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Assay amplification-multiple valent fluorophores

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The synthesis of a dendrimeric, internally quenched, fluorogenic peptide allowed signal amplification following enzymatic cleavage.

Biological assays and screens utilise a variety of reporter systems, for example the detection of, or a change in, a fluorogenic property, the position of a labelled band on a gel and so forth. These systems are inherently limited in their sensitivity by the nature of the fluorophore or reporter system. Multivalency systems could, in theory, be detected at much lower concentrations enhancing applications in many areas of biology, from DNA sequencing to HT screens.[1]

One specific assay system that has been developed for high-throughput screening is fluorescence resonance energy transfer (FRET), a convenient, rapid and sensitive method, which has been widely exploited in many areas, in particular the assaying of proteolytic activity.[2] This assay is based on a phenomenon in which excitation energy is absorbed and then transferred from a fluorescent donor to a quenching acceptor. The energy transfer efficiency is mainly dependent upon the spectral overlap of the quencher and the donor and the distance between the two dyes.[3] When the two dyes are separated, for example by proteolytic cleavage, the fluorescence emission increases allowing direct, real time, monitoring of the cleavage.

With the aim of improving enzyme assay sensitivity in the area of protease detection, the concept of using multiple valency in a dual label biological assay was explored. The study was performed using a tri-branched system of donors and one acceptor group and was compared with a conventional FRET substrate of the same composition. FRET-amplified dendrimers are described in the literature by Fréchet[4] and Müllen[5] and their coworkers as light harvesting systems but they are never used in biologic assays. Many donor–quencher couples are described in the literature.[6] The dansyl–dabsyl couple has been used by Hartwig to monitor Heck reactions during catalyst screenings,[7] in addition these dyes are inexpensive, and can easily be used in solid or solution phase synthesis and display efficient energy transfer properties (dansyl λ_{ex} = 350 nm, λ_{em} = 550 nm, dabsyl λ_{ex} = 530 nm).

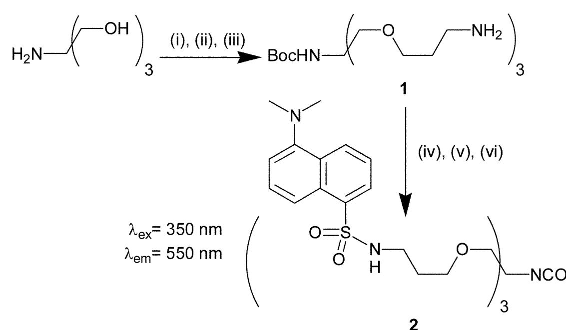
The multivalent tri-dansyl labelled isocyanate **2**, with a 5-atom spacer between the branching center and the fluorophore, was synthesised as shown in Scheme 1.[8] Thus Michael addition of 1,1,1-tris(hydroxymethyl)aminomethane onto acrylonitrile was followed by Boc-protection and nitrile reduction with borane–THF to give **1**. The dansyl group was attached to the three amino groups, the Boc group removed and the free amine converted to an isocyanate using stoichiometric amounts of DMAP and Boc_2O.[9,10]

The peptide sequence H-Gly-Pro-Ala-Lys-Leu-Ala-Ile-Gly-OH is readily cleaved with trypsin and due to its availablity this model protease was used for assay development purposes.[11] Peptide synthesis was carried out using solid phase methods following a standard Fmoc-strategy using an HMPA linker attached to aminomethyl PS resin (1.11 mmol g^{-1}, 1% DVB, 75–150 μm).

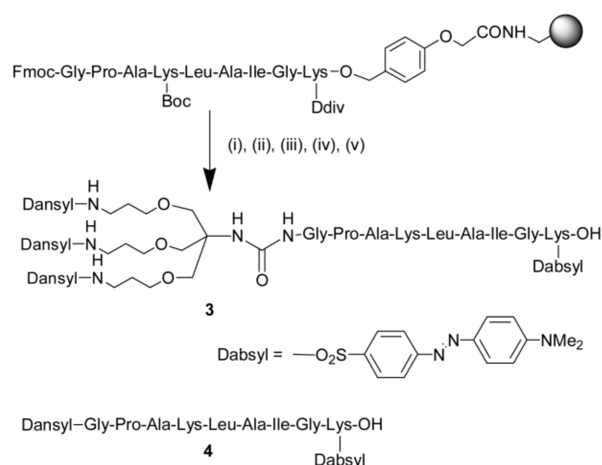
The first amino acid attached was Fmoc-Lys(Ddiv)-OH [Ddiv is 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)isovaleryl][12] which allowed subsequent side chain derivatisation with dabsyl chloride following Ddiv cleavage (2% hydrazine). The labelled dendrimer scaffold was grafted onto the *N*-terminal glycine residue of the peptide using the reactive isocyanate group. Each step of this synthesis was monitored by a ninhydrin test. The attachment at the *N*-terminus was chosen in order to maximize enzyme accessibility. The multilabelled substrate **3** was obtained after TFA cleavage and purification.

The control peptide **4** (one donor and one acceptor), was synthesised following the same procedure, using dansyl chloride instead of monomer **2** (Scheme 2). Both peptides were purified by semi preparative HPLC, lyophilised and characterised by LC-MS.[13]

Pictures A, B, C, D (Fig. 1) were taken at various stages of the synthesis and show the efficiency of the FRET process. Thus



Scheme 1 Synthesis of the tri-dansyl labelled monomer **2**. *Reagents and conditions:* (i) acrylonitrile, KOH 40%, dioxane, 3 d, 55%; (ii) Boc_2O, NEt_3, MeOH, 4 h, reflux, 75%; (iii) BH_3–THF, dioxane, 2 h, reflux; (iv) dansyl chloride, NEt_3, DCM, 4 d, 32% (2 steps); (v) TFA, DCM, 45 min, quantitative; (vi) Boc_2O, DMAP, THF, 90 min, −10 °C.



Scheme 2 Synthesis of the FRET-amplified substrate **3** and the control peptide **4** on solid phase. *Reagents and conditions*: (i) 20% piperidine in DMF, 20 min; (ii) for **3**: monomer **2**, DIPEA, DMAP, DCM, overnight. For **4**: dansyl-Cl, NEt_3, DCM, overnight; (iii) 2% hydrazine in DMF, 2 h ; (iv) dabsyl-Cl, NEt_3, DCM, overnight; (v) TFA–DCM–TIS 95 : 3 : 2, 90 min. The peptides were precipitated with cold Et_2O, centrifuged and washed with Et_2O. Isolated yields: 32% (**3**) and 42% (**4**).

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under UV illumination the bead tri(dansyl)-GPAKLAIGK(D-div)-resin is highly fluorescent whereas the latter one [tri-(dansyl)-GPAKLAIGK(dabsyl)-resin] is completely quenched. This proves the FRET process can occur efficiently on the beads which is encouraging for dendritric enhancing of FRET signals for on bead assays.

The substrates were then treated with the protease and were cleaved as expected, specifically at the Lys–Leu bond, leading to an increase of fluorescence (Fig. 2).[14]

The control peptide **4** (blue line) showed good quenching efficiency (84%) for this dansyl–dabsyl couple despite an interchromophore distance of nine amino acids. Peptide **3** (red line) showed an even higher quenching efficiency (97%). The overall increase in fluorescence in going from the cleaved mono-labelled peptide **4** to the cleaved tribranched labelled peptide **3** was 2.8. However, taking into account the background levels of the non-cleaved peptide and the fluorescence increase (peptide **4**: 1 $\rightarrow$ 6.1, peptide **3**: 1 $\rightarrow$ 28.4), the signal-to-noise ratio is actually increased by a factor of 4.6 (Table 1). The amplification here resulting from the three fluorophore branched emission on the cleaved peptidic derivative, with little or no self-quenching observed in this tri-branched dansyl construct.

In conclusion, a FRET-amplified substrate has been successfully prepared in high purity. Its cleavage shows a significant fluorescence increase over the corresponding monovalent peptide, while the quench efficiency shown is even better than the control peptide consisting of 1 donor and 1 quencher. Clearly amplification enhancement of this magnitude is highly significant in assay terms and is now being explored in other assay systems.

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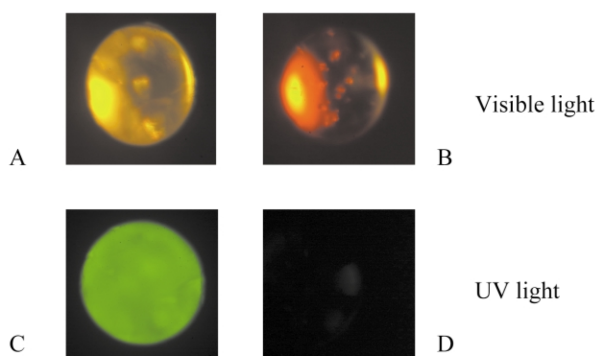

Fig. 1 (A) The tri(dansyl)-GPAKLAIGK(Ddiv)-resin (yellow) and (B) the tri(dansyl)-GPAKLAIGK(dabsyl)-resin (red) under visible light. (C) and (D) The same resin beads under UV illumination (λ = 355 nm). The resin bearing the FRET peptide (red) is not detectable under UV light.

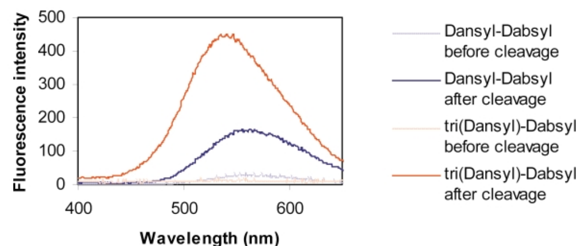

Fig. 2 Fluorescence emission spectra of the substrate **3** tri(dansyl)-GPAKLAIGK-(dabsyl) (red line) and of the control (dansyl)-GPA-KLAIGK-(dabsyl) **4** (blue line) (to measure fluorescence, the peptides were excitated at 335 nm and the emission observed at 560 nm). The spectra of cleaved peptides (continuous line) were registered after no more significant fluorescence increase was noticed. Both peptides had an initial concentration of 10 μM.

Table 1 Fluorescence intensity (arbitrary units) of the subtrates before and after the enzymatic cleavage. The peptides were excitated at 335 nm and the values were registered at 545 nm (**3**) and 560 nm (**4**)

Peptide	Starting fluorescence	Final fluorescence	Amplification
4	27	165	6.1
3	16	455	28

Notes and references

1 M. S. Shchepinov, *The Glen Report*, 1999, **12**, 1.
2 S. Latt, S. A. Auld and B. L. Vallee, *Anal. Biochem.*, 1972, **50**, 56; I. J. White, J. Lawson, C. H. Williams and N. M. Hooper, *Anal. Biochem.*, 1999, **268**, 245; M. W. Pennington and N. A. Thornberry, *Peptide Res.*, 1994, **7**, 72; L. L. Maggiora, C. W. Smith and Z.-Y. Zhang, *J. Med. Chem.*, 1992, **35**, 3727; M. Zimmerman, B. Ashe, E. C. Yurewicz and G. Patel, *Anal. Biochem.*, 1977, **78**, 47; M. Zimmerman, E. C. Yurewicz and G. Patel, *Anal. Biochem.*, 1976, **70**, 258; R. G. Kruger, P. Dostal and D. G. McCafferty, *Chem. Commun.*, 2002, 2092.
3 P. Wu and L. Brand, *Anal. Biochem.*, 1994, **218**, 1.
4 S. L. Gilat, A. Adronov and J.-M. J. Fréchet, *Angew. Chem., Int. Ed.*, 1999, **38**, 1422; A. Adronov and J.-M. J. Fréchet, *Chem. Commun.*, 2000, 1701; A. Adronov, S. L. Gilat, J.-M. J. Fréchet, K. Ohta, F. V. R. Neuwahl and G. R. Fleming, *J. Am. Chem. Soc.*, 2000, **122**, 1175.
5 T. Weil, E. Reuther and K. Müllen, *Angew. Chem., Int. Ed.*, 2002, **43**, 1900.
6 M. Meldal and K. Breddam, *Anal. Biochem.*, 1991, **195**, 141; E. D. Matayoshi, G. T. Wang, G. A. Krafft and J. Erickson, *Science*, 1990, **247**, 954; H. Nagase, C. G. Fields and G. B. Fields, *J. Biol. Chem.*, 1994, **269**, 20952; G. Rossé, E. Kueng, M. G. P. Page, V. Schauer-Vukasinovic, T. Giller, H. W. Lahm, P. Hunziker and D. Schlatter, *J. Comb. Chem.*, 2000, **2**, 461; E. D. Matayoshi, G. T. Wang, G. A. Krafft and J. Erickson, *Science*, 1990, **247**, 954.
7 J. P. Stambuli, S. R. Stauffer, K. H. Shaughnessy and J. F. Hartwig, *J. Am. Chem. Soc.*, 2001, **123**, 2677; S. R. Stauffer, N. A. Beare, J. P. Stambuli and J. F. Hartwig, *J. Am. Chem. Soc.*, 2001, **123**, 4641.
8 S. Lebreton, N. Newcombe and M. Bradley, *Tetrahedron Lett.*, 2002, **43**, 2479.
9 H. J. Knölker, T. Braxmeier and G. Schlechtingen, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 2497.
10 The reaction to convert the amino group into isocyanate was monitored by TLC and the monomer **2** was characterised by LC-MS [(M + H)$^+$: 1018] and by IR (υ_{NCO}: 2253 cm^{-1}).
11 S. Grahn, D. Ullmann and H. D. Jakubke, *Anal. Biochem.*, 1998, **265**, 225.
12 S. R. Chhabra, B. Hothi, D. J. Evans, P. D. White, B. W. Bycroft and W. C. Chan, *Tetrahedron Lett.*, 1998, **39**, 1603.
13 The peptide **3** tri-(dansyl)-GPAKLAIGK-(dabsyl) was characterised by MALDI-TOF-MS [(M + H)$^+$: 2159] and the control peptide **4** (dansyl)-GPAKLAIGK-(dabsyl) by LC-MS [(M + H)$^+$: 1374]. Their purities, determined by HPLC, were >95% (λ = 254 nm).
14 Experimental conditions for enzymatic cleavages: the hydrolysis were performed in a 3 ml reaction volume containing 50 mM Hepes buffer (pH 8.0), 15% DMSO and 10 μM peptide. The reaction was started by addition of the enzyme. The DMSO was added to aid solubility but did not affect enzyme activity. The cleavage rate was measured using a spectrofluorometer (excitation at 335 nm and monitoring emission at 560 nm). Hydrolysis was also followed and checked by RP-HPLC.