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## ASSEMBLY STRATEGIES FOR ENZYME IMMOBILIZATION

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### Introduction

Nature's ability to perform cascade reactions in an effective way is based on the correct assembly of the different biocatalysts in a metabolon. Positional control is especially important in multistep reactions, in which the product of one catalytic reaction is used directly as substrate for the next transformation. Immobilization of catalysts, e.g. adsorption of enzymes on beads or homogeneous catalysts on solid supports has been a topic of investigation for many years.<sup>1</sup> Compared to nature's metabolons, not much control over the spatial distribution of the catalytic sites in these manmade supported catalysts is obtained. In order to have more control new methods for catalyst assembly have to be developed. This can be achieved by using Nature's approach of organization through self assembly. To imitate this we are looking for ways to assemble enzymes on a supramolecular support to produce a synthetic metabolon. Next to enzymes these metabolons can contain non-biological catalysts.

In a model system we are engineering a lipase to introduce chemical handles for covalent immobilization. The lipase explored for this procedure is the lipase *Candida Antarctica* Lipase B (CAL-B). CAL-B is a very stable enzyme, which can tolerate high temperatures and a variety of solvents without losing activity. We will introduce a chemical functionality through incorporation of a non-natural amino acid functioning as a handle for covalent attachment to a scaffold. Using protein engineering it has been shown that the methionine analogues like azidohomocysteine or homo propargyl glycine can be readily incorporated into proteins using a bacterial auxotroph strain.<sup>2</sup> After introduction of an azido function a covalent connection between enzyme and molecules containing a terminal acetylene function can be made by a Cu-catalyzed Huisgen [3+2] cycloaddition.<sup>3</sup>

Virus particles such as *Cowpea chlorotic mottle virus* and *Potatoe virus X* (PVX) are excellent examples of well-defined non-covalent assemblies. The protein shells of viruses provide highly promising scaffolds for the construction of new materials.<sup>4</sup> In addition to possessing nano-scale dimensions overall, the protein subunits that comprise the capsid shell present regularly spaced attachment sites that could order different material compounds.<sup>4,5</sup> It can be envisaged that the capsid proteins can be covalently functionalized via protein engineering with bio active proteins such as enzymes. Like this we can develop a catalyst scaffold based on a virus particle, which will enable us to gain control over catalyst organization via assembly of the support (fig.1). A significant practical advantage is the ease with which large quantities of the catalytically active particles can be obtained due to the reproductive power of viruses, thus giving rise to a one step production method for biocatalyst and scaffold.

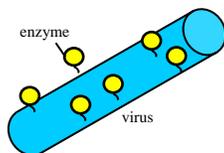


Figure 1. Schematic representation of enzyme decorated virus particle.

A second self assembling scaffold can be developed using the organization properties of surfactant molecules into highly ordered structures, such as rods, ribbons, tubes and helices.<sup>6</sup> The introduction of strong hydrogen bonding moieties has been shown to be instrumental in the structuring and stabilization of such assemblies. The incorporation of bis-ureido groups in molecules and polymers gives rise to strong hydrogen bonding interactions that have been used to form stable organo-<sup>7</sup>, and hydro-gels<sup>8</sup>, to structure inorganic materials<sup>9</sup>, and also polymeric assemblies. We are going to use an ammonium terminated surfactant, in which a bis-ureido group is incorporated in its hydrocarbon chain.<sup>10</sup> This molecule forms well-defined highly ordered ribbon-like bilayer aggregates in water. In addition it has been demonstrated that these ribbons can be functionalized via a modular approach through molecular recognition of other bis-urea containing molecules.<sup>10</sup> Such molecules can be linked to enzymes which can then be ordered through assembly of the support.

Here we report on our progress in developing the different scaffolds for enzyme immobilization and in producing a modified enzyme that can be attached to these scaffolds.

### Results and Discussion

We have produced fusion proteins of PVX capsids with CAL-B. Using molecular biology techniques we have fused the genes encoding the two proteins. In order to prevent steric hindrance of the bulky enzyme during the capsid assembly process the genetic construct was made in such a way that partly the fusion protein and partly the capsid alone was produced. Virus particles produced by plants that were infected with modified viral RNA were decorated with CAL-B (see fig.1), as could be observed by electron microscopy and Western blotting. Unfortunately expressing the fusion protein appeared to be toxic to the plant and this led to a very low amount of virus particles that could be isolated.

In conclusion, our system to produce in one step a catalytically active virus particle works. However the yield is very low. We are now investigating other methods to immobilize enzymes on the virus particle after virus production.

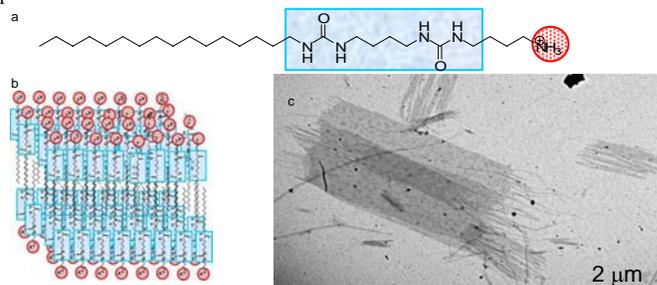


Figure 2. TEM image of ribbons. a. schematic structure of ammonium terminated surfactant. b. molecular model of the ribbon. c. TEM image of ribbons.

As a second self assembling scaffold we are using an ammonium terminated surfactant containing a bis-ureido group.<sup>10</sup> Extensive studies on the aggregation behavior of these ribbon aggregates, performed in Eindhoven, led to a model for the molecular arrangement within the aggregate, in which the surfactant molecules form a fully interdigitated bi-layer. The ribbons can be functionalized by incorporating other bis-ureido-butylene modified molecules. Functionalization of these ribbons with molecules of biological origin was demonstrated by coupling biotin to a ureido-butylene moiety via a PEG spacer. This molecule can be incorporated into the ribbons and incubation with gold-labeled streptavidin led to selective decoration of the ribbons with these biocomolecules.<sup>10</sup>

We can bio functionalize the ribbons after their formation. We are now developing ways to insert molecules that will lead to chemical functionalities on the surface of the ribbons that will allow us to attach enzymes in a bio orthogonal fashion.

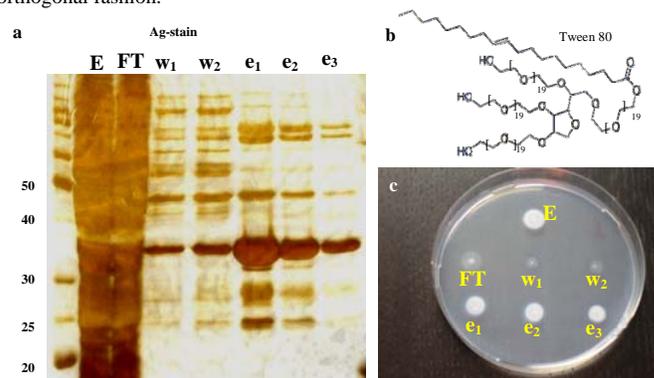


Figure 3. Purification and activity of recombinant CAL-B. a. Total protein stain (silver stain) of polyacrylamide gel electrophoresis analysis of His6 tag purification of CAL-B. E=extract, FT= flow through, w=wash, e=elution fraction. b. structure of Tween 80. c. CAL-B activity assay.

The bio orthogonal reaction that we are going to use is the Cu-catalyzed Huisgen [3+2] cycloaddition. For this we need to engineer enzymes to contain either an azide or acetylene functionality. To do this for our model enzyme we have to express the enzyme in a bacterial system. For this we isolated the gene encoding CAL-B from the yeast *Pseudozyma aphidis* using a PCR method. We cloned the gene into the bacterial expression vector pET22B in which the gene is coupled to a tag of 6 histidines at the C-terminus. The protein was expressed and purified using Ni-NTA column chromatography (fig. 3a). The contaminating proteins in the elution fractions could be removed by size exclusion chromatography. We tested the fractions for esterase activity by

spotting the fractions on agar plate containing Tween 80, which is a substrate for CAL-B. After hydrolysis of this molecule by the enzyme a white precipitate is formed which is visible on the plate. We found that the purified protein indeed has esterase activity (fig 3).

We are now able to produce and purify active CAL-B in a bacterial system. We transformed a methionine auxotrophe strain with our CAL-B expression vector in order to produce the enzyme with incorporated methionine analogs. CAL-B contains four internal methionines. On a small scale we have been able to produce the enzyme in the presence of azidohomoalanine. In a test reaction we have performed a Cu-catalyzed cycloaddition in the presence of an alkynyl functionalized poly ethylene glycol (PEG, Mw=2000 Da). After analysis it using Western blotting it appeared that azidohomo alanine was introduced and that one was available for reaction. We can produce CAL-B containing azide functionalities that can be modified by a Cu-catalyzed cycloaddition in the presence of an acetylene containing reactant. This sets the stage for being able to covalently attach CAL-B to a scaffold that is functionalized with an acetylene moiety.

## Conclusions

We have shown that we can produce enzyme coated virus particles in a one step procedure. However the yields are very low. We are investigating other methods to immobilize enzymes to viruses either covalently or non covalently. We have made progress in developing a different scaffold for enzyme immobilization by using an ammonium terminated surfactant, in which a bis-ureido group is incorporated in its hydrocarbon chain. We have shown that aggregates formed by this amphiphilic molecule can be bio functionalized. Progress is made by engineering our model enzyme into harboring functionalities that can be used to covalently link the enzyme to other structures in a bio orthogonal manner.

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