Lecture Notes in Bioengineering

Hermann Nirschl
Karsten Keller *Editors*

Upscaling of Bio-Nano-Processes

Selective Bioseparation by Magnetic Particles

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Hermann Nirschl · Karsten Keller
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Editors
Hermann Nirschl
Institute of Mechanical Process
Engineering and Mechanics
Karlsruhe Institute of Technology
Karlsruhe
Germany

Karsten Keller
DuPont
Wilmington
DE
USA

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Preface

Despite ongoing progress in nano- and biomaterial sciences, large-scale bioprocessing of nanoparticles remains a great challenge, especially because of the difficulties in removing unwanted elements during processing in food, pharmaceutical, and feed industry at production level. While conventional processing requires a multitude of steps, a new approach was investigated based on the selective separation of proteins by adsorption to functionalized particles and selective separation by magnetic forces.

This book originated in a project called MagPro^{2}LIFE, which was funded by the EU in the seventh Framework Program and conducted from July 2009 to June 2013, and united many experts in the field to create a platform technology. It based on the results of the preceding project NanoBioMag, which laid the basics for the upscale of the process. The aim of the book is to collect the knowledge gathered during the project for use or for further research in the area. It provides a summary of the current state and the latest developments and achievements in the project. Topics include the synthesis of particles and their functionalization by different methods, the development of magnetic separation technology, and the application for In-Situ separation and downstream processing.

Karlsruhe, April 2014 Hermann Nirschl

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We acknowledge the EU for funding our research in the seventh framework program in the project MagPro^{2}LIFE.

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About the Editors

Prof. Dr.-Ing. Hermann Nirschl is head of chair of process machinery at the Institute of Mechanical Process Engineering and Mechanics at the Karlsruhe Institute of Technology, focusing on process machinery in solid liquid separation, blending, nanoscaled particle structures and numerical simulation.

Dr. Karsten Keller is an Engineering Fellow of the Discovery Research group at Solae LLC (JV of DuPont) and has over 20 years of process engineering experience. He is developing novel processes for protein and new product opportunities from the lab-scale to commercial production and is the author of over 70 publications on subjects of separation, modeling, particle technology, biotechnology and nanotechnology. He is the inventor of 8 patents worldwide.

Contributors

Alexander Scholz Institute of Process Engineering in Life Sciences, Karlsruhe Institute of Technology, Karlsruhe, Germany

Rodica Alexandrescu National Institute for Laser, Plasma and Radiation Physics, Bucharest, Romania

Victor Alvan Amasifuen Institute of Mechanical Process Engineering and Mechanics (MVM), Karlsruhe Institute of Technology, Karlsruhe, Germany

Johann Bauer Merck KGaA, Darmstadt, Germany

Martin Cerff Institute of Process Engineering in Life Sciences, Karlsruhe Institute of Technology, Karlsruhe, Germany

Izabell Craciunescu National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania

Niels Dalgaard Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany; DuPont Nutritional Biosciences Aps, Brabrand, Denmark

Helen Engel LinkedIn Corporation, Mountain View, CA, USA

Johannes Feller Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany

Matthias Franzreb Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany

Gunnar Grim Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany

Timothy John Hobley Technical University of Denmark, Lyngby, Denmark

Karl Holschuh Merck KGaA, Darmstadt, Germany

Sebastian Holzapfel LinkedIn Corporation, Mountain View, CA, USA

Karsten Keller DuPont, Wilmington, DE 19980, USA

Valéry Tchanque Kemtchou Bergakademie Freiberg, Institut für Mechanische Verfahrenstechnik und Aufbereitungstechnik, Freiberg, Germany

Johannes Lindner Institute of Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany

Katharina Menzel Institute of Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany

Ion Morjan National Institute for Laser, Plasma and Radiation Physics, Bucharest, Romania

Alexandrina Nan National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania

Christian Fiil Nielsen Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, Karlsruhe, Germany; DuPont Nutritional Biosciences Aps, Brabrand, Denmark

Hermann Nirschl Institute of Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, 76131 Karlsruhe, Germany

Anja Paulus Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany

Urs Alexander Peuker Bergakademie Freiberg, Institut für Mechanische Verfahrenstechnik und Aufbereitungstechnik, Freiberg, Germany

Clemens Posten Institute of Process Engineering in Life Sciences, Karlsruhe Institute of Technology, Karlsruhe, Germany

Susanne Rasmussen DuPont Nutritional Biosciences Aps, Brabrand, Denmark

Elisabeth Rondeau LinkedIn Corporation, Mountain View, CA, USA

Maria Schäfer Bergakademie Freiberg, Institut für Mechanische Verfahrenstechnik und Aufbereitungstechnik, Freiberg, Germany

Vlad-Mircea Socoliuc Romanian Academy-Timisoara Branch, Center for Fundamental and Advanced Technical Research, Timisoara, Romania

Wickie Sondergaard Romanian Academy-Timisoara Branch, Center for Fundamental and Advanced Technical Research, Timisoara, Romania

Rodica Turcu National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania

Ladislau Vékás Romanian Academy-Timisoara Branch, Center for Fundamental and Advanced Technical Research, Timisoara, Romania

Erich J. Windhab LinkedIn Corporation, Mountain View, CA, USA

Abbreviations

AEX	Anion Exchanger
AMTPS	Aqueous Micellar Two-Phase Systems
ATPS	Aqueous Two-Phase Separation
ATRP	Atom Transfer Radical Polymerization
BBI	Bowman-Birk-Inhibitor
BET	Brunauer-Emmet-Teller
CEX	Cation EXchanger
CFD	Computational Fluid Dynamics
CI	Chymotrypsin Inhibition
CIP	Cleaning In Place
CME	Continuous Magnetic Extraction
DLS	Dynamic Light Scattering
EBA	Expanded Bed Adsorption
FEM	Finite Element Modeling
HGMS	High Gradient Magnetic Separation
HGMF	High Gradient Magnetic Filtration
ISMS	In-Situ Magnetic Separation
ISPR	In-Situ Product Removal
LCA	Life Cycle Assessment
LCST	Lower Critical Solution Temperature
LD	Laser Diffraction
MANACO	Magnetic Nano Composites
MANDHALA	Magnetic Arrangement for Novel Discrete Halbach Layout
MEC	Magnetically Enhanced Centrifugation
MEP	Magnetic Extraction Phases
MF	Magnetic Fluid
MNP	Magnetic Nano Particle
NAMPEX	Nano Membrane Pore Extruder
NanoMags	Magnetic Nanoparticles
NMR	Nuclear Magnetic Resonance
NPC	Nanoparticles Clusters

NTU	Nephelometric Turbidity Unit
PBA	Packed Bed Adsorption
ROMER	Rotating Membrane Reactor
SANS	Small Angle Neutron Scattering
SDS-PAGE	Sodium Dodecyl Sulfate Poly Acrylamid-Gel Electrophoresis
SEM	Scanning Electron Microscopy
SIP	Sterilization In Place
SMEP	Smart Magnetic Extraction Phases
SOP	Standard Operating Procedure
SLS	Static Light Scattering
SolPro	Solution Process
TEM	Transmission Electron Micrograph
UF	Ultrafiltration
XPS	X-ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction

Keywords

Selective protein separation · Magnetic particles · Scaling up processes · Superparamagnetic nanoparticles · High gradient magnetic separation · Magnetically enhanced centrifugation · Laser pyrolysis · Magnetic microgels · Vesicles by rotating membrane pore extrusion · Spray drying · Halbach magnet based HGMS · Continuous magnetic extraction · In-Situ magnetic separation

Chapter 1
Introduction

Hermann Nirschl

Biologically-derived materials represent one of the most important sources of new technology food and pharmaceutical products due to their precisely controlled structure, biofunctional properties and potential for inexpensive and sustainable production. Recent advances in a variety of areas of biotechnology, from systems biology to bioreactor technology, have made large-scale production of sophisticated new biomolecular materials possible. However, the costs of producing these exciting new materials can be prohibitive due to separation processing, which typically constitute 80 % of the total cost of production. Bioseparation technology used in industry today is based on principles first discovered over 70 years ago and improvements are needed at all stages of processing, i.e. from pre-treatment of raw materials prior to fermentation, the fermentative product itself, and during subsequent purification and modification to yield the final product.

Functional magnetic (nano)particle composites have the potential to enhance the physical and chemical properties of bioseparation processes, i.e. (nano)particles have extremely high surface areas, rapid binding kinetics and unique physical and chemical properties. However, handling them in complex and demanding bioprocesses where profits can be marginal for 'low-value, high-throughput' products is a great challenge. Such particles with magnetic properties might enable significant improvements in productivity, economic feasibility, sustainability and product quality and its control. However, implementation of functional magnetic particles as adsorbents in the bioprocessing industry requires the synergistic interplay of a host of components. The two major barriers to implementing magnetic nanoparticles and composites are the safe and effective large-scale manufacturing of appropriately functionalised superparamagnetic particles, and the lack of large-scale process technology to separate these particles from

H. Nirschl (✉)
Institute of Mechanical Process Engineering and Mechanics, Karlsruhe Institute of Technology, 76131 Karlsruhe, Germany
e-mail: hermann.nirschl@kit.edu

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the production streams. For this technology to be accepted in industry, it must be safe, capable of scaling to high production rates, reliable, robust and inexpensive.

The goal of the EU funded MagPro^{2}Life project was to address these barriers and demonstrate the use of functional magnetic (nano)particle separation at pilot-scale on biotech pilot lines. The potential impact of selective magnetic separation is significant but adoption by the biotechnology industry hinges on successful initial demonstrations of complete technologies. Therefore, the main objective of the MagPro^{2}Life project is to scale-up innovative nanotechnology-based processes to pilot-line-scale and demonstrate the feasibility of this technology.

The MagPro^{2}Life project was based on a series of focused objectives:

- development of scalable magnetic particle production technologies;
- development of scalable surface functionalization technologies;
- develop large-scale processing technologies that can be used with the adsorbents produced;
- and integrate the previous three objectives to demonstrate that this technology can be implemented for safe, industrial scale bioseparation.

The project produced several magnetic nanoparticle separation technologies that can be applied to bioseparation in the industry, i.e. low-value, low-concentration products to high-value, high-concentration products. This was demonstrated in pilot-scale separation of a set of model biomolecular products.

MagPro^{2}Life was motivated by the fact that magnetic process technology can significantly improve the value chain in industrial production for emerging biotechnological and pharmaceutical markets. Major European companies and SMEs are expected to benefit from pilot line demonstrations of the new magnetic processes.

1.1 State-of-the-Art

The most commonly used industrial methods of biomolecular separation are based on filtration, centrifugation and fixed-bed chromatography. Filtration and centrifugation are most often used for low-resolution removal of solids, frequently combined with precipitation. Chromatography is a proven separation technology that is highly versatile. Biomolecules are purified using reversed phase chromatography utilising hydrophobic interactions. Ion exchange chromatography uses ionic interaction between the support surface and charged groups of the peptide. Gel permeation chromatography separates molecules primarily on the basis of size exclusion. However, fixed-bed chromatography has significant drawbacks when implemented on an industrial scale, i.e. discontinuous operation, intolerance to suspended solids and expensive capital and operating costs. For example in the case of ion exchange chromatography concentrated acids and brines are required

to elute the product and regenerate the separation media. Elution solutions in biotechnology also frequently contain substances to increase elution efficiency that have to be removed by additional separation steps. This results in the need for large volumes of sterile water from which large amounts of industrial waste is generated.

Thus, current bioseparation technologies have low process yields, high costs and require large numbers of discrete unit operations in series (e.g. centrifugation, filtration, ultrafiltration, ultracentrifugation, precipitation, adsorption chromatography as well as several polishing operations). Selective magnetic separation can be considered a standard tool in analytics due to its easy and efficient handling. An increasing number of commercially available isolation and purification kits enable for a wide range of applications, e.g. DNA separation and diagnostics. High levels of purification can be obtained from highly complex feed streams like human or animal blood in a one-step separation. However, the novel technology has not been transferred into industrial scale production of bioproducts yet.

Magnetically enhanced biotechnologies promise to improve the yield and reduce costs of bioprocessing. Research to explore the possibility of implementing magnetic separation for large-scale industrial bioseparation has been supported by the EU and a number of national funding agencies. Several magnetically assisted processes employing new techniques were developed and tested recently in laboratory scale. This includes, for example high-gradient magnetic fishing (HGMF) (Hubbuch and Thomas 2002), smart magnetic extraction phases (SMEPs) (Becker et al. 2009), magnetic field enhanced centrifugation (MEC) (Lindner et al. 2013) and magnetic field enhanced cake filtration (Eichholz et al. 2011). Further, the industrial feasibility of HGMF has also been demonstrated conclusively in a comparative study by the expanded bed adsorption chromatography (EBA) (Hubbuch et al. 2001). HGMF outperformed EBA by at least a factor of 4 based on an ECO-99 indicator regarding resources, ecosystem quality, and human health.

On the particle production side, synthesis methods have been established and optimised to deliver robust and reusable magnetic adsorbents (Franzreb et al. 2006) possessing targeted selectivity and high-target binding capacity. Furthermore, the use of magnetic adsorbents for in situ product recovery (Ottow et al. 2007), intensification of fermentative production and control of industrially relevant enzymatic modification reactions have been developed and large-scale magnetic particle production processes have been screened.

The low process yields and high costs, observed in the manufacture of many of today's approved biopharmaceuticals, are direct consequences of the conventional practice of using large numbers of discrete unit operations placed one after the other, with each performing just a single or limited number of function(s). Manufacturers of biotechnological products now face unprecedented pressure to reduce spending, make their manufacturing processes more cost effective, and bring new products to market more rapidly than ever before. Such pressures, therefore, encourage a departure from traditional thinking and separation methods, and have fuelled interest in the evaluation of newer 'multifunctional'

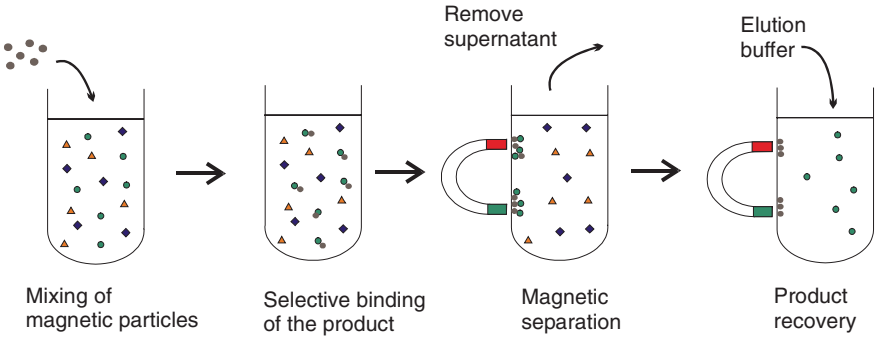

Fig. 1.1 General principle of magnetic separation in millilitre scale

technologies, which have hitherto been considered too avant-garde for adoption within the necessarily conservative biopharmaceutical sector. The implementation of such '*bioprocess intensification*' techniques signifies a reduction in the number of processing steps employed in manufacturing, and brings with it promises of improved yields and throughputs, reductions in plant size and waste generation, and lower manufacturing costs. In this context, the generation of robust adsorptive methods capable of *selectively 'fishing' soluble target molecules directly out of crude unclarifed bioprocess feedstocks*, i.e. those typically encountered during the early stages of a traditional downstream process scheme, has received considerable attention in recent years. Notable examples of such techniques include affinity aqueous two-phase extraction and expanded bed adsorption.

Commercial production of functionalized magnetic supports for use in biotech applications exist for use in batch binding mode with subsequent magnetic collection, typically achieved with simple permanent magnet racks. Useful magnetic support materials can be produced by: *encapsulation, infiltration* or *coating* of magnetic material into natural or synthetic polymers. A wide variety of magnetic particles are available for clinical and small-scale analytical applications. However, there has been no breakthrough to transform the production of the magnetic supports into large-scale with the goal to use them in downstream processing.

The basic principle of the millilitre-scale application of magnetic particle-based separations in biotechnology is illustrated in Fig. 1.1. A pure and concentrated product can be obtained at the end of the procedure involving an adsorption and desorption step. However, only microgram to milligram amounts of product is to be obtained by this technique.

This was an attempt to transfer the above-mentioned analytical technique into production technologies for high-value bioproducts (e.g. pharmaceutical proteins, DNA for gene therapy, etc.). Using a lab-scale 'High Gradient Magnetic Separator' (HGMS), it was demonstrated in the past the feasibility of the combination of

magnetic nanoparticle-based supports and magnet technology for the purification of a bioproduct (e.g. trypsin from a crude porcine pancreatin feedstock) is possible.

Regarding 'Magnetic Nanofluid Extraction' which is based on the principle of 'Magnetic aqueous two-phase separation', the state-of-the-art is defined by the work of two research groups located in Lund, Sweden and Nagoya, Japan. The group in Lund investigated the speed up of the phase separation in 'Aqueous Two-Phase Separation' (ATPS) when an inert magnetic fluid or magnetic particles were incorporated into one of the phases (Ottow et al. 2007). In these articles, the authors report of a **reduction of the separation time by a factor of 35–70** and sometimes in the case of high-viscosity phases by a factor of more than 100. The only article about the use of functionalised magnetic particles in combination with ATPS is by the group in Nagoya (Suzuki et al. 1995). Although the experiments were conducted only in small-scale batch mode, the achieved **improvement of the apparent separation factor was up to 35-fold**.

1.2 New Developments

In MagPro^{2}Life, the goal was to advance and scale-up novel particle and process technologies. A key objective was to support research and development on highly promising new magnetic nanoparticles composites that have unique physical and chemical properties while enabling scaling-up of the production of the highly promising new particles. The research and development projects allowed to produce innovative adsorbants with functional properties that will generate new modes of processing or overcome existing bottlenecks. The scientific and technical challenges of scaling-up particle production were estimated to be high—the price of magnetic particle adsorbents, when the project started in 2009, was a serious limitation in implementing magnetic enable bioprocess technologies in industry.

The implementation of this technology in industry with magnetically enabled bioprocesses has up to now not been established. MagPro^{2}Life established a set of advanced process technologies for magnetically enhanced bioprocessing through the production pilot plants. Feasibility studies were conducted on a set of model products that allow us to define the performance of these new technologies for specific markets. That is the production pilot lines demonstrate the industrial feasibility of the novel processing technology for fermentation and downstream processing. Through fermentation, intensification by in situ magnetic separation of target products or inhibiting products, production rates will be increased. In combination with smart magnetic enzyme catalysts and continuous selective magnetic separation, a production rate increase of 10–20 % was reached in lab-scale. In pilot-scale, downstream processing smart magnetic supports enabled efficient adsorption of target product from the crude fermentation broth. Following magnetic separation of the adsorbent particles recovered the target product and the magnetic bead for recycling after elution.

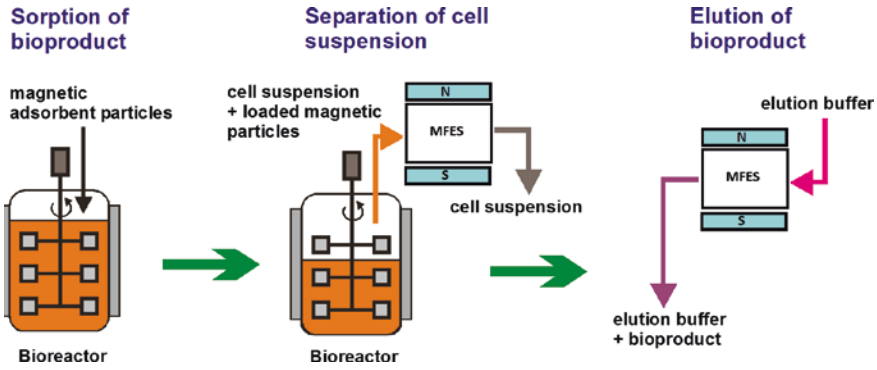

Fig. 1.2 Basic steps necessary to purify a bioproduct by magnetic separation directly from a crude fermentation broth

With the Magnetic Field Enhanced Centrifuge, separation efficiencies of more than 99 % have been reached for magnetic adsorbent particles in the size range of 1 μm and below. Theoretical analysis of the macroscopic and microscopic aspects of these processes using numerical tools made it possible to optimise and predict the performance of this process. MagPro^{2}Life introduced the possibility of having available several versatile separation and extraction techniques that require easily controllable external variables to change properties that are more ideal for selective separation.

One of the primary objectives of the project was to bring together lab-scale magnetic purification techniques already proven feasible in numerous analytical benchtop applications (protein analytics, etc.) and industrial magnetic separation technology being a state-of-the-art technology for some decades in mineral ore processing (High Gradient Magnetic Separation, etc.). This is an attempt to apply the above-mentioned analytical technique for the production of high-value bioproducts (e.g. pharmaceutical proteins, DNA for gene therapy, etc.). A possibility to transfer magnetic small-scale lab techniques into technologies for the industrial production of bioproducts is illustrated in Fig. 1.2. After the mixing of magnetic nanostructure-based adsorbent particles, the target product adsorbs selectively on the particle surface. The magnetic particles are selectively separated by a magnetic separation process and after elution the product is available in a pure and concentrated form. In theory, no prior clarification process is necessary like in conventional chromatographic techniques.

The objective of the project was to even transpose the above-mentioned cyclic process into a fully continuously operating process. Novel continuous magnetic separation and structuring processes were developed. The technologies developed relying on the utilisation of magnetic sub-micron sized particles that are themselves composed of magnetic nanoparticles. Moreover, the project was considered to have an enormous wide range of potential existing and new products as illustrated in Fig. 1.3. The surface of the magnetic particles can be functionalized with a broad range of ligands. Those ligands allow the separation of specific molecules out of a variety of bio, feed and food product sources.

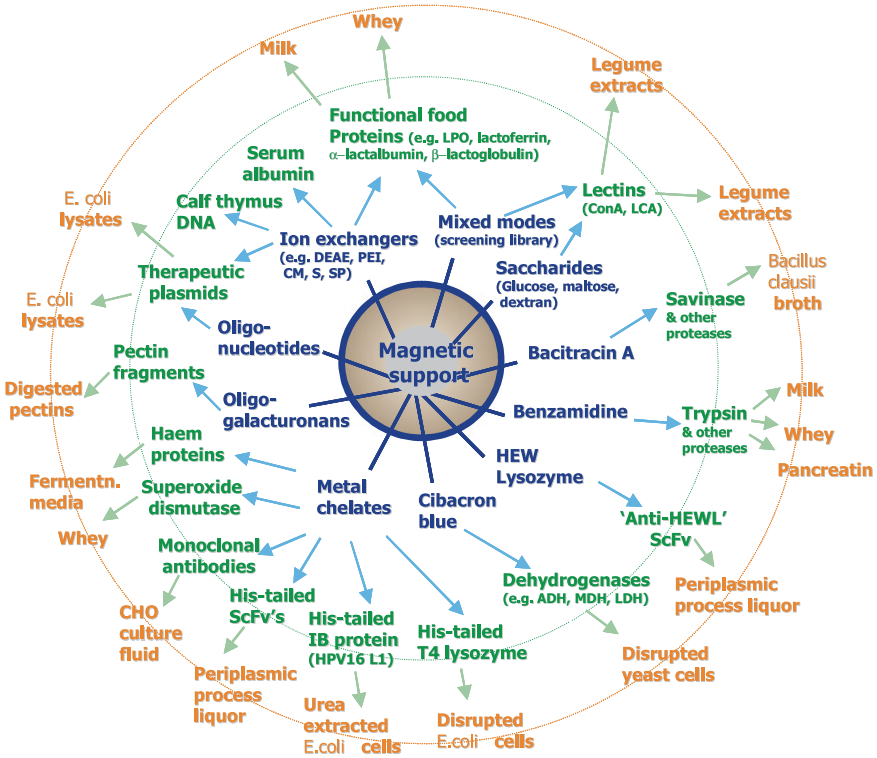

Fig. 1.3 Potential products and product sources for magnetic nanoparticle-based supports (Suzuki et al. 1995)

1.3 Overview

The link to market needs was represented by a preliminary product selection of natural soy-based nutraceuticals/pharmaceuticals such as Bowman-Birk Inhibitor (BBI), a protease inhibitor for MS treatment, and Lunasin which is reported to have anticarcinogenic properties as well as recombinant proteins and nucleic acids produced by microbial fermentation. The main thrust of the project was to develop: advanced and robust magnetic and structured or assembled nanoparticles and composites, industrial large-scale production and process technologies and then link them together to demonstrate safe industrial scale implementation.

The goals of the project were achieved through contributions from 16 partners. The following chart shows the Organisation (Fig. 1.4).

The main S&T objective was therefore to build on knowledge gained through preliminary work so as to produce constituent technologies for the large-scale production of functionalized magnetic particles and pilot-line-scale equipment for the novel processes. In broad terms, the project delivered complete and flexible

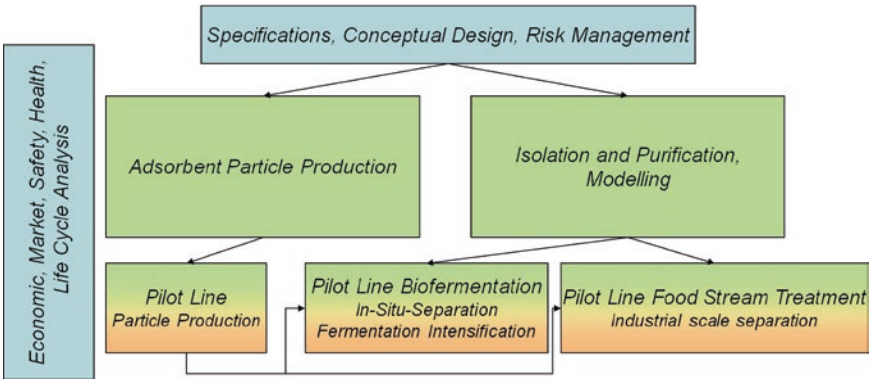

Fig. 1.4 Project organisation

modular manufacturing lines, which might be deployed to produce innovative target products, with applicability and performance sustainability. Both, major companies and European SMEs (in particular, suppliers of machinery, support materials and other consumables) were involved in pilot line demonstrations of the new magnetic processes.

By combining certain key advances in nanotechnology with others in biotechnology, magnetism and materials science, it was aimed to develop a robust and efficient set of smart (i.e. self-adapting) multifunctional and/or biospecific sorbents and macromolecular assembly processes. The planned research activities addressed: fabrication of bespoke magnetic nano- and microparticle assemblies; their functionalisation using advanced 'self-adapting' polymer materials combined with new highly specific biomolecular recognition systems and evaluation of produced adsorbents.

1.3.1 Particle Fabrication and Functionalisation

The consortia followed different approaches for carrier particle development to provide a maximum of flexibility in industrial take-up. The production also incorporates the equipping of the carriers with product-appropriate functionalisations and established Affinity Ligands. In general, two particle production pathways have been investigated. Depending on the target product value and amount to be purified a wet or dry processing can be used. Streamlined and economic manufacturing routes to magnetic nanocomposites based on magnetic nanofluids and synthetic monomers as starting materials have been developed. Commercially available intermediate products have been tested for their applicability in these processes to further reduce production costs. The following adsorbent particles have been produced in large-scale and used.

SolPro Solution Process for pilot-scale particle production is using sonochemical precipitation (magnetite), miniemulsion polymerisation (sorbent) and composite synthesis by spray drying (magnetic bead)

NanoMags Magnetic Nanoparticles are produced by Laser Pyrolysis and precipitation. A functional polymer coating is to provide binding sites

MagPrep Continuous synthesis of magnetic particles in suspension

MANACO Nanoparticle Membrane Pore Extrusion provides smart magnetic vesicles for extraction/separation and drug delivery. Rotating Membrane Extrusion (ROMER) upscales existing vesicle manufacturing equipment

Additionally to established ligands, new approaches for functionalising base support materials were followed after. Magnetic particles can in general be assembled with any ligands already known from chromatography (i.e. affinity, pseudo affinity, ion exchange, mixed mode, hydrophobic, etc.).

1.3.2 Isolation and Purification

The main goal was to design large-scale equipment to extract, classify and separate valuable products in innovative sorption processes based on smart, self-adapting micro-sorbents. To successfully compete with classical production processes, it is crucial to provide highly efficient process equipment especially regarding the recovery of the magnetic adsorbent particles. Ideally in case of a 100 % recovery, the actual costs generated by particle production might play a minor role in the total costs. MagPro^{2}Life therefore dedicated quite some resources into separation knowledge development and process equipment development:

CME Continuous Magnetic Extraction for the separation of target product out of crude bio-feedstocks.

MEC Magnetic Field Enhanced Centrifugation is a solid–liquid separation process using high-gradient magnetic fields to capture the particles on magnetic wires and discharge them continuously by centrifugal forces. Continuous discharge was realised by a decanter design.

1.3.3 Pilot Lines

All of the aforementioned sub-units demonstrate to be the tools of a product separation toolbox. Pilot lines have been realised by combination of the most appropriate tools from this toolbox. Depending on the target product in terms of tonnage, selling price, purity, etc. different carrier particles, different functionalisations and separations tools are needed. By combining afore introduced sub-units, the pilot lines are realised, wherein the sub-unit should be easily interchangeable to promote modular set up. The following pilot lines were set up:

1.3.3.1 Protein Production Stream

Several different types of consortia produced adsorbent particles are tested for their feasibility in industrial processing of soy feed streams. Products to be separated and purified are BBI and Lunasin. Different separation devices like MEC, Halbach magnet based HGMS and CME were tested. Model protein is the Bowman-Birk Inhibitor. It is a soybean-derived protease inhibitor that has anti-inflammatory and anticarcinogenic activities. In clinical studies, it has been shown that the ability to inhibit trypsin and chymotrypsin activities enable BBI as a treatment for multiple sclerosis (MS) and several forms of cancer. It might be a product as pharmaceutical or food additive.

1.3.3.2 Fermentation Intensification

Bioprocess intensification using magnetic particles is an approach developed in previous studies where inhibiting products are removed continuously during fermentation process to increase production rate. Magnetic separation using functionalised magnetic adsorbent particles during cultivation could be an alternative strategy to remove extracellular products in situ in an external loop. Removing, for example inhibiting components during the fermentation can increase yield and productivity of the biosystem. One part of the project has been the investigation of the interactions between the microorganism catalyst, the magnetic adsorbent particles and the separation unit. In addition to inhibiting species, unwanted by-products and/or valuable bioproduct can be magnetically separated and recovered. This pilot line combines E. coli fermentation with simultaneous affinity separation of inhibiting products. The adsorbent particles are produced by the consortia itself in pilot-scale production facilities. Magnetic separation of the adsorbent particles was performed by classic HGMS. As mentioned above, a portfolio of different demonstration products has been defined so far. Each of the products represents at least one of the market sections—food, feed and pharma—that are targeted on.

Model protein is phytase. This is an enzyme that can break down the undigestible phytic acid (phytate) part found in grains and oil seeds and thus release digestible phosphorus, calcium and other nutrients. Phytase is used as an animal feed supplement—often in poultry and swine—to enhance the nutritive value of plant material by liberation of inorganic phosphate from phytic acid (myo-inositol hexakisphosphate) and, thereby, to reduce environmental phosphorus pollution.

1.3.3.3 Economic, Market, Safety, Health and Life Cycle Analysis

In 2005, it has been estimated that the European Biotechnology Market could be worth over 100 billion EUR with a global market potential of above 2000 billion EUR in 2010 (EU 2004). Within the next decade, the market shares of relevant products are expected to more than double, thus the benefits of the proposed process