

### Novel, Targettable Bioimaging Probes Using Conjugates of Quantum Dots and DNA

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### THESE DE DOCTORAT

#### **DE L'UNIVERSITE PIERRE ET MARIE CURIE**

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> Présentée par Anusuya BANERJEE

Pour obtenir le grade de Docteur de l'Université Pierre et Marie Curie

# Novel, Targettable Bioimaging Probes Using Conjugates of Quantum Dots and DNA

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### Introduction

In this growing era of development of interdisciplinary approaches to understand fundamental biological processes, semiconductor nanoparticles such as Quantum Dots (QDs) have emerged as novel fluorescent probes with infinite potential. The photophysical properties of QDs such as broad absorption, narrow emission spectrum, reduced blinking and enhanced photostability make them advantageous over organic fluorophores for several bioimaging applications. Additionally, high surface-to-volume ratio along with customizable and multiple reactive ligands on the surface make these nanoparticles ideal candidates for biosensing and delivery. However, using QDs for biological applications is a multistep process. For all biological applications, QDs need to be transferred from organic solvents to aqueous buffers. This is carried out by systematic over-coating or displacement of ligands on the surface of QDs by more amphiphilic ligands. The QDs thus obtained are readily dispersed in water. The next step involves conjugation of specific biomolecules such as therapeutic ligands, nucleic acids (DNA or RNA) and proteins. Bioconjugation of specific ligands provide QDs with a biological identity, which then helps in specific targeting, tracking, sensing or delivery related applications. Bioconjugates of QDs truly reflect the immense potential of a syntheticbiological scaffold for customizable applications.

Despite considerable progress in development of new surface chemistries and conjugation strategies for QDs, there are several issues that limit their widespread applicability. Low yield of specific bioconjugates, reduced quantum yield and stability and increase in nanoparticle size are few of such limitations. Therefore, there is continued interest to develop novel conjugation strategies to consummate several of these issues. One of the specialties of the LPEM group is in development of highly robust surface chemistries that not only enable QDs to be dispersed in water but also provide additional scope for bioconjugation. Given the opportunity to work in such a dynamic group in my PhD under the joint supervision of Dr. Benoit Dubertret (ESPCI, ParisTech) and Prof. Yamuna Krishnan (University of Chicago), I have worked towards the development of QDs for specific applications in biology. In this manuscript, I report my original findings along with a comprehensive overview of the existing

literature. I also discuss extensively the methods employed to obtain these results and how they may be further optimized, wherever necessary.

The FIRST CHAPTER starts with an overview of QDs as the next generation of fluorescent probes for biology. After this, I discuss a pedagogical overview of different state of the art ligands used to transfer QDs from organic media to water. This is followed by a discussion of highly successful strategies to conjugate DNA to QDs discussing their advantages and limitations in a case to case basis. I also include a progressive description of the different methods of purification and range of applications where such QD-DNA conjugates can be used. The goal of this chapter is to provide the readers a perspective of the plenary developments in the field.

The SECOND CHAPTER describes a novel method of coupling DNA to QDs to synthesize QD-DNA conjugates. Starting with an overview of already existing strategies, I describe systematic experiments carried out to carefully evaluate this strategy and optimize the reactions for high yield and photophysical stability of these conjugates. I will also discuss how this strategy has been successfully adapted to nanoparticles other than QDs and to other biomolecules like proteins, making this a robust and highly generalizable method of conjugation.

The THIRD CHAPTER describes utilization of these QD-DNA conjugates or bioimaging of specific intracellular pathway. Herein, I will also discuss how ligand design and conjugation chemistry can enable the synthesis of QD-DNA-protein conjugates with novel properties. These conjugates could be additionally used to visualize specific receptor dynamics for over 20 minutes, overcoming several issues of previously employed methods from literature.

The FOURTH CHAPTER provides a comprehensive examination of QD based probes with variable surface chemistries for applications in biology. Using a library of versatile ligands developed in the lab, I examine the bulk behavior of QDs in complex media both *in-vitro* and *in-vivo*. This chapter aims to compare the ligands to enable better designing of QD based probes for biology.

The FIFTH CHAPTER discusses in detail the instrumentation, materials, methods and analysis strategies employed to carry out these experiments.

# List of abbreviations

Ab	Antibodies
Acn	Acetonitrile
ALBR	Anionic Ligands Binding Receptor
APS	Ammonium Per Sulfate
BME	ß-Mercaptoethanol
Вр	Base Pairs
Bt	Biotin
Cy3/5	Cyanine-3 or Cyanine 5
Dh	Hydrodynamic diameter
DHLA	Dihydrolipoic Acid
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTMam	5-(1,2-dithiolan-3-yl)-N-(3-methacrylamidopropyl)pentanamide
DTT	Dithiothretol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
Em	Emission
EtOH	Ethanol
Ex	Excitation
Ext Coeff	Extinction Coefficient
FA	Fluorescamine
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FWHM	Full Width of Half Maxima
GNP	Gold Nanoparticles
HPLC	High-Performance Liquid Chromatography
IgG	Immunoglobulin G
kDa	Kilo Dalton
LAMP-1	Lysosomal Associated Membrane Protein-1
LE	Late Endosome

MAPEG	Poly(ethylene glycol) methyl ether methacrylate
MPA	3-Mercaptopropionic Acid
NA	Numerical Aperture
NHS	N-hydroxysuccinimide
NP	Nanoparticle
NPL	Nanoplatelets
PEG	Poly(Ethylene Glycol)
Pen-Strep	Penicillin-Streptomycin
polyHis	Poly-Histidine
QD	Quantum Dots
QY	Quantum Yield
RE	Recycling Endosome
RPM	Rotations Per Minute
SAB	Streptavidin Agarose Beads
SDS	Sodium Dodecyl Sulfate
SE	Sorting Endosome
SEC	Size Exclusion Chromatography
sSMCC	sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
sSPDP	sulfosuccinimidyl 3-(2-pyridyldithio)propionate
SPP	3-sulfopropyldimethyl-3-methacrylamidopropylammonium
<i>t</i> BuOK	tert-butoxide
TEM	Transmission Electron Microscope/y
Tf	Transferrin
TRITC	Tetramethyl Rhodamine Isothiocyanate
Zw	Zwitterion

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# **Chapitre 1 Quantum Dot-DNA Conjugates – An Overview**

Quantum dots (QDs) are semiconductor nanomaterials with unique material and optical properties that lend in tremendous potential for applications in biology.<sup>1-4</sup> Properties such as size tunable emission, high fluorescent quantum yield and resistance to photobleaching make QDs highly desirable candidates for several biosensing and tracking applications. Additionally, high surface to volume ratio, multiple chemically reactive ligands on the surface and high biomolecule loading capacity have augmented the use of QDs in targeted delivery and therapeutics. For most of these specific applications, QDs are first conjugated with biomolecules such as nucleic acids, proteins or signaling molecules. Last couple of decades has seen a lot of development in the routes of functionalization of QDs with these biomolecules. In this chapter, a brief introduction to QDs as strong bioimaging probes is provided. The discussion is further continued to systematic evolution of QDs from synthesis to transition into aqueous media, subsequent bioconjugation and eventual applications. The primary focus of this chapter is to highlight different approaches of bio-functionalization of QDs with nucleic acids and applications of these conjugates. Quantum dot-DNA conjugates bring together the material and biological properties of both QD and DNA. As discussed in subsequent sections, these conjugates can be tailored for multiple physical, chemical and biological applications.

In this thesis, focus is levied upon biological applications of QD with particular focus on conjugates of QD and DNA. Addition of a biomolecular layer of DNA further increases the scope of applicability of QDs for very specific biological applications. In this chapter, common physico-chemical properties of QD are discussed, along with detailed discussion of transition of QDs from organic to biological media, strategies of conjugation of DNA and some applications of thus generated conjugates.



**Figure 1-1** Comparison of the size of commonly used semiconductor nanoparticles with respect to fluorophore (FITC) and commonly used proteins. *Reproduced with permission from Michalet et al. Science 307, 538 (2005); DOI: 10.1126/science.1104274* [3].

### 1.1 Quantum Dots as fluorescent probes

In this section several properties of QDs are discussed, particularly with focus to the ones useful in biology.

### 1.1.1 Advantages of QDs over organic fluorophores

Quantum Dots are new age fluorescent probes with several advantages over commonly employed organic fluorophores, with typical sizes comparable to commonly used proteins (**Figure 1-1** and **Figure 1-2**). Compared to organic fluorophores, QDs are substantially larger and have higher electron density. These two advantages make QDs ideal probes for correlative fluorescence and electron microscopy. Additionally, in comparison to organic fluorophores, QDs have broad absorption with narrow and symmetrical emission peaks. This enables simultaneous excitation of two (or more) QD based probes and sharper non-overlapping emissions. Using QD conjugated to biomolecular markers, up to five different components within single cell can be simultaneously imaged. The QDs additionally have delayed emission decay in comparison to fluorophores. This property can also be used for designing and imaging probes that can be visualized using Fluorescent Lifetime IMaging (FLIM). A very interesting work by Ruedas Rama *et al.* show that the fluorescence life time of QDs was sensitive to concentrations of analytes such as chloride and hence can be used as a nanosensor.<sup>5</sup> These studies show that QDs can be visualized using several types of imaging approaches and high great scope in biology.

The other highlight of QDs over fluorophores is their remarkable photostability. In specific comparative *in-vitro* and *in-cellulo* experiments, QDs have been imaged for several minutes wherein other fluorophores have been shown to bleach within this time (**Figure 1-3**). Due to substantially enhanced photostability in comparison to fluorophores, these nanobioconjugates

could also be used to carry out single molecule studies such as vesicle dynamics.<sup>6,7</sup> Another limitation of fluorophores is the photo-blinking *i.e* random fluctuation in fluorescence intensity that causes transient (or permanent) loss of signal. QDs have substantially reduced photo-blinking than organic dyes. Along with others, recent works from our lab has demonstrated the synthesis and spectroscopy of highly photo-stable QDs with non-blinking emission and 100% QY at room temperature.<sup>89</sup> Thus optical and photophysical properties of QDs position these nanocrystals as very strong alternatives for diverse applications in biology.

#### **1.1.2 Electronic properties**

As mentioned before, QDs are semiconductor colloidal nanocrystals of 5-10 nm diameter. A semiconducting element has properties between those of a conducting and an insulating element. These properties are defined by the electronic structures. In an atom, the electrons possess discrete energies. A valence band (VB) is defined as a state of energy where the electrons of the last orbital of any element reside. Electrons in the VB can absorb quantized energy and get localized in the higher energy conducting band (CB), leaving a hole or a unit of positive charge in the valence band. This defined energy difference between the VB and the CB is a characteristic property of every element, and called the band gap. As expected, insulating materials have a higher band gap regarding the conducting materials. Semiconducting materials have intermediate band gap between the conducting and the insulating materials (Scheme 1-1). These properties can be used to also describe the electronic structure of a nanocrystal comprising of 100-10000 atoms in a confined volume. The difference between the electronic structure of a bulk material (single atoms) and a nanocrystal (several hundreds of atoms) is the additional quantization of energy levels within the CB and the VB, wherein an electron-hole pair can delocalize. Also, the energy corresponding to the band gap of bulk element and the nanocrystal is slightly different. In a 3D confined nanocrystal such as QD, this band gap difference can also be synthetically engineered by altering the packing of atoms (crystallinity) and/or introducing minor amount of atoms of different elements (doping).



**Figure 1-2** Properties of QDs used for different types of imaging. (A) Size tunable fluorescent emission. (B) Simultaneous multicolor imaging of intracellular targets using QDs emitting at different wavelength. (C) Imaging of QDs complexed by GroEl protein using transmission electron microscopy. (D) Difference in the emission decay of QDs compared to organic dye. (E) Use of lifetime of QDs for sensing environmental chloride concentrations *in-vitro* (left) and corresponding lifetime distribution (right). *(Reproduced with permission from Quantum Dot Corp* (**A**,**B**); *from Ishii et al. Nature 243,628-32 (2003); DOI: 10.1038/nature01665.1.*<sup>10</sup> (**C**); *from Gao et al., Current Opinion in Biotechnology (2005), 16:63–72 DOI:10.1016/j.copbio.2004.11.003)*<sup>11</sup> (**D**); *and from Ruedas-Rama et al., Analyst, (2012), 137, 1500–1508 ; DOI: 10.1039/c2an15851e)*<sup>5</sup> (**E**)



**Figure 1-3** Photophysical properties of QDs. (A) Comparison of time-dependent photobleaching of intracellular fluorescent probes. Nuclear antigens were labelled with QD 630–streptavidin (red), and microtubules with AlexaFluor 488 (green) (top). Microtubules were labelled with QD 630– streptavidin (red), and nuclear antigens were stained green with Alexa 488 (bottom). Continuous exposure times in seconds are indicated (B) Comparison of changes in fluorescence intensities of intracellular targets labelled with QD 608–streptavidin and Alexa 488–streptavidin. (C) The cells were observed by differential interference contrast (DIC) (C, top left) and epifluorescence (C, top right) showing single QD on the cell membrane. (C, bottom left) trajectory of diffusion of the QD localized in the indicated region. (C, bottom right) The fluctuations in corresponding QD intensity show successive blinking events characteristic of a single nanocrystal. *(Reproduced with permission from* Wu, Xingyong *et al.* Nature Biotechnology *21*, *1* (*2003*); *DOI: 10.1038/nbt764*<sup>12</sup> (A and B), from *Michalet et al. Science 307*, *538* (*2005*); *DOI: 10.1126/science.1104274*)<sup>3</sup>(C)

Due to the size of QDs in nanometer regime, quantum confinement effects govern the electronic properties of QDs Quantum confinement effect explains that as the nanoparticle size increases, the band gap decreases by the factor of  $1/a^2$ , where a is the radius of Bohr's exciton. Thus the fluorescence emission shifts towards higher wavelengths (Scheme 1-2). This relates the electronic properties of QD to the size of the nanoparticle.



Scheme 1-1 Band gap arrangements of materials showing the difference between insulators, semi-conductors and conductors.



Scheme 1-2 Size-dependent change in band gap energies in QDs (and thus emission wavelength)

#### **1.1.3 Optical properties**

For ensemble QDs, there are discrete energy levels where the electron can be excited. Each of these electronic transitions appears as distinct peaks in the absorption spectrum of QDs. The first excitonic peak or the highest wavelength of absorption is the reflection of the band gap energy. The absorption at lower wavelength is more continuous, and reflects the overlapping energies of the transition states. The electronic properties of QDs lend into size tunable fluorescence. As the size increases, band gap energy decreases and the emission shifts more towards the higher wavelengths. Also the breadth of the fluorescence peak (FWHM) can give useful insights into polydispersity in the size of nanoparticle synthesis.



**Figure 1-4** Optical properties of QDs (A) Fluorescence emission spectra of QDs in the visible range (400-700 nm) (B) Representative image of QDs in hexane showing high TEM contrast Scale bar represents 20 nm.

For biological imaging, the two most popular ranges of fluorescence wavelengths of interests are the visible region (400-700 nm) and the near infrared region (NIR, 700 nm to 1000 nm). In the context of this thesis, we focused on QDs emitting in the visible range. For readers interested in NIR imaging using QDs, the work of Gao *et. al.* provides a comprehensive overview.<sup>11</sup> For bioimaging limited to visible region, QDs derived from CdSe are interesting nanomaterials. The emission wavelength of CdSe nanocrystals can be tuned within 450 nm to 700 nm for particle size between 2 and 7 nm. A representative image of the electron dense QDs (emitting at 610 nm) with near-homogenous size distribution is also shown ().

#### 1.1.4 Organic synthesis of core-shell heterostructures

Quantum dots are semiconductor nanomaterials synthesized by systematic formation of a core and a capping shell. Several photophysical and optical properties of QDs arise from the core. The shell assists in passivation of the surface defects of the core, enabling to thus preserve the properties of the core and prevent environmental damage, along with further tuning of the photophysical properties. Detailed overview of process of synthesis and factors that affect the material properties of QDs can be found in classical works by groups of Alivisatos, Bawendi, Owen, Guyot-Sionnest etc.<sup>13–16</sup> Organic ligands are bound to the shell of QDs, which need to be systematically over-coated or displaced by amphiphilic moieties in order to make the nanocrystals water-soluble. A step wise scheme to generate QDs dispersed in water is shown in **Scheme 1-3**. In the next section, different strategies to make QDs water-soluble are described in detail.



Scheme 1-3 Steps to synthesize Quantum Dots for biological applications.

### 1.2 Methods to disperse QDs in aqueous media

Commonly employed methods to transfer QDs from organic to aqueous media are discussed in this section. These methods are chosen such that the nanoparticle diameters are <30 nm, which is considered desirable for range of biological applications including targeting and therapeutic delivery.

#### **1.2.1 Encapsulation**

Encapsulation with phospholipids remains one of the most fundamental methods to solubilize QDs in aqueous media.<sup>17,18</sup> Pioneered by Dubertret *et al.*, this strategy involves over-coating the existing organic ligands with amphiphilic phospholipids (Scheme 1-4, A). Phospholipids comprise of aliphatic chains and polar head groups. The aliphatic chains can organize within the organic ligands on the surface of QDs (like trioctylphospine (TOP) and tri-noctylphosphine oxide (TOPO)) by the virtue of hydrophobic interactions. The composition of the phospholipid formulations can be tailored for specific applications. In this study by Dubertret et al., these QD-phospholipid formulations remained stable for up to a month at a broad range of salt conditions at high concentrations. Though upon inception, it was one of the most radical approaches to transfer ODs in aqueous solutions, but the strategy has some inherent disadvantages. Since the strategy is based on over-coating the existing ligands, the inherent size of dispersed particles is large. Also phospholipids can easily fuse to biological membranes and thus these encapsulated QDs remain in cytosol, and cannot be delivered to specific organelles. Additionally, these formulations are poorly stable in diluted conditions. In order to overcome several of these issues, Pellegrino et al. developed another strategy of encapsulation via the use of polymeric ligands based on poly(maleic anhydride-alt-1tetradecene).<sup>19</sup> Herein QDs were coated with these oligomeric ligands and these ligands were subsequently crosslinked on the surface of QDs (by specific linker molecules), followed by hydrolyzation of the unreacted anhydride groups (Scheme 1-4, B). This step made the polymeric shell amphiphilic. This strategy generated compact QDs of 11-13 nm diameter. In this work however no bioconjugation or targeting was shown. In another work soon after, Kairdolf et al. demonstrated that QDs encapsulated with poly(acrylic acid)-octylamine modified copolymer can be cross linked and hydroxylated by reaction with 1,3-diamino-2propanol (DAP) in presence of EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide).<sup>20</sup> This strategy neutralized the charge on QDs, maintained small  $D_h$  (13-14 nm) and conserved high QY up to 60-65%. Additionally due to near neutral surface potential, the QDs had negligible non-specific adsorption on HeLa cells. Encapsulation via the polymeric shell in general has since then acknowledged as a fairly robust method to generate QDs with high QY and preserved solution stability and minimal non-specific adsorption on cell membranes.



Scheme 1-4 Two approaches for encapsulation of QDs in hydrophilic shells.

#### 1.2.2 Ligand exchange

The above described strategies over-coated 'new' ligands on the already 'existing' ligands on the surface of QDs. An alternate approach to disperse QDs in aqueous solutions involves systematic change of surface ligands. The nature of these exchanging ligands determine the properties of QDs including hydrodynamic radius, fluorescent quantum yield and colloidal stability. A typical procedure to exchange ligands on the surface of QDs involves precipitation of the nanocrystals out of the organic medium and then re-dispersion in presence of excess of the amphiphilic ligands. The resuspended solution is usually kept at high temperature (60-90°C) for 5-18 hours to facilitate exchange and enable sufficient surface coverage. After ligand exchange, the QDs display polar functional groups that impart solubility in aqueous media. These ligands are bound to the QD surface by non-covalent bonds. Several designs of these ligands have been tuned to increase bond strength, stability, and solution properties of QDs. Similarly, the procedure for ligand exchange may additionally influence the polydispersity of the water solubilized QDs.<sup>21,22</sup>

In the next section typical design and properties of several ligands with affinity towards specific functional groups for the QD surface are discussed. The chemical groups which have been shown to have high affinity for the QD surfaces include thiols (discussed below),

carboxylic acids<sup>23</sup>, phosphonic acids<sup>24</sup> and amines. For the scope of this thesis, selected thiolbased ligands are discussed in detail. The ligands that are commonly used to coat the surface of QDs can be broadly classified into two groups - small molecule ligands and higher molecular weight polymeric ligands.

*Small molecular weight ligands.* These ligands are generally derivatives of mercaptoacetic acids or mercaptoethyl amines (Scheme 1-5). First demonstrated by Chan *et al.* close to a decade back, the most popular mercaptoacetic acid-based ligand is mercaptopropionicacid (MPA)<sup>2,21</sup>. The others include for instance, mercaptohexanoic acid (MHA)<sup>25</sup> or mercaptoundecanoic acid (MUA)<sup>26</sup>. Initially demonstrated by Liu *et al.*, an alternate type of small molecule ligand includes mercaptoacetamine-based ligands include cysteine<sup>27</sup> or cysteamine<sup>4</sup>. The general design of both these categories is based on the affinity of the thiol functionality towards the surface of QDs. The other functional group (acid/amine) imparts hydrophilicity and can also be used for conjugation.

Monothiol-based ligands are widely used for ligand exchange of QDs. However they also suffer from inherent disadvantages. The anchoring of monothiol ligands to the QD surface is mitigated by a single non-covalent bond which is prone to oxidation. This causes reduced stability of QD in aqueous solutions. Due to desorption, surface coverage of these passivating ligands on the QD are reduced, that results into aggregation of the dots. Also, protonation of thiolate (at lower pH) or UV irradiation causes removal of these ligands from the QD surface. In order to circumvent these issues of instability, dithiol bearing ligands are being increasingly used. Pioneered by Clapp et al., the most popular dithiol containing ligand for capping the surface of QDs is dihydrolipoic acid (DHLA).<sup>28–33</sup> Dihydrolipoic acid is a small ligand obtained by the reduction of a-Lipoic acid. The thiolate groups thus generated associate with the surface of QDs via chemisorption. The binding and long-term stability of DHLA-coated QDs is higher than monothiol ligands. Owing to high negative charge density at physiological pH, DHLA-coated QDs show often high non-specific adsorption of proteins that in turn affect their biological applicability. In order to specifically address this issue variants of DHLA have been developed. These variants often include a passivating layer of Polyethylene Glycol (PEG) along with the dithiol.<sup>34,35</sup> Another variant of DHLA-based small molecule ligand includes zwitterions instead of PEG, that impart hydrophilicity and antifouling properties to the QDs. <sup>32</sup> Other small molecule/oligomeric ligands with three coordinating thiols (tridentate) have also been developed in past. <sup>36,37</sup>. However for it has been

noticed that QDs modified with tridentate ligands have lower QY (QY= 25-30%) in comparison to bidentate ligands (QY up to 60%)



Scheme 1-5 Small molecule ligands for affinity towards the surface of QDs

Polymeric ligands. Using the design principles from small molecule ligands, several polymeric ligands have been synthesized. These polymeric ligands comprise of several monomers with affinity for QD surface, several charged or hydrophilic groups that impart water solubility and some reactive groups that can be used for bioconjugation (Scheme 1-6). Polymers can thus be considered as unified scaffold that comprises of several desirable modalities. The synthesis is often carried out by copolymerization of stoichiometrically predefined monomers. Post synthesis, the polymers are incubated with the QDs preexchanged with amphiphilic ligands such as MPA. Another design used an existing backbone of polymer on which desirable entities are grafted. For disulfide containing ligands, the presence of a strong reducing agent such as NaBH<sub>4</sub> in the media assists in generation of thiol and favors dynamic interactions with the QD surface.<sup>38,39</sup> For some imidazole or pyridinebased polymers, the activation steps is not required.<sup>40,41</sup> There have been multiple designs of such polymers with various compositions of surface anchoring and water-solubilizing monomers. The interested readers are referred to a review by Sperling et al. for a comprehensive description of various polymeric scaffolds used to disperse QDs in aqueous media.42

Surface exchange via polymeric ligands has several advantages over mono/bidentate small molecule-based ligands. Owing to the multidentate nature of interactions between the surface and the polymers, QDs display higher stability in dilute conditions for prolonged periods of time. A very interesting comparison by our group in past has discussed the time-dependent desorption of dithiol *vs* poly-thiol ligands from the surface of QDs via ligand competition

experiments. It was shown that the rate of desorption  $(k_{off})$  of polymeric ligands was more than 300 lower than that of dithiol-based ligands. <sup>38</sup> Secondly the polymers derived from thiol-based linkers have higher tolerance to pH (4.5-9 or higher), whereas polyhistidine-based ligands are often unstable below pH 6. Despite the nature of ligands, QDs coated with hydrophilic ligands have both high stability and QY in neutral to basic pH. Nevertheless, both of these classes are more stable than the comparative small molecular building blocks. Additionally, QDs coated with polymeric ligands also have additional functional groups incorporated for conjugation where specific covalent attachment of biomolecules could be obtained. Polymer based ligands have also expanded the intracellular stability of the conjugates. An inherent issue with the use of polymeric ligands is the concomitant increase in the size of QDs. The amphiphilic coating can easily increase the hydrodynamic radius 2-3 folds. Therefore, current efforts are attempted to devise polymeric scaffolds that not only provide the advantages discussed above, but also retains smaller size of nanocrystals. This section covered several broad strategies employed for assisting to transfer QDs from organic media to aqueous media. In the next section, different methods to conjugate DNA to QDs will be discussed.



Scheme 1-6 Typical schematic of polymeric scaffolds for ligand exchange of QDs.

The strategies mentioned above discuss the types of ligands and their assembly onto the surface of QDs by different exchange methods. The overall goal of different ligand exchange strategies is to achieve bright and compact QDs in biological buffers. Some of the desirable properties of afore-mentioned QDs in biological buffers include

- (i) Homogenous monodisperse population of QDs
- (ii) Retaining high fluorescence quantum yield
- (iii) Long-term colloidal stability
- (iv) Small size
- (v) Minimal non-specific interactions with biological materials
- (vi) Synthetic reactivity to chemical conjugation.

Actively pursued research aims to achieve water solubilized QDs with several of these properties. In general, transition from organic solvent to aqueous media is accompanied by reduction in quantum yield. Type of ligands and the exchange procedure tends to affect the state of aggregation of the capped QDs. The ligand exchange procedures generate major population of single nanocrystals homogenously coated with the polymeric ligands. However, along with these QDs, there are subpopulations of dimers or oligomeric aggregates of QDs that form, and increase the heterogeneity of samples. Bigger aggregates can be removed by centrifugation, but often smaller clusters (dimers etc) cannot be further removed. Also, smaller ligands maintain compactness of the QDs, but display poor stability in dilute conditions and long durations. Contrarily, QDs coated with polymeric ligands have higher colloidal stability, but the QDs are both heterogeneous and substantially bigger. Current efforts are dedicated to optimize several of these parameters based on the specific target applications. After dispersing QDs in aqueous solutions, the next step is to functionalize them with specific biomolecules, such as nucleic acids. The following section discusses different strategies of conjugation of DNA to QDs and their advantages and limitations.

# **1.3 Strategies to conjugate DNA to QDs dispersed in aqueous** media

In the previous section, properties of QDs particularly advantageous to the biological community was discussed, followed by an overview of different methods to disperse QDs in aqueous media. For specific applications in biology, QDs are conjugated to biomolecules such as DNA. In this section, a comprehensive overview of different methods to conjugate DNA to QDs, with case-specific advantages and limitations are discussed.

#### 1.3.1 Properties of DNA

Nucleic acids, particularly DNA are one of the most stable and chemically unreactive class of biomolecules. The natural selection of DNA as the hereditary material in due course of evolution, is attributed to this remarkable chemical un-reactivity. There are several very interesting characteristics of DNA. Three of the most relevant in the context of this discussion

include (i) polyanionic nature (ii) hybridizability and (iii) persistence length. In subsequent sections, the use of these properties for conjugation, detection and material design using QD-DNA conjugates will be discussed. These approaches are not only applicable to conjugation of DNA, but also to other nucleic acids (RNA, PNA and LNA), to proteins and peptides and other biomolecular ligands. Specific conjugation of DNA to QDs (and other NPs) is brought about by conjugation of particular functional groups on DNA with those on QD. As such, several types of 'reactive' chemically-modified DNA are commercially available. Incorporation of these reactive functional groups is carried out during solid phase synthesis of growing oligonucleotide chain of desired sequence. These modifications produce specifically end-labelled DNA, where single functional groups (such as -NH<sub>2</sub>, -SH, -N<sub>3</sub>) can be chemically inserted along with hydrocarbon linker chain of desired length. This linker chain projects the functional group away from the oligonucleotide chain, and hence increases the solvent accessibility and reactivity of the functional group. Several different lengths of linkers are available commercially. These are synthesized by systematic addition of blocks of C3 linkers during oligonucleotide synthesis. Several reports suggest that linker length is consequential towards the chemical conjugation of DNA to NPs and thus should be carefully evaluated. Additional chemical groups such as biotin or PEG can be incorporated during the synthesis of DNA. These groups add to the water solubility, reactivity and/or the intracellular uptake of DNA.

Despite similar chemical mechanisms of conjugation of DNA and proteins to QDs (or other NPs), there are multiple factors to consider. First and most important is the number of 'reactive' functional groups. Since these groups (particularly NH<sub>2</sub> and SH) are incorporated by highly controlled reactions during solid phase synthesis of oligonucleotides, only one functional group per DNA molecule is reactive. In contrast, in proteins several copies of these functional groups are present due to high amino-acid content (NH<sub>2</sub> due to lysine and arginine, SH due to cysteine). This makes proteins more susceptible to in-solution conjugation than DNA. On the other hand, since the position of the functional group in DNA is fixed, the conjugates produced using DNA are inherently oriented and homogenous, in comparison to proteins.

Second very important factor is the charge on biomolecule. Polyanionic nature of DNA often plays a consequential role in conjugation reactions. Several types of nanoparticle surfaces, including QDs, have negative electrostatic potential that resist solution proximity of DNA molecules. Such electrostatic repulsion causes reduced reactivity and poor yield of conjugates.

To address this issue, surface modification approaches that passivate the QDs surfaces (by groups such as PEG and zwitterions) have improved and facilitated conjugation of DNA. Alternately, coating the QDs surfaces with highly positively charged peptides has results into electrostatic attraction between the DNA and the QDs, facilitating conjugation. Several of these factors are discussed later in this chapter.

There are two modes of conjugation of DNA on QD. The first is conjugation of DNA *to* the metallic shells of QDs (buried within the amphiphilic ligand layer). The second mode of conjugation involves conjugation *on* the existing layer of amphiphilic ligands. These two modes are discussed in the subsequent sections.

#### 1.3.2 Conjugation of DNA to shells of QD

Several ligands such as thiols, amines and imidazoles have affinity towards the inorganic shells of QDs (**Scheme 1-7**). These interactions have been utilized to attach DNA to QDs as described in the subsequent section.

*Thiolated DNA*. This is the most popular category of ligands for the non-covalent attachment to the surface of QDs. As discussed in the above sections, thiols have intrinsic property to self-assemble on the surface of nanoparticles including QDs. To conjugate thiolate DNA on QD, the latter are first dispersed in water using small molecule ligands such as MPA. Excess of MPA is removed by repeated centrifugation and resuspension in basic buffers (pH>8), where deprotonation of MPA makes the nanocrystals hydrophilic. Then thiol-labelled DNA is added in excess, such that dynamic equilibrium can replace the original ligands with DNA. First shown by Mitchell *et al.*, alkylthiol labelled DNA (of different lengths) have been self-assembled on colloidal nanoparticles such as QDs in high yield and reproducibility  $^{43-45}$  In the first report, the exchange took 1 to 2 days and several length and sequences of DNA were sensitive to pH, photooxidation and dilutions. Since then several reports have conjugated thiolated DNA on QDs within shorter times.<sup>46,47</sup> Similar to thiol-based hydrophilic ligands, the issues of photo-oxidative ligand loss and pH sensitivity is a fundamental limitation of this approach.



**Scheme 1-7** Conjugation of DNA to the shell of QDs (A) via thiols (top) and polyHistidine (bottom) and (B) via modifications in DNA backbone.

**Peptide tagged DNA:** Imidazoles are another class of functional groups that have high affinity toward shells of QDs. This affinity has been harvested in the use of polyhistidine peptide tags of various lengths to conjugate DNA to QD. Sapsford et al. demonstrated that polyhistidine tags could self-assemble over the QDs surfaces with almost instantaneous kinetics.<sup>48</sup> Several reports from Mattoussi's group have also demonstrated that DNA conjugation can be carried out on peptide tags with terminal functional groups such as lysine (-NH<sub>2</sub>), aspartate (-COOH) or cysteine (-SH) using specific reaction conditions.<sup>49</sup> These conjugates were then purified mixed with ligand exchanged QDs. These conjugates are state of the art and easy to assemble. However, there are certain limitations. Affinity of Imidazole group towards the QD surface is highly pH-dependent. Protonation of the N-hydrogen of imidazole functional group (at pH <6) destabilized the conjugates. Additionally, the cationic nature of polyhistidine-tagged peptides and anionic nature of DNA renders additional complexity to conjugation reactions in solution. Additionally pH-sensitivity of the reaction often results in low yield and aggregation of the interacting biomolecules. <sup>50</sup> These issue can however be circumvented by using DNA with a neutral polypeptide backbone (PNA). Peptide nucleic acids are neutral and can hybridize to complementary DNA strands. However, PNA can be used only for limited applications in biosensing as PNA-DNA duplexes have higher thermodynamic stability than DNA-DNA<sup>51</sup> and are far more expensive than DNA.

*Phosphorothioate modified DNA*. The backbone of DNA comprise of polyphosphates. These polyphosphates can be synthetically modified to phosphorothioates wherein one of the nonbridging atoms of oxygen is replaced with Sulphur. Sulphur has substantially higher affinity (~3000 X) for Cd<sup>2+</sup> than oxygen and forms stronger complexes with the inorganic shells of nanoparticles. First demonstrated by Kumar *et al.* on colloidal gold, this affinity has been utilized to conjugate phosphorothioate modified DNA to GNPs<sup>52,53</sup> and later to QDs<sup>54–56</sup> either post or during synthesis. This method has also been extended to generate homogenous monofunctionalized QDs.<sup>57</sup> This method is advantageous to link DNA on QDs, but suffers from the fundamental limitation that the 'attached' DNA is conformationally distorted and often not hybridizable to complementary DNA. However, this issue can be avoided by introducing a DNA overhang with phosphodiester backbone, displaying the target sequence. Using a combination of phosphorothioate backbone (for binding) and phosphodiester backbone for molecular assembly, these pre-functionalized QDs can be further used for hybridization, biosensing, cellular targeting and other applications.

#### 1.3.3 Conjugation of DNA to ligands on QD

In the previous section, the conjugation strategies were based on the affinity of the functional groups on DNA towards the shell of the QDs. Another popular category of conjugation can be based on affinity of DNA for specific ligands coating the surface of the QDs (**Scheme 1-8**). Some of the examples are discussed below and summarized.

*Lipid encapsulated conjugates.* As discussed in the previous section, amphiphilic phospholipids can be used to impart water solubility to QDs. As shown by Dubertret *et al.*, the composition of encapsulating phospholipids can designed to display functional groups that can be used for conjugation of DNA.<sup>17,18</sup> More recently, Aimé *et al.* have shown that lipid oligonucleotide conjugates (LONs) can embed within the amphipathic capping layer on QDs by hydrophobic interactions.<sup>58</sup> In this method of conjugation of DNA, the oligonucleotides are first conjugated to the amphiphilic lipids and then added to the overall encapsulating mixture to directly display conjugated DNA on the surface of QDs. These methods however lack control over the conjugation efficiency. These conjugates also have less applicability in

context of cellular processes, since phospholipid preparations often result into unambiguous fusion with the cell membrane.



Scheme 1-8 Conjugation of DNA to specific ligands on QD

*Electrostatic interactions with ligands on QD.* The phosphate backbone of DNA is highly anionic. This makes DNA highly prone to association with positively charged surface coatings by the virtue of electrostatic attraction. There are several examples where QD-DNA conjugates were synthesized using this method. This approach is very simple, instantaneous and results in high DNA loading. Mirkin *et al.* demonstrated for the first time that DNA loading capacity on GNP can be further enhanced by step-wise increase in the osmolarity of the reaction. <sup>59</sup>

Phosphate backbone mediated electrostatic attachment of DNA on polymer coated QDs has been shown. <sup>44</sup> In a report by Peng *et al.*, QDs were ligand-exchange with cationic polymers such as poly(diallyldimethylammonium chloride) and associated with DNA by electrostatic interactions. <sup>60</sup> In another report by Lee *et al.*, QDs were first ligand-exchanged with amine-modified DHLA and passivated with a layer of high molecular weight PEG. This DHLA-PEG hybrid layer has moderate cationic nature along with antifouling properties and can be used to quantitatively load DNA mediated by electrostatic interactions. <sup>61</sup> Other strategy involves using small molecule ligands such as MPA (negative charge) followed by PEI (polyethylenimine). <sup>62–64</sup> These high to moderately cationic QDs have been extensively used to electrostatically assemble excess of DNA for biosensing and therapeutic delivery.

However, electrostatics mediated attachment of DNA to QD and other nanoparticles have several limitations. Affinity of phosphate towards the positive surface of QDs often leads to conformational distortion of DNA. This prevents the complementary DNA strands to form classical Watson and Crick base pairs and hence conjugated DNA loses its potential to hybridize. Also, the preparations are often heterogeneous with variations in conjugation yield. Additionally, affinity of interactions of phosphate backbone of DNA with the QD surface can vary with the pH and the conjugates tend to aggregate upon long storage.

*Surface-Modified QDs.* In a recent report, Kwon *et al.* have demonstrated that DNA can be conjugated on QD based on affinity of polyimidazoles for Ni-NTA.<sup>65</sup> In this approach, QDs displaying carboxylic acid functional groups have been conjugated with polyglycine – polyhistidine peptide tag. In parallel, DNA is conjugated to thiolated NTA. Post conjugation,  $Ni^{2+}$  chelation of the NTA is carried out, that makes the DNA reactive towards the polyhistidine tag. These two reactants have been shown to self-assemble instantaneously and the complexation can be reversed in presence of excess imidazole. However, this study does

not discuss the natural propensity of polyhistidine tags to self-assemble on the inorganic surface of QDs (and other NPs). The specificity of conjugation of the polyglycine-polyhistidine peptide to the carboxylic acid ligands is thus debatable. This approach is conceptually similar to the design of using either unmodified or NTA modified QDs to associate polyhistidine tagged proteins<sup>48,66</sup> where tighter control over the number of DNA per QDs is imposed.

Affinity for already-conjugated ligands. Biotin-streptavidin binding is one of the strongest non-covalent interactions known in biology. This high affinity has been exploited in multiple scenarios to attach biomolecules to each other. This is also one of the widely used strategies to conjugate DNA to QDs. Herein QDs are first functionalized with streptavidin by either affinity (peptide) or covalent conjugation methods. <sup>3,44,67,68</sup> Then, these QDs are purified from unconjugated proteins and simply mixed with commercial biotinylated DNA. The biotinylated DNA can simply attach on the QD by the streptavidin linker. Since each streptavidin has 4 biotin binding sites, it is possible to assemble more than one DNA per protein. In order to further control the stoichiometry of biotin on streptavidin, molecularly engineered variants of the protein have been used, which have affinity for only one biotin (and hence biotinylated DNA) that permit monolabelling (per streptavidin molecule) with biotinylated DNA. This is also one of the most popular varieties available commercially. Zhang et al. showed that different lengths of DNA could be easily assembled on the QD surface by simple one step mixing protocol.<sup>69</sup> However, the only limitations of this approach is that the additional layer of protein often results into increase in the size of the particle, that eventually limits its applications in contexts of particle tracking at neuronal synapses or ion channels.

Conjugation of DNA to ligands on QDs has been demonstrated by different strategies. Each strategy has its own merits and demerits, as described above and listed in **Table 1-1**. Most reactions are designed based to the need of specific types of conjugates, such as QY, size, poly-functionalization, ease of preparation etc. Rapidly expanding library of ligands is paving way for development of newer generation of conjugates with highly desirable properties for applications in biology.

S.No	Approach	Modification on QD	Modification on DNA	Advantages	Disadvantages	References
1	Phospholipid encapsulation	Organic ligands	Amine/Thiol	<ul><li>One step procedure</li><li>Compatible with range of synthesis</li><li>DNA retains hybridizability</li></ul>	<ul><li>Stability issues</li><li>Large size of particles</li><li>Random fusion with membranes</li><li>Low QY</li></ul>	17,18
2	Electrostatic attraction with polymer on QD	Positively charged hydrophilic ligands	Unmodified. Interaction with phosphate backbone	<ul><li>Rapid</li><li>High DNA loading efficiency</li><li>Reversible</li></ul>	<ul><li>Difficult to control stoichiometry of DNA</li><li>Issues with stability and aggregation</li></ul>	61,64
3	Affinity for cationic shell of QD	Positively charged or neutral	Thiol /Polyhistidine/ Phosphorothioate	<ul><li>Rapid assembly</li><li>High yield</li><li>compact</li></ul>	<ul> <li>Unstable in high dilutions</li> <li>Loss of DNA by photoxidation</li> <li>pH sensitive</li> <li>Inteactions with DNA backbone can cause conformational change</li> <li>Low QY due to charge transfer</li> </ul>	43–45,48,55
4	Affinity for specific ligand	Streptavidin conjugation	Biotinylated DNA	<ul> <li>Fast and easy</li> <li>Conjugation of DNA independent of length</li> <li>Stoichiometric control</li> <li>Uich OV</li> </ul>	• Increase in conjugate size du to presence of protein	3,35,44,67–69
		Ni-NTA modified	Polyhistidine modified	<ul><li>One step</li><li>Efficient</li><li>High QY</li></ul>	• pH sensitive stability	65
5	Covalent conjugation with	QDs with carboxylic acids	Amine modified	<ul><li>Highly stable</li><li>pH insensitive</li></ul>	<ul><li>Multistep</li><li>Low yield</li></ul>	70–72
	ligands on QD	QDs with amines	Thiol modified	<ul><li>DNA hybridization preserved</li><li>Moderate-high QY</li></ul>	Nanoparticle size increases	73 74
		QDs with thiols QDs with azide	Amine modified cyclooctyne	• Reactants are not prone to hydrolysis	• Loss of QY	75

# **Table 1-1** Comparison of different methods for conjugation of DNA to Quantum Dots.

# **1.4 Types of covalent conjugation reactions**

Commercially available linkers are most commonly employed to form covalent bonds between the ligands on QDs and DNA. These linkers (i) either assist in the 'activation' of the reacting molecules by forming transient intermediates (using carbodiimide chemistry) or (ii) have two functionally reactive groups on the same linker, one for each reacting molecule (bifunctional linkers). The following section describes the use of these linkers in several examples of covalent conjugation of DNA to QDs based on the reacting functional groups ().

Amine to carboxylic acid conjugation. This is one of the extensively used strategies for conjugation of DNA to QDs (Scheme 1-9, A). The QDs are first coated with ligands that bear carboxylic acid functional groups. These ligands are either small molecules such as MAA, MPA, MHA and DHLA 70,71,76,77 or derived polymeric scaffolds terminating in carboxylic acid.<sup>72,78</sup> To all of these QDs 'terminating' in solvent exposed carboxylic acid functional groups, conjugation of amine-functionalized DNA is carried out in the presence of EDC and NHS. First, the carboxylic acid group of QDs is activated by reaction with EDC. This step produces a highly reactive (and labile) O-acylisourea intermediate. This intermediate can be immediately mixed with NHS that results into QD-NHS. These NHS derived QD are then reacted immediately with amine-DNA to form a stable amide bond between the capping ligand and DNA. In case of failure of reaction with NHS, the intermediate hydrolyses and the carboxylic acid on QDs can be regenerated. This reaction offers several advantages. First, owing to fast rate of hydrolysis ( $t_{1/2} = 4$  hours at pH 7.4°C), this reaction is completed very fast. Second, this conjugation method retains the small size of QDs. This reaction is additionally very sensitive to pH. The activation of QDs using EDC works better in acidic pH and the subsequent reaction with NHS requires basic pH. Hence the swift tuning of pH is not only tricky but also difficult to control during the reaction. This along with fast hydrolysis of NHS causes reduced yield.

*Amine to thiol reactive linkers.* There are two popular types of amine to thiol reactive cross linkers used for bioconjugation on QDs. The first is sulfosuccinimidyl-4-(N-maleimido-methyl) cyclohexane-1-carboxylate (sSMCC) which has a terminal NHS and a terminal maleimide (**Scheme 1-9**, B). In this reaction, amine-labelled DNA is first reacted with NHS

to give DNA maleimide. The DNA maleimide is then conjugated with polymer-capped QDs which have been reduced to display thiols.<sup>74</sup> The same design could also be used to conjugate proteins and antibodies to QDs.<sup>79</sup> Alternately, a more classical utilization of this approach involves functionalizing QDs displaying primary amines to thiol modified DNA.<sup>18</sup>

Another bifunctional linker called succinimidyl 3-(2-pyridyldithio)propionate (SPDP) can also be used to functionalize amine terminating DNA to QDs.<sup>73</sup> This linker has an NHS group on one end and a cleavable pyridyl disulfide of the other. General template to use this linker is to first functionalize amine-DNA with the linker. In the second step, the disulfide is cleaved in presence of a reducing agent to generate reactive thiols. The reducing agent and the pyridine-2 thione group (by product) thus generated are removed and the DNA functionalized with a reactive thiol is mixed with QD coated with PEG-maleimide. The thiol-maleimide reaction results into conjugation of DNA. The specific advantages of using SPDP over SMCC are the control over utilization of the pyridyl disulfide. After reaction of the linker with the primary amine containing biomolecule, the modified biomolecule can be easily stored for long periods without loss of function of the linker. However in case of SMCC, the maleimide derivative should be reacted within a day due to hydrolysis of the maleimide. A comparative disadvantage of SPDP over SMCC is the formation of disulfide crosslinking in the case of former. In case of reactions carried out in pH 7-8, reformation of disulfide can lead to cross linking of biomolecules instead of reaction of maleimide of QD to the thiols of biomolecule. Hence tight regulation of pH in case of this reaction is highly sought after.

*Click chemistry.* Click chemistry is the new age biorthogonal labelling technique employed to conjugate range of biomolecules onto another. The reaction comprises of covalent conjugation of a molecule with functional azide to another molecule with alkyne via cycloaddition (**Scheme 1-9**, C). The reaction is often catalyzed by Cu<sup>2+</sup>. However, Cu<sup>2+</sup>cations are known to quench the photoluminescence of QDs.<sup>80</sup> In order to circumvent this issue, Cu free click chemistry has been employed to conjugate DNA to QD while maintaining high fluorescent quantum yield.<sup>75</sup> Briefly, QDs coated with DHLA-PEG-N<sub>3</sub> were mixed with cyclooctyne modified DNA (thrombin binding aptamer) in a 1:30 ratio and left overnight at 4°C. With this approach approximately 67% of total DNA was conjugated. This is one of the highest yields reported so far.



Scheme 1-9 Covalent conjugation reactions used to synthesize QD-DNA conjugates

Covalent conjugation of biomolecules on the surface of QDs is by far the most popular methods to prepare biofunctionalized QDs. The linkers are cheap and easily available and most of the reactions can be carried out without the need of highly specialized conditions.

These methods also ensure that the DNA (or other biomolecule) remains tightly bound to the QDs and the stability is derived from the innate stability of the encompassing ligands. After preparation of QD-DNA conjugates by various methods discussed above, the conjugates need to be purified from the excess uncoupled reactants. Few of the most popularly employed methods are discussed below.

# **1.5 Purification of conjugates of QD-DNA**

The search for ideal method of purification of functionalized QDs from the excess reactants is as old as the hunt for best strategy of conjugation. Almost no biological or chemical application of these specific conjugates can be carried out without purification of excess (uncoupled) DNA. There are three broad methods of separation discussed herein.

*Electrophoresis.* Electrophoretic migration of QDs depends on both the type of QD structure and the type of polymer coating. Ligand such as MPA, DHLA and polymeric impart negative charge on QDs, that facilitate migration towards positive terminals. <sup>38,76</sup>Conjugation of DNA on QDs results in further decrease of the zeta potential of the surface of QDs without massively altering the molecular weight. This results into faster migration towards the positive terminal. This property has been utilized to separate QDs conjugated with DNA from the unconjugated ones.<sup>69,81</sup> This is one of the most simple and routinely used methods. QDs conjugated to DNA have even been purified by the extent of labelling stoichiometry. <sup>57</sup>. However, in order to extract conjugated QDs from the gel, tedious extraction processes need to be carried out. The relevant bands of interest should be first excised out of the agarose gel followed by either melting of the gel or prolonged incubation of the gel fragments in relevant buffers. QDs are then re-collected back by centrifugation and concentration. Passing QD-DNA conjugates from several of these methods often result into poor yields and issues with long-term stability.

*Ultracentifugation.* Owing to the presence of multiple metallic atoms in confined volume, QDs (and other NPs) have high density in core and at the shell. Additional coating of organic polymeric layer does not increase the density of particles. However, general ligand exchange procedures can change the state of aggregation of the samples. Further conjugation of biomolecules on this layer does not contribute towards increase in the density of the biomolecules. This allows for separation of QDs from fluorescent or colorless biomolecule layer.<sup>17,73</sup>

*Chromatographic separation.* Several examples of purification of QD-DNA conjugates using variety of chromatographic techniques have been reported in past. For example, in reactions with high yield of conjugation, the nanoparticle size increases due to addition of layer of biomolecules. This facilitates purification of QD-DNA conjugates from unreacted DNA using size exclusion chromatography (SEC).<sup>74,82</sup> Another approach to purify QD-DNA conjugates from unreacted DNA is by the use of Anion exchange chromatography (AEC).<sup>83</sup> The affinity of diethyl-aminoethyl (DEAE) based matrices for anionic ligands such as DNA is well known.<sup>84</sup> This affinity is used as a method to purify QDs conjugated with DNA from the unconjugated ones. First, column prepacked with DEAE cellulose beads is equilibrated with buffers of low ionic strength. This enables interaction of the DNA-functionalized QDs with the matrix whereas the unconjugated QDs elute out at this time. Then the entrapped QD-DNA conjugates can be eluted by simply passing buffer of higher ionic strength through the column. A further modification of this strategy is the use of DEAE modified magnetic beads.<sup>85</sup> Herein, Uddayasankar *et al.*, tuned the purification strategies to particularly separate OD-DNA conjugates from unreacted ODs by binding to DEAE on the magnetic beads. In this particular study, QD conjugated to different lengths of DNA were purified by tunic the ionic strength of the interacting and eluting media. The rapid purification times (within minutes) and reduced number of interacting interfaces in case of magnetic beads were far more efficient method of purification over cellulose beads.

# **1.6 Applications of QD-DNA conjugates**

The predictable base pairing properties and persistence length of DNA make it an ideal template for nanoconstruction. Conjugation of electron dense nanoparticles such as gold or QD to DNA opens up routes for novel designs of synthetic nano-assemblies.<sup>68,86</sup> These DNA

functionalized GNP/QD templates can then be used to synthesize novel materials for engineering, charge transport and photo physics. In parallel, these scaffolds could also be used for biological applications. In the beginning of this chapter, several applications of QDs as reagents for bioimaging were discussed. Within this umbrella term, three categories of specific applications of QD-DNA are discussed.

Synthetic programmable scaffolds. With the increasing popularity and ease of generation of QDs (and other NPs) functionalized by DNA, these synthetic scaffolds have been recently used to develop highly complex, tunable and reconfigurable macromolecular scaffolds (Scheme 1-10, A). Conjugates of QD-DNA have been assembled into various geometries for about 15 years now. In one of the first reports by Mitchell et al., QD and GNPs assemblies were augmented by DNA hybridization.<sup>43</sup> In rather recent examples, ODs emitting at different wavelengths were conjugated to DNA of different lengths and assembled into reversibly programmable structures with controlled valency and orientation by Tikhomirov et al.<sup>87</sup> These structures could be tuned to mitigate electron transfer thereby allowing control on photophysical properties. In another report by Ko et al., QD-DNA conjugates have been precisely positioned on DNA origami templates using hybridization and used to tune fluorescence lifetime of QDs via this synthetic scaffold.<sup>88</sup> In yet another approach electrostatic interactions have been used to encapsulate QDs within a DNA nanocage by Wang et al., to conserve single molecule aggregation-free state of QDs in solutions.<sup>89</sup>In a similar approach by Wang et al., QD-DNA conjugates have been used for detection of correctly 'folded' 3D DNA origami cage.<sup>90</sup>

These conjugates are not only novel materials for design and assembly, but also display interesting electronic and chemical properties and therefore valuable for understanding the macromolecular behavior of complex nanomaterials.

*Biosensing application.* The conjugates of QD-DNA have been widely utilized for detection of nucleic acids, and other molecular ligands. The biosensing modality is introduced to QD-DNA conjugates by use fluorescently labelled complementary probes that can hybridize and ensure a bimolecular process like FRET (Scheme 1-10, B). <sup>91–93</sup>The FRET signal can be induced by simple mixing of target polynucleotide sequences <sup>94</sup>. Another variation of this process is when loss of FRET is coupled to the biosensing. An interesting example of this 'FRET-loss' method is the work by Zhang *et al.* where a thrombin binding aptamer was



Scheme 1-10 General application of QD-DNA conjugates

conjugated to QD and blocked by partially complementary sequence. Terminal fluorophore on this complementary sequence formed a FRET pair with QD. Binding of a longer complementary ssDNA or conformational change induced by thrombin to the aptamer, displaced the fluorophore labelled DNA, thereby causing loss of FRET. <sup>75</sup> In another classical example by Kay *et al.* use conformational change of DNA induced FRET to quantify pH in endosomes of live cells.<sup>95</sup>

Another biosensing application of QD-DNA conjugates is in Fluorescence In Situ Hybridization (FISH). The purpose of FISH is to locate specific gene targets in metaphase chromosomes of model organisms for karyotyping and biomarker detection. The fundamental principle involves searching for 'target' sequences mediated by hybridization of fluorescent complementary sequence. The probe DNA conjugates to QD are incubated with cells (in metaphase) denatured and fixed on glass slides. The probe DNA is allowed to hybridize with the 'target' gene. The slides are thoroughly washed and stained with DAPI (to mark the chromosomes) and imaged using total internal microscopy. Almost at the same time, Bentolila *et al.*, and Xiao *et al.*, demonstrated that the FISH assay using QD-DNA probes have much higher sensitivity and signal to noise ratio in comparison to organic fluorophores.<sup>96,97</sup>

Another category of biosensing and theranostics application of QD-DNA conjugates not discussed herein is the use of QD-aptamers. Aptamers are synthetic sequences of DNA that can be selected to have specific recognition motifs binding to particular targets. These aptamers are designed and selected by SELEX (Systematic evolution of ligands by exponential enrichment) where DNA sequences with specific affinity for targets of interest are screened and mutated to improve the affinity by several iterations of synthetic evolution. Functionalization of QDs with these DNA aptamers paves way for using QDs for molecular diagnostics.

Using QDs as alternatives to fluorophores for biosensing assays have definitely improved the reliability of detection due to high signal to noise and also substantially increased the limit of detection of analytes, thereby improving the current state of the art. These QD-DNA-fluorophore FRET-based detection methods are robust, sensitive, ratiometric and highly quantitative. The existing challenge is to carefully control the number of DNA and generate

homogenously labelled populations of conjugates. This area is currently actively being investigated.

**Delivery and therapeutics.** There are a few examples where QD-DNA conjugates have been used for intracellular delivery of biologically active materials for therapeutic applications. These examples can be broadly divided into nanoparticle assisted gene regulation and gene therapy (Scheme 1-10, C). One of the earliest examples by Srinivasan et al. showed intracellular gene regulation by tracking and delivery of plasmid using QD-DNA conjugates.<sup>73</sup> A plasmid encoding for EGFP is labelled with several QDs via PNA-DNA hybridization and transfected into cells. The intracellular and subsequent nuclear delivery of the labelled plasmid is tracked by time lapse imaging of the QD signal. Also, time-dependent analysis of intracellular GFP signal could be used as a read out for plasmid induced protein expression. Similar design of QD-DNA conjugates have been used for by Wu et al. for transfection of plasmid and over expression of a number of proteins in cellulo.<sup>98</sup> An alternative approach by Li et al. involved transcriptional activation of the 'conjugated' plasmid in response to spatial stimulus.<sup>99</sup> This design has been implemented in loading plasmid encoding EGFP onto QD surfaces and transfecting this complex into cells. The complexes localize in nucleus and remain transcriptionally inactive. However addition of glutathione in the media triggers plasmid release from the QD surface, which in turn makes the plasmid transcriptionally more active. This design provides additional control over the transcription can is a classic example of biosensing and therapeutic application of QD-DNA conjugates.

Another class of therapeutic applications is the use of QD-DNA conjugates for siRNA mediated gene silencing.<sup>100–102</sup> First demonstrated by Derfus *et al.*, QDs were labelled with siRNA against EGFP and transfected in stable cell lines. The concomitant silencing of EGFP gene was observed by comparison of EGFP signal in trypsinized cells. This design has been further tuned by others to show QD-DNA mediated gene silencing in other cellular contexts. For these therapeutic approaches, a careful evaluation of nanoparticle surface parameters is essential, as different nanoparticles can often get trapped in the endosomal pathway and get degraded. This impedes their full potential in therapeutics.

# **1.7 Conclusions**

This chapter summarizes how QD-DNA conjugates are new generation probes with great potential in biology. The photophysical properties, methods to transition QDs in aqueous solutions and major strategies to conjugate DNA to QDs were discussed in detail. Most of these conjugation strategies could also be used to attach proteins to QDs, which are explained in subsequent chapters. Additionally, scope of these conjugates for applications such as biosensing and intracellular delivery were discussed. These applications can be considered partly within the umbrella term 'bioimaging' using QDs. The goal of this chapter was to provide a comprehensive overview of the potential of QDs as probes in biology and discussion of recent trends and developments in this direction. Rapidly developing infrastructure, ligand chemistry and understanding of interactions between material and biological interface have set stage for challenging applications of QD-DNA conjugates such as multiplex biosensing, simultaneous tracking of cellular pathways and rapid and efficient delivery of therapeutic payload. Undoubtedly, the time is ideal for use of these probes for novel, high through-put and multifarious applications is biology. These advances continue to demonstrate and inspire superior performance of QDs for diversified applications ranging from material design to molecular therapy. The subsequent chapters discuss several of some of the original contributions to this actively growing field of QD-DNA conjugates in biology. Impartial overviews of the advantages and case specific limitations have been critically discussed wherever necessary.

# **Chapitre 2 A Novel Method to Conjugate DNA to Quantum Dots**

Quantum Dot conjugates with DNA are very interesting candidates for several types of applications. For example, QD-DNA conjugates have been used for siRNA<sup>103</sup>, FISH<sup>47</sup>, detection of DNA mismatch and femto-molar (rare) nucleic acids.<sup>104,93</sup> QD-DNA have also been used for exploring bimolecular FRET<sup>46,94,95</sup> and in photovoltaics.<sup>105,106</sup> Ease of chemical modification<sup>107</sup> and predictable Watson-Crick base pairing properties of DNA enables use of QD-DNA conjugates to form designer heteromolecular structures with precise control.<sup>87</sup> Therefore, it is interesting to investigate methods to generate stable QD-DNA conjugates, in a reproducible manner. As discussed in the first chapter, DNA has been conjugated to QD (CdSe/ZnS) with several handful of methods. Briefly, the most common methods of conjugating DNA to QD have been via (1) a coupling between the carboxyl group on a polymer-coated QDs and an amine-functionalized DNA by reaction between 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) <sup>108,72</sup>(2) a ligand exchange between initially MPA (mercapto propionic acid)- coated QD with a thiol-functionalized DNA directly  $^{43,45,109}$  or mediated by a linker  $^{110}$  and (3) the use of QD-conjugated streptavidin functionalized with biotinylated DNA.<sup>103,94</sup> Nevertheless, each strategy has its own limitations. EDC-NHS coupling suffers from low conjugation efficiencies due to hydrolysis of NHS esters. Ligand exchange with thiol-labelled DNA is both limited to loss of DNA by desorption into aqueous media over time and dependence of stability on pH of reaction. Most thiolated ligands have further been reported to quench the photoluminescence of QDs thereby compromising their generic applicability.<sup>111,112</sup> Finally, the biotin-streptavidin strategy needs at least two steps of conjugation/purification and increases the size of QDs substantially. These issues result into limited targettability and hence scope. These factors maintain interest in investigation of novel methods for conjugation of DNA to Quantum Dots.

In the previous chapter, detailed outline of methods of ligand exchange of QDs and subsequent conjugation to DNA were discussed. In this chapter, a novel method to conjugate DNA to QDs is provided. This method overcomes several issues of the previously discussed strategies and is additionally highly adaptable to different nanoparticles and biomolecules.

Most of this work is carried out on QDs emitting at 610 nm coated with the (20-80)<sub>20</sub>-Zw polymer, unless specified otherwise. The novel conjugation strategy described here couples amine labelled DNA (or other biomolecules) to thiols on the polymers coating the surface of the QDs. Step wise development of this novel conjugation strategy is described in detail. The work is further supported by investigation of different chemical and biochemical parameters that affect the reaction with respect to the conjugation yield. After thorough optimization of the reaction parameters, the same strategy has been extended to conjugate DNA of different lengths and to different nanoparticles including polymer-capped Gold nanoparticles (GNPs) and Nanoplatelets (NPLs). The same strategy can also be used to graft proteins on QDs. This method is a robust, highly adaptable and widely applicable strategy to conjugate diverse variety of biomolecules to colloidal nanoparticles coated with the designated polymer, and has great potential for widespread use.

# 2.1 Dispersion of Quantum Dots in aqueous media

Before beginning any conjugation reactions with biomolecules, QDs need to be first ligand exchanged and dispersed in aqueous buffers. This section describes briefly the steps for the same.

#### 2.1.1QDs in organic solvent

For this project, QDs were obtained in hexane from Nexdot. In brief, the QDs were CdSe/CdS/ZnS core-shell-shell heterostructures. The obtained QDs were characterized by absorbance, fluorescence and TEM (**Figure 2-1**). These QDs had first excitonic peak at ~595 nm and emission maximum around 610nm with FWHM ~35 nm. From TEM analysis of >250 particles, mean diameter of this batch was estimated to be  $7.7\pm0.8$  nm. Immediately after synthesis, QDs had high fluorescence quantum yield (QY) close to 65-70%.



**Figure 2-1** Characterization of QD emitting at 610 nm in hexane. (A) Absorption (black) and emission (grey) spectra of QDs in hexane (B) TEM image (C) size distribution of the batch of QD used in this work.

#### 2.1.2 Dispersion of QDs in aqueous buffers

Organic synthesis of QDs renders the nanocrystals coated with ligands incompatible with biological solvents and buffers. In order to conjugate any biomolecule to QDs, the nanocrystals need to be first dispersed in water. There are several methods to transfer QDs from organic media to aqueous media. These methods have been discussed in great detail in Chapter 1. Briefly, two of the most popular methods to disperse QDs in water include coating them with amphiphilic small molecule ligands (such as Mercaptopropionic acid, MPA) or with higher molecular weight polymers. Small molecule ligands such as MPA have a terminal thiol and a carboxylic acid group. The terminal thiols can attached to the surface of QDs and subsequently displace the original ligands. Additional conjugation reactions can be carried on the carboxylic acid groups. However, small molecule coated QDs have issues with long-term stability. The thiols anchoring to QD shells are prone to photo-oxidation and can desorb in dilute solutions. This exposes hydrophobic patches on the surface of QDs that eventually lead to aggregation. In order to circumvent these issues, multidentate polymeric ligands are used, those bind to the surface of QDs by several appendages and also display several polar groups to impart water solubility.

Previous work from our group have carefully developed and characterized several polymers with exceptional properties. Of particular interest is the thiotic acid-sulfobetaine containing zwitterionic polymer detailed by Giovanelli *et al.*<sup>38</sup> Dots coated with this polymer are soluble in water, retain high QY, compact dimensions and can be easily biofunctionalised. These dots are also stable in wide range of pH, salinity and higher dilution. The design of this polymer also had terminal carboxylic acid functional groups, on which specific bioconjugation

reactions were carried out. In the work described in this chapter, the terminal functional group was not utilized for conjugation. In fact systematic investigation was carried out to test conjugation on the thiols of the polymer. Thiols on the polymers anchor to the surface of QDs by the thiolate anion, and keep the polymer in place. This strategy is the first report demonstrating that several of these thiols can be actually used for bioconjugation as well.

*Synthesis of polymer.* For this work,  $(20-80)_{20}$ -Zw was used. Briefly, the polymer synthesis was carried out by co-polymerisation of a monomer 1 (20%) and of 3-[3 methacrylamidopropyl(dimethyl)ammonio]propane-1-sulfonate (SPP, 80%) in presence of (<5%) MPA for chain termination. Monomer 1 was synthesised by peptidylic coupling of thiotic acid and N-(3-aminopropyl) methacrylamide exactly as described in the previous work. Detailed protocol for synthesis of this polymer is described in Section 5.1.3 (Chapter 5). The monomer conversion was assessed by NMR and the polymer was obtained as an off-white solid.

#### 2.1.3 Ligand exchange of QDs

Dispersion of QDs in water was carried out by classical two step ligand exchange procedure described previously.<sup>38</sup> The detailed protocol is given in Section 5.1.3 (Chapter 5). Briefly, QDs in organic solvent were first exchanged with small amphiphilic ligand Mercaptopropionic acid (MPA) by overnight incubation at 60°C. The following day, excess of MPA was removed by repeated centrifugation and QDs were dispersed in DMF. Addition of a base in excess (potassium tert-butoxide) deprotonates the MPA (on the surface of QDs), making them insoluble in DMF. The DMF is removed by centrifugation and the QDs could be dispersed in basic (pH>7) buffers. In the next step, QDs coated with MPA were exchanged with the zwitterionic polymer. The polymer was first reduced to convert disulfide to thiols, and then left to facilitate dynamic interactions with the surface of the QDs, that eventually displace the MPA and homogenously coat the surface of QDs.

In this work all QDs used were coated with the  $(20-80)_{20}$ -Zw polymer and emitted at 610 nm, unless specified otherwise.

*Characterization of QDs with (20-80)20-Zw polymer.* After the above mentioned ligand exchange procedure, QDs were soluble in aqueous medium such as 0.2 M NaHCO<sub>3</sub> (pH 8.3), 1X PBS (pH 7.4) and 20mM NaCl. For long-term stability buffers such as 0.2M NaHCO<sub>3</sub> and 20 mM NaCl were observed to preserve the quantum yield (QY) of QDs better that PBS.

Freshly ligand exchanged QDs (610 nm) had conserved QY (~60% with respect to Rhodamine) for more than 3 months. These QDs were loaded on a Size Exclusion Chromatography (SEC) column for characterization of profile of elution. These QDs eluted with four overlapping peaks (fitted with Gaussian function in Origin) spanning over 15-30 mins, with peak of maximal elution around 25 min (**Figure 2-2**). The elution profile was exactly similar in both absorbance and fluorescence mode on the SEC, indicating that all four populations retain their fluorescence. Additionally, several iterations of ligand exchange followed by SEC on different batches of QDs (with same polymer) showed that the elution profile on SEC and ratio between the four populations of QDs remained consistent (**Table 2-1**)



**Figure 2-2** Elution chromatogram of QDs on a Size Exclusion Chromatography column. QDs coated with  $(20-80)_{20}$ -Zw was monitored on the SEC using absorbance (black) and fluorescence (grey) detection mode.

Table	2-1	Charact	eristics	of elution	of QDs	coated	with	$(20-80)_{20}$	-Zw po	lymer (	on SE	C after
ligand	exc	hange.										

Population	Elution range (min)	Elution time <sup>a</sup> (min)	Relative fraction <sup>b</sup> (%)
P1	15.5-16.3	15.9	2.7
P2	17.4-21.2	19.3	16.4
P3	20.8-23.0	21.9	15.6
P4	24.0-27.0	25.5	65.2

<sup>a</sup> peak of maximum elution. <sup>b</sup> fraction of selected peak with respect to the total QD elution

# 2.2 Conjugation of amine labelled DNA on thiols of QD

From one of the very first reports of QDs in biological applications, Dubertret et al. demonstrated that specific ligands on QDs can be used to conjugate DNA to these nanocrystals<sup>18,91</sup>. Since then, there have been a range of strategies employed to conjugate DNA to QDs. Several of these are discussed in detail in Chapter 1. For example, amine functionalized DNA (DNA-NH<sub>2</sub>) has been coupled to carboxyl group on a polymer-coated QDs by reaction with 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). 72,108 Another method to generate QD-DNA conjugates involves ligand exchange of ODs first with mercaptopropionic acid (MPA) followed by displacement of MPA by thiol-functionalized DNA (DNA-SH) directly, 43,45,108 or mediated by a linker.<sup>110</sup>Alternately, QDs have been functionalized with streptavidin first, followed by addition of a biotinylated DNA.<sup>103,113</sup> Nevertheless, each strategy has its own limitations. EDC-NHS coupling suffers from low conjugation efficiencies due to hydrolysis of NHS esters. Ligand exchange with thiol-labelled DNA is limited because the surface bound thiols can be easily oxidized by light or oxygen. Finally, the biotin-streptavidin strategy increases the size of QDs substantially thereby limiting its applicability. Also, conjugation of DNA on the QD surface is limited by the number of streptavidin available on the QD. Along with these limitations specific to chemical reactions, purification of QD-DNA conjugates is often tricky. Most conventional method is gel purification and centrifugation, which are both labor intensive and tend to decrease vields.<sup>93,114,115</sup>

In this work, the polymer used to coat the QDs displays both anchoring (thiol-functionalized monomer) and water-solubilizing (zwitterion-based monomer) groups. Thiols, in general, have very high affinity towards metal ions on the shell of nanoparticles such as QDs, GNP, SNPs and NPLs and thus are intricate part of various polymer designs. It was thus interesting to investigate whether some of the thiols present on this zwitterionic polymer could be used to conjugate DNA-NH<sub>2</sub> on QDs. Utilizing the thiols eliminates the need for incorporation of any additional functional groups specifically for conjugation, thereby simplifying coupling of biomolecules on QDs. Similarly, abundance of –NH<sub>2</sub> functional group in various biomolecules such as DNA, proteins and peptides additionally merits the general applicability of this work.<sup>74,79</sup>

**Conversion of amine-labelled DNA to DNA-maleimide.** The details of all the DNA sequences used are listed in Appendix 2. In the first step of the reaction, amine labelled DNA was reacted with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC), a commonly utilized heterobifunctional linker (sSMCC/DNA=25). This linker has an *N*-hydroxysuccinimide group on one end (reactive towards amine) and a reactive maleimide group (affinity towards thiols) on the other end. The reaction was kept for 30-45 min at room temperature under vigorous stirring. Excess of unreacted sSMCC was removed by precipitation of DNA-maleimide in absolute ethanol. The DNA-maleimide could be clearly seen as a pellet in the bottom of the tube post centrifugation. Quantification at this step using Cy5 labelled DNA showed recovery of >95% of DNA after precipitation, implying that no DNA is lost due to precipitation (**Figure 2-3**). The DNA-maleimide hence generated was used immediately for reaction.



**Figure 2-3** Synthesis of DNA-maleimide. (A) Reaction between amine labelled DNA and sSMCC to produce DNA maleimide. (B) DNA-maleimide pellets down upon ethanol precipitation. (C) Quantitative recovery of DNA after Ethanol precipitation.

*Activation of QDs by reduction of surface thiols.* In parallel to the reaction above, QDs need to be activated to expose thiols of the polymer which react to the maleimide-functionalized DNA. In our understanding, on the polymer, two species of thiols coexist: first, as S<sup>-</sup> linked to the surface of the QDs *via* complexation, and second are "free" dithiols or disulfides that can be converted to dithiols. In this work, focus is laid upon utilization of some of these thiols for conjugation with biomolecules. In order to increase the reactivity of QDs towards maleimide

functionalized biomolecules, QDs were first reduced. For this QDs were incubated with Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (TCEP/QD=400) (**Scheme 2-1**). TCEP was chosen over other reducing agents such as DTT since it prevents re-oxidation of the hence generated thiols, thereby prolonging the half-life of the transient thiol species. The solution was left under constant stirring for 30 min at RT. After reduction, the solution was passed through a size exclusion column in PBS to remove excess TCEP. The QD were then concentrated by ultra-filtration (100kDa) and used immediately.



Scheme 2-1 Reduction of the thiols on the polymer of QDs by TCEP.

*Reaction of DNA-maleimide with QD-thiols.* The reduced QDs were mixed with a solution of DNA-maleimide in PBS, 1X with DNA/QD=25/1 and left for stirring overnight at RT. The reactions was purified and analyzed after 12-16 hrs (Scheme 2-2).



Scheme 2-2 Reduction of the thiols on the polymer of QDs by TCEP.

*Purification of Conjugates.* Broadly, two methods were employed for purification of conjugates. First method used sucrose density gradient ultracentrifugation of QD-DNA reactions (40,000 RPM for 30 minutes). The QD-DNA conjugates (or unconjugated QDs) were separated from excess unreacted DNA owing to higher density. Post centrifugation, the fluorescent fraction (comprising of QD-DNA and QD) was then re-collected and buffer exchanged with NaHCO<sub>3</sub>by ultrafiltration and stored for further processing. The second method involved using SEC to purify QD-DNA/QD from unreacted DNA. SEC of only DNA (ss, 15 mer) shows a sharp elution after 35 mins (Figure 2-4). On the other hand the peak of

max absorption (smallest  $D_h$ ) of QD elutes close to 25 min. Hence this significant difference in time of elution can be reliably used to purify the conjugates from unreacted DNA.



**Figure 2-4** Chromatographic separation of QD and DNA. Difference in elution time between QD and DNA on the size exclusion column.

# 2.3 Validation of QD-DNA conjugates

After purification of the conjugates using SEC, it was important to validate the presence of DNA on the QDs. This was carried ut using both qualitative and quantitative approaches as discussed below.

#### 2.3.1 Qualitative approaches

*Elution on SEC.* Coupling of several strands of DNA on QD ideally changes the size of the QDs (and therefore the molecular weight). This difference would be reflected in the change of elution time of the most intense peak of QD on the SEC chromatogram and thus could be used for qualitative validation of conjugation. The sample of QD-DNA conjugates eluted marginally faster than the unconjugated QD sample on a size exclusion column, suggesting increase in the hydrodynamic diameter of the QDs due to conjugation of DNA (**Figure 2-5**, B).

*Electrophoretic migration.* Another qualitative test for successful coupling of DNA to QD was carried out by assessing the electrophoretic mobility of QD-DNA conjugates compared to QD. QD-DNA exhibited faster migration towards the positive terminal on 0.8% agarose gel in 50 mM Borate buffer (pH 8.5) comparison to QD, due to addition of negatively charged DNA (**Figure 2-5**, C).

Affinity beads based assay. To test the presence of DNA on the surface of QD, a simple affinity beads based assay was designed. It was observed that the polymer-coated QDs had minimal non-specific binding towards commercial agarose beads in NaHCO<sub>3</sub>. The QD-DNA conjugates were purified after reaction, and mixed with biotinylated DNA (DNA-Bt) having a complementary sequence to the DNA conjugated on the QDs. If there were DNA on the QDs post purification, then by hybridization, they were expected to be labelled by biotin (QD-DNA-Bt). These biotinylated QDs would ideally have affinity towards streptavidin agarose beads (SAB) (Figure 2-5, D). These QDs were mixed with SAB and the beads were washed three times to remove any non -specific adsorption of QDs on SAB. These beads were then observed under a fluorescence microscope with  $\lambda_{exc} = 450\pm25$  and  $\lambda_{ems} = 610\pm20$ , that enabled selective visualization of QDs. A clear fluorescent halo around the beads was visible as it can be seen on Figure 2-5, E(iii). (i) Unconjugated QD, (ii) QD-DNA without DNA-Bt and (iii) QD-DNA with random (non-complementary) DNA-Bt all showed minimal non-specific fluorescent signal with the SABs. This SAB based assay could be used for rapid validation of conjugation of DNA on QD. Further controls and detailed protocol are also provided in Section 5.2.2 (Chapter 5).

*Aggregation assays.* To test for the presence of DNA on the surface of QD, a simple aggregation based assay was designed. Purified QD-DNA conjugates were incubated with equivalent concentration of complementary DNA-Bt in presence of 10 mM MgCl<sub>2</sub>. These QDs were then washed by ultra-filtration (100kDa) twice to get rid of excess DNA-Bt. After this, the QDs were incubated with 0.25-0.5 molar excess of streptavidin solution for 5 mins. Then the conjugates were centrifuged at 12000 RPM for 30 mins. For negative controls, conjugates were hybridized with DNA-Bt and centrifuged (without addition of streptavidin).

In presence of QD-DNA-Bt, conjugates aggregated and pelleted to the bottom of the tube. In absence of streptavidin, QD-DNA-Bt remained in solution (**Figure 2-6**). After centrifugation, the supernatant was removed and the pellet was resuspended and seen under microscope (100X). Aggregates of QDs were seen where protein was added and not in the other tube. Additionally, no aggregation was seen in QDs (no DNA) mixed with streptavidin (data not shown).

In this section several qualitative methods to detect the presence of DNA on QDs have been discussed. Several of these methods, such as electrophoresis, have been routinely used in past by different groups. The issue with electrophoresis is that often the changes in the

electrophoretic migration would depend not only on the original ligands at the surface of QDs, but also on the number of DNA conjugated. Other methods such as difference in elution time can be used only in case of reactions with high conjugation yield. Similarly, aggregation based assay would also be successful if QDs should have more than 1 biotinylated DNA on the surface of QDs. Additionally aggregation may be caused by several factors such as loss of solution stability, which may lead to falsified conclusions. In our experience the agarose beads based assays are rapid, very sensitive and reliable for detection of success conjugation of DNA. However, two fundamental aspects need to be carefully evaluated. First is the non-specific adsorption of different polymeric coatings to the SABs. Second it has been observed that the non-specific adsorption may also be dependent on the pH of the buffer. Upon careful evaluation of these two parameters, this assay appears to be the most sensitive and reliable method to detect DNA on QD.



**Figure 2-5** Qualitative validation of conjugation of DNA on QD. (A) Schematic of reaction of conjugation of thiols on QD to maleimide functionalized DNA. (B) Difference in elution time of QD (black) from QD-DNA (grey). (C) Faster elution of QD conjugated to DNA (ss or ds) in comparison to QD. (D) Schematic of principle of streptavidin beads assay. (E) Beads are fluorescent with QD-DNA-Bt, validating the assay. In all other cases, fluorescence is low to

negligible. Beads were imaged with  $\lambda_{exc}$ = 450±25 and  $\lambda_{ems}$ =610±20 and the indicated scale bar is 100µm.



**Figure 2-6** Aggregation assay using streptavidin protein. (A) Principle of assay (B) photograph of tubes of QD-DNA-Bt with (indicated by +) or without (indicated by -) streptavidin protein before (left) and after (right) centrifugation under UV illumination. Microscopic image (100X) of solutions with protein shows aggregation

#### 2.3.2 Assessing non-specific interactions of DNA with QD

As discussed in the previous chapter, specific charged ligands may cause. To further support the above qualitative tests, specific experiments to ascertain non-specific adsorption of biomolecules to QDs were done. The non-specific interaction of DNA with the polymer coat on QDs was assessed by two tests. First was based on SABs binding assays. Solutions of (i) uncoupled QD mixed with DNA-Bt (ii) QD-DNA with non-complementary DNA-Bt were incubated with SAB and visualized under fluorescent microscope. The beads remained non-fluorescent, thereby confirming the absence of non-specific interaction between DNA and QDs (**Figure 2-7**, A). Several other non-specific absorption controls are also discussed in chapter.

Another approach used dual mode of detection on SEC. Samples of (i) QD mixed with Cy5 labelled DNA and (ii) QD-DNA mixed with Cy5 labelled non-complementary DNA were

loaded on the SEC and monitored using absorbance at two wavelengths – 350 nm (black, for QD) and 630 (grey, for Cy5). Then the presence of Cy5 signal during the elution of QD was estimated. No Cy5 fluorescence was detected corresponding to the QD and QD-DNA, validating that there is minimal non-specific adsorption of DNA-Cy5 on QDs (**Figure 2-7**, B).



Figure 2-7 Comparison of specific vs non-specific adsorption (A) QD-DNA with complementary DNA-Bt (i) QD-DNA with non-complementary DNA-Bt (ii) QD-DNA with non-complementary longer DNA-Bt (iii) QD only mixed with DNA-Bt (iv). (B) HPLC elution chromatogram of QD mixed with DNA-Cy5 (i) and QD-DNA mixed with non-

complementary DNA-Cy5 (ii). Chromatogram corresponding to absorbance at 350 nm for QD and QD-DNA (black line) and absorbance at 630nm nm DNA-Cy5 (grey line) is plotted for the corresponding mixtures. No absorbance of Cy5 is seen at retention volumes of QD/QD-DNA (15-27mL), suggesting absence of co-elution of DNA thereby proving lack of non-specific absorption in both.

#### 2.3.3 Quantification of number of DNA conjugated on QD

For quantification of reactions described in this work, two approaches were taken – first, the DNA labelled with fluorophore at C-terminal was conjugated to the QDs. Alternately for reactions with high conjugation yield, change of absorbance at 260nm of QD upon conjugation of DNA was measured (**Figure 2-8**).



**Figure 2-8** Quantification of DNA conjugated to QD. Absorption spectrum of QD (black) and QD-DNA (red) after SEC purification is shown. DNA is labelled with Cy5.5. The presence and quantification of DNA on QD can be carried out both by the absorbance of fluorophore at 680nm (inset) and by DNA at 260 nm.

# 2.4 Parameters that affect the yield of conjugation of DNA to QDs

In the previous section, several methods to validate the presence of DNA on QDs after conjugation were discussed. Reaction with dual labelled DNA with 5'primary amine and 3' Cy5 on QD showed that in these conditions, approximately 2-3 DNA molecules could be

conjugated to QDs. The ability to conjugate biomolecules on the surface of QDs could depend on variables such as total number of functional groups accessible for conjugation, pH of the reaction media, dimensions of the biomolecule (mostly relevant for proteins), total charge on the biomolecule etc. It was then interesting to see whether this conjugation yield could be improved by further optimizing several of these conditions.

#### 2.4.1 Role of the polymer coat

As discussed in the introduction chapter, conjugation of DNA is very different from conjugation of proteins to QDs (and other NPs). One of the causative factors for such differences is the total number of functional groups available for conjugation. Reactive groups such as NH<sub>2</sub> and COOH are abundant on proteins, owing to amino acids such as lysine, arginine, aspartic and glutamic acid. Contrarily, the functional groups on DNA are synthetically incorporated. Hence by design, oligonucleotides are monofunctionalized. Therefore, there is only one reactive group per DNA (unless synthesized otherwise). In the reaction discussed in this chapter, the second reactant is thiol of the polymer on the QDs. By intrinsic design, the polymer synthesis comprises of 20% monomer derived from a-Lipoic acid and 80% zwitterions with average chain length of approximately 20 monomers. This lends into statistically 2-4 dithiol groups per polymer chain. Some of these thiols from the lipoic acid will anchor the QD surface and keep the polymer attached to the QDs. Others remain as disulfides, which can be conjugated to biomolecules. Additionally, the dynamic nature of thiol-metal interactions may have some transient reactive thiols that also facilitate covalent conjugation of biomolecules on the polymer. It was then interesting to investigate whether the QDs could be made more reactive, by incorporation of additional thiols. With that motivation  $(50-50)_{10}$ -Zw was synthetized as described in Section 5.1.3 (Chapter 5). As such, polymer should possess approximately 5 (or more) monomer units derived from a-Lipoic acid instead of 2-4 for the (20-80)<sub>20</sub>-Zw. Further increase in the mole fraction of the thiotic acid derived monomer (and concomitant reduction in mole fraction of sulfobetaine) reduced the solubility of polymers in aqueous buffers and therefore could not be used (data not shown).

In the same conditions (QD/TCEP: 1/400, DNA/QD:25/1, DNA length:15 mer) DNA conjugation was carried on QDs coated with this new polymer. As it can be seen in **Table 2-2**, the conjugation efficiency on the QDs comprising of higher mole fraction of dithiol monomer

is higher. This implies that the polymer design can be further fine-tuned to improve the conjugation yield of this reaction.

Ta	ble	2-2	Conj	ugation	of ss	DNA	15	bp	length	on	different	types	of poly	mer-co	ated	QD
(re	actic	on co	onditi	on: TC	EP/QI	D = 40	0/1,	, DN	NA/QD	=25	5/1 in PBS	5 pH 7	.4)			

Polymer	% dithiol <sup>a</sup>	Length/ polymer <sup>b</sup>	#dithiol/ polymer <sup>c</sup>	Conjugation yield <sup>d</sup>
$(20-80)_{20}$	20	20	2-4	20
$(50-50)_{10}$	50	10	5	60

<sup>a</sup> % mole fraction of monomer 1 derived from a-Lipoic acid in the initial synthesis reaction. <sup>b</sup> average number of monomers per polymer chain. <sup>c</sup> statistical number of dithiols per polymer chain. <sup>d</sup> Percentage of total DNA finally conjugated with respect to initial excess of DNA calculated using absorbance at 260 nm.

#### 2.4.2 Effect of reducing agent

In previous sections, an outline of reaction of primary amine labelled DNA to thiols on the polymer of QDs was described. Since the reaction involved thiols, it was hypothesized that reducing agents may have a detrimental role in the efficiency of coupling of DNA to the polymers.

In general, it was seen that DNA-maleimide can be conjugated to QDs in absence of reducing agent, albeit with very low efficiency (<10%). Addition of a strong reducing agent like TCEP substantially improves the efficiency of conjugation. This effect however is not linear. On experimentation with different (100X-1000X) molar excess of TCEP with respect to QD, it was found that the effective increase in number of DNA conjugated was *not* stoichiometric. Several possible factors could explain this effect.

(1) Polydisperse nature of the polymer synthesis: the *co*-polymerization reaction results in synthesis of a range of polymers with statistical average length and composition. Within this average, there are several populations of polymers with a heterogeneous distribution of dithiol bearing groups.

(2)Thiol-dithiol equilibria during reduction and purification procedure: the effective number of thiols, available for conjugation, was insensitive to concentration of TCEP beyond a given regime.


**Figure 2-9** Effect of TCEP on photostability of QDs (A) change of intensity of a layer of QD on glass slide upon UV excitation for is shown in presence of 400 X TCEP (top) and 40000 X TCEP (below). (B) Time-dependent loss of fluorescence intensity of a fixed region of slide upon continuous excitation is shown. The graph shows progressive and faster loss of fluorescence in case of 40000X TCEP (black) in comparison of no TCEP (red) or 400 X TCEP (blue) (C) QY of QDs exposed to different molar excess of TCEP is shown as a function of time. Sample with 40000 X TCEP loses 50% of its QY within 24 hours of incubation.

On further experiments with much higher excess of TCEP (5E4-5E6 molar excess) it was seen that the conjugation efficiency improved only marginally; however the QDs became highly susceptible to photobleaching (**Figure 2-9**). These QDs where drop-casted on glass slide and visualized under microscope (at 100X). It was observed that the fluorescence intensity was rapidly reduced and the QDs photo-bleached within minutes. Also, at  $\sim 5X10^6$ 

molar excess of TCEP (per QD), the QDs could be seen to precipitate out of solution within 30 minutes. This can be explained by simple desorption of polymers from the surface of the QDs caused by the following two factors.

- The phosphine in TCEP is a very strong ligand for the QD surface. At very high concentrations, TCEP may sufficiently displace the thiol from the surface of QDs, hereby causing total desorption of the polymer.
- (ii) In higher concentrations, TCEP can cause abrupt reduction in the pH of the medium (<4.5). At this pH also, QDs have been shown to have lower QY and solution stability, possibly due to instability of the polymer coat on the QD.

These experiments conclude that though the reducing agent assists in increasing to conjugation yield of this reaction, but must be used after proper optimization. In these reactions, a range between 100-1000X of TCEP per QD was seen to not affect the solution stability of QDs. Therefore, for subsequent experiments 400X molar excess of TCEP used as an optimal excess. At this excess of TCEP, conjugation efficiency was higher without compromising on the photostability of the QD.

#### 2.4.3 Effect of pH of medium

The classical reaction of DNA conjugation on QD is carried out at pH 7.4 and buffered with PBS pH 7.4 at room temperature. For this experiment, the pH of DNA + sSMCC reaction was not changed and kept at pH 7.4. Since thiol-maleimide reaction is favored at pH 6-6.5, reactions were set spanning pH 6-8.5 in increments of 0.5 unit of pH (in HEPES). It was observed that pH does not improve the conjugation efficiency at all (**Table 2-3**). A similar study carried out in the group in past demonstrated that in this pH regime, physico-chemical properties of QDs remain fairly similar. It was thus concluded that within the physiological regime, this reaction was insensitive to pH.

**Table 2-3** Conjugation of ss DNA 15 bp length on QDs coated with  $(20-80)_{20}$ -Zw at varying pH (reaction condition: TCEP/QD = 400/1, DNA/QD = 25/1)

рН	6	6.5	7	7.5	8	8.5
#DNA/QD <sup>a</sup>	2.5	3	2.2	3.2	3	2.4

<sup>&</sup>lt;sup>a</sup> calculation of number of DNA conjugated per QD post purification by SEC using absorbance at 260 nm.

#### 2.4.4 Effect of salt concentration

During the conjugation reaction of DNA on QDs carried out at  $\sim$ pH 7.4, the zwitterionic polymer was expected to be electrostatically neutral, but charged. The solvent exposed sulfobetaine group of the polymer should be negatively charged at this pH. The phosphate groups of DNA should also be negatively charged at this pH. The similarly charged species might exhibit limited interaction in solution. In order to test this hypothesis, the effect of salt in screening the negative charges (and thus influencing the reaction efficiency) was tested (**Figure 2-10**)



**Figure 2-10** Effect on salt on conjugation of ss DNA on QD. Schematic showing conditions of (A) low salt and (B) high salt in conjugation of DNA to QD. Analysis of QD (dotted lines), QD-DNA-Cy5 in low salt (dashed lines) and QD-DNA-Cy5 in high salt (solid lines) by different approaches is shown. (C) Elution chromatogram from SEC. Inset shows zoom in between 22.5-25.5 min (D) Normalized absorbance spectra (at 450nm) after purification. Inset

shows zoom in between 550-700 nm corresponding to Cy5 absorbance. (E) Emission spectra of conjugated DNA (Cy5). (F) Saturation of conjugation efficiency of DNA to QD at high salt conditions. Inset shows number of DNA conjugated per QD.

Typically for these experiments, the requisite pellet of DNA-maleimide was resuspended in NaCl solution of higher than desired strength, and mixed with reduced QD to obtain the desired NaCl concentration in the final solution and left overnight for reaction.

On increasing the salt concentration to 1400 mM in the final reaction, several interesting properties in the conjugates were observed. In the elution chromatogram, the peak of maximum absorption shifted by more than 60 seconds towards left, *i.e* towards faster elution (**Figure 2-10**, C). Since this was an SEC column, this shift indicated substantial change in the molecular weight/size of the conjugation, and hence greater conjugation efficiency. Similar high signal was seen for Cy5, when ssDNA labelled with this fluorophore was conjugated on the QDs (**Figure 2-10**, C). In parallel, QDs were simply mixed with 1400 mM NaCl, desalted and injected in SEC to test whether faster elution was an effect of organization of the zwitterionic polymer in specific configuration. The QDs treated with salt eluted exactly as untreated QDs, confirming that the elution shift is an effect of conjugation of DNA to QDs. (data not shown).

On quantification using absorbance spectroscopy it was found that the number of DNA conjugated per QD could be increased to  $14\pm 2$  from  $3\pm 1$  at 140mM NaCl (**Table 2-4**) in the best conditions. Hence it was concluded that salt dramatically improved the conjugation efficiency of DNA to QD in this reaction.

**Table 2-4** Conjugation of ss DNA 15 bp length on QDs coated with  $(20-80)_{20}$ -Zw at varying salt conditions (reaction condition: TCEP/QD = 400/1, DNA/QD = 25/1 in PBS pH 7.4 additionally supplemented with indicated excess of salt)

NaCl conc (mM) <sup>a</sup>	Excess DNA added <sup>b</sup>	#DNA/QD°
140	40	3±1
1400	25	12±4

<sup>&</sup>lt;sup>a</sup> concentration of NaCl in the final reaction. <sup>b</sup> initial excess of DNA added. <sup>c</sup> final number of DNA conjugated per QD after purification by SEC using absorbance at 260 nm.

Another interesting experiment was to find out whether conjugation efficiency scales linearly with salt concentration. To test this, reduced QDs were mixed with 25X excess of ssDNA-maleimide in presence of different concentrations of NaCl at pH 7.4. These reactions were left over night and desalted next day before purification via the SEC. Post purification, quantification of the conjugated excess of DNA showed that the maximum number of DNA/QD conjugated is 12±4 and the % conjugation efficiency cannot be increased over 40% (**Figure 2-10**, F). Nevertheless this was the highest conjugation efficiency observed in optimized conditions. The improvement in conjugation efficiency by addition of salt can be explained by three possibilities

- (i) The polymer used in this reaction is zwitterionic with negatively charged functional group exposed to the solvent. Similarly the phosphodiester backbone of DNA is highly negatively charged. These two similar charges are highly repellant and may prevent *solution proximity* and thus reactivity of the DNA-maleimide with QD-SH. However addition of excess of salt shields the highly charged surfaces and increases proximity and local concentration of reactants.
- (ii) Several reports in past have demonstrated that additional of salts improves solvation of the zwitterion, which may positively interact with *'incoming'* reactive ligands. Similarly, organized rearrangement of zwitterions around polyelectrolytes such as DNA can help in increasing the local concentration of DNA in proximity to the QDs, facilitating better reaction yields.
- (iii) In presence of excess salt, the conformation of ssDNA further rigidifies and orients the maleimide group in a more accessible (and hence reactive) configuration facilitates the reaction.<sup>116,117</sup>

To further confirm the extent to which the zwitterionic polymer affects the conjugation reaction, an electrostatically neutral polymer was designed (Scheme 2-3). In this case, the sulfobetaine group was replaced by PEG (polyethylene glycol, n=8). The polymer obtained is thus  $(20-80)_{20}$ -PEG. This polymer ensured that there are no patches of local charge. This design enabled to further confirm the detrimental role of local charges on the conjugation yield of this reaction. The results are summarized below (Table 2-5).



**Scheme 2-3** Chemical structures of two types of functionalizing monomers used for these experiments namely sulfobetaine with zwitterionic nature (top) and polyethylene glycol (PEG) with neutral nature (bottom)

**Table 2-5** Conjugation of ss DNA 15 bp length on QDs coated with different polymers (reaction condition: TCEP/QD = 400/1, DNA/QD =25/1 in PBS pH 7.4 additionally supplemented with indicated excess of salt)

Co-monomer <sup>a</sup>	[NaCl](mM) <sup>b</sup>	#DNA/QD <sup>c</sup>
Zw	140	2
Zw	1400	8
PEG	140	5
PEG	1400	6

<sup>&</sup>lt;sup>a</sup> monomer of sulfobetaine (Zw) or polyethylene glycol (PEG) copolymerized with dithiol monomer in 4:1 ratio respectively <sup>b</sup> Concentration of NaCl in the final reaction <sup>c</sup> Final number of DNA conjugated per QD after purification by SEC using absorbance at 260 nm.

These experiments suggested that charges on the QDs as well as DNA can impact the conjugation efficiency. Several additional inferences could be drawn.

- (i) In general, neutral surface like PEG is more reactive towards conjugation of DNA in comparison to relatively charged zwitterions. This suggests that, the relative conformations and solvent accessibility of local patches of polymers *on* the surface of QDs can severely impact the conjugation efficiency.
- (ii) For the PEG polymer, the conjugation efficiency is similar irrespective of the salt concentration. This implies that the conjugation yields, at least under these conditions, are widely dependent on the surface properties of the QDs (and the polymers).

#### 2.4.5 Effect of DNA length

Since DNA is a polyanion, it was then interesting to assess the variation in conjugation efficiency with respect to the length of DNA. The experiments for coupling different length of DNA on QD were carried out at NaCl concentration of 1400mM. It was found that despite screening of charges by addition of high salt, the coupling is both size and charge limited. The total number of DNA/QD for single stranded DNA was  $12\pm4$  for 15 bases and  $6\pm2$  for 45 bases length (**Table 2-6**). Similar total charge limited conjugation was also seen in reaction of single *vs* double stranded DNA labelled with Cy5. Upon conjugation ds DNA of 15bp length (same as before), the total number of DNA conjugated was  $4\pm1.3$  per QD.

**Table 2-6** Conjugation of DNA of different length and strandedness on QDs (reactioncondition: TCEP/QD = 400/1, DNA/QD = 25/1 in 1400mM NaCl)

DNA length <sup>a</sup>	strandedness <sup>b</sup>	#DNA/QD <sup>c</sup>
15	SS	12±4
15	ds	4±1.3
45	SS	6±2

<sup>a</sup> number of nucleotides per strand of DNA. <sup>b</sup> number of strands of DNA- ss: single stranded, ds: double stranded <sup>c</sup> final number of DNA conjugated per QD after purification by SEC using absorbance at 260 nm.

Similar experiments were done on QDs emitting at different wavelengths also, and will be discussed in the subsequent sections.

# 2.5 Experiments on stability of QD-DNA conjugates

After the conclusive validation of conjugation of DNA to QDs, it was then interesting to test the photophysical and solution stability of these conjugates. These experiments are described in the following section.

#### 2.5.1 Quantum yield measurements on QD-DNA conjugates

Many articles have discussed the loss of QY of QDs upon conjugation with biomolecules, including DNA. In order to test this, QY of QDs were recorded for NaHCO<sub>3</sub> for up to 1 month (

**Table 2-7**). DNA were grafted using the conditions explained above (TCEP:400/1, DNA/QD:25, salt:140 mM). The loss of QY measured was <20% for up to 15 days, and <30% for about a month on different batches of reaction.

#### 2.5.2 Solution stability of QD-DNA conjugates

Many reports in past have discussed the loss of stability of QD due to desorption of polymers in dilute solutions. A detailed account is provided in the review by Sperling *et al.*<sup>42</sup> To assess the stability of QD-DNA conjugates in dilute solutions, fluorescence of dilute solutions of 25 nM were monitored for over a week in biological buffers such as PBS and NaHCO<sub>3</sub>. The samples remained fluorescent even in dilute conditions. To further assess the stability of QD-DNA conjugates in solution, the presence of fluorescence of QD-DNA-Bt on streptavidin beads was monitored for three days (**Figure 2-11**). The beads remained fluorescent over time, hereby suggesting that the polymer were not lost from the QD surface. These results also confirmed, that the specific polymer strands that were coupled to DNA, were also stable on the QD in dilute solutions over days.



**Figure 2-11** Stability of QD-DNA on SABs after repeated washing on (i) day 1 (ii) day 2 and (iii) day 3. The beads retain their fluorescence validating the stability of QD and the polymer covalently coupled to DNA on agarose beads in solution.

### 2.6 Applicability of the coupling strategy

As mentioned in the beginning of the chapter, there is continued interest in investigating conjugation strategies that can be extended to different types of nanoparticles and biomolecule. Since (i) thiols are an intrinsic component of several types of ligands for nanoparticles and (ii) amines are abundant in biomolecules, it was interesting to investigate the scope of this strategy to different nanoparticles and biomolecules. The following section covers some of these studies.

#### 2.6.1 On QDs emitting at different colors

To test the applicability of this strategy to graft DNA on different types of QDs irrespective of their synthesis protocols, ligand exchange was carried out on QDs emitting at 4 different wavelengths -550 nm, 580 nm, 610 nm and 650 nm. A brief characterization of these QDs (in hexane) is described in Appendix 1. For these reactions, QDs were ligand exchanged with (20-80)<sub>20</sub> -Zw and conjugation of ssDNA (15mer) was carried out as discussed above, in PBS pH 7.4. The presence of DNA on QD was tested with streptavidin agarose assay as described before (**Figure 2-12**). All four types of QD-DNA conjugates could be seen on the agarose beads when hybridized with biotin labelled complementary DNA. In absence of complementary DNA labelled with biotin, QD-DNA conjugates did not associate with the streptavidin agarose beads, thereby confirming the presence of DNA on the surface of the QDs. Only qualitative analysis of the conjugation of DNA on commercially available QDs of different types was carried out for this work.



**Figure 2-12** Generality of coupling strategy to different types of Quantum dots. the conjugation strategy was tested on four different types of commercially available QDs coated with  $(20-80)_{20}$ Zw polymer. Fluorescent microscopy images of SABs mixed with QD-DNA-Bt withQD550 (Green), QD580 (Yellow), QD610 (Orange), QD650 (Wine Red) ( $\lambda$ exc=450±25 nm,  $\lambda$ ems=Long pass 485nm). The images have been colored using ImageJ. Insets are QD-DNA-Bt on SABs after 3 washes in tubes, illuminated using UV-lamp without any image processing.

Similar to QDs emitting at 610 nm coated with  $(20-80)_{20}$ -Zw polymer, all the QDs emitting at different wavelengths upon conjugation to DNA with the above method retained high QY post conjugations. (**Table 2-7**). It could thus be concluded that the QD-DNA conjugates retained their QY for long durations and hence valuable for downstream applications.

**Table 2-7** Quantum yield of QDs upon conjugation of ssDNA stored in NaHCO<sub>3</sub> pH 8.3 in the dark at 4°C for indicated period of time.

$\lambda_{em} Q D^a$	QY <sup>b</sup>	QY <sub>QD-DNA</sub> <sup>c</sup>	$QY_{QD-DNA}^{d}$
550	37	<u>(Day1)</u> 26	<u>(Day10)</u> 21
580	20	15	27
610	54	46	43
650	15	14	17

<sup>a</sup> Wavelength of emission of QD dispersed in water after ligand exchange with Zw(20-80)<sub>20</sub>. <sup>b</sup> Quantum yield of unconjugated QDs. <sup>c</sup> Quantum yield immediately after conjugation with DNA. <sup>d</sup> Quantum yield of conjugates after storage for 10 days. All Quantum yield measurements were carried out in NaHCO<sub>3</sub> pH 8.3 with reference to rhodamine 6G

#### 2.6.2 Conjugation of DNA to other nanoparticles

To further investigate the generality of this conjugation strategy, the conjugation of DNA was additionally carried out on (i) gold nanoparticles (GNP) and (ii) Cd-based nanoplatelets, both commercially obtained from Nexdot. These nanoparticles were first coated with the (20-80)<sub>20-</sub> Zw polymer using two step ligand exchange procedure. After this step, conjugation reactions with ssDNA (15 mer) were carried out as described and the presence of DNA on the purified conjugate was validated by SAB assay.

For conjugation on GNP, the protocol was specifically optimized. Initial conjugation reactions with 400X TCEP did not result into desired product. But when the molar excess of TCEP was increased to 50,000 and with an excess of DNA/GNP of 50 (in 1400 mM NaCl), signs of positive conjugation of DNA on the GNP were visible by SAB based assay (**Figure 2-13**). It was seen that the minimum concentration of TCEP required for conjugation of DNA on GNP was at least 125 times higher than for QD. This result is explained by the understanding that since thiols have higher affinity for GNP than QD, stronger reducing environment may be necessary to make thiols available for conjugation. Hence huge excess of TCEP helps enhance the coupling with the polymer.<sup>118</sup> The reaction was not optimized further. Nevertheless it was conclusively demonstrated that the same strategy could be used to couple DNA to GNPs with minor customization.

Similar experiments were done to detect the presence of DNA on NPLs (by SABs visualized using with microscopy, data not shown).



**Figure 2-13** Validation of conjugation of DNA to GNP using SABs. GNP-DNA-Bt attaches to SABs and is not removed after several washes (left). However, GNP-DNA in absence of complementary DNA-Bt do not show any affinity for the beads (right).

#### 2.6.3 On different lengths of DNA

As shown in section 2.4.5, the same strategy of conjugation described herein could be used to conjugate DNA of different length and strandedness on QDs displaying similar surface chemistry. These conjugates with saturating conjugation yield of DNA/QD ( $12\pm4$  for 15mer and  $6\pm2$  for 45 mer) eluted at similar times, suggesting the boundary conditions for maximally optimized conjugation was attained ().



**Figure 2-14** Conjugation of different length of DNA on QD. Chromatogram showing change in retention time based on length of DNA conjugated on QD

#### 2.6.4 Conjugation of proteins using similar strategy

This coupling strategy could be extended to conjugating proteins such as streptavidin, BSA and transferrin on QDs (Chapter 5). Experiments shown by others in the group have also demonstrated that this strategy could be extended to conjugation of protein A, that was then used to attach antibodies with preserved epitope binding orientations.<sup>79</sup> These results point towards the potential for use of this strategy for various applications.

# **2.7 Conclusions**

Functionalization of biomolecules on nanoparticles is a topic of emerging interest. Particularly, applications of functional molecules like nucleic acids on QDs have seen a lot of progress in terms of synthesis and characterization recently. The QD-DNA conjugates have also found numerous applications in biomedical imaging, biosensing and also in optoelectronics.

In this work a new approach to conjugating DNA on surface of polymer coated QDs was described, and various parameters that influence the reaction were discussed. These results demonstrate (1) a novel method to conjugate DNA covalently to the polymer coating the surface of QDs (2) promising role of higher salt concentrations to improve coupling efficiency (3) the accessibility of the hence coupled DNA for hybridization despite the presence of a highly charged polymer (4) minimal non-specific interactions of DNA with QD. The QD-DNA conjugates prepared by the presented method have high efficiency of coupling (40% of total DNA excess), higher QY than reported before (80-100% of the uncoupled QD) and enhanced solution stability. This method could be easily adapted to a range of QDs and other nanoparticles, different lengths of DNA and types of proteins. Hence this work presented an exhaustive evaluation of a novel method of conjugation that is invaluable for the synthesis of biofunctionalized nanoparticles for applications like cellular imaging and biosensing.

In the next chapter an interesting application of these QD-DNA conjugates for bioimaging is discussed.

# **Chapitre 3 Quantum Dot-DNA Conjugates for Controlled Assembly of Transferrin**

# **3.1 Introduction**

In previous chapter, a novel method to couple DNA to Quantum dots (QDs) to synthesize conjugates of QD-DNA has been discussed in detail. Careful material design and evaluation of the conjugation strategy has set basis for QD based probes with high quantum Yield (QY) and compact size. In this chapter a novel method to conjugate proteins to QDs is discussed. In this method, the conjugates of QD-DNA (as described before) are used to assemble proteins labelled with complementary DNA mediated by DNA hybridization. This work is carried out with a model protein Transferrin (Tf) such that Tf labeled QDs (QD-DNA-Tf) are produced. Further, the biochemical and intracellular properties of these conjugates have been investigated in detail. These probes have been shown to recapitulate several of the features of endogenous transferrin. These probes have additionally been used to visualize endosomal dynamics for several tens of minutes, paving way for a more comprehensive use of QDs for bioimaging. Careful design of the probe (selection of surface chemistry of QDs) and the conjugation strategy and post synthetic evaluation has resulted in development of new generation of probes that can be easily used to harness specific pathways for durations seldom achieved by organic dyes.

#### 3.1.1 Quantum dots as bioimaging agents

As discussed in the previous chapters, Quantum Dots (QDs) are very interesting probes for numerous biological applications such delivery of bioactive molecules<sup>103,119</sup> and bioimaging<sup>3,28</sup>. Specific properties like broad absorption with narrow emission make them very useful for multicolor imaging. Additionally very low photobleaching and reduced photo blinking enable long duration bioimaging at time-scales unsurpassed by organic fluorophores.<sup>7,120</sup> Despite such exceptional properties, the imaging of intracellular processes using QDs have limited success, due to numerous complexities arising from conjugating QD to proteins. First, for most applications, QDs are conjugated to specific targeting agents like

proteins. The conjugation strategies need to be carefully evaluated so as to preserve the properties of both conjugated proteins and QD. For example, QDs should retain their high fluorescence QY and stability in solution. Second, the conjugated proteins should retain their intracellular properties such as mechanisms of uptake (endocytosis), intracellular fate (steady-state localization) and clearance (degradation or recycling). Lastly, the conjugates is also dependent on the ease and reproducibility of the strategy. These factors affect the global use of QD-protein conjugates for biological applications. In this section, the current issues and the state of the art of using QD conjugates is detailed.

#### 3.1.2 Methods to conjugate proteins to QD

In Chapter 1, numerous methods to conjugate DNA to QDs are discussed in detail. Most of these methods of conjugation can be also used to link proteins to QDs. Before addressing the novel conjugation strategy mediated by DNA hybridization developed in this thesis, a general overview of existing methods is provided. Briefly, the methods can be divided into covalent and non-covalent methods.

*Covalent methods.* Covalent methods of conjugation involve the use of commercially available linkers such as EDC, NHS, SMCC, SPDP etc. to conjugate specific functional groups of proteins to ligands on QDs. QDs are first solubilized in water by coating them with amphiphilic ligands. These ligands can be both small molecular ligands and bigger polymer based ligands. These ligands have been discussed in detail in Chapter 1. The ligands can be additionally developed to facilitate conjugation of biomolecules. Proteins are made up of hundreds of amino acids, with several functional groups exposed to the solvent and available for conjugation (**Scheme 3-1**). Based on the target functional group on the protein, two most popular reactions are described below.

(i) Carboxylic acid on QD to amine of protein: QDs coated with ligands displaying carboxylic acid are first activated with an excess of EDC (5 < pH < 7.5). This activation step prepares a reactive O-acylisourea intermediate that can react with and amine functional group of proteins presence of NHS to form a stable amide bond. This is one of the most popular and frequently used methods of conjugation of proteins to QDs.<sup>2,121</sup>

(ii) Thiol of QD to amine of protein: reactive linkers like SMCC have an NHS functional group (reactive to amine) and a maleimide functional group (reactive to thiol). These zero length linkers conjugate proteins to QD without additional increase in size <sup>79,122</sup> Another class of thiol to amine cross linker is SPDP.<sup>123</sup> Herein the amine on QD can be reacted with the NHS of SPDP to give a pyridyl-disulfide derivative. This molecule can be cleaved in presence of a reducing agent to generate reactive thiol, which can be conjugated to exposed and reduced cysteine of the concerned protein.



Scheme 3-1 Amino acids with functional groups commonly used for conjugation of proteins to QDs.

*Non-Covalent methods.* There are several types of non-covalent methods used for conjugation of proteins to QDs:

(i) Polyhistidine tag: proteins are often genetically engineered to incorporate a hexahistidine tag to simplify the purification using affinity chromatography. This tag is preferably introduced either in the N or the C terminal of the protein, and can bind to Ni<sup>2+</sup> chelated Nitrilotriacetic acid (Ni-NTA). It was found out that polyhistidine has very high affinity towards to cationic shell of QDs as well.<sup>29,35,124</sup> This affinity has been successfully used to attach polyhistidine tagged protein to QDs.

This method generates compact and spatially oriented protein conjugates rapidly. However, the affinity of polyhistidine tags is pH-dependent. Additionally proteins with functional N or C terminals cannot be attached to QDs via polyhistidine tag for steric consideration.

- (ii) Biotin streptavidin affinity: QDs conjugated with biotin can be instantaneously mixed with streptavidin to assemble proteins on QD.<sup>31,114,115</sup> Another layer of biotinylated protein can be subsequently added on this streptavidin layer, giving rise to protein functionalized QDs. Alternately, streptavidin can be conjugated on the QD (via covalent or non-covalent approaches described above), followed by addition of biotinylated protein. This is one of the most popular strategies. However, as can be seen by the design the average size of conjugates is much bigger. This can limit specific applicability of the conjugates, particularly in context of neuronal targeting etc.
- (iii) Fragment affinity: this is a special strategy to specifically couple functional antibodies on the surface of QDs. In the first step, the linker protein (such as protein A or G) is coupled to QDs. Then the antibody of interest is mixed which attaches to this layer of protein by cognate hydrogen bonding. In this manner, a range of antibodies can be assembled on QD-protein A complexes with preserved orientations.<sup>125</sup> These conjugates can then be used to identify specific biomolecular targets. However, there are several limitations of this strategy. Only immunoglobulins can be assembled by this method. Additionally conjugation of an intermediate layer of protein increases the size of the conjugates. Since the binding of immunoglobulins to protein A is based on H-bonding, the stability of these conjugates is often dependent on pH.

S.No	Approach	QD	protein	Advantages	Disadvantages	Refs.
Covale	nt Conjugation		-	~	~	
1	EDC-NHS	Carboxylic acid	Amine	Rapid, one step	Poor yield	2,121
			(Lysine/arginine)	Easy to perform	Protein crosslinking	
				• Compact	No orientation control	
2	SMCC/MBP	Thiol	Amine	• Rapid	• Two step procedure	79,122
			(Lysine/arginine)	Moderate vield	• Each step requires purification	
				Compactness retained	No orientation control	
3	SPDP	Amine	Thiol (Cystein)	Moderate vield	• Two step process	123
				Compact	• Disulfide prone to reduction	
				I I I I I I I I I I I I I I I I I I I	• Protein can denature and functionality upon cleavage of cysteine disulfide	
					<ul> <li>No orientation control</li> </ul>	
Non-C	ovalent Conjugation					
4	Poly(Histidine) tag	Metallic shells	N/C terminal histidines	<ul><li>Instantaneous assembly</li><li>High conjugation efficiency</li></ul>	• Not applicable to proteins with functional N/C terminal	29,35,124
				<ul><li>Controlled orientation</li><li>Compact</li></ul>	• Proteins 'buried' within existing legends on QD surface	
				·	• Proximity to highly charged QD surface can cause local denaturation	
					• Stability depends on pH	
5	Streptavidin-	Biotin modified	Streptavidin	• Facile and rapid	• Two step	31,114,115
	biotin		L	• High vield	• Only valid for biotin modified OD with	
				Monovalency possible	streptavidin or biotin modified protein with streptavidin OD	
					• Large size of conjugates	
					High aggregation and cross linking	
5	Fragment affinity	proteinA/G/M	immunoglobulins	• Rapid assembly	• Conjugation yield depends on yield of	125
	- •			Controlled orientation	intermediate protein	
				Controllable reversible binding	<ul> <li>Binding of only specific proteins</li> </ul>	
				6	• Large size of conjugates	
					• pH-dependent binding	

# **Table 3-1** General methods to conjugate proteins to QDs.

#### 3.1.3 Desirable properties of QD-protein conjugate for bioimaging

As discussed in the introduction chapter in detail, bioimaging is one of the major applications of QDs in biology. In that regard, there are several 'quality checks' that the final conjugates should pass. First is that the QDs after conjugation and purification should still retain high fluorescence QY and solution stability. Second the protein conjugated on the QD should not undergo conformational change due to the chemistry of the conjugation and the proximity of the highly charged QD surface. Structural/conformational damage to conjugated protein impairs its biological function and thus the applications. The conjugates should also retain small size and stability within the range of physiological pH and temperature. Additionally for bioimaging, it is also essential to maintain good photophysical properties, including resistance to photobleaching and minimal photo-blinking.

#### 3.1.4 Transferrin as a model protein system

Transferrin (Tf) derives the name from the physiological function of intracellular transport of iron. In most eukaryotic cells, Tf acts as the major transporter of iron in diferric (Fe<sup>2+</sup>) form. Tf is a ~80kDa glycoprotein with two distinct lobes, the N and the C lobe (corresponding to the N and the C terminal). Each lobe has two subdomains, the interface of which harbors the iron binding pocket. Ligand (iron) binding induces a conformational change in Tf. The protein without iron is called the Apo-transferrin (apo-Tf) and the form with bound iron is called the holo-Transferrin (holo-Tf). In holo-Tf, the iron binding site in each lobe is organized by octahedral arrangement of conserved amino acids - two tyrosines, one aspartic acid and a histidine. Neighboring amino acids further assist in stabilization of iron and pH responsive release (**Figure 3-1**).<sup>126</sup>



**Figure 3-1** X-ray crystal structure of (A) transferrin (1a8e) and (B) detailed iron binding site with conserved residues. (*Reproduced with permission from Gomme et al.* Drug Discovery Today, *10 267-273 (2005); DOI: 10.1016/S1359-6446(04)03333-1*, (A))<sup>127</sup>

*Iron in physiology.* Iron is a very important metal for biological homeostasis. It is an essential co-factor in several biological reactions including oxygen transport by hemoglobin and DNA replication. However, free iron (Fe) is prone to autoxidation and promotes generation of free radical and therefore highly toxic. Thus iron should be transported in an inactive form. This transport is widely carried out by Tf, where iron is associated in diferric (Fe<sup>2+</sup>) form. Iron binds to transferrin at near neutral pH and is released at acidic pH.

*Iron transport by Transferrin.* The cycle of iron transport *via* Tf is exquisite (Scheme 3-2). Holo-transferrin binds to the transferrin receptor (TfR) on the cell surface. After binding Tf-TfR complex is sorted at the cell membrane (<1-2 min) followed by internalization by clathrin coated pits into early endosomes (EE). From EE, Tf-TfR is sent indiscriminately to slow or fast maturing population of recycling endosomes (RE). The fast maturing RE population is localized closed to the cell surface from which it can directly fuse with the membrane, thus releasing the endocytosed cargo outside and replenishing the surface pool of TfR ( $t_{1/2} = 2-5$  mins). The slower maturing population on the other hand is concentrated close to the nucleus in a more defined punctate like structure ( $t_{1/2} = 8.5$  mins).<sup>127–129</sup> Generally the RE in cells have lower pH than the SE. This gradual drop in pH assists in release of iron from the holo-Tf. Thus, formed apo-Tf has lower affinity for the TfR upon recycling. Tf and TfR containing endosomes reach the cell periphery and subsequently fuse with the membrane. At the membrane, the pH of surrounding medium is close to 7, which further destabilizes the Tf

associated with the TfR, resulting into release of the apo-Tf. This apo-Tf can again chelate iron from the extracellular media and repeat this cycle.



Scheme 3-2 Intracellular circulation of transferrin and transferrin receptor. (*Reproduced with permission from Mayle et al.* Biochimica et Biophysica Acta, 3 264-81 (2012); DOI: 10.1021/nl070363y)<sup>127</sup>

*Transferrin conjugated nanoparticles.* In general, conjugation of Tf to several synthetic scaffolds has enabled the intracellular delivery of these materials. The Tf has been used for targeting of synthetic DNA based devices <sup>130</sup>, therapeutic agents <sup>131</sup>, magnetic or gold nanoparticles<sup>132,133</sup> and QDs<sup>134–136</sup> with several types of surface modifications. However, upon adsorption or specific conjugation to NPs, Tf have been shown to undergo differences in endocytosis, intracellular routing and recycling.<sup>137–141</sup> The endocytosis of NP conjugates of Tf (NP-Tf) is delayed. In general, fluorophore tagged Tf is endocytosed within minutes, whereas

NP-Tf uptake happens over 30 mins to few hours. However similar to fluorophore conjugated Tf, the conjugates are endocytosed by receptor mediated endocytosis (RME) by clathrin coated pits. Intracellular routing is also different. As discussed above Tf-TfR traverses from cell membrane to the sorting/early endosome (EE) followed by the (RE) before being released out of the cells. However for NP-Tf particles, several reports have indicated their presence in the endolysosomal system at steady-state. Since such conjugates were en-route to the degradation pathway, the rapid recycling was not observed.



**Figure 3-2** Mechanism of uptake of nanoparticle-transferrin conjugates (A-D) schematic. Electron microscopy image of gold nanoparticle conjugated to Tf uptaken by clathrin coated pits (E and F). Have letters bigger especially on TEM. (*Reproduced with permission from Chithrani et al.* Nano Letters 7, 6 (2007); DOI: 10.1021/nl070363y)<sup>139</sup>

There is a lack of consensus over the definitive cause of these differences. Factors such as surface chemistry, charge on the polymer, accessibility of Tf to the TfR, size and shape of the NP and conjugation chemistry have been suggested responsible for ambiguous intracellular behavior of conjugates (**Figure 3-2**).<sup>137,140–142</sup> However it is understood that the difference in the rate of endo/exocytosis and intracellular localization may be a cumulative effect of several of the above factors operating in a case specific manner.

In the above section, function, physiological role and cycle of iron delivery Tf were discussed. Additionally, differences between the intracellular behavior of Tf *vs* NP-Tf were discussed. In the next section, an overview of the novel conjugation strategy presented in this work and the specific advantages are discussed.

#### 3.1.5 A novel method to conjugate Transferrin on QDs

In this section, a brief description of a novel method to conjugate Tf (generalizable to other proteins) to QD is provided. The goal is to interface readers with the motivation and the experimental tools in hand before leading though with specific experiments and results.

*The method.* In this work, a novel strategy to conjugate proteins to QDs mediated by DNA hybridization is demonstrated (**Scheme 3-3**). To synthesize these conjugates, two interacting modalities are separately generated. First module is QD conjugated to single stranded (ss) DNA of a defined sequence. The second module is Tf conjugated to DNA of sequence complementary to the above. These two are purified, characterized and then mixed together in predefined ratio to synthesize QD-DNA-Tf

Post synthetic yield and properties of these conjugates are carefully evaluated. This strategy is further extended to different proteins to expand the general applicability of such conjugates.



**Scheme 3-3** Stepwise synthesis of Quantum Dot-transferrin conjugate mediated by DNA hybridization. (A) conjugation of DNA to QD, (B) conjugation of complementary DNA to transferrin and (C) assembly of QD-Tf via DNA hybridization.

*DNA as a templating scaffold.* In this work, DNA has been used as a templating scaffold on QDs. There are several advantages of using DNA like a linker.

- (i) Due to single reactive functional group on DNA, it is easier to control the number of DNA conjugated on QDs. This helps in limiting the 'hybridizable' entities on QD (Scheme 3-4)
- (ii) Using this method, by simply altering the sequences of DNA, several macromolecules can be assembled on a single QD scaffold. This strategy may be particularly useful to assemble NPs with multiplexing capacities.<sup>143</sup>
- (iii) A lot of proteins conjugated to NPs have been shown to undergo altered fate and misrouting. When NPs are mixed in cell media (comprising of buffers, amino acids, serum etc), a lot of proteins from the serum adsorb on the surface of NPs. This adsorption is based on several factors, including surface ligands and charge. This non-specific adsorption of proteins change the surface of NPs exposed to the cells. Several reports indicate that altered fate of NP-protein conjugates arise from the loss of visibility of the target protein, within this protein corona. <sup>140</sup>

Persistence length and polyanionic nature of DNA makes it a programmable, universally rigid and charged linker.<sup>144</sup> It is possible that the conjugation mediated via this linker affects the formation of protein corona on the QD surface. Additionally, ~8.5 nm rigid linker can actually protrude the conjugated protein 'out' of the sphere of non-specifically adsorbed proteins around the QD. Therefore, it was interesting to test, whether conjugates prepared by DNA hybridization show biological properties similar to differently prepared conjugates.



**Scheme 3-4** Schematic of example of DNA mediated templating of QDs synthesis of macromolecular complexes with defined properties. (*Reproduced with permission from Tikhomirov et al.* Nature nanotechnology, 6, 8 485-90 (2011); DOI: 10.1038/nnano.2011.100)<sup>87</sup>

# 3.2 A novel method to functionalize QDs with proteins: synthesis and characterization

#### 3.2.1 Synthesis of QD-DNA-Tf

*Hydrophilic Quantum Dots.* QDs emitting at 650 nm were obtained in hexane from Nexdot. A hydrophilic, multidentate polymer comprising 50% of dithiols and 50% of zwitterions ((50-50)<sub>10</sub>-Zw) was synthesized by copolymerization reaction. The detailed stepwise synthesis is described in Chapter 2 section 2.1.3. The zwitterionic functional groups enabled dispersion of QDs in water whereas the dithiol bearing functions could anchor the polymer on the QD surface, and additionally used for conjugation. Ligand exchange of QDs with this polymer was carried out by classical two-step process as described before.<sup>38</sup>

**Conjugation of DNA to QDs.** The details of all the DNA sequences used are listed in Appendix 2. It has been shown in past that water-solubilized QDs coated with the above mentioned polymer contains several thiols (from polymer) bound to the surface of QDs, and few available for conjugation.<sup>74,79</sup> The conjugation of DNA (25mer, ss) on thiols of QDs was carried out by the strategy explained in Chapter 2.<sup>74</sup> Briefly, amine-labelled DNA was reacted with sSMCC. In parallel, QDs were reduced with TCEP (TCEP/QD = 400). Excess of sSMCC and TCEP were purified by precipitation and gel filtration respectively. The reactive DNA (DNA-maleimide) was mixed with reduced QDs to synthesize QD-DNA. Several reactions were carried out to optimize initial excess of DNA required to obtain up to 1 DNA/QD. For a single stranded 25mer DNA, an initial excess of 5 DNA/QD resulted into a final conjugation of ~1.5±0.5 DNA/QD (**Figure 3-3**), with up to 40% conjugation efficiency in best cases. Additionally this efficiency scales non-linearly with increasing initial excess of DNA. These conjugates were purified by size exclusion chromatography (SEC) or ultracentrifugation (UC) and stored at 4°C till further use.



**Figure 3-3** Conjugation of DNA to QD. (A) Calibration curve of conjugation efficiency of number of DNA/QD (final) based on the initial excess of DNA in the reaction (B) The elution of QD-DNA (~1DNA/QD) conjugates is marginally faster than QD on the SEC column.

Conjugation of complementary DNA to Transferrin. DNA functionalized Tf (Tf-DNA), was synthesized by first reacting Tf with sSMCC to form Tf-maleimide. In parallel, thiol labelled DNA was reduced in presence of TCEP. Excess of sSMCC and TCEP were removed by ultrafiltration. After this, reduced DNA-SH and Tf-maleimide were mixed together (DNA/Tf = 20) to form Tf-DNA. DNA conjugated Tf (Tf-DNA) was purified by SEC. Several conjugation reactions to standardize the dependence of final conjugation efficiency to initial excess of DNA were carried out. These reactions were carried out on FITC labelled Tf (~2 FITC/Tf) to accurately quantify the final conjugation efficiency of DNA on Tf and similarly recapitulated on unlabeled Tf (Figure 3-4, A). With increasing conjugation efficiency, the peak of maximum absorption peak changed to 260 nm (DNA specific) instead of 280 nm (protein specific) (Figure 3-4, A, Inset). With an initial excess of DNA/Tf of 20, ~0.5±0.1 DNA could be covalently conjugated to Tf (Figure 3-4, B). The purified conjugates were also characterized by electrophoretic mobility shift assay. First, the conjugates were loaded on 3% agarose gel along with unlabeled DNA and stained with EtBr (Figure 3-4, C). The Tf-DNA migrated slower than DNA and stained positively with EtBr. Unconjugated Tf on the other hand did not stain with EtBr. The purified conjugates were also characterized using polyacrylamide gel electrophoresis (PAGE). The statistically monofunctionalised conjugates of Tf-DNA were loaded on a gel along with unconjugated Tf and DNA. The migration of the ~1DNA/Tf conjugate towards the positive terminal was faster than that of unconjugated Tf on a 4-12% gradient PAGE. The Tf and the Tf-DNA conjugates stained with Coomassie based staining reagent. The uncoupled DNA was not stained (Figure 3-4, D).



**Figure 3-4** Conjugation of Transferrin (Tf) to complementary DNA. (A) conjugation of DNA to fluorophore-labelled Tf. With increase in the conjugation efficiency of DNA, absorbance at 260 nm increases. (inset shows the peak of maximal absorbance changes from 280 nm to 260 nm with increase in conjugation efficiency). (B) Calibration curve of the initial excess of DNA to the final conjugation yield on Tf. (C) Agarose gel electrophoresis shows that DNA functionalized Tf is stained by EtBr and migrates slower than uncoupled DNA on a 3% agarose gel. Only Tf does not show up on the gel. (D) 12% PAGE gel stained with Coomassie staining reagent showing change in migration of Tf upon conjugation to DNA. Unconjugated DNA does not stain in this gel.

*Synthesis of QD-DNA-Tf conjugates.* After synthesis and SEC purification of QD-DNA and complementary DNA-Tf, the conjugates were mixed in 1:5 ratio (QD-DNA: Tf-DNA) in presence of 10 mM MgCl<sub>2</sub> in PBS pH 7.4. These conjugates were heated to 40°C in water bath and left to equilibrate to RT and then stored at 4°C. The conjugates self-assembled within <5 hours and could be used immediately after. The conjugates were characterized by SEC and absorption spectroscopy and 'templated' protein could be reliably computed (**Figure 3-5**). Based on initial excess of Tf-DNA, 1.3±0.6 Tf-DNA can be assembled on QD-DNA with 14-38% conjugation efficiency, presumably limited by the initial #DNA/QD. This conjugation efficiency was found to be independent of molecular weight of proteins (data not shown). This statistically monolabelled QD-DNA-Tf elutes about 15-20 sec before QD, suggesting minimal change in the size of nanoparticle.



**Figure 3-5** Assembly of Quantum Dot – transferrin conjugate by DNA hybridization (A) change in the peak of conjugates with  $\sim$ 1 Tf/QD on SEC (B) change in absorption used for quantification of conjugates.



**Figure 3-6** Conjugation of Tf-DNA to QD-DNA probed by dual color imaging.(A) schematic of assay. Biotinylated QDs ( $QD_{bt}$ , top), QD-DNA conjugate ( $QD_{bt}$ -DNA, middle) and QD-DNA-Tf conjugate ( $QD_{bt}$ -DNA-Tf, bottom) were mixed with FITC (reactive towards primary Amine on Tf) and purified using SEC. (B) Streptavidin beads labelled with  $QD_{bt}$ , (top),  $QD_{bt}$ -DNA (middle) and  $QD_{bt}$ -DNA-Tf (bottom) were imaged in QD channel (red), FITC channel (Green) and digitally merged (yellow). Only  $QD_{bt}$ -DNA-Tf conjugates show fluorescence in both the QD and FITC channel.

Another assay for more sensitive detection of the QD-DNA-Tf conjugates was developed (**Figure 3-6**). Biotinylated QDs ( $QD_{Bt}$ ) were used for stepwise assembly of QD-DNA-Tf ( $QD_{bt}$ -DNA-Tf). In this experiment,  $QD_{Bt}$ ,  $QD_{Bt}$ -DNA and  $QD_{bt}$ -DNA-Tf were mixed with

primary amine reactive FITC in 0.2M NaHCO<sub>3</sub> buffer. Since both the QDs and DNA lack primary amines these conjugates show negligible reactivity towards FITC. These reactions were individually purified using SEC and products were loaded on streptavidin agarose beads (SABs). Only the SABs loaded with QD<sub>bt</sub>-DNA-Tf showed fluorescence in both QD and FITC channel, and beads labelled with QD<sub>Bt</sub> and QD<sub>Bt</sub>-DNA were fluorescent only in the QD channel. This assay further confirms the presence of Tf on QDs.

#### 3.2.2 Biochemical characterization of QD-DNA-Tf

As describe in the above section, the QD-DNA-Tf conjugates eluted marginally faster than QDs on SEC, indicating that the compact size of nanoparticles conjugates were preserved. This result was additionally supported by the electrophoresis. It was seen that QD-DNA-Tf conjugates migrated very similar to QD-DNA (~1.5DNA/QD) and unconjugated QD on a 0.8% agarose gel (**Figure 3-7**, A)

*Quantum yield of conjugates.* Different strategies of purification often cause loss or rearrangement of ligands on the surface of QDs which may leads in a diminution of the QY. In general, conjugation of proteins to QDs is followed by purification. During this step, a loss of stabilizing ligands from surfaces of QDs can result into exposed hydrophobic patches that results into aggregation and affect the QY and stability of the final conjugates. To test if this grafting strategy also resulted in loss of QY of the conjugates during and after the reaction; QY was measured at every step of the process. The QD-DNA and QD-DNA-Tf conjugates retained >80% QY compared to unconjugated QDs post purification (**Figure 3-7**, B). The conservation of high fluorescence QY post conjugation on multidentate-zwitterion coated QDs is in consistence with previous from our group.  $^{10,38,39}$ 



**Figure 3-7** Biochemical characterization of QD-DNA-Tf. (A) electrophoresis of QD, QD-DNA (~1.5 DNA/QD) and QD-DNA-Tf (~1 Tf/QD) in Sodium Borate pH 8.5. (B) QD-DNA-

Tf retain high QY post synthesis. (C) QD ( $\blacksquare$ ), QD-DNA (•) and QD-DNA-Tf ( $\Delta$ ) all retain high QY (>50%) in the physiological pH range of 4.5-8.5.

*Fluorescence of QD-DNA-Tf conjugates at different pH.* Endocytosis involves intracellular passage of cargos from different endocytic vesicles or endosomes. The maturation of these endosomes over time is facilitated by rapid change in vesicular pH.<sup>145–147</sup> Several organic probes such as fluorescein have pH-sensitive fluorescence. This limits their applications in context of endocytic pathways. To further investigate the scope of QD-DNA-Tf conjugates as probes for endocytic pathways, their QY was compared with respect to pH. The QD, QD-DNA and QD-DNA-Tf retained >50% of their QY within the pH range of 4.5 to 8.5 after incubation for 8 hours in the buffered media (**Figure 3-7**, C)<sup>1</sup> This enhanced stability and relative insensitivity of QY over a range of pH is highly desirable and can be easily applied in context of model systems with over acidified endosomes where pH sensitive probes such as fluorescein prove ineffective.

# **3.3 Biological properties of QD-DNA-Tf**

So far in this chapter a new method to assemble conjugates of QD and Tf has been described. After carefully characterizing the biochemical properties of these conjugates, it was then interesting to explore their intracellular properties. To characterize the uptake, intracellular dissemination and the eventual fate of these conjugates, several interesting microscopy-based experiments were designed. These experiments were carried out on IA2.2F cells. IA2.2F is a specific variant of Chinese Hamster Ovarian (CHO) cells that over-expresses human transferrin receptor and folic acid receptor on the cell membrane.<sup>130</sup> This cell line was a kind gift from the laboratory of Prof. Yamuna Krishnan (University of Chicago).

#### 3.3.1 Receptor-mediated endocytosis of QD-DNA-Tf

For specific biological applications, the conjugates of NP-proteins should retain the molecular recognition properties of the target protein. For proteins that undergo intracellular traffic, the binding and internalization *via* specific pathways is very important. This section describes several tests to determine the specificity of uptake of QD-DNA-Tf.

<sup>&</sup>lt;sup>1</sup> For buffers, pH 4.5-5.5 in 0.1M MES, pH 6.5-8.5 in 0.1M HEPES and pH 9.5 in 0.1M Sodium Tetraborate.

*Endocytosis of QDs.* NPs including QDs are endocytosed by cells by various mechanisms.<sup>148,149</sup> To use QDs (or other NPs) for bioimaging, it is essential that the intracellular pathway taken by the conjugates mimics endogenous receptors. In this regard, an important aspect to consider is the extent of the non-specific endocytosis of the QDs. Since QDs without protein ligands are also endocytosed in bulk,<sup>141</sup> it is important to determine how much of the internal labelling is specifically from the pathway of interest, and not from the non-specific uptake of QDs. The non-specific fluorescence signal was estimated by incubating cells with 100 nM QD and QD-DNA and compared to QD-DNA-Tf in similar conditions. The total intensity with QD was up to 90% lower than that of QD-DNA-Tf, suggesting that despite prolonged incubation (3 hours), non-specific endocytosis of QDs was minimal (**Figure 3-8, A**). Conjugation of ssDNA to QDs resulted in higher non-specific uptake. Relative to QD, QD-DNA had approximately 4 fold higher intracellular labelling. This non-specific endocytosis has been observed with DNA-modified NPs, due to the affinity of DNA for anionic ligand binding scavenger receptors on the cell surface.<sup>150</sup> This enhanced endocytosis could be significantly reduced by passivating these cells with unlabeled ssDNA.

*Endocytosis of QD-DNA-Tf via transferrin receptor.* Another important aspect for specific applications of the conjugates of QD and proteins is the role of the conjugated protein. The general expectation of using QD conjugates of specific proteins is that the conjugates will follow the pathway of the coupled protein. This is possible only if the conjugate is recognized by the cognate receptor on the cell surface and uptaken by similar mechanisms. In other words, the molecular recognition of the attached protein to its receptor should be retained. These two parameters were assessed as follows. To further confirm that endocytosis of QD-DNA-Tf is specifically mediated by TfR, competition experiments were carried out. Cells were pulsed with 100 nM QD-DNA-Tf with 1000 fold excess of unlabeled Tf (specific ligand) or ssDNA (non-specific ligand) and the uptake was compared to QD-DNA-Tf in absence of any additional ligands (**Figure 3-8**, B). The uptake of QD-DNA-Tf was reduced by more than 90% in presence of Tf, whereas addition of DNA did not significantly affect the intracellular intensity.



**Figure 3-8** Endocytosis of QD-DNA-Tf. (A) QD and QD-DNA have minimal non-specific endocytosis in comparison to QD-DNA-Tf. (B) Competition experiments showing that QD-DNA-Tf is endocytosed specifically by Transferrin receptor (TfR). Fluorescence intensity per cell is individually estimated (n=60). Mean fluorescence intensity per dish is normalized to cells treated with QD-DNA-Tf. Each bar graph represents % Mean fluorescence intensity and SEM of two independent experiments.

Receptor-mediated endocytosis of NPs such as QD and GNP conjugated to Tf have been investigated in the past.<sup>137,139</sup> Such conjugates are recognized by specific transferrin receptors (TfR) and internalized into clathrin-coated vesicles. However the endocytosis of GNP-Tf conjugates has been shown to be dependent on both size and shape of the NPs. Nanoparticles can additionally be endocytosed in absence of protein-based ligands, often in a concentration and time-dependent manner.<sup>151,152</sup> Therefore careful evaluation of the extent of non-specific internalization of QD by bulk endocytosis or scavenger receptor mediated endocytosis is essential. In context of this work, the polymer coating the QDs is locally charged but electrostatically neutral. The zwitterionic polymers of similar design have been shown to have minimal non-specific absorption on the cell membranes.<sup>40,79</sup> Additionally, the synthesis of QD-DNA-Tf is carried out by hybridization of conjugated DNA with Tf-functionalized complementary strand. It is expected that the DNA strands are sandwiched between the QD and the protein by design, and thus not exposed or easily accessible to the receptors. The constitutive effect of polymer design and bioconjugation strategy may explain the specific uptake of QD-DNA-Tf conjugates in cells.

#### 3.3.2 Kinetics of endocytosis of QD-DNA-Tf

Transferrin internalizes into EEs within the first 4-7 minutes, followed by subsequent transport into RE. However as discussed in the introduction for nanoparticle conjugates of Tf, different groups have found that the endocytosis of conjugates is often delayed. Similar results were found in this work, wherein it was observed that endocytosis of QD-DNA-Tf occurs with a  $t_{1/2}$  of ~123.7 minutes. In similar types of experiments with Tf<sub>647</sub>, Tf endocytosed with  $t_{1/2}$  =5.1±1.3 mins, similar to those previously obtained by fluorescence and/or radioactivity based assays (**Figure 3-9**and **Table 3-2**)



**Figure 3-9** Kinetics of endocytosis of QD-DNA-Tf ( $\bullet$ ) in comparison to Tf<sub>647</sub> ( $\Box$ ). For each point on the curve, mean fluorescence intensity of 60 cells is estimated. Mean and SEM of 2 independent experiments is plotted.

Table 3-2 Kinetics of endocytosis of Tf<sub>647</sub> and QD-DNA-Tf

Endocytosing cargo	t <sub>1/2</sub> (min)
Tf <sub>647</sub>	5.1±1.3
QD-DNA-Tf	123.7

It was then interesting to estimate whether slower binding of the conjugate to the receptor causes the delay in endocytosis of QD-DNA-Tf. Cells were incubated with 100 nM QD-DNA-Tf at 4°C for 30 mins. At this temperature Tf binds to TfR but no endocytosis takes place. These cells were then warmed to 37°C for 3 hours as normal, surface stripped and fixed. For control, cells were directly pulsed with 100 nM QD-DNA-Tf for 3 hours, surface stripped and fixed. It was found that the efficiency of endocytosis with and without the pre-incubation was remarkably similar (**Figure 3-10**). This concluded that the binding of QD-DNA-Tf to TfR was not the rate-limiting step. Contrarily, in case of Tf conjugated to QD directly, cell intensity increases almost 2.5 fold upon pre-incubation (data not shown). Above results indicated that receptor binding kinetics is not detrimental in endocytosis of QD-DNA-Tf.



**Figure 3-10** Endocytosis of QD-DNA-Tf does not improve by pre-binding to TfR. Mean and SD of fluorescence intensity for 60 cells is plotted.

Uptake of nanoparticle conjugates with Tf are influenced by several parameters such as nanoparticle size, proximity of Tf to the NP, the surface charge on the polymer of NP, ligand density etc. In the above experiment, the facilitated binding of QD-DNA-Tf does not increase the uptake whereas a similar experiment with QD-Tf improves the efficiency of uptake. This can be explained by energetics of receptor-ligand binding. In case of QD-DNA-Tf, Tf 'protrudes' out of the QD surface by a rigid ~8.5 nm DNA linker. Additionally, polyanionic nature of DNA can cause local rearrangements of the zwitterion polymer around the Tf,

making it more accessible to the solvents, and also to the receptor. Further, this design also allows for sufficient conformational freedom to the conjugated ligand to screen and bind to the receptor instantaneously. In contrast, Tf conjugated to QD directly is not 'protruded' away from the surface of QDs, and neither is it expected that the polymers may rearrange due to electrostatic factors. Additionally, proximity to the QD surface further restricts the conformational search for 'induced fit' binding to TfR. This may explain the substantial increase in uptake of QD-Tf upon pre-incubation with the cells.

#### 3.3.3 Steady-state localization of QD-DNA-Tf

To further evaluate whether the intracellular fate and trajectory of QD-DNA-Tf is similar to Tf, the steady-state intracellular distribution of the conjugates were determined. For this, two types of experiment were done: colocalization and immunofluorescence. Since for these experiments dual color imaging is required, several fluorophores and instrumentation (compatibility of filters) were screened. The microscopic filters are described in Appendix 4. For the dual color fluorescence imaging with QD-DNA-Tf emitting at 650 nm, the fluorophore with minimal spectral bleed-through was FITC. Hence, for the colocalization experiment,  $Tf_{FITC}$  was used and for immunofluorescence, secondary antibody labelled with FITC was used.

**Colocalization experiment:** Intracellular behavior of the conjugates of QD-DNA-Tf should ideally be like Tf. Hence for colocalization experiment QD-DNA-Tf were compared to Tf<sub>FITC</sub>. (**Figure 3-11**, A) To determine intracellular distribution of QD-DNA-Tf, cells were first pulsed for 3 hours and then surface-stripped in ascorbate buffer. Following this, cells were pulsed with Tf<sub>FITC</sub> for 7 mins and chased for 10 mins. The  $t_{1/2}$  for Tf recycling is ~8 mins and therefore a majority of the internalized cargo should be routed towards recycling by the end of chase period. Then the cells were shifted to ice and their surface was re-stripped as before. The cells were fixed with ice cold methanol and imaged in both the QD-DNA-Tf channel and the FITC channel. The timing of pulse for both QD-DNA-Tf and Tf<sub>FITC</sub> is very important. Fluorophore-labelled Tf has fast endocytosis and recycling kinetics. Hence, in this design, the pulsing of second color is timed after the saturation with the first color. Also in this step, pre-removal of surface bound QD-DNA-Tf before pulsing Tf<sub>FITC</sub> ensures that all TfR occupied on

the surface of the cells are made available to bind to  $Tf_{FITC}$ . Using this experimental design, it was seen that QD-DNA-Tf colocalized within the RE, similar to  $Tf_{FITC}$ .

*Immunofluorescence experiment:* Since most of the NP-Tf conjugates reported in literature are misrouted to the endolysosomal system instead of RE like Tf, it was essential to evaluate whether the perinuclear organization of QD-DNA-Tf was additionally overlapping with the Late Endosome (LE). For these experiments, an antibody against a lysosomal resident protein called 'Lysosomal Associated Membrane Protein-1' (LAMP-1) was used (**Figure 3-11**, B). For immunofluorescence experiments of QD-DNA-Tf with LAMP-1, QD-DNA-Tf was internalized and surface labelling was removed. Cells were washed on ice for 10 mins followed by fixation with ice cold methanol. Then the cells were washed with PBS (3X) and permeabilised with 1X saponin buffer for 30 minutes. Cells were incubated with anti LAMP-1 (Mouse) followed by secondary antibody labeling with Anti-mouse-Alexa488. (Experiments with anti-mouse-fluorescein showed very poor labelling and hence Anti-mouse-Alexa 488 was used). This experiment demonstrated clear demarcation between localization of QD-DNA-Tf and LE within a single cell, confirming that these conjugates do not end up in lysosomal system for subsequent degradation.



Figure 3-11 Steady-state localization of QD-DNA-Tf. (A) colocalization experiment with RE marker  $Tf_{FITC}$  (B) Immunofluorescence experiment with LE marker LAMP-1


**Figure 3-12** Pearson's correlation coefficient analysis suggests QD-DNA-Tf reside in the RE, not the LE. White bars indicate colocalization indices of images in two channels superimposed in original orientation and grey bars indicate colocalization indices when image corresponding to the green channel (FITC) is digitally rotated by 90°. Mean and SD of PCC from 20 cells is plotted

*Quantification of localization of QD-DNA-Tf.* In CHO cells, RE is a distinct perinuclear compartment. It was observed that steady-state distribution of both QD-DNA-Tf and Tf<sub>FITC</sub> is perinuclear. From the colocalization experiments, the QD-DNA-Tf conjugates colocalize upto  $65\pm15\%$  (Pearson's colocalization coefficient, PCC) with Tf suggesting that at steady-state, QD-DNA-Tf also resides in the RE. Immunofluorescence studies with antiLAMP-1 show that although both QD-DNA-Tf and LAMP-1 appear to be widely perinuclear, however, the PCC of colocalization is negative to <20%. Clear demarcation is seen in both the staining patterns (**Figure 3-12**). This suggests that QD-DNA-Tf does not go to late endosome lysosome-like compartment. The PCC is further reduced upon digitally rotating the image corresponding to one channel by 90° and following similar algorithm using ImageJ.

Since Steady-state localization of QD-DNA-Tf in RE could be altered by microtubule disrupting agents. For these experiments, cells were pre-treated with nocodazole, a small molecule drug that destabilizes microtubules.<sup>153,154</sup> Using this drug, endocytosing cargo can be arrested in the sorting/early endosome, and prevented from trafficking further to RE or late endosome-lysosome. Naturally, the fluorescent cargo would appear close to the cell periphery

than nucleus. In experiments with cells pre-treated with nocodazole, QD-DNA-Tf accumulated to distinct punctate close to the cell membrane (**Figure 3-13**). This distinct patterning was similar to cells pulsed with  $Tf_{647}$ . In control untreated cells, majority of fluorescence signal was localized to the perinuclear region for both QD-DNA-Tf and  $Tf_{647}$ . These results also indicate that the endocytosis of QD-DNA-Tf is specifically due to receptor-mediated endocytosis.



**Figure 3-13** Microtubule dynamics affects steady-state localization of QD-DNA-Tf. (A) Chemical structure of drug Nocodazole (B) Schematic of pathway blocked by nocodazole. Nocodazole treated cells pulsed with (C)  $Tf_{647}$  (D) and QD-DNA-Tf. Untreated cells for comparison pulsed with (E)  $Tf_{647}$  (F) and QD-DNA-Tf. Scale bar is 10µm.

Ligand density has been considered as an important factor in determining the initial rate of endocytosis of NP conjugates of proteins. However, the role of ligand density in determining the intracellular fate of cargo has seldom being investigated. It was found that oligomerized Tf is endocytosed but destined for degradation and hence send to late endosome lysosome-like compartment.<sup>155</sup> Endocytosis of GNP-Tf also resulted into steady-state localization of conjugates in the LE when multiple Tf molecules were adsorbed on the GNP.<sup>139</sup> In this work, a tight control on the labelling of Tf on QD was elucidated and it was demonstrated that QD-DNA-Tf goes to the RE. Based on already existing evidence in literature and these findings, it appears that the ligand density can significantly impact the ultimate intracellular localization

of NPs. However it cannot be denied that steady-state localization of NP-protein conjugates may not be exclusively driven by ligand density. NP synthesis, type of surface polymer, chemistry of conjugation and nature of ligands can significantly affect the final fate, and need to be investigated on a case to case basis.

# 3.3.4 Endocytosis of QD-DNA-Tf does not affect the uptake of subsequent

#### Tf-TfR

Endocytosis of NP-Tf conjugates involve initial binding of ligand to the receptor that facilitates additional receptors to bind to the nanoparticle and eventually wrap the cell membrane around the conjugate and internalize it (**Figure 3-14**).<sup>139</sup> This binding of multiple TfR can cause an imminent deficit of the receptor at the membrane. Additionally delayed routing and recycling of NP conjugates of Tf could potentially perpetuate the deficit at the cell membrane, affecting the endocytosis of 'fresh' ligands. To check whether intracellular presence of QD-DNA-Tf grossly alters the TfR population at the cell membrane, cells were re-pulsed with Tf<sub>FITC</sub> at pre-defined time intervals and the intracellular fluorescence intensity was compared to control cells which have not been pulsed with QD-DNA-Tf first (**Figure 3-14**). Detailed pulse chase experiments on cells incubated with (i) QD-DNA-Tf followed by Tf<sub>FITC</sub> and (ii) Tf<sub>647</sub> followed by Tf<sub>FITC</sub> show that the second round of endocytosis (Tf<sub>FTIC</sub>) has similar kinetics with  $t_{1/2} = 4.6\pm1.7$  and  $6.0\pm0.5$  min respectively. This suggests that normal cellular trafficking and recycling of TfR is retained in both the cases.



**Figure 3-14** Endocytosis of  $Tf_{FITC}$  is similar when cells are first pulsed with  $Tf_{647}$  ( $\Box$ ) or QD-DNA-Tf ( $^{\circ}$ )

#### 3.3.5 QD-DNA-Tf recycle out of cells over time

Steady-state localization of QD-DNA-Tf in RE suggests that these conjugates are not 'prepared' for degradation. For experimental validation, cells were pulsed with 100 nM QD-DNA-Tf for 3 hours, surface-stripped and chased with unlabeled Tf for 20 mins. Clear difference in the total intracellular fluorescence intensity was seen. It was then interesting to investigate the kinetics of recycling of QD-DNA-Tf.

*Kinetics of recycling.* The endocytosed cargo after reaching recycling endosomal compartment is trafficked back to the plasma membrane. This helps in recycling of the receptor to further engage its ligand and repeat the intracellular transport. Since several reports have suggested that conjugates of NP-Tf are misrouted and prone to degradation, it was interesting to see whether a pressure of incoming ligands (unlabeled hoto-Tf) can trigger recycling. In order to test the design of this assay, experiments were first carried out with Tf<sub>647</sub>. Cells were pulsed with Tf<sub>647</sub> for 30 minutes. In this time, all endosomal vesicles (EE/SE and RE) of the cells were fluorescent. Then the surface labelling was removed and cells were chased by Tf for desired period of time and fixed. Systematic pulse chase and fixation

experiments were carried out to study the recycling of  $Tf_{647}$  already endocytosed. Detailed design is explained in Chapter 5 Section 5.4.6.  $Tf_{647}$  was observed to recycle out of the cells (more than 80% of initial) with a  $t_{1/2}$  of ~9 mins, a value similar to those obtained by other groups using radiolabeled Tf. <sup>127,128</sup>



**Figure 3-15** Pulse-chase-fix type experiments can be used to estimate the kinetics of recycling of Tf647 ( $\Box$ ) or QD-DNA-Tf( $\circ$ ).

Table 3-3 Kinetics of recycling of Tf<sub>647</sub> and QD-DNA-Tf

Recycling cargo	t <sub>1/2</sub> (min)
Tf <sub>647</sub>	8.9±4.7
QD-DNA-Tf	8.9±3.1

Similar experiments are carried out with QD-DNA-Tf. Cells were pulsed with QD-DNA-Tf for 3 hours, followed by chase with Tf for indicated periods. It was seen that QD-DNA-Tf recycles out of cells with a  $t_{1/2}$  of ~9 mins, remarkably similar to that of Tf (**Figure 3-15**, **Table 3-3**). This is to our understanding the first report of such fast recycling of NP conjugate of Tf. In fact in past, even after perinuclear localization of QD-Tf, they were shown to not recycle after 4 hours.<sup>138</sup>

## 3.3.6 Photostability of QD-DNA-Tf in endosomes

To further confirm that the loss of fluorescence of QD-DNA-Tf with time was due to recycling and not photobleaching, the photostability of QD-DNA-Tf was tested. In these experiments the intracellular fluorescence intensities of cells under different pulse-chase conditions was compared. Herein, the cells were incubated with QD-DNA-Tf for 3, 3.5 and 4 hours the intracellular intensity was compared to cells which have been additionally chased for 30 mins post 3 hours of pulse. The intracellular intensity remained consistent (80-100%) in the 'only pulsed' cells. Strikingly, in the control cells with additional chase period of 30 mins, the intracellular intensity had reduced by 75-90%. This suggests that prolonged incubation of QD-DNA-Tf in cells does not change the photophysical stability of the conjugates (**Figure 3-16**). **Table 3-4**). This further supports that recycling of conjugates by 'reduction in fluorescence over time' probed herein is devoid of any artifacts from photophysical instability of the QD.



**Figure 3-16** Intracellular fluorescence intensity of QD-DNA-Tf for different time periods. Each bar graph represents mean and SD of fluorescence intensity for 60 cells is plotted.

**Table 3-4** Photostability of QD-DNA-Tf in RE compared to recycling.

Experiment	% intensity
4 hours pulse $+ 0$ hr Chase	83.5±35.8
3.5 hours pulse + 0 hr Chase	100.0±26.3

3 hours pulse $+$ 0.5 hr Chase	25.2±15.4
3 hours pulse $+ 0$ hr Chase	89.5±18.5

# 3.3.7 Recycling of conjugates of QD-Tf is affected by several factors

After carefully confirming the recycling of the conjugates in cells, it was then interesting to determine whether the routing of QD-DNA-Tf to RE followed by fast recycling is an exclusive effect of the surface chemistry or conjugation methodology. To test that, similar experiments were repeated with QDs conjugated directly to Tf (QD-Tf). Direct synthesis of QD-Tf is detailed in Chapter 2. These conjugates were localized in the perinuclear region, similar to QD-DNA-Tf. However, the kinetics of recycling was about four times slower than QD-DNA-Tf (Data not shown). These results suggest that endocytosis, steady-state localization and eventual fate of QD conjugates of Tf are governed by complex interplay of several parameters.

Recycling of macromolecular ligands is caused by several factors, such as global changes in pH, positive pressure of incoming ligands, deficit of certain metabolites etc. However, the recycling of NP conjugates may be further governed by additional parameters, including surface charge and ligand structures, adsorption of biomolecules etc. At this point, it is concluded that this recycling of QD-DNA-Tf is a complex interplay of several material, chemical and biological factors. Detailed investigation in due course will enable specific understanding of factors that lead to similar recycling of conjugates.

In this work, QD-DNA conjugates have been used to assemble proteins to generate fluorescent probes with very interesting biological properties. In the previous section, detailed characterization of these probes and their intracellular properties were discussed. These experiments were carried out on IA.2.2F cells, which were mostly fixed and imaged to study fundamental intracellular properties of these probes. After careful understanding of these properties it was then interesting to assess the potential of these conjugates for bioimaging. In the next section use of these conjugates for live imaging is discussed.

# 3.3.8 Recycling of QD-DNA-Tf - insights from long duration live imaging

In the above section, recycling of QD-DNA-Tf was probed using carefully designed assays on cells which were fixed. It was then interesting to gain insights into the real-time dynamics of recycling of these conjugates. Thus, several experiments were carried out using confocal live imaging.

# *Live imaging of QD-DNA-Tf conjugates – set up.*

For long duration imaging, spinning disc confocal microscopy was used. Detailed description is provided in chapter 2. Briefly, the imaging software settings were fixed such that defined number of confocal Z stacks / time frame was acquired. This selection was based on several factors such as thickness (height) of cell, region of interest (intracellular or membrane) and intensity of probes. After acquisition of data, the images were rearranged into time series of each confocal slice by an in-house MATLAB program. The time series of each confocal section was manually selected for a stack where the cell membrane and the perinuclear region were both in focus and loaded into Metamorph (**Scheme 3-5**). All the data was treated with a noise reduction program called Multidimensional Image Analysis (MIA) developed by the imaging platform at Curie Institute. The details of the programs are also discussed in Chapter 2. After this step, the time series was loaded into ImageJ and analyzed as described in the next section.



Evolution of selected plane over time

Scheme 3-5 Schematic for imaging and data processing using Spinning Disk Confocal microscope.

#### *Recycling of QD-DNA-Tf conjugates – probing distribution.*

In the previous section, the kinetics of recycling of QD-DNA-Tf was estimated using measurements of fluorescence intensity of cells fixed at a predefined chase period. These results were also supported by visualizing the recycling of QD-DNA-Tf with live imaging using the set up described above. Cells were pulsed with QD-DNA-Tf for three hours, surface stripped and chased in presence of unlabeled Tf. Within 5 minutes of the start of chase period, cells were imaged live. The number and distribution of endosomes labelled with QD-DNA-Tf changed massively within the cells. A representative time course of confocal images after every 5 minutes is shown with the perinuclear (white arrows) and membrane-proximate (red arrows) regions highlighted (**Figure 3-17**). The intracellular distribution of endosomes changes dynamically over time. A comparison of the initial (t=5.00 min, top left) and final (t=30 min, bottom left) is distribution is shown (**Figure 3-18**). The total time spend between these two images (recycling time) is close to 24 minutes. The two images are pseudo-colored (initial-purple, final-yellow) and digitally merged (right). The change in distribution can be

further seen by negligible overlap (white) between the two colors. So using live imaging, not only the recycling of QD-DNA-Tf was confirmed, but also insights on temporal distribution was obtained.



**Figure 3-17** QD-DNA-Tf relocates from the perinuclear region (white arrow) to the cell membrane (red arrow) and recycles out of cells over time.



**Figure 3-18** Live imaging of recycling of QD-DNA-Tf. (A) cells at the beginning of chase period. (B) same cells after ~24 mins. (C) Digital superimposition of (A, purple) and (B, yellow) in pseudo-colors to show change in the intracellular location of populations of fluorescently labelled endosomes over time. Scale bar represents  $10\mu m$ .

#### Probing single vesicle recycling.

These experiments additionally lend in the possibility of using QD-DNA-Tf conjugates as probes for visualizing gradual recycling of endosomes with time. After organization of data from the steps described above, the images were processed on a single vesicle level to evaluate individual trajectories. Using particle tracking tools in ImageJ, individual trajectories of endosomes loaded with QD-DNA-Tf *recycling out of cells* were observed. These endosomes were seen to traverse towards cell membrane at different velocities without photobleaching. Several vesicles could be followed from perinuclear region to the cell periphery and the vesicles at the cell periphery were seen to disappear over time. Representative image of whole cell pulsed with QD-DNA-Tf with indicated ROI is shown. Insets are digital zoom of the ROI at indicated time. (**Figure 3-19**, A) In the top panel, individual trajectories of (shown in yellow) within t=0 to t=2 min is seen. Below this, representative images after every 26 sec show clear evolution of the trajectory of this vesicle. This demonstrates the use of QD-DNA-Tf to monitor recycling dynamics of individual endosomes.

#### Evaluating endosomal photostability of QD-DNA-Tf

In the experiments mentioned above, live cells were imaged. However due to the design of the experiment, each cell had several hundreds of endosomes which were fluorescently labelled. Nevertheless, for specific comparison of photostability, less number of endosomes needed to be tracked. Due to the numerous endosomes and rapid recycling, the previous experimental design could not be used to estimating the long duration photostability of the probes. Hence, in a new approach, cells were treated with a drug called Monensin. Monensin is an ionophore that chelates monovalent ions such as Na<sup>+</sup>. In mammalian cell lines, this drug has been shown to abolish intracellular pH gradient thus homogenizing the pH within the endosomal system. Since pH is a major biochemical factor driving endosomal maturation (endocytosis, recycling and degradation) this drug thereby affects intracellular transport of materials. In this experiment, cells were first treated with Monensin for 20 minutes and pulsed with QD-DNA-Tf and incubated for 3 hours. As expected, the intracellular delivery on conjugates was diminished up to 13% of the original (this lowering of uptake upon treatment with Monensin also confirms that the bulk of fluorescence intensity seen in cells is *specifically* due to internalized conjugates). As expected in this system, there were far less number of

endosomes labelled with QD-DNA-Tf. These individual endosomes were imaged for up to 20 minutes. Representative images from the first 10 minutes are shown (**Figure 3-19**, B).

The endosomal dynamics in these cells were significantly different from those in the above experiment. In this recycling compromised system, not only the number of vesicles was low, but also individual vesicles demonstrated massively altered mobility. They did not traverse long distances and were not visibly routed towards the cell periphery. The apparent diffusion coefficient of endosomes within the Monensin treated cells were more than an order of magnitude lower than those in normal cells (**Figure 3-20**).



Figure 3-19 Long duration tracking of endosomes using QD-DNA-Tf nanobioconjugates. For these experiments, Ia2.2 cells were pulsed with 100 nM of indicated sample for 3 hours, surface stripped, chased with unlabeled Tf and imaged live using spinning disc confocal microscope. See SI for details of the imaging, data filtering and analysis algorithms used. Representative image of whole cell pulsed with QD-DNA-Tf with indicated ROI is shown. Insets are digital zoom of the ROI at indicated time. (A) Cell showing normal recycling of QD-DNA-Tf. Inset (red, top) shows that the endosome of interest can be tracked over time. Also, it recycles out within first 2 minutes of chase (representative images shown after every 2 mins). Inset (red, below) shows the tracking of this endosome of interest within short intervals of time (representative images after every 26 sec). (B) Cell in presence of 30µM Monensin show compromised recycling of QD-DNA-Tf and enable long duration imaging. Inset (green, top) shows tracking of the endosome of interest for up to 10 minutes (representative images shown after every 2 mins). Inset (green, below) shows the tracking of this endosome of interest within short intervals of time shows limited mobility in response to Monensin treatment (representative images after every 26 sec). Images were processed using ImageJ. Scale bar represents 10µm.



Figure 3-20 Comparison of diffusion coefficient of REs of normal cells in comparison to cells treated with Monenisn. Inset shows the chemical structure of Monensin

This recycling compromised system set basis for evaluation of the intra-endosomal photophysical stability of these conjugates. From this experiment it was concluded that owing to the high photostability the vesicles could be imaged for long duration, which was seldom achieved by organic fluorophores. Therefore, these conjugates can be widely applicable as robust probes for bioimaging.

# **3.4 Conclusions**

In this work, a novel strategy to conjugate proteins to QD with tight stoichiometric control is demonstrated. It was also shown that for QD-DNA-Tf conjugates both the probe (QD) and the protein (Tf) retain their molecular recognition properties. This study additionally outlines several key features of bioconjugates of QDs. Several factors affect internalization and post internalization behavior of QDs. Most importantly, controlling the stoichiometry of conjugated ligands may facilitate normal routing of conjugates of QD and Tf. In these experiments, it was conclusively shown that careful ligand design could generate conjugates of QD and Tf which mimic several of the intracellular properties of Tf. Additionally, QD-DNA-Tf undergoes slow endocytosis and recycling – indicating that the uptake and release of NP-Tf conjugates may be governed by mutually exclusive synthetic and biological sets of parameters. This is also the first report where bioimaging of recycling endosomes for >20 mins was shown.

These results indicate that a careful design of surface properties, choice of ligands and conjugation chemistries can give rise to more predictable behavior of bioconjugates of QDs and facilitate their context-specific application particularly for bioimaging. In future, this strategy can be easily tuned to different proteins and other ligands such as RNAs, peptides, small molecules without need for customization. Additionally, this same design can be adapted to template several ligands on the surface of the same QD by tuning DNA sequences, thereby facilitating multiplexing applications.

This work thus is a strong example for generation of tailor-made QD bioconjugates for bioimaging of key cellular processes in bulk and on single endosome level.

# Chapitre 4 Systematic Evaluation of Quantum Dot Surface Chemistries for Biological Applications

In this thesis so far, several aspects of functionalization and applications of QDs in diverse biological contexts have been discussed. In the first chapter, a brief description of strategies to disperse QDs in aqueous media, followed by a comprehensive overview of strategies to functionalized DNA to QDs was discussed. In the second chapter, development of a novel method to conjugate DNA to QDs was extensively discussed. One of the fundamental advantages of using QDs is the remarkable photostability over organic fluorophores. This property is specially promising in context of bioimaging. However, in order to image specific intracellular processes such as cellular trafficking, QDs need to be conjugated with functional proteins. With this overview, in the third chapter a novel strategy to conjugate proteins to QD-DNA conjugates were described. The biological behaviors of these conjugates were exhaustively studied and eventually their potential in bioimaging was demonstrated. During these studies, it was observed that the biological properties of QDs are often associated with the surface properties, *i.e.* the nature of amphiphilic ligands on the surface of QDs. In this last experimental chapter, a systematic assessment of QDs coated with four different kinds of multidentate ligands is discussed. This study is aimed to better understand how electrostatically neutral ligands affect the interactions of biomolecules with QDs. The overall goal is to be able to screen surface chemistries based on the case specific applications of interest, including biosensing and bioimaging.

# **4.1 Introduction**

The interactions of nanoparticles (NPs) with the biological interface are dynamic and complex. It is well established now, that the surface of NPs get irreversibly altered after interaction of complex biological media like serum.<sup>156</sup> Current line of NP research supports that cell culture media or serum influence several physico-chemical and biological behavior of NPs.<sup>157–159</sup> This occurs due to adsorption of protein components from the serum on the NP surfaces.<sup>77,140,151,157,160,161</sup> These non-specifically associated proteins are globally termed as 'protein-corona' around NPs. The protein corona has several interesting properties. On the

surface of NPs, several proteins may adsorb strongly and form a stable and irreversible NPprotein interface or the 'hard' corona. Onto this layer, several additional proteins may associate and form a rather loosely held 'soft' corona, comprising of mostly protein-protein interactions.<sup>140,161,162</sup> These two physicochemical distinct layers have their unique properties and dynamic behavior. For example, proteins conjugated to NPs can become 'invisible' to cognate receptors if they are surrounded by the hard corona of tightly adsorbed, biochemically similar proteins.<sup>163</sup> On the other hand, if the covalently conjugated protein is 'protruded' out of this layer (by use of different length linkers), such that it lies within the softly bound proteinaceous layer, its visibility to cognate receptors (or antibodies) can be improved.<sup>160,164</sup>

Serum adsorption also affects the uptake of NPs within cells.<sup>165–167</sup> One of such examples is when adsorption of serum proteins on the NP surfaces causes reduced non-specific cellular uptake of untargetted NPs.<sup>168–170</sup> This reduction in uptake has been attributed among other things, to lower adhesion to the cell membranes which concomitantly results into lower uptake.<sup>171</sup>

Although so far it is not possible to provide a concise prediction of nanoparticle behavior in presence of biological media, several general conclusions can be made. The composition of serum and the nature of NPs both dictate the interactions at this dynamic nanomaterial-biology interface. The protein adsorption is determined by factors such as composition of the NP, amphiphilic ligands on the surface, pH, temperature etc.<sup>172,173</sup> As an effect of protein adsorption, the NPs undergo change in size, surface potential, colloidal stability, stealth character etc. The adsorbed proteins have also been shown to undergo conformational change, local (reversible or irreversible) denaturation particularly on spherical NPs.<sup>156,167,174,175</sup> Needless to say, two things are clear –(i) The nature and extent of non-specific adsorption of serum proteins on NP surfaces is governed by physicochemical properties of the nanomaterials in concern . And (ii) this resultant protein corona in turn changes several physical, chemical and biological properties of NPs.

In this chapter, spherical QDs coated with several electrostatically neutral ligands are studied. In this section, several interesting *in-vitro* experiments to understand the behavior of QDs of different surface chemistries in complex media are described. Before understanding how surface chemistry affects the properties of QDs inside cells; it was interesting to compare several of these parameters in cell-like media, *i.e.* buffers in presence of serum. The broad objective is to compare the physicochemical properties of QDs with pre-defined surfaces upon interaction with complex media (*in-vitro*). Additionally, the intracellular behaviors of these proteins-modified NPs are studied in CHO cells. The subsequent sections describe in detail the experimental approaches and eventual conclusions from this work.

# 4.2 Quantum dots and the surface chemistries of interest.

## 4.2.1 Photophysical characterization of QDs in organic solvent

For this work, QD emitting at 610 nm (hereafter called QD) were obtained in hexane from Nexdot. They photophysical properties included broad absorption and narrow emission with FWHM of ~35 nm peaking at 610 nm (**Figure 4-1**). Using Transmission Electron Microscopy, the diameter of NPs were estimated to be  $7.7\pm0.8$  nm (up to 250 QDs were individually counted) and the batch was observed to be highly monodispersed. As discussed early, for all types of biological experiments, QDs need to be first dispersed in aqueous media. These QDs were ligand-exchanged using the two-step protocol described before.<sup>38</sup> The details of the four types of statistical copolymers used for ligand exchange of four aliquots of the same batch of synthesis of the QDs is briefly described below. These polymers (3 out of 4) comprised of different ratio of monomer with methacrylamide derivatives of *a*-Lipoic acid (DTMAm) and sulfobetaine (Zw) or (1 out of 4) of *a*-Lipoic acid (DTMAm) and Polyethylene Glycol (PEG) (**Table 4-1**).



**Figure 4-1** Properties of QDs in Hexane. (A) Absorbance (black) and Fluorescence (Grey) spectra of QD emitting at 610 nm. (B) TEM image showing monodisperse preparation of QDs. Scale bar indicated 20 nm.

Name	Monomer 1	Monomer 2	Chain length
(20-80) <sub>20</sub> -Zw	DTMAm <sup>a</sup> (20%)	$\text{SPP}^{b}(80\%)$	20
(20-80) <sub>10-</sub> -Zw	DTMAm (20%)	SPP (80%)	10
(50-50) <sub>10</sub> -Zw	DTMAm (50%)	SPP (50%)	10
(20-80) <sub>20</sub> -PEG	DTMAm(20%)	MAPEG <sup>c</sup> (80%)	20

Table 4-1	List and	composition	of different	copolymers	s used in th	nis studv
						2

<sup>a</sup> DTMAm - 5-(1,2-dithiolan-3-yl)-N-(3-methacrylamidopropyl)pentanamide. <sup>b</sup> SPP - 3-sulfopropyldimethyl-3methacrylamidopropylammonium inner salt. <sup>c</sup> MAPEG- Poly(ethylene glycol) methyl ether methacrylate. Overall molar monomer percentage in the copolymers are indicated in brackets.

# 4.2.2 Types of polymers, brief characterization and method of ligand exchange

The detailed synthesis, purification and characterization of four types of copolymer used in this work, method for ligand exchange and storage of QDs is discussed in detail in Materials and Methods. After ligand exchange, QDs coated with different copolymers displayed different physico-chemical properties. In this section, individual characteristics of these QDs will be described.

*Quantum yield.* Transition of QDs to aqueous solution by ligand exchange is often accompanied by change of Quantum Yield (QY). This batch of QDs had high QY with respect to Rhodamine 6G (~65%) in hexane. From QDs in hexane, all water dispersed QDs maintained a high QY >70% (**Figure 4-2**, A) Relative to each other, the QD coated with (20-80)<sub>10</sub>-Zw showed marginal (<20%) decrease in the QY in 0.2M NaHCO<sub>3</sub>. These experiments were run at a QD concentration of 25 nM, (in triplicate). Mean and SEM of 2 individual ligand exchange batches is plotted.



Figure 4-2 Quantum yield of QDs in aqueous media. (A) Relative QY of QDs after ligand exchange with indicated copolymers in 0.2M NaHCO<sub>3</sub> pH 8.3. (B) Dependence of QY of

QDs with indicated polymers on pH of media. All data are normalized to the value QY of  $(20-80)_{10}$ -Zw at pH 7.

*pH-dependent fluorescence emission.* For applications of QDs in biology especially in context of cellular targeting, the dependence of fluorescence emission on the pH of the media is very important. For multiple cellular pathways, endosomal maturation is accompanied by a change in pH (see chapter 3 for details). Therefore, it was essential to evaluate whether the fluorescence QY of each of these QDs was vastly affected by pH. For these experiments, 30 nM solutions of QDs were prepared in the following buffers: 4 < pH < 5.5 in 0.1 M MES, 6.5 < pH < 8.5 in 0.1M HEPES and pH = 9.5 by 0.1M Sodium tetra-Borate. All data are normalized to the QY of  $(20-80)_{10}$ -Zw at pH 7.5. In these experiments, it was found that QY of QDs (all types) is higher in basic media than in acidic media (**Figure 4-2**, B). Also the difference in QY could vary up to 50% between pH 4.5 to 9.5 for each type. These two trends were consistent over all the four types of surface chemistry of the QDs. This suggests that the pH sensitivity of the fluorescence of the QDs does not depend on the nature of copolymer (within the types screened herein) on its surface but arises from other physico chemical parameters such as the alteration of the crystalline surface of the QDs by the protons.

*Heterogeneity in ligand-exchanged populations of QDs.* As seen by the TEM image, the batch of synthesis used for this experiment was highly monodisperse. However, the nature of the ligands and the procedure of ligand exchange can substantially affect the overall populations the QDs in water, generating subpopulations of partially aggregated (dimers /oligomers) nanocrystals. This aggregation is caused by the entropic need to minimize the hydrophobic patches on the surface of nanocrystals (Scheme 4-1) and is complex to control. When  $100\mu$ L of  $1\mu$ M concentration of each sample was injected, these populations eluted at different times on the size exclusion chromatography (SEC) column (Figure 4-3). Several attempts in this thesis to alter the ratio of these subpopulations by changing the concentration of ligands and time of exchange have been widely unsuccessful (data not shown). It was found that the individual populations pertaining to each Ligand Exchange was widely a signature of the type of polymer coating the surface of the QDs.



Scheme 4-1 Heterogeneity in QD populations after ligand exchange



**Figure 4-3** Profile of elution of QDs on Size exclusion chromatography column with indicated ligands. The peaks are fitted using Origin with big aggregates (pink), small aggregates (light red) and single (dark red) QDs after ligand exchange

From the chromatograms of elution of these QDs coated with different ligands, the following observations could be made: i) The population eluting out the first, *i.e.* close to 15 mins had

the maximum hydrodynamic size  $(D_h)$  and therefore indicated the peak of maximal aggregation; ii) This peak appeared in all the QDs coated with zwitterionic polymer at higher concentration (data not shown). After storage for a year (or more), the percentage contribution of this peak increased (**Figure 4-4**). These two results indicated that this chromatogram could be actually used to compare the state of aggregation and the 'age' of the QDs in aqueous buffers. On the other hand, the almost sharp elution of QDs coated with the PEG-based copolymer suggested that this polymer generated a more homogenous population of nanocrystals in water. Continuous nature of peaks made it difficult to isolate individual fraction using SEC. Also, re-concentration of the first peak (most aggregated) further resulted in loss of stability of the fractions and the QDs were visibly adsorbed to the ultrafiltration membrane (data not shown). Additional attempts to characterize these peaks were not pursued.



Figure 4-4 Elution profile of ligand exchanged QDs change with ageing of the sample.

These populations could be removed (at least the most aggregated peak) from the bulk batch by ultracentrifugation. However re-concentration of the QDs from classical ultrafiltration further removed several ligands from the surface and changed the state of heterogeneity. Hence for all experiments, these batches were used as it is, without any additional purification of the aggregated NPs. The state of aggregation of QDs is consequential in applications such as single molecule tracking. However for more bulk applications (like monitoring endocytosis pathways as used in this work) it is not an absolute requirement to remove these populations.

*Electrophoretic mobility*. These ligand-exchanged QD samples were then loaded on 0.8% agarose gels to compare the electrophoretic mobility. All types of QDs migrated towards the positive terminal, displaying the negative surface potential, despite electrostatically neutral polymer coatings. The zwitterions are locally charged but overall neutral, whereas PEG is neutral. This suggests that in context of these QDs, surface potential of the QDs vary substantially based on the nature of these polymers coating (**Figure 4-5**). However, within this global anionic behavior, there are evidently different rates of migration among the four samples. The QDs coated with PEG had the slowest rate of migration, most likely due to reduced surface charge and less counter ions. The (20-80)<sub>10</sub>-Zw migrated faster than the (20-80)<sub>20</sub>-Zw, possibly due to more compact size of former imparted by the length of the polymer chain. Surprisingly, the 50-50 had the highest mobility towards positive terminal. This can be accounted as an effect of two scenarios:

- i) First higher density of dithiols ( $\sim$ 5/QD) and thus greater population of thiolate anions on the surface of QDs may increase the affinity towards positive terminal.<sup>176–179</sup>
- ii) A second scenario can be imagined if most of the thiols are anchored to the surface of QDs at steady-state, then the polymer is more compactly arranged on the surface, resulting in smaller  $D_h$  of the nanocrystals. The real scenario could also be a complex outcome of both of these parameters.



**Figure 4-5**Electrophoretic mobility of QDs coated with indicated polymers on a 0.8% agarose gel run in 50mM Borate buffer pH 9.

# 4.3 Probing biological characteristics of the QDs in-vitro

In this section, QDs synthesized with exactly similar mechanism but variable surface ligands (copolymers) were tested for non-specific adsorption of protein. The goal was to compare how the nature of surface ligands dictates the protein corona and thereby affect NP properties. Several of these parameters such as electrophoretic mobility, hydrodynamic diameter, surface potential, change in untargeted uptake were compared.

For these experiments, Fetal Bovine Serum (FBS) from Gibco was used. Typical experiments were carried out on  $1\mu M$  solutions of QDs within total volume of  $100\mu L$  after addition of requisite volume of FBS solutions.

# 4.3.1 Electrophoretic mobility of QDs upon incubation with serum

In these experiments, each type of QD were added to solutions with various percentage of FBS  $(OD = \sim 100 \text{ pmols})^2$  and incubated for 24 hours to allow maximum protein adsorption. <sup>180</sup> The following day, the QDs were run on a 0.8% agarose gel in 50mM borate buffer (pH =8.5). The images of individual gels to compare the migration of respective QDs (in PBS, extreme left of each image) to varying concentrations of FBS are shown on Figure 4-6. This experiment lend into some very interesting observations. First, all samples still retained their negative surface potentials, indicated by the bulk migration towards positive terminal. Second, increase in the percentage of FBS did not necessarily increase the net electrophoretic mobility (between 10-75% of FBS). This suggests that beyond a specific concentration of proteins, the extent of non-specific adsorption of proteins on these QDs is not governed by the abundance of proteins in surrounding medium. This is a promising lead, since many conjugation reactions require QDs to be incubated with increasing excess of biomolecules of interest, and may often result into high non-specific adsorption of proteins on QDs. Third, the most striking alteration in electrophoretic mobility was shown by  $(50-50)_{10}$ -Zw and the (20-80)<sub>20</sub>-PEG. Addition of FBS to (50-50)<sub>10</sub>-Zw reduced its electrophoretic mobility towards the positive terminal. This could be an effect of both association of positively charged proteins to the QDs surface and increase of the NP size. On the other hand, incubation of QDs coated with PEG increased the mobility towards positive terminal suggesting that net charge on the

<sup>&</sup>lt;sup>2</sup> These concentrations were chosen as 50-100 nM of QDs is typical concentration used in most biological assays.

particles increased due to adsorption of proteins. Fourth, and probably the most striking observation, was the electrophoretic mobility of the other two zwitterionic polymers It was observed that increasing concentration of serum proteins on QDs coated with (20-80)-Zw polymers (both chain lengths, 10 and 20 monomers on average) had no effect on the electrophoretic mobilities of the QDs. This indicated that this design of polymers probably had the ideal mole fraction of thiol-zwitterionic components for preserved anti-fouling properties in complex media.



**Figure 4-6** Interaction of QDs of different surface chemistries with Fetal Bovine Serum (FBS). (A) Schematic showing association of serum proteins with QDs (B) Electrophoretic mobility of QDs is affected upon non-specific adsorption of proteins. The types of ligands are indicated below each gel and the % of FBS is indicated in the key. (C) Change of Quantum Yield of QD upon incubation with FBS in 1x PBS pH 7.4. All data are normalized to individual QDs types in PBS pH 7.4 in absence of FBS.

# 4.3.2 Hard corona and electrophoretic mobility

In the above section, the dependence of electrophoretic mobility of QDs upon the concentration of serum was discussed. In these experiments QDs were first incubated with indicated concentration of serum for 24 hours and then directly loaded on-to the agarose gel. In an experiment like this it can be imagined that the QDs are coated with two types of layers of proteins: i) directly strongly adsorbed proteins on the surface of the QDs *i.e* 'hard corona'; ii) proteins adsorbed on this first layer of proteins *i.e* soft corona'.<sup>3</sup> Obviously, the first layer is held more tightly than the second layer. In the electrophoresis results discussed above, the overall migration properties were a cumulative effect of both hard and soft corona over the QDs. it was then interesting to observe whether the 'removal' of soft corona re-alter the electrophoretic properties. For these experiments, QDs incubated with 10% FBS were centrifuged 55,000 RPM for 20 minutes on an ultracentrifugation system in 1X PBS buffer pH 7.4. The nanocrystals centrifuged as a thick pellet, and the unbound and loosely bound proteins remained in the supernatant. The supernatant was discarded, and QDs were resuspended in aqueous media. This process was repeated thrice to get rid of majority of loosely bound proteins. The NPs obtained after this purification comprised exclusively of QDs and proteins tightly bound on their surface (Figure 4-7, A). These QDs were then run on 0.8% agarose gel. The electrophoretic migration of these QDs was very similar to those seen in the previous section, further confirming that the change in electrophoretic migration was an effect of protein corona on NPs (Figure 4-7, B).

<sup>&</sup>lt;sup>3</sup> In this context 'layer' may not exclusively imply a monolayer of proteins.



**Figure 4-7** Electrophoresis of QDs complexed with hard corona of proteins. (A) Schematic showing preparation of QD-protein complexes. (B) Electrophoretic migration of QDs in PBS (left) and after protein adsorption (right) for indicated surfaces.

# 4.3.3 QY of QDs upon incubation with serum

The non-specific adsorption of proteins on the QD surface can lead to several effects including change in the QY. One of the possible causes for this is that adsorption of proteins often tend to replace original ligands on the surface of QDs, often in a concentration-dependent manner. This process can additionally cause loss of solution stability of QDs over time. In experiments which involve internalization of QDs by cells (such as electroporation, microinjection or endocytosis), the NPs are often surrounded by several cytosolic or targeting proteins that would essentially coat the surface of QDs. In this scenario, it is absolutely essential that QDs retain their high QY. With this objective in mind, QY of QDs incubated with 10%, 25% and 75% FBS was assessed in the media with indicated %FBS diluted using 1X PBS (**Figure 4-7**, **C**). The QY of QDs coated with (20-80)<sub>20</sub>-Zw, (20-80)<sub>20</sub>-PEG and (50-50)<sub>10</sub>-Zw remained within 85-100% of the original after incubation with 10-25% FBS for about 24 hours, and decreased steadily at 75% FBS. On the other hand, the QD of (20-80)<sub>10</sub>-Zw was observed to increase with higher concentrations of FBS. This last observation cannot be explained with our current understanding of the system. Nevertheless, it could be

concluded from this data that all the four types of QDs retained their high QY even after 24 hours of incubation with 10-25% FBS and thus invaluable for applications of intracellular processes.

# 4.3.4 Quantification of the protein content.

In the above section, several results were discussed that concluded that incubation of NPs with high concentrations of serum resulted into modification of the surfaces with protein. It was then interesting to estimate this protein content. For this purpose, Fluorescamine reagent was used. This reagent has been used for quantification of proteins from over decades. Fluorescamine is a colorless compound that reacts with primary amine containing molecules to generate a pyrrolidinone derivative which is fluorescent (**Scheme 4-2**). This reagent can thus quantify proteins containing solvent exposed basic amino acids such as lysine and arginine. Another advantage of this compound is that the fluorescent product emits at 495 nm, well separated from the emission peak of the QDs of interest ( $610\pm18$  nm). Therefore there was minimal spectral overlap for individual detection.



Scheme 4-2 Chemical reaction between Fluorescamine and primary amine-containing molecule.

Sensitivity of detection response. Before quantification of the fluorescence of proteins bound on the surface of QDs, the detection response of the reagent was estimated. Since the protein solution used herein in FBS, and different commercial batches can vary based on supplier and sources, all the results are reported in terms of %FBS. Secondly the adsorption of proteins on QDs is random, and the orientation of binding to the NP surface is in-homogenous and uncontrolled. Therefore, several lysine/arginine groups may remain bound to the surface in a manner that the reagent in solution may not be able to access it. Hence, the quantification will be variable and error-prone. Hence only the *relative* quantification is carried out. The sensitivity of the reagent to increasing percentages of FBS was estimated: on a microtiter plate, to  $20\mu$ l of  $300\mu$ g/ml solution of Fluorescamine,  $40\mu$ L of FBS solution was added (such that the desired percentage of FBS is obtained in total volume of  $60\mu$ l). The reaction occurs within few milliseconds, and the fluorescent product is stable for hours. However, for the sake of this experiment, the reaction was left for 15 minutes under stirring before measurement. The fluorescence emission was measured at 465 nm ( $\lambda_{ex}$ =390) on a microplate reader. All measurements were carried out in quadruplet, and mean and SEM of two independent experiments is plotted. The fluorescence of the reagent showed a linear response between 0-10% of FBS (**Figure 4-8**). This region of linear sensitivity was chosen since maximum percentage of FBS in cell culture media is 10%, and hence this is the cut-off of the total protein context in all biological assays. At higher % of FBS (75%) the fluorescence signal saturated (data not shown).



**Figure 4-8** Evolution of fluorescence intensity of Fluorescamine with increasing percentage of FBS in solutions. Fluorescence was recorded using  $\lambda_{ex}$ =390 nm and  $\lambda_{em}$ =465 nm in 1X PBS pH 7.4. Mean and SEM of 2 independent experiments is plotted Fluorescamine reagent shows linear response to FBS concentration within region of interest.

*Background reactivity with different ligands on QDs.* In this work, four different types of polymers were used. Before reliably computing the proteins corona, it was essential to ensure that these polymers (and any remaining ligands on the surface of QDs) do not have some erroneous background reactivity to Fluorescamine, which may result into false detection. To

estimate this, three different concentrations of each sample of QD were reacted with Fluorescamine and the background responses were estimated. For all polymer coatings, even at high QDs concentrations (1 $\mu$ M), Fluorescamine showed negligible conversion to fluorescent product. This sets basis for reliable computation of protein corona on all QDs (**Figure 4-9**).

After ensuring that (i) conversion to fluorescent product is linear within the region of interest (ii) there is no background reactivity from any of the QDs; this assay was applied to the QDs coated with a hard corona of proteins. Solutions of  $0.1\mu$ M concentration of QD (with adsorbed proteins) of each type of sample (different polymer coating) were added to the microtiter plate in presence of the Fluorescamine reagent. Emission was recorded in two specifications  $\lambda_{em}$ =465 nm ( $\lambda_{ex}$ =390 nm) for Fluorescamine and  $\lambda_{em}$ =610 nm ( $\lambda_{ex}$ =350 nm) for QDs. The Fluorescamine signal was computed in to protein concentration (in %FBS) by calibration curve explained previously. After this step, the relative protein adsorption was plotted for all the QDs. All measurements were done in quadruplet, and mean and SEM of two independent experiments was plotted.



**Figure 4-9** Evolution of fluorescence intensity of Fluorescamine upon increasing concentration of QDs (log scale) of indicated type. Fluorescence was recorded using  $\lambda_{ex}$ =390

nm and  $\lambda_{em}$ =465 nm in 1X PBS pH 7.4. Mean and SEM of 2 independent experiments is plotted. Fluorescamine reagent shows negligible background fluorescence signal to range of concentration of QDs.

Using this experiment, it was found out that all the QDs coated with zwitterionic polymers had significantly lower (approx. half) non-specific adsorption of the proteins than of QDs coated with the PEG-based polymer (Figure 4-10). This is very interesting observation since PEG-based coatings are exhaustively used to impart antifouling properties to ODs. Having a scaffold with further lower non-specific adsorption of proteins definitely presents an interesting alternative for surface functionalization of QDs. Several comparative reports on physico-chemical properties such as hydration, solvation, binding energy between ligand derivatives of PEG and zwitterions also support these observations.<sup>181–184</sup> In essence of these existing studies and the results obtained herein, several conclusions can be made. The Zwitterionic polymers have better antifouling properties than PEG-based polymers. And second is that on the basis of NP coverage, the different zwitterionic polymers (with different mole fractions of zwitterionic monomer) may impart antifouling properties in unique yet corelative manner.<sup>184,185</sup> Though the subtle difference in the protein corona within the different zwitterionic coatings could not be highlighted by the methods described above, but the difference between uncharged (PEG) and locally charged (zwitterionic) coating could be conclusively addressed.



**Figure 4-10** Relative adsorption of proteins on QDs coated with different polymers as quantified using Fluorescamine assay. Fluorescence was recorded using  $\lambda_{ex}$ =390 nm and  $\lambda_{em}$ =465 nm (Fluorescamine) and  $\lambda_{em}$ =610 nm and  $\lambda_{ex}$ =350 nm (QD) in 1X PBS pH 7.4. Mean and SEM of 2 independent experiments is plotted. All data are normalized to the Fluorescamine/QD signal for QD-protein corona sample with (20-80)<sub>10</sub>-Zw polymer.

*Nature of proteins adsorbed on QD* After confirming that all the above four surface chemistries have some proteins associated with them upon incubation with the serum, it was then interesting to find out the exact nature of these proteins. With this idea in mind, these protein-QD complexes were heat-digested in presence of reducing agent ( $\beta$ -Mercaptoethanol) and loaded on an SDS-PAGE gel (4% stacking, 12% resolving). For calibration, various dilutions of FBS were also added (**Figure 4-11**).



**Figure 4-11** Non-specific adsorption of proteins on QD samples as determined by SDS-PAGE with 4% stacking and 12% resolving gel. Proteins migrate to 12% gel whereas remnants of QD (indicated by arrow) are stuck at the boundary between 4% and 12%. Comparative dilutions of FBS (left) and different samples of QDs (right) are shown.

The results from PAGE analysis conclusively suggested two things - (i) the protein that is present in maximal abundance binds the most -i.e., non-specific binding to QDs is dependent on serum concentration of the protein. Based on the 'apparent' molecular weight and the serum composition (Suppliers catalogue) this unknown protein is most likely Albumin in this case) (ii) Taking the intensity of the input (amount of QD in sample stuck within 4% gel see Figure 12, arrow) as an estimated of net QD concentration, the (20-80)<sub>10</sub>-Zw showed minimal non-specific adsorption of proteins on the surface. This result was additionally confirmed by running the same gel at higher concentration of samples (data not shown). This also helps in delineating that within the zwitterionic polymers, (20-80)<sub>10</sub>-Zw had the least nonspecific adsorption of proteins on the surface. An alternate way to confirm whether serum proteins massively stick on the surface of QDs is by monitoring the change in size of the NPs. As discussed in the previous sections, the heterogeneity in individual batches of QDs can be easily monitored using SEC. Therefore, comparison of 'before' and 'after' spectra of QD samples could enable facile estimation in both change in net size and state of aggregation upon incubation with serum. With this idea, QD-protein corona complexes were injected in the SEC column. Figure 4-12 shows the overlap of the pre- and post-serum complexation with QDs. As clearly seen, 3 out of 4 QDs (covered by (20-80)<sub>20</sub>-Zw, (20-80)<sub>20</sub>-PEG, (50 $50)_{10}$ -zw) have characteristic features such as (i) loss of peak with smallest D<sub>h</sub> (ii) increase in the contribution of the most aggregated peak (close to 15 min elution) (iii) change of features of the intermediate peak. And further supporting the result from the PAGE analysis,  $(20-80)_{10}$  –Zw withstands most of these changes – the overall shape of elution is conserved, the percentage contribution of the most aggregated peak is still the lowest. However, the smallest  $D_{\rm h}$  peak is slightly shifted towards faster elution, suggesting that a homogenous population of QDs is obtained.



**Figure 4-12** Profile of elution of freshly ligand exchanged QDs (black) and purified QDs coated with protein corona (blue) on Size exclusion chromatography column with indicated ligands. In all QDs the relative fraction of most aggregated peak is increased. The  $(20-80)_{10}$ -Zw show the most conserved elution profile than all other type of QDs

*Change of physico-chemical properties.* Additionally, the physico-chemical properties of QD complexed with serum proteins were also assessed my measurement of  $D_h$  (by DLS) and zeta-potential (**Table 4-2**). Though all samples indicate towards an approximately 100% increase in the  $D_h$ , it must be understood that these measurements are often subjective and less reliable due to inherent heterogeneity in the samples.

Table 4-2 Physico-chemica	al properties of	QDs change a	fter adsorption	of proteins
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QD-Type	$\frac{\text{DLS}}{(\text{D}_{h}, \text{nm})}$	PDI	ζ-potential (mV)	$\frac{\text{DLS}}{(\text{D}_{h}, \text{nm})}$	PDI	ζ-potential (mV)
$(20-80)_{20}$ -Zw	17.4	93%	-5.65	28.52	95.7%	-7.72
$(20-80)_{10}^{20}$ -Zw	12.4	44%	-9.66	30.15	100%	-9.34
(20-80) <sub>20</sub> -PEG	10.0	83%	-4.93	30.08	97.4%	-7.79
$(50-50)_{10}^{20}$ -Zw	14.1	47%	-9.58	34.83	96.6%	-9.34

# 4.4 Probing biological characteristics of these QDs in-cellulo

After careful evaluation of the properties of QDs with biological media, it was then interesting to estimate whether the interaction with cells (membrane/uptake etc.) was dependent on the nature of surface ligands, and how these interactions vary with the concentration of QDs and time of incubation. For all the cellular experiments I.A.2.2F cells were used. The cells were seeded on LabTek Chambered slides such that on the day of experiments, at least 10<sup>6</sup> cells were available.

# 4.4.1 Dependence on concentration.

For this experiment, cells were incubated with  $0.01\mu$ M,  $0.1\mu$ M,  $0.5\mu$ M or  $1\mu$ M sample of each type of QDs for 2 hours.<sup>4</sup> After the incubation period, cells were washed, stripped in acidic media (to remove loosely bound QDs) and fixed using methanol (see chapter 5 for details). Cells appearing morphologically rounded (indicating loss of health/cell death) were excluded from the analysis. For analysis, 80cells/dish were individually quantified. The points on the graph represent mean of fluorescence intensity (normalized to maximum

<sup>&</sup>lt;sup>4</sup> These concentrations were chosen as 50-100 nM of QDs is typical concentration used in most biological assays.
concentration) and SEM of two independent experiments. The bar graph represents absolute mean intensity for two different concentrations after 3 hours (Figure 4-13).



**Figure 4-13** Cellular labelling as a function of concentration of QDs coated with different ligands in culture medium (Hams-F12 supplemented with 10% FBS) (A) schematic of assay (B) comparison of trends of individual samples (C) Relative comparison of cellular labelling of QDs at indicated concentrations.

For all QDs increasing concentration correlates with increasing in cellular fluorescence. Within this trend, total labelling by QDs with longer polymeric chains (20 monomers) saturated within 3 hours. On the other hand, the uptake of QDs with shorter chains (10 monomers) continued to increase till this time. However, much to our surprise, substituting PEG with zwitterion did not massively change the interactions with cells. At this point it is not possible to differentiate between the contributions of non-specific adsorption at the membrane to the intracellular fluorescence. Further studies in this direction may be able to quantitatively discern the extent to which surface charge on NPs affects interactions with cells.

#### 4.4.2 Dependence on time

In Chapter 3, detailed mechanism of endocytosis of NPs was discussed. As seen in several reports in literature, QDs with different surface chemistries can be up-taken by cells even without the presence of any endogenous targeting ligand.<sup>141,151</sup> In general, QDs can be uptaken into cells due to binding of several receptors (specific or otherwise) to the surface of the nanocrystals followed by wrapping of the membrane around them. The time taken for completion of this multistep process depends on several factors such as shape, size, surface chemistry of QDs (or nanocrystals) and the type of cells. Thus, both dose of the NP and the period of incubation are consequential. Hence, experiments were designed to test how duration of pulse with QDs (of different surface chemistry) may affect the cellular fluorescence. These cells were pulsed with 100nM QD samples for 30 mins, 1 hour, 2 hours and 3 hours. These pulse durations were selected based on incubation periods of typical cellular assays. For all the surface chemistries, the cellular fluorescence had saturated within the first two hours suggesting that the maximum cellular labelling is done within this period (Figure 4-14). The absolute intensity of cells labelled with (20-80)<sub>10</sub>-Zw was the highest while surprisingly, the one with the PEG was the lowest. Further studies to outline mechanisms of uptake may enable better understanding of these results.



**Figure 4-14** Dependence of cellular labelling with the duration of pulse. (A) Schematic of assay (B) Plot of normalized intensity as a function of duration.

## **4.5 Conclusion**

In this chapter, several surface chemistries of QDs were investigated for biological applications. The type of ligands definitely dictates the overall fate of NPs in biological applications. The surface chemistry can affect the non-specific adsorption of proteins on the NP surface, which results into the change in the overall size, surface proteins and interaction with orphan receptors on cellular surfaces. In this chapter, a systematic study of how surface chemistries affects interaction of QDs with complex media was provided. Based on these results, several generalizations can be made. QDs with different surface chemistries can have massively different fate in complex cellular media. The type of polymeric ligands affects the overall non-specific adsorption of serum proteins on the NPs. In these experiments, it was also found that zwitterions have lesser non-specific adsorption of proteins than the PEG-based polymers. This is a valuable result, since PEG-based polymers are extensively used for imparting antifouling properties to QDs (and other NPs) in cellular assays. It was additionally observed that cellular fluorescence from QDs coated with polymers with longer chain length saturate faster than those of the smaller counterparts. However, additional experiments

evaluating mechanisms of uptake of these samples may provide better insight into additional physico-chemical parameters that control these interactions.

This study thus sets basis for careful evaluation of few parameters which are essential to better prepare QDs for challenging applications in biology.

# **Chapitre 5 Materials and Methods**

In this chapter, details of the instrumentation, materials, methods and analysis strategies used to carry out the experiments described in this thesis.

# **5.1 Characterization techniques**

## 5.1.1 Absorption

Optical absorption spectroscopy was performed on UV-3600, Shimadzu UV-Vis.-NIR double beam spectrophotometer ranging from 200 nm to 1100 nm. A quartz cuvette with minimum volume of 80  $\mu$ L and an optical path of 1 cm was used to record both reference and absorption spectra of sample. Calculations of samples concentrations are made on samples having absorption between 0.1-1, *i.e.* with the range of Beer-Lambert's law.

### 5.1.2 Fluorescence

Emission spectra were measured on a Fluoromax-3 fluorimeter (Jobin Yvon, Horiba). A rightangle configuration was used. Optical density of the samples was checked to be less than 0.1 to avoid reabsorption artifacts. All of the fluorescence spectra are automatically corrected by the apparatus function and the apparatus is always used in its right angle configuration (90° between the excitation and emission beam). All of the fluorescence spectra are recorded on samples having absorption below 0.1 at the chosen excitation wavelength ( $\lambda_{exc}$ ).

### 5.1.3 Measurement of Quantum yield

For all QD samples, QY are measured in reference to Rhodamine 6G ( QY = 95% in ethanol)<sup>186</sup> and relative QY can be estimated based on the following formula.<sup>187</sup>

$$QY(x) = QY(ref) \times \frac{\sum F(ech) * A(ref)}{\sum F(ref) * A(ech)} \times (\frac{n(x)}{n(ref)})^2$$

Where:

*ref* is reference, rhodamine x is sample (QD or other)  $\sum F$  is integration of the fluorescence signal A is absorbance n is refractive index of the medium (mainly water or hexane for samples, Ethanol for reference)

Even though QY measurements are independent of concentration, QY were made on 10 nM solutions of QD for the sake of homogeneity.

#### 5.1.4 Epifluorescence microscope

All cells were imaged with Olympus IX71 microscope fitted with a 100W Mercury Lamp. Before imaging, the lamp was switched ON and left for stabilization for at least 15-20 minutes. The microscope is fitted with a bias stabilized QuantEM:512SC EMCCD camera with regulated cooling to -20°C from Photometrics. Three types of objectives 10X (air), 60 (water) and 100X (oil) were used with 1X and 1.5X field diaphragm induced magnification. Custom made/optimized filters from Chroma scientific and/or Shemrock were assembled based on target fluorescence excitation and emission wavelength of interest. Optical set-up, complete list of filters and target fluorophores is given in Appendix 4 and 5.

For all fluorescence imaging, each field was viewed in respective channels. The exposure settings were optimized based on the intensity of fluorophores on that day. For each experiment, all settings for a given fluorophore were kept constant. Additionally, to have more reliable boundary and topology of cells, each field of view was imaged in DIC contrast (under white light). For live cell experiments, a similar set up fitted with temperature controller was used. Filter cubes were not changed.

#### 5.1.4 Spinning disk confocal microscope

Inverted spinning disk confocal Roper/Nikon microscope with temperature and CO<sub>2</sub> control for live cell imaging was used from the imaging platform from Institute Curie. The spinningdisc system is set up on an inverted microscope Nikon TiE, equipped with a piezo stage MCL mounted on a XYZ encoding motorized scanning stage. This system also includes a Photo Ablation module in the form of the Roper iLAS2 system, images being recorded on a CoolSNAP HQ2 camera. The system includes 4 laser lines 405,491nm, 561nm and 633nm, allowing imaging in DAPI, CPF, GFP, YFP, Cy3, mCherry and Cy5 (all common fluorophores). For imaging using QDs, excitation corresponding to mCherry with 50 ms exposure provided excellent fluorescence signal. The microscope is embedded within a thermo-regulated enclosure and  $CO_2$  from Life Imaging Services (LIS Cube Box). It is provided to allow long-term recording of live samples. For imaging in the context of this thesis, 60X oil objective with NA 1.4 was used.

## 5.1.5 Transmission electron microscopy (TEM)

TEM was done using JEOL 2010. For preparation of samples, carbon-coated Cu grids (200 mesh, TedPella Inc) were used. Samples ( $\sim$ 5-10µl) were deposited with concentration in the range of 1-100nM and dried for 5 mins at 60°C (for samples in water). For samples in aqueous buffer dilution was carried out in water, since dried salts often appear as dense patches and NPs 'appear' aggregated.

*Estimation of size and polydispersity of QDs using TEM.* The size distribution of QDs batches was measured by TEM images of > 250 QDs in different imaging planes and grids.

## 5.1.6 Ultracentrifugation

Quantum Dots and other nanomaterials have high density that enables separation of these particles from relatively low density biomolecules such as DNA and proteins. In the LPEM laboratory, two types of ultracentrifugation methods are employed: (i) differential centrifugation (ii) rate zonal centrifugation.

**Differential centrifugation**. For rapid purification of proteins from QDs, differential centrifugation has been frequently used in this thesis. The separation is based on the principle that particles with different densities/size sediment at variable rates. The largest and most dense particles sediment the fastest followed by less dense and smaller particles. Briefly, sample (50-150µl) is carefully diluted to 2mL in desired buffer (PBS pH 7.3/0.2M NaHCO<sub>3</sub> pH 8.3). Weight-balanced (with 0.1-0.5mg precision) tubes are centrifuged in Beckman Optima Ultracentrifuge using fixed angle rotor (MLA-130) at 75,000-120,000 RPM for 15-20 mins. The QDs centrifuge down as a lightly held pellet whereas the biomolecule remains in the supernatant. The supernatant can be carefully discarded to collect only the QD pellet for subsequent use.

*Rate zonal centrifugation using a sucrose gradient*. This method has been used to purify QDs from proteins and DNA. Briefly, 10-40% density gradient of sucrose (in 20mM NaCl) is prepared in polypropylene tubes (5 mL, Beckman Coulter) using Gradient Station (Biocomp

Instruments) and then the sample (50-150µl) are loaded carefully on the top. Weight-balanced (with 0.1-0.5mg precision) tubes are centrifuged in Beckman Optima Ultracentrifuge using swinging bucket type rotor (MLS-50) at 35,000 to 50,000 RPM for 20-30 mins. The QD-conjugates can be separated from unconjugated biomolecules and easily extracted under illumination of Gradient Station. Post extraction, the conjugates can be buffer-exchanged and concentrated using ultrafiltration with vivaspin (100kDa), and stored for future use.

## 5.1.7 Size Exclusion Chromatography

Two different types of SEC have been used in this thesis. Commercial NAP columns (Illustra, GE Healthcare Lifesciences) for rapid desalting and purification; or SEC on High performance Liquid Chromatography (HPLC) for simultaneous purification, quantification and spectroscopic characterization.

*NAP-5/10.* These are commercial SEC columns that have been routinely used for purification of QDs/DNA from reducing agent, QDs from reactive biotin/fluorophore, DNA/protein from fluorophores etc (**Scheme 5-1**). These SEC columns are prepacked with Sephradex G-25 beads of 20-80 $\mu$ m diameter. These columns are first equilibrated with 3 column volume of buffer (PBS, NaHCO<sub>3</sub> etc) and then sample of interest is applied (500 $\mu$ l for NAP-5, 1000 $\mu$ l for NAP-10) and allowed to pass through the column. The small molecules get trapped in the beads and the higher molecular ligands can be easily eluted with additional 500-1000 $\mu$ l elution buffer.



Scheme 5-1 Purification of reduced QDs from excess TCEP using size exclusion column NAP-5.

*High Performance Liquid Chromatography*. Several of conjugates including QD-DNA, DNA –protein and QD-protein were routinely analyzed on a SEC column adapted on a High Performance Liquid Chromatography (HPLC) apparatus. The HPLC system Prominence from Shimadzu equipped with 150W xenon lamp with holographic concave diffraction grating monochromators for both excitation and emission between 200-650 nm range for fluorescence spectra was used. For absorbance, the system was equipped with D2 and W lamp that enabled multi-wavelength UV detection simultaneously, obtaining a range of absorbance spectra between 190-800nm. Superose 6 10/300 GL prepacked columns (GE Healthcare) were used for purification (preparative column). The column was first equilibrated with PBS, pH 7.4 (Sigma) at the flow rate of 0.5mL/min for one hour followed by injection of the prepared sample within 50-200 $\mu$ L volume. For purification, the samples were prepared in PBS, filtered with 0.2  $\mu$  PVDF filters (Merck) and then injected into HPLC. Reference retention times of individual proteins, QD, DNA, conjugates etc were individually determined routinely to calibrate for column performance (Figure 5-1). Typical elution profiles to show difference in elution time of individual conjugates is shown in (Figure 5-2).



**Figure 5-1** Superimposed chromatograms of elution profile of typical conjugates of QD-DNA (red), Protein-DNA (Blue) and ssDNA (Black) obtained from SEC-HPLC are shown.



**Figure 5-2** Recovery of QDs as fractions from HPLC. Fractions under white light (top), UV (bottom) and on chromatogram.

### 5.1.8 Gel Electrophoresis

Two types of gel electrophoresis approaches were used.

For characterization of QDs and related conjugates, **agarose gel electrophoresis** was used (AGE). Typical preparation included gelation of 0.8-1 g of agarose powder (Sigma) in 100 mL of 50mM Borate buffer (0.8 to 1% gels at pH 8.5) by heating in microwave for 30 secs, shaking and reheating for 20 secs. The agarose gel was allowed to set/solidify for half an hour in a dedicated apparatus and ready to use. The electrophoresis was carried out on Apelex electrophoretic chambers and typically run at 110V.

QDs coated with different ligands have different electrophoretic migration. The following table summarizes the trends of electrophoretic migration (**Table 5-1**).

Type of ligand	Terminal	<b>Relative migration</b>
MPA	+	++
Zwitterionic polymer (20-80) <sub>20</sub>	-	++
Zwitterionic polymer $(20-80)_{20}$ + DNA	-	+++
Zwitterionic polymer $(20-80)_{20}$ + transferrin (protein)	-	++
Zwitterionic polymer $(50-50)_{10}$	-	+++
Zwitterionic polymer $(20-80)_{20}$ + DNA/protein	-	++
PEG polymer $(20-80)_{20}$	-	+
PEG polymer (20-80) <sub>20</sub> + DNA/protein	-	++

 Table 5-1 Type of ligands for QD and electrophoretic migration.

For characterization of DNA, proteins and related conjugates, **polyacrylamide gel electrophoresis** (PAGE) was used. The QDs do not migrate in acrylamide gels due to narrow porosity, even at low percentages of acrylamide (<4%). Typical preparation involves cross-linking of acrylamide with bis-acrylamide (29.5:0.5) in presence of initiator (10% Ammonium Persulfate) and catalyst (Tetramethylethylenediamine, TEMED). Additional compositions are described in Appendix 3. Alternately pre-cast gradient PAGE (4-15%) gels were also used. Gels were run in Bio-Rad Electrophoresis Units (mini-PROTEAN tetra cell) at 200V potential with 110A current.

### 5.1.9 Dynamic Light Scattering

Dynamic light scattering (DLS) measurements of ligand-capped QDs were carried out on a CGS-3 goniometer system equipped with a HeNe laser (633 nm) and an ALV/LSE-5003 correlator. All samples were initially filtered through 0.2  $\mu$ m Millipore syringe filters. Data was collected by monitoring the light intensity at 90° scattering angle. The hydrodynamic size distribution was obtained using the CONTIN algorithm (ALV software).

### 5.1.10 Zeta Potential

Zeta Potential (ζ-Potential) were determined using a Malvern Zetasizer Nano ZS90 instrument using disposable folded capillary cell cuvettes (DTS1070, Malvern instruments Ltd). Samples were prepared in 0.2M NaHCO<sub>3</sub> buffer (or water) at 30-150 nM concentration range for QDs.

Samples were freshly prepared before measurements and additional filtered with  $0.2\mu$  Millipore syringe filters.

#### 5.1.11 Affinity beads assays

For fast and sensitive detection of conjugation of DNA/protein to QD or protein to DNA or DNA to Gold NPs, commercial affinity beads (Sigma Aldrich) have been frequently used. The basic principle of this type of assay is the affinity of NP with target ligand towards target on the commercial beads. These agarose beads (20-100µ diameter) are invisible under UV illumination. Successful labeling of these beads with one or more fluorescent targets imparts characteristic halo-like fluorescence on the beads (**Figure 5-3**). This design can be used to qualitatively assess the success of conjugation reactions. A detailed example is described below. The parameters of reaction (concentration, buffers, and excesses) need to be optimized on a case to case basis.



**Figure 5-3** Typical image of fluorescently labelled agarose beads under microscope at 10X and 60 X magnification

This example is an assay for rapid detection of DNA on surface of QD. Biotin-streptavidin affinity is one of the strongest biological interactions known (Kd  $\sim 10^{-15}$ M) and the same was used for detection of DNA. Streptavidin agarose beads (SAB) (50µl, 4% solution) were washed and equilibrated three times with 20mM NaHCO<sub>3</sub> pH 8.3 buffer before use. The washed beads were aliquoted separately and individual samples (20µl, 20-40nM QD sample) were added to each aliquot. Volume was adjusted to 500µl with desired buffer. The samples were left to interact with the beads for 5 min under mild rotation. The beads were then centrifuged at 300RPM for 30 sec and supernatant were removed. This was repeated thrice to

ensure all non-specifically associated ligands are removed. The samples of QD-DNA-Bt remain attached to the SAB due to presence of biotin and unconjugated QDs were removed during washes. The beads were then imaged on microscope at 10X magnification with  $\lambda_{exc}$ = 450±25 and  $\lambda_{ems}$ =610±20 with 50 ms exposure. The nature of the assay allows only quantitative measurements. A relative comparison of fluorescence intensity of beads for a typical experiment is tabulated in **Table 5-2**.

It is imperative to characterize the non-specificity of this assay based on different polymers and different nanoparticles also. Generally the following controls are tested to assess the degree of reliability of this assay for each new type of reaction. It was observed that this assay has minimum non-specific interactions when carried out in NaHCO<sub>3</sub> pH 8.3 than PBS pH 7.4 for QDs coated with all three zwitterionic polymers mentioned above (**Figure 5-4**).

 Table 5-2 Typical qualitative assessment of the SAB based assay based on Relative fluorescence intensity of agarose beads.

S.No	Test sample	Ideal interaction <sup>a</sup>	Obtained signal <sup>b</sup>
1.	QD	No	-
2.	QD-DNA	No	+
3.	QD-DNA+cDNA-Bt <sup>1</sup>	Yes	+++
4.	QD-DNA+ncDNA-Bt <sup>2</sup>	Negligible	+
5.	QD-DNA+nclongDNA-Bt <sup>3</sup>	Negligible	+
6.	$QD + DNA-Bt^4$	No	-

<sup>a</sup> Interaction with Streptavidin agarose beads

<sup>b</sup> whether or not QD fluorescence was seen on beads (in 0.2M NaHCO<sub>3</sub> buffer)

<sup>1</sup> cDNA-Bt: perfectly complementary biotinylated DNA

<sup>2</sup> nc-DNA-Bt: non-complementary biotinylated DNA

<sup>3</sup> nc-longDNA-Bt: non-complementary biotinylated DNA of longer length

<sup>4</sup> DNA-Bt: biotinylated DNA of random sequence



**Figure 5-4** A typical streptavidin agarose beads with biotin DNA- QD based assay is shown. For this set of experiments, NaHCO<sub>3</sub> has lesser non-specific interactions with samples than PBS for various controls.

## 5.2. Chemical methods

#### 5.2.1 Materials

QDs emitting at different wavelength (550-650 nm) were obtained from Nexdot in hexane and used as it is. Proteins such as Human holo Transferrin (Tf), Bovine Serum Albumin, Streptavidin, Fluorescein isothiocynate (FITC), tris(2-carboxyethyl)phosphine (TCEP), electrophoresis grade agarose, Ethidium Bromide (EtBr), were obtained from Sigma Aldrich, France. Alexa<sub>647</sub> NHS ester was obtained from FluoProbes, Interchim. Sulfo succinimidyl-4-(N-maleimidomethyl cylcohaxane-1-carboxylate) (sSMCC), Hams F-12 media, trypan blue, fetal bovine serum (FBS), trypsin, cell culture grade PBS and HEPES, Streptomycin, Penicillin, Hygromycin and G418 antibiotics were obtained from Gibco, ThermoFisher Scientific. Syringe filters were obtained from Merck, Millipore. NAP-5/10 and SEC column were obtained from GE Healthcare. Glass bottom LabTek-II imaging chambers and vivaspin centrifugal filters (100kDa, 30kDa) were purchased from IDT, Belgium and re-quantified before use. LAMP-1 antibody was a generous gift from Prof. Ludger Johannes (Institute Curie, Paris, France). The Ia.2.2 cell line was a generous gift from Prof. Yamuna Krishnan (University of Chicago, Illinois, USA).

	Name	Company	Purity
1.	3-Mercaptopropionic acid	Sigma Aldrich	>99%
2.	a-Lipoic acid	Sigma Aldrich	>99%
3.	<i>N</i> -(3-aminopropyl)methacrylamide	Sigma Aldrich	98%
4.	2-(N-Morpholino)ethanesulfonic acid sodium salt	Sigma Aldrich	>99%
5.	All salts (NaCl, KCl, CaCl <sub>2</sub> , MgCl <sub>2</sub> , NaHCO <sub>3</sub> )	Sigma Aldrich	95- 99.5%
6.	Human holo Transferrin	Sigma Aldrich	>98%
7.	Bovine Serum Albumin	Sigma Aldrich	>96%
8.	Fluorescein Isothiocyanate	Sigma Aldrich	>90%
9.	tris(2-carboxyethyl)phosphine	Sigma Aldrich	>98%
10.	Ethidium Bromide	Sigma Aldrich	~95%
11.	Agarose	Sigma Aldrich	>90%
12.	sulfosuccinimidyl 4-(N- maleimidomethyl)cyclohexane-1- carboxylate	ThermoFishcher Scientific	>95%
13.	succinimidyl 3-(2-	ThermoFishcher	>90%
	pyridyldithio)propionate	Scientific	
14.	Antibiotics (Penicillin, Streptomycin)	ThermoFishcher	>90%
		Scientific	
15.	Streptavidin	Biospa	-
16.	DNA (amine modified)	IDT	>80%
17.	DNA (fluorophore modified)	IDT	>90%
18.	Antibiotics (G418, Hygromycin)	InvivoGen	>85%
19.	Cell Culture Media related	Gibco,	-
		ThermoFishcher	
		Scientific	

**Table 5-3** List of commercially available reagents commonly used in this thesis with indicated purity.

## 5.2.2 Nomenclature of the amphiphilic polymers

In this thesis, four different types of polymers have been used. Their reference names and monomer compositions are tabulated in **Table 5-4**. The broad scheme of synthesis is shown in Scheme 1. These polymers can be easily stored as solid powder for several years without any stability or re-suspension related issues. Detailed synthesis procedure and characterization is described subsequently.

Name	Monomer 1	Monomer 2	Monomer 3	Chain length
$(20-80)_{20}$ -Zw	DTMAm <sup>a</sup> (20%)	SPP <sup>b</sup> (80%)	$MPA^{c}(0.5\%)$	20
$(20-80)_{10}$ -Zw	DTMAm (20%)	SPP (80%)	MPA (<1%)	10
$(50-50)_{10}$ -Zw	DTMAm (50%)	SPP (50%)	MPA (<1%)	10
(20-80) <sub>20</sub> -PEG	DTMAm	MAPEG <sup>d</sup>	MPA (0.5%)	20

 Table 5-4 Different amphiphilic polymers used in this thesis

<sup>a</sup> DTMAm - 5-(1,2-dithiolan-3-yl)-N-(3-methacrylamidopropyl)pentanamide. <sup>b</sup> SPP - 3-sulfopropyldimethyl-3methacrylamidopropylammonium inner salt. <sup>c</sup> MPA- Mercaptopropionic acid. <sup>d</sup> MAPEG- Poly(ethylene glycol) methyl ether methacrylate 950g/mol

#### 5.2.3 Synthesis of monomer DTMAm

The thiol-based monomer (5-(1,2-dithiolan-3-yl)-N-(3-methacrylamidopropyl)pentanamide (DTMAm) was synthesized according to previously results from the LPEM.<sup>38</sup> Briefly, a peptide coupling is proceeded between the thioctic acid and the*N*-(3-aminopropyl)methacrylamide) in anhydrous solvent. The polymer is purified and dry to obtain a yellow powder (yield > 60%).

#### 5.2.4 Synthesis of (20-80)<sub>n</sub>-Zw copolymer

Copolymer synthesis was carried out according to reference. <sup>38</sup> The polymer was synthesized *via* a static copolymerization of DTMAm (monomer 1, 20%) and of 3-[3 methacrylamidopropyl(dimethyl)amino]propane-1-sulfonate (SPP, 80%) in presence of MPA (10 mol% regarding to the monomers for (20-80)<sub>10</sub>-Zw) as terminating functional group. The polymerization is performed in acetic acid:NaCl 20mM (9:1) solution at 70°C after previous degassing of the solution under argon for 30 min The polymerization is proceeded overnight. For the purification, first acetic acid is evaporated under reduce procure at 60°C. The copolymers are then precipitated twice in ethanol and recover in milli-Q water prior to lyophilization (**Scheme 5-2**).

The synthesis of the  $(20-80)_{20}$ -Zw copolymer is done following the same procedure, only the amount of MPA changes (5mol% regarding to the monomers).

The synthesis of the  $(50-50)_{10}$ -Zw copolymer is done following the same procedure, only initial amount of DTMAm:SPP was adjusted accordingly (50mol% each). The amount of MPA was as  $(20-80)_{10}$ -Zw (10mol% with respect to the monomers).



**Scheme 5-2** General scheme of synthesis of polymer with varying ratios of both monomers for zwitterion (top) and PEG-based (bottom) is shown. The reaction is carried out in acetic acid at 70°C with constant stirring.

#### 5.2.5 Synthesis of (20-80)20-PEG copolymer

The copolymer is synthesized as the  $(20-80)_{20}$ -Zw by replacing the zwitterionic-based monomer (SPP) by the poly(ethylene glycol) methyl ether methacrylate ( $M_n = 950$  g/mol, Sigma Aldrich). The polymerization is conducted in toluene and the copolymer is precipitated twice in cold diethyl ether before lyophilization (**Scheme 5-2**).

## 5.2.6 Ligand exchange of QDs (MPA protocol)

For this work, a classical two-step ligand exchange has been used to transfer QDs from organic solvents (typically hexane) to aqueous media

**Step 1.** A 50 $\mu$ L solution of stock solution of QD (10  $\mu$ M) in hexane is precipitated in 6 mL ethanol by centrifugation at 6000 RPM for 10 minutes. Excess EtOH is discarded. The QDs are dispersed in ethanol by vortexing and re-precipitated again. This step ensures removal of excess organic ligands in the solution. The nanocrystals are then resuspended in 200 $\mu$ L of MPA (>95%) and left overnight at 60°C to carry-out ligand exchange (**Scheme 5-3**).<sup>38</sup>

Step 2. QDs (in MPA) are centrifuged to remove excess of MPA. The QDs are then resuspendend in 200µl of dimethylformamide. This homogeneous solution is treated with a base such as potassium tert-butoxide (2 mg). This step deprotonates the QDs, causing reprecipitation. The mixture is thus centrifuged to remove the excess DMF and QDs are washed twice with EtOHto remove excess of base. The deprotonated QDs are dispersed in 200µl of sodium tetraborate (10 mM, pH 9.5). In parallel, the polymer of choice is solubilized in water (~10mg/ml) and reduced with NaBH<sub>4</sub> (1mg NaBH<sub>4</sub> per mg of polymer) for 30 min. The QDs dispersed in sodium tetra-borate are simply mixed with this reduced polymer solution and left at 60°C overnight (Scheme 5-3). Post-polymer capping, the solution is washed with 20mM NaCl two times and concentrated on ultrafiltration membranes (100kDa). The solution is further washed with MilliQ water twice before lyophilization for long-term storage. Alternately QDs can be stored in buffers such as 0.2M NaHCO<sub>3</sub> and 20mM NaCl at 50  $\mu$ M.



Scheme 5-3 Two step ligand exchange procedure (MPA procedure).

# **5.3 Conjugation methods**

The underlined procedures describe the conjugation of chemically modified dyes to proteins, DNA, peptides and also for QDs in some cases. For the sake of simplicity, the macromolecule to be conjugated is referred to as "target" in the subsequent section.

## 5.3.1 Bioconjugation of QDs with dyes or small molecules

*Labelling with dyes.* In this thesis, several types of chemically modified dyes have been used to label variety of targets. Three broad categories of modifications are (i) NHS (ii) ITC and (iii) maleimide (Scheme 5-4).

Several of the NHS-functionalized fluorophores (Alexa<sub>488</sub>, Alexa<sub>647</sub>, Cy5.5, DyLight 680) have been used to label types of targets *via* their accessible primary amines. The basic procedure to label targets involves two steps: (i) solubilization of stock of fluorophore in anhydrous DMSO at a typical concentration around 10mg/mL. (ii) Dispersion of target in 0.2M NaHCO<sub>3</sub> pH 8.3-9. The reaction can also be carried in other basic non-amine containing buffers including HEPES and borate buffers. Typical excesses used are: dye/protein: 50-100X, dye/peptide: 100-200X, dye/DNA: 500-1000. The exact same conditions are also followed for isothiocynate (ITC) derivatives of fluorophores such as Fluorescein isothiocyanate (FITC) and Tetramethyl Rhodamine Isothiocyanate (TRITC) to label targets *via* their amine functional groups. In several experiments it was observed that NHS dyes have better labelling efficiency than ITCs, but the solution stability of ITCs was much higher than NHS. In a typical reaction 5-10 mg of protein is dissolved in 1 mL of 0.2M NaHCO<sub>3</sub> pH 8.3 in a low binding eppendorf (<200 $\mu$ M) and mixed with 20-30 $\mu$ l of 10mg/ml dye. This reaction is left at RT in dark for 6 hours to overnight.



Scheme 5-4 Commonly used reactions for labelling small dyes to macromolecular target

For labelling of targets with dyes functionalized with maleimide, the first step involves resuspension in with mildly acidic buffers (pH  $\sim$  6-7). Another essential step in this reaction involves reducing the disulfides into maleimide reactive thiols, typically with agents such as TCEP or DTT. Generally, 5 mM TCEP/DTT solution can effectively reduce up to up to 5 mg/mL protein solution. The reducing agent is then removed from the solution by ultrafiltration and the exposed thiols can be immediately mixed with the maleimide functionalized dye in typical rations described above.

All labelled targets were purified using NAP-5 or 10 columns as described earlier in this chapter. Protein and peptide targets were preferably stored in -20°C (in 10% glycerol solutions) and DNA and QDs were stored in 4°C in buffers such as PBS pH 7.4 and NaHCO<sub>3</sub> pH 8.3 respectively.

*Biotinylation of targets.* For several experiments it was also important to biotinylate targets such as proteins and QDs. Biotinylation reactions were either carried out with Biotin-NHS (reactive towards primary amines), Biotin-hydrazide (reactive towards carbonyl groups or carboxylic acids in presence of EDC) or Biotin-maleimide (reactive towards thiols)

For these experiments, the targets are carefully weighted and resuspended in basic buffers (pH  $\sim$  8-9.5) and mixed with 50×molar excess of biotin-NHS or biotin hydrazide<sup>5</sup>. For reaction of targets with biotin-maleimide, targets are resuspended in PBS 1 × pH 7.4 and mixed with 100× molar excess biotinylating reagent and left for reaction at room temperature overnight. All labelled targets are purified using NAP-5 or 10 columns as described later in this chapter and concentrated using ultrafiltration columns (>30 kDa).

## 5.3.2 Conjugation of DNA to QDs

For all types of chemical reactions, DNA with 5' terminal functional group ( $NH_2$  or SH wherever necessary) conjugated via a C6 linker was used.

Typical reaction is carried out in 100 $\mu$ l of final volume. Tight control in the total volume of the reaction helps in maintaining similar yields across all reactions. The protocol described here is optimized for conjugation of 15-45 mer ss DNA-NH<sub>2</sub> to QDs coated with zwitterionic polymer [ (20-80)<sub>20</sub>-Zw ] The detailed discussion of this methodology is provided in Chapter 3.

**Preparation of DNA-maleimide**. For this reaction, amine-labelled DNA has to be converted to maleimide. Briefly, 300 nmol of sSMCC is added to a solution of 12.5 nmol DNA-NH<sub>2</sub> in 0.2M NaHCO<sub>3</sub>, pH 8.3 (sSMCC/DNA=25/1). The reaction is kept for 45 min at room temperature under vigorous stirring to yield DNA-maleimide. Excess of unreacted sSMCC is removed by precipitation in cold ethanol at 14000 RPM for 30 mins.

**Reduction of QD with TCEP.** A fresh stock solution of TCEP is prepared (5mg/mL) in PBS  $1 \times$  immediately before use. To a solution of 5µM QD<sub>610</sub> in PBS, TCEP is added such that the

<sup>&</sup>lt;sup>5</sup> For reaction with biotin-hydrazide, the carboxylic acid groups on the target need to be first activated using EDC. This activation is carried out in acidic buffer (such as MES pH 5) followed by generation of *O*-acylisourea intermediate that can then react with the biotin hydrazide derivative. However, essential step here is to change the buffer swiftly from acidic to basic pH, such as to promote nucleophilic addition of biotin hydrazide to this reactive intermediate.

final molar ratio of TCEP/QD was 400/1. The QD-TCEP solution is left for reduction at pH 7.4 under constant stirring for 30 mins at RT. After reduction, the solution is passed through a size exclusion column (NAP-5) equilibrated with PBS as per discussed in Section 5.1.7

*Reaction of DNA-maleimide with reduced QD.* The QDs-thiols are mixed with DNA-maleimide such that DNA/QD=25/1 in PBS pH 7.4. The solution is left to react overnight at RT to form stable covalent bond between QD and DNA.

#### 5.3.3 Conjugation of proteins to QDs

Similar to the detailed method described above, proteins can also be conjugated to QD *via* the heterobifunctional linker sSMCC. Typical reaction involves 50 nmol of protein with 500 nmol of sSMCC (sSMCC/protein = 10) in 0.2M NaHCO<sub>3</sub>, pH 8.3 for 1 hour under gentle rotation. This step enable reaction of primary amines on the amino acids (such as lysine) to react with the NHS group on sSMCC to give rise to protein-maleimide. The excess of sSMCC is removed by passing the reaction through vivaspin 30 kDa at least 5 times (molecular weight of proteins >30 kDa). In parallel, 10 nmol QD is reduced with 4  $\mu$ mol TCEP (TCEP/QD = 400) in PBS pH 7.4 for at least 45 min with constant stirring. Excess of TCEP is removed from the reaction using NAP-5 size exclusion columns. The reduced QD are then mixed with protein-maleimide (protein/QD=5) and left for reaction in PBS, pH 7.4. Excess of unconjugated protein is purified by ultracentrifugation or SEC (HPLC). The conjugates are stored at 4°C in NaHCO<sub>3</sub> until further use. The basis and optimization of this reaction is described in detail in Chapter 3.

#### 5.3.4 Conjugation of proteins to thiolated DNA

Thiol-labelled DNA (DNA-SS) can be conjugated to protein-maleimide.<sup>130</sup> Briefly, 50 nmol of protein is reacted with 500 nmol of sSMCC (sSMCC/protein = 10) in 0.2M NaHCO<sub>3</sub>, pH 8.3 for 1 hour under gentle rotation. The excess of sSMCC is removed by passing the reaction through vivaspin 30 kDa at least 5 times to synthesize protein-maleimide. In parallel, 250 nmol DNA-SS is reduced with 12.5  $\mu$ mol TCEP (TCEP/DNA = 50) in PBS pH 7.4 for at least 45 mins with constant stirring to get pure DNA-SH. Excess TCEP is purified by passing the reaction through vivaspin 3 kDa atleast 5 times. The DNA-SH is mixed with the protein-maleimide (DNA/protein = 5) and left for reaction in PBS pH 7.4 overnight under mild stirring. The conjugates (protein-DNA) were purified from excess DNA by SEC purification.

## **5.4 Biology experiments**

#### 5.4.1 Cell maintenance

IA2.2 cells (derivative of Chinese Hamster Ovary, CHO cells) were used for all experiments. Cells were maintained in Ham's F12 complete media (Gibco, Life Technologies) supplemented with 10% heat inactivated FBS,  $100\mu g/mL$  Streptomycin,  $100\mu g/mL$  Penicillin,  $100\mu g/mL$  Hygromycin and  $100\mu g/mL$  G418 in T-25 air vented flask. Passage of cells were carried out by incubation of confluent cells with 500  $\mu$ L of 0.25% Trypsin-EDTA (Gibco, Life Technologies) in T-25 for 5 minutes in 37°C. Post-trypsinisation, 50 $\mu$ L of cell suspension was added to 7 mL of fresh media and cells were allowed to grow in 37°C before repassing after 3 days. Cell media was changed every 30 hours.

#### 5.4.2 Plating of cells for experiments

Cells were seeded at a density of  $0.25 \times 10^6$  in Nunc Lab-Tek chambered slides ( $0.8 \text{ cm}^2$ ) and grown for 2 days at 37°C. For experiments, cells were washed in M1 buffer (140 mM NaCl, 20 mM HEPES, 1mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM KCl pH 7.4).

#### 5.4.3 Uptake experiments

All experiments were carried on cells seeded on glass chambers (as described above). For uptake of specific cargo (fluorophore labelled protein, DNA functionalized proteins, FITC dextran etc), cells were pulsed with cargo in cell medium (typically 100-500 nM) and left for desired period of time.

### 5.4.4 Stripping of surface ligands

For cellular imaging, fluorophore-labelled cargo should be stripped from the cell surface first. These cargos may be bound to specific receptors or non-specifically associated with membrane. These interactions can be significantly reduced by incubation in acidic pH for short time. In general, surface-bounded ligands were stripped by incubating cells in ascorbate buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM MgCl<sub>2</sub>, pH 4.5) for 10 mins on ice followed by rigorous washing with ice cold M1/PBS buffer also on ice. It is essential to maintain cells in ice at these steps so that endocytosis and micropinocytosis of highly acidic

buffer is minimized. Following this, the cells were thoroughly washed and processed for either imaging or further manipulation (Scheme 5-5).



Scheme 5-5 Step-wise procedure to remove non-specific labelling from cell surface.

#### 5.4.5 Fixation of cells

In most cases, after specific experimental protocols, cells need to be fixed before imaging. For fixation, two typical protocols were used. First involves cell fixation using **Paraformaldehyde (PFA).** Cells were incubated with a solution of 2% PFA solution for 20 minutes at RT. This method works by cross-linking proteins, particularly basic amino acids to each other. The second method uses fixation by **Methanol**. Cells are incubated with chilled absolute Methanol at -20°C for 10 minutes followed by rinsing with PBS pH 7.4 (**Figure**)

**5-5**). Cells are fixed due to disruption of hydrophobic interactions within proteins and instantaneous precipitation of soluble proteins. In quantitative imaging experiments, it was seen that fluorescence intensity loss (for QD labelling) upon fixation with Methanol is lower than with PFA. Therefore for all experiments, fixation was carried out with methanol. Methanol fixation is also beneficial for immunofluorescence experiments, since methanol induces pores on the cell membrane by dissolution of the lipids. This removes the necessity to additionally permeabilize cells for antibody labelling of intracellular targets.



Figure 5-5 Fixation of cells in Methanol. Fixation in Methanol does not alter morphology or labelling patter of cells. However fixation causes reduction in fluorescence intensity. Scale bar represents  $10 \mu m$ .

### **5.4.6 Kinetics experiments**

Kinetics experiments (both uptake and recycling) were done by pulsing cells with cargo (typically 100 nM for QD based and 500 nM for fluorophore based) for pre-defined periods of time.

*Uptake experiments*. Cells were pulsed with cargo for defined period of time. After the pulse period was over, surface bound labelling was removed by acid stripping on ice. Cells were washed thoroughly in M1 or PBS buffer to get rid of excess of non-specifically bound ligands on ice. After this step, all solution from the chambers was aspirated and ice cold methanol was added and cells were immediately shifted to -20°C. Cells were incubated for 10 minutes and the methanol was removes by 2 washes with PBS, cells were imaged in PBS.

*Recycling experiments.* Cells were pulsed with cargo for defined period of time. After the pulse period was over, surface bound labelling was removed by acid stripping on ice. Cells

were washed thoroughly in M1 or PBS buffer to get rid of excess of non-specifically bound ligands on ice. After this step, cells were pulsed with second cargo (mostly 500 nM transferrin, unlabeled or fluorophore labeled) for indicated period of time and surface stripped. Surface stripping step can be avoided in case of unlabeled cargo. After this step, all solution from the chambers was aspirated and ice cold methanol was added and cells were immediately shifted to -20°C. Cells were incubated for 10 minutes and the methanol was removed by 2 washes with PBS. Cells were imaged in PBS.

## 5.4.7 Dual-labelling experiments

Dual-labelling experiments in cells were done to either determine the intracellular localization of the defined cargos or to simultaneously probe both uptake and recycling of cargos with time. For these experiments, two parameters were carefully evaluated. First, for dual color imaging, the probes should have minimum overlap between the excitation and emission spectra. And second, the choice of imaging filters. The filters using for fluorescent imaging should have narrow band-width such that only selective and specific fluorescence emission is collected (**Scheme 5-6**)





In our experience, the best non overlapping fluorophore pairs were Alexa<sub>488</sub>/FITC with Alexa<sub>647</sub>. Similarly, the best non-overlapping pair of fluorophore and QDs was FITC and QD<sub>650</sub>. These pairs of fluorophores/QDs were used whenever dual color labeling experiments were carried out.

### 5.4.8 Colocalization experiment

For colocalization experiments of QD-DNA-Tf with  $Tf_{FITC}$ , QD-DNA-Tf was internalized and surface labelling was stripped by incubating in ascorbate buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM MgCl<sub>2</sub>, pH 4.5) for 10 mins on ice followed by rigorous washing with ice cold M1 buffer on ice. Then cells were pulsed with warmed 500 nM  $Tf_{FITC}$  for 7 minutes and chased for 10 mins at 37°C. Then the cells were shifted to ice and the surface was re-stripped as before. The cells were fixed with ice-cold methanol.

Colocalisation experiments were also done with FITC dextran – 10kDa (marker for late endosome) but the fluorescence intensity of LE marked with FITC-Dex<sub>10</sub> was very poor, possibility due to acidic pH-dependent quenching of fluorescence of FITC. This effect thus necessitated the use of immunofluorescence for marking the LE compartments.

#### 5.4.9 Immunofluorescence experiment

For colocalization experiments, cells were labelled with cargo, surface-stripped and fixed in ice-cold methanol. Then the cells were washed with PBS (3 times) and permeabilized with  $1 \times$  saponin buffer for 30 minutes. Cells were incubated with primary antibody (mouse anti-LAMP-1, BD Biosciences) in saponin buffer (1:100) for 1 hour followed by 3 times washing in PBS. Fluorophore-labelled secondary antibody (rabbit anti mouse Alexa488, BD Biosciences) was added in saponin buffer was added (1:100) for 45 mins followed by washing with PBS. Cells could be imaged for up to 3-4 days without additional preparation.

Immunofluorescence experiments were also tried with mouse anti-Rab11 (marker for recycling endosomes) but revealed poor labelling of intracellular targets in exactly same conditions.

# **5.5 Data Analysis**

## 5.5.1 Analysis of data from epifluorescent microscope

All images were processed using ImageJ or Fiji. Fiji was used in case of specific plugins such as Co-loc2, TrackMate etc. For analysis, exclusively healthy cells with visible pseudopodia were selected, while morphologically distinct dead cells were eliminated. In several experiments (data not shown) it was however observed that dying/dead cells have higher cellular labelling with QDs, presumably due to initial increase in cell surface area (during onset of program cell death) and porosity and acidity of the cell membrane. Concomitantly, auto-fluorescence signal is also increased.

## 5.5.2 Background subtraction and marking of cell boundary

Since cells experiments involved incubation with labelled biomolecules (fluorophores or QDs), there was minimal background staining on the glass slides which needed to be removed for reliable estimation of intracellular fluorescence intensities. Cell boundary was marked using DIC image of the corresponding field of interest. Background was selected from each image on a region with no cell. This selection needs to be carefully done since during the procedure of cell washing, several adherent cells can be get removed from their original location, leaving an un-exposed surface. This region, if selected, can lead to under estimation of the background.

### 5.5.3 Quantification of fluorescence intensity

After background subtraction, individual cell intensity is measured. For estimation of mean intensity, at least 50-60 healthy cells are individually measured for reliable statistics. The intensities are copied on Origin 8.0 and relevant analysis is carried out.

### 5.5.4 Colocalization of dual colored images

For co-localization experiments, same field of view is imaged in two different channels. The imaging settings as described above are individually optimized for each channel (each fluorophore). First, the backgrounds are subtracted and then, the cells are digitally merged using ImageJ. Following this, co-localization algorithm called Co loc-2 (Fiji.sc) is run. The

program provides several options (based on different models of colocalization estimation). In context of dual labelling shown in this thesis, Pearson's Correlation coefficient (PCC) is the most relevant. The PCC is not sensitive to differences in mean signal intensities or range, implying it does not depend upon intensity of different fluorescent probes in altering channels. The result ranges from +1 to -1 for perfect to anti-correlation. An absolute requirement for using PCC (and co-loc2) is to have negligible bleed through in the two channels of interest. Bleed through leads to false positive colocalization coefficients. For statistical analysis, the PCC is estimated for atleast 20 cells and mean and SD is plotted wherever necessary. The PCC was tested to be sensitive to digital rotation (90°) of the image of one channel. This further supports the sensitivity of the algorithm to real *vs* pesudo colocalization

### 5.5.5 Analysis of data from spinning disc confocal microscope

Single time course imaging experiment on the spinning disc microscope in principle generates several gigabytes of data. In default setting, the images are obtained as systematic Z stacks for a given time, over entire duration of imaging. However, not all planes in Z stack have entities/plane of interest. In order to reduce the time for data processing, confocal slices were rearranged into time stacks of a given confocal plane of interest. This selection was carried out by using a custom-made Matlab® program that converts a z-stack into t-stack for a given xy plane (**Scheme 5-7** and **Scheme 5-8**).

After this transformation of data, the stacks are individually analyzed for plane of interest, and confocal planes are selected such that vesicles/organelles/other ROI are in focus and necessary analysis is carried out.



Evolution of selected plane over time

Scheme 5-7 Overall procedure for analysis data from spinning disc confocal imaging using Matlab®.



Scheme 5-8 Demonstration of matrix transformation by custom-built Matlab® program for image analysis

## 5.5.6 Particle tracking from live imaging experiments

*Noise filtering using Multidimensional Image analysis.* Multidimensional Image Analysis or MIA is a noise reduction and segmentation algorithm developed by the Curie Institute for simplification of fluorescence imaging and data processing. It is particularly useful in identifying single particle (cells/bacteria/endosome/fluorophores) due to ability to deconvolute small structures with low intensity from big structures with high intensity<sup>188</sup> This deconvolution was particularly required for detection of single endosomal vesicles *away* from perinuclear region (where most of the QD labelled ligands accumulated), as discussed in Chapter 4.

#### Single-endosome tracking.

After threshold processing using MIA, stacks of images were prepared for particle tracking experiments. Single-endosome particle tracking experiments were done using "Manual tracking" plugin ImageJ. Several automated algorithms such as *Particle tracker classic, Spot tracker 2D, Trackmate* etc. were also tried. However, automated vesicle detection was seen to be error-prone, specifically in tracking endosomes which marginally drifted out of plane over few milliseconds and returned back. Hence it was concluded that manual tracking was although laborious but a reliable approach for assessing endosomal movement.

The full stack of images of the ROI assembled as time stacks were opened in image J and the manual tracking plugin was selected (ImageJ < Plugin < Manual Tracking). A dialogue box titled "Tracking" should open. On this box, the "add track" option was selected. Then the vesicle of interest was manually selected by single click on the fluorescent point of interest (FPOI). The stack automatically shifts to the next frame. The same step was repeated for all the consequent stacks till which the FPOI can be tracked/followed. Then the trajectory is saved and the trajectory can be visualized using the 'overlay dots and lines' in the same box.

This chapter summaries all of the most important experiments and analytical approaches used in this thesis.

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## Conclusions

Developments in novel routes of chemical synthesis, better ligands and state-of-the-art instrumentation have definitely expanded the scope of semiconductor nanoparticles, particularly Quantum Dots (QDs) in biology. Despite considerable and continued progress in this emergent nanomaterial-biology interface, several issues still limit the widespread applicability of QDs for biosensing and bioimaging applications. Inadaptable and stringent conjugated biomolecules and erroneous intracellular behavior of these conjugates remain persistent challenges that need attention.

During the past three years of my PhD in LPEM under the joint supervision of Dr. Benoit Dubertret and Prof. Yamuna Krishnan (University of Chicago), I have worked towards attending several of these issues, to further expand the scope of these fascinating fluorescent nanomaterials in biology. Building upon the highly sought after and robust thiol-zwitterion based polymeric ligands developed in the group; I developed a novel method to conjugate functional biomolecules to QDs. The advantage of this method over existing strategies is the fact that the conjugation is carried out on the thiols present on the polymers. Since thiols are intrinsic parts of several ligands designed for different types of nanoparticles, conjugation reactions on thiols of QDs can be additionally extended to other nanoparticles. On the biomolecule this reaction utilizes primary amines, which are abundant in DNA, proteins, antibodies, peptides and diverse biological targets. Thus this strategy could be easily adapted to different nanoparticles and biomolecules and thus highly generalizable. A meticulous characterization of this strategy resulted in several conclusions. This reaction was found to be highly sensitive to salt conditions, moderately sensitive to concentration of reducing agents and insensitive to pH of the media. In optimized conditions, upto ~12 DNA molecules could be conjugated per QD. This yield decreased as the length of DNA increased. The conjugates thus generated retained >80% of their Quantum yield, and could be stored in solution for several months. This strategy could also be extended to conjugate DNA of varied lengths on QDs emitting at different wavelengths, on Gold nanoparticles, Nano-platelets and additionally for conjugation of proteins and antibodies on QDs.

In the second part of my thesis, I used these QD-DNA conjugates to assemble proteins on QDs mediated by DNA hybridization. The QD-DNA conjugates were synthesized as

described in the first chapter. In parallel, a model protein Transferrin (Tf) was conjugated with complementary DNA. The individual reaction yields were carefully evaluated and conjugates could be assembled with stoichiometric control. This novel method of conjugation of proteins to QD could be easily adapted to other protein systems. After careful evaluation of the biochemical properties, these QD-DNA-Tf conjugates were used to label CHO cells that bore Tf receptors (TfR) on the cell surface. These conjugates were readily internalized and labelled specific endosomal vesicles. Though QD conjugates of Tf (and other such model proteins) have been synthesized in past, one of the major limitations of these probes is the misrouting within cells. Instead of trafficking to recycling endosomes (RE) like endogenous Tf, these conjugates get trapped in late-endosome (LE)-lysosome systems. This misrouting is additionally accompanied by degradation or massively delayed recycling. In my results with statistically mono-assembled QD-DNA-Tf conjugates I find that though the endocytosis of these conjugates is delayed, but the steady state localization of these conjugates is in RE and the kinetics of recycling is similar to endogenous Tf. These results point towards the role of ligand density in dictating the biological fate of conjugates of QDs. After careful evaluation of the intracellular behavior of these conjugates, these probes were further used to track the recycling dynamics of TfR using spinning disc confocal microscopy. These conjugates demonstrate new generation of fluorescent probes with strong potential in bioimaging.

In the last part of my thesis, I screened a library of polymeric scaffolds on QDs to investigate the performance of these probes in complex biological environment. Using a host of *in-vitro* and *in-cellulo* approaches, I demonstrate that short length zwitterionic polymers have higher stability and greater antifouling properties than the longer chain or PEG based polymers. These experiments also highlight that zwitterionic polymers have lower propensity of non-specific adsorption of biomolecules on the surface. I also find that neural (both charged and uncharged) ligands have similar trends of non-specific uptake within cells. This work particularly highlights the superior performance of zwitterionic ligands over PEG based ligands. Given the popularity of PEG based ligands in existing literature in imparting stealth properties to nanocrystals, zwitterions are competitive scaffolds with great potential as novel ligands for colloidal nanoparticles, particularly QDs in biology.

This thesis is thus poised to further expand the scope of synthetic QD based probes for multiple applications in biology.

# Appendices

#### Appendix 1: Different QDs used in this study

**Absorbance and fluorescence spectra:** Quantum Dots emitting at four different wavelengths have been used in this thesis. The absorbance (350-first excitonic peak) and full emission spectrum of all the QDs is plotted (Figure 1).



**Figure 1.** Absorbance and fluorescence spectra of QDs emitting at 550 nm (green), 580 nm (yellow), 610 nm (orange), and 650 nm (red) in hexane are shown.

**Estimation of nanoparticle size:** to estimate the typical size of the QDs Transmission electron microscopy (TEM) was carried out. The size distribution of QDs was measured by TEM images of > 250 dots in different imaging planes and grids. The average diameter of QDs emitting at different wavelengths is summarized in the Table 1 below.

Table 1: Characterization of QDs with different emission wavelength

$\lambda^{1}(nm)$	FWHM <sup>2</sup> (nm)	Size <sup>3</sup> (nm)		
550	44	8.9±1.4		
580	33	8.2±0.0		
610	39	9.7±1.6		
650	25	7.8±2.1		

<sup>1</sup> Emission wavelength of QD

<sup>2</sup> Full Width of Half Maxima of the fluorescence emission peak

<sup>3</sup> Size of Quantum Dots measured by TEM of >200 individual nanoparticles

### **Appendix 2: Compositions of different buffers used in this thesis**

Buffer	pH (at 25°C)						
For conjugation reactions							
PBS	7.4						
NaHCO <sub>3</sub>	NaHCO3200mM NaHCO3 in deionized water						
HEPES	<b>HEPES</b> 115 mM NaCl, 1.2 mM CaCl <sub>2</sub> , 1.2 mM MgCl <sub>2</sub> , 2.4 mM K <sub>2</sub> HPO4, 20 mM HEPES						
MES buffer 0.1M MES (N-Morpholinoethane sulfonic acid), 0.9% w/v NaCl,		4.5-5					
For cell culture							
M1	M1 140 mM NaCl, 20 mM HEPES, 1mM CaCl <sub>2</sub> , 1 mM MgCl <sub>2</sub> , 5 mM KCl						
Ascorbate buffer 160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM MgCl <sub>2</sub>		4.5					
For electrophoresis							
Borate buffer50mM sodium Borate dissolved in deionized waterSDS-PAGE running24.8 mM Tris base, 192 mM glycine and 0.1% w/v SDS dissolved in deionized water		8.5-9					
		8.3					
buffer							

<sup>1</sup>Indicates compositions of working solutions of individual buffers

### **Appendix 3: Sequences of DNA used in this thesis**

Experiment		Sequence $(5' \rightarrow 3')^1$ , Name		Chemical
			(bases)	Modification
1.	Conjugation to QD	TTGCTCTGGTGTCAA, DNA-Amine	15	5' NH <sub>2</sub>
2.	Biotinylation of QD-DNA conjugates	TTGACACCAGAGCAA, DNA-Bt	15	5' Biotin
3.	Quantification of QD-DNA conjugates	TTGACACCAGAGCAA, DNA-Cy5	15	5' Cy5
4.	Quantification of conjugation efficiency	TTGACACCAGAGCAA, Amine-DNA-Cy5	15	5' NH <sub>2</sub> , 3' Cy5
5.	Conjugation to QD	GGAAATGTATGCTATATATATCAGTT, 4AT-25	25	5' NH <sub>2</sub>
6.	Conjugation to QD	TGTCAATCTTGGAAATGTATGCTATATATATCAGTT, 4AT-35	35	5' NH <sub>2</sub>
7.	Conjugation to QD	TTGCTCTGGTGTCAATCTTGGAAATGTATGCTATATATAT	45	5' NH <sub>2</sub>
8.	Quantification of conjugation		25	5' Cy5
	efficiency	AACTGATATATATAGCATACATTTCC, 4AT-25-comp-Cy5		-
9.	Biotinylation of QD-DNA		25	5' Biotin
	conjugates	AACTGATATATATAGCATACATTTCC, 4AT-25- comp-Bt		
10.	Hybridization to Seq	AACTGATATATATAGCATACATTTCC, 4AT-25- comp-Bt	25	5' Biotin
11.	Hybridization to Seq	AACTGATATATATAGCATACATTTCCAAGATTGACA, 4AT-35- comp-Bt	35	5' Biotin
12.	Hybridization to Seq	AACTGATATATATAGCATACATTTCCAAGATTGACACCAGAGCAA,	45	5' Biotin
		4AT-35- comp-Bt		
13.	Conjugation to proteins	AACTGATATATATAGCATACATTTCC, 4AT-25- comp	25	5' NH <sub>2</sub>

<sup>1</sup> Indicates the orientation of the sequence based on the synthesis. DNA hybridization is orientation specific

#### **Appendix 4: Fluorophores and related filters**

	Fluorophore	Similar probes	Λex <sup>1,2</sup> (max) nm	Λem <sup>3</sup> (max) nm	Excitation filter	Emission filter
1.	QD	QDs with emission maximum at 550/580/610/650 nm	200-500	Indicated wavelengths	450±25	$485 (LP)^4$
2.	QD (610)	-	200-500	610	450±25	605±20
3.	QD (650)	-	200-500	650	450±25	655±10
4.	Fluorescein	FITC, Alexa 488	470<483	515	485±10	524±12
5.	Cy3	TRITC, Rhodamine	517<554	574	525±25	593±23
6.	Alexa 647	Texas Red	550<595	614	560 ±25	645 - 35

<sup>1</sup> Indicates range of wavelength with high absorbance cross section for QDs. <sup>2</sup> indicates wavelengths of two primary excitonic peaks in the absorbance spectrum of commercial fluorophores.<sup>3</sup> Wavelength of maximal emission. <sup>4</sup> Indicates Long Pass filter.





**Diagram of the optical set-up used for imaging on Olympus IX71**. The path of excitation and emission light is indicated by black and purple arrows. Instrumental components are labeled in blue.

#### Abstract

Quantum dots (QD) are new generation of versatile probes for biology, particularly for bioimaging. For specific applications, QDs are conjugated to biomolecules such as nucleic acid or proteins and subsequently targeted to unique intra-cellular pathways.

Building upon the state-of-the-art ligands for water-dispersible QDs developed by the lab, a novel and highly generalizable method to conjugate DNA to QD is developed in this thesis. This method employs thiols present on polymers on QDs for conjugation to maleimide-functionalized DNA. Extensive characterization of parameters affecting this reaction is carried out and the strategy is extended to other nanoparticles and biomolecules. Following this, a novel method to conjugate proteins to QD via DNA hybridization is discussed. Using a model protein Transferrin (Tf), the unique properties of thus generated QD-DNA-Tf conjugates are studied *in-vitro* and *in-cellulo*. These conjugates are subsequently used for tracking endosomal dynamics for up-to 20 minutes, exploiting the fullest potential of QDs for live imaging. In the last part, additional studies on factors affecting the 'biological performance' of QDs are carried out. Using a range of highly adaptable polymeric ligands developed by the group, interactions of surface-modified QDs with the biological interface are probed. Systematic biochemical and cellular experiments demonstrate that QDs coated with zwitterionic polymers have superior antifouling properties compared to poly(ethylene glycol)-based polymers and stability in diverse biological contexts.

#### Résumé

Les boîtes quantiques (ou Quantum Dots en anglais - QD) sont une nouvelle génération de sondes polyvalentes pour la biologie, en particulier pour l'imagerie. Pour des applications de marquage des voies intra-cellulaires, les QDs peuvent être conjugués à des bio(macro)molécules telles que des acides nucléiques ou des protéines.

En partant des travaux du laboratoire LPEM portant sur le développement de ligands permettant la dispersion des QDs dans l'eau et leur fonctionnalisation, une nouvelle méthode de conjugaison de l'ADN sur les QDs a été développée dans cette thèse. Cette méthode utilise les motifs thiols présents sur les polymères des QDs pour le greffage d'ADN à fonctions maléimides. Les paramètres affectant cette réaction ont été étudiés et cette stratégie de couplage a été étendue à d'autres nanoparticules et biomolécules. En partant de ces QDs greffés ADN, des protéines modifiées ADN ont pu être à leur tour attachées aux QDs en utilisant le principe de complémentarité de l'ADN. Les propriétés uniques des conjugués ainsi générés ont été mises en évidence en utilisant la Transferrine (QD-ADN-Tf) et ces complexes ont été étudiés in vitro et in cellulo. Ces conjugués ont ensuite été utilisés pour le suivi de la dynamique des endosomes jusqu'à 20 minutes, exploitant ainsi pleinement le potentiel des QDs pour l'imagerie directe. Dans la dernière partie, des études supplémentaires sur les facteurs influençant la «performance biologique» des QDs ont été réalisées. Pour cela, une large gamme de ligands polymères développée par le groupe a été utilisée pour sonder l'interaction de la surface des QDs avec l'interface biologique. Des expériences biochimiques et cellulaires systématiques ont permis de démontrer que les QDs revêtus de polymères zwitterioniques ont des propriétés d'adsorption non spécifique et de stabilité supérieures aux polymères poly(éthylène glycol) dans divers environnements biologiques.