CHIMIA **2004**, *58*, No. 10

Chimia 58 (2004) 711–717

ISSN 0009–4293

Concepts and Prototypes for Formulation and Delivery of Biopharmaceuticals and in Tissue Engineering

Lorenz Meinel, Bruno Gander, and Hans P. Merkle*

Abstract: Novel technologies in the discovery of new therapeutic or immunogenic moieties in the fields of low molecular weight and biomacromolecular pharmaceuticals have led to an increasing demand for delivery systems capable of protecting, transporting, and selectively depositing those therapeutic agents at their sites of action, with the aim to control their pharmacological profiles, reduce frequencies of administration, limit side effects and toxicity, and, last but not least, foster patient compliance. This applies particularly to biomacromolecules, *i.e.* peptide, protein, and nucleic acid biopharmaceuticals. Our group's prime interest in this area is to improve efficacy and safety of drug treatment and vaccination, and contribute to patient-friendly administration modes. In particular, we focus on peptide and protein therapeutics, vaccines and gene medicines. In this summary, we will specifically address three areas: (i) our long-term involvement in the biomedical engineering of particulates as vaccine delivery systems, (ii) skeletal tissue engineering applying human mesenchymal stem cells, and (iii) our engagement in research on cell penetrating peptides (CPP) as carriers for drug delivery.

Keywords: Biodegradable microspheres · Cell penetrating peptides · Human mesenchymal stem cells · Microencapsulation technologies · Tissue engineering · Vaccine delivery systems

Introduction

The engineering of systems to deliver therapeutic agents efficaciously and safely to the desired sites of action at appropriate rates and doses has grown into an independent field in its own right which exceeds the range of traditional pharmaceutical disciplines and attracts the interest of an interdisciplinary community in both academic and industrial research. Novel technologies in discovering new therapeutic or immunogenic moieties in the fields of low molecular weight and biomacromolecular pharmaceuticals have led to an increasing demand for delivery systems capable of protecting, transporting, and selectively depositing those therapeutic agents at their sites of action, with the aim to control their pharmacological profiles, to reduce frequencies of administration, limit side effects and toxicity, and, last but not least, foster patient compliance. This applies particularly to biomacromolecules, *i.e.* peptide, protein and nucleic acid biopharmaceuticals. Nevertheless, the contribution of the delivery system is sometimes overlooked in view of the predominant attention placed on the exploding role of target and lead discovery, even more so in the postgenomic era. Notwithstanding, as subsumed under the slogan *'No payoff without delivery'*, drug discovery may be biotechnology's raison d'être, but its therapeutic and economic success is utterly dependent upon versatile drug delivery, as highlighted in a previous *Nature Biotechnology* editorial [1].

A selection of typical problems that are encountered when approaching drug delivery solutions are briefly summarized in Table I. The answers to drug delivery issues range from stealth formulations, *e.g.* through PEGylation of biopharmaceuticals or surface modification of drug delivery systems, slow release implants in biodegradable polymers, liposomal formulations, and systems for pulmonal or nasal delivery, to transdermal patches, ligand-mediated approaches for selective targeting, vaccine delivery systems and others. Our group's prime interest in drug and antigen delivery is to improve efficacy and safety of drug treatment and vaccination, and to contribute to patient-friendly administration modes. In particular, we focus on peptide and protein therapeutics, vaccines and gene medicines. In this summary, we will specifically address three areas: (i) our long term involvement in the biomedical engineering of particulates as vaccine delivery systems, (ii) skeletal tissue engineering applying human mesenchymal stem cells, and (iii) our engagement in research on cell penetrating peptides (CPP) as carriers for drug delivery.

*Correspondence: Prof. Dr. H.P. Merkle
Institute of Pharmaceutical Sciences
Drug Delivery & Formulation Group
Swiss Federal Institute of Technology Zürich
ETH Hönggerberg
Wolfgang-Pauli-Strasse 10
CH–8093 Zurich
Tel.: +41 44 633 73 10
Fax: +41 44 633 13 14
E-Mail: hmerkle@pharma.ethz.ch

Engineering of Particulates for Antigen and DNA-Vaccine Delivery

Vaccine Delivery Systems

Modern vaccine development increasingly involves formulation and delivery strategies. It is now fully recognised that a

Table 1. Selection of current issues, implications, and solutions in drug delivery research

Clinical and pharmaceutical issues	Clinical and pharmaceutical implications	Drug delivery solutions
Chemical and physical instability	Rapid inactivation of therapeutic agents: chemical degradation, physical inactivation, denaturation	Use of stabilizers, water-free formulations (freeze drying), non-aqueous media
Poor aqueous solubility	Low dissolution rates and incomplete bioavailability	Stabilized colloidal dispersions, salt formation, inclusion complexes, emulsions, microemulsions, self-dispersing lipid formulations
Poor oral absorption	No oral bioavailability	Colonic delivery; pulmonal or nasal delivery
Unfavourable pharmacokinetics	Rapid clearance of drug requiring high frequency of administration, or higher but potentially toxic doses, or continuous infusion	Slow release injectables through crystallization, precipitation or embedding in biodegradable polymer implants, transdermal delivery; oral slow release formulations
Unfavourable biodistribution	Preferential distribution of pharmaceuticals to normal tissue can lead to systemic side effects or toxicity that restrict the amount of drug that can be administered	Localized drug delivery, ligand-mediated targeting to exclude non-specific drug activity
Rapid enzymatic cleavage	Loss of therapeutic activity, high frequency of administration	Stealth formulations as PEG-, dextran- or PVP-conjugates; slow release particulates
Rapid biological clearance by the mononuclear phagocyte system (MPS)	Loss of therapeutic activity, high frequency of administration	Stealth formulations
Systemic toxicity	Systemic toxicity may override local benefits, *e.g.* of systemically administered growth factors	Stealth formulations, slow release formulations for localized delivery, liposome formulations
Poor immunogenicity	Low or biased immunogenicity of synthetic or subunit vaccines	Immune adjuvants, micro- and nanoparticulates, liposomes, virosomes, emulsions
Unwanted antigenicity	Recognition of therapeutic biomacromolecules as foreign may activate the body's immune response and cause neutralization through antibodies	Formulation approaches, protein engineering, stealth formulations

successful vaccine not only requires an optimal immunogen or DNA, but also depends on the particular delivery route, delivery kinetics, formulation and stability of the biomacromolecules. Therefore, polymers and polymeric delivery systems play an increasingly important role in vaccine development. Our own activities have significantly contributed to the progress made in the area of antigen delivery from biodegradable microspheres [2].

Vaccines represent the most cost-effective approach to control and even eradicate microbial infections and hold promise for immunotherapeutic purpose in the fight against cancer diseases. Difficulties associated with the efficient use of certain vaccines are (i) the necessity of multiple injections for primary immunization followed by periodic boosters throughout life time to maintain immunity; (ii) a generally very short shelf-life at room temperature so that refrigeration and cool chains are required; (iii) a strong humoral, but often modest cellular response which is inappropriate for many indications such as intracellular viral, bacterial and parasitic infections or tumor immunotherapy; and (iv) the difficulty to ascertain efficiency and safety with combination vaccines. Antigen delivery technologies are considered essential to remedy some of these limitations; at the same time, antigen delivery technologies are expected to foster the development of new and improved vaccines.

A major milestone in the development of antigen delivery systems for new and more efficacious vaccines was the use of biodegradable poly(lactide) and poly(lactide-*co*-glycolide) (PLA/PLGA) microspheres, within a special WHO Programme for Vaccine Development, initiated in the late 1980s [3]. PLA/PLGA microspheres can be loaded with one or several antigens, which are released *in vitro* in a continuous or pulsatile manner, thereby potentially mimicking the repeated injections of conventional vaccination schedules. Immunisation of small animals such as mice and guinea pigs with a single injection of microencapsulated tetanus or diphtheria toxoid afforded protective and long-lasting antibody responses [3]. PLGA-microspheres have also proved their inherent immunostimulating properties for synthetic immunogenic peptides, and, very interestingly, they can also assist in the elicitation of cellular effector responses, *e.g.* so-called cytotoxic T cell (CTL) responses [3]. This type of response is instrumental to fight intracellular infections and for cancer vaccination.

Our recent and future efforts are in the targeting of nano- and microparticles to dendritic cells (DC), which are the most professional and efficient antigen presenting cells (APC). PLGA microparticles of sizes below 10 μm are avidly phagocytosed in an unspecific way by phagocytes such as macrophages and DC, both *in vitro* and *in vivo* (Fig. 1) [4]. Thereby, presentation of exogenous antigens through MHC-class I molecules is mediated, which may lead to the generation of CTL responses (Fig. 1). Targeting of DC is considered to be of importance to promote efficient immune response *via* both (i) selective uptake of antigen loaded microparticles and (ii) cell

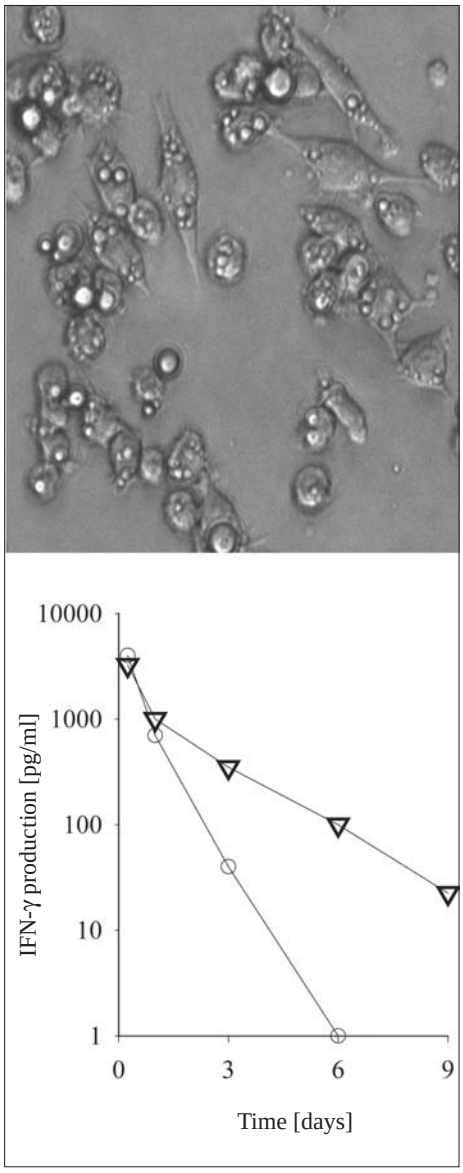

Fig. 1. Uptake *in vitro* of PLGA microspheres by macrophages (upper panel) and presentation *in vitro* of dissolved and microencapsulated malaria peptide (PfCS334-342) by human dendritic cells isolated from fresh blood to antigen-specific cytotoxic T lymphocytes (lower panel). The PLGA MS were co-incubated overnight with the dendritic cells, and the antigen-specific cytotoxic T cells were added at indicated times. Antigen presentation was measured by the production of interferon-γ by the cytotoxic T cells. Dendritic cells (triangles) presented the microencapsulated antigen substantially longer than the dissolved antigen (circles). Fig. adapted from [4d] with permission from Elsevier Science Ltd.

specific activation. DCs express a broad spectrum of cell surface molecules which could be targets for selective uptake of nano- and microparticles containing antigens. They include chemokine-, Fc-, complement-, Toll-like- and scavenger-receptors, integrins as well as C-type lectins. Those receptors fulfill a variety of functions such as uptake or phagocytosis of pathogens and interactions with microbial ligands which lead to host cell activation (Toll-like receptors, *e.g.* TLR9 for CpG motifs). The mannose receptor (MR) is a well-characterized endocytic receptor that binds mannosyl/fucosyl or N-acetylglucosaminyl terminated glycoconjugate ligands. Those ligands are present on a range of bacteria, fungi, virus-infected cells and parasites. There are several mannose receptor-like molecules including Dec205 and DC-SIGN.

Immature DCs and macrophages represent most efficient phagocytes. Prior to internalization, the adsorption of specific plasma proteins (opsonins) is a key factor that leads to unspecific uptake and clearance of microparticulate matter in the tissue. Unspecific uptake can be drastically reduced by surface modification with highly hydrated poly(ethylene glycol) (PEG) chains [4]. Phagocytosis has been demonstrated to be significantly influenced by the surface characteristics of the microparticles. When appropriately covered with PEG chains, *e.g.* through the adsorption of suitable surfactants, a highly hydrophilic and protein repellent surface is created. Owing to the brush-like conformation of the PEG chains anchored on the microspheres, their surface becomes sterically stabilized, and both opsonization and unspecific phagocytosis by phagocytes is inhibited or blocked. Indeed, inhibition of unspecific phagocytosis is a prequisite to allow specific phagocytosis mediated by suitable ligands, the aim of a present study in our laboratory.

We expect that surface modifications with the above-described ligands and PEG will address specific sub-cellular pathways of uptake, maturation and activation of dendritic cells. It is envisaged that in conjunction with genomic profiling by microarray and PCR technologies, promising vaccine formulations can be identified and selected prior to *in vivo* testing.

Thus, in addition to the present state of the art, polymeric microparticulates represent a versatile technology to allow the bioengineering of vaccine delivery systems of complex nature and beyond our current anticipation.

Technologies for Manufacturing Particulate Delivery Systems

The therapeutic or immunologic benefit of micro- or nano-encapsulated drugs and vaccines brought forth the need to prepare such microparticulate carriers in larger quantities and sufficient quality suitable for clinical trials and commercialization. Very commonly, microencapsulation processes are based on the principle of so-called solvent extraction/evaporation, spray-drying or coacervation. While lab-scale experiments can be mostly conducted in simple beaker/stirrer set-ups or laboratory spray-driers, products for clinical trials or the market require economic, robust and well-controllable technologies, preferably allowing for aseptic production. To this aim we have developed novel technologies such as solvent extraction using extrusion through micro-fabricated micro-channel devices for non-turbulent static mixing, so-called micromixers, and ultrasonic atomization in reduced pressure atmosphere [5]. Here, we would like to describe briefly the micromixer technology.

Solvent extraction technology based on extrusion through static micromixer is an appealing approach for industrial, aseptic microsphere manufacturing [5]. In this technology, a solution of the microsphere-forming polymer is processed along with an extraction fluid for the solvent of the polymer solution. Upon mixing in a micromixer, the polymer solution forms small droplets in the extraction fluid, whereby the polymer solvent partitions into the extraction fluid. This gives birth to embryonic microspheres, which are hardened during further solvent diffusion along their pathway into the collection fluid. The final microspheres are separated from the collection fluid by filtration and dried under vacuum or freeze-dried. The technology offers several advantages over common reactors in that the mixer is quite small, can be accommodated in any isolator, and the set-up is simple and suitable for continuous production. All equipment can also be readily sterilized. In contrast to a reactor-based process, scaling-up does not cause changes in the processing and hydrodynamic conditions of the necessary droplet formation process. Very interestingly, the process is also suitable to prepare core-shell type microspheres with a release controlling central polymeric matrix, typically made of PLGA, and a shell of a second polymer carrying specific ligands for targeting or maturation stimuli for specific cell types, as outlined above.

Skeletal Tissue Engineering

Skeletal deformities due to congenital defects, disease and injury have a significant impact on the psychological well being, appearance, and function of patients. Temporomandibular disorders alone are estimated to affect 30 million individuals in the US, with an incidence of more than one million patients per year. Current treatments of refractory cases reflect the inadequacy of existing techniques to restore the skeleton. In many cases, tissue loss is treated by autologous tissue grafting, a method limited by the harvesting difficulties, donor site morbidity and the clinicians' ability to contour delicate 3D shapes. Novel approaches include conduction (by a scaffold), induction (by bioactive molecules) of

cell migration to repair relatively small defects, and cell transplantation into the defect site (with or without biomaterial) to repair larger defects [6]. All these methods are inadequate for most parts of the craniofacial skeleton because of the complexity of the structures being replaced. The most demanding challenge in the context of craniofacial defects involves the presence of multiple connective tissues incorporating fibrous joints and/or bone-cartilage interfaces. Therefore, ideal repair would involve tissue grafts that allow directed *differentiation of stem cells* to achieve the desired structure and functionality involving the generation of complex tissues. This is the context of skeletal tissue engineering. One approach in the field is to recapitulate some aspects of the environment present *in vivo* during tissue development, and thereby stimulate the cells to regenerate functional tissue structures. This involves cells, a biomaterial, and facilitated delivery of chemical species and biochemical regulatory signals into the scaffolds, in combination with physical stimuli. Our envisioned scenario involves the use of *adult stem cells* obtained from the patient's bone marrow, *biomaterial scaffolds* to serve as structural and logistic template and drive tissue development, and *bioreactors* designed to provide precise control over the cellular environment. References to this subject are summarized in [7].

Mesenchymal stem cells have the capacity to differentiate along different lineages, including cartilage and bone. This feature, their ease of availability, along with their proliferation capacity makes them an interesting choice for the engineering of complex tissues on a single implant, such as autologous osteochondral plugs. We and others have shown that the cells can deposit a 3D organized cartilage- or bone-like matrix on different biomaterials, including partially demineralized bone, unmodified collagen, cross-linked collagen, poly-lactide, and silk [7]. In our studies, silks demonstrated promising *biomaterial* characteristics, such as excellent mechanical strength rivaling high-performance synthetic fibres such as Kevlar, good biocompatibility exceeding those of collagen and poly(lactide) scaffolds and biodegradability [7]. Recently, we demonstrated in cooperation with Prof. David Kaplan from Tufts University in Boston that bone formation on silk scaffolds can be predetermined by the scaffold's geometry (L. Meinel, unpublished data). This is interesting because architectural features of native bone have been shown to play a major role in mechanical stability in general and mechanotransduction in bone cells in particular. Bone geometry also plays an important role in the context of ostechondral plugs, where complex bone geometries are found with a more plate-like geometry of the immediate subchondral bone supported by an underlying trabecular network (Fig. 2).

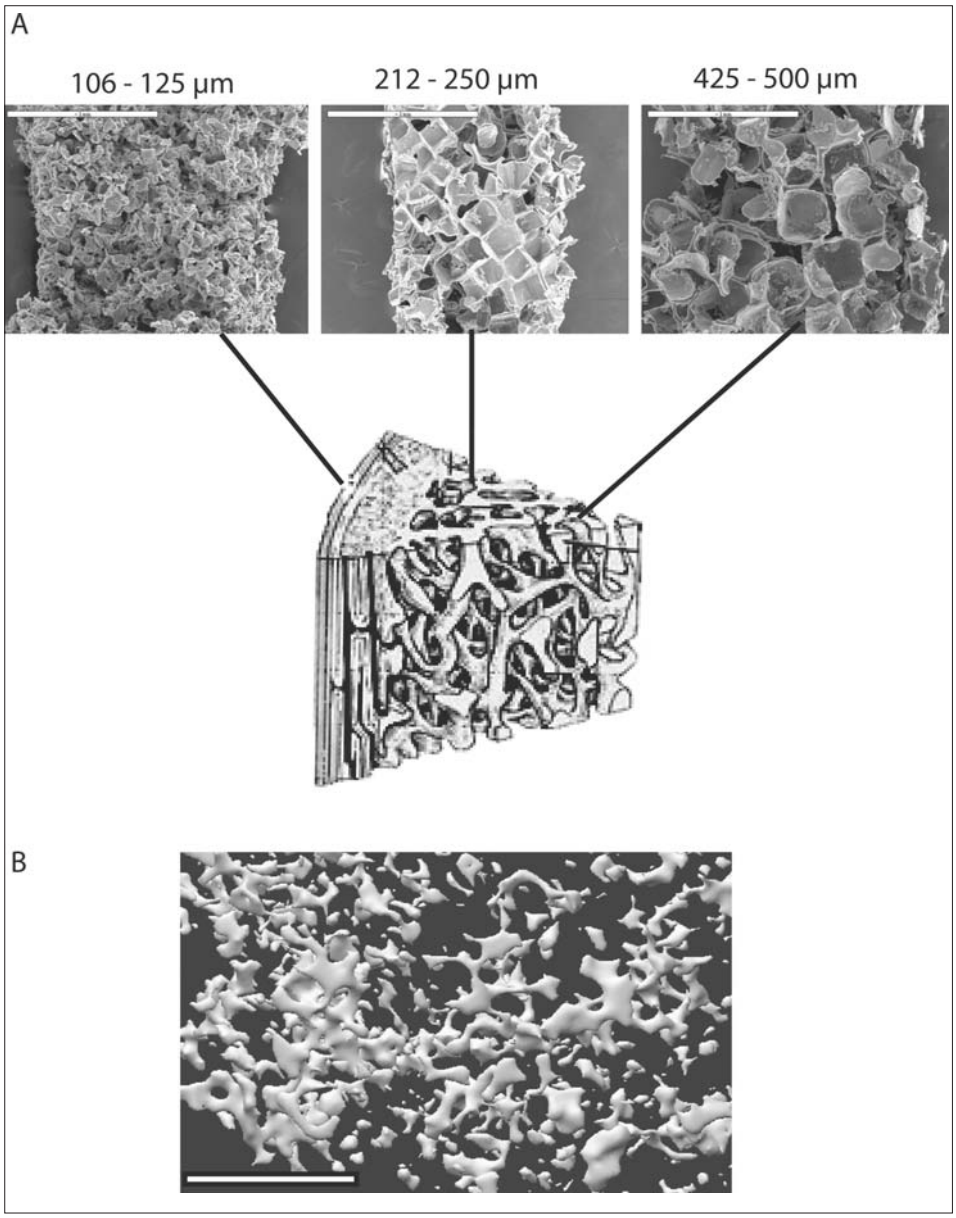

Fig. 2. Tissue engineering of bone based on human mesenchymal stem cells (hMSC) cultured on silk scaffolds. (A) Porous degradable silk scaffolds prepared with a range of pore sizes corresponding to those found in various regions of native bone provided an excellent template for mineral deposition by hMSC (numbers indicate pore size range in µm). (B) Engineered trabeculae resembled those found in native bone. After 4 weeks of cultivation, engineered bone constructs were approximately 1 cm in diameter and 2 mm thick, and contained a dense trabecular matrix; bar length = 1 mm. The formation of mineralized bone was markedly improved over that achieved using collagen scaffolds [7].

The engineering of 3D tissues usually requires *bioreactors* designed to (i) establish uniform distribution of cells throughout the scaffolds, (ii) control culture parameters (*e.g.* temperature, pH, osmolarity, levels of oxygen, nutrients, metabolites, regulatory molecules), (iii) facilitate mass transfer between the cells and the culture medium, and (iv) provide physiologically relevant physical signals (*e.g.* interstitial fluid flow, shear, pressure, mechanical compression) [7]. We have demonstrated that the composition, morphology and mechanical properties of engineered tissues grown in mechanically active environments were generally better as compared to static environments, presumably due to enhanced mass transport at tissue surfaces whereas molecular diffusion remained the dominant mechanism of mass transport within the tissue. In ongoing efforts in cooperation with Prof. Ralph Müller, ETH Zürich, we develop advanced bioreactors linked with imaging devices, thereby providing immediate feedback control through monitoring the progression of mineralization in the developing tissues.

Very few publications describe the engineering of ostechondral plugs. Usually such studies were performed on the basis of in-

dividually engineered tissues, which were then subsequently sutured together, and implanted into osteochondral defects. The engineering of osteochondral structures on a single scaffold using mesenchymal stem cells (which have the potential to differentiate into chondroctes or osteblasts) requires the spatially restricted presentation of factors that specifically guide chondrogenic and osteogenic differentiation, respectively, of mesenchymal stem cells. Our current approaches evaluate the possibility for physical entrapment of growth factors into silks. Initial data demonstrated the feasibility of this approach showing the sustained release of a chondrogenic growth factor – insulin-like growth factor I – from silk films (L. Meinel, unpublished data).

Taken together, our results suggest that osteochondral tissues can be engineered *in vitro* by directed biophysical regulation of human mesenchymal stem cells on 3D silk scaffolds delivering in a localized fashion physically entrapped sequences of regulatory factors, in advanced bioreactors designed to provide environmental control, interstitial flow, mechanical loading and feedback mechanisms to assess tissue growth and quality.

Cell Penetrating Peptides (CPP) as Carriers for Cellular Delivery of Biopharmaceuticals

As a result of the rapid enhancement of the discovery process towards novel bioactive agents, increasing numbers of potential peptide, protein- or nucleic acid based biopharmaceuticals are considered for therapeutic development. Nevertheless, due to their large molecular size, charge and polarity, the clinical development of these biomacromolecules is likely to be problematic. This is a result of their mostly insufficient ability to cross cellular membranes or reach intracellular targets, and explains their often poor or zero bioavailability when administered in test animals or clinically. The discovery of various 10 to 30-mer cell penetrating peptides (CPP), with the ability to translocate cellular membranes has, therefore, opened new perspectives in biomedical research. Chemical ligation or physical assembly of CPP with biopharmaceuticals of poor cellular access is currently an important avenue in engineering delivery systems that could mediate the non-invasive import of such problematic cargoes into cells. Various oligocationic cell penetrating peptides, *e.g.* the HIV-1 derived Tat peptides, the Antennapedia homeodomain derived penetratin, or synthetic peptides have been well reviewed in the literature [8]. They are widely considered for the therapeutic delivery of peptides, proteins, oligonucleotides, plasmids, peptide nucleic acids (PNAs) and even nanoparticles.

Besides these oligocationic CPP, enhanced translocation of the cellular membrane has also been reported for more weakly cationic peptides, *e.g.* hCT(9-32), a 24-mer human calcitonin (hCT) derived CPP with the sequence LGTYTQDFNKFHTFPQTAIGVGFA–NH_2 that we have introduced in a cooperative effort with Prof. Annette Beck-Sickinger's group at the University of Leipzig, and which represents the lead compound for a novel class of CPP (see Table 2). The discovery of the hCT derived CPP originated from the observation of the cellular delivery of an N-terminal fragment of hCT, hCT(9-32), into epithelial cells of excised bovine nasal mucosa [9]. The key requirements for the cellular translocation of hCT derived CPP was further studied in a collaborative effort [10]. Specifically we found that truncated sequences of hCT, from hCT(9-32) to hCT(18-32), penetrated the plasma membrane of a fully organized epithelial model, namely differentiated MDCK monolayers, and resulted in a sectoral, vesicular cytoplasmic distribution. The uptake process was temperature-, time- and concentration-dependent, indicating that translocation may follow an endocytic pathway. Amino acid substitutions of hCT(18-32) suggested that both the Pro in position 23 and the positive charge of Lys in position 18 are crucial for peptide uptake. The reverse sequence hCT(32-18) did not penetrate the membrane, indicating the importance of sequence orientation. We further investigated whether hCT derived CPP, in addition to cellular uptake, have the capacity to deliver an N-terminally conjugated fluorescent cargo through epithelial barriers [10]. For comparison, more classical CPP like Tat(47-57) and penetratin(43-85) were also investigated (see Table 2). Again we used fully organized confluent epithelial cell models for this purpose, *i.e.* MDCK, Calu-3 and TR146 cell cultures. We observed large differences in the uptake pattern between the peptides and the epithelial models (Fig. 3). The results are largely contrasting to a current dogma in CPP research, *i.e.* the more or less uniform capability of CPP to translocate into virtually any type of cells or tissue. Instead translocation was found to depend crucially on the CPP and the epithelial cell type. Moreover, the investigated CPP did not show significant potential for permeation of the model cargo across the epithelial models. The transepithelial permeability of all tested CPP and of their conjugated cargo was even lower than that of usual markers for paracellular permeation, *e.g.* mannitol or mean molecular weight polyethyleneglycols. This questions another dogma in current CPP research, namely the hypothesis that CPP have the potential to cross epithelial and endothelial barriers and deliver CPP ligated therapeutic cargos into virtually every tissue or organ, even across the blood brain barrier [11]. Obviously this is not the case with the currently investigated CPP, at least not to any pharmaceutically relevant or significant extent. The result strongly supports our claim that for the sake of relevance CPP should be investigated in well-differentiated epithelial models, rather than proliferating cell cultures. Nevertheless, the distinct patterns of cellular entry between the investigated CPP suggests potential for targeted epithelial delivery of selected tissues.

The metabolic fate of CPP and their cargo is a third subject of our current research in this area. For this purpose we investigate the metabolic cleavage kinetics and patterns of various CPP in contact with well-differentiated epithelial barrier models [10]. For this purpose hCT derived CPP, Tat(47-57) and penetratin(43-58), were incubated with well-differentiated confluent epithelial models. The metabolic degradation kinetics of the tested CPP in contact with three models, MDCK, Calu-3 and TR146, was evaluated by RP-HPLC. Identification of resulting metabolites of fluorescence labelled hCT(9-32) was through RP-HPLC fractionation and peak allocation by MALDI-TOF mass spectrometry (MS), or direct MALDI-TOF MS of the incubates. The levels of proteolytic activity were highly variable between the investigated epithelial models and the CPP. The Calu-3 model exhibited the highest proteolytic activity. But the patterns of metabolic cleavage of hCT(9-32) were similar in all three models. Initial cleavage of this peptide occured in the N-terminal domain, possibly by endopeptidase activity yielding both the N-terminal and the C-terminal counterparts. Further metabolic degradation was by aminopeptidase, endopeptidase and/or carboxypeptidase activities. In conclusion, when in contact with epithelial models the studied CPP are subject to efficient metabolism, a prerequisite of cargo release on the one hand, but with potential for premature cleavage and loss of the cargo as well. The result sheds light on the importance of the metabolic fate of CPP in contact with epithelia and the need for specific structure modifications to improve CPP performance.

A fourth aspect of our current CPP research is on the cellular mechanisms by which CPP enter cellular compartments. The mechanisms underlying the cellular translocation of CPP are yet incompletely understood and even subject to controversial discussions in the literature [8]. For instance, the cellular translocation of penetratin and Tat peptides was initially assigned to a passive, temperature-independent process or to the inhibition of endocyto-

Table 2. Code, sequence and origin of currently investigated cell penetrating peptides (CPP) [10][11]. Throughout peptides are carboxyfluoresceine labelled at the N-terminus for confocal laser scanning microscopy. Tat and penetratin CPP are included for comparison.

Code	CPP sequence	Origin
hCT(9-32)	LGTYTQDFNKFHTFPQTAIGVGFA–NH_2	Human calcitonin derived
hCT(9-32)-br	LGTYTQDFNKFHTFPQTAIGVGFA–NH_2 | GPDEVKRKKKP	Human calcitonin derived, in combination with simian virus derived nuclear localization sequence in side chain
SAP	VRLPPP–VRLPPP–VRLPPP–OH	Modified from γ-zein, a storage protein of maize
Tat(47-57)	GYGRKKRRQRRRG	HIV-1 derived
Penetratin(43-58)	RQIKIWFQNRRMKWKK	Antennapedia homoeodomain of Drosophila

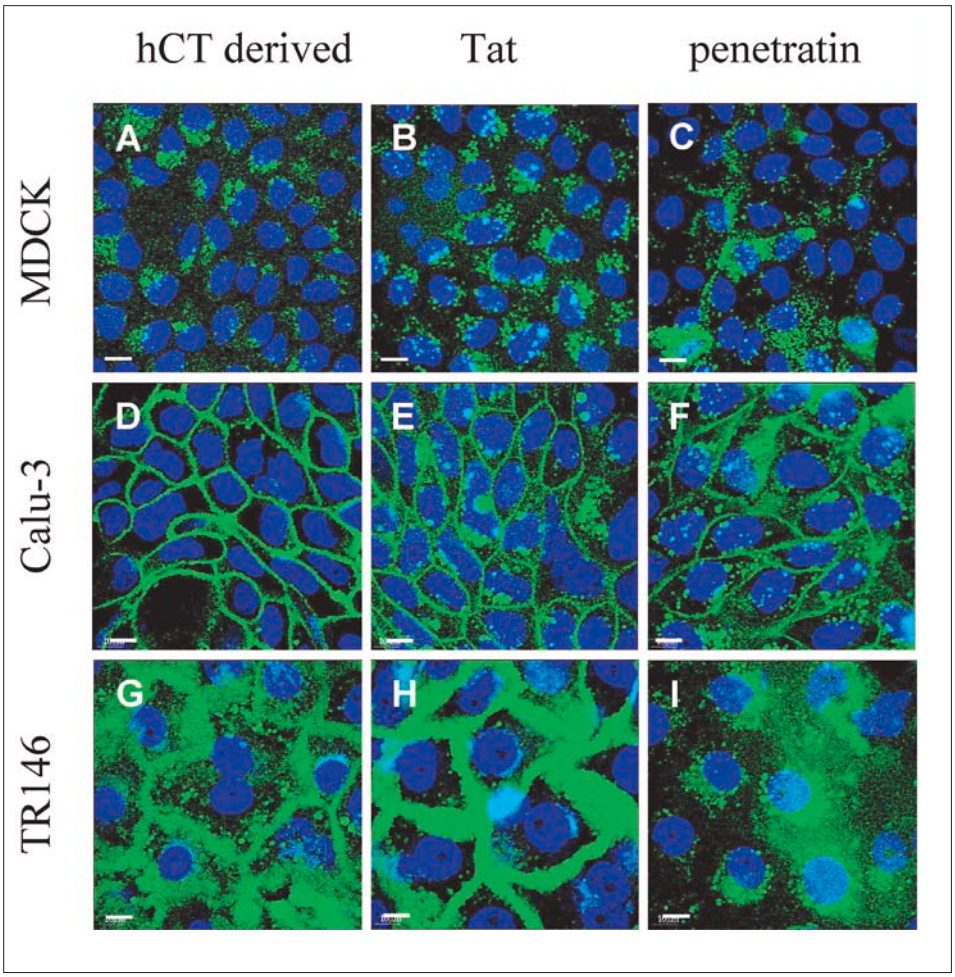

Fig. 3. Cell line dependent cellular uptake of carboxyfluorescein labelled hCT derived peptides, Tat(47-57) and penetratin(43-58) in MDCK and Calu-3 cell monolayers and TR146 cell multilayers. Confocal laser scanning microscopy. Confluent MDCK (panels A-C), Calu-3 (panels D-F) and TR146 (panels G-I) layers incubated at 37 °C for appropriate time periods with 40 μM of hCT(12-32) (panel A) or hCT(9-32) (panels D and G), 10 μM Tat(47-57) (panels B, E, H) and 10 μM penetratin(43-58) (panels C, F, I). Note the cell line dependent contrasts between formation of cytoplasmic vesicles (MDCK/A-C; TR146/I), exclusively paracellular deposition (Calu-3/D) and combined cytoplasmic vesicular and paracellular deposition (Calu-3/E-F and TR146/G-H). The cell nuclei are given in blue and the CPP in green. Bars: 10 μm. Fig. derived from [10c] with permission from Kluwer Academic Publishers.

sis. The observations were thought to be consistent with a theoretical model for a CPP induced physical perturbation of the lipid membrane leading to a direct translocation of the plasma membrane. More recently, however, the related hypotheses involving direct translocation have been challenged following several reports on artefactual results that were caused by cell fixation prior to confocal laser scanning microscopy (CLSM) of cells incubated with fluorescence labelled CPP, or by experimental difficulties to distinguish cell surface-associated CPP from CPP internalized in cytosolic compartments [8]. In a collaborative approach we investigated two novel CPP in this respect: the modestly cationic sweet arrow peptide (SAP; VRLPPP–VRLPPP–VRLPPP–OH) and a branched human calcitonin derived peptide, hCT(9-32)-br, carrying a oligocationic, simian virus 40 derived cationic nuclear localisation sequence GPDEVKRKKKP that was conjugated to the side chain of Lys^{18} [12]. We observed (C. Foerg, unpublished data) that both peptides were readily internalized by HeLa cells through an energy-dependent pathway *via* cell membrane lipid rafts. Cellular uptake into cytoplasmic compartments was typically associated with a vesicular fluorescence pattern indicating an endocytic process into cytoplasmic vesicles. Confirmatory information was provided by reduced uptake at lower temperature and in the presence of endocytosis inhibitors. The conclusion of lipid-raft mediated endocytosis was supported by colocalization of translocated SAP and hCT(9-32)-br with biochemical markers for clathrin-independent pathways as well as with the early endosome antigen 1 (EEA1). Translocation was further impaired by agents that disrupt lipid rafts. Endosomal escape of the peptides could be demonstrated by means of a pulse-chase study. Our results are of mechanistic interest for further development of CPP as tools for the cellular delivery of biomacromolecular therapeutics.

Received: July 13, 2004

[1] Anonymus (Editorial), *Nat. Biotechnol.* **1998**, *16*, 115.
[2] P. Johansen, Y. Men, H.P. Merkle, B. Gander, *Eur. J. Pharm. Biopharm.* **2000**, *50*, 129.
[3] a) C. Thomasin, G. Corradin, Y. Men, H.P. Merkle, B. Gander, *J. Control. Release* **1996**, *41*, 131; b) Y. Men, B. Gander, H.P. Merkle, G. Corradin, *Vaccine* **1996**, *14*, 1442; c) Y. Men, H. Tamber, R. Audran, B. Gander, G. Corradin, *Vaccine* **1997**, *15*, 1405; d) B. Boehm, M. Peyre, D. Sesardic, R.J. Huskisson, F. Mawas, A. Douglas, D. Xing, H.P. Merkle, B. Gander, P. Johansen, *Pharm. Res.* **2002**, *19*, 1330; e) M.

Peyre, D. Sesardic, H.P. Merkle, B. Gander, P. Johansen, *J. Pharm. Sci.* **2003**, *92*, 957.

[4] a) Y. Men, R. Audran, C. Thomasin, G. Eberl, S. Demotz, H.P. Merkle, B. Gander, G. Corradin, *Vaccine* **1999**, *17*, 1047; b) M. Peyre, R. Fleck, D. Hockley, B. Gander, D. Sesardic, *Vaccine* **2004**, *22*, 2430; c) S. Faraasen, J. Vörös, G. Csucs, M. Textor, H.P. Merkle, E. Walter, *Pharm. Res.* **2003**, *20*, 237; d) R. Audran, K. Peter, J. Dannull, Y. Men, E. Scandella, M. Groettrup, B. Gander, G. Corradin, *Vaccine* **2003**, *21*, 1250.

[5] a) S. Freitas, A. Walz, H.P. Merkle, B. Gander, *J. Microencapsulation* **2003**, *20*, 67; b) S. Freitas, H.P. Merkle, B. Gander, *J. Control Release* **2004**, *95*, 185.

[6] a) L. LeResche,. *Crit. Rev. Oral. Biol. Med.* **1997**, *8*, 291; b) L. Meinel, O.E. Illi, J. Zapf, M. Malfanti, H.P. Merkle, B. Gander, *J. Control. Release* **2001**, *70*, 193; c) L. Meinel, E. Zoidis, J. Zapf, P. Hassa, M.O. Hottiger, J.A. Auer, R. Schneider, B. Gander, V. Luginbühl, R. Bettschart-Wolfisberger, O.E. Illi, H.P. Merkle, B. von Rechencherg, *Bone* **2003**, *33*, 660; d) E. Alsberg, E.E. Hill, D.J. Mooney, *Crit. Rev. Oral Biol. Med.* **2001**, *12*, 64.

[7] a) G. Vunjak-Novakovic, *Novartis Foundation Symposium* **2003**, *249*, 34; b) L. Meinel, V. Karageorgiou, R. Fajardo, B. Snyder, V. Shinde-Patil, L. Zichner, D. Kaplan, R. Langer, G. Vunjak-Novakovic, *Ann. Biomed. Eng.* **2004**, *32*, 112; c) L. Meinel, V. Kareourgiou, S. Hofmann, R. Fajardo, B. Snyder, L. Zichner, R. Langer, G. Vunjak-Novakovic, D. Kaplan, *J. Biomed. Mater. Res.* **2004**, *71A*, 25. d) L. Meinel, S. Hofmann, V. Kareourgiou, L. Zichner, R. Langer, D. Kaplan, G. Vunjak-Novakovic, *Biotechnology & Bioengineering* **2004**, in press; e) L. Meinel, S. Hofmann, V. Karageorgiou, C. Kirker-Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. Vunjak-Novakovic, D.L. Kaplan, *Biomaterials* **2005**, *26*, 147, f) G.H. Altman, F. Diaz, C. Jakuba, T. Calabro, R.L. Horan, J. Chen, H. Lu, J. Richmond, D.L. Kaplan, *Biomaterials* **2003**, *24*, 401; g) J.R. Mauney, J. Blumberg, M. Pirun, V. Volloch, G. Vunjak-Novakovic, D.L. Kaplan, *Tissue Eng.* **2004**, *10*, 81.

[8] R. Trehin, H.P. Merkle, *Eur. J. Pharm. Biopharm.* **2004**, *58*, 209.

[9] a) S. Lang, B. Rothen-Rutishauser, J.C. Perriard, M.C. Schmidt, H.P. Merkle, *Peptides* **1998**, *19*, 599. b) M.C. Schmidt, B. Rothen-Rutishauser, B. Rist, A.G. Beck-Sickinger, H. Wunderli-Allenspach, W. Rubas, W. Sadee, H.P. Merkle, *Biochemistry* **1998**, *37*, 16582.

[10] a) R. Trehin, U. Krauss, R. Muff, M. Meinecke, A.G. Beck-Sickinger, H.P. Merkle, *Pharm. Res.* **2004**, *21*, 33; b) R. Trehin, H.M. Nielsen, H.G. Jahnke, U. Krauss, A.G. Beck-Sickinger, H.P. Merkle, *Biochem. J.* **2004**, *382*, 945. c) R. Trehin, U. Krauss, A.G. Beck-Sickinger, H.P. Merkle, H.M. Nielsen, *Pharm. Res.* **2004**, *21*, 1248.

[11] a) J. Fernandez-Carneado, M.J. Kogan, S. Castel, E. Giralt, *Angew. Chem. Int. Ed.* **2004,** *43*, 1811; b) U. Krauss, M. Muller, M. Stahl, A.G. Beck-Sickinger, Bioorg. *Med. Chem. Lett.* **2004,** *14*, 51.

[12] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, *Science* **1999**, *285*, 1569.