TECHNICAL NOTE

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FRET study in oligopeptide-linked donor–acceptor system in PVA matrix

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Abstract

An oligopeptide: Lys-Gly-Pro-Arg-Ser-Leu-Ser-Gly-Lys-NH_2, cleaved specifically by a matrix metalloproteinase 9 (MMP-9) at the Ser-Leu bond, was labeled on the ε-NH_2 groups of lysine with donor (5, 6 TAMRA) and acceptor (HiLyte647) dye. The donor control was a peptide labeled with 5, 6 TAMRA only on the C-terminal lysine, and the acceptor control was free HiLyte647. Following three products were studied by dissolving in 10% (w/w) poly(vinyl alcohol) and dried on glass slides forming 200 micron films. Absorption spectra of the films show full additivity of donor and acceptor absorptions. A strong Fluorescence Resonance Energy Transfer (FRET) with an efficiency of about 85% was observed in the fluorescence emission and excitation spectra. The lifetime of the donor was shorter and heterogeneous compared with the donor control.

Introduction

Forster Resonance Energy Transfer (FRET) has been used successfully to detect metalloproteinase-9 (MMP-9) enzyme activity [1, 2] and to visualize MMP-9 presence in cells [3]. The detection is based on a selective cleavage of a peptide whose partial sequence is recognized by MMP-9. The target sequence, Lys-Gly-Pro-Arg-Ser-Leu-Ser-Gly-Lys-NH_2, was optimized by Kridel *et al* [4].

The ability to control the concentration or activity of MMP-9 is key in many biomedical applications because the enzyme plays a crucial role in the destruction of basement membranes during tumor progression. This process requires the degradation of the basement membrane and the extracellular matrix (ECM) surrounding the tumor [5], which is thought to be perpetrated by proteases, in particular, matrix metalloproteinases (MMP) [5–8]. So far, over 26 MMPs have been identified. They are zinc-dependent endopeptidases which can be grouped based on structural differences [8]. MMPs are upregulated in almost all types of human cancers, and increased levels of MMPs are often associated with poor prognosis [7, 8]. In particular, MMP-9, also known as gelatinase B, plays a role in degrading the ECM and the basement membrane. An increased activity of MMP-9 has been associated with a many types of epithelial cancers, including breast, ovarian, colon, and prostate cancers [6]. Thus, techniques for accurately and quickly measuring MMP-9 levels can lead to rapid, non-invasive detection and early treatment of cancers.

The FRET phenomenon and its applications have been described in many publications and books [9–15]. Today, FRET is commonly used in spectroscopy/microscopy to study molecular interactions. In the present manuscript, we characterize the fluorescence properties, FRET in particular, of the MMP-9-specific peptide mentioned above. The donor dye is 5, 6 TAMRA and the acceptor dye—HiLyte 647. We selected these two fluorophores because of their brightness, stability and insensitivity to the pH of the environment. The dyes were conjugated to the lysine residues of the peptide: 5, 6 TAMRA on the ε-NH_2 group of the C-terminal lysine and HiLyte 647 on the N-terminal lysine (figure 1). A donor control was the peptide labeled with (5, 6 TAMRA) only, and the acceptor control was free HiLyte647. The three molecules, i.e. the peptide carrying the donor–acceptor system (DA), the peptide

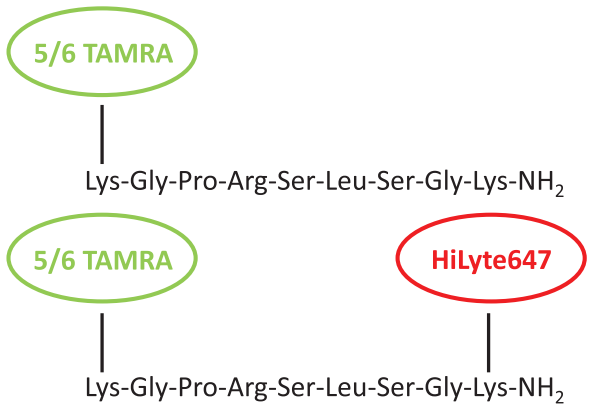

Figure 1. Structures of the donor (D, 5/6 TAMRA) and donor–acceptor linked system (DA). As an acceptor (A) was taken free HiLyte647.

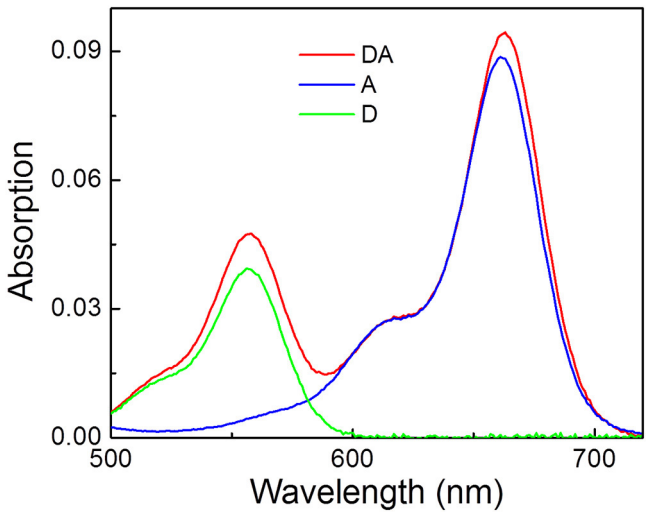

Figure 2. Absorption spectra of the donor (D, 5/6 TAMRA), acceptor (A, free HiLyte647) and donor–acceptor system (DA). Concentrations were the same for all compounds (20 μM). The DA absorption is well approximated by the combination of D and A spectra.

with the donor only (D), and the free acceptor (A) were dissolved in poly(vinyl alcohol) (PVA). The solution was spread on glass slides and dried, resulting in films approximately 200 μm thick. The films were isotropic, stable and easy to handle and store. The fluorescence properties of all films stored in a cabinet did not change significantly over a one month period. We chose PVA matrices for the reasons mentioned above and to eliminate potential end-to-end diffusion. Polymer matrices, in form of nanoparticles, are of great interest because of their frequent use for drug delivery as well as in sensing and monitoring of disease progression. The goal of our study was to characterize the fluorescence properties and FRET in the DA system on a peptide suitable for a selective cleavage by MMP-9.

Materials and methods

All chemicals used were of analytical grade. The biotin-C6_Linker-Lys(5-FAM)-Gly-Pro-Arg-Ser-Leu-Ser-Gly-Lys(Cy5)-NH_2 peptide and free HiLyte647 were purchased from Anaspec (Freemont, CA). After cleavage from support, the peptide was purified by preparative HPLC. The molecular weight (MW = 2266.8 Da) and purity (>96%) of the peptide were verified by mass spectrometry and analytical HPLC, respectively. Poly(vinyl alcohol) (PVA) (MW 130 000) was purchased from Sigma-Aldrich (St. Louis, MO). Deionized water used for the experiments was produced by a Millipore filtration system.

Steady state measurements

Absorption spectra were measured using a Cary 50 Bio UV–visible spectrophotometer (Varian Inc., Australia). Absorption spectra were scanned from 500 nm to 750 nm using PVA film reference as a baseline.

Steady-state fluorescence intensity measurements of all the samples were measured using a Cary Eclipse spectrofluorometer (Varian Inc., Australia) with a front-face-geometry custom-build attachment. Using 475 nm excitation, the emission was scanned from 540 nm to 775 nm using a 530 nm long pass filter on the emission side. Excitation was scanned from 475 nm to

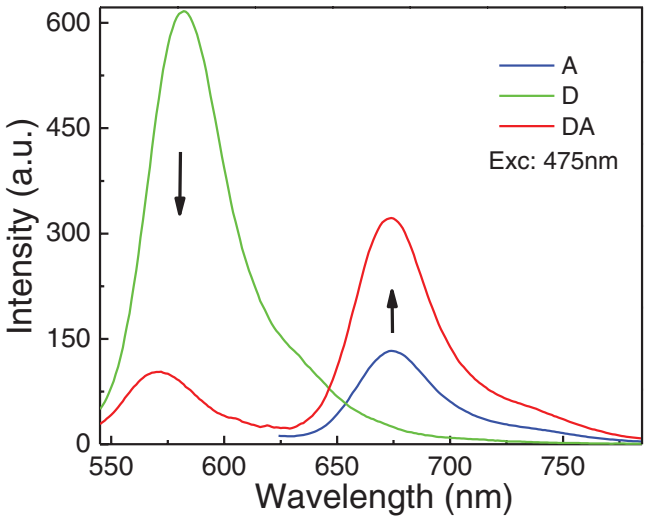

Figure 3. Fluorescence emission spectra of the donor (D, 5,6 TAMRA), acceptor (A, HiLyte647) and donor–acceptor linked system (DA). Whereas fluorescence of the donor in the DA system decreased about 6 fold, the emission of the acceptor increased 2.4 times which indicates very efficient FRET.

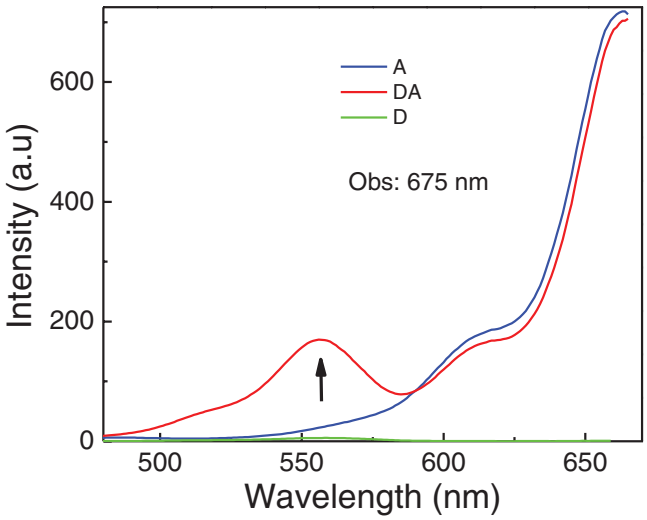

Figure 4. Excitation spectra of the donor (D, 5/6 TAMRA), acceptor (A, free HiLyte647) and donor–acceptor system (DA) observed at 675 nm (acceptor emission). The donor emission at 675 nm is minimal (green). However, in the DA system the emission at 675 nm is strongly enhanced (red). This indicates that acceptor excited state is strongly populated through FRET from the donor (5,6 TAMRA).

675 nm and steady-state excitation spectra were measured by observing the emission at 665 nm.

Time resolved measurements

A FluoTime 200 (PicoQuant, Gmbh, Berlin, Germany) was used to measure fluorescence lifetime of the samples. The fluorometer was equipped with an ultra-fast multichannel plate detector (Hamamatsu, Japan). Lifetimes were measured using a front-face geometry setup. A 475 nm pulsed laser diode was used for excitation, and the lifetimes were measured at 575 nm. The lifetime decays were measured under the magic angle conditions, and FluoFit software v. 4.5.3 from PicoQuant was used for data analysis using the exponential re-convolution procedure with non-linear regression (multi-exponential deconvolution model) expressed as:

$$I(t) = \int_{-\infty}^{t} \mathrm{IRF}(t') \sum_i \alpha_i e^{\frac{-t-t'}{\tau_i}} \quad (1)$$

where IRF(t') represents the instrument response function at time t', τ_i is the lifetime of the ith component, and α_i is the amplitude of decay of the ith component at time t. The average values were calculated as:

$$\overline{\tau} = \sum_i f_i \tau_i, \; f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \quad (2)$$

where f_i is a fractional intensity. The amplitude averaged lifetime is:

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i \quad (3)$$

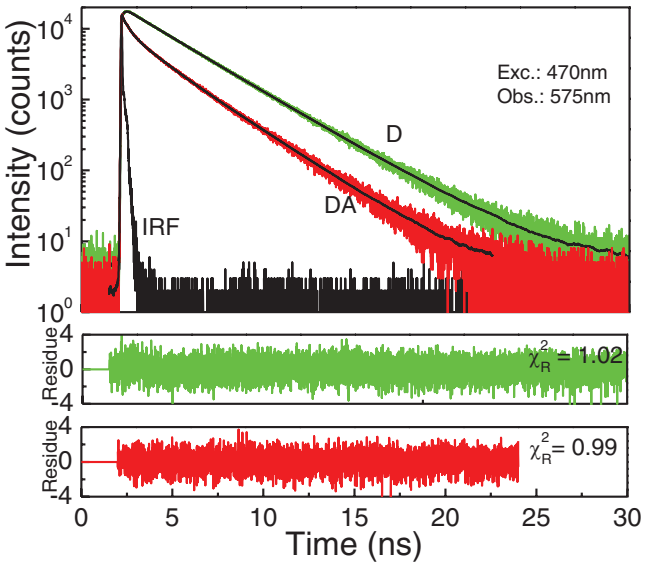

Figure 5. Fluorescence intensity decays of the donor (D, 5, 6 TAMRA) and donor–acceptor system (DA). The donor intensity decay can be well approximated by a single exponent fit with the lifetime of 3.78 ns. In contrast, the emission of the donor (at 575 nm) in DA system is very heterogeneous and can be approximated with four exponents fit yielding an amplitude averaged lifetime of 0.76 ns.

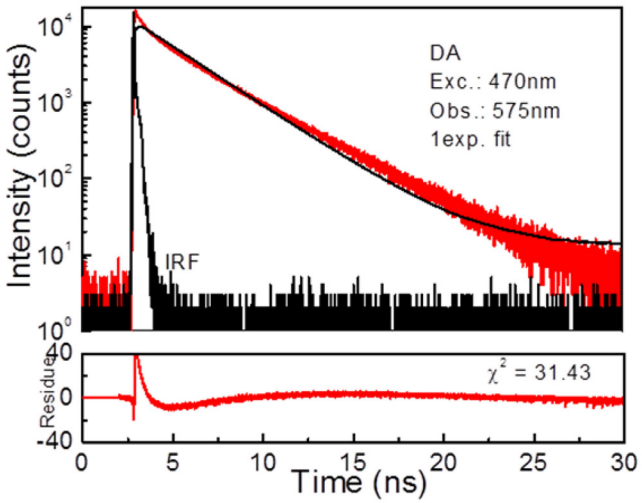

Figure 6. Fluorescence intensity decay of donor in DA system (at 575 nm) approximated with a single exponent fit.

Results

Preparation of doped PVA films

First, we estimated that a 10% (w/w) PVA solution changes its volume (contracts) 12-fold upon drying. Next, we prepared 10 ml of DA, D, and A samples, each at a concentration of 1.7 μM, aiming at a concentration of about 20 μM after drying. A 10 ml aliquot of PVA only was dried and used for obtaining the baseline/background reference.

Steady-state FRET measurements

Absorption spectra of the donor (D) and donor/acceptor (DA) with the peptide linker as well as free acceptor (A) are presented in figure 2. The DA spectrum shows two bands corresponding to the donor (555 nm) and the acceptor (665 nm) fluorescence. With a good approximation, the DA spectrum is a superposition of the D and A spectra, confirming the lack of chemical interactions between the D and A chromophores. All samples have an absorption below 0.1 which simplifies corrections for inner-filter effects.

Emission spectra of D, A, and the DA system are presented in figure 3. The D and A spectra are very well separated, allowing a selective observation of donor emission without an interference from the acceptor. At 475 nm excitation, the emission intensity of the donor is more than 4-fold higher than the acceptor emission. In the DA system, these intensities are reversed. The donor emission decreased about 6-fold and the acceptor emission increased more than 2-fold. These simultaneous changes in the donor and acceptor emission spectra observed in the DA system clearly demonstrate an efficient FRET.

An unambiguous proof of energy transfer is the presence of the donor excitation in the acceptor excitation spectrum. The excitation spectra of the samples were measured at 675 nm, where the acceptor emission peak is found (figure 4). Consequently, the donor only sample should have negligible emission at this wavelength. However, due to FRET, a donor peak is clearly visible in the excitation spectrum of the DA system. The donor only sample shows minimal excitation at this wavelength, with negligible emission. The acceptor only sample shows an overlapping excitation spectra with the acceptor in the DA system from 600–650 nm. FRET parameters for DA system (spectral overlap, Forster R_0, transfer efficiency and the apparent D to A distance) are provided in supporting materials (stacks.iop.org/MAF/4/047002/mmedia).

Time-resolved measurements

Lifetime measurements of DA and D are presented in figure 5. The donor only sample has a lifetime of 3.78 ns; the spectrum can be accurately approximated with a single exponential fit. The donor lifetime in the DA system is reduced to 0.76 ns (amplitude-averaged) and is heterogeneous, requiring a multi-exponential fit (see Supporting Materials). The 5-fold decrease of the lifetime of the donor is consistent with the fluorescence intensity change. The observed heterogeneity of the fluorescence intensity decay is a result of the presence of various conformers of the DA system in the sample [13, 16–18]. Such conformers result in the different distances and orientations between the donor and the acceptor. Should there be a single donor-to-acceptor distance in DA system, the fluorescence lifetime would be shorter, but homogeneous. As seen in figure 6, the donor lifetime in the DA system cannot be approximated with a single exponential fit.

Conclusions

We studied FRET in a donor–acceptor system formed within a peptide carrying with two fluorescent dyes. The peptide can be specifically cleaved by the MMP-9 enzyme. In this DA system, the fluorescence of the donor is strongly quenched and the acceptor emission is enhanced, consistent with about 85% energy transfer efficiency. The fluorescence intensity decay of the donor becomes shorter and heterogeneous in the DA system when compared to the donor alone. This suggests that the DA systems created in the PVA matrix have different conformations of D and A. The distance and orientation distribution of the D and A transition moments result in a non-exponential fluorescence intensity decay.

The fluorescence properties of proposed donor-peptide-acceptor system, including its convenient spectral range, brightness, stability and pH insensitivity, make the system a potentially valuable tool for the MMP-9 sensing and imaging.

We demonstrated the cleavage of Donor-oligopeptide-Acceptor system in Supplementary Materials.

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