



Project no. **222422**

Project acronym: **ECCell**

Project title: Electronic Chemical Cell

Instrument: STREP/FET OPEN

Thematic Priority: Theme 3 Information and Communication Technologies

**Deliverable 1.1: report, public (PU)**

**Development of replication chemistry running in the electronic cell.**

Due date of deliverable: 1. 11. 2010

Actual submission date: 03. 11. 2010

Start date of project: **1.09.2008**

Duration: **3 years**

Organisation name of lead contractor for this deliverable:

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

# 1. Deliverable n. 1.1: Development of replication chemistry running in the electronic cell

## 1.1 Introduction

In WP 1 - Programmable Replication – the main objectives include the chemical ligation of DNA via disulfide bonds and a pH controlled copy release. The advantages of the disulfide ligation chemistry are high reaction rates, the pH- and redox-control potential, reversibility and its structural similarity to the native phosphodiester bond. Since the methods for chemical preparation of thiol modified DNA building blocks are well established in our work group the specific efforts were directed to the development of highly sensitive FRET detection methods for microfluidic experiments, sequence design and examination of reaction conditions.

## 1.2 Syntheses of Thiol-Oligonucleotides

To utilize the disulfide-exchange reaction in the context of ECCell we started to develop new ligation systems to verify the availability of this chemistry in the replication process and to outline the reaction conditions compatible with gelation, pH-jumps and analysis in microfluidics.

For the synthesis of 3'- and 5'-thiol modified oligonucleotides 5'-thio-desoxycytidine and 3'-thio-desoxyguanosine nucleosides were synthesized by Mitsunobu reaction introducing a thioacetate.<sup>[1]</sup> The conveniently protected thiol-precursors were immobilized on thiol-CPG respectively (Figure 1). 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 or activated disulfides.

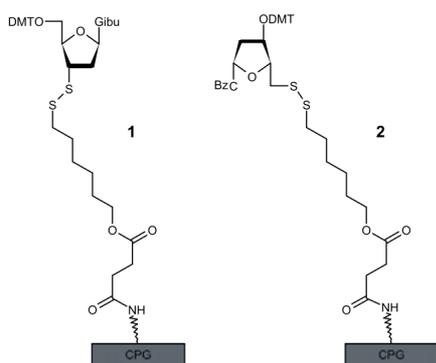


Figure 1: Solid supports for phosphoamidite syntheses.

Activated disulfides were obtained by reaction of the free thiol-oligonucleotide with a twenty fold excess of modified Ellman's reagent in a buffered solution at pH 7 and subsequent HPLC workup.

## 1.3 Implementation of Fluorescence Quenching Systems

One objective was the development of an activator independent chemical DNA ligation or replication system and to combine it with a suitable analytical method like FRET or fluorescence quenching for monitoring in microfluidic systems. We therefore chose thiol-disulfide exchange reaction as ligation chemistry and a Xanthene-Dabcyl pair for ligation detection at 490nm (Figure 1). We synthesized a 6-FAM-Dabcyl pair for ligation experiments in bulb furthermore an Alexa488-Dabcyl pair for a widely pH independent detection with minimized bleaching. The choice of the fluorophore in the FRET quenching system was made corresponding to the needs of microfluidic laser detection and the quencher compatibility.

The Dabcyl Quencher was introduced as an activating disulfide leaving group. Synthesis starts from orthogonally protected lysine, which is subsequently deprotected and modified with

Ellman's reagent and Dabcy acid (Figure 2).

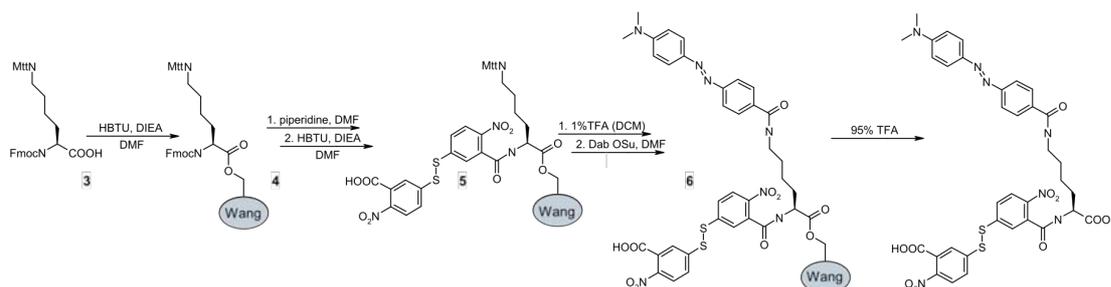


Figure 2: Synthesis of Dabcy-Lys Quencher.

The fluorophore was introduced in the same precursor molecule as the quencher in order to monitor the ligation step with increasing fluorescence (Figure 1). We followed two concepts of insertion for the fluorophore. For monitoring FRET quenching in precursor molecules up to 10mers we introduced 6-FAM as a phosphoramidite or Alexa488 as an active ester via an amino modifier phosphoramidite. This terminal insertion gives a strong fluorescence signal but it makes the signal intensity dependent on sequence length and hybridisation (Figure 3-2A).

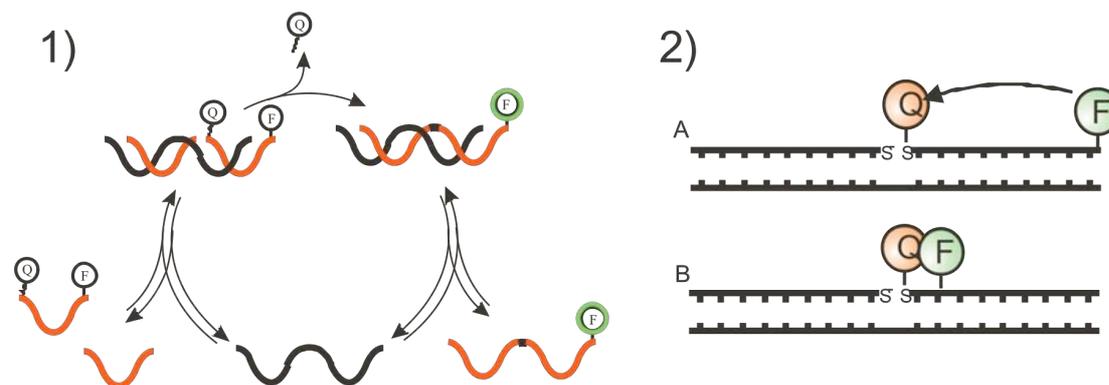


Figure 3: 1) Illustration of a ligation cycle, 2) concepts of quenching.

Alternatively we introduced the fluorophore Alexa488 via an active ester in close proximity to the quencher in the DNA strand. An applicable amino modification was synthesized, starting from *O*<sup>6</sup>-Phenyl-2'-desoxyinosin phosphoramidite in oligonucleotide synthesis.<sup>[2,3]</sup> After DNA workup the desoxyinosin was converted with 1,4-diaminobutane and functionalized with Alexa488 (Figure 4).

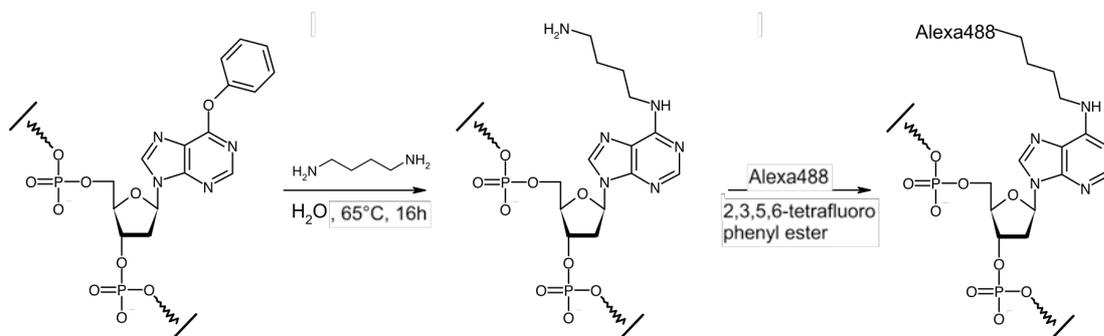


Figure 4: Alexa488 modification of adenine.

The latter contact quenching system (Figure 3-2B) is expected to be more sensitive than the FRET quenching system and provides the opportunity to freely choose sequence length in replicating systems.

## 1.4 Disulfide replicating systems

Utilizing the FRET detection methods we designed and synthesized a number of model systems to perform and analyze self-replication and templated oligonucleotide ligation experiments. For self-replication experiments we designed symmetric systems (A) an unsymmetric systems (B) and crosscatalytic systems (C) (Table 1).

**Table 1.** Sequence design for replication experiments.

Model Systems	3'-Thiol-Oligonucleotides	5'-Thiol-Oligonucleotides	Templates
(A) symmetric	ATTATACCG-SH	<b>Dab</b> -CGGTATAAT- <b>F</b>	ATTATACCGCGGTATAAT
	<b>F/A</b> -ATTATACCG- <b>Dab</b>	HS-CTTGAAGTA	TACTTCAAGCGGTATAAT
	TATCTCAA <sup>A</sup> G- <b>Dab</b>	HS-CGGTATAAT	TATCTCAAGCGGTATAAT
(B) unsymmetric	<b>F</b> -TGATTGCG- <b>Dab</b>	HS-CAATCA	ACTAACGCGTTAGT
	CATAA <sup>A</sup> G- <b>Dab</b>	HS-CGCTTATG	CATAAGCGCTTATG
(C) crosscatalytic	<b>F/A</b> -ATTATACCG- <b>Dab</b>	HS-CTTGAAGTA	ATTATACCGCTTGAAGTA
	TACTTCAAG-SH	PyS-CGGTATAAT	TACTTCAAGCGGTATAAT
	TATCTCAA <sup>A</sup> G- <b>Dab</b>	HS-CGGTATAAT	ATTATACCGCTTGAGATA
	ATTATACCG-SH	PyS-CTTGAGATA	TATCTCAAGCGGTATAAT

**F**: 6-FAM, **A**: Alexa488, **Dab**: Dabcyl quencher, PyS: 2-Thiopyridyl

The systems labelled with 6-FAM as fluorophore were used for experiments in bulb at pH 6-8 due to its strongly pH dependent fluorescence and its bleaching sensitivity. For measurements under varying pH-value in microfluidics Alexa488 was chosen.

Replication experiments for all types of model systems were performed at micromolar concentrations and indicated a template effect and a contribution of side reactions to the product formation.

## 1.5 Self-replication experiments

We performed self-replicating experiments with a self-complementary 9-18mer system under variation of pH- value and initial template concentration. Besides a templated ligation the experiments revealed a strong background reaction from the precursor complex AB (Figure 5).

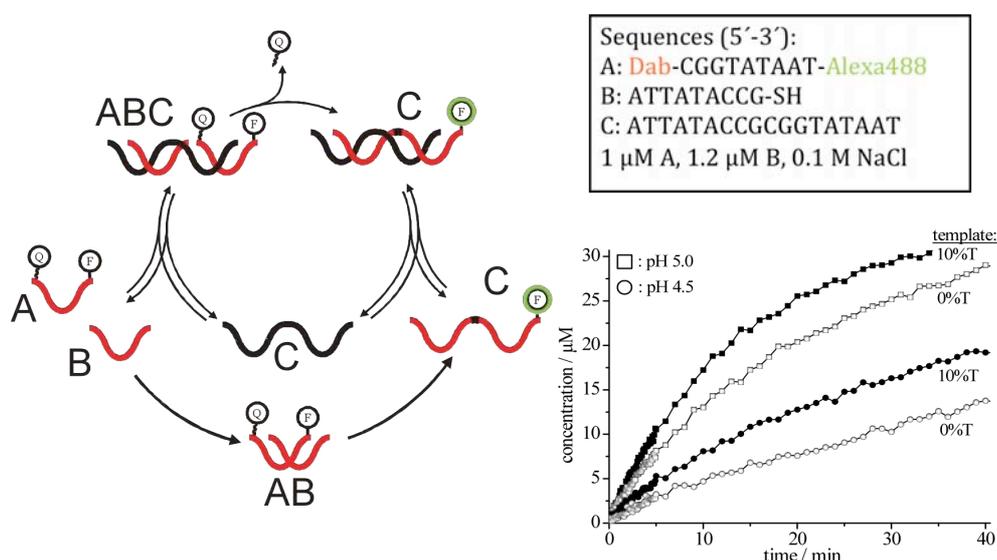


Figure 5: Illustration and online kinetics of a self-replicating system. Side reactions and pH-dependency of the ligation are demonstrated.

Beside this undesired reaction pathway we could demonstrate a strong influence of the pH-value on the ligation rate. As the thioate mainly acts as a nucleophile in the disulfide exchange reaction the ligation rate decreases with the grade of protonation which makes the ligation reaction controllable by pH-jumps to a certain extend.

### 1.6 Templated chemical DNA ligation experiments

To explore reaction conditions for the templated oligonucleotide ligation and its programmability we chose the symmetric 9-18mer system with a 3'-activation and a 5'-FAM fluorescent dye for studies on reaction rates under various conditions. The ligation experiments were performed at micromolar concentrations with a variation of the initial template concentration, temperature and pH-value (Figure 6).

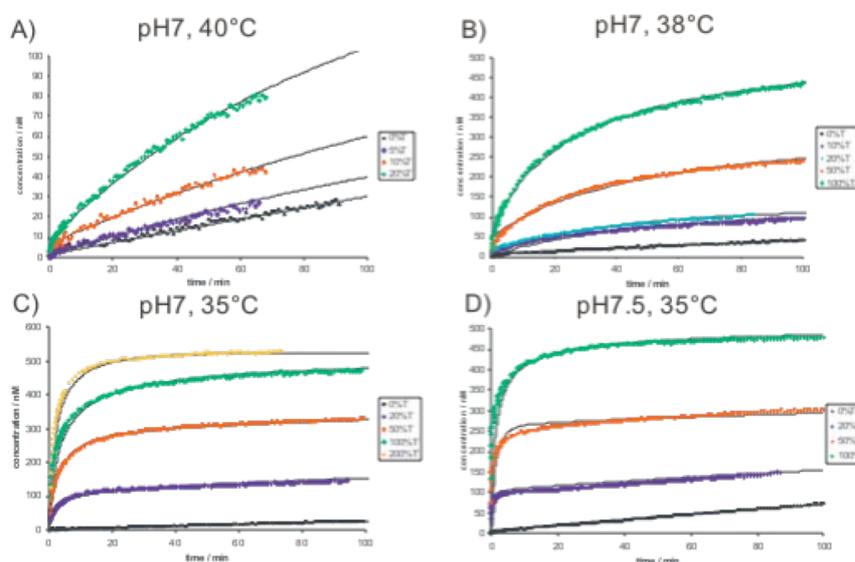


Figure 6: FRET online kinetics for templated DNA ligation, 0.5  $\mu$ M precursors, 100 mM NaCl.

Near melting point there was a considerable contribution of the untemplated reaction pathway to the product formation observed (Figure 6A/6B). Below melting point a fast templated ligation reaction was observed. Under these conditions the untemplated ligation reaction makes little contribution to the product formation (Figure 6C/6D). We could demonstrate that the ligation rate increases with increasing pH-value as well.

### 1.7 Comparison of 3'- and 5'-activation in disulfide ligation

Besides a 3'-activated disulfide ligation system we also examined the corresponding 5'-activated complementary system under similar reaction conditions. Surprisingly, here we found a much stronger background reaction than for a 3'-activation. Comparing the 3'-versus the 5'-activation for the non-templated background reactions we observed strongly different rates under similar conditions. In the case of a 3'-activation and a 5'-nucleophile we observed significantly lower rates than for a 5'-activation and a 3'-nucleophile. These results cannot be explained by nucleophilicity, since the 5'-thioate is expected to be the stronger nucleophile. Experiments with interchanged nucleophiles, leading to 3'-3'- and 5'-5'-ligations also gave lower yields for the 3'-activation. The effect apparently originates from sterical reasons, since in the case of the 3'-activated disulfide, a random backside attack for the 3'-end appears hindered. We also found

that, within a reasonable range, there is only little influence of the reaction temperature observable for the 3'-activation motif (Table 2).

**Table 1.** Oligonucleotide sequences and temperature effects for non-templated ligation experiments.[a] constants not determined

		3'-activation	5'-activation
3'-S-ODN	5'-S-ODN	<sup>F</sup> ATTATACCG <sup>Q</sup> SCTTGAAGTA	TACTTCAAG <sup>S</sup> QCGGTATAAT <sup>F</sup>
T / °C		k <sub>i</sub> [M <sup>-1</sup> ·s <sup>-1</sup> ]	
	35	41	391
	38	25	547
	40	22	718
	43	21	a
	45	23	844
	55	a	1413

On the other hand, an increase of reaction temperature greatly increases the reaction rates for the 5'-activation (Table 2). Therefore, for the 3'-activation, temperature can be used as an effective tool for the regulation of the templated ligation by influencing hybridisation. We could demonstrate that, for the 3'-activation, the templated ligation can be affected by temperature variation via hybridisation whereas the untemplated background is apparently unaffected by a temperature rise due to a sterical hindrance at the activated side.

### 1.8 Single point substitution experiment

The templated ligation and its selectivity towards slight template modifications were to be demonstrated. Therefore we examined the 3'-activated system combined with a template with a G vs T substitution. For the modified template **TM** the observed reaction rates are comparable to the untemplated case, whereas the complementary template **T** shows the expected effect under similar conditions.

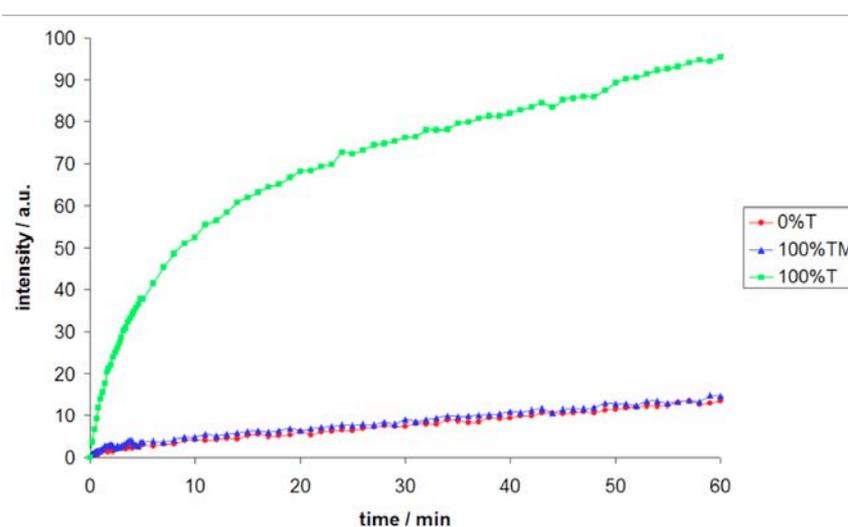


Figure 7: Influence of template on the ligation; reaction conditions: 0.5  $\mu$ M conc.; pH 7, 0.1M NaCl, 30°C.

**T:** TACTTCAAGCGGTATAAT; **TM:** TACTTCAAGCTGTATAAT.

The ligation experiments indicated no significant effect of the modified template on the ligation. A mutation close to the ligation site seems to have a big effect on the complex formation under the chosen conditions. Due to the high reaction rates and the sensitive FRET-detection method the single point mutation analysis is a promising application for the system. Nevertheless, further experiments with various modified sequences have to be performed.

### ***1.9 Attachment of DNA to synthetic block copolymers***

One main objective in WP1 includes the combination of DNA-scp with oligonucleotide replication and the inline coupling of DNA to scp. Together with the Herrmann group we synthesized a poly(propylenoxide)-phosphoramidite (PPO) ( $M_w = 6800$  g/mol) and utilized it for the preparation of a DNA-scp template for chemical ligation and for the preparation of scp-thiol-oligonucleotides.<sup>[6]</sup> We demonstrated the compatibility of the DNA block copolymer synthesis with thiol oligonucleotide chemistry. We synthesized PPO-thiol-oligonucleotides and analyzed them via gel electrophoreses.

### ***1.10 Replication chemistry for the electronic cell***

Our developments on the disulfide exchange reaction as a ligation motif for the chemical DNA replication led to promising results for the electronic cell. For the 3'-activation we have in hand the probably fastest DNA ligation reaction which shows only little side reactions due to the sterical hindrance in the activated precursor molecule, which can be overcome best in the termolecular complex of the templated reaction. For this system, temperature variations therefore mainly influence the hybridization and not the reaction rates directly, which makes temperature a suitable tool to directly influence the ligation via hybridization. We also demonstrated that the pH-value determines the reaction rate by protonation/deprotonation and therefore by activation/deactivation of the nucleophile.

Combined with the pH-independent, photo stable Alexa488/Dabcyl-detection method the disulfide chemistry can be used as a chemical DNA ligation system in microfluidics and be manipulated in an electronic cell. The system does not yet provide an efficient full replication circle due to background reactions but the high rates, the temperature independence of the ligation and the pH influence give a potential of external interference in the electronic cell.

Furthermore the block copolymer chemistry is compatible with the thiol-oligonucleotides and demonstrates the connection to compartmentation and vesicle formation.

Good progress has been made concerning the implementation of replication in the electronic cell. In principle the DNA ligation system is applicable for a pH driven replication and with a potentiostat precursor molecules could be electronically provided for the ligation.

### ***1.11 References***

- [1] V. Patzke, PhD thesis, Ruhr-Universität Bochum, 2005 <http://www-brs.ub.ruhrunibochum.de/netahtml/HSS/Diss/PatzkeVolker/Inhaltsverzeichnis.pdf>.
- [2] A. E. Ferentz, T. A. Keating, G. L. Verdine, *J. Am. Chem. Soc.* **1993**, *115*, 9006–9014.
- [3] A. E. Ferentz, G. L. Verdine, *Nucleosides & Nucleotides* **1992**, *11* (10), 1749-1763.
- [4] M. Roth, *Anal. Chem.* **1971**, *43*, 880.
- [5] P. de Montigny, J. F. Stobaugh, R. S. Givens, R. G. Carlson, K. Srinivasachar, L. A. Sternson, T. Higuchi, *Anal. Chem.* **1987**, *59*, 1096.
- [6] F.E. Alemдарoglu, K. Ding, R. Berger, A. Herrmann, *Angew. Chem. Int. Ed.* **2006**, *45*, 4206.
- [7] J. T. Goodwin, D. G. Lynn, *J. Am. Chem. Soc.* **1992**, *114*, 9197.