The Effects of Design Parameters on Nanoparticle Cellular Uptake, Nuclear Transport and Accumulation

by

Peter Shih-Yi Tang

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science

Institute of Biomaterials and Biomedical Engineering

University of Toronto

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University of Toronto
2014

Abstract

Studying the effects of the physicochemical properties of nanomaterials on cellular uptake, toxicity, and exocytosis can provide the foundation for designing safer and more effective nanoparticles for clinical applications. However, an understanding of the effects of these properties on subcellular transport, accumulation and distribution remains limited. The present study investigates the effects of surface density and particle size of semiconductor quantum dots on cellular uptake as well as nuclear transport kinetics, retention, and accumulation. The current work illustrates that cellular uptake and nuclear accumulation of nanoparticles depends on surface density of the nuclear localization signal peptides with nuclear transport reaching a plateau at 20% surface nuclear localization signal peptide density in as little as 30 minutes. These intracellular nanoparticles have no effects on cell viability up to 72 hours post treatment. These findings will set a foundation for engineering more sophisticated nanoparticle systems for imaging and manipulating genetic targets in the nucleus.
To my granny who just had her hundredth birthday, her health and longevity.

獻給我剛過完一百歲生日的奶奶，並祝她身體健康、平安喜樂！

To Candace, for her support and wisdom.

To my brother and parents, for everything.
“If all difficulties were known at the outset of a long journey, most of us would never start out at all” by William Buckley Jr.
Acknowledgments

**First, I would like to thank Dr. Warren Chan** for his support, open-mindedness, wisdom and guidance. If some day I have a chance to lead a group, he will be my role model. He taught me to think critically, evaluate objectively, and decide confidently. This thesis marks the start of my next journey, in which I will follow my heart and enjoy as much as this one.

**In addition, I would like to sincerely thank people at school:**

My committee members: Dr. Jon Rocheleau and Dr. Eli Sone, who trained me to critically review my data and supported me throughout the program.

The external examiner: Dr. Penney Gilbert, for helping me complete this thesis.

My collaborators: Dr. Fayi Song for the nanobarcode and its pending US patent; Dr. Alexandre Albanese for our review article, which is the most downloaded Annual Review of Biomedical Engineering article in 2013 (see Appendix 11); and Drs. Linda Penn, Jumi Shin, Antonia DeJong, Sarmitha Sathiamoorthy, and Romina Ponzielli as well as Lindsay Lustig for our collaborative projects.

Lab members: Dr. Kun Chen for cheering me up all the time, his technical support and critical analysis; Ed Sykes and Abdullah Syed for helping me with my experimental design and analysis; Dr. Kim Tsoi for QD projects; Dr. Leo Chou, Bill Dai, Dr. Alex Albanese, and Dr. Carl Walkey for giving me second opinions in research and life; and the rest for making the learning experience fun and memorable.

Instrument assistance: Dan Mathers and Ying Lei for ICP, Drs. Feng Xu and Chris Yip for confocal microscopy, and Dr. Rodrigo Fernandez-Gonzalez for microinjection.

My friends at IBBME: Kyryl Zagorovsky, Noel Wu, Dr. Stephen Czarnuch and Alex Posatskiy for my first design project; Lauren Lin, Alex Yu, Alphonsus Ng, Jocelyn Lo, Gabrielle Lam, and Dr. Amy Won on our floor; Hart Levy and Kenneth Ng for Biomedical Engineering Student Association’s juice fridge.
Funding agencies for scholarships: the Queen Elizabeth II Graduate Scholarship in Science and Technology, Ontario Graduate Scholarship, and International Graduate Scholarship from the Ministry of Education, Taiwan.

**Most importantly, I gratefully appreciate these people in my life outside school:**

My dearest family: Candace’s family for their support and belief in me since we got married, my extended family for their unreserved love, and my cousin Noreen Li in Toronto.

My friends in management consulting: Jason Ng, Dr. Lydia He, Dr. Mary Rose Bufalino, Xavier Cheng and Dr. Preethy Prasad at Graduate Management Consulting Association; Justin Xu, Dr. Vicky Tung, Cherry Rose Tan and Ibrahim Baig at Case Advisory Inc., and our amazing and accommodating nonprofit and corporate clients; Dr. Lingli He and Dr. Juan Li at McKinsey & Co. Insight Asia Pacific program in Chicago; Ian Xiao and Jeff Wu at Toronto Case Practicum; my consulting mentor at Life Science Ontario, Dr. Mary Argent-Katwala; and my colleague at GenScript USA Inc., Afra Wang, during our internship.

My friends in Taiwan: those I met at National Chiao Tung University, Song-Jin Tsai, Dr. Yu-Jing Liu, Chia-Hsuan Li, Ching-Yun Chang, and Ling-Ya Chiu; at Development Center for Biotechnology, Ben Liu, Lucy Chou, and Yingzi Chen; and at National Chengchi University’s Management of Technology for the Executives program, Terry Sheng, Casper Tu, and Linhua Lin. Through our conversations, I feel connected to my past and my future.

My friends from Taiwan in Toronto: Dr. Wes Chiang, Dr. Kuochieh Liao, Dr. Wei-Jiun Su, Joe Yen, Ruyun Chiang, Summer Chang and Steve Yang, and Dr. Chih-Kuang Chen. We had great food and great times together. Toronto is a great city to meet all of you!

Sincerely yours,

Peter Tang

July 2014 in Toronto, Canada
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<th>Description</th>
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<td>CPP</td>
<td>cell penetrating peptide</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma-atomic emission spectroscopy</td>
</tr>
<tr>
<td>MAA</td>
<td>mercaptoacetic acid</td>
</tr>
<tr>
<td>mNLS</td>
<td>mutated NLS</td>
</tr>
<tr>
<td>mPEG</td>
<td>methoxy-polyethylene glycol</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(D,L-lactide-co-glycolide)</td>
</tr>
<tr>
<td>QD</td>
<td>quantum dot</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>scNLS</td>
<td>scrambled NLS</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
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Chapter 1
Thesis Overview

1.1 Nuclear Transport of Nanoparticles

Nano-bio interaction has received significant attention in recent years as research groups aim to correlate the physicochemical parameters of nanoparticles with biological responses.¹ Such correlation will enable rationale design of nanoparticles with desired functionalities. These parameters have been demonstrated to affect cellular uptake of nanoparticles. Most studies to date have focused on elucidating the biodistribution and cellular interactions of nanoparticles of different physicochemical properties.¹

The nucleus is the primary target of many chemotherapeutics, probes and small molecules for effectively treating and diagnosing diseases.²⁻⁴ Therefore, this thesis work focused on developing targeting strategies for the nucleus as it would have the greatest impact on cancer therapy. Such targeting is often achieved by conjugating the nuclear localization signal (NLS) peptide to the therapeutics, probe, or nanoparticle. The NLS is essentially a positively-charged peptide sequence that interacts with importin proteins to actively deliver the payload to the nucleus. While the NLS has been successfully demonstrated to assist the delivery of nanoparticles into the nucleus, few studies have investigated the role of the surface ligand density and nanoparticle size on nuclear transport. For example, the size of gold nanoparticles has been shown to dictate their intracellular destination.⁵ In the absence of NLS, nanoparticles with diameters of 5.5 nm were unable to enter the nucleus while those with diameters of 2.4 nm were found inside the nucleus. The upper limit for active nuclear transport has been demonstrated with 26 nm gold nanoparticles coated with proteins containing NLS,⁶ although the specific physicochemical properties for optimal nuclear transport remain unknown.

This NLS-based strategy is often limited by the minimal cellular penetration ability of NLS previously used, so most studies are only proof-of-concept and require artificial conditions, such as cell membrane permeation⁷ and microinjection,⁸ to bypass the cell membrane barrier. Such conditions not only limit the potential of nanoparticles as nuclear targeting nanocarriers, but also hinder investigation of their interaction with the biological system. Nano-bio interaction has received significant attention in recent years, as research groups aim to correlate the physical parameters of nanoparticles with biological responses.¹ Such correlation will enable rational
design of nanoparticles with desired functionalities. These parameters include size, ligand density, and surface charge, which have been demonstrated to affect cellular uptake of nanoparticles. However, less is known about their effects on nuclear transport due to limited nanoparticle designs capable of both cellular uptake and nuclear transport.

1.2 Motivation
There have been limited studies on the role of nanoparticle design in mediating subcellular transport. My thesis work aims to address these issues and to enable future studies along this line, which will provide a basis to optimally design nanoparticles for targeting specific organelles. This is important for delivering therapeutics to subcellular organelles where they may be more effective (e.g., the nucleus or mitochondria) or probes that may allow us to study biological processes in living cells.

1.3 Specific Aims
Aim 1: Design Nanoparticles Capable of Nuclear Transport
Aim 2: Manipulate these Nanoparticles’ Design Parameters
Aim 3: Analyze Design Parameters’ Effects on Nuclear Transport

1.4 Thesis Outline
In this thesis, I used quantum dots (QDs) as a model nanoparticle for assessing the effects of NLS ligand density and particle size on nuclear targeting. QDs are fluorescent nanoparticles that can be coated with NLS peptides and quantified in cells using techniques such as inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and flow cytometry. QDs have also been used as carriers of therapeutics or imaging agents to characterize and analyze intracellular processes. Not only will using QDs provide a better understanding of nuclear transport, my study may provide an added benefit to guiding their design as nuclear probes for
imaging genetic processes or carriers of therapeutics that can interact with nuclear components. This work has been accepted and published by the journal *Small*.\(^1\)

In the Appendices, I include summary of collaborative projects that have been published and my contribution, including a review article on the effects of nanoparticle design parameters on cellular interactions,\(^1\) and a research article on the cellular interaction of nonblinking plasmonic QD assemblies and their application in multiplex biological detection.\(^19\)
Chapter 2
Introduction

2.1 Subcellular Delivery

2.1.1 Importance

Cancer is one of the most devastating diseases worldwide with more than 10 million new cases reported every year.\textsuperscript{20} Mortality of cancer has decreased in the past few years as a result of better knowledge of tumour biology and improved diagnostics and therapeutics.\textsuperscript{21,22} Current cancer intervention consists of surgical intervention, radiation, and chemotherapeutics that often remove and kill healthy cells around cancer cells, causing adverse effects to the patients.\textsuperscript{22} Only limited knowledge in basic cancer biology has been translated to clinical application due to the inability to deliver therapeutics specifically to the target site with marginal or no collateral damage.\textsuperscript{23,24} For example, only 1 to 10 parts out of 100,000 monoclonal antibodies reach their parenchymal targets \textit{in vivo}.\textsuperscript{25} Efficacy per unit of therapeutic agents shall be increased with greater targeting specificity.\textsuperscript{26}

2.1.2 Current Progress

Currently, drug delivery to these subcellular compartments is achieved by engineering or screening drugs that diffuse through cell membranes and spread the entire cell. However, such drug diffusion does not target specific organelles and might cause non-specific effects.\textsuperscript{27} In contrast, an alternative approach exploits endocytosis-mediated uptake for intracellular delivery, but the internalized drugs are often entrapped inside the endocytic vesicles, impeding trafficking to their subcellular site of action and limiting therapeutic effects.\textsuperscript{3} Subcellular drug targets comprise of the majority of organelles (Table 2.1) with the nucleus being the most critical one. Subcellular targeting may be achieved by taking advantage of the intracellular sorting signals and mechanisms. The approach first delivers particles into the cytosol, where the conjugated sorting signals are recognized and facilitated movement of the particles to the designated organelles, such as the nucleus and mitochondria.
Table 2.1. Subcellular Targets of Common Drugs and their Applications

<table>
<thead>
<tr>
<th>Subcellular target</th>
<th>Drug or compound</th>
<th>Mode of action</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>Enalivudine</td>
<td>Lipid linkage promotes membrane targeting of inhibitors and better inhibition of HIV fusion complex</td>
<td>HIV fusion inhibitors</td>
</tr>
<tr>
<td></td>
<td>C34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myr-proS1</td>
<td>Myristoylated or stearoylated ProS1 domain of HIV targets the fusion complex</td>
<td>HIV fusion inhibitor</td>
</tr>
<tr>
<td></td>
<td>Pertuzumab</td>
<td>Palmitoylation of the β loop of GPCRs efficiently modulates GPCR signaling</td>
<td>GPCR modulator</td>
</tr>
<tr>
<td>Early endosomes</td>
<td>Cholesterol-linked β-secretase inhibitor</td>
<td>Addition of cholesterol promotes membrane tethering and endocytosis into endosomes, in which the active enzyme is localized</td>
<td>Efficient inhibition of the β-secretase enzyme; a therapeutic target in Alzheimer’s disease</td>
</tr>
<tr>
<td></td>
<td>Anticancer drugs</td>
<td>Ligands of cell surface receptors (folate, LDL, cholesterol and transferrin) mediate endocytosis of the heterologous conjugates</td>
<td>Effective transport of anticancer drugs to the interior of the cell</td>
</tr>
<tr>
<td></td>
<td>Denileukin diltitox, IL-2 and IL-13</td>
<td>Diphtheria toxin conjugates and Pseudomonas exotoxin conjugates enable the uptake of the III and release in the intracellular site</td>
<td>Malignant lymphomas</td>
</tr>
<tr>
<td>Late endosomes and lysosomes</td>
<td>β-glucosidase, β-hexosaminidase</td>
<td>Replacement of lysosomal enzymes directly or by mannose-6-phosphate mediated uptake</td>
<td>Enzyme replacement therapy for lysosomal storage diseases (Gaucher’s and Fabry’s disease)</td>
</tr>
<tr>
<td></td>
<td>Cycler-2</td>
<td>Cholesterol-sequestering agent</td>
<td>Niemann-Pick disease</td>
</tr>
<tr>
<td>Endoplasmic reticulum and Golgi</td>
<td>Antigenic peptides</td>
<td>Delivery of conjugated antigens for presentation on MHC class I complex by conjugation to STX-B</td>
<td>Malignant lymphomas, ovarian cancer and intestinal adenocarcinomas</td>
</tr>
<tr>
<td>complex</td>
<td>Fluorescent cancer imaging dyes</td>
<td>Imaging of tumours by STX-B conjugates of fluorescent dyes, as STX-B binds to GB3, which is overexpressed by tumours</td>
<td>Colon cancer, liver metastasis and ovarian tumours</td>
</tr>
<tr>
<td></td>
<td>Shiga holotoxin</td>
<td>Selective killing of GB3 overexpressing tumours</td>
<td></td>
</tr>
<tr>
<td>Cytosolic delivery</td>
<td>Anticancer drugs, siRNAs and plasmids</td>
<td>Conjugation with cell-penetrating peptides such as Tat and VP12 enables transport of heterologous conjugates into the cell</td>
<td>Delivery of anticancer drugs, siRNAs, plasmid DNA and proteins</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Ganetixib</td>
<td>Selective targeting of the HSP90 network in cancerous mitochondria</td>
<td>Rapid tumour cell death</td>
</tr>
<tr>
<td></td>
<td>Antioxidant (ubiquinol and α-tocopherol)</td>
<td>Lipophilic cations such as triphenylphosphonium cations conjugated with antioxidants target mitochondria and confer protection</td>
<td>Neurodegenerative diseases</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Antitumour drugs, cisplatin, doxorubicin and DNA</td>
<td>Delivery of genes by viral mediated vectors, viral-like particles or liposomes Nanoparticles encapsulate the drug and enable slow and effective release Targeted charge-reversal nanoparticles carry conjugates to the nucleus</td>
<td>Carcinomas</td>
</tr>
</tbody>
</table>

GB3, globotriaosylceramide (also known as CD27); GPCR, G protein-coupled receptor; HIV, hepatitis B virus; HSP90, heat shock protein 90; II, interleukin; LDL, low-density lipoprotein; MHC, major histocompatibility complex; siRNA, small interfering RNA; STX-B, Shiga toxin subunit B; Tat, transactivator of transcription protein; VP12, viral protein 22.
2.1.3 Cytosolic Delivery

The cytosol is among the most critical subcellular targets for its various metabolic, signaling and pathogenic involvements. Cell penetrating peptides (CPPs) have been used to facilitate translocation across the cell membrane and to specific organelles inside cells, and many of them are derived from natural sequences. For example, Tat is a CPP derived from HIV-1 virus, and it has been commonly used to deliver different nanoparticles into cells. Intracellular destinations of Tat-mediated delivery include the nucleus, vesicles, and endolysosomal compartment, depending on the nature of nanoparticles delivered. To evade endolysosomal sequestration, Tat can be further modified with pH-sensitive proteins, such as the influenza virus hemagglutinin protein HA2. The Tat-HA2 peptide has been shown to destabilize the endosomal membrane without causing cytotoxicity, and deliver gold nanoparticles into cytosol.

In addition to pH-sensitive proteins, a class of polymeric nanocarriers receives increasing attention for endosomal escape. For example, negatively charged poly(D,L-lactide-co-glycolide) (PLGA) based-nanocarriers become positively charged in acidic environment such as late endosomes and lysosomes. The underlying mechanism involves accumulation of positively charged PLGA nanocarriers to the negatively charged vesicular membranes, membrane destabilization, and escape of these nanocarriers into cytosol. In a pilot study by our group, QDs were delivered into the cytosol by a PLGA nanocarrier. However, QD encapsulation efficiency awaits further characterization and optimization.

2.1.4 Nuclear Targeting

Most chemotherapeutic drugs fall into the following categories: alkylation agents, antimetabolites, anthracyclines, plant alkaloids, and topoisomerase inhibitors. These drugs interfere with cell division, DNA synthesis and function, and induce cell death. Nuclear targeting will greatly enhance their therapeutic effects, so it receives increasing attention for delivering chemotherapeutic drugs in the past decades. However, such nuclear targeting is often hindered by the nuclear envelope. Nucleus is surrounded by the double-layered nuclear envelope decorated with specialized channels called nuclear pore complex (NPC) which allows
passive diffusion of certain sizes of nanoparticles (Figure 2.1). For example, in the absence of NLS, nanoparticles with diameters of 5.5 nm were unable to enter the nucleus while those with diameters of 2.4 nm were found inside the nucleus.\(^5\) This suggests that the upper size limit for passive diffusion to be between 2.4 and 5.5 nm. However, nanoparticles between 2.4 and 5.5 nm were not tested, so further investigation is required to determine the upper size limit for nanoparticles capable of nuclear entry through passive diffusion. On the other hand, the upper limit for active nuclear transport has been demonstrated with 26 nm gold nanoparticles coated with proteins containing NLS.\(^6\) Passage through nuclear envelope is mainly mediated by the importin \(\alpha\) and \(\beta\) transporters. Importin \(\alpha\) is an adaptor protein that binds importin \(\beta\) and recognizes the NLS of its cargo. Detailed mechanisms for transport through nuclear pore complexes are introduced in Section 2.1.5.

The first NLS (PKKKRKV) was discovered in Simian virus 40 (SV40) Large T-antigen NLS.\(^39\) Since then, many other NLS sequences have been discovered, but the SV40 NLS is perhaps the most commonly used. However, NLS-coated nanoparticles often get trapped inside the endosomes that prevent their release into cytosol and result in low nuclear transport efficiency. To improve this, CPP was used to investigate its role in facilitating endosomal escape of NLS-coated nanoparticles.\(^40\) This CPP (CKKKKKKSEDEYPYVPN) was derived from an adenoviral fiber protein and has been demonstrated to enter cells through receptor-mediated endocytosis.\(^41\) As expected, nuclear transport was absent for gold nanoparticles coated with NLS peptides alone because they were trapped inside the endosomes after cellular uptake. Nuclear transport was seen for gold nanoparticles coated with a long peptide that contains both NLS and CPP sequences in tandem, supporting the role of CPP in facilitating endosomal escape into cytosol, which is required for subsequent nuclear transport. Interestingly, much efficient nuclear transport was seen when using CPP and NLS as two types of short peptides instead.

However, previous studies using the NLS/CPP mixture strategy had minimal control over the ligand density due to intermediate proteins required (bovine serum albumin\(^{33,40}\) or streptavidin\(^{42}\)) or the surface coating method used.\(^{43}\) In addition, these studies relied on qualitative analysis, making it difficult to compare and conclude. To better investigate nuclear transport of nanoparticles, the present study builds on this dual peptide strategy and improves control of ligand density through direct peptide attachment onto nanoparticles.
2.1.5 Mechanisms for Nuclear Transport through Nuclear Pore Complexes

The nucleus is surrounded by the nuclear envelope that contains two membranes which connect to NPC. Figure 2.1 illustrates structure of NPC, which is composed of 30-50 proteins called nucleoporins that are functionally conserved from yeast to mammals.\textsuperscript{44,45} NPC is asymmetrical – fibrils stretch from the outer nuclear membrane, while fibrils are basket-like on the nucleoplasmic surface.\textsuperscript{46} The central channel is rich in a subset of nucleoporins with amino acid repeats (FXFG or GLFG) that interact with karyopherins and facilitate their transport through NPC.\textsuperscript{47} Karyopherins are transport receptors that recognize cargoes for transport. Depending on the direction of transport, karyopherins can be further categorized into importins and exportins which facilitate transport into and out of the nucleus, respectively.

Figure 2.2 illustrates roles of other proteins and their interaction with importins during transport into the nucleus. The process begins when the adaptor protein importin-\(\alpha\) recognizes and binds to NLS on a cargo that can be either a protein or a nanoparticle. Subsequently, importin-\(\beta\) binds and shuffles the importin-\(\alpha\)/cargo complex through NPC into the nucleus, where RanGTP binds to importin-\(\beta\), dissociates the tripartite complex, and releases the cargo. Importins are re-shuffled back into the cytoplasm with the aid of RanGTP. Importin-\(\alpha\) requires an additional nuclear export receptor, CAS, for this translocation.\textsuperscript{48} Directionality of importins transport across NPC is determined by the balance between RanGTP and RanGDP.\textsuperscript{49,50} RanGEF is a RanGTP exchange factor that converts RanGDP to RanGTP. RanGEF resides in the nucleus, where it maintains high levels of RanGTP.
**Figure 2.1. Nuclear Pore Complex.** The NPC (~200 nm in length) is composed of nucleoporins and is located on the nuclear membrane with a central channel and openings to the cytoplasmic and nuclear sides. On the cytoplasmic side, cytoplasmic filaments are anchored to a cytoplasmic ring at the plasma membrane. On the nuclear side, there is a basket-like structure composed of an inner ring and a distal ring that extends into the nucleus. The ribosome (~150 Å) is included as scale. © Adapted with permission from reference,\(^\text{48}\) Copyright (2004) Nature Publishing Group.
Figure 2.2. Nuclear Transport of NLS-coated Proteins/Nanoparticles. Nuclear transport of NLS-containing proteins or nanoparticles (both labeled as P-NLS) begins when importin-α (α) recognizes their NLS and forms a complex, which subsequently binds to importin-β (β) and translocates through the nuclear pores into the nucleus. Inside the nucleus, RanGTP dissociates the protein from these importins. RanGEF maintains a high concentration of RanGTP by converting RanGDP to RanGTP. The dissociated importin-α and -β are then transported back to the cytoplasm through the pore. Importin-β is exported with RanGTP to the cytoplasm, where RanGTP hydrolyses to RanGDP. Export of importin-α requires the nuclear export receptor CAS, which subsequently binds RanGTP and forms a tripartite complex. Adapted with permission from reference,48 Copyright (2004) Nature Publishing Group.
2.2 Nanoparticles

2.2.1 General Information

Nanotechnology is in the position to further advance cancer therapeutics by improving the targeting specificity.\(^{51}\) Nanotechnology is defined by the British Standards Institution as ‘design, characterization, production and application of structures, devices and systems by controlling shape and size at nanoscale,’ and ‘nanoscale’ refers to ‘having one or more dimensions of the order of 100 nm or less.’\(^{52}\) Nanoscale materials are comparable in size to biological molecules and systems (Figure 2.3), and possess unique properties not found in atoms or macroscopic materials, such as a high surface area-to-volume ratio, and tunable optical, electronic, magnetic, and biologic properties. In addition, they can be engineered for desired physical properties (sizes, shapes), chemical characteristics (compositions, surface chemistry), and geometric structures (hollow vs. solid).\(^{22,53}\) Their unique properties present opportunities in advancing medical applications as summarized in Table 2.2.

Nanotechnology has exploited the enhanced permeability and retention effects for ‘passive targeting’\(^{54}\). ‘Active targeting’ further improves the targeting specificity by conjugating these nanocarriers to ligands that bind to specific receptors or antigens on the surface of cancer cells.\(^{22}\) Both targeting strategies have provided the foundations for drug delivery at the organizational and cellular level, but failed to address critical issues at the subcellular level, such as subcellular availability and accessibility of drug targets.

An ideal nuclear transport carrier must:\(^{33}\) (1) cross cell membrane, e.g. by CPP or receptor-mediated endocytosis; (2) evade endocytic entrapment; (3) posses NLS and interact with the importins to pass nuclear pores (size threshold: 30-50 nm); and (4) minimally affect cell viability and function. The size requirement makes nanoparticles an ideal model to study the effects of design parameters on nuclear transport.

While gold nanoparticles provide clues to nuclear transport of nanoparticles in earlier studies,\(^{5,6,33,40}\) these gold nanoparticles’ nuclear presence was not validated by confocal microscopy because they were not fluorescent, so ideal nanoparticles for this type of study should possess intrinsic fluorescence for such validation. In addition, ideal nuclear targeting
nanoparticles shall possess facile surface modification procedures for CPP and NLS peptides, and minimal effects on cell viability and function. These parameters make QDs ideal nanoparticles for nuclear targeting.
Figure 2.3. Nanotechnology and Medicine. The schematic illustrates the size scale of nanomaterials (right), their progress toward clinical applications (top left), and their proof-of-concept application in research laboratories (bottom left). © Reproduced with permission from reference,\textsuperscript{55} Copyright (2010) Massachusetts Medical Society.
Table 2.2. Clinical Phases of Nanomaterials

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Trade Name</th>
<th>Application</th>
<th>Target</th>
<th>Adverse Effects</th>
<th>Manufacturer</th>
<th>Current Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metallic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron oxide</td>
<td>Feridex</td>
<td>MRI contrast</td>
<td>Liver</td>
<td>Back pain, vasodilation</td>
<td>Bayer Schering</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Resovist</td>
<td></td>
<td>MRI contrast</td>
<td>Liver</td>
<td>None</td>
<td>Bayer Schering</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Combidex</td>
<td></td>
<td>MRI contrast</td>
<td>Lymph nodes</td>
<td>None</td>
<td>Advanced Magnetics</td>
<td>In phase 3 clinical trials</td>
</tr>
<tr>
<td>NanoTherm</td>
<td></td>
<td>Cancer therapy</td>
<td>Various forms</td>
<td>Acute urinary retention</td>
<td>MagForce</td>
<td>In phase 3 clinical trials</td>
</tr>
<tr>
<td><strong>Gold</strong></td>
<td>Verigene</td>
<td>In vitro diagnostics</td>
<td>Genetic</td>
<td>Not applicable</td>
<td>Nanosphere</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Aurimmune</td>
<td></td>
<td>Cancer therapy</td>
<td>Various forms</td>
<td>Fever</td>
<td>Cytimmune Sciences</td>
<td>In phase 2 clinical trials</td>
</tr>
<tr>
<td>Nanoshells</td>
<td>Aurosshell</td>
<td>Cancer therapy</td>
<td>Head and neck</td>
<td>Under investigation</td>
<td>Nanospectra Biosciences</td>
<td>In phase 1 clinical trials</td>
</tr>
<tr>
<td><strong>Semiconductor</strong></td>
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<td>Fluorescent contrast, in vitro diagnostics</td>
<td>Tumors, cells, tissues, and molecular sensing structures</td>
<td>Not applicable</td>
<td>Life Technologies, eBioscience, Nanoco, CrystalPlex, Cytodiagnosics</td>
<td>Research use only</td>
</tr>
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<td>Cancer therapy</td>
<td>Breast</td>
<td>Cytopenia</td>
<td>Abraxis Bioscience</td>
<td>FDA approved</td>
</tr>
<tr>
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<td>Doxil/Caelyx</td>
<td>Cancer therapy</td>
<td>Various forms</td>
<td>Hand-foot syndrome, stomatitis</td>
<td>Ortho Biotech</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Polymer</td>
<td>Oncaspars</td>
<td>Cancer therapy</td>
<td>Acute lymphoblastic leukemia</td>
<td>Urticaria, rash</td>
<td>Rhône-Poulenc Rorer</td>
<td>FDA approved</td>
</tr>
<tr>
<td>CALAA-01</td>
<td></td>
<td>Cancer therapy</td>
<td>Various forms</td>
<td>Mild renal toxicity</td>
<td>Calando</td>
<td>In phase 2 clinical trials</td>
</tr>
<tr>
<td>Dendrimer</td>
<td>VivaGel</td>
<td>Microbicide</td>
<td>Cervicovaginal</td>
<td>Abdominal pain, dysuria</td>
<td>Starpharma</td>
<td>In phase 2 clinical trials</td>
</tr>
<tr>
<td>Micelle</td>
<td>Genexol-PM</td>
<td>Cancer therapy</td>
<td>Various forms</td>
<td>Peripheral sensory neuropathy, neutropenia</td>
<td>Samyang</td>
<td>In phase 4 clinical trials</td>
</tr>
</tbody>
</table>

2.2.2 Quantum Dots

QDs are highly luminescent semiconductor nanoparticles, stable against photobleaching, and narrow in spectral line width.\textsuperscript{56} QDs possess high quantum yield and high molar extinction coefficients (~ 2 orders of magnitude higher than that of organic dyes).\textsuperscript{57,58} In comparison to organic dyes, these QDs possess unique properties — size-dependent fluorescence emission and broad excitation spectra (Figure 2.4). These properties enable simultaneous excitation for QDs of various emissions at a single-wavelength.\textsuperscript{16}

Biological applications require QDs that are stable in the biological environment, such as QDs made of CdSe cores with an additional layer of ZnS. The ZnS layer protects QDs from oxidation, keep cytotoxic Cd or Se metals from leaching into biological environment, passivates the surface and substantially improves the photoluminescence yield.\textsuperscript{58,59} High-quality QDs are synthesized using high-temperature growth solvents/ligands — mixture of trioctyl phosphine and trioctyl phosphine oxide — and pyrolysis of organometallic precursors. This method resulted in CdSe QDs with highly crystalline cores and improved size distributions of 8–11%.\textsuperscript{60} This method has been further developed to coat the CdSe core with an additional layer of wide-bandgap semiconducting material (e.g., ZnS, CdS) by including appropriate organometallic precursors to the reaction.\textsuperscript{58,59,61} QDs synthesized by this method are not soluble in aqueous biological environments, and require surface modification with hydrophilic ligands. In addition to QDs’ solubility, these hydrophilic ligands also allow biomolecules to attach to QD surfaces. Figure 2.5 illustrates the generic strategies to render organic QDs water soluble and summarizes common hydrophilic ligands as well as the linking chemistry for direct attachment of biomolecules to QDs.

Conjugation to biomolecules such as DNA, peptides, and proteins, confer these QDs specific biological functions. Biological applications of QDs include cell labelling, biosensing and energy transfer, \textit{in vivo} imaging, and diagnostics.\textsuperscript{62} Near-infrared QD imaging has been demonstrated to successfully guide a major cancer surgery, the sentinel lymph node mapping, in large animals.\textsuperscript{63} However, no clinical application of QDs has yet been reported due to lack of consensus and conclusive studies regarding potential cytotoxicity of QDs. In fact, many other nanoparticles are faced with similar challenges, limiting their further biomedical application. It
is therefore important to investigate the effects of nanoparticles on cell functions before their application in biomedical engineering.
Figure 2.4. Size-dependent Optical Properties of Quantum Dots. QDs of indicated CdSe core sizes were illuminated under ultraviolet light at 365 nm, demonstrating size-dependent fluorescence properties (a) and spectra (b). The black line represents the absorbance spectrum for QDs with emission at 510 nm. © Adapted with permission from reference,16 Copyright (2009) Oxford University Press.
Figure 2.5. Strategies for Quantum Dot Solubilization and Surface Modification. (a) As-synthesized QDs are capped with hydrophobic ligands, which replaced by excess hydrophilic ligands that render these QDs soluble in aqueous solution. Generic strategies to further modify these aqueous QDs with biomolecules (b) and commonly used biomolecules (c). Color legend: pink (linkage to QDs), blue (water solubility), and green (biomolecule linking functionality). © Adapted with permission from reference,16 Copyright (2005) Nature Publishing Group.
2.3 Biological Interactions

2.3.1 General Information

Safety concerns arise at the interface between nanomaterials and biological systems. It is important to gain comprehensive understanding of the biological interactions of these nanoparticles. Such knowledge would aid in controlling their toxicity, and potentially confer therapeutic effects to these nanoparticles, rather than limit their application.

Nanoparticles may interact with biological systems via epithelial translocation (skin exposure), gastrointestinal tract (ingestion), and respiratory tract (inhalation). Biomedical application of nanoparticles requires knowledge of such interaction. Biological interactions of nanoparticles attribute to their physicochemical characteristics, intracellular concentrations, contact time, subcellular localizations, and interactions with molecules inside the cell. Despite much effort was made, such knowledge is barely available. This is because most studies focused on the endocytic routes or limited organelles, and failed to relate the biological responses to the physicochemical properties of the nanoparticles. Effects of these nanoparticles on cell division and daughter cells are also often neglected. For example, many of these studies used cell viability as the sole readout, but overlooked the underlying mechanisms. Microarray profiling has demonstrated such subtle changes induced by carbon nanotubes and gold nanoparticles in the absence of detectable change in cell viability. For example, gold nanoconstructs modified with nuclear targeting aptamers decreased cell viability even though gold nanoparticles are generally considered inert. Few such studies have been reported, so these effects must be evaluated on a case-by-case basis before a consensus is reached. While the associated cytotoxicity seems to prevent biomedical application of nanoparticles, this may also grant nanoparticles novel chemotherapeutic properties.

2.3.2 Biological Interaction with Quantum Dots

The effects of intracellular QDs on cell viability have been previously demonstrated. However, the results were inconclusive regarding the toxicity and characteristics of cytoplasmic nanoparticles, hence requiring further work to fully understand the intracellular fate and effects of QDs. While proper coating (e.g. ZnS shell) renders QDs nontoxic, there is still concern that
such coating may degrade in the biological systems over a sufficiently long period of time, exposing their CdSe-core, releasing cytotoxic Cd$^{2+}$ and subsequent reactive oxygen species (ROS). Experimental conditions that trigger Cd$^{2+}$ release have also been identified, such as oxidation and ultraviolet exposure. In another study, CdSe/ZnS core/shell QDs were conjugated to nucleic acids, and light-activated ROS was demonstrated to break and damage nucleic acids. On the other hand, micelle-QDs injected into *Xenopus* embryos showed minimal effects on cells even at dosages of more than $10^9$ QDs per cell. Therefore, studies investigating nanoparticle-cell interactions must take into account these potential effects of QDs on cells and ensure the experimental conditions are as physiological as possible.

### 2.4 Design Parameters

#### 2.4.1 General Information

The concept of design parameters comes from the nature, which has evolved molecules, viruses, bacteria, and other biological structures into precise sizes, shapes, and chemistries to mediate interactions and functions. Common design parameters for nanoparticles include sizes, shapes, surface chemistries, surface activities and aggregation states.

There are increasing interests in investigating nano-bio interactions, which are interactions between nanomaterials and biological systems. These studies are carried out with a panel of nanoparticles with all design parameters kept constant except the parameter of interest in the experimental framework (Figure 2.6). Then these nanoparticles are exposed to biological systems and assessed for their biological responses. Systematic analyses enable scientists to attribute a specific biological response to a single or combination of design parameters. As a result, these studies allow for setting design rules that facilitate the engineering of nanodevices.

#### 2.4.2 Design Parameters vs. Cellular Uptake

Cellular interactions of nanoparticles on the cell membrane include the scenarios illustrated in Figure 2.7. Cellular uptake of nanoparticles depends on key design parameters such as surface charges, ligand densities, and nanoparticle sizes. In terms of the effects of surface charges, positively charged nanoparticles are found to enter cells much faster than their counterparts that are neutrally or negatively charged. Negative charges on the cell membrane have been
suggested to drive higher cellular uptake of positively charged nanoparticles through electrostatic attractions.\textsuperscript{74,75} One recent report has shown that this electrostatic attraction between positively charged nanoparticles and negatively charged cellular membrane favors association of nanoparticles onto the cell’s surface, and results in their subsequent cellular uptake.

For nanoparticles with ligands, their interaction with cellular receptors depends on the densities of these ligands on nanoparticle surface. A multivalent effect has been demonstrated for nanoparticles with multiple ligands that interact with several receptors on the cell membrane. Such association affects the binding avidity of these ligands to the receptors. For instance, the binding affinity of herceptin to the ErbB2 receptor is $10^{-10}$ M in solution, and increases to $5.5 \times 10^{-12}$ M and $1.5 \times 10^{-13}$ M on 10nm and 70 nm nanoparticles, respectively.\textsuperscript{76} The increased ligand-receptor binding affinity was attributable to a higher protein density on the surface of larger nanoparticles. In support, the effects of ligand density on cellular uptake have been demonstrated by other studies with larger nanoparticles (100 — 300 nm), such as aptamers,\textsuperscript{77} poly-Arginine,\textsuperscript{78} and Tat peptides.\textsuperscript{79} Earlier studies have demonstrated both linear and exponential relationships between ligand densities and cellular uptake, but only a small range of densities was used. For example, Gu et al. prepared polymer-based nanoparticles (ca. 160 nm) with various aptamer ligand densities and showed a 5-fold increase in cellular uptake by increasing the ligand density from 0.5% to 5% (10-fold) in the formulation.\textsuperscript{77} The effects were linear between aptamer densities of 0.5 and 5% and diminished above 5%. On the other hand, Zhao et al. prepared iron oxide nanoparticles with Tat peptides and demonstrated an exponential increase in cellular uptake, by approximately 100-fold, for nanoparticles with 15 Tat peptides on each nanoparticle compared to those with only one peptide on each nanoparticle (15-fold).\textsuperscript{79} However, ligand densities were expressed differently across these studies, making it difficult to conclude. Future studies on ligand density effect would require full characterization of ligand densities on nanoparticles for these results to be comparable across research groups. In addition, these reports used nanoparticles larger than 100 nm, and it requires further investigation to determine whether similar effects are also observed for nanoparticles smaller than 100 nm.

For nanomaterials with a given geometric shape, their cellular uptake strongly depends on their dimensions. The 50 nm diameter has been demonstrated for maximal rate of uptake and intracellular concentration in certain mammalian cells for spherical gold nanoparticles, silica
nanoparticles, single walled carbon nanotubes, and QDs. The size effect on cellular uptake is attributable to the so-called “wrapping time” that describes the process where a particle is enclosed by the cellular membrane.

2.4.3 Design Parameters vs. Nuclear Transport

Despite extensive studies on the effects of these design parameters on the cellular uptake, reports of these effects on nuclear transport are scarce and often qualitative. One study investigated the size effect on nuclear transport using NLS-tagged gold nanoparticles that were microinjected into the cytoplasm of *Xenopus* oocytes and characterized for their subcellular distribution by electron microscopy. Nuclear fraction, or the ratio of gold nanoparticles found inside the nucleus to total intracellular gold nanoparticles, decreased from 36% to 28% as the size increased from 22 nm to 26 nm, respectively. The size effect was attributed to the slower diffusion of larger nanoparticles in the cytoplasm before reaching the nuclear pore complex, compared to the diffusion rates of their smaller counterparts. However, the opposite was observed in another earlier study that used NLS-tagged proteins constructs with multiple copies of green fluorescence proteins (GFP). Nuclear transport was assessed by the ratio between nuclear and cytoplasmic fluorescence of GFP. The ratio was 1 for GFP protein, suggesting equilibrium between its transport across the nuclear and cytoplasmic compartments. NLS-tagged GFP showed an average ratio of 3.3, showing its nuclear accumulation driven by NLS-mediated active nuclear transport. This average ratio increased to 15 and 40 for NLS-tag constructs with 2 or 4 copies of GFP, showing a positive correlation between the construct lengths and their nuclear accumulation. The authors attributed this correlation to a reduction in passive diffusion from the nucleus back to the cytoplasm with increasing construct lengths. It is unclear how an additional copy of GFP affects the overall protein size since GFP is a cylindrical protein that is 4.2 nm in length and 2.4 nm in diameter. It remains to be determined whether this is also the case for nanoparticles with similar sizes, such as QDs.

The effects of ligand density on nuclear transport are inconclusive because most studies were conducted under non-physiological conditions with minimal characterization of nanoparticles. For example, Kurihara et al. prepared liposomes with various NLS densities and demonstrated positive correlation between NLS densities and these liposomes’ association with isolated
nuclei. It is uncertain whether similar NLS density effects on nuclear transport can be observed in intact cells.

In summary, it is important to illustrate the effects of design parameters on nuclear transport of nanoparticles to facilitate design of optimal nuclear targeting nanoparticles with desired properties.

Results and findings of this present study contributed to this field through a design that allowed for straightforward manipulation of ligand density, systemic investigation of these parameters’ effects on nuclear transport, and quantitative evaluation of these effects under physiological conditions.
Figure 2.6. Synthesis and Characterization of Design Parameters. Nanomaterials of desired design parameters are first synthesized and characterized for their physical and chemical properties (a). Then these nanomaterials are exposed to biological systems to evaluate their effect on biological responses (b, c). Databases can be created with information generated from correlating biological responses to a particular design parameter that is varied one at a time. Predictive software can be developed to simulate biological responses to nanomaterials (d) and facilitate design of next-generation nanodevices (e). Examples are listed for the most common design parameters, biological systems, biological responses investigated, and possible applications. © Adapted with permission from reference 1. Copyright (2012) Annual Reviews.
Figure 2.7. Effects of Design Parameters on Cellular Interaction with Nanoparticles. (a) Examples of design parameters that can affect interaction between nanoparticles and biological systems. (b) Schematic of cellular interaction of ① ligand-coated nanoparticles that can trigger signaling cascades by binding to receptors on the cell membrane. ② Some nanoparticles can enter cells after binding to membrane receptors, remain inside vesicles, and then leave the cell. ③ Other nanoparticles can leave these vesicles after cellular entry, and subsequently interact with organelles or translocate to subcellular structures of interest. ④ Nanoparticles can also interact with cells through non-specific association with cell membrane, and subsequently enter cells. © Reproduced with permission from reference,1 Copyright (2012) Annual Reviews.
Chapter 3
Materials and Methods

3.1 Preparation of Water-soluble Quantum Dots

Chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise specified. CdSeS/ZnS alloyed core/shell QDs were purchased from Cytodiagnostics (emission peak wavelength at 575 nm with a diameter of 4.5 nm) and CdSe/ZnS core/shell QDs from Ocean Nanotech (emission peak wavelengths at 450, 575, 665 nm for diameters of 3.0, 4.8, 8.0 nm, respectively). CdSeS/ZnS QDs (diameter = 4.5 nm) were used unless otherwise specified. These organic QDs were rendered water-soluble by 4 hours of ligand exchange with mercaptoacetic acid (MAA) as previously described, and purified as following. In a typical preparation, 500 μL QD solution was added to 500 μL acetone containing 40 μL aqueous solution of tetramethylammonium hydroxide (25% w/w), which was included to facilitate phase transfer of water-soluble QDs from organic to aqueous phase. Excess MAA was removed by centrifugation (1000 g, 5 minutes) and disposal of the supernatant. The process was repeated another 2 times with 1 mL acetone. Trace acetone was left to completely dry before the QD pellet was re-dispersed in phosphate-buffered saline (PBS), adjusted to pH 7.4, filtered through 0.22 μm syringe filter, and stored at 4°C before use.

3.2 Characterization of Quantum Dots

Absorbance and fluorescence spectra were measured using a UV-1601 spectrophotometer (Shimadzu) and a FluoroMax-3 fluorimeter (Jobin Yvon Horiba), respectively. QD concentrations were determined by absorbance at the first excitonic peak using extinction coefficients provided by the manufacturers. The diameters of QDs were determined by Tecnai-20 transmission electron microscopy (TEM) from FEI using carbon film coated copper grids (300 mesh, Ted Pella Inc.), and at least 50 nanoparticles were measured for each sample.

Hydrodynamic diameters of these QDs were measured by dynamic light scattering (DLS) on a Nano ZS Zetasizer (Malvern) with a 633 nm laser. Zeta potential values of these QD bioconjugates (20 nM) were also measured using this Nano ZS Zetasizer, but in 10 mM HEPES
buffer (final pH 7.5). The DIP-CELL electrode was cleaned between each measurement by batch sonication. Each sample was limited to 10 runs of measurement to minimize potential damages of the electrical current to the QD bioconjugates. Both hydrodynamic diameters and zeta potentials were presented as the average number from at least 3 batches of preparation.

3.3 Preparation of Quantum Dot-Peptide Bioconjugates

Water-soluble QDs were further stabilized with 1 kDa methoxy-polyethylene glycol-sulfhydryl (mPEG-SH; CreativePEGWorks) through ligand exchange (60°C, 1 h) in 25 mM borate buffer (pH 8.3), followed by removal of excess PEG with Amicon® Ultra Centrifugal Filters (molecular weight cut-off: 30 kDa, Millipore). The process was repeated another 2 times with pure water. Custom peptides were synthesized by BioMatik (for peptide sequences, see Table 4.1). Peptide adsorption was carried out in the same borate buffer (37°C, 1 h) with a constant surface density of total peptide (NLS and Control), which was 250 for the 4.5 nm QDs, and adjusted accordingly for other sizes of QDs to account for their different surface areas.
3.4 Characterization of Quantum Dot-Peptide Bioconjugates

3.4.1 Gel electrophoresis

Successful PEGylation and peptide adsorption were confirmed with 0.7% agarose gel electrophoresis (15 minutes, 135 V) in 0.5X Tris/borate/EDTA buffer and imaged with a Typhoon Trio variable mode imager (GE Healthcare).

3.4.2 Peptide Quantification

Peptide adsorption onto QDs was quantified by a depletion assay after removing excess, unbound peptides from QD-peptide bioconjugates with a tabletop ultracentrifuge (Beckman Optima MAX-XP), as previously described. In brief, QD-peptide bioconjugates were removed from the supernatant by ultracentrifugation (200,000 g, 60 minutes) and confirmed by UV-Vis. Proper controls were included to account for peptide loss due to ultracentrifugation. The remaining peptides in the supernatant were quantified by a fluorescence-based protein assay using a FluoroProfile® Protein Quantification Kit (Sigma) according to the manual. Alternatively, a custom FITC-labeled NLS peptide (BioMatik) was used and quantified by a calibration curve of known FITC-labeled NLS concentrations.

3.5 Cellular Uptake of Quantum Dots

3.5.1 Cell culture

The human cervical cancer HeLa cell line (ATCC CCL-2) were seeded at 5 x 10^4 cells/well into 8-well chambered cover glass slides (Nunc), or at 10^6 into 6-well plates (Nunc) for microscopic imaging and ICP quantification, respectively, and then incubated overnight to allow for cell attachment before treatment with the indicated QD-peptide bioconjugates. These cells were washed thoroughly with 1X phosphate buffered saline containing Mg^{2+} and Ca^{2+} to remove extracellular QDs. HeLa cell line was chosen because it is most commonly used in this type of study with much information available for reference in experimental designs. Information obtained from this study can also be widely applicable to and comparable with past and future literature. HeLa cells will be cultured by adapting methods I previously developed. In brief,
cells are maintained in DMEM with 10% FBS supplemented with 5% CO₂ at 37°C. Cells are seeded the day before treatment.

3.5.2 Confocal Microscopy

Intracellular QDs were visualized with counterstaining and controls using the FluoView 1000 laser scanning confocal microscope (Olympus). Cells will be seeded onto Lab-Tek™ Chamber Slides™ (Nunc) for imaging purposes. After treatment, cells are fixed, permeabilized and counterstained for nuclei (Hoescht dye), and endosomal/lysosomal markers. Then confocal images are acquired with an Olympus FluoView 1000 laser scanning confocal microscope. This will verify the biological function of QD conjugates, and their intracellular distribution (e.g. free in cytosol or trapped inside endosomes and lysosomes).

3.5.3 Transferrin Conjugation with Alexa Fluor® 488

For endocytosis experiments, human holo-Transferrin (Sigma) was labeled with an Alexa Fluor® 488 Protein Labeling Kit (Life Technologies) according to manufacturer’s specifications.

3.5.4 Inductively Coupled Plasma-Atomic Emission Spectroscopy

Intracellular QDs were quantified by ICP-AES using the Optima 7300V system (PerkinElmer) after acid digestion in HNO₃ (70°C, 1 hour). QD concentrations were converted from measured Cd concentrations using Cd-QD calibration curves established for each QD size. Baseline concentration of free Cd ions in QD solution was accounted for and subtracted from intracellular Cd concentration prior to conversion into QD concentrations. Cell numbers were converted from measured Mg concentrations using Mg calibration curves with known numbers of cells using the technique developed by Albanese et al.

3.5.5 Cell Viability Assessment

The effects of intracellular QDs on cell viability were determined by the Cell Proliferation Kit II (Roche Applied Science). Their values were expressed as percentage cell viability of non-treated cells with identical culture conditions. Cells were seeded into 96-well plates one day before treatment. Cells were next treated with increasing concentrations of QDs for 24 hours. At the end of treatment, extracellular and membrane-associated QDs were removed and cells were thoroughly rinsed thrice with PBS. XTT is a tetrazolium salt that is cleaved to water-soluble formazan products by a metabolic mechanism only inside viable cells. Thus, the amount of
colorimetric formazan is proportional to the number of viable cells. In brief, cells will be cultured for 2 hours with XTT reagent mixture. Absorbance between 450-500 nm with a reference wavelength of 600 nm will be measured with an ELISA plate reader. Sample wells with culture medium and XTT reagent mixture only will be included as absorbance baseline. Absorbance will be compared to that of control cells not receiving QDs (100% cell viability).

3.6 Nuclear Accumulation of Quantum Dots

3.6.1 Nucleus Isolation

Nuclei of the QD-treated cells were isolated using the Nuclei EZ Prep kit from Sigma. The nuclear isolation procedures were validated as following. Isolated nuclei were stained with Hoescht 33342 and Alexa Fluor® 488 phalloidin (Life Technologies) for nuclear and actin labeling, respectively. Confocal microscopy showed only stains for the nucleus, but not actins, which are the major component of the cytoskeleton, suggesting that the collected samples contained isolated nuclei without cytosolic components. Confocal microscopy also demonstrated that the washing protocol removed loosely bound QDs on the nuclear membrane, and confirmed the presence of QDs inside these isolated nuclei, which were subsequently quantified using ICP as described above.

3.7 Cytoplasmic Microinjection of Quantum Dot-Peptide Bioconjugates

Microinjection studies were carried out as previously described. HeLa cells were seeded at 10^5 cells per 50 mm PELCO® clear wall glass bottom dishes (Ted Pella Inc.), and then incubated to allow for optimal cell morphology for microinjection with solution containing QD-peptide bioconjugates and a co-injection marker (FITC-tagged 72k Da Dextran; Sigma). The solution was microinjected using an Eppendorf Micromanipulator 5171 combined with an Eppendorf Transjector 5246 mounted on a Nikon TE2000 inverted fluorescence microscope. After microinjection (time 0), cells were imaged under the FluoView 1000 confocal microscope at the indicated time points. During live cell imaging, these cells were maintained at controlled temperature (37°C) and CO₂ level (5%). Microinjection began with the following settings: the
pressure of injection (150 hPa), time of injection (0.5 seconds) and the compensation pressure background pressure (30 hPa) for comparable injection volumes between experiments. However, these settings are adjusted to accommodate variations in microneedle opening sizes, so injection volumes cannot be accurately quantified. Therefore, translocation of QD-peptide bioconjugates from the cytoplasm to the nucleus was compared qualitatively. First, cells with successful microinjection into the cytoplasm, as demonstrated by clear cytoplasmic distribution of the green FITC-Dextran marker, were counted (N₁). Other cells with this marker inside the nucleus were excluded from analysis. Cells with translocation of cytoplasmic QDs into the nucleus, as demonstrated by QD fluorescence in the nucleus, were then quantified (N₂) and expressed as the percentage of cells capable of translocating cytoplasmic QDs into the nucleus (N₂/N₁). At least 10 cells were analyzed per measurement.

3.8 Statistics

Unless otherwise specified, data shown are mean ± standard error results from n independent experiments (n ≥ 3). Statistical comparison using ANOVA and Tukey’s post-hoc analysis was determined by Prism 5 software (GraphPad).
4.1 Design and Preparation of Quantum Dot Bioconjugates

QDs were used as the model nanoparticle to investigate the effects of ligand density and particle size on nuclear transport. Commercially available QDs were chosen. These QDs were made of cores of either CdSeS alloyed composition or CdSe, and both were coated with an inorganic ZnS shell. Those with alloyed cores had an additional proprietary metal coating on top of the ZnS shell. These QDs had sizes ranging from 3.0 to 8.0 nm and their optical properties and hydrodynamic sizes were characterized prior to use in all biological studies (Figure 4.1). CdSeS/ZnS QDs (diameter = 4.5 nm) were used unless otherwise specified. A SV 40 large T antigen-derived NLS peptide sequence was chosen for its well established ability to deliver small drugs and nanoparticles into the nucleus. A space filler peptide was used to maintain a constant surface density of total peptides. A panel of space filler peptides was tested (Table 4.1 and Figure 4.2).

In this thesis work, ‘nuclear transport’ is defined as following to account for different cellular uptake efficiencies of QD-peptide bioconjugates with various ligand densities or particle sizes:

\[
\text{Nuclear transport} = \frac{\text{Quantity of nuclear quantum dots}}{\text{Quantity of intracellular quantum dots}}
\]
Figure 4.1. Characterization of Quantum Dots. (a) Fluorescence (solid line) and absorbance (broken line) spectra of QDs of indicated sizes. (b) Summary of the physical sizes of QDs measured by TEM, and their hydrodynamic sizes determined by DLS. \( n = 3 \) with 150 particles analyzed by TEM for each QD size. (c) Representative electron micrographs for these QDs. These QDs were composed of either CdSeS/ZnS alloyed core/shell (diameter = 4.5 nm) or CdSe/ZnS core/shell (3.0, 4.8, 8.0 nm).
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Description</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>NLS</td>
<td>native NLS</td>
<td>PPKKKRKV-HHHHHH</td>
</tr>
<tr>
<td>mNLS</td>
<td>mutated NLS</td>
<td>PPKTKRKV-HHHHHH</td>
</tr>
<tr>
<td>scNLS</td>
<td>scrambled NLS</td>
<td>KPKVKPKR-HHHHHH</td>
</tr>
<tr>
<td>CPP</td>
<td>cell penetrating peptides</td>
<td>KKKKKKSEDEYPVVPN-HHHHHHH</td>
</tr>
<tr>
<td>Control</td>
<td>non-functional, scrambled CPP</td>
<td>SKEKDKKEYPKPKVKNY-HHHHHH</td>
</tr>
</tbody>
</table>

Notes:
1. NLS: Native NLS derived from SV40 Large T Antigen.\(^90\)
2. Mutated NLS (mNLS): Lysine to Threonine mutation (underlined).\(^91\)
3. Scrambled NLS (scNLS): by alternating positive amino acids (underlined) and re-arranging the other amino acids of the native NLS.
4. CPP derived from an adenoviral endocytosis peptide.\(^40\)
5. The Control peptides: by alternating positive amino acids (underlined) and re-arranging the other amino acids of the above adenoviral receptor mediated endocytosis peptide.\(^40\)
6. Poly-histidine (His\(_6\)) is included as a linker to the ZnS surface of CdSeS/ZnS and CdSe/ZnS QDs.
Figure 4.2. Design and Characterization of Peptides. Cellular uptake, nuclear accumulation and nuclear transport of QDs coated with NLS, mNLS, scNLS, and the Control peptide were first characterized. Nuclear transport, as defined earlier, was used to account for different cellular uptake efficiencies of QD-peptide bioconjugates. Quantitative values from these measurements were normalized to that of the CdSeS/ZnS QD-NLS bioconjugates to obtain a relative nuclear transport value after 1 hour of incubation (a). Cellular uptake was also analyzed for CdSeS/ZnS QDs coated with NLS, CPP, NLS and CPP after 1 day of incubation (b) and their nuclear accumulation (c). * $p < 0.05; n = 3.$
Interestingly, neither a mutated or scrambled NLS sequence was able to completely abolish nuclear transport of QDs, so they were not used in the rest of the study. The Control peptide used in this study was an irrelevant peptide sequence that showed no nuclear transport activity. Both NLS and Control peptides were coated onto the QD surface via poly-histidine interactions where the imidazole side chains of the histidines bind to the Zn ions. Figure 4.3a provides a schematic of the peptide bound to the QDs and the linking chemistry. This self-assembly approach for coating peptides onto the QD surface has been shown to be rapid (< 30 minutes), with high affinity (equilibrium binding constant, $K_d^{-1} \sim 1 \times 10^9 \text{ M}^{-1}$) and a low dissociation rate ($k_{off} < 0.001 \text{ s}^{-1}$). It also enables control over the surface valence of biomolecules on the QD surface by adjusting the molar ratios of various peptides to QDs during the reaction. In this study, the NLS density was manipulated by adjusting stoichiometric ratios of NLS and the Control peptides while keeping the surface density of total peptides constant (Figure 4.3b). I first demonstrated that the affinities of NLS and Control peptides to QD surface were identical using a fluorescence-based protein assay (Figure 4.4a). I next confirmed that this approach was capable of producing QDs with desired NLS densities using fluorescently labeled NLS peptides, and showed a strong correlation ($R^2=0.9972$) between the expected and measured surface NLS densities (Figure 4.4b).
Figure 4.3. Engineering Surface Peptide Densities of Quantum Dots. (a) Schematic for peptide adsorption onto CdSeS/ZnS QDs through metal-affinity coordination between its histidine appendage (His$_6$) and the Zn ions on QD surface. The strategy enables facile self assembly of His$_6$-appended peptides onto QDs with high affinities. (b) Schematic showing control of surface peptide density by adjusting stoichiometric ratios of NLS peptides and the Control peptides.
Figure 4.4. Characterizing Surface Peptide Densities of Quantum Dots. (a) The NLS and Control peptides have identical affinity between the His$_6$ and QDs as determined by a protein assay. (b) Manipulation of NLS peptide density was further confirmed by quantifying fluorescently labeled NLS peptides on QD surface in the presence of the Control peptides. Red line: linear regression, R$^2$: correlation coefficient. The total peptide (NLS + the Control peptide) number per QD was kept at 250, so the expected NLS/QD ratios between 15.625 and 250 correspond to NLS densities between 6.25% and 100%. n.s., not statistically significant; n = 3.
4.2 Cellular Interaction of Quantum Dot-NLS Bioconjugates

Previous studies have shown that nanoparticles can enter cells and the nucleus. However in most of these studies, the amount of nanoparticles that entered the nucleus was not quantified. In the present work, a simple method was used to determine the number of nanoparticles in the nucleus. I isolated the nuclei from cells incubated with QDs using a commercial nucleus isolation kit and measured the metal content of the isolated nuclei using ICP. The measured metal concentration allows for the determination of the number of QDs. However, this technique cannot differentiate QDs inside the isolated nuclei from those that adsorbed onto the surface of these isolated nuclei. Therefore, confocal fluorescence microscopy was used to confirm these QDs were inside the isolated nuclei rather than simply adsorbed on the nuclear membrane.

The percentage of QDs that entered cells and subsequently the nucleus was determined by comparing the amount of QDs in the nuclei to that in the whole cells. In addition, confocal fluorescence microscopy was used to demonstrate their cellular uptake and nuclear transport (Figure 4.5). In the initial experiments, CdSeS/ZnS QDs were coated with CPP and NLS peptides (Table 4.1). I initially considered this design because I thought the CPP would deliver the QDs into the cells through receptor-mediated endocytosis, escape the endo-lysosomal vesicles, enter the cytoplasm and NLS peptides will actively transport the QDs into the nucleus by interacting with the importin proteins. The NLS peptides alone were found to deliver the QDs into the cytoplasm and then into the nucleus, while the CPP alone was found 50% less efficient for nuclear transport (Figure 4.2b,c). Therefore, the CPP was omitted in the rest of this present study, as multiple peptides were not required for nuclear delivery. Cytoplasmic delivery of QDs by the NLS peptides was likely attributable to the positive zeta potential of the QDs modified with these peptides (Figure 4.6a), which showed a positive correlation with cellular uptake of the QDs. However, the underlying mechanisms await further investigation. It is well known that cationic nanoparticles can enter cells via endocytosis. For example, a positively charged Tat peptide has been shown to deliver its QD bioconjugates into cytoplasm by endocytosis.

To determine whether cellular uptake of QD-NLS bioconjugates was mediated by endocytosis, cells were simultaneously treated with green endosome markers (Alexa 488-tagged transferrin)
and red QD-NLS bioconjugates. The green Alexa tag was shown to colocalize with the red fluorescence from the QD-NLS bioconjugates, as demonstrated by yellow fluorescence under confocal microscopy (Figure 4.6b). The endosome/QD complexes were mostly found in the perinuclear area. Such perinuclear accumulation of QD-loaded vesicles has been previously reported for QD-Tat peptide bioconjugates, which remained trapped inside vesicles for up to 24 hours.32 In contrast, the QD-NLS bioconjugates were capable of endosomal escape as evidenced by red QD fluorescence observed outside the endosomes (Figure 4.6b). These escaped QD-NLS bioconjugates were capable of subsequent nuclear transport as illustrated by their red fluorescence inside the nucleus (Figure 4.6b’), which was more diffuse and dimmer compared to their counterpart inside endosomes. The diffuse pattern suggested that these QDs remained disperse after endosomal escape and likely entered the nucleus as single QDs. However, the underlying mechanisms remain to be further established despite the observed colocalization.
Figure 4.5. Intracellular Distribution of Quantum Dot Bioconjugates. Confocal images in the present study were cross-sections through representative cells and their nuclei as illustrated in the schematic showing the three axes (a): a representative confocal image (b) and the orientation of cross-sections on the three axes (c). (d) Confocal microscopy demonstrated nuclear transport of CdSeS/ZnS QD-NLS bioconjugates, which were found mostly inside the nucleus. (e) In contrast, CdSeS/ZnS QDs with the Control peptides showed minimal cellular uptake and negligible nuclear transport. Color legends: nucleus (blue), actin (green) and QD (red). Blue color was removed to highlight the nuclear QD fluorescence (d’, e’). Scale bars equal 10 μm. The images were acquired with 60x UPlanApo water objective (NA = 1.2) with indicated Excitation (Ex) and Emission (Em) wavelengths for nuclei ($\lambda_{ex} = 405$ nm; $\lambda_{em} = 450/50$), actins ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 527.5/55$) and QDs ($\lambda_{ex}$= 405 nm; $\lambda_{ex} = 560$ long-pass filter).
Figure 4.6. Zeta Potential and Intracellular Fate of Quantum Dot-NLS Bioconjugates. (a) The average zeta potential values were 0, +2, and +6 mV for CdSeS/ZnS QDs with 0%, 50% and 100% NLS densities, respectively, * $p < 0.05$; $n = 3$. (b) Confocal microscopy demonstrated the colocalization of endosome markers (green) and CdSeS/ZnS QD-NLS bioconjugates (red), shown as yellow fluorescence (arrows). Arrowheads highlight red fluorescence, suggesting endosomal escape of CdSeS/ZnS QDs. These CdSeS/ZnS QDs were found mostly inside the nuclei (blue). Blue color was removed to highlight the nuclear QD fluorescence ($b'$). These confocal images were cross-sections as illustrated in Figure 4.5a. Scale bars equal 10 μm. The images were acquired with 60x UPlanApo water objective (NA = 1.2) with indicated Excitation (Ex) and Emission (Em) wavelengths for nuclei ($\lambda_{\text{ex}} = 405$ nm; $\lambda_{\text{em}} = 450/50$), endosome markers ($\lambda_{\text{ex}} = 488$ nm; $\lambda_{\text{em}} = 527.5/55$) and QDs ($\lambda_{\text{ex}} = 405$ nm; $\lambda_{\text{ex}} = 560$ long-pass filter).
4.3 Effects of Ligand Density on Nuclear Transport of Quantum Dots

To examine the effect of the ligand density on nuclear transport, CdSeS/ZnS QDs were prepared with seven ligand densities: 0, 6.25, 12.5, 18.75, 25, 50 and 100% NLS. The QD surface peptide density was kept constant with space filler peptides as illustrated earlier (Figure 4.3). Cells were incubated with the CdSeS/ZnS QD-NLS bioconjugates for 1 hour, washed thoroughly, collected, and analyzed by ICP. The effects of NLS density on cellular uptake and nuclear accumulation showed linear correlation ($R^2=0.9995$, 0.9937, respectively, see Figure 4.7a,b). The present study showed a drastic and linear increase in nuclear transport with NLS densities up to 20% ($R^2=0.9276$), at which approximately half of total intracellular CdSeS/ZnS QDs were transported into the nucleus (Figure 4.7c). Any increase above 20% NLS density only resulted in a modest increase in nuclear transport, and deviation from the initial linear relationship between NLS density and nuclear transport.

These QD-NLS bioconjugates were capable of nuclear transport up to 2 hours post treatment, and their nuclear transport reached a plateau at 30 minutes (Figure 4.8). The observed timeframe is consistent with previous studies using different nanoparticle systems. One study investigated polyplex-mediated cytosolic delivery of oligonucleotides, and showed cellular uptake and endosomal rupture as early as 32 minutes.96 Another group studied nuclear transport of poly-Arginine protein nanoparticles, and demonstrated cellular entry to be the major barrier for nuclear transport.97 The sizes are approximately 100 and 20 nm for the polyplex and poly-Arginine nanoparticles, respectively. This suggests that the observed timeframe is not specific to the QDs used in this study.
Figure 4.7. Effects of NLS Density on Cellular Uptake and Nuclear Transport. Cells were incubated with CdSeS/ZnS QD-peptide bioconjugates of indicated NLS densities for 1 hour, trypsinated, and collected for ICP analysis, showing results for the cellular uptake of QDs with different densities of NLS (a) and their respective nuclear accumulation (b). * $p < 0.05$ compared to the next lower NLS density; ** $p < 0.05$; $n = 3$. (c) The NLS density effect on nuclear transport showed a steep increase up to approximately 20% NLS followed by a plateau. Red line: linear regression, $R^2$: correlation coefficient between NLS density of 0% and 20%; $n = 3$. 
Figure 4.8. Cellular Uptake and Nuclear Transport over Time. Cells were treated with CdSeS/ZnS QD-peptide bioconjugates of the indicated NLS density for the indicated time before collection for ICP analysis. The results demonstrated that cellular uptake (a) and nuclear accumulation (b) occurred in the first 30 minutes with modest accumulation afterward. (c) Nuclear transport showed no further increase between 30 and 120 minutes post treatment. n.s., not statistically significant; \( n = 3 \).
To confirm nuclear transport of QDs, we used microinjection to deliver QD-peptide bioconjugates into the cytoplasm, followed by assessment of their nuclear transport based on their fluorescence. Cells were first microinjected with the green co-injection marker fluorescein isothiocyanate (FITC)-tagged dextran (which labels the initial injection site) and red emitting QD-NLS bioconjugates, and then analyzed using confocal fluorescence microscopy. This co-injection marker does not passively diffuse across the nuclear membrane and is used here to identify cells that received microinjection into the cytoplasm. Representative frames of videos for QD-bioconjugates of NLS and the Control peptides at indicated time points were shown (Figure 4.9). Image analysis demonstrated that nuclear transport of cytoplasmic QDs was enabled by the NLS peptides, but not the Control peptide (Figure 4.10). These results also confirmed the ICP results that the measured nuclear transport of QDs was attributable to their presence inside the nucleus, and not due to their non-specific binding to the surface of the nucleus.
Figure 4.9. Nuclear Transport of Cytoplasmic Quantum Dot-Peptide Bioconjugates (Representative Images). Green emitting FITC-Dextran (72kDa) was mixed with red emitting CdSeS/ZnS QD-peptide bioconjugates and microinjected into cells (which appeared orange). Once injected, these cells were imaged using confocal fluorescence microscopy from 5 to 30 minutes post injection. (a,d) At 5 minutes, fluorescence resided in the cytoplasm, suggesting successful microinjection. NLS peptides facilitated nuclear transport of QDs over time (a-c) whereas, in contrast, the Control peptide did not (d-f). This was observed up to 2 hours post injection (data not shown). The white dashed lines highlight the nuclear-cytoplasmic boundary. Scale bars equal 10 μm. The images were acquired with 60x UPlanApo water objective (NA = 1.2) with indicated Excitation (Ex) and Emission (Em) wavelengths for nuclei (λ_{ex} = 405 nm; λ_{em} = 450/50), FITC-Dextran (λ_{ex} = 488 nm; λ_{em} = 527.5/55) and QDs (λ_{ex}= 405 nm; λ_{ex} = 560 long-pass filter).
Figure 4.10. Nuclear Transport of Cytoplasmic Quantum Dot-Peptide Bioconjugates (Quantification). (a) FITC-Dextran (green) does not enter the nucleus. In these experiments, the monitoring of the green fluorescence of the cell will determine if the QDs are accidentally microinjected into the nucleus. If such a case occurred, those cells would be excluded from quantitative analysis. The white dashed lines highlight the nuclear-cytoplasmic boundary. Scale bars equal 10 μm. The images were acquired with 60x UPlanApo water objective (NA = 1.2) with indicated Excitation (Ex) and Emission (Em) wavelengths for nuclei (λ<sub>ex</sub> = 405 nm; λ<sub>em</sub> = 450/50), FITC-Dextran (λ<sub>ex</sub> = 488 nm; λ<sub>em</sub> = 527.5/50) and QDs (λ<sub>ex</sub> = 405 nm; λ<sub>ex</sub> = 560 long-pass filter). (b) Representative images of microinjection experiments (Figure 4.9) were quantified as the ratio of the number of cells with nuclear accumulation of QDs at 30 minutes post injection. The results suggested that NLS peptides facilitated nuclear transport of QDs over time whereas, but the Control peptide did not. ** p < 0.01. n = 3 with 30 cells analyzed per peptide.
4.4 Effects of Particle Size on Nuclear Transport of Quantum Dots

A second set of QDs made of CdSe cores and coated with an inorganic ZnS shell were used for the subsequent size effect studies as the fluorescence of the alloyed CdSeS/ZnS QDs used above are tuned by composition and not by size. Three sizes (3.0, 4.8, 8.0 nm) were chosen to investigate the effects of particle size on nuclear transport. Cells were treated with these CdSe/ZnS QDs for 1 hour before cell collection and nucleus isolation for ICP analysis. In the absence of NLS, cellular uptake was seen in all three sizes of CdSe/ZnS QDs and was the highest for the 4.8 nm CdSe/ZnS QDs (Figure 4.11a), but nuclear accumulation was only seen in the 3.0 nm CdSe/ZnS QDs (Figure 4.11b,c). Since the NLS was absent, the observed nuclear accumulation can be attributed to passive diffusion of these small 3.0 nm CdSe/ZnS QDs.
Figure 4.11. Effects of Particle Size on NLS-Independent Nuclear Transport. Three sizes of CdSe/ZnS QDs (diameters = 3.0, 4.8, 8.0 nm) were used to study the size effects on NLS-dependent nuclear transport. Cells were treated with these QDs for 1 hour before collection for ICP analysis. The particle size effects were first determined using three sizes of QDs with 0% NLS, showing optimal size of 4.8 nm for NLS-independent cellular uptake (a). NLS-independent nuclear accumulation was only seen for the smallest 3.0 nm QDs (b). (c) A similar trend was seen for nuclear transport of the QDs. The effects of NLS peptides on nuclear transport of these QDs were further characterized in Figure 4.12. *p < 0.05; n.s., not statistically significant; n = 3.
In contrast, NLS-independent passive diffusion was not seen for the larger 4.8 and 8.0 nm CdSe/ZnS QDs, suggesting an upper size limit between 3.0 and 4.8 nm for nuclear entry via a passive diffusion mechanism. The presence of NLS enhanced the cellular uptake and appeared to facilitate the nuclear accumulation of the 4.8 and 8.0 nm CdSe/ZnS QDs (Figure 4.12), but higher NLS density did not further increase the percentage of CdSe/ZnS QDs transported into the nucleus from the cytoplasm. In our density effect study above, a NLS density plateau was also seen for the 4.5 nm CdSeS/ZnS QDs, above which NLS density had minimal effects on nuclear transport, suggesting the observed effects were not specific to these 4.5 nm CdSeS/ZnS QDs.
Figure 4.12. Effects of Particle Size on NLS-Dependent Nuclear Transport. Three NLS densities were used to study their effects on nuclear transport of three sizes of CdSe/ZnS QDs (diameters = 3.0, 4.8, 8.0 nm). Cells were treated with QD-peptide bioconjugates with indicated sizes and NLS densities for 1 hour before collection for ICP analysis. The NLS density effects on each size of QDs’ cellular uptake (d), nuclear accumulation (e), and nuclear transport (f) were determined. NLS peptides increased cellular uptake and enabled nuclear accumulation of the 4.8 and 8.0 nm QDs, but their effects on nuclear transport reached a plateau at 6.25% NLS. In contrast, the effects of NLS peptides on the 3.0 nm QDs were negligible. *p < 0.05; n.s., not statistically significant; n = 3.
4.5 Cytotoxicity of Intracellular Quantum Dots

The QD bioconjugates had no effects on cell viability during the nuclear transport study, or up to 72 hours post treatment (Figure 4.13). The ZnS shell has been demonstrated to render QDs minimally cytotoxic. This suggests that the cells remained physiologically viable in the presence of these QDs, and the observed effects on nuclear transport were indeed attributable to their design parameters, such as ligand density and particle size, not cytotoxic effects.

A parallel study demonstrated minimal exocytosis of these intracellular QD-NLS bioconjugates over time (Figure 4.14) and that they were retained in the cells, which is consistent with previous work. Previous studies investigated three areas of cellular interaction with nanoparticles—cellular uptake, mitotic partitioning, and cell tracking—and established that the internalized nanoparticles remain inside cells and are transferred to daughter cells when parent cells divide.
Figure 4.13. No Effect of Quantum Dot-NLS Bioconjugates on Cell Viability over Time. (a) Cells were treated with 5 nM CdSeS/ZnS QD-NLS bioconjugates for 1 or 2 hours before cell viability measurement and the results showed no cytotoxic effect. The term “Non-treated” referred to cells that were not incubated with the QDs. (b) Cells were incubated with CdSeS/ZnS QD-NLS bioconjugates for 1 hour to allow for their cellular uptake and rinsed thoroughly to remove bioconjugates in the media. These cells were cultured for 24, 48, or 72 hours before cell viability measurement. There were no effects of QD-NLS bioconjugates on viability of the daughter cells. n.s., not statistically significant; \( n = 3 \).
Figure 4.14. Retention of Quantum Dot-NLS Bioconjugates. Cells were incubated with CdSeS/ZnS QD-NLS bioconjugates for 1 hour to allow for their cellular uptake and rinsed thoroughly to remove bioconjugates in the media. Then these cells were cultured for 24, 48, or 72 hours before collection and subsequent ICP analysis. The results showed that the amount of QDs per cell decreased over time (a). However, the total quantity of QDs within the cell culture remained the same (b). The total cell number increased from 24 to 72 hours (c). n.s., not statistically significant; n = 3.
5.1 Effects of Ligand Density on Nuclear Transport of Quantum Dots

Earlier studies on nuclear targeting nanoparticles provided proof of concept for nuclear transport, but these studies did not correlate the nuclear transport process and the design parameters of the nanoparticles, such as ligand density and particle size. The effects of these parameters have been mainly reported on cellular uptake of nanoparticles,\(^5\,\,9,\,\,11\) and studies on nuclear transport are often qualitative and require physical methods such as microinjection or electroporation to facilitate cellular delivery of NLS-QD bioconjugates.\(^8,\,\,10\) Gerion et al. published one of the first studies on nuclear transport of silanized CdSe/ZnS QDs coated with NLS peptides.\(^10\) In terms of their study, they showed that 15% of their CdSe/ZnS QDs entered the nucleus while the other 85% was in the perinuclear space. They rationalized that the nuclear pore size, shape, and permeability might change during a cell’s life cycle, and subsequently alter the size limit for nuclear transport of QDs. In 2004, techniques to characterize nanoparticle physicochemical properties were in early development and, hence, it was logical to assume the biological system was responsible for the differential uptake. The present thesis work complements the Gerion et al. finding and suggests that the QD physicochemical properties are also important in mediating nuclear transport. Likely, the defined physicochemical properties for optimal uptake are related to properties of the particles and the nuclear pores. The present study contributes to this field through a design that allowed for facile manipulation of ligand density, systemic investigation of the effects of these parameters on nuclear transport, and quantitative evaluation of these effects. These design parameters included seven ligand densities and three nanoparticle sizes, and the results further refined the upper size limit for NLS-independent baseline nuclear entry through NPC, most likely through passive diffusion.

Here, I show a linear correlation of NLS ligand density with cellular uptake and nuclear accumulation (Figure 4.7). The effects on nuclear transport were more profound below 20% NLS density, above which the effects became moderate. This could be attributable to the rate limitation of the active nuclear transport process with two major phases: (1) recognition of NLS-tagged cargos by adaptor proteins (importin \(\alpha\) and \(\beta\)) and (2) the passage of this tripartite complex through the NPC (Figure 2.2).\(^10\) While the recognition phase might be facilitated by
the presence of more NLS, the passage phase is limited to the capacity of the NPC.\textsuperscript{103} The increased nuclear transport might also be attributable to the rate of endosomal escape, which has been previously reported to be the rate-limiting step for nuclear delivery by nanoparticles.\textsuperscript{104} The NLS peptide used in the current study is rich in amino acids that have been shown to facilitate endosomal escape. There are 11 such amino acids (lysine, arginine, and histidine) in this NLS peptide of 16 amino acids. Both arginine and lysine facilitated endosomal escape of nanoparticles through fusion with endosomal membranes.\textsuperscript{105} On the other hand, histidine facilitates endosomal escape through its imidazole ring (pK\textsubscript{a3}=6.04) that increases the buffering capacity in endosomes and lysosomes.\textsuperscript{106} Poly-histidine appendage with as few as 4 histidines is sufficient to serve as a linker between peptides and QDs.\textsuperscript{94} Poly-histidine appendage used in the current work contains 6 histidines, and it is possible that the imidazole rings of these 2 additional residues were not involved in QD binding but rather in facilitated endosomal escape. Further investigation is required to assess the effects of ligand density on each of these steps, in particular during endosomal escape and nuclear entry. I anticipate the results to be applicable to other nanoparticles, such as gold nanoparticles, which can be similarly modified with peptide ligands through poly-histidine mediated self assembly.\textsuperscript{107} In addition to facile self assembly, the experimental designs in the current study demonstrate the following advantages: comprehensive ligand densities (0-100%), a constant total peptide density by including an additional Control peptide as fillers, and their minimal effects on cell viability up to 72 hours post treatment.
5.2 Effects of Particle Size on Nuclear Transport of Quantum Dots

In addition to the ligand density, the size of the QDs also plays an important role in nuclear transport. The double-layered nuclear envelope presents a barrier to entry into the nucleus. NPC controls the transport process, and actively transport gold nanoparticles up to 26 nm in size, and allow for passive diffusion of smaller nanoparticles in the absence of NLS. There is discrepancy for the upper limit for passive diffusion (5.2 nm or 9 nm) using non-metallic probes such as dextran or protein probes, respectively. In a previous study, QDs with diameters between 2.1 and 4.4 nm were used to investigate the upper limit for passive diffusion of metallic nanoparticles across NPC, and the limit was found to be approximately 3.1 nm. However, cells were fixed and permeabilized prior to incubation with QDs in that study, so it is unclear whether this limit can be also observed in live cells. In contrast, the present study investigated this limit in live cells under physiological conditions, and demonstrated the limit to be approximately 3.0–4.8 nm based on the direct assessment of the physical sizes of the nanoparticles (Figure 4.1c). NLS is required for nuclear transport of QDs that are larger than this size. In the presence of NLS, the 4.8 nm CdSe/ZnS QDs demonstrated the highest cellular uptake and nuclear accumulation (Figure 4.12a,b). Size alone was insufficient to explain this phenomenon because this optimal size was an intermediate one between 3.0 and 8.0 nm. Rather it should be attributable to the balance between NLS-dependent transport and nuclear retention. For larger nanoparticles > 20 nm, an increase in size led to slower diffusion in the cytoplasm to reach the nuclear pore complex, which subsequently resulted in less nuclear transport and lower nuclear retention. On the other hand, smaller protein constructs with a physical size less than 3 nm in one cross-section are capable of diffusing from the nucleus back to the cytoplasm, which reduced their nuclear retention. Their increase in length reduces this diffusion, maintains their nuclear retention, and subsequently increased nuclear accumulation. In support, the present study demonstrates NLS-independent nuclear entry of the 3.0 nm CdSe/ZnS QDs. However, other factors — such as surface curvature, which is a function of particle sizes — have been reported to influence the binding affinity of ligands on these particles. These factors cannot be excluded from this present study. It is also unclear whether 4.8 and 8.0 nm CdSe/ZnS QDs without NLS peptide coating were trapped inside the endosomes, and consequently prevented from entering the nucleus passively. This requires further investigation.
5.3 Conclusion

In summary, the present work demonstrates the effects of design parameters on the nuclear transport of nanoparticles, using a strategy designed for facile manipulation of these parameters. The process of nanoparticles nuclear transport is composed of three major steps: endocytosis, endosomal escape, and nuclear entry (Figure 5.1). This study demonstrated that cellular uptake was mediated through endocytosis and was linearly correlation with ligand density and attributed the correlation to elevating surface charges with the increasing NLS density. The endocytosed nanoparticles then escaped from endosomes and entered the nucleus. The nuclear transport showed a positive correlation with the NLS ligand density up to 20%, at which point the rate of nuclear transport became steady. The nuclear accumulation was the result of cellular uptake and nuclear transport, so cellular uptake and nuclear accumulation became linearly dependent at NLS densities up to 20%, above which the rate of nuclear transport became constant. However, further investigation is required to assess how ligand density affects each of these steps, in particular endosomal escape and nuclear entry.
Figure 5.1. Schematic for Nuclear Transport of Quantum Dot-NLS conjugates. The process includes three major steps—(i) endocytosis of QD-NLS conjugates, (ii) their endosomal escape, and (iii) their nuclear entry through interaction between NLS and the nuclear pore complex (NPC). The illustration is not to scale.
Chapter 6
Future Plan

While this thesis work clearly demonstrates the effects of particle size and ligand density on cellular uptake and nuclear transport, it did not consider the length difference between the Control peptide and the native NLS peptide, or the effect of peptide length on nuclear transport. I attempted to use scrambled and mutated NLS peptides of similar lengths to the native NLS peptides but both scrambled and mutated NLS peptides were incapable of inhibiting nuclear delivery of CdSeS/ZnS QDs. Based on these early results, I was mostly concerned about finding peptides that can coat the surface of the QDs and completely abolish their nuclear transport. With the Control peptide, I cannot exclude the influence of its length on interfering with NLS-mediated nuclear transport process of QDs. The longer length of the Control peptide may block the interaction of the NLS peptide with the importin proteins in an active transport process if the density of the Control peptide is high. Indeed, a recent study from our research group showed that the polymer PEG could fill the space between herceptin antibodies on the nanoparticle surface. If the PEG is too long, it can negatively influence the herceptin targeting specificity to cell receptor.\textsuperscript{112} Identification of optimal filler peptides is challenging, as any change in the length of peptides could alter their isoelectric point, hydrophobicity, and functional groups. These changes in chemical properties could affect how they are coated on the QDs as well as their stability and overall surface charge. Future work is needed to identify proper filler peptides as well as to study the effect of peptide coating heterogeneity on cellular uptake and subcellular transport. In addition, our future work will assess the effects of other design parameters and the intra-parameter relationships. Knowledge of their effects and relationships will facilitate development of nuclear-targeting carriers. Finally, \textit{in vivo} application of these nuclear targeting nanoparticles shall be investigated using biologically inert materials, such as gold nanoparticles.
References


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Appendices

Appendix 1. Curriculum Vitae

EDUCATION

Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada
- Master of Applied Science, Faculty of Engineering, 2014
- Director of Case Development, Graduate Management Consulting Association
- Facility Manager, Biomedical Engineering Students’ Association

Medical Science, University of Toronto, Toronto, Ontario, Canada
- Master of Science, Faculty of Medicine, 2006
- Secretary, Medical Science Students’ Association
- Social Representative, Medical Science Students’ Association

ACHIEVEMENTS & AWARDS

- Ontario Graduate Scholarship (Canada): CAD$15,000
- The Queen Elizabeth II Graduate Scholarships in Science and Technology (Canada): CAD$15,000
- International Graduate Scholarship (Taiwan): USD$30,000
- International BioPharmaceutical Association Award
- American Society for Cell Biology Pre-Doctoral Travel Award (USA)

PUBLICATIONS


Google Scholar statistics:
- Citations: 460, h-index: 4, i10-index: 4 (as of July 26, 2014)
- For a most recent list of publications: go to http://goo.gl/ABm0oC
Appendix 1. Curriculum Vitae (continued)

PATENTS

Song, F., Chan W.C.W., and Tang, P.S., Title of invention: Nonblinking plasmonic quantum dot multifunctional nanobarcodes for multiplex biological detections (patent submission to USPTO by Innovations and Partnerships Office, University of Toronto).

CONFERENCES

Appendix 2. Summary of Collaborative Work:

In addition to the thesis work, I contributed to a few other projects. Two of these projects are most relevant to the thesis and therefore included at the end after Copyright Acknowledgements.

In the first publication,¹ we reviewed and summarized current understanding of nano-bio interactions, including the effects of nanoparticle design parameters on their cellular uptake. Through the review process, I found very few studies about the effects of these design parameters on nuclear transport of nanoparticles. These studies were often qualitative, proof-of-concept, and not conclusive. Such finding motivated me to select this research topic to further advance the understanding of nano-bio interactions at the subcellular level in a quantitative manner. This collaborative work was the most downloaded article in the Annual Review of Biomedical Engineering in 2013 (see Appendix 3). © This work is attached at the end and reproduced with permission from reference,¹ Copyright (2012) Annual Reviews.

In the second publication,¹⁹ we developed a nonblinking plasmonic QD nanobarcode for multiplex biological labeling. The work was based on a nanobarcode system that had been previously developed by Dr. Fayi Song. Then we further characterized this system using single particle spectroscopy and two-photon excitation fluorescence correlation spectroscopy. These spectroscopic experiments were conducted with our friends and collaborators in Dr. David Cramb laboratory in the University of Calgary. Next, we demonstrated the biological application of this system. My contribution was mainly on designing and carrying out these biological experiments to showcase their application in multiplex biological labeling. This work would not be complete without the validation work by Dr. Kun Chen of the Chan group showing that nanobarcodes inside microbeads can be distinguished by commercial flow cytometry systems. The nanobarcode system had been submitted for a US patent application. © This work is attached at the end and reproduced with permission from reference,¹⁹ Copyright (2012) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

The current thesis work has also been published¹⁸ and attached at the end for your reference.