advancements and a unique feature of the CDTM series is the integration of 'Technical Application' sessions. For CDTM2010 Olav Scheimann (St Andrews University, UK) presented a session on EPR spectroscopy and its value in resolving conformations and dynamics of membrane associated proteins. Werner Witke (Leica Microsystems, DE) presented a session on Total Internal Reflection Microscopy (TIRF) and its capacity to study crucial events that occur on and very close to the plasma membrane. Dries Vercauteren (Ghent University, BE) presented a session comparing pharmacological and molecular approaches to inhibit endocytic pathways and the requirements to integrate both to gain a more accurate picture of uptake pathways.

CDTM2010 welcomed over 180 registered delegates from 27 different countries and from five continents. Over 90 delegate posters were presented, with six of these also selected to highlight their work via short talks. The publication of the CDTM2010 abstracts in the journal *Drug Discovery Today* is a major landmark for the symposia series reflecting its international standing in the scientific community, its maturation into an important event in the Drug Delivery calendar and its ability to consistently deliver high quality science both on the podium and through the delegate contributions. It is hoped that all who attended CDTM2010 departed with renewed energy for the scientific challenges they face.

We look forward to CDTM2012.

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DELEGATE ABSTRACTS

A1

Design and development of polymeric nanoparticles for targeted delivery of nucleic acid-based therapeutics to tumor sites

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Nucleic acids are widely used as potent therapeutics in cancer research. They can either promote gene expression by bringing a gene either not expressed or under-expressed into tumor cells (cDNA), or alternatively silence expression of genes such as oncogenes (RNAi mediators). However, before they can be efficiently translated to the clinic, this technology requires some optimization: nucleic acids and their vehicles need for instance to be protected from rapid elimination from the bloodstream (opsonization, clearance, and nuclease-mediated degradation) and the specificity of tumor addressing has to be validated. Hence a polymeric nanoparticular carrier encapsulating nucleic acids, either plasmid DNA or siRNA, was developed. Nanoparticles are composed of (1) PLGA, a well tolerated and biodegradable polymer, (2) PEG groups to avoid opsonization, (3) PEI moieties to complex nucleic acids and to enhance cytosolic delivery and (4) RGD sequence for active tumor targeting. Nanoparticles were formulated by double emulsion or water-in-oil-in-water method. Physical properties of such nanoparticles were assessed by dynamic light scattering (size and polydispersity index) and laser doppler electrophoresis (zeta potential). The efficiency of nucleic acid encapsulation into the carrier was determined by the Picogreen assay. Cytotoxicity and transfection capacity were assessed in an *in vitro* model of B16F10 melanoma cells. To date, various designs of nanoparticles were successfully formulated with appropriate size, surface charge and encapsulation efficiency. The PLGA nanoparticles did not show cytotoxic effects on cells and, although less efficient than PEI alone, allowed DNA delivery into tumor cells.

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A3

Pulmonary delivery of mRNA: *in vitro* and *in vivo* evaluation

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Gene therapy is a very promising field of research in medicine. The success of gene based therapeutics will depend on a well thought-out and well-designed delivery system, which should guide the nucleic acids into the desired compartment of the selected cells. However, humans and other organisms have developed natural barriers that protect their body against different kinds of pathogens or intruders. During the evolution of the human being, these barriers have become almost perfect and difficult to overcome. The nuclear membrane, one of the final barriers that protect our genes, appears to be the most important and the crucial one to overcome in non-viral gene delivery. In this work we try to avoid the need to overcome this barrier by intracellular delivery of mRNA instead of pDNA. mRNA delivery has many advantages. First, mRNA does not have to overcome the nuclear barrier and therefore mRNA can transfect also non-dividing cells or dividing cells independent of their cell cycle. Second, mRNA cannot integrate in the genome. Consequently, mRNA mediated gene expression is transient and the risk of insertional mutagenesis can be excluded. Third, there is no need to select a promoter [1]. In this work we evaluate whether mRNA complexed with cationic liposomes (composed of e.g. the cationic lipid GL67) are able to transfect the respiratory tissue of mice. The efficacy of the mRNA:liposome complexes and the gene expression kinetics will be studied and compared with pDNA:liposome complexes. In this study we focus in particularly on GL67-based liposomes. GL67 is an amphiphile consisting of a cholesterol anchor lined to a spermine headgroup in a 'T-shape' configuration. It was proven that GL67 based liposomes are the most effective non-viral pulmonary gene delivery systems [2]. Evaluation of the

in vitro luciferase gene expression in A549 lung adenocarcinoma cells and selection of the most optimal mRNA:liposome ratios are the first steps towards this goal. The efficiency of mRNA/GL67 complexes will be compared to its 4th generation plasmid counterparts–pCpG-CMV-Luc/GL67 lipoplexes. This non-viral mRNA delivery system is potentially a more efficient way for delivering therapeutic genes specifically and directly to the respiratory tract. The respiratory tract is a very interesting and important target organ for gene therapy as it is affected by many acute and chronic diseases, such as cancer, cystic fibrosis, asthma, alpha-1-antytrypsin deficiency or respiratory infections. It is quite a special organ with the possibility of non-invasive, topical administration of a drug through the airways.

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A4
siRNA containing nanoparticles: stability of encapsulation and particle size

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A large effort is currently put into the development of nano-scaled carrier systems that can guide siRNA molecules to their target cells after intravenous injection. One of the main issues in this research is the integrity of the siRNA containing nanoparticles in the blood stream. The integrity of the nanoparticles comprises both the particle size and the stable encapsulation of siRNA. Techniques currently available for studying the disassembly and size distribution of siRNA containing nanoparticles are time-consuming and incompatible with biological fluids. We initially developed a fluorescence fluctuation spectroscopy (FFS) based method which allows us to monitor the integrity of siRNA-carrier complexes in less than one minute in complex biological media and at very low siRNA concentrations. Second, while the size distribution of the complexes can be easily measured in a clear dispersion by dynamic light scattering or electron microscopy, it cannot be measured in more complex biological media such as plasma or whole blood, which contain several different interfering components. To address this issue, we have developed a novel technique, based on single particle tracking (SPT) microscopy, for studying the size distribution (and aggregation) of nanoscopic drug complexes in biological fluids. For stabilization of the particle size of cationic lipid based nanoparticles, inclusion of lipids conjugated with PEG is widely used to sterically hinder aggregate formation. We have demonstrated that in order to obtain remaining siRNA complexation to the cationic liposomes, effective encapsulation inside the liposome, or in between lipid multilayers is required, since siRNA electrostatically bound to the outer side of the liposomes is quickly pushed away by the ubiquitous albumin molecules in blood which leads to siRNA degradation and loss of effectiveness. Formation of siRNA protecting multilayers is hindered by inclusion of PEG-lipids, a hurdle that needs to be overcome either by post-insertion of the PEG-lipid into multilayer containing siRNA-liposome complexes, or by efficient encapsulation of the siRNA inside the aqueous core of the PEGylated liposome. Size stabilization in buffer can be easily achieved by inclusion of minor percentages (~1%) of PEG-lipids. In whole blood however, we demonstrate that much higher percentages of PEG–lipids (5–10%) are required to achieve size stabilization. This requirement has not been previously considered because of the lack of a suitable technique to study the aggregation phenomena in whole blood. In our work we demonstrate that assaying the physicochemical properties of siRNA encapsulating nanoparticles should always be carried out in the biological media they are designed to be employed in. Two novel microscopy based methods were developed that enable such characterization in biological fluids such as serum, plasma or even whole blood.

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A5
Investigating the effects of cationic lipid-mediated toxicity and how to optimize liposomal systems for transfection purposes

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For magnetic resonance imaging (MRI) of therapeutic cells, these cells are often prelabelled in culture with iron oxide nanoparticles, enabling them to be non-invasively monitored by MRI following transplantation *in vivo*. Magnetoliposomes (MLs) are nanosized Fe_3O_4-cores (14 nm diameter) each surrounded by a lipid bilayer [1]. Different types of MLs have been utilised for biomedical research applications [2,3], where cationic MLs are more optimally suited for *in vitro* cell labelling [1]. Unfortunately, cationic lipids display several inherent properties which, to date, have not been clearly defined [4]. In the present work, cationic MLs as well as their non-iron oxide-containing vesicular counterparts were used to label NIH 3T3 fibroblasts. Using distearoyltrimethyl ammoniumpropane (DSTAP) as the cationic lipid the effects on cell physiology of the different particles was compared. Different amounts of DSTAP were used, indicating that when the cationic lipids exceed a certain safe threshold (3.33%), this affects cell viability by different mechanisms that are dependent and independent of actual nanoparticle internalization. Internalization-dependent mechanisms are closely linked to the induction of reactive oxygen species and altered Ca^{2+} homeostasis; the indirect mechanisms appear to indicate plasma membrane destabilization by means of transfer of the cationic lipid from the nanoparticles to the plasma membrane. The extent of cationic effects could be modified by: (1) the size of the liposome, (2) the presence of a stabilising iron oxide core, (3) the use of reactive oxygen species or Ca^{2+} channel inhibitors, (4) the nature of the cationic lipid and (5) the nature of the neutral matrix lipids. Based on these results, a novel cationic peptide-lipid conjugate (dipalmitoylphosphatidylethanolamine-

succinyl-tetralysine [DPPE-succ-(Lys)4]) was synthesized, which efficiently reduced cytotoxic effects and further augmented the internalization efficiency of the MLs [5]. In conclusion, the results indicate that the use of cationic lipids for transfection purposes should be carefully considered as they can induce severe cytotoxic effects. By carefully controlling the physicochemical properties of the liposomal systems used, many of the cytotoxic effects can immediately be reduced. These data highlight the need for careful optimization of cationic liposome formulations and that great advances can still be made with respect to diminished toxicity and enhanced internalization.

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A6
Intracellular iron oxide nanoparticle coating stability determines nanoparticle usability and cell functionality

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Iron oxide nanoparticles are routinely exploited as T2/T2* contrast agents [1]. One of the most active topics in this biomedical research area is the non-invasive imaging of pre-labelled stem or therapeutic cells upon transplantation *in vivo* in [2]. To this end, commercial particles such as Endorem® are frequently employed, however, the particles display several characteristics which makes them less suitable for *in vivo* labelling [3]. In the present work, the effects on cell physiology of in-house produced cationic magnetoliposomes (MLs), that is, 14-nm diameter iron oxide cores each individually enwrapped by a lipid bilayer containing 3.33% of distearoyltrimethyl ammoniumpropane (DSTAP)[4] – a cationic lipid – are compared with the effects of Resovist (carboxydextran), Endorem (dextran) and VSOP (citrate) iron oxide particles. When the particles are incubated at high dosages, reaching high intracellular iron levels, this results in a transient decrease in cell cycle progression, actin cytoskeleton remodelling and focal adhesion formation and maturation [5]. The extent of these effects is in line with the intracellular iron concentration and appears to be common for all particles. When reaching similar intracellular iron concentrations and when verifying that the different particles are routed along the same way and are therefore exposed to similar intracellular microenvironment at fixed time points, it is shown that intracellular stability of the coating molecules is of high importance. The results *in vitro* show that citrate-coated particles are rapidly degraded, whereas those coated with dextran are more stable, but still less than the MLs. The degradation of the particles can be shown by the increase in free ferric ions, and the distorted *r*1/*r*2 ratio of the particles, hampering their use for long-term imaging. Labelled cells further show increases in reactive oxygen species and transferrin receptor expression in C17.2 neural progenitor cells and impeded functionality of PC12 rat pheochromocytoma cells. The extent of these effects is in line with the degradability of the particles *in vitro*. The MLs appear to be the most stable particles and further show a high persistence of the label in continuously proliferating C17.2 cells. In conclusion, the results indicate that the type of coating material used is highly important with regard to maintaining cell functionality and stability of the label. Further characterization of cell-nanoparticle interactions is both warranted and needed [1].

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A7
Nuclear inclusion of inert and chromatin-targeted polystyrene beads and plasmid DNA containing nanoparticles

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Introduction and aim: The nuclear membrane is currently one of the major cellular barriers to the effective delivery of plasmid DNA (pDNA). Cell division has a positive influence on the transfection efficiency from naked pDNA and nanoparticles containing pDNA. At the end of mitosis, the pDNA near the chromatin is probably randomly included in the nuclei of daughter cells during reassemby of the nuclear envelope around chromatin. However, very little is known on the nuclear inclusion of nanoparticles during cell division. We were interested if inert nanospheres get randomly enclosed in artificial Xenopus nuclei and in nuclei of dividing cells. We investigated nanospheres with a different size and charge, and whether the enclosure could be enhanced by the use of chromatin binding peptides such as AT-hooks. *Material and methods*: Non-targeted positively charged, poly-ethyleneglycol (PEG)-ylated and negatively charged green fluorescent polystyrene nanospheres (Molecular Probes) of 100, 200 or 500 nm were used. The 100 nm nanospheres were also modified with Mel-28 (GPSKPRGRPPKHKAKT), mutated Mel-28 (GPSKPGGGPPGHKAKT) or HMGA2 (SPKRPRGRPKGSKNKS), containing an AT-hook or a mutated AT-hook (targeted nanospheres). Artificial nuclei were obtained with the 'Xenopus egg extract (XEE) nuclear assembly reaction'. The enclosure of the nanospheres in the artificial nuclei and upon microinjection was visualised by confocal fluorescence microscopy. *Results and conclusions*: Periodically the non-targeted nanospheres were able to get enclosed in the artificial nuclei but enclosure was rather limited. The enclosure of the positively charged spheres is higher than that of the negatively charged and the PEG-ylated variants, likely as a result of aspecific interactions with the nett negatively charged chromatin. Size is also important: spheres with a diameter of

200 nm and 100 nm are better enclosed than the 500 nm variants. The enclosure of spheres modified with chromatin binding peptides is indeed higher than the enclosure of the non-targeted spheres and the spheres modified with the mutated AT-hook. When polystyrene spheres were injected in the cytoplasm of HeLa cells, initially, the nanospheres spread homogenously in the cytoplasm. Upon cell division, however, the nanospheres accumulated in a specific perinuclear region and enclosure in the nuclei of divided cells was never observed. Therefore, reaching the nucleoplasm seems to be very difficult and we question whether the chromatin binding peptides are able to target the nanospheres into the daughter nuclei of living cells. It thus seems that nuclear inclusion in the XEE assay does not represent the situation in living cells

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A8
mRNA delivery to cervical carcinoma and mesenchymal stem cells mediated by cationic carriers

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We complexed mRNA encoding luciferase (mLUC) to either one of the cationic lipids Lipofectamine (LF) or DOTAP/DOPE, or to linear poly(ethyleneimine), a cationic polymer (linPEI). After incubating the resulting lipo- or polyplexes with HeLa cells for different periods of time, we determined luciferase expression by a bioluminescence assay. Both extent and duration of luciferase expression were dependent on the type of complex used. With LF, mRNA expression lasted for about 9 days maximally, which is not significantly shorter than what can be achieved with pDNA polyplexes. When electroporation was used to transfer mLUC into the cells, luciferase expression lasted for 12 h only. An important characteristic of mRNA-mediated transfection by means of all three complexes is that it could already be detected 30 min after adding the complexes to the cells. In order to estimate the number of positive cells, we transfected the cells with an mRNA encoding Green Fluorescent Protein (GFP) and compared the results with transfection by means of pDNA. With transfection by means of mRNA complexed to LF or DOTAP/DOPE a substantially larger fraction of cells (>80%) was transfected than with pDNA (40%). After establishing the characteristics of mRNA-mediated transfection by means of expression of reporter proteins, we tested the carriers for their ability to mediate expression of a functional protein in mesenchymal stem cells. For that purpose we complexed an mRNA encoding CXCR4, a receptor binding stromal derived factor 1, to the cationic lipids and the polymer. The resulting complexes were incubated with mesenchymal stem cells and CXCR4 expression was assayed. The fraction of CXCR4-positive cells was approximately 80% and 40% for mRNA-cationic lipoplexes and lin-PEI polyplexes respectively. The results of these experiments indicate that mRNA, under certain conditions, may be preferable to pDNA to achieve transfection, particularly in cases requiring transient protein expression.

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A9
Cellular uptake of long-circulating pH-sensitive liposomes: evaluation of the liposome and its encapsulated material penetration in cancer cells

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Print 3G, a peptidic antagonist of oncoprotein involved in breast cancer, could reduce the angiogenic development of breast tumors, leading to tumor dormancy. The necessity of intravenous administration of Print 3G led to the development of long-circulating liposomes as drug carriers. Pegylated liposomes, too large to be collected by fenestrated organs, accumulate passively in solid tumors thanks to the EPR effect. The strategy was to combine the protective properties of PEG with the transfection properties of pH-sensitive lipids that could promote the uptake of liposomes by cells and avoid lysosomal sequestration and degradation of entrapped materials such as peptides. In this study, we compare two formulations in terms of cellular uptake using confocal microscopy. The first one is composed of SPC:CHOL:mPEG-750-DSPE (47:47:6), used as 'standard' liposomes, and the second one composed of DOPE:CHEMS:CHOL:mPEG750-DSPE (43:21:30:6), used as pH-sensitive liposomes.

Firstly, we evaluated the penetration of an encapsulated model molecule, calcein, in Hs578t human breast cancer epithelial cells. When calcein was encapsulated in standard liposomes, its penetration was effective only in a few cells. On the contrary, the majority of cells were fluorescent when calcein-loaded pH-sensitive liposomes were applied on cells for three hours. Secondly, we studied the penetration of liposomes themselves in Hs578t cells using 25-[(nitrobenzoxadiazolyl)methylamino]norcholesterol (NBD-CHOL) as a fluorescent marker of the phospholipid membrane. The obtained results were comparable to those obtained with calcein: a higher penetration of liposome was observed for pH-sensitive liposomes. Finally, the cellular uptake of liposomes using both NBD-CHOL and rhodamine encapsulated in the inner cavity of vesicles was evaluated with Hs578t cells and compared with WI26 human diploid lung fibroblast cells. This experimental design allowed us to follow simultaneously the cell distribution of the encapsulated material and of the liposome itself. Confocal pictures obtained with pH-sensitive liposomes on both WI26 and Hs578t cells allowed us to visualize co-localized red and green of rhodamine and NBD-CHOL, with a higher degree of colocalization in an area close to the nucleus. In comparison with 'standard' liposomes, we observed a higher penetration of the encapsulated material and of the liposome itself in breast cancer cells. Moreover, we visualized a colocalization near the nucleus of liposomes components. From results obtained with fibroblastic cells, there was no difference in terms of cellular uptake between the two formulations. In perspective, we would like to compare these results, obtained with model molecules, with experiments performed with biotinylated Print 3G to assess its cellular distribution. Moreover, it would be interesting to correlate results obtained with confocal microscopy with a possible increase of the peptide efficacy against cancer cells when it is encapsulated in long-circulating pH-sensitive liposomes.

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A10

Role of dynamin-dependent and clathrin-dependent uptake pathways in nonviral gene delivery studied by chemical and genetic means

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Introduction: Endocytosis is known to be a major cell uptake mechanism for non-viral gene delivery vehicles. Several mechanisms of endocytosis have been described and it seems that not all of them are equally beneficial in terms of gene delivery efficiency. According to the literature the preferential cell uptake pathway is both carrier and cell type dependent. Rational design of effective and safe gene delivery vectors requires deeper understanding of the cellular uptake mechanisms of gene delivery vehicles. The purpose of our study was to clarify the role of dynamin-dependent cell uptake pathways, including both clathrin-dependent and caveolae-dependent endocytosis, in non-viral gene delivery. *Methods*: The studies were performed with three widely used non-viral gene delivery systems: cationic polymer branched polyethyleneimine (PEI), cationic lipid N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethyl ammonium methylsulfate (DOTAP) and calcium phosphate (CaP) precipitates. The internalization pathways of these gene delivery vehicles were studied by using genetically modified cell lines: HeLaK44A cells with inducible block of dynamin-dependent endocytosis and BHK21-tTA cells with inducible block of clathrin-dependent endocytosis. As an alternative approach chemical blockers chlorpromazine, dansylcadaverine, nystatin and dynasore were used to inhibit specific endocytic pathways. Relevant concentration of each inhibitor was determined by MTT cell viability assay. Size of the complexes was measured, and expression of marker protein at different timepoints from 0 to 72 hours after exposure to complexes was determined in intact cells and cells with blocked endocytic pathway(s). Results: The obtained data indicated that in both HeLaK44A and BHK21-tTA cell lines for DOTAP-based nanoparticles clathrin-dependent endocytic pathway seemed to be predominantly responsible for successful gene delivery, whereas for efficient PEI-mediated transfection caveolae-mediated pathway was important. In HeLaK44A cells block of dynamin-dependent endocytosis resulted only in moderate (40–50%) decrease of transfection efficiency of both PEI and DOTAP complexes. This suggests that other pathways, not dependent of dynamin, participate in the uptake of both PEI- and DOTAP-based nanoparticles in this cell line. In HeLaK44A cells blockage of dynamin-dependent endocytosis by genetic means increased transfection efficiency of Ca-phosphate precipitates 4-fold whereas chemical blockage of dynamin-dependent pathway by dynasore reduced transfection efficiency of Ca-phosphate precipitates almost completely. However, in general, the results obtained by using genetic means were comparable with results obtained by using chemical inhibitors.

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A11

Enhanced intracellular delivery by guanidinium functionalized ROMP-polymers

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Intracellular delivery of therapeutic molecules has always been a challenge due to the poor permeability of cell membrane to large, negatively charged macromolecules and their restricted biodistribution. In the past decades, cell penetrating peptides (CPPs) are shown to improve the intracellular delivery of bioactive molecules and among the CPPs, arginine-rich peptides are highlighted as the most effective subclass. In the light of this information, we designed and synthesized guanidinium functionalized polyoxanorbornenes which can adopt cell penetrating activity and show superior uptake properties compared to peptide analogues (i.e. nonaarginine, R9). The structure–activity relationship was studied by mono-guanidinium and di-guanidinium functionalized monomers and a specific trend was observed for each cell line studied. In addition to intracellular uptake profiles of molecules, their exceptional ability to deliver bioactive cargo, such as DNA, siRNA and intact proteins, into both adherent and suspension cell lines, as well as in primary cells has been demonstrated. A non-covalent complexation approach was utilized for the delivery of bioactive molecules, instead of covalent attachment. Non-covalent interactions are highly favored over covalent attachment of cargo, in terms of simplicity, efficiency of delivery and stability of bioactive cargo. Furthermore, structural requirements and optimal experimental conditions have been investigated for an efficient intracellular delivery agent.

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A12

Engineering functional chitosan for delivery of drugs or RNAs

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In the last decade, considerable studies on preparation of nanocarriers with cationic liposomes or polymers have been reported for intracellular delivery of DNA and siRNA [1]. Particle uptake has been proven through several kinds of endocytosis pathways, but the uptake efficiency varies depending on the property of carrier materials, particle size, and cell types. Using biocompatible and biodegradable chitosan (CTS) as carrier material, we designed and synthesized functional chitosan derivatives (such as amphiphilic CTS, ligand-targeted CTS), and then developed different technologies to prepare CTS nanoparticles for the potential application of loading, delivering and releasing anti-cancer drugs or RNA therapeutics (siRNA and microRNA). In one system, we initially conjugated a fatty acid (LA) to CTS to obtain amphiphilc CTS-LA, and then synthezised CTS-LA-TM by quaternization. Subsequently nanoparticles with size less than 200 nm can be easily formed by self-assembly of CS-LA-TM in biological solution or neutral solution [2]. These loaded PTX with encapsulation efficiency of 60–90% and showed sustained release in 1 week without burst release. Alternatively,

we formulated CTS-RNAs (siRNA or microRNA) nanoparticles by direct complexation. The nanoparticles with sizes of 120–200 nm and surface charge of ~20 mV showed complex stability and efficiency of protecting RNAs from RNase degradation. These nanoparticles can both transfer RNAs into cells and protect entrapped intracellular RNAs, in 2–4 hours without apparent critical cytotoxicity. Moreover, cell adhesive peptide GRGDY has been grafted to CTS by photosensitive crosslinker [3], and PEGylation has been carried out for target transportation to tumor cells with overexpressed integrin receptors and for efficient delivery of drugs or RNA therapeutics.

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A13
Incorporation of 2,3-diaminopropionic acid in linear cationic amphipathic peptides produces pH sensitive vectors

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Non-viral vectors that harness the change in pH in endosomes are increasingly being used to deliver cargoes, including nucleic acids, to mammalian cells. Here we present evidence that the pK_a of the β-NH2 in 2,3-diaminopropionic acid (Dap) is sufficiently lowered, when incorporated in peptides, that its protonation state is sensitive to the pH changes that occur during endosomal acidification. The lowered pK_a around 6.3 is stabilised by the increased electron withdrawing effect of the peptide bonds by inter-molecular hydrogen bonding and from contributions arising from the peptide conformation, including mixed polar/apolar environments, Coulombic interactions and inter-molecular hydrogen bonding. Changes of the charged state are therefore expected between pH 5 and 7 and large-scale conformational changes are observed in Dap rich peptides, in contrast with analogues containing lysine or ornithine, when the pH is altered through this range. These physical properties confer a robust gene delivery capability on designed cationic amphipathic peptides that incorporate Dap. Recent results investigating the link between hydrophobicity, number of charges, Coulombic interactions and side chain pK_a are considered in terms of the efficiency of gene delivery.

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A14
Octaarginine mediated delivery of fluorescent cargo to human smooth muscle cells

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The high incidence and severity of diseases involving smooth muscle dysfunction, which include cardiovascular diseases and premature labour, dictates the need for our continued search for novel therapeutic strategies to treat these conditions. Cell penetrating peptides (CPP) are a class of non-viral vectors that show considerable promise for drug delivery purposes yet their suitability for uptake, and delivery of biologically active cargo, to human native cells and tissues remains unresolved. For any new drug delivery strategy, including the use of CPPs, to reach fruition this needs to be elucidated. We have begun to explore this issue for CPPs applied to human uterine cells and tissues (including myometrium and blood vessels) obtained from biopsies collected, following LREC-approved written informed consent, from patients undergoing elective Caesarean section at the end of pregnancy. Primary cultured human myometrial cells were prepared on glass-bottomed culture dishes, grown to 80–90% confluence and exposed to serum-reduced conditions overnight before exposure to CPP (or, separately, were methanol-fixed for subsequent immunofluoresence staining of protein localisation). Cellular uptake of fluorescently labelled (Alexa 488) D-Octaarginine (R8, 2 μM) was assessed in the first series of experiments for 24, 48 and 72 hours ($n = 2$). At each time point, z-section confocal microscopy revealed punctate intracellular fluorescence (indicative of vesicular compartmentalisation) particularly dense in the perinuclear area. A second series of experiments assessed the time-course of intracellular delivery up to 24 hours. Punctate intracellular loading was observed by 4 hours. More dense perinuclear and plasma membrane-localised fluorescence was observed at later time points. Immunofluoresence labelling revealed that human myometrial cells possessed expected cytoskeletal (α-smooth muscle actin, tubulin), plasma membranous and perinuclear localised components of endocytotic pathways (Caveolin-1, Clathrin Heavy Chain, Early Endosomal Antigen-1, Lysosomal Associated Membrane Protein-1 and 2 and Flotillins). Next, small segments of native (non-cultured), human uterine tissue were incubated with 2 μM D-R8 and nuclear dye Hoechst 33342 (1 μM) for 4 hours. Confocal microscopic examination revealed peptide entry into smooth muscle cells of both the myometrium and uterine blood vessels with homogenous intracellular fluorescence in many cells but some with more punctate perinuclear/nuclear fluorescence. In uterine tissues incubated with a similar, putatively cell-impermeant, Alexa488 control peptide $(GS)_4GC$, no intracellular fluorescence was observed. These preliminary investigations illustrate that an octameric cationic CPP can successfully enter primary cultured and native human smooth muscle cells and tissues. This opens up a new avenue for targeted delivery of cellular therapeutics in human tissues and in particular to human smooth muscles.

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A15
Endocytic DNA and siRNA delivery mediated by pH sensitive peptides

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Amphipathic peptides have emerged as promising candidates for both DNA and siRNA delivery. Consisting of both hydrophilic and hydrophobic domains, they bind to nucleic acids and at the same time provide pH dependent membrane destabilising activity, promoting endosomal escape. The aim of this study was to investigate the efficiency of such peptides in delivering siRNA and plasmid DNA, and to improve our understanding of how the structural difference between the peptides could affect the uptake mechanism and intracellular trafficking of the system. A series of structurally related histidine-rich amphipathic peptides (LAH4-L1, LAH6-X1L, LAH6-X1-26 and LAH6-X1-W) were investigated. The LAH peptides are 25–26 amino acids in length and comprise cationic lysines to allow electrostatic interaction and complexation with the negatively charged nucleic acids. Each of the peptides also contains four or six histidine residues. With a starting pK_a around 6.0, the imidazole group of histidine may allow buffering and subsequently destabilise endosomes, thus enhancing endosomal escape of the nucleic acids. The LAH peptides demonstrated pH responsive character whish is classically manifested as a conversion from an alpha helical conformation at neutral pH to a disordered conformation at acidic pH. Differences in the number of charges and the hydrophobicity in the four peptides affect the nature and pH dependence of this transformation. Luciferase reporter gene studies showed that the *in vitro* DNA transfection efficiency of the LAH peptides were comparable to commercially available lipofectamine in both A549 and MCF-7 cells. These peptides, in particular LAH6-X1L, also showed high resistance to serum in MCF-7 cells. In addition, both LAH4-L1 and LAH6-X1L mediated significant knockdown of GAPDH enzyme in siRNA transfection studies in the presence of serum. Live cell confocal imaging was carried out to study the intracellular trafficking of the peptide/nucleic acid complexes. Co-localisation experiments were performed with LAH6-X1L- DNA/siRNA complexes and dextran in A549 cells, with the nucleic acids labelled with rhodamine (red), nucleus labelled with Hoechst (blue) and dextran lablled with Alexa-fluor-488 (green). Dextran is known to be internalised through fluid-endocytosis and end up in endososmes and later lysosomes. At an early stage (within the first hour of post-transfection) there was a high level of co-localisation between LAH6-X1L complexes and dextran (shown as orange in colour). At later stages (300 minutes post-transfection), the degree of co-localisation significantly reduced as the siRNA (red) and dextran (green) was shown to be clearly separated from each other. Our results indicate that the histidine-rich peptides offer great promise as siRNA delivery vectors with the ability to promote endosomal/lysosomal escape.

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A16
Role of polymer architecture on polycation induced cell death: systematic study on molecular mechanism

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More than 3000 references can be found in the literature that relate to the application of polycations for drug or gene delivery. However, systematic studies that try to correlate polymer molecular weight, architecture and/or composition with polycation induced cell toxicity are scarce, and the underlying biomolecular mechanisms remain largely unknown. In this contribution new findings are presented on the mechanisms of polycation induced cell death and its correlation with the polymer architecture and degradation rate. For our studies, we firstly synthesized a polymer library based on L-lysine monomer units. The library contained linear, hyperbranched and dendritic L-lysine analogues in a broad range of molecular weights. We then investigated the effect of molecular weight (Mn), degree of branching and polydispersity on the mechanism of short and long term cell toxicity *in vitro*. The molecular mechanisms underlying cell death at various stages of cell exposure to polycation were identified. The onset and extent of these specific modes of cell death were shown to be dependent on the size and degree of branching of the polycations. Simultaneously the *in vitro* degradation profile for analogues was assessed and correlated with the process of cell death. For the first time the factors contributing to the differential toxicity profile of the L-lysine analogues are analyzed and discussed.

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A17
A novel 3D model for the study of functionalised-nanoparticle penetration into human tissue

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The advancing field of nanotechnology is progressing rapidly towards the development of multifunctional nanoparticles for use in biomedicine. These nanoparticles benefit from functional biomolecules attached to their surface and can act as unique carrier systems. However, the impermeable nature of both the plasma and nuclear membranes hinders their potential. Two current methods to enhance uptake are using external magnetic fields to remotely control particle direction, and functionalising the nanoparticles with a cell penetrating peptide; both of which facilitate cell entry. To date, studies have largely adopted traditional 2D cell monolayers, the results of which cannot reliably be translated to a human body. This study has focused on using 3D collagen gels seeded with human fibroblast cells as a tissue equivalent model for the study of nanoparticle penetration into human tissue. Iron oxide nanoparticles were employed, which have an attached cell penetrating peptide (penetratin); are magnetic (to allow external control via magnetic fields); and are fluorescent (to allow visualisation). Various analytical techniques were used including fluorescence staining, TEM and histology to compare nanoparticle penetration into gel models both with/without penetratin attachment, and with/without the presence of a magnetic field; both of which have previously been shown to increase nanoparticle uptake in monolayer cul-

tures. This study has provided essential insight into the biomedical potential and possible problems of functionalised-nanoparticle tissue penetration.

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A18
Hybrid nanoparticles from cationic lipid and polyelectrolytes as antimicrobial agents

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Cationic lipids and polyelectrolytes with the quaternary ammonium moiety in their chemical structure are potent antimicrobial agents. In this work, cationic bilayer fragments prepared from dioctadecyldimethylammonium bromide (DODAB), carboxymethylcellulose (CMC) and polydiallyldimethylammonium chloride (PDDA), added in this sequence, produced potent antimicrobial particles that were characterized by dynamic light-scattering and tested against two bacteria species: *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Two different diameters for particles were obtained depending on DODAB concentration. At 0.1 or 0.5 mM DODAB, cationic hybrid particles of DODAB/CMC/PDDA presented final mean diameters of 108 or 500 nm, respectively and zeta-potentials of 30 or 50 mV, respectively. Both particulates yielded the same activity against *P. aeruginosa*: 0% of cell viability at 1–2 μg/mL PDDA as the outermost cationic layer. For *S. aureus*, at 2 μg/mL PDDA, cell viability for larger particles was 0%, while for smaller particles, 12–15% of cell viability was still obtained. The antimicrobial effect was dependent on the amount of positive charge on particles and independent of particle size. PDDA revealed a high potency as antimicrobial agent and *P. aeruginosa* was more sensitive to all cationic assemblies than *S. aureus*.

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A19
Novel formulations for tuberculostatic drugs based on cationic lipid

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Cationic bilayers in form of bilayer fragments (BF) or large vesicles (LV) provide adequate environment for solubilization and stabilization of antimicrobial drugs with the advantage of being also antimicrobial agents. In this work, BF or LV interaction with two tuberculostatic drugs, rifamicin (RIF) and isoniazide (ISO) is characterized and the assemblies tested against *Mycobacterium smegmatis*. Methods were employed to determine cell viability, minimal bactericidal concentration and entrapment efficiency for both drugs from dialysis experiments. The occurrence of synergism between cationic lipid and rifamicin was a major result of this investigation. The cationic lipid alone killed *M. smegmatis* over a range of low concentrations. Rifamicin drug particles above its solubilization limit could be solubilized by BF at 0.5 mM lipid. LV were leaky to isoniazide whereas Rifamicin could be incorporated in the cationic bilayer at high percentiles. The novel assemblies may become useful in chemotherapy against tuberculosis.

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A20
Antibody targeting of polymeric nanoparticles for cancer therapy

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Antibodies are now the most common form of therapeutic compound under preclinical and clinical development. Normally these proteins are clinically employed for their ability to bind to their cognate antigen and elicit biological effects such as receptor antagonism. However, the application of antibodies as drug delivery agents is also an area of keen interest. This strategy has successfully reached the clinic in the form of drugs such as the radioimmunoconjugates ibritumomab tiuxetan (Zevalin®), [131I]-tositumomab (Bexxar®) and the drug conjugate gemtuzumab ozogamicin (Mylotarg®). Despite the clinical application of these drugs, direct drug/radionuclide conjugation has many drawbacks such as the necessity for a linker that does not inactivate the drug compound and possible hapten immunogenicity concerns that may arise from systemic administration. To circumvent these issues we have investigated the development of novel drug-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles, coated with a layer of targeting antibodies. This approach avoids direct linkage of the antibody to the drug. We have shown that the conjugation of nanoparticles to antibodies targeting the death receptor Fas can be employed for the specific targeting of colorectal carcinoma cells. Furthermore, we have demonstrated that Fas-targeted nanoparticles encapsulating camptothecin (CPT) elicit an >50-fold improvement in the IC50 of the chemotherapy alone. This improved efficacy is due to several factors including the improved uptake and internalisation of CPT and up-regulation of Fas receptor expression by CPT. The ability to exploit antibodies not only for targeting of drug-loaded nanoparticles, but also to elicit therapeutic effects themselves is an exciting approach to drug delivery. The application of this methodology in cancer and other diseases, where appropriate drug and antibody combinations can be identified, has the potential to synergistically improve their efficacies.

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A21
Cationic PLGA nanoparticles loaded with DNA for gene delivery delivery

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Nonviral gene delivery vectors such as liposomes, dendrimers and polymeric nanoparticles have recently been developed as alternatives to virus-based vectors in order to reduce immunogenicity and toxicity risks. In most formulations, anionic nucleic acids are bound to the positively charged vector surfaces through charge–charge interactions. However, a recent *in vivo* study has shown that in endosomes the DNA:nanoparticles complexes can disso-

ciate and whilst the nanoparticles can reach the cytoplasm, the cargo DNA is ineffectually retained in endo/lysosomal vesicles and thus unable to perform its therapeutic action. Based on these observations, we have developed a novel poly(lactic-co-glycolic acid (PLGA) nanoparticle formulation to encapsulate and deliver target DNA into the cytoplasm of target cells. Our formulation is based on combining salting out and emulsion-evaporation processes to reduce sonication steps in an attempt to overcome DNA destruction by shearing effect. Using this formulation we have produced a uniform population of 250 nm nanoparticles entrapping plasmid DNA in both supercoiled and open circular structures. Transformation assays using plasmids released from the particles demonstrated retention of DNA functionality in these formulations. As nude anionic nanoparticles particles have previously been shown to preferentially localise in late endosomes, we have also formulated nanoparticles bearing a low cationic charge to provoke their release from the endo/lysosomal pathway. Didodecyl dimethyl ammonium bromide (DMAB) coating results in only a 10% increase in size and no significant alteration of DNA release. Furthermore, study of the localisation of fluorescent DMAB coated NP demonstrated their ability to escape from endosomal compartments into the cytosol. Finally, *in vitro* transfection assays performed on mammalian cells using these positively charged nanoparticles entrapping a GFP coding plasmid have exhibited significantly improved transfection profiles than anionic particles or liposomal reagents.

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A22
Click chemistry for the generation of cell permeable apoptotic peptides

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The use of proteins and peptides as drug molecules has been held back by their proteolytic instability and inability to cross-cellular membranes. Proteins and long peptides are often produced by expression in *E. coli* rather than by solid phase peptide synthesis. A drawback of *in vivo* protein and peptide synthesis is the difficulty to selectively modify the product peptide by the attachment of fluorescent dyes or ligation to other macromolecules like polysaccharides, lipids or peptides. Here we present a facile method to modify an expressed protein or peptide to create a C-terminal alkyne group. This functionality is then used inter alia for conjugation to the cell-penetrating peptide octa-arginine. This will provide a vector for delivery across the plasma membrane of cells. To demonstrate our method, we have produced in *E. coli* a peptide derived from the Bak protein; one of the key regulators of apoptosis in eukaryotic cells. In the cell it is usually found bound to Bcl-xL at the outer mitochondrial membrane. If this interaction is disrupted, Bak oligomerizes and forms pores which trigger mitochondria dependent apoptosis through cytochrome c release. Small peptides derived from the BH3 helix of Bak have been shown to induce apoptosis. We have expressed such a peptide in *E. coli* as a fusion protein. The ketosteroid isomerase fusion protein is insoluble and readily purified from cell extracts. The peptide is then cleaved from the fusion protein by reaction with cyanogen bromide at a strategically inserted methionine residue to generate a homoserine lactone at the C-terminus of the Bak peptide. This lactone is then used for direct amide formation with inexpensive propargylamine. The resulting alkynyl peptide serves as a reagent for highly efficient 'click' reactions to couple to a wide range of azides. Since the Bak peptide is not able to cross the cell membrane, the well-known octa-arginine cell penetrating peptide sequence was added as a delivery vector. Here we discuss the synthesis of this semi-synthetic peptide and its interaction with, and uptake into, cancer cell lines.

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A23
Protein delivery through the intestinal epithelium: a vitamin B12-mediated approach

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The vitamin B12 transport pathway offers potential for enhancing the uptake of orally administered biologicals, including proteins, peptides and immunogens. The oral delivery of these large molecules is often impeded by the epithelial cell barrier and proteolysis occurring at the mucosal surfaces. Research efforts have been made to enhance oral delivery by employing carrier molecules or ligands conjugated to the pharmaceutically active component, capable of exploiting specific receptor-mediated uptake (RME) to provide their co-absorption. One of the few potential ligands available for enabling transcytosis across the epithelium is vitamin B12. There are several sites on vitamin B12 molecule that are suitable for modification to form bioconjugates. The route followed in this work examined the preactivation of the 5′-hydroxyl group on the ribose moiety by the use of carbonyldiimidazole (CDI), followed by attack of a nucleophile to furnish the hexanediamine spacer. The resultant α-ω-aminohexylcarbamate VB12 derivative was conjugated to fluorescent carboxy-functional nanoparticles (<200 nm size), for use as a model for potential therapeutic carriers. These systems were applied to confluent Caco-2 monolayers, which characteristically form tight junctions. Although several cell lines express the IF-B12 receptor responsible for the binding, internalisation and transcytosis of VB12, the Caco-2 cell line was chosen as the preliminary *in vitro* model to study the potential of the VB12 transport system for the delivery of VB12-conjugated nanoparticles. Immunostaining and confocal microscopy were used to verify receptor/transport protein expression by the cells, as an essential prerequisite for ligand-based transcytosis. We demonstrate that the surface modification of nanoparticles with the α-ω-aminohexylcarbamate derivative of vitamin B12 enables their resultant uptake and transport in the apical-basolateral direction of

Caco-2 cells through exploitation of the natural receptor governed processes involved in VB12 absorption.

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A26

Four-wave mixing imaging to study protein entry and release in mammalian cells

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Optical microscopy is a powerful tool for tracking the binding, internalisation and subcellular trafficking of delivery vectors to mammalian cells. By exploiting multiphoton processes, subcellular structures can be imaged with intrinsic three-dimensional (3D) spatial resolution. Common fluorescent labels in multiphoton microscopy include organic fluorophores, which suffer from photobleaching, and quantum dots which are more photostable but contain cytotoxic elements (such as Cd or In). Gold nanoparticles (GNPs) are ideal optical labels in terms of photostability and bio-compatibility, but emit weak fluorescent signal. We have developed a novel multiphoton microscopy technique that exploits the third-order nonlinearity called four-wave mixing (FWM) of GNPs in resonance with their surface plasmon. In terms of imaging performances, FWM microscopy features a spatial resolution better than the one-photon diffraction limit and optical sectioning capabilities. We show high-contrast background-free imaging of gold-labels (down to 5 nm size) and sensitivity to the single particle level. We are also able to demonstrate a directed dissociation of the GNP from bound proteins at their surface. These results pave the way for active tracking of conjugated nanoparticles, before the controlled release of therapeutically relevant proteins to a localised site of interest.

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A27

Efficient gene delivery using acid-responsive lipid envelopes for adenovirus

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Gene therapy involves the delivery of a functional gene by a vector into target cells, resulting in a desired therapeutic effect. Adenovirus (Ad) has shown a great promise in gene therapy [1,2]. However, *in vivo* studies have reported an immunogenic response and an overwhelming accumulation and gene expression in the liver resulting in significant hepatoxicity. These issues currently inhibit the use of this vector for use in clinical therapies. Such limitations have been overcome by engineering artificially enveloped Ad using zwitterionic and cationic lipid bilayers [3,4]. However, this resulted in a significant reduction of gene expression *in vitro*. We observed that this may be due to poor release of the Ad from its lipid envelope. In the present work, we have explored the use of pH-sensitive DOPE:CHEMS lipid-envelopes to stimulate the virus release from the envelope and consequently result in higher levels of gene expression. Artificially enveloped Ad (DOPE:CHEMS:Ad) were prepared by lipid film hydration followed by sonication. The physicochemical characteristics of the resulting hybrid biomaterials were characterised by transmission electron microscopy, atomic force microscopy, dot blot, dynamic light scattering and zeta potential measurements. The enveloped viruses exhibited good stability at physiological pH (7.4) but immediately collapsed and released naked virions at pH 5.5. Furthermore, recombinant Ad encoding for beta-galactosidase (β-gal) enveloped in DOPE:CHEMS showed comparable levels of gene expression to naked Ad in different cell lines. These transfection results were further confirmed by studying the intracellular trafficking of fluorescently labelled, Cy3-Ad using confocal laser scanning microscopy (CLSM). Interestingly, Cy-3 Ad enveloped in DOPE:CHEMS showed a uniform fluorescence distribution within the cytoplasm indicating Ad endosomal release. In addition, pH-sensitive enveloped Ad injected directly into human cervical adenocarinoma (C33a) xenografts grown on the flank of nude mice showed similar levels of gene expression to naked Ad. In conclusion, this type of artificially enveloped Ad offers a promising tool in gene delivery since high level of Ad gene expression can be maintained while one can expect to dramatically improve the innate Ad immunogenicity and hepatotoxicity *in vivo*.

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A28

In vitro silencing of TGFβ1 in a corneal epithelial cell line using nanoparticles

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Introduction: Severe ocular inflammatory disorders constitute a sight-threatening group of diseases that present treatment difficulties due to the intrinsic barriers of the ocular surface. Previous work in our group has demonstrated that epithelial cells from human cornea (HCE cell line) basally secrete TGFβ1 (a commonly detected cytokine in ocular inflammatory diseases). At present, gene therapy (including siRNA-based therapies) holds promise for the treatment of several diseases, including ocular disorders. However, the development of safe and effective delivery vehicles still remains a major challenge for its clinical application. *Purpose*: This work is a proof-of-concept study meant to evaluate the efficacy of the *in vitro* gene silencing technique for different siRNAs targeting relevant pro-inflammatory cytokines

involved in ocular surface inflammation. We also want to determine whether the use of nanoparticulated drug delivery systems, based on cationized gelatine and chondroitin sulfate, as carriers for siRNAs improve the level of gene silencing. *Methods*: HCE cells were transfected with specific siRNAs against TGFβ1 and its Receptor 2 (TGFBR2) or against GAPDH as a negative control. Lipofectamine was used at 1.6 μl/well in 24-well plates and different siRNA concentrations from 20 to 300 nM were assayed. Silencing efficacy was tested, comparing Lipofectamine2000- or Nanoparticle-based transfection, at protein and RNA levels. Potential toxicity was evaluated by means of the XTT test. *Results*: TGFβ1 and TGFBR2 silencing reached 70% at the RNA level (measured by quantitative real-time-PCR) when using Lipofectamine. Lower silencing was detected at the protein level (measured by Western blotting or ELISA). However, the use of nanoparticles did not significantively improve the silencing efficacy of the evaluated siRNAs. siTGFβ1- and siTGFBR2-transfected cells showed viability percentages equivalent to those of control untransfected cells. CONCLUSION: It is possible to silence *in vitro* TGFβ1 and TGFBR2 expression in a corneal epithelial cell line by conventional techniques obtaining acceptable silencing levels while maintaining high cell viability. The use of nanoparticles as siRNA vehicles to improve silencing levels requires further studies

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A29
Efficient siRNA delivery and effective gene silencing by lipoplexes

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siRNA is double-stranded RNA typically 21–24 nucleotide base-pairs long. Gene silencing by siRNA has gained wide acceptance in genomics and is already in different phases of clinical trials as a potential therapeutic. Long chain fatty acid conjugates of spermine have previously been synthesized and evaluated in our research group for both gene and siRNA delivery [1,2]. We report the synthesis of two novel unsymmetrical N4,N9-difatty acid conjugates of the naturally occurring polyamine spermine with the aim of developing structure–activity relationships for their potential as non-viral, self-assembly vectors for siRNA delivery. After transfection with lipoplexes of Alexa Fluor® 647-labelled siRNA (a 24-mer from Qiagen), silencing EGFP expression, both the efficiency of delivery and the effectiveness of knock-down (gene silencing) were evaluated in HeLa cells stably expressing EGFP. Analysis was by FACS 48 hours post transfection. All transfection experiments were carried-out in DMEM containing 10% foetal calf serum. The efficiency of intracellular delivery was measured by the (normalized) fluorescence of Alexa Fluor® 647-labelled siRNA; N4,N9-dioleoylspermine (DOS) showed 150% of the delivery efficiency achieved with N4-linoleoyl-N9-oleoylspermine (LOS). However, knock-down results show that LOS is more effective with a reduction of EGFP expression levels from control (100%) to 25 ± 3% at a concentration of 3 μg/well (N/P = 11, $n = 3$ and triplicate replicates). Under the same experimental conditions, DOS reduced EGFP expression to 27 ± 2% at a concentration of 6 μg/well (N/P = 22) and to 32 ± 2% at a concentration of 3 μg/well (N/P = 11). Cell viability was measured as the percentage of viable cells using the Alamar Blue® assay [3]. The results show that at 3 μg/well LOS cell viability is 83 ± 4%, at 6 μg/well LOS cell viability is 46 ± 8%, while at 6 μg/well DOS cell viability is only 32 ± 9%. Transfection of cells with Lipofectamine™2000 resulted in reduction of EGFP expression to 37 ± 3%, with cell viability of 91 ± 6%. We conclude from these results that the unsymmetrical lipopolyamine LOS is an excellent transfecting agent for the delivery of siRNA producing effective gene silencing in the presence of 10% foetal calf serum.

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A30
Peptide dendrimer based drug delivery system

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In the past decades, dendrimers have been extensively studied for their unique properties such as spherical nanostructure, monodistributed size and numerous peripheral functional groups. Peptide dendrimers, which were synthesized from amino acids, have been reported as biomaterials for disease diagnosis and treatment due to their excellent biocompatibility and degradability. Herein, we reported the synthesis of peptide dendrimers and their biomedical applications as molecular probes for magnetic resonance imaging (MRI) and carriers for drug/gene delivery. The synthesis of peptide dendrimers was according to a previously reported method [1]. The dendrimers with different generations were synthesized and functionalized. Targeting moieties, mPEG, Ga-DTPA complexes and anti-tumor drugs were immobilized on the peripheral groups of the dendrimers. The dendrimers immobilized Ga-DTPA complexes were used as MRI molecular probes and the relaxivity of contrast was tested on 1.5 T MRI both *in vitro* and *in vivo*in. The generations of dendrimers were 2, 3, 4, and galactosyl moiety was used as targeting ligand for liver imaging. The relaxivity of the contrasts were measured and for G4 dendrimer was 100.8 mM-1•S-1, which was much higher than that of the commercial Ga-DTPA product. The signal intensities were determined by choosing an appropriate region of interest in mouse liver tissue. After 10 minutes injection, the SI increase in liver tissue was observed with an averaged enhancement of 43% for G3T and 37% for G4T, respectively. The non-specific dendritic agents G2, G3 and G4 showed low SI increases. The dendritic probes of G2T, G3T and G4T showed 25%, 35% and 34% relative enhanced SI after 1 hour injection. The peptide dendrimers were fabricated gene vectors and gene transfections of generation 3, 4 and 5 of peptide dendrimers were compared, the

results demonstrated that G5 showed the highest gene transfection efficiency both in the medium with or without serum. Peptide dendrimer based drug delivery system was with dual targeting and pH-sensitive functions. Dendrimer–doxorubicin conjugates were synthesized via a pH sensitive bond. The drug release at pH 5.0 was much faster that that at pH 7.4. The sustained release time was as long as 20 hours and more than 90% of the immobilized drugs were released at pH 5.0. The *in vitro* anti-tumor effects of the dendrimer drug delivery system were investigated and it showed that the peptide dendrimer was a promising carrier for drug delivery.

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A31
Pyridylhydrazone-based PEG for pH-reversible lipopolyplex shielding

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PEGylation that is reversed after the therapeutic agent reaches the target cell presents an attractive feature for drug, protein or nucleic acid delivery. Amine-reactive, endosomal pH cleavable ω-2-pyridyldithio poly(ethylene) glycol α-(butyraldehyde)-carboxypridylhydrazone N-hydroxysuccinimide ester (OPSS-PEG-HZN-NHS) was synthesized and applied for bioreversible surface shielding of DNA lipopolyplexes. N1-cholesteryloxycarbonyl-1,2-diaminoethane was reacted with pH-sensitive (OPSS-PEG-HZN-NHS) or the corresponding stable (OPSS-PEG-NHS) reagent. Both types of micelles remained shielded at pH 7.4 as demonstrated by size exclusion column separation after 4 hours of incubation at 37 °C. But only disruption of OPSS-PEG-HZN-Chol micelles was observed at endosomal pH 5 in 30 min, while OPSS-PEG-Chol was almost stable for 8 h in the same conditions. Lipopolyplexes composed of DNA condensed with polyethylenimine (PEI), dioleoyl phosphatidylethanolamine (DOPE) and hydrazone linked pH labile lipid Chol-HZN-PEG were prepared by the ethanol injection technique, with particle size of 160 nm and zeta potential of 8 mV. Pyridylhydrazone-based PEGylated lipopolyplexes was as stable as their non-pH sensitive counterparts at physiological conditions, and had smaller size compared with non-PEGylated variants. At pH 5.4, increasing size was only detectable in pH-reversible lipopolyplexes. Both luciferase and EGFP gene transfections of pH-reversible lipopolyplexes showed an up to 40-fold enhancement in gene expression with reversibly shielded polyplexes compared to stably shielded lipopolyplexes. Investigation of cellular association and uptake by flow cytometry, together with intracellular tracking by CLSM reveal the probability of intracelluar deshielding of PEG. Incorporation of a ligand for transferrin receptor targeting further improved the transfection.

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A32
The 5th generation of poly(L-lysine) dendrimer is a potential carrier for *in vivo* in gene delivery

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Poly(L-lysine) dendrimers have been widely used as reagents for *in vitro* gene transfecion. Here, different generations of dendritic poly(L-lysine)s were synthesized, including G3, onium salt G3 (OG3), G4 and G5, and their characteristics for *in vitro* gene transfection and potentials as *in vivo* gene delivery carriers were evaluated. Gel retardation assays proved that the dendrimers could form complexes with plasmid DNA, and dendrimer G3 could inhibit the migration of pDNA at an N/P ratio of 0.5, G4 and G5 at N/P ratio of 1.0 and onium salt G3 at N/P ratio of 2.0. A DNase I protection assay with G5 showed acquired resistance from combining pDNA with dendrimer; this can resist the nuclease-catalyzed degradation, and the protection capacity of G5 was even stronger than that of PEI. Atomic force microscopy demonstrated that all the 4 generations of dendrimer/DNA complexes showed similar particle size within 100–200 nm. At N/P ratios from 1 to 25, zeta potentials of the 4 dendrimer/pDNA complexes gradually changed from negative to positive with a tendency that the higher generation and higher potential value variants gave a stronger combination potency of the complex with negatively charged cell membranes. *In vitro* cytotoxicity evaluation showed good biocompatibility of each dendrimer within N/P ratios of 1–25. Body weight evaluation of BABL/c mice, together with tissue section observation, blood routine detection and blood biochemistry analysis (liver and kidney function, myocardial enzymes and electrolytes, etc.) of dendrimer G5 also showed good *in vivo* biocompatibility 2 and 7 days after tail vein injection. *In vitro* gene transfection comparison revealed that G5 had an obvious higher efficiency than other dendrimers. Transfection efficiencies of each dendrimer were not influenced by the presence of serum, which is a very important merit for *in vivo*in gene delivery. Quantitative analysis in mRNA and protein level showed that the transfection efficiency of dendrimer G5 was ~60% of PEI's, but PEI had obvious toxicity to cultured cells and its transfection efficiency would be greatly reduced by the presence of serum. Considering that dendrimer G5 had almost the same *in vitro* gene transfection efficiency as G6, we concluded that the fifth generation of poly(L-lysine) dendrimer should be a suitable carrier for *in vitro* gene transfection and, more importantly, a potential carrier to construct *in vivo* gene delivery system.

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A33
Muscle-targeted HIF-1a gene expression system for therapeutic angiogenesis in ischemic limbs

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Therapeutic angiogenesis is expected to be a promising treatment for patients with ischemic disorders such as cardiac and limb ischemia. However, recent clinical trials failed to show much expectant benefits, largely due to suboptimal therapeutic genes and delivery strategies. Herein, we focused on the development of a hypoxia inducible factor-1a (HIF-1a) gene induced muscle-specific angiogenesis strategy that would improve safety and effi-

ciency. (1). A muscle-specific eukaryotic gene expression plasmid, pSV40E/MCK-HIF1a, was constructed by integrating SV40-enhancer with MCK promoter to regulate HIF-1a gene expression. (2) *In vitro* and *in vivo* studies both indicated that, compared with the natural MCK promoter, the SV40E/MCK hybrid promoter significantly increased HIF-1a gene expression, while retaining a good muscle-cell specificity. Although less efficient than the nonspecific CMV promoter, the hybrid promoter provided more stable gene expression and represented a good compromise between transcriptional activity and muscle specificity. (3) *In vitro* biological effects of increasing HIF-1a gene expression were analyzed in myoblasts to evaluate the function of the muscle-specific gene expression system. Real-time PCR showed up-regulation of several critical angiogenic genes expression, such as VEGF, ANGPT-1, MMP-2 and SDF-1, which were previously demonstrated to facilitate new blood vessel formation and/or maturation. Transwell cell migration assay revealed that pSV40E/MCK-HIF1a transfected L6 cells could recruit progenitor cells derived from bone marrow and muscle tissue. These observations suggested the muscle-specific gene expression system may be useful for stimulating new blood vessel growth and maturation in ischemic limbs while restricting the therapeutic effect to muscle tissue. (4) When reporter gene was transferred into mice limb skeletal muscles, using various nonionic natural polymers, including hyaluronic acid, alginic acid and dextran, the formulated plasmid/polyer resulted in different levels of reporter gene expression, depending upon the type and concentration of the polymers. Some of them showed better performance than naked DNA and these results indicated that the pSV40E/MCK-HIF1a combined with a suitable nonionic polymer may provide a safe and efficient gene therapy system for treatment of limb ischemia.

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A34
Amphipathic CPPs upregulate Ca in cells' cytosol and induce lysosomal exocytosis

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Cell penetrating peptides (CPP) promote the uptake of different bioactive cargo molecules that makes the harnessing of CPPs a promising strategy for drug design and delivery. The translocation mechanism of CPPs into cells, however, has still remained elusive. Direct passage of peptides across the plasma membrane might interfere with its integrity and introduce disturbances. In our study we assessed how cells compensate the disturbances and which processes are induced in response to CPP uptake. Applying fluorometry, flow cytometry and fluorescence microscopy we demonstrate that the uptake of various CPPs enhances the calcium levels in Jurkat and HeLa cells' cytoplasm. The elevated cytoplasmic free calcium concentration evokes downstream effects of membrane repair response and lysosomal exocytosis. Our results indicate that ten of the most commonly used CPPs can be divided into three groups based on their interaction with plasma membrane, the induction of calcium influx, and downstream responses: (1) primary amphipathic CPPs (e.g. MAP, TP) that modulate plasma membrane integrity inducing influx of calcium ions into cells and activate membrane repair and lysosomal exocytosis starting, from low concentrations; (2) arginine-rich, secondary amphipathic, CPPs (e.g. Penetratin, pVEC) that induce changes in the intracellular calcium concentration or subsequent responses at relatively high concentrations and (3) non-amphipathic CPPs (e.g. Tat, Arg9) that do not evoke changes in the intracellular calcium concentration or subsequent responses even at high concentrations. Triggering of the plasma membrane repair response may help cells to recover by replacing the misorganized or membrane active CPPs containing plasma membrane regions, whereas non-amphipathic CPPs could infiltrate without subsequent cellular responses.

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A35
Cellular delivery of oligonucleotides by PepFect

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PepFect (PF) series of peptide based transfection reagents have been developed for the delivery of oligonucleotides (ON) and plasmids into cells. Some PFs are also capable of nuclear delivery of oligonucleotides, for example phosphorothioate 2′-O-methyl RNA oligomers translocate into nucleus and rescue the luciferase expression in the splicing redirection assay after coupling to PF. The optimal ratio of ON with PF for obtaining the functional complexes has been described earlier, but it is not known how such particles interact with the cell surface, enter cells, and reach nucleus. In order to characterize the oligonucleotide delivery by PFs, we labelled 2′-OMe ON with 1.4 nm Nanogold (NG) particles. The membrane interaction, uptake, and intracellular traffic of ON–NG after complexing with PFs were mapped by transmission electron microscopy to unravel their internalization mechanism. PFs pack the Nanogold-labelled ON into small (~200 nm) particles in solution. Smaller particles of ON–NG–PF complexes associate later to form bigger assemblies at the surface of HeLa cells and are taken up by cells in vesicles. The size, electron density and regularity of ON–NG–PF containing structures vary largely depending on the PepFect and its concentration. In cells the majority of the complexes locate in the endosomal/lysosomal vesicles after four hours of incubation. However, the vesicles often have a discontinuous membrane and the Nanogold-labelled oligonucleotides can be found in the cytosol. In addition, with the help of some PFs, the oligonucleotides also reach the cell nucleus. Our results demonstrate that non-covalent complexes of Nanogold-labelled oligonucleotides with PepFects form particles that concentrate at the cell surface and enter cells by endocytotic mechanism. The finding that oligonucleotides have reached nucleus suggests that ON–PF complexes could induce the destabilization of endosomal mem-

branes, followed by the escape of ON from vesicles and translocation into nucleus. Our electron microscopy results are in line with data published earlier regarding the redirection of splicing with oligonucleotides delivered into cells by PFs.

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A36
Live-cell imaging and single-particle tracking of polyplex internalization

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Systemic delivery of therapeutic genes for gene therapy or cancer gene therapy requires gene vectors that overcome several barriers. The vector has to enable tissue-selective delivery, internalize efficiently and finally release its cargo reliably within the target cell. Tissue specificity and enhanced internalization can be achieved by cell-specific ligands that bind to certain surface markers that are upregulated in, for example, solid cancers. Functionalization with pH-sensitive and redox-sensitive linkers or polymers allows the vector to 'sense' external stimuli that will trigger their activation in temporally and spatially controlled manner. We investigate the uptake of targeted and untargeted polymeric gene vectors (polyplexes) by highly sensitive fluorescence microscopic methods on a single cell level [1]. The epidermal growth factor receptor (EGFR) is overexpressed on a high percentage of human carcinomas and is therefore an attractive therapeutic target for tissue-specific targeting by non-viral vectors in cancer gene therapy. Comparing uptake kinetics and internalization dynamics, single particle tracking in combination with quenching experiments revealed typical three-phase dynamics of the uptake process independent of targeting. Phase I was characterized by slow, actin-cytoskeleton-mediated movement of the particles with drift and included the internalization process. During phase II, particles displayed increased velocities with confined and anomalous diffusion in the cytoplasm. Phase III was characterized by fast active transport along microtubules. Targeting of polyplexes for receptor-mediated endocytosis by the EGF receptor resulted in shortening of phase I and strongly accelerated internalization. Targeted as well as untargeted particles were transported in early endosomes marked by Rab5–GFP and accumulated in late endosomes marked by Rab9–GFP. The endosomal release dynamics of polyplexes consisting of DNA condensed with the cationic polymers linear polyethyleneimine (LPEI), poly-(L)-lysine (PLL) or poly-(D)-lysine (PDL) were studied by photochemical release in living cells [2]. Using double-labeled polyplexes, DNA and polymer were imaged simultaneously by dual-color fluorescence microscopy. Our results demonstrate that the characteristics of the cationic polymer significantly influence the release behavior of the polyplexes. For LPEI/DNA particles, LPEI quickly spread throughout the cytosol, whereas DNA was released slowly and remained immobile thereafter. In the case of PLL particles, both DNA and polymer showed quick release. PDL particles remained condensed upon photosensitizer activation.

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A38
Vascular endothelium remodeling in human African trypanosomiasis

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Molecule movement into the central nervous system (CNS) is restricted by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid (CSF) barrier. Human African trypanosomiasis (HAT) or sleeping sickness, caused by the parasites, *Trypanosoma brucei* (T.b.) gambiense or *T.b. rhodesiense*, is fatal if untreated. The first disease stage is associated with trypanosome proliferation in the periphery. The second stage is when the parasites reach the CNS. HAT treatment is stage specific with drugs, which are assumed to cross the BBB, used to treat CNS stage disease. Since the treatment of CNS-stage HAT is more toxic than that of early-stage, it is vital to stage HAT [1]. Staging requires a CSF sample. Lumbar puncture under field conditions is difficult and invasive. Improved tests for staging HAT are required [2]. Our studies have established that *T.b. brucei* crosses the murine blood–CNS interfaces at ~day 11 post-infection (p.i.) and the animals died at day 37.9 ± 1.23. At day 7, 14 and 21 p.i. no loss of barrier integrity was measurable using the inert tracer, [^{14}C]sucrose (342 Da; radius 4.6 Å), nor was there any endothelium remodeling (including transporter up/downregulation) as measured with eflornithine, pentamidine or nifurtimox [3,4]. BBB, but not choroid plexus, dysfunction, occurred at days 28 and 35 p.i. with resultant increases in [^{14}C]sucrose space [3,4]. Suramin (1429 Da) brain distribution increased at day 35 p.i., suggesting considerable BBB breakdown as this molecule is highly albumin (60 kDa; radius 35.5 Å) bound [4,5]. Furthermore, the increased [^{14}C]sucrose association with the endothelial cell at day 35 p.i. compared to the non-infected and other infected time groups suggested an increase in vesicular trafficking [3]. This loss of integrity may be a sign of terminal disease. However, perhaps there was an earlier loss of blood–CNS barrier integrity (possibly when the parasites entered the CNS) that was not measurable using [^{14}C]sucrose (an inert tracer with smaller molecular dimensions being needed) and/or this was a reversible process that was undetectable at the times studied. Furthermore, endocytosis may be a sensitive marker of endothelium remodeling. The characterization of vesicular expression in a murine model of HAT may be the first step towards vesicle targeted staging strategies. Overall understanding blood–CNS barriers breakdown in HAT could contribute to the development of therapeutics and therapeutic targets to control brain injury and to the characterization of biomarkers for safer staging of the disease.

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A39

Preparation of solid DNA nanoparticles for use in gene therapy

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Background: Non-viral gene therapy, based on nanosized particles, is a potential therapeutic option in various diseases. The success is mainly dependent on an efficient gene delivery vector. Aerosol synthesis can provide pure solid DNA particles with substantial high dose of DNA per particle and thereby increase the amount of DNA delivered into cells as compared to commonly used DNA polymer complexes. The purpose of this study was to test the suitability of plasmid DNA alone or in complex with cationic polymers for the preparation of solid DNA nanoparticles by an aerosol flow reactor method. *Methods*: The sample solutions contained either plasmid DNA (pDNA) alone or complexed at a ratio of 1:1 (w/w) with branched or linear polyethylenimine (PEI) with the molecular weight of 25 kDa. The additive agents, L-leucine and mannitol, were added to PEI/DNA complexes at a ratio of 1:8 (w/w). The aerosol flow reactor method [1] involved atomization of sample solutions to nanosized droplets, which were immediately dried in a heated flow reactor tube by the evaporation of the solvent. The dried nanoparticles were collected with a low-pressure impactor and the size distribution was determined by a differential mobility analyzer. The surface morphology was analyzed using field emission scanning electron microscopy and the structural integrity of pDNA was evaluated by agarose gel electrophoresis. *Results*: The produced pure pDNA nanoparticles were spherical and had a mean diameter of 125 nm. However, pDNA in such nanoparticles did not preserve its supercoiled structure due to the shearing stresses caused by the atomization process. The complexation of pDNA with PEIs before atomization allowed the maintanence of pDNA integrity. The further addition of either L-leucine or mannitol to initial sample solution, stabilized nanoparticles structure and prevented them from water uptake and subsequent deformation. The resulting solid nanoparticles had a mean size between 65 and 125 nm and the loading content of pDNA in a single nanoparticle was approximately 10% (w/w). *Conclusions*: The aerosol flow reactor method provides an effective way of producing solid DNA nanoparticles with a size optimal for cell uptake and for potential use in non-viral gene delivery

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A40

Exploiting a bacterial toxin translocation domain for the endosomal escape of CPP-imported cargoes

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The clinical impact of CPP-based delivery agents has yet to be realized due to a lack of delivery efficiency to the cell cytoplasm. Polycationic cell penetrating peptides are a major class of CPPs. However, upon internalization via endocytosis, these CPPs are typically trapped in endosomes and are subsequently degraded or recycled out of cells. To promote endosomal escape, we investigated the use of a bacterial protein domain derived from *Pseudomonas aeruginosa*, Exotoxin A (ETA253-412), capable of translocating known protein domains out of vesicular compartments. We constructed, expressed, and purified a series of CPP–ETA253-412–eGFP fusion proteins. We used confocal microscopy and flow cytometry to confirm the internalization of CPP (poly-arginine or TAT)-containing constructs at 37°C in human cervical carcinoma (HeLa) cells. Additionally, we observed the time-dependent relocation of CPP-ETA253-412–eGFP constructs from the endosome to the cytosol. These experiments demonstrate the potential of the ETA253-412 translocation domain in relocating cargoes, such as protein therapeutics, siRNA and vaccine formulations, to the cytosol of target cells.

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A41

Investigation of microsphere-mediated cellular delivery

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Recently we have developed a polystyrene microsphere-based system designed to efficiently deliver biological materials into a broad range of cell lines [1,2]. This versatile delivery system is capable of transporting any biological cargo from small molecules to oligonucleotides and bulky proteins into cells [3–5]. However, the specific mechanism of cellular entry is largely unknown and widely varies from study to study. As such, chemical, biological and microscopic methods have been used to elucidate the mechanism of cellular uptake for these nanoparticles in several cell lines. Additionally, gene expression profiling has been used to determine if there is a transcriptional response to 'beadfection' [6,7].

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A42
Cytosolic delivery of macromolecules using pH-dependent fusogenic peptide

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The plasma membrane plays critical roles in maintaining cellular homeostasis. It serves as a barrier against unfavorable attack on cells from an unpredictable external world. However, the membranes are also barriers to intracellular delivery of various therapeutic molecules. For improving their translocation, we developed a novel method using GALA peptide/cationic lipid complexes. The GALA peptide (amino acid sequence: WEAALAEALAEALAEHLAEALAEALEALAA) is a 30-residue amphipathic peptide with a repeat sequence of glutamic acid–alanine–leucine–alanine, and designed to mimic the function of viral fusion protein sequences that mediate escape of virus genes from acidic endosomes into cytosol [1]. The GALA peptide converts its structure from random to helical when the pH is reduced from 7.0 to 5.0, and this leads to destabilization of the membranes. When attached with bioactive cargoes, the GALA peptide may thus serve as intracellular vector bearing efficient endosomal escape function. However, the negative charges from glutamic acids (seven residues) in the GALA sequences reduce the efficiency of binding to a negatively charged cell surface. To overcome this problem, a cationic lipid was employed as an 'adhesive' for pasting the GALA peptide onto cell surface to accelerate its cellular uptake. We examined the ability of GALA peptide as a delivery vector using FITC as a model of membrane-impermeable low-molecular weight drugs. When FITC–GALA (1 μM) was administrated to HeLa cells, co-addition of cationic lipid, Lipofectamine 2000 (LF2000), significantly increased uptake efficiency. In a time-dependent manner, FITC–GALA escaped from endosomes, and diffuse fluorescent signals were observed in both cytosol and nucleus, suggesting that the cytosolic translocation proceeds along with endosomal acidification. The GALA/cationic lipid system was also applied for the intracellular delivery of FITC–avidin protein (68 kDa). When FITC–avidin (250 nM) was mixed with biotinylated-GALA (1 μM)/LF2000 complexes, FITC–avidin effectively internalized into cells, and diffuse signals of the FITC–avidin in cytosol were observed. In the absence of these complexes, efficiency of cytosolic diffusion of the FITC–avidin was quite low. These results suggest the usefulness of our approach for intracellular delivery of macromolecules using GALA peptide and cationic lipid [2].

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A44
A dual uptake mechanism for the peptide Tat–LK15

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Knowing the mechanism of uptake is fundamental in developing new and refining existing drug delivery systems. Recently, Tat–LK15 peptide resulting from the fusion of Tat peptide and the synthetic amphipathic LK15 has shown greater transfection efficiency than Tat alone [1]. However, its uptake mechanism is ambiguous as the influence of the amphipathic peptide (LK15) and Tat peptide upon binding remains unclear. To elucidate this issue, the present study investigates the effect of temperature and peptide concentration on the cellular uptake mechanism of TAMRA–Tat–LK15 peptide. HeLa and HT29 cell lines were incubated with 1, 2.5 or 5 μM TAMRA–Tat–LK15 and TAMRA–Tat peptide solutions at different temperatures (4°C, 20°C, and 37°C) or in the presence of sodium azide (a metabolic inhibitor). A Zeiss LSM510 microscope was used to monitor cellular uptake in using a thermoelectric controlled temperature chamber. Our data indicate clearly TAMRA–Tat–LK15 peptide uptake (diffuse distribution in the cells) at 4°C for a 5 μM bulk solution while images do not suggest uptake in these conditions at lower concentrations (1 and 2.5 μM). At higher temperatures (20°C and 37°C) TAMRA–Tat–LK15 was observed in cells at all concentrations (a mixture of diffuse and punctuated fluorescence in cells). Interestingly, pre-incubation with 10 mM sodium azide did not completely block peptide uptake in cells for 1 and 2.5 μM bulk peptide solutions, and, had no effect at 5 μM and above. Overall, the results suggest the presence of concentration dependent uptake mechanisms of the Tat–LK15 peptide in cells.

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A45
Visualizing the effect of integrin targeting and surface shielding on gene vector uptake by live cell imaging

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Nonviral vectors enable the safe delivery of transgenes into target tissues but are still less efficient than viral gene vectors. To develop novel artificial systems with enhanced efficiencies a detailed understanding of the cellular uptake and intracellular trafficking is essential. By visualizing the entire pathway of a single nanoparticle, from its first contact with the cell surface to the delivery of the DNA to the cell nucleus, detailed information about the mechanisms of uptake, intracellular trafficking and DNA release can be gained. Here we study the effect of integrin targeting and surface shielding on the internalization of gene vectors by live cell imaging with highly sensitive fluorescent microscopy. αvβ3 and αvβ5 integrin receptors are attractive targets for antiangiogenic cancer gene therapy as they play a pivotal role in angiogenesis and proliferation of malignant tumors. A cyclic RGD peptide specifically binds to those receptors and thus can be used for specific targeting of gene vectors such as polyplex micelles. In this study the analyzed micelles consisted of a thiolated PEG-block-poly(lysine) copolymer complexed with fluorescently labeled DNA [1]. To analyze the influence of shielding, two types of micelles containing a differently sized PEG shell were compared. To directly compare the internalization of targeted and untargeted micelles without knowing the details of their internalization pathway, we simultaneously added both micelle types with different fluorescent labels onto cells and evaluated their

colocalization degree over time. Additionally the internalization kinetics of integrin targeted micelles was compared to EGF targeted polyplexes that are well-known for their fast uptake kinetics [2]. The internalization pathway was then studied with inhibitor experiments and by colocalization with specific marker proteins. Our results reveal a strong competition between unspecific electrostatic interactions and specific receptor–ligand interactions that determines successful targeting of the micelles. Enhanced PEG shielding of the micelles leads to the reduction of electrostatic interactions resulting in a specific and faster internalization of the targeted micelles. Additionally we observed a considerable effect of the applied micelle concentration as well as the micelle size on their internalization properties. Our data lead to a more detailed understanding of the targeting effect than can be observed by conventional bulk instruments. The gained knowledge enables to maximize the therapeutic benefit of future gene vectors for clinical application.

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A46
A designer biomimetic vector for breast cancer gene therapy

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Introduction: Gene therapy holds the potential to cure many diseases, provided that the genetic or molecular basis is understood. In cancer, the delivery of therapeutic genes via viral vectors has proven more effective than the current alternative non-viral methods. However tissue specificity, high costs of production and safety remain major concerns with viral delivery. This study examines the use of a designer biomimetic vector (DBV), that is essentially a recombinant fusion protein, to deliver the therapeutic inducible nitric oxide synthase (iNOS) gene to breast cancer. The DBV is composed of several discrete motifs each designed with single function architecture including: (a) a DNA condensing motif (DCM) obtained from the adenovirus mu peptide, (b) a ZR-75-1 breast cancer cyclic targeting peptide (TP) for specific delivery of the nanoparticles, (c) an endosomal disruption motif (EDM) that mimics the influenza virus fusogenic peptide and (d) a nuclear localization signal (NLS), rev, obtained from the human immune-deficiency virus type-1. We now use this DBV to deliver the cytotoxic iNOS gene *in vitro* and the GFP reporter gene *in vivo* to ZR-75-1 tumours. *Methods*: The DBV was expressed in *Escherichia coli*, extracted with affinity chromatography and purified using size exclusion chromatography. The DBV was complexed to piNOS to form nanoparticles which were used either for characterisation via electrophoretic mobility shift assays, serum stability assays or dynamic light scattering analysis. ZR-75-1 breast cancer cells were transfected with DBV/piNOS nanoparticles and toxicity was quantified using the WST-1 cell toxicity and clonogenic assays. Over expression of iNOS was also confirmed via western blotting and greiss test. Finally ZR-75-1 intradermal tumours were grown using SCID models and the DBV/pEGFP-N1 nanoparticles were delivered both intratumourally and intravenously. Tumours and organs were excised and the GFP distribution was determined. *Results*: The DBV was effectively expressed in *E. coli* at approximately 3 mg/l yield. The DBV condenses piNOS into spherical nanoparticles between N:P ratios of 4–10. At a N:P ratio of 9, piNOS was fully condensed with an average size of 75.1 nm. Transfection with the DBV/piNOS nanoparticles resulted in a maximum of 62% cell kill. INOS overexpression was confirmed and total nitrite levels were in the range of 18 μM and comparable with lipofectamine/piNOS. Finally 48 h after i.v. injection of the DBV/pEGFP-N1 nanoparticles GFP protein was detected in all the organs. The addition of chloroquine (30 mg/kg I.P.) did not enhance the expression of GFP indicating functionality of the EDM. Furthermore the addition of nocodazole (3 mg/kg I.P.) resulted in a reduction in GFP expression again indicating NLS functionality *in vivo*. *Conclusions*: The DBV/piNOS nanoparticles gave significant cytotoxicity in ZR-75-1 breast cancer cells *in vitro* and with less than 20% transfection this indicates a bystander effect. Despite a lack of tumour targeting by the DBV vector *in vivo*, the data indicates that the DBV/pEGFP-N1 nanoparticles do not aggregate and can travel through the bloodstream with confirmation of gene expression in all the organs. Future studies will concentrate on using the human osteocalcin promoter (hOC) to transcriptionally target the iNOS plasmid to ZR-75-1 breast tumours.

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A47
Cellular delivery and biological activity of metall complex-peptide conjugates

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Bioorganometallic chemistry has become more and more important in several fields, especially in the development of new drugs for cancer treatment. A number of metal-based building blocks have promising features for applications in therapy and diagnosis. Introduction of a metal centre could add new features that may help to overcome some problems in cancer treatment. However the low water solubility and bioavailability of these organometallic compounds inhibits their therapeutic use in medicine. Therefore intracellular delivery of therapeutics is the challenging task in medicinal chemistry research. Recently, so-called cell-penetrating peptides (CPP) have emerged as potent tools to introduce substances into cells. CPP are an inhomogenic group of peptides that share the ability to translocate in a large number of different cell-lines without the need of any receptor or transporter molecule. Thereby they are capable to transport various cargos inside cells, like proteins, oligonucleotides, nanoparticles or small organic drugs. This work describes the coupling of metal-based building blocks to cell-penetrating peptides based on an antimicrobial peptide cathelicidin CAP18 or on the human peptide hormone calcitonin (hCT). Synthesis was achieved by solid phase peptide synthesis using standard Fmoc chemistry and activation by HOBt/DIC. Several different metal complexes have been investigated, for example, half-sandwich-complexes of different metals as iridium, manganese, rhodium or iron. To introduce the potential metal-specific activity to the bioconjugate, up to two organometal moieties were coupled either N-terminally, to a

amino acid side chain or in between two amino acids. Cellular uptake of the new bioconjugates was investigated with different methods like fluorescence microscopy, atom absorption spectroscopy or flow cytometry. High accumulation could be observed in different tumour cells. Furthermore, cell viability assays showed that those organometallic peptide conjugates are very potent and possess promising cytotoxic properties.

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A48
Polyelectrolite complex based microspheres for colon specific anticancer drug delivery

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Localized delivery of chemotherapeutic agents has long been the aim of clinical colon cancer therapy in order to limit the indiscriminate activity of many anti-cancer drugs on rapidly dividing cells, including normal tissues. The ideal drug delivery system (DDS) is envisioned to selectively and efficiently transport the anticancer drug to the target cells. It will not only minimize side effects associated with inappropriate drug distribution, but will also enhance therapeutic efficacy by increasing local drug concentration. The goal of our study was to develop wheat-germ aglutinin (WGA) functionalized chitosan-Ca-alginate microspheres (MS) loaded with acid-resistant nanoparticles (NP) of 5-FU, as colon targeting DDS and evaluate its *in vitro* efficacy and *in vivo* biodistribution. The rationale behind the design of the formulation is the presence of high level of polysaccharides of microbial origin in the human colon and the possibility of direct binding of MS to the mucosal surface by nonspecific or specific ligand–receptor interactions using biological molecules (WGA), thus enabling active uptake of 5-FU in the target cancer cells. A simple one-step spray drying procedure was used to produce polyanion/polycation MS loaded with acid-resistant NP of 5-FU with mean diameter of ~14.74 μm, high production yield (~50%) and encapsulation efficiency (~72%). Using 1,1′-Carbonyl-diimidazol as a surface group activation agent, successful conjugation of WGA to MS surface was achieved (~50%). Haemagglutination test confirmed that WGA, treated by covalent coupling procedure, still retained its specific carbohydrate binding activity on the surface of MS. *In vitro* efficiacy was evaluated by investigating 5-FU permeability and [methyl-3H]thimidine uptake in Caco-2 cells. The cumulative amount of transported 5-FU through Caco-2 cells was 15.1% and 6.5% for 5-FU solution and WGA conjugated MS, respectively. Cell culture studies also indicated a marked decrease in [methyl-3H]thimidine uptake for WGA decorated MS compared to 5-FU solution, suggesting that immobilization of WGA onto MS surface, due to the improved interaction and enhanced tissue accumulation of 5-FU could led to improved efficacy in targeted anticancer colon therapy. *In vivo* biodistribution studies were conducted with oral administration of ^{99m}Tc labeled MS on fasted male Wistar rats. The imaging was performed at different time intervals post administration. The results showed that MS traversed fairly quickly through upper part of GI tract and resided in the colon for relatively longer period of time, probably due to the particle size, pH dependent swelling and surface properties of the MS. Overall, the results of this work showed that cross-linked polycation/polyanion MS loaded with 5-FU and decorated with WGA, were able to effectively deliver 5-FU to colon region, thus affecting the transport of 5-FU into the cells and consequently improving the efficacy.

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A50
Engineering macrophages to synthesize recombinant adenoviruses in hypoxic areas of human prostate tumours

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Background: Like many other forms of human malignancy, prostate carcinomas contain multiple regions of transient and chronic hypoxia. New therapies targeting the hypoxic areas of tumours need to be designed as these sites are highly resistant to conventional cancer therapies. We have recently shown that macrophages accumulate in these hypoxic areas of prostate tumours, so we investigated the possibility of using these cells to deliver therapeutic genes to these otherwise inaccessible sites. *Materials and methods*: We designed a novel system in which macrophages are used to deliver hypoxia-regulated therapeutic adenovirus. In this approach, macrophages are co-transduced with a hypoxically activated E1A/B plasmid and an a hypoxia-regulated E1A/B construct and an E1A-dependent oncolytic adenovirus, whose proliferation is restricted to prostate tumor cells using prostate-specific promoter elements from the TARP, PSA and PMSA genes. *Results*: When co-cultured with prostate tumour spheroids, these 'armed' macrophages migrated into the hypoxic centres of the 3D tumour masses where E1A/B protein expression was upregulated, resulting in replication of the latent E1A/B-deficient adenovirus. Multiple copies of the virus (~5000/macrophage) were released and infected neighbouring prostate tumour cells, resulting in widespread gene expression. Systemic administration of co-transduced macrophages into mice bearing human prostate xenografts resulted in their subsequent trafficking into the hypoxic areas of tumours leading to viral replication and widespread infection of neighboring tumour cells, resulting in the marked inhibition of tumor growth and reduction of pulmonary metastases. *Conclusions*: We show for the first time that macrophages can be engineered to express high titres of a therapeutic adenovirus

specifically in hypoxic areas of human prostate tumours and that expression of the gene being delivered in the adenovirus can be restricted to prostate tumour cells by placing it under the control of a prostate-specific promoter (PSA). This novel approach employs three distinct levels of tumour-specific targeting; the homing of the macrophages to tumours, the synthesis and release of therapeutic adenovirus only in hypoxia tumour areas, and the targeting of therapeutic gene expression to prostate tumour cells.

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A51
Targeted nanodrug delivery systems for the treatment of tuberculosis

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South Africa currently has the highest incidence of TB in the world at 358 per 100,000 people. In 2007 alone 112,000 people died of TB in South Africa, of which 94,000 (72%) were co-infected with HIV [1]. Although TB treatments exist, poor patient compliance and drug resistance pose a great challenge to programs worldwide. To improve the current inadequate therapeutic management of TB, a polymeric anti-TB nanodrug delivery system, for anti-TB drugs, was developed that could enable entry, targeting, sustained release for longer periods and uptake of the antibiotics in the cells, hence reducing the dose frequency and simultaneously improve patient compliance. The aim was to prepare functionalised polymeric nanodrug delivery vehicles to target TB infected macrophage cells. Successful nanoencapsulation of anti-TB drugs and a targeting agent, mycolic acids (MA) was achieved. MA (a lipid molecule on the cell wall of M.tb) was explored due to its cholesteroid properties [2] that could attract it to the infected macrophages/foam cells. The nanoparticles were characterized and subjected to *in vitro* analyses in THP-1 and U937 cells in order to determine their uptake and localization. Cytotoxicity in different cell lines was also analysed. In another approach targeting was achieved via attaching nucleic acid aptamers [3], onto the surface of drug-carrying PLGA nano-particles. The aptamers were prepared via the SELEX process [4], specifically against the mannose receptor (MR), which is significantly over-expressed during the activation of the macrophages in the presence of M.tb. Uptake of the MA PLGA nanoparticles was achieved where little co localization was observed with endocytic markers, indicating that they could be localized in the cytosol. Vesicles bearing these particles were also observed in the cell membrane of these cells. We will report the uptake of the aptamers to THP-1 cells illustrating the feasibility of using the nucleic acid species for active targeted drug delivery. The success of these two approaches of anti-TB drug targeting will greatly address the challenges of poor bioavailability, reduced efficacy and adverse side effects for diseases such as TB.

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A52
Targeted SAINT-O-Somes, a novel type of liposomes for improved delivery of siRNA

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Gene silencing by siRNA has become a powerful technique with a great potential for therapeutic application. Increased insight in the role of endothelial cells in the pathology of cancer and inflammatory diseases has shifted the interest in the development of siRNA drug delivery devices for pharmacological intervention towards these cells. Additionally, endothelial cells are readily accessible for substances transported by the blood and their heterogeneity allows for specific drug targeting approaches. Liposomes represent a drug-carrier system for the delivery of siRNA that can be tailored on demand to introduce cell specificity. However, unlike in macrophages or in many tumor cells, in endothelial cells the processing of liposomes and subsequent release of drug content is inefficient due to the absence of adequate intracellular processing machinery which limits pharmacological efficiency. Therefore, we developed a lipid based drug delivery system with a superior intracellular release characteristic which is suitable for the *in vivo* delivery of siRNA. The design of the carrier is based on long circulating conventional liposomes that were formulated with a cationic amphiphile, 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride (SAINT-18). These so-called SAINT-O-Somes have a diameter of 100 nm and showed a 10-fold higher encapsulation efficiency for siRNA compared to liposomes without SAINT and protect the siRNA from degradation for at least 6 weeks. Moreover, SAINT-O-Somes are fully stable in a biological relevant milieu (i.e. presence of serum), but are destabilized in the lower pH in endosomes of endothelial cells, enabling release of siRNA into the cytoplasm of the cell. In order to efficiently target activated endothelial cells, SAINT-O-Somes were equipped with antibodies against E-selectin or VCAM-1 adhesion molecules that are (over)expressed at sites of inflammation.

Coupling of these ligands showed a highly beneficial effect for transfection efficacy to TNF-α activated endothelial cells compared to non-targeted SAINT-O-Somes. The intracellular delivery of anti VE-cadherin siRNA SAINT-O-Somes to activated endothelial cells resulted in a specific, 70% down-regulation of VE-cadherin gene expression. In conclusion, we demonstrated that SAINT-O-Somes are stable, high capacity carriers for effective siRNA delivery into endothelial cells that present the requirements for *in vivo* application.

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A53
Toxin assisted intracellular delivery of gold nanoparticles

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Targeted intracellular delivery of biomolecules using nanoparticles has attracted many of the science disciplines. Nanoparticles because of their tuneable size and unique optical properties are emerging not only as imaging probes but also serving as intracellular cargo delivery carriers. Gold nanoparticles are best candidate for all these applications because of their not particularly reported cytotoxicity and ease of biofunctionalization. For intracellular cargo delivery application, it is necessary that a carrier is not only has the capacity to carry the biomolecule efficiently but also able to deliver it to the cytosol which is the main site for all physiological and chemical activities inside the cell. It is well documented that on intracellular delivery, bioconjugated gold nanoparticles are trapped by endolysosomes where their biomolecular coating degrades eventually. For avoiding this fate and for gaining access into the cytosol, we used a new approach, that is, toxin assisted delivery for gold nanoparticles. A bacterial toxin streptolysin-O is a secreted protein of 61 kDa which forms pores in plasma membrane of host cell for gaining access into the cytosol. It has been used as a simple and rapid mean of transfection for intracellular delivery of oligonucleotides and siRNA. Our results confirm that SLO treated cells showed an increased cellular uptake of gold nanoparticles then untreated cells. We also studied the effect of poly ethylene glycol (PEG) on SLO assisted cellular uptake by increasing the PEG amount gradually and found that PEG affects the cellular uptake adversely. We are currently combining fluorescence microscopy, photothermal microscopy and transmission electron microscopy to fully understand the mechanism, localization and fate of gold nanoparticles during SLO assisted uptake.

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A54
Quantifying uptake and distribution of arginine rich peptides at therapeutic concentrations using fluorescence correlation spectroscopy and image correlation spectroscopy techniques

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Due to an apparent ability to enter cells in an energy independent manner, cell-penetrating peptides (CPPs) are increasingly being used as vectors for the delivery of macromolecules into cells. But 20 years on, their uptake and intracellular distribution are still debated [1] especially as most studies have been carried out at relatively high concentrations (micromolar), while therapeutic doses more likely to be in the nanomolar range. Thus, we hypothesised that taking advantage of fluorescence correlation spectroscopy (FCS) and image correlation spectroscopy (ICS) should help to understand the delivery mechanisms (especially the intracellular distribution) of arginine rich peptides TAMRA-Tat and TAMRA-nona-arginine (R9) at therapeutic doses. TAMRA-Tat and TAMRA-R9 peptides were incubated for one hour with both Caco-2 and HeLa cells. Initial observation of uptake was carried with a Zeiss LSM510 Meta Confocor 2. FCS and ICS were then used to measure peptide concentrations (density of particles per beam waist area) in distinctive areas and in the whole cell (cartography). ICS, implemented in parallel to FCS, was developed in house based on the work of P. Wiseman's group [2,3]. Subcellular distribution was analysed with confocal microscopy revealing two main areas – punctate and cytoplasmic regions – sampled initially with FCS to obtain diffusion times and concentration. Diffusion times in the punctate areas are very long (300 ± 50 μs) compared to the cytoplasm (26 ± 8 μs) at 500 nM, suggesting a bound component compared to free peptide. As FCS cannot sample the whole cell, ICS provided a more complete view of the distribution of TAMRA-Tat and TAMRA-R9 in which large areas of the cells behave as the 'cytoplasmic' area used in FCS. Our results indicate that arginine rich peptides are observed at nanomolar concentrations in all areas sampled. At concentrations below 500 nM, punctate and discrete areas are clearly labelled suggesting a possible entry via an endocytosis only mechanism. Finally, as the bulk concentration increases the fraction detected in the cytoplasm increases suggesting the simultaneous presence of a non-endocytotic mechanism of entry. Overall, FCS and ICS demonstrate that they provide invaluable information on the cellular delivery of peptides at therapeutic levels.

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A55
Tat-LK15, a Tat-fusion peptide, to deliver therapeutic siRNA in chronic myeloid leukemic cells

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Chronic myeloid leukaemia (CML) is caused by the reciprocal translocation of chromosomes 9 and 22 resulting in the formation of the BCR-ABL fusion protein, which exhibits deregulated tyrosine kinase activity. Hence, BCR-ABL would be a key target for developing a therapy for CML. We have used the potential of RNA interference to study the silencing of this oncoprotein. siRNA has been used to target wide range of genes in various cell types using cell penetraing peptides (CPPs). In this study we have evaluated the ability of the Tat fusion peptide, Tat-LK15 [1] to study uptake of

siRNA and also silencing of the BCR-ABL protein in K562 CML cells. Tat-LK15 peptide [1], a fusion of Tat and membrane lytic peptide LK15, was used to non-covalently complex siRNA targeting the BCR-ABL mRNA (b3a2 isoform). Complexation of siRNA by Tat-LK15 was studied using fluorescence correlation spectroscopy (FCS) in the presence of the intercalating dye YOPRO-1. Cy5 labelled siRNA was used to study uptake in K562 cells using flow cytometry and confocal microscopy. The reduction in BCR-ABL protein levels was observed by Western blot. Results were compared with K562 cells transfected with lipofectamine/siRNA complexes. MTT assay was performed to study the cytotoxicity of the Tat-LK15/siRNA complexes. The YOPRO-1 competitive binding assay revealed efficient condensation of siRNA by Tat-LK15 and Lipofectamine™ at charge ratios higher than 3:1 (less than 10% of YOPRO-1 labelled siRNA). Flow cytometry studies using varying amounts of siRNA showed an increase in intracellular existence of Cy5-siRNA also leading to an increase in percentage positive transfected cells. Confocal microscopy confirmed the increase in intracellular localization upon transfection with higher amount of siRNA 4 hours and 24 hours post-transfection. Finally RNAi was observed using siRNA, which resulted in 70–80% reduction in BCR-ABL protein levels at lower concentrations. However, silencing observed using siRNA did not last longer than 48 hours. Cytotoxicity studies show that Tat-LK15/siRNA complexes are not toxic when lower concentrations of siRNA are used. Here, we show that Tat-LK15 can be a potential vector in delivering siRNA targeting genes of clinical significance.

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A56

Carbon nanotube-dendron series for siRNA delivery: mechanisms of cellular internalisation

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Carbon nanotubes have been attracting attention as tools for various biomedical applications. Chemical surface functionalization of multi-walled carbon nanotubes (MWNT) has shown remarkably increased aqueous solubility and debundling of nanotube aggregates that makes this material a promising candidate for biological applications. In this work, a series of dendron-MWNT derivatives were synthesized as potential vectors for siRNA delivery [1]. To elucidate the mechanism of cellular internalization characteristics of the dentron-MWNT:siRNA complexes, a fluorescence probed, non-coding siRNA sequence was used and its nanotube-mediated cytoplasmic delivery was studied in comparison to that by cationic liposomes. siRNA delivered by the dendron-MWNT was found throughout the cytoplasm including the nucleus. The siRNA delivered by cationic (DOTAP:cholesterol) liposomes was co-localized with endosomal markers indicating primarily an endocytosis pathway for internalization as previously described in the literature. The cellular transport of the siRNA was significantly increased with higher dendron generations conjugated on the nanotube surface at physiological conditions (37 °C) as well as under endocytosis-inhibiting conditions (4 °C). This work demonstrated that clathrin-coated endocytosis is a contributing but not the major pathway for the cellular internalization of the dentron-MWNT:siRNA complexes and could offer a great advantage via direct cytoplasmic delivery of siRNA for effective gene silencing.

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A57

Cellular internalisation of humanized IgG antibody changes by functionalization onto multi-walled carbon nanotubes

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Antibodies have been extensively used as anti-neoplastic therapeutics clinically and preclinically as they allow for therapeutic and specific targeting to specific cell receptors. The humanized CTMO1 IgG antibody was raised against the membrane-associated antigen of human milk fat globules (HMFG) derived from the anti-HMFG mouse monoclonal antibody CTMO1, but with similar affinity to the polymorphic epithelial mucin-1 (MUC-1). Anticancer drugs derived from murine HMFG1 have been under development in phase III clinical trial [1]. Carbon nanotubes have remarkable physicochemical properties offering an array of interesting features. In the context of this study, their large surface area offered a template for conjugation with a variety of monoclonal antibodies. Multi-walled carbon nanotubes (MWNT) were chemically functionalized with humanized CTMO1 IgG. The MWNT-IgG constructs were observed to target MUC-1 positive cells, but were retained at the plasma membrane with limited internalization. In contrast, a time-dependent cell surface binding and internalization was observed for the humanized CTMO1 IgG alone. The co-localization of the fluorescently labeled IgG with markers of specific cellular compartments was also studied using confocal laser scanning microscopy, to determine its mechanism of cellular uptake and trafficking pathway. The results here indicated that the size and aggregation state of the MWNT-IgG constructs played a determinant role in their interaction with cells. The design and development of CNT-antibody con-

structs needs further optimization in order to constitute a viable novel platform for cancer treatment with the purpose of combinatory therapeutic/diagnostic functionality.

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A58

Role of cell-surface carbohydrates and plasma membrane components in the internalization of cell-penetrating peptides

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Among cell-penetrating peptides, penetratin is widely used as a molecular device to cross membranes and transport biologically active molecules inside cells [1,2]. But, the underlying internalization mechanisms for such behaviour is still studied and discussed [3]. The idea is now well accepted that the physico-chemical properties of the cargo [4], the cell-penetrating peptide [5], and the disulfide-bridge in the conjugate [6], have an impact in the intracellular delivery pathways of the conjugate. Therefore, it is obvious that the internalization pathways and the final localization of conjugates within cells can hardly be anticipated. We have previously reported that penetratin internalizes in cells at 37 °C and 4 °C, thus through translocation and endocytosis pathways [7]. The translocation process occurs at low micromolar penetratin, while endocytosis is activated at higher concentrations. We have now studied the impact of cell-surface (GAG, sialic acid) and plasma membrane (cholesterol) components in the temperature-dependent cell internalization efficiency [8] and pathways [7] of penetratin and other well-studied cell-penetrating peptides. These results will be presented and discussed.

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A59

Development of a microwell device for correlative light and electron microscopy

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New innovations and techniques are constantly being developed within the field of microscopy with the aim of generating higher through-put analysis and/or gaining the maximum data out of a single sample. Correlative light electron microscopy or CLEM involves bringing together the two most common aspects of microscopy, fluorescence and electron microscopy. The weaknesses in fluorescence microscopy, low resolution, can be counteracted by the highly detailed electron microscopy images. On the other hand, the weakness of electron microscopy for live intracellular tracking, can be counteracted using fluorescence microscopy. This project involves the development of a microwell array technique to allow a user to correlatively image the same cell under both fluorescence microscopy and scanning electron microscopy (SEM). Microwell arrays were ablated into borosilicate glass and PDMS (silicone elastomer) cover slips using 193 nm and 157 nm excimer lasers (MetaFAB, Cardiff University). The surface of the substrate is first coated with a sacrificial layer before ablation thus providing an important step in helping to remove ablation debris during sonication. PDMS surfaces were further modified to optimise cell adhesion by oxidizing the surface using UV/ozone treatment and reacting with APTES (aminopropyltriethoxysilnae) to create an amine modified surface. Initially, for proof of concept, KG1a (acute myelogenous leukaemia) cells were allowed to settle into the microwells before being exposed to transferrin as an endocytic marker or a pro-apoptotic peptide linked to the cell penetrating peptide R8 to determine whether apoptosis can be monitored. The cells were then imaged by confocal microscopy then fixed, dehydrated, dried and splutter coated for imaging by SEM. We have successfully imaged uptake of transferrin and the effects of a pro-apoptotic peptide whilst cells were resting within the microwells. We have also obtained correlative images of KG1a cells imaged before fixation under light microscopy and the same cells under SEM. By comparing cell number and their position within the microwells before and after fixation we are confident of achieving correlative microscopy. For adherent cells we are able to create microwell arrays of varying sizes in both glass and PDMS. Post-ablation processing increased microwell quality whilst the auto-fluorescence in glass was reduced by various cleaning steps. However, switching to PDMS provided a much lower auto-fluorescent substrate on which to work. PDMS is naturally very hydrophobic (contact angle ~105°); using UV/ozone we were able to reduce the hydophobicity of the surface (contact angle ~40°). This formation of hydroxyl groups on the surface allowed for further modification using APTES, which improved cell adhesion. We can now obtain correlative images using confocal microscopy and SEM of the same cells and are developing further methods for TEM correlative light electron microscopy studies.

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A60
Formulation of new reducible liposomes for gene delivery

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Gene therapy aims to eradicate causes rather than symptoms of diseases and is believed by many to be the therapy of the future. Improved liposome formulations are a valuable alternative to viral gene delivery vectors and the rapid disulfide linkages cleavage by the intracellular reductive environment can induces fast reducible lipoplex dissociation and efficient DNA release, yielding increased gene expression. On the light of these findings, we developed four different liposome formulations based on SS14, a reducible cationic gemini-like surfactant. SS14 was previously synthesized by our group [1,2]. Helper lipids bearing different alkyl chain and/or polar head types were chosen and four formulations were investigated: DMPC/SS14:0.75/0.25; DOPC/SS14:0.75/0.25; DMPC/DMPE/SS14:0.5/0.25/0.25; DOPC/DOPE/SS14:0.5/0.25/0.25 molar ratios. SS14-containing liposomes were prepared by repeated extrusions through polycarbonate filters of 100 nm pore diameter. Three out of four liposome formulations showed a size distribution with a monodisperse population (polydispersity index, P.I. ≤ 0.3) while in DMPC/DMPE/SS14 liposomes, large aggregates ($\varnothing > 1$ μm) were found together with the main liposome population, possibly due to fluctuating lamellar sheets. All liposome dimensions were between 95 and 120 nm. Zeta potential, within experimental error, was the same for all the formulations, ranging from $+39 \pm 7$ mV and $+55 \pm 8$ mV. By monitoring the displacement of SYBR-Green I from DNA, a negative trend of fluorescence in function of CR was noticed for each formulation with a plateau reached beyond CR5. Since between the reducing intracellular space and the oxidizing extracellular environment a high redox potential difference exists (~100–1000-fold), by agarose gel electrophoresis we demonstrated the ability of GSH to enable DNA release. Transfection activity and cytotoxicity of the four formulations were compared at CR5 and CR15 on U87-MG, Cos-7, HeLa and MG63 cell line using pEGFP-N1 as plasmid DNA. Firstly, liposome effectiveness was not inhibited by the presence of serum in transfection experiments. Secondly, the introduction of helper lipids bearing PE polar heads in two-component liposome formulations increased significantly transfection efficiency up to 7-fold ($p < 0.05$). This may be due to the high fusogenic properties of their phosphoethanolamine (PE) polar head. Finally, three-component formulations were more cytotoxic. In particular, DOPC/DOPE/SS14:0.5/0.25/0.25 CR5 liposomes demonstrated superior transfection efficiency (24.4 ± 2.7% by FACS analysis on U87-MG cells) and modest cytotoxicity. The mechanisms beneath intracellular reduction, transfection enhancement and increased cytotoxicity will be the subject of further investigation.

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A61
Strategies for microsphere-mediated delivery of oligonucleotides

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An efficient intracelllar delivery of oligonucleotides is a vital step for gene therapy. Many technologies have been developed to design efficient transfection agents. Many of these agents are promising tools *in vitro* but they fail when *in vivo* assays are carried out. Recently we have developed a polystyrene microsphere-based system designed to efficiently deliver biological materials into a broad range of cell lines. Additionally, these particles have been successfully test *in vivo*. The fact that these polymer particles are easy to functionalise with high controllability over the cargo loading, showing any undesired cytotoxic effect, make them enormously attractive as delivery system. Our recent advances in the design of strategies for the delivery of oligonucleotides using microspheres as transfection system will be presented.

See reference below for additional reading
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A62
Microsphere-mediated delivery of therapeutic peptides on neuronal cells

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Many proteins exert their biological roles as components of complexes, and the functions of proteins are often determined by their specific interactions with other proteins. The identification of inhibitory peptides and derived peptidomimetics has been developed as potent inhibitors of protein–protein interaction. More specifically protein–protein interaction domains that couples the NMDA receptor to intracellular proteins are potential targets for the development of new therapies to combat neurodegenerative diseases [1]. Different studies of the PDZ domain in nNOS inhibitors have been carried out. The peptidic nature of these compounds has obstructed their uptake into the cell. Amino cross-linked microspheres have been used previously for the delivery of therapeutic molecules [2–5]. The design, synthesis and biological evaluation of microspheres as carrier systems to facilitate the cellular uptake of these peptidic sequences on SH-SY5Y neuroblastoma cells will be presented.

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A63
siRNA versus pharmacological inhibition of endocytic pathways for studying cellular uptake of cell penetrating peptides and other drug delivery vectors

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Cell-penetrating peptides (CPPs) have the potential to deliver numerous therapeutic macromolecules into cells including peptides, proteins, and nucleic acids. Under defined conditions endocytosis is thought to be of significant importance for CPP entry but identifying the exact uptake mechanism and pathway(s) involved has been difficult. Multiple pathways have been reported to contribute to uptake, including macropinocytosis and those regulated by clathrin and cavaeolin-1. This project aims enhance the use of cell penetrating peptides as drug delivery vectors by developing new technologies to study their mechanisms of uptake. Traditionally studies investigating the uptake of these molecules, and other drug delivery vectors, have been performed using chemical inhibitors but these are often toxic and lack specificity [1]. We have developed siRNA-based assays to silence endocytic proteins that have previously been shown to regulate distinct endocytic pathways. The effect of depleting these proteins was then assessed to investigate their roles in mediating the uptake of well characterised endocytic probes and CPPs. Two cell lines were predominantly used, HeLa (cervical cancer epithelial) and A431 (human epithelial carcinoma). Endocytic proteins clathrin heavy chain, flotillin-1, dynamin II, caveolin-1 and P21-activated kinase (PAK-1) were depleted using single siRNA sequences; siRNA against GFP was used as a control. In siRNA treated cells the uptake of fluorescent endocytic markers including; Alexa488-transferrin (clathrin mediated endocytosis), 40 kDa FITC Dextran (fluid phase uptake and macropinocytosis), FITC conjugated anti-CD59 antibody (flotillin-1 dependent uptake) and the uptake of Alexa488 CPPs (RRRRRRRRGC-Alexa488-R8, and GRKKRRQRRRPPQ-Alexa488-HIV-TAT) were measured by flow cytometry. Protein depletion was assessed from protein lysates using SDS PAGE and Western blotting. Overall, the data shows that siRNA transfection method could effectively reduce expression of clathrin heavy chain, caveolin-1 and flotillin-1 from HeLa cells and this then allowed for us to study effects on endocytosis of various probes. PAK-1 has been shown to regulate macropinocytosis and we show that the induction of macropinocytosis and PAK-1 expression are highly cell line dependent. Paralleled with this was our findings that cationic CPPs induce an increase in fluid phase uptake of dextran and the extent of this was cell line dependent. Comparative analysis of these experiments with those performed using pharmacological inhibitors, allowed us to determine the usefulness of this approach for drug delivery research.

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A64
SAINTargs, a novel lipid-based targeting device for siRNA delivery

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The endothelium represents an important therapeutic target because its pivotal role in many diseases such as chronic inflammation and cancer and its accessibility for systemic administration. RNA interference by small interfering RNA (siRNA) has become in the last decade a very powerful tool in basic research, and has huge potential to become an important new class of therapeutics for humans. However, due to their size and charge, siRNAs have no bioavailability to enter unperturbed cells. To overcome this problem, our laboratory developed a non-viral lipid-based targeting device which efficiently and specifically delivers siRNA into endothelial cells. Molecular determinants expressed on the surface of inflammation-activated endothelial cells, like certain adhesion molecules and receptors involved in endocytosis, are excellent candidates to increase carrier-mediated siRNA uptake. Therefore we conjugated monoclonal anti-E-selectin antibodies to the cationic amphiphilic lipid, 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride (SAINT-18) which was complexed in a 1:2000 molar ratio with the transfection agent SAINT-MIX (SAINT:DOPE, 1:1), and siRNA, resulting in a siRNA containing lipoplex called anti-E-selectin-SAINTarg [1]. Our findings demonstrate that anti-E-selectin-SAINTargs maintained the antigen recognition capacity of the parental antibody and showed increased siRNA uptake in otherwise difficult-to-transfect primary human umbilical vein endothelial cells (HUVEC) as compared to non-targeted SAINT-MIX. Moreover, anti-E-selectin-SAINTargs superior binding and uptake efficiency was corroborated by improved silencing of both gene- and protein expression of VE-cadherin in activated HUVEC. The VE-cadherin gene expression could be silenced up to 95% by VE-cadherin specific siRNA, at low siRNA concentrations (30 pmol/ml). Furthermore, no non-specific silencing by scrambled or VE-cadherin specific siRNA was observed. To optimize siRNA delivery into activated endothelial cells we also synthesized anti-VCAM-1-SAINTargs which were as efficient in VE-cadherin silencing as anti-E-selectin-SAINTargs. Because of the heterogeneous expression of adhesion molecules on inflammation-activated endothelial cells *in vivo*, a combination of these two SAINTargs may result in enhanced siRNA effects. Taken together, SAINTargs demonstrate specific and efficient targeting to inflammation-activated difficult-to-transfect primary endothelial cells and results in strong siRNA specific gene silencing at low siRNA concentrations.

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A65
Cationic star homo- and co-polymers for gene delivery

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Several groups of star polymers were synthesized and evaluated as gene delivery vehicles. All polymers were synthesized by group transfer polymerisation and were based on 2-(dimethylamino)ethyl methacrylate (DMAEMA). In particular, one group of DMAEMA star homo-polymers of different molecular weights and three groups of star copolymers of different architectures were prepared. The three groups of copolymers were based on the DMAEMA monomer and a second hydrophilic monomer comprising either poly(ethylene glycol) methacrylate, methacrylic acid or glycerol methacrylate. All series of star polymers were characterized by gel permeation chromatography and nuclear magnetic resonance spectroscopy. Aqueous solutions of the star polymers were studied by turbidimetry, hydrogen ion titration, and dynamic light scattering. All but the most recent star polymers were evaluated for their ability to transfect cells. The transfection efficiency was affected by the molecular weight of the star polymer, the star architecture and the nature of the second co-monomer.

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A66
Gene electrotransfer: comparison between 2D cultured cells and multicellular tumor spheroid model

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Electroporation is a physical method to deliver molecules into cells and tissues. Clinical applications have been successfully developed for antitumoral drug delivery and clinical trials for gene electrotransfer are underway [1]. However, little is known about the mechanisms involved in these processes. The main difficulties stem from the lack of cell models which reliably replicate the complex *in vivo* environment. To increase our understanding of the DNA electrotransfer mechanisms, we recently exploited multicellular tumor spheroids (MCTS) as an *ex vivo* model of tumor [2]. This 3-dimensional model can replicate the *in vivo* in complex environment and therefore enables us to develop new strategies for studying mechanisms of molecules delivery by electric field pulses. In the present study, we observed cells response to electric field pulses for propidium iodide and plasmid DNA delivery. HCT116 cells were pulsed either in suspension (2D culture) or in MCTS (3D culture) and 10 pulses lasting 5 ms were applied at different voltages. Confocal and biphotonic microscopy allowed us to visualize the repartition of permeabilized and transfected cells in MCTS subjected to electric pulses. Flow cytometery analysis was used to obtain quantitative analysis both on cells pulsed in suspension or on cells pulsed in MCTS (in that case, cells were dissociated by an enzymatic treatment). Results show differences in electric field sensitivity between cell in suspension and MCTS. Permeabilization process (revealed by propidium iodide uptake) is affected only the first cell layers of MCTS. A maximum of 30% of cells being permeabilized was obtained at 400 V cm^{-1}. Increasing the field strength above that value did not further increase the number of permeabilized cell. On the contrary, in the case of cells pulsed in suspension, up to 90% of cells were shown to be permeabilized at 700 V cm^{-1}. DNA delivery process (revealed by GFP expression) showed that less than 5% cells were transfected when present in the spheroid model while, under the same conditions, about 25% of them were transfected when pulsed in suspension. These results point out the difficulty DNA has to cross the multicellular barrier and give an explanation for the different of responses of cells *in vitro* and *in vivo* [3]. Taken together, these results are in agreement with the ones obtained in tumors and indicate that the spheroid model is more relevant to an *in vivo* situation than cells cultured as monolayers. They validate the spheroid model as a way to study electromediated gene delivery processes.

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A68
Combination of a triblock copolymer L64 with electrotransfer increases gene delivery *in vitro*

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Gene transfer into muscle cells is a key issue in biomedical research. Indeed, it is important for the development of new therapy for many genetic disorders affecting this tissue and for the use of muscle tissue as a secretion platform of therapeutic proteins. Electrotransfer is a promising method to achieve gene expression in muscles. However, this method can lead to some tissue damage especially on pathologic muscles. Therefore there is a need for the development of new and less deleterious methods. Triblock copolymers as pluronic L64 are starting to be used to improve gene transfer mediated by several agents into muscle tissue.

Their mechanism of action is still under investigation. The combination of electrotransfer and triblock copolymers, in allowing softening electric field conditions leading to efficient DNA transfection, could potentially represent a milder and more secure transfection method. In the present study, we address the possible synergy that could be obtained by combining the copolymer triblock L64 and electroporation. The synthesis of fluorescent probes L64-rhodamine and DNA-rhodamine is presented here. These probes allowed us to gain some insights into the mechanism of transfection of the combined physical and chemical methods. We have found that a pretreatment of cells with L64 could improve the transfection efficiency. Neither interaction of DNA with the cell membrane, nor L64 membrane interaction seemed to be related to the gain obtained in these transfecting conditions.

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A69
A receptor-mediated gene delivery system using CXCR4 ligand-conjugated cross-linking peptides

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Application of DNA as therapeutics requires efficient cell and tissue-specific targeting which can be achieved by modification of vehicles with a ligand for certain receptor. CXCR4 is a receptor of chemokine SDF-1 and is expressed on some types of cancer and stem cells. Cystein-flanked peptides which are capable of forming small and stable DNA condensates because of cross-linking are considered to be a perspective group of non-viral vehicles. The aim of this project is to characterize a CXCR4 ligand-conjugated cross-linking peptides as a receptor-mediated gene delivery system. We studied four types of DNA/peptide complexes with different ratio between cystein-flanked arginine-rich peptide modified with N-terminal sequence of the chemokine SDF-1 (residues 1–17) and peptide (CHRRRRRRHC) – 100%, 50%, 10% and 0% (ligand-free control). The peptides modification with histidine residues facilitates the escape of DNA from endosomes. Template polymerization of cross-linking peptides was used to form DNA/peptide complexes. EtBr exclusion and DNA retardation assays proved peptides ability to condense DNA. Transfection activity was studied in CXCR4(+),(A172 and HeLa) and CXCR4(−) (CHO) cell lines with lacZ as a reporter gene. Transfection efficacy of ligand-conjugated vehicles in CXCR4(+) HeLa and A172 cells was 10-times higher compared to control peptide. The level of transgene expression with ligand-conjugated peptides in low N/P ratios was comparable with the efficacy of control PEI. Otherwise transfection efficacy of ligand-conjugated peptides on CXCR4(−) CHO cells was lower than in control PEI. Thus these results demonstrate that ligand-conjugated peptide-based vehicles reported can be a perspective approach for effective gene delivery to CXCR4 expressing cells.

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A70
Antibody targeting of lipid nanocapsules for directed drug delivery: physico-chemical characterization and *in vitro* study

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Lipid nanocapsules are recently developed as nanocarriers for lipophilic drugs delivery. The surface characteristics of these colloidal particles are determinant in order to provide a controlled and directed delivery on target tissues with specific markers. We report the development of immuno-nanocapsules, in which antibodies are conjugated to nanocapsules offering the promise of selective drug delivery to specific cells. Several nanocapsule systems were prepared by the solvent displacement technique obtaining an oily core surrounded by a functional shell with surface carboxylic groups. Antibodies were conjugated with nanoparticles by the carbodiimide method that allows it the covalent immobilization of protein molecules through these carboxylic surface groups. A complete physico-chemical characterization of the immuno-nanocapsules was developed confirming the immobilization of protein molecules on the colloidal nanoparticles via electrokinetic and colloidal stability experiments. The immunoreactivity of the protein–nanocapsules complexes was studied following the changes in the turbidity after addition of specific antigens, showing an adequate surface disposition of the covalent bound antibodies in order to a specific immunological recognize. Finally, nanocapsules were conjugated to a specific antibody to HER2 oncoprotein. In this case, in addition to the colloidal characterization, an '*in vitro*' study was developed using this surface modified system with different lipophilic anti-cancer drugs entrapped in their oily core. Flow cytometry experiments were used in order to evaluate the cytotoxicity (IC50) of our modified nanocapsules with wild-type and HER2 over expressing tumoral, cell lines. The obtained results have shown the capacity of the immuno-nanocapsules to increase their uptake in tumoral cells, suggesting their ability to a selective deliver drugs.

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A71
Characterization of polymer-coated nanoparticles based on DNA condensation via spermine

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The combination of the complete human genome sequence and the understanding of molecular pathways of some diseases including cancer, could lead to develop several interesting new treatments, such as gene therapy. But one of the major obstacles preventing this therapy from being used is the lack of specific and efficient delivery systems. The uptake of vectors by living cells depends on the degree of DNA condensation, thus we used a demonstrated condensing agent of nucleic acids: spermine. Nanoparticles based on DNA condensation by this natural polyamine were synthesized. In order to protect DNA against DNase degradation, these nanoparticles were coated with the positive charged polymers chitosan or polyethyleneimine (PEI). Folic acid was covalently bound to chitosan with the aim of enhance nanoparticle endocytosis via folate receptor, which is over-expressed in cancer

cells. Nanoparticles were characterized and some preliminary *in vitro* studies were done, showing that nucleic acids are efficiently condensed with this system, which appears to have a potential use in cancer gene therapy.

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A72
Reduced transgene persistence and trafficking to nuclear periphery are barriers to transfection in lipid substituted nonviral cationic polymer

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Background: Polyethylenimine (PEI) is one of the most sought after cationic polymer for nonviral gene delivery owing to its ability to transfect a variety of cell types efficiently. The amine groups found on the polymer renders high density of cationic charges, which facilitates efficient binding to DNA, while allowing polymer to be derivatized conveniently. Recently, our lab has derived a novel amphiphilic polymer by grafting linoleic acid (LA) to a low molecular weight PEI (2 kDa). The resulting polymer, PEI2k-LA, displayed significant improvement in transfection efficiency in HEK 293T cells over the ineffective, unmodified 2 kDa PEI. However, when PEI2k-LA was used to transfect rat bone marrow stromal cell (rBMCS), low transfection was observed despite 80% of the cells showing polyplex uptake. We aim to further improve PEI2k-LA transfection efficiency in primary cell line by gaining better understanding of its intracellular kinetics in transfection. In this study, we compared the efficiency of polyplexes trafficking to the nuclear periphery with respect to cellular uptake and transgene expression. Polyplexes routing to the nuclear periphery may facilitate passive nuclear uptake of transgene DNA following mitosis, which may increase the probability of transgene expression. *Methods*: A mammalian expression vector encoding the green fluorescent protein is covalently labeled with Cy5 (Mirus Bio Label IT® Tracker). Plasmid DNA labelled using this method maintains transcriptional activity, permitting simultaneous tracking of DNA and transgene expression. Labelled DNAs are complexed with PEI2k-LA or 25 kDa branched PEI (bPEI25k) to transfect tissue cultured rBMSC; cells and nuclei are processed for analysis by flow cytometry at 0.16, 1, 4, and 7 days to assess for DNA uptake and transgene expression. *Results and discussion*: GFP-expression was detected in bPEI25k transfected cells, but not with PEI2k-LA treated cells. PEI2k-LA was able to deliver DNA with similar efficiency as bPEI25k; both carriers delivered DNA to >90% of cells by Day 1. However, the percent of cells with DNA uptake reduced to <50% at an earlier time point with PEI2k-LA than with bPEI25k (~1.7-fold difference between carriers by Day 4). There were significantly fewer nuclei with plasmid DNA associated from PEI2k-LA treated cells than bPEI25k (6% versus 43%, Day 7). Further, the nuclei from PEI2k-LA treated cells had, on average, fewer amounts of DNA associated (~11-fold lower). Taken together, these data suggest that the lack of transfection in rBMSC by PEI2k-LA may be attributed to reduced transgene trafficking to the nuclear periphery and reduced intracellular retention of transgene DNA. Carrier efficiency in transfection may be improved by concurrently enhancing its DNA protective ability and nuclear routing capability.

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A74
Utilising the fluorescent properties of Laurdan to study plasma membrane fluidity in cells treated with the cell penetrating peptide R8

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Despite a large body of research, the mechanism of CPP translocation across biological barriers remains unclear. CPP interactions with membrane lipids have been studied by numerous groups and are hypothesised to be critical determinants for internalisation into cells. The possibility exists that cationic CPPs such as octaarginine (R8) and HIV-TAT, at certain concentrations, affect the phase behaviour of the membrane bilayer [1,2]. This phenomenon may explain our earlier studies with leukaemia cells; R8 freely crosses the plasma membrane at concentrations >5 μM, in cells depleted of cholesterol and also at low temperatures [3]. We therefore determined what effects different temperatures, and cholesterol manipulations had on the fluidity and phase behaviour of the plasma membrane of leukemic KG1a and K562 cells and then compared the data with that obtained from experiments in cells incubated with R8. Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is a fluorescent membrane probe that possesses different spectral properties depending on the phospholipid phase state of the membrane. Upon passing from the gel phase to the liquid crystalline phase a shift of the emission maxima is observed, from 440 nm to 490 nm and the emission/excitation values obtained can be used to determine membrane fluidity. The results confirm that for both cell lines, over the temperature range of 4–37 °C, the plasma membrane fluidity increased with increasing temperature. Extraction of plasma membrane cholesterol results in an influx of R8-Alexa488 into the cytosol of cells incubated at 37 °C with 2 μM peptide but this effect can be reversed by adding back cholesterol to cholesterol depleted cells. MβCD treatment caused an increase in plasma membrane fluidity but this was unchanged in cells in which had been incubated with MβCD:Chol. Direct plasma membrane translocation of R8-Alexa488 was previously seen in the majority of both KG1a and K562 cells within 10 min of peptide addition (10 μM) while the peptide was restricted to intracellular vesicles at 2 μM thus raising the possibility that translocation at high concentration was the result of peptide induced effects on membrane fluidity. This was however not the case as no effects on membrane fluidity were observed when similar Laurdan measurements were performed in R8 treated cells. Overall the data show that under conditions where direct translocation of R8 is observed, the fluidity of the plasma membrane is unperturbed.

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A75

Alkylglyceryl chitosan nanoparticles for drug delivery across the blood–brain barrier

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Targeting therapeutic compounds to the central nervous system (CNS) via systemic administration requires crossing the blood–brain barrier (BBB). This is currently one of the most challenging problems in CNS drug development. A series of alkylglyceryl chitosans with systematically varied degrees of grafting were prepared through synthetic steps that involved the protection of amino moieties via the formation of phthaloyl chitosan. These alkylglyceryl-modified chitosans were formulated into nanoparticles via a standard ionic gelation technique using sodium tripolyphosphate; the stability and size distribution profiles of nanoformulations were determined using dynamic light scattering. The mean diameter of the particles was found to range between 200 and 350 nm, with the zeta potential between +37 and +41 mV. The stability of nanoformulations was investigated under physiological conditions: it was found that an increase in pH from 4 to 7.4 resulted in a raised hydrodynamic diameter of particles and in a corresponding decrease of their zeta potential. A further chemical modification involving a partial quaternisation of the alkylglyceryl-modified chitosan improved the stability of the formulation at neutral pH, as shown by the changes in the zeta potential and particle size. Preliminary *in vitro* tests using mouse-brain endothelial cells demonstrated no toxicity and an efficient uptake and indicated that butylglyceryl chitosan and butylglyceryl N,N,N-trimethyl chitosan nanoparticles are promising formulations for BBB targeting.

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A76

A study of the interaction of novel, coated microparticles with alveolar macrophages and their application in tuberculosis treatment via inhalation

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Introduction: Mycobacterium tuberculosis (MTb) is a pathogenic mycobacterium and the main causative agent of tuberculosis infection in humans. Current treatment involves a multi-dose drug regimen for a minimum of 6–9 months. Approximately 80% of all MTb cases affect the pulmonary region. Despite this fact therapy is currently based on oral and parenteral formulations [1]. Aerosol delivery of anti-tubercular agents aims to reduce the systemic toxicity associated with conventional therapy, to maximise local concentrations of therapeutics in the alveolar region and target alveolar macrophages (AM), the niche environment of the MTb bacilli. We have bioengineered novel, inhalable microparticles designed to efficiently target drugs intracellularly to alveolar macrophages using opsonic coatings. The aims of this study were: (i) to determine the effect of the coatings on the uptake and intracellular trafficking of the microparticles in AMs and (ii) to assess the effect of coated and uncoated microparticles on macrophage activation. *Materials and methods*: Poly-lactide-co-glycolide (PLGA) microparticles were manufactured using a solvent evaporation method and coated with a number of opsonic proteins. THP-1 cells were differentiated using phorbol 12-myristate-13-acetate (PMA) into a macrophage-like cell and where necessary infected with MTb. Non-infected or infected cells were treated with fluorescently labelled microparticles, fixed and counterstained using LAMP-1 and DAMP. Their uptake and intracellular trafficking was visualised using confocal laser scanning microscopy (CLSM). THP-1 blue cells were used to assess the effect of the microparticles on AM activation. This cell line produces a reporter protein when NFkB is activated. These cells were also differentiated using PMA and subsequently treated with microparticles. *Results*: The coated microparticles were efficiently internalised by infected THP-1 cells and showed some degree of co-localisation with MTb after 1 h. Microparticle-treatment led to significant activation of NFkB. The degree of activation was found to be microparticle size and coating dependent. *Conclusion*: Opsonic coating of inhalable microparticles significantly increases their uptake into TB-infected AMs and facilitates co-localisation with the mycobacterium. Previous work by us and others has shown that empty microparticle treatment of MTb infected cells can decrease mycobacterial viability. The increase in NFkB expression associated with microparticle treatment may explain this phenomenon via induction of pro-inflammatory cytokines important for mycobacterium control. Overall this work suggests that microparticles may have immunopotentiator applications in MTb control.

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A77

Development of a high throughput method for screening of novel nanotechnologies for siRNA transfection of airway cells using high content screening (HCS)

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Introduction: RNA interference (RNAi) is an endogenous system in eukaryotic cells whereby sequence-specific RNAs are able to bind and degrade their complementary mRNA. Properly applied, this system could potentially be used to control and treat a wide range of respiratory diseases including cystic fibrosis, lung cancer and inflammatory lung disease. However, siRNA delivery problems encountered in the lungs include poor airway mucus penetration, insufficient cell uptake, poor cell-type specific targeting and rapid clearance. To overcome these problems, we have developed a range of novel nanotechnologies for transfection of airway epithelial cells and alveolar macrophages. The aim of this study was to develop a high throughput method for screening novel nanotechnologies for siRNA transfection of airway cells using high content screening (HCS). *Materials and methods*: A range of polyethyleneimine-polyethyleneglycol (PEI-PEG) polymers was synthesised and complexed with fluorescent siRNA (fl-siRNA) and

used to transfect an airway epithelial cell line, Calu-3 cells. A range of targeted, mannosylated liposomes were also synthesized, fl-siRNA was encapsulated therein and these complexes were used to transfect an alveolar macrophage-like cell line, THP-1. Each of the systems was characterised for size, zeta-potential and encapsulation efficiency prior to transfection. To determine the efficiency of fl-siRNA transfection facilitated by these nanoparticles a protocol was specifically designed to qualitatively and quantitatively monitor siRNA uptake using InCell 1000 high content screening. *Results*: A number of the PEI-PEG nanoparticles significantly increased siRNA uptake into Calu-3 cells and a number of the mannosylated liposomes were capable of efficiently transfecting alveolar macrophages, a particularly difficult to transfect cell type. *Conclusion*: HCS facilitated the screening of a large number of novel nanoparticles rapidly and comprehensively for siRNA delivery efficiency, providing both high quality cell images and quantitative data on siRNA uptake, thereby avoiding the need for separate microscopy and quantification studies.

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A78
Endosomal DNA release studies using giant unilamellar vesicles as model endosomal membranes

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Endosomal DNA release is one of the main barriers to successful non-viral gene delivery, since the inability of DNA to escape from the endosome at an early stage leads to its degradation through trafficking to the lysosomal compartment. It is therefore essential to understand the interactions between commonly used gene delivery vectors and endosomal membranes. While membrane interactions are often studied using small unilamellar vesicles (SUVs) as model bilayers, it is proposed that giant unilamellar vesicles (GUVs) present more realistic models due to their larger size, their superior lipid packing due to reduced surface curvature and the ability to visualise them using light or confocal microscopy. GUVs composed of a mixture of neutral or neutral and negatively charged lipids, representing early or late stage endosomal membranes respectively were prepared by electroformation in calcein, followed by the addition of cobalt chloride to quench background fluorescence. GUVs were then observed by confocal fluorescence microscopy before and after the addition of lipid:DNA complexes composed of equimolar mixture of dimethyldioctadecylammonium bromide (DDAB) with the helper lipid dioleoylphosphatidyl-ethanolamine (DOPE) incorporating a 10 mol% rhodamine-labelled DOPE at a 4:1 lipid:DNA charge ratio. Furthermore, in order to visualise the DNA in relation to the encapsulated calcein (green) and the lipid (red), 4′,6-diamidino-2-phenylindole (DAPI) was added to highlight the DNA blue. Both endosomal models formed spherical GUVs approximately 10–90 μm in diameter and were visible as green calcein-encapsulating vesicles. Upon the addition of lipid:DNA complexes to the early endosomal model, a large number of GUVs were shown to lose fluorescence due to calcein leakage, which was concentration dependent first order kinetics. This was also associated with visible alignment of the lipid (red) and the DNA (blue) around the GUV with possible pore formation and DNA translocation across the endosomal membrane. When lipid:DNA complexes were added to the late endosomal membrane model (which incorporated a small percentage of anionic lipid), calcein leakage and pore formation on the surface of the GUV membranes were clearly visible. Additionally, and exclusively to this model, however, a high number of GUVs were shown to deform after the addition of the complexes with or without calcein leakage. This was thought to be due to electrostatic interactions between the cationic DDAB and the anionic lipid domains of the endosomal membrane. In conclusion, it is thought that DDAB-DOPE:DNA complexes interact with both early and late endosomal membranes, causing pore formation and DNA translocation across the membrane, however the nature of the interaction changes as the endosomes traffic from early to late stages.

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A79
Characterisation of a cytosolic shuttle based upon ricin toxin

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We have cloned and codon optimised both modified ricin B chain (containing N-terminal 6 His and V5 motifs) and disarmed ricin A chain (containing either a deletion (deleted amino acids 177–183) or mutation (amino acids 177–183 mutated to Gly) within the active site). These molecules were expressed in *Escherichia coli* BL21*DE3 and affinity purified from *E. coli* lysate using Talon affinity resin. Following an initial round of characterisation by SDS PAGE and Coomassie brilliant blue staining, Western blotting (using commercially available anti-ricin A or B chain antibodies as well as antibodies specific for N- and C-terminal epitopes) was successfully used to confirm the production of both species of molecule. Both ricin A and B chains were tested for toxicity against a panel of cell lines either individually, after mixing the A and the B chains, or after re-folding using published protocols. Having ascertained that, relative to wild-type ricin A chain, minimal toxicity was displayed by the disarmed A chain analogues, further controls were undertaken to investigate the character of the recombinant B chain. These studies are reported here and show that the recombinant B chain demonstrates both lectinic activity and the ability to translocate to the Golgi, being localised to GM130 positive structures as depicted by epifluorescence microscopy. Further, crude subcellular fractionation and Western blotting of Vero cells exposed to refolded ricin toxin containing disarmed A chain show the disarmed A chain in the cytosol and the differential sedimentation of the B chain within membrane delimited structures. This data suggests the potential of these materials as cytosolic delivery vehicles suitable for use with gene medicines such as antisense oligonucleotides or RNAi agents.

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A80

Microcalorimetric and spectroscopic studies on the mechanism of interaction between novel peptoids and lipid bilayers - effect of length, charge and N-terminal end group

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Biomacromolecules as proteins and nucleic acids are promising drug candidates. However, one problem with biomacromolecules is that they usually have to pass the cell membrane to exert their effect. Utilization of cell penetrating peptides (CPPs) might be a way to transport biomacromolecules across the cell membrane. It is becoming increasingly evident that CPP uptake pathways may vary depending on the physico-chemical properties of the CPP and the cargo they deliver, the specific cell types and the specific experimental conditions. Nevertheless, the interaction between CPPs and membrane is the very first step of the internalization. Analysis of the CPPs interaction with liposomes is expected to provide information about the CPPs interaction with the cell membrane. We have performed a thermodynamic characterization and spectroscopic of the binding between a series of novel CPPs and anionic liposomes. Recently, we described a new class of CPPs, which seem to show superior biological effect compared to the well described CPPs. The molecular design of these alpha-peptide-beta-peptoid chimeras is based on alternating repeats of (-amino acids and (-peptoid residues. The rationale was to benefit from the structure-promoting effects and lipophilicity from the unnatural chiral (-peptoid residues, and the (-amino acid residues providing cationic properties and hydrogen bonding possibilities. The chimeras are very stable toward proteolysis, non-hemolytic, possess antibacterial activity and promising cell-penetrating potential. Interpretation of the data obtained in ITC-experiments showed that an increased number of basic residues in the novel CPPs sequence resulted in a more favorable interaction with the anionic liposomes. Additional experiments revealed that a hydrophobic interaction was a part of the binding. From CD spectra it was concluded, that no major structural changes occurred in the novel CPPs when they were in the presence of anionic liposomes. The initial electrostatic attraction in CPPs internalization mechanism was confirmed by comparing Gibbs free energy ((*G*) with the number of basic residues. Furthermore, it is proposed that the hydrophobic interaction registered could be between hydrophobic groups on the novel CPP and the hydrophobic region of the liposome. Another possibility could be simultaneously increased lipid-lipid interaction in the hydrophobic region of the liposome. In conclusion, when comparing the novel CPPs with results obtained for the well described CPP penetratin it seems, that the binding to anionic liposomes is more favorable for all novel CPPs investigated.

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A81

Studies towards improved cell-penetrating peptide-promoted macromolecular drug delivery

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The general concept of drug delivery facilitated by cell-penetrating peptides (CPPs) is well-known; however its practical utility for delivery of biopharmaceuticals necessitates further development concerning *in vivo* stability and efficiency of these peptidic carriers. In the present project, the aim is to increase the stability towards enzymatic degradation as well as to improve membrane translocation properties by incorporating novel unnatural amino acids into the naturally occurring CPP penetratin. The CPP efficiency of these penetratin analogues will be tested upon conjugation to a therapeutic biomacromolecule. Nine novel and unique amino acid building blocks have been synthesized from enantiopure aziridines to form amino acids with additional cationic charges as compared to natural amino acids. An increased number of cationic charges in CPPs have been shown to improve the interaction between CPPs and the cell membrane. The novel amino acids will be incorporated into penetratin to increase its cationic charge and to generate more efficient and stable CPPs. The enzymatic stability of penetratin is estimated by testing its resistance towards degradation by intestinal juice from rats. The metabolites are analyzed by an Orbitrap MS to identify the initial sites of cleavage and the largest non-degradable fragment as well. Thereby the optimal sites for incorporation of the novel amino acids may be revealed. The modified penetratin molecules will be tested for stability and CPP efficiency.

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New configuration of an *in vitro* blood–brain barrier model

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It is an undeniable fact that neuroscience has an urgent need for a reliable and translatable *in vitro* model to investigate the human blood–brain barrier (B3). The use of human primary cerebral capillary endothelial cells is considered to provide such a model. The aim of the present study was to compare a B3-model based on two novel immortalized human primary brain endothelial cell (hBEC) lines. The human cerebral cortex microvascular endothelial cell (hCMEC-D3) and the human brain capillary endothelial cell line (NKIM-6) were used. These cell lines were used to investigate the potential transport of large molecules across the cell monolayer. The B3 is unique in that it consists of highly selective endothelial cell interface that create tight junctions around the capillaries separating the bloodstream from the brain parenchyma. Brain endothelial cells in association with astrocytes display complex tight junctions, polarized expression of enzymes, transporters and receptors. In order to take advantage of the influence associated with astrocytes we established an *in vitro* co-culture model of hBECs with primary human astrocytes. The co-culture was performed either by growing the cells on either side of a permeable membrane or growth in direct contact. Using a cell-based kinetic profiling approach

we studied the optimal conditions for attachment and proliferation of the astrocytes and hBECs. Furthermore, we monitored the effect of hBEC growing directly on the surface of an adherent astrocytic monolayer. The tight junctions between the brain endothelial cells forms a diffusion barrier that is responsible for the high paracellular resistance which is a crucial characteristic for any B3-model. In order to test the integrity of this barrier in the B3-model and simultaneously measure the transcellular transport we combined fluorescent compounds and dye labelled large molecules to test the permeability across the barrier. This strategy allows for the discrimination between transcellular and paracellular transport.

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A83
Solid lipid nanoparticles for gene delivery into prostate cancer cells

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Prostate adenocarcinoma is the most common cancer occurring in male. The aim of this study is to develop a gene delivery system based on solid lipid nanoparticles (SLNs) for the transfer of tumor suppressor genes that are able to induce death into prostate cancer cells. Formulations of cationic SLNs, consisting of stearic acid/DOTAP/pluronic, were produced. Additionally, formulations with and without 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in various molar ratios were tested. The SLNs produced were approximately 100 nm in size and showed a positive surface charge (+40 mV) in water. The SLNs showed excellent stability, as evidenced by size, zeta potential, transfection efficiency over 140 days, and possibility of lyophilization and/or sterilization without loss of efficiency. The SLNs were able to protect genetic material against DNase digestion and showed a transfection capacity comparable to that of Lipofectamine 2000®, a commercially available gene carrier. Interestingly, we found that the transfection efficiency of SLNs in prostate cancer PC3 cells was significantly higher when compared to that in normal human prostate PNT2-C2 cells. Further examination revealed that this is due to enhanced endosomal escape rather than enhanced internalization of SLNs in prostate cancer cells. These results indicate that cationic SLNs are a promising tool for gene delivery into prostate cancer cells.

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A84
Kinases in cationic lipid/polymer-mediated gene delivery

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Cationic lipids/polymers, complexed with DNA (also called lipo/polyplexes), are promising tools for gene delivery or transfection. Lipo/polyplexes have low toxicity, a relative low immunological response and can be synthesized on large scale. Lipo/polyplexes are internalized by cells via endocytosis. The endocytotic pathway that is used by lipo/polyplexes depends on the cell type and the type of lipo/polyplexes, and likely contributes to transfection efficiency. We have recently shown that adhesion receptors are involved in binding and endocytosis of lipoplexes. Cell receptors also have been described for the endocytosis of polyplexes. Receptor occupation can initiate signaling cascades, commonly mediated by kinases, which in turn tightly regulate endocytosis and endocytotic processing. The elucidation of cellular signaling signatures, initiated by lipo/polyplexes and/or those that allow or preclude gene delivery, will be instrumental in understanding the interaction between lipo/polyplexes and cells at the molecular level and contribute to the design of protocols with improved gene delivery efficiency. In this study we have performed a screen with a wide range of validated pharmacological kinase inhibitors, and evaluated their effects on lipo/polyplex transfection efficiency. In this screen a kinase is identified that specifically influences the transfection efficiency of a polyplex. It is further demonstrated that, as a part of the underlying mechanism, this kinase regulates the endocytotic processing of the polyplex and, as a consequence, controls its endosomal escape.

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A85
Peptide-based nano-particle for *in vivo* delivery of siRNA

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The development of short interfering RNA (siRNA), has provided great hope for therapeutic targeting of specific genes responsible of pathological disorders. However their clinical application remains limited by their poor cellular uptake, low bioavailability, and insufficient capability to reach targets *in vivo*. We have designed a novel approach, based on short amphipathic peptides 'CADY' that promotes efficient delivery of siRNA into wide variety of mammalian cell lines and *in vivo* upon systemic and topical administrations. This carrier consisting of a balance between hydrophobic and hydrophilic domains and forms stable discrete 'nanoparticles' with siRNA, through non-covalent interactions. Cellular uptake mechanism of CADY/siRNA nanoparticles is dependent on the size of the particle and involves membrane potential and dynamic, which enables a rapid release of the siRNA into the cytoplasm and promotes a robust down-regulation of target mRNA. CADY-carriers were applied to the delivery of siRNA targeting the cell cycle regulatory protein Cyclin B1 into cancer cells. We demonstrated that when associated with CADY, sub-nanomolar concentrations of siRNA Cyclin B1 significantly knocked down Cyclin B1 protein levels resulting in cell cycle arrest in G2 arrest and blocked cancer cell proliferation. The surface of CADY particles can be functionalized and addition of cholesterol-moiety significantly improves siRNA stability *in vivo*, thereby enhancing the efficiency of this technology for systemic administration following intravenous injection. We have validated the therapeutic potential of this strategy for cancer treatment by targeting cyclin B1 in various mouse tumour models and demonstrate that CADY-mediated delivery of cyclin B1 siRNA prevents tumour growth *in vivo* following systemic intravenous injection. Moreover, we showed that functionalization of CADY particles with other chemical groups or biological moieties can be applied to generate formulations to target specific cell types or tissues which can

be of a major interest for future development. Given the biological response yielded through this approach, we propose that non-covalent, peptide-based delivery technologies hold a strong promise for therapeutic administration of siRNA.

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A86
High-efficient transfection using cationic lipids with programmed biodegradability

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Delivery of nucleic acids into cells has an ever-increasing number of applications with outstanding advances in both gene therapy and biotechnology, highlighting the induction of pluripotency in somatic cells. While the use of viral vectors is currently the most efficient transfection method, their antigenicity along with the risk of potential mutagenesis, among other inconvenients, are important limitations that hinder its application in medicine. Non-viral delivery systems (cationic lipids and polymers) represent an attractive alternative, particularly because of their low-cost, tuneable design and procedural simplicity. However, the *in vivo* efficacy of these carriers needs to be increased for both research purposes and clinical application. As repetitive dosing would be required in any gene therapy treatment, the cytotoxicity due to the use of these chemicals needs to be reduced, ideally by regulating their metabolic fate. To address these issues, a tripodal cationic lipid [1] was specifically designed to undergo complete intracellular metabolisation into naturally occuring compounds aiming to minimise the toxicity associated with its cytoplasmatic residence. Besides the toxicity issue, the incorporation of hydrolysis-prone linkages was addressed to enhance the cationic lipid-DNA dissociation once the lipoplexes have entered the cell by endocytosis. The novel compounds showed remarkable transfection efficiency along with reduced toxicity in a variety of immortalized cells and stem cells. Moreover, preliminary *in vivo* studies underlined the potential applicability of these non-toxic reagents for the delivery of DNA into mouse lung. These reagents, contrary to the most of chemical carriers commercially available, might offer a viable chemical alternative to viral transfection.

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A87
Immune stimulation following microneedle delivery of influenza virus-like particle (VLP) vaccines to human skin

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Virus-like particles (VLPs) possess a number of features that make them attractive vaccine candidates for immunization against infectious disease. Efficient intra-epidermal delivery of VLP vaccines would exploit the abundance of Langerhans cells (LCs) that reside within the skin epidermis to generate an efficient host immune response. Microneedles (MNs) are currently being developed for the convenient and pain-free delivery of drugs and vaccines across the skin barrier layer. Whilst MN-based vaccines have demonstrated proof-of-concept in mice, it would be extremely valuable to understand how MN targeting of influenza VLP vaccines to the skin epidermis affects activation and migration of LCs in the real human skin environment. MNs with lengths of 700 μm were laser-etched from stainless steel sheets and surface-coated with either influenza H1 (A/PR/8/34) or H5 (A/Viet Nam/1203/04) VLPs. The coated MNs easily and reproducibly penetrated freshly excised human skin, depositing approximately 80% of the vaccine load within 60 s. Experiments conducted in cultured human skin showed that H1 and H5 VLPs, delivered via MNs, stimulated LCs causing morphological changes and a significant decline in total LCs number in epidermal sheets at 24–48 hours compared to untreated skin at the same time points. Histological sections showed that LCs in VLP treated samples were more dispersed throughout the epidermis with substantial numbers in the vicinity of the basement membrane. The response made by LCs was more manifest in human skin treated with H1 VLPs, compared with H5 VLPs. These findings corroborate observations in mouse studies, where H1 VLPs were shown to be significantly more immunogenic than H5 VLPs. Our data provide strong evidence that MN-facilitated delivery of influenza VLP vaccines initiates a stimulatory response in LCs in human skin epidermis. The results complement and support data gained from animal models, suggesting dendritic cells (DCs), including LCs, targeted through intra-epidermal or intra-dermal deposition of the vaccine generates immune response. This study also emphasizes the value of cultured human skin alongside animal studies for informative preclinical testing of intra-dermal vaccines.

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A88
Electrically based transdermal techniques for delivery of therapeutic macromolecules

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Advances in molecular biology have given us a wide range of protein and peptide based drugs that are unsuitable for oral delivery because of their high degree of first-pass metabolism. Though parenteral delivery is successful for developed and commercially available protein and peptide based drugs, chronic and self administration formulations are not the ideal choice through this route. Transdermal delivery is emerging as the biggest application target for these agents, however, the skin is extremely efficient at keeping out such large molecular weight compounds and therapeutic levels are never going to be realistically achieved by passive absorption. Therefore novel transdermal drug delivery systems have been developed with the aim to achieve the objective of systemic medication through topical application to the intact skin surface with benefits of deliver therapeutic macromolecules in desire therapeutic doses to overcome the difficulties associated with the oral route, namely poor bioavailability of drug and the tendency to produce rapid blood

level. Some newly active rate controlled electrically based transdermal techniques including: iontophoresis, electroporation, ultrasound and photomechanical waves have been developed and commercialized for the delivery of troublesome therapeutic protein and peptide based macromolecular drugs. This study covers the development of different electrically based transdermal techniques for delivery of therapeutic protein and peptide based macromolecular drugs, current status and assesses the pros and cons of each technique and summarises the evidence-base of their drug delivery capabilities.

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A89
Molecularly imprinted polymers: macromolecule recognition

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Molecular imprinting is a technique used to engineer synthetic antibody mimics by the polymerisation of so-called functional monomers and cross-linkers around a target (template) species. Following removal of the template from the polymer matrix, cavities remain which display both chemical and steric selectivity for the imprinted molecule. To date the imprinting of biologically relevant macromolecules has been somewhat unsuccessful due to the inherent complexity of imprinting such moieties in aqueous media. Unlike small, organic molecules that are typically employed as templates, macromolecular structures such as peptides and proteins can exist in a multitude of conformations which leads to the development of heterogeneous binding sites as opposed to the well defined cavities formed during the regular imprinting process. The proteins will denature in traditional imprinting environments due to the presence of organic solvents and elevated temperatures. Additionally, the size of these biomolecules means that removal from the polymer matrix and subsequent re-binding is often inefficient. As a consequence, molecular imprinting has yet to achieve its true potential as efficacious, robust, reliable and cost-effective alternatives to the currently used antibody-based recognition systems. Projects currently underway within our laboratories aim to utilise target-selective peptides, derived from a phage display library, as a high affinity 'functional monomers' in a hybrid peptide-polymer molecularly selective system. Targets include lipopolysaccharide (LPS), the major pathogenic determinant of Gram negative bacteria and prion protein which is believed to be the causative agent of a group of invariably fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs). Work to date has focused on optimisation of surface chemistries. Bifunctionalised polystyrene resin and glass surfaces have been synthesised to facilitate the independent immobilisation of peptide moiety and an initiator species. Polymer growth from the surface has been monitored by Fourier transform infra-red spectroscopy and atomic force microscopy. Future work will involve optimising a number of polymerisation variables and incorporating the phage-display derived peptide into the system to fully evaluate its potential as an antibody mimic.

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A90
Interference of mycobacterium tuberculosis with the endocytic pathways on macrophages and dendritic cells from healthy donors: role of cathepsins

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Antigen-presenting cells (APC) such as macrophages and dendritic cells (DCs) play a pivotal role in tuberculosis pathogenesis. Macrophages are also key effector cells in mycobacteria killing. In order to survive inside the host immune cells mycobacteria developed different strategies. Among them blocking of phagosome-lysosome fusion and consequential reduced phagosome acidification assumes a crucial role allowing mycobacteria to escape acidic pH and destruction by proteolytic enzymes present in phagolysosomes. Since phagosome acidification varies between macrophages and DCs this may allow different kinetics of acquisition and activity for the enzymes involved. The aim of the present study was to compare the distribution of two key cathepsins: the exopeptidase cathepsin B and the endopeptidase cathepsin S inside human monocyte derived macrophages and DCs infected with *Mycobacterium* spp. Infected immune cells were collected after 3 hours and 1 day post-infection and prepared either for immunofluorescence confocal microscopy or for immunogold electron microscopy on ultrathin cryo sections. In macrophages we did not observe significant co-localization between either BCG or Mycobacterium tuberculosis and cathepsins B or S indicating that phagosome-lysosome fusion was strongly hindered. Similar results were observed for Mycobacterium tuberculosis after infection of DCs. In DCs the acquisition of cathepsin B into the phagosomes containing BCG was different from the acquisition of cathepsin S. Cathepsin S content was decreased by 30% after 1 day of infection whereas cathepsin B content inside BCG-positive phagosomes was increased. Our data indicate that cathepsins might be involved in differential mycobacterial persistence in macrophages compared to dendritic cells.

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A91
Role of mycobacterium tuberculosis outermembrane porins in bacterial survival within macrophages

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Mycobacterium tuberculosis (Mtb) is the etiologic agent of tuberculosis a major worldwide health concern. One important feature in Mtb virulence is the ability to withstand the detrimental conditions of the phagosome within macrophages. Most of the virulence factors of Mtb are PAMPS from the outer membrane of the bacilli. Outer membrane porins participate in the inflow of hydrophilic compounds and we have shown that they are important for mycobacteria intracellular survival. Several porins have already been described as a means for nutrient acquisition but also as a possible pathway for antibiotic inflow. Previous studies showed that mutant

Mycobacterium smegmatis lacking the MspA porin grow defectively due to the lack of glucose uptake but display increased resistance to several antibiotics and also to nitric oxide. Nitric oxide burst is a well described bactericidal mechanism in mouse macrophages and the inducible nitric oxide synthase is the enzyme responsible for NO release. In this study, we describe a novel putative outer membrane protein conserved between *M. tuberculosis* and *Mycobacterium bovis* BCG. We show that the absence of this protein limits bacterial growth *in vitro* but results in increased BCG survival within macrophages. We also demonstrate that although interferon-gamma stimulation of macrophages induces ten times increased killing of BCG, bacteria lacking this protein remain unsusceptible to this stimulation. Furthermore, quantification of iNOS and IL-1beta expression through qRT-PCR revealed that those genes were less upregulated during infection with the mutant bacteria compared to the WT strain suggesting that the increased survival of the mutants is due to lower macrophage activation and release of nitric oxide. We conclude that MtpA from Mtb complex is important to release virulence factors required for macrophage activation.

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A92

In vivo phage display to identify peptides that target the brain

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The delivery of novel macromolecular therapeutics into brain parenchyma to treat central nervous system disorders (CNS) is hindered by the blood–brain barrier (BBB). The BBB is comprised of microvascular endothelial cells that line the capillaries traversing the brain. The existence of highly restrictive tight junctions and the relatively low abundance of morphologically evident endocytic vesicles restricts both paracellular and transcellular access to the brain of therapeutic proteins, peptides and nano-medicines [1]. As part of an ongoing programme to identify novel ligands that mediate endocytotic and transcytotic events within the BBB we report here the use of a Phage Display library to identify small cyclic peptides (-7mer) that traverse the *in vivo* rat BBB. A Phage Display Library (Ph.D.-C7C™ New England Biolabs) representing 1.2×10^9 unique genotypes encoding random-7mer disulphide constrained peptides genomically fused to the pIII coat protein of the filamentous phage M13 was utilised in all studies. A synchronous selection strategy [2] was employed to select for peptides homing to a range of organs before undertaking a final selection for peptides that home to brain grey matter. In this final selection the library was injected i.v. into a rat and circulated for 15 minutes before perfusion with saline to remove freely circulating phage and then glycine buffer (pH 2.2) to strip the vasculature of binding phage. The brain was removed and the white matter and capillaries depleted before the grey matter (brain parenchyma) was homogenised and phages recovered. The recovered phages were gene sequenced to determine the corresponding peptide library sequence displayed. From the sequenced population a conserved motif AC-SXTSSTX-CGGGS was identified at a frequency of 25%; secondary phage studies and bioinformatic analysis of a large population of sequenced clones (>500) corroborated this sequence. *In vivo* biodistribution studies of a clone displaying the conserved motif (AC-SYTSSTM-CGGGS) revealed a selective homing to brain grey matter as demonstrated by a 4-fold increase in AUC0-∞ and 3.5-fold increase in Cmax in brain grey matter compared to insertless phage (no displayed phage). Studies are addressing the molecular pathways of entry of this peptide phage into the CNS.

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A93

Phage display identification of a lung transduction peptide that affords enhanced macromolecule transport across the intact lung epithelium

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Evolutionary technologies based upon the screening of combinatorial libraries, for example, phage display, are used to survey the molecular diversity of target cell surfaces with the aim of identifying peptide motifs that promote target cell binding or internalisation [1]. Here, an M13 phage peptide library displaying cyclic 7-mer peptides was biopanned against the luminal surface of primary cultures of rat lung alveolar epithelial cells. 'Cell associated' phage were isolated after 4 rounds of biopanning, with the peptide library repertoire contracting from 1.2×10^9 clones to a maxium of 2×10^3 clones. DNA sequencing of 'cell associated' phage clones indicated peptide sequences to be largely composed of hydrophillic amino acids with isoelectric points approximating neutrality. The most frequent phage clone bore the peptide sequence C-TSGTHPR-C (termed LTP-1) and displayed enhanced (>1000-fold) transport (versus phage control vector) across restrictive *in vitro* alveolar epithelial monolayers [2]. When the LTP-1 phage clone (LTP-1) was administered as a coarse aerosol into the airways of an isolated perfused rat lung IPRL preparation [3] the extent of phage absorption across the pulmonary epithelium was 8.6% by 120 min, some 1500-fold greater than either the insertless vector control or a library clone that displaying a control peptide sequence (C-PLLAPGI-C, termed NB-3) that was isolated from the first biopanning round. When LTP-1 phage was co-administered with a 100-fold molar excess of the synthetic LTP-1 peptide sequence (syn-LTP-1) the extent of LTP-1 phage was competitively inhibited (LTP-1 phage absorption reduced to 0.1% by 120 min, $p < 0.05$). In contrast, the synthetic NB-3 peptide (syn-NB-3) displayed no inhibitory effect (7.6% LTP-1 phage absorbed dose absorbed by 120 min, $p > 0.05$). The syn-LTP-1 peptide sequence was grafted onto the surface of an anionic PAMAM G5.5 dendrimer

at a 1:1 stoichiometry to test the lung transduction functionality of the peptide using the 53 kDa dendrimer as a model macromolecular cargo. Phage peptide-dendrimer conjugates were labelled with a fluorophore and characterised by ^{1}H NMR and quantitative amino acid analysis prior to administration into the airways of the IPRL model. The extent of absorption of PAMAM G5.5 alone equalled 17 ± 6% of lung deposited dose absorbed by 90 min. G5.5 dendrimers displaying one syn-LTP-1 peptide per polymer (termed G5.5-syn-LTP-1) displayed a 1.8-fold greater extent of absorption ($p < 0.05$) cf. G5.5 alone; G5.5 dendrimer displaying one equivalent of the syn-NB3 peptide showed no evidence of enhanced absorption ($p > 0.05$). The enhanced absorption of G5.5-syn-LTP-1 absorption was competitively inhibited by co-administration of 100-fold molar excess of syn-LTP-1 peptide ($p < 0.05$) but not by syn-NB-3 peptide ($p > 0.05$), an observation consistent with the participation of a specific receptor-mediated transport mechanism. As such the LTP-1 peptide motif may serve as a platform for enhancing macromolecule absorption from the airways.

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A94
Differential transport of anionic PAMAM dendrimers across *in vitro* biological barriers

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Polyamidoamine (PAMAM) dendrimers are a class of branched polymers that have the potential to serve as drug carriers. This is primarily due to their extremely low polydispersity index, the ability to precisely control their size and charge, and the multiple functional groups that they bear on their surfaces giving the ability to conjugate a wide range of therapeutic molecules. The transport across *in vitro* biological barriers of cationic PAMAMs has been widely studied with reports often indicating high barrier permeability, although interpretation of such data in the context of cation-induced barrier toxicity is often omitted. We are investigating the intrinsic biological activity of intact stable anionic dendrimer-drug conjugates where the dendrimer moiety not only confers a backbone for attachment of multiple phamacological ligands but also offers a means to physically modulate *in vivo* tissue disposition, for example, affording access to intestinal submucosa but excluding BBB penetration. In this abstract we report the differential *in vitro* barrier permeability of a molecular weight series of anionic PAMAM dendrimers, that is, G1.5, 2935 Da; G3.5, 12,931 Da; G5.5, 52,907 Da which has supported our ongoing *in vivo* investigations. Dendrimers were fluorescently labelled and added to the apical surface of epithelial cell monolayers grown on a semi-permeable inserts (Transwell). Permeability coefficients (ρ) were determined for transport in the apical to basal direction. The epithelial models included the highly restrictive MDCK-I (TEER 5000 Ω cm^2), the moderately restrictive Caco-2 (TEER 600 Ω cm^2) and the low restrictive MDCKII (TEER 200 Ω cm^2). For CACO-2 and MDCKII an inverse relationship was evident between dendrimer transepithelial transport and dendrimer molecular size, with dendrimer ρ decreasing approx. 5-fold G1.5 ⇒ G3.5, and decreasing approx. 10-fold G1.5 ⇒ G5.5. The permeability of the cell models to dendrimer transport declined as the paracellular restrictiveness of the monolayers increased. Indeed, for MDCKI monolayers dendrimer concentrations in the basal chamber remained at all times below the limit level of detection, but could be readily enhanced by briefly adding EDTA to the media. Nevertheless, predicted (based upon LLQ) ρ for dendrimer transport across MDCKI were at least ×10–15-fold lower than in the other cell models. Significantly, even for the smallest dendrimer, that is, G1.5, the maximum predicted (based on LLQ) ρ across MDCKI was no greater than 15% of the ρ obtained for the paracellular marker F-Na. Whereas ρ for G1.5 was 51% and 56% of that for F–Na in CACO-2 and MDCKII models, respectively. Biocompatibility studies show no affect of the anionic dendrimers upon overall barrier properties. The paracellular route is the major pathway of dendrimer transport across biological barriers. Stable pharmacologically active conjugates of dendrimer – drug are an interesting experimental therapeutic with potential to provide differential tissue distribution/exclusion based upon physical characteristics.

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A95
Non-toxic, highly efficient delivery of nucleic acids into challenging cells using safectin transfection reagent

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Deliverics Ltd. has developed a novel cationic lipid-mediated transfection reagent for DNA and siRNA delivery into both easy and challenging to transfect eukaryotic cells: SAFEctin Transfection Reagent. This reagent is a water-based formulation of cationic and neutral lipids with programmed biodegradability. SAFEctin allows for the highest transfection efficiency of nucleic acids into many cell types (e.g. immortilized cells, mESC, hMSC) with the simplest-to-use and fastest procedure in the market: (i) mix SAFEctin and the nucleic acid (ii) followed by direct addition to cells, either in the presence or absence of serum and antibiotics. The formulation has been developed to have very low toxicity to cells and as such it is not necessary to remove or change culture medium following transfection. Combination of the highest/safest transfection rates on the market with the simplest to use protocol ensures optimal performance and fast results. The SAFEctin Transfection Reagent is a universal system that outperforms competitor's products in each of the three defining features any researcher seeks in this kind of product: efficacy, safety and ease of use.

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