Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 5092

www.rsc.org/obc

PAPER

Effect of surfactants on the chemiluminescence of acridinium dimethylphenyl ester labels and their conjugates†

Anand Natrajan,* David Sharpe and David Wen

Received 7th February 2011, Accepted 12th April 2011
DOI: 10.1039/c1ob05543g

Chemiluminescent acridinium dimethylphenyl esters, containing two methyl groups flanking the phenolic ester bond, display excellent chemiluminescence stability and are used as labels in automated immunoassays for clinical diagnostics. Light emission from these labels is triggered with alkaline peroxide in the presence of the cationic surfactant cetyltrimethylammonium chloride. Under these conditions, light emission is rapid and is complete in <5 s. In the present study we examined the effect of various surfactants on light emission from acridinium dimethylphenyl ester labels and their conjugates containing hydrophilic linkers derived either from hexa(ethylene)glycol or a sulfobetaine zwitterion. Sulfobetaine zwitterions are very polar and incorporation of these functional groups in acridinium dimethyphenyl esters and their conjugates represents a new approach to improving the aqueous solubility of these chemiluminescent labels. Our results indicate that in general, surfactants affect light emission from these labels and their conjugates by two discrete mechanisms. Cationic surfactants, but not anionic or non-ionic surfactants, accelerate overall light emission kinetics and a more modest effect is observed with zwitterionic surfactants. Surfactants also enhance total light output and the magnitude of this enhancement is maximal for cationic surfactants and a sulfobetaine zwitterionic surfactant. These observations are the first to clearly delineate the role of the surfactant on the chemiluminescence reaction pathway of acridinium esters and can be rationalized based on known effects of surfactant aggregates on bimolecular and unimolecular reactions.

Introduction

Chemiluminescence has emerged to be a dominant detection technology in the clinical diagnostics industry and chemiluminescent labels are widely used in automated instruments.[1] Among the different chemiluminescent labels, acridinium phenyl esters are especially attractive because of their high sensitivity with detection limits in the attomole range. Moreover, they exhibit fast light emission using simple triggering reagents. The relatively small size of acridinium esters is also beneficial for minimizing steric interference in binding reactions.[2–5] Chemiluminescent acridinium ester labels containing two methyl groups flanking the phenolic ester bond[2–5] (Fig. 1) are used in automated, immunochemistry instruments for clinical diagnostics. These acridinium ester labels demonstrate significantly improved chemiluminescence stability compared to unsubstituted, acridinium phenyl esters at physiological pH.[3] In addition, acridinium dimethylphenyl esters are fairly easy to synthesize with a variety of structural modifications to increase their aqueous solubility, lower their non-specific binding and to increase their quantum yields.[2] Light emission from these labels is triggered in two steps. An initial treatment with acid rapidly converts the non-chemiluminescent pseudobase[6] (water adduct) **1** (Fig. 2) to the chemiluminescent acridinium ester **2** (Fig. 2). Subsequent reaction of the acridinium ester with alkaline hydrogen peroxide generates chemiluminescence. The cationic surfactant cetyltrimethylammonium chloride which enhances overall light output[7] is included in the triggering reagents. Under these conditions, light emission from acridinium dimethylphenyl esters is quite rapid and is essentially complete in <5 s.[2]

Excited state acridone **6** (Fig. 2) is believed to be the light emitting species in the chemiluminescence reaction of acridinium phenyl esters and is presumably formed by addition of hydroperoxide ions to C-9 of the acridinium ring followed by scission of the phenolic ester bond.[8,9] A high energy dioxetanone intermediate **5** (Fig. 2) has been proposed in the reaction pathway to the excited state acridone,[8,9] but computational studies have suggested that its direct formation from the dioxetane **4** (Fig. 2) is energetically more favorable.[10] This latter study[10a] also identified the formation of this dioxetane as the slow step in the overall reaction sequence but this hypothesis has not been experimentally verified. Detailed kinetics parameters for the chemiluminescence of various acridinium esters as well as fluorescence measurements of the corresponding

Siemens Healthcare Diagnostics, Advanced Technology and Pre-Development, 333 Coney Street, East Walpole, MA 02032, USA. E-mail: anand.natrajan@siemens.com; Fax: +1-508-660-4591; Tel: +1-508-660-4582

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1ob05543g

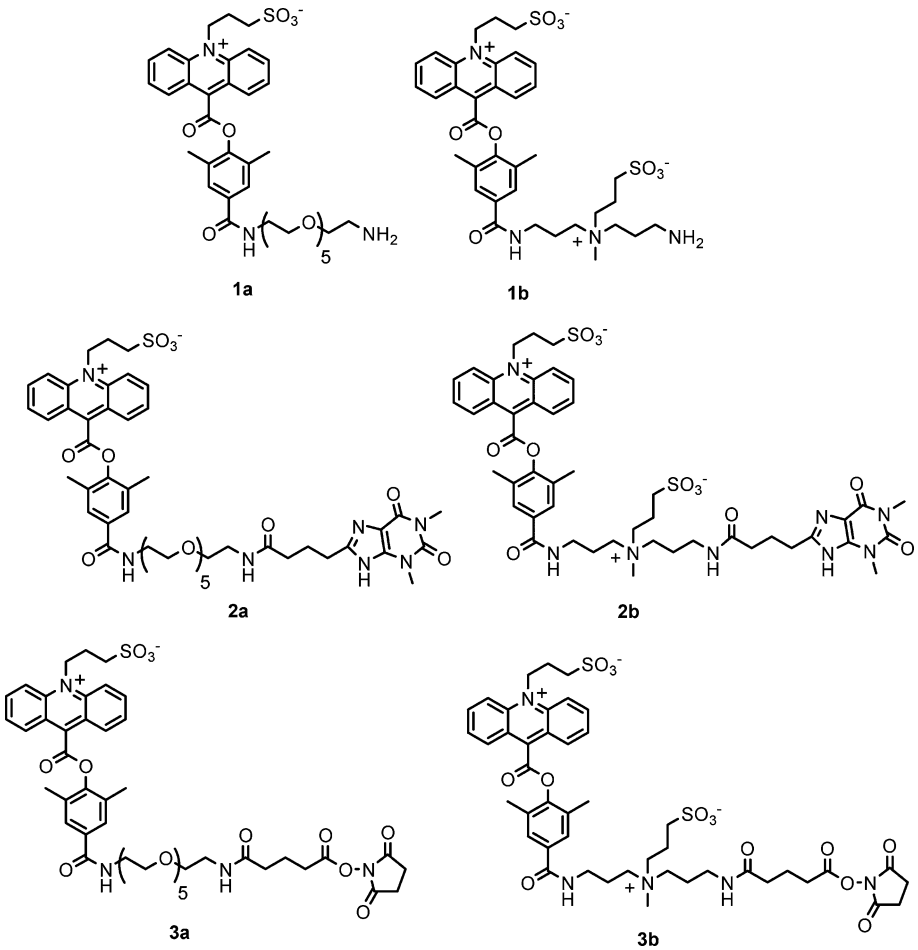

Fig. 1 Structures of acridinium ester labels **1a** and **1b**, theophylline conjugates **2a** and **2b** and, NHS ester labels **3a** and **3b** used for protein labeling.

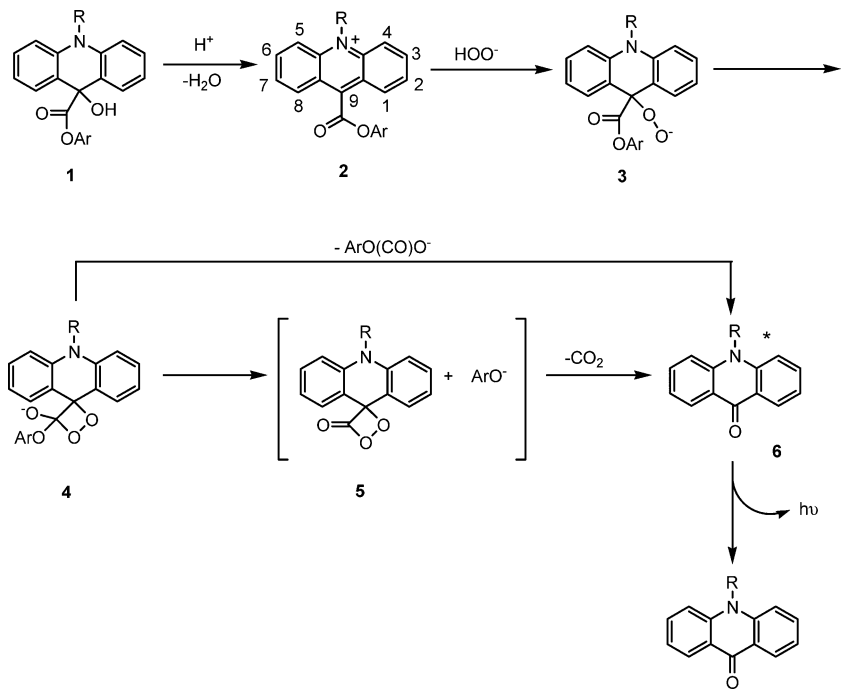

Fig. 2 Simplified reaction pathway for chemiluminescence from acridinium esters. R = $–CH_2CH_2CH_2SO_3^-$ for compounds **1a**, **1b**, **2a**, **2b** and protein conjugates of **3a** and **3b**. Formation of both dioxetane **4** and dioxetanone **5** involve dispersal of negative charge in the transition states of these reactions.

acridones in various solvents have also been recently reported[10b,c] and they support the notion that excited state acridone is the primary emitter. Decomposition of the dioxetane **4** or dioxetanone **5** is postulated to occur by electron transfer from the acridine nitrogen to the peroxide bond[9–11] by a mechanism analogous to the CIEEL (chemically initiated electron-exchange luminescence) mechanism proposed by Schuster.[12] A simplified version of the reaction pathway leading to chemiluminescence from acridinium esters is illustrated in Fig. 2.

In a previously reported study, the cationic surfactant cetyltrimethylammonium chloride was observed to enhance overall light output from an acridinium dimethylphenyl ester containing an *N*-methyl group.[7] Similar effects of surfactants in enhancing the luminescence of other chemiluminescent labels such as luminol,[13] peroxyoxalates[14] and dioxetanes[15] have also been observed. In a study that examined the effect of surfactants on the chemiluminescence of acridinium phenyl ester labels lacking the dimethyl groups, antibody conjugates of these labels were observed to exhibit the greatest increase in chemiluminescence in the presence of the non-ionic surfactant triton X-100, whereas cetyltrimethylammonium chloride was most effective for albumin conjugates.[16]

In the current study, we examined the effect of cationic, zwitterionic, anionic and non-ionic surfactants on light emission from two hydrophilic acridinium dimethylphenyl ester labels as well as their conjugates (Fig. 1) that are currently used in automated immunoassays. The purpose of this study was mainly to elucidate the role of the surfactant on the chemiluminescence from these hydrophilic labels especially pertaining to the reaction pathway illustrated in Fig. 2. We felt that a better understanding of the role of the surfactant was needed to determine whether there is scope for further enhancement of the chemiluminescence of these labels. From a practical point of view, increased light output from these labels would be beneficial in improving assay sensitivity of clinically important analytes.

Results and discussion

Acridinium esters and conjugates

Hydrophilic acridinium dimethylphenyl esters (Fig. 1) display excellent chemiluminescence stability and low non-specific binding,[2,5] and are used as labels in automated immunoassays. In the current study, the free labels **1a** and **1b** in Fig. 1 were used for the preparation of small molecule theophylline conjugates **2a** and **2b**. Protein conjugates were prepared using the NHS (N-hydroxysuccinimidyl) ester derivatives **3a** and **3b**. Compound **1a** with an unsubstituted *N*-sulfopropyl acridinium ring contains a hydrophilic linker derived from hexa(ethylene)glycol which is attached *para* to the phenolic ester bond. This linker increases aqueous solubility of the label and facilitates conjugate synthesis and protein labeling.[5] Compound **1b** is structurally distinct from **1a** and contains a hydrophilic linker with a sulfobetaine zwitterion. Zwitterions are highly hydrophilic and appear to complement poly(ethylene)glycol in their ability to reduce non-specific binding of proteins.[17] Recent studies have shown that surfaces functionalized with zwitterions such as sulfobetaines and carboxybetaines are extremely resistant to protein adsorption, fouling and biofilm formation.[17] The incorporation of a zwitterionic linker such as that present in compound **1b** thus represents a complementary approach towards increasing the aqueous solubility and lowering the non-specific binding of acridinium ester labels.

The syntheses of compounds **1a–3a** have been described previously.[2,5] Syntheses of compounds **1b**, **2b** and **3b** containing the zwitterionic sulfobetaine linker are illustrated in Fig. 3 and were accomplished in a straightforward manner using commercially available reagents. The zwitterionic linker **iv** itself was synthesized in three steps from *N*,*N*-bis(3-aminopropyl)methylamine as described in the Experimental section. The primary amines in *N*,*N*-bis(3-aminopropyl)methylamine **i** were first converted to the benzyl carbamates, following which the protected derivative **ii** was *N*-alkylated at the tertiary amine with 1,3-propane sultone to give compound **iii**. Deprotection of the primary amines in **iii** gave the zwitterionic linker **iv**. The linker **iv** was next coupled to acridinium ester **v**[4,5] to give compound **1b** which was purified by preparative HPLC. Conversion of **1b** to the theophylline conjugate **2b** was accomplished in one step by coupling to commercially available 8-carboxypropyltheophylline using (benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) followed by HPLC purification. Conversion of **1b** to the NHS ester label **3b** was accomplished by first condensing **1b** with glutaric anhydride followed by activation of the resulting carboxylate derivative with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU). Compound **3b** was purified by HPLC prior to protein labeling.

Protein conjugates of the labels **3a** and **3b** were prepared, as described in the experimental section, using three different proteins; a murine anti-TSH monoclonal antibody with an acidic pI = 5.6 (TSH = Thyroid Stimulating Hormone); a murine anti-HBsAg monoclonal antibody with a pI = 7 (HBsAg = Hepatitis B Surface Antigen) and egg white avidin with a basic pI = 10.5.[18] All three labels displayed similar reactivity towards the three proteins and the extent of label incorporation was very similar as described in the Experimental section. Using an input of 10 equivalents of the acridinium ester labels **3a** and **3b**, approximately 5 labels were incorporated in each protein as measured by MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry.

Surfactants and light measurement protocol

Previous studies[7,16] that examined the effect of surfactants on acridinium ester chemiluminescence noted an enhancement of chemiluminescence in the presence of surfactants but the mechanism leading to this enhancement was not defined clearly. Micellar catalysis of bimolecular reactions largely results from increased local concentrations of the reactants in a small volume of the micellar phase.[19] An important property of ionic micelles, that makes them effective catalysts of bimolecular reactions, is their ability to bind various substrates and attract oppositely charged reactive ions to their surfaces. The increased local concentration of the two reactants is manifested by an increase in the observed reaction rate. Surfactant aggregates also have significant effects on the rates of unimolecular reactions such as the decarboxylation of 6-nitrobenzisoxazole-3-carboxylate, the intramolecular cyclization reactions of *ortho*-haloalkyl-substituted phenoxides and 1,2-elimination reactions.[20,21] All these reactions involve charge dispersal in their transition states leading to products and are

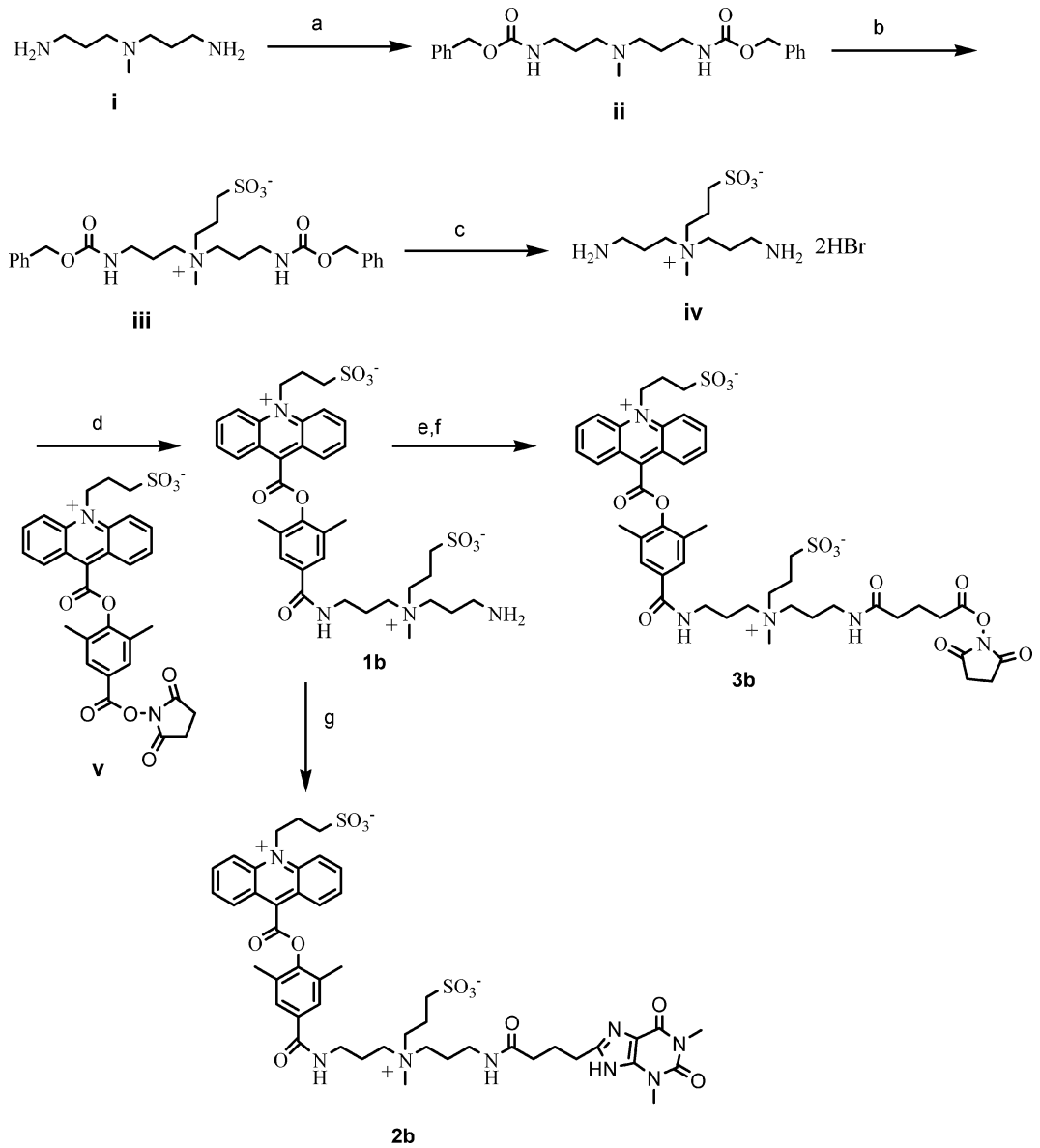

Fig. 3 Synthetic scheme for **1b**, **2b** and **3b**. Reagents: (a) *N*-(benzyloxycarbonyloxy)succinimide, chloroform; (b) 1,3-propane sultone, ethyl acetate; (c) 33% hydrogen bromide in acetic acid; (d) 0.25 M sodium bicarbonate/dimethyl formamide; (e) glutaric anhydride, diisopropylethylamine, water/methanol; (f) TSTU, diisopropylethylamine, water/dimethyl formamide; (g) 8-carboxypropyltheophylline, BOP reagent, diisopropylethylamine, dimethyl sulfoxide.

catalyzed by various cationic and zwitterionic surfactants resulting from reduced medium polarity of the micellar environment.[20,21]

Light emission from acridinium dimethylphenyl esters that are used as chemiluminescent labels in automated immunoassays is triggered by the sequential addition of equal volumes (0.3 mL) of two reagents. An initial treatment with 0.1 M nitric acid containing 0.5% hydrogen peroxide (Reagent 1) is followed by the addition of 0.25 M sodium hydroxide containing 7 mM of the cationic surfactant cetyltrimethylammonium chloride (CTAC) (Reagent 2).

In the current study, we examined the effect of different surfactants in Reagent 2 on emission kinetics as well as total light emitted from the acridinium ester labels and conjugates. Specifically, light emission from each label or conjugate was measured for a period of two minutes (in 0.5 s intervals) which was sufficiently long for >90% emission under all conditions. Emission kinetics, as reflected by the time required for >90% emission of light from the labels and their conjugates, are tabulated in Tables 1 and 2 for the free labels **1a** and **1b**, the theophylline conjugates **2a** and **2b** as well the different protein conjugates of the labels **3a** and **3b**. These emission times reflect the effect of the surfactant in either accelerating or inhibiting the chemiluminescence rate of the acridinium ester labels and conjugates. The total amount of light emitted at the two minute measurement time, in the presence of various surfactants, was used to calculate the effect of the surfactant on light output (relative quantum yield). Total light output in the absence of surfactant was normalized to a value of one for all labels and conjugates. Relative light output values in the presence of surfactants are listed in Tables 3 and 4 for the free labels, the theophylline conjugates and the protein conjugates.

Table 1 Time (seconds[a]) for emission of >90% total light of acridinium esters **1a** and **1b** and theophylline conjugates **2a** and **2b** in the presence of different surfactants

Compound	No surfactant	CTAC	CTAOH	CTPAC	CTBAC	DDAO	DDAPS	SDS	Triton X-100
1a	60.0	1.5	1.5	1.5	4.0	40.5	10.0	67.0	57.5
1b	67.5	1.0	1.0	1.5	3.0	40.0	7.0	63.0	61.0
2a	55.5	2.5	2.5	1.5	1.5	42.5	14.0	60.0	56.0
2b	62.0	1.5	1.5	1.5	1.5	42.0	9.5	60.5	59.5

[a] Average of five replicates. Abbreviations used: CTAC = cetyltrimethylammonium chloride (cationic); CTAOH = cetyltrimethylammonium hydroxide (cationic); CTPAC = cetyltripropylammonium chloride (cationic); CTBAC = cetyltributylammonium chloride (cationic); DDAO = *N*,*N*-dimethyldodecylamine oxide (zwitterionic); DDAPS = *N*,*N*-dimethyldodecylammonio-1,3-propane sulfonate (zwitterionic); SDS = sodium dodecyl sulfate (anionic); Triton X-100 (non-ionic).

Table 2 Time (seconds[a]) for emission of >90% total light of acridinium ester–protein conjugates of labels **3a** and **3b** in the presence of different surfactants

Conjugate	No surfactant	CTAC	CTAOH	CTPAC	CTBAC	DDAO	DDAPS	SDS	Triton X-100
Anti-TSH Mab-**3a**	64.5	2.5	3.0	3.0	3.5	46.5	14.5	89.0	62.5
Anti-TSH Mab-**3b**	61.5	1.5	3.0	2.0	2.5	40.0	9.5	86.5	59.5
Anti-HBsAg Mab-**3a**	64.5	3.0	3.0	5.0	5.5	46.0	13.5	90.5	61.0
Anti-HBsAg Mab-**3b**	60.5	2.0	3.0	3.0	4.0	39.0	9.0	88.0	58.5
Avidin-**3a**	89.5	3.5	3.0	21.0	9.0	72.5	61.0	87.5	89.0
Avidin-**3b**	74.5	1.0	1.5	2.5	2.0	53.0	29.0	80.0	73.5

[a] Average of five replicates.

Table 3 Observed enhancement of light output of acridinium esters **1a** and **1b** and theophylline conjugates **2a** and **2b** in the presence of different surfactants at a measurement time of two minutes

Compound	No surfactant	CTAC	CTAOH	CTPAC	CTBAC	DDAO	DDAPS	SDS	Triton X-100
1a	1	3.2	3.4	3.6	3.3	2.4	3.9	1.3	1.5
1b	1	2.9	3.2	3.2	3.0	2.3	3.7	1.3	1.4
2a	1	2.7	3.1	3.5	3.3	2.1	3.4	1.4	1.4
2b	1	2.9	3.0	3.3	3.3	2.4	3.8	1.3	1.4

Table 4 Observed enhancement of light output of acridinium ester–protein conjugates of labels **3a** and **3b** in the presence of different surfactants at a measurement time of two minutes

Conjugate	No surfactant	CTAC	CTAOH	CTPAC	CTBAC	DDAO	DDAPS	SDS	Triton X-100
Anti-TSH Mab-**3a**	1	3.1	3.2	3.4	3.6	2.6	4.1	1.2	1.4
Anti-TSH Mab-**3b**	1	2.1	2.3	2.2	2.4	2.4	3.7	1.1	1.4
Anti-HBsAg Mab-**3a**	1	3.2	3.2	3.5	3.6	4.6	7.3	1.1	1.5
Anti-HBsAg Mab-**3b**	1	2.4	2.6	2.7	2.8	2.5	3.9	1.1	1.4
Avidin-**3a**	1	4.9	4.8	5.6	5.5	3.5	5.7	1.5	1.4
Avidin-**3b**	1	2.7	2.6	3.2	3.2	2.8	4.4	1.3	1.4

Thus, Tables 3 and 4 reflect the ability of the various surfactants tested to enhance the total light emitted by the acridinium ester labels and conjugates.

Chemiluminescence measurements were made using 10 femtomoles (femtomole = 10^{-15} mole) of acridinium labels **1a** and **1b** and theophylline conjugates **2a** and **2b** and 2.5 femtomoles of protein conjugates in a total volume of 0.6 mL. Surfactant concentrations in Reagent 2 were five times its reported CMC (critical micelle concentration) in water. Final concentration of reagents was 0.25% (~80 mM) peroxide in 0.125 M sodium hydroxide and the final concentration of the surfactant was 2.5 times its reported CMC in water. Thus, concentrations of peroxide and surfactant were in vast excess compared to concentrations of the acridinium ester labels and their conjugates. Details of these measurements can be found in the experimental section.

Among the different cationic surfactants, we examined the effect of the counterion of cetyltrimethylammonium salts as represented by the surfactants CTAOH (with hydroxide counterions) and CTAC (with chloride counterions). Hydroxide ions are reported to bind weakly to cetyltrimethylammonium micelles and this is reflected in the increased CMC and ionization constant α of CTAOH when compared to CTAC.[22] The reactive ions in the chemiluminescence reaction of acridinium esters are hydroperoxide ions which have been reported to display similar affinity to cetyltrimethylammonium micelles as hydroxide ions.[23] The effect of head group size in cationic surfactants was also tested by including the surfactants cetyltripropylammonium chloride (CTPAC) cetyltributylammonium chloride (CTBAC).[24] Aggregates of both these surfactants have been reported to bind anions less strongly than cetyltrimethylammonium micelles owing to reduced

polarity of their head groups which is reflected in lower CMC values of these surfactants compared to CTAC.[24] Two zwitterionic surfactants, DDAO (*N*,*N*-dimethyldodecylamine oxide) and DDAPS (*N*,*N*-dimethyldodecylammonio-1,3-propane sulfonate) were included in the current study to examine their effects on acridinium ester chemiluminescence. While aggregates of these surfactants are reported to attract anions only weakly,[25,26] they offer a non-polar environment similar to cationic micelles.[20,21] Finally, the effects of the anionic surfactant SDS (sodium dodecyl sulfate) and the non-ionic surfactant triton X-100 on acridinium ester chemiluminescence were also investigated.

Effect of surfactants on light emission kinetics

The effect of surfactants on emission kinetics of the two acridinium ester labels and their conjugates is illustrated in Tables 1 and 2. Light emission profiles for the labels **1a** and **1b** are illustrated in Fig. 4 and 5, in the presence and absence of surfactants. Emission profiles of the theophylline and protein conjugates were similar and are illustrated in Fig. 6–13 of the ESI.† In the absence of surfactant, light emission was quite slow for the labels and their conjugates requiring a minute or more for >90% light emission. In the presence of the cetyltrialkylammonium surfactants, CTAC, CTAOH, CTPAC and CTBAC, light emission was observed to be

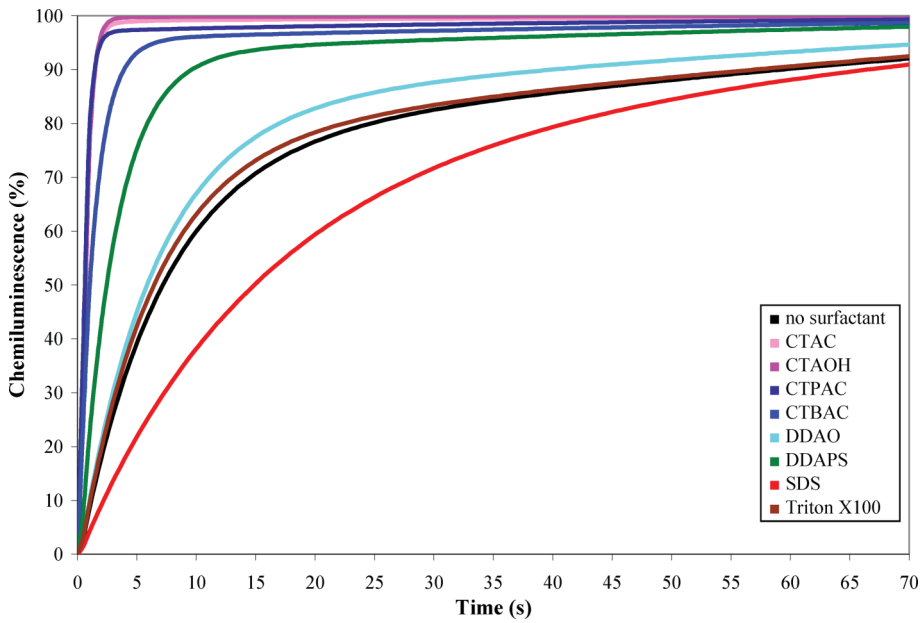

Fig. 4 Light emission profiles of acridinium ester label **1a** in the absence and in the presence of various surfactants.

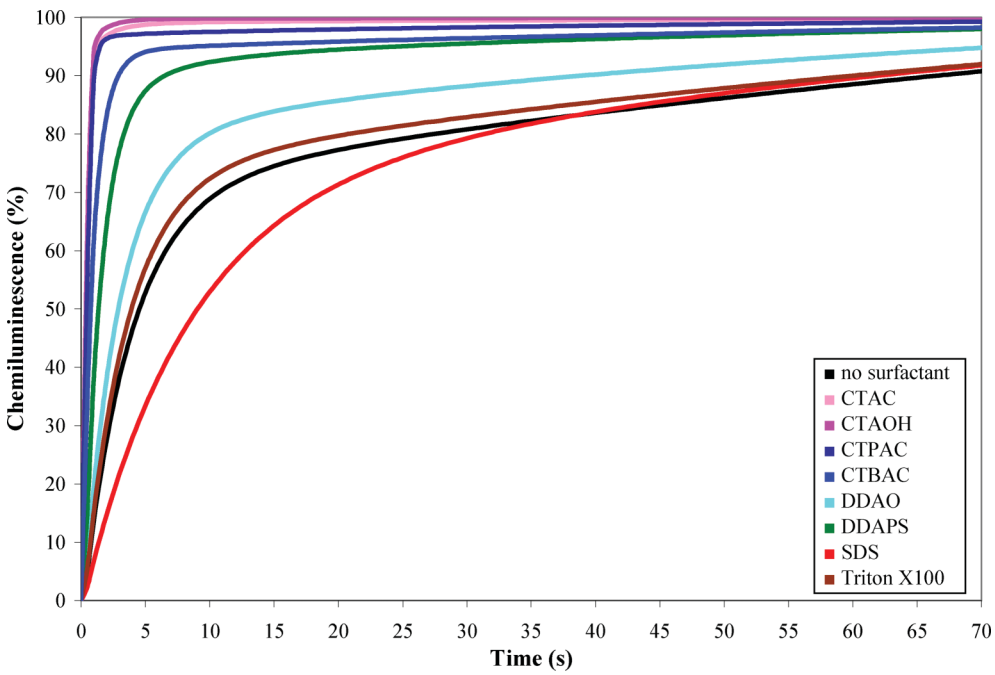

Fig. 5 Light emission profiles of acridinium ester label **1b** in the absence and in the presence of various surfactants.

significantly faster and emission times were compressed to ~5 s for the labels and their conjugates. The only exception was the avidin conjugate of **3a** which exhibited relatively slower emission in the presence of CTPAC and CTBAC. Although aggregates of CTPAC and CTBAC were expected to bind hydroperoxide ions less strongly than micelles of the two cetyltrimethylammonium surfactants, with the exception of the avidin conjugate of **3a**, we did not observe a significant attenuation in emission kinetics (Tables 1 and 2) because the surfactants were in vast excess. Protein pI in general had little effect on the ability of the cationic surfactants in accelerating emission kinetics. From the emission times in Tables 1 and 2, relative rate enhancements can be estimated by comparing the emission times in the absence of surfactant and in the presence of surfactant. These ratios indicate that the cationic surfactants, especially CTAC and CTAOH, increase emission rates by at least an order of magnitude for acridinium dimethylphenyl ester labels and their conjugates.

Zwitterionic micelles are reported to attract anions weakly[25,26] and both the zwitterionic surfactants, DDAO and DDAPS were less effective than CTAC and CTAOH in enhancing the light emission kinetics of the labels and their conjugates. The amine oxide surfactant, which has a more covalent head group, was observed to be significantly less effective than the sulfobetaine surfactant in enhancing light emission kinetics. Finally, no enhancement in light emission kinetics could be observed in anionic micelles of sodium dodecyl sulfate (SDS) as well as non-ionic surfactant triton X-100.

Cetyltrimethylammonium hydroperoxide (CTAOOH) has been shown to catalyze phosphate ester hydrolysis by concentrating the reactive components in the micellar phase thereby leading to faster observed rates.[23,27,28] Our observations on the effects of aggregates of cetyltrimethylammonium salts on the light emission kinetics of the acridinium dimethylphenyl ester labels **1a** and **1b** as well as their conjugates are consistent with these reports. The reaction of hydroperoxide ions with these acridinium esters and their conjugates appears to be a slow step (conversion of **2** to **3** in Fig. 2) in the chemiluminescence reaction pathway that is catalyzed by cetyltrimethylammonium micelles, resulting from (a) binding of these labels and their conjugates to the aggregates and, (b) increased local concentration of hydroperoxide ions at the positively charged micellar surface. Attenuation of this positive charge as exemplified by the two zwitterionic surfactants decreases the local concentration of hydroperoxide ions leading to slower light emission. Aggregates of the anionic surfactant SDS, as well as the non-ionic surfactant triton X-100 are not expected to concentrate negatively charged hydroperoxide ions at their surfaces and consequently both these surfactants did not accelerate emission kinetics. Comparison of the emission profiles of **1a** and **1b** (Fig. 4 and 5) indicate that initial rates decrease in the order CTAC (cationic) ~ CTAOH (cationic) ~ CTPAC (cationic) ~ CTBAC (cationic) > DDAPS (zwitterionic) > DDAO (zwitterionic) > triton X 100 (non-ionic) ~ no surfactant > SDS (anionic). Similar conclusions can be drawn from comparing the emission profiles of the theophylline conjugates **2a** and **2b** (Fig. 6 and 7, ESI†) and the antibody conjugates of **3a** and **3b** (Fig. 8–13, ESI†).

Effect of surfactants on light output

In addition to emission kinetics, surfactants were also observed to exert significant effects on the total light emitted by the acridinium ester labels and their conjugates as illustrated in Tables 3 and 4. In the present study, the total amount of light was measured for two minutes for each label and conjugate both in the absence of surfactant and the presence of various surfactants. The relative quantum yields of the labels **1a** and **1b** and their conjugates were assigned a value of one in the absence of surfactant to facilitate interpretation of surfactant effects on total light output. Light output on different proteins was similar for the two labels.

As illustrated in Tables 3 and 4, aggregates of the cetyltrimethylammonium surfactants, which were the most effective in enhancing light emission kinetics, were observed to enhance light output approximately 3-fold from the labels and theophylline conjugates although more variation was observed for the protein conjugates. Chemiluminescence enhancement of the labels **1a** and **1b**, the two theophylline conjugates **2a** and **2b** and the protein conjugates of **3a** and **3b** was also slightly greater in the presence of the two cationic surfactants CTPAC and CTBAC with larger head groups.

Micelles of the two zwitterionic surfactants DDAO and DDAPS were observed to be less effective than cationic cetyltrimethylammonium surfactants in enhancing emission kinetics but both surfactants enhanced light output from the two labels as well their conjugates. The magnitude of this enhancement in DDAO was slightly lower compared to the cationic surfactants. However, the sulfobetaine surfactant DDAPS was more effective than CTAC and CTAOH in enhancing light output from the two labels and their conjugates as illustrated in Tables 3 and 4.

Micelles of the non-ionic surfactant triton X-100, which did not enhance emission kinetics of the acridinium ester labels and their conjugates, were also only marginally effective in enhancing light output. Similarly, the anionic surfactant SDS showed only marginal enhancement of light output for the labels **1a** and **1b** and their conjugates.

What is the mechanism by which cationic and zwitterionic surfactants enhance chemiluminescence of acridinium esters **1a** and **1b** (Fig. 1) and their conjugates? When considering plausible mechanisms, the impact of the micellar environment on all the reaction steps outlined in Fig. 2 must be assessed. Clearly, as noted earlier, formation of the hydroperoxide adduct **3** (Fig. 2) from the acridinium ester **2** is accelerated by cationic surfactants which is manifested as faster light emission. Formation of excited state acridone **6**, which is the light emitting species, is postulated to occur either from dioxetane **4** or dioxetanone **5** (Fig. 2) by the CIEEL mechanism[9–11] and the micellar environment may influence not only the formation of all three chemical species but may also affect emission from excited state acridone **6**.

Chemiluminescence from the decomposition of dioxetanes *via* the CIEEL mechanism results in the formation of singlet excited states of the primary emitters in high quantum yields.[9,11,12,29] Chemiluminescence emission spectra of acridinium esters are identical to the fluorescence spectra of the corresponding acridones which display mono-exponential fluorescence decay reflecting emission from a single excited state.[10b] Chemiluminescence emission spectra of acridinium ester conjugates of fluorescent dyes, resulting from very efficient energy transfer from excited state acridone to the fluorescent moieties, are also identical to the fluorescent spectra of the dyes.[30]

Published studies[31] on the fluorescence of acridones in various media are thus useful in understanding the impact of the micellar environment on emission from excited state acridone **6** (Fig. 2).

Hinze *et al.* have reported that the fluorescence quantum yields, fluorescence lifetimes and fluorescence emission spectra of *N*-methylacridone (NMA) in water, as well as in solutions of cationic, zwitterionic, anionic and non-ionic surfactants are very similar.[31a] The fluorescence quantum yield of this acridone was close to unity in these different media as well as in alcoholic solvents (methanol, ethanol and 2-propanol) but decreased in less polar solvents such as dimethyl sulfoxide and dimethyl formamide. For example, the emission wavelength maximum, fluorescence quantum yield and fluorescence lifetime for NMA in water was observed to be 431 nm, 1.00 and 15.8 ns, respectively. In a 5 mM solution of CTAC, the emission wavelength maximum, fluorescence quantum yield and fluorescence lifetime were 427 nm, 0.97 and 15.2 ns, respectively. In a previous study, we had also observed that the chemiluminescence emission wavelength maximum of an *N*-sulfopropyl acridinium ester, which forms electronically excited *N*-sulfopropylacridone, in the presence of CTAC is 426 nm.[2]

Siegmund *et al.* studied singlet–triplet dynamics of NMA in a wide range of solvents[31b] and also observed lower fluorescence quantum yields in non-polar solvents for NMA as well as for acridone and *N*-phenylacridone.[31b,c] In halogenated solvents where the fluorescence quantum yield of NMA was significantly lower, kinetics of intersystem crossing were 5–10 fold higher.[31b] A related study by Mory *et al.* on a series of acridones with different *N*-alkyl groups showed that the first singlet excited state is populated in polar solvents and the first triplet state is populated in non-polar solvents.[31d]

The fact that the fluorescence quantum yield of NMA is the same in water and surfactant solutions[31a] along with data published by Siegmund *et al.*[31b] on singlet–triplet dynamics of acridones suggests that in the chemiluminescent reaction of acridinium ester, (a) singlet–triplet dynamics of excited state acridone **6** are not influenced by surfactants and, (b) solubilization of excited state acridone **6** occurs in a relatively polar region of the micelle, *i.e.*, the Stern layer of the micellar phase which is considered to be 'alcohol-like' in polarity.[19] Thus, published studies strongly suggest that surfactants can only influence the chemiluminescence reaction steps of acridinium esters preceding the formation of excited state acridone **6** but not its emission.

To understand how surfactant aggregates might affect formation of dioxetane **4** and dioxetanone **5**, it is useful to examine published reports on their impact on analogous unimolecular reactions. Unimolecular reactions such as the decarboxylation of 6-nitrobenzisoxazole-3-carboxylate,[19] the intramolecular cyclization reactions of *ortho*-haloalkyl-substituted phenoxides[19] and 1,2-elimination reactions[20] all involve dispersal of negative charge in the transition states, and are catalyzed by aggregates of cationic and zwitterionic surfactants because of reduced polarity (alcohol-like) at the micellar phase. These observations are consistent with classical studies by Hughes, Ingold and co-workers on the effects of solvent polarity on organic reactions.[32] In the intramolecular cyclization reactions of *ortho*-haloalkyl-substituted phenoxides, from a comparison of the cyclization rates, relative rate enhancement in CTAC aggregates was observed to be similar to that observed in ethanol whereas rate enhancement in CTPAC and CTBAC micelles, with more hydrophobic surfactant head groups, was greater and similar to that observed in 2-propanol.[20d] It was postulated that zwitterionic micelles derived from DDAPS offered a less polar environment than micelles of cetyltrimethylammonium bromide based on the magnitude of the observed catalysis which was slightly greater for DDAPS. Catalysis was only very modest in the presence of non-ionic surfactants presumably because of increased hydration of these surfactant aggregates.

Formation of both dioxetane **4**, from intramolecular cyclization of hydroperoxide adduct **3** as well as dioxetanone **5** from elimination of the phenol from **4** (Fig. 2), also involve dispersal of negative charge in the transition states and are expected to be facilitated by reduced medium polarity. Either of these intermediates may be the immediate precursor to excited state acridone **6**[8–10] and therefore, an increase in their yields should result in a concomitant increase in the yield of excited state acridone. Consistent with this expectation, we have observed that both cationic micelles of cetyltrialkylammonium salts as well as zwitterionic micelles of DDAPS enhance the chemiluminescence of acridinium esters and their conjugates but significant chemiluminescence enhancement was not observed in aggregates of the non-ionic surfactant triton X-100 because of increased hydration of the latter. Chemiluminescence enhancement was maximal for the zwitterionic surfactant DDAPS as well as the two cationic surfactants CTPAC and CTBAC with large head groups indicating that medium-polarity is an important factor affecting formation of excited state acridone **6**. In the unimolecular decarboxylation of 6-nitrobenzisoxazole-3-carboxylate, little catalysis was noted in the presence of anionic SDS micelles.[33] Similarly, we observed minimal enhancement in chemiluminescence of acridininium dimethyl esters and their conjugates in the presence of this anionic surfactant.

Finally, it is also important to examine the potential impact of the micellar environment on the CIEEL mechanism leading to excited state acridone **6** (Fig. 2). Details of the reaction steps involved in the conversion of dioxetane **4** or dioxetanone **5** to excited state acridone **6** are presently unclear,[10] and there is also controversy regarding the efficacy of an intermolecular *versus* an intramolecular electron transfer process on the chemiluminescence of dioxetanes. For example, in the triggered luminescence of spiroadamantyl-substituted dioxetanes, Adam *et al.* observed an increase in the excitation yield of the light emitting species with increased solvent viscosity.[34] These observations were attributed to the 'solvent-cage' effect on the CIEEL mechanism of this dioxetane and were considered to support the intermolecular back-electron transfer (BET) mechanism as opposed to an intramolecular process leading directly to the excited state phenolate anion. However, Ciscato *et al.*[35] recently reported that an intermolecular BET reaction is a low yield process compared to an intramolecular BET reaction for acridinium-dioxetanes. The latter study is more relevant to our work and since surfactants enhance the light emission of acridinium esters, therefore it is unlikely that they would promote an intermolecular BET reaction for decomposition of dioxetane **4** or dioxetanone **5** (Fig. 2).

Conclusions

We have examined the effects of surfactants on light emission from two hydrophilic acridinium dimethylphenyl ester labels that are currently used in automated immunoassays for clinical diagnostics. Our results indicate that surfactants influence the chemiluminescence reaction pathway (Fig. 2) at two different steps of the overall process. The initial reaction of hydroperoxide ions with the acridinium ester is accelerated by cationic surfactants

leading to faster light emission. Surfactants also enhance total light output by facilitating formation of the dioxetane **4** and/or the dioxetanone **5** if the latter is indeed a true reaction intermediate. Literature evidence[31] strongly suggests that surfactants do not affect the fluorescence quantum yield, the fluorescence lifetime, emission spectrum or singlet–triplet dynamics of excited state acridone, which is the light emitting species.

From a practical point of view, cationic surfactants such as CTAC, that are effective in both enhancing light output and accelerating emission kinetics of acridinium dimethylphenyl esters are the most useful for automated instruments where a fast read time is required to maintain high throughput.

Experimental

General

Chemicals and surfactants were purchased from Sigma–Aldrich (Milwaukee, Wisconsin, USA) unless indicated otherwise. *N*,*N*-Bis(3-aminopropyl)methyl amine was purchased from TCI America. The syntheses of the acridinium compounds **1a** and **3a**, and the theophylline conjugate **2a** have been described previously.[5] Cetyltripropylammonium chloride (CTPAC) and cetyltributylammonium chloride (CTBAC) were synthesized using a literature procedure.[24]

All final acridinium esters and theophylline conjugates were analyzed and purified by HPLC using a Beckman-Coulter HPLC system. MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry was performed using a Voyager DE™ Biospectrometry™ Workstation from Perkin–Elmer. This is a benchtop instrument operating in the linear mode with a 1.2 meter ion path length, flight tube. Spectra were acquired in positive ion mode. For acridinium esters and theophylline conjugates, α-cyano-4-hydroxycinnamic acid was used as the matrix and spectra were acquired with an accelerating voltage of 20 000 volts and a delay time of 100 ns. For protein conjugates, sinapinic acid was used as the matrix and spectra were acquired with an accelerating voltage of 25 000 volts and a delay time of 85 ns.

For HRMS (High Resolution Mass Spectra), samples were dissolved in HPLC-grade methanol and analyzed by direct-flow injection (injection volume = 5 μL) ElectroSpray Ionization (ESI) on a Waters Qtof API US instrument in the positive ion mode. Optimized conditions were as follows: Capillary = 3000 kV, Cone = 35, Source T = 120 °C, Desolvation T = 350 °C. NMR spectra were recorded on a Varian 500 MHz spectrometer.

Synthesis of compounds 1b, 2b and 3b (Fig. 3)

(a) *N*,*N*-Bis(3-benzyloxycarbamoylpropyl)methylamine, compound ii. A solution of *N*,*N*-bis(3-aminopropyl)methyl amine (1 g, 6.9 mmol) in chloroform (40 mL) was treated with *N*-(benzyloxycarbonyloxy)succinimide (3.78 g, 15.2 mmol, 2.2 equivalents). The reaction was stirred at room temperature for 16 h. TLC analysis of the reaction mixture on silica using 15% methanol in ethyl acetate showed the formation of a polar product in a clean reaction. The reaction mixture was diluted with chloroform (40 mL) and washed with saturated aqueous sodium bicarbonate solution. It was then dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure to afford a viscous oil that solidified upon storage to a waxy solid. HPLC analysis of the product was performed using a Phenomenex, 10 micron, C_{18} 3.9 mm × 25 cm column and a 30 min gradient of 10 → 70% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA; TFA = trifluoroacetic acid) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. Product was observed eluting at 21.6 min as a broad peak. Yield = 3.2 g (quantitative); δ_H (500 MHz, $CDCl_3$) 1.59–1.70 (m, 4 H), 2.16 (s, 3 H), 2.37 (t, 4 H, J = 6.5), 3.24 (q, 4 H, J = 6.0), 5.07 (s, 4 H), 5.55 (br s, 2 H), 7.27–7.40 (m, 10 H); δ_C (125 MHz, $CDCl_3$) 26.77, 39.84, 41.66, 55.81, 66.45, 127.98, 128.06, 128.44, 136.71, 155.45; MALDI-TOF MS m/z 414.4 (M + H)$^+$; HRMS m/z 414.2385 (M + H)$^+$ (414.2393 calculated).

(b) *N*,*N*-Bis(3-benzyloxycarbamoylpropyl)methylammonium-1,3-propane sulfonate, compound iii. A solution of *N*,*N*-bis(3-benzyloxycarbamoylpropyl)methylamine (1.2 g, 2.9 mmol) in anhydrous DMF (15 mL) was treated with 1,3-propane sultone (0.71 g, 5.8 mmol, 2 equivalents). The reaction was heated at 145 °C under nitrogen for 1 h. HPLC analysis of a small portion of the reaction mixture was performed using a Phenomenex, 10 micron, C_{18} 3.9 mm × 25 cm column and a 30 min gradient of 10 → 70% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA; TFA = trifluoroacetic acid) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. Product was observed eluting at 19.6 min (~80% conversion) with starting material eluting at 21.6 min. The reaction mixture was concentrated under reduced pressure and the recovered oil was dissolved in methanol (20 mL). TLC analysis on silica using 40% methanol, 60% ethyl acetate indicated clean separation of product ($R_f \approx 0.2$) from starting material ($R_f \approx 0.3$). The above reaction was repeated on the same scale and the combined reaction mixture was purified by flash chromatography on silica using 40% methanol, 60% ethyl acetate as eluent. Yield = 1.55 g (60%); white foam; δ_H (500 MHz, CD_3OD) 1.87–1.96 (m, 4 H), 2.06–2.14 (m, 2H), 2.84 (t, 2 H, J = 6.5), 2.99 (s, 3 H), 3.21 (t, 4 H, J = 6.2), 3.24–3.30 (m, 4 H), 3.44–3.50 (m, 2 H), 5.08 (s, 4 H), 7.27–7.33 (m, 2 H), 7.35 (br d, 8 H); δ_C (125 MHz, CF_3COOD) 17.65, 20.18, 37.54, 47.18, 48.20, 59.34, 60.02, 70.81, 128.15, 128.40, 129.07, 132.45; MALDI-TOF MS m/z 536.4 (M + H)$^+$; HRMS m/z 536.2435 (M + H)$^+$ (536.2430 calculated).

(c) *N*,*N*-Bis(3-aminopropyl)methylammonium-1,3-propane sulfonate, compound iv. *N*,*N*-Bis(3-benzyloxycarbamoylpropyl)-methylammonium-1,3-propane sulfonate (0.8 g, 1.49 mmol) was stirred in 15 mL of 33% HBr/AcOH at room temperature for 24 h. Ether (100 mL) was then added and a white, granular solid separated out. The product was allowed to settle and the ether was decanted. This process was repeated twice with ether (2 × 50 mL). Finally, the product was dried under vacuum. The recovered viscous oil was dissolved in 5–6 mL water, frozen at –80 °C and lyophilized to dryness to afford a glassy solid. TLC analysis on silica using 25% ammonia, 75% methanol and ninhydrin for visualization showed a single spot of $R_f \approx 0.2$.

Yield = 0.766 g (quantitative); δ_H (500 MHz, CF_3COOD) 2.77–2.83 (m, 6H), 3.45 (s, 3H), 3.59 (br t, 2H), 3.70 (br s, 4H), 3.96 (br s, 6H), 7.42 (br s, 6H); δ_C (125 MHz, CF_3COOD) 18.15, 20.73, 38.03, 47.78, 48.76, 59.49, 61.30; MALDI-TOF MS m/z 268.2 (M + H)$^+$; HRMS m/z 268.1686 (M + H)$^+$ (268.1695 calculated).

(d) Compound 1b. A solution of 2′,6′-dimethyl-4′-carboxylphenyl *N*-sulfopropylacridinium-9-carboxylate[2,5] (30 mg, 0.061 mmole) in DMF (3 mL) was treated with diisopropylethylamine (0.016 mL, 0.0917 mmole, 1.5 equivalents) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) (22 mg, 0.0732 mmol, 1.2 equivalents). The reaction was stirred at room temperature. After 15 min, HPLC analysis of a small portion of the reaction mixture was performed using a Phenomenex, 10 micron, C_{18} 3.9 mm × 25 cm column and a 30 min gradient of 10 → 70% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. Product NHS ester (compound **v**) was observed eluting at 20 min and was the major component. This DMF solution of the NHS ester, compound **v**, was added dropwise to a solution of *N*,*N*-bis(3-aminopropyl)methylammonium-1,3-propane sulfonate, compound **iv**, (0.136 g, 0.0304 mmol, 5 equivalents, HBr salt) dissolved in DMF (1 mL) and 0.25 M sodium bicarbonate (1 mL). The reaction was stirred at room temperature. After 3 h, HPLC analysis showed clean conversion to the product **1b**, eluting at 12.4 min. Using a 40 min gradient of 10 → 40% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA), the product eluted at 19.2 min. The product was purified by preparative HPLC using a YMC, 10 micron, C_{18} 30 × 250 mm column and 40 min gradient of 10 → 40% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a solvent flow rate of 20 mL min^{-1} and UV detection at 260 nm. The HPLC fractions containing product **1b** were combined and concentrated under reduced pressure to yield a yellow, sticky solid. Yield = 45 mg (86%, TFA salt); δ_H (500 MHz, CF_3COOD) 2.40 (br s, 2 H), 2.56 (br s, 10 H), 2.95 (br s, 2 H), 3.24 (s, 3 H), 3.46 (br t, 4 H), 3.65 (br s, 4 H), 3.72–3.86 (br m, 6 H), 5.87 (br t, 2 H), 7.71 (s, 2 H), 8.15 (t, 2 H), 8.57 (t, 2 H), 8.76 (d, 2 H, *J* = 8.6), 8.92 (d, 2 H, *J* = 9.1); δ_C (125 MHz, CF_3COOD) 16.3, 17.7, 20.2, 22.2, 23.2, 37.4, 37.8, 47.4, 48.1, 48.2, 50.0, 59.1, 60.1, 60.9, 118.3, 123.4, 124.9, 127.9, 128.4, 129.5, 130.6, 131.9, 140.5, 141.5, 148.8, 151.5, 171.8; MALDI-TOF MS *m*/*z* 743.2 (M + H)$^+$; HRMS *m*/*z* 743.2786 (M + H)$^+$ (743.2784 calculated).

(e) Compound 3b. A solution of compound **1b** (45 mg, 0.0525 mmol) in methanol (3.6 mL) and water (0.4 mL) was treated with diisopropylethylamine (0.053 mL, 0.303 mmol, 5.8 equivalents) and glutaric anhydride (34.5 mg, 0.303 mmol, 5.8 equivalents). The reaction was stirred at room temperature. After 15 min, HPLC analysis of a small portion of the reaction mixture was performed using a Phenomenex, 10 micron, C_{18} 3.9 mm × 25 cm column and a 30 min gradient of 10 → 70% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. The glutarate derivative was observed eluting at 14 min and was the major component. The solvent was then removed under reduced pressure. The residue was dissolved in DMF (3.6 mL) and water (0.4 mL). This solution was treated with diisopropylethylamine (0.106 mL, 10 equivalents) and TSTU (182 mg, 10 equivalents). The reaction was stirred at room temperature. After 10 min, HPLC analysis showed complete conversion to the product **3b** eluting at 15.3 min. The product was purified by preparative HPLC using a YMC, 10 micron, C_{18} 30 × 250 mm column and 40 min gradient of 10 → 40% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a solvent flow rate of 20 mL min^{-1} and UV detection at 260 nm. The HPLC fractions containing product **3b** were combined, frozen at –80 °C and lyophilized to dryness to a yellow, fluffy powder. Yield = 28.7 mg (50%); δ_H (500 MHz, CF_3COOD) 2.27 (br m, 4 H), 2.44 (br s, 2 H), 2.62 (s, 8 H), 2.71 (br s, 2 H), 2.87 (br s, 2 H), 3.03 (br s, 2 H), 3.13 (s, 4 H), 3.26 (s, 3 H), 3.50 (br s, 2 H), 3.64 (m, 6 H), 3.83 (br s, 4 H), 3.91 (br s, 2 H), 5.94 (br t, 2 H), 7.78 (s, 2 H), 8.21 (t, 2 H), 8.63 (t, 2 H), 8.82 (d, 2 H, *J* = 8.6), 9.00 (d, 2 H, *J* = 8.9); δ_C (125 MHz, CF_3COOD) 16.3, 17.7, 19.8, 20.1, 22.1, 23.1, 24.5, 24.9, 29.1, 31.9, 33.8, 37.4, 47.5, 48.2, 50.1, 60.0, 60.2, 118.3, 123.4, 124.9, 127.9, 128.5, 129.4, 130.6, 131.9, 140.4, 141.5, 148.7, 151.5, 168.8, 173.9, 176.7, 177.5, 180.2; MALDI-TOF MS *m*/*z* 955.2 (M + H)$^+$; HRMS *m*/*z* 954.3274 (M + H)$^+$ (954.3265 calculated).

(f) Theophylline conjugate 2b. A solution of compound **1b** (22 mg, 0.0257 mmol, TFA salt) in DMSO (2 mL) was added to 8-carboxypropyltheophylline (5.5 mg, 0.0207 mmol, Sigma) followed by diisopropylethylamine (0.0088 mL, 0.0505 mmol, 2 equivalents) and (benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (13.7 mg, 0.031 mmol, 1.2 equivalents). The reaction was stirred at room temperature for 16 h and then analyzed by HPLC using a Phenomenex, 10 micron, C_{18} 3.9 mm × 25 cm column and a 40 min gradient of 10 → 40% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. Product was observed eluting at 23.6 min and was the major component. The product was purified by preparative HPLC using a YMC, 10 micron, C_{18} 30 × 250 mm column and 40 min gradient of 10 → 40% B (A = water with 0.05% TFA, B = MeCN with 0.05% TFA) at a solvent flow rate of 20 mL min^{-1} and UV detection at 260 nm. The HPLC fractions containing product **2b** were combined, frozen at –80 °C and lyophilized to dryness to a yellow, fluffy powder. Yield = 7.5 mg (37%); δ_H (500 MHz, CF_3COOD) 2.36 (br s, 2 H), 2.50 (br s, 4 H), 2.62 (br s, 2 H), 2.67 (s, 6 H), 2.79 (br s, 2 H), 3.06 (m, 2 H), 3.30 (s, 3 H), 3.46 (t, 2 H, *J* = 7.8), 3.51 (t, 2 H, *J* = 7.2), 3.65 (br s, 4 H), 3.70 (s, 3 H), 3.81–3.89 (m, 4 H), 3.91 (s, 3 H), 3.94 (t, 2 H, *J* = 6.4), 5.99 (m, 2 H), 7.82 (s, 2 H), 8.25 (t, 2 H), 8.67 (t, 2 H), 8.87 (d, 2 H, *J* = 8.6), 9.04 (d, 2 H, *J* = 9.5); MALDI-TOF MS *m*/*z* 992.0 (M + H)$^+$; HRMS *m*/*z* 991.3680 (M + H)$^+$ (991.3694 calculated).

General procedure for protein labeling with acridinium esters

Three proteins were used for labeling with the acridinium ester labels **3a** and **3b**; a murine anti-TSH monoclonal antibody with an acidic pI = 5.6 (TSH = Thyroid Stimulating Hormone), a murine anti-HBsAg monoclonal antibody with a neutral pI = 7 (HBsAg = Hepatitis B Surface Antigen) and egg white avidin, pI = 10.5. The following is a typical procedure for labeling the three proteins with 10 equivalents input of acridinium ester label.

The anti-TSH murine monoclonal antibody (1 mg, 6.67 nanomoles, stock solution 5 mg mL^{-1}, 0.2 mL) was diluted with 0.2 mL of 0.1 M sodium carbonate, pH 9. The protein solution was treated with DMSO solutions of acridinium esters as follows: for labeling with 10 equivalents of **3a**, 0.0129 mL of a 5 mg mL^{-1} DMSO solution of the compound was added and, for labeling with 10 equivalents of compound **3b**, 0.0127 mL of a 5 mg mL^{-1} DMF solution was added. The labeling reactions were stirred at 4 °C for 16 h and were then transferred to 4 mL Amicon™ filters (MW 30 000 cutoff) and diluted with 3.5 mL de-ionized water.

Table 5 Acridinium ester label incorporation in protein conjugates

Protein/conjugate	Observed mass	Observed Iincrease in mass	# of labels
Unlabeled anti-TSH Mab	151336	—	—
Anti-TSH Mab-**3a**	155238	3902	4.6
Anti-TSH Mab-**3b**	155898	4562	5.4
Unlabeled anti-HBsAg Mab	149947	—	—
Anti-HBsAg Mab-**3a**	153834	3887	4.6
Anti-HBsAg Mab-**3b**	154167	4220	5.0
Unlabeled avidin	63928	—	—
Avidin-**3a**	68024	4096	4.8
Avidin-**3b**	68516	4588	5.5

The volume was reduced to ~0.1 mL by centrifuging at 4000*g* for 10 min. The concentrated conjugate solutions were diluted with 4 mL de-ionized water and centrifuged again to reduce the volume. This process was repeated a total of four times. Finally, the concentrated conjugates were diluted with 0.1 mL de-ionized water. The conjugates were analyzed by MALDI-TOF mass spectrometry, using the Voyager-DE instrument from Perkin–Elmer, to measure acridinium compound incorporation. Typically, this entailed measuring the molecular weight of the unlabeled protein and the labeled protein. The acridinium compound label contributed the observed difference in mass of these two measurements. By knowing the molecular weight of the specific acridinium compound label, the extent of label incorporation of that specific acridinium compound could thus be calculated. Label incorporation in each protein is tabulated in Table 5.

Light emission

Chemiluminescence of acridinium esters was measured on an Autolumat LB953 Plus luminometer from Berthold Technologies. Acridinium esters and their conjugates were diluted for chemiluminescence measurements in an aqueous buffer of 10 mM disodium hydrogen phosphate, 0.15 M NaCl, 8 mM sodium azide, 0.015 mM BSA, pH 8.0. Protein conjugates were serially diluted to a final concentration of 25 pM based on protein concentration measured using the BCA Protein Assay from Pierce. Acridinium ester labels **1a** and **1b** and theophylline conjugates **2a** and **2b** were likewise diluted to a final concentration of 100 pM. A 0.010 mL volume of each diluted acridinium ester sample was dispensed into the bottom of a cuvette, representing the chemiluminescence from 2.5 femtomoles of acridinium ester-labeled protein conjugate and 10 femtomoles of acridinium ester labels **1a** and **1b** and theophylline conjugates **2a** and **2b**. Cuvettes were placed into the primed LB953 and the chemiluminescence reaction was initiated with the sequential addition of 0.3 mL of Reagent 1, a solution of 0.5% hydrogen peroxide in 0.1 M nitric acid followed by the addition of 0.3 mL of Reagent 2, a solution of 0.25 M sodium hydroxide containing surfactant at 5 times its reported critical micelle concentration (CMC) in water. Each chemiluminescence flash curve was measured in 240 intervals of 0.5 s (2 min total time) from the point of chemiluminescence initiation with the addition of surfactant solution in 0.25 M NaOH. Each chemiluminescence reaction was carried out five times, averaged and converted to a percentage of the chemiluminescence accumulated up to each time interval. Chemiluminescence values for 2 min collection times were also normalized for comparison to reactions without surfactant with reactions with the various surfactants. The output from the luminometer instrument was expressed as R.L.U.s (Relative Light Units). For evaluation of surfactants, the surfactant was either omitted or replaced in Reagent 2 with one of the following surfactants at five times its reported critical micelle concentration (CMC) in water.

(a) Cetyltrimethylammonium chloride (CTAC), CMC = 1.4 mM;[22,24]

(b) Cetyltrimethylammonium hydroxide (CTAOH), CMC = 2.3–3.4 mM;[22]

(c) Cetyltripropylammonium chloride (CTPAC), CMC = 0.65 mM[24]

(d) Cetyltributylammonium chloride (CTBAC), CMC = 0.52 mM,[24]

(e) N,N-Dimethyldodecylamine oxide (DDAO), CMC = 2 mM;[25]

(f) N,N-Dimethyldodecylammonio-1,3-propane sulfonate (DDAPS), CMC = 3.17 mM;[36]

(g) Sodium dodecyl sulfate (SDS), CMC = 8 mM;[37]

(h) Triton X-100, CMC = 0.24 mM.[38]

Acknowledgements

We thank Mary Foltz for pI measurements and Drs. Qingping Jiang and Alan Burkhardt for helpful suggestions with the manuscript.

References

1 L. J. Kricka, *Anal. Chim. Acta*, 2003, **500**, 279–286.
2 A. Natrajan, D. Sharpe, J. Costello and Q. Jiang, *Anal. Biochem.*, 2010, **406**, 204–213.
3 S.-J. Law, T. Miller, U. Piran, C. Klukas, S. Chang and J. Unger, *J. Biolumin. Chemilumin.*, 1989, **4**, 88–98.
4 S.-J. Law, C. S. Leventis, A. Natrajan, Q. Jiang, P. B. Connolly, J. P. Kilroy, C. R. McCudden and S. M. Tirrell, *US Pat.* 5,656,426, 1997.
5 (*a*) A. Natrajan, D. Sharpe and Q. Jiang, *US Pat*. 6,664,043 B2, 2003; (*b*) A. Natrajan and D. Wen, *Green Chem.*, 2011, **13**, 913–921.
6 I. Weeks, I. Behesti, F. McCapra, A. K. Campbell and J. S. Woodhead, *Clin. Chem.*, 1983, **29/8**, 1474–1479.
7 S. C. S. Chang and T. E. Miller, *US Pat.* 4,927,769, 1990.
8 F. McCapra, *Acc. Chem. Res.*, 1976, **9/6**, 201–208.
9 F. McCapra, D. Watmore, F. Sumun, A. Patel, I. Beheshti, K. Ramakrishnan and J. Branson, *J. Biolumin. Chemilumin.*, 1989, **4**, 51–58.
10 (*a*) J. Rak, P. Skurski and J. Błażejowski, *J. Org. Chem.*, 1999, **64**, 3002–3008; (*b*) K. Krzymiński, A. Ożóg, P. Malecha, A. D. Roshal, A. Wróblewska, B. Zadykowicz and J. Błażejowski, *J. Org. Chem.*, 2011, **76**, 1072–1085; (*c*) K. Krzymiński, A. D. Roshal, B. Zadykowicz, A. Białk-Bielińska and A. Sieradzan, *J. Phys. Chem. A*, 2010, **114**, 10550–10562.
11 (*a*) F. McCapra, *J. Chem. Soc., Chem. Commun.*, 1977, 946–948; (*b*) F. McCapra, I. Beheshti, A. Burford, R. A. Hann and K. A. Zaklika, *J. Chem. Soc., Chem. Commun.*, 1977, 944–946.
12 G. B. Schuster, *Acc. Chem. Res.*, 1979, **12**, 366–373.
13 J. Lasovský, M. Rypka and J. Slouka, *J. Lumin.*, 1995, **65**, 25–32.
14 N. Dan, M. L. Lau and M. L. Grayeski, *Anal. Chem.*, 1991, **63**, 1766–1771.
15 P. J. Sheridan, *US Pat.* 5,853,974, 1998.
16 F. J. Bagazgoitia, J. L. Garcîa, C. Diéquez, I. Weeks and J. S. Woodhead, *J. Biolumin. Chemilumin.*, 1988, **2**, 121–128.
17 (*a*) J. Ladd, Z. Zhang, S. Chen, J. C. Hower and S. Jiang, *Biomacromolecules*, 2008, **9**, 1357–1361; (*b*) Y. Chang, S. Chen, Z. Zhang and S. Jiang, *Langmuir*, 2006, **22**, 2222–2226; (*c*) Z. Zhang, T. Chao, S. Chen and S. Jiang, *Langmuir*, 2006, **22**, 10072–10077; (*d*) W. K. Cho, B. Kong and I. S. Choi, *Langmuir*, 2007, **23**, 5678–5682; (*e*) G. Cheng, Z. Zhang, S. Chen, J. D. Bryers and S. Jiang, *Biomaterials*, 2007, **28**, 4192–4199;

(*f*) H. Kitano, A. Kawasaki, H. Kawasaki and S. Morokoshi, *J. Colloid Interface Sci.*, 2005, **282**, 340–348.
18 A. T. Marttila, K. J. Airenne, O. H. Laitinen, T. Kulik, E. A. Bayer, M. Wilchek and M. S. Kulomaa, *FEBS Lett.*, 1998, **441**, 313–317.
19 (*a*) C. A. Bunton, F. Nome, F. H. Quina and L. S. Romsted, *Acc. Chem. Res.*, 1991, **24**, 357–364; (*b*) P. Scrimin, P. Tecilla, U. Tonellato and C. A. Bunton, *Colloids Surf., A*, 1998, **144**, 71–79; (*c*) L. S. Romsted, C. A. Bunton and J. Yao, *Curr. Opin. Colloid Interface Sci.*, 1997, **2**, 622–628.
20 (*a*) P. D. Profio, R. Germani, G. Savelli, G. Cerichelli, N. Spreti and C. A. Bunton, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1505–1509; (*b*) G. Cerichelli, L. Luchetti, G. Mancini, M. N. Muzzioli, R. Germani, P. P. Ponti, N. Spreti, G. Savelli and C. A. Bunton, *J. Chem. Soc., Perkin Trans. 2*, 1989, 1081–1085; (*c*) G. Cerichelli, G. Mancini, L. Luchetti, G. Savelli and C. A. Bunton, *J. Phys. Org. Chem.*, 1991, **4**, 71–76; (*d*) G. Cerichelli, L. Luchetti, G. Mancini, G. Savelli and C. A. Bunton, *Langmuir*, 1996, **12**, 2348–2352.
21 (*a*) L. Brinchi, P. D. Profio, R. Germani, G. Savelli and C. A. Bunton, *Langmuir*, 1997, **13**, 4583–4587; (*b*) L. Brinchi, R. Germani, G. Savelli, N. Spreti, R. Ruzziconi and C. A. Bunton, *Langmuir*, 1998, **14**, 2656–2662; (*c*) L. Brinchi, R. Germani, G. Savelli and C. A. Bunton, *J. Phys. Org. Chem.*, 1999, **12**, 890–894.
22 L. Sepúlveda and J. Cortés, *J. Phys. Chem.*, 1985, **89**, 5322–5324.
23 J. Toullec and M. Moukawim, *Chem. Commun.*, 1996, 221–222.
24 R. Bacaloglu, C. A. Bunton and F. Ortega, *J. Phys. Chem.*, 1989, **93**, 1497–1502.
25 A. Natrajan and C. N. Sukenik, *J. Org. Chem.*, 1988, **53**, 3559–3563.
26 C. A. Bunton, M. M. Mhala and J. R. Moffat, *J. Org. Chem.*, 1987, **52**, 3832–3835.
27 V. K. Balakrishnan, E. Buncel and G. W. Vanloon, *Environ. Sci. Technol.*, 2005, **39**, 5824–5830.
28 C. A. Bunton and H. J. Foroudian, *Langmuir*, 1993, **9**, 2832–2835.
29 (*a*) C. Lee and L. A. Singer, *J. Am. Chem. Soc.*, 1980, **102**, 3823–3829; (*b*) A. P. Schaap, T.-S. Chen, R. S. Handley, R. DeSilva and B. P. Giri, *Tetrahedron Lett.*, 1987, **11**, 1155–1158.
30 Q. Jiang, J. Xi, A. Natrajan, D. Sharpe, M. Baumann, R. Hilfiker , E. Schmidt, P. Senn, F. Thommen, A. Waldner, A. Alder and S.-J. Law, *US Pat*. 6,165,800, 2000.
31 (*a*) W. L. Hinze, T. E. Riehl and H. N. Singh, *Anal. Chem.*, 1984, **56**, 2180–2191; (*b*) M. Siegmund and J. Bendig, *Ber. Bunsenge. Phys. Chem.*, 1978, **82**, 1061–1068; (*c*) M. Siegmund and J. Bendig, *Z. Naturforsch*, 1980, **35a**, 1076–1086; (*d*) S. Mory, H.-J. Weigman, A. Rosenfeld, M. Siegmund, R. Mitzner and J. Bendig, *Chem. Phys. Lett.*, 1985, **115**, 201–204.
32 (*a*) E. D. Hughes and C. K. Ingold, *J. Chem. Soc.*, 1935, 244–255; (*b*) E. D. Hughes, *Trans. Faraday Soc.*, 1941, **37**, 603–631; (*c*) E. D. Hughes and C. K. Ingold, *Trans. Faraday Soc.*, 1941, **37**, 657–685; (*d*) K. A. Cooper, M. L. Dhar, E. D. Hughes, C. K. Ingold, B. J. MacNulty and L. I. Woolf, *J. Chem. Soc.*, 1948, 2043–2049.
33 C. A. Bunton and M. J. Minch, *Tetrahedron Lett.*, 1970, **44**, 3881–3884.
34 W. Adam, I. Bronstein, A. V. Trofimov and R. F. Vasil'ev, *J. Am. Chem. Soc.*, 1999, **121**, 958–961.
35 Luiz Francisco, M. L. Ciscato, F. H. Bartoloni, D. Weiss, R. Beckert and W. J. Baader, *J. Org. Chem.*, 2010, **75**, 6574–6580.
36 Y. Wang, X. Huang, Y. Li, J. Wang and Y. Wang, *Colloids Surf., A*, 2009, **333**, 108–114.
37 P. Horowitz, *J. Colloid Interface Sci.*, 1977, **61**, 197–198.
38 (*a*) S. Ross and J. P. Olivier, *J. Phys. Chem.*, 1959, **63**, 1671–1674; (*b*) G. Valdés-Díaz, S. Rodríguez-Calvo, A. Pérez-Gramatges, M. Rapado-Paneque, F. A. Fernandez-Lima, C. R. Ponciano and E. F. da Silveira, *J. Colloid Interface Sci.*, 2007, **311**, 253–261.