A SIGNAL-RESPONSIVE HYDROGEL FOR CONTROLLED DRUG RELEASE

A Thesis in
Bioengineering
by
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ABSTRACT

Precisely controlling the release of pharmaceutical compounds is necessary in order to achieve maximum therapeutic benefit. Controlled drug release systems of different sizes, shapes, structures, and functionalities have been widely investigated for drug delivery and tissue engineering applications. The most promising drug release systems are on-demand release systems in which drugs are released in response to environmental stimuli, traditionally through dramatic physical or chemical changes of the carrier material. In particular, self-regulated drug release systems, which respond to changes in the concentration of specific analytes, are especially intriguing as the systems can be applied to maintain homeostasis by counteracting pathological events as they occur. Though current techniques to engineer self-regulated systems show promise, they are not generalizable for complex pathological situations where numerous signals are present.

Hence, a dual aptamer-based hydrogel system that is potentially able to respond to any specific signal molecule was engineered. The dual aptamer system includes a drug-loading oligonucleotide strand and a signal molecule-binding strand. In the absence of the signal molecule, the two strands are hybridized and the system sequesters the drug. In the presence of the signal molecule, the two strands separate and the system releases the drug.

In the thesis, human vascular endothelial growth factor (VEGF) and adenosine were used as the model drug and signal molecule, respectively. First, the drug-loading strand was designed and its activated and deactivated states were optimized for VEGF sequestration and release. Then, the signal molecule-binding strand was generated and the association and dissociation of the two strands in response to adenosine investigated. Finally, the dual aptamer-based superporous hydrogel system was established and the behavior of adenosine-triggered VEGF release was characterized.
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1. Background

Precisely controlling the release of pharmaceutical compounds is necessary in order to achieve maximum therapeutic benefit\(^1\,^2\). Controlled release of pharmaceutical compounds can reduce side effects and decrease the frequency of administration by maintaining the concentration of drugs in an effective and nontoxic range and by improving the biodistribution of drugs\(^3\). Therefore, controlled drug release systems have been widely investigated for drug delivery and tissue engineering\(^4\,^5\).

**Controlled drug release systems**

The common mechanisms of controlled drug release are diffusion-controlled release, degradation-controlled release, and affinity-controlled release depending on whether release is controlled by diffusion rate of the drug, degradation rate of the carrier, or dissociation rate of the drug from the carrier\(^6\). Accordingly, drug release can be controlled by regulating the formulations of drug carriers to adjust drug solubility in the carrier\(^7\), drug diffusivity through the carrier\(^8\), degradation rate of the carrier materials\(^9\), the affinity between the drug and the carrier\(^10\,^11\), and the ratio of binding sites and the drug molecules. Thus, various synthetic and natural materials with different sizes, shapes, structures and functionalities have been developed\(^12\). Widely investigated materials include lipids\(^13\,^14\), proteins\(^15\), polysaccharides\(^16\), synthetic polymers\(^17\,^18\) (e.g. polylactide (PLA), Poly(Lactide-co-Glycolide) (PLGA), and poly(ethylene glycol) (PEG)), and inorganic
materials\textsuperscript{19,20,21} (\textit{e.g.} carbon nanotubes, silica, graphene, iron oxides and gold). Organism such as viruses, bacteria and eukaryotic cells are also used as complex materials\textsuperscript{22}. In addition, the sizes of drug carriers are manipulated to vary from nanoscopic scale\textsuperscript{23,24}, microscopic scale\textsuperscript{25,26}, to macroscopic scale\textsuperscript{27,28} depending on the specific application of the system.

\textit{On-demand drug release systems}

With the development of techniques to manipulate the properties of drug carriers, it appears practical to engineer a system to realize drug release at a designed time point with a desired rate. However, the accurate time and rate of drug release required in real pathological conditions can be difficult to predict\textsuperscript{29}. To tailor the drug release with precise spatial, temporal and dosage control, on-demand drug release systems were developed.

On-demand drug release systems can release drug in response to environmental stimuli\textsuperscript{30,31}. The feasibility of on-demand drug release is traditionally accomplished by developing a carrier material that undergoes dramatic physical or chemical changes when stimuli are applied\textsuperscript{32,33}. The changes usually alter the confirmation and stability of carriers or the interactions between carriers and drugs, which eventually lead to drug release. For instance, in a pH-responsive nanosphere, the amino-group protonation induces chitosan swelling and subsequently the anti-cancer drug leaking in the local acidic environment of tumor tissues\textsuperscript{34}. A magnetically responsive ferrogel composed of Pluronic-F127 micelles and superparamagnetic iron oxide nanoparticles releases drugs when a magnetic field is applied as iron oxide nanoparticles approach each other and squeeze the micelles\textsuperscript{35}. A dendrimer–drug conjugates containing thiol-cleavable bonds releases drugs in the presence of intracellular glutathione due to the breakage of the covalent bond between the dendrimer and the drug\textsuperscript{36}. 
The concept of on-demand drug release systems was brought up in 1970s with the use of thermosensitive liposomes\textsuperscript{37}. Since then, materials that respond to a variety of stimuli, including temperature\textsuperscript{38}, magnetic and electric field\textsuperscript{39,40}, ultrasound\textsuperscript{41}, light\textsuperscript{42}, pH\textsuperscript{43}, and specific analytes\textsuperscript{44} have been investigated to develop on-demand drug delivery systems. Particularly, self-regulated systems\textsuperscript{29}, which are responsive to changes in the concentration of specific analytes, are most intriguing as the systems mimic the natural feedback systems in living organism and control the drug release without expensive and inconvenient machines. Thus, the systems can potentially be applied as an approach to precisely deliver drugs temporally and spatially and as a non-invasive strategy to maintain homeostasis which is crucial for treating numerous diseases such as diabetes.

Though current techniques to engineer self-regulated systems show promise, they are not generalizable for complex pathological situations where numerous signal molecules are present because traditionally, the systems are diffusion-controlled or degradation-controlled which require the signal molecules to have specific functions such as shift the charges of drug carriers to cause confirmation changes of the carriers, catalyze the degradation reaction of drug carriers, or cleave the chemical bond between drugs and carriers. Hence, we engineered an affinity control-based dual aptamer hydrogel system, which is potentially able to respond to any specific signal molecule.

**Aptamers**

Aptamers are single-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) molecules that specifically bind to their targets with high affinities\textsuperscript{45}. Aptamers are generated by an upstream process termed systematic evolution of ligands by exponential enrichment (SELEX) and a downstream truncation\textsuperscript{46,47}. In the upstream process, a synthesized oligonucleotide library with a variety of $10^{13}$-$10^{15}$ different sequences that span 20-100 residues in length is incubated
with target molecules. The sequences that bind to the target are isolated from the unbound and weakly bound oligonucleotides. Subsequently, the oligonucleotide sequences are eluted from the ligand-target complexes and amplified by polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR. Also, the selectivity to specific target can be enhanced by counter-SELEX using analogue target to eliminate aptamers recognizing close-related targets. Usually after 8-15 rounds of selection, aptamers can be identified.

![Diagram](image)

Figure 1-1. Identification of aptamers through the systematic evolution of ligands by exponential enrichment (SELEX).

In the downstream truncation, the aptamers selected from the SELEX process are shortened on the basis of essential and nonessential nucleotides (nt). The essential nucleotides are the nucleotides that either directly interact with the target or facilitate the formation of functional structure of the aptamer while the nonessential nucleotides are not
necessary for target binding. With algorithms based prediction of the secondary structure of aptamers and synthesis and characterization of aptamer segments, nonessential nucleotides can be identified and tailored\textsuperscript{52,53,54}.

Figure 1-2. Aptamer truncation. The essential and non-essential regions of the aptamer are shown by the orange and grey color, respectively. The function of the different domains are distinguished by the background color.

Aptamers have numerous advantages over other affinity ligands\textsuperscript{55,56}. First, they can interact with target molecules or cells with high specificity and affinity. For example, aptamers are able to distinguish between chiral molecules or to recognize a distinct epitope of a target molecule\textsuperscript{57,58}. The binding affinities of most aptamers against their targets are comparable to those of antibodies for antigens. However, antibodies are produced \textit{in vivo} or from \textit{in vitro} cell culture whereas aptamers are obtained and synthesized without the involvement of living organism. Targets that are not immunogenesis or tolerated for generation of antibody are unrestricted for selection of aptamer. Since the invention of the selection process in 1990\textsuperscript{59,60}, aptamers that interact with a broad section of targets including small molecules\textsuperscript{61}, large molecules\textsuperscript{62}, and cells\textsuperscript{63} have been identified. In addition, aptamers are tolerant to harsh conditions which allows them to be chemically modified or conjugated to a support material without losing their functional structure and binding capability. Aptamers also routinely avoid the immunogenicity concerns of protein antibodies even when administered in excess of therapeutic doses\textsuperscript{64,65}. The binding
between aptamer and target is reversible. For instance, when introducing a complementary sequence of an aptamer, the formation of nucleic acid hybridization can destroy the functional secondary and tertiary structure of the aptamer and thereby dramatically reduces its binding ability to the target. Vice versa, binding of the target to an aptamer can facilitate the recovery of the functional structure of the aptamer and consequently decrease the stability of the nucleic acid base pairs between the aptamer and its complementary sequence, which ultimately separate the hybridization.

The high specificity and affinity, broad range of target, ease of identification and synthesis, chemical stability, little immunogenicity and reversible and controllable binding ability enable aptamers to be an extremely promising candidate for developing the signal-responsive system for drug controlled release.

**Hydrogels**

Hydrogels are networks of hydrophilic polymers which absorb water while maintaining their structures. Hydrogels are widely investigated in tissue engineering and drug delivery systems as they are generally biocompatible due to their similarity to the native extracellular matrix. Hydrogels possess porous structures which permit loading of drugs and provide aqueous environment for the loaded drugs. Particularly, superporous hydrogels, the hydrogels with interconnected microscopic pores, are advantageous for drug loading and signal responding because of the ease of diffusion within the hydrogels. With these features, the superporous hydrogels were chosen as the scaffold of our controlled drug release.
2. **Outline of the thesis**

The dual aptamer system includes a drug-loading strand and a signal-binding strand (Figure 1-3). When the two strands hybridize, the drug-loading strand is activated and captures the drug. When the two strands separate, the drug-loading strand is deactivated and releases the drug. The signal molecule controls the hybridization and separation of the two strands through the signal-binding strand. When the signal molecule is absent, the two strands hybridize. When the signal molecule is present, the two strands separate. The system is based on the reversible binding of aptamers to their targets and the reversible hybridization between aptamers and their complementary sequences.

![Figure 1-3](image)

Figure 1-3. Schematic for the mechanism of the dual aptamer system to load and release drugs in response to signals. The protein structure is obtained from the RCSB PDB [www.rcsb.org](http://www.rcsb.org)\(^\text{72}\).

In the thesis, human vascular endothelial growth factor (VEGF) and adenosine were used as the model drug and signal molecule, respectively. The drug-loading strand has a VEGF-binding domain, and a complementary domain. The VEGF-binding domain, adapted from a
VEGF aptamer sequence, is able to bind to VEGF. The complementary domain forms intramolecular hybridization with the VEGF-binding domain which destroys its functional structure and consequently deactivates its binding functionality. However, when a hetero-sequence is introduced to form stable intermolecular hybridization with the complementary domain, the self-hybridization is disrupted. Consequently, the functional structure of the VEGF-binding domain is recovered and its VEGF-binding functionality is activated. In the absence of the adenosine, the signal-binding strand acts as the hetero-sequence that hybridizes with the complementary domain of the drug-loading strand and activates the VEGF-binding functionality. In the presence of the adenosine, however, the signal-binding strand, adapted from an adenosine aptamer, interacts with its target. Thus, the secondary and tertiary structure of the signal-binding strand is altered which destabilizes the intermolecular hybridization which results in the reformation of the intramolecular hybridization of the drug-loading strand. As a consequence, the VEGF-binding functionality of the drug-loading strand is deactivated.

In the thesis, we first designed the drug-loading strand, then generated the signal-binding strand, and finally established the dual aptamer hydrogel system for controlled drug release. To design the drug-loading strand, we studied the VEGF-binding sequence and the complementary sequence separately and then incorporated them into a single strand as the VEGF-binding domain and the complementary domain, respectively. Next, we examined the deactivated and activated states of the drug-loading strand for VEGF sequestration. To generate the signal-binding strand, we adapted the adenosine aptamer and investigated the functionality of the sequence to associate with and dissociate from the drug-loading strand in response to adenosine. To establish the dual aptamer-based hydrogel system, we examined the incorporation of the aptamers into the hydrogel and tested the VEGF sequestration functionality of the system. Finally, we investigated the adenosine responding functionality of the system and characterized the behavior of adenosine-triggered VEGF release.
Chapter 2

METHODOLOGY

1. Materials

All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and their sequence are listed in Table 2-1. Recombinant human vascular endothelial growth factor (VEGF165, MW = 38.2 kDa) and the VEGF165 enzyme-linked immunosorbent assay (ELISA) kit were purchased from Peprotech (Rocky Hill, NJ). Streptavidin-coated polystyrene particles (MPs, diameter = 5.04 μm) were obtained from Spherotech (Lake Forest, IL). Ammonium persulfate (APS), phosphate-buffered saline (PBS), tris-borate-EDTA (TBE) buffer, Tween 20, acetic acid, sodium bicarbonate, and N, N, N', N'-tetramethylethylenediamine (TEMED) were purchased from Fisher Scientific (Waltham, MA). Poly(ethylene glycol) diacrylate (PEG700DA, Mn = 700), Pluronic F-127, adenosine, uridine, and cytidine, were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA), SYBR® Safe DNA Gel Stain, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Life Technologies (Grand Island, NY).

Table 2-1. The oligonucleotides.

<table>
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<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
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</tr>
<tr>
<td>Vapt1</td>
<td>5'-biotin-AGATTTAGTTAGGTTC CCAGACTCAAGAGTGCAGGG-3'</td>
</tr>
<tr>
<td>Vapt2</td>
<td>5'-biotin-CCCGTCTTTCCAGACAAGAGTGCAGGG TTAGGTAGTAGTAG-3'</td>
</tr>
<tr>
<td>CV1-0</td>
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</tr>
<tr>
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<td>VS'</td>
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</tr>
<tr>
<td>Ctrl-VS</td>
<td>5'-FAM- CTACTCTAAACCTAACCTTCCGCTTCCAGACAAGAGTGCAGGG AAGGT -3'</td>
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</tbody>
</table>
2. Methods

2.1 Generation of DNA sequences

The adapted aptamer sequences with randomly generated tails were designed by NUPACK. The detailed coding was revealed in the appendix. Briefly, the tail sequence is calculated to have a linear structure and to remain the original structure of the aptamer.

2.2 Prediction of DNA structures

The secondary structure and the Gibbs free energy of the DNA sequences were acquired by RNAstructure version 5.7. The predicted structures with lowest Gibbs free energy were presented.

2.3 Synthesis of DNA-functionalized microparticles

All oligonucleotides were annealed at 95 °C for 3 min and then rested at room temperature for 30 min. Prior to the immobilization of DNA onto microparticles (MPs), the MPs were passivated with 1% BSA and washed with 500 μL wash buffer (PBS buffer+0.05% Tween 20) once. To synthesize the DNA-functionalized particles, 50 μg MPs were incubated with 10
pmol biotin-labeled DNA in 20 uL reaction buffer (PBS buffer + 0.05% Tween 20 + 0.1% BSA) for 1 h at room temperature. To remove free DNA from the microparticle suspension, 500 uL wash buffer was added. The MPs were then centrifuged at 1,000 x g for 7 min and the 500 uL of the supernatant was collected and replaced. The MPs were washed 2 times. These microparticles are referred to as the single-stranded DNA-functionalized MPs (ss-MPs). To prepare MPs with the double-stranded DNA (ds-MPs), the ss-MPs were incubated with 10 pmol complementary oligonucleotides in 20 uL reaction buffer overnight at room temperature and then washed thoroughly with 500 uL wash buffer to remove the free DNAs.

2.4 Quantification of VEGF capture on particles

The various DNA-functionalized MPs (50 ug) or non-functionalized MPs (50 ug) were incubated with 3 ng VEGF in 13 μL reaction buffer for 24 h at room temperature. The supernatant was collected by centrifuging the MPs at 1,000 x g for 7 min. The amounts of VEGF remained in the supernatant were measured by ELISA following the protocol provided by the company. Measured VEGF concentrations were normalized with untreated VEGF. The amounts of VEGF captured on the microparticles were calculated by subtracting the amount of VEGF in the supernatant from the total amount of VEGF (3 ng).

2.5 Gel electrophoresis

The ssDNA and dsDNA samples were prepared by incubating DNA (20 pmol) with or without its complementary oligonucleotide (20 pmol) in 20 uL PBS at room temperature overnight. The various DNA samples were electrophoresed through a 10% polyacrylamide gel in TBE buffer at 100 V for 60 min. The gels were then stained with TBE buffer containing 0.01%
SYBRsafe at room temperature with shaking at 70 rpm for 45 min. The gels were imaged with the Maestro Fluorescence imaging system (Cambridge Research & Instrumentation, Inc. (CRi), Woburn, MA) with an excitation filter from 445 to 490 nm and a 515 nm longpass emission filter.

2.6 Flow cytometry

To examine the hybridization between two DNA strands, signal-binding strands functionalized microparticles (AS-MPs or (Ctrl-AS)-MPs) were incubated with PBS with or without drug-loading strands (VS or VS’) overnight and washed twice with 500 μL wash buffer. The ss-MPs and the ds-MPs were treated with SYBRsafe for 30 min and washed twice with 500 μL wash buffer. The signal intensity of the particles were measured by flow cytometry. To test the separation of two DNA strands, the VS strand labeled with 6-carboxy-fluorescein (denoted as FAM-VS) was used to prepare the ds-MPs. The ds-MPs (50 μg) were incubated in 50 μL wash buffer containing various concentration of adenosine (0.5, 1, 2, 5, 10 mM), 10 mM uridine, 10 mM cytidine, or saturated guanosine (approximately 2.6 mM) overnight at room temperature. The particles were washed thoroughly with wash buffer and run through flow cytometry using the Guava easyCyte™ Flow Cytometer (EMD Millipore, Billerica, MA) with an excitation wavelength of 488 nm and a fluorescent channel of 525/30 nm.

2.7 Fluorescence Spectroscopy

The 2’-aminopurine modified sequence VS (AmPr-VS) (500 pmol) was hybridized with AS (500 pmol) in PBS buffer (50 μL). The 50 μL of AS-VS hybrid solution was transferred into a fluorescence 96-well plate (Perkin Elmer, Waltham, MA) and mixed with 50 μL PBS buffer (with or without adenosine). The final concentration of aptamer-hybrid and the adenosine was 5 μM
and 10 mM, respectively. The fluorescence intensities (307/370 nm (Excitation/Emission)) of the VS, AS-VS complex (with/without adenosine) were recorded by a Tecan M200 Pro Micro-plate reader (Tecan US Inc., San Jose, CA) at predetermined time intervals.

2.8 Synthesis of superporous hydrogels

Superporous hydrogels were prepared following an adapted protocol previously described73. Briefly, 1 μL PEG700DA, 1 μL Pluronic F-127 (10%, w/v), 0.25 μL acetic acid, 0.1 μL TEMED, 0.004 g sodium bicarbonate, 1.65 μL APS (10%), and 1 μL water were mixed with or without methacrylamide-functionalized AS (100 pmol) to generate the aptamer-functionalized hydrogel and the native hydrogel, respectively. The hydrogels were washed with water for 1 h, 70% ethanol overnight, and PBS for 8 h. After washing and drying the hydrogels, 20 μL PBS solution containing 120 pmol VS was added to the hydrogels and incubated overnight at room temperature. The hydrogels were then washed with 1.8 mL reaction buffer for 8 h.

2.9 Fluorescence imaging of hydrogels

The FAM-VS was used to prepare the dual aptamer-functionalized hydrogels. After the synthesis, the hydrogels were imaged under the Maestro Fluorescence imaging system with an excitation filter from 445 to 490 nm and a 515 nm longpass emission filter.

2.10 Measurement of VEGF sequestration

To load VEGF, 50 nanogram VEGF in 20 μL reaction buffer was added to the aptamer-functionalized hydrogels and the native hydrogels and incubated overnight at room temperature.
The hydrogels were shaken at 70 rpm in 1 mL reaction buffer at room temperature for 1 h. The supernatant was then collected and stored at -20 °C. The amount of VEGF in the supernatant was measured by ELISA.

2.11 Examination of VEGF release

To measure the VEGF release, after VEGF loading, the hydrogels were washed with reaction buffer to discard the burst release. Subsequently, 1 mL release buffer (reaction buffer with different concentrations of adenosine or 10 mM various nucleosides) was added accordingly. After 1 h incubation at room temperature with 70 rpm shaking, the release buffer was totally collected and stored at -20 °C until analyzed through ELISA.
Chapter 3
RESULTS

1. Design the drug-loading strand

The drug-loading strand (VS) has to two states, the activated state and the deactivated state. At the activated state, the strand binds to VEGF while at the deactivated state, the strand unbinds from VEGF. To design the drug-loading strand, we studied the VEGF binding sequence and its complementary sequence separately and incorporated them into a single strand as the VEGF binding domain and the complementary domain, respectively. Next, we optimized the deactivated and activated states of the drug-loading strand for VEGF sequestration and release.

1.1 Investigation of VEGF-binding sequences

To test the ability of the sequences to capture VEGF, the oligonucleotides were immobilized on the streptavidin-coated polystyrene microparticles through the biotin functional group. The particles was then incubated with the VEGF and the amount of VEGF remained in the supernatant was measured by the enzyme-linked immunosorbent assay (ELISA) and the amount of VEGF captured on the particles was calculated by subtracting the quantity of VEGF in the supernatant from the total amount.
Figure 3-1. Sequestration of VEGF by VEGF-binding sequences. a. The illustration of the binding between VEGF and the VEGF aptamer. b. The quantification of the amount of VEGF captured by particles functionalized with/without VEGF aptamers. c. The predicted secondary structure of the VEGF-binding sequences. d. The amount of VEGF captured by various VEGF binding sequences. The sample size N=3. The error bar stands for the standard deviation (STD).
First we demonstrated the reliability of this method by incubating 3 ng VEGF with 50 μg non-functionalized particles or particles functionalized with the VEGF aptamer (Vapt0) reported in a previous literature. The non-functionalized particles only captured 0.03% ±9.6% VEGF while the particles functionalized with the aptamer sequestered 92%±0.69% VEGF (Figure 3-1. b), indicating the particles themselves don’t interact with VEGF and VEGF bond to the aptamer on the particles.

Then we adapted the VEGF binding sequences by adding 15nt tail sequences on either 5’ end (Vapt1) or 3’ end (Vapt2) of the 26 nucleotides (nt) VEGF aptamer sequence (Vapt0). The tail sequences were generated using NUPACK and the codes used were in the appendix. The adapted aptamer sequences Vapt1 and Vapt2 captured 90.0%±1.1% and 90.4%±2.5% VEGF, respectively (Figure 3-1. d). The result suggests that the adapted aptamers are able to bind to the VEGF with a similar binding affinity as the original aptamer.

1.2 Investigation of complementary sequences

In the adapted aptamer sequences, the 26 nt VEGF aptamer sequence generated through SELEX and truncation is considered essential and the 15 nt tail sequence randomly created by a software non-essential. Correspondingly, the hybridization between a complementary sequence and the essential sequence of the aptamer is considered as essential hybridization and the hybridization between a complementary sequence and the non-essential sequence of the aptamer as non-essential hybridization.

A serial complementary sequences (CVs) were designed to find the efficient essential hybridization for VEGF release. The complementary sequences fully hybridize with the non-essential sequence to form 15 base pairs (bp) and partially hybridize with the essential sequence of the aptamer. The gel electrophoresis result demonstrated that all of the complementary
sequences can hybridize with the corresponding aptamer as the fluorescence intensity of the bands increased significantly, indicating the formation of DNA base pairs. When approaching from the 5’ end, the amount of released VEGF increased gradually while the length of the essential hybridization increased from 0 bp to 7 bp (Figure 3-2. b). By comparison, when approaching from the 3’ end, the amount of released VEGF increased dramatically with the length of the essential hybridization increasing from 0 bp to 4 bp (Figure 3-2. b). The differences of the VEGF release when approaching from the opposite directions are not related to the Gibbs free energy (ΔG) of the hybridization reaction since we have purposely saturated the ΔG with the 15bp non-essential hybridization. The complementary sequence that hybridizes with the first 4 nucleotides on the 3’ end of the essential aptamer sequence (CV2-4) induced 75.6±2.5% VEGF to be released (Table 3-1). Thus, the 4 base pairs formed on the 3’ end of the aptamer were identified as efficient essential hybridization.

Table 3-1. Release of VEGF by complementary sequences.

<table>
<thead>
<tr>
<th>Hybridization length (nt)</th>
<th>15</th>
<th>17</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
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<tr>
<td>Essential hybridization length (nt)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-essential hybridization length (nt)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Approach from 3’</td>
<td>VEGF release %</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approach from 5’</td>
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<td></td>
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</tr>
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<td>ΔG (kcal/mol)</td>
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<td></td>
<td></td>
<td></td>
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<td>Approach from 5’</td>
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<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

<table>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
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<tr>
<td>VEGF release %</td>
<td>5.5±1.1</td>
<td>22.3±1.6</td>
<td>31.4±1.4</td>
<td>32.0±3.7</td>
<td>55.7±4.1</td>
<td>53.1±3.7</td>
</tr>
<tr>
<td>ΔG (kcal/mol)</td>
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<td>-20.6</td>
<td>-23.1</td>
<td>-25.1</td>
<td>-27.3</td>
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<td>-23.0</td>
<td>-23.6</td>
<td>-25.5</td>
<td>-26.2</td>
</tr>
</tbody>
</table>
Figure 3-2. Release of VEGF by complementary sequences. 

a. Illustration of VEGF release from the aptamer. 

b. Percentage of VEGF released by various complementary sequences. The sample size N=3. The error bar stands for the standard deviation (STD). 

c. Images of gels after electrophoresis showing hybridization between aptamers and their corresponding complementary sequences of varying length.
1.3 Investigation of the deactivated state of the drug-loading strand

We incorporated the VEGF binding sequence and the complementary sequence together to create drug-loading strands. First, we only added 4 nucleotides in the complementary domain (VS-0) to form the 4 bp essential hybridization with the aptamer. The VEGF sequestration was still significant as 74.8%±4.7% VEGF was sequestered (Table 3-2).

Then the non-essential hybridization was supplemented to find the sufficient intramolecular hybridization length to deactivate the VEGF-binding functionality. When the non-essential hybridization length was lengthened to 5 bp (VS-5), the sequestration of VEGF decreased to 18.8%±5.5% (Table 3-2). Further increase of the non-essential hybridization length, however, did not reduce VEGF binding. Therefore, 9 bp intramolecular hybridization in which 4 base pairs are essential and 5 base pairs are non-essential was identified as an adequate length to deactivate the VEGF-binding functionality.

Table 3-2. Deactivation of drug-loading strands affected by the length of intramolecular hybridization.

<table>
<thead>
<tr>
<th>Intramolecular hybridization length (nt)</th>
<th>13</th>
<th>11</th>
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</thead>
<tbody>
<tr>
<td>Essential hybridization length (nt)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Non-essential hybridization length (nt)</td>
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<td>7</td>
<td>5</td>
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<tr>
<td>VEGF sequestration %</td>
<td>24.1±7.5</td>
<td>18.6±5.9</td>
<td>18.8±5.5</td>
<td>31.5±3.5</td>
<td>74.8±4.7</td>
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<tr>
<td>ΔG (kcal/mol)</td>
<td>-11.2</td>
<td>-9.9</td>
<td>-7.9</td>
<td>-4.1</td>
<td>-2.1</td>
</tr>
</tbody>
</table>
Figure 3-3. Deactivation of drug-loading strands. 

a. Illustration of incorporation of the aptamer and the complementary sequence. 

b. Predicted secondary structure of the deactivated drug-loading strands. 

c. The Gibbs free energy of the drug-loading strands when in its deactivated state. 

d. Percentage of VEGF sequestered by various drug-loading strands when in its deactivated state. The sample size N=3. The error bar stands for the standard deviation (STD).
1.4 Investigation of the activated state of the drug-loading strand

We examined the activated state of the drug-loading strand by introducing a 20 nt complementary sequence (CS20) to hybridize with the complementary domain of the drug-loading strand (VS-5). The VEGF-binding functionality was affectedly restored as 92.4±0.9% VEGF was sequestered after the hybridization.

Then we shortened the complementary sequence to find the effective length of intermolecular hybridization to activate the VEGF-binding functionality. The complementary sequences (CS19, CS18, CS17, CS16, and CS15) which form 19, 18, 17, 16, and 15 base pairs with the drug-loading strand recovered the sequestration of VEGF to 93.8±1.1%, 93.2±1.5%, 94.9±1.0%, 87.5±1.3%, and 55.7±3.4%, respectively (Table 3-3). Sixteen base pairs of intermolecular hybridization was, therefore, considered necessary to activate the VEGF-binding functionality of the drug-loading strand.

Table 3-3. Activation of drug-loading strands affected by the length of intermolecular hybridization.

<table>
<thead>
<tr>
<th>Intermolecular hybridization length (nt)</th>
<th>20</th>
<th>19</th>
<th>18</th>
<th>17</th>
<th>16</th>
<th>15</th>
</tr>
</thead>
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<tr>
<td>VEGF sequestration %</td>
<td>92.4</td>
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<td>93.2</td>
<td>94.9</td>
<td>87.5</td>
<td>55.7</td>
</tr>
<tr>
<td>±0.9</td>
<td>±1.1</td>
<td>±1.5</td>
<td>±1.0</td>
<td>±1.3</td>
<td>±3.4</td>
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</tr>
<tr>
<td>ΔG (kcal/mol)</td>
<td>-23.8</td>
<td>-22.8</td>
<td>-22.1</td>
<td>-20.2</td>
<td>-18.8</td>
<td>-18.8</td>
</tr>
</tbody>
</table>
Figure 3-4. Activation of drug-loading strands. 

a. Illustration of the hybridization between the drug-loading strand and the complementary sequence. 

b. The predicted secondary structure of the activated drug-loading strand when hybridized with different complementary sequences. 

c. Images of electrophoresed gels showing hybridization between the drug-loading strand and a complementary sequence. 

d. Percentage of VEGF sequestered by various drug-loading strands when in the deactivated state. The sample size N=3. The error bar stands for the standard deviation (STD).
2. Generate the aptamer duplex

To generate the aptamer duplex, based on the adenosine aptamer reported in a previous literature\textsuperscript{75}, we designed the signal-binding strand and adjusted the drug-loading strand. Then we investigated the association and dissociation of the two strands in the absence and presence of adenosine.

2.1 Adaptation of the adenosine-binding sequence

The signal-binding strand (AS) has two functions, hybridizing with the drug-loading strand to activate it for VEGF capture and separating from the drug-loading strand in response to adenosine which eventually deactivates the drug-loading strand to release VEGF. To create the signal-binding strand, we adapted the adenosine binding sequences by adding a 10 nt tail sequence on the 3’ end of the 27 nucleotides (nt) adenosine aptamer sequence. The tail sequence was generated using NUPACK and the codes used were in the appendix. The 27 nt adenosine aptamer sequence is considered essential and the 10 nt tail sequence non-essential. Correspondingly, the hybridization on the essential nucleotides is considered as essential hybridization and the rest as non-essential hybridization.

To hybridize with the signal-binding strand, the non-essential nucleotides in the complementary domain and in the VEGF-binding domain of the drug-loading strand were altered accordingly. As 9 intramolecular base pairs were sufficient to disrupt the binding between aptamer and VEGF and 16 intermolecular base pairs were adequate to sabotage the intramolecular hybridization and recovered the VEGF capture ability, when we adapted the drug-loading strand, the self-hybridization remained 9 bp and the hetero-hybridization between the two strands was designed to be 16 base pairs.
Two drug-loading strands were generated (Figure 3-5). The sequence VS forms 10 bp essential hybridization and 6 bp non-essential hybridization with the sequence AS. The sequence VS’ forms 7 bp essential hybridization and 9 bp non-essential hybridization with the sequence AS.

2.2 Investigation of the association between the drug-loading strand and the signal-binding strand

First, we examined the hybridization between the drug-loading strands and the signal-binding strand in the absence of adenosine with flow cytometry. The results demonstrated that both VS and VS’ hybridized with the AS as when VS or VS’ was added, the distribution of the fluorescence intensity shifted to the right, indicating the increase of the signal of the DNA dye which suggested the formation of DNA base pairs. Then the VEGF-binding functionality of the
aptamer duplex was tested. The ELISA result showed that the hybrid of VS-AS sequestered 76.9%±0.5% VEGF while the hybrid of VS’-As only sequestered 36.9%±4.6% VEGF (Figure 3-6). Possibly, the VEGF sequestration by the VS’-AS hybrid is hindered by unexpected base pair matches between the two strands which affects the proper structure formation of VEGF-binding domain after hybridization. The sequence VS is selected as the drug-loading strand and the sequence AS as the signal-binding strand.

Figure 3-6. Association of the drug-loading strand and the signal-binding strand. a. The illustration of the binding between a drug-loading strand and a signal-binding strand. b. Flow cytometry histograms of the hybridization between the drug-loading strand and the signal-binding strand. c. Percentage of VEGF sequestered by the hybridized drug-loading strand and the signal-binding strand. The sample size N=3. The error bar stands for the standard deviation (STD).
2.3 Investigation of the dissociation of the drug-loading strand and the signal-binding strand

First, the dissociation of the aptamer duplex in response to the presence of adenosine was examined. The signal-binding strand was immobilized on a microparticle surface and the drug-loading strand was labeled with a fluorescent group (Em. 518 nm). The aptamer duplex was treated with 10 mM adenosine, uridine, cytidine, and saturated guanosine (approximately 2.6 mM), respectively. The fluorescence intensities on the particles were measured by flow cytometry. The adenosine-responding functionality of the aptamer duplex was demonstrated as the fluorescent intensity decreased after the treatment of adenosine while remained constant after treating the hybrid with the control substrates (guanosine, uridine, or cytidine), which suggested that the fluorescence-labeled drug-loading strand separated from the signal-binding strand when adenosine was present. To demonstrate the adenosine-responding functionality was corresponding to the binding between adenosine and the adenosine aptamer, we scrambled the adenosine binding sequence (Ctrl-AS). The hybrids of AS-VS and (Ctrl-AS)-VS were treated with 10 mM adenosine and only AS-VS with the correct adenosine binding sequence dissociated in the presence of adenosine.

Figure 3-7. Specificity of the adenosine-responsive dissociation of the duplex. a. Flow cytometry histogram showing substrate specificity. b. Flow cytometry histogram showing sequence specificity.
Next, we tested dissociation percentage of the hybrid in various concentrations of adenosine. To describe the concentration dependency, we fitted the data with a regression line \( x = (a-y)^2/y^b \), where \( x \) is the adenosine concentration and \( y \) is the fluorescence intensity, in Matlab as the measured fluorescence intensity has a linear relationship with the concentration of the aptamer duplex and the relationship between the adenosine concentration and the aptamer duplex at equilibrium is

\[
[S] = \frac{([AC]_0 - [AC])^2}{[AC] \cdot K}
\]

Where \( S \) is the adenosine concentration; \([AC]\) represents the concentration of the aptamer duplex at equilibrium; \([AC]_0\) is the initial concentration of the aptamer duplex; \( K \) is a constant.

Figure 3-8. Concentration dependency of adenosine-responsive dissociation of the duplex. a. The flow cytometry histogram showing sequence dissociation of the duplex in different concentration of adenosine. b. The curve fitting in Matlab.
Finally, we examined the response time of the aptamer duplex to the presence of adenosine (10 mM). The drug-loading strand was synthesized with a 2’-aminopurine located on the unhybridized domain. The fluorescence of 2’-aminopurine can be quenched when changing from an unhybridized state to a hybridized state. Therefore, the fluorescence intensity indicates the hybridization and separation of the two strands. The fluorescence spectroscopy result suggested that the response time to adenosine of the aptamer duplex is short as the fluorescence intensity reached plateau within 1 min which suggested that the dissociation of the aptamer duplex reaches equilibrium within 1 min.

Figure 3-9. Response time to adenosine of the duplex.
3. Establish the dual aptamer-based hydrogel system

To establish the dual aptamer-based superporous hydrogel system, we examined the incorporation of the aptamers into the hydrogel and tested the VEGF sequestration functionality of the system. Then we investigated the adenosine responsive functionality of the system and characterized the behavior of adenosine-triggered VEGF release.

3.1 Incorporation of the aptamer duplex and loading of VEGF into the system

The superporous hydrogels were prepared using an adapted protocol described in the previous literature\(^73\). In brief, the Poly(ethylene glycol) diacrylate undergoes a free radical polymerization with the initiator, ammonium persulfate, and the catalyst, TEMED, while the acetic acid and sodium bicarbonate produces carbon dioxide. The gas is trapped and consequently creates porous structures in the hydrogel networks.

The signal-binding strand was modified with methacrylamide to react with the polymer backbone of the hydrogel. After the formation of the hydrogel, the drug-loading strand was added to hybridize with the signal-binding strand. To examine the incorporation of the aptamer duplex, we labeled the drug-loading strand with a fluorescent group (Em. 518 nm) and added to the hydrogels prepared with or without the methacrylamide-modified signal-binding strand. After washing thoroughly, the hydrogels were imaged. The hydrogel with methacrylamide-modified signal-binding strand exhibited intense fluorescent signal while the native hydrogel showed no signal in the green channel, indicating the aptamer duplex was successfully incorporated.

To test the loading of VEGF into the system, VEGF was incubated with the hydrogels with or without the aptamers. Approximately, 7%±9.5% and 97%±0.7% of 50 ng VEGF were
sequestered by the native hydrogel and the aptmer-functionalized hydrogel, respectively. The VEGF sequestration functionality of the system was successfully realized.

Figure 3-10. Establishment of the dual aptamer-based superporous hydrogel system. a. Fluorescence image of the superporous hydrogels with/without aptamer. b. Percentage of VEGF sequestered by the superporous hydrogels with/without aptamer. The sample size N=4. The error bar stands for the standard deviation (STD).

3.2 The adenosine-responsive release of VEGF

To investigate the dissociation of the aptamer duplex and consequent VEGF release in response to the presence of adenosine, we prepared the hydrogels with fluorescence-labeled drug-loading strands, loaded VEGF into the hydrogels, and incubated the hydrogels in buffers with or without adenosine. The fluorescence intensity decreased approximately 60% after the treatment of adenosine, indicating more than half of the aptamer duplex dissociated. The amount of released VEGF increased approximately 10 times after the treatment of adenosine suggesting that the adenosine-responsive release of VEGF was accomplished.
Figure 3-11. The adenosine-responsive system. a. Fluorescence of the aptamer-hydrogels treated with/without adenosine. b. Amount of VEGF released by the hydrogels treated with/without adenosine. The sample size N=4. The error bar stands for the standard deviation (STD).

The ability of the system to control VEGF release in response to adenosine was further investigated. First, the specificity of the adenosine controlled release was studied by using uridine, guanosine, and cytidine as control substrates. As 2.4, 0.55, 0.24, and 0.13 ng VEGF were released after treated with adenosine, uridine, cytidine, and guanosine, respectively, the system demonstrated to be specific to adenosine. Then, the dose control on the released VEGF by the concentration of adenosine was examined through measuring the amount of released VEGF in response to various adenosine concentrations. As the adenosine concentration rose from 0 mM, 1 mM, and 5 mM to 10 mM, the amount of VEGF released increased from 0.25 ng, 1.1 ng, and 1.6 ng to 2.7 ng.
Figure 3-12. Adenosine-controlled VEGF release. a. Specificity of the system in responding to adenosine. b. Triggered VEGF release as a function of adenosine concentration. The sample size N=4. The error bar stands for the standard deviation (STD).

Finally, we generated the profile of multi-triggered VEGF release by adding adenosine to the release buffer at designed time points (1h, 5h, 9h). The ELISA result showed that without the trigger of adenosine, the hourly release of VEGF was very low, while with adenosine triggering, the amount of released VEGF increased significantly.

Figure 3-13. Profile of hourly release of VEGF. Adenosine triggered release (orange) and sustained release (grey). The sample size N=4. The error bar stands for the standard deviation (STD).
Chapter 4

CONCLUSION AND FUTURE WORK

In this thesis, a dual aptamer-based hydrogel system was successfully engineered. The drug, human vascular endothelial growth factor (VEGF), was sequestered into the hydrogel with high efficiency. The release of the drug in response to the specific signal molecule, adenosine, was achieved based on the mechanism we proposed. The dose of released drug was also demonstrated to be dependent on the concentration of the signal molecule.

The loading ability of the system can be adjusted by altering the amounts of incorporated aptamer duplex to fulfill the clinic drug dose requirement. The more drug-loading strands incorporated, the more drugs can be loaded as the concentration of the loaded drug is related to the concentration of the drug-loading strand with the equation:

\[ K_d = \frac{([A]_0 - [AP]) \times ([P]_0 - [AP])}{[AP]} \]

Where [A]₀ represents the concentration of the incorporated drug-loading strand; [AP] stands for the concentration of the loaded drug; [P]₀ is the concentration of the input drug; Kₐ is a constant.

The dose of released drug is also controlled by the concentration of the aptamer duplex. The less drug-loading strands remained in the hydrogel after adenosine trigger, the more drugs can be release. The concentration of adenosine has a relationship with the concentration of the incorporated drug-loading strand as:

\[ K = \frac{([A]_i - [A])^2}{[A] \cdot [S]} \]
Where \([A_i]\) is the initial concentration of the incorporated drug-loading strand; \([A]\) represents the concentration of the incorporated drug-loading strand after adenosine trigger; \(S\) is the adenosine concentration; \(K\) is a constant.

The constant \(K\) can be manipulated by altering the binding affinity of the adenosine aptamer to adenosine and the hybridization stability of the drug-loading strand and the signal-binding strand. The adjustment of the constant \(K\) will realize the release of desired drug dosage in response to the physiological adenosine concentration.

The features of an ideal controlled drug release system, besides precise control of the drug release, include efficient drug loading and preserving the bioactivity of drugs through loading, storage, and release process. Therefore, in the future, the bioactivity of the released VEGF should also be studied. To prove the generalizable feature of the methodology we invented in this thesis, other molecular or even cellular models can be used.

In real pathological conditions, various signal molecules can be present and multiple drugs may be required for the treatment of a disease, so the system can be further developed to achieve multiple signals-triggered single drug release, single signal-triggered multiple drugs release, or multiple signals-triggered multiple drugs release by employing multiple aptamer-duplexes. The methodology used to engineer our controlled release system can eventually be applied to mimic biosystems to accomplish the sequential release of different drugs in response to the sequential presence of various signals.

To further study the feasibility of the system we engineered, animal studies can be performed. However, the unmodified oligonucleotides are susceptible to the degradation of nuclease in the body. Modified nucleotides should be introduced to stabilize oligonucleotides against nuclease-mediated degradation. Commonly used substitutions include 2′-amino pyrimidines, 2′-fluoro pyrimidines, and 2′-O-methyl ribose purines and pyrimidines. The modified oligonucleotides may behave differently on target-binding and oligonucleotide-
hybridization so further *in-vitro* studies have to be performed first to study the thermodynamic and kinetic behaviors of the modified oligonucleotides before *in-vivo* study was conducted.
Appendix

NUPACK codes

To generate Vapt1
material = dna
temperature[C] = 25.0   # optional units: C (default)
trials = 10
sodium[M] = 0.14   # optional units: M (default)
dangles = some

# target structure using DU+ notation
structure stickfigure = U15U26

# sequence domains
domain a = N15
domain b = CCCGT CTTCC AGACA AGAGT GCAGG G

# thread sequence domains onto target structures
stickfigure.seq = a b

# specify stop conditions for normalized ensemble defect
# default: 1.0 (percent) for each target structure
stickfigure.stop = 1.0

# prevent sequence patterns
prevent = AAAA, CCCC, GGGG, UUUU
To generate Vapt2
material = dna
temperature[C] = 25.0  # optional units: C
trials = 10
sodium[M] = 0.14  # optional units: M
dangles = some

# target structure using DU+ notation
structure stickfigure = U26U15

# sequence domains
domain b = N15
domain a = CCCGT CTTCC AGACA AGAGT GCAGG G

# thread sequence domains onto target structures
stickfigure.seq = a b

# specify stop conditions for normalized ensemble defect
# default: 1.0 (percent) for each target structure
stickfigure.stop = 1.0

# prevent sequence patterns
prevent = AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR,
SSSSSS, WWWW, YYYYYY
To generate AS

material = dna

temperature[C] = 25.0  # optional units: C (default) or K

trials = 10

sodium[M] = 1.0  # optional units: M (default), mM, uM, nM, pM

dangles = some

# target structure using DU+ notation

structure stickfigure = U1D2U4U4D2U7U3U10

# sequence domains

domain a = ACCT GGG GGA GT ATT GC GGA GGA AGGT

domain b = N10

# thread sequence domains onto target structures

stickfigure.seq = a b

# specify stop conditions for normalized ensemble defect

# default: 1.0 (percent) for each target structure

stickfigure.stop = 1.0

# prevent sequence patterns

prevent = AAAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, YYYYY
Bibliography


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