# Interaction of Sugar and Anion in Water via Hydrogen Bonding: Chain-Length Dependent Agglutination of Oligosaccharide Clusters Induced by Multivalent Anion Binding

Osamu Hayashida, Masaki Kato, Kazuyuki Akagi, and Yasuhiro Aoyama*

*Institute for Fundamental Research of Organic Chemistry*
*Kyushu University, Hakozaki, Higashi-ku*
*Fukuoka 812-8581, Japan*
*CREST*
*Japan Science and Technology Corporation (JST)*

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Oligosaccharides on the cell surfaces play important roles in various cellular recognition processes.[1] However, the chemical basis of information storage in oligosaccharides is still poorly understood. Simple saccharides (sugars), like simple salts, are too hydrophilic (extensively hydrated) to form hydrogen-bonded complexes in water. They can be strongly bound only under solvophobic conditions, i.e., in organic media.[2] The binding of such hydrophilic species in water is one of the most intriguing problems in molecular recognition. The present work is concerned with sugar−anion interaction, where sugar is a macrocyclic oligosaccharide cluster.[3] We report here that the anions, especially the phosphates, are readily incorporated into clustering oligosaccharide pools via hydrogen bonding, thereby inducing chain-length dependent agglutination of the clusters.

Calix[4]resorcarene, with four alkyl (undecyl) tails in the present case, is a bowl-shaped resorcinol cyclic tetramer. As reported,[3b] reaction of the aminoethoxy derivative with lactone of α(1−4)-linked diglucose (maltose) gives an amide-linked octakis-(diglucose) compound (G2, Figure 1a). A similar reaction with that of pentaglucose (maltopentaose) in methanol at 60 °C for 6 h affords (61% yield) the corresponding pentaglucose cluster G5 (molecular weight, 8065) having 40 glucose residues.[4] Figures 1b (G2) and 1c (G5) show the schematic structures and space-filling CPK models. Cluster G5, like G2, is miscible with water (solubility, >1 g/mL, i.e., ≥0.1 M) but is readily agglutinated by a phosphate salt such as $Na_2HPO_4/NaH_2PO_4$, $Na_2RP$ (RP = D-ribose-5-phosphate), and nucleotides such as $Na_2GMP$ (GMP = guanosine-5′-monophosphate) (Figure 1d) to give precipitates of a composition of G5:($HPO_4^{2-}/H_2PO_4^-$) ≅ 1:50 or G5:$RP^{2-}$ ≅ 1:10.

NMR studies reveal that complexation of the phosphate anions is taking place. (1) The $^{31}P$ NMR signals for dianionic guests $Na_2HPO_4$ (line a) or $Na_2RP$ (line b) 2 mM in $D_2O$-DMSO-$d_6$ (9:1)[5] undergo upfield shifts in the presence of host G5 until saturation is reached at $[H]_t/[G]_t \cong 0.3$ (Figure 2a) (H = host, G = guest, and t = total). The complexation-induced shifts (CIS) of 2.2 ppm for $HPO_4^{2-}$ and 3.4 ppm for $RP^{2-}$ amount to 88 and 94%, respectively, of the chemical shift differences ($\Delta\delta_P$) between dianion and monoanion (2.5 ppm for $HPO_4^{2-}/H_2PO_4^-$ and 3.6 ppm for $RP^{2-}/HRP^-$). While concurrent turbidity precludes any

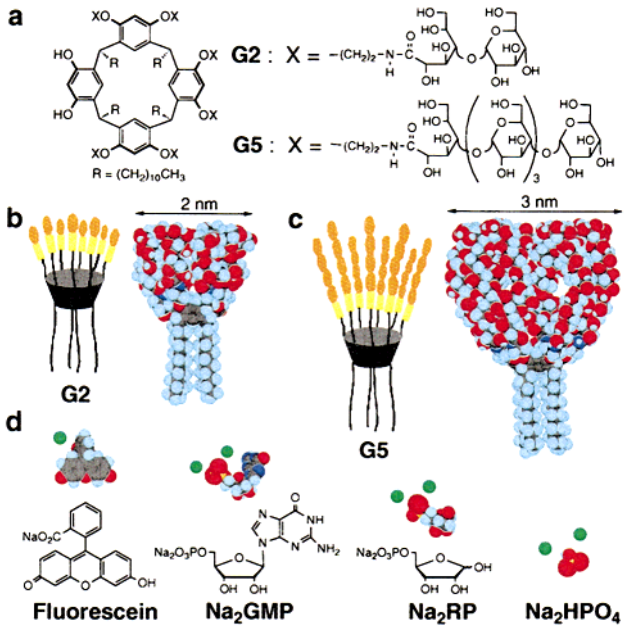


**Figure 1.** Structures of hosts (**a**−**c**) and guests (**d**). The saccharide moieties in schematic cluster structures in **b** and **c** are colored. The C, O, N, H, and P atoms and $Na^+$ ions in space-filling models are shown in black, red, dark blue, light blue, orange, and green, respectively.

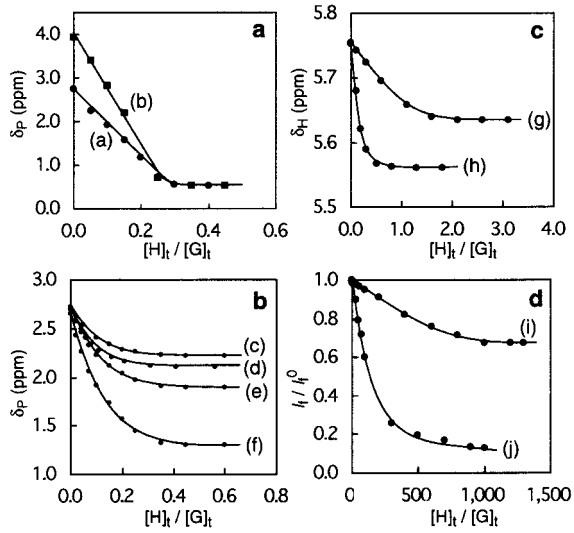


**Figure 2.** NMR and fluorescence titration of guests with host G2 or G5 at 25 °C: (**a**) $^{31}P$ NMR (at 243 MHz) chemical shifts ($\delta_P$, in reference to external $H_3PO_4$ in $D_2O$ ($\delta_P$ = 0)) for $Na_2HPO_4$ (a) and $Na_2RP$ (b) (2 mM) with host G5 in $D_2O$-DMSO-$d_6$ (9:1). (**b**) $^{31}P$ NMR chemical shifts ($\delta_P$) for $Na_2HPO_4$ (2 mM) with host G2 in $D_2O$−DMSO-$d_6$ (10:0, c), (9:1, d), (7:3, e) or (5:5, f). (**c**) $^1H$ NMR (at 600 MHz) chemical shifts ($\delta_H$) for 1′-H of the ribose ring of GMP (0.2 mM) with host G2 (g) or G5 (h) in $D_2O$−DMSO-$d_6$ (9:1). (**d**) Relative fluorescence intensities ($I_f/I_f^0$) for fluorescein (0.1 μM) with host G2 (i) or G5 (j) in $H_2O$ at pH 7.0 (HEPES) ($I_f^0$ refers to $I_f$ in the absence of host). The change in pH, if any, resulting from addition of the host is too small to account for the host-induced change in chemical shifts.

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(4) Anal. Calcd for $C_{328}H_{552}N_8O_{216}\cdot 2H_2O$: C, 48.60; H, 6.92; N, 1.38. Found: C, 48.40; H, 6.98; N, 1.55.

(5) Dynamic light scattering (DLS) shows that larger aggregates result from G5 (0.1 mM) and $Na_2HPO_4$ (10 mM) with higher contents of DMSO as an organic cosolvent; average diameter of aggregates in μm (% DMSO content) = 3 (0) < 6 (10) < 11 (20). In the absence of $Na_2HPO_4$, there is no DLS-detectable aggregation of G5 in $H_2O$−DMSO (9:1).

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quantitative analysis, the data show that a large number ($\gg 1$) of dianions $HPO_4^{2-}$ or ribose-5-$OPO_3^{2-}$ are simultaneously bound to host G5 via strong host-to-guest hydrogen-bonding or even protonation to lead essentially (~90%) to the monoanionic state, $H_2PO_4^-$ ($\delta_P = 0.15$) or ribose-5-O(H)$PO_3^-$ ($\delta_P = 0.45$), as if the guests were in an environment of pH $\leq$ 6 ($pK_a = 7.0$ for $HPO_4^{2-}/H_2PO_4^-$). Fifty percent complexation occurs for both guests at $[H]_t/[G]_t \cong 0.15$ (Figure 2a), i.e., at host concentration of $[H]_{50} \cong 0.3$ mM ($[G]_t = 2$ mM). The implication of $[H]_{50}$ is that $K_{min} = 1/[H]_{50} \cong 3 \times 10^3$ $M^{-1}$ represents the lower limit of the binding constant of H for a molecule of G, $H + G \leftrightarrows H{\cdot}G$.[6](2) The short-chain host G2 also binds the guests, keeping apparently transparent solutions. Dianionic guests $Na_2HPO_4$ and $Na_2RP$ in $D_2O$-DMSO-$d_6$ (9:1)[5] exhibit a CIS of 0.6 ppm for $Na_2HPO_4$ (Figure 2b, line d) or 0.57 ppm for $Na_2RP$ (not shown for clarity), corresponding to ~20% protonation. The saturation of $Na_2HPO_4$ occurs at $[H]_t/[G]_t \cong 0.4$, $K_{min} \cong 7 \times 10^3$ $M^{-1}$ being evaluated from $[H]_{50} \cong 0.15$ mM in a similar manner as above. As regards the solvent effects, % protonation (CIS/$\Delta\delta_P \times 100$) increases with increasing contents of DMSO, in accord with the host-to-guest (G2-to-$HPO_4^{2-}$) hydrogen-bonding; % protonation (CIS, $\Delta\delta_P$, % DMSO content) = 20 (0.49, 2.43, 0) < 24 (0.60, 2.53, 10) < 30 (0.83, 2.79, 30) < 45 (1.36, 3.06, 50) (Figure 2b). (3) The complexation of nucleotides at 0.2 mM can be followed also by $^1H$ NMR, i.e., by monitoring the diagnostic upfield shifts (0.1−0.2 ppm) for 1′-H on the ribose ring. The titration curves, as typically shown for GMP (Figure 2c, line g), fit with a 1:1 host:guest stoichiometry (also confirmed by Job plots) with $K = 4.0 \times 10^3$ ($Na_2AMP$), $1.6 \times 10^4$ ($Na_2GMP$), $2.9 \times 10^4$ ($Na_2ADP$), $3.7 \times 10^4$ ($Na_2ATP$), $1.3 \times 10^4$ (AMP), and $3.8 \times 10^4$ $M^{-1}$ (GMP) from computer-aided least-squares curve-fitting (AMP and GMP are free acids in the zwitterionic form having a monoanionic phosphate group and AM(D)(T)P = adenosine-5′-mono(di)(tri)phosphate). The phosphate groups are responsible for the binding, since nucleoside adenosine lacking the phosphate group neither shows any detectable affinity to the host nor inhibits the complexation of nucleotides. (4) Long-chain host G5 binds GMP and AMP more efficiently with a saturation occurring at $[H]_t/[G]_t \cong 0.8$ for GMP (Figure 2c, line h); $K_{min} \cong 3 \times 10^4$ $M^{-1}$ for GMP and $2 \times 10^4$ $M^{-1}$ for AMP. (5) Thus, the larger host G5 exhibits 1:*n* H:G stoichiometries ($n > 1$) even for the largest nucleotide guests, while that of the smaller host G2 is 1:1 except for the smallest $Na_2HPO_4$ guest. This may be easily understood on the basis of the sizes of hosts and guests (Figure 1). A Scatchard analysis[7] of the titration data gives the values of $n \cong 10$ for $Na_2HPO_4$ with G2 and $n \cong 4$ for GMP and AMP with G5.

Other relevant points are as follows. (6) DLS (dynamic light scattering) and TEM (transmission electron microscopy) show that $Na_2HPO_4$ immediately induces aggregation of both G2 and G5 (0.1−1 mM in water) to give spheres/vesicles ($d = 100-400$ nm), which further grow to $\mu$m-sized particles either very rapidly (in minutes) in case of G5[5] or slowly (in days) in case of G2.[8] (7) Host G5 is agglutinated/precipitated by various salts such as sulfate ($Na_2SO_4$), sulfonate (2,4,6-$(O_2N)_3C_6H_2SO_3Na$), borate ($Na_3BO_3$), perchlorate ($NaClO_4$), and dicarboxylate (sodium oxalate) in addition to phosphates but not by monocarboxylate (NaOAc) or halide (NaCl). (8) Simple saccharides such as maltose and maltopentaose and *N*-methylacetamide as single-chain or amide-linkage references of the present cluster hosts are inert to $Na_2HPO_4$, causing neither shifts in $\delta_P$ nor agglutination. (9) Fluorescein (Figure 1d) with $pK_a = 6.7$ forms a 1:1 complex with host G2 ($K = 1.1 \times 10^4$ $M^{-1}$ from Benesi−Hildebrand analysis) or G5 ($K = 5.4 \times 10^4$ $M^{-1}$) under conditions of host excess at pH = 7.0. The bound guest thereby loses 33% (with G2) or 87% (with G5) of the original fluorescence intensity ($I_f{}^0$) (Figure 2d) as if it were in an environment of pH = 6.6 (G2) or 5.4 (G5). This is in accord with the NMR results (items 1 and 2).

Combined evidence indicates that (a) anions are incorporated in the clustering oligosaccharide pools of hosts G2 and G5 via hydrogen bonding and (b) the hosts thereby undergo coaggregation with multivalent anions in a biphasic manner, i.e., rapid formation of submicrometer-sized particles followed by their oligosaccharide chain-length dependent agglutination.

Known examples of saccharide complexation in water are driven by hydrophobic (CH-$\pi$) forces.[9] The phosphates form salts with cationic (mostly ammonium) hosts in both organic[10] and aqueous[11] media. In this regard, the present interaction between the saccharide host (neutral) and an inorganic anion $HPO_4^{2-}$ (by no means hydrophobic) in water (item a) must be free from any hydrophobic/CH-$\pi$ or electrostatic assistance. Still, the binding affinity of $K \gg K_{min} = 3 \times 10^3$ $M^{-1}$ for G5 is comparable with those in the order of $10^{1-4}$ $M^{-1}$ for the hydrogen-bonded saccharide-phosph(on)ate complexes in organic media.[2e,12] In view of their amphiphilic nature, it is not surprising that the present hosts form aggregates, where the multiple hydrogen-bond forming anions may cross-link or glue the oligosaccharide chains in an intramolecular (intracluster), intermolecular (intercluster), or interaggregate fashion. The chain-length dependent agglutination (item b) may be taken in light of oligosaccharide-triggered cell adhesion.[13]

To summarize, the clustering oligosaccharide chains fixed on a rigid macrocycle act as a *macrosolvent* for anions. Simple salts which are otherwise soluble only in water can be readily extracted from water into the clusters. The present work may thus open an important yet unexplored area of molecular recognition of highly polar species in water and may also provide a clue to understand how oligosaccharides can be informative, e.g., adhesive, particularly in reference to the so-called saccharide cluster effects.[14]

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(6) If we assume that successive addition of G to H having *n* unit-binding sites (h) occurs independently ($h + G \leftrightarrows h{\cdot}G$), the equilibrium constant can be expressed as $K_{unit} = k_{a(hG)}/k_{d(hG)} = [h{\cdot}G]/[h][G] = 1/(n[H]_{50} - 0.5[G]_t)$ at 50% complexation ($[h{\cdot}G] = [G]$) ($k_a$ and $k_d$ are association and dissociation rate constants). For the equilibrium $H + G \leftrightarrows H{\cdot}G$, $k_{a(HG)} \cong nk_{a(hG)}$ for a statistical reason and $k_{d(HG)} \cong k_{d(hG)}$ and hence $K = k_{a(HG)}/k_{d(HG)} \cong nk_{a(hG)}/k_{d(hG)} = nK_{unit} = n/(n[H]_{50} - 0.5[G]_t) = 1/([H]_{50} - 0.5[G]_t/n) > 1/[H]_{50}$ ($n < \infty$).

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