Development and application of a novel acridinium ester for use as a chemiluminescent emitter in nucleic acid hybridisation assays using chemiluminescence quenching

Richard C. Brown,[a] Zhaoqiang Li,[a,b] Andrew J. Rutter,[a] Xiaojing Mu,[b] Owen H. Weeks,[a] Keith Smith†[b] and Ian Weeks*[a]

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Chemiluminescent acridinium esters (AEs) permit the development of high sensitivity ligand binding assays due to a combination of high intensity light emission and very low backgrounds. Here these advantages are exploited for use in homogeneous nucleic acid hybridisation assays using quenched chemiluminescence. AE chemiluminescence is conventionally initiated at highly alkaline pH. Novel "active" AEs were designed that permit initiation under conditions compatible with maintenance of nucleic acid hybrids (*i.e.* pH less than 9). Methyl red was found to be a dark quencher species capable of functioning at this pH. Practical application of the chemiluminescence quenching assay system has been demonstrated using two model nucleic acid hybridisation assays based on intra- and intermolecular emitter/quencher pairs.

Introduction

Acridinium esters (AEs) have been used extensively as end-point labels in ligand binding assays such as immunoassays and nucleic acid hybridisation assays.[1] Conventionally, AE chemiluminescence is measured as a rapid flash type emission (typically one second duration) of visible light (wavelength approximately 430 nm). Ligand binding assays can be "heterogeneous" or "homogeneous" depending respectively on whether or not the binding complexes formed between the target molecule and binding reagent need to be physically separated from non-bound reagent, for example by solid-phase adsorption, prior to end-point measurement. AEs have been used successfully in both types of assay but have not thus far been widely applied to homogeneous assay systems based on energy transfer quenched chemiluminescence. The more commonly encountered quenched fluorescence methods use a matched fluorescent emitter/quencher pair to monitor ligand-binding reactions. However, the performance of simple, solution-phase fluorescence-based assays is limited by the low sensitivity of detection of the emitted signal as a consequence of the background due to scattered incident light and endogenous sample fluorescence.[2] In contrast, quenched AE chemiluminescence offers greatly increased sensitivity as a result of its inherently lower background.

We were particularly interested in exploiting the potentially high sensitivity of detection of AE chemiluminescence quenching systems in nucleic acid probe hybridisation assays. Depending on the nature of the probe/target design, conformational change of the AE/quencher labelled probe induced by hybridisation with the analytical target nucleic acid will have the effect of either separating or bringing closer the emitter/quencher pair such that the emitted signal intensity proportionately reflects the degree of hybridisation and thus the quantity of analyte nucleic acid present. However, simple substitution of a chemiluminescent AE label for a fluorescent label as presently used in analogous fluorescence quenching assays will not result in a workable system since, in contrast to fluorescence, AE chemiluminescence requires an altered chemical environment (*e.g.* elevated pH) to effect signal emission. This will impact on the components of the assay system for several reasons. Firstly the hybridised nucleic acid duplex cannot survive exposure to elevated pH (>9)[3] because of dissociation. Secondly the desired optical absorption characteristics of the chosen matched quencher molecule must be retained under the conditions used to initiate the chemiluminescent reaction.

The general rules for the use of AE as a biomolecular labelling species are that the chemiluminescent signal is easily triggered, that the AE can be readily coupled to ligand and that the chemiluminescent and biochemical properties of such AE–ligand conjugates are essentially the same as the individual components. The use of *N*-hydroxysuccinimide ester derivatives of AE have been shown to permit efficient coupling with selected amine groups in proteins or modified oligonucleotides using established labelling methods and post-labelling purification techniques.[1] This allows production of high specific activity labelled species that can be used at low concentrations (1–10 femtomoles per reaction) in sensitive ligand binding assays.

For application to nucleic acid chemiluminescence quenching assays several other, more specific, criteria also need to be achieved. Firstly the AE chemiluminescence must be sufficiently intense to permit high sensitivity measurements over a conveniently short time period when initiated under conditions compatible with the maintenance of the labelled probe-nucleic acid target duplex which, evidence suggests, is less than or equal to a pH of 9.[3]

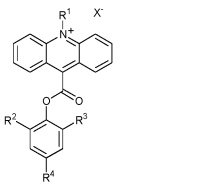
[a]*Molecular Light Technology Research Limited (A subsidiary of Gen-Probe Incorporated, 10210 Genetic Center Drive, San Diego, CA 92121, USA), 5 Chiltern Close, Cardiff Industrial Park, Cardiff, Wales, UK CF14 5DL*
[b]*Department of Chemistry, University of Wales, Swansea, Wales, UK SA2 8PP*
† Present address: School of Chemistry, Cardiff University, Cardiff, Wales, UK, CF10 3AT.

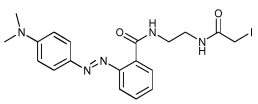
Secondly, the AE labelled probe must also display sufficient stability under conditions of storage and use such that there is no appreciable loss of activity. This is of particular relevance in the context of assay incubation conditions where the ambient pH may itself be alkaline. Finally the chemiluminescent reaction must allow the emitting species to remain in sufficiently close proximity to the quencher for energy transfer to occur. This combination of features has not previously been demonstrated nor applied to AE quenched chemiluminescence assays by commonly used AEs. Thus, achievement of a functional chemiluminescence quenching-based system has necessitated the design and synthesis of a novel AE compound that has this desired combination of properties. Additionally, a further requirement was to ensure that the proposed quencher was capable of quenching the emission under the chemical conditions used to bring about the chemiluminescent reaction.

Here we describe the design and synthesis of a novel AE/quencher pair and demonstrate the successful application of the chemiluminescence quenching principle to model assay systems. Molecular "hairpin probes"[4] were used to demonstrate the application of this principle to intramolecular emitter/quencher pairs and complementary oligonucleotide pairs were used to demonstrate the principle as applied to intermolecular hybridisation, in this example as a model helicase substrate. These systems demonstrate that AE chemiluminescence quenching can be successfully used as the basis of highly sensitive nucleic acid hybridisation assays and their associated applications.

Results and discussion

Design and synthesis of the chemiluminescent emitter and quencher





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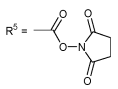
1 R^1 = $(CH_2)_3R^5$; $R^2 = R^3$ = Br; R^4 = H; X = I

2 R^1 = $(CH_2)_{10}R^5$; $R^2 = R^3$ = NO_2; R^4 = H; X = CF_3SO_3

3 R^1 = $(CH_2)_{10}R^5$; $R^2 = R^3$ = CF_3; R^4 = H; X = CF_3SO_3

4 R^1 = CH_3; $R^2 = R^3$ = Br; R^4 = $(CH_2)_2R^5$; X = CF_3SO_3

5 R^1 = CH_3; $R^2 = R^3$ = H; R^4 = $(CH_2)_2R^5$; X = CF_3SO_3



AE labels used in existing chemiluminescent ligand binding assays are exposed to highly alkaline hydrogen peroxide to produce chemiluminescence. Most such labels produce little or no emission at pH $\leq$9[5a] and thus cannot satisfy the criteria required for the present application. It has previously been established that the chemiluminescent reaction rate of the AE can be varied by changing the substituents of the phenyl ring.

Stabilisation of the phenolate anion leaving group, which is produced during the chemiluminescent reaction, with electron-withdrawing substituents (accompanied by the reduction in pK_a of the corresponding phenol[5c]), increases the rate of the reaction and thus renders the molecule more "active". Although presently established AEs do not yield chemiluminescence at pH $\leq$9 (which is necessary to preserve nucleic acid duplexes), we reasoned that "activated" AEs would be chemiluminescent under such conditions. However, such substitutions are also likely to render the phenyl ester bond more prone to hydrolysis and thus unstable in aqueous solution and so a compromise would be needed in this regard. It has also been shown that some stabilisation towards hydrolysis can be achieved by incorporating bulky groups at the 2 and 6 positions of the phenol.[5d] Our strategy was to examine structures where electron withdrawing groups were also used to create some element of steric inhibition of hydrolysis by incorporating them at these positions. Several structures were investigated and three candidate structures (compounds **1** to **3**) were selected for further study.

Selection of optimum AE

Fig. 1 shows typical chemiluminescent emission kinetics of compounds **1–3** when the chemiluminescent reaction is initiated (here at pH10).

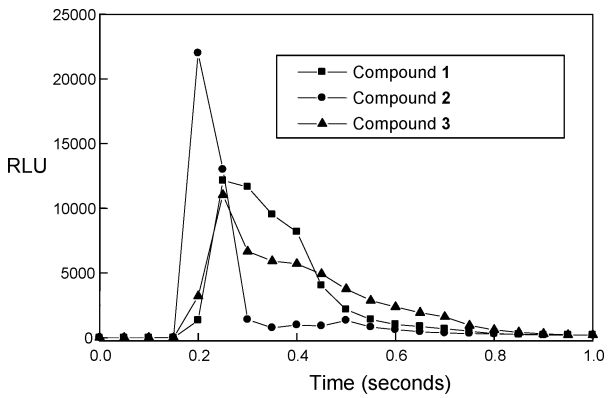


Fig. 1 Kinetics of chemiluminescence of compounds **1**, **2** and **3** initiated at pH 10 using a 1 second measurement time. Chemiluminescence signal is expressed as arbitrary relative light units (RLU).

It can be seen that compound **2** exhibits the fastest kinetics and so appears to be a good candidate to exhibit lower pH chemiluminescence emission. However, studies of the stability of buffered aqueous acetonitrile solutions of the compounds (Table 1) showed that compounds **2** and **3** are inherently less stable than compound **1** and therefore less likely to be suitable for routine use.

A good compromise between the reactivity and stability of the substituted phenyl ester was achieved with the 2, 6-dibromophenyl

Table 1 Stability of substituted phenyl AE compounds **1–3**[a]

Compound	0 days[b]	4 days[b]	6 days[b]
1	100	98	100
2	100	95	71
3	100	74	57

[a] Hydrolysis of the phenyl acridinium ester results in the formation of a non-chemiluminescent product which is reflected by loss of chemiluminescent activity. [b] Data are RLU expressed as a percentage of the emission intensity of the freshly prepared solution.

ester (compound **1**). Compound **4** is an analogue of compound **1** in which the chemiluminescent reaction results in the acridone emitter no longer being covalently attached to the ligand. This property has been utilised in the past with established AEs such as compound **5** in heterogeneous assays to minimise unwanted loss of quantum yield of the primary emitter due to possible non-specific quenching effects exerted by the attached ligand[1] and to allow maximum sensitivity of detection to be achieved.

However, for the present purpose, where specific quenching is the basis of the assay, such a property represents a disadvantage since maximal chemiluminescence quenching is required. Fig. 2 and 3 show typical chemiluminescent kinetic profiles, produced at pH 14 and 9, respectively, of generic oligonucleotides labelled with compounds **1**, **4** and **5**.

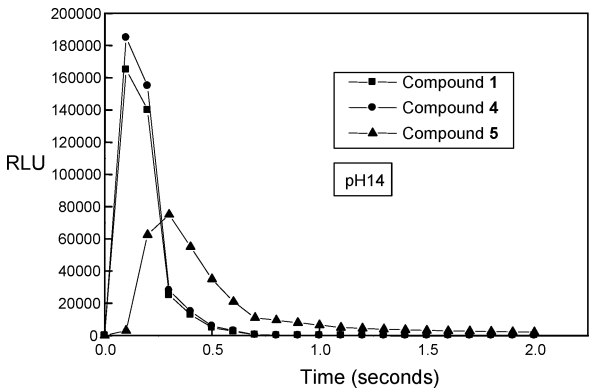


Fig. 2 Kinetics of chemiluminescence of compounds **1**, **4** and **5** initiated at pH 14.

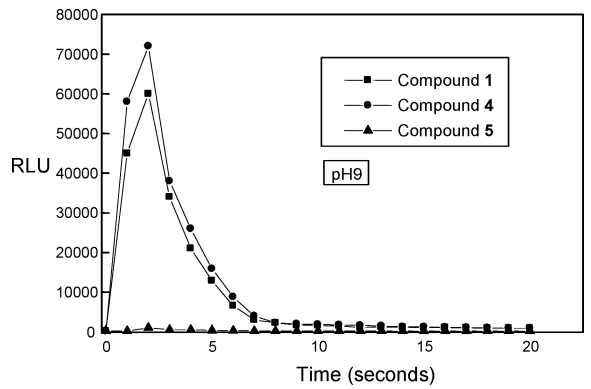


Fig. 3 Kinetics of chemiluminescence of compounds **1**, **4** and **5** initiated at pH 9.

As can be seen from Fig. 2, all three compounds readily undergo a chemiluminescent reaction at pH 14. However, as shown in Fig. 3, although compounds **1** and **4** yielded greater than 95% of the total chemiluminescent signal within 5 seconds at pH 9, compound **5** was inactive at this pH and is thus unsuitable for use in situations where the integrity of ligand binding complexes must be preserved. Compounds **1** and **4** displayed similar chemiluminescent kinetics, though compound **1** exhibited slightly lower light emission intensity when coupled to the oligonucleotide ligands used here. This was of little practical significance in this study and so compounds **1** and **4** were therefore both selected for further investigation as oligonucleotide labels for a chemiluminescence quenching assay, which permitted side-by-side comparison of the effect of the geometry of attachment to oligonucleotides on the quenching efficiency. However, it was first necessary to identify a suitable quencher of the chemiluminescent emission.

Selection of optimum quencher

The key requirement for a suitable quenching molecule is that it must act as a dark quencher, which causes the electronically excited state of the primary emitter of the chemiluminescent reaction to reach its ground state by radiationless decay rather than by photonic emission. In the present situation, this property must be a feature of the quencher molecule under the chemical conditions that exist at the point of excited state product formation during the chemiluminescent reaction. By contrast, this is not a consideration for the design of fluorescence quenching systems where there is no net chemical change during the photophysical process. Accordingly, molecules known to quench fluorescence may be ineffective as chemiluminescence quenchers. It is known from fluorescence studies[6] that quenching can occur by either resonance energy transfer or contact-mediated quenching depending on the system concerned. The latter has less constraint in terms of the overlap of the emission spectrum of the donor and the absorption spectrum of the quencher compared to the former. However, in general terms, it is desirable to maximize spectral overlap with a quencher having a high extinction coefficient in the event that resonance energy-transfer is an important contributor to quenching in a given system. Fig. 4 shows an overlay of the emission spectrum of *N*-methylacridone and the absorption spectra of dabcyl and methyl red (as terminal 3′ poly$A_{(10)}$ oligonucleotide conjugates) obtained under conditions corresponding to those required for the chemiluminescent reaction (pH 9). The emission spectrum is representative of the acridone emitters of the chemiluminescent reactions studied here and displays a relatively narrow emission bandwidth of 415 nm to 470 nm with a maximum at approximately 430 nm. It can be seen that, under the chemical conditions used here for the chemiluminescent reaction, the wavelength overlap of the acridone emission with the absorption of a methyl red oligonucleotide conjugate (λ_{max} = 460 nm) was marginally

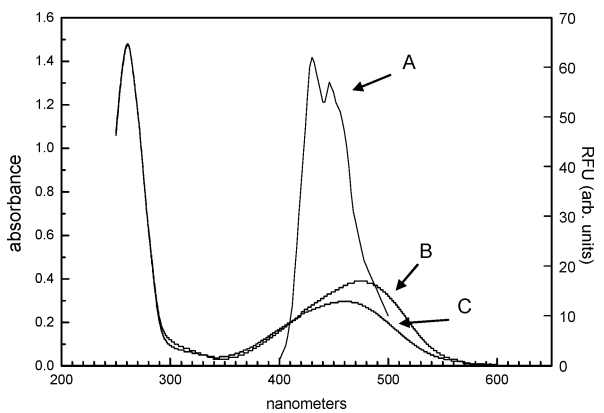


Fig. 4 Absorption spectra of poly$A_{(10)}$dabcyl (**B**) and poly$A_{(10)}$methyl red (**C**) both at approximately 20 μM (normalised by OD 260 nm) in Tris buffer (0.2 M, pH 9.0). (**A**) corresponds to the fluorescence emission spectrum of *N*-methylacridone in arbitrary fluorescence units.

greater than that with a dabcyl oligonucleotide conjugate (λ_{max} = 480 nm). In practice, both compounds appeared suitable though here methyl red was selected as the candidate quencher for further investigation.

Use of AE compound 1 and methyl red quencher derivative 6 as an intramolecular emitter/quencher pair demonstrated by a model chemiluminescence quenching hairpin probe

In order to demonstrate the use of the selected emitters and quencher a labelled "hairpin" oligonucleotide probe sequence 5′*CCGGTC*CAGGTGGAGCAATGATCTTGATCTTCAT-*GACCGG* 3′ (sequence **1**) was used. The model hairpin probe was a conventional stem-loop structure[4] consisting of a 28-nucleotide target binding sequence (underlined) flanked by 6-nucleotide complementary stem-binding sequences (italics). The oligonucleotide was initially created with an incorporated 3′ tritylthio group and a free 5′ amino group. The probe was labelled with either compound **1** or compound **4** at the 5′ amino group and both probes were then de-protected to reveal the free thiol group and labelled with quencher **6** at the 3′ position. In its normal state the labelled probe (sequence **1**) adopts a "hairpin" configuration as a result of intramolecular duplex formation of the stem-binding regions, resulting in juxtaposition of the emitter and quencher. Consequently, when the chemiluminescent reaction was initiated, quenching occurred as demonstrated by the absence of emitted light. However, following incubation with its complementary target, 5′ATGAAGATCAAGATCATTGCTCCACCTG 3′ (sequence **2**), emission of light occurred when the chemiluminescent reaction was initiated. This is consistent with the assumption that the target-binding region of sequence **1** forms an intermolecular duplex with sequence **2** which disrupts the existing stem-binding (intramolecular) duplex of sequence **1**. This, in turn, results in separation of the emitter and quencher. Fig. 5 shows dose-response curves of the binding of the target (sequence **2**) with sequence **1** labelled with quencher **6** and either compound **1** or compound **4**. Although both displayed a target dose-related increase in chemiluminescence intensity, the zero target signal of the compound **1** probe was much lower than that of the compound **4** probe. The high signal observed with the compound **4** labelled probe in the absence of target (*i.e.* the background signal) is probably the result of the covalent dissociation of the primary emitter from the rest of the molecular complex during the chemiluminescent reaction, which reduces the efficiency of specific quenching. By contrast, this does not occur with compound **1** since the primary emitter remains attached to the oligonucleotide. Thus the background signal for compound **1** labelled probe was less than 1% of the signal observed when a 10-fold molar excess of target was present. Accordingly, an approximate sensitivity of detection of less than 2 fmol was achieved.

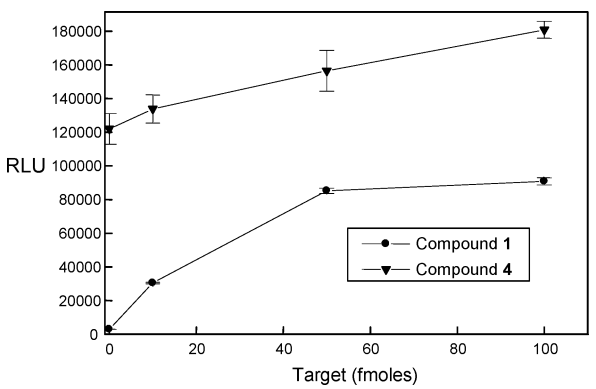


Fig. 5 Dose-response of the binding of target (sequence **2**) to the model hairpin probe (sequence **1**) labelled with either compound **1** or compound **4**. Data points are mean ± SD (n = 3).

The dose-response curve of the assay of the target using the compound **1** labelled probe was also compared using pH 14 and pH 9 chemiluminescence conditions. At pH 14 the zero target signal was much higher than that obtained at pH 9 (Fig. 6) and the assay exhibited a narrower dynamic range.

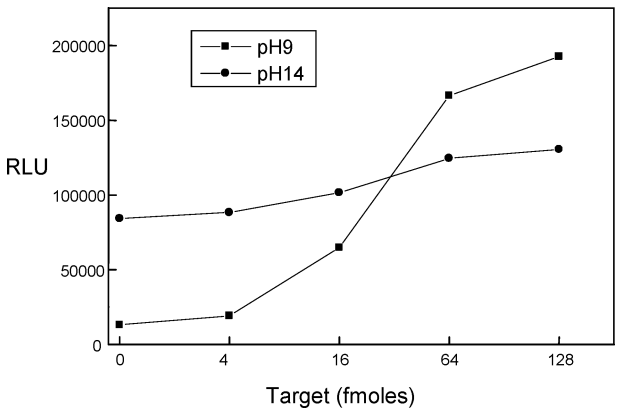


Fig. 6 Dose-response of the binding of target (sequence **2**) to the model hairpin probe (sequence **1**) labelled with compound **1** using chemiluminescence conditions at pH 9 and pH 14.

This observation is consistent with the assertion that with conventional AE chemiluminescence at pH 14, the stem-loop structure is "de-hybridised" non-specifically with the resultant loss of emitter/quencher proximity.

In order to demonstrate the use of the selected chemiluminescent emitter and quencher in an intermolecular emitter/quencher pair we first obtained a 44mer oligonucleotide, 5′CGCGAAGCGTTCGCACTTCGTCCCGCCTTCCTGCG-CCTTCCTGT 3′ (sequence **3**), and a 36mer oligonucleotide, 5′CATGCTCGCAGCGGGACGAAGTGCGAACGCTTCGC-G 3′, (sequence **4**), part of which is complementary to part of sequence **3** (underlined). The first 26 nucleotides of the 5′ end of the 44mer are complementary to the first 26 nucleotides of the 3′ end of the 36mer and the remainder of the nucleotides comprises a non-complementary ragged end with a 3′overhang of the longer strand. The 44mer oligonucleotide was designed with a 5′ NH_2 moiety for subsequent AE conjugation whereas the 3′ end of the 36mer oligonucleotide was provided with the quencher already attached. The 5′ end of the 44mer (sequence **3**) was labelled with either compound **1** or **4**. When hybridised to each other, these oligonucleotides form a duplex with both blunt and ragged ends, consistent with the secondary structure that enables the duplex to act as a substrate for the 5′ DNA helicase enzyme from *B. stearothermophilus*[7] (Cambio Ltd., UK). The emitter/quencher moieties are thus juxtaposed at the blunt end of the duplex.

In order to study the quenching of the emission of compound **1** labelled sequence **3** by quencher labelled sequence **4**, we firstly

incubated the former with a 1000-fold molar excess of the latter at 43 °C for 10 minutes following which chemiluminescence was measured at pH 9 and pH 14.

Fig. 7 shows that co-incubation of the two complementary oligonucleotides resulted in loss of chemiluminescent signal when measured at pH 9. This quenching was not observed using chemiluminescence measurement at pH 14 nor with replacement of sequence **4** with a non-complementary (irrelevant) quencher labelled oligonucleotide with chemiluminescence measured at pH 9, indicating that the loss of signal was due to specific, hybridisation-mediated chemiluminescence quenching.

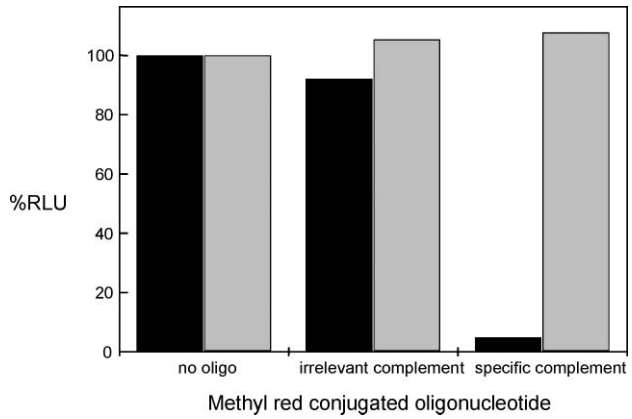


Fig. 7 Intermolecular hybridisation induced chemiluminescence quenching. Data are normalised to allow for increased efficiency of the chemiluminescent reaction initiated at pH 14 (shaded bars) relative to pH 9 (black bars). The response axis is expressed as the RLU as a percentage of the signal obtained with no quencher oligonucleotide present ("no oligo").

In order to permit comparison of the relative performance of compound **1** and compound **4** labelled sequence **3**, a 12.5 fmol aliquot of each labelled probe respectively was incubated with various amounts of the quencher labelled sequence **4** for 60 minutes at 50 °C. Fig. 8 shows that increased duplex formation was associated with a quenched chemiluminescent signal at pH 9 in both cases.

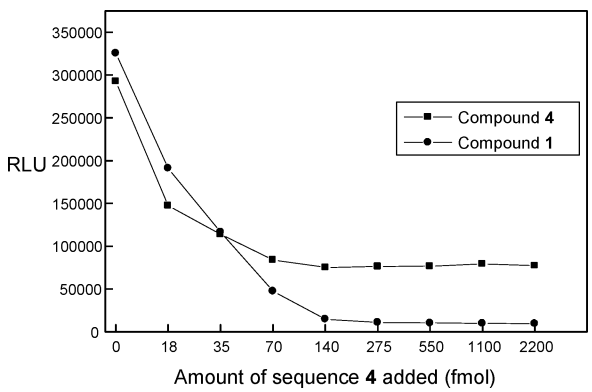


Fig. 8 Comparison of the chemiluminescence emission intensity of compound **1** and **4** labelled sequence **3** following hybridisation with various doses of quencher labelled sequence **4**.

However, in common with the hairpin probe described above, the maximum degree of quenching of the compound **1** labelled oligonucleotide was greater than that observed with the corresponding compound **4** labelled oligonucleotide. Accordingly, the duplex formed by the association of compound **1** labelled sequence **3** and quencher labelled sequence **4** was subsequently used as a substrate for helicase enzyme activity studies in which the action of the enzyme is to unwind nucleic acid duplexes.

Initial studies of AE-labelled sequence **3** (performed in the absence of quencher labelled sequence **4**) indicated that the recovery of signal following the prescribed incubation was only 43% and 47% (relative to the RLU obtained at 0 minutes) for compound **4** and compound **1**, respectively. This suggested that the combination of even slightly alkaline pH (here pH 7.5) and elevated temperature (50 °C) resulted in a loss of signal due to partial hydrolysis of the AE. In order to overcome the potential loss of specific chemiluminescence activity due to AE hydrolysis under the above conditions, the production of substrate was undertaken in mildly acidic buffer (annealing reagent), which was used with extended incubation times at lower temperature. This yielded a substrate comprising a duplex with a quenching efficiency of greater than 99% as measured by comparison of the chemiluminescence yield obtained using pH 9 (maximally hybridised) and pH 14 (maximally single stranded) chemiluminescence conditions. This substrate was thus expected to demonstrate low background and high yield when used in helicase enzyme activity assays.

The suitability of the above duplex as a substrate for helicase enzyme was demonstrated by incubating mixtures of various concentrations of the enzyme with the substrate thence measuring chemiluminescence at pH 9 of 10 μL samples taken from the reaction mixture at defined times. The enzyme incubation was performed in the presence of the required co-factors at pH 6.8 which is optimal for this particular enzyme. Fig. 9 shows the reaction time course for helicase activity, which demonstrates a progressive increase in chemiluminescent signal consistent with the predicted duplex unwinding and consequent separation of the respective emitter/quencher labelled oligonucleotide strands. At maximal unwinding there was an approximately 40-fold increase in chemiluminescent emission relative to the corresponding background signal in the absence of enzyme. Omission of either magnesium or ATP co-factors from the incubation buffer resulted in loss of enzyme activity (data not shown) confirming that the change in chemiluminescence was mediated specifically by enzyme activity.

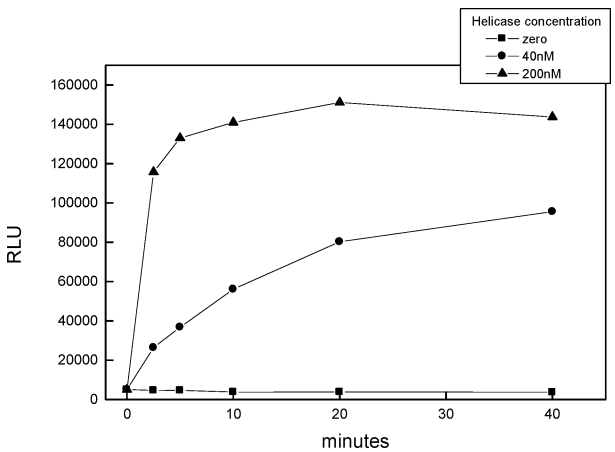


Fig. 9 Time course of helicase-dependent formation of single stranded compound **1** labelled 44mer (sequence **3**).

Conclusion

Fluorescence quenching has been widely used as a means of detecting and quantifying nucleic acids in solution-based ligand-binding assays, but suffers from relatively poor sensitivity of detection in this context. By contrast, chemiluminescent molecules can form the basis of simple, highly sensitive detection systems. However, we have demonstrated that the simple view of taking a known fluorescence quenching system and replacing the fluorescent molecule with a chemiluminescent molecule is an untenable one since other factors need to be considered. Acridinium esters (AE) are widely recognised as highly successful chemiluminescent labels and offer many advantages over other labels. Despite this fact, no workable consideration has been given previously as to how AE might be used in a quenched chemiluminescence, ligand-binding assay since the highly alkaline conditions used to initiate AE reactions are not compatible with maintaining the integrity of the binding complex, which is crucial for modulating the quenching process. We have demonstrated this by showing non-specific loss of quenching when chemiluminescence quenched binding complexes are exposed to the elevated pH normally associated with conventional AE chemiluminescent systems. Moreover, many fluorescence quenchers are potentially incompatible with the change in chemical conditions required to initiate AE chemiluminescence such that performance predicted from fluorescent systems may be reduced under the conditions required for certain chemiluminescence reactions. In fact, we have found it necessary to design a novel AE/quencher pair capable of functioning within the restrictive chemical boundaries required for maintenance of the integrity of biological binding complexes which is crucial for modulation of specific quenching and which is sufficiently stable for routine use.

Chemiluminescence of oligonucleotide probes labelled with compound **1** was empirically optimised to allow an acceptably rapid flash (5 seconds duration) at a pH that allowed maximal signal generation but with minimal disruption of the secondary structure of the hybridised probe. Under the conditions employed here, 0.2 M Tris pH 9.0 was found to give a rapid signal with minimal apparent disruption of the duplex. We also found that derivatives of compound **6** were highly effective quenchers of the chemiluminescence emission under these conditions. Increasing the chemiluminescent reaction pH above 9 showed a progressive increase in background signal consistent with loss of secondary structure and concomitant non-specific loss of quenching.

We have demonstrated the ability of the optimised quenched chemiluminescence system to form the basis of highly sensitive, homogeneous detection systems for oligonucleotide binding processes and related applications in which the intensity of chemiluminescence emission is modulated by either intramolecular or intermolecular duplex formation.

Experimental

All reagents were of the highest grade, purchased from established commercial sources, and were dried where required using recommended methods. Oligonucleotides designed to our own specifications were purchased from Eurogentec (Southampton, UK) and were certified 95% pure.

Synthesis of AEs 1–5 and quencher 6

The various substituted phenyl acridine ester synthetic intermediates of compounds **1–5** were synthesised as described previously.[8] Briefly, the acridine-9-carboxylic acid was converted to the acid chloride by the action of thionyl chloride. A suspension of the acid chloride in anhydrous pyridine was then stirred under anhydrous conditions with the required substituted phenol to yield the ester. Compounds **4** and **5** were prepared as described previously.[8]

Synthesis of 9-(2,6-dibromophenoxycarbonyl)-10-(3-succinimidyloxycarbonylpropyl)acridinium iodide (Compound 1). A solution of 4-iodobutanoic acid (480 mg, 2.24 mmol), *N*-hydroxysuccinimide (247 mg, 2.14 mmol) and dicyclohexylcarbodiimide (523 mg, 2.53 mmol) was stirred in tetrahydrofuran (12 ml) under anhydrous conditions at 0 °C for 3 h then at room temperature overnight. Following filtration of the mixture, the filtrate was concentrated on a rotary evaporator and chromatographed on a silica column eluted with toluene–ethyl acetate (4 : 1 v/v). Fractions at Rf = 0.48 were combined and evaporated to yield succinimidyl 4-iodobutanoate as a yellow solid (567 mg, 84% yield, mp 86–87 °C; ν_{max}/cm^{-1} 1739 and 1657 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 3.22 (2 H, t, *J* 7, $IC\mathit{H}_2CH_2$), 2.78 (4 H, s, $COC\mathit{H}_2C\mathit{H}_2CO$), 2.71 (2 H, t, *J* 7, $CH_2C\mathit{H}_2COO$), 2.18 (2 H, quintet, *J* 7, $CH_2C\mathit{H}_2CH_2$); δ_C (400 MHz, $CDCl_3$, Me_4Si) 4.2, 26.0, 28.5, 32.2, 168.0, 169.4; *m*/*z* (EI) 311 (M^+, 1%), 197 (100), 184 (29), 169 (52)). A portion of this material (51.6 mg, 0.17 mmol) was mixed with 2,6-dibromophenyl acridine-9-carboxylate (57.2 mg, 0.13 mmol) in nitrobenzene (2 mL) and heated in a glass tube on an oil bath at 150 °C for 0.5 h. Acetonitrile (0.5 mL) was added and the crude product precipitated by addition of diethyl ether (2 mL) followed by repeated washing with ethyl acetate (10 × 2 mL). Compound **1** was obtained in low yield (approximately 3%) as a red powder (ν_{max}/cm^{-1} 1816, 1746, 1721 (CO); δ_H (400 MHz, acetonitrile-d_3, Me_4Si) 8.93 (2 H, d, *J* 9, 2 × NCC*H*CH), 8.62 (2 H, d, *J* 8, 2 × CCC*H*CH) 8.40 (2 H,m, 2 × NCCHC*H*), 8.01 (2 H, m, CCCHC*H*), 7.73 (2 H, d, *J* 8, C(Br)C*H*CHC*H*C(Br)), 7.20 (1 H, t, *J* 8, C(Br)CHC*H*CHC(Br)), 5.33 (2 H, t, *J* 8, $NC\mathit{H}_2CH_2CH_2CO$), 3.02 (2 H, t, *J* 7, $NCH_2CH_2C\mathit{H}_2CO$), 2.69 (4 H, s, $COC\mathit{H}_2C\mathit{H}_2CO$), 2.45 (2 H, m, $NCH_2CH_2C\mathit{H}_2CO$); *m*/*z* (ES^+) 638.9769 (M^+ $C_{28}H_{21}{}^{79}Br_2N_2O_6$) requires 638.9761.

Synthesis of 9-(2,6-dinitrophenoxycarbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate (Compound 2). An anhydrous mixture of 11-bromoundecanoic acid (2.192 g, 8.27 mmol) and sodium iodide (3.04 g, 20.3 mmol) was stirred in acetone (20 mL) for 20 h. The mixture was then poured into water (20 mL) and extracted with diethyl ether (4 × 25 mL). The combined ether extracts were washed with saturated sodium thiosulfate solution (20 mL), dried over anhydrous magnesium sulfate, filtered and evaporated to yield a pale pink solid which was purified on a silica column eluted with hexane–ether–formic acid (100 : 100 : 0.1 v/v). Fractions at Rf = 0.49 were combined and evaporated to yield 11-iodoundecanoic acid as a white solid (2.23 g, 83% yield, mp 63–64 °C (lit. 64–65 °C); ν_{max}/cm^{-1} 1693 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 3.12 (2 H, t, *J* 7, $IC\mathit{H}_2CH_2$), 2.23 (2 H, t, *J* 7, $CH_2C\mathit{H}_2CO$), 1.75 (2 H, quintet, *J* 7, $ICH_2C\mathit{H}_2$), 1.56 (2 H, quintet, *J* 7, $CH_2C\mathit{H}_2CH_2CO$), 1.28–1.18 (12 H, m, ICH_2CH_2 $(C\mathit{H}_2)_6CH_2CH_2CO$); δ_C (400 MHz, $CDCl_3$, Me_4Si) 7.5, 25.0, 28.9, 29.4, 29.7 (3 lines), 30.8, 33.9, 34.3, 179.9; *m*/*z*

(EI) 313 ([M + H]$^+$, 3%). A portion of this material (556 mg, 1.78 mmol) was mixed with *N*-hydroxysuccinimide (440 mg, 3.83 mmol) and dicyclohexylcarbodiimide (770 mg, 3.76 mmol) in tetrahydrofuran (20 mL) under anhydrous conditions at 0 °C for 3 h then at room temperature overnight. Following filtration of the mixture, the filtrate was concentrated on a rotary evaporator and chromatographed on a silica column eluted with toluene–ethyl acetate (4 : 1 v/v). Fractions at Rf = 0.59 were combined and evaporated to yield a white solid which was recrystallized from hexane + ethyl acetate to yield succinimidyl 11-iodoundecanoate (387 mg, 53% yield, mp 84 °C; ν_{max}/cm^{-1} 1725 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 3.16 (2 H, t, *J* 7, ICH_2CH_2), 2.81 (4 H, s, $COCH_2CH_2CO$), 2.57 (2 H, t, *J* 7, CH_2CH_2COO), 1.79 (2 H, quintet, *J* 7, ICH_2CH_2), 1.71 (2 H, quintet, *J* 7, $CH_2CH_2CH_2COO$), 1.41–1.22 (12 H, m, $ICH_2CH_2(CH_2)_6CH_2CH_2COO$); δ_C (400 MHz, $CDCl_3$, Me_4Si) 7.7, 25.0, 26.0, 28.9, 29.1, 29.4, 29.6, 29.7, 30.9, 31.3, 34.0, 169.1, 169.6; *m/z* (CI) 427 (M + NH_4^+, 15%), 301 (15), 225 (16), 149 (30) 132 (100). This material (188 mg, 0.460 mmol) was mixed with silver trifluoromethanesulfonate (214 mg, 0.836 mmol) in benzene (1.5 mL) under anhydrous conditions for 18 h at room temperature and the mixture chromatographed on a short (2 g) silica column eluted with dichloromethane. Fractions at Rf = 0.48 were combined and evaporated to yield a white gum consisting of succinimidyl 11-(trifluoromethanesulfonyloxy)undecanoate (133 mg, 67%); ν_{max}/cm^{-1} 1819, 1787, 1725 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 4.44 (2 H, t, *J* 7, $SOCH_2CH_2$), 2.74 (4 H, s, $COCH_2CH_2CO$), 2.51 (2 H, t, *J* 7, CH_2CH_2COO), 1.73 (2 H, quintet, *J* 7, $SOCH_2CH_2$), 1.65 (2 H, quintet, *J* 7, $CH_2CH_2CH_2COO$), 1.37–1.17 (12 H, m, $SOCH_2CH_2(CH_2)_6CH_2CH_2COO$); δ_C (400 MHz, $CDCl_3$, Me_4Si) 24.9, 25.4, 26.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 31.3, 78.1, 119.0, 169.0, 169.6; δ_F (400 MHz, $CDCl_3$) −74.8. A portion of the above product (57 mg, 0.13 mmol) was mixed with 2,6-dinitrophenyl acridine-9-carboxylate (53 mg, 0.13 mmol) in 1,2-dichloroethane (2 mL) and refluxed under nitrogen for 20 h. The solvent was evaporated and the residue dissolved in dichloromethane and chromatographed on a small silica column (0.5 g) eluted with dichloromethane followed by dichloromethane–acetonitrile (3 : 1 v/v). Fractions having Rf = 0.17 were pooled and evaporated to yield compound **2** in low yield (15.8 mg, 14%) as a yellow oil. ν_{max}/cm^{-1} 1812, 1778, 1735 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 8.71–8.60 (4 H, m, 2 × NCC*H*CH, 2 × CCC*H*CH), 8.41 (2 H, dd, *J* 8, 7, 2 × NCCHC*H*CH), 8.34 (2 H, d, *J* 8, 2 × NO_2CCHCH), 7.92 (2 H, dd, *J* 9, 7, 2 × CCCHC*H*CH), 7.78 (1 H, t, *J* 8, $NO_2CCHCHCH$), 5.48 (2 H, t, *J* 8, NCH_2CH_2), 2.69 (4 H, s, $COCH_2CH_2CO$), 2.45 (2 H, t, *J* 7, CH_2CH_2COO), 2.08 (2 H, m), 1.68–1.53 (4 H, m) and 1.35–1.10 (10 H, m) ($NCH_2(CH_2)_8CH_2COO$). δ_C (400 MHz, $CDCl_3$, Me_4Si) 24.9, 26.0, 26.9, 29.0, 29.2, 29.4, 29.5, 29.6, 30.1, 31.3, 53.1, 119.6, 124.1, 128.9, 129.7, 130.2, 131.1, 136.6, 140.7, 141.6, 143.9, 144.8, 161.1, 169.1, 169.7; *m/z* (ES$^+$) 671.2352 ($M^+ - CF_3SO_3^-$) (100%) $C_{35}H_{35}N_4O_{10}$ requires 671.2348), 519 (15), 505 (23), 491 (42), 361 (77), 246 (44).

Synthesis of 9-(2,6-bis(trifluoromethyl)phenoxycarbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate (Compound 3). A mixture of succinimidyl 11-(trifluoromethanesulfonyloxy)undecanoate (75 mg, 0.17 mmol), 2,6-bis(trifluoromethyl)phenylacridinium-9-carboxylate (75 mg, 0.17 mmol) and 1,2-dichloroethane (1.6 mL) was refluxed under nitrogen for 21 h. The yellow residue formed upon evaporation of the solvent was extracted with dichloromethane (1 mL) and the extract chromatographed on silica gel eluted with dichloromethane–acetonitrile (3 : 1 v/v). The fractions having Rf = 0.19 were pooled and evaporated to give compound **3** as a yellow solid in low yield (15.9 mg, 10.7%, mp 164–165 °C; ν_{max}/cm^{-1} 1815, 1779, 1733 (CO); δ_H (400 MHz, $CDCl_3$, Me_4Si) 8.78 (2 H, d, *J* 9, 2 × NCC*H*CH), 8.71 (2 H, d, *J* 9, 2 × CCC*H*CH), 8.49 (2 H, dd, *J* 9, 7, 2 × NCCHC*H*CH), 8.06 (2 H, d, *J* 8, 2 × CF_3CCHCH), 7.98 (2 H, dd, *J* 9, 7, 2 × CCCHC*H*CH), 7.12 (1 H, t, *J* 8, $CF_3CCHCHCH$), 5.60 (2 H, t, *J* 8, NCH_2CH_2), 2.76 (4 H, s, $COCH_2CH_2CO$), 2.51 (2 H, t, *J* 7, CH_2CH_2COO), 2.29–2.18 (2 H, m), 1.84–1.69 (4 H, m) and 1.51–1.26 (10 H, m) ($NCH_2(CH_2)_8CH_2COO$). δ_C (400 MHz, $CDCl_3$, Me_4Si) 24.9, 26.0, 26.9, 29.0, 29.3, 29.4, 29.5, 29.6, 30.1, 31.3, 53.3, 119.8, 124.3, 125.7, 126.0, 128.5, 129.0, 130.0, 132.4, 140.6, 141.7, 144.9, 161.8, 169.1, 169.7; δ_F (400 MHz, $CDCl_3$) −78.4 ($CF_3SO_3^-$), −59.8 (CF_3); *m/z* (ES$^+$) 717.2390 ($M^+ - CF_3SO_3^-$) (100%) $C_{37}H_{35}F_6N_2O_6$ requires 717.2394), 519 (17), 505 (8), 491 (9).

Synthesis of 2-(4-dimethylaminophenylazo)-*N*-[2-(2-iodoacetylamino)ethyl]benzamide (Compound 6). 2-[4-(Dimethylamino)phenylazo]benzoic acid (1.251 g, 4.65 mmol) in THF (30 mL) was cooled to 0 °C and dicyclohexylcarbodiimide (1.103 g, 5.3 mmol) was added, followed by *N*-hydroxysuccinimide (0.568 g, 4.94 mmol). The mixture was stirred at 0 °C for 2 h, thence overnight at room temperature. The mixture was filtered and the filtrate evaporated to dryness to give a red solid which was recrystallised from acetone–diethyl ether and dried under vacuum to give succinimidyl 2-[4-(dimethylamino)phenylazo]benzoate (0.849 g, 50%, mp 124–126 °C; FAB-MS *m/z* 361 [(M + H)$^+$, 23%], 252 [17], 133 [100]). A portion of this (74 mg, 0.20 mmol) in dioxane (2 mL) was mixed with *t*-butyl *N*-(2-aminoethyl)carbamate (99 mg, 0.63 mmol) in methanol (1 mL). The mixture was shaken at room temperature in the dark for 4 h. Tlc (toluene–EtOAc 1 : 1) showed that all the starting material (Rf = 0.85, violet) was converted into a new component (Rf = 0.60, orange). Combined fractions from chromatography on a silica gel column eluted with toluene–EtOAc 1 : 1 yielded *N*-[2-(*t*-butoxycarbonylamino)ethyl]-2-[4-(dimethylamino)phenylazo]benzamide as an orange solid (75 mg, 91%, mp 131–134 °C; FAB-MS *m/z* 434.2 [(M + Na)$^+$, (6%), 412 [(M + H)$^+$, 50%], 252 (82), 133 (100). A portion of this material (70 mg, 0.17 mmol) in dioxane (2 mL) was treated with concentrated hydrochloric acid (0.6 ml). The orange solution turned to violet. The mixture was stirred at room temperature for 15 min and then evaporated to dryness. The residue was washed with dioxane (2 × 3 mL) and diethyl ether (3 mL) and was then dissolved in MeOH (4 mL). Aqueous saturated sodium bicarbonate solution (2 mL) was added followed by dichloromethane (20 mL) and then water (20 mL). The organic layer was isolated, dried over anhydrous magnesium sulfate and evaporated to give *N*-(2-aminoethyl)-2-[4-(dimethylamino)phenylazo]benzamide as a red solid (42 mg, 79%), mp 119–124 °C; FAB-MS *m/z* 312 [(M + H)$^+$, 13%), 252 (31), 133 (100). To this material (60 mg, 0.19 mmol) in dichloromethane (5 mL) was added triethylamine (20 mg, 0.20 mmol). The mixture was cooled to 0 °C and iodoacetyl chloride (40 mg, 0.20 mmol) was added. After 5 min at 0 °C, tlc (CH_3Cl–MeOH, 4 : 1) showed that most of the starting material (Rf = 0.4) was converted into a new

component (Rf = 0.6). Stirring at 0 °C was maintained for another 5 min and the resulting mixture was then evaporated to dryness. The residue was redissolved in dichloromethane (3 mL) and loaded onto a silica gel column, which was then eluted with CH_3Cl–MeOH, 7 : 1. The desired fractions were combined and evaporated to yield 2-(4-dimethylaminophenylazo) *N*-[2-(2-iodoacetylamino)ethyl]benzamide (25 mg, 27%, mp 140–142 °C; δ_H (400 MHz, $CDCl_3$, Me_4Si) 9.52 (exch.), 8.35 (1 H, dd, *J* 8, 2), 7.80–7.73 (3 H, m), 7.54 (1 H, td, *J* 8, 2), 7.48 (1 H, td, *J* 8, 2), 6.79 (2.h, d, *J* 9), 3.70 (2 H, m), 3.61 (2 H, s), 3.53 (2 H, m), 1.70 (exch.); FAB-MS *m/z* 480 [$(M + H)^+$, 75%)], 252 (52), 107 (100).

Chemiluminescence kinetics and stability measurements

Compounds **1**, **2** and **3** were dissolved in acetonitrile (50 μL) and diluted in 1 mM hydrochloric acid to yield a concentration between 1 and 10 ng/mL. The chemiluminescence of 10 μL samples of these solutions was measured in a luminometer ("Lumino", Stratec, Pforzheim, Germany) equipped with *in situ* addition of initiating reagents. Chemiluminescence was activated by sequential dispensing of 80 mM H_2O_2 (200 μL) and 0.2 M glycine pH10 (200 μL) with signal measurement for 1 s (following the second reagent addition) at a 50 ms sample rate. In order to obtain an approximate, relative measure of stability of the compounds, the above acidic aqueous solutions of compounds **1**, **2** and **3** were held at 4 °C and further 10 uL samples removed and measured at intervals of 4 and 6 days. AEs **1**, **4** and **5** (1–10 ng) were compared at pH 9 and pH 14. Here the second reagent addition in the luminometer was either respectively 0.2 M Tris pH 9.0 or 0.5 M NaOH.

Comparison of the absorption spectra of quenchers

Initial experiments investigated the overlap between the known fluorescence emission spectrum of *N*-methylacridone (which approximates to the chemiluminescence emission spectra of the AEs being studied here) and the absorption spectra of $polyA_{10}$ dabcyl (17 μM) and $polyA_{10}$ methyl red (21 μM). Absorption spectra were recorded in Tris buffer (0.2 M, pH 9) over the range from 250 nm to 600 nm using a Cecil 3000 spectrophotometer.

Conjugation of AE to oligonucleotides

Oligonucleotides for labelling with AEs were synthesised with a terminal 5′ or 3′ NH_2. The oligonucleotide (10 nmol) in labelling buffer (10 μL, HEPES 0.125 M, pH 8.0) was added to a solution of the AE in DMSO (10 μL). The contents were gently mixed and incubated for 20 min at 37 °C, following which excess *N*-hydroxysuccinimide ester on the AE was deactivated by addition of lysine (5 μL, 0.125 M in labelling buffer). The oligonucleotide was precipitated by adding sequentially aqueous sodium acetate (30 μL, 3 M), water (245 μL) and aqueous glycogen (5 μL, 40 mg/mL) and chilled (–20 °C) ethanol (640 μL), then was incubated on dry ice for 10 min prior to centrifugation at 15̄000 rpm in a 0 °C centrifuge. The supernatant was carefully aspirated and the pellet taken up in probe storage reagent (20 μL, sodium acetate, 0.1 M, containing lithium lauryl sulfate, 0.1%, w/v).

The labelled oligonucleotide was further purified by conventional gradient RPLC (C_{18}), using aqueous triethylammonium acetate buffer (0.1 M, pH 7.0)/acetonitrile. Absorbance was monitored at 260 nm and 0.5 mL fractions were collected into aqueous lithium lauryl sulfate (10 μL, 10%, w/v). Chemiluminescence (1 μL/fraction) was measured at pH 14 and the peak fractions were pooled. The labelled oligonucleotide was re-precipitated by sodium acetate/chilled ethanol as above, then taken up in probe storage reagent and stored at –70 °C. AE conjugated oligonucleotide yields and concentrations were determined by conventional absorption spectroscopy using a GenQuant spectrophotometer (Pharmacia) as recommended by the manufacturer.

Conjugation of quencher 6 to AE-labelled oligonucleotides

Oligonucleotides for dual-labelling were synthesised by established methods and obtained commercially with an amine linker to facilitate AE labelling and a trityl-protected thiol to facilitate coupling of the quencher **6**. The desired quantity of AE-labelled oligonucleotide prepared as described above (typically 10 nmol in 20 ul probe storage reagent) was diluted with an aqueous solution of triethylammonium acetate (200 μL, 0.1 M, pH 7.0). The protective trityl group was removed by the addition of aqueous silver nitrate solution (6.7 μL, 0.01 M) and the mixture incubated for 30 minutes at room temperature. An aqueous solution of dithiothreitol (10 μL, 0.01 M) was added and the mixture incubated for 5 minutes at room temperature with shaking following which it was centrifuged at 10̄000 rpm for 2 minutes. The supernatant was carefully removed and transferred to a fresh test tube to which an aqueous solution of sodium bicarbonate (195 μL, 0.1 M, pH 9.0) was then added. Quencher **6** (1 mg) was dissolved in DMSO (30 μL) and 6.6 μL of this was added to the AE-labelled oligonucletide solution with mixing. Following incubation for 90 minutes at room temperature, the mixture was clarified by centrifugation at 10̄000 rpm for 2 minutes. The supernatant was removed and the oligonucleotide recovered by sodium acetate/ethanol precipitation as described above. The pellet was dissolved in probe storage reagent and stored at –70 °C.

Intramolecular dual-labelled probe experiments

For hybridisation experiments using the hairpin probe, AE quencher labelled sequence **1** (10 fmol) in hybridisation buffer [50 μL, aqueous lithium succinate (0.1 M, pH 5.1) containing lithium lauryl sulfate (8.5% w/v), LiOH (125 mM), EDTA (1.5 mM) and EGTA (1.5 mM)] was mixed with various known quantities of oligonucleotide target (sequence **2**) in water (50 μL) and incubated at 60 °C for 15 min followed by room temperature incubation for 15 min. Chemiluminescence of an aliquot of the hybridisation assay solution (10 μL) was then measured in the luminometer. For pH 14 measurement the luminometer reagents were (1) aqueous H_2O_2 (0.2 mL, 80 mM) followed immediately by (2) aqueous NaOH (0.2 mL, 0.5 M). Measurement duration was 1 to 2 seconds as appropriate. For pH 9, a single addition of aqueous Tris/HCl (0.2 mL, 0.2 M) containing H_2O_2 (80 mM) was used, with a measurement duration of 1 to 5 seconds as appropriate.

Intermolecular dual-labelled probe experiments

Initial experiments were designed to investigate the optimal conditions for maximal annealing and to demonstrate that the

intermolecular quenching worked in practice. These experiments used dilutions of the two labelled single stranded oligonucleotides (AE labelled sequence **3** and quencher labelled sequence **4**) in aqueous HEPES buffer (0.1 M, pH 7.4) containing sodium chloride (10 mM) and glycerol (10% v/v). The AE labelled sequence **3** was incubated with excess quencher labelled sequence **4**. It was determined that the optimal annealing protocol for helicase substrate generation consisted of incubation, in annealing reagent [aqueous sodium succinate (20 mM), sodium chloride (50 mM), magnesium chloride (1 mM), Triton-X100 (0.1% w/v), pH 5.1)], of quencher labelled sequence **4** (3 μM) with the AE labelled sequence **3** (2 μM) for 18 hours at room temperature, which yielded 200 pmol of substrate. Aliquots of the substrate solution were stored at −80 °C. The enzyme assay buffer was aqueous HEPES (0.1 M, pH 6.8), containing sodium chloride (10 mM), magnesium chloride (5 mM), bovine serum albumin (500 μg/mL), adenosine triphosphate (ATP, 1 mM). A mixture of various concentrations of enzyme (0, 40 and 200 nM) and substrate (2.5 nM) diluted in enzyme buffer was incubated at room temperature and the reaction stopped by the addition of one volume of stop reagent (enzyme buffer plus Triton X-100, 1% w/v). Chemiluminescence was measured at pH 9 in a 10 μL sample aliquot of the incubation solutions using the method described above for the intramolecular labelled probe experiments.

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