A novel nanocompartment system named Synthosome for biotechnological applications

by

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Abstract

The functionalized nanocompartment system, Synthosome, has been developed for putative biotechnological applications. A Synthosome is a hollow sphere consisting of a mechanically stable vesicle with a block copolymer membrane and an engineered transmembrane protein acting as the selective gate. Among other functions, the interior contains an enzyme catalyzing a reaction or a charged macromolecule species as trap for compounds. Two areas of application were targeted as proof of principle: A) Selective product recovery in nanocompartments and B) Enzymatic conversion in nanocompartments. Selective recovery of negatively charged compounds has been achieved in the example of sulforhodamine B by using positively charged polylysine molecules as a trap inside the nanocompartment. Conversion in nanocompartments has been achieved by 3,3’,5,5’-tetramethylbenzidine oxidation employing horseradish peroxidase (HRP). In the second part of this thesis, the encapsulation of nanophosphor particles in Synthosomes for selective recovery of single stranded DNA is reported. The nanophosphors based FRET system presents to our knowledge the first use of polymer vesicles for monitoring DNA translocation through a polymer mimic of a biological membrane. Four channel proteins (OmpF, FhuA and two engineered FhuA variants) were investigated by time resolved fluorescence (TRF) measurements for their ability to translocated single stranded DNA.
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I dedicate this thesis to my parents and to my wife
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<tbody>
<tr>
<td>abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammoniumpersulfate</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-Desoxynucleotide-5’-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleicacid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithioerithol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetate</td>
</tr>
<tr>
<td>FhuA</td>
<td>Ferric hydroxamate uptake protein component A</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Water</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
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<td>mole</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomole</td>
</tr>
<tr>
<td>OD</td>
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</tr>
<tr>
<td>OmpF</td>
<td>Outer membrane protein F</td>
</tr>
<tr>
<td>oPOE</td>
<td>Octyl Polyoxoethylene</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly dimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PMOXA</td>
<td>Poly (2-methyloxazoline)</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RTS</td>
<td>Rapid Translation System</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (-hydroxymethyl)-ammonomethane</td>
</tr>
<tr>
<td>µmol</td>
<td>Micromole</td>
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1. Concept

Nanobiotechnology is a young and rapidly evolving field of research at the crossroads of biotechnology and nanoscience, two interdisciplinary areas which combine advances in science and engineering. Although often considered one of the key technologies of this century, nanobiotechnology is still in a fairly embryonic state.

In recent years much progress has been achieved in the synthesis of hollow nano sized compartments (Meier et al. 2000). Vesicles, micelles, onion or spongic phases are, for example, formed in a self-assembly process out of lipids or amphiphilic compounds (Antonietti and Forster 2003). Traditional phospholipid membranes, like those found in cells, are widely used in laboratories for forming both spherical vesicles and planar membranes (Collier and Messersmith 2001). Lipid vesicles with incorporated proteins represent a powerful system for many applications such as drug delivery, pharmacology, material science, and biotechnology. However, while they do have excellent assay characteristics, the long-term stability of lipid vesicles is intrinsically limited in vitro and in vivo due to mechanical weakness (Choi et al. 2005).

For applications in biotechnology, we need stable vesicles. Therefore, it has been a goal to replace lipid membranes with synthesized polymers for the development of stronger nanoscale hybrid devices. Self-assembled, amphiphilic triblock copolymers have been suggested as a potential building material for new biomimetic membranes and for forming vesicles. These vesicles are superior to liposomes in terms of mechanical and chemical stability (Discher et al. 1999), especially at low pH and principles for shape engineering (Grumelard et al. 2004) of vesicles are emerging (Antonietti and Forster 2003).
Here we explore the potential of nanoscale vesicles and the assembly of natural transporters available in *E. coli* for applications in biotechnology. We propose a spherical vesicle embedded with membrane channels as nanocompartments. We report applications of nanocompartments in biotechnology and in diagnostics.

### 1.1 Nanocompartments

Lipid bilayers are the basic constituent of biological membranes. The lipids serve as a fluid matrix for membrane or membrane-associated proteins, which are responsible for various key functions such as signaling or transport (Meier et al. 2000). In addition to their biological functions, lipid membranes have unique material properties; such as high flexibility and mechanical stability. These phospholipid membranes are widely used in laboratories for forming both spherical vesicles and planar membranes.

Vesicles are microscopic sacs that enclose a volume with a molecularly thin membrane. The membranes are generally self-directed assemblies of amphiphilic molecules with a dual hydrophilic-hydrophobic character. Biological amphiphiles form...
vesicles principally by lipids of molecular weight less than 1 kDa for cell functions. In contrast, artificial, freestanding membranes are fragile and thus of little technological interest. Block copolymers that mimic lipid amphiphilicity can also self-assemble into vesicles in dilute solution, but polymer molecular weights can be orders of magnitude greater than those of lipids. Structural features of the vesicles, as well as properties such as stability, fluidity and intermediate dynamics, are greatly influenced by characteristics of the polymers (Discher et al. 1999).

In a number of applications, the loading of vesicles has been accomplished using a concept from nature, where cell membrane proteins allow for the transport of various species to the inside of a cell, and for the removal of compounds to the outside medium. Following this idea, incorporation of (cell) membrane channel proteins in the polymer vesicles wall was performed. As previously mentioned, membrane proteins offer an excellent channel for transporting small molecules and ions, either specifically or non-specifically. The transport may be directed or the substances can move freely in both directions via diffusion through the channel. Insertion of membrane proteins in polymer-stabilized lipid membranes has been successful. However, it has generally been thought that they are an inappropriate system for such insertion, due to incompatibilities in thickness. Namely, lipid membranes offer a ‘universal’ thickness of ~4 nm, which is also the height of the general protein channels. On the other hand, polymer membranes are at least two-fold thicker, and their size is very much dependent on the polymer used, preparative method and environmental conditions. Therefore, their dimensional mismatch was considered to be too large to create a chemically favorable environment for the inserted protein. Indeed, one could rather imagine adsorption of the protein to the membrane or the formation of protein domains within the membrane instead of random insertion.

Recently, it has been shown experimentally that functional incorporation of membrane proteins into block copolymer membranes is feasible, yet the mechanism of such an insertion remained unclear. Indeed, the experimental approaches employed planar membranes from a triblock copolymer (Nardin et al. 2000), which was also found to form vesicles in solution. The BLM technique allows for the measurements of conductance across the membrane, which varies upon the insertion of pore-forming...
species into the film. Two well-characterized channel proteins: OmpF and LamB, naturally found in Gram-negative bacteria were subject to this study, which focused not only on the insertion of proteins themselves, but also their functionality within the polymer membranes. A fully functional incorporation of porins into the artificial (non-physiological) environment of a polymer membrane is possible. Further applications are foreseen, such as creating protein-polymer hybrid materials for diagnostics, sensors and drug delivery.

Pata and Dan theoretically considered the problem stated above (Pata and Dan 2003) and proved via mean field calculations that such insertion can be possible. Conventional lipid bilayers are relatively (vertically) incompressible, due to the limited number of possible conformations of lipid molecules in the membrane. Therefore, they cannot support a perturbation in thickness and a small dimensional mismatch will result in a huge energy penalty that prohibits protein insertion. In block copolymer membranes, however, the hydrophobic chains are in an unfavorable, stretched conformation inside the membrane core. Therefore, a local compression of the membrane around a protein (Figure 2) increases local surface tension energy but decreases the stretching energy. This facilitates the protein incorporation. The successful incorporation of membrane proteins into planar polymer membranes and support from theoretical considerations served as motivation to generate a protein-reconstituted polymer vesicular membranes (Kita-Tokarczyk et al. 2005). Reconstitution of integral membrane proteins into polymer vesicles to form functioning proteovesicles provides a powerful tool in many research fields.
Figure 2: Conformation of AB diblock copolymer chains near a protein inserted in a polymeric bilayer. Matching a protein whose height, which is half the thickness of the membrane, is easily obtained through polymer chains stretching (Pata and Dan 2003).

### 1.1.1 Outer membrane proteins of *E. coli*

*E. coli* is surrounded by two concentric lipid bilayer membranes which confine the periplasmic space containing the murein sacculus. Both membranes contain proteins that assist in the passage of matter and information. Still, the two membranes differ markedly with respect to composition and function. The lipid component of the inner (cytoplasmic) membrane is exclusively composed of phospholipids, mainly phosphatidylethanolamine (70-80 %), phosphatidylglycerol and cardiolipin, which are equally distributed in the inner and outer leaflet. In contrast, the outer membrane is highly asymmetric, with its inner leaflet showing the same lipid composition as the cytoplasmic membrane and the outer leaflet consisting of lipopolysaccharides. With respect to function, the major difference between the inner and outer membrane is that the outer membrane, due to the presence of pore-forming proteins (porins), is considerably more leaky than the cytoplasmic membrane (Koebnik et al. 2000).

The outer membrane also protects the cell from harmful chemicals and proteins such as proteases, bile salts, antibiotics, toxins and phages, and against drastic changes in osmotic pressure (Cowan et al. 1992). The proteins embedded in the outer membrane fulfill a number of tasks that are crucial to the bacterial cell, such as solute and protein
translocation, and signal transduction. Unlike membrane proteins from all other sources, integral outer membrane proteins do not consist of transmembrane $\alpha$-helices, but instead fold into antiparallel $\beta$-barrels. Over recent years, the atomic structures of several outer membrane proteins belonging to six families have been determined (Figure 3). They include the OmpA membrane domain, the OmpX protein, phospholipase A, general porins (OmpF, PhoE), substrate-specific porins (LamB, ScrY) and the TonB-dependent iron siderophore transporters FhuA and FepA.

Figure 3: The $E.\ coli$ cell envelope. Crystallographic data from the soluble and membrane proteins now generally depict the structural organization of bacterial cell envelope (Cao and Klebba 2002).

Transport of solutes across the outer membrane of $E.\ coli$ is mediated and controlled by three classes of membrane proteins: general diffusion porins, solute-specific porins, and high affinity receptors (Lambert et al. 1999). The general diffusion pores formed by porins allow the diffusion of hydrophilic molecules (<600 Da) and show no particular substrate specificity, despite some selectivity for either cations or anions. High molecular weight outer membrane proteins (TonB-dependent receptors), are involved in the uptake of large substrates, such as iron-siderophore complexes or vitamin $B_{12}$. Intriguingly, some of these receptors (e.g. FecA of $E.\ coli$ and PupB of $Pseudomonas\ putida$) contain an N-terminal extension that enables them together with a protein of the
cytoplasmic membrane, to signal the presence of substrates from the cell surface into the cytoplasm.

TonB-dependent receptors depend on the electrochemical potential of the cytoplasmic membrane and a proteinaceous, energy-transducing module, the TonB-ExbBD complex. By analogy with the porins and based on genetic and biochemical studies, it was assumed that these large proteins are built up from huge β-barrels that are gated by a flexible loop. However, solving the three-dimensional structure revealed an unexpected structural organization (Koebnik et al. 2000). These proteins formed a C-terminal 22-stranded β-barrel and an N-terminal plug domain, which is located inside the barrel and thus obstructs the channel interior. One of these large proteins is FhuA, which is studied extensively both in vivo and in vitro.

1.1.2 Selected outer membrane protein – FhuA (Ferric hydroxamate uptake protein component A)

FhuA (Mw 78.9 kDa), the receptor for ferrichrome-iron and the energy transducing protein TonB, mediates the active transport of ferric siderophores across the outer membrane. Microorganisms secrete siderophores to acquire iron. FhuA is one of the more complex members of the superfamily of bacterial outer membrane proteins. It is a multifunctional protein in the outer membrane of E. coli that actively transports [Fe$^{3+}$] ferrichrome, the antibiotics albomycin and rifamycin CGP 4832, and mediates sensitivity of cells to the unrelated phages T5, T1, φ80 and UC-1, and to colicin M and microcin J25 (Killmann et al. 2002).

FhuA is monomeric and composed of a COOH-terminal β-barrel domain (residues 161 to 723) and an NH$_2$-terminal cork domain (residues 1 to 160), which is located inside the barrel and thus obstructs the channel interior (Figure 4). The plug domain is tightly attached to the barrel interior by nine salt bridges and more than 60 hydrogen bonds. The FhuA barrel is larger than any barrel formed by the porins with 22 antiparallel transmembrane β strands. It is 69 Å in height and has an elliptical cross section of 46 by 39 Å (Ferguson et al. 1998). The cork domain, consisting mainly of a mixed four-stranded β sheet, is arranged in the barrel with the β sheet plane inclined by ~45° to the membrane normal, so that it sterically occludes most of the cross section.
The presence of the cork domain suggests that the direct passage of ferrichrome-iron and small molecules through FhuA is not possible. In the cork domain (residues 1 to 160), the first four amino acid residues are involved in ferrichrome binding.

In order to make FhuA a passive diffusion channel, the FhuA (Δ5-160) mutant was created and incorporated in the outer membrane. Cells that synthesized FhuA (Δ5-160) displayed a higher sensitivity to large antibiotics such as erythromycin, rifamycin, bacitracin and vancomycin indicating non-specific diffusion of compounds across the outer membrane of cells (Figure 5). FhuA (Δ5-160) is as stable as wild-type FhuA, and is not degraded by trypsin and proteinase K added to an outer membrane preparation (Braun et al. 1999). From differential scanning calorimetry experiments, removal of the cork destabilized the protein unfolding at 61.6°C unlike wild-type FhuA. This indicated that the cork and the β-barrel behave as autonomous domains, unfolding at 65°C and 75°C respectively (Bonhivers et al. 2001).

FhuA (Δ5-160) supported diffusion of ions through an artificial planar lipid bilayer forms fluctuating channels in the lipid bilayers. It might be that the removal of the globular domain increases the flexibility of loop 4 and other surface exposed loops, so that the rapidly moving loops cause the frequent transition of open and closed states of FhuA (Δ5-160). In addition, with more than 60 residues that are exposed to the interior of the β-barrel channel and fixed to the globular domain, some amino acid side chains may become flexible when the globular domain is removed (Braun et al. 2002).
From a biotechnological point of view the ability to create a selective and regulated pathway into a cell is an extremely interesting prospect with direct relevance to biotherapeutics and biosensors applications. One approach for biotherapeutics is to directly exploit the innate properties of pore-forming proteins. This usually requires little or no modification of the original molecule with efforts expended on developing cost-
effective means of synthesis and testing. A more challenging approach is to apply “re-engineering” techniques to create custom pore-forming molecules that permit controlled passage of a desired molecule (Panchal et al. 2002). Significant progress has been made in membrane protein engineering over the last five years, based largely on the redesign of existing scaffolds by genetic engineering (Bayley and Jayasinghe 2004).

After carefully analyzing the channel using PDB viewer, we designed a FhuA (Δ1-129) mutant with a β strand inside the barrel, which may help to stabilize the channel (Figure 6). FhuA barrel has a surface area of 1409 Å², which provides a large channel for biotechnological applications.

Figure 6: Structural models of FhuA (Δ1-129) looks like an open channel after leaving two-beta strands for mechanical support in the barrel designed with PDB viewer (GlaxoSmithKline, Basel, Switzerland).

1.1.3 Block copolymer vesicles: Principles of formation and application

Due to their potential for the encapsulation of guest molecules into their interior, the preparation of hollow sphere structures with dimensions in the submicrometer range is of increasing interest for fundamental and applied reasons. Usually, the controlled formation of such nanometer- or micrometer-sized structures can only be achieved by using templating techniques or self-assembly mechanisms. A typical example, known for more than 30 years, is the aggregation of individual lipid molecules in water into
spherically closed lipid bilayers, i.e., vesicles or liposomes. In the recent years, these vesicular morphologies have found a multitude of applications in various scientific and applied fields.

The broad variety of self-organizing structures of surfactants and block copolymers is shown in Figure 7. Particulate structures such as spherical and cylindrical micelles as well as vesicles form in dilute solution. Spherical micelles with cubic packing (FCC, BCC), hexagonally packed cylindrical micelles (HEX), and lamellar phases form in the solid, lyototropic, and in ternary systems. There are also modulated (MLAM) and perforated layer phases (PLAM) as well as cubic bicontinuous structures such as the gyroid. Using the phase diagrams one can specifically adjust the morphology of these structures via block lengths and polymer concentration. Thus it is possible to prepare tailor made nanostructured materials (Antonietti and Forster 2003).

Other approaches for preparation of nanometer- to micrometer-sized spherical polymer shells involve layer-by-layer deposition of polyelectrolytes on the surface of a charged nanoparticle followed by the dissolution of the templating particle or the self-assembly of amphiphilic diblock copolymers into micelles, selective cross-linking of their hydrophilic shell, and subsequent degradation of the hydrophobic core.
Figure 7: Self-organization structures of block copolymers and surfactants: spherical micelles, cylindrical micelles, vesicles, fcc- and bcc-packed spheres (FCC, BCC), hexagonally packed cylinders (HEX), various minimal surfaces (gyroid, F-surface, P-surface), simple lamellae, as well as modulated and perforated lamellae (MLAM, PLAM) (Antonietti and Forster 2003).

Although it has been known for several years that under suitable conditions amphiphilic block copolymers can aggregate spontaneously into vesicular structures, it is a complex process. Meier and coworkers developed the idea of preparing amphiphilic diblock copolymers and suitable ABA triblock copolymers consisting of hydrophilic A blocks and a hydrophobic B can also self-assemble in water into vesicular structures. The synthesis and the characterization of a highly flexible poly (dimethylsiloxane) (PDMS) middle block and two water-soluble poly (2-methyloxazoline) (PMOXA) side blocks has been previously described (Figure 8) (Nardin et al. 2000). This copolymer forms vesicular structures in dilute aqueous solution (Figure 9).
Meier and coworkers proved that the OmpF transmembrane protein could be incorporated into an ABA-polymer nanocompartment loaded with β-lactamase for enzymatic conversion (Nardin et al. 2001) and that viral DNA can be injected into a nanocontainer by the assistance of LamB transmembrane protein. Interestingly the membrane proteins remained fully functional despite this artificial environment, even after a subsequent polymerization of their reactive block copolymer matrix (Graffe et al. 2002).

We propose to refer functionalized nanocompartments as **Synthosomes**. A Synthosome is a hollow sphere consisting of a mechanically stable vesicle with a block copolymer membrane and an engineered transmembrane protein acting as selective gate. Among other functions, the interior contains an enzyme catalyzing a reaction or a charged macromolecule species as a trap for compounds.
1.2 Functionalization of nanocompartments: Nanophosphors DNA conjugates

Clinical diagnostics, drug discovery and microbiological research often rely on fluorescent labels for their readout system. These labels are employed in multifaceted detection schemes such as indicating the presence of specific antigens, e.g. in immunoassays, staining tissues for fluorescence microscopy, quantifying DNA strands in Polymerase Chain Reactions, or even tracking biomolecules in living organisms. Semiconducting nanocrystals (quantum dots) represent a novel and interesting class of fluorophores that are applied if high photostability or multiplex capabilities are of importance. They attracted significant attention when the groups of A. P. Alivisatos (Bruchez et al. 1998) and S. Nie (Chan and Nie 1998) proposed them as fluorescent labels. In research groups they are already widely used (Michalet et al. 2005; Smith et al. 2004) and can be regarded as established due to their commercial availability.

Despite their many advantages the biggest drawback of quantum dots is their chemical composition, which consists of mostly toxic materials such as CdSe and InAs. Nanophosphor particles such as CePO₄:Tb³⁺ and YVO₄:Eu³⁺ have been introduced as an alternative to quantum dots due to their less toxic composition (Hoheisel, W., Christoph, P., Bohmann, K., Haase, M. & Riwotzki, K. Preparation of luminescent-doped inorganic nanoparticles and usage as labels for biomolecule probes, Bayer 2001: Patent WO 2001086299) (Figure 10). Nanophosphor particles consist of crystalline oxidic nanoparticles which are doped with ~8-12 mol % of lanthanides ions. Nanophosphor particles are highly stable (photo, colloidal, thermal, oxidation, shelf-life), cost-effective
as well as scalable in production, and neither require a core-shell structure nor an exact size control for synthesis. CePO₄:Tb³⁺ particles have an average diameter of ~7 nm and a high quantum yield of ~50% (Riwotzki and Haase 1998). Excitation at low wavelengths <300 nm is efficient (ε_{Max} ~500,000 M⁻¹ cm⁻¹) and the four narrow banded emission lines with the main emission peak at 542 nm correspond to the ⁵D₄⁻⁷F₅ transition of terbium (Riwotzki et al. 2001) (Figure 11).

Nanophosphor particles are capable of efficient fluorescence-resonance-energy-transfer (FRET) with organic dyes and are applicable in homogeneous assay formats (Bohmann, K., Hoheisel, W., Koehler, B. & Dorn, I. Resonance energy transfer assays based on luminescent inorganic doped nanoparticles, Bayer 2003: Patent WO 2003040024).

The organic dye rhodamine (TAMRA) is a preferred acceptor for CePO₄:Tb³⁺ nanophosphors acting as donors. Rhodamine’s absorption band centers at the ⁵D₄⁻⁷F₅ emission of terbium at 542 nm ensuring an effective spectral overlap. The particle’s fluorescence life-time is in the millisecond range and allows a nearly background-free readout of the acceptor emission by using time gated fluorescence (TGF) detection mode with a delay time of a few microseconds.
Figure 11: Photoluminescence emission spectra of CePO₄:Tb nanophosphor particles recorded in time gated fluorescence mode. The delay time after the excitation pulse at $\lambda_{\text{ex}} = 273.5$ nm was chosen to 40 $\mu$s. Solid line: Luminescence of the nanophosphor DNA conjugates; dotted line: Luminescence of the nanophosphor DNA conjugates after hybridization with the complementary TAMRA labeled primer. Luminescence of the organic dye, which is sensitized by the 542 nm nanophosphor peak and overlaps with the nanophosphor $^5D_{4-}^7F_{4-}$ emission. Inset: TEM-image of CePO₄:Tb nanophosphor particles.

Encapsulation of fluorescent nanoparticles in vesicles is desirable for applications in which the nanoparticles have to be protected from quenching molecules or require complex surface modifications such as antibodies or fluorescence tags.
Objectives

2. Objectives

Inspired by Meier’s work, we aimed in a collaborative effort to expand the scope of their nanocompartment system to functionalized nanocompartment systems designed for biotechnological applications. For biotechnological applications, we have to eliminate the diffusion barrier due to the small channels such as OmpF, OmpC, and PhoE porins are small in diameter (7-11 Å) and allow the only molecules with a molecular weight >600 g/mol (Koebnik et al. 2000). We therefore used the larger channel FhuA and tailored it by genetic engineering.

Here we report the proof of principle for two areas of application in biotechnology: A) As positively charged nanocompartments (loaded with polylysine) for selective product recovery of negatively charged compounds and B) As enzymatically loaded nanocompartments for bioconversion.

We also report the encapsulation of nanophosphor particles in Synthosomes for the selective recovery of single stranded DNA. The nanophosphors based FRET-system represents to our knowledge the first use of polymer vesicles for monitoring DNA translocation through a polymer mimic of a biological membrane. Four channel proteins (OmpF, FhuA and two engineered FhuA variants) were investigated by time resolved fluorescence (TRF) measurements for their ability to translocate single stranded DNA.
3. Materials and Methods

3.1 Cloning, expression and extraction of FhuA and its mutants

3.1.1 Bacterial strains and constructed plasmids

The *fhuA* gene was provided on the pHK 763 vector by Prof. Volkmar Braun (Killmann et al. 1993). The *fhuA* gene was sequenced and re-cloned in pPR1-IBA1 vector (IBA GmbH). *E. coli* strains/plasmids constructed and used are shown in Table 1.

<table>
<thead>
<tr>
<th>E. coli strains/Constructed Plasmids</th>
<th>Expressed Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α/pPR1FhuA/Omp8/Omp9</td>
<td>FhuAwt</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-129)/Omp8/omp9</td>
<td>FhuA (∆1-129)</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-160)/Omp8/Omp9</td>
<td>FhuA (∆1-160)</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-129) neg/Omp8/Omp9</td>
<td>FhuA (∆1-129) neg</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-129) pos/Omp8/Omp9</td>
<td>FhuA (∆1-129) pos</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-160) neg/Omp8/Omp9</td>
<td>FhuA (∆1-160) neg</td>
</tr>
<tr>
<td>DH5α/pPR1FhuA (∆1-160) pos/Omp8/Omp9</td>
<td>FhuA (∆1-160) pos</td>
</tr>
<tr>
<td>BL 21/pGOMP/Omp8/Omp9</td>
<td>OmpFwt</td>
</tr>
</tbody>
</table>

Table 1: *E. coli* strains/Constructed plasmids and expressed proteins FhuAwt (wild-type), FhuA (∆1-129) deletion mutant (without 1-129 amino acids in the mature protein), FhuA (∆1-160) deletion mutant (without 1-160 amino acids in the mature protein), FhuA pos and neg mutants (amino acids substitutions as given in Table 2) and OmpFwt (wild-type).

3.1.2 Cloning of FhuA and FhuA mutants using pPR-IBA1

a) *FhuA* wild-type

The *fhuA* gene was cloned from the pHK763 vector into our standard isopropyl-β-D-thiogalactopyranoside (IPTG) inducible expression system pPR-IBA1 using an EcoRI restriction site at the 5’-end and an XhoI restriction site at the 3’-end. The PCR (94°C for 3 min, 1 cycle; 94°C for 1 min/ 60°C for 1 min / 72°C for 3 min, 25 cycles; 72°C for 10 min, 1 cycle) was performed using the above described conditions and two primers: FhuAEcoRI-F 5’-ACCAGGAATTCCGATGGCGCCTCCAAAACGTCGAT-3’ and FhuAXhoI-R 5’-CATGCAATGGCTCGAGTTAGAAAACGGAAGGTGTCTG-3’. The amplified DNA fragments were purified by using a QIAquick Gel Extraction Kit
Materials and Methods

(Qiagen, Hilden, Germany), digested with EcoRI and XhoI (both New England Biolabs; 20 U for 4 h), cloned into the pPR-IBA1 plasmid using standard molecular biology techniques, and transformed into *E. coli* DH5α (F φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ- thi-1 gyrA96 relA1) (Invitrogen, Karlsruhe, Germany) using the TSS transformation protocol (See also Appendix for detailed protocol) (Chung et al. 1989).

**b) FhuA (Δ1-129) and FhuA (Δ1-160) mutants**

The pPR1-FhuA (Δ1-129) and pPR1-FhuA (Δ1-160) plasmids were constructed by using the above stated FhuAXhoI-R reverse primer and two different forward primers: for the FhuA (Δ1-129) variant the FhuA129-F primer 5’-ATGGGT CTCGAATTCCGGA-TGCGCGCTGAAATTATGCGTG-3’ and for the FhuA (Δ1-160) variant the FhuA160-F 5’-ATGGGTCTCGAATTCCGGATGCCGCTGAAAG AAGTTCAG-3’. For both FhuA deletion variants the above-described PCR protocol with annealing temperatures of 68°C (FhuA (Δ1-129)) and 66°C (FhuA (Δ1-160)) was employed.

![Schematic representation of PCR using overlap extension PCR](image)

1- signal sequence
2- Truncated FhuA gene

Figure 12: Schematic representation of PCR using overlap extension PCR.

The signal sequence for directing FhuA variants to the outer membrane of *E. coli* was subsequently attached in front of the truncated *fhuA* genes by overlap extension PCR as shown in the Figure 12 (Ho et al. 1989).

In a first PCR the signal sequence was amplified from the pHK763 for both FhuA variants with a complementary region of the *fhuA* genes ((Δ1-129), (Δ1-160)) at the
5’end. For FhuA (Δ1-129), FhuAEcoRI-F was used as the forward primer and FhuASS129-R 5’-CATAATTCAGCGCGTGCCTGTGCATAAAC-3’ as the reverse primer. For FhuA (Δ1-160), FhuAEcoRI-F was used as the forward primer and FhuASS160-R 5’-CTGAACTTCTTTCAGTGCTGTGCATAAAC-3’ as the reverse primer. In a second PCR reaction the fhuA genes ((Δ1-129), (Δ1-160)) were amplified using FhuASS129-F 5’-GTTTATGCACAAGGCACGCGCTGAAATTATG-3’ and FhuASS160-F 5’-GTTTATGCACAGGCACGTGAAAGAAGTTCA-3’ as forward primers for FhuA (Δ1-129) and FhuA (Δ1-160) respectively and FhuAXhoI-R as reverse primers. In a concluding third step the PCR products from the first and second PCR were combined and amplified using flanking primers (FhuAEcoRI-F and FhuAXhoI-R). For both FhuA deletion variants the above-described PCR protocol with annealing temperatures of 63.3°C (FhuA (Δ1-129)) and 61.3°C (FhuA (Δ1-160)) were employed.

The amplified DNA fragments of FhuA mutants were purified by using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), digested with EcoRI and XhoI (both New England Biolabs; 20 U for 4 h), and cloned into the pPR-IBA1 plasmid using standard molecular biology techniques as described earlier.

c) FhuA neg and FhuA pos mutants

FhuA negative (FhuA neg) and positive (FhuA pos) mutants were constructed by site directed mutagenesis on the loops in anti-parallel β-strands at the surface for protein-protein interaction by the salt bridge approach by substituting for positively or negatively charged as described in Table 2.
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<table>
<thead>
<tr>
<th>FhuA mutants with negative charges in the loop areas</th>
<th>FhuA mutants with positive charges in the loop areas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acid changes</strong></td>
<td><strong>Amino acid changes</strong></td>
</tr>
<tr>
<td>Lys226Met</td>
<td>Asp292Lys</td>
</tr>
<tr>
<td>Lys226Met, Arg543Met</td>
<td>Asp292Lys, Asp498Lys</td>
</tr>
<tr>
<td>Lys226Met, Arg543Met, Arg679Met</td>
<td>Asp292Lys, Asp498Lys, Asp635Lys</td>
</tr>
</tbody>
</table>

Table 2: Substitution of amino acids in the salt bridges for constructing FhuA neg and FhuA pos mutants.

FhuA neg and pos mutants were generated by Stratagene QuiKChange mutagenesis method.

Figure 13: Principle of the Stratagene QuiKChange-Mutagenesis method. Plasmid was denatured, primers containing the desired mutation were annealed and amplified using *pfuTurbo* Polymerase. Methylated and non mutated parental DNA template were digested with Dpn I and transformed in *E. coli*.
The principle of QuiKChange mutagenesis method is that the DNA template is denatured. After denaturation, the mutagenic primers containing the desired mutations are annealed to the template and extended using pfuTurbo DNA polymerase. After extension, the parental methylated and hemimethylated DNA is digested with Dpn I, and the mutated DNA is transformed into *E. coli* as shown in Figure 13.

The above mentioned amino acids were accumulated on the same gene to give the FhuA neg and FhuA pos mutants by using the following PCR recipe: the PCR volume was 50 µl and consisted of 39 µl dd H₂O, 1 µl of each primers (forward and reverse primer, 20 pmol), 2 µl plasmid pPR1-FhuA (∆1-129) (1:20) (concentration of the template should be 5 to 50 ng (usually the miniprep has 200 to 300 µg/ml of DNA template)), 1 µl dNTP (10 mM) and 1 µl pfuTurbo (2.5 U). The PCR conditions were: 95°C for 30 sec, 1 cycle; 95°C for 30 sec/Tm (of primer)-5°C for 1 min / 68°C for 6 min, 18 cycles. Following the temperature cycling, the product was treated with Dpn I. The Dpn I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA.

For the FhuA neg mutant, the following primers were used:

- **FhuA N1_F1**: 5’- GGC GTC CGG ATG ATA TGA CCA ATT TTA CC -3’
- **FhuA N1_R1**: 5’- GGT AAA ATT GGT CAT ATC ATC CGG ACG CC -3’
- **FhuA N2_F1**: 5’- GTA CCG GAA GAT ATG CCG ATT GTA GTT GC -3’
- **FhuA N2_R1**: 5’- GC AAC TAC AAT CGG CAT ATC TTC CGG TAC -3’
- **FhuA N3_F1**: 5’- GTA CCG GAA GAT ATG CCG ATT GTA GTT AC -3’
- **FhuA N3_R1**: 5’- GTA ACT ACA ATC GGC ATA TCT TCC GGT AC -3’

For changing the amino acid Lys 226 to Met 226, FhuA N1_F1 and FhuA N1_R1 were used with an annealing temperature of 61°C. After transformation, the same plasmid was used for second mutation (Arg543Met) by using FhuA N2_F1 and FhuA N2_R1 with an annealing temperature of 55°C. The third mutation of Arg 679 to Met was done in the same plasmid with an annealing temperature of 66°C.

For FhuA pos mutant, the following primers were used:
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For changing the amino acid Asp292Lys, FhuA P1_F1 and FhuA P1_R1 were used with 55°C for annealing. After transformation, the same plasmid is used for the second mutation (Asp498Lys) by using FhuA P2_F1 and FhuA P2_R1 with an annealing temperature of 54°C. The third mutation of Asp635Lys is done with an annealing temperature of 59°C.

For the FhuA neg and FhuA pos mutants, each mutation was introduced at the desired position using the above set of primers. After Dpn I digestion and transformation in *E. coli*, clones were picked and sequenced for mutations to check for the desired mutations stated in Table 2. Likewise, the FhuA neg and pos mutants were created as shown in Figure 14.

![Figure 14: Structural models of FhuA (Δ1-129) neg and FhuA (Δ1-129) pos mutants with amino acids changes as mentioned in the Table 2, designed with PDB viewer (GlaxoSmithKline, Basel, Switzerland).](image)
After sequencing results, the plasmids containing the respective genes were transformed into *E. coli*, and freshly grown overnight cultures were mixed with 50% glycerol (500 µl of *E. coli* overnight culture and 500 µl of 50% glycerol) and stored at –80°C for further use.

### 3.1.3 Expression, extraction and purification of FhuA and FhuA variants

For high expression of FhuA it is crucial to freshly transform the pPR1-FhuA plasmid into the expression host *E. coli* B<sup>E</sup> strain BL 21 (DE3) omp8 (F<sup>−</sup> hsdSB<sup>−</sup> <i>trH<sup>−</sup> mH<sup>−</sup></i>) gal ompT dcm (DE3) Δ<sup>lamB ompF::Tn5 ΔompA ΔompC</sup>) (Prilipov et al. 1998). A 5 ml overnight culture was prepared in TY medium (Bactotryptone 10 g/l, Yeast extract 5 g/l and NaCl 5 g/l) and 3 ml were used to inoculate 300 ml TY medium for FhuA production (1 L shaking flask: Infors HT Multitron, Bottmingen, Switzerland; 250 rpm, 37°C). When OD<sub>578</sub> reached 0.5, FhuA-protein expression was induced by adding IPTG to a final concentration of 1 mM. Cells were grown further at 37°C to an OD<sub>578</sub> of 1.0 and harvested by centrifugation (3200 g, 20 min; Eppendorf 5810R; Hamburg, Germany). Cells were resuspended in 30 ml of lysis buffer (0.2 M Tris/HCl pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM phenylmethanesulfonyl fluoride (PMSF; protease inhibitor)), cooled on ice and disrupted by passing three times through a high-pressure homogenizer (Emulsiflex-C3, Avestin Inc, Ottawa, Canada) at 2000 bar. The disrupted cell suspension was mixed with 30 ml of FhuA extraction buffer (50 mM Tris/HCl pH 8.0, 10 mM MgCl<sub>2</sub>, 2% Triton X-100) and gently vortexed (Heidolph Reactop, Schwabach, Germany) for 10 min at room temperature until homogeneity of the sample was achieved. The outer membrane fractions were isolated by centrifugation (48384 g, 45 min, 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA).

To remove the Triton X-100, the outer membranes were washed three times with 15 ml of water and centrifuged (48384 g, 45 min, 4°C; Avanti J-20XP). Outer membrane fractions were resuspended in 12 ml FhuA-solubilisation buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 1% Octyl-Polyoxoethylene (oPOE), 1 mM PMSF) (Locher and Rosenbusch 1997) and shaken in 15 ml tubes for 1 h at 37°C (250 rpm, Infors HT Multitron) followed by 10 h at 12°C (250 rpm, Infors HT Aquatron, Bottmingen, Switzerland) (Braun et al. 2002). Membrane fractions were subsequently removed by
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centrifugation (48384 g, 15 min, 4°C; Avanti J-20XP) and supernatant containing FhuA-protein was concentrated using ultrafiltration (Centricon YM30, Millipore; Bedford, USA, 3220 g, 20 min; Eppendorf 5810R; Hamburg, Germany). The purity of extracted fractions was controlled by protein gel electrophoresis and was comparable to previously reported values (See also Appendix for detailed protocol). (Killmann and Braun 1992).

3.2 Expression, extraction and purification of OmpF

The \textit{ompF} gene was provided on the pGompF vector by Dr. Ralf Koebnik. pGompF was freshly transformed into the expression host \textit{E. coli} B\textsuperscript{E} strain BL 21 (DE3) omp8 (Prilipov et al. 1998). 4 ml of overnight culture were used to inoculate 400 ml of LB medium (1 L shaking flask: Infors HT Multitron, Bottmingen, Switzerland; 250 rpm, 37°C). When \textit{OD}_{578} reached 0.5, OmpF-protein expression was induced by adding isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were grown further at 37°C until \textit{OD}_{578} reached 1.0 and harvested by centrifugation (3200 g, 20 min; Eppendorf 5810R; Hamburg, Germany). Cells were resuspended in 400 ml of breaking buffer (2 % SDS, 20 mM Tris/HCl pH 8.0, 1 mM phenylmethanesulfonyl fluoride (PMSF; protease inhibitor)), cooled on ice and disrupted three times by passing through a high-pressure homogenizer (Emulsiflex-C3, Avestin Inc, Ottawa, Canada) at 2000 bar. The cell envelopes were recovered by centrifugation (48384 g, 40 min, 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA), supplemented with 200 ml of preextraction buffer (0.5 % Octyl-Polyoxoethylene (oPOE), 20 mM NaH\textsubscript{2}PO\textsubscript{4} pH 7.3), and shaken (Infors HT Multitron, Bottmingen, Switzerland; 250 rpm, 37°C) for 45 min to achieve homogeneity. The outer membrane fractions were isolated by centrifugation (48384 g, 40 min, 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA). Outer membrane fractions were further treated to solubilize the OmpF protein with 200 ml of extraction buffer (3 % oPOE, 10 mM EDTA, 20 mM NaH\textsubscript{2}PO\textsubscript{4} pH 7.3) and incubated for 40 min (250 rpm, 37°C; Infors HT Multitron). Membrane fractions were subsequently removed by centrifugation (48384 g, 40 min, 4°C; Avanti J-20XP) and the supernatant containing OmpF protein was concentrated using ultrafiltration (Centricon YM30, Millipore; 3220 g, 20 min; Eppendorf 5810R; Hamburg, Germany). After concentrating to 5 ml, the OmpF protein was solubilized and dialyzed against 100 volumes of oPOE.
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buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 1% oPOE, 1 mM PMSF) using a cellulose membrane (12-14 kDa MWCO; Spectrum Laboratories Inc, Rancho Dominguez, USA) at 4°C for 12 h. OmpF extraction was confirmed by SDS/PAGE visualization (Prilipov et al. 1998).

3.3 Characterization of FhuA channel

3.3.1 Protein insertion in polymer monolayers by Langmuir trough experiment

Transmembrane protein-polymer and transmembrane protein-lipid interactions were measured in a monolayer experiment through a surface pressure increase according to the Wilhemy plate method (Graff et al. 2002). A 22 µl aliquot of the polymer (1 mg/ml dissolved in chloroform) was spread onto a subphase consisting of PBS buffer. The isotherm cycles were registered at a barrier speed of 15 mm/min. The barrier was compressed to a surface pressure of 25 mN/m and 100 µl of FhuA Δ1-129 (0.6 mg/ml) was injected into the subphase. As a control experiment, 100 µl of the detergent oPOE that is used in FhuA solubilisation was injected into the subphase after the polymer monolayer was compressed to 25 mN/m.

3.3.2 Liposomes and Calcein assay

10 mg of egg-phosphatidylcholine (PC; Lipoid GmbH) lipids were dissolved in 1 ml of chloroform, dried under a nitrogen stream, and residual chloroform was removed by vacuum in a desiccator overnight for lipid film formation. The lipid film was suspended in 1 ml of Tris-buffer (100 mM KCl, 10 mM Tris, pH 7.4) and uni-lamellar vesicles were formed by 10 freeze-thaw cycles as previously described (Traikia et al. 2000). Liposomes were subsequently extruded six times through a disposable filter (Millipore; diameter of 0.2 µm) and purified through a Sephadex G-25 column (PD-10; Amersham Biosciences, Uppsala, Sweden). The average diameter of the liposomes was determined using a Zeta-Sizer (Zeta-Sizer Nano Series; Malvern, Worcestershire, United Kingdom).

Fluorescence measurements employing calcein used a wavelength of 480 nm for excitation and emission was recorded at 520 nm. In each experiment the liposomes were loaded with a self-quenching concentration of 50 mM calcein (M_w: 622.54 g/mol) (Jayaraman et al. 2001). The diameter of the liposomes was not affected by calcein.
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incorporation using Tris-buffer (50 mM Calcein; 10 mM Tris pH 7.4, 100 mM KCl). 50 µl of the chromatographically purified liposomes mixture was supplemented with 150 µl of Tris-buffer (100 mM KCl, 10 mM Tris pH 7.4) and used for each experiment. In all subsequent experiments the liposomes were finally broken by adding 10 µl of 10 % Triton X-100 and the fluorescence was compared to ensure comparable calcein and liposome loads in each experiment.

The detergent oPOE affects the liposome stability and causes release of calcein into the surrounding medium that results in increased calcein fluorescence. The influence of oPOE on calcein fluorescence was determined by stepwise adding 10 µl FhuA-solubilisation buffer (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 % oPOE, 1 mM PMSF) to the calcein loaded liposomes.

1.62 nmol OmpF and 2.1 nmol FhuA (Δ1-129; Δ1-160) variants were added stepwise in 10 µl batches to the liposome suspension and the fluorescence increase was recorded at 520 nm. After reaching a stable fluorescence signal, identical amounts of OmpF and FhuA variants were supplemented at the positions indicated by the arrow in Figure 21A. Finally, the liposomes were broken by the addition of Triton X-100 (final concentration 6.43 µM).

3.4 Nanocompartment system for biotechnological applications

3.4.1 ABA polymer Nanocompartment formation

a) Direct Dispersion method

50 mg of ABA (PMOXA-PDMS-PMOXA) triblock copolymer (S131) (Nardin et al. 2000) were suspended in 4.95 ml of PBS (0.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄ 8 H₂O, 14 mM KH₂PO₄ 2 H₂O, pH 7.4) and stirred gently at room temperature (RCT basic IKAMAG, IKA-Werke GmbH, Staufen, Germany) overnight to form self-assembled nanocontainers. For forming uniformly sized nanocompartments the mixture was extruded three times through a 0.45 µm disposable filter (Millipore) using a 2 ml syringe (Norm-Ject, Henke Sass Wolf GmbH, Tuttlingen, Germany).

Triton X-100 was subsequently added to achieve a final concentration of 0.5 % and the suspension was sonicated twice for 10 s (Branson 5510E-DTH, Branson...
Ultrasonic Corporation, Danbury, USA) and stirred (RCT basic IKAMAG, 300 rpm; 5h). The Bio-Beads method (Rigaud et al. 1995) was applied to reconstitute FhuA variants in nanocompartments. For nanocompartments harboring FhuA variants 100 µl of FhuA (Δ1-129 (0.6 mg/ml)) were added shortly after the sonication step. The mixture was stirred for 3-4 hrs. Prior to addition of the Bio-Beads, they were first cleaned two times with methanol and then washed several times with water (Holloway 1973). The amount of Bio-Beads depends on the adsorptive capacity of beads for detergents, i.e. the determination of the maximal amount of detergent that can be adsorbed per gram of beads. This adsorptive capacity was determined by analyzing the time course of detergent removal from a solution containing a fixed amount of detergent and various amounts of beads. The adsorption capacities of Bio-Beads was determined for various detergents (Rigaud et al. 1998). Bio-Beads (Bio-Beads SM-2, Bio-Rad Laboratories GmbH, Munich, Germany) were added after 1 h in three 1 h intervals while the sample was stirred for 6-8 hrs at 4°C.

The supernatant containing the nanocompartments harboring FhuA was finally centrifuged (Eppendorf, 3200 g during 20 min) to remove Bio-Beads.

b) Ethanol method

50 mg of ABA (PMOXA-PDMS-PMOXA) triblock copolymer (S131) were dissolved in 250 µl of ethanol (99.8 %) and stirred for 30 min. The clear solution was added drop wise into 5 ml of PBS and stirred at room temperature for 3 to 4 h. During this incubation period nanocompartments were formed by self-assembly and ethanol was evaporated. Six time extrusion through a 0.45 µm filter (Millipore) ensures uniformly sized nanocontainers.

For nanocompartments harboring FhuA or FhuA variants, a 100 µl solution containing 9.2 nmol of FhuA or FhuA variants was mixed with the ethanol solution of ABA polymer and dropwise added into 5 ml of PBS as described above. Nanocompartments formed by both methods were purified by gel filtration using a Sepharose 4B (Sigma-Aldrich) (Graff et al. 2002).
3.4.2 Selective product recovery of charged compounds

As a proof of principle for the recovery of negatively charged compounds, Synthosomes were prepared by the Ethanol method with the modification that the solution of ABA polymer in Tris-buffer (10 mM, pH 7.4) contained positively charged polylysine molecules (0.5 mg/ml (final concentration); $M_w$: 15-30 kDa) as electrostatic traps. The polylysine molecules are large in size and cannot diffuse out of the Synthosomes.

a) Sulforhodamine B model

As a control experiment, sulforhodamine B was added to different concentrations of polylysine to investigate the effect of fluorescence quenching due to the sulforhodamine B and polylysine interaction. This experiment was repeated with varied concentrations of polylysine and a constant sulforhodamine B concentration.

A 5 µl aliquot of sulforhodamine B (final concentration 8 mM; Tris/HCl 10 mM pH 7.4) was added to four batches of 2 ml suspension of purified Synthosome: batch 1. Synthosome with FhuA ($\Delta$1-129) & loaded with polylysine; batch 2. Synthosome with FhuA ($\Delta$1-129) & not loaded with polylysine; batch 3. Synthosomes without FhuA ($\Delta$1-129) & loaded with polylysine; and batch 4. Synthosomes without FhuA ($\Delta$1-129) & polylysine. The Synthosome suspension was slowly stirred overnight in the presence of sulforhodamine B at room temperature. To remove non-entrapped sulforhodamine B, Synthosome suspensions were centrifuged using Centricon YM filters (30 kDa cut-off; Millipore GmbH, Schwalbach, Germany; 2000 g, 20 min; Eppendorf 5810R; Hamburg, Germany) and washed with 9 ml Tris-buffer (10 mM pH 7.4). All samples were finally resuspended in 2 ml Tris-buffer (10 mM pH 7.4) and the sulforhodamine B fluorescence was determined (excitation 540 nm; emission spectra recorded from 580-700 nm). The same procedure was applied for Synthosomes prepared according to the Direct Dispersion method (results not shown).

b) DNA entrapment

50 µl of DNA (60 mer, 20 pmol) was added to two batches of 1 ml suspensions of purified Synthosomes: batch 1. Synthosomes with FhuA ($\Delta$1-129) and loaded with polylysine; batch 2. Synthosomes without FhuA ($\Delta$1-129) and loaded with polylysine.
The Synthosomes suspension was slowly stirred overnight in the presence of the ssDNA. To remove non-entrapped ssDNA, Synthosome suspensions were centrifuged using Centricon YM filters (30 KDa Cut-off; Millipore GmbH, Schwalbach, Germany) and washed with PBS buffer. After washing, the samples were concentrated to 1 ml and 60 µl of SYBR gold was added to this 1 ml of Synthosomes suspension. SYBR gold fluorescence was determined (excitation 495 nm; emission spectra recorded from 510 to 600 nm).

3.4.3 Bioconversion in Synthosome employing Horseradish Peroxidase (HRP) and TMB as model system

Synthosomes were prepared by the Direct Dispersion and Ethanol Method for bioconversion reactions employing HRP. For encapsulation inside the Synthosomes, 14.4 U of HRP dissolved in 4.95 ml PBS were used during nanocompartment formation. Ready to use 3,3’,5,5’-Tetramethylbenzidine (TMB)/H₂O₂ solution (Sigma Cat nr: T0440) is used for kinetic measurement of TMB oxidation by the HRP (Josephy et al. 1982). 10 µl of TMB/H₂O₂ solution were added to a 100 µl dispersion consisting of 50 µl purified Synthosomes and 50 µl of PBS buffer. The soluble blue reaction product of the one-electron oxidation of TMB was recorded at 370 nm.

Further oxidation of TMB in acid solution yields a yellow diimine reaction product with an absorbance maximum at 450 nm (Josephy et al. 1982). This end-point assay was used to compare the Synthosome preparation methods (Direct Dispersion and Ethanol method) by terminating the HRP-catalyzed conversion of TMB (50 µl purified Synthosomes and 50 µl of PBS buffer) after 5 min by supplementing 0.25 mM of HCl (final concentration). The resulting yellow reaction product was recorded after a 3 minute incubation at 450 nm.

3.5 Nanocompartment system for analytical applications

3.5.1 Preparation and encapsulation of nanophosphor DNA conjugates in Synthosomes

Primers for labeling nanophosphor particles and FRET measurements were purchased at Thermo Electron GmbH (Ulm, Germany). The nanophosphor particles were
prepared by Bayer Technology Services GmbH (Leverkusen, Germany) with carboxylic
groups at the surface. Standard EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide)
coupling procedures were used for the first coupling EDC to the nanophosphor particles
(4.5 mg corresponding to a concentration of 13 nM). followed by the primer binding (5’-ATCAGCCCCTCATTGTG-3’; primer concentration 200 nM) through an amine group at
the 5’-end. The unbound primer was subsequently removed by various washing steps
(Walsh et al. 2001).

Nanophosphor DNA conjugates were encapsulated in Synthosomes using a
similar protocol to the one developed for polylysine encapsulation. 10 mg of ABA
(PMOXA-PDMS-PMOXA) triblock copolymer (S131) (Nardin et al. 2001) were
dissolved in 50 µl of ethanol (99.8 %) and stirred for 30 min. The clear solution was
added dropwise into 1 ml of Tris-buffer (100 mM KCl, 10 mM Tris, pH 7.4) containing a
water suspension of CePO$_4$:Tb-nanophosphor DNA conjugates (~14.3 µM) and stirred
overnight at room temperature. During this incubation period the ethanol evaporated and
Synthosomes were formed by self-assembly. Six time extrusion through a 0.45 µm filter
(Millipore) ensured uniform spherical shaped Synthosomes (Selvin 1995) as proven by
average diameter determination using a Zeta-Sizer (Zeta-Sizer Nano Series; Malvern,
Worcestershire, United Kingdom).

For Synthosomes harboring OmpF, FhuA or FhuA variants, a 30 µl solution
containing 4.9 nmol of OmpF, 6.3 nmol of FhuA or FhuA variants was mixed with the
ethanolic solution of ABA triblock copolymer and dropwise added as described above to
1 ml of Tris-buffer containing nanophosphor DNA conjugates. Formed Synthosomes
were purified as previously reported by gel filtration employing Sepharose 4B (Sigma-
Aldrich) (Graff et al. 2002).

3.5.2 FRET-measurements of DNA hybridization

500 µl of the chromatographically purified nanophosphor DNA conjugates
loaded Synthosomes with 2.5 µl of a complementary oligonucleotide 5’-TTTTCACAA
TGAGGGGCTGAT-3’ (0.25 nmol final concentration) with a TAMRA 3’-modification
were used in FRET-hybridization experiments. After hybridization, the TAMRA-dye is
in close proximity to the nanophosphor particles. 20 min after addition of the TAMRA
labeled oligonucleotide, the fluorescence lifetime of the nanophosphor particles was measured in TRF mode using a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc, Palo Alto, USA). After pulsed excitation at 273.5 nm, the decay of the fluorescence was detected at the main peak of the Tb\(^{3+}\) ion light emission at 542 nm for 4 ms. By using the TGF mode, a full fluorescence spectrum was recorded after a delay time of 40 µs to prevent any light originating from the Xe-flash lamp (excitation), from autofluorescence of organic materials like polymers or DNA sequences, or from direct excitation of the organic dyes entering the detector. Light emission from the organic dye in TGF mode can only be detected when it is sensitized by a FRET-process with the nanophosphors acting as the donor (Figure 11). Finally, shells of the loaded Synthosomes were broken by the addition of Triton X-100 (final concentration 6.16 µM) and sonicated for 1 min (Branson 5510E-DTH, Branson Ultrasonic Corporation, Danbury, USA) and incubated at 80°C using a heating block (Thermomixer comfort; Eppendorf, Hamburg) for 30 min. After cooling to room temperature, the FRET-process was recorded as described. The latter experiment ensured an encapsulation of the nanophosphor conjugates in Synthosomes and proved the integrity of the primer as well as the conjugated DNA.

3.6 Preliminary experiments for scaling up the nanocompartment system

3.6.1 AB polymer Nanocompartment formation

10 mg of AB polymer (PI-PEO-3) (Stephan Förster group, Hamburg) were dissolved in 200 µl of chloroform with 100 µl of PBS and the chloroform was allowed to evaporate. After the chloroform is evaporated, 900 µl of PBS buffer was added and the solution stirred overnight at room temperature. During this incubation period nanocompartments were formed by self-assembly.

For nanocompartments harboring FhuA or FhuA variants, a 100 µl solution containing 9.2 nmol of FhuA or FhuA variants was mixed with AB polymer solution and added to the buffer.

For calcein encapsulation, AB polymer nanocompartments were formed as described above, but with the addition of 900 µl self quenching concentration of calcein (50 mM) in Tris buffer (100 mM KCl, 10 mM Tris, pH 7.4) and stirred overnight. The
Materials and Methods

calcein encapsulated in the AB polymer nanocompartments was purified through a Sephadex G-25 column (PD-10; Amersham Biosciences, Uppsala, Sweden).

For the scaling up of Synthosomes, AB polymer (P2VP-PEO-8) (Poly (2-vinylpyridine-b-polyethyleneoxide) (Stephan Förster group) was used. 200 mg of AB polymer was dissolved in 2 ml of 99.8% ethanol. A clear solution of polymer was added dropwise into the buffer and stirred overnight for vesicle formation. Once the vesicles are formed, they were extruded using a high-pressure homogeniser (Avestin Inc, Ottawa, Canada) through a 1 µm filter and then through a 800 nm filter. After two to three cycles of circulation through the 800 nm filter, the Synthosomes were passed through a 400 nm and a 100 nm filter. Synthosomes collected after extrusion through the 100 nm filter were analysed by dynamic light scattering measurements and by cryo TEM pictures.

For applications in biotechnology, we must produce the nanocompartment system in 20 ml scale. Keeping this in mind, we began optimising the solubilisation protocol of FhuA from outer membrane fractions.

3.6.2 Optimization of solubilisation of FhuA from outer membrane fractions

For optimizing the solubilisation of FhuA from outer membrane fractions, the procedure mentioned in section 3.1.3 was followed with some modifications. After disruption of cells, they were mixed with equal amounts of FhuA extraction buffer 1 (50 mM Tris/HCl pH 8.0, 10 mM MgCl₂, 4% Triton X-100) and gently vortexed (Heidolph Reax top, Schwabach, Germany) for 10 min at room temperature until homogeneity of the sample was achieved. The outer membrane fractions were isolated by centrifugation (48384 g, 45 min, 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA). To remove the excess Triton X-100 the outer membranes were washed three times with 15 ml of water and centrifuged (48384 g, 45 min, 4°C; Avanti J-20XP). Outer membrane fractions were resuspended in 5 ml of Tris buffer (0.2 M Tris/HCl pH 8.0, 10 mM MgCl₂, 1mM PMSF). Equal amounts of the solution were divided into 25 portions and centrifuged to remove Tris buffer. 1 ml of solubilisation buffer (1 M Tris-HCl, pH 7.5), with different membrane protein solubilizing detergents (JBSolution Detergent Test kit, Jena Bioscience GmbH, Jena, Germany) were tried for solubilisation instead of using oPOE at different temperatures and times. After every experiment the
samples were centrifuged (11000 g, 10 min; Eppendorf, Germany), and the supernatant containing FhuA was collected and analyzed in SDS gel.
4. Results

4.1 Cloning, expression and extraction of FhuA and its mutants

FhuA and its mutants were cloned as explained in the Materials and Methods Section 3.1.2. After ligation of \( fhuA \) genes in pPR-IBA1 (Figure 15), the ligation mixture was transformed and clones were picked and grown overnight in LB media for plasmid extraction. Plasmids were digested with EcoRI and XhoI to check the insert of the \( fhuA \) gene.

Figure 15: Cloning scheme of \( fhuA \) gene in pPR-IBA1.
Results

Figure 16: (A) 1 – DNA ladder, 2 – pPR1FhuA, 3 – pPR1FhuA digested, 4 – pPR1FhuA (Δ1-129), 5 – pPR1FhuA (Δ1-129), 6 – pPR1FhuA (Δ1-129) digested and 7 – pPR1FhuA (Δ1-129) digested. (B) pPR1FhuA and FhuA mutants plasmids and digested with EcoRI and XhoI. From left: 1 – DNA ladder, 2 – pPRIBA1, 3 – pPR-IBA1 FhuA (Δ1-129), 4 – pPR1 FhuA (Δ1-160), 5 – pPR-IBA1 FhuA (Δ1-129) pos, 6 – pPRIBA1 FhuA (Δ1-129) neg, 7 – pPR-IBA1, 8 – pPR1-FhuA (Δ1-129) pos digested, 9 – pPR1-FhuA (Δ1-129) pos digested, 10 – pPR1-FhuA (Δ1-129) neg digested.

From Figures 16A&B, it is shown that the fhuA genes were inserted in the plasmid. FhuA wild-type and FhuA (Δ1-129), FhuA (Δ1-160), FhuA neg and FhuA pos mutants were sequenced and did not harbor any mutation compared to the sequence deposited in the PDB-database (Ferguson et al. 1998) except an additional his6-tag (PDH6DLA) after aa-position P405 (Braun et al. 2002). This proved the sequence integrity of FhuA (Δ1-129) neg and FhuA (Δ1-129) pos.

FhuA and its mutants were freshly transformed and expressed in *E. coli* BL21 as described in the Materials and Methods. FhuA and its mutants were overexpressed and after solubilizing FhuA from the outer membrane, other Omp proteins were still present in the supernatant (Figure 17). In order to purify, His-tag purification was attempted, but did not yield pure protein (data not shown).
Figure 17: SDS gel of over expressed FhuA and its mutants. From left 1 – membrane fraction of FhuA (Δ1-129), 2 – membrane fraction of FhuA wild-type, 3 – solubilized FhuA (Δ1-129), 4 – solubilized FhuA (Δ1-160) and 5 – protein ladder.

FhuA mutants were transformed in *E. coli* BL21 (DE3) Omp8 (Prilipov et al. 1998) (a mutated strain used to block the expression of other outer membrane proteins). By using this strain, we could see overexpression of only FhuA and its mutants (Figure 18 A and 18 B).

Figure 18: (A) FhuA (Δ1-129) before and after IPTG induction, (B) FhuA (Δ1-160) before and after induction and (C) FhuA (Δ1-129) and FhuA (Δ1-160) after solubilisation.

After solubilizing the outer membrane fraction, the supernatant yields a clear band of FhuA (Δ1-129) and FhuA (Δ1-160) (Figure 18C).
4.2 Expression, extraction and purification of OmpF

pGompF was freshly transformed into the expression host *E. coli* K-12 strain BL21 (DE3) omp8 (Prilipov et al. 1998) as described in the Materials and Methods. Figure 19 shows that OmpF is overexpressed and present in the outer membrane fractions. After discarding the membrane pellet, OmpF can be seen in the supernatant of solubilisation buffer.

![Image of SDS gel](image)

Figure 19: SDS gel of OmpF overexpression in omp8, lane – 1, protein ladder, lane – 2, cell sample before induction, lane – 3, cell sample after induction, lane – 4, first extracted membrane fraction, lane – 5 supernatant after first extraction, lane – 6, solubilized OmpF.

4.3 Characterization of FhuA channel

4.3.1 Functional reconstitution of FhuA (Δ1-129) in PMOXA-PDMS-PMOXA triblock copolymer

Protein-lipid interactions can be investigated with monolayer techniques. A change in the surface pressure resulting from injection of a protein beneath a lipid-monolayer indicates an interaction between the monolayer and the protein of interest. A protein molecule that penetrates into the monolayer at the air-water interface laterally compresses the monolayer by its insertion and increases the surface pressure (Schwarz and Taylor 1999). For Langmuir isotherm measurements, a monolayer of ABA (PMOXA-PDMS-PMOXA)- triblock copolymer was formed (Graff et al. 2002) and the surface pressure increased to 22.5 mN/m immediately after FhuA (Δ1-129) addition. Control experiments with solubilisation buffer containing the oPOE detergent caused an
increase in surface pressure of 5 mN/m and proved that the high increase in surface pressure can mainly be attributed to FhuA (Δ1-129) insertion into the block copolymer film (Figure 20).

![Figure 20: Surface pressure versus time diagrams for insertion of FhuA (Δ1-129) into a ABA triblock copolymer (■) and the control with oPOE (1%) (○).](image)

4.3.2 Functional reconstitution of FhuA (Δ1-129) in liposomes

In a first experiment, liposomes loaded with a self-quenching concentration of calcein (50 mM) were prepared and purified to homogeneity as described in Materials and Methods. The size of liposomes was determined (Zeta-Sizer Nano Series, Malvern) after purification to be 220 nm ± 10 nm.

In a first control experiment, 2.1 nmol FhuA (Δ1-129), 2.1 nmol FhuA (Δ1-160), and 1.62 nmol OmpF were added to the calcein loaded liposomes in a stepwise manner as indicated by an arrow in Figure 21. Functional incorporation of the channel protein into the liposomes enabled translocation of calcein molecules through the channel protein and increases the fluorescence signal. The insertion of FhuA mutants and OmpF into the calcein loaded liposomes occured within a few seconds (Figure 21), proving the
structural integrity of the β-barrels. The transmembrane channel preparations contain 1% of oPOE-detergent that affects the liposomes integrity in contrast to supplemented buffer (Figure 21A). The higher fluorescence values for FhuA (Δ1-129) and FhuA (Δ1-160) compared to OmpF can be attributed to the larger pore size and higher number of FhuA molecules. There is no difference between the translocation of calcein between FhuA (Δ1-129) and FhuA (Δ1-160) at the macroscopic level. Finally, all liposomes were destroyed by adding 6.43 µM Triton X-100 (final concentration). The increase in calcein fluorescence to comparable levels showed that comparable amounts of liposomes have been employed in each experiment.

Figure 21A: Incorporation of FhuA (Δ1-129), FhuA (Δ1-160) and OmpF into liposomes loaded with 50 mM calcein. Increase in fluorescence intensity is caused by dilution of the self-quenching as a result of its diffusion through the transmembrane protein channels into the external environment (100 mM KCl, 10 mM Tris pH 7.4). Three transmembrane channels (2.1 nmol Fhu (Δ1-129) (○), 2.1 nmol FhuA (Δ 1-160) (X), 1.62 nmol OmpF (▲), FhuA solubilisation buffer (◼) (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 % oPOE, 1 mM PMSF) and buffer (◇) (100 mM
KCl, 10 mM Tris pH 7.4) were added step-wise to the calcein loaded liposomes (as indicated by an arrow). In the last step all liposomes were destroyed by adding Triton X-100.

CoCl$_2$ is an effective quencher of calcein fluorescence (Kendall and Macdonald 1982) and small cobalt ions should diffuse with less steric hindrance than calcein molecules through the OmpF channels. A control experiment employing calcein loaded liposomes and CoCl$_2$ as quencher confirmed the rapid OmpF, FhuA (Δ1-129) and FhuA (Δ1-160) insertion in liposomes (Figure 21B).

![Figure 21B: Incorporation of FhuA (Δ1-129), FhuA (Δ1-160) and OmpF into liposomes loaded with 50 mM calcein. Quenching of fluorescence by cobalt diffusion through the channels causes a decrease in fluorescence intensity. Three transmembrane channels (2.1 nmol Fhu (Δ1-129) (○), 2.1 nmol FhuA (Δ 1-160) (X), 1.62 nmol OmpF (▲), FhuA solubilisation buffer (■) (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 % oPOE, 1 mM PMSF) and buffer (◇) (100 mM KCl, 10 mM Tris pH 7.4) were added step-wise to the calcein loaded liposomes (as indicated by an arrow). In the last step all liposomes were destroyed by adding Triton X-100.](image)

The average size of the liposomes measured at 220 nm remained within a ± 5 % variation after addition of FhuA variants or OmpF.
4.4 Nanocompartments (Synthosomes) for biotechnological applications

4.4.1 Selective product recovery of negatively charged molecules

Figure 22: Schematic representation of two Synthosomes designed for A) Selective product recovery by loading Synthosomes with positively charged macromolecules as traps for negatively charged compounds, and B) Biocatalytic conversions of substrates by enzymes encapsulated in Synthosomes.

Figure 22A illustrates the separation principle of a Synthosome loaded with positively charged polylysine molecules (0.5 mg/ml of M<sub>w</sub> 15-30 kDa) as a trap for negatively charged compounds. Negatively charged compounds such as sulforhodamine B bind electrostatically to polylysine if they can diffuse through the FhuA transmembrane channel or penetrate the shell wall consisting of ABA-polymer.

a) Sulforhodamine B entrapment

From the control experiments, which check the interaction between polylysine and sulforhodamine B, it can be seen that the fluorescence is not quenched by the addition of different amounts of polylysine; instead it increases two folds as shown in the Figure 23.
Figure 23: Interaction of sulforhodamine B with polylysine (different concentrations). Sulforhodamine B with 0.5 mg/ml of polylysine (x), sulforhodamine B with 0.25 mg/ml of polylysine (○), sulforhodamine B with 0.125 mg/ml of polylysine (▲) and as control, sulforhodamine B in Tris buffer (◊).

The fluorescence signal of the Synthosomes harboring FhuA (∆1-129) and polylysine is 15 times higher compared to Synthosomes not harboring the FhuA (∆1-129) gate (excitation at 540; absorbance at 590 nm; Figure 24). This result clearly indicates that sulforhodamine B enters in the Synthosome nearly exclusively through the FhuA (∆1-129) channel. It further shows that polylysine is an effective trap for negatively charged molecules such as sulforhodamine B. Without an electrostatic trap, the sulforhodamine B can diffuse in and out of Synthosomes. For Synthosomes containing FhuA (∆1-129) without a polylysine trap, the recorded fluorescence signal at 590 nm is approximately two times higher compared to the controls lacking a FhuA (∆1-129) channel protein. The increased fluorescence might be attributed to sulforhodamine B molecules binding unspecifically to the ABA-triblock copolymer. In the presence of a FhuA (∆1-129) channel, the accessible surface for sulforhodamine B molecules is
increased (nearly doubled) by not only offering the vesicle’s external surface, but also the internal vesicle’s surface for unspecific binding. The fluorescence signal of FhuA (Δ1-129) without a polylysine trap is still five times lower compared to Synthosomes harboring FhuA (Δ1-129) and polylysine as an electrostatic trap (Figure 24). These differences prove that polylysine is an effective electrostatic trap.

Figure 24: Entrapment and interaction of sulforhodamine B with Synthosomes harboring FhuA (Δ1-129) & loaded with polylysine (●), harboring FhuA (Δ1-129) & not loaded with polylysine (○), not harboring FhuA (Δ1-129) & loaded with polylysine (▲), and not harboring FhuA (Δ1-129) & not loaded with polylysine (△).

b) DNA entrapment

Figure 25 illustrates the separation principle of Synthosomes loaded with polylysine molecules as traps for ssDNA. From Figure 25, it is clear that ssDNA diffuses through the FhuA (Δ1-129) channel and binds electrostatically to the polylysine present.
inside the Synthosomes, whereas the synthosomes without FhuA show no peak of SYBR Gold to ssDNA.

Figure 25: (A) Schematic representation of nanotraps for selective product recovery of ssDNA. (B) Emission spectra of SYBR gold bound to ssDNA trapped inside the nanocompartments harboring FhuA (Δ1-129) & loaded with polylysine (■), not harboring FhuA (Δ1-129) & loaded with polylysine (○).
4.4.2 Bioconversion in enzymatically loaded nanoreactors

Figure 22B describes the principle of using enzymes trapped in Synthosomes for enzyme catalysis. For a versatile use of Synthosomes in biotechnological applications, the Direct Dispersion and Ethanol method were both investigated. As a bioconversion example the oxidation of TMB by encapsulated HRP was chosen as a proof of principle (Figure 26; bioconversion monitored at 370 nm). A soluble blue reaction product is formed initially due to one-electron oxidation of TMB (Figure 26). The kinetic data of the TMB oxidation shows that the activity in the presence of a FhuA (Δ1-129) channel is significantly higher than the background observed in the Synthosomes loaded with HRP, but lacking a FhuA (Δ1-129) channel (Figure 26).

![Graph showing absorbance over time for TMB oxidation by HRP encapsulated in Synthosomes with and without FhuA (Δ1-129)](image)

Figure 26: Conversion of TMB oxidation by HRP encapsulated inside the Synthosomes with FhuA (Δ1-129) (■) and without FhuA (Δ1-129) (○) recorded at 370 nm.

The TMB example proves again that FhuA (Δ1-129) is incorporated in the ABA-polymer and acts as a gate for compound flux to the HRP catalyst, which is confined in the interior of the Synthosome. The low background activity might be attributed to a few HRP enzymes that are still bound to the outer surface of the Synthosomes after
purification, and/or a very slow diffusion of TMB molecules through the polymer membrane.

Figure 27: Comparison of Direct Dispersion method (A/A*) with Ethanol method (B/B*) for the conversion of TMB by HRP encapsulated inside the Synthosomes. The reaction is quenched after five min by HCl addition and colored product formation is recorded at 450 nm after three min (R: background without HRP and without FhuA).

In order to compare two Synthosome preparation procedures the HRP enzyme was inactivated after a 5 min incubation time by the addition of hydrochloric acid (HCl, 0.25 mM final concentration). The absorbance of a stable yellow diimine reaction product in acidic pH was measured at 450 nm for product quantification (Josephy et al. 1982) (Figure 27). The Direct Dispersion method (A: Synthosomes with HRP and without FhuA/A*: Synthosomes with HRP and with FhuA) and the Ethanol method (B: Synthosomes with HRP and without FhuA/B*: Synthosomes with HRP and with FhuA) show different background levels of absorbance. The higher absorbance background with the Direct Dispersion method (abs (A) = 0.38) > (abs (B) = 0.29) can mainly be attributed to more turbid vesicle dispersions formed by this method (Figure 27). The Direct
Dispersion method, despite the background absorbance, is superior in terms of encapsulation of functional HRP enzymes in Synthosomes. The differences in absolute absorbance values were 1.78 for the Direct Dispersion method (abs (A*)-abs (A)) as compared to 0.91 (abs (B*)-abs (B)) for the Ethanol method (Figure 27).

4.5 Nanocompartment system for analytical applications

Synthosomes encapsulated with nanophosphor DNA conjugates were prepared as described in the Material and Methods and always purified to homogeneity (Broz et al. 2005; Nardin et al. 2001). The average diameter of the Synthosomes encapsulated with nanophosphor DNA conjugates was determined (Zeta-Sizer Nano Series, Malvern) in each preparation after purification to be 220 nm ± 10 nm.

4.5.1 Detection principle for DNA translocation into Synthosomes

Synthosomes were loaded with nanophosphor DNA conjugates. Transmembrane channel proteins such as FhuA or OmpF (trimer) were embedded in the triblock copolymer membrane (Nardin et al. 2001) acting as selective filters by controlling the compound flux in and out of the Synthosome. Small DNA fragments containing a TAMRA-fluorescence dye at the 3’-end hybridize after translocation through the channel protein to the complementary sequence of the DNA-labeled nanophosphor particles. Hybridization events enable an energy transfer (FRET) from the nanophosphors to the TAMRA-dye upon excitation. The distance between both FRET-partners is within the Förster radius (6.5 nm) between Tb-ions and rhodamine (Selvin 1995). As a result, the emission life-time of the nanophosphors was reduced in case DNA molecules translocate through the channel protein and hybridize with the DNA-label nanophosphor particles (Figure 28 A-E).
Figure 28: Time resolved fluorescence decay in hybridization experiments of a TAMRA-labeled primer with nanophosphor particles that have been encapsulated in Synthosomes: A) Nanophosphor DNA conjugates in the absence of a transmembrane protein, B) with OmpF (trimer), C) with FhuA wild-type, D) with FhuA (Δ1-129) and E) with FhuA (Δ1-160) as a
selective channel protein. Fluorescence decay was recorded at 542 nm before hybridization in the absence of the TAMRA-labeled primer (■), after hybridization with the TAMRA-labeled primer (●; 0.25 nmol) and after destroying the Synthosome shell with a combined heat/detergent treatment (80°C, 30 min/Triton X-100; 6.16 µM; ▲). The curves were normalized to an initial fluorescence for comparison.

4.5.2 Control experiments for hybridization of DNA

In a first control experiment it was proven that hybridization of nanophosphor DNA conjugates with a TAMRA-labeled primer reduces the half-life by 40 % whereas the lifetime remains unchanged by adding a TAMRA-labeled primer (5’-TTTTCACAA TGAGGGGCTGAT-3’) with a non-complementary sequence (data not shown).

In a second control experiment, it was proven that the TAMRA-labeled primers couldn’t diffuse through the polymer shell into Synthosomes loaded with DNA labeled nanophosphors. The fluorescence half-life remained unchanged (Table 3) and upon destabilizing the ABA polymer membrane by Triton X-100 addition, the half-life was reduced by 23 %, proving a FRET between the nanophosphors and the hybridized primer (Table 3).

<table>
<thead>
<tr>
<th>Synthosomes loaded with nanophosphor DNA conjugates and</th>
<th>Normalized fluorescence half-life of nanophosphor DNA conjugates with a complementary TAMRA-labeled primer</th>
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<td>Before hybridization</td>
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<td>without channel protein with OmpF</td>
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<td>with FhuA (Δ1-129)</td>
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Table 3: Half-life of fluorescence before and after hybridization of a TAMRA-labeled primer with a complementary nanophosphor DNA conjugate that has been encapsulated inside of Synthosomes. The half-lifes were in the range of 0.40-0.92 ms and have been normalized for comparison to the respective fluorescence values before hybridization.

4.5.3 Translocation of single stranded DNA through FhuA, FhuA variants and OmpF

The FhuA monomer has an elliptical cross section of 39 to 46 Å resulting in a pore area that is more than 23 times larger than the pore area of OmpF (elliptical cross
Results

Figure 29 shows engineered FhuA variants in which the “cork” domain has been removed (FhuA (Δ1-160) (Braun et al. 1999) or partially removed (FhuA (Δ1-129)). The engineered FhuA (Δ1-160) variant is characterized as a passive diffusion channel (Braun et al. 2002).

Figure 29: Schematic representation of the nanophosphors based FRET-system for monitoring DNA translocation into Synthosomes with the following channel proteins acting as selective gates: A) FhuA wild-type / FhuA deletion variants and B) OmpF trimer.

OmpF forms a trimer in contrast to the monomeric FhuA protein in biological membranes (Nardin et al. 2001; Tieleman and Berendsen 1998). The small diameter of OmpF represents a diffusion barrier that prevents efficient translocation of primers with a height of ~13 Å and length of ~71.4 Å in linear form. The half-life of nanophosphors is close to the error-margin and is reduced by ~4.5 % in Synthosomes with an OmpF gate (Table 3). FhuA wild-type harboring the “cork” domain inside the β-barrel shows a similar result to the OmpF channel (half-life of nanophosphors is slightly reduced by 6.6 %; Table 3). In the FhuA (Δ1-129) a detectable primer translocation occurs (half-life reduction by 16.0 %). The FhuA (Δ1-160) variant lacking the cork domain shows a fast primer translocation (half-life reduction by 39.0 %).
4.6 Preliminary results of scaling up the nanocompartment system

4.6.1 AB polymer Nanocompartment formation

Synthosomes encapsulated with calcein were prepared using AB polymer and purified to homogeneity. Non-encapsulated calcein was removed as described in the Materials and Methods. Confocal microscopic images show calcein encapsulated Synthosomes (excitation 480 nm and emission at 520 nm).

Figure 30: Confocal image of Calcein encapsulated Synthosomes prepared using AB polymer.

20 ml of Synthosomes were prepared using AB polymer (P2VP-PEO-8) as described in the Material and Methods. The average diameter of the Synthosomes was determined (Zeta-Sizer Nano Series, Malvern) to be around 100 nm ± 20 nm. Cryo TEM images shows uniform size of unilamellar vesicles in the range of ~150 nm (scale bar) (Figure 31).
4.6.2 Optimization of solubilisation of FhuA from outer membrane fractions

27 detergents (JBSolution Detergent Test Kit, Jena Bioscience GmbH, Jena, Germany) were tested for the improved solubilisation of FhuA. SDS and Sodium cholate show improved FhuA solubilisation. SDS solubilises FhuA in less than one hour at any temperature and at a concentration of 8.5 mM. However, SDS appears to denature the FhuA (Hoffmann et al. 1986). Na-cholate, with a 12 h solubilisation period at 15°C, yields more solubilized protein and appears to be an alternative method for solubilisation (Figure 32).
Figure 32: SDS gel of FhuA wild-type solubilisation at 15°C for 12 h, lane – 1, solubilised with 10 mM Sodium cholate, lane – 2, protein ladder and lane – 3, solubilised with 1% oPOE.
5. Discussion

The Synthosome system represents a nanocompartment with a broad range of promising applications in biotechnology. A main prerequisite for efficient product recovery using Synthosome systems is to limit diffusion barriers by employing transmembrane channels with large pore diameters. For this prerequisite, one of the largest outer membrane protein, FhuA, was selected. The FhuA monomer has an elliptical cross section of 39 Å to 46 Å resulting in a pore area that is more than 23 times larger than the one of OmpF (elliptical cross section of 7 Å to 11 Å). In addition, a deletion mutant FhuA (Δ5-160) was prepared in order to remove the iron transfer domain and to form a large passive diffusion channel (Braun et al. 1999). This truncated FhuA channel, however, showed fluctuations in ion transport (Braun et al. 2002) indicating a contracting/expanding channel or a flipping loop at the channel exit. We engineered apart from the deletion mutant FhuA (Δ5-160) a novel deletion mutant by removing the first 129 amino acids (Δ1-129), leaving an additional β-strand in the β-barrel (using PDB viewer). This β-strand is intended to provide a mechanical support within the β-barrel to prevent the contraction of the FhuA channel in the ABA-polymer or in the lipid membrane (Braun et al. 2002) (Figure 4-6). However, we must measure single channel conductance of FhuA (Δ1-129) incorporated into artificial bilayer membranes to prove the presence of this β-strand inside the barrel and compare the stability of single channel conductance with FhuA (Δ1-160). Control experiments in liposome (Figure 21A&B) revealed no detectable difference for small molecules such as calcein on the macroscopic level.

We proved the functional reconstitution of FhuA channels (FhuA (Δ1-129) and FhuA (Δ1-160)) in liposomes as well as in triblock copolymer vesicles (Figure 20-21) and explored subsequently the potential use of Synthosomes in biotechnological applications such as selective product recovery and bioconversions. Selective product recovery was achieved by encapsulating a positively charged polymer (polylysine) in the Synthosome and trapping negatively charged sulforhodamine B molecules inside the Synthosome.
Discussion

The HRP experiments showed that the enzyme activity is retained inside the Synthosomes. However, encapsulation efficiencies of HRP enzymes are ~15% and require improvement. Control experiments further confirmed that A) FhuA (Δ1-129) acts as a gate for compound flux into the Synthosome, and B) ABA triblock copolymer vesicles are barely or not at all penetrable for small molecules such as calcein or sulforhodamine B within the duration of the experiments (24 h). The latter property is an important prerequisite for biotechnological applications.

To ensure a broad range of Synthosome applications, it is important to encapsulate enzymes in their active form. Enzymes during encapsulation are exposed to block copolymer molecules and organic solvents required for polymer dissolution. For the preparation of Synthosomes, it is therefore important to develop methods allowing different encapsulation strategies. For the HRP we employed two methods: the Direct Dispersion method and the Ethanol method. Of the two methods, the Direct Dispersion method proved to affect HRP activity less. A further advantage of Synthosomes is the protection of enzymes against proteolytic degradation.

Both methods are easy to perform, reliable and have been used for the production of Synthosomes in milligram scale. For bringing Synthosomes closer to industrial applications, a scale up is required as well as further developments in selective product recovery.

Synthosomes encapsulated with nanophosphor particles for the selective recovery of single stranded DNA has a broad application in diagnostics. Figure 28 shows the schematic representation of FRET based monitoring of DNA translocation into Synthosomes with encapsulated nanophosphor DNA-conjugates. Upon primer translocation through the transmembrane channel protein and hybridization of the primer to the complementary DNA strand, the fluorescence half-life of the nanophosphor particles was reduced due to FRET from the nanophosphor particle to the TAMRA-labeled primer.

Our long term goal is to develop Synthosome applications in the field of PCR-/RNA-diagnostics that allows us to detect by FRET measurements specific PCR-/RNA-products in complex mixtures with high sensitivity. The advantages of the Synthosome
Discussion

system compared to direct measurements in solution include: a) large molecules such as proteins cannot enter and therefore cannot interfere with FRET measurements, b) target DNA is concentrated inside the Synthosome, c) entrapped DNA or RNA is protected from degrading enzymes, d) subsequent separation procedures through gel-filtration chromatography is simple and boosts sensitivity, e) possible use in organic solvents (depending on the block copolymer), and f) channel translocation is tunable through methods of protein engineering.

As a proof of concept we had to ensure that a) single stranded primer DNA translocates through the transmembrane channels and not through the ABA coblock polymer shell, and b) a fluorescence signal can be detected through the polymer shell of Synthosomes.

For the first concept proof we showed that the TAMRA-labeled primer cannot diffuse through the ABA triblock copolymer (Table 3, Figure 28A) and that a small channel protein such as OmpF will not sterically permit primer translocation through the transmembrane channel (Figure 28B). These experiments additionally showed that a fluorescence signal from the nanophosphor particles can be detected through the polymer shell of the Synthosomes. Compared to the values obtained in absence of a triblock copolymer, the quantum yield of the fluorescence through the Synthosome shell is reduced by 26 %, achieving a detection of primer DNA concentration as low as 49 nM.

All obtained half-lifes correlate well with the steric demands of the primer DNA and the space available inside the channel proteins for primer DNA translocation. The well-characterized FhuA (Δ1-160) variant, which lacks the “cork domain”, enables a rapid primer DNA translocation (Table 3; Figure 28E) in contrast to the FhuA wild-type (Figure 28C). A translocation of single stranded DNA through FhuA or an engineered FhuA variant has previously not been reported. The FhuA (Δ1-129) deletion mutant contains an additional β-strand spanning the β-barrel in the pdb-crystal structure (Ferguson et al. 2000). FhuA (Δ1-129) shows a lower translocation efficiency than FhuA (Δ1-160) (Table 3; Figure 28D-28E).

Further protein engineering by removing positively charged clusters and groups that cause steric hinderance in the β-barrel of FhuA might further improve DNA
translocation and thinner polymer shells might further improve the quantum yield. Currently we are working on a protocol to release upon purification the concentrated and hybridized primers in order to boost the sensitivity to pM concentrations.

We hope that the Synthosome platform for selective recovery will lead, in combination with the nanophosphors based FRET-system, to novel applications in diagnostic, drug discovery and microbiological research.
6. Summary and Outlook

For validating Synthosomes application in biotechnology, we engineered one of the largest outer membrane proteins FhuA and reconstituted it in nanocompartments. We also created FhuA mutants with positive and negative charges at the loops of the FhuA β barrel potentially to connect these two channels with electrostatic interactions. We have shown a proof of principle of bioconversion in Synthosomes by encapsulating HRP inside the nanocompartments embedded with FhuA channel. Still protocols must be established for encapsulating active enzymes at high level. Depending on the enzyme used and on the application, the chemical nature of the vesicle-forming amphiphile and the preparation method can be selected (Walde and Ichikawa 2001). Synthosomes were loaded with polylysine and shown as an effective trap for charged molecules such as ssDNA. However, we must quantify the amount of encapsulation of polylysine inside the nanocompartments and also the trapped DNA inside Synthosomes.

We are currently working on the solvent resistance characteristics of the enzymes inside Synthosomes. For industrial application of Synthosomes, we scaled up Synthosomes preparation to 20 ml scale without any functionalisation using AB polymer (P2VP-PEO-8), which can be synthesized in the Kg scale.

We have proven that the TAMRA-labeled primer can translocate through FhuA deletion mutants, whereas OmpF does not permit the primer translocation. We hope that this novel nanocompartment system will lead to applications in DNA-/RNA- diagnostics.
7. Reference


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Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM,
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8. Appendix

8.1 Cloning of FhuA and FhuA mutants using pPR-IBA1

8.1.1 PCR amplification, digestion and ligation of FhuA and FhuA mutants

Detailed protocol for amplification of FhuA and FhuA mutants, insertion in pPR-IBA1 and transformation in *E. coli* DH5α

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Appendix

Ligation of FhuA in pPR-IBA1

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8.1.2 FhuA (∆1-129) neg and FhuA (∆1-129) pos mutants by site directed mutagenesis

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#### 8.1.3 Transformation of FhuA and FhuA mutants in Omp8

1. 5 ml overnight culture of *E. coli* K-12 strain BL21 (DE3) (Omp8) were prepared by inoculating LB Media with *E. coli* glycerol stocks at -80°C. The culture was grown overnight by shaking at 250 rpm and 37°C.

2. 500 µl of the overnight culture was used to inoculate 50 ml of LB media and the OD$_{578}$ was measured using a spectrophotometer.

3. The culture was allowed to grow in the shaker (Infors HT Multitron) at the same conditions, i.e. 250 rpm and 37°C.

4. The OD$_{578}$ was constantly monitored until the value reached between 0.4 and 0.5.

5. When the OD$_{578}$ became greater than 0.4, the cells were centrifuged at 3200 g for 10 min at 4°C.

6. The supernatant was discarded and the pellet was resuspended in 2 ml of TSS buffer. The TSS buffer has the following composition:

   For 100 ml of LB media:

   - PEG 10 g
   - DMSO 5 ml
   - MgSO$_4$ 0.6 g
7. 200 µl of the resuspended pellet was then aliquoted into Eppendorf tubes and the rest was discarded.

8. The respective plasmid (FhuA wild-type and FhuA mutants) was then added to the 200 µl resuspended pellet aliquot tubes.

9. The tubes were kept on ice for 20 min.

10. The tubes were then given a “heat shock” at 42°C for 45 s using a water bath and were immediately put on ice.

11. 800 µl of LB Media was then added to the suspension containing plasmids and the tubes were incubated for 1 hour at 180 rpm and 37°C.

12. The tubes were centrifuged at 600 g using a tabletop centrifuge (Eppendorf 5415 D) for 2 min.

13. 900 µl of the supernatant from each tube was thrown away and the cells were gently resuspended.

14. The cells were spread on a LB agar plate with Ampicillin (100 µg/ml) and kept overnight in an incubator at 37°C.

8.2 Expression, extraction and purification of FhuA and FhuA mutants

8.2.1 Expression of FhuA and FhuA mutants

FhuA and the FhuA mutants were picked from the transformed plates and expressed for final extraction and solubilisation. The expression is carried out in TY media using the following protocol:

1. Freshly transformed clones were picked by using a toothpick from the agar plates after the transformation and inoculated in 5ml LB media with Ampicillin (100 µg/ml) overnight at 250 rpm and 37°C.

2. 3 ml of the overnight culture was used to inoculate 300 ml of TY Media (1:100 dilutions) and the cells were allowed to grow at 250 rpm and 37°C until the OD\textsubscript{578} reached 0.5.

3. At OD\textsubscript{578} 0.5, the cells were induced with 300 µl of IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM final concentration).
4 A 1 ml sample was collected before induction and centrifuged at 15243 g and 4°C for 10 min and the pellet was stored at -20°C.
5 The cultures were further grown up to an OD\textsubscript{578} of approximately 1.4.
6 When OD\textsubscript{578} reached 1.4, the cells were harvested by centrifugation at 3200 g at 4°C for 20 min.
7 A 1 ml sample was collected before harvesting and centrifuged at 15243 g and 4°C for 10 min for checking the protein expression on the SDS Gel. The pellet was stored at -20°C.

8.2.2 Analysis of overexpressed FhuA and FhuA mutants by SDS PAGE Gel Electrophoresis

Expression of FhuA and FhuA variants was checked using SDS Polyacrylamide Gel Electrophoresis. The sample for the SDS Gel was prepared using the following protocol:

1. Lysis buffer (4% SDS, 20 mM Tris-HCl, 0.2 mM EDTA, pH 8.0) was added to the samples (taken before induction and after expression) on the basis of their OD\textsubscript{578} value (25 µl of lysis buffer for an OD value of 1.0) and mixed properly.
2. The same volume of the 3X loading dye was added to the sample.
   The 3X loading dye had the following composition:
   - 150 mM Tris-Cl (pH 6.8)
   - 100 mM DTT
   - 6 % SDS (electrophoresis grade)
   - 0.3 % bromophenol blue
   - 30 % glycerol
3. The sample was heated at 95°C for 3 min for denaturation.
4. 1.5 µl of DNAse I was added (If the samples were too sticky).
5. Sonication was performed for 30 s to reduce the viscosity of the samples.
6. 10 µl of each sample was loaded on the 10 % SDS Gel, along with 10 µl of the standard protein marker.

7. The SDS gel was run at 200 Volts for 70 min.

8. Gel was stained for 45 min followed by destaining for 1.5 h.

9. The gel was then left overnight in water (If the gel is not destained properly).
8.3 In vitro evolution of FhuA-GFP fusion protein in cell free expression system

We constructed the *fhuA-gfp* fusion gene for monitoring the expression of FhuA by fluorescence and tried to express this fusion protein *in vivo*. But we could not express *in vivo*, possibly due to misfolding of the fusion protein. Hence, we used a cell free expression system because *in vivo* expression of foreign or synthetic genes can be subject to certain restrictions such as protein aggregation, degradation and toxicity. These limitations have been overcome with high-yield *in vitro* protein production (Rapid Translation System RTS 100, *E. coli* HY Kit, Roche Applied Science, Mannheim, Germany). The components of the lysate, including substrates, the energy regenerating system and factors required for regulation of the coupled reactions, are optimized to ensure high synthesis rates. RTS eliminates the need for laborious up- and down-stream steps (e.g. host cell transformation, culturing, or lysis) typically associated with cell-based expression systems. The RTS *E. coli* HY lysate contains all the machinery needed to drive coupled transcription and translation of a DNA template in a single reaction vessel. Cell-free systems are usually based either on a crude cell extract including ribosomes and all soluble enzymes, translation factors and tRNAs (S30 fraction), or on a combination of ribosome-free extract (S100 fraction) plus isolated ribosomes. The DNA templates such as plasmid, an isolated gene, or a synthetic DNA fragment is added to the translation mixture, and the corresponding mRNA is synthesized *in situ* by the endogenous RNA polymerase present in the bacterial extract or its supernatant fraction. Under these conditions translation is going on while mRNA is still elongating, and the rates of transcription and translation are coordinated. This is why such systems are called *coupled transcription-translation systems* (RTS Application Manual, Roche Diagnostics, Mannheim, Germany).
Appendix

8.3.1 Cloning and expression of FhuA-GFP into pGFP Vector

![Cloning scheme of FhuA-GFP fusion in pGFP Vector.](image)

Figure 33: Cloning scheme of FhuA-GFP fusion in pGFP Vector. *fhua* (*Δ1-129*) gene is amplified using forward primer with restriction enzyme Hind III and reverse primer with 10 alanine linker and Kpn I. After PCR amplification the *fhua* gene with linker is digested with respective enzymes and cloned in the pGFP vector.
In a first approach, reverse primer FhuAFus-R 5’-GGCCGCTGCAGCGGCTGCAGCCGCTGCAGCGAAACGGAAGGTTGC-3’ for FhuA (Δ1-129) was designed having an overhang, coding downstream for a 10 alanine α helix. Similarly, a forward primer FhuAFus-F 5’-GCTGCAGCGGCTGCAGCGCCATGGTGAGCAAGGGC-3’ for eGFP having a complementary upstream overhang was constructed. Amplification of the *gfp* and *fhuA* mutant genes with the respective primers enabled a subsequent annealing of the complementary parts during an *fhuA-gfp* fusion PCR, and thus a PCR product coding for eGFP connected via an α helix to the C-terminus of a FhuA mutant. The aim was to clone the product into a suitable vector for subsequent expression of the fusion construct. However, that this approach could not yield a fusion product may be due to the lengthy annealing section between the two genes (data not shown).

Alternatively, it was thought to directly clone the FhuA (Δ1-129) mutant into pGFP (Figure 33) using the forward primer PGFP-F 5’-ATGCCCAAGCTTGGGATGCGCGCTGAAATTATGCGTG-3’ and the reverse primer PGFP-R 5’-AGGCGGGTACCCCAGCGGCAGCAGCGGCGGCAGCGGCGGCAGCGAAACGGAAGGTTGC-3’. Both vector and FhuA (Δ1-129) mutant PCR products were digested with Kpn I and Hind III, followed by T4 DNA ligase mediated insertion of the *fhuA* gene aside *gfp*. Clones were picked and digested to check the insert of *fhuA* (Δ1-129) and *gfp* fusion gene. FhuA proteins were over expressed using pPR-IBA1 vector. *fhuA* (Δ1-129)-*gfp* fusion gene was therefore recloned in pPR-IBA1 vector using the forward primer FhuA129-F 5’-ATGGGTCTCGAATTCCCGGATGCGCGCTGAAATTATGCGTG-3’ and the reverse primer CFGFP-R 5’-CCGCTCGAGCGGTTACTTGTACAG-3’ for overexpression. *fhuA* (Δ1-129)-*gfp* fusion gene was prepared with and without signal sequence of FhuA in order to check the effect of fusion protein expression.
8.3.2 Expression of FhuA (Δ1-129)-GFP using *in vitro* system RTS 100 *E. coli HY Kit*

*pPR-IBA1* expression vector, coding FhuA (Δ1-129)-GFP fusion construct, was used for the *in vitro* protein synthesis. The vector includes T7 regulatory elements. In a coupled *in vitro* reaction, the template DNA was first transcribed into mRNA by T7 RNA polymerase, followed by translation into protein by the ribosomal machinery present in the *E. coli* lysate. Expressed protein accumulates during the reaction and was harvested after 4 hours.

In parallel to the working solution a GFP control reaction was prepared with 1 µg of control vector coding for GFP. Reaction components were mixed as shown in the table 1 and the reaction tubes were incubated at 30°C for 4 hours. After this the fluorescence was checked in comparison with the control GFP. SDS-PAGE with Coomassie staining was used for subsequent analysis of protein preparations (‘soluble’ and ‘pellet’ fractions).

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Lysate</td>
<td>12</td>
</tr>
<tr>
<td>Reaction Mixture</td>
<td>10</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>12</td>
</tr>
<tr>
<td>Methionin</td>
<td>1</td>
</tr>
<tr>
<td>Reconstitution buffer</td>
<td>5</td>
</tr>
<tr>
<td>Plasmid encoding FhuA (Δ1-129)-GFP or control vector GFP</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

Table 4: Reaction volume and components for cell free expression (Rapid Translation System RTS 100, *E. coli HY Kit*, Roche Applied Science) of FhuA (Δ1-129)-GFP.

The samples were precipitated with acetone prior to the addition of SDS-PAGE sample buffer. In order to distinguish ‘soluble’ and ‘pellet’ fractions, 5 µl samples of the reaction solution were centrifuged for 5 min at 15243 g. Supernatants and pellets were placed in separate tubes and 50 µl of ice cold acetone were added to each of them. The samples were incubated on ice for 5 min and centrifuged for 5 min at 10000 rpm. Supernatants were discard and pellets were dried for 10 min. After adding 20 µl of SDS-PAGE sample buffer and heating for 5 min at 95°C, 10 µl of the samples were applied onto an SDS gel.
8.3.3 Cell free expression using *E. coli* lipid mixture

The reaction protocol is the same as described above with the only difference that 100 µg of *Egg PC* lipid mixture were added in order to facilitate correct protein folding in reaction tubes before the incubation step. Three reaction solutions were prepared with plasmids encoding FhuA (∆1-129), FhuA (∆1-129)-GFP and GFP. As a control experiment, two reactions with vectors encoding FhuA (∆1-129) and GFP without *E. coli* lipid mixture were started.

In both cases, the fluorescence couldn’t be observed or the fusion protein was not expressed by *Rapid Translation System RTS 100, E. coli HY Kit*. Hence we tried to remove the signal sequence of *fhuA (∆1-129)-gfp* fusion gene and expressed in both cell free system and in *E. coli* BL21 strain.

![Image of gel electrophoresis](image)

Figure 34: (A) lane 1 – protein ladder, lane 2 – FhuA (∆1-129)-GFP in cell free mixture, lane 3 – FhuA (∆1-129)-GFP in cell free mixture (supernatant), lane 4 – FhuA (∆1-129)-GFP in cell free mixture (pellet), lane 5 – GFP-pPRIBA1 in cell free mixture, lane 6 – GFP-pPRIBA1 in cell free mixture (supernatant) and lane 7 – GFP-pPRIBA1 cell free mixture (pellet). (B) lane 1 – protein ladder, lane 2 – GFP-pPRIBA1 after induction and lane 3 – FhuA (∆1-129)-GFP in pPR-IBA1 after induction.

We could see from Figure 34, the fusion protein overexpressed. However that no fluorescence could be observed indicates that the protein may be folded incorrectly.
8.4 Preliminary experiments of entrapping PCR product in Synthosomes

25 µl of 100 bp PCR Product was added to two batches of 1 ml suspension of purified Synthosomes (prepared by Ethanol method): batch 1. Synthosomes with FhuA (Δ1-129) and loaded with polylysine; batch 2. Synthosomes without FhuA (Δ1-129) and loaded with polylysine. The Synthosome suspension was slowly stirred for one hour. To remove non-entrapped 100 bp PCR product, Synthosomes were purified using Sepharose 4B. 5 µl of SYBR gold was added to this mixture of Synthosomes suspension. SYBR gold fluorescence was determined (excitation 495 nm; emission spectra recorded from 510 to 600 nm).

From Figure 35, it is clear that the 100 bp PCR product diffuses through the FhuA (Δ1-129) channel and binds electrostatically to the polylysine present in the Synthosomes. The fluorescence intensity is 3 times higher in the case of Synthosomes harboring FhuA (Δ1-129) and loaded with polylysine than in the Synthosomes not harboring FhuA (Δ1-129) and loaded with polylysine. However, we could observe a small peak of SYBR gold bound to DNA in the case of Synthosomes not harboring FhuA (Δ1-129) and loaded with polylysine. This could be due to the non-specific binding of PCR product to the outer surface of Synthosomes.
Figure 35: Emission spectra of SYBR gold bound to PCR product trapped inside the Synthosomes with FhuA (Δ1-129) & loaded with polylysine (■) and without FhuA (Δ1-129) & loaded with polylysine (○).