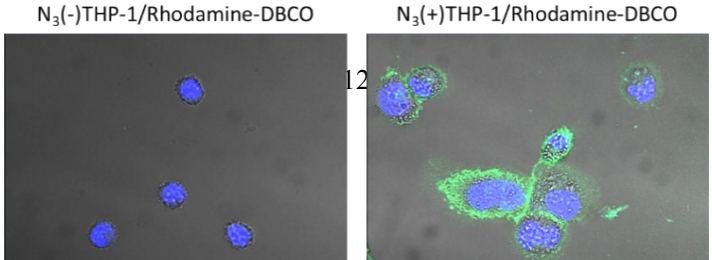


Fig. S3. CLSM images of THP-1 cells treated with (right) or without (left) 100 μM Ac_4ManNAz for 3 days and followed by reacted with DBCO-PEG4-Carboxyrhodamine 110 for 1 hour. Scale bars indicate 20 μm. Green: Rhodamine, Blue: Hoechst.

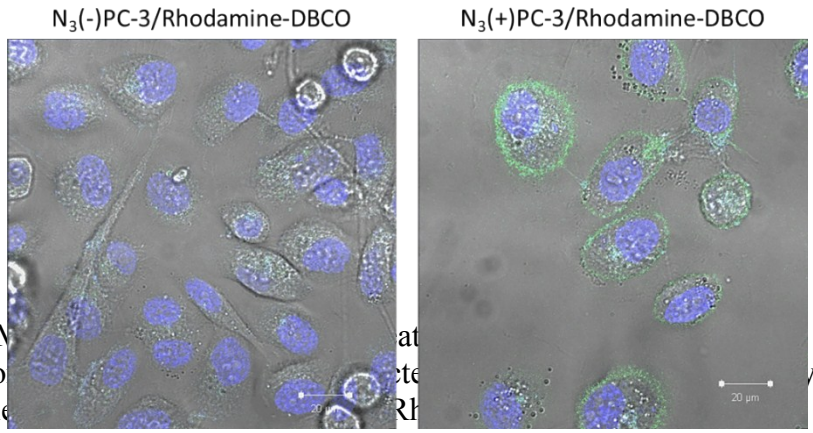
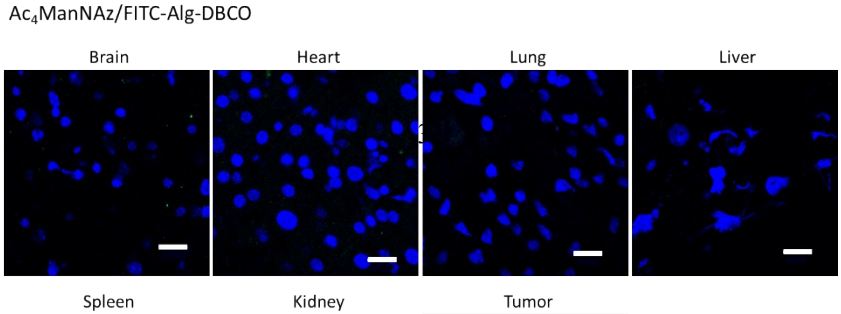


Fig. S4. CLSM at (left) 100 μM Ac_4ManNAz fo te yrhodamine 110 for 1 hour. Scale Rh



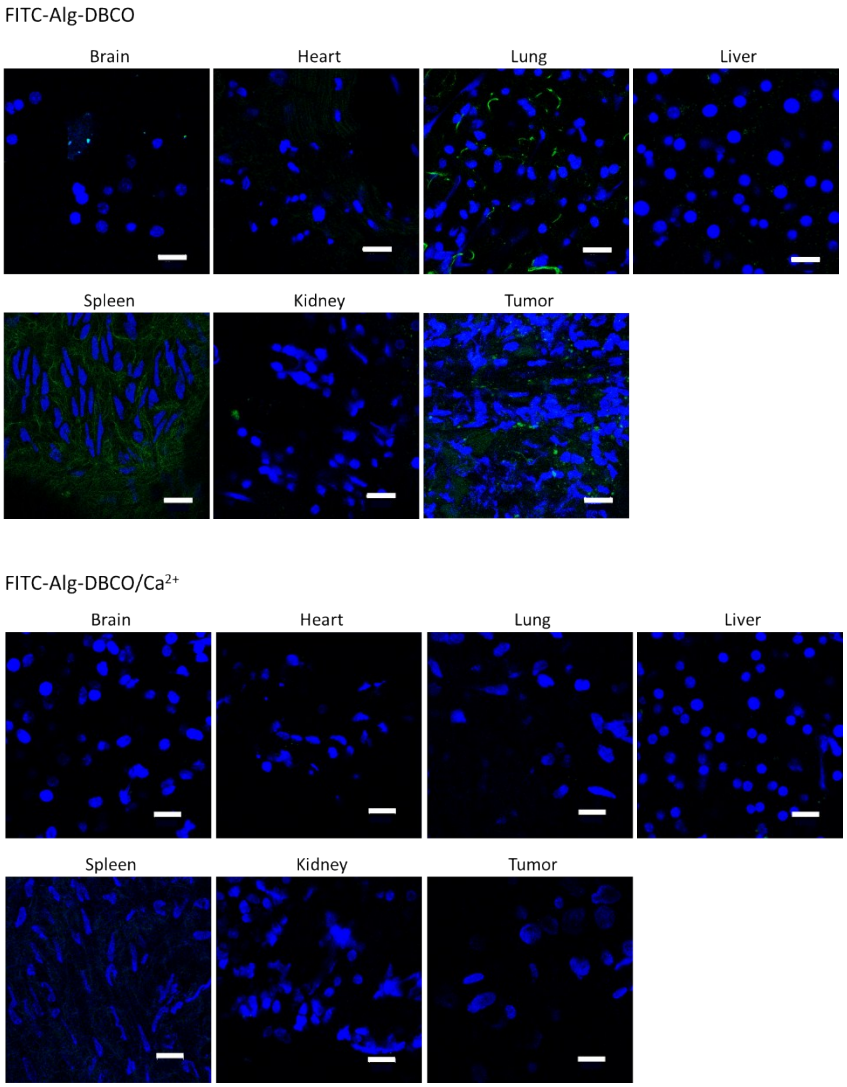


Fig. S5. CLSM images of organs and tumors removed from the tumor-bearing mice at day 7 after treatment with Ac_4ManNAz/FITC-Alg-DBCO, FITC-Alg-DBCO, or FITC-Alg-DBCO/calcium chloride. Scale bars indicate 20 μm. Green: FITC, blue: Hoechst.