Single Molecule Microscopy Is Useful Technique For DNA Hairpin FRET Systems.

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Abstract

Nanometre sized semiconductor quantum dots as fluorescent materials are now becoming as ubiquitous as the organic dyes that preceded them. By virtue of their tuneable optical properties, narrow emission profiles and photostability, QDs have proved themselves as resilient and multifarious in both their applications and their underlying photophysics compared to their organic dye counterparts. Single Molecule Microscopy techniques (SM) are especially relevant to macromolecular biochemistry as chemical processes occurring within large molecules that are often too complicated to study in the ensemble. Using both total internal reflection (TIR) and Confocal Microscopy (CM) and both image processing and photon counting to achieve understanding of the binding kinetics and dynamic properties exhibited by the molecule via FRET studies. Several versions of the system have been examined to optimize the FRET pairing of donor/acceptor tags, achieve quantitative and ratiometric bioconjugation to nanocrystals and perfect techniques of microscopy and image processing.

Keywords: Total Internal Reflection, DNA Haipin, FRET, Confocal Microscopy and QDs.

Introduction

SM optical microscopy utilises fluorescent species. Single molecule fluorescence microscopy (**SMFM**) measures the radiative emission of a fluorophore relaxing from an excited state to its ground state. In SMFM, this is achieved using coherent laser light to excite the fluorophore to a singlet excited state, from which it can relax rapidly. However this can be followed by either absorption of additional photons, eventually lead to an ionised or "photo-bleached" state, or spin-inversion and non-radiative intersystem crossing to a triplet state. Relaxation from this triplet state (phosphorescence) is spin-forbidden and therefore occurs much more slowly than fluorescence.

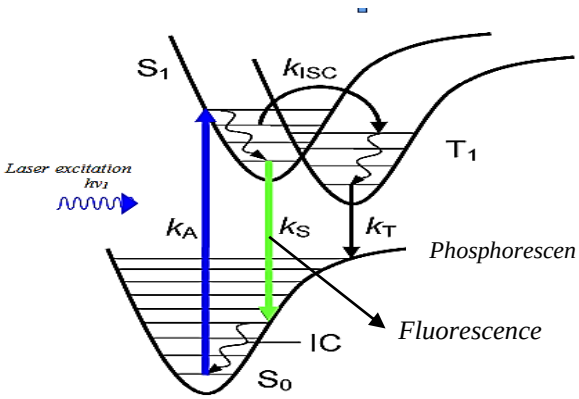

Fig.(1): shows cycle of a fluorophore. $h\nu_1$ is absorbed, causing an electron to be promoted to an excited state. Decay from excited state occurs either by direct emission (fluorescence) or intersystem crossing followed by phosphorescent decay. Both routes produce emitted radiation that is Stokes shifted from the laser excitation wavelength due to rapid internal conversion. Ref [1].

The cycle of excitation/emission of a fluorophore can be seen in Fig.(1) total internal reflection fluorescence microscopy (TIRFM) is highly suited to molecules that can be immobilised on a substrate, as it confines the illuminated volume along the z axis; allowing a wide field to be studied (the TIR footprint) while still reducing the excitation

volume to picolitres. This is achieved by an artefact of total internal reflection- the evanescent wave Fig.(2). The evanescent wave generated by the TIR beam at the glass coverslip/ sample interface was exploited to reduce the excitation volume. This wave penetrates the medium with an exponential decay in field strength from the interface with an approximate penetration depth of ~120 nm in intensity with distance from the interface:

$$I(z) = I(0)e^{-\frac{z}{d}} \quad \text{.....Eq 1}$$

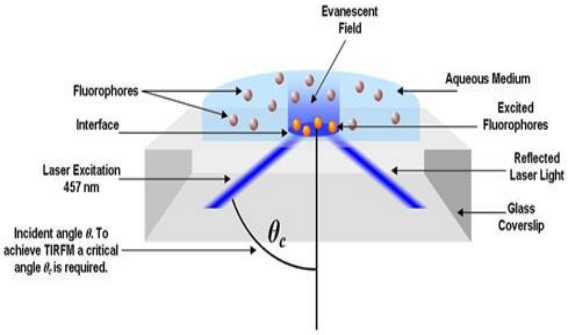

Fig.(2): Schematic representation of system undergoing total internal reflection. Incident radiation approaches interface between glass coverslip and aqueous sample containing fluorophores at critical angle and is totally reflected, propagating an evanescent wave. A wide field of illumination is achieved in a thin optical slice, exciting fluorophores contained within a picolitre excitation volume.

Where *I(0)* is the intensity of the light at the interface and the penetration depth, *d*, is defined as the distance into the second medium, n_2, normal to the interface at which the light intensity of the evanescent wave has decreased to *1/e* of its interfacial value. The Parameter *d* is related to the wavelength of the excitation radiation, λ, and both n_1 and n_2, according to equation 2. *d* is defined as the distance into the sample that *E(z)* has reduced to *(1/e)E(0)* and is related by the following reaction to:

$$d = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2\theta_i - n_2^2}} \quad \text{.....Eq 2}$$

Where: λ= the wavelength of the incident illumination, θ = the incident angle, *n(1) and n(2)* = the refractive indices of the media at the interface (in the case of Fig (2), the coverslip is defined as having refractive index n(1) and the sample has refractive index n(2)).

Requirements for TIR

Light approaches the interface at incident angle θ. At and above a critical angle θ_c (dependent on the media either side of the interface) the incident beam is reflected by the interface, back into the first medium. For total internal reflection to occur and an evanescent wave propagated, there must be a reduction in refractive index (*n*) across the interface between slide and sample (n_2 to n_1). The relationship between refractive indices and critical angle is described by Snell's Law:

$$\theta_c = sin^{-1}(n_1/n_2) \quad \text{.....Eq 3}$$

For the example, where n_2 = glass n_1 = water, the critical angle can be calculated as 60.1°.

To produce quality data from TIR microscopy the incident beam must be clean (having a Gaussian distribution with no high-frequency noise), circularly polarised (due to the dependence of photon absorption on correct dipole orientation, in TIRM molecules are not free to rotate and therefore show a distribution of orientations) and collimated (producing a beam of consistent width).

Quantum Dots as FRET Donors

Fluorescence Resonance Energy Transfer (FRET) is a method often utilized infolding and dynamic studies of proteins and nucleic material. Two fluorescent species are required with an overlap in the area under the curve of the emission of one species, the donor, with the absorbance of another, the acceptor Fig.(3).

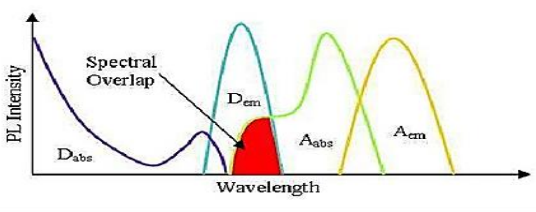

Fig.(3): Schematic representation of absorption and emission spectra for donor QD and organic dye acceptor, showing spectral overlap between emission of donor and absorption of acceptor necessary for FRET [3].

When the donor species is excited, it transfers energy non-radiatively to the acceptor species within 10- 100 nm via a dipole interaction, exciting the acceptor. The fluorescence intensity of both species can be measured to calculate the FRET efficiency E. This can be related to the distance between the two fluorophores by:

$$E = R_0^6 / R_0^6 + r^6 \quad \text{.....Eq 4}$$

Where r is the distance between fluorophores and R_0 is the Förster distance, related to the spectral overlap integral between species by:

$$k_T(r) = 1 / \tau_D (R_0 / r)^6 \quad \text{.....Eq 5}$$

Where $k_T(r)$ is the rate of energy transfer and τ_D is the lifetime of the donor in the absence of energy transfer. To achieve accurate calculations of r, good spectral separation between the donor and acceptor excitation wavelengths is critical. Cadmium Selenide semiconductor Quantum Dots (nanocrystals, crystallites, QDs) have been studied extensively since the 1980s. Quantum Dots are crystals of semiconductormaterial within the nanometre regime.[2] They display characteristics of quantum confinement, meaning that their physical diameter lies below the Bohr exciton radius of the bulk material. [3] This leads to tunable emission, with the diameter prescribing the emission wavelength. QDs have broad excitation/ narrow emission profiles, high quantum yields, are more photostable than organic dyes and can easily adsorb onto the surface of a glass slide, making them ideal for TIR single molecule microscopy. CdSe QDs with their broad absorption spectra and narrow, focused emission spectra are ideal donor species; highly tunable for a specific acceptor molecule. Quantum dots have been conjugated to biological molecules such as single strand DNA hairpins to achieve single molecule studies of binding kinetics [4-6] with some success. This project intends to lay the foundations of biological conjugation, bulk and SM microscopy techniques with the aim of performing accurate kinetic and dynamic measurements on biological macromolecules.

Experimental Work

Synthesis of Quantum Dots

Quantum dots can be synthesised as colloidal solutions and by self-assembly on solid substrates.15 Early syntheses of QDs utilised salts as precursor materials but later methods using first organometallic precursors, then safer metal oxides produced narrower emission bands and more reliable results.[3] Coordinating and non coordinating solvents have been utilised, with varying results. [4-5] A shell of lattice- matched semiconductor material is often used to increase quantum yield and photostability of QDs. CdSe QDs are often coated withZnS.

TIR microscope FRET

Optical equipment used for this purpose is illustrated below Fig.(4). Once the laser beam (of appropriate wavelength to excite the fluorophore under study) has passed through the optical track it enters the microscope Fig.(4) shows a cutaway schematic of objective-type TIR microscopy. The incident laser beam illuminates the rear focal plane of the high numerical-aperture (NA–the cone angle within which a lens can collect light) oil immersion objective lens; is focussed and directed off-axis to the front focal plane of the objective and is then refracted at an angle that equals or exceeds the calculated critical angle onto the interface between slide and sample. Fluorescence is collected along the axis of the objective lens and is separated from the excitation beam and any laser scatter by a long-pass dichroic mirror before being reflected off a mirror and collected by a CCD (charge-coupled display) camera connected to a computer. Long-pass (LP), short-pass (SP) and band-pass (BP) filters can also be included to improve signal to noise ratio before emitted light reaches the camera.

A Coherent laser light is produced by a Beamlok 2080 Argon/Krypton gas phase laser at power of less than 40 mW Fig.(4). On leaving the laser, the beam is plane polarised and can feature unwanted high frequency noise, undesirable features for SM microscopy. Fluorophore absorption of photons is dependent on dipole orientation, meaning that only a small proportion of molecules correctly aligned would be excited

by plane polarised light. To obtain high quality results, a degree of beam attenuation is required before the microscope. After leaving the laser the beam is first redirected by reflection from a kinematically mounted mirror. Kinematic mounting is advantageous as it allows fine adjustment to the direction of the beam/position of the footprint.

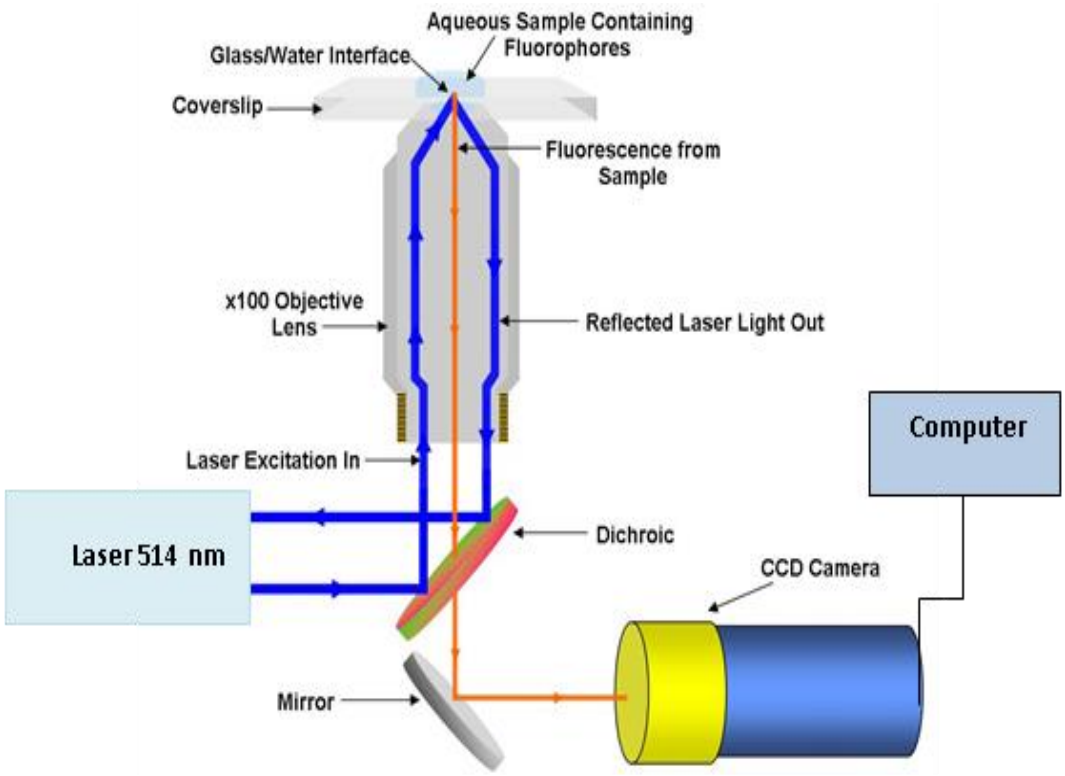

Fig.(4): Schematic representation of a cutaway schematic of objective-type TIR microscopy. The incident laser beam illuminates the rear focal plane of the high numerical-aperture (NA).

Bulk Measurements on DNA hairpin FRET system as a precursor to single molecule measurements.

Ensemble measurements were conducted on an 18-mer *ss*-DNA (single strand DNA) hairpin of structure **5' CCC-AAA-AAA-AAA-AAT-GGG 3'** labelled with a single Cy5 molecule in the 5' position and conjugated to a molecule of biotin at the 3' end Fig.(5). Biotin was included due to its strong binding to streptavidin. The streptavidin was decorated with Alexafluor® providing a FRET pair with Alexafluor® donating to Cy5. The system showed a weak acceptor signal. The lack of strong signal may be due to old materials or inappropriate distance between the Alexafluor® and Cy5 FRET pair as the optimum separation range for FRET measurements is 10-100 nm. Differently labelled versions of the same hairpin were sourced to try to boost the signal. Investigations were also started into a shorter link from the hairpin to the QD.

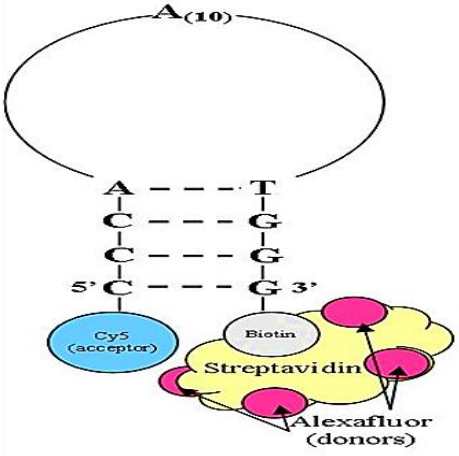

Fig.(5): Initial FRET pair including organic Cy5 dye and Quantum dot (QD).

Fig.(6) shows a commercially sourced replacement for the system. The link via biological molecules has been replaced with organic TAMRA dye. This system shows stronger FRET than the system in Fig.(5).

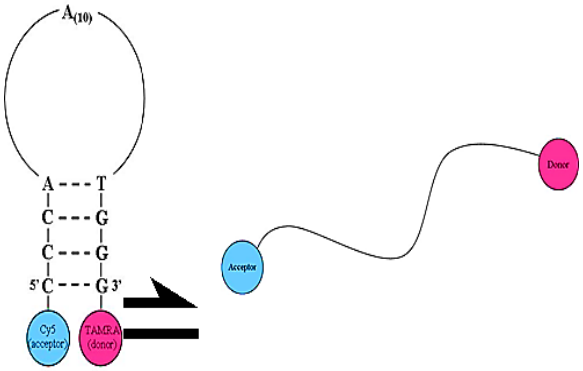

Fig.(6): 18-mer DNA hairpin dual-labelled with donor TAMRA and acceptor Cy5 shown reversibly melting.

Results and Discussion

CdSe Quantum Dots

Cadmium Selenide semiconductor Quantum Dots (nanocrystals, crystallites, QDs) have been studied extensively since the 1980s. Quantum Dots are crystals of semiconductor material within the nanometre regime.[5] They display characteristics of quantum confinement, meaning that their physical diameter lies below the Bohr exciton radius of the bulk material.[6] This leads to tunable emission, with the diameter prescribing the emission wavelength. Fig.(7) shows photographs and PL spectra of CdSe QDs synthesised in-house. QDs have broad excitation/narrow emission profiles, high quantum yields, are more photostable than organic dyes and can easily adsorb onto the surface of a glass slide, making them ideal for TIR single molecule microscopy.

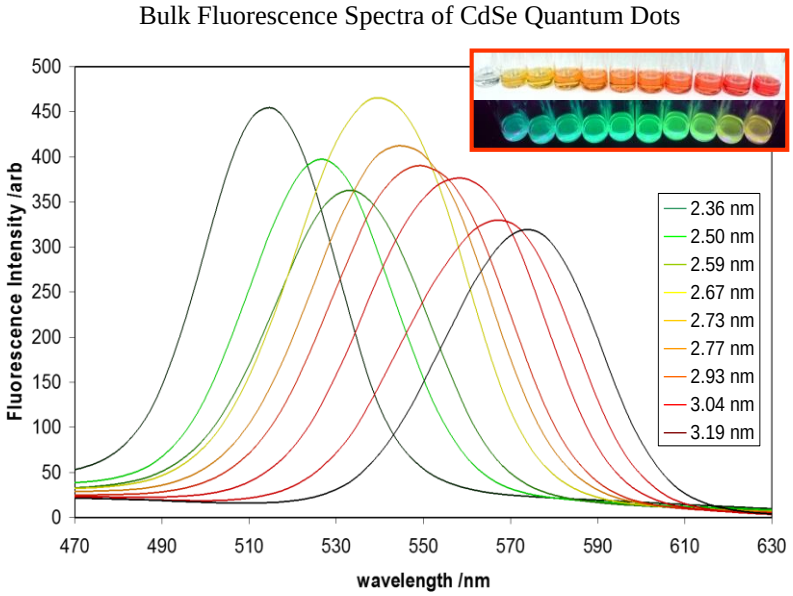

Fig.(7): PL spectra of colloidal solutions of CdSe QDs dispersed in octadecene (ODE) photographed in ambient light (top) and the same sample under broad UV light (bottom). Redshift with increasing diameter can be clearly seen.

Bulk Measurements on DNA hairpin FRET system

Bulk spectroscopic measurements were performed on this hairpin system to measure FRET intensity ratios during binding/ unbinding of the hairpin. The hairpin was forced into a denatured (unbound) state via 2 methods; addition of the complementary DNA strand and heating. Both methods produced rough results that indicate the system could be

successfully studied more closely at the single molecule level. Fig.(8) shows the absorption spectra of the dual labelled hairpin and the complementary strand. Fig.(9) shows absorption and fluorescence spectra for the same system and Fig.(10) shows bulk PL spectra obtained over a range of temperatures. The changing FRET ratio shows the changing distance between the dye labels, indicating binding/unbinding of the hairpin.

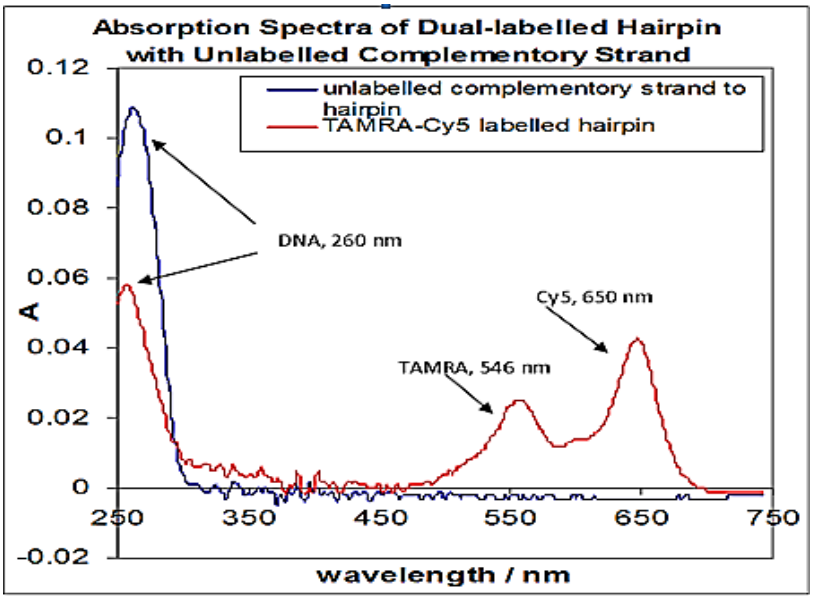

Fig.(8): Absorption spectra of dual labelled (TAMRA Cy5) DNA. Unlabelled complementary strand is included for comparison. Complementary strand shows peak at 260 nm consistent with DNA but does not show absorbance peaks typical of the fluorescence tags.

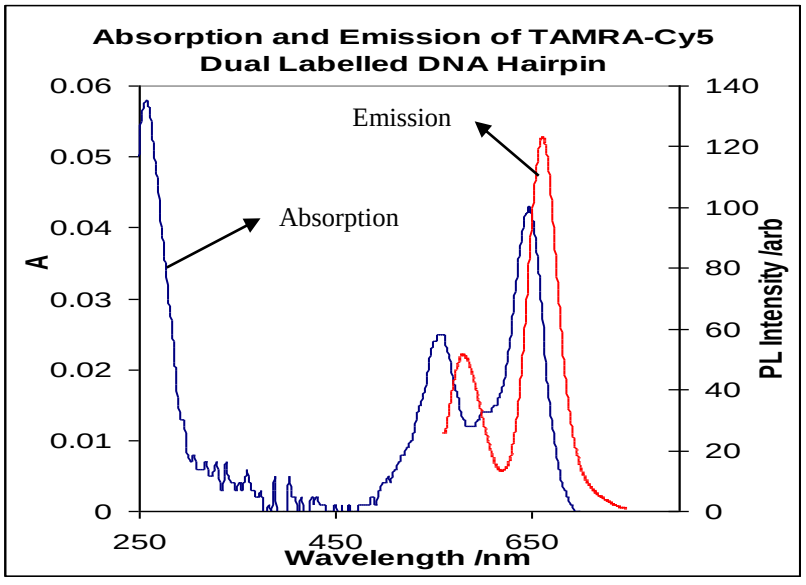

Fig.(9): Shows Absorption and emission spectra of TAMRA-Cy5 labelled hairpin. Overlap between TAMRA emission and Cy5 absorption is not precisely matched but the system allows for good separation of both emission profiles, making it highly suitable for future single molecule studies.

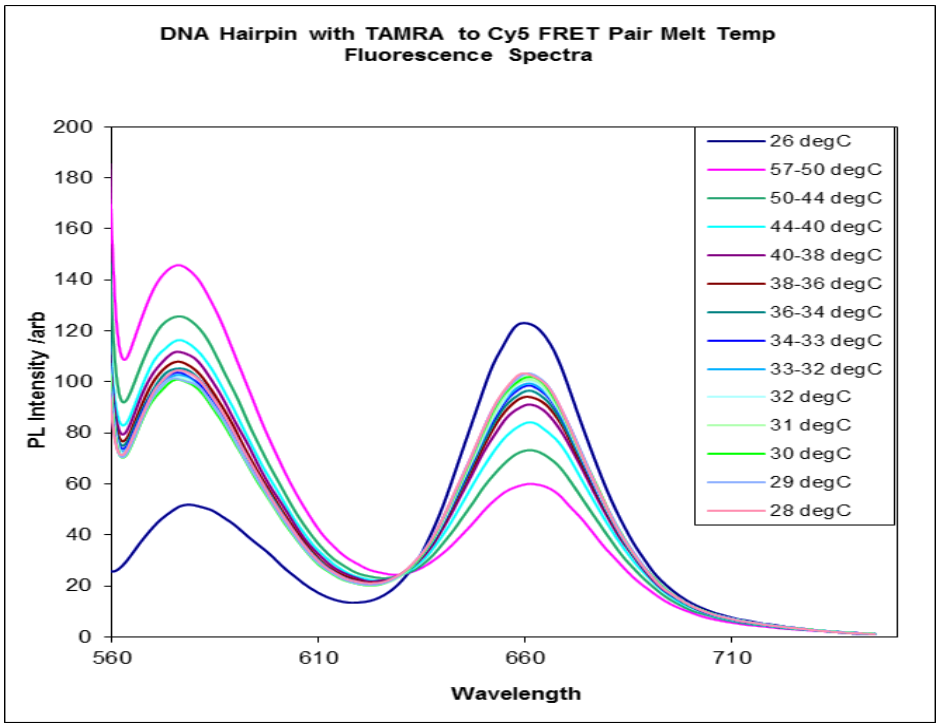

Fig.(10): PL spectra obtained from hairpin system with changing temperature. The change in FRET intensity ratio shows change in distance between the FRET dye pair.

In order to test the suitability of quantum dots as a FRET donor with the hairpin under study a system is required that places the QD within a known suitable distance of the acceptor dye molecule. Conjugation via biological molecules may constitute a distance greater than the 10-100 nm regime that FRET may be observed within. Combining the promising results from our facile method of aqueous transfer with the DNA hairpin under study may yield better results. To this end, a DNA hairpin with a Cy5 label at the 5' end coupled with a thiol at the 3' end may result in a molecule that can bind directly to the surface of the dot Fig.(11). Before acquiring a new sample of DNA a test is proposed to determine the feasibility of conjugating a dye molecule to the surface of a QD. To this end a dye is required with a thiol functional group. This is not commercially available but an alternative is a dye with a hydrazide functional group that can be reacted with a carboxylic acid such as the one available on mercaptopropanoic acid. This would lead to a thiolated dye that theoretically can be conjugated to a QD via the method described above. Fig.(14) shows the structure of the Alexafluor® hydrazide dye chosen, as well as a schematic representation of the conjugated dye-QD system.

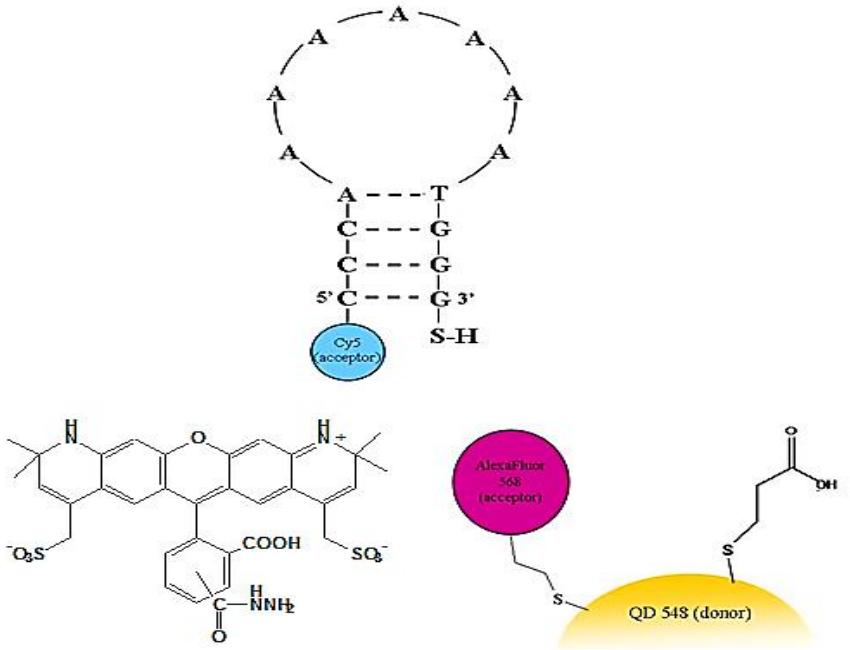

Fig.(11): Proposed DNA hairpin with thiol terminal group for direct conjugation to QD.

Conclusion

Nanometre sized semiconductor quantum dots as fluorescent materials are now becoming as ubiquitous as the organic dyes that preceded them. By virtue of their tuneable optical properties, narrow emission profiles and photostability, QDs have proved themselves as resilient and multifarious in both their applications and their underlying photophysics compared to their organic dye counterparts. Using both TIR and confocal microscopy and both image processing and photon counting to achieve understanding of the binding kinetics and dynamic properties exhibited by the molecule via FRET studies. Several versions of the system have been/will be examined to optimise the FRET pairing of donor/acceptor tags, achieve quantitative and ratiometric bioconjugation to nanocrystals and perfect techniques of microscopy and image processing. Bulk spectroscopic measurements were performed on this hairpin system to measure FRET intensity ratios during binding/ unbinding of the hairpin. The changing FRET ratio shows the changing distance between the dye labels, indicating binding/ unbinding of the hairpin. In order to test the suitability of quantum dots as a FRET donor with the hairpin under study a system is required that places the QD within a known suitable distance of the acceptor dye molecule. Conjugation via biological molecules may constitute a distance greater than the 10-100 nm regimes that FRET may be observed within. Combining the promising results from our facile method of aqueous transfer with the DNA hairpin under study may yield better results.

References

[1] Axelrod, D., Burghardt, T.P., Thompson, N.L., "Total Internal Reflection Fluorescence", *Annu. Rev. Biophys. Bioengin*, 13, 247-268, 1984.

[2] Axelrod, D., "Total Internal Reflection Fluorescence Microscopy in Cell Biology", Traffic, 2, pp.764-774, 2001.

[3] Merrifield, C.J., Moss, S.E., Ballestrem, C., Imhof, B.A., Giese, G., Wunderlich, I., Almers, W., "Endocytic Vesicles Move At The Tips Of Actin Tails In Cultured Mast Cells". Nature Cell Biology, 1, pp.72-74, 1999.

[4] Meyer, T., Teruel, M.N., "Fluorescence Imaging of Signaling Networks", TRENDS in Cell Biology, 13(2), pp.101-106, 2003.

[5] Schmoranzer, J., Goulian, M., Axelrod, D., Simon, S.M., "Imaging Constitutive Exocytosis with Total Internal Reflection", Journal of Cell Biology, 149(91), pp.23, 2000.

[6] Radivoyevitch T, Loparo KA, Jackson RC, Sedwick WD: "On Systems and Control Approaches to Therapeutic Gain". *BMC Cancer*, 6(1):104, 2006.
[7] Manegold C, "Gemcitabine (Gemzar) In Non-Small Cell Lung Cancer". *Expert Rev Anticancer Ther*, 4(3):345-360, 2004.
[8] Kroep JR, Van Moorsel CJ, Veerman G, Voorn DA, Schultz RM, Worzalla JF, Tanzer LR, Merriman RL, Pinedo HM, Peters GJ: "Role of Deoxycytidine Kinase (Dck), Thymidine Kinase 2 (TK2), and Deoxycytidine Deaminase (Dcda) in the Antitumor Activity Of Gemcitabine (Dfdc)". AdvExpMedBiol, 431:657-660, 1998.
[9]Ting Qiu, Dan Zhao, Guohua Zhou, Yuan Liang, Zhike He, Zhihong Liu, Xiaoniu Peng and Li Zhou, "A positively charged QDs-based FRET probe for micrococcal nuclease detection", *Analyst*, 135, 2394-2399, 2010.
[10] LI Xin, XUE Bing, LI Yang & GU Yue Qing, "Protease-activated quantum dot probes based on fluorescence resonance energy transfer", Nano-Biomedical Optoelectronic Materials and Devices, Vol.58 No.21: 2657–2662, 2013.
[11] Lin Qiu, Yanhua Bi, Cheli Wang, Jingyan Li, Peilin Guo, Jinchen Li, Weijiang He, Jianhao Wang,* and Pengju Jiang, "Protein A Detection Based on Quantum Dots-Antibody Bioprobe Using Fluorescence Coupled Capillary Electrophoresis", Int. J. Mol. Sci., 15, 1804-1811, 2014.

الخلاصة

تقنيات الجزي الواحد المجهرية هي تقنيات ذات صلة بالكيمياء الحيوية المتعلقة بالجزئيات الماكروية كعمليات لكيميائية التي تحدث داخل الجزيئات الكبيرة والتي تعتبر عمليات معقدة. باستخدام تقنيات الانعكاس الكلي الداخلي (TIR) ومتحد البؤر المجهري (CM) و معالجة الصور و فوتون العد للتحقيق لفهم طاقة الربط والخصائص الديناميكية ملزمة للجزيء خلال تقنية انتقال الطاقة الرنيني. عدة انواع من لانظمة تم اختبارها لتحديد الامثل لزوج المانح والمكتسب في تقنية انتقال الطاقة الرنيني, انجاز تناسبات معيارية نوعية لتقنيات مجهرية ومعالجة صورية مثلى للربط البايولوجي الامثل لزوج المانح والمكتسب في تقنية انتقال الطاقة الرنيني والتركيب البلوري النانوي.