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Creation of anticancer nanomedicine targeting lysosomal glycosidases

(がん細胞のリソソーム糖分解酵素を標的とするナノ医薬の創出)

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Doctor Thesis

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Table of Contents

Table of Contents.....	2
Abbreviations.....	4
Chapter 1 General Introduction.....	7
1-1. Lysosomal Metabolism in Cancer Cells.....	8
1-2. Nanosomes.....	15
1-3. Reference.....	19
Chapter 2 Efficient Synthesis of Glycosidase Inhibitor-Carrying Nanosomes And Evaluation of Their Functions.....	24
2-1. Introduction.....	25
2-2. Result and discussion.....	29
Evaluation of nanosomes behavior in serum.....	29
Nanosomes facilitate nanoparticle design without losing antiadhesive surface.....	34
2-3. Summary.....	42
2-4. Experimental.....	43
2-5. Reference.....	60
Chapter 3 Evaluation of Nanosomal Anti-Tumor Activity And Revealing Mechanism.....	64

3-1. Introduction.....	65
3-2. Result and discussion.....	66
Evaluation of lysosomal GlcNAc salvage pathway by utilizing glycomics.....	66
Following nanosomal intracellular dynamics by living cell imaging.....	70
Evaluation of nanosomal toxicity for cancer cells.....	74
Nanosomal antitumor activity for liver cancer bearing mice.....	80
Molecular mechanism in the enhanced anti-tumor effect of nanosomes.....	84
Evaluation anti-tumor effect for invasive and non-invasive cancer cells.....	91
Comparing anti-cancer effect between cancer cells and normal cells.....	96
3-3. Summary.....	101
3-4. Experimental.....	103
3-5. Reference.....	112
Chapter 4 Conclusion Remarks.....	117
Acknowledgement.....	121

Abbreviations

1 D	1 dimension
2 D	2 dimension
Ao	aminoocy
Ac	acetyl
AcOH	acetic acid
Boc	Tert-butyl
CHCl ₃	chloroform
CMP	Cytidine monophosphate
DIC	<i>N,N</i> -diisopropylcarbodiimide
DHB	Dihydroxybenzoicacid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
eq.	Equal
ESI	electro spray ionization
Euc	Fucose
Gal	galactose
GlcNAc	<i>N</i> -acetylglucosamine
GalNac	<i>N</i> -Acetylgalactosamine
K _i	binding constant
K _{inact}	inactivation rate
Man	mannose
MALDI	Matrix assisted laser desorption ionization

Me	methyl
MeCN	acetonitrile
MeOH	methanol
MS	mass spectra
NANA	N-acetyl neuraminic acid
NaOMe	sodium methoxide
NAG	N-acetylglucosaminidase
Neu5Ac	sialic acid
NMR	nuclear magnetic resonance
<i>p</i> -NSA	<i>p</i> -Nitrosalicylaldehyde
<i>p</i> -NP	<i>p</i> -nitrophenol
NP	Nano-Particle
NS	Nanosome
PC	Phosphocholine
QDs	Quantum dots
r.t.	room temperature
SAMs	self-assembled monolayer-coated
$t_{1/2}$	half-time for inhibition
THF	tetrahydrofuran
TOF	time of flight
TOPO	trioctylphosphine oxide
UDP	uridine-5'-phosphate
UV	ultra violet

Chapter 1

General Introduction

1-1 Lysosomal Metabolism in Cancer Cells

All roads lead to lysosome.¹ Endocytic trafficking of the extracellular macromolecules toward lysosome is a critical metabolic pathway to provide cancer cells with many fuels such as amino acids, monosaccharides, lipids, and intermediates re-used for the synthesis of macromolecules during extremely accelerated growth and malignant transformation.^{2,3} A burgeoning body of evidence has indicated that cancer cells synthesize rapidly high level of multi-antennary *N*-glycans and densely clustered *O*-glycans, as well as various glycosaminoglycans and hyaluronic acid than normal cells during proliferation and metastasis.⁴ Notably, posttranslational protein glycosylation in cancer cells needs a vast amount of sugar resources supplied from lysosomal degradation systems. An increase in the synthesis of glycoproteins having hyperbranched *N*-glycans is a hallmark of proliferating cancer cells to construct the galectin lattice that contributes to neoplastic transformation, tumor cell survival, angiogenesis and tumor metastasis.⁵ Increased hyper-branching *N*-glycan expression during malignant transformation may be due to enhanced activities of *N*-acetylglucosaminyltransferases IV and V (GnT-IV and GnT-V).⁶ However,

given that GnT-IV and GnT-V show considerably lower affinity ($K_m = 5.0$ mM and 11.0 mM) with a common substrate, uridine 5'-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc), than other GnTs such as GnT-I ($K_m = 40$ μ M) and GnT-II ($K_m = 960$ μ M),⁷ it is clear that increasing intracellular concentration of UDP-GlcNAc is crucial for the rapid synthesis of hyperbranched *N*-glycans. Hexosamine biosynthetic pathway (HBSP) is a central *de novo* biosynthetic process to produce UDP-GlcNAc from D-glucose supplied through its transporters. Alternatively, GlcNAc is salvaged significantly into HBSP from the turnover of glycoconjugate by the degradation processes in lysosomes, where they can be phosphorylated by GlcNAc-6-kinase and subjected directly to the synthesis of UDP-GlcNAc, and utilized subsequently for *de novo* synthesis of CMP-Neu5Ac and UDP-GalNAc, respectively (**Figures 1-1**).⁸ Therefore, it should be emphasized that cancer cells use extensively the salvage pathway to produce a large amount of GlcNAc for the synthesis of UDP-GlcNAc as fundamental material.

Hexosamine synthetic pathway

GlcNAc salvage pathway

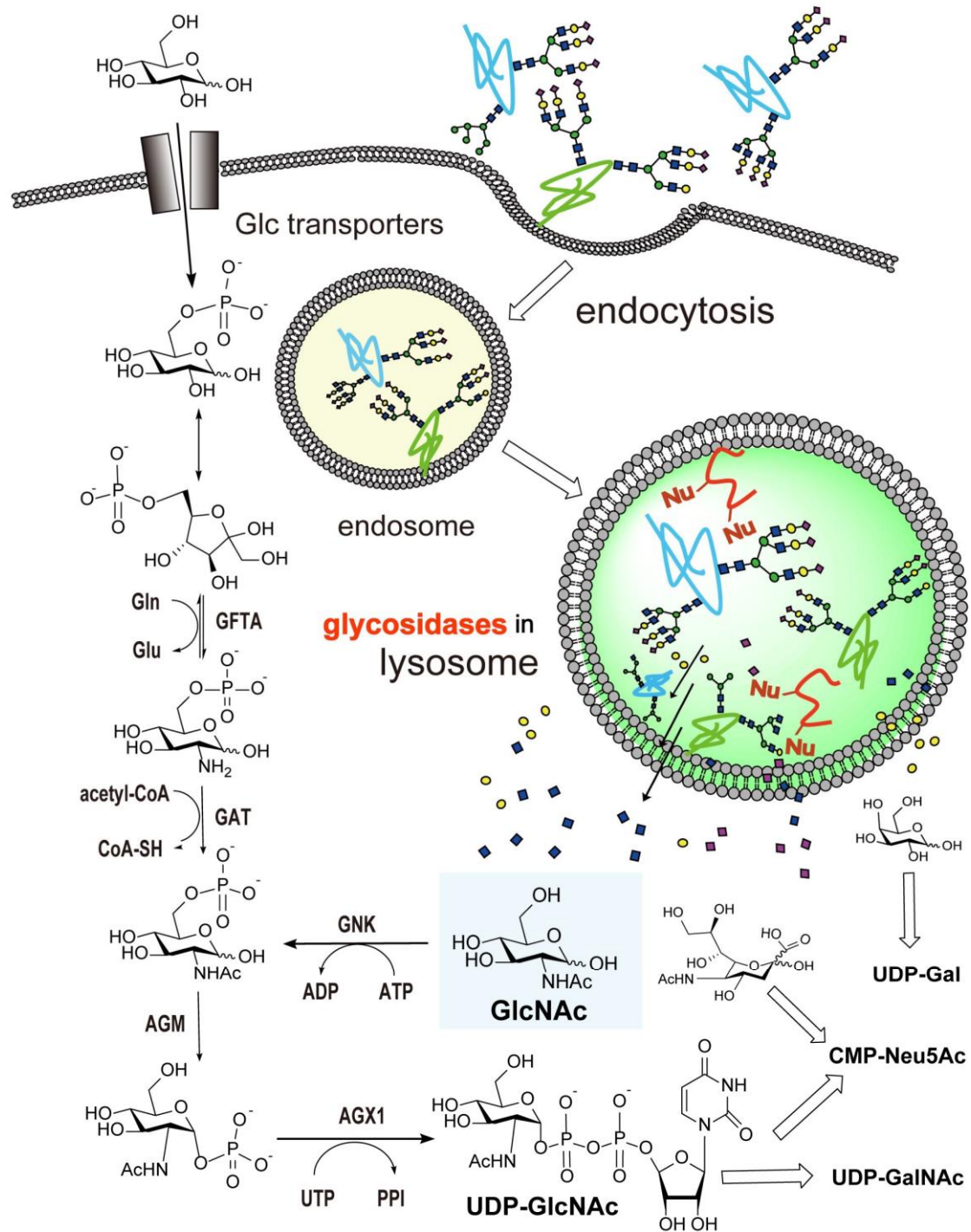


Figure 1-1-1. Glycan metabolism and biosynthetic pathway

Indeed, Vocadlo et al.⁹ and our previous results¹⁰ demonstrated that unnatural GlcNAc analogs such as 5S-GlcNAc and 4F-GlcNAc act as metabolic glycosyltransferase inhibitors by hijacking a salvage pathway to convert into unnatural UDP-GlcNAc analogs. Intracellular accumulation of the unnatural UDP-GlcNAc then shuts down the synthesis of natural UDP-GlcNAc, which inhibits the activities of β -*N*-acetylglucosaminyltransferase (OGT), GnT-IV and GnT-V, resulting in the decrease of *O*-GlcNAc modification of the intracellular proteins⁹ and hyperbranched *N*-glycans on cancer cell surface.¹⁰ Similarly, it was also reported that fluorinated analogs of fucose and sialic acid (2F-Fuc, 6F-Fuc, and 3F-Neu5Ac) taken up by cells are converted preferentially into the corresponding nucleotide sugars through salvage pathways, which inhibit the synthesis of GDP-Fuc and CMP-Neu5Ac.¹¹ Despite significant inhibitory effects on various glycosyltransferases and unique hijacking mechanism shown by cell-based experiments, the clinical potency of the metabolic inhibitors appears to be limited due to highly inefficient cellular uptake and difficulties in the *in vivo* enhanced retention in the circulation and targeted delivery of these small sugar mimics.¹² Importantly, the degradation of *N*-glycans attached to

proteins accumulated in lysosomes determines the efficacy of GlcNAc salvage pathway to provide cancer cells with a sufficient amount of nucleotide sugars. It is, therefore, not surprising that glycosidases reside in lysosomes to hydrolyze *O*-glycosidic linkages in major glycan structures (**Figures 1-2**) may become highly potential and alternative targets for glycosyltransferases^{13,14} when the inhibitors are incorporated efficiently into the intracellular space. Our present results demonstrate for the first time that lysosomal glycosidases are promising anticancer targets when smart antiadhesive nanoshuttles transport the mechanism-based covalent inhibitors (**Figures 1-3**) through the derailed endocytic route of cancer cells.

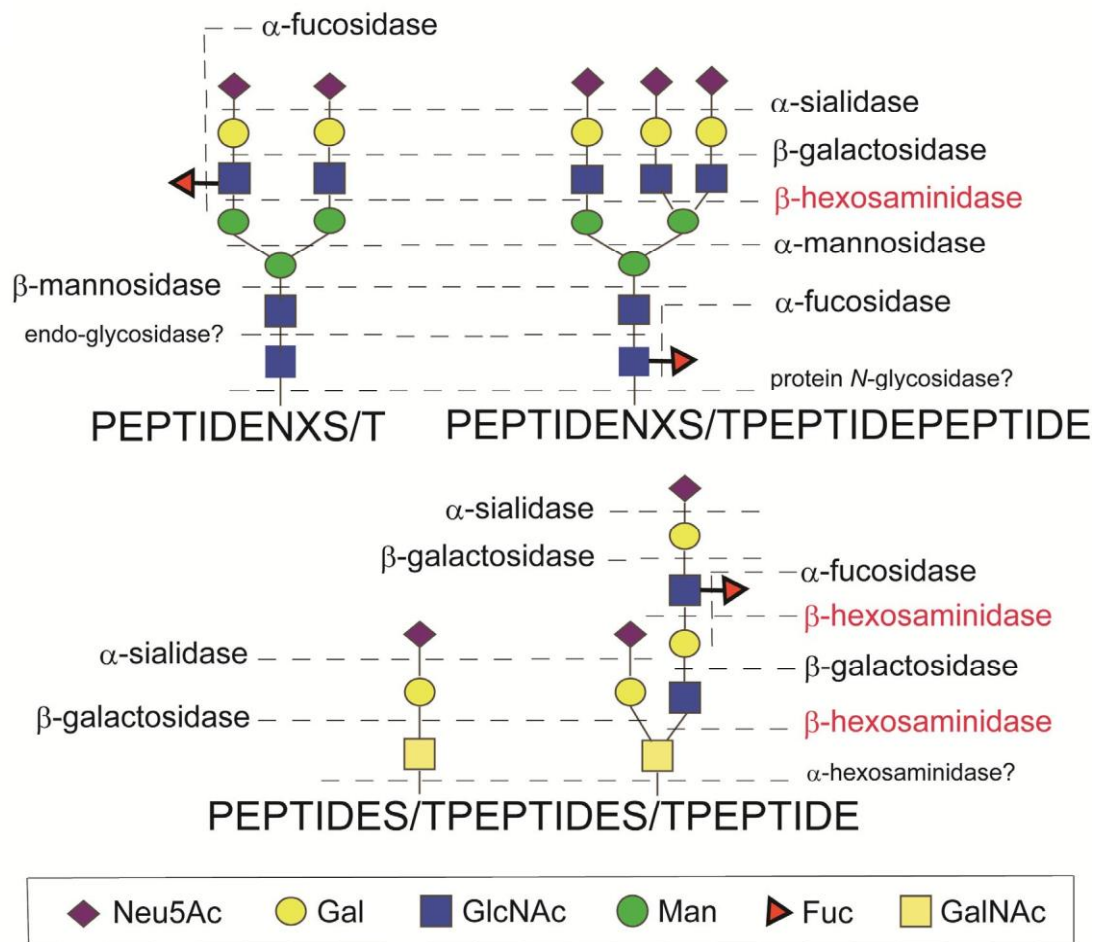


Figure 1-1-2. *N*- and *O*-glycans attached to the proteins are digested consecutively from non-reducing end by *exo*-glycohydases such as α -sialydases, β -galactosidases, β -hexosaminidases and some other important glycosidases such as α -mannosidases, α -fucosidases, and β -glucuronidase,.

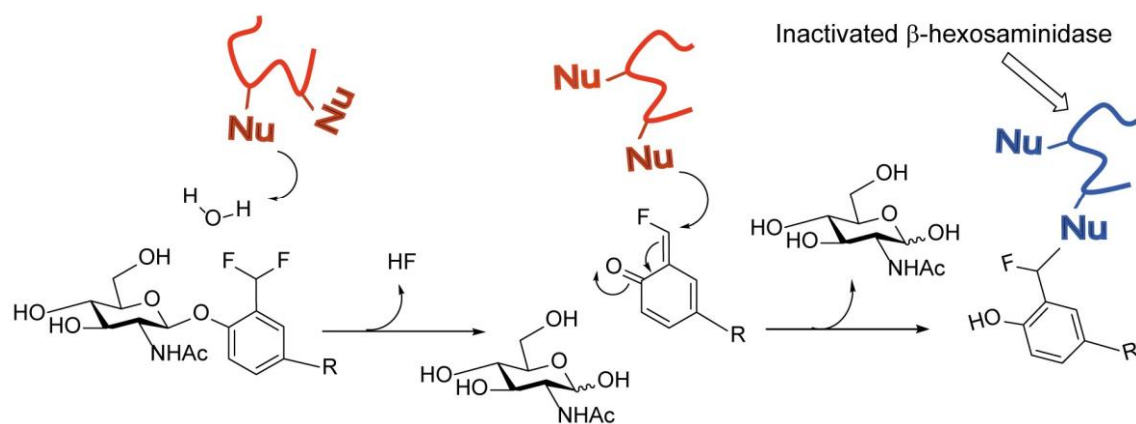


Figure 1-1-3. Mechanism-based inhibition of *exo*-glycosidases represented by an irreversible covalent inhibitor (a suicide substrate) for β -hexosaminidase. The aglycone moiety inactivates enzyme by modifying covalently a critical nucleophile (Nu) at the active site.

1-2 Nanosomes

Nanoparticles (NPs) have been focused as very strong tools for drug delivery system. There are a variety types of NPs such as gold nanoparticles, and quantum dots, magnetic beads. These nanoparticles have various features depending on their size. Firstly, NPs easily accumulate in tumors by enhanced permeability and retention (EPR) effect.^{15,16} Secondly, cancer cells incorporate NPs by highly activated endocytosis.^{17,18} However, nanoparticles still have main challenges related to nonspecific “protein corona”. To solve these problems, “nanosomes” were developed as new drug delivery tools.¹⁹ The cellular membranes that define interfaces between individual cells and interactions with extracellular proteins naturally lead supramolecular platforms to control many biological processes such as intercellular adhesion, receptor-ligand recognition mediated endocytosis, and subsequent intracellular signal transductions. Cell membranes are mixed self-assembled lipid bilayer composed of two major classes of structural lipids: (a) glycerophospholipids: phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns), and phosphatidic acid (PA), and (b)

sphingolipids: sphingomyelin (SM) and glycosphingolipids (GSLs). Importantly, PtdCho accounts for more than 50% of the phospholipids in mammalian cells and self-organizes spontaneously as a planar and stable bilayer with a cylindrical molecular geometry, while the composition of other lipids in different organelle membranes varies among cells, and this may impose a curvature stress on the membrane.²⁰ Therefore, it is likely that highly organized geometry of the zwitterionic phosphorylcholine (PC) headgroup of PtdCho and SM is essential for achieving antifouling property of the cell surfaces, in which various membrane receptors can interact with specific ligands while avoiding the influence of nonspecific protein adsorption. The pioneering work by the Whitesides's group has demonstrated that coating gold surfaces with self-assembled monolayer (SAM) of alkanethiolates having a zwitterion headgroup enables their surfaces to resist the nonspecific adsorption of proteins.²² Remarkably, the concept of mixed SAMs containing the zwitterionic and other functional headgroups could allow generating surfaces with specific ligands and antifouling nature, concurrently.²³ These discoveries and importance of well-organized PC headgroups of PtdCho and SM in biological membranes greatly encouraged us to explore an ideal mixed

SAM coating that enables nanoparticles to display glycosidase inhibitors without losing antifouling property.

This thesis focuses on lysosomal glycosidases as the target enzymes to suppress cancer growth, and nanosomes are used as drug delivery carrier to target lysosomal enzymes.

In chapter 2, glycosidase inhibitor displayed nanosomes were synthesized and characterized. Firstly, the behavior of NPs that coated various ratios of PC and PEG linker was observed by FCS to evaluate protein corona formation, and 20% of PC linker was enough to prevent protein corona. Then, glycosidase inhibitors targeting β -hexosaminidase and galactosidase were synthesized and displayed on nanosomes (**NS-8101** and **NS-8102**). Furthermore, **NS-8101** was evaluated inhibition activity to human placenta β -hexosaminidase, and covalent inhibitor activity was increased by displaying on nanosomes.

In chapter 3, anticancer activity of NSs were evaluated with *in vitro* and *in vivo*. Firstly, intracellular dynamics of nanosomes were followed by living cell imaging. From the imaging, **NSs** localized in lysosome after 8h. However, **NS-8101** and **NS-8102** were escaped from lysosome. Then, the toxicity of nanosomes for cancer cells were evaluated by MTT assay. As a result, **NS-**

8101 induced lysosomal membrane permeabilization yielding apoptotic cell death in HepG2 (IC₅₀ = 10.7 nM) and MDA-MB-231 (IC₅₀ = 31.8 nM). **NS-8102** showed strong toxicity for A549 cells. Furthermore, treatment with **NS-8101** suppressed tumor growth in the human liver cancer xenograft mice.

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Chapter 2

Efficient Synthesis of
Glycosidase Inhibitor-Carrying
Nanosomes
And Evaluation of Their Functions

2-1 Introduction

The pioneering work by Whitesides demonstrated that coating gold surfaces with self-assembled monolayer (SAM) of alkanethiolates having a zwitterionic headgroup enables their surfaces to resist the nonspecific adsorption of proteins.¹ This finding and importance of well-organized phosphorylcholine (PC) headgroups of phosphatidylcholine (PtdCho) and sphingomyelin (SM) of biomembranes² encouraged us to explore an ideal SAM coating that enables nanoparticles to display glycosidase inhibitors without losing antifouling property. In 2011, we established a facile and generalizable method for the preparation of such antiadhesive nanoparticles made of hard-core metal such as quantum dots (QDs), gold nanoparticles (AuNPs), and softshell mixed SAM of 11-mercaptoundecylphosphorylcholine (PC linker)^{1,3} and an aminooxy-functional headgroup, 11,11'-dithio bis[undec-11-yl 12-(aminooxyacetyl)amino hexa(ethyleneglycol)] (AO linker),⁴ namely nanosomes (NSs) as new class of smart nanoparticulate platform (**Figures 2-1-1**).^{5,6} Oxime-based surface loading of the aldehyde/ketone auxiliary⁷ (**Schemes 2-1-1**) is expected to facilitate multimodal design of antiadhesive nanoparticles carrying various cargos in a designated avidity.

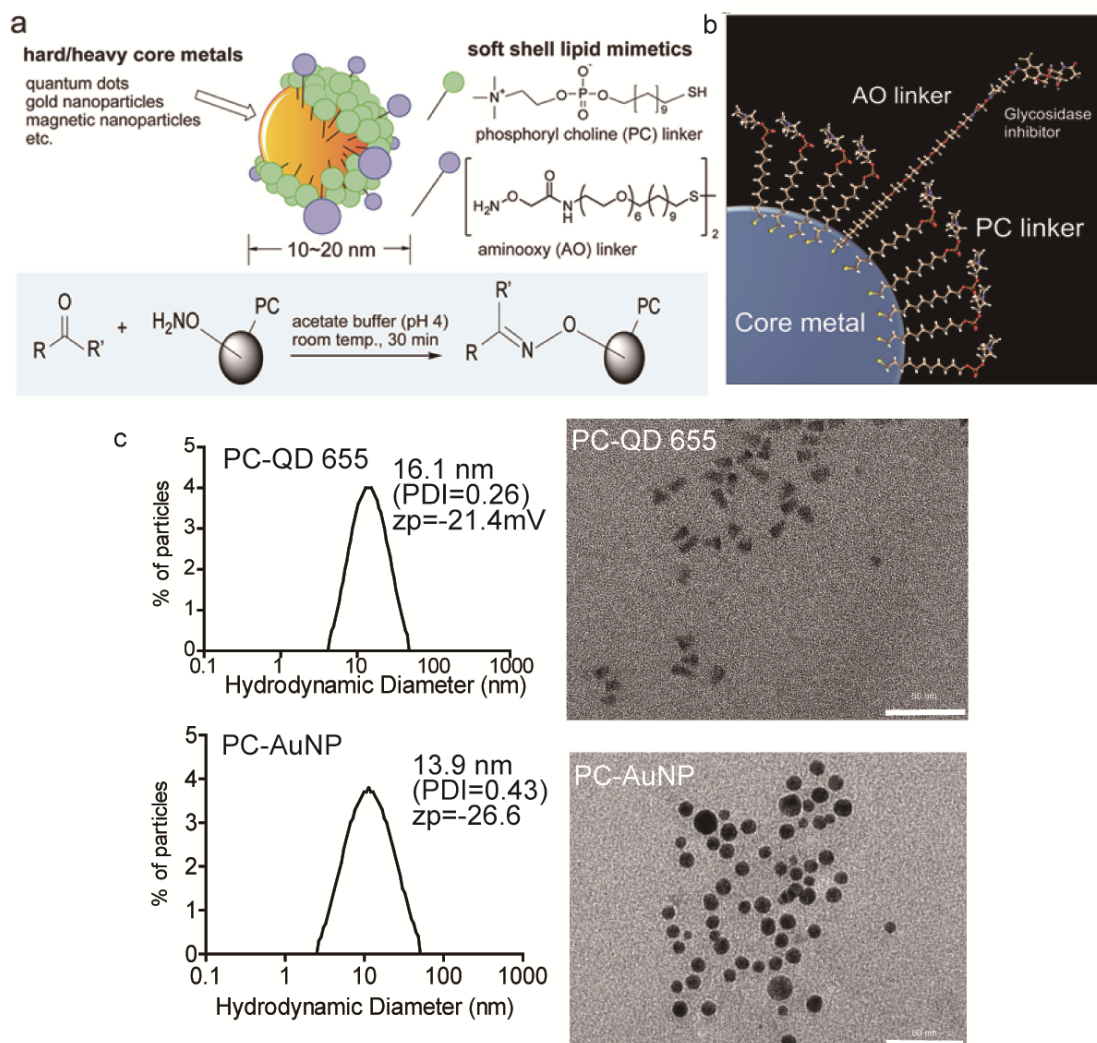
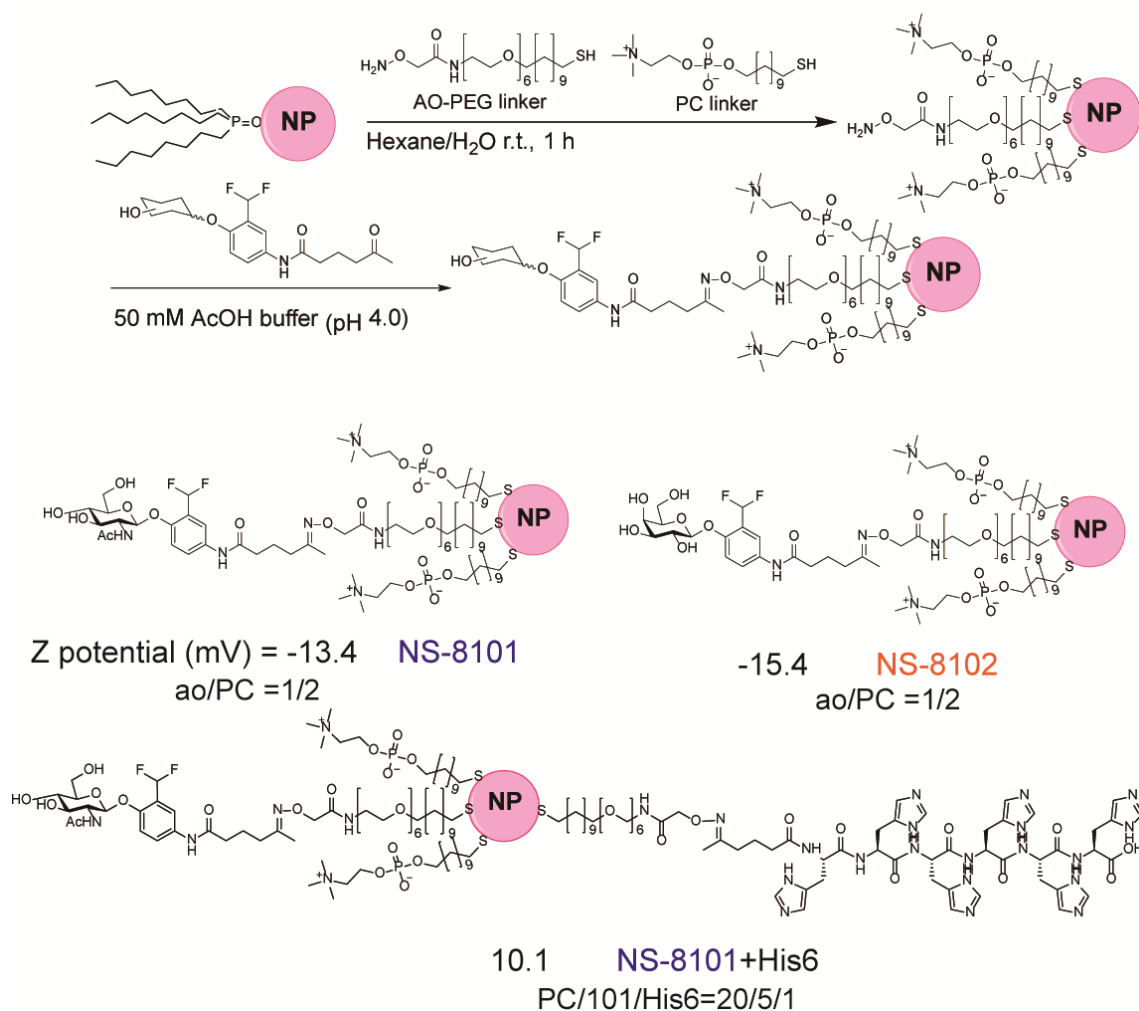


Figure 2-1-1. (a) Schematic representation of nanosome composed of core metals and shell of mixed SAM of PC and AO linkers, and chemistry of the surface loading of aldehyde/ketone auxiliary. (b) Surface chemistry of NSs representing highly packed mixed SAM composed of AO linker and PC linker. Density of PC linkers of the single component SAM coating QD655 or AuNP nanoparticle surface in case of QD/Au nanoparticles of an average diameter of 10 nm was estimated to be 67.1 PC linker/nm² according to our previous results determined by means of ¹H-NMR. On a flat plane gold substance, it was reported that 1 PC linker in the PCSAM theoretically occupies 0.05042 nm² corresponding to 19.8 PC linker/nm². (c) Transmission electron micrograph (TEM) and dynamic light scattering (DLS) of NSs showing average hydrodynamic diameter and zeta potential, respectively.



Scheme 2-1-1. Coating QDs surface by PC and ao linker. Compounds were displayed on NPs under acid conditions. Each compound displaying NPs zeta potentials.

In this chapter, **NS-8101** and **NS-8102** were synthesized as carrier of covalent inhibitors that target β -hexosaminidase and β -galactosidase (**Scheme 2-1-1**), highly expressed in human cancer cells.¹⁰ Compounds **101** and **102** are designed to serve dual functionalities: “a suicide substrate” liberates an active intermediate (**Figure 2-1-1b**)¹¹ and a ketone linker allows a conjugation with nanoparticles. FCS of NS-8101 in 100% FBS were performed to evaluate antiadhesive effects on natural physiological environments (**Figure 2-1-2b**). Targeted delivery of NS-8101 and NS-8102 allows achieving much higher potency in the inhibition than those of free inhibitors. To investigate the relationship between intracellular traffic of NS-8101 and antitumor effect, NS-8101/His6 was also prepared (**Scheme 2-1-1**), in which His6 helps evasion of early endosomes.⁵

2-2 Results and discussion

Evaluation of nanosomes behavior in serum

Our previous study has shown that NSs composed of quantum dot (QD) core and PCSAM shell exhibits highly excellent solubility (dispersibility) in protein-free saline and long-term stability in acidic aqueous solution (pH>2) without losing quantum yields.⁵ In the present study, we tested more precisely antifouling nature of the NSs coated by the mixed SAM composed of PC linker and PEG linker [(1-mercaptoundec-11-yl) hexa (ethyleneglycol)]⁸ in physiological environments by fluorescence correlation spectrometry (FCS) analysis in 100% FBS (**Figure 2-2-1**). The results clearly showed that the antifouling property of NSs depends strongly on highly organized surface PC headgroups of the mixed SAM coating (**Figure 2-2-2**). In 100% FBS, nanoparticles completely covered by SAM of the alkanethiolate having an hexa (ethylene glycol) headgroup aggregated immediately to form precipitation, indicating that protein adsorption onto this surface from highly heterogeneous biological fluids is an entirely irreversible process.⁹ In contrast, the mixed SAM of PEG linker with PC linker provided NSs with strikingly enhanced dispersibility independent of surface “protein corona” in 100% FBS

throughout the tested conditions (PC=20~100%) as evidenced by good fitting curves with a single-component diffusion model (**Figure 2-2-2**). We have shown that the mixed SAM formed by combined use of AO linker containing the hexa(ethylene glycol) moiety with PC linker facilitates surface loading of various cargo molecules having an aldehyde/ketone auxiliary without losing antiadhesive nature of PCSAM coated NSs (**Figure 2-2-1**). Indeed, near-infrared (NIR) fluorescence imaging of QD-based NSs displaying synthetic glycan derivatives after intravenous administration of the mouse has revealed the significance of the terminal sugar residues in the biodistribution and *in vivo* circulation of the glycosylated-NSs.⁵ It has also been uncovered that most NSs carrying synthetic glycopeptides can be internalized endocytically by cultured human cancer cells and accumulated consequently in lysosomes, whereas they can avoid an early endosomal trap when NSs are modified concurrently with a ketone capped histidine hexapeptide (His₆).⁶ It is important to note that surface passivation by zwitterionic PCSAM coating dramatically reduces nonspecific absorptions of serum proteins. (**Figure 2-2-2**). It is considered that glycans displayed through oxime bond on the mixed AO/PCSAM were capable of showing the abovementioned functions as ligands

of cell surface lectins¹² that may ultimately determine *in vivo* biodistribution²⁸ independent of nonspecific protein adsorption.

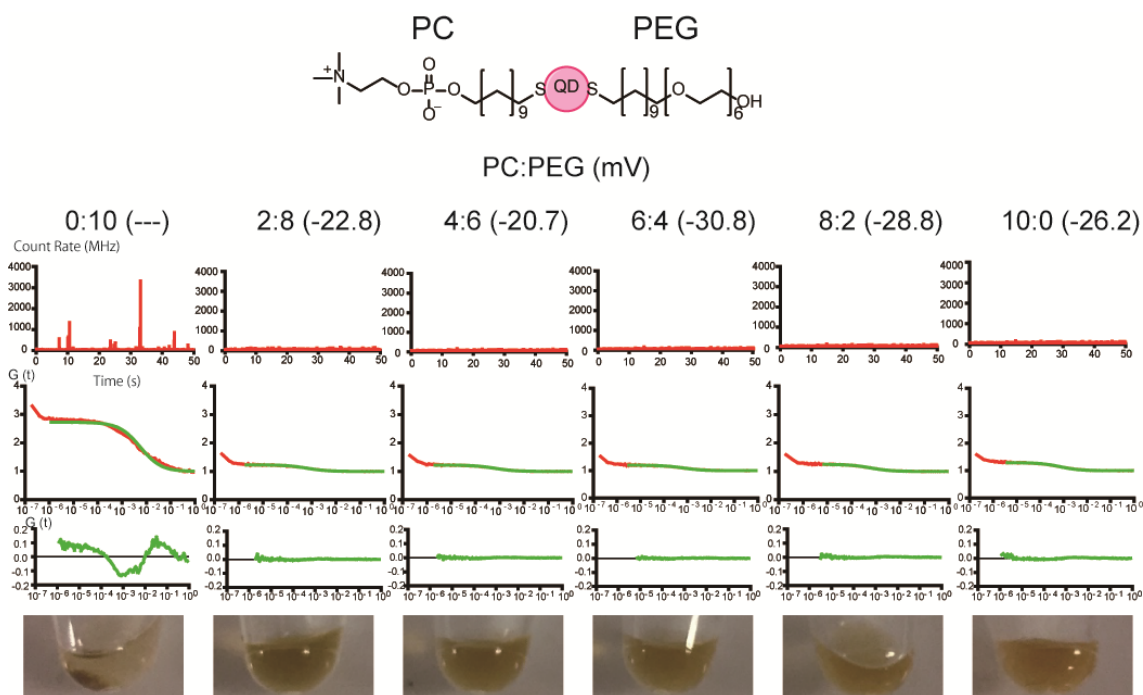


Figure 2-2-1. Pictures represent behavior in 100% fetal bovine serum (FBS) of nanoparticles (QD655) coated by mixed SAM composed of PC linker and PEG linker, in which only nanoparticles coated by SAM of PEG linker (PEG/PC=100/0) formed a large aggregation as indicated by red arrows. FCS analysis shows fluorescence fluctuations of these nanoparticles in 100% FBS. The autocorrelation curves were fitted by using one component diffusion model.

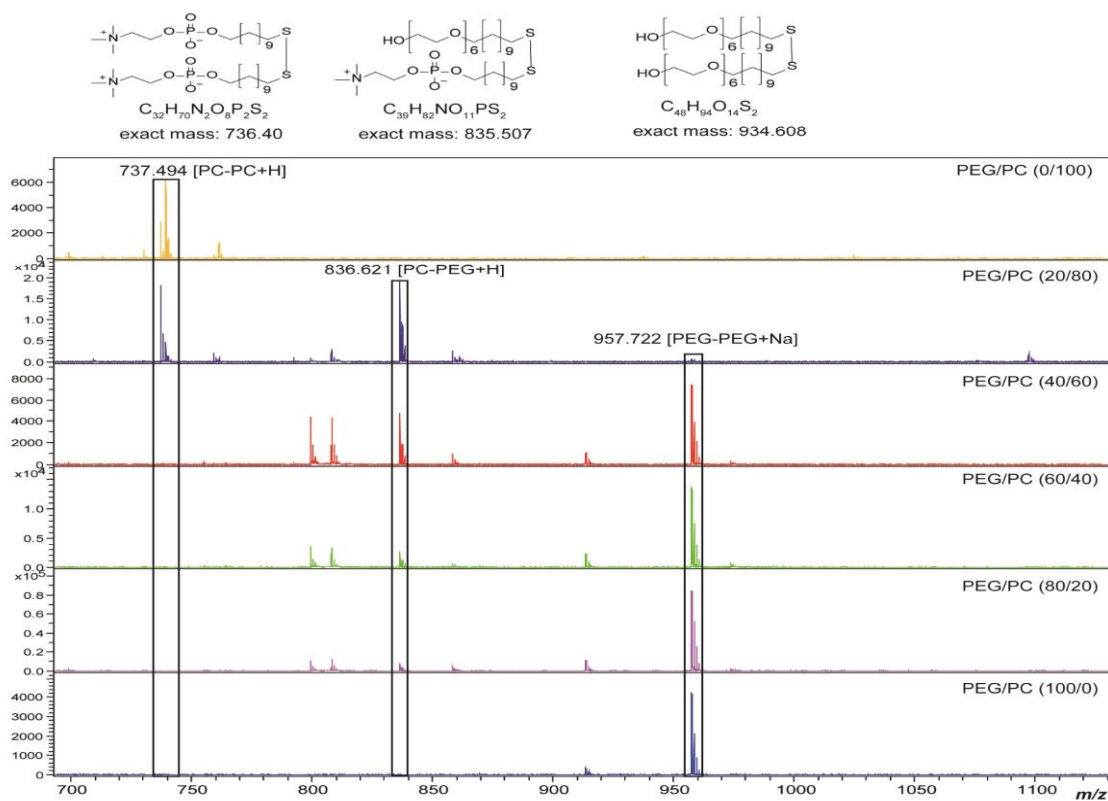


Figure 2-2-2. Characterization of nanosomes coated by mixed SAM composed of PEG and PC linkers used in **Figure 2-2-1** by MALDI-TOFMS. The signals at m/z 737.494, m/z 835.507, and m/z 934.608 corresponding to the three structures derived from PEG linker and PC linker can be used for the estimation of the molar ratio of PEG/PC of the mixed SAM.

Nanosomes facilitate nanoparticle design without losing antiadhesive surface.

Merits of oxime-based surface loading strategy using mixed SAM-coated nanoparticles are evident because nucleophilic addition of highly reactive AO group to the aldehyde/ketone auxiliary proceeds specifically in an aqueous solution without any coupling reagents^{7,13} and gives quantitatively antiadhesive nanoparticles carrying cargoes in a designated surface density according to the molar ratios between PC linker and AO linker in the mixed SAM coating (**Figure 2-1-1a**).^{5,6} With this strategy, we could prepare NSs (**NS-8101** and **NS-8102**) carrying covalent inhibitors targeting two major lysosomal glycosidases: β -hexosaminidase and β -galactosidase (**Table 2-4-1**), which are highly expressed in human cancer cells.^{16,17} Aglycone of compounds **101** and **102**, 2-(difluoromethyl)-4-(5-oxo-hexane-1-yl) phenoxy moiety, was designed to serve dual functionalities such as (a) “a suicide substrate” liberates an active intermediate to inactivate enzymes only after specific cleavage an *O*-glycoside bond between the aglycone and GlcNAc β 1 $\rightarrow$ or Galb1 $\rightarrow$ by the targeted glycosidases (**Figure 1-1-1c**),^{14,15} and (b) a ketone linker enables small covalent inhibitors to combine with nanoparticles through an oxime bond (**Figure 2-1-1a**, and **Figure 2-2-3**). We considered that

targeted delivery of **NS-8101** and **NS-8102** to lysosomes in cancer cells allows for achieving much higher potency and selectivity in the inhibition than those of the free covalent inhibitors (**Figure 2-2-7**). In addition, our recent advances in multicomponent surface loading protocol enabled us to elaborate **NS-8101/His₆** (**Figure 2-2-3**), in which **101** moiety inactivates selectively lysosomal β -hexosaminidases and **His₆** moiety helps rapid escape of NSs from early endosomal compartment after endocytosis.⁶ Accordingly, we postulated that characterization of intracellular traffic and cytotoxicity of **NS-8101/His₆** might permit insight into molecular mechanism in the antitumor activity of nanomedicines targeting lysosomal glycosidases. Importantly, chemical ligation of AO linker with compound **101** and **His₆** derivative did not alter hydrodynamic diameter (19.5 nm) and antifouling property of NSs, when PC linker is a major component of the mixed SAM, while zeta potentials of NSs may be affected significantly by the balance of surface density between the compounds displayed concurrently (**Figure 1-1-1b**, **Table 2-2-1**, **Figure 2-2-3**, **Figure 2-2-4**, **Figure 2-2-5** , **Figure 2-2-6** and **Table 2-2-1**). Given that the density of PC linker in the single component SAM on the core QD of 10 nm in an average diameter was estimated to be 67.1 PC linkers/nm² (0.0149 nm²/PC

linker),⁵ much smaller area occupied by 11-mercaptoundecyl moieties of the mixed AO/PCSAM on the curved surface of such small core nanoparticles (~10 nm) than that reported for flat gold surface (0.05042 nm²/PC linker)³ may be essential for the formation of highly packed and stable innermost alkanethiolate phase. As a result, surface curvature of core metal nanoparticles can preserve the configurational flexibility of zwitterionic PC headgroups leading to cell membrane-like “antifouling property” based on minimized electrostatic energy and total dipole moment in outer surface phase of the mixed SAMs. Notably, hexa(ethylene glycol) moiety of AO linker will provide distal glycosidase inhibitors **101** and **102** as well as **His₆** with sufficient flexibility and distance from antifouling surface of PCSAM, thus allowing for a direct interaction both with cell surface lectins and lysosomal glycosidases (**Figure 2-1-1b**).

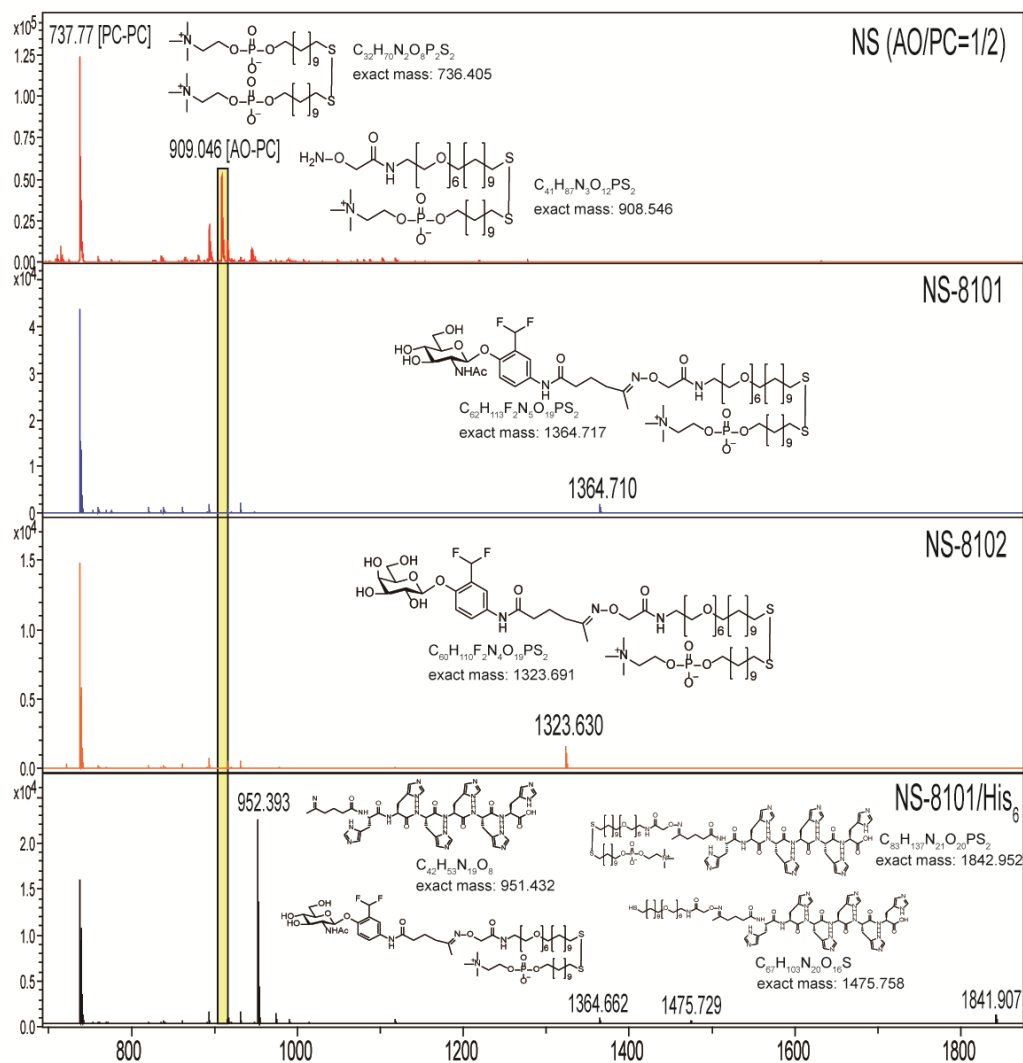


Figure 2-2-3. The signal at m/z 909.0 of MALDI-TOFMS corresponding to the ion composed of AO and PC linkers can be used as a convenient marker to monitor the reaction with cargo molecules having a ketone/aldehyde auxiliary.

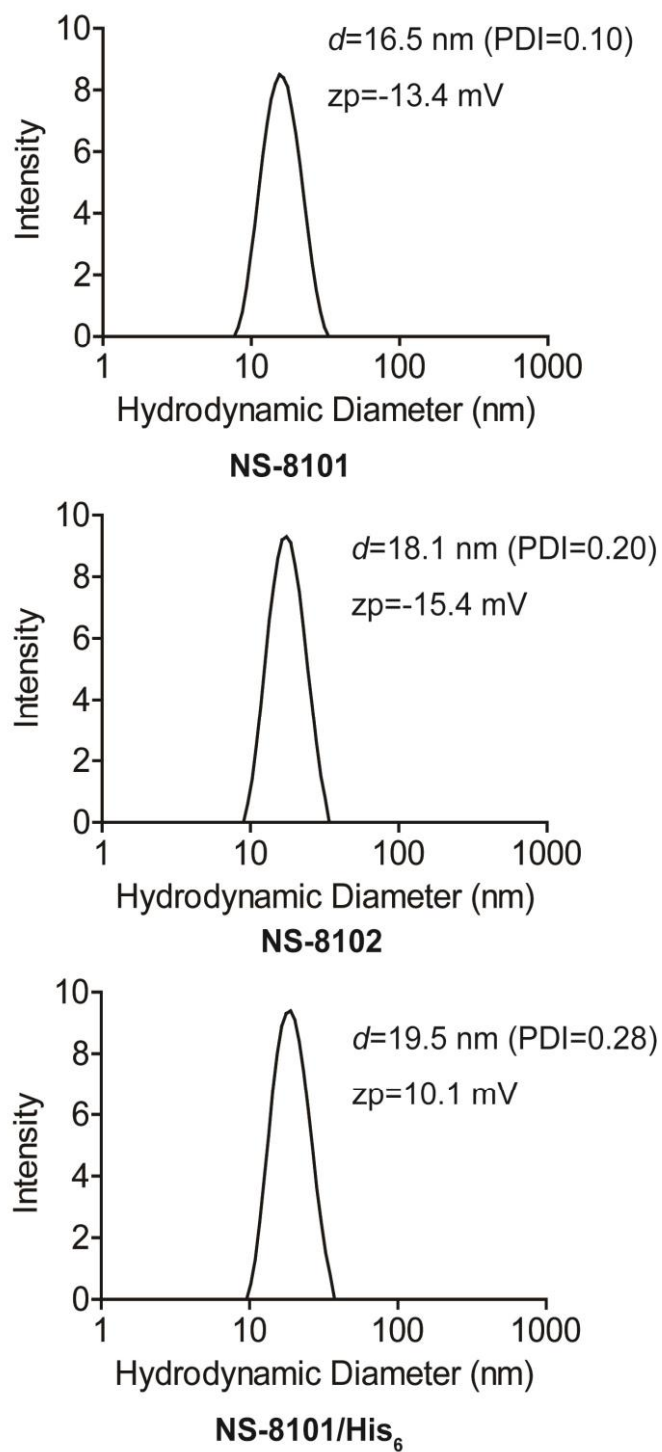
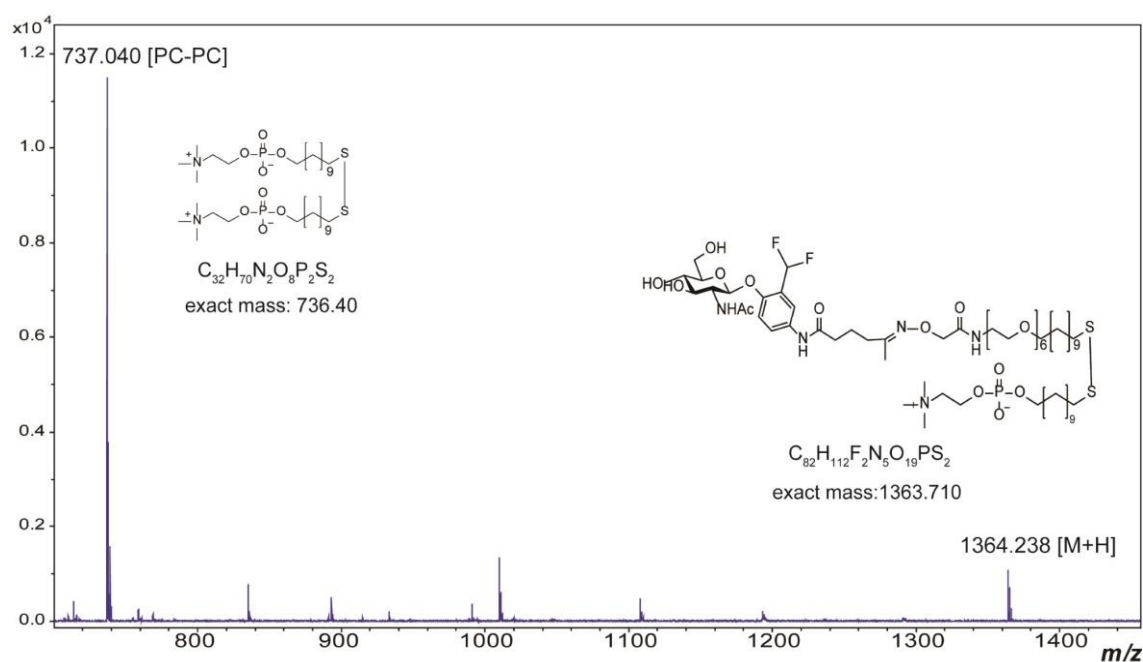


Figure 2-2-4. Dynamic light scattering (DLS) showing average hydrodynamic diameter. “zp” means zeta potential.

a



b

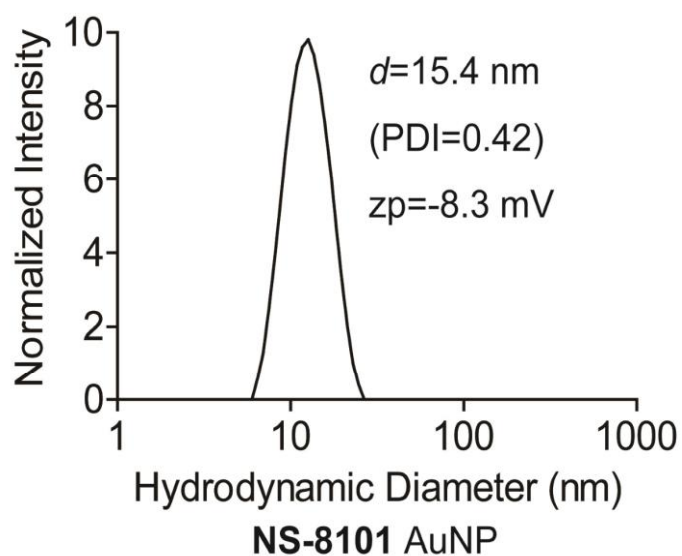


Figure 2-2-5. Characterization of NS-8101 prepared from AuNP as a biocompatible counterpart of QD. (a) MALDI-TOFMS. (b) Dynamic light scanning (DLS) showing average hydrodynamic diameter. “zp” means zeta potential.

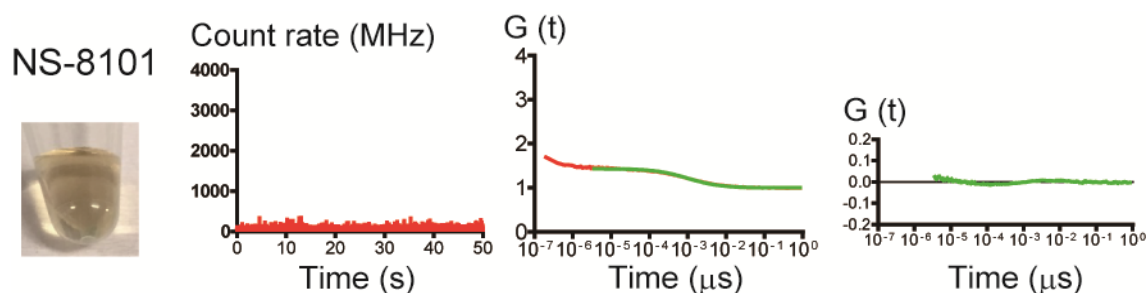


Figure 2-2-6. Picture represents behavior in 100% fetal bovine serum (FBS) of NS-8101 (PC/101=2/1). FCS analysis shows fluorescence fluctuations of NS-8101 (PC/101=2/1) in 100% FBS. The autocorrelation curves were fitted by using one component diffusion model.

Nanosome (PC/cargo)	Surface Chemistry	Zeta potential (mV)	HD diameter (nm, PDI)
NS (control)	67.1 PC/nm ²	QD: -21.4 Au: -26.6	QD: 16.1 (0.26) Au: 13.9 (0.43)
NS-8101 (2/1)	44.7 PC/nm ² 22.4 101/nm ²	QD: -13.4 Au: -8.3	QD: 16.5 (0.26) Au: 15.4 (0.42)
NS-8102 (2/1)	44.7 PC/nm ² 22.4 102/nm ²	-15.4	18.1 (0.20)
Ms-8101/His6 (20/5/1)	51.6 PC/nm ² 12.9 101/nm ² 2.5 His6/nm ²	10.1	19.5 (0.28)

Table 2-2-1. Nanosomes carrying mechanism-based glycosidase inhibitors.

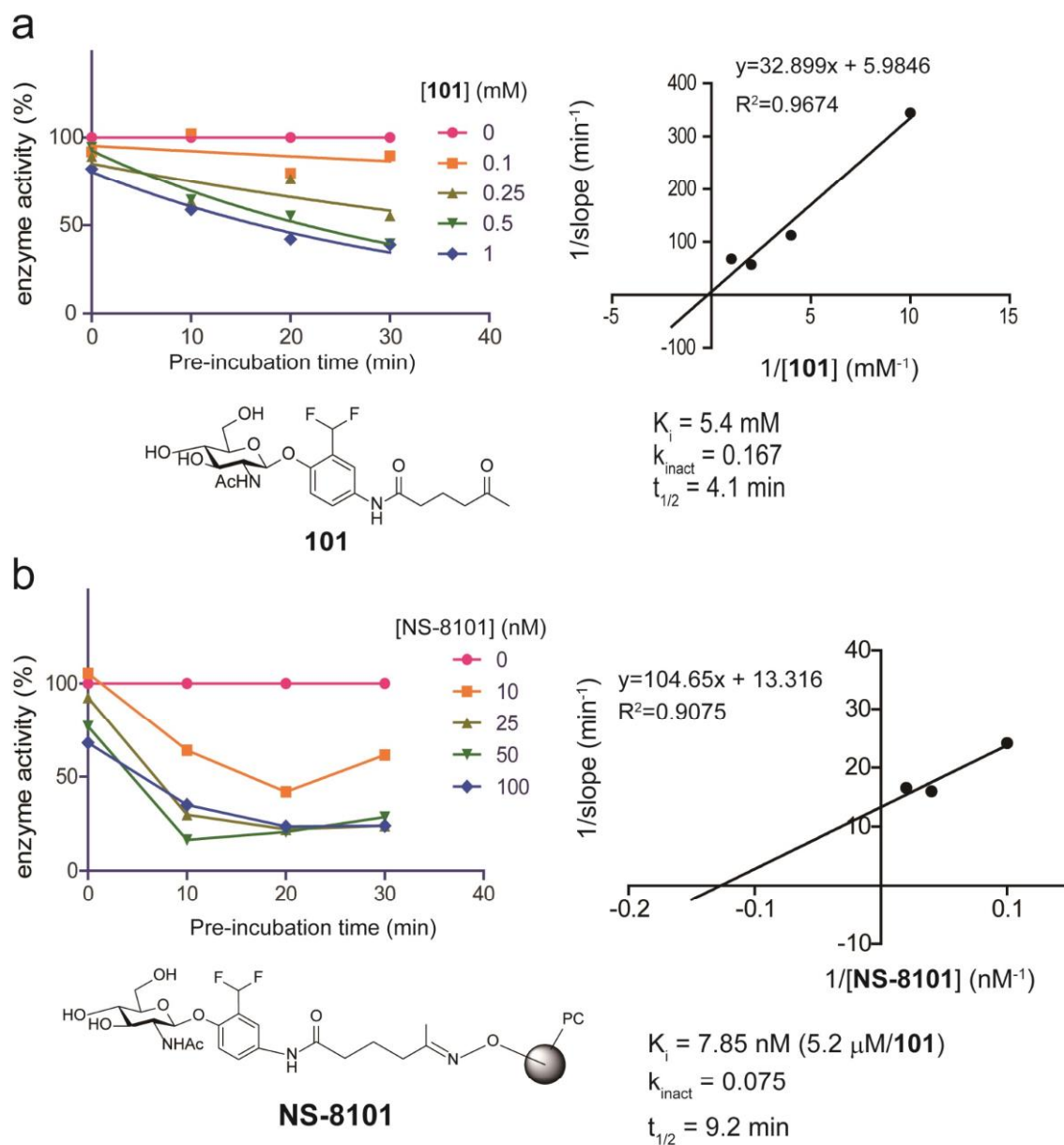


Figure 2-2-7. Kinetic analysis shows dramatically enhanced inhibition of NS-8101 against human β -*N*-acetyl-D-glucosaminidase by cluster glycoside effect when compared with that of small molecular irreversible **101**.

2-3 Summary

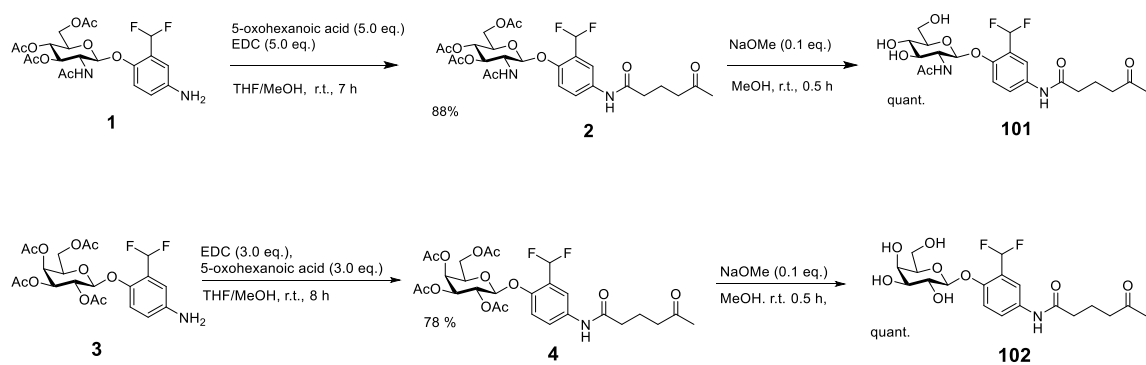
In this chapter, irreversible inhibitors (**101**, **102**) and these displayed nanoparticles (**NS-8101**, **NS-8102**) were synthesized and characterized. From the FCS results, PC coated QD655 showed long-term stability in every serum. These facts show that PC coating prohibited nonspecific protein adhesion and forming protein corona. Furthermore, **NS-8101** showed a very strong inhibition activity for NAG compared with free **101**. So, nanosome not only prohibited formation of protein corona but also increased inhibition activity of irreversible glycosidase inhibitor. These results indicate that nanosome may become a strong tool for drug delivery system.

2-4 Experimental

General Methods and Materials. All solvents and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Chemical Industry Co. Inc. (Tokyo, Japan), Wako Pure Chemical Industries Co. Inc. (Tokyo, Japan), Funakoshi Co., Inc. (Japan, Tokyo), Medicinal Chemistry Pharmaceuticals, Co., Inc. (Hokkaido, Japan), Thermo Fisher Scientific K. K. Co. Inc. (Tokyo, Japan), Kanto Chemical Co. Inc. (Tokyo, Japan) and used without further purification. TOPO-coated QDs (655 nm and 800 nm) were purchased from Thermo Fisher Scientific K. K. Co. Inc. (Tokyo, Japan). Microcon YM50 was purchased from Millipore Co. Inc (Tokyo, Japan). Human placenta *N*-acetylglucosaminidase (NAG) was purchased from Sigma-Aldrich Japan Co. Inc. (Tokyo, Japan). Proton and carbon NMR were recorded at 25°C on BRUKER AVANCE 600 MHz (¹H: 600 MHz, ¹³C: 150 MHz) Bruker AVANCE (Bruker Biospin Co., Germany). Chemical shifts are indicated by ppm. Electro-spray ionization mass (ESI-MS) spectra were obtained with a JMS-T100LP mass spectrometer (JEOL Co. Inc., Tokyo, Japan). Matrix Assisted Laser Desorption Ionization time-of-flight mass (MALDI-TOFMS) spectra were obtained with an Ultraflex III TOF mass spectrometer (Bruker Daltonik

GmbsH Co. Inc, Germany) equipped with a reflector and controlled by the Flexcontrol 3.0 software package. Reactions were monitored by thin layer chromatography (TLC), and TLC was carried out by Merck silica gel glass plates (20 × 20 cm; layer thickness, 0.25 mm; Silica gel 60F254, Merck Co. Inc, USA); spots were visualized by treatment with a solution of 5 % H₂SO₄ in MeOH and heating at 200°C, and by UV lights (256 or 365 nm) when applicable. Column chromatography was performed on Kanto Chemical silica gel N60 (40-50 µm).

Synthesis of glycosidase inhibitors



Scheme 2-4-1. Synthesis of covalent glycosidase inhibitors **101** and **102**.

2-Difluoromethyl-4-*N*-(5-oxo-hexane-1-yl)phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranoside (2). Compound **1**¹ (314 mg, 0.64 mmole) was dissolved in THF (20 mL) and MeOH (5 mL), then 5-oxohexanoic acid (0.384 mL, 3.2 mmole), EDC (610 mg, 3.2 mmole) were added to the solution and stirred at room temperature for 7 h. After evaporation to remove solvents, the residual syrup was dissolved in CHCl₃. The organic layers were washed with 1 N HCl aq, saturated NaHCO₃ aq, and brine. Then, solvents were dried by MgSO₄ and evaporated under reduced pressure. Silica-gel chromatography (hexane/EtOAc, 1:3) of the crude product gave pure compound **2** (0.56 mmole, 88 %).

¹H NMR (600 MHz, DMSO-d₆) δ 10.02 (s, 1 H), 8.1 (d, 1 H, *J*=9.1 Hz), 7.86 (s, 1 H), 7.68 (d, 1 H, *J*=8.3 Hz), 7.24 (d, 1 H, *J*=9.1 Hz), 6.89 (t, 1 H, *J*=55.9 Hz), 5.21 (m, 2 H), 4.95 (t, 1 H, *J*=9.44 Hz), 4.24 (m, 1 H), 4.1 (m, 3 H), 2.49 (t, 2 H, *J*=7.55 Hz), 2.29 (t, 2 H, *J*=7.55 Hz), 2.09 (s, 3 H), 2.03 (s, 3 H), 2.0 (s, 3 H), 1.97 (s, 3 H), 2.06 (s, 3 H), 1.79 (s, 3 H), 1.76 (m, 2 H).

¹³C NMR (150 MHz, DMSO-d₆) δ 123.34, 116.89, 116.89, 111.18, 100.02, 71.74, 71.24, 68.51, 61.07, 61.073, 53.13, 41.97, 35.27, 29.55, 22.37, 20.38, 20.138, 18.898. HR-ESI-MS: C₂₇H₃₄F₂N₂O₁₁Na [M+Na]⁺ expected 623.20284, found 623.20319

2-Difluoromethyl-4-*N*-(5-oxo-hexane-1-yl)phenyl 2-acetamido- 2-deoxy- β -D-glucopyranoside (101). Compound **2** (150 mg, 0.24 mmole) was treated with sodium methoxide (1.3 mg, 0.024 mmole) in MeOH (20 mL). After 0.5 h, sodium methoxide was neutralized by DOWEX-50. Solvent was filtered and dried up to afford pure **101** as white powder (114 mg, 0.24 mmole, quantitative yield).

¹H NMR (600 Hz, D₂O) δ 7.52 (s, 1 H), 7.41 (d, 1 H, *J*=8.8 Hz), 7.18 (d, 1 H, *J*=8.8 Hz), 6.79 (t, 1 H, *J*=55.46 Hz), 5.04 (d, 1 H, *J*=8.5 Hz), 3.9 (m, 2 H), 3.71 (m, 1 H), 3.56 (m, 2 H), 3.47 (t, 1 H, *J*=9.39 Hz), 2.56 (t, 2 H, *J*=7.0 Hz), 2.32 (t, 2 H, *J*=7.2 Hz), 2.1 (s, 3 H), 1.97 (s, 3 H), 1.82 (m, 2 H).

^{13}C NMR (150 MHz, D_2O) δ 125.94, 119.98, 116.13, 111.71, 99.40, 75.94, 73.25, 69.40, 60.17, 60.17, 54.78, 42.09, 35.36, 28.82, 21.90, 18.82. HR-ESI-MS: $\text{C}_{21}\text{H}_{28}\text{F}_2\text{N}_2\text{O}_8\text{Na}$ $[\text{M}+\text{Na}]^+$ expected 497.17114, found 497.16879.

2-Difluoromethyl-4-*N*-(5-oxo-hexane-1-yl)phenyl 2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranoside (4). Compound **3²** (200 mg, 1.63 mmole) was dissolved in THF (50 mL) and MeOH (5 mL), then 5-oxohexanoic acid (0.583 mL, 4.89 mmole), EDC (765 mg, 4.89 mmole) were added to the solution and stirred at room temperature for 8 h. After evaporation to remove solvents, the residual syrup was dissolved in CHCl_3 . The organic layers were washed with 1 N HCl aq, saturated NaHCO_3 aq, and brine. Then, solvents were dried by MgSO_4 and evaporated under reduced pressure. Silica-gel chromatography (hexane/EtOAc, 1:2) of the crude product produced pure compound **4** (1.28 mmole, 78 %).

^1H NMR (600 MHz, DMSO-d_6) δ 10.02 (s, 1 H), 7.86 (s, 1 H), 7.68 (d, 1 H, $J=9.06$ Hz),

7.17 (d, 1 H, $J=8.69$ Hz), 6.86 (t, 1 H, $J=55.52$ Hz), 5.42 (d, 1 H, $J=7.93$ Hz), 5.36 (d, 1 H, $J=2.64$ Hz), 5.3 (dd, 1 H, $J=12.46, 3.4$ Hz), 5.24 (m, 1 H), 4.46 (t, 1 H, $J=6.42$ Hz), 4.17 (m, 1 H), 4.11 (m, 1 H), 2.49 (t, 2 H, $J=7.55$ Hz), 2.28 (t, 2 H, $J=7.55$ Hz), 2.16 (s, 3 H), 2.09 (s, 3 H), 2.04 (s, 3 H), 2.03 (s, 3 H), 1.96 (s, 3 H), 1.76 (m, 2 H).

^{13}C NMR (150 MHz, DMSO-d_6) δ 123.14, 116.58, 111.72, 111.64, 98.76, 70.79, 70.31, 68.35, 67.50, 61.68, 42.47, 35.65, 21.00, 20.83, 19.55. HR-ESI-MS: $\text{C}_{27}\text{H}_{33}\text{F}_2\text{NO}_{12}\text{Na}$ $[\text{M}+\text{Na}]^+$ expected 624.18685, found 624.18922.

2-Difluoromethyl-4-*N*-(5-oxo-hexane-1-yl)phenyl β -D-galactopyranoside (102).

Compound **4** (189 mg, 0.314 mmole) was treated with sodium methoxide (5.0 mg, 0.031 mmole) in MeOH (40 mL). After 0.5 h, sodium methoxide was neutralized by DOWEX-50. Solvent was filtered and dried up to afford pure **102** as white powder (136 mg, 0.314 mmole, quantitative yield).

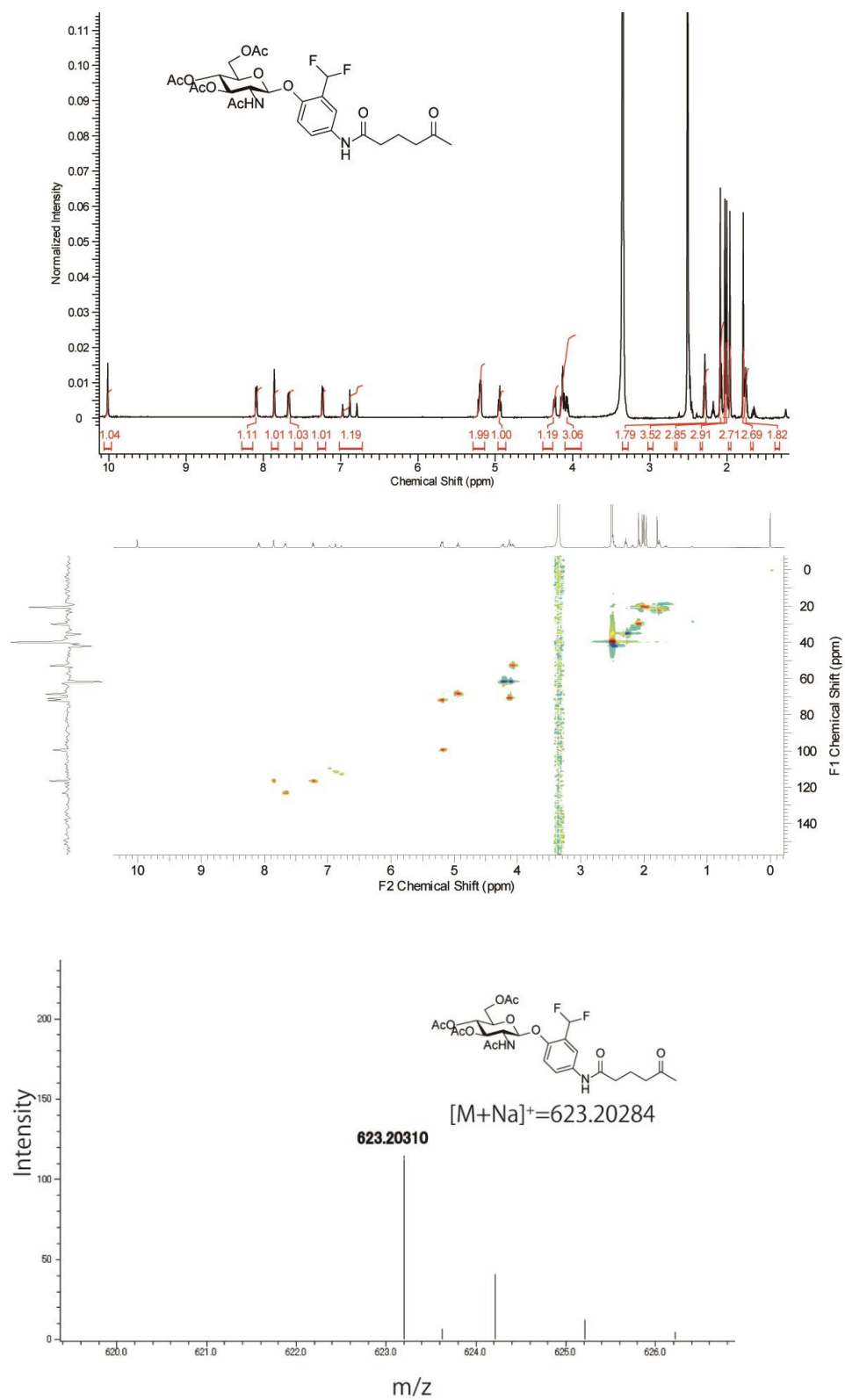
^1H NMR (600 Hz, CD_3OD) δ 7.8 (s, 1 H), 7.65 (d, 1 H, $J=8.9$ Hz), 7.29 (d, 1 H, $J=9.1$ Hz), 7.17 (t, 1 H, $J=55.3$ Hz), 4.85 (d, 1 H, $J=7.6$ Hz), 3.92 (d, 1 H, $J=3.0$

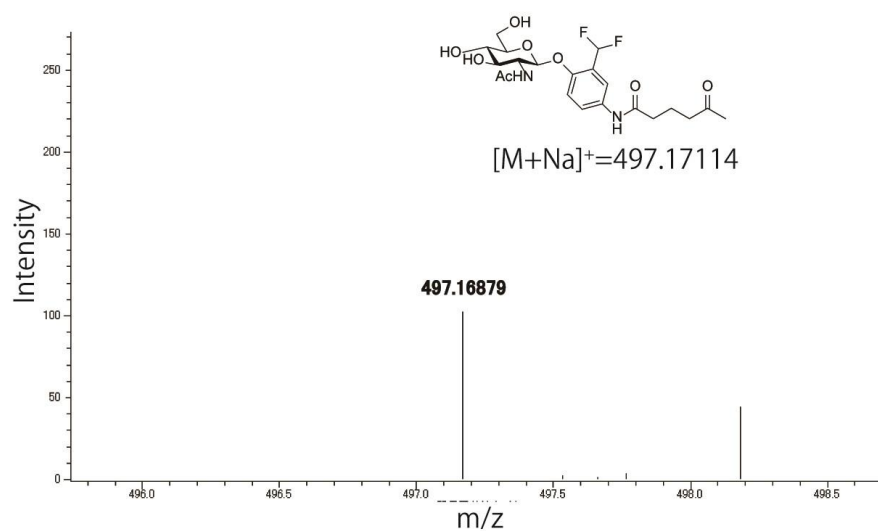
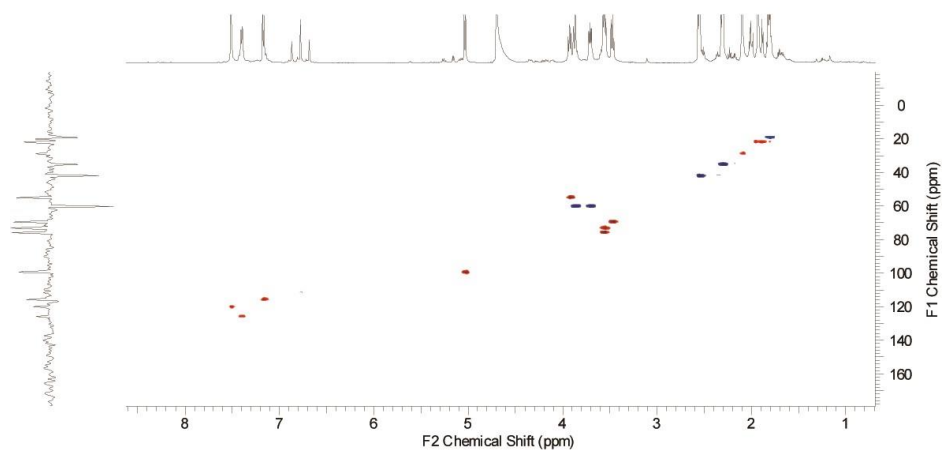
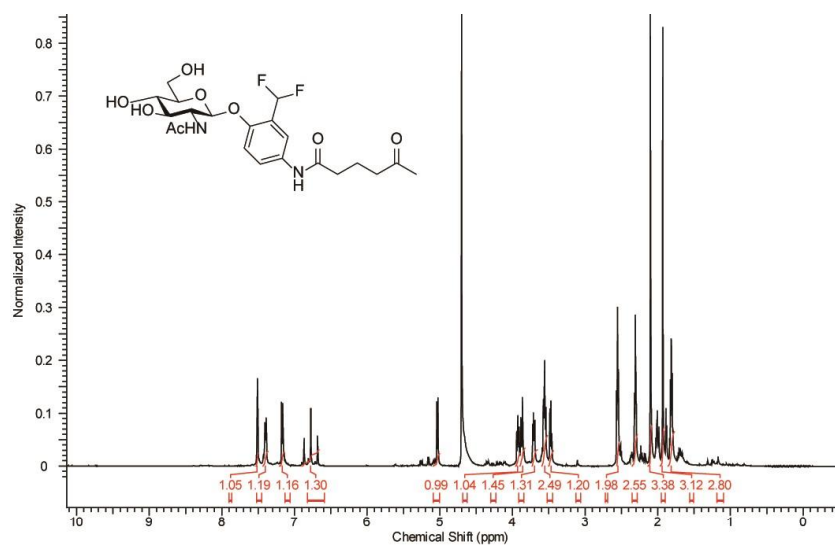
Hz), 3.88 (m, 3 H), 3.69 (t, 1 H, $J=5.67$ Hz) 3.59 (dd, 1 H, $J=9.6, 3.2$ Hz), 2.61 (t, 2 H, $J=7.2$ Hz), 2.4 (t, 2 H, $J=7.4$ Hz), 2.17 (s, 3 H), 1.94 (m, 2 H). ^{13}C NMR (150 Hz, CD_3OD) δ 132.27, 116.73, 110.86, 102.52, 75.52, 73.36, 70.72, 68.80, 60.88, 41.77, 35.31, 28.58, 19.34. HR-ESI-MS: $\text{C}_{19}\text{H}_{25}\text{F}_2\text{NO}_8\text{Na}$ $[\text{M}+\text{Na}]^+$ expected 456.14459, found 456.14770.

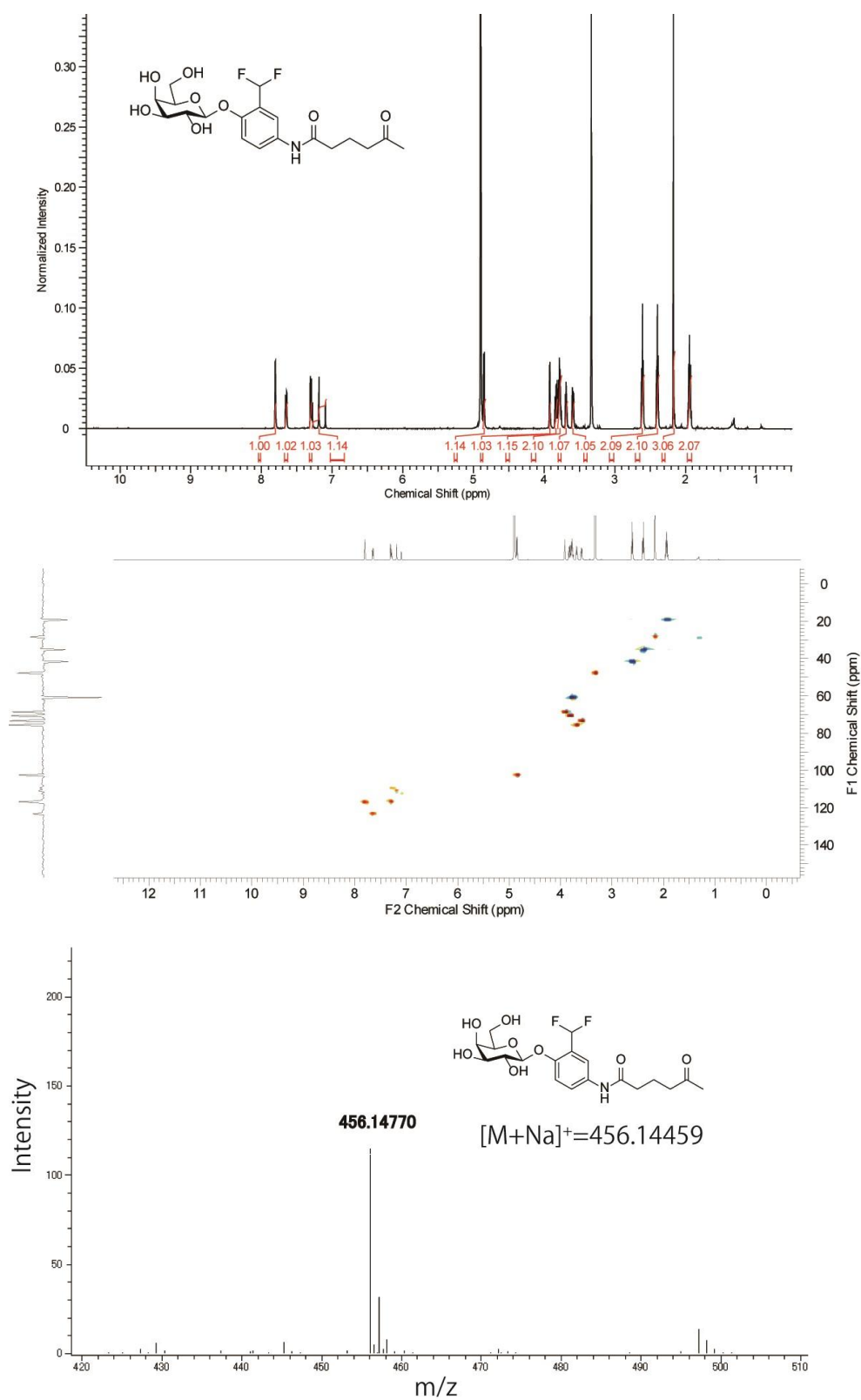
compound	2		101		4		102	
nuclear	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C
C1-H1	5.21	100.02	5.04	99.4	5.42	98.76	4.85	102.52
C2-H2	4.1	53.13	3.9	54.78	5.24	68.37	3.88	70.72
C3-H3	5.21	71.74	3.56	75.94	5.3	70.31	3.59	73.36
C4-H4	4.95	68.51	3.47	60.4	5.36	67.5	3.92	68.8
C5-H5	4.1	71.24	3.56	73.2	4.46	70.79	3.69	75.52
C6-H6a	4.1	61.07	3.71	60.17	4.11	61.68	3.88	60.88
C6-H6b	4.24	61.07	3.9	60.17	4.17	61.68	3.88	60.88
Ketone linker	2.49	41.97	2.56	42.09	2.49	42.47	2.61	41.77
Ketone linker	2.29	35.27	2.32	35.36	2.28	35.65	2.4	35.31
Ketone linker	1.76	18.89	1.82	18.82	1.76	19.55	1.94	19.34
Me	1.79	22.37	1.97	21.9	1.96	21.0	2.17	28.58
Difluoromethyl C-H	6.89	111.18	6.79	111.71	6.86	111.72	7.17	110.86
NH	10.02				10.02			
NH	8.1							
NHAc	2.09	20.38	2.1	28.82				
Ac	2.03	20.38			2.16	20.83		
Ac	2.00	20.38			2.09	20.83		
Ac	1.97	20.38			2.04	20.83		
Ac					2.03	20.83		
Ph C-H	7.86	116.89	7.57	119.98	7.86	111.64	7.8	116.73
Ph C-H	7.68	123.34	7.41	125.94	7.68	123.14	7.29	123.27
Ph C-H	7.24	116.89	7.18	116.13	7.17	116.58	7.17	116.73

Table 2-4-1. Chemical shifts of compounds **2**, **4**, **101**, and **102**

NMR spectra & HR-MS







Preparation of Nanosomes using quantum dots (QDs) as a core material.

CdSeS/ZnS quantum dots (QDs) with an emission peak of 655 nm (Thermo Fisher Scientific K.K., Tokyo, Japan) were solubilized using an excess trioctylphosphine oxide (TOPO) in decane. TOPO-QDs (50 μ L, 1 μ M/decane) was added to MeOH (50 μ L) and i-PrOH (100 μ L), then centrifuged for 5 min, 15000 rcf. to precipitate TOPO-QDs. The pellet-shaped TOPO-QDs were dissolved in n-hexane (100 μ L). QDs were coated by using phosphocholine (PC) linker and aminooxy (AO) linker according to the method described in our previous reports.^{28,29} In brief, the lyophilized PC linker were dissolved in MeOH to a final concentration of 100 mM and the lyophilized AO linker was also dissolved in water to a final concentration of 10 mM. The solution of PC linker (4 or 8 μ L, 100 mM/MeOH) was added to AO linker (10 μ L, 10 mM/Milli Q), NaBH₄ (1 μ L, 12 wt%/14 N NaOH) and water (50 μ L) and then added to QDs solution on the ratio of a $2 \times 10^5/1$ (linker/QDs). The hexane/water two-layer solution was mixed for 30 min at room temperature. The n-hexane layer containing TOPO was removed and excess TOPO were washed by hexane. Excess linker was removed by washing four times with Milli Q through an Amicon Ultra-0.5 centrifugal Filter Unit with Ultracel-50 membrane (MWCO

of 50kDa, Millipore UFC5050).

Conjugation of cargo with Nanosomes. The conjugation of surface AO linker and aldehyde/ketone auxiliary were performed by oxime formation reaction in mild acid condition.^{28,29} The AO/PC mixed SAM coated QDs (50 μ L, 1 μ M/Milli Q) were added to compounds **101** and **102** (10 μ L, 30 mM **101** or **102**/MeOH, AO/PC=1/2), or **101** and **His₆** (6 μ L, 30 mM **101**/MeOH and 4 μ L, 10 mM **His₆**/Milli Q, AO/PC=1/4) and sodium acetate buffer (200 μ L, 100 mM, pH4.0). Then, intensely vortexed for 30 min at room temperature. Excess compounds were removed by washing four times with MilliQ through an Amicon Ultra-0.5 centrifugal Filter Unit with Ultracel-50 membrane (MWCO of 50 kDa, Millipore UFC5050).

Characterization of Nanosomes. Size and dispersion of NSs were determined with dynamic light scattering (Milli Q, Fiber-optics dynamic light scattering spectrophotometer, Otsuka Electronics Co., Osaka, Japan) and transmission electron microscopy (JEM-2100F, JEOL Inc., Tokyo, Japan). Ligand exchange and oxime formation reaction was confirmed by MALDI-TOFMS analysis (Ultraflex III TOF mass spectrometer, Bruker Daltonik GmbsH Co. Inc,

Germany) and zeta potential (Milli Q, Zeta Potential and Submicron Particle Size Analyzer, Beckman Coulter Co. Inc., Tokyo, Japan).

Preparation of Nanosomes using gold nanoparticles (AuNPs) as a core material. Gold nanoparticles with 10 nm with an average diameter were synthesized according to the method reported by Green *et al.*⁵¹ TOPO (25 g) and octadecylamine (10 g) were added to 100 mL of cyclohexanol in a 300 mL flask and the mixture was heated to 80°C. Under vigorous stirring, $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ (1 mL, 1 M/EtOH) was injected and the mixture was heated to 150°C. Then, the mixture was added to NaBH_4 (0.5 mL, 100mM/EtOH) dropwise and stirred for 30 min. The reaction mixture was added to MeOH (200 mL), and the solution was centrifuged at 3500 rpm for 10 min. Then, supernatant was removed, and the precipitates were distributed in toluene (30 mL). The concentration of AuNP solution was determined from the mean diameter of AuNP from TEM images. Briefly, the average Au atoms per nanoparticle were calculated as $N = \frac{\pi \rho D^3}{6M} = 30.896 D^3$, where ρ was the density for face-centered cubic gold (19.3 g/cm³), M was molecular weight of gold (197 g/mol), and D was the mean diameter of AuNPs from TEM images. AuNPs were

coated by mixed SAM composed of PC linker and AO linker in a similar protocol described above. TOPO-AuNPs (100 μ L, 1 μ M/toluene) were added to MeOH (200 μ L), then centrifuged at 15,000 rcf to precipitate AuNPs. The pellet shaped TOPO-AuNP were re-dissolved in n-hexane (200 μ L). The solution of PC linker (16 μ L, 100 mM/MeOH) were added to AO linker (40 μ L, 10 mM/Milli Q), NaBH₄ (10 μ L, 12 wt %/14 N NaOH) and water (50 μ L) and then added to TOPO-AuNPs on a ratio of $8 \times 10^5/1$ (linker/AuNPs). The hexane/water two-layer solution was mixed for 30 min at room temperature. The n-hexane layer containing TOPO was removed and washed vigorously by using n-hexane. Excess linkers were removed by washing with MilliQ through an Amicon Ultra-0.5 centrifugal Filter Unit with Ultracel-50 membrane (MWCO of 50 kDa, Millipore UFC5050). The conjugation of surface AO linker with aldehyde/ketone auxiliary were performed by oxime formation reaction in mild acid condition as described above.^{28,29} The AO/PC mixed SAM coated AuNPs (100 μ L, 1 μ M/Milli Q) were added to compound **101** (10 μ L, 30 mM **101**/MeOH, AO/PC=1/2) and sodium acetate buffer (200 μ L, 100 mM, pH4.0). Then, the mixture was intensely vortexed for 30 min at room temperature. Excess inhibitors were removed by washing four times

with Milli Q through an Amicon Ultra-0.5 centrifugal Filter Unit with Ultracel-50 membrane (MWCO of 50 kDa, Millipore UFC5050). **NS-8101 (Au)** was characterized by general methods employed for the characterization of NSs derived from QDs (**Figure 2-2-5**) and used for *in vivo* experiments.

Behavior of Nanosomes in fetal bovine serum characterized by fluorescence correlation spectroscopy. Nanosomes (QDs) coated with mixed SAM composed of any ratio of PC and PEG linker (**Figure 2-2-1**) and **NS-8101 (Figure 2-2-6)** (2 μ L, 1 μ M/Milli Q) were added to 200 μ L of 100% fetal bovine serum (FBS, Nichirei Biosciences Inc., Tokyo, Japan, protein concentration is 3.0~4.5 g/mL). Then, the solution was subjected directly to the measurement of fluorescence fluctuation of the nanoparticles in 100% FBS according to the previously reported method²⁸ by using Fluorescence Correlation Spectroscopy (LSM 510 META Confocor 2, Carl Zeiss Inc., Germany).

Cluster glycoside effect of NS-8101 on the inhibition of human placenta hexosaminidase. Kinetic constants of the inhibitory effect by **101** and **NS-8101** on human placenta hexosaminidase activity (**Figure 2-2-7**) were determined by using *p*-nitrophenyl (pNP) GlcNAc as a substrate according to

the previously reported method.¹³ In brief, several concentration of **101** (1 μ L, 1~10 mM/MilliQ) or **NS-8101** (1 μ L, 10~1000 nM/MilliQ, AO/PC=1/2) were added to Triton-X (1 μ L, 0.1%/MilliQ), Britton-Robinson buffer (1 μ L, 500 mM/MilliQ, pH4.25), MilliQ (6 μ L), and human placenta hexosaminidase (1 μ L, 15 mU/mL). The mixture was pre-incubated at 25°C and at 10 minute intervals (0~30 min), aliquots (3 μ L) of this pre-incubated solution were transferred into the assay medium and monitored the residual enzyme activity of this solution as follows: the aliquots of pre-incubated enzyme solution (3 μ L) were added to the assay medium [Britton-Robinson buffer (3 μ L, 500 mM/MilliQ, pH4.25), Triton-X (3 μ L, 0.1%/Mill Q), *p*-nitrophenyl GlcNAc (3 μ L, 4.8 mM/MilliQ) and MilliQ (18 μ L)]. The solution was incubated at 25°C at 30 minute intervals (30~90 min), aliquots (8.5 μ L) of the assay medium were transferred into the reaction stop medium (8.5 μ L, 0.5 M Na₂CO₃ aq.). The residual hexosaminidase activity was monitored with the increase of absorbance (450 nm) due to the hydrolyzed *p*-nitrophenolate from *p*-NP GlcNAc by microplate reader (spectraMax M5, Molecular Devices Co., Sunnyvale Co.).

2-5 References

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Chapter 3

Evaluation of Nanosomal Anti-Tumor Activity And Revealing Mechanism

3-1 Introduction

In Chapter 2, **NS-8101** and **NS-8102** were synthesized as carrier of covalent inhibitors targeting β -hexosaminidase and β galactosidase. Here, we evaluated biological activity of NSs. To assess whether human cancer cells internalize **NS-8101** and **NS-8102** displaying covalent inhibitors against lysosomal glycosidases, we first determined the efficacy in cellular uptake of NSs by cancer cells cultured in D-MEM supplemented with 10% FBS. Cell viability, measured by the MTT assay, was performed to evaluate NSs anticancer activity. Then, prompted by the substantial in vitro cytotoxic effect of NS-8101 on tumor cell growth, we further assessed whether antiadhesive NS-8101 shows antitumor activity in vivo mouse model (a BALB/c murine xenograft tumor model with HepG2 cells) after intravenous administration.

3-2 Results and discussion

Evaluation of lysosomal GlcNAc salvage pathway by utilizing glycomics

The concept of targeting lysosomal glycosidases was inspired by evidence showing the importance of highly activated endocytic GlcNAc salvage pathway in cancer biology.^{1~4} Indeed, we confirmed that synthesis of total *N*-glycans of cancer cells, particularly major complex-type *N*-glycans with sialic acid residues, can be suppressed dramatically when human liver cancer cells (HepG2) cultured for 48 h in the serum-free medium instead of normal medium supplemented with 10% fetal bovine serum (FBS) (**Figure 3-2-1**). This observation clearly indicates that endocytic uptake and subsequent lysosomal degradation of the extracellular serum glycoproteins is a critical metabolic mechanism for providing cancer cells HBSP with a sufficient amount of GlcNAc needed for the rapid synthesis of UDP-GlcNAc (**Figure 1-1-1a**). It is likely that lysosomal exo-glycosidases, particularly galactosidases and hexosaminidases whose expression levels are elevated in a wide range of tumor types,⁵ are promising targets for blocking GlcNAc salvage pathway of cancer cells. However, it is clear that most glycosidase inhibitors tested as anticancer reagents exhibit diminished clinical potency with significant side effects both *in vitro* cell-based assay *In vivo* assessment uses animal models owing to highly ineffective delivery of small molecule inhibitors to cancer cells and extremely poor cell

membrane permeability to sugar-mimic inhibitors, whereas glucose is the only sugar that can be internalized by cells through its specific transportation system. (**Figure 1-1a**). Considering that cancer cells uptake rapidly a vast amount of extracellular secretory glycoproteins through aberrantly activated endocytosis and macropinocytosis,^{1,3,23} it seemed that engineered nanoparticles⁶ of approximately 10~20 nm in a globular protein-like hydrodynamic diameter might be a potential carrier for a more effective delivery of small-molecule glycosidase inhibitors as cargoes for endolysosomal compartment of cancer cells. However, it is important to note that the biological identity of nanoparticles that determines the interaction among cancer cells can be defined when they are exposed to a physiological environment containing a highly complex mixture of proteins.^{26,27} When nanoparticles are administered intravenously into a body, the surfaces of nanoparticles will interact immediately with serum proteins to form protein corona and may aggregate unless they have a specific antifouling surface to avoid non-specific protein adsorption.⁷ Given accumulated evidence that surface protein coronas of nanoparticles affect strongly *in vivo* biodistribution, a wide range of endogenous cellular uptake/phagocytosis,⁸ and drug delivery efficiency after intravenous administration,⁹ our interest has been centered on a more rational design of antiadhesive nanoparticles to avoid or reduce surface nonspecific protein adsorption.

This may lead to new methods that allow for cancer specific and highly efficient targeting of nanomedicines to the intracellular space using aberrantly activated endocytic routes.

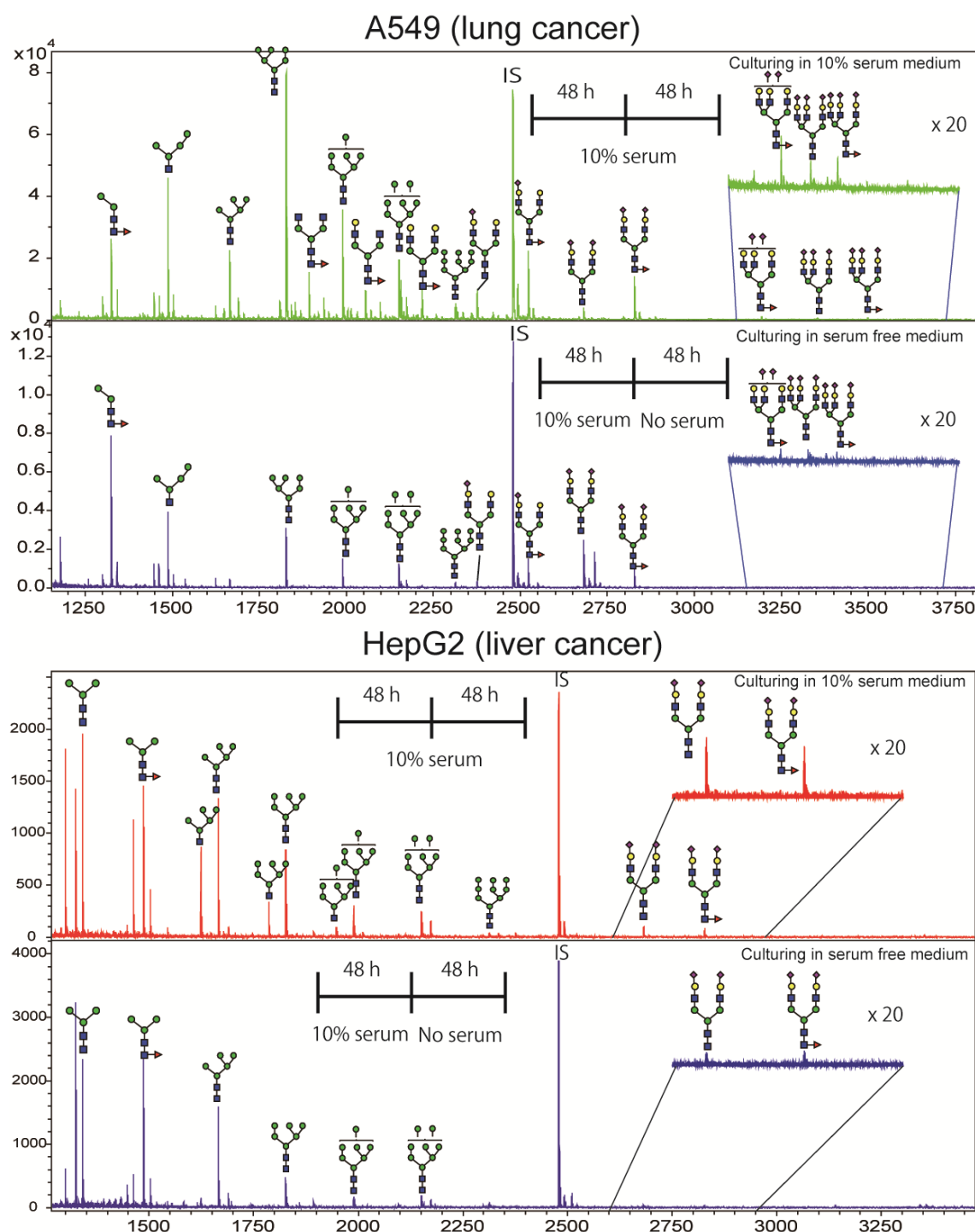


Figure 3-2-1. Effect of extracellular nutrition during cell culture on *N*-glycan synthesis of human liver cancer HepG2 cells and lung cancer A549 cells. *N*-Glycan structures of HepG2 and A549 cells cultured in a general DMEM supplemented with 10% FBS for 96 h and in a serum-free medium for 48 h before the analysis was profiled by means of glycoblotting-assisted protocol.

Following nanosomal intracellular dynamics by living cell imaging.

To assess whether human cancer cells internalize **NS-8101** and **NS-8102** displaying covalent inhibitors against lysosomal glycosidases, we first determined the efficacy in cellular uptake of NSs by HepG2 cells cultured in D-MEM supplemented with 10% FBS. As shown in **Figure 3a**, confocal fluorescence imaging of cells after NSs exposure indicated that HepG2 cells immediately internalize **NS-8101**, **NS-8102**, and control NS (PCSAM coated QD655) by endocytosis. However, after an incubation period of 8 h, fluorescence intensity due to intracellular NSs (red) showed a markedly enhanced uptake of **NS-8101** and **NS-8102** when compared with control NS. Live cell imaging during co-incubation of 10 nM NSs with HepG2 cells provided evidence that endocytic uptake of **NS-8101** and **NS-8102** (red) induces lysosomal membrane permeabilization (LMP) and complete evasion from endolysosomal compartment (green) within 2 h after NSs exposure while control NS did not (**Figure 3-2-2**, **Figure 3-2-3** and **Figure 3-2-4**). We hypothesized that LMP induced by **NS-8101** and **NS-8102** may facilitate a leakage of cathepsins to the cytosol, which is a critical step in lysosome-mediated apoptosis.^{10, 11}

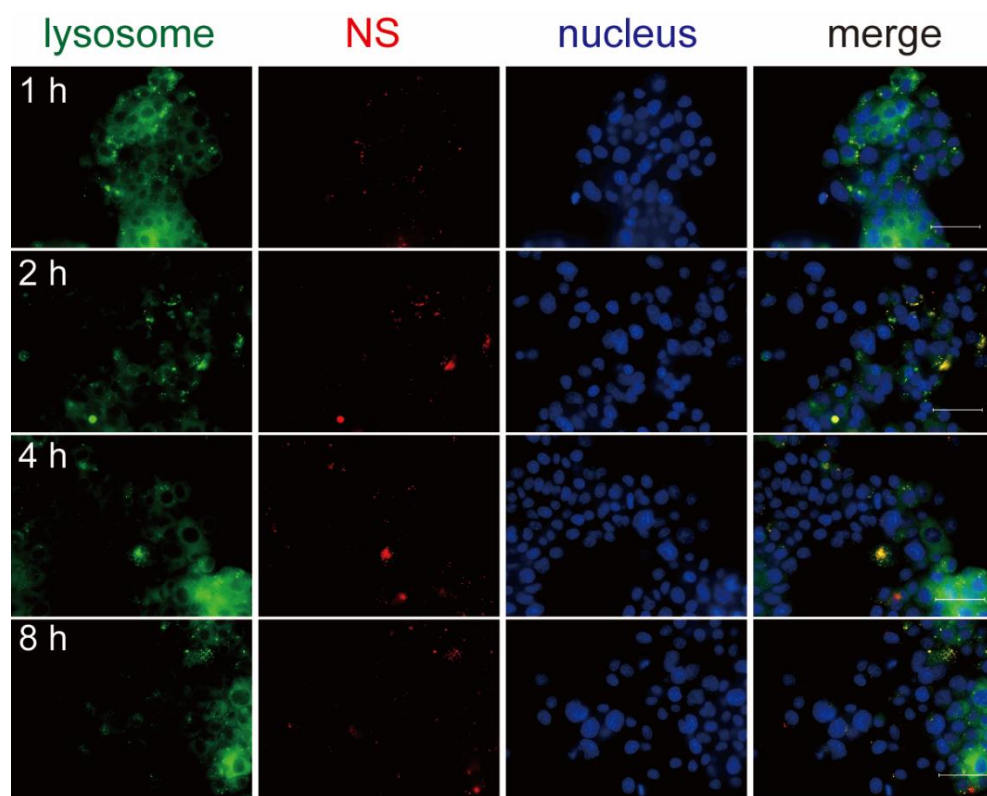


Figure 3-2-2 Scale bar, 50 μm . HepG2 cells were co-incubated with 10 nM NS (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

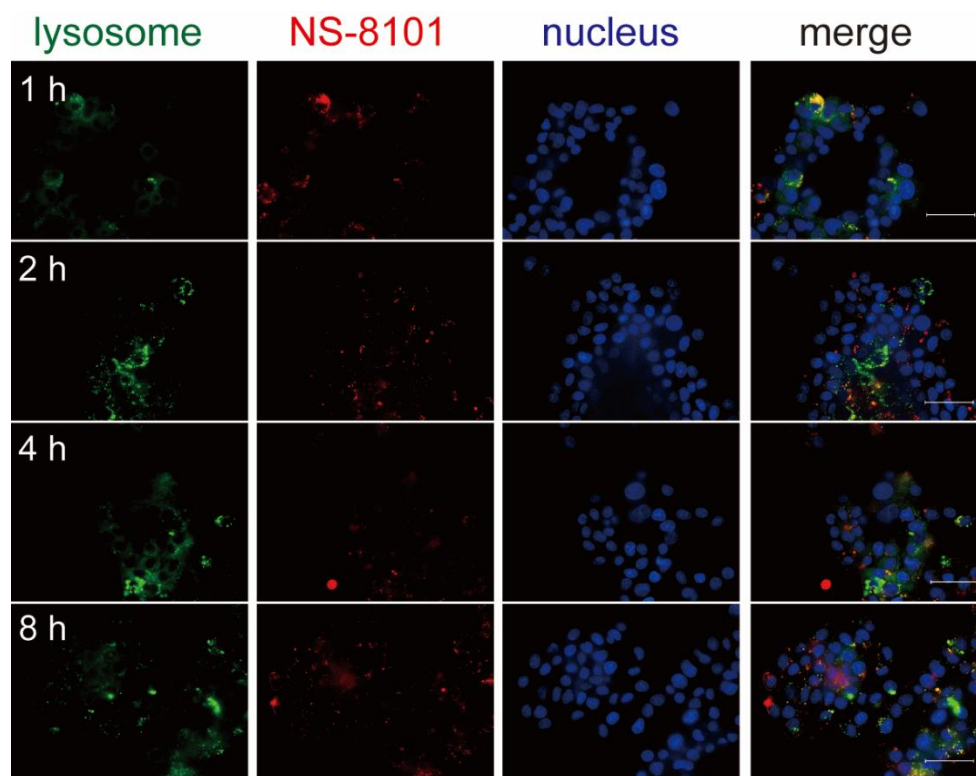


Figure 3-2-3 Scale bar, 50 μm . HepG2 cells were co-incubated with 10 nM NS-8101 (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

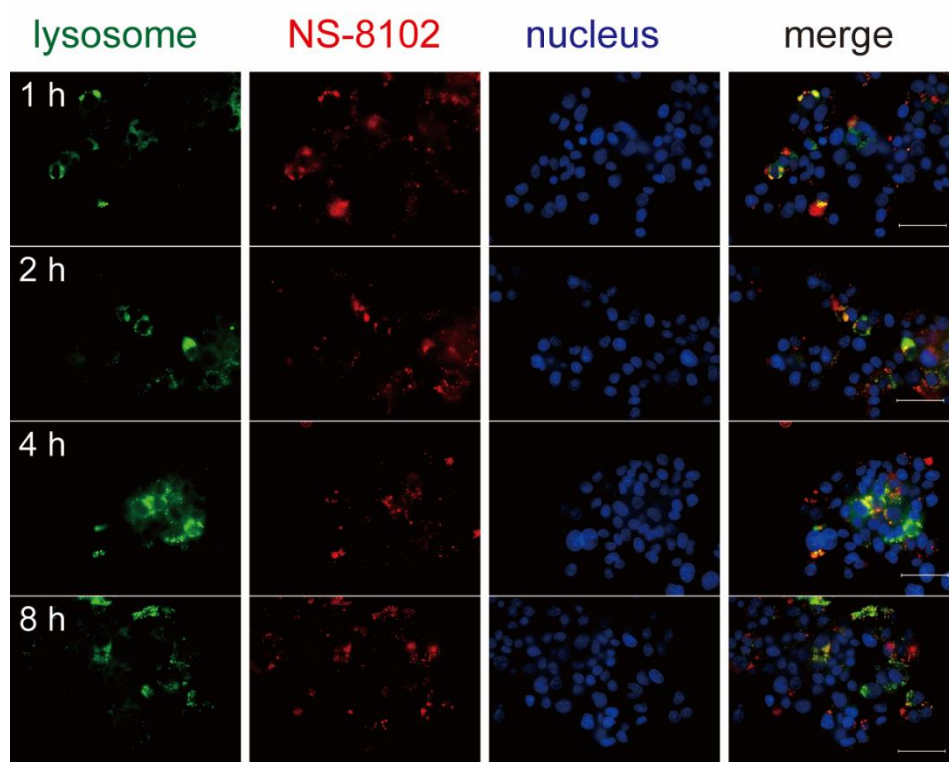


Figure 3-2-4 Scale bar, 50 μm . HepG2 cells were co-incubated with 10 nM NS-8102 (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

Evaluation of nanosomal toxicity for cancer cells.

To test this hypothesis, we determined whether LMP induced by **NS-8101** and **NS-8102** stimulates an apoptotic pathway leading to HepG2 cell death. Cell viability measured by the MTT assay was found to decrease markedly in the presence of **NS-8101** and **NS-8102** in a dose-dependent manner whereas control **NS** as well as small molecular **101** and **102** did not affect cell viability of HepG2 (**Figure 3-2-5** and **Figure 3-2-6**). Remarkably, **NS-8101** exhibits potent and much higher antitumor activity ($IC_{50} = 10.7$ nM) to HepG2 cells than **NS-8102** ($IC_{50} = 59.3$ nM) and a clinically approved 5-FU, demonstrating that lysosomal β -hexosaminidase is a promising target for the development of new class of anticancer therapeutic nanomedicines inducing LMP-mediated cell death pathway. In addition, it is important to note that potency in the LMP-mediated apoptosis triggered by **NS-8101** appears to depend significantly on the surface avidity of the displayed covalent inhibitors (**Figure 3-2-7** and **Figure 3-2-8**). This result clearly shows that there is an optimal surface chemistry [approximately (10~20 **101** + 50~40 PC)/nm²] giving a potent activity ($IC_{50} = 10\sim 20$ nM). Indeed, lowering the surface density of **101** [(3~6 **101** + 60 PC)/nm²] reduced significantly the cytotoxicity

(IC₅₀ = 70~80 nM), suggesting that optimization of surface chemistry is crucial for achieving a desired nanomedicine that has potent biological activity and antiadhesive nature, concurrently.

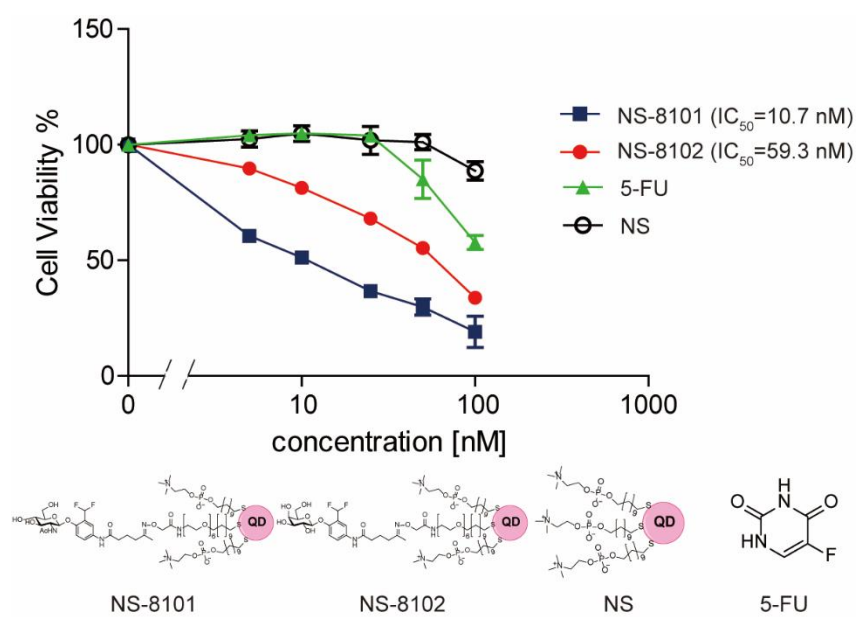


Figure 3-2-5 Detection of IC_{50} value for HepG2 cells. NS-8101 IC_{50} was 10.7 nM nad NS-8102 IC_{50} was 59.3 nM.

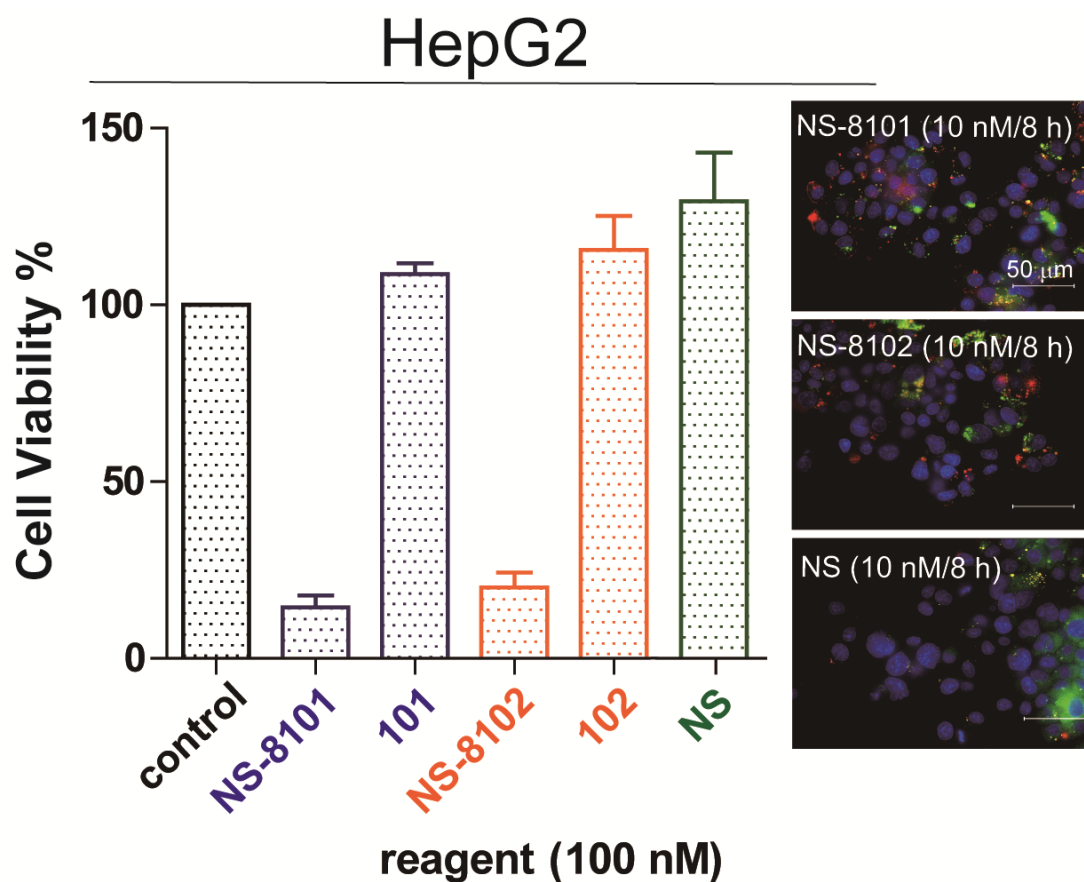


Figure 3-2-6 Loss of viability of HepG2 cells after 98 h coincubation with 100 nM reagents (101, 102, NS-8101, and NS-8102) measured by MTT assay represents cluster glycoside effect on the inhibition against lysosomal glycosidases.

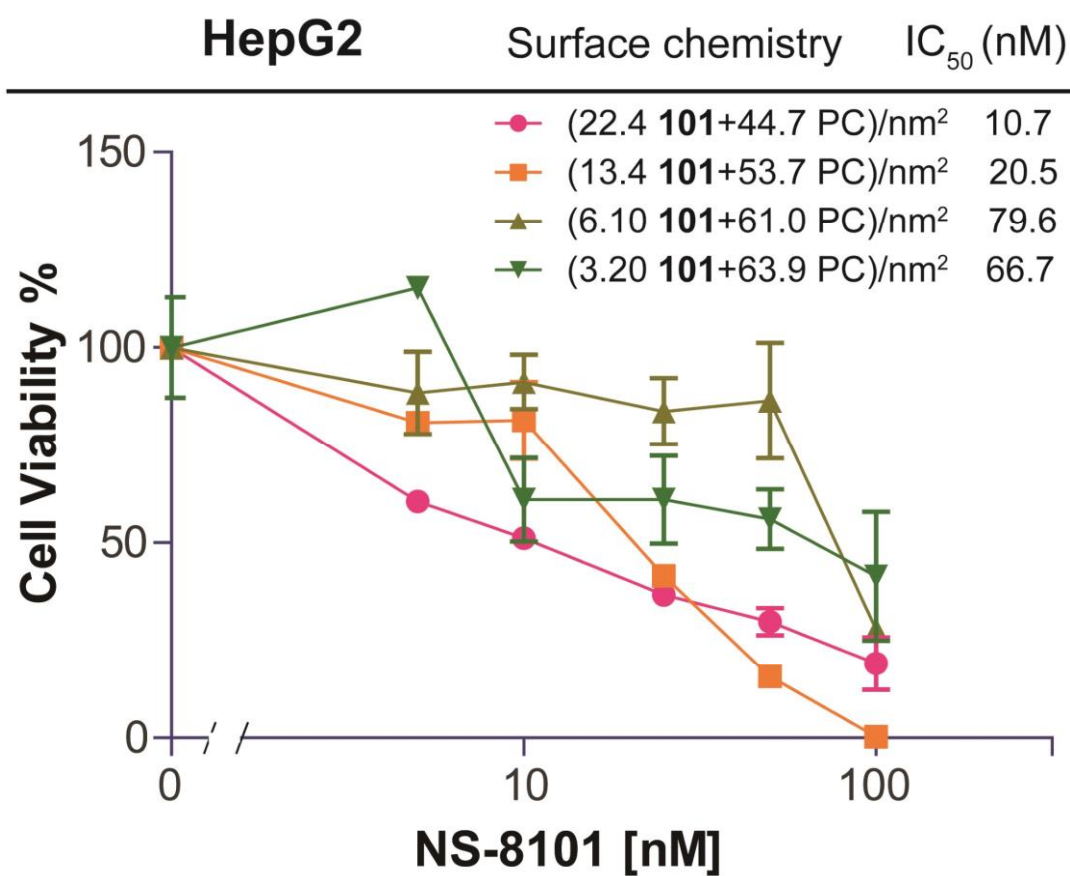


Figure 3-2-7. Potency in the LMP-mediated apoptosis triggered by NS8101 depends on the surface density of the covalent inhibitor.

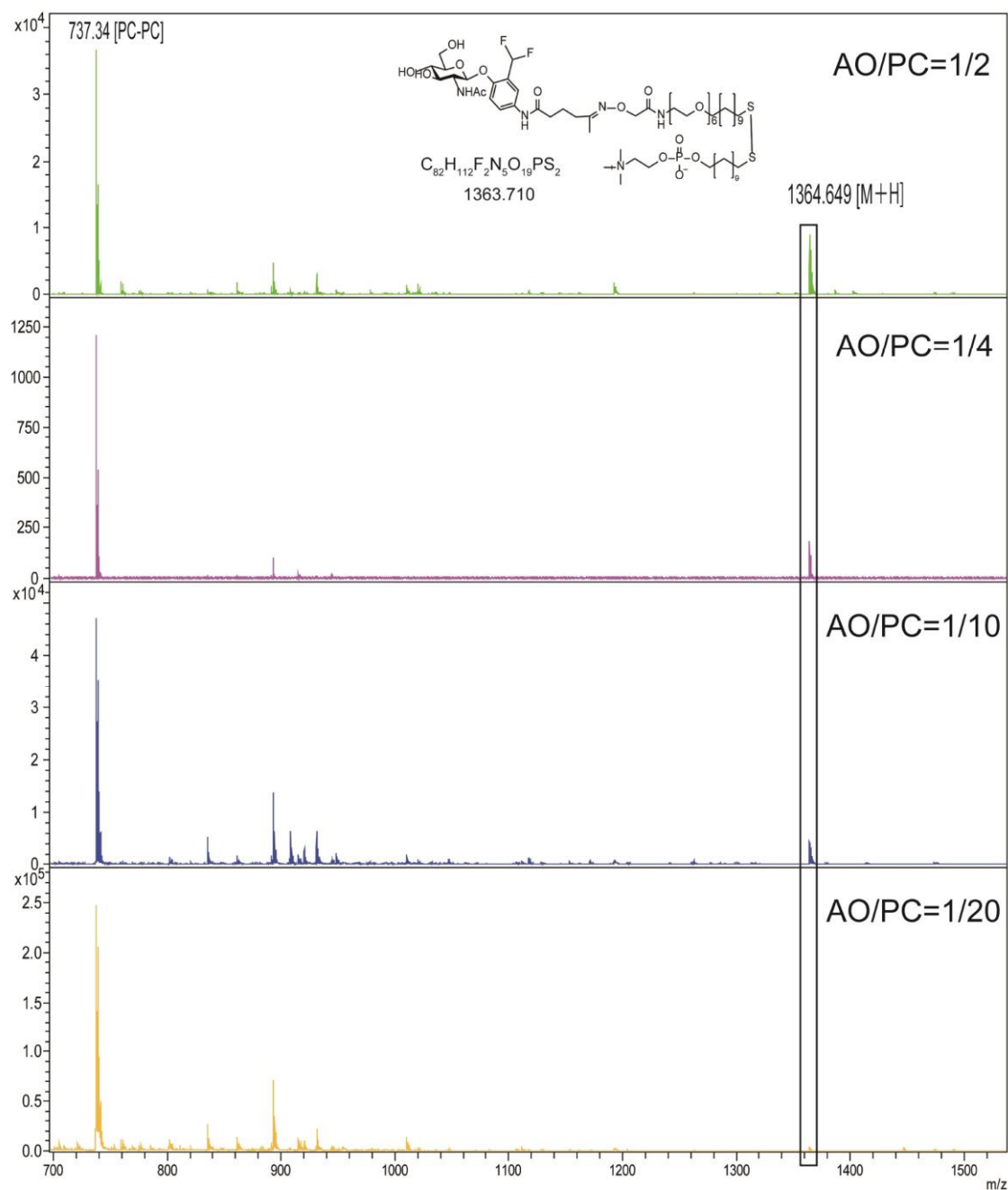


Figure 3-2-8. Characterization of NS-8101 having a different surface chemistry in terms of the density of **101**. The mass signal at m/z 1364.649 of MALDI-TOFMS corresponding to the ion composed of **101**-conjugated AO and PC linkers can be used as a convenient marker to monitor and estimate the modification with **101**.

Nanosomal antitumor activity for liver cancer bearing mice.

Prompted by the substantial *in vitro* cytotoxic effect of **NS-8101** on tumor cell growth, we further assessed whether antiadhesive **NS-8101** shows antitumor activity *in vivo* mouse model (a BALB/c murine xenograft tumor model with HepG2 cells) after intravenous administration. Saline, control NS (PCSAM-AuNP as a biocompatible counterpart of PCSAM-QD), or **NS-8101** (Au) prepared similarly from AuNP core instead of QD core (**Figure 2-2-5**) were injected 6 times into the tail vein of the tumor-bearing mice at a dose of 5 mg/kg every 4 days. On day 21, the size of tumors treated with **NS-8101** was significantly smaller than other groups (**Figure 3-2-9**), indicating that NSs carrying covalent glycosidase inhibitors were endocytosed efficiently by tumor cells and showed LMP-mediated antitumor activity in the mouse model of HCC, consistent with *in vitro* experiments (**Figure 3-2-5**). In addition, the systemic administration of **NS-8101** did not cause an apparent body weight loss in the treated mice (**Figure 3-2-9**), demonstrating that **NS-8101** exhibited no detectable toxicity under the examined conditions regardless of the presence of covalent inhibitors. It is interesting to note that live animal near-infrared (NIR)⁷ fluorescence imaging of **NS-8101** prepared

from QD800 (1 μ M x 100 μ L) after intravenous injection and surgical dissection 2 h after administration uncovered the circulation of **NS-8101** in the whole body and distribution in tumor site and major tissues such as liver, lung, kidney, spleen, and brain (**Figure 3-2-10**).

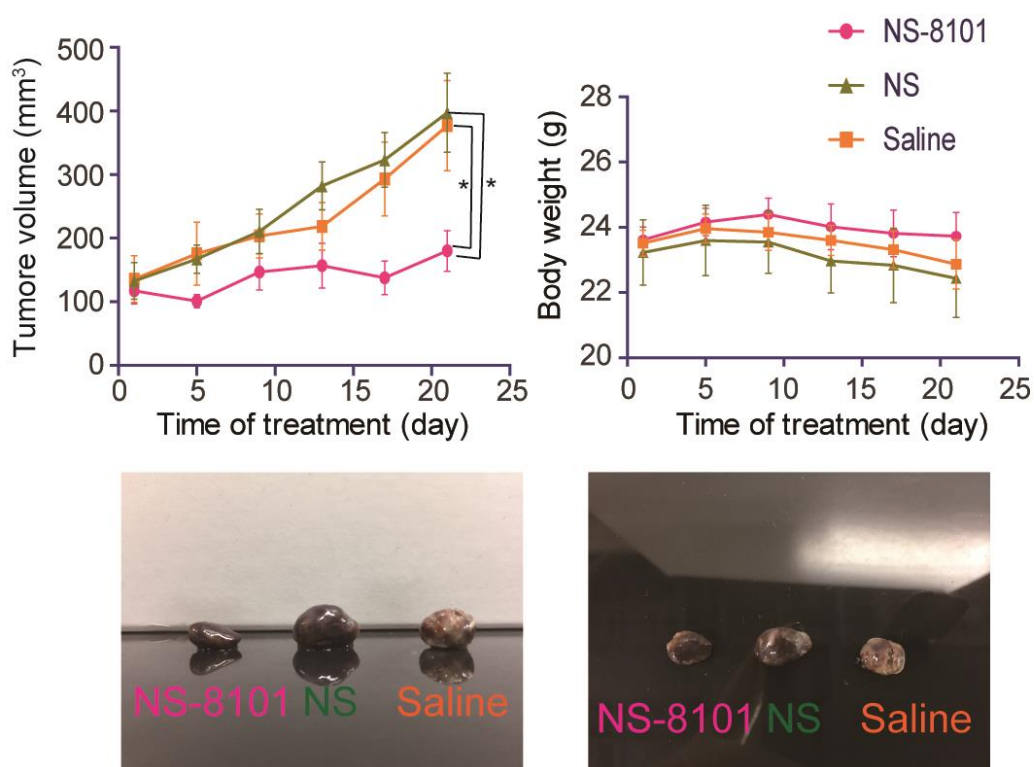


Figure 3-2-9. *In vivo* antitumor effects of **NS-8101** on HepG2 tumor growth in nude mice. Tumor sizes in the mice treated with **NS-8101** (Au), control NS (Au), or saline (n=6) *P < 0.05 (Student t test). Body weights of the mice in the same experiment as the pictures of the removed tumor samples at the termination of the experiments.

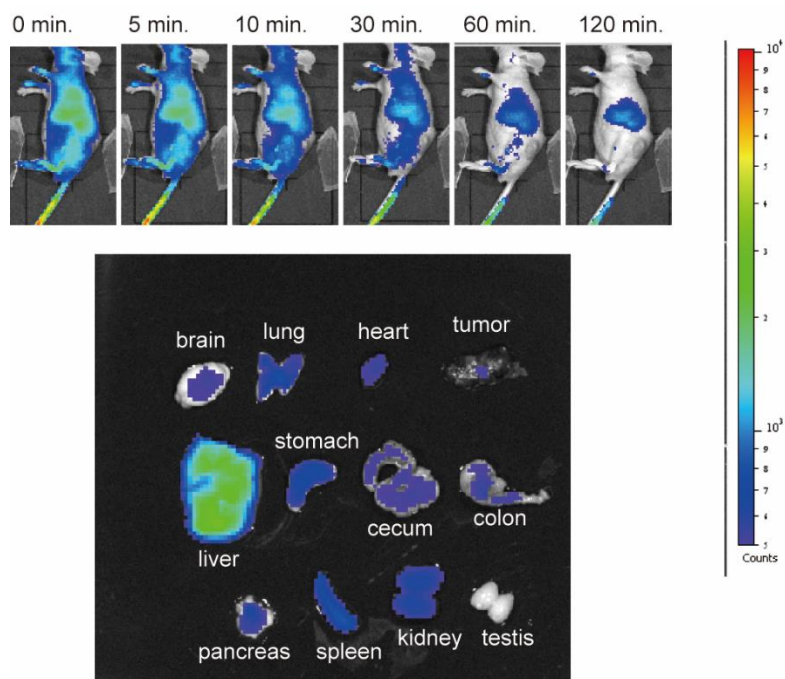


Figure 3-2-10. *In vivo* imaging of NS-8101 injecting to tumor bearing mice.

Molecular mechanism in the enhanced anti-tumor effect of nanosomes

Our interest was focused on molecular mechanisms in the antitumor activity of **NS-8101**, particularly significance of the clustered surface GlcNAc residues of **NS-8101** in the endocytosis by cancer cells. Given the distinctly enhanced uptake of **NS-8101** and **NS-8102** by HepG2 cells comparing with that of control NS (**Figure 3-2-11**), we thought that polyvalent GlcNAc β 1 $\rightarrow$ moieties of the covalent inhibitors loaded densely on **NS-8101** may contribute to efficacy of the endocytosis mediated by cell surface lectins. Notably, it has been documented that relatively weak affinity of glycans with cell surface lectins as well as various enzymes can be strengthened dramatically by increasing the valency of carbohydrates or glycan-ligands, namely “cluster glycoside effect”.^{12~14} Indeed, internalization of 10 nM **NS-8101** by HepG2 cells was completely blocked when cells are cultured in the medium containing 10 mM GlcNAc (**Figure 3-2-12**), demonstrating that the uptake of **NS-8101** is conducted by the interaction between tumor cell surface lectins and clustered GlcNAc residues displayed in the external phase of the mixed SAM of **NS-8101**. It was also uncovered that the destruction of the clathrin-coated pits by depletion of potassium ion (K⁺)¹⁵ suppresses strongly

internalization by HepG2 cells and instead, induces cell surface localization of **NS-8101** (**Figure 3-2-12c**). These observations clearly indicate that the uptake of **NS-8101** by HepG2 cells is initiated by the recognition of GlcNAc residues of **NS-8101** by membrane-associated lectins and proceeds subsequently in a clathrin-mediated endocytic mechanism. In contrast, our previous study revealed that the cellular uptake of PCSAM coated nanoparticles (control NSs) occurs in a passive manner, which is independent of clathrin- and caveolae-mediated pathways.⁸ Considering that antifouling PCSAM surface completely avoids nonspecific interaction of NSs with highly complicated serum proteins as shown in **Figure 2-2-1**, it is worthy to speculate that much higher specific gravities and stiffness of core metals such as QDs and AuNPs than mammalian cells could become a crucial trigger for invagination and wrapping of NSs on the cell surface, namely nonspecific physical endocytosis. More importantly, the antiadhesive property of mixed SAM surface of **NS-8101** (**Figure 2-2-6**) allows achieving highly amplified affinity in the specific interaction between clustered sugars and cell surface receptors (lectins) even in the presence of abundant serum proteins such as albumin, apolipoproteins, complement components, and immunoglobulins.

HepG2

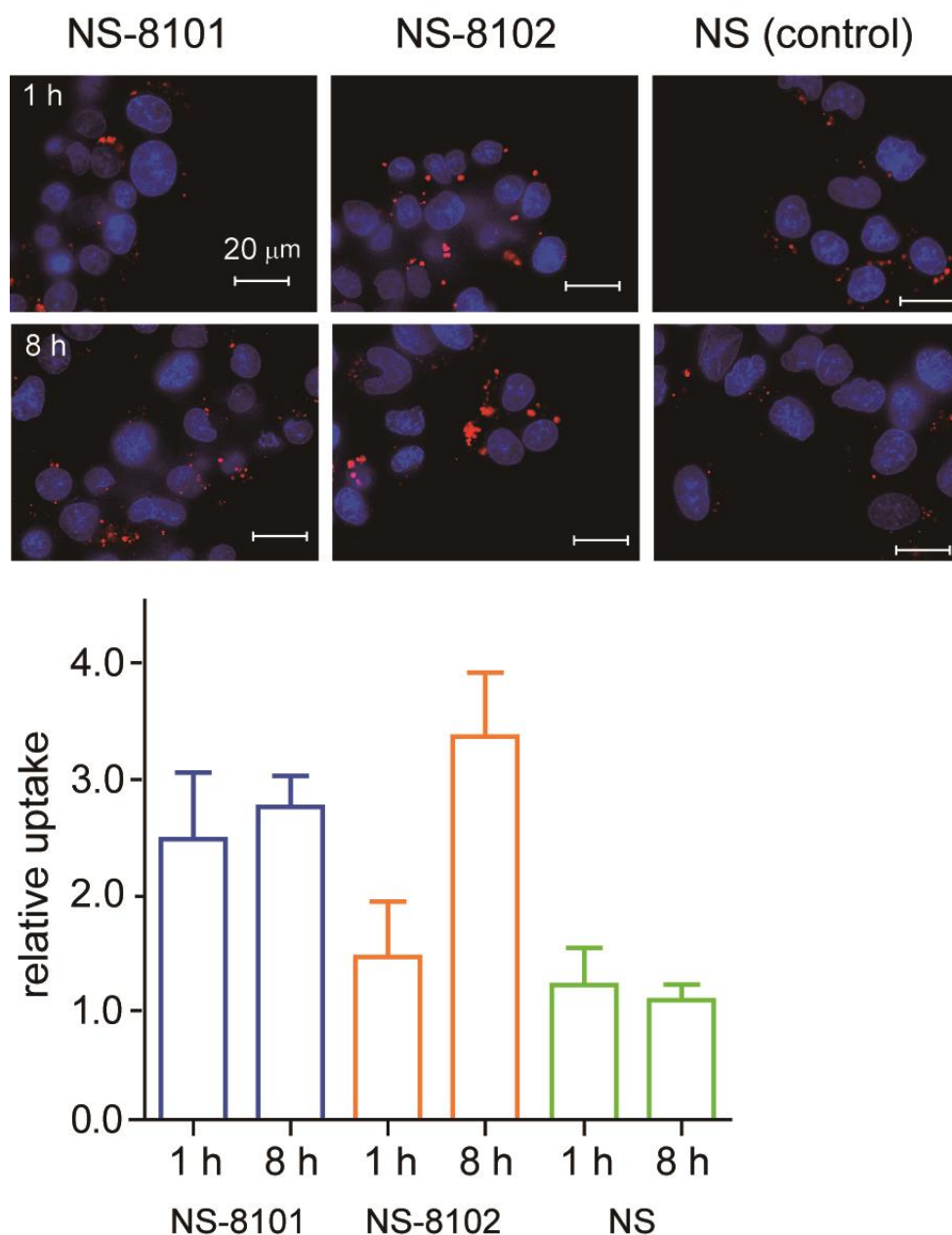


Figure 3-2-11. Confocal cell images showing uptake of NSs prepared from QD655 (red) by HepG2 cells after 1 h and 8 h incubation with 10 nM NSs. Nuclei were stained with Hoechst 33342 (purple). Scale bar, 20 μm . The graph shows mean fold uptake relative to that of control NS after 1 h and 8 h (n=3).

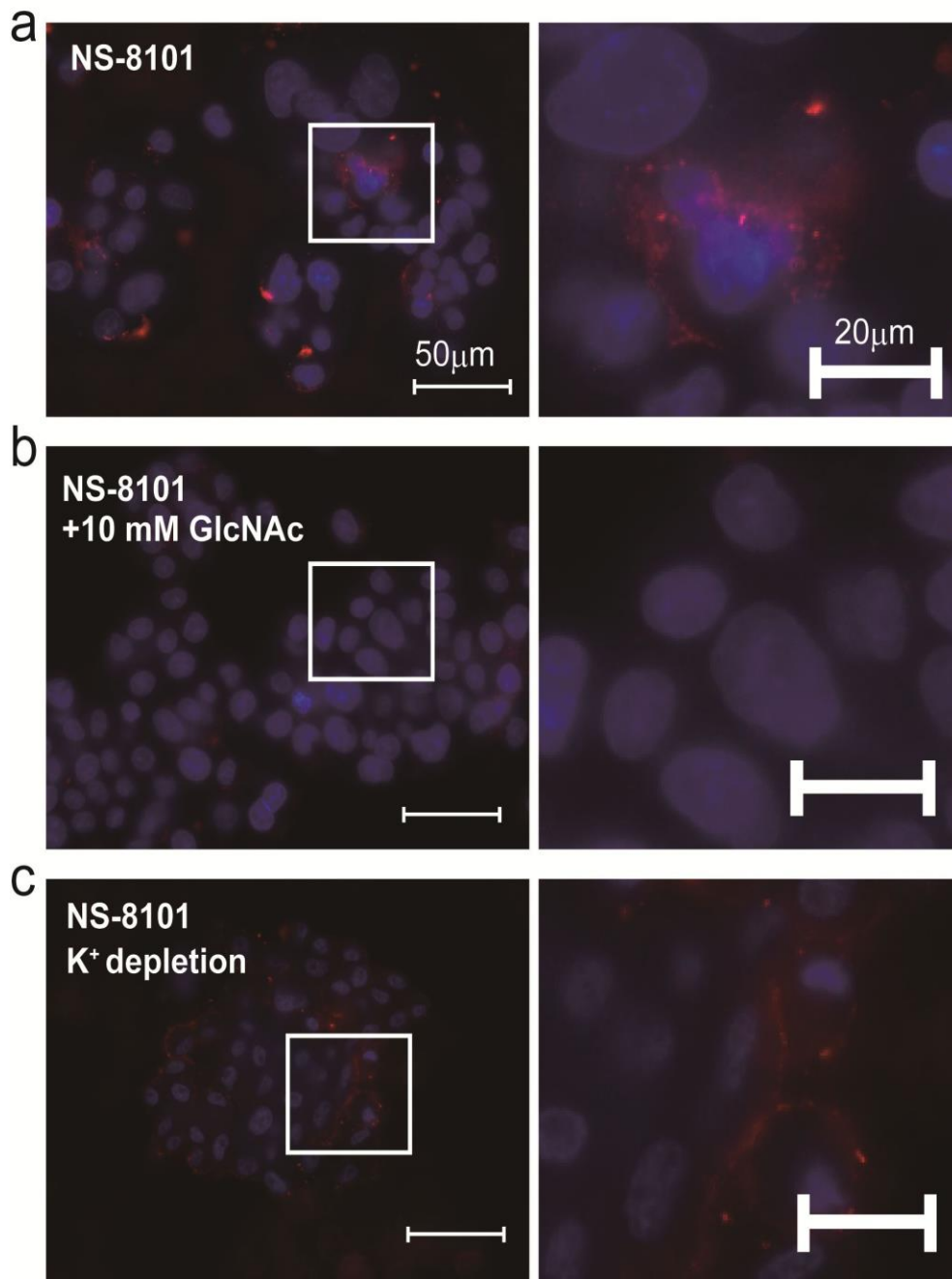


Figure 3-2-12. (a) Representative cell images showing intracellular distribution of **NS-8101** (red) 2 h after exposure of HepG2 cells to 10 nM **NS-8101** (control). Scale bar, 50 μm (left) and 20 μm (right) showing a higher magnification of the boxed areas. (b) Endocytic uptake of **NS-8101** is abrogated when HepG2 cells are co-cultured in the medium containing 10 mM GlcNAc. (c) Disruption of the formation of clathrin-coated pits by K⁺ depletion inhibits uptake of **NS-8101**.

To gain an insight into the molecular mechanisms of LMP and cellular cytotoxicity induced by **NS-8101** after lectin-mediated active transportation, we assessed the endocytic traffic and cytotoxicity of **NS-8101/His₆** (**Table 2-2-1**), in which **His₆** moiety can induce LMP through “proton sponge effect”¹⁶ in acid organelles as reported previously.⁸ Consistent with our previous result, **NS-8101/His₆** (10 nM) appeared to escape immediately from endosome 30 min after coincubation with HepG2 cells (**Figure 3-2-13** and **Figure 3-2-14**) while LMP caused by **NS-8101** was mostly observed 2 h after coincubation (**Figure 3-2-13** and **Figure 3-2-3**). Surprisingly, cell viability assay unveiled that **NS-8101/His₆** has no inhibitory effect on the growth of HepG2 cells in the concentration range tested for **NS-8101** (**Figure 3-2-14**). This result clearly indicates that antitumor activity of **NS-8101** depends strongly on the irreversible inactivation of lysosomal β -hexosaminidases by covalent inhibitors. It seems that generation of NSs with drastically increased particle size owing to covalently harboring multiple β -hexosaminidases may affect significantly the integrity of the lysosomal membranes, which causes LMP to release lysosomal cathepsins into the cytosol, which is a critical step for cathepsin-mediated killing of tumor cells.^{10,11} In contrast, it is clear that membrane permeabilization induced by a rapid escape of **NS-8101/His₆** from early endosome before being developed into lysosome cannot release any lysosomal enzymes.

HepG2

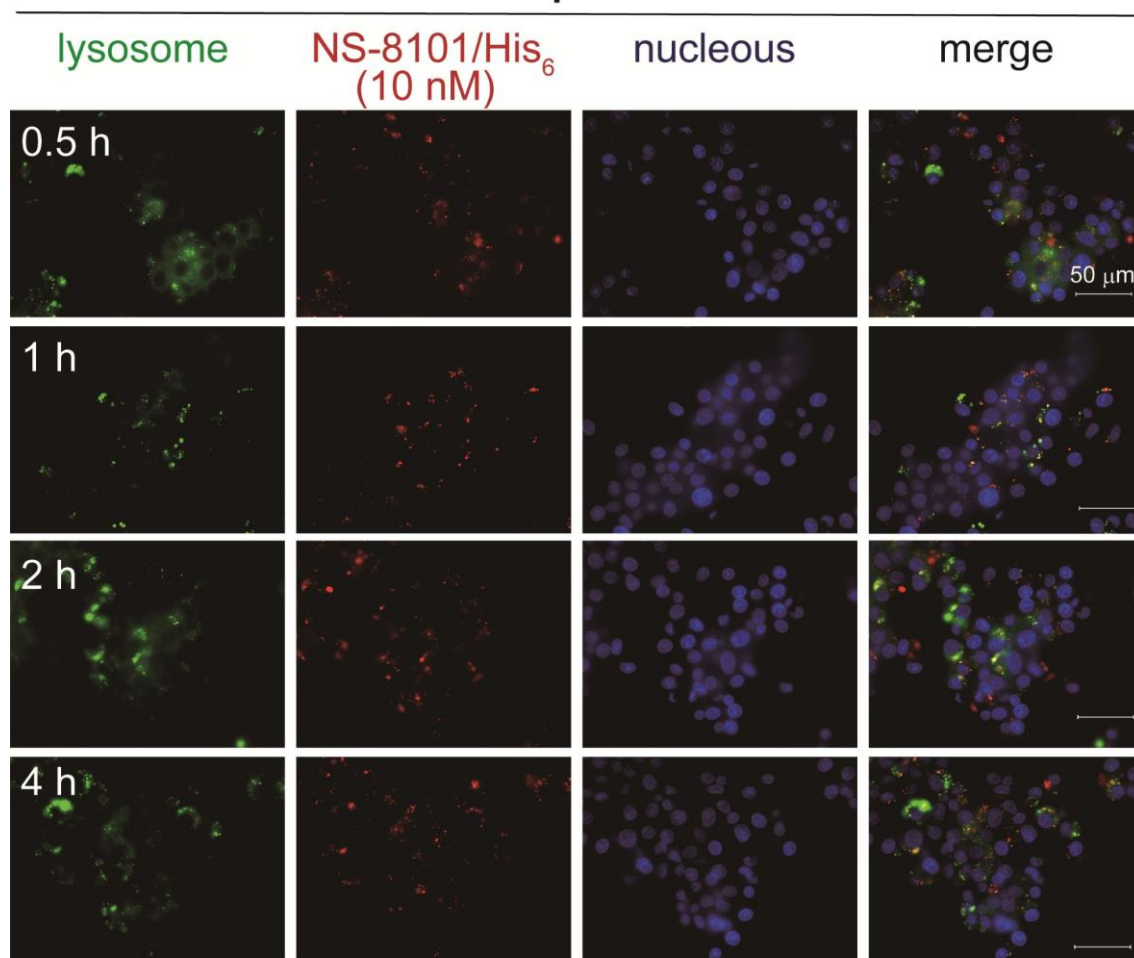


Figure 2-2-13. Representative cell images showing intracellular distribution of 10 nM NS-8101/His₆ at different times after exposing HepG2 cells to NS-8101/His₆. Scale bar, 50 μm. Cells were co-incubated with NS-8101/His₆ (red) for 0.5, 1, 2, and 4 h and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple). Merged pictures clearly show that an escape of NS-8101/His₆ from endosomal entrapment has already occurred after a 0.5 h coincubation with HepG2 cells.

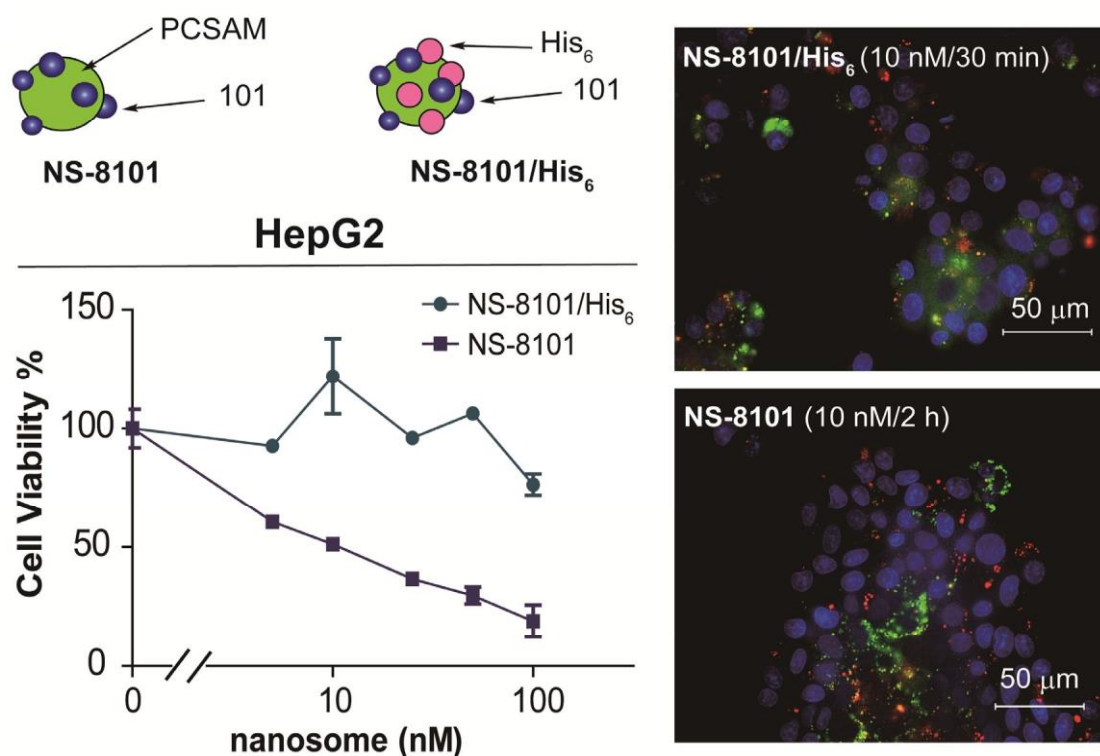


Figure 3-3-14. Viability of HepG2 cells after 98 h coincubation with 1~100 nM **NS-8101/His₆** measured by the MTT assay shows that attachment of **His₆** moiety abrogates antitumor activity of **NS-8101** (left). Representative cell images showing intracellular distribution of **NS-8101/His₆** and **NS-8101** (right). Scale bar, 50 μm. HepG2 cells were co-incubated with 10 nM **NS-8101/His₆** (red) for 30 min or with 10 nM **NS-8101** (red) for 2 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

Evaluation anti-tumor effect for invasive and non-invasive cancer cells

To assess the feasibility of antitumor effect of **NS-8101** in other tumor cells, we examined the effects of **NS-8101** on two typical human breast cancer cells: MCF7 and triple negative MDA-MB-231 cells. As shown in **Figure 3-2-15**, glycan analysis provided evidence that MDA-MB-231 cells distinctly synthesize various bi- and tri-antennary complex-type *N*-glycans with terminal sialic acid residues while MCF7 cells seem to have only some high mannose-type *N*-glycans as major glycoforms. Clearly, highly aggressive MD-MBA-231 cells use extensively UDP-GlcNAc in the rapid synthesis of glycoproteins bearing such bi- and tri-antennary complex-type *N*-glycans, suggesting that a large amount of GlcNAc may be salvaged into HBSP from lysosomal degradation system (**Figure 1-1-1**). We considered that expression levels of lysosomal *exo*-glycosidases in endocytic GlcNAc salvage pathway in MDA-MB-231 cells can be elevated significantly when compared with those of MCF7 cells. Interestingly, live cell imaging in the presence of 10 nM **NS-8101** indicates that **NS-8101** (red) is endocytosed into both MCF7 and MDA-MB-231 cells and induces LMP 2 h after coincubation (**Figure 3-2-16**) in a similar manner to the result observed in HepG2 cells (**Figure 3-2-3**). MTT assay showed that the viability of both tumor cells appears to decrease significantly in the presence of **NS-8101** (**Figure 3-2-17**). However, it is important to note that **NS-8101** kills

MDA-MB-231 cells more efficiently ($IC_{50}=31.8$ nM) than MCF7 ($IC_{50}=95.3$ nM), suggesting highly activated endocytosis and elevated expression levels of lysosomal β -hexosaminidases in MDA-MB-231 cells.

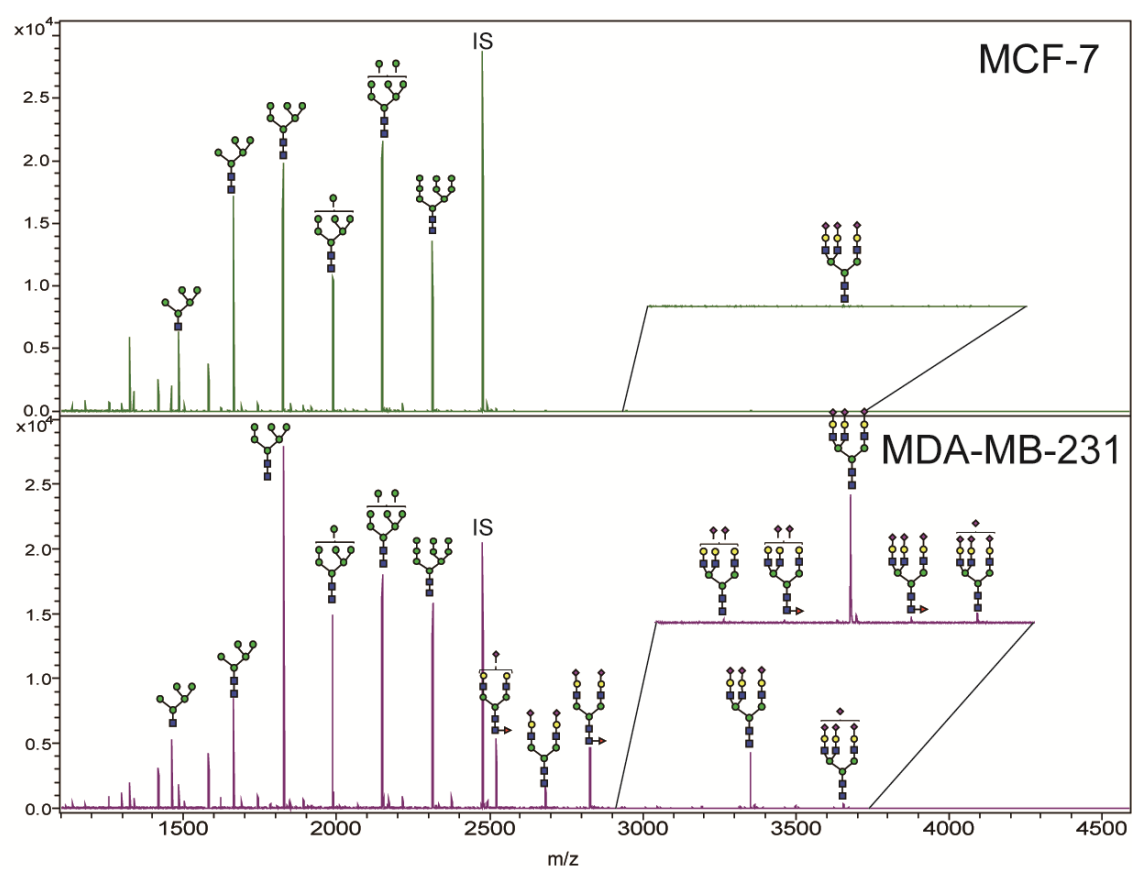


Figure 3-2-15. Major *N*-glycans of human breast cancer cells (MCF7 and MDA-MB-231 cells) uncovered by glycoblotting-assisted glycomics.¹⁷

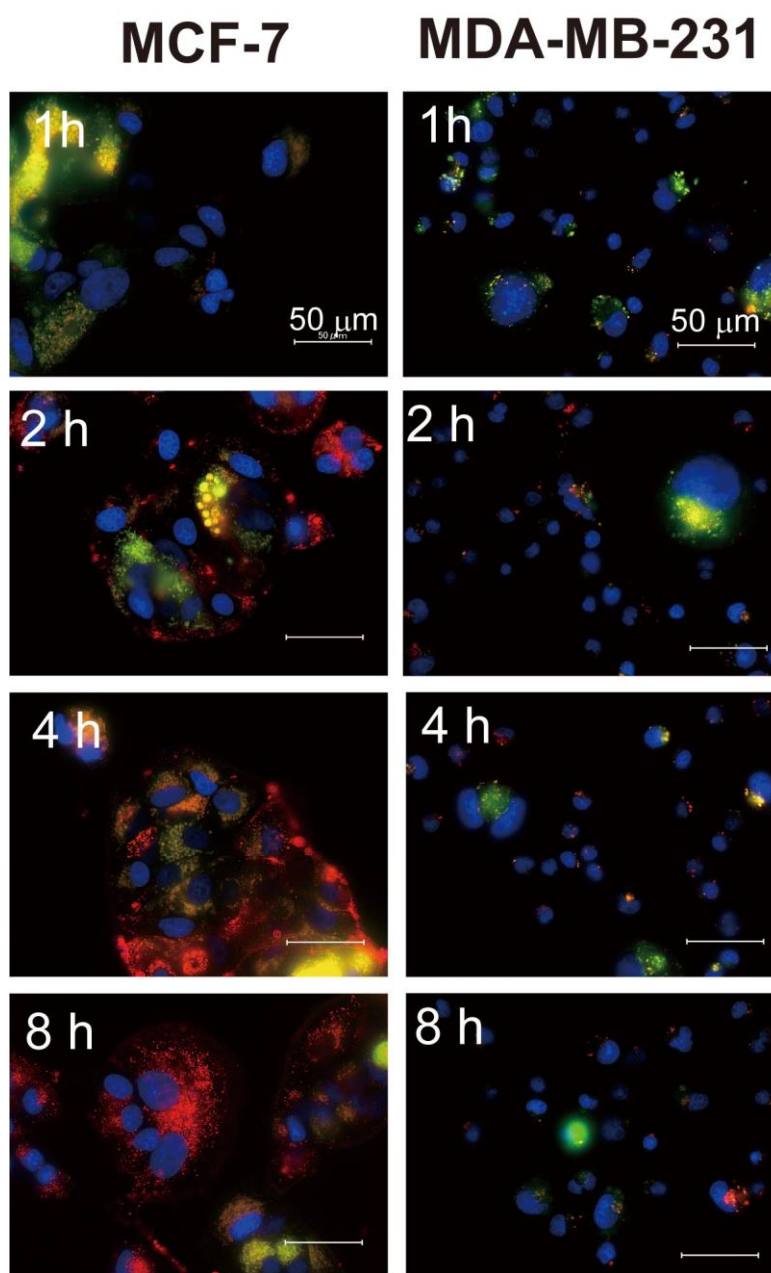


Figure 3-2-16 Scale bar, 50 μm . MDA-MB-231 and MCF-7 cells were co-incubated with 10 nM NS-8101 (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

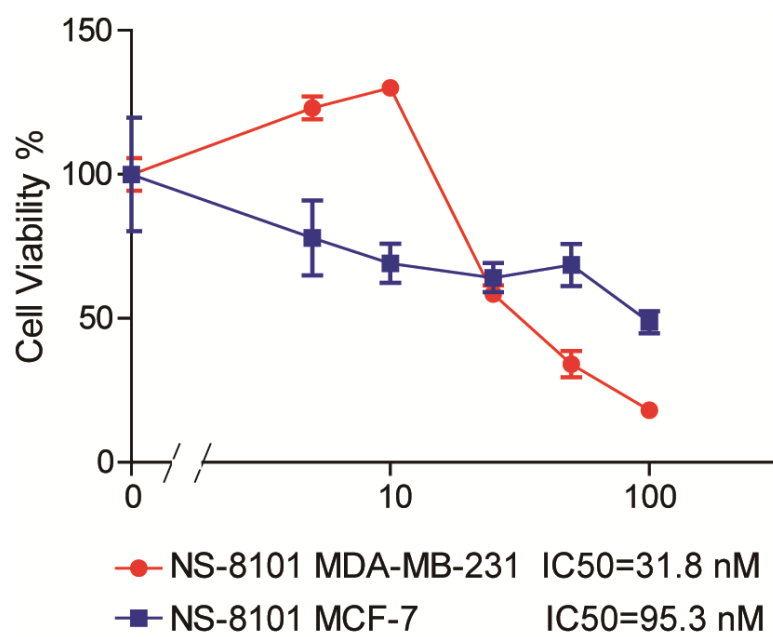
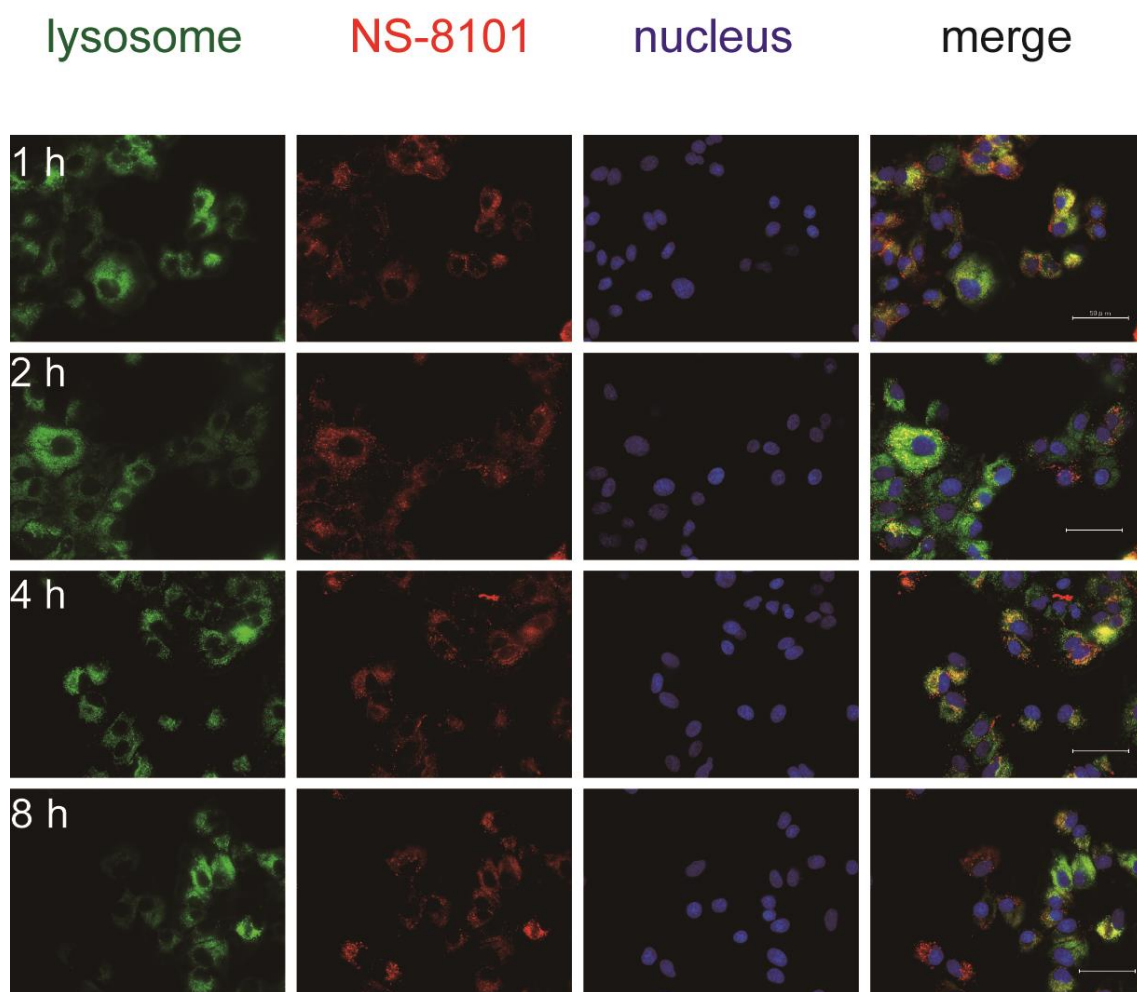


Figure 3-2-17 Comparing IC₅₀ values between MCF-7 and MDA-MB-231 cells.

Comparing anti-cancer effect between cancer cells and normal cells.

To assess the feasibility of antitumor effect of **NS-8101** in other cells, we examined the effects of NSs on normal and cancer lung cells, A549 and normal lung OUS-11 cells. Interestingly, live cell imaging in the presence of 10 nM **NS-8101** and **NS-8102** indicates that **NS-8101** and **NS-8102** (red) is endocytosed into A549 cells and induces LMP 4 h after coincubation (**Figures 3-2-18** and **Figures 3-2-19**), however, in OUS-11 cells, LMP were not induced after 8 h (**Figures 3-2-20**). MTT assay showed that the viability of A549 cells appears to decrease significantly in the presence of **NS-8102** (**Figures 3-2-21**). However, it is important to note that **NS-8102** kills only A549 cells, and OUS-11 cells are not killed, suggesting highly activated endocytosis and elevated expression levels of lysosomal enzymes in cancer cells.



Figures 3-2-18 Scale bar, 50 μ m. A549 cells were co-incubated with 10 nM NS-8101 (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

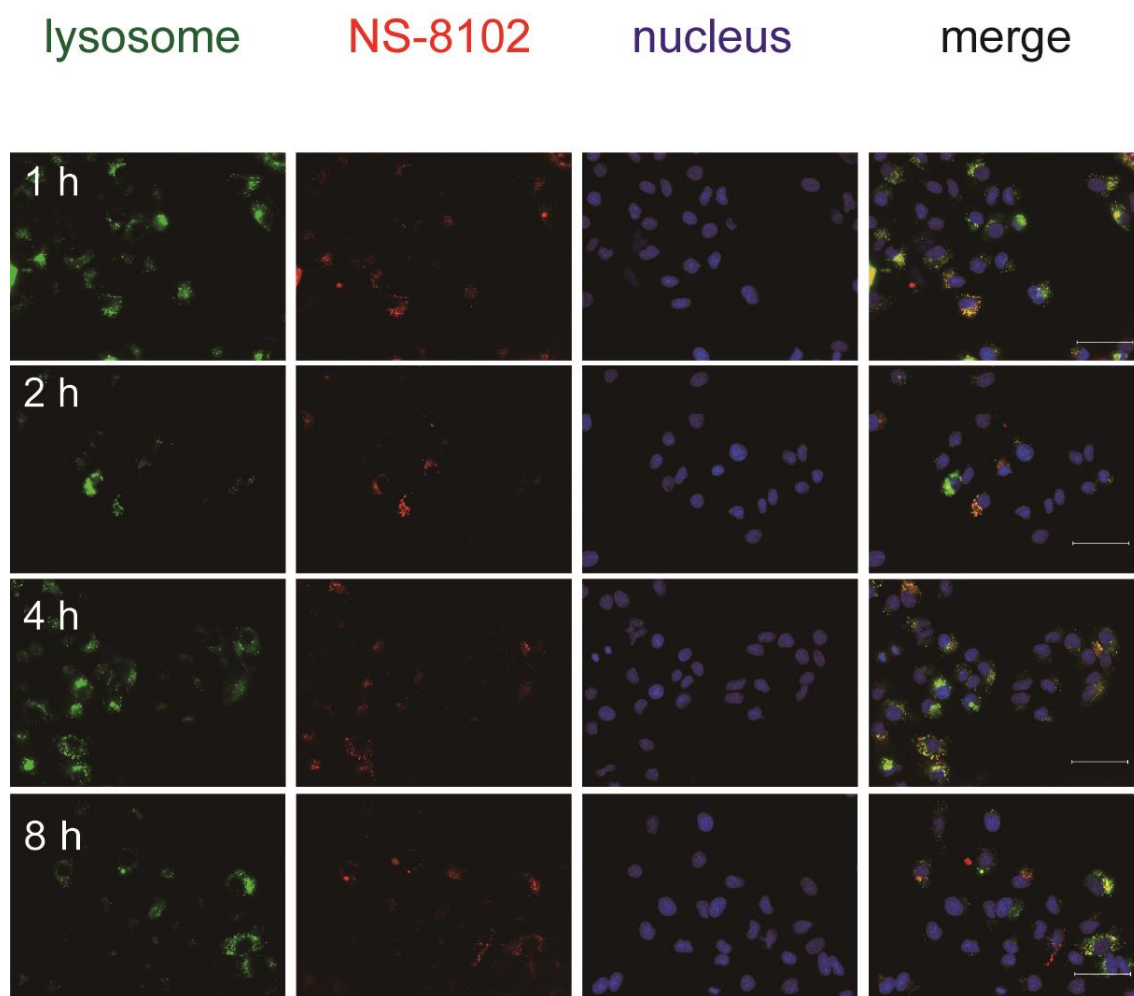


Figure 3-2-19 Scale bar, 50 μ m. A549 cells were co-incubated with 10 nM NS-8102 (red) for 1-8 h, and fixed, respectively. Lysosomes were stained with LysoTracker DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

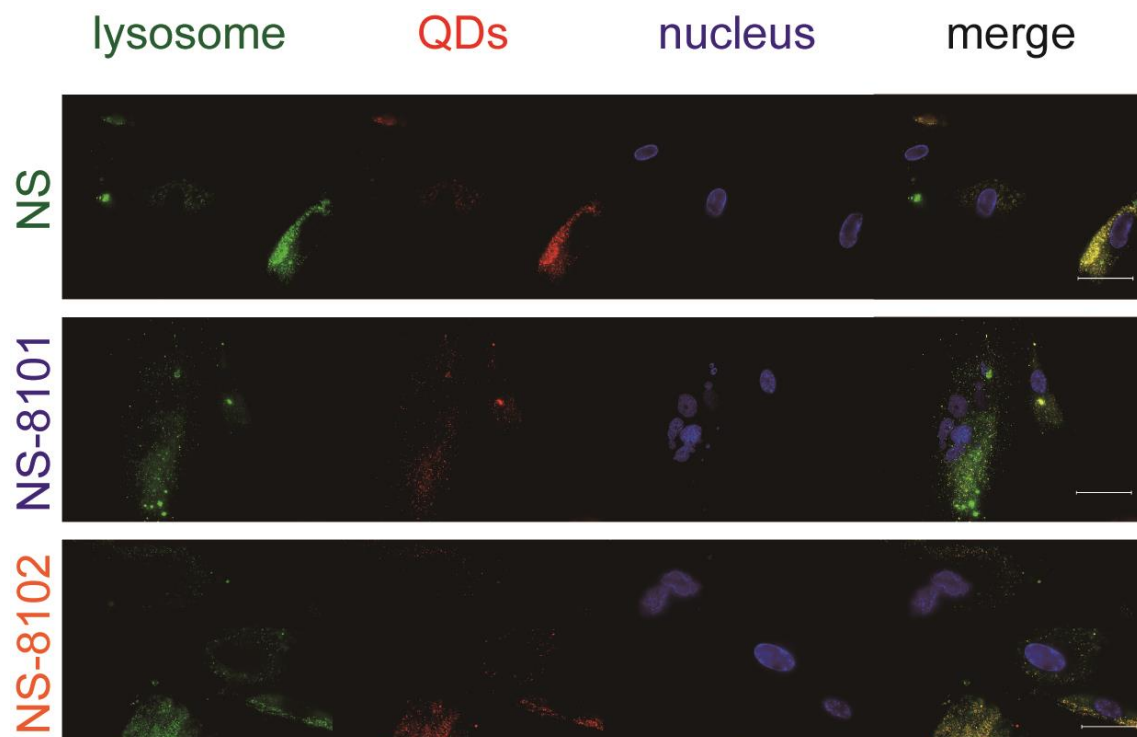


Figure 3-2-20 Scale bar, 50 μ m. OUS-11 cells were co-incubated with 10 nM NS (red) or NS-8101 or NS-8102 for 8 h, and fixed, respectively. Lysosomes were stained with LysoTracker Green DND-26 (green), while the nuclei were stained with Hoechst 33342 (purple).

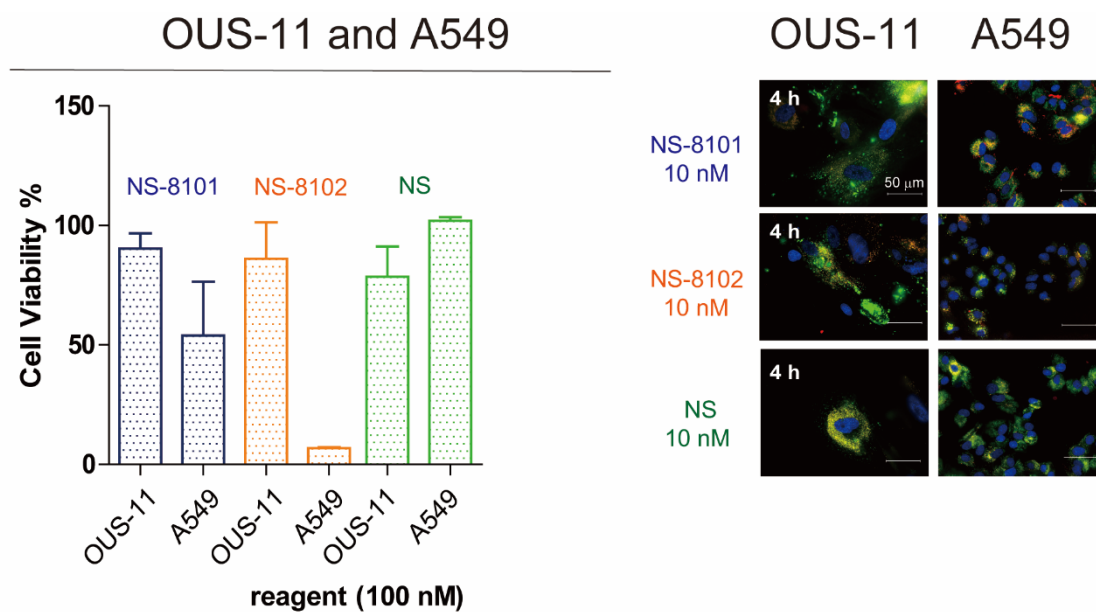


Figure 3-2-21. The viability of each compound assay of A549 and OUS-11. All reagents were 100 nM.

2-3 Summary

In this chapter, anti-cancer effects of glycosidase inhibitor displayed nanosomes. live cell imaging and cell viability assay using human liver cancer cells revealed that antiadhesive NSs carrying covalent glycosidase inhibitors (**NS-8101** and **NS-8102**) allow for enhanced uptake by tumor cells, targeted inactivation of lysosomal glycosidases, and inducing LMP-mediated apoptotic cell death [**NS-8101** (PC/101=2/1): $IC_{50} = 10.7$ nM and **NS-8102** (PC/102=2/1): $IC_{50} = 59.3$ nM]. Uptake of **NS-8101** is completely blocked by 10 mM GlcNAc or K^+ depletion (**Figure 3-2-12**), demonstrating that HepG2 cells internalize **NS-8101** through lectin-mediated endocytosis in a clathrin-dependent manner. The underlying molecular mechanisms of *in vitro* antitumor effect by **NS-8101** is also supported by evidence that escape of **NS-8101/His₆** from early endosomal trap completely loses its cell growth inhibitory effect (**Figure 3-2-14**). Furthermore, consistent with *in vitro* experimental results, **NS-8101** prepared from AuNP as a biocompatible counterpart of QD core dosed at 5 mg/kg/4 days gives complete tumor growth inhibition in the HCC xenograft mice model after systemic intravenous administration (**Figure 3-2-10**), although there remains much to be addressed

about pharmacokinetics, delivery efficiency to tumor site, clearance, and toxicity *in vivo*.²²

2-4 Experimental

General Methods and Materials. All solvents and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Chemical Industry Co. Inc. (Tokyo, Japan), Wako Pure Chemical Industries Co. Inc. (Tokyo, Japan), Funakoshi Co., Inc. (Japan, Tokyo), Medicinal Chemistry Pharmaceuticals, Co., Ltd. (Hokkaido, Japan), Thermo Fisher Scientific K. K. Co. Inc. (Tokyo, Japan), Kanto Chemical Co. Inc. (Tokyo, Japan) and used without further purification. TOPO-coated QDs (655 nm and 800 nm) were purchased from Thermo Fisher Scientific K. K. Co. Inc. (Tokyo, Japan). Microcon YM50 was purchased from Millipore Co. Inc (Tokyo, Japan). Nude mouse (BALB/c Slc-nu/nu) was purchased from Sankyo Lab. Service Co. Inc. (Tokyo, Japan). The fluorescence microscope used for image acquisition and image analysis was an all-in-one fluorescence microscope BIOREV BZ-9000 series generation II (Keyence Co. Inc., Osaka, Japan). Living animal imaging was performed by IVIS imaging system (Summit Pharmaceuticals International Co. Inc., Tokyo, Japan) equipped with CCD camera.

Cell Culture. Human hepatocellular carcinoma HepG2 (RIKEN cell bank, Saitama, Japan) and human breast cancer cells MCF-7 (American Type Culture Collection, Manassas, VA) and A549 were maintained in D-MEM High-glucose (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Tokyo, Japan), and cultured in a humidified incubator at 37°C in a humidified incubator with 5% CO₂. Human invasive breast cancer cells MDA-MB-231 (RIKEN cell bank, Saitama, Japan) were maintained in Leibovitz's L-15 (Wako, Osaka, Japan), supplemented with 10% FBS, and cultured in an incubator at 37°C, without CO₂ injected condition. OUS-11 were maintained in MEM (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Tokyo, Japan), and cultured in a humidified incubator at 37°C in a humidified incubator with 5% CO₂.

Effect of extracellular nutrition on *N*-glycan profiles of cultured human cancer cells. Human cancer cells (HepG2 and A549) were plated in two 10 cm dishes at a seeding density of 1 x 10⁶ cells/dish, and cells were incubated at 37°C in a humidified incubator with 5% CO₂. After 48 h, the medium was

removed, and the cells were washed with 10 mL of 200 mM D-PBS (Wako, Osaka, Japan). Then, one dish was added 10 mL of D-MEM High-glucose (Wako, Osaka, Japan) without FBS (condition B), and 10 mL of D-MEM High-glucose supplemented with 10% FBS (condition A) was added to the other dish, and they were incubated at 37°C in a humidified incubator with 5% CO₂ for 48 hour. After incubation, the cells were washed with ice-cold 200 mM D-PBS (5 mL) and scraped with cell scraper (BM Equipment Co., Ltd., Tokyo, Japan) in 5 mL of D-PBS containing 10 mM EDTA. Then, the cell suspensions were centrifuged at 10,000 g for 10 min at 4°C. After removing supernatants, pellets were dissolved by 1000 µL of 0.1 % SDS, 1 % triton-X 100, 100 mM ammonium hydrogen carbonate (AHC), and followed by heat-incubation for 10 min at 90°C. After being cooled to the room temperature, the amount of protein in cell lysis solution was quantified by BCA assay. The solution containing 100 µg of protein was added to 500 U PNGaseF from *Flavobacterium meningosepticum* (New England BioLabs Japan Inc, Tokyo, Japan) and incubated at 37°C, overnight. Then the sample mixture was dried up by SpeedVac. N-glycan analysis was performed by glycoblotting method.¹⁷ Glycoblotting reactions were performed by SweetBlot 7 (System Instruments,

Tokyo, Japan). BlotGlyco beads (200 μ L, 10 mg/mL suspension, Sumitomo Bakelite Co., Tokyo, Japan) were aliquoted on the well of a MultiScreen Solvinert filter plate (Millipore, Billerica, MA, USA). Then, PNGaseF treated cancer cell samples (100 μ g of protein, 4~5 x 10⁵ cells), sialylated glycan (SG) as a standard (20 μ L, 100 pmol) and 2% ACN solution (180 μ L) were applied to the well and the mixture was incubated for 45 min at 80°C to enrich glycans by beads surface hydrazide groups. The plate was washed with 200 μ L of 2 M Guanidine-HCl in 16.6 mM AHC followed by 200 μ L MilliQ and 200 μ L MeOH containing 1% trimethylamine. All washing steps were performed twice. Then, unreacted hydrazide group was capped by being treated with 100 μ L of 10% Ac₂O in MeOH and incubated for 30 min at room temperature. After incubation, the solution was removed by vacuum and the plate was washed with 200 μ L of 10 mM HCl aqueous solution, MeOH and dioxane at twice. Then, methyl esterification of sialic acid carboxyl groups was performed by being treated with 100 μ L of 1-methyl-3-*p*-tolyltriazene (MTT) solution (100 mM/dioxane) and incubated at 60°C for 90 min. After methylation, the plate was washed with dioxane, MeOH and MilliQ twice. Then, the surface of BlotGlyco trapped glycans were tagged by *trans*-iminization reaction with of

ao-WR (aminooxy functionalized peptide reagent, 20 μ L, 20 mM/MilliQ) in 2% ACN solution (180 μ L) for 45 min at 90°C. WR-tagged glycans were eluted by adding water (150 μ L), and purified by Mass PREPTM HILIC μ Elution Plate (Waters, Milford, MA, USA) according to the manufacture's description. After purification, these glycans were analyzed by MALDI-TOF MS as shown in **Figure 3-2-1**.

Cellular uptake of Nanosomes. Cells were suspended in medium supplemented with 10% FBS and plated on the wells of 8-well plate (Fisher Scientific) at a seeding density of 5×10^4 cells/well. Cells were allowed to adhere overnight at 37°C in a humidified incubator with 5% CO₂. Nanoparticle (NSs) stock solution was diluted in autoclaved MilliQ and added to existing media. NSs derived from QDs were added to yield final concentrations of 10 nM and they were co-incubated with cells. At the designated timepoints, cells were washed with Opti-MEM (gibco) three times to remove any adherent NSs and cells were stained with lysosomal marker LysoTracker Green DND-26 (200 μ L, 5 nM/Opti-MEM) and incubated at 37°C in a humidified incubator with 5% CO₂ for 30 min. Then, the nuclei were

stained with Hoechst 33342 (2 μ L, 1 ng/ μ L/Opti-MEM) and incubated at 37°C in a humidified incubator with 5% CO₂ for 15 min.⁸ Stained cells were imaged by an all-in-one fluorescence microscope BIOREV BZ-9000 series generation II (Keyence, Osaka, Japan) which was equipped with a 60 $\times$ lens. Imaging pictures were analyzed and quantified of QDs fluorescence by BZ-II Analyzer software (Keyence, Osaka, Japan). Images of cellular uptake after 2 h co-incubation with **NS-8101** under different conditions in order to study underlying mechanism were observed by fluorescence microscopy as follows:

(1) GlcNAc-mediated endocytosis: Cells were co-incubated with 10 nM **NS-8101** in the medium supplemented with 10% FBS and 10 mM GlcNAc at 37°C in a humidified incubator with 5% CO₂ for 2 h, followed by stained nuclei with Hoechst 33342 (2 μ L, 1 ng/ μ L/Opti-MEM) and fluorescence image were taken by all-in-one fluorescence microscope. (2) Clathrin-dependent endocytosis: K⁺ depletion was achieved by pre-incubation of cells in the K⁺-free HEPES buffer (140 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/mL D-glucose)¹³ at 37°C in a humidified incubator with 5% CO₂ for 1 h. The cells were quickly washed with K⁺-free HEPES buffer, and were incubated with 10 nM **NS-8101** in K⁺-free HEPES buffer at 37°C in a

humidified incubator with 5% CO₂ for 2 h, followed by stained nuclei with Hoechst 33342 (2 µL, 1 ng/µL/Opti-MEM) and fluorescence image were taken by all-in-one fluorescence microscope.

Cell viability assay. HepG2, MCF-7, MDA-MB-231, A549 and OUS-11 cells were seeded in 96-well plate (Thermo Fisher), at 5 x 10⁴ cells/well, and incubated at 37°C with 5% or without CO₂ for 24 h. The cells were treated with NSs for 96 h. Cell growth was determined by adding 10 µL of a Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan), and the mixture was incubated for 1.5 h and the optical density was measured using SUNRIZE Thermo microplate reader (Tecan Japan Co., Inc., Kawasaki, Japan). All values are expressed as means ± the standard error of the mean (SEM), and the assays were repeated three times. The bar graphs in each picture were generated with Prism 4.0 (GraphPad Software, Inc., San Diego, CA).¹⁹

Evaluation of *in vivo* tumor growth inhibition. Four-week old male BALB/c Slc-nu/nu (15~19 g) were purchased from Sankyo Lab. Service (Tokyo, Japan). Subcutaneous HepG2 tumor bearing mice were prepared by implanting

subcutaneously into the right flank with HepG2 cells (1.0×10^7 cells in 0.2 mL PBS per mice).¹⁹ When tumors grew 100 mm³, the mice were randomly divided into 3 groups (n=6). Saline, NS (Au), **NS-8101** (Au) were injected into the tail vein of the tumor-bearing mice (200 µL, 1 µM/saline, concentration of **101** was estimated to be 600 µM) on days 1, 5, 9, 13, 17, and 21. Tumor size was measured on days 1, 5, 9, 13, 17, and 21, and calculated based on the following equation: volume = $0.5 \times (\text{major diameter}) \times (\text{minor diameter})^2$.²⁰

Living animal imaging in tumor bearing mice. BALB/c Slc-nu/nu (5-week old, male) were obtained from Sankyo Lab. Service (Tokyo, Japan). Subcutaneous HepG2 tumor bearing mice were prepared by implanting subcutaneously into the right flank with HepG2 cells (1.0×10^7 cells in 0.2 mL PBS per mice). When tumors grew 200 mm³, **NS-8101** derived from QD800 (100 µL, 1 µM/saline) was injected into the tail veins of HepG2 tumor bearing mice. Then, the mice were anesthetized by isoflurane, and were imaged by IVIS imaging system (Summit Pharmaceuticals International Co., Tokyo, Japan). The exposure time is 1 sec. and the length of excitation and emission wave length are 710 nm and 820 nm, respectively. The mice were imaged for 2 h after

intravenous injection. After 2 h imaging, the mice were dissected, and the fluorescence images were taken. Major organs were isolated from all mice and organ fluorescence image were also observed as shown in **Figure 3-2-10**.⁷

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Chapter 4

Conclusion remarks

In this thesis, we focused on the lysosomal hydrolysis inhibitors to control cancer metabolism. All roads lead to lysosome. Endocytic trafficking of the extracellular macromolecules toward lysosome is a critical metabolic pathway to provide cancer cells with many fuels such as amino acids, monosaccharides, lipids, and intermediates reused for the synthesis of macromolecules during extremely accelerated growth and malignant transformation. Of note, recent studies unveiled the importance of UDP-GlcNAc as a pivotal sugar nucleotide for the synthesis of cancer-relevant sialylated complex-type *N*-glycans of glycoproteins as well as a variety of glycoconjugates containing GlcNAc residues. In addition, it should be noted that UDP-GlcNAc is also a common intermediate for *de novo* synthesis of UDP-GalNAc and CMP-Neu5Ac, key sugar nucleotides needed for cancer specific protein glycosylation both in mucin-type *O*-glycans and complex-type *N*-glycans.⁴ Given that potency of endocytic GlcNAc salvage pathway affects strongly intracellular concentration of UDP-GlcNAc, it has been considered that lysosomal glycosidases such as β -hexosaminidases and β -galactosidases become promising antitumor targets only when their inhibitors could be delivered efficiently into the intracellular lysosomal compartment of the cancer cells.

Although targeted delivery of nanoparticles with such inhibitors is expected to achieve this goal, the clinical translation and benefit of nanomedicines has been limited owing to physical and biological barrier faced upon exposure of nanoparticles to complex biological fluids.

In chapter 2, nanosome (NS) were developed as new drug delivery tools for preventing the formation of “protein corona”. We demonstrate that a mixed SAM coating of core metals such as QDs and AuNPs of 10~20 nm in a hydrodynamic diameter with PC and AO linkers allows establishing a novel strategy toward therapeutic nanomedicines having such an ideal surface chemistry. Notably, FCS analysis provides evidence that NSs coated with mixed SAM containing PC linker exhibit a remarkably superior antifouling property in 100% FBS while nanoparticles coated with SAM of PEG linker as a single component, an alkanethiol having a hexa(ethylene glycol) headgroup, aggregate immediately and form a precipitation when exposed to 100% FBS . It is important to note that zwitterionic PC linker is fundamental material for achieving antifouling property of mixed SAM coating, and AO linker facilitates surface loading of functional cargoes such as ligands and inhibitors involving an aldehyde/ketone auxiliary onto nanoparticles. In enzyme

kinetics experiments, covalent inhibitor activity for β -hexosaminidase was improved by being displayed on nanoparticle. The phenomena was also induced by the surface to prevent nonspecific protein adhesion.

In chapter 3, biological evaluation of NSs were performed. Indeed, live cell imaging and cell viability assay using human liver cancer cells revealed that antiadhesive NSs carrying covalent glycosidase inhibitors (**NS-8101** and **NS-8102**) allow for enhanced uptake by tumor cells, targeted inactivation of lysosomal glycosidases, and inducing LMP-mediated apoptotic cell death [**NS-8101** (PC/101=2/1): $IC_{50} = 10.7$ nM and **NS-8102** (PC/102=2/1): $IC_{50} = 59.3$ nM]. Uptake of **NS-8101** is completely blocked by 10 mM GlcNAc or K^+ depletion (**Figure 4a~c**), demonstrating that HepG2 cells internalize **NS-8101** through lectin-mediated endocytosis in a clathrin-dependent manner. The underlying molecular mechanisms of *in vitro* antitumor effect by **NS-8101** is also supported by evidence that escape of **NS-8101/His₆** from early endosomal trap completely loses its cell growth inhibitory effect. Furthermore, consistent with *in vitro* experimental results, **NS-8101** prepared from AuNP as a biocompatible counterpart of QD core dosed at 5 mg/kg/4 days gives complete tumor growth inhibition in the HCC xenograft mice model after

systemic intravenous administration, although there remains much to be addressed about pharmacokinetics, delivery efficiency to tumor site, clearance, and toxicity *in vivo*.

In this thesis, new anti-cancer drug candidates were successfully developed. NSs prevented nonspecific protein adherent and forming protein corona. These facts may solve problems about the nanoparticle drugs. Actually, by utilizing anti-adhesive nanoparticles, lectin or asialoglycoprotein receptor mediated endocytosis can be observed. Furthermore, lysosomal enzyme targeting was successful via these endocytosis pathway. We expect, therefore, that combining the merits of our antiadhesive nanomedicine platform and high technologies for nanoparticle design will provide dramatic improvements in the therapeutic index and reduction of systemic toxicity toward the clinical translation of patient care.

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