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Optimization of Dye Molecules for Use in Studying Inflammatory Caspase Enzymes

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ABSTRACT Inflammatory caspases are important enzymes in the initial immune response to pathogens. A common method of studying caspase activity is through the use of fluorogenic assays, in which the cleavage of a substrate by an enzyme results in the emission of fluorescence. Fluorogenic assays require the use of fluorophores: molecules that will fluoresce upon the cleavage of a substrate. Different fluorophores have different characteristics, and these characteristics can be analyzed in order to determine which fluorophores will be most suitable for activity assays. Two commercially available fluorophores were compared for use in fluorogenic assays of inflammatory caspases: MCA and EDANS. MCA was determined to be the better one overall, while EDANS provided better discrimination against background biological fluorescence.

INTRODUCTION

Inflammatory caspase enzymes are a family of proteins that are involved in triggering the immune response upon exposure to pathogens. Caspase enzymes are cysteine dependent proteases that cleave after an aspartic acid residue. Production of these enzymes in a cell increases when cell damage or microorganism invasion are detected. This can lead to the activation of inflammatory caspases (icaspases), causing them to cleave a molecule known as gasdermin D. When cleaved, gasdermin D aggregates in the inner leaflet of cell membranes, producing pores that cause efflux of potassium ions from the cell and an influx of water, leading to swelling and bursting of the cell. This form of programmed cell death, which leads to an inflammatory response, is known as pyroptosis (Shi et al., 2017). While this process plays an important role in defending the body against invading pathogens, it can also lead to hyperinflammatory disease states (Guo & Callaway, 2015). The three inflammatory caspase enzymes in humans are C1, C4, and C5. Characterizing the mechanisms of these three icaspases is the first step in facilitating the development of medications that can prevent diseases associated with maladaptive icaspase activation. However, the only caspase for which the mechanism is well understood is C1; the process through which C4 and C5 trigger inflammation is largely unknown. Due to the unique role that C4 and C5 play in causing inflammation, determining the mechanism through which C4 and C5 trigger pyroptosis is an important scientific question.

Current research has studied the activity of icaspases using peptide substrates that are not selective for each icaspase. In order to characterize the mechanism of each icaspase—

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rather than understanding how icaspases function broadly—peptides that are selective for each icaspase must be developed. The peptides that are currently used to study inflammatory caspases react most strongly with C1, which is the most active icaspase (Ramirez et al., 2018). There are currently no peptide molecules that react selectively with C4 or C5.

There are different methods of studying the reaction between an enzyme and a substrate, but one commonly used approach involves attaching two different dye molecules to a substrate: a donor and a quencher (Poreba et al., 2013). This approach is known as a Förster Resonance Energy Transfer, or FRET-based assay (Figure 1). When the quencher is present, it prevents the donor from producing fluorescence.

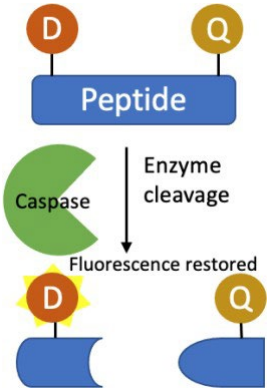

Figure 1. Schematic of the FRET-based assay used to study inflammatory caspases. When the peptide is cleaved, the quencher *Q* can diffuse away from the donor *D,* allowing *D* to fluoresce.

The key aspect of this process is that it is distance-dependent; the energy transfer occurs when the quencher is in close proximity to the donor. When the distance between the donor and the quencher is great enough, the probability of energy transfer between the donor and the quencher decreases and the donor releases the energy as fluorescence. Thus, when an icaspase enzyme cleaves a substrate with a donor and a quencher in close proximity, the distance between the donor and the quencher will increase, restoring the fluorescence of the donor. This leads to an experimentally observable signal of enzyme activity.

FRET-based peptides can be used to discern the activity of each icaspase when all are present within a cell if peptides that are both selective for individual icaspases and produce fluorescence signals at significantly different wavelengths can be developed. Here, we focus on examining peptide substrates with different donor molecules to determine if these donors will be suitable for activity assays of icaspases. Specifically, the photophysical properties of the donor molecules were examined to determine the signal-to-noise ratio for icaspase activity assays. The donor dyes selected are commercially available as side chains of amino acids, allowing them to be incorporated into FRET-based peptides.

One complication in FRET-based assays occurs due to high substrate concentrations. Under these conditions, the distance between the donor and the quencher lessens because of the large number of substrate molecules present in solution. Thus, the donor molecules can still be quenched, even when the substrate has been cleaved. This process is known as the inner filter effect (Liu et al., 1999). The inner filter effect can hinder the accuracy of a fluorogenic assay by quenching the signal. We examined 7-methoxycoumarin-4-acetic acid (MCA) and 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) in order to determine the extent of the inner filter effect for these fluorophores. The fluorophores are paired with a quencher in a fluorogenic assay, for which 4-dimethylaminoazobenzene-4′-sulfonyl chloride (Dabsyl) was used as the quencher.

These dye molecules can be compared in terms of intrinsic properties that are relevant to their use as fluorophores. One of the key properties in designing any assay for use in biological systems is the ability to discriminate between background biological fluorescence and fluorescence produced by the fluorophores in the assay. Most biological fluorescence is due to the amino acid tryptophan, which fluoresces between 310-350 nm (Pan et al., 2011) — therefore fluorophores with emission maxima at wavelengths longer

than 350 nm are desirable. Hence, MCA and EDANS were compared in terms of their brightness, fluorescence quantum yields, extent of the inner filter effect, and Förster radii, as these are all relevant properties to the quality of the fluorophores.

METHODS

Absorbance and Fluorescence Spectra and Fluorescence Quantum Yield

For all spectra measurements, MCA and E-EDANS were dissolved in 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.4) using concentrations of 200 μM for both sample and standard fluorophore absorbance measurements and concentrations of 2 μM for fluorescence measurements. The standard fluorophore was 7-amino-4-methylcoumarin (AMC) for both EDANS and MCA. EDANS was modified such that it was a side chain to glutamic acid, known as E-EDANS. This is the true molecule that is present when used in assays, as EDANS is usually attached to glutamic acid in the peptide substrate. A difference was noted in the spectral behavior of EDANS and E-EDANS, so E-EDANS was used to provide data that are more representative of the substrate. A similar effect was not observed for MCA, so modifications were unnecessary. Buffer was used as a blank for both the fluorescence and absorbance measurements. The fluorescence measurements were taken using a Cary Eclipse Fluorescence Spectrophotometer and the absorbance measurements were taken using a NanoDrop One spectrophotometer. The quantum yield Φ_x can be calculated by using the quantum yield of a fluorophore for which the value is known Φ_{st}, the integrated fluorescence of the sample F_x, the integrated absorbance of the standard λ_{st}, the integrated fluorescence of the standard F_{st}, and the integrated absorbance of the sample λ_x (Equation 1). All calculations were performed in Igor Pro 8 version 8.04 and Microsoft Excel for Mac 16.57

$$\Phi_x = \Phi_{st} \frac{F_x \lambda_{st}}{F_{st} \lambda_x} \qquad (1)$$

Inner Filter Effect

To calculate the inner filter effect, fluorescence experiments were performed in 50 mM HEPES buffer (pH 7.4) with 500 nM free fluorophore combined with concentrations of substrate ranging from 0 – 400 μM for E-EDANS and 0 – 40 μM for MCA for a total assay volume of 100 μL in a well plate. Each of the specified concentrations of substrate had six replicates. Mca-HDGK(Dabsyl) and E(EDANS)-EHDGK(Dabsyl) were the substrates used for MCA and E-EDANS, respectively (Figure 2). The fluorescence of the substrate at each of the concentrations was also determined and subtracted from the fluorescence of the fluorophore with the respective substrate concentration to yield the true fluorescence from the fluorophore. The experiments were performed using a Synergy H1 Hybrid Multi- Mode Reader with a 96 well plate and the fluorescence intensity was reported in relative fluorescence units (RFU). The excitation wavelengths were 330 nm for MCA and E- EDANS, and the emission wavelengths were 390 nm and 490 nm for MCA and E-EDANS, respectively.

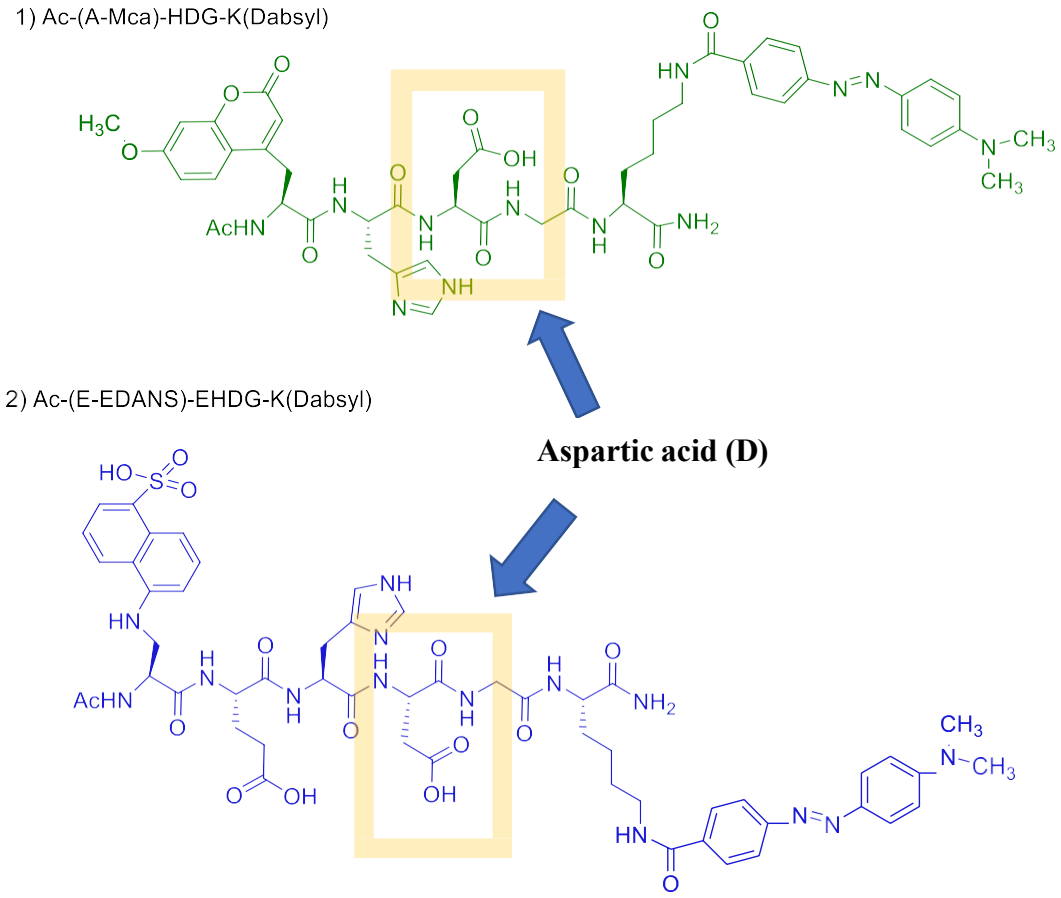

Figure 2. Chemical structures of 1) Mca-HDGK(Dabsyl) and 2) E(EDANS)-EHDGK(Dabsyl) peptides used in inner filter effect studies. The aspartic acid (D) is the residue after which caspase enzymes cleave.

The fluorescence of the substrate *f(S)* was subtracted from the fluorescence of the fluorophore and the substrate *f(F +S)* to provide the actual fluorescence produced by the fluorophore *f(F)* (Equation 2).

$$f(F) = f(F+S) - f(S) \qquad (2)$$

The correction percentage *Corr%* was then calculated by dividing the fluorescence of the fluorophore *f(F)* in the presence of the substrate by the fluorescence of the fluorophore in the absence of the substrate $f(F)_0$ (Equation 3).

$$Corr\% = \frac{f(F)}{f(F)_0} \qquad (3)$$

Förster Radii

The Förster radii were calculated by measuring the fluorescence of the fluorophore and the absorbance of Dabsyl from 340-600 nm in 50 mM HEPES buffer (pH 7.4). The excitation wavelength for the fluorescence measurements was 304 nm. The fluorescence and absorbance of the buffer was subtracted to remove any background fluorescence and absorbance. The concentration of the fluorophore was 2 µM, and the concentration of Dabsyl was 2 µM. The fluorescence measurements were taken on a Cary Eclipse Fluorescence Spectrophotometer, and the absorbance measurements were taken on a Cary 60 UV-Vis Spectrophotometer. The Förster radius was calculated using the orientation factor κ^2, the refractive index of the solvent n, the fluorescence quantum yield of the donor Φ_D, and the overlap integral of the donor emission and the quencher absorbance spectra J_{DA} (Equation 4) Calculations were performed in Igor Pro 8 version 8.04 and Microsoft Excel for Mac 16.57.

$$R_0^6 = (8.8 \times 10^{-5})\kappa^2 n^{-4} \Phi_D J_{DA} \qquad (4)$$

Computational Structures

The 3-D energy minimized structures of Mca-HDGK(Dabsyl) and E(EDANS)-EHDGK(Dabsyl) were determined using a molecular mechanics (MM2) force field method with PerkinElmer ChemDraw Chem3D software.

Fluorescence Enhancement Factor

To determine the fluorescence enhancement factor (FE), fluorescence experiments were carried out using 2 µM samples of substrate and 2 µM samples of free fluorophore in 50 mM HEPES buffer (pH 7.4). The fluorescence was measured using a Cary Eclipse Fluorescence Spectrophotometer. The background buffer fluorescence was subtracted, and the fluorescence of the free fluorophore F_{fl} was then divided by the fluorescence of the substrate F_{sub} (Equation 5) All calculations were performed in Microsoft Excel for Mac 16.57.

$$FE = \frac{F_{fl}}{F_{sub}} \qquad (5)$$

RESULTS AND DISCUSSION

The fluorescence and absorbance spectra for EDANS and MCA were taken in order to compare the spectral properties of the fluorophores (Figure 3). Both MCA and E-EDANS had peak absorbances in the same wavelength range, but MCA had much greater absorbance and fluorescence. E-EDANS fluoresced at a longer wavelength than MCA, which provided better discrimination against biological fluorescence.

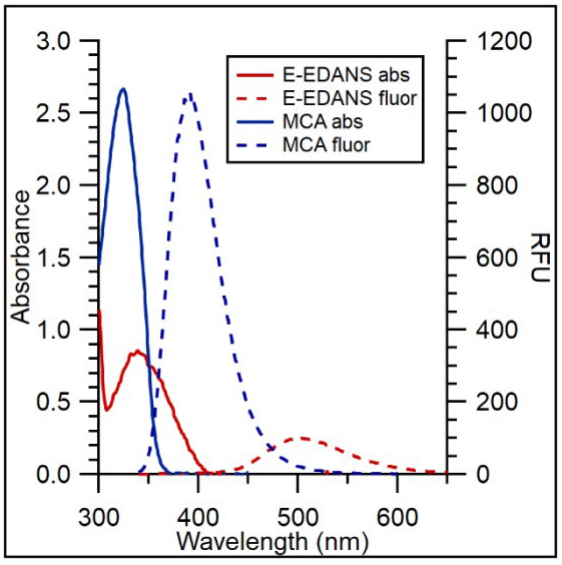

Figure 3. Absorbance spectra of 200 μM E-EDANS and MCA. Fluorescence spectra of 2 μM E-EDANS and MCA with a 330 nm excitation wavelength.

The inner filter effect was determined by using a consistent concentration of free fluorophore coupled with an increasing substrate concentration. The substrate concentrations selected were similar to those used in enzymatic assays. This provided the ability to compare the fluorescence of the fluorophore in the presence and absence of the substrate, as well as the baseline fluorescence of the substrate itself. The fluorescence of the substrate *f(S)* was subtracted from the fluorescence of the fluorophore and the substrate *f(F +S)* to provide the actual fluorescence produced by the fluorophore *f(F)* The correction percentage *Corr%* was then calculated by dividing the fluorescence of the fluorophore *f(F)* in the presence of the substrate by the fluorescence of the fluorophore in the absence of the substrate $f(F)_0$. The inner filter effect was determined using the correction percentage, which showed the percent of the fluorescence present after the addition of the substrate.

E-EDANS saw a significant decrease in fluorescence as substrate concentration increased (Table 1). There was a significant drop in true fluorescence even at 20 µM substrate, and 400 µM substrate resulted in only 17.9% of the fluorescence remaining. While this strong correction effect was expected, the low levels of

fluorescence at high substrate concentrations led to uncertainty in the reproducibility of the values.

The inner filter effect for MCA was not as significant at the concentrations used in enzymatic assays, though an effect was still visible. At 40 µM there was 71.5% of the fluorescence remaining, so a correction would still be necessary when analyzing data (Table 2).

[S] µM	*f*(S)	*f*(E-EDANS+S)	*f*(E-EDANS)	Corr%
0	66	1746	1680	1.000
20	178	1392	1214	0.723
40	187	1167	979	0.583
80	317	961	644	0.384
160	320	749	429	0.255
200	320	768	448	0.267
400	404	703	300	0.179

Table 1. Inner filter effect data for 500 nM free E-EDANS reported in RFU.

[S] µM	*f*(S)	*f*(MCA+S)	*f*(MCA)	Corr%
0	846	20938	20092	1.000
2	1512	19343	17831	0.887
5	2540	22238	19697	0.980
10	4429	20852	16423	0.817
20	6476	22788	16312	0.812
40	11320	25695	14375	0.715

Table 2. Inner filter effect for 500 nM free MCA reported in RFU.

The lower fluorescence levels of E-EDANS in relation to MCA are quite clearly seen in Tables 1 and 2. In order to quantify those differences, the fluorescence quantum yields and brightness for each fluorophore were determined. The fluorescence quantum yield is a ratio of the photons emitted by a molecule to the photons absorbed. A higher quantum yield thus indicates that the fluorophore will fluoresce more photons per photons absorbed.

The quantum yields for E-EDANS and MCA were determined using Equation 1 with 7-amino-4- methylcoumarin (AMC) as the standard (Table 3).

Fluorophore	Φ_x
MCA	0.14
E-EDANS	0.14

Table 3. Quantum yields for E-EDANS and MCA using AMC as the standard.

Because the fluorescence quantum yields were the same for MCA and E-EDANS, that may give the impression that they have equivalent utility as fluorophores, which is not the case. Fluorescence quantum yields provide the ratio of photons emitted over photons absorbed, so if the fluorophore absorbs fewer photons, it will emit fewer, even with equivalent quantum yields. The true brightness B of the fluorophore can be calculated using the fluorescence quantum yield Φ_x and the extinction coefficient at 330 nm ε, as this is the most common wavelength used for MCA and EDANS fluorophores in enzyme assays (Equation 6).

$$B = \Phi_x * \varepsilon \qquad (6)$$

The brightness values for E-EDANS and MCA are compared in Table 4.

Fluorophore	Brightness (M^{-1}•cm^{-1})
MCA	1.8×10^3
E-EDANS	5.5×10^2

Table 4. Brightness of MCA and E-EDANS donors.

The brightness of MCA has a significantly larger value than that of E-EDANS, which explains the

much stronger signal produced by MCA. Despite equal quantum yields, the higher absorbance of MCA results in greater total fluorescence, providing clearer indications of activity in enzyme assays.

Another important feature of fluorophores in fluorogenic assays is the degree to which the fluorescence of the donor is quenched in the intact peptide. This can be predicted before purchasing peptide substrates by calculating the distance between the donor and quencher and comparing this distance to the Förster radius of the donor-acceptor pair. The Förster radius of a fluorophore pair is the distance at which energy transfer is 50% efficient. For effective quenching to occur, the donor and the quencher must be closer in proximity than the Förster radius R_0. In particular, a distance of $<R_0/2$ leads to complete quenching. The Förster radii were calculated using Dabsyl as the quencher, as this is the most common quencher used for MCA and E-EDANS in peptide substrates (Table 5).

Fluorophore	Förster Radius (Å)
MCA	26
E-EDANS	30

Table 5. Förster radii for MCA and E-EDANS using Dabsyl as a quencher.

Since the Förster radii were similar, this indicates that similar peptides can be used for both fluorophores while still achieving quenching.

Two common substrates that are used in studying icaspases are Mca-HDGK(Dabsyl) and E(EDANS)-EHDGK(Dabsyl). It is important for these substrates to have sufficient quenching of fluorescence in order to have a strong signal to noise ratio. The distance between the donor and the quencher in these substrates was computationally determined and compared to the Förster radii. The structures of the substrates were determined using Chem3D modeling software, which produced an energy minimized structure using a force field calculation. The distance between the donor and the quencher was then approximated from the calculated structure (Table 6).

Peptide	Distance (Å)
Mca-HDGK(Dabsyl)	15.3
E(EDANS)-EHDGK(Dabsyl)	15.5

Table 6. Distance between the donor-quencher pair in two common inflammatory caspase substrates.

The distance between the donor-quencher pair in the MCA peptide (Figure 4) does not quite meet the $R_0/2$ criterion but is within 2 Å of the cut-off. The $R_0/2$ for MCA is 13 Å, while the distance between the donor-quencher pair in the substrate is 15.3 Å. The E-EDANS (Figure 5) peptide is almost exactly at the $R_0/2$ criterion, which supports the use of these peptides in fluorogenic assays.

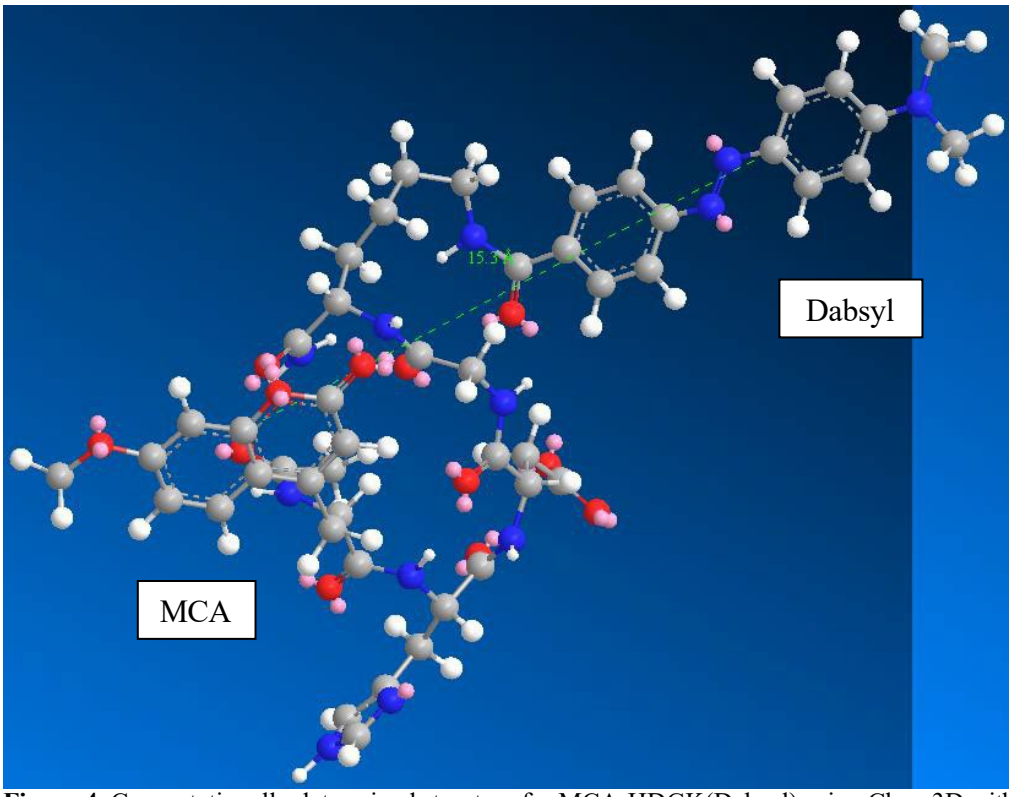

Figure 4. Computationally determined structure for MCA-HDGK(Dabsyl) using Chem3D with the distance between the donor (MCA) and quencher (Dabsyl) shown. The approximate distance of 15.3 Å is on the order of $R_0/2 =$ 13 Å, supporting near complete quenching of MCA fluorescence by Dabsyl in this peptide.

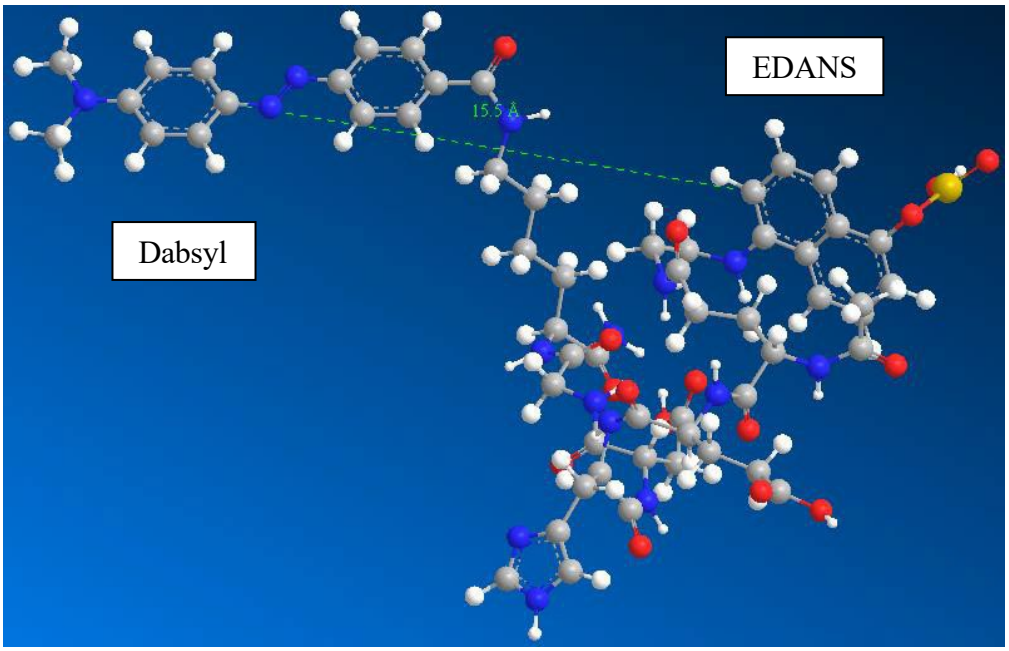

Figure 5. Computationally determined structure for E(EDANS)-EHDGK(Dabsyl) using Chem3D with the distance between the donor E-EDANS and quencher (Dabsyl) shown. The approximate distance of 15.5 Å is on the order of $R_0/2 =$ 15 Å, supporting near complete quenching of EDANS fluorescence by Dabsyl in this peptide.

The theoretical quenching suggested using the computational structures can be experimentally confirmed through the use of the fluorescence enhancement factor (FE), which is a measure of how much greater fluorescence of the free fluorophore is compared to that of the fluorophore in the substrate. It is calculated by dividing the fluorescence of the free fluorophore by the fluorescence of the substrate with the donor and the quencher attached (Equation 5). The FE was calculated for both the MCA and E-EDANS substrates (Table 7).

Peptide	FE
Mca-HDGK(Dabsyl)	28
E(EDANS)-EHDGK(Dabsyl)	38

Table 7. FE for two common peptides using MCA and E-EDANS as fluorophores.

Since the FE indicates that there was much greater fluorescence for the free fluorophores, as expected, it is clear that significant quenching occurs within the peptide, confirming the computational predictions. In general, an FE value of greater than 20 is required to have a viable fluorogenic assay. E-EDANS may have had a higher FE due to MCA having a shorter Förster radius, but MCA still had a high enough FE to be viable in a fluorogenic assay. Despite the lower overall fluorescence for E-EDANS, both of the peptides studied met the criterion.

CONCLUSION

MCA proved to be a superior fluorophore to E-EDANS, providing much better brightness and enhanced signal for use in assays. Despite equivalent fluorescence quantum yields, E-EDANS absorbs much less light, thus providing much less fluorescence as well. The Förster radii were comparable, allowing for the use of similar peptides while still achieving sufficient quenching of the donor. E-EDANS did have one advantage, however, in that it emits at a longer 490 nm wavelength compared to a 390 nm wavelength for MCA, providing better discrimination between background biological fluorescence and the fluorescence generated by the assay. Further fluorophores will be explored by looking for an EDANS mimic that has better brightness, thus retaining the best aspects of each fluorophore.

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