Project no. 026723-2

NanoBioPharmaceutics

Nanoscale Functionalities for Targeted Drug Delivery of Biopharmaceutics

Integrated Project (IP)

Thematic Priority 3: NMP

Publishable Summary

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Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	☒
PP	Restricted to other programme participants (including the Commission Services)	☐
RE	Restricted to a group specified by the consortium (including the Commission Services)	☐
CO	Confidential, only for members of the consortium (including the Commission Services)	☐

General summary

The NanoBioPharmaceutics project aimed at the development of innovative multidisciplinary approaches for the design, synthesis and evaluation of molecular, nano- and micro-scale functionalities for targeted delivery of therapeutic peptides and proteins (biopharmaceutics). The development of functionalized nanocarriers and nanoparticle-based microcarriers for Protein/Peptide (P/P) delivery is both an important scientific challenge and potentially a business breakthrough for the biopharmaceutical industry. NanoBioPharmaceutics focused on the development of functionalized nanocarriers for the treatment of various diseases based on targeted, controlled delivery of P/P drugs. More specifically, NanoBioPharmaceutics addressed the following scientific and technological objectives:

- Design, synthesis and functionalization of novel nanocarriers and nanoparticle-based microcarriers for targeted delivery of P/P drugs via oral, nasal and Blood Brain Barrier (BBB) crossing administration routes.
- Toxicological screening of the nanocarriers and investigation of the release profile of P/P drugs under various environmental conditions as well as the assessment of the biocompatibility and biodegradability of the new formulations.
- Design and functionalization of carriers to meet the requirements for nasal delivery of P/P drugs for vaccination.
- Oral nanoparticulate P/P carrier systems capable of adhering to the gastrointestinal mucosa and also displaying protective and permeation enhancing properties.
- Design and functionalization of carriers to meet the requirements for transportation of P/P drugs through the Blood Brain Barrier including the establishment of an *in vitro* model for the assessment of nanocarriers permeability through the BBB.

Generally, these objectives as well as the project milestones have been successfully met in full accordance with the workplan, the Consortium Agreement and all ethical guidelines. Developments within or outside the project made adjustments of the workplan necessary and reasonable to guarantee maximum success of NanoBioPharmaceutics. Decisions on these matters were taken by the General Assembly and followed up by the entire Consortium in a very cooperative and constructive manner.

Summarizing the scientific results it can be ascertained that the project was very successful in terms of producing, testing and implementing numerous nanoparticulate carrier systems, the “NanoToolbox”. These systems combined with peptides have been the basis for *in vitro* and *in vivo* tests addressing the oral, nasal and BBB administration route. A deep understanding and sound knowledge has been established at the partners´ facilities in order to continue this successful development of carrier/protein systems for crossing different barriers.

For the oral administrative route a real breakthrough has been achieved and a patent has been filed. For the nasal and the Blood-Brain-Barrier route very promising systems have been developed which will be the basis for further developments in order to establish systems which might be used also in clinical testing. In addition to these application-oriented developments a deep understanding of possible interactions of the nanocarriers with cell systems has been generated by *in vitro* and theoretical experiments. This is a basis for a systematic and knowledge based design of future nanocarrier systems.

In addition to these successful scientific results the project also turned out to be very effective from a management point of view. The project, with partners coming from different disciplines and working in completely different fields, succeeded in bringing together the expertise of these partners in a synergistic and constructive way. A common understanding and a common language of chemists, biologists and physicists has been established. This will be clearly the basis for further research activities of these partners in the cross-disciplinary area of nanomedicine. Furthermore, the project managed to continuously increase the

number of female researchers also in higher management positions. This positive development has been supported by specific measures like training courses and specifically designed events.

Summaries of the workpackage results

WP12 – Polymer-Protein/Peptide Nano-Aggregates and Systems for Targeted P/P Delivery

Although in general, there are many promising drug candidates currently in the research pipeline, there are some common problems with them. (i) Many drugs, although highly active against their intended disease also affect healthy tissue; (ii) Small molecules, once in the circulation (blood) are rapidly removed by the renal (kidneys and liver) system, lowering their concentration and hence efficacy. This means drugs must be given in higher doses, or more often; (iii) Protein-based drugs are rapidly degraded in the body by proteases; (iv) Although a drug is active against a disease, it must first reach its target organ and therefore needs a targeting group. Considering these problems, the aim of this workpackage was to devise strategies to solve these problems and improve the delivery of therapeutic agents to the disease sites in the body. Two general strategies were devised for this, which are summarised in Figure 1. All the strategies involved attaching/associating the therapeutic to either a polymer or a nano/micro particle in some manner. The rationale for this is that these structures are able to reduce the toxicity of the drug, prevent enzymatic degradation, increase the circulation time in the body and add the possibility of including targeting ligands for selective organ uptake (e.g. the brain).

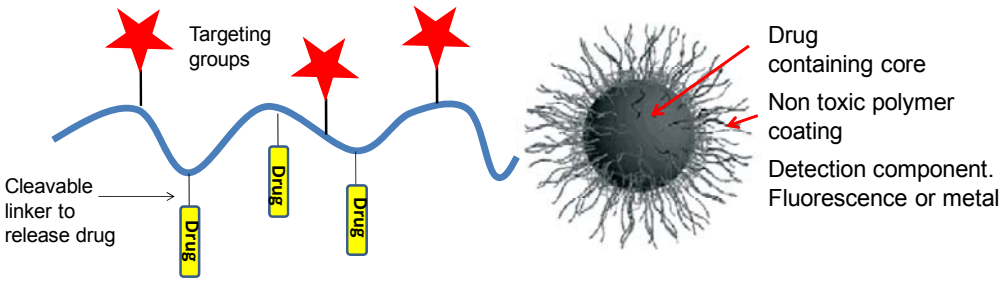

Figure 1. Schematic showing the two general strategies used in this workpackage

One approach involved directly combining drugs with polymers: A short peptide fragment was designed to be capable of preventing HIV virus from entering cells, and conjugated to a non-toxic synthetic polymer, maintaining peptide activity and reducing its degradation by enzymes. A similar strategy was used with a large protein drug, TNF alpha.

A library of peptide mutants has been constructed which are not only effective in inhibiting the entry of HIV-1_{HXB2} but also Fuzeon® resistant mutants. All peptides and peptide-polymer conjugates produced, showed to have an inhibitory efficacy in the 1-100 nanomolar range, are not cytotoxic in the 1-1000 micromolar range and are good candidates as Fuzeon alternatives in HAART (Highly Active Antiretroviral Therapy), and may be suitable for application in patients with Fuzeon-resistant strains of HIV-1.

In summary, WP12 successfully created a plethora of new, well defined, delivery vehicles for polypeptide drugs. Instead of focusing on a single material with a high chance of failure, the whole spectrum of polymer-protein conjugates, their attachment to inorganic nanoparticles, or inclusion to micron sized spheres were evaluated and their utility tested.

WP3 – Toxicity, Immunogenicity and Degradation Testing

Workpackage 3 was dedicated to the identification and characterization of potential adverse effects of the engineered nanoparticles on human health. Testing of nanoparticles is more complex than that of conventional substances because light scattering, light absorption and chemical (redox-)activity can interfere with the assay signal in unpredictable ways and cause false-positive and false-negative results.

All nanoparticles produced by the consortium were investigated for their toxic effects. In addition also various standard particles were used to validate newly established assays and to compare the action of the engineered nanoparticles to that of well-known particles. To reduce the use of animals extensive *in vitro* testing was performed prior to application in animals. Toxicity was tested in blood cells and plasma and in isolated cells and (immortalized) cell lines of different origin. Screening assays to identify toxic particles and more specific assays to identify physicochemical parameters, which induce certain toxic mechanisms, were used. Prior to any toxicity testing, however, lack of contamination was ascertained. To prevent misinterpretation of the toxicological effects of the nanoparticles, additional controls were included to the standard assay set-up and several assays were used for the evaluation of one toxicological parameter (Figure 2).

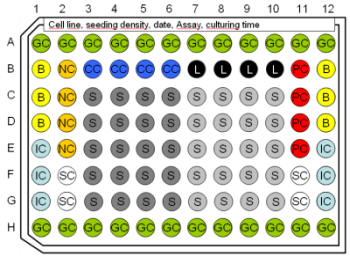

Figure 2: Standard layout for the evaluation of the cytotoxic effect of two samples in different concentrations including various controls (cellular growth control, blank, negative control, positive control, color control, interference control, and for certain assays lyses).

As loading of the nanoparticles may change the toxic potential, unloaded and loaded nanoparticles were tested in parallel. Adverse action on cells was identified using cytotoxicity screening assays. After successful passage of the cytotoxicity screening, influence on plasmatic coagulation and platelets and on white and red blood cells was studied. Additional tests evaluated the effect of the nanoparticles on various physiological functions of macrophages like chemotaxis, cytokine secretion and phagocytosis. Activation of the immune system either in the form of complement activation or as activation of lymphocytes was seen in several samples. The characterization of the toxic effect helped the producers to optimize their particles.

Causes for endotoxin contamination of samples were identified and eliminated. The particle-producing partners were specifically trained to deal with endotoxin and cytotoxicity testing.

The nanocarriers, which were evaluated as pyrogen-free, non cytotoxic and hemocompatible, showed no adverse effects in animals demonstrating that the screening was effective to prevent toxic particles from being used *in vivo*.

WP4 - Interaction between Cells and Nanostructures

The main objective of WP4 was to examine the interaction of nanoparticles (NPs) developed in WP12 with human cells and biomimetic lipid membranes which resemble cell membranes. The cells were representative of the cells making up the barriers in the human body that NPs would encounter and must pass in order to get into the body. These included epithelial cells and endothelial cells. An epithelial cell line was used to represent the barrier that NPs would encounter taken up orally and arriving in the gut. Three different endothelial cell types were examined. These included *microvascular endothelial cells* representing cells making up the microcapillary blood vessels in nearly all tissues of the body. These are the cells that would

first be encountered after the passage of a NP through epithelial cells in the lung, nose, and after injection of NPs directly into the blood. In addition, *macrovascular endothelial cells* representing endothelial cells lining the larger vessels of the body and *brain microvascular endothelial cells* were used. The brain is separated from the rest of the body by the blood-brain barrier that is made up by a unique population of endothelial cells that form a very tight barrier allowing the passage of only very specific compounds. Finally, biomimetic lipid membranes that are synthetically generated and form structures very similar to those surrounding cells can be used to determine and measure the interaction of NPs with the membrane models.

The goal of this WP was to examine how a NP's physicochemical makeup, (that is, its size, shape, structure, chemical composition, charge, etc.) affects its adhesion, uptake and transport across the unique different cell types and to biomimetic membranes and to determine if there were differences based on these characteristics. To follow the interaction of the NP with cells, NPs were generally labeled with a fluorescent marker or were directly visible in cells using high magnification microscopy.

Nearly 200 NPs were developed and examined for uptake in the various cell culture and biomimetic membrane models developed. Most of the NPs rapidly adhered to and were taken up within the cells. With time, most NPs passed through the cell membrane and were generally closely associated with various intracellular organelles responsible for the uptake of nutrients and proteins in the cells. Due to the extremely small size of NPs, it was difficult to determine whether NPs were able to transfer through cells.

In some cases a good correlation of *in vitro* and *in vivo* results was observed. Therefore, these *in vitro* models may be used to predict the outcome of NPs administered to the body and thus may serve as alternatives to animal studies.

WP5 Nasal delivery

WP5 focused on nanoparticle-aided nasal vaccination and pulmonary delivery of P/P drugs.

In recent years, the lungs have been also studied as a delivery route for proteins because they have a large surface area and allow rapid absorption due to close contact between alveoli in the deep lung and the circulation. The *pulmonary delivery* of proteins may be limited by proteases in the lung, which reduce the overall bioavailability. Novel carriers with improved delivery features might overcome the administration difficulties and increase efficiency of protein delivery to the deep lung.

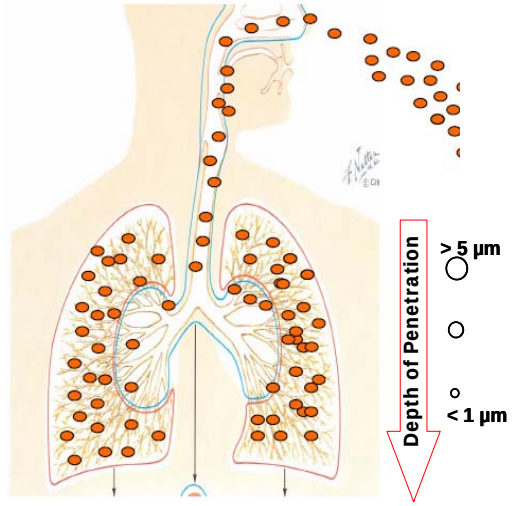

Figure 3: Size-dependent particle distribution in the lung

One of the main objectives of WP5 with respect to pulmonary delivery was to design carriers to meet the requirements of pulmonary administration (e.g., size, aerosol characteristics, cell uptake, protein bioavailability). Various types of nanocarriers (e.g., nanoparticles, nanogels, polyelectrolyte complexes) were thus prepared for pulmonary delivery of insulin. A computational model for simulating the particulate fluid flow in the lungs was also developed. Particles in the size range of 1 to 5 µm in diameter are deposited in the small airways and alveoli.

Due to the withdrawal of Exubera as a pulmonary insulin delivery system by Pfizer, in the second year of the project the main objectives of WP5 have been changed to nasal delivery of vaccines.

Vaccination is considered to be the most effective way of fighting infectious diseases like HIV, malaria, influenza, etc. Among the potential needle-free administration routes, nasal vaccination is particularly attractive. The nose is easily accessible and the nasal cavity is equipped with a high density of dendritic cells that can mediate strong systemic and local immune responses. In addition, vaccination via the intranasal administration route requires much lower doses of antigen, because of the limited dilution of the vaccine formulations by the nasal fluids and minimal exposure of vaccine to low pH or/and secreted degradative enzymes. However, since intranasal administration of free antigens cannot readily elicit immune responses, a nanocarrier-based vaccine delivery system may have the potential to improve the antigen's efficacy.

In this respect, the aim of WP5 was to design nanocarriers to meet the requirements for nasal vaccination in terms of antigen/adjuvant loading, stability, toxicity, haemocompatibility, cell uptake and induction of immune responses. Various types of nanocarriers containing antigen and adjuvant were thus designed according to the specific requirements and tested *in vitro* for crossing the nasal barrier and *in vivo* for their immunogenicity. Selected nanocarrier formulations were found to exhibit immune responses, however presently not of an adequately high level. Two *in vitro* models, one of the upper and one of the lower respiratory tract were developed for examining the nanocarriers ability to cross the epithelial and endothelial barriers. Finally, a computational model was developed to predict the particle flow and deposition in the nasal cavity.

WP6 – Oral P/P Delivery Systems

The objective of WP6 was to establish *in vitro* models to predict the oral drug uptake of therapeutic peptides, to perform *in vivo* bioavailability and biofeedback studies and to evaluate gastrointestinal transit times of various nanoparticulate drug delivery systems.

In order to reach a sufficiently high oral bioavailability, various barriers encountered with the gastrointestinal (GI) tract have to be overcome. These are mainly the enzymatic barrier, the mucus gel layer barrier and the absorption barrier. Strategies to overcome these barriers include the design of more stable peptide and protein drugs, the co-administration of enzyme inhibitors and permeation enhancers as well as various formulation approaches. Among different types of formulations micro- and nanoparticulate delivery systems seem to be most promising. Due to their comparatively small size they can penetrate in the mucus gel layer avoiding a pre-systemic metabolism of the incorporated peptide/protein on the way between the delivery systems and the absorption membrane.

A considerable number of studies focusing on the mucoadhesive properties of a wide range of polymeric materials have been performed using different *in vitro* methods and techniques. In particular the novel thiolated chitosan (chitosan-mercaptonicotinic acid) showed strongly improved mucoadhesive properties. In comparison to the unmodified polymer at least 60-fold higher adhesive properties were achieved.

Finally, rat *in vivo* analysis of blood glucose lowering was carried out for three formulations within WP6. An ELISA system for quantification of human insulin in rat plasma was established. The great potential of certain nanoparticles was demonstrated by *in vivo* studies. Particles based on bioreducible poly(amido amine)s, for

instance, showed an oral bioavailability of human insulin of 0.16%. Using thiolated chitosan nanoparticles improved the relative oral bioavailability of human insulin 20-fold up to 1.5% compared to unmodified chitosan nanoparticles.

These systems also showed 60-fold higher adhesive properties to the mucus layer. Furthermore, mucoadhesion studies with particles of increasing size showed a clear correlation between particle size and particle adhesion. The smaller the particles were the higher were their adhesive properties on intestinal mucosa.

Additionally, a device designed to administer nanoparticles in dry form to enhance mucoadhesion to the gastrointestinal mucosa, was developed within WP6 and a patent has been filed.

Results generated within WP6 provide essential information for the effective oral administration of insulin and likely numerous further therapeutic peptides.

WP7 P/P Delivery Systems for Crossing the Blood Brain Barrier

The brain is an exceptional organ. It is rich of intercellular contacts, of which many function via peptidic transmitters. Moreover, cells in the brain communicate in a paracrine fashion with each other – also in many cases via peptidic messengers – and send signals in the body. Many of the peptidic communication pathways in the brain are described and understood and could potentially be used for pharmacological purposes like prolongation of cellular life, pain relief, axon guidance, anti-degenerative measures. Major diseases like neurodegenerative diseases (Alzheimer, Parkinson, and Huntington) could potentially be influenced in a helpful and positive way. Access to the brain is controlled by the blood brain barrier (BBB).

A major road block for a broader application of peptides and proteins in the treatment of brain diseases is thus the limited passage of the blood brain barrier. Although methods are known to use the existing transport pathway for active transport, these pathways are usually too slow and ineffective for pharmacological purposes. It is thus the major objective of WP7 to increase efficacy of existing transport systems while using nanoparticles loaded with P/P drugs. Per transport event, a whole targeted nanoparticle will be transported, loaded with an as high amount of P/P drug as possible.

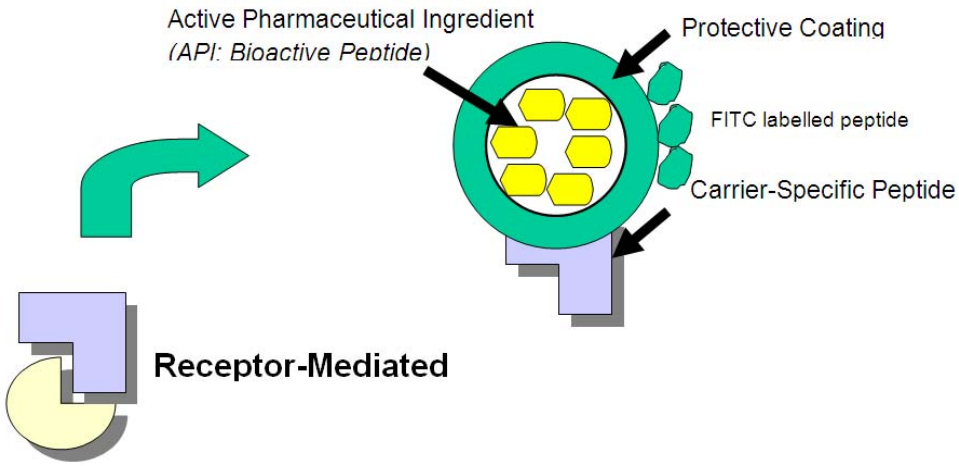

Figure 4: Schematic representation of a NP labelled with a FITC (fluorescein isothiocyanate) peptide on the surface

To enhance the possibility of brain uptake, the nanocarriers were modified with specific brain-targeting peptides as well as reporter peptides. To enable the detection within cells both the carrier and the targeting peptide were fluorescently labelled in different ways.

In vitro and *in vivo* methods were used for the estimation of brain uptake index and brain permeation ability of the carriers.

The efficacy of newly developed or adapted methods/models/technologies for nanocarrier analysis was tested in WP7. A highly specific *in vitro* BBB model has been developed, characterized and used to study the penetration of NPs across the BBB. Additionally, an ELISA method has been developed to detect labeled NPs which allow tracking them after injection in the animals. The sensitivity of the method allowed measurements of labeled NPs in different organs, blood and Cerebrinal Spinal Fluid (CSF). A specifically developed LC/MS analysis has been used to confirm the ELISA results. Open-flow microperfusion (OFM), a novel technique for sampling Brain Interstitial Fluid (BIF) has been also established within this WP.

The work within WP7 focussed on the evaluation of the most successful NP formulation for passing the BBB using the targeting peptide with the highest potential. Summarizing the results, the *in vitro* studies show that most of NPs labelled for brain targeting were taken up by other endothelial cells as well as brain endothelial cells. The obtained *in vivo* data lead to the conclusion that the nature of the NPs may be of extreme importance for a more efficient brain delivery.

Dissemination, Exploitation and Training Issues

A public website www.nanobiopharmaceutics.org was established and continuously updated. A project brochure was also prepared and distributed at many different occasions.

The Consortium was very active in disseminating the project results. Until now, far more than 100 scientific articles were published in peer-reviewed journals of various disciplines. NanoBioPharmaceutics was widely represented in form of lectures and poster presentations at many scientific events throughout the entire life time of the project.

Two major conference events were organized within NanoBioPharmaceutics. The International Conference on Nanomedicine took place on September 9-11, 2007 in Porto Carras, Greece and was mainly organized by partner CPERI. As a midterm conference, the EuroNanoMedicine 2009 was organized jointly by the FP6 Integrated projects MediTrans, NanoEar and NanoBioPharmaceutics to benefit from the synergies of the three drug delivery projects and to foster the scientific exchange. EuroNanoMedicine 2009 took place on September 28-30, 2009 in Bled, Slovenia, with DECHEMA as the main and NIC as the local organizer and 225 participants from 25 countries. As a satellite event to the public conference programme, a Young Researchers Technical Workshop was organized for the young researchers of NanoBioPharmaceutics, NanoEar and MediTrans. A press release announcing EuroNanoMedicine 2009 and introducing NanoBioPharmaceutics was released.

NanoBioPharmaceutics was also invited on several occasions to present itself at political or strategic events, e.g. by the European Commission or the ETP Nanomedicine.

The specific need for trainings was assessed and trainings and workshops on different topics were planned accordingly, taking into account the interdisciplinary knowledge of the partners. A focus was set on meeting the needs of the young researchers.

Topics of workshops/trainings included:

- Endocytosis/Transcytosis
- Endotoxin and cytoxicity testing
- Particle size analysis
- Voice and presentation training for female researchers

Additionally, the NBP tour, a student exchange programme was organized.

Many different exploitable results were generated during the project that may also be of great interest for use for other than pharmaceutical purposes.

- Partner THIOMATRIX has filed a patent on a technology developed within WP6 designed to administer nanoparticles in dry form to enhance mucoadhesion to the gastrointestinal mucosa.
- Partner IQS is currently preparing a patent on a method for the preparation of thermosensitive acrylamides. IQS has founded a Spin-Off company named Sagetis to develop its technology to link both targeting and reporting peptide on the surface of nanoparticles designed with characteristics to cross the blood brain barrier.
- A novel series of peptides designed to inhibit entry of HIV into human cells have been designed and synthesized.

Management Issues

The management of the project was very successful due to good communication of the Management Team, the WP leaders and the Coordinator.

DECHEMA as the coordinator strongly supported the WP leaders in their scientific leadership and in the communication with their WP partners by organizing regular WP meetings or telephone conferences and following up the results of these meetings very closely.

During the project, several adjustments of the workplan were considered necessary due to the development of the project or to react to influences from outside the Consortium, e.g. the problems with the Pfizer drug Exubera. These changes were managed successfully due to good communication and cooperation between the partners, WP leaders and the coordinator. The integration of WP12 into the application workpackages after 30 months can be considered an example for an outstanding management success. This restructuring was very beneficial for the project partners as well as for the outcome of the project as it fostered the interdisciplinary work and for the particle producers tremendously enhanced the understanding of pharmaceutical and medical as well as industrial requirements.

Additionally, the Gender Action Plan defined at the beginning of the IP can be considered very successful and efficient, as involvement of female researchers, especially in leading and management positions, has increased throughout the project.