1. Introduction

Electron transfer (ET) between proteins plays a crucial role in all living organisms. Within the respiratory chain the redox protein cytochrome c (cyt c) is an important electron mediator between enzymes. Due to this property cyt c can be used as the central building block of artificial ET chains. For the assembly of these fully electro-active architectures, the layer-by-layer technique is applied [1]. This technique enables the construction of a redox-active cyt c•DNA multilayer system, by using DNA as the second building block [2]. The multilayer system can be coupled an enzyme, like the pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) and is therefore applicable as analytical signal chain with sensitivity towards glucose [3].

One important feature of DNA containing multilayer system, compared to other systems, is it’s very high surface loading of electro-active protein, which can only be achieved by assembling at pH 5. However, the assembly process and the structure of the system, is not entirely understood, yet. Until now, the role of electrostatic interactions in the stabilization of cyt c•DNA assembly has been reported in literature [4, 5]. Furthermore the cyt c•DNA interaction has been investigated from the perspective of DNA, indicating a 15 bp interaction site on the nucleic acid [6].

In our previous work we have found evidence for a special interaction between cyt c and DNA. CD spectroscopy revealed a change in the α-helical structures of the protein. This effect was dependent on the DNA concentration, the pH-value and the ionic strength of the buffer.

Based on these results, we intended to elucidate the cyt c•DNA interaction, by NMR spectroscopy. We focused on gaining a fundamental understanding of the interaction mechanism between cyt c and oligonucleotides (DNA of a defined size), which will finally give us an idea of the structural properties of the cyt c•DNA multilayer system.

Due to their expertise in NMR-spectroscopy and experience in the work with horse heart (hh) cyt c, we performed this study in cooperation with the Crowley group at the National University of Ireland in Galway (NUIG). The STSM was executed from the 28.08.2013 – 24.09.2013.
Before the start of the measurements the project was discussed with the group leader, Dr. Peter Crowley and the co-worker Mrs. Ciara Kyne BSc. The issue was discussed in detail. Potential problems, economical use of the limited amount of oligonucleotides and the variation of the DNA and salt concentration were figured out. As a result of the discussion the preliminary work plan was adapted.

The results of our first STSM with the Crowley group gave us an orientation concerning the optimal conditions for the measurements, e.g. the cyt c concentration of 50 μM yeast cyt c was also valid for hh cyt c. We also decided to keep as close as possible to the molecular ratio of cyt c, DNA and buffer concentrations. Furthermore, 15N labelled horse heart (hh) cyt c was previously expressed and purified from *E. coli*.

### 2. Results

#### 2.1. Experiments performed at pH 5

Experiments performed at pH 5 and low salt concentrations (0, 10 and 20 mM NaCl), resulted in the formation of sticky cyt c•DNA agglomerates, which occurred already at a low DNA concentration of 3 μM. These precipitated and stuck to the walls of the Eppendorf tubes, colouring them red. Therefore, the complexes were not accessible for HSQC spectroscopy. In order to find the optimal NaCl concentration, stabilizing the cyt c•DNA complexes in solution and making them accessible for NMR-spectroscopy, the remaining solution was investigated by UV/Vis spectroscopy.

We found that the agglomeration can be reversed by increasing the salt concentration. This was a) indicated by the gradual colouring of the Eppendorf tube. The strong red colour of the 0 mM NaCl sample indicated that a substantial amount of cyt c•DNA was stuck to the surface of the Eppendorf tube, when no salt was present. With increased NaCl concentration the intensity of the colour decreased, indicating that the sticky complex was disrupted and cyt c got in solution. The agglomerates clearly started to come off the surface at NaCl concentrations of 30 mM and 40 mM. At 50 mM NaCl all cyt c molecules seemed to be back in solution, since no red colour was left.

The solution has been analyzed by UV/Vis spectroscopy at 550 nm. Here the highest absorption was found at 0 mM NaCl. This may be due to the formation of cyt c /DNA agglomerates, which scatter the irradiated light. First, the absorption dropped after addition of 10 mM and 20 mM NaCl. We assume that the cyt c•DNA complexes in solution are disrupted while the agglomerates kept sticking to the Eppendorf tube. At 30 mM NaCl the adsorption increased again, indicating a loss of stickiness of the cyt c•DNA agglomerates and their stabilisation in solution. A further increase of the salt
concentration resulted in a decline of the adsorption, which may mean, that the agglomerates in solution were finally disrupted by the high salt concentration. The measured adsorptions does not clearly correlate to the amount of cyt c●DNA attached to the Eppendorf tube and does not fully return to the value of the cyt c control, even at high salt concentrations. Despite this ambiguity we relied on our observation by eye and concluded that cyt c●DNA agglomerates can be stabilized in solution most effectively at salt concentrations between 30 mM and 40 mM.

HSQC spectra were recorded with the same NaCl concentrations as described above (except 20 mM NaCl). Cross peaks sensitive to the change of the pH value and the ionic strength were spotted by a pH and a NaCl-titration and a peak list was set to assign each peak to its respective amino acid residue. The spectra recorded from cyt c●DNA samples with no NaCl and 10 mM NaCl lead to weak cyt c signals, since a substantial amount of cyt c was stuck to the Eppendorf tube and was therefore not accessible for HSQC. However, the spectra gave evidence that csp’s may occur at certain cross peaks due to DNA binding. Increasing the NaCl concentration to 30 mM resulted in the weakening of the cyt c●DNA interaction and subsequently in stronger cyt c spectra. Despite this no csp’s were found here. This indicates that the cyt c●DNA agglomerates kept sticking to the surface and only unbound cyt c remained in solution.

The NaCl concentration was further increased to 40 mM and 50 mM NaCl, which resulted in cyt c spectra of good quality. Csp’s can be observed at some NH-cross peaks, again. Here the effect can be clearly related to DNA binding, since salt and pH effects can be excluded. We also tried to show that the disruption of the cyt c●DNA complexes is possible by increasing the NaCl concentration to 100 mM. Observing the samples, we found that the increased NaCl concentration brought the precipitated cyt c back in solution. The NMR-spectrum provided csp’s, but these had to be related to the high salt concentration, since only peaks which are known to shift in dependency on the salt concentration were affected.

Since no clear prove of the cyt c●DNA interaction was found at pH 5, the pH value of the cyt c●DNA samples was altered for further investigation. A pH titration from pH 5 towards pH 6 was executed in order to destabilise the complexes. But during the first titration step, even more cyt c precipitated,
which made the preparation of a useable sample impossible. Further experiments were performed at pH 7 and pH 6.

2.2. Experiments performed at pH 6 and pH 7

During the preparation of the cyt c●DNA at pH 6 and pH 7, no precipitation and sticking of the complexes to the Eppendorf tubes was observed. HSQC spectra of the cyt c●DNA samples prepared with 30 mM NaCl and pH 6 or pH 7, showed clear csp’s compared to the respective cyt c control spectrum. A pH titration of the cyt c●DNA complex was performed at an ionic strength of 30 mM, going from pH 7 to pH 6. The csp’s changed in dependency on the pH value, which indicated a dependency of the cyt c●DNA interaction on the pH value. The effect could also be related to the sensitivity towards the pH change. However, the comparison to the cyt c control spectrum showed that some peaks are clearly shifted due to DNA binding. Besides many others the AA residues listed in the following were affected at pH 7: G6, K7, K8, Q12, Q16, H33, Y48, K72, K87, T89, E90, A96, Y97, and E104.

When performed at pH 6 the cyt c●DNA spectrum showed csp’s at almost all of the above listed peaks. But additionally, more csp’s were observed here at peaks with basic AA-residues, like H18, K22, K86, R91, K99 and K100. Besides this uncharged AA located in the close environment of the listed ones were also affected.

Labelling of the affected AA-residues in a graphical cyt c model revealed their location on the protein surface. We found that mainly AAs located at the alpha helical structures of the protein were attacked by DNA. This correlates to our findings from CD spectroscopy, which indicated that alpha helical structures are affected in presence of DNA. These secondary structures may be inserted in major groove of the DNA molecule. This way of binding DNA is already known from histones, the cells’ DNA binding proteins. Like histones, cyt c shows a high content of alpha helical structures, supporting our hypothesis of cyt c●DNA binding via insertion of an alpha helix in the major groove of DNA.

A DNA titration at pH 7 and 30 mM NaCl proved the dependency of the csp’s on the DNA concentration. Csp’s increased from 3 µM, 6 µM to 18 µM DNA. A saturation effect occurred at around 18 µM DNA, since increasing the DNA concentration to 24 µM did not result in more intense csp’s.
We made sure that the csp’s refer to cyt c●DNA binding, since comparison with the respective controls showed that pH and NaCl sensitive cross peaks shift stronger in presence of DNA. Some of them even shifted in a different direction. Furthermore, additional csp’s of none pH and salt sensitive peaks were found after DNA binding.

In order to investigate the reversibility and the nature of the binding process, a spermine titration was applied on a cyt c●DNA sample with 24 µM DNA. However no back-shift of the affected peaks was found, since the chosen spermine concentration was too low. This experiment has to be repeated at higher spermine concentrations and a DNA concentration below the threshold of saturation.

3. Summary

All together we managed to show the interaction between cyt c and DNA by HSQC spectroscopy and identified specific DNA binding sites on the protein.

At pH 5 the agglomeration and precipitation of the cyt c●DNA complexes was very efficient at a low ionic strength. The subsequent precipitation made HSQC analysis almost impossible, since the spectra obtained were found to be weak and consequently hard to evaluate. However, we managed to adjust the salt concentration in a way that the cyt c●DNA complexes were stabilized in solution, avoiding precipitation and enabling HSQC analysis. In the recorded spectra we observed csp’s, caused by DNA binding at salt concentrations of 40 mM and 50 mM.

Going towards the neutral pH range, the spectrum quality was improved even in presence of DNA. Clear csp’s were observed in these spectra, proving DNA binding to cyt c. Specific DNA-binding sites were spotted on the cyt c surface, indicating that mainly alpha helical structures of the protein are involved. This result is in agreement with our results from CD spectroscopy, which we wanted to validate by NMR spectroscopy.

Beyond this, a DNA saturation effect was observed at around 18 µM DNA. This proves that the csp’s were effectively caused by DNA binding.
4. References

[1] F. Lisdat et al. (2009), Chemical Communications, 3:274-283
[6] XQ. Ding et al. (2005), Analytical Biochemistry, 339