# Hyaluronic acid-based pH-sensitive polymer-modified liposomes for cell-specific intracellular drug delivery systems

# Hyaluronic acid-based pH-sensitive polymer-modified liposomes for cell-specific intracellular drug delivery systems

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**Abstract**

For the enhancement of therapeutic effects and reduction of side effects derived from anticancer drugs in cancer chemotherapy, it is imperative to develop drug delivery systems with cancer-specificity and controlled release function inside cancer cells. pH-Sensitive liposomes are useful as an intracellular drug delivery system because of their abilities to transfer their contents into the cell interior through fusion or destabilization of endosome, which has weakly acidic environment. We earlier reported liposomes modified with various types of pH-sensitive polymers based on synthetic polymers and biopolymers as vehicles for intracellular drug delivery systems. In this study, hyaluronic acid (HA)-based pH-sensitive polymers were designed as multi-functional polymers having not only pH-sensitivity but also targeting properties to cells expressing CD44, which is known as a cancer cell surface marker. Carboxyl group-introduced HA derivatives of two types, MGlu-HA and CHex-HA, which have a more hydrophobic side chain structure than that of MGlu-HA, were synthesized by reaction with various dicarboxylic anhydrides. These polymer-modified liposomes were stable at neutral pH, but showed content release under weakly acidic conditions. CHex-HA-modified liposomes delivered their contents into CD44-expressing cells more efficiently than HA-modified or MGlu-HA-modified liposomes or unmodified liposomes, whereas the

same liposomes were taken up only slightly by cells expressing CD44 proteins less. Competition assay using free HA or other polymers revealed that HA derivative-modified liposomes might be recognized by CD44. Therefore, HA-derivative-modified liposomes are useful as cell-specific intracellular drug delivery systems.

*Keywords*: hyaluronic acid / pH-sensitive liposome / CD44 / drug delivery system / endosome / cancer chemotherapy

## Introduction

Cell-specific drug delivery is crucially important to develop highly effective therapeutic systems with less-adverse effects. Furthermore, most bio-pharmaceuticals should be delivered to target cell interior and to target organelles to express their drug efficacy. For this purpose, drug-loaded nanocarriers composed of polymeric materials or lipid-based materials have been studied intensively.[1–3] For cancer treatment, nano-sized drug carriers present benefits of reducing the spread of anticancer drugs to normal tissues or normal cells and of accumulating into tumor tissues via enhanced permeation and retention (EPR) effects.[4] For example, polymeric micelle-based systems having various sizes efficiently accumulated to tumor tissues. Furthermore, smaller polymeric micelles penetrated into tumor tissues more effectively.[5] Poly(ethylene glycol) (PEG)-modified liposome encapsulating doxorubicin (DOX), Doxil, is an example of a commercially available lipid-based nanocarrier that can achieve DOX delivery into tumor tissues via the EPR effect.[6] However, such a “passive targeting” approach requires more precise delivery of drugs directly to target cells only, in addition to control of drug release.[2,7] The insertion of ligand molecules having specificity to tumor-specific surface receptors or endothelial cells directly into neovascular vessels of a tumor is regarded as effective approach providing cancer cell specificity to

nanocarriers.[8–11] To control drug release profiles, external stimuli-sensitive properties such as temperature, pH, magnetic field and light have been applied for nanocarriers.[12–21] ThermoDox is one example of a temperature-responsive liposome designed to achieve drug release at tumor tissues under local heating of tumor tissues.[22–24] Temperature-sensitive polymer-modified liposomes are another platform to develop temperature-responsive liposomes.[11,13] The use of functional polymers might be beneficial to control temperature-sensitivity and temperature-regions to release anticancer drugs by changing polymer chemical structures. To obtain stimulus-responsive nanocarriers, pH is also an important external stimulus because tumor tissues possess lower pH than physiological pH. Moreover, weakly acidic pH intracellular compartments (endo/lysosomes) exist inside of cells. To deliver drugs or macromolecules into cytosol of target cells, pH-responsive liposomes have been developed. Mixtures of dioleoylphosphatidylethanolamine and amphiphiles having carboxyl groups act as pH-responsive liposomes because, at neutral pH, this mixture forms a bilayer structure by hydration derived from carboxyl groups, whereas a rapid transition to hexagonal II phase is generated after protonation of carboxyl groups, leading to membrane fusion or drug release.[25] Modification of polymers having carboxyl groups to liposomes is another strategy to obtain pH-responsive liposomes.

Poly(acrylic acid) derivative-modified liposomes show vigorous membrane disruptive activity under acidic pH because polymers become hydrophobic after protonation of carboxyl groups. The polymers interact with a lipid membrane via hydrogen bond formation with phosphate groups and hydrophobic interactions.[26,27] Furthermore, the pH region can be controlled by changing the hydrophobicity of poly(acrylic acid) derivatives, which changes the pKa of carboxyl groups.[27] We also prepared carboxylated poly(glycidol)s as a pH-responsive polymer for pH-sensitization of liposomes.[28–30] pKa and pH-sensitivity of carboxylated poly(glycidol)s were controlled by spacer units next to carboxyl groups: poly(glycidol) derivatives having more hydrophobic spacer structures exhibited higher pKa and stronger membrane disruptive properties.[29] We further introduced these pH-sensitive units to naturally occurring polysaccharides such as dextran, mannan, and curdlan.[31–33] Carboxylated polysaccharides also showed pH-responsive properties. These polysaccharide-modified liposomes delivered model proteins into cytosol of target cells effectively via membrane fusion with endosomes.[31–33] Polysaccharides are important as base materials because of their biodegradability and easy functionalization. In addition, the cell surface has many kinds of lectins, polysaccharide-specific receptors, which are useful for targeting ligands to specific cells.[34–37]

Considering this background, we conceived multifunctional polysaccharide derivatives having both specificity to tumor cells and pH-responsive properties for this study. Integration of DDS functionalities into one molecule or one nanocarrier is effective strategy to develop multifunctional DDS. Hyaluronic acid (HA) was selected as a backbone of the multifunctional pH-responsive polymer.[38] HA is a biocompatible material: it is a main component of the extracellular matrix. Moreover, also it is known to bind to CD44 proteins specifically as a surface receptor on cancerous cells.[34,35,39–41] However, in most cases, raw HA was used for just providing targeting properties to nanocarriers. Here, we extended our strategy for development of pH-responsive polymers to HA: 3-methyl glutarylated (MGlu) units or 2-carboxycyclohexane-1-carboxylated (CHex) units were introduced to HA, and their pH-responsive capabilities were assessed (Figure 1). Furthermore, cell-specific anticancer drug delivery using HA derivative-modified liposomes was examined.

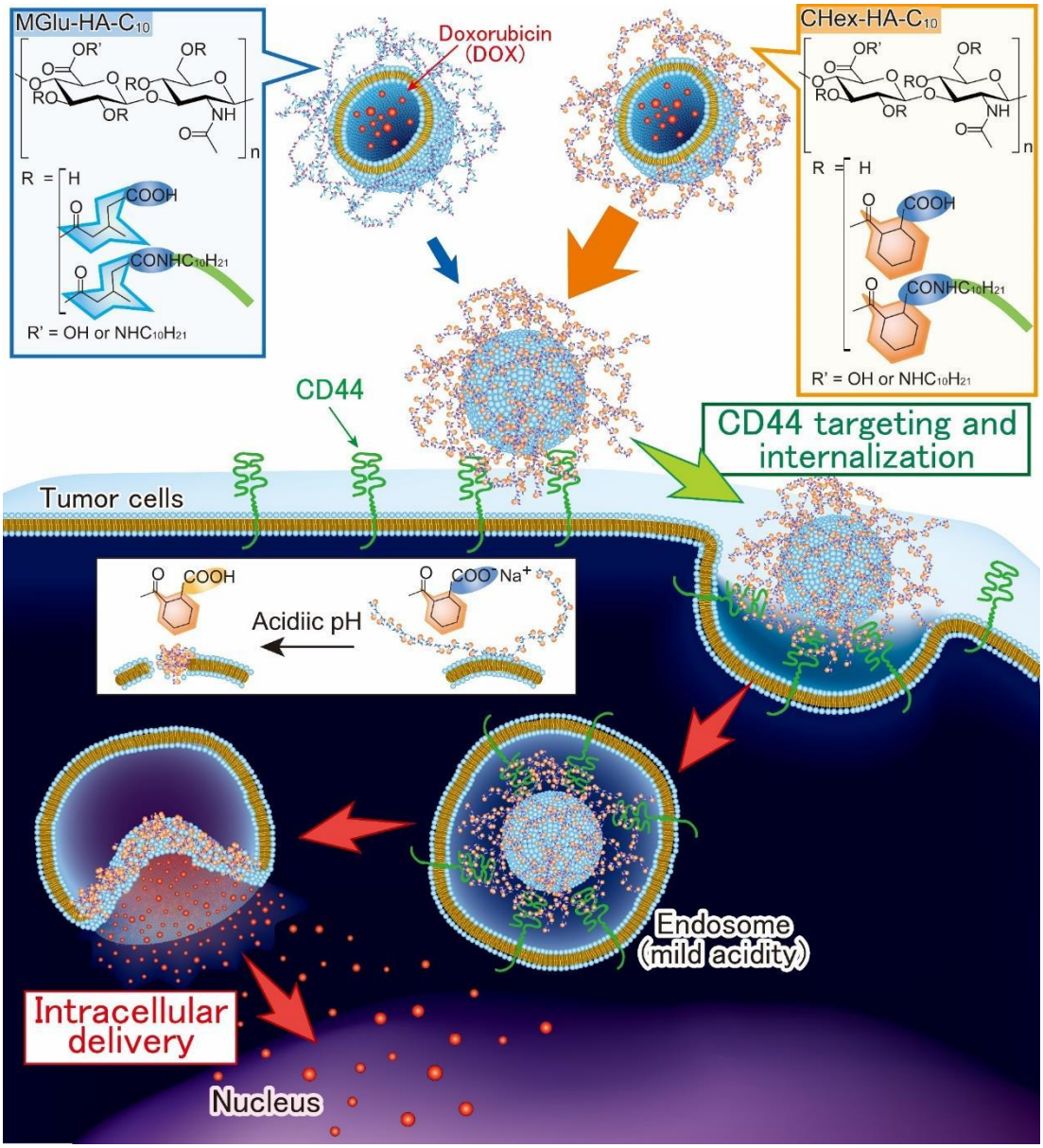


**Figure 1.** Design of hyaluronic acid derivative-modified liposomes for CD44-expressing cell-specific intracellular drug delivery. These liposomes are taken up by cells *via* endocytosis and trapped in endosome. Its weakly acidic environment triggers destabilization of the liposome, which induces release of drugs in endosome and their transfer to cytosol *via* destabilization of endosome.

## Results and Discussion

**Characterization of Hyaluronic Acid Derivatives.** Hyaluronic acid derivatives with different contents of MGlu groups or CHex groups as pH-sensitive moiety were synthesized by reacting HA with various amounts of 3-methylglutaric anhydride or 1,2-cyclohexanedicarboxylic anhydride (Figure 2 and Table 1). Decyl groups were further introduced to HA derivatives by reaction of decylamine with carboxyl groups in HA derivatives for fixation of these polymers onto liposome membrane (Figure 2 and Table 2). As a control, carboxyl groups of HA were reacted directly with decylamine to obtain anchor group-having HA (HA-$C_{10}$). The obtained HA derivatives were characterized using $^{1}$H NMR. Figures 3A–3C respectively represent $^{1}$H NMR spectra of HA, $MGlu_{52}$-HA, and $MGlu_{57}$-HA-$C_{10}$. In comparison to spectra for HA (Figure 3A) and for $MGlu_{52}$-HA (Figure 3B), the introduction of MGlu groups to HA was confirmed from the existence of new peaks corresponding to MGlu groups (0.9 ppm, 1.9–2.3 ppm except for acetyl group of HA (2 ppm)) in Figure 3B. From the integration ratio of peaks of MGlu residues to those of sugar backbone (2 ppm, 3.3–4.8 ppm), 52% of hydroxyl groups of HA was estimated as combined with MGlu residues. Similarly, from the integration ratio between sugar backbone, MGlu

residues, and decyl groups (0.9–1.4 ppm), decyl-amidated MGlu residues and MGlu residues were found to be combined to 2% and 57% of hydroxyl groups of HA, respectively, in the product, which is designated as $MGlu_{57}$-HA-$C_{10}$. CHex-HA derivatives were also evaluated using the same procedure (Figures 3D and 3E). The synthesis of HA-$C_{10}$ was also confirmed by the presence of decyl-amidated moieties (0.9–1.4 ppm, Figure 3F). Compositions of HA derivatives prepared in this study are presented in Tables 1 and 2.

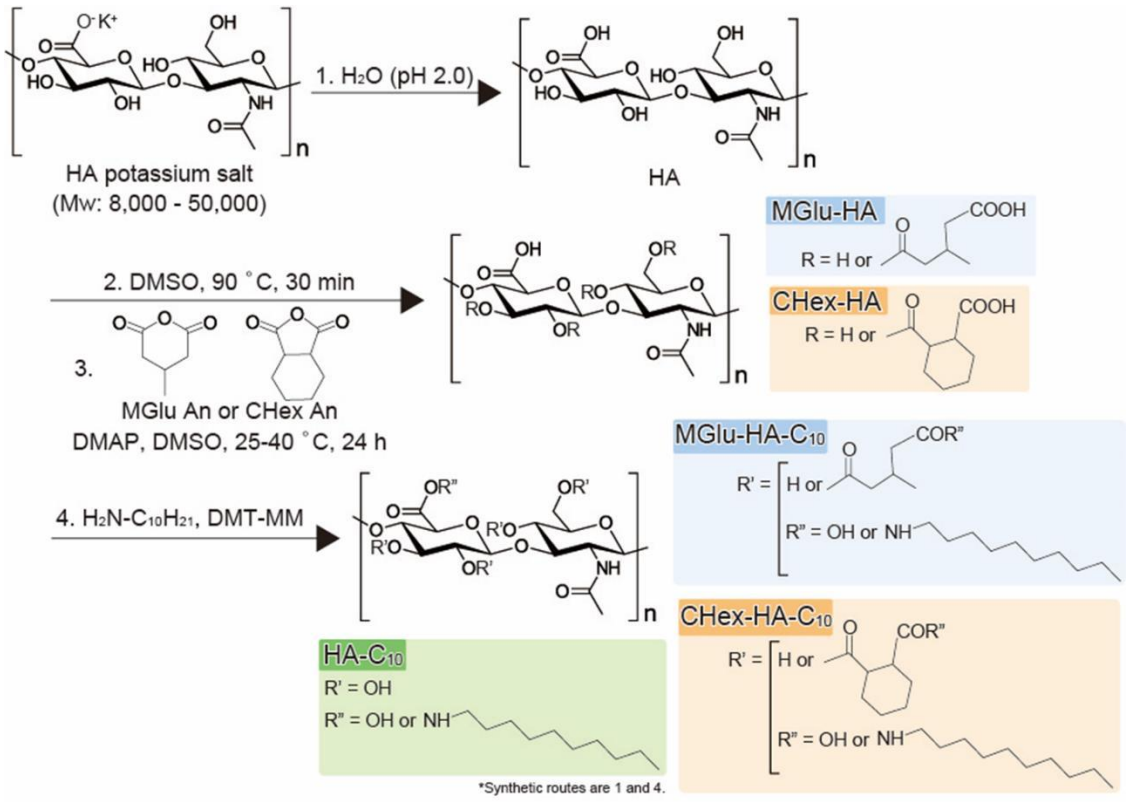


**Figure 2.** Synthetic route for hyaluronic acid derivatives having carboxyl groups and alkyl chains as anchor units to liposomal membrane.

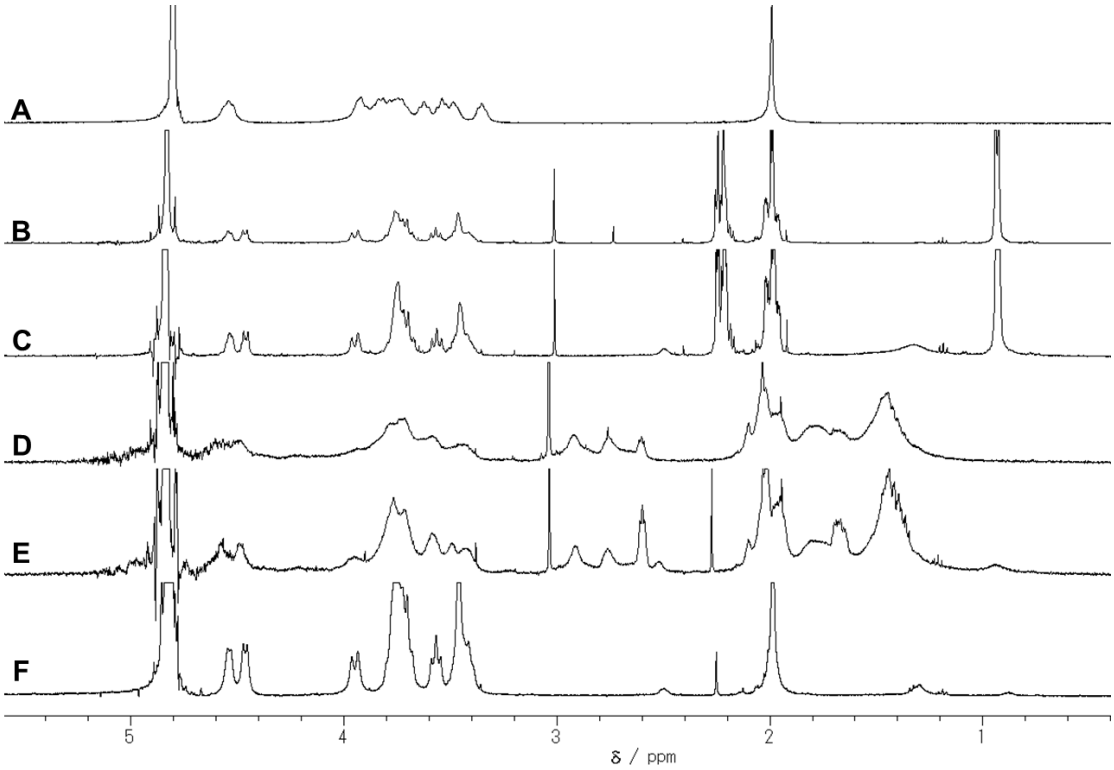


**Figure 3.** $^{1}$H NMR spectra of (A) hyaluronic acid, (B) $MGlu_{52}$-HA, (C) $MGlu_{57}$-HA-$C_{10}$, (D) $CHex_{60}$-HA, (E) $CHex_{50}$-HA-$C_{10}$, and (F) HA-$C_{10}$ in $D_2O$/NaOD.

**Table 1. Synthesis of Hyaluronic Acid Derivatives**

| Polymer | HA (mg) | DMAP (mg) | Dicarboxylic acid anhydride (mg) | DMSO (mL) | Reaction temperature (°C) | Yield (mg) | Yield (%) | MGlu or CHex (%)* |
|---|---|---|---|---|---|---|---|---|
| $MGlu_{11}$-HA | 266.2 | 161.8 | 286.5 | 25 | 25 | 201.4 | 73.4 | 11 |
| $MGlu_{18}$-HA | 250.1 | 161.1 | 254.4 | 25 | 25 | 272.6 | 87.5 | 18 |
| $MGlu_{43}$-HA | 104.8 | 66.1 | 210.7 | 10 | 40 | 144.2 | 120.2 | 43 |
| $MGlu_{52}$-HA | 509.8 | 327.1 | 4852.9 | 50 | 40 | 647.8 | 87 | 52 |
| $CHex_{18}$-HA | 1022.6 | 658.3 | 333.9 | 100 | 40 | 891.4 | 81.2 | 18 |
| $CHex_{27}$-HA | 307.2 | 197.4 | 103.3 | 30 | 40 | 377.2 | 85.3 | 34 |
| $CHex_{34}$-HA | 305.3 | 196.6 | 153.2 | 30 | 40 | 393.7 | 83 | 34 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $CHex_{60}$-HA | 310 | 194.9 | 261 | 30 | 40 | 490.7 | 80 | 60 |

*% for OH groups of HA determined by $^{1}$H NMR.

**Table 2. Synthesis of Hyaluronic Acid Derivatives Having Anchor Moieties**

| Polymer | MGlu-HA, CHex-HA or HA (mg) | n-Decylamine (mg) | DMT-MM (mg) | Water (mL) | Reaction time (h) | Yield (mg) | Yield (%) | Conversion (%)* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | MGlu or CHex | $C_{10}$ |
| $MGlu_{20}$-HA-$C_{10}$ | 206.6 | 24.9 | 42.3 | 12 | 17 | 210 | 95.5 | 20 | 4 |
| $MGlu_{57}$-HA-$C_{10}$ | 524.9 | 42.9 | 73.4 | 26 | 10 | 510 | 91.3 | 57 | 2 |
| $CHex_{27}$-HA-$C_{10}$ | 302.4 | 33.5 | 65.5 | 18 | 23 | 270.9 | 84.4 | 27 | 6 |
| $CHex_{50}$-HA-$C_{10}$ | 304.8 | 25.1 | 47.6 | 18 | 23 | 316.7 | 108.6 | 50 | 5 |
| HA-$C_{10}$ | 268.4 | 11.3 | 21.3 | 13 | 23 | 251.3 | 93.2 | 0 | 3 |

*% for OH groups of HA determined by $^{1}$H NMR.

Acid–base titration was conducted to assess the protonation behaviors of carboxyl groups on HA derivatives (Figure 4). MGlu-HA and CHex-HA changed their protonation state in the range of pH 4–10. Considering that p*K*a of carboxyl groups in the parent HA is reported as 3.04 and that the protonation degree of these carboxyl groups is less than 0.1 at pH 4.5,[42] the carboxyl groups in MGlu unit or CHex unit mainly changed their protonation state in the range of pH 4–10. The protonation behaviors of HA derivatives were affected strongly by MGlu/CHex contents or a spacer unit structure next to carboxyl groups. p*K*a of MGlu-HA/CHex-HA increased concomitantly with increasing MGlu/CHex unit contents in HA derivatives (Table S1).

161 This result might derive from the promotion of protonation by a proximity effect of

162 carboxyl groups, as described in our previous reports about poly(glycidol) derivatives or

163 dextran derivatives.[29,31,33] In addition, CHex-HA showed higher p*K*a value than that of

164 MGlu-HA having the same amount of carboxylated units (Table S1), indicating that the

165 more hydrophobic structure of CHex unit might promote the protonation of carboxyl

166 groups more than the MGlu unit did. These HA derivatives had p*K*a 5.4–6.7 (Table S1),

167 which corresponds to pH of early/late endosomes. Therefore, HA derivatives are

168 expected to change their protonation state in these intracellular acidic compartments.

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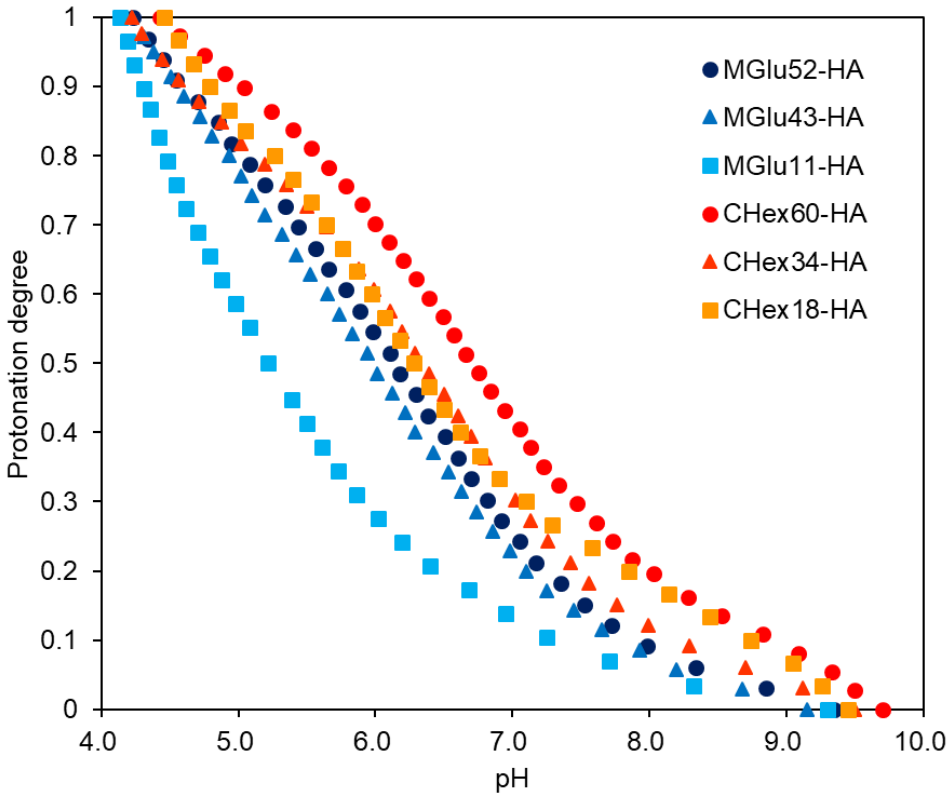


170

171 **Figure 4.** Acid-base titration curves for HA derivatives.

172

173 To elucidate the pH-sensitivity of HA derivatives, we evaluated the interaction
174 of HA derivatives with a liposomal membrane. First, HA derivatives were added to
175 pyranine-loaded egg yolk phosphatidylcholine (EYPC) liposomes at various pH. Then,
176 the release of pyranine was monitored (Figure 5). Addition of HA-$C_{10}$ to liposome
177 affected the pyranine leakage from liposomes at any pH only slightly, which indicates
178 that the interaction of HA-$C_{10}$ with liposomal membrane might be low, irrespective of
179 the environmental pH. In contrast, addition of $MGlu_{20}$-HA-$C_{10}$ and $CHex_{27}$-HA-$C_{10}$
180 suppressed the pyranine leakage at neutral pH compared with untreated liposomes
181 (Figures 5A and 5B), but gradually induced content release with decreasing pH (Figures
182 5C-E). These results suggest that the attachment of these polymers stabilized the
183 liposomal membrane but that the liposome membrane was destabilized gradually after
184 protonation of carboxyl groups in HA derivatives. However, the release percentage was
185 almost identical to that in the case of untreated liposomes, even after carboxyl groups
186 were protonated completely at pH 4 (Figure 4). For $MGlu_{20}$-HA-$C_{10}$ and $CHex_{27}$-HA-
187 $C_{10}$, the MGlu or CHex unit contents might be insufficient to destabilize the liposomal
188 membrane fully via hydrophobic interactions. $MGlu_{57}$-HA-$C_{10}$ and $CHex_{50}$-HA-$C_{10}$ also

189 only slightly affected the pyranine release at neutral pH (Figures 5A and 5B), but they

190 exhibited significant content release at acidic pH within 5 min (Figures 5C and 5D).

191 Considering the protonation curves of HA derivatives (Figure 4), more than 80% of

192 carboxyl groups might be protonated at pH 5.1 or 5.7, where significant content release

193 was induced respectively by $MGlu_{57}$-HA-$C_{10}$ or $CHex_{50}$-HA-$C_{10}$ (Figure 5E). After

194 protonation of most carboxyl groups, these polymers might become hydrophobic and

195 destabilize liposomal membrane within a few minutes. Results show that side chain

196 structures and their contents can control the interaction of HA derivatives with

197 liposomes. $MGlu_{20}$-HA-$C_{10}$ and $CHex_{27}$-HA-$C_{10}$ had p*K*a values of 5.4-6.4 (Table S1)

198 but could not induce content release, whereas $MGlu_{57}$-HA-$C_{10}$ or $CHex_{50}$-HA-$C_{10}$,

199 which have p*K*a values of 6.1-6.7 (Table S1), showed significant contents release.

200 Relatively high p*K*a values and high MGlu/CHex contents might be required for

201 efficient destabilization of liposomal membranes.

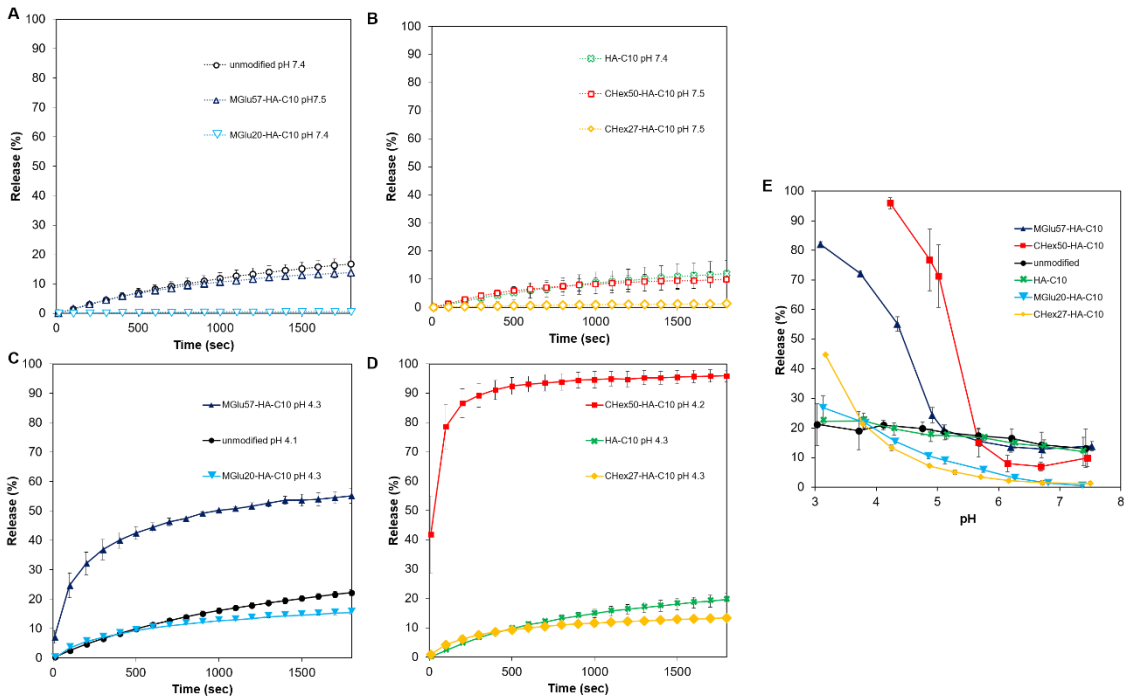


**Figure 5.** Time-dependence (A-D) and pH-dependence (E) of pyranine release from egg yolk phosphatidylcholine liposomes induced by various HA derivatives. Percent release at neutral pH (A, B) and acidic pH (C, D) and release of pyranine after 30 min-incubation (E) were shown. Polymer and lipid concentrations were 0.1 mg/mL and 2.0 × $10^{-5}$ M, respectively. Each point is the mean ± SD ($n$ = 3).

**Preparation of Hyaluronic Acid Derivative-Modified Liposomes.** HA derivative-modified liposomes were prepared using film hydration with a mixed thin film of EYPC and HA derivatives. The liposome suspension was extruded further through a polycarbonate membrane with 100 nm pore size. The size and zeta potential

of liposomes were investigated (Table 3 and Figure S1). All liposomes had narrow size distribution and average sizes were 130–200 nm, which is a suitable size for cellular uptake. HA derivative-modified liposomes showed more negative zeta potentials than those of unmodified liposomes or HA-$C_{10}$-modified liposomes, indicating the modification of carboxylated HA derivatives onto the liposome surface. Pyranine-loaded liposomes were used to evaluate pH-responsive content release for liposomes (Figure 6). As shown in Figures 6A and 6B, all liposomes retained their contents at neutral pH, whereas $MGlu_{57}$-HA-$C_{10}$-modified liposomes and $CHex_{50}$-HA-$C_{10}$-modified liposomes induced content release within 10 min at acidic pH (Figures 6C and 6D), similarly to the results depicted in Figure 5. Figure 6E represents the pH-dependence of pyranine release from liposomes. Liposomes modified with HA-$C_{10}$ or HA derivatives having low MGlu/CHex units showed only slight content release under experimental conditions. In contrast, liposomes modified with HA derivatives having high MGlu/CHex units showed remarkable content release. Especially, $CHex_{50}$-HA-$C_{10}$-modified liposomes induced content release at a higher-pH region than that of $MGlu_{57}$-HA-$C_{10}$-modified liposomes, probably because intracellular compartments have weakly acidic pH (endo/lysosome). Therefore, $CHex_{50}$-HA-$C_{10}$-modified liposome is expected to respond to intracellular pH after internalization to cells.

**Table 3. Particle Sizes and Zeta Potentials of Liposomes**

| Liposome | Size (nm) | PdI | Zeta potential (mV) |
|---|---|---|---|
| Unmodified | 193.5 ± 26.7 | 0.160 ± 0.107 | -4.6 ± 0.3 |
| HA-$C_{10}$ | 160.9 ± 14.7 | 0.179 ± 0.071 | -3.1 ± 0.3 |
| $MGlu_{20}$-HA-$C_{10}$ | 191.5 ± 3.8 | 0.256 ± 0.030 | -47.2 ± 0.4 |
| $MGlu_{57}$-HA-$C_{10}$ | 144.8 ± 8.5 | 0.282 ± 0.007 | -40.7 ± 2.1 |
| $CHex_{27}$-HA-$C_{10}$ | 128.6 ± 6.1 | 0.133 ± 0.029 | -46.7 ± 1.0 |
| $CHex_{50}$-HA-$C_{10}$ | 140.8 ± 8.5 | 0.148 ± 0.019 | -37.8 ± 1.9 |

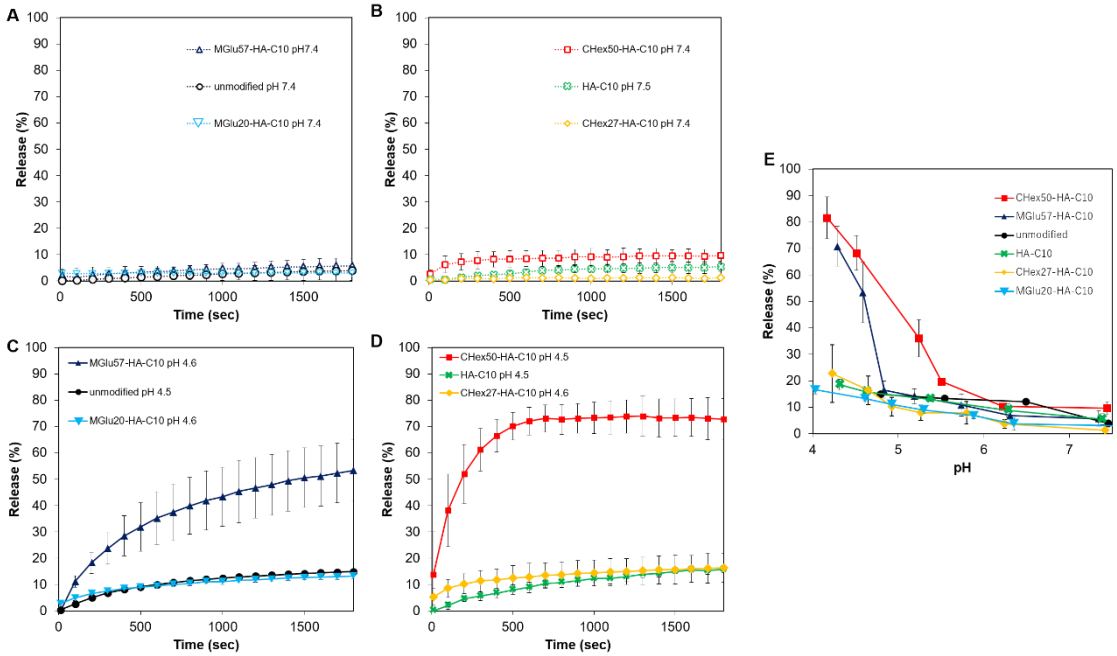


**Figure 6.** pH-Sensitive contents release behaviors of HA derivative-modified liposomes. Time courses at neutral pH (A, B) or at acidic pH (C, D) and pH-dependence after 30 min-incubation (E) of pyranine release from liposomes modified with or without HA derivatives were shown. Lipid concentrations were 2.0 × $10^{-5}$ M. Each

point is the mean ± SD ($n$ = 3).

**Interaction of Hyaluronic Acid Derivative-Modified Liposomes with Cells.** Next, interaction of HA derivative-modified liposomes with cells was investigated. CD44 protein on the cell surface might affect cellular association of HA derivative-modified liposomes. Therefore, CD44 expression in various cells was evaluated using fluorescence-labeled antibody for CD44. As shown in Figure S2, HeLa cells and colon26 cells showed higher expression of CD44 than either MCF-7 cells or NIH3T3 cells. Considering these results, HeLa cells and colon26 cells were used respectively as $CD44^{high}$ human-derived cells or mouse-derived cells, whereas MCF-7 cells and NIH3T3 cells were used respectively as $CD44^{low}$ human-derived cells or mouse-derived cell in the following experiments. After these cells were treated with DiI-labeled liposomes modified with or without HA derivatives, their cellular fluorescence intensity was ascertained using flow cytometric analysis. In the case of $CD44^{high}$ cells, modification of HA-$C_{10}$ to EYPC liposomes increased the cellular association of liposomes twice (Figures 7A and 7C), whereas cellular association of HA-$C_{10}$-modified liposomes was identical to that of unmodified liposomes in the case of $CD44^{low}$ cells (Figures 7B and 7D). These results indicate that HA on the liposome surface was

recognized by CD44 on HeLa cells or colon26 cells and that internalization of liposomes were promoted. MGlu-HA-$C_{10}$-modified liposomes exhibited almost equal or less cellular association than that of unmodified liposomes, irrespective of the CD44 expression on the cells (Figure 7). This equivalence suggests that introduction of MGlu groups to HA interferes with the interaction of HA derivatives with CD44 protein. Alternatively, the negative charge of MGlu-HA-$C_{10}$-modified liposomes (Table 3) suppressed cellular association of liposomes. By contrast, CHex-HA-$C_{10}$-modified liposomes showed much higher cellular uptake by $CD44^{high}$ cells alone than by unmodified liposomes (Figures 7A and 7C). Particularly, three-times-higher fluorescence intensity was observed from CHex-HA-$C_{10}$-modified liposome-treated HeLa cells than from HA-$C_{10}$-modified liposomes (Figure 7A). According to our earlier report, CHex unit-introduced dextran derivatives exhibited much higher cellular association than MGlu unit-introduced dextran because of its hydrophobic spacer structure.[33] However, in the case of HA derivatives, introduction of CHex units to HA showed enhanced cellular association to $CD44^{high}$ cells but no effect to $CD44^{low}$ cells (Figure 7). Hydrophobicity of CHex units might be suppressed by hydrophilic HA backbone compared with dextran. These results suggest that not hydrophobic interaction but interaction via CD44 contributes to the cellular association of CHex-HA-$C_{10}$-

modified liposomes.

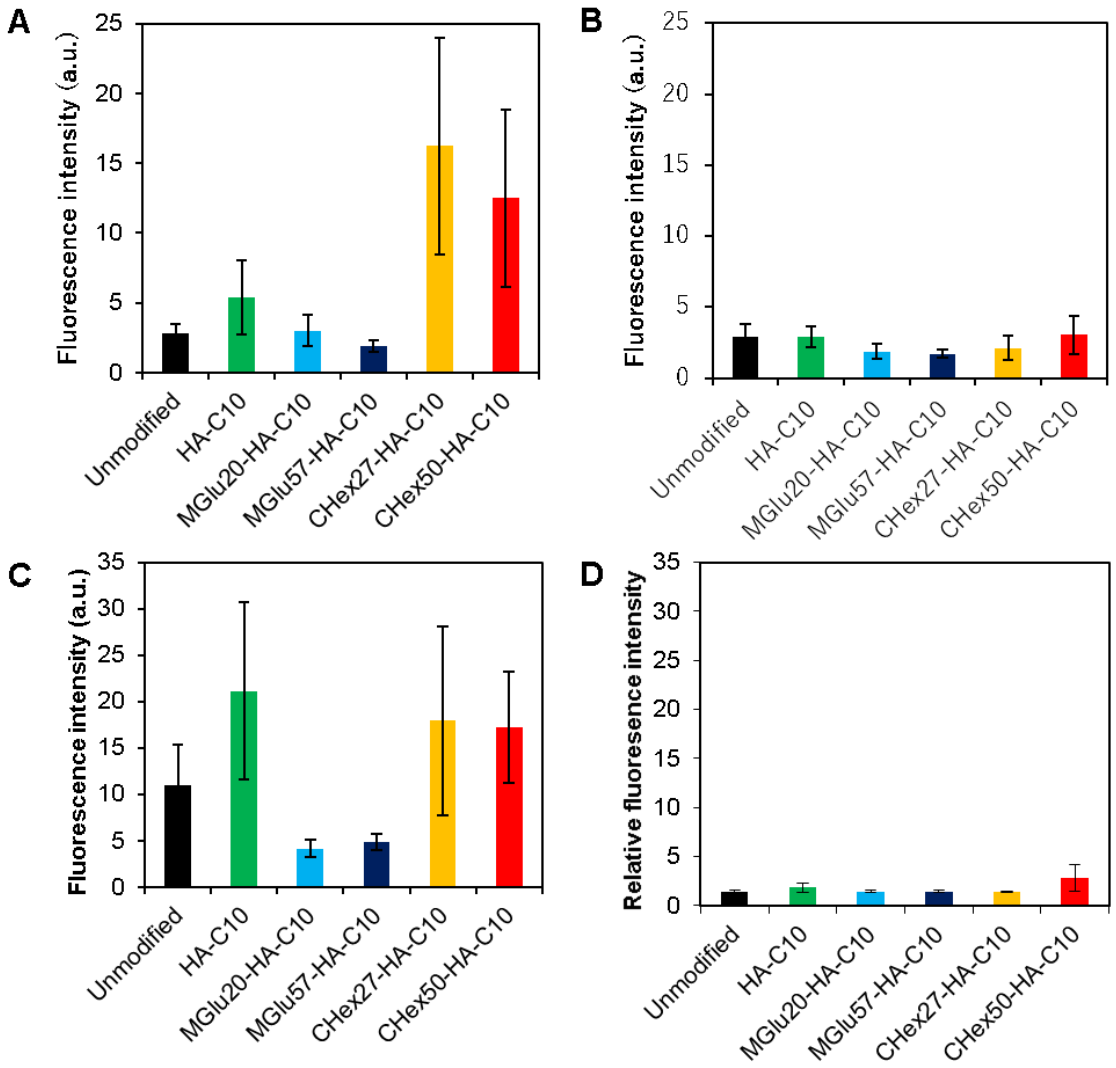


**Figure 7.** Fluorescence intensity for HeLa cells (A), MCF-7 cells (B), Colon26 cells (C), and NIH3T3 cells (D) treated with DiI-labeled EYPC liposomes modified with or without HA derivatives. Cells were incubated with liposomes (lipid concentration: 0.5 mM) for 4 h at 37 ºC in serum free medium. Cellular auto fluorescence was corrected.

To elucidate the uptake mechanism of HA derivative-modified liposomes, competition assay was applied (Figure 8). HeLa cells were treated with HA-$C_{10}$-modified liposomes or $CHex_{27}$-HA-$C_{10}$-modified liposome in the presence of various inhibitors. Cellular association of HA-$C_{10}$-modified liposomes was suppressed strongly in the presence of free HA, which indicates that HA-$C_{10}$-modified liposomes were surely taken up via CD44 on HeLa cells. Cellular association of $CHex_{27}$-HA-$C_{10}$-modified liposomes was also suppressed by free HA, indicating that these liposomes were also recognized by CD44 and that introduction of CHex units did not interfere with the interaction with CD44. For the case in which that $CHex_{27}$-HA without decyl groups were used as an inhibitor, cellular association of HA-$C_{10}$-modified liposomes decreased slightly but that of $CHex_{27}$-HA-$C_{10}$-modified liposome was reduced considerably. These results suggest that not only CD44 recognition but also CHex groups contribute to the cellular association of $CHex_{27}$-HA-$C_{10}$-modified liposomes. To evaluate the effects of CHex groups on cellular association, CHex group-introduced dextran ($CHex_{40}$-Dex)[33] was used for comparison. Results show that $CHex_{40}$-Dex did not affect the cellular association of $CHex_{27}$-HA-$C_{10}$-modified liposome to any great degree. The same tendency was obtained when using colon 26 cells (Figure S3). Therefore, both CHex group and HA backbone might be necessary to enhance the

cellular association of liposomes to CD44-expressing cells. CHex units might promote the binding of HA backbone to CD44 proteins via hydrophobic interaction or cyclohexyl structure itself, which is the same backbone structure with polysaccharides. These results suggest that the introduction of CHex groups to HA is effective for promoting liposome uptake by CD44-expressing cells.

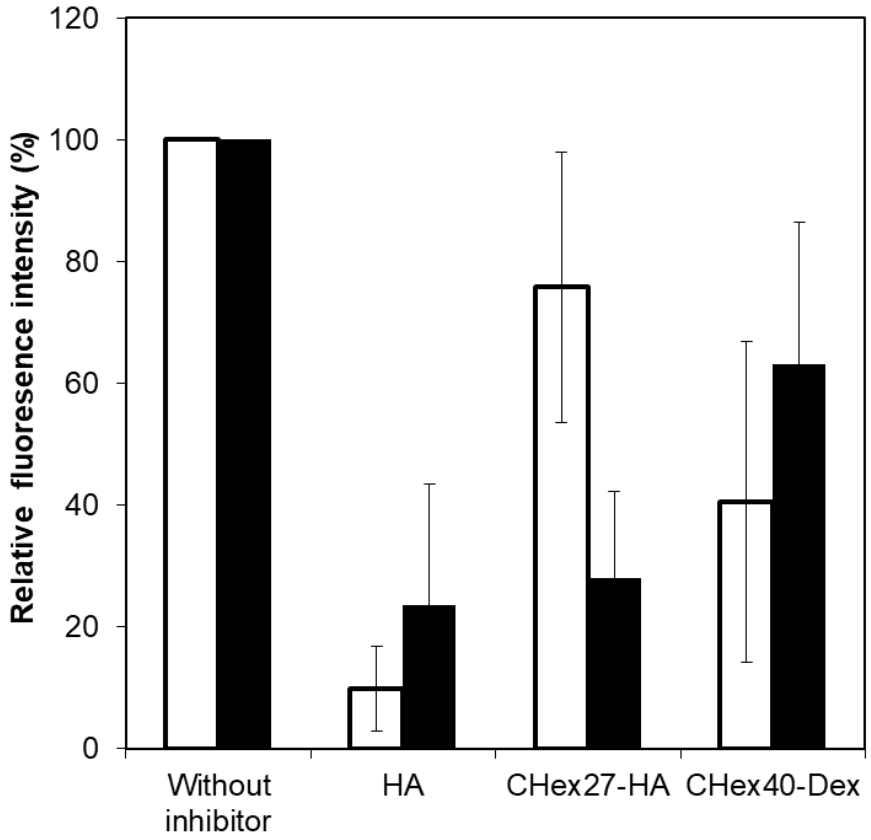


**Figure 8.** Inhibition of cellular association of HA-$C_{10}$-modified liposomes (open bars) and $CHex_{27}$-HA-$C_{10}$-modified liposomes (closed bars) by various inhibitors. HeLa cells were pre-incubated with various inhibitors for 1 h before liposome treatment. Relative

fluorescence intensity was calculated as the ratios of the amount of association in the presence of inhibitor to that in the absence of inhibitor.

**Intracellular Delivery of Anticancer Drugs by Hyaluronic Acid Derivative-Modified Liposomes.** Finally, the intracellular distribution of liposomes was examined. DiI-labeled liposomes were applied to HeLa cells. Then DiI fluorescence in the cells were detected using confocal laser scanning microscopy (Figure 9). For cells treated with unmodified liposomes and $MGlu_{57}$-HA-$C_{10}$-modified liposomes, most DiI fluorescence was observed from the periphery of cells (Figures 9A and 9C). In the cases of HA-$C_{10}$-modified liposomes and $CHex_{50}$-HA-$C_{10}$-modified liposomes, DiI fluorescence was found to exist not only at the cellular periphery but also inside of the cells as punctate fluorescence (Figures 9B and 9D). These results indicate that the recognition of HA-$C_{10}$-modified liposomes and $CHex_{50}$-HA-$C_{10}$-modified liposomes by CD44 better promote the internalization of liposomes than the recognition of other liposomes. Intracellular distribution of liposomes were further analyzed by staining of intracellular acidic compartments (endosomes and lysosomes) (Figure S4). Liposome-derived fluorescence dots inside of cells were overlapped with fluorescence derived from LysoTracker, which indicates that liposomes were trapped in endosome or

lysosomes after internalization to the cells.

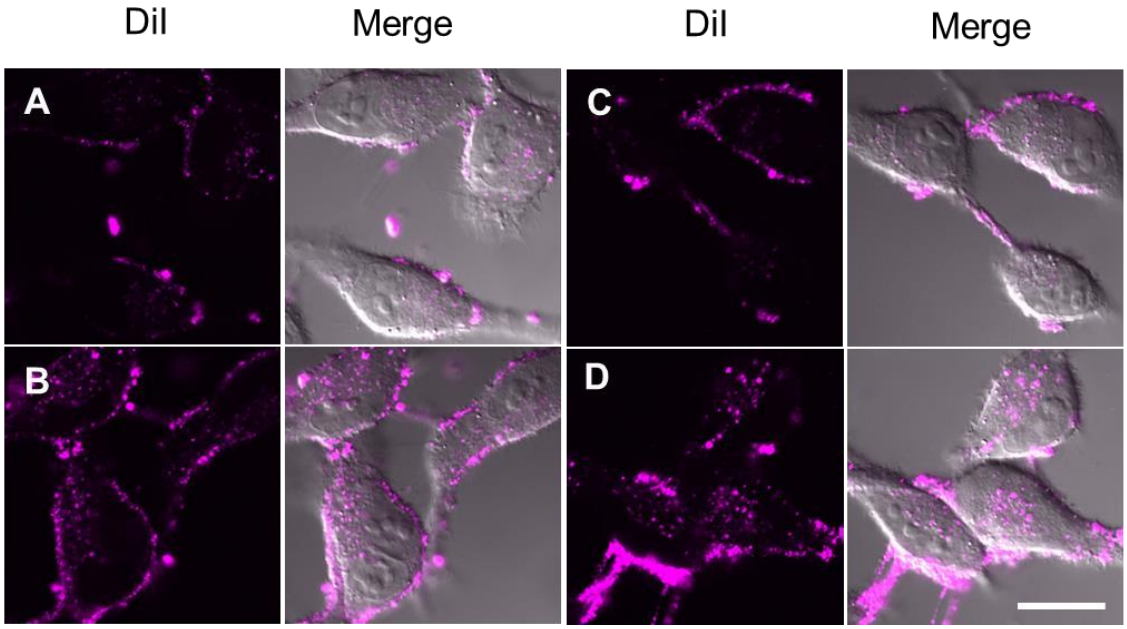


**Figure 9.** Confocal laser scanning microscopic (CLSM) images of HeLa cells treated with DiI-labeled EYPC liposomes modified without (A) or with HA-$C_{10}$ (B), $MGlu_{57}$-HA-$C_{10}$ (C) and $CHex_{50}$-HA-$C_{10}$ (D) for 4 h at 37 °C in serum-free medium. Bar represents 20 μm.

Intracellular drug delivery performance was investigated further. An anticancer drug (DOX) was encapsulated to liposomes using remote loading method.[43] The encapsulation efficiency of DOX was higher than 95% for unmodified liposomes and HA-$C_{10}$-modified liposomes, but 70–80% for $MGlu_{57}$-HA-$C_{10}$-modified liposomes and

$CHex_{50}$-HA-$C_{10}$-modified liposomes (Table S2). This result might derive from slight destabilization of liposomal membrane during the preparation of $MGlu_{57}$-HA-$C_{10}$- or $CHex_{50}$-HA-$C_{10}$-modified liposomes because the lipid membrane was dispersed in pH 6.0 aqueous solution for preparing pH gradient to encapsulate DOX. However, these liposomes retained nanometer size and negative zeta potentials after DOX loading (Figure S5 and Table S2). HeLa cells were incubated with DOX-loaded liposomes. Then the intracellular distribution of DOX was detected using CLSM (Figure 10). For cells treated with unmodified liposomes, DOX fluorescence was observed from the cell periphery as with Figure 9 (Figure 10A). In the case of HA-$C_{10}$-modified liposome-treated cells, dotted DOX fluorescence was observed from the periphery and inside of the cells (Figure 10B). This observation suggests that HA-$C_{10}$-modified liposomes were taken up by cells, but they were trapped in endo/lysosomes because of their pH-insensitive properties. By contrast, cells treated with HA derivative-modified liposomes showed punctate fluorescence within cells and diffused fluorescence in the nucleus (Figures 10C and 10D). Particularly, $CHex_{50}$-HA-$C_{10}$-modified liposomes exhibited high performance to deliver DOX into the nucleus. These results reflect the pH-responsive membrane disruptive ability (Figures 5 and 6) and high cellular association (Figure 7) of HA derivatives. Results show that HA-derivative-modified liposomes

were internalized to cells and were trapped in endo/lysosomes (Figure S4). Subsequently, HA derivatives might become hydrophobic responding to acidic pH in endo/lysosomes and might destabilize liposomes and endo/lysosomal membrane, leading to delivery of DOX into cytosol and accumulation of DOX into the nucleus, whereas DiI fluorescence was observed from the same location with endo/lysosomes because of its hydrophobic property (Figure S4). Compared with $MGlu_{57}$-HA-$C_{10}$-modified liposomes, $CHex_{50}$-HA-$C_{10}$-modified liposomes better induced content release in a high-pH region (Figure 6). Therefore, $CHex_{50}$-HA-$C_{10}$-modified liposomes might respond to pH decrease in endosomes with earlier timing and might show higher intracellular delivery performance than $MGlu_{57}$-HA-$C_{10}$-modified liposomes show. Consequently, liposomes modified with HA derivatives, particularly CHex group-introduced HA derivatives, might be effective as intracellular drug delivery carriers to CD44-expressing cancerous cells. To elucidate DDS performance of CHex group-introduced HA derivative-modified liposome, cytotoxicity against HeLa cells was investigated (Figure S6). Compared with DOX-loaded liposomes without HA derivatives, DOX-loaded $CHex_{50}$-HA-$C_{10}$-modified liposomes exhibited strong cytotoxicity, which is comparable with free DOX. These results reflect the efficient DOX delivery to nucleus by $CHex_{50}$-HA-$C_{10}$-modified liposomes (Figure 10D).

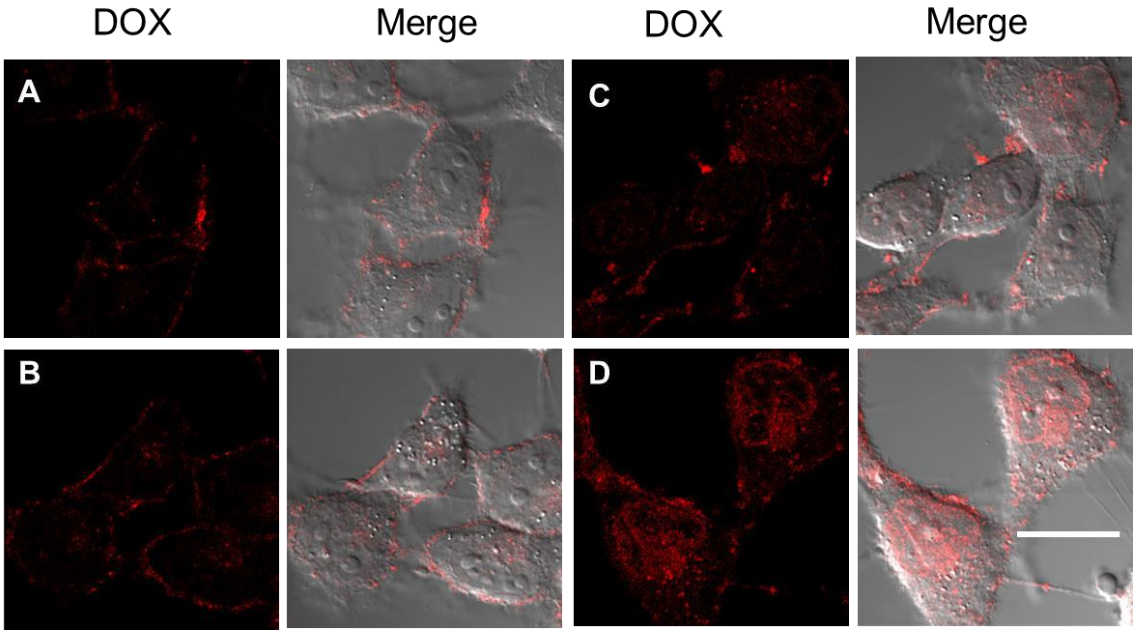


**Figure 10.** CLSM images of HeLa cells treated with DOX-loaded EYPC liposomes modified without (A) or with HA-$C_{10}$ (B), $MGlu_{57}$-HA-$C_{10}$ (C) and $CHex_{50}$-HA-$C_{10}$ (D) for 4 h at 37 °C in serum-free medium. Bar represents 20 μm.

## Conclusion

For this study, hyaluronic acid derivatives having pH-sensitivity and targeting ability were developed. MGlu unit-introduced or CHex unit-introduced hyaluronic acid derivatives induced lipid membrane disruptive activity in response to acidic pH. CHex unit-introduced hyaluronic acid derivative-modified liposomes exhibited high cellular association to highly CD44-expressing cells, whereas the same liposomes were taken up

only to a slight degree by CD44-low cells. These liposomes can deliver anticancer drugs to the interior of cells via pH-responsive membrane disruptive ability in endo/lysosomes. Therefore, pH-sensitive hyaluronic acid derivative-modified liposomes are promising as CD44-positive cell-specific intracellular drug delivery systems.

## Materials and Methods

**Materials.** Egg yolk phosphatidylcholine (EYPC) was kindly donated by NOF Co. (Tokyo, Japan). 3-Methylglutaric anhydride, 1,2-cyclohexanedicarboxylic anhydride and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Sigma (St. Louis, MO.). Hyaluronic acid potassium salt (Mw:8,000-50,000), 1-aminodecane, pyranine and Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). 4-Dimethylaminopyridine (DMAP) was obtained from nacalai tesque (Kyoto, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was from Life technologies. pH-sensitive dextran derivative ($CHex_{40}$-Dex) was prepared as previously reported.[33] Doxorubicin (DOX) was kindly donated by Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

**Synthesis of Hyaluronic Acid Derivatives.** Hyaluronic acid potassium salt was first converted to free acidic form by addition of hydrochloric acid (pH 2.0) and subsequently lyophilized. 3-Methyl-glutarylated hyaluronic acid (MGlu-HA) and 2-carboxycyclohexane-1-carboxylated hyaluronic acid (CHex-HA) were prepared by reaction of hyaluronic acid with 3-methylglutaric anhydride and 1,2-

411 cyclohexanedicarboxylic anhydride, respectively. A given amount of dimethyl sulfoxide

412 (DMSO) was added to hyaluronic acid and stirred at 90 °C for 30 min under argon

413 atmosphere. After cooling to room temperature, DMAP and dicarboxylic anhydrides

414 were added to DMSO solution of HA. The mixed solution was kept at a given

415 temperature for 24 h with stirring under argon atmosphere. Then, saturated sodium

416 hydrogen carbonate aqueous solution was added to the reaction mixture for

417 neutralization and the reaction mixture was dialyzed against water for 3 days. The

418 product was recovered by freeze-drying. $^{1}$H NMR for MGlu-HA（400 MHz,

419 $D_2O$+NaOD）: δ 0.9 (s, -CO-$CH_2$-CH(C*H₃*)-$CH_2$-), 1,9 – 2.3 (br, -CO-C*H₂*-C*H*($CH_3$)-

420 C*H₂*-, -NH-CO(C*H₃*)), 3.3 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6-4.8 (br, glucose

421 1*H*). $^{1}$H NMR for CHex-HA（400 MHz, $D_2O$+NaOD）: δ 1.3-2.2 (m, -cyclo-C*H₂*, -NH-

422 CO(C*H₃*)), 2.6-3.0 (m, cyclo-CH), 3.3 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 – 4.8

423 (br, glucose 1*H*).

424 As anchor moieties for fixation of MGlu-HA, CHex-HA and HA onto

425 liposome membranes, 1-aminodecane was combined with carboxyl groups of MGlu-

426 HA, CHex-HA and HA. Each polymer was dissolved in water around pH 7.4, and 1-

427 aminodecane (0.1 equiv. to hydroxyl group of polymer) was reacted to carboxyl groups

428 of the polymer using DMT-MM (0.1 equiv. to hydroxyl group of polymer) at room

temperature for 10-23 h with stirring. The obtained polymers were purified by dialysis in water. The compositions for polymers were estimated using $^1$H NMR. $^1$H NMR for MGlu-HA-$C_{10}$（400 MHz, $D_2O$+NaOD）: δ 0.9 (s, -CO-$CH_2$-CH(C*H₃*)-$CH_2$-, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.2 – 1.4 (br, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$), 1.9 – 2.3 (br, -CO-C*H₂*-C*H*($CH_3$)-C*H₂*-, -NH-CO(C*H₃*)), 2.5 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6-4.8 (br, glucose 1*H*). $^1$H NMR for CHex-HA-$C_{10}$（400 MHz, $D_2O$+NaOD）: δ 0.9 (br, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.3-2.2 (m, -cyclo-C*H₂*, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$, -NH-CO(C*H₃*)), 2.6-3.0 (m, cyclo-CH), 3.2 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 – 4.8 (br, glucose 1*H*). $^1$H NMR for HA-$C_{10}$（400 MHz, $D_2O$+NaOD）: δ 0.9 (br, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.2-1.4 (m, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$) 1.9-2.0 (s, -NH-CO(C*H₃*)), 2.5 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 – 4.8 (br, glucose 1*H*).

**Titration.** To 40 mL of an aqueous solution of each polymer (carboxylate concentration: $3.0 \times 10^{-4}$ M) was added an appropriate amount of 0.1 M NaOH solution to make pH 11.0. The titration was carried out by the stepwise addition of 0.01 M HCl solution at 25 °C, and pH and conductivity of the solution were monitored.

**Cell Culture.** Human cervix adenocarcinoma-derived HeLa cell, human breast cancer-derived MCF-7 cell and murine embryo fibroblast-derived NIH3T3 cell were grown in DMEM supplemented with 10% FBS (MP Biomedical, Inc.) and antibiotics at 37 °C. Murine colon adenocarcinoma-derived Colon-26 cell was grown in RPMI-1640 supplemented with 10% FBS and antibiotics at 37 °C.

**Preparation of Liposomes.** To a dry, thin membrane of EYPC (10 mg) was dispersed in aqueous 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4, 1.0 mL). The liposome suspension was further hydrated by freezing and thawing, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was purified with ultracentrifugation for 2 h at 4 °C twice. Polymer-modified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with polymers (lipids/polymer = 7/3, w/w). For encapsulation of DOX, dry membrane of a lipid mixture with polymers was dispersed in 300 mM ammonium sulfate (pH 6.0). The obtained liposome suspension was extruded through a polycarbonate membrane with a pore diameter of 100 nm and outer phase of liposome was substituted to PBS (pH 7.4) for formation of pH gradient. Then aqueous

DOX solution (10 mg/mL) was added to the liposome suspension at DOX/lipid (g/mol) ratio of 75 and the mixed solution was incubated for 1 h at 30 °C. Free DOX was removed from the liposome suspension by ultracentrifugation for 2 h at 4 °C. Encapsulation efficiency of DOX by liposomes was estimated from absorbance of DOX at 499 nm for the DOX-loaded liposomes dissolved in 0.3 M HCl (50%) –ethanol (50%) before and after purification with ultracentrifugation.

**Dynamic Light Scattering and Zeta Potential.** Diameters and zeta potentials of the liposomes (0.2 mM of lipid concentration) were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data was obtained as an average of more than three measurements on different samples.

**Release of Pyranine from Liposome.** Release of pyranine from liposome was measured as previously reported.[29-33,44] To a suspension of pyranine-loaded EYPC liposomes (lipid concentration $2.0\times10^{-5}$ M) in PBS of varying pHs was added varying HA derivatives dissolved in the same buffer. For evaluation of polymer-modified liposomes, pyranine-loaded liposomes (lipid concentration: $2.0 \times 10^{-5}$ M) were added to PBS of varying pH at 37 °C and fluorescence intensity (512 nm) of the mixed

suspension was followed with excitation at 416 nm using a spectrofluorometer (Jasco FP-6500, FP-6200). The percent release of pyranine from liposomes was defined as

Release (%) = $(F_t - F_i) / (F_f - F_i) \times 100$

where $F_i$ and $F_t$ mean the initial and intermediary fluorescence intensities of the liposome suspension, respectively. $F_f$ is the fluorescent intensity of the liposome suspension after the addition of TritonX-100 (final concentration: 0.1%).

**Immunostaining of CD44 on cells.** Cells ($2 \times 10^5$ cells) cultured for overnight in 35-mm glass-bottom dishes were washed with PBS, and then incubated in phenol red-free DMEM containing 10% FBS and 1% BSA (1 mL). PE-labeled anti-human CD44 antibody (10 μL, BD Biosciences) was added gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with PBS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.). For flow cytometric analysis, cells ($1 \times 10^6$ cells) were suspended in PBS containing 2% FBS (100 μL). Subsequently, PE-labeled anti-human CD44 antibody (10 μL, BD Biosciences) or anti-mouse CD44 antibody (0.2 μg/μL, 1 μL, BD Biosciences) was added gently to the cells and incubated for 30 min at 4 °C in the dark. The cells were washed with PBS containing 0.1% BSA three times.

Fluorescence intensity of these cells was determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.). Cellular auto fluorescence was subtracted from each data.

**Cellular Association of Liposomes and Inhibition Assay.** Liposomes containing DiI were prepared as described above except that a mixture of polymer and lipid containing DiI (0.1 mol%) was dispersed in PBS. Cells ($5 \times 10^4$ cells) cultured for overnight in 24-well plates were washed with PBS, and then incubated in serum-free DMEM (0.25 mL). The DiI-labeled liposomes (0.5 mM lipid concentration, 0.25 mL) were added gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with PBS three times. Fluorescence intensity of these cells was determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.). Cellular auto fluorescence for each cell was subtracted from each data. DiI fluorescence of each liposome was measured and cellular fluorescence shown in Figure 7 was corrected using liposomal fluorescence intensity. For inhibition assay, free HA (10 mg/mL), CHex-HA (1 mg/mL) and CHex-Dex (1 mg/mL) were pre-incubated to cells for an hour before the incubation of DiI-labeled liposomes for 4 h.

519 **Intracellular Behavior of Liposomes.** Cells ($2 \times 10^5$ cells) cultured for overnight in

520 35-mm glass-bottom dishes were washed with PBS, and then incubated in serum-free

521 DMEM (1 mL). The DiI-labeled liposomes (1 mM lipid concentration, 1 mL) or DOX-

522 loaded liposomes (4 μg/mL DOX concentration, 1 mL) were added gently to the cells

523 and incubated for 4 h at 37 °C. After the incubation, the cells were washed with PBS

524 three times. CLSM analysis of these cells was performed using LSM 5 EXCITER (Carl

525 Zeiss Co. Ltd.).

## Associated content

## Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

Figures and Tables showing the p*K*a of HA derivatives, size distribution of liposomes, CD44 expression in various cells, inhibition assay for colon 26 cells, intracellular distribution of liposomes, characterization of DOX-loaded liposomes and cytotoxicity of liposomes.

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## Notes

The authors declare no competing financial interest.

**Acknowledgments**

This work was supported by JSPS KAKENHI Grant number (15H03024) and the Kao Foundation for Arts and Sciences.

**Abbreviations**

HA, hyaluronic acid; DOX, doxorubicin; MGlu, 3-methyl glutarylated; CHex, 2-carboxycyclohexane-1-carboxylated; EYPC, egg yolk phosphatidylcholine; Dex, dextran; CLSM, confocal laser scanning microscopy

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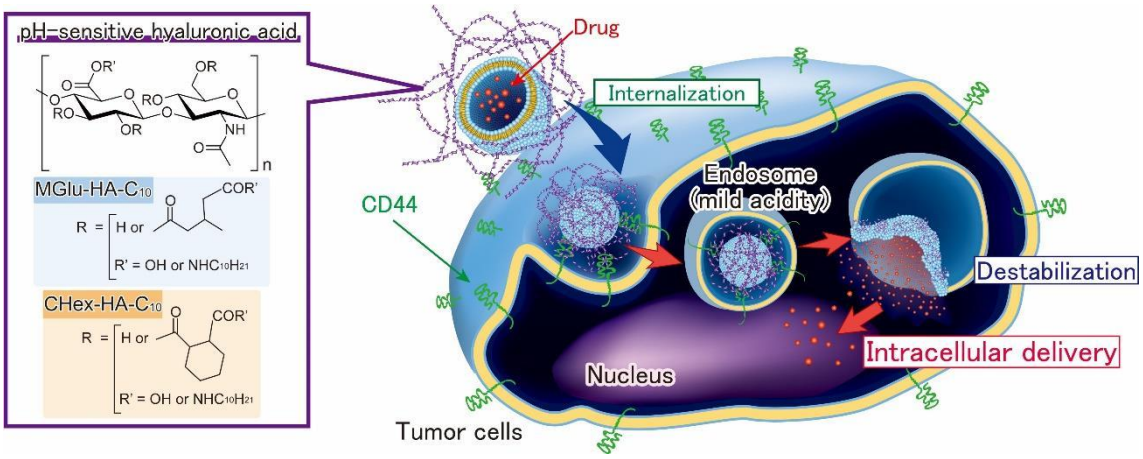

**Supporting information**

# Hyaluronic acid-based pH-sensitive polymer-modified liposomes for cell-specific intracellular drug delivery systems

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**Table S1. p*K*a of HA derivatives**

| Polymer | p*K*a |
|---|---|
| MGlu10-HA | 5.37 ± 0.16 |
| MGlu43-HA | 6.01 ± 0.04 |
| MGlu52-HA | 6.13 ± 0.02 |
| CHex18-HA | 6.33 ± 0.05 |
| CHex34-HA | 6.37 ± 0.03 |
| CHex60-HA | 6.70 ± 0.01 |

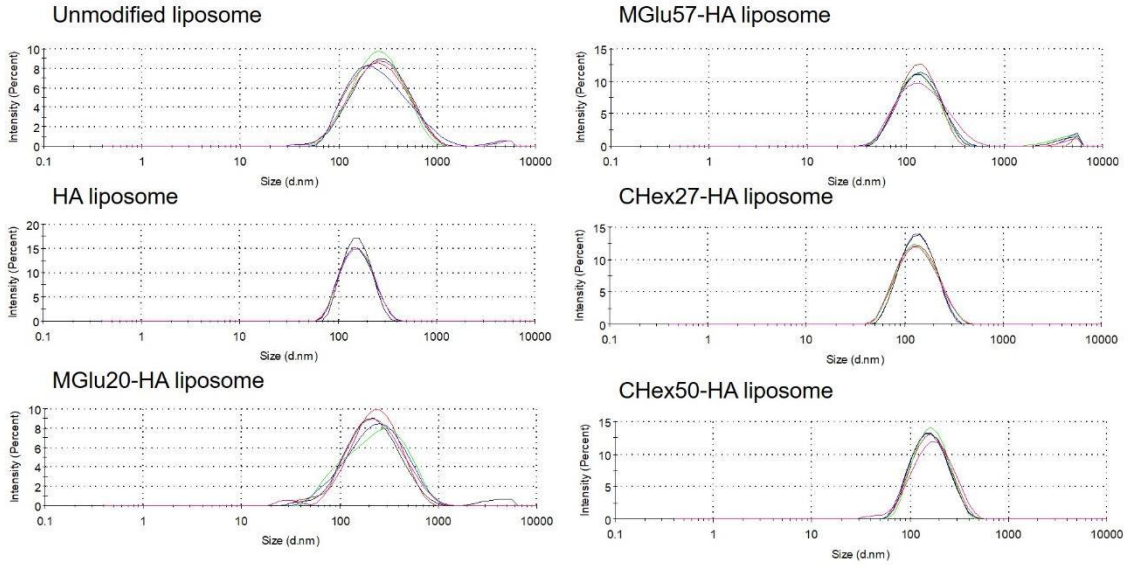



**Figure S1.** Size distribution of liposomes modified with or without HA derivatives.

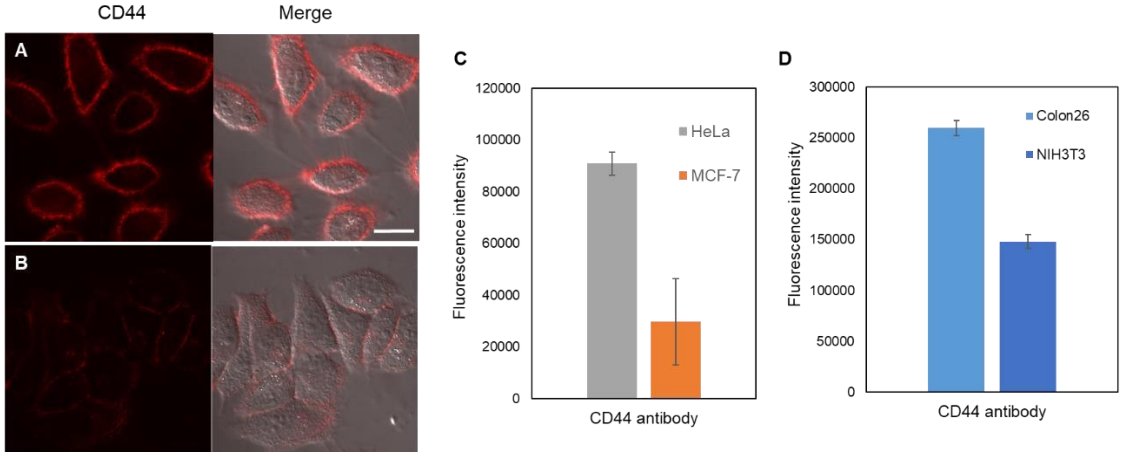
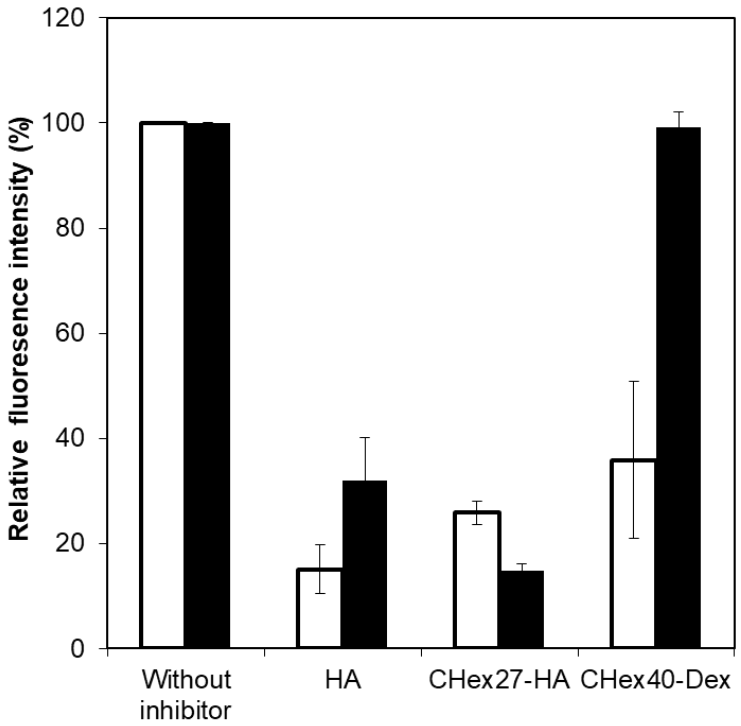



**Figure S2.** Confocal laser scanning microscopic (CLSM) images for HeLa cells (A) or MCF-7 (B) cells treated with anti-human PE-CD44 antibody for 4 h at 37 °C in phenol red-free 10% FBS medium. Scale bar represents 20 μm. (C, D) Fluorescence intensity for human cell lines treated with anti-human PE-CD44 antibody (C) or mouse cell treated with anti-mouse PE-CD44 antibody (D). Cellular auto fluorescence was corrected.

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724 **Figure S3.** Inhibition of cellular association of HA-$C_{10}$-modified liposomes (open bars)
725 and $CHex_{27}$-HA-$C_{10}$-modified liposomes (closed bars) by various inhibitors. Colon26
726 cells were pre-incubated with various inhibitors for 1 h before liposome treatment.
727 Relative fluorescence intensity was calculated as the ratios of the amount of association
728 in the presence of inhibitor to that in the absence of inhibitor.

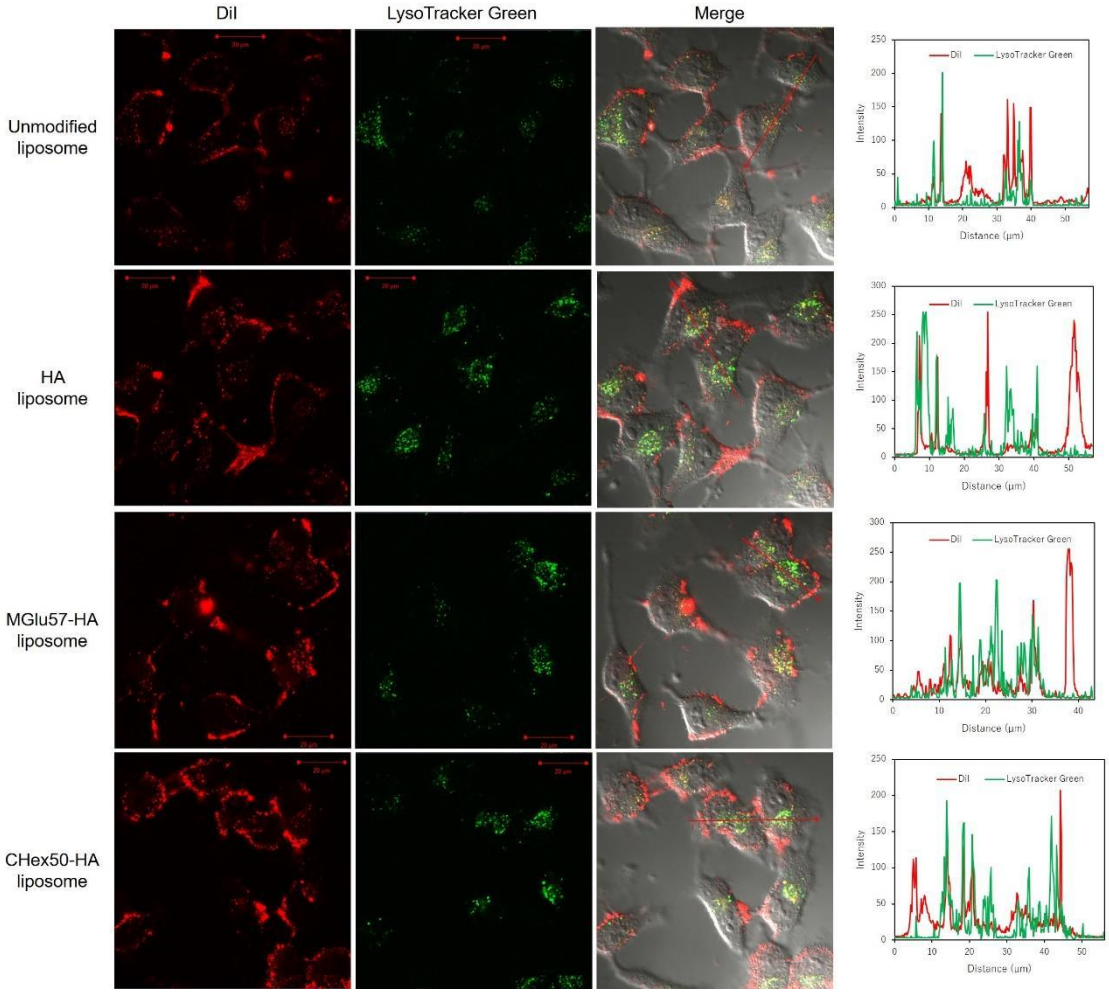


**Figure S4.** CLSM images of HeLa cells treated with DiI-labeled liposomes with or without HA derivatives for 4 h. Intracellular acidic organelle was stained by LysoTracker Green. Scale bars represent 20 μm. Line profiles of fluorescence intensity in merged images were also shown.

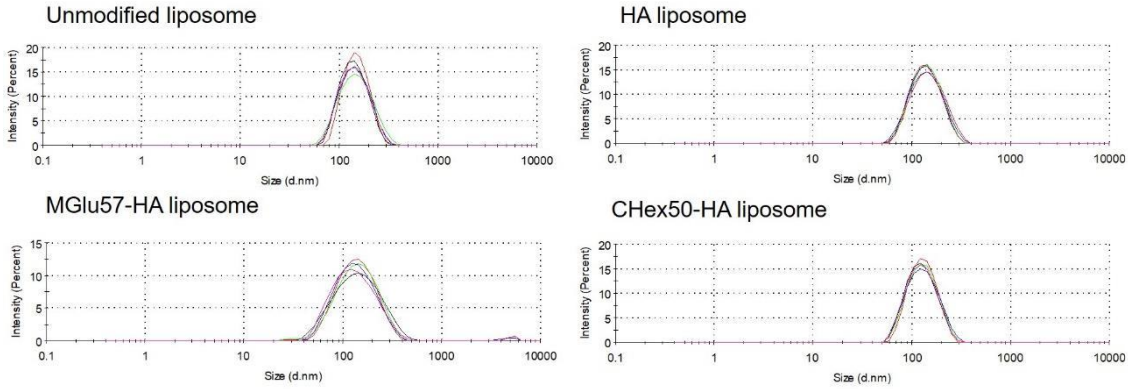


**Figure S5.** Size distribution of DOX-liposomes modified with or without HA derivatives.

737 **Table S2. Particle Sizes and Zeta Potentials of DOX-Loaded Liposomes**

| Liposome | Encapsulation efficiency (%) | Z-average (nm) | PdI | Zeta potential (mV) |
|---|---|---|---|---|
| Unmodified | 99.7 ± 3.5 | 138.4 ± 1.2 | 0.096 ± 0.023 | -8.2 ± 0.8 |
| HA-$C_{10}$ | 97.9 ± 9.6 | 137.3 ± 2.8 | 0.103 ± 0.011 | -8.8 ± 0.2 |
| $MGlu_{57}$-HA-$C_{10}$ | 73.3 ± 12.6 | 122.2 ± 2.0 | 0.167 ± 0.038 | -36.7 ± 1.3 |
| $CHex_{50}$-HA-$C_{10}$ | 79.5 ± 6.8 | 131.9 ± 12.2 | 0.153 ± 0.055 | -39.8 ± 0.1 |

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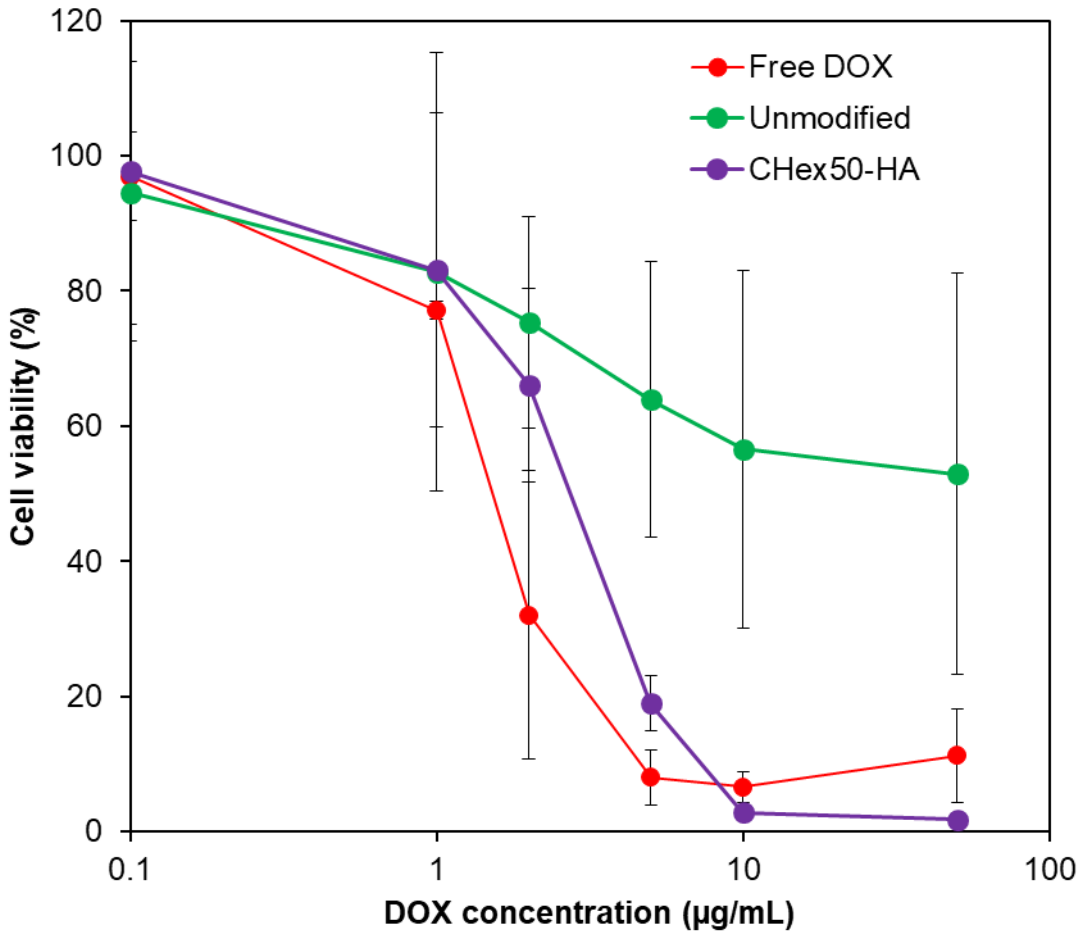


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740 **Figure S6.** Cell viability of HeLa cells treated with free DOX or DOX-loaded liposomes
741 modified with or without CHex50-HA as indicating DOX concentrations for 24 h.

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