Final Publishable Summary Report

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The aim of the project was to show that bead surface display (BeSD) which was originally designed to access optimized protein binders after directed evolution can also be used to monitor enzymatic catalysis. The assay would thereby provide a novel high throughput method for the directed evolution of catalytic enzymes. Especially post-translational modifications triggered by enzymes like the condensation (C)-domains of the nonribosomal peptide synthetases are a promising target since those modifications are involved in numerous signalling pathways in humans and are a prime target in drug research. At the beginning of the project the most important post translational modifications were evaluated according to their importance and suitability for the BeSD assay:

1.) Evaluation of suitable catalytic reactions that can be developed using BeSD

The following reactions were shortlisted:

- NRPS condensation domains
- Glycosyltransferases
- Protein kinases or kinase substrates

The kinase reactions were finally chosen due to two major reasons. First they are currently the most important enzyme target in for the pharmaceutical industry since alterations in their activity are linked to several forms of disease in humans. Second by using fluorescent labelled antibodies that specifically target the phosphorylated form of kinase substrates a straightforward method to combine a selective binding reaction with a catalytic reaction will be available, which is a prerequisite for a successful assay. Out of more than 500 known kinases the kinases MEK1 and ERK2 present in the MAPK pathway were chosen. Alterations in this pathway can be found in more than 60 % of melanomas and are also present in other forms of cancer. The main idea was to evolve MEK1 in the presence of an MEK1 inhibitor to see if an artificial resistance could be generated by directed evolution which would be monitored using the BeSD assay. Furthermore kinase peptide substrates can be evolved with the display system which could lead to novel kinase peptide inhibitors or fluorescent peptide substrates that could be used to monitor kinase reactions.

2.) Optimization of the BeSD assay reaction conditions

At the beginning of the project the BeSD assay conditions had to be optimized. Different emulsification methods, surfactants, bead sizes and reaction times and protocols were tested. In addition bead losses, which would decrease the amount of variants that can be analysed per selection round were monitored in washing steps. Those steps led to more stable droplets after the emulsion PCR reactions and losses during de-emulsification and washing could be minimized.

3.) Cloning, Mutagenesis and Expression of Protein Kinases and their Substrates

The protein kinases MEK1, ERK2 and MKKK1 were cloned and expressed in *E. coli*. Furthermore constitutively active mutants of MEK1 were designed that could be used for positive selections in our display system. ERK2 which is a substrate for MEK1 was expressed with an avi-tag as well as chemically biotinylated which allows it to be displayed on beads.

4.) Development of the BeSD assay for the evolution of post translational modification reactions

The potential development of enzymatic resistances against inhibitors is a very important factor in the assessment of the success of enzyme inhibitors. The BeSD assay could be used to evaluate this factor by screening mutant libraries of the target enzyme for their phosphorylation efficiency in the presence of known or novel inhibitors. The process cycle for one selection round is shown in Figure 1.

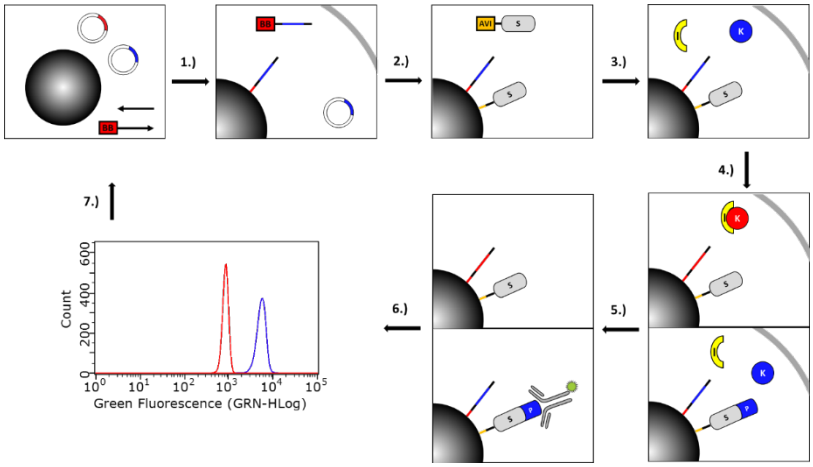

Figure 1: BeSD assay cycle for the evolution of resistant MEK1 mutants in the presence of inhibitor. 1.) Emulsification of template DNA, modified primers (3'dual biotinylated forward primer) and streptavidin coated beads and subsequent ePCR. 2.) De-emulsification and binding of avi-tagged substrate, 3.) Addition of inhibitor, emulsification and IVTT. 4.) Only resistant mutants of MEK1 phosphorylate the substrate bound to beads. 5.) De-emulsification and binding of fluorescent antibody specific for phosphorylated protein. 6.) Evaluation of protein phosphorylation on FACS. 7.) Sequencing of evolved kinase variants and application in new cycle.

Several steps of the assay have so far been investigated. Step one has been tested with different protein kinases and substrates and showed that the DNA is displayed on beads. Quantification of the results by real time PCR led to the expected amount of DNA displayed per beads. The binding of avi-tagged protein shown in step two was tested with ERK2 as well as with chemically biotinylated ERK2 which was thought as an alternative possibility to bind the protein to the beads. Avi- tagged protein was found to be superior with the suitability of biotinylated substrate decreasing with increasing biotinylation state. For steps 3 to 5 initial tests using active and inactive mutants of MEK1 were conducted without emulsification. Several antibodies detecting either phosphorylated Tyr or the phosphorylated or non-phosphorylated form of ERK2 were tested. These tests were conducted in multiple reaction conditions and showed that phosphorylated ERK2 can clearly be distinguished from the non-phosphorylated form which is a prime requisite for the success of the assay.

In summary the suitability of the assay for the detection of protein phosphorylation on beads could be shown. The results obtained in this project will be used in a follow up project and will be strengthened by the collaboration with Prof. Kevin Dalby of the University of Austin, who has long term experience in the MAPK pathway. This assay could be used in the initial evaluation of the efficiency novel kinase inhibitors which would be of high demand for the pharmaceutical industry.

5.) Development of an alternative microfluidic based assay.

To extend the applicability of the BeSD assay for other kinase reactions where no suitable antibody is known an alternative assay on microfluidic chips is in current development. This assay uses water in oil in water double emulsions and could hence monitor catalytic reactions without the necessity of an antibody bound to beads. So far the setting of the microfluidic system was optimized for the use of streptavidin coated beads with and without bound protein. The major obstacle of beads settling in tubing and microfluidic chips could be prevented by using density enhancing medium, increasing the flow rate of both aqueous and oil phase and movement of the tubing.

Further development of this assay will further broaden the application spectrum of the BeSD assay and would represent a novel high throughput early stage screening system for the success of kinase inhibitors.