공학석사학위논문

Water Soluble Polymerized Metabolic Precursors Based on PEG-PAMAM-Ac_3ManNAz System for Epigenetic Targeting

수용성 고분자화 대사 전구체를 이용한 후성 표적화 전략

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ABSTRACT

Water Soluble Polymerized Metabolic Precursors Based on PEG-PAMAM-Ac_3ManNAz System for Epigenetic Targeting

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Over the past several decades, various drug delivery systems have been studied to minimize the side effects of anticancer agents and to increase delivery efficiency to cancer. Despite the development of numerous targeting moiedties, several obstacles remain. Tumor heterogeneity and quantitative limitation of targeted biomolecules are reasons why chemotherapy failed to suppress cancers completely.

In this study, we developed hydrolysable polymerized metabolic precursors (pMPs) by conjugating PEG-PAMAM linear dendritic block copolymers (LDBC) and triacetylated N-azidoacetyl-mannosamine

(Ac_3ManNAz) via *Steglich* esterification. As the carboxylic acids of the dendron disappear, pMPs became amphiphilic block copolymers and formed self assembly of 150 nm in aqueous condition. Regardless of genetic phenotypes of tumor cells, pMPs can generate 'receptor-like' azide groups on the cancer cell surface. Furthermore, the azide groups were visualized by ADIBO-Cy5.5 via copper-free click chemistry *in vitro* condition. It is expected that pMPs have synergy with the enhanced premeability and retention (EPR) effect and hypersialylation of tumor cells to enable tumor cell specific glycan labeling *in vivo* condition.

Keywords: Tumor Heterogeneity, Metabolic Glycoengineering, Epigenetic Targeting, Copper-Free Click Chemistry, Linear-Dendritic Block Copolymer, Self-Assembly

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CONTENTS

List of Figures and Tables

Figure 8. Azide group generation on cell surface in various concentration of pMPs (a) Visualization of azide groups on the surface of HT-29 tumor cells (b) Coomassie staining and wetern blot analysis of HT-29 tumor cells

Figure 9. Time dependent azide group generation on cell surface after pMPs treatment (a) Visualization of azide groups on the HT-29 tumor cell surface (b) Coomassie staining and wetern blot analysis of HT-29 tumor cells

1. INTRODUCTION

Metabolic oligosaccharide engineering was pioneered by Reutter and co-workers to modify the acyl side chains on cell surface sialic acids in living cells using unnatural sugars as the metabolic precursors[1-2]. After that Carolyn Bertozzi's group reported bioorthogonal functional groups could incroporated into cell membrane sialic acid treating with N-acetylmannosamine derivatives[3-4]. Once the unnatural functional group is integrated into sialic acid, it is treated with a probe molecule bearing complementary bioorthogonal fuctionality for precise glycan imaging[5]. As of now, many groups employed this technique for studying various purposes like glycoproteomics, embryogenesis[6-7].

As research on drug delivery systems continues, lots of targeting moieties have been developed that can target only tumors[8-10]. Despite the numerous targeting moieties developed, tumor heterogeneity is one of the reasons why chemotherapy failed to completely suppress cancer[11]. Recent studies reported extensive diversity of genetic phenotype both between and within tumors[12-13]. For this reason, conventional active targeting method using specific biomolecule could not deliver therapeutic agent to the entire cancer. In addition, the limited number of biological receptors on tumor cells is an obstacle to increasing delivery efficiency[14].

To overcome these limitations, we employed a strategy to artificially introduce target receptors into tumor cells. When cancer cells ingest azido sugar as a metabolite, the azide group is expressed on the cell surface regardless of the genetic phenotype of the cells (Fiugre 1a). Furthermore tumor cells showed increased expression of sialic acid sugars on the surface. Owing to their high expression of sialic acids, this approach could improve efficacy[15]. In order to selectively express azide groups in cancer cells, it is important to transport metabolites intensively to cancer tissues.

In our previous study, we developed polymerized metabolic precursors (pMPs) by conjugating triacetylated N-azideoacetyl-mannosamine (Ac_3ManNAz) and generation 4 poly(amido- amine) (PAMAM [G4.0]). However, this materials have some drawbacks about water solubility and targeting ability. Herein, we chose PEG-PAMAM linear dendritic block copolymer(LDBC) to overcome the defects. We easily synthesized PEG-PAMAM LDBC using mPEG-NH_2 as a core initiator. Since the properties of mPEG-PAMAM are mainly determined by PEG, the purification required for the synthesis of PEG-PAMAM was carried out by simple precipitation method. Polymerized metabolic precursor(pMPs) were synthesized by conjugating PEG-PAMAM and triacetylated N-azidoacetyl-mannosamine(Ac_3ManNAz) via Steglich esterification reaction. Since the solubility of the dendritic block is determined by its terminal surface, it becomes hydrophobic after the coupling with Ac_3ManNAz[16]. As a result,

amphiphilic nature of pMPs led to form self-assembly in aqueous condition. The size of self-assembly on the aqueous condition was characterized to be about 150 nm, which is a suitable size to exhibit the EPR effect (Figure 1b).

The human colon adenocarcinoma cells (HT-29) were treated with pMPs. Ester bonds of pMPs were hydrolyzed and release free form Ac_3ManNAz. Our method generates azide groups on the tumor cell surface and these cells including subpopulation of tumor cells. Azide groups on the cell membrane is visualized by copper free click reaction with ADIBO-Cy5.5.

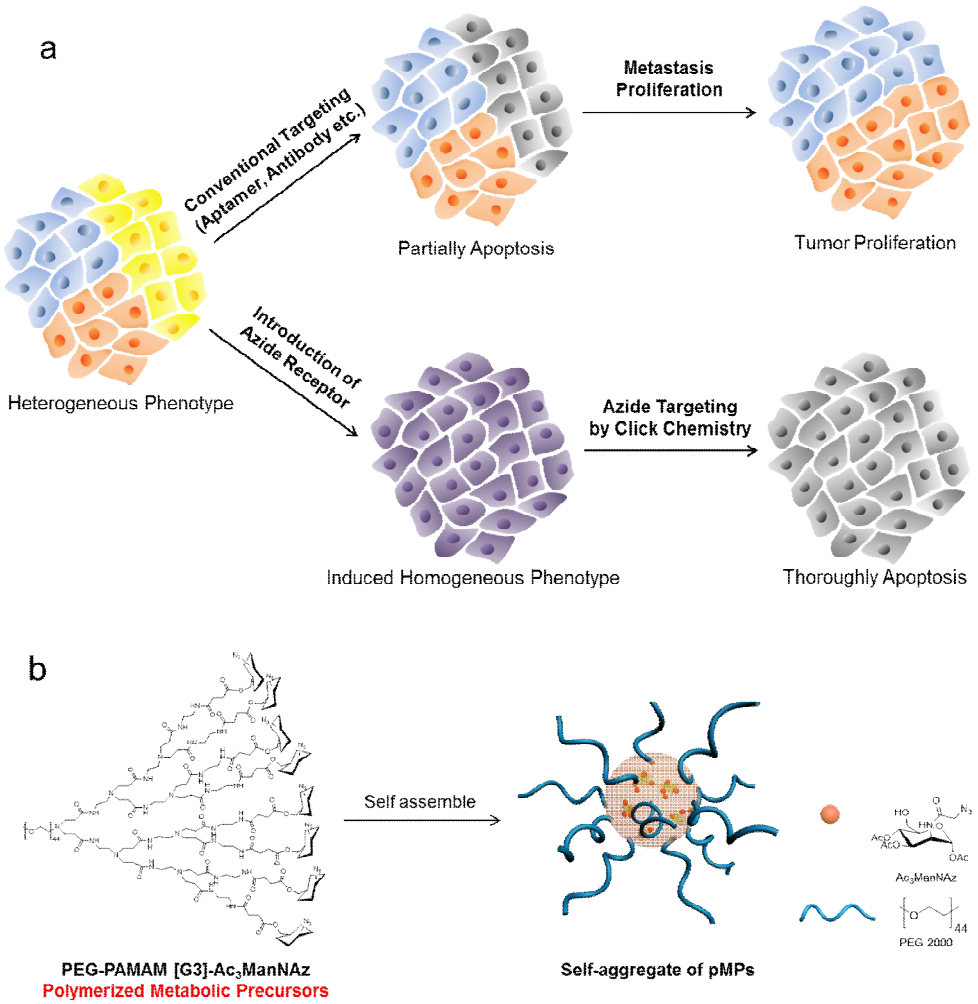


Figure 1. (a) Schematic illustration of the targeting strategy to overcome tumor heterogeneity using metabolic glycoengineering and copper-free click chemistry. (b) Chemical structure of pMPs and its self-assembly foramtion

2. EXPERIMENTS

2.1 Materials

Methoxy PEG amine (MW : 2K) (mPEG-NH_2) was obtained from Sunbio (Gunpo, Korea). Anhydrous methanol, Methyl acrylate, Ethylenediamine (EDA), Succinic anhydride, N,N'-Dicyclohexylcarbodiimide (DCC), 4-(Dimethylamino) pyridine (DMAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDA was distilled with calcium hydride before use in the experiments. Dichloromethane (MC) wase obtained from Daejung Chemicals and Metals Co. (Goryeong, Korea). MC was dried over calcium hydride. Triacetylated *N*-azidoacetyl-*D*-mannosamine (Ac_3ManNAz), azadibenzocyclooctyne-Cy5.5 (ADIBO-Cy5.5) were achieved from FutureChem (Seoul, Korea).

2.2 Instruments

^{1}H NMR analysis was performed by Bruker Advance 300 MHz spectrometer using $CDCl_3$-d_6 or D_2O as a solvent at room temperature. Molecular weight change due to hydrolysis was measured by gel permeation chromatography (GPC) with Shimadzu RID-10A refractometer equipped with Styragel HR 3, HR 4, HR 4E columns in series. THF was used as an eluent with the flow rate of 1.0 mL/min and PEG standards were used for

calibration. Measurement of size was performed by Otsuka ELS-Z (Taipei, Taiwan). All cellular images were obtained by a confoal laser microscope (Leica TCS SP8) (Wetzlar, Germany) with 405 diode (405 nm) and He-Ne (633 nm) lasers.

2.3 Synthetic Procedure for PEG-PAMAM Linear Dendritic Block Copolymers

Synthesis of the PEG-PAMAM linear dendritic block copolymers is a two step process (1) Michael addition of amine groups and (2) exhaustive amidation of the resulting methyl esters with large excess of ethylenediamine.

2.3.1 Michael addition of amines

First, 2-neck round bottom flask was charged with methyl acrylate (100.0 equiv of amine). Amine terminated compounds/methanol solution was added at room temperature under nitrogen atmosphere. This reaction was carried out at 40 ^{o}C. Whether the reaction is complete was confirmed by using nihydrin test and chloranil test. After the Michael addition reaction was finished, the solvent and excess methyl acrylate was evaporated with a rotary evaporator. The white sticky residue was precipitated in cold diethyl

ether to remove residual impurities and filtered off to give the product, a white solid. The solid product was dried under vaccum.

2.3.2 Exhaustive amidation of methyl esters

Ester terminated coumound was dissolved in anhydrous methanol and added to ethylenediamine (200.0 equiv of ester) slowly at room temperature under nitrogen atmosphere. The reaction is carried out at 45 ^{o}C. After 4 days, methanol and ethylenediamine were removed under vacuum. The crude product was precipitated in cold diethyl ether and filtered off to give a product. The product was a white solid and was dried over vacuum.

2.3.3 Synthesis of PEG-PAMAM [G0.5] Dendron

mPEG-PAMAM [G0.5] was synthesized using the procedure described in section 2.3.1., starting from mPEG-amine (1.00 g, 0.5 mmol) and methyl acrylate (8.61 g, 100.00 mmol) in 5.0 mL anhydrous methanol. The mixture was purified by precipitation in cold ether to give white solid (1.02 g, 93.9 %).

^{1}H NMR in $CDCl_3$-d_6 : δ_{PEG}(C**H$_3$**O) = 3.3684 (s) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.6327 (m) ; δ_{PAMAM}(-COOC**H$_3$**) = 3.6552 (s) δ_{PAMAM} (-C**H$_2$**COOCH$_3$) = 2.4396 (t) ; δ_{PAMAM} (protons next to tertiary amines) = 2.6 - 2.8 (m)

2.3.4 Synthesis of PEG-PAMAM [G1.0] Dendron

mPEG-PAMAM [1.0] was synthesized using the procedure described in section 2.3.2., starting from mPEG-G0.5 (0.92 g, 0.42 mmol) and ethylenediamine (10.18 g, 169.41 mmol) in 5.0 mL anhydrous methanol. The mixture was purified by precipitation in cold ether to give white solid (0.85 g, 88.0 %).

^{1}H NMR in $CDCl_3$-d_6 : δ_{PEG}(C**H$_3$**O) = 3.3766 (m) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.6403 (m) ; δ_{PAMAM} (-C**H$_2$**CONH) = 2.3824 (t) ; δ_{PAMAM} (-CONHC**H$_2$**) = 3.3042 (q) ; δ_{PAMAM} (protons next to primary and tertiary amines) = 2.64 - 2.84 (m)

2.3.5 Synthesis of PEG-PAMAM [G1.5] Dendron

mPEG-PAMAM [G1.5] was synthesized using the procedure described in section 2.3.1., starting from mPEG-G1.0 (0.81 g, 0.36 mmol) and methyl acrylate (12.52 g, 145.40 mmol) in 4.0 mL anhydrous methanol. The concentrate was purified by precipitation in cold ether to give white solid (0.86 g, 91.8 %).

^{1}H NMR in $CDCl_3$-d_6 : δ_{PEG}(C**H$_3$**O) = 3.3700 (s) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.6340 (m) ; δ_{PAMAM}(-COOC**H$_3$**) = 3.679 (s) ; δ_{PAMAM} (-C**H$_2$**CONH) = 2.3593 (t) ; δ_{PAMAM} (-CONHC**H$_2$**) = 3.2616 (q) δ_{PAMAM} (-C**H$_2$**COOCH$_3$) = 2.4227 (t) ; δ_{PAMAM} (protons next to tertiary amines) = 2.50 – 2.83 (m)

2.3.6 Synthesis of PEG-PAMAM [G2.0] Dendron

mPEG-PAMAM [2.0] was synthesized using the procedure described in section 2.3.2., starting from mPEG-G1.5 (0.78 g, 0.30 mmol) and ethylenediamine (14.58 g, 242.55 mmol) in 4.5 mL anhydrous methanol. The mixture was purified by precipitation in cold ether to give white solid (0.68 g, 84.0 %).

^{1}H NMR in $CDCl_3$-d_6 : δ_{PEG}(C**H$_3$**O) = 3.3809 (s) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.6444 (m) ; δ_{PAMAM} (-C**H$_2$**CONH) = 2.3689 (m) ; δ_{PAMAM} (-CONHC**H$_2$**) = 3.2677 (m) ; δ_{PAMAM} (protons next to tertiary and primary amines) = 2.52 - 3.00 (m)

2.3.7 Synthesis of PEG-PAMAM [G2.5] Dendron

mPEG-PAMAM [G2.5] was synthesized using the procedure described in section 2.3.1., starting from mPEG-G2.0 (0.69 g, 0.26 mmol) and methyl acrylate (17.69 g, 205.60 mmol) in 8.0 mL anhydrous methanol. The concentrate was purified by precipitation in cold ether to give white solid (0.75 g, 87.0 %).

^{1}H NMR in $CDCl_3$-d_6 : δ_{PEG}(C**H$_3$**O) = 3.3744 (s) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.643 (m) ; δ_{PAMAM}(-COOC**H$_3$**) = 3.672 (m) ; δ_{PAMAM} (-C**H$_2$**CONH) =

2.375 (t) ; δ_{PAMAM} (-CONHC**H$_2$**) = 3.273 (q) ; δ_{PAMAM} (-C**H$_2$**COOCH$_3$) = 2.435 (t) ; δ_{PAMAM} (protons next to tertiary amines) = 2.51 – 2.81 (m)

2.3.8 Synthesis of PEG-PAMAM [G3.0] Dendron

mPEG-PAMAM [3.0] was synthesized using the procedure described in section 2.3.2., starting from mPEG-G2.5 (0.70 g, 0.21 mmol) and ethylenediamine (19.95 g, 331.99 mmol) in 5.0 mL anhydrous methanol. The mixture was purified by precipitation in cold ether to give white solid (0.47 g, 63.0 %).

^{1}H NMR in D_2O : δ_{PEG}(C**H$_3$**O) = 3.298 (s) ; δ_{PEG}(C**H$_2$**C**H$_2$**O) = 3.620 (m) ; δ_{PAMAM} (-C**H$_2$**CONH) = 2.364 (q) δ_{PAMAM} (-CONHC**H$_2$**) = 3.216 (m) δ_{PAMAM} (protons next to tertiary and primary amines) = 2.54 – 2.87 (m)

2.3.9 Synthesis of Succinamic Acid Terminated PEG-PAMAM [G3.0]

25 mL round bottom flask was charged with mPEG-G3.0 (0.5 g, 0.14 mmol) and succinic anhydride (0.13 g, 1.25 mmol) and dissolved in anhydrous MC (8.00 ml). The solvent was evaporated by rotary evaporator and the concentrate was dissolved in H_2O to degrade succinic anhydride. H_2O is removed under vaccum and precipitated in diethyl ether 3 times to remove succinic acid.

^{1}H NMR in D_2O : δ_{PEG}(C**H**$_3$O) = 3.310 (s) ; δ_{PEG}(C**H**$_2$C**H**$_2$O) = 3.618 (m) ; δ_{PAMAM} (-C**H**$_2$CONH) = 2.404 (m) ; δ_{PAMAM} (-CONHC**H**$_2$) = 3.296 (m) δ_{PAMAM} (-CONHC**H**$_2$C**H**$_2$NHCO-) = 3.235 (m) δ_{PAMAM} (-NHCOC**H**$_2$C**H**$_2$COOH) = 3.420 (t) δ_{PAMAM} (protons next to tertiary amines) = 2.66 – 2.80 (m)

2.4 Conjugation of Ac_3ManNAz to PEG-PAMAM [G3.0]

25 mL round bottom flask was charged with succinamic terminated mPE-G3.0 (55.0 mg, 0.0125 mmol), DCC (46.46 mg, 0.225 mmol), DMAP (27.50 mg, 0.225 mmol) and dissolved in anhydrous chloroform(6.00 mL) at ice bath under nitrogen atmosphere. Add Ac_3ManNAz (58.29 mg, 0.150 mmol) to round bottom flask. Remove DCU by using syringe filter and evaporate chloroform. The excess reactants were dialyzed in DMSO for 72 hours using a cellulose membrane (MWCO 2,000 : Spectrum Laboratories, TX, USA). After dialysis, the Ac3ManNAz conjugated PEG-PAMAM [G3.0] was obtained by lyophilization.

2.5 Preparation of self assembly

In 10 mL round bottom flask, pMP(10.0 mg) was dissolved in 3 mL chloroform. Chloroform was evaporated by roatary evaporator at 55 ^{o}C. Add 1 mL of H_2O and stirred at 60 ^{o}C for 30 min. The solution was passed through 0.22 um syringe filter. Before DLS analysis, the sample was diluted with 2 mL of H_2O. Size of self assembly was characterized by DLS.

2.6 *In vitro* release of Ac_3ManNAz

To observe of release of Ac_3ManNAz from pMP in vitro, Ac3ManNAz (10.0 mg) was dissolved in DPBS(1x) (2.0 ml) solution. The samples were sampled at 0, 3, 12, 24 h and dried by lyophilization. Obtained samples were dissolved in THF and salt was removed by PVDF syringe filters (0.22 um). The samples were analyzed by GPC system.

2.7 Cell culture

HT-29 (Human colon 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℃.

2.8 Cell viability Assay

HT-29 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 pMPs with various concentration of Ac_3ManNAz: 1.25, 2.5, 5, 10, 20 μM. 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).

2.9 Cellular imaging to determine the generated azide groups

U87 and HDF cells were seeded onto 35 mm glass-bottom dishes at a density of 2.5 x 10^5 cells in 2 mL of media with variuos concentration of pMPs (0, 2.5, 5.0, 10.0, 20.0 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 4 % paraformaldehyde for 10 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.

2.10 Western blot analysis of cells

HT-29 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_4ManNAz (20 μM) or pMPs (20 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).

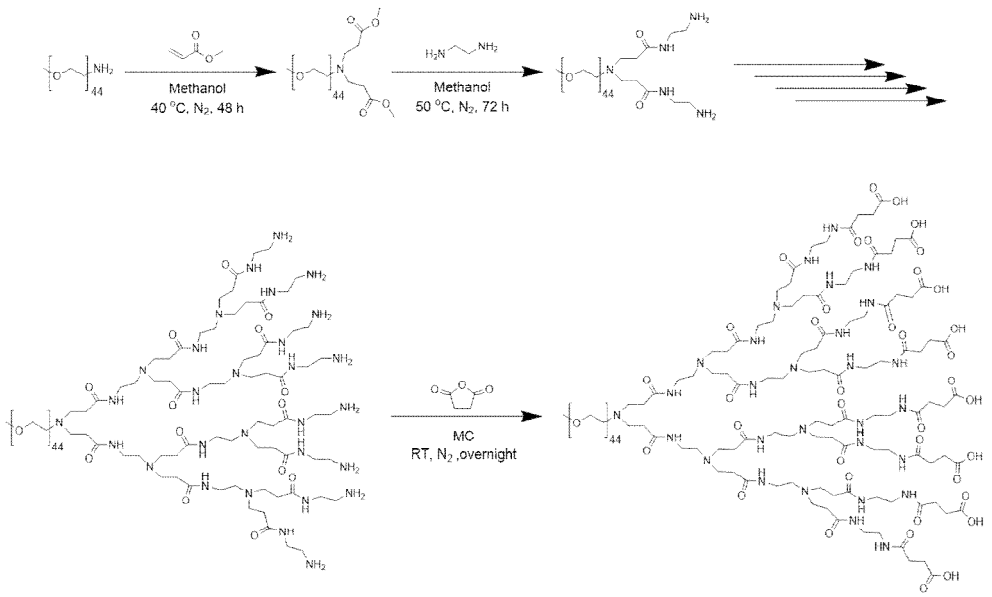
3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of succinamic acid terminated PEG-PAMAM [G3.0]

PEG-PAMAM linear dendritic block copolymers (LDBC) were used as a backbone structure of the polymerized metabolic precursors (pMPs). Despite the advantages of dendrimers in high suface functionality and delivery efficiency, there was a limit to its use due to the synthetic barriers such as difficult purification process. By using mPEG-NH_2 as a core initiator, PEG-PAMAM LDBCs were easily synthesized. Purification is accomplished by simple precipitation.

PEG-PAMAM LDBC were scuccessfully synthesized up to 3rd generation via iterative michael addition and amidation reaction. Synthetic schemes of PEG-PAMAM LDBC are illustrated in scheme 1. In Michael addition reaction, we confirm the all of primary and sceondary amines were consumed using the nihydrin and chloranil test. The 100 % conversion in the amidation reaction was determined by disappearance of hydrogen peak of methyl esters in ^{1}H-NMR spectra. Terminal amine groups of PEG-PAMAM [G3] were converted to carboxylic acid by reacting succinic anhydride and characterized by ^{1}H-NMR and ninhydrin test.

^{1}H-NMR spectra of each compounds are shown in Figure 2.



Scheme 1. Synthetic Scheme of Succinamic Terminated PEG-PAMAM [G3] Linear Dendritic Block Copolymers

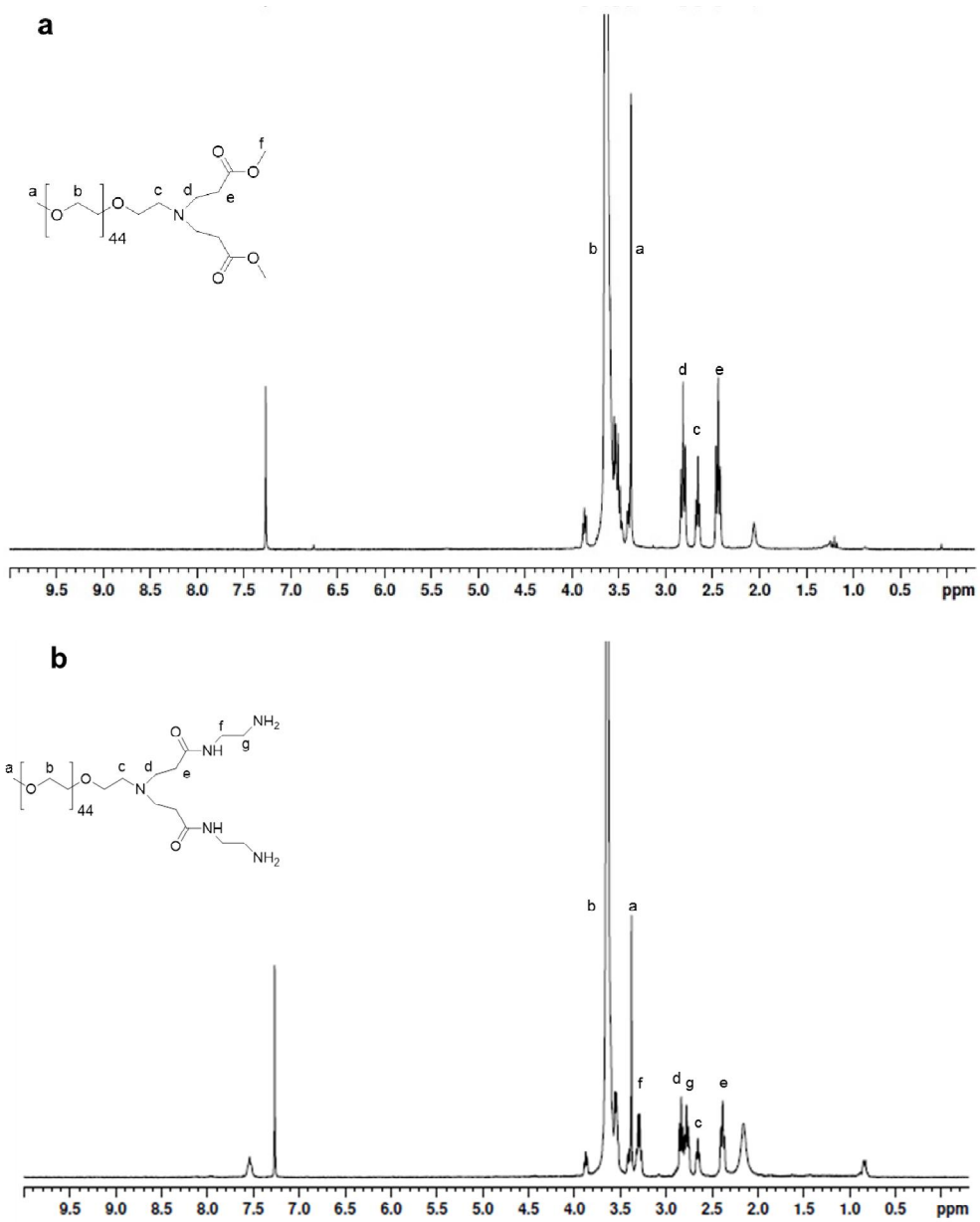


Figure 2. ^{1}H NMR spectra of (a) mPEG-G0.5 and (b) mPEG-G1.0 in $CDCl_3$

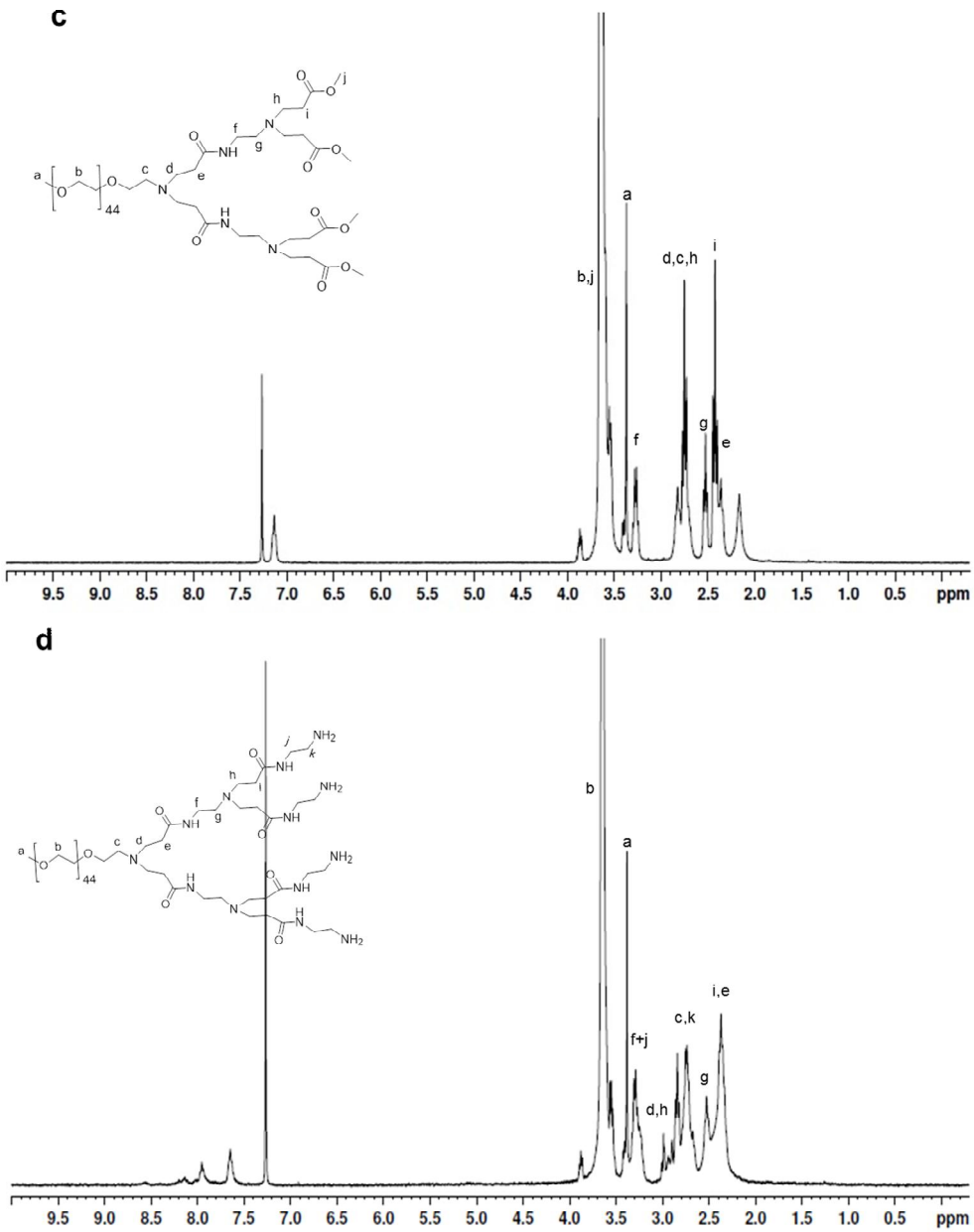


Figure 2. ^{1}H NMR spectra of (c) mPEG-G.1.5 and (d) mPEG-G2.0 in $CDCl_3$

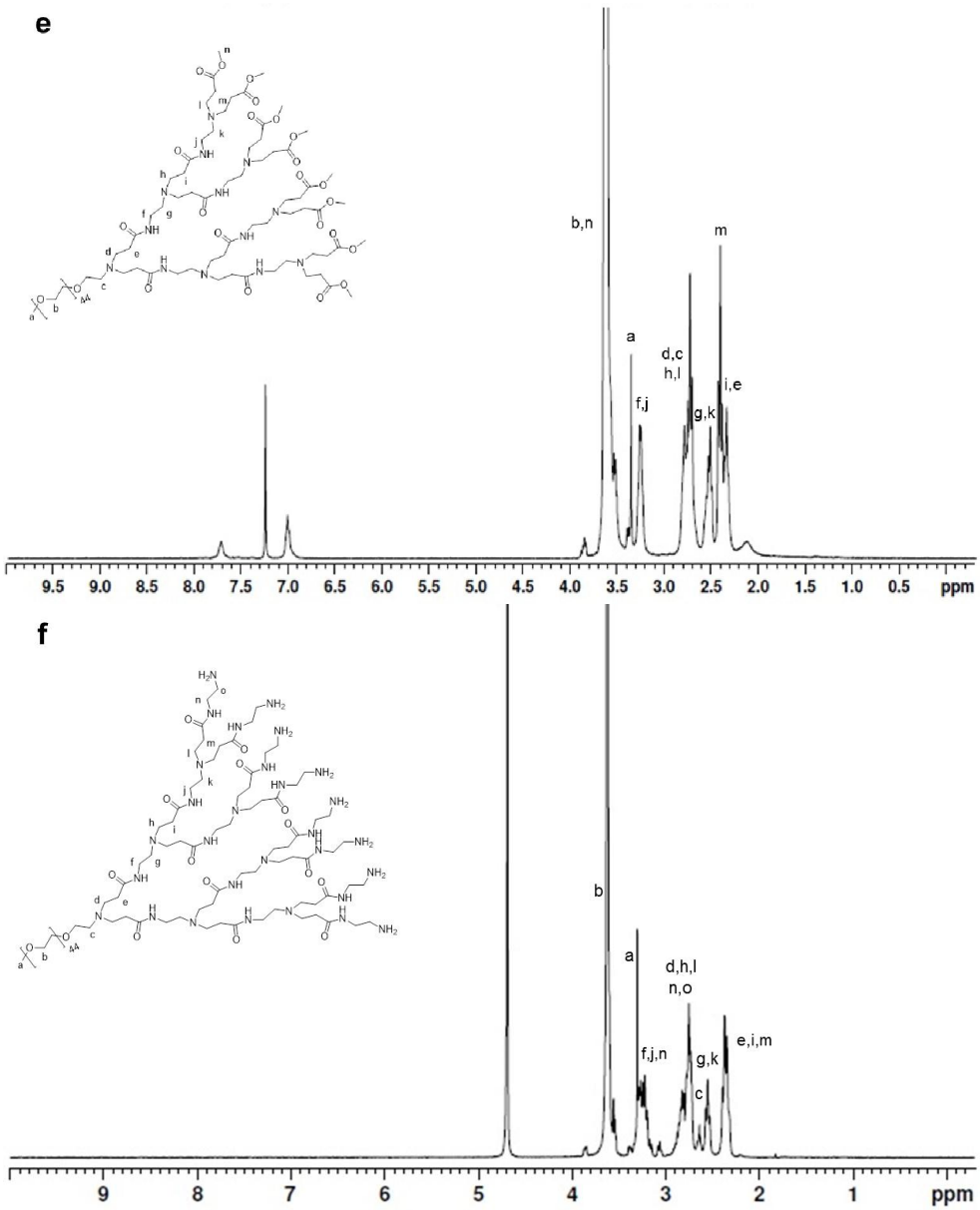


Figure 2. ^{1}H NMR spectra of (e) mPEG-G2.5 in $CDCl_3$ and (f) mPEG-G3.0 in D_2O

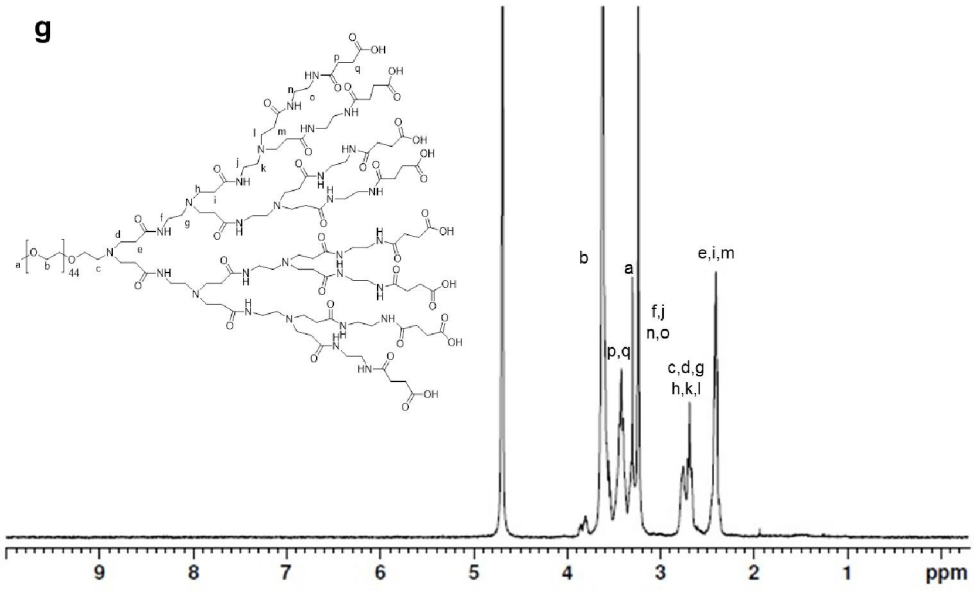
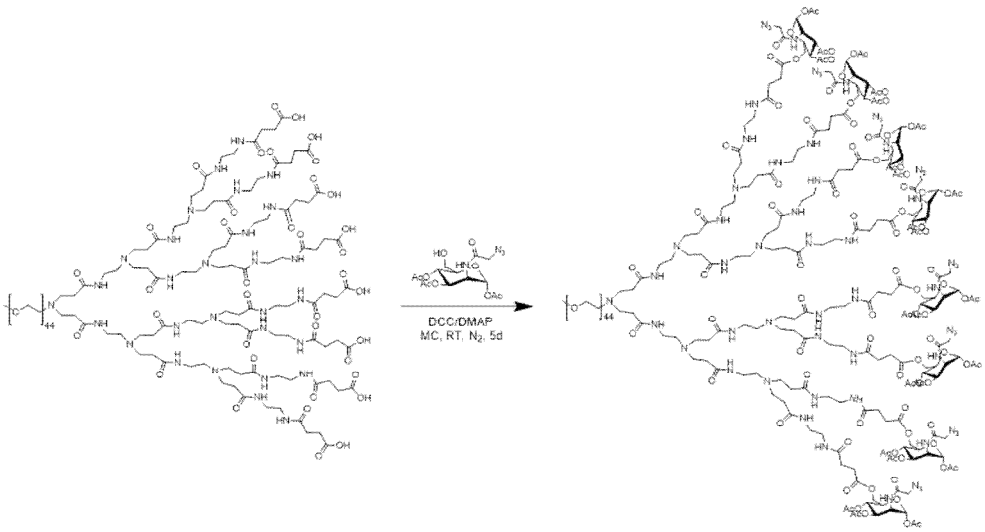


Figure 2. ^{1}H NMR spectra of (g) mPEG-G3.0-COOH in D_2O

3.2 Synthesis and characterization of pMPs

For metabolic labeling of tumor cells, we uses Ac_3ManNAz as a metabolic building block. Because Ac_3ManNAz can easily can easily produce unnatural sialic acid, overexpressed in various kind of tumors.

PEG-PAMAM [G3] LDBC and Ac_3ManNAz was conjugated by *Steglich* esterification (Scheme 2). The reaction was carried out for 5 days. Crude products were dialyzed in DMSO to inhibit hydrolysis of ester bonds for 3 days. After the reaction, pMPs were analyzed by ^{1}H NMR (Figure 3) and the ^{1}H NMR spectrum promised that 5.7 Ac_3ManNAz was conjugated. Also, shift of the peak molecular weight without noticeable shoulders in the trace of GPC indicated succesful conjugation of mPEG-G3.0 and Ac_3ManNAz (Figure 4).



Scheme 2. Synthetic Prodcedure of pMPs.

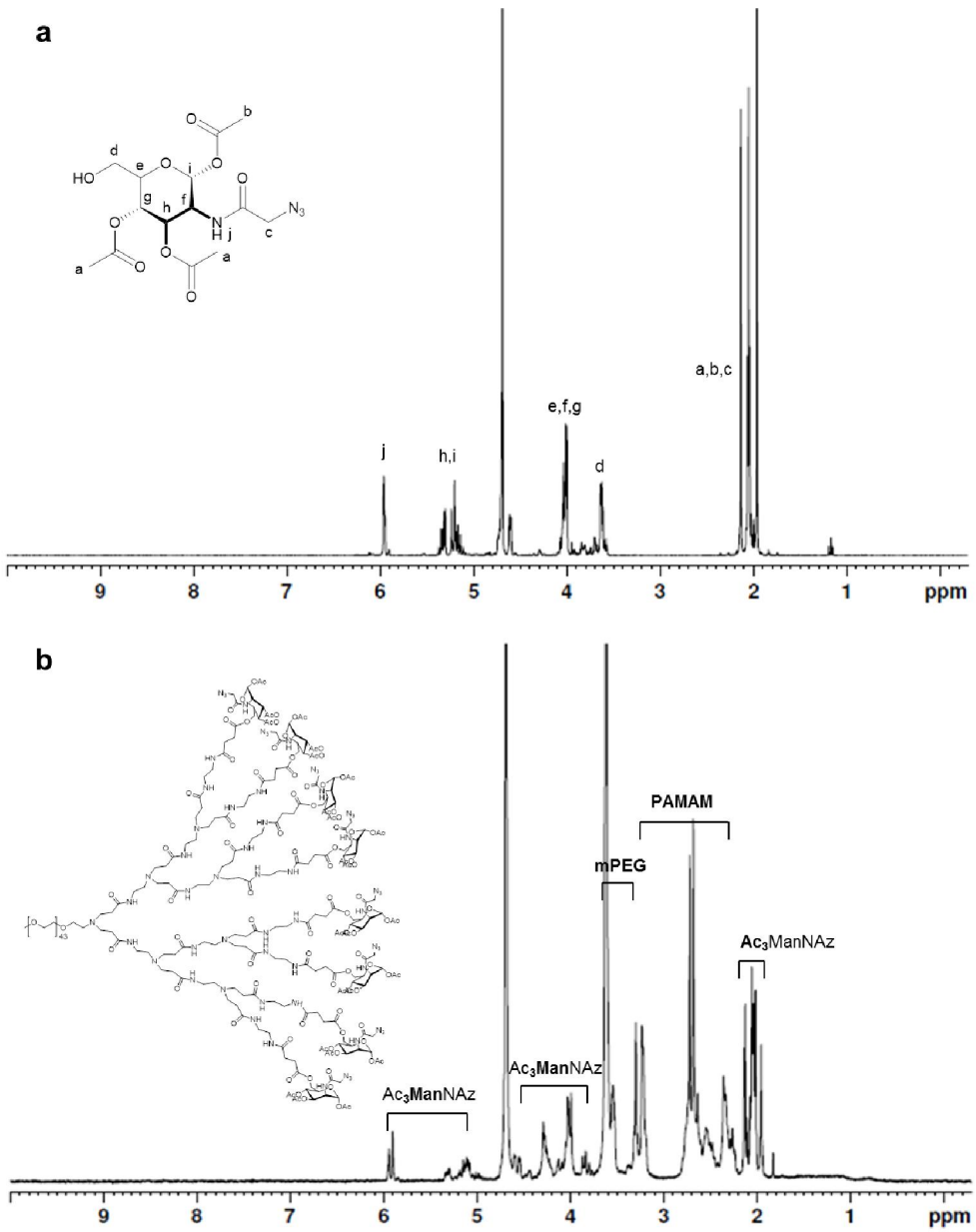


Figure 3. ^{1}H NMR spectra of (a) $Ac_3ManNAz$ and (b) pMPs in D_2O

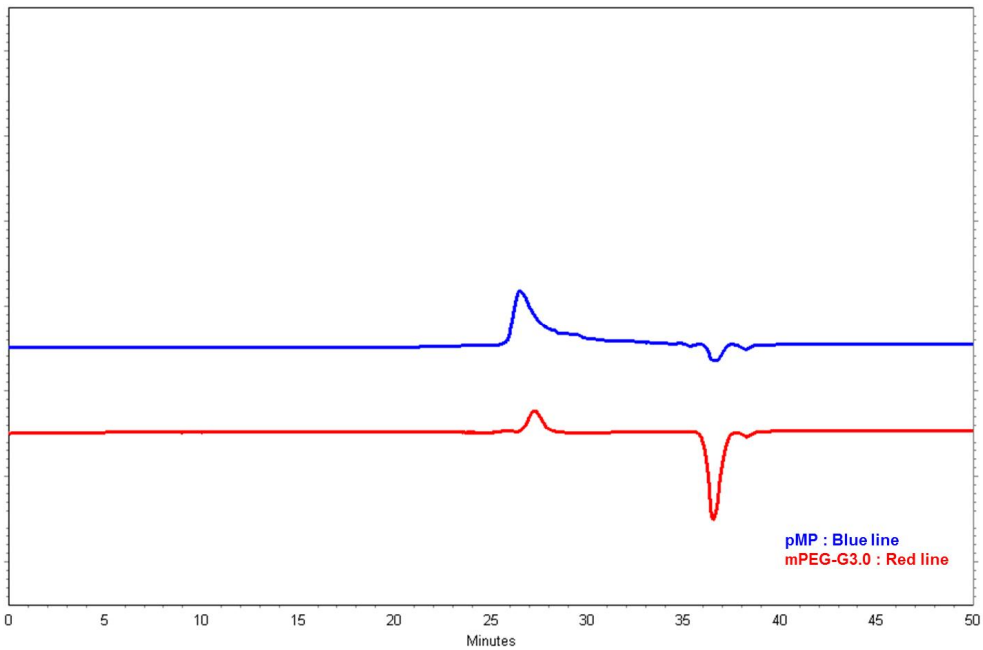


Figure 4. GPC spectra of pMPs and mPEG-G3.0.

3.3 Formation of Self-assembly

By conjugating Ac_3ManNaz to PEG-PAMAM, the carboxylic acid at the terminal of the dendron disappears and the pMPs become amphiphilic block copolymers that can form self assemblies in aqueous condition.

The size of self-assembly was analyzed to be about 150 nm by DLS, which is a suitable size to exhibit the EPR effect (Figure 5). Its size was measured relatively larger than size of linear block copolymers. This is consistent with previous research about self assemble behavior of LDBC[17-18].

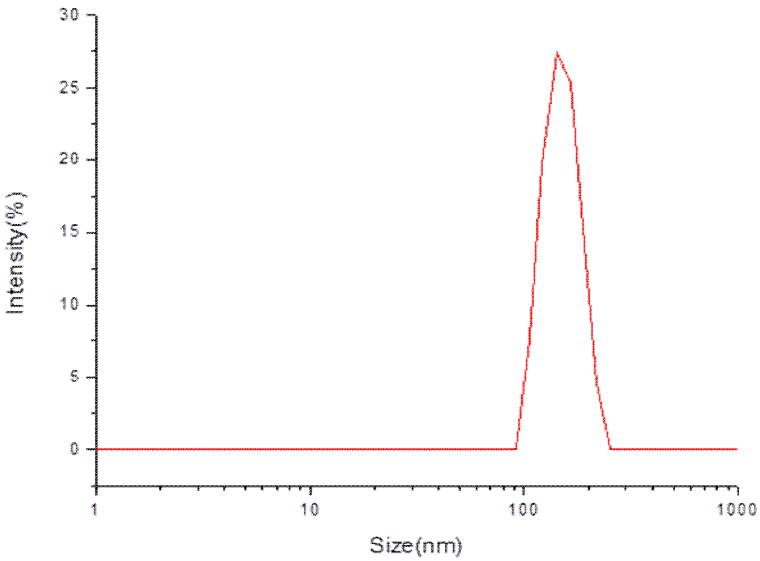


Figure 5. Size distribution of self-aggregates

3.4 *In vitro* release of Ac_3ManNAz

Only free form of Ac_3ManNAz can act as a building block in cell metabolism, therefore establishing the release of free Ac_3ManNaz is important. Since tertiary amines in PAMAM play role as a catalyst, degradation rate of ester bonds in pMPs was relatively faster than other ester bonds[19].

Hydrolysis of the ester bonds leds to a decrease in molecular weight and secretion of Ac_3ManNAz. These phenomenons were obserbed by GPC (Figure 6). Relase of Ac_3ManNAz was started from 3 hours, and amount of it was gradually increased to 24 hours.

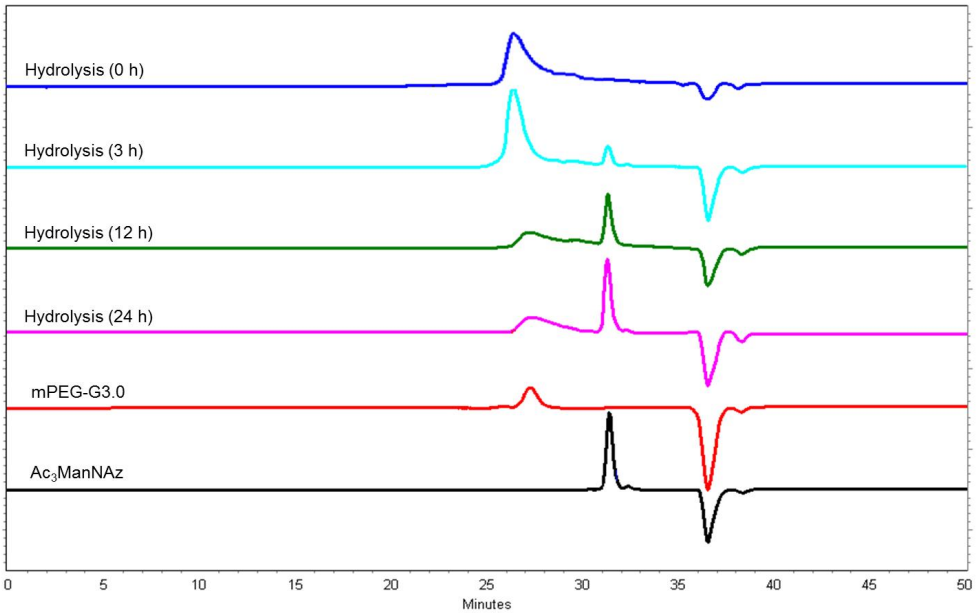


Figure 6. GPC spectra of pMPs on time-dependent hydrolysis

3.5 *In vitro* studies of pMPs

Purpose of *in vitro* studies is proving the expression of chemical receptors on tumor cell surface. Despite treatment with 20 uM of pMPs for 48 hours, no cytotoxicity was observed (Figure 7). Generation of azide groups was visualized by ADIBO-Cy5.5. Since ADIBO has high reactivity to azide groups in physiological condition without any catalysts[20], ADIBO is chosen as complementary bioorthogonal functional groups. Fluorescence intensity was depends on concentration of pMPs, and we decided that 20 uM was the optimal concentration for cellular imaging (Figure 8a). Western blot analysis using phosphine-PEG-biotin and streptavin-HRP demonstrated the generation of azide containing glycoprotein on the tumor cell surfaces after treatment of pMPs (Figure 8b). In addition, when the expression of the azide groups was visualized with time, it did not expressed until 6 hours and reached the peak at 48 hour after pMPs treatment. Time-dependent azide group expression was also proved by western blot analysis (Figure 9 a,b). The tendency of expression of azide groups with time is consitent with *in vitro* release experimental data.

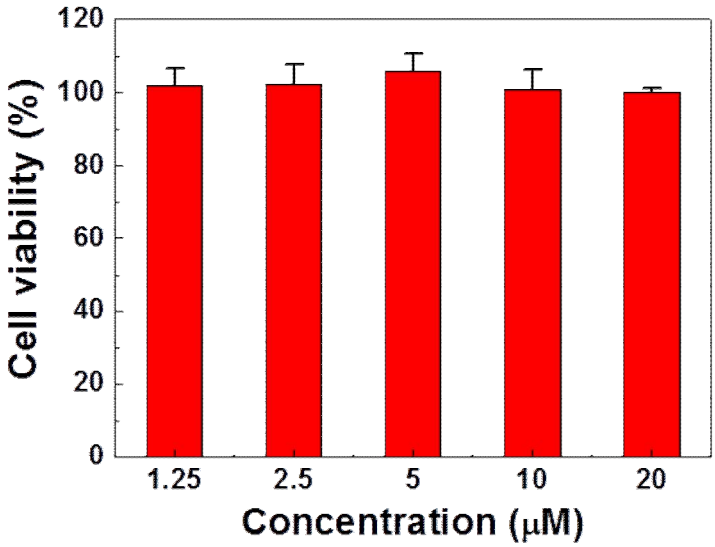
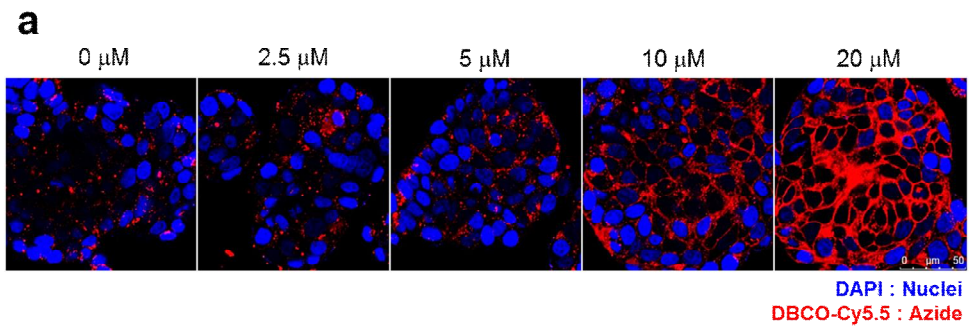


Figure 7. Viability of HT-29 cells treated with pMPs for 48 hours



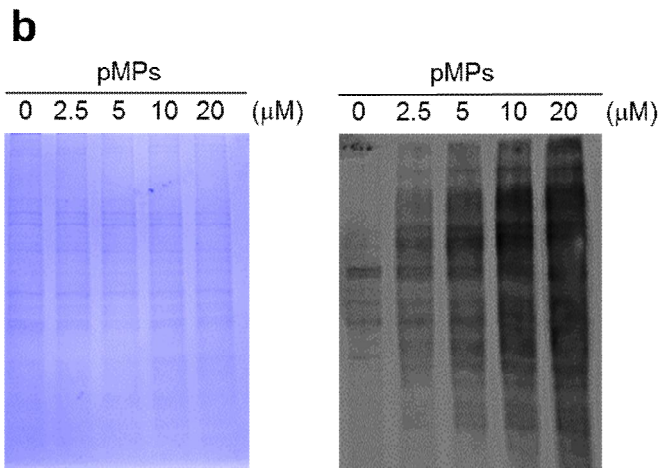


Figure 8. Azide group generation on cell surface in various concentration of pMPs (a) Visualization of azide groups on the surface of HT-29 tumor cells (b) Coomassie staining and wetern blot analysis of HT-29 tumor cells

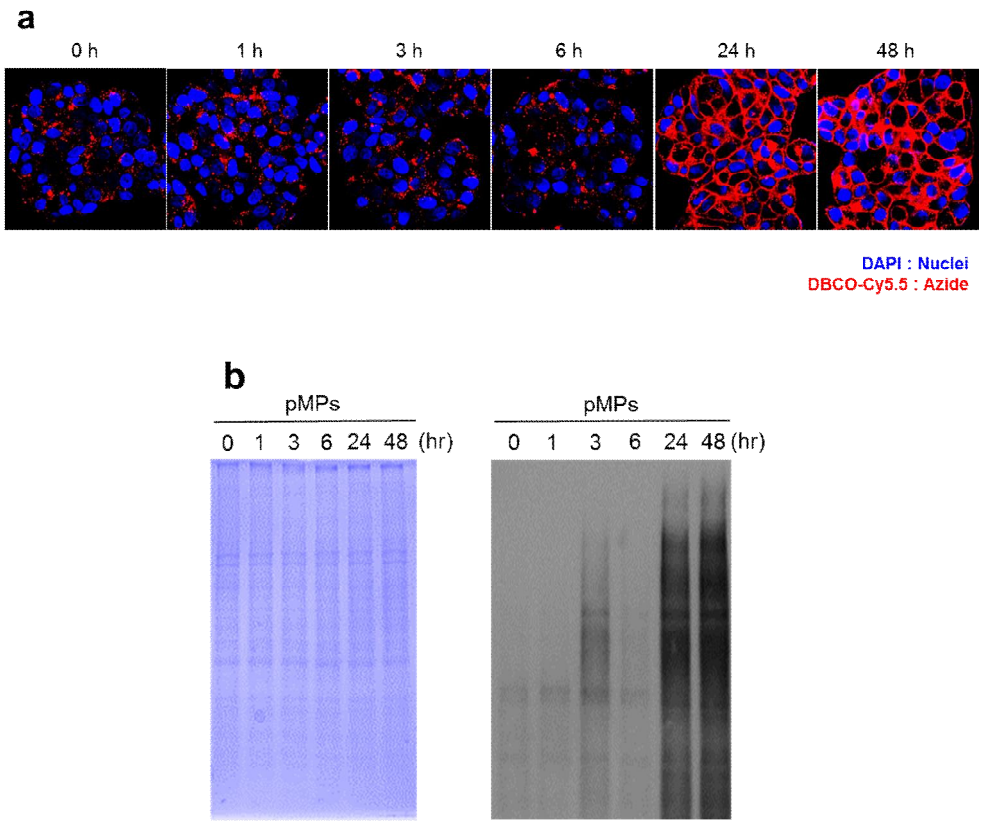


Figure 9. Time dependent azide group generation on cell surface after pMPs treatment (a) Visualization of azide groups on the HT-29 tumor cell surface (b) Coomassie staining and wetern blot analysis of HT-29 tumor cell

4. CONCLUSION

In this study, we developed polymerized metabolic precursors (pMPs) using PEG-PAMAM LDBC and Ac_3ManNAz. PEG-PAMAM [G3] was used as a backbone structure of the pMPs. By using mPEG-NH_2 as a starting material, all the purification steps required for the PEG-PAMAM synthesis were carried out by the simple precipitation method. PEG-PAMAM LDBCs were conjugated to Ac_3ManNAz via *Steglich* esterification to produce hydrolyzable pMPs. Since the amphiphilic nature of pMPs, it can form self aggregates in aqueous condition. The size of self assembly was about 150 nm, applicable size to exhibit the EPR effect.

In vitro studies showed that pMPs did not show cytotoxicity up to 20 uM and generation of azide groups on the tumor cell surface was confirmed by western blot analysis. Furthermore the 'receptore-like' azide groups could be labeled with ADIBO-Cy5.5 via copper free click chemistry.

The results from *in vitro* studies supported succesful preparation of pMPs and this strategy based on metabolic glycoengineering and copper free click chemistry will provide platform technology to overcome tumor heterogeneity.

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요약문

화학요법은 항암제를 이용하여 암을 치료하는 것이다. 항암제는 암세포에 작용한다면 약이지만, 정상세포에 작용한다면 독성을 야기한다. 따라서 종양에만 선택적으로 약물을 전달하는 약물 전달 체계가 화학요법에 있어 매우 중요하다. 약물 전달 체계에 대한 다양한 연구가 진행되면서 종양 세포만을 표적으로 할 수 있는 타게팅 모이티들이 많이 개발되었다. 하지만 종양세포의 이질성과 표적화 생체 분자의 양적한계 때문에 화학요법을 통한 암의 완치에 실패하였다.

이러한 한계점들을 극복하기 위하여, 본 연구에서는 고분자화 대사전구체를 이용하여 암 조직에 인공 표적 수용체를 도입하였다. PEG-PAMAM 과 Triacetylated-N-azido acetyl-D-mannosamine($Ac_3ManNAz$)의 화학적 결합을 통해 수용성 고분자화 대사 전구체를 합성하였으며, 이 물질은 수용액 상에서 150 nm 의 자가 조립체를 형성하였다. 세포 실험을 통하여 암의 표현 형질과 관계 없이 모든 종양 세포가 azide 기를 발현하는 것과 생리 조건에서 azide 기가 무동 클릭 화학을 통해 표적화 될 수 있음을 확인하였다. 본 연구에서 개발된 고분자회 대사

전구체를 생체 실험에 사용할 경우, 자가 조립체의 enhanced permeability and retention (EPR) 효과와 종양세포의 시알산 과다 발현을 통해 종양세포에만 특이적으로 azide 기를 도입할 수 있으며, 무동 클릭 화학을 통한 azide 기 표적화를 통해 약물 전달 효율을 증가시킬 수 있을 것으로 기대된다.

주요 어: 종양 이질성, 후성 표적화 전략, 고분자화 대사 전구체, 당 대사 공학, 무동 클릭 화학, 광학 이미징

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