Molecular structural requirements, dye specificity, and application of anionic peptide amphiphiles that induce intense fluorescence in cationic dyes†

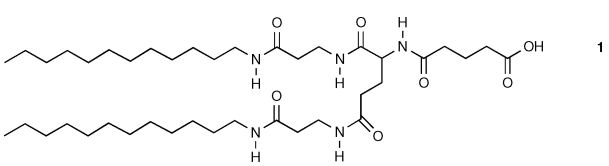
Hiroshi Hachisako,*[a,b] Naoya Ryu[b] and Ryoichi Murakami[a,b]

Received 15th October 2008, Accepted 9th February 2009
First published as an Advance Article on the web 15th April 2009
DOI: 10.1039/b818206j

We have previously reported that a double-chain anionic amphiphile capable of intermolecular triple hydrogen bonds could form extremely hydrophobic sites in water and specifically incorporated stilbazolium-based compact hemicyanine dyes as monomeric species, resulting in induction of intense fluorescence emission in the dyes. In this paper, the structural requirements of the intense fluorescence-inducing amphiphiles were investigated. It is noted that the introduction of β-Ala residues into two long-chain alkyl group moieties was most effective for the amphiphiles derived from L-glutamic acid with relatively shorter side-chain methylenes. The dye specificity in terms of induction of the intense fluorescence was also investigated using hemicyanines (stilbazolium *etc.*), cyanine, carbocyanine, thiacarbocyanines, and azo dye. The amphiphile with the shortest octanoyl-β-alanyl double-chain alkyl groups, longer side-chain, and shorter spacer was found to show increased sensitivity to alkali metal ions, especially Li^+. This could be a potential OFF-ON type fluorescence sensor for Li^+.

Introduction

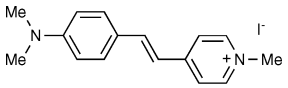
Fluorescent functional dyes are known as very useful materials. In general, the fluorescence property is largely dependent on the chemical structure of the dye itself. Of the fluorescence spectral parameters (excitation wavelength λ_{ex}, maximum emission wavelength λ_{em}, fluorescence intensity, and quantum yield), fluorescence quantum yield (Φ_F) associated with fluorescence intensity can be improved to some extent by lowering the polarity of organic solvents. However, many kinds of water-soluble ionic dyes are too polar to dissolve in nonpolar organic solvents. Moreover, the molecular design of dyes for improved properties generally causes problems in terms of a complicated synthesis procedure, chemical stability, cost performance, *etc.* Also, solvatochromic dyes are very useful materials that can be used to probe microenvironments. In general, those dyes possessing push-pull substituents responsible for intramolecular charge transfer (ICT) character show low fluorescence efficiency in water.[1] Therefore, it is of great significance to utilize practically non-fluorescent dyes as highly fluorescent ones. In this respect, a promising method would be to increase the local hydrophobicity in water using oppositely charged aqueous amphiphile assemblies. This methodology would enable us to readily utilize numerous kinds of commercially available, water-soluble ionic dyes as highly fluorescent ones. However, since there has been a limitation on lowering the polarity of local hydrophobic regions in aqueous molecular assemblies using conventional peptide amphiphiles,[2] a novel design is desired. Peptide synthesis is suitable for the design of complementary multiple intermolecular hydrogen bonding. Double-chain peptide amphiphiles capable of triple hydrogen bonding would provide much more highly specific and hydrophobic environments for oppositely charged water-soluble dyes in water than conventional ones capable of double hydrogen bonding.[2a] Unexpectedly, there have been no examples of the introduction of amide groups into the long-chain alkyl moieties of double-chained peptide amphiphiles. In our previous communication,[3] we have reported that the newly designed L-glutamic acid-derived anionic peptide amphiphile **1** induced intense fluorescence emission in an incorporated cationic monomeric dye, *trans*-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (abbreviated as St-4C_1) that originally fluoresces very weakly in water.[1b,4] This methodology would enable us to readily utilize already existing dyes as highly fluorescent ones. In this paper, we would like to report the molecular structural requirements, dye specificity, and application of the intense fluorescence-induction in cationic dyes by anionic amphiphiles.



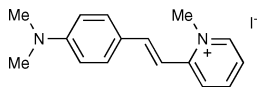
1

[a]*Department of Nanoscience, Faculty of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan. E-mail: hatisako@nano.sojo-u.ac.jp*
[b]*Division of Applied Chemistry, Graduate School of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan*

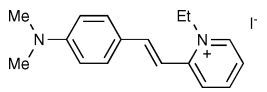
† Electronic supplementary information (ESI) available: (1) Experimental details; (2) Tables of spectral data; (3) Supplementary figures and spectra. See DOI: 10.1039/b818206j



St-4C$_1$



St-2C$_1$

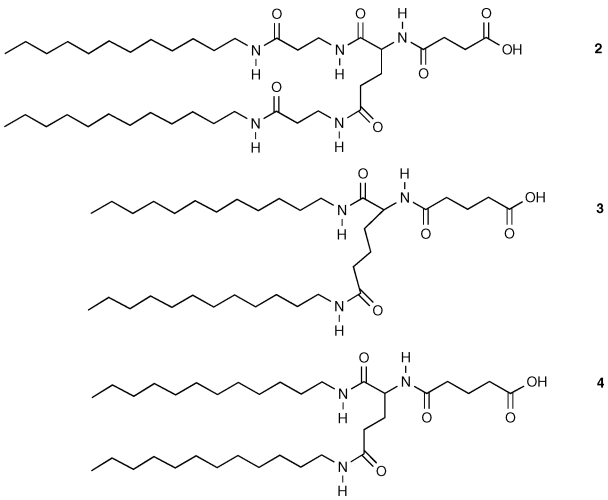


St-2C$_2$

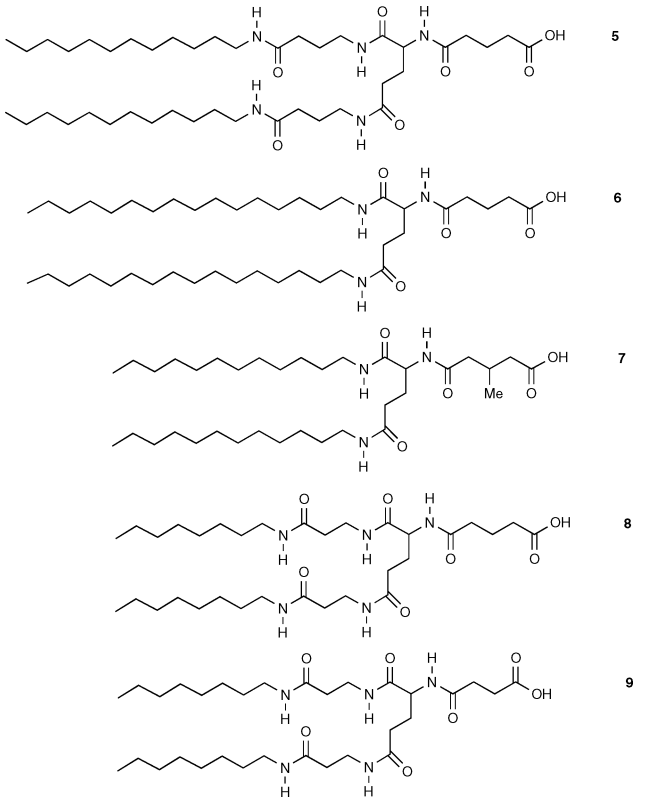
Results and discussion

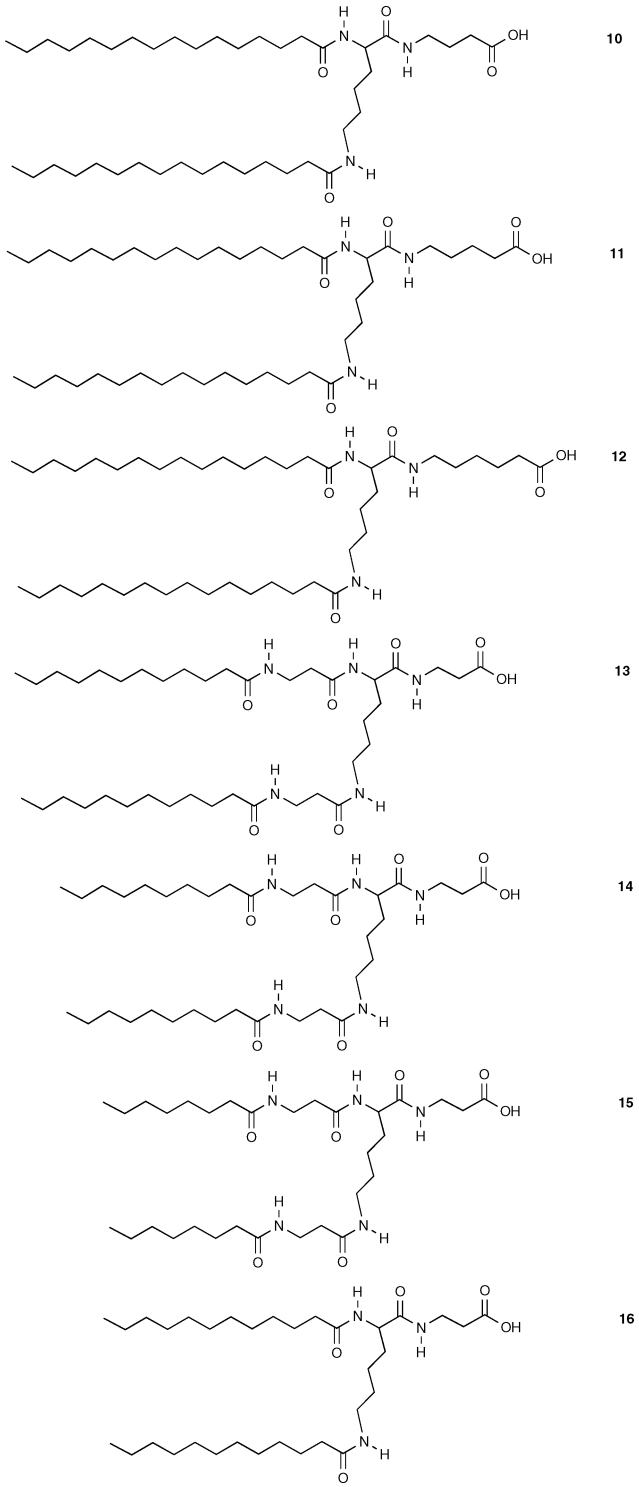
Spectral behaviour of St-4C$_1$ in the presence of various amphiphiles

Cationic hemicyanine dye, St-4C$_1$ is known to exhibit negative solvatochromism, *i.e.*, the nature of having a red-shift in the absorption λ_{max} with decreasing solvent polarity. The λ_{max} of St-4C$_1$ (0.15 mM) in water and in methanol (containing 10 vol% water) were 448 and 475 nm,[2,3] respectively. St-4C$_1$ was used in this study as a solvatochromic probe in order to investigate the inner hydrophobicity of self-assemblies of **1–16** in water at 20 °C. When **1** was added to an aqueous solution of St-4C$_1$ and the pH was adjusted to 10, the λ_{max} shifted to 517 nm.[3] Similarly, the λ_{max} of St-4C$_1$ were 498 nm and 511 nm with **2** and **3**, respectively.[3] Although the local polarities of conventional amphiphiles such as **4** and SDS in water are comparable to that in methanol (λ_{max}; *ca.* 475 nm),[2,3] the polarities of inner hydrophobic regions of self-assemblies of **1** and **3** are comparable to those of nonpolar aprotic solvents, *e.g.*, chlorobenzene.[3] It was found for the first time that the L-glutamic acid-derived amphiphile **1** induced unprecedentedly red-shifted λ_{max} and intense fluorescence[3] in St-4C$_1$ by introducing additional amide bonding into double-chain alkyl groups in conjunction with conventional molecular structural requirements.[2a] As shown in Fig. 1, the effect of the additional amide bonding of **1** on a further red-shift of λ_{max} accompanied by remarkable fluorescence enhancement is apparent when compared with the results of the corresponding amphiphile **6** in which –NHCO- is replaced by –CH_2CH_2-. A similar trend was observed for the corresponding **8** and **4** with –NHCO- and –CH_2CH_2-, respectively (see Table 1). It is noted that L-Lys-derived amphiphile **16** was found to induce a remarkable red-shift of λ_{max} and intense fluorescence even without introducing the additional amide bonding, indicating that the introduction of hydrogen bonding moieties into the double-chain alkyl groups is not necessarily effective for L-Lys-derived amphiphiles with longer side-chains. The introduction of β-Ala residues into two long-chain alkyl group moieties was found to be effective for the amphiphiles derived from L-glutamic acid with shorter side-chains. In fact, L-glutamic acid-derived amphiphiles which were designed based on conventional molecular structural requirements had limitations on the induction of considerable bathochromic and hyperchromic effects in St-4C$_1$.[2] On the other hand, when the β-Ala residues of **1** were replaced by GABA residues (**5**), λ_{max} was slightly blue-shifted to 516 nm as compared with 517 nm for the **1**–St-4C$_1$ system.‡ This indicates that elongation of the β-Ala moiety using GABA is not necessarily suitable for a further λ_{max} red-shift of St-4C$_1$. The λ_{max} of 498 nm for **2** indicates that a shorter spacer (spacer methylene number, x = 2) is not suitable for the formation of specific hydrophobic sites in which St-4C$_1$ is incorporated, as compared with the λ_{max} of 517 nm for **1** (x = 3).[3] The same conclusions were drawn from the results of the related amphiphiles **8** and **9**, and Lys-derived amphiphiles **10**, **11**, and **12**. The longer spacer responsible for increased hydrophobic interaction is not necessarily suitable for an absorption red-shift as suggested from the λ_{max} data of L-Lys-derived amphiphiles **10**, **11**, and **12** with x = 3, 4, and 5, respectively, because amphiphile **10**, with spacer methylene number x = 3, induced the maximum λ_{max} value as shown in Fig. S2.† Table 1 summarizes the λ_{max}, λ_{ex}, λ_{em}, and relative fluorescence intensity (F.I.) of St-4C$_1$ in the presence



‡ The previously reported λ_{max} value (445 nm)[1] of St-4C$_1$ in the presence of **5** should be changed to 516 nm as seen in Table 1. It is noted that the λ_{max} of St-4C$_1$ in amphiphile systems seems to depend, to some extent, on the solubilization procedure of the amphiphile in water using conc. NaOH aq. to maintain a highly alkaline pH, *e.g.*, approximately above pH 12. This was caused, *e.g.*, when a relatively large drop of conc. NaOH (*e.g.*, 3.5 N) aq. was added to the solution. The amphiphile seemed to be surely hydrolyzed to some extent by a combination of conc. NaOH and heating before preliminary sonication of the aqueous suspension of amphiphile at a somewhat higher alkaline pH such as 12, resulting in an insufficient bathochromic λ_{max} shift in the added St-4C$_1$. On the other hand, the amphiphile induced sufficient λ_{max} red-shift in added St-4C$_1$ by heating at mild alkaline pH between pHs 9–10 after preliminary sonication of the aqueous suspension of **5** using a bath-type sonicator. The suppressed decomposition/hydrolysis is ascribed to the moderately/slightly lowered pH when **5** with a free carboxylic acid was ionized and solubilized at the expense of NaOH. The resulting pH seems to be insufficient to hydrolyze amide groups buried inside amphiphile self-assemblies. Therefore, first sonication of the solution of amphiphile at room temperature (at pHs 9–10), followed by heating, is recommended instead of the opposite procedure at highly alkaline pH, that is, first heating followed by sonication and at highly alkaline pH above 12, even if a bath-type sonicator were to be used. Aqueous solutions of peptide amphiphile–St-4C$_1$ seem to be visually stable at pH 10 and at room temperature as reported previously.[3] In this paper, all the aqueous amphiphile–dye samples were prepared by the former method, *i.e.*, heating after preliminary sonication of the amphiphile–dye mixture at alkaline pH around 10 to minimize decomposition of the peptide amphiphiles used.





of **1–16** in water. It is concluded that introduction of additional hydrogen bonding moieties into the two long-chain alkyl groups[3] is quite an effective molecular design for the L-glutamic acid-derived amphiphiles with shorter side-chains, in conjunction with the conventional structural requirements.[2a]

Binding constants of St-4C$_1$ to representative anionic amphiphile aggregates

The binding constant (K_s) was tentatively determined from the equation suggested by Sepúlveda *et al.*[5] Calculations were based on the following equation: $f/(1–f) = K_s\{[D_t]–[S_t]f\}–K_s[\mathrm{cac}]$, in which f denotes the fraction [aggregate-incorporated dye]/[total dye],§ D_t is the total amphiphile concentration, S_t is the total St-4C$_1$ concentration, and cac is the critical aggregation concentration of the amphiphile, respectively. For the calculation of f, variation of λ_{max} was used instead of the variation of absorbance, because absorbance was less reproducible for the dyes used in this study. K_s values for St-4C$_1$ are considered to contain considerable experimental errors because only two data points were used for the calculation of the slope (K_s) due to the drastic change of λ_{max} for St-4C$_1$ at the narrow molar ratio range. However, these K_s values may be used as comparisons for systematic interpretation. The K_s values for **1**–St-4C$_1$, **2**–St-4C$_1$, and **4**–St-4C$_1$ were 9×10^4 M^{-1}, 3×10^4 M^{-1}, and 3×10^5 M^{-1},[2a] respectively. These results indicate that the shorter spacer of **2** is not suitable for the incorporation of St-4C$_1$, as reported previously.[2a] It is noted that the K_s value of **4**–St-4C$_1$ is larger than that of **1**–St-4C$_1$. This indicates that introduction of β-Ala residues into the double-chain moiety led to the induction of more intense fluorescence in St-4C$_1$ at the sacrifice of binding constant. This suggests that the amphiphile **1** is so tightly packed that it is difficult for St-4C$_1$ to be incorporated, but St-4C$_1$ fluoresces intensely once it is incorporated into the self-assembled amphiphile **1**.

§ Fraction f was determined according to the following equation $f = (\lambda – \lambda_a)/(\lambda_m – \lambda_a)$, where λ, λ_a and λ_m denote the λ_{max} value of St-4C$_1$ in the presence of varying concentrations of amphiphile in water, the λ_{max} value of St-4C$_1$ alone in water, and the λ_{max} value of St-4C$_1$ completely incorporated into an amphiphile assembly in water, respectively. The slope (K_s) could be obtained by plotting $f/(1 – f)$ against $\{[D_t] – [S_t]f\}$.

Table 1 UV-visible absorption and fluorescence spectral data of St-4C$_1$ in the presence of various peptide amphiphiles in water; 20 °C, pH 10, [St-4C$_1$] = 0.15 mM, [amphiphile] = 3.0 mM

	UV-visible absorption spectral data				Fluorescence spectral data[c]			
Amphiphile	λ_{max}/nm	Absorbance [a]	Molar extinction coefficient (ε_{max})/dm^3 mol^{-1} cm^{-1}	Dye species[b]	λ_{ex}/nm	λ_{em}/nm	Intensity[e]	Φ_F[f]
1	517	0.846	56400	M_i	575	604	295	0.36
2	498	0.656	43733	M_i	566	603	120	0.14
3	511	0.747	49800	M_i	570	603	270	0.32
4	478	0.458	30533	M_i	555	598	97.9	0.12
5	516	0.848	56533	M_i	575	603	141	0.17
6	490	0.560	37333	M_i	560	601	174	0.21
7	517	0.827	55133	M_i	570	602	270	0.32
8	517	0.896	59733	M_i	577	605	205	0.24
9	498	0.511	34067	M_i	561	604	44.2	0.05
10	531	0.860	57333	M_i	582	605	424	0.50
11	526	0.800	53333	M_i	579	603	372	0.44
12	509	0.747	49800	M_i	571	602	224	0.27
13	524	0.895	59667	M_i	578	603	398	0.47
14	512	0.812	54133	M_i	571	601	354	0.42
15	454	0.391	26067	M_i	548	596	29.8	0.04
16	530	0.978	65200	M_i	583	605	434	0.51
None (in water)	448	0.405	27000	M_d	532	595	0.74	0.0009
None (inMeOH)[d]	475	0.644	42933	M_d	545	595	1.8	0.002

[a] Path length: 0.1 cm. [b] M_d: molecularly dispersed monomer of St-4C$_1$. M_i: incorporated monomer of St-4C$_1$. [c] Path length: 1.0 cm, band width: 1.5 nm. [d] Contains 25 vol% of water (MeOH:water = 75:25 v/v). [e] These values are equipment-dependent relative fluorescence intensities measured on RF-5300PC. Note that these values are dependent upon the actual spectrofluorophotometer used for measurement and its settings. Therefore, all the same settings were used throughout this study to interpret results systematically. [f] Since fluorescence quantum yield is obviously quenched at this concentration, Φ_F should be referred to as "relative fluorescence efficiency" in this study. It has been reported that Φ_F of St-4C$_1$ alone in water is 0.0009.[4b] Using this value, the Φ_F values of the respective amphiphile–St-4C$_1$ systems ([amphiphile] = 3.0 mM and [St-4C$_1$] = 0.15 mM, [amphiphile]/[St-4C$_1$] = 20) were tentatively estimated, assuming that the Φ_F value of aqueous amphiphile–St-4C$_1$ complex is proportional to fluorescence intensity. Although quenching does play a significant role in the range of concentrations used, these values could be used for systematic interpretation.

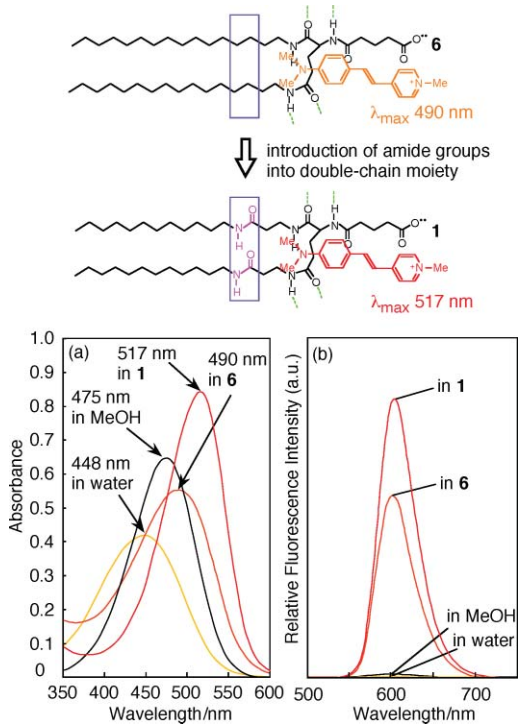
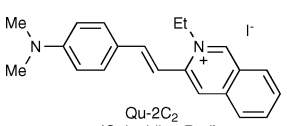


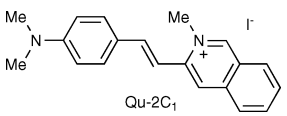
Fig. 1 Relationships between chemical structure of amphiphiles (**1** and **6**) and visible absorption spectra (a) and fluorescence spectra (b) of St-4C$_1$ in the presence of the amphiphiles in water; 20 °C, pH 10, [St-4C$_1$] = 0.15 mM, [**1**] = [**6**] = 3.0 mM. Path length of quartz cell for visible absorption spectra measurement: 0.1 cm. Path length of quartz cell for fluorescence spectra measurement: 1.0 cm. Methanol solution of St-4C$_1$ contains 25 vol% water (methanol:water = 75:25 v/v).

Dye specificity

The dye specificity of the intense fluorescence-inducing amphiphiles was investigated using representative amphiphiles **1–5**, **7**, **10**, **11**, and **13**. Fig. 2 shows digital photographs of aqueous solutions of various cationic dyes in the presence of 3.0 mM of amphiphiles under black light irradiation in the daytime (left) and under black light irradiation in the dark (right). It is noted that **1** induces intense fluorescence in most of the dyes (a)–(j) except for azo dye (k) as shown in Fig. 2. This indicates that the present amphiphile systems cannot convert an intrinsically non-fluorescent azo dye to a fluorescent one. It is also noted that amphiphile **5** exhibited similar versatility in terms of intense fluorescence induction in various cationic dyes. However, no similar versatility was observed for structurally related amphiphiles **2** and **4**. Amphiphile **3** could not induce intense fluorescence in DE9ETCC (j). Therefore, among L-glutamic acid-derived amphiphiles, **1** and its analogue **5** induced intense fluorescence in many kinds of cationic dyes used in this study but not azo dye MDEPAP.



Qu-2C$_2$
(Quinaldine Red)



Qu-2C$_1$

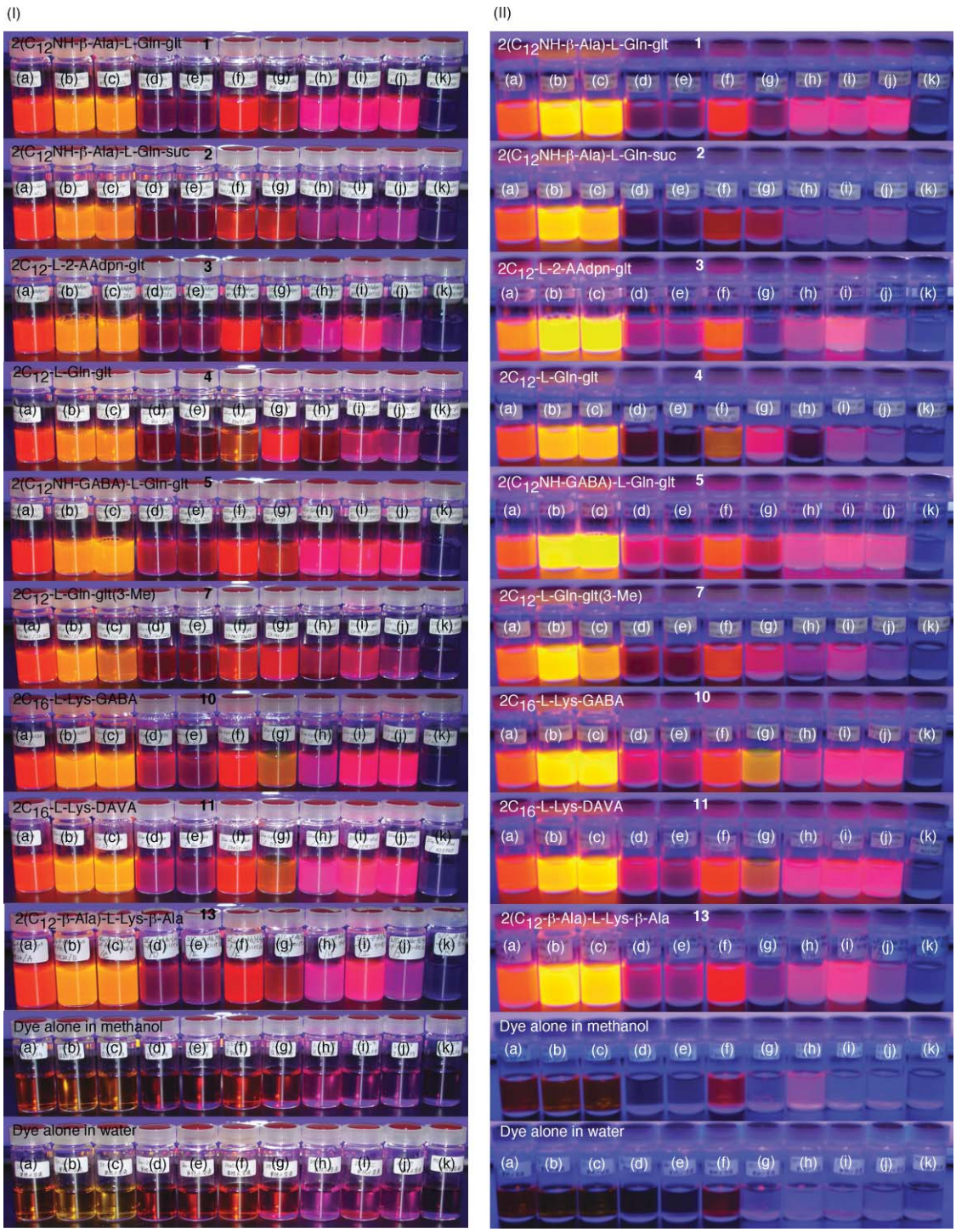
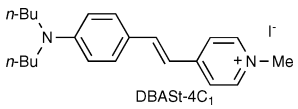
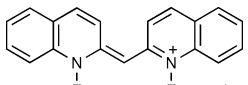


Fig. 2 Photographs of aqueous cationic dyes in the presence of various anionic peptide amphiphiles in water; [amphiphile] = 3.0 mM, [dye] = 0.15 mM, pH 10, 20 °C. (I) under black light irradiation in the daytime. (II) under black light irradiation in the dark. The abbreviations of dyes used in this study are as follows: (a) St-4C_1, (b) St-2C_1, (c) St-2C_2, (d) Qu-2C_2 (Quinaldine Red), (e) Qu-2C_1, (f) DBASt-4C_1, (g) DEC, (h) DPTCC, (i) DE9MTCC, (j) DE9ETCC (NK-737), and (k) MDEPAP.

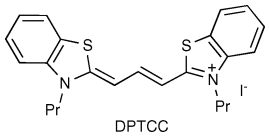
The compactness of the dye molecule seems to predominate over hydrophobicity in terms of preferential incorporation.[3] The following results obtained from visible absorption spectra are consistent with the conclusion. (*i*) Stilbazolium dye, DBASt-4C_1 (solution (f)) with a bulky *N*,*N*-dibutylamino group equilibrates between two different environments in the presence of self-assembled **1** (see Table S1 in the ESI-2†), that is, incorporated monomer with a λ_{max} of 520 nm and non-incorporated monomer with a slight shoulder at 477 nm in spite of its increased hydrophobicity. (*ii*) The dye (St-4C_{22}) with an *N*-docosyl group



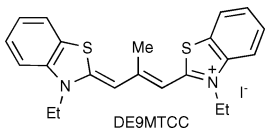
DBASt-4C$_1$



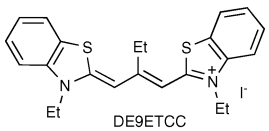
DEC



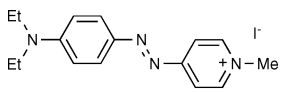
DPTCC



DE9MTCC

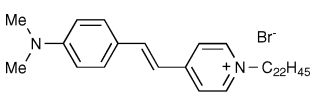


DE9ETCC
(NK-737)



MDEPAP

was practically non-fluorescent even in the presence of self-assembled **1**. In this case, it is natural that the docosyl group is incorporated into the hydrophobic region of self-assembled **1**, and therefore, the stilbazolium moiety should be electrostatically bound to the carboxylate and in contact with bulk water as schematically shown in Fig. S1 (ESI-3).† In fact, St-4C$_{22}$ exhibited a λ_{max} value at 442 nm ascribable to monomeric species of St-4C$_{22}$ in water with a negligible slight shoulder at around 508 nm. It is noted that azo dye MDEPAP which is essentially non-fluorescent did not show fluorescence induction at all, although MDEPAP was regarded as being incorporated in the highly hydrophobic supramolecular cavities (see Table S3 in ESI-2).† It is reported that azobenzene itself virtually does not fluoresce.[6] In view of these results, it is concluded that amphiphile **1** can, to some extent, recognize compact dyes with relatively small substituents.[2,3] The similar versatility in terms of intense fluorescence induction in cationic dyes was observed even for L-lysine-derived amphiphiles such as **10** and **11** as shown in Fig. 2. For these amphiphiles (**1**, **5**, **10**, and **11**), the dyes in which intense fluorescence was induced almost coincides. However, amphiphiles **1**, **5**, **10**, and **11** could not recognize the subtle difference between DE9MTCC and DE9ETCC (NK-737) as compared with amphiphiles **3** and **13**. In other words, with amphiphiles **1**, **5**, **10**, and **11** we seem to attain our initial aim of developing highly nonpolar local microenvironments in water using newly designed anionic peptide amphiphiles that can induce unprecedentedly intense fluorescence in various kinds of cationic dyes practically insoluble in nonpolar organic media. Amphiphiles **3** and **13** induced less intense fluorescence in DE9ETCC ((j) in Fig. 2, and Table S9 in ESI-2†), possessing an ethyl group at the 9-position, than in the corresponding methyl group-containing DE9MTCC ((i) in Fig. 2, and Table S8 in ESI-2†). As shown in Table S9†, the solutions of DE9ETCC show additional peaks attributable to H- and/or J-aggregates. This indicates that, in the self-assemblies of these amphiphiles, the equilibrium between incorporated monomer and non-incorporated H- or J- aggregates of DE9ETCC is displaced to the right in spite of the increased hydrophobicity of DE9ETCC over DE9MTCC. In other words, amphiphiles **3** and **13** recognized whole compactness over hydrophobicity more specifically than **1**, **5**, **10**, and **11**. In general, formation of the H- and/or J-aggregates of ionic dyes bound to oppositely charged amphiphile assemblies is ascribed to non-incorporation of the dyes into the inner hydrophobic region of amphiphile assemblies. Especially, it is believed that the J-aggregates are a result of head-to-tail stacking of less planar dyes.[7] If we prefer versatility in terms of induction of intense fluorescence to dye selectivity, amphiphiles such as **1**, **5**, **10**, and **11** or their analogues would be useful. Among L-glutamic acid-derived amphiphiles, the one most likely to be effective for recognition of molecular compactness was **7**, which possesses a methyl group on the spacer moiety as compared with **4**. As clearly seen in Fig. 2, amphiphile **7** could distinguish subtle differences between St-2C$_1$ (b) and St-2C$_2$ (c), and between DE9MTCC (i) and DE9ETCC (j). In view of these results, the intense fluorescence-inducing amphiphiles **1**, **5**, **10**, and **11** tend to exhibit the least dye specificity in terms of remarkable differences in fluorescence intensity. The visible absorption and fluorescence spectral data are listed in Tables S1–S9 (see ESI-2†). Although the degree of fluorescence enhancement in the presence of amphiphiles as compared with that of dye alone in water depends on the combination of amphiphiles and dyes, the dyes that exhibited the remarkable fluorescence enhancement in water were St-4C$_1$, St-2C$_1$, St-2C$_2$, and DE9MTCC in the presence of amphiphiles **13**, **10**, **5**, and **3**, respectively. The fluorescence of St-2C$_2$ in the presence of **5** was most enhanced up to 684 times (see Table S1 in ESI-2†). Yao *et al.* have reported induction of enhanced fluorescence in St-2C$_2$ using bulky counteranion and poly(vinylpyrrolidone).[8] They also attributed the enhanced fluorescence to both the high rotational resistance for the single bond in St-2C$_2$ and the matrix polarity effect that can suppress the nonradiative processes. In our study, it is likely that the increased hydrophobicity inside aggregates is almost parallel with the rigidification of the incorporated dye molecules, resulting in induction of bathochromic shift and intense fluorescence in the dye.



St-4C$_{22}$

Design of anionic peptide amphiphile showing increased sensitivity to alkali metal ions

We have previously predicted that amphiphile **1** could have potential application in fluorescent sensory systems because **1** afforded many other cationic dyes enhanced fluorescence that would enable sensing by reduced fluorescence intensity.[3] The intense fluorescence of St-4C$_1$ induced by amphiphile **1** should be quenched when hydrogen bond-breaking solutes are added to the solution. However, it was found that the amphiphile **1** maintained

intense fluorescence even after addition of any hydrogen bond-breaking solutes such as urea, nucleosides, and nucleotides. The addition of NaCl resulted in a slight increase in the original intense fluorescence of **1**–St-4C_1. These results indicate that the original hydrophobic interaction arising from the long alkyl chains of **1** is too strong to allow the molecular packing to loosen in water even if inter-amphiphile hydrogen bonding is destroyed by added solutes. Therefore, it seemed difficult to apply these host–guest complexes to construction of ON-OFF type fluorescent sensing systems towards appropriate hydrogen bond-breaking analytes. Therefore, we have designed a novel type of amphiphile **15** that has shorter double-chain alkyl groups and a longer side-chain than **1**, not only to attain looser molecular packings than **1** but also to transform into highly hydrophobic aggregates due to the long side-chain as suggested from the sufficiently red-shifted λ_{max} and enhanced fluorescence of St-4C_1 by simple **16** (see Table 1). Although amphiphile **16** possesses less amide bonding than **1** and **15**, it induces more intense fluorescence in St-4C_1 than **1** and **15**. This indicates that the long side-chain of the Lys residue contributes to the remarkable red-shift and enhanced fluorescence of St-4C_1. As expected, when amphiphile **15** was added to the aqueous solution of St-4C_1 at pH10, the λ_{max} only slightly red-shifted from 448 nm to 454 nm ($\Delta\lambda_{max}$; 6 nm). When the pH was lowered to 6.1, 5.1, and 3.8, the λ_{max} red-shifted from 448 nm to 518 nm, 518 nm, and 516 nm, respectively, and the solutions turned highly fluorescent (see (a) and (b) in Fig. S3†). This indicates that electrostatic repulsion between adjacent carboxylate headgroups led to looser packing of **15** at pH 10 and the repulsion was relaxed due to partly restored COOH at acidic pH. Such a slight red-shift from 448 nm to 454 nm in the presence of **15** under alkaline conditions and a large red-shift from 454 nm to 518 nm ($\Delta\lambda_{max}$; 64 nm) by lowering the pH was not observed for the corresponding L-glutamic acid derived **9** under the similar conditions. Amphiphile **9** is a structural isomer of **15**. Therefore, the only slightly red-shifted λ_{max} (454 nm) from 448 nm indicates that the longer side chain of the Lys residue of **15** plays an important role in the formation of no highly hydrophobic microenvironments at pH 10. This could be ascribed to expansion of the side-chain of the Lys residue due to cooperation of the electrostatic repulsion between adjacent carboxylate headgroups and the decreased hydrophobic interaction among shorter double-chain alkyl groups that contain hydrophilic amide groups. We have found that, upon addition of 30 mM of alkali metal salts (LiCl, NaCl, KCl, RbCl, and CsCl) to the aqueous solution of **15**–St-4C_1 at pH 10, a remarkable bathochromic shift and fluorescence enhancement were observed as shown in Fig. 3.

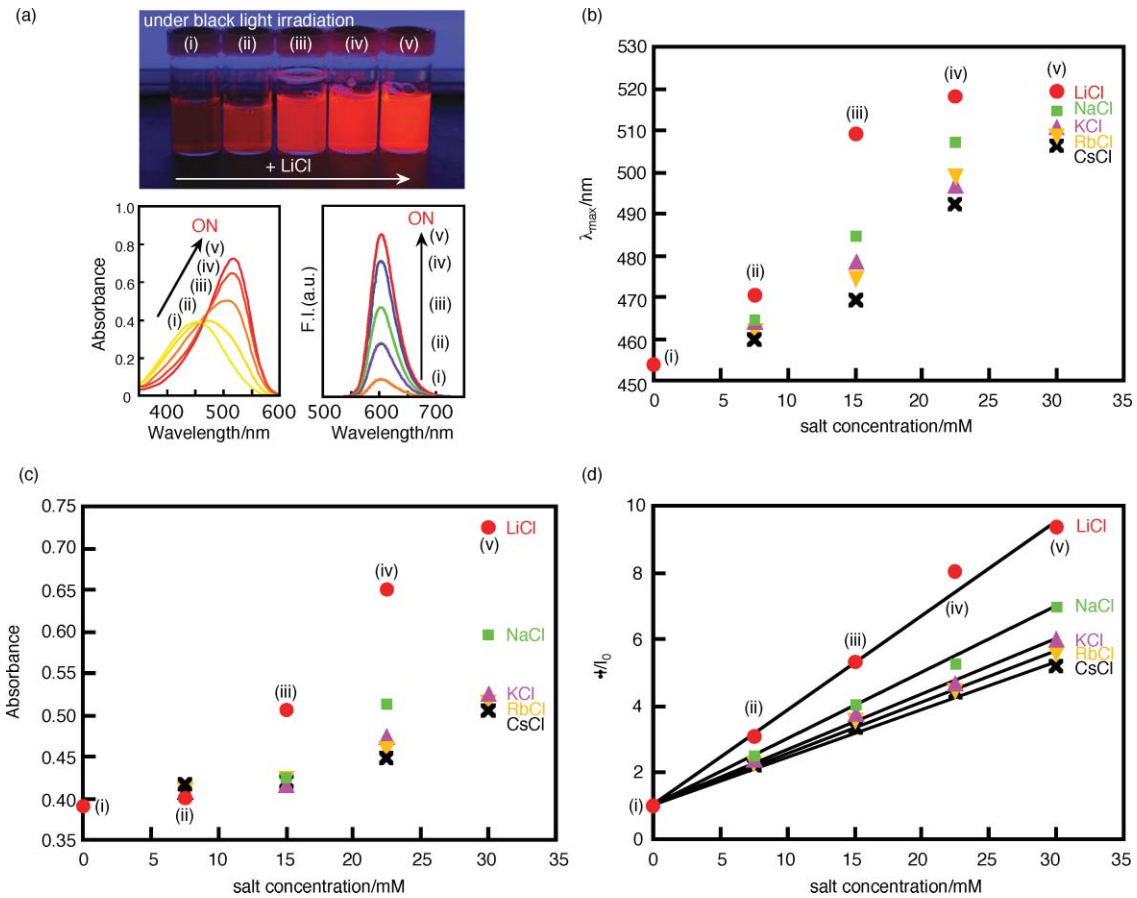


Fig. 3 (a) Digital photograph of aqueous solutions of host–guest complex of **15** and St-4C_1 and their visible absorption and fluorescence spectra with varying LiCl concentration. Relationship between salt concentration and visible absorption λ_{max} (b), absorbance (c), and relative fluorescence intensity (d), respectively; 20 °C, [St-4C_1] = 0.15 mM, [**15**] = 3.0 mM. I_0 denotes the fluorescence intensity before addition of salts at pH 10. The pH was adjusted by using conc. NaOH aq.; initial concentration of Na^+ is *ca.* 0.1 mM for all the aqueous solutions.

Similar fluorescence enhancements were observed when the corresponding alkali hydroxides (LiOH, NaOH, KOH, RbOH, and CsOH) were added, indicating that not Cl^- but alkali metal ions are triggers of bathochromic shift and fluorescence enhancement. It is noted that only fluorescence intensity gave good linearity against alkali salt concentration without precipitation. Monovalent metal ion Cu^+, multivalent metal ions Ca^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+} led to precipitation of the anionic amphiphiles. Among alkali metal ions, Li^+ induced the most intense fluorescence as well as the largest bathochromic shift in St-4C_1. The differences in the fluorescence intensities were very small for K^+, Rb^+, and Cs^+. The effectiveness of the alkali metal ions for induction of intense fluorescence is in the following order: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. This order is in good agreement with the lyotropic series (Hofmeister's series: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$) which indicates the order of ability of salting-out effect. It is generally accepted that neutralization of charge and dehydration of solutes/dispersoids by added electrolytes are responsible for the salting-out effect. Although precipitations were not observed in the present systems, the strongest dehydration and relaxation of electrostatic repulsion by Li^+ must have happened simultaneously with increasing alkali salt concentration at pH 10. It is likely that this process displaces the equilibrium in Fig. 4 to the right, and that the electron density distribution in the sensitive dye molecule will be changed in this process, as will the geometry of the amphiphile matrix, and both effects would be expected to induce spectroscopic and fluorescence changes in the dye. A novel type of amphiphile **15** may be potentially applicable to a OFF-ON type fluorescence sensor for monovalent alkali metal ions, especially for Li^+. Such salting-out-like effects that promotes hydrophobic interaction between adjacent amphiphiles by addition of alkali metal cations is consistent with the fact that aggregate morphologies of **15** in water changed from small particles to helical ribbon upon addition of LiCl and NaCl, respectively, at pH 10 (see Fig. S5 in ESI-3†). Original small particles and fragmentary aggregates (a) were converted to the mixture of sea urchin-like assemblies of divergently assembled nanotubes and untwisted tapes (b), and helical aggregates (c) in the presence of Li^+ and Na^+, respectively. Also, DSC thermograms showed increased T_c and ΔH values upon addition of LiCl and NaCl, respectively (see Fig. S6 in ESI-3†). These results are consistent with the increased molecular packing of **15** due to the cooperation of dehydration and relaxation of electrostatic repulsion by addition of alkali metal ions. Amphiphiles **13** and **14** with two dodecanoyl-β-alanyl groups and two decanoyl-β-alanyl groups, respectively, originally induced much more enhanced fluorescence than **15** at pH 10 even without addition of alkali metal ions. In view of these results, it is concluded that the Lys-derived anionic double-chain amphiphile **15** with two octanoyl-β-alanyl groups and a β-Ala-headgroup enabled the formation of a loosely packed self-assembly at pH 10, and changed to densely packed aggregates upon addition of alkali metal ions, accompanied by remarkable fluorescence enhancement, especially by addition of Li^+. The increased hydrophilicity by having additional amide groups must have been compensated by the intramolecular hydrogen bond formation responsible for the denser molecular packing as schematically shown in Fig. 4. To the best of our knowledge, there has been no example of double-chain peptide amphiphiles behaving like this.

Conclusions

We have demonstrated that it is possible to induce intense fluorescence in cationic dyes using appropriately designed double-chain anionic amphiphiles that can form extremely hydrophobic sites in water. In this study, it could be concluded that the more hydrophobic the inside of the amphiphile assembly is, the closer the molecular packing of amphiphile attained. This results in rigidification of the dye molecules upon binding to the inside of the aggregates (even through simple hydrophobic interactions). Such rigidification will result in increased planarity (hence higher conjugation, therefore red absorption shift), as well as minimizing the role of vibrationally coupled nonradiative deactivation of the excited state (hence higher fluorescence intensity). It is noted that the introduction of β-Ala residues into two long-chain alkyl group moieties was most effective for the amphiphiles derived from L-glutamic acid with relatively shorter side-chain methylenes. The related L-Lys-derived amphiphiles with a longer side-chain were found to be capable of inducing intense fluorescence even without such additional amide groups in the double-chain alkyl groups. As a whole, it seems difficult for the intense fluorescence-inducing amphiphiles to recognize detailed dye structure. The general trend that increased fluorescence intensity led to decreased dye specificity was observed. The cationic dyes in which intense fluorescence was induced were hemicyanines and thiacarbocyanines with planar and relatively compact molecular structures. An azo dye that is essentially non-fluorescent did not show fluorescence induction at all, although it was incorporated in the highly hydrophobic supramolecular cavities. These results indicate that whether the dye is essentially fluorogenic or not is important in induction of intense fluorescence as well as the whole molecular planarity and compactness of the dye molecule. The amphiphile with the shortest octanoyl-β-alanyl double-chain alkyl groups, longer side-chain, and shorter methylene spacer was found to be most sensitive to Li^+, probably depending on lyotropic series. Therefore, an aqueous amphiphile–dye complex system that originally does not fluoresce so intensely was found to be potentially applicable to an OFF-ON type fluorescence sensor for Li^+.

In conclusion, newly designed amphiphiles such as **1** were found to form highly specific sites hydrophobic enough to induce intense fluorescence emission in solvatochromic stilbazolium dyes with compact push-pull substituents and other related cationic hemicyanine, and thiacarbocyanine dyes except for the corresponding azo dye. The largest fluorescence enhancement of St-4C_1 through amphiphile **15** in the presence of Li^+ is of current intense interest to us and we hope it could be extended to a selective recovery system of Li^+ by *e.g.*, immobilization of self-assemblies of amphiphile **15** or coprecipitation of its analogues with Li^+. Further investigations including improved molecular design are now ongoing.

Experimental

Materials and methods

All the amphiphiles except for **4** were newly synthesized and identified by Fourier transform infrared spectroscopy (FTIR)

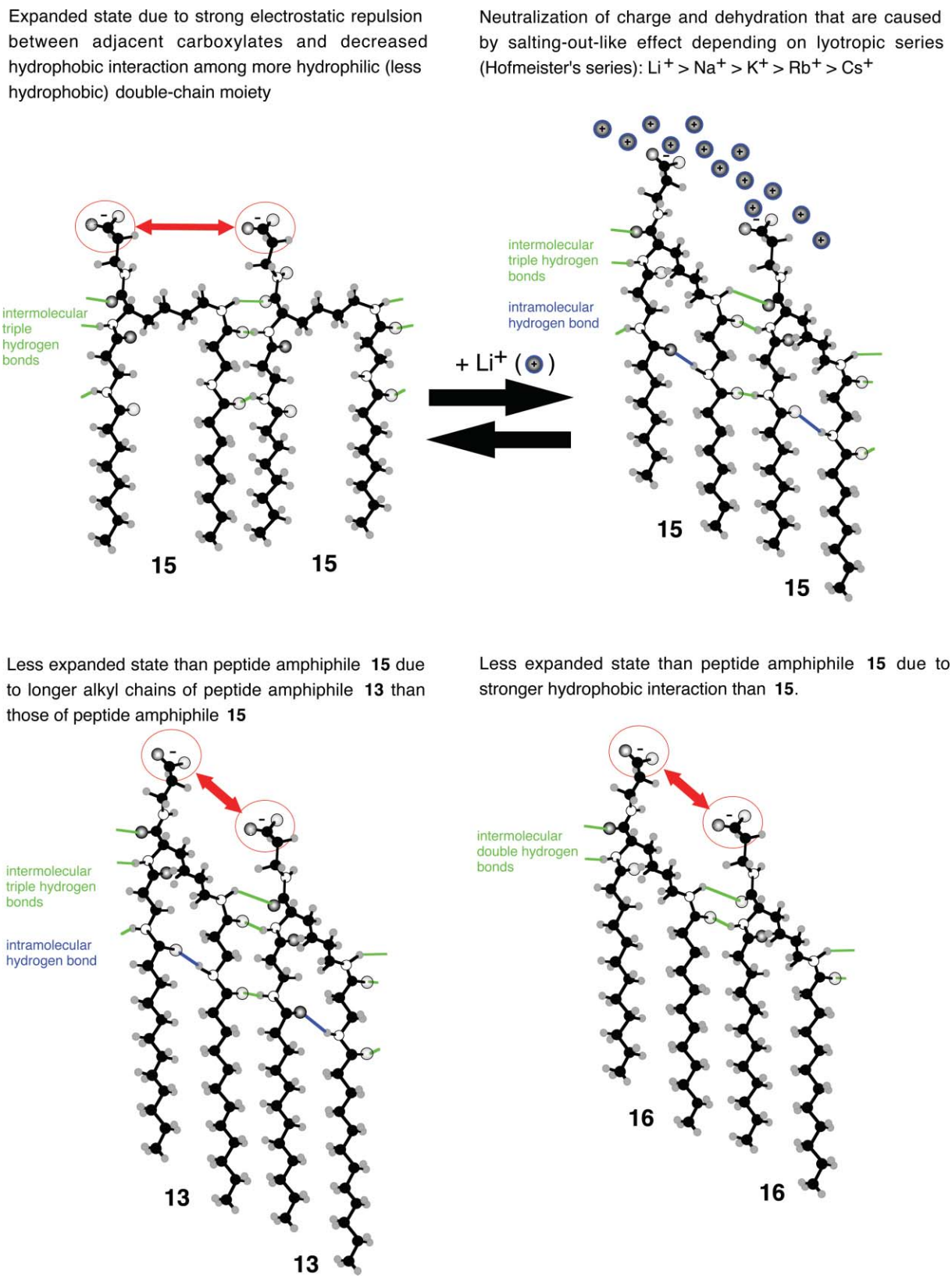
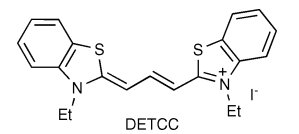
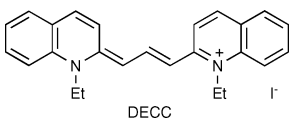


Fig. 4 Schematic illustration of the salting-out-like phenomenon of peptide amphiphile **15** in the presence of Li^+ in water at pH 10, depending on lyotropic series (Hofmeister's series: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$). In general, the salting-out effect is caused by simultaneous charge neutralization and dehydration of electrolytes, resulting in precipitation of dispersoids. It is noted, however, that amphiphile **15** did not precipitate in the solutions in Fig. 3. Counterion (Cl^-) is omitted for clarity because all the alkali metal salts used in this study were chlorides. The arrows in orange denote the electrostatic repulsion between carboxylates. Amphiphiles **13** and **16** are not originally expanded at pH 10 due to the strong hydrophobic interaction arising from dodecanoyl groups.

measurement,¶ ^{1}H-NMR measurement with a JEOL JNM-EX-270, and elemental analyses. Amphiphiles derived from α-amino acids denote L-isomers unless otherwise specified. The dyes used in this study are as follows: *trans*-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (abbreviated as St-4C$_1$), *trans*-2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (abbreviated as St-2C$_1$), *trans*-2-[4-(dimethylamino)styryl]-1-ethylpyridinium iodide (abbreviated as St-2C$_2$), *trans*-4-[4-(dimethylamino)styryl]-1-docosylpyridinium bromide (abbreviated as St-4C$_{22}$), 2-[4-(dimethylamino)styryl]-1-ethylquinolinium iodide (Quinaldine Red or abbreviated as Qu-2C$_2$), 2-[4-(dimethylamino)styryl]-1-methylquinolinium iodide (abbreviated as Qu-2C$_1$), *trans*-4-[4-(dibutylamino)styryl]-1-methylpyridinium iodide (abbreviated as DBASt-4C$_1$), 1,1′-diethyl-2,2′-cyanine iodide (abbreviated as DEC), 1,1′-diethyl-2,2′-carbocyanine iodide (abbreviated as DECC), 3,3′-diethylthiacarbocyanine iodide (abbreviated as DETCC), 3,3′-dipropylthiacarbocyanine iodide (abbreviated as DPTCC), 3,3′-diethyl-9-methylthiacarbocyanine iodide (abbreviated as DE9MTCC), 3,3′-diethyl-9-ethylthiacarbocyanine iodide (NK-737 or abbreviated as DE9ETCC), and 1-methyl-4-(4-diethylaminophenylazo)pyridinium iodide (abbreviated as MDEPAP). All the dyes were commercially available and used as received.



DETCC



DECC

Preparations of peptide amphiphiles

All the peptide amphiphiles **1–16** including intermediates **17–58** were prepared according to conventional peptide syntheses (see ESI†).

Characterization of amphiphiles

The chemical structures of all the compounds synthesized were confirmed by Fourier transform infrared spectroscopy (FTIR) measurement with a JASCO FT/IR-7000,¶ by ^{1}H NMR measurement with a JEOL JNM-EX-270, and by elemental analysis with a Yanaco CHN Corder MT-3. It is noted that most of the amphiphiles and their precursors with more than three amide bonds per molecule formed organogels in $CDCl_3$, and therefore, ^{1}H NMR signals were considerably broadened. Therefore, ^{1}H NMR assignment was restricted to main signals, and no appreciable existence of impurities was confirmed to ensure the validity of elemental analyses.

Preparation of aqueous dispersions of amphiphiles

The amphiphiles were suspended in water (pH 10) and quickly sonicated in a water bath using Ultrasonic Cleaner US-2R produced by AS ONE Co. Ltd., followed by heating in hot water. The procedure was repeated several times until homogeneous dispersions were obtained. Then the pH was adjusted with sodium hydroxide.

Preparation of aqueous amphiphile–dye mixtures

All the solutions of the amphiphile–dye complexes were prepared by addition of stock solution of the dyes to aqueous dispersions of the amphiphiles and subsequent sonication in a water bath and heating. After adjustment of the pH to 10.0 with sodium hydroxide at 20 °C, the solutions were allowed to stand at room temperature in the dark. The solutions were subjected to visible absorption and fluorescence spectra measurements.

Preparation of aqueous amphiphile–dye–salt mixtures

All the solutions of the amphiphile–dye complexes and 30 mM of alkali metal chlorides were prepared by direct addition of salt crystals to the aqueous amphiphile (3.0 mM)–dye (0.15 mM) solutions. Aqueous solutions with lower salt concentration were prepared by dilution of the amphiphile (3.0 mM)–St-4C$_1$ (0.15 mM)–salt (30 mM) solutions with amphiphile (3.0 mM)–St-4C$_1$ (0.15 mM) solution adjusted at pH 10 with sodium hydroxide and subsequent sonication in a water bath and heating. After being allowed to stand at room temperature in the dark, the solutions were subjected to visible absorption and fluorescence spectra measurements.

Visible absorption and fluorescence spectra measurements

The samples in a 0.1 cm quartz cell were incubated in a sample holder for 10 min at 20 °C. The visible absorption spectra were measured with a JASCO Ubest V-530 spectrophotometer. Similarly, the samples in a 1.0 cm quartz cell were incubated in a sample holder for 10 min at 20 °C. Fluorescence spectra were measured with a SHIMADZU RF-5300PC spectrofluorophotometer at 20 °C.

Characterization of amphiphile aggregates

The formation of highly ordered amphiphile self-assemblies in water was confirmed by using transmission electron microscopy (TEM) with a JEOL 2000FX transmission electron microscope or Phillips TECNAI F20 S-TWIN. The aqueous samples (0.6–1.0 mmol dm^{-3}, pH 10) were spotted onto carbon-coated copper grids. The samples were air-dried at room temperature, after which they were post-stained with 2 wt% aqueous ammonium molybdate. Aggregate morphologies of **1–16** in water were observed using TEM. All the amphiphiles formed bimolecular layer-based aggregates in water at pH 10: tubules and ribbons for **1** and **2**, tubules for **3**, tubules and helices for **4**, long tubes for **5**, small particles and vesicles for **6**, fibrillar aggregates for **7**, fibrillar aggregates and small particles for **8** and **9**, fibrillar aggregates

¶ FT-IR measurements are useful for assignment of peptide compounds. The main peaks for peptide amphiphiles are as follows: *ca.* 3300 cm^{-1} ($\nu_{N\text{-}H}$: stretching vibration of hydrogen-bonded N-H of amide), around 3000 cm^{-1} ($\nu_{C\text{-}H}$: C-H stretching vibrations of long-chain alkyl groups), around 1730 cm^{-1} ($\nu_{C=O}$: C=O stretching vibration of ester), around 1700 cm^{-1} ($\nu_{C=O}$: C=O stretching vibration of carboxylic acid), 1690 cm^{-1} ($\nu_{C=O}$: C=O stretching vibration of carbamate (urethane) of Z-group), around 1630 cm^{-1} ($\nu_{C=O}$: C=O stretching vibration of amide), around 1550 cm^{-1} ($\delta_{N\text{-}H}$: N-H deformation vibration of amide). Other main peaks characteristic of the compound were also listed.

for **10**, ambiguous assemblies of small particles and fragmentary tubules for **11**, vesicular aggregates for **12**, untwisted tapes for **13**, tubular aggregates for **14**, small particles and vesicles for **15**, and tubular and vesicular aggregates for **16**. No particular relationship was found between aggregate morphologies of amphiphiles and the spectral behaviours of St-4C_1.

Differential scanning calorimetry (DSC) measurements of 20 mM aqueous dispersions also supported the existence of crystalline bilayer structures. The phase transition temperature was measured by DSC with a SEIKO I & E DSC 120. The sample solution (20 mmol dm^{-3}, pH 10) was sealed in an Ag capsule and scanned using a heating rate of 2.0 °C min^{-1}. Crystalline-to-liquid crystalline phase transition temperature (T_c) and transition enthalpy (ΔH) of bilayer-based aggregates estimated by DSC measurements were as follows: T_c, 89 °C (ΔH; 2.6 kcal mol^{-1}) and 102 °C (ΔH; 5.4 kcal mol^{-1}) for **1**; T_c, 83 °C (ΔH; 3.9 kcal mol^{-1}) and 107 °C (ΔH; 5.1 kcal mol^{-1}) for **2**; T_c, 45 °C (ΔH; 2.9 kcal mol^{-1}) for **3**; T_c, 49 °C (ΔH; 5.0 kcal mol^{-1}) for **4**; T_c, 40 °C (ΔH; 9.5 kcal mol^{-1}) for **5**; T_c, 68 °C (ΔH; 11 kcal mol^{-1}) for **6**; T_c, 40 °C (ΔH; 8.9 kcal mol^{-1}) for **7**; T_c, 44 °C (ΔH; 9.7 kcal mol^{-1}) for **8**; T_c, 53 °C (ΔH; 11 kcal mol^{-1}) for **9**; T_c, 78 °C (ΔH; 14 kcal mol^{-1}) for **10**; T_c, 79 °C and 86 °C (ΔH; 12 kcal mol^{-1}) for **11**; T_c, 84 °C and 91 °C (ΔH; 13 kcal mol^{-1}) for **12**; T_c, 65 °C and 80 °C (ΔH; 10 kcal mol^{-1}) for **13**; T_c, 44 °C (ΔH; 3.2 kcal mol^{-1}) for **14**; T_c, 49 °C (ΔH; 0.72 kcal mol^{-1}) for **15**; T_c, 44 °C (ΔH; 1.7 kcal mol^{-1}) and 57 °C (ΔH; 9.4 kcal mol^{-1}) for **16**.

Acknowledgements

This work was supported in part by The Ministry of Education, Culture, Sports, Science and Technology, JAPAN (S0801085). We are grateful to Dr. H. Ihara and Dr. M. Takafuji of Kumamoto University for elemental analyses, and Dr. R. Tomoshige of Research Center for Advances in Impact Engineering, Faculty of Engineering, Sojo University for taking transmission electron micrographs using Phillips TECNAI F20 S-TWIN. We are also grateful to undergraduate students, Y. Miyazono, Y. Hirayama, M. Nakashima, Y. Oh-ishi, T. Okamoto, Y. Takemura, Y. Isomoto, and T. Mizuguchi for amphiphile preparations and other technical assistances.

References

1 (*a*) R. Badugu, J. R. Lakowicz and C. D. Geddes, *J. Am. Chem. Soc.*, 2005, **127**, 3635–3641; (*b*) X. Cao, R. W. Tolbert, J. L. McHale and W. D. Edwards, *J. Phys. Chem. A*, 1998, **102**, 2739–2748.
2 (*a*) H. Hachisako, Y. Murata and H. Ihara, *J. Chem. Soc., Perkin Trans. 2*, 1999, 2569–2577; (*b*) H. Hachisako, T. Yamazaki, H. Ihara, C. Hirayama and K. Yamada, *J. Chem. Soc., Perkin Trans. 2*, 1994, 1671–1680; (*c*) H. Hachisako, T. Yamazaki, H. Ihara, C. Hirayama and K. Yamada, *J. Chem. Soc., Perkin Trans. 2*, 1994, 1681–1690.
3 H. Hachisako and R. Murakami, *Chem. Commun.*, 2006, 1073–1075.
4 (*a*) A. L. Lusk and P. W. Bohn, *Langmuir*, 2000, **16**, 9131–9136; (*b*) Y. Shiraishi, R. Miyamoto and T. Hirai, *Langmuir*, 2008, **24**, 4273–4279.
5 (*a*) C. Hirose and L. Sepülveda, *J. Phys. Chem.*, 1981, **85**, 3689–3694; (*b*) C. A. Bunton, F. Rivera and L. Sepülveda, *J. Org. Chem.*, 1978, **43**, 1166–1173.
6 (*a*) H. Rau, *Angew. Chem., Int. Ed. Engl.*, 1973, **12**, 224–235; (*b*) J. Yoshino, N. Kano and T. Kawashima, *Chem. Commun.*, 2007, 559–561; (*c*) Q. Bo and Y. Zhao, *Langmuir*, 2007, **23**, 5746–5751, and references therein.
7 (*a*) E. E. Jelley, *Nature*, 1936, **138**, 1009–1010; (*b*) G. Scheibe, *Angew. Chem.*, 1939, **52**, 631–637; (*c*) A. H. Herz, *Advances in Colloid and Interface Science*, 1977, **8**, 237–298; (*d*) A. L. Lusk and P. W. Bohn, *Langmuir*, 2000, **16**, 9131–9136; (*e*) N. Vranken, P. Foubert, F. Köhn, R. Gronheid, I. Scheblykin, M. V. Auweraer and F. C. D. Schryver, *Langmuir*, 2002, **18**, 8407–8417; (*f*) H. Berlepsch, M. Regenbrecht, S. Dähne, S. Kirstein and C. Böttcher, *Langmuir*, 2002, **18**, 2901–2907.
8 H. Yao, M. Yamashita and K. Kimura, *Langmuir*, 2009, **25**, 1131–1137.