To my parents
ACKNOWLEDGMENTS

It has been more than ten years since I obtained my bachelor’s degree from University of Science and Technology and China in 1999. There have been many impediments and obstacles during this long journey, which has eventually led me to my dream of obtaining a Ph.D. degree in Chemistry. This is truly the best moment for me to review every twist in my travels during this period, and express my appreciation to everyone who has helped and encouraged me.

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Since the 1950s, traditional scientific research in physics, chemistry and biology has turned towards artificial manipulation of biological activities and construction of molecular level objects. Among all the molecules scientists investigate, nucleic acids and their natural hybridization behavior have aroused tremendous attention. Scientists have explored the chemistry and composition of isolated nucleic acids and have extended the research to the critical and versatile functions of these molecules in biological systems.

The first goal of this research was development of a drug delivery system utilizing aptamers for targeted chemotherapy. The aptamers that have used were discovered recently through cell SELEX with high binding selectivity and specificity to target cancer cells. These aptamers were grafted to a liposome particle surface to form a targeting carrier, which selectively bound to targeted cancer cells and released loaded drug molecules.

Another area of investigation was the development of advanced biomaterials for tissue engineering and drug delivery. A DNA hybridization mechanism was used to build a DNA-polymer hybrid hydrogel for potential biomedical and bioengineering applications by combining the advantages of both the polymer and DNA. By introducing an extra photoresponsive element,
this hybrid hydrogel is photocontrollable and able to encapsulate and release payloads via sol-gel conversion. This is a promising biomaterial for tissue engineering and drug carriers.

The third individual research project focused on developing DNA-based nanomotors with the goal of engineering a reversible photo-driven molecular motor. A photoresponsive single DNA hairpin structure was engineered by incorporating photoresponsive moieties, azobenzenes, in the DNA backbone. The azobenzene incorporated DNA nanostructures can absorb at two different wavelengths, and displays reversible motor movement with higher energy conversion efficiencies than linear DNAs. The future goal is to develop high efficiency photo-driven molecular nanomotors using artificial light sources or even solar energy.

Overall this research has applied the basic chemistry and physics of nucleic acids, as well as their functionalities in special situations. The ultimate goal of these projects was to use nucleic acids and nanotechnology to design, develop and investigate functional and smart materials, which we envision will be useful in biomedical and pharmaceutical applications.
CHAPTER 1
INTRODUCTION

Nucleic Acids and Molecular Engineering

Nucleic acids are bioactive macromolecules which are crucial to life. In addition to transferring genetic information across generations, they also have extremely important roles in regulation of biological reactions, as well as expression of numerous functional biological components.\textsuperscript{1} The basic components of assembling nucleic acids include several nucleotides which share the same backbone structure, yet bear different bases. Different assemblies of these bases contribute to the specification of the sequences, which can either specifically interact with complementary oligonucleotides to form duplex structures such as DNA, or can exist as single strands with structural folding. The natural recognition character endows numerous biological and artificial functions \textit{in vivo} and \textit{in vitro}.

In recent years, there have been many innovative research designs making use of nucleic acids for molecular engineering by designing and developing special oligonucleotides on both sequences and three-dimensional structures, such as molecular beacon biosensors\textsuperscript{2,3}, aptamer targeting ligands\textsuperscript{4,5}, DNA tweezers\textsuperscript{6}, single DNA nanomotors\textsuperscript{7,8}, and a three-dimensional DNA cube\textsuperscript{9}. All of these nucleic acid-based biomolecules have the merits of precise controllability, reproducibility, biocompatibility, and have been widely used in bioengineering and biotechnology, biomedical research, tissue engineering and biomedicine.\textsuperscript{10} The understanding of nucleic acids on their physical behaviors under different conditions and the possible chemical modifications is the critical factor to produce artificial building units for both fundamental research and application. These studies would further enrich these molecules and enable them for use in numerous applications under the subject of “nucleic acids based molecular engineering”.

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In order to further explore the molecular engineering based on nucleic acids in more details, it is necessary to study these molecules from very fundamental aspects. This chapter will first describe the composition and structure of short nucleic acids, oligonucleotides, which will be proceeded by the discussion of their chemistry and synthesis.

**Composition and Structure of Nucleic Acids**

One of the most important functions of nucleic acids is storing and expressing genetic information, generally through a physical pairing process between oligonucleotides and their targets. A better grasp of nucleic acid composition and structure will help in understanding the mechanism of their physical properties, and possibly open up avenues to control these properties and biological functions by modifying the structures of components.

DNA was first isolated by Friedrich Miescher in 1869.\(^{11}\) The composition and linear structure of this oligonucleotide was investigated by several scientists.\(^{12,13}\) However, it was not until 1953 that the DNA anti-parallel duplex structure was first discovered by James Watson and Francis Crick.\(^ {14}\) The basic units of nucleic acids are nucleotides, which include a phosphate, an ester bond linked sugar ring, and a base linked to the sugar ring. All nucleotides have the same backbone (phosphate and sugar ring) and differ only in the bases tethered to the backbone. There are five types of natural bases to compose DNA and RNA sequences: adenine (abbreviated A), cytosine (C), guanine (G), thymine (T) only found in DNA, and uracil (U) only found in RNA (Figure 1-1). Each of these bases (A, G, C and T in DNA; A, G, C and U in RNA) can be tethered to the same backbone to form a unique nucleotide sequence which can be further linked together to compose oligonucleotides. Compared to mega-base long DNAs from living cells bearing genetic codes, oligonucleotides used in molecular engineering are generally from tens to
hundreds of nucleotides in length, so their structure and function can be feasibly controlled by sequence design and studied for functionality.

DNA is composed of A, G, C and T with a D-deoxyribose backbone while RNA is composed of A, G, C, U with a D-ribose backbone. All single strand polynucleotides have similar structures, but with the different bases. For DNA strands, double helix structures are formed when complimentary oligonucleotides are in close proximity and stabilized by both base-stacking and hydrogen bond base-pairing between two base pairs: G-C and A-T (Figure 1-2).\textsuperscript{15} This is generally called complementary base pairing and the interaction strength is mainly decided by stacking effects dependant on the sequences. It should be noted that G-U pairing occurs often in RNA and G-T occur in DNA, even though the pairing mechanisms are similar. Noticeably, hydrogen bonds between pairing bases contribute to the sequence specificity instead of stability.\textsuperscript{16} However, the number and ratio of G-C base pairs which has three hydrogel bonds as well as the length of DNA strands are two main factors of determining the stability of double strand DNAs.\textsuperscript{17}

In summary, the composition and structure of DNA sequences determine the stability of a DNA duplex. As the interactions between complementary strands are not covalent, the hybridization and dehybridization is totally reversible by varying physical parameters such as temperature and buffer conditions. In molecular engineering, one general strategy uses self-assembly of different DNA sequences which possess complete or partially complementary regions to construct nanostructures or to trigger molecular movement by strand displacement.

**Synthesis of Chemically Modified Nucleic Acids**

Natural nucleic acids are synthesized within the biological environment through complicated enzymatic driven reactions. One of the advantages of nucleic acids is their robust
structure that is convenient for in vitro replication, while maintaining bioactivity. Currently, we can synthesize not only nucleic acids identical to those from nature (albeit shorter), but also chemically modified derivatives for novel functions. The most commonly used method of synthesizing artificial DNA sequences includes using phosphoramidite derivatives of different composition units with an automated instrument, called a DNA synthesizer. This method is also called the solid-support synthesis method for oligonucleotide.

Briefly, nucleotide phosphoramidite units bearing different bases are individually synthesized by organic chemistry, and linked together in a DNA synthesizer to produce oligonucleotides. These phosphoramidites are composed of a base, a sugar ring and protection groups (Figure 1-3). Along with the sugar ring and phosphate, there are several functional groups (dimethoxytrityl (DMT), diisopropylamino, and 2-cyanoethyl) to order the synthesis direction. The DMT caps the 5’ oxygen for selective activation of 5’-hydroxyl groups under acidic conditions. The phosphate is also protected by diisopropylamino and 2-cyanoethyl groups for selective activation under proper conditions. The base linked to the sugar ring is also converted to a benzamide (except thymine) to protect the 1’-amine group during synthesis.

The phosphoramidite bearing different bases can be loaded into a commercially available DNA synthesizer to synthesize required DNA sequences. This process is carried out with a solid controlled-pore glass (CPG) support, and includes four chemical reactions: detritylation, coupling, capping and oxidation (Figure 1.4). For every cycle through these four steps one nucleotide is added to the sequence and the growing oligonucleotide is ready for the next nucleotide conjugation. The 3’-hydroxyl of the first nucleotide is attached to the CPG beads through a spacer as the first base. There are many types of CPG beads connected with normal
bases or other chemical species such as fluorophores, quenchers, or functional chemical groups such as biotin.

After many cycles of this synthesis, individual nucleotides are linked together and form an oligonucleotide. As shown in Figure 1-4, the produced oligonucleotides solution from DNA synthesizer is actually a mixture of multiple oligonucleotide species in different length due to incomplete synthesis during the process. Therefore, the mixture is generally transferred for deprotection and purified by HPLC to obtain designed oligonucleotides. Specifically, following the completion of synthesis, the DNAs tethered on the beads are cleaved by reaction with weakly basic conditions (generally ammonium hydroxide or a mixture with other additives) at a high temperature. The collected DNAs are then purified by high-performance liquid chromatography (HPLC). A typical mobile phase for separation is 0.1M triethylammonium (TEAA) water solution with reverse phase C-18 column. The purified DNA sample is incubated with weak acid (generally 80% acetic acid) to remove the DMT group, and the concentration is measured by the absorbance at 260nm.

**Fluorescence and Applications**

Fluorescence is the emission of electromagnetic radiation (EMR) generally induced by absorption of another wavelength of EMR. In most cases, this emission wavelength is longer or lower in energy than the absorbed photon. However, in the case of multiple photon absorption or in light harvesting systems, the emission can be at shorter wavelength. Scientists have discovered that a lot of biological materials are fluorescent under certain circumstances, and can thus be used as a character for signaling or differentiation. As the fields of organic and bioorganic chemistry development, more and more synthetic compounds are being synthesized with strong fluorescence *in vitro* and *in vivo*. 
In analytical and bioanalytical chemistry, fluorescence is extremely important for signaling and detection due to its high sensitivity, safety, and ease of measurement. Artificial fluorophores (fluorescent chemical species) can be incorporated into target systems for monitoring events. Related mechanisms and phenomena have also been intensively studied: fluorescent quenching and fluorescence resonance energy transfer (FRET), fluorescence upconversion, and fluorescence anisotropy. Moreover, fluorescence spectroscopy is a very powerful tool in life sciences for sensitive and nondestructive tracking or analyzing biomolecules. As for bioengineering and molecular engineering on DNA, fluorophore-labeled nucleic acids are commonly used to characterize, identify, and demonstrate molecular level responses and behaviors.

**Fluorescence and FRET**

Molecules have various states from an energy point of view referred to as energy levels. Fluorescence is primarily the relaxation mechanism with electronic and vibrational states when the molecules absorb photon energy. Generally, the molecules will be in the ground vibrational level of the ground electronic state (a low energy state). The relative energy ordering of excited states can be displayed in the form of a *Jablonski diagram*, as shown in Figure 1-5 A. The singlet ground, first and second electronic states are depicted by $S_0$, $S_1$, and $S_2$, respectively. When the molecule is excited by absorbing a photon, it is promoted from the ground electronic state to an upper vibrational electronic state in $S_1$ or $S_2$.

By collision with other molecules, the excited molecule undergoes vibrational relaxation and possible internal conversion to the lower vibrational level in $S_1$. Then the molecule returns to the ground electronic state, emitting a photon. This emission is called fluorescence. In a typical
fluorescence measurement, the different frequencies of fluorescent emitted by molecules are measured, under different conditions to obtain emission and excitation spectra.

Molecules that display fluorescence are called fluorophores or donors; popular fluorophores include fluorescein, Coumarin and Cy3. The energy of emitted light of some molecules can also be converted to heat. This type of molecules is named as quencher or acceptor such as Dabcyl or Black Hole Quencher (BHQ). As opposed to fluorophores which absorb and emit light, quenchers can absorb a wide range of wavelengths and convert the photon energy to heat. As a result, quenchers can be regarded as an energy receptor fluorescent resonance energy transfer (or Förster resonance energy transfer, FRET). The basic mechanism can be simply described in two main steps: normal fluorescence emission of the fluorophore by direct excitation of the first fluorophore, followed by a rapid energy transfer to an acceptor or quencher followed by the transfer of photon energy released in the form of light or heat (Figure 1-5 B).

Two criteria must be met for efficient FRET: compatibility and proximity. Compatibility can be defined by the overlap between the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecules. FRET can only happen if the overlap exists and the greater the degree of the overlap, the more efficient the FRET process (Figure 1-5 C). Proximity refers to the distance between donor and acceptor. Usually, Förster radius (the distance where energy transfer is 50% efficient) is used to judge an efficient FRET when the donor-acceptor pair is in proximity. In molecular biology, the proximity is generally a distance of maximum length of a biosensing probes between each end as donor and acceptor are brought together. Compatibility and proximity will determine whether the FRET is efficient enough for a reliable assay.
**Fluorescence Spectrometer**

The fluorescence spectrometer is a complex instrument with multiple components to detect a fluorescence signal (emission light). These basic components includes a broad band light source, two monochromators, a sample cell, and photo detector as shown in Figure 1-6 (and with some other components such as an appropriate grating, a diode array sensor and associated electronics, not displayed in the figure). The light beam from the source first passes through the excitation monochromator to isolate the wavelengths used to excite the samples. The excitation light is therefore focused onto the sample inside a suitable cell and the fluorescence is collected through the grating and then detected by a diode array sensor. A fluorescence spectrometer generally contains two monochromators, excitation monochromator and emission monochromator. Both of these monochromators are programmable so that either an excitation spectrum of a sample at a fixed emission wavelength or the emission spectrum at a fixed excitation wavelength can be recorded. The whole fluorescence spectrometer is extremely versatile and its high sensitivity makes it one of the most powerful and sensitive detection tools available.

FRET-based signaling methods have been widely used in genomics, proteomics, bioengineering, biomedical diagnosis and microbiology. Fluorescence spectroscopy and related technologies have been developed with diverse methodologies and instrumentation to address basic scientific studies, as well as biomedical and clinical research.

**Systematic Evolution of Ligands by Exponential Enrichment (SELEX)**

The organic synthesis of oligonucleotides by the DNA synthesizer has been described previously so that the design and synthesis of diverse and massive DNAs are possible for biological studies and material science. Besides the genetic information storage function, short-
sequence DNAs have been discovered to selectively recognize protein targets with high binding affinity and specificity. These special sequences, called aptamers, are single stranded oligonucleotides, typically 80-100mers, which can interact with a variety targets, such as proteins, peptides, and ions. They have advantages over protein-based targeting ligands such as a robust structure, ease of reproduction and retention of binding characteristics in different conditions. Aptamers are selected from a large library of oligonucleotides through a process named Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process, which was first proposed and demonstrated in 1990 and recently further developed into cell SELEX technique. The cell SELEX method can efficiently discover those special oligonucleotide sequences which can recognize target cells without the knowledge of the target identity, typically a protein or sugar on the membrane surface.

The basic theory underlying SELEX is that each short oligonucleotide possesses a unique sequence and corresponding secondary structure. These oligonucleotides can interact with the target molecules or cells, in the same manner as antibodies interact with their antigens. As opposed to selections with pre-obtained targets, there is no need to pre-determine the exact binding mechanism and receptors on the cells in order to discover the aptamers. The main SELEX strategy is to select the potential sequences from a large quantity of library sequences \(10^{15}-10^{16}\) through many rounds of selective binding with positive cells and negative targets, with amplification by polymerase chain reactions. The positive cells are the targeted cells, which can interact with specific DNA sequence(s); meanwhile, the negative cells are the nontarget cells used to exclude non-specific binding DNA sequence(s), forcing the selected sequences to only specifically bind with target cells.
A typical cell SELEX process used two types of cells to screening aptamer sequences, positive and negative cell lines.\textsuperscript{30} A sequence library is first produced by a DNA synthesizer with random DNAs. The library sequences and target cells are then incubated under proper conditions to allow maximum binding events. The subsequent washing and purification steps are intended to retain all possible aptamer candidates, and eliminate unbound or weakly bound sequences. These two steps can refine the pool leaving only the sequences binding tightly to the target cells. After the undesired sequences are removed, the bound sequences are recollected from the target cells by elution and amplified via the polymerase chain reaction for the next round of selection. The new pool will go through another round of incubation and elution to remove unbound and weakly bound sequences. The conditions of incubation and elution are critical steps since it is strongly desirable to retain sequences with high binding affinity for enrichment and to eliminate those sequences with low binding affinity. After 15-30 rounds of repeated selection, a small subset of sequences remains in the pool to be identified. These potential aptamer sequences are characterized by screening with target and nontarget cells to verify their binding affinity and specificity.

Cell SELEX is simple, reproducible, and straightforward. The selected aptamers can specifically bind to target cells with dissociation constants ($K_d$) in the nanomolar-to-picomolar range.\textsuperscript{31} More importantly, this strategy can be applied universally to any group of cells without knowing the details of targeting interactions. Studying the interaction between aptamers and their targets has not only developed specific molecular probes for disease diagnosis, but has also facilitated clinical therapy to identify new cancer drugs and tumor treatment.\textsuperscript{32-34}
Targeted Drug Delivery Systems

Targeted drug delivery is a method of delivering pharmaceuticals to the specific regions of patients’ bodies in a controllable manner. The purpose of this method is to accumulate drugs locally to increase the therapeutic efficacy and reduce unexpected toxicity. Traditionally, drugs are taken by oral ingestion or intravascular injection. In this way, the drug is distributed throughout the body via systemic blood circulation. There is only a small portion of drug that can reach the diseased region by this passive method; this significantly lessens the efficiency for treatment for malignant diseases. Simply increasing the dosage is not feasible, because almost all of the drugs are toxic for healthy cells, as well as cancer cells. Targeted drug delivery systems are therefore developed to improve efficacy while reducing side effects, especially for cancer and tumor chemotherapy and diagnosis.

There are two kinds of targeted drug delivery pathways: active targeted delivery such as some antibody conjugated drugs, and passive targeted delivery, which makes use of Enhanced Permeability and Retention (EFR) effect.\(^\text{35}\) Currently, both methods have been developed individually with some overlap. For example, liposomes and some macromolecule based drugs tend to aggregate preferentially in tumor tissues than in normal tissues due to their compromised vasculature.\(^\text{36,37}\) This phenomenon can be explained by the unusually fast growth rate of tumor cells that stimulate the proliferation of blood vessels locally. These newly formed vessels have abnormal composition and architecture, which facilitate the accumulation of liposomes and macromolecules, thereby magnifying the therapeutic drug effect through EFR. In order to maximize the drug transduction and efficiency, chemical modification of liposomes and macromolecules with targeting units have been used in combination with both active and passive characters for drug delivery.
Ligands for Targeting Function

In biological interactions, the proper ligand is able to bind to its target and form a stable complex. The binding between ligands and targets is generally caused by intermolecular forces, such as hydrogen bonds and ionic bonds, and the process is usually reversible. Rarely, covalent binding occurs in biological systems.

There are two main types of targeting molecules for most of the targeting delivery systems, antibodies and ligands. Antibody-based targeting mainly uses protein-based units that have been discovered to bind with targets, especially receptors on cell surfaces. Another group of targeting molecules is composed mostly of artificial synthetic molecules, such as peptides, oligonucleotides, and organic ligands. Although antibodies and peptides have been widely used by tethered with delivery vehicles for targeted drug transduction, aptamers are recently introduced to drug delivery systems to improve ligand-facilitating function.

Liposomes

Liposomes are microscopic phospholipid spheres with a bilayered structure. They have received much attention during the last thirty years as pharmaceutical carriers. Liposomes are composed of self-assembling phospholipids, which automatically form spherical structures. Since phospholipids consist of a hydrophilic end and a hydrophobic end, the liposomes structures have different affinities to external molecules in different regions: water-loving core, and a water-repealing shell (Figure 1-7).

From the first observation that phospholipids in aqueous solution can form a closed bilayered structure, scientists have developed liposomes from cell-like microstructures to powerful pharmaceutical carriers of small objects. The initial clinical applications using plain liposomes have one main drawback: the liposomes are quickly eliminated from circulation. To
increase the retention time of liposomes in vivo, the first important strategy is to coat the liposome surface with a biocompatible polymer. Polyethylene glycol (PEG) has been widely used since 1990 for this purpose; it can protect liposomes from recognition by the immune system and can slow down liposome clearance.\textsuperscript{39,40} Nowadays, PEG coating of liposome structures is widely used, and PEG-linked phospholipids are commercially available. Some recent research has further engineered the PEG liposomes with removable features on the target site so that transportation is more efficient and the payload is more susceptible to be released.\textsuperscript{41} Continuing interest in long-circulating liposomes for cancer therapy have left to the development of other polymer molecules not only for protection, but also for biodegradation and controllability.\textsuperscript{42-44}

Liposome surface modification with chemical species, though not particularly important in formation of functional liposomes, is critical for ligand conjugation. In general, conjugation methodologies include carboxyl-amino, pyridyldithiol-thiol and maleimide-thiol reactions, all of which are commercially available in kit forms.\textsuperscript{45} Facilitated by surface modification chemistry, several types of ligands, can be conjugated to the liposome surface to aid in targeting. For example, major research on antibody mediated liposome targeting utilizes available antibodies to target cancer cell surface receptors. Combination of this antibody conjugation with an endosome-disruptive peptide can improve cytosolic delivery of the carried drug.\textsuperscript{46} Another important ligand is folate. Folate receptors are often found and overexpressed in many tumor cells, so that folate mediated liposomes are also popular approaches for cancer therapy.\textsuperscript{47} Very recently, liposomes bearing DNA sequences (aptamers) though chemical bonds have been formulated and successfully used to target cancer cells, and deliver anticancer drugs.\textsuperscript{48,49}
Hydrogels and Their Applications

Hydrogels

A hydrogel is a polymer material that can display both sol (liquid) and gel (solid) states under different conditions. They were originally introduced by Wichterle and Lim for biological uses in the early 1960s. After that, numerous studies have explored and advanced the potential applications of hydrogels for material sciences and bioengineering. Recently, more and more research has focused on pharmaceutical and biomedical functions of this type of material due to its unique three-dimensional polymeric structure that can contain significant amounts of water or biological fluids. Similar to natural tissues, hydrogels also possess a degree of elastic flexibility under external pressured. Therefore, they have been regarded as ideal biomaterials for tissue engineering and drug delivery, as well as device fabrication and controllable materials in industry.

Although hydrogels can maintain the morphology in gel state, they contain approximately 99% of water. The affinity from encapsulating water is attributed to the presence of hydrophilic groups (e.g., -OH, -COOH, -CONH-, -SO3H, -NH2, etc) in polymers forming stable hydrogel structures. However, the polymer networks that are more hydrophobic (e.g., poly(lactic acid), poly(lactide-co-glycolide)) contain less aqueous phase (5-10%). A typical hydrophilic hydrogel structure is shown on Figure 1-8 with a crosslinked polymer network. The water molecules are encapsulated inside the network and maintain a stable gel state. The small water molecules are able to travel throughout the network by diffusion, which is limited by the physical properties on the network. Therefore, by adjusting the polymer characteristics and liquid content, hydrogels can be easily tuned to mimic physical properties as natural tissue assemblies.
Traditional-polymer based hydrogels show a swelling behavior in an aqueous environment upon stimulus. More recently, totally reversible sol-gel convertible hydrogels were constructed using a polymer backbone and DNA crosslinkers. This type of totally reversible and dissolvable material is able to be engineered at the molecular level due to the involvement of DNA strands. By simple modification of the DNA sequences with stimuli-sensitive hybridization, reactive chemical species, signaling units and other biological active moieties, these hydrogels can be developed into smart and controllable materials. In addition to bulk hydrogels, hydrogel nanoparticles (nanogels) have also been synthesized for drug delivery. Nanogels display the properties of both nanoparticles and hydrogels, depending on the applications. And both properties can be controlled separately to adjust functions. The pharmaceutical applications based on nanogels can benefit from the hydrophilicity, flexibility, versatility, and biocompatibility of the nanosized particles. The ease of injection of nanogels with encapsulated medications allows access to diseased regions for chemotherapy.

Hydrogels and their derivatives have provided numerous pharmaceutical platforms and solutions for problems in tissue engineering and drug delivery. After liposomes, hydrogel based drug delivery systems are among the most efficient and promising fields to develop cancer drug carriers and chemotherapy.

Hydrogels for Drug Delivery

Hydrogels are often used for localized drug delivery because of their hydrophilicity, biocompatibility and controllability. The most common drug delivery system using a hydrogel platform has two steps: encapsulation and release. The loading and transportation characteristics of hydrogels are determined by the backbone chemical components, side groups, crosslinking features, and physical properties. As chemotherapeutics are either hydrophobic or hydrophilic,
they have different solubilities in the aqueous fluid that fills the hydrogel matrix. In most cases, the drug molecules are loaded during the preparation of the hydrogel by physical mixing with the buffer solutions.

The release processes are comparatively more sophisticated and complicated in response to external stimuli especially under intentionally positioned controlling factors. By controlling the degree of swelling, crosslinking density, and degradation rate, delivery kinetics can be manipulated according to the desired drug release profiles. Most polymer hydrogels release their loads by swelling induced network size changes. Some DNA-based hydrogels, however, can totally dissolve under specific conditions and release their drug payload.62

Because the most common mechanism of drug release from hydrogels is passive diffusion, size difference and physicochemical characters play a critical role during the loading and storage periods. Drug diffusion out of a hydrogel matrix is primarily dependent on the cage sizes (repeated network unit) within the matrix of the gel or when applicable on external stimuli.66 Although the cage size is generally in the range of tens to hundreds of nanometers, which is much bigger than small molecule drugs or macromolecules, a slow diffusion rate is always observed, due to the complicated interactions between the drug molecules and hydrogel matrix.67

Controlled drug release strategies are intended to provide artificially controlled parameters to fulfill the specific therapeutic needs. There is a great interest in stimuli-induced release systems compared to passive, time-controlled systems.68 Some of these responsive systems include physically-induced release systems, chemically-induced systems, and other stimuli-induced release systems. The commonly used physical stimuli include temperature, electric potential, light, pressure, sound, and magnetic fields, while pH, solvent composition, ionic strength, and specific molecular recognition events are common chemical stimuli. Hydrogels’
unique physicochemical and biological characteristics, along with their highly diversified adjustment and modification, have drawn significant attention as excellent candidates for drug delivery systems of pharmaceuticals.69

**Nucleic Acids Based Nanomachines**

One of the most remarkable characteristics of nucleic acids is the reversible hybridization between complementary nucleotides, which can be manipulated through DNA engineering to create novel building materials. The construction of branched DNA junctions between helices enables assembly of complex three dimensional structures and functional objects.70,71 Strategies using precisely controllable DNA structures have positioned and moved molecules using electronic circuits72 and near-field optical devices73. One clear direction for DNA engineering is to develop nanomachines. Although the DNA sequences alternate between flexible single strands or more rigid duplex structure, they are not able to display regulated mechanical movements by themselves. There is always a need to apply external stimuli or fuels to initiate repeatable movements.

The most commonly used triggering mechanism for this repetitive movement is the addition of completely or partially complementary DNA strands (fuels) to drive the nanomachines through competing hybridization.6,7 One representative is the molecular switch, in which organic molecules transition between two or more molecular states.74 The nanoscale dimensions and the abundant synthetic routes for these organic molecular switches offer the ability to synthesize artificial two-state nanostructures. However, using these fuels to drive the machines requires addition and removal of DNA strands from the system, which will inevitably damage the efficiency and lifetime of the nanomachines. As alternatives, other energy sources have been explored to drive molecular motors. Although some artificial nanomotors can utilize
alternative energy sources, including hydrolysis of the DNA backbone\textsuperscript{75} and ATP\textsuperscript{76}, applications of an electromagnetic field as an energy source is highly desired, especially from the viewpoint of collecting solar energy. Therefore, photon-driven DNA nanomotors have aroused great scientific interest for use in functional microdevices. As the nucleic acids themselves have low response to photons, artificial photosensitive units can be tethered to the DNA sequences by chemical methods to make them react to external photon energy.

**Azobenzene**

Azobenzene is a chemical compound composed of two phenyl rings linked by a N=N double bond, yet, the name is often used to refer to a wide class of molecules that share the core azobenzene structure with different chemical functional groups extending from the phenyl rings.\textsuperscript{77} Photo-isomerization, a common property of these molecules, is facilitated by the absorption of UV and Visible light such that a \textit{trans-cis} conversion occurs as shown in Figure 1-9.

The azobenzene isomerizes from its planar \textit{trans}- form to the non-planar \textit{cis}- form after UV-light irradiation (300nm ~ 400nm), and back to the \textit{trans}- form after irradiation with visible light (400nm ~ 500nm). This process is completely reversible under UV and visible irradiations. Additionally, the \textit{cis}-to-\textit{trans} isomerization of azobenzene is extremely rapid, occurring on a picosecond timescale, but the rate of the reverse process varies greatly, depending on the compound: usually hours for azobenzene-type molecules, minutes for aminoazobenzenes, and seconds for the pseudo-stilbenes.\textsuperscript{78} The ability to control the isomerization state of azobenzene derivatives is so useful that these molecular switches are now used frequently in triggering photo-initiating or photo-regulating processes.\textsuperscript{79}
As photoisomerization of azobenzene has been used in diverse fields for a photocontrollable function at the molecular level, one limitation of this molecule on photoswitching wavelengths occur in the UV range, where the UV can damage the biological system in vivo. In order to extend the wavelengths to visible or even near-IR range, which has the least absorption and highest penetration capability, a series of azobenzene derivatives were synthesized with a longer switching wavelength up to 530nm. These derivatives can be used for either photo-driven molecular motors, or photoswitches of biomolecular functions.

**Azobenzene Incorporated DNAs**

To harness the photosensitive response of azobenzene in DNA nanostructures, azobenzene is chemically modified into the format of a phosphoramidite derivative, so that it can be covalently tethered to DNA strands. Afterwards, the azobenzene DNA becomes photoresponsive and hybridization/dehybridization with complementary strands is controlled by simply irradiating with UV or visible light. The mechanism of this photo-controlled DNA behavior has been shown to result from the isomerization of azobenzene moieties, which further induces hybridization/dehybridization between complementary stands. When the azobenzene incorporated DNA is irradiated with visible light, the azobenzene moieties take planar trans-conformation and stabilize the hybridization by stacking interactions. When irradiated with UV light, the azobenzene moieties isomerize to the non-planar cis-conformation and destabilize the duplex structure by steric hindrance (Figure 1-10).

Based on the influence of azobenzene incorporation into DNA strands, complex DNA nanostructures and nanomachines have been built using azobenzene incorporated linear DNAs. These photoresponsive nanostructures have been further developed mainly to produce photocontrollable nanomachines and photoswitches for bioactivities.
Recently, a DNA nanomachine powered by light irradiation was reported using azobenzene incorporated DNAs to control a nanotweezers movements. Originally such nanotweezers was constructed with plain DNAs, driven by adding “fuel” DNA strands and “antifuel” strands. In this case, one azobenzene incorporated DNA was mixed with several other single strand DNAs to form a tweezers structure. By irradiating with UV and visible light in turn, the azobenzene incorporated DNA can be controlled to either dehybridize or hybridize with other sequences, with tweezers undergoing “on” and “off” motions. It is therefore one simple but straightforward model of photo-driven DNA-based molecular motors. However, highly efficient, inexpensive, and long-lived photoresponsive DNA nanomachines are still under further investigation.
Figure 1-1. DNA bases and their corresponding nucleotide structures. DNA nucleotides are shown as A, G, C, and T (deoxy-D-ribose sugar), while U is shown in an RNA nucleotide (D-ribose sugar).
Figure 1-2. Structure of a DNA oligonucleotide and base pairing.
Figure 1-3. Structure of phosphoramidite and four nucleic acid phosphoramidite monomers.
Figure 1-4. Nucleic acid synthesis through DNA synthesizer.
Figure 1-5. Fluorescence mechanism and fluorescence resonance energy transfer (FRET). (A) Jablonski diagram of fluorescence, (B) Fluorescence resonance energy transfer (FRET), (C) The relationship between the absorbance and emission spectra of the FRET pair.
Figure 1-6. The scheme of a typical fluorescence spectrometer with main components.

Figure 1-7. Multifunctional liposome nanostructure for targeted delivery.
Figure 1-8. Scheme of a regular crosslinking hydrophilic hydrogel network.

Figure 1-9. Photo-induced isomerization of an azobenzene molecule.
Figure 1-10. Scheme of the reversible hybridization/dehybridization of an azobenzene incorporated DNA duplex. The azobenzenes are in *trans*-form after irradiation with visible light and can stabilize the duplex structure. The azobenzenes are in *cis*-form after irradiation with UV light and can dissociate the hybridized duplex structure.
CHAPTER 2
LIPOSOME-BASED NANOSTRUCTURE FOR TARGETING DRUG DELIVERY

Introduction

The background of liposomes and their development for biomedical and pharmaceutical applications was introduced on Chapter 1. The major function of these nanostructures is drug delivery \textit{in vitro} and \textit{in vivo} with low toxicity, biocompatibility, and high carrying efficiency.\textsuperscript{87-89} Modifications to the liposome structure with multifunctional approaches in recent years have increased interest in their use for tumor and cancer therapy. Some of these modifications include: conjugation to an antibody, tethered with peptide units on the liposome surface, chemical modification of the dual lipid bilayer to encapsulate both hydrophilic and hydrophobic drugs, and physical/chemical modification of the internal and external environments for controllable decomposition.\textsuperscript{90-94} Today, there are many basic liposome nanostructures that can accomplish functions such as long circulation time and accumulation on specific tumor or cancer cells.\textsuperscript{95,96} However, most of those models have limitations for fully targeting selective sites and lack of consistency. Therefore, further exploration on multifunctional liposomes as drug carriers with high selectivity, stability, capacity, and controllable and fast release rates, as well as fewer side effects are strongly desired.

Currently, one of the major drawbacks for liposome delivery is the nonspecific binding and delivery due to the lack of highly specific targeting ligands. Antibodies can provide high specificity, but they are usually unstable and difficult to obtain. As a result, cancer chemotherapy associated with targeting capability remains a huge challenge and always results in compromised drug efficiency.\textsuperscript{97,98} To address this issue, we use aptamers that were generated from cell SELEX as targeting ligands to build liposome-based drug delivery systems. Previous research has demonstrated that aptamers for leukemia cells (sgc8 and tdo5) discovered in the Tan group can
be used as satisfactory targeting ligands in several nanostructures. The goal of the present research is the construction of an aptamer-conjugated multifunctional liposome nanostructure for potential targeted drug delivery. In order to achieve this goal, an aptamer ligand was conjugated to the liposome surface and several other biological factors such as PEG coating, were also introduced. The sgc8 aptamer was chosen because of its high binding affinity ($K_d = 0.8\text{nM}$) toward leukemia CEM-CCRF cells. And the sgc8 aptamer can be covalently linked to the liposome via a spacer. The liposome and loaded model drug molecules (FITC-Dextran) were labeled with different fluorophores to monitor the transportation and delivery of the drug to the cells. As more and more aptamers have been discovered in recent years, they display advantages for targeted drug delivery compared to antibodies, peptide and chemical-based binding ligands. Thus, this aptamer liposome conjugate was developed not only as a specific tool for cancer treatment, but also as a model for aptamer based cancer therapy.

**Materials and Instrumentation**

Hydrogenated soy phosphatidyl choline (HSPC), cholesterol (Chol), methoxypoly(ethylene glycol)- distearoyl-phosphatidyl-ethanolamine (MPEG-DSPE) and maleimide-terminated PEG-DSPE (MalPEG) were purchased from Avanti Polar Lipids (Alabaster, AL). Liposome extruder and extrusion membranes were also purchased from Avanti Polar Lipids. FITC-Dextran (FD), Sephadex G-50 beads, sodium bisulfite, sodium sulfite, 1-amino-2 naphthol-4-sulfonic acid, ammonium molybdate, perchloric acid, sulfuric acid, ammonium molybdate solution, and other solvents were purchased from Sigma-Aldrich Chemical. DNA synthesis reagents and TMR modifier were purchased from Glen Research (Sterling, VA). All the solutions were made using nanopure water obtained from a Millipore system (18.2MΩ/cm resistivity).
An ABI3400 DNA/RNA synthesizer (Applied Biosystems) was used for sgc8 aptamer synthesis. The purifications were carried out on a ProStar HPLC system equipped with gradient unit (Varian) with C-18 column (Econosil, 5U, 250 × 4.6mm) (Alltech Associates). The concentrations of all DNAs were obtained using the absorbance at 260nm measured with a Cary Bio-300UV spectrometer (Varian). Fluorescence of FD and TMR-sgc8 was monitored with a plate reader (Safire, TECAN). Dynamic light scattering of liposomes was performed by Coulter LS Particle Analyzer. Other instrumentation included a Vortex Minimixer (Fisher Scientific), a FACScan cytometer (Becton Dickinson Immunocytometry Systems), and a FluoView 500 Confocal microscopy (Olympus).

CEM-CCRF cells (human acute lymphoblastic leukemia) were obtained from ATCC. NB4 cells (acute promyelocytic leukemia) were obtained from the Department of Pathology at the University of Florida.

**Design and Experiments**

Designing the liposomes included three main steps: preparation of the liposome particles (with and without loading model drugs), conjugation of the liposomes with aptamers, and controlled delivery to target cells (Figure 2-1). The details of the liposome composition and structure are described on Figure 2-2. A well-established liposome preparation protocol was used to construct the aptamer-conjugated liposomes with minor modification according to the specialty of aptamer ligands.\(^\text{101}\) The dye labeled sgc8 aptamer was synthesized and purified separately and tethered with a functional group for future linkage to the liposome. The model drug can be loaded to liposomes during liposome synthesis. After characterization and purification of the aptamer-conjugated liposome solution, two types of cancer cells, CEM (positive binding cells) and NB4 (negative nonbinding cells) were incubated with liposomes...
under different conditions. The delivery specificity with target and nontarget cells was determined by Flow cytometry and Confocal microscopy.

**Synthesis and Purification of Liposomes**

Hydrogenated soy phosphatidyl choline (HSPC), cholesterol (Chol), methoxypoly(ethylene glycol)-distearoyl-phosphatidyl-ethanolamine (MPEG-DSPE) and maleimide-terminated PEG-DSPE (MalPEG) (molar ratio 2:1:0.08:0.02, respectively) were mixed together inside a clean 100mL round bottom flask under N$_2$ protection. The flask was sealed and vortexed for 30 minutes at room temperature. The mixture solution was subjected to a slow rotating evaporation at 40RPM, 55$^\circ$C and 600mg Hg until an evenly spread layer of thin lipid film was obtained on the bottom of the flask. The flask was placed under vacuum with a slow and constant N$_2$ flow for at least 2 hours or overnight to remove all the chloroform.

FITC-Dextran was dissolved in 20mM HEPES buffer (150mM NaCl, pH7.4) at 30mg/mL. FD solution (3mL) was used to rehydrate the lipid film by swirling in a 55$^\circ$C water bath. After the entire lipid was removed from the flask bottom, the solution was transferred to a cryo-freezing tube, which was subjected to 8 freeze/thaw cycles alternatively in liquid nitrogen and a 55$^\circ$C water bath. The solution was allowed to set overnight at 4$^\circ$C.

The solution was extruded on a 50$^\circ$C heating plate through 0.4µm, 0.2µm and 0.1µm polycarbonate membranes twice each. Dynamic light scattering (DLS) was performed to examine the size distribution of the liposomes. A small portion of such liposome solution was saved as a pre-column sample for later calculation of lipid concentration. The rest of the liposome solution was transferred to the prepared Sephadex G-50 column to eliminate free lipid, unincorporated FD and other impurities. The collected fractions (0.5mL each) were applied for phosphate assaying and dye assaying as described below:
Phosphate assaying: Standard solutions of 0, 25, 50, 100, 150, 200, 500nM of PO$_4^{3-}$ were prepared from a 1mM NaH$_2$PO$_4$ solution in replicates of 3. Approximately 100nmol (about 7μL) of each fraction from the Sephadex column was added to separate test tubes. A 0.7mL aliquot of perchloric acid was added to each tube, which was vortexed and placed in a 180ºC-220ºC oil bath for 90 minutes. The test tubes were cooled and 0.7mL Fiske-Subbarow reagent and 7mL ammonium molybnate solution were added to the tubes and heated for 15 minutes in boiled water bath. Absorption was measured at 815nm on the plate reader. The lipid concentrations were calculated by comparing them with standard solutions.

Dye loading assay: Standard solutions of both FITC and TMR dyes were prepared using the same method as phosphate standard solutions. The liposomes were burst with Tritonx100 in 1:1 ratio and added to a 383 well plate with 3 replicates of each. The plate was analyzed for both FITC fluorescence ($\lambda_{ex} = 488$nm, $\lambda_{em} = 520$nm) and TMR fluorescence ($\lambda_{ex} = 545$nm, $\lambda_{em} = 580$nm). The FD loading percentage was determined by using the equation:

$$\text{Day loading (\%) } = \frac{(100 \times \text{Dye/Lipid of each sample})}{\text{(Dye/Lipid of pre-column sample)}}$$  

2.1

The calculated data were plotted by comparing them with standard FD solutions.

Liposomes were then ready for aptamer conjugation.

**Synthesis of Dye Labeled Aptamer**

The original unmodified sgc8 aptamer has the sequence, 5′-ATC TAA CTG CTG CGC CGG GAA AAT ACT GTA CGG TTA GA-TMR-3′. The sequence can be synthesized by DNA synthesizer and purified by HPLC. In order to conjugate to liposomes, sgc8 aptamer was synthesized with the several modifications. The 5’ end was modified with a thiol (S-S) for future reaction with the M alPEG on the l iposome surf ace. T he 3’ end was labe led with tetramethylrhodamine (TMR) fluorophore to quantitatively characterize the amount of aptamers
linked on the liposome surface and to monitor the liposome-cell interaction. Thus, the final modified aptamer sequence was 5’-Thiol-ATC TAA CT G CT G CG C CGC C GG GAA AAT ACT GT A CGG T TA GA -TMR-3’. All oligonucleotides were synthesized by solid-state phosphoramidite chemistry on a 1μmol scale. The completed sequences were then deprotected in AMA (ammonium hydroxide/40% aqueous methylamine 1:1) at 65°C for 20 minutes and further purified with reverse-phase HPLC on a C-18 column.

**Aptamer Conjugation to Liposomes and Purification**

The conjugation of sgc8 aptamer to liposomes was performed by adding a 5-fold excess of sgc8 aptamer (compared to the amount of MalPEG estimated from phosphate assay) to the liposome solution. Before the mixing of aptamer and liposomes, the 3’ thiol-modified aptamer was first activated by 100mM TCEP solution at 4°C for 30 minutes. Then 5-fold of the activated aptamer was mixed with the liposome solution and incubated overnight at 4°C. Following this, 2 mM β-mercaptoethanol (BME) was added to quench the unreacted maleimide group. The aptamer-liposome solution was again passed through a Sephadex column to remove free aptamer, and the lipid/FITC/TMR assays were repeated to characterize liposome composition. The liposome solution was added to dialysis tubing and placed inside 500mL HEPES buffer solution at 4°C (buffer was changed after each measurement). The fluorescence was measured every 24 hours (FITC: λ<sub>ex</sub> = 488nm, λ<sub>em</sub> = 520nm; TMR: λ<sub>ex</sub> = 545nm, λ<sub>em</sub> = 580nm).

**Flow Cytometry and Confocal Microscopy Imaging**

The cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 100IU/mL penicillin-streptomycin. The cell density was determined using a hemocytometer. Approximately 1 × 10⁶ cells were dispersed in buffer for each flow cytometry test and 20,000
events were counted. Cell sorting allows for quantitative analysis of different cell species. The cells were kept in an ice bath at 4°C during all experiments.

The cellular fluorescent images were collected using the confocal microscope immediately after the flow cytometry experiments. Three lasers sources from flow cytometry spectrometer were used to provide excitation wavelengths at 458nm, 488nm, 514nm, 543nm, and 633nm. The liposome samples included both FITC (from FD) and TMR (from sgc8) labeling, so the TMR was imaged by excitation at 543nm and collected at 570nm without interference from FITC. Images were generally taken after 10 seconds to allow for instrumentation setup and focusing. The images were colored with green to represent the dye distribution (color is not indicative of actually emission wavelength).

**Results and Discussion**

**Design of Multifunctional Liposome Nanostructures for Drug Delivery**

The components of liposomes have been selected based on several previous protocols. The two main aspects are the selection of the main components and the preparation method. Here HSPC, Chol, MPEG-DSPE and MalPEG were chosen to construct a stable liposome bilayer sphere structure. HSPC, Chol are two main components to support the lipid bilayer, MPEG-DSPE is an amphiphilic compound used to obtain long-acting properties. The hydrophilic-lipophilic balance of MPEG-DSPE is essential to maintain liposome structures with increased circulation time in the body. With MPEG, the liposome sphere structure is maintained without early decomposition. MalPEG is a maleimide-terminated PEG-DSPE with a maleimide group linked on one end. After the preparation of liposomes, TCEP can be added to the solution to activate maleimide, so that thiol-modified DNA sequences can be tethered by a covalent bond. The ratio of the four components is important to balance the liposome stability with respect of
each functional unit. The ratio was designed by considering the number of aptamers on the surface, so that the particles could tightly bind ligands for increased specificity and decreased steric interference. Compared to antibodies and peptides, a high density of aptamer targeting ligands tethered on liposome surface was achieved because of the smaller size and decreased interference.

**Characterization of the Liposome**

Some of the characterization procedures have been published elsewhere. The uniformity of the synthesized liposomes was first confirmed by DLS, and the size distribution was 100±20nm (Figure 2-3 A). Free phospholipid and FD were removed by passing the liposomes through a size-exclusive Sephadex gel column preconditioned with HEPES buffer. The fractions were collected for both phosphate and two-dye assays. As in Figure 2-3 B, tubes from No.1 to No. 17 were the collected tubes. The No. 18 contained unreacted ligands and was used for determining the concentration of liposomes based on lipid concentration. The collected fraction with highest lipid absorption and fluorescence intensity (tube 6) was the liposome sample. The FITC and TMR assays were also performed to further confirm that the tube 6 contained the highest amount of both types of dye molecules (Figure 2-3 C,D). Free lipid and liposomes outside the 80-120nm range, were excluded by this separation method.

In the mean time, based on the lipid and aptamer concentrations, we can estimate that each liposome has approximately 250 aptamers tethered to its surface. This leads to multiple aptamer-receptor interactions and potential strong binding to the membranes of target cells. However, the high density of aptamers on the particle surface may not increase the binding efficiency due to electronic interactions among closely spaced oligonucleotides. The ideal density of aptamer ligands on the liposome surface could be obtained by experimental methods; however, this is not
the focus of the current study. The stability of unmodified liposomes and sgc8-modified liposomes is a very important factor for evaluation of liposomes as carriers. Therefore, both modified and plain liposomes were compared by screening for the variations in size and size distribution. There was no observable decomposition of either liposome structure within ten days of storage in buffer solution at 4°C.

In order to further assess the purity of the samples, the liposomes were dialyzed in dialysis tubing with 200nm pores. This allowed only small dye molecules, such as free aptamer and FD, to pass through into the surrounding buffer, which was monitored by the fluorescence. As shown in Figure 2-4, FD leakage became insignificant and stable after 5 days with an overall loss of 15-20% FD to the outer buffer solution. Also, there were small amounts of free TMR-sgc8 aptamer and TMR-sgc8-linked phospholipid detected in the buffer solution. Based on these data, liposomes were generally dialyzed for 3 to 5 days before cell experiments to eliminate unbound small molecules. The amount of loaded FD inside the liposomes was determined by breaking the vesicle structure by diluting the solution 1:100 with methanol and sonication for 10 minutes. The FD concentration was calculated by comparing it to standard FD solutions. In each liposome, there were approximately several thousand FD molecules, a very high loading capacity.

**Delivery to Target Cells**

The sgc8 aptamer binds Protein Tyrosine Kinase 7 (PTK7), a protein which is overexpressed on some cancer cell membranes. Two types of leukemia cancer cells were chosen to take advantage of this specificity: target (positive binding) CEM cells and non-target (non-binding) NB4 cells. NB4 cells were used as the negative control since this cell line lacks the membrane protein receptor that strongly binds to sgc8. The delivery of FD was monitored by screening the fluorescence of drug molecules by flow cytometry and confocal microscopy at
different concentrations and temperatures. Previous research has demonstrated that the sgc8 aptamer can selectively bind to CEM cells at 4°C within 20 minutes, and the liposomes are also stable at this temperature. Therefore, all the experiments were performed under conditions which favor both liposome stability and aptamer binding.

The results from flow cytometry clearly confirmed that the liposomes were selectively bound to target CEM cells and not to non-target NB4 cells (Figure 2-5) at all concentrations. Higher concentrations of liposome were able to transport larger amounts of loaded FD model drug into cells (a rightward shift). In the control experiment, plain liposomes without sgc8 displayed only a slight shift under the same conditions due to the negligible nonspecific interaction between the liposome and the cells. In order to visualize the delivery of drug molecules, confocal microscopic images were taken on 500nM sgc8-liposomes incubated with both CEM and NB4 cells. As Figure 2-6 shows, the same trend of selective binding and possible internalization were observed by the fluorescence signals, as the majority of FD fluorescence could be observed inside the CEM cells, with no corresponding fluorescence for NB4 cells. These phenomena suggested that the liposomes first bound to the surface of the cell membrane by aptamer recognition and then the FD molecules were delivered into the cells in a dose-dependent manner.

Based on previous research of sgc8 aptamer interaction with different cell lines, it is very likely that the sgc8-liposomes directly bind to the target cell surface through the PTK7 receptor, overexpressed on CEM cells but not on NB4 cells. The liposomes first approach to the cell surface by free diffusion, and then specifically bind to target CEM cells, and their cargo is released by one or both of two main delivery routes: 1) fusion with the cell membrane and subsequent release of the drug; or 2) delivery of liposomes by the endosome into the lysosome,
resulting in drug release into the cell cytoplasm.\textsuperscript{103} While most nanoparticle carriers could follow the second route, it is very likely that our liposomes followed the first route, due to the proven fast and direct fusion of the liposome bilayer with the cell membrane and the intracellular internalization of aptamers. On the other hand, a longer time-course study suggested that the alternate route of delivery may have a significant impact on FD delivery.\textsuperscript{104} It is nevertheless apparent that sgc8 aptamers do facilitate the process of liposome delivery to the target cells, irrespective of the route taken for FD release.

The relationships between incubation time and the specificity and capability of liposome targeting delivery were also investigated. CEM cells were treated with the same batch of liposomes and incubated for 3 or 6 hours while the FD fluorescence was monitored. As shown in Figure 2-7, aptamer-directed delivery still seemed to be the prevailing phenomenon in the liposome-cell interaction for both incubation periods. The NB4 cells also showed slight absorption of liposomes after an incubation time of slightly more than 30 minutes (data not shown). Although the same concentration-dependent delivery was observed, the amount of fluorescent model drug delivered inside the CEM cells was not improved upon extending the incubation time. Since the flow cytometry results can only differentiate the binding step and do not provide information to the internalization process, we can only conclude that the fast binding process is maintained for CEM cells after 30 minutes and the longer incubation times are not useful. Another interesting result is that nonspecific binding between sgc8-liposome and CEM cells became more apparent as incubation time increased. This can be explained by the passive absorption between liposomes and target cells. In other words, longer incubation times will introduce more events of this nonspecific interaction between any liposome nanostructures and biological cells.
Conclusion

In summary, the preparation and characterization of a targeted liposome platform with drug delivery capability has been demonstrated. The principle of targeted drug delivery into cells has been established with selectivity to target cancer cells with our aptamer-modified liposome delivery system. Under proper conditions, aptamer-liposome conjugates demonstrate specific binding with target cells and release the loaded model drug. The delivery is facilitated by sgc8 aptamers and confirmed by the aptamers’ recognition of target cells. However, it should be noted that the phenomenon of increased nonspecific binding and delivery correlates with increased incubation time (usually longer than 6 hours), whereby liposome structures would start to attach and fusion with any cells.

The addition of aptamers, which are easy to synthesize and modify to increase target cell specificity and sensitivity, make our DNA-mediated liposome carrier an efficient new material for drug transportation. Moreover, by simply replacing the aptamers used in this work with any new aptamers, our design can be universally applied to a variety of target cells with high drug delivery efficiency and specificity. Although the exact mechanism for this process remains to be elucidated by further kinetic studies, the results strongly support our hypothesis of a two-step process: binding and delivery. The current liposome system will require further optimization for stability and loading capacity before its application in vivo. While limitations must be considered, our model system indicates a promising trend for the successful development of liposome-based drug delivery systems.
Figure 2-1. Scheme of liposome-based drug delivery to target cells. The liposome can be surface modified with targeting moieties and loaded with hydrophilic or hydrophobic drugs. The whole liposome can specifically bind with target cells and release the drug.

Figure 2-2. Liposome nanostructures showing composition and lipid bilayer structure. The liposome bilayer is composed of four components (HSPC, Chol, MPEG-DSPPE and MalPEG) with sgc8 aptamers linked by chemical bonds.
Figure 2-3. Characterization of liposomes after synthesis and purification. (A) DLS of liposome solution for size distribution with 100 ± 20nm. Assays of lipid (B), FITC(C) and TMR (D) content of collected fractions after size exclusive column purification.

Figure 2-4. Dialysis of liposomes on FITC (A) and TMR (B) for 10 days. The concentration of either FITC and TMR in buffer solution was monitored by fluorescence. Three parallel experiments were performed for each dye dialysis.
Figure 2-5. Binding assays of liposomes at different concentrations with CEM and NB4 cells. The liposomes and cells were incubated for 30 minutes at 4 °C followed by flow cytometry to monitor the binding with (A) target CEM cells and (B) non-target NB4 cells. The black arrow points to shift position of control cells without liposomes, the skyblue arrow points to 500nM sgc8-liposomes, and the green arrow points to 500nM liposomes without aptamer.
Figure 2-6. Confocal microscopic images of 500nM sgc8-liposome incubated with CEM cells and NB4 cells. The left side is the optical image and right side is the fluorescence image.
Figure 2-7. Binding assays of liposomes at different concentrations with CEM and NB4 cells. The liposomes and target CEM cells were incubated for (A) 3 hours and (B) 6 hours followed by flow cytometry. The black arrow points to control cells without liposomes, the yellow arrow points to 500nM liposomes, and the skyblue arrow points to 500nM liposomes without aptamer.
CHAPTER 3
DNA-POLYMER HYBRID HYDROGELS FOR PHOTOCONTROLLABLE CANCER THERAPY

Introduction

Hydrogels have been explored extensively as biomaterials in complex functional devices\textsuperscript{105}, tissue growth\textsuperscript{106}, and pharmaceutical carriers.\textsuperscript{107} Besides being building materials for device fabrication and biosensor development, hydrogels have been developed to respond only when exposed to external stimuli, such as temperature changes,\textsuperscript{108} photons,\textsuperscript{109} ions,\textsuperscript{110} proteins\textsuperscript{111} and DNA.\textsuperscript{112} Hydrogels that undergo physicochemical changes upon the application of stimuli are of great interest for biomedical research, drug development and clinical applications.\textsuperscript{113} The application of electromagnetic radiation (photon) has been reported to induce morphological changes in the shape of polymers and gels.\textsuperscript{109} To date, however, there have been only a few reports using photon energy to control the molecular release of loads from DNA hydrogels.\textsuperscript{114} Nonetheless, the use of light energy to drive hydrogel gel-sol conversion can be easily and effectively performed with photons of different wavelengths. Moreover, photon-initiated response can induce precisely localized changes in physical and chemical properties with excellent spatial resolution. Electromagnetic radiation is also a clean energy source, and it can be applied in otherwise inaccessible environments by the easy transportation of light through optical fibers and waveguides. In addition, photons with longer wavelengths can be introduced for faster and deeper penetration through biological samples, including human tissue. The ability to use photons to control the release of loads from hydrogels has many important applications in basic research, controlled release of drugs, and clinical practice.

Currently, both single components, such as polymer chains or pure DNA,\textsuperscript{115} and hybrid materials, such as the combination of DNA and DNA-polymer conjugates, have been used to
In this research, a new type of DNA crosslinked polymer hydrogel with reversible photocontrollability was designed. These hydrogels were functionalized by the incorporation of azobenzene moieties (Azo-s) into DNA strands to control the hybridization and dehybridization between complementary strands. Upon UV-Vis light irradiation, azobenzene changes its conformation between the trans- and cis- states. By this means, a novel type of photoregulated hydrogel with controlled reversibility was developed whereby sol-gel conversion can be utilized to encapsulate and release different loads. The controlled release capability was successfully tested using small molecules, proteins and even nanoparticles, demonstrating that the hydrogel is a promising biomaterial for delivering multiple types of pharmaceuticals and reagents of interest with excellent localization and controllability.

**Materials and Instrumentation**

The azobenzene phosphoramidite (Azo-) was synthesized according to a previously created protocol. All the materials to synthesize acrydite phosphoramidite were purchased from Aldrich Chemical, Inc. The materials for DNA synthesis, including CPG columns and reagents for DNA modification and coupling, were purchased from Glen Research Co. The DNA-Polymer conjugates were synthesized by a DNA synthesizer (Applied Biosystems) from the 3′ end to 5′ end with acrydite phosphoramidite coupled on the 5′ end.

The purification of chemical compounds was performed by using silica gel chromatography column and monitored by thin layer chromatography (TLC) (silica gel 60F254; Merck) and NMR spectrometry (Mercury 300). An ABI3400 DNA/RNA synthesizer (Applied Biosystems) was used for all the DNA-related synthesizes. Purifications were carried out on a ProStar HPLC system equipped with a gradient unit (Varian) and a C-18 column (Econosil, 5U, 250 × 4.6mm) (Alltech Associates). The concentrations of all DNAs were determined from the
absorbance at 260nm using a Cary Bio-300UV spectrometer (Varian). The melting properties were studied with a MyiQ single-color RT-PCR system (Bio-Rad). A Fluorolog-Tau-3 Spectrofluorometer with a temperature controller (Jobin Yvon) and quartz cell (Starna Cells, Inc.) were used for all steady-state fluorescence measurements. The UV light source was a portable 6W UV-A fluorescent lamp (FL6BL-A; Toshiba), and the visible light source was a general table lamp with a 60W lamp and optical filters (Asahi Technoglass).

CEM-CCRF cells (CCL-119 T-cell, human acute lymphoblastic leukemia) were obtained from ATCC. NB4 cells (acute promyelocytic leukemia) were obtained from the Department of Pathology at the University of Florida.

**Design and Experiments**

The main goal of developing the hydrogel is the reversible controllability under light irradiations. The designed hydrogels were composed of three major components to form a hydrogel: two DNA-polymer conjugates and a DNA linker. The modified DNA linker (24-base) was prepared following the previous method of synthesizing and incorporating azobenzene phosphoramidite (Azo-) onto a DNA backbone. UV light (~350nm) can photoisomerize the azobenzene moieties to the cis- isomer, while visible light (~450nm) can switch the conformation back to the trans- form. The isomerization of azobenzene is capable of regulating the hybridization between two complementary strands such that the trans- state can stabilize the hybridization, and the cis- state can destabilize it. As shown in Figure 3-1, the Azo- incorporated DNA strand, which is 24 bases in length with 11 Azo- insertions, serves as a photoresponsive crosslinker (ADL).

The crosslinks are formed by linking two polyacrylamide chains, each having 12-base DNA pendent groups. The pendent groups of one polymer chain (DPC-A) are complementary to
one half of the ADL; the pendent groups of the other polymer chain (DPC-B) are complementary
to the other half of the ADL. Thus, the ADL crosslinks DPC-A and DPC-B polymer act as a
crosslinker. The DNA polyacrylamide conjugates (DPC-A, DPC-B) were synthesized
individually by photo-initiated polymerization of 5'-acyrdite-modified oligonucleotide monomer
mixed with acrylamide (4%, w/v, mole ratio Oligo:acrylamide = 1:200). The polymer conjugates
were not further characterized here since the focus was demonstration of how sol-gel conversion
can drive encapsulation and release functions. By mixing the three components together (DPC-
A, DPC-B and ADL), the ADL can crosslink the two polymer chains and form a hydrogel. The
irradiation of UV light can drive the isomerization of \textit{tran}- azobenzene to \textit{cis}--, which may
dehybridize the crosslinking between complimentary DNAs. Therefore, the three components are
dissociated and the gel is dissolved. The irradiation of visible light will reform the gel since the
\textit{cis}-- azobenzene will isomerize back to \textit{tran}- and the hybridization takes effect again. And the
whole process is expected to be reversible due to the reversible azobenzene isomerization
(Figure 3-2).

\textbf{Synthesis of Azobenzene Phosphoramidite (Azo-)}

The details of synthesizing Azo- are depicted in Figure 3-3 A. Compound 1. In a round-
bottom flask, a solution of D-threoninol (0.91g, 9.0mmol), 4-(phenylazo) benzoic acid (2.25g,
10.0mmol), DCC (2.05g, 10.0mmol) and HOBt (1.32g, 10.0mmol) in DMF (50mL) was stirred
under an argon atmosphere at room temperature for 24 hours. The reaction mixture was filtered
and then concentrated by evaporation. The residue was purified by column chromatography
(ethyl acetate/methanol 20/1) and dried to afford the title compound (2.34g, 7.48mmole, 83%) as
an orange solid. $^1$H NMR (CDCl$_3$): $\delta$ 7.96-7.38 (m, 9H), $\delta$ 7.12 (d, 1H), $\delta$ 4.33 (m, 1H), $\delta$ 4.09
(m, 1H), $\delta$ 3.98 (d, 2H), $\delta$ 1.29 (d, 3H). Compound 2. To a solution containing 1 (0.8g, 2.4mmol)
and 4-dimethylaminopyridine (DMAP) (0.015g, 0.12mmol) in dry pyridine (10mL) at 0°C, DMT-Cl (1.0g, 3.0mmol) in CH₂Cl₂ (4mL) was added dropwise. The mixture was stirred for 1 hour at 0°C and then at room temperature for another 24 hours. The solvent was evaporated, and the residue was an orange-red oil, which was purified by column chromatography (ethyl acetate/hexane/triethylamine 50:50:3) and dried to afford 2 (0.76g, 1.24mmol, 52%) as an orange-red solid. ¹H NMR (CDCl₃): δ 8.00-6.78 (m, 23H), δ 4.25 (m, 1H), δ 4.17 (m, 1H), δ 3.77 (s, 6H), δ 3.60 and 3.42 (dd, 2H), 1.23 (d, 3H). Compound 3. To a solution containing 2 (0.62g, 1.0mmol) in anhydrous CH₃CN (20mL) at 0 °C, N, N' diisopropylethylamine (DIPEA) (0.39g, 3.0mmol) was added over 15 minutes. Then, 2-cyanoethyl diisopropyl chlorophosphoramidite (290µL, 1.3mmol) was added dropwise, and the reaction mixture was stirred at 0°C for 5 hours. After removing the solvent, the residue was dissolved in ethyl acetate, and the organic phase was washed with NaHCO₃ and NaCl solutions and dried over anhydrous magnesium sulfate. The solvent was evaporated, and the residue was purified by column chromatography (ethyl acetate/hexane/triethylamine 40:60:3) and dried to afford 3 (0.52g, 0.64mmol, 64%) as an orange-red solid. ¹H NMR (CDCl₃): δ 8.00-6.79 (m, 22H), δ 6.62 (d, 1H), δ 4.48 (m, 1H), δ 4.39 (m, 1H), δ 4.21-4.10 (m, 2H), δ 3.77 (s, 6H), δ 3.57-3.34 (m, 4H), δ 2.76-2.72 (m, 2H), δ 1.30-1.25 (m, 15H). ³¹P (CDCl₃): δ 149.

**Synthesis of Acrydite Phosphoramidite (4)**

The compound 4 was synthesized by two steps (Figure 3-3 B). First, a mixture of 6-amino-1-hexanol (9.32g, 0.08mol) and TEA (16.16g, 0.16mol) in 100 mL dichloromethane was cooled to 0°C. Methacryloyl chloride (10g, 0.0957mol) was then added slowly, and the reaction was stirred at 0°C for 2 hours, after which 100mL water was added to quench the reaction. The organic layer was washed with 5% HCl and dried. After evaporating all of the solvent, the crude
6-hydroxyhexyl methacrylamide was used for the next step without further purification. Second, to a solution containing 6-hydroxyhexyl methacrylamide (2g, 10.8mmol) in anhydrous CH3CN (40mL) at 0°C, N, N' Diisopropylethylamine (DIPEA) (3.9g, 30.0mmol) was added over 15 minutes. Then, 2-cyanoethyl diisopropyl chlorophosphoramidite (2.9mL, 13mmol) was added dropwise, and the reaction mixture was stirred at 0°C for 5 hours. After removing the solvent, the residue was dissolved in ethyl acetate, and the organic phase was washed with NaHCO3 and NaCl solutions and dried over anhydrous magnesium sulfate. The solvent was evaporated, and the residue was purified by column chromatography (ethyl acetate/hexane/triethylamine 40:60:3) and dried to afford 4 (3.33g, 8.64mmol, 80%) as a colorless oil. ¹H NMR (CDCl₃): δ 5.92 (br, 1H), 5.63 (m, 1H), 5.27 (m, 1H), 3.86-3.72 (m, 2H), 3.66-3.49 (m, 4H), 3.30-3.23 (m, 2H), 2.61 (t, 2H), 1.92 (m, 3H), 1.58-1.50 (m, 4H) 1.37-1.32 (m, 4H) 1.17-1.13 (m, 12H). ¹³C NMR (CDCl₃): δ 168.6, 140.4, 119.3, 118.0, 63.8, 63.6, 58.6, 58.3, 43.2, 43.1, 39.8, 31.3, 29.7, 26.8, 25.8, 24.9, 24.8, 24.7, 19.0. ³¹P (CDCl₃): δ 148.

**Synthesis of Azobenzene DNA Linker (ADL) and DNA linker (DL)**

ADL was synthesized by using a DNA/RNA synthesizer ABI3400 (Applied Biosystems). A solid-phase synthesis method was used to couple FAM to the 5’ ends. The synthesis started with a 3’-Dabcyl controlled-pore glass (CPG) column at the 1µmol scale. A routine coupling procedure was used to link the normal bases starting at the 3’ end on Dabcyl CPG, as shown in Figure 3-4. The Azo- was dissolved in acetonitrile and loaded to the DNA synthesizer as a normal coupling reagent with a sleeping time of 900 seconds. After synthesis, the DNAs were cut by weak base and eluted from the silica beads and chemically treated before being transferred to the HPLC for purification. The final sample was dried and stored at -20°C for future use. The DL was prepared by the same method. The sequences of ADL and DL were
5’AC*TC*AT*CT*GT*GA*AG* AGAA*CC*TG*GG-3’ and
5’ACTCATCTGTGAAGAGATTTGCCGG-3’, respectively, where * is the Azo- moiety. Both
crosslinkers have the same normal DNA sequence and ADL contains 11 Azo- moieties.

Synthesis of DNA-Polymer Conjugates (DPCs)

The DNA-polymer conjugates were synthesized on the ABI3400 synthesizer with reagents:
acrydite phosphoramidite and normal DNA bases (Figure 3-4). Acrydite phosphoramidite was
dissolved in acetonitrile and loaded into the DNA synthesizer for preparation of the two types of
acrydite-modified oligonucleotides (Sequence A: 5’-acrydite-TTTTCACAGATG AGT-3’;
Sequence B: 5’-acrydite-TTTTCCAGGTTCTCT-3’). The synthesized acrydite-modified
oligonucleotide monomers were further purified by reverse-phase HPLC and quantitatively
characterized by absorption at 260nm. DPC-A and -B were prepared separately at 3mM DNA
concentrations. The stock solution contained 10mM Tris buffer (pH8.0), 50mM NaCl, 10mM
MgCl2, 4% acrylamide, 1% MW Ciba IRGACURE 2959 and 3mM DNA Sequence A or B.
After mixing, UV light from a portable UV lamp (350nm) was applied 5cm away from this
mixed solution for 18 minutes for copolymerization of the DNA and the acrylamide. DPC-A and
-B were obtained as clear yellow solutions. The number-average molecular weight was estimated
by GPC to be approximately 100,000.

Hydrogel Preparation

DNA linkers (ADL or DL), DPC-A and DPC-B were mixed in stoichiometric 3mM DNA
concentrations in Tris buffer (10mM Tris (pH 8.0), 50mM NaCl, 10mM MgCl2). Crosslinked
hydrogels (yellow color) or DL crosslinked hydrogel (colorless) were formed immediately after
mixing. All hydrogels were incubated at 60ºC for 5 minutes before tests. Other concentrations of
hydrogels were prepared by direct dilution with buffer solution followed with annealing and visible light irradiation.

**Encapsulation and Release of Hydrogels**

The temperature of loaded hydrogels was carefully controlled by positioning the light sources at a distance that would prevent direct heating. The temperature was monitored for maximum 2 degree variation from 50ºC and the thermal effect was negligible. Encapsulation of loads was the same for fluorescein and gold NPs. Both materials were mixed with hydrogels by incubation at 50ºC for 5 minutes. The horseradish peroxidase (HRP) enzyme was incubated at 40ºC in order to maintain bioactivity. The release of fluorescein from hydrogels was monitored by observation and imaging by a CANON SD870 digital camera. To accomplish this, 200μM fluorescein was homogeneously dissolved in 300μM hydrogel. A 5μL aliquot of gel mixture was cut and placed on a transparent plastic plate with an additional 100μL of blank buffer. Visible and UV light were applied on top of the small reservoir, respectively.

The release of gold NPs was monitored by absorption spectroscopy. The release curves of gold NPs were obtained by measuring the absorbance at 520nm. For both light-driven and thermal-driven release, 20μL of hydrogel was placed on the bottom of the quartz cuvette and allowed to sit for 10 minutes. Then, 80μL Tris buffer was added on top, followed by 5 to 10 minutes of visible light irradiation by a 60W lamp and 450nm optical filters. Then the loaded cells were further irradiated on incubation in a water bath. After irradiation/heating, the samples were immediately transferred to the fluorospectrometer for absorption measurements.

The release of HRP utilized the same set of microcells. In this case, the hydrogel was placed on the bottom of a small vial, and buffer was added on top. After each light irradiation, 1μL supernatant was transferred to a 2mL vial and mixed with luminol and hydrogen peroxide in
buffer and stirred for 10 minutes. The emission curves were obtained by measuring the chemiluminescence at 410nm after 10 minutes. Since the luminescence intensity is proportional to the HRP concentration, the released HRP can be quantitated using the chemiluminescence intensity emitted from oxidation reaction.

**Biocompatibility of ADL and ADL Hydrogels**

ADL and ADL hydrogels were prepared with different concentrations and mixed with the same amount of CEM cells (1 million/mL). The cytotoxicity of each sample was calculated by counting cell proliferation at 0, 12, 24, 48 and 72 hours. The proliferation was obtained by counting living cells under the microscope. The distribution of cells at different stages was monitored by Vybrant Apoptosis Assay Kit #2 (Invitrogen) and flow cytometry (FACScan cytometer, Becton Dickinson Immunocytometry Systems).

**Cell Viability Study**

DL and ADL crosslinked hydrogels were loaded with doxorubicin (10mg/mL) by mixing the drug with sol state hydrogels. The hydrogels were placed inside the bottom of a small quartz cuvette with cells on top (CEM cells, 20,000/well). After irradiation by visible/UV light (only on hydrogel portion), the cells were incubated for 48 hours before the viability test. The cell samples were mixed with MTS Cell Proliferation Assay and the living cells were determined by monitoring the absorbance at 490nm.

**Results and Discussion**

**Preparation of Photocontrollable Hydrogel**

The ADL has two half segments, which are complementary to the 12-base DNA pieces from DPC-A and DPC-B, and are linked to the polyacrylamide backbone by a four-T-base spacer. The photoisomerization of Azo- on the DNA linker upon light irradiation was
characterized by absorption spectroscopy. To maximize the photoregulation efficiency, one Azo-moiety was incorporated after every two nucleotides of the ADL. The synthesized and purified ADL is water soluble and displays a yellow color in buffer solution. In the presence of the ADL linker with the Azo- unites in the trans- configuration, the crosslinking of two polymer chains between ADL and complementary strands from DPC-A and DPC-B immediately occurred to yield a yellow-colored hydrogel (Figure 3-5). To prevent possible incomplete hybridization that results from the cis- state Azo-, a pretreatment of annealing (10 minutes in a 50-60°C water bath) and visible light irradiation (5 minutes, 450nm) was always performed. Because of the reversible cis-trans isomerization of the azobenzene moieties, the DPC-A and DPC-B crosslinks are expected to associate/dissociate with suitable visible/UV irradiations. Thus, the hydrogel can be transformed between the gel and sol states.

**Reversible Sol-gel Conversion**

The gel-sol conversion and its reversibility were investigated through repeated UV and visible light irradiations. The ADL crosslinked hydrogel sample was prepared by directly mixing ADL, DPC-A and DPC-B in stoichiometric 3mM concentrations based on DNA quantity. At this concentration, the sample formed a robust yellow gel. The initially formed 3mM hydrogel could be homogeneously diluted to other concentrations by annealing at 50°C with buffer. In order to investigate concentration-dependent properties, such as photo-sensitivity and efficiency of encapsulation and release, the reversible photoconversion was investigated with a 300μM ADL hydrogel from dilution, which was first irradiated by visible light and then treated with either UV or visible light. A portable UV lamp (350nm) was used for the UV light source, and a 60W table lamp with a 450nm filter was used as the visible light source. The 350nm UV light irradiation induced a melting behavior after 2 minutes (Figure 3-6 A, top row). The irradiation process
lasted for approximately 20 minutes before the gel completely converted to liquid. The melted gel could be rapidly re-gelled with 450nm visible light irradiation within 2 minutes. This gel-sol conversion could be repeated at least ten times without noticeable loss of conversion rate (data not shown). By contrast, the hydrogel formed by DL linker did not display such melting under continuous UV light irradiation (Figure 3-6 A, bottom row).

The control experiment was carried out on a 300μM hydrogel crosslinked with plain DNA linker (DL) (Figure 3-6 B). The DL has a sequence identical to ADL, except with no Azo-moiety. The DL crosslinked the polymer chains, DPC-A and DPC-B, in the same manner as the ADL hydrogels did. UV irradiation up to 20 minutes induced only a very slight tilting by the effect of gravity. The inert response to UV light of this plain DNA- crosslinked hydrogel validates the influence of the Azo-moiety, which is the key component underlying the photocontrollability and reversibility of the hydrogel. Aside from the effect of gravity, ADL- and DL-constructed hydrogels may also absorb a small amount of visible light energy standard for DNA-based materials, and thereby induce slight gel deformation. Both phenomena can aid in a slow deformation of hydrogels, but they are insignificant compared to the strong light absorption from azobenzene moieties. Since DNA hybridization is sensitive to temperature, the light sources were placed 5cm away from the gel, and the temperatures on all gel samples were always monitored to be 25 ± 2°C to prevent thermal effects. Such precautions were also taken for all photo-induced gel-sol conversions.

Figure 3.7 displays the SEM images of hydrogels prepared by DL and ADL. These images demonstrate that both types of materials have regularly cellular structures with macropore sizes of approximately 10μm. However, the microstructures of these two types of hydrogels are different. The DL crosslinked hydrogel has tangled network with more diversified aperture size
and thinner reticular structure. In contrast, the ADL crosslinked hydrogel appears to have parallel tube-like structure with regular aperture size. Since the images were obtained after visible light irradiation to ensure all the azobenzene moieties were in the \textit{trans-} form, it seems that \textit{trans-} Azo- can induce regulated hybridization, as well as a more uniform and regularly tube-like cellular structure.

By their very nature, the robustness of hydrogels will decrease with lower density of crosslinked scaffold. Previous synthesized DNA crosslinked hydrogels have generally required a DNA concentration above 1mM to maintain a well-defined gel state and biofunction.\textsuperscript{62} However, Figure 3-5 clearly demonstrates that both sol and gel states are stable at the much lower concentration of 300\(\mu\)M. In addition, the concentration could be reduced to 50\(\mu\)M, while keeping enough rigidity to maintain a gel-like morphology, the hydrogel in a 1mL inverted microtube took more than 1 minute to flow from top to bottom (data not shown). At the same time, it should be noted that the loosely crosslinked hydrogel network can cause low-concentration hydrogels to deform rapidly, which will damage the encapsulating capability. On the other hand, high-concentration hydrogels may require a longer time for gel-sol conversion and thus reduce the release rate. As a consequence, it is necessary to balance the gel and sol states equally via suitable dilution with buffer, so that the gel rigidity and conversion are both optimized in order to further develop their photocontrollable load-carrying function.

\textbf{Theoretical Calculations and Modeling of the Hydrogel Microstructure}

To demonstrate the photon-triggered release of loads from photosensitive ADL crosslinked hydrogels, a series of ADL crosslinked hydrogels with different concentrations were prepared to explore the controllability, all other conditions remained unchanged. The doping process was achieved by simply mixing the loads with sol-state hydrogels by either heating or UV light
irradiation under stirring. The solutions were then cooled and irradiated at 450nm to reform the gel and encapsulated the load.

The encapsulation capability based on stability and immobility is related to the hydrogel matrix. One advantage of our hydrogel is that the crosslinking is determined solely by DNA hybridization between two different polymer chains, in contrast to the random crosslinking observed in polymer-based hydrogels. It is therefore possible to estimate some physical properties of this matrix. To evaluate individual particle entrapment by physical size and interaction, the term “cage” was used to represent the hydrogel network pore size. Cage sizes are generally difficult to obtain in normal polymer-based hydrogels due to the wide distribution of molecular sizes and structures, making their crosslinking difficult to predict mathematically. However, in DNA-based hydrogels, the crosslinking is due to precisely synthesized DNA chains, which allow cage size estimation from the chain lengths on a regulated 2-D hydrogel structure. Two neighboring DPC polymer chains are assumed to extend on one plane and along the same direction, crosslinked by an intermediate ADL strand as shown in Figure 3-8 A. By modeling the chemical bond length of the cage structure, the calculated distance between two neighboring DNA branches is 49.25nm, and the distance between two parallel polymer chains is 7.33nm. Because of the softness of these linkages, the hydrogel is expected to have a circular structure, 36.02nm in diameter, with maximum containing capability and minimum interior stress. The isometric mixing of 3mM concentration ensures a compact crosslinking, and the hydrogel has an average cage size of approximately 10.1nm in diameter. The 300, 100 and 50μM hydrogels have the size of 17.42, 25.12 and 31.65nm from calculation, respectively. Compared to the 3mM hydrogel, these lower concentration hydrogels by direct dilution can still keep the regularity. Although we have applied pre-treatment and concentration adjustment to all the hydrogels,
irregular crosslinking among multiple polymer chains is inevitable on all branched polymer materials due to random crosslinking events (Figure 3-8 B).

Photocontrollable Releasing

In order to study the capability and efficiency of hydrogels for controlled release, three hydrogel concentrations (300, 100, 50μM) were prepared and pre-loaded with the following: small molecule fluorescein (<1 nm), gold nanoparticles (NPs) (13nm), and bioactive horseradish peroxidase (HRP) enzyme (≈ 6nm) (Figure 3-9), represent a diverse set of pharmaceutical candidates: small molecule drugs, chemo- and phototherapeutic reagents, and protein-based nanomedicine, respectively.

The small-sized fluorescein molecule is a commonly used material for labeling and tracking other chemicals or biomolecules because of its observable bright orange color and detectable strong fluorescence. More importantly, the size and physical properties of fluorescein are very similar to many chemotherapy drugs. For the fluorescein-loaded hydrogel, there was no observable color diffusion from gel to surrounding buffer solution after more than 30 minutes of visible light irradiation at room temperature (25ºC). After applying UV light, however, the hydrogel mixture started to melt, and fluorescein molecules were observed to rapidly diffuse out to the buffer solution (Figure 3-10). This diffusion was not caused by a strong absorption of UV light by the fluorescein molecules, as confirmed by a control experiment in which fluorescein dissolved with DL crosslinked hydrogel had no response to either visible or UV light. It is interesting to observe the fluorescent’s stable encapsulation and induced release, since the fluorescein molecule is much smaller than the calculated cage size. We believe that one main factor underlying this phenomenon is the limit mobility of fluorescein molecules in the large hydrogel pocket without an external driving force for self-diffusion. This lack of movement
significantly prolongs the retention time of the trapped molecules. Two other hydrogels with concentrations of 100 and 50μM were tested under the same conditions, and the diffusion processes seemed faster with uncontrollable leaking as a consequence of the larger hydrogel cage.

Water-soluble BSA-modified gold NPs (13nm) were used to further study the entrapment/release capabilities of our hydrogels. In order to specifically study the relationship between hydrogel concentration and doped loads, three different hydrogels were prepared based on DNA concentrations of 300μM, 100μM, and 50μM. These hydrogels were made in the presence of a 500nM gold NP suspension, entrapping the gold nanoparticles. A small portion of gel mixture was placed on the bottom of a quartz microcell (Figure 3-11 top) with buffer solution on top. When irradiated with UV light, the hydrogel melted, and the trapped gold NPs were released to the top of the buffer solution and quantitatively monitored at several time intervals by the strong gold NP absorption at 520nm. The hydrogels were initially irradiated with thirty minutes of visible light to see if there was any gold NP leaking before applying UV light.

The absorption curves in Figure 3-11 show that the UV light dissolved the hydrogels rapidly after 1 minute, releasing gold NPs into the buffer solution for absorption measurement. Both 300μM and 100μM hydrogels can steadily encapsulate NPs without leaking (low absorbance prior to UV irradiating), while the 50μM hydrogel seems to enclose the particles much less tightly. The UV light dissolved the hydrogels rapidly after 1 minute, and gold NPs were released to buffer solution. The absorption curves also demonstrate different release rates of gold NPs under UV light irradiation. All hydrogels seem to have an initial bursting release period following by a much slower release. For the 300μM hydrogel, the release rate was comparably slow and lasted for more than 15 minutes before reaching a plateau with an average rate of (1.96
± 0.19) x 10^{-4} \text{nmole/min}. The 100\mu M hydrogel seemed to have a higher rate of release upon UV irradiation. The average release rate was (3.95 ± 0.36) x 10^{-4} \text{nmole/min} before reaching a plateau at 15 minutes. Despite a serious problem with uncontrollable leaking, the evenly diluted 50\mu M hydrogel is the fastest to reach the plateau with a release rate of (6.88 ± 0.70) x 10^{-4} \text{nmole/min} in 5 minutes. This difference among the three hydrogels clearly demonstrated the concentration-dependent encapsulating and releasing capability under light irradiation. It seems that the 100\mu M hydrogel has the best balance overall, with stable containment and rapid release rate, properties which actually correlate well with the stability of the gel and sol states in hydrogels.

Besides the release rate, the net amount of gold NPs released from each hydrogel is also an important factor in evaluating the delivery capacity. The 300\mu M hydrogel displays high resistance in releasing gold NPs by 60 minutes of UV illumination, and only 38.1% of NPs were released after 30 minutes of visible and UV light, while the 100 and 50\mu M hydrogels separately released up to 66.9% and 48.4 % (68% - 20 %) during the same period. The 100\mu M DL crosslinked hydrogel control displayed weak response to both visible and UV light regarding both release rate and amount (less than 10% overall released). Although the size of gold NPs is smaller than that of the theoretically calculated cage size for an ideally crosslinked hydrogel, the interaction between the cage skeleton and gold NPs is able to balance the retention and diffusion rates.

The small molecule fluorescein and the larger gold NPs are generally structurally stable and will not change activity in most transporting methods or materials. However, bioactive enzymes and macromolecules which require chemical binding or strong physical adsorption for stability, may be irreversibly altered upon hydrogel encapsulation, damaging their bioactivities. To show this was not the case hydrogels were used to deliver HRP as a bioactive drug model.
HRP is a commonly used enzyme which enhances weak signals by releasing chemiluminescence after substrate oxidation. Similar to the visible/UV irradiation applied to gold NPs, the release from HRP-loaded hydrogels was examined, and the luminescence profiles were recorded by spectrofluorometer. The luminescence intensities were further converted to HRP amount released to buffer solution as shown in Figure 3-12. All three ADL crosslinked hydrogels with different densities have typical UV switch-on releasing profiles. The 100μM hydrogel was better at releasing HRP while the more dilute 50μM hydrogel had the fastest release rate. The calculated results demonstrated a net release of 46.7%, 59.2% and 56.0% of active HRP after 60 minutes of UV irradiation released from 300μM, 100μM and 50μM ADL hydrogels, respectively. The control 100μM DL hydrogel, by comparison, only released 4.8%. These results demonstrate the successful delivery of a bioactive protein by our photocontrollable hydrogels.

The activity of the enzyme molecules was confirmed by the enzymatic reaction. Of the different formulations, the 100μM hydrogel has the best balance of enzyme storage under visible light and rapid release under UV light.

**Thermodynamic Profiles**

Aside from the controllability brought by the photosensitive Azo- moiety for multiple-load releasing, the DNA crosslinked hydrogels may also have reversible thermo-sensitive melting properties. The melting property can also be regarded as a gel-sol conversion independent of the Azo- moiety and external light energy and defined only by DNA sequences. To investigate this hypothesis, three ADL hydrogels, 300μM, 100μM, 50μM, and one 100μM DL hydrogel were evaluated for encapsulation and release of gold NPs (Figure 3-13). As expected, all DNA-crosslinked polymer hydrogels including the DL crosslinked gel had melting profiles very similar to that of the pure DNA duplex without polymer backbone. The results of thermal gold
NP release have a similar trend to that of visible/UV release, where a temperature lower than the melting temperature ($T_m$) can be regarded as visible irradiation, while temperatures higher than $T_m$ can be regarded as UV irradiation. Since this thermo-responsive property of ADL-crosslinked hydrogel is independent of Azo- modification, it provides an additional parameter in controlling the release of loads.

**Biocompatibility**

Biocompatibility of the hydrogels was also investigated by incubating hydrogel samples with cells. Different concentrations of DNA linkers and hydrogels were mixed with human leukemia CEM cells, and cell proliferation was monitored by counting living cells and monitoring cell apoptosis at different stages from 12 to 72 hours.

ADL and ADL hydrogels were prepared with different concentrations and mixed with the same batch of CEM cells (1 million/mL). The cytotoxicity under each condition was calculated by proliferation profiles. CEM cell samples incubated with ADL or ADL hydrogels were treated with apoptosis reagent, Alex Fluor 488 annexin V/propidium iodide (Vybrant Apoptosis Assay Kit #2, Invitrogen) and analyzed with flow cytometry.

Biocompatibility of ADL: Leukemia CEM cells was evaluated by incubating at 37°C in cell culture medium. The number of cells was maintained at 100,000 in each well for cytotoxicity investigation. When the cells were mixed with DNA linkers and hydrogels, apoptosis reagent was added to cell medium at the same time to label dying and unhealthy cells. The cell proliferation-based cytotoxicity was monitored by flow cytometry from zero to 72 hours (Figure 3-14 A). The toxicity of ADL to CEM cells modeled final conditions when the ADL hydrogel totally dissolved and all ADL were released to cell culture solution. Only at concentrations higher than 100μM and incubation time of more than 48 hours do the results
show that a significant toxicity was observed. This indicates that the Azo-DNA linker is safe to be used for this cell line at concentrations less than 100μM.

Biocompatibility of ADL hydrogels: Four different concentrations of ADL crosslinked hydrogels were investigated for cytotoxicity to CEM cells. Each gel was mixed with an identical cell aliquot, and the cell proliferations were monitored from zero to 72 hours (Figure 3-14 B). The calculated final concentrations of ADL and DNA-polymer conjugates were 1, 10, 30 and 100μM, respectively. The results in Figure 3-13 show a comparable low toxicity for low- and high-concentration hydrogels. At the concentration of 10μM, the hydrogel is very dilute in cell medium, and the crosslinking ratio is very low. As a result, the pore size of this gel is large enough to permit cells to go inside the gel matrix and freely make contact with DPC and ADL. For this reason, it has a slightly lower toxicity than the sample containing 1μM ADL in cell medium, while the DPC should contribute the rest of toxic effect. For high-ratio crosslinked ADL hydrogels, such as 1mM, the highly crosslinked polymer network can maintain a robust rheology and prevent the cells from entering the hydrogel. Therefore, the effective concentrations of ADL from these hydrogels are much less than those containing ADL alone. It is only when the gel is totally dissolved, that a large amount of free ADL is available. The middle concentrations of gels (100 and 300μM) have higher cytotoxicity compared to the 100μM sample. This is caused by the comparably looser structures and higher effective concentrations of ADL.

Photocontrollable Cancer Drug Delivery

After demonstrating the hydrogel’s ability to carry and deliver model drug molecules, the efficacy of the photoresponsive hydrogel was tested for cancer cell treatment. An anticancer drug, doxorubicin, was selected for the chemotherapy study using the photocontrollable hydrogel
as a carrier. The same strategy described above was followed for encapsulation and release. The 100μM hydrogel was homogeneously mixed with 10mg/mL doxorubicin in the sol state, followed by visible irradiation to reform the gel. The doxorubicin encapsulated hydrogel was first evaluated for photocontrollability. The released doxorubicin was quantitatively measured to mimic the release under three conditions: dark, visible irradiation, and UV irradiation. As shown in Figure 15 A, the 100μM hydrogel system displayed an approximately 5.0% leaking ratio after 20 minutes in the dark. Irradiation with visible light for 20 minutes induced an additional 4.1% release. The leakage in the dark and with visible irradiation is mainly due to the self-diffusion of the small drug molecule within the matrix, especially, close to the surface region, producing a rapid escape rate in the early stages. The UV light, as expected, triggered an immediate rapid release of the drug for about 10 minutes, followed by slower release approaching a plateau. The net amount of the drug molecules released within 20 minutes of UV irradiation is approximately 65.1%.

The same Dox-loaded hydrogel was tested with CEM cancer cells to study the drug’s efficacy. Both DL crosslinked and ADL crosslinked hydrogel with and without drug were compared for photocontrollable chemotherapy. In each case, an aliquot of cells was loaded on top of a hydrogel layer inside a cuvette. Each hydrogel layer was irradiated with visible or UV light for 10 minutes, then removed and incubated with cells followed by a viability study as shown in Figure 3-15 B. Compared to the control cells (without hydrogel), all DL hydrogels show less than 10% higher cell death rate due to the combination of material toxicity and drug leakage. The ADL hydrogels have similar profiles under visible light, indicating the slim influence of the Azo- moiety on cell viability. However, the UV light irradiation of the ADL hydrogel melted the gel and released the loaded Dox drug. It induced a very high death ratio of
cancer cells up to approximately 80%, which is 69% higher than normal cells without hydrogels and drug, or 59% higher any than other situations. These results clearly validate the sensitive photoresponse of the ADL-crosslinked hydrogel to UV light, resulting in release of a large amount of drug and inhibition of cell proliferation.

**Conclusions**

Reversible UV/visible controlled DNA crosslinked hydrogels have been prepared, and they display controllable encapsulation and release by a variety of loaded materials. The hydrogels are easy to synthesize and can be made with controllable sizes and structures. In contrast to chemically triggered hydrogels, they can be reversibly photoregulated by photons. The ADL crosslinked hydrogel system has been demonstrated as a very efficient carrier for encapsulating and delivering small molecules, large-sized nanoparticles and bioactive enzymes. More importantly, this hydrogel-based carrier has been successfully used to carry and release a cancer drug with good photocontrollability in vitro. By rational design of the DNA crosslinker and DNA polymer conjugates, this hydrogel system can be applied to a much wider range of pharmaceutical carriers for precise, spatially photocontrollable delivery. Moreover, the additional aspect of temperature control could be used to drive a similar temperature dependent. Future investigation of multiple-factors and coherent design of the hydrogels can extend this biomaterial to applications involving drug and gene delivery and tissue engineering.
Figure 3-1. Design of ADL- and DNA-polymer conjugates. The two 12-base DNA segments (green and blue), which are complementary to the 24-base ADL, can hybridize under proper conditions and crosslink the polymer chains, resulting in a hydrogel.
Figure 3-2. Scheme of photoregulated hydrogel. Azo-incorporated DNA linkers can crosslink two types of DNA-polymer conjugates (the DNAs sequence from each type of conjugate are different) and form the hydrogel (a-b). The loaded hydrogel can be reversibly controlled by visible and UV light to release previously encapsulated loads.
Figure 3-3. Scheme of synthesis of (A). azobenzene-tethered phosphoramidite monomer (Azo-) and (B). acrydite phosphoramidite monomer.
Figure 3-4. Scheme of incorporation of Azo- or acrydite units to DNA sequences by DNA synthesizer.
Figure 3-5. Preparation of ADL crosslinked hydrogel. Mixing of equal amount of DPC-A, DPC-B results in a liquid solution. Addition of ADL causes incomplete gel formation with bubbles. The hydrogel was incubated at 50°C for 5 minutes to produce a homogeneous yellow-color gel.

Figure 3-6. Responses of ADL and DL crosslinked hydrogels under electromagnetic radiation at two wavelengths. (A) Reversible gel-sol conversion from UV-visible irradiation on a 300μM hydrogel. The gel began to respond after 2 minutes illumination at 350nm and totally melted after a 20-minute illumination period. The sol state can be rapidly regelled by illumination at 450nm. The same hydrogel irradiated solely by visible light displayed slight tilting. (B) Response to continuous UV light of the 300μM DNA linker (DL, without Azo-) crosslinked hydrogel.
Figure 3-7. SEM images of the dried DNA hydrogels. (A) DL crosslinked hydrogel and (B) ADL crosslinked hydrogel.
Figure 3-8. Modeling and theoretical calculation of hydrogel matrix dimensions (Not drawn to scale). (A) Theoretical model of hydrogel matrix in regularly crosslinked condition. (B) Scheme of reversible sol-gel conversion by UV and visible light. The irregular crosslinking between random complementary DNA strands varies according to actual cage size and transition conditions.
Figure 3-9. Model of different types of loads for hydrogel-controllable encapsulation and release.

Figure 3-10. Ultraviolet irradiation of fluorescein-encapsulated ADL hydrogel (300μM).
Figure 3-11. Controllable release of 500nM 13nm gold NPs mixed with three different concentrations of ADL hydrogels. The mixtures were each retained inside a quartz microcell with buffer solution on top and were irradiated by visible light and UV light. The absorbance at 520nm was monitored. The absorbance values were normalized by setting pure buffer absorption as 0% and 100nM NPs solution as 100% (The right top insertion is the setting of the microcell for absorption measurement). All experiments were repeated at least three times.
Figure 3-12. Controllable release of HRP enzyme encapsulated in hydrogels quantitatively calculated by catalyzing luminol oxidation. (A) The HRP catalyzed oxidization of luminol with chemiluminescent intermediate. (B) Chemiluminescence profile of the produced intermediate which is the monitored at 410nm. All experiments were repeated at least three times.
Figure 3-13. Thermal release of gold NPs. Gold NPs (13nm) were mixed with hydrogels at an initial concentration of 500nM. All hydrogels were irradiated by visible light for 10 minutes at 20°C before increasing the temperature. The absorption at 520nm was monitored every 10 degrees. 100% absorbance was obtained by measuring 100nM gold NPs in buffer solution. All experiments were repeated at least three times.
Figure 3-14. Biocompatibility of CEM cells with ADL and ADL crosslinked hydrogels. Different concentrations of ADL and ADL crosslinked hydrogels were incubated with the same amount of CEM cells. CEM cells at 37°C generally can double their population every 24 hours with sufficient culture media. (A) Living cells under different concentrations of ADL (1, 10, 100, 300μM). (B) Living cells under different concentrations of ADL hydrogels (10, 100, 300, 1000μM).
Figure 3-15. *In vitro* doxorubicin (Dox) release from hydrogel. (A) Photocontrollable release of Dox from ADL crosslinked hydrogel in buffer. The drug loaded hydrogel was kept in the dark for 20 minutes, then irradiated with visible light for 20 minutes, followed by UV light for another 20 minutes. (B) Cell viability after treatment with Dox-loaded hydrogels under different photoirradiation conditions.
CHAPTER 4
DEVELOPMENT OF SINGLE DNA MOLECULE NANOMOTOR REGULATED BY PHOTONS

Introduction

As described in Chapter 1, functional DNA sequences and their structures have drawn considerable attention. DNA self-assembly based on spontaneous hybridization between complementary strands is an effective way to construct multidimensional nano-objects, such as macromolecular architectures\textsuperscript{70,71,124-127}, biochips\textsuperscript{128}, photonic wires\textsuperscript{129}, enzyme assemblies\textsuperscript{130}, and DNA probes\textsuperscript{131,132}. Similar to RNA or protein-based nanostructures\textsuperscript{133-135}, DNA-based nanomachines have been developed with the ability to change their conformations upon external stimulation.\textsuperscript{6,136} The first single DNA molecular motor developed a few years ago in the Tan lab can switch between two conformations and perform extending-shrinking motion with stable, convenient operation and high efficiency.\textsuperscript{7} However, this single-DNA nanomotor requires addition and removal of fuel and waste strands for motor function.\textsuperscript{6,7,137} Although some artificial nanomotors can utilize alternate energy sources, including hydrolysis of the DNA backbone\textsuperscript{75} and ATP\textsuperscript{76}, application of an electromagnetic field as a convenient energy source is highly desired, especially with the goal to utilize solar energy. Therefore, a photon-driven single-DNA nanomotor would be of great scientific interest as well as contribution to the applications in nanoscience.

Azobenzene molecules have been extensively studied due to their reversible isomerization between the planar \textit{trans} form and non-planar \textit{cis} form under UV and visible light irradiations.\textsuperscript{78,79,122} The first light-powered molecular device using an azobenzene polymer proved the concept of optomechanical energy conversion in a single device.\textsuperscript{138} Several DNA-based nanostructures incorporated with azobenzene moieties for photoregulated action have also
been constructed and investigated.\textsuperscript{85,86,139-144} A model of light driving azobenzene-DNA molecular motor based on exchange of multiple DNA strands has been recently demonstrated.\textsuperscript{85} Although the concept of a single-molecule nanomotor was introduced in these studies, several DNA sequences were actually needed to perform the motor movement. Overall, the advent of a single-DNA nanomotor and the knowledge of special optical properties of azobenzene have made developments of a photo-regulated single molecule nanomotor feasible.\textsuperscript{145}

Inspired by the photoregulation capability of azobenzene to DNA hybridization, we have designed a single-molecule DNA nanomotor regulated by photons. The design makes use of the dehybridization (open state) and hybridization (closed state) of a hairpin structure by controlling the photoresponsive azobenzene moieties integrated in the DNA hairpin’s duplex stem segment. Because of its reversible extension-contraction behavior, the open-closed cycle of the hairpin molecule is considered as a molecular motor, and such motion can be characterized by monitoring fluorescence variation between a fluorophore and a quencher on both ends of the stem. Compared with previous DNA motors, which involve bimolecular or multimolecular interactions among several independent DNA strands, the hairpin-structured DNA motor is a single-molecule nanomotor that can facilitate the open-closed cycling by virtue of its own unique structural architecture. In this single-molecule DNA nanomotor, the motor can act as a simple, yet precisely functionalized, molecular motor. Because of its simplicity and intramolecular interaction, a single-DNA nanomotor with reversible photoswitching ability is expected to possess novel properties superior to those consisting of multiple-component DNA nanostructures. Because the single-DNA molecule motor relies on intramolecular interactions within the molecule instead of disjunctive DNA strand exchange, the photoregulation process is expected to be concentration-independent and thus ideal for developing high-density molecular motors.
Materials and Instrumentation

All the chemicals for synthesis of the phosphoramidite monomer were purchased from Aldrich Chemical, Inc. The materials for DNA synthesis, including CPG columns and reagents for DNA modifications and coupling, were purchased from Glen Research Co. All the chemicals were used without further purification, except as otherwise explained. The DNA probes were synthesized by a DNA synthesizer from the 3′ end to 5′ end, starting with CPG column labeled with a Dabcyl quencher (Dabcyl CPG) and with a FAM fluorophore coupled on the 5′ end. The target complementary DNA (cDNA) for the photoswitchable molecular motors (PSMMs) is a partially complementary strand with the sequence complementary to the PSMMs from 3′ end throughout the entire loop. The linear DNAs were synthesized with the same Dabcyl CPG on azobenzene-incorporated sequences, and general bases were CPG-coupled with FAM on the 5′ end for the complementary sequences.

The purification of chemical compounds was performed by glass column for the silica gel chromatography and identified by thin layer chromatography (TLC) (silica gel 60F254; Merck) and NMR spectrometry (Mercury 300). An ABI3400 DNA/RNA synthesizer (Applied Biosystems) was used for all DNA-related synthesis. The purifications were carried out on a ProStar HPLC system equipped with gradient unit (Varian) with a C-18 column (Econosil, 5U, 250 × 4.6mm) (Alltech Associates). The concentrations of DNAs were determined from the absorbance at 260nm with a Cary Bio-300UV spectrometer (Varian). The melting properties were studied with a MyiQ single-color RT-PCR system (Bio-Rad). A Fluorolog-Tau-3 Spectrofluorometer with a temperature controller (Jobin Yvon) was used for all steady-state fluorescence measurements. The samples were loaded with a quartz cell for fluorescent measurements (Starna Cells, Inc.). The UV light source was a portable 6W UV-A fluorescent
lamp (FL6BL-A; Toshiba), and the visible light source was a general table lamp with a 60W bulb and 350nm optical filters (Asahi Technoglass).

**Design and Experiments**

The photoswitchable motor design requires the choice of DNA backbones and the artificially positioning of photoresponsive azobenzene moiety (Azo-) (Figure 4-1). In this case, a 31-base DNA probe, which displayed a stable hairpin structure in buffer solution, and fits most of the requirements for a simple and highly responsive nanodevice, was selected. From previous research on inserting Azo- to DNA sequences, it is possible to insert multiple Azo- onto the stem duplex to adjust the photocontrollability. In order to monitor the molecular open and closed states, a Dabcyl quencher was tethered to the 3’ end and a FAM fluorophore was attached to the 5’ end to avoid interference from free dye molecules. Since the single molecule DNA motors make use of intramolecular interactions, comparable linear DNA probes inserted with Azo- were designed to compare with hairpin structured DNA motors in an energy conversion efficiency studies. Other hairpin structured DNAs with different loop sequences were also synthesized to further evaluate the significance of the motor molecules.

The single-molecule nanomotor was constructed as a fully functionalized DNA molecule driven by absorption of electromagnetic energy. The main components of PSMM include a hairpin backbone, azobenzene moieties (azobenzene phosphoramidite, Azo-) and a fluorophore/quencher (F/Q) pair for signaling motor movement. Under UV and visible irradiances, the PSMM can reversibly switch between the open (unhybridized) and closed (hybridized) states. The reversible extension (open) and contraction (closed) states of the “arms” (forming the stem duplex in the closed state) functions as a molecular motor, because of the cycling and stretching actions of the DNA.
In order to demonstrate PSMM regulated by photons, a 31-base DNA was selected for the construction of the nanomotor. This DNA has a stable hairpin structure including a 6 base pair stem and a 19 base loop, with a Fluorescein (FAM) at the 5’ end and a Dabcyl quencher at the 3’ end: 5’ FAM-CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-Dabcyl 3’ (underlined bases represent stem sequences). In the closed state, the DNA forms a hairpin structure, and fluorescence is quenched due to the proximity of fluorescein and Dabcyl. The fluorescence intensity is related to the distance between the F/Q pair, and indicates the closed or open states of the PSMM. The completely open state with fully extended structure occurs when complementary DNA (cDNA) is bound to the molecule with strong binding affinity, resulting in maximum fluorescent intensity due to the largest separation between fluorescein and Dabcyl. The F/Q pair was included to serve as an indicator to monitor the molecular structural changes. Hence, the difference in separation distance between the closed and fully open states provides a functional “open” operational range for the molecular motor when the PSMM is driven by light irradiations in a reversibly contracting and extending movement. In other words, the fluorescence intensity of the open state will vary within the operational range due to limitations of extension capability of a single DNA strand. In this manner, the hairpin-to-extension structure exchange can be regarded as a single molecule’s motion, where the push-and-pull dynamics can be achieved by photons. Thus, a well-controlled photoswitchable DNA nanomotor can be produced based on this design.

The positioning of Azo- moieties follows the basic principle from the literature. To maintain loop flexibility, they are inserted only in the stem duplex at three selected sites on each end (Figure 4-2). The nomenclature of these possible sequences is given in Figure 4-3, for single to triple Azo- incorporation on either arm of the stem duplex.
Synthesis and Purification of Photoswitchable Molecular Motors (PSMMs)

The azobenzene phosphoramidite (Azo-) was synthesized with the same protocol as described on Chapter 3. Hairpin molecules were synthesized by using a DNA/RNA synthesizer ABI3400 (Applied Biosystems). A solid-phase synthesis method was used to couple FAM to the 5’ end. The synthesis started with a 3’-Dabcyl controlled pore glass (CPG) column at 1µmole scale. A routine coupling procedure was used to couple the normal bases starting at 3’ end on the Dabcyl CPG. A proper amount of Azo- was dissolved in dry acetonitrile (20mg/200µL) in a vial connected to the synthesizer. A 20mg sample of Azo- can make a single incorporation in the DNA at 1µmole synthesis scale. The coupling step can be performed at room temperature immediately after the Azo- reagent preparation. It then can be used as a normal base for insertion in the synthesizer program, allowing at least 600s reaction time. A 900s reaction time was applied to couple the FAM fluorophore at the 5’ end. After the synthesis, the CPG substrate was transferred to a glass vial, and standard AMA (ammonium hydroxide: methylamine = 1:1) deprotection solution was added after incubated in water bath at 50°C for 12 hours, the sample was centrifuged for 30 minutes to separate the solid beads from the oligonucleotide, and the clear supernatant was carefully collected. Then, the oligonucleotide was concentrated by ethanol precipitation. The precipitate was redissolved by TEAA solution (0.1M) and injected into a reverse-phase HPLC using a C-18 column. The product was eluted using a linear 30-minute gradient from 19% acetonitrile/(0.1M TEAA solution) to 55%. The collected product was then vacuum dried, detritylated, and stored at -20°C for future use.

Characterization of PSMMs

The concentration of each DNA was calculated by the absorbance at 260nm by Beer’s Law:
\[ c = \frac{A_{260}}{b(\varepsilon_{\text{nat}})} \]  
(Eq. 4-1)

c is the concentration of the modified DNA (M), \( A_{260} \) is the absorbance at 260nm, b is the thickness of the cell (cm), and \( \varepsilon_{\text{nat}} \) is the molar extinction coefficient of the unmodified DNA.

The concentration of the Azo- PSMMs can be calculated as:

\[ c = \frac{A_{260}}{d(\varepsilon_{\text{nat}} + n\varepsilon_{\text{azo}})} \]  
(Eq. 4-2)

n is the number of Azo- incorporated to the DNA and \( \varepsilon_{\text{azo}} \) is the extinction coefficient of the azobenzene moiety (4,100 M\(^{-1}\) cm\(^{-1}\)).

Thermal denaturizing profiles of PSMMs and linear DNAs were measured by RT-PCR. To cover all the possible transition states, the temperature was slowly increased at 1°C/min from 10°C to 80°C. The buffer solution for all the measurements was: 20mM Tris buffer, pH8.0, 20mM NaCl, 2mM MgCl\(_2\).

**Photoregulation of PSMMs and Optimization of Operation Conditions**

PSMMs were prepared with to equal concentration (100nM) for the entire experiment at room temperature (25°C) using the selected buffer solution. For the photoregulation of PSMMs, irradiations by UV light at 350nm and visible light at 450nm were applied for all experiments, although the irradiation times were varied. Fluorescence intensity was recorded under excitation at 488nm. Once the temperature had stabilized at 25°C, the quartz cell with samples was maintained at the set temperature for at least 5 minutes before each test. The steps for UV/Vis irradiation measurements were: 1). The sample is diluted and added in quartz cell; 2). The quartz cell was set in the holder and maintained in place for 5 min.; 3). Irradiation was performed with visible light at 450nm for 1 min.; 4). The fluorescence spectrum was measured (excited at 488nm); 5). Irradiation was performed with a UV lamp at 350nm for 5 min.; 6). Fluorescence
spectrum was again measured (excited at 488nm); 7). Steps 1 to 6 were repeated to confirm the reversibility of the photoregulation.

The intensity changes were compared for 5 to 20 rounds of irradiation measurements, and ended with addition of 5-fold cDNA. For optimization experiments, the UV light irradiation time varied from 1 to 20 minutes. An fluorescence recovery of around 50% over irradiated by visible light for PSMM3 (with three Azo- insertions) was obtained after 10 minutes UV irradiation time. Longer periods can improve the efficiency slightly, but prolonged irradiation using the current portable UV lamp over 30 minutes tends to result in photobleaching of the fluorophore and decomposition of the structure (data not shown). For the reversibility test, the UV irradiation time was set at 3 minutes for all the tests, except as otherwise noted.

**Buffer Optimization**

The buffer solution conditions were optimized based on PSMM3 photoresponse under UV/Vis irradiation. Generally, a solution with high ion-strength is able to stabilize the duplex structure (closed state), but it will also hinder the conversion to the open state.

A high salt concentration aids base pairing and improves the hybridization rate and stability of duplex (open to closed), but it also induces a uni-directional nanomotor with poor balance or non-reversibility. On the other hand, a very low salt concentration of buffer solution will not be able to initiate a large amount of fully closed hairpin structure, although it does facilitate conversion between closed and open states. Additionally, low salt concentration impedes the conversion rate kinetically.

Therefore, proper ion-strength is needed to balance this closed-open conversion by setting up conditions that would favor both states equally. A typical DNA buffer solution was used (Tris-HCl (pH8.0), NaCl and MgCl₂). The concentrations of Na⁺ and Mg²⁺ were varied from
1mM to 500mM, and the Tris-HCl concentration was varied from 2mM to 200mM, as a function of photoresponse of the PSMM duplex with UV/Vis irradiation. The optimized buffer contained the following: 20mM Tris-HCl (pH8.0), 20mM NaCl, and 2mM MgCl₂. Under these conditions, PSMM3 nanomotor operation displayed a well-balanced kinetic rate of open and closed states.

**Light Sources Optimization**

In order to examine the sensitivity of the hairpin structure to the azobenzene isomerization upon irradiation, two groups of light sources were selected. For both groups, a 60W table lamp with a 450nm filter had enough power to trigger fast cis- to trans- conversion and was therefore chosen as the visible light source. A Fluorolog-Tau-3 Spectrofluorometer set to excite at 350nm was chosen for group one as the UV light source, and a portable 6W UV light source (350nm) was chosen for group two. The power of the two UV light sources was measured by a power meter for group one at 0.028mW (±0.002) and for group two 0.197mW (±0.003) at the sample irradiation position. A ten-round test was performed with these two groups of light sources in the previously selected buffer solution. Reversible photoregulation was carried out by repeated irradiations at 450nm and 350nm, followed by emission scans (λ_ex= 488nm). In all cases, the low power spectrofluorometer could not initiate a fluorescence variation of more than 5% with up to 20 minutes of irradiation. However, the high-power portable light source was observed to drive the variation up to 60%, depending on buffer solutions and irradiation time. Therefore, the portable UV lamp was selected as the UV light source for the subsequent experiments. It is likely that an even stronger UV light source will help improve the trans- to cis- conversion, even though its use raises serious problems in terms of damaging the DNA structure and photobleaching the fluorophore. Photobleaching was observed with the present portable UV lamp for irradiation time longer than 30 minutes.
Design, Synthesis and Photoregulation of Linear DNAs

The 12-base linear sequence 5’GGTCGCGCTAGG-Dabcyl 3’, and the 10-base sequence 5’TCGCGCTAGG-Dabcyl 3’ were incorporated with multiple Azo-moieties on chosen positions. All of these DNAs and the FAM labeled cDNAs, 5’ FAM-CCTAGCGCGACC 3’ and 5’ FAM-CCTAGCGCGA 3’, were prepared using the general procedure described above. For Azo- incorporated sequences, Dabcyl CPG was used for synthesis, and FAM fluorophore was labeled only to cDNA.

The specialized hairpin structure of PSMMs has been compared with another hairpin structures. While conventional DNA nanomotors involve only linear DNAs with single strand and duplex structures, PSMMs have a hairpin structure on the loop moiety that amplifies the impact of external stimuli (in this case, isomerization of azobenzene) on the open-closed conversion, as determined in this study. The hairpin structure can stabilize the stem duplex for comparable Tₘ with shorter base pairs than linear DNAs, with and without azobenzene moieties.

To further examine the impact of the special hairpin structure on nanomotor efficiency, we designed a similar hairpin structure, PolyT(A3), for comparison. The PolyT(A3) has 31 bases with the same stem duplex as PSMM3, but it has only T bases in the loop segment: 5’ FAM-CCT AGC TTT TTT TTT TTT TTT T-Azo-GC-Azo-TA-Azo-G G-Dabcyl 3’ (underlined bases represent stem moieties). Three Azo-moieties were incorporated at the same positions as those in PSMM3.

Results and Discussions

Engineering of Azo-Moieties on PSMMs

Because of their flexibility, the sequence in the loop strand may have little impact on stem duplex stability. However, the deliberate design of hairpin molecules with specific stem
modifications may have a direct impact on stem duplex stability. In this experiment, several Azo- moieties were incorporated to the PSMM stem in vary amounts and at different positions. The six designed motors were named PSMM1 to PSMM6 as shown in Figure 4-3. These PSMMs were then screened for fluorescence changes under light irradiations to observe the photoconversion between open and closed states.

**Characterizations of Azo- DNA**

Since the azobenzene has a similar structure as the Dabcyl quencher, a suspected quenching effect should be noted when Azo- moieties are spatially close to the fluorophore. Indeed, a simple fluorescence measurement revealed that PSMM 4-6 with Azo-s close to the FAM showed no fluorescence increased when cDNA was added, while PSMM 1-3 with the Azo-far away from FAM displayed the expected “on-off” fluorescence accompanying the motor’s open-closed cycles.

The hairpin structured molecules bearing the Azo- moieties have the basic functions of a molecular beacon, which is in stable hairpin structure under normal conditions and a linear structure when the temperature is higher than the melting temperature (T_m) or in the presence of cDNA. Without UV or visible irradiation, the inserted Azo- should not influence the melting nature of the molecules regardless of the the positions of the Azo- moieties.

The melting curves shown in Figure 4-4 display distinct difference between PSMM1-3 and PSMM 4-6. The lack of fluorescence response in PSMM 4-6 arises from fluorescence quenching by azobenzene may due to a similar to a quenching mechanism as Dabcyl. The fluorescence and quenching mechanism is depicted in Figure 4-5 with possible energy transfers between fluorophore and quencher. Significant fluorescence enhancement for PSMM1-3 was observed when cDNA was added, thus confirming the selective quenching of FAM fluorescence from both
Dabcyl and Azo- at close distance. Because of the difficulties of monitoring structural changes by fluorescence intensity variations, the investigation was narrowed to PSMM1-3 with Azo- incorporated at the quencher end. By this selection of positions of Azo- incorporation, the photoregulation cycle of the PSMMs can be investigated by comparing fluorescence intensities from the closed state to the fully open state. It may be recalled that the distance variations between the closed and fully open states provides a necessary and functional operational range for the molecular motors because the PSMM is driven by light in a reversible contracting and extending movement.

To optimize the motor function, two main factors that were suspected to impact the efficiency and reversibility of our PSMM1-3 nanomotors were investigated: buffer composition and light sources. A buffer solution was chosen after optimizing the balance to favor both open and closed states: 20mM Tris-HCl pH8.0, 20mM NaCl, 2mM MgCl₂. Additionally, a 60W table lamp with a 450nm filter was chosen as the visible light source in all cases, and a portable 6W UV light source (emission at 350nm) was chosen for the UV light source. Reversible photoregulation was carried out by repeated irradiations at 450nm and 350nm at fixed time periods, followed by emission scans (λetect= 488nm).

**Photoregulation of the PSMMs and Their Energy Conversion Efficiency Comparison**

With the selected light sources and buffer conditions, PSMM1-3 were tested as described in the experimental section. The three nanomotors displayed different fluorescence recovery after visible irradiation followed by UV irradiation, as shown in Figure 4-6 and Table 3-1.

As expected, motors with more Azo- incorporations, such as those incorporated in PSMM3, resulted in higher fluorescence recovery, indicating that increasing of the amount of Azo- moiety can introduce a higher impact on azobenzene isomerization and hairpin structure
stability. A five-fold of cDNA was added at the end of the photoregulation experiment for each type of nanomotor in order to compare the fluorescence intensity of all PSMMs in their fully open states (induced by cDNA). By setting the fluorescence intensity when azobenzene takes the trans-form as a baseline (0%) and the intensity after addition of excess amount of cDNA as 100%, the number of PSMMs in the open state was calculated for each photoregulation process. In these calculations, the fluorescence intensity in closed state at 488nm was used as the baseline (blue curves) and the fluorescence intensity for the fully open state (green curves) as 100%. The fluorescence recovery parameter, Recovery (%), based on the three states was calculated in order to evaluate the closed-open conversion efficiency:

\[
\text{Recovery} \ (%) = \frac{I_{uv} - I_o}{I_t - I_o} \tag{Eq. 4-3}
\]

\(I_{uv}\) is the fluorescence intensity of DNA solution after UV irradiation; \(I_o\) is the fluorescence intensity after visible irradiation; \(I_t\) is the fluorescence intensity of DNA solution after adding extra cDNA. The higher the recovery value, the higher the number of molecules in open state were driven by photonic energy, and the better the efficiency of the conversion from closed to open state. Since the reversible open-to-closed conversion step is very fast for each of the PSMMs, the Recovery (%) for the closed-to-open conversion was used to evaluate the efficiency.

The Recovery (%) was used to compare the efficiency of energy conversion from photonic energy to molecular motion for PSMM1-3 under the same conditions. The closed-to-open conversions were approximately 14.2%, 26.3% and 54.7% for PSMM 1-3, respectively. This result supported the assumption that multiple azobenzene incorporation will introduce a higher impact on hairpin structure stability or photoregulation capability. The improvement of this Recovery (%) is, however, not proportional to the number of Azo-moieties, although it does
gradually increase as the number of Azo- increases. Specifically, the triple Azo- nanomotor (PSMM3) displayed a much higher open-to-closed ratio than PSMM1 and PSMM2, which supports this argument. Furthermore, the result is consistent with previous studies of the relationship between Azo- moiety and duplex association/dissociation conversion on linear DNAs. Because the energy barrier of azobenzene isomerization from trans- to cis- is higher than cis- to trans-, the trans- to cis- conversion requires a longer UV light irradiation time to drive this conversion. The results demonstrate that Recovery (%) can reach about 60% (±3%) after 20 minutes of UV irradiation before photobleaching of FAM fluorophore appears to become a serious problem. Thus, for realistic usage which balances input and output energy, a UV irradiation time from 2 to 10 minutes is satisfactory for the PSMM operating in numerous cycles without losing apparent functionality and efficiency.

Theoretical calculation of the extension and contraction forces is based on the free energy of hybridization and extending capability of single- and double-stranded DNA. We estimated the force based on Gibes free energy and the distance variation of the closed (L₁) and open (L₂) structures. The L₁ and L₂ were theoretically calculated by chemical bonds and persistent length, which results with approximately 10.2 and 2.2nm, and give the estimated values of two forces of 1.5 and 3.1pN (the average forces based on the irradiation timeline), respectively. Noticeably, single-stranded DNA has the tendency to form random coils in solution instead of an extended structure.

The total input photon energy on extending the nanomotor can be calculated by UV lamp power (0.197mW) and irradiation time (5 minutes) with:

\[ E_{\text{input}} = P \times t = 5.91 \times 10^{-2} \text{J} \quad \text{(Eq. 4-4)} \]
Based on our previously calculated extension force (1.5pN) and distance (8nm), each nanomotor has the extension work of $1.2 \times 10^{-20}$ J ($w_{output} = F_s$). We can regard the extension work as the output mechanical energy. Therefore, the total output work $W_{output}$ for each type of nanomotor under our conditions can be calculated by:

$$W_{output} = [(\text{Extended molecule})\%] \times [\text{Total molecule number}] \times [w_{output}] \quad \text{(Eq. 4-5)}$$

where the total molecular number is $7.22 \times 10^{12}$ (100 nM $\times$ 120μL $\times$ $N_A$). From the equation, total output works for PSMM 1-3 are 14.2%, $1.23 \times 10^{-8}$ J; 26.3%, $2.28 \times 10^{-8}$J, and 54.7%, $4.74 \times 10^{-8}$J, respectively (the front numbers are the percentage of extended molecules based on recovery). Accordingly, the energy conversion efficiencies ($W_{output} / E_{input}$) are: $2.09 \times 10^{-7}$, $3.85 \times 10^{-7}$ and $8.02 \times 10^{-7}$, respectively.

**Reversibility**

Figure 4-7 shows the reversibility of the PSMM3 nanomotor for ten rounds of closed-open cycles. For each cycle, 1 minute visible irradiation and 3 minutes of UV irradiation were applied. Compared to an approximately 40% decrease in cycling recovery for the previous DNA-fueled nanomachines under more favorable conditions (higher temperatures, higher number of Azo-), the cycling of PSMM3 maintains its recovery consistency, which shows no tendency to decrease after ten cycles. Additionally, all the cycles were performed at room temperature (25ºC) where the closed state is more favored. Thus, the conversion efficiency should be further improved at higher temperatures (close to $T_m$), where the stem is close to the transition point from duplex to single-strand state.

These results demonstrate that the hairpin nanomotor possesses high close-open conversion efficiency and molecular stability under the current operating conditions. Repeated nanomotor cycles displayed no obvious decomposition of the motor for up to 20 cycles. Overall, these
results demonstrate a long-lasting molecular motor with high conversion efficiency using a clean energy input.

**Energy Conversion Efficiency: Comparison with Linear DNAs**

A comparably high fluorescence recovery seems very interesting from the perspective of energy application, since it is related to energy conversion efficiency. That is, under the same operating conditions for similar molecular nanomotors labeled with F/Q, a higher fluorescence variation illustrates that higher ratios of molecules are driven from one state to another state. This comparison is applicable to hairpin structures as well as linear DNA strands as long as conditions are normalize and parameters are correlated. In this case, the previously defined Recovery percentage can be used as an indicator of energy conversion efficiency. Under these conditions, a higher Recovery percentage means that comparable DNA motor systems can convert more absorbed photon energy to drive the structure changes. This efficiency can therefore be related to the capability of fulfilling motor-like function. Besides the composition of the molecules, the structures of different molecular may play a role in absorbing photon energy under the same conditions, we can regarded it as a part in contributing to the overall energy conversion capability for specific molecules.

In order to compare the conversion efficiency of our hairpin single-molecule nanomotors to that of previously investigated linear DNAs, a series linear DNAs, as well as another hairpin structure were designed, and the melting temperature \( T_m \) was used as the correlated parameter for comparison. The definition of \( T_m \) for short DNA duplex can be expressed by:

\[
T_m (^\circ C) = \frac{\Delta H^\circ}{\Delta S^\circ + R \ln C_{DNA}} - 273.15
\]  

(Eq. 4-6)

where \( \Delta H^\circ \) and \( \Delta S^\circ \) are the melting parameters, \( R \) is the ideal gas constant, and \( C_{DNA} \) is the molar concentration. For DNA sequences with the same concentrations, \( C_{DNA} \) is the same, while
ΔS° varies slightly. Therefore, T_m is approximately proportional to ΔH°, which is the energy absorbed by the DNA molecule to dissociate into single strand. Therefore, T_m value can be regarded as the capability of absorbing sufficient energy to dissociate DNA duplex structures. For DNA nanomotors involved in duplex dissociation, T_m can be used as the standard to evaluate the structural conversion (i.e., motor operation) achieved by absorbing external energy. Different duplex structures, such as hairpin and linear duplex, with the same T_m value are expected to display the same Recovery percentage under the same photoregulation conditions.

Two groups of linear DNAs bearing Azo- and Dabcyl and their cDNA bearing FAM have been synthesized as shown in Figure 4-8: L12-1, L12-2 and L12-3/L12-cDNA; L10-1 and L10-2/L10-cDNA. The sequences of linear DNAs were designed in a manner similar to the truncation of the hairpin DNA from the 3’ end with the insertion of 0, 1, or 2 extra Azo-, and have a T_m comparable to that of PSMM1-3 (Table 4-1). For L12-1 to L12-3, all three linear DNAs have almost the same T_m (53.7–55.2°C) as PSMMs (55–57°C). Therefore, they should be able to dissociate the duplex structure with the same recovery as PSMMs under the same conditions. L10-1 and L10-2 were intentionally selected with much lower T_m (47.5–48.6°C) than PSMMs. Thus, L10-1 and L10-2 should show higher Recovery (%).

The fluorescence spectra of all the linear DNAs under the same conditions as PSMM are displayed in Figure 4-9, and the Recovery (%)s are summarized in Table 4-1. Under the same conditions, the recovery is from 14.2 to 54.7% for PSMM motors compared to 2.9-11.5% for L12 DNAs and 6.4% to 13.8% for L10 DNAs. These findings indicated that neither linear DNAs with similar thermal response (L12s), nor those with thermal response at lower temperatures (L10) have the same response to light energy as PSMMs. If it is assumed these linear DNAs can do the same work as PSMM (actually less than PSMM due to smaller size changes), the energy
conversion efficiencies based on Recovery percentage values for L12 DNAs (L12-1, L12-2, L12-3) are $4.26 \times 10^{-8}$, $8.38 \times 10^{-8}$ and $1.69 \times 10^{-7}$, respectively, and for L10 DNAs (L10-1, L10-2) are $9.26 \times 10^{-8}$, and $2.03 \times 10^{-7}$, respectively. Linear DNAs cannot produce a comparable recovery by absorbing light energy from the same input energy as PSMM3, and they display a lower photon to mechanical energy conversion efficiency. Moreover, the hairpin structure has the same number or fewer Azo-moieties tethered on the backbone compared to linear DNAs. The PSMM3 should theoretically absorb fewer photons per molecule than L12 DNAs, but PSMM3 displays a high Recovery percentage. These results strongly validate the expectation that the PSMMs have much higher energy conversion efficiency than linear DNA structures and that this directly results from the hairpin structure. These efficient hairpin-structured nanomotors are operated under mild conditions of room temperature, illustrating that improvement of recovery can be further achieved by increasing the operational temperature. Although the recovery for PSMMs at temperatures closer to their $T_m$ was not systematically examined, higher conversion recoveries were observed when the operational temperature was increased.

The fluorescence recoveries of the L12 DNAs, when compared with the PSMMs, demonstrate that the PSMMs have much higher efficiency response to photon energy. Since both PSMMs and linear DNAs have the same nucleotide sequence and Azo-components, the main factor contributing to their variable efficiencies is the difference between their respective structures. Specifically, the structure of the PSMM is folded hairpin, in which the loop moiety affects the stability of stem duplex, while linear DNAs have an extended duplex structure which is only affected by strand exchange. The short stem duplex of PSMM3 with triple Azo-incorporation is highly sensitive to conformational changes of Azo-isomerization, resulting in a 54.7% change in conformation. In contrast, the linear DNAs seem to unzip only from the end
incorporated with multiple Azo-, resulting in partial dehybridization (2.9% for L12-1).

Moreover, even with the saturated Azo- loading of linear DNAs (L12-3), the duplex dissociation is still very low (11.5%) at room temperature. Therefore, based on the mechanism of fluorescence variation, there are fewer linear DNA duplexes dehybridized under these experimental conditions compared to PSMM molecules. Also, the low efficiency of linear DNAs is a function of the T_mS of both the trans- and cis- conformations, which are much higher than room temperature T_(RT): T_m(trans-) > T_m(cis-) > T(RT), whereas the T_mS of PSMMs, when azobenzene takes cis- conformations, are significantly lowered and are below room temperature: T_m(trans-) > T_(RT) ≥ T_m(cis-). This result demonstrates that hairpin-based nanomotors are more energy efficient than motors based on linear DNAs.

**Energy Conversion Efficiency Comparison with PolyT Loop DNAs**

While conventional DNA nanomotors involve only linear DNAs with single strand and duplex structures, PSMMs have a hairpin structure on the loop moiety that amplifies the impact of external stimuli (in this case, isomerization of azobenzene) on the open-closed conversion. The hairpin structure can stabilize the stem duplex for comparable T_m with shorter base pairs than linear DNAs, with and without azobenzene moieties. To further examine the impact of the special hairpin structure on nanomotor efficiency, a similar hairpin structure, PolyT(A3), was designed for comparison. The PolyT(A3) has 31 bases with the same stem duplex as PSMM3, but only T bases in the loop moiety: 5’ FAM-CCT AGC TTT TTT TTT TTT TTT TTT T-Azo-GC-Azo-TA-Azo-G G-Dabcyl 3’ (underlined bases represent stem moieties). Three Azo-moieties were incorporated at the same positions as those in PSMM3. The photoregulation of this nanomotor also displayed high efficiency and photoreversibility as shown in Figure 4-10. Moreover, the PolyT(A3) nanomotor had an average efficiency of 38.9% with at least five cycles
of UV/Vis irradiation. As opposed to the structure of linear DNAs, these results illustrate that molecular motors based on hairpin structures display higher conversion efficiencies. At the same time, their stability is not affected. The PolyT(A3), which has a T base loop, has a tendency to form a regular and symmetric structure, while PSMM3 molecules have a specific loop structure because of their asymmetric base sequences. Nevertheless, both the PolyT(A3)- and PSMM3-based nanomotors displayed high nanomotor efficiency, giving conclusive evidence that the hairpin structure enables DNA nanomotors to display highly efficient conversion.

**Conclusion**

Molecular interactions are believed to play a key role in the significance of PSMMs. First, for linear DNA nanomotor systems, at least two pieces of DNA are needed to trigger a motor movement. Therefore, the hybridization process is based on an intermolecular interaction, and the concentration of each DNA strand contributes to the overall hybridization rate and, hence, the motor efficiency. For PSMMs, there is only one DNA strand that functions as a single-molecule motor. Thus, the hybridization within the PSMMs takes place by intramolecular interaction. The photoregulation of PSMM3 was compared to that of the ten-base linear DNAs (L10-1) at different concentrations. As shown in Table 4-2, the constant distance of paired hybridizing moieties within a single hairpin-structured molecule produces a type of concentration-independent nanomotor which maintains high conversion efficiency (from 54.7 to 44.7% on recovery), while, on the other hand, the linear L10-1 displays a significant concentration-dependence Recovery (%) (6.3 to 0.7%). Therefore, the hairpin nanomotors are expected to overcome the low efficiency problem experienced by linear DNA nanomotors, especially in a situation where high density DNA motor packing is required.
In conclusion, we have designed a photoswitchable single-molecule DNA nanomotor, the first fully reversible single-molecule DNA nanomachine driven by photons without any additional DNA strands as fuel. The incorporation of photosensitive Azo- moieties successfully produces a reversible photoregulated DNA nanomotor. This clean, photon-fueled nanomotor holds promise for applications that require the conversion of photonic energy into other forms of energy, such as mechanical movement. In addition, photoregulated DNA nanomotors can be easily manipulated and reproduced, as the design is simple and straightforward in terms of synthesis and operational strategy. Furthermore, PSMM is a novel single-molecule nanomotor system, which does not require several DNA strands for operation. Thus, the intramolecular interaction that occurs in this system circumvents the complexity introduced by intermolecular reactions needed in previous DNA nanomotors. Compared to concentration-dependent linear DNAs, the concentration-independence of the PSMM allows high efficiency at any concentrations and in situations that would otherwise be difficult to achieve as a result of interference by multi-molecular interactions. Continued investigation of the relationship between hairpin structure and energy conversion efficiency will further elucidate the mechanisms underlying the energy conversion process.
Table 4-1. Comparison of all DNA sequences with and without Azo- incorporation

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>base number</th>
<th>Azo- number</th>
<th>Azo-/base ratio (%)</th>
<th>Tm (°C) ±0.5°C</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>57.5</td>
<td>N/A</td>
</tr>
<tr>
<td>PSMM1</td>
<td>31</td>
<td>1</td>
<td>3.2</td>
<td>57.0</td>
<td>14.2 ±2.5</td>
</tr>
<tr>
<td>PSMM2</td>
<td>31</td>
<td>2</td>
<td>6.5</td>
<td>56.3</td>
<td>26.3 ±3.3</td>
</tr>
<tr>
<td>PSMM3</td>
<td>31</td>
<td>3</td>
<td>9.7</td>
<td>55.0</td>
<td>54.7 ±3.1</td>
</tr>
<tr>
<td>PolyT</td>
<td>31</td>
<td>3</td>
<td>9.7</td>
<td>54.9</td>
<td>31.3 ±2.5</td>
</tr>
<tr>
<td>L12-1</td>
<td>12</td>
<td>3</td>
<td>25</td>
<td>55.2</td>
<td>2.9 ±0.8</td>
</tr>
<tr>
<td>L12-2</td>
<td>12</td>
<td>4</td>
<td>33.3</td>
<td>54.8</td>
<td>5.7 ±1.1</td>
</tr>
<tr>
<td>L12-3</td>
<td>12</td>
<td>5</td>
<td>41.7</td>
<td>53.7</td>
<td>11.5 ±2.3</td>
</tr>
<tr>
<td>L10-1</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>47.5</td>
<td>6.3 ±1.6</td>
</tr>
<tr>
<td>L10-2</td>
<td>10</td>
<td>4</td>
<td>40</td>
<td>48.6</td>
<td>13.8 ±2.2</td>
</tr>
</tbody>
</table>

Table 4-2. Conversion efficiencies of PSMM3 and L10-1 at different concentrations

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Conversion efficiency by Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100nM</td>
</tr>
<tr>
<td>PSMM3</td>
<td>54.7 ±3.1</td>
</tr>
<tr>
<td>L10-1</td>
<td>6.3 ±1.3</td>
</tr>
</tbody>
</table>
Figure 4-1. Scheme of photoswitchable single-molecule DNA motor (PSMM) with three azobenzene moieties inserted. The PSMM exists in a contracted state when azobenzene is in the *trans*- conformation under visible light irradiation, and an extended state when azobenzene is in the *cis*- conformation under UV light irradiation.
Figure 4-2. Scheme of available positions for Azo- incorporation in the stem region.

Figure 4-3. The sequences of the six types of PSMM (PSMMs 1-6). PSMM1, PSMM2, and PSMM3 (or named PSMM1-3 for all three types) are hairpin structures with one to three Azo- on the 3’ end, and PSMM4, PSMM5, and PSMM6 (or named PSMM4-6 for all three types) are hairpin structures with one to three Azo- on the 5’ end. The blue bases are stem moieties; red are Azo- units, and black bases are on loop moieties.
Figure 4-4. Melting temperature profiles of PSMM1-6 under the same conditions (The melting temperatures of PSMM1-3 are summarized in Table S1). The measurements were repeated twice for each sample. The concentration was 100nM in buffer solution (buffer: 20mM Tris buffer pH8.0, 20mM Na\(^+\), 2mM Mg\(^{2+}\)).
Figure 4-5. Suggested mechanisms of photoresponse of PSMM1-3 and PSMM4-6. Pretreatment of visible light ensured the hairpin structure formation and the lowest fluorescence background at the starting point.
Figure 4-6. Fluorescence spectra of PSMM1-3 (λ<sub>ex</sub> = 488nm) from top to bottom after irradiations. UV light: 6W UV lamp (350nm), visible light: 60W desktop lamp with 450nm filter; the temperature is at 25°C; Red curve: 100nM DNA in buffer after visible irradiation (450nm), 1 minute; Green curve: 100nM DNA in buffer after UV irradiation (350nm, 5 minutes); Blue curve: 100nm DNA in buffer after irradiations, and then 5-fold cDNA is added (buffer: 20mM Tris buffer pH8.0, 20mM Na<sup>+</sup>, 2mM Mg<sup>2+</sup>).
Figure 4-7. Closed-to-open cycling by repeated visible and UV irradiations at 25°C. Vis (450 nm): 1 min; UV (350 nm): 3 min. Fluorescence intensities at the maximum emission (525 nm) were recorded immediately after each irradiation ([PSMM3] = 100nM, [cDNA] = 500nM, buffer: 20mM Tris buffer pH8.0, 20mM Na+*, 2mM Mg2+).

Figure 4-8. The sequences of (a) 12-base Azo- incorporated linear sequences and cDNA; (b) 10-base Azo- incorporated linear sequences and cDNA
Figure 4-9. Fluorescence spectra of Linear DNAs (A) L12-1, L12-2 and L12-3 from top to bottom, respectively; and (B) L10-1 and L10-2, from top to bottom, respectively. $\lambda_{ex}$= 488nm; UV light: 6W UV lamp (350nm), visible light: 60W desktop lamp with 450nm filter; the temperature is at 25°C; Red curve: 100nM DNA in buffer after visible irradiation (450nm), 1 minute; Green curve: 100nM DNA in buffer after UV irradiation (350nm, 5minutes); Blue curve: 100nm DNA in buffer after irradiations, and then 5-fold cDNA is added (buffer: 20mM Tris buffer pH8.0, 20mM Na+, 2mM Mg$^{2+}$).
Figure 4-9. Continued
Figure 4-10. Fluorescence spectra of PolyT(A3); $\lambda_{ex}= 488$nm; UV light: 6W UV lamp (350nm), visible light: 60W desktop lamp with 450nm filter; the temperature is at 25°C; Red curve: 100nM DNA in buffer after visible irradiation (450nm), 1 minute; Green curve: 100nM DNA in buffer after UV irradiation (350nm, 5 minutes); Blue curve: 100nm DNA in buffer after irradiations, and then 5-fold cDNA is added (buffer: 20mM Tris buffer pH8.0, 20mM Na$^+$, 2mM Mg$^{2+}$).
CHAPTER 5
SUMMARY AND FUTURE WORK

Summary of the Molecular Engineering of Nucleic Acids

Deoxyribonucleic acid (DNA) is not only the key means of genetic expression, but also is a critical component of many molecular biosensors and biomaterials. Numerous researchers have taken advantage of DNA’s adaptability to develop probes specifically targeting a wide range of candidate biomolecules. This has led to the design of various DNA based detection and signal amplification probes, such as aptamers and molecular beacons (MBs). Additionally, DNA self-assembly (based on spontaneous hybridization between complementary strands) is an effective way to construct multidimensional nano-objects. Further, DNA-based nanomachines have been artificially constructed with the ability to change their conformation upon external stimulus and produce mechanical motion. Recently, DNA based biomaterials were investigated and introduced to fabricate smart materials for tissue engineering and drug delivery.

This research has focused on molecular engineering utilizing DNA towards functional and smart materials with three key projects: 1) the design and construction of liposome-based nanostructures as pharmaceutical carriers for potential therapeutic applications; 2) the development of polymer-DNA hybrid hydrogels for the photocontrollable release of loaded pharmaceuticals and tissue engineering; and 3) the design and characterization of single-strand, hairpin-structured DNA molecules as molecular nanomotors. Recently, we further developed the hydrogel material into a nanogel particle, which can absorb NIR light for gel-sol conversion and release loads.

A Liposome-based Nanostructure for Aptamer-Directed Delivery

Many new candidate drugs display low solubility in biological environments. Therefore, effective delivery requires a solubilizing and specific delivery system to provide sufficient drug
availability and facilitate clinical research. In recent years, liposomes have been recognized as drug delivery vehicles with controlled drug release ability, large loading capacity, high biocompatibility, and suitable solubility, thus, replacing otherwise poorly soluble and highly toxic drugs. By conjugation with the sgc8 aptamer (which has high binding affinity with target leukemia CEM-CCRF cells), we created a therapeutic liposome drug delivery system. We investigated the preparation and characterization of an aptamer sgc8-liposome platform, and we demonstrated the principle of targeted delivery of FITC-Dextran (FD) into cells. Under mild conditions, the results demonstrated that our designed drug delivery system could specifically and rapidly bind with target cells and release the loaded model drug. Due to the abundance of aptamer candidates and their stable chemical properties, aptamer-liposome conjugates may provide an important model for targeted drug delivery with high efficiency and excellent selectivity.

**DNA Crosslinked Hydrogels for Photocontrollable Release**

Hydrogels are hydrophilic materials composed of polymer skeletons and crosslinkers. They are able to offer controllable encapsulation and release of pharmaceuticals for drug delivery. We created a DNA crosslinked hydrogel using DNA polymer conjugates and a DNA linker, resulting in a photocontrollable assembly that displays reversible sol-gel conversion. The conversion occurs because photosensitive azobenzene moieties, which are incorporated into the DNA sequences, respond to different light sources. Consequently, azobenzene isomerization induced by visible and UV light can either destabilize or stabilize the duplex structure, resulting in a sol or gel state, respectively. As a result, the sol-gel transition can be achieved and the process is completely reversible. Accordingly, three different materials (fluorescein, horseradish peroxidase, and gold nanoparticles) were encapsulated inside the gel and controllably released by
light irradiation. The ability to use clean and localized light for such controllable release of multiple loads provides a promising stimulus-sensing biomaterial for drug delivery. Moreover, we also intend to encapsulate living cells inside the hydrogel for controllable growth and regeneration, with the goal of achieving advanced tissue engineering.

A Single-Component DNA Nanomotor Regulated by Photons

In our previous study in *Nano Letters* (2002, 2 (4), 315-318), we reported the design of a single molecular nanomotor driven by complementary strands (fuel). Recently a novel single molecule nanomotor was redesigned to be driven by photon energy instead of DNA fuel. It is a hairpin-structured, single-strand DNA molecule, which is incorporated with azobenzene moieties to facilitate reversible photoswitching. Upon repeated UV/Vis irradiation, this nanomotor displayed 40-50% open-closed energy conversion efficiency. This type of nanomotor displays well-regulated responses and can be operated under mild conditions with no output of waste. In contrast to multiple-component DNA nanomachines, the intramolecular interaction in this single-molecule system offers unique concentration-independent motor functionality. Moreover, the hairpin structure of the motor’s backbone can significantly improve the efficiency of light-to-movement energy conversion with high efficiency. These results suggest that azobenzene-incorporated, hairpin-structured, single-molecule DNA nanomotors have promising potential for applications which require highly efficient light-driven molecular motors.

In summary, this research has focused on investigating, designing, and constructing nanoscale multifunctional structures and biomaterials for biomedical and bioengineering applications. The current outcomes have successfully demonstrated the basic concepts of those objectives. Promising applications can be foreseen based on current results, such as implementation of liposomes and nanogels to carry anti-cancer drugs or imaging reagents for
target cancer cells \textit{in vivo}. Future work will involve building highly regulated DNA nanomotors, which can convert solar energy to mechanical energy to enable the construction of complex nanomachines. In addition, we are using our DNA-based hydrogels to build “smart” biomaterials which will be highly controllable and functional. With our approaches, DNA based chemotherapy and artificial tissues are being developed for clinical trials.

**Future Work: NIR Light Controlled DNA Crosslinked Hydrogels for Drug Release**

Currently, we are developing a photocontrollable nanogel based on our DNA crosslinked hydrogels as shown in Figure 5-1. Au-Ag nanorods with the size of approximately 250 nm have been successfully coated with DNA-polymer hydrogel. Aptamer moieties were conjugated to the surface of the hydrogel nanostructure to permit the entire particle to recognize and bind to the target cells. By using an NIR laser to irradiate the solution after the binding process, the loaded drug molecules were released to the local area around the target cells and inhibited cell viability. This nanogel delivery platform was successfully produced and is therefore being tested for chemotherapy on target cells. Furthermore, we are working to engineer and optimize this system with multiple functions not only to carry and deliver drug molecules, but also to encapsulate genes or enzymes for targeted delivery.
Figure 5-1. NIR photocontrollable nanogels.


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BIOGRAPHICAL SKETCH

Huaizhi Kang was born in Beijing, China in 1975. He spent his childhood mainly in Hefei, Anhui. He obtained his bachelor’s degree in polymer physics from University of Science and Technology of China in 1999. Inspired by the environment of the school and his family, he decided to go abroad to continue his research career. He entered Bowling Green State University at the same year. In 2002, he earned a master’s degree in photochemical science under the supervision of Dr. Felix N. Castellano. After a short period of graduate study from 2002 to 2003 at University of Michigan in medicinal chemistry, Huaizhi returned to China, where he worked as a senior consultant on education and technology in a private company. In 2005, he decided to continue his graduate studies and pursue a Ph.D. degree, and joined Dr. Weihong Tan’s group at the University of Florida. Huaizhi’s research interests are the applications of nanoscience and nanotechnology, especially on molecular engineering of oligonucleotides for drug delivery, biomaterials and functional DNA structures. After four and a half years, Huaizhi Kang completed his Doctor of Philosophy in analytical chemistry in December 2009.