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Relay FRET Event in a Designed Trichromophoric Pentapeptide Containing *o-, m*-Aromatic-Amino Acid Scaffold

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The concept of relay FRET event is established in a designed trichromophoric pentapeptide containing *o-,m*-aromatic amino acid scaffold in the backbone as a novel a β-turn mimetic β-sheet folding nucleator. This system would find applications in studying fundamental processes involving interbiomolecular interactions in chemical biology.

Förster resonance energy transfer (FRET), between a donor and an acceptor chromophore in a close proximity of 0-10 nm, occurs through non-radiative dipole–dipole interation.[1] The FRET is extremely sensitive to small changes in distance and is used as a research tool to investigate molecular level interactions in chemistry and biology. Advanced research now resulted in the design of many FRET pairs aiming at uncovering the structure, function, dynamics and interactions involving biomolecules inside a cell. [2-3] To date, a myriad of FRET pairs and FRET based strategies have been developed to investigate inter-biomolecular interactions and in several other chemical/biochemical applications.[4] However, most of these examples restricts FRET occurrence among two chromophors or two unit of biomolecules having one chromophore in each and with a limited distance of 10 nm. However, it is a matter of realization that inside a cell the inter-biomolecular interaction is not restricted to only two molecules rather occurs among other large biomacromolecular complexes and often at distances more than 10 nm. [4a, 5] Therefore, study of long range multimolecular interactions in a cell or for multiplex biological assays has fueled efforts to expand traditional two-dye FRET system to multichromophoric interacting partners. Recently, several investigations have been reported that focused on FRET between three distinct fluorescent molecules.[5] As for an example, a sequential energy transfer system in oligonucleotides containing three chromophores in the three separate strands has been reported in 1999.[6a] Turro and coworkers have synthesized trichromophoric DNA that can serve the purpose of multiplex biological assays[6b] and other applications such as biological labeling and imaging. Few more examples exist in literature in the field of DNA.[4a, 5d, 6c]

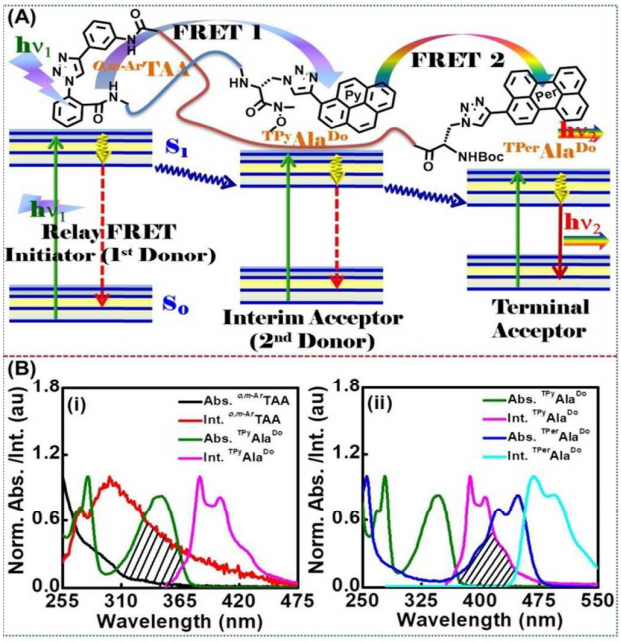

Figure 1. (A) Three chromophores in a peptide and the interactions of two coupled FRET pairs-the concept of relay FRET. (B) (i-ii) The absorption and emission spectra of shown FRET pairs with shaded regions showing overlap in spectra of donors and acceptors.

It is thus clear that most of the reported triple-dye-FRET systems are based on DNA scaffolds. Though, several bi-

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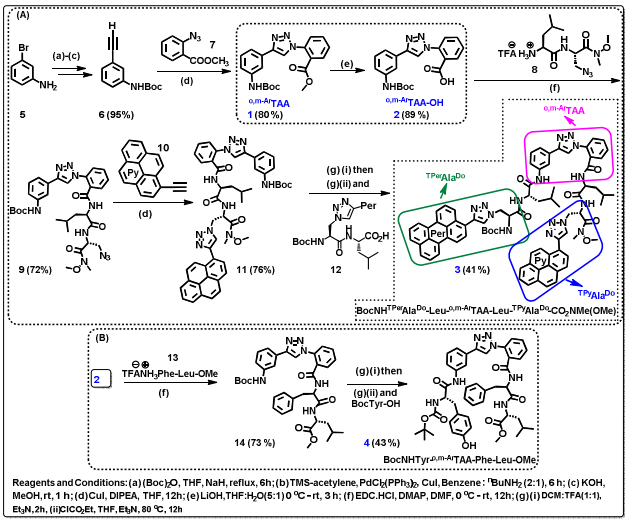
chromophoric-traditional-FRET based peptides/proteins have been reported only very little attention has been paid to expand the two-dye FRET system into three. Only recently, the three-dye systems on a flow cytometer and a three-fluorophore FRET in protein for live cell imaging have been reported.[6d] The triple-dye FRET system exhibiting relay FRET process could enable to study the long range energy transfer that is not allowed by the constraints of one-step FRET process. Moreover, such sequential energy-transfer mechanism has been used to elucidate the efficient energy transfer in the photosynthetic light-harvesting arrangements[6c] and can be utilized in a multiplex biological assay. Though there are few reports on triple-FRET system in complex biological ansembles, novel and simpler design in a short peptide is worthwhile for understanding relay FRET event in a particular conformation.

As a part of our ongoing research activity toward the design of constrained turn mimetic molecular scaffold, we thought that, *o,o*- or *o,m*-aromatic triazolyl amino acid could serve as interesting scaffold for inducing a peptide secondary structure and for spatially orienting terminal chromophores very close in a peptide for showing interesting photophysics.[7] In particular, we, envisioned that a pentapeptide with the scaffold in the backbone if decorated with triazolylperylene amino acid (**^{TPer}Ala^{Do}**) at the *N*-terminus and triazolylpyrene amino acid (**^{TPy}Ala^{Do}**) at the *C*-terminus, we could achieve a FRET relay process from the scaffold **$^{o,m\text{-}Ar}$TAA** to **^{TPy}Ala^{Do}** to **^{TPer}Ala^{Do}** upon exciting the peptide at the absorption (λ_{max} = 290 nm) of scaffold wherein no or negligible absorption is exhibited by other two chromophore (Fig. 1).



Scheme 1. (A) Synthesis of *o,m*-aromatic triazolyl amino acid scaffold, **1** (**$^{o,m\text{-}Ar}$TAA**) and its fluorescent pentapeptide, **3** and (B) Leu-Enkephalin analogue tetrapeptide, **4**.

With this light we want to report herein the design (a) of triazolyl *o,m*-aromatic amino acid scaffolded tetrapeptide, a Leu-enkephalin analogue to study the conformational induction by the scaffold and (b) of trichromophoric fluorescent pentapeptide to establish a two steps FRET relay process. Thus, both the non-fluorescent Leu-enkephalin analogue peptide as well as fluorescent pentapeptide adopted β-strand structure induced by the scaffold's turn shape. The relay FRET was also established wherein the FRET-source is the scaffold (**$^{o,m\text{-}Ar}$TAA**), the interim chromophore is the triazolyl pyrene (**TPy**) of **^{TPy}Ala^{Do}** and the ultimate emitting (FRET terminus) is the triazolyl perylene (**TPer**) of **^{TPer}Ala^{Do}**. Such a relay FRET in a short designed peptide is conceptually efficient in shedding light for newer design of probe for studying complex inter-biomolecular interaction beyond the limit of traditional FRET dimension inside a cell.[4a, 5-6]

The synthesis of the scaffold **1** (**$^{o,m\text{-}Ar}$TAA**)started with preparation of m-[(^{t}butyloxycarbonyl)amido]-phenyl acetylene **6** from *m*-bromo aniline **5** followed by 1,3-azide-alkyne dipolar cycloaddition reaction between **6** and *m*-azidomethyl benzoate **7**. The scaffold amino acid with free acidic group, BocNH**$^{o,m\text{-}Ar}$TAA**-OH (**2**) was then obtained via hydrolysis of **1** using LiOH (**Scheme 1**). Next, we incorporated the scaffold **1** into a trichromophoric fluorescent pentapeptide **3**, [BocNH-**^{TPer}Ala^{Do}**–Leu–**$^{o,m\text{-}Ar}$TAA**–Leu–**^{TPy}Ala^{Do}**-CONMe(OMe)] to demonstrate our design concept of FRET relay process (**Scheme 1**). We also incorporated the scaffold into a tetrapeptide sequence **4**, analogous to Leu-enkephalin [BocNH-Tyr–**$^{o,m\text{-}Ar}$TAA**–Phe–Leu-OMe] to test the conformational induction by the scaffold. All intermediate and final peptides were synthesized following a solution phase peptide coupling protocol and were purified by column chromatography using silica-gel (60-120 mesh) and characterized via NMR, IR and HR-mass spectrometry.

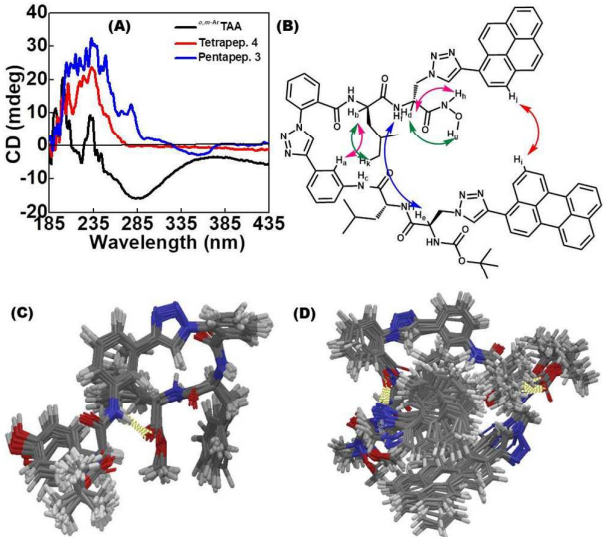


Figure 2. (A) CD spectra of **$^{o,m\text{-}Ar}$TAA** (**1**), fluorescent pentapeptide **3** and tetrapeptide **4** in methanol (50 μM; rt). (B) Backbone and side chain interactions as reflected from ROESY spectra of peptide **3**. (C-D) Clustering of structures (within 21 kJ/mole global minima) obtained from molecular dynamics simulation for tetrapeptide **4**, and pentapeptide **3**, respectively.

The secondary structure elucidation by CD spectropolarimeter revealed a hairpin turn structure (-196, +200 and +207 nm)[7c, 8] of the scaffold **1** with negative induced CD signals corresponding to its absorption wavelengths at 250

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and 283 nm in polar protic solvent MeOH (Fig. 2A). On the other hand both the fluorescent pentapeptide **3** and Leu-enkephalin analogue tetra peptide **4** containing $^{o,m\text{-Ar}}$**TAA** scaffold in the backbone, showed predominantly turn induced β-strand like structures (Fig. 2A) indicating that the scaffold in the turn conformation acted as a β-turn mimetic β-sheet folding nucleator.[7b, 9]

That the β-strands are associated with intramolecular H-bonding interactions among the two strands was evident from the amide -NH stretching absorptions in FTIR spectra for both the peptides (ESI†, Section 2.2).[7b, 10] The C=O stretching at 1722/1654 and 1743/1658 cm^{-1}, respectively, for peptide **3** and **4** which did not depend on the sample concentration supported β-sheet structures in both the peptides.[10] The support for moderate intramolecular hydrogen bonding interactions in both the peptide **3** and **4** came from VT-NMR analysis in d_6-DMSO showing moderate $\Delta\delta/\Delta T$ (3-6 ppb/k) values for the amide NH's and supported the predominant turn induced β-sheet like structure of the peptides (ESI†, Section 2.3).[7b, 11] Furthermore, the interaction of various protons in the solution conformations was revealed from NOESY and ROESY spectra in d_6-DMSO of both the peptides **3** and **4** (ESI†, Fig. S6-S7) revealing spatial H-H interactions. The 2D NMR supported the close proximity of the two terminal fluorescent unnatural amino acids and thus the possibility of a photophysical interaction between ^{TPer}**Ala**^{Do} and ^{TPy}**Ala**^{Do} in pentapeptide **3** (Fig. 2B).[7] Next, MD simulations using an OPLS 2005 force field in Schrodinger Macromodel (Maestro vs. 9.1) software package supported the turn induced β-sheet conformationin in both the peptides (Fig. 2C-D).[10b, 12]

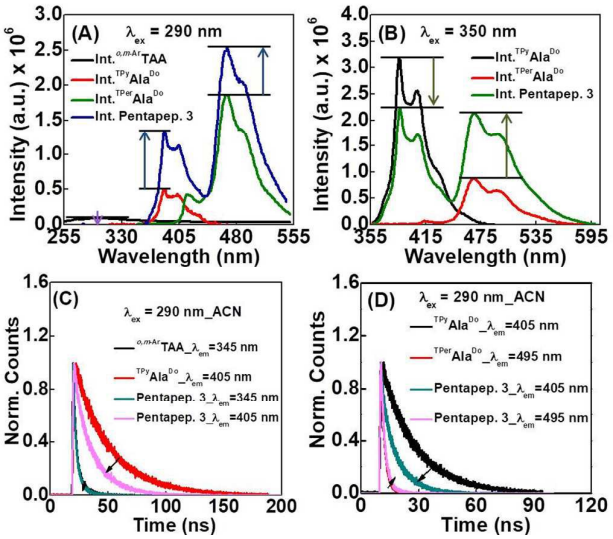

Figure 3. Fluorescence emission spectra of (A) donor $^{o,m\text{-Ar}}$**TAA,** interim acceptor ^{TPy}**Ala**^{Do}, end-acceptor ^{TPer}**Ala**^{Do} and the pentapeptide **3** and (B) donor ^{TPy}**Ala**^{Do}, acceptor ^{TPer}**Ala**^{Do} and the pentapeptide **3** in acetonitrile. (C-D) Time resolved fluorescence of $^{o,m\text{-Ar}}$**TAA** , ^{TPy}**Ala**^{Do} and pentapeptide **3** (10 μM each, rt; λ_{ex} = 290 nm).

To test our hypothesis of FRET relay process, we, next, proceeded to study the photophysical properties of pentapeptide **3** in details. The UV-visible and fluorescence photophysical properties of scaffold $^{o,m\text{-Ar}}$**TAA,** fluorescent triazolyl amino acid monomers, ^{TPy}**Ala**^{Do} and ^{TPer}**Ala**^{Do} indicated a possibility of FRET process from donor scaffold to acceptor ^{TPy}**Ala**^{Do} and then to the end acceptor ^{TPer}**Ala**^{Do} (Fig. 1b and ESI†, Section 4, Fig. S13).[1] Thus, upon excitation at the scaffold (λ_{ex} = 290 nm) we observed three emission bands at 320, 405 and 495 nm corresponding to the emission from the scaffold $^{o,m\text{-Ar}}$**TAA**, **TPy** of ^{TPy}**Ala**^{Do} and from **TPer** of ^{TPer}**Ala**^{Do}, respectively (Fig. 3A). Comparative intensities revealed a negligible emission from the scaffold compared to its free state, while an increase in intensities by about 2.6 and 1.4 times were observed from **TPy** and **TPer**, respectively, compared to their corresponding free monomer emissions in acetonitrile indicating two consecutive FRET events (Fig. 3A).[5] Furthermore, upon excitation at 347 nm (absorption of **TPy**), the decrease in **TPY** and increase in **TPer** emission intensities evidenced the 2nd FRET from **TPy to TPer** in peptide **3** (Fig. 3B). The time resolved fluorescence study follows the same trend as steady state fluorescence. Thus, a decrease in average life time was observed for the donor scaffold from 4.8 to 3.9 ns ($^{o,m\text{-Ar}}$**TAA**, λ_{ex} = 290 nm, λ_{em} = 350 nm, in ACN) with an increase in interim acceptor life time from 15.2 ns to 16.2 ns (^{TPy}**Ala**^{Do}; λ_{em} = 405 nm) and increase in end acceptor (^{TPer}**Ala**^{Do}; λ_{em} = 495 nm) from 3.8 ns to 4.7 ns (Fig. 3C-D, Table 1). All observations suggested a consecutive two FRET processes from scaffold to **TPy** (1st FRET) and then to **TPer** (2nd FRET).

Table 1. Summary of time resolved fluorescence in ACN.

Entry	Φ_f	λ_{em} [nm]	τ_1 [ns]	τ_2 [ns]	<τ> [ns]
$^{o,m\text{-Ar}}$**TAA**	0.25	350	2.3 (59 %)	8.3 (41 %)	4.8
^{TPy}**Ala**^{Do}	0.14	405	15.2 (100 %)	---	15.2
^{TPer}**Ala**^{Do}	0.65	495	3.8 (100 %)	---	3.8
Tripep. 11	0.01	350	3.62 (100 %)	---	3.6
	0.17	405	3.8 (3 %)	17.3 (97 %)	16.9
PentaPep. 3	0.001	350	1.9 (58 %)	6.5 (42 %)	3.9
	0.12	405	2.9 (6 %)	16.9 (94 %)	16.2
	0.33	495	3.67 (83 %)	9.5 (17 %)	4.7

For lifetimes of the fluorescent amino acids and peptides λ_{ex} = 290 nm; Concentration of each fluorescent amino acids and peptides = 10 μM; <τ>, are weighted means from the bi-exponential fits: $<\tau> = 1/(\alpha_1/\tau_1 + \alpha_2/\tau_2)$.

Further evidence of 1st FRET from scaffold to **TPy** came separately from the decrease in intensity as well as life time of the scaffold and increase in intensity and life time of the **TPy** upon excitation at 290 nm in the interim tripeptide **11** (ESI†, Fig. S14-S15, Table S10-11). The second FRET was also separately supported both from steady state as well as from time resolved fluorescence (Fig. 3B, D and ESI†, Table S10-S12). Using the values of κ^2= 2/3, we calculated the FRET efficiencies which were found to be 89 % and 30 % for FRET pairs $^{o,m\text{-Ar}}$**TAA/TPy** and **TPy/TPer**, respectively (SI, Section 4.2). Thus, we established a relay FRET process with excellent to good efficiency in our designed fluorescent pentapeptide **3** with the novel scaffold, $^{o,m\text{-Ar}}$**TAA**, in the middle of the backbone inducing an overall β-hairpin structure.

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As an application the interaction of the fluorescent pentapeptide with only protein BSA was tested (Fig. 4).[7] Our aim was just to see whether in a protein microenvironment the FRET photophysics is maintained by the pentapeptide or not. The absorption increases as a solution of the probe peptide 3 was titrated with BSA (Fig. 4A). The overlapping of emission spectra of BSA protein and absorbance of pentapeptide **3** indicated that there might be a possibility of energy transfer from Trp unit of BSA to pyrene unit of pentapeptide **3** (ESI†, Section 5.6, Fig. S18a). Thus, as we excited at absorption of BSA (280 nm) we observed a decrease in BSA emission intensity as well as an increase in pyrene emission intensity indicating a visual FRET from BSA protein to pentapeptide **3** (Fig. 4B). Time resolved fluorescence study also supported this FRET phenomenon (ESI†, Section 5.7). The association constant was found to be 5.3 x 10^4 M^{-1} with free energy of binding (ΔG) -6.5 kcal/mol (ESI†, Section 5.8).

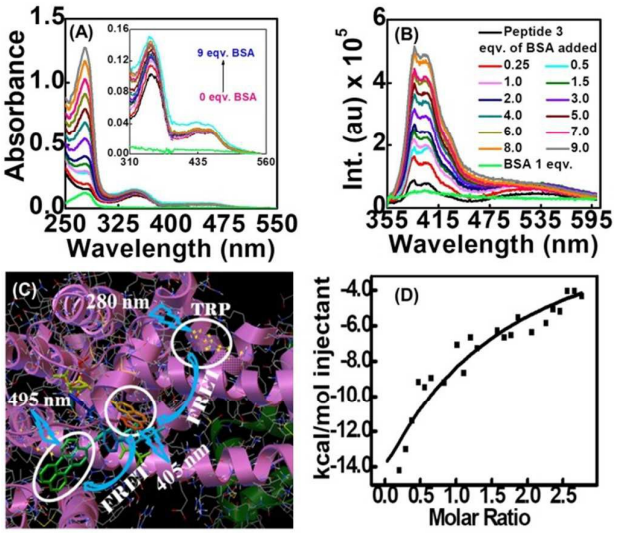

Figure 4. Titration of probe pentapeptide **3** with increasing amount of BSA (a) UV visible and (b) fluorescence emission spectra (λ_{ex} = 280 nm; peptide = 5 μM in 20 mM phosphate buffer, pH 7.0, rt]. (C) Docking pose of pentapeptide **3** in presence of BSA showing interaction with the hydrophobic pocket of BSA. (D) Plot from isothermal titration calorimetry.

To have much information on the interaction a docking calculations was carried out using Autodock4 (Fig. 4C). [13] Docking pose clearly indicated the close proximity of **TPy** of probe **3** and Trp of BSA and hence the possibility of occurrence of FRET process was supported. The probe peptide **3** was located in the vicinity of tryptophan (Trp-134) and remained surrounded by other hydrophobic amino acids of the hydrophobic pocket of site I of BSA protein. Hydrophobic binding without perturbing the α-helix secondary structure of BSA (ESI†, Section 5.9) was also supported from an anisotropy study (ESI†, Section 5.11).

We also carried out Isothermal titration calorimetric measurement to investigate the thermodynamic characterization of the interaction of **pentaeptide 3** with BSA in phosphate buffer (pH 7.0) at 298 K. The negative deflections suggested the binding process is exothermic in nature (ESI†, Section 5.12).The binding isotherm was fitted to a sigmoidal curve involving one binding site mode (Fig. 4D). The binding is favoured enthalpically with binding constant (K) 1.1 X 10^5 M^{-1} and free energy of binding (ΔG) -6.9 kcal/mol both of which are found to be closer to the experimental values (ESI†, Section 5.11).[7]

In conclusion, we successfully introduced a FRET relay system peptide with a β-sheet inducing turn mimetic *o,m*-triazolyl aromatic scaffold (**$^{o,m\text{-}Ar}$TAA**) which acts as FRET origin. The FRET enegy from the scaffold then transferred to the interim acceptor, **TPy** and then to terminal acceptor, **TPer** which ultimately emits fluorescence light. The FRET relay peptide was also able to interact with BSA leading to the same FRET process. This newly designed fluorescent peptide might find wider application in chemical biology.

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Relay FRET Event in a Designed Trichromophoric Pentapeptide Containing *o-, m*-Aromatic-Amino Acid Scaffold

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