RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Synthesis and properties of chemiluminescent acridinium esters with different N-Alkyl groups

Shenliang Wang and Anand Natrajan*

Siemens Healthcare Diagnostics

Advanced Technology and Pre-Development

333 Coney Street

East Walpole, MA 02032

* Author to whom correspondence should be directed.

E-mail: **anand.natrajan@siemens.com**

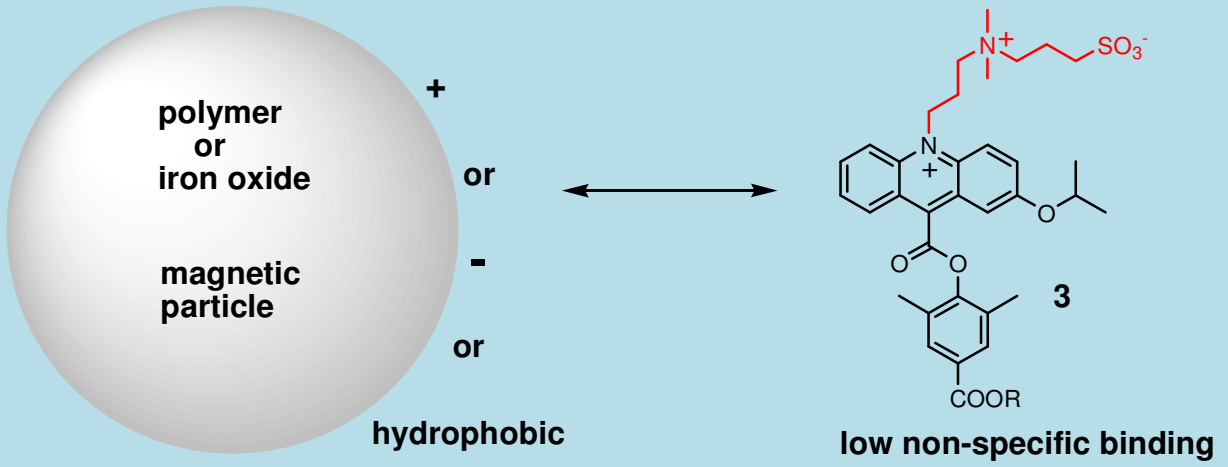
Phone: 1-508-660-4582

Fax: 1-508-660-4591

Abstract

Chemiluminescent acridinium dimethylphenyl esters are highly sensitive labels that are widely used in automated assays for clinical diagnostics. Light emission from these labels and their conjugates is triggered by treatment with alkaline peroxide. In the current study, we provide a comprehensive examination of how polarity of the N-alkyl group in acridinium dimethylphenyl esters and their protein conjugates affect their properties. We describe a new synthetic strategy for the manipulation of the N-sulfopropyl group in acridinium esters which facilitates the attachment a structurally simple sulfobetaine zwitterion to the acridinium nitrogen. Our studies are the first and most detailed to date and they indicate that N-alkyl groups containing charge-neutral sulfobetaines in acridinium dimethylphenyl esters as exemplified by the labels **3** and **6** offer significant advantages over commonly used N-sulfopropyl groups. These include faster emission kinetics, improved chemiluminescence stability and lower non-specific binding to magnetic microparticles with different surfaces. Acridinium ester **3** is a particularly attractive new label because it contains a structurally simple N-alkyl group with a sulfobetaine zwitterion and its synthesis is relatively straightforward from the corresponding N-(3-chloropropyl) substituted acridinium ester precursor.

Graphical Abstract



Acridinium esters containing N-alkyl groups with charge-neutral sulfobetaine zwitterions when compared to N-sulfopropyl groups exhibit faster light emission, improved chemiluminescence stability and lower non-specific binding to different magnetic microparticles.

Introduction

Chemiluminescent acridinium dimethylphenyl esters[1] are highly sensitive labels that are used in automated immunoassays for a wide range of clinically important analytes in Siemens Healthcare Diagnostics' ADVIA Centaur® systems.[1a-d] Light emission from acridinium ester labels and their conjugates is triggered by the sequential addition of two reagents. An initial treatment with 0.1 M nitric acid containing 0.5% hydrogen peroxide converts the non-chemiluminescent water adducts (commonly referred to as pseudobases [1g,h,j,2]) to the chemiluminescent acridinium forms of the labels (Figure 1). Subsequent treatment with 0.25 M sodium hydroxide containing the cationic surfactant cetyltrimethylammonium chloride (CTAC), neutralizes the acid and ionizes the hydrogen peroxide. Hydroperoxide ion addition to C-9 (see Figure 1 for numbering system) is followed by a series of reactions that ultimately lead to excited state acridone (**III** in Figure 1) which is the primary emitter.[3] Dioxetanes and dioxetanones have been proposed as reaction intermediates in the overall reaction pathway leading to chemiluminescence from acridinium esters.[3] In our previous studies, we provided evidence that the surfactant CTAC affects light emission from acridinium esters by two discrete mechanisms.[1e] CTAC micelles provide a high local concentration of hydroperoxide ions at the cationic micellar surface which accelerates light emission from acridinium dimethylphenyl esters and their conjugates. CTAC micelles also provide a low-polarity environment which is conducive to the formation of dioxetanes and/or dioxetanone intermediates which are precursors to excited state acridone.

Acridinium esters are typically synthesized from their acridine ester precursors by quaternization of the acridine nitrogen with alkylating reagents. Because of severe steric hindrance from the periplanar hydrogens at C-4 and C-5 (see Figure 1 for numbering system), N-alkylation of acridine esters is difficult to achieve in good yields especially with complex alkylating reagents. The N-alkylation of acridine esters in variable yields with reagents such as

simple alkyl iodides, benzyl bromide and *tert*-butyl iodoacetate has been reported in the literature.[4] In these reactions, the alkylating reagent was also used as the solvent. More powerful alkylating reagents such as trifluoromethanesulfonates (triflates) have been reported to afford better yields in some cases[5] but in more recent studies[6], reported yields were quite poor for the N-alkylation of different acridine ester precursors using both triflates as well as iodides. In our experience too, with the exception of powerful methylating reagents such methyl triflate or the reagent 1,3-propane sultone, the N-alkylation of acridine dimethylphenyl esters is very difficult to achieve in good yields. Recently, we outlined a three-step strategy to improve the reactivity of acridine esters towards alkylating reagents which entailed reduction of the acridine ester to the more reactive acridan ester followed by N-alkylation of the acridan and subsequent oxidation of the N-alkylated acridan to the N-alkyl acridinium ester.[7]

Chemiluminescent acridinium dimethylphenyl esters with N-methyl groups were first described in the literature as stable chemiluminescent labels suitable for applications in automated immunoassays.[8] These labels are quite hydrophobic and in recent publications, we have described the syntheses and properties of a range of structurally diverse and useful acridinium ester labels with N-sulfopropyl groups and containing other hydrophilic functional groups as well appended either to the phenol or at the acridinium ring.[1] We discovered in our studies that the N-sulfopropyl group can easily be introduced in excellent yields by N-alkylation of a wide range of acridine ester precursors with limited quantities of 1,3-propane sultone using ionic liquids such as 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] and tetrafluoroborate [BMIM]BF_4] as reaction media.[1,9]

Conjugation of acridinium dimethylphenyl esters to biomolecules such as proteins is typically carried out at the phenol which places the acridinium ring furthest from the protein surface and most exposed for interactions with the surrounding environment including serum/blood components as well as with the surfaces of microparticles. Because of the

synthetic constraints outlined above, the N-sulfopropyl group has been commonly used to improve the water solubility and alleviate the hydrophobicity of the acridinium ring. This strategy is quite effective, especially when hydrophilic linkers containing poly(ethylene) glycol or sulfobetaine zwitterions are attached to the phenol in the N-sulfopropyl acridinium ester labels.[1] At physiological pH, which is also typically used for immunoassays, addition of water to C-9 of the N-sulfopropyl acridinium ring (Figure 1) neutralizes the positive charge on the acridinium nitrogen and imparts a net negative charge to N-sulfopropyl acridinium esters. Clearly, the presence of a negative charge in the label can be a drawback because of the potential for charge interactions that can exacerbate both non-specific binding as well as elicit assay interference.

In a recent study, we reported that the N-sulfopropyl group in acridinium esters can be used as a handle for the attachment of other functional groups via the activated N-sulfonylpentafluorophenyl ester.[1h] Using this chemistry, we synthesized a charge-neutral acridinium ester containing an N-alkyl group with a sulfobetaine zwitterion and no net charge.[1h] This acridinium ester exhibited the same high light yield but lower non-specific binding to magnetic microparticles compared to a structurally analogous acridinium ester with an N-sulfopropyl group.[1h] This study illustrated that besides overall water solubility, the charge of the N-alkyl group also plays an important role in affecting the properties of the acridinium ester label.

In the current study, we provide a comprehensive examination of the effect of the polarity of the N-alkyl group in acridinium dimethylphenyl esters and their protein conjugates on their properties including light yield, chemiluminescence stability as well as non-specific binding to four different kinds of magnetic microparticles with different surface compositions and charge. We describe a new synthetic strategy for the manipulation of the N-sulfopropyl group in acridinium esters which facilitates the attachment of other functional groups to the acridinium

nitrogen. Our study is the first and the most detailed to date on how the N-alkyl group in acridinium dimethylphenyl esters affects the properties of these commercially-useful labels and also offers a new paradigm for acridinium ester design.

Results and Discussion

Synthesis of acridinium esters and bovine serum albumin (BSA) conjugates

The structures of acridinium dimethylphenyl ester labels synthesized in the current study are shown in Figure 2. Acridinium ester **1** containing an N-sulfopropyl group and compound **6** containing a charge-neutral N-alkyl group with a sulfobetaine have been described previously[1g,h] and were used as reference compounds. Because we wanted to evaluate only the effects of the polarity of the N-alkyl group in the acridinium ring on the properties of the corresponding acridinium ester labels, to avoid confounding effects we did not add any hydrophilic linkers to the phenols in the new labels **2**-**5**. Additionally, we wanted to ensure that the acridinium rings in the pseudobases of the new labels **2**-**5** would be charge-neutral. Towards that end, only charge-neutral but hydrophilic functional groups namely poly(ethylene) glycol or zwitterions were incorporated in the new labels **3**-**5**. Acridinium esters **2a,b** containing an N-alkyl group with a tertiary amine were also included in the study and these labels were synthesized from a common synthetic intermediate which was also used for sulfobetaine zwitterion-containing labels **3a**,**b**.

The synthetic scheme for acridinium ester labels **2**-**5** is shown in Figure 3 starting from the common intermediate **8** containing an N-(3-chloropropyl) group. Acridinium ester **8** was synthesized from compound **7**, the corresponding N-sulfopropyl acridinium ester.[1g] Conversion of the N-sulfopropyl group in **7** to the sulfonyl chloride was achieved by refluxing in neat thionyl chloride for ≤ 30 minutes. Condensation of this sulfonyl chloride with pentafluorophenol resulted in the formation of **12** as described previously (Figure 3). [1h]. Prolonged heating of **7** in thionyl chloride for ≥ 2 hours resulted in cleavage of the sulfonate moiety and formation of the N-(3-

chloropropyl) acridinium ester **8**. Presumably, **8** is formed from the initially formed sulfonyl chloride by displacement with chloride ion. Manipulation of the easily introduced N-sulfopropyl group in **7** to either give the sulfonyl chloride or the N-(3-chloropropyl) derivative **8**, expands the potential for possible chemical transformations that can be carried out at the acridinium nitrogen as illustrated in Figure 3. As mentioned earlier, alkylation of the acridine nitrogen with complex or bulky alkylating reagents is remarkably challenging. For example in our previous studies, our attempts to directly introduce the N-(3-chloropropyl) or N-(3-bromopropyl) group by N-alkylation of acridine ester precursors with either 3-chloropropyl triflate or 3-bromopropyl triflate met with no success (unpublished data).

Displacement of the chloro group in **8** directly with dimethylamine or the hexa(ethylene) glycol derivative **ii**[10b] was unsuccessful but treatment of **8** with potassium iodide lead to the unstable but more reactive N-(3-iodopropyl) acridinium ester which was used without purification after a simple filtration to remove salts. In the presence of silver triflate (AgOTf), this N-(3-iodopropyl) acridinium ester reacted readily with dimethylamine and **ii** to give the acridinium esters **9** and **11** respectively. We had originally envisaged using commercially-available hexa(ethylene) glycol monomethyl ether instead of amine **ii** to establish an ether linkage but despite the increased reactivity of the N-(3-iodopropyl) acridinium ester derivative, little reaction was observed with the hydroxyl group of the former. Conversion of compound **9** to the final targets **3a**,**b** was accomplished by first N-alkylation of the tertiary amine in **9** with 1,3-propane sultone in [BMIM][PF_6] to give compound **10**. Acid hydrolysis of the methyl ester in **10** gave compound **3a** which was converted to the N-hydroxysuccinimde (NHS) ester **3b** using standard chemistry. In a similar vein, compound **11**, with a methylamino hexa(ethylene) glycol moiety attached to the acridinium nitrogen was subjected to acid hydrolysis to give acridinium ester **2a** which was converted to the NHS ester **2b**.

Synthesis of labels **5a**,**b** was accomplished from the pentafluorophenyl ester **12**. The pentafluorophenyl ester in **12** was easily displaced by treatment with the hexa(ethylene) glycol derivative **i**[10] in refluxing acetonitrile which gave compound **13**. Acid hydrolysis of the methyl ester in **13** gave compound **5a** which was converted to the NHS ester **5b**. Yields of intermediates and final compounds illustrated in Figure 3 were quite good as shown in the figure legend with the exception of the displacement reaction involving compound **ii** where the yield was more modest (37%). HPLC traces, NMR and high resolution mass spectra of all intermediates and final compounds are shown in Figures S1-S13 (supplementary material).

Bovine serum albumin (BSA) conjugates were prepared by treatment of the protein with ten equivalents each of the various acridinium ester labels **1b**-**6b** (Table 1) as described in the experimental section. Label incorporation was measured by mass spectroscopy and was quite similar for the different labels leading to the incorporation of 5-7 labels in the protein.

pH Titrations and chemiluminescence measurements

pH Titrations of acridinium ester labels **1a**-**6a** illustrating conversion of the acridinium forms of the labels to their respective pseudobases (**I** in Figure 1) are shown in Figure 4, panel A. The carboxylic acids **1a**-**6a** were used for these pH titrations to ensure adequate aqueous solubility in buffer. Briefly, as described in our previous studies, the C-2-isopropoxy substituted acridinium ring exhibits a long wavelength absorption band at 384 nm.[1g] As the pH is raised, formation of the pseudobase disrupts conjugation in the acridinium ring which results in the loss of this absorption band. For labels **2a**-**6a** which have bulkier N-alkyl groups compared to **1a** which has an N-sulfopropyl group, pKa for pseudobase formation was shifted to acidic pH in the range 3.5-4.0 as shown in Figure 4, panel A. The greater steric requirements of the bulkier substituents in compounds **2a**-**6a** presumably favor pseudobase formation which should relieve the steric congestion caused by these substituents in the planar acridinium ring.[1h]

Structures of the various pseudobases corresponding to acridinium ester labels **1a**-**6a** are also shown in Figure 4, panel B. An examination of these structures highlights important differences in the properties (charge and hydrogen bonding capabilities) of the various N-alkyl groups in the central rings (pseudobase of acridinium ring) of the labels. The reference compound **1a** has an N-sulfopropyl group and consequently the central ring contains a negatively charged sulfonate moiety. Pseudobases of labels **2a**-**6a** have charge-neutral central rings but with different polar substituents and different capabilities for hydrogen bonding. Acridinium ester **2a** contains only a tertiary amine which can be both a hydrogen bond acceptor and/or carry a positive charge when protonated. On the other hand **3a** has a charge-neutral sulfobetaine zwitterion which is neither a hydrogen bond donor nor an acceptor. Acridinium ester **4a** is similar to **2a** but also has the ability accept additional hydrogen bonds due to the presence of the poly(ethylene) glycol chain. The acridinium esters with sulfonamide linkages namely **5a** and **6a** are hydrogen bond donors due to the sulfonamide nitrogen. In addition, **5a** contains a poly(ethylene) glycol chain that can also accept hydrogen bonds. Acridinium ester **6a**, also a reference compound, has a polar charge-neutral sulfobetaine zwitterion.[1h]

Chemiluminescence measurements of the acridinium ester labels **1a**-**6a** were carried out using a luminometer and a spectral camera as described in the experimental section. Similarly, BSA conjugates of the acridinium ester labels **1b**-**6b** (NHS esters corresponding to **1a**-**6a**) were used for chemiluminescence measurements.

For chemiluminescence measurements of the acridinium ester labels, **1a**-**6a** (precursors of the NHS esters **1b**-**6b**) were dissolved in dimethyl sulfoxide (~ 0.5 mg/mL) and these solutions were serially diluted to nanomolar concentrations in buffer. Chemiluminescence from 0.01 mL samples were initiated by the addition of 0.3 mL of 0.1 M nitric acid containing 0.5% hydrogen peroxide followed by the instantaneous addition of 0.25 M sodium hydroxide with or without CTAC (7 mM). In the presence of CTAC, emission was fast (similar to what we reported

previously[1e]) and light was collected for a period of 10 seconds integrated at 0.1 second intervals (five replicates). Chemiluminescence measurements of protein conjugates were carried out in a similar manner. Aqueous solutions of BSA conjugates were serially diluted to nanomolar concentrations and chemiluminescence from 0.01 mL samples were measured as described above. Additional details can be found in the experimental section.

Emission wavelength maxima of the acridinium ester labels **1a**-**6a** are shown in Table 2 and the complete emission spectra of the labels are shown in Figures S14a-f (supplementary material). All compounds exhibited very similar emission spectra and emission wavelength maxima in the 452-456 nm range (Table 2). These results are consistent with our earlier observations that showed that only the electronic properties of the acridinium ring and not the nature of the acridinium N-alkyl group influence emission spectra of acridinium esters.[1d,h]

Emission profiles of the acridinium ester labels **1a**-**6a** and their BSA conjugates are shown in Figure 5, panels A and B respectively. Both the free labels **1a**-**6a** as well as their BSA conjugates showed fast emission although some differences were noted among the different labels and conjugates (Figure 5 and Table 3). Fastest emission times were observed with the labels **3a** and **6a** containing sulfobetaine zwitterions in the acridinium ring (Table 3) with ≥ 95% emission in < 5 seconds. Acridinium ester labels **4a** and **5a** with poly(ethylene) glycol chains exhibited relatively slower emission with time to 95% emission in < 7 seconds which was similar to the reference compound **1a** with an N-sulfopropyl group. Emissive rates of the BSA conjugates, for the most part, closely paralleled those of the free labels **1a**-**6a** (Table 3).

In addition to emission kinetics, we also measured light yield (specific chemiluminescent **a**ctivity, SCA) of the labels and the conjugates (Table 3). The two acridinium ester labels **2a** and **4a** and their corresponding BSA conjugates (derived from NHS esters **2b** and **4b**) showed depressed light output compared to the reference compounds **1a** and **6a** and their BSA conjugates. For example specific activity of **2a** and **4a** were observed to be 2.5 and 1.5 (units of

10^{19} RLUs/mole, RLU = Relative Light Unit) which was significantly lower than that of the reference compounds **1a** and **6a** whose specific activities were 4.0 and 7.8 respectively. The lower specific activity of **2a** and **4a** and their BSA conjugates probably results from quenching of excited state acridone by electron transfer from the tertiary amines in these compounds. Similar quenching is well known for fluorescent compounds.[12] In the absence of such quenching, new acridinium ester labels **3a**, and **5a** showed specific activities of 4.5 and 5.6 respectively which was quite comparable to **1a** and **6a**. The specific activities of the BSA conjugates of these acridinium esters were also comparable with specific activities of 5.6, 3.7, 6.4 and 5.7 respectively for **1b**, **3b**, **5b** and **6b**. The higher specific activity for **5a** and **6a** as well as their BSA conjugates may reflect the presence of more hydrophobic acridinium rings in these compounds resulting in more effective partitioning into CTAC micelles.[1g]

Non-specific binding and chemiluminescence stability

Non-specific binding of the acridinium ester labels **1a**-**6a** as well their BSA conjugates of these labels (**1b**-**6b**) to four different kinds of particles was measured at neutral pH as described in the experimental section. Results are shown in Figure 6 and tables S1 and S2 (supplementary material). At neutral pH, the various labels exist primarily as the pseudobase (Figure 4, panel B). Magnetic microparticles with different surface functional groups were selected as represented by commercially-available[11] M-270 (amino surface), M-270 (carboxylate surface) and M-280 (hydrophobic surface) particles. These three magnetic particles are derived from a polymer core with different surface functional groups for the attachment of various biomolecules. (In the case of M-280 particles, surface tosylate groups were hydrolyzed to hydroxyl groups prior to use). In addition to these three microparticles, in house paramagnetic particles (PMPs) derived from iron oxide (Fe_3O_4) with an amine functionalized surface were also included in the study. By screening non-specific binding of the various acridinium ester labels **1a**-**6a** and their BSA conjugates to these four magnetic particles

with different surface functional groups and/or core structures (PMPs), we wanted to determine whether we could (a) discern differences in the interaction of the central rings (pseudobase of acridinium ring) of the various labels to these different particles and, (b) determine whether there was an optimal structure of an N-alkyl group in an acridinium ester for low non-specific binding across different types of magnetic microparticles.

The protocol for measurement of non-specific binding is described in the experimental section and entailed measuring residual chemiluminescence associated with the magnetic microparticles after a wash step. This residual chemiluminescence (RLUs) divided by the total chemiluminescence input is defined here as fractional non-specific binding (fNSB) and reflects both, resistance to non-specific binding as well as efficacy of the wash step.

Fractional non-specific binding of the acridinium ester labels **1a**-**6a** and their BSA conjugates measured using the above described protocol are shown in Figure 6, panels A and panel B respectively. For all labels **1a**-**6a**, fNSB was observed to be the highest for M-270 particles with amine surfaces, lowest for the same particles with carboxylate surfaces, with the M-280 hydrophobic particles and PMPs falling somewhere in between. These results are anticipated because of the presence of the phenolic carboxylates in the labels **1a**-**6a**. Of greater interest were the observed differences in the fNSB values of the different N-alkyl-substituted central rings in the labels **1a**-**6a** to the four particles. In the case of **1a**, the presence of an additional negative charge in the pseudobase exacerbated non-specific binding to particles with amine surfaces (M-270 amino surface and PMPs) whereas this label showed low fNSB to particles with carboxylate surfaces (M-270 carboxylate surfaces) and to hydrophobic M-280 particles. These observations suggest that N-sulfopropyl acridinium esters, *in the absence of additional hydrophilic functional groups in the phenol,* are best suited for use as labels with these two types of particles. In contrast, labels **2a** and **4a** with tertiary amines in their N-alkyl groups showed higher fNSB to carboxylate particles, lower fNSB to particles with amine surfaces and comparable fNSB as **1a** to M-280 particles which has a hydrophobic surface.

These results are also expected because of the presence of a positive charge in the partially protonated tertiary amines in **2a** and **4a**. Surprisingly, label **5a** which has an N-alkyl group containing a sulfonamide linkage to methoxy hexa(ethylene) glycol, showed high fNSB to all particles with the exception of PMPs defying generally accepted wisdom that poly(ethylene) glycol confers resistance to non-specific binding. Labels **3a** and **6a** with sulfobetaine zwitterions in their N-alkyl groups showed the lowest fNSB to all four particles thereby demonstrating that these types of polar functional groups are optimal for alleviating the hydrophobicity of the central ring (pseudobase of acridinium ring) without introducing any overall charge to the acridinium ester label. Clearly, the relatively low non-specific binding observed for **6a** suggests that the sulfonamide linkage with its ability to donate a hydrogen bond does not play a significant role in affecting non-specific binding.

BSA conjugates of the labels **1b**-**6b** (Figure 6, panel B) showed similar trends with differing fNSB values as a function of charge of the label and charge on the particle surface as described above for the free labels **1a**-**6a**. The conjugate of label **5b,** containing an N-alkyl group with a sulfonamide linkage to methoxy hexa(ethylene) glycol, once again exhibited high fNSB particularly to hydrophobic M-280 particles and M-270 particles with amine surfaces. Once again, BSA conjugates of labels **3b** and **6b** with sulfobetaine zwitterions in their N-alkyl groups exhibited the lowest fNSB values to all four particles.

Finally, we evaluated another important property of acridinium ester labels which is their chemiluminescence stability (Figure S15, supplementary material). Besides low non-specific binding, which is important for assay performance, chemiluminescence stability is also extremely important both for maintaining long reagent shelf life as well as for preserving assay performance characteristics such as stable dose response curves and assay sensitivity. Figure S15 illustrates the chemiluminescence stability of BSA conjugates of labels **1b**-**6b** at 37^{o}C in buffer at pH = 7.4 over 30 days. The conjugates of the acridinium esters were diluted into phosphate buffer pH = 7.4 and were stored at 37^{o}C protected from light. Chemiluminescence

from 0.01 mL samples was measured periodically (five replicates) and the observed RLUs were averaged and converted to percentages. The initial observed RLUs at the first time point (day 1) was assigned a value of 100% and residual chemiluminescence as a percentage of this initial value reflects the chemiluminescence stability. As can be noted from Figure S15, in general, increased bulk of the N-alkyl group in the acridinium ring led to improved stability. This is because labels **2a**-**6a** form pseudobases at lower pH (Figure 4, panel A) which is conducive to improved stability.[1d,h,j] Conjugates of the two labels **3b** and **6b** with zwitterions in their N-alkyl groups showed comparable and improved stability compared to the conjugate of the N-sulfopropyl acridinium ester **1b**.

Conclusions

In the current study we have described the syntheses of acridinium dimethylphenyl esters with various hydrophilic N-alkyl groups in the acridinium ring using new synthetic protocols. Functionalization of the acridine nitrogen is extremely challenging due to severe steric hindrance and our synthetic approaches should be useful for the assembly of other similar structures. Our studies are the first and most detailed to date on how polarity of the N-alkyl group affect the properties of these commercially-useful chemiluminescent labels and they indicate that N-alkyl groups containing charge-neutral sulfobetaines as exemplified by the labels **3** and **6** offer significant advantages over commonly used N-sulfopropyl groups. These include faster emission kinetics, improved chemiluminescence stability and lower non-specific binding to magnetic microparticles with different surfaces. Acridinium ester **3** is a particularly attractive new chemiluminescent label because it contains a structurally simpler N-alkyl group with a sulfobetaine zwitterion compared to **6** and its synthesis is relatively straightforward from the corresponding N-(3-chloropropyl) substituted acridinium ester precursor.

Acknowledgements

We thank Mr. Richard Wright for NMR spectra.

Experimental

General

All chemicals and solvents were purchased from Sigma-Aldrich and were used without further purification. HPLC was performed on a Beckman-Coulter System. Analytical HPLC was performed with a Phenomenex, Kinetex, C_{18}, 5 micron, 150 x 4.6 mm column and using a 20 minute gradient of 10% → 100% acetonitrile/water (each with 0.05% trifluoroacetic acid, TFA) at a flow rate of 1 mL/min and UV detection at 260 nm. MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) mass spectroscopy was performed on Bruker Microflex mass spectrometer using cyano-4-hydroxycinnamic acid as matrix for small molecules and sinapinic acid for BSA conjugates. ^{1}H-NMR spectra were recorded on a Bruker AVANC III HD 600 MHz High Performance Digital NMR spectrometer. High-resolution mass spectrometry (HRMS) was performed on a Waters Qtof API US instrument in the positive ion mode. Absorbance spectra were obtained on a Cary *100 Bio* UV-Visible spectrophotometer. Preparative HPLC was performed using a YMC, C_{18}, 10 micron, 250 x 30 mm column.

1. Synthesis of acridinium esters

Compound **8**

A solution of compound **7** (500 mg, 0.88 mmole) in thionyl chloride (5 mL) was heated under argon at reflux for 5 hours. The volatiles were evaporated under reduced pressure and the residue was washed with anhydrous hexanes. The reaction was monitored by analytical HPLC until the disappearance of compound **7** (retention time at 11.1 minutes). The product **8** was purified by preparative HPLC (40 minute gradient of 10% → 85% acetonitrile/water each with 0.05% TFA) and the HPLC fractions were frozen at -80°C and lyophilized to dryness. Product **8** was recovered as a light yellow powder. Yield = 382 mg (83%). ^{1}H-NMR (CF_3COOD) δ 1.55 (d, J = 6.1 Hz, 6H), 2.59 (s, 6H), 2.80 (m, 2H), 3.97 (m, 2H), 4.12 (s, 3H), 4.95 (p, 1H), 5.72 (m, 2H), 7.91 (d, J = 2.7 Hz, 1H), 8.04 (s, 2H), 8.14 (dd, J = 8.8, 6.8 Hz, 1H), 8.23 (dd, J =

9.9, 2.7 Hz, 1H), 8.47 (m, 1H), 8.72 (m, 2H), 8.77 (m, 1H). MALDI-TOF MS *m/z* $[M]^+$ 520.339; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{30}H_{31}ClNO_5$, 520.1891; found 520.1895.

Compound **9**

A solution of compound **8** (300 mg, 0.57 mmole) and potassium iodide (1.0 g, 6.0 mmole) in anhydrous acetone (50 mL) was heated at reflux for 16 hours. The reaction was monitored by analytical HPLC until the disappearance of compound **8** (retention time at 14.4 minutes). After cooling to room temperature, the solvent was evaporated under reduced pressure and residue was dissolved in anhydrous dichloromethane and filtered. The filtrate was concentrated under reduced pressure and the crude N-(3-iodopropyl) acridinium ester intermediate and was used as is in the following step without further purification. MALDI-TOF MS *m/z* $[M]^+$ 612.067.

To the solution of the crude N-(3-iodopropyl) acridinium ester intermediate (100 mg, 0.16 mmole) in acetonitrile (10 mL) under argon, dimethylamine in THF (2 M, 0.246 mL) was added via syringe, followed by a solution of silver triflate (150 mg, 0.58 mmole) in acetonitrile (10 mL). The reaction was stirred at room temperature in the dark for 48 hours and quenched with 1 mL of trifluoroacetic acid. The solution was filtered and concentrated under reduced pressure. The product was purified by preparative HPLC (40 minute gradient of 10% → 70% acetonitrile/water each with 0.05% TFA) and the HPLC fractions were frozen at -80°C and lyophilized to dryness. Product **9** was recovered as a red powder Yield = 54 mg (64%). ^{1}H-NMR (CF_3COOD) δ 1.51 (d, J = 6.1 Hz, 6H), 2.55 (s, 6H), 2.88 (m, 2H), 3.13 (s, 6H), 3.84 (m, 2H), 4.08 (s, 3H), 4.92 (p, 1H), 5.61 (m, 2H), 7.88 (d, J = 2.7 Hz, 1H), 8.00 (s, 2H), 8.10 (dd, J = 8.8, 6.8 Hz, 1H), 8.19 (dd, J = 9.9, 2.7 Hz, 1H), 8.43 (m, 1H), 8.57 (m, 2H), 8.74 (d, J = 8.6 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 529.393; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{32}H_{37}N_2O_5$, 529.2703; found 529.2706.

Compound **2a**

A solution of compound **9** (16 mg, 0.030 mmole) in acetonitrile (1 mL) and 2 M HCl (10 mL) was heated at 100°C for 6 hours. The reaction was monitored by analytical HPLC until the disappearance of compound **9** (retention time at 10.1 minutes). The solution was concentrated to a small volume under reduced pressure and purified by preparative HPLC (40 minute gradient of 10% → 55% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness. Acridinium ester **2a** was recovered as a red powder. Yield = 14 mg (90%). ^{1}H-NMR (CF_3COOD) δ 1.57 (d, *J* = 6.1 Hz, 6H), 2.61 (s, 6H), 2.94 (m, 2H), 3.18 (s, 6H), 3.89 (m, 2H), 4.97 (p, 1H), 5.66 (m, 2H), 7.94 (d, *J* = 2.6 Hz, 1H), 8.12 (s, 2H), 8.15 (m,1H), 8.24 (dd, *J* = 9.9, 2.7 Hz,1H), 8.48 (m, 1H), 8.63 (dd, *J* = 10.1, 2.8 Hz , 2H), 8.80 (d, *J* = 8.7 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 515.208; HRMS (ESI, *m/z*) $[M+H]^+$ calculated for $C_{31}H_{35}N_2O_5$, 515.2540; found 515.2534.

Compound **2b**

To a solution of compound **2a** (7 mg, 0.013 mmole) and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU) (25 mg, 0.083 mmole) in anhydrous DMF (3 mL) under argon, diisopropylethylamine (DIPEA, 0.050 mL, 0.29 mmole) was added using a syringe. The reaction was stirred at room temperature protected from light for 20 minutes. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material eluting at 8.4 minutes to product eluting at 9.4 minutes. The product was purified by preparative HPLC (40 minute gradient of 10% → 60% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness. Yield = 6 mg (72%). ^{1}H-NMR (CF_3COOD) δ 1.55 (d, *J* = 6.1 Hz, 6H), 2.60 (s, 6H), 2.92 (m, 2H), 3.15 (s, 6H), 3.18 (s, 4H), 3.87 (m, 2H), 4.93 (p, 1H), 5.64 (t, 2H), 7.90 (d, *J* = 2.5 Hz, 1H), 8.10 (s, 2H), 8.15 (m, 1H), 8.22 (dd, *J* = 9.9, 2.6 Hz ,1H), 8.46 (m, 1H), 8.61 (d, *J* = 9.8 Hz, 2H), 8.77 (d, *J* = 8.7 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 611.488; HRMS (ESI, *m/z*) $[M+H]^+$ calculated for $C_{35}H_{38}N_3O_7$, 612.2704; found 612.2725.

Compound **10**

Compound **9** (50 mg, 0.094 mmole), 1,3-propane sultone (86 mg, 0.70 mmole), 2,6-di-*tert*-butyl-pyridine (0.315 mL, 1.40 mmole) and [BMIM][PF_6] (0.400 mL) were heated in a sealed tube at 155°C for 16 hours. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material eluting at 10.1 minutes to product eluting at 9.9 minutes. The product was purified by HPLC (40 minute gradient of 10% → 60% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a yellow powder. Yield = 37 mg (60%). ^{1}H-NMR (CF_3COOD) δ 1.52 (d, *J* = 6.1 Hz, 6H), 2.52 (s, 6H), 2.64 (m, 2H), 2.96 (m, 2H), 3.29 (s, 6H), 3.44 (m, 2H), 3.82 (m, 2H), 4.07 (m, 5H), 4.91 (m, 1H), 5.61 (t, *J* = 8.4 Hz, 2H), 7.89 (d, *J* = 2.7 Hz, 1H), 8.00 (s, 2H), 8.12 (m, 1H), 8.23 (dd, *J* = 9.9, 2.7 Hz, 1H), 8.46 (m, 1H), 8.62 (dd, *J* = 9.7, 6.2 Hz, 2H), 8.75 (d, *J* = 8.7 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 651.054; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{35}H_{43}N_2O_8S$, 651.2735; found 651.2740.

Compound **3a**

A solution of compound **10** (30 mg, 0.046 mmole) in acetonitrile (1 mL) and 2 M HCl (10 mL) was heated at 100°C for 6 hours. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material eluting at 9.9 minutes to product eluting at 9.0 minutes. The solution was concentrated to a small volume under reduced pressure and purified by HPLC (40 minute gradient of 10% → 55% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give an orange powder. Yield = 26 mg (90%). ^{1}H-NMR (CF_3COOD) δ 1.55 (d, *J* = 6.1 Hz, 6H), 2.60 (s, 6H), 2.67 (m, 2H), 3.00 (m, 2H), 3.35 (s, 6H), 3.47 (m, 2H), 3.89 (m, 2H), 4.11 (m, 2H), 4.96 (p, 1H), 5.66 (m, 2H), 7.93 (d, *J* = 2.6 Hz, 1H), 8.11 (s, 2H), 8.15 (m, 1H), 8.25 (dd, *J* = 9.9, 2.6 Hz, 1H), 8.49 (m, 1H), 8.66 (m, 2H), 8.78 (d, *J* = 7.8 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 637.288; HRMS (ESI, *m/z*): $[M]^+$ calculated for $C_{34}H_{41}N_2O_8S$, 637.2578; found 637.2416.

Compound **3b**

To a solution of **3a** (10 mg, 0.015 mmole) and TSTU (25 mg, 0.083 mmole) in anhydrous DMF (3 mL) under argon was added DIPEA (0.050 mL, 0.29 mmole) using a syringe. The reaction was stirred at room temperature protected from light for 20 minutes. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material eluting at 9.0 minutes to product eluting at 9.2 minutes. The product was purified by preparative HPLC (40 minute gradient of 10% → 60% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give an orange powder. Yield = 8 mg (75%). ^{1}H-NMR (CF_3COOD) δ 1.55 (d, J = 6.0 Hz, 6H), 2.59 (s, 6H), 2.66 (m, 2H), 2.99 (m, 2H), 3.17 (s, 4H), 3.34 (s, 6H), 3.46 (m, 2H), 3.88 (m, 2H), 4.09 (m, 2H), 4.93 (p, 1H), 5.65 (m, 2H), 7.90 (s, 1H), 8.10 (s, 2H), 8.14 (m, 1H), 8.24 (d, J = 9.9 Hz, 1H), 8.48 (m, 1H), 8.65 (m, 2H), 8.77 (d, J = 8.7 Hz, 1H). MALDI-TOF MS *m/z*: $[M]^+$ 734.171; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{38}H_{44}N_3O_{10}S$, 734.2742; found 734.2891.

Compound **11**

A solution of compound **8** (100 mg, 0.19 mmole) and potassium iodide (300 mg, 2.0 mmole) in anhydrous acetone (20 mL) was heated at reflux for 16 hours. The reaction was monitored by analytical HPLC until the disappearance of compound **8** eluting at 14.4 minutes. After cooling to room temperature, the solvent was evaporated under reduced pressure and residue was dissolved in anhydrous dichloromethane and filtered. This solution was evaporated to dryness and the crude N-(3-iodopropyl) acridinium ester was used as is in the following step without further purification. To the solution of previous product in DMF (6 mL) under argon, compound **ii**[10], (100 mg, 0.32 mmole) was added via syringe, followed by DIPEA (0.2 mL, 1.16 mmole). The reaction was heated at 65°C protected from light for 6 hours (the reaction was monitored by analytical HPLC until the disappearance of peak for starting material at 15.0 minutes) and then quenched with 1 mL of trifluoroacetic acid. The reaction solution was

concentrated under reduced pressure and the product was purified by preparative HPLC (40 minute gradient of 10% → 70% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness and the product was recovered as a brown oil. Yield = 47mg (37%). ^{1}H-NMR (CF_3COOD) δ 1.56 (d, *J* = 6.0 Hz, 6H), 2.59 (s, 6H), 2.96 (m, 2H), 3.19 (s, 3H), 3.61 (s, 3H), 3.91 (m, 4H), 3.94 (m, 20H), 4.04 (m, 2H), 4.13 (s, 3H), 4.96 (p, 1H), 5.66 (m, 2H), 7.93 (d, *J* = 2.7 Hz, 1H), 8.05 (s, 2H), 8.16 (m, 1H), 8.24 (dd, *J* = 9.9, 2.6 Hz, 1H), 8.47 (m, 1H), 8.63 (d, *J* = 9.7 Hz, 2H), 8.80 (d, *J* = 8.7 Hz, 1H). MALDI-TOF MS *m/z*: $[M]^+$ 793.520; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{44}H_{61}N_2O_{11}$, 793.4270; found 793.4296.

Compound **4a**

A solution of compound **11** (30 mg, 0.037 mmole) in acetonitrile (1 mL) and 2 M HCl (10 mL) was heated at 100°C for 6 hours. The reaction was monitored by analytical HPLC which indicated conversion of starting material eluting at 10.7 minutes to product eluting at 9.2 minutes. The solution was concentrated under reduced pressure and purified by HPLC (40 minute gradient of 10% → 55% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a brown oil. Yield = 26 mg (90%). ^{1}H-NMR (CF_3COOD) δ 1.56 (d, *J* = 6.1 Hz, 6H), 2.60 (s, 6H), 2.95 (m, 2H), 3.19 (s, 3H), 3.60 (s, 3H), 3.90 (m, 4H), 3.96 (m, 20H), 4.04 (m, 2H), 4.96 (p, 1H), 5.66 (m, 2H), 7.93 (d, *J* = 2.6 Hz, 1H), 8.11 (s, 2H), 8.16 (m, 1H), 8.23 (dd, *J* = 10.0, 2.6 Hz, 1H), 8.47 (m, 1H), 8.63 (d, *J* = 10.1 Hz, 2H), 8.79 (d, *J* = 7.8 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 779.291; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{43}H_{59}N_2O_{11}$, 779.4113; found 779.4128.

Compound **4b**

To a solution of compound **4a** (15 mg, 0.019 mmole) and TSTU (25 mg, 0.083 mmole) in anhydrous DMF (3 mL) under argon was added DIPEA (0.050 mL, 0.29 mmol) via a syringe. The reaction was stirred at room temperature protected from light for 20 minutes. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material

eluting at 9.1 minutes to product eluting at 10.1 minutes. The product was purified by preparative HPLC (40 minute gradient of 10% → 60% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a brown oil. Yield = 8 mg (48%). ^{1}H-NMR (CF_3COOD) δ 1.57 (d, *J* = 6.1 Hz, 6H), 2.62 (s, 6H), 2.96 (m, 2H), 3.20 (s, 7H), 3.61 (s, 3H), 3.88 (m, 4H), 3.97 (m, 20H), 4.04 (m, 2H), 4.95 (p, 1H), 5.67 (m, 2H), 7.92 (d, *J* = 2.5 Hz, 1H), 8.12 (s, 2H), 8.17 (m, 1H), 8.24 (dd, *J* = 9.8, 2.5 Hz, 1H), 8.47 (m, 1H), 8.63 (d, *J* = 9.8 Hz, 2H), 8.79 (d, *J* = 8.4 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 876.485; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{47}H_{62}N_3O_{13}$, 876.4277; found 876.4423.

Compound **13**

To a solution of compound **12**[1h] (124 mg, 0.17 mmole) in DMF (5 mL), compound **i**[10] (50 mg, 0.17 mmole) and DIPEA (0.10 mL, 0.58 mmole) in DMF (1 mL) was added. The reaction was heated at 65°C for 16 hours. The reaction was monitored by analytical HPLC until the disappearance of compound **12** (retention time at 14.3 minutes). The product was purified by preparative HPLC (40 minute gradient of 10% → 70% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a red oil. Yield = 65 mg (45%). ^{1}H-NMR (CF_3COOD) δ 1.53 (d, *J* = 6.1 Hz, 6H), 2.55 (s, 6H), 2.88 (m, 2H), 3.57 (m, 5H), 3.78 (m, 2H), 3.87 (m, 2H), 3.94 (m, 20H), 4.09 (s, 3H), 4.92 (m, 1H), 5.72 (m, 2H), 7.88 (d, *J* = 2.6 Hz, 1H), 8.01 (s, 2H), 8.11 (dd, *J* = 8.8, 6.7 Hz, 1H), 8.22 (dd, *J* = 10.0, 2.7 Hz, 1H), 8.45 (m, 1H), 8.68 (d, *J* = 9.6 Hz, 2H), 8.74 (dd, *J* = 8.8, 1.2 Hz, 1H). MALDI-TOF MS *m/z* [M]+ 843.430; HRMS (ESI, *m/z*) [M]+ calculated for $C_{43}H_{59}N_2O_{13}S$, 843.3738; found 843.3741.

Compound **5a**

A solution of compound **13** (50 mg, 0.059 mmole) in acetonitrile (1 mL) and 2 M HCl (10 mL) was heated at 100°C for 6 hours. The reaction was monitored by analytical HPLC which indicated conversion of starting material eluting at 13.1 minutes to product eluting at 11.3 minutes. The solution was concentrated under reduced pressure and purified by HPLC (40

minute gradient of 10% → 65% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a red oil. Yield = 40 mg (82%). ^{1}H-NMR (CF_3COOD) δ 1.56 (d, *J* = 6.1 Hz, 6H), 2.60 (s, 6H), 2.92 (m, 2H), 3.61 (m, 5H), 3.81 (m, 2H), 3.90 (m, 2H), 3.97 (m, 20H), 4.96 (p, 1H), 5.77 (m, 2H), 7.92 (d, *J* = 2.6 Hz, 6H), 8.11 (s, 2H), 8.15 (m, 1H), 8.25 (dd, *J* = 10.0, 2.6 Hz, 1H), 8.48 (m, 1H), 8.72 (d, *J* = 10.1 Hz, 1H), 8.78 (d, *J* = 8.8 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 829.467; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{42}H_{57}N_2O_{13}S$, 829.3576; found 829.3599.

Compound **5b**

To a solution of compound **5a** (15 mg, 0.018 mmole) and TSTU (25 mg, 0.083 mmole) in anhydrous DMF (3 mL) under argon was added DIPEA (0.050 mL, 0.29 mmole) via a syringe. The reaction was stirred at room temperature protected from light for 20 minutes. The reaction was monitored by analytical HPLC which indicated complete conversion of starting material eluting at 11.3 minutes to product eluting at 12.2 minutes. The product was purified by preparative HPLC (40 minute gradient of 10% → 70% acetonitrile/water each with 0.05% TFA). The HPLC fractions were frozen at -80°C and lyophilized to dryness to give a red oil. Yield = 11 mg (66%). ^{1}H-NMR (CF_3COOD) δ 1.87 (d, *J* = 6.1 Hz, 6H), 2.93 (s, 6H), 3.23 (m, 2H), 3.50 (s, 4H), 3.92 (m, 5H), 4.11 (m, 2H), 4.20 (m, 2H), 4.27 (m, 20H), 5.24 (p, 1H), 6.07 (m, 2H), 8.21 (d, *J* = 2.5 Hz, 1H), 8.41 (s, 2H), 8.46 (m, 1H), 8.55 (dd, *J* = 9.9, 2.5 Hz, 1H), 8.78 (m, 1H), 9.02 (d, *J* = 9.8 Hz, 2H), 9.08 (d, *J* = 8.8 Hz, 1H). MALDI-TOF MS *m/z* $[M]^+$ 926.125; HRMS (ESI, *m/z*) $[M]^+$ calculated for $C_{46}H_{60}N_3O_{15}S$, 926.3740; found 926.3919.

*2. Preparation of BSA conjugates of acridinium esters **1b**-**6b***

Briefly, a solution of bovine serum albumin (0.5 mg BSA for each reaction as 0.2 mL of 2.5 mg/mL solution, 7.5 nanomoles) in carbonate buffer (0.1 M, pH 8) was mixed with the DMSO solution of compounds **1b-6b** (~0.5 mg/mL) corresponding to 10 equivalents excess over BSA. The reactions were incubated on a tilt shaker at 4°C for 4 hours. After the indicated

reaction time, the solutions were transferred onto 4 mL Amicon filters (30,000 molecular weight cutoff) and de-salted with de-ionized water three times using a bench top centrifuge (Thermo Scientific Sorvall Legend X1R centrifuge). The final concentrated solutions (0.5 mL) were transferred into amber glass vials and adjusted to ~ 0.5 mg/mL with de-ionized water after determining the original concentrations by BCA protein assay. Label incorporation was determined by MALDI-TOF mass spectrometry based on the observed differences in molecular weights of unlabeled BSA and the conjugates.

3. pH Titrations

Compounds **1a-6a**, prepared as a ~2.5 mM solution in DMSO, were diluted 50-fold with different pH buffers (0.1 M HCl for pH 1.0; 0.1 M H_3PO_4/NaH_2PO_4 buffer for pH 2.0, 2.5 and 3.0; 0.1 M HOAc/NaOAc buffer for pH 3.5, 4.0, 4.5, 5.0 and 5.5; and 0.1 M NaH_2PO_4/Na_2HPO_4 buffer for pH 6.0, 6.5 and 7.0). The solutions were allowed to stand for 1 hour at room temperature before measurement of the UV-Visible absorption spectra of the solutions. The absorption band at 384 nm decreased with increasing pH indicative of pseudobase formation.

4. Emission spectra

Chemiluminescence emission spectra of acridinium esters **1a–6a** were measured using a SpectraScan PR-740 spectroradiometer (camera) from Photo Research Inc.

Briefly, the acridinium ester labels were dissolved in 1:1 water/acetonitrile to approximately ~ 0.01mM. In each measurement, 0.01 mL of the acridinium ester solution was mixed with 0.3 mL of 0.1 M nitric acid containing 0.5% hydrogen peroxide in 0.1M nitric acid. Prior to the addition of 0.3 mL of 0.25 M sodium hydroxide containing 7 mM CTAC, the shutter of the camera was opened and light was collected for 5 seconds after addition of base and surfactant. The spectroradiometer measured the luminescence intensity as a function of wavelength from 380-780 nm.

5. Chemiluminescence measurements

Chemiluminescence of acridinium esters **1a–6a** and their BSA conjugates from **1b**-**6b** was measured on Autolumat LB953 Plus luminometer from Berthold Technologies. Briefly, the acridinium esters **1a-6a** in DMSO at ~ 2.5mM were serially diluted 5 million-fold into buffer (10 mM NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, 8 mM NaN_3 and 0.015 mM BSA, pH= 8). Emission from 0.01 mL samples were measured in triplicate by the addition of 0.3 mL of 0.1 M nitric acid containing 0.5% hydrogen peroxide in 0.1M nitric acid followed by 0.3 mL of 0.25 M sodium hydroxide containing 7 mM CTAC. Light was collected for a total of 10 seconds integrated at 0.1 second intervals. Similarly, BSA conjugates of **1b-6b** at 0.5mg/mL were serially diluted 100,000-fold into buffer and chemiluminescence from 0.01 mL samples were measured in triplicate. The output of the luminometer was in RLUs (Relative Light Units).

6. Fractional non-specific binding (fNSB)

The fractional nonspecific binding of acridinium esters and their BSA conjugates were evaluated on the automated ADVIA Centaur® XP system using Dynabeads M-270 amine, M-270 carboxylates, M-280 tosyl-activated microparticles from LifeTechnologies Inc., as well as in house paramagnetic microparticles (PMPs with amine surfaces). The M-280 beads were first hydrolyzed with 0.1M $NaHCO_3/Na_2CO_3$ buffer (pH = 9) at 37°C and hydrolysis of the tosylate groups was monitored by UV-Visible spectroscopy until there was no residual absorption in the supernatant. All particles were rinsed with buffer (0.1M HEPES, 0.15 M NaCl, 1mM EDTA, 0.076 mM BSA, 7.7 mM NaN_3, pH 7.4) three times and diluted to 0.500 mg/mL. The acridinium esters **1a-6a** were diluted with the same buffer to 0.1 µM while the BSA conjugates of **1b-6b** were diluted to 1.3µg/mL. Briefly, 0.05 mL of acridinium ester reagent and 0.200 mL of each particle suspension were incubated for 5.5 minutes at 37°C, and the particles were then separated, aspirated and washed with 10 mM potassium phosphate, 0.14 M sodium chloride, 14 mM sodium azide, and 1.0 mM EDTA, pH 7.2 in water also containing 0.8 mM Tween-20 (non-ionic surfactant). Chemiluminescence associated with the particles (five replicates) was measured by the addition of addition of 0.3 mL of 0.1 M nitric acid containing 0.5% hydrogen

peroxide in 0.1M nitric acid followed by 0.3 mL of 0.25 M sodium hydroxide containing 7 mM CTAC. Fractional non-specific binding (fNSB) was calculated as the ratio of residual chemiluminescence associated with the particles following the wash divided by the total chemiluminescence input.

7. Chemiluminescence Stability

The BSA conjugates of **1b**-**6b** were stored at 37°C in phosphate buffer, pH 7.4, protected from light. Chemiluminescence from 0.01 mL samples (triplicates) were measured periodically as described above. Residual chemiluminescence as a function of time was monitored periodically to assess chemiluminesence stability.

References

1. (a) 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; (b) A. Natrajan, D. Sharpe and Q. Jiang, *US Pat*. 6,664,043 B2, 2003; (c) A. Natrajan, Q. Jiang, D. Sharpe and J. Costello, *US Pat*., 7,309,615, 2007; (d) A. Natrajan, D. Sharpe, J. Costello and Q. Jiang, *Anal. Biochem.,* 2010, **406**, 204-213; (e) A. Natrajan, D. Sharpe and D. Wen, *Org. Biomol. Chem*., 2011, **9**, 5092-5103; (f) A. Natrajan, D. Sharpe and D. Wen, *Org. Biomol. Chem*., 2012, **10**, 1883-1895; (g) A. Natrajan, D. Sharpe and D. Wen, *Org. Biomol. Chem*., 2012, **10**, 3432-3447; (h) A. Natrajan and D. Sharpe, *Org. Biomol. Chem*., 2013, **11**, 1026-1039; (i) A. Natrajan, D. Wen and D. Sharpe, *Org. Biomol. Chem*., 2014, **12**, 3887-3901; (j) A. Natrajan and D. Wen, *RSC Advances*, 2014, **4**, 21852-21863; (k) A. Natrajan, D. Sharpe and Q. Jiang, *US Pat* 8,778,624, 2014.
2. (a) I. Weeks, I. Behesti, F. McCapra, A.K. Campbell and J.S. Woodhead, *Clin. Chem*., 1983, **29/8**, 1474-1479; (b) J.W. Bunting, V.S.F. Chew, S.B. Abhyankar and Y. Goda, *Can. J. Chem*.,1984, **62**, 351-354; (c) P.W. Hammond, W.A. Wiese, A.A. Waldrop III, N.C. Nelson and L.J. Arnold Jr, *J. Bioluminescence and Chemiluminescence*, 1991, **6**, 35-43.
3. (a) F. McCapra*, Acc. Chem. Res*., 1976, **9/6**, 201-208; (b) F. McCapra, D. Watmore, F. Sumun, A. Patel, I. Beheshti, K. Ramakrishnan and J. Branson, *J. Bioluminescence and Chemiluminescence*, 1989, **4**, 51-58; (c) J. Rak, P. Skurski and J. Błażejowski, *J. Org. Chem*., 1999, **64**, 3002-3008; (d) K. Krzymiński, A.D. Roshal, B. Zadykowicz , A. Białk-Bielińska and A. Sieradzan, *J. Phys. Chem. A*, 2010, **114**, 10550-10562; (e) K. Krzymiński, A. Ożóg, P. Malecha, A.D. Roshal, A. Wróblewska, B. Zadykowicz, and J. Błażejowski, *J. Org. Chem*., 2011, **76**, 1072-1085.

4. (a) G. Zomer and J.F.C. Stavenuiter, *Analytica Chimica Acta*, 1989, **227**, 11-19; (b) S. Batmanghelich, J.S. Woodhead, K. Smith and I. Weeks, J*. Photochem. Photobiol. A: Chem*., 1991, **56**, 249-254.
5. (a) N. Sato, *Tetrahedron Lett*.,1996, **47**, 8519-8522; (b) M. Adamczyk, P.G. Mattingly, J.A. Moore and Y. Pan, *Org. Lett*., 2003, **5**, 3779-3782.
6. (a) R.C. Brown, Z. Li, A.J. Rutter, X. Mu, O.H. Weeks, K. Smith and I. Weeks, *Org. Biomol. Chem*., 2009, **7**, 386-394; (b) K.A. Browne, D.D. Deheyn, G.A. El-Hiti, K. Smith and I. Weeks, *J. Am. Chem. Soc*., 2011, **133**, 14637-14648; (c) K.A. Browne, D.D. Deheyn, R.C. Brown and I. Weeks, *Anal. Chem*., 2012, **84**, 9222-9229.
7. A. Natrajan and D. Wen, *Green Chem. Lett. Rev*., 2013, **6**, 237-248.
8. S.-J. Law, T. Miller, U. Piran, C. Klukas, S. Chang and J. Unger, *J. Bioluminescence and Chemiluminescence*, 1989, **4**, 88-98.
9. A. Natrajan and D. Wen, *Green. Chem.,* 2011, **13***,* 913-921.
10. (a) M.R. Infante, J. Seguer, A. Pinazo and M.P. Vinardell, *J. Dispersion Science and Technology*, 1999, **20**, 621-642; (b) J. Riggs-Sauthier, W. Zhang, T.X. Viegas and M.D. Bentley, *WO2008/112257 A1.*
11. For a description of magnetic particles used in the current study see the section on magnetic microparticles at www.**lifetechnologies**.com.
12. *Principles of Fluorescence Spectroscopy, second edition*, J.R. Lakowicz: Ed, Kluwer Academic/Plenum Publishers, New York, 1999.

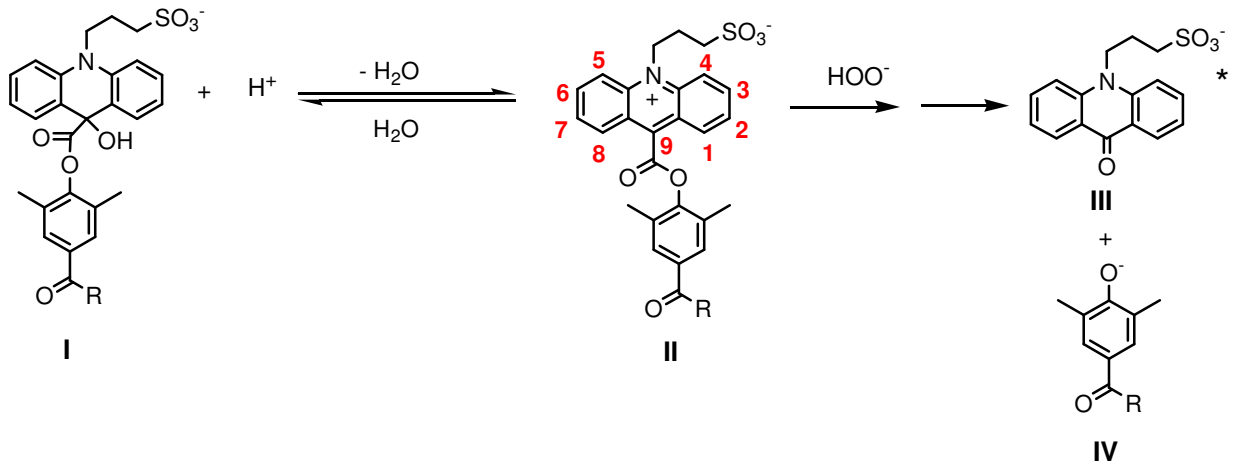


Figure 1. Simplified chemiluminescence reaction pathway of an N-sulfopropyl acridinium dimethylphenyl ester. Acid treatment with 0.1 M nitric containing 0.5% hydrogen peroxide converts the water adduct (pseudobase) **I** to the acridinium form **II**. Subsequent treatment with 0.25 M NaOH containing cetyltrimethlammonium chloride (CTAC) ionizes the hydrogen peroxide which initiates light emission. Excited state acridone **III** is the primary emitter. CTAC accelerates emission kinetics and enhances light yield.

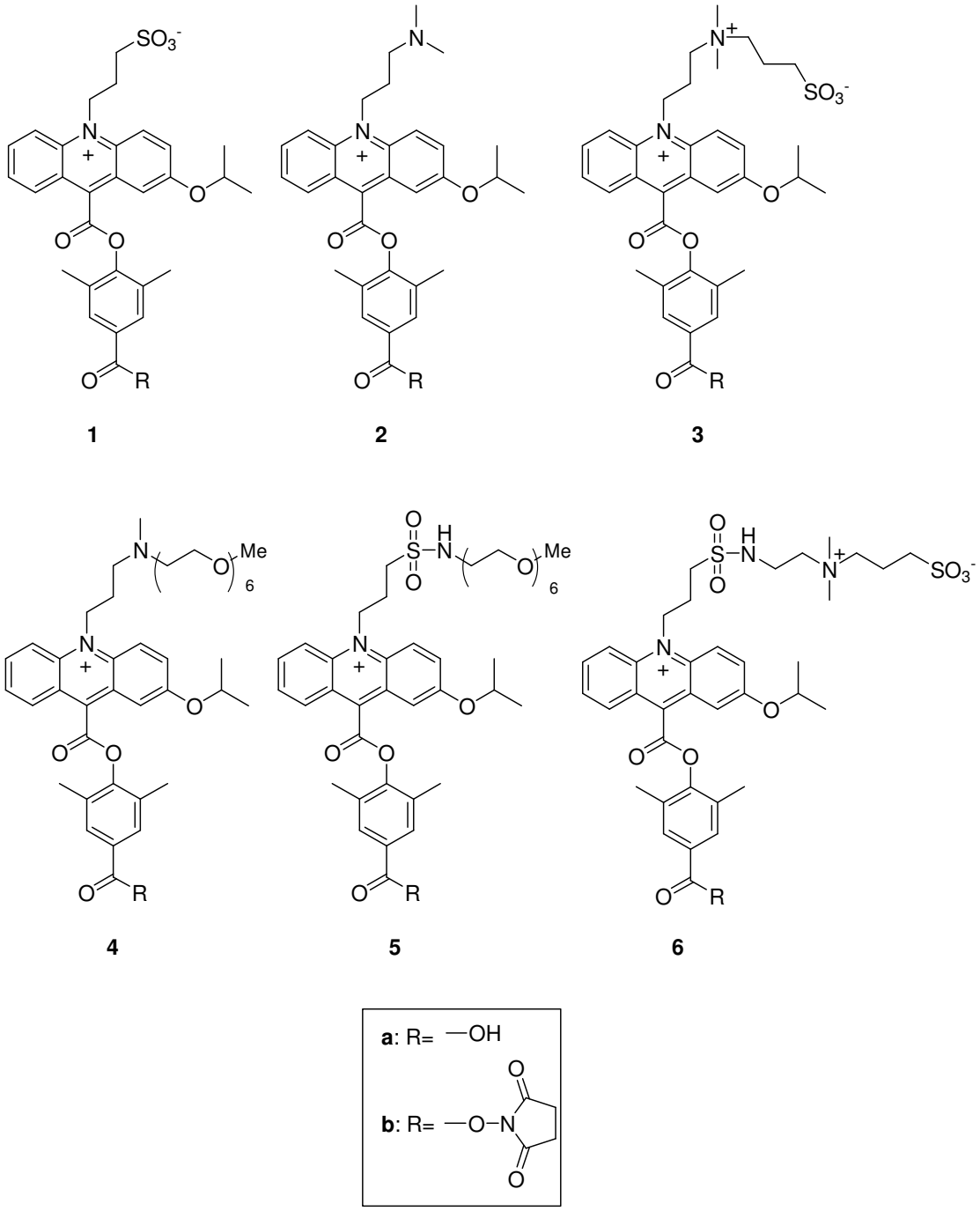


Figure 2. Structures of acridinium dimethylphenyl esters with different N-alkyl groups. Acridinium esters **1** and **6** were described in our earlier work[1h,g] and contain an N-sulfopropyl group and an N-alkyl group with a charge-neutral sulfobetaine respectively. Acridinium esters **2**-**5** were synthesized in the current study.

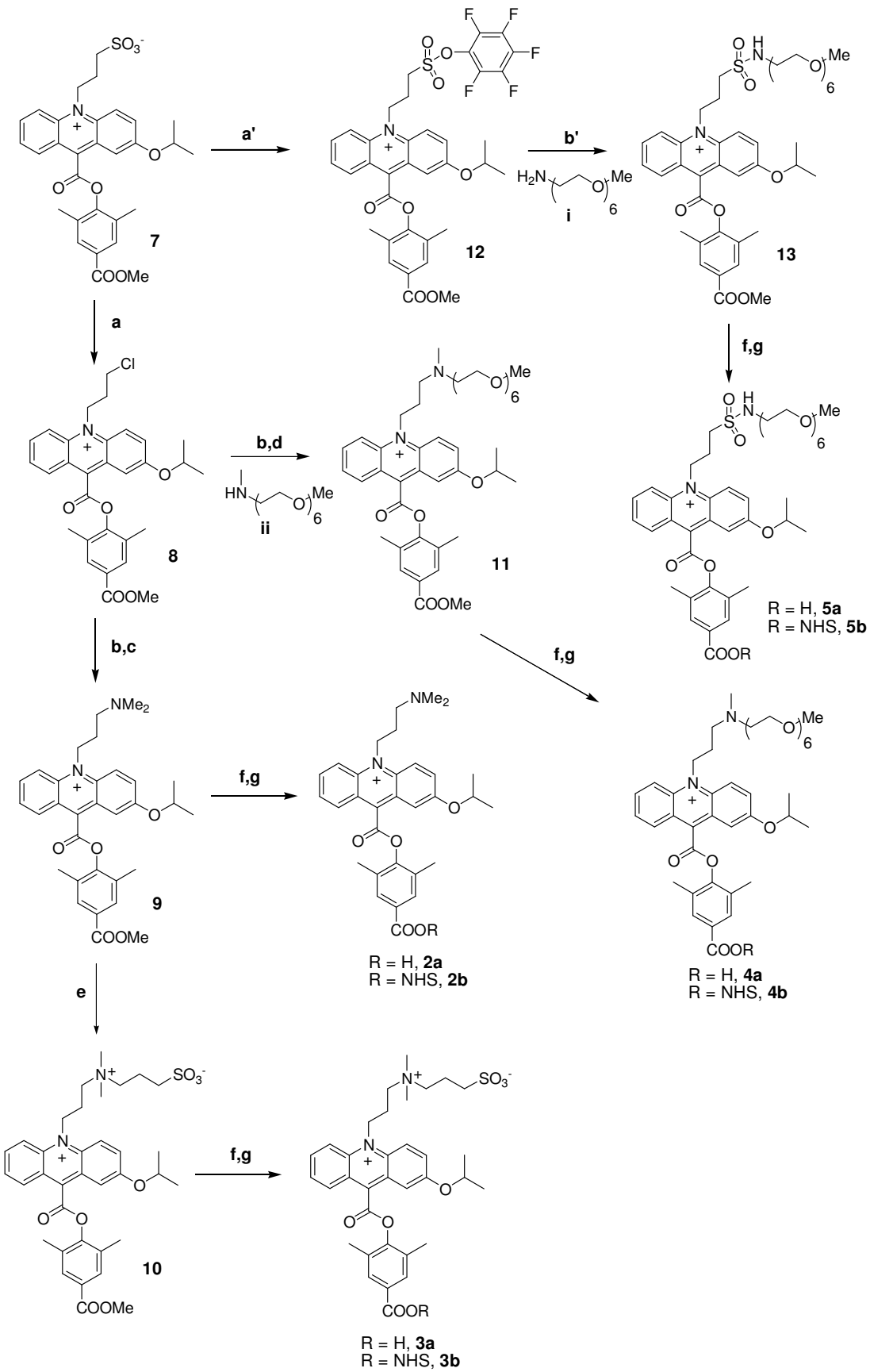
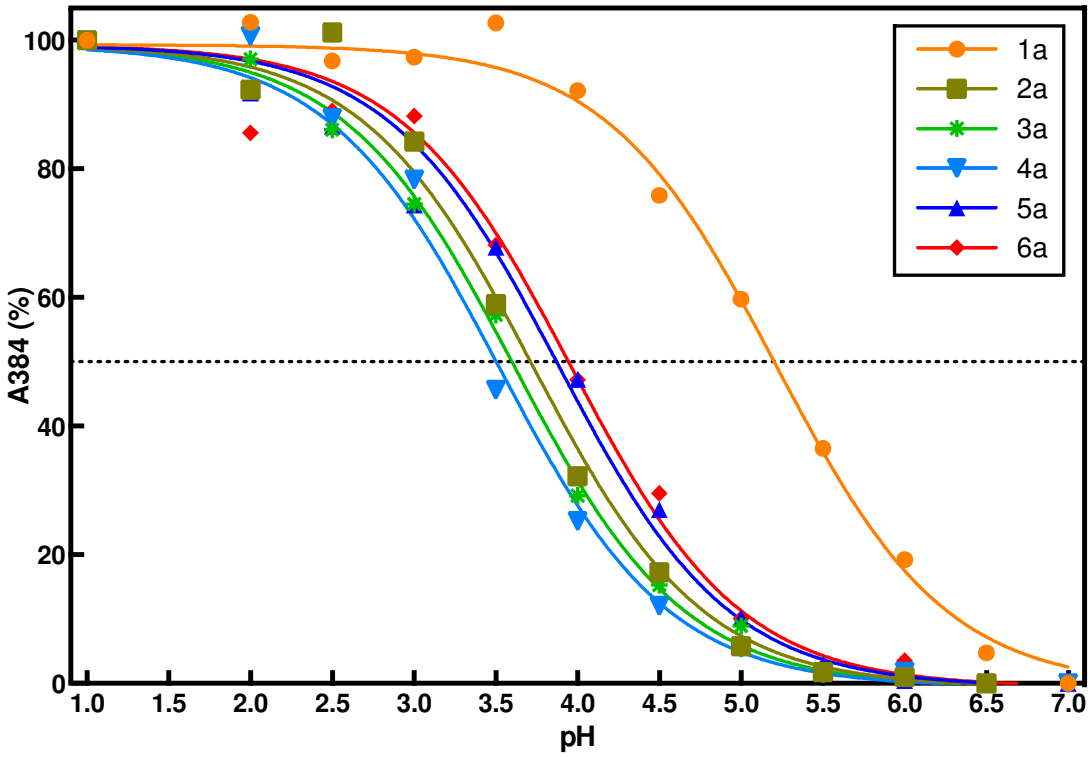


Figure 3. Synthetic scheme for acridinium esters **2a**,**b**; **3a**,**b**; **4a**,**b** and **5a**,**b**. Reagents: (**a**) thionyl chloride, reflux, ≥ 2h, 83%; (**a'**) thionyl chloride, reflux, ≤ 30 minutes; pentafluorophenol, triethylamine, dichloromethane[1h] (**b**) KI, acetone, reflux; (**b'**) MeCN, reflux, 60%; (**c**) 2M $NHMe_2$ in THF, silver triflate (AgOTf), MeCN, 64% over 2 steps; (**d**) **ii**, AgOTf, MeCN, 37%, 2 steps; (**e**) 1,3-propane sultone, 2,6-di-*tert*-butylpyridine, 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], 155°C, 60%; (**f**) 1N HCl, reflux, **2a**,**3a**,**4a**, 90%, **5a**, 82%; (**g**) N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU), diisopropylethylamine (DIPEA), DMF, **2b** 72%, **3b**,**4b**, 70%, **5b**, 66%. NHS = N-hydroxysuccinimide.

A



B

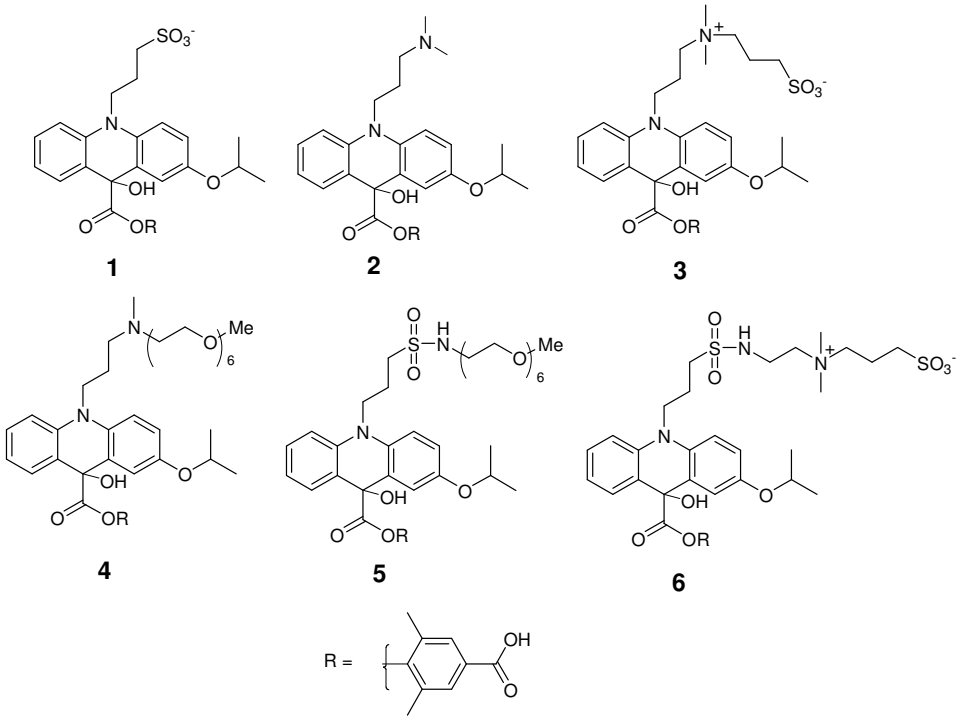


Figure 4. **Panel A** : pH Titrations illustrating conversion of the acridinium forms of the labels **1a**-**6a** (carboxylic acids) to their respective pseudobases (water adduct **I** in Figure 1). Increased steric bulk of the N-alkyl substituents in acridinium esters **2a**-**6a** induced a shift in the pKa for pseudobase formation to acidic pH. **Panel B** : Structures of the pseudobases of labels **1a**-**6a**.

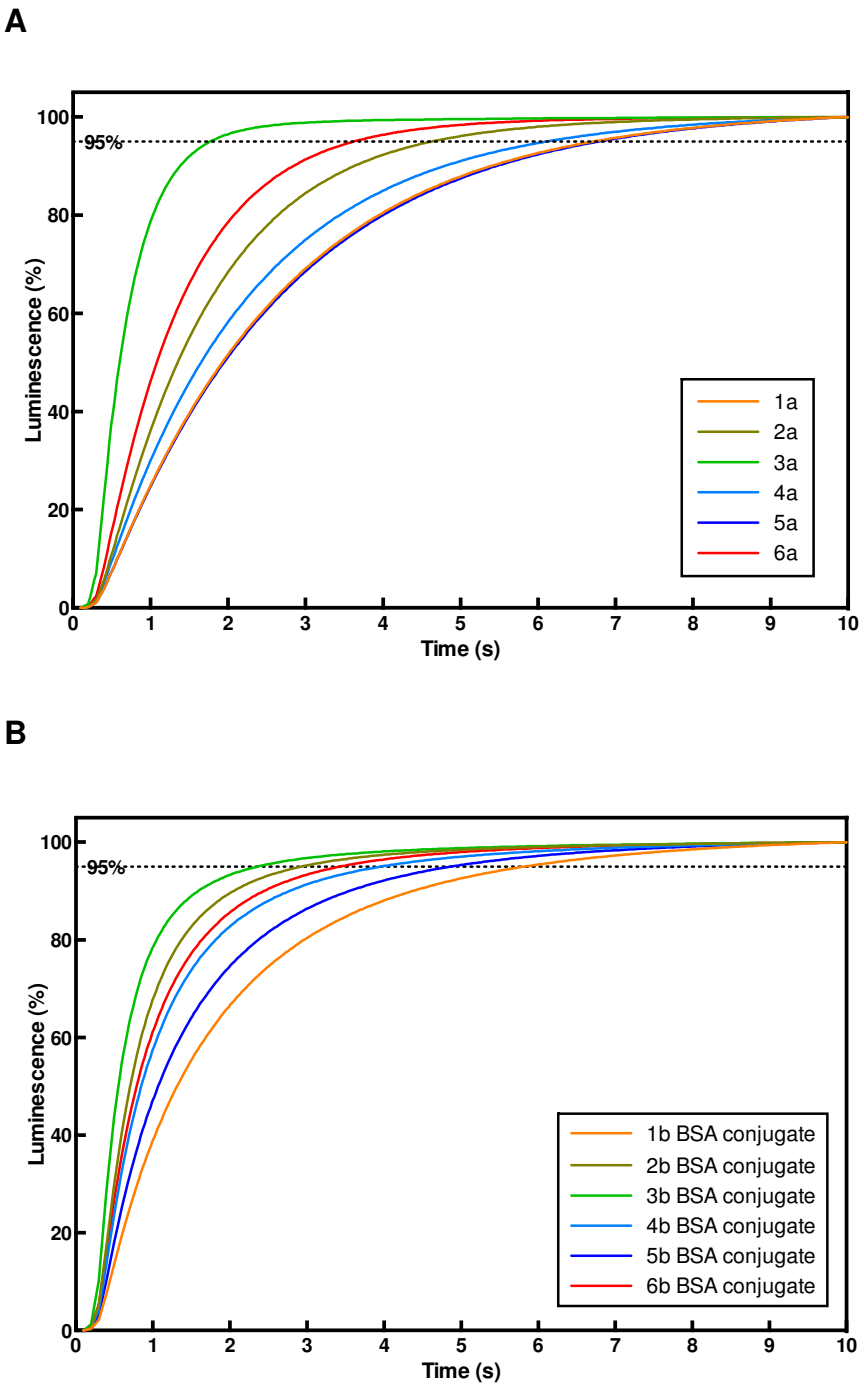
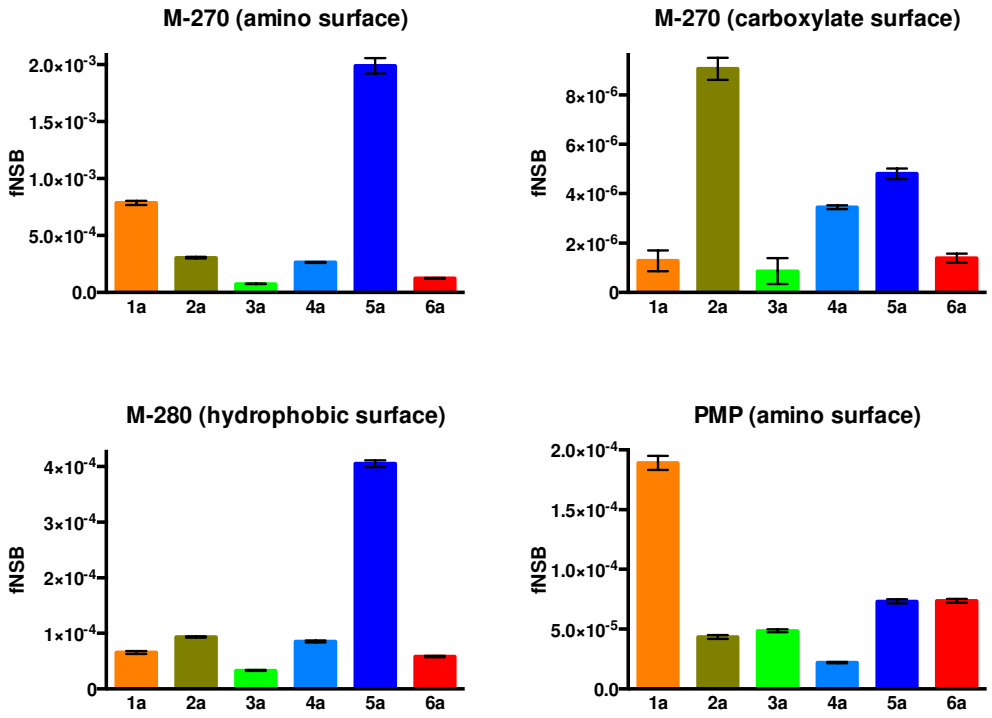


Figure 5. **Panel A**: Chemiluminescence emission profiles of acridinium esters **1a**-**6a**. Dimethyl sulfoxide solutions of the labels were serially diluted into buffer as described in the experimental section. **Panel B**: Chemiluminescence emission profiles of BSA conjugates of acridinium esters **1b**-**6b**. The conjugates were serially diluted into buffer as described in the experimental section. Chemiluminescence was initiated by the addition of 0.3 mL of 0.1 M nitric acid containing 0.5%

hydrogen peroxide followed by the addition of 0.3 mL of 0.25 M sodium hydroxide containing 7 mM CTAC. Light was collected for 10 seconds integrated at 0.1 second intervals.

A



B

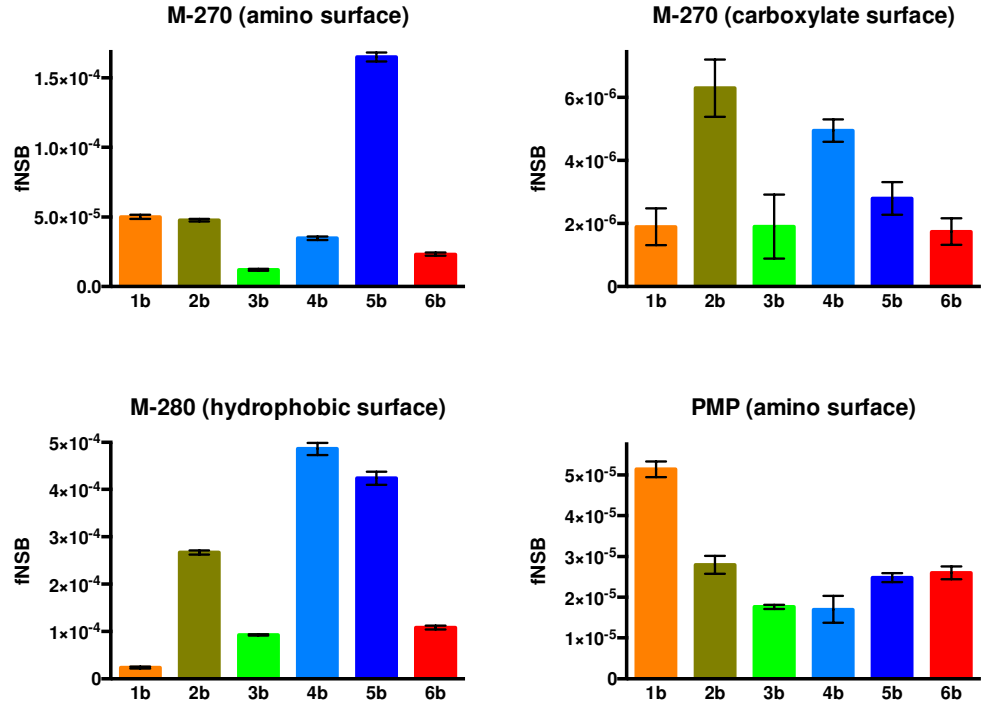


Figure 6. **Panel A**: Fractional non-specific binding (fNSB) of acridinium ester labels **1a**-**6a** to four different kinds of magnetic microparticles measured on the ADVIA Centaur® system. Dimethyl sulfoxide solutions of acridinium ester labels **1a**-**6a** were serially diluted into buffer and mixed with the magnetic microparticles. The particles were washed once with phosphate buffer pH 7.2 containing a non-ionic surfactant and residual chemiluminescence associated with the particles was measured by the sequential addition of 0.3 mL of 0.1 M nitric acid containing 0.5% hydrogen peroxide followed by 0.3 mL of 0.25 M sodium hydroxide containing 7 mM CTAC. Residual chemiluminescence divided by the total chemiluminescence input is the fractional non-specific binding. M-270 and M-280 are magnetic particles from Life Technologies[11]. PMPs (paramagnetic particles) were in-house particles. Tosylate groups on M-280 particles were hydrolyzed prior to use. **Panel B**: Fractional non-specific binding (FNSB) of BSA conjugates of acridinium ester labels **1b**-**6b** to four different kinds of magnetic microparticles measured on the ADVIA Centaur® system.

Table 1. Label incorporation in BSA (rounded to the nearest integer) measured by mass spectroscopy using 10 equivalents acridinium ester input in the labeling reaction.

Acridinium ester	No. of labels
1b	6
2b	7
3b	5
4b	6
5b	6
6b	7

Table 2. Emission spectra maxima (λ_{max}) of acridinium esters **1a**-**6a**. Spectra were recorded in >90% aqueous media using a PR-740 spectroradiometer (camera) from Photo Research Inc. Bandwidth slit: 2 nm; Aperture: 2 degrees; Exposure time: 5000 msec.

Acridinium ester	λ_{max} (nm)
1a	452
2a	452
3a	456
4a	452
5a	454
6a	456

Table 3. Observed specific chemiluminescence activity (SCA) of the acridinium esters **1a**-**6a** and BSA conjugates prepared from the corresponding NHS esters **1b**-**6b**. Light emission was triggered by the sequential addition of 0.3 mL reagent 1 (0.1 M nitric acid + 0.5% peroxide) followed by 0.3 mL reagent 2 (7 mM CTAC in 0.25 M NaOH). (RLU = Relative Light Unit). Measuring time was 10 seconds.

Acridinium ester/ BSA conjugate	SCA (RLUs/mole x 10^{-19})	Time(seconds) for >95% emission
1a	4.0	6.8
2a	2.5	4.7
3a	4.5	1.8
4a	1.5	6.2
5a	5.6	6.8
6a	7.8	3.7
1b BSA conjugate	5.6	5.9
2b BSA conjugate	2.3	3.0
3b BSA conjugate	3.7	2.4
4b BSA conjugate	1.3	4.0
5b BSA conjugate	6.4	4.9
6b BSA conjugate	5.7	3.5