



Title	Fluorescence photoswitching based on a photochromic pKa change in an aqueous solution
Author(s)	Fukaminato, Tuyoshi; Tateyama, Emi; Tamaoki, Nobuyuki
Citation	Chemical Communications, 48(88), 10874-10876 <a href="https://doi.org/10.1039/c2cc35889a">https://doi.org/10.1039/c2cc35889a</a>
Issue Date	2012-11-14
Doc URL	<a href="http://hdl.handle.net/2115/53186">http://hdl.handle.net/2115/53186</a>
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Type	article (author version)
File Information	CC48-88_10874-10876.pdf



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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

# Fluorescence photoswitching based on a photochromic $pK_a$ change in aqueous solutions

Tuyoshi Fukaminato,\*<sup>a</sup> Emi Tateyama,<sup>a</sup> and Nobuyuki Tamaoki\*<sup>a</sup>

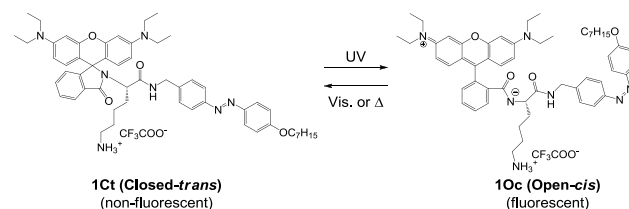
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Reversible fluorescence photoswitching of RSA-Azo dyad **1** was clearly demonstrated in an acidic aqueous solution. The fluorescence photoswitching mechanism is based on the reversible ring opening/closing reactions of RSA unit induced by a photochromic  $pK_a$  change along with the photoisomerization of Azo unit.

Fluorescent photochromic small molecules and proteins have attracted increasing interest owing to their potential applications to optical data storages and molecular switches.<sup>1,2</sup> In addition, biological applications such as bio-markers or super-resolution fluorescence microscopes are becoming one of the trends in this field.<sup>3</sup> For the latter applications, the fluorescence photoswitching property in aqueous conditions is indispensable. Although some fluorescence photoswitching in aqueous conditions including intracellular environments have been demonstrated by utilizing the assembled systems such as nanoparticles or vesicles containing photochromic molecules,<sup>4</sup> (non-assembled) water-soluble fluorescent photochromic molecules are still rare<sup>5,6</sup> and it is desired to improve their fluorescence properties and functionalities.

Rhodamine spiroamide (RSA) fluorophores<sup>6</sup> are one of the prominent molecules of water-soluble fluorescent photoswitchable molecules for super-resolution microscopic applications because of their high fluorescence quantum yields and robust photochemical stability in aqueous conditions. The derivative undergoes the photoinduced ring-opening reaction and the thermal closing reaction. The closed-state is transparent in the visible region and is practically non-fluorescent. Upon irradiation with UV light, the open-state, which absorbs in the green region and emits at around 580 nm, is produced. Although the open-state is highly fluorescent and enables to detect the fluorescence signal even at the single-molecule level, the fluorescent state cannot be maintained for a long time due to the rapid thermal back reaction (in a few milliseconds in polar solvents).<sup>6a</sup> The fast thermal recovery restricts its photoswitching application only to the super-resolution fluorescence microscopy. On the other hand, the ring opening/closing processes of RSA depend on the pH or the polarity of surrounding environments, that is, the open- and the closed-states of RSA exist in equilibrium depending on the local pH or polarity.<sup>6b, 6d</sup> With decreasing pH of the solvent, the ratio of the fluorescent open-state is increasing, and vice versa. In this study, we focused on this pH dependence of ring-opening/closing reactions of RSA. It

Scheme 1 Photoisomerization of RSA-Azo dyad **1**

is expected that ring opening/closing reactions of RSA can be indirectly induced by connecting a photochromic unit, which enables to reversibly control the local conditions such as polarity or acidity along with its photoisomerization. This approach may allow us to keep the photoinduced fluorescent open-state of RSA unit stable, which makes it possible not only to increase the detectable photon number in super-resolution microscopic applications but also to utilize the derivative for other applications such as fluorescent photoswitchable bio-markers. Here we report on the molecular design and the synthesis of a water-soluble fluorescent photoswitchable RSA derivative based on a photochromic  $pK_a$  switching.

In order to prepare our desired fluorescent photoswitchable molecules, it is necessary to avoid the spectral overlap between the absorption bands of both isomers of a photochromic unit and the fluorescence band of the RSA unit to deter the fluorescence quenching due to an intramolecular energy transfer. Azobenzene (Azo) derivatives are one of the most popular photochromic compounds and have been extensively used for the photocontrol of biomolecular structures and biological functions.<sup>7</sup> Azos show the reversible *cis-trans* photoisomerization upon alternate irradiation with appropriate UV and visible light even in aqueous conditions and both isomers have the absorption band within relative short-wavelength regions ( $\lambda < 500$  nm). In addition, it is well known that Azos reversibly change molecular polarity or basicity along with the photoisomerization.<sup>8,9</sup> Taking these advantages into account, we selected Azos as a suitable photoswitching unit in our molecular design.

It has been challenging to develop the highly fluorescent molecule connecting with Azo unit, since a pico-second photoisomerization dynamics of Azo easily quenches the excited state of fluorophore and therefore Azos are commonly utilized as the efficient fluorescence quencher.<sup>10</sup> For these problems, Eshow et al.<sup>11</sup> recently reported that the highly fluorescent property can be achieved for Azo connecting fluorescent molecules by

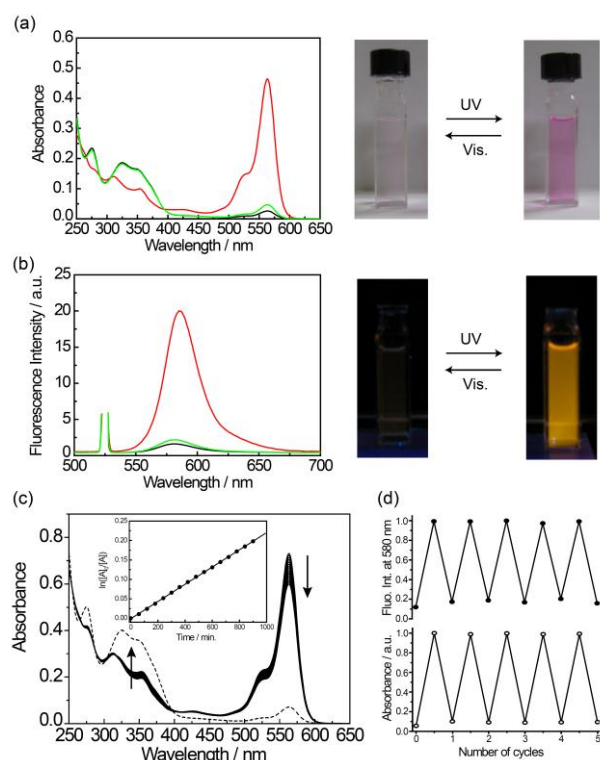
carefully selecting a fluorescence unit, whose absorption and fluorescence bands are far from the absorption band of Azo unit. According to their strategies, we designed and synthesized a RSA-Azo dyad **1** (Scheme 1), in which an Azo derivative is connected to Rhodamine B fluorophore through an amino-acid lysine spacer. In this molecular design, the lysine spacer plays an important role in the separation of electronic states of both RSA and Azo units and the high water-solubility. Dyad **1** was prepared according to Scheme S1 in ESI.<sup>†</sup> Synthetic and structural characterization details are provided in ESI.

Left sides of Fig. 1a and 1b show absorption and fluorescence spectral changes of **1** upon alternate irradiation with UV (365 nm) and visible (490 nm) light in an aqueous solution (pH 3.8 ± 0.1). The weak absorption band at 563 nm and the pronounced absorption bands around 300–400 nm, which correspond to the absorption band of the open-state of RSA unit and the  $\pi$ - $\pi^*$  transition band of Azo unit, are observed. The absorption spectrum suggests that the equilibrium between the open- and the closed-state of RSA unit almost leans towards the closed-one before photoirradiation. Upon irradiation with 365 nm light to

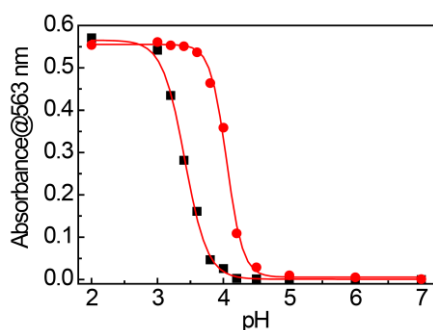
the **1Ct** (Closed-*trans*) solution, the absorption band at around 300–380 nm is gradually decreased and the characteristic absorption band at 563 nm is dramatically increased and the solution color changes from nearly colorless to pink as shown in the right side of Fig. 1a. These spectral and color changes suggest that the ring-opening reaction of RSA unit takes place and the equilibrium between the open- and the closed-state of RSA unit leans towards the open-one. From HPLC analyses, the conversion ratio from the *trans*- to the *cis*-isomer of Azo unit of dyad **1** upon 365 nm light irradiation at the photostationary state (PSS) was estimated to be > 95 % (see Fig. S1 in ESI). Upon irradiation with 490 nm light to the PSS solution, the absorption spectrum nearly recovers to initial one (Fig. 1a). The rate constant of thermal-back relaxation ( $k$ ) for **10c** (PSS under the irradiation with 365 nm light) was determined to be  $2.2 \pm 0.1 \times 10^{-4} \text{ min}^{-1}$  at 25 °C by plotting the absorbance at 563 nm (Fig. 1c) (Detailed explanation is described in ESI). The small  $k$  value indicates that the photoinduced open-state of RSA-Azo dyad **1** can be maintained for a reasonably long time in comparison with the photoinduced open-state of conventional RSA.<sup>6a</sup>

As shown in the left side of Fig. 1b, reversible fluorescence intensity changes were also observed along with the photoisomerization. Before photoirradiation, very weak orange fluorescence centred at 580 nm was observed under excitation with 520 nm light. When the solution reaches to the PSS under irradiation with 365 nm light, the fluorescence intensity increases to around 15 times of the initial one and a strong orange emission is readily visible (right side of Fig. 1b). The fluorescence intensity decreases again upon visible (490 nm) light irradiation. These absorption and fluorescence spectral changes are repeatable at least more than five-cycles (Fig. 1d). Fluorescence quantum yield ( $\Phi_f$ ) for the PSS state under 365 nm light irradiation was estimated to be  $0.37 \pm 0.05$  in an aqueous solution (pH 3.8) (see “General” in ESI), which is almost similar to that of typical RSA derivatives.<sup>6d</sup> Therefore, this result indicates that the negligible quenching effect of Azo unit on fluorescence property of RSA unit.

These reversible absorption and fluorescence spectral changes in dyad **1** may be attributed to the change of the equilibrium constant between the open- and the closed-state of RSA unit along with the photoisomerization of Azo unit. In order to confirm the origin of the photoinduced ring-opening/closing reaction of RSA unit, pH dependences of absorption spectra of RSA were measured for both *cis*-Azo and *trans*-Azo isomers. In an aqueous solution of pH 5.0, the absorption band at 563 nm was not clearly observed, while a characteristic strong absorption band at 563 nm was observed in a solution of pH 3.0 (see Fig. S2a in ESI). In both conditions, however, the absorption spectral change at 563 nm band was not remarkably observed along with the photoisomerization of the Azo unit (see Fig. S2b in ESI). The titration curves of an absorbance at 563 nm against the pH value of the solvent were plotted for both **1Ct** and **10c** solutions (Fig. 2). From these titration curves,  $pK_a$  values of the protonated open-form of RSA were estimated to be 3.4 for the *cis*-Azo and 4.1 for the *trans*-Azo isomer, respectively. Therefore, the useful pH range for the fluorescent photoswitchable molecule **1** can be estimated to be around 3.5–4.0. These results suggest that the observed ring-opening and closing reactions of RSA unit upon



**Fig. 1** (left): (a) Absorption and (b) fluorescence spectral changes in an aqueous solution ( $[C] = 8.8 \times 10^{-6} \text{ M}$ , pH 3.8 ± 0.1) upon alternate irradiation with UV (365 nm) and visible (490 nm) light; before photoirradiation (**1Ct**, black-line), PSS under 365 nm light irradiation (red-line), PSS under 490 nm light irradiation (green-line); (right): photographs of the color and fluorescence changes along with photoisomerizations. (c) The change of absorbance at 563 nm of a PSS solution (pH 3.8 ± 0.1, 25 °C) under the dark condition; dashed-line is corresponding to the absorption spectrum before photoirradiation and each line is monitored at 1 hour intervals. Inset: the thermal fading of **10c** in an aqueous solution (pH 3.8 ± 0.1) at 25 °C under the dark condition. (d) Photoswitching cycles of **1** in an aqueous solution (pH 3.8 ± 0.1) upon alternate irradiation with UV (365 nm) and visible (490 nm) light. The changes of absorbance at 563 nm (open-circle) and fluorescence intensity at 580 nm (solid-circle) were monitored in an aqueous solution (pH 3.8 ± 0.1).



**Fig. 2** Titration curves of *trans*-Azo isomer (black-square) and *cis*-Azo isomer (PSS under 365 nm light irradiation) (red-circle). The absorption change at 563 nm was monitored in the pH range from 7.0 to 2.0 in aqueous solutions.

alternate irradiation with UV and visible light are induced by  $pK_a$  changes along with the photoisomerization of Azo unit.<sup>9</sup>

Supplementary, RSA-Azo dyad **1** has a possibility to achieve the non-destructive fluorescence readout capability<sup>12</sup> because absorption spectra of both isomers of Azo unit do not overlap with the fluorescence spectrum of the open-state of RSA unit and the selective excitation of only RSA unit is possible by using the light longer than 530 nm, where both isomers of Azo unit have no absorption bands. However, unfortunately, the excitation ( $\lambda_{ex}$  = 532 nm) of the fluorescent isomer (**10c**) induces the photoisomerization reaction from *cis*- to *trans*-isomer in Azo unit and efficiently produces the non-fluorescent isomer (**1ct**). This probably attributes to the photosensitized reaction of Azo unit via the triplet-state of RSA unit.<sup>13</sup>

In conclusion, a water-soluble fluorescent photoswitchable molecule, RSA-Azo dyad **1**, was designed and synthesized, and the reversible fluorescence photoswitching was successfully demonstrated in aqueous solutions. The mechanism in the fluorescence photoswitching of dyad **1** is based on the reversible change of molecular  $pK_a$  induced by the photoisomerization of Azo unit. It is clearly confirmed that the fluorescence switching mechanism based on a photochromic  $pK_a$  change is useful for the design of water-soluble fluorescent photoswitchable molecules. Optimization of the switching efficiency and the useful pH range in this system are under progress.

This work was supported by JSPS KAKENHI; Grant-in-Aid for Challenging Exploratory Research (No. 23655111) and Young Scientist (A) (No. 24685021).

## Notes and references

<sup>30</sup> <sup>a</sup> Research Institute for Electronic Science, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan. Fax: +81-11-706-9350; Tel: +81-11-706-9350; E-mail: tuyoshi@es.hokudai.ac.jp (T. F.); tamaoki@es.hokudai.ac.jp (N. T.)

<sup>35</sup> <sup>†</sup> Electronic Supplementary Information (ESI) available: Detailed synthetic procedure of dyad **1**, HPLC analyses of **1** upon irradiation with 365 nm light and absorption spectra of dyad **1** in other pH conditions. See DOI: 10.1039/b000000x/

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