Galactosylated Cucurbituril-inclusion polyplex for hepatocyte-targeted gene delivery†

Soo Kyung Kim, Kyeng Min Park, Kaushik Singha, Jeeyeon Kim, Youngjoo Ahn, Kimoon Kim*, and Won Jong Kim*

1. Materials

Dextran with an average molecular weight of 40 kDa was obtained from TCI (Tokyo, Japan). Spermine tetrahydrochloride, sodium borohydride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,4-dihydroxybenzaldehyde and Potassium bromide for FT-IR were purchased from Sigma-aldrich (St Louis, MO, USA). Potassium periodate was obtained from Acros Co. (Belgium). A syringe pump model-KDS 100L (Holliston, MA, USA) was used for slow and reproducible addition of reactants. Dulbecco’s Modified Eagles’ Medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and Dulbecco’s phosphate buffered saline (DPBS) were purchased from Invitogen-Gibco (Carlsbad, CA). A luciferase assay system with reporter lysis buffer 5X was supplied by Promega (Madison, WI), and a bicinchoninic acid (BCA) protein assay reagent kit was obtained from Pierce Chemical Co. (Rockford, IL). The Human hepatoma-derived cell (HepG2) and Human cervix epithelial carcinoma cell (HeLa) were obtained from the Korean Cell Line Bank (KCLB).

2. Synthesis


The polysaccharide dextran of 40 kDa in average molecular weight was oxidized following the procedure reported by Azzam et al. [Macromolecules, 2002, 35, 9947-9953]. In Brief, to a solution of dextran (500 mg, 3.08 mmol with respect to glucose residues) in deionized water (15 mL) was added a solution of sodium periodate (66 mg, 3.08 mmol) drop wise, and the mixture was vigorously stirred in dark at room temperature for 18 h. A resulting clear yellow solution was dialyzed against distilled water (D.W.) (MWCO: 12 kDa) at 4°C for 2 days.
Oxidized dextran (Oxi-Dex) was lyophilized to obtain a white powder. The formation of aldehyde groups of dextran were confirmed by FT-IR spectroscopy, which showed the characteristic peak of aldehyde groups at 1726 cm\(^{-1}\) conforming to the reported value (Macromolecules, 2002, 35, 9947-9953).

2.1.2. Determination of aldehyde content in Oxi-Dex (2)

The aldehyde content in oxidized dextran (Oxi-Dex) was determined according to standard protocol [Pharm. Res. 1991, 8, 400-402.] with a slight modification. Briefly, Oxi-Dex 2 (20 mg) was dissolved in hydroxyl-amine hydrochloride solution (5 mL, 0.25 M) of pH 3.26. The mixture was sonicated for 2 h, and stored overnight at room temperature. Degree of periodate cleavage was estimated by titration of the HCl, generated on treating the aldehyde functionality with measured amount of hydroxylamine hydrochloride. The titration was carried out with standardized NaOH (0.1 N) solution till the end point has been reached at pH 3.26 as recorded on a digital pH meter. In the end, the aldehyde content of the samples were calculated by comparing the titer values of NaOH with that obtained from standard curve generated by plotting the volume of NaOH (0.1 N) against the amount of 2,4-dihydroxybenzaldehyde. The degrees of cleavage of the three samples were estimated as 70%, 79% and 84% for reaction time 18 h, 30 h and 55 h respectively. We have continued the subsequent studies with the sample (2) having 70% aldehyde content.
2.2.1 Synthesis of Dextran-graft-spermine (Dex-spm) copolymer

Oxi-Dex 2 (250 mg) was taken up in D.W. (15 mL) and kept overnight to make a completely clear solution. The solution was added dropwise into borate buffer (7 mL, 0.1 M, pH 11) containing 3 equimolar amount of spermine (with respect to cleaved aldehyde group on Oxi-Dex), using syringe pump at an addition rate of 3 mL/h. The mixture was stirred at room temperature in dark for 24 h, and was subsequently reduced by sodium borohydride (4 equimolar with respect to aldehyde of dextran). The stirring was continued for another 48 h in dark at room temperature followed by dialysis against de-ionized water using cellular membrane tube (MWCO 3.5 kDa) for 2 days. Subsequent freeze-drying afforded the desired Dex-spm conjugate 3 as fluffy yellow powder in 28% average yield.

Fig. S1 The standard titration curve obtained by plotting the volume of NaOH solution against the amount of the standard aldehyde (2,4-dihydroxy-benzaldehyde). The percentage of cleavage was calculated by comparing the respective titer value of NaOH for different samples.

### Table 1: Titration Data for NaOH Solution

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>mmol (NaOH)</th>
<th>uL (NaOH)</th>
<th>Cleavage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxi-Dex/18h</td>
<td>0.172915</td>
<td>1690</td>
<td>70.01</td>
</tr>
<tr>
<td>Oxi-Dex/30h</td>
<td>0.196448</td>
<td>1920</td>
<td>79.53</td>
</tr>
<tr>
<td>Oxi-Dex/55h</td>
<td>0.207702</td>
<td>2030</td>
<td>84.09</td>
</tr>
</tbody>
</table>
\(^1\)H NMR (D\(_2\)O, 300 MHz): 1.587 (\(m\), 4H, dextran-CH\(_2\)NH(CH\(_2\))\(_3\)NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)NH(CH\(_2\))\(_3\)NH\(_2\)), 1.786 (\(m\), 4H, dextran-CH\(_2\)NHCH\(_2\)CH\(_2\)CH\(_2\)NH(CH\(_2\))\(_3\)NHCH\(_2\)CH\(_2\)CH\(_2\)NH\(_2\)), 2.760-3.109 (\(m\), 14H, dextran-CH\(_2\)NHCH\(_2\)CH\(_2\)CH\(_2\)NHCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)NHCH\(_2\)CH\(_2\)CH\(_2\)NH\(_2\)), 3.467-3.827 (\(m\), glucose methine protons), and 4.923 (\(m\), 1H, anomeric proton).

2.2.2 Determination of Dex-spm conjugation ratio:

The elemental analysis of Oxi-Dex (N 0.048, C 38.40) and Dex-spm conjugate (N 11.4507, C 46.4484) revealed 33.7% conjugation with respect to total dextran residues oxidized or unoxidized.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxi-Dex</td>
<td>0.048%</td>
<td>38.40%</td>
</tr>
<tr>
<td>Dex-spm</td>
<td>11.4507%</td>
<td>46.4484%</td>
</tr>
</tbody>
</table>

Fig. S2 Elemental composition of Oxi-Dex and Dex-spm

2.3 Preparation of Dextran-graft-spermine-galactosylated CB (Dex-spm-gCB)

The preparation of Dex-spm-gCB was conducted following the standard protocol reported in the literature. Briefly, 10 mg and 5 mg of galactosylated cucurbituril (gCB) were added to a solution of Dex-spm (2 mg) separately. The final solutions were gently shaken and kept for 2 h for host-guest interaction. The resulting supramolecular assemblies having dextran and CB residues in molar ratio of 100:25 and 100:12 were denoted as Dex-spm-gCB-25 and Dex-spm-gCB-12 respectively. Similarly 10 mg and and 5 mg of hydroxyl cucurbituril (hCB) on treatment with 2 mg of Dex-spm gave rise to Dex-spm-hCB-25 and Dex-spm-hCB-12 respectively. The formation of the inclusion complexes were confirmed by \(^1\)H-NMR study.
2.4. Plasmid preparation.

pCMV-Luc plasmid was prepared from 24 h bacterial cultures and purified by using the Qiagen plasmid Maxi kit (Qiagen, Valencia, CA). The concentration of pDNA solution was quantified by O.D. measurement in 260 nm (1 OD corresponds to 50 \( \mu \)g/ml pDNA).

2.5. Agarose Gel Electrophoresis.

Electrophoresis was performed to evaluate DNA condensation ability of the cationic polymers. The cationic polymers/pDNA complexes with different N/P ratios ranging from 0 to 50 in DPBS buffer were prepared and incubated for 30 min at room temperature. Then, 5 \( \mu \)L of complex suspension containing 200 ng of DNA was electrophoresed on the 0.1% (w/v) agarose gel with ethidium bromide (0.5 \( \mu \)g/mL, EtBr) in 0.5 X of Tris-acetate-EDTA (TAE) running buffer at 100 V for 20 min. The gel was then analyzed on a UV illuminator.
(WiseUV®WUV, DAIHAN Scientific, Seoul, Korea) to observe the position of the complexed pDNA relative to that of the naked pDNA.

2.6. Particle size and zeta-potential measurements.

Polyplexes with Dex-spm, Dex-spm-gCB-25, and Dex-spm-hCB-25, were prepared at a variety of N/P ratios ranging from 1 to 10 by adding the polymer solution to the pDNA solution. The concentration of pDNA was adjusted to 50 μg/mL. The mixtures were then incubated for 30 min at room temperature. The particle size of each samples were measured by particle size analyzer using Zetasizer Nano S (Malvern Instruments, Malvern, UK). Surface charge was measured by determination of zeta potential using a Zetasizer Nano Z (Malvern Instruments, Malvern, UK) in D.I water.

Fig. S4 Determination of polyplex formation by gel retardation assay
2.7. Cell culture and in-vitro transfection study.

The Human Hepatoma-derived cell (HepG2) and Human cervix epithelial carcinoma cell (HeLa) were selected for this study and maintained at 37°C with a humidified atmosphere containing 5% CO₂ in DMEM supplemented with 1% penicillin/streptomycin(P/S) and 10% (v/v) fetal bovine serum (FBS). The cells were subcultured prior to confluence using 0.25% trypsin-EDTA. Cells were seeded in 24-well plates at an initial density of 4 x 10⁴ cells/well with 0.5 mL of complete medium (DMEM) and incubated overnight at 37°C in 5% CO₂ so that confluence could attain 70% at the time of transfection. The polymer/DNA complexes were formed at different N/P ratios ranging from 1 to 10. However, each formulation of polyplexes having different N/P ratios, contained 1 μg of DNA as prepared according to the conditions describe above. The cells were washed with Dulbecco’s phosphate buffered saline (DPBS) prior to transfection, and incubated with the complexes in 0.3 mL of serum-free culture medium for 4 h. Then the medium was replaced completely with serum containing medium, followed by an additional incubation for 24 h in serum medium. For determination of transfection efficiency, the cells were rinsed gently with warm DPBS after removal of

Fig. S5 (a) Particle size and (b) zeta potential measurements for Dex-spm, Dex-spm-gCB-25 and Dex-spm-hCB-25
medium, and were then subjected to lysis with 150 μL/well of reporter lysis buffer (Promega). An aliquot of cell lysates were allowed to react with 100 μL of luciferin substrate and were analyzed by detecting the light emission, and the obtained values were integrated by using a microplate spectrofluorometer (VICTOR3 V™ Multilabel Counter, Perkin Elmer – Wellesley, MA, USA). The protein content of the cell lysates was evaluated by BCA protein assay kit. Triplicate measurements regarding all experiments were carried out to get the average value.

2.8. Cytotoxicity Assay.

The cell viability of polymer/pDNA complexes was evaluated by using the standard MTT assay protocol. Briefly, Cells were seeded onto 24-well plates at a density of 6 x 10⁴ cells/well and cultured overnight in a humidified atmosphere of 5% CO₂ at 37°C with 500 μL of complete DMEM. The growth medium was replaced with 300 μL serum-free DMEM culture medium, and to this serum-free medium were added the complexes i.e., BPEI25K, Dex-spm, Dex-spm-gCB-25, and Dex-spm-hCB-25 which were prepared separately by 30 mins incubation prior to the addition. The cells were incubated for another 24 h with complete medium, and then treated with 50 μL of 5 mg/ml MTT solution. After incubation at 37°C for 4 h further, the medium containing MTT solution was removed, and 300 μL of DMSO was added to dissolve the formazan salt produced by living cells. For the assessment of the cytotoxicity, an aliquot of 100 μL from each 24-well was transferred into a 96-well plate, and the absorption was measured at 570 nm by using a microplate spectrofluorometer (VICTOR3 VTM Multilabel Counter, Perkin Elmer-Wellesley, MA, USA). The relative percentage of the control (untreated) cells, which were not subjected to any treatment but underwent the identical culturing and washing procedures, were used to represent 100% cell viability.
2.9. Galactose inhibition study

Cells (HepG2 and HeLa) were cultured and seeded in the same manner as performed in *in-vitro* transfection study. The stock solutions including free galactose at various molar ratios of dex-spm-gCB-25 per free galactose ranging from 1/0 to 1/200 (prepared with fixed N/P ratio of 7 in complexes) were added to each of the 24-well plates, 2 hours prior to the transfection. After transfection, the cells were incubated for additional 3.5 h, and serum free DMEM removed subsequently to replace that with fresh DMEM containing complete medium conditions. Then, the cells were kept under incubation for 24 h. All experimental conditions remained same as described in section 2.7 (supporting information).
2.10. Analysis of cellular uptake of the polyplexes by FACS

HepG2 cells were seeded on 12-well culture plate (120,000 cells/well) with 1 mL of complete DMEM and incubated in a humidified 5% CO₂ atmosphere at 37 °C overnight. pCMV-Luc was labeled with TOTO-3 on ice at a final concentration of 100 nM by mixing them for 30 min before complexation of polyplexes. For transfection the complexes with N/P ratio of 7, were incubated for various time periods such as 15 min, 30 min, 1 h and 4 h. After each incubation time, serum free medium was removed and cells were washed twice with DPBS. Then, the cells were incubated with 200 μL of 0.25% trypsin/EDTA for 10 min at 37 °C for detachment. The harvested cells were centrifuged, and the supernatant was removed. Cell suspensions were kept in 200 μL of 4% para-formaldehyde solution for 15 min of

Fig. S7 Galactose inhibition study of (a) Dex-spm-gCB-25 and (b) Dex-spm on HeLa cells
fixation and centrifuged again to remove the solution. PBS solution (400 μL) supplemented with 2% of FBS and 0.1% of sodium azide, was added into the cells for measurement of fluorescence inside of the cells (1x10^4) for each sample.

2.11. Intracellular translocation study of the polyplexes

HepG2 cells were seeded on cover glass coated with 0.01% poly-lysine solution in a 12-well plate at a density of 6 x 10^4/well in 1 mL of complete DMEM containing serum and incubated in a humidified 5% CO_2 atmosphere at 37°C for 24 h. TOTO-3 iodide(1 mM) was diluted to proper concentration to stain pDNA at a DNA bp : dye ratio of 200:1 for 30 min at room temperature. The polymer/pDNA complexes at N/P ratio of 7 were incubated for another 30 min before transfection. The cells were washed twice with DPBS, and incubated with the complexes in 0.5 mL of serum-free culture medium for 4 h. The incubated cells were washed twice gently with warm DPBS, followed by being fixed with 4% (w/v) para-

Fig. S8 Cellular uptake study by FACS for the polyplexes on HepG2 cells.
formaldehyde solution for 15 min. To determination of intracellular translocation, the cells were analyzed by a confocal laser scanning microscope using an excitation wavelength of 633 nm.

Fig. S9 Cellular uptake study by confocal laser scanning microscopy on HepG2 cells