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NanoBioMag

Magnetic Field Assisted Biomaterials Processing

Specific Targeted Research Project (STREP)

Priority 3 NanoMatPro

Publishable Final Activity Report
(Final Executive Summary)

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List of Participants:

1) Solae/DuPont (DuPont)
2) Technical University of Denmark (DTU)
3) Swiss Federal Institute of Technology (ETH)
4) Forschungszentrum Karlsruhe GmbH (FZK)
5) University of Birmingham (Bham)
6) Universität Karlsruhe (UniKa)
7) Bühler AG (Bühler)

Project Coordinator:
Dr. Thomas Friedmann
Solae Denmark A/S
Sydhavnsgade 7
DK-8100 Aarhus C
Denmark

The growing demands for efficient and economical production processes, and continued improvements in product quality call for permanent improvement of existing processes. The use of magnetic field based operations provides a unique additional degree of freedom to optimize such processes. The magnetic properties lead to the potential for multifaceted approaches for novel macromolecular bioprocessing such as external manipulation, self-assembly, transport, and separation. In the last decade the application of magnetic fields emerged from separation processes in the minerals industry into a wide range of industries including water-treatment, highly selective bio-separation, hyperthermia, drug delivery and Magnetic Resonance Imaging (MRI). The NanoBioMag project combines biotechnology with nanotechnology via the use of magnetic fields.

The overall objective of the NANOBIOMAG project was to enhance the competitiveness of the European biomaterials-, pharmaceutical- and food industries by developing unique and novel materials, and related process technologies. These technologies are located at the intersection of nanomaterials, biomaterials and magnet technologies. As a first step, multifunctional 'smart' magnetic materials were fabricated, while in the second step, new processes that utilise these new materials in order to produce novel materials and products and significantly reduce the current high number of processing steps for the production of these materials, were developed. Another component to this work was to explore the effects of strong magnetic fields on molecular structures with intentions to tailoring properties of complex biomolecules.

'Smart' nano- to micron-sized magnetic particles with multifunctional properties are of key importance. Their properties allow external manipulation, provide a means for self-assembly, transport and separation through a combination of magnetic and other forces. Highly selective particle coatings allow the capture of target materials out of complex environments. Considerations of size and surface properties can lead to new breakthroughs in adsorption capacities and reduced processing time. The integration of all these properties into 'smart' and multifunctional materials together with the possibility of their scaleable production lays the ground work for enabling technologies for industrial materials.

In order to benefit from this 'multifunctional magnetic materials manufacture', the development of novel processing technologies was required in the project. For the purposes of this study these processing technologies was broadly divided into two research efforts, BIOSEPARATION and BIOSYNTHESIS. In the area of bioseparation, two new processes were developed. In the biosynthesis area, new knowledge was acquired on the use of the magnetic field on the molecular level to enhance and change properties. The outcomes from the NanoBioMag project are described and summarized below.

Work Package 1: Multifunctional Magnetic Materials Manufacturing (4M)

The fabrication of multifunctional 'smart' magnetic materials was the focus of Work Package 1 (WP1). The institutions involved in this work were The University of Birmingham and Solae Company. The synthesis of smarter magnetic multifunctional adsorbents promising new properties and possibilities for future applications, for example 'self cleaning', 'self elution', 'combined solid-supported purification and macromolecule assembly', 'solid-supported purification coupled with chemical and/or enzymic modification' was carried out by researchers at the University of Birmingham. This group built on their existing knowledge in the manufacture of novel adsorbent materials for use in magnetic separation and chromatographic processes and in particular their know-how in surface functionalization, to produce materials with high target binding strength and capacity.

Smart Polymer -Magnetic Adsorbent Phases (SP-MAPs) and Smart Molecular Imprinted Polymer-Magnetic Adsorbent Phases (SMIP-MAPS) were designed. To begin this work, 'Blueprints' of various SP-MAPs & SMIP-MAPs were 'sketched out'. All of the SP-MAPs envisaged at the beginning of the NanoBioMag project have been created. Additionally, during the course of the project, new types and functions were also created and validated. An example is the all-or-nothing switchable binding & tunable SEC. Furthermore, during the course of study the number of synthesis routes for the SP-MAPs was also expanded.

A significant achievement from this work package was the evolution and validation of a flexible 'generic assembly platform' for the manufacture of all SP–APs (A schematic is shown in Figure 1). Extensive 'proof-of-principle' experimentation verified the feasibility and potential of this SP-AP manufacturing platform. The fabrication process employed a 'two-stage activation/surface patterning' procedure at its foundation, which allowed for a vast array of subsequent 'graft to' and graft from' reactions to be used. This platform chemistry also prompted macroporous as well as nonporous support designs, whereby using chemistry that gives patterning in 2-D on nonporous supports can yield patterning in 3-D on macroporous supports. This allowed for different functions installed to be spatially 'uncoupled/segregated' from one another, for example exterior having a self cleaning shell and a ligand binding interior core. The characterization of the supports at the various stages provided invaluable knowledge and allowed proof of principles of design of SP-MAPs.

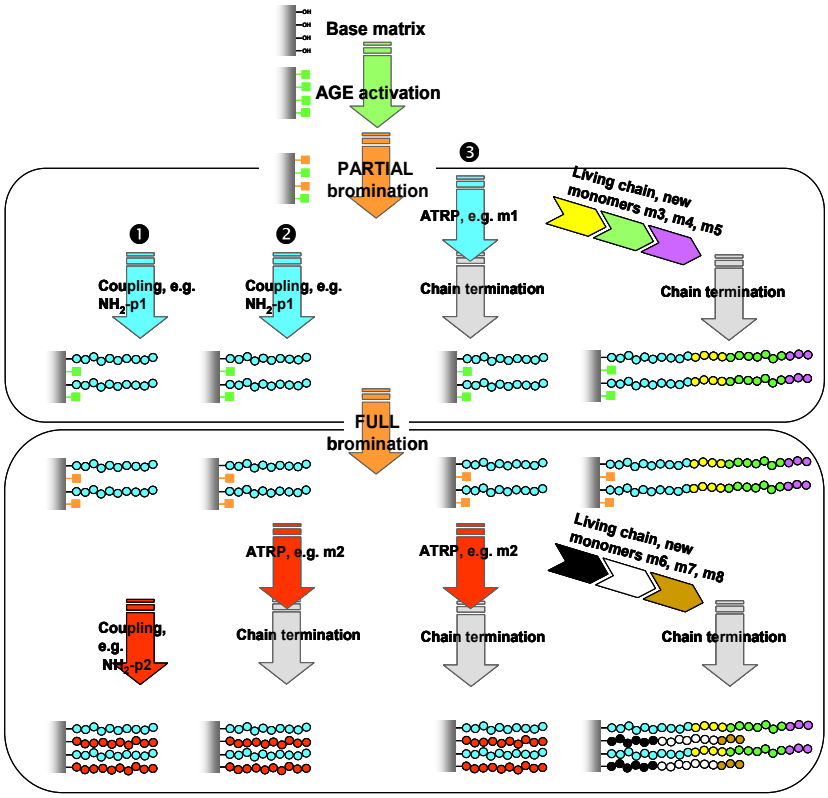

Fig. 1. A Schematic illustration of a generic platform for the assembly of SP-APs. Three general synthetic paths can be taken, i.e. ❶ Sequential 'graft to'→'graft to'; ❷ Sequential 'graft to' → 'graft from'; and ❸ Sequential 'graft from' →'graft from'. Common features of the platform are the use of a two step surface patterning/priming reaction based on activation with allyl glycidyl ether and subsequent partial conversion of the allyl groups to brominated intermediates able to react with incoming nucleophiles or function as an immobilized initiator of atom transfer radical polymerization reactions. In the case of polymers with protecting groups (e.g. ptBA) a deprotection step would be necessary to obtain the functional polymer.

While SMIP-MAPs materials were not created within the timeframe of the project, a new 'inverse emulsion' based approach has been designed to fabricate these materials. Work will continue to achieve this deliverable after the NanobioMag project has ended.

Work Package 2: Smart Magnetic Extraction Phases (SMEP)

Downstream processing is a bottleneck in many industrial processes for the production of recombinant proteins. Aqueous two-phase systems have been considered a protein-friendly and scalable extraction method for many years, but they are not yet widely used for industrial applications. The main limitations are the high costs of phase-forming polymers which are difficult to recycle, long durations of phase separation steps, and the lack of theories to predict protein partitioning. It has been suggested in literature that by introducing magnetic adsorbents into ATPS, some of these limitations could be overcome.

Within WP2, a new process concept for the downstream processing of proteins based on the use of magnetic adsorbents combined with micellar aqueous two-phase systems was developed. A new procedure called SMEP (Smart Magnetic Extraction Phases), where the phase separation is accelerated by using inhomogeneous magnetic fields, was developed and tested. The feasibility of the process was shown by applying it to separate binary protein mixtures of hen egg white lysozyme and ovalbumin.

Figure 2 summarizes the steps which are carried out in one full cycle of the SMEP process. The employed SMEP phases consisted of the non-ionic surfactants and magnetic beads based on polyvinyl alcohol functionalized with polyacrylic acid, giving weak acid cation exchangers (work done in cooperation with WP 1). Protein partitioning was achieved by adsorbing them selectively onto magnetic adsorbents which accumulate in the disperse phase of the system. The magnetic adsorbents adsorb the target protein and pull it out of the 'surfactant-depleted' phase into the 'surfactant-rich' phase.

Nearly all of the phase-forming polymer may be recycled. This polymer recycling may be achieved by using aqueous solutions of non-ionic surfactants, which separate above the so-called cloud point temperature (T_{CP}) into two co-existing phases, one enriched (containing >99% of total surfactant), the other depleted in surfactant (containing < 1% of total surfactant). At low temperatures (20°C), the solution is homogeneous. While the ionic strength is low, lysozyme binds selectively to the adsorbents. At elevated temperature (30°C), an aqueous two-phase system is formed and the adsorbents accumulate in the dispersed phase which sediments quickly when switching on the magnetic coil. After removing the supernatant waste, high-ionic-strength elution buffer is added and the temperature lowered to carry out elution in an homogeneous solution. Then, the temperature is raised again to induce phase separation.

After separating the phases, the target protein is found in the supernatant and recovered from the vessel, while the eluted adsorbents and nearly all of the surfactants remain in the reactor and can be mixed with a new feed solution. In our example, the ATPS formation is controlled via the temperature, protein binding via the ionic strength, and the phase separation rate via the magnetic field which makes the process flexible and easy to operate. In the SMEP process, adsorption and elution of the target protein were carried out in a batch-wise manner. All steps were done in one vessel which was placed in a magnetic coil, such that the magnetic field can be switched on and off.

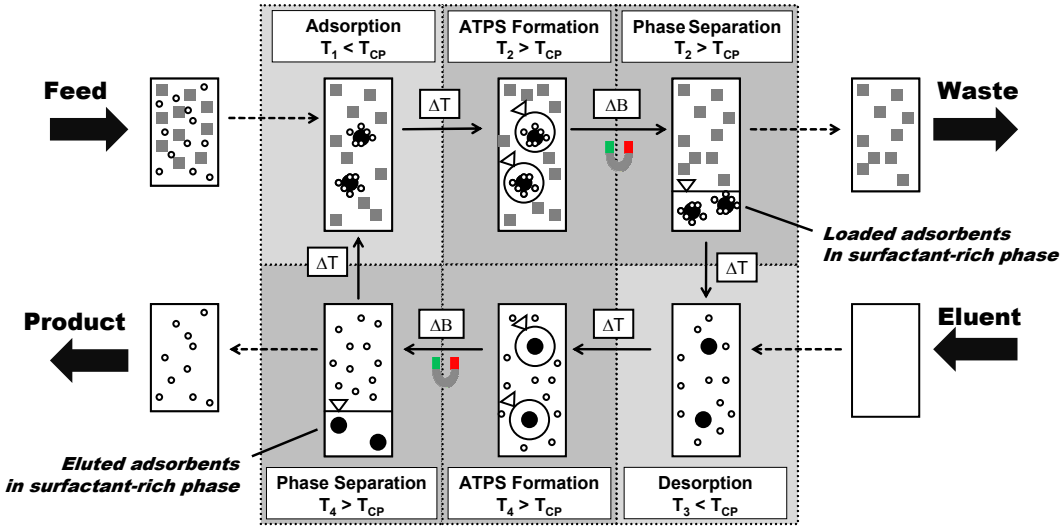

Fig. 2: Process scheme for using SMEP for protein separation

The SMEP ("Smart Magnetic Extraction Phases") were used in laboratory and pilot-scale (200 mL) experiments over three full cycles. The Pilot-scale SMEP-reactor with a liquid volume of 200 mL is shown in Figure 3. Before running pilot-scale experiments, lab-scale studies were conducted regarding liquid-liquid-phase equilibria, protein adsorption & partitioning equilibria, and phase separation kinetics in magnetic fields.

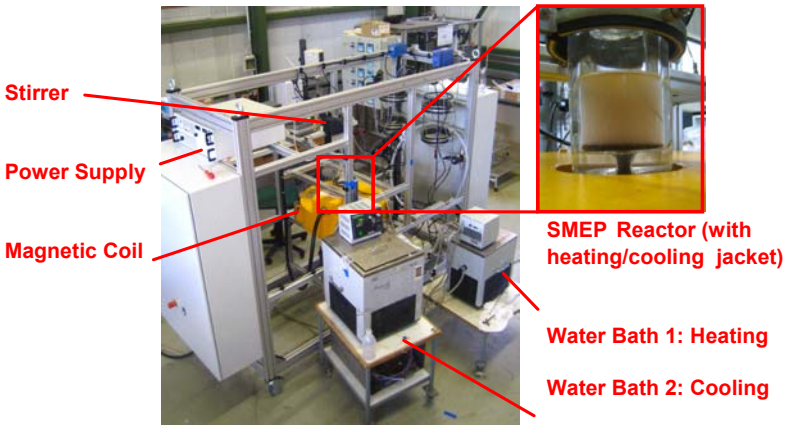

Fig. 3: Pilot-scale SMEP-reactor with a liquid volume of 200 mL. The magnetic adsorbents-containing phase (brown) settles to the bottom

Parallel to the experiments, analytical expressions were derived to predict the liquid-liquid phase separation rate in magnetic fields as well as the protein separation performance for two-component system. Both parameters are used for evaluating the feasibility of the process.

Work Package 3: Magnetic Field Enhanced Centrifugation (MEC)

The focus of WP3 of the NanoBioMag project was bioprocessing especially cultivation, in-situ magnetic separation and analytics using magnetic supports. Biotechnological production processes using genetically modified bacteria have been established as a standard method. By utilizing magnetic carrier particles with specific surface functionalization selective separation of target product out of the gross bio broth allows highly efficient product recovery at a minimum of process steps. Furthermore the fermentation can be enhanced by in-situ magnetic separation of inhibiting products during fermentation. Since there are no currently existing large scale

magnetic separation equipment the transfer into industrial application has been inhibited. In lab-scale units High Gradient Magnetic Fishing (HGMF) is used to separate magnetic carrier particles out of fermentation broth. In HGMF the external magnetic field is distorted by magnetic wires. This results in high field gradients and high magnetic forces to the particles.

An outcome of WP3 was a novel magnetic separator utilizing a rotating magnetic matrix within an external magnetic field. The MEC (Magnetic Field Enhanced Centrifuge), a lab-scale prototype for the large-scale separation of magnetic adsorbent particles for the selective magnetic separation out of fermentation broth and liquid process streams was developed, built and successfully tested in an integrated bio process with in-situ magnetic separation of the product. The combination of the HGMS principle with its high separation efficiency at low particle sizes with continuous centrifugation allows for large scale selective separation. The capacity limitation of classical HGMS filters was avoided by continuous cleaning of the magnetic matrix due to centrifugal forces. Additionally the fermentation process could be intensified by applying in-situ magnetic separation during fermentation to separate proteases, which is documented to inhibit the production. The model system which was investigated was *Bacillus licheniformis*. This is a strain that is used industrially for the production of extracellular hydrolases. The secreted product, largely during nitrogen limitation, was an alkaline serine protease (Subtilisin). The Experimental set-up for the in-situ magnetic separation process is shown in Figure 4.

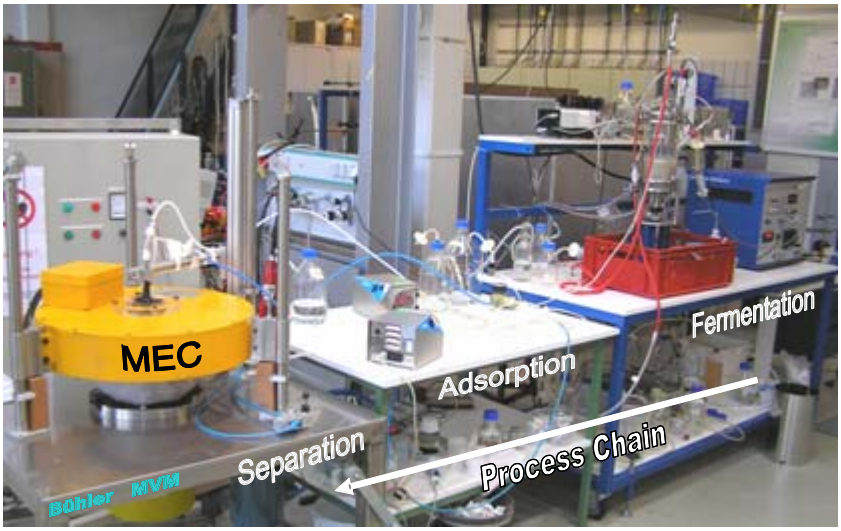

Fig. 4: Experimental set-up for in-situ magnetic separation process (left: MEC, right: fermentation)

The main component of the Magnetic Field Enhanced Centrifuge is a rotating magnetizable wire rod that distorts the external magnetic field generated by an electro magnet. A mixture of bio broth and magnetic particles loaded with target product enters the centrifuge. The magnetic particles are attracted by the rotating magnetic matrix and attach to it. Due to centrifugal forces the particles slide along the matrix wires and detach at the end of the wire to be separated into a collection chamber, from where they can be removed either batchwise (the lab-unit is shown in Figure 4) or continuously (future industrial centrifuge). By adjusting field strength, rpm and differential rpm of magnetic matrix and beaker the new test equipment offers a maximum of control of the separation. The "waste" broth floats over an orifice and is continuously removed into a non-rotating collector ring to be re-fed into cultivation or wasted.

In order to make the bioprocess industrially relevant, the concentration of both biomass and the protease product Subtilisin was increased. The bio dry mass concentration was elevated to

a value of nearly 30 g/l and protease concentration was increased to more than 1g/l in the extracellular liquid. Especially under nitrogen limitation productivity significantly increased. The fed-batch phase, started after a batch growth phase (see Figure 5: Fed-batch phase after batch growth phase).

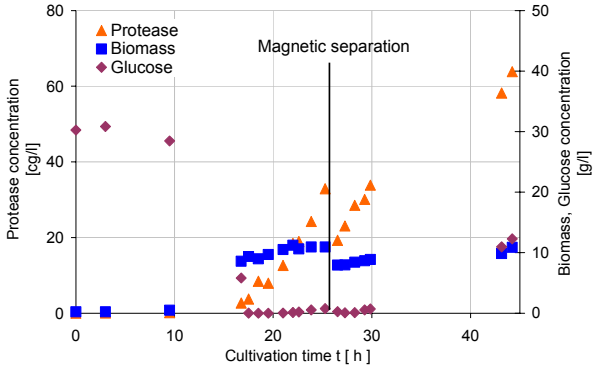

Fig. 5: Cultivation of *Bacillus licheniformis* under three different nitrogen limitations

Separation of commercially available Chemagen magnetic beads (x_{50}=1.5 µm, M_S=43 Am²/kg) out of water was also tested in the MEC and showed a strong influence of magnetic field (as shown in Figure 6). In total about 97 % of the particles could be separated at 0.3 T field strength using a triple layer matrix with 15 wires per layer.

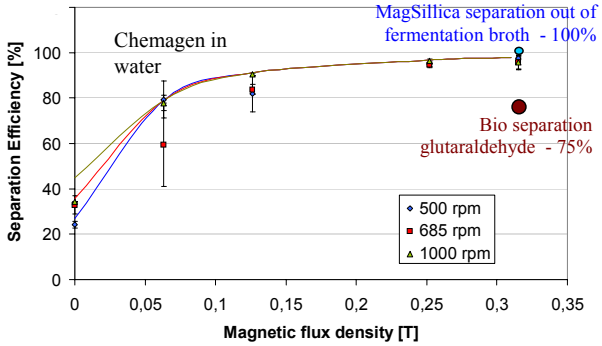

Fig. 6: Magnetbead separation at different rpm

The MEC was also used to separate Subtilisin loaded glutaraldehyd particles (x_{50}=5µm, M_S=20 Am²/kg) out of the broth. The first experiment with a MEC coupled to a bioreactor process showed that about 81% of the particles could be separated from a 20 g/l *B. licheniformis* broth with an external magnetic field of 0.3 T. Additional tests at higher biomass concentration at about 30g/l showed slightly lower separation efficiencies of 75% due to increased viscosity. The separation was performed with a high selectivity, meaning that no bio film was visible inside the collection chamber of the centrifuge. 100% separation can be realised for slightly bigger magnetic particles as shown with Merck MagSillica particles (x_{50}=7µm, M_S=45 Am²/kg). By increasing the number of wires and field strength also weaker or smaller magnetic material like Chemagen beads etc. can be separated with highest efficiency.

Work Package 4: Nano-Biostructure synthesis (NABIS)

Another aspect to the NanoBioMag project with regards to assisted biomaterials processing was to investigate the effects if any, of the use of strong magnetic fields as a new structuring force on biomolecules, with desirable outcomes being biostrucutures with tailored properties. Biomolecules such as Triglycerides, Phosphatidylcholine, DNA and certain proteins can be influenced by strong magnetic fields under very specific conditions. Although these molecules are not magnetic in a classic sense, they do experience a weak form of magnetism, called diamagnetism. This force competes with the thermal fluctuation, kT, which is usually stronger than any effect induced by magnetic fields. However, if molecules are mutually oriented through self-assembly or an external force, the magnetic effect on each molecule adds up, and can eventually overcome the disorienting Brownian motion.

Within the scope of this project an experimental set up was built to test the influence of coupled strong magnetic and flow fields on structure and crystallization behavior of biomolecules. Of particular interest were self-assembly systems. The set up comprises a superconducting magnet with a temperature controlled Searle rheometer geometry in the bore (Figure 7). It allows combining the influences of a magnetic field and a structuring or destabilizing shear force. Information on the system can be gathered over the scattering pattern of a laser beam through the gap and the rheological data.

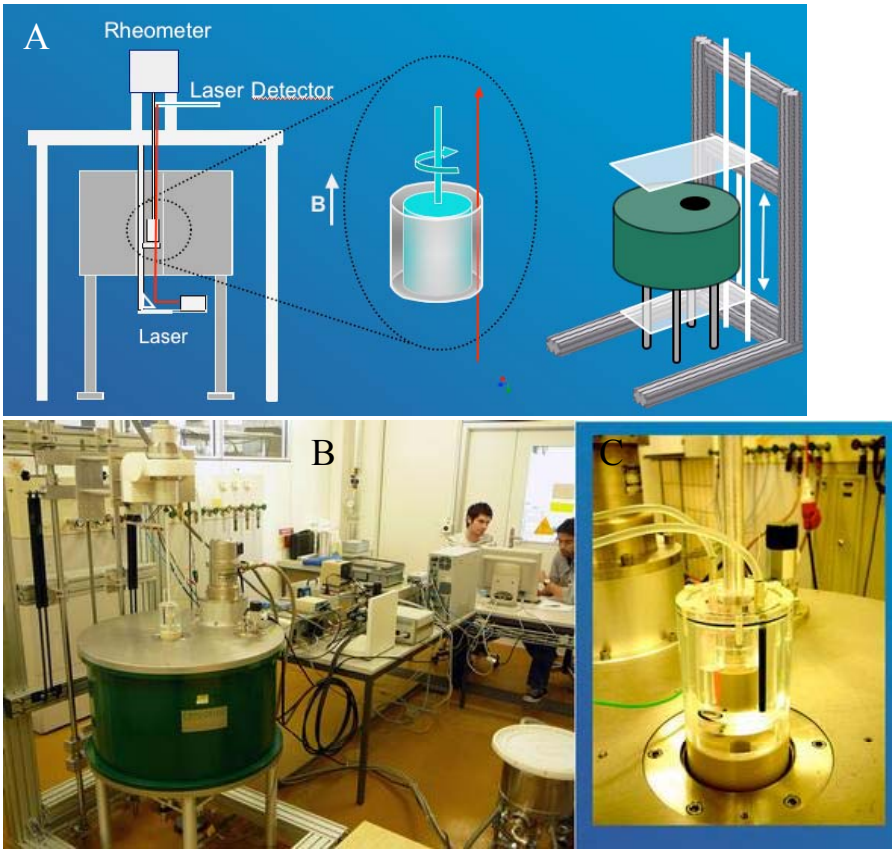

Fig.7 Magnet Rheomoeter Lab (A and B), and close up of the shear cell (C)

The set up was mainly used for two systems: Triglycerides and modified phospholipid vesicles. A food grade triglyceride system was used which exhibits six kinds of crystal morphologies. The melt was crystallized under different shear, temperature and magnetic field conditions. No magnetic field effect was found for crystallization under shear. Under static conditions, however, the recrystallization from one crystal morphology to another was

accelerated by a strong magnetic field. More experiments are on the way to characterize this effect.

Additionally, as part of this work package, continuing research aims at creating smart nanocapsules which release their content under magnetic field. With an eye on targeted drug delivery, this project is situated at the interface between food, life-sciences, and material science. A method was developed to produce phospholipids-based vesicles with a modified surface to make them responsive to magnetic fields.

Work Package 5: Construction using new smart supports (CUSS)

Similarly to WP4, the aim of Work Package 5 was to investigate the effects of strong magnetic fields on molecular structures. The exciting outcome form this work that for the first time it was conclusively shown that a 'magnetic handle' offered by the use of magnetic particles can be exploited for the scaleable modification of biological complexes. This work is likely to have far reaching implications for the assembly line like modification and construction of nano-sized biological entities in the future.

To examine the main aim of the work, the focus was the construction and study at small scale of a generic, scaleable, integrated continuous processing system that employs magnetic particles to control the extent of modification of proteins (as shown schematically in Figures 8 and 9). The concept was proven by the attachment of polyethylene glycol groups (PEGylation) to the enzyme trypsin and subsequent analysis by a range of methods including mass spectrometry. The apparatus and methods developed involved reversible binding of the enzyme to a ligand (which protected the active site of the enzyme) on the magnetic particles in a specially developed continuous pipe reactor. This was followed by a second pipe reactor in which activated polyethylene glycol (PEG) was added. Subsequently the magnetic particles with the PEGylated protein attached were captured in a magnetic separator and eluted, which not only permitted control of the reaction, but also purification of the complex from excess PEG. By varying process parameters such as time, concentrations, PEG molecular weight and temperature, the extent and type of PEGylation was controlled in one integrated process.

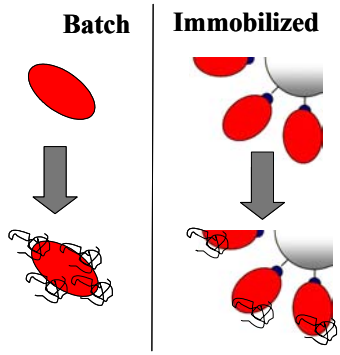

Fig. 8 Concept of magnetic support control of modification

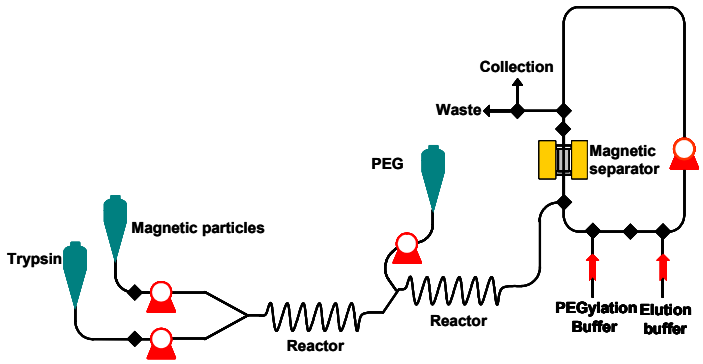

Fig. 9 Process schematic for magnetic support mediated control of protein modification

This workpackage contained an extremely high risk, blue skies activity, namely the construction of biological complexes using magnetic particles to be examined in a variation of the system described above. At the writing of this report that work has only been partially completed. The reason for only partially achieving this goal has been the choice of ambitious model systems, which have proven to be difficult to put into practice, not a deficiency in the scientific concept which these model systems are to prove. The final model system chosen was the construction of an ATPsynthase nano-motor (shown in Figure 10). The protein components of the motor with appropriate affinity tags for reversible attachment to magnetic

supports have been cloned, expressed and purified. With the aid of magnetic particles, assembly of an active motor has been attempted, but is yet to be demonstrated. Work is continuing on this and successful demonstration of the objective is expected after the project finishes.

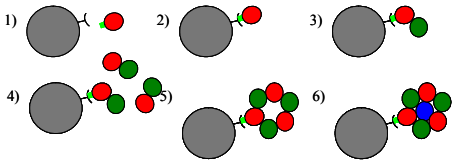

Fig. 10: Concept of ATPsynthase assembly in a magnetic particle based system

In addition the process concept was extended to successfully demonstrate for the first time the stabilization of fermentation broths by the direct use of magnetic particles during cultivation of the bacterium *Bacillus*. This is schematically illustrated in Figure 11.

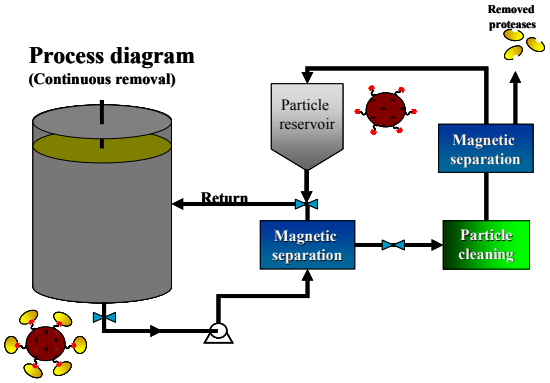

Fig. 11: Schematic for use of magnetic particles for stabilisation of fermentation broths.

The execution of the NanoBioMag Project involved the large collaborative effort between work packages and project partners resulting in favourable outcomes such as training of PhD students, postdoctoral fellows and employment of research staff. The generated knowledge coming out of this project has been presented at numerous technical conferences and seminars. Publication in peer reviewed journal articles and conference proceedings has been the main avenue for the dissemination of the generated knowledge.

The NanoBioMag project (STREP) program was concluded in spring 2008.