Novel Interaction between Glutamate and the Cu^{2+}/DMABN/β-CD Complex

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A new fluorescence-quenched ternary complex, Cu^{2+}/DMABN/β-CD, has been synthesized using β-cyclodextrin (β-CD), copper ions (Cu^{2+}), and 4-(dimethylamino) benzonitrile (DMABN). The significant quenching of DMABN fluorescence is realized through a photoinduced electron-transfer process between DMABN and Cu^{2+}. On the basis of the photochemical properties of the ternary complex, we have proposed a Cu^{2+}/DMABN/β-CD complex structure with a 2:2:2 molar ratio. To further investigate the ternary complex and explore its potential applications in bioanalysis, we have adopted a novel strategy to restore the quenched fluorescence of DMABN when the complex interacts with an important neurotransmitter, glutamate. The restoration of DMABN fluorescence in the ternary complex is complete and fast upon glutamate interaction. The new complex synthesis and the restoration of the quenched fluorescence processes enable a better understanding of cyclodextrin-based chemistry and also the exploration of novel bioanalysis techniques for neurotransmitters and amino acids. GABA and NMDA have also been used to interact with the ternary complex to restore its fluorescence. Different binding capabilities with the complex show that there are significant differences in binding affinity with different ligand molecules. The new complex and the restoration process will be further explored for bioassays for amino acids and neurotransmitters.

Introduction

In an earlier communication, we have reported the synthesis of a ternary complex, Cu^{2+}/pyrene/β-CD, and its photochemical behavior, which shows preliminary promise in developing direct amino acid analysis methodologies.[1] We are reporting here the synthesis of a new ternary complex by a similar strategy, using β-cyclodextrin (β-CD), copper ion (Cu^{2+}), and 4-(dimethylamino)benzonitrile (DMABN). A detailed study of the photochemical mechanism for the restoration of the fluorescence of this interesting complex, Cu^{2+}/DMABN/β-CD, upon interaction with a few important neurotransmitters and amino acids has been carried out spectroscopically. The strategy to synthesize this new complex and the involved fluorescence restoration mechanism are mainly based on the current knowledge of the three involved species: DMABN, Cu^{2+}, and β-CD.

The DMABN molecule has a unique spectroscopic property that allows the elucidation of its different conformational states. Upon electronic excitation, DMABN initially forms a locally excited (LE) or "nonpolar" state with geometry and dipole moment (6D) similar to those in the ground state. In polar media, an electron is transferred from the donor group (dimethylamino) to the acceptor group (cyanobenzene), and simultaneously, the donor group undergoes a twist. This twist makes the donor orbital orthogonal to the acceptor orbital, thus ensuring a complete electron transfer. The resulting state is a highly polar state with a dipole moment of about $16D^2$ and is known as a twisted intramolecular charge transfer (TICT) state. The fluorescence spectrum of DMABN in polar media displays two distinct bands.[2] The highly polar state and a nonpolar state are responsible for the long- and short-wavelength bands, respectively. The relative positions and intensities of these two bands are affected by the solvent polarity (which stabilizes the polar TICT state by solvation) and steric environment as well as the medium viscosity (which hinders the twisting motion of the rotating dimethylamino group).

Cyclodextrins (CDs) are cyclic oligosaccharides that possess hydrophobic cavities, which can encapsulate organic and organometallic molecules in aqueous solutions.[3,4] The geometry of cyclodextrin gives it the shape of a cone. Upon the inclusion of a fluorophore, CDs offer a more protective microenvironment and generally enhance the luminescence of the guest molecule by shielding the excited species from quenching and nonradiative decay processes that would otherwise occur in bulk solution. In general, the guest molecule loses its hydration spheres upon entering the cyclodextrin cavity, and water molecules are simultaneously expelled from the cavity. The restricted shape and size of the hydrophobic cavity of CD geometrically constrains the solute molecule and, therefore, markedly affects its properties.

Copper (II) ion is known to be able to quench the fluorescence via a photoinduced electron transfer (PIET) process, which includes the transfer of an electron from the fluorophore in the excited state to the metal ion.[5] It can form complexes with CDs in alkaline solutions.[6] Cu^{2+}/CD binary complexes are capable of forming inclusion complexes with organic molecules in the cavity.[7] It is known that ethylenediamine tetraacetic acid can strongly bind with the Cu^{2+} in bulk alkaline solution, which drives the Cu^{2+}/CD binding equilibrium to the direction of dissociation, leaving free CDs in the solution.[8] Copper (II) ion can also bind with glutamate, asparate, etc.[9]

On the basis of these interesting mechanisms and phenomena occurring in the aqueous medium, we have synthesized a new ternary complex and studied it spectroscopically upon its interactions with glutamate and a few other ligand molecules. The ligands used in this study are important in neurochemistry and neurotransmitter studies:[10,11] glutamate (neurotransmitter, amino acid), NMDA (a neuro-stimulant amino acid, *n*-methyl-

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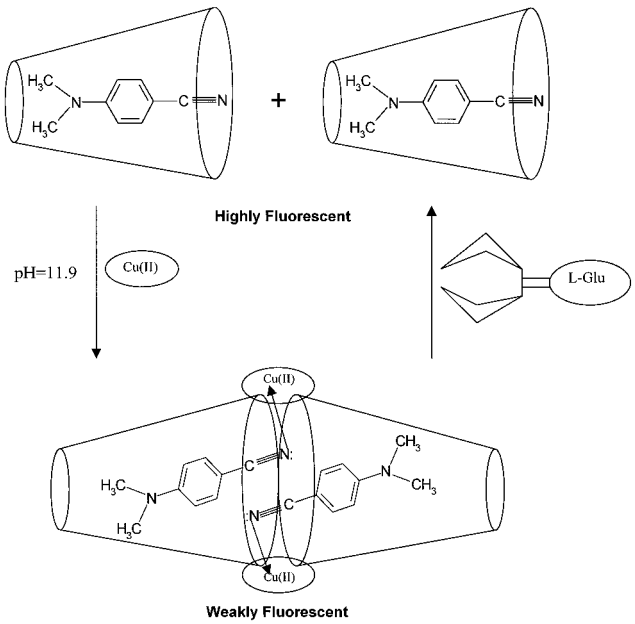

Figure 1. Schematic representation of the formation of the 2:2:2 Cu^{2+}/DMABN/β-CD ternary molecular complex.

D-aspartic acid), and GABA (a neurotransmitter, γ-amino-*n*-butyric acid). The scheme of synthesis of the ternary complex and the molecular interaction mechanism between the weakly fluorescent ternary molecular complex and glutamate are shown in Figure 1. Complete fluorescence restoration of DMABN is observed when the ternary molecular complex interacts with the glutamate. The photophysical and photochemical properties of DMABN during the stepwise formation of the ternary complex and its interactions with glutamate will be discussed.

Experimental Section

DMABN (Aldrich Chemical Co.) was purified by recrystallization from ethanol. β-CD (Aldrich Chemical Co.), copper sulfate ($CuSO_4$-$5H_2O$) and sodium hydroxide (Fisher Scientific), glutamate (Acros Organics), and NMDA and GABA (Sigma Chemical Co.) were used as received. Ultrapure deionized water (Easy Pure LF, Barnstead) was used for the preparation of aqueous solutions. Absorption and emission spectra were recorded with a Hewlett-Packard Diode Array Spectrophotometer [Model 8450A] and a Perkin-Elmer Luminescence Spectrometer [Model LS 50B, UK], respectively. A stock solution of DMABN was prepared in 95% ethanol. The final concentration of DMABN in the solution was 0.01 mM, which contains less than 0.1% ethanol. We have not observed any change in the fluorescence spectrum recorded in freshly prepared DMABN solution containing 10 mM β-CD and in the solution after storage for 12 to 24 h. This indicates that the DMABN molecule is stable in β-CD solution at a pH of 11.9. The formation of Cu^{2+}/β-CD and Cu^{2+}/ligand binary complexes is fast. Therefore, all experiments were carried out with freshly prepared solutions. The excitation wavelength for DMABN is 295 nm, and the slit width for both excitation and emission was 10 nm. Excitation and emission spectra were used for both qualitative and quantitative analysis.

Results and Discussion

A. Synthesis of the Ternary Complex Cu^{2+}/DMABN/β-CD. *a. Synthesis of the Complex.* The synthesis of Cu^{2+}/DMABN/β-CD was carried out in the aqueous solution. This is a two-step synthesis, as shown in Figure 1, with each step being monitored by spectroscopic methods. First, a binary complex of DMABN/β-CD was prepared and confirmed by fluorescence measurements. As shown in Figure 2A, there was a significant fluorescence enhancement when β-CD was added to a DMABN aqueous solution. Second, the newly formed binary complex was used to react with Cu^{2+}. Figure 3 shows the quenching of fluorescence of the complex, which is [Cu^{2+}]-dependent. The newly formed complex is further studied by reaction with glutamate and other amino acids or neurotransmitters. The complex formation is greatly influenced by the concentrations of β-CD and Cu^{2+}, which is discussed below.

b. Effect of β-CD on the Absorption and Emission Spectra of DMABN. DMABN is fluorescent in aqueous solution, and its fluorescence intensity increases when it is encapsulated inside the β-CD cavity. Both fluorescence and absorption spectra have been recorded at different stages of complex formation in order to investigate its photochemical properties. The absorption spectra of a DMABN aqueous solution were recorded as a function of [β-CD] in the range between 0.01 and 10 mM at pH 11.9. Upon addition of β-CD, the absorbance measured at the absorption maximum (296 nm) gradually increases, with slight broadening and blue shift ($\sim$2$-$3 nm). No isosbestic point is observed. The absence of an isosbestic point excludes the possibility of a single equilibrium involving only one type of DMABN (guest)/β-CD (host) complex formation with 1:1 (host:guest) stoichiometry. The increase in the absorbance with the increase of [β-CD] is believed to be due to the encapsulation of the DMABN molecule inside the β-CD cavity.[12]

In contrast to the absorption spectra, the emission spectra of DMABN undergo a remarkable change with the increase of [β-CD], as shown in Figure 2A. In the absence of β-CD, the TICT emission intensity (I_T, measured at 510 nm) is 1.3 times higher than that of the normal emission band (I_N, measured at 360 nm). As [β-CD] increases, the intensity ratio of the TICT emission to the normal emission (I_T/I_N) increases and reaches a maximum value of 2.05 (Figure 2B) when [β-CD] is 1.0 mM, i.e., 100 times the concentration of DMABN. This intensity ratio then gradually decreases to 0.08 at 10.0 mM [β-CD]. Distinctly different behaviors of both bands have also been observed. The intensity of the TICT emission increases exponentially, while that of the normal emission increases linearly with the increase of [β-CD]. Our experimental results match excellently with the similar study done by Nakamura et al.[13a] on DMABN and 6-O-glucosyl-β-cyclodextrin (G_1-β-CD). It is noteworthy that the TICT emission intensity of DMABN (0.01 mM) is lower than that of normal emission in 10 mM β-CD solution at pH 11.9, which is contrary to the similar experimental results obtained by Nakamura et al. in both neutral and basic media.[13a] They always observed higher intensity for the TICT emission. This discrepancy can be explained on the basis of the polarity of the microenvironment of DMABN. It is well understood that polarity affects the TICT emission in two opposing ways.[2,14] With an increase in polarity, the TICT emission initially increases, and after reaching a maximum, it decreases with a further rise in polarity. This decrease in TICT emission is due to the increase in the nonradiative decay rate (intersystem crossing, ISC) from the TICT singlet state to the low-lying triplet state. Therefore, the maximum value of I_T/I_N is obtained in medium polar solvents such as methanol (Figure 7). Nakamura et al.[13a] recorded emission spectra of DMABN (0.01 mM) in a solution of water and acetonitrile (9:1 by volume) containing 10 mM β-CD, which was sufficient to increase the polarity of the microenvironment and, hence, the TICT emission. On the basis of their experimental results, which are very close to our

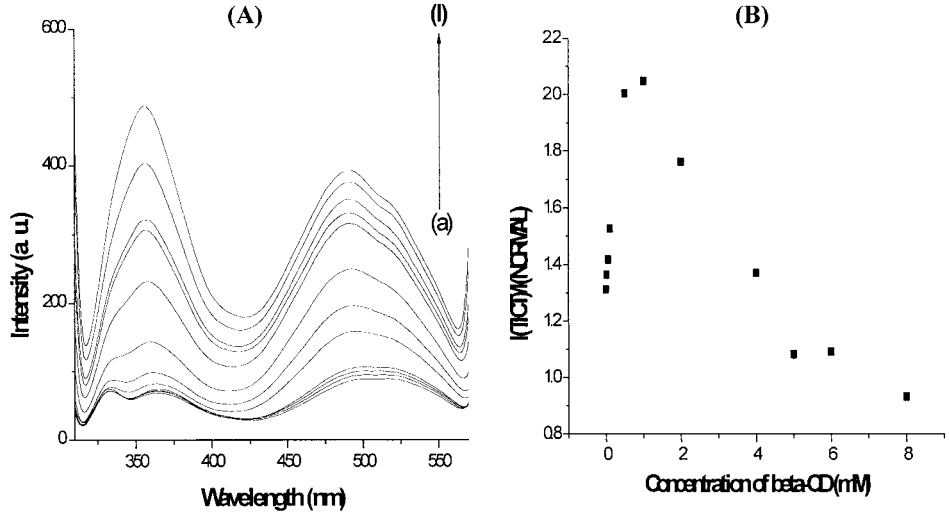

Figure 2. (A) Effect of β-CD concentration on the fluorescence spectra of DMABN at pH 11.9: (a) 0.0, (b) 0.01, (c) 0.05, (d) 0.1, (e) 0.5, (f) 1.0, (g) 2.0, (h) 4.0, (i) 5.0, (j) 6.0, (k) 8.0, and (l) 10.0 mM β-CD. [DMABN] = 0.01 mM. (B) Effect of β-CD concentration on the intensity ratio of the TICT emission to the normal emission (I_T/I_N) at pH 11.9.

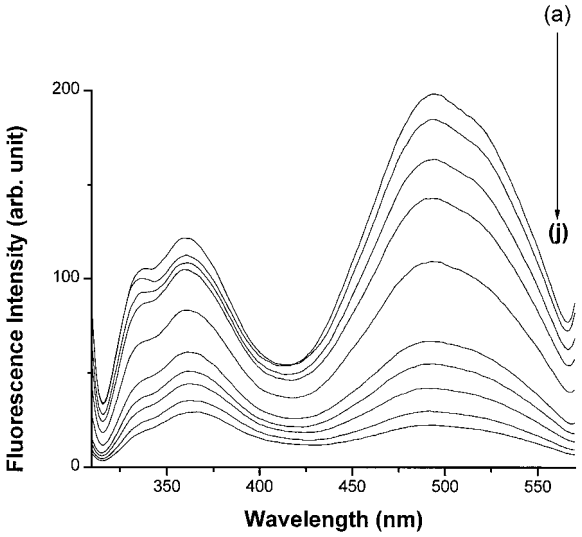

Figure 3. Effect of Cu^{2+} ion concentration on the fluorescence spectra of DMABN/β-CD binary complex at pH = 11.9: (a) 0.0, (b) 0.01, (c) 0.05, (d) 0.1, (e) 0.2, (f) 0.4, (g) 0.5, (h) 0.6, (i) 0.8, and (j) 1.0 mM Cu^{2+} ion. [DMABN] = 0.01 mM, [β-CD] = 1.0 mM.

observations, we assign 1:1 DMABN/β-CD inclusion complex as the major species at the maximum value of I_T/I_N, i.e., at 1.0 mM β-CD concentration, although there is a possibility of the 2:2 complex occuring as a minor species. Further increase of β-CD concentration leads to the formation of a 2:2 DMABN/β-CD complex (homodimer), as observed by Nakamura et al.[13a]

The band maxima of both the TICT and normal emissions are blue-shifted with the increase of [β-CD]. We have observed 10 and 18 nm blue shifts for the normal and TICT emission bands, respectively, when [β-CD] increases up to 10 mM. The blue shift is probably due to the transfer of DMABN molecules from the polar aqueous medium to the less polar and relatively hydrophobic β-CD cavity. It is known that the TICT emission of the DMABN molecule is very sensitive to the polarity and viscosity of the medium. In this study, we mainly concentrate on monitoring TICT emission to investigate the spectral changes during the complex formation of Cu^{2+}/β-CD and Cu^{2+}/ligands. Therefore, for further study, we will be keeping β-CD concentration fixed at 1.0 mM, where the I_T/I_N ratio was maximum for 0.01 mM DMABN.

The fluorescence excitation spectra, in the absence and in the presence of β-CD (1.0 mM), have also been recorded with fluorescence monitored at both the normal and TICT band maxima. No noticeable change has been observed in the absence of β-CD. However, in the presence of 1.0 mM β-CD, two different spectra are obtained, with band maxima located at 285 nm when monitored at normal emission (λ_{max}flu = 360 nm) and at 298 nm when monitored at TICT emission (λ_{max}flu = 510 nm). We suggest that in the absence of β-CD, the origin of the two fluorescence bands has the same ground-state precursor, and the conformational change from the planar to the twisted structure occurs only in the excited state. However, in the presence of β-CD, there are at least two different types of inclusion complexes present in the ground state (further explained later).[13]

c. Effect of Cu^{2+} Ion on the Photochemical Properties of the DMABN/β-CD Complex. Cu^{2+} ions can quench the fluorescence of the DMABN/β-CD complex through PIET. To study the effect of Cu^{2+}, we optimized the complex formation with [β-CD] = 1.0 mM and [DMABN] = 0.01 mM, for the following reasons: (i) at 1.0 mM β-CD, I_T/I_N has a maximal value, which makes it a very sensitive indicator for the slight changes in the microenvironment inside the β-CD cavity during the formation of Cu^{2+}/β-CD/DMABN and Cu^{2+}/ligand complexes; (ii) the number of free β-CD in the solution is maintained small, which is very important to obtain better sensitivity for the determination of glutamate, as explained below. The β-CD/DMABN complex is in equilibrium with free β-CD and DMABN. It is, therefore, very reasonable that free β-CD will bind with a Cu^{2+} ion at pH 11.9 to form a Cu^{2+}/β-CD complex. This complex does not contain DMABN, and it will bind to glutamate without producing a fluorescence signal. This type of complex will, therefore, hinder a sensitive and accurate determination of glutamate.

To determine whether there is any chemical interaction between DMABN and Cu^{2+} ion, we recorded the absorption spectra of DMABN with respect to appropriate reference solutions. The absorption spectra of DMABN, both in the absence and in the presence of β-CD at neutral pH, show that the absorption band maximum is located around 296 nm, and the band position remains intact in the presence of Cu^{2+} ion. However, the absorption spectra recorded in the presence of Cu^{2+} ion and β-CD at pH 11.9 do not show any peak at 296

nm, characteristic of the DMABN molecule. Instead, a broad band at ~685 ± 5 nm, close to the characteristic Cu^{2+} ion absorption,[8] and a very strong band at 245 nm are observed. This result indicates that there exists some chemical interaction between DMABN and the Cu^{2+} ion which has either completely changed the electronic states of the DMABN molecules in the presence of β-CD at pH 11.9 or overlapped in such a way that the 296 nm band is buried under the strong absorption band of the ternary complex. The possible chemical interaction between DMABN and Cu^{2+} in the presence of β-CD could be the ligand−metal coordination, where the DMABN molecule behaves like a ligand and coordinates to the Cu^{2+} ion via a nitrogen atom in its nitrile functional group. Thus, Cu^{2+}/DMABN interaction is possible only when the ternary molecular complex is formed at pH 11.9.

The fluorescence spectra of DMABN in the presence of Cu^{2+} ions reveal interesting information about the photochemistry of the complex. As [Cu^{2+}] increases, the fluorescence intensity of both the normal and TICT emission decreases (Figure 3). The intensity of the TICT band decreases exponentially with a slight blue shift (3 nm), whereas the normal emission decreases linearly with a slight red shift (3 nm) when the [Cu^{2+}] increases up to 1.0 mM in the presence of 1.0 mM β-CD. Further increase of [Cu^{2+}] leads to its precipitation as $Cu(OH)_2$. This result clearly shows the formation of a 1:1 complex between a Cu^{2+} ion and β-CD, which is consistent with the previous results.[1,6,8] The decrease in the fluorescence intensity is due to the PIET process, as described earlier. It is interesting to note that the different behavior of the two bands, TICT and normal, during the addition of Cu^{2+} might be due to the difference in the mode of interaction of the Cu^{2+} ion with the inclusion complexes, i.e., 1:1 and 2:2 β-CD/DMABN complexes.

The value of I_T/I_N decreases significantly when [Cu^{2+}] increases from 0.01 to 0.2 mM and then decreases linearly when [Cu^{2+}] increases up to 1.0 mM. We believe that the first decrease of I_T/I_N is due to the interaction of the Cu^{2+} ion with the 1:1 β-CD/DMABN complex (the major species, as described earlier) and that the second decrease is due to the interaction of the Cu^{2+} ion with the 2:2 β-CD/DMABN complex (the major species at higher Cu^{2+} concentration). This also shows that the TICT emission is more sensitive than the normal emission toward the quenching process by Cu^{2+}. Therefore, we expect a more sensitive response in the fluorescence restoration of the TICT emission when the complex reacts with glutamate. More interestingly, we have not observed any fluorescence quenching of DMABN emission by the Cu^{2+} ion in the absence or even in the presence of β-CD at neutral pH. This indicates that the quenching mechanism is operated only when the DMABN molecule is encapsulated inside the β-CD cavity. A similar phenomenon was observed by Corradini et al.[15] in a mono-functionalized β-CD containing an encapsulated dansyl moiety in the presence of a Cu^{2+} ion. Harris et al.[16] have also shown the quenching of the luminescence intensity of tris(2,2′-bipyridyl)dichlororuthenium(II) by copper (II) cyclodextrin complexes. In both cases, they observed static quenching. We believe that a similar quenching mechanism is valid in the present study. This again supports (i) the formation of the ternary complex and (ii) the PIET process operating in a very short range; i.e., only when the DMABN molecule is encapsulated by β-CD can Cu^{2+} interacts with it.

There is little change in the fluorescence excitation spectra when monitored at either the normal or the TICT band maxima of DMABN under the following experimental conditions: (i) 1.0 mM Cu^{2+} ion at neutral pH and (ii) 1.0 mM Cu^{2+} and 1.0 mM β-CD at pH 11.9. In the first case, the excitation spectrum peaked at 296 nm, which matched well with that for the free DMABN solution at neutral pH. In the second case, the excitation peak appeared at 310 nm. It is thus evident that the ternary complex exists only in one conformation in the ground state (2:2:2 Cu^{2+}/DMABN/β-CD complex, further explained later). The dimethylamino group of DMABN can still twist inside the ternary complex to show the TICT emission. The red shift in the excitation spectra most likely corresponds to the interaction between DMABN and the Cu^{2+} ion. We have not observed any absorption band at 310 nm for the ternary complex. This could be due to the presence of the adjacent strong band at 245 nm, under which weak 310 nm band is buried. It is also worthwhile to point out that even though there are two distinctly different DMABN/β-CD species (both 1:1 and 2:2) existing in the absence of Cu^{2+}, there is only one species in the presence of Cu^{2+} (i.e., the 2:2:2 Cu^{2+}/DMABN/β-CD complex). At [Cu^{2+}] lower than 1.0 mM, the formation of a 1:2:2 Cu^{2+}/DMABN/β-CD complex might be possible, which would show incomplete fluorescence quencing of DMABN and therefore would not be suitable for biosensor applications. Interactions between the DMABN's dimethylamino nitrogen atom and Cu^{2+} is improbable because the lone-pair electrons of the nitrogen atom is delocalized via conjugation (resonance effect) with the aromatic ring in the presence of a strong electron-withdrawing (inductive effect) nitrile group at the para position. The steric effect of the two methyl groups can further prevent this process from happening. On the other hand, coordination through the nitrile nitrogen atom is favorable. Therefore, the dimethylamino group of the DMABN molecule projected toward the larger rim of the β-CD may flip over 180° while forming a complex with Cu^{2+}, producing only one kind of capsule-like ternary complex (Figure 1).

d. Effect of pH on the Stability of the Cu^{2+}/DMABN/β-CD Complex. In basic solutions, CDs dissociate protons of secondary hydroxyl groups to adopt an anionic form.[17] The deprotonated form of CD binds to the Cu^{2+} ion tetragonally to form a stable complex, Cu^{2+}/β-CD. With decreasing pH, the Cu^{2+}/β-CD complex becomes unstable. For the biosensor application, it is desirable to have a system that is stable at or very close to the neutral pH. We have prepared Cu^{2+}/DMABN/β-CD complexes at different pH's in the range of 11.0−13.0 and also in 1 M NaOH and studied them spectroscopically (both absorption and emission). We have chosen an optimal pH at 11.9, where the Cu^{2+}/DMABN/β-CD complex is stable. The basicity of the solution is reduced, and the spectroscopic results are similar to those at higher pH, including 1M NaOH. As expected, more quenching is observed for solutions having pH higher than 11.9, but we did not observe complete quenching even at 1M NaOH solution. In this study, therefore, we have made all aqueous β-CD solutions keeping pH constant at 11.9.

e. Structure of the Cu^{2+}/DMABN/β-CD Complex. Although the structure of the Cu^{2+}/β-CD complex has not been fully elucidated yet, both 1:1 and 2:1 molar ratios have been proposed on the basis of results from potentiometric, spectrophotometric, polarimetric, and conductometric methods.[8,18] It has been suggested that the two pairs of C2 and C3′ secondary hydroxyl groups of contiguous glucose units of β-CD are cross-linked by the $Cu(OH^-)(O^{2-})Cu$ ion bridge, supporting a 2:1 Cu^{2+}/β-CD complex, especially with a high β-CD concentration. However, our experimental result rules out the possibility of a 2:1 Cu^{2+}/β-CD complex structure at pH 11.9 because, as mentioned earlier, a 1 mM β-CD solution only consumes 1 mM Cu^{2+} ion, and excess addition of Cu^{2+} leads to its precipitation

as $Cu(OH)_2$. The following two binary complexes both have been confirmed to have 1:1 molar ratio: DMABN/β-CD and Cu^{2+}/β-CD. Thus, the stoichiometry of Cu^{2+}, DMABN, and β-CD in the ternary molecular capsule should be 1:1:1. There are two possible conformations for a 1:1:1 complex: DMABN "inside" or "outside" the cavity. The "inside" one will have to have the DMABN molecule tightly packed inside the β-CD cavity; while the "outside" one will have DMABN molecule totally outside of the cavity. If the "inside" 1:1:1 complex is formed, a single Cu^{2+} ion has to bind to the deprotonated secondary hydroxyl groups of two adjacent glucose units of the same β-CD, thereby covering a small portion of the larger rim of the β-CD. The length of the DMABN molecule[19] and β-CD[2] is about the same (8 Å). If it forms a complex with the Cu^{2+} ion and CD on a 1:1:1 ratio, the cavity-packed DMABN molecule will have its dimethylamino group extremely close to the narrower rim of the cone. The dimethylamino group's free rotation will thus be severely hindered. Therefore, it would be difficult to observe the TICT band in the fluorescence spectrum, which is contradictory to our observation, a very strong TICT band in Figure 3. If the "outside" 1:1:1 complex is formed, the DMABN molecule shall bind to Cu^{2+} outside the β-CD cavity. Then there should be little fluorescence quenching of the complex, as shown in our control experiments, which yielded no quenching if DMABN was not inside the CD cavity. We have observed significant fluorescence quenching when Cu^{2+} is added to the mixture of DMABN and β-CD at pH 11.9, as shown in Figure 3. Thus, our results do not support an "inside" nor and "outside" 1:1:1 Cu^{2+}/DMABN/β-CD complex formation.

On the basis of the β-CD titration curve against DMABN, which maintains a 1:1:1 stoichometry, and in light of the spectroscopic results, we are proposing a 2:2:2 Cu^{2+}/DMABN/β-CD complex at pH 11.9, with $[Cu^{2+}] = 1.0$ mM, [DMABN] $= 0.01$ mM, and [β-CD] $= 1.0$ mM. Our experimental results can be explained with a 2:2:2 Cu^{2+}/DMABN/β-CD complex, as schematically shown in Figure 1. Two similar 1:1 β-CD/DMABN units are connected together (homodimer) by two Cu^{2+} ions to form a molecular capsule-like complex. Two Cu^{2+} ions is required to keep the Cu^{2+}/β-CD stoichiometry constant. Nakamura et al.[13a] showed that 2:2 β-CD/DMABN complex decomposes to a 1:1 complex in basic solutions because the anionic CDs do not associate to form a dimer due to Coulombic charge repulsion. In our case, it is the Cu^{2+} ion, which forms a stable complex with anionic β-CD and drives the equilibrium toward the complex formation. In this molecular capsule, DMABN remains inside the β-CD cavity. The DMABN molecules remain slightly tilted to be coordinated with Cu^{2+}. The dimethylamino group of DMABN is away from the narrowest part of the CD cavity and thus can be twisted upon excitation to produce TICT emission. Therefore, fluorescence quenching takes place when Cu^{2+} is added. Each Cu^{2+} is tetracoordinated and linked to two glucose units of two different β-CD molecules and thus becomes pentacoordinated after binding to a single DMABN molecule. Further inclusion of ligand molecules, such as glutamate, inside the molecular capsule cavity will be extremely difficult.

B. Novel Mechanism for the Restoration of the Quenched Complex Fluorescence. *a. Fluorescence Restoration of the Complex by Glutamate.* We have applied a novel photochemical mechanism for the restoration of DMABN fluorescence by interacting the complex with glutamate (Figure 1). As shown in Figures 4 and 5, glutamate was able to react with the complex and bind with Cu^{2+}, which is the source of fluorescence quenching through PIET. The restoration of DMABN fluorescence is complete and fast. This process could be used for glutamate bioassay.

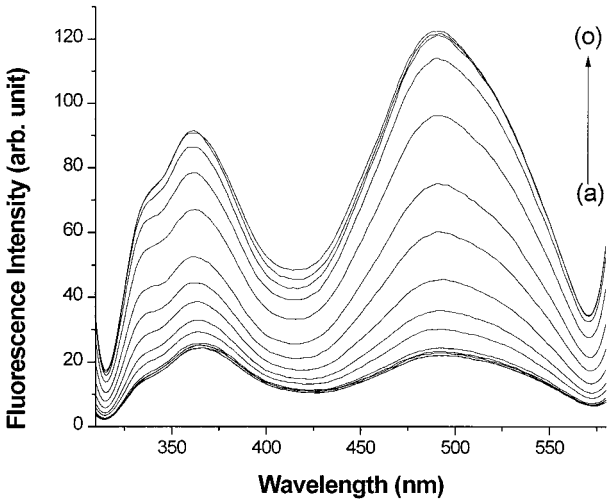

Figure 4. Effect of glutamate concentration on the fluorescence spectra of Cu^{2+}/DMABN/β-CD ternary molecular complex at pH=11.9: (a) 0.0 μM, (b) 10.0 μM, (c) 50.0 μM, (d) 100.0 μM, (e) 200.0 μM, (f) 0.9 mM, (g) 1.5 mM, (h) 2.3 mM, (i) 3.0 mM, (j) 4.0 mM, (k) 5.6 mM, (l) 7.7 mM, (m) 10.6 mM, (n) 16.1 mM, and (o) 24.3 mM glutamate. [DMABN] $= 0.01$ mM, [β-CD] $= 1.0$ mM, $[Cu^{2+}] = 1.0$ mM.

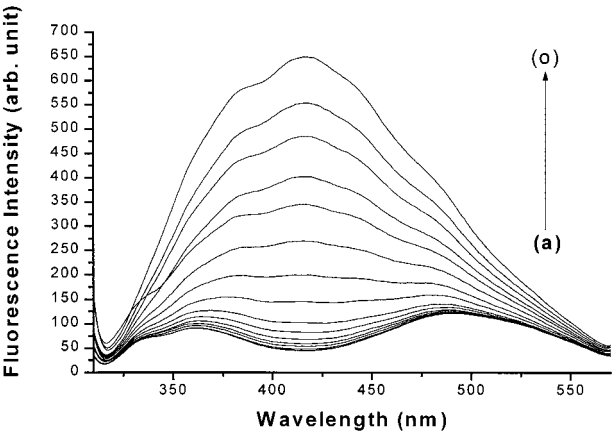

Figure 5. Effect of high glutamate concentration on the fluorescence spectra of the Cu^{2+}/DMABN/β-CD ternary molecular complex: (a) 0.016, (b) 0.024, (c) 0.041, (d) 0.057, (e) 0.084, (f) 0.1, (g) 0.2, (h) 0.3, (i) 0.5, (j) 0.8, and (k) 1.0 M glutamate. [DMABN] $= 0.01$ mM, [β-CD] $= 1.0$ mM, $[Cu^{2+}] = 1.0$ mM.

b. Effect of Glutamate on the Spectroscopic Properties of Cu^{2+}/DMABN/β-CD complex. We have studied the effect of glutamate on the emission spectra of the ternary complex. The spectroscopic properties have displayed different behaviors in a large glutamate concentration range, from 10 μM to 300 mM. When glutamate interacts with the Cu^{2+}/DMABN/β-CD complex, the restoration of DMABN fluorescence has been observed, as shown in Figure 4. Depending on glutamate concentration range, there are different ranges of sensitivities. In the concentration range of 10−200 μM, both the normal and TICT emission increase linearly (Figure 4). This small but perceptible change indicates that glutamate molecules are capable of interacting with the ternary complex even at very low concentration. There have been reports about the inclusion of glutamate inside the β-CD cavity based on the fact that β-CD/pyrene emission increases further upon interaction with a second guest molecule, such as linear and branched chain alcohols and amino

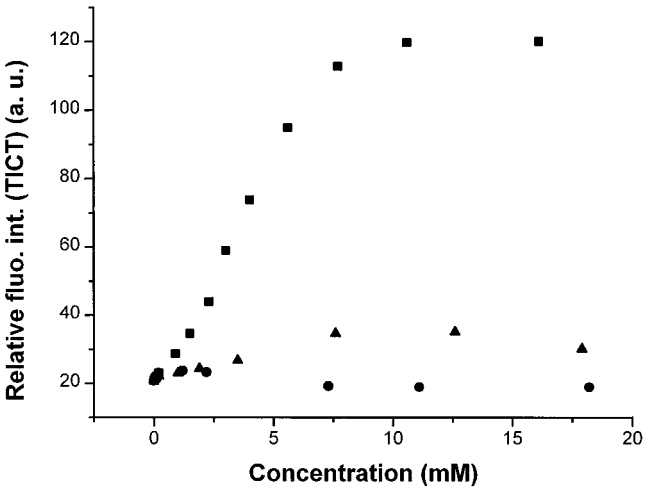

Figure 6. Interaction of glutamate (■), GABA (●), and NMDA (▲) with the ternary molecular complex. The fluorescence intensity is measured at the TICT emission band maximum..

acids.[20,21] In this study, however, the addition of glutamate in the above-mentioned concentration range did not show any fluorescence enhancement of the solution containing only 0.01 mM DMABN and 1.0 mM β-CD (in fact, the TICT emission decreases while the normal emission remains constant). Therefore, the observed fluorescence enhancement is indeed due to the interaction between the Cu^{2+} ion and the glutamate molecule in the solution, which drives the equilibrium toward the dissociation of the Cu^{2+}/DMABN/β-CD complex. When the glutamate concentration range is between 200 μM and 20 mM, a dramatic fluorescence enhancement of the TICT emission is observed. Similar restoration is also observed for the normal emission, although as expected, the TICT emission restoration is more pronounced than that for the normal emission band. The restoration of DMABN fluorescence by glutamate interaction with this molecular complex can be useful for the detection of glutamate. Preliminary experiments show that the detection limit of glutamate is in the sub-μM range when a conventional spectrometer with a mercury lamp is used.

The spectroscopic properties of DMABN have also been changed after the complex interacts with glutamate. When glutamate concentration is low, the positions of the two bands in the spectrum are intact. When higher glutamate concentration is used (between 20 and 100 mM), the two distinct bands gradually merge to form one band (Figure 5). There are significant shifts of the band positions of both the normal (toward red) and TICT emissions (toward blue) with a constant increase of the fluorescence intensity when glutamate is added. The disappearance of the bands is probably due to solution viscosity changes (see below).

c. Comparison of Spectral Properties of Other Ligands upon Interaction with the Ternary Complex. We have also studied the interaction between the ternary complex and GABA and NMDA, both of which are important in neurochemistry and neurotransmitter studies. The interactions of these ligand molecules greatly depend on the ligand concentration. In the range between 200 μM and 20 mM, different ligand molecules behave differently, as shown in Figure 6. Upon the addition of GABA, the fluorescence intensity of DMABN first increases slightly and then decreases. A slight decrease in DMABN fluorescence intensity is also observed when NMDA is added. It is clear that the ternary complex is most sensitive to glutamate in this concentration range. High concentrations of the ligand molecules have generated more complicated spectroscopic observations for these three molecules. When a higher concentration of the ligand molecule is used (between 20 and 100 mM), a continuous decrease in DMABN fluorescence intensity up to a GABA concentration of 50 mM is observed. For NMDA, there is an abrupt but less pronounced fluorescence restoration at a concentration of about 20 mM that continues up to 50 mM, which then becomes constant.

C. Discussion. In formulating the molar ratio of the new complex, there is the possibility of an intermediate state of partial inclusion of the DMABN molecule inside the CD cavity. However, it is unlikely for one DMABN molecule to be partially included in one CD cavity while still being complexed with Cu^{2+}, which is simultaneously complexed with two different hydroxyl groups on the CD larger ring. It is possible that the DMABN molecule would be able to stretch out of the CD cavity with a short distance (e.g., one bond length) if two CDs are linked together, such as that shown in Figure 1, to provide extra cavity space. In this way, one DMABN molecule and two deprotonated hydroxyl groups from each of the two linked CDs will be complexed with Cu^{2+} simultaneously to form a stable molecular capsule.

Since glutamate and NMDA are both anionic amino acids, they bind similarly with Cu^{2+}. Both can restore DMABN fluorescence upon interaction with the Cu^{2+} ion. Glutamate is more reactive than NMDA as glutamate induces dramatic fluorescence restoration. It requires a much higher concentration, up to 20 mM, for NMDA to interact with the Cu^{2+} ion. At lower NMDA concentrations, no appreciable fluorescence restoration has been observed. It has been reported that an anionic amino acid molecule can interact with two Cu^{2+} ions at a time.[9] Thus, we expect that either the glutamate or the NMDA molecule can make a bridge with two Cu^{2+} ions. The poor reactivity of NMDA is possibly due to the steric hindrance caused by the presence of an *N*-methyl group at the binding site and the shorter chain length of the NMDA molecule, which cannot bind to two Cu^{2+} ions effectively. The same does not apply to GABA. Each GABA molecule can only bind to one Cu^{2+}. Our results show that the addition of GABA leads to a slight restoration of the DMABN emission, which is an indication of its poor binding capability with the Cu^{2+} ion in solution.

With six different solvents, cyclohexane, dioxane, acetonitrile, methanol, water, and glycerol, we have confirmed that the value of I_T/I_N of DMABN is extremely sensitive to the polarity and viscosity of the medium, as discussed earlier (Figure 7). Both the normal and TICT fluorescence bands are well separated in all solvents but glycerol. The spectroscopic properties of the DMABN molecule in glycerol solution are found to be very similar to those recorded in aqueous solutions with high concentrations of the ligands ($\sim$1 M). We thus suggest that the shift of the band position at higher concentrations of GABA and glutamate is likely due to the change in the microenvironment (both polarity and viscosity) of DMABN in the medium which is very similar to that in glycerol. The inset of Figure 7 shows clearly that the emission spectral behavior of DMABN at very high concentration of glutamate ($\sim$1.4 M) is very similar to that in glycerol even in the absence of β-CD. As we have mentioned earlier, in our control experiment, the addition of glutamate in a solution containing 10 mM β-CD and 0.01 mM DMABN neither changes the emission band maxima nor increases the emission intensity when the glutamate concentration is below 20 mM. Therefore, this spectral change is indeed due to the change of the polarity and viscosity of the medium around the DMABN molecule. From the inset of Figure 7, it is also confirmed that such spectral behavior is not due to the

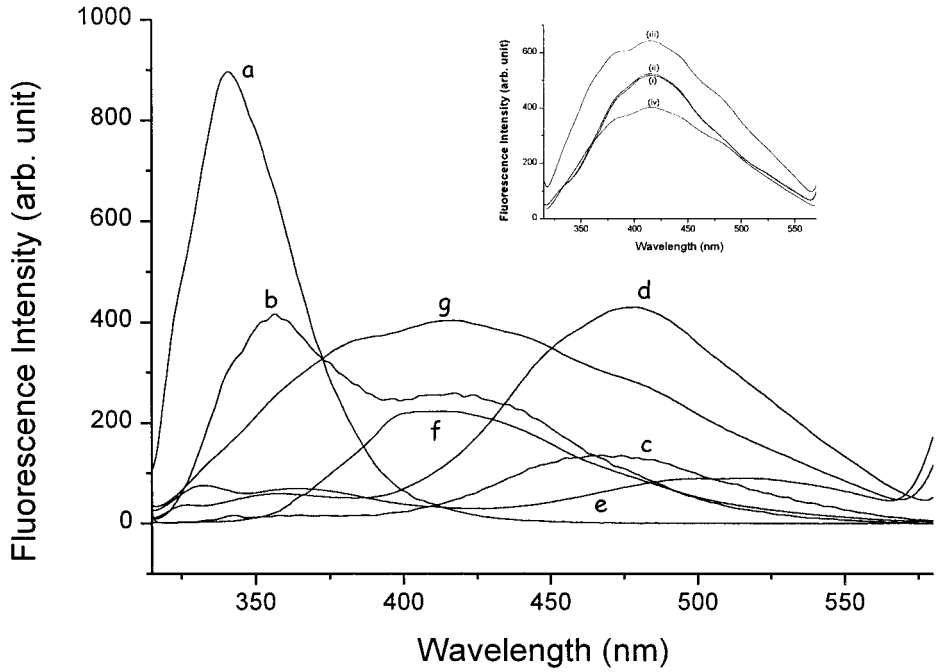

Figure 7. Fluorescence emission spectra of DMABN (0.01 mM) in pure solvents when excited at the absorption band maximum: (a) cyclohexane, (b) dioxane, (c) acetonitrile, (d) methanol, (e) water (pH = 11.9), (f) glycerol, (g) same as (iv) in inset. Inset shows the effect of glutamate (~1.4 M) on the emission spectra of DMABN (0.01 mM) in (i) water at pH 7.0, (ii) water at pH 11.9, (iii) 1.0 mM β-CD at pH 11.9, (iv) 1.0 mM β-CD + 1.0 mM Cu^{2+} at pH 11.9; excitation wavelength = 295 nm.

β-CD/DMABN/neurotransmitter ternary complex formation by the additional inclusion of neurotransmitters in to the β-CD cavity.

The feasible concentration range for fluorescence restoration is very different for different ligand molecules because of their different binding capability with the Cu^{2+} ion in solution. Depending on their binding capability with the Cu^{2+} ion, the present study shows that it is still possible to realize the selectivity required in specific bioassays. Therefore, the key for selectivity depends on the types of ligand molecules, their binding capability, and the conformation around the binding site. We are presently designing a few Cu^{2+}/β-CD-based molecular complexes for better selectivity and sensitivity, especially for glutamate detection, on the basis of the same principle of fluorescence restoration. Another way to use this new complex is to combine the assay with separation techniques such as capillary electrophoresis and/or HPLC, by which similar ligand molecules can be separated before their interactions with the complex for fluorescence detection. There are several potential advantages of this approach over the existing amino acid analysis by separation technique.[22] For example, a variety of dye molecules can be chosen, since there is no requirement for specific functional groups on the dye for binding with the analyte (ligand molecule). The interaction between the ligand and the complex is fast and complete. The complex itself does not fluoresce strongly, thus showing a very low background signal. This will lead to higher signal-to-noise ratio. We expect that the new strategy developed here will be useful for sensitive and selective glutamate analysis as well as for the bioanalysis of other amino acids and neurotransmitters.

Conclusion

We have reported here for the first time the synthesis of a new, fluorescence-quenched complex, Cu^{2+}/DMABN/β-CD. On the basis of the photochemical properties of the ternary complex, we have proposed the complex structure to have a 2:2:2 molar ratio. When the complex interacts with neurotransmitters and amino acids such as glutamate, GABA, and NMDA, the quenched fluorescence of DMABN is restored completely and quickly. The new complex synthesis and the restoration of the quenched fluorescence processes enable a better understanding of cyclodextrin-based chemistry and the exploration of a novel bioanalysis for neurotransmitters and amino acids. Synthesis of the complex capsule can be carried out in the solution phase, which should facilitate the usage and study of this interesting molecular capsule.

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