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# Lactose-appended schizophyllan is a potential candidate as a hepatocyte-targeted antisense carrier†‡

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**A schizophyllan (β-1,3-glucan) derivative carrying lactose-appendages prepared by reductive amination can form stable macromolecular complexes with polynucleotides, shows excellent affinity with a lactose-binding lectin, and effectively mediates gene transfection into hepatocytes.**

Over the past decade, researchers in the area of supramolecular chemistry have achieved impressive progress towards the development of efficient antisense delivery systems for clinical therapy.[1] To further develop these delivery systems with the minimum side effects and reduced cost, carriers should show the following properties: (1) easy preparation of carrier/antisense complexes, (2) low cytotoxicity, (3) no immunogeneity and (4) high selectivity for target cells. Artificial carriers reported so far include poly-L-lysines,[2] polyethyleneimines,[3] polyamidoamine dendrimers,[4] *etc.*, which mostly utilize electrostatic interactions to form carrier–antisense complexes.

Schizophyllan (SPG), β-1,3-glucan, has been of great interest for many researchers because of its gel-forming ability as well as its anticancer activity.[5] Physical and structural studies have revealed that SPG exists in a unique triple-stranded helical structure (t-SPG) in aqueous solution, whereas it is dissociated into the individual single strands (s-SPG) in dimethyl sulfoxide (DMSO).[6] Recently, we found that unique triple-stranded macromolecular complexes are formed when s-SPG in DMSO is mixed with polynucleotides in aqueous solution (Fig. 1).[7] This finding encouraged us to develop new SPG-based antisense carriers, in which carrier–antisense complexes are formed by the unique 'shape fitting' between two helical components (SPG and polynucleotide), instead of the conventional electrostatic interactions. In the series of our research, we found several advantages of the macromolecular complexes as antisense carriers over the conventional ones: (1) the macromolecular complexes are stable under physiological conditions, (2) complexed polynucleotides are protected against degradation by DNase[8] and (3) the complexed polynucleotides can be quickly released in the presence of the complementary RNA. In addition to the expected long blood-circulation time arising from the lack of β-1,3-glucanase in human body, these characteristics suggest the potential utility of SPG to mediate antisense transfer in human cells. Our next step should be, therefore, to develop a *cell-targeted* carrier based on SPG. In this respect, SPG-derivatives carrying oligosaccharide appendages are of great interest, since oligosaccharides can act as specific ligands for carbohydrate-binding proteins (lectins) on the target cell surface.[9] Herein, we report such a new example that a SPG-lactose conjugate can strongly bind asialoglycoprotein receptors on hepatocytes and mediate the following receptor-dependent endocytosis as well as effective transfection.

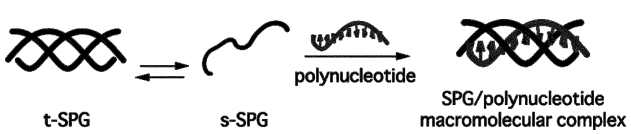


**Fig. 1** Formation of the SPG/polynucleotide macromolecular complex.

SPG bearing lactose-appendages (SPG-Lac, Chart 1) were prepared through the following procedure.[10] Pendent glucosides of native SPG (MW 150 kDa) were selectively oxidized by treatment with aqueous $NaIO_4$ to afford an aldehyde-functionalized SPG, in which the β-1,3-glucan main-chain without the $NaIO_4$-sensitive 1,2-diol group remains intact. Schiff base formation between aldehyde-functionalized SPG and aminoethyl-lactoside was attained in DMSO and then, an excess amount of $NaBH_4$ was added to yield SPG tethering lactosides through secondary imino-linkages. We estimated the conversion ratio (*n*) based on the nitrogen contents by elemental analysis. SPG having a moderate content of lactose-appendages ($n = 0.14$) was thus obtained.

Macromolecular complexes composed of SPG-Lac and polynucleotides were easily prepared by mixing SPG-Lac in DMSO and polynucleotides in water. Proof of complex formation was obtained by CD spectra (Fig. 2(a)): The SPG-Lac/poly(dA) complex shows CD spectra in which a predominant negative peak (250 nm) observed for free poly(dA) was suppressed and new negative (265 nm) and positive (282 nm) peaks appeared. The observed CD spectral change is similar to that of the SPG/poly(dA) complex, indicating the formation of a hetero-triple-stranded macromolecular complex.[8] We measured the CD spectra at various temperatures (5–80 °C) to assess the thermal stability of the complex. The CD spectra of the SPG-Lac/poly(dA) complex are independent of the temperature up to 45 °C, at which point they suddenly changed with increasing temperature at around 50 °C into the CD spectra attributable to free poly(dA).[11] The finding proves

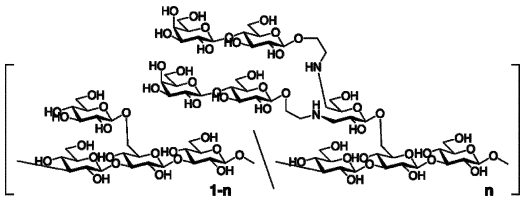


**Chart 1** Structure of SPG carrying lactose appendages (SPG-Lac).

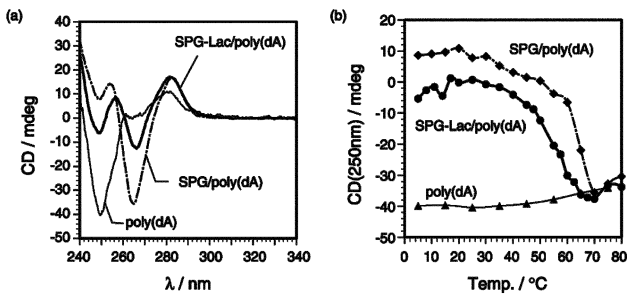


**Fig. 2** (a) CD spectra of the SPG-Lac/poly(dA) complex, SPG/poly(dA) complex and poly(dA): [poly(dA)] = 0.08 mg ml$^{-1}$, [SPG-Lac] or [SPG] = 0.42 mg ml$^{-1}$, 5 °C, $d = 1.0$ cm, Tris-buffer (0.83 mM, pH 8.0). (b) Temperature dependence of the CD (250 nm) intensities.

† Electronic supplementary information (ESI) available: Procedures for the synthesis of aminoethyl-lactoside and a confocal fluorescent microscopic image. See http://www.rsc.org/suppdata/cc/b3/b313426a/
‡ Polysaccharide–Polynucleotide complexes, Part 26.

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that the complex is dissociated cooperatively at this temperature. The SPG-Lac/poly(dA) complex showed a melting temperature ($T_m$) at around 52 °C (Fig. 2(b)), which is lower than that (63 °C) of the SPG/poly(dA) complex but still sufficiently higher than physiological temperature.

The emission intensity of fluorescein isothiocyanate-labeled ricinus communis agglutinin (FITC-$RCA_{120}$, lactose-specific) was suppressed in the presence of the SPG-Lac/poly(dA) complex, suggesting their binding (Fig. 3(a)).[12] Together with the fact that neither poly(dA) nor the SPG/poly(dA) complex induces such fluorescence suppression, we can conclude that specific interactions occur between the complex and FITC-$RCA_{120}$.[13] Fig. 3(b) shows the relative fluorescence intensities ($I/I_0$) of FITC-$RCA_{120}$ plotted against various concentrations of the macromolecular complexes. We estimated the $RCA_{120}$ affinity ($K_{1/2}$) of the SPG-Lac/poly(dA) complex to be 14 μg $ml^{-1}$ by using computational curve fitting.[14] The $K_{1/2}$ value based on [lactose-unit] is $1.7 \times 10^{-6}$ M which is 120 times higher than that of monomeric lactose ($2.1 \times 10^{-4}$ M) owing to the clustering effect, and comparable to that of multi-lactosylated polyacrylamides ($9.1 \times 10^{-7}$ M).[12] These data clearly show that the SPG-Lac/poly(dA) complex can be strongly recognized by lactose-binding lectins and therefore can act as a hepatocyte-specific antisense carrier.

Specific lectin-binding of the SPG-Lac/poly(dA) complex was also demonstrated by confocal microscope observation using lectin-labeled agarose beads (~50 μm) and rhodamin-labeled $(dA)_{45}$. $RCA_{120}$-agarose was stained by the SPG-Lac/Rho-$(dA)_{45}$ complex (Fig. S1, ESI†), whereas agarose bearing ConA (α-Man/Glc specific) was not. These data also reveal that carrier/polynucleotide complexes are *not* dissociated upon binding to lectins and therefore, one can expect that the complexed polynucleotides are up-taken by the target cell *via* receptor-mediated endocytosis without unfavourable release at the cell surface.

Receptor-mediated antisense transfection by SPG-Lac was demonstrated using human hepatocytes (HepG2) and $(dA)_{40}$-tagged phosphorothioate-type antisense (AS-c-myb: 5′-GTGCCGGGGTCTTCGGGC-$(A)_{40}$-3′) which blocks the proto-oncogene mRNA translation.[15] HepG2 cells were cultured in the presence of the SPG-Lac/As-c-myb complex, SPG/As-c-myb complex or free As-c-myb ([As-c-myb] = 30 μg $ml^{-1}$) and then, the cell-numbers were counted by using the Cell Counting Kit-8 (Dojin).[16] The SPG-Lac/As-c-myb complex was found to suppress the cell-growth effectively (52 ± 4%) in comparison to the SPG/As-c-myb complex (81 ± 6%) and free As-c-myb (91 ± 6%). Together with the facts that HepG2 expresses lactose-receptors on the cell surface and that monomeric lactose (20 mM) reduces the transfection effect (79 ± 5%), specific lactose-protein interactions and the following receptor-mediated endocytosis should be responsible for the enhanced transfection effect observed for the SPG-Lac/As-c-myb complex.

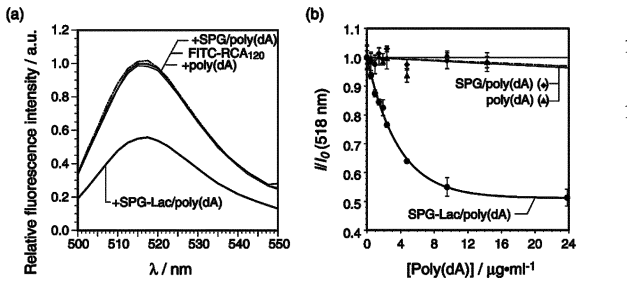


**Fig. 3** (a) Fluorescence spectra of FITC-$RCA_{120}$ in the presence of free poly(dA) (9 μg $ml^{-1}$) and that complexed with SPG derivatives (46 μg $ml^{-1}$): Tris-buffer (20 mM, pH 7.2), 25 °C, $\lambda_{ex}$ = 490 nm. (b) Relative fluorescence intensities ($I/I_o$) of FITC-$RCA_{120}$ at various concentrations of the SPG-Lac/poly(dA) complex, SPG/poly(dA) complex and free poly(dA).

In conclusion, a SPG-derivative carrying lactose-appendages was prepared *via* oxidation followed by reductive amination. Easy preparation, high stability, excellent lectin-affinity and effective antisense-transfection ensure the potential of SPG-lactose conjugates as new hepatocyte-targeted antisense carriers.

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10 Aminoethyl-lactoside was synthesized from *per*-acetyllactose *via* glycosylation with 2-bromoethanol, azidation and hydrogenation followed by deacetylation. See ESI† for detailed procedures and characterization.

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