Triple-FRET Technique for Energy Transfer Between Conjugated Polymer and TAMRA Dye with Possible Applications in Medical Diagnostics

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Abstract Three-component Förster resonance energy transfer (FRET) has been used to obtain efficient FRET between the cationic conjugated polymer (CCP) as donor and 5-carboxy tetramethylrhodamine (TAMRA) dye as acceptor, by using an intermediate donor, fluorescein. In spite of the fact that there is enough overlap between the emission spectra of CCP and absorption spectra of TAMRA, the efficiency of FRET between CCP and TAMRA is poor. The reason for this is that while the Förster critical distance is not very sensitive to the overlap, the FRET efficiency is extremely sensitive to it. However, it is observed that the FRET efficiency between CCP and TAMRA improves considerably when fluorescein is introduced in the solution. The triple FRET so obtained can be used for deoxyribonucleic acid sequence detection in medical diagnostics because the fluorescence emission from TAMRA is pH-insensitive.

Keywords Triple-color FRET (triFRET) · Light-emitting polymer · Fluorescent dye · UV-visible absorption · Photoluminescence

1 Introduction

The Förster resonance energy transfer (FRET) is extensively used by many workers for medical diagnostics using sequence detection of deoxyribonucleic acid (DNA) of the infected person [1, 2]. The basic principle of this diagnostic is that when a peptide nucleic acid (PNA) probe labeled with a fluorescent dye (PNAC*) is hybridized with DNA, having a sequence complementary to that of PNA, the complex acquires negative charge from the DNA. This negatively charged hybridized DNA–PNAC* complex becomes electrostatically attracted toward the cationic conjugated polymer (CCP), e.g., polyfluorene phenylene (PFP) as described in the present work. If the concentration of PFP (donor) and

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the hybridized complex (acceptor) is appropriate, the distance between the two may be within the critical distance required for FRET between them, resulting in the quenching of blue photoluminescence (PL) intensity of CCP and significant enhancement of PL of the dye. On the other hand, if the sequences of single-stranded DNA (ssDNA) and PNAC* are noncomplementary, the two will not be hybridized, and FRET will not occur [1].

In order that FRET may be efficient, it is necessary that there should be maximum overlap between the PL spectrum of the donor and absorption spectrum of the acceptor [3–5]. Also, the distance between the acceptor and the donor must be within the Förster radius. The donor–acceptor separation distance R is described by the equation:

$$R = R_0 \left[(1/E_T) - 1 \right]^{1/6} \quad (1)$$

where R_0 is the Förster radius (in nm) at which efficiency of energy transfer reaches 50% and E_T is the efficiency of energy transfer [5, 6]. Also, FRET will be most efficient when the number of donor and acceptor molecules is of the same order. Besides all the abovementioned requirements, the dye to be used for labeling PNA should be highly photostable [7, 8], should have low Stokes shift and high extinction coefficient, and its PL emission should not be sensitive to the pH of the solution. Many researchers have used 5-carboxy-tetramethylrhodamine (TAMRA) dye to label PNA [9]. Though it meets most of the abovementioned requirements, it suffers from the disadvantage that the overlap between the PL spectrum of the polymer and absorption spectrum of the dye is low. Some workers have therefore used fluorescein for labeling PNA in spite of the fact that it does not meet all the criteria described above.

We have attempted to sort out this problem by using the triple-color FRET (triFRET) technique, which consists of two donor–acceptor pairs sharing a common chromophore and can be used to study long-range molecular interactions [10]. The three-chromophore system offers many advantages over conventional FRET, such as higher efficiency for long-range energy transfer and better detection sensitivity for acceptor fluorescence enhancement due to FRET [11].

In conventional FRET, the energy is transferred nonradiatively from an excited donor molecule to an acceptor in ground state via an induced dipole–dipole interaction [12, 13]. The time scale of FRET is a function of distance between the donor and acceptor. However, when the distance between the two is close to the Förster critical radius, the process of FRET occurs within a few femtoseconds. In contrast, the time interval between absorption and emission by the acceptor is typically of the order of nanoseconds. The efficiency of FRET therefore depends primarily on the distance between the donor and acceptor fluorophores and is not influenced by short-range solvent effects such as dielectric characteristics of the solvent and positioning of host macromolecules between the involved fluorophores [6]. On the other hand, the triFRET system can be used to study interactions between the two molecules (donor and acceptor), which may even be beyond the Förster radius.

In the triFRET system consisting of donor (1), intermediate donor (2), and acceptor (3), the following possible energy transitions may occur:

- Energy going from the principal donor to two different acceptors simultaneously ($1 \rightarrow 2$ and $1 \rightarrow 3$), called two-parallel one-step FRET
- Energy going from the principal donor to the second donor and subsequently to the final acceptor, and energy going directly from first donor to final acceptor ($1 \rightarrow 2 \rightarrow 3$ and $1 \rightarrow 3$), called parallel one- and two-step FRET
- Energy going from the principal donor to the second donor and subsequently to the acceptor ($1 \rightarrow 2 \rightarrow 3$), which may be called two-step FRET

These three scenarios for a triFRET system depend on the positional arrangement of the three components involved [14]. The triFRET system can be demonstrated to work with proper chromophore selection and is favorable in a cascade process, i.e., sequential energy transfer from the principal donor to the intermediate donor and subsequently to the final acceptor. The FRET system under study consists of a blue light-emitting polymer poly [9,9-di (6,6' - *N,N'*-trimethylammonium) hexylfluorenyl-2,7-diyl)-alt-*co*-(1,4-phenylene)] dibromide salt (PFP) along with the two species of the fluorescent dyes, fluorescein and TAMRA. The dyes are chosen for their well-known spectral properties [8]. Fluorescein acts as the second or intermediate donor, TAMRA as the final acceptor, and the third component, PFP, acts as the principal donor. This polymer (PFP) possesses unique combination of optoelectronic properties and also serves as a light-harvesting molecule [1]. Due to its optical amplification property, the polymer has found wide applications [15]. The structure of this polymer allows effective electronic coupling and provides a tool for ultrafast intra- and interchain energy transfer. We report here the successful design of a light-harvesting system based on two-step FRET, using a polymer–dye combination, which will find application in FRET and DNA detection.

2 Experimental Details

Fluorescein was purchased from Sigma Aldrich, USA, and TAMRA from Ana Spec, USA. The water-soluble cationic conjugated polymer, PFP, was commercially procured from American Dye Source, Canada. The solution of the polymer was prepared in deionized (DI) water and ethanol in the ratio 1:1. TAMRA dye was dissolved in methanol and fluorescein in DI. DI water was obtained from a TKA water filtration system. The absorption spectra was measured using a Shimadzu UV-Visible Spectrophotometer, model no. UV-2450, while the fluorescence measurements were made with Shimadzu Spectrofluorophotometer, equipped with a xenon lamp excitation source, model RF-5301PC. The spectra were recorded in the range 350 to 700 nm. FRET between PFP and TAMRA was studied in the absence of fluorescein, and no significant energy transfer was observed from PFP to TAMRA. PL measurements of the composite solution of fluorescein and TAMRA dyes were also carried out to achieve the donor–acceptor ratio where maximum energy is transferred from fluorescein to TAMRA. For triFRET analysis, PFP was added successively to the solution of fluorescein and TAMRA to determine the optimum ratio of energy transfer from the principal donor (PFP) to the final acceptor (TAMRA). After each addition of the polymer, the solution containing all the three components was mixed thoroughly for about 15 min to achieve homogeneity, and PL spectra of the complex (PFP/Fluorescein/TAMRA) was then observed to measure the energy transfer. The process of addition of PFP followed by mixing was repeated until we obtained saturation of FRET, which is found to be at 20 μ l of PFP. The optimum FRET was obtained when the concentration of PFP was 2.5×10^{-7} M, and that of the dyes was 10^{-4} M.

3 Results and Discussion

The spectral overlap of the PL and absorption spectra of the three components was observed to examine the feasibility of the triFRET process and is shown in Fig. 1, where the absorption and emission spectra of CCP and both the dyes have been plotted (symbol A for absorption and F for emission). It is found that while there is a good overlap between the PL emission spectra of CCP (curve F1) and the absorption spectra of fluorescein (curve A2) and

Fig. 1 Absorption and emission spectra of the light-emitting polymer and fluorescent dyes—PFP: A1 ($\lambda_{\max}$) = 376 nm; F1 ($\lambda_{\max}$) = 410 nm; fluorescein: A2 ($\lambda_{\max}$) = 492 nm, F2 ($\lambda_{\max}$) = 520 nm; TAMRA: A3 ($\lambda_{\max}$) = 547 nm, F3 ($\lambda_{\max}$) = 573 nm

also between the PL emission spectra of fluorescein (curve F2) and the absorption spectra of TAMRA (curve A3), the overlap between the PL spectra of CCP (curve F1) and the absorption spectra of TAMRA (curve A3) is comparatively poor.

The Förster critical radius R_0 (in nm) is related to the spectral overlap by the relation:

$$R_0 = 2.11 \times 10^{-2} [K^2 J(\lambda) \eta^{-4} Q_D]^{1/6} \quad (2)$$

where K^2 is a factor describing the relative orientation in space between the transition dipoles of the donor and acceptor, $J(\lambda)$ is the spectral overlap integral of donor emission and acceptor absorbance (with wavelength in nanometers), η is the refractive index of the medium, and Q_D is the quantum yield of the donor. The efficiency of FRET is expressed by the relation:

$$E_T = 1 / (1 + R/R_0)^6 \quad (3)$$

where E_T is the energy transfer efficiency and R_0 is the Förster radius (in nm) at which the efficiency of energy transfer becomes 50%, R being the separation distance between the donor and acceptor [5]. Using (2) and the observed spectral overlap (from Fig. 1), we have calculated the values of R_0 for the triFRET pairs: CCP–TAMRA, fluorescein–TAMRA, and CCP–fluorescein as 4.3, 5.8, and 6.4 nm, respectively. Though these values of R_0 are not widely different, the FRET efficiency, E_T , is extremely sensitive to the Förster critical distance as given by (3). Thus, the degree of spectral overlap is important where efficiency is concerned.

The effect of the presence of fluorescein between CCP and TAMRA for increasing values of polymer concentration is shown in Fig. 2. It is found that the PL of TAMRA increases with the addition of polymer until a saturation of PL is achieved.

A significant enhancement in the fluorescence intensity of the final acceptor, with almost complete quenching of the second donor as the donor concentration is varied ensures that energy transfer occurs in two steps from the polymer to fluorescein and from fluorescein

Fig. 2 Emission spectra of polymer PFP, fluorescein (FL), and TAMRA in a composite solution for different concentrations of PFP (from 0 to 20 μl) at an excitation of 376 nm

to TAMRA as indicated by Fig. 2 (possibility 3 of the triFRET system). This shows that fluorescein has transferred all its energy, as well as that obtained from the polymer, to TAMRA. The comparison of the two-FRET system, i.e., conventional FRET, between PFP and TAMRA, and the two-step FRET, i.e., between PFP, fluorescein, and TAMRA, is shown in Fig. 3. It is observed that PL intensity of TAMRA increases about sevenfold in the presence of the intermediate donor fluorescein in two-step FRET. On the other hand, a very weak FRET is obtained in the conventional single-step FRET between PFP and TAMRA.

Fig. 3 Comparison of the emission spectra of conventional FRET (PFP/TAMRA, crosses) and two-step FRET (PFP/FL/TAMRA, open squares)

Fig. 4 Effect of fluorescein on the PL intensity of TAMRA for different concentrations of CCP

In order to examine the role of fluorescein on the efficiency of FRET between CCP and TAMRA, the variation of the intensity of PL emission from TAMRA has been plotted as a function of concentration of CCP with and without fluorescein, as shown in Fig. 4. It is found that for a given concentration of CCP, there is a significant increase in the emission intensity of TAMRA with the addition of fluorescein. This shows that when the distance between CCP and TAMRA is constant, the addition of fluorescein improves the efficiency of FRET between the two.

Implementing the idea of triFRET in DNA sequence detection would lead to improvement in the efficiency of energy transfer from CCP to TAMRA when used in a biosensor in which the PNA probe (labeled with TAMRA) will act as an acceptor and CCP as a donor. However, due to electrostatic attraction, the energy transfer occurs from CCP to the hybridized complex of ssDNA/PNAC* (for a complementary DNA sequence), but the efficiency of FRET observed is not so significant and can be improved by introducing an intermediate component, viz., fluorescein dye, which will mediate the energy transfer and thus will enhance the efficiency of FRET.

4 Conclusions

In spite of little overlap between the absorption spectrum of the acceptor, TAMRA dye, and the emission spectrum of the donor, CCP, we have demonstrated an efficient FRET between the two by introducing an intermediate donor, fluorescein dye. In the present case, the FRET is obtained by a two-step process, where the energy is transferred from the principal donor (CCP) to the acceptor (TAMRA) via an intermediate donor (fluorescein). The three species come in close proximity with each other resulting in efficient energy transfer to long-range distances due to the presence of the intermediate donor, which becomes possible by adjusting their concentrations. The triFRET technique allows us to use TAMRA dye with CCP for FRET, which is highly photostable and gives pH-insensitive emission, and is therefore a better option than fluorescein to be used in biosensors for DNA sequence detection.

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