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Notes

Lactosaminated *N*-Succinyl-chitosan: Preparation and Biodistribution into the Intestine, Bone, Lymph Nodes and Male Genital Organs after I.v. Administration

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Abstract: Reductive amination of *N*-succinyl-chitosan (**1**) and lactose using sodium cyanoborohydride in 1/15 M phosphate buffer (pH 6.0) for 6 d was suitable for the preparation of lactosaminated *N*-succinyl-chitosan (**2**). At 8, 24 and 48 h after i.v. administration of fluorescently labeled **1** (**1'**) or **2** (**2'**), Peyer's patch, mesenteric lymph nodes, testes, prostate, preputial grand, intestine (small intestine plus cecum), femoral muscle, backbone and peritoneum were taken. Peyer's patch and mesenteric lymph nodes were put together as lymph nodes. Over 10% of dose/g tissue was distributed to the prostate and lymph nodes at 48 h post-administration in both **1'** and **2'**. **2'** was easily distributed into not only the liver but also prostate, intestine, preputial gland and lymph nodes. Although galactose receptors are known to exist not only on the liver parenchymal cells but also on prostate and testes, the selective distribution of **2'** into the prostate and the testes were not observed clearly. This study suggested that **1** and **2** should have possibilities for both the prevention and cure of lymph node metastasis as drug carriers.

Keywords: lactosaminated *N*-succinyl-chitosan, *N*-succinyl-chitosan, biodistribution, lymph nodes, prostate, intestine.

Introduction

In the field of drug delivery, chitosan and its derivatives have received much attention as potential drug carriers.[1] *N*-Succinyl-chitosan (**1**) being one of chitosan derivatives is expected as a useful macromolecular drug carrier showing long-term retention in the body, especially systemic circulation.[2] In contrast, lactosaminated *N*-succinyl-chitosan (**2**) synthesized by reductive amination using sodium cyanoborohydride was mainly distributed to the liver after i.v. administration[3] because the liver parenchymal cells have asialoglycoprotein receptors which specifically recognize the galactose moiety;[4,5] however, it was debatable whether the reaction condition described in the previous report was the best one. Moreover, galactose receptors are known to exist not only on the liver parenchymal cells but also on prostate and testes.[6] The distributions into these tissues were not reported at other carriers with galactose residue. Further, the distribution of carrier into backbone is meaningful as an index of side effects in the case of anticancer drugs, and that of carrier into the lymph systems might prevent cancer metastasis. Thus, in the present study, the degree of lactosamination of **2** was checked at various reaction conditions, and the biodistribution characteristics of **1** and **2** into the tissues, especially intestine, lymph nodes, bone and male genital organs were investigated.

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Experimental

Materials, Animals and Statistical Analysis. *N*-Succinyl-chitosan (**1**) sodium salt (MW: 3.4×10^5 g/mole; succinylation degree: 0.81) was a gift from Katakura Chikkarin Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of reagent quality.

Male C57BL/6 mice (6 weeks old, specific pathogen-free) weighing ca. 20 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All experiments in the present study conformed to the Guidelines for Animal Experimentation of Hoshi University.

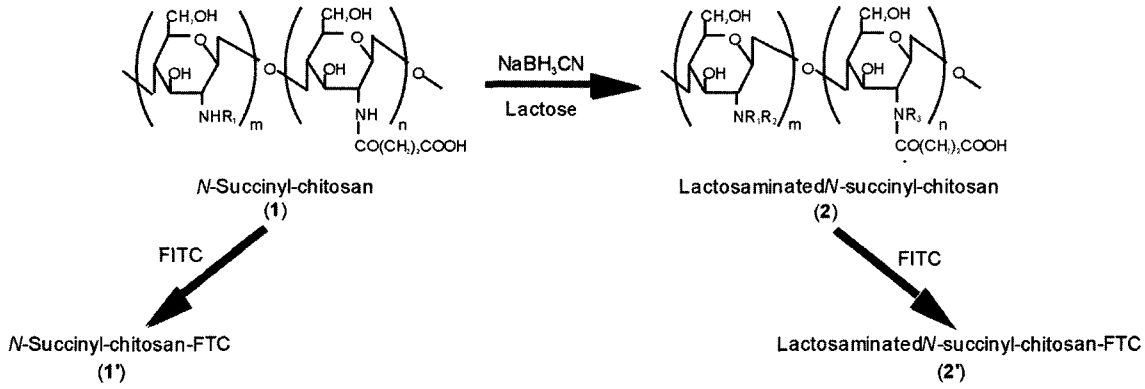
Students *t*-test or the one-way ANOVA was used for statistical comparison using the StatView computer program (StatView software, SAS Institute Inc., USA). Differences were considered significant when the *p*-value was less than 0.05.

Lactosamination of *N*-Succinyl-chitosan. The synthetic approach of **2** is briefly summarised in Figure 1. **1** (100 mg) was dissolved in 6 mL of 1/15 M phosphate buffer (pH 6.0, 7.0 or 8.0), and then 6 mL of methanol was added. To the solution was added lactose (640 mg) dissolved in 2 mL of the same buffer. $NaBH_3CN$ (320 mg) dissolved in 2 mL of the same buffer was added to the solution, followed by

stirring at room temperature at 400 rpm for several days. At predetermined time points, aliquots of 5 mL of the reaction mixture were collected and evaporated. The following procedures were performed as described in the previous report.[3] Further, **2** was prepared in the same manner using 1/15 M phosphate buffer (pH 6.0) but with addition of various amounts of lactose. The content of lactosamine residues was determined by the anthrone-sulfuric acid method.[7] The standard calibration curve was made using galactose and lactose every time.

Biodistribution Studies. Fluorescently labeled **1** and **2**, termed here as **1'** and **2'**, were prepared according to the method reported previously.[2] The fluorescein thiocarbamyl (FTC) content of **1'** and **2'**, examined pursuant to the previous report,[2] were 2.3 and 1.5% (w/w), respectively.

1' was administered to mice at the dose of 0.2 mg (0.2 mL) by injection into the tail vein. Mice were euthanized at 8, 24 and 48 h post-injection, Peyer's patch, mesenteric lymph nodes, testes, prostate, preputial grand, intestine (small intestine plus cecum), femoral muscle, backbone and peritoneum were enucleated. The contents in intestinal tract were washed out with phosphate buffered saline of pH 7.4 (PBS) before measurement. Peyer's patch and mesenteric lymph nodes were put together and investigated as lymph nodes. The following procedures were carried out as described before.[2] As for backbone, the homogenization was performed after ultrasonication at 28 kHz for 20 min. Similarly, the biodistribution of **2'** was examined as stated above. The concentration was corrected by the recovery ratio. The recovery ratios of **1'** or **2'** from each tissue homogenates are shown in the following: Recovery ratio of **1'**: 75.3% (lymph node), 100.0% (testes), 69.6% (preputial grand), 74.5% (intestine), 44.6% (backbone), 80.4% (peritoneum), 102.0% (femoral muscle) and 80.6% (prostate); Recovery ratio of **2'**: 68.4% (lymph node), 102.1% (testes), 87.5% (preputial grand), 47.2% (intestine), 55.4% (backbone), 94.5% (peritoneum), 101.4% (femoral muscle) and 74.7% (prostate). The difference of the individual data was very little.



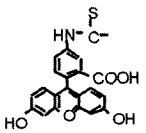
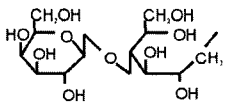
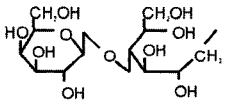
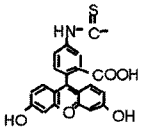
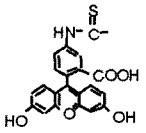
Substitution	1	1'	2	2'
R_1	H			
R_2	——	——	H	H *or* 
R_3	——	——	H	H *or* 

Figure 1. Chemical structures and synthetic approach for polymers (**1**, **1'**, **2** and **2'**) used in this study. Degrees of succinylation and deacetylation of **1** were ca. 0.81 and 1.00, respectively. Degree of lactosamination of **2** used in vivo study was ca. 0.30 (w/w). FTC contents of **1'** and **2'** were ca. 2.3 and 1.5% (w/w), respectively. The detailed substituent information of **2** was described in the text (discussion section).

Results

Lactosamination of *N*-Succinyl-chitosan. Figure 2 shows the relationships between the lactosamine content of **2** and the pH of the reaction mixture or reaction time. The reaction progressed slowly at pH 7.0 and 8.0, and the lactosamine content reached 5.0 and 4.2% (w/w) even for 6 d reaction, respectively. In contrast, the lactosamine content increased more rapidly at pH 6.0 with reaction time, and reached ca. 18.9% (w/w) at 6 d. Buffer below pH 6.0 were not employed because the use of acidic medium with low pH for a long time might cause the degradation of MW.[8] When the amounts of lactose added were changed at pH 6.0, the lactosamine content varied as shown in Figure 3. The lactosamine content increased with addition of greater amounts, and reached 30.3% (w/w) when 640 mg of lactose was added (the amount of **1** was 50 mg). However, the content decreased when more than 640 mg of lactose was added. Therefore, in the following experiment, **2** was produced at a lactose/**1** ratio of 12.8 in 1/15 M phosphate buffer (pH 6.0) for 6 d. The degree of lactosamine in **2** determined by the anthrone-sulfuric acid method was consistent with the value calculated from the C/N ratio in elemental analysis (data not shown).

Biodistribution Studies. Figure 4 shows the biodistribution of **1'** and **2'** after i.v. administration. The biodistribution of **1'** into the liver, kidney and spleen was below 5% of dose/g tissue as reported earlier.[2] Over 10% of dose/g tissue was transferred to the prostate and lymph nodes at 48 h post-administration. In contrast, **2'** appeared to be easily distributed into prostate, intestine, preputial gland and lymph nodes as well as the liver. The distribution of **2'** into intestine and lymph nodes were higher than that of **1'**.

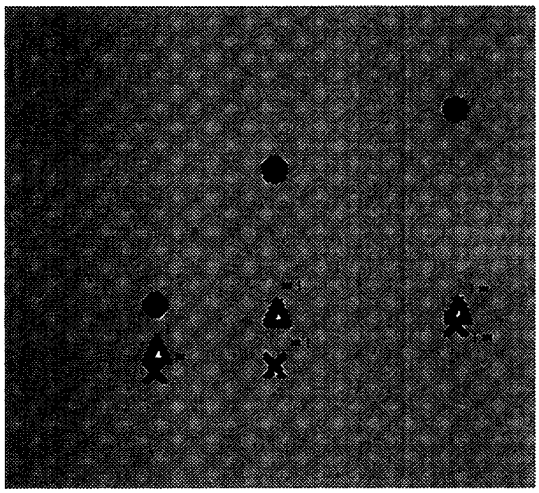


Figure 2. Effect of pH of 1/15 M phosphate buffer and reaction time on lactosamine content in **2**. Closed circles, open triangles and crosses represent pH 6.0, 7.0 and 8.0, respectively. Each point represents the mean ± discrepancy/$\sqrt{2}$ (n = 2). *: $p < 0.05$, **: $p < 0.01$ vs. pH 6.0.

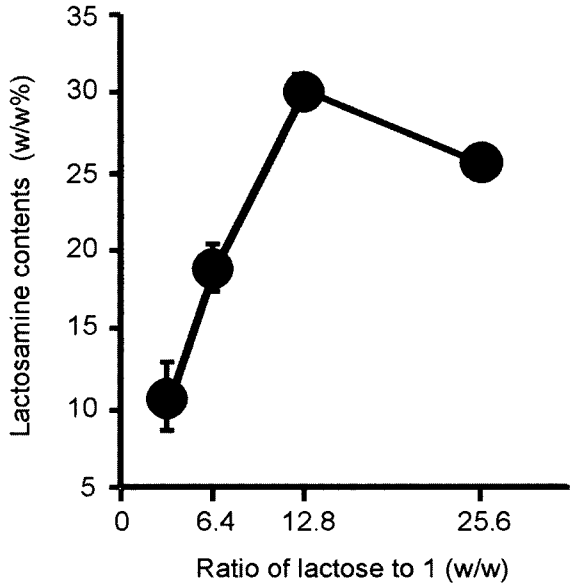


Figure 3. Effect of lactose amount on lactosamine content in **2**. When the lactose/**1** ratio is 12.8, lactose amount is 640 mg (Suc amount is 50 mg). The 1/15 M phosphate buffer (pH 6.0) was used. Each point represents the mean ± discrepancy/$\sqrt{2}$ ($n = 2$).

Discussion

$NaBH_3CN$ can reduce a wide variety of organic functional groups such as aldehyde, ketone and imine.[9,10] Schwartz and Gray synthesized disaccharide-loaded glycoproteins by reductive amination of a protein and reducing disaccharide using $NaBH_3CN$, and reported that the reaction proceeded somewhat slowly at weakly acidic and neutral pH and was faster under weakly alkaline conditions.[10] However, in the present study, the reaction progressed slowly in neutral and alkaline buffer, and the reductive amination proceeded much faster in acidic buffer. Our results were consistent with those reported by Borch and collaborators, indicating that the reductive amination proceeded well at pH 6-7 where Schiff base formation was optimal.[9] Schwartz and Gray suggested that Schiff base formation was not the rate-limiting step in the reaction process,[10] but the rate-limiting process in our reaction appeared to be based on Schiff base formation. These differences may have been due to differences in reaction conditions and the materials used. The detailed mechanism of this reaction remains to be resolved in future studies. As shown in Figure 3, more lactose could be introduced to **1** by increasing the amount of lactose added, but that amount introduced was lower than the maximum level (30% (w/w) at 12.8-fold) with addition of more than 12.8-fold lactose. The introduction of lactose was therefore considered to be saturated at 12.8-fold lactose under these conditions, and further addition of lactose seemed to reduce the efficiency of the reaction. The reason for this is not yet clear. The maximum level of 30% (w/w) corresponded to about 18%

(mol/sugar unit) substitution, suggesting that almost all the primary amino groups in **1** underwent reductive amination and the newly produced secondary amines scarcely underwent further reductive amination. **2** was considered to possess many carboxyl groups (81% (mol/sugar unit)), galactose (13% (mol/sugar unit)), secondary amines (18% (mol/sugar unit)) and few primary amino groups.

It was reported previously that the bond between **1** and fluorescein moiety was stable in the body[11] and that **2'** was little quenched in vitro for 48 h.[3] Therefore, in this experiment, the stability influence and quenching effect of both fluorescent-carriers were considered to be negligible. The detection limit was 0.05 ng/mL for fluorescein isothiocyanate.

The distribution of drug-polymer conjugates is known to depend on that of carrier (polymer) in the case of the low molecular weight drugs without organ-specificity.[12] This study clarified that **1'** was not only retained in the systemic circulation but also distributed to the prostate and lymph nodes (Figure 4(A)). For **2'**, nearly 30% of dose/g tissue was distributed to the liver.[3] In this study, the concentration of **2'** distributed was relatively high at the prostate, intestine, preputial gland and lymph nodes as compared with the others tested; however, the distribution to the prostate and the testes was not marked (Figure 4(B)). The distribution of **2'** into the prostate and the testes might not be selective because the distribution into the prostate was observed not only **2'** but also **1'**. From these findings, one may say that the galactose receptors existing on the prostate and testes are distinct from those located on the liver parencymal cells. The distribution of **2'** into the muscle was low similar to galactosylated poly(L-lysine).[13] The concentration of **2'** distributed to the intestine was above 10% of dose/g tissue, which was higher than that of **1'** distributed. For lactosaminated human serum albumin, the distribution to the intestine was at a similar or lower level compared with that to the spleen and kidney, and was markedly lower than that to the liver.[14] However, intestine was also affected as well as the liver in the case that a large amount of carrier was used.[15] As for the distribution into the backbone, **1'** showed around 4% of dose and **2'** did about 2% of dose (data not shown). Most anticancer drugs do damage to bone marrow which is an important tissue as the hematopoietic system. As a consequence, they often invite myelosuppression. As for **1** and **2**, their small distribution into the backbone may lead to suppression of such side effect. Further, **1**- and **2**-drug conjugates are supposed to be preventive and curative against lymph node metastasis

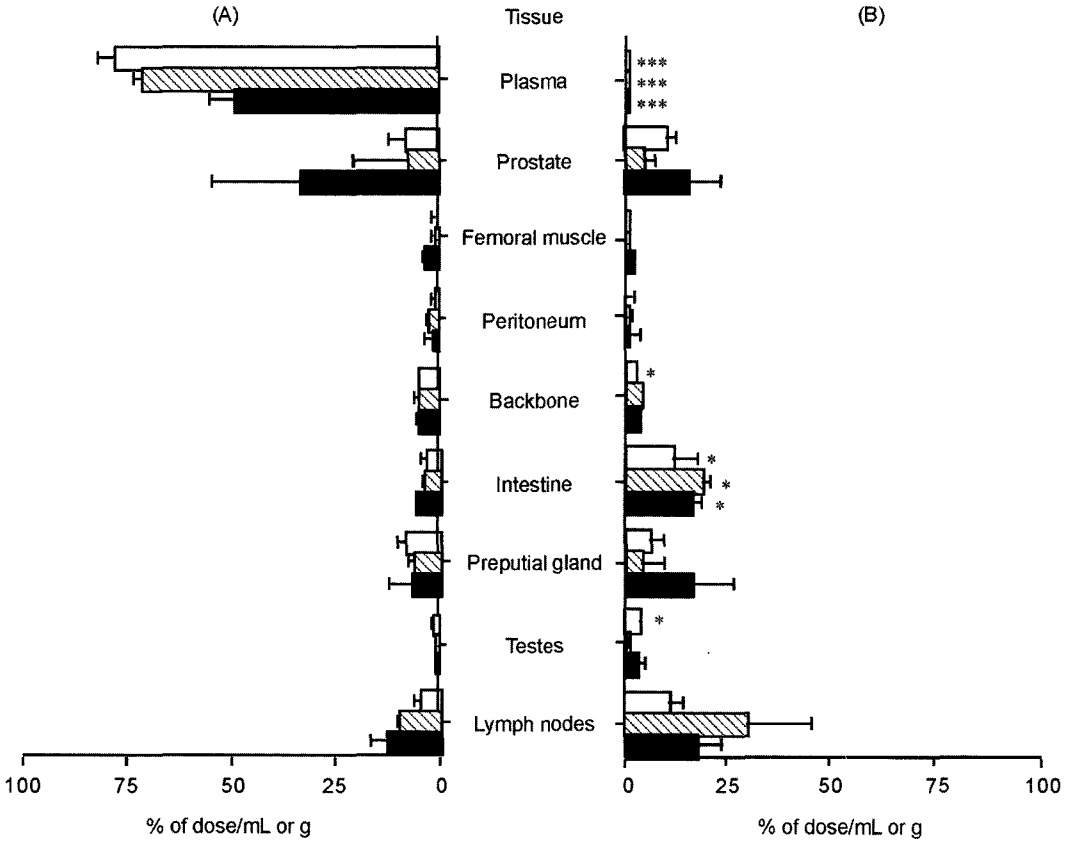


Figure 4. Biodistribution of **1'** (A) and **2'** (B) after i.v. administration at a dose of 0.2 mg per mouse. Open, hatched and closed columns indicate 8, 24 and 48 h post-administration, respectively. X-axis shows the concentration of **1'** or **2'** distributed into each tissue. Each column represents the mean ± standard deviation (n = 5). *: $p < 0.05$, ***: $p < 0.001$ vs. **1'**.

because **1'** and **2'** were transferred to the lymph systems at a fair concentration. Even the physical mixtures of **1** or **2** and MMC were also more effective for M5076 liver metastatic tumor than MMC alone,[16] which might be related to the distribution of these carriers to the lymph systems because some chitin and chitosan derivatives tend to stimulate the immune system including macrophages.[17,18]

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