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# Integration of high information content replication in ECCells.

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Organisation name of lead contractor for this deliverable:

Ruhr-Universität Bochum (RUB-BioORG), Günter von Kiedrowski

Hebrew University (HUJI), Itamar Willner

Ruhr-Universität Bochum (RUB-BioMIP), John McCaskill

# 1. Deliverable 1.2: Integration of high information content replication in ECCells

## *1.1 Introduction*

In WP 1 - Programmable Replication – the outstanding objectives include the programmable chemical ligation of DNA and a programmable pH-controlled copy release. So far in ECCell, disulfide chemistry was newly introduced in templated chemical DNA ligation. The advantages of the disulfide ligation chemistry are high reaction rates, reversibility and its structural similarity to the native phosphodiester bond. A number of sequences were tested, as well as different ligation sites (3´-thio-dG vs 3´-thio-xG), different monitoring and a variation of activation from 3´- to 5´-end. Furthermore, a FRET-quench detection system, based on a Fluorescein-Dabcyl-pair, was developed for online monitoring of the ligation reaction in a cuvette or in a microfluidic environment. A 9mer-18mer ligation system with a 3´-activation site was found to be most effective in the templated ligation since the untemplated background reaction was efficiently suppressed here due to a sterical hindrance. For the system we have demonstrated that temperature variation can be used as an effective means of control as the template effect is reduced with increasing temperature.

We demonstrated the compatibility of the DNA block copolymer synthesis with thiol oligonucleotide chemistry by synthesizing PPO-thiol-oligonucleotides and utilizing scp-DNA in chemical ligation experiments as a template. Nevertheless, for the integration of chemical replication and its control in the ECCell the immobilization process in the microfluidic system via sdp-DNA and the replication process were separated in different strands for reasons of higher flexibility in sequence design. The interaction of immobilization and replication can thereby be achieved via DNA helper-strands, binding the ligation part to the polymer-DNA on hybridization.

The requirements for a programmable spatially resolved replication-system in the ECCell are:

- Fast, templated ligation reaction with limited background- and side-reactions including a pH-independent online detection method.
- compatibility with an electrochemically induced pH-switch from pH 5.8 to pH 7.2 and v.v. (WP3).
- sequence design must be chosen in correspondence with scp-DNA (synthetic copolymer DNA) or amphiphilic lipid-DNA, designed for retention or immobilisation in a pluronic matrix in microfluidics (WP2).

For the established disulfide-DNA ligation system, based on Watson-Crick base pairing and a CG-ligation motif, the pH-variation is insufficient for an effective product-template-separation. On the other hand, the triplex formation of DNA in a parallel motif requires a pH value of 6 for an effective base pairing, due to the protonation of cytosine. The triplex-strand release is achieved at pH 7 and therefore a triplex replication can be induced and controlled via hybridization events under pH-switch in the given range. Most importantly, this process also applies to longer DNA sequences, so that high information content ligation chemistry, which arise from multiple ligations, can be turned over without the product completely inhibiting further access to the template for subsequent copying.

Our aim was to develop a triplex-DNA replication-system which is implementable in the ECCell. The system is based on the self replication of a palindromic duplex DNA, previously described by Li and Nicolaou.[1] The system consists of a homopyrimidine-homopurine-homopyrimidine shape and the ligation sites are phosphordiester bonds formed next to thymine and adenine nucleobases. The replication of the DNA duplex herein proceeds via two

ligation steps on the Hoogsteen- and the Watson-Crick-side of the symmetrical triplex-DNA, respectively. Both ligation steps could be separately performed under varying pH conditions. To utilize the concept of triplex-replication, localized and under pH-control in a microfluidic environment, disulfide chemistry was to be introduced to the system. Since previous replication experiments with disulfide chemistry were based on a CG-ligation motif, new nucleoside modifications had to be synthesized to provide a TT-ligation for the Hoogsteen-side and an AA-ligation for the Watson-Crick-side of the system. We focussed on the syntheses of the Hoogsteen-ligation, since a Watson-Crick-disulfide ligation was already shown in previous experiments with the CG-motif. A significant variety of reactivity was not to be expected here by the introduction of thiol-adenosine and the synthetic effort for the full system appeared too high for the full replication circle. Nevertheless a full replication- or amplification-circle is accessible without Watson-Crick ligation by a spatially resolved and pH-controlled amplification process in the microfluidic environment (Figure 1).

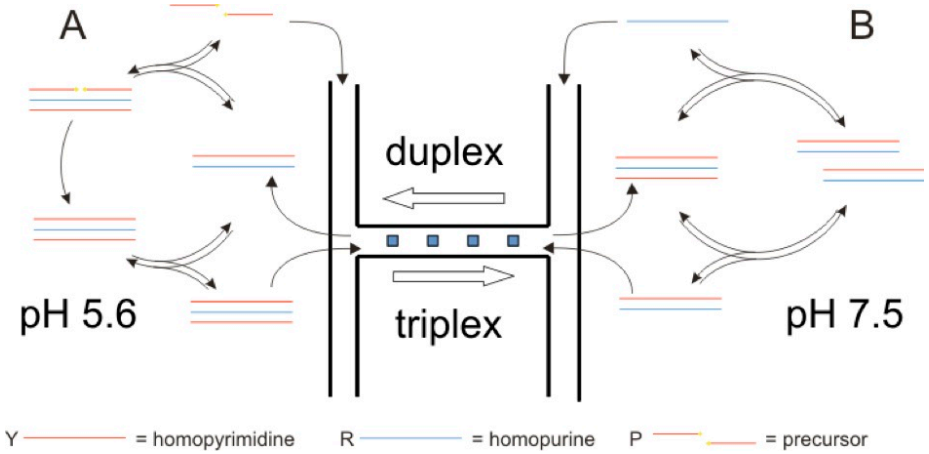


Figure 1: Programmable Chemical Replication in the ECCell; triplex ligation and duplex amplification result in self replication of double stranded DNA.

A full exponential replication circle is achieved when triplex DNA, synthesized by chemical disulfide ligation at the Hoogsteen-side of duplex DNA at pH 5-6 in position A, is transferred to position B, where pH 7.5 and the homopurine strand R is given. Hereby, one triplex strand gives two duplex strands which can, after transfer to position A, both serve as templates for triplex ligation.

The sequence design was performed according to the Nicolaou-System.[1]

*Table 1:* ***Sequence pool for triplex-amplification in the ECCell.***

| | |
|---|---|
| A-strand | 5´ TTT TGC GGA TTC |
| B-strand | 5´ GAG AAA AAA GAG GAA GGG GAA TCC GCA AAA |
| C-strand | 5´ CTC TTT TTT CTC CTT CCC GAA TCC GCA AAA |
| R-strand | 5´ GGG AAG GAG AAA AAA GAG GAA GGG |
| Y-strand | 5´ CCC TTC CTC TTT TTT CTC CTT CCC |

The sequence pool was extended for the immobilisation process with an amphiphilic lipid-DNA (A-strand) strand and two counterstrands (B- and C-strand), binding the homopurine- or the homopyrimidine-strand, respectively (Table1). The retention mechanism (Figure 2, exemplary for the homopyrimidine binding) further contains a toe-hold of 6 base pairs for a controlled release via strand displacement.

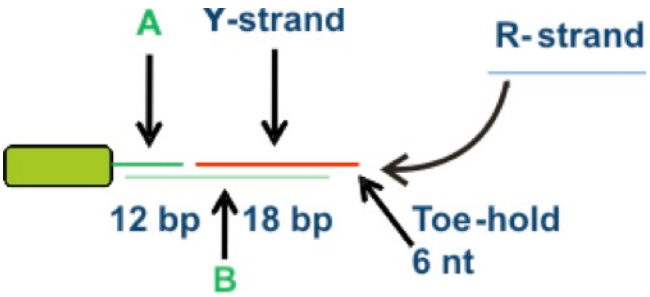


Figure 2: Retention mechanism on amphiphilic lipid-DNA with helper-strand B.

Both DNA-strands of the replication system can herewith be localized in the pluronic matrix of the ECCell and released as a duplex- or triplex-strand for cycling events (see also WP2, WP4).

## *1.2 Syntheses for a triplex-DNA-replication system in an microfluidic environment*

To create a triplex-DNA based replication system in a microfluidic environment a number of new inventions were necessary. For a homopyrimidine triplex-ligation sulfur-modifications at the 3´- and 5´-position of the C- or T- nucleoside had to be introduced. Prior to this, in the disulfide ligation systems the ligation side was restricted to a C-G-motif since base stacking is known to be strongest here, which is important for an effective templated ligation. Although a 5´-C modification was therewith already available we decided to maintain the sequences of the Nicolaou-system with a T-T-ligation side. For the Hoogsteen-ligation, fully protected dT-thiol-nucleosides were synthesized.

### *Thiol-nucleosides of thymidine*

The synthesis of the 5´-thiol-dT starts from thymidine **1** with the introduction of sulfur as a thioester at the 5´-end via Mitsunobu reaction. The following DMT-protection of **2** at the 3´-OH gives the fully protected nucleoside **3**. After saponification of the ester, the nucleoside was immobilized on a newly synthesized polystyrene-solid support as a starter nucleoside for automated DNA synthesis to give **5**.[2]

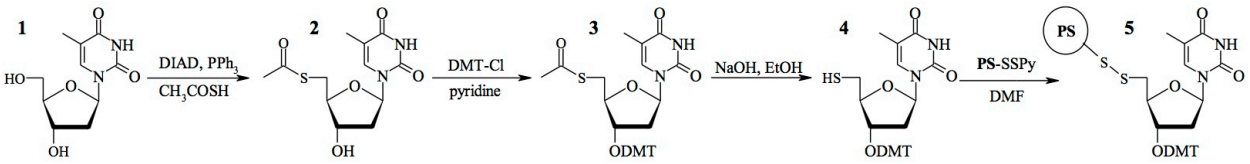


Figure 3: synthetic route of the solid support **5** for 5´-thiol-oligonucleotide synthesis.

The polystyrene-support (PS-SSPy) has shown advantages over the formerly used CPG-support concerning the loading and the coupling efficiency in DNA synthesis. The thiol modification was introduced as a succimidyl-ester of mercaptohexanol, activated as a thiopyridyl-disulfide.

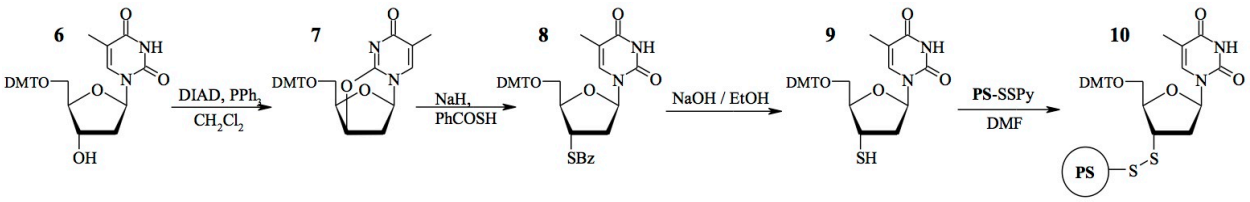


Figure 4: synthetic route of the solid support **10** for 3´-thiol-oligonucleotide synthesis.

The synthesis of a 3´-thiol-dT **9** also starts from thymidine with the DMT-protection of the 5´-OH (**6**).[3] The following Mitsunobu reaction gives the 2,3´-anhydronucleoside **7** which is

transferred to the 3´-thioester **8** with sodium-thiobenzoate in the following step. Subsequent saponification gives the 3´-thiol-nucleoside **9** which is immobilized on solid support and used as a starter nucleoside **10** in oligonucleotide synthesis.

***Oligonucleotide syntheses***

Phosphoamidite syntheses yielded thiol-oligonucleotides with the starter nucleosides in 3'- or 5'- position. Standard deprotection and purification followed by reduction using tris-(2-carboxyethyl)-phosphine (TCEP) gave 3'- and 5'-thiol-oligonucleotides, which were utilized in chemical ligation experiments as free thiols **12** or activated disulfides **11**. Besides thio- and amino-modified oligonucleotides **11** and **12** unmodified template molecules **13** and **14** were synthesized for ligation experiments on a duplex template. Oligonucleotide **15** was provided by RUBa for triplex ligation experiments on a preorganized duplex-strand.

*Table 2:* ***Oligonucleotides for triplex-ligation.***

| | | |
|---|---|---|
| 3´-Thiol-ODN | 5´ nCCC TTC CTC TTTSH | **11** |
| 3´-Thiol-ODN | 5´ Alexa488CCC TTC CTC TTTSH | **11a** |
| 3´-Thiol-ODN | 5´ Alexa488CCC TTC CTC TTTSEll | **11b** |
| 3´-Thiol-ODN | 5´ Alexa488CCC TTC CTC TTTSDabcyl | **11c** |
| 5´-Thiol-ODN | 5´ HSTTT CTC CTT CCC | **12** |
| Homopurin-Template | 5´ GGG AAG GAG AAA AAA GAG GAA GGG | **13** |
| Homopyrimidine-Template | 5´ CCC TTC CTC TTT TTT CTC CTT CCC | **14** |
| Looped-Template | 5´ CCC TTC CTC TTT TTT CTC CTT CCC-TTTTT-GGG AAG GAG AAA AAA GAG GAA GGG | **15** |

After purification, the 3´-thiol-ODN **11** was further modified with a pH-independent fluorescent dye at the 5´-amino-linker. Alexa 488 was introduced via its succinimidly-ester and purification was achieved by precipitation and RP-HPLC. The resulting 3´-thiol-ODN **11a** was then reduced with TCEP, desalted and activated with Ellman´s reagent and the Dabcyl-modification thereof, respectively. After HPLC- purification and characterization with MALDI-TOF, the activated oligonucleotides **11b** and **11c** were obtained and used in triplex-ligation experiments.

## *1.3 Triplex-ligation experiments*

For the triplex-ligation experiments we chose the looped template **15** in order to avoid Watson-Crick base pairing events with the precursor molecules. First experiments for the determination of suitable reaction conditions in the ECCell were performed at pH 5.3 and 50mM NaCl with the activated oligonucleotide **11b** and analysed by PAGE-gel. Low salt concentrations were necessary to allow a pH-switch in the ECCell. The system is, in principle, suitable for the microfluidic environment, applying CG-electrophoresis as an analytic tool.

After a preannealing step of **A (11b)** and **T (15)**, the ligation experiments were started by the addition of 2 eq of 5´-ODN **B (12)**.

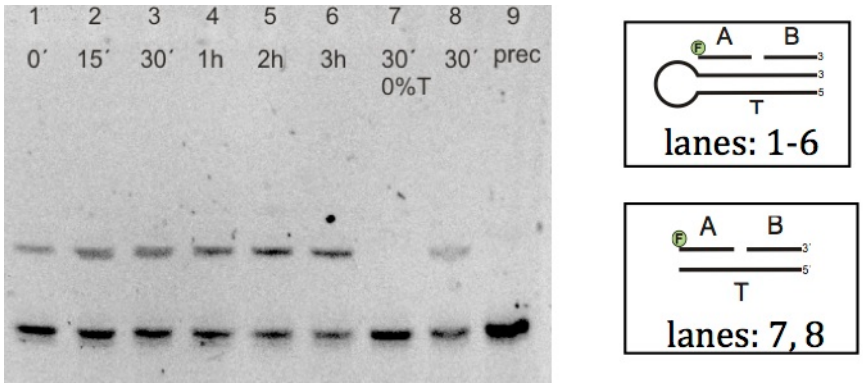


Figure 5: Triplex-ligation; reaction conditions: pH 5.3, 50mM NaCl, 1$\mu$M A + T, 2$\mu$M B ; preannealing of A + T, 90°C (5min)=> 15min=> 20°C.

Gel analysis revealed a fast and clean templated ligation reaction (Figure 3). From the start (lane 1) a continuous product formation was observed for 3h (lanes 1-6) without any side products detectable. Furthermore, the control experiment without template addition showed no product band (lane 7), which indicates that the product formation undergoes the templated reaction path, only. The control experiment with the Watson-Crick template **13** showed less product formation (lane 8) at pH 5.3 than for the Hoogsteen template.

In another experiment, we compared the Watson–Crick and Hoogsteen-ligation at pH 5.3 (Figure 6).

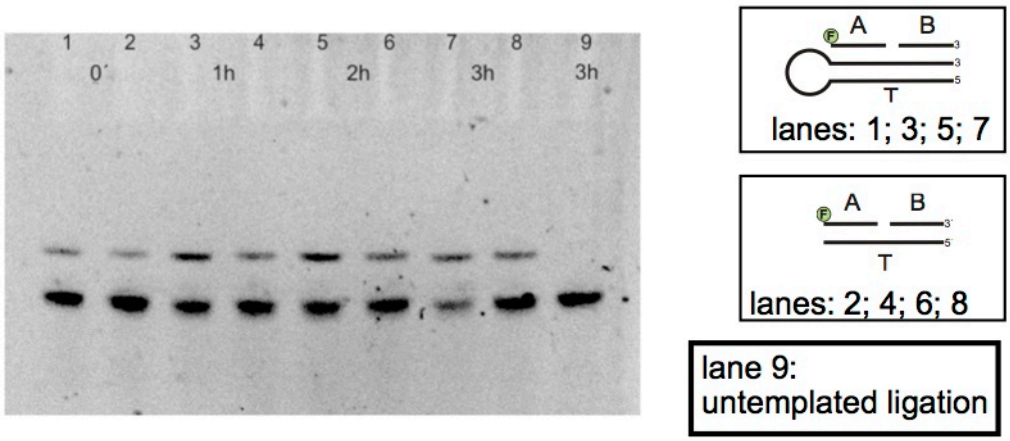


Figure 6: Hoogsteen vs Watson-Crick; ligation at pH 5.3 with template variation; reaction conditions: pH 5.3, 50mM NaCl, 1$\mu$M A + T, 2$\mu$M B ; preannealing of A + T, 90°C (5min)=> 15min=> 20°C.

Here we also found the Hoogsteen ligation to be more effective at pH 5.3 than Watson-Crick ligation. Furthermore, even after 3h reaction time no untemplated product formation was observed and no side products are detected. This indicates that the triplex ligation exclusively follows the templated reaction pathway without any significant background reaction.

In further experiments we could also demonstrate that the preannealing time effects the product formation in the triplex ligation. We observed less product formation in the gel when we shortened the preannealing time from 15 to 5 min (Figure 7).

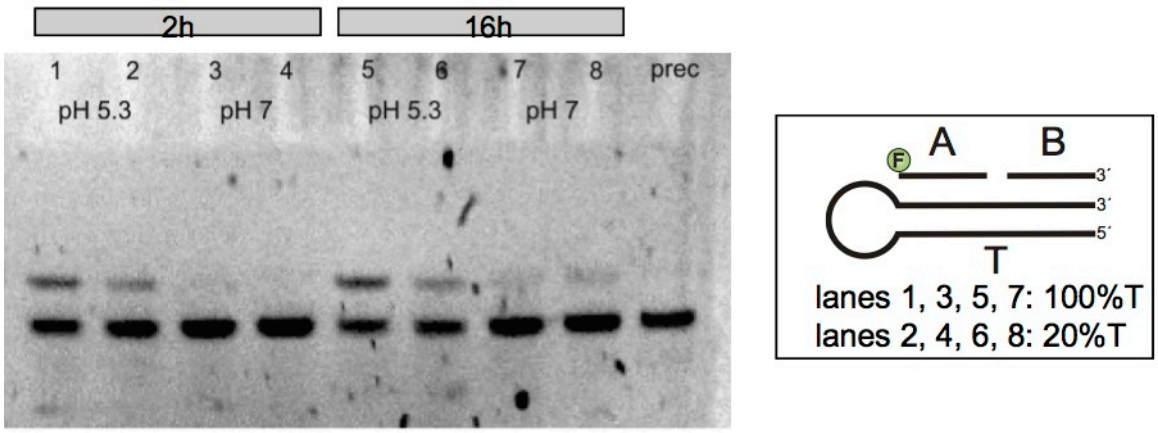


Figure 7: Triplex-ligation with pH-switch, 50mM NaCl, 1$\mu$M A + T, 2$\mu$M B ; preannealing of A + T, 90°C (5min)=> 5min=> 20°C.

We also performed a pH-switch from pH 5.3 to pH 7 and observed that the template effect of the looped template **15** is drastically reduced at pH 7 and only very little product formation becomes monitorable after 16h (lane 7 + 8). The variation of template concentration also showed the expected effect.

These results on the triplex ligation prove the programmability of the chemical ligation and the pH-controllability of the copy release. We have shown that the pH-switch from pH 5.3 to pH 7 effectively prohibits the product formation because the triplex-hybridisation gets prevented. Consequently, the pH-switch enables the copy release and simultaneously stops the templated ligation at the Hoogsteen-side.

To test the FRET-Quencher system in a 12mer precursor molecule under Hoogsteen-basepairing, we also investigated the triplex-Ligation with the looped template **15** and the 3´-Dabcyl-Thiol-ODN **11c**. Experiments were performed in a cuvette at pH 6 with and without template addition and preannealing steps (Figure 8). In the untemplated case, no conversion was observable. On template addition, we observed a product formation which was further increased by the introduction of a preannealing of the precursors **A** and **T**.

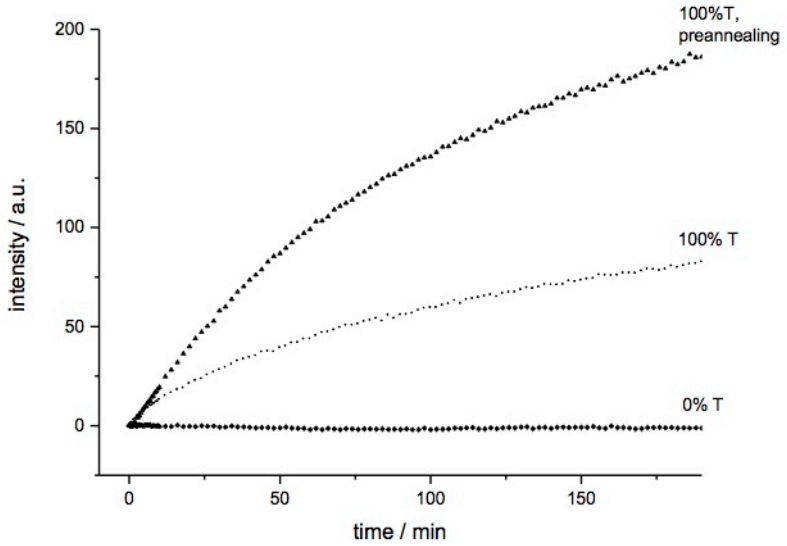


Figure 8: **O**nline monitoring of the triplex ligation; pH 6, 50mM NaCl, 1µM A, 2µM B; preannealing of A + T, 90°C (5min)=> 15min=> 20°C.

With the online measurements we could verify the absence of a background reaction as well as the influence of a preannealing on the ligation rate. The relatively weak Hoogsteen-basepairing and the low salt concentrations appear to retard the formation of the precursor

complex. This can be compensated by the preannealing.

We demonstrated that the templated disulfide ligation of DNA is highly efficient and fast as a ligation chemistry, without significant side reactions. Untemplated background reactions were efficiently suppressed (Watson-Crick-ligation) or not observed (Hoogsteen-ligation). We developed two means of control for a programmable replication in the ECCell, temperature- and pH-switch. In the case of the triplex-ligation we are able to switch the ligation by pH variation from pH 5.3 to pH 7 and to induce a programmable copy release with this mechanism. The combination of this programmable ligation with the induced immobilization, transport and pH-switch in the ECCell leads to a programmable replication system. Most importantly for this deliverable, this process also applies to longer DNA sequences, so that high information content ligation chemistry, which arise from multiple ligations, can be turned over without the product completely inhibiting further access to the template for subsequent copying.

Note that the DNA wire polymerization in D3.2 also goes some way towards addressing the high information content target of this deliverable.

## *1.4 References*

[1] T. Li, K.C. Nicolaou, Nature 1994, 369, 218-221.

[2] V. Patzke, PhD thesis, Ruhr-Universität Bochum, 2005 http://www-brs.ub.ruhr-uni bochum.de/netahtml/HSS/Diss/PatzkeVolker/Inhaltsverzeichnis.pdf.

[3] J. W. Gaynor, J. Nentley, R. Costick, Nature Protocols 2007, 2 (12), 3122-3135.