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A Structural Remedy toward Bright Dipolar Fluorophores in Aqueous Media

Subhankar Singha, Dokyoung Kim, Basab Roy, Sunderraman Sambasivan, Hyunsoo Moon, Alla Sreenivasa Rao, Jin Yong Kim, Taiha Joo, Jae Woo Park, Young Min Rhee, Taejun Wang, Ki Hean Kim, Youn Ho Shin, Junyang Jung, and Kyo Han Ahn*

The donor–acceptor (D–A) type dipolar fluorophores, an important class of luminescent dyes with two-photon absorption property, generally emit strongly in organic solvent but poorly in aqueous media. To understand and enhance the poor emission behaviour of dipolar dyes in aqueous media, we undertake a rational approach that includes a systematic structure variation of the donor, amino substituent of acedan, an important two-photon dye. We identify several factors that influence the emission behaviour of the dipolar dyes in aqueous media through computational and photophysical studies on new acedan derivatives. As a result, we can make acedan dyes emit bright fluorescence under one- and two-photon excitation in aqueous media by suppressing of the liable factors for poor emission: 1,3-allylic strain, rotational freedom, and hydrogen bonding with water. We also validate that these findings can be generally extended to other dipolar fluorophores, as demonstrated for naphthalimide, coumarin and NBD dyes. The new acedan and naphthalimide dyes thus allow us to obtain much brighter two-photon fluorescent images in cells and tissues than in their conventional forms. As an application of these findings, a thiol probe is synthesized based on a new naphthalimide dye, which shows greatly enhanced fluorescence from the widely used N,N-dimethyl analogue. The results disclosed here provide essential guidelines for the development of efficient dipolar dyes and fluorescence probes for studying biological systems, particularly by two-photon microscopy.

Introduction

The donor–acceptor (D–A) type, dipolar fluorophores have been widely used in molecular probes and biological tags owing to their highly emissive nature. Representative classes of dipolar dyes include acedan (2-acetyl-6-(dimethylamino)naphthalene), naphthalimide (4-amino-1,8-naphthalimide), coumarin (7-amino-coumarin), benzocoumarin, NBD ((4-nitro-2,1,3-benzoxadiazol-7-yl)amine), dansyl (5-amino-naphthalene-1-sulfonyl), rhodol, Nile red and blue, and so on. These dyes used so far mostly contained a dialkylamino group as the electron-donor group and an electron-withdrawing moiety (acetyl, nitro, cyanide, dicyanovinyl, etc.), both conjugated to an aromatic system in such a way that the donor and acceptor are electronically conjugated. Such dipolar dyes generate intramolecular charge transfer (ICT) excited states upon irradiation with light, which bestows the dipolar dyes with environment-sensitive photophysical properties: typically, they emit at the longer wavelength as the polarity of media increases. This environment-sensitive property of the dipolar fluorophores has been importantly utilized in the development of polarity-sensitive molecular probes. On the other hand, they emit weakly in polar and poorly in aqueous media, in contrast to the “symmetric” dyes that have symmetric resonance structures, such as BODIPY, rhodamine, cyanine, and fluorescein derivatives, which generally show medium polarity-insensitive emission properties. In spite of numerous applications of the dipolar fluorophores in areas of chemistry, biology, and medical sciences, those factors that make them emit poorly in aqueous media have not been fully understood. Considering that those dipolar dyes have been used in various types of molecular probes and tags for biological analytes, a better understanding on those factors that influence their emission properties in water is of fundamental importance. Furthermore, if we could improve their poor emission properties in water, the results would hold a great promise for the development of molecular probes, in particular, two-photon imaging probes for biological systems as dipolar dyes constitute an important class of two-photon excitable fluorophores.
Among the dipolar dyes, acedan and its analogues constitute an important class of two-photon excitable fluorophores for bioimaging of tissues, as they are small in size, can be readily modified, and show good two-photon absorption properties. Two-photon (absorbing) dyes that can be excited in the near-infrared wavelength region have received great interest as they enable deeper tissue imaging with reduced autofluorescence as well as with very high spatial and temporal resolution by two-photon microscopy (TPM). In spite of the promising two-photon properties of acedan and its derivatives, and other various dipolar fluorophores in general, their poor emission behaviour in aqueous media has not been fully addressed yet.

Our interest in the development of two-photon fluorescent probes based on acedan and its analogues; instead, a larger 1,3-allylic strain (Fig. 2). The third factor that can affect the fluorescence intensity of acedan derivatives is the rotational freedom of the amino group (Fig. 2). A bulky amino group is expected to undergo a slower or hindered internal rotation in the excited state, which can also contribute to the fluorescence enhancement of acedan derivatives by reducing the nonradiative decay. With the aforementioned rationales in mind and confirming their computational results (details in ESI†), we have prepared various N-alkyl- and N,N-dialkyl-substituted acedan derivatives with different steric bulkiness, and compared their photophysical properties. A series of compounds 2–9 including acedan (Fig. 3a) are thus synthesized by adopting our improved method of introducing the acetyl group, and their photophysical properties are thoroughly evaluated.
dichloromethane, and cyclohexane) (Fig. S1, ESI†). We confirmed that all the compounds are soluble in the media; the fluorescence was measured by excitation at the maximum absorbance wavelength (λ<sub>abs</sub>) of each compound. The uncertainty is less than ±10% for all the measurement. (c) Photos of acedan (365 nm).

Photophysical properties of acedan and its derivatives

Absorption spectra for acedan (1) and its derivatives 2–9 were obtained in solvents with a wide polarity span (HEPES buffer, water, ethanol, acetonitrile, N,N-dimethylformamide, dichloromethane, and cyclohexane) (Fig. S1, ESI†). We confirmed that all the compounds are soluble in the concentration range evaluated. A comparison of the absorbance for compounds 1–9 in different solvents (Table S1 and S2, ESI†) show that acedan derivatives with N-cyclohexyl groups (5 and 6) have increased absorbance, particularly in water; compound 5 shows more than 1.5-fold enhanced molar absorptivity (ε) from that of acedan (Table 1).

Emission spectra for compounds 1–9 in various solvents show a significant variation in the intensity, particularly in aqueous media (Fig. S2, ESI†). All the compounds emit more strongly in the organic solvents than in water as usual, except for nonpolar cyclohexane. A comparison of the emission intensity for compounds 1–9 in aqueous media shows significant improvement for compounds 5 and 6 over acedan (Fig. 3b).

Clearly N-monoalkyl derivatives (3–7) emit enhanced fluorescence from those of N,N-dialkyl derivatives (1, 2, 3 and 9), which confirm that the N-substitution pattern significantly influences the formation of the ICT states and their nonradiative decay processes.<sup>5</sup> Although a further study is required to argue about the emissive state, acedan derivatives with an N-monoalkyl donor seem to attain a PICT state that emits strongly, rather than a TICT state that emits poorly.<sup>6,7</sup> N-Cyclohexanol analogue 5 give the largest enhancement (threelfold) among the acedan derivatives, emitting much brighter fluorescence in water compared with that of acedan when excited at 365 nm (Fig. 3c). Its N-(4-acetamido)cyclohexyl analogue 6 also shows a similar but slightly lower enhancement. On the other hand, compound 9 shows very weak fluorescence in water, probably through a TICT excited state caused by a strong allylic strain. Then, we compared quantum yields of compounds 1–9 measured in different solvents (Table 1 and Table S5, ESI†). The high quantum yields of acedan in organic solvents (Φ<sub>F</sub> = 0.48 in CH<sub>2</sub>Cl<sub>2</sub>; 0.52 in CH<sub>3</sub>CN) drop in water (Φ<sub>F</sub> = 0.20). In contrast, compound 5 still shows a comparable quantum yield (Φ<sub>F</sub> = 0.40) in water to those observed with acedan in organic solvents. A similar level of quantum yields is observed in the case of N-acetyl analogue 6. Other N-monoalkyl derivatives (3, 4 and 7) show in-between values of those of acedan and 5. In fact, with enhanced absorbance and quantum yield, the brightness (ε × Φ<sub>F</sub>) for compound 5 improved more than three times from that of acedan in water (Table 1).

Table 1 Spectroscopic data for acedan (1) and its derivatives 2–9 in water.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (nm)</th>
<th>ε&lt;sup&gt;a&lt;/sup&gt; (M&lt;sup&gt;−1&lt;/sup&gt; cm&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (nm)</th>
<th>Φ&lt;sub&gt;F&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Brightness</th>
<th>τ&lt;sup&gt;f&lt;/sup&gt; (ns)</th>
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<td>2240 1.29</td>
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<td>515 0.30</td>
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<td>501 0.31</td>
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<td>4</td>
<td>355 13,500</td>
<td>501 0.32</td>
<td>4320 n.d.</td>
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<td>6960 3.15</td>
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<td>503 0.39</td>
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<td>819 n.d.</td>
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</table>

<sup>a</sup>The maximum absorbance wavelength. <sup>b</sup>Molar extinction coefficient. <sup>c</sup>The maximum emission wavelength. <sup>d</sup>Fluorescence quantum yield, measured by one-photon excitation at 370 nm. <sup>f</sup>Fluorescence life time, measured at the center region of the emission spectra. n.d. = Not determined. The uncertainty is less than ±10% for all the measurement.
whose conformation in the ground state is close to that of the ICT excited state may undergo ultrafast ICT, whereas a molecule whose conformation is distinct from that of the ICT excited state may undergo slower ICT process via locally excited (LE) state, which involves the twisting motion of the amino group. Also, the potential energy surface of the ground state along the twisting coordinate of the amino group is rather shallow to allow a distribution of the torsional angle ($\phi$) in the ground state. From our molecular dynamics (MD) simulations results, compound 5 shows a nearly flat conformation ($\phi = 1.66^\circ$) plausibly due to the reduced allylic strain, whereas the calculated ground state conformation of acedan shows a small torsional angle ($\phi = 10.47^\circ$). Upon excitation, compound 5 thus seems to attain a PICT state readily; whereas acedan may initially generate a partially-twisted ICT state, which is further twisted to form a fully-twisted ICT state (a TICT state) that can be stabilized in water to give poor emission. Again, the PICT state would become more emissive as the amino donor group experiences a restricted rotational freedom by favourable structural factors (as in case of 5 and 6). Thus, the allylic strain and the rotational freedom associated with the donor amino group govern the emission behaviour of the dipolar dyes in water. Additionally, the H-bonding to the donor amino group by water affects the ICT at both the ground and excited states, and also provides the solvent-mediated relaxation route for the excited state, thereby affecting the emission behaviour of dipolar dyes in water. Although it is difficult to separate the effect of one factor from others and also the emissive state is under debate, still the allylic strain seems to play the major role based on a rough analysis of our experimental results (see ESI† for more detail analysis).

The dipolar dyes generally emit strongly in most organic solvents through the ICT excited states. Accordingly, compound 9, which emits poorly in water, fluoresces strongly in CH$_2$Cl$_2$ and CH$_3$CN. The H-bonding-mediated relaxation of the excited state is absent in organic media, which leads to strong fluorescence in general. As we pointed out above, compound 9 seems to emit via a partially-twisted ICT state in organic solvent, rather than through the fully twisted charged-separated ICT state (the TICT state) that is stabilized in the highly polar water. In organic solvent, the emission intensity of dipolar dyes seems to be dependent mostly on the degree of the ICT; the more electron-donating amino group would cause more ICT and thus cause the corresponding dye to emit more strongly.

On the basis of the structural effects on the emission behaviour of acedan derivatives in water, we have further synthesized several derivatives of dye 5 and compared their emission behaviour; these compounds have an additional substituent on the cyclohexyl ring, either at nearby the amino group (compounds 13–15) or at the remote site (compound 16) (Fig. 4a). Also, their parent cyclohexylamine analogue (compound 17) was compared with a pyrrolidine analogue 18. Dyes with a pyrrolidine as the donor are known to emit strongly. Compounds 13 and 15 with an additional substituent nearby the donor amine causes a small but further increase in the emission intensity from that of compound 5 (Fig. 4b and Fig. S10, ESI†), indicative of positive substituent effects: a reduction in the rotational freedom or additional blocking of the water hydrogen bonding to the donor nitrogen, or both. Finally, it should be noted that the pyrrolidine analogue 18 shows slightly lower but comparable fluorescence to that of compound 17, although they are poorly soluble in water which limited further evaluation. The enhanced fluorescence of compound 18 from that of acedan (1) can be rationalized by evoking the reduced allylic strain caused by the five-membered pyrrolidine ring, which, on the other hand, would enhance the ICT. Indeed, compound 18 shows the absorption and emission maxima red-shifted by 22 nm and 8 nm (Table S10, ESI†), respectively, from that of acedan. Although dyes with pyrrolidine and azetidine donors also show a comparable level of emission to those dyes with the monoalkylamino donors, the latter type of donors has an advantageous feature over the former type; it can be further functionalized to develop fluorescent probes, in particular activatable probes through a carbamate linker.$^4_{,}^{4c,4d}$

Two-photon absorbing properties of acedan derivatives

Of particular note is that the substituent effects on the emission property of acedans are more pronounced under the two-photon excitation conditions. As mentioned previously, acedan and its derivatives have been recognized as an important class of two-photon dyes owing to their structural simplicity and good two-photon absorption properties.$^3$ We have compared the two-photon absorption cross-sections (TPACS, $\delta$) for selected compounds in different solvents and by changing the excitation wavelength (Fig. 5 and Fig. S11, ESI†). The TPACS value of $N$-cyclohexyl derivative 5 (297 GM) is three-times higher than that of acedan in water (99 GM) when obtained under excitation at 740 nm (Fig. 5a and Table S11, ESI†). In the case of an analogue 6, a little lower TPACS values than those of 5 were observed (247 GM). With the enhanced quantum yields, both compounds 5 and 6 thus show greatly enhanced two-photon action cross-section ($\delta\Phi_\delta$) values from those of acedan: 6- and 5-fold-enhanced values in water,
respectively (Fig. 5b). As a result, the new acedan derivative 5 is expected to show bright fluorescence that is not much sensitive to an environment as in the case of acedan, an important but hitherto an unexplored feature from such dipolar fluorophores. The conventional environment-sensitive D–A type fluorophores can yield undesired artifacts from the environmental changes that they would experience in different biological compartments. In this context, our approach provides a solution to this issue. Again, compound 7, a (monoalkyl)amine derivative, shows two-photon properties better than those of acedan in water, but inferior to those of 5 and 6.

Extension to other dipolar fluorophores

Next, we validate that the above approach can be generally extended to other dipolar fluorophores. For this purpose, three well-known classes of dyes are selected: 4-amino-1,8-naphthalimide, 7-aminoacoumarin and NBD. The corresponding N,N-diarylaminocoumarin (10a, 11a, and 12a), N,N-dimethylaminocoumarin (10b, 11b, and 12b) and N-cyclohexylamino (10c, 11c, and 12c) derivatives were synthesized and their emission intensities were compared (Fig. 6 and Fig. S4–S6, ESI†). We have confirmed that naphtalmide 10a, a model compound of widely used N,N-dialkylaminonaphthalimides, shows poor fluorescence in a polar solvent such as acetonitrile and, particularly in water. In contrast, N-cyclohexyl-derivative 10c shows greatly enhanced fluorescence in the polar solvent as well as in water. Fluorescence quantum yields determined for naphtalmides in water confirmed a dramatic change (up to 750-fold increase) from 10a (ΦF = 0.0004) to 10c (ΦF = 0.30) (Table S5, ESI†). Again, with one-photon absorption maxima around 450 nm, the naphtalmide dyes can be excited under two-photon excitation at the longer wavelength (~900 nm) than that of acedans. However, naphtalmide dyes have been scarcely used in two-photon probes so far, likely owing to negligible TPACS values in water observed for the widely used N,N-dialkylaminonaphthalimides such as 10a (Table S12, ESI†). In this respect, naphtalmide 10c (λ = 33 GM) and its analogues are highly promising for two-photon imaging applications.

Again the same trend in the quantum yield was also observed in case of 7-aminoacoumarin derivatives (11a–11c) (Fig. 6 & Table S6, ESI†). N,N-diethylaminocoumarin 11a has a very low quantum yield in water (ΦF = 0.05), but the corresponding N-cyclohexyl-derivative (11c; ΦF = 0.59) has ~12-fold enhanced quantum yield and thus shows strong fluorescence even in water. In the case of another D-A type dye, NBD derivatives (12a–12c), again we can confirm significantly enhanced fluorescence in water (6.6-fold in the case of 12c) as well as in polar acetonitrile (21-fold in the case of 12c) (Fig. 6 & Table S6, ESI†).

The poor emission behaviour of the common N,N-disubstituted derivatives of 4-amino-1,8-naphthalimide, 7-aminoacoumarin, NBD, and other related D–A type fluorophores has obviously limited their use as two-photon absorption dyes. However, it is now evident that these dipolar dyes can be developed into one- and, in particular, two-photon fluorescent probes and molecular tags for biological analytes in an aqueous environment.

Evaluations of the new dyes in cells and tissues imaging

To show an important applicability of these new dyes to fluorescence imaging of biological systems, we have further evaluated the new acedan and naphthalimide fluorophores, 5 and 10c respectively, for imaging of cells and tissues by two-photon microscopy (TPM), in comparison with their N,N-dimethyl analogues.21 Through the experiments, we show that the naphtalmide dyes can be used as good or even better two-photon dyes in comparison with the acedan derivatives. Both the new acedan and naphtalmide derivatives make a marked difference from the N,N-dimethyl analogues in their cellular images when observed by TPM (Fig. 7a). From the relative fluorescence intensity data, compounds 5 and 10c show significantly enhanced fluorescence compared to acedan...
Fig. 7 Evaluations of the new and old types of acedan and naphthalimide dyes in cells and tissues imaging by two-photon microscopy (TPM). (a) The upper row: TPM images of HeLa cells treated with acedan 1 (100 µM) and its N-cyclohexyl derivative 5 (100 µM) under excitation at 880 nm with 16.25 mW laser power. The lower row: TPM images of HeLa cells treated with N,N-dimethyl-naphthalimide 10a (100 µM) and its N-cyclohexyl analogue 10c (100 µM) under excitation at 900 nm with 15 mW laser power. The images were taken after incubation for 30 min. Cells untreated with any fluorophore were used as controls. (b) TPM images of mouse tissues (brain, liver and kidney) obtained after incubation with acedan 1 (100 µM) and its N-cyclohexyl derivative 5 (100 µM) for 10 min, respectively, under excitation at 880 nm. Tissues without treatment of any fluorophore are used as controls. Laser power: 75 mW. (c) TPM images of mouse tissues (brain, liver and kidney) obtained after incubation with naphthalimides 10a (100 µM) and 10c (100 µM) for 10 min, respectively, under excitation at 900 nm. Laser power: 70 mW. Scale bar: 50 μm. (d, e, f) Relative intensity plot of the respective TPM images shown in (a), (b) and (c), which were obtained by collecting and averaging of all the data pixels. The error bars indicate ± SD.

and 10a, respectively (Fig. 7d). An even more striking difference was resulted when the new D–A type fluorophores 5 and 10c were used for imaging of tissues by TPM. To lower the significant autofluorescence otherwise, we have excited the acedan and its derivatives at 880 nm, rather than at 740 nm (Fig. S14 and S15, ESI†). The new acedan derivative 5 exhibits much brighter TPM images in brain, liver, and kidney tissues (Fig. 7b). As a result, 15–17-fold brighter fluorescent images with respect to that of the autofluorescence are realized with compound 5, whereas only 5–6-fold brighter images with acedan (1) (Fig. 7e).

Naphthalimide 10c again shows much brighter two-photon images in the brain, liver, and kidney tissues, when compared to the N,N-disubstituted derivative 10a (Fig. 7c): 13–19-fold brighter images with respect to that of the autofluorescence are realized with 10c, whereas only 4–5-fold brighter images with 10a (Fig. 7f). The relative fluorescence intensity data highlight the dramatic substituent effect on the two-photon emission property of such D–A type fluorophores.

Applications to molecular probes for biological analytes

To demonstrate the potential applicability of the new dyes in the development of molecular probes, finally we have synthesized two maleimides P1 and P2 containing N,N-dimethylamino- and N-(4-hydroxycyclohexyl)amino-naphthalimide dyes, respectively, for fluorescent sensing of biothiols (Fig. 8a). These thiol probes are initially in the quenched state due to the photo-induced electron-transfer (PET) process from the maleimide moiety to the naphthalimide dye, but are expected to show fluorescence upon treatment with biothiols such as cysteine (Cys) as the Michael addition of the thiol to the maleimide blocks the PET process.22 P2 indeed shows a large enhancement in the fluorescence upon treatment with Cys, whereas P1 shows negligible enhancement (Fig. 8b). The contrasting results again highlight that the amine donor in dipolar dyes can play a crucial role in governing their fluorescence behaviour in aqueous media. These probes were then applied for imaging of biothiols in HeLa cells by TPM. The HeLa cells treated with P2 show much brighter fluorescence images than those treated with P1 at the micro molar concentrations (Fig. 8c). The signal enhancement
is caused by the Michael addition of biothiols (cysteine, glutathione, homocysteine and hydrogen sulfide) in cells to the probe. At the probe concentration of 5 µM, P2 gives turn-on type signal enhancement (>13-fold) in sensing cellular biothiols whereas P1 gave only threefold enhancement (Fig. 8d), again demonstrating the powerfulness of our approach to boost the two-photon emission intensity of dipolar dyes in water to a practically useful level.

Conclusions
We have disclosed that the emission properties of dipolar fluorophores in aqueous media can be significantly altered by changing the donor amino substituent. Both theoretical computations and photophysical studies on acedan derivatives have identified those factors that affect their emission properties in water, affording highly fluorescent acedan derivatives, in particular, with greatly enhanced two-photon emission properties. We have validated that the rational approach can be generally extended to other donor-acceptor type dyes with amine donor to dramatically enhance their poor fluorescence in polar and aqueous media, as demonstrated for 1,8-naphthalimide, 7-aminocoumarin, and NBD derivatives. Furthermore, we have compared the new fluorophores with their commonly used N,N-dialkylamino forms in cell and tissue imaging and in fluorescence sensing of biothiols with the corresponding molecular probes, the results of which highlight the potential applicability of our findings to the common dipolar dyes in the development of bright molecular probes, in particular two-photon probes, for biological analytes.

Experimental Section
The experimental procedures for the synthesis of all the dyes and the biothiol probes P1 and P2, one-photon spectroscopic analysis, two-photon spectroscopic analysis, tissue and cell imaging experiments, and theoretical computations are described in the ESI†. The experimental procedures regarding mouse tissues herein were performed in accordance with protocols approved by the Kyung Hee University Committee on Animal Research and followed the guidelines for the use of experimental animals established by The Korean Academy of Medical Science. We made every effort to minimize animal suffering and reduce the number of animals used to prepare samples for imaging (for details of sample preparation, see ESI†).

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Notes and references


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Structural factors that govern the poor emission behaviour of dipolar dyes in aqueous media are identified, leading to new acedan derivatives with brighter fluorescence and, in particular, significantly enhanced two-photon properties. The same approach can be generally extended to other dipolar fluorophores, showing promising two-photon tissue imaging and sensing applications.