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Chapter 1: Introduction & Overview
Dissertation Synopsis

Objectives
The aims of this investigation are to (i) prepare hybrid quantum dot (QD)-polymer complexes, (ii) manipulate structural and chemical properties of the hybrids and characterize their effects on biocompatibility, and (iii) assemble diverse, heterostructured complexes for enhancing and fluorescently tracking gene therapy.

Hypothesis
Controlling both the chemical functionalities and hierarchal nanostructuring of the QD-polymer complex may enable the design of fluorescent, synergistic systems that are more effective than their constituent components, therefore providing efficient solutions to complex biological problems such as gene therapy.

Rationale
The properties of biological systems depend on the controlled assembly of highly specialized materials into evolved nanostructures. Examples include DNA-containing systems like viruses and chromosomes that demonstrate highly concerted interplay between their material properties and structural organization. Therefore synthetic systems designed to mimic their biological counterparts must possess similar synergies between, and enable fine tunability of, their chemical functionalities and hierarchal structuring. The use of QD-polymer complexes may enable integration of diverse materials into ordered nanostructures whose biocompatibility and cellular interactions can be manipulated by controlling the properties of the individual components.

Results
Preparation of the QD-polymer core included both aqueous synthesis of inherently water soluble QDs and modification of commercially available hydrophobic QDs. Optically active CdS nanocrystals were successfully synthesized using cationic polymers and histidine oligopeptides, but high cytotoxicity and poor optical properties when integrated with cells precluded their use in bioapplications. Commercially available CdSe-ZnS QDs were made water-miscible by exchanging their hydrophobic surface ligand with mercaptoacetic acid, and assembling cationic single-layer and anionic multi-layer polymer-coated complexes. Layer-by-layer coating of QDs was robust enough to enable integration of polymers with diverse chemical functionalities, independent of the inorganic core.

Biocompatibility of the QD heterostructures integrated with diverse polymers was investigated, and primarily depended on the chemical structure of the integrated polymers. The QD cores did not cause significant toxicity, as was demonstrated by using cores with different chemical compositions. Single layer complexes containing cations with high charge densities were highly cytotoxic, while those coated with bio-derived cations with low charge density were more biocompatible. These properties were independent of the composition of the nanoparticle core. Cytotoxicity of the anionic
multi-layer complexes depended on the chemical composition of both the anionic and the cationic polymers, demonstrating that the composite properties of the heterostructures depend on those of the individual polymers.

Cationic dextran coated QDs were selected to complex with DNA, due to the low cytotoxicity and high transfection efficiency of the complex. The use of these complexes yielded more than 15-fold higher levels of protein production than the use of equivalent amounts of polymer, DNA and QD containing complexes simultaneously mixed together, without first coating the nanocrystals with the polymer. Integrating functionalized gene therapy polymers into the hybrid complex resulted in 2.5-fold increase in protein production. Characterizing the basis of gene delivery enhancement revealed that the cationic QD cores condensed DNA more effectively than their unstructured and free polymer counterparts, forming beads-on-a-string morphologies similar to nucleosomes. These heterostructures were used to continuously track the delivery of DNA containing complexes from cellular internalization to protein production, and used as different color labels for tagging the delivery of multiple genes.

**Conclusions**

Herein we demonstrated that: (i) water soluble QDs can be coated with oppositely charged polyelectrolytes to assemble single-layer and multi-layer structures, (ii) biocompatibility and cellular interactions of the hybrids depend primarily on the chemical structure of the integrated polymers, and (iii) QD-polymer-DNA complexes assemble into hierarchal structures that demonstrate better gene delivery properties than their unstructured counterparts, enabling optical tracking of the delivery complex.
1.1 Introduction to Quantum Dots

Colloidal semiconductor quantum dots (QDs) are single crystals that are a few nanometers in diameter (< 15 nm), which demonstrate discretization of energy levels (Section 1.1.1)\(^1\). They are generally composed of periodic groups of II-VI (e.g. CdSe) or III-V (e.g. GaN) materials, although several other semiconductors may also be used. They are too large to be atomic/molecular systems and too small to demonstrate bulk properties, and therefore possess novel physical characteristics specific to their intermediate size (Fig 1.1)\(^6\). Due to their large surface area to volume ratios, their properties are dominated by the surface functionalities, and not their bulk characteristics\(^7\). The optoelectronic properties and fine morphological control of the QDs make them ideal candidates for use in highly interdisciplinary fields, including applications to complex biological problems. In the subsequent sections the physics of quantum confinement is discussed, followed by the optical properties of QDs, and some current and future bioapplications.

Figure 1.1 Size of quantum dots (QDs) relative to other biological elements. QDs are larger than atoms and molecules, but smaller than cells and organelles. They are similar in size to proteins and biological fluorophores (source: Quantum Dot Technologies, reference 6).
1.1.1 Brief Physics of Quantum Confinement

The small size of the quantum dots (less than Bohr radius) leads to three-dimensional confinement of free electrons and holes, leading to discretization of their energy states, different from the continuous energy states of the bulk material\(^9\) (Fig 1.2a)\(^10\). This discretization limits the mobility of the free charge carriers and leads to the development of discrete conduction and valence bands\(^11-13\). As the size of the nanoparticle decreases, the energy levels get further discretized and the band-gap between the conduction and valence band increases\(^13\). Therefore, the energy of the band-gap can be controlled by both, tuning the size of the QD, and using materials with different bulk band-gap values.

![Diagram showing the energy bands and quantum dots](image)

**Figure 1.2** Effect of the size of quantum dots (QDs) on the bandgap energy and fluorescence. (a) Semiconductors have intrinsic bandgaps defined by the energy difference between the conduction band (bonding orbitals) and the valence band (antibonding orbitals). As the QD size decreases, energy levels are discretized, and the bandgap increases (reproduced based on reference 10). (b) Smaller QDs have larger bandgaps, which causes blue shifting of the fluorescence. By varying the QD size, a rainbow of QD colors can be prepared. (source: Quantum Dots Technologies, reference 15)

1.1.2 Optical Properties of QDs

Absorption of a photon of energy above the band-gap energy results in the creation of an electron-hole pair (exciton)\(^7\). The negatively charged electrons are excited from the valence bands to the conduction bands, while positive holes are left behind, leading to separation of charge. Radiative recombination of an exciton leads to the emission of a photon\(^1\). Based on the energy released, the recombination may be in the visible spectrum of light, and therefore detectable by the naked eye. Tuning both the size and composition of the QD allows control of the band-gap energy levels, enabling preparation of QDs with a spectrum of resolved emission. For example, the CdSe QDs have a Bohr radius of approximately 5.4 nm and a bulk band-gap of 1.7 eV, corresponding to deep red emission (~650 nm)\(^14\). As the size of the QD decreases, the energy of the band-gap
increases and the emission spectra become more blue-shifted (Fig 1.2b). The emission of the QD can be further enhanced by epitaxially growing a shell of higher band-gap material around the semi-conductor core. This increases the brightness of the QD emission, and eliminates the broad trailing red tails (700 – 800 nm) by decreasing the number of surface defects which cause trapping and partial quenching of the released photon. Thus epitaxial capping with a higher band-gap material enhances the brightness of the QD by minimizing non-radiative recombination of the exciton pair, as demonstrated by capping CdSe QDs with ZnS shells.

The optical properties of the QDs make them better fluorescent markers than their organic counterparts. These include (i) absorption and emission characteristics, (ii) stability and resistance to photobleaching, and (iii) bright fluorescence. As evidenced by the absorption spectra (Fig. 1.3a), QDs have broad absorption generally in the UV region, which allow excitation of multiple colors using a single UV source. They have narrow, symmetric and finely resolved fluorescence spectra (Fig 1.3b) without the broad trailing red tails characteristic of organic dyes. Therefore QDs are ideal probes for simultaneous labeling of multiple targets; broad absorption enables excitation using a single source while narrow, resolved emission enables distinct characterization of multiple targets. QDs are very stable fluorophores; they are resistant to photobleaching and maintain their fluorescence over extended periods of time and through repeated exposure. This allows their use as multi-dimension probes for z- and time-resolved microscopy. Since both require repeated measurements over extended periods of time, loss of fluorescence over the course of data collection may compromise the integrity of the results. Therefore the use of QD enables reliable, extended characterization of multi-dimensional systems. Lastly, QDs are much brighter than organic dyes with higher quantum yields. They also have much longer fluorescence lifetimes. Combined, these properties increase the signal to noise ratio of the image, enabling high sensitivity and resolution. Therefore, small amounts of target molecule can be detected, allowing single molecules to be labeled and tracked.

1.3 Potential for Biological Applications

QDs are well suited for biological applications; their small size enables efficient conjugation to intracellular organelles and proteins and their optical properties allow (i) immunolabelling and single molecule localization, (ii) optical multiplexing, and (iii) continuous multi-tracking. Due to their bright and stable fluorescence, QDs have been used for immunofluorescence staining of F-actin, heat shock proteins, and several other cellular markers in live and fixed cells. They have been used for optical multiplexing by the simultaneous labeling of multiple biomolecules based on color and intensity. QDs were used to produce optical barcodes in polystyrene beads that encapsulate nanoparticles of different colors at varying ratios. Multi-peptide mediated internalization was used to specifically incorporate multi-colored QDs into different mammalian cells, generating a unique and spectrally resolvable code for each cell type. Different color QDs were modified with specific cell-recognition ligands for tumor and normal cell types, and targeted cells in co-cultures. QD surfaces were modified with
organelle-specific targeting sequences, which selectively labeled cellular structures. QDs are resistant to photobleaching, which enables continuous acquisition of high-quality images over extended periods of time. This enables tracking of intracellular structures and mechanisms, which may help elucidate cellular pathways and provide a more fundamental, detailed understanding of cellular processes. Continuous, long term visualization of cellular processes was demonstrated by preparing serotonin, glycine receptor and epidermal growth factor-conjugated QDs, and visualizing their internalization and cellular transport through the cells. Continuous progression of respiratory syncytial virus infection of Hep-2 cells was also visualized using QD-virus conjugates. Real-time tracking of small, labeled molecules inside single cells was also demonstrated by labeling mammalian cells with transferrin coated QDs.

![Figure 1.3 Optical spectra of CdSe-ZnS QDs as a function of their size. (a) As the size of the QD decreases, absorbance becomes more blue-shifted, indicating higher energy absorption. All spectra are highly defined and demonstrate indiscriminate, broad absorption in the UV region (< 350 nm). (b) Fluorescence becomes more blue-shifted as the QD size decreases. All fluorescence spectra are narrow and finely resolved. (source: Evident Technologies, reproduced with permission, reference 21)
In addition to their optical and structural properties, QDs used for biological applications should (i) be hydrophilic, (ii) allow integration of diverse chemical functionalities, and (iii) be biocompatible and non-toxic to cells. Hydrophilicity is important since cellular environments are aqueous, whereas commercially available QDs are produced in organic solvents with hydrophobic surface coatings. Integration of various chemical functionalities enables manipulation of the QD properties and tailoring for specific cellular functions. However, this integration is challenging since most QDs have bulky, organic ligands on their surface that must be passivated or removed to allow covalent or electrostatic interactions with biomolecules\textsuperscript{19,22} (e.g. antibodies, localizing proteins). Finally, biocompatibility is a significant concern, as leaching of metal ions from the QD surface (e.g. Cd\textsuperscript{2+}) may result in cytotoxicity and mutagenesis\textsuperscript{40,41}. Therefore, hybrid QD systems must be multi-functional if they are to be used as effective probes for bioapplications.
1.2 Introduction to Gene Therapy

Gene therapy is the delivery of exogenous nucleotide-based drugs to manipulate endogenous protein levels, thereby regulating imbalances in the levels of functional protein production\(^{12,43}\). In the subsequent sections the potential of gene therapy, cellular barriers impeding delivery, and the hierarchal nanostructuring of biological DNA delivery vectors (virus) and DNA packing systems (chromosomes) are discussed.

1.2.1 Potential of Gene Therapy

The human genome is comprised of 3164.7 million nucleotide bases, less than 2\% of which account for the estimated 30,000 genes that encode for proteins\(^{44}\). Advances in human genome mapping and the molecular understanding of the fundamental disease mechanisms has lead to the potential use of combinatorial nucleic-acid based therapies\(^{45,46}\). Inherited and acquired genetic diseases can result from mutations in the genetic sequence, which then causes modified gene expression and altered protein production. Either an increase or a decrease in protein levels may manifest itself as abnormal cellular activity, leading to diseased behavior. Return to normal protein levels may nullify the effects of the disease, and restore the cell to its normal function; this can include increasing suppressed protein expression or decreasing excessive protein production (Fig 1.4)\(^4\). Gene therapy holds great promise for the compensation of deleterious genetic disorders.

**Figure 1.4** Potential of gene therapy. (a) Cellular functions are controlled by their proteins, which are encoded by the DNA. (b) Exogenous genetic material can be delivered to cells using gene delivery vectors like viruses, which control the endogenous protein levels and manipulate the functions of the cell (source figure reproduced from reference 47).

Therapeutic applications of gene therapy are still in the experimental phase\(^48\). The first clinical trials were conducted in 1990, where lymphocytes of patients suffering from a form of severe combined immunodeficiency disease were isolated from the patient’s body, treated with a retrovirus carrying the therapeutic gene, and then returned\(^{49}\). However, since then progress has been slow due to the complications associated with the use of viruses as the DNA delivery platforms. Their use is plagued by potentially toxic
immunological and inflammatory responses\textsuperscript{50,51}. Alternative synthetic systems are being investigated, but their use is restricted due to low levels of gene product expression due to impedance and arrest by the cellular barriers to gene delivery\textsuperscript{42}.

### 1.2.2 Barriers to Gene Therapy

There are several barriers that impede the delivery of therapeutic nucleic acids from outside the cell to the nucleus, where they can be transcribed\textsuperscript{52}. These include low cellular uptake of the complex, limited endosomal escape, degradation by intracellular non-specific nucleases, lack of nuclear targeting, restricted passage across the nuclear membrane, and inefficient release of DNA from the delivery complex (Fig. 1.5).

![Figure 1.5 Barriers to gene delivery. The first bottleneck is (a) efficient assembly of the DNA-vector complexes, followed by (b) delivery of the complex to the cells, (c) cellular internalization of the complexes, (d) endosomal escape of the complex, versus (e) endosomal degradation, (f) dissociation of the DNA from the delivery vector, (h) nuclear localization of the gene, versus (g) degradation of the free nucleic material, and (i) transport of the free nucleic material across the nuclear membrane and inside the nucleus (reproduced with permission from Nature Publishing Group, reference 52).](image)

Cellular internalization can occur via several mechanisms, the most common being non-specific endocytosis, whereby the cell periodically samples its environment by non-specifically engulfing the extracellular material. While these complexes are internalized within the cell, they remain trapped in membrane-bound organelles (endosomes, and lysosomes) where they are eventually degraded, thus never reaching the nucleus. Therefore, efficient endosomal escape of the complex is essential for mediating high levels of gene delivery\textsuperscript{53}. This may be achieved by disrupting the endosomal membrane via association with the lipid bilayer\textsuperscript{54}, or by causing osmotic swelling and rupture of the endosome\textsuperscript{55}.

Once a material is released in the cytosol, it must be trafficked to the nucleus, where gene transcription may occur. However, cytoskeletal elements (microfibers and filaments) impede this diffusion, and cytoplasmic endonucleases act to degrade the
DNA. Nuclear localization sequences, which consist of stretches of cationic amino acids, aid in cellular trafficking and nuclear targeting. Fast and efficient transport of the complex is necessary for high levels of sustained gene expression.

Upon reaching the nucleus, the complex must pass through the nuclear membrane and be internalized into the nucleoplasm. The transport of DNA across the nuclear envelope occurs through the nuclear pore which is approximately 55nm in diameter. Particles less than 25nm in diameter can diffuse through the membrane pores, while larger particles enter via the formation of a nuclear pore complex. The complex formation is controlled by the presence of short stretches of basic amino acids, known as the nuclear localization sequences. Once inside, the delivery vector must dissociate from the DNA to allow transcription of the delivered gene and subsequent production of the protein. If the complex remains bound to the DNA it can not be transcribed by the enzyme and is functionally inactive.

1.2.3 Viruses: Natural Gene Delivery Vectors

Several types of viruses can be used for nucleotide-based drug delivery, including retrovirus, adenovirus, and adenoassociated virus. Among them, the most common one is the adenovirus, for which the structure and infection mechanism have been extensively studied. Adenovirus is a non-enveloped, double-stranded DNA virus approximately 150 MDa in mass. The capsid forms an icosahedron with 12 vertices and 20 facets and has a diameter between 60 - 90 nm. At each vertex are highly negatively charged glycoprotein fibers approximately 35 nm in length which have ligands grafted to their ends to serve as markers for receptor mediated endocytosis. It contains a linear genome of ~ 36 kbp that is stabilized by basic (cationic) proteins, and forms the viral core.

While the details may vary, the overall infection mechanisms of different types of adenovirus are similar. The virus enters the cell via receptor-mediated endocytosis of the fiber proteins, and in the endosome undergoes a series of cooperative structural changes in the capsid. The acidic environment of the endosome leads to structural changes in the protein capsid, resulting in association of the proteins with the endosomal membrane, and release the capsid-encapsulated DNA into the cytoplasm. The complex is transported to the nucleus where it is internalized via the formation of a nuclear pore complex. The DNA is released from the complexing proteins, and is ready for transcription. Both the structural organization and the material properties of the virus are precisely controlled to produce efficient gene delivery vectors.

While viruses achieve high efficiencies of gene delivery, there are several problems associated with their use. They evoke inherent cytotoxic and immunogenic responses, are prone to mutations, and have size limitations on the genes that they can deliver. Furthermore, most viruses are not cell-specific and so, not cell selective. Therefore, non-viral methods of gene delivery are being developed so that delivery vector properties can be systematically manipulated to meet the specifications of the application. Based on the viral mechanism, it is clear that both material properties and
internal nanostructure of the delivery vehicle are important for achieving high delivery efficiency.

1.2.4 Chromosomes: Natural DNA Reservoirs

Human cells contain twenty three pairs of chromosomes, each between 1.7 and 8.5 cm long, and 2 nm in diameter, totaling approximately 2 billion nm when completely extended. Since the nucleus is spherical, typically 5 – 20 μm in diameter, unstructured packing of DNA within the nucleus may cause several problems: (i) physical confinement of a large amount of DNA in a small nucleus, (ii) entanglement of the long, thin DNA strands, and (iii) rigidity of the highly charged DNA backbone limiting the ease with which DNA can be manipulated. To overcome these problems, highly efficient condensing mechanisms have evolved that compact DNA, based on both electrostatic interactions and ordered structuring of the nucleic acid and the complexing materials.

Several hierarchies of organization are necessary to effectively compact DNA. The first order of packing is the assembly of the fundamental unit of DNA condensing, the nucleosome, which comprises of DNA and highly basic proteins called histones. Between 150 and 200 basepairs of double stranded DNA wrap around the histone octamer to form a complex approximately 11 nm in diameter, and 100 kDa in molecular weight (Fig 1.6). A second histone (H1) fastens the DNA to the octamer histone core. Nucleosomes demonstrate characteristic beads-on-a-string morphologies, as visualized using transmission electron microscopy. Condensed histone-DNA units are connected by segments of uncomplexed, exposed linker DNA resulting in an approximate packing ratio of 6. The second order of packing is further condensation of beads-on-a-string chromatin with the aid of the H1 protein, to form chromatin fibers 30 nm in diameter. Each helical turn of the chromatin fiber contains approximately 1200 basepairs of DNA, condensed into six nucleosomes. This results in an approximate packing ratio of 40. Finally, the DNA is further packaged by organizing into extended and condensed loops, scaffolds, and domains, giving a packing ratio of between 10,000 and 50,000.

![Figure 1.6 Organization of chromatin. Coiling of approximately 150 basepairs of DNA around histone core to form approximately 200 base pair nucleosome in a beads-on-a-string morphology (figures reproduced based on reference 78).](image-url)
1.3 Specific Aims

The general aims of this dissertation were to (i) prepare hybrid quantum dot (QD)-polymer complexes, (ii) manipulate hybrid structural and chemical properties and characterize their effects on biocompatibility, and (iii) use diverse, heterostructured complexes for gene therapy. This was accomplished by the following specific aims.

**Aim 1: Prepare stable, hybrid QD-polymer cores**

- **Aim 1a: Synthesize CdS QDs in aqueous solutions**
  - Use cationic polymers as capping agents
  - Test photostability, biocompatibility and tunability of synthesized QDs
  - Select QD core with better optical properties and tunability for subsequent investigations

- **Aim 1b: Modify CdSe-ZnS QDs with organic polymers**
  - Optimize modification procedure to make QDs hydrophillic
  - Use histidine oligopeptides as capping agents
  - Develop polymer coating techniques to assemble anionic QD-polymer complexes
  - Characterize optical properties and morphology of modified QDs
Aim 2: Characterize biocompatibility of polymer coated QDs

- Determine inherent biocompatibility of the QD core
- Assemble single-layer systems with different polymers
- Assemble multi-layer structures with different polymers
- Characterize biocompatibility of composite complexes and determine contribution of nanoparticle core and coating polymer layers

Aim 3: Investigate use of QD-polymer-DNA hybrid

- Select QD-polymer cores mediating high levels of protein production and low cytotoxicity
- Characterize morphology of hybrid complexes
- Investigate effect and mechanism of nanostructuring on gene delivery
- Use as fluorescent probes for constant monitoring of gene delivery
- Use as labels for tagging multi-gene systems
- Evaluate utility and potential of hybrid gene delivery vehicles
1.4 Specific Rationale

Polymer modified semiconductor QDs were used for assembling gene delivery nanostructures since they (i) enable fine morphological control of the QD core, (ii) possess inherent fluorescence of the QDs, and (iii) allow easy integration of diverse chemical functionalities. These properties are important for the assembly of finely tuned delivery vectors for mediating efficient gene delivery and tracking.

The small size and uniform size distribution make QDs ideal platforms for gene delivery. They are similar in size and shape to histones, natural DNA condensing proteins, which might help them effectively condense DNA. They are small enough to enable hierarchical organization of the QD-polymer-DNA complexes, which may enhance delivery properties due to nanostructuring. Their size may facilitate the assembly of small complexes that can be efficiently endocytosed by the cells and encounter less impedance during cellular trafficking. Furthermore, QD size, shape and size distribution can be readily manipulated, allowing their morphology to be systematically varied for optimization of the delivery properties of the hybrid complex.

The inherent fluorescence of QDs makes them excellent probes for optical characterization of complex systems, and can be easily monitored using a fluorescence microscope. Their fluorescence may be used to develop semi-quantitative models for predicting protein expression, which could help determine therapeutic doses. QDs demonstrate high photostability and do not hydrolyze or degrade in water, therefore enabling multi-dimensional and continuous tracking of complexes. Continuous, time-resolved three dimensional imaging may enable elucidation of gene delivery pathways and identification of gene delivery barriers, aiding in the design of highly efficient gene delivery vectors. QDs have highly resolved emission spectra and therefore multiple colors can be used to tag different genes, enabling simultaneous characterization of combinatorial, multi-gene systems.

Integrating the QDs with polymers can enable assembly of complexes with diverse functionalities, including bio-derived and synthetic materials. The efficiency of different materials in mediating gene delivery and their escape from or arrest by different cellular barriers may be characterized. This can help identify materials best suited for avoiding arrest by specific cellular barriers. Systematic integration of different materials with complimentary functionalities may enable the assembly of highly synergistic gene delivery systems, which mediate escape from different cellular barriers. Highly characterized delivery systems also have the potential to be optimized for very specific gene therapy applications.
1.5 References

44. National Human Genome Research Institute, http://www.genome.gov/ 
47. Food and Drug Administration, http://www.fda.gov/ 
75. Chromosomal Packing, http://sgi.bl.s.umkc.edu/waterborg/chromat/chroma02.html
77. Hierarchal Organization of DNA, http://nano.chem.emory.edu/~dlm/chromatin.html
Chapter 2: Aqueous Synthesis of CdS Quantum Dots
Chapter Synopsis

**Objective**
The aim of this section was to synthesize and characterize optical properties and biocompatibility of CdS quantum dots (QD), prepared in aqueous solutions using cationic polymers and histidine oligopeptides of varying molecular weights and chemical structures as the capping agents.

**Hypothesis**
Varying the chemical properties of the capping agents may enable synthesis of CdS QDs with controlled optical properties and morphologies, which may be subsequently used for bioapplications.

**Rationale**
The use of aqueous phase synthesis will enable production of inherently hydrophilic QDs that can be used for biological applications, while providing an alternate technique that is more environmentally benign than the organic synthesis. The use of cationic polymers and histidine rich motifs has previously demonstrated stabilization of optically active CdS QDs. However, no systematic investigation has been conducted characterizing the effect of varying the polymer structure and molecular weight on the properties of the synthesized QDs, and the interactions of the synthesized QDs with biological systems.

**Results**
The optical properties and morphology of the synthesized QDs depend on (i) the molecular weight of the polymer, (ii) its mass concentration during synthesis, and (iii) the molecular structure of the monomer. Cationic branched polyethyleneimine (bPEI) polymers with molecular weight exceeding 2 kDa effectively passivated optically active CdS nanoparticles, within effective concentration ranges of the polymer. Increasing the mass concentration within these effective ranges resulted in the synthesis of QDs with blue-shifted and more defined absorbance and fluorescence spectra, indicating the synthesis of smaller QDs with tighter size distributions. Increasing the molecular weight of the polymer resulted in broadening of the effective concentration window over which optically active QDs are prepared, moving it to higher concentrations, and enhancing the brightness of QD fluorescence. Using linear PEI and polyallylamine (PAA), other synthetic polycations, as the surface capping agents led to the synthesis of QDs with less defined absorbance spectra, and with decreased fluorescence, as compared to those synthesized using bPEI. All QDs had highly cationic surfaces and resulted in widespread cytotoxicity and when incubated with COS-7 cells, therefore precluding their use for bioapplications.

Histidine oligopeptides with three or more covalently linked residues were effective stabilizers for producing optically active CdS QDs. The number of covalently linked histidine residues affected the morphology and optical properties of the synthesized QDs; as the histidine chain length increased, the size of the QDs decreased, size distribution became tighter, and the absorbance and fluorescence became more blue-shifted. The QDs
had crystalline cores, as revealed by transmission electron microscopy. Integration of the QDs with cells demonstrated poor optical resolution and aggregation of the particles in solution.

**Conclusions**

Controlling the chemical properties of the capping agents enabled tailoring of the optical properties of the QDs. However, these QDs were either cytotoxic or demonstrated poor optical properties like photobleaching and aggregating in solution, and were therefore precluded from further use as bioprobes.
2.1 Background

Semiconductor QDs prepared using different synthesis techniques have varied structures and properties; high quality II-VI nanocrystals, exemplified by CdS, have been prepared in organic and aqueous environments using thermal decomposition and ligand stabilization techniques\(^1\)\(^-\)\(^3\). While nanoparticles produced in organic solvents at elevated temperatures exhibit finely tunable optoelectronic properties (Fig 1.3), their surfaces are capped with large, hydrophobic ligands which make them immiscible with polar and aqueous solvents\(^4\)\(^-\)\(^7\). Therefore, they require extensive modification if they are to be interfaced with aqueous environments common in biological applications\(^8\)\(^-\)\(^10\). Organic synthesis also generates large quantities of toxic, environmentally harmful organic waste. Alternative aqueous synthesis methods that use benign conditions to produce water-compatible QDs are currently under investigation\(^11\)\(^-\)\(^12\). Using these methods optically active nanocrystals with varying core compositions, including CdSe\(^13\) and CdS\(^11\)\(^-\)\(^14\), have been successfully prepared\(^12\). However applications of these QDs have been limited since their optical properties, including the absorption and emission spectra, are neither as finely tunable nor as resolved as of those produced in organic solvents.

Aqueous synthesis of CdS QDs has been previously demonstrated using a variety of nanoparticle surface passivating agents, to produce intrinsically water-miscible nanocrystals. Typical synthesis schemes include initial incubation of the cationic metal precursor salts (e.g. CdCl\(_2\)) with the stabilizing agent and rapid addition of the anionic precursor salt (e.g. Na\(_2\)S) to form nanoparticles\(^12\). The properties of the synthesized QDs depend on the reaction conditions and the composition and concentration of the precursor salts and the capping agents. Varying these conditions and materials, especially the chemical structure of the stabilizers, has a significant effect on the optical properties of the QDs. Synthetic and biological ligands and polymers have been tested as surface passivators, including thiol-containing ligands\(^15\), phytochelatin peptides\(^16\)\(^-\)\(^17\), cationic polymers\(^14\)\(^-\)\(^18\)\(^-\)\(^19\) and dendrimers\(^20\)\(^-\)\(^21\), producing optically active CdS nanocrystals.

The use of two material groups as surface passivators for CdS QD synthesis, cationic polymers and histidine oligopeptides, is particularly promising. Previous investigations have demonstrated that cationic polymers with high charge densities, like high molecular weight cationic branched polyethyleneimine and polyamidoamine (PAMAM) dendrimers, are effective stabilizers for producing CdS QDs\(^14\)\(^-\)\(^18\)\(^-\)\(^21\). The surfaces of CdS particles are anionic due to adsorption of negatively charged hydroxyl groups to the cationic dangling cadmium bonds (Fig 2.1a)\(^22\), to which the cationic polymers electrostatically associate. Histidine oligopeptides were effective at mediating synthesis of noble metal nanoclusters; silver, gold, platinum and copper nanoparticles were previously synthesized using histidine amino acids and histidine-rich motifs\(^23\). Recent work using yeast surface display demonstrated that histidine rich sequences promote binding to CdS surfaces\(^24\). Nanoparticle stabilization is likely mediated by formation of metal-imidazole bonds, formed by donating the electron pair of the imidazole ring to stabilize the cations\(^25\) (Fig 2.1b).
Herein we have used cationic polymers and histidine oligopeptides as capping agents for producing CdS QDs. Cationic bPEI, linear polyethyleneimine (IPEI) and polyallylamine (PAA) were tested as capping agents (Appendix). bPEI is a highly branched structure with primary, secondary and tertiary amines in its backbone, while IPEI and PAA are linear polycations with secondary amines in the backbone and primary amines grafted to the backbone, respectively. Histidine oligopeptides with varying number of covalently linked histidine residues were also tested. Since the capping agents electrostatically associate with the CdS nanocrystals, changing any parameters that might affect these electrostatic interactions can affect the properties of the final QD produced. To the best of our knowledge, no systematic investigation of the effects of varying the molecular weight and structural architecture of these capping agents on the optical properties of the nanoparticles has been previously demonstrated. Furthermore, biocompatibility of polycation stabilized CdS nanoparticles with cells has also not been previously characterized.
2.2 Specific Aims

2.2.1 Cationic Polymer Mediated Synthesis
- Optimize experimental conditions (pH, temperature, salt concentration) for CdS QD synthesis using bPEI polymer as the capping agent.
- Determine the effect of varying bPEI chain length on the size and optical properties of the synthesized nanoparticle.
- Investigate the effect of varying polymer structure on the size and optical properties of the synthesized QD, by testing bPEI, IPEI and PAA as the capping agents.
- Test the biocompatibility of the “best” cationic QDs synthesized using cationic polymeric surface capping agents.

2.2.2 Histidine Oligopeptide Mediated Synthesis
- Optimize experimental conditions (solution pH) for CdS QD synthesis using His 10 as the capping agent.
- Investigate the effect of varying the number of covalently linked histidine residues (His 1, 3, 5, 10, 80 – 100) on the properties of synthesized QDs
- Test the biocompatibility of histidine-coated QDs synthesized using different length histidine capping agents.
2.3 Materials & Methods

2.3.1 Experimental Setup

Materials

CdCl₂ and Na₂S were purchased from Alfa Aesar. PAA, bPEI and lPEI were obtained from Sigma Aldrich. Histidine amino acid (His1) and polypeptide chain (pHis, 70-90 units) were obtained from Sigma Aldrich. Oligopeptide chains with 3, 5 and 10 histidine units (His3, His5 and His10, respectively) were synthesized at the MIT Biopolymers Lab. The pH of the solutions was adjusted by adding HCl and NaOH purchased from Sigma Aldrich. All other reagents were also purchased from Sigma Aldrich.

QD Synthesis

Stock solutions of 100 mM CdCl₂ and Na₂S were prepared in deionized water and further diluted to 0.5mM in pH adjusted water (values discussed later)\textsuperscript{12}. Capping agent (CA) solutions were prepared in deionized water at 10mg/mL. Varying volumes of CA were added to 2mL of diluted CdCl₂ while stirring vigorously in a heated water bath maintained at 80°C. The volumes were varied to obtain capping agent unit to cadmium molar ratios over wide ranges, and was based on the molar concentration of the monomer residues, and not that of the terminators polymer chains. CdCl₂ and CA were equilibrated for 10 minutes and followed by the rapid addition of 3mL diluted Na₂S (in excess). The solution was stirred on heat for 10 minutes, and cooled for 4 hours to allow the optical properties to stabilize.

Particle Characterization & Size Estimation

The absorbance spectra of undiluted, particles were obtained using a UV/Vis spectrometer (Beckman Coulter, DU 800). For fluorescence measurements, the samples were diluted in equal volume deionized water, excited at 325nm (unless noted otherwise), and their emission spectra obtained using a fluorimeter (Hitachi 2500). The peak positions of the absorbance and fluorescence spectra were obtained by determining the maxima of the corresponding curves. The nanoparticle size was estimated using the effective-mass model including Coulomb interactions, which correlates the absorbance fronts to the CdS particle size\textsuperscript{26}. Surface charge of the nanoparticles was determined in deionized water, using the Smoluchowski method (ZetaPals Analyzer, Brookhaven).

TEM Characterization

Nanoparticles were purified to remove excess reactants by ultrafiltration through microsep centrifugal columns (Pall, 3 kDa and 30kDa cutoff). The purified QD’s were diluted in deionized water and adsorbed on carbon coated copper grid (Ladd Research Industries) for 20 minutes, rinsed with water, dried, and imaged using a JEOL 2010 at an accelerating voltage of 200 kV.
**Incubating Cell with QDs**
Cos-7 cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin/Amphotericin B. They were incubated at 37 °C in 5% CO₂. For QD incubations, cells were plated at a density of about 75,000 cells per well in a 24-well plate, and allowed to grow overnight. Prior to incubation with QDs, cells were incubated in serum-free medium (0.5 mL per well). QD complexes were introduced and the cells were incubated for 2 hrs following they were supplemented with fresh, complete medium.

**Fluorescence Imaging**
The cells were imaged using an inverted IX51 Olympus microscope with GFP (ex 450/40, em 525/50), TRITC (ex 535/50, em 610/75) and DAPI (ex 360/40, em 460/50) filters. Images were obtained using SimplePCI software.

**2.3.2 Experimental Design: Cationic Polymer Mediated Synthesis**

**Optimizing Experimental Conditions**

1. Determine optimal synthesis temperature (25°C, 50°C, 80°C)
2. Determine optimal synthesis pH (0.63, 1.25 and 2.5 mM HCl/NaOH solutions)
3. Determine optimal salt concentration (1.25, 2.5 and 5 mM NaCl buffers)
4. Test effect of PEG addition (0.125, 0.625, 1.25, 3.125, 6.25 μM buffers)

Select optimal synthesis conditions
Varying Chain Length of bPEI

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<th>Dose (µg/mL)</th>
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Characterizing Polymer Structure

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<tr>
<td>1250</td>
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</table>

Characterize:
- absorbance

Testing biocompatibility of cationic QDs

1. Select “best” QDs from previous steps
2. Deliver to cells
3. Characterize results with fluorescence microscopy

2.3.3 Experimental design: Cationic Polymer Mediated Synthesis

Optimizing experimental conditions (solution pH)

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Select optimal conditions
Varying number of covalently linked histidine residues in the stabilizing agent

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</table>

Characterize:
- absorbance
- fluorescence

Testing biocompatibility of histidine-coated QDs

1. Select “best” QDs from previous steps
2. Deliver to cells
3. Characterize results using fluorescent microscopy
2.4 Results & Discussion: Cationic Polymer Mediated Synthesis

The use of synthetic polycations, bPEI, IPEI and PAA, to synthesize CdS QDs was investigated. First, reaction conditions including temperature, pH, salt and surfactant concentration were optimized, using bPEI as the capping agent. The effect of varying the molecular weight (MW) of bPEI on the absorbance and fluorescence properties of the QDs was characterized. Next, QDs were prepared using IPEI and PAA as the capping agents, and their optical properties were characterized and compared to the properties of QDs synthesized using bPEI. Lastly, biocompatibility of the “best” QDs synthesized using bPEI, IPEI and PAA was determined.

2.4.1 Optimizing Synthesis Conditions

The effect of varying reaction temperature on the optical properties of bPEI capped CdS QDs was determined by synthesizing nanoparticles in deionized water at 25°C, 50°C and 80°C (Fig. 2.2). QDs were synthesized by equilibrating CdCl₂ (dissolved in water) with bPEI (25 kDa) for 10 mins, followed by rapid addition of Na₂S (dissolved in water). The particles were aged for 4 hours after synthesis, and optical properties were characterized. The absorbance spectra (Fig 2.2a) of QDs prepared at all three temperatures are similar in shape and intensity of absorption. They are very well defined with two distinct absorption peaks, one at 320 nm and the other at 345 nm. These peaks are due to the discretization of energy levels as a result of quantum confinement of CdS (Section 1.1.1). Visual characterization of fluorescence under a UV lamp demonstrated a significant difference in the brightness of the QDs synthesized at different temperatures; those produced at 80 °C were bright blue, while those synthesized at 25 °C were faint blue-green. Subsequent characterization of the QD fluorescence spectra (ex. 325 nm) yielded similar results (Fig 2.2b); the emission peaks of all three QDs were between 450 – 460 nm, those produced at 80 °C being slightly more blue-shifted (445 nm) than those produced at 50 °C (453 nm) and 25 °C (456 nm). There was a significant difference in brightness, with QDs produced at 80 °C being more than 1.5-fold brighter than those produced at 50 °C and 25 °C. This enhanced fluorescence may be due to more efficient capping of the QD by the polymer at higher temperatures, leading to a decrease in the number of surface defects. Since QDs synthesized at 80°C have the brightest fluorescence, all subsequent syntheses were carried out at that temperature, unless stated otherwise.

The effect of solution pH on the optical properties of the QDs was determined by synthesizing QDs in pH adjusted solutions; precursor salts were dissolved in 0.63, 1.25 and 2.5 mM HCl or NaOH solutions. Synthesis was conducted as before and the absorbance spectra obtained 4 hours after synthesis (Fig. 2.3). QDs produced in pure water have the split-peak absorbance profiles, but as the precursor salt solutions become increasingly acidic or basic, the peaks flatten out and the absorbance spectra lose their definition. At high HCl concentrations, all quantized absorbance is lost and the spectra resembles that of unstructured, bulk CdS. Visual characterization of the QD fluorescence showed a decrease in fluorescence brightness and red-shifting of the emission color with pH adjustment of the precursor salt solutions. The data demonstrate that QDs with the
best optical properties are synthesized in unadjusted deionized water, and that the addition of acid or base compromises the optical quality of the QD produced. Therefore all subsequent reactions for cationic polymer synthesis were conducted in plain deionized water, unless stated otherwise.

Figure 2.2. Characterizing the optical properties of bPEI capped CdS QDs synthesized at varying temperatures. (a) Absorbance spectra of the nanoparticles prepared at different temperatures are similar in shape and peak positions, and demonstrate the characteristic split-peak absorbance. (b) Fluorescence spectra become significantly brighter and slightly blue-shifted with increasing synthesis temperatures.

The effect of salt concentration on the optical properties of the QDs was determined by characterizing nanoparticles prepared by dissolving the CdCl₂ and Na₂S in 1.25, 2.5 and 5 mM NaCl. Analyzing the absorbance spectra (Fig. 2.4) revealed that the addition of salt resulted in loss of definition as the peaks flattened out. Visual characterization of the fluorescence revealed that QDs synthesized in increasingly higher salt concentrations had
progressively decreased fluorescence. These data demonstrate that addition of salt to the reaction solutions is detrimental to the optical properties of the synthesized QDs. Since the associations between the QD and the capping agent are electrostatic, and addition of salt causes charge shielding, presence of salt may decrease the strength of interactions between the QD surface and the cationic polymer resulting in inefficient passivation of the QD surface, leading to poor optical properties. All subsequent syntheses were performed without the addition of salt, unless stated otherwise.

Figure 2.3 Absorbance spectra of CdS QDs synthesized in acidic and basic buffers, at 80 °C using bPEI as the capping agent. The absorbance becomes less defined as acid or base are added to the synthesis solution.

The effect of surfactant concentration on the optical properties of the synthesized QDs was determined by supplementing the precursor salt solutions with polyethylene glycol (PEG) at concentrations ranging between 0.25 and 6.25 μM. The absorbance spectra of QDs prepared at varying PEG concentrations (Fig 2.5) are similar, with no significant differences between the peak positions and absorption profiles. Visual characterization of the QD fluorescence did not reveal any differences between the color and the brightness of the QDs synthesized at varying PEG concentrations. Therefore, the addition of PEG does not affect the optical properties of the synthesized QDs within the concentrations tested. Subsequent syntheses were conducted without the addition of the surfactant, unless stated otherwise.
Figure 2.4 Absorbance spectra of CdS QDs synthesized in buffers with varying NaCl concentration, at 80 °C using bPEI as the capping agent. The addition of salt appears to have no effect on the absorbance properties of the synthesized QDs.

Figure 2.5 Absorbance spectra of CdS QDs synthesized with varying concentrations of polyethylene glycol surfactant, at 80 °C using bPEI as the capping agent. Addition of the surfactant does not appear to have a significant effect on the absorption properties.
The data presented in this section demonstrates that within the ranges investigated, optimized QDs were synthesized at 80 °C in deionized water, without the addition of acid, base, salt, or surfactant. All subsequent cationic polymer syntheses were carried out at these optimized conditions, unless stated otherwise.

2.4.2 Investigating the Effect of Chain-Length

The effects of varying the concentration and molecular weight of the capping agent on the optical properties of the synthesized QDs were investigated. QDs were synthesized by equilibrating CdCl₂ (dissolved in water) with bPEI (25 kDa) for 10 mins, followed by rapid addition of Na₂S (dissolved in water). Increasing concentrations of bPEI (25 kDa) ranging between 2.5 and 1250 μg/L, were added to the cadmium salt solution. The particles were aged for 4 hours after synthesis, and optical properties were characterized (Fig. 2.6). At low concentrations of bPEI (2.5 μg/mL) the spectra are relatively flat and featureless, indicating the absence of quantum confinement of CdS. Furthermore, after mixing the reactants a fine, yellow precipitate formed that eventually settled down to the bottom of the reaction vessel. The particles were likely large aggregates of bulk CdS that formed due to insufficient polymer to mediate synthesis of finely dispersed QDs. As the concentration of bPEI was increased (25 to 250 μg/mL), the absorption spectra became more defined and distinct peaks corresponding to exciton absorptions were visible. The peaks also become more blue-shifted, indicating a decrease in particle size. The CdS solution remained visibly clear, and the large, yellow aggregates did not form. At very high bPEI concentrations (1250 μg/mL), the absorption spectra became unfeatured again, as the profile associated with quantized absorption was lost. However, the yellow precipitate did not form, indicating that CdS did not aggregate. This suggests that when the polymer concentration was very high, the nanoclusters formed did not demonstrate fluorescence in the visible region. These data demonstrate that there is a range of concentrations (concentration window) between which the polymers act as effective capping agents to produce optically active CdS quantum dots. By varying the concentrations within these ranges, the optical properties of the QDs can be manipulated.

Figure 2.6 Absorbance profiles of QDs synthesized at 80 °C, with varying concentrations of 25kDa bPEI. At very low and high bPEI concentrations, optically active QDs with well-defined absorption spectra are not prepared. There is a range within which bPEI acts as an effective capping agent; increasing the concentration of bPEI within this range causes blue-shifting of the absorbance spectrum.
To determine the effect of the molecular weight of the polymer on the optical properties of the synthesized QDs, effective concentration windows of 2, 10, 25 and 70 kDa bPEI were determined. Synthesis was conducted at polymer concentrations ranging between 2.5 and 1250 μg/mL, and the positions of the first QD absorption peaks were determined (Fig 2.7). Blue-shifting of the first peak from the bulk value (~460 nm) reflects a decrease in particle size, indicating effective capping. CdS treated with 2 kDa bPEI did not demonstrate any blue-shifting, and visual inspection revealed the formation of yellow precipitate that lacked fluorescence. The use of bPEI polymers (> 2kDa) within the respective effective concentration windows resulted in blue-shifting of the absorbance fronts. Using 10 kDa bPEI as the capping agent at concentrations between 2.5 and 250 μg/mL produced optically active QDs, while higher molecular weight polymers were effective at higher doses. 10 kDa bPEI passivated QDs demonstrated the most blue-shifting at lower bPEI concentrations than those prepared using 25 and 70 kDa polymers. However, the maximum value of the blue-shift was approximately the same for all three higher molecular weight bPEI’s. This suggests that a critical minimum size of polymer is needed, beyond which bPEI can act as an effective stabilizing agent. Above this size (> 2 kDa), the maximum blue-shifting of the peak position is approximately the same for all polymers, but observed at different polymer concentrations. Smaller chain length polymers also have smaller effective concentration windows and appear to form the most blue-shifted QDs at lower concentrations than large molecular weight polymers.

![Figure 2.7](image)

**Figure 2.7** First absorption peak position as a function of bPEI molecular weight and concentration. Polymers greater than 2 kDa are capable of producing optically active QDs. Increasing the molecular weight of the polymer broadens the effective concentration window.

The effects of chain length on the optical properties of the synthesized QD may be explained by considering two different factors that affect QD stabilization: electrostatic association of the cationic polymer chain with the anionic nanocrystal, and the molar concentration of the polymer. Since bPEI chains likely interact with the growing QDs by
weak electrostatic interactions, increasing the polymer chain length reinforces these interactions by enabling multiple electrostatic interactions, making the binding stronger. For very short chain lengths the binding may not be strong enough to enable effective passivation of the QD surfaces, resulting in the formation of CdS aggregates without quantum confinement. As the chain length increases the binding is reinforced, resulting in stronger interactions between the QD and the polymer, therefore facilitating the synthesis of optically active CdS QDs. This effect is likely more pronounced at smaller chain lengths, since progressively increasing the polymer chain length may have diminishing enhancement on the polymer-QD binding. However, as the chain length of the polymer is increased, its molar concentration decreases, at constant mass concentrations. Therefore fewer chains are available to bind to the QDs. This could cause the effective concentration window to broaden and move to higher concentrations, and explain why maximum blue-shifting occurs at higher mass concentrations. While the binding between each polymer chain and QD increases in strength with increasing molecular weight of the polycation, there are fewer chains available to bind to the QDs. This mechanism predicts that once the higher molecular weight polymers bind to the QDs, the association will be very strong and the particles will be more effectively stabilized than by smaller molecular weight polymers.

Stronger interactions between the passivating polymer and the QD surface may lead to reduction in the number of defects on the QD surface, resulting in brighter fluorescence. This hypothesis was tested by comparing the fluorescence of QDs synthesized at the same mass concentration (125 μg/mL) using bPEI of different molecular weights (Fig 2.8). The sample prepared using 2 kDa bPEI did not show any fluorescence, while those prepared using 10, 25 and 70 kDa bPEI showed increasingly brighter fluorescence. The positions of the peak maxima were similar, located between 525 – 535 nm. This data further supports that higher molecular weight bPEI binds more tightly to the surface of the QDs, than does lower molecular weight bPEI.

![Fluorescence spectra of QDs synthesized using different molecular weights bPEI, at the same mass concentrations. As the molecular weight of the QD-capping polymer increases, the fluorescence of the nanoparticle becomes brighter and more blue-shifted.](image)

**Figure 2.8** Fluorescence spectra of QDs synthesized using different molecular weights bPEI, at the same mass concentrations. As the molecular weight of the QD-capping polymer increases, the fluorescence of the nanoparticle becomes brighter and more blue-shifted.
The data presented in this section demonstrates that both the mass concentrations and the chain length of the capping polymer have a significant effect on the optical properties of the synthesized QDs; bPEI polymers with low molecular weights (< 2 kDa) are not effective capping agents, while higher molecular weight polymers are effective within defined concentration ranges. As the molecular weight of the polymer increases, this effective window increases and moves to higher mass concentrations. QDs produced using higher molecular weight capping agents demonstrate brighter fluorescence than those prepared using low molecular weight ones.

2.4.3 Determining Effect of Molecular Structure

The effect of varying the molecular structure of cationic polymers was investigated by using lPEI (25 kDa) and PAA (19 kDa) as capping agents. QD were synthesized by equilibrating CdCl$_2$ (dissolved in water) with the capping agent for 10 mins, followed by rapid addition of Na$_2$S (dissolved in water). The particles were aged for 4 hours after synthesis, and optical properties were characterized. The effective concentration ranges of the IPEI and PAA were determined by synthesizing QDs at polymer concentrations ranging between 2.5 and 1250 μg/mL, and analyzing the positions of the first absorption peak of the synthesized QDs (Fig. 2.9). Both polymers demonstrate blue-shifting of the absorption peaks but to different extents, when present above a critical minimum concentration. Maximum blue-shifting of IPEI capped QDs (350 nm) is less than that of PAA capped QDs (325 nm), and both occur at approximately the same concentration of the polymer (250 μg/mL), under the conditions tested. Therefore IPEI forms QDs that are larger in size than those produced by using PAA. The absorbance fronts of PAA capped QDs show similar blue-shifts as those synthesized with similar molecular weight bPEI (25 kDa, 325 nm), and therefore likely produce QDs similar in size. Both IPEI and PAA have broader effective concentration ranges than bPEI, indicating that they are not as effective as bPEI in mediating the synthesis of very small nanoclusters. This may be due to the rigidity of their linear polymer backbones which limit their wrapping around CdS nanoparticles. It could also be because the branched polymer has higher hydrodynamic radius, enabling more efficient encapsulation of the QDs. This data demonstrates that the optical properties of the QDs depend on both the chemical and molecular structures of the polymer.

To further investigate the optical properties of the IPEI and PAA capped QDs, absorbance and fluorescence spectra of QDs synthesized using IPEI and PAA at 125 mg/mL were analyzed (Fig. 2.10). The absorbance spectra (Fig 2.10a) have one peak each; IPEI stabilized QDs have an absorbance peak at 340 nm, while PAA terminated QDs have a peak at 320 nm. They do not have the split-peak absorbance characteristic of bPEI passivated CdS QDs. However their peaks individually correspond to those obtained using bPEI (320 and 345 nm). It is possible that association of the secondary amine with the CdS results in the more red-shifted peak at ~340 nm (PEI, bPEI), while association of the primary amine with the CdS QDs results in the formation of the more blue-shifted peak at ~325 nm (PAA, bPEI). Furthermore, IPEI capped QDs have sharp absorbance profiles, whereas PAA coated QDs show a trailing rise. This suggests that the QDs obtained using IPEI are more homogenous in size than those obtained using PAA.
Analysis of the fluorescence spectra (Fig 2.10b) reveals that IPEI coated QDs are not as brightly fluorescent as PAA coated QDs, and both are less bright than bPEI capped QDs (Fig 2.8). This further suggests that linear IPEI and PAA do not passivate the QD surface as strongly as bPEI.

\[ \text{Absorbance (A. U.)} \]

\[ \text{Wavelength (nm)} \]

\[ \text{IPEI} \quad \text{PAA} \]

**Figure 2.9** First absorbance peak positions of QDs synthesized using IPEI and PAA as a function of polymer concentrations. Both polymers are capable of mediating optically active CdS QD synthesis. However, the absorption peaks are not as blue-shifted as those obtained when bPEI is used as the capping agent.

\[ \text{Absorbance (A. U.)} \]

\[ \text{Wavelength (nm)} \]

\[ \text{IPEI} \quad \text{PAA} \]

**Figure 2.10** Optical properties of CdS QDs prepared using IPEI and PAA as the capping agents. (a) Absorbance spectrum of the IPEI capped QDs is more defined and red-shifted than that of the PAA capped QDs. (b) Fluorescence spectra of PAA capped QDs is significantly brighter and slightly blue-shifted than that of the IPEI capped QDs.

The data presented demonstrates that the molecular structure of the polymer has a significant effect on the properties of the synthesized QDs. QDs obtained using bPEI are small with a sharp, well defined absorbance and bright fluorescence. QDs prepared using
IPEI have well defined absorbance, but are not as bright. QDs prepared using PAA are small and bright, but do not have well defined absorbance spectra. The “best” QDs are those produced using bPEI, IPEI and PAA as the capping agents, synthesized at polymer concentrations of 125 μg/mL.

2.4.4 Biointegration of cationic QDs

The surface charge of QDs capped with bPEI was characterized prior to integration with cells. The zeta potentials were determined by measuring the electrophoretic mobility of QDs in deionized water, prepared using bPEI (2, 10, 25 and 70 kDa), IPEI and PAA at polymer concentrations of 125 mg/mL (Fig 2.11). The surface potential of all optically active QDs are positive; samples synthesized with 2 kDa bPEI form large aggregates that do not have a well defined surface charge, while nanocrystals produced with bPEI > 2 kDa, IPEI and PAA are cationic. This demonstrates that the surfaces of the optically active QDs are coated with the cationic polymers.

Cellular interactions of the cationic polymer coated CdS QDs were probed by characterizing the treated cells with microscopy (Fig 2.12). Cellular morphologies were analyzed using light microscopy (left panel), cytotoxicity was determined by treating with propidium iodide (PI) (red, middle panel), and complex uptake was monitored by localizing the fluorescence of the nanoparticles (blue, right panel). PI is a cell impermeant dye that undergoes 50-fold enhancement in fluorescence when it binds to single-stranded DNA, and is therefore an indicator of dead or dying cells with compromised cellular membranes and dissociated DNA. Cells treated with bPEI, IPEI and PAA passivated QDs were small and rounded with fragmented membranes, lacked distinct cellular organelles, and stained densely with PI, indicating widespread cell death. There were low levels of blue fluorescence in the cells, indicating limited QD-complex uptake or poor optical stability. These data demonstrate that the cationic QDs tested were highly cytotoxic, and their subsequent use for bioapplications was not pursued.

![Figure 2.11](image_url) Zeta potentials of CdS nanoparticles synthesized using cationic polymers. All samples except those prepared using bPEI 2 demonstrate positive zeta potentials, indicating that the surfaces of the QDs are coated with cationic polymers. CdS treated with 2 kDa bPEI (bPEI 2) are neutral, likely due to the aggregation of CdS (as demonstrated previously).
Figure 2.12 Cells treated with CdS nanoparticles synthesized using (a) bPEI, (b) IPEI, (c) PAA and (d) untreated cells. The fragmented morphology of the cells (left panel) and the intense PI staining (middle column) demonstrate that cationic polymer capped CdS QDs are highly cytotoxic. Faint blue fluorescence (right column) show QDs associating with cells.
2.5 Results & Discussion: Histidime Mediated Synthesis

The use of histidine oligopeptides to passivate CdS nanoparticles was investigated. Synthesis conditions were reoptimized and the effects of varying peptide chain length on nanoparticle optical properties, size and morphology were characterized. Biocompatibility of histidine-passivated QDs was tested by incubating the “best” nanoparticles with COS-7 cells.

2.5.1 Optimizing Synthesis Conditions

The reaction conditions of histidine oligopeptide mediated CdS QD synthesis were optimized by varying the temperature and the pH of the reaction. QDs were synthesized by equilibrating CdCl₂ (dissolved in water) with the capping agent for 10 mins, followed by rapid addition of Na₂S (dissolved in water). The particles were aged for 4 hours after synthesis, and optical properties were characterized. Preliminary investigations demonstrated that varying the temperature had the same effect on histidine-oligopeptide mediated synthesis as it did on bPEI mediated synthesis (Fig 2.2); synthesis at higher temperatures yielded QDs with better optical properties. Therefore synthesis was conducted at 80 °C. However, varying the pH of the precursor salt solutions had a very different effect on the optical properties of the histidine-oligopeptide passivated QDs than it did on bPEI capped QDs. Since histidine moieties likely mediate synthesis by a different mechanism than bPEI QDs, a different response to solution pH is expected. Histidine oligopeptides likely mediate synthesis by stabilizing the cationic metal ion with the lone electron pair of the imidazole ring forming metal cation-imidazole bonds (Fig 2.1b).

To determine the effect of varying precursor salt solution pH on the optical properties of the QDs, nanoparticles were prepared using salts dissolved in pH adjusted water, with 10-mer histidine oligopeptide (His 10) as the capping agent. Cadmium salts were dissolved in pH 3, 5 and 7 solutions, and sulfide salts were dissolved in pH 7, 9 and 11 solutions, and the absorbance of the synthesized QDs was characterized (Fig. 2.13). QDs prepared from precursor cadmium salts dissolved in mildly acidic to neutral pH buffers (pH 5 – 7) have more defined absorbance spectra than those prepared using cadmium salts in low pH, highly acidic buffers (pH 3). Conversely, the absorbance spectra of QDs synthesized with the sulfide salt dissolved in buffers at higher pH values (pH 7 – 9) show better absorbance profiles than those prepared using sulfide salts in buffers at low pH values (pH 5). Visually characterizing the fluorescence of the QDs under a UV lamp (Table 2.1) demonstrated that QDs with more defined absorbance profiles have brighter, more blue-shifted fluorescence. Therefore QDs produced from cadmium salts dissolved at pH 5 or 7, and sulfide salts dissolved at pH 9 or 11 produced QDs with bright blue, fluorescence. However, if both the salt solutions had high pH (pH 7 and 11), the QDs precipitated out of solution. Therefore the best combination of cadmium/sulfide solution pH was 5/11, since it produced QDs with good absorption profiles, bright fluorescence, and high water stability. All subsequent histidine-mediated syntheses of CdS QDs were conducted at 80 °C, with the cadmium and sulfide salts dissolved in water with pH adjusted to 5 and 11, respectively, without the addition of salts or surfactants.
2.5.2 Investigating Histidine Oligopeptide Mediated Synthesis

The effect of varying the concentration of histidine peptides on the optical properties of the synthesized CdS QDs was investigated (Table 2.1). QD were synthesized by equilibrating CdCl₂ (dissolved in water) for 10 mins. with histidine oligopeptide, followed by rapid addition of Na₂S (dissolved in water). The particles were aged for 4 hours after synthesis, and absorbance and fluorescence spectra of the nanoparticles synthesized at different His10 concentrations were obtained. The spectra were representative of nanoparticles synthesized using peptides with three or more histidine residues. Concentration of the histidine peptide was expressed as the molar ratio of histidine residues to cadmium ions, to enable investigation of the effect of covalently linked histidine residues in the capping agent chain, on the optical properties of the synthesized QD.

The absorbance spectra of CdS nanoparticles (Fig 2.14a) synthesized using varying concentrations of His10 become more defined as the concentration of the peptide increases (from 0 to 12). In the absence or very low concentrations of histidine peptides (ratio between 0 and 2), spectra with low levels of broad absorption increasing monotonically at wavelengths less than 500nm, are observed. This supports that non-crystalline, non-homogeneous aggregates form in the absence of a stabilizer. As the concentration of stabilizers is increased (ratio 4 and above), the absorbance spectra become more defined. Distinct absorption peaks between 300nm and 500nm emerge, indicating the formation of crystalline particles with progressively smaller sizes and
tighter size distributions. The peak positions of the absorbance fronts blue-shift with increasing peptide concentrations, from 333nm at a ratio of 2, to 310nm at a ratio of 12.

<table>
<thead>
<tr>
<th>pH of CdCl₂</th>
<th>pH of Na₂S</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>blank</td>
<td>green</td>
<td>green/blue</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>orange</td>
<td>blue</td>
<td>blue</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>blue</td>
<td>blue</td>
<td>blue (pt)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Fluorescence of CdS QDs synthesized with the cadmium and the sulfide salts dissolved in different pH buffers, using His 10 as the capping agent. Fluorescence is most blue-shifted when sulfide salts are dissolved in high pH solutions (pH 11), and cadmium salts are dissolved in neutral pH solutions.

Figure 2.14 Optical properties of CdS QDs prepared at varying concentrations of the His10 capping oligopeptide. (a) Absorbance spectra become more defined and blue-shifted with increasing concentrations of the oligopeptide. (b) Fluorescence spectra become slightly more blue-shifted with increasing concentrations of His10 peptide.
This blue-shift indicates increased quantum confinement of the energy levels which is indicative of a decrease in the size of the synthesized nanoparticle. Analyzing the fluorescence spectra (Fig 2.14b) reveals that in the absence of a terminator (ratio 0), the samples demonstrate no detectable fluorescence. As the concentration of the histidine peptide increases, the fluorescence of the particles becomes brighter and more blue-shifted. At low doses of His10 (ratio 2), the particles display broad, dull fluorescence between 400 nm to 600 nm, with a peak at 498 nm. As the concentration of His10 is increased, the fluorescence intensity of the particles increases, and the peaks become blue-shifted, to 442 nm (ratio 12). This may be due to a decrease in particle size as more capping agent is available to passivate the nanoparticles with the peptide chain. The decrease in nanoparticle size with an increase in terminator concentration may be explained by probing the growth mechanism of the nanoparticles. Growth of the QD is in competition with termination due to binding with the histidine peptide. As the concentration of the stabilizer increases, growth is hampered and the termination is facilitated resulting in a shift of the equilibrium toward termination. This results in the synthesis of smaller nanoparticles.

The effects of varying the chain length of the histidine peptides on the optical properties of the QDs were also investigated. QDs were synthesized at different molar ratios of histidine units to cadmium using histidine amino acid (His1), oligopeptides with 3, 5 and 10 histidine residues (His3, His5, His10), and poly histidine (pHis, 70-90 units), as the capping agents. The absorbance spectra were obtained and used to determine the position of the first absorbance fronts (Fig. 2.15a). CdS particles synthesized using histidine amino acid did not have absorbance fronts (448-457 nm) that were significantly more blue-shifted than those observed in bulk CdS (~450 nm). Visual inspection of the solution revealed that CdS precipitated out as yellow aggregates. Nanoparticles prepared using pHis had very blue-shifted absorbance fronts (308-331 nm) and did not produce the yellow precipitate. As the number of covalently linked histidine units in the peptide increases, the absorbance fronts became more blue-shifted. This effect was more pronounced at shorter chain length oligopeptides, and diminished significantly as the peptide length exceeded ten residues. At corresponding molar concentration of histidine units to cadmium, QDs capped with peptides of higher chain lengths demonstrated more blue-shifted absorbance spectra, indicating production of smaller sized nanoparticles. Fluorescence of the nanoparticles was also obtained and used to determine the position of the fluorescence fronts (Fig. 2.15b). Nanoparticles prepared using His1 showed no detectable fluorescence. As the chain-length of the peptide increased, the position of the fluorescence peak at the corresponding molar concentration of histidine units to cadmium became more distinct and blue-shifted. His3 capped QD showed fluorescence at 547 nm - 527 nm, while pHis capped nanoparticles had peaks at 469 nm - 415 nm.

The blue-shifting of the absorbance and fluorescence spectra indicate the synthesis of smaller nanoparticles as the number of covalently linked histidine residues in the capping agent increases. Increasing the chain length may change the dynamic binding strength of the peptide to the cadmium ions on the surface of the growing nanoparticle. Data in literature suggests that histidine imidazole ring coordinates the divalent metal cations via labile electrostatic interactions (Fig 2.1b). At any given time, dynamic equilibrium is
maintained between the associated and dissociated molecules. If several histidine residues are covalently linked, the probability of association between the chain and the growing crystal increases, since there are multiple possible association sites. Furthermore, as some of the chains coordinate cadmium, other non-associated histidine residues are brought in close contact with the surface of the growing nanocrystal, thus facilitating attachment. Thus several covalently linked histidines may reinforce labile imidazole-cadmium bonds, facilitating stronger binding between the terminator and the growing nanocrystals, and resulting in early termination\textsuperscript{24}. This hypothesis is further supported by the diminishing decrease in particle size as the chain length is increased; cooperativity effects are greatest at short chain lengths, and become less pronounced as the chains elongates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.15}
\caption{Optical first peak positions of CdS QDs as a function of histidine oligopeptide chain length and concentration of capping agent. (a) Absorbance and (b) fluorescence peaks are blue-shifted as the number of covalently linked histidine residues in the capping oligopeptide increases, and as the concentration of the peptide is increased.}
\end{figure}

The nanoparticle sizes scale as a function of the concentration and length of the capping agents, and were determined using the position of the absorption fronts (Table 2.2)\textsuperscript{26}. The particle sizes decrease with increasing concentrations of the stabilizer. As the amount of
His10 is increased from histadine to cadmium ratio 2 to 12, the size of the particles decreased from 2.60 nm to 2.36 nm. Increasing the number of histidine residues in the terminator also decreases the particle size; the size of His3 capped nanoparticles ranges between 3.40 nm – 2.98 nm, while pHIs capped QDs range between 2.58 nm – 2.34 nm. While the nanoparticle size decreases with an increase in both, the histidine peptide dose and chain length, the latter appears to have a greater effect on controlling the particle size.

<table>
<thead>
<tr>
<th>concentration ratio</th>
<th>Particle Diameter (nm) (±0.2)</th>
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<tbody>
<tr>
<td></td>
<td>His1</td>
</tr>
<tr>
<td>2</td>
<td>5.10</td>
</tr>
<tr>
<td>4</td>
<td>5.42</td>
</tr>
<tr>
<td>6</td>
<td>5.31</td>
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<tr>
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</tr>
<tr>
<td>10</td>
<td>5.17</td>
</tr>
<tr>
<td>12</td>
<td>5.07</td>
</tr>
</tbody>
</table>

Table 2.2 Sizes of CdS QDs synthesized using histidine oligopeptides of different lengths, and at different concentrations. As the number of covalently linked histidine residue and the concentration of the capping agent increase, the sizes of the synthesized QDs decrease.

The morphology, crystallinity and size of the QDs were further probed by using transmission electron microscopy (TEM) to characterize QDs prepared using His3 and pHis peptides, at a ratio of 6:1 (Fig. 2.16a & b). The QDs are spherical in shape and appear as discrete particles, not visibly aggregated. There are visible differences between the two samples; those produced using His3 are larger (3.9nm ±1.1nm) and have greater size variations than those produced using pHis (2.9 nm ±0.6 nm). These trends are in accordance with those inferred from the blue-shifts of the absorbance and fluorescence fronts (Figure 2.14), and the respective sizes are similar to those calculated using the absorbance fronts (3.1 nm and 2.4 nm respectively, Table 2.2). High resolution TEM images determined the lattice spacing and crystalline structure of the QDs (Fig. 2.16c). The lattice spacing of the QDs imaged are 3.1 Å (left) and 2.7 Å (right), and correspond to cubic CdS, which has lattice spacings of 3.16 Å and 2.73 Å. Cubic packing has been previously observed in CdS nanoparticles synthesized in aqueous solutions, and is different from the hexagonal packing observed in bulk CdS. This demonstrates that the histidine polypeptides do not act exclusively as passive surface coatings, but may be involved in the nucleation and stabilization of the QDs.
Figure 2.16 Transmission electron microscopy (TEM) images of CdS QDs prepared using histidine oligopeptides. QDs prepared using (a) His\textsubscript{3} as the capping agent are larger and more polydisperse than those prepared using (b) His\textsubscript{10} as the capping agent (scale bar 20 nm). (c) High resolution TEM images reveal lattice fringes, which demonstrate the crystallinity of the QDs core (scale bar 5 nm).

2.5.3 Biointegration of histidine mediated QDs

Cellular interactions of the histidine oligopeptide passivated CdS QDs were probed by characterizing cells treated with nanoparticles synthesized using His3, His5, His10 and pHis, at histidine to cadmium molar ratios of 10:1 (Fig 2.17). Cells were incubated with QDs for 1 hr, and imaged 12 hours post treatment. Cellular morphologies were analyzed using light microscopy (left panel), and QD uptake was monitored by localizing the fluorescence of the nanoparticles (blue, right panel). The cells are spread out with intact cellular and nuclear membranes and distinct cellular organelles, indicating that the QDs are not highly toxic. However, fluorescent imaging reveals that the QDs have poor optical properties; the fluorescence diminishes within a few hours post treatment and the particles aggregate in solution, as demonstrated by the diffuse blue fluorescence (right panel). Therefore their subsequent use for bioapplications was not pursued.
Figure 2.17 Cells treated with CdS QDs prepared using (a) His 3, (b) His 5, (c) His 10, and (d) untreated controls. The cells (left panel) are not curled up and fragmented, but extended and sprouting processes, and therefore viable. Faint blue fluorescence (right column) indicates the presence of QDs which are aggregated in solution.
2.6 Summary & Conclusions

2.6.1 Cationic polymer-mediated synthesis
- Both mass concentrations and chain lengths of the capping polymer have an effect on their QD-passivating properties.
  - bPEI (> 2 kDa) produce optically active CdS nanoparticles
  - Polymers have effective concentration ranges within which they mediate synthesis of CdS QDs
  - Increasing molecular weights result in increasing the effective concentrations range of the polymer
  - Higher molecular weight polymers yield QDs with brighter fluorescence
- Molecular structure of the polymer affects the optical properties of QDs.
  - bPEI coated QDs are small with tightly controlled size distribution, have sharp, well defined absorbance and bright fluorescence
  - IPEI passivated QDs have well defined absorbance and tight size distributions, but are larger and not as bright
  - PAA terminated QDs are small and bright, but do not have well defined absorbance spectra or controlled size distributions
- QDs prepared using the cationic polymers have positive surfaces
- Cationic QDs are all highly cytotoxic and therefore not suitable for bioapplications.

2.6.2 Histidine oligopeptide-mediated synthesis
- Histidine oligopeptides with 3 or more covalently linked residues can be used as effective stabilizers to produce optically active CdS QDs.
- Number of covalently linked histidine residues affects the properties of the synthesized QDs.
  - the size of the synthesized particle and the particle size distribution decreases with increasing chain length
  - Absorbance and fluorescence become more blue-shifted with increasing chain length
- CdS QDs have crystalline cores and cubic packing.
- Histidine mediated CdS QDs can not be used as bioprobes since they do not demonstrate good optical properties when incubated with cells, and aggregate in solution.

Therefore, the use of aqueous-based QDs as bioprobes was not pursued further, and the use of commercially available QDs was investigated.
2.7 References


22. Manahan, S. E. *Environmental Chemistry*.
Chapter 3: Layer-by-Layer Modification of CdSe/ZnS & GaN Nanocrystals
Chapter Synopsis

Objective
The aim of this section was to deposit oppositely charged polymer layers on the surface of quantum dots (QDs), demonstrate use of the charged complex as building blocks for assembling hierarchal structures, and test versatility of the technique by using nanoparticle cores and polyelectrolytes with different chemical compositions.

Hypothesis
Coating QDs with polymers may enable functionalization of their surfaces and manipulation of their chemical properties, enabling assembly of diverse nanoparticle-polyelectrolyte hybrid complexes stable enough to be integrated into ordered arrays.

Rationale
The use of QDs for biological applications requires modification of the nanoparticle with diverse functionalities to allow tailoring of QD-cellular interactions for specific applications. Electrostatic layer-by-layer deposition of oppositely charged polyelectrolytes is a fast, versatile technique that enables integration of multiple functionalities and has been demonstrated for nanomaterials such as gold nanoparticles. Assembling the QD-polymer complexes into ordered structures will demonstrate stability and manipulability of the hybrids.

Results
Here we optimize a procedure for CdSe-ZnS QD surface ligand exchange by displacing hydrophobic trioctyl phosphine oxide (TOPO) ligands with mercaptoacetic acid (MAA), enabling the QDs to be dispersed in water. Surface potential measurements revealed that the QD-MAA were anionic, and subsequent coating with cationic polyallylamine (PAA) polymer rendered them positively charged. The single-layer, cationic QD-polymer complexes were subsequently coated with another layer of anionic polvinylsulfonate (PVSA) polymer, resulting in a second surface charge reversal. Analyzing the absorbance spectra and morphologies of the heterostructures revealed that the optical properties did not change significantly, and that the QDs were individually coated.

Integration of polymer coated QDs into ordered heterostructures was also demonstrated. Cationic QD-PAA and anionic QD-MAA selectively adsorbed to oppositely charged substrates, and were sequentially deposited on anionic hyaluronic acid (HA) patterned glass substrates to form glass/HA/QD-PAA/QD-MAA bilayers. Charged QDs show preferential binding to oppositely charged islands, and not to the uncharged, non-adhesive glass substrates. These patterns were stable, reproducible over multiple length scales, and showed high fidelity.

The robustness of the layer-by-layer modification technique was tested by coating GaN QDs with different cationic and anionic polyelectrolytes. Monitoring the surface charge
revealed characteristic surface charge reversals, and deposition on patterned substrates demonstrated selective adsorption on oppositely charged substrates.

**Conclusions**

Layer-by-layer deposition of polyelectrolytes on QDs enabled surface functionalization of nanoparticles, while preserving the properties of the individual, inorganic QD cores. These hybrids were stable enough to be integrated into ordered heterostructures, and the technique was versatile enough to enable incorporation of nanoparticles and polymers with diverse chemical compositions. These hybrids may be tailored for bioapplications.
3.1 Background

Commercially available high quality CdSe-ZnS QDs with finely tunable morphologies and optical properties are prepared in organic solvents, with bulky, organic surface capping ligands like trioctyl phosphine/trioctyl phosphine oxide (TOP/TOPO). QDs coated with these ligands are hydrophobic and therefore not miscible with aqueous environments, limiting their applications; biointegration of QDs requires easy suspension of the nanocrystals in aqueous solutions, and ready integration with functionalized materials. Several modification schemes render the TOPO-coated QDs soluble in water: (i) exchanging the hydrophobic surface ligand, (ii) encapsulating QD-TOPO complexes in amphiphilic functionalized materials, or (iii) hybrid combinations of the two techniques. Some common techniques used to make hydrophobic QDs water compatible, their advantages and limitations, and ease with which diverse functionalities can be integrated are discussed in the subsequent sections.

The most common method of rendering QDs miscible in water is by replacing the hydrophobic TOPO with hydrophilic ligands. TOPO molecules passivate the QD surfaces by coordinating the lone pair of electrons of phosphorous, with the dangling surface metal cations. However, these interactions are labile with the coupling between TOPO molecules and the metal surfaces in dynamic equilibrium. The introduction of a second ligand which binds to the dangling surface metal cations more strongly than the TOPO phosphine moiety results in displacement of TOPO, and association of the new ligand. Thiol-, imidazole- and other phosphine-containing ligands demonstrate high affinity for the metal nanocrystals, and effectively displace the TOPO molecules. However, the ability of these modified QDs to remain suspended in water depends on the hydrophillicity of the functionalities attached to the metal-coordinating moieties; polar, charged side groups facilitate dispersion of the QD-ligand complexes aqueous environments, and render them dispersible and stable in water, while amphiphilic or hydrophobic functionalities can cause aggregation. Several ligands have been used for TOPO ligand displacement, of which the thiolated-carboxylates are the most common. These bifunctional ligands have a nucleophilic thiol on one end that coordinates with the dangling surface metal cations, and a charged hydrophilic carboxylate on the other end that renders the modified QDs water soluble. While this technique is quick and inexpensive, the thiol-cadmium interactions are also labile and the ligands bound to the surface are in dynamic equilibrium with the solution. Repeated modifications and extended exposure to aqueous environments causes aggregation of modified nanoparticles. This aggregation is due to depletion of the surface ligands and the formation of disulfide bonds between them, and compromises the structural and optical integrity of the QDs. The use of polydentate ligands to coordinate with the metal cations has been investigated, and while they bind more strongly to the QD surface, such materials require preparation and modification procedures that are time and resource consuming.
Figure 3.1. Displacement of TOPO from the QD surface, and suspension of the modified QD in solution depends on the chemical properties of the displacing ligand. If the ligand has a strong nucleophillic moiety (b, c), it can replace TOPO from the QD surface by coordinating with the metal cation. However, if it does not have such a functionality, then the QD will remain passivated with TOPO (a). The stability of the modified QDs in aqueous solution depends on the ability of the modifying ligand to cause repulsion between the QDs, to overcome intrinsic van der Waals forces. Ligands with strongly charged moieties (b) enable QDs to remain individually suspended in solution, while those capped with uncharged ligands (c) aggregate in solution.

Coating and encapsulating hydrophobic QDs with functionalized materials has been previously demonstrated. Amphiphillic materials including phospholipid micelles, organic dendrons, simple and block co-polymers have all been used to produce water-miscible QDs. The lipophillic regions of the amphiphillic materials associate with the organic ligands through hydrophobic interactions, while the hydrophilic regions coordinate with water to facilitate suspension in aqueous solutions. While the individual hydrophobic interactions coordinating the amphiphillic material to the QD-TOPO cores are weak, they are collectively reinforced forming stable envelopes around the inorganic core. These modified QDs remain suspended over extended periods of time, and can undergo chemical modification without aggregating. Diverse chemical functionalities integrated in the hydrophilic region of the amphiphillic coating can help produce water miscible QDs with varied surface functionalities. However preparation of functionalized materials requires several modifications steps that are time and resource intensive, and encapsulating the bulky hydrophobic QD-ligand significantly increases the size of the modified nanoparticle complex.

Hybrid techniques combining ligand exchange and QD encapsulation have also been demonstrated, the most common involving the growth of silica shells around modified QD cores (Fig 3.3). The hydrophobic QD surface ligands are first exchanged with thiol-containing silane (e.g. mercapto-trimethoxysilane) followed by cross-linked of the silanes to form a siloxane shell. Diverse chemical functionalities can be incorporated into the
shell to tailor the surface chemistry, including amino, phospho- and polyethylene glycol moieties. However, this method is very labor intensive involving extensive QD modification and the synthesis of functionalized materials. Furthermore, the polymer shells dissolve after extended exposure to aqueous environments.

Figure 3.2 Aqueous instability of QDs coated with thiol-containing ligands. Nucleophilic thiols coordinate with metal cations forming labile thiol-metal bonds. However, oxidation of the thiols leads to the formation of disulfide bonds, which leave the surface of the QDs unpassivated. These uncoated nanoparticles oxidize in solution and aggregate due to high van der Waals forces (reproduced from reference 14 with permission).

In addition to the aforementioned techniques, layer-by-layer deposition of polyelectrolytes on particle surfaces for coating colloids, microparticles and gold nanoparticles has been previously demonstrated. This technique entails electrostatically depositing layers of oppositely charged polyelectrolytes on functionalized particle surfaces, leading to the assembly of multi-layered structures (Fig 3.4). Each step in the deposition cycle is characterized by charge reversal due to the overcompensation of charge neutralization, characteristic of electrostatic layer-by-layer assembly systems. This is a simple and versatile technique that enables integration of several functionalities, but was not used to modify QDs at the time this research was initiated, likely due to the
small size, high surface curvature and difficult surface chemistries involved in QD manipulation, making it tedious and time-consuming.

Herein we (i) optimized a method for exchanging the QD hydrophobic ligands to make the modified nanoparticles miscible with water, (ii) developed surface coating techniques using layer by layer deposition of polymers on QDs to tailor nanoparticle surface chemistries, (iii) integrated the QDs into ordered heterostructures to demonstrate their use as building blocks, and (iv) tested the robustness of the technique by integrating QDs and polyelectrolytes of varying compositions. This technique can enable ready assembly of multi-layered structures with diverse chemical properties that can be integrated with biological systems.

Figure 3.3 CdSe-ZnS QD stabilization by silanization. (a) Silane-containing thiols displace TOPO from the nanoparticle surface. (b) Silanes are polymerized to form primary siloxane shells. (c) Additional silanes with versatile surface chemistries are subsequently incorporated and cross-linked with the primary siloxane core (reproduced from reference 22 with permission).
Trioctylphosphine oxide (TOPO)
Mercaptoacetic Acid (MAA)
Polyallyl Amine (PAA)
Polyvinyl Sulfonic Acid (PVSA)

**Figure 3.4** Layer by layer deposition of polyelectrolytes on QD surfaces. Hydrophobic TOPO is displaced using MAA, which renders the QDs water miscible and anionic. These QDs are subsequently coated with cationic and anionic polymers, resulting in the assembly of hybrid nanocomplexes (reproduced from reference 15 with permission).
3.2 Specific Aims

Optimize CdSe-ZnS Modification Protocol

- Select TOPO-exchange ligand for passivating CdSe-ZnS QD surfaces thereby enabling suspension in aqueous solutions.
- Determine the effect of temperature, resuspension buffer and purification techniques on the optical properties and miscibility of the modified QDs.

Demonstrate layer-by-layer deposition of polyelectrolytes on QD surfaces to assemble multi-layered structures.

- Prepare and characterize single- and multi-layer structures with alternating charges
- Determine the effect of polymer coating on the optical properties and morphology of the QD.

Manipulate QD-polymer hybrids

- Assemble bi-layers of oppositely charged QDs on patterned substrates to produce ordered structures.
- Test the reproducibility and fidelity of electrostatically assembled structures.

Test Versatility of the Technique

- Investigate robustness of the coating technique by integrating inorganic cores and organic polyelectrolytes of varying composition.
- Assemble and characterize bi-layers of oppositely charged QDs on patterned substrates.
3.3 Materials & Methods

3.3.1 Experimental setup

Materials
CdS-ZnS QDs were purchased from Evident Technologies. Polyelectrolytes were purchased from Sigma Aldrich and Fluka Chemicals. All chemicals were purchased from Sigma Aldrich at reagent grade.

Characterizing Physical Properties
The absorption spectra of undiluted particles were obtained using a UV/Vis spectrometer (Beckman Coulter, DU 800). Surface charge of the polyelectrolyte coated QDs suspended in 50 mM NaCl in deionized water was determined using the Smoluchowski method (ZetaPals Analyzer).

Substrate Patterning with HA
The hyaluronic acid (HA) patterned glass slides were prepared as described elsewhere. Polydimethylsiloxane (PDMS) stamps were prepared by casting against silicon masters fabricated using photolithography. The stamps and the glass surfaces were both plasma cleaned for 2 mins (model PDC-001, Harrick Scientific Inc.), the slides were spin coated (model CB 15, Headaway Research, Inc.) with 5 mg/mL of HA and brought into conformal contact with the PDMS stamp. The HA was allowed to evaporate overnight before the stamp was peeled off and freshly exposed patterned surfaces were washed three times by immersing in deionized water. A thin film of QDs-PAA (cationic) was deposited on the substrate for 30 minutes and excess QDs were removed by washing the substrate with deionized water. A subsequent layer of QDs-MAA (anionic) was similarly deposited on QDs-PAA coated surfaces, and the sample washed and allowed to air dry.

Microscopic Characterization
Fluorescence images were obtained using an Olympus microscope (IX51) with GFP (ex 450/40, em 525/50) and TRITC (ex 535/50, em 610/75) filters using SimplePCI software. Fluorescence was quantified using Scion Image software. The AFM was operated in the tapping mode at a scan rate of 0.5 Hz using 300 kHz (MikcoMasch) tips. Images were taken on a NanoscopeIV (Digital Instruments) and data was manipulated using Nanoscope IV (version 6) software (Veeco Instruments Inc.).
3.3.2 Experimental design: CdSe-ZnS QD Modification

Optimizing QD Ligand Exchange Protocol

1. Select surface modification ligand (MAA, MEA, MEtOH, Cystine)

2. Determine optimal modification temperature (30 °C, 50 °C, 80 °C)

3. Test different resuspension buffers of varying pH (2 – 12) and salt concentrations

4. Optimize secondary purification of QD-ligand from organic impurities

5. Select optimal conditions

Optimizing QD polymer coating Protocol

1. Determine effective concentrations and purification protocols for cationic polymer coated QD-ligand

2. Test coating with bPEI of varying molecular weights (0.7, 2, 10, 25 & 75 kDa)

3. Determine effective concentrations and purification protocols for anionic polymer coated QD-polymer
Integrating Modified QDs into Devices

1. Coat CdSe-ZnS QDs with cationic PAA and anionic PVSA
2. Characterize QD properties (surface charge, absorbance, morphology)
3. Test electrostatic deposition & device assembly
4. Repeat for GaN QDs to test robustness of the technique
3.4 Results & Discussion

3.4.1: Optimizing CdSe-ZnS Modification Protocol
Surface ligand exchange of TOPO capped CdSe-ZnS QDs with different mercapto-containing molecules was investigated. Ligand exchange was optimized by varying the temperature, pH of the resuspension buffer, and the modification protocol. The effects of varying modification parameters on the ease of suspension in aqueous solutions and on the fluorescence properties of the modified QDs were characterized.

Selecting ligand for QD surface passivation
The first step in making QDs water-miscible was to exchange the bulky, hydrophilic TOPO group with another ligand that is: (i) strongly nucleophilic to coordinate with the dangling surface metal cations, (ii) hydrophilic to enable suspension in aqueous environments, and (iii) self-repellent to prevent aggregation of the QD in solution. For this exchange, thiol-containing ligands were tested since the sulphydryl is strongly nucleophilic, and such ligands conjugated with different pendant functionalities are readily available. Ligands tested included mercapto acetic acid (MAA), mercapto ethylamine (MEA), mercapto ethanol (MEtOH) and cysteine (Cys).

Modification of hydrophobic TOPO capped CdSe-ZnS QDs with MAA, MEA, MEtOH and Cys was carried out, and stability in aqueous solutions was investigated. QDs were modified by suspending in chloroform, mixing with ligands reconstituted in DMSO (if necessary), sonicating at 80 °C, precipitating with ethanol, and resuspending in deionized water. The ease with which the modified QDs resuspended in water, and the intensity of their fluorescence when observed under a UV lamp, were used as indicators of QD stability in the aqueous environments (Table 3.1). QDs treated with MAA were the easiest to resuspend, showed the least amount of aggregation, and demonstrated the brightest fluorescence of all modified QDs. MEA treated QDs also resuspended easily, but were not as brightly fluorescent as MAA-treated QDs, and settled out of solution a few hours post-treatment. MEtOH and Cys treated QDs did not resuspend but remained aggregated and demonstrated very limited fluorescence.

The difference in the ease of resuspension and the optical properties may be attributed to both, the inherent properties of the QDs, and the surface passivation ability of the exchange ligands, which depends on the chemical functionalities of their pendant chains. QDs suspended in solution are subject to both attractive and repulsive forces, and whether they aggregate or remain finely dispersed depends on which forces dominate\(^1,^5\). Their small sizes leads to the development of large van der Waals forces, causing hydrophobic attraction between the QDs. However, the surfaces of the nanoparticles are highly negatively charged due to adsorption of hydroxyl molecules to the dangling bonds of the surface metal cations (Fig 2.1a, Section 2.1). The presence of the polar/charged pendant chains of the thiol-containing ligand, or the bulky organic side group of TOPO lead to electrostatic or steric repulsion between the suspended QDs, thus facilitating dispersion in the solvent. If the pendant chains are not strongly charged, as in the case of
MEtOH and Cys, the modified QDs do not have enough repulsion between them to enable dispersion in water, and therefore aggregate. However, if the ligands are functionalized with highly charged moieties, as are MEA and MAA, there is sufficient repulsion between nanoparticles to enable dispersion in aqueous solutions. Therefore MAA and MEA treated QDs resuspend in water, while MEtOH and Cys treated QDs aggregate. Furthermore, MAA is anionic which may enhance the inherent negative charge of the QD and enable the modified QD to remain suspended in aqueous solutions. MEA is positively charged, which may compete with the inherent negative charge of the QD and therefore produce modified QD that are not as stable as those treated with MAA.

<table>
<thead>
<tr>
<th>Surface Ligand</th>
<th>Suspension</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>MAA</td>
<td>clear</td>
<td>high</td>
</tr>
<tr>
<td>MEA</td>
<td>clear</td>
<td>medium</td>
</tr>
<tr>
<td>MEtOH</td>
<td>aggregated</td>
<td>low</td>
</tr>
<tr>
<td>Cystiene</td>
<td>aggregated</td>
<td>low</td>
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Table 3.1 Testing different thiol containing ligands as stabilizers for QD. MAA stabilized QDs are the easiest to resuspend, and the brightest in solution, when compared to QDs treated with MEA, MEtOH and cysteine.

To test the differences in surface charges of MAA and MEA treated QDs, their electrophoretic mobilities were measured over a range of values between pH 2 and 7 (Fig 3.5). MAA treated QDs were predominantly negative over the entire range, while MEA treated QDs went from being positively charged to negatively charged at an approximate isoelectric point of 5.5. Since the pH of the resuspending deionized water was approximately 5.0, QD-MAA are expected to be highly negatively charged (-40 mV), while QD-MEA are only slightly positively charged (10 mV). This further demonstrates that the difference in stability of the MAA and MEA treated QDs was due to differences in electrostatic surface potentials. Based on the data presented, MAA was selected as the TOPO displacement ligand for all subsequent modifications.
Figure 3.5 Surface zeta potentials of MAA and MEA treated QDs over a range of pH values. MAA passivated QDs are highly negative at all pH values above 3, while MEA passivated QDs undergo charge inversion at approximately pH 5.5. (Error bars between 2 – 7 mV)

Optimizing ligand exchange

Once the exchange ligand was selected, the exchange protocol was optimized to allow assembly of stable, finely dispersed QDs. The first step was to vary the temperature of the sonicating water bath to determine its effect on the dispersion and fluorescence of the modified QDs (Table 3.2). QDs modified at higher temperatures (80 °C) were more readily dispersed, and had brighter fluorescence than those modified at lower temperatures (50 and 30 °C). Therefore higher temperatures are better for surface ligand exchange reactions. Since association of TOPO with the QD is a labile equilibrium reaction, increasing the temperature likely increases the rate at which the ligands dissociate/associate with the surface. However, once TOPO dissociates, it is likely that MAA associates with the QDs instead of TOPO, since the former is available in excess and has a higher affinity for the metal cation than TOPO. Therefore increasing the temperature shifts the equilibrium towards MAA binding to the QD surface, and all subsequent ligand exchange reactions were carried out at 80 °C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Suspension</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>aggregated</td>
<td>low</td>
</tr>
<tr>
<td>50</td>
<td>clumpy</td>
<td>medium</td>
</tr>
<tr>
<td>80</td>
<td>clear</td>
<td>bright</td>
</tr>
</tbody>
</table>

Table 3.2 Investigating the effect of temperature on QD ligand exchange. QDs modified at high temperatures (80 °C) are easier to resuspend in solution and have brighter fluorescence than those modified at low and moderate temperatures (30 and 50 °C).
The second step in optimizing the exchange protocol was to vary the pH values of the QD-MAA resuspension buffers. Excess MAA and TOPO were removed by precipitating the ligand exchange solution with ethanol, centrifuging to pellet the displaced QD-MAA, and removing the supernatant. The pellet was subsequently resuspended in pH adjusted water, and the fluorescence of the solution characterized (Fig 3.6). The results demonstrate that increasing pH results in enhancement of fluorescence and blue-shifting of the emission peak. This effect is very pronounced at low pH values, and becomes less significant at higher pH values; the fluorescence reaches 90% of its maximum value when QD-MAA are suspended in pH 9 solution. Addition of small amounts of salt to the pH adjusted water further facilitated QD-MAA resuspension, without affecting their fluorescence. Therefore, high pH buffers prepared at low concentrations were tested as resuspension solutions. Tris base buffer (50 mM, pH 9) demonstrated excellent resuspension and bright fluorescence of the modified QD-MAA, and was therefore selected as the resuspension buffer and used for all subsequent modifications.

![Figure 3.6](image)

**Figure 3.6** Varying pH of the resuspension buffer of MAA modified QDs. QDs resuspended in high pH solutions (> 9) demonstrate significantly brighter fluorescence.

The final step in optimizing the ligand exchange was purification of the QD-MAA from residual organics. If this is not performed, too many organic impurities remain in the solution causing it to turn cloudy. If the QD-MAA solution is purified too many times then the MAA ligand is stripped from the QD surface causing the QDs to aggregate. Since both of these can interfere with subsequent QD coating with polyelectrolytes, it is important to optimize the separation procedure. The QD-MAA suspended in tris buffer were precipitated with twice the volume equivalent of isopropanol (polarity index 3.9). Ethanol does not precipitate the QD-MAA from tris solution since it is highly polar (polarity index 5.2), and therefore not strong enough to displace QD-MAA. The volume of isopropanol is critical, since too little does not displace the QD-MAA, and too much results in aggregation of the QDs, likely due to stripping of MAA from the QD surface. The displaced QDs were centrifuged briefly, the supernatant removed, and QD-MAA
resuspended in tris-base buffer. The purified QDs were subsequently modified by coating with cationic polymers.

3.4.2 Demonstrating layer-by-layer deposition of polyelectrolytes on QD surfaces to assemble multi-layered structures

The protocol\textsuperscript{26} for layer-by-layer (LbL) coating of CdSe-ZnS QDs (3.2 nm, green) was optimized and surface charge, optical properties and morphology of the complex were characterized.

Optimizing polymer deposition technique

Preparing disperse, individually wrapped, cationic polymer coated QD-MAA required optimization of the coating and purification procedures. The first step was to determine optimal concentrations of the QD-MAA and cationic polymers. QD-MAA were suspended in aqueous solutions at 30 – 50 µg/mL, as determined using optical measurements (Appendix I). More concentrated QD solutions resulted in aggregation when added to the cationic polymer, and lowering the QD concentration increased the purification time. High concentrations of the polyelectrolyte were necessary since it must be in excess to over-compensate for the anionic QD-MAA. Branched polyethyleneimine (bPEI, 25 kDa) was used coating polymer, and solutions were maintained at 10 mg/mL; lower concentrations resulted in QD clumping while higher concentrations made some polymer solutions very viscous.

Next, QD-MAA were rapidly mixed with 10 mg/mL polymer solution. It was important to add the QD-MAA to the polymer so that the latter is always in excess, which facilitated individual coating of the particles. The QD-MAA/cationic polymer solutions were vigorously vortexed, and incubated on benchtop for 20 minutes to ensure complete electrostatic association between the QD and the polyelectrolyte. The excess polymer was removed using Amicon filters (100 kDa cutoff), according to manufacturer’s protocols. Several other purification methods were tested (centrifugation, dialysis, microspin columns and gel filtration) but none were successful as they caused aggregation of the QDs.

The effect of varying molecular weight on the ability of the polymer to coat QDs was also investigated. Cationic bPEI polymers of different molecular weights (0.7, 2, 10, 25 and 75 kDa) were used to coat QD-MAA surfaces, and the ease of resuspension and fluorescence were characterized (Table 3.3). QD-MAA coated with bPEI with a molecular weight of 10 kDa or greater did not aggregate after purification and exhibited bright fluorescence. However, QD-MAA coated with bPEI less than 10 kDa did not resuspend well after purification, and had very weak fluorescence. The data demonstrates that larger molecular weight polymers are more efficient at coating and stabilizing QD-MAA surfaces than shorter chain polymers. Since individual electrostatic interactions are weak, increasing the chain length may facilitate multiple interactions thereby reinforcing the electrostatic interactions. This can make it easier for high molecular weight polymers to overcompensate for charge neutralization, mediating assembly of positively charged polymer coated QD that are stable in water\textsuperscript{27}. For all subsequent modifications, polymers with molecular weights between 10 to 70 kDa were used.
Table 3.3 Effect of polymer chain length on the coating QD-MAA. Polycationic bPEI coated QD-MAA are stable in solution when coated with high molecular weight polymers (> 2 kDa).

<table>
<thead>
<tr>
<th>MW bPEI</th>
<th>Suspension</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>700</td>
<td>aggregated</td>
<td>bright</td>
</tr>
<tr>
<td>2,000</td>
<td>aggregated</td>
<td>bright</td>
</tr>
<tr>
<td>10,000</td>
<td>clear</td>
<td>bright</td>
</tr>
<tr>
<td>25,000</td>
<td>clear</td>
<td>bright</td>
</tr>
<tr>
<td>70,000</td>
<td>clear</td>
<td>bright</td>
</tr>
</tbody>
</table>

The final step was to test assembly of multiple layers. The QD concentrations were further reduced to keep the solution from turning turbid due to aggregation and cross-linking of highly cationic QDs and anionic polymers. Purified QD-bPEI were suspended at a concentration of 5 μg/mL. These were added to 10 mg/mL anionic PVSA polymer. It is important to maintain the anionic polymers in significant excess, to ensure that the cationic QDs are individually coated and to minimize cross-linking. The cationic QD solution was mixed slowly with the anionic polymers while vortexing to facilitate electrostatic interactions. The solution was incubated on the bench top for 20 minutes, and excess anion was removed by purifying through Amicon filter (100 kDa), as described previously. All subsequent QD modifications were made according to this optimized procedure, unless stated otherwise.

Characterizing properties of QD-polymer complexes

Once the procedure for polymer coating the QDs was optimized, surface charge, optical properties and morphology of the modified nanoparticles were analyzed. QDs were sequentially coated with cationic polyallylamine (PAA, 19 kDa) and polyvinylsulfonic acid (PVSA, 25 kDa). The surface charge of modified QDs suspended in 50 mM NaCl solutions was determined by measuring their electrophoretic mobility (Fig 3.7). Zeta potentials of two different sized CdSe-ZnS QD, green 3.4 nm (solid bars) and red 7.2 nm (hatched bars), were measured. The zeta potential of bare mercaptoacetic acid modified QDs are -29.5 mV (green) and -37.5 mV (red), likely due to the presence of anionic MAA and hydroxyl molecules adsorbed to the dangling surface zinc ions. The PAA coated QDs are 23.6 mV (green) and 31.6 mV (red), while the PVSA coated ones are -53.8 mV (green) and -84 mV (red). Both QD complexes show characteristic patterns of surface charge reversals caused by excess polymer deposition due to overcompensation of charge neutralization. These values are in agreement with those of other polyelectrolyte-coated colloidal systems found in the literature. The absolute values of surface charges appear to be higher for the larger red (7.2 nm) QDs, particularly for the PVSA coat. This may be due to their lower surface curvature which allows the stiff polymer chains to fold around the nanoparticles more easily, and therefore facilitate polyelectrolyte adsorption. The data demonstrate that polyelectrolyte deposition on QDs was successful.
Next, the effect of the polymer coating on the absorption properties of the QDs was investigated. The absorption spectra of red and blue QD-MAA, -PAA, -PVSA and QD-TOPO (in DMSO) were obtained (Fig 3.8). For both red and green QDs, the positions of the first observable peak (607 nm and 508 nm, respectively) shift slightly after MAA exchange, and remain blue shifted after polyelectrolyte adsorption. As expected, the dominant shifts for both modifications occur after MAA exchange, as previously reported\textsuperscript{11,12}. The shifts are attributed to the differences in ligand binding affinities; the thiol-zinc bond is stronger than the TOPO-zinc bond, and may cause distortion of the energy levels, leading to reorganization of the electronic density and increase in the confinement energy, thus resulting in a slight blue-shift. While photooxidation may also cause a blue-shift, it is unlikely to be the cause since the peak positions and QD optical densities remained constant for several days post modification. Other than peak positions shifts, the overall shape of the spectra was unchanged; there is no observable peak broadening which suggests that the structure of the QDs is preserved throughout the several processing steps and there is no clumping or fragmentation. Therefore the polyelectrolyte deposition does not appear to be detrimental to the absorbance properties or size homogeneity of the QDs.
Figure 3.8: Absorbance of red (light) and green (heavy) polymer coated QDs is not affected by coating with polyelectrolytes. The shapes of the absorbance spectra remain the same, with slight blue shift in of the first absorbance peak, attributed to the displacement of TOPO by MAA (reproduced with permission from reference 33).

The size and morphology of the modified QDs were further investigated using transmission electron microscopy (TEM) to visualize red QDs modified with MAA, PAA and PVSA coatings (Figure 3.9). The dense cores of the nanoparticles are approximately 7nm in diameter, and their sizes and shapes do not vary after polyelectrolyte deposition. This reinforces that the QDs do not photooxidize or fragment due to polymer coating, and that the blue shifts in the absorption spectra (Figure 3.8) are likely due to tighter thiol-zinc bonding. The QDs do not aggregate but remain distinct, which indicates that the polyelectrolytes form layers around individual particles. While the deposited layers are too thin to be visualized directly\textsuperscript{32}, their presence is supported by the existence of a halo around the PVSA coated QDs (Figure 3.9c) which is not visible in the MAA treated QDs (Figure 3.9a). There is also an apparent increase in the interparticle spacing as additional polymer layers are deposited on the QD surface. Therefore the overall morphology of the nanoparticles is preserved, and they appear to be individually coated by the polymers.
Figure 3.9 TEM images of (a) MAA capped, (b) PAA, and (c) PVSA coated red QDs. The morphology and size of the QDs is not affected by polyelectrolyte deposition. The QDs do not aggregate but remain individually dispersed. (scale bar 25 nm) (reproduced with permission from reference 33).

### 3.4.3 Manipulating QD-polymer hybrids

The ability of polymer-coated QDs to selectively assemble into hybrid heterostructures was investigated. The specificity of charge interactions was determined by quantifying the fluorescence of anionic (QD-MAA) and cationic (QD-PDMC) nanoparticles deposited on positive and negative substrates (Fig 3.10). Anionic and cationic substrates were prepared by spin coating hyaluronic acid (HA, 1MDa) and bPEI (70 kDa) on plasma cleaned glass surfaces, incubating overnight, and removing the excess, unbound polymer by rinsing three times with deionized water. The QD-layers were imaged using fluorescence microscopy, and the fluorescence intensity was quantified using Scion Image software (Fig 3.10). Approximately 10-fold more cationic red QDs deposited on anionic substrates than did anionic red QDs (40 vs. 4), and 6-fold more cationic green QDs deposited on anionic substrates than did anionic green QDs (115 vs. 20). Similarly, 9-fold more anionic red QDs deposited on cationic substrates than did cationic red QDs.
(180 vs. 20), and 3.5-fold more anionic green QDs deposited on cationic substrates than did cationic green QDs (170 vs. 50). Therefore both anionic and cationic, red and green QDs preferentially deposited on oppositely charged substrates, with low levels of non-specific binding.

![Bar chart showing fluorescence intensity (A.U.) for QDs on HA and bPEI substrates.](image)

**Figure 3.10** Characterizing specificity of binding of polyelectrolyte coated QDs to oppositely charged substrates. Red and green, cationic and anionic QDs show preferential adsorption to oppositely charged substrates, with very low levels of non-specific binding.

Anionic (QD-MAA, green) and cationic (QD-PAA, red) QDs were electrostatically adsorbed onto anionic polymer (HA) patterned substrates, and visualized using fluorescence microscopy (Fig 3.11). Patterned substrates were prepared by spin coating HA on plasma cleaned glass substrates, bringing them in conformal contact with polydimethyl siloxane (PDMS) moulds, allowing HA to evaporate overnight, peeling off the stamp and washing off the excess, unbound polymer prior to QD deposition. Thin films of cationic and anionic QDs were sequentially deposited on the anionic, HA patterned substrates for 30 mins and excess, unbound QDs were washed off by rinsing three times with deionized water. Glass substrates with three layers were imaged: (i) patterned (anionic) HA layer adsorbed on glass, (ii) red (cationic) QD-PAA layer electrostatically associated with the HA/glass substrate, and (iii) green (anionic) QD-MAA layer assembled on the positive PAA-QD/HA/glass substrate. Fluorescence images of the patterned bilayer images show excellent overlap between the green anionic (Fig. 3.11a) and red cationic (Fig. 3.11b) QD layers. The fluorescent areas are where QD binding occurred, and the dark squares correspond to the non-adhesive glass surface. The modified QDs selectively adhere to oppositely charged substrates but not to the untreated glass, and do not wash off even after several rinses. The electrostatic forces are strong enough to enable QD assembly on oppositely charged polyelectrolyte (HA) and QD surfaces, even after washing. The films appear smooth and show conformal coverage with very little clumping or pattern distortion, even after drying. The pattern fidelity is
very high: the features have sharp edges, the layers are precisely aligned, and the patterns are reproducible over large areas. The data demonstrates that the properties of the QDs can be consistently and reproducibly controlled, and that the modified QDs are stable building blocks for integrating into heterostructured complexes and devices.

Figure 3.11 Electrostatic deposition of polymer-coated cationic (green) and anionic (red) QD bilayers on charged patterned substrates visualized through (a) green (FITC) and (b) red (TRITC) filters. Charged QDs specifically bind to oppositely charged patterns (fluorescent regions), and not to the uncharged substrate (dark regions) (reproduced with permission from reference 33).

Feature integrity and the height profiles were further characterized by imaging the QD bilayers using atomic force microscopy (AFM, Fig. 3.12). The features appear to be the same size (3μm squares) with an aspect ratio close to unity. There is some non-specific adsorption on the untreated glass surface, which could be due to the residual debris from the PDMS mold or the polyelectrolytes. The height difference between the bilayer and the non-adhering regions, as indicated by the height profile (inset), is approximately 15 nm. The HA layer is approximately 3 - 6 nm in height while the PAA and MAA modified QDs are approximately 7 nm and 3 nm respectively, which implies that monolayers of QDs may be deposited on the surface.
Figure 3.12 Electrostatic deposition of polymer-coated cationic (green) and anionic (red) QD bilayers on charged patterned substrates visualized using atomic force microscopy. Patterns demonstrate high fidelity and smooth coverage. Inset measures the height profile across the patterned feature, along the dotted line (reproduced with permission from reference 33).

3.4.4 Testing Versatility of the Technique

The robustness of the layer-by-layer surface deposition technique was tested by integrating inorganic QDs and polymers with different chemical compositions. Anionic colloidal GaN QDs (3nm, blue) were produced in-lab without any surface passivating layer, and were coated with cationic polydiallyldimethylammonium chloride (PDMC, 15 kDa) and anionic polyacrylic acid (PAAc, 16 kDa). The same coating methods optimized for PAA coating of CdSe-ZnS QDs were used. Briefly, QDs were introduced into the cationic polymer solutions, incubated on benchtop for 30 mins, and purified from the excess unbound polymer by spinning twice through an Amicon filter (100 kDa). The surface charges of the nanoparticles were characterized over a range of pH values (Fig. 3.13). Both uncoated (green) and anionic PAAc coated (red) GaN QDs are negatively charged at all pH values above 4, while cationic PDMC coated QDs are positively charged for all values tested (up to pH 12). This characteristic surface charge reversal is due to excess deposition of polyelectrolytes, due to overcompensation of charge reversal, as discussed previously. The data demonstrate that polyelectrolyte deposition on GaN QD surfaces was also successful.
Figure 3.13 Zeta potentials of polymer-coated GaN QDs over a range of pH values. Uncoated (UC) and polyanion coated (PAAc) QDs are highly negatively charged above pH values of 4. Polycation coated (PDMC) QDs are highly positively charged over the entire range tested. The curves were drawn in by hand to guide the eye.

The controlled integration of polymer-coated GaN QDs into ordered arrays was also investigated by adsorbing GaN nanoparticles on oppositely charge patterned substrates. Patterned substrates were prepared by spin coating HA on plasma cleaned glass substrates, bringing them in conformal contact with polydimethyl siloxane (PDMS) moulds, allowing HA to evaporate overnight, peeling off the stamp and washing off the excess, unbound polymer prior to QD deposition. Thin films of cationic GaN QDs were deposited on the anionic, HA patterned substrates for 30 mins and excess, unbound QDs washed off by rinsing three times with deionized water. Glass substrates with two layers were imaged (Fig 3.14): patterned (anionic) HA layer adsorbed on glass, and blue (cationic) GaN QD-PDMC layer electrostatically associated with the HA/glass substrate. The fluorescent blue lines display where QD binding occurred, and the dark lines correspond to the non-adhesive glass surface. The modified QDs selectively adhere to oppositely charged substrates but not to the untreated glass, and do not wash off even after several rinses. The electrostatic forces are strong enough to enable QD assembly on oppositely charged polyelectrolyte (HA), even after washing. The films appear smooth and show conformal coverage with very little clumping or pattern distortion, even after drying. The pattern fidelity is very high: the features have sharp edges, the layers are precisely aligned, and the patterns are reproducible over large areas. Therefore polymer coated nanoparticles can be electrostatically manipulated, independent of their inorganic core.
Figure 3.14 Electrostatic deposition of cationic polymer-coated GaN QDs on oppositely charged HA patterned substrates. Cationic QDs bind specifically to the anionic HA patterns (blue fluorescence) and not to the uncharged substrate (dark regions) forming features with high pattern fidelity (scale bar 50 μm).

The data demonstrates that layer-by-layer deposition of polyelectrolytes on QD surfaces enables control of their surface chemistries, without affecting the morphology or optical properties of the inorganic core. These polymer-coated QDs were stable enough to electrostatically assemble into heterostructures, and the technique is robust enough to enable integration of QD and polymers, independent of the chemical compositions.
3.5 Summary & Conclusions

3.5.1 Optimized CdSe-ZnS Modification Protocol

1. QD-TOPO were displaced with 10 x volume of ethanol and centrifuged at 10x kg for 2 mins (manufacturer’s protocol).
2. Supernatant was discarded and QD-TOPO resuspended in 10x (original volumes) of chloroform (manufacturer’s protocol).
3. Equivolume of MAA was added to the QD-TOPO solution, and sonicated at 80 °C for 30s.
4. QD-MAA were precipitated using equivolume ethanol and centrifuged at 10x kg for 2 mins.
5. Supernatant was discarded and QD-MAA resuspended in 10 x (original volume) 50 mM tris-base buffer (pH 9).
6. QD-MAA were precipitated using 2x volume of isopropanol and centrifuged at 10x kg for 2 mins.
7. Supernatant was discarded and QD-MAA were suspended in 10x (original volume) 50 mM tris-base buffer (pH 9).

3.5.2 Demonstrating layer-by-layer deposition of polyelectrolytes on QD surfaces to assemble multi-layered structures

- Optimizing polymer deposition technique
  - QD-MAA were diluted to 30 – 50 mg/mL, added to equivolume 10 mg/mL cationic polymer solution (10 – 70 kDa), and incubated for 20 mins.
  - QD-polymer were purified twice using Amicon columns (100 kDa).
  - Cationic QD-polymer were diluted to 5 mg/mL and added to 10 mg/mL anionic polymer (10 – 70 kDa), and incubated on benchtop for 20 mins.
  - QD-polymer were purified twice using Amicon columns (100 kDa).
- Layer by layer deposition of oppositely charged polyelectrolytes on QD surface was successfully demonstrated by characteristic surface charge reversals.
  - Coated inorganic QD were not fragmented or degraded and retained their optical properties and morphology.
  - QDs were coated individually and did not form aggregates or clumps.
- Electrostatic assembly of charged QDs and oppositely substrates was demonstrated.
  - Charged QDs were preferentially deposited on oppositely charged substrates, with low levels of non-specific binding.
  - Adsorption to charged, patterned substrates produced ordered, heterostructured templates with high pattern fidelity and stability.
- Coating technique was robust enough to combine inorganic cores and organic polyelectrolytes of varying compositions that could be integrated into ordered nanostructures.
3.6 References


Chapter 4: Effect of Surface Functionalization on Biocompatibility of QDs
Chapter Synopsis

Objective
The aim of this section was to investigate the biocompatibility of (i) uncoated quantum dots (QD), (ii) single-layered QD-cation and (iii) multi-layered QD-anion complexes, and determine the effect of varying individual core and polymer chemical compositions on the cellular interactions of the hybrid complexes.

Hypothesis
Integration of different materials may allow manipulation of the cellular interactions of the QD-polymer hybrids, enabling semi-rational design of complexes tailored for specific bioapplications.

Rationale
Novel optoelectronic properties of QDs make them ideal candidates for bioprobes, but their surfaces must be functionalized prior to biointegration. Layer-by-layer coating of nanoparticles allows fast, easy and versatile surface modification of the nanoparticles, while preserving their core properties. Integration of polyelectrolytes previously used for biological applications may allow control of QD complex properties and enable design of hybrids with specific properties.

Results
Incubation of uncoated nanoparticles including anionic CdSe-ZnS and GaN QDs, and polystyrene nanoparticles with epithelial Cos-7 cells revealed that the nanoparticles are not inherently cytotoxic, but demonstrate different degrees of cellular uptake. Coating these nanoparticles with different cationic polymers forms complexes with varying biocompatibilities; branched polyethyleneimine (bPEI), polyallylamine (PAA), polydiallyldimethyl ammonium chloride (PDMC), polylsyline (pLys) and polyarginine (pArg) coated nanoparticles are highly cytotoxic, diethylaminoethyl dextran (dextran) coated nanoparticles demonstrate dose dependent toxicity, and quaternized hydroxyethylthioxy cellulose (cellulose) coated nanoparticles are non-toxic.

Multi-layered heterostructures were assembled by coating cationic QD-dextran cores with different anionic polymers, and their biocompatibility was determined by incubating with COS-7 cells. The complexes demonstrated varying levels of cytotoxicity that depended on the chemical compositions of the anionic polymers; polyvinylsulfonic acid (PVSA) and polyacrylic acid (PAAc) containing complexes were highly cytotoxic and caused extensive distortion of cellular morphologies, while dextran sulfate (DS) containing complexes had limited cytotoxicity and low cellular uptake. Further investigations using QD-cation-DS multi-layered complexes with different cations revealed that biocompatibility of heterostructures also depended on the chemical composition of the integrated cations; PAA containing complexes remained highly cytotoxic but bPEI and PDMC containing complexes demonstrated reduced toxicity when coated with DS, as compared to their corresponding single-layer complexes.
Conclusions

The overall biocompatibility of the multi-layered complexes depends primarily on the chemical composition of all the integrated polymers, and is relatively independent of the nanoparticle core. Therefore, rationally designed complexes with controlled properties may be prepared.
4.1 Background
Commercially available hydrophobic quantum dots (QDs) with excellent optical properties can be made water miscible by exchanging the bulky, organic surface passivating ligands with hydrophilic mercaptoacetic acid (MAA), producing anionic QD-MAA cores (Chapter 3). Subsequent deposition of oppositely charged polyelectrolytes on the QD-MAA cores results in the assembly of heterostructured complexes, which maintain the structural integrity and optical properties of the inorganic core, while enabling surface functionalization of the complex. The chemical compositions of both, the nanoparticle core and the coating polymers may be varied, thus enabling the assembly of complexes with diverse chemical functionalities that are tailored for specific bioapplications. The chemical structures, properties, and biointegration of several nanoparticle cores and cationic and anionic polyelectrolytes are discussed in the following section.

4.1.1 Nanoparticle Cores
High quality QDs with finely resolved emission spectra, stable fluorescence, and uniform morphology are exemplified by CdSe-ZnS nanocrystals\(^1\text{-}\text{4}\). However, their use in bioapplications raises concerns regarding the potential inherent cytotoxicity of the nanoparticles\(^5\text{-}\text{7}\). Several studies have demonstrated that uncapped CdSe or CdS QDs are prone to surface oxidation, as indicated by the red-shift of the optical properties, and deterioration of the crystallinity of the inorganic core\(^5\text{,}\text{8}\). Oxidation leads to the release of free cadmium ions in solution (Fig 4.1) whose cytotoxic effects have been extensively characterized\(^9\text{-}\text{10}\). Capping the QDs with a layer of ZnS greatly decreases the cytotoxic effects by limiting the release of cadmium ions, as indicated by preservation of the optical properties and morphology of the CdSe core\(^4\text{,}\text{6}\). In addition to the chemical toxicity, the nanoparticles may also demonstrate indiscriminate cytotoxicity due to their small size and high mass, which can cause perforation of the cell membranes and arrest of cellular functions by complexing with the organelles and proteins\(^5\text{,}\text{11}\).

Figure 4.1 Oxidation of the CdSe nanoparticle. The non-metal anions react to form oxides in the presence of an oxidizing agent, leaving behind free cadmium ions (Reproduced based on schematic in reference 5)
To test the inherent cytotoxicity of the QDs due to their composition and size, CdSe-ZnS (3 nm), GaN (3 nm) and polystyrene (PS, 20 nm) nanoparticles were used as the anionic nanoparticle cores. GaN nanoparticles are similar in size, morphology and structure to the CdSe-ZnS QDs: both are small, spherical, crystalline, anionic and composed of semiconductor materials. However, the gallium-nitride bond is stronger and not as easily hydrolyzed as the cadmium-selenide bond, therefore limiting the release of free metal cations in solution. In addition, free gallium ions are significantly less cytotoxic than cadmium ions. Therefore the use of GaN nanoparticles should mimic any indiscriminate, non-chemical cytotoxicity caused by CdSe-ZnS nanoparticles. The structure and morphology of the PS nanoparticles are very different from those of CdSe-ZnS QDs; they are larger, non-crystalline and composed of biologically inert organic materials. Their surfaces are coated with carboxylic acid moieties to mimic the anionic charge of the CdSe-ZnS QDs modified with MAA. These nanoparticles are known to have very low inherent cytotoxicities, and therefore their use should enable direct observation of the effects of nanoassembly of the polymers, on the cellular interactions of the complexes.

4.1.2 Materials for QD Coating

Cationic and anionic polyelectrolytes with different chemical structures (Appendix II) were used to coat the oppositely charged nanoparticle cores. Polymers selected for polyelectrolyte coating were (i) highly charged to facilitate electrostatic interactions with the oppositely charged nanoparticle-complexes, (ii) between 10 – 100 kDa in molecular weight so that they were large enough to strongly bind to the nanoparticle, but small enough to enable efficient separation of the unbound polymer from the complex (Section 3.4.2), (iii) commercially available and not requiring additional preparation for ease of modification, and (iv) sufficiently hydrophilic to enable complex miscibility with aqueous environments. Seven cations and three anions were selected, including synthetic and biologically derived polymers with high and low charge densities. Their structures, chemical properties, integration into layer-by-layer assemblies, and previous bioapplications are discussed below. Charge densities are expressed as the number of charges per monomer unit mass, and are inversely proportional to monomer molecular weight; for monomers with the same number of charges, higher molecular weight units have lower charge densities than lower molecular weight ones.

Cationic Polymers

The cationic polymers selected included three synthetic polymers, branched polyethylenimine (bPEI), polyallylamine (PAA) and poly diallyldimethyl ammonium chloride (PDMC), and four bio-derived polymers, polyllysine (pLys) and polyarginine (pArg) polypeptides, amine-derivatized diethylaminoethyl dextran (dextran), and quaternized hydroxyethylthoxy cellulose (cellulose). Their properties as discussed below, and structures are illustrated in Appendix II.

Cationic bPEI is randomly branched with primary, secondary and tertiary amines separated by ethyl moieties. When completely ionized, it has one charge per monomer unit (44 amu). Since it has the lowest molecular weight monomer, it has the highest
charge density of all polymers tested. PAA is a linear polymer with pendant methyl-
primary amine moieties grafted to the hydrocarbon backbone. It is completely ionized
with one charge per monomer unit (56 amu). PDMC is a stiff, linear polymer with
quaternary ammonium groups in rings that form part of the backbone of the polymer
chain. It is completely ionized, and has one charge per monomer unit (157 amu). The
linear polypeptides, pLys and pArg, have amide backbones, and primary amine and
guanidine moieties respectively in the pendant side chains. pLys has one charge per
monomer (129 amu), while pArg has two charges per monomer (157 amu); pArg has
approximately the same charge density as PDMC, while pLys has a lower charge density.
Dextran and cellulose are both linear, glucose based polymers modified with pendant
quaternized amines. They have one charge per monomer unit (424 and 539 amu,
respectively) and much lower charge densities than the other cationic polymers.
Therefore the polymers, in order of decreasing charge density, are bPEI, PAA,
PDMC/pArg, pLys, dextran and cellulose.

The cationic polymers selected were previously incorporated into electrostatic layer-by-
layer assemblies: bPEI was adsorbed onto poly-lactic-acid particles in. PAA was
deposited on PS colloid particles, PDMC was coated on gold nanoparticles, pLys was
deposited on condensed DNA particles, and pArg, dextran and cellulose were
deposited on anionic thin films. They formed stable, electrostatically assembled
structures with finely controlled surface properties that were stable in aqueous
environments.

The polymers were also used for cellular delivery of biological materials: bPEI, PAA,
PDMC, pLys, pArg and dextran were complexed with DNA and
delivered to cells, yielding varying levels of gene-product. There was a tradeoff between
the efficiency of delivery and invoked cytotoxicity; optimized bPEI complexes yielded
the highest levels of gene delivery, but also caused highest levels of cytotoxicity. Varying
the polymer to DNA mass ratios greatly affected the delivery properties; an initial
increase in the amount of polymer yielded significant enhancement in the amount of gene
expression with low levels of cytotoxicity, but continued increase in the amount of
polymer resulted in decreased gene expression and elevated levels of cytotoxicity. At
high polymer doses, the cationic polymers become increasingly cytotoxic; bPEI, PAA,
PDMC, pLys, pArg and dextran all demonstrated elevated cytotoxicity at high polymer
concentrations. Cytotoxicity also increased with the charge density of the polymers:
block-co-polymers of cationic bPEI and uncharged polyethylene glycol (PEG),
demonstrate progressively increasing cytotoxicity as the ratio of the charged bPEI to the
uncharged PEG increases. Changing the molecular architecture also affected the
cytotoxicity of the polymers; dendrimer analogues of PEI, branched PEI, and linear PEI
demonstrated progressively decreasing levels of gene transfection and cytotoxicity at the
equivalent polymer to DNA mass ratios, indicating that ordered, highly branched
structures are more cytotoxic than their linear, unstructured analogues. Therefore the
biocompatibility of the cationic polymer complexes depends on the chemical structure,
properties, and the effective dose of the polymers.
Several mechanisms may account for the elevated cytotoxicity levels of the different cationic polymers, including cell membrane perforation, endosomal rupture, and arrest of anionic cellular proteins. Cationic complexes attach to and puncture cell membranes, compromising their integrity and triggering cell death, as observed for amine-containing dendrimers. The small size and high positive charges facilitate binding of the complexes to the anionic cell membranes. Endocytosed cationic bPEI-containing complexes escape endosomal confinement by osmotic swelling and rupture; sequestering of hydrogen ions by the polymer leads to an excessive influx of hydrogen and chloride ions and water, leading to swelling and vesicle rupture. However, endosomal rupture also releases degradative enzymes into the cell cytoplasm, which can trigger cell death.

**Anionic Polymers**

Three anionic polymers were selected to coat the cationic QD-polymer complexes (Appendix II); synthetic polyvinylsulfonic acid (PVSA) and polyacrylic acid (PAAc), and bio-derived dextran sulfate (DS). PVSA and PAAc are linear polymers with pendant sulfonate and carboxylate groups grafted to their backbones. They have one anionic charge each per monomer unit (molecular weights 123 and 72, respectively), and have high charge densities. DS is a glucose based polymer with anionic sulfate grafted to the glucose ring backbone. It has three anionic charges per monomer repeat unit (molecular weight 351), and therefore has similar charge density as PVSA and PAAc. These materials have also been previously incorporated into layer-by-layer assemblies; PVSA was deposited on the surface of PAA coated CdSe-ZnS QDs, PAAc was adsorbed onto PAA coated PS particles, and DS was deposited on planar films. Delivery of anionic polymers to cells with compromised cellular membranes has resulted in cytotoxicity; DS added to lymphocytes caused gelatination of the cellular nuclei by extraction of histone proteins by the anionic polymer and complexing of cationic histone-DS with anionic DNA. The swelling pressure caused by these cross-linking gels results in the distortion of cellular morphology, and the rupture of the cells.
4.2 Specific Aims

Uncoated & Single Layer Systems

- Test the biocompatibility of CdSe-ZnS-MAA, GaN and PS-COOH nanoparticles.
- Investigate cellular interactions of cationic polymer-coated nanoparticle complexes.
  - Incorporate bPEI, PAA, PDMC, pLys, pArg, dextran and cellulose into single-layer complexes.
  - Characterize cationic QD complexes using zeta potentials.
  - Determine biocompatibility of cationic complexes.
- Investigate the effect of varying the core composition on biocompatibility of the complexes, by using CdSe-ZnS, GaN and PS nanoparticles as deposition platforms.

Multi-Layer Systems

- Investigate cellular interactions of anionic polymer-coated QD complexes.
  - Deposit PVSA, PAAc and DS polymers on suitable QD-cation core.
  - Characterize anionic QD complexes using zeta potentials.
  - Determine biocompatibility of anionic complexes.
- Determine the effect of varying cationic polymers on the properties of the multi-layered system.
  - Assemble QD-cation-anion multi-layered systems using bPEI, PAA, PDMC and dextran as cations.
  - Investigate the effect on biocompatibility
4.3 Materials & Methods

4.3.1 Experimental design

**QD-PE Modification**
CdSe-ZnS core-shell QD were modified with mercaptoacetic acid, and coated with cationic polymers, as described in section 3.4.1 and 3.4.2.

**QD-PE Cell Incubation**
Monkey kidney COS-7 cells maintained at 37 °C and 5% CO₂, were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B. Cells were seeded at a density of about 75,000 cells per well in a 24-well plate, and 30,000 cells per well in an 8-well collagen treated cover-glass chamber, and allowed to grow overnight. QD-polymer complexes were delivered to cells in serum-free medium for 2 hrs, and then replaced with fresh serum-supplemented medium.

**Cell viability quantification**
QD-polymer treated cells were washed with phosphobuffered saline (PBS), incubated on ice for 15 min with trypsin (2.5 mg/mL) and propidium iodide (PI, 5 μg/mL) supplemented-PBS, and analyzed using a FACScan (Becton Dickinson) flow cytometer, equipped with an argon ion laser. PI treated cells were detected using a PE/PI band-pass filter (585/42). The cells were appropriately gated by forward and side scatter. Normalized cell viability was determined as the percentage of viable cells, relative to untreated cells exposed to the same conditions.

**Zeta potential measurements**
Surface electrophoretic mobilities of hybrid complexes were determined using a zeta potential analyzer (ZetaPlus, Brookhaven) with a HeNe laser at 632 nm. Complexes were dispersed in 50 mM NaCl in deionized water, and measurements were repeated three times. The Smoluchowski method was used to calculate the zeta potentials.

**Fluorescence microscopy imaging**
Low magnification (40x) images were obtained using an inverted Olympus microscope (IX51) with GFP (ex 450/40, em 525/50), TRITC (ex 535/50, em 610/75) and DAPI (ex 360/40, em 460/50) filters, using SimplePCI software.
4.3.2 Experimental setup

*Testing biocompatibility of inherent nanoparticle cores*

Prepare CdSe-ZnS-MAA, GaN and PS-COOH nanoparticles and test biocompatibility

*Test biocompatibility of cationic nanoparticles: effect of varying polymer and core compositions*

1. Coat nanoparticles with bPEI, PAA, PDMC, pLys, pArg, dextran and cellulose

2a. CdSe-ZnS cores  
2b. GaN cores  
2c. PS cores

3. Characterize by determining zeta potentials

4. Deliver to cells and characterize cytotoxicity using visual assay

5. Determine cellular interactions by using light and fluorescence microscopy

6. Quantify cytotoxicity using polymer coated CdSe-ZnS QDs
Test biocompatibility of anionic multi-layered systems: effect of varying anion composition

1. Prepare QD-DEAE-anion nanoparticles using PVSA, PAAc and DS

2. Deliver to cells and characterize results using light and fluorescence microscopy

Determine if biocompatibility is independent of internal cation or depends on the composite properties of both polymers

1. Prepare QD-cation-DS nanoparticles using bPEI, PAA, PDMC and DEAE

2. Deliver to cells and characterize results using light and fluorescence microscopy
4.4 Results & Discussion

Cellular interactions of (i) uncoated, (ii) single layer cationic, and (iii) multi-layered anionic complexes with COS-7 cells were investigated. COS-7 cells are immortalized African green monkey kidney epithelial (fibroblast) cells that grow in monolayers. They were selected because they are robust, have easily characterizable morphologies, actively endocytose extracellular materials, and are very well characterized. The effects of varying the compositions of the nanoparticle core and coating polymers on biocompatibility and cellular morphology were determined.

4.4.1 Uncoated & Single Layer Systems

Prior to biointegration, the surface charges of modified CdSe-ZnS (green, 3.4 nm), GaN (blue, 3 nm), and PS (green, 20 nm) nanoparticles suspended in 50 mM NaCl were determined using zeta potential measurements (Fig 4.2). The surfaces of all three nanoparticles were highly anionic. Modified CdSe-ZnS QDs and PS nanoparticles had carboxylic acid moieties grafted to their surfaces, which contributed to the negative surface charge. GaN nanoparticles were negatively charged likely due to the adsorption of anionic hydroxyl molecules to the dangling bonds of the surface gallium atoms, as observed for other metal nanoparticles (Fig 2.1a)³². Therefore any differences in cellular interactions were not due to variations in surface charges of the nanoparticles.

![Figure 4.2 Zeta potential of CdSe-ZnS-MAA, uncapped GaN, and PS-COOH nanoparticles are all highly anionic due to the presence of carboxylic acid moieties and adsorbed hydroxyl groups.](image)

Inherent biocompatibility of anionic, uncoated CdSe-ZnS, GaN, and PS nanoparticles was determined by incubating them with COS-7 cells for 2 hrs, and characterizing the results 24 hrs post-treatment (Fig 4.3). Cellular morphologies were analyzed using light microscopy (left panel), and nanoparticle uptake was monitored by visualizing the fluorescence (right panel). The cells treated with nanoparticles are extended and sprout cellular processes, have distinct cellular and nuclear membranes, large nuclei, and dense nucleoli. All these are indicators of cell viability, therefore demonstrating that the cells are healthy and metabolically active after treatment with the nanoparticles. Analyzing the fluorescence of the treated cells revealed differences in nanoparticle uptake by the cells; those treated with CdSe-ZnS QDs demonstrated both, diffuse widespread fluorescence and concentrated bright spots, indicating that while some QD may be spread throughout the cells, others aggregated to form clumps. Cells treated with GaN particles showed blue
fluorescence, indicating significant levels of non-specific uptake of the QDs. Cells treated with PS nanoparticles exhibited discretized bright green fluorescence, demonstrating that the endocytosed nanoparticles remained vesicle bound. The data demonstrated that nanoparticles are not inherently cytotoxic, and are internalized by cells to varying degrees.

Figure 4.3 COS-7 cells treated with CdSe-ZnS-MAA, GaN, and PS-MAA nanoparticles, characterized using light (left panel) and fluorescence (left panel) microscopy. All cells appear elongated with distinct organelles and intact membranes, and are therefore healthy. Fluorescence microscopy (right panel) reveals that the GaN and PS are readily taken up by the cells, but the CdSe-ZnS particles show limited uptake (scale bar 25 μm).

Single-layer, cationic nanoparticle complexes were prepared by coating anionic CdSe-ZnS, GaN and PS nanoparticles with cationic bPEI (25 kDa), PAA (19 kDa), PDMC (17 kDa), pLys (15 – 23 kDa), pArg (15 – 23 kDa), dextran (<100 kDa) and cellulose (17 kDa). Nanoparticles were introduced into the cationic polymer solutions, incubated on
the benchtop at room temperature for 30 mins, and purified from the excess unbound polymer by spinning through an Amicon filter (100 kDa) twice. The surface potentials of the assembled complexes suspended in water were determined by measuring their respective zeta potentials (Fig. 4.4). All nanoparticles were positively charged, indicating successful deposition of the cationic polymers. The variations in zeta potentials between different samples were within the experimental error of the instrument, and not necessarily reflective of differences in the surface charge concentrations.

Biocompatibility of the single layer, cationic complexes was determined by incubating the heterostructures with COS-7 cells for 2 hrs, and determining the resulting cytotoxicity 24 hrs post-treatment. A visual assay was used to characterize cell viability; the treated samples were stained with propidium iodide (PI), and visualized under the microscope. PI is a cell impermeant dye that undergoes 50-fold enhancement in fluorescence when it binds to single-stranded DNA, and is therefore an indicator of dead or dying cells with compromised cellular membranes and dissociated DNA. Based on the apparent cytotoxicity of the cationic complex treated cells, a value of 0, 0.5, or 1 was assigned to the sample. If most of the cells were dead then a value of ‘0’ was assigned, if the cells looked similar to untreated controls with minimal toxicity, then a value of ‘1’ was assigned, and if the cytotoxicity was somewhere in-between, then a value of ‘0.5’ was assigned. The results (Fig 4.5) show that all the nanoparticles coated with bPEI, PAA, PDMC, pLys, and pArg were highly cytotoxic and caused widespread cell death. Nanoparticles coated with dextran caused some toxicity, while those coated with

Figure 4.4 Zeta potential of CdSe-ZnS-MAA, GaN and NP-COOH coated with cationic polymers (bPEI, PAA, PDMC, pLys, pArg, dextran, cellulose). All the tested nanoparticles have highly positive surface, indicating the successful deposition of cationic polymers (error bars between 2 – 8 mV).
cellulose were non-toxic and did not result in significant cell death. Since the cellular interactions are independent of the core material, and the nanoparticles are not cytotoxic, the data demonstrates that biocompatibility of the nanoparticles depends primarily on the polymer coatings. Surface material properties dominate, due to the high surface area to volume ratios of the nanoparticles.  

**Figure 4.5** Cytotoxicity of cationic polymer-coated CdSe-ZnS, GaN and PS nanoparticles determined using visual assay. All nanoparticles coated with bPEI, PAA, PDMC, pLys and pArg are highly cationic, those coated with dextran show intermediate levels of toxicity, while those coated with cellulose are not toxic. Effect of polymer coating is independent of the nanoparticle core.

Cellular interactions of the cationic polymer coated CdSe-ZnS QDs were further probed by characterizing the treated cells with microscopy (Fig 4.6). Cellular morphologies were analyzed using light microscopy (left panel), cytotoxicity was determined by treating with PI (red, right panel), and complex uptake was monitored by localizing the fluorescence of the nanoparticles (green, right panel). Cells treated with bPEI, PAA, PDMC, pLys and pArg coated QDs were small and rounded with fragmented membranes, lacked distinct cellular organelles, and stained densely with PI, indicating widespread cell death. They had significant green fluorescence in the cell debris, indicating that the QDs likely associated with the cells, prior to cell death. Cells treated with dextran-coated QDs demonstrated moderate levels of cytotoxicity; most of the cells were healthy, but PI staining revealed higher levels of cytotoxicity than observed in the untreated cells. There were low levels of green fluorescence in the cells, indicating limited QD-complex uptake. Cells treated with cellulose-coated QDs appeared healthy, and PI staining did not reveal higher levels of cytotoxicity than observed in the untreated cells. These complexes demonstrated particulate green fluorescence inside the cells, indicating high levels of QD-complex uptake. These data demonstrate that all cationic QDs interact with the cells, but their cytotoxicity and cellular uptake depended on the chemical composition of the coating polymer.
Figure 4.6 Cells treated with cationic polymer-coated CdSe-ZnS QDs, characterized using light (left panel) and fluorescence (right panel) microscopy. Cells treated with bPEI, PAA, PDMC, pLys and pArg coated QD are curled up with fragmented membranes (left panels) and show intense PI staining (red, right panel), which indicate widespread cell death. Residual QD-complexes (green) are localized in dead cell debris. Dextran treated cells demonstrate low levels of toxicity and PI staining, and limited localization of QD-complexes in cells. Cellulose coated complexes have very low levels of cytotoxicity, show virtually no PI staining, and a lot of QD localization in healthy cells (scale bar 25 μm).
Biocompatibility of the cationic polymer coated CdSe-ZnS QDs was further characterized by quantifying their relative cytotoxicity. Cells were treated with varying doses of the polymer-coated nanoparticles for 2 hrs, incubated for 24 hrs., then treated with PI, and their viability determined using fluorescence activated cell sorting (FACS). The results (Fig 4.7) were expressed as percent normalized cell viability, relative to cell viability of untreated cells maintained under the same conditions. The results show that QDs coated with bPEI, PAA, PDMC, pLys, and pArg were highly cytotoxic at all doses, dextran (DEAE) coated QDs demonstrated dose-dependent toxicity, while cellulose treated QDs (HEC) were non-toxic at all doses. These results further demonstrate that the cytotoxicity of the cationic QD-polymer complexes depends on the polymer coating.

![Figure 4.7 Cell viability of cationic polymer-coated CdSe-ZnS QDs. QDs coated with bPEI, PAA, PDMC, pLys and pArg are highly cytotoxic at all doses, dextran coated QDs demonstrate dose-dependent toxicity, while cellulose coated QDs are non-toxic at all the doses tested (error bars between 3 – 8 %).](image-url)

The different biocompatibilities of the polymer-coated QD complexes are due to the differences in chemical properties and structures of the coating polymers. Branched PEI, PAA, PDMC, pLys and pArg have high charge densities and demonstrated the highest cytotoxicities, dextran has low charge density and demonstrates moderate, dose-dependent cytotoxicity, while cellulose has the lowest charge density and is relatively non-toxic. Therefore, the higher the charge density of the coating polymer, the greater is the evoked cytotoxicity. This direct correlation between the charge density and cytotoxicity of the complexes is similar to the trends observed in co-polymer systems with varying charge densities, and may be a result of increased cell-permeating efficiency or increased endosomal escape of the cationic complex (Section 4.1.2.1)\textsuperscript{35}. Another reason for the high cytotoxicity of the polymer-coated QDs can be the physical localization of the polymer on the QD surface, leading to increased effective charge concentrations. The polymer is readily accessible to the cellular environment, and the close proximity of the cation chains may promote synergistic electrostatic interactions.
This is similar to the effective concentration enhancement observed when the cytotoxicity of linear polymers is compared to that of their branched and dendrimer analogues, as fewer charges are buried inside the polymer\textsuperscript{29} (Section 4.1.2.1). It is also analogous to the increase in cytotoxicity of higher molecular weight polymers, compared to mass-equivalent doses of their lower molecular weight counterparts.\textsuperscript{24}

The data presented in this section demonstrate that biocompatibility of the nanoparticles depends primarily on the polymer coating, and is relatively independent of the core. For all subsequent investigations CdSe-ZnS QDs were used as the inorganic cores.

\section*{4.4.2 Multi-Layer Systems}

Anionic, multi-layered complexes were prepared using PVSA (15 kDa), PAAc (16 kDa) and DS (20 kDa) to coat cationic CdSe-ZnS QD-polymer complexes. Based on the results presented in the previous section, dextran and cellulose coated QD complexes were used as the cationic core, since they demonstrated moderate to low levels of cytotoxicity. While both cationic cores were successfully coated with the anionic polymers, QD-cellulose containing complexes aggregated during purification. This could be due to the lower charge density of the cellulose polymer leading to deposition of thinner anionic films around the cationic QD cores, resulting in nanoparticles that are not very stable\textsuperscript{15,16}. Therefore QD-dextran complexes were used as the cationic cores for subsequent modifications, unless noted otherwise.

Anionic PVSA, PAAc and DS coated QD-dextran complexes were characterized by determining their surface zeta potentials (Fig 4.8). The surface charges of multi-coated hybrids suspended in 50 mM NaCl were all highly negative, demonstrating the successful adsorption of the anionic polymers on the QD-dextran surface\textsuperscript{33}. These reversals in surface charges are characteristic of layer-by-layer deposition, as discussed previously (Section 3.4.2). The variations in the zeta potentials of the different samples are within the instrument error, and not necessarily reflective of differences in the charge concentrations.

Biocompatibility of the multi-layer, anionic complexes was determined by incubating the heterostructures with COS-7 cells for 2 hrs, and determining the resulting cytotoxicity 24 hrs post-treatment (Fig 4.9). Cellular morphologies were observed using light microscopy (left panel), cytotoxicity was determined by treating with PI (red, middle panel), and complex uptake was monitored by localizing the fluorescence of the nanoparticles (green, right panel). Treatment with both PVSA and PAAc containing heterostructures demonstrated widespread cytotoxicity; the cell membranes were fragmented and merged together, cellular morphologies distorted, discrete organelles were not readily visible, and cells were intensely stained with PI. The cells also demonstrated bright green fluorescence, indicating uptake of the QD-containing heterostructures. Cells treated with DS coated complexes demonstrated much lower toxicity, as evidenced by the intact cellular morphologies and limited PI staining, and showed less green fluorescence indicating a decrease in QD-complex uptake. Therefore the cellular interactions of the multi-layered complexes varied in the degree of cytotoxicity and cellular uptake. Since
all three assemblies have the same cationic QD-dextran cores with limited cytotoxicity, the differences in cellular responses are due to the different chemical functionalities of the anionic polymer coatings.

The morphologies and PI staining patterns of the anionic, multi-layered complex treated cells (Fig 4.9) are different from those of the cationic, single-layer complex treated cells (Fig 4.6). While both samples show fragmented cell membranes, discrete intense PI staining, and QD-complex internalization, the former also demonstrates globular cellular morphologies where cell membranes appear to merge together, and DNA (stained with PI) is distributed throughout the cells, arrested in gelatinous networks. Therefore it is likely that the two complexes mediate cell death via different mechanisms. The cell-permeation and endosomal rupture mechanisms of cationic polymer-mediated cell death were previously discussed. It is possible that the anionic, multi-layer systems cause cell death by displacing DNA from histones, sequestering the proteins, and then complexing with the free DNA to form gelatinous cross-linking networks, as observed for other anionic polymers.
Figure 4.9 Cells treated with CdSe-ZnS-MAA-dextran complexes coated with PVSA, PAAc and DS, characterized using light microscopy (left panel), PI staining (middle panel) and QD localization (right panel). Cells treated with complexes containing PVSA and PAA have distorted cell morphologies and intense PI staining, demonstrating that they are dying or dead, and show intense QD complex (green) localization in the cell debris. Cells treated with DS containing complexes show low levels of cytotoxicity and limited QD-complex uptake (scale bar 50 μm).

The final step was to determine if the biocompatibility of the multi-layer QD-complexes depended primarily on the outer anionic polymer layer, or on both the anionic and the cationic polymer layers. Multi-layered complexes with different cationic polymers (bPEI, PAA, PDMC and dextran), but the same anionic polymer coating (DS), were prepared. Based on the results presented in Fig. 4.9, DS containing complexes demonstrated the lowest levels of cytotoxicity, and therefore DS was selected as the anionic polymer. Anionic DS coated, bPEI-, PAA-, PDMC- and dextran-containing, multi-layered complexes were incubated with COS-7 cells for 2 hrs., and results were analyzed 24 hours post-treatment (Fig 4.10). Cellular morphologies were observed using light microscopy (left panel), cytotoxicity was determined by treating with PI (red, middle panel), and complex uptake was monitored by localizing the fluorescence of the nanoparticles (green, right panel). Cells treated with bPEI containing complexes demonstrated low levels of cytotoxicity, as evidenced by some distortion and fragmentation of cellular morphology and limited PI staining. They also demonstrated low levels of QD uptake, evidenced by limited green fluorescence. Cells treated with
PAA containing complexes had highly fragmented cell membranes and PI staining revealed high levels of cytotoxicity, with moderate levels of QD complex uptake. Cells treated with PDMC demonstrated moderate levels of cytotoxicity, and QD complex uptake. Compared to their cationic single-layered counterparts (Fig 4.6), multi-layered complexes containing bPEI and PDMC demonstrated significantly reduced cytotoxicity. The data demonstrate that the properties of the inner, cationic polymers in multi-layered complexes may be partially shielded by the outer anionic polymer, but the composite properties of the heterostructures depend on the properties of both the polyelectrolytes. Since the anionic polymers do not form a tight shell around the cationic QD but assemble into a loose mesh, it is likely that both the polymers are exposed to the cellular environment, and therefore influence cellular interactions. The degree to which the behavior of one polymer dominates or shields the properties of the other depends on the properties and chemical compositions of the constituent polymers.

Figure 4.10 Cells treated with CdSe-ZnS-MAA-cation-DS complexes containing bPEI, PAA, PDMC, and dextran, characterized using light microscopy (left panel), PI staining (middle panel) and QD localization (right panel). Cells treated with PAA containing complexes show widespread toxicity and diffuse QD complex remains in dead cell debris. Cells treated with bPEI and PDMC-containing complexes demonstrate moderate toxicity and some uptake of QD complexes. Those treated with dextran-containing complexes have low toxicity and show limited QD-complex uptake (scale bar 25 μm).
Here we demonstrate that cellular interactions of anionic and cationic QD complexes can be specifically tailored by incorporating polymers with different functionalities. The properties of both the cationic and anionic polymers affect the composite properties of the system, and are relatively independent of the inorganic QD cores. Therefore, rationally designed complexes with controlled properties can be prepared.
4.5 Summary & Conclusions

4.5.1 Uncoated & Single Layer Systems

- Cellular delivery of uncoated, anionic CdSe-ZnS-MAA, PS-MAA and GaN nanoparticles did not cause excessive cell death demonstrating that the nanoparticles were not inherently cytotoxic.
- Cationic polymers with varying chemical structures were used to coat the anionic nanoparticles, forming cationic single-layered complexes.
- Biocompatibility of the cationic single-layer complexes depended on the chemical structure of the polymer, and was relatively independent of the inorganic core:
  - QDs coated with high charge density polymers (bPEI, PAA, PDMC, pLys, pArg) caused widespread cell death.
  - Dextran coated nanoparticles demonstrated dose dependent toxicity.
  - Quaternized cellulose nanoparticles were non-toxic at all ratios tested.
- Cationic QDs associated with the cells, and depending on their surface modifications, were either localized in dead cell debris or in healthy, viable cells.

4.5.2 Multi-Layer Systems

- Dextran-coated QDs were coated with anionic PVSA, PAA and DS polymers to form stable anionic multi-layered structures.
- Biocompatibility of anionic multi-layered complexes depended on the chemical compositions of the anionic polymers.
  - QD-dextran-PVSA and QD-dextran-PAAc were highly cytotoxic and showed extensive cellular uptake leading to distortion of cellular morphologies.
  - QD-dextran-DS demonstrated limited cytotoxicity and low cellular uptake.
- Cellular interactions of multi-layered structures depended on the chemical structures of both the cationic and the anionic polymers.
  - QD-PAA-DS remain highly cytotoxic.
  - QD-bPEI-DS and QD-PDMC-DS demonstrate decreased cytotoxicity and low cellular uptake, as compared to their single-coated cationic counterparts.
4.6 References


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Chapter 5: QD-Based Gene Delivery
Chapter Synopsis

Objective
The aim of this section was to assemble and characterize multi-layered quantum dot (QD)-cationic polymer-DNA heterostructures, investigate the effect of nanostructuring on gene delivery properties, and visually track and characterize multi-gene systems.

Hypothesis
The use of multi-layered QD-polymer-DNA complexes with diverse chemical functionalities and highly defined nanostructures may enable assembly of highly efficient gene delivery vehicles capable of mediating high levels of protein production and optically tracking the gene delivery complex.

Rationale
Polymer coated semiconductor QDs were used for assembling gene delivery nanostructures since they (i) enable fine morphological control of the QD core, (ii) possess inherent fluorescence of the QDs, and (iii) allow easy integration of diverse chemical functionalities. These properties are important for the assembly of finely tuned delivery vectors for mediating efficient gene delivery and tracking.

Results
Diethylaminoethyl dextran (dextran)-coated QDs were selected as the DNA condensing cores based on their gene delivery properties; when delivered to COS-7 cells they yielded the highest levels of gene-product production and low levels of cytotoxicity. Characterization of the electrophoretic mobilities demonstrated that QD-dextran complexes condensed and over-compensated for the anionic DNA, elicited high levels of protein production, and could be easily visualized using fluorescence.

Multi-layered heterostructures were assembled by integrating functionalized poly β-amino esters (C32) with the QD-dextran/DNA complexes, yielding approximately 2.5 fold enhancement in protein production. Characterization of hybrids using transmission electron microscopy revealed ordered beads-on-a-string assemblies of QDs wrapped with the polymers and DNA, analogous to naturally occurring nucleosomes. The heterostructures were more effective at condensing DNA and mediated higher levels of protein production than their unstructured and free polymer counterparts, at the same mass ratios.

Heterostructured QD-dextran/DNA/C32 complexes were used to characterize DNA delivery. Colocalization of QD and labeled DNA inside cells revealed that the nucleic material remained bound to the QD, and could therefore be used for monitoring gene delivery. Continuous imaging of a single cell tracked the intracellular route of the heterostructures, from delivery to protein production. Multi-colored heterostructures (red and blue) were used to monitor delivery of different genes (nuclear and mitochondrial...
localizing GFP), and co-visualization with the gene products demonstrated that they were effective labels for tagging gene delivery.

**Conclusions**

Multi-layered QD-dextran/DNA complexes demonstrate hierarchal nanostructuring which leads to more effective DNA condensation and assembly of enhanced gene delivery vehicles than their unstructured counterparts. Incorporation of the functionalized C32 polymer further increases the gene delivery efficiency of the multi-layered complexes. Hybrids also enabled the continuous tracking of the delivery complex inside cells, and the simultaneous tagging of multiple genes.
5.1 Background

Inorganic-organic complexes can be manipulated to allow design of diverse hybrid platforms; integration of polymers with different functionalities allows chemical diversity, while manipulation of the quantum dot (QD) size and morphology enables control of the structural organization (Chapter 4). Assembly of highly tunable heterostructures incorporating multiple functionalities may provide efficient solutions to multi-faceted biological problems like gene therapy, the delivery of exogenous DNA to manipulate endogenous protein levels. To date, several specialized materials have been developed for mediating gene therapy which should present high levels of gene-product expression but low levels of non-specific cytotoxicity. However, these synthetic systems are not as efficient as viruses, the biological gene delivery vectors (Section 1.2.3). The difference in efficiency could be due to differences in structural organization and limited material functionalities, leading to arrest by cellular barrier. In the following sections the characterization of unstructured synthetic cationic polymer-DNA delivery systems, and nanostructured hybrid systems for gene therapy, are discussed.

5.1.1 Unstructured Gene Delivery Systems

The use of different synthetic materials for mediating gene delivery has been extensively investigated. These materials must (i) complex with DNA and facilitate cellular uptake of the complex, (ii) protect DNA from enzymatic degradation while in transit within the cell, and (iii) dissociate from DNA after nuclear localization, to enable gene transcription. Some of the commonly used cationic polymers for gene delivery applications include branched polyethylenimine (bPEI), polylysine (pLys), polyarginine (pArg) and diethylaminoethyl dextran (dextran) (for discussion of chemical structures and properties, see section 4.1.2.1). These materials electrostatically interact with the highly anionic phosphate moieties of the DNA backbone, and lead to condensation of the nucleic acid. While the use of these polymers can avoid some of the inflammatory and immunogenic responses evoked by viruses, their application is limited by low transfection efficiencies due to their arrest by cellular barriers to gene delivery. The inability of these complexes to escape cellular impedances may be due to lack of both materials with specialized chemical functionalities, and hierarchal nanostructuring of the complex.

Functionalized materials specifically designed for mediating enhanced gene delivery, target specific barriers that impede the delivery of the DNA complex (Section 1.2.2). Nucleic acids complexed with bPEI covalently linked with arginine-glycine-aspartate (RGD) residues or glucosyl units demonstrated increased levels of protein expression due to increased cellular uptake of the polymer/DNA complex, mediated by association of the ligand with cell surface receptors. Thermo and pH responsive polymers, which undergo a sharp coil-globule transition in water at 32 °C, being hydrophilic below this temperature and hydrophobic above it, reversibly condense with DNA at lower temperatures, and facilitate release of plasmid at physiological temperatures. Biodegradable polymers with reducible disulfides or hydrolyzable bonds facilitated
the release of polymer-bound DNA, therefore mediating higher levels of gene transcription.

The use of one particular class of hydrolyzable polymers, poly-β amino esters, has demonstrated very high levels of protein production as compared to other synthetic, cationic polymers\textsuperscript{17,18}. These polymers are prepared via the conjugate addition of primary amines to diacrylates, which results in the synthesis of cationic polyesters that are hydrolyzed in aqueous environments\textsuperscript{18}. These polymers can reversibly condense DNA; in their undegraded form they are cationic enough to effectively condense DNA, but after hydrolysis they fragment and dissociate from the nucleic acid, returning it to its original, uncondensed state\textsuperscript{19}. Libraries of different poly-β amino esters were synthesized by varying the constituent amines and diacrylates, with the resulting polymers demonstrating varying levels DNA delivery efficiency\textsuperscript{19,20}. Of the polymers tested, C32 (Fig 5.1) demonstrated the best gene delivery properties\textsuperscript{21}; it mediated the highest levels of protein production while eliciting low levels of cytotoxicity, and was more effective than bPEI as a gene delivery vector.

![Figure 5.1 Synthesis of C32 from constituent diacrylate and amine.](image)

Structural morphology of several polymer-DNA complexes was investigated using atomic force microscopy (AFM) and transmission electron microscopy (TEM). Analyzing the morphologies revealed that the complexes formed rods, toroids and spheroids, depending on the relative concentrations of the polymer and DNA, the chemical structure of the condensing polycation, and the counter ion and the solvent used for the complexing solution\textsuperscript{22-25}. The size of the condensed complexes varied between ~50 and 300 nm, and lacked defined structuring on the nanometer length scale\textsuperscript{25}. The size and morphology of the polymer-DNA complexes was affected by the charge density of the complexing polymers with highly charged polymers binding DNA more effectively.
The geometric and structural organization of the cationic charge also influenced DNA condensation; equivalent amounts of dendrimer, branched, and linear structural analogues of polyethyleneimine, had different DNA condensation abilities, with the highly branched, ordered dendrimers being the most effective\(^{26}\). Polymers that more efficiently compacted DNA mediated higher levels of protein production.

Therefore previous investigations demonstrate that chemical composition, structural organization, and hierarchal assembly of the delivery complexes affect their size and morphology, which leads to differences in transfection properties.

### 5.1.2 Nanostructured Gene Delivery Vehicles

The use of alternate hybrid inorganic, polymer and DNA heterostructured complexes as platforms for efficient delivery and optical characterization of multi-gene systems was previously investigated. To date, examples of hybrid materials complexed with DNA include cationic polymers grafted to gold nanoparticles\(^{27}\), dendrimers attached to silica nanoparticles\(^{28}\), amine-modified fluorescently labeled silica nanoparticles\(^{29}\), fluorescent silica nanotubes\(^{30}\), and fluorescently labeled Ni/Au nanorods\(^{31}\). These structured hybrids show more efficient gene expression than their unstructured analogues. While the delivery efficiencies of these structures are low, their properties may be manipulated by controlling the hierarchal assembly and integration of multiple functionalities.

Herein the use of nanostructured polymer-coated quantum dots (QDs) to complex with DNA is investigated. The use of QD-based platforms for gene delivery applications has not been previously demonstrated, and confers several advantages on the hybrid complex. Layer-by-layer deposition of different polymers enables the incorporation of multiple functionalities that can systematically target escape from various gene delivery barriers. The use of morphologically defined, nanostructured QD-cores may facilitate the assembly of complexes with controllable hierarchal organization. The combination of integrating multiple functionalities in an ordered heterostructure may lead to the synergies in gene delivery efficiency. Finally the bright, resolved, stable fluorescence of the QD core can enable continuous tracking of the gene delivery complexes, and simultaneous characterization of multi-gene systems.
5.2 Specific Aims

Nanostructural Enhancement

- Select cationic core for gene delivery
  - Select biocompatible polymer coating for assembling cationic hybrid cores
  - Characterize gene delivery properties of QD-polymer/DNA complex, and select optimal QD to DNA mass ratio
  - Integrate functionalized polymers to enhance protein production
- Characterize morphology of hybrid QD-polymer/DNA complexes and compare with their unstructured and free polymer counterparts
- Determine effect of nanostructuring on gene delivery properties of the complex.
  - Investigate DNA condensation & protection from enzymatic degradation
  - Characterize levels of protein expression and cytotoxicity

Fluorescent Tracking

- Demonstrate continuous tracking of QD-polymer-DNA complexes inside single cells, from delivery to protein production.
- Monitor multi-gene delivery by using different color QDs as platforms for simultaneously delivering multiple plasmids.
5.3 Materials & Methods

5.3.1 Experimental design

Materials
CdSe-ZnS core-shell QDs were purchased from Evident Technologies in red, green and blue (4 nm, 2.1 nm and 1.9 nm diameters) preparations. Enhanced green fluorescence proteins (EGFP, 3.4 kbp), rhodamine labelled plasmid (pGeneGrip/LUX) and nuclear and mitochondrial localizing GFP (pCMV/myc/nuc and pCMV/myc/mito) plasmids were obtained from BD Biosciences, Gene Therapy Systems, and Molecular Probes, respectively. COS-7 cells were purchased from American Type Culture Collection, cell culture supplies from Gibco BRL, and Nhe 1 enzyme from New England Biolabs. All other materials were purchased from Sigma Aldrich.

QD surface modification and nanoassembly preparation
CdSe-ZnS core-shell quantum dots (QD) were modified with mercaptoacetic acid (MAA), and coated with cationic polymers, as described previously. Briefly, trioctylphosphine oxide capped QDs were sonicated with MAA, purified twice by ethanol precipitation, and suspended in 50 mM tris base buffer. The anionic QDs (30 – 50 µg/mL) were mixed with two volume equivalents of cationic polymer solutions (10 mg/mL), incubated on benchtop for 20 mins, and purified twice by spinning through an Amicon separating column (100 kDa cut off). To prepare nanostructured complexes (NSC), cationic polymer-coated QDs (30 – 50 µg/mL) were mixed with DNA (1 mg/mL) at QD to DNA mass ratio of 1.5:1 (unless specified otherwise) and incubated on benchtop for 20 mins. To form heterostructured complexes (HSC), C32 (10 mg/mL) was mixed with the NSC at C32 to DNA mass ratio of 10:1 (unless stated otherwise) and incubated on benchtop for 20 mins. Unstructured complexes (USC) were prepared by adding dextran (5 mg/mL) to MAA-capped QD (30 – 50 µg/mL) and DNA mixture, at QD to dextran to DNA mass ratio of 1.5:3:1. Free polymer complexes (FPC) were prepared by adding dextran directly to DNA at dextran to DNA mass ratio of 3:1. The QD, dextran and DNA mass ratios of all the complexes were equivalent.

QD-Cation/DNA cell transfection
Monkey kidney COS-7 cells maintained at 37 °C and 5% CO₂ were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B. Cells were seeded at a density of about 75,000 cells per well in a 24-well plate, and 30,000 cells per well in an 8-well collagen treated cover-glass chamber, and allowed to grow overnight. QD-polymer-DNA complexes with 1 µg and 0.5 µg of DNA were delivered to cells in 24-well and 8-well, respectively, in serum-free medium. Cells were treated for 2 hrs (unless stated otherwise), and then loaded with fresh serum-supplemented medium.

Cell viability and transfection quantification
QD-polymer-DNA treated cells were analyzed 2 days post-treatment (unless stated otherwise). Cells were washed with phosphobuffered saline (PBS), incubated on ice for 15 min with trypsin (2.5 mg/mL) and propidium iodide (PI, 5 µg/mL) supplemented-
PBS, and analyzed using a FACScan (Becton Dickinson) flow cytometer, equipped with an argon ion laser. PI treated cells were detected using a PE/PI band-pass filter (585/42), and GFP expressing cells using a FITC band-pass filter (530/30). The cells were appropriately gated by the forward and side scatter. Relative cell viability was determined as the percent of viable cells, relative to untreated cells exposed to the same conditions. Relative transfection was expressed as the percent of cells expressing the gene product, relative to those treated with lipofectamine 2000, at an optimized LPF to DNA ratio of 1.5:1.

Zeta potential measurements
Surface electrophoretic mobilities of hybrid complexes were determined using a zeta potential analyzer (ZetaPlus, Brookhaven) with a HeNe laser at 632 nm. Complexes were dispersed in deionized water, and measurements were repeated three times. The Smoluchowski method was used to calculate the zeta potentials.

Enzymatic cleavage & agarose gel electrophoresis
Equal amounts of free DNA, and DNA condensed in NSC, FSC and FSC* were cleaved with Nhe 1 restriction enzyme for 1 hr at 37 °C, according to manufacturer’s protocols. The enzyme was subsequently denatured by heating at 70 °C for 5 mins. Equal amounts of untreated and restriction enzyme treated complexes were loaded in 1% agarose gel with 0.5 mg/mL ethidium bromide, and run at 100 mV for 60 mins in 1x TAE buffer. Images were obtained using UV BioRad transillumination imaging system, and ChemiDoc software.

Thermogravimetric Analysis
QD-dextran in tris base buffer were lyophilized for 2 days, loaded in a platinum well, and analyzed using the thermogravimetric analyzer (TG/DTA 320 Seiko). Temperature was increased from 30 °C to 400 °C at 2 °C/min.

Transmission electron microscopy characterization
Complexes were adsorbed on carbon coated copper grids (Ladd Research Industries) for 20 minutes, allowed to dry and imaged using a JEOL 2010 at an accelerating voltage of 200 kV.

Fluorescence microscopy imaging
Low magnification (40x) images were obtained using an inverted Olympus microscope (IX51) with GFP (ex 450/40, em 525/50), TRITC (ex 535/50, em 610/75) and DAPI (ex 360/40, em 460/50) filters, using SimplePCI software. Cell tracking images (100x) were obtained with a Zeiss Axiovert epifluorescence time-lapse systems using a GFP (ex 480/40, em 528/38) filter, with Metamorph software. Cells were maintained at 37 °C and 5% CO₂, using an environmental chamber. Confocal images were obtained using a Zeiss LSM510 system with a 100x oil immersion objective, using FITC (bp 505 – 530) and rhodamine (bp 565 - 615) filters with Argon (488 nm) and HeNe (543 nm) lasers. All images were digitally enhanced.
5.3.2 Experimental setup: Nanostructural Enhancement

1. Prepare bPEI, PAA, PDMC, dextran and cellulose coated QD complexed with DNA and deliver to cells at varying QD to DNA mass ratios (1 to 10).

2. Select QD-polymer system with highest cell transfection and lowest cytotoxicity.

3. Optimize QD-polymer/DNA complex by monitoring (i) zeta potential, (ii) agarose gel shifts, (iii) protein production, (iv) cytotoxicity and (v) cell morphology at varying ratios.

4. Integrate functionalized polymer, C32, and characterize (i) protein production, (ii) cytotoxicity and (iii) cell morphology at varying ratios.

5. Analyze morphology by visualizing using transmission electron microscopy.

6. Prepare nanostructured, unstructured and free polymer complexes and deliver to cell.

7. Characterize gene delivery efficiency by monitoring (i) zeta potential, (ii) protection from enzymatic degradation, (iii) protein production, and (iv) cytotoxicity.
5.3.3 Experimental setup: **Fluorescent Tracking**

1. Complex green QD-polymer with rhodamine labeled DNA and observe colocalization 6 and 12 hrs post delivery

2. Continuously track intracellular route of QD-polymer/DNA complex from delivery to protein expression (14 hours)

3. Observe colocalization of red QD and gene product green fluorescent protein, inside cells

4. Observe multiple gene delivery (nuclear and mitochondrial localizing GFP) using different colored QDs (red and blue)
5.4 Results & Discussion

In the subsequent sections, the selection, optimization, and characterization of QD-polymer/DNA complexes, effect of nanostructuring on the gene delivery efficiency, and the use of hybrids as fluorescent probes for tracking and characterizing multi-gene delivery in COS-7 cells, were investigated. For all subsequent investigations, enhanced green fluorescent protein plasmid (pEGFP) DNA was used as the reporter gene because (i) the gene product can be easily detected and assayed due to its bright green fluorescence, (ii) has high transcription efficiency, (iii) is a medium sized plasmid, (iv) is completely characterized, and (v) can be easily engineered to integrate additional genes. COS-7 cells are immortalized African green monkey kidney epithelial (fibroblast) cells that grow in monolayers. They were selected because they are robust, have easily characterizable morphologies, actively endocytose extracellular materials, and are very well characterized.

5.4.1 Optimizing QD-polymer-DNA cores

The first step in assembling hybrid delivery vectors was to select a suitable cationic polymer coating for the QD-MAA, to produce effective QD-cation complexes. Suitable QD-cation complexes must (i) facilitate complexing of DNA, (ii) be capable of mediating gene delivery and protein production, and (iii) demonstrate low cytotoxicity. Cationic branched polyethyleneimine (bPEI, 25 kDa), Polyallylamine (PAA, 19 kDa), polydiallyldimethylammonium chloride (PDMC, 17 kDa), diethylaminoethyl dextran, (dextran, < 100 kDa) and hydroxyethylthoxy cellulose (cellulose, 25 kDa) were used to coat Red CdSe-ZnS QDs (4.2 nm) QDs. These cationic QD-polymer cores were used to complex with pEGFP DNA at varying QD to DNA mass ratios, and delivered to COS-7 cells for 2 hours. The cell viability and protein expression were quantified 48 hours post-treatment. Cell viability was reported as the relative cell viability, relative to the viability of untreated cells that were maintained under the same conditions. Cell viability was determined by using fluorescence activated cell sorting (FACS) to quantify the number of dead and live cells in propidium iodide treated cell samples. PI is a cell impermeant dye that undergoes 50-fold enhancement in fluorescence when it binds to single-stranded DNA, and is therefore an indicator of dead or dying cells with compromised cellular membranes and dissociated DNA. Transfection efficiency was determined by measuring the percentage of cells with green fluorescence (gene product) and relative transfection efficiency was expressed relative to the transfection observed when equivalent amounts of DNA were delivered using lipofectamine 2000 (LPF), a commercially available gene transecting agent.

Analyzing cell viability results (Fig 5.2a) demonstrates that QD-polymer/DNA complexes containing bPEI, PAA and PDMC were highly cytotoxic at all QD to DNA ratios with their use resulting in almost complete cell death (< 5% viability). Cells treated with dextran containing complexes displayed dose-dependent toxicity (30 - 100% viability), displaying that these complexes become increasingly toxic at higher QD to DNA ratios. Cells treated with cellulose-containing complexes were relatively non-toxic at all QD to DNA ratios (> 95 % viability). The differences in cytotoxicity are likely due...
to differences in charge densities of the polymers (Section 4.1.2.1); bPEI, PAA and PDMC have very high charge densities, and likely caused cytotoxicity by cell membrane perforation and endosomal rupture (Section 4.4.1). Dextran has lower charge density than the synthetic bPEI, PAA and PDMC, and therefore QD-dextran complexes demonstrated varying levels of cytotoxicity. Cellulose has the lowest charge density, and QD-cellulose complexes were non-toxic at all doses tested\(^8\). Next, the corresponding levels of transfection were analyzed (Fig 5.2b). Cells treated with dextran containing complexes were the only ones that demonstrated significant levels of protein production and were relatively independent of the QD to DNA ratios. The variations in the levels of transfection are within the experimental error and are not necessarily reflective of differences in gene expression. The lack of protein production in cells treated with complexes containing bPEI, PAA and PDMC was likely due to the high cytotoxicity of the complexes – most of the cells were dead and therefore unable to express the gene product. Cells treated with cellulose-containing complexes did not demonstrate GFP expression at any QD to DNA ratio, likely due to arrest of the delivery complex by cellular barriers\(^9\).

![Figure 5.2](image)

**Figure. 5.2** Gene delivery efficiency of cationic QD-DNA hybrid complexes, characterized by determining the normalized (a) cell viability and (b) protein production of treated COS-7 cells. Complexes containing bPEI, PAA and PDMC are highly cytotoxic and do not mediate gene product production. Complexes containing dextran demonstrate dose-dependent cytotoxicity and mediate protein expression. Cellulose-containing complexes do not evoke any cytotoxicity and do not mediate protein production.

The results indicate that dextran containing hybrid complexes mediated efficient gene expression at low QD to DNA ratios. To further optimize delivery, QD-dextran/DNA complexes were prepared at low QD to DNA ratios (<4), and their electrophysical properties and gene delivery efficiency were characterized. The surface zeta potentials of the complexes dispersed in deionized water were positive at all ratios (Fig 5.3a), and similar (30 mV ± 4 mV) to those of uncomplexed QD-dextran cores (25 ± 5 mV). The highly positive values demonstrate that the DNA was completely condensed by the cationic QD. Gel shift assays were conducted in 1% agarose gels, and the results (Figure 5.3b) demonstrate that free DNA migrated towards the anode (bottom of the gel), while DNA complexed with cationic QD-dextran moved slightly towards the cathode (top of
the gel). This data also demonstrates that free DNA was negatively charged, while QD-dextran/DNA complexes were positively charged. The low mobility of the QD-dextran/DNA complexes was likely due to their high mass, which may impede movement of the complex through the agarose gel. These data demonstrate that the electrophoretic mobilities of the QD-dextran DNA complexes prepared at QD to DNA ratios between 0.25:1 and 2.5:1 were positive, indicating complete, efficient condensing of the anionic nucleic material.

Figure 5.3 Characterizing the electrophoretic mobilities of QD-dextran-DNA complexes. (a) Zeta potentials of complexes at all QD to DNA mass ratios are positive, indicating complete condensation of DNA. (b) Gel shifts demonstrate that while free DNA migrates towards the anode (bottom of the gel), DNA complexed with QD-dextran at all ratios, remains arrested in the wells.

Gene transfection properties of QD-dextran/DNA complexes at low QD to DNA mass ratios were characterized by delivering the complexes to COS-7 cells for 2 hours, and characterizing normalized cell viability and protein production 48 hours post-treatment. Analyzing the relative transfection of the QD-dextran/DNA complexes (Figure 5.4, bars) indicates that GFP levels are relatively independent of the QD to DNA ratios. While the
levels of protein production vary at the different ratios, the error bars are large, and there appears to be no systematic trend in GFP production with respect to variations in QD to DNA ratio. Therefore these variations are likely due to systematic errors, and not reflective of differences in transfection levels. Cell viability (Figure 5.4, line) was high at all ratios tested, and also independent of the QD to DNA ratio. These data suggest that protein expression and cell viability did not depend on the QD to DNA mass ratios at low values (< 2.5:1).

![Graph](image)

**Figure 5.4** Characterization of gene delivery efficiency of CdSe-ZnS QD-dextran-DNA complexes at varying ratios. High levels of normalized viability (line) indicate low cytotoxicity at all QD to DNA ratios. High levels of protein production do not demonstrate a clear dependence on the QD to DNA ratio.

Cellular interactions of QD-dextran/DNA complexes were probed by fluorescent characterization of treated cells 48 hours post-treatment. The cellular distributions of the expressed green fluorescent protein (GFP, green) and QDs (red) were analyzed (Fig 5.5). Since the QDs were delivered at very low doses, they were difficult to detect; the lowest QD to DNA ratio at which red fluorescence was observed was 1.5:1. Therefore this ratio was subsequently used for assembling QD-dextran/DNA gene delivery complexes.

### 5.4.2 Preparing and characterizing morphology of heterostructured complexes

To further enhance gene delivery properties of the QD-dextran/DNA complex C32 was integrated into the assembly, since previous use of C32 with DNA yielded high levels of protein production. Heterostructured complexes (HSC) were prepared by assembling QD-dextran/DNA and QD to DNA ratio of 1.5:1, and adding varying amounts of C32 polymer. These complexes were incubated on benchtop for 20 mins., and delivered to cells for 2 hours. The percent relative cell viability and transfection were determined using FACS, 48 hours post-treatment. Analyzing the cell viabilities of the treated cells (Figure 5.6, line) demonstrated that cytotoxicity increased as the amount of C32 in the
complexes increased, indicating that C32 was toxic at higher ratios. Protein production (Figure 5.6, bars) initially increased with increasing C32, reaching a maximum value of 350% at a ratio of 25:1. At higher C32 ratios transfection decreased, likely due to the elevated cytotoxicity levels.

Figure 5.5 Cells treated with CdSe-ZnS QD-dextran DNA complexes, characterized using fluorescence microscopy. EGFP gene product (green) is visible at all QD to DNA ratios. QD complex (red) is visible at higher QD to DNA ratios (> 0.75)
Cells treated with HSC at varying C32 to DNA mass ratios were further characterized by treating with propidium iodide (PI) and imaging using fluorescence microscopy (Fig 5.7). Cellular morphologies were analyzed using light microscopy (left panel), cytotoxicity was determined by treating with PI (red, right panel), and gene delivery efficiency was monitored by localizing GFP fluorescence (green, right panel). Analyzing the morphology of the cells demonstrates that as the C32 to DNA ratio was increased, the cells became increasingly curled up, the cellular and nuclear membranes began to fragment, and cellular organelles disappeared, all indicators of decreased cell viability. The cells also demonstrated increased PI staining, an indicator of increased cytotoxicity. GFP production of cells initially increased in number and intensity of color with increasing amount of C32 until a ratio of C32 to DNA of 25:1 was reached, following which protein production appeared to remain constant.

The results (Fig 5.6 and 5.7) demonstrate that there was an optimal C32 to DNA mass ratio at which transfection was enhanced, while increase in cytotoxicity was limited. 10:1 was selected as the optimal C32 to DNA ratio since transfection is increased approximately 2.5-fold, while cell viability decrease is limited to 72%. For all subsequent preparations of heterostructured complexes, QD to DNA to C32 ratios of 1.5:1:10 were used.
Figure 5.7 Cells treated with QD-dextran-DNA-C32 complexes at varying C32 to DNA ratios, characterized using light (left panel), and fluorescence (middle and right panels) microscopy. As the amount of C32 used increases, cell viability decreases, as evidenced by the fragmented morphology of the cells, and increased PI staining (red). The amount of protein expression (green) initially increases with increased amounts of C32, and appears to plateau at C32 to DNA concentrations of 25:1.

Prior to analyzing the morphologies of the QD-dextran/DNA/C32 complexes, the relative mass ratios of dextran polymer and the inorganic semiconducting materials were determined using thermo gravimetric analysis. The sample was tested over a temperature range of 30 – 400 °C, and the results showed three regions of weight loss (Figure 5.8). The first was observed between 200 – 250 °C, accounted for approximately 12% reduction in mass and was due to the loss of tris base (bp 220 °C). The second occurred between 260 – 340 °C, accounted for approximately 58 % loss in mass, and was due to the organic polymer burning off. The final loss in mass occurred after 370 °C, and was due to loss of inorganic material, as reported for other inorganic/organic hybrid...
Therefore, the mass ratio of the organic dextran polymer to QD was approximately 2:1.

![Figure 5.8](Image)

**Figure 5.8** Loss in mass of CdSe-ZnS-dextran complex as a function of temperature, determined using thermogravimetric analysis. There are three regions of temperature drop, around 170 °C, 325 °C, and following 350 °C, which correspond to loss in weight due to loss of tris base, dextran polymer and the inorganic material, respectively.

Next, the effect of polymer localization to the QD surface, on the overall morphology of the hybrid was investigated (Fig 5.9). Adsorbed polymer chains cluster on the QD surface, and can lead to an increased local concentration of the polyelectrolyte. This is in contrast to unbound polymer chains that, in the absence of an adsorption substrate, are freely dispersed in solution. This ‘concentration localization’ on the nanocrystal surface may promote more efficient DNA condensation and hierarchal structuring of the QD-polymer/DNA hybrids to form nanostructured complexes (NSC), that are different from the rod and torroid structures observed when free polymer/DNA complexes are assembled. The small size, high curvature and surface localization of cationic charges of the polymer-coated QDs are similar to those of histone proteins and dendrimers, both of which, when complexed with DNA, form strings of dense, cationic DNA-nanoparticle “beads” connected by anionic, uncomplexed “linker” DNA. The structure of the NSC is expected to be similar to the beads-on-a-string morphology of nucleosomes and dendrimers-DNA complexes. Furthermore, any hierarchal nanostructuring effects as
a result of polymer localization to the QD surface should be lost if corresponding amounts of QD/polymer/DNA are simultaneously mixed together to form unstructured complexes (USC), without first adsorbing the polyelectrolyte on the QD core. Conversely, incorporation of additional polymers to the NSC to assemble heterostructured complexes (HSC) is unlikely to affect the bead-on-a-string morphology of the hybrid, but may promote cross-linking of the constituent NSC and further condensation of the hybrid.

**Figure 5.9** Characterizing self-assembly of hybrid complexes. (a) Schematic representation of hybrid assemblies. Nanostructured complexes (NSC) comprise dextran (yellow) coated QDs (red) complexed with DNA (green). Heterostructured complexes (HSC) have additional C32 polymer (purple). Unstructured complexes include QD, DNA and dextran mixed at the same mass ratios as NSC, without first coating QD with dextran.

Morphologies of structured and unstructured cationic polymer-coated CdSe-ZnS core-shell QDs (4 nm diameter) and anionic DNA (3.4 kbp, pEGFP) complexes were characterized using transmission electron microscopy (TEM, Fig. 5.10). NSC comprised of dextran-coated QD complexed with DNA, and were assembled at an optimized QD to DNA mass ratio of 1.5:1. HSC were prepared by mixing NSC with poly-β amino ester C32, at an optimized C32 polymer to DNA mass ratio of 10:1. USC were assembled by simultaneously mixing QD, DNA and dextran in the same mass ratios as NSC, without first coating QD with the polymer. The relative mass ratio of dextran to QD in dextran-coated QD assemblies was 2:1, determined using thermo gravimetric analysis. Therefore USC were prepared at QD to DNA to dextran mass ratios of 1.5:1:3. TEM characterization of these complexes revealed that all three assemblies had dense, spherical cores approximately 5 nm in diameter, and were surrounded by diffuse material (Fig. 5.10a-c). Further characterization of the dense cores using high resolution TEM revealed the presence of lattice fringes (Fig. 5.10d), characteristic of crystalline QD cores. Based on their size, shape and crystalline structure, the dense cores were identified as QDs, while the diffuse material comprised cationic polymer and DNA.
However, the morphologies of the complexes was different from each other; NSC and HSC form QD-polymer/DNA strings, while USC form large aggregates, 200 – 300 nm in diameter. The QD-chains vary in size; NSC complexes are 50 – 100 nm in length while HSC form larger, more elaborately branched structures. Previous characterization of polymer-coated QDs without DNA revealed lack of hierarchal structuring and lower amounts of the diffuse material\textsuperscript{35}. These data demonstrate that the localization of the polymer on the QD surface has a significant effect on the morphology and nanostructure of the QD-polymer/DNA hybrids, and results in the assembly of complexes with defined hierarchal structures. This is likely due to enhanced DNA complexing ability of the cationic QD core, caused by an increase in the local concentration of the polyelectrolyte due to clustering of the polymer chains adsorbed on the QD surface.

Figure 5.10 Transmission electron microscopy characterization reveals that (a) NSC and (b) HSC form complexes with defined secondary structures of linked QD chains, not found in their (c) USC analogues. (d) High resolution lattice fringes reveal the crystalline QD core. Black and white scale bars are 20 nm and 5nm, respectively.

The data presented in this section demonstrates that QD-dextran nanoparticles formed effective cores to condense and deliver DNA, and their efficiency was enhanced by incorporating functionalized gene delivery polymers like C32. These hybrid heterostructured systems demonstrated secondary ordering to form quantosomes, biomimetic nucleosomes.

5.4.3 Determining effect of nanostructuring on gene delivery properties

The effect of polymer localization to the QD surface, on the DNA complexing ability of the dextran-coated QD core, was investigated. In addition to the NSC, HSC and USC, free polymer complexes (FPC) which comprised unbound dextran polymer and DNA at the same dextran to DNA mass ratio (2:1) as NSC, were also prepared. DNA condensation was monitored by determining the electrophoretic mobilities of the QD-dextran/DNA complex using gel electrophoresis. Free DNA and prepared complexes were loaded in 0.5 mg/mL mM ethidium bromide (EtBr) agarose gels, run for 1 hour, and
imaged. The positions of DNA complexes on the agarose gel were revealed by EtBr staining, and were visible as fluorescent bands (Fig 5.11). Analysis of the migratory patterns of the bands revealed that DNA complexed in various assemblies had altered electrophoretic mobilities. Naked DNA migrated towards the positive electrode at the bottom of the gel; uncomplexed DNA is anionic and freely dispersed in solution, therefore demonstrating high mobility through the gel. DNA complexed in NSC and HSC appeared to move slightly towards the negative electrode (top of the gel); the cationic QDs appear to effectively overcompensate for charge neutralization of anionic DNA, and the limited mobility is likely due to the high mass of the complexes. DNA incorporated in the USC is partially arrested; free dextran condensed only some of the DNA which remains in the wells, while the rest is uncomplexed and migrates the same distance towards the negative electrode, as does naked DNA. Therefore free dextran in USC does not facilitate complete DNA condensing. DNA in FPC remains arrested in the well and does not appear to move toward either electrode; it is unclear whether the FPC is positively charged or neutral. This data demonstrates that analogous assemblies containing equal amounts of QD, polymer and DNA demonstrate different electrophoretic mobilities as a result of nanostructuring the QD-dextran DNA complexing core.

![Figure 5.11 Gel electrophoresis results show anionic DNA migrates towards the anode, cationic NSC, HSC and FPC* towards the cathode, and uncharged FPC remains arrested in the well.](image)

The surface charges of the hybrid assemblies were further characterized by measuring the zeta potential of the DNA-containing complexes (5.12). NSC and HSC are highly cationic, and their surface charge is similar to that of dextran-coated QD without the addition of DNA (27 ±3 mV). This shows that dextran-coated QDs are effective at
overcompensation of charge neutralization of DNA, forming cationic assemblies. USC have highly negative surfaces which may be due to excess, uncomplexed DNA or uncoated QDs (-43 ±6.9), which are not electrostatically neutralized by the free polymer in the complex. The FPC are neutral which demonstrates that while the free polymer complexes with DNA, it does not overcompensate for the anionic charge. The data demonstrate that analogous assemblies containing equal amounts of QD, polymer and DNA have very different complexing properties as a result of different nanostructures; localization of polymer to QD surfaces makes it more effective at electrostatically complexing with and overcompensating for charge neutralization of the DNA, than equivalent amounts of free polymer.

Figure 5.12 Zeta potentials of complexes reveal that NSC, HSC and free polymer complex (FPC*) at optimized polymer to DNA mass ratio effectively condense DNA, while USC and FPC at original mass ratios do not. Error bars represent standard error.

There may be several reasons that contribute to the enhancement of the DNA complexing ability of the polymer, when it is localized on QD surfaces. The first is effect of polymer chains clustering on the surface of the QD, leading to local physical increase in concentration. Physical proximity of the cationic charges may also promote cooperative coulombic interactions between the polymer and the oppositely charged DNA. These effects are analogous to those observed when the molecular weight of DNA complexing polymers is increased; at equivalent mass amounts, higher molecular weight cationic branched polyethyleneimine are more effective at electrostatically condensing DNA and forming smaller, more defined complexes, than their lower molecular weight counterparts 42,43. Complexing ability may also be enhanced because of minimization of steric hindrance due to surface localization of the polymer, facilitating ready availability of the cationic charge for electrostatic interactions. This effect is analogous to when dendrimers
are used to complex DNA instead of their linear or branched counterparts; at equivalent mass amounts, polyamidoamine dendrimers are more effective at condensing DNA and forming complexes for gene delivery, than linear polyethyleneimine²⁶.

The effect of nanostructuring the QD-dextran core and integrating C32, on relative transfection (Fig 5.13, white bars) and viability (Fig 5.13, hatched bars) of treated cells, was determined. The transfection efficiency of NSC treated cells was 66%; dextran-coated QDs mediated approximately two-thirds as much transfection as that obtained using LPF. The transfection efficiency increased to 260% with the addition of C32 to the complex; HSC mediated approximately 2.5-fold as much transfection as that obtained using LPF. Transfection efficiency dropped to < 5% when USC and FPC were used to deliver DNA; these unstructured and free polymer complexes were much less effective at mediating gene delivery than LPF and their nanostructured counterparts (NSC). Analysis of the relative viability demonstrates that 84% of NSC treated cells were alive; treatment with QD-dextran/DNA complexes resulted in slight decrease in cell viability, as compared to untreated cells. Cells treated with HSC demonstrate 71% effective viability; addition of C32 to the complexes increases cytotoxicity of the DNA containing complex. Treatment with USC and FPC demonstrate 86% and 98% effective viabilities, demonstrating that treatment with QD-containing complexes causes slight increase in cytotoxicity, while treatment with polymer/DNA complex is relatively non-toxic. Therefore both relative transfection and viability increase due to nanostructuring of the QD-dextran core prior to DNA complexing, and are further promoted by the incorporation of C32 to form HSC.

![Bar chart](image)

**Figure 5.13** Gene product expression (white bar) and cell viability (hatched bar) demonstrate that NSC treated cells yield more gene expression than USC and FPC, and similar expression levels as FPC*. HSC mediated delivery results in further enhancement of gene expression. Error bars represent standard deviation.
The differences between the transfection and cytotoxicity properties of the different DNA-containing complexes may be due to several reasons. The higher transfection mediated by the NSC as compared to the unstructured and free polymer counterparts may be attributed to more efficient DNA complexing by the QD-dextran core, as demonstrated by TEM analysis and electrophoretic characterization. NSC are small and electrostatically condense and overcompensate for anionic DNA, resulting in the assembly of cationic complexes that can readily associate with, and be endocytosed by, the negatively charged cell membrane. The negatively charged and neutral USC and FPC are not electrostatically attracted to the anionic cell membrane. Furthermore, complete condensation of the DNA within the NSC may protect it from enzymatic degradation and arrest by intracellular gene delivery barriers. Addition of C32 to the complex further increases its transfection efficiency, due to the intrinsic gene delivery properties of the polymer. Decreased relative cell viability caused by the QD-containing complexes (NSC and USC) as compared to those containing only the polymer (FPC) demonstrate that there may be a slight increase in toxicity when QDs are delivered to cells. Several studies have previously demonstrated that CdSe-ZnS core-shell QDs have limited inherent cytotoxicity, and biocompatibility of the QDs depends primarily on the surface coating of the QDs. However, delivery of large complexes to cells has previously demonstrated elevated cytotoxicity levels, irrespective of the core material. Therefore is it likely that the increase in cytotoxicity is not due to the delivery of QDs, but due to the indiscriminate delivery of large complexes. Addition of C32 decreases cell viability further, and could be due to increased cationic charge of the complex, or due to increased uptake of the complex. There appears to be a trade-off between transfection and cell viability, as observed for other cationic polymer-based gene delivery systems. However HSC appear to mediate higher levels of transfection and less toxicity as compared to several commercially available cationic gene delivery polymers.

Analysis of gene transfection data clearly demonstrates that the addition of C32 polymer and QD-dextran/DNA to form HSC results in increased transfection and decreased cell viability, but the interaction mechanism between the polymer and NSC is unclear. Since both the polymer and the composite NSC are cationic, C32 do not electrostatically adsorb to the entire complex. Instead, the two may form physical blends, or C32 may attach to anionic, uncomplexed DNA regions. Discussing the latter involves further probing the binding mechanism between the cationic QD-dextran and the anionic DNA. It is unclear whether contiguous segments of DNA complex with and wrap around cationic QD-dextran cores, multiple strands interact with single QD-dextran core, or whether they associate via other mechanisms. Whichever the case may be, it is likely that most of the DNA is condensed by the dextran-coated QDs, forming cationic beads. However, there may be exposed regions of anionic DNA between adjacent QD-dextran cores that are not condensed. This structure is analogous to that of DNA condensed with histones to form nucleosomes, and DNA complexed with dendrimers. It is possible that cationic C32 attaches to these unbound DNA segments, resulting in further cross-linking and complexing of the NSC. This mechanism of C32 incorporation would also explain the more elaborate branching observed in HSC (Fig. 5.10b), as compared to NSC (Fig. 5.10a).
5.4.4 Fluorescent Tracking

The ability of the dextran-coated QDs and DNA to remain associated inside cells post-delivery was investigated, to validate the use of QDs as labels for tracking DNA delivery. QD-dextran (green) and rhodamine labelled DNA plasmid (red, 5.1 kbp,) complexed as NSC and HSC were delivered to cells for 1 hour, the cells were fixed 6 and 12 hrs post treatment, and the cellular distribution of the QDs and labelled DNA characterized (Fig. 5.14). For NSC treated cells (top panel), the QD and DNA were either colocalized, or in very close proximity to each other, distributed punctuately throughout the cell. With increasing time, they appeared to localize towards the center of the cells, indicating nuclear localization. HSC treated cells (bottom panel) also showed both QDs and DNA, but with the QDs in excess. In the cytoplasm, most of the DNA is adjacent to (red-green), or complexed with (yellow), QD-dextran. Their punctuated distribution indicates that the complexes may be endosome-bound. In the nucleus, more free (red) than complexed (yellow) DNA is observed, both diffusely spread and localized around the nucleoli. Since protein production is first detected approximately 6 hrs after incubation of cells with HSC (see Fig. 5.15), some DNA dissociation is expected at that time.

![Figure 5.14](image)

**Figure 5.14** Confocal microscopy (100x, scale bar 10 μm) of QD-dextran (green) and rhodamine labelled DNA plasmid in fixed cells 6 and 12 hrs after treatment with HSC. Both dissociated (red) and complexed (yellow) DNA is observed in the nucleus and the cytoplasm.
Optical tracking of QD-dextran/DNA complexes and the gene product, from delivery to protein production, was demonstrated by visualizing HSC treated COS-7 cells. Cells were treated for 15 mins with HSC comprising QD-dextran (green) and unlabelled pEGFP DNA encoding for enhanced green fluorescent protein (EGFP). Post-treatment, they were incubated in an environmental chamber and continuously visualized using fluorescence microscopy. Since both the QD and expressed protein were green, they could not be independently tracked inside cells based on color. Instead, their trajectories were inferred by comparison (data not shown). The route of HSC within the EGFP expressing cell was compared with that of HSC within other cells, treated under the same conditions, but not expressing EGFP. The evolution of EGFP production was compared to that of cells treated with red QD-containing HSC (red QDs were not distinctly visualized using continuous imaging). Analyzing the fluorescence of the cells (Fig. 5.15) demonstrates that the complexes are initially present diffusely over the entire cell (data not shown), indicating attachment to the cell surface. Approximately 2 hr 20 mins post treatment punctuate green vesicles begin to appear, likely due to endocytosis of the complexes. At 3 hrs post treatment, several such green vesicles are visible, localizing around the perinuclear region (asterisk), suggesting continued endocytosis and nuclear localization. By 3 hr 40 mins (arrow) green particulates begin to appear inside the nucleus, where they continue to localize. At 5 hrs post treatment substantially more green is observed in the cytoplasm and is distributed more diffusely than the endocytosed quantosomes, likely indicating the beginning of EGFP production. The color becomes more intense and widespread with time, and is stable for at least 3 days post treatment (data not shown). These data demonstrate that QDs do not hydrolyze or photobleach over the observed period, and may be repeatedly imaged to track gene delivery in individual cells. Tracking the HSC intracellularly will help elucidate delivery routes and identify cellular barriers impeding delivery. Understanding these mechanisms can subsequently aid in the design of more efficient delivery vehicles.

Long term fluorescent labeling and semi-quantification of gene delivery complexes with HSC was investigated by visualizing QD-dextran (red) and EGFP (green) in fixed cells (Fig 5.16). The cells were treated with HSC for 2 hours, and results were obtained 36 hours post treatment. Projections (left panels) and their respective orthogonal sections (right panels) show EGFP spread diffusely throughout the cytoplasm and concentrated densely in the spherical nucleus. QD particulates in the cytoplasm (red) and nucleus (yellow) were brightly fluorescent, and resisted oxidative degradation even after extended exposure to intracellular environments. This demonstrated that QDs can be visualized simultaneously with the gene product.
Figure 5.15 Continuous tracking of HSC (particulate green) cellular internalization and gene product expression (bright, diffuse green), using time-resolved fluorescence microscopy (100x, scale bar 10 μm). HSC are endocytosed (2 hr 20 min), trafficked to the nucleus (3 hr, asterisk) and enter the nuclear region (3 hr 40 min, arrow). EGFP expression is first observed 5 hrs post treatment, and remains stable for at least 14 hrs.
Figure 5.16 Confocal projection and (b) orthogonal sections (100x, scale bar 10 µm) of HSC treated fixed cells show EGFP (green) and QD-dextran (red). EGFP is distributed diffusely in the cytoplasm and concentrated densely in the nucleus, while QDs are found in the nuclear/perinuclear region. Orthogonal slices XZ (b1) and YZ (b2) show QDs localizing inside the cell.
Multi-gene delivery was demonstrated and characterized using different color HSC, to deliver DNA encoding for GFP variants. Live cells treated for 2 hours with red HSC (QD 4.5 nm) comprising DNA encoding for nuclear localizing GFP (5.7 kbp) were imaged 2 days post treatment (Fig. 5.17a). Results show intense GFP localization in the large spheroid nucleus, and diffuse spread in the perinuclear region, indicating GFP en route to the nucleus. Red QDs are visible in and around the nucleus, indicating nuclear localization of the HSC. Cells treated with blue HSC (QD 2.5 nm) delivering DNA encoding for mitochondrial localizing GFP (5.7 kbp) were observed 2 days post treatment (Fig. 5.17b). Results show GFP punctuately distributed in the cytoplasm, concentrating in the perinuclear region and absent from the nucleus, indicative of mitochondrial localization. Blue QDs are seen inside the cells, localizing in and around the nucleus. Simultaneous delivery of red and blue HSC delivering nuclear and mitochondrial localizing GFP, respectively, was also investigated (Fig. 5.17c). The results show GFP localization in the nucleus, perinuclear region and mitochondria, with both red and blue HSC visible in and around the nucleus. These data demonstrate the use of HSC as optical labels to simultaneously characterize multi-gene delivery systems.

The data presented demonstrated that QD-based, fluorescent gene delivery assemblies can be used to track DNA-containing vehicles to identify barriers to gene delivery, and may be used to simultaneously visualize multi-gene delivery using different colored QDs.
5.5 Summary & Conclusions

5.5.1 Nanostructural Enhancement

- Several cationic, single-layer QD complexes were used to condense DNA and delivered to cells; based on high levels of protein expression and low levels of non-specific cytotoxicity, dextran coated nanoparticles were selected as the delivery cores.
- Cationic dextran coated QDs effectively condensed DNA to form nanostructured complexes (NSC), and when delivered to cells yielded higher levels of protein expression than their unstructured and free polymer counterparts.
  - Increasing the QD to DNA ratio increased the toxicity of the complexes
  - Optimized QD to DNA mass ratio was at 1.5:1
- Integrating functionalized gene delivery polymers with NSC yielded heterostructured complexes (HSC) which demonstrated significant enhancement in protein production levels and increase in cytotoxicity, when delivered to cells.
  - Optimized heterostructures were assembled at C32 to DNA ratio of 10:1 where protein production was enhanced to 250% and cell viability decreased to 72%.
  - Lower ratios did not yield as much enhancement in protein production, while higher ratios yielded excessive cytotoxicity
- Hybrid NSC and HSC complexes had defined secondary nanostructures with beads-on-a-string morphologies biomimetic of the nucleosomes, not observed in their free polymer and unstructured counterparts.

5.5.2 Fluorescent Tracking

- HSCs were used to continuously track the intracellular route of QD-polymer-DNA complexes inside single cells, from delivery to protein production.
  - Colocalization with labeled DNA demonstrated that DNA was bound to QD
  - Continuous long-term visualization did not cause photobleaching of QDs
- Multi-colored HSCs were used to tag the delivery of multiple genes, using different colors as labels for genes expressing different proteins.
5.6 References

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Chapter 6: Conclusions & Future Recommendations
6.1 Main Results

**Aim 1a: Synthesis of CdS QDs**
The use of both cationic polymers and histidine oligopeptides mediated the synthesis of optically active CdS QDs, as discussed below.

**Cationic polymer-mediated synthesis**
Optically active CdS QDs were successfully synthesized, using cationic polymers as the capping agents, with the properties of the nanoparticles dependent on (i) chemical structure, (ii) mass concentration, and (iii) chain length of the polymers used. Using bPEI produced QDs with the most defined, split-peak absorbance spectra, and brightest fluorescence. Using IPEI and PAA for QD capping resulted in the production of QDs with single-peak absorbances, and reduced fluorescence as compared to bPEI produced QDs. For all the polymers tested, there was an effective concentration range within which the polymer formed optically active QDs. These polymer-mediated QDs had highly cationic surfaces, and cause high toxicity when incubated with cells. Due to their cytotoxicity they were not investigated for subsequent use in bioapplications.

**Histidine oligopeptide-mediated synthesis**
Histidine oligopeptides with 3 or more covalently linked residues were effective in producing optically active CdS QDs. Varying the number of covalently linked histidine residues affected the properties of the synthesized QDs; increasing the chain length resulted in blue-shifting of the absorbance and the emission spectra. Varying the peptide dose also affected the properties of the nanoparticle, but to a lesser extent. CdS QDs had crystalline cores, with lattice spacing indicating wurtzite crystal structure. However the emission peaks of these QDs were not very finely resolved, and when incubated with cell, they aggregated in solution. Their subsequent use as bioprobes was not investigated.

Therefore despite being optically active, the use of aqueous-based QDs as bioprobes was not pursued further, and the use of commercially available QDs was investigated.

**Aim 1b: Modification of QDs with organic polymers**
QD surface ligand exchange with mercaptoacetic acid (MAA) was optimized to make hydrophobic, commercially available QDs miscible in water. These modified QDs were negatively charged, and subsequently modified by layer-by-layer deposition of oppositely charged polyelectrolytes. Cationic, single-layer and anionic, multi-layer complexes were prepared using PAA and PVSA, respectively. The use of anionic GaN cores, and the subsequent deposition of PDMC and PAAc demonstrated that this technique was robust enough to allow integration of polyelectrolytes on semiconductor QDs, independent of the core-polymer chemical compositions. The charged QD-polymer assemblies were electrostatically assembled on oppositely charged, patterned substrates with high selectivity and pattern integrity.
**Aim 2: Investigation of biocompatibility of polymer-coated QDs**

Biocompatibility and cellular interactions of uncoated, cationic single-layer, and anionic, multi-layer QD assemblies were investigated, as discussed subsequently.

**Uncoated & Single Layer Systems**

Cellular delivery of uncoated, anionic CdSe-ZnS-MAA, polystyrene-MAA and GaN nanoparticles did not cause excessive cell death demonstrating that the nanoparticles were not inherently cytotoxic. The anionic nanoparticle cores were coated with different cationic polymers, forming cationic single-layered complexes. Biocompatibility of these cationic complexes depended on the chemical structure of the polymer, and was relatively independent of the inorganic core; polymers with high charge density (bPEI, PAA, PDMC, pLys, pArg) caused widespread cell death, while bio-derived cationic polymers with lower charge densities (N-dextran, N-cellulose) were more biocompatible.

**Multi-Layer Systems**

Dextran coated QD were subsequently coated with anionic polymers to form stable anionic multi-layered structures; QD-dextran were selected due to their low cytotoxicity and high surface charge and stability. Biocompatibility of anionic multi-layered heterostructures predominantly depended on the chemical composition of the outermost, anionic polymer; synthetic anions with high charge densities (PVSA, PAAc) caused cell death and distortion, while bio-derived polymer with lower charge density (DS) was more biocompatible. Cellular interactions of multi-layered structures depended on the chemical structures of both the cationic and the anionic polymers; coating cytotoxic cationic QD cores (bPEI, PAA, PDMC coated QDs) with biocompatible polyanionic DS resulted in partial shielding of the cytotoxicity.

Therefore, the overall biocompatibility of the single-and multi-layered systems depends primarily on the chemical compositions of the coating polymers, and is almost independent of the nanoparticle core.

**Aim 3: Assembly of QD-polymer-DNA hybrids for gene delivery**

**Nanostructural Enhancement**

Several cationic, single-layer QD complexes were used to condense DNA and mediate delivery to cells; based on high levels of protein expression and low levels of cytotoxicity, dextran coated nanoparticles were selected as the delivery cores. Cationic dextran coated QDs condensed DNA more effectively and when delivered to cells yielded higher levels of protein expression than their unstructured and free polymer counterparts. Integrating functionalized gene delivery polymers with NSC yielded heterostructured complexes (HSC) with significantly enhanced protein production levels with a slight increase in cytotoxicity when delivered to cells. Hybrid NSC and HSC complexes had defined secondary nanostructures with beads-on-a-string morphologies.
mimetic of the nucleosomes, not observed in their free polymer and unstructured counterparts.

**Fluorescent Tracking**

HSCs were used to continuously track the intracellular route of QD-polymer-DNA complexes inside single cells, from delivery to protein production; the particles did not photobleach or cause cytotoxicity, and were internalized inside the cell. Multi-colored HSCs were used to tag the delivery of multiple genes, using different colors as labels for genes expressing different proteins; red and blue QDs were used to assemble complexes delivering GFP plasmids localized in the nucleus and the mitochondria, respectively.

Hybrid QD-polymer complexes demonstrated enhanced gene delivery with biomimetic nanostructuring of DNA plasmid, and their efficiency could be further increased by integrating specialized gene delivery polymers in the complex. These fluorescent probes can be used to continuously track and characterize multi-gene delivery.
6.2 Future Extensions

The QD-polymer coated complexes prepared using layer-by-layer deposition were used for gene therapy, but their applications can be easily extended to other biological and non-biological systems. Some of these potential applications are subsequently discussed.

6.2.1 Gene therapy

Optimize structural morphology of the complex

The nanoparticle core has a significant effect on enhancing the gene delivery properties of the complex, by enabling more efficient interactions between the cationic QD and the anionic DNA. These properties may be further enhanced by using QDs of different sizes and shapes, and characterizing their effect on DNA condensation and gene delivery efficiency.

Develop semi-quantification models for elucidating gene delivery pathways, identifying cellular barriers, and designing functionalized delivery platforms

The relative concentrations of the QD-polymer/DNA complexes can be optimized to enable semi-quantification of the delivered plasmid, by using the fluorescence of the QD cores as a marker for DNA concentration. Based on this fluorescence, the amount of plasmid inside a cell can be quantified, and its cellular distribution be characterized. This can enable elucidation of the gene delivery pathways, and help model the specific bottlenecks of gene delivery systems; the relative amounts of plasmid arrested in cellular barrier, en route to the nucleus, and present inside the nucleus can be determined. By varying the chemical compositions of the QD-polymer complexes, their effects on cellular distribution of delivered DNA may be characterized. This will allow understanding how the structure of the polymer affects its transit inside a cell, and enable identification of functionalities that are efficient at mediating escape from specific cellular barriers. Integration of different functionalities into a multi-functional system that can systematically by-pass the cellular barriers can lead to the design of a highly efficient DNA delivery system.

Use semi-quantification methods to characterize multi-gene arrays

Semi-quantification of the delivered plasmid can also be used as an indicator for the protein expression. By carefully controlling the amounts of DNA delivered, the minimum levels required for effective therapeutic doses may be elucidated. The combinatorial effects of delivering multiple genes can also be investigated, by using different color QDs. Since most genetic disorders are complex manifestations involving several different genes, hybrid QD-polymer delivery systems may help determine optimal combinations and doses for subsequent therapeutic applications.
6.2.2 Other applications

QD-polymer assemblies may be used for other bioapplications, in addition to gene delivery. These include:

- Live-cell and *in vivo* imaging using modified QDs functionalized with ligands to specifically recognize cells with characteristic morphologies.
- Immunolabelling and biological labeling of proteins, organelles and other biological structures; since their surface chemistries can be easily manipulated, they are stable in aqueous environments, and their optical properties are preserved, the QD-cores may act as efficient bioprobes. Furthermore, successful DNA delivery indicates release of QD-DNA complex inside the cellular cytoplasm, which is a barrier for several other QD-labeling systems.
- Detection of biological species; the nanoparticle surfaces can be easily tailored to express affinity for specific biological molecules. Nanoparticle cores (e.g. gold) can be used that elicit specific changes as a result of the ligand binding to the nanoparticle surface, therefore enabling quantification of the species in solution.
- FRET systems can be assembled by tailoring the surfaces of the nanoparticles to facilitate quenching interactions. By varying the properties of the core and the surface ligands, high throughput systems capable of detecting several different species may be assembled.

Non-biological systems may also be developed. These include:

- Nanostructured microelectronics which can combine QDs, and similarly modified nanotubes and nanorods, to produce patterned, structured arrays with controlled electronic properties to produce nano-circuits.
- Photophysics systems that utilize the inherent optoelectronic properties of the QDs.
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To my family and friends, thanks for putting up with me!!!
Appendices
Appendix I. Quantification of QD concentration based on absorbance

Fig A1.1 Quantifying CdSe-ZnS Adirondack red QDs based on absorbance

Fig A1.2 Quantifying CdSe-ZnS Birch green QDs based on absorbance
Appendix II. Figures of Polyelectrolytes

Fig A2.1. Structures of cationic polymers (a) bPEI, (b) IPEI, (c) PAA, (d) PDMC, (e) pLys, (f) pArg, (g) dextran (source: from reference 1), (h) cellulose (reference 2). For detailed discussion of structure and charge density, see Section 4.1.2.1.
Fig A2.2. Structures of anionic polymers (a) PVSA, (b) PAAc and (c) DS (source: reference 3). For detailed discussion of structure and charge density, see Section 4.1.2.2.

**References**

1. [http://www.dextran.dk/deae_index.htm](http://www.dextran.dk/deae_index.htm)
2. [http://www.sigmaaldrich.com](http://www.sigmaaldrich.com)
Appendix III. List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>bPEI</td>
<td>Branched polyethyleneimine</td>
</tr>
<tr>
<td>C32</td>
<td>Poly-β-amino ester, gene delivery polymer</td>
</tr>
<tr>
<td>CA</td>
<td>Capping agent</td>
</tr>
<tr>
<td>COS-7</td>
<td>Monkey kidney epithelial cells</td>
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<tr>
<td>Cys</td>
<td>Cysteine</td>
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<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole filter (ex 360/40, em 460/50)</td>
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<tr>
<td>Dextran</td>
<td>Diethyl aminoethyl dextran</td>
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<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek</td>
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<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<td>DS</td>
<td>Dextran sulfate</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>FPC</td>
<td>Free polymer complex</td>
</tr>
<tr>
<td>FPC*</td>
<td>Optimized free polymer complex</td>
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<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>GFP</td>
<td>Green fluorescence protein, filter (ex 450/40, em 525/50)</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HEC</td>
<td>Quaternized hydroxylethyl ethoxy cellulose</td>
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<td>His-n</td>
<td>Histidine oligopeptide with ‘n’ covalently linked units</td>
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<td>HSC</td>
<td>Heterostructured complex – C32 integrated with NSC</td>
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<td>IPEI</td>
<td>Linear polyethyleneimine</td>
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<tr>
<td>LPF</td>
<td>Lipofectamine</td>
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<td>MAA</td>
<td>Mercaptoacetic acid</td>
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<td>MEtOH</td>
<td>Mercaptoethanol</td>
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<td>Nanostructured complex – dextran coated QDs assembled with DNA</td>
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<td>Poly arginine</td>
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<td>QD(s)</td>
<td>Quantum dot(s)</td>
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<td>QD-material</td>
<td>Outermost layer of the QD-organic complex</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TOPO</td>
<td>Trioctylphosphine oxide</td>
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