Carbohydrate-appended curdlans as a new family of glycoclusters with binding properties both for a polynucleotide and lectins

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β-1,3-Glucans having carbohydrate-appendages (α-D-mannoside, *N*-acetyl-β-D-glucosaminide and β-lactoside) at the C6-position of every repeating unit can be readily prepared from curdlan (a linear β-1,3-glucan) through regioselective bromination/azidation to afford 6-azido-6-deoxycurdlan followed by chemo-selective Cu(I)-catalyzed [3 + 2]-cycloaddition with various carbohydrate modules having a terminal alkyne. The resultant carbohydrate-appended curdlans can interact with polycytosine to form stable macromolecular complexes consistent with two polysaccharide strands and one polycytosine strand. Furthermore, these macromolecular complexes show strong and specific affinity toward carbohydrate-binding proteins (lectins). Therefore, one can utilize these carbohydrate-appended curdlans as a new family of glycoclusters.

Introduction

Carbohydrates are recognized as key substances to construct major cell surface antigens, to mediate cell–cell recognition and to control signal transductions through cell membranes.[1] The carbohydrate chains on cell surfaces also function as receptors for various viruses in the first stage of infection. These functions are highly dependent on the density of carbohydrate chains on cell surfaces: that is, clustered or multivalent carbohydrate chains are essential to induce strong and specific carbohydrate recognition events.

The clustered carbohydrate arrays on artificial molecular systems (artificial glycoclusters) also induce strong and specific carbohydrate recognition processes owing to their multivalent carbohydrate arrays, and their applications have been expanded into a vast research area including therapeutics.[2] For example, artificial glycoclusters based on simple linear polymers (polyacrylamide,[3] polystyrene,[4] *etc.*) have great potential to: 1) neutralize fatal viruses/toxins,[5] and 2) inhibit cancer metastases,[6] mainly by blocking interactions between carbohydrate-recognition proteins on their surfaces and the carbohydrate chains on target cell surfaces.

Besides the enhanced affinity toward carbohydrate-recognition proteins, additional functions originated from their polymeric scaffolds make the glycoclusters quite interesting biomaterials, and various glycoclusters based on functional polymers have been reported. For example, polythiophene-based glycoclusters were designed as potential sensory systems for lectins, viruses and toxins.[7] Furthermore, cyclodextrin-,[8] fullerene-[9] and DNA-based glycoclusters[10] have been also developed for cell-specific drug delivery, cell-specific photodynamic therapy and cell-targeted gene therapy, respectively.

Recently, we have been interested in natural polysaccharides as scaffolds for artificial glycoclusters, since they have unique bioactivities, such as activation of immune systems (chitin/chitosan) and anti-blood-coagulation (heparin/heparin sulfate).[11] Polysaccharide-based glycoclusters should be therefore potential biomaterials in pharmaceutical and medicinal purposes. The design and synthesis of polysaccharide-based glycoclusters with homogeneous chemical structures is quite important to reveal their structure–function relationship and to improve their functions.

One potential approach to obtain polysaccharide derivatives having functional groups at the desired positions is a chemo-enzymatic strategy reported by S. Kobayashi *et al.* and others.[12] In this strategy, chemically modified carbohydrate monomers are enzymatically polymerized to the corresponding polysaccharides (the bottom-up approach, Fig. 1a). However, the molecular weights of the resultant polysaccharides are relatively low due to enzyme-mediated hydrolysis (reverse reaction), and polysaccharides with large molecular weights are, therefore, hardly ever obtained. Furthermore, since the enzymes (glycosynthases) have strong specificity for their substrates, the functional groups attached onto the carbohydrate monomers are limited to small and therefore less functional ones (such as the methoxy group), and introduced bulky substituents (carbohydrate units) strongly lower the binding affinity of the corresponding carbohydrate monomers to the enzymes. Tedious synthetic routes to the modified carbohydrate monomers are also a serious obstacle, especially for those who are not expert at synthetic organic chemistry. No glycocluster has therefore been developed through this bottom-up strategy so far.

Direct introduction of carbohydrate modules onto polysaccharide scaffolds (the polymer-modification approach, Fig. 1b) is an

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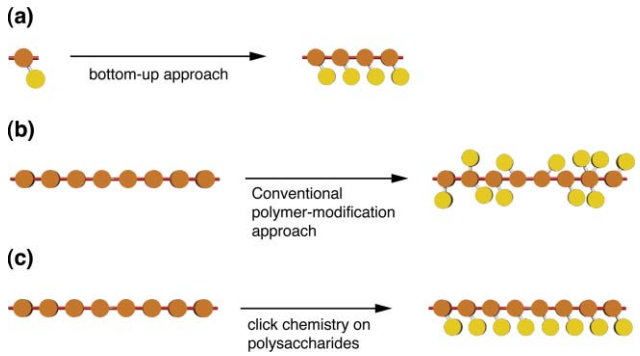


Fig. 1 Three approaches to prepare polysaccharide-based glycoclusters: (a) the enzymatic polymerization of chemically modified carbohydrate monomers (bottom-up approach), (b) the direct glycosylation of polysaccharides (polymer-modification approach) and (c) the click chemistry on polysaccharides, discussed in this paper.

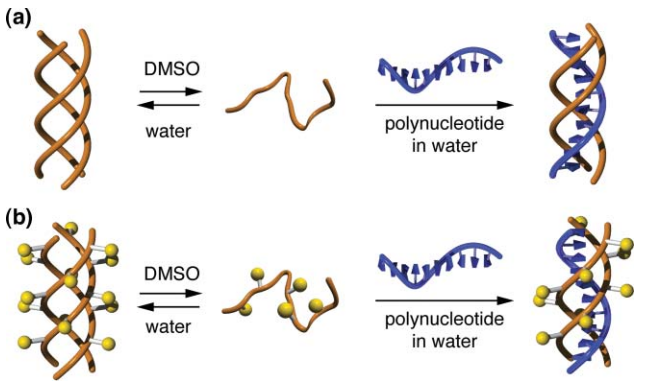


Fig. 2 Solvent-induced structural transition of (a) curdlan and (b) carbohydrate-appended curdlans and their complexation with polynucleotides.

alternative approach to access polysaccharide-based glycoclusters; however, is quite difficult to obtain glycoclusters with a well-defined chemical structure by regioselective glycosylation of the native polysaccharides.[13] Since their hydroxy groups have similar reactivities toward electrophiles, regioselective and quantitative reactions are hardly ever accomplished. Many research groups have directed intense research effort into exploiting chemical glycosylation procedures to obtain polysaccharide-based glycoclusters with homogeneous structure; however, no successful example has been reported so far.

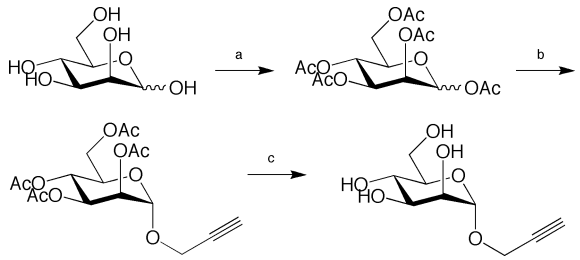
Recently, two new synthetic methods have emerged. One is a direct bromination/azidation reaction of a linear β-1,3-glucan (curdlan) to afford 6-azido-6-deoxy-curdlan.[14] A great advantage of this reaction is its exclusive C6 regioselectivity and high yield (100% conversion). The other method is a Cu(I)-catalyzed chemo-selective coupling between organic azides and terminal alkynes which has attracted increasing research interest owing to its convenient, quick and quantitative reaction.[15] This chemo-selective coupling, now termed "click chemistry", has been used for various applications, including chemical modification of self-assembled monolayers (SAMs),[16] ligation between polymer strands,[17] *etc.* These excellent studies encouraged us to establish "click chemistry" on polysaccharides, that is, chemo-selective coupling between azido-appended polysaccharides and alkyne-terminated oligosaccharide modules to provide polysaccharide-based glycoclusters having both chemically pure structures and large molecular weights (Fig. 1c).[18]

Among the natural polysaccharides, β-1,3-glucans are one of the most interesting polysaccharide families, because they have unique structural and conformational properties: that is, the β-1,3-glucans exist as triple-stranded helices in water but they dissociate into single-stranded random coils in dimethylsulfoxide (DMSO) (denaturing process, Fig. 2a).[19] This solvent-induced conformational transition is entirely reversible, and addition of water to the β-1,3-glucans in DMSO induces re-construction of their triple-stranded helical structures (re-naturing process). Recently, we found that, when certain polynucleotides (polycytosine, polydeoxyadenosine, *etc.*) are present during the re-naturing process, unique triple-stranded macromolecular complexes composed of one polynucleotide strand and two polysaccharide strands are formed.[20] In the course of our research, we found several advantages of the macromolecular complexes: 1) they are thermally stable under physiological conditions,[21] 2) the complexed polynucleotides are protected against degradation by DNase/RNase,[22] and 3) the complexed polynucleotides can be quickly released in the presence of their DNA/RNA complement.[23] In addition to the expected long blood-circulation time arising from lack of β-1,3-glucanase in human body, these characteristics suggest a potential utility of β-1,3-glucans as polynucleotide-carriers toward human cells. Furthermore, since a variety of mammalian cells have their unique patterns of carbohydrate-binding proteins on their cell surfaces, β-1,3-glucan-based glycoclusters should have great potential as cell-specific polynucleotide carriers.[24] Recently, we reported that a curdlan modified with β-lactoside appendages can form a triple-stranded macromolecular complex with poly(C), and that the resultant complex can interact with a specific lectin.[18] Herein, we report the synthesis of various β-1,3-glucan-based glycoclusters having α-D-mannoside- (CUR-Man), *N*-acetyl-β-D-glucosaminide- (CUR-GlcNAc) and β-lactoside-appendages (CUR-Lac) and their binding properties both with polycytosine and lectins (Fig. 2b).

Results and discussion

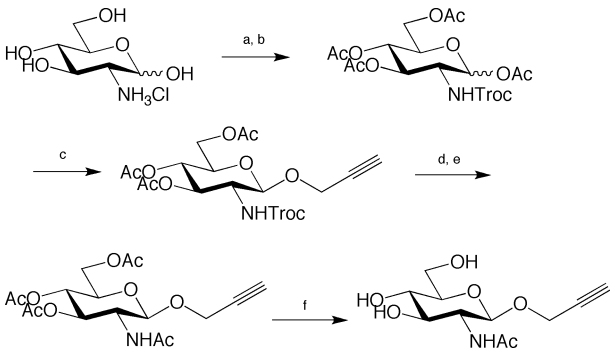
Synthesis of alkyne-terminated carbohydrate-modules

Carbohydrate modules carrying a terminal alkyne group were prepared through two different synthetic routes.[25] The first was direct acetylation of all hydroxy groups of the corresponding natural carbohydrates and subsequent glycosylation with propargyl alcohol (mediated by boron trifluoride) followed by deacetylation. 1-*O*-(2′-Propargyl)-α-D-mannoside and 1-*O*-(2′-propargyl)-β-lactoside were prepared through this synthetic procedure (Scheme 1 for 1-*O*-(2′-propargyl)-α-D-mannoside as a representative). The other was applied to D-glucosamine hydrochloride to afford an alkyne-terminated *N*-acetyl-β-D-glucosaminide module. In this synthetic procedure, the amino-functionality of D-glucosamine hydrochloride was first protected with 2,2,2-trichloroethyl chloroformate (TrocCl) in the presence of sodium bicarbonate. The subsequent acetylation of all hydroxy groups and the following glycosylation with propargyl alcohol afforded 3,4,6-tri-*O*-acetyl-1-(2′-propargyl)-2-*N*-(2′,2′,2′-trichloroethoxy)-β-D-glucosaminide. The subsequent zinc-mediated deprotection of the Troc group followed



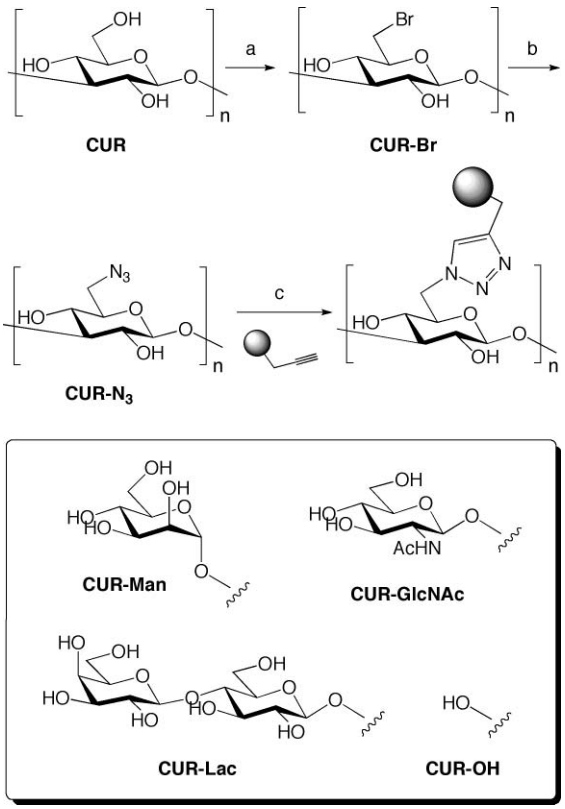
Scheme 1 Synthesis of the α-D-mannoside module. *Reagents and conditions*: a) Ac_2O, Py, r.t., 6 h, 94%; b) 2-propargyl alcohol, $BF_3{\cdot}OEt_2$, CH_2Cl_2, r.t., 45 h, 52%; c) aqueous ammonia, THF, MeOH, r.t., 2 h, 95%.

by acetylation of the amino group afforded 2-*N*-acetyl-3,4,6-tri-*O*-acetyl-1-(2′-propargyl)-β-D-glucosaminide. Deacetylation of all its *O*-acetyl groups afforded 2-*N*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosaminide (Scheme 2).



Scheme 2 Synthesis of the *N*-acetyl-β-D-glucosaminide module. *Reagents and conditions*: a) TrocCl, $NaHCO_3$, water, 1 day, 87%, b) Ac_2O, Py, r.t., 6 h, 98%; c) 2-propargyl alcohol, $BF_3{\cdot}OEt_2$, CH_2Cl_2, r.t., 46 h, 32%; d), Zn powder, acetic acid, r.t., 1 h; e) acetic anhydride, acetic acid, r.t., 2 h, 68% (2 steps); f) aqueous ammonia, THF, MeOH, r.t., 2 h, 91%.

Preparation of 6-azido-6-deoxy-curdlan. Curdlan (CUR) was first converted into 6-bromo-6-deoxycurdlan (CUR-Br) according to the literature, namely, activation of the primary hydroxy groups with triphenylphosphine followed by bromination with carbon tetrabromide (Scheme 3). The resultant CUR-Br shows a simple ^{13}C NMR spectrum, in which the peak due to the bromomethyl group (-*C*H_2Br, 44.27 ppm) has replaced that of the hydroxymethyl group (-*C*H_2OH, 60.90 ppm). There are no unassignable peaks, confirming the quantitative and selective bromination of all the C6 positions of native curdlan. The resultant CUR-Br can then be quantitatively converted into 6-azido-6-deoxycurdlan (CUR-N_3) through nucleophilic substitution with sodium azide in DMSO (85 °C, 36 h). The subsequent reprecipitation in methanol afforded pure CUR-N_3 as a brown powder. The quantitative and exclusive conversion of primary hydroxy groups into azido groups was confirmed by a ^{13}C NMR spectrum of the product, in which a peak due to the azidomethyl group (-*C*H_2N_3, 50.72 ppm) was observed, and there were no peaks assignable to the hydroxymethyl or bromomethyl groups. Gel permeation chromatography (GPC) analyses of CUR-Br and CUR-N_3 show decreases in molecular weight for the bromination/azidation steps, indicating that the β-1,3-glucan main-chain is cleaved to some extent under these reaction conditions. It should be, however, emphasized that the



Scheme 3 Synthesis of curdlan derivatives having α-D-mannoside (CUR-Man), *N*-acetyl-β-D-glucosaminide (CUR-GlcNAc), β-lactoside (CUR-Lac) and hydroxy groups (CUR-OH). *Reagents and conditions*: a) triphenylphosphine, DMF, LiCl, r.t., 3 h, and then carbon tetrabromide, 60 °C, 24 h; b) sodium azide, DMSO, 85 °C, 36 h; c) alkyne-terminated carbohydrate modules, $CuBr_2$, ascorbic acid, propylamine, r.t., 12 h, DMSO.

molecular weight of CUR-N_3 is still high enough (MW = 82 kDa) to be a scaffold for the artificial glycoclusters.

Cu(I)-catalyzed [3 + 2]-cycloadditions of the alkyne-terminated saccharide modules onto CUR-N_3. Addition of the alkyne-terminated carbohydrate modules onto CUR-N_3 was achieved in DMSO containing $CuBr_2$, ascorbic acid and propylamine. Other solvent systems, such as methanol and water, are also widely recognized as being excellent for this coupling reaction, but the low solubility of CUR-N_3 in these solvent systems, however, strongly restricts utilization of these solvents. In contrast, DMSO can easily dissolve CUR-N_3 to provide a homogeneous solution, and therefore the reaction proceeds smoothly. We confirmed that *N*-methyl-2-pyrrolidone (NMP) is also a good solvent to dissolve CUR-N_3 and to smoothly mediate this reaction. It should be noted that, as we already reported in the literature,[18] propylamine acts as a base to accelerate the reaction, and the reaction is completed within one hour. This coupling reaction has several advantages as mentioned below. 1) Since the procedure for this reaction is quite easy and no stringent conditions (*viz.* water-free or degassed solvents) are required, this synthetic strategy can be widely accepted even for non-specialists. 2) Since CUR-N_3 shows a strong IR peak at 2100 cm^{-1} and no other functionality shows any peaks around this region, the reaction can be easily monitored *in situ* by an ATR-IR spectrometer. The resultant

curdlan derivatives having various carbohydrate-appendages can be obtained as white powders after purification through dialysis (MWCO8000) and subsequent lyophilization.

In addition to their IR spectra showing no residual azido-peak, the ^{13}C NMR spectra clearly showed quantitative conversion of CUR-N_3 into the corresponding CUR-derivatives (Fig. 3 for CUR-GlcNAc as a representative), which is clearly evidenced by: 1) no residual signal of the azido-methyl group, 2) new peaks assignable to the carbohydrate-appendages, 3) two new peaks (145 and 124 ppm) assignable to the 1,4-triazole-linker and 4) no unassignable peaks. It should be emphasized that these extremely bulky carbohydrate modules cannot be introduced into polysaccharides through the chemo-enzymatic bottom-up strategy. Furthermore, GPC analysis revealed that the carbohydrate-appended CUR-derivatives have large molecular weights (MW = 123 kDa, 129 kDa and 145 kDa for CUR-Man, CUR-GlcNAc and CUR-Lac, respectively), which cannot obtained through the chemo-enzymatic bottom-up approach. Here, it should again be emphasized that our approach is the only one enabling access to chemically pure multivalent β-1,3-glucan-based glycoclusters.

Macromolecular complex formation between the carbohydrate-appended curdlans and polycytosine. Macromolecular complexes composed of the β-1,3-glucan-based glycoclusters and polycytosine (poly(C)) were easily prepared by mixing the β-1,3-glucan-based glycoclusters in DMSO and poly(C) in water. Evidence for complex formation was obtained from the CD spectra (Fig. 2). For example, the CUR-Man/poly(C) complex shows CD spectra, in which: 1) the predominant negative peak (275 nm) observed for free poly(C) was strongly enhanced and slightly blue-shifted (~3 nm) and 2) a new positive (242 nm) peak appeared (Fig. 4). The observed CD spectral changes are consistent with those of a CUR/poly(C) complex with two CUR strands and one poly(C) strand. Together with this CD spectral change, stoichiometric analysis based on job plots indicate the formation of triple-stranded macromolecular complexes composed of two CUR-Man strands and one poly(C) strand.

We measured the CD spectra at various temperatures to assess the thermal stability of the complexes (Fig. 5). For example, the

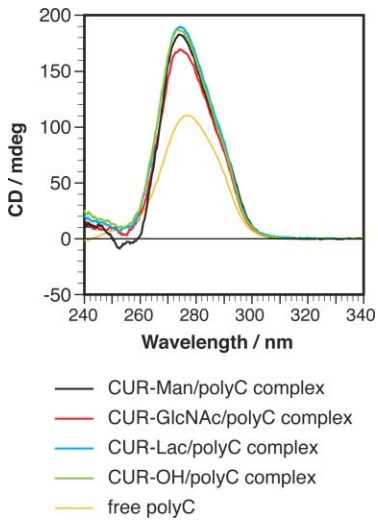


Fig. 4 CD spectra of CUR-Man/poly(C) (black line), CUR-GlcNAc/poly(C) (red line), CUR-Lac/poly(C) (blue line), CUR-OH/poly(C) (green line) and free poly(C) (orange line). [poly(C)] = 0.08 mg ml^{-1}, [CUR-derivatives] = 20.4 $mM_{repeating\text{-}unit}$ ml^{-1}, [DMSO] = 8.4% v/v, 5 °C, d = 1.0 cm, Tris-buffer (0.83 mM, pH 8.0).

CD spectrum of the CUR-Man/poly(C) complex was independent of the temperature up to 41 °C; however, it suddenly changed at around 45 °C into that assignable to free poly(C), indicating the *cooperative* dissociation of the complex. The CUR-Man/poly(C) complex showed a melting temperature (T_m) at around 44 °C, which is sufficiently higher than physiological temperature.

The other β-1,3-glucan-based glycoclusters (CUR-GlcNAc and CUR-Lac) can also interact with poly(C) to form triple-stranded macromolecular complexes with similar CD spectra to CUR-Man/poly(C). Their melting temperatures are, however, different from each other depending on the structure of their carbohydrate appendages; that is, the melting temperatures of CUR-Man/poly(C) (44 °C) and CUR-GlcNAc/poly(C) (43 °C) are significantly higher than that of CUR-Lac/poly(C) (36 °C). At the present time, we have no decisive information to explain

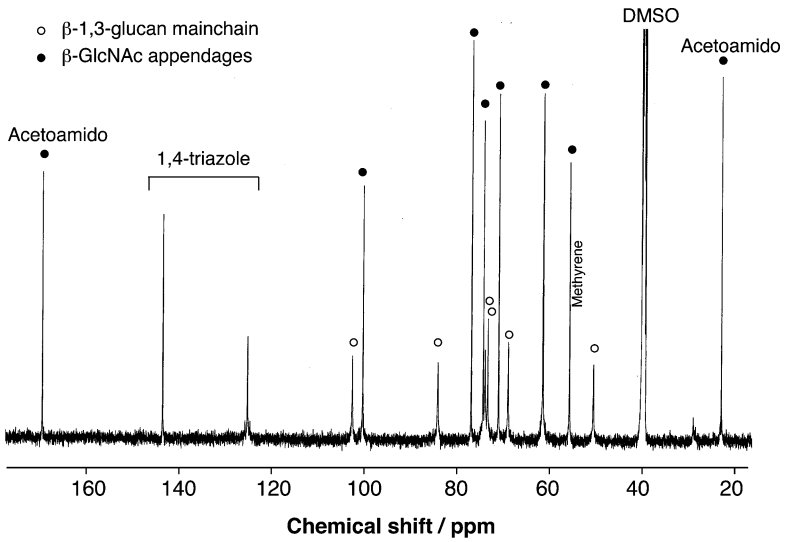


Fig. 3 ^{13}C NMR spectrum of CUR-GlcNAc: 600 MHz, DMSO-d_6, 60 °C.

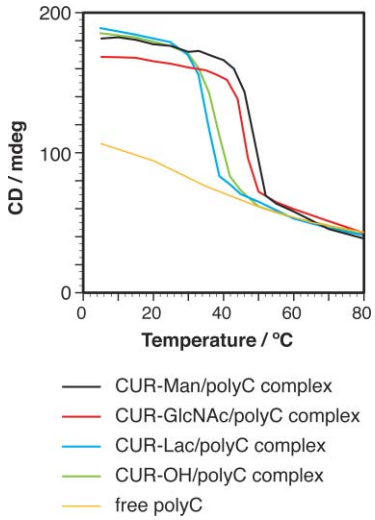


Fig. 5 Temperature-dependent CD intensities of CUR-Man/poly(C) (black line), CUR-GlcNAc/poly(C) (red line), CUR-Lac/poly(C) (blue line), CUR-OH/poly(C) (green line) and free poly(C) (orange line). [poly(C)] = 0.08 mg ml^{-1}, [CUR-derivatives] = 20.4 mM$_{\text{repeating-unit}}$ ml^{-1}, [DMSO] = 8.4% v/v, 5 °C, d = 1.0 cm, Tris-buffer (0.83 mM, pH 8.0).

this difference in T_m values, but we consider, however, that comparison between the T_m values of the macromolecular complexes composed of these curdlan-derivatives and that composed of curdlan without a carbohydrate-appendage (CUR-OH) can supply useful information to explain the difference.[26] Since CUR-OH/poly(C) shows a relatively low T_m value, the high T_m values of CUR-GlcNAc/poly(C) and CUR-Man/poly(C) could arise from multiple hydrogen-bonding between the carbohydrate appendages and the complex main-chains, stabilizing the macromolecular complexes. The relatively low T_m value of CUR-Lac/poly(C) could be attributable to steric hindrance between the bulky disaccharide appendages and the triple-stranded complex main-chain, which should compensate for the stabilization due to multiple hydrogen-bonding between the β-lactoside appendages and the complex main-chain.

Specific affinity between the carbohydrate-appended complexes and carbohydrate-binding proteins. Affinities between the carbohydrate-appended macromolecular complexes and carbohydrate-binding proteins (lectins) were assessed through surface plasmon resonance (SPR) assays using lectin-immobilized Au surfaces. In this assay, we used concanavalin A (ConA), wheat germ agglutinin (WGA) and *Ricinus communis* agglutinin (RCA_{120}) as lectins that specifically bind to α-Man-, β-GlcNAc- and β-Lac-residues, respectively.

As shown in Fig. 6, an injection of the CUR-Man/poly(C) complex induced a rapid increase in the resonance unit for ConA-immobilized Au surfaces, indicating a strong binding of this macromolecular complex onto the ConA-immobilized Au surface. On the other hand, no such increase was observed for any other macromolecular complexes, including CUR-GlcNAc/poly(C), Lac/poly(C) and CUR-OH/poly(C). These results clearly indicate that: 1) the binding between CUR-Man/poly(C) and ConA-immobilized Au surface is specific, and 2) this specific interaction is mediated by the α-Man-appendages of CUR-Man/poly(C) and the immobilized ConA on the Au surface. Such specific

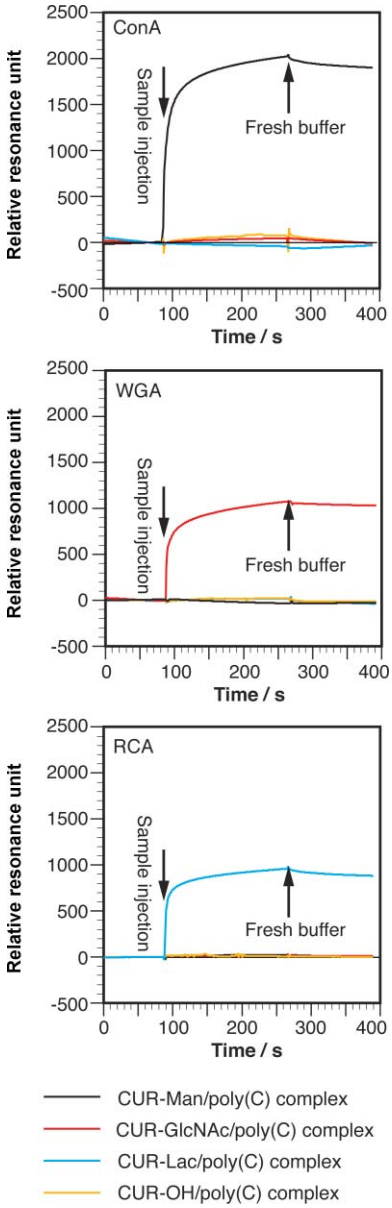


Fig. 6 SPR sensorgrams obtained by ConA-, RCA_{120}- and WGA-immobilized Au surfaces: 0.83 mM Tris-HCl buffer (pH 8.0) containing $CaCl_2$ (8.3 mM), $MnCl_2$ (8.3 mM) and DMSO (4.2 wt%), 25 °C, flow rate = 20 μl min^{-1}, [poly(C)] = 0.04 mg ml^{-1}, [CUR-Lac], [CUR-Man], [CUR-GlcNAc], or [CUR-OH] = 10.2 mM$_{\text{repeating-unit}}$.

interactions can be also observed for other lectins: CUR-GlcNAc/poly(C) specifically interacts with WGA, and CUR-Lac/poly(C) specifically interacts with RCA_{120}. Association (k_a) and dissociation rate constants (k_d) of these specific bindings were estimated through computational curve fitting on the association and dissociation phases, and their affinity constants ($K_a = k_a/k_d$) were estimated to be 1.7×10^6 M^{-1} (CUR-Man/poly(C)), 8.5×10^5 M^{-1} (CUR-GlcNAc/poly(C)) and 5.5×10^5 M^{-1} (CUR-Lac/poly(C)). These high K_a values should arise from the enhanced affinity between the clustered carbohydrate appendages of these macromolecular complexes and the lectins on the Au surfaces.

The specific lectin-affinity of these macromolecular complexes is also confirmed through staining assay of lectin-immobilized agarose beads. Here, it should be noted that the agarose beads have a spherical shape with an average size (~50 nm) similar to that of most native cells, and therefore, lectin-immobilized agarose beads can be recognized as the simplest model of native cells. In this assay, rhodamine-labeled macromolecular complexes were used to fluorescently stain the agarose beads. The carbohydrate-appended curdlan-derivatives were first dissolved in DMSO and then mixed with water containing rhodamine-labeled poly(C) (Rho-poly(C)) to give the corresponding macromolecular complexes carrying fluorescent probes. The resultant macromolecular complexes were incubated with the lectin-immobilized agarose beads in Tris-HCl buffer (pH 7.2). The resultant agarose beads were then washed with fresh Tris-HCl buffer repeatedly to remove non-specifically adsorbed macromolecular complexes. Confocal laser scanning microscopic (CLSM) images of the resultant agarose beads show specific interactions between the carbohydrate-appended macromolecular complexes and the lectin-immobilized agarose beads (Fig. 7). For example, ConA-immobilized agarose beads were fluorescently stained by CUR-Man/Rho-poly(C) although no other macromolecular complex, such as CUR-GlcNAc/Rho-poly(C) or CUR-Lac/Rho-poly(C), could stain them. These data clearly indicate that the observed binding of CUR-Man/Rho-poly(C) onto ConA-immobilized agarose beads is attributable to the specific interaction between the α-Man-appendages of CUR-Man/Rho-poly(C) and the ConA-units of ConA-immobilized agarose beads. Such specific binding properties were also observed for other carbohydrate-appended macromolecular complexes: WGA- and RCA_{120}-immobilized agarose beads were fluorescently stained by CUR-GlcNAc/Rho-poly(C) and CUR-Lac/Rho-poly(C), respectively, in a specific manner.

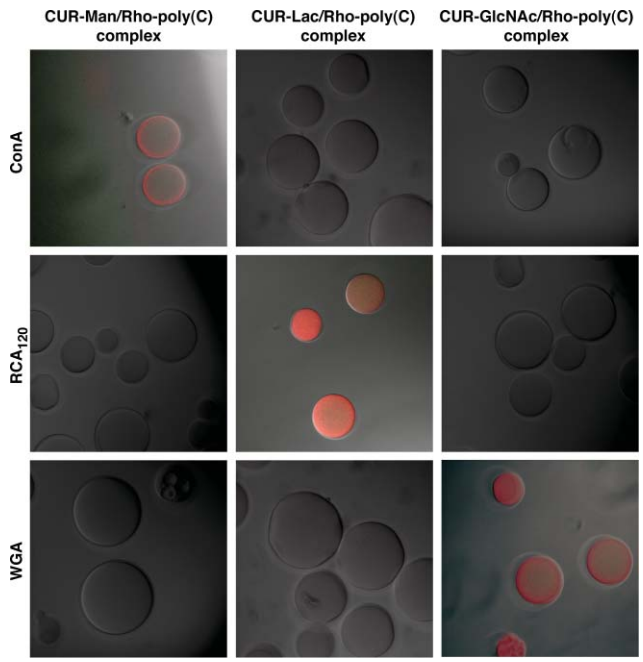


Fig. 7 Confocal fluorescent microscopic images of (top) ConA- (middle) RCA_{120}- and (bottom) WGA-agarose beads stained by (left) CUR-Man/Rho-$(C)_{45}$ complex, (middle) CUR-Lac/Rho-$(C)_{45}$ complex and (right) CUR-GlcNAc/Rho-$(C)_{45}$ complex: 25 ºC, Tris-buffer (20 mM, pH 7.2) containing $CaCl_2$ (10 mM) and $MnCl_2$ (10 mM), $\lambda_{ex} = 548$ nm.

Since the fluorescent probes are attached onto the polynucleotide components in the macromolecular complexes, these CLSM images indicate that the oligosaccharide-appended curdlans deliver the complexed polynucleotides onto the surface of agarose beads based on the specific carbohydrate–lectin interactions. Together with the fact that natural cells express carbohydrate-binding proteins on their surfaces in a cell-specific manner (*e.g.*, the asialo-glycoprotein receptor in hepatocytes), these data strongly support the potential of oligosaccharide-appended curdlans as cell-specific polynucleotide carriers.

Conclusions

We prepared glycoclusters with a β-1,3-glucan main-chain through the direct addition of multiple copies of carbohydrate modules onto curdlan. Since the reaction is highly selective for primary hydroxy groups, the glycoclusters are homogeneous in the chemical structures of their repeating units: that is, the carbohydrate modules are attached not onto the C2 or C4 positions but onto the C6 position of every repeating unit. Such structural homogeneity cannot be achieved through conventional glycosylation onto natural polysaccharides (the polymer-modification approach). Furthermore, the glycoclusters have large molecular weights that are not possible through the chemo-enzymatic polymerization of carbohydrate-monomers (the bottom-up approach). Since our glycoclusters have both homogeneous structures and large molecular weights, they show unique properties arising from the β-1,3-glucan main-chain; namely, they interact with poly(C) to form stable macromolecular complexes composed of two polysaccharide strands and one polynucleotide strand. Furthermore, SPR and CLSM assays using the lectin-immobilized Au surfaces and agarose beads show the strong and specific lectin-affinity of these macromolecular complexes. Along with these unique binding properties, various great advantages of β-1,3-glucans, such as non-cytotoxicity and long-blood circulation time, ensure the potential of β-1,3-glucan-based glycoclusters as polynucleotide carriers.

Experimental

General

^1H and ^{13}C NMR spectra were acquired on a Bruker DRX600 spectrometer in $CDCl_3$, D_2O or DMSO-d_6 at 600 MHz. The chemical shifts were reported in ppm (δ) relative to Me_4Si. IR spectra were recorded on a Perkin–Elmer Spectrum One Fourier transform infrared spectrometer attached with a universal ATR sampling accessory. Circular dichroism (CD) spectra were measured on 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 (TLC) was carried out with Merck TLC aluminium sheets pre-coated with silica gel 60 F_{254}.

CD spectral measurements. CUR with oligosaccharide-appendages (24.5 $mmol_{repeating\text{-}unit}$) were dissolved in DMSO (100 μl)

and then mixed with a poly(C) aqueous solution (1 mg ml^{-1}, 100 μl). The resultant mixtures were then diluted with Tris-buffer (10 mM, pH 8.0), incubated for 2 days at 4 °C and then used for CD spectral measurements.

SPR sensorgram measurements. Au surfaces immobilized with carboxymethyl dextran were used for the SPR measurements. Before the measurements, the carboxyl groups on the Au surfaces were first activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) and then rinsed with pure water several times. The lectins in pure water were then immobilized onto the Au surfaces by coupling with the activated carboxy groups. After rinsing and stabilizing with 0.83 mM Tris-HCl buffer (pH 8.0) containing $CaCl_2$ (8.3 mM), $MnCl_2$ (8.3 mM) and DMSO (4.2 wt%), the macromolecular complexes composed of CUR-derivatives and poly(C) in the same buffer systems were loaded and the sensorgrams obtained.

Synthesis of 2,3,4,6-tetra-*O*-acetyl-1-(2′-propargyl)-α-D-mannoside. $BF_3{\cdot}OEt_2$ (10 ml) was added to 1,2,3,4,6-penta-*O*-acetyl-D-mannose (2.1 g) and propargyl alcohol (5.0 ml) in anhydrous CH_2Cl_2 (5.0 ml) at room temperature, and stirring continued for 45 h under a nitrogen atmosphere. The resultant mixture was diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness. Although the residue was subjected to the purification by silica gel column chromatography (hexane only → hexane–ethyl acetate (1 : 1)), the R_f values of the starting material and the product were so close that the pure product could not be obtained. Thus, a deacetylation (in a mixture of aqueous ammonia, MeOH and THF) of this product was performed for purification purposes (silica gel, $CHCl_3$–MeOH–H_2O (4 : 5 : 1)). Re-acetylation of the compound was carried out by treatment with a mixture of pyridine (200 ml) and acetic acid (150 ml). An excess of EtOH was added to the resultant mixture to quench the reaction and the solution was concentrated *in vacuo*. The mixture was diluted with ethyl acetate and the organic layer was washed with 0.5 N aqueous HCl and saturated aqueous $NaHCO_3$ several times. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated to dryness to give 2,3,4,6-tetra-*O*-acetyl-1-(2′-propargyl)-α-D-mannoside as a white powder in 52% yield. ^{1}H NMR ($CDCl_3$, TMS): 5.34–5.28 (m, 3H), 5.04 (s, 1H), 4.31–4.28 (m, 3H), 4.12 (dd, $J = 2.13$ and 12.2 Hz, 1H), 4.04–4.02 (m, 1H), 2.49 (s, 1H), 2.12 (s, 3H), 2.11 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H); $[M + Na]^+ = 409.12$ (calc. 409.11); IR (KBr, cm^{-1}) 1756.

Synthesis of 1-(2′-propargyl)-α-D-mannoside. Aqueous ammonia (50 ml) was added to 2,3,4,6-tetra-*O*-acetyl-1-(2′-propargyl)-α-D-mannoside (1.0 g) in THF–MeOH (200 ml, 1 : 1) and stirring was continued for 2 h at room temperature. The solvent was evaporated and the resultant residue was subjected to the purification by silica gel column chromatography ($CHCl_3$–MeOH (4 : 1) → $CHCl_3$–MeOH–H_2O (4 : 5 : 1)) to obtain 1-(2′-propargyl)-α-D-mannoside in 95% yield as a white powder after lyophilization. ^{1}H NMR (DMSO-d_6 containing a small amount of D_2O, HOD): 4.96 (d, $J = 1.03$ Hz 1H), 4.28 (dd, $J = 2.07$ and 15.9 Hz, 1H), 4.23 (dd, $J = 2.02$ and 15.9 Hz, 1H), 3.87 (dd, $J = 1.51$ and 3.36 Hz, 1H), 3.81 (dd, $J = 1.40$ and 12.4 Hz, 1H), 3.73–3.67 (m, 2H), 3.61–3.58 (m, 2H), 2.84 (s, 1H); $[M + Na]^+ = 241.09$ (calc. 241.07); IR (KBr, cm^{-1}) 2970.

Synthesis of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-1-(2′-propargyl)-β-lactoside. $BF_3{\cdot}OEt_2$ (10 ml) was added to 1,2,3,6,2′3′4′6′-octa-*O*-acetyllactose (3.3 g) and propargyl alcohol (5.0 ml) in anhydrous CH_2Cl_2 (5.0 ml) at room temperature, and stirring continued for 44 h under a nitrogen atmosphere. The resultant mixture was diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness. Although the residue was subjected to purification by silica gel column chromatography (hexane only → hexane–ethyl acetate (1 : 1)), the R_f values of the starting material and the product were so close that the pure product could not be obtained. Thus, a deacetylation (in a mixture of aqueous ammonia, MeOH and THF) of this product was performed for purification purposes (silica gel, $CHCl_3$–MeOH–H_2O (4 : 5 : 1)). Re-acetylation of the compound was carried out by treatment with a mixture of pyridine (200 ml) and acetic acid (150 ml). An excess of EtOH was added to the resultant mixture to quench the reaction and the solution was concentrated *in vacuo*. The mixture was diluted with ethyl acetate and the organic layer was washed with 0.5 N aqueous HCl and saturated aqueous $NaHCO_3$ several times. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness to give 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-1-(2′-propargyl)-β-lactoside as a white powder in 49% yield. ^{1}H NMR ($CDCl_3$, TMS): 5.35 (d, $J = 2.81$ Hz, 1H), 5.23 (t, $J = 9.34$ Hz, 1H), 5.11 (dd, $J = 8.31$ and 10.3 Hz, 1H), 4.96 (dd, $J = 3.40$ and 10.4 Hz, 1H), 4.92 (t, $J = 8.40$ Hz, 1H), 4.74 (d, $J = 7.98$ Hz, 1H), 4.50 (dd, $J = 1.52$ and 12.8 Hz, 1H), 4.49 (d, $J = 8.08$ Hz, 1H), 4.34 (s, 1H), 4.34 (s, 1H), 4.15–4.07 (m, 3H), 3.88 (t, $J = 6.80$ Hz, 1H), 3.82 (t, $J = 9.49$ Hz, 1H), 3.64 (m, 1H), 2.47 (s, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.06 (s, 6H), 2.06 (s, 3H), 2.05 (s, 6H), 1.97 (s, 3H); $[M + Na]^+ = 697.31$ (calc. 697.21); IR (KBr, cm^{-1}) 1752.

Synthesis of 1-(2′-propargyl)-β-lactoside. Aqueous ammonia (50 ml) was added to 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-1-(2′-propargyl)-β-lactoside (1.1 g) in THF–MeOH (200 ml, 1 : 1) and stirring was continued for 3 h at room temperature. The solvent was evaporated and the resultant residue was subjected to purification by silica gel column chromatography ($CHCl_3$–MeOH (4 : 1) → $CHCl_3$–MeOH–H_2O (4 : 5 : 1)) to obtain 1-(2′-propargyl)-β-lactoside in 93% yield as a white powder after lyophilization. ^{1}H NMR (D_2O, HOD): 4.53 (d, $J = 7.93$ Hz, 1H), 4.35 (dd, $J = 1.90$ and 8.66 Hz, 1H), 4.32 (d, $J = 7.72$ Hz, 1H), 3.85 (d, $J = 11.5$ Hz, 1H), 3.80 (d, $J = 3.03$ Hz, 1H), 3.70–3.58 (m, 5H), 3.55–3.52 (m, 3H), 3.49–3.48 (m, 1H), 3.42 (t, $J = 8.11$ Hz, 1H), 3.32 (t, $J = 8.28$ Hz, 2H), 2.82 (s, 1H); $[M + Na]^+ = 403.09$ (calc. 403.12); IR (KBr, cm^{-1}) 2971.

Synthesis of *N*-acetyl-3,4,6-tri-*O*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosaminide. To glucosamine hydrochloride in saturated aqueous $NaHCO_3$, 2,2,2-trichloroethoxycarbonyl chloride (TrocCl) was added and the resultant mixture was stirred at room temperature for 1 day. The white precipitate was filtered off and washed with water and ethanol to give *N*-(2′,2′,2′-trichloroethoxycarbonyl)-D-glucosamine as white powder. The resultant white powder was treated with acetic anhydride in pyridine for 6 h at room temperature. After the reaction was complete,

the resultant mixture was diluted with ethyl acetate and washed with 0.5 N aqueous HCl. The organic layer was dried, filtered, concentrated and chromatographed (silica gel, toluene–ethyl acetate (2 : 1)) to give 1,3,4,6-tetra-*O*-acetyl-*N*-(2′,2′,2′-trichloroethoxycarbonyl)-D-glucosamine. $BF_3{\cdot}OEt_2$ (10 ml) was added to 1,3,4,6-tetra-*O*-acetyl-*N*-(2′,2′,2′-trichloroethoxycarbonyl)-D-glucosamine (2.5 g) and propargyl alcohol (5.0 ml) in anhydrous CH_2Cl_2 at room temperature and stirring continued for 46 h under a nitrogen atmosphere. The resultant mixture was diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness. The obtained crude product was treated with zinc powder in acetic acid at room temperature for 1 h. After the reaction was complete, acetic anhydride was added and the mixture was stirred for 2 h at room temperature. The mixture was filtered, diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ several times. The organic layer was dried over anhydrous $MgSO_4$, filtered, concentrated and chromatographed ($CHCl_3$–MeOH (25 : 1)) to give *N*-acetyl-3,4,6-tri-*O*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosaminide as a white powder in 23% yield (6 steps). ^{1}H NMR ($CDCl_3$, TMS): 5.52 (br s, 1H), 5.28 (t, $J = 9.62$ Hz, 1H), 5.10 (t, $J = 9.63$ Hz, 1H), 4.86 (d, $J = 8.41$ Hz, 1H), 4.39 (s, 2H), 4.27 (dd, $J = 4.70$ and 12.3 Hz, 1H), 4.15 (dd, $J = 1.80$ and 12.2 Hz, 1H), 3.96 (dd, $J = 8.78$ and 19.0 Hz, 1H), 3.74–3.71 (m, 1H), 2.47 (s, 1H), 2.09 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H); $[M + Na]^+ = 408.21$ (calc. 408.13); IR (KBr, cm^{-1}) 1751, 1649, 1531.

Synthesis of *N*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosamine. Aqueous ammonia (50 ml) was added to *N*-acetyl-3,4,6-tri-*O*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosaminide (1.0 g) in THF–MeOH (200 ml, 1 : 1) and stirring was continued for 2 h at room temperature. The solvent was evaporated and the resultant residue was subjected to the purification by silica gel column chromatography ($CHCl_3$–MeOH (4 : 1) → $CHCl_3$–MeOH–H_2O (4 : 5 : 1)) to obtain *N*-acetyl-1-*O*-(2′-propargyl)-β-D-glucosaminide in 91% yield as a white powder after lyophilization. ^{1}H NMR (CD_3OD): 4.50 (d, $J = 8.10$ Hz 1H), 4.27 (s, 1H), 4.26 (s, 1H), 3.79 (dd, $J = 2.1$ and 12.0 Hz, 1H), 3.61–3.15 (m, 5H), 2.75 (m, 1H), 1.88 (s, 3H); $[M + Na]^+ = 282.21$ (calc. 282.10); IR (KBr, cm^{-1}) 2970, 1655, 1535.

Synthesis of 6-bromo-6-deoxycurdlan (CUR-Br). Native curdlan (0.51 g) and LiCl (1.3 g) in dry DMF (30 ml) were stirred at 80 °C for 1 day to obtain a homogeneous curdlan solution. After cooling the resultant solution to room temperature, triphenylphosphine (2.0 g) in DMF (10 ml) was added to it and the resultant mixture stirred at room temperature for 3 h. Carbon tetrabromide (2.5 g) in DMF (6.0 ml) was then added and the resultant mixture stirred at 60 °C for 12 h. Subsequent reprecipitation from methanol and centrifugation gave CUR-Br as a brown powder in 31% yield. ^{13}C NMR (DMSO-d_6): 102.29 (Glc), 84.23 (Glc), 73.95 (Glc), 72.62 (Glc), 68.17 (Glc), 44.27 (Glc).

Synthesis of 6-azido-6-deoxycurdlan (CUR-N_3). CUR-Br (0.50 g) and NaN_3 (2.0 g) in DMSO (50 ml) were stirred at 85 °C for 48 h. The subsequent reprecipitation from water and centrifugation gave CUR-N_3 as a brown powder in 76% yield. ^{13}C NMR (DMSO-d_6): 102.26 (Glc), 83.71 (Glc), 73.98 (Glc), 72.90 (Glc), 68.29 (Glc), 50.72 (Glc).

Chemo-selective coupling between 6-azido-6-deoxycurdlan and functional modules. $CuBr_2$ (2.7 mg), ascorbic acid (25 mg), propargyl glycoside (400 mg) and propylamine (100 μl) were added to 6-azido-6-deoxycurdlan (50 mg) in DMSO (2.0 ml) and the mixture incubated at room temperature for 12 h. The mixture was dialyzed (water, MWCO8000) followed by lyophilization to give the desired curdlan derivatives as white powders with yields of 65–82%.

CUR-Man. ^{13}C NMR (DMSO-d_6): 143.76 (triazole), 125.35 (triazole), 102.06 (Glc), 99.05 (Man), 83.94 (Glc), 73.67 (Glc), 73.44 (Man), 73.24 (Glc), 70.77 (Man), 70.25 (Man), 68.78 (Glc), 66.93 (Man), 61.13 (Man), 55.29 (methylene), 50.48 (Glc).

CUR-GlcNAc. ^{13}C NMR (DMSO-d_6): 169.65 (acetoamide), 143.76 (triazole), 125.35 (triazole), 102.60 (Glc), 100.35 (GlcNAc), 84.14 (Glc), 76.88 (GlcNAc), 74.27 (GlcNAc), 73.67 (Glc), 73.24 (Glc), 70.89 (GlcNAc), 68.78 (Glc), 61.35 (GlcNAc), 61.29 (GlcNAc), 55.58 (methylene), 50.43(Glc), 22.98 (acetoamide).

CUR-Lac. ^{13}C NMR (DMSO-d_6): 143.61 (triazole), 125.60 (triazole), 103.70 (Lac), 102.60 (Glc), 101.88 (Lac), 84.10 (Glc), 80.51 (Lac), 75.51 (Lac), 74.95 (Lac), 73.83 (Glc), 73.33 (Lac), 73.19 (Glc), 70.73 (Lac), 68.88 (Glc), 68.20 (Lac), 61.59 (Lac), 60.76 (Lac), 60.46 (Lac), 59.61 (Lac), 55.14 (methylene), 50.46 (Glc).

CUR-OH. ^{13}C NMR (DMSO-d_6): 147.71 (triazole), 123.97 (triazole), 102.49 (Glc), 83.94 (Glc), 74.04 (Glc), 73.19 (Glc), 68.91 (Glc), 55.02 (methylene), 50.38 (Glc).

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26 A molecular dynamics calculation of the triple-stranded helix of schizophyllan (a β-1,3-glucan having a β-1,6-glucoside-appendage every three repeating units) showed hydrogen-bonding networks between the glucoside-appendages and the triple-helix main-chain. No such hydrogen-bonding network was observed for curdlan. This result provides a reasonable explanation for the higher thermal stability of the triple-helix of schizophyllan than that of curdlan (unpublished data).

