UNIVERSITY OF LEEDS

RecA-based patterning of DNA scaffolds

[redacted]

Student number: [redacted]

Submitted in accordance with the requirements for the degree of:

BSc Nanotechnology

The University of Leeds

School of Electronic and Electrical Engineering

[redacted]

17th of May 2013

Declaration of Academic Integrity

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Submitted in accordance with the requirements for the degree of:

Bachelor of Science

The University of Leeds

©2013

School of Electronic and Electrical Engineering
University of Leeds, Leeds LS2 9JT

Abstract

Currently all the CMOS devices are made using top-down manufacturing processes, which consist of different lithography and etching steps. [1, 2] An alternative to top-down manufacturing technologies is bottom-up approach with its capabilities of self-assembling nano-structures. [1, 3, 4] Particularly appealing candidate of self-assembling tool is DNA due to its lock-and-key recognition properties and the large number of different enzymes and proteins available to alter DNA. [5, 6] It has been shown that it is possible to form higher order DNA structures such as DNA origami, through careful design of DNA. [4] However, if such structure is to be altered, complications arise as the whole structure needs to be re-engineered again.

RecA is a protein in E.coli bacteria, which is responsible for homologous recombination of DNA strands - it provides strand exchange between damaged and new DNA strands. [5] However, this process can also be used to produce intermediate DNA structures such as Holliday junctions, which are three or four-hand DNA structures. [5, 6] It also has been shown that RecA protein can be used as nano-scale patterning tool of DNA molecules for the process of DNA metallisation. [6, 7, 8]

The goal of this project is to show that it is possible to form a longer DNA molecule using two short and non-complementary DNA fragments and RecA protein. This project if successful can be applied in the field of DNA origami, as it would allow to create longer DNA strands with controlled sequence starting from smaller fragments. This would be an improvement as sometimes the desired longer DNA sequence cannot be easily found *in vivo* or cannot be chemically synthesised. In order to show that the process of forming longer DNA structures is successful, gold nanoparticles were attached to the short DNA fragments. The AFM images of the formed DNA-nanoparticle conjugates indeed show that RecA protein can be used to create longer DNA strands starting from two shorter DNA molecules. This method could be used as a tool for controlled and programmable nanoscale object self-assembly in further research.

Acknowledgments

From the bottom of my heart I would like to say big thank you for all the Bioelectronics research group members for their energy, understanding and help throughout my project, especially to [redacted] for the guidance throughout the gel extraction, [redacted] for the help with AFM imaging and [redacted] for advices on DNA deprotection process. It truly has been very, very good time in this lab. I also would like to say special thank you to [redacted] and [redacted], without your help and wise guidance this project would have not been the same!

Abbreviations

AFM - Atomic Force Microscopy

ATPγS: Adenosine 5′-O-(3-thio)triphosphate

AuNP - Gold nanoparticles

bp - Base pairs

DTT - Dithiothreitol

DNA - Deoxyribonucleic acid

dsDNA - Double stranded deoxyribonucleic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

NEB - New England Biolabs

NPF - Nucleoprotein filament

PCR - Polymerase Chain Reaction

ssDNA - Single stranded deoxyribonucleic acid

Table of Contents

1. Introduction

For already more than forty years semiconductor industry has been able to keep up the miniaturisation of electronic devices according to the Moore's law, however, more and more quantum mechanical problems become apparent as the gate length of transistors approaches length of 10 nm. [1, 2, 3]

So far all the CMOS integrated circuit fabrication processes have been exclusively top-down manufacturing processes, which consist of series of masking, lithography and etching steps. [2] Lithography steps allow to define structures on the mask, which will be etched away or will stay on the surface depending on the mask type. [9] From this it follows that lithography is one of the key steps that define the minimum feature size in a top-down process. Up until now semiconductor industry has been using excimer lasers (KrF and ArF lasers with 224 nm and 193 nm emission wavelengths) along with phase-shift masks, which have allowed to reach gate lengths of 28 nm. [2, 10] However, further miniaturisation may require completely different manufacturing methods, as the current top-down technologies struggle to obey further miniaturisation laws due to problems such as device size fluctuation. [1]

There is a big potential in developing bottom-up manufacturing methods, which utilise inherently small combinatorial elements to form nanostructures, this approach has a lot of advantages over conventional top-down processes - such as less wasted material. [11] In the field of molecular electronics there already have been seminal papers that show the possibility of creating a transistor just from a singe molecule or nanoparticle, however, still an issue persists of connecting these elements into a circuit, which would mimic electronic circuit. [12, 13] A promising route of connecting nano-circuit elements together is through molecular self-assembly and DNA self-assembly in particular. [14]

DNA molecule exhibits excellent lock-and-key recognition properties - two single stranded DNA molecules will form a double stranded DNA molecule only if there is a high order of complementarity of these structures, this is due to the fact that T-bases bind to A-bases and G-bases will only form hydrogen bonds between C-bases. [7] Due to this property DNA seems to be a promising base material for self-assembled electronic circuits. [15] Double stranded DNA (dsDNA) is a flexible molecule in terms of applications - it can be used as controllable length metallic nanowire or it is possible to use it in much more sophisticated way as it is done in nature, where it is an information carrier for self-assembly. [6, 16, 17] Somewhat middle ground between using DNA as a metal wire and

the way it is used in nature is using its lock-and-key recognition properties to assemble controlled nano-structures. [14]

A good example of using the self-assembly capabilities of DNA is DNA-origami, which in its principle is building nanoscale structures through sophisticated use of complementary DNA parts and different enzyme digestion methods to create overhangs on DNA. [18, 19, 20] Typically the methodology of creating DNA origami consists of using a long template ssDNA and short fragments of ssDNA, which in certain fragments are complementary to regions of the long template ssDNA. [19] Due to the fact that the ssDNA molecules are short and bind to the ssDNA only in certain, known places, this structure can be folded as a result producing larger structures such as seen in **Figure 1**. [19]

Figure 1[19]
DNA origami structure. This smiley face is a great example of the capabilities of DNA origami technology. The only problem is that these structures require very careful engineering as the small fragments must be complementary to the long template DNA. The scale bar is 100 nm.

The problem with DNA origami is that the production of these structures require a lot of engineering as the small fragments used in creation of these structures must be either cut from complementary strand of dsDNA using different enzymes or very carefully found in completely different DNA template, although the molecular toolbox of DNA systems is large and well researched it not always is possible. [19, 20] Also, if the DNA origami structure is to be altered, it cannot be done easily as changing the template or ssDNA fragments will introduce non complementary parts in the strands, which will completely alter the way DNA origami folds and will not produce the desired structure. Therefore a protocol that allows to easily extend DNA molecules with desired bases would be highly applicable in the field of DNA origami.

Another approach of forming smaller electronic circuits is to use a nanoscale structures that already can act as a transistor themselves and then assemble such structures in a larger entity to form an electronic circuit through the use of molecular self-assembly. It has been shown that molecules, carbon nanotubes and single nanoparticles can be used as a working field effect transistors. [21, 22, 3] Approach of assembling these entities could be through the use of DNA self-assembly. There are papers that show a controlled nanoparticle network formation through the use of DNA self-assembly. [23, 3]

Binding of DNA to nanoparticles is fairly common method now and consists of using thiolated DNA primers, which allow incorporation of thiol group in the DNA through the PCR process, so there is some preliminary work done to create self-assembled nanoparticle networks. [23]

The approach of creating nanoparticle networks using DNA self-assembly requires either finding different DNA molecules with complementary fragments in them [24], or using sufficiently short DNA strands, which can be synthesised in the lab. [23] Work by done by Chad Mirkin's group is much closer to what the aim of this project is. Two different types (A and B) ssDNA molecules were attached to gold nanoparticles in separate solutions, these ssDNA molecules have no complementary regions. [23] When these two types of conjugates are brought together in one solution with a third ssDNA molecule, which has 'sticky ends' (fragments complementary to A and B), a dsDNA region is formed due to the complementary sequences in ssDNA molecules, this binding event can be confirmed by the colour change of the solution, as the nanoparticles form clusters. [23] This paper shows that it is possible to controllably assemble gold nanoparticle networks, but more importantly it shows that this process can be reversible and can withstand several temperature cycles. [23]

The main goal of this project is to create a protocol that would allow to bind together two shorter strands of dsDNA, which do not have a complementary region in them through the use of RecA protein. The principle used in this project is slightly different from that what was used in Reference 23 - introduction of a ssDNA with 'sticky ends' will not initialise the formation of longer dsDNA structures due to the exact fact that dsDNA is used, which already has two complementary strands. RecA protein can create triple-strand structures by winding in ssDNA in a dsDNA structure in the region where these two strands are complementary to each other. [7, 25] This phenomenon usually is exploited, when making Holliday junctions which are three (or four) arm DNA junctions and it will be applied in this project as well. [6] RecA protein will be responsible for creating controlled assembly of dsDNA strands by winding in a complementary ssDNA strand. The benefit of such protocol is that it can be applied in the field of DNA origami, as this protocol would allow easy combination of short single stranded or double stranded DNA molecules with desired complementary regions, as a result it would be much easier to form the origami structures due to the fact that the sequences do not have to be found *in vivo*, but can be made from shorter fragments.

In this project two dsDNA fragments of different sequences and lengths will be used without a complementary fragment in between them, workflow of the project is shown in **Figure 2**. Further, a short ssDNA molecule, with half of its length complementary to fragment of the first dsDNA, while the other half would be complementary to fragment of the second dsDNA, will be used to 'glue' two non-complementary dsDNA strands together forming a longer dsDNA structure using RecA protein (**Fig.2**, Step 3). RecA protein will be allowed to polymerise the ssDNA (forming nucleoprotein filament, NPF) and further protein will wind the ssDNA together with two non-complementary dsDNA strands creating a longer dsDNA structure as shown in the final step of **Fig.2**. In order to observe the binding event, dsDNA will be functionalised with gold nanoparticles (AuNPs), therefore investigation of dsDNA/AuNP conjugate structures will be done as it will ease the imaging process in AFM, since the particles can be readily distinguished from dsDNA (**Fig.2**, Step 2).

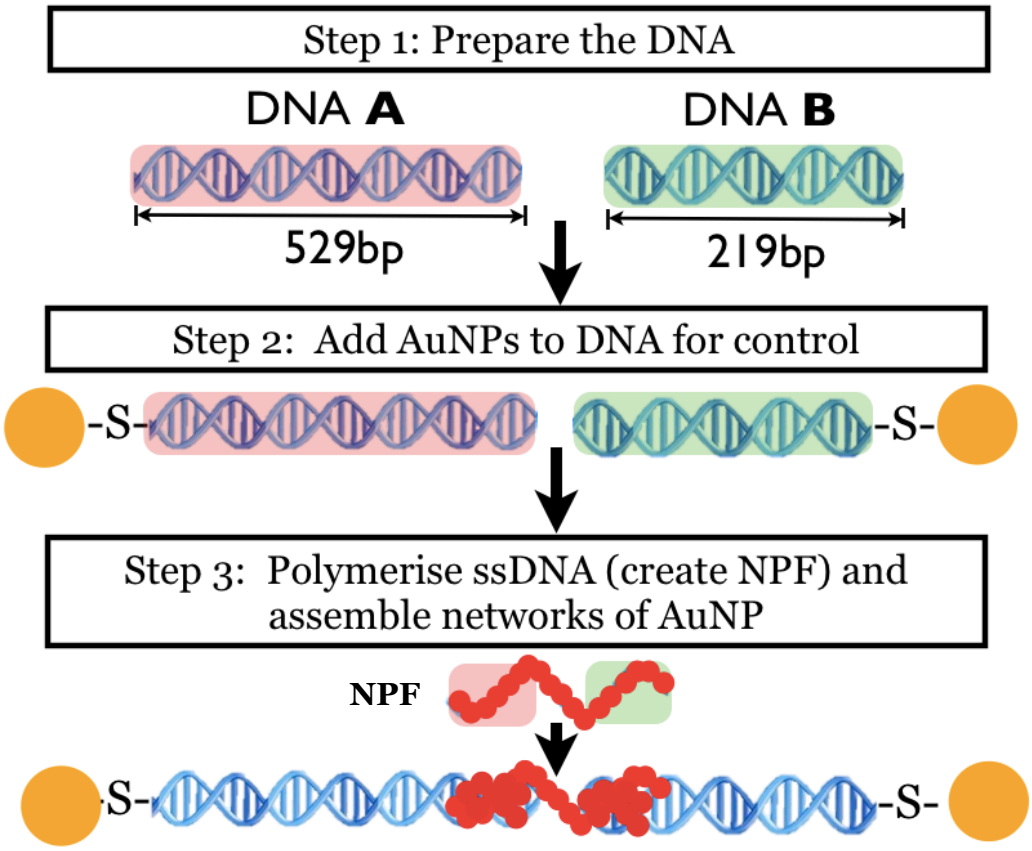

Figure 2

The expected workflow of the project. AuNP attachment is necessary to readily distinguish assembled dsDNA structures from the background of an AFM image.
Similarly to the work done in Ref.23, nanoparticles are functionalised with two non-complementary sequenced DNA molecules and a third complementary molecule is used to bind together the particles with different DNA molecules on them. However, the main difference of this project is that dsDNA is used to functionalise the AuNPs (instead of ssDNA), therefore RecA protein is used to wind in the nucleoprotein filament (NPF), which allows to form assembled dsDNA structures.

2. Materials and Methods

2.1 Basic materials

All the primers for PCR processes were bought from Integrated DNA Technologies (IDT). Enzymes, buffers as well as dNTPs required for PCR experiments were purchased from New England Biolabs (NEB). The ssDNA molecules used in the 'bridging' experiments were bought from IDT. **Figure 3** shows the location of 219 bp, 313 bp and 529 bp DNA primer locations on N2 Plasmid DNA template.

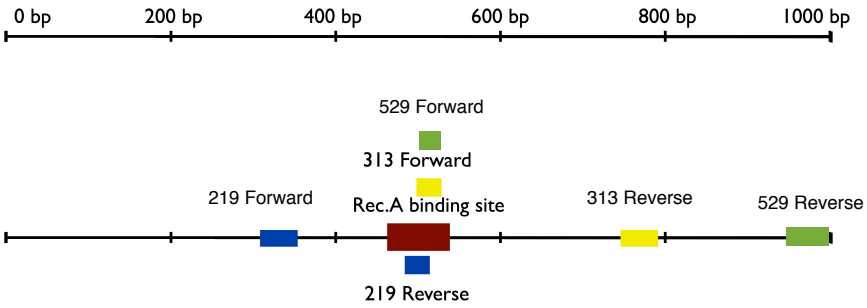

Figure 3
An illustrative comparison of the primer locations of dsDNA strands on N2 plasmid template.

5 nm (5 nm in diameter, OD 1, stabilised suspension in citrate buffer, MKBK2487) citrate stabilised AuNPs were bought from *Sigma Aldrich* company, while 12 nm (12 nm in diameter nanoXact gold, EAW1170, 0.05 mg/ml) citrate stabilised AuNPs, 5 nm (5 nm in diameter Tannic nanoXact gold, KJW1306, 0.05 mg/ml) and 12nm (12nm in diameter Tannic nanoXact gold, JMW1017, 0.05 mg/ml) tannic acid stabilised AuNPs were bought from *nanoComposix* company. All the nanoparticles were resuspended in HEPES buffer (50 mM, pH 7.5), by centrifuging the particles, followed by the removal of the supernant, which contains the original buffer and resuspended in equivalent amount of HEPES buffer (50 mM, pH 7.5). Particles were stored in the fridge at 4^{o}C afterwards.

NAP-5 columns (illustra™NAP™-5 columns Sephadex™ G-25, DNA grade, product code 17-0853-01) for deprotection of dsDNA were bought from *GE Health Care/Life sciences*. Agarose powder was bought from *Invitrogen*, all gels were run using *BioRad* power pack with different experimental set-ups for each of the experiments. PCR products were run on agarose gels against a 100 bp ladder purchased from *NEB*. The agarose gels were prepared using 1X TAE buffer.

Once PCR experiment was done, the samples were purified using QIAquick PCR Purification kit from *Qiagen* and eluted in 100 µl of nuclease-free water. After purification, quantification of DNA was done with Nanodrop 2000C Spectro-photometer in Nucleic acid

mode, the PCR product was also run on 1% agarose gel for 40 min at 80V against 100 bp ladder.

2.2 Basic methods

Table 1 shows the denaturation, annealing and extension temperatures for dsDNA fragments used in the PCR reaction. All of the PCR experiments were prepared in 200 µl reaction tubes and run on Eppendorf Mastercycler ep Gradient 5345. The reaction mixture prepared had the final concentration of 1X LongAmp Reaction buffer, 1.2 mM of dNTPs, 400 nM of Forward and 400 nM of Reverse primers, 100 pg/µl of template DNA and 0.01X of LongAmp Taq DNA polymerase.

DNA fragment	Denaturation temperature, ^{o}C	Annealing temperature, ^{o}C	Extension temperature, ^{o}C
219 bp dsDNA	94	64	65
313 bp dsDNA	94	42	65
529 bp dsDNA	94	61	75

Table 1
Information on primers used in the PCR process.

The thiolated primers had protection group on them to protect dsDNA from forming disulphide bonds between strands themselves. In order to remove these protection groups DNA was incubated with 0.1 M DTT in volume ratio of DNA:DTT of 1:4. The problem arrises in the fact that DTT causes nanoparticle aggregation due to the fact that it has two thiol groups, as a result DTT must be removed from the DNA solution, this is done through the use of NAP-5 columns. The protocol of DNA deprotection acquired courtesy to Mr *Samuel T. Houlker*. [26]

In order to prepare AuNPs for the formation of the DNA/AuNP conjugates, the particles were spun at 16 krpm for 35 min and 60 min for 12 nm and 5 nm particles respectively. Afterwards the supernant was removed from the eppendorf tube and the particles were suspended in equal amount of HEPES buffer (50 mM, pH 7.5) to ensure that the buffer of DNA and the nanoparticles match. About 100 µl of deprotected DNA was mixed with 100µl of AuNPs in HEPES buffer (50 mM, pH 7.5) and left for incubation at room temperature. To protect the conjugates from possible aggregation they were stored away from light. [27]

For further control of experiments it was encouraged to find a method how to separate AuNPs with different number of DNA strands on them. Gel electrophoresis followed by band extraction was found to be the process to obtain sufficiently high DNA/

AuNP conjugate concentrations. Sample preparation for gel electrophoresis was as follows. Conjugates after incubation were centrifuged at 7.5 krpm for 35 min after incubation. The supernant was removed from the eppendorf tube after centrifugation and the conjugates left at the bottom of the tube were resuspended in 20 µl of HEPES buffer (50mM, pH 7.5). Two such tubes were mixed together along with 10 µl of 6X loading dye, which made the total volume to be loaded on the gel. Total volume of 50 µl of conjugates were loaded on 0.8% agarose gel. Later it was tested that it is possible to load the conjugates without any loading dye as the nanoparticles themselves are heavy enough not to escape the gel bed. The gel electrophoresis kit, extraction kit and the protocol was acquired courtesy to Mr *Devesh Nandal*. [28] After running conjugates on agarose gel for 40 min at 80V, the red band of conjugates was further run and extracted. After extraction of the band was finished all the fractions obtained from the gel extraction were centrifuged at 7.5 krpm for 35 min to remove the supernant and were resuspended in 20 µl HEPES buffer (50 mM, pH 7.5). After every centrifugation step described, the conjugates were quantified using Nanodrop 2000C Spectrophotometer to control the concentration of DNA throughout the process.

In order to form DNA/AuNP conjugate networks, ssDNA molecules need to be polymerised with RecA to form NPF. Polymerisation reaction mixture is as follows in **Table 2**.

The reaction tubes were placed at 37^{o}C for 15 min and then used in Section 3.2.6.

Once the NPF were made further experiments of conjugate network formation was done. The solution for conjugate network formation can be seen in **Table 3**. Once all the reagents were put together, the tubes with the Reaction mixture were placed at 37^{o}C for 60 minutes and then the solution was used for AFM imaging.

Reagent	Concentration	Volume, µl
70 bp ssDNA	10 µM	1
RecA	2 mg/mL	4.7
Tris-Ac	300 mM	1
Mg-Ac	20 mM	1
ATPγS	5 mM	2
Nuclease-free H_2O	0	0.3
Reaction mixture		**10**

Table 2
Reaction mixture of NPF formation. Further deviations from this protocol are described in Results and Discussion section.

Reagent	Concentration	Volume, µl
70 bp ssDNA	10 µM	5
RecA	2 mg/mL	4.7
Tris-Ac	300 mM	2
Mg-Ac	20 mM	2
ATPγS	5 mM	2
Nuclease-free H_2O	0	4.3
Reaction mixture		**20**

Table 3A

Reagent	Concentration	Volume, µl
Reaction mixture from Table 2A	-	2
Tris-Ac	300 mM	2
Mg-Ac	20 mM	2
Nuclease-free H_2O	0	16
Reaction mixture		**20**

Table 3B

Table 3

Reaction mixtures used in previous experiments (Table 3A) and the reaction mixture to be used in the further experiments. Reaction mixture on the RHS (Table 3B) will be used as the solution of NPFs, this mixture is used to 'mimic' the LHS mixture, while reducing the NPF concentration and reducing free-RecA concentration.

All the imaging with AFM was done with mica sample holders. The sample preparation method was as follows:

Step 1: Take volume equivalent to 7.5 ng of dsDNA from DNA/AuNP conjugates or DNA/AuNP bridging experiment solution and pipete-mix with nuclease-free water to create 20 µl of solution.

Step 2: Mica surface was prepared by removing the top few layers of mica with Sticky tape, washing the new surface three times with nuclease-free water.

Step 3: Ni^{2+} ions helps the DNA to bind to the mica surface, since the DNA as well as mica surface is negatively charged. 20 µl of 10 mM $NiCl_2$ was deposited on the surface and left in humid chamber for 60 s. Afterwards, the $NiCl_2$ was removed by flicking the sample holder.

Step 4: Take the solution from **Step 1**, deposit it on the mica surface and leave in humid chamber for 10 minutes.

Step 5: After 10 minutes, wash the mica surface three times with 50 µl of nuclease-free water and blow-dry it using nitrogen.

3. Results and Discussion

3.1 PCR results

In order to form the DNA/AuNP conjugates the first step is to prepare the dsDNA, which will be bound to AuNPs. The preparation of DNA is done through the PCR processes. In order to confirm that the DNA obtained from PCR process is the DNA required, gel electrophoresis was used as a confirmation tool, **Figure 4A** and **4B** show 219 bp & 313 bp and 529 bp DNA gels respectively. As it can be seen in the pictures, the bands are smooth and quite sharp, meaning that the PCR process has been successful and the necessary fragments have been amplified exponentially comparing to the template, as there is only one band present in the lanes of the samples. However, occasionally for 219 bp & 313 bp fragments vague band of about 500 bp was visible on the gel, which most likely represents the 532 bp template DNA used in the PCR process, this band was not seen, when 219 bp and 313 bp fragments were used as templates for their respective PCR processes.

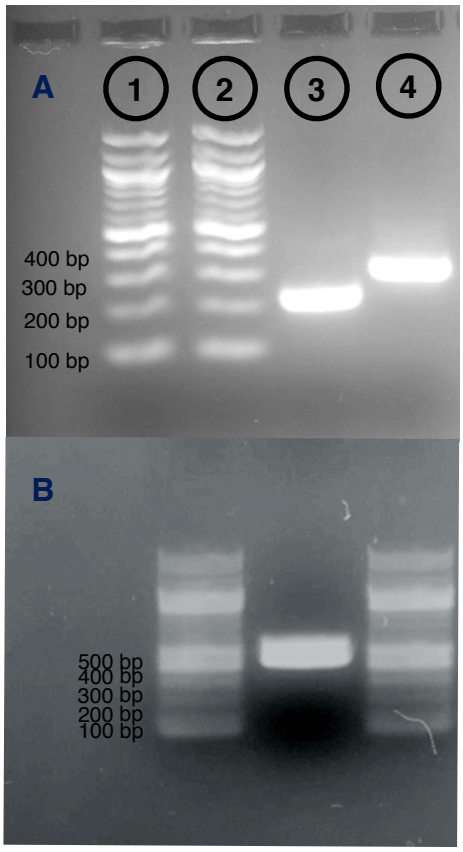

Figure 4

Fig.4A: Gel electrophoresis experiment of 219 bp (#3) and 313 bp (#4) dsDNA fragments run against 100 bp ladders (Fig. 4A). As it can be seen the fragments run slightly slower than 200 bp and 300 bp fragments, confirming that the fragments indeed are 219 bp and 313 bp long.

Fig.4B: 529 bp long DNA fragment is right next to 500 bp fragment of 100 bp ladder, the PCR process has produced the right length DNA.

3.2. Results of deprotection of dsDNA

In order to remove the protection group from the thiol group on the dsDNA, dithiothreitol (DTT) was used as the agent, following the protocol by Mr *Samuel T. Houlker.* [26] The reason why the process of removing DTT from the solution is necessary is that DTT itself has two thiol groups, as a result DTT will cause the formation of AuNP aggregates as described by Kim et al. [29]

In order to remove the DTT from the solution, NAP-5 columns were used as described in Methods section. Initially the experiments were carried out as in the protocol by Mr *S.T. Houlker*, however, the dsDNA concentration after the deprotection was so low, that no conjugate bands could be seen in the gel extraction step. Since the concentration using protocol described in Ref.26 produced insufficiently low dsDNA concentration after deprotection step, it was proposed to load higher amount of dsDNA in the columns to increase the dsDNA concentration after the deprotection process. Contrary to what was expected, increasing amount of DNA loaded in the column indeed

produced higher concentration dsDNA samples after deprotection step, shown in **Figure 5**, maintaining Gaussian distribution of the DNA across the fractions, which correlates with the theory that DNA regardless of its concentration moves through the column with a constant speed. A comparison between the initial protocol (25µl of dsDNA loaded in the gel, I.P. in **Fig. 5,** described in Ref. 26) and the new protocol, where all the PCR product is loaded in the column, can be seen in **Figure 5.**

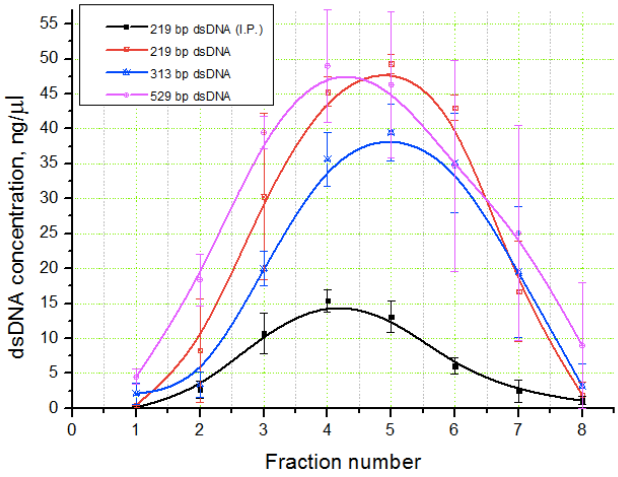

Figure 5
Average values with mean errors of deprotection experiments. As it can be seen there is a Gaussian distribution of concentration of dsDNA, this distribution represents the speed of DNA in the column, meaning that most of the DNA moves with similar speed through the column. Also comparison to the previous deprotection (I.P.) can be made - the new protocol yields much higher concentration of dsDNA after the deprotection process.

Equation 1 shows the calculation done to acquire the efficiency of the deprotection process for the average of 219 bp dsDNA deprotection results as described in **Materials and Methods section**.

$$\eta = \frac{V_{recovered} \cdot c_{recovered}}{V_{loaded} \cdot c_{loaded}} = \\ = \frac{100\mu l \cdot (0.5 + 8.3 + 30.3 + 45.3 + 49.3 + 43.0 + 16.7 + 1.9)\frac{ng}{\mu l}}{100\mu l \cdot 260.2\frac{ng}{\mu l}} = \\ \approx 75\% \qquad (1)$$

The main problem is that the concentration of DNA for each of the fraction is low (due to added volume of DTT and elution step in deprotection process, which dilutes the dsDNA solution), comparing to the concentration of DNA after the PCR process, which is the reason why high concentration of DNA after the PCR process is required and it also explains why only fractions with the highest dsDNA concentration are used for the formation of DNA/AuNP conjugates.

After the deprotection process of dsDNA was finished, the AuNPs are added to the solution right after the deprotection process, so that the exposed thiol groups of oligos do not form disulphide bonds. Formation of disulphide bonds is an issue, because the bond dissociation energy of a disulphide bond at the room temperature is 425.30 kJ/mol, while the Au-S bond dissociation energy is 253.6 kJ/mol. [30]

Relatively large difference in bond strength is the reason, why 12 nm AuNPs must be mixed with deprotected DNA as soon as possible.

Typically incubation times of 72 h seem to be sufficient to produce DNA/ AuNP conjugates, which can be seen by AFM. Lower incubation times were tried, such as an hour, two hours and 24 hours, however, the results were not satisfactory. As a result, throughout the project DNA was incubated with AuNP for at least 72 hours.

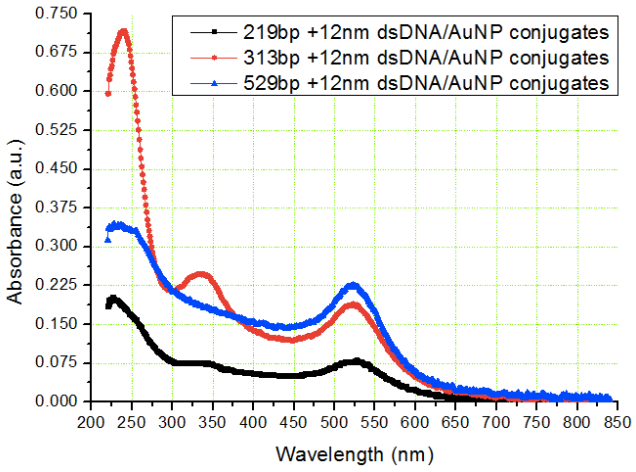

Figure 6
UV-Vis spectrophotometry experiments with dsDNA/AuNP conjugates after the deprotection experiments and centrifugation step, which allows to concentrate the conjugates. As it can be seen, there are peaks present at 260 nm (the slight shift towards shorter wavelengths was also present after the PCR reaction) for all dsDNA lengths and there are peaks at 525 nm, which represent the AuNP presence.

After the incubation period, the samples were centrifuged in order to concentrate the DNA/AuNP conjugates and to remove the excess unbound dsDNA. Further UV-Vis spectrophotometry was used to observe, whether peaks from dsDNA and AuNPs can be observed. From literature it is known that the characteristic peak of the dsDNA in the UV-Vis spectrum is at 260 nm. [31] The AuNPs have a distinct peak at 550 nm, which is the main tool to characterise their presence in the solution. [32] **Figure 6** shows the UV-Vis absorption spectra for 219 bp and 12 nm; 313 bp and 12 nm and 529 bp and 12 nm dsDNA/AuNP conjugates, black, red and blue lines respectively.

3.3. Separation of DNA/AuNP conjugates using gel extraction

A major problem for the AuNP functionalisation is the efficiency of DNA binding to the particles. An AFM picture of samples after dsDNA deprotection and incubation is shown in **Figure 7**. There is a a lot of unbound dsDNA in the background of the image, which interacts with the sample during the bridging experiments, therefore it is necessary to remove this unbound dsDNA from the sample.

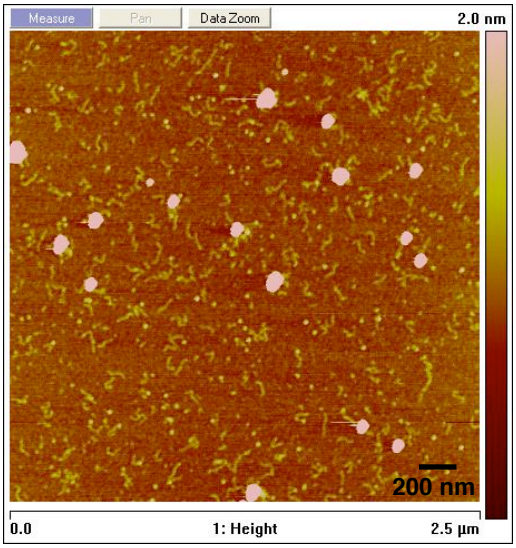

Figure 7
An AFM image of the samples after DNA deprotection and incubation with AuNPs. The large background interferes with the samples during the bridging experiment.

In the field of biochemistry gel electrophoresis is used as means to separate differently sized DNA oligos according to their length. [33] It was proposed

that in a similar fashion it would be possible to separate AuNPs with different number of dsDNA strands on them and, more importantly, separate AuNPs with dsDNA on them from just unbound DNA and AuNPs without any dsDNA strands on them. **Figure 8** shows an idealised gel electrophoresis experiment, where the conjugates are loaded in the wells and due to the different numbers on each of the particles, conjugates are separated.

The experimental set-up for the separation of conjugates was designed specifically by Mr *Devesh*

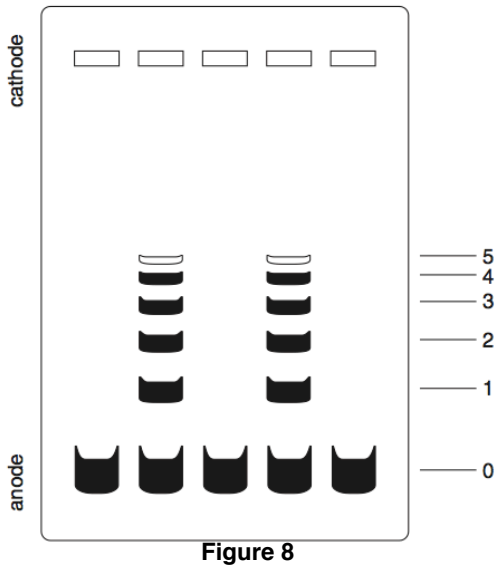

Figure 8
An idealised experiment of a gel experiment, which allows to separate the particles with different number of strands on them.

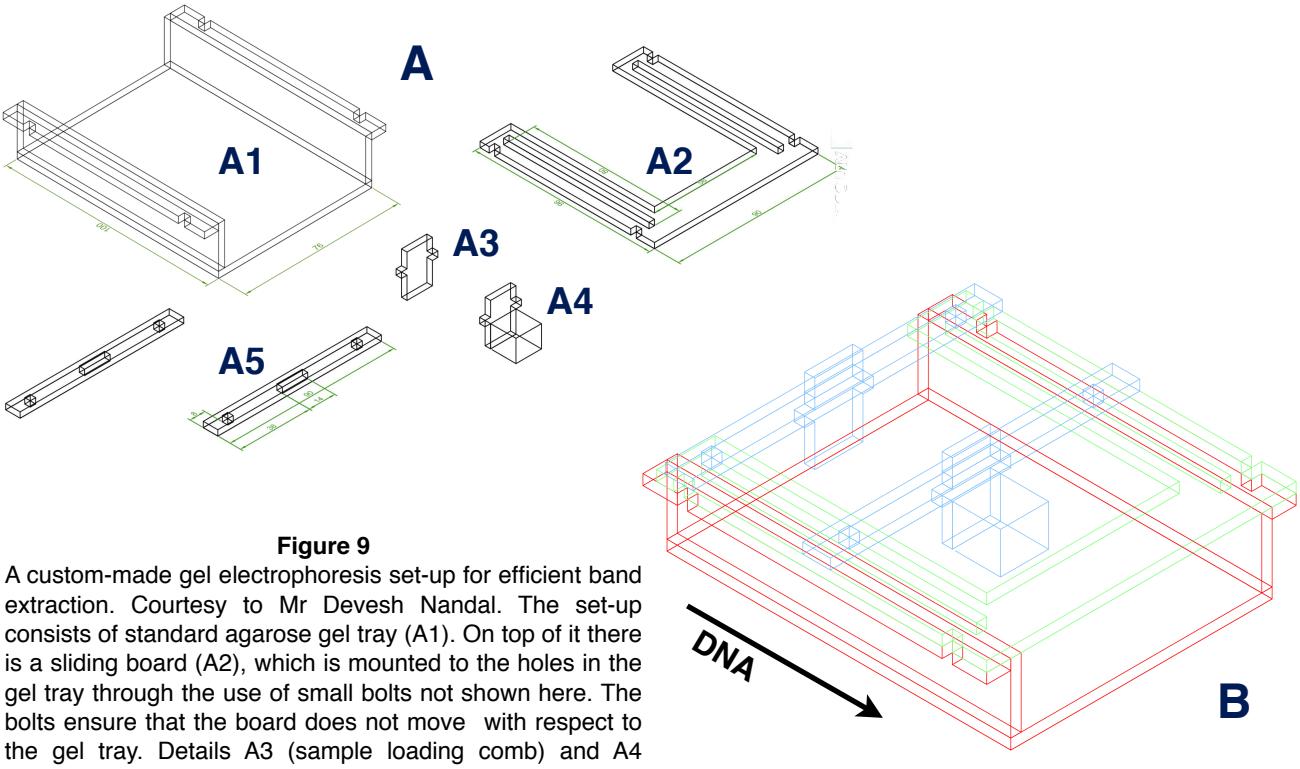

Figure 9
A custom-made gel electrophoresis set-up for efficient band extraction. Courtesy to Mr Devesh Nandal. The set-up consists of standard agarose gel tray (A1). On top of it there is a sliding board (A2), which is mounted to the holes in the gel tray through the use of small bolts not shown here. The bolts ensure that the board does not move with respect to the gel tray. Details A3 (sample loading comb) and A4 (extraction comb) are mounted to detail A5 through the use of double-sided tape as it can be seen in Figure 6B. Similar type of bolts used to hold board on top of gel tray are put in side holes of detail A5. These bolts will ensure that the detail A5 can move along the tracks of detail A2 only, as a result the sample comb and extraction combs are perfectly aligned.

Agarose gel is poured, then detail A2 is mounted in the tray and combs along with their holders are placed in gel. Once gel is rigid, both combs are removed from the gel and comb A3 is placed in the place of comb A4, therefore allowing space to pour in 20% polyacrylamide gel (PA gel), which will work as the block of conjugates, while allowing some degree of buffer motion through it. Once polyacrylamide gel has settled down, all combs are removed and it is proceeded further as it would be done with an ordinary gel. As the desired band has reached the extraction well, part of the buffer is removed such that the top of the gel is not in solution. The buffer in the well is exchanged for desired one and the band is allowed to enter extraction well. Once all of the band has gone in, the voltage is stopped and the DNA is extracted from the well.

Nandal for the extraction process of the DNA/AuNP conjugates. [28] Conventional extraction methods rely on the fact that the wanted strand is cut out from the agarose gel and then using different methods, the agarose is dissolved. However, in the current extraction method bands are separated and they can be easily extracted through another well, like it is shown in **Figure 9**.

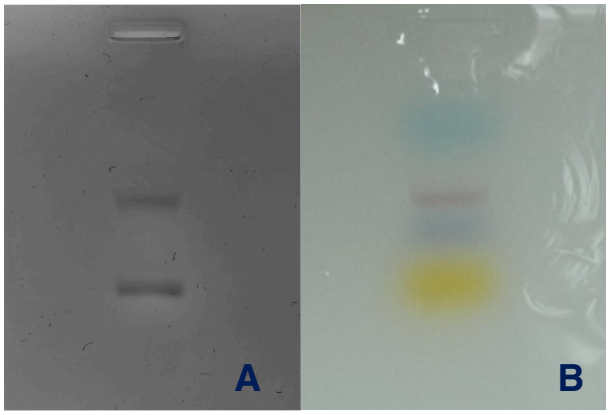

Figure 10
Conjugate extraction experiment. LHS - gel under the UV light illumination, RHS - daylight illumination. The conjugates with the same number of DNA strands run together during gel electrophoresis experiment (red band on RHS), while the unbound DNA, which cannot be seen in daylight, but can be seen under UV.

Key in this set-up is that the conjugates run through the gel and they can be seen in daylight as it can be seen in **Figure 10** due to their typically red colour, which is because of the AuNPs, which are red. [29] Once the band of interest has reached the extraction well, the level of buffer in the cell is lowered, so that the top of the gel is not covered in buffer. The buffer in the extraction well is replaced by the buffer of interest, in this case nuclease-free water. The electrophoresis is started again and the band is allowed to enter the extraction well, since the extraction block right after the well is made from 20% polyacrylamide gel, the conjugates cannot enter the blocking gel, as the polyacrylamide gel has much smaller pore size than the agarose gel, as a result all the conjugates stay in the well. Once all the band has entered the extraction well, the solution simply is removed from the well.

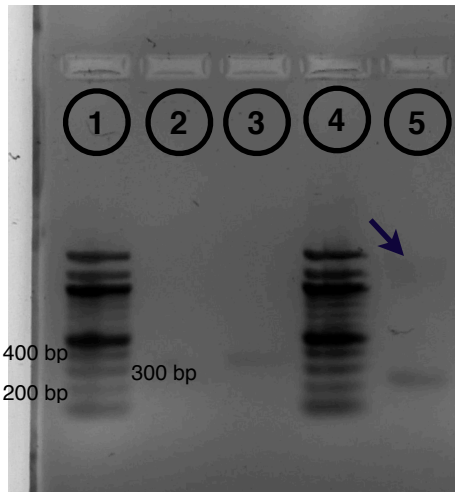

Figure 11
Conjugate separation experiment using conventional gel electrophoresis kits. As it can be seen, there is a vague presence of additional band of conjugates in the lane 5, however, the concentration of conjugates in this band is too low, as most of it would be lost during the extraction experiment.

Initially it was tried to use conventional gel extraction set-up to separate nanoparticles with different number of strands on them, however this method turned out to be ineffective. The results from the experiments can be seen in **Figure 11,** 20 µl of the conjugates from Section 3.2 were loaded with 5 µl of 6X loading dye, there is barely any band from conjugates present, meaning that the conjugate concentration with respect to unbound dsDNA is too low. Therefore extra centrifugation

step was introduced as explained in Materials and Methods**.** Since the band present in **Figure 11** is so vague, it also was decided to use custom-made combs and experimental set-up (see **Fig. 9** and **10**) to load more sample in the gel and to extract it more efficiently. To load more conjugates in the gel, the conjugates from Materials and Methods were centrifuged and then two fractions were mixed together to increase the total amount of conjugates loaded in the agarose gel.

The gel results from this extraction method can be seen in **Figure 10**, where **Fig.10A** is a picture of the DNA in the UV light and **Fig.10B** is a picture of the gel in daylight. As it can be seen in both figures there are two distinct bands present. The red band seen in the daylight corresponds to DNA/AuNP conjugates, as the particles add this band its red colour, while the lower band seen in UV must be the band from unbound DNA molecules, as it cannot be seen in daylight, but can be seen under UV light source. Once the red band representing DNA/AuNP conjugates was visible on the gel, the conjugates were moved through the gel until the extraction well was reached, conjugates then were extracted and centrifuged to remove the supernant and to concentrate the DNA/AuNP conjugates. Gel separation has another positive effect on the conjugates besides separating conjugates according to the number of strands per particle, as it can be seen in **Figure 10**, the unbound DNA molecules run much faster than the conjugates, as a result, the extracted solution has a very little concentration of background DNA. An AFM image before extraction can be seen in **Figure 12A**. There is a large excess of the background even after centrifugation, which allowed to remove some of the unbound DNA.

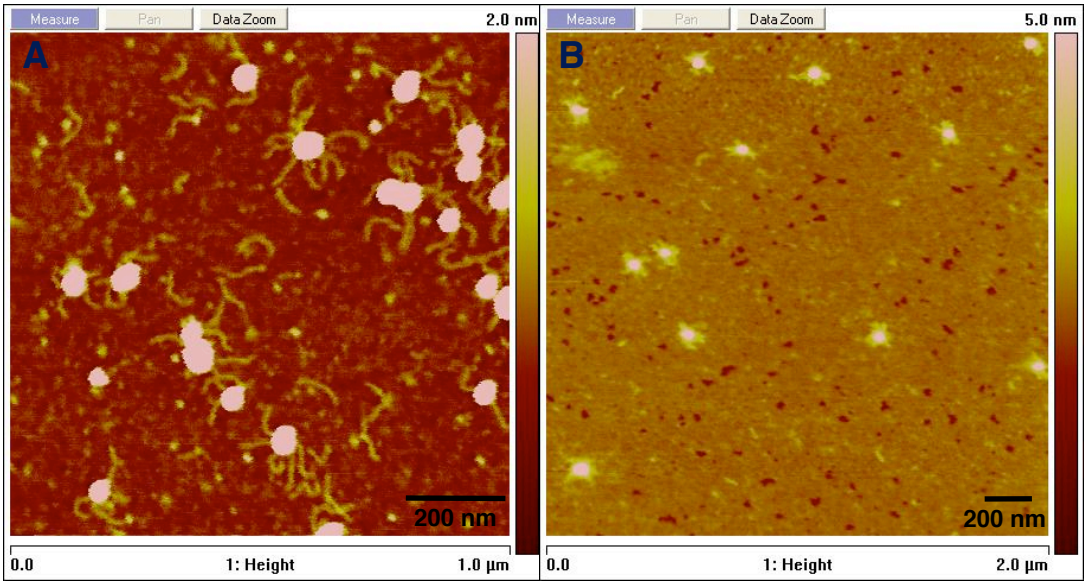

Figure 12

AFM images of DNA/AuNP conjugates before (Fig.12A) and after gel extraction (Fig.12B). As it can be seen after the gel extraction there is a low background of unbound DNA, while every AuNP seems to have a few strands of dsDNA on them, while before the gel extraction there is significantly higher background DNA.

Figure 12B shows the AFM image of the conjugates after the gel extraction, as it can be seen, there is quite low background of the unbound DNA, while it seems that every particle has been conjugated with a few strands of DNA.

Since the band was moving together through the gel, each particle should have the same number of DNA strands on it, as the length of dsDNA strands were found to be equal, meaning that the only difference between conjugates is the number of dsDNA strands per particle, which ultimately increases the weight of the conjugate. [34]

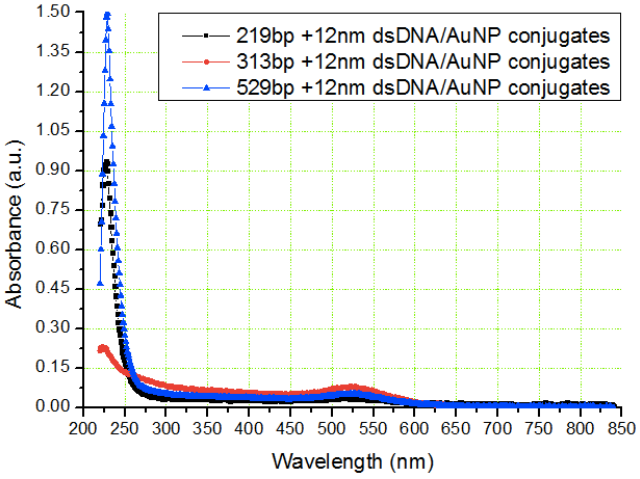

Figure 13

UV-Vis spectrophotometry experiments with dsDNA/AuNP conjugates after the gel-extraction experiments and centrifugation step, which allows to concentrate the conjugates. The structures seen using the AFM indeed are dsDNA/AuNP conjugates, as both the dsDNA and AuNPs peaks are present. The vague peak of AuNP can explain, why the colloidal solution in the eppendorf tube looks only slightly red.

To ensure that the images seen on AFM are images of dsDNA/AuNP conjugates, additional experiments with spectrophotometer was done in the UV-Vis region to observe the presence of peaks at 260 nm and 550 nm, which represent the dsDNA and AuNPs respectively. **Figure 13** shows the UV-Vis absorption results from the three types of conjugates used throughout the project, as it can be seen, the absorption spectra confirms the presence of both - dsDNA and the particles. The suspension also has a slight hint of red colour, which is characteristic to colloidal gold nanoparticles in the size range up to 100 nm. [29]

3.4 Formation of DNA/AuNP conjugate networks

The final step of this project is to use RecA polymerised ssDNA bridge to bind together 219 bp and 12 nm conjugates and 529 bp and 12 nm conjugates. A 70 bp ssDNA is used to accomplish this, a illustrative diagram of the structure of the bridge is shown in **Figure 14**.

From literature it is known that 1 RecA monomer binds to three bases of ssDNA or base pairs of dsDNA. [9] Meaning that in order to polymerise 70 bp bridge and form NPFs approximately 24 RecA monomers are required per bridge, resulting in the necessary concentration of RecA of 0.94 µg/µl per 1 µl of ssDNA at a concentration of 350 ng/µl. However, experimental images show that such concentration is too high and the excess RecA also coats the dsDNA bound to the AuNPs. Development of a protocol, which would allow to bind the conjugates together using ssDNA, but not coating the dsDNA of the

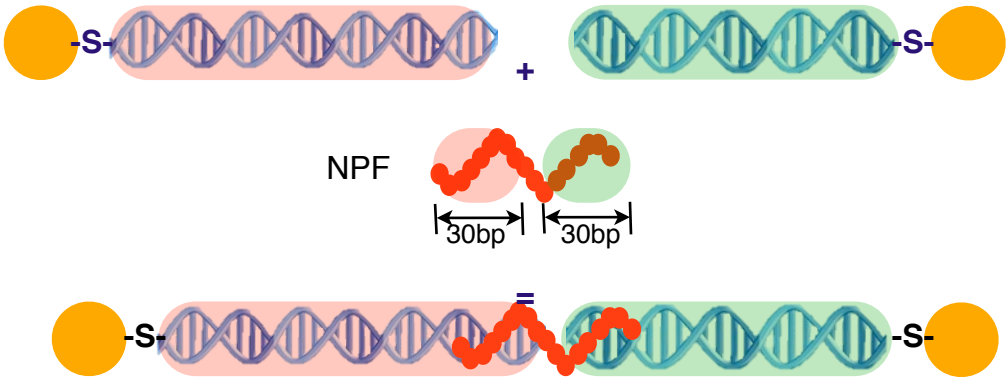

Figure 14
Illustrative diagram of the final step of the project. The ssDNA used to 'bridge' the two conjugates has 30 bp complementary to 219 bp dsDNA and 30 bp complementary to 529 bp (and 313 bp) dsDNA, as it is shown in Figure 14.

conjugates, was the the main goal of this project, therefore the experiments were performed accordingly. Precise reaction mixtures of NPF formation and network forming can be found in Materials and Methods Sections **Table 3**.

The initial experiments consisted of proving the theoretical predictions of a possibility to actually create networks of conjugates, series of repeated experiments were done in order to confirm that assembly event indeed is due to RecA polymerised ssDNA bridges rather than incidental accident. **Figure 15** shows the first AFM images of the bound conjugates, where the reaction mixture consisted of 2 pmol of NPFs. There is a lot of excess RecA molecules, which coat not only ssDNA bridges, but also dsDNA found on AuNPs, this can be confirmed by the large height profile of the structures, as dsDNA has a height profile of about 2 nm. Big structures that can be seen in **Fig.15A** can also be

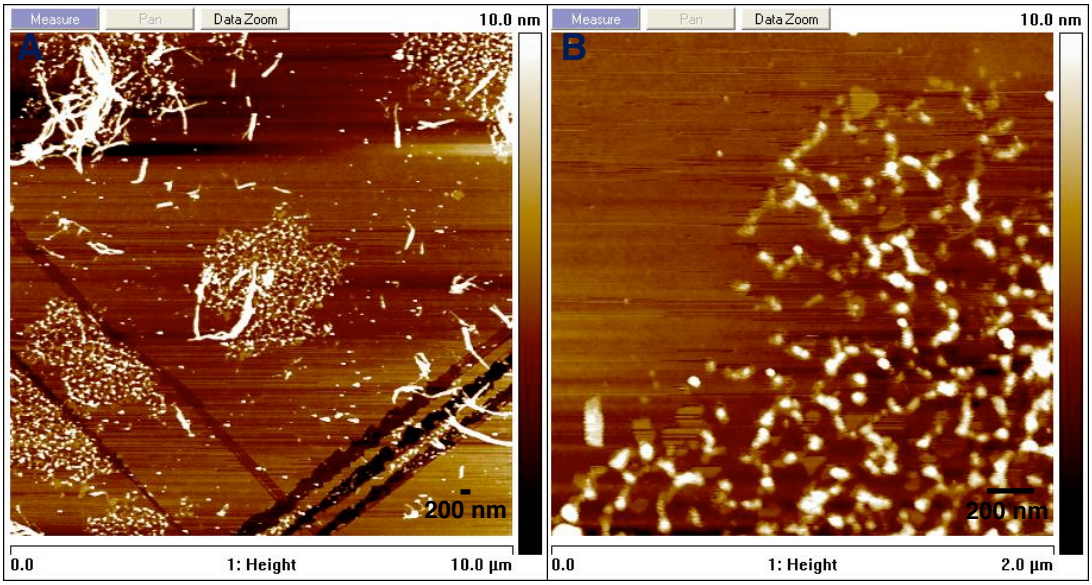

Figure 15
AFM images of bridged DNA/AuNP conjugates of a 10x10 µm^2 image (Fig.9A) and smaller 2x2 µm^2 image (Fig.9B). There seems to be a large excess of RecA molecules in the solution of NPFs, as not only the bridges seem to polymerised, but dsDNA on the conjugates as well, which is confirmed by the height of the structures, which is about 5 nm, comparing to pure dsDNA shown further.

explained in terms of RecA coverage, as NPF are more rigid than classical dsDNA, meaning that it is less likely for the conjugates to fall apart, if all the DNA is polymerised instead of only 70 bp. [35, 36]

Fig.15B clearly shows that RecA has coated not only ssDNA but dsDNA of conjugates as well, as the height profile of the structures seen is above 5 nm. For comparison, **Figure 16** shows the image of the same experimental parameters used in **Fig. 15**, however, during the NPF formation, a completely non-complementary ssDNA was used, which has no regions complementary to either of the dsDNA strands. It clearly can be seen that no structures similar to those of **Fig.15A** can be seen, meaning that the formation of the nanoparticle arrays is governed by the complementary ssDNA bridge, which follows the initial idea of the project.

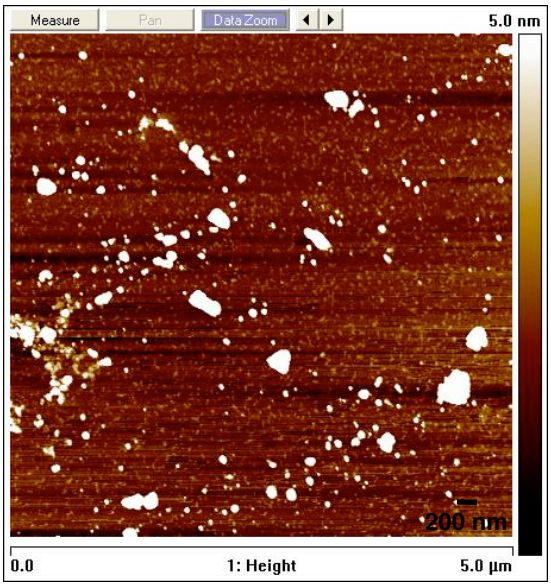

Figure 16
A 5x5 µm² image of an experiment, where non-complementary 70 bp ssDNA was used to form NPF. Since there are no structures as in Figure 12, it can be said that the nanoparticle arrays are formed due to the complementary ssDNA bridge.

Further experiments were done to find the best parameters of the experiments, which would produce conjugate networks using RecA, while not affecting the dsDNA strands on the AuNPs optimisation experiments were done using just the corresponding dsDNA and the 70 bp ssDNA bridges.

The first modifications of NPF solution were done with an idea in the mind that more ssDNA molecules in the solution would be able to bind the excess RecA. This approach lead to using 5 times more ssDNA molecules than the original calculations predicted, at the same time less NPF were used, so that if there is any excess RecA molecules, they would only slightly affect the dsDNA found on the conjugates. First thing that was noticed after implementing these changes is that there are a lot less structures on the surface. **Figure 17A** shows one of few areas found to have structures on it. There is a less RecA polymerisation present in the images comparing to initial results seen in **Fig. 15**, however, there still are excess RecA. molecules in the solution, which was determined by looking at the height profile of dsDNA seen in **Fig.17B**, which is less than 5 nm, however, it still is not comparable to the height profile of pure dsDNA.

Although the experiments provided some interesting structures, further investigation was done through the use of dsDNA on its own to save the conjugates and to ease the experiment preparation. As the 'NPF protocol' used in preparation of **Figure 17** seemed to

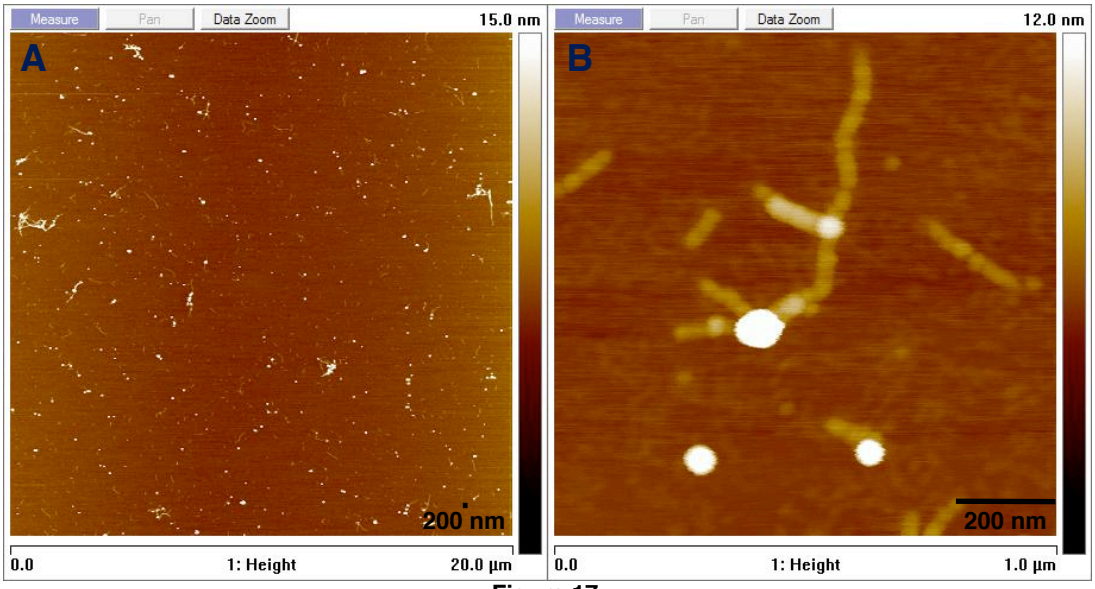

Figure 17

AFM images of bridged DNA/AuNP conjugates of a 20x20 µm^2 image (Fig.17A) and smaller 1x1 µm^2 image (Fig.17B). Not so explicit coating of dsDNA molecules can be observed, as the RecA has been mopped-up by extra amount of ssDNA bridges, also there are lot less NPFs in the solution, which decreases the RecA concentration in the solution. However, it still seems insufficient to avoid any dsDNA polymerisation. The distance between from the AuNP to the extensive dot (which might be smaller AuNP due to the height profile) on the dsDNA is 180 nm, which corresponds to 532 bp, which is expected. The extra length after 180 nm structure might be another dsDNA attached to the end through the disulphide bond.

reduce free RecA concentration sufficiently, it was taken as the ground for further experiments. It was proposed that further dilution of NPFs would be a good way how to prepare dsDNA structures without excessive RecA coating. **Table 3** (Materials and Methods) shows the reaction mixture used in **Figure 17** (LHS, Table 3A) and the further reaction mixture that to be used in the following experiments (RHS, Table 5B).

Further experiments consisted of reducing the volume of NPF reaction volume in the production of conjugates to 1 µl of Reaction mixture seen in **Table 3A** and trying to alter the RecA molecule number in the same Reaction mixture. **Figure 18A** and **18B** show the bridging experiment of 219 bp + 12 nm dsDNA/AuNP and 529 bp + 12 nm dsDNA/AuNP conjugates with 0.94 µg RecA protein in Reaction mixture shown in **Table 3A** and 0.6 µg of RecA protein in Reaction mixture shown in **Table 3A** respectively. Although there seems to be some excess RecA in the solution, the images seem promising as the binding site of ssDNA is visible and the distances between particles correspond to the expected distances.

Finally it was thought not to decrease the RecA concentration during the filament formation, but to stick to further dilution as it can be seen in **Table 3B**, as decreasing the RecA concentration would make the ssDNA to be polymerised only partially, which would cause problems afterwards. From this Reaction mixture then NPFs were taken and experiments were done with pure dsDNA, to see at what volume of Reaction mixture seen

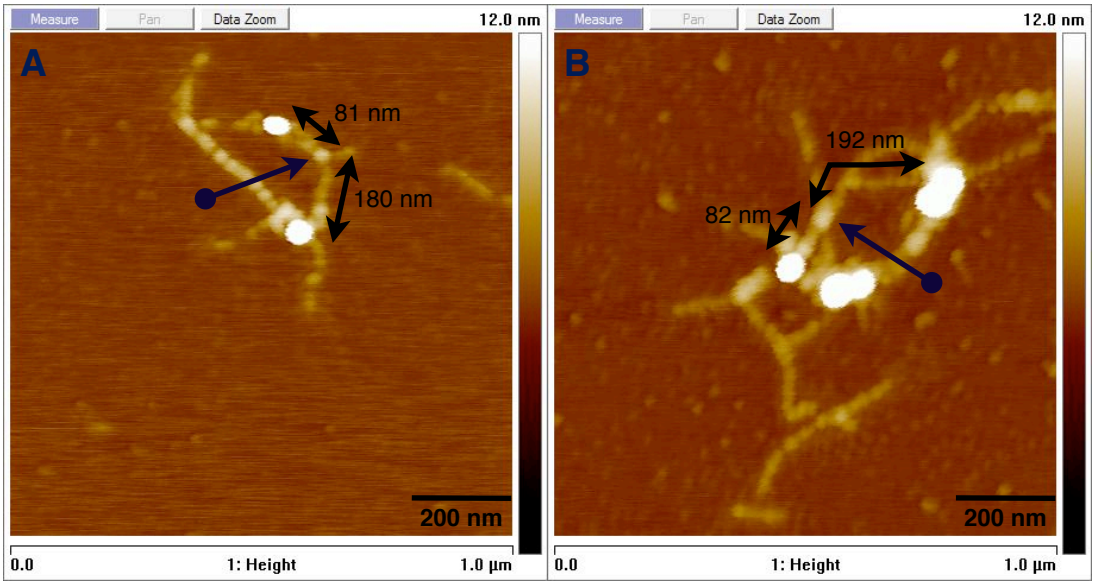

Figure 18

AFM images of bridged DNA/AuNP conjugates of 219 bp and 12 nm and 529 bp and 12 nm conjugates with different RecA concentrations in the NPF Reaction mixture, 1x1 µm^2 image. The total distance between two particles in both images is about 250 nm, which corresponds to 748 bp, which is the right distance. Also it is interesting to notice that there is a 'blob' (navy blue arrow with a dot at the end) on the dsDNA, at the distance, which corresponds to the bridging region, where there should be the binding event. The reason, why the images seem less polymerised with excess RecA might be that the 529 bp dsDNA is much longer than the 313 bp, so that it can absorb more RecA molecules before getting completely polymerised.

in **Table 3B**, the NPFs would be present, while no excess RecA polymerisation could be noticed on the dsDNA molecules. **Figure 19** shows the AFM image of 219 bp and 529 bp dsDNA on their own, with no bridges or RecA added. The pure dsDNA image is taken as the reference to compare whether the conjugates are polymerised by free RecA in the solution. If no difference in height of dsDNA is visible after addition of particular volume of Reaction mixture seen in **Table 3B**, then the same volume would be used afterwards when bridging the conjugates. The final set of fine-tuning experiments were done with just dsDNA strands and NPFs from the Reaction mixture seen in **Table 3B.** Once the volume of NPF solution, which does not coat the dsDNA was found, further experiments was done with the respective volume. **Figure 20** shows the fine-tuning experiments done with complementary and non-complementary dsDNA strands, **Figure 20A** and **Figure 20B** respectively. There were two reasons why experiments with non-complementary ssDNA was done - first of them - as it

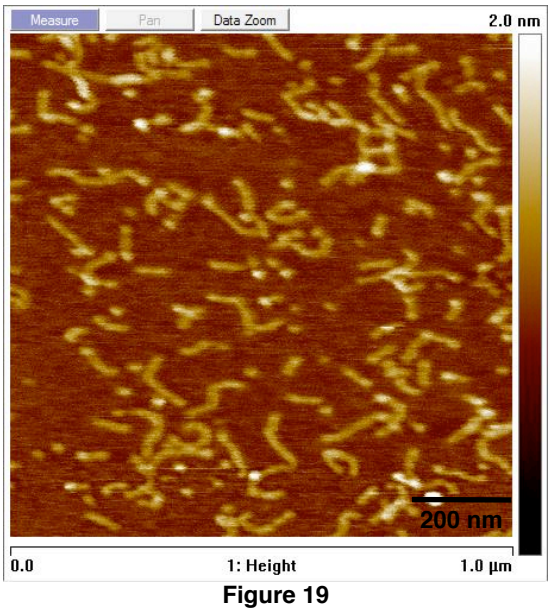

Figure 19

A 1x1 µm^2 image of bare dsDNA - both 219 bp and 529 bp in equal amounts. The height of pure dsDNA is taken as reference to compare, whether RecA in further bridging experiments also polymerise the dsDNA of conjugates at a particular volume of Reaction mixture seen in Table 5B.

can be seen in **Figure 20A**, the complementary strands do form structures of dsDNA, however, it cannot be very well distinguished whether the structures formed are due to the RecA polymerisation or due to the bridging of complementary ssDNA. The second reason why non-complementary ssDNA bridges were used was that it worked as control experiment, as well as means to evaluate the number of filaments in the solution. In preparation of **Figure 20A**$^{+}$ and **Figure 20B**$^{+}$ 49.5 fmol NPFs from the NPF Reaction mixture in **Table 3B** were used, while for preparation **Figure 20A*** and **Figure 20B*** 82.5 fmol of NPFs were used. There is a very fine line between having RecA polymerisation on dsDNA as it can be seen with 82.5 fmol of NPFs. Due to this fact, 49.5 fmol number of NPFs Reaction mixture was chosen to be utilised in further bridging experiments. Further

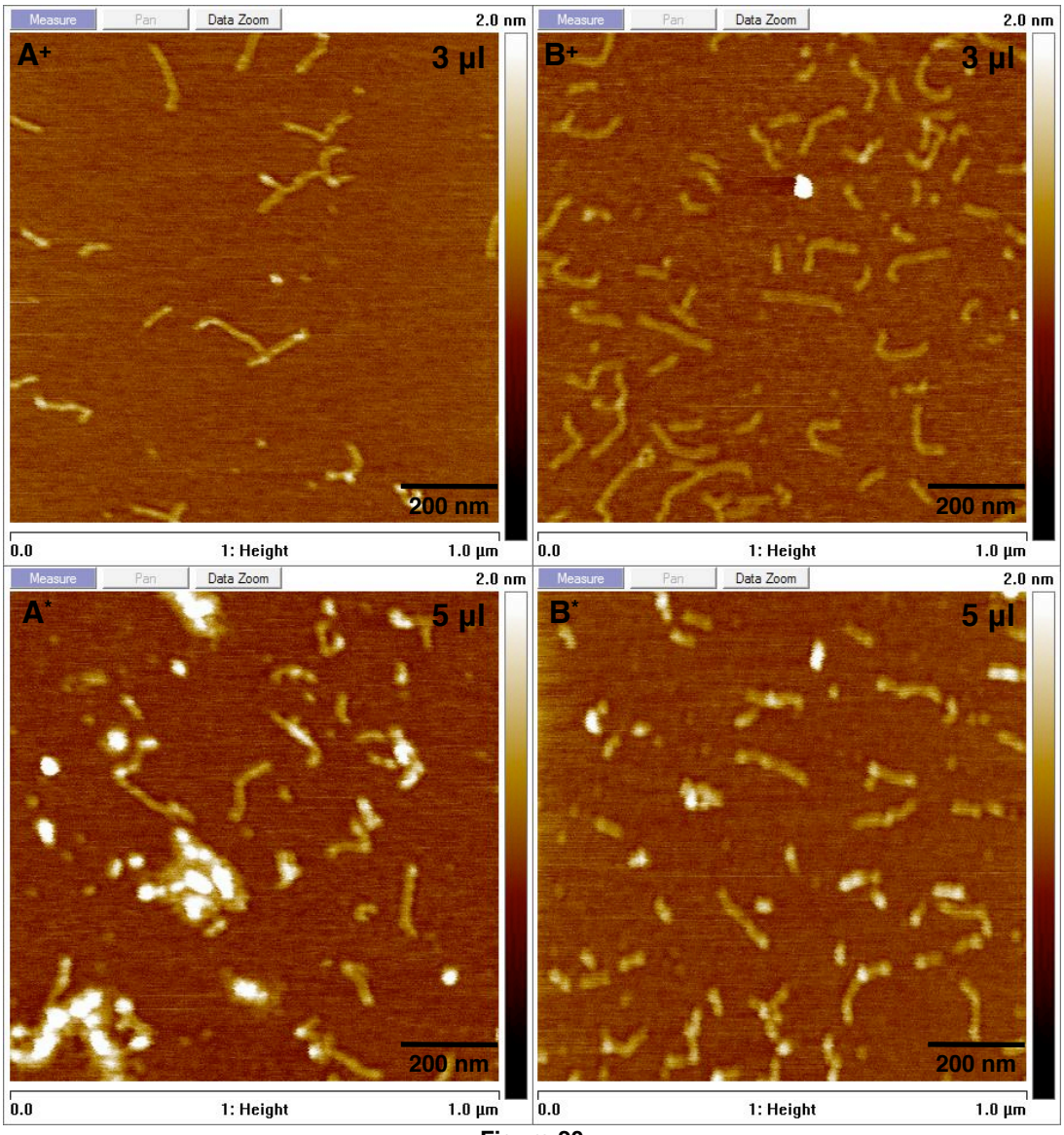

Figure 20

AFM images of experiments done to find the right volume of NPFs, such that there is no polymerisation of the dsDNA strands, while there should NPFs present. LHS represents the experiments done with complementary ssDNA strand, while RHS represents the experiments done with non-complementary ssDNA strands. In the case of both complementary and non-complementary ssDNA, 3 µl of NPFs seem to produce the best results - there are NPFs present in the image, while the dsDNA seems to be unchanged comparing to pure dsDNA seen in Figure 19.

experiments were aimed to obtain good quality images with the new protocol of producing NPFs.

4. Conclusion and Further work

DNA seems to be a promising candidate for the use in self-assembled nanostructures and subsequently self-assembled nanocircuits. [1] The excellent self-assembly capabilities of the DNA can be seen in the field of DNA origami, which allows to design higher order DNA structures in two- or even three-dimensions. [19] However, such structures are very hard to design and if any changes need to be made, the structure in principle has to be redesigned completely. If a protocol that allows easy DNA strand extension and connection were to be designed, it would have a big potential in applications within the field of DNA origami.

During this project the previous technique of removing the protection group on dsDNA was improved, yielding higher concentrations of dsDNA afterwards as shown in **Figure 5**. The basis of this improvement was the fact that loading more dsDNA in the column did not result in equal distribution of dsDNA across the fractions as it was expected, but there still was a Gaussian distribution of concentration of dsDNA across different fractions, which means that dsDNA molecules penetrate through the column with equal speed regardless of the concentration of them.

It was shown that it is possible to repeat the previous results of dsDNA/AuNP conjugate formation even with shorter fragments of dsDNA, with the only problem being relatively long incubation times of at least 72 hours. [26] Further research could be done how to accelerate the formation of conjugates.

Gel extraction seems to be an efficient process of removing the unbound dsDNA from the solution of conjugates and it seems that running the conjugates on agarose gel indeed allows to separate the particles with different strands on them. The described extraction process with custom made experimental set-up indeed was efficient way how to extract the conjugates from agarose gel.

Experiments with different NPF concentrations were done in order to remove the excess RecA protein in the solution. It seems that Reaction mixture described in **Table 5B** does not polymerise the dsDNA molecules, while there is a sufficient number of conjugate networks found in the solution. Maybe further work of NPF formation could be directed towards using another completely random DNA strand to absorb the excess RecA and afterwards these strands could be removed from the solution. For instance using biotin labelled dsDNA, which later can be removed using magnetic beads.

Further work could include investigation how to produce stable conjugates with 5 nm gold nanoparticles. It might be interesting to introduce larger nanoparticles, instead of working with smaller, 5 nm ones, as the contrast comparing to 12 nm particles is what is necessary. Also further work research could be done towards finding how to control the number of dsDNA strands per particle. One such method might be introducing small concentrations of an agent that competes with dsDNA for a place on nanoparticle, such agent could be mercapto-undecanol.

Concluding all the work described here, it is indeed possible to form dsDNA/AuNP conjugate networks in RecA protein mediated process, which was the task of the project. The protocol found in this project can indeed be used to produce longer dsDNA fragments from two short non-complementary dsDNA fragments; these results may find applications in the field of DNA origami.

References

[1] W. Lu and C.M. Lieber, *Nat.Mat.,* **6,** 842 (2007).

[2] D. J. Frank, et al., *Proc. IEEE,* **89,** 259 (2001).

[3] K. Keren et al., *Science,* **297**, 72 (2002).

[4] K. Sanderson, *Nature,* **464**, 158 (2010).

[5] S.C. West et al, *Cell*, **60** (2), 329 (1990).

[6] E. Braun, K. Keren, *Advances in Physics*, **53** (4), 441 (2004).

[7] R. Sharma, A.G. Davies, C. Wälti, *J. Nanosci. Nanotechnol.*, **11** (12), 1 (2011).

[8] T. Carell, *J. Am. Chem. Soc.*, **128** (5), 1398 (2006).

[9] M. Madou, Fundamentals of Microfabrication, Boca Raton, Florida: *CRC Press*, 9 (2002).

[10] A. Tritchkov, S. Jeong, C. Kenyon, *Proc. SPIE,* **5754**, 215 (2005).

[11] A. Kuzyk et al, *Nature,* **483**, 3111 (2012).

[12] H. Song et al, *Nature*, **462**, 1039 (2009).

[13] K. Wolff, U. Hilleringmann, *ESSDERC '09. Proceedings of the European CD-ROM,* **460** (2009).

[14] R. Sharma, A.G. Davies, C. Wälti, *Nanotechnology,* **23**, 365301 (2012).

[15] M. Hamedi, A. Elfwing, R. Gabrielsson, O. Inganäs, *Small,* **9** (3), 363 (2013).

[16] C. Nugent, V. Lundblad, *Genes Dev,* **12** (8), 1073 (1998).

[17] N.C. Seeman,A.M. Belcher, *Proc. Nat. Acad. Sci. USA,* **99**, 6451 (2002).

[18] R.J. Kershner et al, *Nature Nanotechnology,* **4***,* 557 (2009).

[19] P. W. K. Rothemund, *Nature*, **440**, 297 (2006).

[20] Z. Kreptic, *J. Am. Chem. Soc.*, **134**, 8356 (2012).

[21] C. Dekker, S.J. Tans, A. R. M. Verschueren, *Nature,* **393** (6680), 49 (1998).

[22] D. C. Ralph, C. T. Black, M. Tinkham, *Superlattices and Microstructures*, **20**(3), 389 (1996).

[23] C.A. Mirkin et al, *Nature,* **382**, 607 (1996).

[24] N.C. Seeman, *J. Am. Chem. Soc.*, **126** (33), 10230 (2004).

[25] G. Bertucat, R. Lavery, C. Prevost, *Biophys J.,* **77** (3), 1562 (1999).

[26] S.T. Houlker, *Master thesis*, University of Leeds (2012).

[27] J.Z. Zhang et al, *Physical Review B*, **73**, 165405 (2006).

[28] D. Nandal, R. Sharma, C. Wälti, A.G. Davies, *Article in preparation*.

[29] J.Y. Kim, J.S. Lee, *Nano Letters*, **9**(12), 4564 (2009).

[30] Y.R. Luo, Comprehensive Handbook of Chemical Bond Energies, *CRC Press*, Boca Raton, FL (2007).

[31] A.M Nowicka *et al*, *Bioelectrochemistry,* **70**(2),440 (2006).

[32] F.A.A. Arockiya, *Spectrochim Acta A Mol Biomol Spectrosc,* **99**,166 (2012).

[33] J.H. Kim, S. Chaudhary, M. Ozkan, *Nanotechnology,* **18**, 195105 (2007).

[34] V.V. Kushnirov, *Journal of Biological Chemistry,* **278** (49), 49636 (2003).

[35] C. Dekker et al, *Nucl. Acids Res.,* **37** (12), 4089 (2009).

[36] R.M. Story, I. T. Weber, T.A. Steitz, *Nature,* **355**, 318 (1992).