

**APPLICATIONS OF GEL ELECTROPHORESIS
IN QUANTUM DOT CONJUGATES' SEPARATION AND PURIFICATION**

A Thesis presented to the Faculty of the Graduate School
University of Missouri

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
LUXIN WANG

Dr. Xudong Fan and Dr. Azlin Mustapha, Thesis supervisors

JULY 2009

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

**APPLICATIONS OF GEL ELECTROPHORESIS
IN QUANTUM DOT CONJUGATES' SEPARATION AND PURIFICATION**

Presented by Luxin Wang

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

Dr. Xudong Fan, Associate Professor, Biological Engineering

Dr. Azlin Mustapha, Associate Professor, Food Science

Dr. Fu-hung Hsieh, Professor, Biological Engineering

ACKNOWLEDGEMENTS

My sincere thanks and appreciation go to Dr. Xudong Fan and Dr. Azlin Mustapha, for their support and guidance to my Biological Engineering graduate studies. Without their encouragement and assistance, I wouldn't have finished my research and course work on time.

Special THANKS to Dr. Mustapha, for her generous financial support during my studies.

I would like to express my gratitude to Dr. Fu-hung Hsieh, for serving as a committee member, providing suggestions and help during my studies.

At the same time, I gratefully acknowledge Jay Cupps, Chung-Shieh Wu, Dr. Ya Guo and my other graduate friends for their friendship and help.

Finally, I am wholeheartedly grateful to my parents, for their love, caring, and support.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF FIGURES	v
LIST OF TABLES	vii
ABSTRACT.....	viii
Chapter	
1. INTRODUCTION	1
1.1 Fabrication of quantum dots	1
1.1.1 Colloidal synthesis of quantum dots	3
1.1.2 The “TOP/TOPO” based synthesis method.....	6
1.2 Properties of quantum dots	6
1.2.1 Optical properties of quantum dots	7
1.2.2 The core-shell structures of quantum dots.....	8
1.3 Application of quantum dots.....	10
1.3.1 Quantum dots and <i>in vivo</i> imaging.....	10
1.3.2 QD application in bacteria and DNA detection.....	11
1.4 Toxicity of quantum dots.....	13
1.5 Water soluble quantum dots.....	15
1.6 Bioconjugation techniques.....	16
1.6.1 Zero-length crosslinkers.....	16
1.6.1.1 EDC.....	16
1.6.2 Avidin-biotin system.....	19
1.6.3 Protein A.....	22
1.7 Antibody modification.....	22
1.8 Application of labeled antibodies.....	24
1.9 Gel electrophoresis.....	24
1.10 <i>E. coli</i> O157:H7.....	25
2. MATERIALS AND METHODS.....	27

2.1	Property study of Quantum dots.....	32
2.1.1	Calculation of quantum dot concentration.....	32
2.2	Surface modification of QDs.....	33
2.3	Gel electrophoresis.....	34
2.4	QD protein A complex recollection from agarose gel	35
2.5	Cell based microarray.....	37
3.	RESULTS.....	39
3.1	Gel electrophoresis application.....	41
3.2	QD EDC protein A complexes.....	41
3.3	QD protein A recollection from agarose gel.....	46
3.4	Biological property of QD protein A complex recovered from the gel.....	46
4.	CONCLUSIONS.....	51
5.	REFERENCES.....	53

LIST OF FIGURES

Figure		Page
1.1	TM-APM image of GaAs QDs made by nanolithography	2
1.2	Water beads spreadyed on glass surfaces	3
1.3	Colloidal synthetic scheme demonstration.	5
1.4	Absorpthion spectra of different quantum dots	7
1.5	The fluoresence/emission spectra of different quantum dots	8
1.6	Chemical structure of EDC.....	17
1.7	EDC crosslinker	17
1.8	Chemical structures of CMC and DCC	19
1.9	Chemical structures of biotin and biotein/avidin system.....	21
1.10	Fc region and antigen binding sites on antibodies	22
2.1	Scheme for synthesis QD core structures.	29
2.2	Scheme for CdZe/ZnS core/shell structure synthesis.	30
2.3	Scheme for making water soluble QDs	31
2.4	GeBa \textit{flex} tube.....	35
2.5	Diagram for eulutin QD protein A complexes out of the gel	36
2.6	Glawss slide with circles on top.....	37
2.7	Cell based microarray	38
3.1	Fresh red quantum dots.....	39
3.2	QD EDC protein A complexe structure	42
3.3	Gel electrophoresis of QDs in different buffers.....	43

3.4	Gel electrophoresis of QD EDC protein A complexes	44
3.5	Gel electrophoresis of QD EDC with various protein A amounts....	45
3.6	Recollection of QD EDC protein A complexes from the gel.	47
3.7	Microarray study of eluted green QD EDC protein A complexes....	49
3.8	Microarray study of eluted red QD EDC protein A complexes.....	50

LIST OF TABLES

Table		Page
2.1	Chemicals used in QD synthesis.....	27
2.2	Different volume ratios of protein A and QDs	34
2.3	Chemical used in gel electrophoresis.....	34

APPLICATIONS OF GEL ELECTROPHORESIS
IN QUANTUM DOT CONJUGATES' SEPARATION AND PURIFICATION

Luxin Wang

Dr. Xudong Fan and Dr. Azlin Mustapha, Thesis Supervisors

ABSTRACT

The objectives of this study were to build Quantum dot (QD) crosslinker complexes for antibody conjugation usage, to purify QD crosslinker complexes by gel electrophoresis and to check the biological functionalities of eluted QD crosslinker complexes recovered from gel electrophoresis by cell based microarray. Zero-length crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was chosen to be the first crosslinkers, followed by the conjugation with secondary crosslinker protein A. The purpose of adding secondary crosslinkers was to make uniform QD crosslinker complexes. Due to the high affinity between protein A and the Fc region of antibodies, QD EDC protein A complexes were in uniform structures and all antigen binding sites faced outwardly. Gel electrophoresis is a method used for separating DNA, RNA or proteins in biological studies. In this study, gel electrophoresis was adopted to check the

complete conjugation between QDs and protein A. In addition, it was successfully used as a separation method for purifying conjugated QDs. GeBaflex tubes were used to elute the conjugated QDs from the gel, these recovered QD EDC protein A complexes showed their biological functionalities in following cell based microarray studies. Fluorescence gave out by the cells labeled with QD antibody conjugates successfully proved that gel electrophoresis can be used as an efficient separation and purification method for QD crosslinker complexes. The recovered purified QD crosslinker complexes keep their biological functionalities and can be used for future antibody conjugation.

CHAPTER 1

INTRODUCTION

In 1932, Rocksby found that the red or yellow color of some silicate glasses could be linked to microscopic inclusions of CdSe and CdS (Rocksby, 1932) and in 1985, Ekimov and others discovered that these changes in color were linked to the energy states determined by quantum confinement in CdSe or CdS “quantum dots”.

The term “quantum dots” (QDs) refers to crystal structures with extremely small dimensions. Due to its small size, the electronic states begin to resemble and its electronic characteristics and optical properties are changed (Woll and others, 2002). Compared to bulk (three-dimensional) materials and quantum well (two-dimensional) structures, QDs are zero-dimensional systems (Huang and others, 2002) and their electronic states are fully quantized, which are similar to a single atom or atomic system. This fully quantized system is stable against any thermal perturbation.

Due to those unique properties quantum dots have, considerable efforts have been made to fabricate quantum dots and use QDs in different research fields. The desire to identify, understand, and explore the size-dependent properties of QDs at the nanometer scale has literally been simulated since 1985 and motivated quantum dots research development.

1.1 Fabrication of Quantum dots

Techniques used to fabricate Quantum dots had been modified and optimized by many scientists. At the beginning, techniques like lithographic patterning and quantum

well (QW) structure etching were used to make semiconductor hetero-structures, which provided carrier confinement in all three directions and behaved as electronic quantum dots (QDs). Unfortunately, the sizes of structures made by electron beam lithography or focused ion beam lithography were larger than the desirable level and the distances between QDs were large and generated additional emission from sidewalls and QW or QWR structures (Lee, 2002). A picture of GaAs quantum dots made by a lithography method is shown in Figure 1.1.

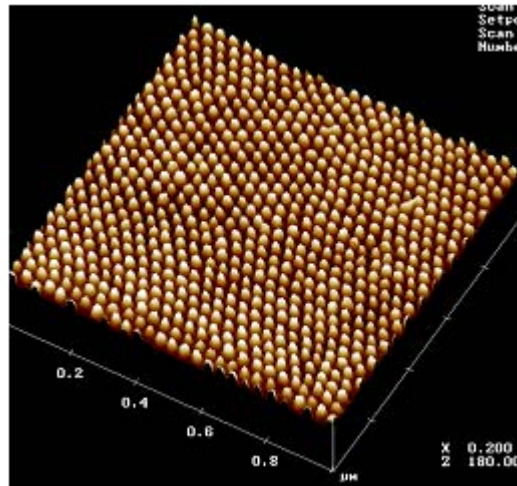


Figure 1.1 Tapping-mode atomic force microscope (TM-AFM) image of an array of GaAs quantum dots made by block copolymer nanolithography. (Adapted directly from: <http://www.princeton.edu/~polymer/nano.html>, Accessed on April 5, 2009)

Recently, quantum dots have been made through molecular beam epitaxy. In this process, chemicals are evaporated and sprayed into small objects by condensing onto a substrate surface (Molecular Beam Epitaxy, 2005). This process likes spraying water on glass surfaces (Figure 1.2). When water falls onto glass surfaces, the water condenses

into many tiny water balls. As more layers are sprayed, the size of the balls starts to grow. When the balls reach a specific size, they become quantum dots.



Figure 1.2 Water beads sprayed on glass surfaces. (Adapted from <http://www.scienceimage.csiro.au/index.cfm?event=site.image.thumbnail&id=6202&viewfile=f&divid=BU>, Accessed on April 5, 2009)

One drawback that both the lithographic method and epitaxy method could not overcome is that quantum dots generated from these methods are hard to use. Because those dots are attached to a substrate. Thus, a fabrication method which can make “free”, uniform in composition, size, shape, internal structure, and surface chemistry QDs is essential to further successfully understanding their size-dependent properties.

One method that is considered to be easier, cheaper, and shows good productivity is the colloidal synthesis method. Due to the advantages of the colloidal method, it has been widely used in industry and scientific research labs for routinely manufacture of quantum dots.

1.1.1 Colloidal synthesis of quantum dots

The colloidal synthesis method is a process in which particles are made from precursor compounds dissolved in solutions. There are three components in the system; they are precursors, organic surfactants and solvents. When the reaction system is heated

to a sufficiently high temperature, the precursors will transform into monomers (Wikipedia.com, Accessed on July 29, 2008). A general scheme for preparing monodisperse nanoparticles contains a single, temporally short nucleation event and a slower growth period on existing nuclei. The nucleation process can be done by a rapid injection of reagents into a reaction system containing hot solvent (Murray and others, 1993). A colloidal synthesis system setup sample is shown in Figure 1.3.

In the colloidal synthesis process, the temperature of the solution is the most critical factor. First of all, this temperature needs to be high enough to decompose the reagents, forming a supersaturation species in solution. These species will be used in nucleation of quantum dots. After the nucleation process, the concentration of these species in solution will drop below the critical concentrations for nucleation, thus further materials will only be added to the existing nuclei (Murray and others, 2001). Secondly, in order to realize the growth of the nuclei, the proper temperature needs to be adjusted and maintained. The temperature needs to be low enough to promote further nuclei growth.

In addition to the temperature, incubation time, concentrations of different chemical compounds, and surfactants are also important factors that can contribute and influence the quantum dot sizes. In general, quantum dots' sizes increase with the increasing of reaction time. The addition of more ingredients will increase the growth rate of the existing nuclei.

During nanoparticles' growth, the surfactants in solution can be adsorbed reversibly to the surfaces of nanoparticles, providing dynamic organic shells (also called

capping layer). This capping layer can stabilize the nanoparticles in solution and control their growth. Surfactants that bind to the core surfaces can slow down materials' addition rate to the core/nuclei surfaces, resulting in smaller sizes. After nanoparticles reach the desired sizes, further growth can be stopped by cooling the solution (Murray and others, 2001). It has been found that colloidal semiconductor quantum dots can have a very narrow size distribution (Parak and others, 2004) with the proper control of temperature, time, concentrations and surfactants.

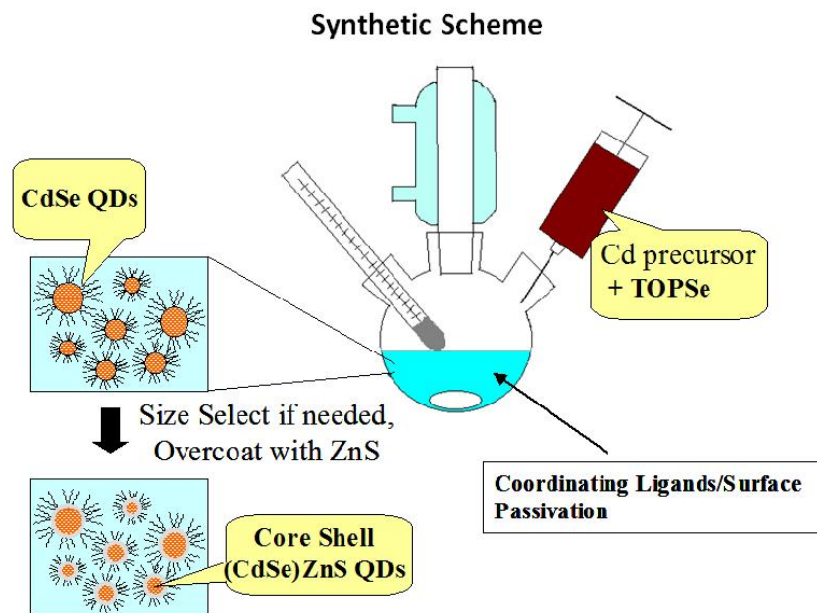


Figure 1.3 Colloidal synthetic scheme demonstration. (Adapted from Dr. Bawendi, M. website: <http://nanocluster.mit.edu/research.php>, Accessed on August 5, 2008)

Since colloidal nanocrystals are well dispersed in solution and not bound to any solid support or surfaces, they can be produced in large quantities in a reaction flask and be transferred as well as used in any solutions for various purposes. QDs produced by

colloidal method can be easily modified on their surfaces for adding biological molecules or active crosslinkers for a variety of usage.

1.1.2 The “TOP/TOPO” based synthesis method

“TOP/TOPO” synthesis method is an organometallic approach to synthesizing CdE (E = S, Se, Te) nanoparticles. The preparation method is based on the pyrolysis of organometallic reagents (for example, dimethylcadmium and bis selenium) by injecting them into the hot solvents (TOPO or TOP) (Matsumoto and others, 2004). This method is widely used due to its versatility, reproducibility and high quality of the particles.

1.2 Properties of quantum dots

By shrinking the bulk material to a small quantum dot, the properties of quantum dots become very different with three dimensional systems (bulk materials), two dimensional systems and one dimensional systems (quantum wires). Being a zero dimensional system, the charge carriers and excitations of QDs are confined in all three dimensions. Quantum dots are smaller than the De Broglie wavelength; their properties depend on their exact number of atoms. For large clusters or bulk materials, they have a well-defined lattice; properties no longer depend on their exact number of atoms (Parak and others, 2004). In addition, a quantum dot is a semiconductor. In semiconductors, the electronic properties are directly related to the transitions between the edges of the valence bands and the conduction bands. In a dot, the electrons have larger energies than electrons in a bulk at the conduction bands, although those electrons are in ground states (Parak and others, 2004).

1.2.1 Optical properties of quantum dots

The most striking effect in semiconductor nanoparticles is the widening of the gap E_g between the highest occupied electronic states and the lowest unoccupied states (Trindade and others, 2001). E_g is the minimum energy needed to create an electron-hole pair, energy lower than E_g cannot be absorbed by quantum dots. Thus, different quantum dots have different absorption spectra based on their different diameter and sizes, because the band gaps depend on the sizes of QDs. Figure 1.4 shows the absorption spectra of different sizes quantum dots.

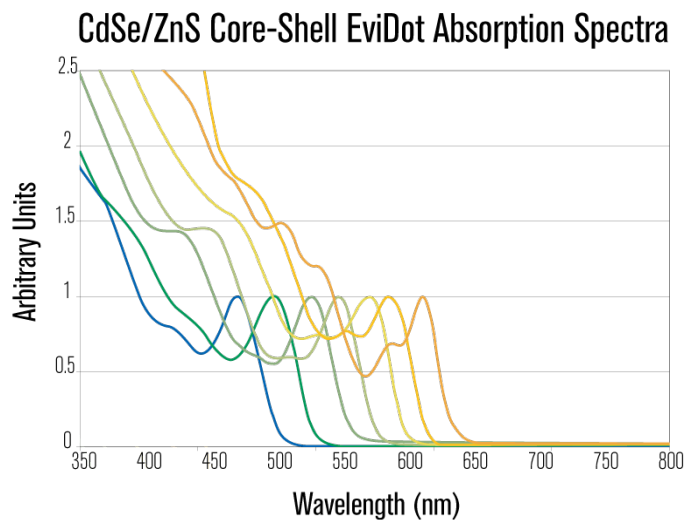


Figure 1.4 Absorption spectra of different quantum dots. The bigger the quantum dots size the bigger absorption wavelength. (Adapted directly from Evident Tech website: <http://www.evidenttech.com/products/evidots/evidot-specifications.html>, Accessed on August 2008)

The fluorescence/emission wavelengths of Quantum dots are longer than those of the absorbed light. In Figure 1.5, these are emission wavelengths of quantum dots. The position of the luminescence peak depends on the average quantum dot sizes and its width is correlated to the nanocrystals' size distribution. By measuring the maximum of

emission spectra and their width, those peaks and their values can be used to estimate the sizes or size distributions of quantum dots.

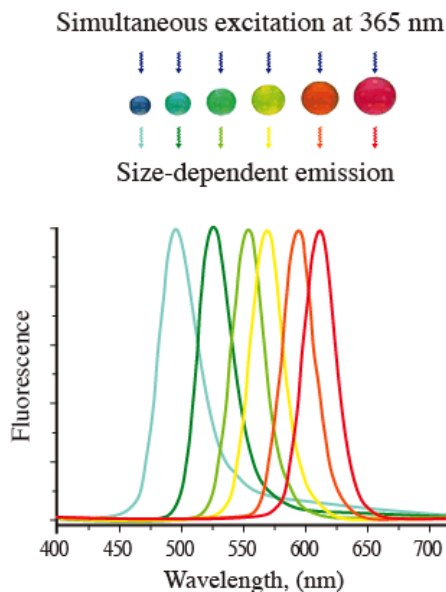


Figure 1.5 The fluorescence/ emission spectra of different quantum dots. (Adapted from website: <http://cnmt.kist.re.kr/main/pr/newsletter/save/60/sub22-3.html>, Accessed August 2008)

1.2.2 The Core-Shell structures of quantum dots

Coating a nanoparticle by another material will generate the “core-shell” nanoparticle structure. In “core-shell” structures, the cores are made from colloidal process. They can be made by metal or other materials. The shell parts of the quantum dots are made by organic materials. Usually, the core materials should be capped by materials that have a larger band gap. The conduction band energy of capping material is higher than that of the core material and the valence band energy of the capping material is lower than that of the core material (Eychmüller, 2004). There are many core/shell

systems are available, like hydrophobic monolayers, positively or negatively charged monolayers, or polymer layers (Katz and others, 2004).

One purpose of adding this capping layer is to prevent the excitons from spreading over. By adding shell layers, the excitons are forced to recombine and confined to the core. After adding the shell layer, the luminescence/fluorescence efficiency will be changed. According to Banin and Millo (2004), although for InAs/InP core/shell structures, the adding of shell led to the quenching of fluorescence, for InAs/CdSe core/shell structures, the quantum yield (fluorescence strength) actually increased up to a maximum value of 21% after the addition of shell layers (Banin and Millo, 2004).

Another reason to build the core/shell structure is to increase the stability of core structures of quantum dots. Core/shell structures with a shell made of materials having a larger band will provide protection and increased stability than just the core structure itself. One experiment done by Banin and Millo in 2004 proved this hypothesis. The stability of InAs core structures and InAs/CdSe core/shell structures was tested by measuring their absorption and emission spectra after a long storage time (ten months) under daylight. They found that the emission of InAs core structures was quenched by a factor of 40 compared to the fresh cores. For the core/shell structures, there was only a 16% to 13% reduction in emission strength (Banin and Millo, 2004).

Last but not least, the core/shell structure can prevent the aggregation of the nanoparticles into bulk materials and control the final dimensions of nanoparticles.

Colloidal CdSe/ZnS core-shell nanocrystals are one core/shell structure nanoparticles. They have attracted great attention from scientists for long time. These structures demonstrated high quantum yield and high photostability at room temperature and made them promising emitters for quantum information and potential biological applications (Alivisatos, 2004). Core/shell structures can also be used for devices like thin-film LEDs or nanocrystal quantum dot (QD) lasers (Klimov and others, 2000 and Mikhailovsky and others, 2002). CdSe/ZnS core/shell quantum dots have been the best understood quantum dots and commonly used.

1.3 Applications of quantum dots

Quantum dots are emerging as a new class of fluorescent probes for biomolecular and cellular imaging, target cells detection and DNA labeling dyes. They have unique optical and electronic properties such as “size-tunable light emission, improved signal brightness, resistant against photobleaching and simultaneous excitation of multiplex fluorescence colors” (Gao and Dave, 2007). The following part will talk about the application of quantum dots in biology and medical fields.

1.3.1 Quantum dots and *in vivo* imaging

Quantum dots have been widely tested for its application for *in vivo* imaging. In 1998, Chan and Nie demonstrated that QDs could be conjugated to a membrane-translocating protein called transferrin. The QDs retained their bright fluorescence *in vivo* after conjugation and were not noticeably toxic. This phenomenon revealed a potential application of QDs as intracellular labels for living cell studies. Another

example, in one cell motility test experiment, cells migrated over a substrate covered with silica-coated QDs, as the cells moved across the substrate; they got labeled with QDs, causing an increase in fluorescence from cells and a nonfluorescent “dark” path in their trails (Pellegrino and others, 2003). These phagokinetic tracks can be used to accurately assess invasive potential of different types of cancer cells.

Quantum dots have also been used to image the vascular systems in various animal models. In a report by Larson et al, the injected QDs could remain fluorescence and detectable when they circulated into capillaries in adipose tissues and the skin of a living mouse (Larson and others, 2003). The advantage to use quantum dots instead of organic dye is that quantum dots demonstrated longer lifetime than organic dyes. The circulation lifetime of an injected molecule depended on the size of the molecule and its chemical properties. Organic dyes can be quickly eliminated from circulation minutes after injection due to their small sizes and renal filtrations. QDs and other nanoparticles are larger than dyes and won't be cleared through the kidneys. Thus, quantum dots can keep its viability, brightness after they were injected to live mouse bodies.

1.3.2 QD applications in bacteria and DNA detection

One of the most popular ways to use nanoparticles in bacteria or DNA detections is to conjugate QDs to antibodies or complementary DNA bands, forming a fluorescence-based reporting assay. Zhao and others (2004), used QD antibody conjugates to detect *E. coli* O157:H7. In this study, nanoparticles of 60 nm in sizes were conjugated to monoclonal antibodies that are specific to *E. coli* O157:H7. Those antibody nanoparticle conjugates bound to antigens present on surfaces of *E. coli*. From that research, scientists

found that the signals produced by antibody nanoparticle conjugates are >1,000 times greater than that produced by the dye molecule-labeled antibodies (Zhao and others, 2004).

In recent years, the development of new technologies, like microarrays and Polymerase Chain Reaction (PCR), has provided powerful tools to help researchers solving biological problems. Nanoparticles, with their advantage of intense fluorescence signal, have been tried and tested their application in DNA based technologies, like in DNA hybridization process. Those nanoparticles are used as reporter dyes for targets detection and identification purpose.

In this DNA microarray study, the scheme includes three parts. They are 1. DNA immobilization on glass surfaces, 2. DNA probes labeling by nanoparticles and 3. DNA hybridization between the capture DNA and the probe DNA. One example, Rubpy-doped silica nanoparticles have been used in “one color” microarray, this novel microarray showed good correlation with the traditional phycoerythrin labeled method, but with a 20 times lower detection limits (Zhou and Zhou, 2004).

The usage of nanoparticles in the detection of bacteria, DNA and other cells, have been tested via different ways. Most of the studies showed promising results and discoveries. Those exciting developments in nanoparticle applications stimulated the further efforts on this area. Nanoparticles with their great potential as a better replacement for current widely used organic dyes opened another door for biological studies.

1.4 Toxicity of quantum dots

The potential toxic effects of semiconductor quantum dots have recently become a topic of considerable importance and discussion. For potential application *in vivo* tests, toxicity is the key factor to determine whether quantum dot imaging would be approved by regulatory agency and used for human beings (Gao and Dave, 2007), especially QDs have showed longer circulation time in living animals (Larson and others, 2003). The law community and scientists are on opposite sides. One reason for this disagreement is the lack of sufficient knowledge regarding different nanoparticles. Studies directly related to toxicity analysis have only started to gather some information. Detailed information about unwanted side effects in live cells is still needed, especially after hours of continuous exposure.

Some studies showed that QDs with stable polymer coatings are nontoxic to humans and animals (Dubertret and others, 2002; Gao and others, 2004; Lidke and others, 2005; Larson and others, 2003; Ballour and others, 2004; Stroh and others, 2005; Parungo and others, 2005; Jaiswal and others, 2003; Voura and others, 2004). But, very little is known about the effects of long-term exposure of different nanoparticles of different cell types and tissues. Surface modifications of nanoparticles have been thought as a good way to keep the QDs' stability and prevent the release of core/heavy metal materials (Maysinger and Lovric, 2007).

The toxicity of quantum dots, when tested *in vitro*, depends on several factors, like QD properties and cell status. From the QD's perspective, the core structure, when not protected, can gradually disintegrate and cause the release of toxic ions (like

cadmium ions) into to the medium (Derfus and others, 2004). Secondly, “the diffusion of oxygen is facilitated by the loose shell around the core, this can cause electron or energy transfer, which on the other hand, results in the formation of toxic reactive oxygen species (ROS)” (Lovric and others, 2005; Ipe and others, 2005; Samia and others, 2003; Green and Howman, 2005), leading to the cell damage or death (Ipe and others, 2005). Nanoparticles can induce the generation of Reactive Oxygen Species (ROS) in the cellular environment. Unfortunately, although it has been found that high levels of free material ions leading to the formation of ROS and oxidative damage have been detected in live cells that were exposed to the nanomaterials, the exact mechanisms for ROS production are still not well understood (Maysinger and Lovric, 2007). More research that includes *in vivo* long term exposure tests are needed.

In the mean time, the cellular environment and cell status play critical roles in their response to quantum dots. For example, cells exposed to quantum dots are more vulnerable when they don't have enough nutrients or growth factors, whereas cells cultured in the presence of serum are more resistant to QD induced cell death. One hypothesis stated that Quantum dots can interact with serum and other biological fluid components and change (reducing or increasing) their damaging effects on cells (Maysinger and Lovric, 2007).

As mentioned in the previous paragraph, cells in serum demonstrated better resistance to QD toxicity. “One of the best documented components used in cell culture medium that can modify nanoparticles' entry and the extent of nanoparticle-induced cytotoxicity is serum albumin. This biomolecule can reduce the internalization of

quantum dots and delay or reduce cells' death induced from the exposure to QDs” (Maysinger and Lovric, 2007).

Although debate exists about the application of QDs in biological studies and its toxicity, research related to its novel application is still going on. In the following part, the methods used for QDs modification for potential biological application will be discussed.

1.5 Water soluble quantum dots

Making the quantum dots water soluble is the first step for QD conjugation. As cells can not survive in an organic solvent, water soluble quantum dots is the format that can be used in biological related studies.

There are two general strategies for making water soluble quantum dots. One approach is to exchange the hydrophobic monolayer of ligands on the QD surface with hydrophilic ligands (Chan and Nie, 1998). Unfortunately, this method can cause particle aggregation and decreases the fluorescent efficiency. In addition, possible desorption of labile ligands from the QD surfaces can increase toxicity due to exposure of toxic QD elements. The second method is that the native hydrophobic ligands will be retained on the QD surfaces and rendered water-soluble through the adsorption of amphiphilic polymers with hydrophobic segments. There are several polymers that have been applied, octylamine-modified polyacrylic acid (Wu and others, 2003) and PEG-derivatized phospholipids (Dubertret and others, 2002). The hydrophobic domain can

interact with alkyl chains of the ligands present on the QD surfaces, whereas the hydrophilic groups face outwards and make the QDs water soluble.

Once water soluble QDs are available, conjugation which can link the biological compounds to QDs can be realized by the usage of various crosslinkers.

1.6 Bioconjugate techniques

In order to use the quantum dots, different crosslinkers have been tested and used to conjugate the quantum dots to different biological functional groups. In this section, three common bioconjugate techniques that have been widely proven to generate promising results in biological studies will be introduced.

1.6.1 Zero-length crosslinkers

The smallest available reagent systems for bioconjugation are zero-length crosslinkers. Those crosslinker compounds can realize the conjugation of two molecules by forming a bond without adding a new atom in between.

1.6.1.1 EDC

EDC, short for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, is the most popular carbodiimide for use in conjugating biological substances. EDC is a water soluble chemical, so it is easy to add EDC to any kind of reaction without treatment. The structure of EDC is showing in the following picture, downloaded from www.Wikipedia.com.

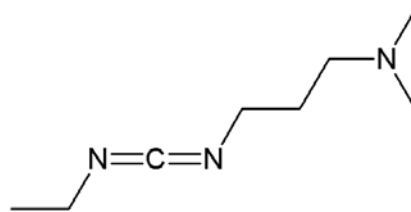


Figure 1.6 Chemical structure of EDC. Adapted from Wikipedia website on: http://upload.wikimedia.org/wikipedia/commons/8/87/EDC_Structure.png. Accessed on April 16, 2009.

EDC can cause a variety of chemical conjugation. It can link two molecules, one containing an amine group and the other one containing a carboxylate group, together. But, using EDC as a crosslinker has one drawback. That is the presence of both carboxylates and amines on one molecule will result in self-polymerization. The scheme of how an EDC crosslinker works is shown in Figure 1.7.

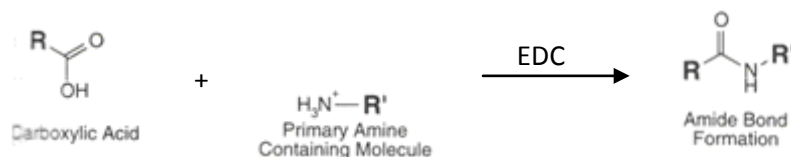


Figure 1.7 EDC reacts with carboxylic acids to create an active-ester intermediate. This intermediate can react with amine containing compounds to create conjugates. (Adapted from Hermanson, 1996).

The following EDC crosslinker advantages and applications were adapted from www.piercenet.com Accessed on April 19, 2009.

Advantages of EDC crosslinkers

- Efficiency of EDC-mediated coupling is increased with the presence of Sulfo-NHS

- Amide bonds formed with EDC crosslinking reaction can provide a neutral linkage
- Excess reagent and crosslinking byproducts can be easily removed by washing with water or diluted acid
- EDC is water soluble, so this crosslinking reaction can be done in physiologic solutions without adding an organic solvent
- High purity, crystalline EDC can be used to create high-purity activated derivatives

Applications of EDC

- Conjugate carboxyl to amine groups in peptides and proteins
- Convert carboxyls to amine-reactive Sulfo-NHS esters
- Crosslink proteins to carboxyl coated beads or surfaces
- Activate nanoparticles with amine-reactive Sulfo-NHS esters
- Couple haptens to carrier proteins (e.g. attach a peptide to KLH)
- DNA labeling through 5' phosphate groups

There are other chemicals that can be used to build zero crosslinkers. For example, CMC, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide, is a water-soluble reagent that can be used to form amide bonds between one molecule containing a carboxylate and another molecule containing an amine group. Another example is DCC, dicyclohexyl carbodiimide. DCC has been used for peptide synthesis and creating peptide bonds (Sheehan and Hess, 1955; Barany and Merrifield, 1980). EDC, CMC,

DCC, DIC all belong to the carbodiimides group in the zero-length crosslinker. Other zero-crosslinkers are like Wookward's reagent K, N, N'-carbonyldiimidazole, etc.

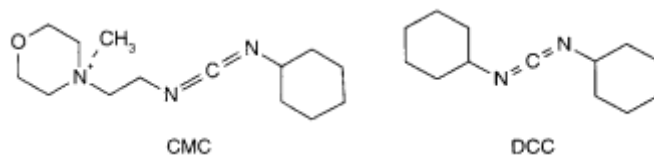


Figure 1.8 Chemical structures of CMC and DCC. (Adapted from Hermanson, 1996).

1.6.2 Avidin-biotin systems

One of the popular methods for conjugation is to use natural strong binding of avidin with a small molecule biotin. Modification reagents can add functional biotin groups to proteins, nucleic acids and other molecules in different shapes and relativities. Commercial kits are also available for adding biotin. The interaction between biotin and avidin is among the strongest non-covalent affinities, with a dissociation constant of approximately $1.3 \times 10^{-15} \text{M}$. When the biotin is bound to avidin, the interaction promotes great stability to the complex.

“Avidin is a glycoprotein found in egg whites that contains four identical subunits of 16,400 Daltons each. The total molecular weight is approximately 66,000 Daltons” (Green, 1975; Hermanson, 1996). Each avidin molecule can contain as many as four biotin binding sites. Biotinylated molecules and avidin conjugates can find and bind to each other under extreme conditions. The specificity of this binding is like antibody to antigen. “The only disadvantage of using avidin is that avidin has the potential to bind nonspecifically to components other than biotin due to its high pI and carbohydrate

content” (Hermanson, 1996). These nonspecific interactions can lead to increased background signals in some assays and inhibit the usage of the avidin-biotin system. However, the avidin-biotin system is still one of the most popular crosslinkers used in biological compound conjugation.

Compared with other ligand-binder interaction systems, the advantages of the avidin-biotin system have been summarized as following:

- The noncovalent interaction of avidin with biotin is characterized by a formation constant of 10^{15} L/mol. This is among the highest formation constants reported and is about 10^3 - 10^6 times greater than the interaction of ligands present on antibody and antigen. This high affinity can ensure that stability of the formed complex will not be disturbed by pH changes or presence of debris in the environment.
- Avidin and biotin binding is very specific. The binding process is directed only to the target of interest.
- Both avidin and biotin have four binding sites per molecule. This useful property makes it possible to use multiple biotinylated molecules and avidins to create mixtures consist of various functional compounds.
- The molecular weight of biotin is 244.31 Daltons. Because of its small size, when biotin is introduced to macromolecules, the binding of biotin won't influence the function of the macromolecules.

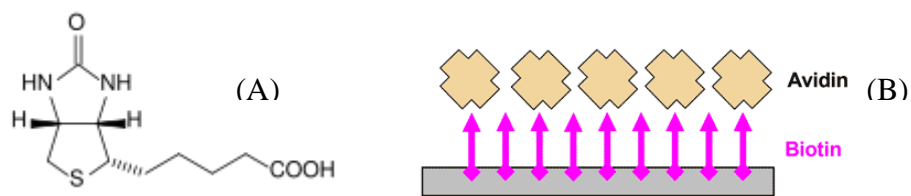


Figure 1.9 Chemical structure of biotin (A) (wikipedia.com) and biotin-avidin system (B). (URL: www.azom.com/details.asp?ArticleID=3753 Accessed on April 19, 2009)

1.6.3 Protein A

Protein A is a 40-60 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It is encoded by the *spa* gene. It has been widely used in biochemical research because of its ability to bind immunoglobulins. It can bind proteins from many mammalian species, most notably IgG. It binds with the Fc region of immunoglobulins through interaction with the heavy chain. Because of this special binding between protein A and the antibody, it makes the labeling of the antibody easier. In Figure 1.10, there are pictures of the Fc region of the antibody and antigen binding site on the antibody.

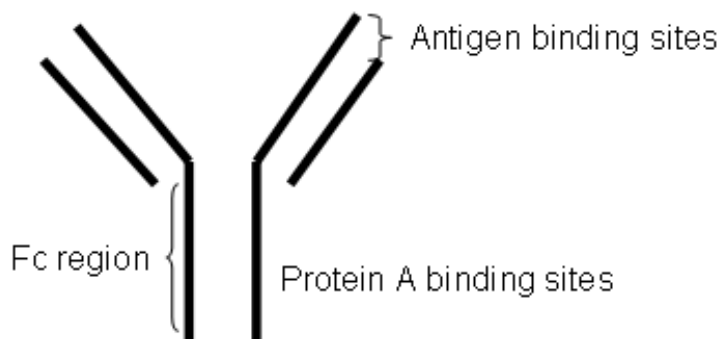


Figure 1.10 Fc region of antibodies and antigen binding sites on antibodies.

1.7 Antibody modification

Methods for antibody modification have been developed in many previous studies. It is an important step for any further conjugation between antibodies and nanoparticles, as well as future applications in biological researches. Site-specific delivery of drugs or site-specific binding is established based on the successful conjugation.

In order to keep the functionality of the antibody after conjugation, modification is needed to avoid the conjugation to the immunoglobulin fractions. The Immunoglobulin G molecule is composed of two light and two heavy chains. The two chains are held together by noncovalent interactions and disulfide bonds. The heavy chains of immunoglobulin molecules are similar, with a molecular weight of subunits from approximately 450 to 550 amino acids with three dimensional structures. Many functional groups can be used for modification - for example - the Lysin ϵ -amine group, N-terminal α -amine groups and carboxylate groups. “Although both amine and carboxylate groups are as plentiful in antibodies as they are in most proteins, the distribution of them within the three-dimensional structure of antibody is nearly uniform throughout the surface topology. Due to this reason, the conjugation processes that use these groups will crosslink randomly to nearly all parts of the antibody molecule, which in turn, leads to a random orientation of the antibody within the conjugate structure”. This randomly conjugation may block the antigen binding sites and prevent the binding between antibodies and antigens (Hermanson, 1996).

Crosslinkers that can selectively react with antibodies attracted attentions. For example, the disulfides in the hinge region that hold the heavy chains together have been chosen and cleaved with a reducing agent (like MEA, DTT or TCEP, etc.) to create two half-antibody molecules, with an antigen binding site on each of them. Then, these half antibodies with disulfides will be labeled and used.

1.8 Applications of labeled antibodies

The successful developments of bioconjugation techniques accelerated the usage of QD antibody conjugates, due to its high brightness and high resistance to photobleaching. One of the most common applications of QD antibody conjugates is to use them as secondary reporters to detect the presence of target pathogen from food or environmental samples. The flow chart of pathogens detection from food was divided into the following procedures: 1. Separation of target pathogens from food samples; 2. Binding of QD antibody complexes to the surfaces of targets; 3. Fluorescence detection.

Quantum dot antibody conjugates have also been used in flow cytometry. Quantum dots demonstrated greater thermal stability than traditional low molecular weight phycobiliprotein fluorophores. Quantum dot antibody conjugates against monocyte and T cell antigens retained most of their fluorescence under the high temperature annealing step, allowing simultaneous fluorescent immunophenotyping and telomere length measurement in flow cytometry. While the traditional fluorescent probes are severely damaged at the 82°C hybridization step, making simultaneous immunophenotyping difficult, QDs showed great potential to be the best replacement for traditional fluorescent compounds (Kapoor and others, 2009).

1.9 Gel electrophoresis

Gel electrophoresis is a technique used for DNA, RNA and protein separation. Based on the charges present on the molecules, loaded DNA, RNA and protein will move toward the positive side at different speeds. The movement speed is based on the

molecular weights of segments; the smaller the molecule weight, the faster the movement speed is.

Gel electrophoresis can be used as an analytical method for separation and comparison of different size molecules. Agarose gel is a crosslinked polymer. The gels used in separating proteins or small nucleic acids (DNA, RNA, or oligonucleotides) are usually composed of different concentrations of acrylamide. When separating larger nucleic acids (greater than a few hundred bases), purified agarose gives better results (Wikipedia.com 2009).

In recent years, using the gel electrophoresis to analyze nanoparticles has been tested and proven successful. It has been used to separate the QD-DNA conjugates (Fu and others, 2004). Again, it was proven from these tests that gel electrophoresis can be used for QDs and QD-biomolecules conjugate separation. By comparing the size of different conjugates, complete conjugation and non-complete conjugation can be separated.

One purpose of this study is to apply a gel electrophoresis related technique to purify the QDs and QD protein A complexes that will be used to make QD antibody conjugates used in foodborne pathogen detection. The target pathogen in this study is *E. coli* O157:H7.

1.10 *E. coli* O157:H7

Escherichia coli is a large and diverse group of bacteria. Although most strains of *E. coli* are harmless, approximately 10% to 15% of them can make you sick (Sojka and

Carnaghan, 1961; Gross, 1994; Horne and others, 2000). Some *E. coli* can cause diarrhea, while others can cause urinary tract infections, respiratory illness and pneumonia, etc. Some kinds of *E. coli* cause disease by making a toxin called the Shiga toxin. The bacteria that make these toxins are called “Shiga toxin-producing” *E. coli*. *E. coli* O157:H7 infection, which was first identified as a pathogen in 1982, remaining on the top of the annual foodborne diseases list. Although the effective intervention methods have decreased the outbreaks related to *E. coli* O157:H7, the number of people who got sick by consuming *E. coli* O157:H7 contaminated foods still exist.

A more accurate, sensitive and portable detection device for foodborne pathogen on-site checking is needed in order to develop efficient intervention methods to prevent future outbreaks. We hope that, through this study, more reliable QD *E. coli* O157:H7 antibody conjugates can be made for future usage. By adopting the gel electrophoresis method, purified conjugates can be available. All these will be the first step for the future development of on-site detection devices.

CHAPTER 2

MATERIALS AND METHODS

Methods used to make water soluble quantum dots were adopted from Jay Cupps (former Masters Student in Dr. Fan's Lab at the University of Missouri). Materials are listed in the following table.

Table 2.1 Chemicals used in QD synthesis.

Chemicals	Catalog No.	Company
Dimethylcadmiun	48-5040	Strem Chemicals
Cadmium oxide (CdO)	244783	Sigma-Aldrich
Lauric acid	L4250	Sigma-Aldrich
Trioctylphosphine oxide (99%)	223301	Sigma-Aldrich
Trioctylphosphine oxide (90%)	346187	Sigma-Aldrich
Hexadecylamine (HDA)	H7408	Sigma-Aldrich
Trioctylphosphine (TOP)	117854	Sigma-Aldrich
Elemental selenium (Se)	229865	Sigma-Aldrich
Hexamethyldisilothioane ((TMS) ₂ S)	52643	Sigma-Aldrich
Dimethylzinc	417246	Sigma-Aldrich
Zinc stearate	307564	Sigma-Aldrich
Anhydrous methanol	322415	Sigma-Aldrich
Methanol	-	ChemStores UMC ¹
Chloroform	-	ChemStores UMC ¹
Hexane	-	ChemStores UMC ¹
Toluene	-	ChemStores UMC ¹

¹ChemStores is located at the Chemistry department at the University of Missouri.

The method used in this study is a modification of the method developed by Hines and Guyot-Sionnest (1996). The schemes used to make quantum dot core CdSe and core/shell structures are listed in Figure 2.1 and 2.2.

Special notes for making quantum dots:

- The bubbler should always be on while heating the system to prevent the buildup of pressure.
- The flask should be turned off when purging the line to a storage tube.
- The bubbler has to be closed when escaping N₂ is used to prevent air from entering the system (for example: adding chemicals to the flask and using the storage tubes)
- Toluene degrades the plunger of the syringe, so the plunger should be removed and allowed to dry as quickly as possible to lessen this degradation
- When the contents in the flask are liquid, stir the mixture at maximum sustainable speed

The usages of QDs usually begin with the fictionalizations of the QD surfaces. One of the most common fictionalization of QDs is to make the QDs water soluble, because most of the biological application of QDs requires water solubility. In our study, we also used water soluble QDs. The scheme to make water soluble QDs is listed in Figure 2.3.

Flush and Flame Nitrogen Line

- Turn Ar tank On (leave brass switch closed)
- Turn vacuum pump & vacuum gauge on
- Put valve on right side of T into operating position, open valve on left side
- Let vacuum down to <800, open stopcock on far right
- Let vacuum down to <800, close stopcock, open Ar valve (brass switch)
- Wait ~3 min, close Ar valve, open stopcock
- Repeat d & e at least 4 times

STEP 1**Flush and Flame Reaction Flask**

- Assemble reaction flask assembly using a septum on the left neck instead of the extender septum combo
- Pull vacuum on setup
- Turn to N₂
- Repeat b & c (flame the assembly when vacuum is pulled)
- Leave the flask with N₂ on
- Turn Water flow on, bottom of condenser to inlet
- Insert temperature probe through right side septum, put small amount of grease around the septum-probe interface.

STEP 2**Add 5g TOPO, 5g of HDA, 30 mg CdO and 0.6g Lauric Acid**

- Remove the left septum, being sure N₂ is still on
Purge Flask at room temperature (pull vacuum, then refill with N₂ a few times)

STEP 3**Raise temperature to 290°C**

- Solution changes from dark red to clear, if some redness remains the temperature can be raised to until it is clear (stay below 360°C)
- Once solution has turned clear, maintain temperature at about 280°C

STEP 4**Purge Se/TOP line, load 2.0mL (181mg Se in 2ml TOP) into syringe**

- purge needle first by repeatedly drawing N₂ out of the flask and expelling it outside the flask
- put needle into stopper to prevent air contamination

STEP 5

Set temperature controller to 140°C and remove the mantle

Inject Se/TOP at desired temperature

(200°C for blue, 240°C for green, 270 °C for yellow and 300 °C for orange/red)

STEP 6

Replace the heating mantle once the temperature is below 180°C
Allow the QDs to remain at ~150° for 30 min
(Quantum dots core structure forms)

STEP 7

Figure 2.1 Scheme for synthesis QDs core structure. (Adapted from Jay Cupp's protocol).

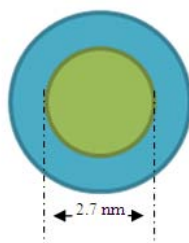
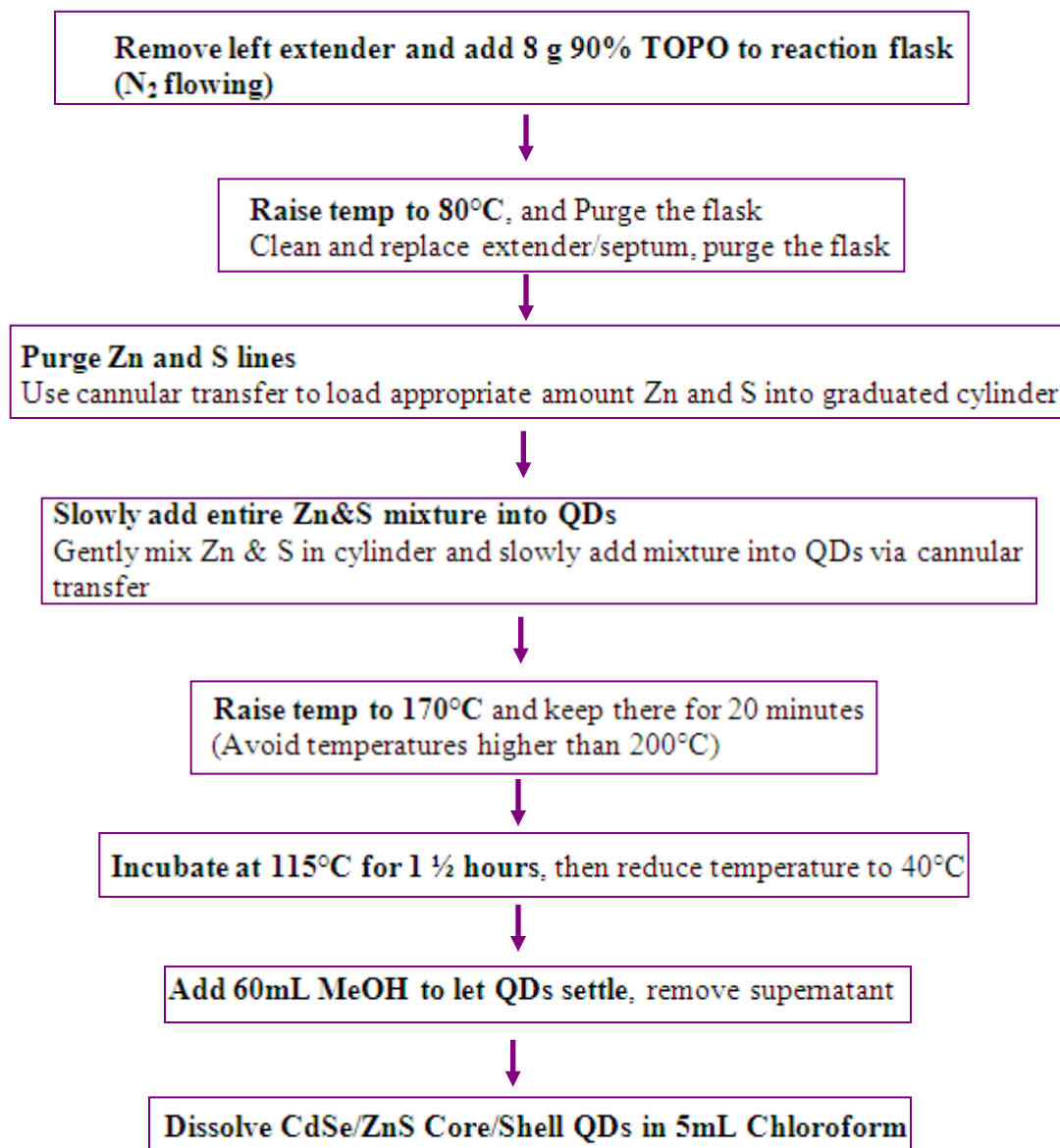


Figure 2.2 Scheme for CdSe/ZnS Core/Shell structure synthesis.

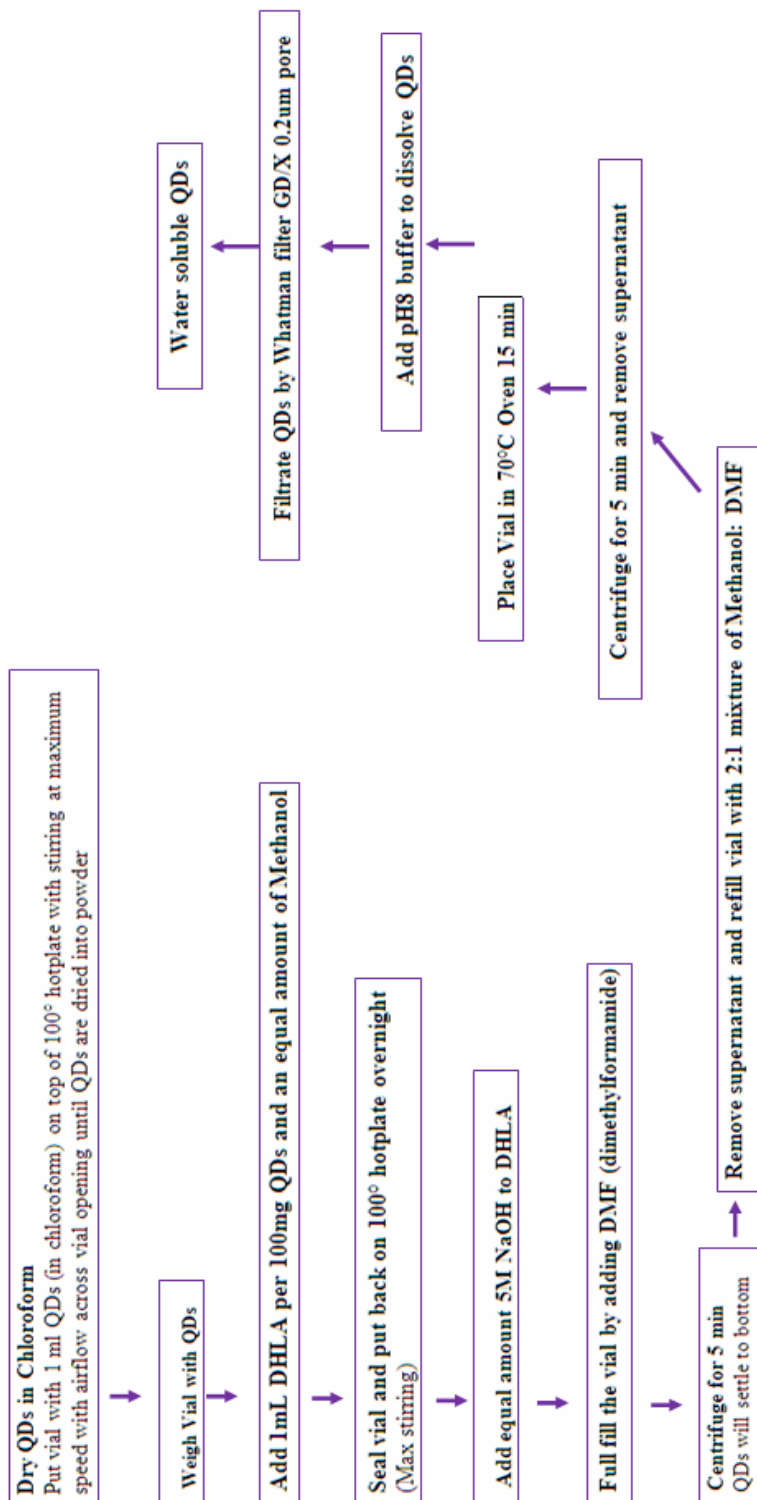


Figure 2.3 Scheme for making water soluble QDs.

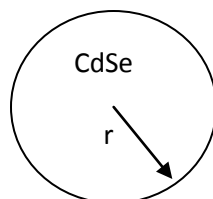
2.1 Property study of quantum dots

Instrumental Neutron Activation Analysis (INAA) is a method to determine the concentration of trace and major elements in a variety of matrices. In INAA, the sample is subjected to a neutron flux, thus radioactive nuclides are produced. As radioactive nuclides decay, they emit gamma rays whose energies are specific for each nuclide. By comparing the intensity of gamma rays emitted by the sample and the rays emitted by a standard, the concentration of one particular element can be quantitatively measured.

In this study, the QDs synthesized were diluted and sent to the chemistry department located on the University of Missouri campus. The purpose was to check the amount of end product of our QD synthesis procedure and calculated the synthesis efficiency as well as the final product concentration. According to the process provided by the chemistry department, 100 μg of liquid sample was pipetted onto 5 filter papers and analyzed. The concentration of Selenium was reported.

2.1.1 Calculation of quantum dot concentration

CdSe quantum dots



$$\frac{4}{3}\pi r_{QD}^3 = V$$

$$N_{Se} Per QD = \frac{\frac{3}{4} \pi r_{QD}^3}{X^3} = atom / QD$$

X=Lattice constant (6.050 Å at 300 k)

$$Concentration(Se) = \frac{atom}{ml}$$

$$\frac{Concentration(Se)}{N_{Se} Pe QD} = \frac{QD}{ml}$$

2.2 Surface modification of QDs

Quantum dots with a DHLA water soluble surface were modified with EDC for further biological application. Ten milligrams of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma) was dissolved in 0.5 ml MES (2-(N-Morpholino) ethanesulfonic acid) solution to make EDC stock solution. One hundred microliters of QDs-DHLA were modified by adding 25~30 µl of EDC stock solution. After that, different amounts of protein A were added and the mixtures were incubated in the cold room (4°C) for 2 h to make QD protein A complex before the further conjugation with antibodies.

Different amounts of protein A were tested to check the minimum amount protein A that is needed for fully binding EDC on QD surfaces. The concentration of protein A is 0.5 mg/ml.

Table 2.2 Different volume ratios of protein A and QDs were tested in this study.

	QDs : Protein A (0.5mg/ml)				
volume	1 μ l : 3 μ l	1 μ l : 15 μ l	1 μ l : 30 μ l	1 μ l : 45 μ l	1 μ l : 60 μ l

2.3 Gel electrophoresis

A10 X stock TAE buffer was made by adding 48.8 g of Tris-base, 10.9 g of Glacial acetic acid, 2.92 g EDTA and 1 L of water (pH 8.18-8.29). A 1 X diluted TAE buffer was used for making gel and served as the running buffer during electrophoresis.

Table 2.3 Chemicals used in gel electrophoresis.

Chemicals	Catalog No.	Company
Tris-base	H5131	Promega
Glacial acetic acid	A38-212	Fisher
EDTA	BP120-500	Fisher
Agarose low EEO	BP160-100	Fisher
Protein A	P6031	Sigma
Load buffer (6X)	N313-KIT	Ameresco

Agarose gel (0.5%) was used for QDs, QD EDC and QD EDC protein A electrophoresis. Two microliters of loading buffer (Envision™ DNA dye loading buffer 6 \times from Amersco®, Solon, Ohio) were mixed with 10 μ l of QD samples and the electrophoresis was done by Bio-rad electrophoresis unit under 100 V for 50 min.

2.4 QD protein A complex recollection from agarose gel

Gel electrophoresis showed its great potential in separating QDs and QD protein A complexes, re-collecting the QD protein A complexes and QDs back from the gel would make gel electrophoresis a potential purification method rather than just being a separation method.

GeBAflex-tube is a tube that can be used to extract DNA or protein out of the gel. The process of extracting QD protein A complexes out of the gel is shown in Figure 2.5.



Figure 2.4 GeBAflex tubes.

(<http://www.bioxys.com/images/42843%20SIZE%20TUBES.jpg> accessed on April 20,

2009).

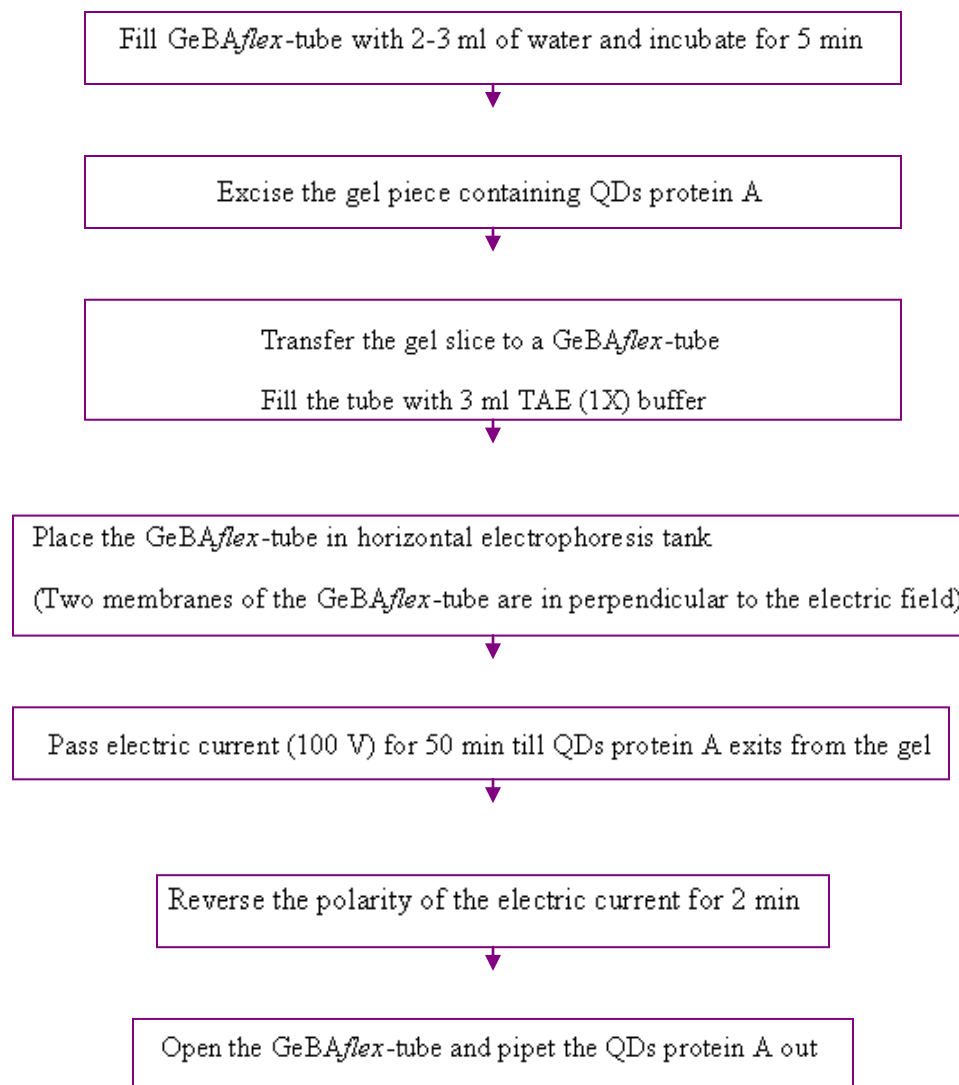


Figure 2.5 Diagram for eluting QD protein A complexes out of the gel.

2.5 Cell based microarray

One glass slide was cleaned by plasma cleaner with oxygen flowing in the machine. After the cleaning step, the glass slide was immersed in 1% 3-Aminopropyl-trimethoxysilane (97%, APTS) for 6 h at room temperature on a shaker. The APS will be washed off after 6 h with a water/ethanol mixture and baked in a 100°C oven for overnight.

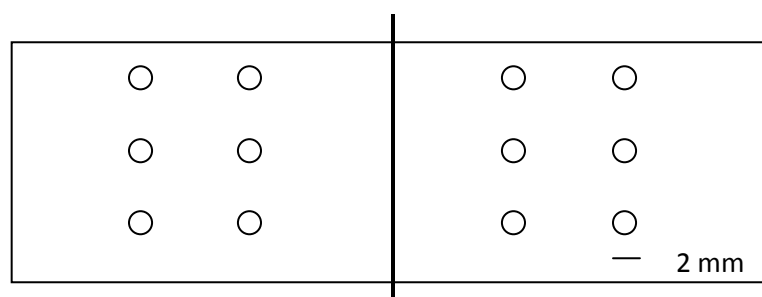


Figure 2.6 Glass slide was divided into two sides. Circles in 2 mm diameter were drawn by marker pen on the back of the slide.

The glass slide was divided into two sides. Six circles were drawn on each side, as shown in Figure 2.6. In each circle, 10 μ l of freshly prepared EDC solution were added, following by mixing another 10 μ l of cells suspension. The slide was placed in a clean petri dish and incubated in a cold room (4°C) for 2 h. During the 2 h incubation, the EDC served as a crosslinker and bound the cells onto the slide surface.

Slides labeled with cells was washed by PBS to clean the excess unbound cells. Ten microliters of QD protein A antibody conjugates were added into the circles and were incubated in a cold room again for another 2 h. There are two sets of QD protein A antibody conjugates, one used freshly prepared QD protein A complexes; while the other one used QD protein A complex recollected after gel electrophoresis. After a 2 h

incubation, excess QD protein A antibody conjugates were washed out by PBS. Fluorescence was detected by microscopy located in the Molecular Cytology Core (MCC), Life Science Building, University of Missouri. The scheme is shown in Figure 2.7.

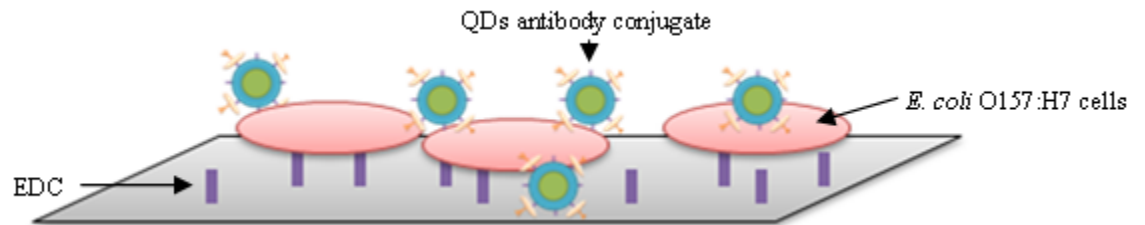


Figure 2.7 Cell based microarray. Pathogenic *E. coli* O157:H7 cells were immobilized on a glass slide with an EDC crosslinker. After the immobilization, cells stayed on the slide surface. QD conjugated antibodies were added to detect cells. If QD antibody conjugates can successfully attach to cell surfaces and detect the cells by giving green fluorescence, it means that the QD antibody conjugates with are functional.

CHAPTER 3

RESULTS

Green and red quantum dots were synthesized in Dr. Fan's lab. Four grams of 99% TOPO, 4 g HDA, 30 mg CdO, 0.6 g Lauric Acid and 9 g of 90% TOPO were used to make the quantum dots. By adjusting the temperature, quantum dots with different colors were made (240°C for green, 300°C for red). Figure 3.1 shows the product container part of the whole fabrication system.

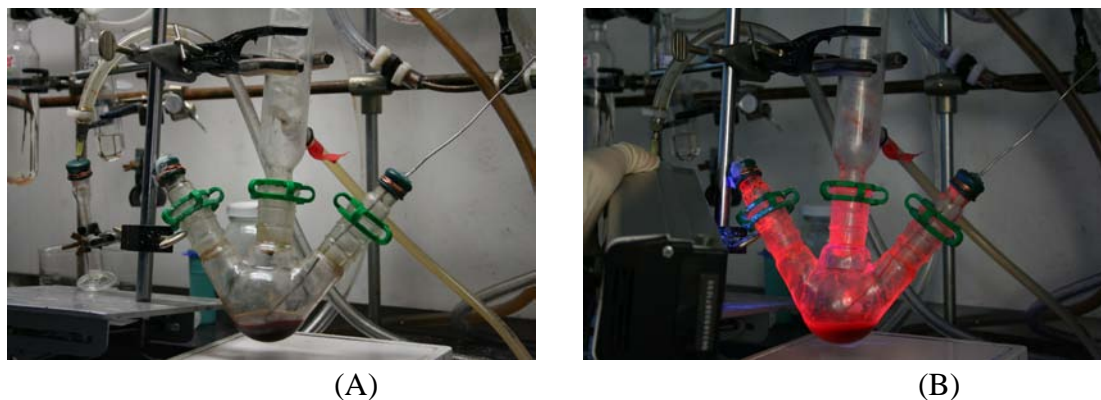


Figure 3.1 Fresh red quantum dots are made in the reaction flask. (A) Fresh red quantum dots in room light. (B) Fresh quantum dots under UV light.

Core parts of quantum dots were made by Cd and Se. To determine the concentration of water soluble QDs, 100g samples were sent to the Chemistry Department at the University of Missouri to test the concentration of individual chemical compounds by instrumental neutron activation analysis (INAA). In this project, INAA was used to detect the concentration of Se. In order to calculate the concentration of QDs, the absorbance wavelength and the diameter of QDs were also used. The formula used to calculate the QD diameter was adopted from Yu and others (2003).

$$CdSe: D = (1.6122 \times 10^{-9})\lambda^4 - (2.6575 \times 10^{-6})\lambda^3 + (1.6242 \times 10^{-3})\lambda^2 - 0.4277\lambda + 41.57$$

D= diameter λ =absorbance wavelength (Yu, et al. 2003)

The absorbance wavelength of the green quantum dots is 530 nm. Thus, by plotting 530 nm in the equation above, the diameter of green quantum dots is approximately 2.7 nm.

Results from the INAA analysis showed that the concentration of Se is 0.5097 PPM of the QD sample. By using the method provided in the following link, the concentration of QDs was calculated.

http://www.sos.siena.edu/Chemistry/files/doc/Exp_6_Number_of_Shells.doc

$I_{\max} = 530 \text{ nm (green)}$ Diameter= 2.4 nm Cd-Se bond length 0.36 nm

Calculation of Formula units of CdSe across diameter of QD:

Diameter/CdSe bond length: $2.7\text{nm}/0.36 \text{ nm} = 7.5 \text{ units}$

Calculation of # of CdSe units in a sphere of d=2.7 nm diameter:

$(4/3)\pi(d/2)^3 = (4/3)(3.14)(7.5/2)^3 = 220.78 \text{ units of CdSe in the Q.D.}$

Calculation of Molar Mass of QD:

$(220.78 \text{ units of CdSe})(\text{molar mass Cd } 112.4118 + \text{molar mass Se } 78.96) = 42,251$

g/Mol, the molar mass of CdSe Q.D. that has a diameter of 2.7 nm.

Calculation of QD concentration:

$$(0.5097 \text{ milligram/L} / 1000) / 42.251 \text{ g/mol} = 1.2 \times 10^{-5} \text{ mol/L}$$

As the sample was diluted by 10 times, the concentration of water soluble QDs is 1.2×10^{-4} mol/L.

3.1 Gel electrophoresis application

Five milliliters of QDs-DHLA and 25 ml of buffer (with various pH values from pH 4 to pH 10) were mixed and stored in a cold room for two days. Ten millimeters of mixture were loaded into 0.5% agarose gel. Comparing the gel picture to the one got with fresh QDs-DHLA, we found that the QDs DHLA is stable in buffer with a pH from 6 to 8. When the pH values are 4, 5, 9, 10, the properties of QDs DHLA are changed by showing slower movement speeds than those mixed with pH 6 to 8 buffers. One potential reason might be the pH 4, 5, 9, 10 buffer changed the surface charges of QDs-DHLA and thus they moved at different speeds. Another reason might be due to the potential aggregation that happened between QDs in buffers with pH values of 4, 5, 9, 10, their molecular weight increased following the aggregation. Figure 3.2 (a) and (b).

3.2 QD EDC protein A complexes

QDs were modified with EDC and protein A to build uniform complexes.

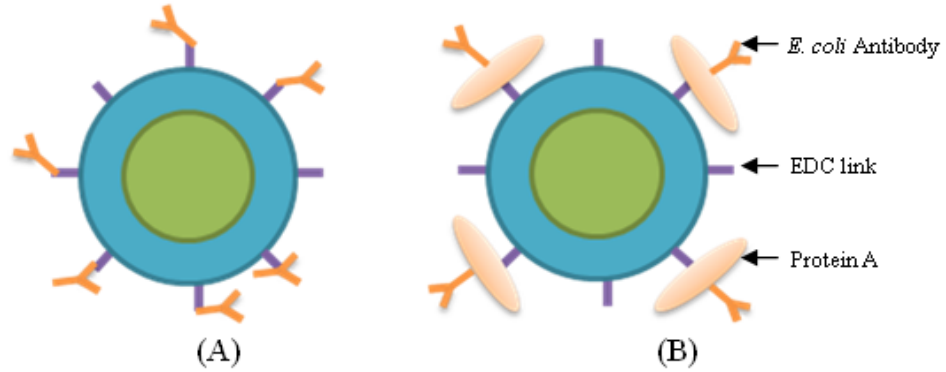


Figure 3.2 By adding protein A, the conjugates of antibodies and quantum dots are in uniform structures. (A) QD EDC antibody random conjugates; (B) QD EDC protein A antibody conjugates in uniform structures.

Gel electrophoresis was used to check the conjugation status between QDs EDC and protein A. Agarose gel (0.5%) was used for electrophoresis. After running 50 min in the gel under 100 V, QDs with EDC and protein A moved at different speeds due to different molecular weights. As shown in Figure 3.3, adding protein A to the surfaces of quantum dots intended quantum dots bigger molecular weight. The movement of QD+EDC+protein A was slower in the gel.

To check the minimum amount of protein A needed for complete conjugation for all EDC crosslinkers present on QDs surfaces, various amounts of protein A were added. After a 2 h reaction, the conjugates were separated and checked on a 0.5% gel, as shown in Figure 3.4. The concentration of protein A used was 0.5 mg/ml. The conjugation process was done in PBS solution. Figure 3.4 shows that the v:v ratio of QDs and protein A of 1:30 can ensure the complete binding of protein A to all potential EDC linkers. Volume ratio less than 1:30 may have some unbounded EDC active linker left on the surfaces of QDs, which may cause self-polymerization.

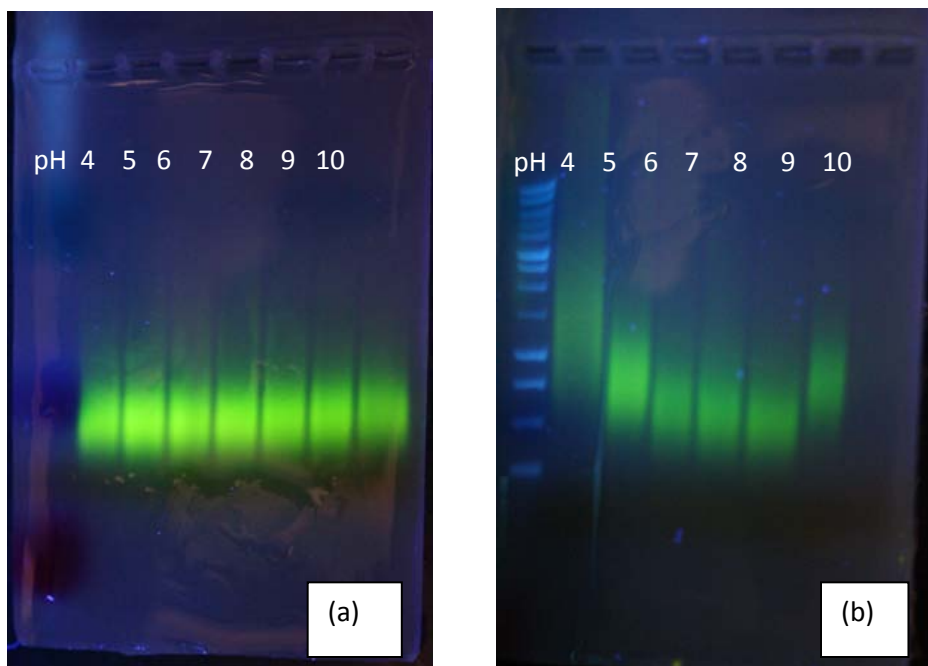


Figure 3.3 (a) gel electrophoresis picture of freshly mixed QDs DHLA in different buffers. From left to right, there are QDs DHLA in buffer 4, followed by pH 5 to pH 10. (b) gel electrophoresis picture of mixed QDs DHLA in buffers (2 days old). From left to right, there are molecular marker (1kb DNA ladder, Promega) and QDs DHLA in buffers (pH 4 to pH 10).

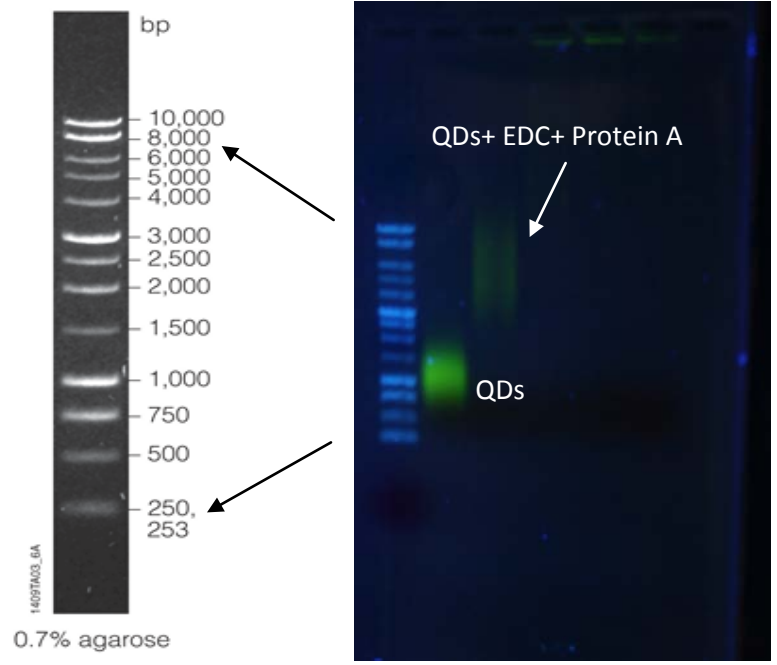


Figure 3.4 Gel electrophoresis of Quantum dots and quantum dots with EDC+protein A. 1 kb DNA ladder was used as a marker.

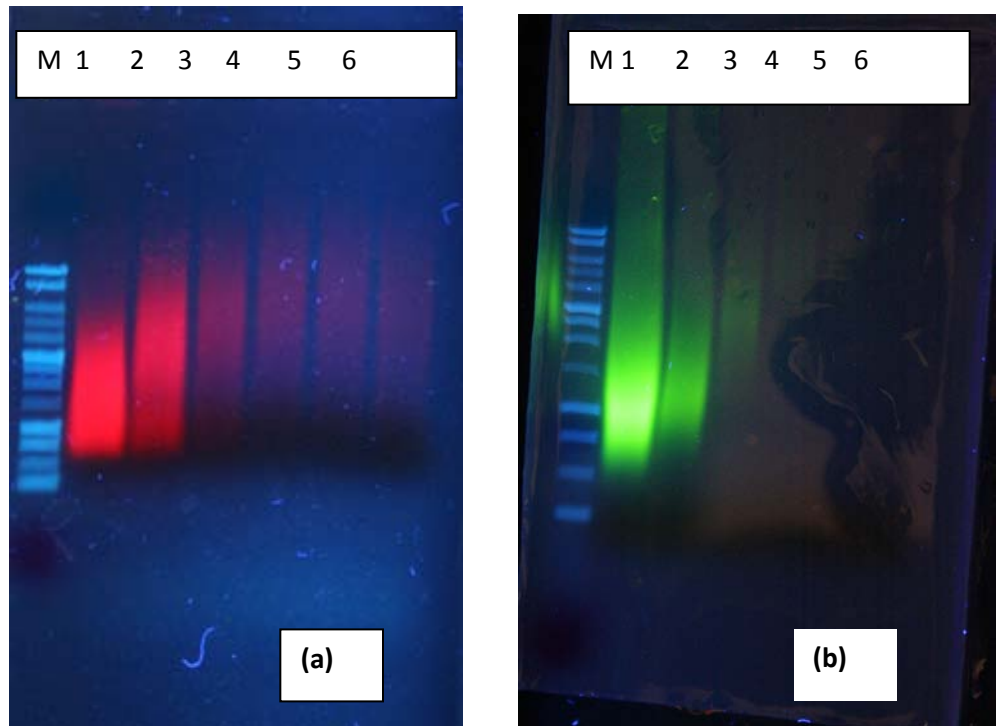


Figure 3.5 Gel electrophoresis of Quantum dots and quantum dots with various EDC protein A crosslinker. (a) Lane 1, 1 kb DNA ladder (Promega); Lane 2, pure QDs with EDC linker; Lane 3, QDs with EDC and protein A (QDs:protein A= 1:3 (v:v)); Lane 4, QDs with EDC and protein A (QDs:protein A= 1:15 (v:v)); Lane 5, QDs with EDC and protein A (QDs:protein A= 1:30 (v:v)); Lane 6, QDs with EDC and protein A (QDs:protein A= 1:45 (v:v)); Lane 7, QDs with EDC and protein A (QDs:protein A= 1:60 (v:v)). (b) has the same arrangement as (a), except green QDs were used.

3.3 QD EDC protein A complexes collection from an agarose gel

QD EDC protein A complexes were purified on a piece of gel by electrophoresis. Due to the changes and difference in molecular weights, pure QDs moved faster than QDs EDC protein A complexes. QD EDC protein A complexes were recollected by cutting the gel containing the complexes and extracting them from the gel by a GeBAflex-tube. Cubes with edge lengths of 6 mm were cut from the gel and put in a GeBAflex-tube, 2 ml of TBE buffer was added to the tube and 100 V was applied for 50 min. After that, the polarity of the electric current was reversed and applied to the tube for 2 min. TBE buffer in the tube will contain QD EDC protein A complexes (Figure 3.5).

3.4 Biological property of QDs protein A complex recovered from the gel

One glass slide was cleaned by plasma cleaner with oxygen flowing in the machine. After the cleaning step, the glass slide was immersed in 1% 3-Aminopropyl-trimethoxysilane (97%, APTS) for 6 h in room temperature on a shaker. The APS was washed off after 6 h with water and ethanol and baked in a 100°C oven overnight. Cells were immobilized on the slide surfaces to check the biological properties of QD EDC protein A complexes recovered from the gel.

E. coli O157:H7 monoclonal antibodies were purchased from Genway (Genway, San Diego, CA). Antibodies and QD conjugation process was started with adding 100 µg of antibodies to QD EDC protein A complexes, followed by 2 h of incubation at 4°C in a cold room.

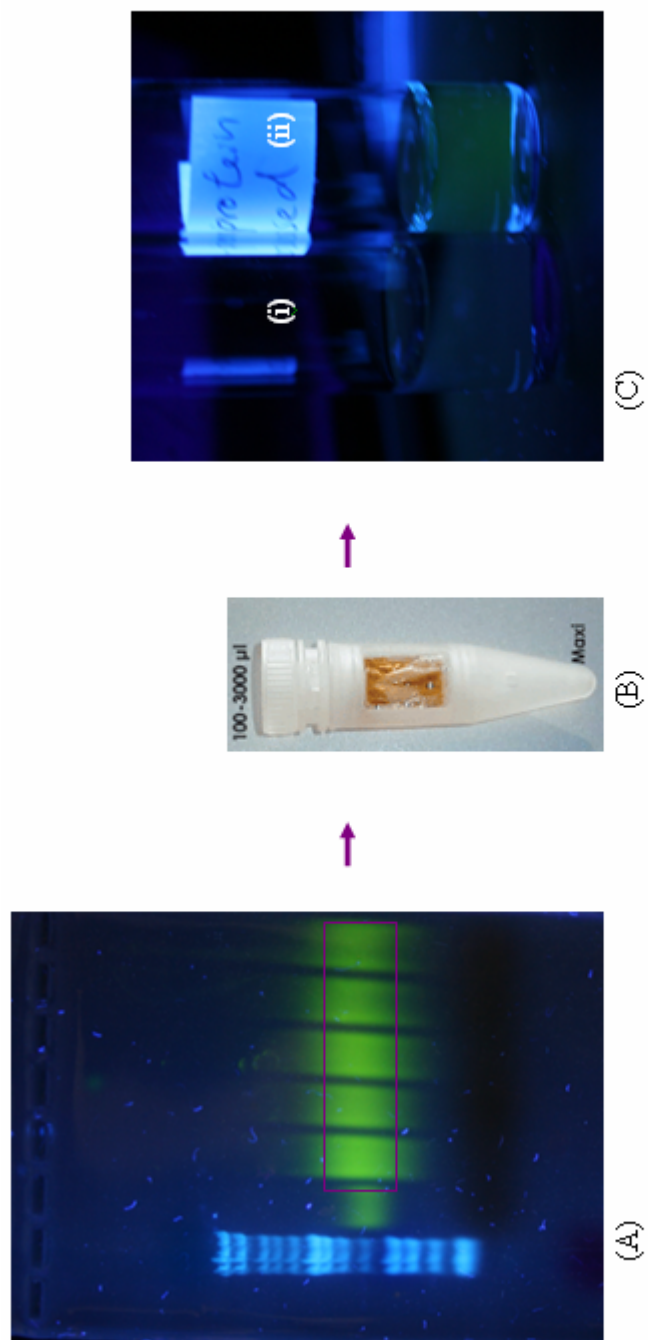


Figure 3.6 Recovery of QD EDC protein A complexes from the gel. Quantum dots with crosslinkers have higher molecular weights (A). Quantum dots with crosslinkers were cut from the gel and put in GeB.Aflex tube for elution (B). After elution, quantum dots with crosslinkers were recovered from the gel and store in tube ii (C). In (C), (i) is negative control (only TAE buffer); (ii) are recovered quantum dots with crosslinkers. Those recovered QD EDC protein A complexes gave out green fluorescence under UV light.

Ten microliters of QD protein A antibody conjugates were added to the circles on a glass slide, which had *E. coli* O157:H7 cells labeled on top, and incubated in a cold room for 2 h. Excess QD protein A antibody conjugates were washed off by PBS. Fluorescence was detected by microscopy located in Molecular Cytology Core (MCC), Life Science Building, University of Missouri. In Figure 3.6, it shows the principle of testing biological properties of QDs, EDC and protein A recovered from gel electrophoresis.

After washing off the excess QD antibody conjugates, the glass slide was checked under microscopy. Figure 3.7 shows images from the microscopy. It proved that the QD EDC protein A complexes recovered from the gel still kept their biological properties. Protein A can bind to antibodies and the following QD antibody conjugates could be successfully synthesized and used in cell detection.

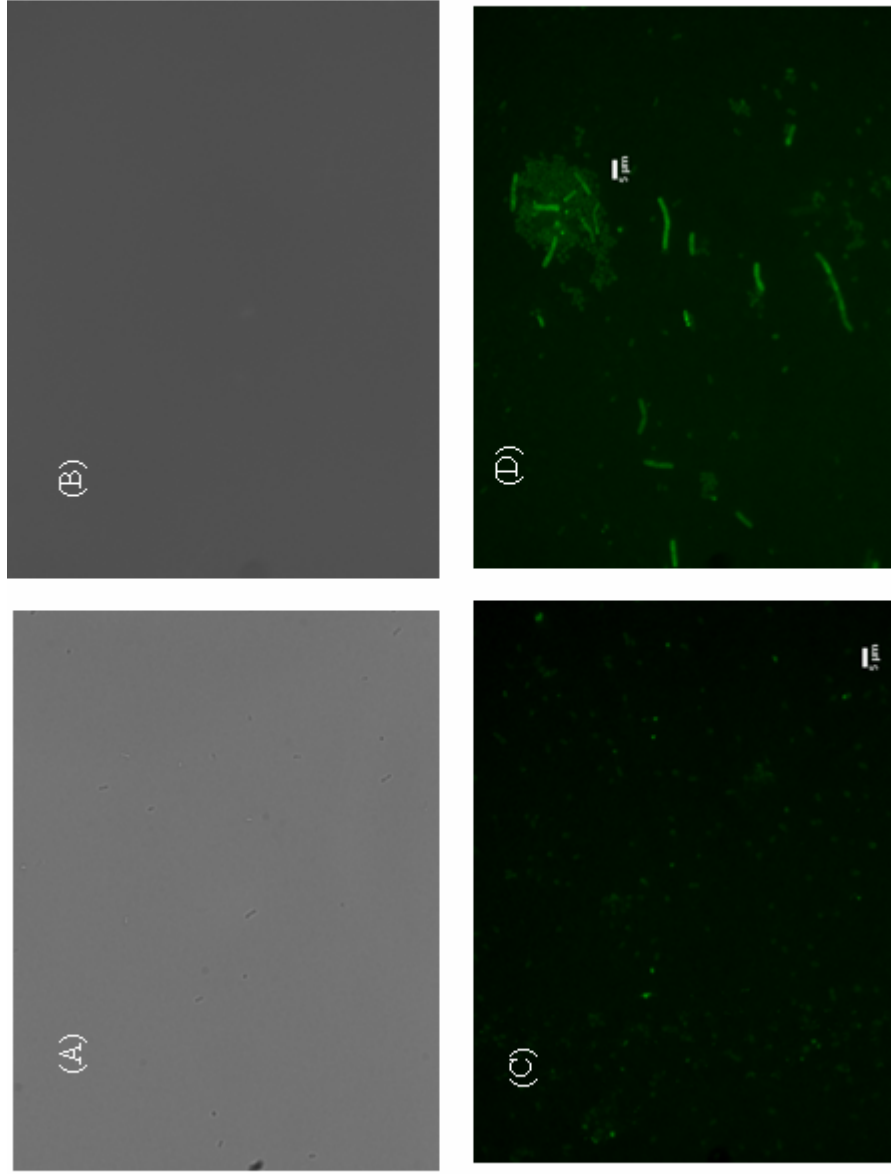


Figure 3.7 (A) is the brightfield vision of immobilized *E. coli* O157:H7 cells only, (B) UV light excitation of immobilized *E. coli* O157:H7 cells only, (C) Detection of *E. coli* O157:H7 by antibodies labeled with eluted QD EDC protein A complexes. (D) Detection of *E. coli* O157:H7 by antibodies labeled with fresh made QD EDC protein A complexes.

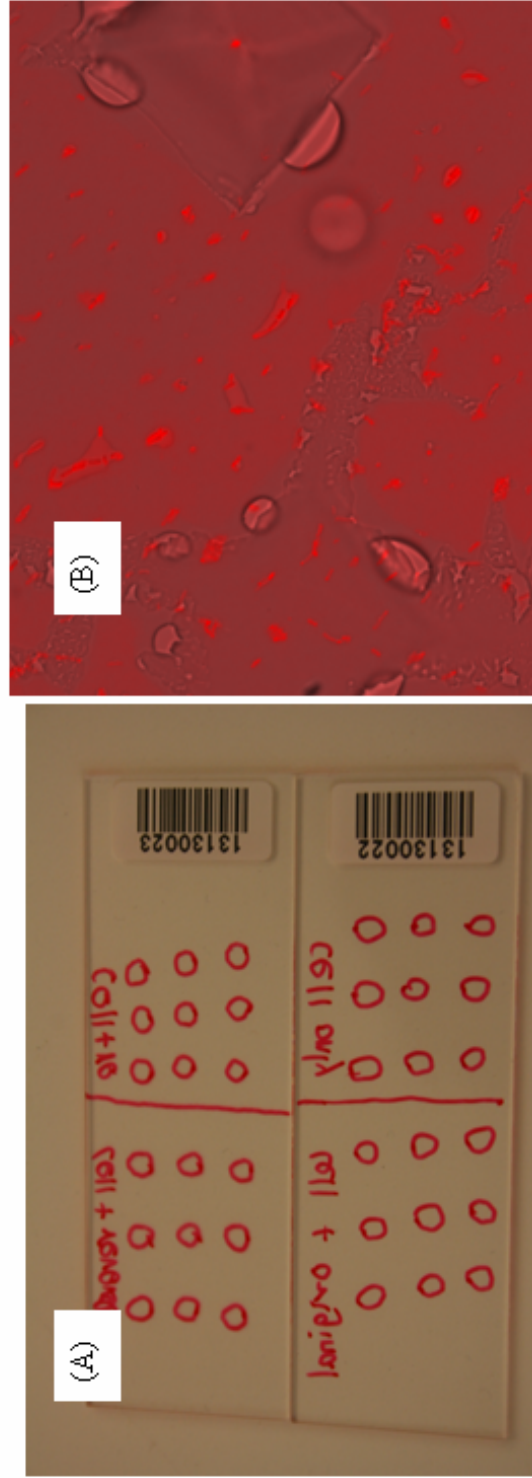


Figure 3.8 (A) Microarray slides used to test the biological function of QD EDC protein A complexes recovered from gel electrophoresis. (B) Overlay of the brightfield picture taken with cells labeled with QD antibody conjugates and picture taken under UV excitation. The red QD EDC protein A complexes were recovered from gel electrophoresis first and then used for making QD antibody conjugates. The fluorescence given out by the cells indicated the successful recovery of QD protein A complexes and their active biological functions.

CHAPTER 4

CONCLUSIONS

In this study, green QDs and orange QDs were synthesized using a Colloidal method in Dr. Fan's lab. The physical properties and concentrations of QDs were analyzed and calculated. To investigate the application of QDs in biological studies, QDs with different colors were modified to be water soluble by adding DHLA to their shell structures. EDC crosslinkers were used to make QD EDC protein A complexes. These QD EDC protein A complexes were used to make QD antibody conjugates. In order to find out the proper amount of protein A needed for completely binding of all active EDC active linkers, gel electrophoresis was used to check the molecular weight changes of the complexes. *GeBaflex* tube is a tube that can be used to recollect DNA sequences from the gel after electrophoresis. In this study, this tube was adopted to see if it can be used for purification of QD EDC protein A complexes. After checking the biological activity of QD EDC protein A complexes recovered from the gel by cell based microarray method, the results showed that:

1. Gel electrophoresis can be used as a purification method to separate the unbound QDs from the QD EDC protein A complexes.
2. The concentration of the QDs synthesized is approximately 120 nmol/L. Volume ratio between QDs and protein A (0.5mg/ml) of 1:30 can realize the complete binding of protein A to EDC linkers present on QDs.
3. *GeBaflex* tube can be used to extract QD EDC protein A complexes from gel after electrophoresis and the QD EDC protein A complexes recovered

by GeBaflex tube kept their biological functions and can be used for further studies.

REFERENCES

- Alivisatos P. 2004. The use of nanocrystals in biological detection. *Nat. biotech.* 22: 47-52.
- Ballour B, Lagerholm BC, Ernst LA, Bruchez MP, Waggoner AS. 2004. Noninvasive imaging of quantum dots in mice. *Bioconjug. Chem.* 15(1): 79-86.
- Banin U, Millo O. 2004. Properties. Nanoparticles: from theory to application. Schmid G. (Editor). Wiley-VCH, Weinheim.
- Barany G, Merrifield RB. 1980. The peptides. Gross E, Meienhofer J. (Editors). Academic Press, New York.
- Chan WCW. 2007. Bio-application of nanoparticles. Series: Advances in experimental medicine and biology. V620. Chan WCW. (Editor). Springer, New York.
- Chan WCW, Nie SM. 1998. Quantum dots bioconjugates for ultrasensitive nonisotopic detection. *Science.* 281(585): 2013-16.
- Derfus AM, Chen WCW, Bhatia SN. 2004. Probing the cytotoxicity of semiconductor quantum dots. *Nano lett.* 4(1): 11-18.
- Diamandis EP, Christopoulos TK. 1991. The biotin-avidin system: principles and applications in biotechnology. *Clin. Chem.* 37(5): 625-36.
- Dubertret B, Skouride P, Norris DJ, Noreaux V, Brivanlou AH, Libchaer A. 2002. In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science.* 298(5599): 1759-62.
- Ekimov AI, Efros AL, and Onushchenko AA. 1985. Quantum size effect in semiconductor microcrystals. *Sol. State. Comm.* 56: 921-24.
- Eychmüller A. 2004. Syntheses and characterizations. Nanoparticles: from theory to application. Schmid G. (Editor). Wiley-VCH, Weinheim.
- Fu A, Micheel CM, Cha J, Chang H, Yang H, Alivisatos AP. 2004. Discrete nanostructures of quantum dots/Au with DNA. *J. Am. Chem. Soc.* 126: 10832-33.
- Gao X, Dave SR. 2007. Quantum dots for cancer molecular imaging. Bio-application of nanoparticles. Series: Advances in experimental medicine and biology. V620. Chan WCW. (Editor). Springer, New York.

Gao XH, Gui YY, Levenson RM, Chung LW, Nie S. 2004. In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat. Biotechnol.* 22(8): 969-76.

Green M, Howman E. 2005. Semiconductor quantum dots and free radical induced DNA nicking. *ChemComm.* 1: 121-3.

Green NM. 1975. Avidin. *Adv. Protein Chem.* 29: 85-133.

Gross WG. 1994. Diseases due to *Escherichia coli* in poultry. In: *Escherichia coli* in domestic animals and humans. Gyles CL. (Editor). CAB international, Wallingford, UK.

Hermanson GT. 1996. Bioconjugate techniques. Academic Press, New York.

Hines MA, Guyot-Sionnest P. 1996. Synthesis and Characterization of Strongly Luminescing ZnS-Capped CdSe Nanocrystals. *J. Phys. Chem.* 100(2): 468-71.

Huang D, Reshchikov MA, Morkoc H. 2002. Growth, structures and optical properties of III-nitride quantum dots. *Int. J. High Speed Electron. Syst.* 12(1): 79-110.

Horne SM, Pfaff-McDonough SJ, Giddings CW, Nolan LK. 2000. Cloning and sequencing of the *iss* gene from a virulent avian *Escherichia coli*. *Avian Dis.* 44: 179-84.

Ipe BI, Lehning M, Niemeyer CM. 2005. On the generation of free radical species from quantum dots. *Small.* 1(7): 706-9.

Jaiswal JK, Mattoussi H, Mauro JM, Simon SM. 2003. Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat. biotechnol.* 21(1): 47-51.

Kapoor V, Hakim FT, Rehman N, Gress RE, Telford WG. 2009. Quantum dots thermal stability improves simultaneous phenotype-specific telomere length measurement by FISH-flow cytometry. *J. Immunol. methods.* Published online http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T2Y-4VS40FR-1&_user=3419478&_rdoc=1&_fmt=&_orig=search&_sort=d&_view=c&_acct=C000049994&_version=1&_urlVersion=0&_userid=3419478&md5=39f0848142e8dcd06d931f8c1b66b790

Katz E, Shipway AN, Willner I. 2004. Biomaterial-Nanoparticle hybrid systems: Synthesis, properties and applications. Nanoparticles: from theory to application. Schmid G. (Editor). Wiley-VCH, Weinheim.

Klimov VI, Mikhailovsky AA, McBranch DW, Leatherdale CA, Bawendi MG. 2000a. Quantization of multiparticle Auger rates in semiconductor quantum dots. *Science.* 287: 1011-3.

Klimov VI, Mikhailovsky AA, Xu S, Malko A, Hollingsworth JA, Leatherdale CA, Eisler H, Bawendi MG. 2000b. Optical gain and stimulated emission in nanocrystal quantum dots. *Science*. 290: 314-7.

Larson DR, Zipfel WR, Williams R, Clark SW, Bruchez MP, Wise FW, Webb WW. 2003. Water soluble quantum dots for multiphoton fluorescence imaging in vivo. *Science*. 2(1): 50-64.

Lee JS. 2002. Growth of self-organized quantum dots. *Semiconductor quantum dots: physics, spectroscopy, and applications*. Masumoto Y., Takagahara T. (Editors). Springer, New York.

Lidke DS, Lidke KA, Rieger B, Jovin TM, Arndt-Jovin DJ. 2005. Reaching out for signals: filopodia sense EGF and respond by directed retrograde transport of activated receptors. *J. cell Biol.* 170(4): 882-5.

Liu Y, Li, Y. 2006. Simultaneous detection of *E. coli* O157:H7 and *Salmonella Typhimurium* using quantum dots as fluorescence labels. *Analyst*. 131(3): 394-401.

Lovric J, Cho SJ, Winnik FM, Maysinger D. 2005. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. *Chem. Biol.* 12(11): 1227-34.

Matsumoto T, Maenosono S, Yamaguchi Y. 2004. Organometallic synthesis of InP quantum dots using Tris (dimethylamino) phosphine as a phosphorus source. *Chem. Lett.* 33(11): 1492-3.

Maysinger D, Lovrić, J. 2007. Quantum dots and other fluorescent nanoparticles: Quo Vadis in the cell? *Bio-application of nanoparticles*. Series: Advances in experimental medicine and biology. V620. Chan WCW. (Editor). Springer, New York.

Mikhailovsky AA, Malko AV, Hoollingsworth JA, Bawendi MG, Klimov VI. 2002. Multiparticle interactions and stimulated emission in chemically synthesized quantum dots. *Appl. Phys. Lett.* 80: 2380-2.

Molecular beam epitaxy. 2005. Accessed on July 14, 2008, from Wikipedia: the Free Encyclopedia Web site: http://en.wikipedia.org/wiki/Molecular_beam_epitaxy.

Murray CB, Norris DJ, Bawendi MG. 1993. Synthesis and Characterization of Nearly Monodisperse CdE (E = S, Se, Te) Semiconductor Nanocrystallites. *J. Amer. Chem. Soc.* 115: 8706-15.

Murray CB, Sun S, Gaschler W, Doyle H, Betley TA, Kagan CR. 2001. Colloidal synthesis of nanocrystals and nanocrystal superlattices. *IBM J. Res. & Dev.* 45(1): 47-56.

Parak WJ, Manna L, Simmel FC, Gerion D, Alivisatos P. 2004. Quantum dots. Bio-application of nanoparticles. Series: Advances in experimental medicine and biology. V620. Chan WCW. (Editor). Springer, New York.

Parak WJ, Boudreau R, Le Gros M, Gerion D, Zanchet D, Micheel CM, Williams SC, Alivisatos AP, Larabell C. 2002. Cell motility and metastatic potential studies based on quantum dot imaging of phagokinetic tracks. *Adv. Mater.* 14(12): 882-5.

Parungo CP, Ohnishi S, Kim SW, Laurence RG, Soltesz EG, Chen FY, Colson YL, Cohn LH, Bawendi MG, Frangioni JV. 2005. Intraoperative identification of exphageal sentinel lymph nodes with near-infrared fluorescence imaging. *J. Thorac. Cardiovasc. Surg.* 129 (4): 844-50.

Pellegrino T, Parak W, Boudreau R, Le Gros MA, Gerion D, Alivisatos AP, Larabell CA. 2003. Quantum dot based cell motility assay. *Differentiation.* 71(9-10): 542-8.

Rocksby HP. 1932. *J. Soc. Glass Tech.* 16, 171.

Samia AC, Chen X, Burda C. 2003. Semiconductor quantum dots for photodynamic therapy. *J. Am. Chem. Soc.* 125(51): 15736-7.

Sheehan JC, Hess GP. 1955. A new method of forming peptide bonds. *J. Am. Chem. Soc.* 77: 1067-8.

Sojka WJ, Carnaghan RBA. 1961. *Escherichia coli* infection in poultry. *Res. Vet. Sci.* 2: 340-52.

Stroh M, Zimmer JP, Duda DG, Levchenko TS, Cohen KS, Brown EB, Scadden DT, Torchilin VP, Bawendi MG, Fukumura D, Jain RK. 2005. Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. *Nat. Med.* 11(6): 678-82.

Trindade T, O'Brian P, Pickett NL. 2001. Nanocrystalline Semiconductors: Synthesis, Properties, and Perspectives. *Mater. Chem.* 13: 3843-58.

Voura E, Jaiswal J, Mattoussi H, Simon SM. 2004. Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. *Nat. Med.* 10(9): 993-8.

Wikipedia.com. 2008. Accessed on July 29, 2008. From Free encyclopedia Web site: http://en.wikipedia.org/wiki/Quantum_dot .

Woll AR, Rugheimer P, Lagally MG. 2002. Self-organized quantum dots. *Int. J. High Speed Electron. Syst.* 12(1): 45-78.

Wu XY, Liu HJ, Liu JQ, Haley KN, Treadway JA, Larson JP, Ge N, Peale F, Bruchez MP. 2003. Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nat. Biotechnol.* 21(1): 41-6.

Yu W, Qu L, Guo W, Peng X. 2003. Experimental Determination of Extinction Coefficient of CdTe, CdSe, and CdS Nanocrystals. *Chem. Mater.* 15 (14): 2854-60.

Zhao W, Wang L, Tan W. 2007. Fluorescent nanoparticle for bacteria and DNA detection. Bio-application of nanoparticles. Series: Advances in experimental medicine and biology. V620. Chan WCW. (Editor). Springer, New York.

Zhao X, Hilliard LR, Mechery SJ, Wang Y, Bagwe RP, Jin S, Tan W. 2004. A rapid bioassay for single bacterial cell quantitation using bioconjugated nanoparticles. *Proc. Natl. Acad. Sci. USA.* 101: 15, 027-32.

Zhou X, Zhou J. 2004. Improving the signal sensitivity and photostability of DNA hybridization on microarrays by using dye-doped core shell silica nanoparticles. *Amal Chem.* 18: 5302-12.