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Possibility of application of quaternary chitosan having pendant galactose residues as gene delivery tool

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Since chitosan is a cationic natural polysaccharide having the formation ability of a polyelectrolyte complex with DNA, it is expected to be used as a carrier of DNA in gene delivery systems. So, in order to achieve an efficient gene delivery via receptor-mediated endocytosis, the synthesis of a novel polycationic polysaccharide derivative having recognizable saccharide residues, *N,N,N*-trimethyl(TM)–chitosan/galactose conjugate, was performed. The formation of a polyelectrolyte complex with DNA and the cellular recognition ability of TM–chitosan/galactose conjugate were tested, and then the possibility of its application as a gene delivery tool was investigated. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

It is well known that some kinds of saccharide play important roles in biological recognition on cellular surfaces. So, they are expected to be applied to cellular recognition devices. Since many polysaccharides are biodegradable and biocompatible, they are useful as biomedical materials. Recently, gene therapy by delivering genes to target cells has became a topic in medical chemistry. Gene-transfer methods that adopt natural receptor-mediated endocytosis pathways for the delivery of DNA into target cells have been developed. Ligands for cellular receptors, such as transferrin (Cotten *et al.*, 1990; Wagner *et al.*, 1990), viral proteins (Cotten *et al.*, 1992), insulin bound to albumin (Huckett *et al.*, 1990), and asialoorosomucoid (Wu *et al.*, 1987, 1989) have been used for import of the DNA molecule. For this purpose, these ligands have been conjugated to DNA-binding compounds, such as a polycation and an intercalating agent. Incubating DNA with these protein conjugates generates ligand-coated DNA, which can bind receptors on the cellular surface and is subsequently internalized. Chitosan is a cationic natural polysaccharide which can form polyelectrolyte complexes with DNA. So, it is expected to be used as a carrier of DNA in gene delivery systems.

In order to achieve an efficient gene delivery via receptor-mediated endocytosis, the present paper is concerned with the synthesis of novel polycationic polysaccharide derivatives having recognizable saccharide residues, *N,N,N*-trimethyl(TM)–chitosan/galactose conjugate, its polyelectrolyte complex formation with DNA, and the fundamental studies of cellular recognition ability and gene delivery.

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EXPERIMENTAL

Materials

Chitosan was provided by Kimitsu Chemical Industries, Ltd. pSVluc and Maker 1 (Lambda phage DNA/*Hind III* digest) was purchased from Wako Pure Chemical Industry. Fluorescein isothiocyanate (FITC) labeled lectin from *Abrus Precatorius* agglutinin (APA) was purchased from Sigma Chemical Company and pSVβGal was purchased from Promega. Organic solvents were purified by usual distillation. Other materials were commercial grade and used without further purification. HepG2 and Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku Co.) containing 10% heat-inactivated fetal calf serum (FCS) (Hazeleton Biologics, Inc.), 4 mM of L-glutamine, 36 mM of sodium bicarbonate and 60 mg/l of kanamycin at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells used in each test were cultured in 96-well flat-bottomed plates (Corning Laboratory Sciences Company) in 200 μl of culture medium.

Synthesis of TM–chitosan

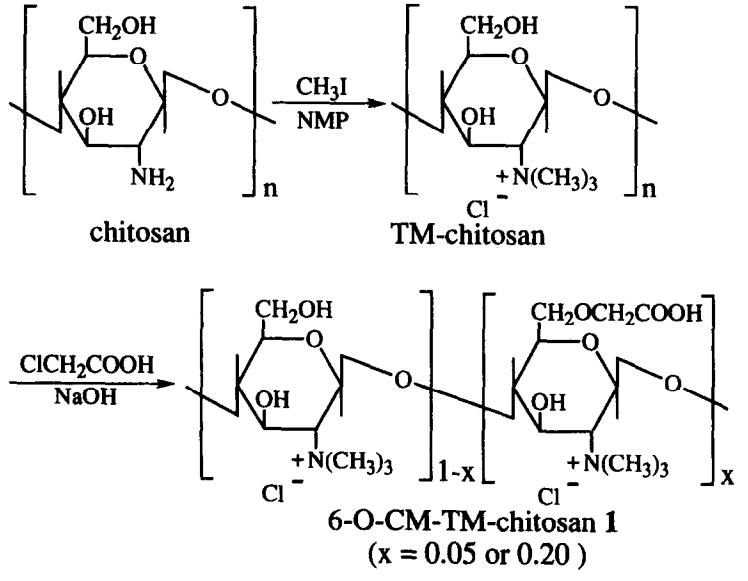
The synthesis of the TM–chitosan unit was performed through two reaction steps shown in Scheme 1. TM–chitosan chloride was prepared from chitosan according to the method described by Domard *et al.* (1986). By changing the reaction condition, the ratio of monochloroacetic acid to TM–chitosan in the reaction, 6-*O*-carboxymethyl(CM)-chitosan (**1**) having two degrees of substitution of carboxymethyl group per sugar unit (DCM) was synthesized.

Synthesis of galactose residue

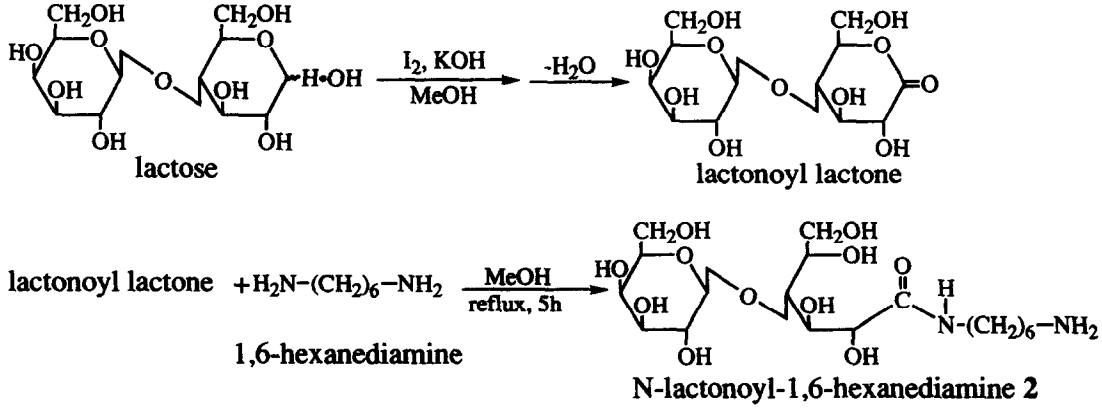
The synthesis of the galactose residue unit was performed through three reaction steps shown in Scheme 2. Lactonoyl lactone was prepared from lactose according to the method described by Kobayashi *et al.* (1985). *N*-Lactonoyl-1,6-hexanediamine (**2**) was prepared according to the following method. 1,6-Hexanediamine (0.17 g, 1.5 mmol) and lactonoyl lactone (0.51 g, 1.5 mmol) were dissolved in methanol (10 ml), and refluxed for 5 h. This reaction mixture was evaporated and then reprecipitated from water and ethanol. *N*-Lactonoyl-1,6-hexanediamine was isolated by ion-exchange chromatography (SP-Sephadex, conditioning: water, eluent: 0.1 M ammonium bicarbonate), and freeze-dried to be afforded as powder, 0.35 g, 0.77 mmol (51%).

Synthesis of TM–chitosan/galactose conjugate

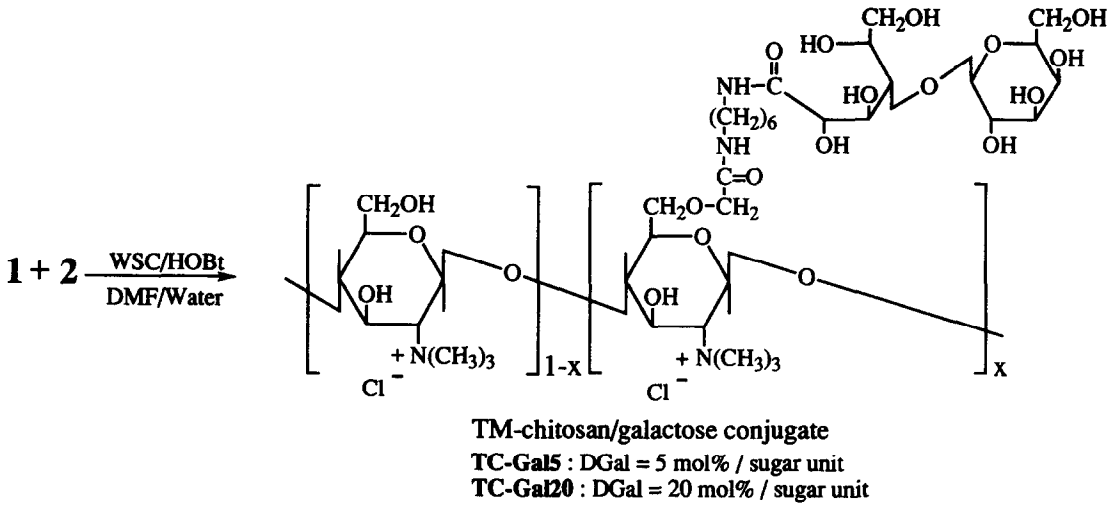
The synthesis of the TM–chitosan/galactose conjugate was performed as shown in Scheme 3. In the case of the TM–chitosan/galactose conjugate (degree of substitution of galactose group per sugar unit (DGal) = 5 mol%/sugar unit: TC-Gal5), **1** (DCM = 5 mol%/sugar unit, 0.2 g, 842 μmol/sugar, 42 μmol/COOH) was dissolved in DMF/water (1:1 v/v) and added to WSC (15 mg, 79 μmol) and HOBt (11 mg, 82 μmol) at 0°C for 1 h. Then, **2** was added and stirred at room temperature overnight. After this reaction mixture was evaporated and dissolved in water, it was ultrafiltrated to remove unreacted reagents. The



Scheme 1. Synthesis route of TM–chitosan.



Scheme 2. Synthesis route of galactose residue.



TM-chitosan/galactose conjugate
TC-Gal5 : DGal = 5 mol% / sugar unit
TC-Gal20 : DGal = 20 mol% / sugar unit

Scheme 3. Synthesis route of TM–chitosan/galactose conjugate.

above reaction was repeated to be lacking residual carboxyl groups. The TM–chitosan/galactose conjugate (DGal = 20 mol%/sugar unit: TC-Gal20) was synthesized by the same method as that of TC-Gal5.

Assay of cytotoxic activity

The cytotoxic activity of the polycations was measured against Hela *utrocervical carcinoma* cells *in vitro*. The tumor cell suspension containing 1×10^5 cells in DMEM containing 10% FCS was distributed in a 96-well multiplate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After adding fresh medium containing diethylaminoethyl(DEAE)-dextran or TM–chitosan, they were incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 48 h. The number of viable cells was determined by means of MTT assay (Mosmann, 1983) using a microplate reader (MTP-120, Corona Electric Co.). The cytotoxic activity was calculated by the following equation.

$$\text{Cytotoxic activity (\%)} = (C-T)/C \times 100$$

C = number of viable cells of control cells
T = number of viable cells of treated cells.

Evaluation of formation of polyelectrolyte complex with DNA

Each polyelectrolyte complex with DNA (pSVluc: 6046 bp) was electrophoresed on 1% agarose gel at 50 V for 45 min and stained with ethidium bromide for visualization.

Interaction of polycations with APA lectin

The interaction of polycations with APA lectin was evaluated by means of a fluorescence polarization method (Haber & Bennett, 1962; Maeda, 1980; Yonemura & Maeda, 1982) using an FPIA system (FPIA system and fluorescence spectrophotometer F-4010, Hitachi, Ltd). FITC-labeled APA lectin solution (50 μl, 2 mg/ml) and various concentrations of polycation were added to be adjusted with a 1/15 M phosphate buffer solution in a cuvette. Measurement of fluorescence polarization was performed at 37°C (EX = 495 nm, EM = 520 nm). For the inhibition test, FITC-labeled APA lectin solution (50 μl, 2 mg/ml), lactose solution (300 μl, 710 mM) and various concentrations of polycation were added to be adjusted with a 1/15 M phosphate buffer solution in a cuvette. The index of fluorescence polarization (ΔP) was calculated by the following equation.

$$\text{The index of fluorescence polarization } (\Delta P) = P_{\text{final}} - P_{\text{initial}}$$

P_{initial} = fluorescence polarization value before addition of polycation solution
P_{final} = fluorescence polarization value after addition of polycation solution.

Assay of β-galactosidase activity

The β-galactosidase activity of the conjugates was measured against HepG2 *human hepatoma* cells *in vitro*. The tumor cell suspension containing 1×10^4 cells in DMEM containing 10% FCS was distributed in a 96-well microplate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After adding fresh medium containing 100 μM chloroquine and incubating in a humidified atmosphere containing 5% CO_2 at 37°C for 1 h, they were changed to FCS-free DMEM containing 5 mM $CaCl_2$ and/or 50 mM lactose for the inhibition test, and added to each sample. After incubation for 2 h, they were changed to FCS-free DMEM and incubated for 48 h. After the addition of X-gal solution (Takai *et al.*, 1990a), they were incubated for 48 h and measured using a microplate reader at 420 nm.

RESULTS AND DISCUSSION

Synthesis of TM–chitosan/galactose conjugate

Synthesis of the TM–chitosan/galactose conjugate was performed through reaction steps shown Schemes 1–3. By repetition of trimethylation, completely trimethylated TM–chitosan chloride was obtained. By the feed ratio of monochloroacetic acid to TM–chitosan, 6-*O*-carboxymethyl(CM)-TM–chitosan (**1**), having two degrees of substitution of carboxymethyl groups (DCM), was synthesized. By controlling the ratio of lactonoyl lactone to 1,6-hexanediamine in the reaction and purification by means of ion-exchange chromatography, *N*-lactonoyl-1,6-hexanediamine (**2**) having hetero terminal groups, a galactose residue and an amine was obtained. By repetition of the reaction of conjugation, the objective TM–chitosan/galactose conjugate having no residual carboxyl groups was obtained. We could synthesize TM–chitosan/galactose conjugates having two values of DGal, TC-Gal5 and TC-Gal20, through the coupling reaction of **2** with 6-*O*-CM-TM–chitosan **1**.

Cytotoxic activity of TM–chitosan

It is known that polycations usually have cytotoxic activities. On the other hand, many polysaccharides are biodegradable and biocompatible. DEAE-dextran and TM–chitosan have the character of both polycations and polysaccharides. Since DEAE-dextran has a low cytotoxic activity, it can be used for the polycation–DNA complex method (Takai *et al.*, 1990b). The results of cytotoxic activity of DEAE-dextran and TM–chitosan against Hela cells *in vitro* are shown in Fig. 1. The cytotoxic activity of

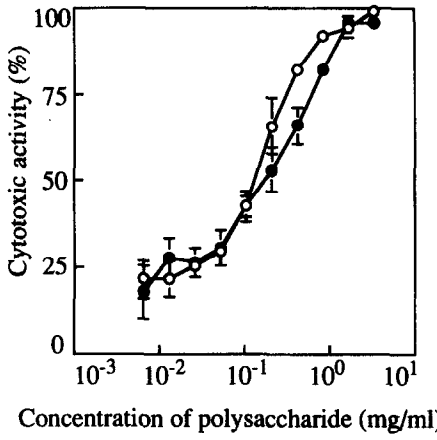


Fig. 1. Results of cytotoxic activity of DEAE-dextran and TM–chitosan against Hela cells *in vitro*. ○: DEAE-dextran; ●: TM–chitosan.

TM–chitosan was as low as that of DEAE-dextran. These results suggested that TM–chitosan could be used for the polycation–DNA complex method.

Evaluation of formation of polyelectrolyte complex with DNA

The results of polyelectrolyte complex formation with DNA are shown in Fig. 2. The molecular weight maker and free DNA (pSVluc) alone were shown at lanes 1 and 2, respectively. Free plasmid DNA bands are revealed as an arrow at lane 2. Free plasmid DNA bands were found at lanes 3–5 (the ratio of cationic group to base pair: 0.25, 0.5, 1) in each panel. There was no free plasmid DNA band at lanes 6–8 (the ratio of cationic group to base pair: 2, 4, 8). Moreover, the bands of complex tended to move

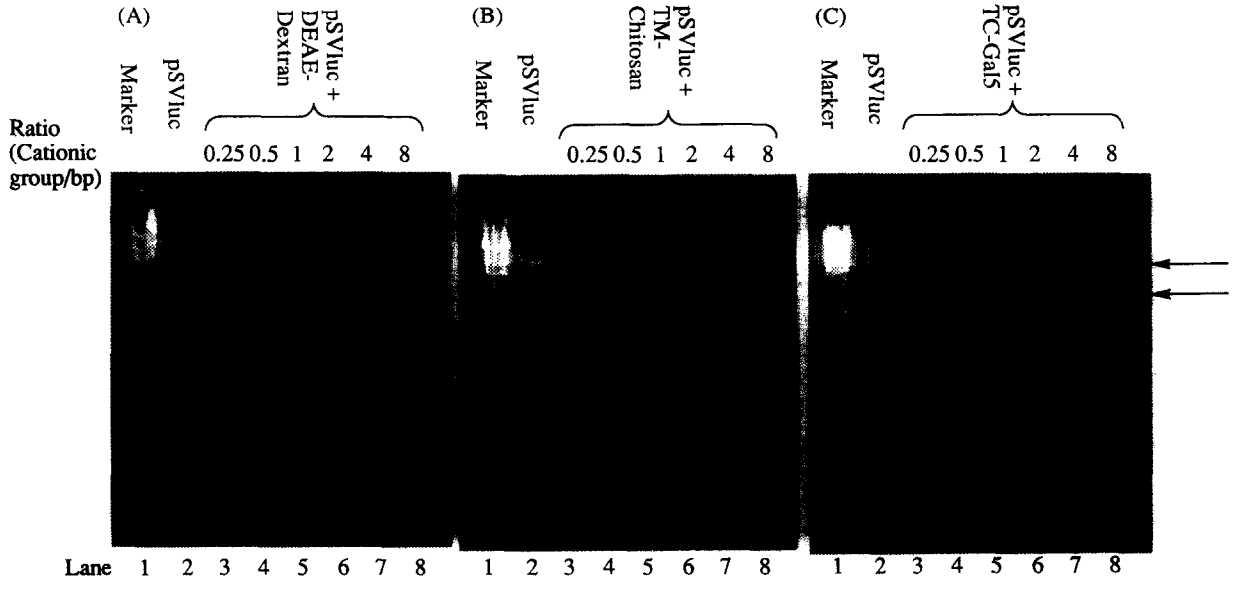


Fig. 2. Results of polyelectrolyte complex formation of cationic polysaccharides with plasmid DNA(pSVluc). Panel A: DEAE-dextran; Panel B: TM–chitosan; Panel C: TC-Gal5. Lane 1: molecular weight marker (Lambda phage DNA/*Hind III* digest), Lane 2: plasmid DNA alone (pSVluc: 6043 bp), Lanes 3 through 8: DNA with progressively increasing proportions of polycation.

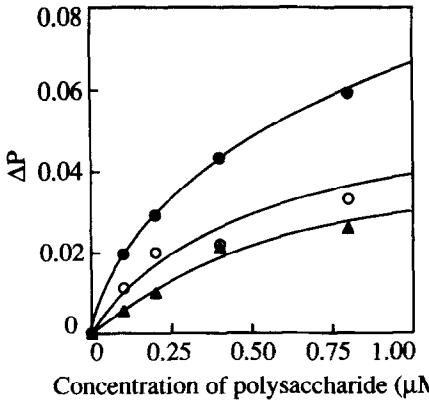


Fig. 3. Interaction of polycations with APA lectin monitored by fluorescence polarization. ●: TC-Gal5; ▲: DEAE-dextran; ○: TC-Gal5 with excess amount of lactose.

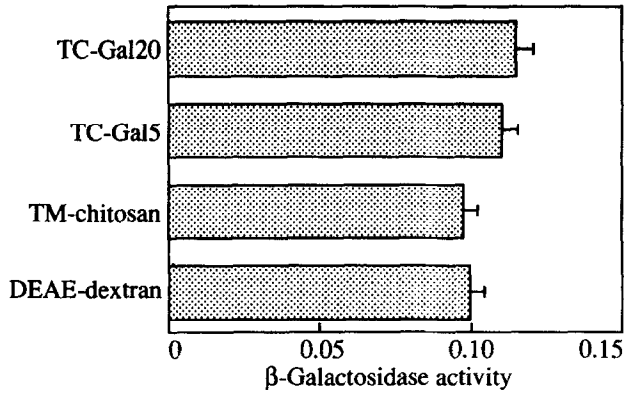


Fig. 4. Results of expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells. DNA dose = 0.5 μg/well. Ratio of polycation/DNA = 2 cationic group/1 bp.

to cathode at lanes 7 and 8. These results suggested that a neutral polyelectrolyte complex was formed when the ratio of cationic group to base pair was 2:1 in each polycation. Thus, this ratio was utilized in all subsequent experiments.

Recognition of TC-Gal5 by APA lectin

The results of interaction of polycations with APA lectin are shown in Fig. 3. The ΔP of DEAE-dextran was a little increased by increasing the concentration of DEAE-dextran. On the other hand, the ΔP of TC-Gal5 was remarkably increased by increasing the concentration of TC-Gal5. Moreover, the ΔP of TC-Gal5 was remarkably decreased by the addition of lactose. These results suggested TC-Gal5 could interact with APA lectin having the recognized ability of a galactose residue.

Expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells

The results of expression of β-galactosidase activity by the polycation–DNA complex in HepG2 cells are shown in Fig. 4. The β-galactosidase activity of TM–chitosan was the same level as that of DEAE-dextran. By increasing the DGal of the conjugate, the β-galactosidase activity of the conjugate tended to increase. These results suggested that the increase of β-galactosidase activity might be caused by the introduction of galactose residues to TM–chitosan.

Effects of inhibitor on β-galactosidase activity by polycation–DNA complex in HepG2 cells

In order to confirm that the increase of β-galactosidase activity was due to specific recognition by the galactose receptor, the effect of inhibitors were investigated. The results of inhibition tests are shown in Fig. 5. The β-galactosidase activities of DEAE-dextran and TM–

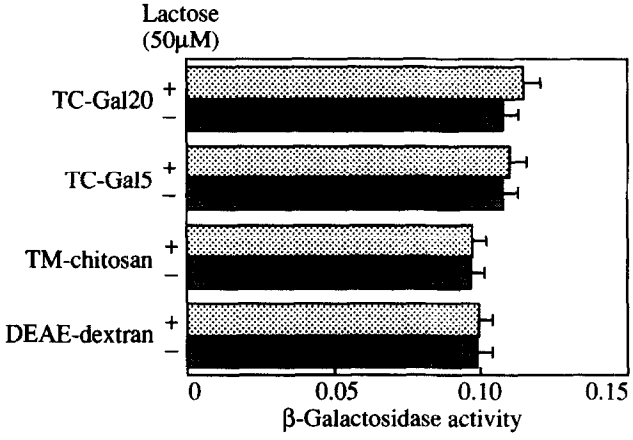


Fig. 5. Effects of inhibitor on expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells. DNA dose = 0.5 μg/well. Ratio of polycation/DNA = 2 cationic group/1 bp.

chitosan were little affected by the addition of lactose in Fig. 5. On the other hand, those of TC-Gal5 and TC-Gal20 tended to decrease a little by the addition of lactose. These results suggested that the increase of β-galactosidase activity of the TM–chitosan/galactose conjugate might be caused by specific internalization via the galactose receptor.

ACKNOWLEDGEMENTS

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