Enhancing Nucleic Acid Detection Sensitivity of Propidium Iodide by a Three Nanometer Interaction Inside Cells and in Solutions

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Most interactions inside living cells take place at nano-length scales. The only way to monitor these nano-scale interactions inside living cells is by utilizing the phenomenon of Fluorescence Resonance Energy Transfer (FRET). Thus, the importance of FRET in visualizing intracellular dynamics has provided continuous motivation for discovery/synthesis of biologically useful molecules exhibiting FRET. While working on intracellular uptake of coumarin loaded biodegradable polymeric nanoparticles, we have discovered a remarkable utility of 6-coumarin (Co) forming a FRET pair with propidium iodide (PI). Based on observations on intracellular behavior of Co and PI, we tested and characterized the Co–PI FRET pair in solutions. We report that Co acts as photon donor to PI (photon acceptor) with a Forster’s distance of ∼3 nanometers. We show a FRET based sensitivity enhancement (30–35%) of PI for nucleic acid detection, both inside cells and in solutions. Our results open a variety of applied avenues by utilizing the newly discovered FRET pair.

Keywords: FRET, Fluorescence Resonance Energy Transfer, Coumarin, HeLa, Nano Drug Delivery.

1. INTRODUCTION

Biophysical advances are pushing the limits for monitoring dynamic events in living cells in real time. The need for direct visualization of several cellular phenomena, previously envisioned as creative and appealing cartoons, has led to several breakthrough inventions and discoveries in microscopy of living cells. Owing to these developments over the years, the advances in cell biology research have benefited several areas of biology. These include development of rigorous experimental systems for in vitro research in drug delivery. While developing one such experimental system in our laboratory, for testing delivery of molecules to cancer cells by biodegradable nanoparticles synthesized from Poly-DL-lactide-co-glycolide (PLGA), we have discovered a novel molecular pair that exhibits the phenomenon of FRET. The most fascinating aspect of our discovery is the extremely well documented importance of both the molecules in general cell biology research.

Propidium iodide (PI) is a fluorescent molecule that binds to nucleic acids,¹⁻³ a property that has led to its widespread use in cell biology research¹⁻⁶ since its discovery. Coumarin and its derivatives have been of interest for several years for their well observed anti-cancer properties in several experimental systems.⁷⁻⁹ Further, fluorescence characteristics of coumarin have enabled its widespread use as a reporter for internalization and degradation of biodegradable nanoparticles during in vitro uptake characterization studies of nanoformulations.¹⁰⁻¹²

One of the most promising nano-drug delivery systems has emerged to be that of biodegradable PLGA nanoparticles,¹⁰⁻¹⁴ especially since PLGA is an FDA approved polymer for drug formulations.¹⁴ In vitro studies of delivery of PLGA nanoparticles can be carried out by encapsulating a model hydrophobic drug molecule like 6-coumarin (Co) due to its fluorescent characteristics.¹¹ In search of experimental data to elucidate the mechanism of uptake and biodegradation of PLGA nanoparticles, we prepared Co loaded PLGA nanoparticles (N-Co) using well established methods.¹⁰ For simplicity of experimentation, we incubated the N-Co with HeLa cells, since this cell line is one of the best characterized in terms of its cell biology.¹⁵ Subsequently, we stained the cells with PI in order to observe the effects of Co, which may have been released from N-Co after uptake, on the cells. Initial observations from our experiments appeared to show much brighter PI signals in the images using a fluorescence microscope for cells incubated with N-Co compared to controls. Further,
cells incubated with free Co did not show any difference in PI signals compared to controls. Therefore, we hypothesized that Co and PI may be forming a FRET pair. FRET occurs when a donor fluorescent molecule in its excited state transfers its energy by a nonradiative dipole–dipole interactions to a neighboring acceptor molecule in close proximity (typically <10 nm), leading to emission of photons by latter. We tested the FRET hypothesis by conducting solution experiments of Co and PI by titrating both the molecules with each other and measuring the impact on their individual fluorescence.

We demonstrate the remarkable results that these experimentally important, and commonly used, molecules form a FRET pair. We report that the Forster’s distance for the FRET behavior of these two molecules is ∼3 nm. We further demonstrate that there is a 30–35% increase in sensitivity of PI while detecting nucleic acids, both inside cells and in solutions. Our discovery of Co and PI as a FRET pair promises to open several new molecular avenues both in applied and basic research. On the one hand, it is obvious that this FRET pair can benefit in vitro drug delivery research by providing kinetics of biodegradation of polymeric encapsulations (like PLGA) inside living cells while delivering molecules to regions rich in nucleic acids (e.g., the nucleus). On the other hand it provides basic cell biology research a strong tool to study the dynamics of interactions between relatively hydrophobic molecules (like Co) and nucleic acids inside living cells.

2. MATERIALS AND METHODS

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA, MW 40,000–75,000, copolymer ratio 50:50), Polyvinyl alcohol (PVA, MW 30,000–70,000) and 6-Coumarin (MW 350.44) were purchased from Sigma Chemical (St. Louis, MO, USA). Dichloromethane (HPLC grade) and Formaldehyde were from Qualigens Fine Chemicals (Mumbai, Maharashtra, India). New Born Calf Serum (NBCS), Dulbecco’s phosphate buffer solution (DPBS) and Dulbecco’s Modified Eagles Medium (DMEM) were purchased from Gibco BRL (Grand Island, NY, USA). Kanamycin acid sulphate, Trypsin-EDTA solution and Propidium Iodide were purchased from Himedia Laboratories Pvt. Ltd (Mumbai, Maharashtra, India). Tissue culture plates (35 mm diameter) and tissue culture flasks (T-25) used were from Corning Life Sciences (Corning, NY, USA). Lambda (λ) DNA used was purchased from Fermentas International Inc (Burlington, Ontario, Canada).

2.2. Formulation of Coumarin Loaded Nanoparticles

Nanoparticles containing a lipophilic fluorescent dye, 6-coumarin [Ex (λ) 450 nm/Em (λ) 490 nm] were formulated by using single emulsion–solvent evaporation technique as described previously. In a typical procedure, 90 mg PLGA was dissolved in 3 ml of dichloromethane. A 2% solution of PVA was prepared in cold distilled water, and centrifuged at 1000 rpm for 5 min and then filtered through a 0.22 μm hydrophilic polysulfonic membrane syringe filter (25 mm Millipore filter unit, Millipore, Bedford, MA, USA) to remove any undissolved PVA. 50 μg of 6-coumarin (stock solution 0.5 mg/ml in dichloromethane) was added to the PLGA solution followed by vortexing. It was then placed on an ice bath for 5 min and emulsified using a microtip probe sonicator (Soniprep 150, MSE Scientific Instruments, Crawley, UK) for 30 s, to obtain a primary emulsion. The primary emulsion was then added in two portions to 12 ml of the PVA solution with intermittent vortexing to obtain o/w emulsion. The emulsion was placed on an ice bath for 5 min and then sonicated for 2 min. The o/w emulsion was stirred overnight on a magnetic stir plate (Spinit, New Delhi, India) to allow the evaporation of dichloromethane and formation of the nanoparticles. The suspension of nanoparticles was stirred in a vacuum desiccator placed on the magnetic stir plate for an additional hour to ensure complete removal of the organic solvent. The suspension was transferred into centrifuge tubes and centrifuged at 27000 rpm (110 000 × g) for 20 min at 4 °C in an ultracentrifuge (Beckman L8-60M Ultracentrifuge, Fullerton, CA, USA). The pellet obtained was resuspended in double distilled water and sonicated for 30 s on an ice bath to disperse any aggregates. Ultracentrifugation was repeated two more times for 20 minutes each. This washing step was meant to remove PVA from the formulation. After the last centrifugation, the coumarin loaded PLGA nanoparticles were resuspended in 7 ml of double distilled water and sonicated for 30 s on an ice bath. The nanoparticles were then centrifuged at 1000 rpm for 10 min at 4 °C to remove any large aggregates. The supernatant was collected and frozen at −70 °C for 45 min and subsequently lyophilized for 2 days (Labconco Freeze Dry system/Free zone 4.5, Kansas City, MO, USA). The lyophilized (powdered form) coumarin loaded PLGA nanoparticles were then stored at 4 °C.

2.3. Nanoparticle Characterization

The diameter of the nanoparticles was obtained with photon correlation spectroscopy (PCS) using quasi elastic light scattering equipment (Brookhaven Instruments Corp., Holtsville, NY, USA) and 90 Plus Particle Sizing Software (Version 3.42). To measure particle size, a dilute suspension (200 μg/ml) of nanoparticles was prepared in double distilled water and sonicated on an ice bath for 30 s to break the aggregates. The sample was filled in a cuvette and subjected to particle size analysis.

2.4. Cell Culture

Human cervical adenocarcinoma (HeLa cells) were used for studying the cellular uptake of coumarin loaded
nanoparticles. HeLa cells were procured from National Centre for Cell Sciences (NCCS), Pune, Maharashtra, India. The cells were cultured regularly in T-25 tissue culture flasks in DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with 10% New Born Calf Serum at 37 °C and 5% CO₂ in a CO₂ incubator (Shellab CO₂ water jacketed incubator, Cornelius, OR, USA). The cells were passaged in a split ratio of 1:2 or 1:3. All the experiments were performed with cells between passages 12 to 14.

2.5. Cellular Nanoparticle Uptake Studies

HeLa cells were seeded in 35 mm diameter tissue culture petridishes and were allowed to grow till 80% confluency. A suspension of nanoparticles (2 mg/ml) in DMEM was prepared as stock. The medium in the petridishes was replaced with the suspension of the nanoparticles (100 μg/ml) and incubated for different time intervals: 15 min, 30 min, 60 min, 90 min and 120 min. After specific time durations the cells were washed three times with DPBS (with Ca/Mg) to remove any non-cell associated nanoparticles. Then the cells were fixed with 4% formaldehyde and were observed using a blue filter (excitation wavelength 460–490 nm, emission 470 nm) in an upright fluorescence microscope (Nikon Optiphot-2, Tokyo, Japan) and images captured using Samsung SDC-312 digital colored camera. To confirm our observations (as discussed in results and discussion) the same samples were stained with propidium iodide (0.03 mg/ml prepared in DPBS) [nucleic acid binding fluorescent dye, Ex (A) 530 nm/Em (A) 615 nm] and observed using an inverted fluorescence microscope IX51 Olympus (Olympus Inc., Tokyo, Japan). Green channel data for Co visualization was acquired by using the mirror unit U-MWB2 (Olympus Inc., Tokyo, Japan) with excitation filter of 460–490 nm, emission filter of 520 nm and a dichromatic mirror at 500 nm. Red channel data for PI visualization was acquired by using the mirror unit U-MWG2 (Olympus Inc., Tokyo, Japan) with excitation filter of 510–550 nm, emission filter of 590 nm and a dichromatic mirror at 570 nm. The images were acquired using a cooled CCD camera (DP70, Olympus Inc., Tokyo, Japan). For control experiment, monolayers of HeLa cells were incubated with suspension of coumarin molecules (0.05 mg/ml in dichloromethane) in DMEM for 15 minutes, prepared by vigorous vortexing. Cells were again observed using IX51 Olympus and images were captured using DP70. Also monolayer of HeLa cells were fixed and stained with propidum iodide (0.03 mg/ml prepared in DPBS), observed using IX51 Olympus and images were captured using Olympus DP70 camera. The specification of the filters used for observing and acquiring images for the above cells using inverted fluorescence microscope IX51 Olympus are mentioned in Figure 1 legend. For each case the exposure time for image acquisition (using DP70) was kept same (fixed manually) to allow signal comparisons.

2.6. Spectrofluorimeter Experiments

For conducting solution experiments, different concentrations (0.01 mg/ml, 0.03 mg/ml, 0.05 mg/ml and 0.1 mg/ml) of coumarin (stock 0.5 mg/ml prepared in Dichromethane) suspended in DMEM (by vigorous
vortexing) were added to PI solutions having fixed concentration of 0.03 mg/ml (prepared in DPBS). Immediately the sample was filled in a cuvette and emission scans were measured at fixed coumarin excitation wavelength (450 nm) and at fixed PI excitation wavelength (530 nm) using Fluorolog-3 Spectrophotometer (Horiba Jobin Yvon, Edison, NJ, USA) respectively. Similarly, different concentrations of PI (0.01 mg/ml, 0.03 mg/ml, 0.06 mg/ml, and 0.1 mg/ml) were added to coumarin solutions having fixed concentration of 0.05 mg/ml and emission scans were measured at fixed coumarin (450 nm) and PI excitation (530 nm) wavelengths respectively. The emission scans were also measured for different concentrations (as mentioned above) for PI and coumarin individually (in the absence of the other molecule). All the experiments were done in triplicates. Blank used were same in all cases comprising of DMEM, DPBS and dichloromethane. The spectral data obtained were analyzed as discussed in results section.

For checking the sensitivity of PI in solutions by measuring its fluorescence with different amounts of λ-DNA (in presence and absence of Co), different concentrations of λ-DNA were prepared in DPBS (0 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml and 500 ng/ml). The respective λ-DNA concentration was added to PI solution with fixed concentration 0.03 mg/ml (prepared in DPBS). The fluorescence intensities were measured at fixed Excitation 530 nm/Emission 590 nm and fixed Excitation 450 nm/Emission 590 nm using Perkin Elmer LS55 fluorescence spectrophotometer (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA, USA). After measuring fluorescence intensities at the above mentioned excitation and emission wavelengths, coumarin solution of concentration 0.05 mg/ml (prepared in dichloromethane) was added to the PI-λ-DNA solution and again fluorescence intensities were measured at fixed Excitation 530 nm/Emission 590 nm and fixed Excitation 450 nm/Emission 590 nm using Perkin Elmer LS55 fluorescence spectrophotometer. The same procedure was repeated for other λ-DNA concentrations. The experiments were repeated in triplicates independently.

2.7. Data Analysis

The FRET Efficiencies (E) and the distances between donor (Co) and acceptor (PI) molecules (R) for different concentrations of donor were calculated as follows. E was calculated for different Co concentrations using the relation as shown in Eq. (1) (results section). For calculation of R, initially the number of Co molecules (N1) and PI molecules (N2) for each Co concentration was calculated by multiplying the respective concentrations (Molecular weight of Co–350.44 and PI–668.40) with the Avogadro’s number (Naw). On the basis of the assumption that the Co and PI molecules are homogeneously distributed in the given volume (i.e., in the cuvette) and considering void volume around each molecule to be a sphere, Void volume per molecule (V) was calculated by dividing volume of total solution in the cuvette by total number of molecules (N1 + N2) for respective donor concentrations. From ‘V,’ the radius (r) was calculated (assuming sphere) for each Co concentration. The distance between the Co and PI molecule (R) for each concentration was therefore obtained by multiplying r with 2 (assuming that the molecules are equally spaced). Thus, the experimental data obtained (E and R values for different donor concentrations) was fitted to Forster’s equation (Eq. (1)) to yield the Forster’s distance for the Co-PI FRET pair (as shown in Fig. 4(A)). The nonlinear fitting was done in MATLAB (The MathWorks, Inc., USA) using the function fminsearch.

3. RESULTS

3.1. Intracellular Uptake of Coumarin Loaded PLGA Nanoparticles Yields Two Phenotypes

Co loaded PLGA nanoparticles (N-Co) were prepared as per existing literature.10 The nanoparticle size (obtained from quasi-light scattering) ranged from 132 to 246 nm with the mean diameter of 183 nm and polydispersity of 0.057. The N-Co preparations were incubated with HeLa cell monolayers for different time intervals, washed repeatedly to separate non cell associated N-Co and fixed for microscopic observations. The fixed samples were then observed using an upright fluorescent microscope. Figure 1(A) shows the fluorescent image of fixed HeLa cells observed after 15 minutes of incubation with our N-Co preparation. All the cells had Co fluorescence associated with them, suggesting rapid “association” of N-Co with the cells. Note that this “association” is a combination of both intracellular uptake and cell surface association. Careful inspection of the samples, as shown in Figure 1(A), showed two distinct phenotypes with respect to the fluorescence associated with the cells. Figure 1(B) shows a higher magnification view of a representative sample, highlighting the two phenotypes by arrows. There was a population of cells, similar to the phenotype indicated by the dashed arrow, which had a weaker fluorescence intensity region (compared to rest of the cell) enclosed within the cell boundary. A second population of cells was also observed, similar to the phenotype indicated by the smooth arrow, that had a much higher fluorescence intensity region (compared to the rest of the cell) enclosed within the cell boundary. To confirm a quantifiable difference between the two phenotypes, we analyzed the total pixels associated with the whole cell in X–Y coordinate system. Figure 1(C) shows a representative plot of pixel values of fluorescence associated with the second phenotype (i.e., the one indicated by a smooth arrow in Fig. 1(B)) in the X–Y plane. Figure 1(D) shows a similar plot for the first phenotype. Firstly it is
clear that the first phenotype shows a valley (Fig. 1(D)) in contrast to the second phenotype that shows a peak (Fig. 1(C)). The remarkable fact is that the size of the peak appears quite similar to the size of the valley (i.e., if we were to invert the peak in Fig. 1(C), it would fit into the valley in Fig. 1(D)). This opened up the possibility that both the phenotypes represented a similar cellular location, which for some cells had much higher amounts of Co associated with it (compared to rest of the cell) and for some had comparatively much less Co associated with it. We also observed that the second phenotype (Fig. 1(C)) increased from ~10 to 20% of the total number of cells with increase in incubation time of N-Co with cells from 15 to 120 minutes. These results were quite intriguing, especially considering that the specific region corresponding to the peak and valley in Figures 1(C and D) appeared to be the cell nucleus. Thus, we hypothesized that the phenotypes were a result of different cells in which Co (or N-Co) had been delivered to the nucleus (Fig. 1(C)) and cells in which the Co (or N-Co) was in the cytoplasm and/or just associated with the cell surface (Fig. 1(D)).

To test the above hypothesis, and to rule out optical artifacts possibly arising due to the use of upright fluorescence microscopy, we first stained the same samples with PI and then observed them using an inverted microscope. If Figure 1(C) represents a phenotype where Co (or N-Co) has been delivered to the nucleus indeed, then we would observe co-localization of Co (or N-Co) with PI (which is expected to give a particularly high signal in the nucleus). Figure 1(E) shows images of cells obtained using the green channel, a wide band filter for Co excitation (see materials and methods). Based on the mirror unit wavelengths, while distinct green fluorescence is observed in the non-nuclear region of most of the cells, with a red nucleus, some cells showed greenish orange nuclear region also indicating a possible co-localization of the green and red signals. On observing similar samples using a filter specific for PI fluorescence, the red channel, as shown in Figure 1(F), we made a remarkable observation. The phenotypes of Figure 1(B) were clearly visible, as shown by the dashed and the smooth arrows. The difference in phenotypes in Figure 1(F) was in terms of a red nucleus (corresponding to Figs. 1(D for B)) for some cells (dashed arrow) in contrast to a bright orange–yellow nucleus for some cells (smooth arrows). Since we had maintained our cooled CCD camera at manual settings (see materials and methods) to obtain quantifiable images, the orange–yellow signal implied an increase in the PI fluorescence inside the cells that was saturating the pixel values. Even more remarkable was the fact that PI fluorescence associated with nucleic acids in the cytoplasm of most of the cells also showed an orange color. Was Co (or N-Co) responsible for this increase in PI fluorescence?

To answer the above question, first we had to carry out an important control experiment. The question was whether we would observe an orange–yellow signal if we incubated the HeLa cells with free Co (i.e., not encapsulated). Figures 1(G and H) show representative images of cells incubated with free Co (concentration equivalent to that used for preparation of our N-Co) for 15 minutes using the same two filters as in Figures 1(E and F). Remarkably, very distinct green and red signals were observed by the respective filters without any orange appearing in even a single cell, indicating a complete lack of any co-localization between Co and PI fluorescence signals. The morphological changes observed in the HeLa cells (Fig. 1(G)) are consistent with literature on effects of free Co on cells in addition to the expectation that a hydrophobic molecule like coumarin would accumulate in cell membranes thereby giving rise to “swollen” cells. Thus, our results in Figure 1 clearly demonstrated that N-Co preparations enable transport of Co into healthy cells, without any observable morphological changes in contrast to free Co, which is not transported inside the cells and remains associated with cell membranes only. While co-localization of Co and PI fluorescence on using N-Co preparation, indicating internalization of N-Co, was not a surprising result, the remarkable orange–yellow signals corresponding to PI, indicating a very high increase in PI fluorescence was indeed very interesting. To confirm whether incubation of cells with N-Co was not leading an overall increase in the nucleic acid content of cells, thus resulting in higher PI fluorescence, we also observed cells stained with just PI, without incubating with either free Co or N-Co. Figure 2(A), a representative image acquired under the same microscope and camera settings as Figures 1(E–H), shows absence of any orange or yellow signals, indicating that the overall amount of PI fluorescence had not increased.

We quantified the pixel data for images obtained from both the green and the red channels to test whether all our visual observations were statistically significant, especially on a single cell level for multiple cells. Figure 2(B) shows total coumarin intensity per cell represented by the green channel fluorescence intensity values per cell. This was calculated as the total green pixel intensity (i.e., G in the RGB images) associated with several individual cells for samples stained with PI after incubation with N-Co for 15 minutes and samples stained with only PI (i.e., no incubation with N-Co). As expected, negligible green intensity was associated with cells that had not been incubated with N-Co. On similar quantification for the red channel (i.e., R in the RGB images), we observed very statistically significant differences (p ≪ 0.01), using two-tailed two sample homoscedastic t-tests, in the PI fluorescence associated with cells that had been incubated with N-Co in contrast to cells that had been stained only with PI (Figs. 2(C and D)). The overall red intensity for N-Co+PI samples was 30% more than the red intensity for samples stained only with PI. Thus, the visual observations were confirmed to be statistically significant. Note that the very significant difference in the red fluorescence intensity was
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Fig. 2. Internalized PLGA nanoparticles loaded with coumarin (N-Co) release coumarin at nucleic acid rich regions. (A) PI stained HeLa cells without N-Co or free coumarin incubation. (B) Green intensity per cell with (N-Co+PI) and without (PI) incubation with N-Co. Open and the gray bars show coumarin fluorescence per cell from two independent experiments (mean ± standard deviation for 25 randomly selected cells in a random field of view). Green bars show mean ± standard deviation of pooled single cell data of the two independent experiments (i.e., n = 50). (C) Total PI fluorescence intensity per cell with (N-Co+PI) and without (PI) incubation with N-Co. The open and the gray bars show the PI fluorescence as in (B). The red bars show mean ± standard deviation of the pooled single cell data as the green bars in (B). The ratio of red intensity in presence of N-Co to that of PI alone is 1.3 based on the mean of the red bars. (D) Logarithm of p-values obtained from t-tests between the red bars in (C) and green bars in (B) respectively. The dashed line shows p = 0.01.

Independent of phenotypes of cells (since Figs. 2B and C do not differentiate in the cell phenotypes). Therefore, we hypothesized an unlikely, but a possible scenario, that subsequent to internalization, N-Co are degraded inside HeLa cells, and the released Co associates with regions rich in nucleic acids (regardless of cytoplasm or the nucleus) where it forms a FRET pair with PI that later binds to the nucleic acids. If this were the case, we would not only expect to see some PI fluorescence while exciting Co (and not PI) while measuring at the emission of PI, we would further expect a much higher PI fluorescence intensity signal while exciting PI due to the additive effect of PI being excited directly by the light source along with some further excitation from small, but significant, emission of photons from Co.

3.2. Co and PI Form a FRET Pair in Solutions

To test the Co–PI FRET pair hypothesis, we conducted solution experiments for the two molecules. First, we measured the fluorescence of solutions, in a spectrofluorimeter, with different PI concentrations in a cuvette in presence and absence of a fixed Co concentration. The fluorescence measurements were done at the emission wavelength of PI (590 nm), with excitation of the samples done at both the excitation wavelength of Co (i.e., 450 nm) and at that of PI (i.e., 530 nm). The fixed Co concentration was chosen to be the same as that used for HeLa cell experiments. PI concentrations were also varied around the same value as that was used for the cell experiments. Figure 3(A) shows, as expected, a very small amount of PI emission in absence of any Co molecules in solution (red bars) at the excitation wavelength of PI (590 nm), with excitation of the samples done at both the excitation wavelength of Co (i.e., 450 nm) and at that of PI (i.e., 530 nm). The fixed Co concentration was chosen to be the same as that used for HeLa cell experiments. PI concentrations were also varied around the same value as that was used for the cell experiments. Figure 3(B) shows a clear increase in PI
Fig. 3. Coumarin (Co) forms a FRET pair with propidium iodide (PI) in solution. (A) PI fluorescence increases significantly at 590 nm (emission wavelength of PI) after exciting at 450 nm (excitation wavelength of Co) in presence of 0.05 mg/ml Co (orange bars) compared to absence of coumarin (red bars). (B) (A) PI fluorescence increases significantly at 590 nm (emission wavelength of PI) after exciting at 530 nm (excitation wavelength of Co) in presence of 0.05 mg/ml Co (orange bars) compared to absence of coumarin (red bars). (C) Co fluorescence decreases significantly at 520 nm (emission wavelength used for monitoring Co associated with HeLa cells using fluorescence microscopy) after exciting at 450 nm (excitation wavelength of Co) in presence of 0.03 mg/ml PI (orange bars) compared to absence of PI (green bars). (D) Co fluorescence decreases significantly at 495 nm (emission wavelength of Co) after exciting at 450 nm (excitation wavelength of Co) in presence of 0.03 mg/ml PI (orange bars) compared to absence of PI (green bars). All data is shown as mean ± standard deviation for independent triplicates (i.e., n = 3).

emission when the solutions are excited at the excitation wavelength of PI in presence of Co (orange bars) compared to absence of Co (red bars). This data confirmed our hypothesis (generated by the HeLa cells data) of increase in PI emission at the excitation wavelength of PI arising from the additive effect of direct light source excitation of PI and some, but significant, excitation by photons emitted from Co. Note that on using PI excitation wavelength, the PI emission is much higher (an order of magnitude), compared to use of Co excitation wavelength (Y-axes of Figs. 3(B and A) respectively), as would be expected. Having found strong evidence in favor of the Co–PI FRET pair hypothesis from one angle, i.e., that of PI emission, we also wanted to test it from the other angle, i.e., that of Co emission.

Thus we measured the Co fluorescence for different concentrations of Co, in presence and absence of a fixed concentration of PI, at excitation wavelength of Co. We used emission wavelengths of 520 nm that is the emission wavelength for detecting Co used in fluorescence microscopy, and 495 nm that is the emission peak for Co. Figure 3(C) shows that Co fluorescence at 520 nm decreases significantly in presence of PI (orange bars) compared to that measured in absence of PI (green bars). This result was particularly important, not only in further supporting the Co–PI FRET pair hypothesis, but it also explains the relatively dull/low intensity green signal observed in Figure 1(E). Finally, Figure 3(D) confirms that Co and PI form a FRET pair since there is a significant decrease observed in Co fluorescence at its own emission peak in presence of PI (orange bars) compared to that in absence of PI. Having clearly proven the hypothesis, formed on the basis of experiments with HeLa cells, that FRET takes place between Co and PI (with Co as the donor and PI as the acceptor), it was important to characterize the FRET pair. Therefore we calculated the Forster’s distance, between Co and PI for FRET to take place. Forster’s distance is the distance between the two
molecules, behaving as a FRET pair, at which the efficiency of transfer of energy from donor to acceptor is 50%. This can be found from the following relation\(^{16,17}\):

\[
E = 1 - \frac{F_{DA}}{F_D} = \frac{R_0^6}{R_0^6 + R^6}
\]

where, \(E\) is transfer efficiency of the FRET pair, \(F_D\) is the fluorescence of donor alone, \(F_{DA}\) is the fluorescence of the donor in presence of the acceptor, \(R_0\) is the Forster’s distance and \(R\) is the distance between the center of the two fluorophores. Required experimental values for the donor fluorescence, more relevant to this equation at the donor peak,\(^{16}\) were obtained from Figure 3(D). \(R\) is a function of concentration of molecules in solutions, assuming the molecules are homogeneously distributed in the solutions. By assuming spherical geometries for the Co and PI molecules in solutions for calculation of \(R\) corresponding to the used concentrations (see materials and methods) we calculated \(R\) and plotted the transfer efficiency (donor fluorescence values obtained from Fig. 3(D)) as a function of \(R\). Then, we used Eq. (1) to fit the data obtained. Figure 4(A) shows the data (open circles) and the fit to the data (smooth curve). Since all the values, except the Forster’s distance are experimental, it was a one parameter fit, giving a value of \(R_0 = 2.92\) nm. Thus, we show that Co–PI forms a FRET pair, with a Forster’s distance of \(\sim 3\) nm.

### 3.3. Sensitivity of PI for Detecting Nucleic Acids is Enhanced by FRET

Having characterized the Co–PI FRET pair, we were interested to test a simple, yet common possible application of our discovery. We had already observed a 30% increase in PI fluorescence in presence of Co inside HeLa cells (Fig. 2(C)), thus indicating an increase in PI sensitivity for nucleic acid detection. Therefore, we checked the sensitivity of PI in solutions by measuring its fluorescence with different amounts of \(\lambda\)-DNA, in presence and absence of Co. Figure 4(B) shows PI fluorescence using the excitation and emission wavelengths of PI. The slope of the line fitting the PI fluorescence in presence of Co was found to be 1.35 times higher than the slope of the line fitting the PI fluorescence alone. This indicates a 35% increase in sensitivity for detecting \(A\)-DNA in presence of Co, since a 35% steeper line indicates a more sensitive response. This result was remarkable since the increase in sensitivity in solution is clearly very close to our observations inside HeLa cells. Another remarkable result was that the slope of the line for PI fluorescence by using an excitation wavelength for Co and emission wavelength of PI was also higher (smooth line, Fig. 4(C)) than the only PI samples from Figure 4(B). This shows that even with Co excitation, FRET between Co and PI allows better sensitivity for measuring DNA by PI.

![Fig. 4.](image)

**Fig. 4.** (A) Forster’s equation fit (smooth curve) to experimental data (open circles, obtained from Fig. 3(D) to calculate transfer efficiencies) yields a Forster’s distance of 2.92 nm. X-axis values were calculated from known concentrations (see data analysis section of materials and methods). (B) PI fluorescence was measured in absence (red circles) and presence (orange squares) of Co with different concentrations of \(\lambda\)-DNA, using excitation/emission of 530/590 nm. Dashed line shows linear fit \((r^2 = 0.96)\) to fluorescence of only PI and DNA yielding \(y = 0.1434x + 65.201\). Solid line shows linear fit \((r^2 = 0.95)\) to the fluorescence of PI and DNA in presence of Co yielding \(y = 0.1932x + 69.871\). Ratio of slopes of the latter to former is 0.1932/0.1434 = 1.35. (C) Emission of samples in (B) measured at 590 nm with Co excitation wavelength (450 nm). Dashed line shows linear fit \((r^2 = 0.98)\) to fluorescence of only PI and DNA yielding \(y = 0.0674x + 34.104\). Solid line shows linear fit \((r^2 = 0.95)\) to fluorescence of PI and DNA in presence of Co yielding \(y = 0.1573x + 58.473\). All data is mean ± standard deviation for independent triplicates (i.e., \(n = 3\)).

### 4. DISCUSSION

Till date the methods utilizing FRET are the only possible way of monitoring molecular interactions taking place in living cells with a spatial resolution of 10 nm and smaller. While several chemical species behave as FRET pairs, their applicability to biological systems is either very limited, or is not existent primarily due to biocompatibility problems (e.g., cytotoxicity). Thus, continued research efforts are invested in developing new FRET pairs with biological applications. In this work, we report FRET behavior of two very important, and commonly used, molecules in biological research. The most surprising aspects of our discovery are that neither the experimental...
system that led us to develop the hypothesis that Co and PI form a FRET pair, nor the molecules themselves (i.e., Co and PI), are new. It is also imperative for us to point out that the two phenotypes of HeLa cells incubated with coumarin loaded biodegradable nanoparticles that we observed in our experiments repeatedly, and that led to the initial formulation of the Co–PI FRET pair hypothesis, have not been repeatable with further passages and even fresh culturing of HeLa cells. Nevertheless, reproducible image data from a phase of experiments led to the formulation of the hypothesis that Co and PI behave as a FRET pair, independent of the phenotypes. Thus, it is interesting to note that a rare phenomenon (in terms of phenotypes) observed with living cells allowed subsequent successful characterization of the Co–PI FRET pair in solutions, with a Forster’s distance of ~3 nm. We are certain that the discovery of this FRET pair will open up several new avenues not only in drug delivery research but also find an invaluable role in basic research cell biology. A key application of our work could allow simultaneous monitoring of uptake and biodegradation of nanoformulations containing drugs targeted at nucleic acids using in vitro cell based experimental systems. This is particularly strengthened by our results that show a 30–35% increase in sensitivity for detection of nucleic acids by PI in presence of Co, both inside cells and in solutions.

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References and Notes

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