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A Targeting Strategy to Overcome Tumor Heterogeneity Using Polymerized Metabolic Precursors

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이 논문을 공학석사 학위논문으로 제출함

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2015 년 6 월

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ABSTRACT

A Targeting Strategy to Overcome Tumor Heterogeneity Using Polymerized Metabolic Precursors

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Historically, drug as a free form has been directly used in cancer treatment. However, while the anticancer drugs can be distributed throughout the whole body, it is very toxic and causes many side effects to normal tissues. To overcome this problem, many nano-technologies have been applied for an effective delivery of anticancer drugs to the targeting cancer cells. In this study, we developed polymerized metabolic precursor (pMPs) to improve tumor-targeting abilities. Furthermore, the pMPs is designed to overcome the intrinsic limitations causing from the limited amount of cellular receptors and the heterogeneity of tumor cells. The pMPs are based on metabolic glycoengineering and click chemistry. Triacetylated *N*-

azidoacetyl-*D*-mannosamine (Ac_3ManNAz) is conjugated to the PAMAM [G4] dendrimer backbone and the compound generates azide groups on the surface of tumor tissue specifically. Regardless of tumor cell types, pMPs create a more uniform cancer cells with artificial glycans on their surface. With the enhanced permeation and retention (EPR) effect followed by metabolic glycoengineering, the pMPs generate 'receptor-like' chemical reporters to produce a homogeneous cancer cell surface in the tumor tissue. Then, they can be labeled by ADIBO-Cy5.5 via copper-free click chemistry *in vivo* condition.

Keywords: Polymerized Metabolic Precursors (pMPs), Tumor Heterogeneity, Metabolic Glycoengineering, Click Chemistry, Optical Imaging

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Figure 13. Fluorescence images of tumor tissues after intravenous injection of ADIBO-Cy5.5 to tumor-bearing mice pretreated with saline (top panel), free Ac_3ManNAz (middle panel), or pMPs (bottom panel).

1. INTRODUCTION

Metabolic glycoengineering is a useful biological technique pioneered by Carolyn Bertozzi's group[1]. It utilizes an intrinsic glycan metabolism of cells to introduce unnatural glycans with various chemical groups[2]. When rationally designed metabolic precursors are treated, cells applicate them as building blocks for glycan metabolism. These precursors are inserted within the structure of glycans[3]. Until today, lots of papers studied about a wide range of applications in this technique from precise analysis of glycans at molecular level to spatiotemporal imaging *in vivo*[4]. Particularly, combined with click chemistry, biorthogonal chemical groups can be easily introduced to glycans by this technique and they enable click reactions on the glycans in cells or bodies as well as in *ex vivo* condition for various purposes[5].

For more than one century, several kinds of nanoparticles have shown promising results in imaging and drug delivery[6]. However, their tumor-targeting ability is still limited, and there are many obstacles to improve their efficacy[7]. In particular, current active-targeting method using biological ligands has intrinsic limitations, because the amount of receptors on tumor cells is limited and the binding of nanoparticles can be saturated[8]. In addition, the heterogeneity of tumor cells makes the situation more complicated since numerous subpopulations of tumor cells express different

kinds and amounts of receptors[9]. Heterogeneity can be observed within cancers of same tissue as well as in different types of cancer. In this point of view, we expect that chemical groups can be introduced onto tumor tissue containing heterogeneous tumor cells. If so, they would be used as artificial active-targeting moieties for the delivery of nanoparticles and provide alternative ways to overcome tumor heterogeneity. Importantly, a targeted delivery of metabolite and tumor cell-specific introduction of chemical groups will be much useful for tumor imaging or drug delivery.

Herein, in order to overcome tumor heterogeneity, we developed polymerized metabolic precursors for biorthogonal targeting strategy in living body (Figure 1a). Polymerized metabolic precursors (pMPs) were synthesized by conjugation of triacetylated *N*-azidoacetyl-*D*-mannosamine (Ac_3ManNAz), the precursor for azide generation, to the succinic acid terminated generation 4 poly (amido amine) (PAMAM [G4])-dendrimer backbone (Figure 1b). The pMPs are intravenously injected to tumor-bearing mice. This injection results in site-specific generation of azide groups on tumor tissue due to the EPR effect. Under the acidic tumor microenvironment, it can be hydrolyzed into free form of Ac_3ManNAz which can introduce azide groups onto tumor cells surface by metabolic glycoengineering. Our approach generates ‘receptor-like’ chemical groups on the surface of target cells by pMPs alone. The advantage of this method

over biological receptors is that the small azide groups can expressed on the cell surface in large quantities regardless of the types or subpopulations of heterogeneous tumor cells.

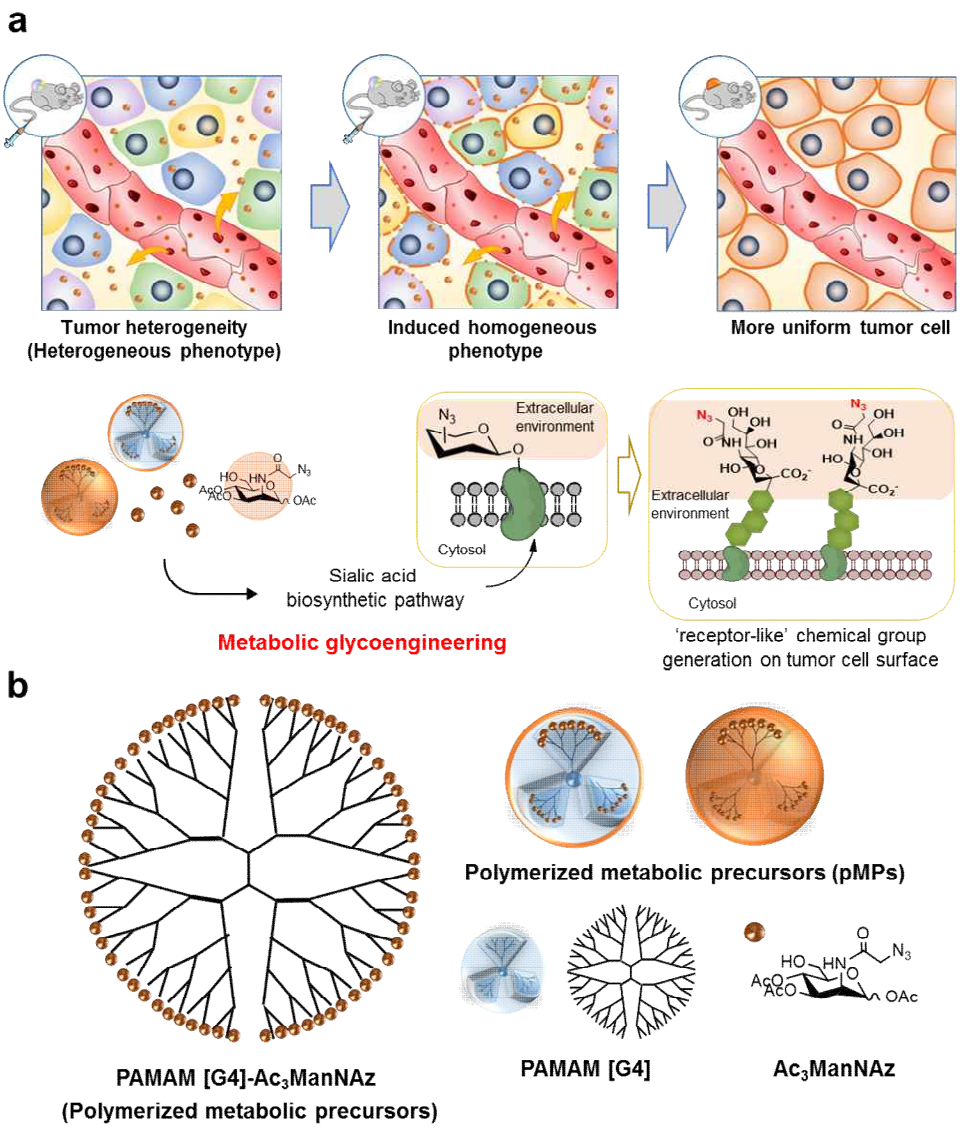

Figure 1. (a) Schematic illustration of the targeting strategy to overcome tumor heterogeneity using polymerized metabolic precursors (pMPs). (b) Chemical structure of pMPs.

2. EXPERIMENTS

2.1 Materials

Succinamic acid terminated generation 4 poly (amido amine) (PAMAM [G4]) dendrimer was purchased as 5 wt% in water from Dendritech, Inc. (Midland, MI, USA). The water was removed by lyophilization before any further experiments. Triacetylated *N*-azidoacetyl-*D*-mannosamine (Ac_3ManNAz) was achieved from FutureChem (Seoul, Korea). 1-ehtyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4-(Dimethylamino) pyridine (DMAP), Dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cy5.5-NHS was acquired from GE Healthcare Life Sciences (Piscataway, NJ, USA). ADIBO-Cy5.5 was purchased from FutureChem (Seoul, Korea).

2.2 Conjugation of PAMAM [G4] Dendrimer-Ac_3ManNAz

Esterification of PAMAM [G4] was carried out according to *Steglich* esterification mechanism. PAMAM [G4] dendrimers were dissolved in 1 ml DMSO in the presence of EDC and DMAP as catalysts at 1 to 1.2 ratios. Then, 3 molar equivalents of triacetylated mannosamine (Ac_3ManNAz) with PAMAM [G4] dendrimers were added to the DMSO solution containing

PAMAM [G4] dendrimers and catalysts. The reactants were stirred at room temperature for 72 hours and were dialyzed in DMSO for another 72 hours using a cellulose membrane (MWCO 3500: Spectrum Laboratories, TX, USA) at room temperature to remove unreacted EDC, DMAP and Ac_3ManNAz. After dialysis, the Ac_3ManNAz conjugated PAMAM [G4] dendrimers were obtained by lyophilization.

2.3 Characterization of PAMAM [G4] Dendrimer-Ac_3ManNAz (Polymerized Metabolic Precursors, pMPs)

Thin Layered Chromatography (TLC) aluminum plates (Silica gel 60 F254, Merck kGaA, Darmstadt, Germany) were used to monitor the progress of the reaction. Reactants and products dissolved in DMSO (0.1 g/ml) were spotted onto a start line on one side of the TLC plates at different time points (1, 2, 3, 5 days). In TLC chambers, chloroform/methanol (2:1, *v/v*) was used as a developing eluent for the plates. After evaporation of developing solvents, chromatographic spots of samples were exposed under uv lamp. ^{1}H NMR analysis was performed by Bruker Advance-300 MHz spectrometer in DMSO-d_6 solution at room temperature (MA, USA). ^{1}H NMR peaks of pMPs were as follows: 2.02 (s; H of acetyl groups from Ac_3ManNAz, 9H), 2.49-2.53 (s; H of CH_2-CH_2 from PAMAM, 4H), 3.06 (s; H of CH_2-CH_2 from PAMAM in between -NH, 4H), 5.04-5.24 (m; H of

Ac_3ManNAz, 1H), 5.99 (s; H of Ac_3ManNAz, 1H), 7.88 (s; H of NH from PAMAM, 3H), 8.50-8.53 (d; H of NH from Ac_3ManNAz, 1H) (Figure 4c). Mass spectra was measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS; PAMAM m/z calculated: 20615, found: 19277, pMPs m/z calculated: 45447, found: 38599) (Figure 5). The conjugated amount of Ac_3ManNAz was determined by high performance liquid chromatography (HPLC).

2.4 Synthesis of Cy5.5-labeled pMPs

The pMPs were labeled with Cy5.5 to trace their movement by fluorescence. Cy5.5-NHS was dissolved in deionized distilled water and added to the water solution containing PAMAM [G4] dendrimer. The mixture was reacted in the dark at room temperature for 72 hours. Then, free Cy5.5-NHS were removed by dialysis against deionized distilled water using a cellulose membrane (MWCO 3500) for 3 days. The final product was lyophilized to give Cy5.5-labeled PAMAM [G4]. After all, Ac_3ManNAz was conjugated to Cy5.5-labeled PAMAM [G4] to give Cy5.5-labeled pMPs, using the same process of preparation of pMPs.

2.5 Cell culture

U87 (Human glioblastoma) cells, HDF (Human dermal fibroblasts) cells, MCF-7 (Human mammary carcinoma) cells, human breast adenocarcinoma cells (MDA-MB-468 and MDA-MB-436), KB (Human epidermoid carcinoma) cells, and A549 (Human lung adenocarcinoma) cells were purchased from ATCC (Manassas, VA, USA). Those cell lines were maintained in RPMI1640 (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS; Welgene, Daegu, Korea), 100 μg/ml streptomycin, and 100 U/ml penicillin (Welgene, Daegu, Korea) in a humidified 5% CO_2 atmosphere at 37℃.

2.6 Cell viability Assay

U87 cells were seeded on 96-well plates ($5x10^3$ cells/well) and incubated for one day. To measure the cytotoxicity of pMPs, the cells were treated with Ac_3ManNAz, PAMAM [G4], or pMPs with various concentration of Ac_3ManNAz: 6.25, 12.5, 25, 50, 100 uM. After incubation for two days, the cells were washed twice with DPBS (pH 7.4) and 20 uL of MTT in serum-free RPMI1640 media (0.5 mg/mL) was added to each well. After further incubation for 2 h at 37 ºC, the media was removed and cells were dissolved in 200 uL of DMSO. Then, the absorbance of each well was measured at

572 nm using a microplate reader (VERSAmaxTM, Molecular Devices Corp., Sunnyvale, CA).

2.7 Western blot analysis of cells

U87 cells were seeded onto 100 x 20 mm cell culture plates at a density of 2 x 10^6 cells per plate in 12 mL of media with Ac_3ManNAz (50 μM) or pMPs (50 μM Ac_3ManNAz). After 2 days of incubation, the cells were washed twice with DPBS (pH 7.4) and harvested from the plates using a cell scraper. The cells were pelleted by centrifugation at 1500 rpm for 5 min, and the cell pellets were re-suspended in 500 μl of lysis buffer (1 % SDS, 100 mM Tris·HCl, pH 7.4) containing protease inhibitor cocktail (Complete, EDTA-free, Roche, NSW, Australia). They were lysed with a probe-type sonifier at 4 ºC. Sonicated lysates were incubated at 4 ºC for 30 min to further solubilized proteins, and insoluble debris was removed by centrifugation for 10 min at 3,000 x g. Final soluble protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) to be 5 mg/ml. Then, 20 μl of the lysate was incubated with 2 μl of phosphine-PEG_3-biotin (5 mM in DPBS) (Pierce, Rockford, IL, USA) for 6 hours at 37 ºC. SDS-PAGE loading buffer was added to each sample and the samples were heated at 95 ºC, before loading onto 10% SPS-PAGE gel. Proteins were transferred to Hybond P membrane (Amercham, St.

Albans, UK), and the membrane was blocked with 5 % bovine serum albumin (BSA) in TBST (50 mM Tris·HCl, 150 mM NaCl, 0.1 % Tween20, pH 7.4) for 2 hours. Then, the membrane was incubated with streptavidin-HRP (diluted 1:2000 in TBST) (Pierce, Rockford, IL, USA) overnight at 4 ºC. The membrane was rinsed three times with TBST and developed by ECL Western Blotting Substrate (Pierce, Rockford, IL, USA).

2.8 Cellular imaging to determine the generated azide groups

U87 and HDF cells were seeded onto 35 mm glass-bottom dishes at a density of 2 x 10^4 cells in 2 mL of media with no sugar, Ac_3ManNAz (50 μM), or pMPs (50 μM Ac_3ManNAz). After incubation for two days, the cells were washed twice with DPBS (pH 7.4) and incubated for 1 hour with ADIBO-Cy5.5 (20 μM, final concentration) (Future Chem, Seoul, Korea) in 37 ºC incubator. They were rinsed with DPBS (pH 7.4) and fixed with a fixative containing formaldehyde-glutaraldehyde for 15 min at room temperature. Then, the cells were washed with DPBS (pH 7.4) again and stained with DAPI (Invitrogen, Carlsbad, CA, USA) to label nuclei. All cellular images were obtained by a confocal laser microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany) with 405 diode (405 nm) and He-Ne (633nm) lasers.

2.9 Cellular imaging of the bindings of click molecules or biological ligands to various tumor cells

ADIBO-Cy5.5 (FutureChem, Seoul, Korea) was used as purchased with no further purification. In order to prepare cRGD-PEG-Cy5.5, similar amide coupling of c(RGDyK) with (t-Boc)-protected PEG-NHS (Mw 2,000), followed by TFA cleavage was carried out. The cRGD-PEG conjugate (8 mg; 2 μmol) was incubated with Cy5.5-NHS (10 μmol) in 500 μL of phosphate buffer (10 mM, pH 7.4) for 2 h in the dark at room temperature. Final product was purified by HPLC. Cetuximab-Cy5.5 was synthesized by the reaction of anti-EGFR monoclonal antibody, Cetuximab (3.3 nmol), and Cy5.5-NHS (40 nmol) in phosphate buffer (0.1 M, pH 8.5). After 2 h reaction, the mixture was purified using PD-10 column to remove unreacted Cy5.5. To prepare folate-PEG-Cy5.5, folic acid (10 mg) was added into 60 mL of ethylenediamine (EDA) and stirred in the dark for 3 h at room temperature. After precipitation in acetone twice, the precipitate was dissolved in water and filtrated to remove the insoluble substance. Then, this folate-EDA (20 mg, 24 μmol) was incubated with (t-Boc)-NH_2-PEG-NHS (Mw 2,000) in 2.5 mL DMSO for 2 h, and treated by TFA for deprotection of t-Boc. The folate-PEG conjugates (10 mg; 4 μmol) was incubated with Cy5.5-NHS (10 μmol) in 500 μL phosphate buffer (10 mM, pH 7.4) for 2 h

in the dark at room temperature. The final product, folate-PEG-Cy5.5 conjugate was purified by HPLC.

All cells were seeded onto 35 mm glass-bottom dishes at a density of 3 x 10^4 cells in 2 mL of growth media with no sugar or pMPs (50 μM Ac_3ManNAz). After 2 days of incubation, the cells were washed twice with DPBS (pH 7.4), and incubated with ADIBO-Cy5.5 (20 μM, final concentration) for 30 min at 37 °C for cellular imaging. The same method was applied to all cell lines: U87, MCF-7, MDA-MB-468, MDA-MB-436, KB, and A549 cells.

For the comparative study using traditional biological binding molecules, all cells were seeded onto 35 mm glass-bottom dishes at a density of 3 x 10^4 cells in 2 mL of growth media and grown to reach 60-80 % confluence. The cells were washed twice with DPBS (pH 7.4), and incubated with cRGD-PEG-Cy5.5 (0.2 μM, final concentration), Cetuximab-Cy5.5 (0.1 μM, final concentration), or Folate-PEG-Cy5.5 (0.2 μM, final concentration) for 30 min at 37 °C for cellular imaging. Fluorescence images were observed using confocal laser microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany) with 405 diode (405 nm) and He-Ne (633nm) lasers.

2.10 Flow cytometry analysis

U87 cells were seeded onto 6-well plates at a density of 2 x 10^4 cells per well in 2 mL of media with no sugar or pMPs (50 μM Ac_3ManNAz) treated with ADIBO-Cy5.5, as described previously. The cells were lifted with DPBS containing 2 % FBS (flow cytometry buffer) and washed twice in FACS buffer (1 mM EDTA). Twenty thousand cells per sample were performed by flow cytometry (BD FACSVerse, BD Biosciences, San Jose, CA, USA), and subsequent data analysis was analyzed using FlowJo software.

2.11 *In vivo*/*ex vivo* imaging

All experiments with live animals were performed in compliance with the laws and institutional guidelines of Korea Institute of Science and Technology (KIST) and approved by institutional committees. For *in vivo* and *ex vivo* experiments, U87 tumor cells (1.0 x 10^7 cells) were subcutaneously planted on flank of 5-week-old male athymic nude mice (20 g, Orient, Gyeonggi-do, Korea). When the tumor reached about 100 mm^2, the experiments were conducted. Cy5.5-labeled pMPs (40 mg Ac_3ManNAz/kg) was injected intravenously once. The mice model was observed over 3 days to display *in vivo* biodistribution of pMPs. Near-

infrared fluorescence (NIRF) images were observed using the IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA).

For analysis of expression of azide groups on tumor tissue, free Ac_3ManNAz or pMPs (40 mg Ac_3ManNAz/kg) were injected daily for 2 days. After that, ADIBO-Cy5.5 (10 mM, 40 μL) was injected (n=3 per each experimental group). All the injections were done intravenously. The biodistribution and time-dependent tumor accumulation profiles were non-invasively imaged by IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA). All data were calculated by the region of interest (ROI) function of the IVIS molecular imaging software.

The major organs and tumors were dissected from mice 72 hours post-injection of Cy5.5-labeled pMPs. For analysis of expression of azide groups on tumor tissue, the tumors were dissected from mice 24 hours post-injection of free Ac_3ManNAz or pMPs (40 mg Ac_3ManNAz). *Ex vivo* images were also obtained with a IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA), and the fluorescence signal intensity at the ROI using IVIS molecular imaging software.

2.12 Histological imaging

For histological evaluation, the dissected tumor tissues were fixed in 4 % (v/v) buffered formalin solution, and embedded in optimum cutting

temperature (OCT) compound (Sakura, Tokyo, Japan) on dry ice. Sections were cut on a cryostat with 6 μm in thickness and picked up on slides with poly-D-lysine, dried at 45 °C under protection from light. Fluorescence of tumor tissue sections was observed (excitation: 673 nm, emission: 692 nm) by IX81-ZDC focus drift compensating microscope (Olympus, Tokyo, Japan).

2.13 Western blot analysis of tumor tissues

Preparation of U87 tumor-bearing mice models and intravenous injection of Ac_3ManNAz (40 mg/kg) or pMPs (40 mg Ac_3ManNAz/kg) were performed by the same method. 48 hours post-injection of Ac_3ManNAz or pMPs, the major organs and tumors were dissected and transferred into 1 ml of lysis buffer (1 % SDS, 100 mM Tris·HCl, pH 7.4) containing protease inhibitor cocktail (Complete, EDTA-free, Roche, NSW, Australia) and homogenized. The lysates were incubated at 4 ºC for 30 min, and insoluble debris was removed by centrifugation for 10 min at 3,000 x g. Using BCA protein assay kit, total soluble protein concentrations were measured. Staudinger reaction with phosphine-PEG_3-biotin, SDS-PAGE, and western blot analysis were performed as described in the previous section.

2.14 Immunohistochemical analysis

The dissected tumor tissues were retrieved from U87 tumor-bearing mice, and fixed in 4 % (v/v) buffered formalin solution. The tissues were dehydrated with a graded ethanol series, and embedded in paraffin. The paraffin tissues were sliced in 4 μm wide slices, and immunohistochemical staining was performed using phosphine-PEG_3-biotin and streptavidin-HRP followed by the standard methods. The paraffin slices were dried and then mounted with Permount SP15-100 Toluene Solution (Fisher Scientific, NJ, USA) mounting media. The samples were observed by a light microscope (BX51, Olympus, Tokyo, Japan), and were photographed using a digital camera photomicroscope (DP71, Olympus, Tokyo, Japan).

3. RESULTS AND DISCUSSION

3.1 Characterization of pMPs

Poly (amido amine) (PAMAM) [G4] dendrimer is used as a basic structure of the polymerized metabolic precursors (pMPs) with a well-established characteristics, and Ac_3ManNAz is used for metabolic glycoengineering of sialic acids (Figure 1b). Ac_3ManNAz can act as a building block in cell metabolism, since it can easily produce unnatural sialic acid, the most abundant glycan on tumor cell surface[10]. Conjugation of PAMAM [G4] dendrimer and Ac_3ManNAz is completed by *Steglich* esterification mechanism (Figure 2). A completion of the reaction was determined by TLC (Figure 3). The free PAMAM [G4] dendrimer was stayed on the start line because of its high polarity, and pMPs was mobilized at maximum height on 3 days after the reaction. There is no further movement of pMPs after 3 days of reaction; the reaction time was fixed as 3 days. After the reaction, free Ac_3ManNAz, free PAMAM, and pMPs are analyzed by ^{1}H NMR (Figure 4). By comparison of the reactants and product, ^{1}H NMR peaks promised that the reaction was successfully completed. In addition, MALDI-TOF determined molecular weight of free PAMAM (m/z calculated: 20615, found: 19277) and that of pMPs (m/z calculated: 45447, found: 38599) (Figure 5). The Ac_3ManNAz conjugation

efficiency was confirmed as 82.58 % using HPLC. Moreover, the size of pMPs was 3.88 ± 0.51 nm in aqueous condition as determined by dynamic light scattering (DLS). It promise that pMPs is small enough to freely move in blood flow and show no further aggregation.

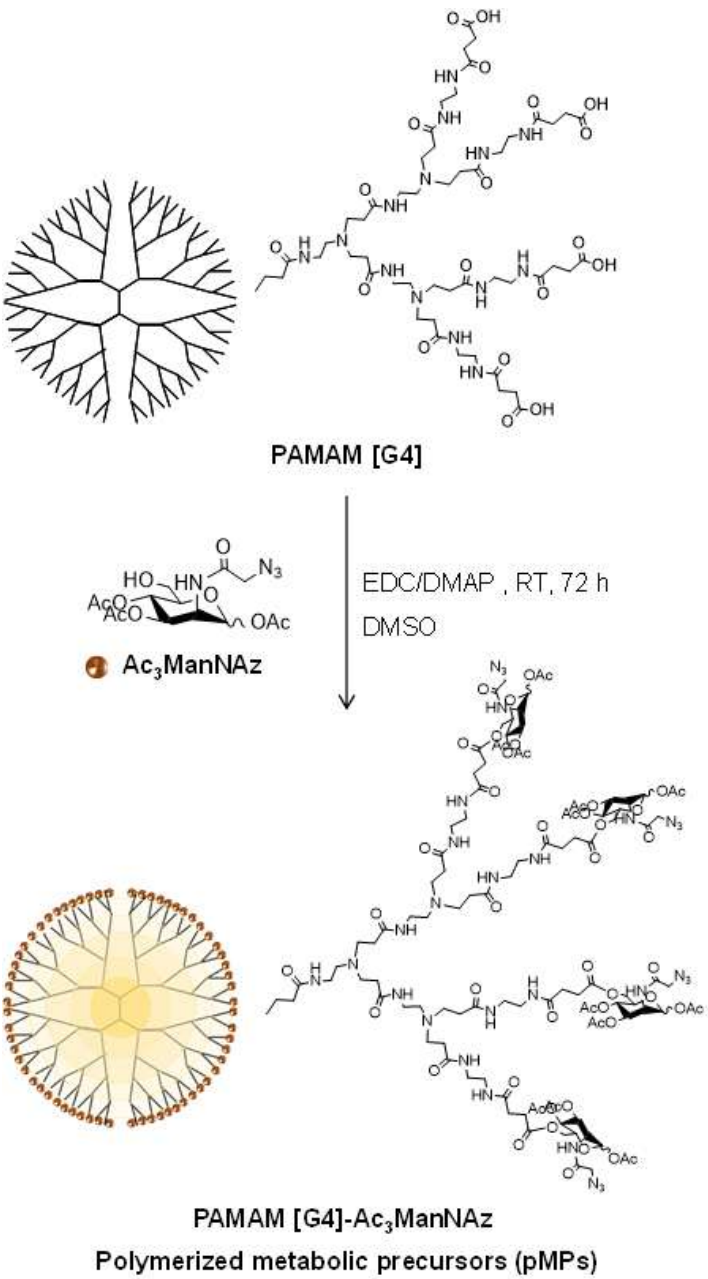

Figure 2. Synthetic scheme of pMPs

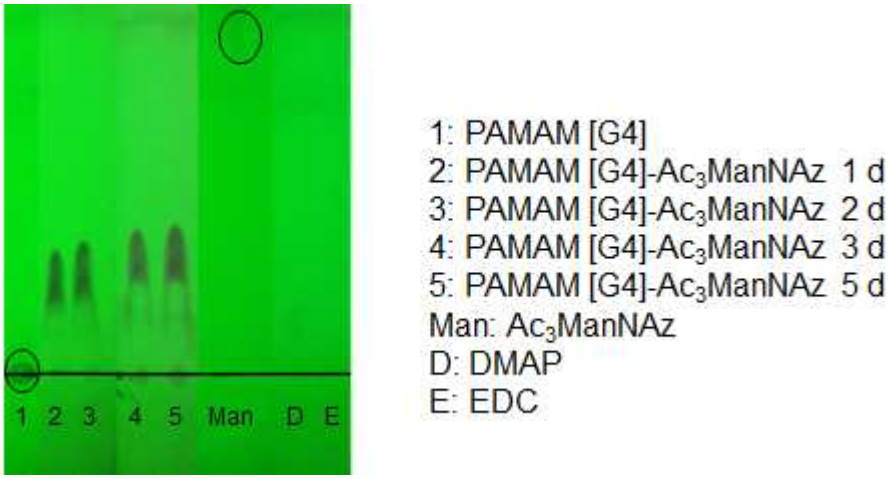

Figure 3. TLC analysis

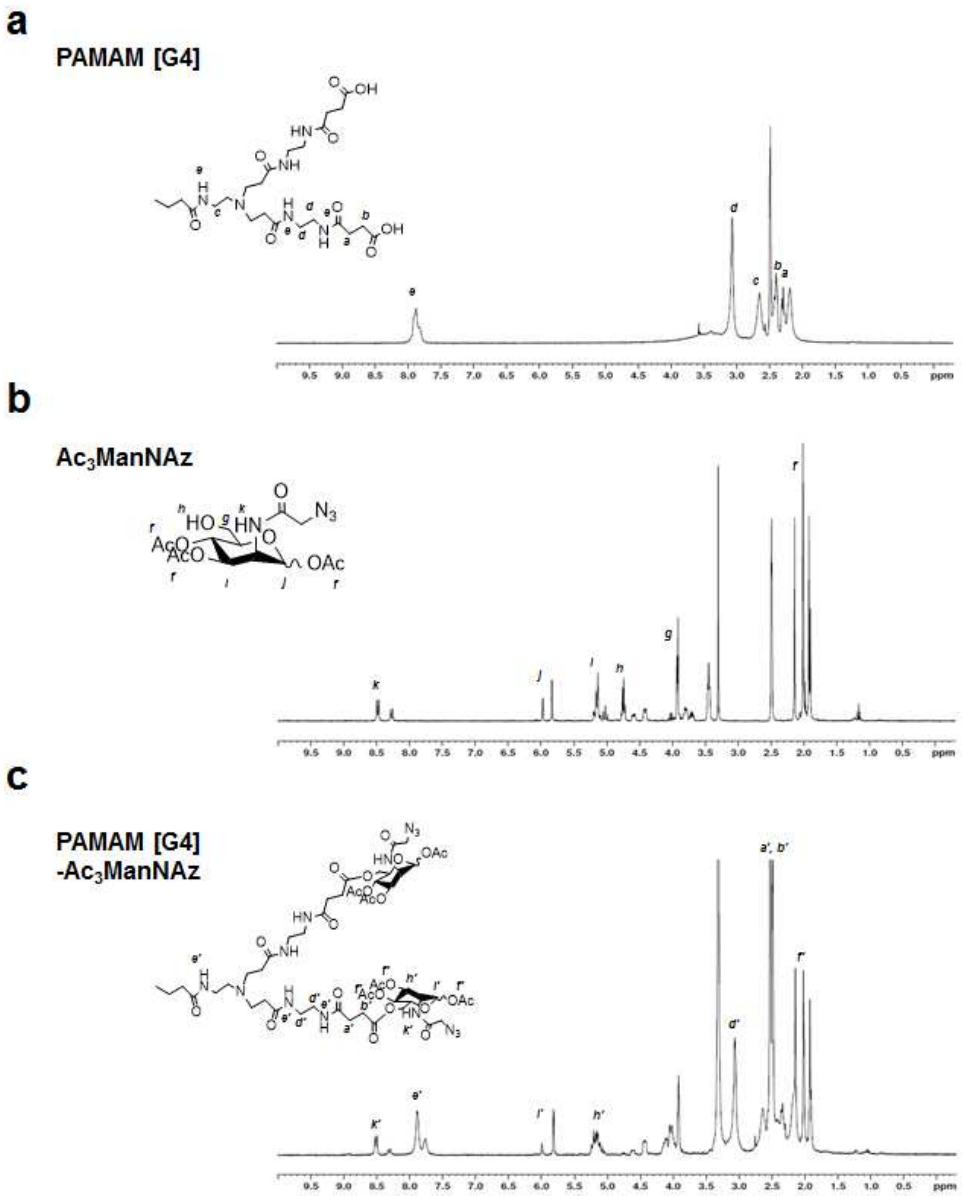

Figure 4. ^{1}H-NMR spectra of (a) PAMAM [G4], (b) $Ac_3ManNAz$, and (c) PAMAM [G4]-$Ac_3ManNAz$. 300 MHz NMR, solvent; DMSO-d_6.

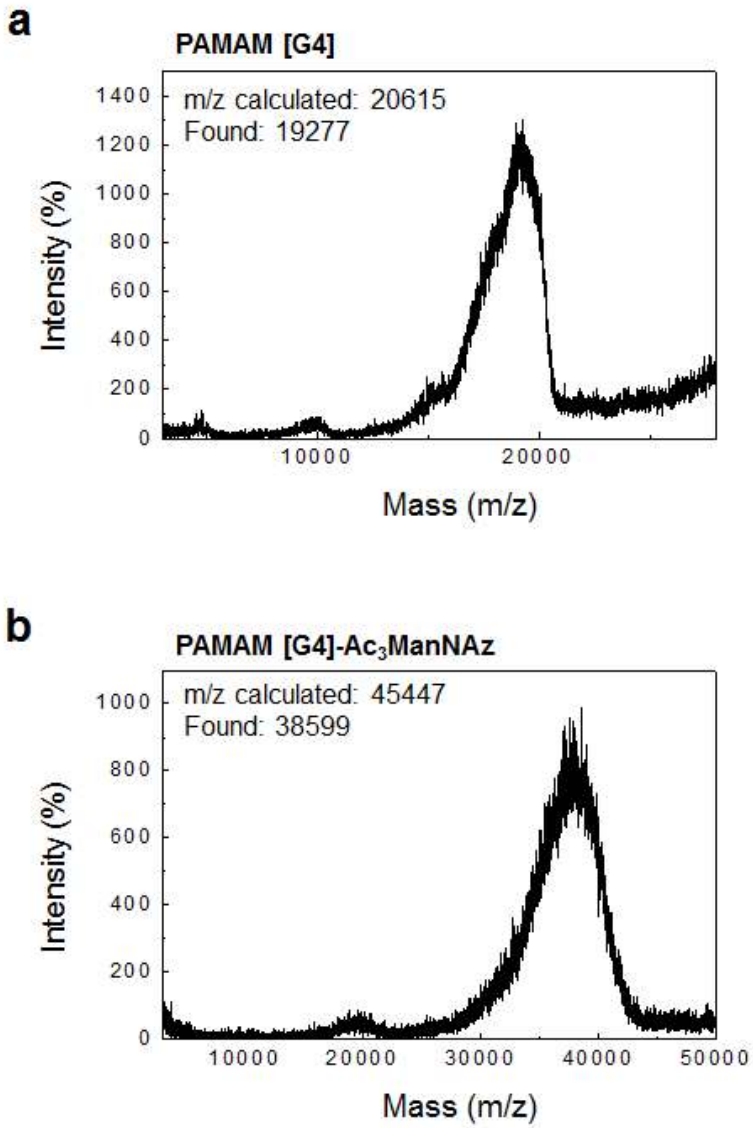

Figure 5. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) data of (a) PAMAM [G4] and (b) PAMAM [G4]-Ac_3ManNAz.

3.2 *In vitro* studies of pMPs

In vitro studies establish the generation of azide groups on the surface of tumor cells. First, azide group generation on cell surface was determined by cellular imaging and western blot analysis. Free Ac_3ManNAz or pMPs was added to U87 tumor cells, and azide groups were determined by binding with ADIBO-Cy5.5 (red fluorescence). After treatment of free Ac_3ManNAz or pMPs for 2 days, the viability of U87 cells was above 90% showing no significant cytotoxicity (Figure 6). ADIBO is chosen as a bioorthogonal chemical group functionalized with its high reactivity to azide groups via copper-free click chemistry[5b]. After treatment with ADIBO-Cy5.5, a higher fluorescence intensity of Cy5.5 was observed in both free Ac_3ManNAz and pMPs treated U87 tumor cells than in untreated cells (Figure 7a). The microscopic images indicated that artificially introduced azide groups on the tumor cell surface were successfully generated by free Ac_3ManNAz and pMPs, and effectively targeted by ADIBO-Cy5.5. Unlike U87 cells, a normal cell line, such as HDF (human dermal fibroblast) cell, did not show a strong signal of fluorescence after treatment of free Ac_3ManNAz or pMPs (Figure 7c). As mentioned earlier, the surface of tumor cell is especially rich in sialic acid. Therefore, even though Ac_3ManNAz can easily make unnatural sialic acid through metabolic glycoengineering, HDF normal cells cannot be visualized as U87 tumor cells. Western blot analysis using

phosphine-PEG_3-biotin and streptavidin-HRP likewise demonstrated the generation of azide groups on tumor cells after the treatment of free Ac_3ManNAz or pMPs (Figure 7b)[11]. Coomassie staining verifies that the total loading amount of proteins for each group of cell lysates is similar.

The heterogeneity of tumor cells complicates the circumstances for various subpopulations of tumor cells expressing different types and amounts of receptors[9]. Tumor is increasingly acknowledged as a combination of many disorders, each with varying causes, prognoses, and appropriate treatments. This diversity is recognizable not only across different types of tumor, but also within tumors of the same tissue. It is now known that cancer cells within the same tumor mass are heterogeneous in many aspects, including morphology or phenotypic expression, exhibition of inherent or acquired drug resistance, and capacity for initiating new tumor growth[9]. The heterogeneity and complexity of tumor is a huge obstruction of present drug delivery systems. However, our strategy can generate 'receptor-like' chemical groups on heterogeneous tumor cells by metabolic glycoengineering and overcome the limitation of current drug delivery systems. To demonstrate, a comparative study using representative click molecules and biological ligands was performed on various cell types: U87, MCF-7, MDA-MB-468, MDA-M-436, KB, and A549. ADIBO was used as a click molecule, and biological ligands such as cRGD (peptide), Cetuximab

(antibody), and folate were used, in order to label the azide group with Cy5.5 dye. The biological receptors for these ligands are known to be expressed in specific tumor cells, and each ligand labeled with Cy5.5 was bound to the targeted cells only as expected. However, after treatment of pMPs, all cell types (U87, MCF-7, MDA-MB-468, MDA-M-436, KB, and A549) were positively visualized on cellular imaging with ADIBO-Cy5.5 (Figure 8a). The azide groups were generated on all cell types by metabolic glycoengineering, so they could bind with ADIBO-Cy5.5. The fluorescence intensity of these cells was quantified using flow cytometry analysis. Compared to the control cells with no treatment, the tumor cells treated with pMPs indicated a higher intensity (Figure 8b). These results show that azide groups are successfully generated from pMPs and formed on the surface of various cell lines. These results demonstrate that polymerized metabolic precursors can overcome the heterogeneity of tumor cells.

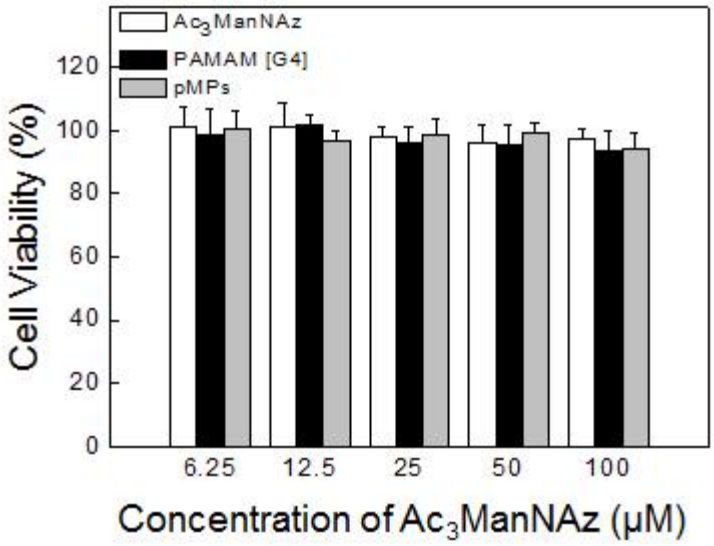

Figure 6. Viability of tumor cells treated with pMPs for 2 days.

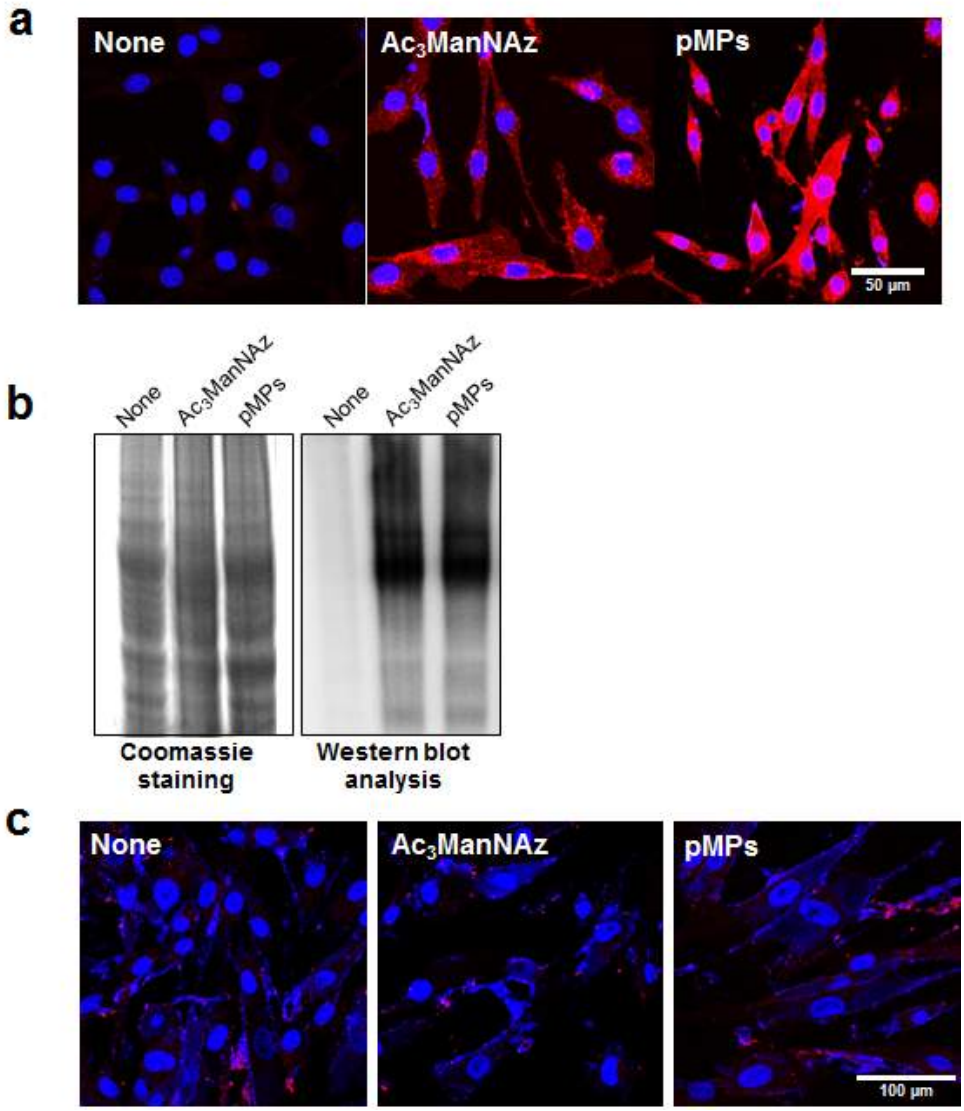

Figure 7. Azide group generation on cell surface after treatment of pPMs. (a) Visualization of azide groups on the surface of U87 tumor cells after treatment of pMPs. (b) Coomassie staining and wetern blot analysis of U87 tumor cells treated with pMPs. (c) Visualization of azide groups on the surface of human dermal fibroblasts (HDFs, normal cells) after treatment of pMPs.

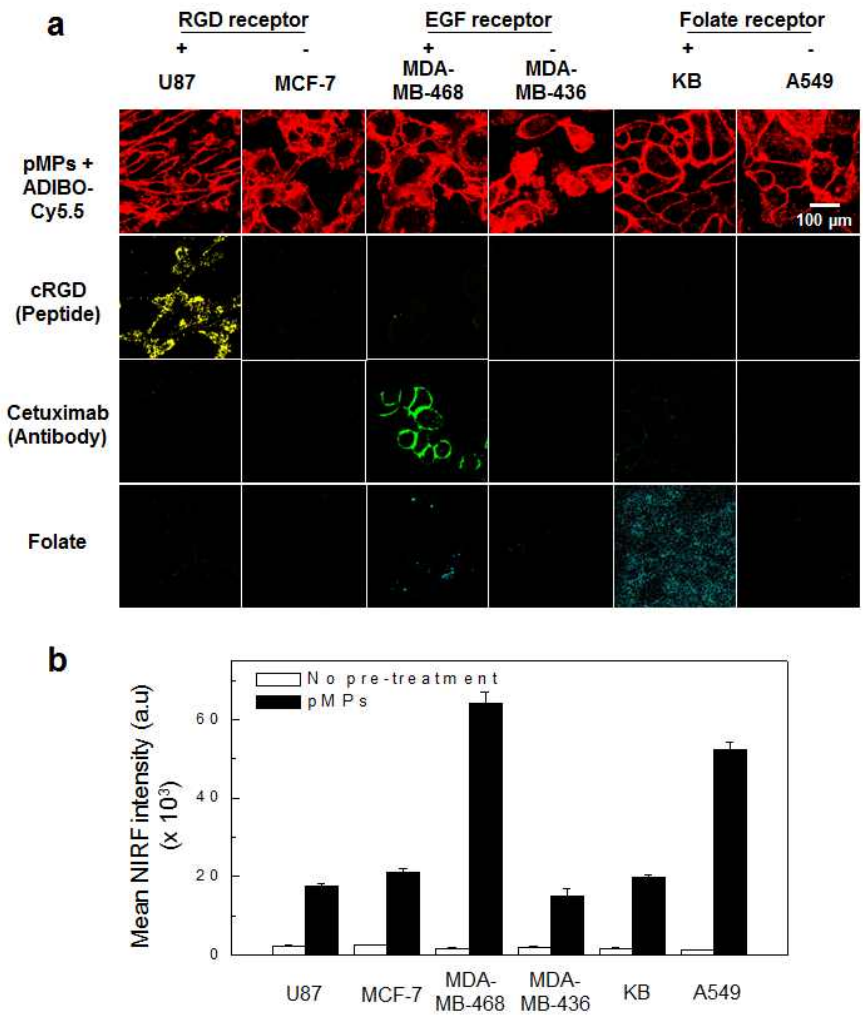

Figure 8. Generation of azide groups by pMPs in various tumor cells. (a) The binding of click molecules and biological ligands to tumor cells. ADIBO-Cy5.5, cRGD-PEG-Cy5.5, Cetuximab-Cy5.5, and Folate-PEG-Cy5.5 were visualized in red, yellow, green, and blue fluorescence (pseudo colors). (b) Flow cytometry analysis of tumor cells after treatment of pMPs and ADIBO-Cy5.5. ADIBO-Cy5.5 was added to tumor cells treated with pMPs (50 μM) for 2 days.

3.3 *In vivo* studies of pMPs

For *in vivo* studies, balb/*c* nude mice models bearing xenograft U87 tumor on one side of flank are prepared. *In vivo* experiments start when the tumor size reached about 100 mm^3. First, targetable glycans, unnatural sialic acids with azide groups, are artificially generated in target cancer cells by an intratumoral injection of the precursor, pMPs. Free Ac_3ManNAz or pMPs were administered to each tumor model by intratumoral injection once a day for 3 days while saline was injected to the control mice model. The coomassie blue staining and western blot analysis of tumor tissues showed that the unnatural sialic acid with azide groups similarly with the cellular conditions (Figure 9).

The biodistribution of pMPs is evaluated by time-dependent whole body imaging and *ex vivo* fluorescence analysis[12]. Whole body distribution was measured using a non-invasive IVIS Lumina K Series III *in vivo* imaging system in live animals. Cy5.5 labeled pMPs was intravenously injected once for fluorescent tracking. The images showed a slow excretion of pMPs over time (Figure 10a). Fluorescence intensities of tumor tissues from the images were quantified and plotted with a mild decreasing curve (Figure 10b). Total photon count in tumor site is distinctively higher in Cy5.5-labeled pMPs than free Cy5.5. Based on the EPR effect[12], pMPs reached to the tumor more specifically and remained there using its characteristics of polymer.

Therefore, compared to small molecules like free Cy5.5 dye, pMPs bring a slower excretion through the whole body, especially from the tumor. In *ex vivo* fluorescence analysis of major organs and tumors, tumor tissue from mice pretreated with pMPs exhibited higher fluorescence intensity than other major organ tissues, except for the liver (Figure 10c and 10d). We expected that a strong signal from the liver was caused as a part of enterohepatic circulation.

For intravenous injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*, western blot analysis, whole body and *ex vivo* fluorescence imaging were evaluated. Three different models of mice were prepared with saline, free Ac_3ManNAz, or pMPs by intravenous injection. After 2 days of injection, ADIBO-Cy5.5 was also injected through tail vein to label the azide groups on cell surface by click chemistry. The tumors were collected and analyzed after all the injections. Compared to saline and free Ac_3ManNAz, the tumor tissue from mice injected with pMPs revealed a strong band in western blot analysis (Figure 11a). The result showed that a large number of azide groups were generated on tumor tissue from pMPs by metabolic glycoengineering. The azide groups can be specifically generated in tumor tissue by pMPs because of its tumor-targeting ability via the EPR effect. The tumor tissue from mice pretreated with pMPs shows significantly more intense fluorescence *in vivo* images than tumor tissues pretreated with

saline or free Ac_3ManNAz (Figure 11b). Even though the number of azide groups generated by Ac_3ManNAz and pMPs were similar *in vitro*, the *in vivo* images were different because free Ac_3ManNAz is a small molecule that has no tumor-targeting ability. The tumors from each group were dissected after 24 hours from injection of ADIBO-Cy5.5. They were analyzed by *ex vivo* fluorescence images and tumor tissue from mice pretreated with pMPs showed distinctively higher intensity of fluorescence than tumors from other group pretreated with saline or free Ac_3ManNAz (Figure 11c and 11d). Furthermore, immuohistological staining supports the metabolic glycoengineering of pMPs. The dark brown spots which show the expression of azide group are intense in tumor tissue, compared to other organ tissues (Figure 12). The immunohistology also demonstrates that metabolic glycoengineering can specifically occur on the tumor tissue after intravenous injection of pMPs. A large number of azide groups can be successfully generated on tumor tissue *in vivo*. More specifically, histological evaluation proves the generation of azide group from pMPs by the accumulation of ADIBO-Cy5.5 in tumor tissue (Figure 13). It was significantly higher in pMPs pretreated mice group than other groups with saline or free Ac_3ManNAz.

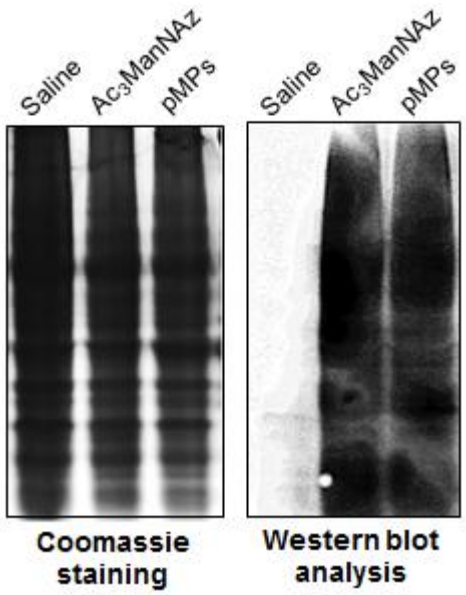

Figure 9. Intratumoral injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*. Coomassie staining and wetern blot analysis of Ac_3ManNAz or pMPs treated tumor tissues.

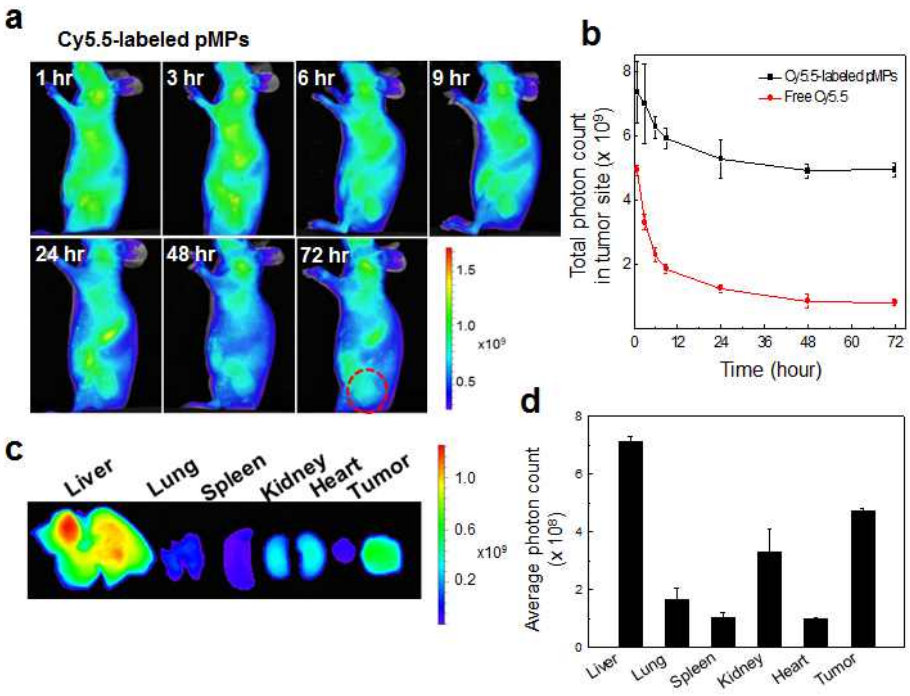

Figure 10. Biodistribution of pMPs in tumor-bearing mice. (a) Whole body images of mice after intravenous injection of Cy5.5-labeled pMPs. (b) Fluorescence intensities of tumor tissues from (a). (c) *Ex vivo* fluorescence images of major organs and tumor from 72 h post-injection of pMPs. (d) Fluorescence intensities of major organs and tumor tissue from (c).

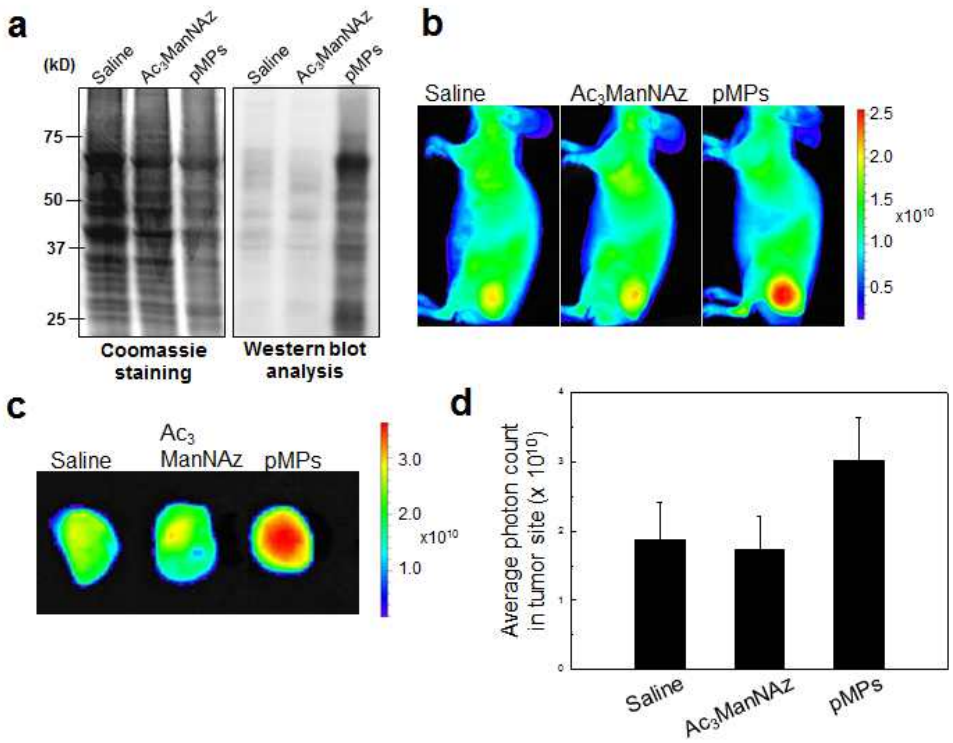

Figure 11. Intravenous injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*. (a) Coomassie staining and wetern blot analysis of tumor tissues after intravenous injection of saline, free $Ac_3ManNaz$, or pMPs. (b) Whole body images of mice after intraveneous injection of ADIBO-Cy5.5, pretreated with pMPs. (c) *Ex vivo* fluorescence images of tumors from each group 24 h postinjection of ADIBO-Cy5.5. (d) Fluorescence intensities of tumor tissues from (c).

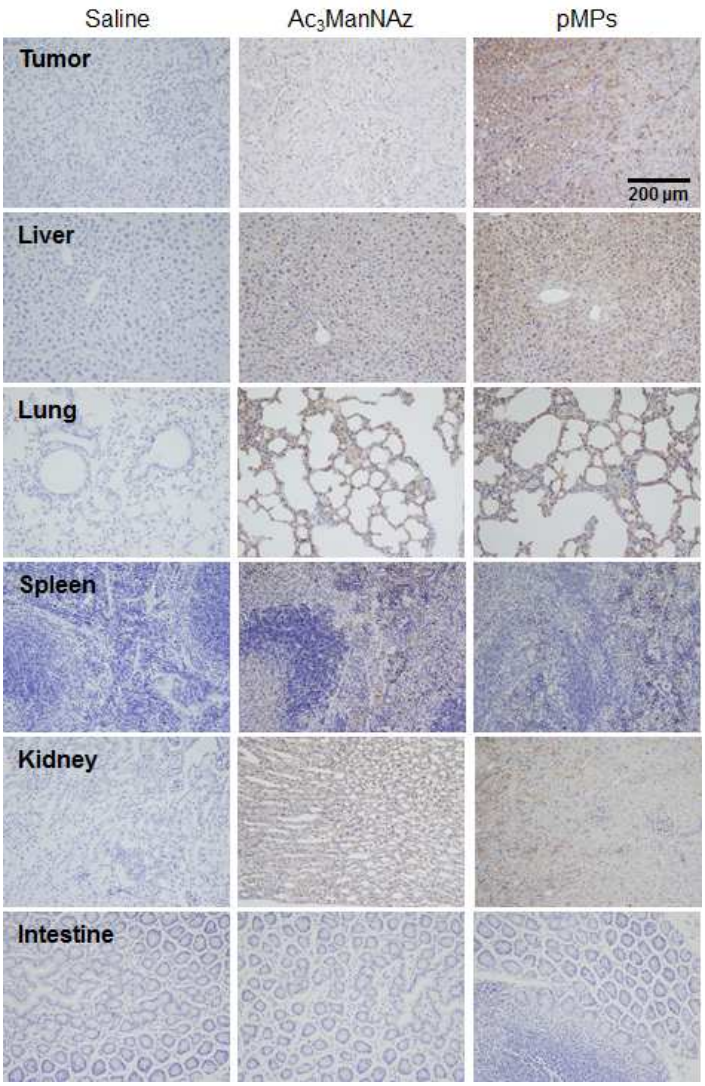

Figure 12. Intravenous injection of saline, free Ac_3ManNAz, and pMPs and metabolic glycoengineering on tumor tissue *in vivo*. Histological staining of major organs and tumor tissues after intravenous injection of saline (left panel), free Ac_3ManNAz (middle panel), and pMPs (right panel).

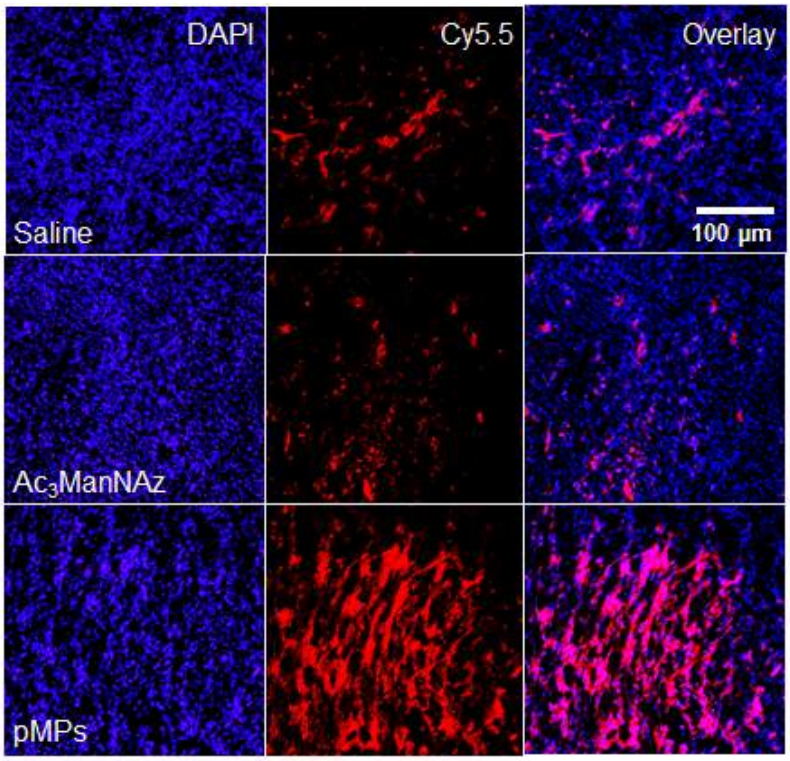

Figure 13. Fluorescence images of tumor tissues after intravenous injection of ADIBO-Cy5.5 to tumor-bearing mice pretreated with saline (top panel), free Ac_3ManNAz (middle panel), or pMPs (bottom panel).

4. CONCLUSION

In this study, we have designed polymerized metabolic precursors (pMPs) to overcome tumor heterogeneity via metabolic glycoengineering and click chemistry. PAMAM [G4] dendrimers are conjugated with Ac_3ManNAz to produce pMPs. The intravenously injected pMPs reach to the tumor cell specifically by EPR effect and successfully produce azide groups on the surface of tumor cells. In particular, an expression of azide groups enables a generation of 'receptor-like' chemical reporters from pMPs itself. Furthermore, by using AIBO-Cy5.5, these azide reporters in the tumor tissue can be labeled via copper-free click chemistry *in vivo* condition. Thus, the tumor can be specifically visualized by optical imaging system. Our results suggest a new targeting strategy which can overcome tumor heterogeneity using site-specific metabolic glycoengineering and copper-free click chemistry. Since the whole procedure is conducted of intravenous injections only, allowing it to be applied to tumor diagnosis and therapy even when the location of the tumor tissue is not well defined. Our strategy significantly demonstrates that it may have a great potential for further biomedical applications as diagnostic or therapeutic probes.

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요약문

기존에 암 치료는 항암제를 단독으로 직접 투여하는 방식을 사용하였다. 하지만 이러한 방법은 암 세포 뿐만 아니라 정상 세포까지 독성을 야기하는 등 다양한 부작용을 나타내었다. 이러한 문제점을 해결하기 위해서 항암제를 표적 암 세포에 전달하는 다양한 나노기술이 개발되어왔다. 본 연구에서는 고분자화 대사 전구체를 이용하여 암 표적화 능력을 향상 시키는 방법에 대한 것이다. 또한, 암 세포가 갖는 고유 수용체의 양적 제한에서 비롯된 표적 능력의 한계 및 암 이질성을 극복할 수 있는 방법에 대한 것이다. 이 방법은 대사 당쇄 조작과 무동 클릭 화학을 이용하였다. PAMAM [G4] 덴드리머와 Triacetylated N-azidoacetyl-D-mannosamine ($Ac_3ManNAz$)의 화학적 결합을 이용하여 암 조직에 특이적으로 인공 표적 수용체인 azide 기를 도입시키는 방법이며, 이 방법을 이용하면 이질성을 갖는 암 세포들을 단일화된 표현 형질로 바꿀 수 있다. 또한 이렇게 도입된 인공 표적 수용체를 표적으로 하는 클릭 화학을 이용하면 암 표적화 능력을 향상 시킬 수 있다. 본 연구에서 개발

된 고분자화 대사 전구체를 이용한 암 표적화 능력 향상 방법을 이용하면 이질성을 갖는 암의 종류에 상관없이 표적 능력을 높일 수 있을 것으로 예상된다.

주요 어: 고분자화 대사 전구체, 종양 이질성, 물질 대사 당공학, 클릭 화학, 광학 이미징

학번: 2013-20621

공학석사학위논문

A Targeting Strategy to Overcome Tumor Heterogeneity Using Polymerized Metabolic Precursors

고분자화 대사 전구체를 이용한 종양 이질성 극복 가능한 표적화 전략

2015 년 8 월

서울대학교 공과대학원

재료공학부

정 슬 희

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지도교수 안 철 희

이 논문을 공학석사 학위논문으로 제출함

2015 년 6 월

서울대학교 공과대학원

재료공학부

정 슬 희

정 슬 희 의 공학석사 학위논문을 인준함

2015 년 6 월

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ABSTRACT

A Targeting Strategy to Overcome Tumor Heterogeneity Using Polymerized Metabolic Precursors

Seulhee Jung

Department of Materials Science and Engineering

College of Engineering

Seoul National University

Historically, drug as a free form has been directly used in cancer treatment. However, while the anticancer drugs can be distributed throughout the whole body, it is very toxic and causes many side effects to normal tissues. To overcome this problem, many nano-technologies have been applied for an effective delivery of anticancer drugs to the targeting cancer cells. In this study, we developed polymerized metabolic precursor (pMPs) to improve tumor-targeting abilities. Furthermore, the pMPs is designed to overcome the intrinsic limitations causing from the limited amount of cellular receptors and the heterogeneity of tumor cells. The pMPs are based on metabolic glycoengineering and click chemistry. Triacetylated *N*-

azidoacetyl-*D*-mannosamine (Ac_3ManNAz) is conjugated to the PAMAM [G4] dendrimer backbone and the compound generates azide groups on the surface of tumor tissue specifically. Regardless of tumor cell types, pMPs create a more uniform cancer cells with artificial glycans on their surface. With the enhanced permeation and retention (EPR) effect followed by metabolic glycoengineering, the pMPs generate 'receptor-like' chemical reporters to produce a homogeneous cancer cell surface in the tumor tissue. Then, they can be labeled by ADIBO-Cy5.5 via copper-free click chemistry *in vivo* condition.

Keywords: Polymerized Metabolic Precursors (pMPs), Tumor Heterogeneity, Metabolic Glycoengineering, Click Chemistry, Optical Imaging

Student number: 2013-20621

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1. INTRODUCTION

Metabolic glycoengineering is a useful biological technique pioneered by Carolyn Bertozzi's group[1]. It utilizes an intrinsic glycan metabolism of cells to introduce unnatural glycans with various chemical groups[2]. When rationally designed metabolic precursors are treated, cells applicate them as building blocks for glycan metabolism. These precursors are inserted within the structure of glycans[3]. Until today, lots of papers studied about a wide range of applications in this technique from precise analysis of glycans at molecular level to spatiotemporal imaging *in vivo*[4]. Particularly, combined with click chemistry, biorthogonal chemical groups can be easily introduced to glycans by this technique and they enable click reactions on the glycans in cells or bodies as well as in *ex vivo* condition for various purposes[5].

For more than one century, several kinds of nanoparticles have shown promising results in imaging and drug delivery[6]. However, their tumor-targeting ability is still limited, and there are many obstacles to improve their efficacy[7]. In particular, current active-targeting method using biological ligands has intrinsic limitations, because the amount of receptors on tumor cells is limited and the binding of nanoparticles can be saturated[8]. In addition, the heterogeneity of tumor cells makes the situation more complicated since numerous subpopulations of tumor cells express different

kinds and amounts of receptors[9]. Heterogeneity can be observed within cancers of same tissue as well as in different types of cancer. In this point of view, we expect that chemical groups can be introduced onto tumor tissue containing heterogeneous tumor cells. If so, they would be used as artificial active-targeting moieties for the delivery of nanoparticles and provide alternative ways to overcome tumor heterogeneity. Importantly, a targeted delivery of metabolite and tumor cell-specific introduction of chemical groups will be much useful for tumor imaging or drug delivery.

Herein, in order to overcome tumor heterogeneity, we developed polymerized metabolic precursors for biorthogonal targeting strategy in living body (Figure 1a). Polymerized metabolic precursors (pMPs) were synthesized by conjugation of triacetylated *N*-azidoacetyl-*D*-mannosamine (Ac_3ManNAz), the precursor for azide generation, to the succinic acid terminated generation 4 poly (amido amine) (PAMAM [G4])-dendrimer backbone (Figure 1b). The pMPs are intravenously injected to tumor-bearing mice. This injection results in site-specific generation of azide groups on tumor tissue due to the EPR effect. Under the acidic tumor microenvironment, it can be hydrolyzed into free form of Ac_3ManNAz which can introduce azide groups onto tumor cells surface by metabolic glycoengineering. Our approach generates ‘receptor-like’ chemical groups on the surface of target cells by pMPs alone. The advantage of this method

over biological receptors is that the small azide groups can expressed on the cell surface in large quantities regardless of the types or subpopulations of heterogeneous tumor cells.

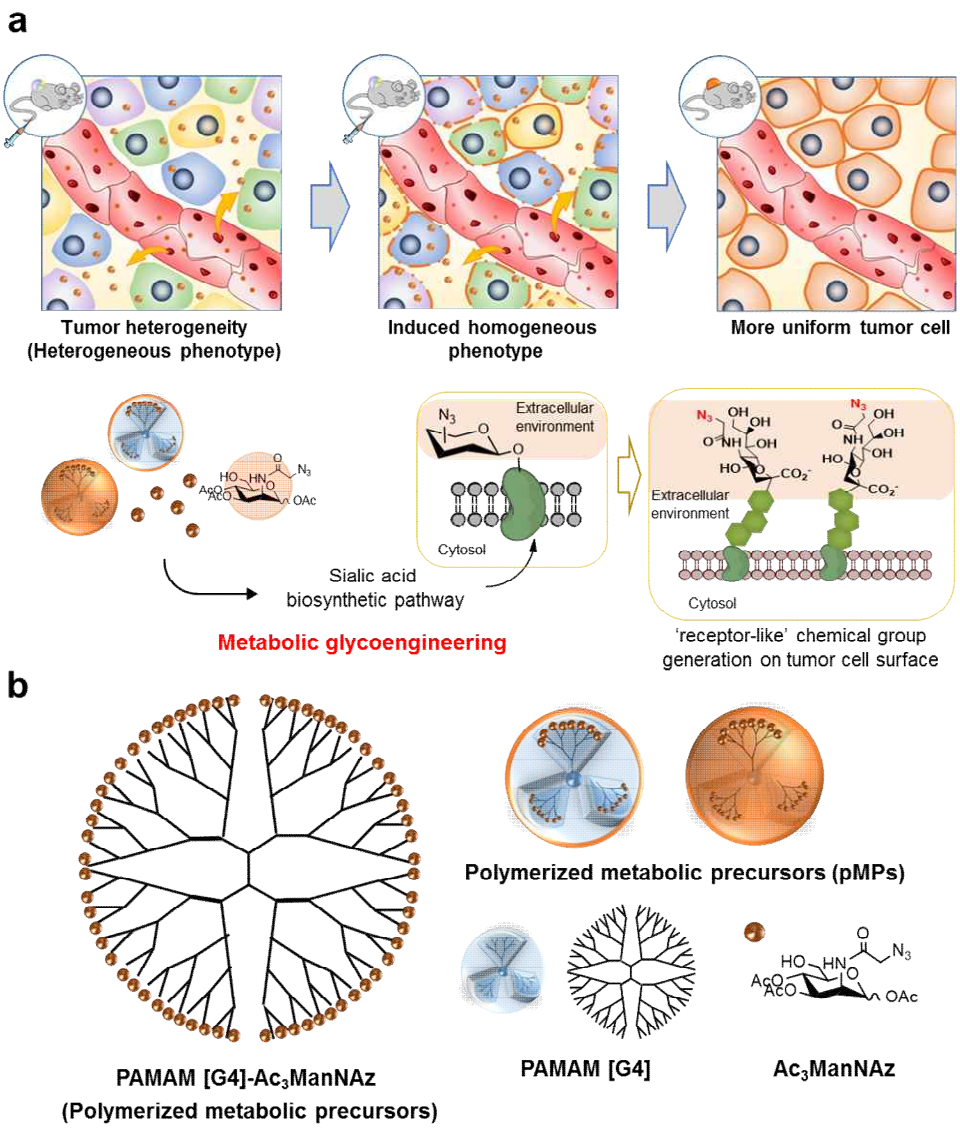

Figure 1. (a) Schematic illustration of the targeting strategy to overcome tumor heterogeneity using polymerized metabolic precursors (pMPs). (b) Chemical structure of pMPs.

2. EXPERIMENTS

2.1 Materials

Succinamic acid terminated generation 4 poly (amido amine) (PAMAM [G4]) dendrimer was purchased as 5 wt% in water from Dendritech, Inc. (Midland, MI, USA). The water was removed by lyophilization before any further experiments. Triacetylated *N*-azidoacetyl-*D*-mannosamine (Ac_3ManNAz) was achieved from FutureChem (Seoul, Korea). 1-ehtyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4-(Dimethylamino) pyridine (DMAP), Dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cy5.5-NHS was acquired from GE Healthcare Life Sciences (Piscataway, NJ, USA). ADIBO-Cy5.5 was purchased from FutureChem (Seoul, Korea).

2.2 Conjugation of PAMAM [G4] Dendrimer-Ac_3ManNAz

Esterification of PAMAM [G4] was carried out according to *Steglich* esterification mechanism. PAMAM [G4] dendrimers were dissolved in 1 ml DMSO in the presence of EDC and DMAP as catalysts at 1 to 1.2 ratios. Then, 3 molar equivalents of triacetylated mannosamine (Ac_3ManNAz) with PAMAM [G4] dendrimers were added to the DMSO solution containing

PAMAM [G4] dendrimers and catalysts. The reactants were stirred at room temperature for 72 hours and were dialyzed in DMSO for another 72 hours using a cellulose membrane (MWCO 3500: Spectrum Laboratories, TX, USA) at room temperature to remove unreacted EDC, DMAP and Ac_3ManNAz. After dialysis, the Ac_3ManNAz conjugated PAMAM [G4] dendrimers were obtained by lyophilization.

2.3 Characterization of PAMAM [G4] Dendrimer-Ac_3ManNAz (Polymerized Metabolic Precursors, pMPs)

Thin Layered Chromatography (TLC) aluminum plates (Silica gel 60 F254, Merck kGaA, Darmstadt, Germany) were used to monitor the progress of the reaction. Reactants and products dissolved in DMSO (0.1 g/ml) were spotted onto a start line on one side of the TLC plates at different time points (1, 2, 3, 5 days). In TLC chambers, chloroform/methanol (2:1, *v/v*) was used as a developing eluent for the plates. After evaporation of developing solvents, chromatographic spots of samples were exposed under uv lamp. ^{1}H NMR analysis was performed by Bruker Advance-300 MHz spectrometer in DMSO-d_6 solution at room temperature (MA, USA). ^{1}H NMR peaks of pMPs were as follows: 2.02 (s; H of acetyl groups from Ac_3ManNAz, 9H), 2.49-2.53 (s; H of CH_2-CH_2 from PAMAM, 4H), 3.06 (s; H of CH_2-CH_2 from PAMAM in between -NH, 4H), 5.04-5.24 (m; H of

Ac_3ManNAz, 1H), 5.99 (s; H of Ac_3ManNAz, 1H), 7.88 (s; H of NH from PAMAM, 3H), 8.50-8.53 (d; H of NH from Ac_3ManNAz, 1H) (Figure 4c). Mass spectra was measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS; PAMAM m/z calculated: 20615, found: 19277, pMPs m/z calculated: 45447, found: 38599) (Figure 5). The conjugated amount of Ac_3ManNAz was determined by high performance liquid chromatography (HPLC).

2.4 Synthesis of Cy5.5-labeled pMPs

The pMPs were labeled with Cy5.5 to trace their movement by fluorescence. Cy5.5-NHS was dissolved in deionized distilled water and added to the water solution containing PAMAM [G4] dendrimer. The mixture was reacted in the dark at room temperature for 72 hours. Then, free Cy5.5-NHS were removed by dialysis against deionized distilled water using a cellulose membrane (MWCO 3500) for 3 days. The final product was lyophilized to give Cy5.5-labeled PAMAM [G4]. After all, Ac_3ManNAz was conjugated to Cy5.5-labeled PAMAM [G4] to give Cy5.5-labeled pMPs, using the same process of preparation of pMPs.

2.5 Cell culture

U87 (Human glioblastoma) cells, HDF (Human dermal fibroblasts) cells, MCF-7 (Human mammary carcinoma) cells, human breast adenocarcinoma cells (MDA-MB-468 and MDA-MB-436), KB (Human epidermoid carcinoma) cells, and A549 (Human lung adenocarcinoma) cells were purchased from ATCC (Manassas, VA, USA). Those cell lines were maintained in RPMI1640 (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS; Welgene, Daegu, Korea), 100 μg/ml streptomycin, and 100 U/ml penicillin (Welgene, Daegu, Korea) in a humidified 5% CO_2 atmosphere at 37℃.

2.6 Cell viability Assay

U87 cells were seeded on 96-well plates ($5x10^3$ cells/well) and incubated for one day. To measure the cytotoxicity of pMPs, the cells were treated with Ac_3ManNAz, PAMAM [G4], or pMPs with various concentration of Ac_3ManNAz: 6.25, 12.5, 25, 50, 100 uM. After incubation for two days, the cells were washed twice with DPBS (pH 7.4) and 20 uL of MTT in serum-free RPMI1640 media (0.5 mg/mL) was added to each well. After further incubation for 2 h at 37 ºC, the media was removed and cells were dissolved in 200 uL of DMSO. Then, the absorbance of each well was measured at

572 nm using a microplate reader (VERSAmaxTM, Molecular Devices Corp., Sunnyvale, CA).

2.7 Western blot analysis of cells

U87 cells were seeded onto 100 x 20 mm cell culture plates at a density of 2 x 10^6 cells per plate in 12 mL of media with Ac_3ManNAz (50 μM) or pMPs (50 μM Ac_3ManNAz). After 2 days of incubation, the cells were washed twice with DPBS (pH 7.4) and harvested from the plates using a cell scraper. The cells were pelleted by centrifugation at 1500 rpm for 5 min, and the cell pellets were re-suspended in 500 μl of lysis buffer (1 % SDS, 100 mM Tris·HCl, pH 7.4) containing protease inhibitor cocktail (Complete, EDTA-free, Roche, NSW, Australia). They were lysed with a probe-type sonifier at 4 ºC. Sonicated lysates were incubated at 4 ºC for 30 min to further solubilized proteins, and insoluble debris was removed by centrifugation for 10 min at 3,000 x g. Final soluble protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) to be 5 mg/ml. Then, 20 μl of the lysate was incubated with 2 μl of phosphine-PEG_3-biotin (5 mM in DPBS) (Pierce, Rockford, IL, USA) for 6 hours at 37 ºC. SDS-PAGE loading buffer was added to each sample and the samples were heated at 95 ºC, before loading onto 10% SPS-PAGE gel. Proteins were transferred to Hybond P membrane (Amercham, St.

Albans, UK), and the membrane was blocked with 5 % bovine serum albumin (BSA) in TBST (50 mM Tris·HCl, 150 mM NaCl, 0.1 % Tween20, pH 7.4) for 2 hours. Then, the membrane was incubated with streptavidin-HRP (diluted 1:2000 in TBST) (Pierce, Rockford, IL, USA) overnight at 4 ºC. The membrane was rinsed three times with TBST and developed by ECL Western Blotting Substrate (Pierce, Rockford, IL, USA).

2.8 Cellular imaging to determine the generated azide groups

U87 and HDF cells were seeded onto 35 mm glass-bottom dishes at a density of 2 x 10^4 cells in 2 mL of media with no sugar, Ac_3ManNAz (50 μM), or pMPs (50 μM Ac_3ManNAz). After incubation for two days, the cells were washed twice with DPBS (pH 7.4) and incubated for 1 hour with ADIBO-Cy5.5 (20 μM, final concentration) (Future Chem, Seoul, Korea) in 37 ºC incubator. They were rinsed with DPBS (pH 7.4) and fixed with a fixative containing formaldehyde-glutaraldehyde for 15 min at room temperature. Then, the cells were washed with DPBS (pH 7.4) again and stained with DAPI (Invitrogen, Carlsbad, CA, USA) to label nuclei. All cellular images were obtained by a confocal laser microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany) with 405 diode (405 nm) and He-Ne (633nm) lasers.

2.9 Cellular imaging of the bindings of click molecules or biological ligands to various tumor cells

ADIBO-Cy5.5 (FutureChem, Seoul, Korea) was used as purchased with no further purification. In order to prepare cRGD-PEG-Cy5.5, similar amide coupling of c(RGDyK) with (t-Boc)-protected PEG-NHS (Mw 2,000), followed by TFA cleavage was carried out. The cRGD-PEG conjugate (8 mg; 2 μmol) was incubated with Cy5.5-NHS (10 μmol) in 500 μL of phosphate buffer (10 mM, pH 7.4) for 2 h in the dark at room temperature. Final product was purified by HPLC. Cetuximab-Cy5.5 was synthesized by the reaction of anti-EGFR monoclonal antibody, Cetuximab (3.3 nmol), and Cy5.5-NHS (40 nmol) in phosphate buffer (0.1 M, pH 8.5). After 2 h reaction, the mixture was purified using PD-10 column to remove unreacted Cy5.5. To prepare folate-PEG-Cy5.5, folic acid (10 mg) was added into 60 mL of ethylenediamine (EDA) and stirred in the dark for 3 h at room temperature. After precipitation in acetone twice, the precipitate was dissolved in water and filtrated to remove the insoluble substance. Then, this folate-EDA (20 mg, 24 μmol) was incubated with (t-Boc)-NH_2-PEG-NHS (Mw 2,000) in 2.5 mL DMSO for 2 h, and treated by TFA for deprotection of t-Boc. The folate-PEG conjugates (10 mg; 4 μmol) was incubated with Cy5.5-NHS (10 μmol) in 500 μL phosphate buffer (10 mM, pH 7.4) for 2 h

in the dark at room temperature. The final product, folate-PEG-Cy5.5 conjugate was purified by HPLC.

All cells were seeded onto 35 mm glass-bottom dishes at a density of 3 x 10^4 cells in 2 mL of growth media with no sugar or pMPs (50 μM Ac_3ManNAz). After 2 days of incubation, the cells were washed twice with DPBS (pH 7.4), and incubated with ADIBO-Cy5.5 (20 μM, final concentration) for 30 min at 37 °C for cellular imaging. The same method was applied to all cell lines: U87, MCF-7, MDA-MB-468, MDA-MB-436, KB, and A549 cells.

For the comparative study using traditional biological binding molecules, all cells were seeded onto 35 mm glass-bottom dishes at a density of 3 x 10^4 cells in 2 mL of growth media and grown to reach 60-80 % confluence. The cells were washed twice with DPBS (pH 7.4), and incubated with cRGD-PEG-Cy5.5 (0.2 μM, final concentration), Cetuximab-Cy5.5 (0.1 μM, final concentration), or Folate-PEG-Cy5.5 (0.2 μM, final concentration) for 30 min at 37 °C for cellular imaging. Fluorescence images were observed using confocal laser microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany) with 405 diode (405 nm) and He-Ne (633nm) lasers.

2.10 Flow cytometry analysis

U87 cells were seeded onto 6-well plates at a density of 2 x 10^4 cells per well in 2 mL of media with no sugar or pMPs (50 μM Ac_3ManNAz) treated with ADIBO-Cy5.5, as described previously. The cells were lifted with DPBS containing 2 % FBS (flow cytometry buffer) and washed twice in FACS buffer (1 mM EDTA). Twenty thousand cells per sample were performed by flow cytometry (BD FACSVerse, BD Biosciences, San Jose, CA, USA), and subsequent data analysis was analyzed using FlowJo software.

2.11 *In vivo*/*ex vivo* imaging

All experiments with live animals were performed in compliance with the laws and institutional guidelines of Korea Institute of Science and Technology (KIST) and approved by institutional committees. For *in vivo* and *ex vivo* experiments, U87 tumor cells (1.0 x 10^7 cells) were subcutaneously planted on flank of 5-week-old male athymic nude mice (20 g, Orient, Gyeonggi-do, Korea). When the tumor reached about 100 mm^2, the experiments were conducted. Cy5.5-labeled pMPs (40 mg Ac_3ManNAz/kg) was injected intravenously once. The mice model was observed over 3 days to display *in vivo* biodistribution of pMPs. Near-

infrared fluorescence (NIRF) images were observed using the IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA).

For analysis of expression of azide groups on tumor tissue, free Ac_3ManNAz or pMPs (40 mg Ac_3ManNAz/kg) were injected daily for 2 days. After that, ADIBO-Cy5.5 (10 mM, 40 μL) was injected (n=3 per each experimental group). All the injections were done intravenously. The biodistribution and time-dependent tumor accumulation profiles were non-invasively imaged by IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA). All data were calculated by the region of interest (ROI) function of the IVIS molecular imaging software.

The major organs and tumors were dissected from mice 72 hours post-injection of Cy5.5-labeled pMPs. For analysis of expression of azide groups on tumor tissue, the tumors were dissected from mice 24 hours post-injection of free Ac_3ManNAz or pMPs (40 mg Ac_3ManNAz). *Ex vivo* images were also obtained with a IVIS Lumina K Series III *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA), and the fluorescence signal intensity at the ROI using IVIS molecular imaging software.

2.12 Histological imaging

For histological evaluation, the dissected tumor tissues were fixed in 4 % (v/v) buffered formalin solution, and embedded in optimum cutting

temperature (OCT) compound (Sakura, Tokyo, Japan) on dry ice. Sections were cut on a cryostat with 6 μm in thickness and picked up on slides with poly-D-lysine, dried at 45 °C under protection from light. Fluorescence of tumor tissue sections was observed (excitation: 673 nm, emission: 692 nm) by IX81-ZDC focus drift compensating microscope (Olympus, Tokyo, Japan).

2.13 Western blot analysis of tumor tissues

Preparation of U87 tumor-bearing mice models and intravenous injection of Ac_3ManNAz (40 mg/kg) or pMPs (40 mg Ac_3ManNAz/kg) were performed by the same method. 48 hours post-injection of Ac_3ManNAz or pMPs, the major organs and tumors were dissected and transferred into 1 ml of lysis buffer (1 % SDS, 100 mM Tris·HCl, pH 7.4) containing protease inhibitor cocktail (Complete, EDTA-free, Roche, NSW, Australia) and homogenized. The lysates were incubated at 4 ºC for 30 min, and insoluble debris was removed by centrifugation for 10 min at 3,000 x g. Using BCA protein assay kit, total soluble protein concentrations were measured. Staudinger reaction with phosphine-PEG_3-biotin, SDS-PAGE, and western blot analysis were performed as described in the previous section.

2.14 Immunohistochemical analysis

The dissected tumor tissues were retrieved from U87 tumor-bearing mice, and fixed in 4 % (v/v) buffered formalin solution. The tissues were dehydrated with a graded ethanol series, and embedded in paraffin. The paraffin tissues were sliced in 4 μm wide slices, and immunohistochemical staining was performed using phosphine-PEG_3-biotin and streptavidin-HRP followed by the standard methods. The paraffin slices were dried and then mounted with Permount SP15-100 Toluene Solution (Fisher Scientific, NJ, USA) mounting media. The samples were observed by a light microscope (BX51, Olympus, Tokyo, Japan), and were photographed using a digital camera photomicroscope (DP71, Olympus, Tokyo, Japan).

3. RESULTS AND DISCUSSION

3.1 Characterization of pMPs

Poly (amido amine) (PAMAM) [G4] dendrimer is used as a basic structure of the polymerized metabolic precursors (pMPs) with a well-established characteristics, and Ac_3ManNAz is used for metabolic glycoengineering of sialic acids (Figure 1b). Ac_3ManNAz can act as a building block in cell metabolism, since it can easily produce unnatural sialic acid, the most abundant glycan on tumor cell surface[10]. Conjugation of PAMAM [G4] dendrimer and Ac_3ManNAz is completed by *Steglich* esterification mechanism (Figure 2). A completion of the reaction was determined by TLC (Figure 3). The free PAMAM [G4] dendrimer was stayed on the start line because of its high polarity, and pMPs was mobilized at maximum height on 3 days after the reaction. There is no further movement of pMPs after 3 days of reaction; the reaction time was fixed as 3 days. After the reaction, free Ac_3ManNAz, free PAMAM, and pMPs are analyzed by ^{1}H NMR (Figure 4). By comparison of the reactants and product, ^{1}H NMR peaks promised that the reaction was successfully completed. In addition, MALDI-TOF determined molecular weight of free PAMAM (m/z calculated: 20615, found: 19277) and that of pMPs (m/z calculated: 45447, found: 38599) (Figure 5). The Ac_3ManNAz conjugation

efficiency was confirmed as 82.58 % using HPLC. Moreover, the size of pMPs was 3.88 ± 0.51 nm in aqueous condition as determined by dynamic light scattering (DLS). It promise that pMPs is small enough to freely move in blood flow and show no further aggregation.

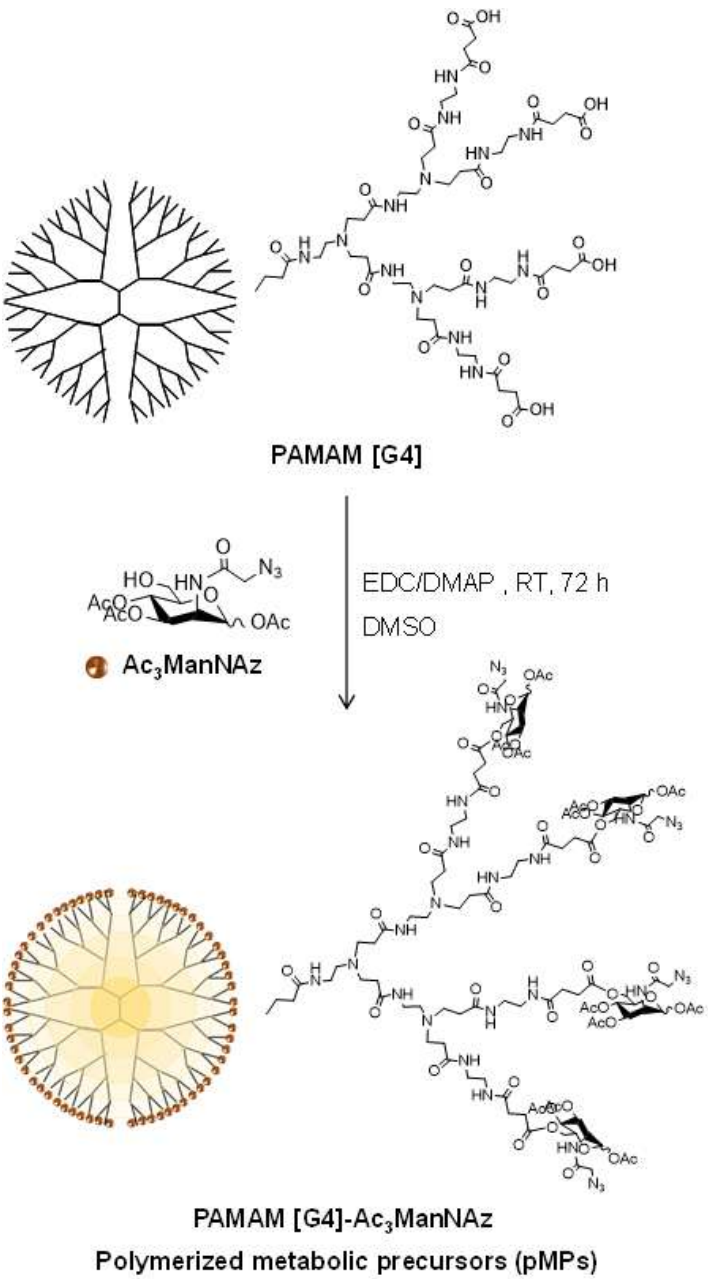

Figure 2. Synthetic scheme of pMPs

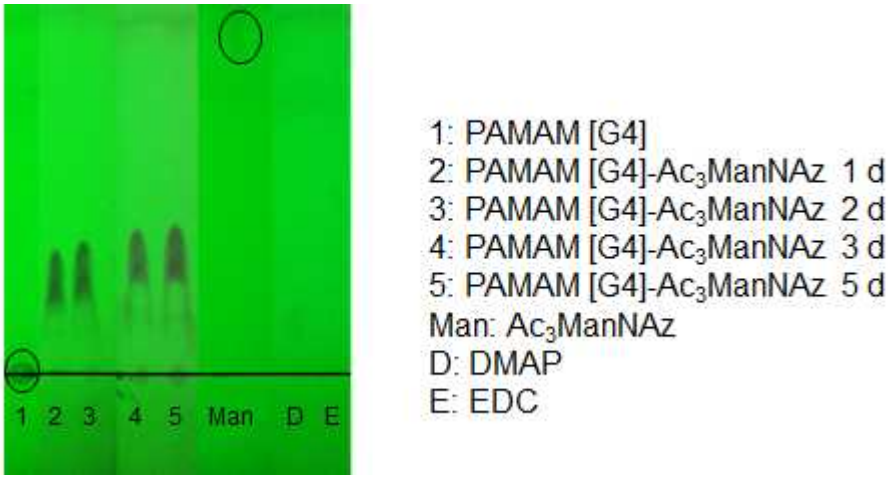

Figure 3. TLC analysis

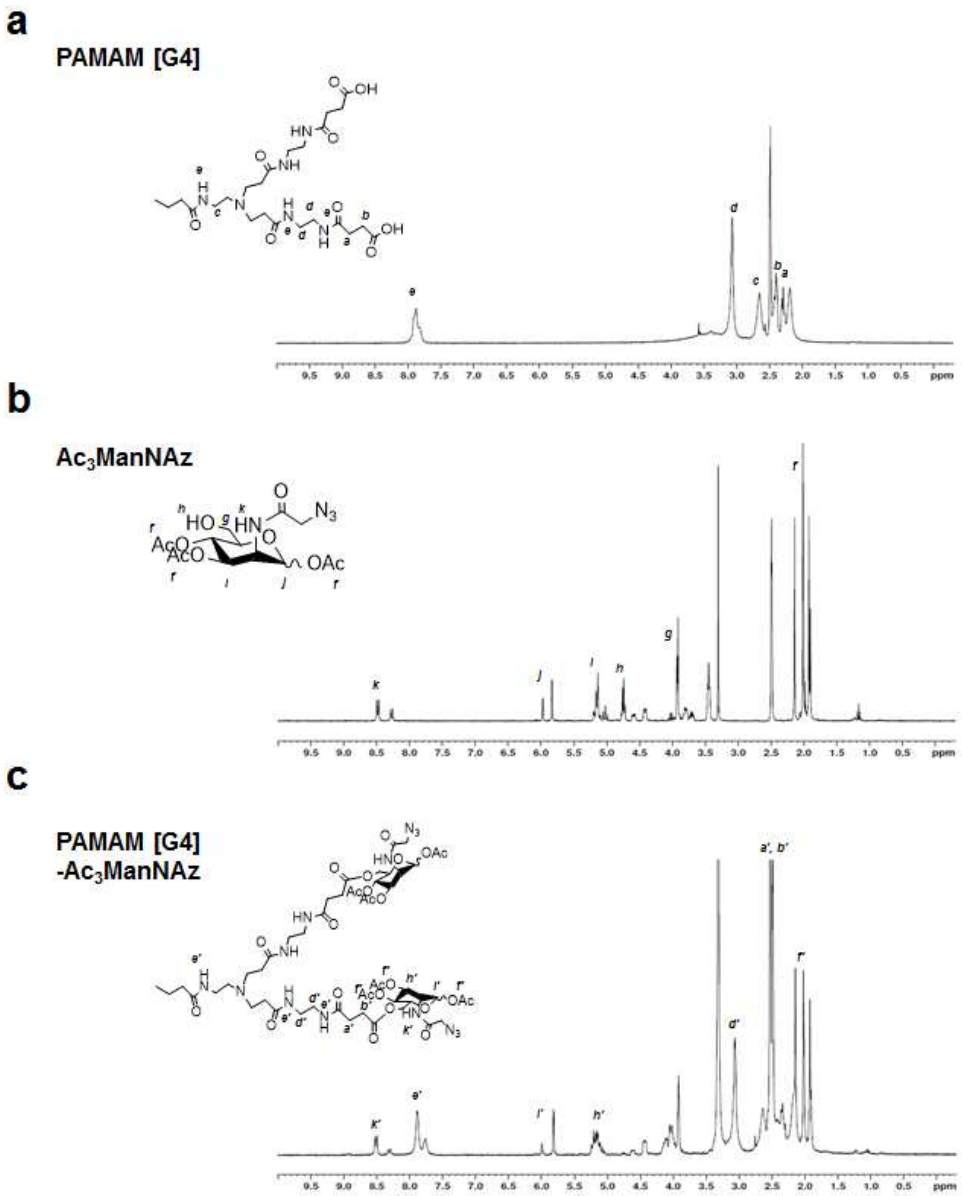

Figure 4. ^{1}H-NMR spectra of (a) PAMAM [G4], (b) $Ac_3ManNAz$, and (c) PAMAM [G4]-$Ac_3ManNAz$. 300 MHz NMR, solvent; DMSO-d_6.

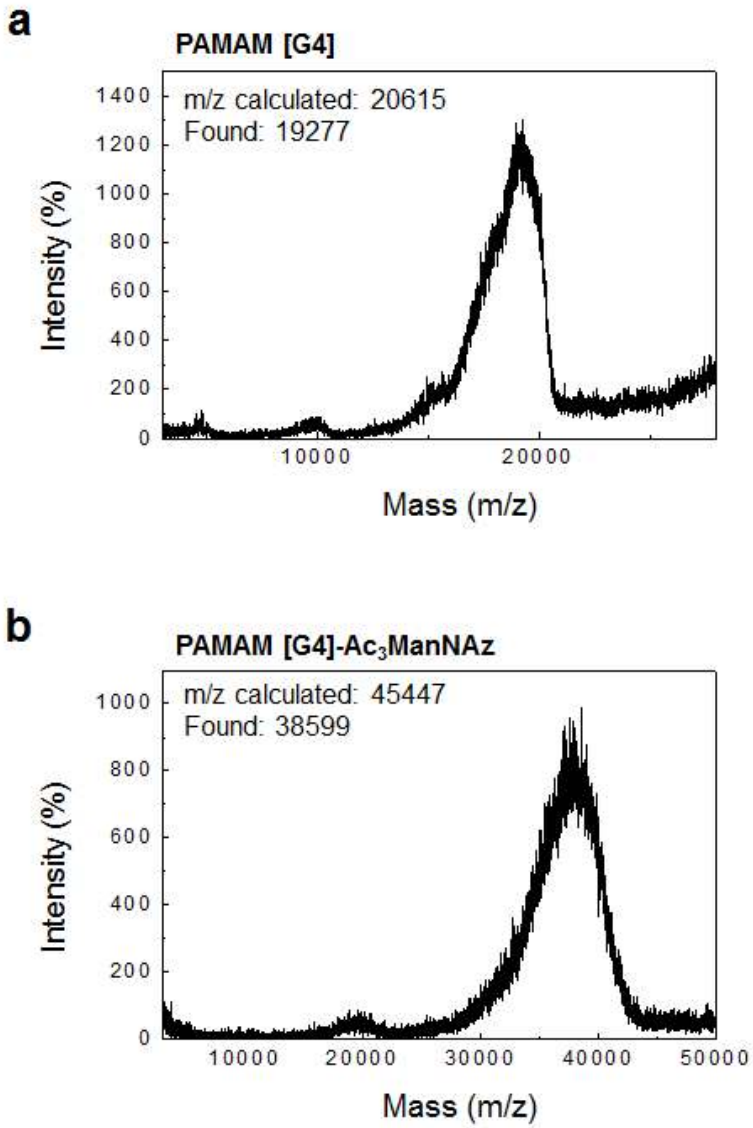

Figure 5. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) data of (a) PAMAM [G4] and (b) PAMAM [G4]-Ac_3ManNAz.

3.2 *In vitro* studies of pMPs

In vitro studies establish the generation of azide groups on the surface of tumor cells. First, azide group generation on cell surface was determined by cellular imaging and western blot analysis. Free Ac_3ManNAz or pMPs was added to U87 tumor cells, and azide groups were determined by binding with ADIBO-Cy5.5 (red fluorescence). After treatment of free Ac_3ManNAz or pMPs for 2 days, the viability of U87 cells was above 90% showing no significant cytotoxicity (Figure 6). ADIBO is chosen as a bioorthogonal chemical group functionalized with its high reactivity to azide groups via copper-free click chemistry[5b]. After treatment with ADIBO-Cy5.5, a higher fluorescence intensity of Cy5.5 was observed in both free Ac_3ManNAz and pMPs treated U87 tumor cells than in untreated cells (Figure 7a). The microscopic images indicated that artificially introduced azide groups on the tumor cell surface were successfully generated by free Ac_3ManNAz and pMPs, and effectively targeted by ADIBO-Cy5.5. Unlike U87 cells, a normal cell line, such as HDF (human dermal fibroblast) cell, did not show a strong signal of fluorescence after treatment of free Ac_3ManNAz or pMPs (Figure 7c). As mentioned earlier, the surface of tumor cell is especially rich in sialic acid. Therefore, even though Ac_3ManNAz can easily make unnatural sialic acid through metabolic glycoengineering, HDF normal cells cannot be visualized as U87 tumor cells. Western blot analysis using

phosphine-PEG_3-biotin and streptavidin-HRP likewise demonstrated the generation of azide groups on tumor cells after the treatment of free Ac_3ManNAz or pMPs (Figure 7b)[11]. Coomassie staining verifies that the total loading amount of proteins for each group of cell lysates is similar.

The heterogeneity of tumor cells complicates the circumstances for various subpopulations of tumor cells expressing different types and amounts of receptors[9]. Tumor is increasingly acknowledged as a combination of many disorders, each with varying causes, prognoses, and appropriate treatments. This diversity is recognizable not only across different types of tumor, but also within tumors of the same tissue. It is now known that cancer cells within the same tumor mass are heterogeneous in many aspects, including morphology or phenotypic expression, exhibition of inherent or acquired drug resistance, and capacity for initiating new tumor growth[9]. The heterogeneity and complexity of tumor is a huge obstruction of present drug delivery systems. However, our strategy can generate 'receptor-like' chemical groups on heterogeneous tumor cells by metabolic glycoengineering and overcome the limitation of current drug delivery systems. To demonstrate, a comparative study using representative click molecules and biological ligands was performed on various cell types: U87, MCF-7, MDA-MB-468, MDA-M-436, KB, and A549. ADIBO was used as a click molecule, and biological ligands such as cRGD (peptide), Cetuximab

(antibody), and folate were used, in order to label the azide group with Cy5.5 dye. The biological receptors for these ligands are known to be expressed in specific tumor cells, and each ligand labeled with Cy5.5 was bound to the targeted cells only as expected. However, after treatment of pMPs, all cell types (U87, MCF-7, MDA-MB-468, MDA-M-436, KB, and A549) were positively visualized on cellular imaging with ADIBO-Cy5.5 (Figure 8a). The azide groups were generated on all cell types by metabolic glycoengineering, so they could bind with ADIBO-Cy5.5. The fluorescence intensity of these cells was quantified using flow cytometry analysis. Compared to the control cells with no treatment, the tumor cells treated with pMPs indicated a higher intensity (Figure 8b). These results show that azide groups are successfully generated from pMPs and formed on the surface of various cell lines. These results demonstrate that polymerized metabolic precursors can overcome the heterogeneity of tumor cells.

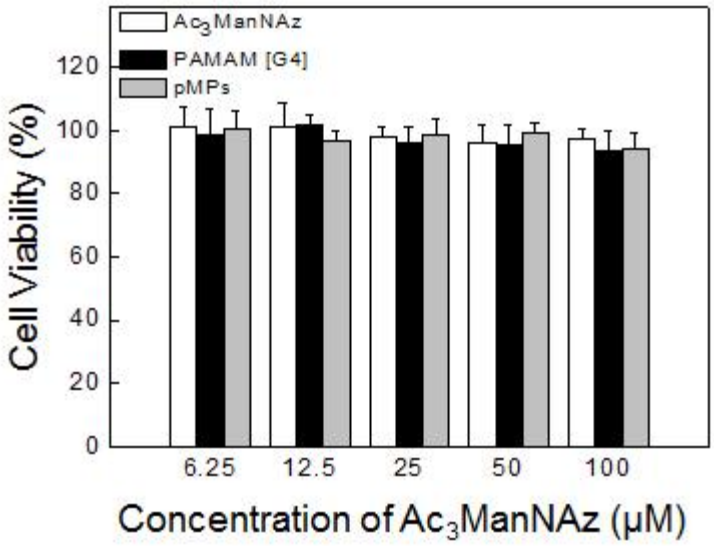

Figure 6. Viability of tumor cells treated with pMPs for 2 days.

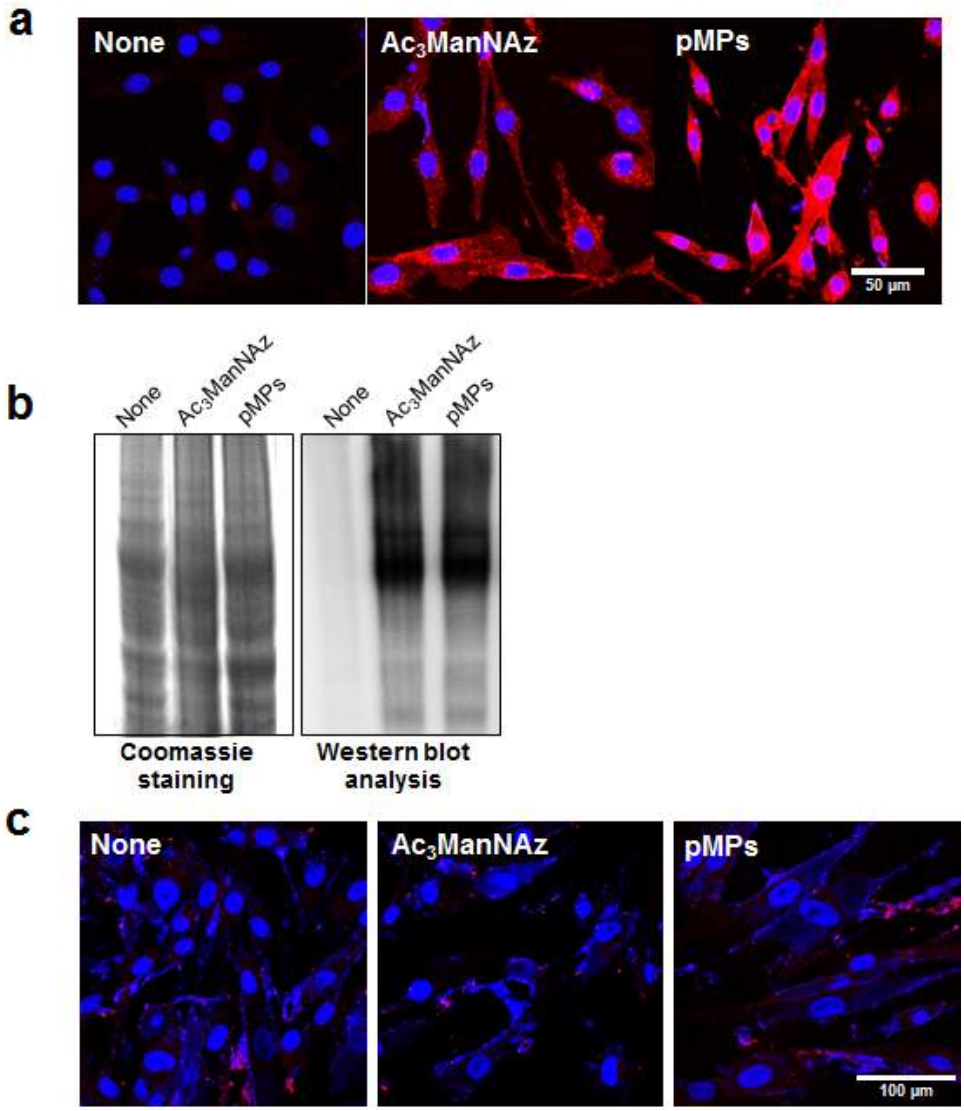

Figure 7. Azide group generation on cell surface after treatment of pPMs. (a) Visualization of azide groups on the surface of U87 tumor cells after treatment of pMPs. (b) Coomassie staining and wetern blot analysis of U87 tumor cells treated with pMPs. (c) Visualization of azide groups on the surface of human dermal fibroblasts (HDFs, normal cells) after treatment of pMPs.

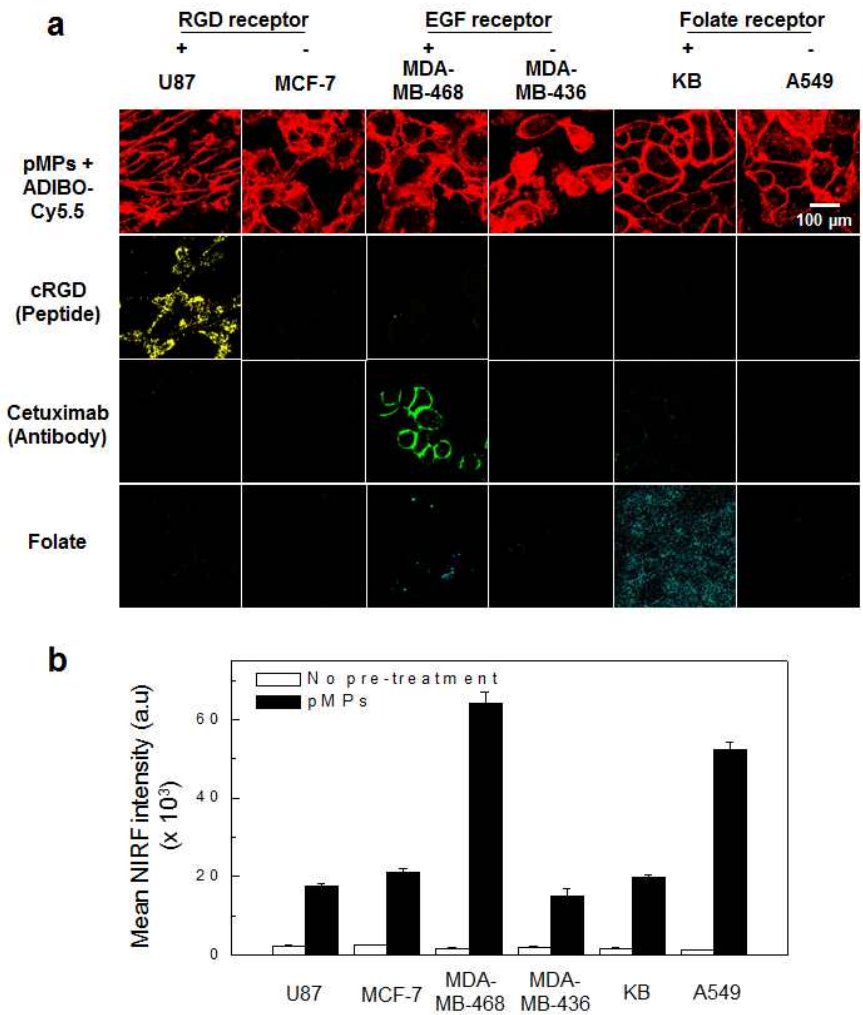

Figure 8. Generation of azide groups by pMPs in various tumor cells. (a) The binding of click molecules and biological ligands to tumor cells. ADIBO-Cy5.5, cRGD-PEG-Cy5.5, Cetuximab-Cy5.5, and Folate-PEG-Cy5.5 were visualized in red, yellow, green, and blue fluorescence (pseudo colors). (b) Flow cytometry analysis of tumor cells after treatment of pMPs and ADIBO-Cy5.5. ADIBO-Cy5.5 was added to tumor cells treated with pMPs (50 μM) for 2 days.

3.3 *In vivo* studies of pMPs

For *in vivo* studies, balb/*c* nude mice models bearing xenograft U87 tumor on one side of flank are prepared. *In vivo* experiments start when the tumor size reached about 100 mm^3. First, targetable glycans, unnatural sialic acids with azide groups, are artificially generated in target cancer cells by an intratumoral injection of the precursor, pMPs. Free Ac_3ManNAz or pMPs were administered to each tumor model by intratumoral injection once a day for 3 days while saline was injected to the control mice model. The coomassie blue staining and western blot analysis of tumor tissues showed that the unnatural sialic acid with azide groups similarly with the cellular conditions (Figure 9).

The biodistribution of pMPs is evaluated by time-dependent whole body imaging and *ex vivo* fluorescence analysis[12]. Whole body distribution was measured using a non-invasive IVIS Lumina K Series III *in vivo* imaging system in live animals. Cy5.5 labeled pMPs was intravenously injected once for fluorescent tracking. The images showed a slow excretion of pMPs over time (Figure 10a). Fluorescence intensities of tumor tissues from the images were quantified and plotted with a mild decreasing curve (Figure 10b). Total photon count in tumor site is distinctively higher in Cy5.5-labeled pMPs than free Cy5.5. Based on the EPR effect[12], pMPs reached to the tumor more specifically and remained there using its characteristics of polymer.

Therefore, compared to small molecules like free Cy5.5 dye, pMPs bring a slower excretion through the whole body, especially from the tumor. In *ex vivo* fluorescence analysis of major organs and tumors, tumor tissue from mice pretreated with pMPs exhibited higher fluorescence intensity than other major organ tissues, except for the liver (Figure 10c and 10d). We expected that a strong signal from the liver was caused as a part of enterohepatic circulation.

For intravenous injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*, western blot analysis, whole body and *ex vivo* fluorescence imaging were evaluated. Three different models of mice were prepared with saline, free Ac_3ManNAz, or pMPs by intravenous injection. After 2 days of injection, ADIBO-Cy5.5 was also injected through tail vein to label the azide groups on cell surface by click chemistry. The tumors were collected and analyzed after all the injections. Compared to saline and free Ac_3ManNAz, the tumor tissue from mice injected with pMPs revealed a strong band in western blot analysis (Figure 11a). The result showed that a large number of azide groups were generated on tumor tissue from pMPs by metabolic glycoengineering. The azide groups can be specifically generated in tumor tissue by pMPs because of its tumor-targeting ability via the EPR effect. The tumor tissue from mice pretreated with pMPs shows significantly more intense fluorescence *in vivo* images than tumor tissues pretreated with

saline or free Ac_3ManNAz (Figure 11b). Even though the number of azide groups generated by Ac_3ManNAz and pMPs were similar *in vitro*, the *in vivo* images were different because free Ac_3ManNAz is a small molecule that has no tumor-targeting ability. The tumors from each group were dissected after 24 hours from injection of ADIBO-Cy5.5. They were analyzed by *ex vivo* fluorescence images and tumor tissue from mice pretreated with pMPs showed distinctively higher intensity of fluorescence than tumors from other group pretreated with saline or free Ac_3ManNAz (Figure 11c and 11d). Furthermore, immuohistological staining supports the metabolic glycoengineering of pMPs. The dark brown spots which show the expression of azide group are intense in tumor tissue, compared to other organ tissues (Figure 12). The immunohistology also demonstrates that metabolic glycoengineering can specifically occur on the tumor tissue after intravenous injection of pMPs. A large number of azide groups can be successfully generated on tumor tissue *in vivo*. More specifically, histological evaluation proves the generation of azide group from pMPs by the accumulation of ADIBO-Cy5.5 in tumor tissue (Figure 13). It was significantly higher in pMPs pretreated mice group than other groups with saline or free Ac_3ManNAz.

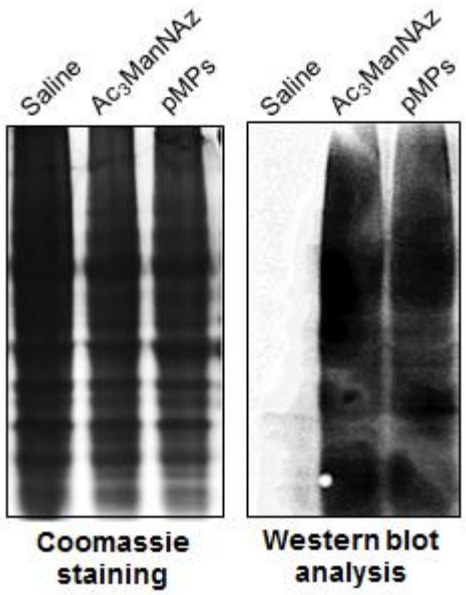

Figure 9. Intratumoral injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*. Coomassie staining and wetern blot analysis of Ac_3ManNAz or pMPs treated tumor tissues.

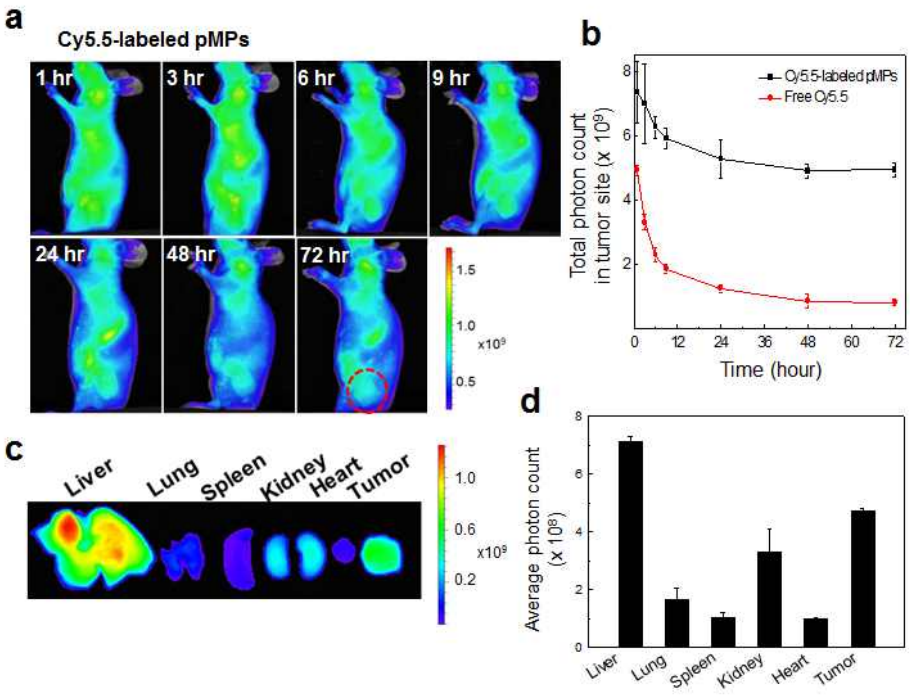

Figure 10. Biodistribution of pMPs in tumor-bearing mice. (a) Whole body images of mice after intravenous injection of Cy5.5-labeled pMPs. (b) Fluorescence intensities of tumor tissues from (a). (c) *Ex vivo* fluorescence images of major organs and tumor from 72 h post-injection of pMPs. (d) Fluorescence intensities of major organs and tumor tissue from (c).

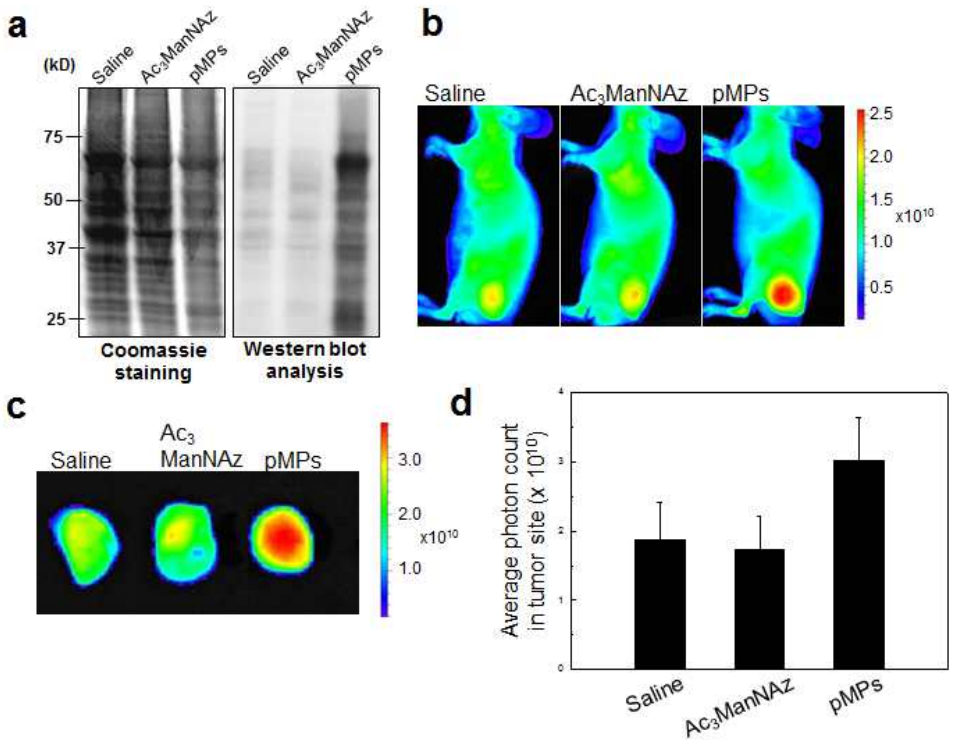

Figure 11. Intravenous injection of pMPs and metabolic glycoengineering on tumor tissue *in vivo*. (a) Coomassie staining and wetern blot analysis of tumor tissues after intravenous injection of saline, free $Ac_3ManNaz$, or pMPs. (b) Whole body images of mice after intraveneous injection of ADIBO-Cy5.5, pretreated with pMPs. (c) *Ex vivo* fluorescence images of tumors from each group 24 h postinjection of ADIBO-Cy5.5. (d) Fluorescence intensities of tumor tissues from (c).

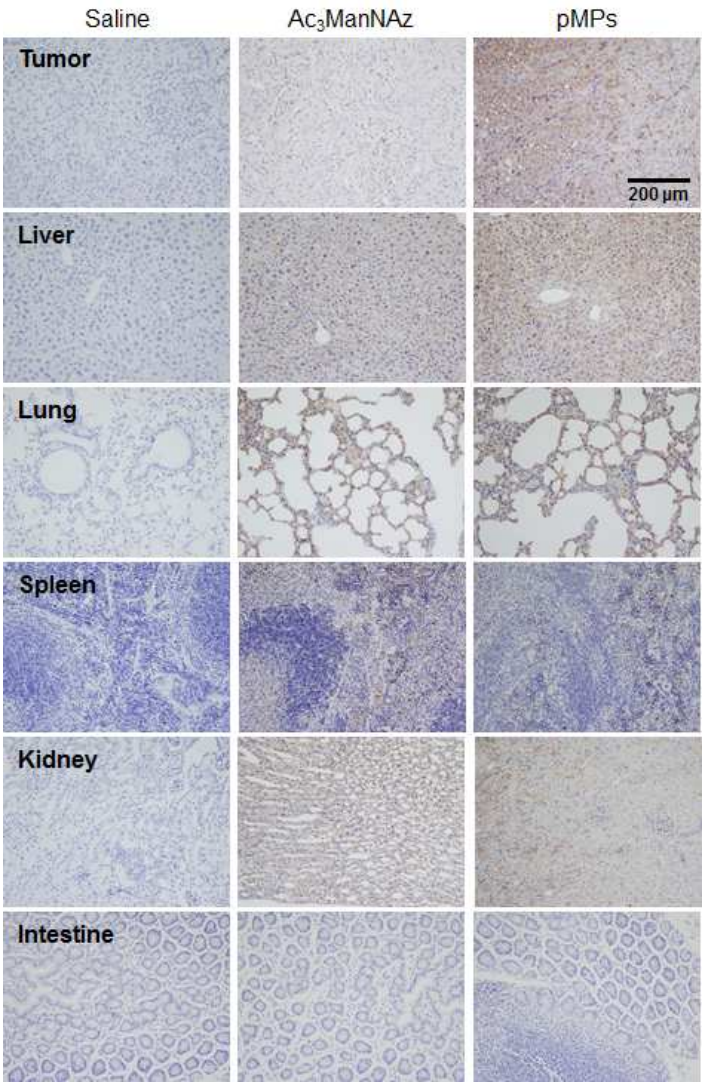

Figure 12. Intravenous injection of saline, free Ac_3ManNAz, and pMPs and metabolic glycoengineering on tumor tissue *in vivo*. Histological staining of major organs and tumor tissues after intravenous injection of saline (left panel), free Ac_3ManNAz (middle panel), and pMPs (right panel).

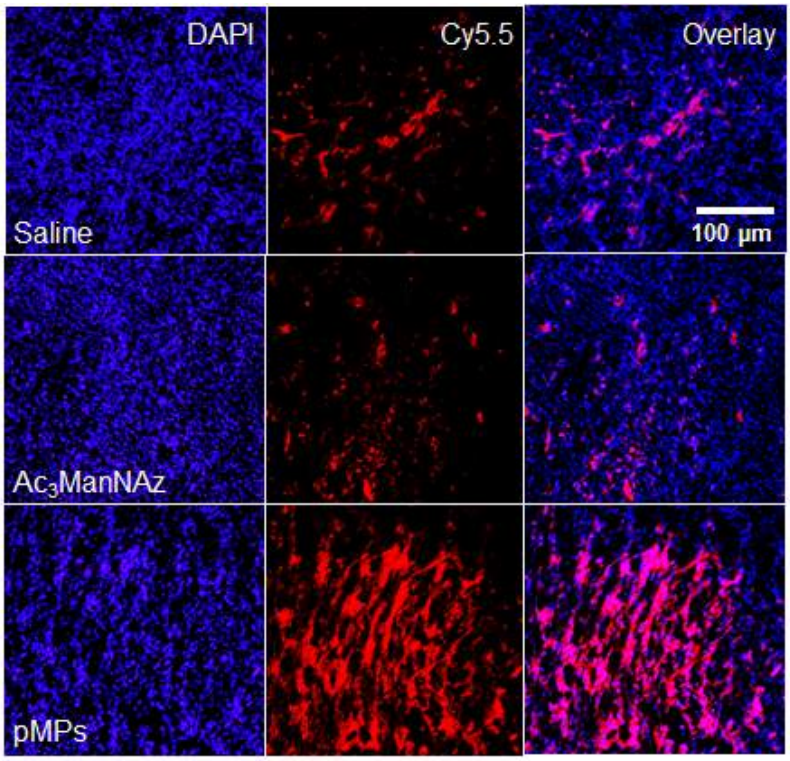

Figure 13. Fluorescence images of tumor tissues after intravenous injection of ADIBO-Cy5.5 to tumor-bearing mice pretreated with saline (top panel), free Ac_3ManNAz (middle panel), or pMPs (bottom panel).

4. CONCLUSION

In this study, we have designed polymerized metabolic precursors (pMPs) to overcome tumor heterogeneity via metabolic glycoengineering and click chemistry. PAMAM [G4] dendrimers are conjugated with Ac_3ManNAz to produce pMPs. The intravenously injected pMPs reach to the tumor cell specifically by EPR effect and successfully produce azide groups on the surface of tumor cells. In particular, an expression of azide groups enables a generation of 'receptor-like' chemical reporters from pMPs itself. Furthermore, by using AIBO-Cy5.5, these azide reporters in the tumor tissue can be labeled via copper-free click chemistry *in vivo* condition. Thus, the tumor can be specifically visualized by optical imaging system. Our results suggest a new targeting strategy which can overcome tumor heterogeneity using site-specific metabolic glycoengineering and copper-free click chemistry. Since the whole procedure is conducted of intravenous injections only, allowing it to be applied to tumor diagnosis and therapy even when the location of the tumor tissue is not well defined. Our strategy significantly demonstrates that it may have a great potential for further biomedical applications as diagnostic or therapeutic probes.

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요약문

기존에 암 치료는 항암제를 단독으로 직접 투여하는 방식을 사용하였다. 하지만 이러한 방법은 암 세포 뿐만 아니라 정상 세포까지 독성을 야기하는 등 다양한 부작용을 나타내었다. 이러한 문제점을 해결하기 위해서 항암제를 표적 암 세포에 전달하는 다양한 나노기술이 개발되어왔다. 본 연구에서는 고분자화 대사 전구체를 이용하여 암 표적화 능력을 향상 시키는 방법에 대한 것이다. 또한, 암 세포가 갖는 고유 수용체의 양적 제한에서 비롯된 표적 능력의 한계 및 암 이질성을 극복할 수 있는 방법에 대한 것이다. 이 방법은 대사 당쇄 조작과 무동 클릭 화학을 이용하였다. PAMAM [G4] 덴드리머와 Triacetylated N-azidoacetyl-D-mannosamine ($Ac_3ManNAz$)의 화학적 결합을 이용하여 암 조직에 특이적으로 인공 표적 수용체인 azide 기를 도입시키는 방법이며, 이 방법을 이용하면 이질성을 갖는 암 세포들을 단일화된 표현 형질로 바꿀 수 있다. 또한 이렇게 도입된 인공 표적 수용체를 표적으로 하는 클릭 화학을 이용하면 암 표적화 능력을 향상 시킬 수 있다. 본 연구에서 개발

된 고분자화 대사 전구체를 이용한 암 표적화 능력 향상 방법을 이용하면 이질성을 갖는 암의 종류에 상관없이 표적 능력을 높일 수 있을 것으로 예상된다.

주요 어: 고분자화 대사 전구체, 종양 이질성, 물질 대사 당공학, 클릭 화학, 광학 이미징

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