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论文题目：HCR Utilization in Triggered Assembly of DNA Nanotube Structure

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# HCR Utilization in Triggered Assembly of DNA Nanotube Structure

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**Abstract：**

As the genetic material of most living organisms, DNA stabilizes inheritance with complementary base pairing. This unique property makes DNA a suitable material for the programmable construction of nanoscale structure that serves variety of functions. This is achieved by artificially designing the base sequence, pairing in a specific manner, thus changing the shape of the structure as a whole. In DNA nanotechnology, one way to realize large-scale DNA self-assembly is single-stranded tiles motif (SSTs) (Seeman 2003). In hybridization chain reaction (HCR), a trigger strand is used to initiate the hybridization of two types of DNA hairpins, achieving linear DNA replication. In this experiment, we combine the assemble structure of SSTs and self-trigger specialty of HCR, using loop trigger to initiate self-assembly of DNA hairpins layer by layer, forming a tube structure. To block the leakage in self-assembly system, we design cover strands to reduce any non-specific interactions between hairpin molecules. By incorporating DNA single-stranded tiles and hybridization chain reaction, this experiment successfully yielded triggerable DNA nanotubes.

**Keyword：** DNA self-assembly, HCR, AFM

## 1. introduction

In structural biology, DNA is readily used to construct desirable structures based on complementary base pairing principle. By artificially arranging the base sequence on the strength of base pairing, we can trigger self-assemble of single-stranded DNA, folding it into two- or three-dimensional nanostructures with controlled shapes and sizes. These structures can perform intended tasks, such as facilitating targeted dug transmission in the human body, detecting and controlling signals in trace analysis, and enabling biocomputers to store sheer volume of information within tiny spaces. Such nanostructures can be easily regulated, come in a variety of forms, and are of high biocompatibility. Thus, as nanoscale biological components, they have great potential in fields including drug transmission, signal control, and biological computation.

The arrangement of single-stranded DNA tiles enables templated single-strand DNA self-assembly, which, when compounding the mechanism with hybridization chain reaction （HCR）, makes possible for artificially triggered DNA self-assembly.

DNA self-assembly with this capability is particularly effective in terms of transforming and magnifying signals. The signal the trigger carries can be amplified when hairpins self-assemble under the presence of trigger strand. This is achieved by connecting the single-stranded DNA to fluorophores. With each DNA hairpin carrying one signal of fluorophores, when this combined structure is triggered into self-assembly process, the fluorophores would converge, thereby magnifying the signal. If each DNA strand is supposedly joined with the same type of fluorophores, the nanostructure assembled with ten DNA hairpins would transmit signals ten times as strong compared

to signals carried by a single hairpin.

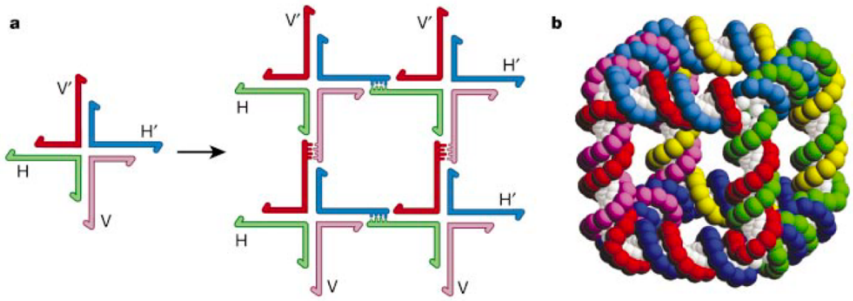


*Figure 1: **a**., According to the designed base sequence, every four single-stranded DNA assemble into a cruciform unit by complementary base pairing, with exposed sticky ends, where multiple cruciform units bind to form a web-like plane. **b.** The base sequence on **t**he cruciform units can be designed to produce complex, three-dimensional structures.*



## 1.1 Research background

### 1.1.1. Single-strand-tile motif :

Previous studies have shown the effectiveness of templated DNA self-assembly in the creating nanostructures with designed shapes. Connected by sticky ends of single-strand DNA or RNA, we can assemble DNA or RNA tiles into periodic and algorithmic two-dimensional lattices, extended ribbons and tubes, three-dimensional crystals, polyhedral, and simple finite two-dimensional shapes. (Wei, 2012) Nevertheless, it is still challenging to construct complex structures from a mass sample of unique, readily locatable DNA modules.

To tackle this issue, in 2012, Peng Yin and his team from Harvard University proposed a simple, alternative pathway to DNA self-assembly. With sticky end covering majority of a single-stranded tile (SST), the separate tiles are allowed to fully connect with each other like pieces of Lego--each one attaches to 4 adjacent SSTs, forming a

complex web-like structure. Designed using base pair principle to secure specific shape of structure beforehand, the desirable nanostructure is obtained by first mixing all the SSTs and then annealing. In this way, DNA self-assembly no longer necessitates a scaffold, in order us to freely set the size and complexity of the structure.

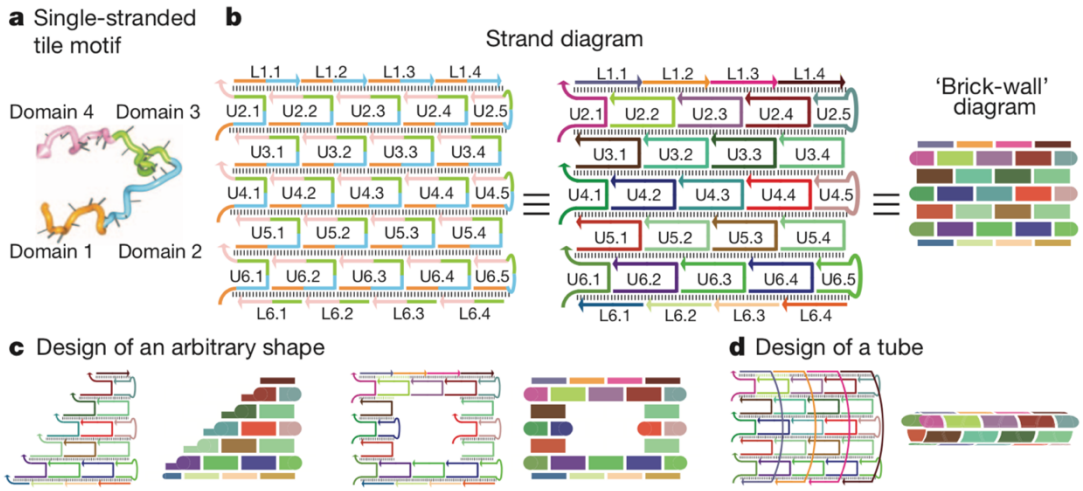


*Figure 2: The formation of a single-stranded tile (SST). **a**. A short strand DNA SST motif. **b**. The rectangular SST design. The left and middle diagram are two different views of the same secondary structure. Each standard (full) tile has a number of bases (labeled U) and each tile on the top and bottom boundary has half the number of bases (labeled L). The diagram on the right is a simplified "brick-wall" diagram. Standard tiles are presented as thick rectangles and boundary tiles as thin rectangles. The unstructured, single-stranded portion of boundary tiles are represented as round corners. Each strand has its unique base sequence, distinguished by color corresponding to the middle diagram in **a**. **c**. Part of the SST in diagram b designed as certain desirable shapes, such as triangles (left) or rectangular rings (right). **d**. A "brick wall" with two ends connected to form a tube with specific width and length.*



### 1.1.2 Hybridization chain reaction (HCR)

Biosensors employ different mechanisms in different procedures, including identifying targets, and transmitting and magnifying signals. Specifically, the increase in number of DNA templates during polymerase chain reaction (PCR) is an example of signal magnification. First, the primer identifies the target DNA template, prompting its algorithmic assembly in the presence of DNA polymerase and deoxynucleoside

triphosphate (dNTP). But PCR is not the only way to amplify signals using DNA. In fact, in 2004, a method suggested that DNA, after binding with the target molecule, can successfully identify and magnify signals without the assistance of external agents. (Evanko, 2004) This is demonstrated in hybridization chain reaction (HCR), which triggers the self-assembly of DNA nanostructure. HCR relies on multiple hairpin molecules recognizing each other, which serves as an enzyme-free substitution to quickly replicate the target DNA strand. The reactants in HCR involve one trigger and two different kinds of DNA hairpin molecules. When the trigger is added to the stable mixture comprising hairpins, a voltage is introduced by complimentary base paring. This difference in electric potential would "open up" the hairpin, bend it straight, and expose a piece of unpaired single DNA strand, which creates a sticky end. This piece of strand, in turn, would "open up" another hairpin molecule, while the latter discloses, again, a piece of single-stranded DNA in a region corresponding with the first one, and the cycle continues until all hairpins are exhausted. This chain reaction is to result in a double helix having a sticky end.

HCR differs from PCR, which promotes algorithmic amplification of DNA, in that HCR stimulates DNA replication in a linear manner. HCR is simple to design, highly sensitive, and free of constant changes of temperature. For these reasons, HCR is involved with applications in the field of bioimaging. (Bi, 2007)

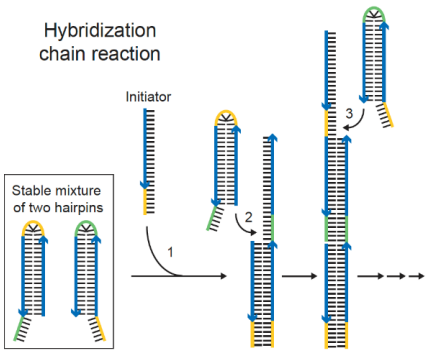


*Figure 3: Visual representation of hybridization chain reaction (HCR). Triggers are added to a stable mixture of two types of hairpins to initiate HCR.*

DNA nanotube structure can be used in trace detection. As showed in the picture, during HCR, the linear trigger strand can induce DNA hairpin to open up and bind, gradually forming a triangle structure with decreasing number of hairpins for each layer. With each hairpin carrying a fluorescent group, separate hairpins bind to trigger strand with limited length through HCR under excited state of certain signal, enabling increase of signal.

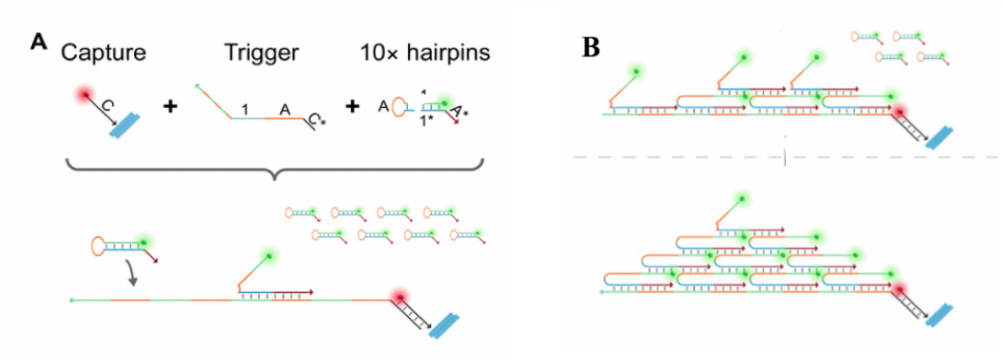


*Figure 4: Triggered assembly of denatured triangular fluorophore cluster consisting of 10 fluorophore-carrying hairpins. (A) Hybridization between the trigger and the hairpins. The trigger consists of 4 connected domains. The hairpins exist metastably without the trigger and self-assemble into the designed structure in the presence of the trigger. (B) The introduction of repeated single-stranded triggers initiates the dynamic capture of hairpins, assembling a fluorophore monomer and producing a second row of binding sites, where a third row of fewer hairpins can attach to, and the pattern continues until all ten hairpins are used up, with no vacant binding site, forming a triangular DNA fluorophore cluster.*

**1.2 Purpose**

This experiment stands out because the development of the designed structure from hairpins can be self-initiated by trigger strand through the combination of SSTs and hybridization chain reaction (HCR). In addition, the nanostructure can be artificially devised in terms of both shapes and sizes. These two advantages give rise to a triggerable structure with great flexibility and a wide range of applications. It can also be further processed into more specialized structures if needed.

If the trigger is linked head-to-tail instead of presenting a linear structure in previous studies, the number of harpins attached would not diminish from row to row, instead making up a tube-like structure with much more compatibility for additional hairpins. On the exposed end of each hairpin, a short base strand called a "cover" is designed to be placed to pair with this area. The intention is to prevent "leakage", which occurs when the sticky end of one hairpin binds to that of another without the initiation of a trigger. The next step is to adjust the concentrations of cover, trigger, and hairpin molecules to specific values so that the nanotube can be successfully produced. After the DNA tube yields, we can observe its shape through atomic force microscope (AFM).

In previous application of HCR, the strength of the fluorescence is restricted by the finite length of the linear trigger and the rule of hairpin binding. Again, the number of hairpins, and thus fluorophores, reduces from one row to the next, constituting a triangle with limited fluorescence. Therefore, an annular trigger is designed to assure that each layer of the tube contains the same number of hairpins. By controlling the number of layers present, we can then adjust the strength of the signal to varying degrees with

respect to expected concentration of molecules under test.

Also, notice that the hairpin molecules used have the same shape and size. Hence, the length of the nanotube can be measured with the number layers it holds. This approach is extremely accurate for nanoscale ruler which can be used in field of structure measurement.

## 2. Method

### 2.1 Sequence Design

#### 2.1.1 Trigger

Due to various limitations of artificially synthesized single-stranded DNA, generally, 200 nt is the maximal length below which DNA strands with acceptable price and accurate sequence can be synthesized. That said, we designed and ordered triggers no longer than 200 nt. The two ends of the trigger are ligation regions, where complimentary short DNA strands would bind to. Then, the entire DNA strand (trigger) would link head-to-tail, undergo ligation, and form a ring-like structure.

On the trigger are 6 spots where hairpin molecules can attach to. These hairpins would go through hybridization chain reaction (HCR) and lie in layers, thereby constructing a DNA nanotube. Each layer of the tube is composed of 6 hairpins, namely, 12 DNA double helices. According to our mathematical model, the diameter of the nanotube is approximately 8 nm.

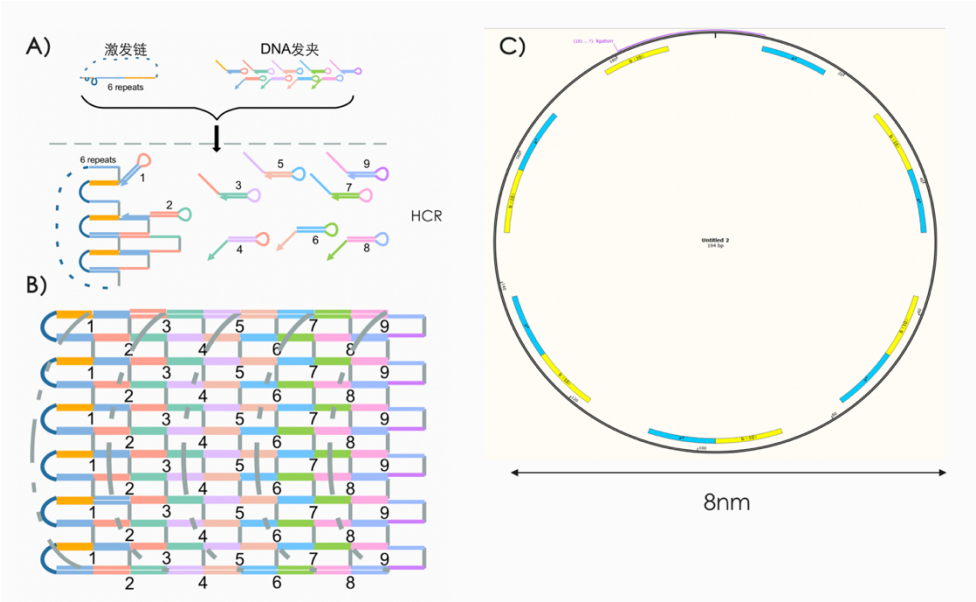


*Figure 5: **A**) HCR between a trigger and DNA hairpins. **B**) Assembled DNA nanotube. **C**) Loop trigger strand.*

### 2.1.2 DNA hairpin molecule

This experiment employed DNA hairpins that are 40 bp long. For convenience, each hairpin can be visualized as comprising four 10-bp-long sites (site 1, site 2, site 3, site 4) from the 5' end to the 3' end. We came up with two distinctive designs for hairpin molecules.

In design A, site 1 is complementary to site 3 and site 2 to site 4. Note that it is virtually impossible for both sets of complementary sites to pair up on a single hairpin for that would distort the molecule's overall shape. Depending on which two sites join together, two types of DNA hairpins are produced after annealing, each having a protruding site (1 or 4) to bind to adjacent layers, respectively. When a trigger is present, these hairpins would undergo HCR, where site 2 and 3 on one layer would pair with

site 4 and 1 on another layer, thus opening up the hairpin, which, in this linear state, acts as the basic unit of the tubular structure.

In design B, site 1 and 3 are still complementary, but site 2 and 4 are not. Consequently, there will be a certain kind of hairpin formed after annealing, with site 1 attaches to site 3, which means that site 4 is the only possible site to which another hairpin molecule could attach. Similar to design A, design B also prompts hairpins to open up and bind to adjacent layers.

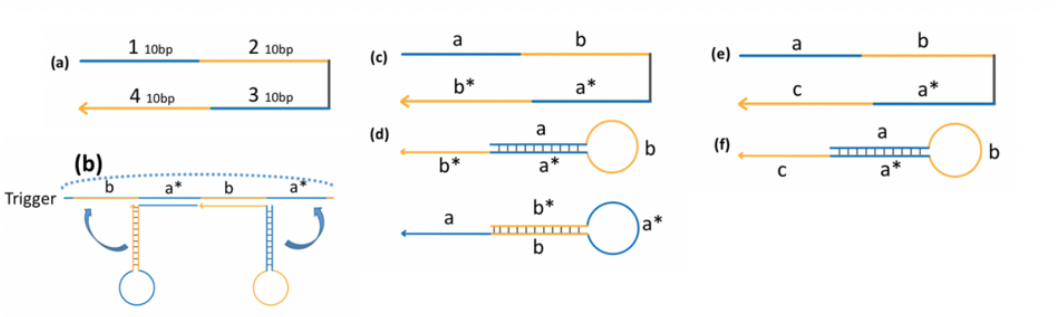


*Figure 6: **a**) Position of the four sites in a DNA hairpin. **b**) Hairpins bind to the trigger. **c**) Design A: site a pairs with site a* and site b pairs with site b* by complementary base pairing. **d**) Two types of DNA hairpins formed in design A after annealing. **e**) Design B: site a pairs with site a*, but site b does not pair with site c because their bases are not complementary. **f**) The single type of DNA hairpin formed in design B after annealing.*

### 2.1.3 Finite and infinite design of DNA tube

If the DNA hairpins on each layer have the same sequence (Figure 6.b), in other word forming the nanotube with cycle of same hairpins, theoretically speaking, we can construct infinitely long DNA nanotube. Whereas by having hairpins with different sequences on each layer, so long as these hairpins are opened up by the complementary bases on the adjacent layer, we are able to adjust the length of the DNA nanotube simply

by altering the number of layers of hairpins.

If the hairpins are to add up infinitely long, their sequences will all be determined by the trigger. This is because the only way to open up a hairpin is for them to pair with one of the 6 spots on the trigger.

In the finite adding up of hairpins, however, the trigger only determines the sequence of the first layer. On any other layers of the nanotube, hairpins' sequences are determined by the layer before them. For both design A and B, we devised the base sequences for all 30 different layers of DNA hairpins.

## 3. Result and Analysis

### 3.1. Reaction condition

#### 3.1.1. Testing effect of buffer solution concentration

This experiment tested how concentration of Mg2+ in buffer solution affects the DNA nanotube yield.

System（20μl）

| Reactant | Volume（μl） |
|---|---|
| Mg2+ buffer solution | 2 |
| DNA hairpin tiles（9 layers）4μM | 4 |
| Trigger 1μM | 1.6 |
| Deionized water | 12.4 |

DNA hairpins are first subjected to annealing and cooled down from 90℃ to 4℃ in 5 minutes. After that, triggers are added to the solution until reaching a 0.08μM final

concentration, meanwhile adjusting hairpins' concentration to 0.8μM. Next, the mixture is transferred to a PCR thermal cycler, where it react for 48 hours. Since the goal was to determine the optimal Mg2+ concentration in the 0.5 X TE buffer, we altered the concentration of magnesium ions to numerous different values (2.5 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM). The solution with each Mg2+ concentration would then separately undergo agarose gel electrophoresis. In this way, according to the brightness and the degree of diffusion of the resulting structures, we were able to determine how well the DNA nanotube could be fabricated under varying magnesium ion buffer concentration and attain the concentration that would yield the most favorable DNA tube.

The results suggested that the DNA nanotube could be well assembled under all the Mg2+ concentrations mentioned (2.5 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM final solution). However, the nanotube would be barely visible under concentration above 50mM. Whereas when the Mg2+ concentration was below 20mM, the nanotube remained relatively clear.

We thus successfully estimated that a final buffer concentration of 20mM best assisted the construction of the DNA nanotube.



**3.1.2 Testing effect of temperature**

This experiment investigated the effect of temperature on the construction of the DNA nanotube.

System (20μl)

| Reactant | Volume（μl） |
|---|---|
| Mg2+ buffer solution（5*TE 200mM） | 2 |
| DNA hairpin tiles（9 layers）4μM | 4 |
| Triggers 1μM | 1.6 |
| Deionized water | 12.4 |

Again, the DNA hairpin molecules first underwent annealing that involved cooling from 90℃ to 4℃. Triggers were added so that hairpins' final concentration became 0.8μM and triggers' final concentration was adjusted to 0.08μM. The mixture was again put into the PCR thermal cycler and given 48 hours to react. We aimed to derive two influence factors: how different temperature and whether constant temperature or decreasing gradient influence the construction of DNA tube. This was achieved by setting the PCR to different temperatures or gradient then record the brightness of the stripe that represent fully developed DNA nanotube in agarose gel electrophoresis.

We first conducted the experiment multiple times with different, fixed temperature. (specifically 30℃, 32.2℃, 36.7℃, 39.8℃, 42℃, 42.9℃, 43.8℃, 45.1℃, 46.4 ℃, 47.6℃, 48.9℃, 50.2℃, and 52℃. due to temperature scale of the PCR thermal cycler.) The observation in agarose gel electrophoresis showed that tube structure can be detected at all temperatures mentioned (range from 30℃ to 52℃) , yet the DNA nanotube was best constructed (most clearly observed) at around 42℃. On the other hand, below 32.2℃, the tube was not clearly visible thus poorly assembled.

We also studied the formation of the DNA nanotube under decreasing temperature over time. Dividing 48 hours into three parts, in the first gradient, we place the solution

was placed under temperatures ranging from 42℃ to 52℃（42℃, 45.1℃, 47.6℃, 50.2℃, 52℃）. The temperature was then changed to 40℃ in gradient 2 and lastly 35℃ in gradient 3. Each gradient lasted 16 hours, therefore totaling 48 hours as previous experiments did. Overall, observation in agarose gel electrophoresis indicated that, at decreasing temperature, the DNA nanostructure was not assembled as well as at constant temperature. Specifically, no recognizable DNA tube formed above 47.6℃. Even when it did at between 42℃ and 45.1℃, the structure was vaguely visible.

The procedure above ascertained the optimal temperature to be constant at around 42℃.

### 3.1.3 Testing effect of reaction time

This experiment analyzed how the duration of annealing affected the assembly of DNA nanotube.

System（20μl）：

| Reactant | Volume（μl） |
|---|---|
| Mg2+ buffer solution（5*TE 200mM） | 2 |
| DNA hairpin tiles（9 layers）4μM | 4 |
| Triggers 1μM | 1.6 |
| Deionized water | 12.4 |

DNA hairpins are first subjected to annealing and cooled down from 90℃ to 4℃ in 5 minutes. After that, triggers are added to the solution until reaching a 0.08μM final concentration, meanwhile adjusting hairpins' concentration to 0.8μM. Next, we set the

temperature of the PCR thermal cycler to be a constant value of 42 ℃. The only variable being manipulated was the time of the reaction. Statistically speaking, the assembly of hairpin molecules is a matter of probability. That said, the longer it took for the mixture to react, the more likely a complete nanotube structure would form. Therefore, we set the reaction time to be relatively long (17h, 24h, and 48h) to ensure successful yielding. The samples were again observed under agarose gel electrophoresis after their respective reaction duration. To analyze the samples, we noted the brightness of stripes that represent developed DNA tube.

According to our observation, the nanotube was clearly visible regardless of the duration. The target stripe in agarose gel is comparatively wide after 17 hours annealing, having a length of 200 to 300 bp with conspicuously diffusion, comprising of smaller substructures of various lengths. 24 hours of annealing produced a more converged target stripe, but diffusion still existed, although to a smaller degree than the 17 hours' result. The DNA tube put to react for 48 hours resulted in the least diffused nanotube of 300 to 400 bp. We concluded that the hairpins assembled most successfully in long period of annealing. For this reason, 48 hours was determined to be the appropriate duration.



### 3.2. Self-assembly of finitely and infinitely long DNA nanotubes

#### 3.2.1 Triggerable self-assembly of finitely long DNA nanotube

By adjusting the number of layers of hairpins, we could alter the length of the DNA nanotube. The following experiment serves to testify this conception.

Using the reactants below, we tested the construction of nanotubes made of less than 9 layers of DNA hairpins.

| Reactants | Volume（μl） |
|---|---|
| Mg2+ buffer solution（5*TE 200mM） | 2 |
| DNA hairpin tiles（4-9 layers）4μM | 4 |
| Triggers 1μM | 1.6 |
| Deionized water | 12.4 |

This experiment, based on hairpin design A (the cycle of a single type of hairpin that able to potentially produce infinite layers), constructed DNA nanotubes consisting of 4 to 9 layers of hairpins. Initially, these hairpins had a concentration of 4μM/layer. After adding to reaction system and adjust to the desirable concentration of 0.8 μM/layer, the hairpin molecules undergo 5 minutes of annealing before triggers are added. Next, the mixture was transferred to the PCR thermal cycler, where it would react at a constant temperature of 42℃ for 48 hours. The sample was then extracted and subjected to electrophoresis of 2% agarose gel under a voltage of 90 V for 90 minutes, generating an electrophoregram.

The result illustrates that, for DNA nanotubes comprising 4 to 9 layers of hairpins, the size of the desirable structure (the upper portion of the DNA band in Figure 7) increases along with the number of hairpin layers. DNA tubes consisting of 4 to 7 layers of hairpins are clearly visible, while those made up of 8 to 9 layers are more diffused but still recognizable.

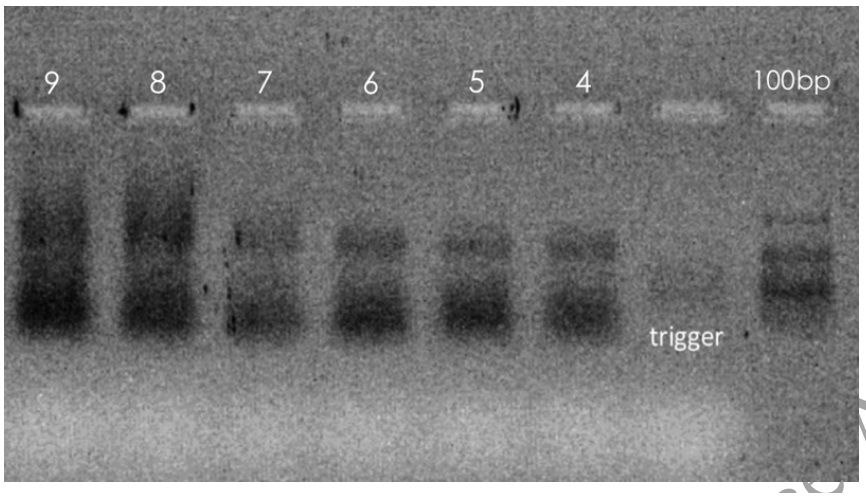


*Figure 7: Electrophoregram of DNA nanotubes comprising 4 to 9 layers of hairpins.*
*From left to right are respectively DNA tubes made up of 9, 8, 7, 6, 5, and 4 layers of hairpins and a control group of 200bp loop trigger strand. Their lengths are measured with a DNA ladder 100 bp long.*

In order to further testify the fabrication of DNA nanotubes with more layers of hairpins, we designed the following experiment using both design A and B. Hairpins with concentration of 4μM/layer would mix together. Thirty layers of hairpins were to split into 7 distinct groups: 1) 1 to 9 layers; 2)10 to 12 layers; 3)13 to 15 layers; 4)16 to 18 layers; 5) 19 to 21 layers; 6)22 to 24 layers; 7) 27 to 30 layers. Initially, only group 1) and 1 μM of triggers were present in the solution. Other groups of hairpin layers were later added to enlarge the DNA structure. Finally, ddH20 were added, making a total volume of 20 μl.

| Number of layers | 9 layers of hairpin | Mg2+ buffer solution | Hairpin | Deionized water | Trigger |
|---|---|---|---|---|---|
| 1-9 | 2μl | 2μl 5*TE | 0 | 14.4μl | 1.6μl |
| 1-12 | | 200mM | 2μl | 12.4μl | |
| 1-15 | | | 4μl | 10.4μl | |

| | | |
|---|---|---|
| 1-18 | 6μl | 8.4μl |
| 1-21 | 8μl | 6.4μl |
| 1-24 | 10μl | 4.4μl |
| 1-28 | 12μl | 2.4μl |
| 1-30 | 14μl | 0.4μl |

Within the solution, the final concentration of one particular type of hairpin was 0.8 μM and that of triggers is 0.08 μM. Hairpins would undergo 5 minutes of annealing, cooled down from 90℃ to 4℃ before triggers were added. Then, the entire mixture was put into the PCR thermal cycler to react at 42℃ for 48 hours. Lastly, the sample was subjected to electrophoresis of 2% agarose gel under 90 V of voltage for 90 minutes.

The result suggested that DNA nanotubes comprising 12 to 30 layers of hairpins do not form clear target stripes in gel result. Fortunately, we can still determine the general trend of size change according to the extent of diffusion. In design B, the structures became more and more diffused as the number of hairpin layers increases from 9 to 30. But for design A, there was not an observable upward trend between diffusion and number of layers: although diffusion did grow more pronounced from 9 to 15 layers, it decreased from 18 to 30, with the nanotube comprising 15 layers being the largest structure. The explanation for this phenomenon is still under research.

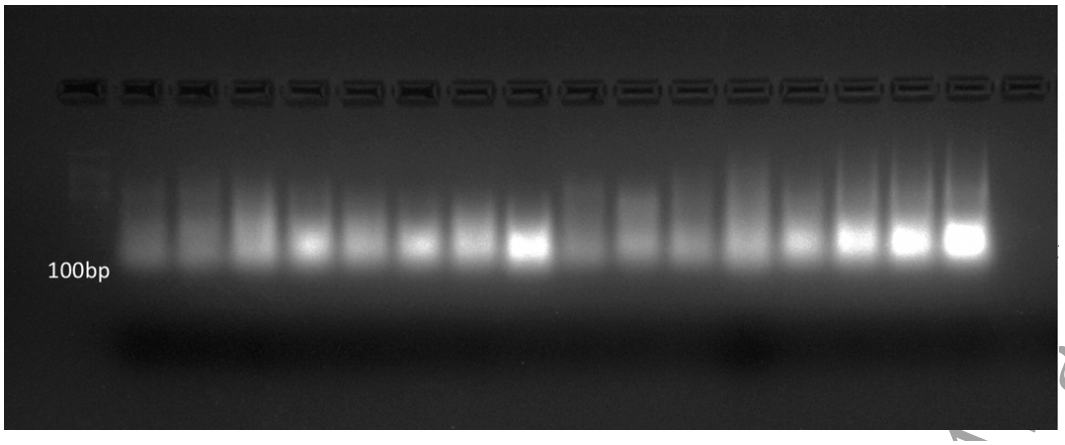


*Figure 8: Electrophoregram of DNA nanotubes comprising 9 to 30 layers. From left to right are respectively DNA ladder (100 bp), DNA nanotubes (comprising 9 to 30 layers) in design A, DNA nanotubes (comprising 9 to 30 layers) in design B.*



### 3.2.2 Triggered self-assembly of infinitely long DNA nanotube

With the goal of exploring the formation of infinitely long DNA nanotube, we designed the following experiment:

System（20μl）:

| Reactants | Volume（μl） |
|---|---|
| Mg2+ buffer solution（5*TE 200mM） | 2 |
| DNA hairpin tiles（infinitely long）4μM | 4 |
| Triggers 1μM | 1.6 |
| Deionized water | 12.4 |

Design A employed cycling of a single kind of hairpin molecule, while design B used cycling of two different types. In design A, the final concentration of hairpin is 0.8 μM. The initial concentration of trigger molecules was 1μM and the final concentration was 0.08μM. Similar to previous procedures, three sample was put into the PCR thermal

cycler and let to react at multiple constant temperatures (40.2℃, 42.5℃, 43.7℃, respectively). After 48 hours, the mixture was subjected to electrophoresis of 2% agarose gel under a voltage 90 V for 90 minutes.

With respected to 100 bp-long DNA ladder, we observed pronounced stratification, inferring the bottom layer to be single-stranded DNA hairpins (shorter than 100 bp) that failed to bind with other hairpin molecules. The top layer is the desirable structure with a length of 300 to 400 bp. The structure yielded under the three temperatures (40.2℃, 42.5℃, 43.7℃) did not display significant differences. Design B is slightly larger than A, but both designs failed to produce the ideal, infinitely long DNA structure. The reason is still under research.


*Figure 9: Image of infinitely long DNA nanotube, shot in agarose gel electrophoresis. The structures, from left to right, are: design A at 40.2C°, 42.5C° and 43.7C°, and design B at 40.2C°, 42.5C° and 43.7C°.*



### 3.3. Preventing leakage with cover strands

#### 3.3.1. The effect of covers

Experimental data showed that after annealing at constant temperature, layers of hairpin could form structures without trigger strand, an issue named “leakage”. To

control the construction of DNA nanotubes, possible combination between hairpins should be avoid when trigger strand is not present in the system. Thus, we design covers to minimize leakage. Cover strands of 10-bp-long pair with exposure end of hairpin, ensuring that the hairpin won't bind with the previous layer without trigger strand's influence.

Design A yields two different kinds of DNA hairpins and therefore two possible exposure sites with distinct base sequences. Hence, for design A, two different covers are designed for the two 10-bp-long site respectively. Design B only produces a single kind of hairpin molecule and thus one site is exposed. Accordingly, a single type of complementary cover is sufficient to prevent leakage.

System（20μl）:

| Mg2+ buffer | 1 to 9 layers of hairpin（4μM） | Cover（4μM） | Deionized water |
|---|---|---|---|
| 2μl 5*TE | 4μl | 1μl | 13μl |
| 200mM | | 2μl | 12μl |
| | | 3μl | 11μl |
| | | 4μl | 10μl |
| | | 5μl | 9μl |

Every layer of hairpins has an initial concentration of 4μM and a final concentration of 0.8μM. The buffer, hairpins and deionized water are mixed and subjected to annealing from 90℃ to 4℃. Covers, each with an initial concentration of 4μM, are added to the mixture, which is then transferred to a PCR thermal cycler at

constantly 42℃ for 48 hours. This procedure aim to test how the ratio between hairpins and covers would affect the degree of leakage.

Observation under agarose gel electrophoresis suggested that diffusion was evident when covers have initial concentration between 1μM and 2μM, final concentration between 0.2μM to 0.4μM. We could observe target strip of DNA tube structure on gel result. When the covers' initial concentration is above 3μM (and the final concentration above 0.6μM), there is no observable target strip, DNA structure longer than 100 bp, successfully blocking leakage. Considering material cost and blocking effect, we choose the proportion of hairpin and cover in the system to be 1:1.

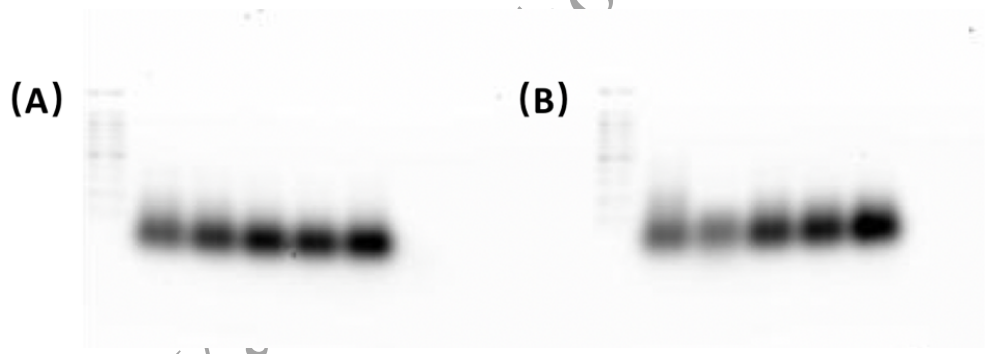


*Figure 10: Electrophoretogram (under agarose gel electrophoresis) under different cover concentrations in the absence of triggers. From left to right in each image are 100-bp-long DNA ladder, and samples with final cover concentration of 0.2uM to 1uM. (A) DNA hairpins and covers in design A. (B)DNA hairpins and covers in design B.*



### 3.3.2. Replacing covers with triggers

When triggers are added, they will bind to the exposure end of hairpin and replace the cover. This is because, compared to covers, triggers are longer, with more bases complementary to the hairpin thus greater energy contained. The sheer attraction

enables triggers to attach and covers to fall off, initiating the assembly of DNA structure.

System A（20μl）：

| Reactant | Volume（μl） |
|---|---|
| Mg2+ buffer（5*TE 200mM） | 2 |
| DNA hairpin tile（infinite）4μM | 4 |
| Covers 4μM | 4 |
| Triggers1μM | 1.6 |
| Deionized water | 8.4 |

System B（20μl）：

| Reactant | Volume（μl） |
|---|---|
| Mg2+ buffer（5*TE 200mM） | 2 |
| DNA hairpin tile（infinite）4μM | 4 |
| Covers 4μM | 4 |
| Deionized water | 12.4 |

This experiment employed a single kind of DNA hairpin (infinite design). In group A, each layer of hairpin had an initial concentration of 4μM, final concentration of 0.8μM. The mixture (Mg2+ buffer, hairpins and deionized water) undergo annealing from 90℃ to 4℃. Covers (initially 4μM and finally 0.8μM) and triggers (initially 1μM and finally 0.08μM) are then added.(Note that triggers are optional) The mixture is transferred to the PCR thermal cycler where two groups of experiments are conducted,

both based on design A where both 1,3 and 2,4 sites in a hairpin can bind.

In the first experiment, we set PCR at constant temperature of 42℃ for 48 hours. After annealing, we compare the formation of DNA nanotubes of both group A and B. Triggers were only added in group A.

Agarose gel electrophoresis reveals that triggers successfully replaced covers and initiated DNA self-assembly. In group A, the nanotube form is larger than the trigger strand in control group, proving the target stripe represent DNA tube structure rather than triggers. When trigger is not present, covers were able to prevent leakage, resulting in nearly no diffusion.

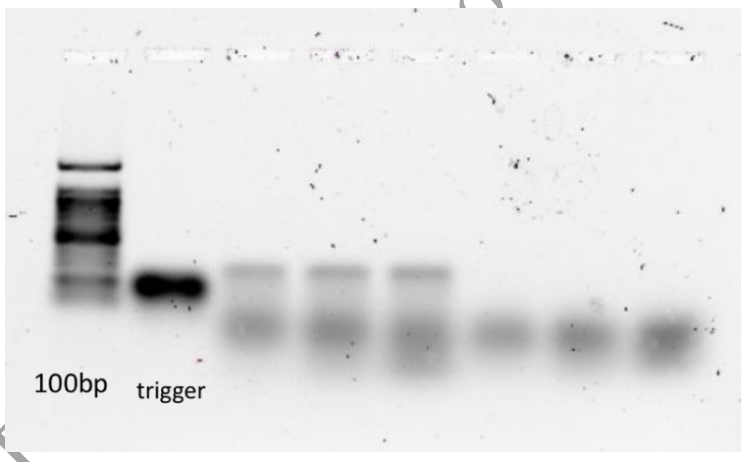


*Figure 11: Electrophoretogram (under agarose gel electrophoresis) after 42 C°, 48-hour annealing. From left to right are 100-bp-long DNA ladder, circular trigger, 3 samples containing both trigger and covers, and 3 containing only covers.*

The second group of experiment explores in impact of temperature on degree of replacement. We set constant temperature 38℃、42℃、45℃、48℃、50℃ on PCR machine, timing 48 hours. We present parallel test to the first group respectively.

Electrophoresis showed that temperature has no decisive impact on how well

triggers can replace covers. Apart from unbind hairpins, there are two observable stripes for group A gel test. The first stripe represents structure 500bp- to 600-bp-long, while the second stripe represent structure 200bp- to 300-bp-long. thus we conclude that the upper layer is the target stripe, DNA tube structure initiated by trigger. As for group B, apart from the unbind hairpins, there is one observable stripe above, length of 200 to 300 bp, without large structure formed. Thus we conclude that at 38℃ to 50℃, covers could successfully block the initiation of DNA nanotube.

The explanation the stripe of 200-300bp long is still under research.

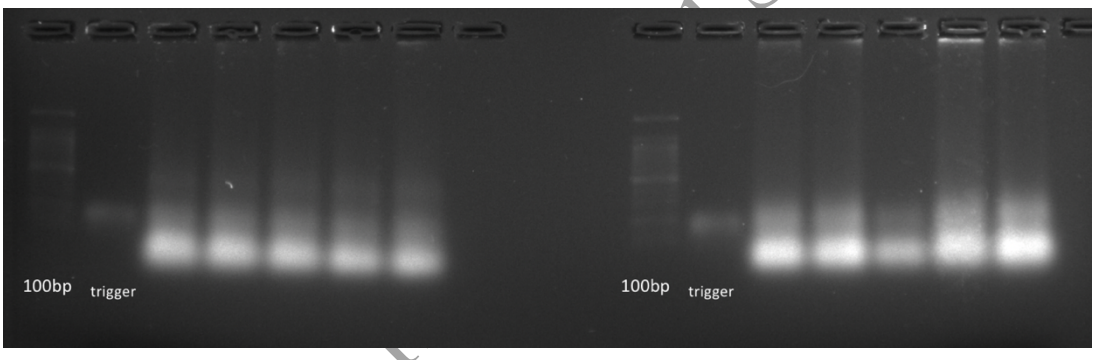


*Figure 12: Group A (left) and group B (right). In each group, from left to right are results of 48-hour annealing at 38C°, 42C°, 45C°, 48C°, and 50C°.*

**3.4 Effect of chain mass on the reaction**

Procedure of DNA purification:

1. Prepare page gel solution (10 ml) of 12% concentration;

2. Run the gel: electrify the solution with agarose gel under voltage of 300V for 2 hours;

3. Dye the gel: use diluted syber safe pigment (1:1000) for 5 minutes to dye the gel for 5 min. The gel is then taken out and dipped in ddH20 for 5 minutes.

4. Recover target gel: Under ultraviolet light, target gel stripe is recovered and crushed

before immersed in 1 milliliter of 1*TE buffer for 30 minutes. The sample is cooled down to -20 ℃ and rests for an hour before transferred to a 4℃ 2000rpm centrifuge machine for one night.

5. The centrifuge machine is precooled to 4℃ and set to 1400 rpm for 10 minutes.500μl of 1*TE buffer is added and the machine is set to 2000 rpm for 1.5 hours.

6. With a syringe, the sample is sent to a 10 ml EP tube, where it is joined by 3 ml of n-butanol. The mixture is centrifuged for at 6000 rpm at 20℃ for 3 minutes. After the liquid becomes stratified, 0.5 ml of the bottom layer is carefully taken and injected into a 1.5 ml EP tube.

7. 55 ul of 3M sodium acetate and 1 ml of absolute ethyl alcohol are added, then transfer to centrifuge machine at 14000 rpm for 10 minutes. Remove the upper liquid.

8. 1 ml of absolute ethyl alcohol (precooled to -20℃) is added, then the sample is transferred into centrifuge machine at 14000 rpm for 10 minutes. The upper layer of the liquid is removed, and the liquid is let to rest for 3min to remove ethanol.

9. 400 ul of ddH2O is added to dissolve the DNA. Its concentration is measured with a NanoDrop spectrometer.

Owing to the lowered concentration of purified single-stranded DNA, the DNA stripes are barely observable in agarose gel electrophoresis. Therefore, we scan the purified 9-layered DNA nanotube using transmission electron microscopy (TEM). According to the design, the nanotube of 9 layers is around 30 nanometers long with a diameter of 8

nanometers. It appears plain under TEM, with clearly identifiable tube structure.

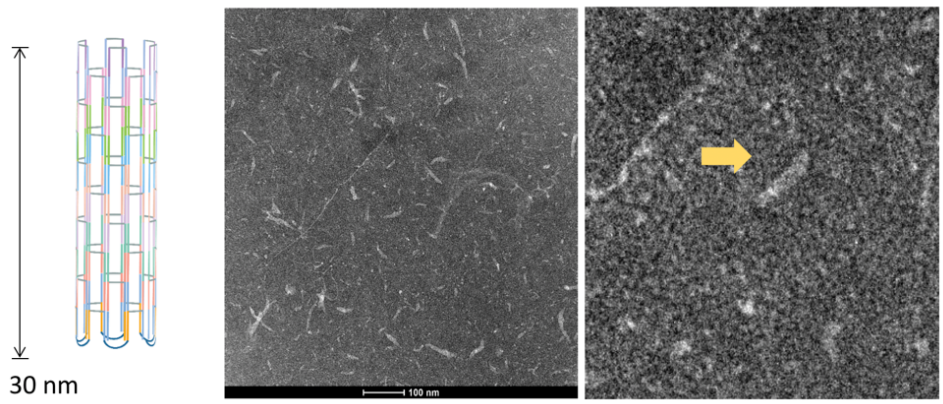


*Figure 13: Diagram of 9-layered DNA nanotube (left) and its image under TEM (right).*



## 4. Discussion

### 4.1. Remaining challenge

#### 4.1.1. Limit on the size of DNA nanotube

Experiments show that, in design A (finite length), the size of DNA nanotube is not solely determined by the number of hairpin layers. Agarose gel electrophoresis shows that, from hairpin layer 12 to 30, the target DNA stripe does not change significantly in appearance as we expected. Diffusion from 12 to 30 layers increases at first but lessens later on, suggesting a curve pattern of the size of DNA nanotube instead of a linear one, which indicates that the length of tube does not increase continuously with layers of hairpins after reaching a specific degree. . However, design B (finite length) produces a different outcome: conclude from level of diffusion from 12 to 30 layers, the structure displays an linear pattern as expected, indicating that the length of DNA nano tube

successfully increase with hairpin layers.

A review on both designs leads to the following speculation: any given hairpin in design A either pairs site 1 with site 3 or site 2 with site 4, spontaneously form two types of hairpins after annealing, exposing site 1 or 4 as binding site. Every hairpin in design B binds site 1 with site 3, exposing site 4 as only possible binding site after annealing. Therefore, hairpins in design A are less likely to bind to adjacent layers of hairpins compares to design B, and this likelihood further diminishes with increasing number of hairpin layers, generating the curve pattern in gel test when layers increase. When the number of layers reaches a certain value, the DNA nanotube ceases to grow in size.

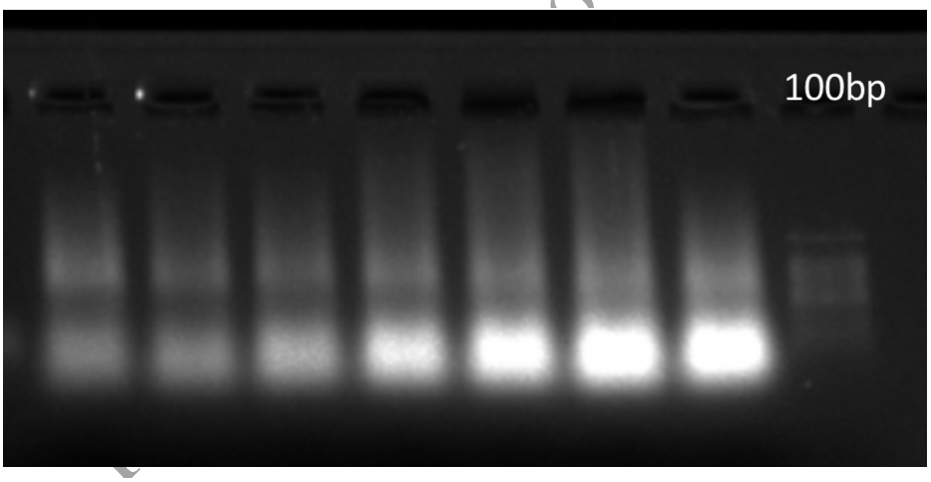


*Figure 14: Electropherogram of target stripes of 12 to 30 layers, showing a curvilinear relationship between the number of layers and diffusion.*



Furthermore, design A (single type if hairpin form infinite structure in theory) fails to yield infinitely long DNA nanotube. Theoretically, such infinite structure can be constructed with either one or two types of hairpins. Our experiment with design A produces obvious target stripe in gel test between 500 to 600 bp, a length comparable

to that of 12 layers structure. Using two kinds of hairpins to circulate, we gain relatively larger structures, some being more than 1000-bp-long, there is no obvious target stripe. We surmise that due to the limited length of trigger strand, limited diameter of DNA nanotube, when the DNA nano tube increase in length, the binding site of hairpins destabilizes or fractures, resulting in structures with generally restricted length. The few successfully formed large structures appear as diffusions in gel test rather than target stripes, while the majority of structures are either less diffused or fractured stripes with a length of 500 to 600 bp.

#### 4.1.2. The formation of unknown stripes

In electropherogram gel test, hairpins that don't construct DNA nano tube exist in the system as stripes at the bottom of gel. The length of tube structure represented by target stripes can be known by number of DNA hairpins it contains. However, result shows that there is an additional stripe between the bottom stripe and the target stripes regardless of the trigger's presence, 100 to 200 bp long, suggesting they are not unbound triggers. In electropherogram gel test, these stripes do not brighten as more hairpin layers are added, which implies number of this unidentified structure in the system remains steady, independent of hairpin layers.

Under atomic force microscope (AFM), the unknown stripes prove not to be DNA nanotubes, but filiform structures instead. We speculate that they form from random binding between hairpin molecules during annealing, as opposed to those binding in layers, forming random structure without specific shapes. The cause of their formation

and ways to avoid them are still under research.

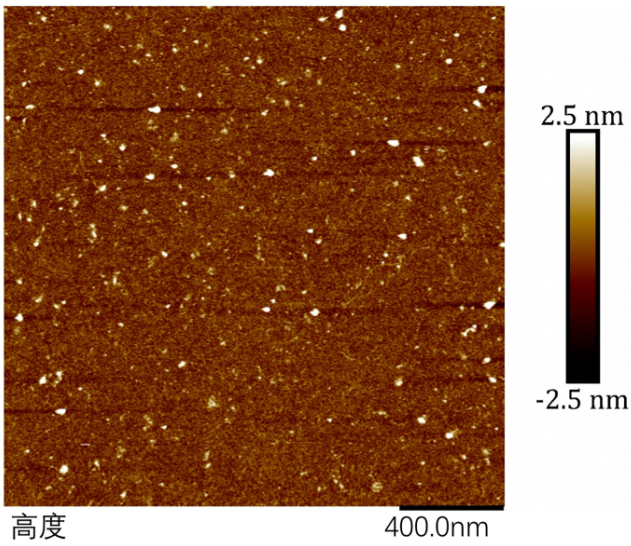


*Figure 15: Image of 100- to 200-bp long unknown stripes under AFM.*

### 4.2. Future prospects

In our next experiment, when the formation for DNA nanotube is further stabilized, we hope to utilize this structure as signal amplifiers. One possible pathway is to connect fluorophores and sensors to triggers and hairpins, triggering the assembly of DNA nanotube when specific signals are detected. After sensor detect the target signal, fluorophore-carrying hairpins will assemble into a readily visible flare available for instrument to detect. We further hope to test how different numbers of hairpin layers affect the result of amplification by altering the signal intensity. More layers of hairpin should be added to the system when the signal is relatively weak and less layers of hairpin should be added when the signal is relatively strong, thereby arriving at a relationship between signal intensity and the number of hairpin layers. (Woehrstein, 2017)

## 5. Conclusion

This experiment combines HCR and DNA nanotechnology, successfully controlled the size of DNA tube structure with layers of DNA hairpin. Through experiments, we find that under 5*TE 200mM Mg ion buffer, 42℃ constant temperature annealing, 48 hour or more reaction time, clear and stable DNA nanotubes can be observed through AGE，AFM and TEM. Assuring specific kind of hairpin for single layer can increase the likelihood of forming the structure, comparing to the design in which two random kinds with hairpin with site 1 and 3, site 2 and 4 can both pair. Because we can operate size of DNA nanotube, different level of signal expansion can be set according to different concentration of target signal, holding potential value to utilize.

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**Appendix**

1.trigger strand （5‘-3‘）:

CGTTATCATTAGGTATCTTTTTTTTTTCGACTTGAACATTAGGTATCTTTTTTTTTTCGACT
TGAACATTAGGTATCTTTTTTTTTTCGACTTGAACATTAGGTATCTTTTTTTTTTCGACTT
GAACATTAGGTATCTTTTTTTTTTCGACTTGAACATTAGGTATCTTTTTTTTTTCGACTTG
AACTAGCTGT

Trigger ligation strand：5’-GATAACGACAGCTAGTTCAA-3’

2. infinite DNA nanotube design（5‘-3‘）:

Single layer circulation：GATACCTAATCGACTTGAACATTAGGTATCGTTCAAGTCG

Double layer circulation：GATACCTAATCGACTTGAACGTCATGACTGGTTCAAGTCG
CGACTTGAACATTAGGTATCGTTCAAGTCGCAGTCATGAC

3. finite DNA nanotube design A

| layer | DNA base sequence（5‘-3‘） | cover（5‘-3‘） |
|---|---|---|
| 1 | GATACCTAATCGACTTGAACCTCCAGTGTCGTTCAAGTCG | ATTAGGTATC |
| 2 | GACACTGGACCACGTGGCACCTCCAGTGTCGTTCAAGTCG | CGACTTGAAC |
| 3 | GACACTGGACCACGTGGCACTTACCTGCATGTGCCACGTG | GTCCAGTGTC |
| 4 | ATGCAGGTAAGAAAGTAGACTTACCTGCATGTGCCACGTG | CACGTGGCAC |
| 5 | ATGCAGGTAAGAAAGTAGACGAGATAGGAGGTCTACTTTC | TTACCTGCAT |
| 6 | CTCCTATCTCCGGAAGTATTGAGATAGGAGGTCTACTTTC | GAAAGTAGAC |
| 7 | CTCCTATCTCCGGAAGTATTGTAGTGAAGGAATACTTCCG | GAGATAGGAG |
| 8 | CCTTCACTACCCGCCATCCGGTAGTGAAGGAATACTTCCG | CGGAAGTATT |
| 9 | CCTTCACTACCCGCCATCCGCCCGCCCATACGGATGGCGG | GTAGTGAAGG |
| 10 | TATGGGCGGGAGCCGTGGACCCCGCCCATACGGATGGCGG | CGGAAGTATT |
| 11 | TATGGGCGGGAGCCGTGGACCGTCCTTTGCGTCCACGGCT | CCCGCCCATA |
| 12 | GCAAAGGACGAGCCCGTGGTCGTCCTTTGCGTCCACGGCT | CCGCCATCCG |
| 13 | GCAAAGGACGAGCCCGTGGTAAGGAGGGTGACCACGGGCT | CGTCCTTTGC |
| 14 | CACCCTCCTTCTGCAGATCAAAGGAGGGTGACCACGGGCT | AGCCCGTGGT |
| 15 | CACCCTCCTTCTGCAGATCAAGGTTAATTTTGATCTGCAG | AAGGAGGGTG |
| 16 | AAATTAACCTTTAGTGGCGTAGGTTAATTTTGATCTGCAG | TGATCTGCAG |
| 17 | AAATTAACCTTTAGTGGCGTCTGCGTCGGAACGCCACTAA | AGGTTAATTT |
| 18 | TCCGACGCAGTCGCGACTAGCTGCGTCGGAACGCCACTAA | TCCGACGCAG |
| 19 | TCCGACGCAGTCGCGACTAGCTAGTCACATCTAGTCGCGA | CTGCGTCGGA |
| 20 | ATGTGACTAGGTTCCGGTGACTAGTCACATCTAGTCGCGA | CTAGTCGCGA |
| 21 | ATGTGACTAGGTTCCGGTGAGCCCTACAATTCACCGGAAC | CTAGTCACAT |
| 22 | ATTGTAGGGCACAGACTCAGGCCCTACAATTCACCGGAAC | AGTGGCCTTG |
| 23 | ATTGTAGGGCACAGACTCAGCCCATACACCCTGAGTCTGT | GCCCTACAAT |
| 24 | GGTGTATGGGACTGGTGGACCCCATACACCCTGAGTCTGT | ACAGACTCAG |

| | | |
|---|---|---|
| 25 | GGTGTATGGGACTGGTGGACGAAGAACCCCGTCCACCAGT | CCCATACACC |
| 26 | GGGGTTCTTCCTAAGCTTTGGAAGAACCCCGTCCACCAGT | ACTGGTGGAC |
| 27 | GGGGTTCTTCCTAAGCTTTGCTAGGACCACCAAAGCTTAG | GAAGAACCCC |
| 28 | GTGGTCCTAGCCGTCCGATTCTAGGACCACCAAAGCTTAG | CTAAGCTTTG |
| 29 | GTGGTCCTAGCCGTCCGATTCGACCACTTAAATCGGACGG | CTAGGACCAC |
| 30 | TAAGTGGTCGTCGGAGTGAACGACCACTTAAATCGGACGG | CCGTCCGATT |

4. finite DNA nanotube design B

| | DNA base sequence (5'-3'): | cover (5'-3'): |
|---|---|---|
| 1 | GATACCTAATCGACTTGAACCTCCAGTGTCGTTCAAGTCG | ATTAGGTATC |
| 2 | CGACTTGAACGAAAGTAGACGTTCAAGTCGGACACTGGAC | GTCCAGTGTC |
| 3 | GTCTACTTTCCGACTTGAAC CGGAAGTATTGTTCAAGTCG | GAAAGTAGAC |
| 4 | CGACTTGAACCCGCCATCCGGTTCAAGTCGAATACTTCCG | CGGAAGTATT |
| 5 | CGGATGGCGGCGACTTGAACAGCCGTGGACGTTCAAGTCG | CCGCCATCCG |
| 6 | CGACTTGAACAGCCCGTGGTGTTCAAGTCGGTCCACGGCT | AGCCGTGGAC |
| 7 | ACCACGGGCTCGACTTGAACAGCCCGTGGTGTTCAAGTCG | AGCCCGTGGT |
| 8 | CGACTTGAACCTGCAGATCAGTTCAAGTCGACCACGGGCT | AGCCCGTGGT |
| 9 | TGATCTGCAGCGACTTGAACTTAGTGGCGT GTTCAAGTCG | CTGCAGATCA |
| 10 | CGACTTGAACTCGCGACTAGGTTCAAGTCGACGCCACTAA | TTAGTGGCGT |
| 11 | TAGTCGCGACGACTTGAACGTTCCGGTGAGTTCAAGTCG | TCGCGACTAG |
| 12 | CGACTTGAACACAGACTCAGGTTCAAGTCGTCACCGGAAC | GTTCCGGTGA |
| 13 | CTGAGTCTGTCGACTTGAACACTGGTGGACGTTCAAGTCG | ACAGACTCAG |
| 14 | CGACTTGAACCTAAGCTTTGGTTCAAGTCGGTCCACCAGT | ACTGGTGGAC |
| 15 | CAAAGCTTAGCGACTTGAACCCGTCCGATTGTTCAAGTCG | CTAAGCTTTG |
| 16 | CGACTTGAACGCATGTCATAGTTCAAGTCGAATCGGACGG | CCGTCCGATT |
| 17 | GCATGTCATACGACTTGAACATCGATCGGTGTTCAAGTCG | GCATGTCATA |
| 18 | CGACTTGAACTTAAGCTAACGTTCAAGTCGACCGATCGAT | ATCGATCGGT |
| 19 | GTTAGCTTAACGACTTGAACGTACCGAAATGTTCAAGTCG | TTAAGCTAAC |
| 20 | CGACTTGAACCTCCGGATGCGTTCAAGTCGATTTCGGTAC | GTACCGAAAT |
| 21 | GCATCCGGAGCGACTTGAACTGAAGGCTGAGTTCAAGTCG | CTCCGGATGC |
| 22 | CGACTTGAACATTTCAAGCTGTTCAAGTCGTCAGCCTTCA | TGAAGGCTGA |
| 23 | AGCTTGAAATCGACTTGAACTGCATACGTGGTTCAAGTCG | ATTTCAAGCT |
| 24 | CGACTTGAACACCCTGCAAGGTTCAAGTCGCACGTSTGCA | TGCATACGTG |
| 25 | CTTGCAGGGTCGACTTGAACTCGATCATTGGTTCAAGTCG | ACCCTGCAAG |
| 26 | CGACTTGAACCCTAGTTAACGTTCAAGTCGCAATGATCGA | TCGATCATTG |
| 27 | GTTAACTAGGCGACTTGAACACGTGTACCGGTTCAAGTCG | CCTAGTTAAC |
| 28 | CGACTTGAACTGCTGAATCA GTTCAAGTCGCGGTACACGT | ACGTGTACCG |
| 29 | TGATTCAGCACGACTTGAACCGTATTCCGAGTTCAAGTCG | TGCTGAATCA |
| 30 | CGACTTGAACATTATCTATTGTTCAAGTCGTCGGAATACG | CGTATTCCGA |