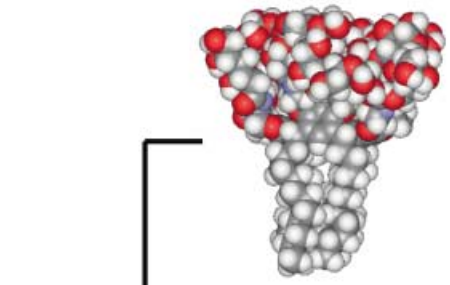
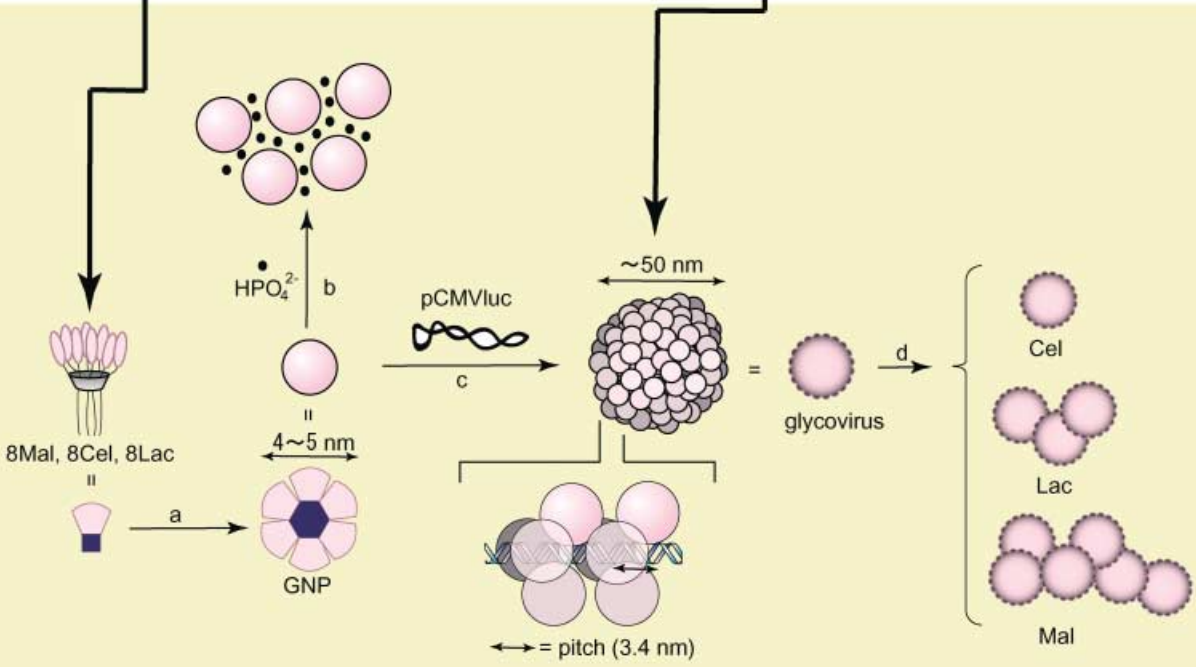
Macrocyclic Glycoclusters

From Amphiphiles through Nanoparticles to Glycoviruses





a: Irreversible Micellization
b: Marriage of Sugar and Salt
c: Size-Controlled Gene Coating
d: Saccharide-Dependent Glycoviral Aggregation

DOI: 10.1002/chem.200305288 *Chem. Eur. J.* **2004**, *10*, 588 – 593

Macrocyclic Glycoclusters: From Amphiphiles through Nanoparticles to Glycoviruses

Yasuhiro Aoyama*[a]

Abstract: Macrocyclic glycocluster amphiphiles are intended to be a covalent-bundle mimic of clustering glycolipid motifs on the cell membrane. They are irreversibly micellized to give glycocluster nanoparticles (GNPs); their masked hydrophobicity endows them with remarkable saccharide specificities in the interactions with biological saccharide receptors. The GNPs also exhibit unprecedented hydrogen-bond capacities; they are agglutinated with Na_2HPO_4 and assembled on plasmid DNA in a number-, size-, and shape-controlled manner to give artificial glycoviral particles capable of transfection. Thus, the intrinsic function of viruses, that is, cell invasion followed by gene expression, is also intrinsic to size-regulated (~50 nm) glycoviruses. The growth of glycocluster amphiphiles through nanoparticles to glycoviruses reveals a hierarchical adhesion control of the saccharide clusters.

Keywords: artificial viruses · gene delivery · macrocyclic glycoclusters · nanostructures · self-assembly

Glycolipid Bundles:[1] An Introduction

Nucleic acids and proteins store information in one-dimensional polynucleotide or polypeptide chains, which form well-defined hydrogen-bond motifs such as Watson–Click base-pairs, α-helices, and β-sheets. Saccharides are also informational biomolecules, playing important roles in a variety of cellular communications.[2] They occur not as one-dimensional polysaccharides but as clustering oligosaccharides on the cell surface. The so-called cluster effects[3] refer to the multivalency of biological saccharide–receptor interactions and have been a subject of considerable studies using artificial multiantennary saccharide derivatives such as polymers/dendrimers and various types of assemblages.[4–6] We, on the other hand, were concerned about characterizable unimolecular glycoclusters with minimized conformational/diffusional freedom. With clustering sphingoglycolipids in cell membranes in mind, we wanted to construct glycolipid-bundle structures by covalently tying up several glycolipid-like molecules in a cyclic array.[1] This is how the work on macrocyclic glycoclusters started. We took advantage of the tailed calix[4]resorcarene framework **1** readily obtained by the condensation of resorcinol and dodecanal.[7] The reactions of macrocyclic octaamine **2**[8] with a lactone derivative of maltose, cellobiose, or lactose afford glycolipid-bundle compounds **1a** (8Mal), **1b** (8Cel), or **1c** (8Lac) with four long-alkyl (undecyl) chains and eight oligosaccharide moieties with terminal α-glucose, β-glucose, or β-galactose residues, respectively, in the opposite sides of the macrocycle (Figure 1).[9] They are monodispersed (molecular weight, 4172) and unimolecularly form a glycocluster motif composed of a definite number of saccharide moieties in a well-defined geometry. The object of our efforts in this area in the last several years is to develop some key concepts to answer the simple question of how saccharides can be adhesive in water.

Undissociable Micelles: Stabilization of Glycocluster Nanoparticles via Lateral Inter(saccharide) Interactions[1]

Amphiphile **1** with a cone shape (Figure 1) forms in water micellar aggregates, hereafter called glycocluster nanoparticles (GNPs), having an aggregation number of ~6 and a DLS (dynamic light scattering) size of 4–5 nm (Scheme 1, step a).[1,9] What is surprising is their unusual stability. As revealed by the lack of surface activity, they do not dissociate into monomers. In other words, the present micellization is irreversible. They are characterizable by means of GPC (gel-permeation chromatography) as well as TEM (transmission electron microscopy) and are readily immobilized upon phosphate coating (see below). Micelles, in contrast to vesicles, do not usually retain integrity as such under GPC conditions and are too dynamic to be frozen.

[a] Prof. Dr. Y. Aoyama
Department of Synthetic Chemistry and Biological Chemistry
Graduate School of Engineering, Kyoto University
Katsura, Nishikyo-ku, Kyoto 615–8510 (Japan)
Fax: (+81) 75-383-2767
E-mail: aoyamay@sbchem.kyoto-u.ac.jp

 DOI: 10.1002/chem.200305288

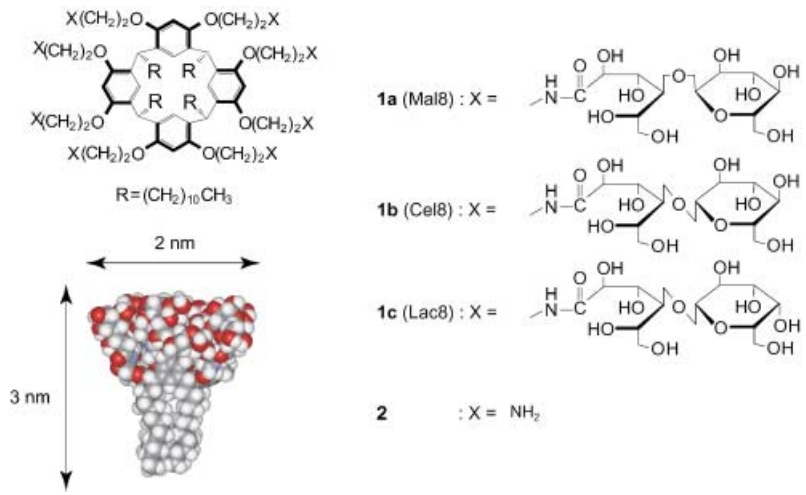
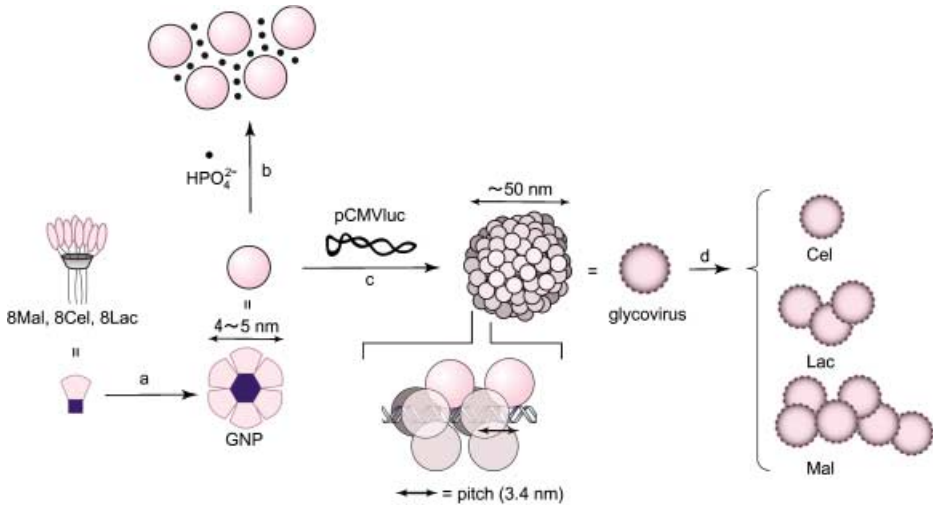


Figure 1. Structures of macrocyclic glycocluster amphiphiles **1a** (8Mal), **1b** (8Cel), and **1c** (8 Lac) and a space-filling illustration in the folded conformation (reproduced after modification with permission from ref. [9]).



Scheme 1. Hierarchical growth of glycocluster amphiphile through nanoparticle (GNP) to glycovirus and its aggregates (reproduced after modification with permission from ref. [9]). Phosphate-induced agglutination of GNPs is also shown.

Masked Hydrophobicity: Saccharide-Directed Cell Recognition[10]

Oligosaccharides play important roles in various cellular activities as signals, targets of bacterial/viral infection, and glues in cell adhesion,[2] where the saccharide–receptor interactions are often specific. This specificity suggests a potential utility of synthetic multivalent saccharide derivatives as carriers of directed drug/gene delivery and blockers/inhibitors of undesired saccharide–receptor interactions. A problem here is nonspecific adsorption which is hydrophobic in origin and is thus promoted by the residual hydrophobicity shown by synthetic compounds even when highly saccharide-substituted. We need to completely mask the hydrophobicity. The present GNP provides a good model to test the idea of saccharide specificity enhancement upon hydrophobicity masking. The results are remarkable.[10,11] An illustration is directed molecular delivery to the hepatic cells which have receptors for the terminal galactose residues of asialoglycoproteins.[10]

In spite of the presence of four long alkyl chains, amphiphile **1** is highly hydrophilic and practically miscible with water (solubility, $>1\,g\,mL^{-1}$). This is because the hydrophobicity of **1** is buried or masked in the core of GNP upon irreversible micellization, the glycocluster part of **1** being thereby rendered unfolded (see above) to give an open aromatic cavity capable of guest binding. The octa(glucose) and octa(galactose) compounds 8Mal (**1a**) and 8Lac (**1c**) in water indeed form stable 1:1 complexes with a variety of guest molecules;[8] the binding constants for phloxine B are reasonably similar for **1a** ($2.0\times10^5\,M^{-1}$) and **1c** ($2.1\times10^5\,M^{-1}$) at 25 °C.[10] With masked hydrophobicity to inhibit nonspecific adsorption, the identity of the saccharide moieties becomes the governing factor; the galactose (**1c**) undergoes specific saccharide–receptor interactions with the hepatic cells, while the glucose (**1a**) is completely rejected by the cells. The guest molecules included are thereby either delivered to the target cells or protected in solution away from the cells.[10] Lectin binding is another example.[11] The glucose and galactose compounds **1a** and **1c** are specifically

We believe that the unprecedented stabilization of the present GNP comes from lateral or side-by-side inter(saccharide) interactions (hydrogen bonding), which fix hydrophobically associated and hence otherwise labile micelles.[1] In support of this, saccharide-free octaamine precursor **2** as octaammonium salt **2**·8(HCl) exhibits no such unusual stability. A rough surface–area calculation suggests that the glycocluster part of amphiphile **1** in GNP takes an unfolded conformation suitable for intermolecular entangling of the saccharide chains. Collaboration of hydrophobic (stacking) association in the core and multiple hydrogen bonding on the surface is a common aspect of DNA duplex formation and protein folding. This may also be the case in the present GNP immobilization. In this context, it is important to note that there is no indication of front-to-front inter(saccharide) interactions that would lead to self-aggregation of GNPs.

bound to concanavalin A (glucose- and mannose-binding) and peanut lectin (galactose-binding), respectively. There is no crossover.

Sticky Saccharides: Marriage of Sugar and Salt to Escape from Otherwise Favorable Hydration Prisons[1,12,13]

An important yet largely unexplored area of molecular recognition is effective complexation driven by hydrogen bonding in highly competitive water as a medium. Owing to their highly hydrophilic nature, saccharides may be regarded as one of the least potential hydrogen-bond makers in water. At the same time, a glance at the structures of amylose and cellulose immediately reveals a hydrogen-bonding potency of saccharides having oligomeric/polymeric nature. The expanded glycolcuster platform immobilized in GNP or on a hydrophobized sensor chip of SPR (surface plasmon resonance) may provide a chance to challenge hydrogen-bond capacity of saccharides in water.[1]

The effective and irreversible adsorption of compound **1a** from an aqueous solution onto a polar solid surface such as quartz provides an early indication of the characteristic feature of **1a** as a polar adsorbate in water.[12] Subsequent work reveals that **1a** and, in a more pronounced manner, its higher (longer saccharide) homologue are co-precipitated with sodium phosphate in water.[13] Sugar and salt are two typical classes of highly water-soluble materials. Here, however, a water-miscible sugar **1a** and a highly water-soluble salt Na_2HPO_4 get married in order to escape from their otherwise favorable hydration shells. As far as conventional analyses are concerned, the present complexation can be expressed in terms of sugar-to-phosphate hydrogen bonding.[13] TEM, on the other hand, clearly shows that this is HPO_4^{2-}-induced agglutination or cross-linking of GNPs with the phosphate ions (H-acceptors) as a glue and the oligosaccharide chains of GNP (H-donors) as a tab for sticking (Scheme 1, step b).[1] The TEM observation of gathering GNPs for a sonicated solution of **1a** and Na_2HPO_4 again confirms the unusual stability of the saccharide-coated GNPs.[1]

Artificial Glycoviruses: Size-Controlled Gene Coating with Glycocluster Nanoparticles[9,14]

The facile phosphate complexation of GNP suggests its application as a new type of DNA binders, especially gene carriers or vectors. Viral vectors have been used in therapeutic trials of gene delivery. Non-viral vectors have so far been exclusively amine-based cationic polymers/dendrimers and lipids.[15] As polycations, they readily bind to polyanionic DNA and also provide an electrostatic driving force for adsorption on the negatively charged cell surface. At the same time, the ease of polycation–polyanion complexation obscures the stoichiometry thereof. More accurately, polycation–polyanion (vector-DNA) complexation occurs at various ratios and is susceptible to crosslink and further hydrophobic aggregation upon charge neutralization, giving rise to polymolecular (with respect to DNA) and huge particles whole size-restricted poor diffusion severely limits their in vivo utility. Endocytosis by which these particles are taken in the cells is also size-controlled.[16] Viruses contain a single genetic (DNA or RNA) molecule which is coated with many but a very definite number of proteins in a compact viral size (20–100 nm in typical cases). In this context, artificial viruses should be monomolecular, should exhibit a definable stoichiometry, and should be of a viral size. These requirements may be met only when the resulting virus, if it forms, is completely free from aggregation. The hydrophobic and electrostatic forces are major complexation drivers in aqueous media. The coating material of artificial virus must be uncharged and least hydrophobic. A potential candidate is saccharides, especially the present neutral GNP with masked hydrophobicity, which fortunately possesses a striking phosphate-complexation ability.

The complexation of 7040 bp (base pair) plasmid DNA pCMVlus, having a reporter gene for a firefly protein luciferase, with GNP occurs in a number-, size-, and shape-controlled manner with a saturation stoichiometry of two GNPs (4–5 nm) per helical pitch (10 bp) with a pitch length of 3.4 nm (Scheme 1, step c).[14] A plausible mode of binding is to place two GNPs in each pitch along the major groove at north and south or east and west pitch by pitch (Scheme 1), thus allowing maximal sugar-phosphate hydrogen bonding and minimal steric interference. When GNPs are derived from octa(β-glucose) compound 8Cel (**1b**), the resulting mononuclear complex pCMVluc•(~1400)GNP (Cel-virus) with a surface (zeta) potential of ~0 mV undergoes compaction into a viral (~50 nm) size (DLS), as also confirmed by transmission electron microscopy (Figure 2b) whose characteristic fine structure (Figure 2e) is indeed indicative of dense GNP-coating of the surface.[9,14] The monomolecularity, stoichiometry, and size criteria for artificial viruses are thus met but this is only when we use 8Cel (**1b**) as a glycocluster. Complexation of pCMVluc with 8Mal (**1a**) or 8Lac (**1c**) with terminal α-glucose and β-galactose residues, respectively, occurs in a similar manner to give respective glycoviruses, which, however, are more or less aggregated, as shown in their TEM images (Figure 2a and d for Mal-virus and Figure 2c and f for Lac-virus).[9] Thus, the aggregation tendencies increase in the order Cel $\ll$ Lac $<$ Mal or β-glucose $\ll$ β-galactose $<$ α-glucose (Scheme 1, step d). Whatever the detailed structural background may be, the present results indicate that an alteration in stereochemistry of a single glycosidic linkage (Mal vs Cel) or a single OH group on a pyranose ring (Mal vs Lac) can result in a drastic change in the adhesion properties of glycoclusters.

Transfectious Glycoviruses: Remarkably Size-Regulated Cell Invasion by Artificial Viruses[9]

The efficiency of gene delivery using cationic vectors depends on particle size and surface charge. In targeted gene delivery, the presence of particular ligands (galactose for hepatic cells as an example) is essential. A problem is that

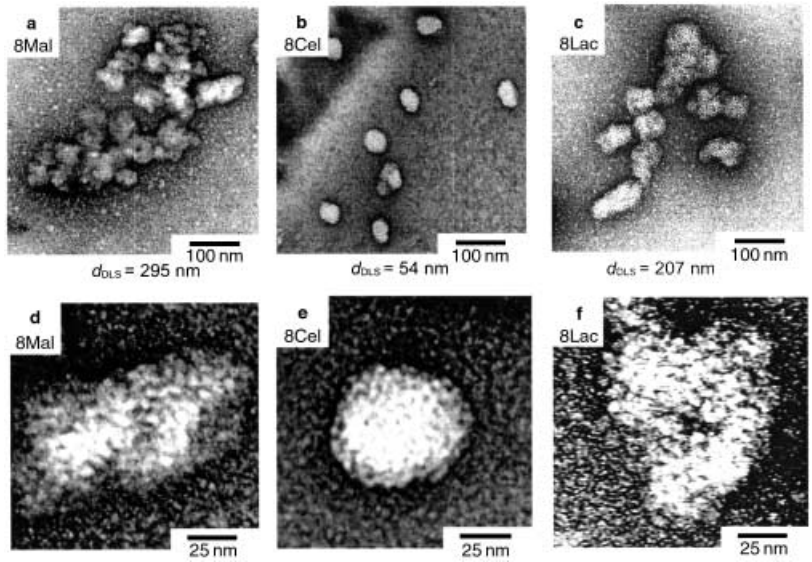


Figure 2. Negative staining TEM (transmission electron microscopic) images of 8Mal (a and d), 8Cel (b and e), and 8 Lac (c and f) complexes (glycoviruses) of pCMVluc with enlargement of a particle in panel d–f (reproduced after modification with permission from ref. [9]).

the size, charge, and ligand factors are interdependent with each other in such a way as an alteration in charge results in that in size and an introduction of saccharide moieties changes the charge. In addition, these indexes can also change as the vector/DNA ratios change. The rigorous correlation of transfection efficacy with any particular factor must be a formidable task. The present glycoviruses are neutral, being primarily free from charge effects, and exhibit a saturation size, where we can expect a straightforward size–efficacy correlation.

They are indeed transfectious. The transfection efficiencies, as obtained by chemiluminescence assay of luciferase expressed in Hela cells, show a semilogarithmic linear correlation (with a negative slope) with the mean DLS sizes of the glycoviruses (Figure 3a).[9] In this correlation are included the data for those derived from partially saccharide-functionalized glycocluster amphiphiles 5Mal, 5Cel, and 5Lac having approximately five saccharide (maltose, cellobiose, or lactose) moieties. Clearly, only monomeric Cel-viruses having an endocytosis-feasible, compact (~50 nm) viral size[16] possesses a substantial transfection ability and those of aggregating Mal- and Lac-viruses may reflect the fractions of monomeric states in glycoviral aggregation equilibria.

The size–activity correlation for hepatic cells HepG2 is shown in Figure 3b.[9] There are two important factors. One is the size factor. The activities of receptor-inert Mal- and Lac-viruses are size-controlled in a similar manner as above for the Hela cells. The other important factor is the receptor. Those of Lac-viruses are higher by a factor of $\sim 10^2$ (shown by a bar in Figure 3b) than expected on the size basis, owing to their receptor-mediated endocytosis. However, they are aggregated and their transfection is size-depressed. The otherwise significant receptor advantage ($\sim 10^2$) is mostly canceled by the size factor. In other words, the specific receptor pathway is still under strict size control.[17] For better hepatocyte targeting, the galactose-cluster motifs have to be manipulated to meet the two requirements of maximal glycovirus–receptor interaction and minimal glycoviral aggregation. We also need a deeper insight into what the driving force of aggregation is and how it is affected by the nature, especially the stereochemistry, of saccharides involved. The most puzzling question raised is why α-glucose (Mal) is highly aggregating while β-glucose (Cel) is not at all.

Hierarchical Adhesion Control: Concluding Remarks

In this paper is described the hierarchical growth of glycocluster amphiphiles through nanoparticles to glycoviruses, where a remarkably manipulated adhesion performance of saccharides is noted. Inter(saccharide) interactions are strong enough to immobilize otherwise labile glycomicelles into nanoparticles (GNPs) but not so strong as to induce self-aggregation of GNPs. Nevertheless, GNPs aligned on the DNA template are compactly packed into glycoviruses which then undergo saccharide-dependent aggregation. The

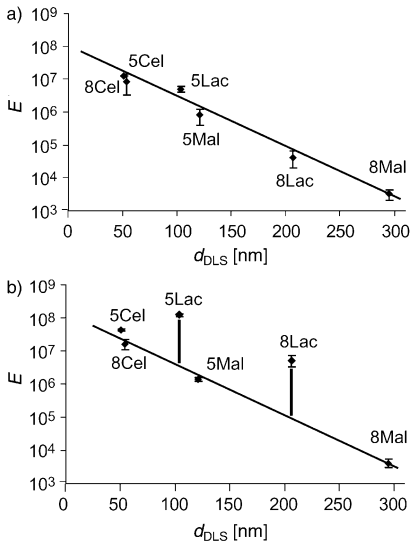


Figure 3. Size dependency of the transfection efficiencies for a) Hela cells and b) hepatic HepG2 cells with a fixed amount (0.6 μg or 4.5 μM P) of pCMVluc at 8Gly/P = 5Gly/P = 2.0 (Gly = Mal, Cel, or Lac and P stands for a base or phosphate moiety of pCMVluc). Luciferase expression efficiencies (E, in arbitrary unit) as a function of mean DLS sizes of the glycoviral particles (reproduced after modification with permission from ref. [9].

apparently fuzzy nature of the saccharides might be better interpreted by suggesting that they become adhesive when and where needed. The essential involvement of anionic (sulfated or carboxylated) saccharides in glycosaminoglycans in extracellular matrices is interesting in view of the strong anion–saccharide (phosphate–glycocluster) interactions revealed here.

Hierarchy is a common aspect of biological structures. There are many examples of number-, size-, and shape-controlled macromolecular associations in biology, from protein subunit associations through viruses, ribosomes, fibrils up to cells and organs. Abiological supramolecular assemblies, on the other hand, have so far been mostly concerned with small convergent systems of the host–guest type or infinite divergent systems such as crystals, gels, and surfaces. What remains to be challenged is finite divergent associations with number, size, and shape control as a nano(bio)technological tool to construct functional nanometric or mesoscopic devices, the bottom-up or template-and-ball approach to artificial viruses being an example. This fascinating area will grow keeping in touch with such topics as number and size control in supramolecular oligomerization,[18] finite macromolecular association,[19] topologically programmed multimolecular metal coordination,[20] and hierarchical self-assembly.[21]

Acknowledgement

This work was supported by RFTF of Japan Society for the Promotion of Science (JSPS), by Grant-in-Aids for COE Research (no. 08CE2005), 21 st COE on Kyoto University Alliance for Chemistry, and Scientific Research (no. 13490021) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and also by CREST of Japan Science and Technology Corporation (JST). The author deeply thanks his collaborators whose names appear in the literatures cited.

[1] O. Hayashida, K. Mizuki, K. Akagi, A. Matsuo, T. Kanamori, T. Nakai, S. Sando, Y. Aoyama, *J. Am. Chem. Soc.* **2003**, *125*, 594.
[2] a) J. Roth, *Chem. Rev.* **2002**, *102*, 285; b) C. E. Ritchie, B. E. Moffat, R. B. Sim, B. P. Morgan, R. A. Dwek, P. M. Rudd, *Chem. Rev.* **2002**, *102*, 305; c) N. E. Zachara, G. W. Hart, *Chem. Rev.* **2002**, *102*, 431.
[3] a) Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321; b) N. Röckendorf, T. K. Lindhorst, *Top. Curr. Chem.* **2001**, *217*, 201; c) D. A. Fulton, J. F. Stoddart, *Bioconjugate Chem.* **2001**, *12*, 655; d) W. B. Turnbull, J. F. Stoddart, *Rev. Mol. Biotechnol.* **2002**, *90*, 231; e) J. J. Lundquist, E. J. Toone, *Chem. Rev.* **2002**, *102*, 555; f) M. J. Cloninger, *Curr. Opin. Chem. Biol.* **2002**, *6*, 742; g) C. O. Mellet, J. Defaye, J. M. G. Fernández, *Chem. Eur. J.* **2002**, *8*, 1982.
[4] a) C. R. Bertozzi, L. L. Kiessling, *Science* **2001**, *291*, 2357; b) J. E. Gestwicki, L. L. Kiessling, *Nature* **2002**, *415*, 81.
[5] a) S. K. Choi, N. Mammen, G. M. Whitesides, *J. Am. Chem. Soc.* **1997**, *119*, 4103; b) D. Zanini, R. Roy, *J. Org. Chem.* **1998**, *63*, 3486; c) D. A. Fulton, J. F. Stoddart, *Org. Lett.* **2000**, *2*, 1113; d) K. Matsuura, M. Hibino, Y. Yamada, K. Kobayashi, *J. Am. Chem. Soc.* **2001**, *123*, 357.
[6] C. Tromas, J. Rojo, J.; J. M. de la Fuente, A. G. Barrientos, R. García, S. Penadés, *Angew. Chem.* **2001**, *113*, 3142; *Angew. Chem. Int. Ed.* **2001**, *40*, 3052.
[7] Y. Aoyama, Y. Tanaka, S. Sugahara, *J. Am. Chem. Soc.* **1989**, *111*, 5397.
[8] T. Fujimoto, C. Shimizu, O. Hayashida, Y. Aoyama, *Gazz. Chim. Ital.* **1997**, *127*, 749.
[9] T. Nakai, T. Kanamori, S. Sando, Y. Aoyama, *J. Am. Chem. Soc.* **2003**, *125*, 8465.
[10] K. Fujimoto, T. Miyata, Y. Aoyama, *J. Am. Chem. Soc.* **2000**, *122*, 3585.
[11] T. Fujimoto, C. Shimizu, O. Hayashida, Y. Aoyama, *J. Am. Chem. Soc.* **1998**, *120*, 601.
[12] T. Fujimoto, C. Shimizu, O. Hayashida, Y. Aoyama, *J. Am. Chem. Soc.* **1997**, *119*, 6676.
[13] O. Hayashida, M. Kato, K. Akagi, Y. Aoyama, *J. Am. Chem. Soc.* **1999**, *121*, 11597.
[14] Y. Aoyama, T. Kanamori, T. Nakai, T. Sasaki, S. Horiuchi, S. Sando, T. Niidome, *J. Am. Chem. Soc.* **2003**, *125*, 3455.
[15] a) J.-P. Behr, *Acc. Chem. Res.* **1993**, *26*, 274; b) *Self-assembling Complexes for Gene Delivery. Laboratory to Clinical Trial* (Eds.: A. V. Kabanov, P. L. Felgner, L. W. Seymour), Wiley, Chichester, **1998**.
[16] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular Biology of Cell*, 4th ed., Garland Science, New York, **2002**, Chapter 13, p. 746.
[17] P. C. Rensen, L. A. Sliedregt, M. Ferns, E. Kieveit, S. M. van Rossenberg, S. H. van Leeuwen, T. J. van Berkel, E. A. Biessen, *J. Biol. Chem.* **2001**, *276*, 37577.
[18] J. M. Rivera, T. Martín, J. Rebek, Jr., *Science* **1998**, *279*, 1021.
[19] a) S. I. Stupp, V. LeBonheur, K. Walker, L. S. Li, K. E. Huggins, M. Keser, A. Amstutz, *Science* **1997**, *276*, 384; b) V. Percec, C.-H. Ahn, G; Ungar, D. J. P. Yeardley, M. Möller, S. S. Sheiko, *Nature* **1998**, *391*, 161; c) J. J. L. M. Cornelissen, M. Fischer, N. A. J. M. Sommerdijk, R. J. M. Nolte, *Science* **1998**, *280*, 1427; d) J. H. K. K Hirschberg, L. Brunsveld, A. Ramzi, J. A. J. M. Vekemans, R. P. Sijbesma, E. W. Meijer, *Nature* **2000**, *407*, 167.
[20] a) B. Olenyuk, J. A. Whiteford, A. Fechtenkotter, P. J. Stang, *Nature* **1999**, *398*, 796; b) G. W. Orr, L. J. Barbour, J. L. Atwood, *Science* **1999**, *285*, 1049; c) N. Takeda, K. Umemoto, K. Yamaguchi, M. Fujita, *Nature* **1999**, *398*, 794; d) D. L. Caulder, K. N. Raymond, *Acc. Chem. Res.* **1999**, *32*, 975.
[21] H. Wu, V. R. Thalladi, S. Whitesides, G. M. Whitesides, *J. Am. Chem. Soc.* **2002**, *124*, 14495.

