Nanocapsules with functionalized surfaces and walls

Nanocapsules

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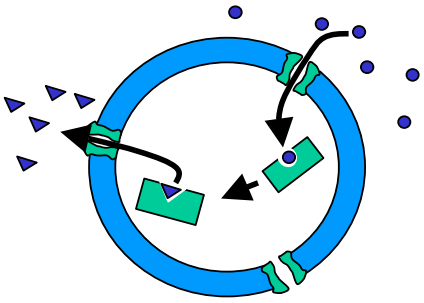

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Part A: Research Results

A.1: Scientific Highlights

CNRS (Toulouse)

The main task of the CNRS team in Toulouse was to provide the consortium with channel forming proteins called porins. This class of proteins are optimised by nature to control the permeability in bacteria for selected molecules across the outer membrane and it was tempting to use them to control the permeability in artificial container. During the first two years we worked out the basic expertise in molecular biology for porin purification and mutagenesis. The main focus was to optimise our planar lipid bilayer set-up, which allows us now to characterise substrate translocation across membrane channels on a single molecular level. For example, the passage of single maltodextrin molecules through Maltoporin. We could quantify the asymmetric translocation on a single molecular level. We determined the energy barriers for molecular transport and the asymmetry in flux from the above experimental input. A similar study was done with antibiotic transport through OmpF. For the latter experiment we performed in addition computer simulation on the antibiotic pathway. The outcome of this type of studies allows the quantification substrate translocation for future engineered channels or may be useful for rational design of new antibiotics. Acetylcholinesterase (AchE) is the main target of insecticides and there is a need for a simple and fast detection method. One current approach is to use the enzymatic inhibition of AchE by insecticides. The current bottleneck is the inherent instability of the enzyme. We have shown that encapsulation of acetylcholinesterase render them resistant to proteases and dilution. Also, our nanocontainer provide an optimal environment, e.g. the capsule may buffer a specific pH different from the outside. A detailed study on acetylcholinesterase allowed us to elucidate different factors influencing the encapsulation ration and a final encapsulation ratio for this enzyme of more than 40% functional encapsulated enzyme was achieved. Current test to functionalise these capsules with proteins for specific recognition are promising.

In the last period we focused on engineering of FhuA and their reconstitution into small lipid container allowing separating specific molecules (Task 1.4). A small side project motivated us to submit a patent. In collaboration with MPI we performed a series of polyelectrolyte encapsulation studies of our containers and the ms is ready. A new project with the MPI beyond our original planning was to encapsulate MCF-7 cell line with the final goal of cell stabilisation to produce a whole cell sensor.

MPI (Golm)

The main task of the MPI was to provide the expertise in encapsulation of various materials in micro- and nanocapsules and in the layer-by-layer electrostatic self-assembly. During the recent years the group has developed a new approach to preparation of micron and sub micron sized capsules using colloidal particles as templates. Encapsulation of various materials, such as polymers, proteins, dyes, drugs in these micro- and nanocapsules has been achieved by different means. Establishing the pH gradient across the capsule wall has been utilized to perform chemical reactions such as inorganic synthesis or precipitation of dyes and drugs with pH-dependent solubility exclusively in the capsule interior. An approach to open and close the walls of polyelectrolyte capsules by pH, salt or solvent exchange was developed. The group has elaborated the method of lipid bilayer coverage of polyelectrolyte capsules and an ion-specific channel reconstruction mimicking natural biological membranes and use these systems as artificial cells. The enzyme encapsulation has also been demonstrated.

Chemical reaction in restricted volumes (task 2.3, **fourth period**). Presence of the polyacid in the capsule volume results in pH values shifted to acidic and the total shift could reach up to 4 pH units. Such conditions inside the capsules are enough to enforce the precipitation of pH sensitive materials exclusively in their interior. The synthesis of inorganic substances exclusively inside polyelectrolyte capsules was performed. Due to high pH inside polycation filled capsules the selective synthesis of magnetic Fe_3O_4 and non-magnetic hematite Fe_2O_3 particles inside the polyelectrolyte capsules filled with polycation was demonstrated. The structure of the resulting particles depends on the ratio Fe^{2+}/Fe^{3+} in the outer solution. Synthesized Fe_2O_3 and Fe_3O_4 core / polyelectrolyte shell composites were characterized by transmission electron microscope (TEM) and (WAXS) techniques. Besides magnetite different ferrites ($CoFe_2O_4$, $ZnFe_2O_4$, $MnFe_2O_4$) were synthesized from the corresponding salts exclusively inside polyelectrolyte capsules of 10 μm diameter. Polyelectrolyte capsules with synthesized ferrites (magnetite) particles possess high enough magnetic activity to be easily manipulated in water solution by an external magnetic field. The usage of hollow

polyelectrolyte capsules as microreactors for spatially restricted inorganic synthesis was shown to be perspective for further investigation.

Adhesion directed nanocapsule arrays (task 2.4, **fourth period**

We succeeded in building arrays of polyelectrolyte nanocapsules on flat surfaces. Such arrays are an important step towards further exploring chemical reactions inside the capsules (see above), since in an array the same chemical reaction can be carried out in many capsules in parallel under different conditions.

Our previous studies allowed us to find ways to coat substrates such that they are nonadhesive or adhesive for PE-nanocapsules. For the adhesive case, we could optimize the mechanical properties and interaction characteristics of nanocapsules such that they adhere to surfaces strong enough to be not detached by typical drag and shear forces in microfluidic systems but at the same time keep their spherical shape (we could avoid capsule collapse due to too strong interactions).

We have used microcontact printing to pattern surfaces with adhesive and nonadhesive regions and incubation of such surfaces with solutions containing capsules lead to the self assembly of the capsules in the form of arrays on the surface. We could show this both for empty capsules and capsules filled with polymers and we could also demonstrate that the interior of individual capsules can be manipulated by illumination or addition of low molecular weight components to the exterior, similar to the results from capsules in bulk. In cooperation with UGE, we could apply the same approach for building arrays of coated living cells. Presently, we are working towards using receptor-ligand type interactions to trigger adhesion, which will allow to achieve reversible binding. Here we want to build up on results obtained in a cooperation with UBW. Specific interactions can additionally be used to create surfaces which have not only adhesive and non-adhesive patches, but rather multiple adhesion sites that interact specifically with the complementary capsules for sorting or creation of capsule libraries.

UGE (Genova)

The original task of the UGE group was the application of Myelin Basic protein for capsule coating. Due to the delay the UGE group finished the predicted tasks ahead of time and on our Midterm meeting we decided a modification of the project. The UGE group extended the investigation to cellular systems and to tasks related to cytotoxicity. In particular we started utilising living yeast cells as permanent core. The viability and the ability to duplicate after encapsulation were demonstrated in a series of experiments that allowed us to find optimal encapsulation conditions. Two-photon and confocal laser scanning microscopy was used in conjunction with the utilisation of flow cytometry and of specific fluorescent compounds to monitor cellular metabolic states. The utilisation of the above-mentioned optical methods also allowed us to check for the three-dimensional integrity of the yeast cells inside a polyelectrolyte nanocapsules and for the homogeneity of coverage. Forward and side optical scattering data and fluorescence signature coming from flow cytometry measurements were in agreement with three-dimensional fluorescence imaging. The success in encapsulation of living cells allowed us to plan other interesting experiments on protein expression (GFP, green fluorescent protein) and enzyme production using polyelectrolyte capsules as the protection of bio-product factories, i.e. the living cell. This hybrid system coupled to the fictionalisation of the capsule surface can be used for an intelligent and highly targeted release.

In the present year we extended our former experiments. The nanocapsules on the CaCO3 cores with different shapes were characterized with respect to preparation conditions (ionic strength and pH changes). Especially the round amorphous calcium carbonate was investigated as a possible permanent core to load with molecules. The encapsulation of living yeast was studied on a strain, expressing GFP (green fluorescent protein) in terms of protein release to the medium. We observed a release trough the capsule under specific conditions. The same encapsulated cells were used for self-assembly experiments on microcontact printed surfaces in collaboration with Dr. A. Fery from MPI Golm, Germany. We found a satisfactorily orientation along the oppositely charged printed structures. Both techniques could give an impact in the field of bioreactors. Furthermore the capsules were used as a model system for FRET (fluorescence resonance energy transfer) and FRAP (fluorescence recovery after photobleaching). The capsules allow us to simulate better the molecular crowding found inside living cells. Furthermore we found a significant influence of the polyelectrolyte binding on the fluorescence properties of the bounded dye (protein or dye). By varying the number of layers as well as the ionic strength of the polyelectrolyte solutions we refined the *Paramecium* model and found conditions for protection against lysosomal enzymes.

ENS (Paris)

Friction. Nanoscale friction is not understood even with simple molecules. A device to study the bidimensional friction of two molecularly smooth surfaces separated by a thin liquid film was constructed by the ENS team. It is based on the surface force apparatus developed by J.N. Israelachvili. Experiments on friction forces between smooth surfaces across alkenes were performed. Several regimes of the friction forces were observed over five decades of speed. A simple statistical model that involves activation barriers is able to account quantitatively for this behaviour. Such a technique can be applied to probe the mechanical properties of nanocapsules or of the polymers they are made of.

One of the polyelectrolytes used to make nanocapsules were studied by means of a friction surface force apparatus and AFM in water and also in air under controlled humidity. The thickness of the layers was 2nm in water and 1nm when dried, as measured with an AFM. The friction and wear properties of the adsorbed polymer layers were characterised at different loads and speeds. The friction in air between the layers was three orders of magnitude higher than in water. The layers showed a good resistance to friction and no surface damage was observed even at high loads.

Dynamic force spectroscopy of weak single bonds between two biomolecules anchored onto spheres. The ENS team has constructed a set-up (based on the development by E. Evans) for measuring dynamic force spectroscopy of a single bond between a receptor and a ligand, which are anchored on beads. It uses read blood cell aspirated in a micropipette as a spring. This system was used to measure the specific interactions between a viral RNA and protein (the REV) involved in AIDS: this interaction occurs at a stage at which the infection might be stopped. The ENS team has performed dynamic force spectroscopy on these weak bonds. The measurements performed with the specific RNA and with a non-specific one (with the same length but not the same sequence) demonstrated the specificity of this interaction. The measurements between REV and the part of the viral RNA involved in the binding indicate that there are two potential wells in this bond. The introduction of some molecules, which can modulate this binding strength, did not allow obtaining reproducible results.

Functionalised spheres. The adhesion between beads functionalised with specific recognition functions BAR and TAP (2,4,6-triaminopyrimidin and barbituric acid) was studied through measurements of the separation force (cooperation with Sergio MOYA who was at the MPI ,Golm). This was done with a red blood cell (RBC) force probe. The beads were coated with mixtures of phospholipids and lipids bearing BAR or TAP in their headgroups. Their adhesion was characterised by aggregation experiments using fluorescent molecules, and also by measuring the force to separate adhering beads.

Adhesion of surfaces bearing metal chelatants. Chelating groups are involved in many biological processes and these particular molecules are used in many nanobiotechnological systems to anchor proteins on surfaces. The adhesion of surfaces functionalised with chelating groups was studied by two techniques: the Surface Forces Apparatus and lipid vesicle micromanipulation. These molecules were nitrilotriacetate groups (NTA) that could be chelated with Nickel ions. The binding energies of two NTA groups by a Nickel ion could be deduced. Both techniques lead to the same value of the binding energy.

Interaction between S-layers (2003-2004). S-layers have been the object of numerous studies regarding their structure. Their self-assembling properties give them a potential for the fabrication of functionalised nanopatterned systems with applications ranging from biotechnologies to non-linear optics. However their interaction has hardly been investigated. Adhesion measurements between S-layers in humidity-controlled air have been reported but the interaction in aqueous media remains to be characterised. The ENS group has studied their interaction by means of an SFA in cooperation with UBW (D. Pum, J. Toca Herrera). The S-layers were formed on mica by adsorption from solution. AFM imaging revealed that they could form ordered protein layers on mica (J. Toca Herrera). The forces measurements revealed a purely repulsive interaction with no adhesion. In pure water, two different exponential regimes are observed. The one at longest distance (from below 100nm) corresponds mainly to double layer forces, with a possible steric forces contribution. A second regime takes place below 40nm and corresponds to the compression of the protein layers. The effect of adding salt is to reduce the decay-length of the first regime. This does not affect the short distance regime. The layers are more fragile at high salt concentration (0.1M) and show signs of damage after high compression. The protein layer thickness is in good agreement with the ones obtained by AFM studies. This shows the feasibility of measurements of interactions between functionalised S-layers.

UBW (Vienna)

The UBW group has long tradition in research on crystalline bacterial cell surface layer (S-layer) proteins and the main task was to provide the consortium with the expertise on S-layer proteins. Besides studies on the molecular sieving, self-assembly and physicochemical properties of S-layer lattices, the interactions between S-layer proteins and cell wall heteropolysaccharides have been investigated. Several S-layer genes have been sequenced and cloned and amino acid positions located on the outermost S-layer surface have been screened to exploit them as fusion sites for generating chimaeric S-layer proteins. By using S-layer specific heteropolysaccharides as biomimetic linkers to solid supports, S-layer fusion proteins incorporating sequences such as affinity tags, streptavidin, IgG-binding domains, allergens, green fluorescent protein or heavy chain camel antibodies were crystallized to generate functional monomolecular protein lattices for label free detection systems, or for coating liposome's or lipid/plasmid particles.

During the recent year the UBW group achieved recrystallization on nanocapsules. We labelled *Bacillus sphaericus* CCM2177 with *Green Fluorescence Protein* (GFP). The protein recrystallization process was studied by transmission electron microscopy (TEM). Laser confocal micrographs confirmed that anionic and cationic capsules are covered by CCM 2177-GFP. Parallel studies were carried out on solid supports. Cationic and anionic polyelectrolyte were adsorbed on hydrophilic silicon wafers. Once the substrates were ready, *Bacillus sphaericus* CCM2177 was recrystallised on polyelectrolytes. AFM tips were modified with secondary cell wall polymer (SCWP). This modification was used to study carbohydrate vs. S-layer interactions. First results show an attractive force of about (1000 pN) when the tip approaches (and "touches") the S-layer. Coexistence of large adhesion forces (2500 pN) with weak non-covalent forces (300 pN) were measured when the AFM tip is retracted from the surface containing the S-layer. Flexibility of various domains of CCM2177 was measured for forces ranging from 100 to 300 pN and different contour lengths. No pattern regularity for the elasticity was detected up to date. We carried out neutron reflectometry measurements on solid supports to determine the thickness of adsorbed protein and the water environment around the polyelectrolyte/S-layer system. Results showed that the protein thickness is in every case about 14 nm. In the same experiment we confirmed former results about the importance of Ca^{2+}-ions in the crystallisation process. The addition of EDTA to the solution led to the loss of the S-layer structure.

Forces between S-layers crystallised on mica surfaces were obtained with the Surface Force Apparatus. First of all AFM imaging was carried out to test the structure of SbpA on mica at different electrolyte concentrations. The results showed that different force regimes (electrostatic and steric; due to the rupture of the protein structure) could be obtained depending on the force load. The thickness of the adsorbed protein layers could be estimated after the experiment. This value, about 15 nm, is in good agreement with neutron reflectivity measurements.

The stability of the S-layer crystal structure on hydrophilic silicon was investigated as a function of ethanol concentration and pH value. Both solvents break weak interactions and led to the loss of the crystalline structure. A complete unfolding/refolding cycle could be obtained when the protein is unfolded with ethanol (40-60 % v/v water-ethanol mixture). The unfolding process seems to be irreversible when the S-layer is treated with pH 3 (citric acid). The stability of silicon/S-layer samples was improved by adding glutaraldehyde. No loss of the structure could be observed for this system. The unfolding/refolding process as a function of the temperature will be measured in future. The crystallisation of S-layers on AFM cantilevers was achieved and further research will be done using the cantilever as a sensor for unfolding and molecular recognition studies.

EPFL (Lausanne)

The task of the EPFL group is to focus on the reconstitution of cell surface receptors and on multiarray techniques. Classically, cellular signaling is investigated either on single cells by microscopy or using microliter volumes of suspensions of live cells and measuring different optical or electrical properties. The EPFL group showed how ligand binding to cell surface receptors and subsequent signaling reactions could be monitored in single (sub-) micrometer sized vesicles derived from biological cells. These vesicles are the smallest autonomous containers capable of performing cellular signaling reactions, thus opening the door to downscale analysis of cellular functions to the micro-/nanometer and femto-/attoliter range. This ultimate miniaturization open novel routes in functional proteomics such as multiplexing single cell bioanalytics or investigating receptor mediated signaling in multiarray format.

During the fourth year we first we realized microarrays of micrometer sized vesicles on glass surfaces. We microcontact printed 100 micrometer sized spots of biotinylated BSA, immobilized in a second step streptavidin on these patterns and finally immobilized with high contrast different vesicles on these micropatterned streptavidin templates. More than 95 % of the streptavidin spots were occupied by micrometer-sized vesicles with perfect suppression of unspecific binding outside of the receptor sites. We have meanwhile extended the approach by micropatterning 20 bases long oligonucleotides on glass surfaces and subsequently depositing site selectively vesicles coated with the complementary oligonucleotides. This approach offers nearly unlimited individual addresses for immobilizing vesicles site-selectively by self-assembly in form of microarrays.

In another project we succeeded to show simple chemical reactions in micrometer sized containers immobilized on glass plates. Lipid vesicles constitute nanocontainer systems ideally suited for the isolation, preservation, transport, and localization of few or single molecules. Their ultra small dimensions (minimal diameters of 20 nm) allow unparalleled reduction of confined volumes to the zeptoliter range (1 zL) 10-21 L). The availability of lipids with variations in the hydrocarbon chains and the polar headgroups permits in addition the optimal design of a container that is tight and inert to the reactants and products of many biochemical processes like protein expression, enzymatic reactions, or mRNA transcription to mention a few. The potential of these systems for miniaturization and bionanotechnology was nevertheless realized only after single vesicles were extracted from the *ensemble* and addressed as individuals, either by means of micromanipulation or by directed assembly on patterned surfaces. Here we present a method that allows the on-demand release and mixing of soluble compounds stored in the interior of individual vesicular nanoreactors. Performing (bio)chemical reactions entails the controlled mixing of reactants. Inspired by cellular processes, we used self-assembly principles to create an integrated device capable of mixing zeptoliter to femtoliter volumes. The principle we employ is thermotropic permeability changes of lipid bilayers to polar solutes. Maximum bilayer permeability (increase of several orders of magnitude) is reached at the characteristic ordered-fluid phase transition temperature (*T*t) of the constituent lipid due to packing defects that create transient pores in the membrane. As illustrated in Figure 1A, we created a nested system of vesicles composed of different lipids, having different *T*t. This enabled us to define conditions under which small unilamellar vesicles (SUVs), trapped in the interior of a large unilamellar vesicle (LUV), release their cargo that is subsequently confined and mixed in the interior of the LUV.

UPO (Porto)

The main task of the UPO group was to set-up the technology of a flow chamber and to determine the viscosity, elasticity and rupture strength of polymeric capsules trough the application of external fields in time and frequency dependence. The first step is to study the shape changes of the capsules under flow shear stress. The first task was to adjust a flow cell to a confocal microscope. The flow cell was designed in order to achieve laminar flow with shear stress rates between 0,1 and 10 N/m^2. It is being built taking into account the confocal laser microscope operation. Osmotically induced deformation of polyelectrolyte capsules has to be further investigated. Some experiments were previously performed using the osmotic pressure method to determine the elasticity modulus of empty polyelectrolyte capsules. More studies have to be carried out to analyse the mechanical properties in time dependence. The partial reversible shrinking-swelling effect observed in capsules filled with osmotically active polymers have still to be understood and quantitative information has to be obtained.

A parallel-plate flow chamber (Task 7.3, **fourth year**) was projected in order to study the shear stress effect on the microcapsules when adsorbed on a modified surface under different flow conditions. The flow cell was designed and set up to a confocal microscope. The observation of the capsules under shear is suggested to extend the understanding of their mechanical behaviour and obtain information about adhesion strength between differently modified surfaces. A microfluidic system was build in order to study capsules deformation and squeeze trough when flowing in channels with dimensions and geometries simulating blood vessels. A silicon chip component was fabricated using lithography and bulk micromachining methods. The device allows the in-situ imaging of the capsules deformation under stimuli.

A.2 Joint publications
(Young researchers paid from the contract in Italic)

All groups
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UBW-MPI

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Toca-Herrera, J.L., Bosio, V., Marek, M., Leporatti, S., Donath, E., Pum, D., Sleytr, U.B. "Carbohydrate-protein recognition: force spectroscopy applied to the system secondary cell wall polymer / S-layer" (manuscript in preparation)

UBW-MPI-ENS

V. Diederichs, *J. L. Toca-Herrera,* S. Küpcü, U. B. Sleytr and D. Pum Fluorescence properties of bacterial green fluorescence fusion protein as a function of temperature, pH and GHCl concentration. (manuscript in preparation)

J. L. Toca-Herrera, V. Bosio, S. Küpcü, A. Fery, U. B. Sleytr and D. Pum Crystallization of bacterial S-layers on flat polyelectrolyte surfaces and hollow polyelectrolyte capsule. (manuscript in preparation)

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UPO-MPI

A. L. Cordeiro, M. Coelho, M.C.S.Pereira,G. B. Sukhorukov and H. Möhwald Development of a flow chamber and microfluid system for studying the mecanical properties of polyelectrolyte capsules. IEEE-Nanobiotechnology (2003)

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A.2 Five most relevant publications

(Young researchers paid from the contract in Italic)

IPBS: Colletier, JP, Chaize B, Winterhalter M, Fournier D: Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. *BMC-Biotechnology* **2** 9 (2002).

MPI*: A.L. Cordeiro*, M.Coelho, G.B. Sukhorukov, F. Dubreuil, H. Mohwald: Effect of shear stress on adhering polyelectrolyte capsules. *J. Coll. Interface Sci.* in press 2004.

UBW: *J. Toca-Herrera*, R. Krastev, *V. Bosio*, S. Küpcü, D. Pum, A. Fery, M. Sara, U. B. Sleytr: Recrystallization of Bacterial S-Layers on Flat Polyelectrolyte Surfaces and Hollow Polyelectrolyte Capsules, *Small, in press,* (2004)

UGE: A.Diaspro, D.Silvano, *S.Krol*, O.Cavalleri, A.Gliozzi: Single Living Cell Encapsulation In Nano-Organized Polyelectrolyte Shells, *Langmuir*, 18, 5047-5050, 2002.

EPFL: D Stamou, C Duschl, E Delamarche and H Vogel: Single vesicle positioning through template-guided self-assembly. *Angew. Chem. Int. Ed. 42*, 5580-5583 (2003).

Part B – Comparison with the Joint Programme of work

B.1 Research Objectives

The objectives of the fourth year were to search for possible application and to optimise the process. As We could fulfil most of the tasks during the third period we had additional possibilities. This was intensively used as briefly outlined in A1.
The CNRS started also to encapsulate a MCF-7 cell line to produce a whole cell detector.

B.2 Methodological Approach and Work Plan

No modification of the research methods was necessary. Due to the late commencement other materials could also be investigated and additional techniques for the characterisation were applied.

B.3 Schedule and Milestones

CNRS

1.4 The CNRS group is currently engaged in preparation of larger quantity of active porins and Appropriated reconstitution protocols. Task completed. The outcome will be used in an ongoing Project

MPI

2.3 continuing the preparation of different nanocapsules depending on the experimental need, inserting inorganic particles as fluorophores or magnets. This task is completed. The outcome will be used in an ongoing project.

2.4 Preparation of specific nanocapsules, creation of artificial bacteria having different layers. Task completed. The results are stimulated a new project.

UGE

3.3 Characterisation of Myelin Basic protein-lipid interaction with surface techniques. Task completed

3.4 The UGE group completed the characterisation of functionalised peptides on lipid surfaces. Task Completed

ENS

4.4 measurement of adhesion between two nanocapsules bearing receptors and ligands. The technology is now available in Paris and will be used in future studies.

4.6 Measurement of single receptor-ligand bond using RBC force probe. The technology was tested and is available at the ENS.

UBW

5.4 characterisation of S-layer proteins with surface techniques
S-layer proteins were characterised with scanning force microscopy, confocal microscopy and zeta potential measurements at the Max Planck Institute of Colloids and Interfaces (Golm). Transmission electron microscopy, contact angle measurements and fluorescence microscopy are established techniques in Vienna (UBW).

Future characterisation of interaction forces between S-layer surfaces with Surface Force apparatus will be carried out in a near future in Paris at *L´Ecole Normal Superier* (ENS).

5.5 Quantification of the solute uptake in presence of different S-layer crystals. This is again part of an ongoing project.

EPFL

6.3 reconstitution of G-protein coupled receptors and the corresponding G- protein
Proof of principles is published and this technique is applied for further studies. (see A1)

6.4 realisation of multiarrays of differently functionalised nanocapsules for the realisation of highly parallel observation of different molecular interaction
Proof of principle is published.

6.5 observation of molecular interaction by different spectroscopic means (ion flow across membranes by FCS)
Proof of principle is achieved in the laboratory and the ms are currently written up.

UPO

7.3 Quantification of the flow field effects on the capsule functionality after docking on specific surfaces.
The effect of the shell thickness and capsule size on their morphology and adhesion under different flow conditions were investigated during a joined thesis (A. Cordeiro) and published by UPO in Collaboration with MPI.

7.4 Quantification of the shear stress influence and Reynolds number on the capsule docking process at a specific surface.
This task was part of the thesis of A. Cordeiro and is in press.

Professional research effort on the network project (4 years)			
Team	Researchers actually financed by the contract in person-months (according the contract) (a)	Researchers actually financed by other sources person-months (b)	Researchers contributing to the project in person-months (according the contract) (a+b)
CNRS	50 (48)	96	146 (48)
MPI	155 (48+24)	150	305 (174)
UGE	58 (48)	96	154 (42)
ENS	50 (48)	24	74 (48)
UBW	26 (48-24)	60	86 (84)
EPFL	53 (48)	24	77 (48)
UPO	63 (48)	24	87 (60)
Total	455 (336)	474	929 (504)

Overall Research Objectives

Nature has created and optimised wonderful tools unmatched to date by classical chemistry. Nature places these tools in nanometer-sized compartments and targets them to a specific site to release their content or to perform specific actions. Our overall objective was to exploit these principles in material science and the initiative of this project started from the knowledge present in the MPI group how to formulate nanometer sized hollow capsules. Our tasks during the past four years was to identify a number of fancy biomolecules, optimise the proteins according to the needs for bioanalytical/industrial applications. A combination of both research areas will create a new type of material towards long-term innovation and exploitation.

Research Methods

The participating partners have their expertise in very different fields. On one hand the MPI group had the expertise in capsule formulation based on the layer-by-layer technique. During the recent years all groups received training in this technique and each group benefited from this technique, either for their current research project or for newly created topics. We combined the material part with functionalisation of

surfaces using biomolecules, here the EPFL provided the technology for receptor reconstitution. The UBW group provided the S-layer as molecular sieves and the IPBS channel proteins. A further expertise was on the characterisation of surfaces on a molecular level and our consortium included several groups controlling state-in-the-art techniques. For example our network got access to very specialised and unique instrumentations like surface force apparatus or micropipette force measurements. Unfortunately such instrumentations are rather specific and the infrastructure is only available in one laboratory (ENS). The network offered also training like AFM which becomes now a widely used technique in this field or rheology (UPO). Especially we like to mention the EPFL and UGE with their unique training facility on fluorescence, a technique being the key technology concerning High Throughput screening.

Breakdown of tasks

We followed the example of nature and the breakdown of the tasked followed the natural schema.
- first to build nanometersized capsules: we tried many different possibilities
- To control the permeability: many proteins are available and may be used as molecular sieves. Here the bottleneck is large production and genetic engineering to modify the specificity. In addition the capsule wall is also selectively permeable.
- Targeting: here we suggested targeting using basic physical interaction like electrostatics or biological recognition.

During the 4-5 years of active collaboration we could provide proof of principle solution to the above-mentioned points. As outline in the previous reports we had to adopt only relative minor points of the originally planed schedule.

Schedule and milestones

The first two years were devoted to combine biomaterial with material science. Building different types of polyelectrolyte shells, to encapsulate enzymes. As our project was delayed by a year we had already a number of expertise prior the official beginning and we were fortunately ahead of schedule.
The second half of the project was devoted to add specificity to our capsules. We tested many possibilities to render the surface specific, to bring the capsule locally to a predetermined spot (see selected publication by the EPFL group). However, this approach continued to be an exiting field. Having now experience with some principles, much more can be done and this will be part of a future project.
Another milestone during this period was devoted on the physical characterisation. In the area of nanometer sized particles many more new technologies are now available and we also participated in the development of techniques and instruments (soft interaction revealed by micropipettes, fluorescence correlation spectroscopy to name a few)

Research efforts

455 man-months were directly financed (40% more than required by the contract) and together with other sources about 930 man months were involved within the network. We were able to produce about 100 publication in peer reviewed journals and some will be written and submitted.

The main scientific highlights were:
IPBS: watching single molecules through a membrane channel and how to get 40% of an enzyme inside a capsule.
MPI: formulation of polyelectrolyte capsules with pH sensitive permeation, opening of the caspules with a outside trigger
UGE: Encapsulation of living cells with a polyelectrolyte layer
ENS: Rupture force measurements on a single bond between one RNA and one protein
EPFL: Budding off of natural vesicles with reconstituted membrane proteins
UBW: Forces between S-layers
UPO: Stability of polyelectrolyte capsules under shear

B.4 Organisation and Management

B 4.1

The network was mainly managed through emailing. This is a very efficient way to inform the individual groups. Furthermore the principle investigator has now visited all groups to have direct contact with the students paid from the network. This turned out to be quite fruitful to stimulate them to benefit more from the network facilities. The coordinator pointed out that long period student may apply for a Marie Curie European Reintegration Grants (ERG). Towards the end of this program most of the group do have now more or less intensive contacts and ongoing collaboration.

B 4.2

A first 2-day meeting was organized in Toulouse in 14.9-16.9.2000 devoted to define and readopt the original plan.

The second meeting was held in Genoa 2001 and was combined with a visit of the UGE Institute with their new Laser facilities..

The third meeting and Mid-term meeting was organized by the MPI and held in Ringberg (24-27.11 2003). This meeting was co-organized with the German-French network on soft condensed matter and we were about more than 60 participants. On our business meeting we decided to organize an international summerschool on this subject.

We organised subsequently a International Summerschool on "Nanocapsules with functionalised surfaces and walls" at the International University Bremen 18-25 July 2003. All groups contributed by tutorial talks on their basic techniques. In addition we were able to have several outside speaker to deliver extra expertise. Altogether we were 45 speakers and about 85 participants. Part of the contributions is published under the responsibility of A. Diaspro (Genova) in a special issue of the IEEE – Nanobiotechnology in January 2004. The business meetings were held during the meeting.

Our final meeting was held in Porto (May 8-9th). We discussed the current state and we spent the entire Sunday for the fine-tuning to submit a future project (STREP 013887/FP6-2003-NMP-TI-3).

The UGE organized in Genoa (June 14-16th) a fluorescence workshop to train PhD and postdoctoral students in this field.

B.4.3 Networking

Overview 2003-2004

From/to	CNRS	MPI	UGE	ENS	UBW	EPFL	UPO
CNRS	**X**	**Several visits,** **participation in a PhD exam** **new joined project**	**CNRS exchange program on Whole cell encapsulation**	**visit**	**visit**	**C. Danelon joined the EPFL group**	**Student exchange, Project on micrfluidic**
MPI	**New project on biodistribution**	**x**	**Encapsulation of cells**		**Delivery of material**		**Student exchange**
UGE	**Exchange programm (CNRS) Whole cell encapsulation**	**Several lab-visits**	**x**			**PhD exchange (D.Silvano)**	**Lab visit**
ENS	**Visit of the lab**		**Visit of the lab**	**x**			**Lab visit**
UBW	**Lab visit J. Toca**	**several lab visits**	**lab visit J. Toca**	**Several lab visits and joined measurements**	**x**		**Lab visit**
EPFL	**Labvisit and Common project on channels**		**Visit of the lab, student exchange**			**x**	**Lab visit**
UPO	**Student exchange**	**Student exchange Ana Cordeiro**	**Student exchange**	**Student exchange J. Gomes**			**x**

IPBS
We continued our collaboration and joint measurements with the MPI. We received support for a follow up project based on the previous network. Concerning whole cell encapsulation we have an ongoing cooperation with Genoa. The CNRS will finance the travel cost for mutual visits. One of our predoctoral students is now in the EPFL group and we started collaboration on channel characterisation. We have an intensive student exchange with Porto to develop new fluidic devices and one student is now in our laboratory.

MPI
The MPI was during the entire period the nucleation center and the driving partner of this subject. This is particularily due to the large number of student working in this field and payed from other sources. The MPI was the main training site or student from MPI came to other laboratories to teach the technique.

UGE
Dr. S. Kroll visited our lab in November to apply the microcontact printing technique to direct the adhesion of coated yeast cells. There is an ongoing collaboration with the MPI and we submitted a new proposal. We started to apply our force-spectroscopy/optical microscope combination to study the deformability of S-layer protein coated vesicles, which are produced in the group of Prof. Sleytr.

EPFL
We started collaboration with the CNRS group on electrophysiological characterisation of channel forming proteins. Currently one postdoctoral student from the CNRS is in Lausanne to learn the technique and micropatterning. Another student from the UGE is performing a post-doc in the group on fluorescence.

UBW
Several team members visited during this period other network partners.
Dr. Jose Toca-Herrera performed several measurements at the *Max Planck Institute of Colloids and Interfaces* (in Golm). During this time he worked with Vera Bosio, who is a member of Dr. Fery´s group, and carried out zeta potential, confocal microscopy and AFM measurements.
He visited *L´Ecole Normal Superier* (ENS) in Paris the second week of March 2003. He had a meeting with Prof. Eric Perez, and they measured forces with Dr. Alberto Martín-Molina between S-layers with surface force apparatus a week in January 2004 and another week in April 2004.
He went three days to Hahn-Meitner-Institute in Berlin to carry out neutron reflectrometry measurements on crystallised S-layers on polyelectrolyte solid supports with Dr. Rumen Krastev.
He also visited the Alberto Diaspro's group in Genoa for a week in June to carry out life-time fluorescence measurements on fusion protein, made at UBW.
Alberto Martin Molina, ENS, visited the UBW group from June 28th until July 2nd, 2004 in order to finish the preparation of a manuscript and to do additional work on S-layer recrystallization.

UPO
We have an intensive collaboration with MPI and IPBS . We have now a new student in Toulouse (IPBS) for a year. We have started a new collaboration with UGE envisaging the studies in the Scanning Laser Confocal Microscopy. Collaboration with ENS concerning the post-doc work of Charles Berger which ended after he left for an indistrial position in The Netherlands.

European benefit

The impact of this particular European training network was to motivate mainly young scientist during their early career to develop a European view of their research. Clearly this effect is less pronounced in the larger already international institution but it was a significant driving force for the smaller groups. Speaking for my own group in Toulouse the students are usually focused on the region only. In my group the students started to asked via email for expertise in partner laboratories, they performed experiments in partner laboratories and performed discussion across Europe. In case of problems they looked up for the expertise and arranged the necessary steps. This was extremely seldom in other groups of our Institute and introduced a real opening of the spirit. The impact was clearly beyond my group and the other students became quite demanding for such working practises. The joined meeting in Ringberg as well as the Summerschool was quite a success and brought many new contacts especially among the young scientist.
One very important aspect and sort of a measure of success is, that 3 of our post-docs (two directly paid and one acting as a group leader) got offers as Professors.

B.5 Training

B. 5.1

During the last period all position were filled. However, the individual groups continued to hire positions financed from other sources. Currently the most appropriated method to find candidates is through the Internet. A further way to get high quality students is the direct advertisement in related groups or posting on relevant meetings.

B.5.2

Participant	Contract deliverable of young researchers to be financed by the contract (person-month)			Young researchers financed by the contract so far (person-month)		
	Pre-doc (a)	post-doc (b)	Total	Pre-doc (a)	post-doc (b)	total
CNRS	20	28	48	50	0	50
MPI	24 (+24)	24	48 (+24)	91	64	155
UGE	20	28	48	9	49	58
ENS	16	32	48	10	40	50
UBW	48 (-24)	0	48 (-24)	0	26	26
EPFL	48	0	48	53	0	53
UPO	28	20	48	51	12	63
Total	204	132	336	264	191	455

CNRS: It was difficult to find an adequate post-doc. However, it was possible to find good predoctoral students. We originally planed to sent J. Gomes (Porto) to Genoa but the preparation of the samples to be investigated in Genoa was more complex than expected. We therefore hired her through Toulouse as she spent most of the time in our group.
MPI: The MPI group is very large and has many international students from all over the world. Therefore finding eligible candidates was not a problem. Several pre as well as post-doctoral students were highly interested to share their knowledge and apply it in other group. This was a very fruit-full exchange and most of the other groups benefited from this exchange. This justified the enlarged budget.
UGE: the group preferred to hire a post-doc. The person qualified in Genoa for a Professorship and has now a position at the University Genoa on Nanobiotechnology.
EPFL: The EPFL group is large and contain many international students eligible for the RTN program fulfilling all national requirements and working in the specific field
UPO: The group has several qualified PhD candidates with high interest to benefit from the exchange program with different partner laboratories. Some were successfully sent to MPI and CNRS/ENS.
UBW: The previously hired post-doc received an offer from Spain and will continue as a Professor.

B. 5.3 Integration of young researcher into the team

IPBS: the young researchers were integrated within the group, they had continuously contact with several newly founded start-up.
MPI: The young researchers are well integrated into the institute having more than 100 foreign researchers. They also take part in courses of the "International Max-Planck Research School on Biomimetic Systems"
UGE: The postdoctoral student was fully integrated within the group and received an offer to continue as a Professor.
ENS: the pre and postdoctoral students are fully integrated in the group and have the same possibilities as the other members
UBW: The postdoc was integrated in the group and was the driving force for the exchange program.

EPFL: the predoctoral student is fully integrated and visited several partner laboratories. This group hired two post-docs from the network and integrated them.
UPO: the students are participating in the group and benefit intensively from visits in partner laboratories. Part of their own students are integrated in MPI, ENS and CNRS.

B. 5.4 During the fourth period we had one summerschool where most of the participating students presented their results and concluded the contribution from the individual group. Several students from the local University participated as well. A special highlight was the fluorescence microscopy workshop (June 14-16) organised by the Italian group in Genoa. A 3-day program included also practical training using the latest instrumentation.

B. 5.6 Special measure to guarantee the interdisciplinary

As outlined each group contributed with their expertise ranging from physics, chemistry, biology, biotechnology and material sciences. The students participated in workshops and summerschools as well as research stays in different laboratories.

B. 5.7 Contact with industry

IPBS:
A new start-up was created: Nanobiotix. Their goal is to construct functional nanocapsules for controlled drug delivery. Students in our lab are participating on the discussion and we have now one joined PhD working partially within the company. The MPI has intensive collaboration with Capsolution, an offspring from their research centre in this area.

B.6 Difficulties

No difficulties requiring special measure occurred.