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Fluorescence Enhancement through Enzymatic Cleavage of Internally Quenched Dendritic Peptides: A Sensitive Assay for the AspN Endoproteinase**

John M. Ellard, Thomas Zollitsch, W. Jon Cummins, Alan L. Hamilton, and Mark Bradley*

Fluorescence resonance energy transfer (FRET) systems in which a peptide sequence contains both a fluorophore and an internal quencher are amongst the best methods available for protease analysis and characterization. Numerous proteases have been studied using this method, including trypsin which cleaves the FRET-based system Dabcyl-Gly-Pro-Ala-Lys-Leu-Ala-Ile-Gly-Edans,^[1] cathepsin B which cleaves Abz-Lys-Leu-X-Phe-Ser-Lys-Gln-EDDnp (where X is Cys(SBz), Thr(OBz), or Ser(OBz) and EDDnp is 2,4-dinitrophenylthylenediamine),^[2] leukotriene D₄ hydrolase which cleaves ϵ -DNP-L-Lys-D-Amp^[3] (where Amp is 2-amino-3-(7-methoxy-4-coumaryl)propanoic acid) and caspase 1 and 3 which cleave the FRET systems BFP-Tyr-Val-Ala-Asp-GFP and BFP-Asp-Glu-Val-Asp-GFP, respectively (where BFP is blue fluorescent protein and GFP is green fluorescent protein)^[4] to name but a few. A host of methods have been developed using this methodology to determine the most appropriate substrate for a particular protease. For example, Meldal et al. have reported a powerful general method for the use of a split-and-mix approach to libraries of FRET-based peptides for on-bead screening to determine optimal substrates for a range of proteases including subtilisin carlsberg,^[5] FRET-based hexapeptide libraries have been used to map the S'-subsite specificity of serine proteases using solution-based assays,^[6] and a positional scanning substrate library has been used to determine the specificity of ICE (= interleukin-converting enzyme).^[7]

In this paper a new method for the detection of proteolytic activity is presented using internal fluorescence quenching between fluorophores of the same type. These are attached to a dendrimer-type structure, thereby giving a high local concentration of fluorophore needed for quenching yet eliminating the need for a separate quenching moiety. This method simplifies the synthesis of substrates for assay compared to traditional FRET, while being very sensitive due to the amplification nature of the assay as multiple

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cleavage sites are available. This self-quenching phenomenon was first observed in a series of dibranched, tribranched, and hexabranched dendrimeric structures, terminally loaded with fluorophores, that showed significantly reduced fluorescence with increasing branching. Thus at equal concentrations of the labeled dendrimer (determined by absorbance) the fluorescence output decreased as shown in Figure 1. This fluorescence quenching is even more significant when one considers that identical molar ratios of the dendrimers were used and that the number of fluorophores is actually increasing along the series.

For these experiments and subsequent loading with labeled peptides “dendrimers” were prepared on the solid phase

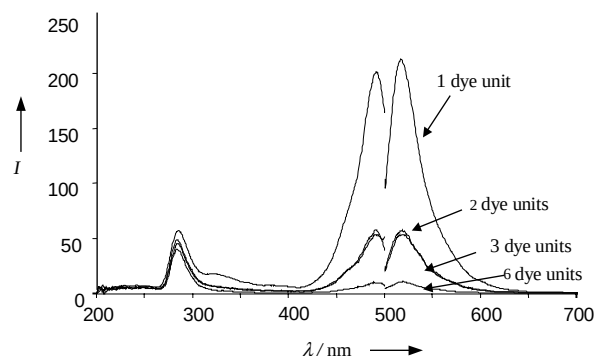
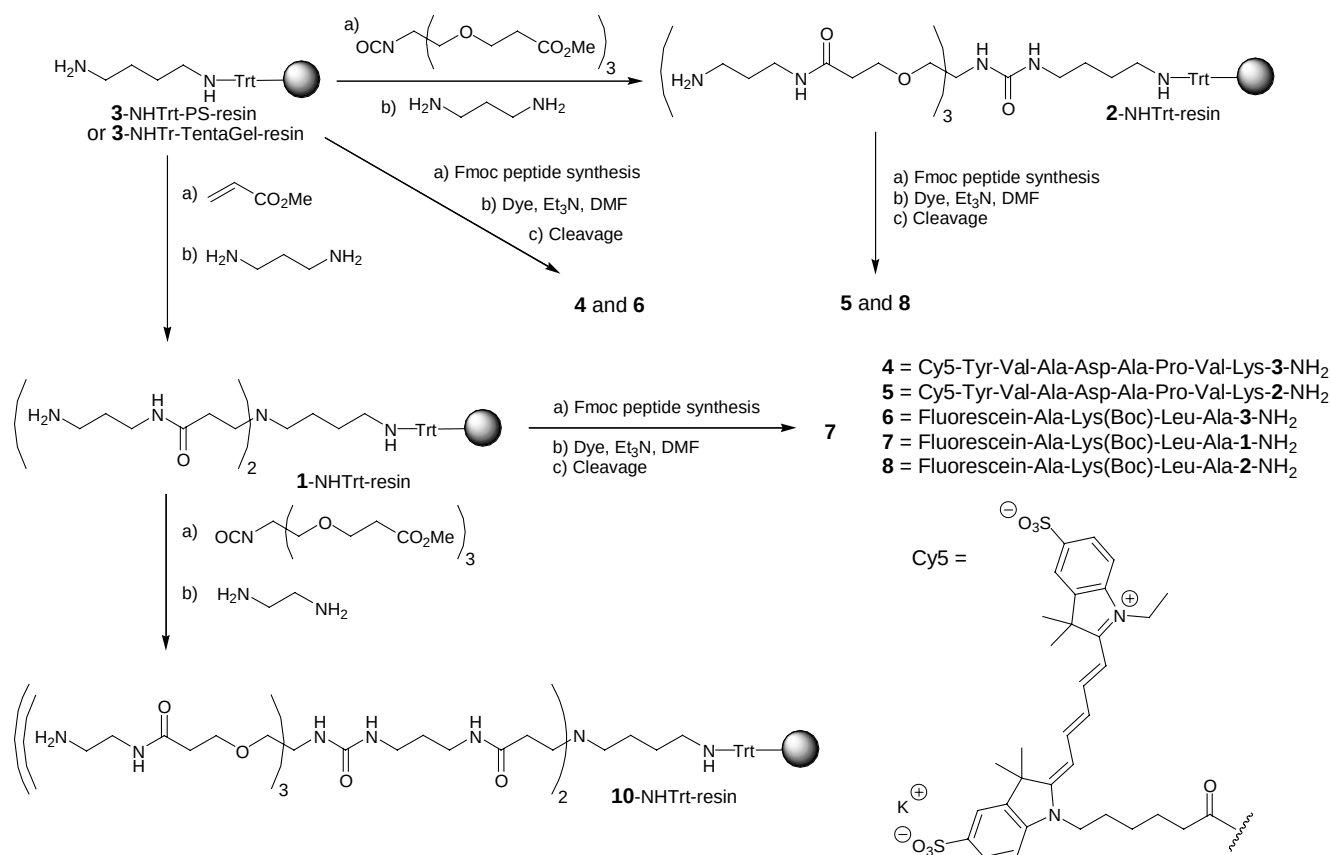


Figure 1. Fluorescence spectra (excitation 492 nm, emission 520 nm) of fluorescein-labeled constructs **3** (1 dye unit), **1** (2 dye units), **2** (3 dye units), and **10** (6 dye units) all at 0.25 μM .

(Scheme 1). Thus resin-bound 1,3-diamino-PAMAM-type dendrimer (**1**-NHTrt-resin) was prepared from 1,4-diaminobutane loaded onto TentaGel resin by a trityl (Trt) linker (0.22 mmol g^{-1} , 90 μm beads) by sequential treatment with methylacrylate and 1,3-diaminopropane.^[8a] **2**-NHTrt-resin was prepared from 1,4-diaminobutane loaded onto polystyrene resin again by a trityl linker (1.5 mmol g^{-1}) by treatment with dimethyl 6-isocyanato-6-(4-carbomethoxy-2-oxabutyl)-4,8-dioxaundecanedioate and 1,3-diaminopropane.^[8b]

The peptide Tyr-Val-Ala-Asp-Ala-Pro-Val-Lys-OH, which is specific for the protease endoprotease AspN, was constructed on the diamine resin **3**-NHTrt-resin and the dendrimer resin **2**-NHTrt-resin (Scheme 1) using standard Fmoc peptide synthesis.^[9] After the final Fmoc deprotection step the amino termini of the peptides were labelled with an *N*-hydroxysuccinimide activated ester of sulfonated Cy5. Finally the peptides were cleaved from the resin by 50% TFA and 3% TIS in CH_2Cl_2 , precipitated with ether, and purified by reversed-phase HPLC (details in Supporting Information) to afford the fluorescent peptides **4** and **5**, respectively (TFA = trifluoroacetic acid, TIS = triisopropylsilane).

Initial enzyme cleavage experiments were carried out on the linear peptide **4** and showed complete and rapid cleavage of the starting peptide. Dendritic peptide **5** was also rapidly consumed affording a fluorescent product with an identical retention time to that previously obtained from **4** (Figure 2), indicating identical cleavage sites of the two peptides. As expected, fluorescence monitoring of the enzymatic cleavage



Scheme 1. Synthesis of dendrimers **1**, **2**, and **10** and peptides **4**–**8**.

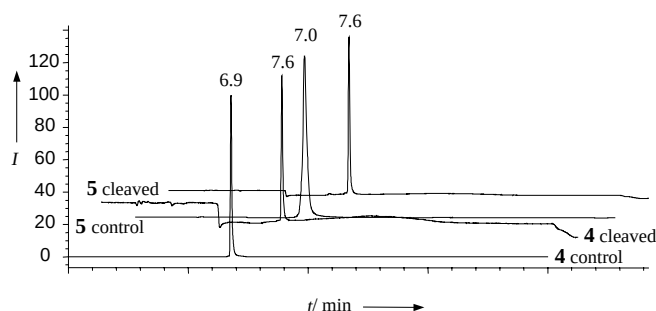


Figure 2. HPLC traces of AspN cleavage of peptides **4** and **5**.

of the linear substrate **4** revealed no increase in fluorescence, while treatment of **5** revealed a fivefold enhancement of fluorescence upon cleavage (Figure 3). From the cleavage

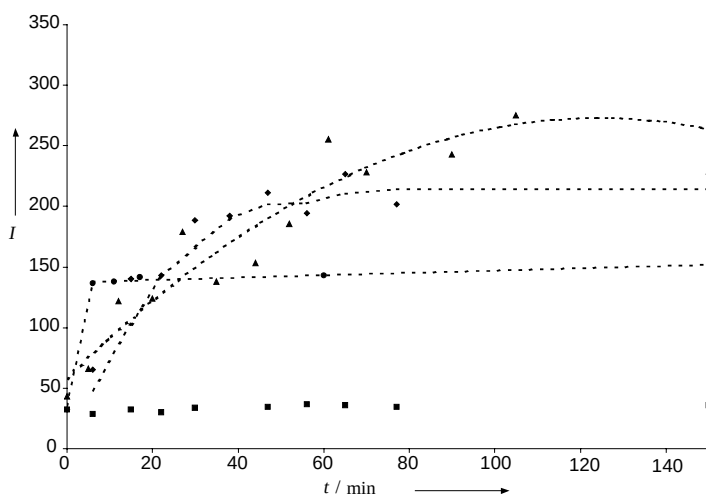


Figure 3. Fluorescence emission over time during AspN cleavage (excitation 640 nm, emission 670 nm) of peptide **5** (●) and chymotrypsin cleavage (excitation 492 nm, emission 520 nm) of peptides **7** (◆) and **8** (▲); ■ controls.

kinetics of substrate **5** a K_m of 4 μM was deduced (Figure 4). However it should be borne in mind that this value is complicated as the tribranched, dibranched and monobranched materials are all substrates for the enzyme.

In order to illustrate the generality of this new technique a second example was sought using a different fluorophore. The peptide Ala-Lys-Leu-Ala-OH was constructed on the diamine resin 3-NHTTrt-resin and the two dendrimer resins 1-NHTTrt-resin and 2-NHTTrt-resin (Scheme 1). Following Fmoc deprotection, fluorescein was introduced by treatment of the resin with fluorescein isothiocyanate and triethylamine. Attempts at TFA-mediated cleavage resulted in decomposition of the peptides (through a typical Edman sequencing cleavage). Resin cleavage was accomplished with 30% HFIP (= 1,1,1,3,3,3-hexafluoro-2-propanol) in CH_2Cl_2 affording the protected peptides **6**, **7**, and **8** (details in Supporting Information).

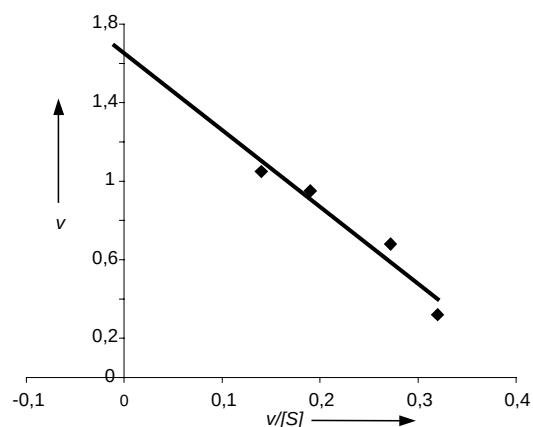


Figure 4. Eadie-Hofstee plot for AspN cleavage of **5**.

The treatment of peptide **6** with chymotrypsin, monitored by HPLC, led to the formation of peptide **9** (FITC-Ala-Lys(Boc)-Leu-OH) by cleavage between the leucine and alanine residues. On the other hand no increase or decrease in fluorescence was observed. Attention was then turned to the cleavage of peptides **7** and **8**. When monitored by HPLC an identical fluorescent peak to that previously obtained was produced (Figure 5) thus indicating that all peptides were cleaved at an identical position.

The fluorescence emission over time for the cleavage of both the PAMAM-peptide dendrimer **7** and the tris(dendrimer) **8** was measured (Figure 3). As can be seen both enzymatic reactions led to a dramatic increase in fluorescence: for **7** there was a six- to sevenfold increase, while for **8** an almost ninefold enhancement was observed.

In conclusion, internally quenched fluorescently labeled dendritic peptides (**5**, **7**, and **8**) have been prepared. Enzymatic cleavage caused a large increase in fluorescence emission, and in the case of dendrimer **8** almost a ninefold fluorescence enhancement was observed. Internally quenched dendritic peptides of this type thus represent a new assay method for proteases avoiding much of the synthetic complexities of FRET-based systems yet with good sensitivity and signal-to-noise ratios.

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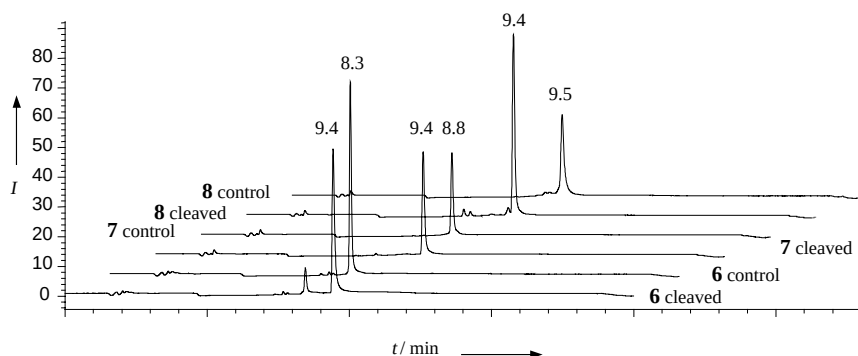


Figure 5. HPLC traces of chymotrypsin cleavage of peptides **6-8**.

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Layer-By-Layer Deposition and Ordering of Low-Molecular-Weight Dye Molecules for Second-Order Nonlinear Optics**

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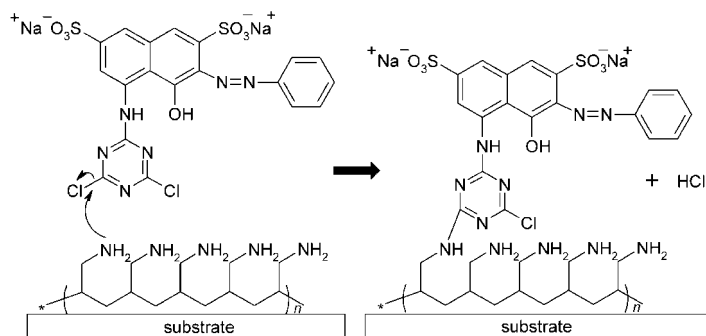
Materials that exhibit second-order nonlinear optical (NLO) properties are key components in electrooptic modulators and frequency-doubling devices.^[1] Second-harmonic generation (SHG), in which incident light at one frequency is converted into light at twice that frequency, is one example of second-order NLO phenomena and is often used as an experimental probe of the second-order susceptibility ($\chi^{(2)}$). A material must have a non-centrosymmetric structure to possess a nonzero $\chi^{(2)}$. Electrooptic modulators have traditionally employed ferroelectric inorganic crystals, such as lithium niobate or potassium dihydrogen phosphate, which are formed at high temperatures. However, organic NLO materials offer several advantages in performance, such as higher nonlinear susceptibilities, higher modulation rates, and potentially lower device fabrication costs.^[2] Organic films

exhibiting nonzero $\chi^{(2)}$ values have been fabricated using a variety of methods, including electric field poling,^[3] Langmuir–Blodgett (LB) films,^[4] and covalent self-assembly.^[5] Both poled polymer systems and LB films have been made with non-centrosymmetric structures that exhibit relatively high values for $\chi^{(2)}$, but poor temporal or mechanical stability restrict their potential applications.^[6] Deposition processes using reactive silane compounds require organic solvents and high temperatures.^[5]

There is a large and growing body of literature on the use of layer-by-layer (LBL) methods for fabricating nanostructured films for a variety of applications. The LBL technique, which relies on purely electrostatic interactions, was first developed by Iler^[7] and further elaborated upon by Decher et al.^[8] Several research groups have demonstrated that the NLO films made by this technique have greater thermal and temporal stability than poled polymer systems.^[9] A related approach that could be employed to fabricate NLO materials involves the use of low-molecular-weight dye molecules and polyelectrolytes as film constituents. Yamada et al. made films of poly(diallyldimethylammonium chloride) and Erichrome Black T that exhibited an SHG intensity that increased only for the first five bilayers and then reached a plateau.^[10] Other research groups, including ourselves, have found that ionic interactions alone are not sufficient for constructing LBL films with low-molecular-weight chromophores.^[11] A combination of low-molecular-weight chromophoric molecules and polyelectrolytes that could be used to construct stable NLO films with the large number of bilayers needed for electro-optic devices is yet to be demonstrated.

The objective of this work was to demonstrate that LBL films made under ambient conditions with a water-soluble, monomeric chromophoric molecule can possess high net polar ordering in each bilayer. By alternating the methods of deposition for each monolayer (covalent reaction and electrostatic interaction) and decoupling the chromophore orientation from the steric constraints of a polymer chain, we hypothesized that the non-centrosymmetric orientation required for nonzero $\chi^{(2)}$ values could be achieved.

The model anionic/reactive species used in this study was Procion Red MX-5B (PR), and the NLO-inactive polycation was poly(allylamine hydrochloride) (PAH, Scheme 1). The pH values of the dipping solutions determine the ionization state of the amine moieties on PAH, which affects both the conformation of the polymer upon adsorption and its



Scheme 1. Reaction between PR and a previously adsorbed monolayer of PAH.

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