pH-Dependent On-off Inclusion Complexation of Carboxymethylated Cyclosophoraoses with Neutral Red

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Cyclosophoraoses, which are a class of unbranched cyclic (1 → 2)-β-D-glucans, are produced as intraoligosaccharides or extraoligosaccharides by many strains of *Rhizobium* and *Agrobacterium.* They form a mixture of large-ring molecules that comprise a variable number of glucose residues (17 to 40) per ring in a neutral or anionic form.[1-4] The first report of cyclosophoraoses came in 1942 with its discovery in the extracellular media of *Agrobacterium tumefaciens* cultures.[5] Cyclosophoraoses generally function in periplasmic places as an osmoprotectant against osmotic stress.[6] They are also reportedly involved in the symbiotic interaction between the *Rhizobiaceae* family and its specific symbiotic plants, such as alfafa, clover and soybean.[1] Throughout this interaction, cyclosophoraoses are suspected of being involved in complexation with various plant flavonoids.[7] In addition, neutral or anionic cyclosophoraoses have been applied as a host molecule in various technologies of inclusion complexation; for example, as a solubility enhancer of poorly soluble guest molecules,[8-12] and as a chiral additive in capillary electrophoresis (CE).[13]

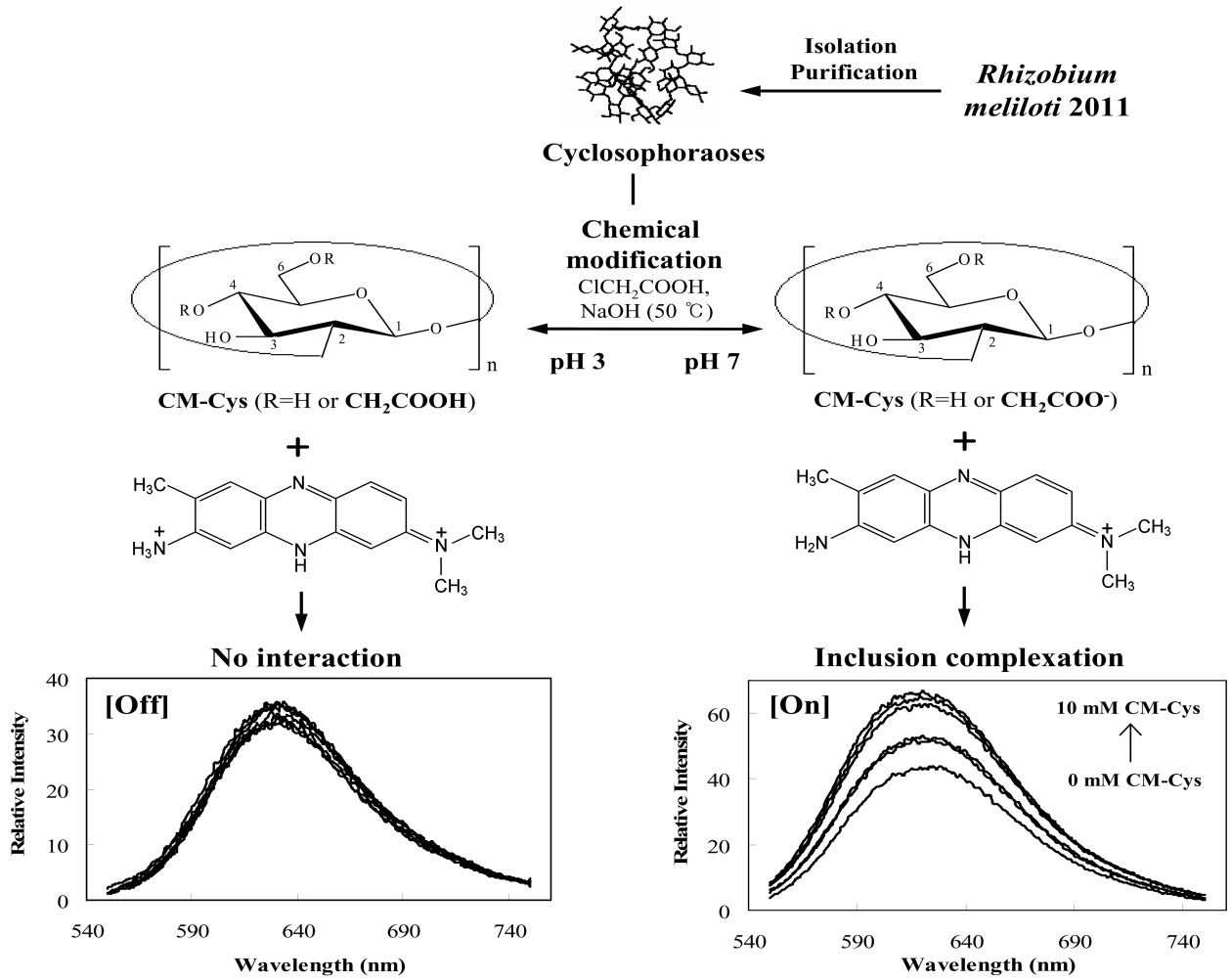
For further application of cyclosophoraoses, carboxymethylated cyclosophoraoses (CM-Cys) was recently synthesized by chemical modification of the cyclosophoraoses and used as a good host for inclusion complexation.[14] The CM-Cys shows a conformational change because of the way the charge distribution of the weak acidic carboxymethyl group varies according to the aqueous pH conditions.[14] In an aqueous solution, neutral red exists in two molecular forms: the acidic form and the neutral form.[15] Neutral red is a readily available biological dye, and it can be used as a fluorescence probe in an acidic medium to investigate the structure of DNA molecules and to construct a sensitive assay of DNA.[16,17] Moreover, neutral red is a good acid-base indicator within a pH range 6.0 to 8.0.[18]

On the basis of the pH-dependent properties of CM-Cys, we investigated the inclusion complexation of CM-Cys with neutral red at the acidic and neutral aqueous conditions. For this purpose, we used UV-Vis and fluorescence spectroscopic analyses (Scheme 1). The binding constant (K_b) of the inclusion complex was determined from fluorescence data with the aid of a modified Benesi-Hildebrand equation.[19]

First, we conducted isolation, purification, and structural analyses of cyclosophoraoses as described previously.[10,12,20] Furthermore, through matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, we confirmed that the ring sizes of the neutral cyclosophoraoses ranged from degree of polymerization (DP) 17 to 27,[11] and that the number-average molecular weight, Mn, of neutral cyclosophoraoses was 3568.6.[12] Although the exact three-dimensional structure of cyclosophoraoses is not known, recent nuclear magnetic resonance (NMR) studies and molecular dynamics simulations have provided molecular models with flexible glycosidic linkage backbones.[21-24] Scheme 1 shows the molecular model of cyclosophoraoses proposed by Jung *et al.*[22] Following this model, we produced CM-Cys by chemical modification with monochloroacetic acid, and we synthesized the CM-Cys though a one-step process in an 85 percent yield. To monitor the reaction, we used thin layer chromatography (TLC). The R_f value of the purified CM-Cys was 0.285. The structure of CM-Cys was characterized with the aid of NMR and Fourier transform infrared (FTIR) spectroscopy and with MALDI-TOF mass spectrometry. The values for the degree of substitution (DS) of CM-Cys were confirmed to range from 0.012 to 0.290 (data not shown).[14] Through NMR spectroscopic analysis, we also identified that the neutral cyclosophoraoses were predominantly substituted with carboxymethyl groups at positions 4-OH and 6-OH, as described previously.[14]

We used a UV-Vis spectroscopic study to confirm the complex stoichiometry. The ΔA values were calculated by measuring the absorbance of neutral red solutions in the absence and presence of CM-Cys. In these standard solutions, the total concentration of two species remained constant but the ratio of the initial concentration, expressed by r, varied between 0 ([neutral red] : [CM-Cys = 0 : 10] and 1 ([neutral red] : [CM-Cys = 10 : 0]). Figure 1(A) shows a continuous variation plot of the $\Delta A \cdot$[neutral red] versus the molar ratio of neutral red and CM-Cys, r, at pH 7. The resulting continuous variation plot clearly demonstrates that the complex has a 1 : 1 stoichiometry at pH 7 because the $\Delta A \cdot$[neutral red] maximum has an r value of 0.5.[25,26]

To identify the pH-dependent conformational change of CM-Cys, we investigated the effects of CM-Cys concentration and pH for the complexation of CM-Cys with neutral red on the fluorescence spectra of neutral red (Scheme 1 and Figure 1(B)). The fluorescence intensity of neutral red was



Scheme 1. Summary of the pH-dependent complexation of CM-Cys with neutral red and fluorescence emission spectra of 4.0×10^{-5} M neutral red at pH 3 and pH 7 in different concentrations of CM-Cys (0 mM, 1 mM, 2 mM, 5 mM, 7 mM, and 10 mM).

enhanced by increasing the CM-Cys concentration from 0 mM to 10 mM (0 mM, 1 mM, 2 mM, 5 mM, 7 mM, and 10 mM) while the neutral red concentration was held constant at 4.0×10^{-5} M.

The fluorescence intensity of neutral red also depends heavily on pH in the presence of CM-Cys. At pH 7, a neutral form of neutral red strongly interacted with CM-Cys as the CM-Cys concentration increased. However, at pH 3, we observed no interaction between CM-Cys and neutral red. At pH 7, CM-Cys might be easily complexed with a neutral form of neutral red by hydrogen bond or electrostatic interactions between -COO^- of CM-Cys and H_2N- of neutral red, which could be stronger than the interactions between -COOH of CM-Cys and ^+H_3N- of neutral red at pH 3.

Given that the binding constant of a complex is a measure of the complexing power, we evaluated the binding constant of a complex of CM-Cys with neutral red at different pH values based on a 1 : 1 (CM-Cys : neutral red) inclusion model. To obtain the binding constant from the fluorescence data, we used the following modified Benesi–Hildebrand equation (double reciprocal plot):[19]

$$(F\text{-}F_0)^{-1} = (Kk[P]_0\,[\text{CM-Cys}]_0)^{-1} + (k\text{Q}[P]_0)^{-1},$$

where F and F_0 represent the fluorescence signals of neutral red in the presence and absence of CM-Cys, respectively, $[P]_0$ and $[\text{CM-Cys}]_0$ represent the initial concentrations of neutral red and CM-Cys, k is an instrumental constant, and Q is the quantum yield for the complex.

Figure 1(C) shows the double reciprocal plots of $(F\text{-}F_0)^{-1}$ versus $[\text{CM-Cys}]_0^{-1}$ at pH 7. The plots exhibit good linearity, and the r value of the equation for the lines of the complex is 0.975. Through the equations of the plots for the complex, the value of K_b was calculated to be 360 M^{-1} for pH 7, though the value of K_b could not be measured for pH 3. This result means that the complexation of CM-Cys with neutral red occurs in the on-off mode, which depends on the pH.

In this study, we synthesized CM-Cys through a one-step chemical modification of cyclosophoraoses that had been isolated by *R. meliloti* 2011. In this case, the CM-Cys was substituted with carboxymethyl groups on the hydroxyl positions of cyclosophoraoses. Furthermore, the absorption and fluorescence measurements have demonstrated that the

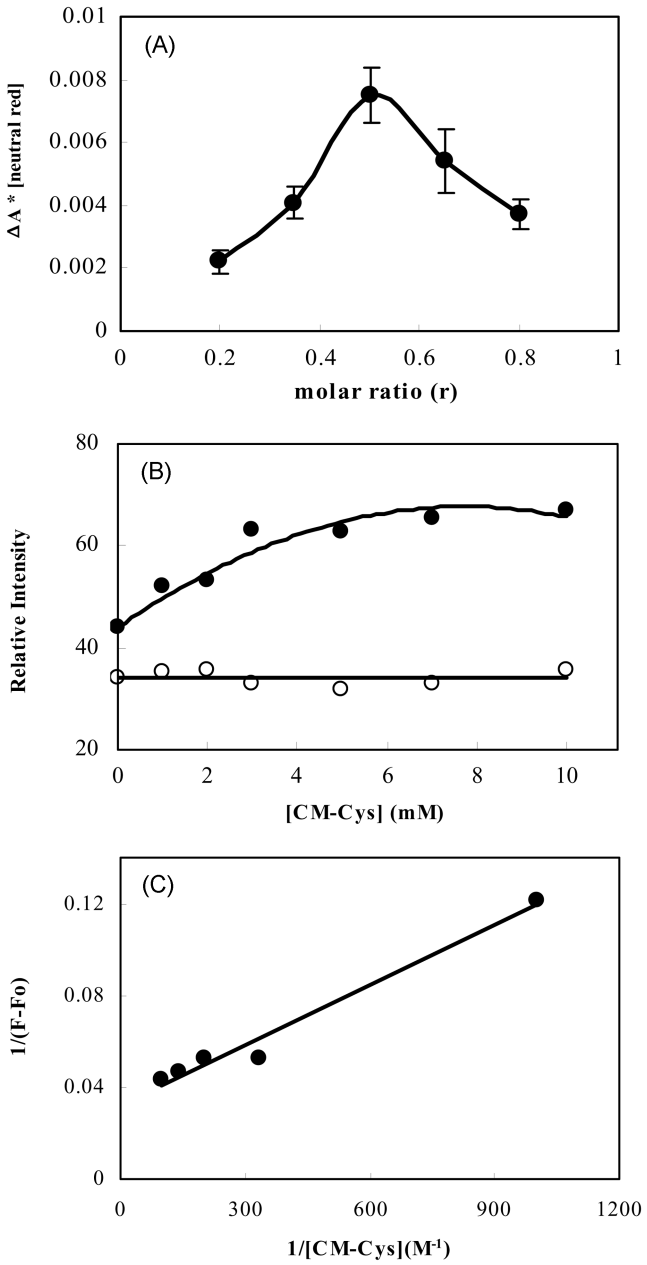


Figure 1. (A) Fluorescence intensities of neutral red at pH 3 (○) and pH 7 (●) on various CM-Cys concentrations; (B) Double reciprocal plots for neutral red complexed with CM-Cys at pH 7; and (C) Job plot of the CM-Cys and neutral red complex at pH 7.

inclusion complexation interaction between CM-Cys and neutral red occurs in the on-off mode, depending on the pHs. The results of UV-Vis and fluorescence studies are consistent with a simple 1 : 1 stoichiometry, and the binding ability of the complex depends critically on the applied pH. In addition, the binding constant of the complex at pH 7 was calculated to be 360 M^{-1}, but no binding was observed at pH 3. This result might also indicate that the binding ability of the complex was affected by changes in the three-dimensional structure of CM-Cys and by the hydrogen bond or electrostatic interactions of CM-Cys and neutral red. We therefore propose that CM-Cys can be applied to the development of biosensors as a kind of conformationally switchable on-off molecule[27,28] in the near future.

Experimental Sections

Materials and apparatus. All the chemicals that contained neutral red (Scheme 1) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The absorption and fluorescence measurements were performed with a U-2000 UV-Vis spectrophotometer (Hitachi, Japan) and an F-2000 fluorescence spectrophometer (Hitachi, Japan).

Preparation of cyclosophoraoses. We cultured *R. meliloti* 2011 in a 5 L jar fermenter containing a GMS medium as previously reported.[29] The isolation and purification of neutral and anionic Cys were achieved as previously reported.[10-12,20,30-32] The structure and molecular weight of neutral Cys were confirmed through NMR spectroscopy,[11,12] electrospray ionization-mass spectrometry,[30] and MALDI-TOF mass spectrometry,[10] as in our previous reports.

Preparation of CM-Cys from neutral cyclosophoraoses. As shown in Scheme 1, CM-Cys was prepared in accordance with a previously reported method.[20] We then added a 16.3 percent monochloroacetic acid solution (8.1 mL) to a mixture of neutral cyclosophoraoses (500 mg) and NaOH (2.8 g) in water (7.4 mL). After stirring for 4.5 h at 50 ^{o}C, we neutralized the mixture with 6 M HCl. We then precipitated the mixture by adding 8 volumes of ethanol and the mixture was kept overnight at 4 ^{o}C. After centrifugation, the precipitate was resuspended in distilled water and concentrated. Finally, the solution was desalted on a column (2 cm × 27 cm) packed with Sephadex G-10. The reaction was monitored with the aid of TLC (ethanol : buthanol : water = 5 : 5 : 4, v/v/v). To identify the CM-Cys, we used NMR spectroscopy and MALDI–TOF mass spectrometry, as described previously.[14]

Determination of the stoichiometry of the complex. The continuous variation method was adopted to determine the stoichiometry of the inclusion complex.[25,26] The total concentration of the two species, CM-Cys and neutral red, was kept constant, and the molar ratio, r, varied from 0 to 1. To measure the differences in absorbance between the free neutral red and the complex for a given mole ratio at 520 nm, we used a UV-Vis spectrophotometer at pH 7.

Determination of the binding constant (K_b) of the complex. Neutral red was dissolved in a KH_2PO_4 buffer at pH 3 and pH 7, and the concentration of the neutral red was kept constant at 4.0×10^{-5} M during the fluorescence experiment. The neutral red was added to the CM-Cys solutions with various concentrations in the KH_2PO_4 buffer at pH 3 and pH 7. After stirring the mixtures for 3 h under darkness, we measured the difference in the fluorescence intensity of neutral red at λ_{ex} 540 nm and λ_{em} 620 nm at pH 3 and at λ_{ex} 450 nm and λ_{em} 620 nm at pH 7.[11]

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