European Workshop on Particulate Systems

June 4-5, 2010

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UMR CNRS 8612
Université Paris-Sud
Châtenay-Malabry

Venue
Cité Internationale
Universitaire de Paris

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Venue

The venue is the "Cité Internationale Universitaire de Paris". The Cité Internationale Universitaire de Paris was founded in the early 1920s in a bid to promote harmony by providing accommodation and facilities to foster ties between talented young people from all over the world.

The Cité Internationale's 40 houses welcome some 10,000 residents a year, including students, researchers, visiting professors, artists and sportsmen from over 140 countries. It provides quality accommodation complete with a wide range of services (featuring a theatre, library, restaurant, sports facilities, support services and more) and aims to create a forum for exchange and debate at the highest level.

Nearly a century has gone by since it was founded, yet the Cité Internationale continues to work tirelessly to provide accommodation and support for international residents. The Cité's park spans 34 hectares and is home to the most extensive range of student accommodation in the Paris region, forming a unique patchwork of linguistic and cultural diversity in the French capital.

PROGRAMME AT A GLANCE

Cité Internationale Universitaire de Paris

Salon Gulbenkian

Friday, June 4th

12:30 Welcome and registration

13:30 Lecture by Prof. Sandy Florence

14:15 Session 1: Nano- and microparticles: design and characterization

16:15 Pause

16:45 Session 2: Tissue engineering and nucleic acid delivery

19:45 Dinner

Saturday, June 5th

9:00 Lecture by Prof. Patrick Couvreur

9:45 Session 3: Vaccine delivery

10:45 Pause

11:00 Session 4: Local routes of administration

12:40 Lunch

14:00 Session 5: Inflammatory diseases

15:40 Pause

16:00 Session 6: Brain delivery

17:00 End of the workshop

DETAILED PROGRAMME

FRIDAY, JUNE 4th

12:30 **Welcome and registration**

13:30 **Lecture by Prof. Sandy Florence**
Interfaces

14:15 **Session 1: Nano- and microparticles: design and characterization**

14:15 **Mounira Hamoudi** (Paris)
Evaluation of the behavior of beads, made of cyclodextrin and oil, in simulated digestive media, for the oral delivery of lipophilic drugs

14:35 **Mohamed Othman** (Paris)
Mechanism of Formation of a Nanoparticulate Self Assembling System and its Interaction with Functionalised Gadolinium Complexes

14:55 **Ranjita Shegokar** (Berlin)
Targeted stavudine lipid nanoparticles: large scale production and long term stability

15:15 **Tamim Chalati** (Paris)
The challenge of entrapment of entirely hydrophilic drugs in porous iron carboxylate nanoparticles

15:35 **Odile Diou** (Paris)
PEGylated nanocapsules of perfluorooctyl bromide as ultrasound contrast agents

15:55 **Jan Möschwitzer** (Berlin)
Optimization of a Novel Combinatory Production Method for Nanosuspenions

16:15 Pause

16:45 **Session 2: Tissue engineering and nucleic acid delivery**

16:45 **Yanhong Wen** (Copenhagen)
Development of activated particulate systems as scaffolds for tissue regeneration

17:05 **Acelya Yilmazer** (London)
Engineering Acid-Responsive Artificial Envelopes around Adenovirus for Efficient Gene Transfer

17:25 **Linda B. Jensen** (Copenhagen)
Elucidating the molecular mechanism of dendriplex formation: effect of dendrimer generation and siRNA concentration

17:45 **Mouna Raouane** (Paris)
Preparation and characterization of squalene nanoparticles for small interfering RNA targeted against the Ret/PTC1 oncogene delivery

18:05 **Amir Varkouhi** (Utrecht)
Gene silencing activity of siRNA complexes based on biodegradable polymers and carbon nanotubes

18:25 **Fernanda Bruxel** (Paris)
Cationic nanoemulsions for delivery of antimalarial oligonucleotides: adsorption and in vitro studies

18:45 End of session 2

19:45 **Dinner**

SATURDAY, JUNE 5th

9:00	**Lecture by Prof. Patrick Couvreur** "Smart" nanocarriers for drug delivery and targeting

9:45 **Session 3: Vaccine delivery**

9:45 **Mette Hamborg** (Copenhagen)
Liposome-Based Cationic adjuvants: Interactions with antigens

10:05 **Medha Joshi** (Utrecht)
Improving anti-tumor responses by DC-SIGN mediated antigen-targeting using glycan modified liposomes: A potential anticancer vaccine

10:25 **Pernille Nordly** (Copenhagen)
A synthetic mycobacterial monomycoloyl glycerol analogue stabilizes DDA liposomes and potentiates their adjuvant effect in vivo

10:45 **Pause**

11:00 **Session 4: Local routes of administration**

11:00 **Cornelia M. Keck** (Berlin)
Silver-Nanolipid Complex (s-NLC): antiinflammatory activity in murine model of atopic dermatitis

11:20 **Nina Knudsen** (Copenhagen)
Penetration of gel and liquid state liposomes into barrier-impaired skin

11:40 **Sasha Nikolić** (Berlin)
Lipid nanoparticles for synergistic skin photoprotection

12:00 **Simona Mura** (Paris)
Toxicological effects of lung exposition to biodegradable nanoparticles

12:20 **Claudia di Tommaso** (Geneva)
A novel drug carrier for the delivery of cyclosporine A to the eye

12:40 Lunch

14:00 **Session 5: Inflammatory diseases**

14:00 **Bart Crielaard** (Utrecht)
Liposomal Targeting in inflammatory disease: Macrophages, friends or foes?

14:20 **Wouter Hofkens** (Utrecht)
Systemically delivered glucocorticoid liposomes Inhibit macrophage mediated cartilage destruction during experimental arthritis

14:40 **Julie Pradal** (Geneva)
P38 MAPK inhibitor-loaded particles for the intra-articular treatment of osteoarthritis: formulation and in vitro activity

15:00 **Maria Coimbra** (Utrecht)
Liposomal delivery of resveratrol, curcumine and N-(3-oxo-dodecanoyl)- L-homoserine lactone to inhibit cancer inflammation

15:20 **Jolanda M. van den Hoven** (Utrecht)
Optimizing the therapeutic index of liposomal glucocorticoids in arthritis

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15:40 **Pause**

16:00 **Session 6: Brain delivery**

16:00 **Inge van Rooy** (Utrecht)
Binding of phage displayed vs synthetic peptides to brain endothelium

16:20 **Davide Brambilla** (Paris)
Nanoparticles against alzheimer's disease: PEG-PACA nanoparticles are able to link the Ab-peptide and influence its aggregation kinetic

16:40 **Antonio Nunes** (London)
In vivo interactions of different functionalized Carbon nanotubes with the brain parenchyma

17:00 End of the workshop

PARTICIPANTS

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Session 1

Nano- and microparticles: design and characterization

EVALUATION OF THE BEHAVIOR OF BEADS, MADE OF CYCLODEXTRIN AND OIL, IN SIMULATED DIGESTIVE MEDIA, FOR THE ORAL DELIVERY OF LIPOPHILIC DRUGS

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Introduction

Poorly-water soluble drugs have often a poor oral bioavailability. One possible strategy to improve the efficiency of such drugs is to encapsulate them into lipid-based systems. A new lipid carrier, namely "beads" made of natural cyclodextrins (CD) and oil has been reported [1]. Very rich in oil, the beads are able to encapsulate lipophilic drugs [2, 3]. Moreover, pharmacokinetics of isotretinoin in rats demonstrated that the drug is successfully released from beads in the digestive tract and that isotretinoin bioavailability is doubled compared to isotretinoin lipid solution [3]. This demonstrates the high potential of this new delivery system for the oral route. However, complementary studies have to be performed. The aim of this study was to evaluate *in vitro* the stability of the beads and the release of progesterone (PG) selected as a model of lipophilic drug in simulated gastric and intestinal fluids (SGIF).

Experimental Methods

Nile red-loaded beads were prepared by adding 5.8 mL of an oily solution of Nile red to 20 mL of an aqueous solution of (8.1% w/v) α-CD. The preparation was continuously shaken at 200 rpm in a gyratory shaker at 28°C until a monodisperse population of beads was obtained. Progesterone was either solubilised or dispersed in soybean oil. PG-loaded beads were prepared as described above.

Bead stability and PG release study from freeze-dried beads were evaluated *in vitro* using the following simulated gastro-intestinal fluids (SGIF): simulated gastric fluid (SGF), simulated intestinal fluid free of sodium taurocholate and sodium taurocholate-containing fluids: Fasted state simulated intestinal fluid (FaSSIF) and Fed state simulated intestinal fluid (FeSSIF).

In stability studies of freeze-dried Nile red-loaded beads in SGIF, 100 beads were introduced in each dissolution vessel. For each considered time and medium, particles were recovered, counted and measured. Confocal microscopy was also employed to visualize the inner structure of beads after incubation in SGIF.

The studies of PG release in the SGIF were performed on the following formulations: beads prepared from PG in oily solution, PG in oily suspension and the content of PG soft capsules. At specific time intervals, samples of SGIF were withdrawn and immediately filtered. The concentration of PG was then determined directly without dilution by HPLC.

Results and discussion

In this work, our results showed that bead diameters decreased when beads were successively incubated in the SGF and then in a simulated intestinal fluid This effect resulted from the hydration and the dissolution of the bead matrix composed of α-CD molecules. Bead erosion and fragmentation allowed the release of PG present in oily droplets dispersed in the matrix. The presence of sodium taurocholate in FaSSIF and FeSSIF significantly influenced the stability of the particles. Indeed, time to reduce the initial bead diameter by half and time to recover 50% of beads were significantly decreased. The effect was more pronounced after the addition of FeSSIF. Our results showed also that sodium taurocholate strongly influenced the % of PG dissolved in SGIF .

In USP SGF/ FeSSIF mixture, the amounts of PG dissolved in the medium were higher with beads prepared from PG in oily suspension than with those obtained from PG in oily solution. Besides both kinds of beads released more PG than the content of PG soft capsule (peanut oil).

Conclusion

The results highlighted the influence of sodium taurocholate on the stability of Nile red-loaded beads and PG release in SGIF. These studies tend to demonstrate that beads are a promising drug delivery system for the oral delivery of lipophilic compounds.

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MECHANISM OF FORMATION OF A NANOPARTICULATE SELF ASSEMBLING SYSTEM AND ITS INTERACTION WITH FUNCTIONALISED GADOLINIUM COMPLEXES

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Introduction

We have established that two neutral polymers, namely polymerised β-CD (pβ-CD) and dextran grafted with lauryl side chains (MD), may completely associate in water, to spontaneously form supramolecular nanoassemblies (NPs) of spherical shape [Gref et al. 2006]. We have synthesized a new gadolinium complex functionalised with an adamantane group, (Gd^{3+} complex of (acid 1,4,7,10- tetra aza cyclododecane-1-[(N-adamantyl) acetamide]- 4,7,10-triacetics), which has been efficiently entrapped into these NPs, leading to a high payload of 1.8×10^5 units of Gd^{3+} complex per nanoparticle and a great relaxivity r_1 enhancement of 48.4 $mM^{-1}.s^{-1}$ at 20 MHz and 37°C [Battistini et al. 2008]. The cohesion of these nanostructures is based upon a “lock and key” mechanism in which the hydrophobic alkyl chains of MD and the adamantyl moieties of Gd^{3+} complex incorporate into the molecular cavities of (pβ-CD). We have investigated here the mechanism of the NPs formation, as well as the inclusion of Gd^{3+} derivative into mono-and pβ-CD by isothermal titration microcalorimetry (ITC) in conjunction with ^{1}H-NMR and dynamic light scattering (DLS).

Experimental Methods

Critical association concentration (CAC) of MD: was estimated by using pyrene as a *fluorescent* probe, by monitoring the changes in the ratio of the pyrene excitation spectra intensities at λ = 333 nm for pyrene in water and λ = 336 nm for pyrene within the micelle core. Excitation spectra were monitored at λ_{em}=390nm.

ITC experiments: A solution of MD or of Gd^{+3} complex, placed into the calorimetric cell, was titrated in a time controlled manner by a concentrated solution of β-CD or pβ-CD, at 25°C. The fit of the final titration curve led to determination of the stoichiometry (N), the stability constant (K) and the enthalpy of the interaction(ΔH).

^{1}H NMR studies: The samples of each polymer and different molar ratios of C_{12} of MD/ CDs of pβ-CD were prepared in deuterium oxide. Chemical shifts were referenced to the solvent values (δ=4.70 ppm for HOD).

The size of NPs formed in ITC experiments was determined by DLS.

Results and discussion

To determine K between MD and pβ-CD by ITC, we have used MD at a concentration lower than the CAC. The obtained K was very high (K=25000 M^{-1}), whereas it was only 1950 M^{-1} between MD and β-CD. These data account for the high affinity of the alkyl chains for the CD cavities. In the case of pβ-CD, the higher K could be explained by the proximity between C_{12} and CDs in the pβ-CD polymer; as soon as one inclusion complex is formed, the alkyl chains on MD become in proximity with other available CD cages in pβ-CD and the probabilities to form new complexes increase. In the case of MD/pβ-CD interaction, the association process was exclusively exothermic (ΔH<0) with positive and favourable entropic contribution (ΔS>0) and mostly entropy driven (|ΔH|<|TΔS|). Large positive entropy changes usually arise from the significantly important translational and conformational freedoms of host and guest upon complexation. The Gd^{3+} complex has a high affinity for pβ-CD; (K= 1460 M^{-1}). The interaction of the Gd^{3+} complex with pβ-CD was lower than that with β-CD (K=11000 M^{-1}). Possibly, steric encumbrance effects might occur from the crosslinked pβ-CD. The interactions of Gd^{3+} complexes with both β-CD and pβ-CD were exclusively exothermic (ΔH<0) with positive entropic contribution (ΔS>0) and mostly enthalpy driven (|ΔH|>|TΔS|). The interactions of Gd^{3+} complex/ CDs are mediated by the formation of van der Waals-type bonds.

^{1}H-NMR investigations showed that the peaks of both CH_3 and CH_2 groups of C_{12} of MD were down-field shifted upon their complexation with pβ-CD, and broadened. The chemical shift demonstrates that almost all the alkyl chains were complexed by the CD cavities, when C_{12} of MD / β-CD of polymer molar ratios were ≥1.

DLS studies confirmed the previous findings, showing that immediately after contact between pβ-CD and MD solutions, stable NPs were formed, whatever the substitution yields of MD.

Conclusion

It was concluded that the C_{12} chains of MD interact with the CDs of pβ-CD with a 1:1 stoichiometry. All interactions were exothermic and spontaneous. The interaction between MD/pβ-CD was entropy driven, while the interactions of Gd^{3+} complex/ β-CD and pβ-CD were enthalpy driven. The association constants of MD/ pβ-CD were high compared to the Gd^{3+} complex/pβ-CD. Total inclusion of C_{12} was confirmed by ^{1}H-NMR studies. Owing to DLS studies, MD and pβ-CD associate in aqueous media to form NPs, whatever their concentrations.

- Gref et al. J. Control. Release (2006) 111, 316–324. - Battistini et al. Chem. Eur. J. (2008) 14, 4551–4561.

TARGETED STAVUDINE LIPID NANOPARTICLES: LARGE SCALE PRODUCTION AND LONG TERM STABILITY

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Introduction
Successful introduction of a drug delivery system to the market needs feasibility of large industrial scale production. Normally from moving up in batch size (factor 10 to 100) various problems occur in obtaining the same product specifications as on the smaller scale. However, it is equally important that batch has reasonable physical stability. Drug delivery such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have the advantage of being produced by high pressure homogenization (HPH) (1). Various nanoparticles are explored for HIV therapy with promising results (2) but none of the research paper is available on scale up of these nanoparticles to industrial scale. To perform *in vivo* studies and/or human trials, qualified medium scale production units to produce clinical batches are essential. In this study we systematically scaled up Stavudine lipid nanoparticles and evaluated its long term stability up to 1 year at various stress conditions.

Experimental Methods
SLN are produced by hot high pressure homogenization techniques, the drug was dissolved in the melted lipid and the melt dispersed in aqueous surfactant solution of the same temperature. The obtained coarse pre-emulsion was passed through a temperature-controlled piston gap APV Gaulin homogenizer (APV Deutschland GmbH) having production capacity of 150 litre/h in continuous mode at 800 bar (= 12,000 psi, 80 MPa). Prepared lipid nanoparticles were then subjected to long term stability at refrigeration, room temperature and 40ºC (1, 3, 6 and 12th months). The nanoparticles were analysed for particle size using photon correlation spectroscopy, PCS (Zetasizer Nano ZS (Malvern Instruments UK)) and laser diffractometry, LD (Mastersizer 2000 (Malvern Instruments, UK)). Biodistribution studies were carried out in Wister rats using Gamma Scintigraphy.

Results and discussion
Stavudine lipid nanoparticles were scaled up successfully using the APV Gaulin 5.5 (20 kg). Maximum dispersivity was obtained within two cycles (63.3 nm), increase in the number of cycles (till 5 cycles) resulted in coalescence/ increase in particle size. The difference of just as small as 3 nm in PCS diameter indicates the excellent scaling ability. Mean PCS particles size differ only a few nm, being outstanding when comparing the scale up to half a ton in 3 hours by the Gaulin 5.5 (scale up factor by 1,250). Lipid nanoparticles showed excellent stability at room and refrigeration storage conditions up to one year. In vivo organ distribution: Drug loaded lipid nanoparticles showed 8.30 fold increase in gamma count over pure drug in the spleen. High spleenic uptake of AUC_0^{24} of 196.605 counts.h/g for stavudine nanoparticles was observed to be much higher than pure drug solution (35.133 counts.h/g). with prolonged mean residence time. Biodistribution studies revealed the targeting potential of prepared lipid nanoparticles to HIV sanctuaries sites in body.

Conclusion
Stavudine lipid nanoparticles (< 100 nm) showed excellent physical stability and targeting potential to HIV reservoirs. The lipid nanoparticles were successfully scaled up to industrial batch size.

References:

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2. Bender A, Schfer V, Steffan AM, Royer C, Kreuter J, Rubsamen-Waigmann H, et al. Inhibition of HIV in vitro by antiviral drug-targeting using nanoparticles. Res Virol. 1994;145(3-4):215-20.

THE CHALLENGE OF ENTRAPMENT OF ENTIRELY HYDROPHILIC DRUGS IN POROUS IRON CARBOXYLATE NANOPARTICLES

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We have investigated the use of porous Metal-Organic Frameworks (MOF) nanoparticles as drug carriers. These MOF nanoparticles are typically built from the assembly of inorganic units (metals) such as Iron and organic linkers with coordinating groups such as carboxylates. They combine a high pore volume and a regular porosity, as well as the presence of organic groups easily tuneable within the framework[1].

Iron(III) carboxylates nanoparticles with several topologies and compositions have been obtained as nanoparticules from a typical hydro or solvothermal synthesis[3] or other methods[2]. The obtained nanoparticles were characterized using XRPD, TGA and FTIR. Particle size was determined by using SEM and light diffusion techniques[2].

Three challenging drugs, Busulfan, AZT-TP and Cidofovir have been successfully entrapped into these hybrid solids. The loadings have been dramatically increased compared with other nanoparticulate systems. Moreover, a sustained release has been achieved in the case of AZT-TP.

Busulfan was encapsulated by soaking the nanoparticles of porous solids into saturated solutions of the drug. Adsorbed Busulfan content was estimated by chemical analysis and XRPD. AZT-TP and Cidofovir were also encapsulated by impregnation using different drug concentrations. AZT-TP loading was estimated by ^{3}H-radioactivity measurements and for cidofovir by ^{14}C-radioactivity measurements[1].

Busulfan loadings were up to 25 wt %. This is much higher than the previously highest reported loadings achieved using polymer systems (5-6% wt/wt) [4].

Concerning the AZT-TP encapsulation, the contents were up to 42 wt%, together with very high entrapment efficiencies. In the case of cidofovir the loadings were up to 44% [2]. First AZT-TP delivery tests under physiological conditions (PBS, 37ºC) have been carried out, observing a controlled drug release after 6 days[2].

The nanoMOFs porous iron carboxylates have several advantages when used as drug nanocarriers. In terms of synthesis, they are obtained in aqueous solutions instead of using organic solvents, thus providing an example of what "green" technology can afford for biomedical applications. In this sense, they act as sponges, encapsulating hydrophilic drugs by immersion in their aqueous solutions. They are not toxic even by using high concentrations up to 250 µg/mL[2].

In addition, the design of the porous hybrid solids, playing with the wide range of compositions and topologies, could allow adapting these porous hybrid matrices to the host molecule, according to its structure and its dosage requirements.

REFERENCES

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KEYWORDS : **Busulfan, Cidofovir, nanoparticles, AZT-TP, MOF, iron Carboxylates.**

PEGYLATED NANOCAPSULES OF PERFLUOROOCTYL BROMIDE AS ULTRASOUND CONTRAST AGENTS

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Introduction

The need to detect cancer at its early stages, as well as, to deliver chemotherapy to targeted site motivates many researchers to build theragnostic platforms which combine diagnostic and therapy. Ultrasonography is a widely available, non invasive and cost-effective imaging modality. The use of contrast agent is often required, because of the weak difference of echogenicity between different tissues. Novel ultrasound contrast agents (UCA) consisting of polymeric nanocapsules of poly(lactide-co-glycolide) PLGA, encapsulating a liquid core of perfluorooctyl bromide (PFOB) have been developed recently. The biodegradable polymeric shell ensures a good stability of the system and could be used as a drug loading compartment. The fluorinated core allows the detection of the capsules by ^{19}F MRI and ultrasonography. The echogenicity of the system has been proved *in vitro* and *in vivo*, in the blood pool. Since they possess a rather hydrophobic surface, nanocapsules are quickly eliminated by the mononuclear phagocyte system. Therefore passive tumor targeting, based on the enhanced permeation and retention effect, cannot be achieved. To increase the plasma half life of nanocapsules and favor passive tumor targeting, one strategy was to decorate them with PEGylated phospholipids. The hydrophilic PEG chains prevent the blood proteins' adsorption, by steric repulsion. Unfortunately, the partial PEG coverage seems to be insufficient to insure a good stealthiness. Another strategy is now considered to increase the surface density of PEG chains: the use of PLGA-PEG block copolymer instead of PLGA.

Experimental Methods

Nanocapsules were prepared either by nanoprecipitation or emulsion-evaporation. Nanoprecipitation was carried out by adding quickly the aqueous phase to the organic one (acetone+CH_2Cl_2), which contained both the polymer and PFOB, followed by solvent evaporation under reduced pressure. Emulsion-evaporation consisted of two steps: 1- Vortex and sonication of the aqueous and the organic (CH_2Cl_2) phases, followed by 2- Evaporation upon stirring at room temperature. Nanocapsules size distribution was measured by Dynamic Light Scattering and correlated to electronic microscopy. The efficacy of PEGylation was assessed by measuring the zeta potential and XPS experiments. PFOB encapsulation was estimated by ^{19}F MRI: freeze-dried nanocapsules were dissolved in $CDCl_3$ containing fluorinated crown ether as an internal standard. The amount of PFOB was determined after integration of the peak at -81ppm, corresponding to the CF_3 group. Nanocapsules compressibility was deduced from measurements of both density, using a DMA 58 vibrating tube densimeter and suspension sound velocity, using an ultrasound resonator cell with piezotransducers at 7.5MHz.

Results and discussion

Objects formed by both methods had similar sizes ($d_H^{nanoprecipitation}$~140nm and $d_H^{emulsion}$~120nm) and polydispersity indexes (~0,2). However, emulsion-evaporation process leads to better encapsulation efficacy (η=80%) than nanoprecipitation (η=25%). This can be due to the lower solubility of PFOB in the mixture of acetone and CH_2Cl_2 as compared to CH_2Cl_2 alone or, the quick diffusion of acetone into the water phase. Transmission Electron microscopy reveals that core-shell structure is preserved with PLGA-PEG. X-Ray Photon Electron Spectroscopy, as well as, zeta potential measurements provides evidence of PEG presence at nanocapsule surface. Scanning Electron microscopy shows that the surface is smooth. Polymeric shell thickness appears to have an influence on capsule compressibility. Preliminary *in-vitro* ultrasound imaging was performed with a clinical ultrasound probe.

Conclusion

This study demonstrates that stealth nanocapsules of PFOB can be produced by a rapid and reproducible method. Echogenicity of the system has to be proved to confirm its potential as a new ultrasound contrast agent.

OPTIMIZATION OF A NOVEL COMBINATORY PRODUCTION METHOD FOR NANOSUSPENIONS

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Introduction

It is a commonly used strategy to apply particle size reduction methods for the formulation development of poorly soluble active pharmaceutical ingredients (APIs). Standard top-down methods, like wet ball milling (WBM) or high pressure homogenization (HPH), have the drawback of long process times. The minimal achievable particle size strongly depends on the API characteristic and several equipment parameters, like e.g. the power density. In order to improve the particle size reduction effectiveness of the standard top methods, a new combinatory process has been developed. It consists of a combination of a freeze drying step (FD) as bottom-up process for solvent extraction and structure modification of the poorly soluble API which is followed by classical top-down processes like WBM or HPH. With this new process very small particles can be produced in a very efficient manner. The work presented here was focused on the assessment of the relevance of various process parameters on the overall particle size reduction effectiveness.

Experimental Methods

Modification of glibenclamide by means of freeze drying

Glibenclamide was used as poorly water soluble model compound. Goal was to assess the influence of the solvent composition and the API concentration during FD on the particle size reduction effectiveness of the top-down step (either WBM or HPH). Therefore glibenclamide was dissolved in mixtures of dimethyl sulfoxide (DMSO): tert-butylalcohol (TBA). Different solvent ratios (90%:10% v/v, 70:30, 50:50, 30:70 and 10:90) were tested. For each solvent ratio three different API concentrations were investigated: 7; 17 and 27 mg per ml of solvent. The API solutions were quench-cooled using liquid nitrogen and lyophilized for 4 days (Tp: -20 °C, Pc < 0.5 mbar).

Characterization of the modified starting material

The morphology of the modified freeze dried API was investigated by scanning electron microscopy (SEM). DSC and XRPD were applied to determine the solid state of the different API powders before and after FD.

Production of nanosuspensions by high pressure homogenization

Suspensions were made with each freeze dried API: 1% w/w glibenclamide, docusate sodium salt (DSS) 0.2% w/w as surfactant. These suspensions were pre-homogenized for one minute at 9000 rpm using an Ultra-Turrax and then homogenized at high pressure (1500 bar) for 20 cycles using a homogenizer Micron LAB 40 (APV Gaulin).

Characterization of the nanosuspensions

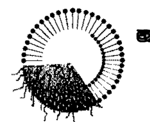
The particle size of the nanosuspensions was measured with photon correlation spectroscopy (PCS) yielding a z-average and a polydispersity index (PI) as well as with laser diffractometry (LD) yielding a volume size distribution.

Results and discussion

It was found, that higher TBA concentrations led to more porous and bulky API cakes after FD. This can be explained by larger crystals that TBA forms during the freezing process and the lower solubility of glibenclamide in TBA. Therefore it can act as a kind of pore former to obtain a more bulky cake. The solid state of the modified API was influenced by the drug concentration and the solvent composition during FD. Lower API concentrations resulted in higher amorphous fraction after FD. A higher DMSO content in the solvent resulted in more crystalline API after FD. Both, the crystallinity as well as the porous structure influenced the particle size reduction effectiveness during the following top-down step. For HPH the best particle size (z-ave 187 nm, PI: 0.180) was achieved with the lowest API concentration (7 mg/ml) and the 10:90 DMSO:TBA ratio during FD. This result is significantly better than from unmodified API (z-ave 772 nm, PI: 0.307). The smallest achievable particle size of the WBM process is less influenced by the modification step (z-ave 166 nm, PI: 0.131(modified API) vs. z-ave 193 nm, PI 0.156 (unmodified API)). However, less production time is needed if modified starting material is used.

Conclusion

It could be shown, that the modification of the API by means of FD can significantly improve the particle size reduction effectiveness of a subsequently performed WBM and HPH step. The solvent and the API concentration used for the FD need to be selected carefully. Ideally the optimal process parameters are selected by applying DoE.



LIPID NANOPARTICLES: DIFFERENCES IN POLYMORHIC STRUCTURES OF SLN &NLC

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Introduction

Lipid Nanoparticles have been invented by R.H. Müller for the encapsulation of lipophilic actives. The first generation (1991) are the “Solid Lipid Nanoparticles (SLN)”. The solid lipid matrix of SLN consists of one solid lipid only. The disadvantage of SLN is their limited drug loading capacity and moreover, the hazard of drug expulsion over the time of storage. The second generation (1999/2000) of lipid nanoparticles are the so called “Nanostructured Lipid Carriers (NLC)”, which consist of a blend of a solid and a liquid lipid (oil). Due to the addition of oil the crystallinity of the lipid matrix is changed. NLC contain imperfections (i.e. nanostructures) which provide more space to host the active molecules. Thus the drug loading capacity can be increased by a factor 2. For NLC it is also known, that drug expulsion during the time of storage is less likely to occur, when compared to SLN. The reason for this is not yet understood. Therefore the aim of this study was to investigate and compare potential differences and changes in crystallinity of SLN and NLC lipid mixtures over time.

Experimental methods

Differences in crystallinity were investigated using differential scanning calorimetry (DSC). Pure lipid (Dynasan 118), as well as mixtures of oil (Miglyol 812) and lipid were investigated immediately after processing and over a period of 4 weeks. Furthermore the influence of the production process on the crystallinity was investigated.

Results and discussion

Solid lipids show polymorphic modifications. Pure Dynasan 118 showed two melting events (56°C and 71°C) when analyzed immediately after processing the lipid. Hence two modifications are coexistent at this time point. Over time the height of the lower melting point decreased, indicating the transformation of the α-modification into the more stable β-modification. This phenomenon is known to cause expulsion of drug actives, because of changes in the lipid matrix from hexagonal to orthorhombic and triclinic.

In contrast to this the NLC lipidic mixture only showed one melting event at 71°C, indicating the absence of the α-modification directly after the production. Neither the melting point nor the height of it changed over the time of storage. Hence no changes over time were observed, and thus the hazard of drug expulsion will be reduced when NLC lipidic mixtures are used.

Moreover, it was found that the production process (e.g. low agitation mixing vrs. high speed mixing vrs. high pressure homogenization) had a strong influence on the crystallinity of the lipid matrix. A higher energy input increased the amount of β-modification and decreased the amount of α-modification. The increase in crystallinity due to high pressure homoginzation has been described for drug nanocrystals before. However, the finding is new for lipid nanoparticles und thus it should be investigated in more detail (e.g. influence on drug loading efficacy, physical stability and drug release) in future work.

Conclusion

The addition of oil to a solid lipid can completely circumvent the re-crystallization of unstable modifications in the lipid matrix. In NLC the hazard of drug expulsion is minimized, as no transformation from unstable to stable modifications occur. Therefore, in respect to physical stability and prevention of drug expulsion, NLC lipid matrices are favourable when compared to SLN.

Session 2

Tissue engineering and nucleic acid delivery

DEVELOPMENT OF ACTIVATED PARTICULATE SYSTEMS AS SCAFFOLDS FOR TISSUE REGENERATION

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Introduction

In tissue regeneration, well-defined scaffolds with therapeutically active substances are essential to structurally support the cell growth and at the same time deliver active components, in order to eventually form a new functional tissue. For this purpose various scaffolds, such as particles, gels or fibers have been investigated with some success. PLGA, which is approved by FDA as scaffold material due to its biocompatibility and biodegradability, is modified by mPEG to obtain a more hydrophilic and flexible surface. In the present project, mPEG-PLGA/PLGA particulate systems are prepared as cell scaffolds, which are potent with drug delivery properties in order to induce efficient autologous cell therapy with e.g. myoblasts for the repair of urinary sphincter and thus treating incontinence, or chondrocytes for cartilage repair.

Experiments

Aiming at developing the most favorable scaffolds, mPEG-PLGA microparticles have been prepared by the double emulsion-solvent evaporation method. The microparticles have been characterized and optimized, in terms of e.g. particle size, surface and internal morphology, and degradation profiles. In order to improve the effect of the scaffold upon injection, the thrombin-related peptide, thrombin receptor agonist peptide (TRAP-6), which is considered as an active component in the process of coagulation and thus for wound healing, has been encapsulated into the microparticles. The encapsulation efficiency and release profile of TRAP-6 in the mPEG-PLGA microparticles have been investigated. In order to achieve a higher degree of sustained release, other polymers with different physicochemical properties have been chosen to prepare nanoparticles by the double emulsion method, which are being developed for efficiently loading the TRAP-6 peptide. The PLGA nanoparticles, which are being optimized with regard to the particle size, encapsulation efficiency, release profile, etc., are prepared for subsequent incorporation into mPEG-PLGA microparticles, which are functional as the active cell scaffolds.

Results and Discussion

So far, these studies have showed that mPEG-PLGA microparticles have been succesfully loaded with 40-60% of TRAP-6. An in vitro study showed good cell adhesion to the freshly prepared mPEG-PLGA microparticles within 36 days, however, the release profile of TRAP-6 indicated a fast burst release occurring within 24 hrs. Various types of nanoparticles have been prepared and optimized to obtain homogenous size distributions in the range 220-300 nm. TRAP-6 loaded nanoparticles are expected to achieve more sustainable release, due to the superior nanostructure as compared to the microparticle structure. Selected batches of nanoparticles will be further incorporated in microparticles.

Conclusion

By optimizing the pharmaceutical formulation processes, nanoparticle-containing microparticles will aid the development of injectable polymeric particulate systems suitable as scaffolds for tissue engineering.

ENGINEERING ACID-RESPONSIVE ARTIFICIAL ENVELOPES AROUND ADENOVIRUS FOR EFFICIENT GENE TRANSFER

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Introduction

Gene therapy involves the delivery of a functional gene by a vector into target cells, resulting in a desired therapeutic effect. Adenovirus (Ad) has shown a great promise in gene therapy [1,2]. However, *in vivo* studies have reported an immunogenic response and an overwhelming accumulation and gene expression in the liver resulting in significant hepatotoxicity. These issues currently inhibit the use of this vector for clinical genetic therapies. Such limitations have been overcome by engineering an artificially enveloped Ad using zwitterionic and cationic lipid formulations [3,4]. However, this resulted in a significant reduction of gene expression *in vitro* due to the poor release of the lipid bilayer enveloped virus from the endosomal compartment that hinders their gene expression capacity. In the present work, we have explored the use of pH-sensitive DOPE:CHEMS (6:4) lipid-envelope to enhance the virus release from the endosome following endocytosis.

Experimental Methods

Artificially Enveloped Ad Preparation
Lipid film hydration technique was used for Ad envelopment. DOPE:CHEMS and DOTAP:Chol lipid films were prepared and hydrated with 10^{10} viral particles/ ml in HEPES pH 8.0 buffer. This was followed by water-bath sonication for 30 min.
Characterization of Enveloped Ad
The surface engineered Ad were characterised by transmission electron microscopy (TEM), atomic force microscopy (AFM), dot blot, dynamic light scattering and zeta potential measurements.
In vitro Transfection and Cellular Traficking Studies
Different cell lines (CAR+ and CAR-) were transfected with Ad (10^8 viral particles/ ml), enveloped Ad in either DOPE:CHEMS or DOTAP:Chol lipid bilayers for 3 h and transgene expression was analyzed by beta-galactosidase (β-gal) assay at 24 h. Confocal laser scanning microscopy (CLSM) was used to track the cellular uptake and endosomal release of Cy3-labelled Ad or enveloped Cy3-Ad vectors in A549 cells.
Intratumoral Enveloped Ad Injection
Nude mice with human cervical adenocarcinoma (C33a) xenografts were intratumorally injected with Ad (10^{10} viral particles/ ml) or enveloped Ad. Levels of transgene expression were analyzed after 24 h.

Results and Discussion

DOPE:CHEMS envelopment exhibited good stability at physiological pH (7.4) but immediately collapsed and released the naked virions at pH 5.5. AFM and TEM were used to elucidate the structure of pH-sensitive enveloped Ad vetors. Dot blotting showed that we can achieve high levels of envelopment efficiencies (>90 %) at high lipid concentrations, such as 10 mM. Furthermore, the gene expression of recombinant Ad encoding for β-gal reporter gene enveloped in DOPE:CHEMS showed high levels of gene expression when tested in different cell lines, comparable to naked Ad. These transfection results were further confirmed by studying the intracellular trafficking of fluorescently-labelled, Cy3-Ad using CLSM. Interestingly, Cy-3 Ad enveloped in DOPE:CHEMS showed a uniform fluorescence distribution within the cytoplasm indicating Ad endosomal release. In addition, pH- sensitive enveloped Ad injected directly into C33a xenografts grown on the flank of nude mice showed same level of gene expression to naked Ad. Thus pH-sensitive envelopes can restore the biological activity of Ad *in vitro* and *in vivo*.

Conclusion

This type of artificially-enveloped Ad offers a promising tool in gene delivery since high level of Ad gene expression can be maintained and expected to dramatically improve the innate Ad immunogenicity and hepatotoxicity *in vivo*.

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ELUCIDATING THE MOLECULAR MECHANISM OF DENDRIPLEX FORMATION: EFFECT OF DENDRIMER GENERATION AND siRNA CONCENTRATION

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Introduction

Efficient delivery systems are highly needed to achieve gene silencing with small interfering RNA (siRNA), since RNA molecules have a short half-life *in vivo*, and their size and charge do not favor cellular uptake. Dendrimers are interesting siRNA carriers due to a well-defined molecular composition, a monodisperse size distribution, and cationic surface groups for multivalent electrostatic interaction with siRNA. Despite the wide application, little is known about the structural characteristics of the dendriplexes in solution.
The aim of the present study was to investigate systemetically the dendriplex formation, elucidating details on the binding between siRNA and poly(amidoamine) (PAMAM) dendrimers with low, intermediate and high charge density.

Experimental Methods

Amino-terminated (PAMAM) dendrimers was used as a model system, and dendriplex formation was performed in 10 mM tris buffer at an amine-to-phosphate ratio (N/P) of 5, 20 and 50, utilizing generation 1, 4 and 7 dendrimers (G1, G4 and G7). The dendrimers have a diameter of 2.2, 4.4 and 8.8 nm, respectively, and a total of 8, 64 and 512 primary amines per molecule for the siRNA interaction. Dendriplex appearance was characterized by dynamic light scattering (DLS) and small angle X-ray scattering (SAXS), and the siRNA protection ability was examined by gel electrophoresis.

Results and Discussion

The G7 and G4 dendrimers were both capable of preventing siRNA migration by electrophoresis, and DLS measurements suggested the presence of larger dendriplexes with an average size of ~150nm with an N/P ratio of 50, which increases slightly at decreasing N/P ratios.
The flexible G1 dendrimer showed no siRNA retardation by gel electrophoresis, and poorly defined structures were observed by DLS, indicating a lack of dendriplex formation.

To obtain adequate statistics for structural information, a final dendrimer concentration of 10mg/mL was chosen for SAXS experiments. The G7 dendriplexes had sperical apearence characteristic for monomeric dendrimers, with an increasing intensity at increasing siRNA concentration. The G7 dendriplex fitted nicely to a sperical model, showing decreased monomer appearance (radius of gyration decreasing from 4.8 nm for dendrimer alone to ~3 nm for dendriplexes) suggesting dendrimer compression upon siRNA binding.
For the G4 dendriplexes, increased structural information was retrieved with increasing amounts of siRNA, indicating a more ordered structure with decreasing N/P ratio, while the information retrieved for G1 dendriplexes were inadequate for further analysis, in acordance with the additional results.

Conclusion

For dendriplex formation to occur, a certain size/charge density of the dendrimer is required. Both G4 and G7 dendrimers were able to prevent siRNA migration, and formed measurable dendriplexes, while G1 dendrimers proved to be inadequate for complexation. The molecular mechanism of siRNA binding was indicated by SAXS measurements for the G7 dendrimer, indicating an increased dendrimer compression, which was dependent on the siRNA concentration.

Preparation and characterization of squalene nanoparticles for small interfering RNA targeted against the Ret/PTC1 oncogene delivery

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Abstract:

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy. The tumour is associated with somatic mutations of Ret proto-oncogene, which is activated by gene rearrangements. The rearranged proto-oncogene, PTC oncogene (Ret/PTC) is the product of the fusion of the tyrosine-kinase domain of the proto-Ret to other genes. The most common variant is Ret/PTC1.

RET/PTC1 rearrangement is the most common genetic alteration identified to date in thyroid papillary carcinomas, it was formed by an intra chromosomic rearrangement which leads to the juxtaposition of the Ret Tyrosine Kinase domain with the genes H4. The fusion Ret/PTC1 oncogene represents an interesting target for small interfering RNA (siRNA) strategies since it is present only in the tumour cells and not in the surrounding normal cells. However, the biological efficacy of the siRNAs is hampered by their short plasmatic half-life due to poor stability in biological fluids and by their low intracellular penetration. In order to protect siRNA from degradation, and to improve their intracellular capture, we have applied the concept of "squalenoylation", ie. consisting in the bioconjugation of a drug substance to squalene, for the delivery of siRNA targeted toward the Ret/PTC1 fusion oncogene.

In this communication, we report the synthesis of siRNA RET/PTC1-Squalene bio-conjugate and their use for nanoparticles preparation. The acyclic isoprenoid chain of squalene has been covalently coupled with siRNA Ret/PTC1 at the 3'-terminus of the sense strand via a stable thioether linkage. The formation of siRNA Ret/PTC1-SQ bio-conjugate was demonstrated by RP-HPLC analysis and was confirmed using MALDI-TOF spectrometer; the molecular weight of siRNA Ret/PTC1-SQ bio-conjugate was 7365 g/mol. The bio-conjugate was obtained in 55 % yield. siRNA Ret/PTC1-SQ bio-conjugate was purified by RP-HPLC and lipophilicity of modified oligonucleotide was investigated by RP-HPLC and showed an enhancement of lipophilicity of the siRNA RET/PTC1. The sense modified sequences and antisense sequences were annealed. Duplex formation was assessed by 4% agarose gel electrophoresis.

The linkage of siRNA Ret/PTC1 to squalene leads to amphiphilic molecule that self-organise in water as siRNA Ret/PTC1-SQ nanoassemblies of 169 nm and Zeta potential of -26.4 mV. The morphology of these nanoassemblies was characterized by observation in Transmission Electronic Microscopy.

Next, the stability of siRNA-SQ Ret/PTC1 nanoparticles in aqueous suspensions was studied, size and zeta potential were monitored for 72 hours and found to be nearly constant over time. Actually, the antitumor activity of the siRNA RET/PTC1-SQ nanoassemblies is under evaluation in human papillary thyroid carcinoma cell line, TPC-1, that expresses the RET/PTC1 oncogene.

GENE SILENCING ACTIVITY OF SIRNA COMPLEXES BASED ON BIODEGRADABLE POLYMERS AND CARBON NANOTUBES

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Introduction

Cationic polymers are used as non-viral vectors for nucleic acid delivery. Due to their positive charge, these polymers are able to complex with anionic nucleic acids to form polyplexes. However, many of these cationic polymers show considerable toxicity both towards in vitro cultured cells and in animal models. The toxicity is attributed to their cationic character, and, additionally, to the fact that, many of the studied polymeric gene/siRNA vectors are non-degradable. In this study, two biodegradable cationic polymers were evaluated for the purpose of siRNA delivery: pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)) and TMC (O-methyl free N,N,N-trimethylated chitosan. Recently, carbon nanotubes (CNTs) have been shown to traverse cellular membranes and shuttle biological molecules, including DNA, siRNA, and proteins, into cancer cells. In this study, the silencing activity and the cellular toxicity of the siRNA complexes based on functionalized CNTs were tested.

Experimental Methods

The silencing activity and the cellular toxicity of complexes based on anti-Luciferase siRNA and either biodegradable polymers or CNTs were tested in H1299 human lung cancer cells expressing firefly luciferase and compared to the silencing activity and the cellular toxicity of siRNA complexes based on non-biodegradable pDMAEMA (poly(2-dimethylamino)ethyl methacrylate) and PEI (polyethylenimine), and the regularly used lipidic transfection agent Lipofectamine. To promote the endosomal escape of the polyplexes based on biodegradable polymers, either the endosomolytic peptide diINF-7 was added to the formulations, or photochemical internalization (PCI) was applied.

Results and discussion

Incubation of H1299 human lung cancer cells expressing firefly luciferase with polyplexes based on pHPMA-MPPM and TMC showed 30-40% silencing efficiency. This silencing activity was equal to or better than that obtained with the standard transfectants. Under all experimental conditions tested, the cytotoxicity of the biodegradable polymers was low. The application of PCI, as well as the addition of the diINF-7 peptide to the formulations increased their silencing activity up to 70-80%. This demonstrates that pHPMA-MPPM- and TMC-based polyplexes benefit substantially from endosomal escape enhancement. Importantly, the polyplexes retained their silencing activity in the presence of serum and they showed low cytotoxicity.
Incubation of H1299 cells with siRNA complexes based on functionalized CNTs showed 20-30% silencing efficiency and a high cytotoxicity. In terms of cytotoxicity and silencing activity, there was no added value of functionalized CNTs over PEI and other established systems.

Conclusion

Biodegradable pHPMA-MPPM and TMC polymers are promising vectors for siRNA delivery and therefore attractive systems for further in vivo evaluations. The functionalized CNTs are highly cytotoxic and not suitable for siRNA delivery.

CATIONIC NANOEMULSIONS FOR DELIVERY OF ANTIMALARIAL OLIGONUCLEOTIDES: ADSORPTION AND *IN VITRO* STUDIES

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Introduction

Malaria is a parasitic disease that affects more than 300 million people in more than 100 countries, mainly in tropical regions. The treatment of malaria has become more complex due to the resistance of the parasites to the antimalarial drugs. A promising strategy based on the antisense oligonucleotides (ON) against the enzyme topoisomerase II of *Plasmodium falciparum* has been considered, given the fact that these single strand nucleic acids can interfere in protein synthesis and inhibit parasite growth. However, the use of these molecules is limited by their low stability and intracellular penetration. As an alternative to these problems, cationic nanoemulsions (NE) have been proposed as delivery systems for this kind of molecules. The aim of this study was to characterize the adsorption of anti-topoisomerase II ON to cationic nanoemulsions, and to study their *in vitro* effects on *P. falciparum* parasites.

Experimental Methods

The NE were produced by spontaneous emulsification procedure and were composed by medium chain triglycerides, egg lecithin, glycerol and MilliQ® water. The cationic lipid (oleylamine or DOTAP) was also present at the cationic ones. Different ON concentrations were added to the emulsion for determination of adsorption isotherms. The systems were characterized before and after ON addition, according to the ζ-potential (electrophoretic mobility), the mean droplet size (photon correlation spectroscopy) and the morphology of the droplets (transmission electron microscopy - TEM).

The hemolytic effect of the NE was evaluated in infected and non infected red blood cells, at different +/- charge ratios. For the *in vitro* evaluation, 10 µM ON solutions or ON/NE complexes were added to early stages parasites, at 0.5% parasitemia at 5% hematocrite. *P. falciparum* 3D7 strain was maintained in O+ human erythrocytes in RPMI 1640 medium supplemented with albumin, 25mM Hepes and 32mM NaHCO3 under continuous culture at 37°C using the candle-jar method. Results were expressed as the percent of parasite growth, compared to the control receiving medium alone.

Results and discussion

The cationic NE showed a positive ζ-potential (> +50 mV) and a mean droplet size of about 200 nm, with a polydispersity index lower than 0.2. After ON addition, no significant differences were detected in size and morphology of the complexes, but an inversion of the zeta potential was evidenced. Moreover, a high electronic density was observed at the interface of NE droplets, suggesting the occurrence of adsorption of ON at the emulsion interface. The ON adsorption isotherms showed a progressive ON adsorption to the nanoemulsions, until it reached a plateau, at higher ON concentration for the double acyl chain cationic lipid DOTAP. The hemolytic effect of the cationic NE was lower than 10% for the +/- charge ratios lower than 4. After 24 hours of treatment, there was a reduction of total parasitemia of approximately 30% for the DOTAP containing ON/NE complexes.

Conclusion

The overall results showed that antisense ON against *P. falciparum* topoisomerase II gene can be efficiently adsorbed to the cationic nanoemulsions, which showed low hemolytic effect, even at final positive charge ratio. Some biological activity could already be observed, showing that these systems are interesting for supplementary studies, which are already in progress.

Session 3

Vaccine delivery

LIPOSOME-BASED CATIONIC ADJUVANTS: INTERACTIONS WITH ANTIGENS

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Introduction

Lipidic particulate delivery systems have been shown to be excellent adjuvants in novel subunit vaccine formulations. Well-known examples are the marketed oil-in-water emulsions MF59™ (Novartis) and AS03 (GlaxoSmithKline) (1). Liposomes are also interesting adjuvants, e.g. the cationic liposomes based on dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose 6,6′-dibehenate (TDB). It is central to understand the interactions between antigen and the lipidic particulate delivery system for the development of new vaccine formulations. The physico-chemical stability of the protein antigen and the association kinetics between antigen and lipidic particulate delivery system are important factors for the stability of the vaccine formulation and potentially also for the induced immune response (2). The aim of the current study was to examine the interactions between DDA/TDB liposomes and the model protein antigen bovine serum albumin (BSA).

Experimental Methods

Dynamic light scattering was used to characterize the size of the DDA/TDB liposomes upon addition of protein. The effect of the protein antigen on the thermotropic phase behaviour of the liposomes was studied by differential scanning calometry (DSC). The structural characteristics of the protein mixed with liposomes were examined by methods such as circular dichroism (CD) and DSC.

Results and discussion

The size and the thermotropic phase behaviour of the DDA/TDB liposomes were altered upon addition of BSA. The size of the liposomes increased with increasing concentration of BSA up to a maximum whereafter further addition of BSA resulted in a decreased size of the liposomes. In addition, the thermotropic phase behaviour of the liposomes was altered upon addition of BSA resulting in a concentration-dependent reduction of the phase transition temperature and loss of the otherwise reversible thermotropic phase behaviour of the liposomes. Structural changes of the protein were observed upon formation of complexes between DDA/TDB liposomes and BSA. The DDA/TDB liposomes caused partial unfolding of BSA as evident by a reduction in the α-helical content. Our results suggest that the underlying mechanism(s) for the observed complexation between DDA/TDB liposomes and BSA is largely based on electrostatic interactions.

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IMPROVING ANTI-TUMOR RESPONSES BY DC-SIGN MEDIATED ANTIGEN-TARGETING USING GLYCAN MODIFIED LIPOSOMES: A POTENTIAL ANTICANCER VACCINE

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Introduction

Dendritic cells (DC) are key antigen presenting cells that have the unique ability to cross present tumor-derived antigens on MHC class I, resulting in effective priming of cytotoxic T lymphocytes (CTL). DC express C-type lectin receptors that bind and subsequently mediate the uptake of carbohydrate structures appended to glycoproteins. Here we have explored the specific expression of the C-type lectin receptor, DC-SIGN to specifically target antigens to DC, to improve anti-tumor responses.

Experimental Methods

Liposome preparation
Glycan-modified stealth and non stealth liposomes containing ovalbumin (OVA) as the model antigen were prepared from a mixture phospholipids and utilizing the thin film hydration method. DID was used a fluorescent marker for the lipid bilayer. The lipid film was hydrated in a buffer solution containing OVA. The liposomes were sized by extrusion till a size of about 200 nm was obtained. Lewis B, the glycans were coupled with these liposomes through the maleimide group for stealth liposomes and phosphoethanolamine (PE) group for non stealth liposomes
Enzyme linked immunosorbant assay (ELISA)
The linkage of the glycans to the liposomes was confirmed in an ELISA by staining the liposomes with anti-Lewis^B antibodies and correct orientation was assessed using DC-SIGN-Fc molecules.
Binding studies
Specific binding and uptake of glycan-modified liposomes to DC-SIGN was assessed by measuring the mean fluorescence of DiD upon incubation of bone marrow derived dendritic cells (BMDCs) with graded numbers of liposomes at 4 and 37^{0}C.
Proliferation assays
To assess the ability of liposomes to deliver the antigen specifically to DCs via DC-SIGN and further leading to presentation via the MHC class I and class II pathways, transgenic (Tg) BMDCs and control non-transgenic (Wt) BMDCs were incubated with graded numbers of liposomes. Four hours later, OVA-specific T cells were added and 72 h later, T cell proliferation was quantified by 3H-thymidine incorporation.

Results and discussion

Both the stealth and non stealth liposomes showed conjugation of ligand on the liposome surface in ELISA when stained with anti-Lewis^B antibodies. However the stealth liposomes failed to show any signal in the ELISA after staining with DC-SIGN-Fc suggesting improper conjugation or unavailability of the ligands for binding to DC-SIGN. In the binding studies the non stealth liposomes showed 8 fold higher binding to BMDCs compared to the stealth liposomes. The binding was DC-SIGN mediated as it could be blocked by addition of high-affinity ligand, mannan or the Ca^{2+}-chelator, EGTA. In the proliferation assays only glycan-modified, but not control-nonmodified, non stealth liposomes liposomes were able to enhance both CD4 and CD8 T cell proliferation.

Conclusion

Our results demonstrate that DC-SIGN targeted glycan modified liposomes could be used for the efficient induction of anti-cancer immune responses and serve a potential anticancer vaccine.

A SYNTHETIC MYCOBACTERIAL MONOMYCOLOYL GLYCEROL ANALOGUE STABILIZES DDA LIPOSOMES AND POTENTIATES THEIR ADJUVANT EFFECT *IN VIVO*

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Introduction

Cationic liposomes are promising delivery vehicles for vaccine antigens and can in combination with immunopotentiators act as potent adjuvants via various mechanisms [1]. Immunopotentiators can be extracted from microorganisms such as mycobacterium species (e.g. *Mycobacterium tuberculosis),* which constitute some of the most immunostimulatory microorganisms. Several compounds from the mycobacterial cell wall, including lipids, are involved in host cell activation. The mycobacterial glycolipid monomycoloyl glycerol extracted from *Mycobacterium bovis* (bacillus Calmette-Guérin) has been shown to be a potent stimulator of the immune system [2]. A synthetic analogue based on 32 carbon atoms (MMG) was found to exhibit comparable immunostimulatory activity. The use of synthetic analogues of mycobacterial lipids in combination with cationic liposomes as vaccine adjuvants provides a promising strategy for exploiting the immunostimulatory activity of mycobacterial cell wall components but without associated toxicity issues. Despite the promising adjuvant activity of MMG, little is known about the biophysical and pharmaceutical properties of MMG-based adjuvants. Thus, we re-synthesized MMG based on 32 carbon atoms and incorporated it into the bilayer of cationic dimethyldioctadecylammonium (DDA) liposomes. In the present study, we provide a thorough pharmaceutical characterization of DDA/MMG liposomes and investigate the immunological consequences of incorporating MMG into the bilayer of DDA liposomes.

Results and discussion

DDA/MMG liposomes were prepared by the thin film method [3]. Incorporation of MMG into DDA liposomes resulted in liposomes with a polydisperse size distribution with an average particle size of approximately 400 nm. The surface charge of the vesicles was not affected by the inclusion of MMG due to its lack of ionizable functionalities. DDA liposomes are unstable in suspension and aggregates within a few weeks [3], and it was therefore investigated whether MMG provides a stabilizing effect on DDA liposomes. Evaluation of the stability of DDA/MMG liposomes indicated that MMG stabilizes the liposomes since their average particle size remained almost constant for up to six months at 4 °C and 25 °C after incorporation of at least 18% MMG. This stabilization of the otherwise unstable DDA liposomes is probably attributed to increased hydration of the lipid membrane as incorporation of MMG into the bilayer enables hydrogen bond formation between the liposomes and the solvent. This was investigated further by obtaining surface pressure/area isotherms of monolayers of DDA and DDA/MMG using the Langmuir-Blodgett technique. The results indicated an increased surface pressure in the presence of MMG when the DDA/MMG monolayer is fully compressed indicating a stronger interaction between the glycerol head group of MMG and water than between the quaternary ammonium head group of DDA and water. Thus, incorporation of MMG into the lipid bilayer of DDA liposomes increases the hydration of the membrane and prevents dehydration of the quaternary ammonium head groups, which may account for the improved colloidal stability of the liposomes. Immunization of mice with the tuberculosis fusion protein Ag85B-ESAT-6 and the DDA/MMG liposomal adjuvants induced antigen-specific IFN-γ responses. The results indicated that incorporation of MMG increased the adjuvant efficacy of DDA liposomes in a concentration-dependent manner.

Conclusion

This study demonstrates that incorporation of MMG into the bilayer of DDA liposomes increases the colloidal stability of the liposomes by enabling hydrogen bond formation between the liposomes and the solvent. In addition, *in vivo* experiments showed that MMG potentiates the adjuvant efficacy of DDA liposomes.

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Session 4

Local routes of administration

SILVER-NANOLIPID COMPLEX (S-NLC): ANTIINFLAMMATORY ACTIVITY IN MURINE MODEL OF ATOPIC DERMATITIS

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Introduction

Recently, the combination of microsilver and Nanostructured Lipid Carriers (s-NLC) was found to be active against atopic dermatitis. The mechanism of action is not yet elucidated. However the action seems to be synergistically.
Thus, the aim of the study was to prove or disprove this estimation in vivo.

Experimental Methods

The *in vivo* efficacy was evaluated using a hapten-induced murine animal model of atopic dermatitis (AD). AD in mice was induced by sensitizing the mice on day zero by applying 2,4-dinitro-1-fluorobenzene (DNFB) as hapten on the shaved back of the animal. This hapten is taken up and processed by epidermal Langerhans cells and presented to T cells in the draining lymph nodes. Re-challenge with the same hapten results in the recruitment of hapten-specific T cells, as a result there is upregulation of cytokines, chemokines and other inflammatory cells in the dermatitis skin.
Mice were challenged on day 5 by application of 0.2% DNFB on both sides of the ear and were rechallenged after two days to get the extensive disease. Thereafter the hapten was applied every other day for 2 more weeks to produce the chronic disease. Once the disease was developed the animals were treated with the test formulations. The weighed amount (100 mg) of formulations were applied daily to the ears and the dermatitis response was determined by the degree of ear swelling using a digital micrometer. Furthermore the degree of erythema was scored on a scale of 0-4. Histology of mice ears was performed at the end of the study.

Results and discussion

Ear thickness
Mice were treated in five groups. Group 1 was the positive, non treated group, group two received the cream base, group 3 received the cream base with NLC, group 4 received the cream base and silver and finally group 5 received the sNLC within the cream base. No significant differences in ear thickness were obtained for the non-treated group and the groups who received the cream base alone or the cream base with NLC only. However, the response to the challenge during the study was still much weaker, e.g. less inflammation when compared to the untreated control. Significant differences in inflammation response were found for the formulations containing silver. The earthicknes of the group treated with the formulation cream base, silver, no NLC reduced the ear thickness by only 49% compared to the control. The s-NLC formulation could reduce the earthickness by 63%.

Reduction in erythema
The reduction in erythema observed in the groups treated with cream base and cream base containing NLC only was not significant. However group 4 (base and silver) and group 5 (base and sNLC) showed a significant reduction in erythema when compared to the positive control. However no difference in erythema reduction was found for the two formulations between each other. In the murine mouse model silver acts against atopic dermatitis, whereas the application of NLC does not. The combination of NLC to silver leads to the formation of sNLC, which was found to be the most effective formulation to reduce erythema.

Conclusion

The combination of microsilver and NLC leads to a synergistic skin healing effect against atopic dermatitis in vivo. Therefore s-NLC have the potential to be a novel and effective therapy regime for the treatment of atopic dermatitis.

Penetration of Gel and Liquid state Liposomes into Barrier-Impaired Skin

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Introduction

Many dermal diseases are characterized by major changes in skin barrier function, making it challenging to deliver drugs into specific layers of diseased skin in a reproducible way. Liposomes can be used to increase the delivery of drugs into the skin upon dermal administration, but to date the mechanism is not fully understood. The drug delivery properties of liposomes are affected by physico-chemical characteristics of the lipid bilayer, and hence the lipid composition is decisive for how the vesicles interact with the skin barrier. The purpose of this study was to elucidate how the fluidity of the liposomal membrane bilayer affected their interaction with intact and diseased skin.

Experimental Methods

Liposome preparation
Unilamellar liposomes composed of binary mixtures of dipalmitoylphosphatidylcholine (DPPC) and dilauroylphosphatidylcholine (DLPC) were prepared by the thin film method, followed by extrusion through 100 nm filters. The thermotropic phase behaviour was determined by differential scanning calorimetry. From these data, two membrane compositions were chosen for the skin penetration studies: A gel state composition (T_m = 39 ºC) with 90% DPPC and 10% DLPC, and a liquid state composition (T_m= 26 ºC) with 40% DPPC and 60% DLPC using ^{14}C-labelled DPPC as a marker.

Skin penetration
The penetration into pig skin was examined using a Franz diffusion cell model. Diseased skin was simulated by an impaired-barrier, which was prepared by tape stripping, removing most of the stratum corneum. For intact skin, a slight increase in the lipid penetration from liquid state liposomes was detected in all skin layers, compared to gel state liposomes.

Results and discussion

For both formulations the penetration into barrier-impaired skin was much more efficient than the penetration into intact skin. Importantly, a very large increase in lipid accumulation in the epidermis was detected in barrier-impaired skin. In addition, penetration into barrier-impaired skin was also slightly increased from liquid state liposomes, compared to gel state liposomes. The amount of lipid permeated through the barrier-impaired skin into the receptor fluid from liquid state liposomes was also enhanced, compared to gel state liposomes.

Conclusion

These results suggest that the membrane fluidity affects the skin penetration of liposomes, whether these are applied onto intact or barrier-impaired skin. Consequently, adjusting lipid composition to control skin penetration appears effective on intact skin as well as on diseased skin, where the skin barrier is distorted.

LIPID NANOPARTICLES FOR SYNERGISTIC SKIN PHOTOPROTECTION

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Introduction

In order to develop a sunscreen product with improved sun protection factor (SPF) and, at the same time, reduce the total amount of the active compounds needed, nanostructured lipid carriers (NLC) were used. NLC can host lipophilic molecules, thus three regulatory approved lipophilc UV filters were used for this study, namely bis-ethylhexyloxyphenol metoxyphenyl triazine, ethylhexyl triazone, and octyl methoxycinnamate. The ability of nine different solid lipids to yield with stable particles was assessed. After the production, optimised NLC were incorporated in semi-solid formulations and *in vitro* SPF was measured, comparing the values of the NLC based products among them and with a traditional emulsion.

Experimental Methods

NLC preparation and characterisation

UV filters loaded NLC were prepared by hot high pressure homogenisation technique. Coarse emulsions were produced by high shear mixing of the melted lipid phase, containing the three UV filters, with the water phase, containing a surfactant (decyl glucoside). The process temperature was kept 10 degrees higher than the melting point of the lipid used. Two cycles of homogenisation applying a pressure of 800 bars gave best results in terms of particle size and polydispersity (PI). All the NLC dispersions contained 20% of lipid phase. A reference nanoemulsion was prepared following the same procedure. Solid state of the lipid matrix, in case of NLC, was assessed by differential scanning calorimetry (DSC), the size was assessed by photon correlation spectroscopy (PCS), and the morphological characteristics of the particles by scanning electron microscope (SEM). In order to pre-select a master formulation to be further analysed, all the stable formulations were scanned within ultraviolet (UV) range.

NLC incorporation into semi-solid formulations and SPF assessment

Selected NLC suspensions and the reference nanoemulsion were incorporated into an amphyphilic cream by means of a high speed stirrer, in order to obtain uniform smooth dispersions. The particle stability within the semi-solid medium was confirmed by DSC. The SPF measurements were performed according to the COLIPA method, measuring the diffuse transmittance as function of wavelength in the UV spectrum.

Results and discussion

All the nine produced NLC formulations had PCS average particle size lower than 200 nm and a PI within 0.2, at the day of production. One month after production and storage at three different temperatures (4°, 25° and 40°C), four NLC dispersions showed good stability in terms of particle size and PI, with almost unchanged parameters. By means of DSC, a melting event with an onset above 40°C was observed for all the formulations, indicating that the NLC formulations are suitable for topical application. By means of SEM, spherical particles with diameter in accordance with PCS data were observed. From the UV scans performed with the four stable NLC formulations and the reference nanoemulsion containing the same amount of the three organic UV filters, noticeable differences could be observed. The highest absorbance was measured for carnauba wax based NLC, followed by bees wax based NLC and the reference nanoemulsion. Other two NLC dispersions showed significantly lower UV absorbance. This difference may be due to different structures of the particles formed by these lipids. The carnauba wax based NLC would correspond to a shell enriched model that is formed when the lipid is solidifying faster than the active components. The bees wax based NLC would correspond to a matrix type particles, while the other two formulations would possess core enriched structure, with the most of the lipid concentrated in the outer part of the particle, preventing the contained organic UV filters from interacting with the UV rays. In case of the nanoemulsion, the UV absorbance is due just to the filtering by organic UV filters. The higher absorbance values obtained for carnauba and partly by bees wax based NLC are a consequence of a synergistic effect of both filtering and scattering of UV rays, the latter due to the solid matrix of the particles. Based on these findings, it was decided to further develop formulations based exclusively on bees wax and carnauba wax. These two formulations were incorporated in the semi-solid product. The DSC runs confirmed the solid state of the particles, with unchanged melting onset temperatures. From the SPF measurements, the superiority of carnauba wax based formulation was confirmed, having and SPF value of 20.19, compared to bees wax and nanoemulsion based formulations with SPF values of 14.13 and 13.76, respectively. Although SPF values cannot be predicted from simple UV scans, a certain degree of analogy between the two groups of data was observed.

Conclusion

This study demonstrated that approximately 45% higher SPF values could be obtained when the organic UV filters were incorporated into nanostructured lipid carriers. The final SPF of the formulation is a result of a synergistic effect of the solid nature of the lipid particles and the presence of organic UV filters that can be achieved by choosing an appropriate solid lipid.

TOXICOLOGICAL EFFECTS OF LUNG EXPOSITION TO BIODEGRADABLE NANOPARTICLES

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Introduction

Pulmonary administration of therapeutic molecules has received a great deal of attention during the past decade. The lungs are an attractive target for non-invasive delivery of many molecules, including fragile and poorly adsorbed drugs, with advantages for both systemic and local applications. The use of nanoparticles (NPs) have attracted interest due to their bioavailability in the lungs, their high retention *in situ* and their low capture by alveolar macrophages when the NPs diameter is lower than 250 nm. Nevertheless, very few data are available about safety and toxicity of biodegradable NPs after inhalation and there is the need to carry out exhaustive studies to assess all the potential risks of their exposure.

In this study, we have prepared biodegradable poly(D,L-lactide-*co*-glycolide) NPs with different surface properties. To investigate the toxicity, internalization and translocation of NPs after pulmonary delivery, we employed an *in vitro* model of the pulmonary epithelium. We used the Calu-3 cell line. This cell line shows the unique property to form confluent monolayers and is characterized by the production of mucus.

Experimental Methods

Nanoparticle preparation

PLGA NPs were prepared by a solvent emulsion evaporaton technique. Uncoated NPs have been modified to obtain cationic and anionic NPs using chitosan and Pluronic F68® respectively. Rhodamine has been introduced in the formulation to obtain fluorescent NPs. PLGA NPs chemically modified with the fluorescent dye (Rhod-NPs) were prepared using PLGA conjugated to Rhodamine.

Cell culture

Calu-3 cells were cultured in 60 cm^2 dishes using 10mL medium and maintained in a humidified, 5% CO2 incubator at 37°C. Cell culture medium was DMEM, supplemented with 10% fetal calf serum and PenStrept® antibiotic solution. For studies of epithelial barrier permeability and uptake Calu-3 cells were seeded on polyester Transwell® cell culture inserts at a density of 3 x 10^5 cells / cm^2. After one day, the medium was removed from the apical compartment allowing Calu-3 to grow at an air-liquid interface.

Cytotoxicity was determined by the MTT assay. The influence of biodegradable NPs on the integrity of cell monolayers was assessed by measuring the transepithelial electrical resistance (TEER).

The study of cellular uptake has been performed incubating cell monolayers with Rhod-NPs at different times. The cellular penetration has been assessed measuring fluorescence intensities by flow cytometry and confocal microscopy. Influence of NPs on inflammatory response, after incubation of cells for different intervals of time, has been investigated by measuring the concentrations of cytokines IL-8 and IL-6 in culture supernatants using the ELISA test. LPS 10µg/ml was used as positive control.

Results and discussion

PLGA NPs with different surface properties have been designed and characterized. Uncoated NPs showed a zeta potential close to neutrality (-4mV), while the use of chitosan and Pluronic® led respectively to positively (+30mV) and negatively (-20mV) charged NPs.

Calu-3 cells were used as a model for pulmonary epithelium. To assess levels of toxicity of NPs an MTT colorimetric assay has been performed. After 72h of exposure to the NPs, the cell viability, even at the highest concentration tested, was higher than 50%. Similar results were obtained both with blank and Rhod-NPs. Incubation of cell monolayers with NPs PVA and NPs Pluronic® doesn't cause any change in the epithelial layer barrier function as compared to control. NPs Chitosan cause a transient effect on tight junctions during the first three hours of incubation: after a reduction, the TEER value returned to baseline levels and increased throughout the following hours. Fluorescence intensity signal increased after incubation of cells with Rhod-NPs compared to non treated cells and demonstrate that nanoparticles can be taken up by Calu-3 with low toxicity. Calu-3 cells secrete a constitutive level of cytokines: stimulation with LPS and NPs led to an increase of cytokines secretion with a maximum output detected at 24h. NPs-induced release was always lower than that achieved with LPS incubation.

Conclusion

This study demonstrated that the Calu-3 lung model provides an interesting methodological platform to better understand the *in vitro* fate of NPs after inhalation.

A NOVEL DRUG CARRIER FOR THE DELIVERY OF CYCLOSPORINE A TO THE EYE

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Introduction

In ophthalmic therapy topical instillation is the mainly used route for drug administration. However, the bioavailability of drugs administered in this way is typically very poor, due to the presence of many protective mechanisms. Biodegradable hexyl-substituted poly(lactides) (hexPLA) are novel polymers[1], which in combination with methoxy polyethylenglycole (MPEG), form diblock copolymers (MPEG-hexPLA), which self-assemble in aqueous environment into micelles and efficiently incorporate poorly water soluble drugs [2]. Here we present the delivery of Cyclosporine A (CsA) into and through the cornea using MPEG-hexPLA micelles as an effective drug carrier for ophthalmic applications such as Dry Eye treatment.

Experimental Methods

Micelle preparation and characterisation
Micelles were prepared by a solvent evaporation method with a copolymer concentration of 3 mg/mL and a CsA concentration of 0.5 mg/mL, and were characterised in terms of size, morphology and drug loading by DLS, TEM and HPLC, respectively.

Toxicity studies
Cytotoxicity test and indirect immunofluorescence were carried out on Human Corneal Epithelial cell (HCE). Unloaded and CsA loaded micelles with different copolymer concentrations were compared. The ocular tolerance was assessed by Confocal Laser Scanning Ophthalmoscopy (CLSO) on rabbit eyes.

Corneal penetration study
Corneal penetration was studied on rat eyes using CsA/Nile Red loaded micelles. After topical instillation, the corneas were flat-mounted and analysed by fluorescence microscopy (FM and CLSM).

Precorneal kinetics study
CsA/MPEG-hexPLA micelle kinetics were determined in lachrymal fluid on rabbit eyes. The results were compared to a commercial CsA formulation.

Results and Discussion

CsA loaded micelles could be prepared with different defined drug concentrations. The prepared micelle formulations were perfectly transparent, with a particle hydrodynamic diameter of around 50 nm.
Cell viability on HCE was evaluated by MTT tests. MPEG-hexPLA polymeric micelles did neither show any toxicity up to 3 mg/mL of copolymer, nor when loaded with the therapeutic level of CsA, 0.5 mg/mL. The ocular tolerance was evaluated by CLSO. Superficial keratitis involved less than 7% of the total corneal area, indicating that the formulations were very well tolerated after topical application on the eye.
The corneal penetration was studied *in vivo* with formulations containing CsA and Nile Red after topical instillation. The results showed that MPEG-hexPLA micelles were able to penetrate the cornea, accumulating in the epithelium and in the stroma. Fluorescent micelles were also observed in the endothelium, proving that the drug carrier micelles are able to reach into all corneal structures.
The precorneal kinetics study revealed a sustained release of CsA, showing that these micelles could work like a drug depot. Such formulations could significantly decrease the number of administrations needed for reaching the therapeutic level of the drug in the eye.

Conclusion

Polymeric micelles based on MPEG-hexPLA have a high capacity to formulate hydrophobic drugs. Their nanosize allows the penetration and the transportation of drugs through the cornea for reaching the anterior segment. CsA/MPEG-hexPLA micelle formulations are very promising novel drug carrier for ophthalmic applications.

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APIGENIN SMARTCRYSTALS FOR NOVEL UV SKIN PROTECTION FORMULATIONS

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Introduction

At the beginning of the 1990s the drug nanocrystals were developed as more efficient approach to increase drug solubility and dissolution velocity. Nanosuspensions exhibit an enhanced penetration characteristic in skin due to their nanoparticle size range. SmartCrystals as second generation of the drug nanocrystals differ in their physicochemical properties. The production has been optimised by introducing modifications to the high pressure homoginization process, i.e. combination technology (CT) [1].

The toxic effects of UV from natural sunlight and therapeutic artificial lamps are a major concern for human health. The major acute effects of UV irradiation on normal human skin comprise sunburn inflammation (erythema), tanning, and local or systemic immunosuppression.

Scientific research has confirmed a wide influence of flavonoid compounds on various cell layers of the skin (e.g. antioxidant, antiallergic and anti-inflammatory effects) [2]. However, the limiting factor of the use of flavonoids is their low water solubility. In order to overcome the formulation problem associated to solubilty, flavonoids can be rendered as nanosuspensions [3]. The aim of this study was to investigate production method for preparation of Apigenin smartCrystals™ using the CT with improved characteristics.

Experimental Methods

Apigenin 5% (w/w) was dispersed by high shear mixing in a 1% (w/w) surfactant solution. The obtained macro-suspension was then milled using the pearl mill Bühler PML-2 (Bühler AG, Switzerland). Milling was carried out discontinuously for seven consecutive cycles. Subsequently, the nanosuspensions were passed through an Avestin C50 (Avestin Europe GmbH, Germany) applying 300 bar, one cycle. Sampling was done at the end of each cycle and analyzed for particle size. Particle size analysis was performed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK) and by laser diffractometry (LD) using a Mastersizer 2000 (Malvern Instruments, UK). Antioxidant capacity was tested using DPPH (1-1 diphenylpicryl-hydrazyle) radical scavenging method.

Results and discussion

Buff coloured Apigenin nanosuspensions were successfully prepared using the CT. Particle size reduction was observed after each milling cycle. Distinct reduction was noticed during the first 5 cycles reaching a PCS diameter of 446 nm and polydispersity index (PdI) of 0.313 which almost remained unchanged after the 6th cycle ending up with z-average (PCS) of 440 nm and a PdI of 0.265. The LD data showed a similar pattern of particle size reduction starting from d(v)99% of 192 µm in raw suspension to 0.565 µm after 5 cycles followed by a minimal reduction in particle size till 0.547 µm after 7 cycles. Subsequent homogenization using Avestin C50 not only further decreased the PCS diameter and d(v)99% to 413 nm and 0.515 µm, respectively, but also aided breaking of possible remaining population of bigger particles giving more homogenized nanosuspension with lower PdI value of 0.202.

As seen from storage data the narrow dispersed nanocrystals from the combination technology are more stable. The antioxidant activity of Apigenin nanocrystals were almost 2 times higher compared to the antioxidant capacity of the original suspension. Furthermore, the developed smartCrystals can be easily incorporated into a gel, which makes Apigenin available for dermal application.

Conclusion

The obtained small particle size with low PdI indicates that the Apigenin crystals are relatively soft. The combination technology can produce smaller nanocrystals (smartCrystals) with improved physical characteristics than nanocrystals produced with the pearl mill alone. Reducing the size to the nanodimension increase the antioxidant effect distinctly (close to factor 2)

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Session 5

Inflammatory diseases

LIPOSOMAL TARGETING IN INFLAMMATORY DISEASE: MACROPHAGES, FRIENDS OR FOES?

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Introduction

In the recent past, liposome-encapsulated corticosteroids have shown some strong beneficial effects in inflammatory diseases, such as arthritis and cancer. Since liposomes and other nanocarriers are mainly phagocytised by macrophages or macrophage-like cells, it is likely that these cells play a pivotal role in the route of effect of drug delivery systems. Although still not completely elucidated, the efficacy of corticosteroid-loaded liposomes is likely due to a direct effect of the drug on the macrophage by downregulating its inflammatory state through glucocorticoid-receptor mediated interference with the NF-κB pathway. In the present study we tested the efficacy of dexamethasone loaded liposomes in animal models multiple sclerosis and Crohn's Disease, illustrating the effect of liposomal targeting on macrophage activity and its possible consequences in inflammatory diseases.

Experimental Methods

Liposome preparation

Empty and dexamethasone phosphate (DXP) loaded PEGylated DPPC/Cholesterol liposomes were prepared by the thin film hydration and extrusion method. Subsequent dialysis against PBS ensured that no free drug was present in the liposomal dispersions.

Experimental Autoimmune Encephalomyelitis (EAE) model for Multiple Sclerosis

SJL female mice were immunized in each flank by s.c. injection of proteolipid protein in Complete Freund's Adjuvant (CFA) containing mycobacterium tuberculosis (day=0), followed by pertussis toxin by i.p. injection. Animals were treated at the first signs of disease (score 1) with a single dose of 10 mg/kg DXP (free or liposomal) or liposomal PBS intravenously. Clinical signs were monitored daily in each group of treatment in a blind fashion using a scale of clinical score ranging from 0 to 5.

Dextran Sodium Sulfate (DSS) model for Crohn's Disease

Chronic experimental colitis was induced in female CL57BL/6 mice: for 5 days they received 1.5% dextran sodium sulfate (DSS) in their drinking water, followed by 10 days of normal drinking water. This cycle was repeated three times. Mice drinking tap water were used as control. During the third cycle, all mice were treated with either PBS, DXP (5 mg/kg), liposomal PBS or liposomal DXP (5 mg/kg, 10 mg/kg or 20 mg/kg). All mice were weighed and scored for their stool consistency and hematochezia on a daily basis, using a score ranging from 0 to 4.

Results and discussion

Experimental Autoimmune Encephalomyelitis model

All mice developed clinical EAE between day 11 and 16 after induction. Mice treated with liposomal DXP showed a lower maximal clinical score when compared to carrier treated control animals (1.6 vs 2.35, $p<0.001$). Free DXP could not show a decrease in such extent (average maximal score 2.1). Liposomal DXP treated animals were during the experiment more days in complete remission and had a lower mean total disease load in comparison to liposomal PBS and DXP treated animals.

Dextran Sodium Sulfate model

The induction of colitis was successful in all mice receiving cycles of DSS and water. After three cycles of DSS, all animals had a combined score for stool and hematochezia between 2 and 3. Treatment with either free DXP (5 mg/kg) or liposomal DXP (5, 10 or 20 mg/kg) did not show reduction of DSS induced colitis in mice. In fact, liposomal DXP increased hematochezia during the first two days, indicating aggravation of the disease.

Discussion

Based on the presented data, we hypothesize that downregulating macrophage activity plays an important role in the (in-)efficacy of corticosteroid-based drug delivery systems. In recent years, there is increasing evidence that the macrophage plays a different role in different physiological and pathological situations. On the one hand, microglia-derived macrophages are part of the pathogenesis of multiple sclerosis: they recognize myelin as a 'foreign' protein and induce an inflammatory response, recruiting blood-borne macrophages and leucocytes. On the other hand, the macrophage plays a defensive and inflammation-suppressing role in physiological intestinal homeostasis. When they fail to fulfil this role, such as in Crohn's Disease, then invading microorganisms are able provoke a, mostly adaptive, immune response, which leads to severe intestinal inflammation. It is therefore important to consider the role the macrophages play in the choice of applying anti-inflammatory drug delivery systems for inflammatory diseases.

Conclusion

This study showed the difference in efficacy of corticosteroid loaded liposomes in animal models for multiple sclerosis and Crohn's Disease, indicating a complex and contrasting role of the macrophage in both diseases.

SYSTEMICALLY DELIVERED GLUCOCORTICOID LIPOSOMES INHIBIT MACROPHAGE MEDIATED CARTILAGE DESTRUCTION DURING EXPERIMENTAL ARTHRITIS

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Introduction

Systemic delivery of pegylated 'stealth' liposomes selectively target macrophages in the inflamed joint during experimental arthritis. Macrophages become activated during inflammation and produce proteases like matrix metalloproteinases (MMP's) and aggrecanases (ADAMTS) which cause cartilage destruction. Also, they release cytokines, e.g. IL-1β, that can stimulate chondrocytes to produces MMP's and ADAMTS. By systemic delivery of liposomally encapsulated anti-inflammatory drugs, like glucocorticoids, macrophage mediated cartilage destruction may be efficiently inhibited.

Objective

The aim of this study was to compare the systemic treatment of liposomally encapsulated prednisolone phosphate (PLP) versus free PLP on cartilage destruction during experimental arthritis.

Experimental Methods

Mice with established antigen-induced arthritis were treated with either a single injection of 10 mg/kg liposomal PLP, 10 mg/kg free PLP, 4 repeated daily injections of 10 mg/kg free PLP, empty liposomes or saline. Inflammation of the knee joints was measured by ^{99M}Tc-uptake. At 5 days after treatment, the mice were sacrificed for histology and biochemical analysis of the knee joints. Cartilage destruction was determined by immunostaining against NITEGE, a neoepitope indicative for early cartilage destruction by aggrecanases activity. The effect of liposomal PLP on gene expression in macrophage and chondrocyte cell lines was studied by quantitative RT-PCR.

Results and discussion

A single intravenous injection with liposomal PLP strongly suppressed the inflammatory infiltrate in the synovium of the knee joint (71% suppression compared to PBS treatment, whereas the same dose of free PLP did not reach a significant effect). This was reflected in a strong suppression of gene expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in the synovium. Liposomal PLP also strongly (>8 fold) suppressed gene expression of MMP 3, 9 and 14 and ADAMTS5 in the inflamed synovium. Moreover, immunostaining for NITEGE in the cartilage matrix was reduced by 49% with liposomal PLP compared to PBS treatment, indicating that liposomal PLP not only suppresses inflammation in the joint, but also the development of cartilage destruction.

In vitro studies showed that liposomal PLP strongly suppressed cytokine, MMP and aggrecanase production by activated macrophages. Moreover, the supernatant of these macrophages was significantly less capable in stimulating chondrocytes to produce MMP 3, 9 and 14 and ADAMTS5, than the supernatant of activated macrophages. This demonstrates that liposomal targeting of macrophages with glucocorticoids inhibits their activation, and thus also that of chondrocytes, resulting in less inflammation and cartilage destruction.

Conclusion

Systemic liposomal delivery of glucocorticoids to macrophages during experimental arthritis strongly inhibits the development of cartilage destruction in the joint.

P38 MAPK INHIBITOR-LOADED PARTICLES FOR THE INTRA-ARTICULAR TREATMENT OF OSTEOARTHRITIS: FORMULATION AND IN VITRO ACTIVITY

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Introduction

Osteoarthritis (OA) is a rheumatic disease characterized by cartilage degeneration, osteophyte formation and pain. Up to now, no curative treatment exists. The management of pain is carried out by oral or systemic administration of analgesics and non steroidal anti-inflammatory drugs. Because of side effects and incomplete effectiveness, these treatments are suboptimal. The intra-articular administration of drugs may limit the occurrence of adverse events and provide high active ingredients concentrations for prolonged periods of time at the site of action.
The imbalance between anabolic and catabolic processes involved in OA leads to an important release of cytokines by the inflamed synovium and activated articular chondrocytes. Thus, MAPK inhibitors, which act on an important pathway of cytokines, show promise for the treatment of OA.
The aim of the present work was to formulate nano- and microparticles loaded with VX-745 (a p38 MAPK inhibitor) with various sizes and to test their *in vitro* activity on human synoviocytes.

Experimental methods

Formulation and characterization of particles:
Nano- and microparticles were produced by a solvent evaporation method. In brief, a solution of poly(lactic-co-glycolic acid) and of VX-745 in dichloromethane was emulsified in an aqueous solution of poly(vinyl)alcohol at different stirring rates and for various durations. Thereafter, the solvent was evaporated for 3 h and particles were washed 3 times by centrifugation and finally freeze-dried for 36 h.
The particles were characterized by laser diffraction, dynamic light scattering, scanning electron microscopy and reverse-phase HPLC.
In vitro activity studies:
Nano- and microparticles were incubated with subconfluent human type B synoviocytes from patients suffering from OA who underwent joint arthroplasty. After 24 and 72 h of activation with IL-1β, supernatants were collected and inhibition of IL-6 biosynthesis was determined by ELISA (eBioscience, San Diego,CA).

Results and discussion

We have produced blank and VX-745-loaded particles ranging from 300 nm up to 25 μm by using the solvent evaporation method. In the initial experiments, a drug encapsulation efficiency of 70% was obtained. The scanning electron microscopy studies revealed that nano- and microparticles were spherical and had a smooth surface.
The *in vitro* experiments on synoviocytes showed that IL-6 production was not induced by drug-free particles. Twenty-four hours after IL-1 activation, in the absence of drug, the production of IL-6 was of 10600 pg/mL. An inhibition of IL-6 production was observed for VX-745-loaded nanoparticles in a dose-dependent pattern. For instance, the release of VX-745 from nanoparticles at 100 nM inhibited the IL-6 down to 5100 pg/mL (52% of control experiment) whereas the IL-6 concentration was of 3100 pg/mL for a drug concentration of 800 nM (71% of control experiment).
The extended drug release properties of the particles were also observed after 72 h incubation on cells. The release of the p38 MAPK inhibitor from nanoparticles at 400 nM inhibited the IL-6 from 4400 pg/mL at 24 h down to 2400 pg/mL at 72h. Microparticles with the mean size of 25 μm released less rapidly VX-745 than nanoparticles due to their smaller specific surface area but still had a significant activity.

Conclusion

Based on the preliminary *in vitro* studies, VX-745-loaded nano- and microparticles display extended release properties and inhibit significantly the production of IL-6 from human synoviocytes. Further *in vitro* and *in vivo* trials are required to demonstrate the advantage of using VX-745-loaded particles for OA treatments.
This work was supported by the De Reuter Fondation, Geneva, Switzerland.

LIPOSOMAL DELIVERY OF RESVERATROL, CURCUMINE AND N-(3-OXO-DODECANOYL)-L-HOMOSERINE LACTONE TO INHIBIT CANCER INFLAMMATION

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The chronic inflammatory environment of tumor areas is nowadays recognized as being the 7th hallmark of cancer and it represents a target for novel antitumor therapeutic strategies.
Natural bioactive compounds are valuable in these strategies and have been studied for a long time for their chemopreventive and therapeutic potential in several chronic inflammatory diseases, including cancer. These agents target multiple signal transduction pathways and modulate gene expression resulting in a broad spectrum of anti-inflammatory, antioxidant, immunomodulatory, pro-apoptotic, and anti-angiogenic activities. As cancers result from complex molecular and cellular deregulations, these compounds may be more likely to have beneficial antitumor effects than agents with selective high-affinity single pathway activity. Moreover, natural bioactive compounds have in general better safety profiles, are well accepted by the public, are chemically characterized and usually cheap. However, their physicochemical properties result in poor in vivo bioavailability. Also there's a lack of human clinical trials that address absorption, distribution, metabolism, and excretion of these compounds in addition to efficacy. These issues limit their use in the clinic.
To improve the bioavailability and control over the absorption and distribution of these compounds we have formulated three anti-inflammatory compounds into liposomes. Resveratrol, curcumin and N-(3-oxo-dodecanoyl)-l-Homoserine lactone (C12) are three different natural compounds derived from plant and microorganism sources respectively. They strongly interfere with a variety of pro-inflammatory cell signalling pathways and therefore may have a role in cancer therapy.
All drugs could be formulated into small pegylated liposomes, but in vitro stability studies suggested that only the curcumin formulation would be stable in vivo, as both resveratrol and C12 were lost from the liposome fraction when incubated with serum albumin. Despite this instability both C12 and resveratrol could inhibit tumor growth for approximately 50% with weekly injections in murine tumor models. At this dosing schedule curcumin decelerated tumor growth by approximately 70%.
Taken together these studies demonstrate that liposomes acting as solubilizers or carriers of anti-inflammatory drugs can improve the efficacy of anti-inflammatory natural compounds.

OPTIMIZING THE THERAPEUTIC INDEX OF LIPOSOMAL GLUCOCORTICOIDS IN ARTHRITIS

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Introduction

Systemic glucocorticoid (GC) delivery within long-circulating liposomes (LCL) strongly increases its therapeutic benefit against inflammation in experimental arthritis as compared to the free drug. However, the systemic adverse effects of liposomal delivery of GC are not known. In this study we compared the efficacy and adverse effects of different doses of liposomal prednisolone and the more potent high clearance corticosteroid budesonide in experimental arthritis to further optimize the therapeutic index of liposomal GC in arthritis

Experimental Methods

Rats with adjuvant arthritis and mice with antigen-induced arthritis were treated with liposomal prednisolone (0.1-10 mg/kg) or liposomal budesonide (0.1-10 mg/kg) or control injections (PBS or empty liposomes). As parameters for systemic activity loss of body weight and increase in blood glucose concentrations were evaluated. A plasma concentration time curve was measured at a dose of 10 mg/kg in healthy rats.

To determine inflammation, suppression of the HPA axis and plasma hormone levels, mice were sacrificed at day 8, 11, 14 or 21. Inflammation of the knee joints was measured by ^{99M}Tc-uptake and histology. Suppression of the HPA-axis was measured by histology of the adrenal gland and plasma levels of corticosterone and ACTH.

Results and discussion

All doses of liposomal GC significantly reduced the inflammation of the joint. However, side effects at the level of body weight and hyperglycemia were noted, related to the sustained free GC level observed after injection of the liposomal GC. Adrenal gland weight was markedly reduced by 10 mg/kg liposomal prednisolone and showed thinning of the adrenal cortex, 5 days after treatment. The suppression of the adrenal gland was associated with a >3-fold reduction in corticosterone and ACTH. Eleven days after treatment, corticosterone levels were still suppressed 2-fold with 5 and 10 mg/kg liposomal prednisolone. Remarkably ACTH levels at this time point were increased by 1.5 and 2-fold with 5 and 10 mg/kg respectively, suggesting a recovery of the HPA-axis. Finally, at 18 days after treatment, adrenal weight, corticosterone and ACTH levels had recovered with 1 and 5 mg/kg of liposomal prednisolone, while synovial inflammation was still suppressed. Our data show that with use of the more potent budesonide, these adverse effects are reduced, while a lower dose is needed to obtain the same therapeutic effect. An inverse relationship with the clearance rate of the GC in question was shown in rats.

Conclusion

Lower dosing of liposomal GC reduces side effects while maintaining efficacy against experimental arthritis. By using a more potent high clearance GC the therapeutic index with liposomal GC can be further improved. Therefore the high-clearance GC budesonide, which until now is only applied in local treatment approaches, may be very useful for the development of novel, highly effective anti-inflammatory preparations for systemic treatment of inflammatory disorders.

Session 6

Brain delivery

BINDING OF PHAGE DISPLAYED VS SYNTHETIC PEPTIDES TO BRAIN ENDOTHELIUM

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Introduction

Previously, we have performed phage display screening as a tool to identify new peptide ligands for targeting to the blood-brain barrier. Two phage clones, displaying 15-amino acid peptides, GLAHSFSDFARDFVA (named GLA) and GYRPVHNIRGHWAPG (named GYR) were identified. These phage were able to bind to human brain endothelial cells *in vitro*, and, after brain perfusion, to the mouse brain *in situ* (5-6x increased binding versus control). After finding that these phage-displayed peptides could bind to brain endothelial cells, the next step was to investigate whether synthetic peptides with these amino acid sequences, when attached to a drug delivery system (i.e. liposome), were able to bind to brain endothelial cells *in vitro* and *in vivo*.

Experimental Methods

Liposome preparation

Liposomes were prepared by the film hydration and membrane extrusion method. For *in vitro* flow cytometry studies, fluorescently labeled 200 nm liposomes with a DPPC:cholesterol:DSPE-PEG$_{2000}$:DSPE-PEG$_{2000}$-maleimide composition were used. For *in vivo* biodistribution and *in vitro* monolayer binding studies, 100 nm liposomes with a EPG:EPC:cholesterol:MPB-PE composition were used, radioactively labeled with [^{3}H]cholesteryl hexadecyl ether.

Coupling of synthetic peptides to liposomes

Peptides were synthesized with a C-terminal cysteine. The thiol group of the cysteine was reacted with the maleimide group on the liposomes. Binding of the peptides to the liposomes was confirmed by UPLC.

Binding of liposomes to bEnd.3 and hCMEC/D3 cells

Human brain endothelial (hCMEC/D3) cells were grown to a monolayer and then brought to suspension by trypsinization. Targeted labeled liposomes were incubated for 1 hour at 4°C with the cells. After washing, binding was investigated by flow cytometry.

Murine brain endothelial (bEnd.3) cells were grown to a monolayer. The layer was incubated with targeted radioactive liposomes for 1 hour at 37°C. Cells were washed and transferred to scintillation vials to count radioactivity.

Biodistribution of liposomes

Targeted radioactive liposomes were injected iv into the tail vein of mice (n=5). After 12 hours, mice were sacrificed and organs were collected and counted for radioactivity. Brains were submitted to capillary depletion to discriminate between liposomes that had bound to brain capillaries and liposomes that had transcytosed into the brain parenchyma.

Results and discussion

When GLA-coupled liposomes were incubated with a monolayer of murine bEnd.3 cells, no increased binding was observed compared to uncoupled liposomes. When GLA and GYR-coupled liposomes were incubated with human hCMEC/D3 cells, an increased binding of GYR-liposomes was observed. While 0.9% of the cells were positive for control and GLA-liposome binding, 1.6% of cells were positive for GYR-liposome binding. This was an 1.8x increase.

The peptide-coupled liposomes were perfused through the mouse brain by *in situ* perfusion. The fraction of liposomes that had bound to the mouse brain was determined. No significant difference was observed between the peptide-coupled and uncoupled liposomes. Finally, GLA-coupled liposomes were injected iv in mice. Brain capillaries were separated from brain parenchyma. In the brain capillary fraction, a 2.4x increased binding of GLA-liposomes was found compared to uncoupled liposomes.

Our previous studies indicated that our identified phage-displayed peptides were able to bind to brain endothelial cells. However, when the peptides were produced synthetically and coupled to a liposome, binding was dramatically decreased or absent. There could be several reasons for this, e.g. particle shape, peptide density, and the conformation of the synthetic peptide on the liposome versus the natural peptide fused to the phage coat protein. As these peptides do show binding to brain endothelial cells when displayed on phage particles we are currently investigating which part of the phage is responsible for this.

Conclusion

When target-binding peptides have been selected by phage display, this does not necessarily mean that the synthetically produced form of the peptide will have the same properties or the same extent of target binding.

NANOPARTICLES AGAINST ALZHEIMER'S DISEASE: PEG-PACA NANOPARTICLES ARE ABLE TO LINK THE Aβ-PEPTIDE AND INFLUENCE ITS AGGREGATION KINETIC

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Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by progressive loss of cognitive functions and specific pathological changes in the brain: the formation of extracellular β-amyloid (Aβ) peptide aggregates and tangles of hyperphosphorylated Tau protein inside neurons. Especially Aβ peptide aggregates are widely regarded as the main cause of neuronal cell degeneration and oligomeric forms are considered as neurotoxic. Despite all scientific efforts, at the moment, effective pharmaco-therapeutic options for prevention and treatment of this dementia are lacking. A possible solution could come from nanotechnology. Especially poly [(hexadecylcyanoacrylate)-co-poly(ethylene glycol cyanoacrylate)] (PEG-PHDCA) nanoparticles (NPs), developed in our laboratories, exhibit not only high in vivo stability (e.g. in bloodstream) but also the ability to reach the CNS. The aim of this study was to prepare PEG-PHDCA NPs, to study their ability to link/adsorb the Aβ peptide 1-42 and to influence its aggregation kinetic.

Experimental methods

The ability of the PEG-PHDCA NPs to link/adsorb the Aβ 1-42 peptide was studied by Capillary Electrophoresis (CE). This technique has been also used to check the ability of our NPs to influence the aggregation kinetic of the peptide. The interaction between the peptide and the NPs has also been studied by Confocal Microscopy, Surface Plasmon Resonance (SPR) and Thioflavin T assays which revealed the aggregation of the peptide.

Results and discussion

As expected, the CE studies showed no alteration of the monomer peak in the positive control (only Aβ peptide). In contrast, a significant decrease of the monomer peak together with the appearance of an unknown peak (with increased intensity as a function of time) was observed in the presence of NPs. CE analyses performed during the peptide aggregation process evidenced a faster decrease of the monomer peak intensity in the presence of NPs compared with the positive control. Moreover, the peak corresponding to the oligomeric forms of Aβ increased in the control while it decreased in the presence of NPs. Again, the NPs induced the appearance of unknown peaks with increasing intensities as time goes. Interestingly, no difference was detected in presence of non-PEGylated PHDCA NPs compared to the positive control.
Surface Plasmon Resonance results clearly showed that PEGylated PHDCA NPs only (i.e. not PHDCA NPs), interacted with Aβ peptide immobilized onto the sensor chip. The binding of PEGylated PHDCA to Aβ 1-42 was specific since it was not detected on immobilized BSA. Very similar results were also obtained from a chip coated with Aβ 1-42 fibrils. Moreover neither non-PEGylated nor PEGylated NPs interacted with the chip coated with BSA. Confocal Microscopy images showed a full colocalization of PEG-PHDCA-Rhodamine NPs and Aβ 1-42 Hilyte™. Objects were formed and were smaller when the peptide concentration was decreased. The images suggest the aggregation of the peptide at the surface of the NPs at high concentration. This hypothesis has been confirmed by Thioflavine T assays.

Conclusion

Capillary Electrophoresis, Confocal microscopy, Surface Plasmon Resonance and Thioflavine T assays experiments clearly confirmed the ability of our NPs to bind the Aβ peptides. Moreover these results suggested a pivotal role played by the PEG chains in this interaction and some degree of specificity of this interaction. All these information allow us to anticipate a possible capture of soluble forms of the peptide by our NPs, both in the bloodstream and in the brain and a subsequent elimination from liver, spleen or microglial cells. This property could prevent or slow down the formation of Aβ toxic oligomers or aggregates under physiological conditions and open new routes in the field of AD therapy.

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IN VIVO INTERACTIONS OF DIFFERENT FUNCTIONALIZED CARBON NANOTUBES WITH THE BRAIN PARENCHYMA

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Introduction

Carbon nanotubes have attracted attention because of their extraordinary properties, such as high electrical and thermal conductivity, great strength and rigidity. In addition, surface functionalization of CNTs constitutes a remarkable development, which renders them readily dispersed in biological solvents, thus expanding their biological capabilities and allows for their exploration in neurological applications. The potential of functionalised(*f*-CNTs) for drug and gene delivery in the central nervous system (CNS) and as neural substrates renders the understanding of their interactions with the neuronal tissue essential. The aim of this study is to systematically explore the uptake of *f*-CNTs by neuronal cells and determine the neuro-toxicological profile of three different types of functionalized MNWT (*f*-MWNT) prepared using three different chemical approaches.

Experimental Methods

Chemical functionalization and characterization of Functionalized Multi-Walled Carbon Nanotubes (f-MWNTs)
Three different types of *f*-MWNTs were tested: i) amino-functionalized MWNT (MWNT- NH_3^+) prepared following the 1,3 dipolar-cycloaddition reaction without prior oxidation (length between 0.5-1µm), (ii) amino-functionalized oxidised MWNT (MWNTox-NH_3^+) following a 2-step chemical treatment consisting of a) oxidation and b) introduction of amine groups by the cycloaddition reaction and (iii) amino-functionalized oxidised MWNT (MWNTox- NH_3^+ amid) following a 2-step chemical treatment consisting of a) oxidation and b) introduction of amine groups by amidation reaction. All types were examined by transmission electron microscopy (TEM).
Diffusion and ultra-microstructural examination of f-MWNT into brain parenchyma in vivo
f-MWNTs were individually administered intracranially into mouse brain cortex as a single dose of 500ng/mouse. The distribution of MWNT-NH_3^+ and MWNTox-NH_3^+ in the brain parenchyma was qualitatively assessed by histological examination of brain sections stained with haematoxylin and eosin (H&E) , toluidine blue and by TEM microscopy at 2 days and 14 days after injection.
Astrocytes and microglia activation
After *f*-MWNT intracranial injection, mice were reared in standard conditions for 30 days before being culled. 50 µm-thick coronal sections were immuno-stained with specific antibodies for different types of glial cells. Antibody mediated staining against glial fibrillary acidic protein (GFAP) was aimed at the identification of activated astrocytes, whereas the microglia/macrophage marker CD11b overexpression was interpreted as a sign of active inflammation.

Results and discussion

TEM analysis indicates that all *f*-MWNTs exhibit good aqueous dispersibility profiles. According to the histological examination of sequential sections of the injected brain after 14 days, it was apparent that clearance of was more efficient for MWNTox-NH_3^+ than MWNT- NH_3^+. *f*-MWNTs were seen as frequently at an early time point of 2 days, indicating that the disappearance of *f*-MWNT from the brain was mainly due to the difference in their clearance profile. This indicates that the interaction of both types of MWNTs followed two different pharmacokinetic profiles within brain tissues. Moreover, at the cellular level, MWNTox-NH_3^+ appeared aggregated within the cytoplasm whileMWNT-NH_3^+ appeared more individualized under the same conditions. Intracellular *f*-MWNT aggregates of both types were found to be surrounded by a membrane-like structure and could also sometimes be seen without a membrane. The influence of MWNT functionalization was assessed in terms of neuroinflammation, by the evaluation of glial activation. More consistently MWNTox- NH_3^+ or MWNTox- NH_3^+ amid (both oxidized) led to microglia activation in the area surrounding the injection which was not seen in MWNT-NH_3^+ injected brains.

Conclusion

The data presented here, suggests that *f*-MWNT properties such as the type of surface functionality have an impact on the *in vivo* behaviour of CNTs, in particular on the interaction with brain tissue that is deemed extremely important for the development of both CNT-based delivery systems or implanted devices in CNS disease applications.