A General Platform for Aptamer Mediated Capture of Specific Targets

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By

JIE XU, B. ENG

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree

Master of Applied Science

McMaster University

MASTER OF APPLIED SCIENCE (2008) **McMaster University**

(Chemical Engineering) **Hamilton, Ontario**

TITLE: A General Platform for Aptamer Mediated Capture of Specific Targets

AUTHOR: JIE XU

SUPERVISORS: Professor Carlos Filipe

Professor Robert H. Pelton

NUMBER OF PAGES: x, 48

Abstract

The purpose of this research is to develop a general method for capturing and separating specific targets. Nucleic acid aptamers are short sequences of single-stranded DNA or RNA which have the ability to bind to the small organic or inorganic molecules such as protein and metal ions with high binding affinity. In this study, a bioconjugate re-usable system was developed. It can reversibly load or unload DNA aptamers.

To allow separation, a thermally responsive polymer (N-isopropylacrylamide, PNIPAM) is used. This polymer can undergo a reversible phase transition upon adding NaCl and/or increasing temperature. A short sequence of single stranded DNA (ssDNA) was coupled to PNIPAM. The ssDNA will experience a reversible phase transition because of the PNIPAM.

The DNA sequence for an aptamer can be extended to contain a sequence that is complementary to that of the ssDNA coupled to PNIPAM. Adding this extended aptamer to the conjugate will result in spontaneous hybridization of the two strands of DNA. These strands can be separated using an agent (e.g. urea) that destroys hydrogen bonding. The conjugate can be recovered using a reversible inverse phase transition.

The same PNIPAM-ssDNA conjugate can be used reversibly for coupling different aptamers. The aptamers did not lose their binding ability when coupled with PNIPAM-ssDNA conjugates. In the process of precipitation separation targets, the

PNIPAM-ssDNA conjugate showed little loss with applied phase transition. Moreover, the coupling efficiency of the ssDNA to PNIPAM conjugates was determined. The binding ability of the ATP aptamers to ATP was also investigated.

Acknowledgements

During the study of my Master's program, I received a great deal of help from many people. I use this opportunity to express my sincere heartfelt thanks to them. My foremost gratitude goes to my supervisors, Dr. Carlos Filipe and Dr. Robert Pelton. I would like to express my deepest appreciation to them. They offered me an inestimable amount of invaluable suggestions, guidance, encouragement and help. Their kindness and patience are greatly appreciated. My sincere thanks also goes to Dr. Yingfu Li for allowing me to use the equipment in his lab to purify DNA samples, to Dr. Xin Ge and Dr. Shunxing Su for their valuable suggestions and discussions, and to Dr. Md. Monsur Ali and Dr. Weian Zhao for their kindness and help.

I received much help from my classmates, both inside and outside my group, in the Chemical Engineering Department. I would like to say thank you to them for their kindness and friendship. I am also grateful to Mr. Doug Keller, Ms. Frances Lima, Mr.James Lei, Ms. Kathy Goodram, and Ms. Andrea Vickers for their help.

Finally, I extend my deep gratitude to my parents and brother for their encouragement and support.

Jie Xu

June.28th, 2008

Hamilton, Ontario

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

For about 40 years, affinity chromatography, invented by Pedro Cuatrecasas and Meir Wilchek[1], has been a widely used technique for separating and purifying specific molecules from complex mixtures. One type of reaction of biological systems is the specific and reversible interaction such as an antibody and an antigen or an enzyme and a substrate. These interactions have been used as the media for affinity chromatography methods which have been utilized to reversibly bind molecules[2]. Compared to antibodies, aptamers have become more and more popular in medical and analytical chemistry applications, because of their many advantages[3].

1.1 DNA aptamer

Nucleic acid aptamers are short sequences of single-stranded DNA or RNA which have the ability to bind to the small target organic or inorganic molecules such as protein, metal ions with high binding affinity[4]. When introducing specific target molecules, aptamers rearrange their original structures to fold to tertiary structures. Aptamers are produced by chemically synthesizing and screening large DNA or RNA molecules by a library technique known as the SELEX (systematic evolution of ligands by exponential enrichment) method[4,5].

Aptamers have advantages over antibodies. They can be more easily produced and applied in vitro experiments rather than in animal bodies in a number of ways.

First, aptamers can be reused multiple times without loss of activity, whereas antibodies are unstable for reversible use. Secondly, aptamers are commercially more profitable than antibodies since antibodies are obtained from animal bodies whose immune systems are defending themselves against foreign attack. In contrast, aptamers can be produced by chemical synthesis. Thirdly, it is easy to modify aptamers with functional groups. However antibodies may lose their biological activity when modified by a functional group. The most attractive application of aptamers is to develop functional aptamers as sensor molecules. Their most frequent use is as molecular recognition tools, while labeled with fluorescent groups.

Romy Kirby, Andrew D. Ellington[6] and co-workers developed an anti-lysozyme aptamer conjugated with streptavidin agarose beads to separate and quantify lysozyme protein[6]. In their research, a detection system using an electronic tongue was applied to measure fluorescent intensity of Cy3-labeled lysozyme bound to anti-lysozyme aptamer conjugated with streptavidin agarose beads. Compare to the analytical applications of antibodies, the advantage of Ellington's method lies in its aptamer usage. In screening aptamer experiments, they used nine selected different anti-lysozyme aptamer sequences, modified as biotinylated aptamers, to conjugate streptavidin agarose beads. After introducing Cy3-labeled lysozyme to the conjugates, they observed that the two anti-lysozyme aptamer sequences 1 and 6 have high binding ability with the fluorescein labeled lysozyme. However, this detection method must be applied by screening the best aptamer, which has a high binding ability with its targets. In the research described by Kirby et al. (2004), the affinity of the aptamer

on the beads was found to be lower than the affinity of that same aptamer when present in solution. A likely reason for this difference may be mass transfer limitations associated with transport of the targets through the boundary layer surrounding the particles. So PNIPAM is used to avoid this bounding limitation in this work. Thus, the use of beads is not a good general platform for separating and purifying specific targets. A more effective way is to build conjugates between stimuli-responsive polymers PNIPAM and biomolecules instead of streptavidin agarose beads. Stimuli-responsive polymers, when conjugated to biological activity molecules, can induce reversible phase separation due to changing environmental conditions, such as pH, ionic strength or temperature[7].

1.2 Stimuli-responsive polymers

Recently, there have been some interesting developments with affinity chromatography using bioconjugates between stimuli-responsive polymers and biomolecules, so as to carry out separation and purification by specific binding to biomolecules and applying polymer phase transition[8]. Stimuli-responsive polymers can change reversibly from a liquid phase to a solid phase as a response to different environmental conditions[9]. This phenomenon can be observed by the swelling or shrinking of the gels[10]. Stimuli-responsive bioconjugates have been applied in the research areas of medicine pharmacology and biotechnology[11]. Bioconjugates have

been used to gradually or suddenly release drugs at particular locations in the human body, as a response to small changes of pH or temperature[12].

The interest of this research is centrered on poly (N-isopropylacrylamide) PNIPAM. PNIPAM is a water-soluble microgel, and undergoes thermally induced shrinking when the temperature of the solution is increased above the LCST (lower critical solution temperature), i.e. 32 °C in pure water[13,14]. When PNIPAM is dissolved in pure water, hydrogen bonds are formed between pure water molecules and the amide groups of the PNIPAM at room temperature. If the temperature of the solution is increased, the hydrogen bond is disrupted and the chain collapses[14]. The PNIPAM state changes from a hydrophilic phase to a hydrophobic phase. The PNIPAM microgel particle network is balanced in organic solvents by elastic forces from cross-links and osmosis force. When these two forces are equal the swelling behavior is equilibrium[15,16,17]. Furthermore, the LCST of PNIPAM can be influenced by salt concentration and pH in the solvent. It will be decreased by addition of high sodium chloride concentration in the solution[18]. In weak acid or base, or low concentration sodium chloride solutions or pure water, hydrogen bonds exist linking the water molecules and the amide groups of the PNIPAM hydrogel network. Thus the PNIPAM hydrogel network is in equilibrium in solution. Thus it cannot swell or shrink in this state. However, increasing the acid or base strength of the solution or sodium chloride concentration, the hydrogen bonds are broken and the network is partly ionized. So the interaction between the network and the water molecules causes the PNIPAM network to swell[19]. Recently, stimuli-responsive PNIPAM has been

considered to be one of the most important polymers in medical applications[19].

1.3 Phase separating polymer bioconjugate design

1.3.1 Polymer oligonucleotide conjugates

A series of papers has presented the development of streptavidin-polymer conjugates[20,21,22]. It is well-known that biotin-streptavidin is a high binding affinity system. The utilization of a biotin-streptavidin system based on the streptavidin-polymer conjugates applied in affinity separating biotin was presented in a number of recent scientific publications. Fong et al. (1999) introduced a method for covalently linking streptavidin to oligonucleotides. They also developed another conjugate by conjugating the complementary oligonucleotides on a linear ester-activated PNIPAM. After hybridizing the complementary DNA sequences in the two conjugates, the streptavidin-PNIPAM complex was formed. This complex bears the affinity precipitation of radiolabeled biotin and phase separation because of the streptavidin and PNIPAM present.

The reversible phase separation takes place in the complex when the environmental temperature is changed. The PNIPAM-oligonucleotide conjugates can be reused by denaturing double stranded DNA sequences and then precipitating the polymer and streptavidin complexes. After phase transition, the PNIPAM-oligonucleotide conjugates were isolated and could be recycled.

Ding et al. (1999) reported that PNIPAM-streptavidin conjugates can reversibly block biotin binding because of the temperature stimulated PNIPAM[23,24].

Takeshi Mori et al. (2001) developed a novel sequence-specific precipitation separation system which is conjugated using PNIPAM and oligonucleotides[25,26]. The PNIPAM- oligonucleotide conjugates have the attractive properties of both the phase transition of PNIPAM and the hybridization functions of oligonucleotide. They prepared the conjugate using PNIPAM and vinyl-$(dT)_8$ which is obtained from the coupling reaction of amino-$(dT)_8$ and *N*-methacryloyloxysuccinimide.

The PNIAPM-$(dT)_8$ conjugate was coupled with $(dA)_8$ by the complimentary DNA hybridization. This conjugate can precipitate $(dA)_8$ because of the phase separation of PNIPAM caused by increasing the salt concentration in the solution to 1.5 M. They also used the PNIAPM-$(dT)_8$ conjugates to precipitate different lengths of the oligonucleotide (dA)s chain. They found that it was more efficient to precipitate the longer (dA)s chain than the short (dA)s chain. They also demonstrated that the PNIAPM-$(dT)_8$ conjugates can be recycled by precipitation, with almost no loss of precipitation efficiency.

To allow reusability, they used PNIAPM-$(dT)_8$ conjugates. Denaturation was applied in double-stranded DNA between the oligonuleotied $(dA)_8$ and $(dT)_8$. They incubated the PNIAPM-$(dT)_8$/(dA)s complex solution in deionized water at 40 °C, and then centrifuged to separate the PNIAPM-$(dT)_8$ conjugates. The conjugate obtained could be used for precipitating (dA)s again. This process can be applied repeatedly.

From the literature reviewing responsive polymer bioconjugates the functioning of therapeutic biomolecules will be controlled if the stimuli-responsive polymer is attached to the biomolecules. In medical research, the most significant characteristic of the stimuli-responsive polymer-biomolecule conjugates is that the biomolecules maintain their biological activities as long as they are attached to a stimuli-responsive polymer[27,28,29].

1.3.2 Polymer enzyme conjugation

Chen et al. (1993) reported that the phase separating polymer-enzyme was formed by coupling NHS-esterified PNIPAM to amino groups on the enzyme β-D-glucosidase[30]. In their investigation, they demonstrated that conjugated β-D-glucosidase also has high enzyme activity, and the PNIPAM-β-D-glucosidase conjugates can be recycled by separating the enzyme with small changes of the surrounding temperature.

They performed recycling precipitation of the model substrate (pNPG) using PNIPAM-β-D-glucosidase. First, PNIPAM-β-D-glucosidase conjugates reacted with the model substrate (pNPG) at room temperature. The conjugates were precipitated from the solution by increasing the temperature. After centrifuging, the conjugate was obtained. The precipitated conjugate was dissolved in the solution again, and was reused for repeated reaction-precipitation cycles. The remaining activity of the conjugates was measured. The results showed that only 30% loss of PNIPAM-β-D-glucosidase conjugate activity after seven cycles.

The activity of native and conjugated β-D- glucosidase was measured. The results showed that the PNIPAM-β-D- glucosidase conjugate is thermally stable, and that the conjugated β-D- glucosidase has better stability against activity loss at 60 °C than the native enzyme.

Ding et al. (1996) conducted a more extensive study of the preparation of PNIPAM-trypsin conjugates and determined the number of olimomer NIPAM chains reacting with primary amine groups in trypsin[31]. They also demonstrated that the thermal recovery of the PNIPAM-trypsin conjugates was 95% in every recycle.

1.3.3 Polymer antibody conjugation

Takei et al. (1994) presented the synthesis of amino modified Immunoglobulin G (IgG) and activated ester of semitelechelic PNIPAM conjugates[32]. They investigated the specific antigen binding activity of PNIPAM-IgG conjugates by measuring FITC fluorescence.

In later work[33,34], they showed that when the temperature is below the critical temperature of the IgG-PIPAAm conjugate, the conjugate was soluble in the solvent. Otherwise, when the temperature is above the critical temperature, the conjugates can be precipitated and separated from the solution. These reversible phenomena are significant in medical research.

Protein A, found in the cell wall of the bacterium Staphylococcus aureus, has the ability to bind immunoglobulins. Therefore researchers in biotechnology use it to

isolate immunoglobulins. Protein A can isolate immunoglobulins because it can bind with the Fc region of immunoglobulins by the interaction of the heavy chain. Jing Ping Chen and Allan S. Hoffman[35] conjugated protein A on PNIPAM and used the conjugates to separate human immunogammaglobulin using the LCST of PNIPAM. Protein A has the ability to specifically bind human immunogammaglobulin so that the PNIPAM-protein A-human immunogammaglobulin complex is formed. This complex can be reversibly separated and precipitated by decreasing or increasing the temperature of the medium solution.

The dissociation constant K_S of the human IgG binding protein A-PNIPAM conjugate has been determined as 3×10^{-6} M by investigating the binding affinity of IgG to conjugated protein A. They determined that about one out of every four conjugated protein A molecules was bound to IgG, and investigated the dissociation constant. These attractive results indicate that the affinity precipitation technique has a promising future in applications of medical research.

1.4 Objective

The objective of this research is to develop a general method for capturing and separating specific targets. A precipitation separation system consisting of a conjugate between PNIPAM and ssDNA was developed. PNIPAM, a stimuli-responsive polymer, is able to precipitate when the salt concentration and/or the surrounding temperature is changed. The phase transition behavior has been used to reversibly precipitate PNIPAM or PNIPAM conjugates[38,39,40] . When the temperature is lower than the LCST, the PNIPAM polymer chains are flexible and form extended hydrophilic coils in the solvent. However, when the temperature is above the LCST the PNIPAM polymer chains collapse and become hydrophobic[41,42,43,44]. The stimuli-responsive polymer PNIPAM has been widely used in biomedical research because of its promising potential. PNIPAM-ssDNA conjugates will also undergo the reversible inverse phase transition because of the PNIPAM phase transition. The advantage of using PNIPAM is that the mass transfer limitation caused by streptavidin agarose beads will be avoided.

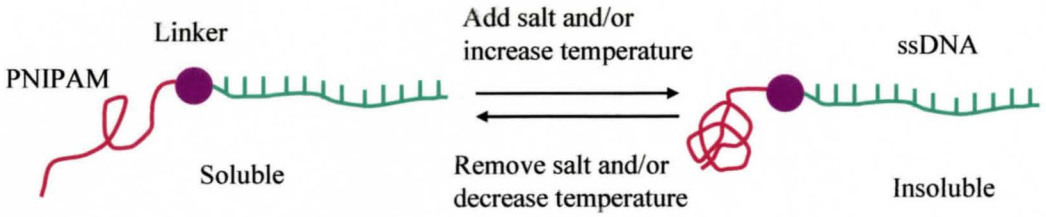


Figure 1.4.1 Phase transition behavior of the bioconjugate. The conjugation is assumed between PNIPAM and ssDNA, using a linker. When changing the temperature or salt concentration, PNIPAM-ssDNA conjugates will change their state from liquid phase to solid phase.

Two properties of DNA were employed to develop a general approach for specific target capture:

1 Short sequences of single stranded DNA (ssDNA) have the ability to specifically bind targets (small molecules, proteins, bacteria and viruses). These are known as DNA aptamers and can be easily developed for a specific target.

2. Two oligonucleotides with complementary sequences can hybridize through hydrogen bonding and form a stable link.

This separation system will be built by hybridizing between an aptamer and ssDNA conjugated to PNIPAM. The DNA sequence for an aptamer can be extended to contain a sequence that is complementary to that of the ssDNA coupled to PNIPAM. Adding this extended aptamer to the conjugate will result in spontaneous hybridization of the two strands of DNA.

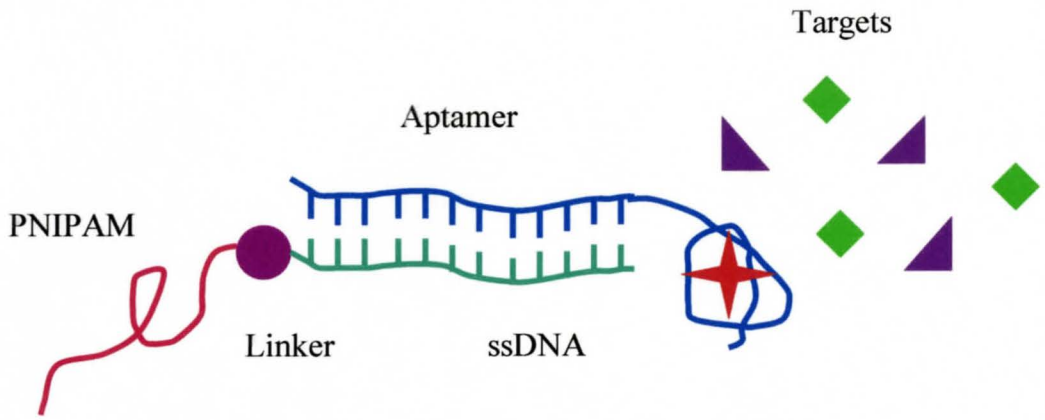


Figure 1.4.2 Aptamer specific capturing targets. The purpose of this work is to build a separation system by hybridizing ssDNA of PNIPAM-ssDNA conjugates and extension containing aptatmer. This system has the ability to capture targets because of the aptamer, and to separate from the complex mixture because of the PNIPAM phase transition.

These double strands can be separated using an agent that can destroy hydrogen bonding (e.g. urea). The conjugate can be recovered using reversible phase transition. It is possible to reversibly load or unload any aptamer to the same conjugate. In this way a reusable system is designed.

Based on the project design, several issues came up and had to be resolved. These issues include the following questions. Does the aptamer labeling functional group still have the ability to couple complementary DNA sequences through hydrogen bonds? Does the fluorescent group labelled aptamer have the high binding ability while coupled with an ssDNA containing conjugate? If this works, will the structure switching behaviour of aptamers bound to PNIPAM-ssDAN be reversible? Will the conjugates in the system influence the aptamers binding affinity? This work was carried out to answer these questions.

Chapter 2. Materials

Amino terminated poly (N-isopropylacrylamide) (PNIPAM) was purchased from polymer Source, Dorval, Quebec. Human thrombin, bovine serum albumin (BSA) and adenosine 5'-triphosphate (ATP) were purchased from Sigma-Aldrich, Oakville, ON. Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) and dithiothreitol (DTT) were purchased from Pierce Biotechnology, Rockford, IL. Urea was purchased from Promega Corporation, USA. The DNA sequences employed are listed in Table 2.1. The concentrations of ssDNA were determined by analytical standard UV spectroscopic methods.

Name of DNA	Sequence	Functionality Description	Purchase
LDNA	5/5ThioMC6-D/ACG TGC TAT CGA TGC TAG TC-3	The short sequence of single stranded DNA (ssDNA) containing thiol modifiers	Integrated DNA Technologies, Coralville, IA. With HPLC purification
FDNA	5'-/56-FAM/TGA CTA GCA TCG ATA GCA CGT-3'	6-carboxyfluorescein labeled short sequence of single stranded DNA	Integrated DNA Technologies, Coralville, IA. With HPLC purification
ssDNA 1	5'-ACG TGC TAT CGA TGC TAG TC-3'	Oligonucleotide sequence	LSB, McMaster University, With de-salted purification
ssDNA 2	5'-GAC TAG CAT CGA TAG CAC GT-3'	Oligonucleotide sequence	LSB, McMaster University, With de-salted purification
ATP Aptamer	5'-/56-FAM/TCA CTG ACC TGG GGG AGT ATT GCG GAG GAA GGT TTT GAC TAG CAT CGA TAG CAC GT-3'	6-carboxyfluorescein labeled ATPbinding DNA aptamer	Integrated DNA Technologies, Coralville, IA. With HPLC purification
QDNA 1	5'-CCC AGG TCA GTG /3Dab/-3'	Quencher labeled short sequence of single stranded DNA	Integrated DNA Technologies, with HPLC purification
Thrombin Aptamer	5'-/56-FAM/TCA CTG TGG TTG GTG TGG TTG GTT TGA CTA GCA TCG ATA GCA CGT-3'	6-carboxyfluorescein labeled thrombin binding DNA aptamer	Integrated DNA Technologies, Coralville, IA. With HPLC purification
QDNA 2	5'-CCA ACC ACA GTG /3Dab/-3'	Quencher labeled short sequence of single stranded DNA	Integrated DNA Technologies, Coralville, IA. With HPLC purification

Table 2.1 DNA sequences employed in these experiments. The DNA descriptions are also listed in this table. FDNA, ATP aptamer and thrombin aptamer were used to couple LDNA. QDNA was coupled to ATP aptamer or thrombin aptamer. Two ssDNA sequences were used to measure the melting temperate of the double-stranded DNA.

Chapter 3. Experimental Methods

3.1 Preparation of ssDNA-PNIPAM conjugates

DNA (ssDNA) containing thiol modifiers 5/5ThioMC6-D/ACG TGC TAT CGA TGC TAG TC-3 was dissolved in MilliQ water to the final concentration of 237 μM by UV spectrophotometer at 260 nm, and 154 mg dithiothreitol (DTT) was dissolved in 1 mL MilliQ water to prepare 1 M DTT solution. DTT was used to reduce the disulfide bonds of ssDNA: 5/5ThioMC6-D/ACG TGC TAT CGA TGC TAG TC-3. Solution of thiol modified oligonucleotides was prepared by suspending 16.8 μL of volume 237 μM thiol modified oligonucleotides in 450 μL 20 mM phosphate buffered saline (PBS) pH 8.4 then 50 μL volumes of 1 M aqueous DTT solution was added, after mixed thoroughly, and the mixture was allowed to react at room temperature for 30 minutes. An NAP-10 column was used for buffer exchange and purification of oligonucleotides. A 1 mL sample was collected from the NAP-10 column. Ethanol precipitation was used to concentrate and purify the deprotected thiol-ssDNA collected from the NAP-10 column. The deprotected thiol-ssDNA was dissolved in 500 μL conjugate buffer for cross-linking.

Amine containing PNIPAM 6 mg was dissolved in 100 μL of 0.15 M NaCl, 0.1M phosphate buffered saline pH 7.2 (the conjugate buffer) and 30 fold molar excess of Sulfo-SMCC (6 mg) was dissolved in 4 mL of conjugate buffer. The solutions of PNIPAM containing amine, and the Sulfo-SMCC solution were mixed gently and allowed to react for 1 hour at room temperature. The solution was

separated into 4 tubes, with volumes of 1mL, 1mL, 1.5 mL and 1.5 mL. In order to remove excess Sulfo-SMCC, 800 μL of 5 M NaCl was added to each of the four tubes, mixed thoroughly and centrifuged for 10 minutes. The supernatant containing unreacted Sulfo-SMCC was removed. The pellets were suspended, and this phase transition was repeated twice to remove the unreacted Sulfo-SMCC completely. After finishing the phase transition, the pellets were dissolved in 1 mL conjugate buffer for cross-linking to oligonucleotides.

Deprotected thiol-ssDNA solution was added to the Sulfo-SMCC-PNIPAM solution, mixed thoroughly and allowed to react at room temperature for 24 hours to form PNIPAM-sSMCC-ssDNA conjugates. Then a high concentration of salt was used to precipitate the conjugate to remove unreacted oligonuleotides. This phase transition was repeated twice to remove unreacted oligonuleotides completely.

3.2 Quantifying coupling efficiency

The ssDNA containing 6-carboxyfluorescein (FAM) FDNA 5'-/56-FAM/TGA CTA GCA TCG ATA GCA CGT-3' was dissolved in MilliQ water. The absorbance of FDNA was measured by a UV spectrophotometer at OD=0.87. Using a DNA Concentration Calculator, the concentration of FDNA was found to be 3.471 μM. Different concentrations of FDNA were used to draw calibration curves. In the following experiments these calibration curves were used to measure the concentration of unreacted FDNA. An excess amount of FDNA was used to couple the PNIPAM-ssDNA conjugate. The final concentration of FDNA was 40 nM. After

combination for one hour at room temperature, free PNIPAM was added to a final concentration of 135 μM, the addition of the unmodified free PNIPAM helped to enhance the precipitation. In order to separate the excess FDNA, applying phase transition to separate the unreacted FDNA from duplex DNA-PNIPAM, 800 μL of 5 M concentration NaCl was added to the solution and mixed gently. After 16000g centrifugation at room temperature for 10 minutes, the supernatant was decanted. The precipitation was the duplex DNA-PNIPAM containing fluorophore, and the unreacted FDNA was in the supernatant. The experimental processes are shown in Figure 3.2.1.

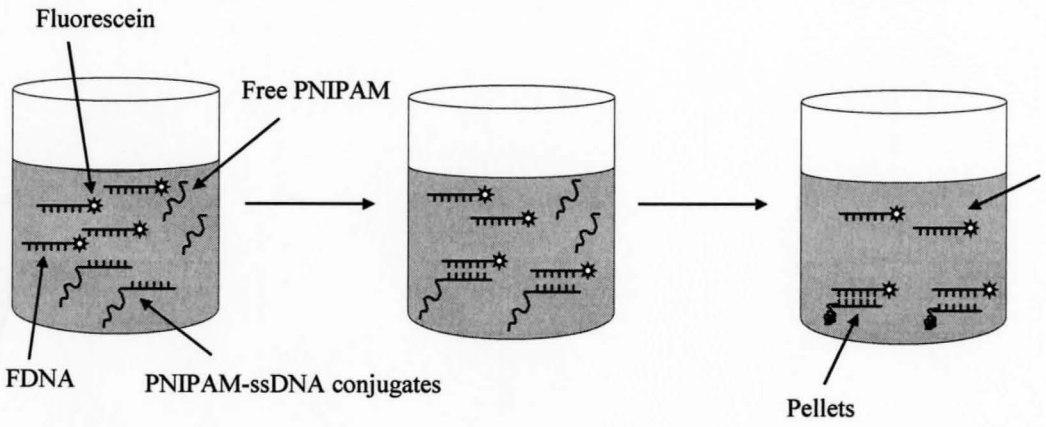


Figure 3.2.1 Experimental process for quantifying coupling efficiency. Certain concentrations of FDNA, PNIPAM-ssDNA and free PNIPAM were in the PBS at pH 7.2. Spontaneous hybridization took place between PNIPAM-ssDNA conjugates and FDNA. After addition of 2 M NaCl and centrifuge of the sample, the supernatant and the pellets were separated. The superntant contains uncoupled FDNA and the precipitate contains free PNIPAM and PNIPAM-ssDNA-FDNA.

3.3 Melting temperature of base paired DNA as a function of urea concentration

In order to optimize denaturation conditions of double-stranded DNA it is necessary to determine the influence of urea on the DNA melting temperature. Short single stranded DNA sequences 5'-ACG TGC TAT CGA TGC TAG TC-3' and 5'-GAC TAG CAT CGA TAG CAC GT-3', which have the same DNA sequences as duplex DNA-PNIPAM systems, were used to measure the DNA duplex melting temperature. The hybridization experiments were performed in 10mM NaCl, 5 mM phosphate buffered saline (pH 7.2) at room temperature. For the following series of melting curve measurements, additional components of urea at different concentrations (0 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M, 8 M, 9 M) were added separately to duplex DNA sample solutions. The melting experiments in solution were performed on a CARY 100Bio UV-visible spectrophotometer. The temperature was increased at the rate of 0.5 $^{\circ}$C per minute from 10 $^{\circ}$C to 70 $^{\circ}$C in 0.15 M NaCl, 0.1 M phosphate buffered saline (pH 7.2). These melting temperature curves were collected at a wavelength of 260 nm.

3.4 The effect of urea concentration on the LCST of PNIPAM

To begin with, 8.5 mg PNIPAM was dissolved in 2 mL of 0.15 M NaCl, 0.1 M phosphate buffered saline (pH 7.2). The measurements of the LCST of PNIPAM were

collected on a UV-visible spectrophotometer equipped with a temperature control system. The temperature was increased from10°C to 50 °C at a rate of 1°C per minute. The solution of PNIPAM in 0.15 M NaCl, 0.1 M phosphate buffered saline (pH 7.2) was prepared at 200 μM. Different concentration of urea (0 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M, 8 M, 9 M) were added separately to nine PNIPAM sample solutions, mixed gently and then measured by spectrophotometer at a wavelength of 500 nm.

3.5 The influence of the presence of urea on the stability of the linker on PNIPAM-ssDNA conjugates

To confirm that PNIPAM-ssDNA is reusable, the sample is designed to be denatured by adding 6 M urea solution. As mentioned in the previous experiments (Section 3.3), the conjugate was linked by sSMCC between ssDNA and PNIPAM. Thus, it is important to verify the influence of urea on the sSMCC linker under the same experimental conditions as the denaturation and rehybridization process. Therefore, urea (9 M urea, 700 μL was added) to 40 nM conjugates (with 200 μM free PNIPAM) to a total volume of 1 mL, mixed gently immediately, and then incubated at room temperature for 3 minutes. To remove the urea, the sample was centrifuged for 10 minutes at 40°C. The conjugate and free PNIPAM were precipitated to the bottom of the tube, with most of the urea remaining in the supernatant. A pipette was used to remove the supernatant carefully without touching the pellets on the bottom. The supernatant was discarded and the pellets were collected and dissolved in a 6 M urea solution again. This denaturation process was repeated. After the pellets were obtained,

a phase transition was performed 3 times to ensure that the urea left in the pellets was removed completely. To demonstrate that the conjugates were still in the pellets, they were dissolved in 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris.HCl (pH 8.3) to prepare the rehybridization experiment. If the conjugate still existed in the pellets it would be confirmed that 6 M urea could not affect its linkage. The sequence of ssDNA 5'-/56-FAM/TGA CTA GCA TCG ATA GCA CGT-3' (3.47 μM, 14.4 uL) was added to the sample to a total volume of 1 mL, mixed gently and rehybridized at room temperature for one hour. This phase transition was repeated twice to separate the supernatant and the precipitate, and to measure the fluorescence intensity of both.

The supernatant of the sample was measured and the fluorescence intensity was found to be 3.9 nM. The pellets were dissolved in 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris.HCl (pH 8.3), and the fluorescence intensities were measured. The result was 34.9 nM. These data demonstrate that 6 M urea cannot damage the conjugate.

3.6 Measuring fluorescence intensity of FDNA-QDNA-conjugates tripartite systems

The tertiary structures were designed for aptamers for selectively binding small molecules (see Figure 3.6.1). The concentration of fluorescein modified ATP aptamers (FDNA) was 40 nM, the concentration of the conjugate was 40 nM, and the concentration of quencher modified oligonucleotides (QDNA) was 120 nM. The hybridization was performed in 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris.HCl (pH 8.3) solution. For fluorescence measurements, different concentrations of ATP

were added after the intensities of the fluorescence became constant. The samples were transferred separately to the same quartz cuvette (with a path length of 1 cm) for measuring. The intensities of the fluorescence were measured on a CARY Eclipse Fluorescence Spectrophotometer with excitation at 490 nm and emission at 520 nm at 20°C.

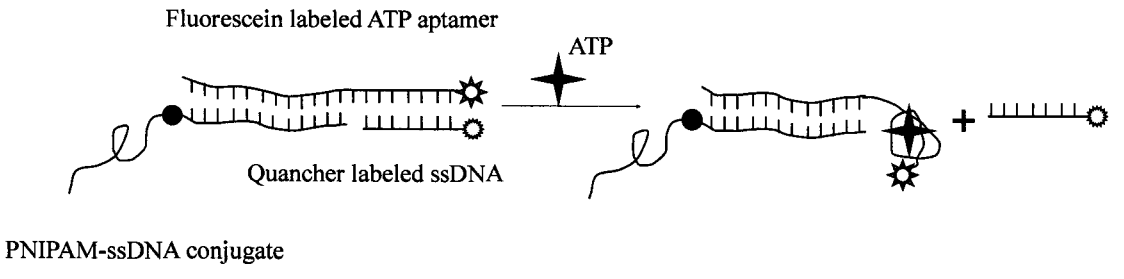


Figure 3.6.1 Tertiary system for specific capture of small molecules. PNIPAM-ssDNA and Quencher (circle) labelled ssDNA were coupled with an extension containing fluorescein (sun) labeled ATP aptamer. ATP (4-point star) was introduced to this tertiary system. This shows that aptamers are more easily bound by ATP molecules than by QDNA.

3.7 Method for replacing aptamers on ssDNA-PNIPAM

A 9 M 700 μL urea solution was introduced into the tertiary complex system solvent, mixed gently, and then left it at room temperature for 3 minutes. The solution obtained was centrifuged at 16,000 g for 10 minutes at 40°C. The supernatant was discarded and the pellets were collected. The DNA denaturation was performed twice. In order to isolate PNIPAM-ssDNA conjugates from the complex mixture, the phase transition was applied. The 2 M NaCl solution was added to the complex mixture

solution, mixed gently, and then centrifuged for 10 minutes. The phase transition was repeated twice to ensure complete isolation, so that the PNIPAM-ssDNA conjugates could recycle to load another aptamer. Thrombin aptamer was used to load on the recovered ssDNA-PNIAPM conjugates. The method was same as the above, loading ATP aptamers to PNIPAM-ssDNA conjugates. This is shown in Figure 3.7.1.

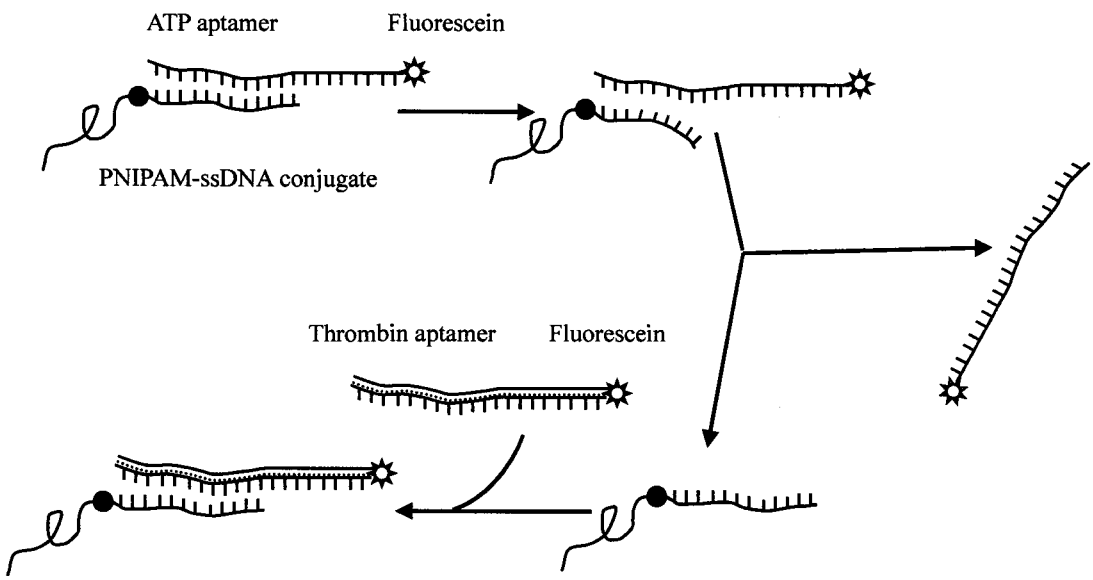


Figure 3.7.1 Replacing aptamers on PNIPAM-ssDNA conjugates. The double strands between aptamers and PNIPAM-ssDNA conjugates were separated using urea. This shows that the recovered conjugates can load fluorescein labeled thrombin aptamers.

Chapter 4. Results and Discussion

4.1 PNIPAM-ssDNA conjugate

To develop PNIPAM-ssDNA conjugates, amine ($-NH_2$) group containing PNIPAM and sulfhydryl (-SH) group containing ssDNA were crosslinked using the linker sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC). Sulfo-SMCC contains an amine-reactive N-hydroxysuccinimide (NHS-ester) and a sulfhydryl-reactive maleimide group. For crosslinking, the NHS ester first reacted with primary amines containing PNIPAM in 0.15 M NaCl, 0.1 M phosphate buffered saline (pH 7.2) to form stable amide bonds. The maleimide group of Sulfo-SMCC reacted with sulfhydryl groups of oligonucleotides in 0.15 M NaCl, 0.1 M phosphate buffered saline pH 7.2 to form stable thioether bonds. The chemical structure of the conjugates is shown in Figure 4.1.1.

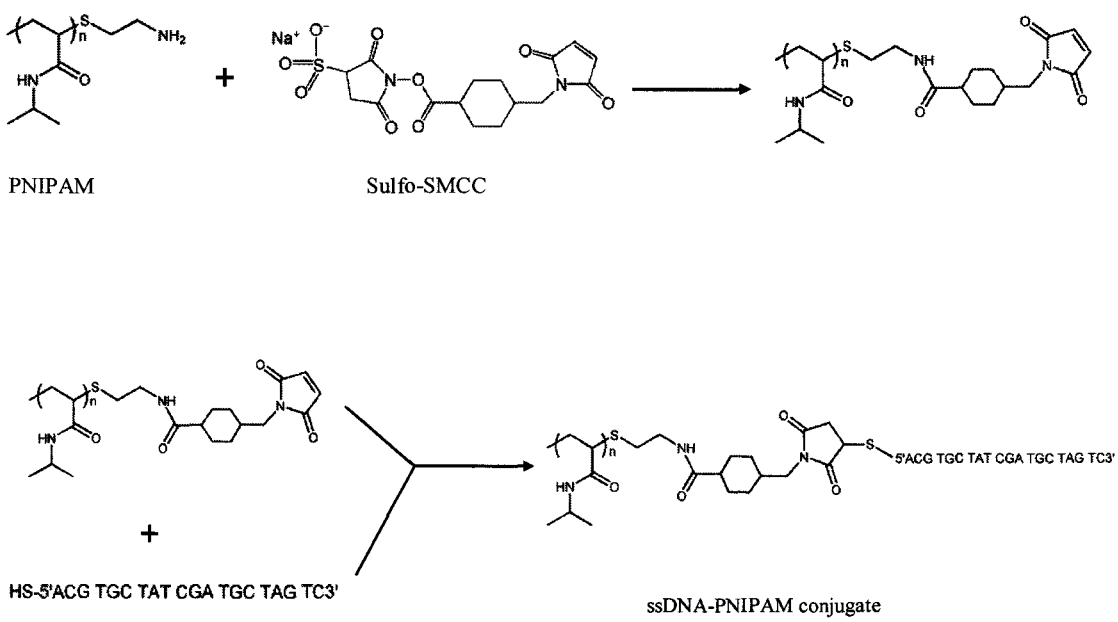


Figure 4.1.1 Structure of PNIPAM-ssDNA conjugate. First, amine terminal PNIPAM reacts with NHS ester of crosslinker Sulfo-SMCC. Second, sulfhydryl groups of ssDNA react with maleimide group of crosslinker Sulfo-SMCC.

The PNIPAM-sSMCC-ssDNA conjugate solution was scanned by a CARY100 Bio UV-Visible Spectrophotometer in the wavelength range from 200 nm to 400 nm. A peak of the absorbance was observed at 260 nm, suggesting that the bioconjugate was successfully obtained. In order to determine coupling efficiency of the conjugates, a new method was developed and described in the next section.

4.2 Quantifying coupling efficiency

A new method was developed to indirectly determine the coupling efficiency. The method consists of hybridizing a complementary ssDNA sequence containing 6-carboxyfluorescein (FAM) FDNA 5'-/56-FAM/TGA CTA GCA TCG ATA GCA CGT-3' to the ssDNA present in the conjugate. The coupling efficiency determination method is shown in Figure 4.2.1.

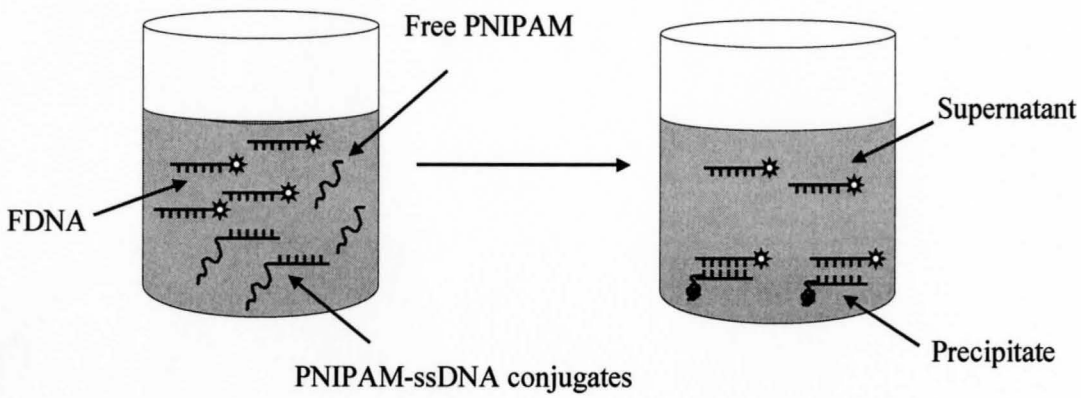


Figure 4.2.1 Coupling efficiency determination. This new method was developed by coupling complementary sequences of FDNA and PNIPAM-ssDNA conjugate and thus measuring the coupling efficiency.

After hybridization between complementary base pairs of two ssDNA sequences, duplex DNA- PNIPAM was obtained. The amount of FDNA in the precipitation was measured using a CARY Eclipse Fluorescence Spectrophotometer

(see Figure 4.2.2).

The coupling ratio of the FDNA: PNIPAM-ssDNA conjugate is 1:1, so the concentration of FDNA coupled with PNIPAM-ssDNA conjugates is equal to the concentration of ssDNA in PNIPAM-ssDNA. The samples of PNIPAM-ssDNA-FDNA were dissolved in the buffer and the fluorescence intensities of the samples were measured. To determine the concentrations of PNIPAM-ssDNA-FDNA, a calibration curve was used to calculate. In this way the concentrations of PNIPAM-ssDNA conjugates were found to be 22.2 nM, 21.7 nM and 23.1 nM, with an average concentration of 22 nM. So, the amount of thio-ssDNA coupled to PNIPAM is equal to 22 nM ×2 mL = 0.044 nanomole. The initial amount of thio-ssDNA added to PNIPAM is 0.132 nanomole. Thus the thio-ssDNA coupling efficiency is equal to 0.044 divided by 0.132. That is

$$(0.044/0.132)\times 100\% = 33\%.$$

Based on the results of coupling efficiency and the experimental process of conjugation between PNIPAM and ssDNA, two main factors considered might lower the ssDNA coupling efficiency. First, the sulfhydryl (-SH) group of oligonucleotide might partly oxidized in air during the purification process of oligonucleotides. Thus, the amount of oligonucleotides containing the sulfhydryl (-SH) group reacting with Sulfo-SMCC would lower the amount of oligonucleotide conjugated to PNIPAM. Secondly, an excess amount of sSMCC reacted with PNIPAM. Unreacted sSMCC is removed by the PNIPAM phase transition after the reaction between PNIPAM and sSMCC is completed. If the unreacted sSMCC is not removed completely, the oligonucleotide may react with this. So, the amount of oligonucleotides conjugated to

PNIPAM will be reduced.

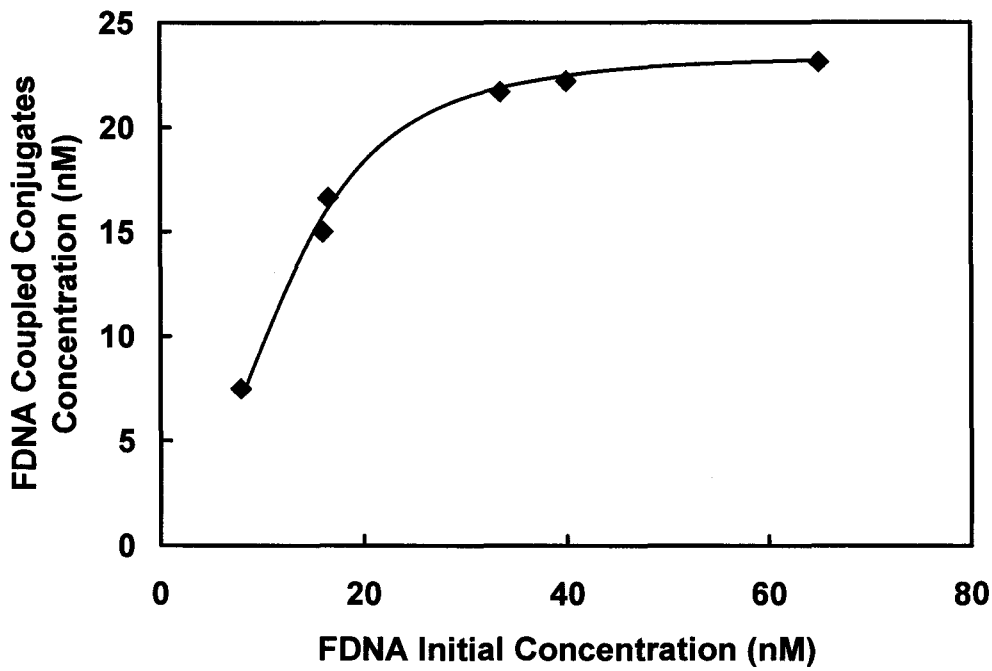


Figure 4.2.2 Concentration of FDNA coupled PNIPAM-ssDNA conjugates as a function of FDNA initial concentration.

4.3 Melting temperature of base paired DNA as a function of urea concentration

The motivation for using urea is that urea can be used in denaturing double-stranded DNA, allowing reusability of the bioconjugates. There are many methods to denature double-stranded DNA in the laboratory. The most traditional and easier way is to melt double-stranded DNA, increasing the temperature above its melting temperature. In the duplex DNA-PNIPAM system, PNIPAM precipitates at high temperatures. Thus when the temperature increases above the LCST (lower critical solution temperature) of the PNIPAM, PNIPAM pellets may affect duplex DNA melting. Another common method for DNA melting is to use urea. In this work, urea was used to separate double-stranded DNA structures into two parts, because it decreases the melting temperature of DNA molecules to about room temperature. It melts the hydrogen bonds between the base pair of double-stranded DNA to release the PNIPAM-ssDNA conjugates.

It is well known that DNA molecules have a strong absorbance at 260nm. With increasing temperature, the absorbance of DNA molecules increases, while all the DNA has been denatured, and the absorbance will be constant. In this experiment, two complementary sequences of ssDNA were used: 5'-ACG TGC TAT CGA TGC TAG TC-3', which has the same sequence as the thio-ssDNA; and 5'-GAC TAG CAT CGA TAG CAC GT-3', which has the same sequence as the extension of ATP aptamer and thrombin aptamer. The melting temperature of DNA depends on the composition and length of the sequence so that melting temperature of the two ssDNA sequences is

equal to the melting temperature of thio-ssDNA and aptamer.

Figrue 4.3.1 shows the DNA melting temperature at various concentrations of urea. The melting temperature of the DNA decreases as the urea concentration increases.

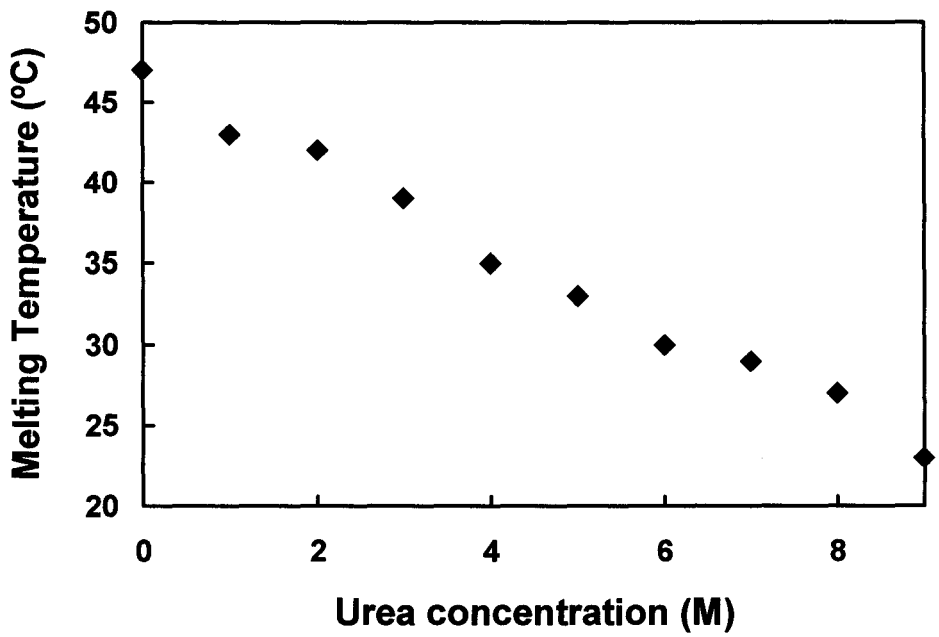


Figure 4.3.1 Melting temperature of double-stranded DNA as a function of urea concentration. The melting temperature decreases as the urea concentration increases.

4.4 The effect of urea concentration on the LCST of PNIPAM

In order to separate double stranded DNA, urea was used. Thermally responsive polymer (N-isopropylacrylamide, PNIPAM) was coupled to ssDNA. Therefore it is necessary to determine the effect of urea on the LCST of PNIPAM. The results are shown in Figure 4.4.1. The LCST of PNIPAM decreases with increasing concentration of urea.

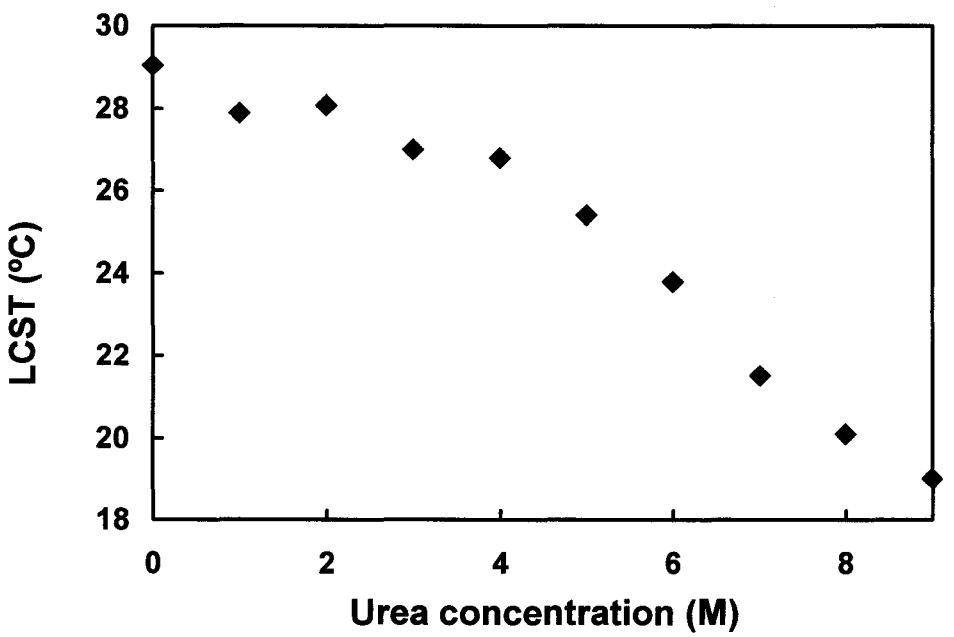
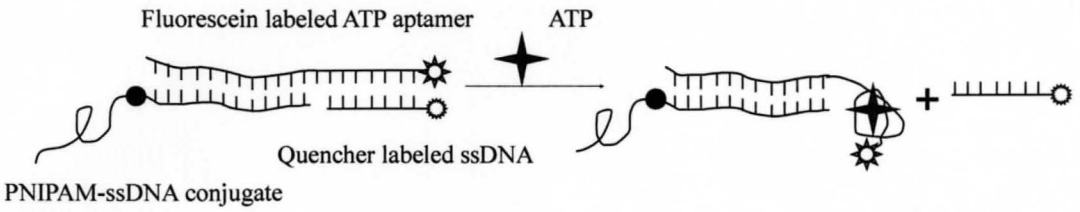


Figure 4.4.1 The LCST of PNIPAM as a function of urea concentration. The LCST of PNIPAM decreases with increasing urea concentration.

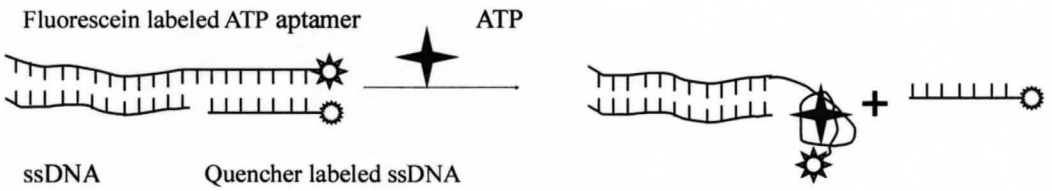
4.5 The effect of PNIPAM on aptamer folding

In order to investigate this question, two DNA sequence systems were designed. The first one is the tertiary structure (Figure 4.5.1 A) containing PNIPAM-ssDNA conjugate (40 nM), ATP aptamer (40 nM) and QDNA (120 nM). The second is the tertiary structure (Figure 4.5.1 B) contains ssDNA (40 nM), ATP aptamer (40 nM) and QDNA (120 nM). After hybridization in a 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris.HCl (pH 8.3) solution for 1 hour at room temperature, various concentrations of ATP (100 μM, 200 μM, 500 μM, 1000 μM, and 1600 μM) were added and the QDNA containing the quencher was separated from fluorophore, resulting in increased fluorescence intensity. The fluorescence intensity was measured on a CARY Eclipse Fluorescence Spectrophotometer with excitation at 490nm and emission at 520 nm. The three parts (A, B and C) of Figure 4.5.1 show almost the same intensities, showing that PNIPAM does not affect the folding of ATP aptamer.

A.



B.



C.

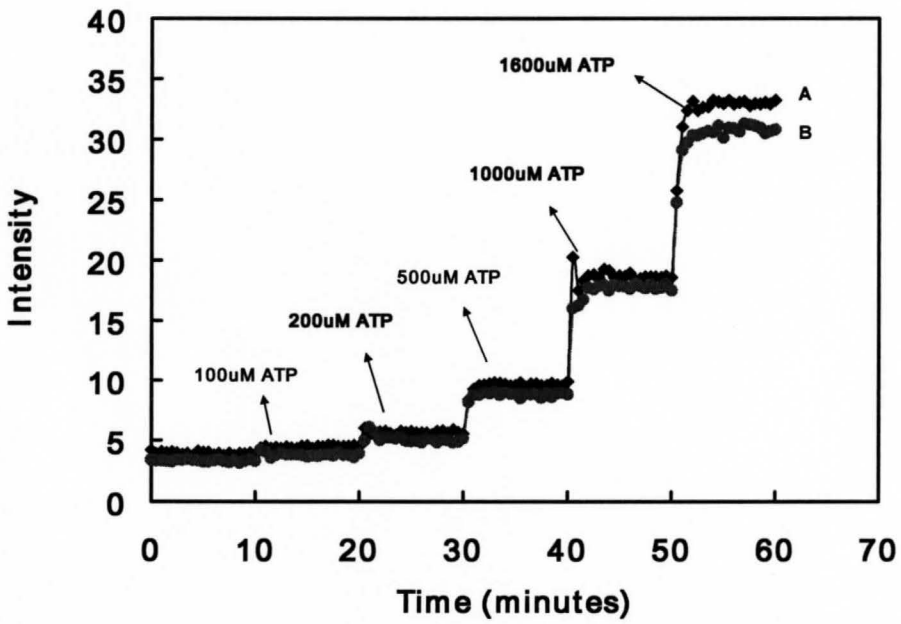


Figure 4.5.1 Effect of PNIPAM on aptamer folding. (A) PNIPAM-ssDNA and ssDNA labelled quencher (circle) were coupled with an extension containing ATP aptamer labeled fluorescein (sun). Different concentrations of ATP (4-point star) were introduced to this tertiary system. Aptamers prefer to bind ATP molecules other than QDNA. The change in intensity of the fluorescence was measured on a CARY Eclipse Fluorescence Spectrophotometer. (B) ssDNA and ssDNA labeled quencher were coupled with an extension containing ATP aptamer labeled fluorescein. After introducing different concentrations of ATP to this tertiary system, the intensity of the

fluorescence was measured by a CARY Eclipse Fluorescence Spectrophotometer. (C) The two figures A and B of the fluorescence intensity are compared, showing almost the same intensity. This shows that PNIPAM cannot affect aptamer structure switching behaviour.

4.6 Quantifying ATP aptamer-PNIPAM bioconjugate behaviour

Aptamer, a single-stranded nucleic acid, has a large number of dimensional shapes, and so it can specifically bind to small molecular targets. Here two complementary DNA sequences are used: aptamer modified by fluorophores, and DNA modified by quencher, to build fluorescence and quenching system. After the small molecules are introduced to the system, the aptamers change their structure to form a complex with the targets, and release QDNA from the aptamer. Because of the structure switching of the aptamer, the fluorescence intensity increases. Two fluorescence and quenching systems were developed, one for the ATP aptamer and the other for the thrombin aptamer. Figure 4.6.1 shows the ATP aptamer capturing specific targets, providing evidence for the presence of PNIPAM-ssDNA conjugates. Different concentrations of ATP molecules were introduced to the FDNA-QDNA-conjugate tripartite systems, so as to get different fluorescence intensities of the systems.

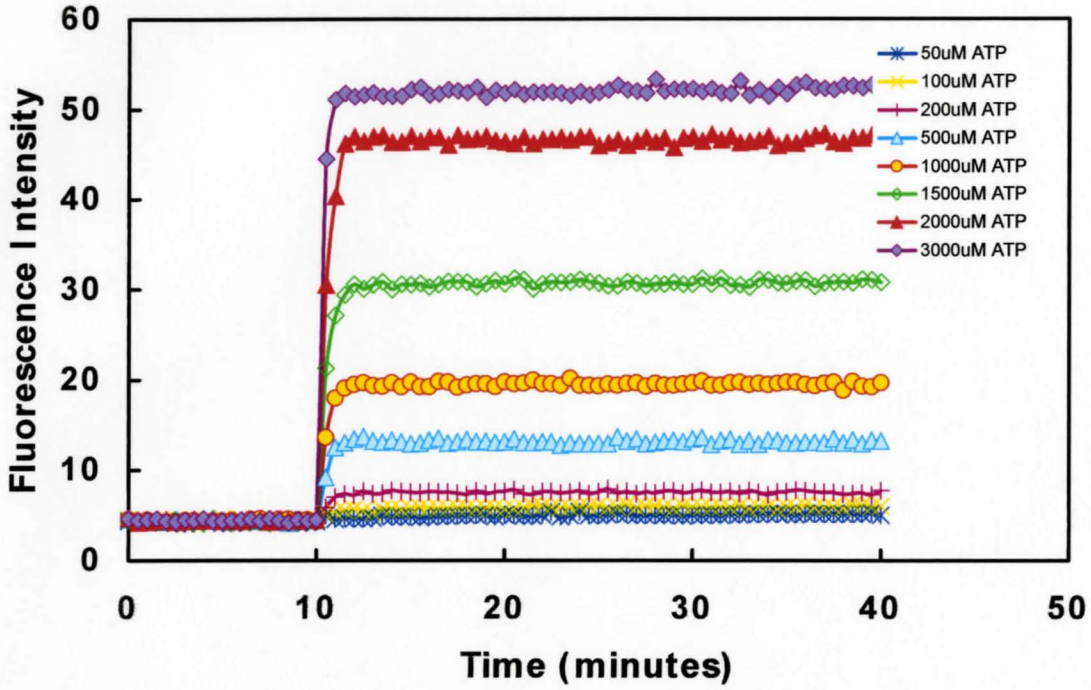


Figure 4.6.1 Fluorescence measurements for the ATP aptamer coupled to the PNIPAM-ssDNA conjugate. The tertiary system of ATP aptamer, PNIPAM-ssDNA conjugates and QDNA was incubated for 10 minutes at 20°C, then different concentrations of ATP were added, and the solutions incubated for 30 minutes.

The fluorescence intensity data (from Figure 4.6.1) were used to determine ATP binding affinity. The fraction of aptamer bound to ATP can be expressed as F/F_0, where F is the fluorescence intensity of the aptamer bound to various concentrations of ATP, and F_0 is the fluorescence intensity of the aptamer not bound to ATP The plot of F/F_0 versus ATP concentration is shown in Figure 4.6.2.

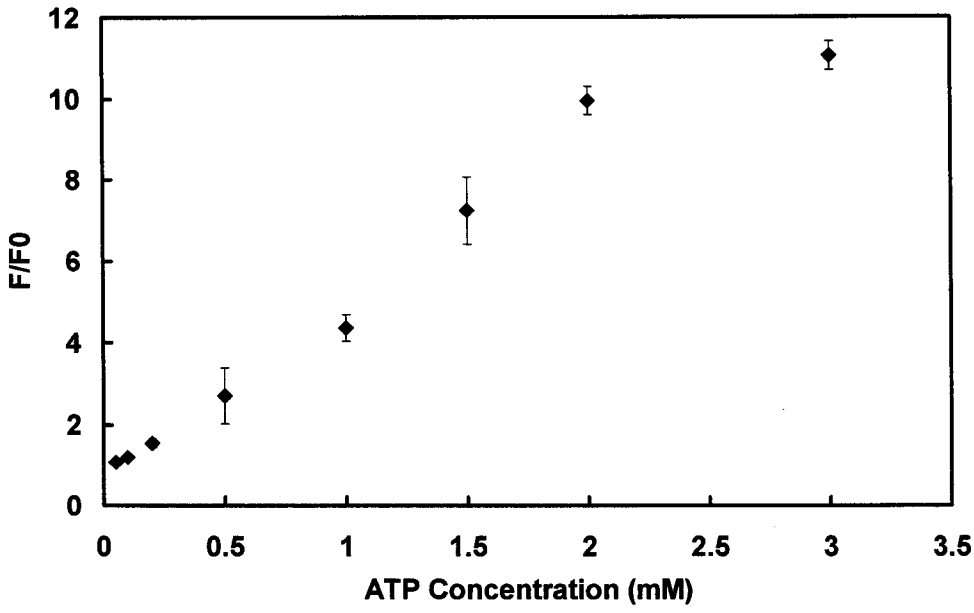


Figure 4.6.2 F/F_0 as a function of ATP concentration. F is the fluorescence intensity of the system consisting of fluorescein labeled ATP aptamer, QDNA and PNIPAM-ssDNA after adding ATP, while F_0 is the fluorescence intensity of the same system without adding ATP. Each point presents the mean value (with standard deviation) of three measurements, obtained from three independent experiments.

When the fraction of aptamer bound by APT is equal to half of the maximum aptamer binding amount, the dissociation constant is equal to the concentration of ATP which is not bound to the aptamer. The fraction F/F_0 is used to denote the fraction of aptamer bound, because the fluorescence intensity of aptamer changes depending on the introduction of ATP targets to the fluorescein labeled aptamer and quencher labeled ssDNA systems. In this way, the dissociation constant (from Figure 4.6.2) was estimated as:

$$K_d = 1300\ \mu M$$

This estimate for K_d is much higher than earlier estimates of ~10 μM[47], and ~600 μM[4].

From this result, the conclusion can be made that the PNIPAM-ssDNA conjugate has little effect on the binding ability of ATP aptamer.

4.7 Reusable conjugate

From the data presented in Figure 4.3.1 and Figure 4.4.1, the conclusion can be obtained that double-stranded DNA can be melted in the presence of 6 M urea at room temperature, while PNIPAM does not precipitate under these conditions in the duplex DNA-PNIPAM system. Therefore, for unloading aptamers, 6 M urea was used to melt double-stranded DNA.

In order to prove that the PNIPAM-ssDNA conjugate is reusable it was applied for precipitation of ATP aptamers or thrombin aptamers. As small molecules, like those of ATP and thrombin, can be specifically captured by the respective aptamers, the conjugates have the ability to precipitate and separate these small molecules. These results showed that the conjugates can be repeatedly used by coupling different aptamers: ATP aptamer or thrombin aptamers. The experimental results were also demonstrated that the conjugate is reusable.

Figure 4.7.1A shows the fluorescence intensity of the fluorescence labeled ATP aptamer as a function of time while 1500 μM APT was introduced to tripartite DNA systems. The tripartite systems of 40 nM ATP aptamers (5'-/56-FAM/TCA CTG ACC TGG GGG AGT ATT GCG GAG GAA GGT TTT GAC TAG CAT CGA TAG CAC GT-3'), 40 nM PNIPAM-ssDNA conjugates and 120 nM QDNA (5'-CCC AGG TCA GTG /3Dab/-3') solution containing 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM

Tris-HCl (pH 8.3), was transferred to a quartz cuvette and incubated for 10 minutes. After that, 1500 μM ATP was introduced in the cuvette and incubated for another 30 minutes at 20°C controlled by the device of temperature controller. The fluorescence intensity was measured on a CARY Eclipse Fluorescence Spectrophotometer with excitation at 490 nm and emission at 520 nm at 20°C. The solution was transferred from the quartz cuvette to a centrifuge tube, added 2 M NaCl to the tube, mixed well and centrifuged. The supernatant was discarded, and the precipitate re-dissolved in 1mL 150 mM NaCl, 100 mM phosphate buffered saline (pH 7.2). In this way, a complex of PNIPAM-ssDNA and ATP aptatmer was obtained. In order to load thrombin aptamers to the same PNIPAM-ssDNA conjugates to which the ATP aptamers were loaded, 6 M urea was used to separate the double-stranded sequences between the ATP aptamers and PNIPAM-ssDNA conjugates. For the experiment, 6 M urea was added to the PNIPAM-ssDNA and ATP complex, and incubated for 3 minutes at room temperature. The samples were centrifuged at 16000g and 40°C. The supernatant and precipitate were separated immediately. The PNIPAM-ssDNA conjugate was obtained after re-dissolving the precipitate and applying the urea denaturation process once again. After precipitation of the PNIPAM-ssDNA conjugates from the ATP aptamer, thrombin aptamers were loaded onto the same conjugate. For repeatedly use of conjugate 40 nM thrombin aptamer (5'-/56-FAM/TCA CTG TGG TTG GTG TGG TTG GTT TGA CTA GCA TCG ATA GCA CGT-3') and 120nm QDNA (5'-CCA ACC ACA GTG /3Dab/-3') were introduced to the recovery PNIPAM-ssDNA conjugate in 5 mM KCl, 1mM $MgCl_2$ and 20 mM Tris-HCl solution (pH 8.3) and incubated for 2 hours at room temperature.

To allow structure switching, 2000 μM thrombin was introduced to the sample, and the data were collected on a CARY Eclipse Fluorescence Spectrophotometer, with excitation at 490 nm and emission at 520 nm at 30°C.

The denaturation and reversible phase transition methods were repeatedly used to recover PNIPAM-ssDNA conjugates. To demonstrate that the conjugate is reusable, the recovered conjugate was applied once again to the precipitation of the ATP aptamer, and again to the precipitation of the thrombin aptamer, using the same procedure described above.

Figure 4.7.1 (A, B, C, D) shows the fluorescence intensity of the aptamer when it is bound by targets and releases QDNA in the presence of PNIPAM-ssDNA conjugates. The results (see Figure 4.7.1), show that PNIPAM-ssDNA conjugates can be reused for several recycles. However, there is certain loss in each recycle. The loss of PNIPAM-ssDNA conjugate activity was found to be 22% after two recycle.

Several reasons were postulated to explain why conjugates cannot be totally reused. First, the DNA denaturation is not completed by the use of urea. Secondly, the two complementary DNA strands (between PNIPAM-ssDNA and aptamer) which had been separated by urea are partly rehybridized. Thus the amount of PNIPAM-ssDNA coupled with the other aptamer decreases. Thirdly, some PNIPAM-ssDNA may be lost during phase transition.

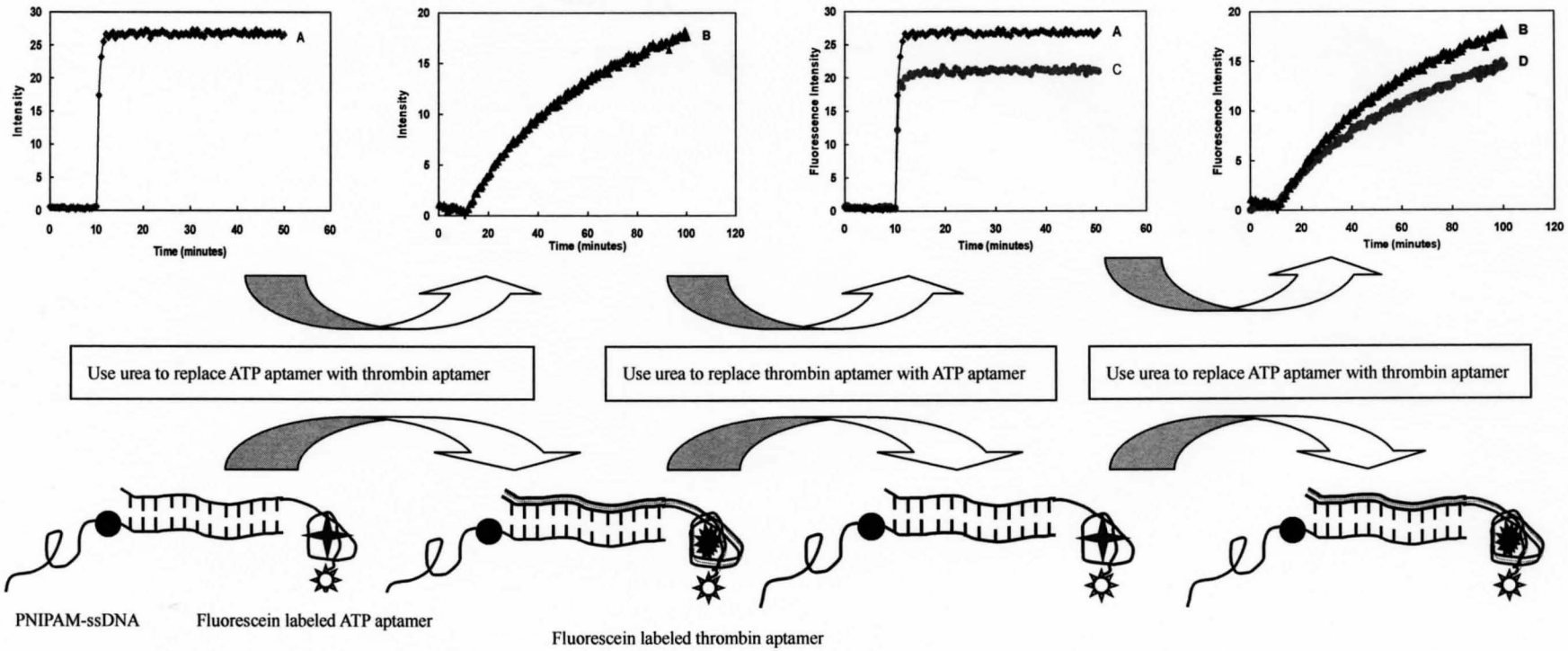


Figure 4.7 1 Reusable conjugates. The four lines on the graphs (A, B, C, and D) show fluorescence intensity change of PNIPAM-ssDNA-ATP aptamer/thrombin aptamer while introducing QDNA. The four drawings show the aptamer structure-switching behaviour. Comparing lines A and C, and lines B and D, we get the result that the conjugates can be reused with only a small amount of loss at each recycle.

Chapter 5. Conclusions and Recommendations

5.1 Conclusions:

(1) This thesis reports the successful conjugation of ssDNA with PNIPAM.

(2) A new method was developed to determine the coupling efficiency of ssDNA to PNIPAM, which was found to be 33%.

(3) It was shown that the LCST of PNIPAM decreases with increasing urea concentration.

(4) The results also demonstrate that PNIPAM does not affect aptamer folding.

(5) The aptamer will not loose its binding ability when coupled with PNIPAM-ssDNA conjugates.

(6) The PNIPAM-ssDNA conjugate is reusable, with 22% loss after two cycles.

(7) The ATP binding ability (or dissociation constant) was estimated to be 1300 μM, significantly higher than previous estimates (~600 μM). This decrease in affinity is likely due to influence of the PNIPAM, or the crosslinker sulfo-SMCC, with the ATP aptamer.

5.2 Recommendations for future studies

(1) The estimate of binding affinity of ATP aptamer with ATP is an important method in this research. The ssDNA-PNIPAM conjugate could be reused to load other aptamers, whose binding affinity would be determined.

(2) The separation system of ssDNA-PNIAPM conjugate could be applied to purify specific targets from complex mixture.

(3) It would be interesting to develop conjugate between PNIPAM and drugs. The LCST of the conjugate could be engineered around 37°C which is the physiology body temperature. In this way, the drug would be deliveried by controlling temperature.

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