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Schizophyllans carrying oligosaccharide appendages as potential candidates for cell-targeted antisense carrier†

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Schizophyllans carrying β-lactoside and α-mannoside appendages were prepared from native schizophyllan through $NaIO_4$ oxidation followed by reductive amination using aminoethyl-β-lactoside and α-mannoside, respectively. The resulting schizophyllans form stable macromolecular complexes with polynucleotides, such as poly(C) and poly(dA). Specific affinity between these macromolecular complexes and saccharide-binding proteins was demonstrated by surface plasmon resonance and agarose gel staining assays. β-Lactoside-appended schizophyllan enhanced an antisense activity in hepatocytes which express lactoside-binding proteins on their cell surfaces.

Introduction

Effective delivery systems for antisense oligonucleotides (AS-ODNs) into eukaryotic cells are of great significance to the establishment of clinical antisense therapy.[1] In earlier investigations, viral vectors were thought to be promising,[2] however, such vectors have been little used in practical therapy so far, because of the inherent dangers arising from their strong immunogeneity and the risks of mutation. Research interest was subsequently shifted to cationic liposome-based delivery systems, in which AS-ODNs were encapsulated inside the liposomes.[3] Although in some cases liposome-based delivery systems showed enhanced antisense effects, in comparison to the administration of naked AS-ODNs, short lifetimes and reduced access to the target tissues were often observed because of accumulation in reticuloendothelial systems. The large size and dense cationic charge of liposome-based carriers also reduces the antisense efficiency. Recently, much effort has been focused on the development of polymer-based synthetic carriers.[4] Several research groups published a large number of papers concerning cationic linear[5] and dendritic[6] polymers, which form polyion complexes with anionic AS-ODNs. However, in spite of their profuse research, no polymer-based antisense delivery system developed so far has been able to replace the injection of naked or virus/liposome-encapsulated AS-ODNs. The most troublesome problem is the high cytotoxicity of these polymers, which arises from their polycationic nature. Since polycationic properties are essential for carrier–AS-ODN complex-formation, many research groups are now concentrating on the search for carriers with an optimum cationic density that show acceptable cytotoxicity and efficiency of delivery.

Schizophyllan (SPG), β-1,3-glucan having a β-1,6-glucoside appendage at every three repeating units, has been of great interest to many researchers because of its gel-forming ability and anti-cancer activity.[7] Physical and structural studies have revealed that SPG exists in an unique triple-stranded helical structure (t-SPG) in aqueous solution, whereas it dissociates into single strands (s-SPG) in dimethylsulfoxide (DMSO).[8] Recently, we reported that, when s-SPG in DMSO is mixed with an aqueous solution of polynucleotide, SPG interacts with the polynucleotides to form a unique hetero-triple stranded helix composed of two SPGs and one polynucleotide.[9] It is of great interest that the macromolecular complex is formed through unique "shape-fitting" between helical SPGs and the helical polynucleotides, in spite of electrostatic interactions. This finding encouraged us to develop a new class of neutral and non-toxic SPG-based antisense carriers that could be potentially free from the conventional dilemmas outlined above. In the course of our research, we found that SPG has excellent properties as an antisense carrier, that is, (i) high thermal stability of the complex under the physiological conditions;[10] (ii) long blood-circulation time of the complex, owing to the lack of β-1,3-glucanase in mammals;[11] (iii) protection of the complexed polynucleotides against degradation by DNases;[12] and (iv) quick release of the complexed polynucleotides to hybridize with target RNA.[13] Together with the expected non-cytotoxicity arising from its neutral nature, these advantages of SPG suggest it has potential utility to mediate antisense transfer into the human cells.

Our next research purpose is to develop SPG-based carriers that can deliver AS-ODNs specifically to the target cells or organs. Such cell/organ-specificity is of great importance to the development of practical antisense delivery systems, since it can increase effective antisense concentrations in the target cells and decrease the required dose, reducing total costs. Furthermore, such cell/organ-specificity can also minimize unfavourable uptake of antisense by non-target cells and reduce the burden on the patient's body. The most effective strategy to attain cell/organ-specificity includes a conjugation of antisense carriers with cell-specific oligosaccharides.[14] Oligosaccharides are unevenly distributed in the human body and play substantial roles in various specific molecular recognition events such as cell–cell adhesion, protein trafficking, immune response, *etc.*. They act as specific ligands for saccharide-binding proteins (lectins) on the target cell surfaces.[15] SPG-derivatives carrying oligosaccharide-appendages should therefore be potential candidates for a nontoxic antisense carrier with cell/organ-specificity. Recently, we reported a short communication in which SPG carrying β-lactoside appendages (SPG-$Lac_{0.14}$)

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effectively mediates cell-uptake of the complexed antisense polynucleotides to increase the antisense activity in hepatocyte-expressing lactoside-binding proteins (or asiallo-glycoprotein receptors) on the cell surface (Fig. 1).[16] In the present paper, we report full experimental data concerning the synthesis and characterization of SPG-Lac$_n$ as well as an SPG carrying α-mannoside appendages (SPG-Man$_n$).

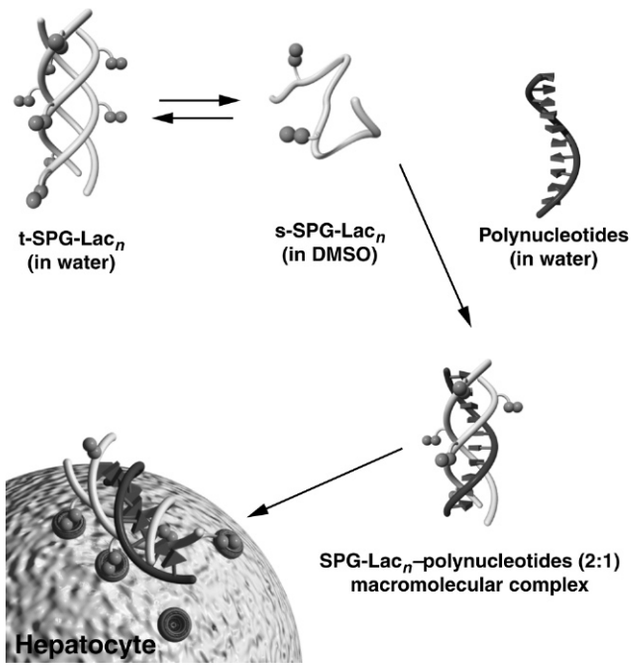


Fig. 1 Formation of a macromolecular complex consisting of two SPG strands with carbohydrate-appendages (SPG-Lac$_n$) and one polynucleotide strand, and the specific interaction between this complex and target cell *via* specific carbohydrate-protein interactions.

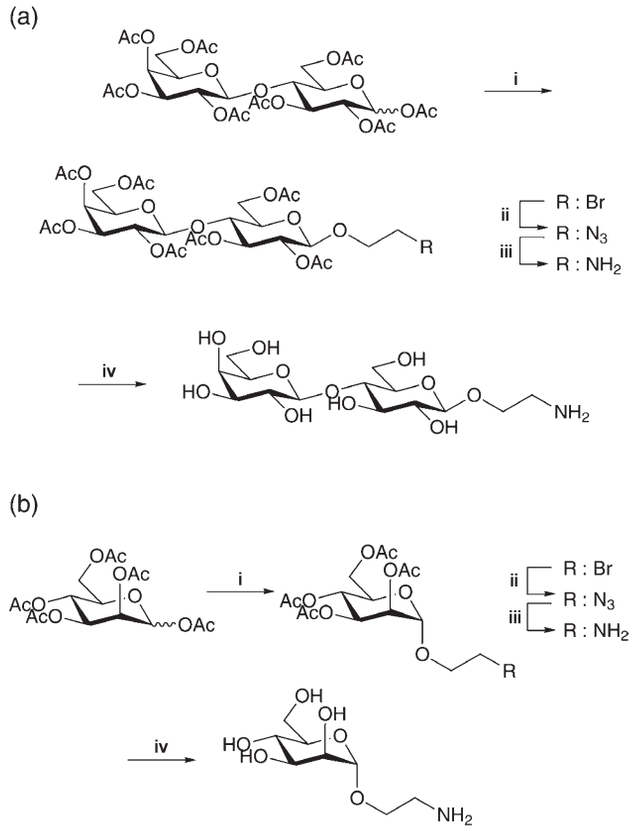
Results and discussion

Synthesis

Syntheses of aminoethyl-glycosides. Aminoethyl-β-lactoside was synthesized from *per*-acetyl lactoside through simple 4-step synthesis (Scheme 1a). *per*-Acetyl lactoside was coupled with 2-bromoethanol by treating with BF_3OEt_2 in dry CH_2Cl_2 to afford *per*-acetyl 2-bromoethyl-β-lactoside. The corresponding β-anomer was exclusively obtained owing to a well-known neighbouring participation from the 2-*O*-acetyl group. The following substitution reaction using sodium azide, hydrogenation using palladium on carbon and deacetylation using ammonia in a water–methanol mixture yielded aminoethyl-β-lactoside. The structural proof was obtained by ^{1}H NMR, IR and MALDI-TOF-MS spectral evidence.

Similar synthetic procedures, starting from *per*-acetyl mannoside afforded aminoethyl-α-mannoside in good yields as shown in Scheme 1b.

Syntheses of schizophyllans with oligosaccharide appendages. SPG derivatives bearing β-lactosides (SPG-Lac$_n$) or α-mannosides (SPG-Man$_n$) were prepared through the following procedure. Native SPG (M_w = 150 kDa) was treated with aqueous $NaIO_4$ to afford aldehyde-functionalized SPG. Since $NaIO_4$ oxidation proceeds in a 1,2-diol-specific manner, the β-glucoside appendages of native SPG were selectively oxidized by this reaction, whereas the β-1,3-glucan main chain (without the 1,2-diol group) remained intact. This appendage-selective oxidation was essential to develop an SPG-based antisense carrier with functional appendages, since perturbation on the structure and conformation of the β-1,3-glucan main chain should destabilize the corresponding SPG–antisense complexes. Schiff base formation between the aldehyde-functionalized SPG and aminoethyl-β-lactoside or -α-mannoside, and the subsequent reduction, yielded SPG-Lac$_n$ or SPG-Man$_n$, respectively, as shown in Scheme 2.[17] The estimated conversion ratio (n) was based on the nitrogen content revealed by elemental analysis. SPG derivatives with various contents of lactosides (n = 0.05, 0.14 and 0.23) or mannosides (n = 0.05, 0.13, and 0.22) were obtained by using different amounts of $NaIO_4$ in the oxidation step.



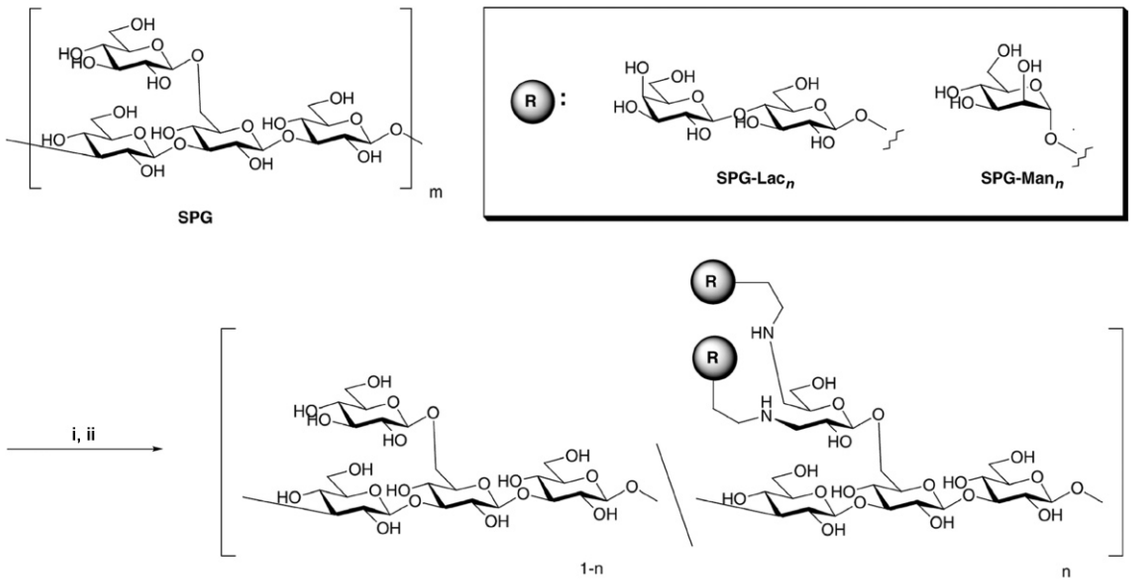
Scheme 1 Synthesis of (a) aminoethyl-β-lactoside and (b) aminoethyl-α-mannoside: (i) 2-bromoethanol, CH_2Cl_2, BF_3OEt_2; (ii) NaN_3, DMF; (iii) H_2, Pd/C, EtOH; (iv) aqueous ammonia in MeOH.

Macromolecular complex formation and thermal stability

Preparation of macromolecular complexes. SPG-Lac$_n$, SPG-Man$_n$ and native SPG were dissolved into DMSO, to dissociate them into the corresponding single-stranded random coils, and then mixed with aqueous solutions containing polynucleotides, such as poly(C) and poly(dA). The resulting mixtures were incubated at 4 °C for 2 d to attain the macromolecular complexes with ordered triple-stranded helical structures.

Macromolecular complex with poly(C). All SPG-Lac$_n$–poly(C) complexes show CD spectra with a positive peak at around 275 nm whose intensity is much more enhanced than that of free poly(C) itself (Fig. 2a). Furthermore, a characteristic new peak appears at around 245 nm for SPG-Lac$_n$–poly(C) complexes. These spectral features of SPG-Lac$_n$–poly(C) complexes are similar to those of SPG–poly(C) complexes, indicating that SPG-Lac$_n$ also forms macromolecular complexes composed of two SPG-Lac$_n$ strands and one poly(C) strand. Similar macromolecular complex formation is also observed for SPG-Man$_n$ (Fig. 2b).[18]

We measured the CD spectra of these complexes at various temperatures (5–80 °C) to assess their thermal stability. CD spectra of the SPG-Lac$_{0.05}$–poly(C) complex are independent of the temperature up to 50 °C, and suddenly change with increasing temperature at around 55 °C into the CD spectra attributable to free poly(C). This sudden spectral change with increasing temperature resembles that of polynucleotide duplexes, indicating that at this temperature the SPG-Lac$_{0.05}$–poly(C) complex is



Scheme 2 Syntheses of SPG-derivatives bearing β-lactosides (SPG-Lac$_n$) or α-mannosides (SPG-Man$_n$); (i) $NaIO_4$ in water, 4 °C, 2 d, (ii) aminoethyl-β-lactoside in DMSO, rt, 2 d, and then $NaBH_4$, rt, 2 d (for SPG-Lac$_n$), or aminoethyl-α-mannoside in water containing $NaBH_3CN$, rt, 3 d (for SPG-Man$_n$).

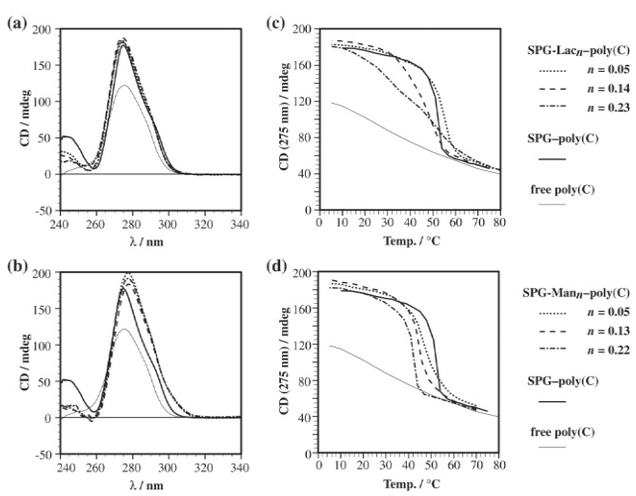


Fig. 2 (a) CD spectra of SPG-Lac$_n$–poly(C) complexes and (b) SPG-Man$_n$–poly(C) complexes along with SPG–poly(C) complexes and free poly(C), and temperature dependent CD (275 nm) intensities of (c) SPG-Lac$_n$–poly(C) complexes and (d) SPG-Man$_n$–poly(C) complexes along with SPG–poly(C) complexes and free poly(C); [poly(C)] = 0.08 mg ml^{-1}, [SPG-Lac$_n$], [SPG-Man$_n$] or [SPG] = 0.42 mg ml^{-1}, 5 °C, d = 1.0 cm, Tris-buffer (0.83 mM, pH 8.0, 8.3 wt% DMSO).

cooperatively dissociated (Fig. 2c). The melting temperature (T_m) is estimated to be 52 °C for SPG-Lac$_{0.05}$–poly(C) which is almost identical to that SPG–poly(C) (51 °C).

A similar temperature-dependent CD spectral change is also observed for the SPG-Lac$_{0.14}$–poly(C) complex, although its thermal stability ($T_m \approx 46$ °C) is lower than that of SPG-Lac$_{0.05}$–poly(C) . Furthermore, cooperative dissociation of SPG-Lac$_{0.14}$–poly(C) is slightly broadened in comparison to that of SPG$_{0.05}$–poly(C) . Similar destabilization and broadened melting behaviour are clearly observed for SPG-Lac$_{0.23}$–poly(C) which shows a continuous decrease in CD intensity with increasing temperature, indicating that excessive lactosylation can destabilize the resulting macromolecular complex.

On the other hand, all SPG-Man$_n$–poly(C) complexes show sharp melting behaviour at around 47 (n = 0.05), 45 (n = 0.13) and 43 (n = 0.22) °C (Fig. 2d). These data clearly show that all SPG-Man$_n$–poly(C) complexes are stable at the physiological temperature.

Macromolecular complex with poly(dA). We also confirmed the macromolecular complex formation between these SPG derivatives and poly(dA). SPG-Lac$_n$–poly(dA) complexes show CD spectra in which the predominant negative peak (250 nm) observed for free poly(dA) is suppressed and new negative (265 nm) and positive (282 nm) peaks appear (Fig. 3a). Although the observed CD spectral changes are similar to those of SPG–poly(dA) complex and indicate the formation of hetero-triple stranded macromolecular complexes, the relatively weak and strong peak intensities at around 265 and 250 nm, respectively, imply conformational perturbations (*i.e.* loose packing) occurring in these macromolecular complexes. These structural perturbations clearly affect the thermal stability of SPG-Lac$_n$–poly(dA) complexes, which dissociate at *ca.* 10 °C lower than SPG–poly(dA) complexes (T_m = 63 °C, Fig. 3c). The macromolecular complex formation was also confirmed for SPG-Man$_n$–poly(dA), however, in this case, no spectral perturbation nor thermal destabilization were observed (Fig. 3b and d).

The data described above indicate that the structure of saccharide appendages affects the stability of the macromolecular complexes, that is, the stability of SPG-Lac$_n$ is lower than that of SPG-Man$_n$ with similar conversion ratio (n).

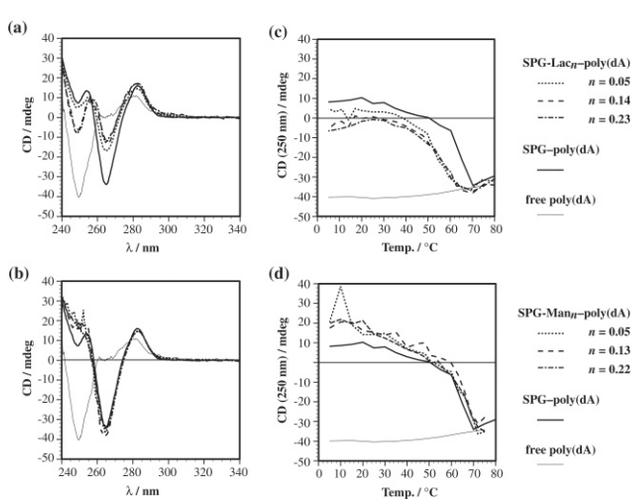


Fig. 3 (a) CD spectra of SPG-Lac$_n$–poly(dA) complexes and (b) SPG-Man$_n$–poly(dA) complexes along with SPG–poly(dA) complex and free poly(dA), and temperature dependent CD (250 nm) intensities of (c) SPG-Lac$_n$–poly(dA) complexes and (d) SPG-Man$_n$–poly(dA) complexes along with SPG–poly(dA) complex and free poly(dA); [poly(C)] = 0.08 mg ml^{-1}, [SPG-Lac$_n$], [SPG-Man$_n$] or [SPG] = 0.42 mg ml^{-1}, 5 °C, d = 1.0 cm, Tris-buffer (0.83 mM, pH 8.0).

Table 1 Kinetic parameters and affinity constants of the macromolecular complexes on binding to lectins immobilized with Au surface[a]

Lectin	Macromolecular complex	k_a /M^{-1} s^{-1}	$k_d \times 10^4$/s^{-1}	$K_a \times 10^{-6}$/M^{-1}
RCA_{120}	SPG-$Lac_{0.05}$–poly(dA)	98.4	0.445	2.21
	SPG-$Lac_{0.14}$–poly(dA)	88.6	0.153	5.79
	SPG-$Lac_{0.23}$–poly(dA)	54.6	16.6	0.0329
ConA	SPG-$Man_{0.05}$–poly(dA)	92.7	0.927	1.02
	SPG-$Man_{0.13}$–poly(dA)	79.0	0.310	2.55
	SPG-$Man_{0.22}$–poly(dA)	104	1.51	0.690

[a] Molarity of nucleotide unit.

Since β-lactoside has a hydrophobic α-face, we assume that the observed thermal destabilization of SPG-Lac_n–polynucleotide complexes should arise from hydrophobic interactions between the β-lactoside appendages and SPG main chain. Hydrophobic interactions between SPG and polynucleotides play substantial roles, in addition to hydrogen bonding networks, to stabilize the SPG–polynucleotide complexes, so that multiple copies of hydrophobic β-lactosides along with the SPG strands should, therefore, perturb the hydrophobic interactions to destabilize these complexes. It should be emphasized, however, that all SPG-Lac_n and SPG-Man_n –poly(dA) complexes show T_m values sufficiently higher than the physiological temperature.

Molecular recognition

Surface plasmon resonance assay. The lectin affinity of the macromolecular complexes was evaluated by surface plasmon resonance (SPR) assays using Au surfaces immobilized with *Ricinus communis* agglutinin (RCA_{120}, β-lactoside-specific) or concanavalin A (ConA, α-mannoside-specific). In the case of the RCA_{120}-immobilized Au surface, all SPG-Lac_n–poly(dA) complexes induce significant increases in resonance, indicating their binding to RCA_{120} (Fig. 4a). Since no such resonance increase is observed for SPG-Man_n–poly(dA) complexes, we can ascribe the origin of the binding to the specific interaction between the β-lactoside appendages of SPG-Lac_n–poly(dA) complexes and RCA_{120}.

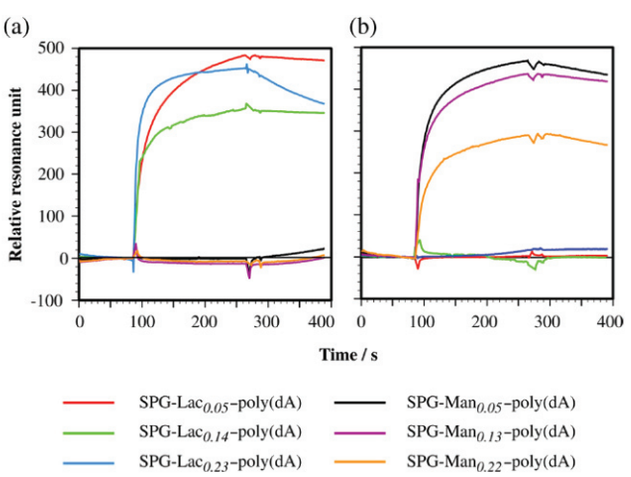


Fig. 4 SPR sensorgrams obtained by (a) RCA_{120}- and (b) ConA-immobilized Au surfaces; 83 mM Tris-HCl buffer (pH 8.0) containing $CaCl_2$ (1 mM), $MnCl_2$ (1 mM) and DMSO (8.3 wt%), 25 °C, flow rate = 20 μl min^{-1}, [poly(dA)] = 83 μg ml^{-1}, [SPG-Lac_n] or [SPG-Man_n] = 420 μg ml^{-1}.

Specific lectin binding of the macromolecular complexes is also demonstrated by using ConA-immobilized Au surfaces, to which SPG-Man_n–poly(dA) complexes bind but SPG-Lac_n–poly(dA) complexes do not (Fig. 4b). We also confirmed that neither SPG–poly(dA) complex nor free poly(dA) (data not shown) binds to the lectin-immobilized surfaces.

Association and dissociation rate constants (k_a and k_d, respectively) for the specific lectin binding of each complex were estimated by using computational curve fitting and summarized in Table 1. The association constants ($K_a = k_a / k_d$) of specific binding were in the range of 0.69–5.79 × 10^6 M^{-1}, except for SPG-$Lac_{0.23}$–poly(dA) complex ($K_a = 3.29 \times 10^4$ M^{-1}), indicating that these macromolecular complexes bind to their specific lectins with excellent affinity. It should be emphasized that the lectin affinity of the other macromolecular complex is highly enhanced in comparison to monomeric lactose ($K_a \approx 10^3$ M^{-1}). This enhanced lectin affinity can be ascribed to well-known saccharide cluster effects producing multiple and intense saccharide–lectin interactions. On the contrary, one of the possible reasons for the less effective lectin affinity of the SPG-$Lac_{0.23}$–poly(dA) complex could be due to the densely-packed lactoside clusters along with a rigid triple-helix backbone, although we can not exclude other possibilities.[19]

As described above, the SPG-$Lac_{0.14}$–poly(dA) complex attains high thermal-stability as well as excellent lectin affinity, so we focused on this macromolecular complex in the following assays.

Agarose gel staining assay

Lectin-specific polynucleotide delivery by saccharide-appended SPGs was clearly demonstrated by confocal laser scanning microscopy (CLSM) observations using agarose beads bearing various lectins. In this assay, we used two kinds of agarose beads, bearing either RCA_{120} or ConA. They were expected to be good artificial models for hepatocytes and macrophages (or dendritic cells), respectively, based on their size (*ca.* 50 μm) and saccharide specificity.

3′-Rhodamine-labelled dA-45mer (Rho-$(dA)_{45}$) was complexed with SPG-Lac_n, SPG-Man_n and SPG to give the corresponding fluorescently labelled macromolecular complexes. The resulting complexes were incubated with agarose beads and then washed several times with buffer to remove unbound macromolecular complexes. The RCA_{120}-bearing agarose beads incubated with fluorescent SPG-$Lac_{0.14}$–Rho-$(dA)_{45}$ show a CLSM image (Fig. 5a) indicating that Rho-$(dA)_{45}$ is attached to the gel. On the other hand, no such image was observed for

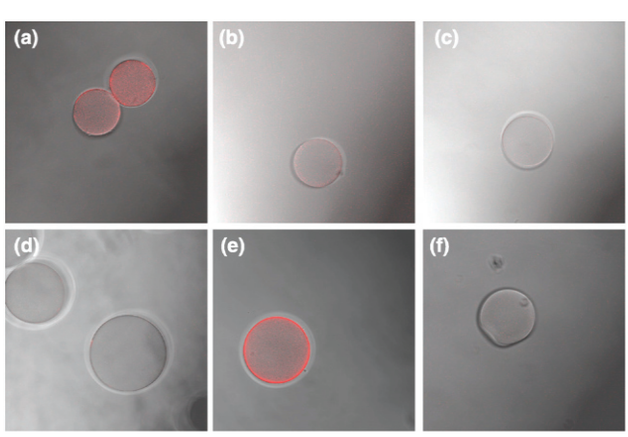


Fig. 5 Confocal laser scanning microscopic image of (a), (b) and (c) RCA_{120}-agarose beads and (d), (e) and (f) ConA-agarose beads stained by (a) and (d) SPG-$Lac_{0.14}$–Rho-$(dA)_{45}$, (b) and (e) SPG-$Man_{0.13}$–Rho-$(dA)_{45}$ and (c) and (f) SPG–Rho-$(dA)_{45}$ complex; 25 °C, Tris-buffer (20 mM, pH 7.2), [$CaCl_2$] and [$MnCl_2$] = 10 μM, Ex = 548 nm. Microscopic and fluorescence images are superimposed in each picture.

ConA-labelled agarose beads, clearly demonstrating that Rho-$(dA)_{45}$ is delivered in a lectin-specific manner (Fig. 5d). Similar lectin-specific delivery is also observed for SPG-$Man_{0.14}$, with which the complexed Rho-$(dA)_{45}$ is preferentially delivered onto ConA-labelled agarose beads (Fig. 5b and e). Furthermore, no such delivery was observed for SPG–Rho$(dA)_{45}$ (Fig. 5c and f). These data clearly indicate that SPG carrying saccharide appendages can deliver polynucleotides onto the agarose gels bearing lectins through specific saccharide–lectin recognition.

These data also reveal that the macromolecular complexes are *not* dissociated upon binding to lectins and, therefore, one can expect that the complexed polynucleotides are taken up by the target cell *via* receptor-mediated endocytosis, without unfavourable release at the cell surface.

Antisense assay

Receptor-mediated AS-ODN delivery was evaluated using human hepatocytes (HepG2) and SPG-$Lac_{0.14}$. In this assay, we used a complementary ODN (5′-GTGCCGGGGTCTTC-GGGC-3′) that is well-known to bind to c-myb mRNA and lead to depression of c-myb.[20] Our previous work revealed, however, that SPG does not form macromolecular complexes with short and hetero-ODNs.[21] We thus used a phosphorothioate-type ODN with a $(dA)_{40}$-tag (5′-GTGCCGGGGTCTTCGGGC-$(A)_{40}$-3′, AS-c-myb).[22]

Briefly, HepG2 cells were cultured in the presence of the SPG-$Lac_{0.14}$–AS-c-myb complex, the SPG–AS-c-myb complex or free AS-c-myb for 2 d and then the cell-numbers (N) were counted by using Cell Counting Kit-8 (Dojin).[23] As shown in Fig. 6a, SPG-$Lac_{0.14}$–AS-c-myb suppresses the cell-growth much more effectively than SPG–AS-c-myb or free AS-c-myb. This increase in antisense activity arises from two individual factors, that is, (i) the protection of complexed AS-c-myb and (ii) an intensified affinity toward hepatocytes. The former effect is clearly demonstrated by comparison between the SPG–AS-c-myb complex and free AS-c-myb, that is, SPG–AS-c-myb shows the enhanced antisense activity in comparison to free AS-c-myb, indicating that the complexed AS-c-myb is protected by SPGs against various deactivation processes including degradation by DNase and/or non-specific interactions with serum albumin.[12] Fig. 6b clearly shows that monomeric galactose (20 mM) reduces the antisense activity of the SPG-$Lac_{0.14}$–AS-c-myb complex to a similar level for the SPG–AS-c-myb complex, whereas it exerts no effect on that of SPG–AS-c-myb and free AS-c-myb. These data certify that enhanced antisense effects observed for SPG-$Lac_{0.14}$–AS-c-myb, in comparison to SPG–AS-c-myb, are clearly attributable to specific recognition between the β-lactoside appendages and asiallo-glycoprotein receptor on hepatocyte cell surfaces.[24]

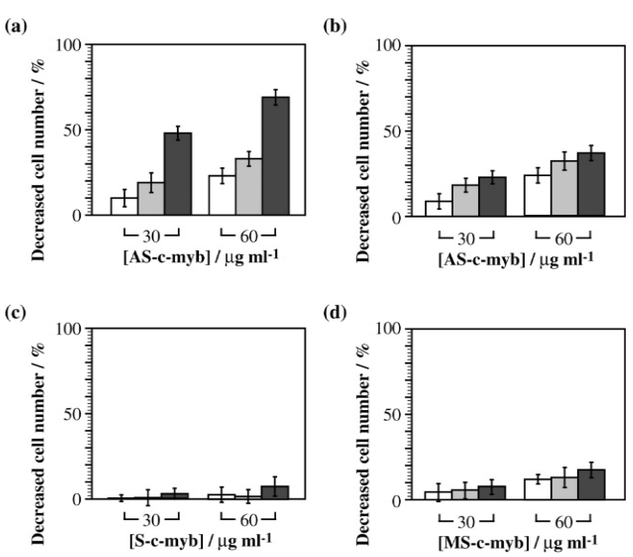


Fig. 6 Percentage of decreased cell number (ΔN) in comparison to the control. $\Delta N = 100 \times (N_{cont} - N) / N_{cont}$, where N is a cell number in the presence of (open bar) free ODNs, (closed grey bar) SPG–ODNs complex and (closed black bar) SPG-$Lac_{0.14}$–ODNs complex, and N_{cont} is that in the absence of ODNs: (a) AS-c-myb; (b) AS-c-myb and galactose (20 mM); (c) S-c-myb; and (d) MS-c-myb.

Similar antisense assays using $(dA)_{40}$-tagged sense (S-c-myb: 5′-CACGGCCCCAGAAGCCCG-$(A)_{40}$-3′) and mismatch sequence (MS-c-myb:5′-GT*CCT*GGGGTC*G*TCGGGC-$(A)_{40}$-3′) show the negligible decrease in the cell number, indicating that the decrease in the cell number observed for SPG-$Lac_{0.14}$–AS-c-myb complexes clearly arises from the antisense activity of AS-c-myb (Fig. 6c and d, respectively). However, we can observe a slight suppression of cell-growth for SPG-$Lac_{0.14}$–S-c-myb and SPG-$Lac_{0.14}$–MS-c-myb. In SPG-$Lac_{0.14}$, lactoside appendages are tethered with imino linkages that should be positively charged under neutral conditions. Although these positive charges are not essential for carrier–ODNs complex formation, they could be partially responsible for the observed cell-growth suppression.

We also confirmed that the observed suppression of cell growth was attributable to the knockdown of c-myb mRNA through reverse transcriptase-mediated PCR (RT-PCR) followed by agarose gel electrophoresis (Fig. 7), in which an amount of c-myb mRNA is strongly suppressed by the SPG-$Lac_{0.14}$–AS-c-myb complex whereas no such effect is observed for β-actin mRNA.[23]

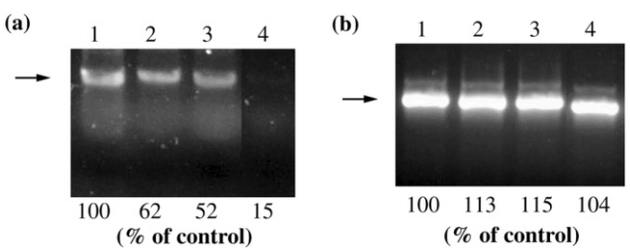


Fig. 7 Agarose gel electrophoresis of (a) c-myb mRNA and (b) β-actin mRNA after RT-PCR; lane 1, control; lane 2, free AS-c-myb; lane 3, SPG–AS-c-myb complex; lane 4, SPG-$Lac_{0.14}$–AS-c-myb complex; HepG2, [AS-c-myb] = 60 mg ml^{-1}, 2% agarose gel, 36 PCR-cycles for c-myb and 28 PCR-cycles for β-actin.

Conclusions

SPG with β-lactoside or α-mannoside appendages were synthesized through reductive amination between $NaIO_4$-oxidized SPG and amino-terminated saccharide derivatives. These SPG derivatives form stable complexes with polynucleotides which can interact with saccharide-binding proteins in a strong and specific manner. Furthermore, an SPG with β-lactosides can act as a potent antisense carrier for hepatocytes. Together with the fact that saccharide-binding proteins are widely distributed in the human body in a cell-specific manner (α-mannoside binding proteins on dendritic cells, *etc.*) and various SPG-derivatives with saccharide appendages are easily accessible through reductive amination, such SPG-derivatives could become potential candidates for non-toxic and cell-specific antisense carriers. We believe that the basic research data collected in this paper would be valuable to the development of such practically useful antisense carriers.

Experimental

General

^1H NMR spectra were acquired on a Bruker DRX600 (Bruker Co., Ltd) in $CDCl_3$ or D_2O at 600 MHz. The chemical shifts were reported in ppm (δ) relative to Me_4Si. IR spectra were recorded on a PerkinElmer Spectrum One Fourier transform infrared spectrometer attached with an universal ATR sampling accessory. Circular dichroism (CD) spectra were measured on a JASCO 720WI circular dichroism spectrometer. Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF)

mass spectra were recorded on PerSeptive Biosystems Voyager-DERP biospectrometry workstation. Silica gel 60 N (particle size 40–50 μm) for column chromatography was purchased from Kanto Chemical Co. Inc. Thin layer chromatography was carried out with Merck TLC aluminum sheets pre-coated with silica gel 60 F_{254}. Lectin-labelled agarose gels and biotin-labelled lectins were purchased from Seikagaku Kougyou (Japan). 3′-Rhodamine-labelled $(dA)_{45}$ was obtained from Hokkaido System Science., Ltd (Japan). AS-c-myb, S-c-myb and MS-c-myb were obtained from Hokkaido System Science (Hokkaido, Japan) and purified through reverse phase high-performance liquid chromatography (RP-HPLC). Native schizophyllan ($M_w = 1.5 \times 10^5$) was kindly supplied by Taito Co. Ltd., (Japan). HepG2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The fetal bovine serum (FBS), minimum essential medium (MEM®), non-essential amino acids and penicillin/streptomycin were purchased from Gibco/BRL. Sodium pyruvate was obtained from Sigma.

CD spectra measurements

SPG-Lac_n, SPG-Man_n and SPG in DMSO (5 mg ml^{-1}, 100 μl) were mixed with an aqueous solution of polynucleotide (1 mg ml^{-1}, 100 μl) and then diluted with Tris-HCl buffer (1 mM, pH 8.0, 1000 μl). The resulting solutions were incubated at 4 °C for 2 d and then subjected to CD spectral measurements.

SPR measurements

SPG-Lac_n, SPG-Man_n and SPG in DMSO (5 mg ml^{-1}, 100 μl) were mixed with an aqueous solution of poly(dA) (1 mg ml^{-1}, 100 μl) and then diluted with Tris-HCl buffer (100 mM, pH 8.0, 1000 μl) containing $CaCl_2$ (1.2 mM) and $MnCl_2$ (1.2 mM). The resulting solutions were incubated at 4 °C for 2 d before they were subjected to SPR assays.

Biacore biosensor and commercially available SPR chip immobilized with avidin–carboxymethyl dextran conjugate (sensor chip SA) was used in the SPR assay. Before the binding assay, avidin-immobilized SPR-tips were allowed to interact with biotin-labelled lectins (50 μg ml^{-1}) to yield lectin-immobilized surfaces.

The solvent system in the SPR assays was 83 mM Tris–HCl (pH 8.0) buffer containing DMSO (8.3 v/v), $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM).

CLSM observations

Rho-$(dA)_{45}$ aqueous solution (1.58×10^{-2} mg ml^{-1}, 100 μl) was mixed with SPG-Lac_n, SPG-Man_n and SPG in DMSO (5 mg ml^{-1}, 10 μl) and Tris–HCl buffer (10 mM, pH 8.0, 10 μl) then incubated at 4 °C for 2 d. The resulting solutions were mixed with lectin-labelled agarose beads in Tris-HCl buffer (10 mM, pH 8.0, [$CaCl_2$] and [$MnCl_2$] = 1 mM, 100 μl) and then incubated at rt for 15 min. The agarose beads were washed several times with buffer over 6 h to remove unbound macromolecular complexes and then subjected to the CLSM observation.

Antisense assays

ODNs (2 mg) were dissolved in Tris buffer (pH 7.8, 10 mM, 1 ml) and then SPG-$Lac_{0.23}$ or SPG in DMSO (9 ml) was added. In this assay, the molar ratio (M_{SPGs} : M_{ODNs}) was controlled at 1.5, where $M_{s\text{-}SPG}$ and M_{ODN} are the repeating molar concentration of SPGs and ODNs, respectively. The resultant mixtures were kept at 5 °C for 1 night for the complexation and then DMSO was removed by ultrafiltration. The final concentration of ODNs was determined based on their absorbance. To confirm the complexation between ODNs and SPGs, we measured the gel electrophoresis migration pattern.

The HepG2 cells were maintained in MEM® supplemented with 10% FBS, 1% non-essential amino acids, and 1 mM sodium pyruvate. All medium contains a 1 wt% penicillin : streptomycin mixture. The cell incubation was always carried out at 37 °C in fully humidified air containing 5 wt% of CO_2.

The HepG2 cells were seeded in 96-well plates (Nunc) at a density of 2×10^3 cells per well and allowed to attach to the plate overnight. On the following day, the medium was changed for a fresh medium, and cells were treated with an appropriate amount of SPG-$Lac_{0.23}$–ODN complex, SPG–ODNs complex, or free ODNs. Subsequently, cells were incubated for 3 d before measurement of the cell growth.

After incubation for 3 d, 10 μl of the Cell Counting Kit-8 (Dojindo, Japan) working solution containing WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolo]-1,3-benzene disulfonate sodium salt) and 1-methoxy-5-methylphenazinium methosulfate were added to each well and incubated for 4 h at 37 °C. Color developing of each well was evaluated by using microplate reader Multiskan JX (Thermo Labsystems, λ = 450 nm). Each sample was tested in triplicate, and the mean and standard deviation of the values were plotted. All growth studies were carried out at least twice.

1-*O*-(2″-Bromoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside

BF_3OEt_2 was added to octa-*O*-acetyl lactose (10.2 g) and 2-bromoethanol (3.0 ml) in anhydrous CH_2Cl_2 (50 ml) at rt and stirring was continued for 40 h under nitrogen atmosphere. The resulting mixture was diluted with ethylacetate and washed with $NaHCO_3$ saturated aqueous solution. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated. Although the residue was subjected to purification by silica gel column chromatography (hexane only, gradually changing to hexane–ethylacetate (1 : 1)), the R_f values of the starting material and the product were so close that we could not obtain the pure product. Structural proof was given by the MALDI-TOF mass spectrum of the crude product. $[M + Na]^+$ = 768.3 (calc. 765.1).

1-(2″-Azidoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside

1-*O*-(2″-Bromoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside (1.21 g, crude) was dissolved in 50 ml of dimethylformamide (DMF) and then sodium azide (0.13 g) was added. The mixture was stirred at 80 °C for 1.5 h, cooled to ambient temperature, poured into ethyl acetate and washed with water and NaCl saturated aqueous solution several times. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The product was purified by chromatography on a silica gel column (toluene–ethylacetate, 3 : 2) to give the product as a colorless solid. ^{1}H NMR ($CDCl_3$, TMS): 5.34 (d, J = 3.3 Hz, 1H), 5.20 (t, J = 9.2 Hz, 1H), 5.10 (dd, J = 8.1 and 10.4 Hz, 1H), 4.97 (dd, J = 3.3 and 10.4 Hz, 1H), 4.92 (t, J = 8.7 Hz, 1H), 4.57 (d, J = 7.9 Hz, 1H), 4.52 (d, J = 7.9 Hz, 1H), 4.52 (dd, J = 1.5 and 8.0 Hz, 1H), 4.14–4.07 (m, 3H), 4.00–3.97 (m, 1H), 3.90 (t, J = 6.7 Hz, 1H), 3.83 (t, J = 9.5 Hz, 1H), 3.70–3.67 (m, 1H), 3.65–3.62 (m, 1H), 3.49–3.45 (m, 1H), 3.29–3.25 (m, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.05 (s, 6H), 2.04 (s, 3H), 1.96 (s, 3H); IR (KBr, cm^{-1}) 1753 (acetyl) and 2110 (azide); $[M + H]^+$ = 706.2 (calc. 706.2).

1-(2″-Aminoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside

1-(2″-Azidoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside (0.35 g) was dissolved in 20 ml of ethanol and then 5% palladium on carbon (0.09 g) was added. The mixture was vigorously stirred under hydrogen atmosphere at rt for 3 h until TLC (chloroform–methanol 4 : 1) indicated complete conversion of the substrate. After acetic acid was added to protect amino function, the palladium on carbon was removed by filtration and the filtrate was evaporated to dryness. The product was purified by chromatography on a silica gel column (chloroform–methanol, 4 : 1 v/v) to give the pure product in a colorless solid (1.33 g, 13%, 3 steps). ^{1}H NMR ($CDCl_3$, TMS): 6.38 (br s, 3H), 5.35 (d, J = 1.5 Hz, 1H), 5.18 (t, J = 9.2 Hz, 1H), 5.10 (t, J = 9.8 Hz, 1H), 5.00 (dd, J = 2.5 and 10.3 Hz,

1H), 4.89 (t, J = 8.5 Hz, 1H), 4.70 (d, J = 11.7 Hz, 1H), 4.61 (d, J = 7.8 Hz, 1H), 4.58 (d, J = 7.8 Hz, 1H),4.16–4.03 (m, 5H), 3.93 (t, J = 6.6 Hz, 1H), 3.93 (t, J = 6.6 Hz, 1H), 3.84 (t, J = 9.4 Hz, 1H), 3.73 (m, 1H), 3.22 (m, 1H), 3.15 (m, 1H), 2.17 (s, 3H), 2.15 (s, 3H), 2.112 (s, 3H, OCOCH_3), 2.07–2.04 (m, 12H), 1.97 (s, 3H); IR (KBr, cm^{-1}) 1751 (acetyl); $[M + H]^+$ = 680.0 (calc. 680.2).

1-(2″-Aminoethyl)-β-lactoside

1-(2″-Aminoethyl)-2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-β-lactoside (2.51 g) was dissolved in 50 ml of ethanol and 10 ml of aqueous ammonia were added. The mixture was stirred at rt for 15 h, concentrated and lyophilized to give the pure product quantitatively without any purification procedure. ^{1}H NMR (D_2O): 4.38 (d, J = 8.0 Hz, 1H), 4.28 (d, J = 7.8 Hz, 1H), 3.98–3.95 (m, 1H), 3.83–3.75 (m, 3H), 3.71–3.55 (m, 4H), 3.52–3.45 (m, 4H), 3.38 (d, J = 8.9 Hz, 1H), 3.21 (d, J = 7.4 Hz, 1H), 3.11–3.09 (m, 1H); Anal. Calcd for $C_{14}H_{27}NO_{11}$: C, 43.63; H, 7.06; N, 3.63. Found: C, 42.38; H, 7.14; N, 3.61; $[M + H]^+$ = 386.4 (calc. 386.2).

SPG-Lac$_{0.14}$

Native schizophyllan (233.8 mg) in water (233.8 ml) was refluxed for 8 h to give homogeneous SPG aqueous solution. The solution was kept at 4 °C for 2 h and then $NaIO_4$ (23.7 mg), dissolved in the minimum amount of water, was added and stirred continuously at 4 °C for 2 d. After dialysis (MWCO 8000) against pure water, 2-aminoethyl-β-lactoside (640 mg) was added and the resultant solution was lyophilized to dryness and re-dissolved into 35 ml of DMSO. After additional 2-aminoethyl-β-lactoside (213 mg) was added and stirring was continued for 2 d, $NaBH_4$ (2.0 g) was added and stirring was kept for 2 d at rt. Afterwards, ethanol (3 ml) was added to destroy $NaBH_4$; the resulting mixture was poured into methanol and the white precipitate was filtered, washed with methanol and acetone repeatedly, re-dissolved into water, dialyzed (MWCO 8000) and lyophilized to give pure SPG-Lac$_{0.14}$ as a white powder. The conversion ratio (n) was estimated based on the following equation, where X is the nitrogen content revealed by elemental analysis and C, H, N and O are atomic weights of carbon, hydrogen, nitrogen and oxygen, respectively.

$$X = 2Nn / \{(24C + 40H + 20O)(1 - n) + (52C + 92H + 2N + 40O)n\} \quad (1)$$

In the preparation of SPG-Lac$_{0.05}$ and SPG-Lac$_{0.23}$, 8.0 and 39.5 mg of $NaIO_4$ were used, respectively, in the oxidation step to obtain SPG derivatives with various β-lactoside contents.

1-*O*-(2′-Bromoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannoside

BF_3OEt_2 (17.6 ml) was added to penta-*O*-acetyl–mannoside (25.0 g) and 2-bromoethanol (13.6 ml) in anhydrous CH_2Cl_2 (200 ml) at rt and the mixture was stirred continuously for 40 h under nitrogen atmosphere. The resulting mixture was diluted with ethyl acetate and washed with $NaHCO_3$ saturated aqueous solution. The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated. Although the residue was subjected to the purification by silica gel column chromatography (hexane : hexane–ethylacetate (1 : 1)), the R_f values of the starting material and the product were so close that we could not obtain the pure product. Structural proof was given by the MALDI-TOF mass spectrum of the crude product. $[M + Na]^+$ = 477.09 (calc. 477.04).

1-(2′-Azidoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannoside

1-*O*-(2′-Bromoethyl)-tetra-*O*-acetyl-α-mannoside (27 g, crude) was dissolved in DMF (200 ml) and then sodium azide (4.1 g) was added. The mixture was stirred at 80 °C for 1.5 h, cooled to ambient temperature, poured into ethyl acetate and washed with water and NaCl saturated aqueous solution several times. The organic layer was dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The product was purified by chromatography on a silica gel column (toluene–ethylacetate, 3 : 2 v/v) to give the product as a colorless solid (4.08 g,). ^{1}H NMR ($CDCl_3$, TMS): 5.37 (dd, J = 3.3 and 10.0 Hz, 1H), 5.31 (m, 2H), 4.88 (s, 1H), 4.30 (dd, J = 5.3 and 12.2 Hz, 1H), 4.13 (d, J = 12.2 Hz, 1H), 4.05 (m, 1H), 3.88 (m, 1H), 3.68 (m, 1H), 3.51 (m, 1H), 3.45 (m, 1H), 2.17 (s, 3H), 2.11 (s, 3H), 2.06 (s, 3H), 2.00 (s, 3H); IR (KBr, cm^{-1}) 2104 (azide), 1742 (acetyl); $[M + Na]^+$ = 440.08 (calc. 440.03).

1-(2′-Aminoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannoside

1-(2′-Azidoethyl)-tetra-*O*-acetyl-α-D-mannoside (2.42 g) was dissolved in ethanol (100 ml) and then a catalytic amount of 5% palladium on carbon was added. The mixture was vigorously stirred under hydrogen atmosphere at rt for 12 h until TLC (chloroform–methanol, 4 : 1) indicated complete conversion of the substrate. After acetic acid was added to protect amino function, the palladium on carbon was removed by filtration and the filtrate was evaporated to dryness. The product was purified by chromatography on a silica gel column (chloroform–methanol, 4 : 1 v/v) to give the pure product in a colorless syrup (2.04 g, 8.1%, 3 steps). ^{1}H NMR ($CDCl_3$, TMS): 5.34 (dd, J = 3.3 and 10.0 Hz, 1H), 5.29 (m, 2H), 4.87 (s, 1H), 4.30 (dd, J = 4.9 and 12.2 Hz, 1H), 4.10 (d, J = 12.2 Hz, 1H), 4.10 (brs, 3H), 3.71 (m, 1H), 3.04 (m, 1H), 2.92–2.01 (m, 2H), 2.21–2.08 (s × 4, 12H); IR (KBr, cm^{-1}) 3554 (amino), 3475 (amino), 1750 (acetyl); $[M + Na]^+$ = 414.13 (calc. 414.14).

1-(2′-Aminoethyl)-α-D-mannoside

1-(2′-Aminoethyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannoside (2.04 g) was dissolved in ethanol (40 ml) and aqueous ammonia (30 ml) was added. The mixture was stirred at rt for 12 h, concentrated and lyophilized to give the pure product without any purification procedure. ^{1}H NMR (D_2O, TMS): 4.80 (s, 1H), 3.91 (m, 2H), 3.87 (m, 2H), 3.70 (m, 2H), 3.63 (m, 2H), 3.52 (m, 1H), 3.12 (m, 2H); IR (KBr, cm^{-1}) 3278 (amino), 1659 (amino); Anal. Calcd for $C_8H_{17}NO_6$: C, 43.04; H, 7.68; N, 6.27. Found: C, 41.03; H, 7.81; N, 5.38; $[M + Na]^+$ = 224.08 (calc. 224.11).

SPG-Man$_{0.13}$

Native schizophyllan (100 mg) in water (100 ml) was refluxed for 3 h to give homogeneous SPG aqueous solution. The solution was kept at 4 °C for 2 h and then $NaIO_4$ (9.97 mg), dissolved in the minimum amount of water, was added and stirring was continued at 4 °C for 2 d. After dialysis (MWCO 15000), aminoethyl-α-mannoside (334 mg), 1 M NaOH (1 drop) and $NaBH_3CN$ (200 mg) was added to the resulting mixture and then stirring was continued for 3 d at rt. After $NaBH_4$ was added and the stirring was continued for additional 8 h at rt, the resulting mixture was dialyzed (MWCO 3500) and lyophilized to give pure SPG-Man$_{0.13}$ as a white powder. In the preparation of SPG-Man$_{0.05}$ and SPG-Man$_{0.22}$, 3.32 and 16.62 mg of $NaIO_4$ were used, respectively, in the oxidation step to obtain SPG derivatives with various α-mannoside contents. The conversion ratio (n) was estimated based on the following equation, where X is the nitrogen content revealed by elemental analysis and C, H, N and O are atomic weights of carbon, hydrogen, nitrogen and oxygen, respectively.

$$X = 2Nn / \{(24C + 40H + 20O)(1 - n) + (40C + 72H + 2N + 30O)n\} \quad (2)$$

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