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PAPER

Zwitterionic reagents for labeling, cross-linking and improving the performance of chemiluminescent immunoassays†

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Improving reagent performance in immunoassays both to enhance assay sensitivity and to minimize interference are ongoing challenges in clinical diagnostics. We describe herein the syntheses of a new class of hydrophilic reagents containing sulfobetaine zwitterions and their applications. These zwitterionic reagents are potentially useful for improving the properties of immunoassay reagents. We demonstrate for the first time that zwitterion labeling is a general and viable strategy for reducing the non-specific binding of proteins to microparticles and, to improve the aqueous solubility of hydrophobic peptides. We also describe the synthesis of zwitterionic cross-linking reagents and demonstrate their utility for peptide conjugation. In automated, chemiluminescent immunoassays, improved assay performance was observed for a hydrophobic, small analyte (theophylline) using an acridinium ester conjugate with a zwitterionic sulfobetaine linker compared to a hexa(ethylene)glycol linker. Sandwich assay performance for a large analyte (thyroid stimulating hormone) was similar for the two acridinium ester labels. These results indicate that zwitterions are complementary to poly(ethylene)glycol in improving the aqueous solubility and reducing the non-specific binding of chemiluminescent acridinium ester conjugates.

Introduction

Improving the aqueous solubility of reagents, preventing aggregation, lowering non-specific binding and minimizing non-specific interactions with sample components in serum or whole blood, are ongoing challenges in clinical diagnostic immunoassays. Commercial immunoassays often use hydrophobic chemiluminescent labels such as luminol or acridinium compounds[1] that have limited aqueous solubility and these labels can exacerbate the non-specific binding of proteins such as antibodies. Recombinant proteins and polypeptides often exhibit poor aqueous solubility as well as a tendency to form insoluble aggregates because of misfolding or denaturation thereby limiting their usefulness in both the pharmaceutical and diagnostics industry.[2] Immunoassay reagents can also interact with sample components in a non-specific manner giving rise to false positives in assays that have been attributed to natural, weakly-binding, polyspecific antibodies.[3] Blocking reagents are often used to alleviate this problem.[3]

One approach that has been used to improve the solubility of peptides and proteins is by labeling with the hydrophilic, non-ionic polymer poly(ethylene)glycol (PEG).[4] PEG-modified proteins exhibit lower non-specific binding to surfaces[5] and PEG modification can confer beneficial improvements in the pharmacokinetic profiles of therapeutic proteins.[6] We have shown that PEG improves the aqueous solubility of chemiluminescent labels such as acridinium esters and that PEG-modified acridinium esters exhibit low non-specific binding and improved immunoassay performance.[7] PEG has also been advocated as a reagent to mitigate interference in immunoassays from endogenous antibodies.[8] In addition to these applications, PEG has also been used to devise inert surfaces that resist protein adsorption.[9]

Zwitterions represent a different class of molecules that are also very hydrophilic and perhaps more so than PEG.[10] Self-assembled monolayers (SAMs) with zwitterionic functional groups have been shown to be extremely resistant to protein adsorption.[11] More recently, a number of studies have reported that surfaces functionalized with zwitterionic polymers containing sulfobetaines and carboxybetaines resist protein adsorption and biofilm formation.[12] A direct comparison of PEG-modified and zwitterion-modified silica surfaces indicated that zwitterions and PEG are equally effective in resisting protein adsorption.[13] It has also been postulated that zwitterions would be chemically more stable than PEG owing to the latter's propensity for oxidative cleavage.[11] Zwitterions such as sulfobetaines with quaternary nitrogens are also electrically neutral (non-ionic) over a wide range of pH.

In addition to surface modification, zwitterionic compounds such as non-detergent sulfobetaines (NDSBs) have been shown

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to be useful as additives in assisting refolding of proteins such as BSA (bovine serum albumin), enzymes and antibodies[14] as well as improving the thermal stability of proteins.[15] Tolbert and co-workers have reported that site-specific modification of two aggregation-prone polypeptides with betaine (trimethylammonium moiety) significantly improved the aqueous solubility of the two polypeptides and inhibited aggregate formation.[16] All these studies suggest that zwitterionic labeling reagents would be useful for reducing the non-specific binding of proteins as well as alleviating the aqueous solubility of hydrophobic labels and antigens.

Although a number of PEG-modified reagents are available from commercial vendors, analogous zwitterionic reagents have not been described in the literature. We describe herein a new class of hydrophilic, labeling and cross-linking reagents containing sulfobetaine zwitterions that are conveniently synthesized using commercially available reagents. These zwitterionic reagents are useful for protein labeling to reduce their non-specific binding to microparticles as well as for improving the aqueous solubility of hydrophobic antigens such as peptides. Our studies are the first to describe zwitterion labeling of proteins as a general strategy for reducing their non-specific binding with practical applications especially in analytical immunochemistry.

We also describe the preparation of zwitterionic cross-linking reagents that are useful for peptide conjugation as well as for the preparation of hydrophilic chemiluminescent acridinium ester conjugates of hydrophobic analytes such as theophylline.[17] A comparison of immunoassay performance of acridinium ester conjugates containing either a PEG linker or a sulfobetaine zwitterion linker in both competitive (theophylline) and sandwich (thyroid stimulating hormone) assays suggest that zwitterions are more effective than PEG in improving assay performance for hydrophobic analytes and, are complementary to PEG in sandwich assays.

Results and discussion

Synthesis of zwitterionic reagents

The structures of zwitterionic reagents **1–5** synthesized in the current study are illustrated in Fig. 1 and the syntheses of the reagents are illustrated in Figures S1–S3 (supplementary material†). The zwitterionic, nucleophilic, cross-linker **3** was described previously and can be easily synthesized in gram quantities in a three step procedure from commercially available *N*,*N*-bis(3-aminopropyl)methylamine.[17] We have observed that this very polar linker is extremely useful for the synthesis of chemiluminescent acridinium ester conjugates (tracers for immunoassays) of hydrophobic analytes where limited aqueous solubility is a concern. The syntheses of the compounds in Fig. 1 were generally accomplished in a straightforward manner using commercially available reagents as described in the experimental section.

Compound **1**, an amine-reactive, zwitterionic reagent was synthesized from 4-dimethylaminobutyric acid as described in Figure S1 (supplementary material†). The carboxylic acid was first converted to the benzyl ester **i** using a published procedure.[18] The tertiary amine in compound **i** was then *N*-alkylated

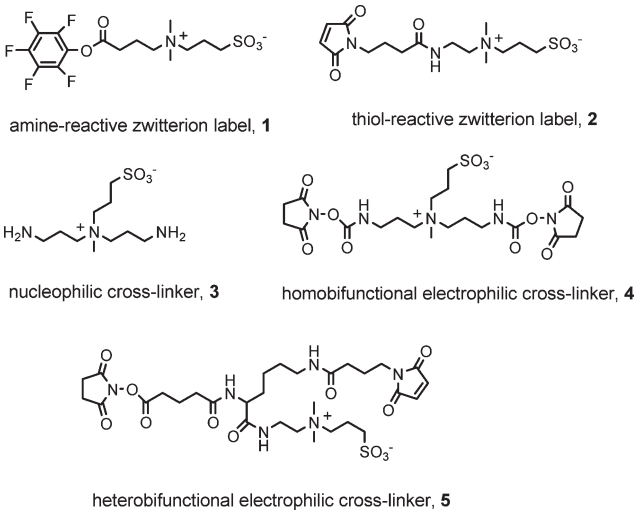


Fig. 1 Structures of zwitterionic reagents.

with 1,3-propane sultone to introduce the sulfobetaine zwitterion (compound **ii**). Hydrogenation of the benzyl ester gave compound **iii** which was converted to the pentafluorophenyl ester **1** and purified by preparative HPLC. The pentafluorophenyl reactive ester was selected instead of a *N*-hydroxysuccinimide ester because it nicely counterbalanced the very polar sulfobetaine moiety such that the final reagent **1** was soluble in polar aprotic solvents such as DMSO for labeling peptides and proteins. Moreover the pentafluorophenyl group also made it relatively easy to purify compound **1** by preparative HPLC with a UV detector.

The synthesis of compound **2**, a thiol-reactive zwitterionic reagent is described in Figure S2 (supplementary material†). The primary amine in commercially available *N*,*N*-ethylenediamine was protected as the benzyl carbamate **iv** followed by *N*-alkylation of the tertiary amine in **iv** using 1,3-propane sultone to give compound **v**. Hydrogenation of compound **v** gave compound **vi** which was converted to compound **2** by coupling with commercially available 4-maleimidobutyric acid followed by HPLC purification.

Syntheses of the cross-linking reagents **4** and **5** are illustrated in Figure S3 (supplementary material†). The homobifunctional cross-linker **4** was synthesized in one step by reacting compound **3**[17] with disuccinimidyl carbonate (DSC) followed by HPLC purification. The NHS ester of this cross-linking reagent is quite stable because of deactivation by the carbamate linkage but was observed to be readily reactive towards the α-amino group of peptides.

The heterobifunctional cross-linker **5** was synthesized using the commercially available lysine derivative *N*-α-*tert*-butoxycarbonyl-*N*-ε-benzyloxycarbonyl-lysine-*N*-hydroxsuccinimide ester as the scaffold. Initial reaction of this amino acid derivative with compound **iv** introduced the sulfobetaine zwitterion to give compound **vii**. Hydrogenation of the benzyl carbamate in **vii** gave compound **viii** with the exposed ε-amine which was then condensed with 4-malemidobutyric acid to give compound **ix**. Removal of the *tert*-butoxycarbonyl protecting group in **ix** followed by reaction of the α-amine of **x** with disuccinimidyl

glutarate (DSG) gave the cross-linking reagent **5** which was purified by preparative HPLC.

All the zwitterionic reagents illustrated in Fig. 1, were observed to be stable when stored at low temperatures (< −10 °C). They were also readily soluble in polar aprotic solvents such as dimethyl sulfoxide (DMSO) for labeling. (HPLC chromatograms of the synthetic intermediates and the final purified materials are shown in Figures S4–S11, supplementary material†).

Peptide and protein labeling with zwitterionic compound 1

The labeling of peptides with the zwitterionic reagent **1** is described in Fig. 2 and Figures S12–S14 (supplementary material†). We selected three peptides, penta(phenylalanine), the tetrapeptide Phe-Gly-Gly-Phe and penta(leucine) as representative of hydrophobic peptides with limited solubility in water and, studied the reactivity of reagent **1** towards the α-amino group of these peptides as well as the impact of zwitterion labeling on their aqueous solubility. The labeling reactions were conducted in a mixture of an organic solvent (DMSO) and sodium carbonate buffer, pH = 9, using a slight excess (approximately 2 equivalents) of zwitterion label **1**. Under these typical conditions used for peptide and protein labeling, hydrolysis of the pentafluorophenyl ester in zwitterion reagent **1** competes with reaction with the α-amino group of the peptides. As can be noted from Figures S12 and S13, the α-amino group in the peptides penta(phenylalanine) and Phe-Gly-Gly-Phe reacted readily with compound **1** affording excellent conversion. Conversion was lower (~60%, Figure S14), for the extremely hydrophobic peptide penta(leucine) presumably because of poorer solubility, accounting for the lower isolated yield (43%) of the zwitterion-labeled peptide **8**. On the other hand, the other two zwitterion-labeled peptides **6** and **7**, derived from penta(phenylalanine) and Phe-Gly-Gly-Phe respectively, were isolated in excellent yield by preparative HPLC.

Solubility was estimated by adding small quantities of deionized water to each of the lyophilized, zwitterion-labeled peptide until a clear solution was obtained. As indicated in Fig. 2, the labeled peptides **6–8** exhibited significant solubility in water at room temperature and at the indicated concentrations which gave homogeneous, clear and somewhat soapy solutions. The calculated solubility[19] of penta(phenylalanine) is reported to be 0.029 g L^{-1} (0.039 mM) in unbuffered water. Zwitterion labeling of the α-amino group with reagent **1** appears to increase the solubility > 10-fold. The calculated solubility of less hydrophobic tetrapeptide, Phe-Gly-Gly-Phe is 2.8 mM whereas the zwitterion-labeled peptide **7** was observed to have the same solubility. The calculated solubility of penta(leucine) in unbuffered water is very low at 0.00016 mM (9.3×10^{-5} g L^{-1}) whereas the zwitterion labeled peptide **8** was observed to be significantly higher at approximately 0.1 mM.

The facile synthesis of the zwitterionic reagent **1** and its reactivity suggests that this reagent could be of general use for improving the aqueous solubility of hydrophobic antigens containing reactive amines. Alternatively, compound **2**, which is a thiol-reactive zwitterionic reagent can be used to introduce the water-soluble sulfobetaine zwitterion for sulfhydryl-containing antigens.

To assess the reactivity of reagent **1** towards proteins, we selected bovine serum albumin (BSA) which is easily analyzed by MALDI-TOF mass spectroscopy for measurement of label incorporation. Following light labeling using 5 equivalents input of the acridinium ester 9-[[4-[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]-2,6-dimethylphenoxy]carbonyl]-10-(3-sulfopropyl)-acridinium (abbreviated as NSP-DMAE-NHS,[7] (Table 1), the labeled protein containing 1.4 labels, was further reacted with compound **1**. Using an input of 10 and 20 equivalents of compound **1**, mass spectral analysis of the labeled protein indicated

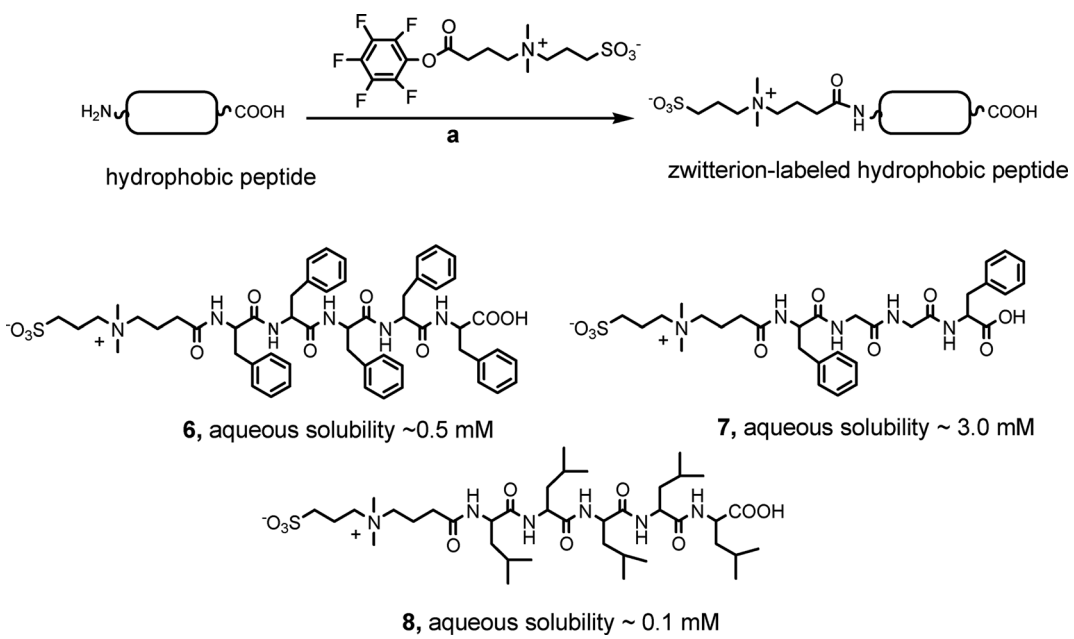


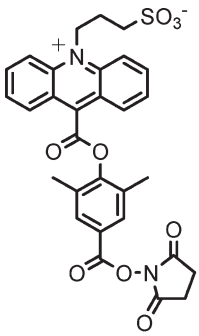
Fig. 2 Hydrophobic peptide labeling with the zwitterionic reagent **1**. Reagents: (a) dimethyl sulfoxide, 0.1 M sodium carbonate, pH 9.

Table 1 Non-specific binding of zwitterion-labeled proteins

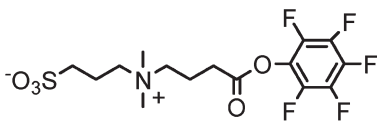
Fractional non-specific bindings for 0.070 mg of three solid phases of 1 pmol zwitterion-modified proteins labeled with NSP-DMAE

Protein	NSP-DMAE-AVD			NSP-DMAE-BGG			NSP-DMAE-FBN		
Solid phase	Unmodified FNSB (%)	Zwitterion-modified FNSB (%)	FNSB Reduction (fold)	Unmodified FNSB (%)	Zwitterion-modified FNSB (%)	FNSB Reduction (fold)	Unmodified FNSB (%)	Zwitterion-modified FNSB (%)	FNSB Reduction (fold)
PMP derivatized with sheep IgG	0.13	0.017	7.6	0.012	0.0068	1.8	0.0094	0.0071	1.3
Dynalbeads M-280 streptavidin	0.016	0.0083	1.9	0.013	0.0081	1.6	0.0062	0.0063	1.0
Dynalbeads M-270 streptavidin	6.3	0.052	121	0.18	0.039	4.6	0.037	0.012	3.1

Abbreviations used: PMP = paramagnetic particle; AVD = avidin; BGG = bovine gamma globulin; FBN = fibrinogen.



NSP-DMAE-NHS



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the incorporation of 5 and 8 zwitterion labels respectively. Similar results were noted using a mixture of bovine gamma globulins (BGG) as described in the experimental section. The reactivity of the pentafluorophenyl ester in reagent **1** towards proteins thus appears similar to the reactivity of *N*-hydroxysuccinimide (NHS) ester derivatives *i.e.* > 40% label incorporation.

Zwitterion-modified surfaces resist protein adsorption[12] and recently, Schlenoff and co-workers,[13] from a study of PEG and zwitterion-functionalized silica surfaces, have proposed a model wherein the main driving force for adsorption is largely entropic in nature. According to this model, protein adsorption to surfaces occurs due to the release of counter ions from both the protein and the surface during the adsorption process. The net free energy change is negative for such a process. For surfaces that are non-ionic, such as those modified by PEG or zwitterions, this mechanism is absent and consequently, the surfaces are resistant to protein adsorption. Regardless of the mechanism, based on an earlier empirical study, Whitesides and co-workers[11] outlined some general principles for designing a protein-resistant surface and they include (a) hydrophilic nature to minimize hydrophobic interactions, (b) overall electrical neutrality to avoid charge interactions and, (c) ability to accept but not donate hydrogen bonds. We have noted that these same principles are also useful for the modification of hydrophobic chemiluminescent labels such as acridinium esters to lower their non-specific binding and to improve their assay performance.[7]

While numerous studies have delineated the properties of zwitterion-modified surfaces,[11–13] there are no studies that have examined the impact of zwitterion labeling of proteins on their non-specific binding to surfaces. Moreover, while studies on protein adsorption to well defined surfaces offer important theoretical insights, nevertheless, in the practice of clinical diagnostics immunoassays (using chemiluminescent or fluorescent labels and magnetic microparticles), such simple situations are rarely encountered. Instead, microparticles are commonly used as the scaffold for the immobilization of a variety of binding molecules such as antibodies with different isoelectric points (pIs) or various antigens. Blocking proteins and surfactants are also usually added leading to a chemically heterogeneous, particle surface. Adding to the complexity is the immunoassay sample which is usually serum or whole blood.

In the current study, we wanted to determine whether zwitterion-labeling of proteins was a general strategy that could be employed for reducing their non-specific binding to magnetic microparticles that are commonly used in immunoassays. High non-specific binding is often encountered in immunoassays and is a limiting factor in improving assay sensitivity. We selected three proteins, egg white avidin (AVD), bovine gamma globulin (BGG) and fibrinogen (FBN) to study non-specific binding to microparticles that are commonly used in immunoassays. AVD is a basic protein[20] and the avidin–biotin interaction is commonly used in immunoassays. BGG is representative of polyclonal

antibodies that are commonly used in immunoassays. Finally, FBN is representative of a large protein that has been used to evaluate the properties of surfaces towards protein adsorption.[11]

For particles, we selected paramagnetic particles (PMPs) as well as two magnetic latex particles (MLPs) coated with streptavidin that are available from a commercial vendor (Invitrogen). The PMPs used in the current evaluation were, 1–10 micron-sized iron(III) oxide particles, coated with an anti-TSH antibody (TSH = thyroid stimulating hormone) on the amino-silanized particle surface using commonly used glutaraldehyde coupling chemistry. The MLPs used for the current evaluation were 2.8 micron, Dynal® M-280-streptavidin particles with bound biotinylated, polyclonal, goat anti-PTH antibody (abbreviated as "M-280" in Table 1) and Dynal® M-270-streptavidin particles with bound biotinylated, monoclonal, mouse anti-cTnI antibody (abbreviated as "M-270" in Table 1). These two MLPs had immobilized streptavidin on their surfaces which was then used to further immobilize, biotin-labeled antibodies capable of binding the analytes PTH (parathyroid hormone) or cTnI (cardiac troponin I) respectively. The intrinsic, surface characteristics of the three types of particles are quite different because of different functional groups. PMPs contain amines on their surfaces whereas M-270 particles have carboxylate surfaces. The M-280 particles have a more hydrophobic surface with a tosylate-activated polystyrene coating (Experimental section). The surfaces of all three particles were also immobilized with different proteins thus creating very heterogeneous surfaces.

The three proteins AVD, BGG and FBN were initially lightly labeled (5 equivalents input) with the chemiluminescent acridinium ester NSP-DMAE-NHS,[7] (Table 1). Chemiluminescence from the acridinium ester label was used to measure non-specific binding of the labeled proteins. A portion of each acridinium ester labeled conjugate was further labeled with 25 equivalents input of the zwitterionic compound **1** as described in the experimental section. The labeled conjugates, with and without zwitterion modification, were used for measurement of non-specific binding to the microparticles by recording the chemiluminescence from the acridinium ester label. The two types of particles (PMPs and MLPs) were mixed with solutions of the acridinium ester and zwitterion-labeled conjugates and were then magnetically separated, washed twice with water and then the chemiluminescence associated with the particles was measured. The results of these measurements are tabulated in Table 1 and details are described in the experimental section. The ratio of this chemiluminescence value in comparison to the total chemiluminescence input is referred to fractional non-specific binding (FNSB) and, in this case is a reflection of the resistance of the protein towards non-specific adsorption to the microparticles. FNSB is defined similarly in assays for analytes and is simply the ratio of background signal to total chemiluminescence input. For small analytes, background signal is measured at extremely high analyte concentration whereas for sandwich assays, chemiluminescence in the absence of analyte is the background signal. (Details of these measurements can be found in the experimental section.)

As indicated in Table 1, zwitterion-labeling of the basic protein AVD and BGG had a significant impact on their non-specific binding to the microparticles. For AVD, labeling with reagent **1** reduced its FNSB by an order of magnitude to PMP, two orders of magnitude to the M-270 microparticles and, by a factor of two for the relatively more hydrophobic M-280 particles. A similar trend was observed for BGG although the magnitude of reduction of non-specific binding was attenuated for this protein. For example on PMP and M-280 particles, an approximately 2-fold reduction in non-specific binding was observed whereas on M-270 particles, a 4.6-fold reduction in non-specific was noted. On the other hand the larger protein FBN showed only a slight attenuation of its non-specific binding to PMP and M-270 particles with zwitterion modification but the trend was similar to that observed for AVD and BGG.

The results outlined in Table 1 have broad implications. These results suggest that labeling of hydrophobic and/or basic proteins with zwitterionic reagents **1** (or **2**) is a viable and general strategy for reducing their non-specific binding to surfaces regardless of the nature of the surface.

Peptide cross-linking using zwitterionic cross-linking reagents 4 and 5

Cross-linking agents are commonly used for the preparation of conjugates and a variety of these reagents are available from commercial vendors. Typical cross-linking reagents contain two reactive groups such as electrophilic, nucleophilic or photoreactive groups that can either be the same (homobifunctional) or different (heterobifunctional). Most cross-linking reagents tend to be hydrophobic although some hydrophilic, PEG-modified reagents with oligo(ethylene)glycol linkers to alleviate aqueous solubility, are also available from commercial vendors. The two zwitterionic cross-linking reagents **4** and **5** (Fig. 1) however are much more polar with excellent aqueous solubility because of the strongly hydrophilic nature of the sulfobetaine zwitterion. We envision that these reagents would be especially useful for cross-linking hydrophobic antigens while at the same time improving their aqueous solubility.

The cross-linking of representative, hydrophobic synthetic peptides using the zwitterionic cross-linking reagents **4** and **5** is illustrated in Fig. 3 and 4. The cross-linking of the α-amino groups of the two peptides penta(phenylalanine) and the tetrapeptide Phe-Gly-Gly-Phe using reagent **4** containing two, amine-reactive, NHS ester groups is described in Fig. 3. Initial reaction of penta(phenylalanine) with a slight excess of **4** resulted in clean conversion to the labeled peptide intermediate **xi** which could be purified by preparative HPLC without compromising the second, reactive NHS ester in **xi**. Subsequent reaction with the second peptide Phe-Gly-Gly-Phe resulted in clean formation of the nonapeptide **9** with the zwitterionic reagent inserted between the two peptides (Figure S15, supplementary material†).

In a similar vein, cross-linking of the peptides penta(phenylalanine) and the sulfhydryl-containing dipeptide Cys-Gly using the zwitterionic reagent **5** was equally facile as described in Fig. 4. Initial reaction of penta(phenylalanine) with **5** gave intermediate **xii** with its maleimide group intact and subsequent reaction (without HPLC purification) with the sulfyhydryl-containing dipeptide resulted in quantitative conversion to the heptapeptide **10** containing the sulfobetaine zwitterion (Figure S16, supplementary material†).

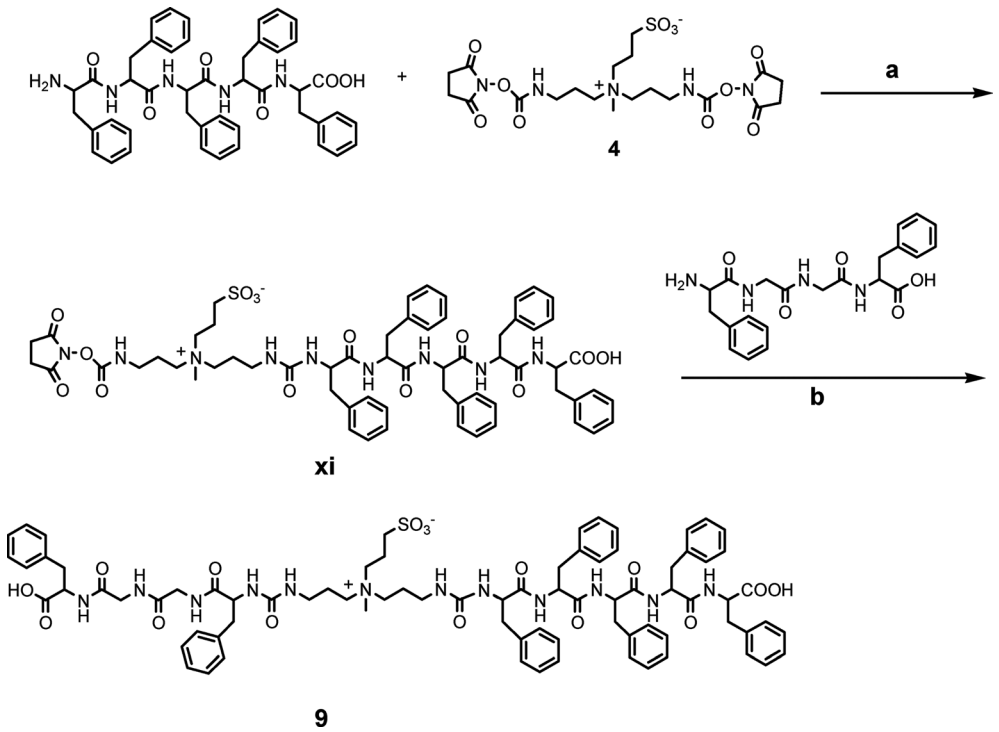

Fig. 3 Synthetic scheme of hydrophobic peptide cross-linking using zwitterionic, homobifunctional cross-linking reagent **4**. Reagents: (a, b) dimethyl sulfoxide, diisopropylethylamine.

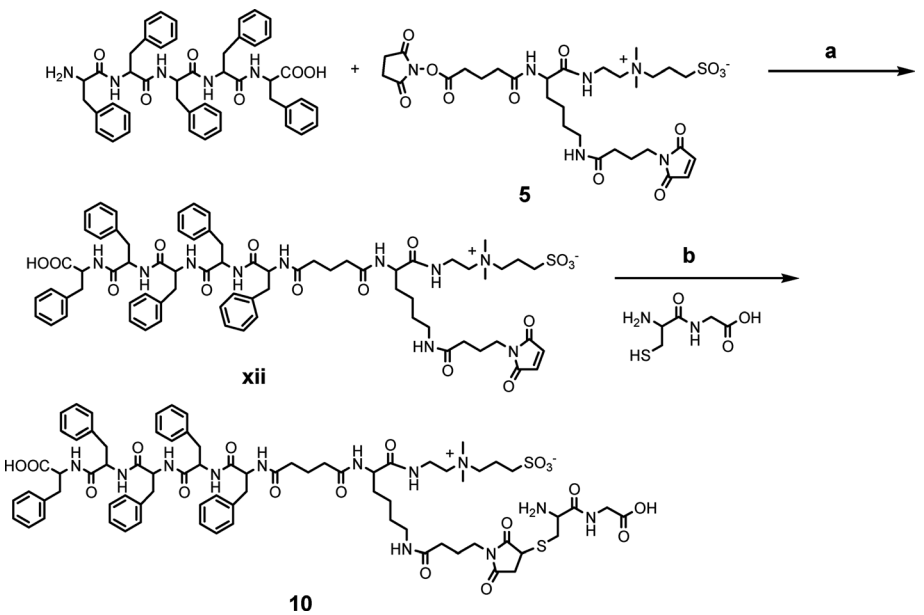

Fig. 4 Synthetic scheme for hydrophobic peptide cross-linking using zwitterionic, heterobifunctional cross-linking reagent **5**. Reagents: (a) dimethyl formamide, triethylamine; (b) dimethyl formamide.

Immunoassay performance

We reported previously that PEG-modified, acridinium ester labels containing alkoxy groups in the acridinium ring exhibit increased output and that these high light output labels can be used to enhance the sensitivity of automated, chemiluminescent, immunoassays.[7] In the current study we compared the effect of two hydrophilic linkers containing either PEG or a sulfobetaine zwitterion on immunoassay performance of acridinium ester labels in both competitive (theophylline) and sandwich (TSH)

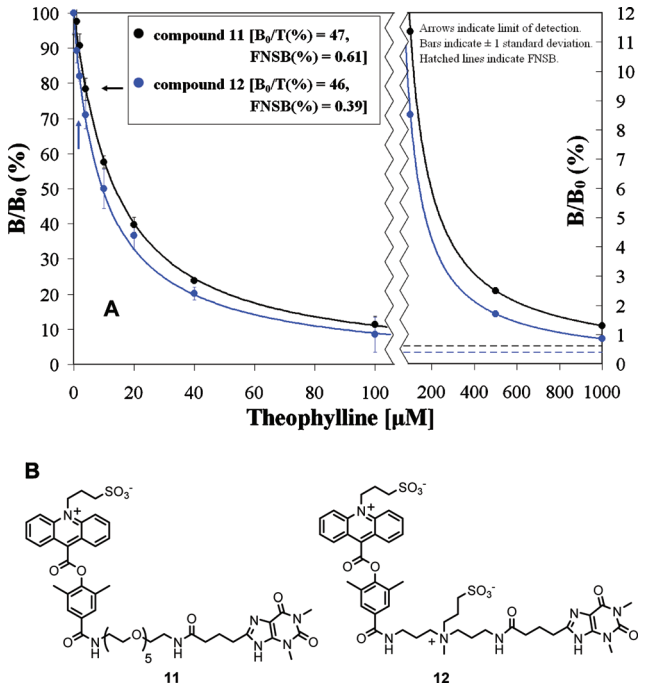

Fig. 5 Structures of chemiluminescent acridinium ester–theophylline conjugates **11** and **12** (panel B) and dose–response curve (panel A) in the automated theophylline assay using these conjugates. Conjugate **12** with zwitterion linker exhibited lower non-specific binding in the assay and improved assay sensitivity. **T** is the total chemiluminescence input; $\mathbf{B_0}$ is the chemiluminescence in the absence of theophylline and **B** is the chemiluminescence in the presence of theophylline. Arrows in the low-dose region of the graph indicate the limit of detection which was observed to be 2 μM for conjugate **12** and 4 μM for conjugate **11** reflecting a two-fold improvement in assay sensitivity. Dotted lines indicate FNSB of conjugates **11** (black line) and **12** (blue line). Standard deviations for assay data were calculated in Microsoft Excel using the built in standard deviation function (STDEV) for five replicates. Microsoft Excel uses the following function to calculate standard deviation

$$\left(\sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}}\right).$$

immunoassays. The purpose of this study was to determine whether the nature of the hydrophilic linker (PEG or zwitterion) would affect assay performance.

Theophylline assay

Theophylline, is representative of a hydrophobic analyte that is commonly measured by immunoassay. In the assay, we compared the immunoassay performance of chemiluminescent acridinium ester–theophylline conjugates containing either a PEG linker (compound **11**, Fig. 5, panel B) or containing a sulfobetaine zwitterion (compound **12**, Fig. 5, Panel B). As can be noted, the two conjugates are structurally very similar, the only difference being the structure of the linker used to conjugate the acridinium ester to the analyte theophylline.

The syntheses of the two acridinium ester–theophylline conjugates have been reported previously.[7,17] Conjugate **12** was prepared using the zwitterionic, nucleophilic cross-linker **3** in Fig. 1. Both conjugates **11** and **12** exhibited similar chemiluminescence profiles.[17]

Assay performance of conjugates **11** and **12** was compared on Siemens' Healthcare Diagnostics' ADVIA Centaur® system, an automated immunochemistry analyzer. In the 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 paramagnetic particles. As indicated in Fig. 5 (panel A), and Table S1 (supplementary material†) in the assay, the measured chemiluminescent signal is inversely correlated with the concentration of theophylline. In the assay comparing the two conjugates **11** and **12**, the latter conjugate with the zwitterion-containing linker exhibited a steeper slope at low dose with equivalent precision, and lower FNSB at high dose. The observed FNSB of 0.39% for conjugate **12** was observed to be lower than that of conjugate **11** whose FNSB was measured to be 0.61%. Thus, by replacing conjugate **11** with **12** in the automated assay, the limit of functional sensitivity decreased from 4 μM to 2 μM reflecting a twofold improvement.

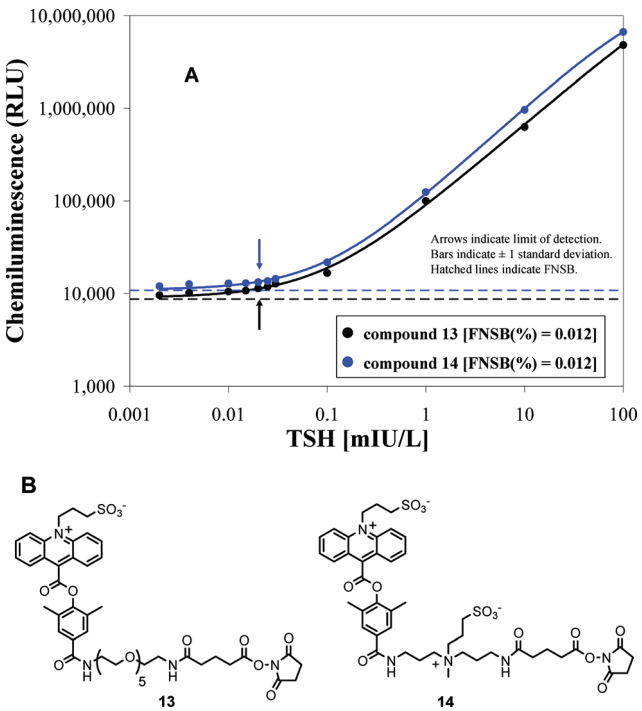

Fig. 6 Structures of chemiluminescent acridinium ester labels **13** and **14** (panel B) and dose–response curve (panel A) in the automated TSH assay using these labels. Both labels had identical assay performance with functional sensitivity of 0.02 mIU L^{-1} indicated by the arrows at the low dose end. Dotted lines indicate FNSB of label **13** (black line) and label **14** (blue line). Standard deviations for assay data were calculated in Microsoft Excel using the built in standard deviation function (STDEV) for five replicates. Microsoft Excel uses the following function to calculate standard deviation

$$\left(\sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}}\right).$$

Both conjugates **11** and **12** also exhibited identical binding affinity of 47% and 46% (B_0/T%) respectively for the antibody immobilized on the solid phase. Our results clearly demonstrate that, in this instance, the sulfobetaine cross-linker is more effective than PEG in improving assay performance and could be useful for other hydrophobic analytes as well.

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 compound **13** with that of the label compound **14** (Fig. 6, panel B), labeled to the same extent (4 labels per antibody) on the same signaling antibody, a murine monoclonal antibody with an acidic pI = 5.6. The syntheses of compounds **13** and **14** were described previously.[7,17] Light output from the two conjugates was similar.[17] The observed dose responses in the TSH assay are shown in Fig. 6 (panel A) and Table S2 (supplementary material†). In the assay, FNSB (0.012%) was observed to be the same for both conjugates of compounds **13** and **14** indicating that the sulfobetaine linker in compound **14** had a similar effect on non-specific binding to paramagnetic particles as the PEG linker in compound **13**. As a consequence of both equivalent assay precision for both labels over the whole standard curve, the combination of equivalent light output and equivalent FNSB, equivalent functional sensitivity in the TSH assay of 0.020 mIU L^{-1} TSH was observed for conjugates of the two labels **13** and **14** (1 IU TSH = 1.58×10^{-8} mol). Thus, in the sandwich assay, chemiluminescent acridinium esters containing either PEG or a sulfobetaine linker were equally effective in reducing the non-specific binding of the labeled antibody and consequently, similar assay performance was observed. Whether these observations would translate to other sandwich assays is difficult to predict, but our earlier observations on the non-specific binding of proteins indicate that antibodies that are more hydrophobic or that are basic would benefit more by labeling with a hydrophilic, zwitterionic chemiluminescent label.

Conclusions

We have described the syntheses and applications of new labeling and cross-linking reagents containing hydrophilic zwitterions. These reagents are easily assembled and are useful for improving the aqueous solubility of hydrophobic antigens as well as for their cross-linking. We had previously reported that PEG is useful for reducing the non-specific binding of chemiluminescent acridinium ester labels and, that linkers derived from PEG are useful for the preparation of acridinium ester conjugates of hydrophobic analytes such as folate and theophylline.[7] Our current results comparing the immunoassay performance of acridinium ester–theophylline conjugates suggest that linkers containing sulfobetaine zwitterions would be more effective than PEG in increasing the aqueous solubility, lowering the non-specific binding and improving immunoassay performance of hydrophobic analytes. Our studies on protein and peptide labeling also indicate that zwitterion labels are useful for decreasing the non-specific binding of hydrophobic and basic proteins as well as alleviating the aqueous solubility of hydrophobic peptides. Although not a focus of our research, we believe, the relatively simple to assemble, zwitterionic reagents that we have described should be useful for other applications as well such as the modification of the surfaces of nanomaterials to make them more hydrophilic.

Experimental

General

Chemicals were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA) unless indicated otherwise. All final compounds were analyzed and purified by HPLC using a Beckman-Coulter HPLC system. HPTLC of synthetic compounds was performed using silica gel 60 CN F_{254}S plates from EMD Chemicals Inc. An iodine chamber was used for visualization of spots. 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 bench top instrument operating in the linear mode with a 1.2 meter ion path length, flight tube. Spectra were acquired in positive ion mode. For small molecules, α-cyano-4-hydroxycinnamic acid was used as the matrix and spectra were acquired with an accelerating voltage of 20 000 volts and a delay time of 100 ns. For protein conjugates, sinapinic acid was used as the matrix and spectra were acquired with an accelerating voltage of 25 000 volts and a delay time of 85 ns. For HRMS (High Resolution Mass Spectra), samples were dissolved in HPLC-grade methanol and analyzed by direct-flow injection (injection volume = 5 μL) ElectroSpray Ionization (ESI) on a Waters Qtof API US instrument in the positive ion mode. Optimized conditions were as follows: Capillary = 3000 kV, Cone = 35, Source T = 120 °C, Desolvation T = 350 °C. NMR spectra were recorded on a Varian 500 MHz spectrometer.

1. Synthesis of zwitterionic reagent 1 (Figures S1, S4 and S5, supplementary material†)

*Compound **i**.* Compound **i** was synthesized from 4-dimethylaminobutyric acid using a literature procedure.[18]

*Compound **ii**.* A solution of compound **i** (0.2 g, 0.905 mmol) in anhydrous DMF (5 mL) was treated with 1,3-propane sultone (0.165 g, 1.5 equivalents) and 2,6-di-*tert*-butylpyridine (0.24 mL, 1.2 equivalents). The reaction was heated at 150 °C under a nitrogen atmosphere. After 1 h, HPLC analysis was performed using a Phenomenex, 10 micron, C_{18}, 3.9 × 300 mm column and a 40 min gradient of 10 → 40% MeCN/water (each with 0.05% trifluoroacetic acid, TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 and 220 nm. Product was observed eluting at 14 min with a minor amount of starting material at 13 min. The reaction was cooled to room temperature and the solvent was removed under reduced pressure. The residue was partitioned between ethyl acetate (50 mL) and water (50 mL). The aqueous solution was separated and washed with ethyl acetate (2 × 50 mL). The aqueous solution was treated with

5 drops of ammonium hydroxide and further extracted with ethyl acetate (2 × 25 mL). The aqueous solution was then concentrated under reduced pressure to afford a sticky gum. Yield = 0.35 g (quantitative); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.55; ^{1}H-NMR (500 MHz, CF_3COOD) δ 2.19 (m, 2H), 2.43 (m, 2H), 2.64 (t, 2H, J = 6.7), 3.07 (s, 6H), 3.34 (m, 4H), 3.59 (m, 2H), 5.23 (s, 2H), 7.33 (s, 5 H); MALDI-TOF MS m/z 344.3 $(M + H)^+$; HRMS m/z 344.1533 $(M + H)^+$ (344.1532 calculated).

Compound 1. A solution of compound **ii** (0.42 g, 1.22 mmol) in methanol (40 mL) was treated with 0.25 g of 10% palladium on carbon. The suspension was hydrogenated in a Parr shaker at 40 psi for 2 h at room temperature. The reaction was then filtered and the methanol solution was concentrated under reduced pressure to afford a white solid, compound **iii** which, was used as such for the next reaction. Yield = 0.234 g (quantitative); MALDI-TOF MS m/z 254.3 $(M + H)^+$ (254.1 calculated).

A solution of compound **iii** (0.114 g, 0.45 mmol) in a 1 : 1 mixture of 0.1 N HCl/MeCN (6 mL) was treated with pentafluorophenol (0.125 g, 1.5 equivalents) and 1-ethyl-(dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 0.230 g, 2.5 equivalents). The reaction was stirred at room temperature. After 1 h, HPLC analysis was performed using a Phenomenex, C_{18}, 10 micron, 3.9 × 300 mm column and a 40 min gradient of 10 → 60% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 and 220 nm. Product was observed eluting at 18 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 40 min gradient of 10 → 60% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. The HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness to afford a gummy solid. Yield = 78 mg (41%); ^{1}H-NMR (500 MHz, CF_3COOD) δ 2.38 (m, 2H), 2.59 (m, 2H), 2.98 (t, 2H, J = 6.7), 3.25 (s, 6H), 3.45 (t, 2H, J = 6.9), 3.58 (m, 2H), 3.77 (m, 2H); ^{19}F-NMR (470 MHz, CF_3COOD) δ −156.6 (d, 1F, J = 17.6), −159.8 (t, 2F, J = 19.8), −165.0 (t, 2F, J = 18.3); MALDI-TOF MS m/z 420.0 $(M + H)^+$; HRMS m/z 442.0720 $(M + Na)^+$ (442.0724 calculated).

2. Synthesis of zwitterionic reagent 2 (Figures S2, S6 and S7, supplementary material†)

Compound v. To a stirred solution of *N*-(benzyloxycarbonyloxy)succinimide (6.30 g, 24.79 mmol, Aldrich) in chloroform (25 ml) under a nitrogen atmosphere and at 0 °C was added drop wise *N*,*N*-dimethylethylenediamine (2.00 g, 21.55 mmol) dissolved in chloroform (15 mL). The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 1 h. The resulting reaction mixture was diluted with chloroform (40 mL) and washed with saturated aqueous sodium bicarbonate (3 × 20 mL). The organic layer was separated and dried over anhydrous sodium sulfate. The filtrate was concentrated to give 5.16 g (quantitative) of viscous oil as the desired product **iv** which was used as such for the next reaction. MALDI-TOF MS m/z 223.5 $(M + H)^+$ (223.1 calculated).

Compound **iv** (1.00 g, 4.5 mmol) was dissolved in anhydrous ethyl acetate (5 mL) in a sealed tube and 1,3-propane sultone (1.10 g, 9.0 mmol) was added under a nitrogen atmosphere. The sealed tube was heated to 90 °C for 16 h and then cooled to room temperature. The precipitated product was filtered and washed with anhydrous ethyl acetate (3 × 20 mL). The resulting white solid was dried under high vacuum to give 1.31 g (85%) of desired product. HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.59; ^{1}H-NMR (500 MHz, CD_3OD) δ 2.20 (m, 2H), 2.85 (t, 2H, J = 6.7), 3.14 (s, 6H), 3.45 (t, 2H, J = 6.6), 3.52–3.62 (m, 4H), 5.11 (s, 2H), 7.27–7.39 (m, 5H); MALDI-TOF MS m/z 344.9 $(M + H)^+$; HRMS m/z 345.1487 $(M + H)^+$ (345.1484 calculated).

Compound 2. Compound **v** (580 mg, 1.7 mmol) was dissolved in methanol/water (95/5, 40 mL) and 10% palladium on carbon (58 mg) was added. The reaction was hydrogenated for 4 h at room temperature using a Parr shaker. The reaction mixture was then filtered and the filtrate was concentrated to dryness to give a white solid as the desired product **vi** which was used as such for the next reaction. Yield = 0.35 g (quantitative). MALDI-TOF MS m/z 211.0 $(M + H)^+$ (211.1 calculated).

To a solution of compound **vi** (21.0 mg, 0.1 mmol) and 4-maleimidobutyric acid (18.3 mg, 0.1 mmol) in anhydrous dimethyl sulfoxide (1 mL) was added diisopropylethylamine (0.035 mL, 0.2 mmol) and benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 66.2 mg, 0.15 mmol). The reaction was stirred at room temperature for 1 h. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 40 min gradient of 0 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. The HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 43 mg (quantitative); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.49; ^{1}H-NMR (500 MHz, CF_3COOD) δ 2.00 (m, 2H), 2.43 (t, 2H, J = 7.5), 2.49–2.57 (m, 2H), 3.24 (s, 6H), 3.39 (t, 2H, J = 7.0), 3.61 (m, 2H), 3.66 (t, 2H, J = 6.6), 3.72 (m, 2H), 3.89 (m, 2H), 6.87 (s, 2H); MALDI-TOF MS m/z 376.7 $(M + H)^+$; HRMS m/z 376.1544 $(M + H)^+$ (376.1542 calculated).

3. Synthesis of compound 4 (Figures S3 and S8, supplementary material†).

A solution of compound **3**[17] (25 mg, 56 μmol) in DMSO (1 mL) was treated with diisopropylethylamine (0.039 mL, 4 equivalents) and this solution was added drop wise to a solution of disuccinimidyl carbonate (DSC, 96 mg, 0.37 mmol) in DMSO (2 mL). The reaction was stirred at room temperature. After 30 min, a portion of the reaction mixture was analyzed by HPLC using a Phenomenex, 10 micron, C_{18}, 3.9 × 300 mm column and a 30 min gradient of 0 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 220 nm. Product was observed eluting at 10 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron C_{18}, 30 × 250 mm column and a 40 min gradient of 0 → 40% MeCN/water (with 0.05% TFA) above at a flow rate of 20 mL min^{-1}. The HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 15 mg (50%); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.73; ^{1}H-NMR (500 MHz, CF_3COOD) δ 2.33 (m, 4H), 2.55 (m, 2H), 3.02 (s, 8H), 3.16 (s, 3H), 3.41 (br t, 2H), 3.52 (m, 6H), 3.62 (m, 2H), 3.69 (m, 2H); MALDI-TOF MS m/z 549.8 $(M + H)^+$; HRMS m/z 572.1639 $(M + Na)^+$ (572.1638 calculated).

4. Synthesis of compound 5 (Figures S3, S9–S11, supplementary material†)

Compound vii. A solution of compound **iv** (105 mg, 0.5 mmol) in anhydrous DMSO (2 mL) was treated with

triethylamine (0.070 mL, 0.5 mmol) and *N*-α-t-BOC-*N*-ε-CBZ-lysine-*N*-hydroxsuccinimide ester (239 mg, 0.5 mmol, Aldrich). The suspension was stirred at room temperature for 24 h and then analyzed by HPLC using a YMC, 3 micron, C_{18}, 4.0 × 50 mm column and a 10 min gradient of 10 → 90% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 nm. Product was observed eluting at 6.2 min. The product was purified by preparative HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 30 min gradient of 10 → 70% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. The product fraction was collected, frozen at −80 °C and lyophilized. The product, compound **vii**, was obtained as a white powder. Yield = 225 mg (79%); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.31; ^{1}H-NMR (500 MHz, CF_3COOD) δ 1.59 (s, 9H), 1.61 (m, 2H), 2.00 (m, 2H), 2.42 (m, 2H), 3.20–3.27 (m, 9H), 3.37 (m, 2H), 3.59–3.79 (m, 6H), 4.26 (m, 1H), 4.37 (m, 1H), 5.19 (s, 2H), 7.30 (m, 5H); MALDI-TOF MS *m*/*z* 572.6 $(M + H)^+$; HRMS *m*/*z* 573.2955 $(M + H)^+$ (573.2958 calculated).

Compound ***ix***. Compound **vii** (200 mg, 0.35 mmol) was added to a mixture of MeOH and H_2O (95/5, 20 mL). Then, 10% of palladium on carbon (20 mg) was added. The resulting suspension was hydrogenated at room temperature using a balloon for 4 h. The reaction mixture was then filtered. The filtrate was concentrated under reduced pressure at room temperature. The desired product, compound **viii**, was obtained as a clear oil and was used as such for the next reaction. Yield = 176 mg (quantitative); MALDI-TOF MS *m*/*z* 440.3 $(M + H)^+$ (439.3 calculated).

A solution of compound **viii** (176 mg, 0.4 mmol) in anhydrous DMSO (4 mL) was treated with 4-maleimidobutyric acid *N*-hydroxsuccinimide ester (50 mg, 0.178 mmol, Thermo) and triethylamine (0.054 mL, 0.4 mmol). The reaction was stirred at room temperature. After 1–2 h, HPLC analysis was performed using a Phenomenex, 10 micron, C_{18}, 3.9 × 300 mm column and a 40 min gradient of 0 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 260 & 220 nm. Product was observed eluting at 27.5 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 40 min gradient of 0 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. The HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 45 mg (40%); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.59; ^{1}H-NMR (500 MHz, CF_3COOD) δ 1.59 (s, 9H), 1.74 (br t, 2H), 2.07 (br t, 4H), 2.44 (m, 2H), 2.62 (br t, 2H), 3.23 (s, 3H), 3.27 (s, 3H), 3.37 (m, 2H), 3.51 (m, 2H), 3.61–3.82 (m, 8H), 4.28 (m, 1H), 4.43 (m, 1H), 6.88 (s, 2H); MALDI-TOF MS *m*/*z* 604.9 $(M + H)^+$; HRMS *m*/*z* 604.3013 $(M + H)^+$ (604.3016 calculated).

Compound ***5***. A solution of compound **ix** (25 mg, 41 μmol) in trifluoroacetic acid (2 mL) was stirred in an ice-bath for 3 h. Anhydrous ether (75 mL) was then added to the reaction mixture to precipitate the product which was collected by filtration and rinsed with ether. The product was then dissolved in methanol (15–20 mL) and analyzed by HPLC as described above. Product, compound **x**, was observed eluting at 13.7 min (complete conversion). The methanol solution was diluted with anhydrous toluene (10 mL) and was concentrated under reduced pressure to afford a white sticky solid which was used as such in the next reaction. Yield = 45 mg (quantitative); MALDI-TOF MS *m*/*z* 504.4 $(M + H)^+$ (504.3 calculated).

A solution of crude compound **x** (24 mg, 0.0386 mmol) and disuccinimidyl glutarate (DSG, 50 mg, 4 equivalents, Thermo) in anhydrous DMSO (2 mL) was treated with triethylamine (6 μL, 1.1 equivalents). The reaction was stirred at room temperature. After 15 min, HPLC analysis was performed as described previously. Product was observed eluting at 24 min. The product was purified by preparative HPLC as described previously. The HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 8 mg (30%); HPTLC (3 : 7, MeCN : water with 0.05% TFA) R_f 0.63; ^{1}H-NMR (500 MHz, CF_3COOD) δ 1.47–1.55 (m, 2H), 1.73 (m, 2H), 1.79–1.95 (m, 2H), 2.02–2.69 (m, 4H), 2.43–2.62 (m, 4H), 2.68 (t, 2H, *J* = 6.7), 2.75 (t, 2H, *J* = 6.7), 3.03 (s, 4H), 3.23 (s, 6H), 3.38 (m, 2H), 3.45–3.56 (m, 2H), 3.61 (br t, 2H), 3.69–3.77 (m, 4H), 3.85 (m, 2H), 3.93 (m, 2H), 4.52 (br t, 1H), 6.88 (s, 2H); MALDI-TOF MS *m*/*z* 717.1 $(M + H)^+$; HRMS *m*/*z* 715.2954 $(M + H)^+$ (715.2973 calculated).

5. Hydrophobic peptide labeling (Fig. 2 and Figures S12–S14, supplementary material†) with zwitterionic reagent 1. The following is an illustrative procedure for the labeling of penta(phenylalanine) with compound **1**, as shown in Fig. 2. A solution of penta(phenylalanine) (2 mg, 2.65 μmol, Aldrich) in DMSO (0.8 mL) was treated with compound **1** (2 mg, 4.8 μmol) dissolved in DMSO (0.2 mL) followed by aqueous sodium carbonate (0.2 mL, 100 mM, pH 9). The reaction was stirred at room temperature for 16 h. HPLC analysis of a small portion of the reaction mixture was performed using a Phenomenex, 10 micron, C_{18}, 3.9 × 300 mm column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 220 and 260 nm. The labeled peptide **6** was observed to elute at 18.5 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) above at a flow rate of 20 mL min^{-1}. HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness to afford a fluffy white solid. Yield = 2.4 mg (92%); MALDI-TOF MS *m*/*z* 988.4 M^+ (988.4 calculated).

The other two peptides Phe-Gly-Gly-Phe and penta(leucine) were labeled in a similar manner.

Compound **7**, Yield = 3.6 mg (quantitative); MALDI-TOF MS *m*/*z* 660.8 M^+ (661.3 calculated).

Compound **8**, Yield = 1.2 mg (43%); MALDI-TOF MS *m*/*z* 820.4 $(M + H)^+$ (819.5 calculated).

Aqueous solubility of the labeled peptides was estimated by dissolving the lyophilized, zwitterion-labeled peptides **6**, **7** and **8** in small quantities of de-ionized water (0.2 mL portions) at room temperature followed by vortexing. Concentrations that afforded homogeneous solutions were used to estimate the aqueous solubility of the zwitterion-labeled peptide.

6. Synthesis of cross-linked peptide 9 from conjugation of penta(phenylalanine) to the tetrapeptide Phe-Gly-Gly-Phe (Fig. 3 and Figure S15, supplementary material†) using zwitterionic, homobifunctional cross-linker 4

*Compound **xi**.* A solution of compound **4** (4.5 mg, 8.3 μmol) in anhydrous DMSO (2 mL) was added to penta(phenylalanine) (2 mg, 2.7 μmol) along with diisopropylethylamine (2 μL, 11.4 μmol). The reaction was stirred at room temperature. After 16 h, HPLC analysis of the reaction mixture was performed using a Phenomenex, 10 micron, C_{18}, 3.9 mm × 300 m column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 220 and 260 nm. Product **xi** was observed eluting at 17 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness to afford a fluffy white solid. Yield = 2.0 mg (64%); MALDI-TOF MS *m/z* 1187.8 $(M + H)^+$ (1187.5 calculated).

*Compound **9**.* A mixture of compound **xi** (2 mg, 1.68 μmol), tetrapeptide Phe-Gly-Gly-Phe (3.6 mg, 5 equivalents) and diisopropylethylamine (1.5 μL, 5 equivalents) in DMSO (2.5 mL) was stirred at room temperature. After 1 h, HPLC analysis was performed as described above. Product **9** was observed eluting at 18 min. The crude reaction mixture was purified by HPLC using a YMC, 10 micron, C_{18}, 30 × 250 mm column and a 30 min gradient of 10 →100% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 1.6 mg (63%); MALDI-TOF MS *m/z* 1500.2 M^+ (1512.7 calculated).

7. Synthesis of cross-linked peptide 10 from conjugation of penta(phenylalanine) with dipeptide Cys-Gly (Fig. 4 and Figure S16, supplementary material†) using zwitterionic, heterobifunctional cross-linker 5. A solution of penta(phenylalanine) (8 mg, 11.2 μmol) and compound **5** (4 mg, 5.6 μmol) in anhydrous DMF (3 mL) was treated with triethylamine (4 μL, 4 equivalents). The reaction was stirred at room temperature for 16 h and then analyzed by HPLC using a Phenomenex, 10 micron, C_{18}, 3.9 × 300 mm column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL min^{-1} and UV detection at 220 and 260 nm. Product **xii** was observed eluting at 18.4 min (MALDI-TOF MS *m/z* 1355.8 $[M + H]^+$; 1353.6 calculated). Starting material **5** eluting at 10 min was completely consumed. To the reaction mixture was added the dipeptide Cys-Gly (2 mg, 11.1 μmol). The reaction was stirred at room temperature. After 20 min, HPLC analysis indicated clean formation of the heptapeptide **10** eluting at 16.5 min with no **xii** at 18.4 min. The heptapeptide **10** was purified by HPLC using an YMC, 10 micron, C_{18}, 30 × 250 mm column and a 30 min gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 20 mL min^{-1}. HPLC fractions containing product were frozen at −80 °C and lyophilized to dryness. Yield = 8.8 mg (quantitative) MALDI-TOF MS *m/z* 1535.3 $(M + H)^+$ (1531.7 calculated).

8. General procedure for the labeling of BSA and BGG with acridinium ester and zwitterion label 1. The following procedure was typical. A solution of BSA (4 mg, 60 nmol) in 1 mL of 0.1 M sodium bicarbonate was treated with 5 equivalents of the acridinium ester, 9-[[4-[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]-2,6-dimethylphenoxy]carbonyl]-10-(3-sulfopropyl)-acridinium abbreviated as NSP-DMAE-NHS[7] ester (see Table 1 for structure) added as a solution in DMF (0.072 mL of a 2 mg mL^{-1} solution in DMF). The reaction was stirred at room temperature for 4 h. The reaction was then transferred to a 4 mL amicon filter (MW 30 000 cutoff) and diluted with 3 mL deionized water. The filter was centrifuged at 4000*g* for 8 min to reduce the volume to ~0.25 mL. This process was repeated two more times. The final conjugate was diluted to 0.8 mL with deionized water to give a 5 mg mL^{-1} solution. Acridinium ester label incorporation, measured by MALDI-TOF mass spectroscopy using sinnapinic acid as matrix indicated the incorporation of 1.4 labels (Figure S17, supplementary material†).

Further labeling of the acridinium ester-labeled BSA conjugate with the zwitterion label **1** was carried out as follows. The conjugate (0.5 mg, 7.5 nmol, 0.100 mL) was diluted with 0.100 mL of sodium carbonate, pH 9. To this conjugate was added 10 and 20 equivalents of the zwitterion label **1** dissolved in DMSO corresponding to 0.0128 mL and 0.0256 mL of a 2.5 mg mL^{-1} solution of **1**. The reactions were stirred at room temperature for 4 h and were then processed as described above. Zwitterion label incorporation was measured by MALDI-TOF mass spectroscopy and the results are listed in Table 2. (Figure S17, supplementary material†)

Table 2 General procedure for the labeling of BSA and BGG with acridinium ester and zwitterion label **1**

Conjugate	Zwitterion reagent **1**, input (eq.)	# of zwitterion labels
BSA-NSP-DMAE	10	5
BSA-NSP-DMAE	20	8
BGG-NSP-DMAE	10	9
BGG-NSP-DMAE	20	13

9. General procedure for the labeling of AVD, BGG and FBN with acridinium ester and zwitterion label 1 for measurement of non-specific binding. A solution of 2 mg mL^{-1} of egg white avidin (AVD), bovine gamma globulin (BGG) and bovine fibrinogen (FBN) was prepared in 1 mL of 0.1 M sodium carbonate, pH 9. The protein solutions were then treated with 5 equivalents of the acridinium ester NSP-DMAE-NHS,[7] (Table 1) added as a solution in DMSO. Specifically, a 2 mg mL^{-1} solution of the acridinium ester in DMSO was prepared and 0.089 mL, 0.04 mL and 0.018 mL of this solution were added to AVD, BGG and FBN respectively. The reactions were stirred at 4 °C for 3 h. A portion (0.5 mL) of each reaction was further treated with a 25 equivalents of compound **1** added as a solution in DMSO. Specifically, a 5 mg mL^{-1} solution of **1** in DMSO was prepared and 0.032 mL, 0.014 mL and 0.006 mL of this solution were added to acridinium ester-labeled AVD, BGG and FBN respectively. The reactions were stirred at 4 °C for 16 h. All three conjugates were then diluted with 0.5 mL each of 0.1 M phosphate, pH 7.4 and transferred to 4 mL centricon filters. The conjugates were further diluted with 3 mL buffer and centrifuged at 4000*g* for 7 min to reduce the volume to ~0.2 mL. This process was repeated two more times. The final conjugates were diluted to a total volume of 0.5–1.0 mL in phosphate buffer and stored at 4 °C.

10. Measurement of FNSB (Table 1). The FNSB of three proteins AVD, BGG and FBN, each labeled with the acridinium ester NSP-DMAE and the zwitterion label **1**, were measured and compared to the FNSB of the three same proteins, labeled only with the acridinium ester. The conjugates were diluted to equivalent concentrations of 10 nM in a solution consisting of 0.1 M sodium *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonate (HEPES), 0.15 M sodium chloride, 7.7 mM sodium azide, 1.0 mM tetrasodium ethylenediaminetetraacetatic acid (EDTA), 12 mM *t*-octylphenoxypolyethoxyethanol (Triton X-100), 0.076 mM BSA and 0.007 mM mouse immunoglobin, pH 7.7. 0.1 mL of these 10 nM protein–acridinium ester conjugate solutions were each mixed with 0.2 mL of horse serum and 0.2 mL of either of three solid phases. The first solid phase was 0.35 g L^{-1} paramagnetic microparticles (PMP) covalently covered with sheep antibody. The second solid phase was 0.35 g L^{-1} of Invitrogen Corporation M-280 magnetic latex microparticle (MLP) Dynalbeads covalently covered with streptavidin. The third solid phase was 0.35 g L^{-1} of Invitrogen Corporation M-270 magnetic latex microparticle (MLP) Dynalbeads covalently covered with streptavidin. The solid phases were magnetically collected and washed twice with water after an incubation of 10 min to allow interaction between the acridinium ester covalently attached proteins and the solid phases. The chemiluminescence (expressed as RLUs, Relative Light Units) of both the input acridinium ester chemiluminescence and the chemiluminescence of the acridinium ester associated with the particles was measured for 5 s on a Berthold Technologies' Autolumat LB953 luminometer with sequential addition of 0.3 mL each of reagent 1 (0.1 M nitric acid and 0.5% hydrogen peroxide) and reagent 2 (0.25 M sodium hydroxide containing 7 mM cetyltrimethylammonium chloride). FNSB was calculated as the ratio of particle-bound chemiluminescence from acridinium ester bound to the solid phases divided by the total chemiluminescence of acridinium ester input.

Invitrogen product literature descriptions of M-270 and M-280 particles: M-270: Hydrophilic bead surface based on glycidyl ether (epoxy) carboxylic acid beads; diameter: 2.8 μm; M-280: Hydrophobic bead surface based on polystyrene tosyl activated beads; diameter: 2.8 μm.

11. Theophylline assay (Fig. 5 and Table S1, supplementary material†). Immunoassay properties including assay sensitivity, FNSB and precision were assessed for acridinium ester–theophylline conjugates **11** and **12**[7,17] (Fig. 5, panel B, Table S1, supplementary material†) using a Siemens Healthcare Diagnostics' ADVIA: Centaur® system, an automated immunoanalyzer. Conjugates **11** and **12** were diluted to concentrations of 0.26 nM in 0.05 M sodium phosphate buffer pH 7.4 containing 0.15 M sodium chloride, 1.1 mM EDTA, 0.22 mM triton X-100, 0.023 mM BSA, and 15 mM sodium azide. The immunoanalyzer automatically dispensed 0.020 mL each of ten serum standards containing 0, 1, 2, 4, 10, 20, 40, 100, 500, and 1000 μM theophylline into reaction cuvettes. Then 0.45 mL of theophylline assay solid phase, containing magnetically separable, PMPs covalently linked with anti-theophylline antibody, was dispensed to each cuvette. To this was added 0.10 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 (expressed as RLUs) corresponding to each theophylline standard concentration were normalized to the percentage of chemiluminescence measured for the zero standard (Fig. 5, panel A). The amount of theophylline in a standard is inversely correlated to the amount of the acridinium ester–theophylline conjugate that will bind to the solid phase in the assay and likewise inversely correlated to the resulting chemiluminescence. The ratio of the bound chemiluminescence of the acridinium ester–theophylline conjugate to the solid phase (B_0) in the absence of unlabelled theophylline to the total chemiluminescence (T) was expressed as a percentage (B_0/T%). Assay sensitivity was assessed as the lowest theophylline concentration whose chemiluminescence was lower than two-standard deviations of the chemiluminescence for the zero standard. FNSB was measured as the ratio of the chemiluminescence corresponding to a very high concentration of theophylline to the total chemiluminescence.

12. TSH (Fig. 6 and Table S2, supplementary material†). Likewise, anti-TSH monoclonal antibody conjugates of the labels compound **13**[7] and compound **14**[17] (Fig. 6, panel B) were compared in the TSH assay. Conjugates (4 labels per antibody for both labels) were prepared as described previously[7a] and were diluted to a concentration of 2.2 nM in a buffer of 0.10 M HEPES, 0.15 M sodium chloride, 7.7 mM sodium azide, 1.0 mM EDTA, 12 mM triton X-100, 76 μM BSA, 97 nM amphotericin B, 5.2 μM gentamicin sulfate, 6.7 μM murine IgG (mouse immunoglobulin G), 67 nM sheep IgG, 5.3 mg L^{-1} murine serum, 50 mg L^{-1} Antifoam B (Sigma Cat. No. A5757) pH 7.7. The automated immunochemistry analyzer performed the following steps for the TSH assay. First, 0.20 ml of each of 12 serum standards of known amounts of TSH was dispensed into separate cuvettes. 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 mIU L^{-1} concentrations of TSH (1 IU = 1.58 × 10^{-8} mol). The instrument then dispensed 0.10 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 PMPs 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 (expressed as RLUs) was determined for each TSH standard. The assay results are illustrated in Fig. 6, Panel A and Table S2 (supplementary material†). 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 curves were fitted using 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 theophylline 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 TSH concentration as a result of using one kind of acridinium ester label relative to another. FNSB is defined here as the ratio of the chemiluminescent signals associated with non-specific binding *versus* specific signal in the assay (ratio of the chemiluminescence corresponding to the 0 mIU L^{-1} TSH standard divided by the total chemiluminescence input per assay).

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