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Molecular Glues Carrying Multiple Guanidinium Ion Pendants via an Oligoether Spacer: Stabilization of Microtubules against Depolymerization

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Adhesive molecules that can stick to proteins or induce their homo- or heterotropic assembly have become of increasing interest not only in protein engineering but also from a therapeutic point of view. For example, paclitaxel, an anticancer drug, may be regarded as an adhesive molecule, which stabilizes microtubules (MTs) against depolymerization by glueing their α/β-tubulin heterodimer components *via* a van der Waals interaction.[1] Here we report Asn(TEG-Gu^+)$_9$ and G1(Gu^+)$_9$R (Figure 1) as a new class of "molecular glues" having guanidinium (Gu^+) ions as sticky pendants for proteins. The adhesion takes place by a salt-bridge formation between the Gu^+ ions and oxyanions that exist ubiquitously in proteins.[2] Since a single Gu^+ ion−oxyanion interaction is too weak to operate under physiological conditions, multiple Gu^+ ions are incorporated *via* a flexible spacer on an oligopeptide or dendron scaffold for realizing "multivalency" of the interaction.[3] Noteworthy, they efficiently stabilize MTs. Protonated forms of some oligoamines such as oligolysine are known to promote GTP-mediated polymerization of tubulins[4] or give rise to bundling of paclitaxel-stabilized MTs.[5] However, in contrast with Asn(TEG-Gu^+)$_9$ and G1(Gu^+)$_9$R, they are unable to stabilize MTs.[4]

Prior to this study, we examined the potential of commercially available arginine nonamer (Arg(Gu^+)$_9$, Figure 1) for the interaction with BSA in 20 mM Tris-HCl buffer at pH 7.0. Isothermal titration calorimetry (ITC),[6] carried out by adding Arg(Gu^+)$_9$ (200 μM) to BSA (12 μM) at 25 °C (Figure S10),[7] displayed an exothermic profile with a progressive decrease in peak intensity upon increase in the amount of Arg(Gu^+)$_9$. Thus, Arg(Gu^+)$_9$ interacts with BSA, where the association constant (K_{assoc}), as evaluated from the ΔH_{ITC} values, was 5.8×10^4 M^{-1}. Of interest, Asn(TEG-Gu^+)$_9$, an asparagine nonamer bearing Gu^+ pendants *via* a triethylene glycol (TEG) spacer, showed a larger affinity ($K_{assoc} = 2.6 \times 10^5$ M^{-1}) than Arg(Gu^+)$_9$ toward BSA. This notable advantage of Asn(TEG-Gu^+)$_9$ stems most likely from a conformational flexibility of the TEG arms, allowing the sticky Gu^+ pendants to adjustably anchor onto the oxyanionic groups of BSA.

Based on these results, we incorporated the same adhesive motif into the periphery of a first-generation (G1) polyether dendron[8] carrying 9 TEG arms for accommodating Gu^+ (G1(Gu^+)$_9$OMe, Figure 1). As a reference, we also prepared lower-generation G0(Gu^+)$_3$OMe (Figure 1) carrying only 3 Gu^+ pendants. K_{assoc} was 3.4×10^5 M^{-1} for G1(Gu^+)$_9$OMe with BSA, as estimated from the ITC thermogram (Figure S11),[7] which is nearly comparable to that of Asn(TEG-Gu^+)$_9$ with BSA. In contrast, the K_{assoc} value of lower-generation G0(Gu^+)$_3$OMe was too small to determine by ITC, suggesting the importance of multivalency for the interaction of

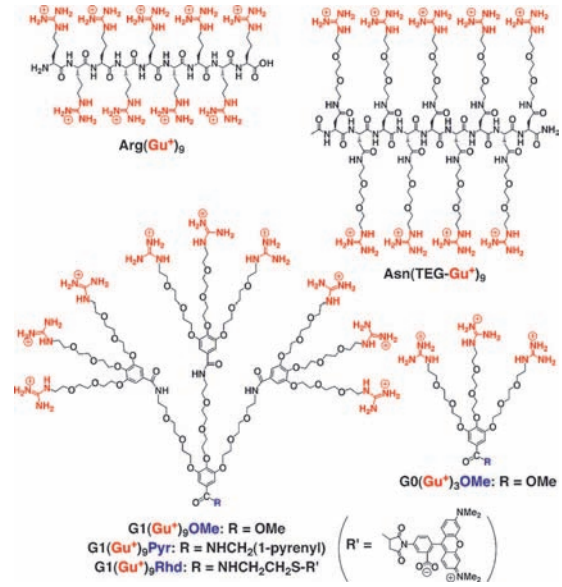

Figure 1. Structures of Arg(Gu^+)$_9$, Asn(TEG-Gu^+)$_9$, G0(Gu^+)$_3$R, and G1(Gu^+)$_9$R. The former two are TFA salts; the latter two are chloride salts.

Gu^+ with BSA in aqueous buffers. Titration of BSA with either Asn(TEG-Gu^+)$_9$ or G1(Gu^+)$_9$OMe (0−10 equiv) hardly changed the circular dichroism (CD) spectral profile of BSA at 200−250 nm[9] (Figure S12),[7] indicating that these molecular glues stick gently to BSA without causing serious denaturation. The design flexibility of dendronized G1(Gu^+)$_9$R allows for the incorporation of desired functional groups at its focal core. Thus, we prepared fluorescent G1(Gu^+)$_9$Pyr and G1(Gu^+)$_9$Rhd bearing pyrene and rhodamine focal groups, respectively (Figure 1). When G1(Gu^+)$_9$Pyr was allowed to interact with BSA, fluorescence resonance energy transfer (FRET) took place from the tryptophan units in BSA to the pyrenyl unit in the molecular glue (Figure S13).[7]

We further investigated the functions of G1(Gu^+)$_9$OMe and Asn(TEG-Gu^+)$_9$ in regard to their potential ability to stabilize microtubules (MTs) against depolymerization. Thus, MTs were allowed to form at 37 °C (incubation time; 20 min) from the α/β-tubulin heterodimer (2.5 mg/mL) in the presence of GTP (1 mM) in PEM buffer [50 mM PIPES buffer (pH 6.9) containing $MgCl_2$ (1 mM) and EGTA (1 mM)].[1] Upon being cooled to 15 °C, MTs alone without stabilizers underwent spontaneous depolymerization, as observed by a decrease in turbidity originating from the light scattering by bundled MTs (Figure 2, blue; OD_{340} = optical density at 340 nm).[1] In sharp contrast, when MTs in PEM buffer (9 μL) were incubated with Asn(TEG-Gu^+)$_9$ (1 mM in Tris buffer, 1 μL;

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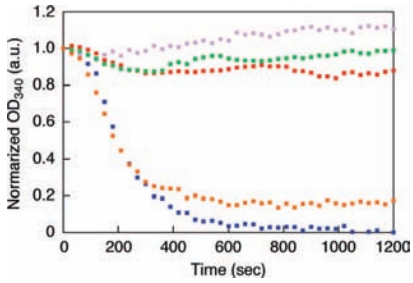

Figure 2. Changes in optical density at 340 nm (OD_{340}) of PEM buffer dispersions of microtubules (MTs; [α/β-tubulin heterodimer] = 2.5 mg/mL, [GTP] = 1 mM), incubated at 15 °C upon being cooled from 37 °C in the absence (blue) and presence of paclitaxel (100 μM; purple), Asn(TEG-Gu^+)$_9$ (100 μM; green), G1(Gu^+)$_9$OMe (100 μM; red), and G0(Gu^+)$_3$OMe (300 μM; orange).[7]

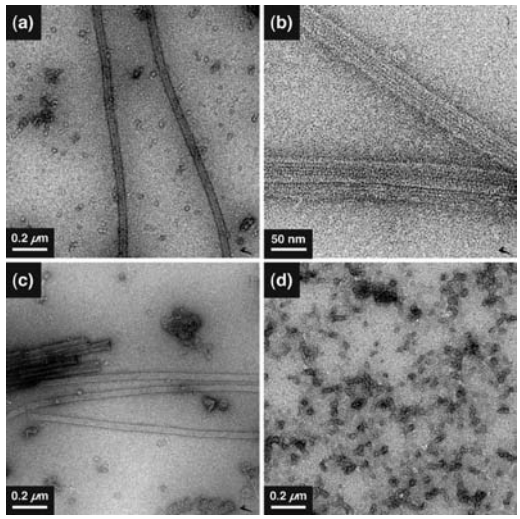

Figure 3. Transmission electron micrographs of microtubules (MTs), prepared with an α/β-tubulin heterodimer (1 mg/mL), GTP (200 μM), and glycerol (10%) in PEM buffer at 37 °C, and then incubated at 15 °C for 20 min with (a), (b) G1(Gu^+)$_9$OMe (40 μM), (c) Asn(TEG-Gu^+)$_9$ (40 μM), and (d) G0(Gu^+)$_3$OMe (120 μM).

green) or G1(Gu^+)$_9$OMe (1 mM in Tris buffer, 1 μL; red) before being cooled to 15 °C, the system remained turbid, suggesting MTs are stabilized against depolymerization. In fact, transmission electron microscopy (TEM) revealed that the resulting mixtures contain nanotubular assemblies (Figure 3a−c). Noteworthy, the inhibitory effects of these molecular glues on the depolymerization of MTs are likely comparable to that of paclitaxel (Figure 2, purple). In contrast, when G0(Gu^+)$_3$OMe (3 mM in Tris buffer, 1 μL) was used under otherwise identical conditions to the above, the turbidity dropped rapidly (orange) at a rate as large as that without stabilizers (blue). In TEM, accordingly, no MTs but only their depolymerized fragments were observed (Figure 3d). These results indicate that the multivalency of the interaction is important also for the stabilization of MTs.

When rhodamine-appended G1(Gu^+)$_9$Rhd was used instead of G1(Gu^+)$_9$OMe, bundled MTs nanotubules were visualized by confocal laser scanning microscopy upon excitation of rhodamine at 543 nm, indicating that G1(Gu^+)$_9$Rhd is colocalized with MTs (TEM: Figure S15).[7] When the resultant MTs were incubated with

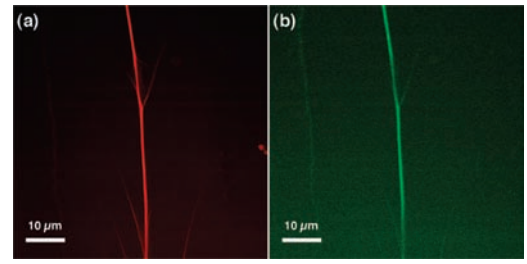

Figure 4. Confocal laser scanning micrographs of microtubules (MTs) upon excitation at (a) 543 and (b) 488 nm. MTs were prepared with an α/β-tubulin heterodimer (1 mg/mL), GTP (200 μM), and glycerol (10%) in PEM buffer at 37 °C, incubated at 15 °C for 20 min with G1(Gu^+)$_9$Rhd (400 μM), and then treated with Oregon Green 488 paclitaxel (1.7 μM).

fluorescently labeled paclitaxel (Oregon Green 488), the bundled nanotubules turned visible upon excitation at 488 and 543 nm (Figure 4). Therefore, G1(Gu^+)$_9$R likely stabilizes MTs by the salt-bridge formation mostly on the protein surface, while paclitaxel is known to utilize a van der Waals interaction upon being incorporated inside the MTs nanotubules.[10]

In conclusion, we developed a new class of molecular glues Asn(TEG-Gu^+)$_9$ and G1(Gu^+)$_9$R, which bear multiple guanidinium ions as sticky pendants via a flexible oligo(oxyethylene) spacer. They can adhere to proteins and also stabilize protein assemblies in aqueous buffers due to a multivalent salt-bridge formation between the sticky Gu^+ pendants and oxyanionic groups in proteins. By using dendronized G1(Gu^+)$_9$R with a designable focal core, one can realize noncovalent functionalization of proteins. Noteworthy is their high ability to stabilize microtubules just like that of paclitaxel, suggesting an interesting chemotherapeutic potential of these molecular glues.

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Supporting Information Available: Synthesis of Asn(TEG-Gu^+)$_9$, G1(Gu^+)$_9$R (R = OMe, Pyr, Rhd), and G0(Gu^+)$_3$OMe. ITC, NMR, MALDI-TOF-MS, and ESI-TOF-MS spectral data, fluorescence and CD spectra, TEM, and confocal laser scanning micrographs. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Zhou, J., Ed. *Microtubule Protocols*; Humana Press: Totowa, NJ, 2007.
(2) Schmidtchen, F. P.; Berger, M. *Chem. Rev.* **1997**, *97*, 1609.
(3) Examples of Gu^+ appended dendrimers: (a) Chen, H.; Neerman, M. F.; Parrish, A. R.; Simanek, E. E. *J. Am. Chem. Soc.* **2004**, *126*, 10044. (b) Wender, P. A.; Kreider, E.; Pelkey, E. T.; Rothbard, J.; VanDeusen, C. L. *Org. Lett.* **2005**, *7*, 4815.
(4) Wolff, J. *Biochemistry* **1998**, *37*, 10722.
(5) Needleman, D. J.; Ojeda-Lopez, M. A.; Raviv, U.; Miller, H. P.; Wilson, L.; Safinya, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16099.
(6) Ladbury, J. E., Doyle, M. L., Eds. *Biocalorimetry 2*; John Wiley & Sons: New York, 2004.
(7) See Supporting Information.
(8) (a) Fréchet, J. M. J., Tomalia, D. A., Eds. *Dendrimers and Other Dendritic Polymers*; Wiley: New York, 2002. (b) Bosman, A. W.; Janssen, H. M.; Meijer, E. W. *Chem. Rev.* **1999**, *99*, 1665. (c) Li, W.-S.; Jong, W.-D.; Aida, T. *Macromol. Eng.* **2007**, *2*, 1057.
(9) Gull, N.; Kumar, S.; Ahmad, B.; Khan, R. H.; Kabir-ud-Din, *Colloids Surf. B* **2006**, *51*, 10.
(10) Amos, L. A. *Org. Biomol. Chem.* **2004**, *2*, 2153.

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