6th European Workshop on Particulate Systems
Geneva, March 23-24, 2006

Organisation

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UNIVERSITÉ DE GENÈVE FACULTÉ DES SCIENCES

Dear EWPS members:

We are pleased to welcome you to the 6th European Workshop on Particulate Systems in Geneva on March 23 and 24, 2006.

The EWPS meeting will be held at the University of Geneva, 30 quai Ernest Ansermet, in the auditoriums A150 and A100. Apart from the scientific program, we have the pleasure to visit two exhibitions at the Museum of Ethnography, a few blocks away from the University, and to share a typical Swiss dinner on Friday. Should you wish to visit the mountain and ski areas during the weekend we have all the necessary information about transportation.

We wish you a fruitful meeting and a pleasant stay in Geneva and are also looking forward to seeing you during the Bio Innovation Day and the World Meeting.

Best regards,

Robert Gurny
Florence Delie-Salmon
Florence von Ow

EWPS 2006
University of Geneva - Sciences II
30 Quai E. Ansermet

Program at a glance

1200 – 1300	Registration / Coffee (entrance hall, Sciences II)

Thursday 23 March 2006 (Room A150)

1300 – 1500	Presentations: *Formulation parameters*
1500 – 1530	Coffee break
1530 – 1800	Presentations: *Delivery of nucleic acids*
1800 – 1845	Invited lecture: Dr. E. Allémann, Bracco Research, Geneva "Microbubbles: from ultrasound contrast agents to drug delivery systems"
1845 – 2000	Diner at the School of Pharmaceutical Sciences
2000 – 2130	Presentations: *Novel concept in delivery*

Friday 24 March 2006 (Room A100)

0900 – 1100	Presentations: *Protein delivery and liposomes*
1100 – 1130	Coffee break
1130 – 1300	Presentations: *Active targeting and surface modification*
1300 – 1350	Lunch at the School of Pharmaceutical Sciences
1400 – 1620	Visit of the Geneva Museum of Ethnography
1630 – 1730	Presentations: *Various routes of administration*
1730 – 1745	Coffee break
1745 – 1845	Presentations: *Vaccine delivery*
1930 →	All together dinner

EWPS 2006

University of Geneva - Sciences II
30 Quai E. Ansermet

Detailed program

Thursday 23 March 2006 Room A150

13.00 – 13.10: Robert Gurny, Florence Delie
Welcome and announcements

Formulation parameters

13.10 – 13.30: Magali Zeisser-Labouèbe (Geneva)
Hypericine loaded nanoparticles for photodynamic therapy of ovarian cancer

13.30 – 13.50: Hervé Hillaireau (Paris)
Encapsulation of antiviral nucleotide analogues cidofovir and azidothymidine-triphosphate in poly(iso-butylcyanoacrylate) nanocapsules

13.50 – 14.10: Richard Kaye (London)
Development and optimisation of biocompatible, particulate formulations for the airway delivery of immunoglobulins

14.10 – 14.30: Nicoleta Butoescu (Geneva)
Coencapsulation of active and superparamagnetic ingredients into PLGA microparticles

14.30- 14.50: Emilia Pisani (Paris)
Polymeric capsules for ultrasound imaging: physical characterization

Coffee break

Nucleic acid delivery

15.30 – 15.50: Camilla Foged (Copenhagen)
Long-circulating liposomes for secretory phospholipase A2 triggered release of siRNA in inflamed tissue

15.50 – 16.10: Haliza Katas (London)
Development of chitosan nanoparticles for siRNA delivery

16.10 – 16.30: Sabrina Oliveira (Utrecht)
Enhanced knockdown of EGFR: improved siRNA endosomal escape and delivery into the cytosol

16.30 – 16.50: Chandrasekaran Ramaswamy (London)
Functionalised lipidic dendrimers for gene delivery

16.50 – 17.10: Holger de Wolf (Utrecht)
Tumor transfection using polyplexes based on biodegradable poly(DMAEA)-phosphazene

17.10 – 17.30: Caroline Roques (Paris)
Characterization and *in vivo* evaluation of different polymer/DNA formulations for gene delivery to the skeletal muscle

18.00 – 18.45 Invited lecture: Dr. E. Allémann,
Bracco Research, Geneva
"Microbubbles: from ultrasound contrast agents to drug delivery systems"

18.45 – 20.00 Diner at the School of Pharmaceutical Sciences (entrance hall)

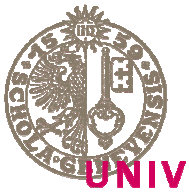
Novel concepts in delivery

20.00 – 20.20: Karine Mondon (Geneva)
Hexyl substituted polylactides used in copolymers for hydrophobic drug incorporation

20.20 – 20.40: Cristianne Rijcken (Utrecht)
Methacrylamide-oligolactates as building blocks for targeted biodegradable polymeric micelles to deliver photosensitizers

20.40 – 21.00: Myrra Carstens (Utrecht)
Self assembly of PEG-Oligolactates with monodisperse hydrophobic blocks

21.00 – 21.20: Laury Trichard (Paris)
Self-assembled semi-solid beads made of cyclodextrin and oil



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Formulation parameters

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HYPERICIN-LOADED NANOPARTICLES FOR PHOTODYNAMIC THERAPY OF OVARIAN CANCER

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Introduction: Ovarian carcinoma is the fourth most frequent cause of cancer-related death in women in the United States. Photodynamic therapy (PDT) has been suggested to improve therapeutic methods and offer the possibility of more effective eradication of this type of cancer [1]. PDT, an alternative treatment modality, is based on the administration of photosensitizers (PS) [2]. Illumination with light at the appropriate wavelength and energy dose induces the selective destruction of diseased tissues via photochemical reactions. Hypericin (Hy), a natural compound extracted from *Hypericum perforatum* is a potentially interesting drug for PDT in the field of cancer [3]. As Hy has an extremely poor water solubility, systemic intravenous administration is problematic and restricts its medical applications. To overcome this problem, colloidal carriers such as nanoparticles (NPs) have been used to produce an injectable suspension of PS [4].
Our objectives are first to produce and characterize Hy-loaded NPs in terms of size and drug loading and then to evaluate *in vitro* the phototoxicity of the encapsulated PS in ovarian cancer cells.

Methods and results: Hy was loaded in biodegradable polymeric NPs of polylactic acid (PLA) and poly(lactic-co-glycolic) acid (PLGA) using the nanoprecipitation method. The NPs were characterized in terms of size and drug loading. NPs in the range of 200 to 300 nm were obtained with a good polydispersity index (PI < 0.1). The nature of the polymer did not have a significant influence on either NP size, charge and drug loading.
Their phototoxicity was assessed on a rat ovarian cancer cell line, NuTu-19 (kindly provided by Dr A. Major, HUG, Geneva Hospital). After incubation of cells with Hy, an irradiation was performed and cell death was assessed by a viability assay (MTT assay) 24 h after. The influence of different parameters on the Hy photoactivity *in vitro* was investigated: (i) Hy concentration; (ii) incubation time of cells with Hy and light dose; (iii) NP drug loading.
The activity of Hy-loaded NPs (loading =0.03%, w/w) was compared to free Hy after 1 h of incubation and a light dose of 2.3 J/cm^2. For all formulations tested, cell viability decreased with increasing Hy concentrations. Only PLA NPs exhibited a higher efficiency than free Hy. Consequently, further experiments were carried out with PLA NPs. Hy photoactivity was increased with incubation time and/or light dose. Interestingly, Hy-loaded NPs efficiency decreased when NP drug loading increased. Several hypotheses should be verified regarding (i) the drug release from NPs, (ii) the physical distribution of Hy in the polymeric matrix, and (iii) the quenching of Hy inside NPs, which could hamper the photoactivity.

References

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2. Sharman et al., *Drug Discov. Today*, **1999**, *4*, 507.
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ENCAPSULATION OF ANTIVIRAL NUCLEOTIDE ANALOGUES CIDOFOVIR AND AZIDOTHYMIDINE-TRIPHOSPHATE IN POLY(ISO-BUTYLCYANOACRYLATE) NANOCAPSULES

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Nucleoside analogues are widely used in the treatment of viral infections, including HIV, herpes virus and cytomegalovirus. After administration, these molecules need to be phosphorylated by cellular kinases into their active triphosphate nucleotide form. Howerver, one of the limitations in the use of nucleoside analogues like azidothymidine (AZT) in anti-HIV therapies lies in the poor efficiency of *in vivo* conversion. The direct administration of the triphosphate form (e.g. azidothymidine triphosphate, AZT-TP) could bypass the metabolic bottleneck, but is currently not yet efficient, due to the excessive hydrophilic character of these molecules, which prevents their cellular penetration. Poly(alkylcyanoacrylate) (PACA) nanoparticles encapsulating AZT have been shown to efficiently deliver this molecule to macrophages, an important reservoir of the infection, after intravenous administration [1]. They are thus interesting candidates for the delivery of AZT-TP by this route.

Besides nucleoside analogues, antiviral nucleotide analogues like cidofovir (CDV) already incorporate a phosphonate group, and thus require only two *in vivo* phosphorylation steps. CDV has recently raised new interest because it holds some promise for the treatment of poxvirus infections, including smallpox, a major bioterrorist threat. Unfortunately, the use of CDV is limited by its poor bioavailability and nephrotoxicity. A new drug delivery approach is thus needed to achieve efficient delivery of CDV. Here again, PACA nanoparticles are promising candidates [1,2].

In this study, we investigated the encapsulation of the nucleotides analogues AZT-TP and CDV in poly(*iso*-butylcyanoacrylate) (PIBCA) aqueous-core nanocapsules, known to efficiently entrap oligonucleotides (ODN) [3]. PIBCA nanocapsules were obtained through interfacial polymerisation in a kinetically stabilized emulsion [3] or microemulsion [4]. The entrapment of AZT-TP as well as CDV was found to be very low. It was close to that of the model nucleotide ATP, whereas ODN, as expected, was more efficiently encapsulated. These results show that the encapsulation of small and hydrophilic molecules such as nucleotide analogues, into aqueous core nanocapsules, remains a challenge that should be addressed with new approaches.

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3. Lambert, G., Fattal, E., Pinto-Alphandary, H., Gulik, A., and Couvreur, P., Polyisobutylcyanoacrylate nanocapsules containing an aqueous core as a novel colloidal carrier for the delivery of oligonucleotides, *Pharm Res* 17 (6), 707-14, 2000.
4. Watnasirichaikul, S., Davies, N. M., Rades, T., and Tucker, I. G., Preparation of biodegradable insulin nanocapsules from biocompatible microemulsions, *Pharm Res* 17 (6), 684-9, 2000.

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DEVELOPMENT AND OPTIMISATION OF BIOCOMPATIBLE, PARTICULATE FORMULATIONS FOR THE AIRWAY DELIVERY OF IMMUNOGLOBULINS

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Poly(lactide-co-glycolide) microspheres, encapsulating a model antibody for pulmonary delivery, have been produced by the spray-drying of double-emulsion formulations. In this work the content of poly(vinylalcohol) (PVA), typically used as an emulsifier, was successfully reduced to zero, by replacement with the component of lung surfactant, dipalmitoylphosphatidylcholine (DPPC). Lactose was also present in all formulations. Microspheres containing DPPC had improved encapsulation efficiencies, and reduced burst-release, even in the absence of PVA. The change of emulsifier had no obvious effect on microsphere geometric diameter or antibody stability. However, DPPC-containing microspheres appeared from SEM images to have potentially beneficial surface properties. Therefore, given both the formulation and safety advantages, non-PVA, DPPC-containing formulations were selected for further investigation.

The effect of several manufacturing variables, on the chosen formulation was assessed by means of two factorial experiments. The first examined spray-drying parameters, and the second involved the homogenisation times and external phase volume of the emulsion. Many of the spray-drying parameters were found to significantly affect yield, encapsulation efficiency and antibody stability, as measured by ELISA. Electron microscopy revealed that the production of spherical microspheres required the resultant outlet temperature to be 50-60ºC. Homogenisation and external phase volume significantly affected the burst release, and geometric diameter of the microspheres, as measured in cyclohexane, and after dispersion in water.

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COENCAPSULATION OF ACTIVE AND SUPERPARAMAGNETIC INGREDIENTS INTO PLGA MICROPARTICLES

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Purpose. The intra-articular (i-a) administration of drugs for the local treatment of osteoarthritis and rheumatoid arthritis has extensively been studied in the last years. Among the most recent approaches to improve drug persistence in the joint, magnetic targeting with superparamagnetic iron oxide nanoparticles (SPION) seems promising[1]. Using an external magnetic field, SPION-containing particles can be guided and retained in a specific organ, while the drug is released and acts locally. Moreover, due to their superparamagnetic nature, these particles do not have remanent magnetization, thus avoiding potentially deleterious aggregation. The aim of this study was to prepare and to evaluate PLGA microspheres encapsulating SPIONs and the anti-inflammatory drug dexamethasone 21-acetate (DXM) as delivery system for i-a administration. Batches of two sizes (1 and 5 µm) of SPION- and drug-loaded PLGA microspheres were prepared by a double emulsion-solvent evaporation method. The particles were evaluated *in vitro* for their size and surface morphology, and compared in terms of iron oxide and DXM content and magnetic retention.

Methods. DXM-loaded PLGA magnetic microspheres were prepared by a double emulsion-solvent evaporation method[2]. Two different microsphere sizes were prepared by changing PLGA concentration in the organic solution as follows: 100 mg/mL to obtain microspheres with a mean diameter of 1 µm and 250 mg/mL for microspheres with mean diameter of 5 µm. Magnetic retention of the microspheres was carried out in a device composed of a tube positioned at a 3 mm-distance away from a Nd-Fe-B magnet, generating a magnetic field of 0.8T. The percentage of retained microspheres was calculated as the ratio of the final microsphere weight (after discarding the remaining suspension and freeze-drying the tube) and the initial weight. Scanning electron microscopy was performed on gold-coated samples with a JEOL JSM-6400 microscope at an accelerating voltage of 15 kV. Iron oxide content was assessed by o-phenanthroline colorimetric assay. Dexamethasone acetate content was assessed by UV absorption spectrophotometry (λ=240 nm).

Results. DXM- and SPION-loaded microspheres could be prepared using two different organic phases: ethyl acetate and ethyl formate, both added with 10% ethanol. Microspheres with a mean diameter of 1 µm or 5 µm could be isolated by centrifugation. Iron oxide content increased with particle size. This could be explained by the higher viscosity of the organic phase for the 5 µm particle preparation compared to the 1 µm particles, leading to a more stable primary emulsion, and hence to a larger iron oxide content. This could also be ascribed to the smaller interfacial surface between the organic phase and the external aqueous phase, limiting SPION loss. The magnetic retention of both microsphere sizes was around 70% for all microsphere batches. As compared to previously evaluated 300 nm SPION-containing PLGA nanoparticles (results not shown), for which the retention in the same conditions was 2%, the higher retention for the microspheres indicates that the strategy of encapsulating SPIONs into microspheres brings a significant improvement in terms of magnetic retention.

Conclusion. Superparamagnetic iron oxide nanoparticles and dexamethasone acetate were successfully encapsulated in PLGA microspheres using selected solvent mixtures. Moreover, the particle size could be tailored by changing PLGA concentration. Both 1 µm and 5 µm particles could be retained by a 0.8 T magnetic field, thus making these particles suitable candidates as a magnetically retained system for intra-articular drug

delivery. Our future research will be focused on the release kinetics of DXM from PLGA microparticles. In a second step, studies of the interaction between the magnetic PLGA microspheres and different macrophage cell lines and *in vivo* studies on animal models of arthritis will be carried out.

References

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POLYMERIC CAPSULES FOR ULTRASOUND IMAGING: PHYSICAL CHARACTERIZATION

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The use of ultrasounds as a diagnostic tool has increased in recent years. Advantages of ultrasound are numerous: low cost of the exam, wide availability, portability, low cost of the equipment and absence of ionizing radiation. Unfortunately, ultrasound signal to noise ratio is usually small. Ultrasound contrast agents are therefore used to enhance the signal to noise ratio.

Commercial ultrasound contrast agents (USCA) usually suffer from a poor stability with time in solution with a mean half life of a few MINUTES. The elaboration of more stable USCA would lead to a more widespread clinical use, and take full benefits of harmonic imaging, which enhances considerably the signal to noise ratio [1,3].

Contrast agents are subjected to insonation, and ultrasound energy has been shown to have a role in increasing polymer degradation rates and therefore drug delivery. The ability to externally control degradation and drug release rates opens many possibilities to non-invasive, targeted drug delivery systems. Additionally, ultrasound is a relatively safe triggering mechanism. These systems have great potential for targeted treatment of diseases such as cancer, for which current systemic treatments have severe toxic side effects.

We have developed a product candidate for ultrasound contrast agent based on biodegradable polymer encapsulating liquid perfluorocarbons.
We have developed an echographic contrast agent candidate based on encapsulation of liquid perfluorocarbon within a biodegradable and biocompatible polymeric shell. The method used to obtain nano/microcapsules composed of a solid polymeric shell encapsulating a liquid perfluorocarbon core is derived from the work of Loxley et al [4]. We use a modified solvent evaporation method from a simple oil-in-water emulsion to obtain cavity-containing particles: Poly(lactide-co-glycolide) is dissolved into methylene chloride along with liquid perfluorocarbon.

Fluorescent optical microscopy and confocal microscopy show spherical particles with an undyed cavity inside. Undyed cavities are well centred within the particles and the shell thickness is homogeneous for each particle.
The morphology and porosity of the capsules have been studied by SEM. The majority of capsules are spherical with smooth surfaces. Few capsules are collapsed, confirming the existence of cavities within the spheres.

For microbubbles contrast agents, echogenicity depends strongly on the size. Our preparation method allows to adjust particle size between 1 and 15 microns only by changing the emulsification speed and the concentration of sodium cholate. Nanoparticles can be obtained using the same preparation process except that emulsification is performed with an ultrasound probe. By freeze-fracture electron microscopy and by transmission electron microscopy we have also verified the presence of a single cavity inside the nanoparticles.

The mechanical properties of our capsules are very important for their use as USCAs since ultrasounds are mechanical waves. In classical mechanics of shells, mechanical properties usually depend on the shell thickness to radius ratio [6]. We have therefore characterized our microcapsules by measuring their thickness and their radius. These measurements have been carried out directly on confocal microscopy slices. In the first set of experiments, the perfluorooctyl bromide volume is fixed as well as the PLGA mass and we vary only the speed of the emulsion blender. Experimental points are well fitted by a straight line: the thickness varies linearly with the capsule radius.

To evaluate the range of thickness to radius ratio available, the PLGA to PFOB proportions are varied. In one set of experiments, the volume of PFOB is fixed and the PLGA mass is varied, keeping everything else constant. Confocal images and the measurements on these images prove that the higher the mass of PLGA the thicker the shells. In another set of experiments, the amount of the polymer in the organic solvent is fixed and the volume of PFOB varies. The measurements on confocal images were carried out and show that the smaller the PFOB volume, the thicker the shells.

We have developed novel echogenic polymeric micro and nanocapsules containing a single liquid core of perfluorocarbon to use as contrast agents for ultrasound imaging. We are able to modulate capsule diameter by modifying the emulsification speed and to vary the shell thickness by changing the polymer to perfluorocarbon ratio. Preliminary results of echogenicity with lyophilized microparticles are very promising.

References

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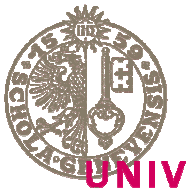

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Nucleic acid delivery

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LONG-CIRCULATING LIPOSOMES FOR SECRETORY PHOSPHOLIPASE A2 TRIGGERED RELEASE OF siRNA IN INFLAMED TISSUE

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Small interference RNA (siRNA) are small RNA duplexes of 19-23 base pairs that have appeared to be potent and highly specific for gene silencing. However, for therapeutic applications, delivery systems are needed to protect siRNA from nuclease degradation, to enhance cellular uptake and for site specific delivery. This presentation describes the preparation and characterization of long-circulating liposomes encapsulating siRNA for silencing of tumor necrosis factor α (TNF-α) in inflamed tissue in a mouse model of rheumatoid arthritis (RA). TNF-α is a cytokine critically involved in the pathogenesis of RA, and TNF-α-neutralizing drugs are currently used for the treatment of RA. The long-circulating liposomes are designed for localized, active release of siRNA in inflamed tissue by secretory phosholipase A2 ($sPLA_2$). $sPLA_2$ acts as a site-specific enzymatic trigger that actively degrades the liposomal carrier in inflamed tissue releasing siRNA. We hypothesize that the $sPLA_2$ generated lysolipids and fatty acid hydrolysis products can function locally as membrane permeability promoters facilitating the delivery of siRNA to the cytoplasm, where it can enter the RNA interference pathway.

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DEVELOPMENT OF CHITOSAN NANOPARTICLES FOR siRNA DELIVERY

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The unique properties of nanoparticulate systems based on biodegradable polymers have gained significant interest and have been studied for decades to overcome the problems of drug and gene delivery systems. Only in recent years, the benefit of these systems have expanded to a new and highly potent gene inhibitor, small interfering ribonucleic acid (siRNA). Among biodegradable polymers, natural polymer chitosan has attracted more attention due to its excellent biodegradability, biocompatibility as well as relatively non-toxic (Lee *et al.* 2005). Here, we studied chitosan as a siRNA vector, prepared by two methods of ionic cross-linking, simple complexation and ionic gelation using tripolyphosphate (TPP) ions. Both methods produced nanosize particles, less than 500nm depending on type, molecular weight as well as concentration of chitosan. In the case of ionic gelation, further two factors namely chitosan to TPP weight ratio and pH solution affected the particle size. The particle surface charge was ranging from -25 to +70 and +20 to +40mV for particles prepared by simple complexation and ionic-gelation, respectively. In vitro studies in CHO-K1 have revealed that preparation method and molecular weight of chitosan play an important role on silencing effect. Chitosan-TPP nanoparticles are shown to be better vectors as siRNA delivery vehicles compared to chitosan-siRNA complex which might due to their higher binding efficiency and loading efficiency determined by agarose gel electrophoresis as well as spectrophotometry. Therefore, Chitosan-TPP nanoparticles have great potential as viable vector candidates for a safer and cost-effective siRNA delivery.

Reference:

Lee, M. et al. (2005) Biomaterials. 26 : 2147-2156

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ENHANCED KNOCKDOWN OF EGFR: IMPROVED SIRNA ENDOSOMAL ESCAPE AND DELIVERY INTO THE CYTOSOL

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The epidermal growth factor receptor (EGFR) has been widely referred as an interesting target for cancer therapy. This interest stands from evidence showing EGFR activation being involved in cell proliferation, migration, development of angiogenesis, and inhibition of apoptosis.
RNA interference (iRNA), a promising technique for silencing genes in a sequence-specific manner, requires delivery of short interfering RNA (siRNA) in the cytosol.
A specific technique developed five years ago, named Photochemical Internalization (PCI), based on light activation of photosensitizer molecules located in intracellular membranes, has proved to release endocytosed molecules in a biologically active form from endocytic vesicles.

In this study, the commercially available carrier for siRNA, Lipofectamine, was tested for its efficiency in delivery of siRNA for silencing EGFR, alone and in combination with PCI. For PCI experiments TPPS2a was chosen as the photosensitizer. Flow cytometry measurements and western blot analysis were performed in order to detect and quantify the decrease of EGFR expression. Confocal microscopy images were taken for intracellular localization studies.

Results showed enhanced silencing of the EGFR when PCI was combined with Lipofectamine-transfection. At lower concentrations PCI enabled the silencing of EGFR. No effect on EGFR expression was detected for naked siRNA in combination with PCI, as well as for non-specific siRNA delivered by Lipofectamine in combination with PCI.
These results demonstrate that the endosomal release is critical and can be an enabling step in the process of silencing by siRNA.

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FUNCTIONALISED LIPIDIC DENDRIMERS FOR GENE DELIVERY

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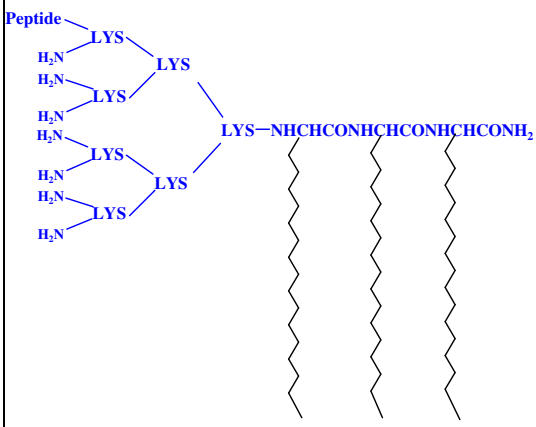
Introduction: The performance of the vector is perhaps the major factor that determines the success of gene therapy. Efficient delivery of DNA first into the individual cells and then into the cell nucleus are crucial steps in gene delivery. Of the different vectors available for gene transfection dendrimers are distinguished from most other non-viral vectors by their precisely controlled topology, uniform molecular weight, and flexibility of functionalization. We have already reported the synthesis of dendrons with lipid chains and free amino groups and have described as vectors for gene delivery[1-2]. Systematic studies on dendritic poly(lysine) with 8 terminal amino groups with or without three lipid chains in the core showed the influence of lipophilicity of these dendrons on transfection properties[3]. Of this series of dendrons those with three C_{18} carbon chains $[(C_{18})_3(L)_7(NH_2)_8]$ show higher gene transfer activity compared to their non lipidic analogues or those with shorter hydrocarbon chains, indicating that the lipophilicity of the dendrons plays a role in dendriplexes formation and thereby transfection. In this study we have modified the structural design by coupling receptor specific peptides such as RGD to one of the amino terminals keeping the remaining amino terminals free to condense the DNA and to form dendriplexes.



Methods: Dendrons containing amino groups and three C_{18} lipid chains with or without peptides are synthesised adopting solid phase peptide synthesis and characterised (Fig.). The ability of these functionalised lipidic dendrons to condense DNA was examined by measuring particle size using dynamic light scattering. The dendriplexes were formed using DNA and dendrons at different charge ratios (+/-) and incubated for 30 min and the mean diameter of the complexes was measured at 25˚C using a Malvern 4700 at 90˚ angle. The surface charge was determined by Zeta potential (Zeta sizer) and shape by Transmission Electron Microscopy. The binding of these dendrons was further confirmed by agarose gel electrophoresis. The targeting of the lipidic dendron molecules carrying nucleic acid to particular cell types were studied by choosing a heterologous model gene (luciferase) to transfer in different cell lines such as BHK and HEK.

Results: The Z- average diameter of dendriplexes formed with plasmid DNA and $(C18)_3\ L_7\ (NH_2)_{8-}$ were ~80nm and the same sized dendriplexes were also obtained by the modified dendrons. The zeta potential showed that the dendrons were incorporated into particles as the zeta potential values became positive after the theoretical point of neutralisation (1:1). The agarose gel electrophoresis also reveals that the amount free DNA decreased as the charge ratio of dendron:DNA increased. The difference in the transfection efficiency was 2-3 times greater than that of the original lipidic dendron $(C18)_3\ L_7\ (NH_2)_8$ without the RGD peptide. Further studies are ongoing to check the no change transfection for a cell line lacking RGD receptors and to determine the receptor specificity as well as the mechanism of their uptake by the cells

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Acknowledgements:
This work is supported by Heptagon proof of concept fund,
http://www.javelin-ventures.com/heptagon.htm

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Geneva, March 23-24, 2006

TUMOR TRANSFECTION USING POLYPLEXES BASED ON BIODEGRADABLE POLY(DMAEA)-PHOSPHAZENE

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Purpose: In the field of non-viral gene therapy, increasing interest is being paid to the design of transfectants based on non-toxic and biodegradable polymers. We tested the tumor transfection characteristics of polyplexes composed of plasmid DNA and the biodegradable p(DMAEA)-phosphazene.

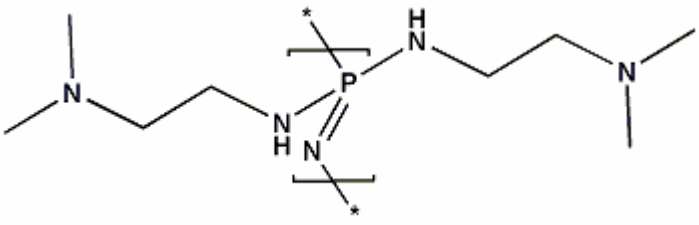

Figure 1: p(DMAEA)-ppz

Methods: Biodistribution and in vivo transfection efficiency of p(DMAEA)-ppz polyplexes were studied after intravenous administration in (Neuro 2A) tumor bearing mice. Data were compared to those of polyplexes based on the non-biodegradable polyethylenimine (PEI22).

Results: Both polyplex systems were rapidly cleared from the circulation and showed considerable disposition in the liver, the lung, and the tumor site. The tumor disposition of both polyplex systems resulted in considerable gene expression levels. In contrast to PEI22 polyplexes, p(DMAEA)-ppz polyplexes did not display substantial gene expression in the lung or in any other major organ.

Conclusion: Intravenous application of p(DMAEA)-ppz enables tumor preferable gene expression. The low cytotoxicity of p(DMAEA)-ppz polyplexes might contribute to this phenomenon.

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CHARACTERIZATION AND *IN VIVO* EVALUATION OF DIFFERENT POLYMER/DNA FORMULATIONS FOR GENE DELIVERY TO THE SKELETAL MUSCLE

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[2]Institut de Myologie - Inserm U582, Groupe hospitalier Pitié-Salpêtrière, Paris, France.

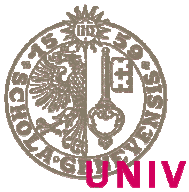
Introduction: So far gene therapy has mainly been based on viral vectors, given their efficiency to transfer DNA *in vivo*. However, virus-derived vectors have numbers of limitations, such as insert size, tissue specificity or immunogenicity, therefore restricting the possibilities of repeat administrations.
Thus, there is a need for rationally designed synthetic formulations. Following this approach, several hurdles have to be overcome, i.e. tissue and cellular barriers. In the current work, we have mainly focused on polymer-based formulations of plasmid DNA. The tissue administration was standardized by direct intramuscular injections. To improve intracellular entry with reference to naked DNA, our approach consisted in associating plasmid DNA with various polymers differing by their charge: a non-ionic block copolymer (PE6400), a molecule displaying one positive charge at physiological pH (Tetronic 304) and a polymer with a high density of positive charges (PEI).
The main goal of this study was to compare the systems obtained with each polymer/DNA association, in terms of physico-chemical characterization. *In vivo* toxicity and efficiency of the various systems have also been assessed after intramuscular injection.

Materials and methods: Reagents: plasmid DNA coding for bacterial β-galactosidase (pCMV-βGal, Invitrogen), PE6400 (BASF), Tetronic 304 (BASF), 25kDa branched polyethyleneimine (PEI, Sigma-Aldrich).
Formulation: PEI/DNA complexes are prepared in water at a N/P ratio of 10. PE6400/DNA (PE6400 0.05 % (w/v)) and Tetronic/DNA (Tetronic 5 % (w/v)) formulations are prepared in Tyrode's salt solution.
For each polymer/DNA association, the morphology of the vectors is assessed by cryo-transmission electron microscopy (cryo-TEM). Characterization of the size and zeta potential of the systems is performed by dynamic light scattering.
The polymer/DNA association is then assessed by gel retardation assay. Concerning the *in vivo* experiments, formulations containing either naked DNA or polymer/DNA associations were injected in tibialis anterior muscle of wild type Syrian hamsters (n = 2 for each group). After one week, animals were sacrificed and the muscles collected. X-Gal and Haematein/Eosin stainings are then performed on serial sections of each muscle.

Results: Characterization of the structures formed by association of DNA with several polymers has shown a great diversity of objects, in terms of size, shape and zeta potential.
PEI/DNA complexes have a relatively small size, with a tendency to aggregate when increasing the complex concentration. These particles exhibit a strongly positive zeta potential. On the opposite, Tetronic/DNA association is leading to systems displaying a negative zeta potential with formation of clusters at various concentrations. Data on PE6400/DNA association revealed a more complex behavior, depending on the DNA concentration and probably forming at low concentration of DNA vesicular structures around lamellar DNA.
Considering the gel retardation assay, no migration of the DNA has occurred when associated to PEI, translating the high association rate of DNA to the polymer. On the contrary, with Tetronic 304 or PE6400, a large amount of DNA is migrating similarly to DNA alone, demonstrating a weaker association.
The *in vivo* injection of plasmid DNA alone did not reveal any toxicity at the site of injection. Concerning the polymer/DNA formulations, *in vivo* experiments have highlighted the extremely high cytotoxicity of PEI towards skeletal muscle. However, no lesions were detected after injection of PE6400/DNA or Tetronic/DNA

formulations.
Due to the high toxicity displayed by PEI/DNA complexes, no efficiency evaluation could be performed on this formulation. Considering the Tetronic/DNA and PE6400/DNA associations, both systems allow transfection, nevertheless PE6400/DNA formulation exhibit superior efficiency.

Conclusion: Rationally designed vectors can be formulated based on polymer/DNA association. Physical characterization of various polymer/DNA vectors highlights a large variety of size, shape and zeta potential.
In vivo experiments have underlined the high toxicity of PEI towards skeletal muscle. However, no toxic effects at the muscular level appeared with formulations based on Tetronic or PE6400.
Furthermore, preliminary tests show some degree of efficiency in gene expression with the highest degree of transfection achieved with the PE6400/DNA association.



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Novel concepts in delivery

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HEXYL SUBSTITUTED POLYLACTIDES USED IN COPOLYMERS FOR HYDROPHOBIC DRUG INCORPORATION

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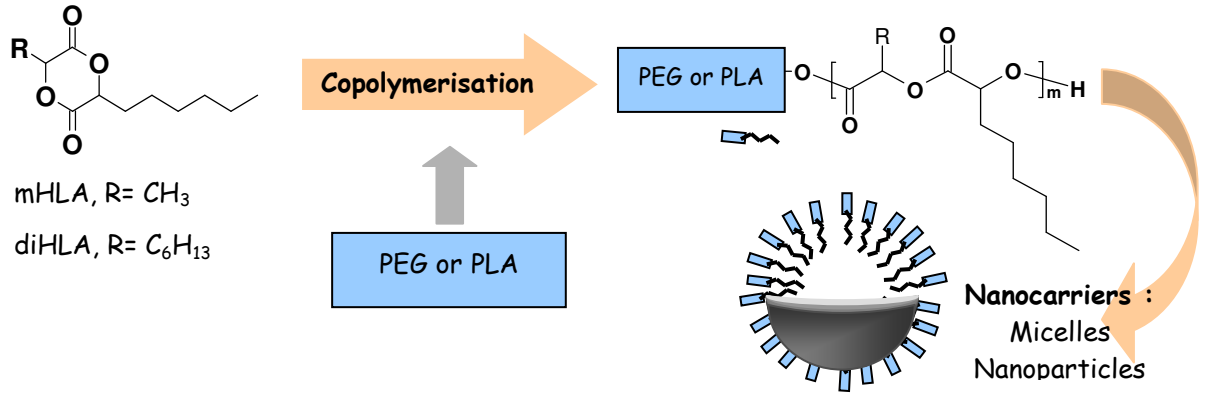
Polylactides are biocompatible compounds and approved by the FDA for medical applications. In combination with other biocompatible polymers, i.e. PEG, they can form micelles or nanoparticles with a hydrophilic shell and a hydrophobic core, in which drugs can be entrapped. Current drug carriers based on PLA and PLGA encounter difficulties to incorporate efficiently hydrophobic drugs. In this perspective we present here the synthesis of novel amphiphilic copolymers with mono and di hexyl substituted lactides (mHLA and diHLA). The substituted polylactides are based on the same polyester backbone as PLA, but have multiple hydrophobic hexyl side chains, which is interesting for incorporating hydrophobic drugs e.g. Griseofulvin.



The novel hexyl-substituted polylactides are synthesised by controlled ring opening polymerisation (ROP) from PEG and PLA, respectively as initiators. Different ratios of the hydrophilic PEG and hydrophobic PmHLA or PdiHLA with various molecular weights are investigated. We will present our initial results on hydrophobic drug incorporation in comparison to standard PLA nanocarriers.

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METHACRYLAMIDE-OLIGOLACTATES AS BUILDING BLOCKS FOR TARGETED BIODEGRADABLE POLYMERIC MICELLES TO DELIVER PHOTOSENSITIZERS

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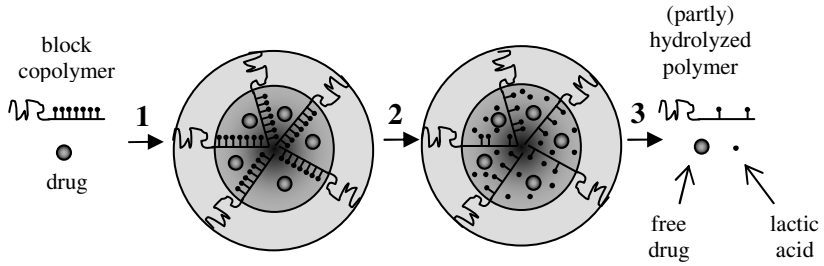
Polymers based on methacrylamide-oligolactates display thermosensitive behavior which is transient under physiological conditions due to hydrolysis of the lactate side chains. Amphiphilic block copolymers synthesized using a PEG-macroinitiator, self-assemble in aqueous media into spherical micelles above the cloud point (CP) of the thermosensitive block.[1,2] Cleavage of the lactic acid side chains causes a gradual increase of the critical micelle temperature (CMT) and leads to controlled destabilization and eventually dissolution of the thermosensitive biodegradable polymeric micelles (Scheme 1).



Scheme 1: General concept of thermosensitive biodegradable polymeric micelles.

The CP and the destabilisation time period can be tailored by varying the methacrylamide backbone and the length of the oligolactate chain.[3] The hydrophobic core of the micelle and physiological destabilization can be exploited for a controlled delivery of hydrophobic drugs.

A class of generally very hydrophobic drugs is the group of photosensitizers, which are currently under investigation in photodynamic therapy (PDT) to treat cancer. Upon irradiation with light of a specific wave length, photosensitizers generate a variety of reactive oxygen species. The radical compounds damage various cell components and ultimately lead to cell death.[4] However, the hydrophobicity of many photosensitizers results in an unfavorable biodistribution (e.g. prolonged skin toxicity) and aggregation with subsequent quenching of the excited state, decreased radical production, etc. These side effects necessitate the photosensitizers to be encapsulated in an adequate carrier system to deliver them specifically to the tumor regions, preferably in a solubilized state. It is the aim of this study to apply biodegradable thermosensitive polymeric micelles as a delivery vehicle for photosensitizers and to target the carrier specifically to neck/head cancer cells.

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SELF ASSEMBLY OF PEG-OLIGOLACTATES WITH MONODISPERSE HYDROPHOBIC BLOCKS

Myrra G. Carstens[1,2], Cornelus F. van Nostrum[1], Aissa Ramzi[1], Leo G.J. de Leede[2], Daan J.A. Crommelin[1], Wim E. Hennink[1]
[1]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands
[2]OctoPlus Technologies BV, Leiden, The Netherlands

Introduction: The nature of the two building blocks of PEG-*b*-PLA, the hydrophilic and nonimmunogenic PEG, and the hydrophobic and biodegradable PLA confer this polymer attractive properties for pharmaceutical applications. The polymer self assembles in water into nanoparticles, which have been extensively studied, for example by Gref *et al* [1] and Yamamoto *et al* [2]. These studies mainly focused on high molecular weight PEG-*b*-PLA. In this study we present the preparation of low molecular weight block oligomers with monodisperse hydrophobic blocks [3]. Their self assembling properties in aqueous environment and the formed nanoparticles were studied in detail.

Results: Fractionation of poydisperse methoxy poly(ethylene glycol)-*b*-oligo-L-lactate (mPEG-*b*-OLA) diblock oligomers resulted in oligomers with an mPEG block with a molecular weight of 350, 550 or 750 Da and a monodisperse OLA block with a degree of polymerization of 4, 6, 8 or 10. DSC demonstrated that the diblock oligomers with a low PEG content were fully amorphous, with glass transition temperatures ranging from –60 to -20°C, and indicated that the blocks were miscible. At concentrations above the critical aggregation concentration (0.4-1 mg/mL), the fractionated oligomers formed nanoparticles in water with a hydrodynamic radius of 130-300 nm, whereas polydisperse mPEG-*b*-OLAs gave formation of large aggregates. Static light scattering measurements showed that the nanoparticles have a low density (0.6-25 mg/mL) which means that the particles are highly hydrated. The particles were stable for two weeks, except for the mPEG350-series and mPEG750-*b*-OLA_4, demonstrating that both the PEG block size and the PEG weight fraction of the oligomers determine their stability. Based on these data, it is suggested that the mPEG-*b*-OLA nanoparticles contain a hydrated core of mPEG-*b*-OLA block oligomers, stabilized by a thin outer PEG layer as shown in figure 1.

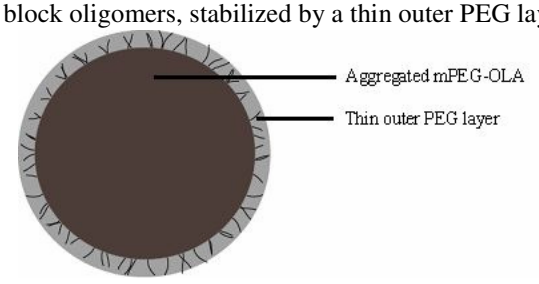

Figure 1 Schematic representation of a mPEG-b-oligo-L-lactate nanoparticle

Conclusion: The presented data demonstrate that mPEG-*b*-oligo-L-lactates with monodisperse hydrophobic blocks self assemble into nanoparticles with a hydrated block oligomer core and a thin outer PEG layer. These self-emulsifying properties, together with their expected biocompatibility and biodegradability, make these systems well suitable for pharmaceutical applications.

References

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2. Yamamoto, Y., et al., J Control Release, 2002. **82**(2-3): p. 359-71.
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SELF-ASSEMBLED SEMI-SOLID BEADS MADE OF CYCLODEXTRIN AND OIL

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[1]UMR CNRS 8612, Faculté de Pharmacie, 5 rue JB Clément, 92296 Châtenay-Malabry Cedex, France.
[2]Groupe de Chimie analytique de Paris-Sud, EA 3343, Faculté de Pharmacie, 5 rue JB Clément, 92296 Châtenay-Malabry Cedex, France

Introduction: Cyclodextrins (CDs) are cyclic oligosaccharides that possess remarkable ability to include lipophilic molecules inside their hydrophobic cavity. It constitutes a true molecular encapsulation. Few years ago, it was also shown that CDs can be used to overcome many problems in micro- or nano-encapsulation[1]. Recently, we have developed a new drug delivery system made of cyclodextrin and oil. Indeed, it is possible to prepare beads by a novel and simple microencapsulation process from a mixture of water, oil and natural cyclodextrins[2].
This work has been firstly focused on the way to produce new beads and on their main characteristics (morphology, structure and components). Finally, to evaluate encapsulation ability of the beads, we have selected a model of lipophilic and fragile drug, the 13-*cis*-retinoic acid or isotretinoin[3,4].

Experimental methods: Unloaded beads were prepared by adding 5.3 mL of Cropure® Soybean oil (Croda, France) to 20 mL of an 8% (w/v) aqueous alpha-cyclodextrin (α-CD) solution (Pharmaceutical grade, Wacker-Chemie, France). The mixture was stirred at 200 rpm in a gyratory shaker (Salvis, Bioblock Scientific, France) at 28°C. Fresh beads were eventually washed and freeze-dried (48h in a Christ LDC-1 alpha1-4 freeze-dryer, Bioblock Scientific, France). Isotretinoin-loaded beads were obtained as described above by dissolving IT (Sigma-Aldrich, France) in the oily phase (0.45%, w/w). Drug-loading efficiency was determined by HPLC with UV-visible detector (λ = 360 nm), after liquid extraction, on fresh and freeze-dried beads. This method made possible the differentiation between intact isotretinoin and its main degradation products.
Bead morphology was determined by different microscopic techniques: optical microscopy, cross polarized optical microscopy, confocal microscopy, scanning electron microscopy and freeze-fracture electron microscopy. Small angle X-ray scattering (SAXS) was also performed at the DCI Synchrotron Facility of LURE (Orsay, France) to identify their crystalline structure. Separation and quantification of triglycerides (TG), known as main components of vegetable oils, were carried out by HPLC equipped with an evaporative light scattering detector. TG composition was determined both from soybean oil as raw material and oil extracted from beads.

Results and discussion: The microencapsulation method leads to the formation of semi-solid beads from two immiscible liquids, i.e. an aqueous solution and a vegetable oil. External stirring of these two liquids, at temperature close to ambient, results in only few hours in a stable milky mixture. The viscosity of the mixture increases as a function of time and, 2 days after, beads dispersed in the aqueous medium are spontaneously obtained. The fabrication yield of beads is around 85% +/- 3%. Bead diameter, determined by optical microscopy, is around 1.6 +/- 0.2 mm. SEM analysis shows that bead surface is rough and irregular. Freeze-fracture electron microscopy and confocal microscopy reveal that beads have an internal hybrid structure made of oily compartments (diameter ranging from about 0.01 to 1.5µm) dispersed in a solid matrix. Indeed, a lipophilic fluorescent probe (Nile Red) previously incorporated in oil was localized sporadically inside the beads. It shows that a high amount of free oil is entrapped inside the matrix.
Cross polarized optical microscopy reveals well-defined and plate crystals and large amounts of birefringent structures. The crystalline organization of the beads is confirmed by X-Ray diffraction analysis, showing a spectrum with at least 2 peaks and 1 large band of diffraction.
HPLC analyses show that compositions of soybean oil and the one extracted from beads are quantitatively and qualitatively the same. C18 triglycerides represent the main components of both samples (> 99% w/w). Pure

inclusion complexes made of C18 TG (Triolein, Trilinolein and Trilinolenin) and α-CD were then prepared and investigated by X-Ray diffraction. The same diffraction positions are localized both in bead and pure inclusion complexes spectra. So, bead crystalline structure seems to result from the formation of C18 TG/α-CD inclusion complexes.
Interestingly, isotretinoin addition to the formulation does not influence bead size and fabrication yield. As seen in Table I, about 98% of intact IT are encapsulated inside fresh beads, demonstrating that beads are highly efficient to encapsulate lipophilic drugs.

Intact Isotretinoin	**Fresh Beads**	**Freeze-dried beads**
Loading ratio	98 (+/- 9) %	84 (+/- 6) %
Loading efficiency	1.3 (+/- 0.3) mg/g	3.6 (+/- 0.6) mg/g

Table I: Isotretinoin-loaded beads characteristics.

Conclusions: This work has described a new microparticulate system, made of safe materials: vegetable oil and α-cyclodextrin. Beads were prepared by a novel and soft process suitable for lipophilic and fragile molecules (no organic solvent, no surface-active agent and no drastic fabrication conditions). Moreover, this process allows high drug-loading, without any change in bead characteristics.
The remarkable properties of beads, and particularly their semi-solid consistency, allow an easy administration on skin. In addition, beads could also present an interest to deliver lipophilic drugs by the oral route.

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Friday 24 March 2006: Room A100

Protein delivery and liposomes

09.00 – 09.20: Stefan Salomon (London)
Spray dried complexes of Poly (γ-glutamic acid) and ovalbumin

09.20 – 09.40: Mingshi Yang (Copenhagen)
Characterization of salmon calcitonin in spray drying powder for inhalation

09.40 – 10.00: Mike Pollitt (London)
Use of field-flow fractionation to study protein adsorption

10.00 – 10.20: Wafa Al-Jamal (London)
Construction of Nanoscale multicompartment liposomes for combinatory drug delivery

10.20 – 10.40: Birgit Romberg (Utrecht)
Enzymatic degradation of a poly(amino acid)-based "stealth" liposome coating

10.40 – 11.00: Laure Lajavardi (Paris)
Effect of intravitreal injection of an immunomodulatory peptide, VIP, encapsulated in sterically-stabilized liposomes, during endotoxin-induced uveitis in rats

Coffee break

Active targeting and surface modification

11.30 – 11.50: Adriana Hapca (Geneva)
Efficacy of antibody labelled nanoparticles for specific tumour targeting

11.50 – 12.10: Karine Andrieux (Paris)
Brain translocation of chitosan nanoparticles overcoated with the monoclonal antibody OX26: application to the delivery of the caspase-3 inhibitor

12.10 – 12.30: Mariagrazia Di Marco (Paris)
Colloidal stability of superparamagnetic iron oxide nanoparticles with different coatings

12.30 – 12.50: Verena Hengst (Utrecht)
Bone-seeking liposomes as drug delivery system for the treatment of bone metastases

13.00 – 13.50 Lunch at the School of Pharmaceutical Sciences (entrance hall)

14.00 – 16.20 Visit of the Geneva Museum of Ethnography (walking distance), Blvd Carl-Vogt 65, Geneva

Various routes of administration

16.30 – 16.50: Orawan Suitthimeathegorn (London)
Drug release from non-aqueous emulsions delivered intramuscularly to rats

16.50 – 17.10: Pakatip Ruenraroengsak (London)
Studies of intracellular transport of dendrimers

17.10 – 17.30: Rainer Müller (Berlin)
Lipid nanoparticles (SLN, NLC)-Present state of development for dermal delivery

Coffee break

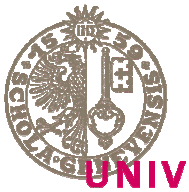
Vaccine delivery

17.45 – 18.05: Sreenivas Pandit (London)
HBsAg loaded PLA NP: adjuvanticity of tocopherol acetate and tocopherol nicotinate in mice

18.05 – 18.25: Man Tsuey Tse (London)
Investigation of PLGA microparticles for DNA vaccine delivery

18.25 – 18.45: Suzie Ribeiro (London)
Genetic immunization using dendriplexes and PLGA/dendriplex carriers against anthrax

19.30 → All together dinner
Cave Valaisanne Chalet Suisse (walking distance)
Blvd Georges Favron 23, Geneva



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Protein delivery and liposomes

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SPRAY DRIED COMPLEXES OF POLY (Γ-GLUTAMIC ACID) AND OVALBUMIN

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Spray drying is widely used in the consumer and healthcare industry. As exemplified by the recent approval of Exubera®, spray dried formulations for the administration of proteins and other particulates has considerable potential [1]. In this study, poly (γ-glutamic acid) (PG), a polyanion, and ovalbumin (OVA) which is a zwitterionic protein were used to form polyelectrolyte complexes. These complexes were utilized to produce particulates that would display systematic changes in particle size and morphology by a controlled spray drying process. The purpose of this study was to deconvolute the effects of spray drying process on final particulate properties with the effects caused by changes in formulation composition. A Niro® SD Micro spray dried was used to prepare the particles in a nitrogen atmosphere. Critical processing parameters that were controlled included nozzle position, drying gas flow and pressure, atomization pressure, inlet an outlet temperatures, and evaporation rate as a function of liquid pump capacity. The PG was produced by fermentation of bacillus subtilis chungkookjang and had a molecular weight of 200-500 kDa. OVA was selected as model protein due to its high water solubility and its IP which is optimum for forming weak polyelectrolyte complexes with PG. [2, 3]. We investigated the ways of forming complex dispersions that could be spray dried. The dispersion was prepared at a pH of 3.3 which is below the isoelectric point of OVA and at where most of the PG is protonated in its free acid form. The rational for using this pH was to form relatively weak complexes that could be spray dried but which was not intractable. A solution of OVA (1% w/v) was added drop wise to a stirred solution of PG (1% w/v) over a period of 15 minutes at 4°C to obtain a fine dispersion. This might be related to a higher viscosity at a lower temperature which stabilizes the particles and avoids aggregation [4]. Preparation at room temperature lead to aggregation due to fibrous structures formed. This dispersion was then spray dried. The spray drying process parameters were systematically varied. The desired results were achieved with an inlet temperature of 160 ±2°C and an outlet temperature of 92 ±2°C. The pump speed was kept constant at a value of 2.4 ±0.2 bar. The fabricated particles had a mean volume diameter (MVD) of 7.4 µm and a standard deviation (STD) of 4.35 and raisin like morphology. The effect of atomization was investigated to find out if a change in atomization pressure affects particle size. The pressure ranging from 3-5 bar did not result in a significant reduction in particle size (6.4-7.4). Since process variation did not reduce particle size, surfactants were added to determine if size and morphology of the PG-OVA microparticles could be reduced more effectively. In an attempt to maintain the same electrostatic interactions between PG and OVA, 6 non-ionic surfactants with different HLB values were examined to determine if they had an effect on particle size and morphology. Surfactants were added in concentrations varying from 2.5-60 mg per formulation. Overall, the particle morphology was not affected by the surfactants. The most effective surfactants were Tween 20 and Tween 21 in highest concentration which reduced the MVD to about 2.25 and 2.20 µm, respectively. The zeta potential did not vary irrespective of type and amount of all the surfactants that were used. If the formation of the dispersion including 60 mg Tween 20 was formed with a homogenizer (Silverstone, 7500 rpm) instead of magnetic stirring the MVD change significantly to 1.6 µm and the size distribution was reduced to a STD of 0.81. This study showed that small amount of surfactant such as Tween 20 was able to reduce particle size as well as particle distribution and the method of preparation of the dispersion effects particle size.

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CHARACTERIZATION OF SALMON CALCITONIN IN SPRAY DRYING POWDER FOR INHALATION

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Objective: Pulmonary drug delivery is considered to be the most promising alternative route of administration for proteins and peptides. Even though a liquid or suspension formulation is usually administered into the lung by either metered dose inhaler (MDI) or Nebulizer, a dry powder inhalation (DPI) may be more feasible in the case of pulmonary administration of proteins and peptides. The objective of this study is particle design of protein containing dry powders for inhalation by spray drying process and characterization of the protein in the dry powder to understand the interplay between stability, formulation and process parameters.

Method: Salmon calcitonin, a 32-amino acid protein with a 1-7 disulfide bond, was employed as a model compound. Disaccharides and chitosan were used as protection agent and absorption enhancer, respectively. After particle formation using a Buchi Mini Spray Drier, the dry power was characterized by SEM, X-ray powder diffraction and DSC. The physicochemical stability of salmon calcitonin in the dry powder was investigated by FTIR, CD and HPLC techniques.

Results: A spherical shaped dry powder was obtained with mannitol as excipient, whereas an irregular shape was obtained with trehalose and lactose. Particle size of the dry powders was around 1-5 micrometer. Addition of sCT in the formulation modified the spray-dried power from a smooth surface to a wrinkled surface. X-ray powder diffraction patterns showed that mannitol retained its crystalline form in dry power, while trehalose and lactose were spray dried into amorphous state. The melting temperature of mannitol was reduced significantly with increased chitosan amount.
HPLC analysis indicated that salmon calcitonin was relatively stable upon spray-drying. However, with the proportion of chitosan in the formulation being increased, dissolution and recovery of salmon calcitonin from the dried powder was decreased. FTIR showed that the secondary structure of salmon calcitonin was slightly changed in the dry powder. Moreover, chitosan did not influence the recovery of salmon calcitonin in an aqueous chitosan formulation, even though CD analysis indicated that the secondary structure of salmon calcitonin was influenced by addition of chitosan in the aqueous formulation.

Conclusion: Salmon calcitonin is stable during the spray drying process under certain conditions. Chitosan, a biodegradable mucoadhesive polymer, negatively affected the physicochemical properties of salmon calcitonin, yet the mechanism remains to be determined.

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USE OF FIELD-FLOW FRACTIONATION TO STUDY PROTEIN ADSORPTION

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Protein adsorption is relevant to various pharmaceutical products, such as minimising wall adsorption of therapeutic proteins, particle based vaccinations and rapid diagnostic kits (studied here). Traditional methods of measuring protein adsorption, such as centrifugation and protein determination by UV adsorption or bicinchoninic acid assay (BCA) and coverage found by depletion do not work well in this system. This is because the gold colloid is prepared by reduction using citrate which leaves excess citrate or oxidation products in the suspension. These absorb at 280 nm and interfere with the BCA assay.

Flow field-flow fractionation (4F) separates entities based on their interaction with a cross flow so separation depends on hydrodynamic diameter. This work aimed to separate protein - particle conjugates from free protein using 4F and use the depletion of protein to determine the coverage.

Gold colloid conjugates were prepared using excess antibody at 25 °C and blocked using casein. They were separated by 4F in 5 mM tris buffer, pH 8.4. The mass of recovered antibody compared to the injected mass corresponded to a coverage of around 10 mg m^{-2}, broadly in agreement with other studies of immunoglobulin adsorption. However, in particle free controls, there was more depletion of antibody than when particles were present. Reasons for this are unknown: possibly the gold colloid is reducing the rate of antibody aggregation or adsorption onto the channel.

Recovery of free antibody depends on concentration and eluent. In 5 mM tris, with casein, antibody recovery is around 55%. With no casein, an additional *ca.* 2 µg of antibody is lost, presumably adsorbed to the channel. Good recovery is achieved in PBS, but this results in aggregation of gold colloid. . Surfactants were not used as they may affect the adsorption and, in trials, tended to increase detector noise.

Thus FFF shows some promise as a means of measuring protein adsorption. Key advantages are that it is performed in native buffer and uses fairly small quantities of the expensive antibodies. Definite conclusions about adsorption can not be made without solving the problem of whether free antibody aggregates or adsorbs to the channel.

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CONSTRUCTION OF NANOSCALE MULTICOMPARTMENT LIPOSOMES FOR COMBINATORY DRUG DELIVERY

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Liposomes are clinically-used delivery systems for chemotherapeutic agents, biological macromolecules and diagnostics. Due to their flexibility in size and composition, different types of liposomes have been developed varying in surface and structural characteristics. Multicompartment liposomes constitute an attractive drug carrier system offering advantages in terms of inner vesicle protection; sustained drug release and possibility for combinatory (cocktail) therapies using a single delivery system. Different therapeutic and diagnostic agents can also be loaded into a single carrier system to achieve combinatory therapeutic/imaging modalities. However, all previously described methodologies for multicompartment or multivesicular liposomes resulted in μm-sized vesicles limiting most pharmaceutical applications. In this work we attempted to overcome this drawback and prepare nanoscale multicompartment liposomes which are applicable for systemic administration and tumor-targeted delivery. A small unilamellar vesicle (SUV) aqueous dispersion (DOPC: DOPG: chol) was used to hydrate a dried film of different lipid content (DMPC: chol), followed by extrusion. The system was characterised by techniques such as photon correlation spectroscopy (PCS), zeta potential measurement, transmission electron microscopy (TEM) and laser scanning confocal microscopy (LSCM). LCSM showed that CF-DOPE-labeled SUVs were surrounding DiI- labeled (DMPC: chol) multilamellar vesicles of the hydrated lipid film. After extrusion, it was observed from TEM that nanoscale mulitcompartment liposomes between the two types of lipid bilayers were formed. We observed a single, multicompartment 'hybrid' vesicle population composed of the two different bilayer types of approximately 200nm in mean diameter rather than a mixture of two independent vesicle populations. In the case of tumor therapy, such multicompartment liposome systems can offer a single carrier for the delivery of two different modalities (drugs, radionuclides, sensitizing agents, diagnostics) based on the hypothesis that therapeutic efficacy will be greatly improved through synergistic/combinatory activity, as well as providing the possibility for imaging the delivered therapeutic if diagnostic molecules are incorporated.

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ENZYMATIC DEGRADATION OF A POLY(AMINO ACID)-BASED 'STEALTH' LIPOSOME COATING

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Purpose: The objective of this study was to evaluate the biodegradability of two poly(amino acid)-lipid conjugates: poly(hydroxyethyl-L-asparagine)-*N*-succinyl-dioctadecylamine (PHEA-DODASuc) and poy(hydroxyethyl-L-glutamine)-DODASuc (PHEG-DODASuc). Upon coupling to the surface of liposomes, both conjugates enhance liposome circulation times to a similar extent as poly(ethylene glycol) (PEG). The *in vivo* fate of PEG after being taken up by the cell is not known and it may cause side effects on a long-term base.

Methods: Enzymatic degradability of the two poly(amino acid)-lipid conjugates in their free form and when grafted to the liposomes surface was investigated using papain, a model protease with a comparable specificity as the lysosomal cathepsin B, pronase E and cathepsin B. Degradation was monitored by following the increase of primary amine groups, which are generated upon cleavage of a peptide bond, with ninhydrin. Final degradation fragments were analysed by MALDI-ToF mass spectrometry.

Results: In contrast to PHEA-DODASuc, incubation of PHEG-DODASuc with the enzymes results in cleavage of the conjugate into low molecular weight peptides. Degradation also occurs when PHEG-DODASuc is coated onto the surface of liposomes.

Conclusion: PHEG-DODASuc is enzymatically degradable even when it is attached to a liposomal surface. Such 'stealth' coating avoids the risk of intracellular accumulation and makes liposomes with a sheddable coating feasible.

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EFFECT OF INTRAVITREAL INJECTION OF AN IMMUNOMODULATORY PEPTIDE, VIP, ENCAPSULATED IN STERICALLY-STABILIZED LIPOSOMES, DURING ENDOTOXIN-INDUCED UVEITIS IN RATS

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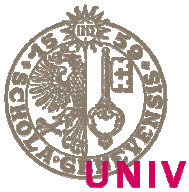
Vasoactive Intestinal Peptide (VIP) is a neuropeptide of the eye, where it induces an immunosuppressive effect that protects this organe against deletary inflammations. However, this protection system can fail, leading to the development of uveitis in the posterior and/or anterior segments of the eye. The intravitreal injection of VIP could be interesting for the treatment of this inflammation. However, it has a short biological half-life. The purpose of the present study was to encapsulate VIP in liposomes that protect the peptide and potentiate its biological immunosuppressive effects. These liposomes-VIP were then tested in vivo in a rat model of uveitis that develops inflammation in a short time (24 hours): endotoxin induced-uveitis (EIU).

Liposomes sterically-stabilized with PEG (100 to 600 nm) were used to encapsulate VIP, with an encapsulation efficiency was 3.1 ± 0.4 mmol VIP / mol lipids. In order to evaluate the inflammatory effect of this formulation, 10 µL of unloaded liposomes were injected at high concentrations in the eye of Lewis rats (60 and 115 mM). After clinical examination and microscopic observation of macrophages and polymorphonuclear (PMN) cells on eye sections, it is observed that liposomes do not cause any intraocular inflammation. Thus, a large amount of liposomes-VIP could be injected without any risk in the small volume required for intravitreal route (10 µL).

The therapeutic effect of VIP was investigated on EIU induced by a footpad injection of lipopolysaccharide (LPS) in Lewis rats. Simultaneously, VIP (0.6 mg/mL) either in saline or in liposomes was injected intravitreally. 24 hours after the injections, liposomes-VIP reduce significantly the clinical score from 3 to 1.5, whereas VIP in solution presents no effect. On liposomes-VIP injected eye sections, the number of ED1 positive cells and PMN cells decreases significantly in both anterior and posterior segments, whereas no difference is noted between saline-VIP and saline. RT-PCR of five inflammatory cytokines RNAs (TNF-α, IL-1β, MCP-1, MIP-2 and IFN-γ) was performed on enucleated eyes. The results show a reduced expression of TNF-α, IL-1β, MCP-1, MIP-2 and IFN-γ RNAs in eyes treated with liposomes-VIP, and a slight reduction in the saline-VIP group, compared to saline and unloaded liposomes.
The intraocular biodistribution of liposomes and VIP was investigated 6 and 24 hours, 7 and 14 days after injection of liposomes labelled by rhodamine linked covalently to phosphoethanolamine. By fluoresence microscopy, liposomes were observed in red (515-560 nm), and either VIP or macrophages were immunostained with FITC and visualized in green (450-490 nm). We show that liposomes diffuse from the vitreous to the internal face of the retina and the anterior segment of the eye (iris and ciliary body). They are eliminated through ciliary muscles, conjunctiva and sclera by the lymphatics. In normal rats, liposomes are present within the retina tissues in higher amounts than in EIU rats, in which the number of inflammatory cells is much higher. Even though they are pegylated, the liposomes can be internalized in macrophages and PMN cells in normal and EIU rats, at all time of experiment. A co-localization of liposomes and VIP is observed at very high level 6 hours after injection in the vitreous. This quantity decreases with time but liposomes-VIP can still be observed 14 days post-injection in the residual inflammatory cells. Since unloaded liposomes and liposomes-VIP have the same behaviour in the eye, the presence of VIP does not influence the biodistribution of liposomes.

To conclude, liposomes can be considered as an adequate formulation of VIP for an intravitreal administration. They prevent the degradation of this peptide, which is essential for its anti-inflammatory effect. Moreover, they offer a distribution in all the tissues affected by uveitis. VIP encapsulation in liposomes seems absolutely necessary to allow a therapeutic effect during EIU.



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Active targeting and surface modification

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EFFICACY OF ANTIBODY LABELED NANOPARTICLES FOR SPECIFIC TUMOR TARGETING

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Purpose: Drug carrier systems such as nanoparticles (NPs) represent a promising approach in the anticancer treatment with important advantages: passive targeting, delivery of hydrophobic drugs, high drug loading capacity, and controlled release rate. To improve the efficacy of these systems, specific targeting can be achieved by covalent modification of NP surface with monoclonal antibodies (mAb). The advantage of such formulations is that the drug carrier capacity of NPs can be combined with the ability of mAb to target tumor tissues (active targeting). In this study we developed poly (lactic acid) (PLA) nanoparticles bearing monoclonal antibodies: either anti-HER2 (trastuzumab, Herceptin®) or anti-CD20 (rituximab, Mabthera®).

Methods. PLA (MW 57 kDa) NPs with free carboxylic acid functions were prepared by a salting-out method(1). For detection the NPs were stained with Dioctadecyloxacarbo-cyanine perchlorate (DiO) 0.01% (w/w). Thiol (-SH) functions were covalently bound to the NPs by a two-step carbodiimide reaction(2). Different concentrations of cystamine were used to vary the -SH concentration on the particle surface. Thiolated NPs were then used to covalently bind mAb via a bifunctional crosslinker, m-maleimidobenzoyl-N-hydroxyl-sulfosuccinimide (sulfo-MBS)(2). The specific interaction between tumor cells and coated NPs (anti-HER2-NPs and anti-CD20-NPs) was determined by flow cytometry and confocal microscopy using two cell lines as models: Daudi lymphoma cells and SKOV-3 human ovarian cancer cells.

Results. After mAb surface modification, the NPs size increased from 170±13 nm to 230±15 nm. The amount of -SH functions on the surface of NPs increased when an increasing concentration of cystamine was used. Results showed that the presence of specific mAb on the NPs surface significantly enhanced the interaction between the mAb labeled-NPs and tumor cells. Confocal microscopy study demonstrated two different types of cellular localization of mAb labeled-NPs. In case of SKOV-3 cells an internalization of the anti-HER2-NPs was observed whereas for Daudi cells a distribution of anti-CD20-NPs on the cellular surface was seen.

Conclusion. This study demonstrated the specific targeting of mAb labeled NPs to tumor cells overexpressing two different antigens. The immunoreactivity of monoclonal antibodies was preserved after covalent reaction with thiolated NPs. Different cellular localizations of anti-HER2-NPs and anti-CD20-NPs were shown in SKOV-3 and Daudi cells, respectively. The mAb coated NPs represent a promising approach to improve the efficacy of NPs in active targeting for cancer therapy.

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BRAIN TRANSLOCATION OF CHITOSAN NANOPARTICLES OVERCOATED WITH THE MONOCLONAL ANTIBODY OX26: APPLICATION TO THE DELIVERY OF THE CASPACE-3 INHIBITOR

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The inhibition of the caspase-3 enzyme is reported to increase neuronal cell survival following cerebral ischemia. The peptide Z-DEVD-FMK is a specific caspase inhibitor, which significantly reduces vulnerability to the neuronal cell death. However, this molecule is unable to cross the blood brain barrier (BBB) and to diffuse into the brain tissue. Thus, the development of an effective delivery system is needed to provide sufficient drug concentration into the brain to prevent cell death [1].

Using the avidin (SA)-biotin (BIO) technology, we describe here the design of chitosan (CS) nanospheres conjugated with polyethyleneglycol (PEG) bearing the OX26 monoclonal antibody whose affinity for the transferrin receptor (TfR) may trigger receptor-mediated transport across the BBB [2, 3]. These functionalized CS-PEG-BIO-SA/OX26 nanoparticles (NPs) were characterized for their particle size, zeta potential, drug loading capacity and release properties. Fluorescently labeled CS-PEG-BIO-SA/OX26 nanoparticles were administered systemically to mice in order to evaluate their efficacy for brain translocation. The results showed that an important amount of nanoparticles were located in the brain, outside of the intravascular compartment. These findings, which were also confirmed by electron microscopic examination of the brain tissue indicate that, this novel targeted nanoparticulate drug delivery system was able to translocate into the brain tissue after iv administration. Consequently, these novel nanoparticles are promising carriers for the transport of the anticaspase peptide Z-DEVDFMK into the brain [4].

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COLLOIDAL STABILITY OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES WITH DIFFERENT COATINGS.

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Introduction: Magnetic nanoparticles are becoming increasingly important for several biomedical applications. We were personally interested in the fate of USPIOs, ultra small iron oxide particles intended for Magnetic Resonance Imaging purposes. The stability and aggregation properties of iron oxide colloids in physiologic media play an important role in controlling the fate, transport, and bioavailability of the carrier. In our study the stability of iron oxide suspensions varying by the type of colloid coating was experimentally determined for different salt concentrations. To our knowledge, such a study was not previously described with so tiny particles (RH around 12nm). The aim was to propose an experimental procedure that could help to determine the part of both an electrostatic and a steric stabilizing effect in the case of different coatings for ultra small iron oxide nanoparticles (USPIO). Hence, the stability of iron oxide particles in the presence of NaCl has been studied by dynamic light scattering, following along time the particle diameter evolution. The results were interpreted using the classical DLVO theory of colloidal stability extended to account for hydration, and interactions between particles. The values of diffuse potential (ψd) and the Hamaker constant (A) could then be obtained[i]. However, in other cases, it became apparent that additional mechanisms should be considered to explain the results.

Experimental methods: Superparamagnetic Iron Oxide Nanoparticles (maghemite) were provided by Guerbet. In our experiments we investigated different types of coatings.
The particle size was measured in different conditions (salt concentration) by photon correlation spectroscopy (PCS) with a Malvern 4700 system (Malvern, England). NaCl was used as electrolytes for all samples and CaCl2 for the NaCl stable samples. All measurements were performed at 20 °C and a scattering angle of 90° was chosen. The coagulation was started by adding directly 20-200 µL of initial iron oxide suspension (concentration 0,2-0,4 mol/l Fe) to an electrolyte solution in the light scattering cell. The resulting dispersion, after a quick shake, was placed in the index matching vat. Individual autocorrelation functions were successively acquired for between 5 and 15 s over the course of 1-4 h. Considering that the initial particle size (RH) was uniformly distributed around 12nm, the calculated values obtained along the time represented a mean hydrodynamic radius, RH, taken over a distribution of colloid monomers, dimers, and higher order aggregates.
Initial colloid aggregation kinetics was then determined by monitoring the increase in RH with time:

That is, the product of the aggregation rate constant k and the colloid number concentration n_0 is proportional to the slope of the RH versus time graph for $t \rightarrow 0$.

Discussion and results: The results obtained for uncoated nanoparticles taken as an example are shown in Fig.1 . It clearly appears that the initial rate of change in the mean aggregate *R*H increases with electrolyte concentration until a maximum rate of aggregation is reached. As described elsewhere[i], the diffusion-limited aggregation is reached at a threshold electrolyte concentration

known as the CCC (critical concentration of coagulation). The CCC depends on the specific cation in solution, and under some experimental conditions, the CCC depends on whether the colloids were coated with different polymers. Colloid stability can then be characterized by the stability factor W [i].

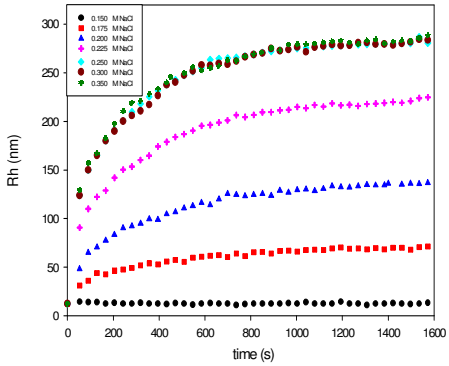

Figure 1: Hydrodynamic radius of uncoated nanoparticles as a function of time measured for different NaCl concentrations.

We also calculated the total interaction energy, $V_{TOT}(H)$, between the iron oxide nanoparticles. An example of Potential curves of uncoated nanoparticles in 0.10 mol/L and 0.40 mol/L NaCl solutions range is reported in Figure 2.

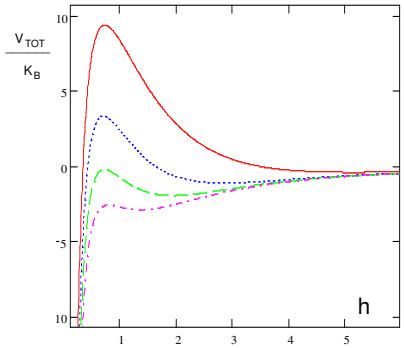

Fig. 2. Interaction energy of uncoated nanoparticles dispersions in 0.10 mol/L and 0.40 mol/L NaCl solutions range.

A worse trend was observed in the Alcohol-coated system, and on the contrary a much better stabilisation with the PEG-coated one. Our PEG-coated particles were stable in the whole range of salt utilised (to 2 M).

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BONE-SEEKING LIPOSOMES AS DRUG DELIVERY SYSTEM FOR THE TREATMENT OF BONE METASTASES

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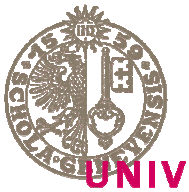
The research project aims to develop bone-seeking liposomes containing bisphosphonic acid derivatives as targeting device on the liposomal surface as novel delivery system for the treatment of bone metastases.
Spread of malignant cells from the primary tumor to the skeleton and subsequent destruction of bone is a frequent and debilitating complication of many cancers, especially breast cancer, prostate cancer and multiple myeloma. Cancer cells that have metastasized to the bone can severely damage the bone. Until today there is no cure for bone metastases. External beam radiotherapy, hormonal therapy and chemotherapy are the available treatment options to control the symptoms and the spread of bone metastases. Newer-generation bisphosphonates have been developed for the treatment of bone metastases to decrease the risk of fractures, reduce bone pain and bone damage.
Developing a bone-seeking carrier system with a high affinity to hydroxyapatite (HAP), the main constituent of bone is proposed here as a promising option for selective bone drug delivery since HAP is only present in hard tissues like bone and teeth. Due to the exceptional affinity of bisphosphonates, which is related to their structural similarity to pyrophosphate (P-C-P portion), bisphosphonic acid derivatives are used in our study as targeting device.
Several strategies using bisphosphonate-targeted conjugates have been developed with the aim to treat diseases like osteoporosis, osteoarthritis or bone cancer. Phosphonate-coupled radiopharmaceuticals for example have been investigated in clinical trials as effective pain palliation treatment for metastatic bone cancer, among them samarium-153 lexidronam (Quadramet®) has already been FDA-approved.
The proposed bone targeting carrier is a bisphosphonate-targeted liposome system with the intention to achieve prolonged local exposure to high concentrations of the active drug, thereby enhancing pharmacological efficacy and minimizing systemic site effects.
Our results encourage further investigation of bisphosphonate-targeted liposomes as potent targeted drug delivery system suitable for the treatment of bone metastases.



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Various routes of administration

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DRUG RELEASE FROM NON-AQUEOUS EMULSIONS DELIVERED INTRAMUSCULARLY TO RATS

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Purpose: Non-aqueous or oil-in-oil (o/o) emulsions composed of two immiscible oil phases have received relatively little attention but been investigated in our laboratory[1]. Such formulations may be used as a potential vehicles to deliver lipophilic or hydrolysable compounds. Although, some *in vitro* experiments have suggested that these non-aqueous emulsions can be used for sustained delivery[2], biological data has not been obtained to date. An *in vivo* study was carried out to evaluate the possibility of non-aqueous emulsions acting as drug reservoirs. Drug absorption after intramuscular injection from the injection site and distribution into various organs of animals was also estimated.

Methods: ^{3}H-Dexamethasone (^{3}H-DEX, 100 µCi / mg) as a model drug was incorporated into the castor oil (internal phase) and this was then used to prepare o/o emulsions. Two experiments were conducted with the same procedure but different type of emulsions: castor oil-in-silicone oil (co/so; non-aqueous) and castor oil-in-water (co/w) emulsions, were administered to rats. ^{3}H-DEX was given in the form of these emulsions (100 µl) at dose of 0.1 mg/ kg. ^{3}H-DEX-co/so or ^{3}H-DEX-co/w emulsions were injected into the lower left hind limb of each anesthetized rat (n=4). At specific time intervals up to 48 h the animals were sacrificed and blood samples were withdrawn. Other organs were then removed, homogenized and measured for radioactivity. The muscle (site of injection) was also collected for detection of dose still remaining to be absorbed.

Results: ^{3}H-DEX was absorbed after intramuscular dosing of castor oil/water emulsions reaching a maximum concentration (C_{max}) at 0.78 µg/ml within 2 h (T_{max}). In the case of castor oil/silicone oil emulsions a C_{max} of 0.48 µg/ml and T_{max} at 4 h was observed. No significant difference was found between two formulations in the area under the plasma concentration-time curve (AUC) and the clearance (CL) ($P > 0.05$). Both the mean residence time (MRT) and the elimination half-life ($t_{1/2}$) were increased following intramuscular administration of ^{3}H-DEX-castor oil/silicone oil emulsions compared with ^{3}H-DEX-castor oil/water emulsions ($P < 0.001$). At the injection site, the clearance of ^{3}H-DEX from castor oil/silicone oil emulsions was slower than that from castor oil/water emulsions. For example, the percentage of ^{3}H-DEX remaining in the muscle at 4 h after administration of castor oil/silicone oil emulsions and castor oil/water emulsions was found to be ca. 33 % and 4 % respectively. For both formulations, high uptake of ^{3}H-DEX was detected in the liver and kidneys whereas minimal amounts were found in other tissues. The amount of ^{3}H-DEX recovered from the liver and kidneys after intramuscular injection of castor oil/water emulsions was higher approximately 1.5 times than that of ^{3}H-DEX-castor oil/silicone oil emulsions.

Conclusions: Administration of ^{3}H-DEX in a castor oil/silicone oil non-aqueous emulsion confirmed slower absorption than from castor oil/water emulsions with a longer T_{max} together with the increase of MRT and $t_{1/2}$. In addition, the slow clearance of ^{3}H-DEX from the muscle injection site was observed. Such emulsions may be considered as a depot formulation for sustained release system but further work in the choice of external oil phase will be necessary to optimize effects.

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STUDIES OF INTRACELLULAR TRANSPORT OF DENDRIMERS

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Purpose: The main problem of tracking nanoparticles is the breakdown of either physical or chemical bonding between the fluorescent *in vivo* probe and nanoparticle. Here we describe the synthesis of an intrinsically fluorescent lysine based cationic dendrimer which lacks a fluorophore, but having sufficient fluorescence intensity to be detected at low concentrations. This avoids the need for fluorescent tagging and its associated problems. To this end, the intrinsic fluorescence of a 6th generation amino terminated polyamide polylysine dendrimer[1] (Gly-Lys_{63} $(NH_2)_{64}$, MW 8149) (***Fig. 1***) was used to study nuclear uptake and mobility inside both fixed and living cells (Caco-2, SKMES-1 and RESF-2 cells) using laser scanning confocal microscopy (LSCM).

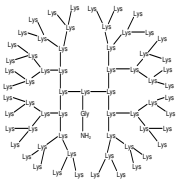

Fig. 1 The chemical structure of the 6th generation poly-lysine dendrimer

Methods: Caco-2 cells were routinely cultured following standard procedures in complete media containing DMEM supplemented with 1% (w/v) MEM, 10% (v/v) FBS and gentamycin (50 µg/ml) at 37°C, 5% CO_2, at 95% relative humidity. After reaching 80% confluence, the cells were harvested and seeded on glass cover slips coated with poly-d-lysine at a density of $1x10^4$cells/cm^2. 24 h after seeding, cells were incubated with the complete medium containing 0.1 % (w/v) 6th generation dendrimers. At different time periods cells were rinsed with serum free media and ice cold D-PBS before they were fixed with 4% paraformaldehyde for 15 min and the reaction quenched with 5 mM ammonium chloride. The cells were then extensively rinsed with ice cold PBS before the cover slips were treated with the antifading medium, Citifluor™, and mounted on slides. For controls, cells were incubated in the same way but in the absence of dendrimer and fixed as described above.
To study the uptake in living Caco-2 cells, SKMES-1 and RESF-2 cells, a similar procedure was carried out except that the cells were first stained with Hoechst 33342 and then transferred into a perfusion chamber. LSCM Z-stack images of the cells were taken every 5 min before and after the dendrimer was added to the media. After the experiment, the cells were re-incubated and cell viability assessed. The diffusion coefficients of the dendrimer relative to their diffusion coefficients in water were calculated.

Results: The uptake of the dendrimers both into the cytoplasm and into the nucleus occurs over a period of time. The same pattern of uptake was found in all types of cell lines. After 15 min a patch of the dendrimers was apparent on the cell surface and at longer time the dendrimers were found in all compartments of the cells. The results in fixed cell indicated that dendrimers were taken up and some could reach the nucleus after 15 min. In living cells the dendrimers were found inside the nucleus within 25-45 min of incubation.
The rapid uptake of the dendrimers into the cell cytoplasm might be initially due to the electrostatic attraction between the positively charged dendrimers and the cell membrane. This allows them to attach to the cell membrane prior to endocytosis. The diffusion coefficients (D) of the dendrimers inside the cells were calculated[2] and found to be between $2.34x10^{-3}$-$6.72x10^{-3}$ $\mu m^2/s$. The diffusion coefficient value of the dendrimer in water is 79.35 $\mu m^2/s$ (from the FRAP technique). It was not unexpected that the mobility of the dendrimers inside the cytosol is hindered by intracellular macromolecules, including the actin-protein complex and actin self assembly[3]. The small size of dendrimer particles (~6.5 nm in diameter) might allow the dendrimers both to diffuse rapidly and to pass easily through the nuclear pores, which have a diameter of about 9 nm [4]. Further

studies of the effect of fluid flow on the uptake of the dendrimer and the dynamics of uptake of the dendrimer-DNA complexes in living cells are being carried out.

Conclusion: This work shows that a fluorescent dendrimer is a versatile probe to study mobility and the uptake of nanoparticles in cells. A novel method for study the dynamic uptake of nanoparticles inside living cells which allows us to calculate diffusion coefficient of the dendrimers inside cells have been developed. Finally the uptake capacity of the dendrimer inside the cells was reduced by the influence of fluid flow.

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LIPID NANOPARTICLES (SLN, NLC) – PRESENT STATE OF DEVELOPMENT FOR DERMAL DELIVERY

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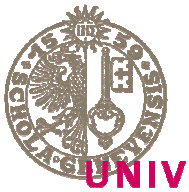
The 1st generation of lipid nanoparticles with solid particle matrix was developed at the beginning of the 90s, the so called solid lipid nanoparticles (SLN). The SLN were developed more or less in parallel by the research group of R.H. Müller in Berlin (SLN by high pressure homogenization) and M.R. Gasco in Turin (SLN by micro-emulsion precipitation method). The SLN combined advantages of the previous traditional carrier systems polymeric nanoparticles, emulsions and liposomes. Identical to polymeric nanoparticles, they possess a solid matrix allowing protection of chemically labile actives and to modulate the release of actives. Identical to emulsions and liposomes, they are composed of well tolerated, regulatorily accepted excipients and can be produced on large scale by high pressure homogenization (the prerequisite for market introduction of any product).

At the turn of the millennium, the second generation technology was developed, the so-called nanostructured lipid carriers (NLC). SLN are produced from solid lipid only, NLC are produced from a blend of a solid lipid with a liquid lipid (oil). The latter leads to lipid matrixes with an imperfect crystal structure, that means plenty of space to accommodate actives. This is what makes the NLC possessing a higher loading capacity for actives than SLN.

For many years lipid nanoparticle research focused on parenteral and oral administration, in the last half decade the emphasis shifted to dermal formulations. The reasons for this are simple: lipid nanoparticles possess special features of high interest for dermal formulations, in addition the time-to-market is much faster with dermal product. The regulatory hurdles are much higher for oral and especially parenteral products. Therefore the strategy was chosen to develop first dermal products. In addition, developments were performed in parallel in pharmacy and cosmetics. There are again 2 simple reasons for this: firstly, the time-to-market is much shorter for cosmetic products (8-12 months). Secondly, there is basically very little difference between cosmetic and pharmaceutical dermal products, that means a cosmetic product proves the “make-ability” of dermal pharmaceutical products (e.g. large scale production of NLC and incorporation of the particles as a stable system into creams or lotions).

At the end of October 2005 at the cosmetic fair “Beauty” in Munich the first 2 cosmetic products were launched (Nanorepair® Q10 cream and serum). Another 5 products are in development. In the pharmaceutical area, the first dermal product entered clinical phase I. From our point of view, the success, performance and applicability of a technology can be judged by the number of years between development/patenting of the technology and introduction of the first product to the market, and secondly by the number of the products appearing on the market after introduction of the first product. Based on these criteria, the NLC performed very well. It took almost 20 years between discovery of the liposomes and introduction of the first product (cosmetic product Capture by Dior), it was just about 5 years between patent application filling of the NLC technology and having the first product on the market.



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Vaccine delivery

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HBSAg LOADED PLA NANOPARTICLES: ADJUVANTICITY OF TOCOPHEROL ACETATE AND TOCOPHEROL NICOTINATE IN MICE

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Hepatitis B infection is still a global problem despite the availability of an effective vaccine. Presently alum is used as an adjuvant for the Hepatitis B surface antigen (HBsAg) which occasionally produces subcutaneous nodules, granuloma and abscesses [1]. The development of a delivery system for the Hepatitis B vaccine with an alternative adjuvant which could induce the desired antibody response from a reduced number of injections would be an enormous benefit. In this work we have investigated the effect of DL-α-Tocopherol acetate (TA) and (±)-α-Tocopherol nicotinate (TN) on the physicochemical characteristics and *in vivo* response of PLA nanoparticles encapsulating Hepatitis B surface antigen (HBsAg).

HBsAg loaded nanoparticles were prepared using PLA, PLA+ 20% TA and PLA+20% TN as a matrix by water-in-oil-in-water (w/o/w) emulsification solvent evaporation method. The internal phase stabilizer was 10% w/v PVA plus HBsAg solution and the external phase stabilizer was 1.25% w/v PVA. The integrity of the antigen and percentage loading of HBsAg in the nanoparticles was determined by SDS-PAGE and BCA assay respectively. The Z average diameter and the zeta potential (mV) were measured using Malvern Zetasizer®. Female BALB/c mice (n=4) were administered intramuscularly and intranasally with 1µg of HBsAg-loaded particle suspension or protein solution alone as appropriate. The animals were boosted on day 21 with 0.5µg of HBsAg-loaded particle suspension or protein solution. Tail vein blood samples were collected after 15, 30, and 45 days and the serum specific antibody responses assessed by ELISA.

No	Organic phase	Z average diameter before freeze drying (nm) [polydispersity)]	Z average diameter after freeze drying (nm) [polydispersity]	Loading efficiency (%) ±s.d
1	PLA	298 [0.205]	338 [0.448]	24.79 ± 2.3
2	PLA + 20% w/w TA	251 [0.234]	320 [0.274]	86.2 ± 0.4
3	PLA + 20% w/w TN	266 [0.146]	341 [0.225]	58.72 ± 0.3

Table 1: Particle size and loading efficiency of PLA nanoparticles with different compositions of TA and TN encapsulating HBsAg

The zeta potential was negative for all the formulations tested (-6 to -10mV). The mean diameter of the particles before the freeze drying was around 250-300nm irrespective of the formulation used. The particle diameter has slightly increased to 320-341nm following freeze drying. When TA and TN used as a part of the particle matrix a substantial increase in the loading efficiency of HBsAg in the nanoparticles was observed compared to PLA alone particles. The increase in loading efficiency of the antigen by the use of TA and TN is desirable since for the antigen carrying nanoparticles, to function as controlled release systems for the generation of long lasting immune responses, it is necessary that they should contain high levels of antigen. The structural integrity of HBsAg in the nanoparticles was not affected by either process parameters or by storage as shown by SDS-PAGE.

The encapsulated antigen has stimulated higher serum specific anti-HBsAg IgG responses compared to the non-encapsulated free antigen especially at the later time points. The serum specific antibody levels for the PLA+TN; PLA+TA blend particles were higher than the free antigen and the PLA alone particles. This is a preliminary study and these results will be confirmed during the long term studies. Nevertheless these data show that TN and TA can act as an adjuvant. In a previous study another it has been shown that another form of tocopherol, α-D-tocopherol polyethylene glycol 1000 succinate (TPGS) has shown adjuvanticity [2, 3].

TA and TN have significantly increased the loading efficiency of HBsAg in the PLA nanoparticles when used as matrix with PLA. HBsAg loaded PLA+TN and PLA+TA blend nanoparticles have influenced the intensity of serum specific antibody response. These studies are ongoing to investigate the long term and cellular immune responses.

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INVESTIGATION OF PLGA MICROPARTICLES FOR DNA VACCINE DELIVERY

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Introduction: The use of buffers to protect DNA during encapsulation has been studied previously, this has been investigated further by observing at which point the DNA degrades during particle manufacture (w/o/w double emulsion solvent evaporation) and the effect of buffers on the size, morphology, encapsulation efficiency and stability of the DNA.

Materials and Methods: Two types of buffers were used, 0.1M $NaHCO_3$ (pH 8.3) and 1% m/v Na_2HPO_4 (pH 9.1). These were selected on the basis that they are generally regarded as safe (GRAS) excipients. Their efficacy was compared with particles made with distilled water alone.
A549 cells were used to assess the viability of DNA encapsulated into the particles.

Results and Conclusions: It was demonstrated that without the protection of buffers, DNA was degraded (i.e. altered supercoiled form into open-circular/linear) in the primary emulsification step.

Both buffers protected DNA in that they conserved the supercoiled conformation of the plasmid DNA, but Na_2HPO_4 conferred more desirable particle characteristics with slightly enhanced loading efficiency and narrower size distribution.

There was a correlation between the degree of supercoiled DNA in the extracts and the transfection efficiency of the A549 cells. DNA extracted from particles made without buffer showed minimal transfection of cells; DNA extracted from particles made with buffer showed comparable transfection ability to each other.

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GENETIC IMMUNIZATION USING DENDRIPLEXES AND PLGA/DENDRIPLEX CARRIERS AGAINST ANTHRAX

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Purpose: Dendrons based on lysine behave as macromolecular polyelectrolytes and as such will attract oppositely charged particles making them ideal for associating with DNA. The behaviour of the cationic polylysine dendrons with DNA (dendriplexes) which were encapsulated in a PLGA matrix[1] has been studied as potential genetic anthrax vaccine (Fig. 1). Recently *Bacillus anthracis* has attracted attention as an agent for bioterrorism, calling for the development of new anthrax vaccines to protect humans against their intentional use as a warfare agent. The virulence of anthrax bacilli is due to the production of three-component protein exotoxin. PA (protective antigen) one of the protein components is singularly the most important antigen required for specific immunity to anthrax. Two types of plasmid DNA were developed, one encoding PA 83 cloned into the eukaryotic expression plasmid pSecTag 2B (7.3kbp) and a control plasmid without PA 83. The plasmids were complexed with dendrons (dendriplexes) condensing the DNA forming particles approximately 80nm in size, depending on the lipophilicity of the dendron. Both dendriplexes and PLGA-dendriplex particles containing the PA DNA were tested by *in vivo* immunological studies.

Methods: A multiple emulsion method was employed to encapsulate the dendriplexes in poly-lactide-co-glycolide (PLGA) particles. Dendriplex was added to 3%w/v PVA solution, which was used to homogenise with PLGA in DCM. This primary emulsion was further homogenised in a PVA solution (1.25%w/v) forming a w/o/w emulsion. Nanoparticle formation was effected by evaporation and harvested via centrifugation. Nanoparticles were characterised by PCS (Malvern Zetasizer) and electron microscopy. Two different mouse strains were used in this study; BALB/c and A/J, 10 of each strain were used with weights ranging from 16-20g. The mice were vaccinated over two months with two sets of dendriplex particle containing PA and control plasmid constructs form each dendron. Each immunization consisted of a dose of approximately 1ug in 20ul PBS per mouse injected into the thigh using a Hamilton syringe. Immunogenicity was determined by the sera collected using ELISA method measuring the anti-PA antibodies.

Results: The immunogenicity is higher for the dendriplexes when compared to naked DNA and control plasmid in both sets of mice but there was no significant difference between the two sets of dendriplexes containing structurally diverse dendrons in the low dose study. Naked PA immunisation with multiple dosing did not induce sufficient antibody response even after secondary boosting post primary i.m immunisation, whereas both dendriplexes and PLGA particles produced strong anti-PA antibody response. The response was dose dependent as depicted by the low and high dose dendriplex treatment groups.

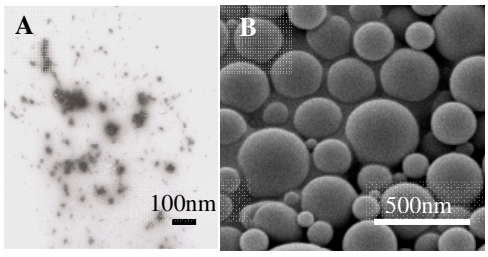

Fig. 1: TEM of PA $(C_{18})_3$ dendriplexes and the PLGA particles in which the dendriplexes is encapsulated.

Throughout the *in vivo* study both dendriplexes and PLGA formulations improved immunomodulatory efficiency in comparison to the naked PA DNA. However, they did not provide protection against lethal toxin challenge in the *in vitro* studies.

Conclusions: To our knowledge this is the first *in vivo* study using dendriplexes and encapsulated dendriplexes as a vaccine against anthrax. Further work needs to be conducted to investigate what levels of antibodies are needed to protect humans against anthrax.[2] The role that antibodies play to induce resistance against this biological weapon needs to be defined.[3]

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