

Selected Paper

Glutathione Nanosphere: Self-Assembly of Conformation-Regulated Trigonal-Glutathiones in Water

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A novel trigonal conjugate of glutathiones with a 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene core was synthesized and its self-assembling behavior was investigated in water. Three glutathione units were regulated to orient on the same side of the benzene ring, through steric repulsions between ethyl groups attached on the benzene core. Concentration dependence of ^{1}H NMR chemical shifts in D_2O revealed formation of molecular assemblies with two affinity constants ($K_a = 4.75 \times 10^2$ and $6.76 \times 10^4\,M^{-1}$), which reflect stepwise assembly directed by electrostatic interactions, hydrophobic interactions, and hydrogen bonding. In scanning electron microscopy, hard spherical assemblies with the size of 310 ± 50 nm were observed at high concentration (10 mM), whereas slightly disordered spherical assemblies were obtained at lower concentrations. The spherical assemblies self-assembled from the conformation-regulated trigonal glutathiones showed regular morphology and enhanced rigidity compared to those formed from conformationally non-regulated trigonal glutathiones.

The self-assembly of biomolecules, such as DNAs and peptides, have attracted much attention because of their potential for the design of elaborated nanostructures.[1] Supramolecular protein assemblies in biological systems, such as viral capsids, fragella, microtubles, amyloid fibrils, and clathrin,[2] provide excellent models for the de novo design of artificial peptide nanoassemblies. Inspired by these biological supermolecules, various kinds of peptide nanostructures have been developed. Amphiphilic peptides have been designed to form vesicle-like assemblies in water[3,4] or monolayers at the oil/water interfaces.[5] α-Helix coiled-coil[6–10] and β-sheet[11–19] structures have been also employed as self-assembling motifs to form peptide nanofibers and gels. For example, Woolfson et al. developed orthogonal coiled-coil peptide units that self-assembled into unique nanofibers, such as zig-zag and branched ribbons.[8] Zhang et al. reported an ionic complementary β-sheet-forming peptide FKFEFKFE which efficiently self-assembled into left-handed helical ribbons.[12a] Chmielewski et al. reported self-assembly of collagen peptide into microstructures via metal coordination.[20]

The self-assembly of conventional peptides depends on their amphiphilicity and capability to form secondary structures. In order to innovate the molecular design of peptide-based molecular assemblies, pre-organization of peptide chains should be introduced since it might reduce the entropy loss during self-assembling and afford unique assemblies even with short peptides.

Such a pre-organization approach has been successfully adopted by biological supermolecules. For example, clathrin[21] and the internal skeleton of tomato bushy stunt virus[2,22] are self-assembled from C_3-symmetric protein subunits. By mimicking these C_3-symmetric protein subunits, we have developed artificial C_3-symmetric self-assembly units for the construction of unique bio-nanoassemblies.[23,24] C_3-symmetric DNA bearing self-complementary sticky-ends spontaneously formed spherical assemblies in water.[23] Trigonal conjugates of β-sheet forming peptides were self-assembled into nanospheres[24a] and nanofibers,[24b] depending on molecular structures. Similarly, C_3-symmetric dipeptide WW conjugate was shown to self-assemble into nanocapsules in methanol–water mixtures, as reported by Gazit et al.[25]

Recently, ubiquitous biological tripeptide, gultathione (γ-Glu–Cys–Gly), has attracted much interest as a useful self-assembly unit. Atkins et al. reported formation of organogel[26a] and hydrogel[26b] from oxidized glutathione and glutathione–pyrene conjugate, respectively. We have developed a C_3-symmetric glutathione conjugate (Trigonal-glutathione, **TG**) which spontaneously self-assembled into nanospheres with the size of 100–250 nm in water,[27] although its symmetric molecular design was totally different from those of conventional amphiphiles. In this paper, we describe synthesis and self-assembly of a novel C_3-symmetric glutathione conjugate in water (Figure 1, **CRTG**). 1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene scaffold was employed, since it has been utilized as C_3-symmetric scaffold to design tripodal receptors.[28] The substituents on the 1,3,5-positions were reported to orient the same side of benzene ring, and this feature was utilized to assemble three glutathione arms on one side of the scaffold.

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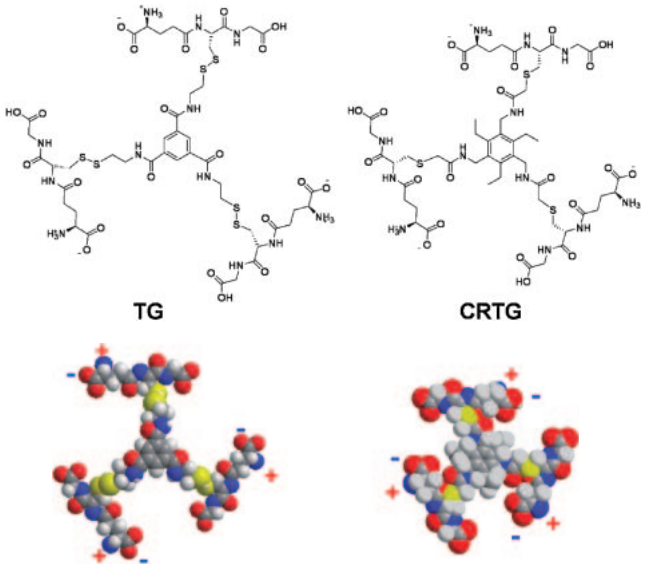


Figure 1. Structures of conventional trigonal-glutathione (**TG**) and conformation-regulated trigonal-glutathione (**CRTG**) at pH 3.

Experimental

General. Reagents were obtained from commercial sources and used without further purification. Deionized water of high resistivity (>18 MΩ cm) purified with a Millipore Purification System (Milli-Q water) was used as a solvent for trigonal-glutathiones. ^{1}H NMR spectra were recorded on a Bruker AV300M spectrometer. Reversed-phase HPLC was performed at ambient temperature with a Shimadzu LC-6AD liquid chromatograph equipped with a UV–vis detector (220 nm, Shimadzu SPD-10AVvp) using GL Science Inertsil ODS-3 columns (4.6 × 250 mm and 20 × 250 mm). MALDI-TOF mass spectra were obtained on a PE Applied Biosystems Voyager System 1180 MALDI-TOF mass spectrometer with dithranol and α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix.

Synthesis. 1,3,5-Tris(aminomethyl)-2,4,6-triethylbenzene (**1**) was synthesized from 1,3,5-triethylbenzene according to a literature method.[28e]

1,3,5-Tris(iodoacetamidomethyl)-2,4,6-triethylbenzene (2). A solution of iodoacetic acid *N*-hydroxysuccinimide ester (1.00 g, 3.53 mmol) in acetone (10 mL) was added to a solution of **1** (0.249 g, 1.00 mmol) in acetone (5 mL) at −5 °C. The mixture was stirred in the dark for 8 h at the same temperature and the resulting colorless precipitate was filtered and dried under vacuum to provide compound **2** (0.225 g, 30%). MALDI-TOF-MS (matrix: dithranol): *m/z* calcd for $C_{21}H_{30}I_3N_3O_3 + Na^+$: 775.93 $[M + Na]^+$, found: 775.79 $[M + Na]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.24 (t, $J = 4.2$ Hz, 3H), 4.29 (d, $J = 4.2$ Hz, 6H), 3.66 (s, 6H), 2.63 (q, $J = 7.2$ Hz, 6H), 1.09 (t, $J = 7.2$ Hz, 9H).

1,3,5-Tris{[2-(carboxymethylcarbamoyl)-2-(γ-glutamylamino)ethylthio]acetamidomethyl}-2,4,6-triethylbenzene (Conformation-Regulated Trigonal-Glutathione, CRTG). Reduced glutathione (0.187 g, 0.608 mmol) was dissolved in degassed water (10 mL). To the solution was added a solution of **2** (0.151 g, 0.200 mmol) in degassed DMF (30 mL) at 0–5 °C under nitrogen atmosphere. After adding diisopropylethylamine (0.40 mL, 2.3 mmol), the mixture was stirred for 3 h under the same conditions. After the solvent was evaporated under vacuum, the residue was dissolved in water and then lyophilized. The resulting colorless powder was purified by reversed-phase HPLC eluting with a linear gradient of acetonitrile/water (15/85 to 30/70 over 60 min) containing 0.1% TFA. The elution fraction containing **CRTG** was lyophilized to a colorless powder (0.183 g, 60%). MALDI-TOF-MS (matrix: α-CHCA): *m/z* calcd for $C_{51}H_{78}N_{12}O_{21}S_3$: 1290.46 $[M]^+$, found: 1290.29 $[M]^+$. ^{1}H NMR (300 MHz, [**CRTG**] = 1 mM in D_2O at 20 °C, TSP): δ 4.42 (dd, $J = 5.1$, 8.7 Hz, 3H), 4.33 (s, 6H), 3.78 (s, 6H), 3.71 (t, $J = 6.6$ Hz, 3H), 3.16 (s, 6H), 2.91 (dd, $J = 5.1$, 14.1 Hz, 3H), 2.77 (dd, $J = 8.7$, 14.1 Hz, 3H), 2.51 (q, $J = 7.5$ Hz, 6H), 2.39 (dt, $J = 2.4$, 7.5 Hz, 6H), 2.02 (dt, $J = 6.6$, 7.5 Hz, 6H), 1.00 (t, $J = 7.5$ Hz, 6H). Elemental Analysis calcd for $C_{51}H_{78}N_{12}O_{21}S_3 \cdot 2CF_3CO_2H$: C, 43.47; H, 5.31; N, 11.06%. Found: C, 43.53; H, 5.30; N, 11.07%.

Molecular Mechanics Calculations. Molecular mechanics calculations were carried out by using OPLS2005 force field in MacroModel 9.1 program (SCHRÖDINGER). Total energy of various conformations of trigonal-glutathiones in water was calculated with the GB/SA solvation model[29] of the program. All geometric parameters (bond lengths, bond angles, and dihedral angles) were optimized without any assumption by using low mode/torsional sampling (MCMM) (step number: 10000) to search the global minimum conformation.

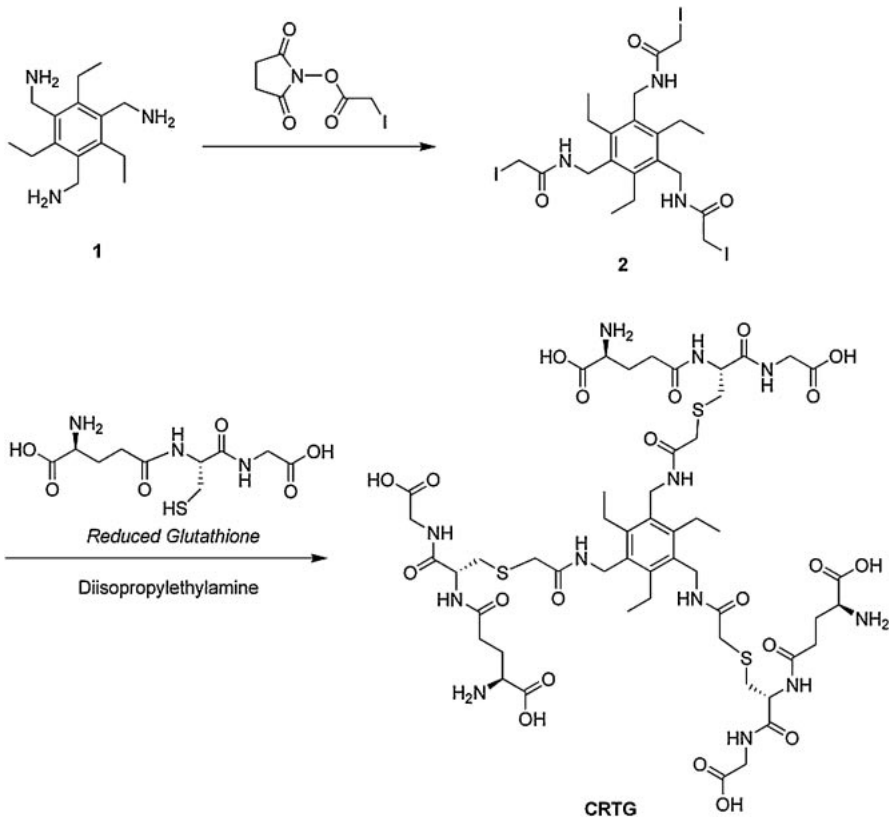
Estimation of Association Constants by ^{1}H NMR. ^{1}H NMR spectra of **CRTG** at different concentration in D_2O were recorded on a Bruker AV300M spectrometer at 18 °C with sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propanoate (TSP) as external standard. The chemical shifts of each proton of **CRTG** were plotted to the concentration. The obtained curves were fitted by nonlinear regression analysis to the isodesmic model (eq 1).[30]

$$\delta_{max} - \delta = \frac{2K_a c + 1 - \sqrt{4K_a c + 1}}{2K_a{}^2 c^2}(\delta_{max} - \delta_f) \qquad (1)$$

δ denotes the chemical shift obtained from the spectra, δ_f and δ_{max} are the chemical shifts for the free and assembled species, respectively, K_a is the association constant, and c is the total concentration of **CRTG** in the sample.

FT-IR Measurements in D_2O. Transmission FT-IR spectrum of **CRTG** at 10 mM in D_2O were recorded with a Shimadzu FT-IR 8400S spectrophotometer using a liquid cell at room temperature under nitrogen atmosphere.

Scanning Electron Microscopy (SEM). Aqueous solutions of trigonal-glutathiones (0.1, 1.0, and 10 mM) were prepared simply by dissolving in water without sonication or heating. The samples were aged at 3 °C for 24 h. The pH was adjusted by adding aqueous NaOH. 10 μL of the solutions were applied to a carbon-coated Cu-grid (Oken Co., Ltd.), left for 60 s, and then removed. The grid was dried in vacuo and coated with platinum (ca. 3 nm, Hitachi E-1030 ion sputter). The grids were observed by SEM (Hitachi S-5000) with an acceleration voltage of 15 kV at tilt angle of 0 and 30°.



Scheme 1. Synthesis of conformation-regulated trigonal-glutathione (**CRTG**).

Results and Discussion

Synthesis of Conformation-Regulated Trigonal-Glutathione. Scheme 1 shows the synthesis of a conformation-regulated trigonal-glutathione (**CRTG**) from 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene (**1**). ^{1}H NMR of compound **1** in $CDCl_3$ at ambient temperature showed that **1** exclusively takes alternate substituent conformation.[28] Compound **1** was converted to the iodoacetamido derivative **2** by condensation with activated ester. **CRTG** was prepared by substituting thiol groups of reduced glutathione for the iodo groups of core molecule **2**. The final product was purified by reverse-phase HPLC and confirmed by MALDI-TOF-MS ($m/z = 1290$) and ^{1}H NMR. Aqueous solution of the purified **CRTG** was pH 3. **CRTG** was soluble in water at the whole range of pH even at high concentration (10 mM). ^{1}H NMR of 1 mM **CRTG** in D_2O showed one set of relatively sharp signals which are assigned to a kind of glutathione, linker arm, and ethyl group at the core, respectively (Figures 3A and 3B). This suggests that substituents on **CRTG** in water preferentially adopted alternative conformation, in which three glutathione arms were oriented to the same side of the benzene ring, because it is known that substituents on 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene scaffold prevent free rotation.[28]

Molecular Mechanics Calculation of Trigonal-Glutathiones. In order to estimate conformations and potential energies of **CRTG** and conventional **TG** in water, molecular mechanics calculations with GB/SA solvation model[29] (MacroModel, OPLS2005 force field, MCMM algorism) were carried out. Figure 2 shows the most stable and metastable conformations of **CRTG** and conventional **TG**, respectively. The "metastable conformation" means conformation at a local minimum potential in which one arm of glutathione units flipped (turned to the opposite face). In the most stable

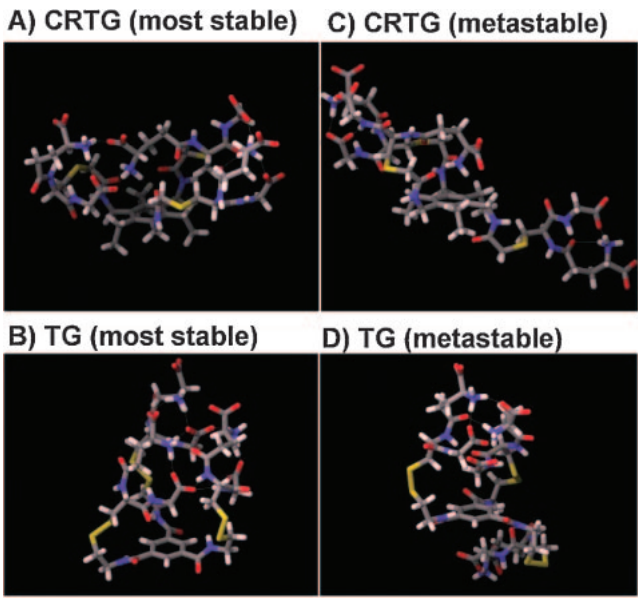


Figure 2. (A, B) Most stable conformation of **CRTG** (A) and conventional **TG** (B) expected by molecular mechanics caluculation (MacroModel). (C, D) Metastable conformation of **CRTG** (C) and conventional **TG** (D) at a local minimum in which one arm of glutathione flipped.

conformations of **CRTG** and **TG**, it was shown that the three glutathione arms were oriented to the same side of the benzene ring by forming intramolecular hydrogen bonds. The tripod structure was more stabilized in **CRTG**, since energy gap ΔE between the most stable and metastable conformations of **CRTG** ($+43.95\,\mathrm{kJ\,mol^{-1}}$) was much larger than that of **TG** ($+6.96\,\mathrm{kJ\,mol^{-1}}$) as summarized in Table 1. As the rotation energy barrier on the C–C bond of ethane is about $12\,\mathrm{kJ\,mol^{-1}}$,[31] the calculated energy gaps indicate that glutathione arms on **CRTG** do not flip at ambient temperature, whereas those on **TG** can flexibly change their orientation. The unique orientation of glutathione units on **CRTG** in Figure 2A is supported by ^{1}H NMR data as described below.

Table 1. Total Potential Energies of Trigonal-Glutathiones Calculated by Molecular Mechanics

	$E_{\text{most stable}}$ /kJ mol^{-1} a)	$E_{\text{metastable}}$ /kJ mol^{-1} b)	ΔE /kJ mol^{-1} c)
CRTG	−4220.95	−4177.00	+43.95
TG	−4243.26	−4236.29	+6.97

a) Total potential energy of most stable conformation shown as Figures 2A and 2B. b) Total potential energy of metastable conformation shown as Figures 2C and 2D. c) $\Delta E = E_{\text{metastable}} - E_{\text{most stable}}$.

Analysis of Self-Assembling Behavior of CRTG Using ^{1}H NMR. Figure 3A shows ^{1}H NMR spectra of **CRTG** at various concentrations in D_2O. As the concentration increases, signals assigned to α-protons of glycine (H_h) and glutamate (H_g) shifted to the lower magnetic field, while the other signals were independent of concentration. These results indicate that **CRTG** is self-assembled by hydrogen bonds and/or electrostatic interactions between glutathione units upon increasing concentration. FT-IR transmission spectrum of 10 mM **CRTG** in D_2O showed peaks at 1729, 1672, 1630, and $1462\,\mathrm{cm^{-1}}$, which are assigned to C=O stretching vibration of COOH, free and hydrogen-bonded amide I bands, and C–N stretching vibration, respectively (Figure 4).[32] It is probable that amide groups on the arms are not dominantly participating in the self-assembly, because the H_i and H_f protons did not show shifts in ^{1}H NMR spectra even at higher concentrations.

Figure 3C shows concentration dependence of chemical shifts of H_h and H_g for **CRTG**. The association constants (K_a) and chemical shifts of free species (δ_f) were calculated by nonlinear regression analysis according to the isodesmic model (eq 1, see Materials and Methods).[30] The analyses of the chemical shifts of H_h and H_g afforded different affinity constants ($K_a = 6.76 \times 10^4\,\mathrm{M^{-1}}$ for H_h and $K_a = 4.75 \times 10^2\,\mathrm{M^{-1}}$ for H_g), respectively, as summarized in Table 2. These results suggest that two different modes of association are involved in the self-assembly of **CRTG**. The intermolecular interactions

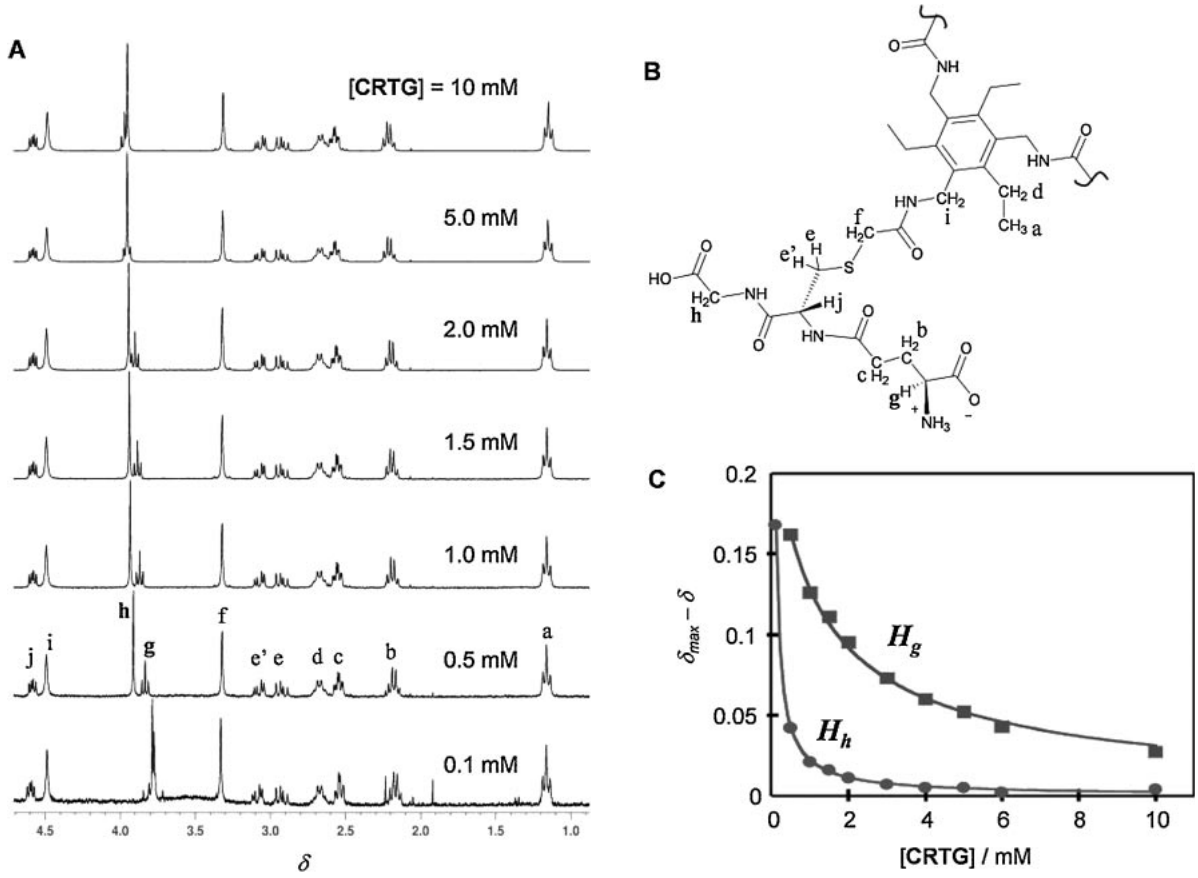


Figure 3. (A) ^{1}H NMR spectra of **CRTG** at various concentrations (0.1–10 mM) in D_2O (pD 3) at 18 °C. (B) Assignment of each proton signal. (C) Dependence of the chemical shifts of H_g and H_h of **CRTG** on the concentration. The solid curves are theoretical according to an isodesmic model (eq 1) using the parameters in Table 2.

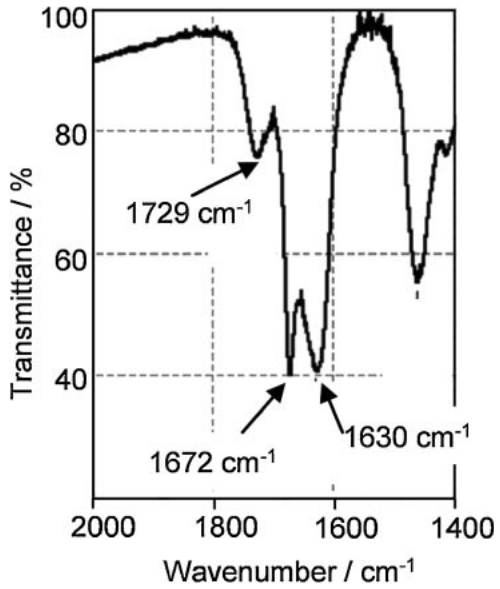


Figure 4. FT-IR spectrum of **CRTG** at 10 mM in D_2O.

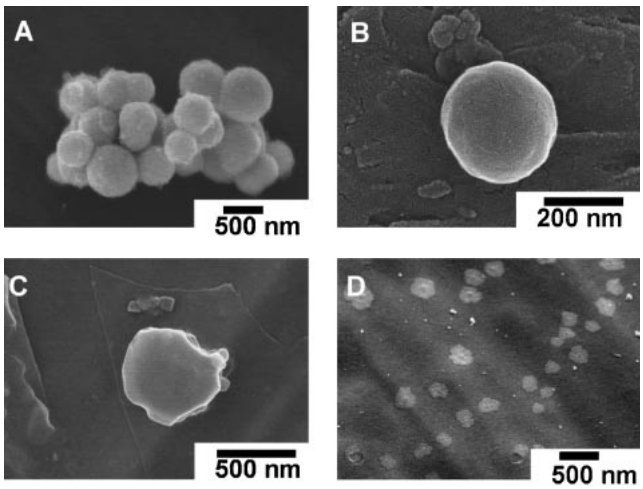


Figure 6. The effect of pH on the nanostructure self-assembled from **CRTG** (1 mM) in water. (A) pH 3, (B) pH 5, (C) pH 7, and (D) pH 10. SEM samples were coated with ca. 3 nm Pt.

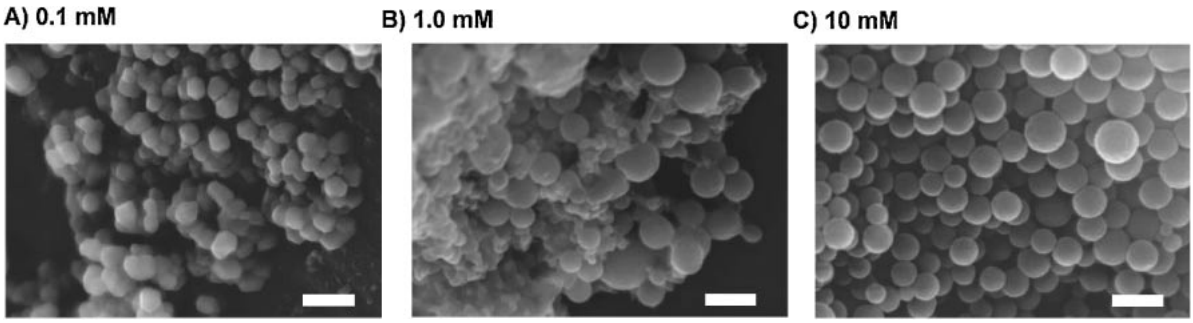


Figure 5. SEM images of aqueous solution (pH 3) of **CRTG** at 0.1 (A), 1.0 (B), and 10 mM (C). SEM sample was coated with ca. 3 nm Pt. Scale bar indicates 500 nm.

Table 2. Apparent Association Constants (K_a) in the Self-Assembly of **CRTG** at 18 °C

	K_a/M^{-1}	δ_f[a)]	δ_{max}[b)]
H_g of **CRTG**	$(4.75 \pm 0.35) \times 10^2$	3.76 ± 0.01	3.996
H_h of **CRTG**	$(6.76 \pm 1.29) \times 10^4$	2.29 ± 0.26	3.955

a) The chemical shift for the free species. b) The chemical shift for the assembled species.

occurring even at lower concentration involve carboxylic acid and amide groups near the H_h proton ($1/K_a = 0.0015$ mM), which might form intermolecular hydrogen bonds. Contribution of glutathione N-terminal groups (ammonium and carboxylate ions) is indicated by the changes in H_g proton signals ($1/K_a = 2.1$ mM), which becomes eminent at higher concentrations. Similar behavior was observed in ^{1}H NMR of conventional **TG**, but the association constant ($K_a = 6.36 \times 10^3\ M^{-1}$) was lower than that of **CRTG**.[27]

Nanostructures of Trigonal-Glutathiones. Scanning electron microscopy (SEM) of **CRTG** showed the presence of spherical assemblies with the size of 200–500 nm at the range of 0.1–10 mM **CRTG** concentration (Figure 5). It is notable that non-collapsed spherical assemblies (diameter, 310 ± 50 nm) with smooth surface were observed at higher concentration (10 mM), whereas irregular spherical assemblies were observed at lower concentrations (0.1 and 1.0 mM). The observed difference in morphology might arise from differences in the assembly mode. It seems that interactions involving glutathione C-termini (hydrogen bonds of carboxylic acid and amide groups), which are dominant at lower concentration (0.1 and 1.0 mM), afford irregular spherical assemblies. At the higher concentration (10 mM), it is probable that **CRTG** molecules in a spherical assembly are rearranged by electrostatic interactions of N-termini (ammonium and carboxylate ions), which afford regular spherical assemblies with smooth surface. Time-dependent morphological changes of the assemblies in the aqueous solutions were hardly observed at room temperature at least for 1 month.

Figure 6 shows the effect of pH on the morphology of **CRTG** (concentration, 1 mM). Upon increasing pH, the spherical assemblies gradually collapsed. It is known that reduced glutathione is ionized with $pK_{a1} = 2.12$ (carboxylic acid of glutaminic acid), $pK_{a2} = 3.59$ (carboxylic acid of glycine), $pK_{a3} = 8.75$ (thiol of cysteine), and $pK_{a4} = 9.65$ (ammonium of glutaminic acid).[33] By using these pK_a values, the isoelectric point (*pI*) of **CRTG** is estimated to be ca. 2.9. It indicates that **CRTG** possess nearly neutral net charge at pH 3, and anionic charge at higher pH. Therefore, the disruption of

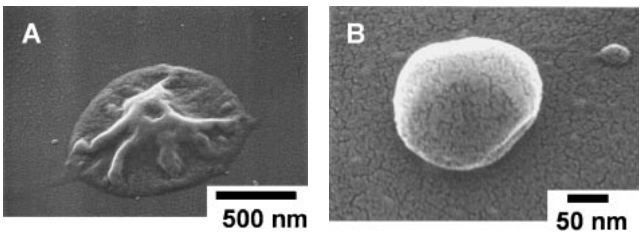


Figure 7. SEM images of the nanostructures self-assembled from conventional **TG** in water (pH 3) at 1 (A) and 10 mM (B). SEM sample was coated with ca. 3 nm Pt.

spherical assemblies at higher pH would be ascribed to the electrostatic repulsion between glutathione units.

The spherical assemblies formed from 10 mM **CRTG** (310 ± 50 nm by SEM, Figure 5C) were larger than those formed from conventional **TG** (112 ± 75 nm by SEM, Figure 7B).[27] In addition, the size distribution observed for **CRTG** is apparently smaller compared to those of **TG**. The nanoassemblies formed from 1 mM **CRTG** (Figures 5B and 6A) are all hard (non-collapsed) spheres, whereas the conventional **TG** (concentration at 1 mM) afforded wrinkly collapsed assemblies (Figure 7A). The difference in hardness between assembled structures of **CRTG** and **TG** might arise from different association constants (**CRTG**: $K_a = 6.76 \times 10^4\,M^{-1}$, **TG**: $K_a = 6.36 \times 10^3\,M^{-1}$). Therefore it is revealed that the regulation of conformation of trigonal glutathiones give rise to the ability to form more regular and harder spherical assemblies.

Conclusion

We have developed a novel trigonal glutathione which takes regulated conformation. This conjugate was self-assembled into hard nanospheres by forming hydrogen bonds and/or electrostatic interaction between glutathione moieties, and the morphology depended on the concentration and pH. It is notable that spherical assemblies were selectively formed without the formation of fibrous assemblies often observed for β-sheet analogs. The spherical assemblies formed from **CRTG** have narrow size distribution compared to those of conformationally non-regulated trigonal glutathiones assemblies,[26] and would be applicable as pH-responding and biodegradable molecular carriers. The present molecular design based on the conformation-regulated C_3-symmetric strategy provides a novel guideline for the design of peptide-based molecular assemblies. Studies of the capability of these nanoassemblies to include guest molecules are currently in progress.

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