



## Enhanced immunoassay sensitivity using chemiluminescent acridinium esters with increased light output

Anand Natrajan \*, David Sharpe, Jim Costello, Qingping Jiang

Siemens Healthcare Diagnostics, Advanced Technology & Pre-Development, 333 Coney Street, E. Walpole, MA 02032, USA

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### ABSTRACT

Chemiluminescent acridinium ester labels are widely used in clinical diagnostics especially in automated immunochemistry analyzers such as Siemens Healthcare Diagnostics' ADVIA Centaur® systems. Although, chemiluminescence from acridinium compounds was discovered more than 50 years ago, details regarding the excitation process are still not well understood particularly in relation to acridinium structure and overall light output. Herein, we report an empirical study that correlates the presence of electron-donating methoxy groups at C-2 and/or C-7 in the acridinium ring with increased light output. We further demonstrate that these high light output labels can be combined with hydrophilic functional groups such as hexa(ethylene)glycol to generate unique acridinium esters that are stable and are useful in improving immunoassay sensitivity for both competitive and sandwich automated immunoassays.

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Chemiluminescence has emerged to be an extremely useful detection technology in clinical diagnostics both for immunoassays and nucleic acid assays [1]. Among the various chemiluminescent labels that are currently employed in diagnostics, acridinium esters are particularly attractive because they have high quantum yields with detection limits in the attomole range [1–3]. Moreover, they exhibit fast light emission with simple chemical triggers and their small size permit simple labeling protocols for proteins and nucleic acids.

Chemiluminescence from acridinium salts [4,5] was observed more than four decades ago, and subsequent studies revealed that acridinium phenyl esters were much more luminescent than simple alkyl esters [6]. Light emission from acridinium phenyl esters when triggered with alkaline hydrogen peroxide is believed to result from addition of hydroperoxide ions to the acridinium ring at C-9 (see Fig. 1 for numbering system) followed by cleavage of the phenolic ester bond, and ultimately resulting in the formation of excited state acridone, which is the light-emitting moiety. The intermediates in this chemiluminescent reaction have not been characterized and although the formation of a dioxetanone intermediate was proposed [6], a later theoretical study [7] has suggested that the direct formation of the excited state acridone from the initial acridinium ester-peroxide adduct is energetically more favorable.

Some early reports describing the applications of acridinium phenyl esters in immunoassays [8] and nucleic acid assays [9]

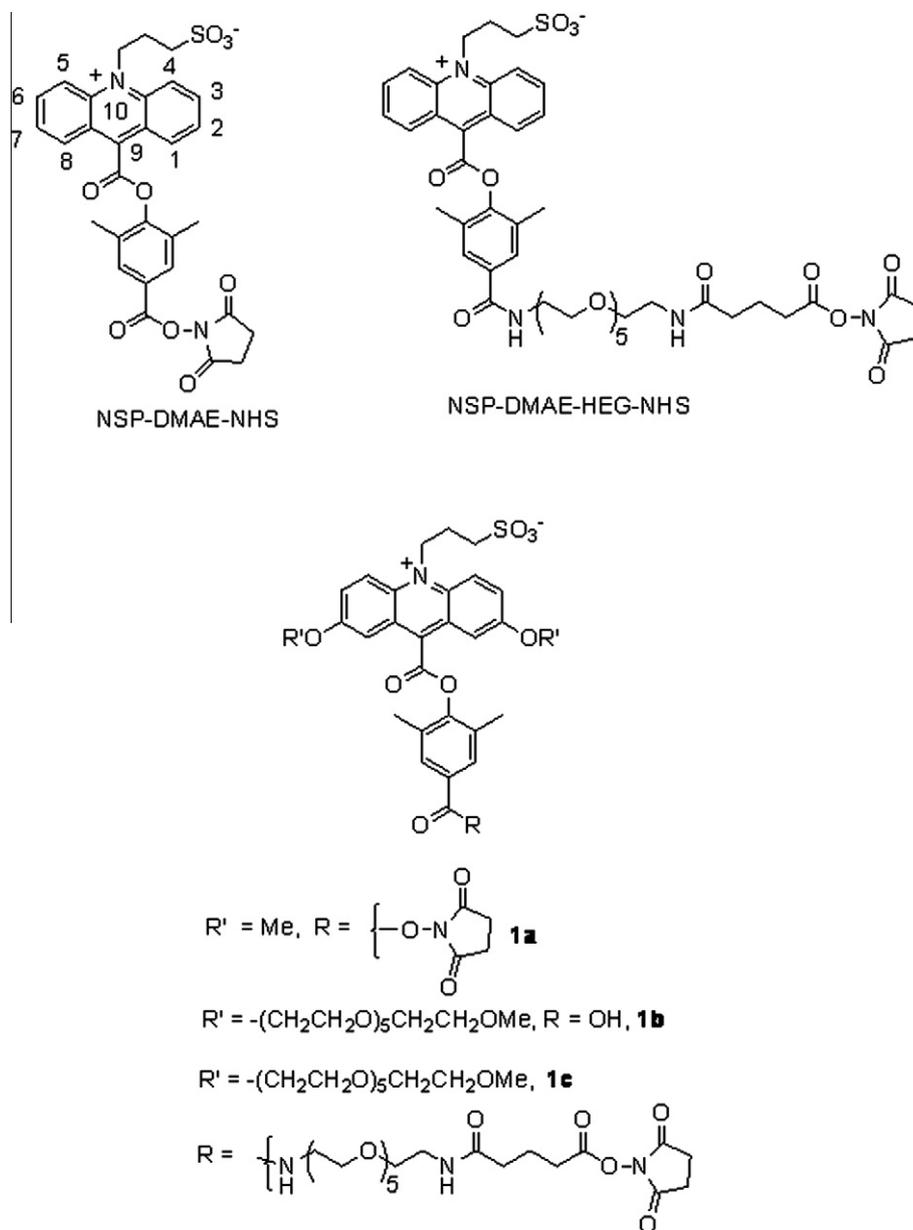
pointed toward their potential as useful labels in clinical diagnostics. The acridinium esters employed in these studies contained functional groups such as *N*-hydroxysuccinimidyl (NHS)<sup>1</sup> esters in the phenyl ring to enable labeling of antibodies and DNA probes. The main drawback of these acridinium esters was their instability resulting from facile hydrolysis of the ester bond between the acridinium ring and the phenol. The hydrolytic instability of these acridinium esters was in fact used rather ingeniously in homogeneous DNA probe assays where it was observed that acridinium ester labeled probes when hybridized to their targets were protected from hydrolysis [10]. Thus, free versus bound labeled probes could be differentiated by selective hydrolysis of the former.

For applications in automated immunoassays, however, the hydrolytic instability of acridinium phenyl esters proved to be a big detriment and although this hydrolytic instability can be alleviated somewhat with the help of additives such as bisulfite [11], it was not until the invention of hydrolytically stable acridinium esters that commercial applications of these compounds in automated instruments could be realized [12]. These hydrolytically stable acridinium esters contain two methyl groups flanking the phenolic ester linkage and were observed to be significantly more stable than acridinium esters lacking these substituents, showing no loss of chemiluminescent activity even after a week at 37 °C at pH 7.0 whereas the latter lost 90% of their activity. Both Law

<sup>1</sup> Abbreviations used: DMAE, dimethyl acridinium ester; FNSB, fractional nonspecific binding; FSSS, fast spectral scanning system; HEG, hexa(ethylene)glycol; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NHS, *N*-hydroxysuccinimidyl, NSP, *N*-sulfolpropyl; TSH, thyroid-stimulating hormone.

\* Corresponding author. Fax: +1 508 660 4591.

E-mail address: [anand.natrajan@siemens.com](mailto:anand.natrajan@siemens.com) (A. Natrajan).



**Fig. 1.** Structures of chemiluminescent acridinium ester labels. Compounds **1a**, **1b**, and **1c** with alkoxy groups at C-2 and C-7 exhibit enhanced light emission compared to NSP-DMAE labels.

et al. [12] and Yin et al. [13] have described the use of a hydrolytically stable acridinium ester called DMAE (dimethyl acridinium ester) in immunoassays. It has also been shown that hydrolytically stable, chemiluminescent acridinium compounds can be devised by replacing the phenyl ester moiety with *N*-sulfonylcarboxamides [14]. These compounds are structurally quite distinct from acridinium phenyl esters but they use the same chemical triggers and exhibit similar emissive properties.

Hydrolytically stable acridinium esters are currently used in automated, high-throughput immunochemistry instruments such as Siemens Healthcare Diagnostics' ADVIA Centaur<sup>®</sup> systems in conjunction with magnetic particles in various immunoassay formats. The structures of two such acridinium esters abbreviated as NSP-DMAE-NHS [15] and NSP-DMAE-HEG-NHS [16] are illustrated in Fig. 1. NSP-DMAE-NHS is more hydrophilic than DMAE-NHS because of the *N*-sulfonylpropyl (NSP) group attached to the nitrogen in the acridinium ring. Both compounds also contain NHS esters, directly attached to the phenyl ring, *para* to the phenolic ester bond. The compound NSP-DMAE-HEG-NHS in Fig. 1 is

even more hydrophilic than NSP-DMAE-NHS owing to the hexa(ethylene)glycol (HEG) linker inserted between the reactive NHS ester and the phenyl ring.

Light emission from the acridinium esters illustrated in Fig. 1 is triggered with alkaline peroxide in the presence of a cationic surfactant and typically lasts 2–5 s. The excited state acridone formed in the chemical reaction emits light with an emission maximum centered at 426 nm. In the current study, we examined the impact of electron-donating methoxy groups, attached at various positions of the acridinium ring, on relative light output and wavelength of light emission. The greatest increase in light output of up to threefold was observed when two methoxy groups were placed at the C-2 and C-7 positions of the acridinium ring (Table 1). The increase in light output was also accompanied with a bathochromic shift in the acridinium ester's emission wavelength maximum from 426 to 484 nm. Replacement of the methyl ethers with hydrophilic methoxyhexa(ethylene)glycol ethers (compound **1b** in Fig. 1) maintained the high light output. Chemiluminescent stability of the parent compound **1a**, with two methoxy groups at C-2 and C-7 of

**Table 1**  
Relative quantum yields and emission wavelength maxima of acridinium esters.

Compound	Relative quantum yield	Emission wavelength maximum (nm)
NSP-DMAE	1	426
NSP-2-OMe-DMAE	2.2	458
NSP-3-OMe-DMAE	0.35	418
NSP-4-OMe-DMAE	0.33	478
NSP-2,4-(OMe) <sub>2</sub> -DMAE	0.35	514
NSP-2,7-(OMe) <sub>2</sub> -DMAE	2.7	484
NSP-2,5-(OMe) <sub>2</sub> -DMAE	0.5	486
NSP-2,4,7-(OMe) <sub>3</sub> -DMAE	1	518
<b>1b</b>	3.3	480

the acridinium ring, was significantly poorer compared to NSP-DMAE-HEG-NHS when the labels were conjugated to an anti-TSH (thyroid-stimulating hormone) monoclonal antibody. However, the analogous conjugate of compound **1c** containing the hydrophilic methoxyhexa(ethylene)glycol ethers instead of methoxy groups was observed to be significantly more stable than the conjugate of **1a** (Fig. 2). This antibody conjugate was observed to significantly improve assay sensitivity in an automated assay for TSH when compared to the NSP-DMAE-HEG-NHS label (Fig. 5). Similarly, in an automated assay for the small analyte theophylline (Fig. 4), the high light output theophylline conjugate **2b** containing the same substituents as acridinium ester **1c** displayed improved assay performance when compared to theophylline conjugate **2a** derived from the NSP-DMAE-HEG label. In summary, the current study outlines structural features in acridinium esters that lead to increased light output and the application of these labels and their conjugates in automated immunoassays leading to enhanced assay sensitivity for both small (theophylline) and large (TSH) analytes.

## Materials and methods

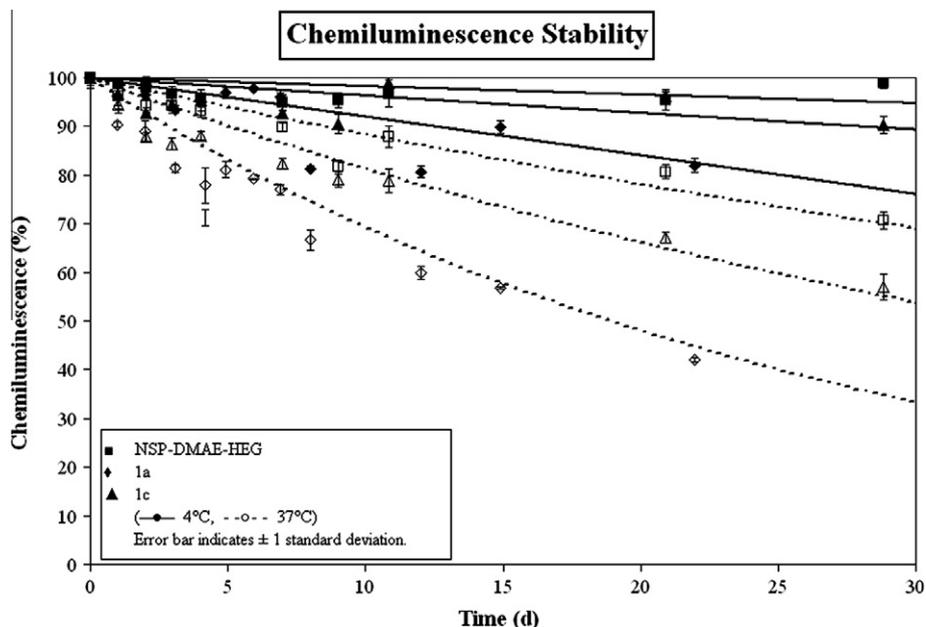
### Synthesis of acridinium esters and conjugates

The syntheses and purification of the acridinium esters listed in Table 1 and Fig. 1 and the theophylline conjugates illustrated in

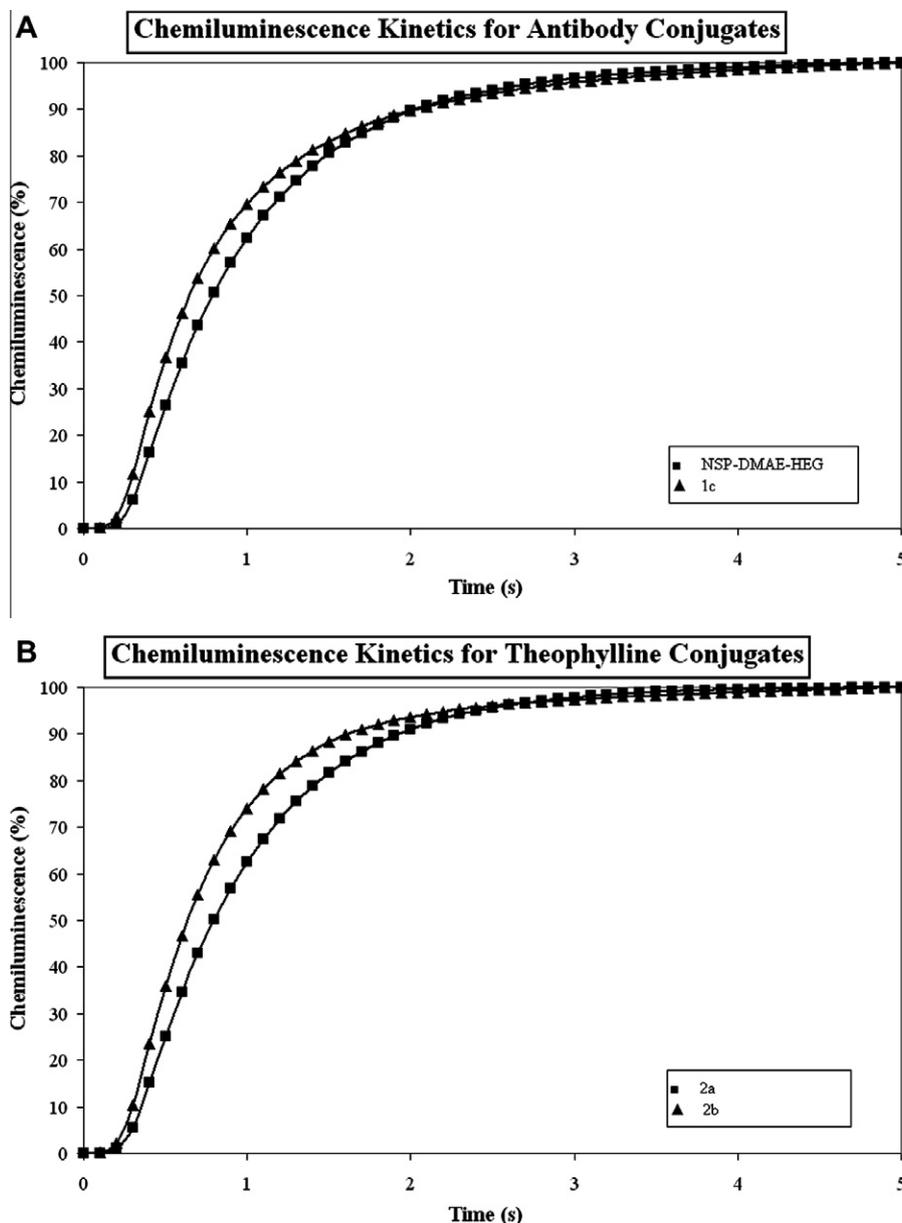
Fig. 4 have been described previously [16,17]. Briefly, methoxy-substituted acridine-9-carboxylic acids were esterified with benzyl-3,5-dimethyl-4-hydroxybenzoate [15] to give the methoxy-substituted acridine esters that were N-alkylated with 1,3-propane sultone. The benzyl protecting group on the phenol of the resulting acridinium esters was then cleaved by acid hydrolysis to give the compounds listed in Table 1. The high light output label **1b** in Table 1 was synthesized from 2,7-dimethoxy acridine-9-carboxylic acid in eight steps [17]. The NHS ester labels NSP-DMAE-HEG-NHS, **1a**, and **1c** were synthesized from the corresponding carboxylic acids as described previously [16,17]. The diamino hexa(ethylene)glycol linker (HEG linker) in NSP-DMAE-HEG-NHS and **1c** was synthesized from hexa(ethylene)glycol [16]. The theophylline conjugates **2a** and **2b** were synthesized from 8-carboxypropyltheophylline and the amine intermediates of NSP-DMAE-HEG and **1c** [16,17].

All final acridinium esters and theophylline conjugates were analyzed and purified by HPLC using a Beckman Coulter HPLC system. Analytical HPLC was performed using a C<sub>18</sub>, 4.6 mm × 30 cm column from Phenomenex and a 30 min gradient of 10→70% acetonitrile in water with 0.05% trifluoroacetic acid in each solvent at a flow rate of 1.0 ml/min and UV detection at 260 nm. For preparative HPLC a C<sub>18</sub>, 30 mm × 250 cm column from YMC was used at a flow rate of 20 ml/min and the same solvent gradient. HPLC fractions of products were frozen at −80 °C and lyophilized to dryness.

MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectroscopy 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-m 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 V 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 V and a delay time of 85 ns. The molecular weights and yields of the various acridinium ester labels and theophylline conjugates are as follows:



**Fig. 2.** Chemiluminescence stability of acridinium ester–antibody conjugates at 4 °C (solid lines) and 37 °C (dashed lines). The conjugates were diluted to a concentration of 0.10 nM in a buffer of 0.10 M HEPES, pH 7.7, and were incubated at 4 and 37 °C for a period of 1 month with periodic 10 μl sampling for measurement of residual chemiluminescence. Residual chemiluminescent activity values for the three labels, NSP-DMAE-HEG-NHS, **1c**, and **1a**, were 95%, 90%, and 78%, respectively, at 4 °C and, 70%, 56%, and 36%, respectively at 37 °C.



**Fig. 3.** Emission kinetics of acridinium ester–antibody (A) and acridinium ester–theophylline conjugates (B). The chemiluminescence kinetics of 0.01 pmol of the anti-TSH conjugates of NSP-DMAE-HEG-NHS label and the high light output label **1c** as well as 1 pmol of the theophylline conjugates **2a** and **2b** were measured using the AutoLumat Plus LB953 luminometer. Chemiluminescence was measured by integration in 50 intervals of 0.1 s for a total light collection time of 5 s. All conjugates emitted  $\geq 90\%$  of total light in 2 s.

NSP-DMAE: Yield = 1.68 g (66%) from acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  494.4 (M+H)<sup>+</sup> (494.4 calculated).

NSP-2-OMe-DMAE: Yield = 28 mg (24%) from 2-methoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  524.7 (M+H)<sup>+</sup> (524.6 calculated).

NSP-3-OMe-DMAE: Yield = 25 mg (47%) from 3-methoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  525.0 (M+H)<sup>+</sup> (524.6 calculated).

NSP-4-OMe-DMAE: Yield = 35 mg (13%) from 4-methoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  525.0 (M+H)<sup>+</sup> (524.6 calculated).

NSP-2,4-(OMe)<sub>2</sub>-DMAE: Yield = 20 mg (11%) from 2,4-dimethoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  555.2 (M+H)<sup>+</sup> (554.6 calculated).

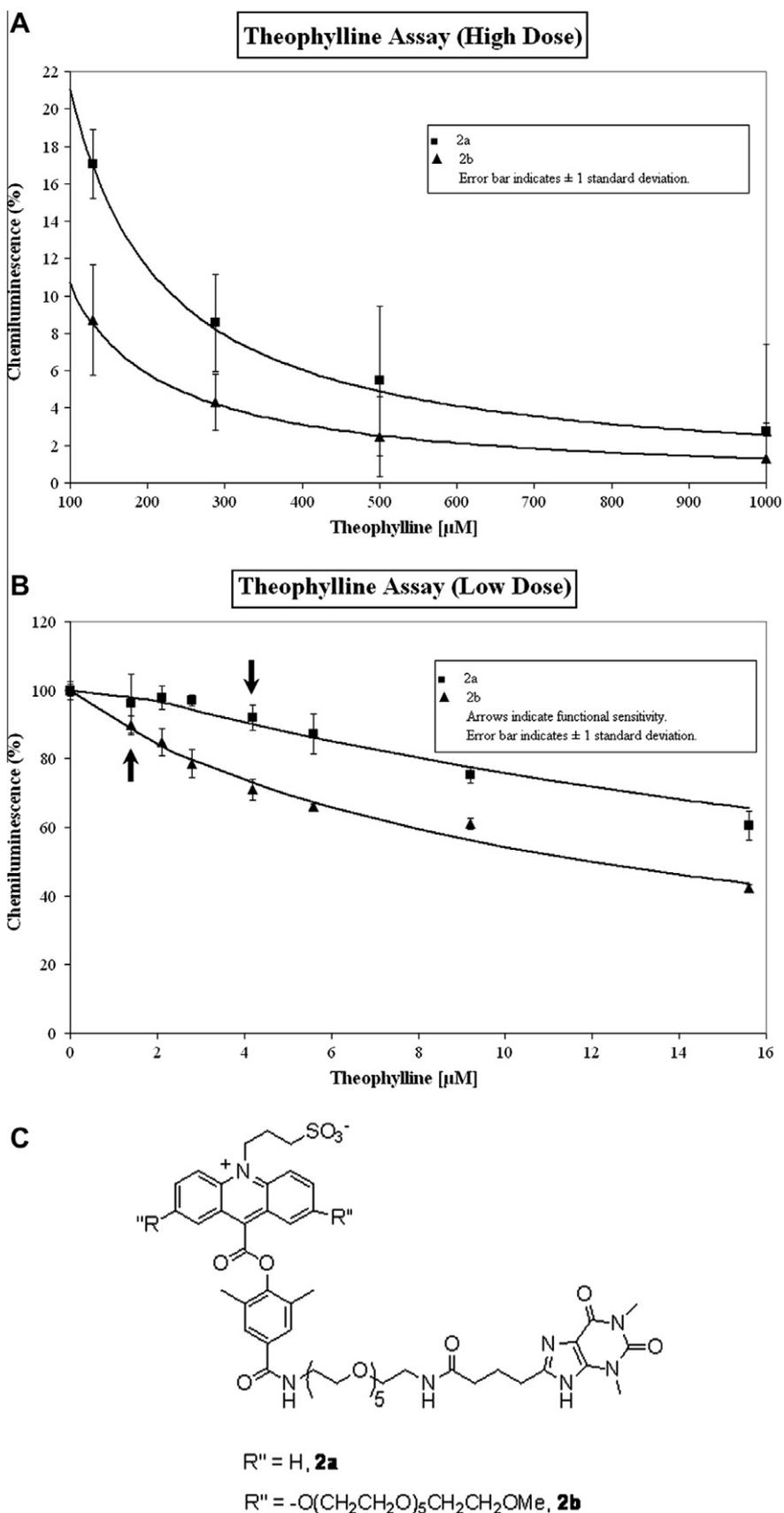
NSP-2,5-(OMe)<sub>2</sub>-DMAE: Yield = 25 mg (5.5%) from 2,5-dimethoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  555.1 (M+H)<sup>+</sup> (554.6 calculated).

NSP-2,7-(OMe)<sub>2</sub>-DMAE: Yield = 25 mg (72%) from 2,7-dimethoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  555.1 (M+H)<sup>+</sup> (554.6 calculated).

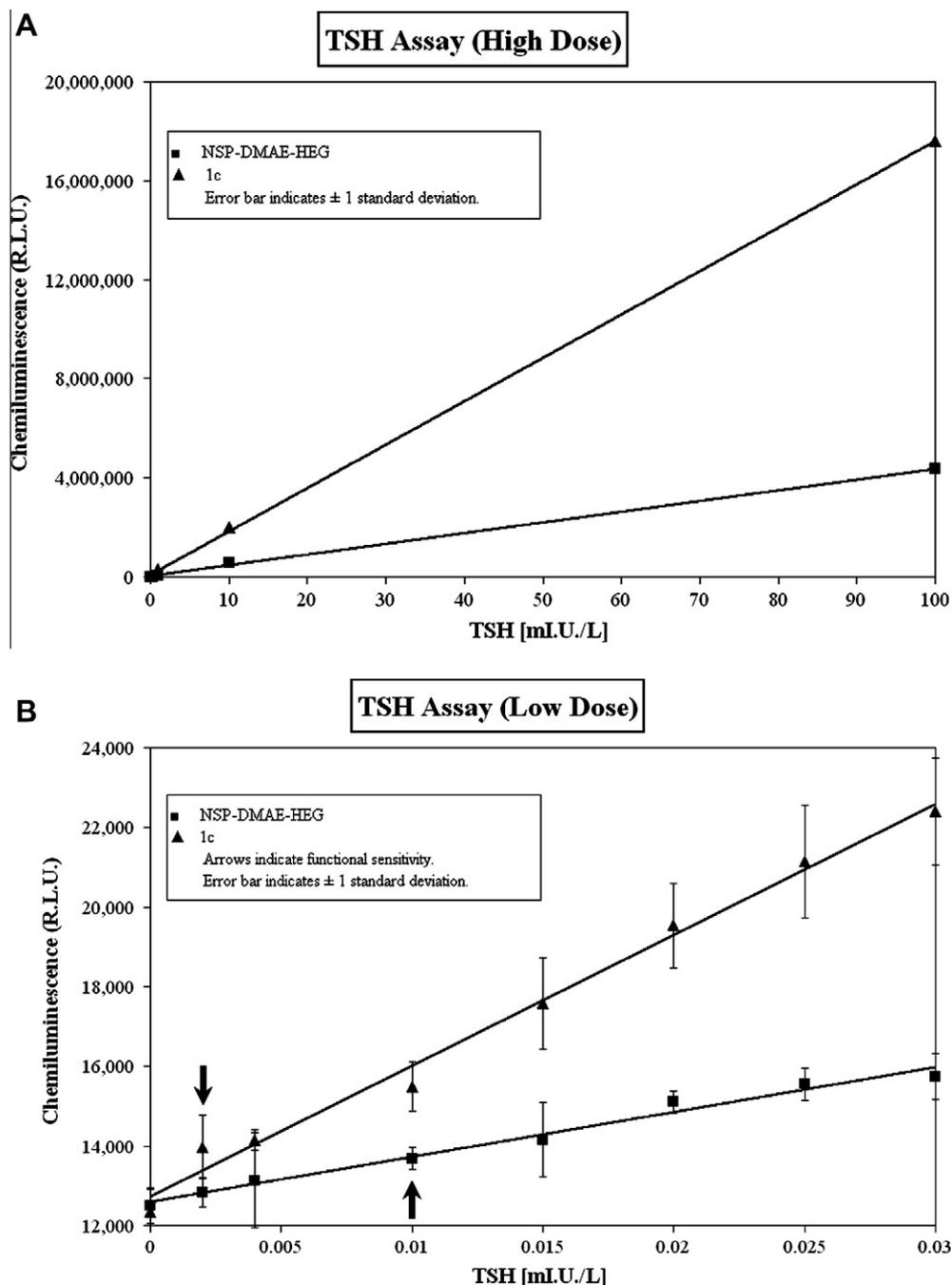
NSP-2,4,7-(OMe)<sub>3</sub>-DMAE: Yield = 20 mg (5%) from 2,4,7-trimethoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  585.0 (M+H)<sup>+</sup> (584.6 calculated).

NSP-DMAE-HEG-NHS: Yield = 120 mg (18%) from NSP-DMAE. MALDI-TOF MS  $m/z$  968.0 (M+H)<sup>+</sup> (968.1 calculated).

NSP-2,7-(OMe)<sub>2</sub>-DMAE-NHS, compound **1a**: Yield = 4 mg (22%) from NSP-2,7-(OMe)<sub>2</sub>-DMAE. MALDI-TOF MS  $m/z$  652.2 (M+H)<sup>+</sup> (651.7 calculated).



**Fig. 4.** Structures of acridinium ester–theophylline conjugates **2a** and **2b** (C) and dose–response curves (A and B) in the theophylline assay using these conjugates. The automated immunochemistry analyzer dispensed 14 serum standards of known amounts of theophylline into separate cuvetts followed by the addition of paramagnetic microparticles covalently derivatized with anti-theophylline antibody. To this was added 0.26 nM theophylline–acridinium ester conjugate solution. The assay proceeded for 7.5 min at 37 °C after which the solid phase was magnetically collected and washed with water to remove unbound acridinium ester conjugate followed by triggering of chemiluminescence. Functional sensitivity indicated by arrows was observed to be 4.2 μM for conjugate **2a** and 1.4 μM for the high light output conjugate **2b**.



**Fig. 5.** Dose–response curves (A and B) in the TSH assay using anti-TSH monoclonal antibody conjugates of the labels NSP-DMAE-HEG-NHS and the high light output label **1c**. The automated immunochemistry analyzer dispensed 12 serum standards of known amounts of TSH into separate cuvettes followed by the addition of 2.2 nM anti-TSH antibody–acridinium ester conjugate solution to each cuvette. The assay then proceeded for 5.0 min at 37 °C. To this was added paramagnetic microparticles covalently derivatized with sheep anti-TSH polyclonal antibody. The assay proceeded for another 5.0 min at 37 °C after which the solid phase was magnetically collected and washed with water to remove unbound acridinium ester conjugate. Functional sensitivity indicated by arrows was observed to be 0.010 mL U/L with the NSP-DMAE-HEG-NHS label and 0.002 mL U/L of TSH with the high light output label **1c**.

Compound **1b**: Yield = 23 mg (15%) from 2,7-dimethoxy acridine-9-carboxylic acid. MALDI-TOF MS  $m/z$  1084.0 (M+H)<sup>+</sup> (1083.2 calculated).

Compound **1c**: Yield = 10.7 mg (39%) from compound **1b**. MALDI-TOF MS  $m/z$  1557.8 (M+H)<sup>+</sup> (1556.7 calculated).

Theophylline conjugate **2a**: Yield = 3.6 mg (42%) from NSP-DMAE-HEG. MALDI-TOF MS  $m/z$  1005.5 (M+H)<sup>+</sup> (1005.1 calculated).

Theophylline conjugate **2b**: Yield = 4.0 mg (67%) from amine intermediate of compound **1c**. MALDI-TOF MS  $m/z$  1595.3 (M+H)<sup>+</sup> (1593.8 calculated).

#### General procedure for protein labeling with acridinium esters

In this study, two conjugate partners were chosen for derivatization with the acridinium esters whose properties were to be compared: theophylline and anti-TSH monoclonal antibody. Theophylline is a small molecule analyte that is amenable to measurement by immunoassay using a competitive assay format. We prepared two acridinium ester conjugates (**2a** and **2b**, Fig. 4) of commercially available 8-carboxypropyltheophylline using the amine derivatives of NSP-DMAE-HEG and the high light output label **1c** [16,17]. Anti-TSH is a murine monoclonal antibody with

binding affinity for TSH. Likewise we prepared two acridinium ester conjugates using NSP-DMAE-HEG-NHS and the high light output label **1c** (Fig. 1). The anti-TSH murine monoclonal antibody (1 mg, 6.67 nmol, stock solution 5 mg/ml, 0.2 ml) was diluted with 300  $\mu$ l of 0.1 M sodium phosphate, pH 8. The protein solution was treated with DMF solutions of various acridinium esters as follows: for labeling with 20 eq NSP-DMAE-HEG-NHS ester, 63  $\mu$ l of a 2 mg/ml DMF solution of the compound was added and, for labeling with 20 eq of compound **1c**, 43  $\mu$ l of a 4 mg/ml DMF solution was added. The labeling reactions were stirred at 4 °C for 16 h and were then transferred to 2-ml Amicon filters (MW 30,000 cutoff) and diluted with 1.5 ml deionized water. The volume was reduced to  $\sim$ 0.1 ml by centrifuging at 4500g. The concentrated conjugate solutions were diluted with 2 ml deionized 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 deionized 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 antibody and the labeled antibody. 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. The number of labels per antibody molecule using NSP-DMAE-HEG-NHS and compound **1c** was the same at eight labels per antibody using a label input of 20 eq. Protein concentration was determined by a commercial colorimetric assay from Bio-Rad [22].

#### *Instrumentation and chemiluminescence measurements of acridinium esters and conjugates*

Chemiluminescence measurements for acridinium esters and their conjugates were made on several instruments, all employing the same chemiluminescence triggering chemistry. Immunoassay data for acridinium ester conjugates were collected on a ACS:180<sup>®</sup> system, an automated immunodiagnosics analyzer from Siemens Healthcare Diagnostics. (The ACS:180 system has now been replaced with a Siemens Healthcare Diagnostics' ADVIA Centaur<sup>®</sup> CP immunoassay system which uses the same acridinium ester labels.) Quantum yield data for acridinium esters and chemiluminescence stabilities for conjugates were measured on the Magic Lite Analyzer<sup>™</sup> (MLA) chemiluminescence analyzer from Siemens Healthcare Diagnostics. Chemiluminescence kinetics was measured on the AutoLumat Plus LB953 chemiluminescence analyzer from Berthold Technologies. Chemiluminescence spectral emission measurements were made using the FSSS (fast spectral scanning system) camera from Photoresearch Inc. On all instruments the chemiluminescence reaction was initiated with the sequential addition of Reagent 1, a solution of 0.5% hydrogen peroxide in 0.1 N nitric acid, and Reagent 2, a solution of 0.25 N sodium hydroxide with an approximately 7 mM concentration of the cationic surfactant cetyltrimethylammonium chloride. Light was measured for a total of 5 s. The output from the luminometer instrument was expressed as RLUs (relative light units). These values were normalized to that of NSP-DMAE carboxylic acid when comparing the acridinium ester compounds or NSP-DMAE-HEG conjugates when comparing the theophylline or anti-TSH antibody conjugates.

#### *Quantum yield measurements*

Chemiluminescence from the various acridinium compounds listed in Table 1 was measured as described above. A solution, typically 1 mg/ml in DMF, of the various HPLC-purified compounds,

was serially diluted into 10 mM phosphate, pH 8, containing 0.15 M sodium chloride, 0.05% BSA, and 0.01% sodium azide and then chemiluminescence measurements were conducted by triggering the chemiluminescence from 25  $\mu$ l of the diluted acridinium compound solution. Likewise, emission measurements for acridinium ester conjugates were made using 10  $\mu$ l of diluted conjugate solution. The relative quantum yield of NSP-DMAE carboxylic acid was assigned a value of 1.0. Relative quantum yields of the acridinium esters in comparison to NSP-DMAE carboxylic acid are listed in Table 1. The relative light outputs for both the theophylline conjugate **2b** and the anti-TSH antibody conjugate of compound **1c** were observed to be double that of the corresponding NSP-DMAE-HEG conjugates.

#### *Emission wavelength measurements*

Visible wavelength emission spectra from the acridinium compounds were measured using the FSSS camera. In a typical measurement, 25–50  $\mu$ l of a 1 mg/ml DMF solution of the acridinium compound was diluted with  $\sim$ 0.3 ml of reagent 1. Just prior to the addition of reagent 2, the shutter of the camera was opened and light was collected for 5 s. The output of the instrument is a graph of light intensity versus wavelength. Emission maximum for each compound is listed in Table 1.

#### *Chemiluminescence stability*

The chemiluminescence stabilities of anti-TSH antibody conjugates of the two high output labels **1a** and **1c** were compared with that of the NSP-DMAE-HEG conjugate. The anti-TSH conjugates of these labels were diluted to a concentration of 0.10 nM in a buffer of 0.10 M Hepes (sodium *N*-2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate), 0.15 M sodium chloride, 7.7 mM sodium azide, 1.0 mM tetrasodium EDTA (sodium ethylenediaminetetraacetate), 12 mM Triton X-100 (*t*-octylphenoxypolyethoxyethanol), 76  $\mu$ M BSA, 97 nM amphotericin B, 5.2  $\mu$ M gentamicin sulfate, 6.7  $\mu$ M murine IgG (mouse immunoglobulin G), 67 nM sheep IgG, 5.3 mg/L murine serum, 50 mg/L Antifoam B (Sigma Cat. No. A5757), pH 7.7, and were incubated at 4 and 37 °C for a period of 1 month with periodic 10  $\mu$ l sampling for measurement of chemiluminescence. Residual chemiluminescence for each sampling time was calculated as a percentage of the initial chemiluminescence.

#### *Emission kinetics*

A chemiluminescence kinetics curve represents the time in which the light from acridinium ester labels is emitted during the chemiluminescence reaction. The chemiluminescence kinetics of 0.01 pmol of the anti-TSH conjugates of NSP-DMAE-HEG-NHS label and compound **1c** as well as 1 pmol of the theophylline conjugates **2a** and **2b** were measured using the AutoLumat Plus LB953 luminometer. Chemiluminescence was measured by integration in 50 intervals of 0.1 s for a total light collection time of 5 s. Chemiluminescence kinetics curves were calculated as a percentage of the total accumulated chemiluminescence for each successive 0.1 s time interval.

#### *Theophylline and TSH assays*

The potential for enhancement of immunoassay functional sensitivity using the higher quantum yield, hydrophilic acridinium ester label **1c** was tested on a Siemens Healthcare Diagnostics' ACS:180 system, an automated immunochemistry analyzer. (The ACS:180 system has now been replaced with Siemens Healthcare Diagnostics' ADVIA Centaur CP immunoassay system which uses

the same acridinium ester labels.) Two different immunoassay formats were used to test for enhancement of functional sensitivity. The theophylline assay was run as an example of a competitive, single-site immunoassay reflecting a general immunoassay design for the detection or quantitation of relatively low molecular weight molecules. The TSH assay was run as an example of a sandwich, two-site immunoassay as a general immunoassay design for the detection or quantitation of macromolecules.

In the theophylline assay, the higher quantum yield, more hydrophilic conjugate **2b**, which was observed to have twice the light output, was compared with the lower quantum yield conjugate **2a**. The automated immunochemistry analyzer automatically performed the following steps for the theophylline assay. First, 0.020 ml of each of 14 serum standards of known amounts of theophylline was dispensed into separate cuvetts. These standards contained respectively 0, 1.40, 2.10, 2.80, 4.20, 5.60, 9.21, 15.6, 32.7, 68.3, 129, 288, 500, and 1000  $\mu\text{M}$  concentrations of theophylline. Then 0.450 ml of theophylline assay solid phase, containing magnetically separable, paramagnetic microparticles covalently derivatized with 8.7 pmol of anti-theophylline antibody, was dispensed to each cuvette. To this was added 0.100 ml of 0.26 nM theophylline–acridinium ester conjugate solution. The assay proceeded for 7.5 min at 37 °C after which the solid phase was magnetically collected and washed with water to remove unbound acridinium ester conjugate. Chemiluminescence values corresponding to each theophylline standard concentration were normalized to the percentage of chemiluminescence measured in the zero standard. The assay results are shown in graphical form in Fig. 4A and B. The amount of theophylline in a standard is inversely correlated to the amount of the theophylline–acridinium ester conjugate that will bind to the solid phase in the assay and likewise inversely correlated to the resulting chemiluminescence. In the competitive-type assay the ratio of the binding capacity of the solid phase for a fixed concentration of acridinium ester conjugate in the absence of unlabeled theophylline is expressed here as the percentage of the bound chemiluminescence for the zero standard to the total chemiluminescence input per assay (B0/T%).

Likewise, anti-TSH monoclonal antibody conjugates of the labels NSP-DMAE-HEG-NHS and **1c** were compared in the TSH assay, where the conjugates were diluted to a concentration of 2.2 nM in a buffer of the same composition as that described above for the analysis of stability. The automated immunochemistry analyzer automatically performed the following steps for the TSH assay. First, 0.200 ml of each of 12 serum standards of known amounts of TSH was dispensed into separate cuvetts. These standards contained respectively 0, 0.002, 0.004, 0.010, 0.015, 0.020, 0.025, 0.030, 0.10, 1.0, 10 and 100 mL U/L concentrations of TSH. The instrument then dispensed 0.100 ml of 2.2 nM anti-TSH antibody–acridinium ester conjugate solution to each cuvette. The assay then proceeded for 5.0 min at 37 °C. To this was added 0.225 ml TSH assay solid phase, containing magnetically separable, paramagnetic microparticles covalently derivatized with sheep anti-TSH polyclonal antibody. The assay proceeded for another 5.0 min at 37 °C after which the solid phase was magnetically collected and washed with water to remove unbound acridinium ester conjugate. Chemiluminescence was determined for each TSH standard. The assay results are illustrated in Fig. 5 at high dose (panel A) and low dose (panel B) TSH. The amount of TSH in a standard is correlated to the amount of anti-TSH antibody–acridinium ester conjugate that will bind to the solid phase in the assay and consequently to the resulting chemiluminescence.

For both assays standard curve data were fitted to four parameter logistic (4PL) splines. Precision was measured as standard deviation of chemiluminescence or the standard deviation relative to the mean chemiluminescence as a percentage. Functional sensitivity for both assays is defined here as the lowest measured the-

ophylline or TSH concentration distinguishable by at least two standard deviations from the zero concentration standard. Enhancement of functional sensitivity is a decrease in the lowest detectable theophylline or TSH concentration as result of using one kind of acridinium ester label relative to another. FNSB (fractional nonspecific binding) is defined here as the ratio of the chemiluminescent signals associated with nonspecific binding versus specific signal in the assay. FNSB in the competitive-type theophylline assay is the ratio of the chemiluminescence corresponding to a very high concentration of theophylline to the total input chemiluminescence per assay. FNSB in the sandwich-type TSH assay is the ratio of the chemiluminescence corresponding to the 0 mL U/L TSH standard to the total input chemiluminescence per assay.

## Results and discussion

### Acridinium ester structure and light emission

Light emission from acridinium esters is triggered with alkaline peroxide which results in the formation of excited state acridone [7]. This excitation process is not well understood especially pertaining to variations in acridinium ester structure and it is difficult to predict how such structural perturbations will affect overall light output. Strong electron-donating groups such as dimethylamino or phenoxide moieties attached to C-2 of the acridinium ring shift the emission wavelength of the acridinium esters into the red (emission wavelength maximum  $\geq 600$  nm) [18], suggesting a smaller energy gap between the ground and the excited state of the light-emitting acridone moiety. However, the response of conventional photomultiplier tube detectors is poor at these wavelengths [19], making it difficult to determine whether this smaller energy gap increases the yield of excited state acridone. Acridinium esters with extended conjugated systems attached to the acridinium ring also shift emission wavelength maxima to longer wavelengths but their conformational flexibility reduces their quantum yields [18]. Consequently, in the current empirical study, we examined the effect of weaker electron-donating methoxy groups in the acridinium ring of acridinium esters, on relative light output and emission wavelength maximum (Table 1). The syntheses of the various acridinium esters and their conjugates have been described previously [16,17] and the numbering system for the acridinium ring is indicated in Fig. 1. The compounds listed in Table 1 are carboxylic acid derivatives, analogous in structure to NSP-DMAE (Fig. 1, carboxylic acid derivative of NSP-DMAE-NHS) with methoxy groups attached to various positions of the acridinium ring. Thus all compounds contained the same phenolic ester leaving group and displayed similar emission kinetics. The total light emitted over 5 s by a fixed quantity of each methoxy-substituted compound was compared with that of the parent compound NSP-DMAE, which was assigned a relative quantum yield value of one. Emission wavelength maxima were measured separately using a FSSS camera as described under Materials and methods.

When compared with the parent compound NSP-DMAE, placement of a single methoxy group at C-2 of the acridinium ring more than doubled the light output and shifted the emission wavelength maximum of the acridinium ester from 430 to 458 nm. On the other hand both the 3-methoxy and the 4-methoxy-substituted, acridinium ester regioisomers exhibited depressed light output which was only a third of the parent compound. The 3-methoxy isomer also exhibited a hypsochromic shift in emission wavelength maximum to 418 nm whereas the 4-methoxy substituent induced a substantial bathochromic shift in the acridinium ester's emission wavelength maximum to 478 nm, despite lowering overall light output. Among the disubstituted compounds, the 2,7-dimethoxy-

substituted acridinium ester displayed the greatest increase in light output of almost threefold compared to the parent compound and its emission wavelength maximum was also shifted to 484 nm. Interestingly, replacement of the methyl ethers in this analog with hydrophilic methoxyhexa(ethylene)glycol ethers (compound **1b** in Table 1) maintained the high light output as well as the emission wavelength maximum. Thus, the increase in light output appears to be correlated to the presence of alkoxy groups at C-2 and C-7 of the acridinium ring rather than any specific type of phenolic ether structure.

Alkaline-peroxide-induced light emission from acridinium esters in the presence of a cationic surfactant, overall, is a complex process involving many steps including partitioning of the reaction components in the surfactant aggregates, chemical reaction, formation of the excited state acridone, its partitioning in the surfactant aggregates, and ultimately light emission. The results in Table 1 thus represent a sum measurement of all these processes. For the sake of simplicity, if we ignore the role of the surfactant, then the quantum yield of an acridinium ester can be considered minimally to be the product of the yield in the chemical reaction, the excitation process, and the fluorescence quantum yield of the light-emitting acridone. The quantum yield of *N*-methylacridone in water has been reported to be close to one [20], so it is unlikely that the enhanced light output observed with the 2-methoxy and the 2,7-dimethoxy-substituted acridinium esters is because of an increase in the fluorescence quantum yields of their respective methoxy-substituted, *N*-methylacridones. Similarly, because both these acridinium esters contain the same leaving group as the parent compound NSP-DMAE, increase in chemical yield is also an unlikely mechanism leading to increased light output. The most likely explanation for the observed increase in light yield for these compounds as well as compound **1b** is that the alkoxy groups at C-2 and/or C-7 of the acridinium ring improve the yield in the excitation process. Thus, compound **1b**, with hydrophilic methoxyhexa(ethylene)glycol ethers in the acridinium ring, behaves analogously to the hydrophobic 2,7-dimethoxy-substituted acridinium ester, because both compounds share the same electronic properties.

From Table 1, methoxy substitution at C-3 of the acridinium ring appears to “deactivate” the ring leading to the observed decrease in light output as well as a shift in emission wavelength maximum to shorter wavelengths. The effect of methoxy substitution at C-4 of the acridinium ring, however, is less clear because this mono-substituted analog shows decreased light output, yet its emission maximum is shifted to a longer wavelength. The deactivating nature (toward light output) of the C-4 methoxy group is also apparent when comparing the relative light yields of the 2,4 and 2,5-disubstituted compounds. The light-enhancing property of the C-2 methoxy group is suppressed by the addition of a second methoxy group either in the same ring at C-4 or at an equivalent position C-5 in the other ring of the three ring system. Similarly, increase in light output observed with the 2,7-dimethoxy-substituted acridinium ester is suppressed by the addition of a third methoxy group at C-4 of the acridinium ring as observed in Table 1.

Although our observations cannot be generalized to chemiluminescent labels that have different reaction mechanisms, other acridinium compound labels with different leaving groups such as acridinium *N*-sulfonylcarboxamides [14] also exhibit increased light output by the placement of alkoxy groups at C-2 and C-7 in the acridinium ring [17].

#### Conjugate stability, light output, and emission kinetics

The chemiluminescence stability of acridinium ester conjugates of a murine anti-TSH monoclonal antibody is shown in Fig. 2 which compares the NSP-DMAE-HEG-NHS label (no acridinium ring sub-

stitution) with the two acridinium esters NSP-2,7-dimethoxy-DMAE-NHS (compound **1a**) and compound **1c**. The conjugates were incubated under identical conditions at 4 and 37 °C in HEPES buffer at pH 7.7. Residual chemiluminescence from the conjugates was measured periodically to determine the extent of acridinium ester decomposition. Compared to the conjugate of the parent label NSP-DMAE-HEG-NHS, conjugates of both the high light output labels **1a** and **1c** were observed to be less stable as indicated by the data in Fig. 2. However, the increased bulk of the methoxy(hexaethylene)glycol ethers in compound **1c** alleviated this instability compared to compound **1a** which only contains simple methyl ether substituents in the acridinium ring. At 30 days at 37 °C, the conjugate of the NSP-DMAE-HEG-NHS label was observed to retain close to 70% of its chemiluminescent activity whereas the conjugate of compound **1c** was slightly less stable and retained approximately 56% of its chemiluminescent activity. The conjugate of compound **1a** (methoxy-substituted acridinium ester) was significantly less stable and retained only 36% of its activity. At 4 °C nominal temperature storage conditions the chemiluminescence stabilities ranked from most stable to least stable were 95% for the conjugates of NSP-DMAE-HEG-NHS label, 90% for compound **1c**, and only 78% for compound **1a**. We attribute the increased stability of compound **1c** over **1a** to a remote steric effect of the bulky methoxy(hexaethylene) glycol ethers which appears to suppress hydrolysis of the phenolic ester. Other alkoxy groups with increased steric demand also displayed improved stability (unpublished data). From a practical point of view, the increased stability and hydrophilicity imparted by the bulky methoxy(hexaethylene)glycol ether substituents in **1c** make it an attractive high light output label for improving immunoassay sensitivity in automated immunochemistry analyzers where reagent stability and extended shelf life of reagents are important.

Emission kinetics of the antibody conjugates of the two labels NSP-DMAE-HEG-NHS and compound **1c** as well as the two theophylline conjugates **2a** and **2b** derived from the same labels, respectively, are shown in Fig. 3. The synthesis of the two theophylline conjugates has been described previously [16,17]. The data indicate that antibody and theophylline conjugates of both labels exhibit similar emission kinetics with  $\geq 90\%$  of the light emitted in 2 s.

#### Theophylline assay

In this assay, acridinium ester–theophylline conjugates are used to measure the concentration of theophylline in a sample through titration of the remaining unoccupied theophylline binding sites of anti-theophylline antibody immobilized on the solid phase. As indicated in Fig. 4, in the assay, the measured chemiluminescent signal is inversely correlated with the concentration of theophylline and therefore it is not readily apparent that increasing the light output of the acridinium ester label would have any effect on assay sensitivity. However, in this assay the hydrophilic, high light output-labeled theophylline conjugate **2b** exhibited a steeper slope at low dose with equivalent precision, and at high dose a twofold lower FNSB (defined here as the ratio of the chemiluminescent signals associated with nonspecific binding versus specific signal in the assay) as seen in Fig. 4. Thus, the observed FNSB of 0.0063 for conjugate **2b** was significantly lower than that of conjugate **2a** whose FNSB was measured to be 0.012. Compared with the conjugate **2a** (NSP-DMAE-HEG label), by using conjugate **2b** in the automated assay, the limit of functional sensitivity decreased from 4.2 to 1.4  $\mu\text{M}$ , reflecting a threefold improvement. Both conjugates **2a** and **2b** also exhibited identical binding affinity of 48% and 48.3% (B0/T%), respectively, for the antibody immobilized on the solid phase but conjugate **2b** was observed to have double the light output of conjugate **2a**. Thus with binding affinity and precision being roughly equivalent, a combination of increased light output and

decreased FNSB of the conjugate **2b** translated to an overall better curve shape in this competitive immunoassay and an improvement in assay sensitivity as seen with the low dose standard curve in Fig. 4 (panel B).

#### TSH assay

TSH is a clinically important marker for thyroid function and is commonly measured by immunoassay. In the current assay, TSH was measured in a sandwich format using two different antibodies directed against different epitopes on the TSH molecule, where the capture antibody resided on the solid phase and the signaling antibody was labeled with acridinium ester. Under identical conditions, we compared the performance of the acridinium ester label NSP-DMAE-HEG-NHS with that of the hydrophilic, high light output label **1c**, labeled to the same extent (8 labels per antibody) on the same signaling antibody. Light output from the conjugate of **1c** was observed to be double that of the NSP-DMAE-HEG-NHS label and both conjugates displayed similar emission kinetics as indicated in Fig. 3. The observed dose responses both at high TSH dose (panel A) and low TSH dose (panel B) are illustrated in Fig. 5. In the assay FNSB decreased by a factor of twofold from  $1.1 \times 10^{-4}$  to  $5.0 \times 10^{-5}$  when comparing the anti-TSH antibody conjugates of the two labels NSP-DMAE-HEG-NHS and compound **1c**, respectively. As a consequence of both equivalent assay precision for both labels over the whole standard curve, the combination of increased light output and lower FNSB for the label **1c** resulted in a fivefold improvement in functional sensitivity in the TSH assay from 0.010 mL U/L down to 0.002 mL U/L of TSH.

#### Conclusions

Chemiluminescent acridinium ester technology has improved tremendously over the years and we now have a much better understanding of the various factors affecting light emission including emission kinetics, wavelength, and quantum yield. In the present study, we have identified structural features in acridinium esters that lead to increased light output. Specifically, placement of electron-donating, hydrophilic alkoxy groups derived from hexa(ethylene)glycol, at C-2 and C-7 of the acridinium ring not only increase light output but also enhance aqueous solubility of the label, leading to lower nonspecific binding. Application of the new label in automated immunoassays led to an increase in assay sensitivity for both competitive and sandwich assays as demonstrated here for theophylline and TSH. Although the assays described in the current study are not commercial assays, an acridinium ester label analogous in structure to **1c** is used in the Siemens Healthcare Diagnostics' ADVIA Centaur, high-sensitivity troponin assay (TnI-Ultra<sup>®</sup> assay) which has a mean detectable concentration of 0.006 ng/ml when compared to 0.03 ng/ml for the ADVIA Centaur troponin assay (cTnI assay), that uses the label NSP-DMAE-NHS.

Further improvements in the quantum yields of acridinium esters will certainly be welcome especially for the measurement of low abundance markers [21]. In this regard we need a better

understanding of the various factors influencing the excitation step during the chemiluminescent reaction of acridinium esters.

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