Universiteit Utrecht

11th European Workshop on Particulate Systems in Nanomedicine

March 13-14, 2014

Program and abstract book

Organizers:
Dr. René van Nostrum & Prof. Gert Storm
Utrecht University
"David de Wied" building
Universiteitsweg 99
3584 CG Utrecht

Program

Thursday, March 13

9:30 Registration and coffee

10:00 Welcome

10:15 **Keynote lecture** (chair: René van Nostrum)
Jean-Christophe Leroux (ETH Zürich)
Colloidal approaches to biodetoxification

Session 1 : triggered release and active targeting
(chair: Rainer Müller)

11:00 **Roel Deckers** (University Medical Center Utrecht)
High intensity focused ultrasound triggered drug release from nano-carriers

11:20 **Neda Samadi** (Utrecht University)
Nanobody-targeted and RNase-loaded nanoparticles based on a hydrophilic polyester aimed for cancer therapy

11:40 **Zahraa S. Al-Ahmady** (University of Manchester)
Antibody-targeted temperature-sensitive liposomes: enhanced in vivo tumor accumulation by heat activation

12:00 **Arianna Gennari** (University of Manchester)
Hyaluronic acid-coated chitosan nanoparticles: the influence of hyaluronic acid presentation

12:20 lunch break

Session 2 : nanoparticles for imaging (chair: Kostas Kostarelos)

13:20 **Céline Xayaphoummine** (University of Geneva)
Synthesis and characterization of a self assembling amphiphilic chelating molecule for molecular MRI

13:40 **Nathalie Stransky-Heilkron** (University of Geneva)
Development of a nanoparticular imaging agent for pancreatic amyloids in type 2 diabetes

14:00 **Neus Lozano** (University of Manchester)
Looking and listening dynamically at the tumour extravasation of sterically stabilized liposomes by multispectral optoacoustic tomography

14:20 **Thomas Blin** (Université Paris-Sud)
Precise engineering of multifunctional pegylated polyester nanoparticles for cancer cell targeting and imaging

14:40 Coffee/thee break

Session 3 : Novel formulations (chair: Elias Fattal)

15:00 **Jorrit Water** (University of Copenhagen)
Hydrogel nanoparticles for delivery of antimicrobial peptides

15:20 **Adam Bohr** (Université Paris-Sud)
Characterization of dendriplexes of cationic phosphorous dendrimers and siRNA

15:40 **Katrin Fuchs** (University of Geneva)
Sunitinib-eluting beads for chemoembolization

16:00 **Andrei Maksimenko** (Université Paris-Sud)
Novel therapeutic modalities of squalenoyl doxorubicin nanoassemblies – a unique long circulating and non pegylated anticancer medicine

16:20 Break with refreshments

Session 4 : Nanocrystals and inorganic nanoparticles
(chair: Gerrit Borchard)

16:40 **Christopher Cadman** (University of Manchester)
Synthesis, functionalization and photooxidative capabilities of TiO2 nanoparticles

17:00 **Gregori Romero** (Free University of Berlin)
Long-term stability of smartcrystals® from poorly soluble plant actives

17:20 **Sven Staufenbiel** (Free University of Berlin)
Protein adsorption patterns on differently produced & sized i.v. amphotericin B nanocrystals

17:40 Closure of the day

19:00 Diner @ restaurant "Inspired", Biltstraat 47, Utrecht

Friday, March 14

9:00 **Keynote lecture** (chair: Gert Storm)
To be announced

Session 5 : Oral applications (chair: Sven Frøkjær)

9:45 **Maria García-Díaz** (University of Copenhagen)
Insulin-lipid loaded poly(lactic-co-glycolic acid) PLGA nanoparticles for oral delivery of insulin

10:05 **Kimberley Span** (Utrecht University)
Development of a novel oral complex for iron delivery: encapsulation of hemin in polymeric micelles and its in-vitro absorption

10:25 **My-Hanh Nguyen** (Free University of Berlin)
Nanocrystalline maghemite: an efficient novel oral phosphate binder

10:45 **Qionghua Wei** (Free University of Berlin)
Amorphous CapsMorph® with high drug loading capacity for oral formulation

11:05 Coffee/thee break

Session 6 : Inflammatory and immunological response of nanomedicines (chair: Cornelia Keck)

11:25 **Nadège Grabowski** (Université Paris-Sud)
Cytotoxicity and inflammatory response following exposure of polymeric nanoparticles to a co-culture of lung cells and macrophages

11:45 **Rie Selchau Kallerup** (University of Copenhagen)
The acyl chain length of trehalose-6,6'dibehenate analogues (TDX) effects the physicochemical properties and immunological profile of DDA/TDX liposomal adjuvante

12:05 **Signe Tandrup Schmidt** (University of Copenhagen)
Formulation of a cationic liposomal adjuvant with different DDA-analogues affects the $CD8^+$ T-cell response

12:25 **Iris Allijn** (University of Twente)
Towards nanoformulations of natural anti-inflammatory compounds

12:45 Closure of the workshop

13:00 Light lunch

List of posters

Free University of Berlin:

Nan Jin
COMPARISION OF METHODS TO PRODUCE DERMAL AZITHROMYCIN NANOSUSPENSIONS TO PREVENT LYME BORRELIOSIS INFECTION

Gregori Romero
NANO LIPID CARRIERS (NLC) & NANOCRYSTAL FORMULATION FOR COUPEROSIS TREATMENT

Patrik Scholz
COMPARISON OF THE NANONIZATION POTENTIAL OF HIGH SPEED STIRRING AND PEARL MILLING

Sven Staufenbiel
ULTRAFINE GELATIN NANOPARTICLES FOR DERMAL DELIVERY OF PEPTIDES & ENZYMES: PRODUCTION AND CHARACTERIZATION

Utrecht University:

Sohail Akhter
RISPERIDONE LOADED PLGA MICROSPHERES: FORMULATION DEVELOPMENT AND DEGRADATION STUDY

Erik Oude Blenke
MEMBRANE FUSION WITH COILED-COIL LIPOSOMES

Burcin Ozbakir
DEVELOPMENT OF METHODOLOGY FOR THE SEPARATION AND QUANTIFICATION OF LIPOSOME-BOUND AND FREE DOXORUBICIN IN PLASMA

Farshad Ramazani
FORMULATION OF IMATINIB INTO PLGA MICROSPHERES AS A PLATFORM FOR INTRATUMORAL DELIVERY OF KINASE INHIBITORS

Yang Shi
MULTIMODAL IN VIVO AND EX VIVO OPTICAL IMAGING TO STUDY BIODISTRIBUTION AND INTRATUMORAL PENETRATION OF DUAL-LABELED CORE-CROSSLINKED POLYMERIC MICELLES

Jos Wennink
AGGREGATION AND GELATION BEHAVIOR OF STEREOCOMPLEXED FOUR-ARM PLA-PEG COPOLYMERS CONTAINING NEUTRAL OR CATIONIC LINKERS

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Keynote lectures

COLLOIDAL APPROACHES TO BIODETOXIFICATION

Jean-Christophe Leroux
Institute of Pharmaceutical Sciences, ETH Zürich, Switzerland

The fields of antidotal medicine and biodetoxification have gained much interest in recent years due to the prevalence increase in drug overdose and intolerance to various food antigens. In this presentation, we will discuss the use of colloidal systems for the treatment of celiac disease (CD), an autoimmune disease triggered by cereal proteins and for the rescue of patients who have been intoxicated with drugs. CD is an inflammatory disease of the intestine triggered by the ingestion of gluten and gluten-like proteins of wheat, rye, and barley in genetically predisposed individuals (~1% of the population). This disease is induced by immunogenic sequences of gluten proteins which are highly resistant to human digestive proteases [1]. The current and only treatment is life-long elimination of gluten from the diet. This dietary regimen is difficult to follow, and is often associated with a decreased quality of life. Poor compliance to a strict gluten-free diet is frequent and predisposes patients to CD complications (*e.g.,* nutritional deficiencies, osteoporosis, secondary autoimmune disorders, malignancies). Hence, there is a need for pharmacological therapies to help managing this common disorder. One of the most interesting therapeutic options consists in administering to CD patients exogenous enzymes ("glutenases") that cleave and detoxify the gluten peptides [1]. While promising, this approach is in part limited by the relative instability of the enzymes in the harsh conditions encountered in the GI tract [2]. We are currently investigating strategies aimed at increasing the stability of glutenases in the stomach and in the small intestine. Using a model immunogenic peptide sequence, we found out that the conjugation of cationic dendronized polymers to glutenases could enhance their mucoadhesion and enzymatic activity in the stomach in rats. On the other hand, the random PEGylation of the enzymes did not improve the stomachal activity, but improved their performance in the small intestine [3]. In addition, we have developed a polymer binder based on hydroxyethyl methacrylate and styrene sulfonate, which reduced the production of immunogenic peptides derived from gluten and decrease its deleterious effect in gluten-sensitive HLA-HCD4/DQ8 mice [4]. This polymer is now in clinical phase 1/2. Finally, we are evaluating the use of transmembrane pH-gradient liposomes, which were initially developed to efficiently load drugs in their inner aqueous core as a means to alter the biodistribution of overdosed drugs and reverse their toxicity [5]. Such liposomes were found to be more efficient than parenteral lipid emulsions that are currently used off label to treat severe drug intoxications. Financial support from the Swiss National Science Foundation is acknowledged.

[1] Pinier, M.; Fuhrmann, G.; Verdu, E.F.; Leroux, J.C. *Am. J. Gastroenterol.* **2010**, *105*, 2551-2561.
[2] Fuhrmann, G.; Leroux, J.C. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9032-9037.
[3] Fuhrmann, G.; Grotzky, A.; Lukic, R. *et al. Nat. Chem.* **2013**, *5*, 582-589.
[4] Pinier, M.; Fuhrmann, G.; Rivard, N. *et al. Gastroenterology* **2012**; *142*, 316-325.
[5] Forster, V.; Luciani, P.; Leroux, J.C. *Biomaterials* **2012**; *33*, 578-3585.

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Session 1:
Triggered release and active targeting

HIGH INTENSITY FOCUSED ULTRASOUND TRIGGERED DRUG RELEASE FROM NANO-CARRIERS

R. Deckers[1], C. Oerlemans[1], J.F.W. Nijsen[1], C.T.W. Moonen[1], G.Storm[2], W.E. Hennink[2]

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Introduction

In recent years there has been growing interest in using nano-sized drug-loaded carriers ('nanomedicines') to improve (cancer) treatment (1). These drug carriers aim for a reduced systemic toxicity and an improved efficacy of particular chemotherapeutic drugs. However, the slow release of the drug from the nano-carriers upon accumulation in the tumor results in low bioavailability of the free drug and an unchanged efficacy. High intensity focused ultrasound (HIFU) is a promising technology for the non-invasive, local triggered drug release from nano-carriers leading to high (local) bioavailability of the drug (2). The interactions of the ultrasound waves with tissue may lead to local heating and mechanical stress, depending on characteristics of the ultrasound wave. Here we show how HIFU can be used to trigger drug release from different nano-carriers (i.e. liposomes and micelles).

Experimental methods

Temperature sensitive (TSL) and non-temperature sensitive liposomes (NTSL) loaded with the drug-mimicking fluorescent dyes fluorescein (hydrophilic) and nile red (hydrophobic) were prepared. Furthermore, non-cross-linked (NCL) and core cross-linked (CCL) polymeric micelles loaded with nile red were prepared. All nano-carrier formulations were subjected to different ultrasound regimes to investigate the role of different ultrasound parameters on the payload release. In order to investigate the underlying mechanism causing the release all nano-carriers were characterized by DLS, GPC and ^{1}H NMR before and after ultrasound application.

Results and Discussion

HIFU mediated temperature increase only allows the release of hydrophilic compounds from TSL. In contrast, HIFU induced mechanical stimuli cause the release of hydrophilic and hydrophobic compounds from TSL and NTSL. Furthermore, the mechanical stimuli cause the release of hydrophobic compounds from NCL and CCL polymeric micelles. We suggest that radiation force induced convection of the nano-carrier dispersion causes shear forces at the walls of the exposure chamber, which leads to a temporary physical destabilization of the nano-carriers and thus release of the payload.

Conclusion

HIFU is a highly promising technique to destabilize nano-carriers on demand and spatiotemporally control the release of hydrophilic and/or hydrophobic compounds.

References:

(1) Allen et al., Science 2004, 303 (5665), 1818–22.
(2) Deckers et al., J. Controlled Release 2010, 148 (1), 25–33

NANOBODY-TARGETED AND RNASE-LOADED NANOPARTICLES BASED ON A HYDROPHILIC POLYESTER AIMED FOR CANCER THERAPY

N. Samadi [1] , M. M. Kijanka [2], S. Oliveira[2], T. Vermonden[1], J. B. vanden Dikkenberg[1], C. F. van Nostrum[1], M. Amidi[1], P. M. P. van Bergen en Henegouwen[2], W.E. Hennink[1]

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Introduction

Ribonucleases (RNases) are small (10–28 kDa) basic proteins, have raised attention for use as novel anti-cancer therapeutics[1,2], due to their ability to catalyze the degradation of cytosolic RNAs and exert cytotoxic effect, when they enter the cell cytosol by endocytosis[3]. However, because of insufficient cellular internalization of these proteins, their cytotoxic efficacy remains relatively poor. Therefore, to fulfill the promising therapeutic potential of RNases tumor selective delivery systems are required. The aim of this study was to develop a nanomedicine based on a hydrophilic polyester (poly lactic-co-glycolic-co-hydroxymethyl glycolic acid; PLGHMGA) for targeted delivery of RNase A as a modality for cancer treatment.

Experimental methods

RNase loaded pegylated PLGHMGA nanoparticles (NPs) were prepared by a double emulsion solvent evaporation method. 10% Maleimide-PEG-PLGA was added to the formulation to graft the Her2 targeted nanobody (11A4) at the surface of the NPs. 11A4-decorated NPs (NB-NPs) were labelled with Alexa Fluor 532. Two different cell types; Her2 over-expressing cancer cells (Skbr3) and Her2 negative cells (MDA-MB-231) were used to study binding, uptake and cytotoxic effect of NPs as well as free RNase. Released RNase was measured by Ultra Performance Liquid Chromatography (UPLC).

Results and Discussion

Results demonstrated 80% release of the loaded RNase over ~10 days. A digestion bioactivity assay showed that the enzymatic activity of released RNase was fully preserved. NB-NPs showed greater binding and uptake by Her2 over-expressing cells compared to NPs devoid of nanobody. Significant and dose-dependent cytotoxicity was observed for RNase-loaded NB-NPs, which can be attributed to either the release of RNase in the endosome and subsequent destabilization of the endosomes resulting in release of the enzyme into the cytosol, or due to destabilization of the endosomes by particles followed by release of the entrapped enzyme in the cytosol (or due to a combination of both).

Conclusion

It can be concluded that targeted PLGHMGA nanoparticles are potential candidates to exploit therapeutic potential of RNases.

References:

(1) De Lorenzo C, et al; Curr Pharm Biotechnol 2008; 9(3):210-214. (2) Krauss J, et. al; Curr Pharm Biotechnol 2008; 9(3):231-234. (3) Youle RJ, et al; Crit Rev Ther Drug Carrier Syst 1993; 10(1):1-28.

ANTIBODY-TARGETED TEMPERATURE-SENSITIVE LIPOSOMES: ENHANCED *IN VIVO* TUMOR ACCUMULATION BY HEAT ACTIVATION

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Introduction

In order to improve the therapeutic potential of liposomal anticancer drugs, interest in developing new generation of liposomes that aim to combine the advantages of both active targeting and triggered release properties has increased. In this study we explored the possibilities to increase the therapeutic specificity of temperature-sensitive liposomes (TSL) by designing anti-MUC-1 targeted vesicles based on the traditional TSL (TTSL) to trigger drug release after specific uptake into cancer cells.

Experimental

We characterized these liposomes by studying their mean diameter, surface properties, serum stability and thermal sensitivity before and after conjugation to anti MUC-1 antibody. Receptor mediated cellular uptake and cytotoxic efficacy of MUC-1 targeted TTSL (TTSL-Ab) were investigated using 2D and 3D cell culture techniques. Taking this system a step further, its biodistribution and therapeutic potential were also examined *in vivo*.

Results and Discussion

TTSL liposomes maintain their physicochemical and thermal properties after conjugation to anti-MUC-1 antibody. Significant enhancement in cellular uptake and cytotoxic activity after 1 h heating at 42°C was observed from TTSL-Ab compared to non-targeted TTSL in MUC-1 over-expressing breast cancer cells (MDA-MB-435). Different heating and administration protocols were applied to explore the effect of mild hyperthermia (HT) on the accumulation of targeted TTSL into the tumor and their therapeutic efficacy. Application of local mild HT (42°C) significantly increased tumor accumulation of targeted TSL compared to non-targeted liposomes. This was associated with moderate improvements in therapeutic activity and overall survival.

Conclusion

The capability of antibody-targeted TTSL liposomes to bind and internalize through receptor-positive tumor cells and their triggerable drug release intracellularly, represent a potential strategy to enhance the therapeutic efficiency of temperature-sensitive liposomal doxorubicin. Ultimately, our results suggested that TTSL-Ab in combination with mild HT can open new possibilities in anticancer therapeutics design.

HYALURONIC ACID-COATED CHITOSAN NANOPARTICLES: THE INFLUENCE OF HYALURONIC ACID PRESENTATION

Dr. Arianna Gennari,[1] Dr. Abdulaziz Almalik,[3] Prof. Nicola Tirelli[1,2]

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Introduction

Hyaluronic acid (HA), a natural component of the extracellular matrix, is also heavily involved in cellular signalling. Due to the overexpression of its main receptor, CD44, in pathological phenomena such as inflammatory reactions and several types of tumors, in the past few years HA has also been seen as a potential means to target the action of an active principle. Two points are discussed here:

- The affinity of HA (in solution or on a substrate) to CD44 depends on its chain length and on its spatial arrangement, therefore the way HA is presented on a carrier structure may have profound effects on the way a carrier would bind to a receptor.
- After binding CD44 is internalized, and then it is represented only very slowly; therefore CD44-mediated endocytosis easily reaches saturation. The amount of internalisable material would therefore depend on the interplay between limited number of available receptors and their affinity to the material, with the paradoxical result that increased affinity, i.e. also higher number of receptor clustered around a carrier structure, may lead to lowed internalisation.

Experimental methods

Nanoparticles were prepared by polyelectrolyte complexation of chitosan (CS) of variable molecular weight with triphosphate (TPP), and the successive coating by HA.

Results and Discussion

CS molecular weight (MW) influences the nanoparticle structure, with higher cross-link density with lower CS MW. This influences the complexation with HA, with low MW (25 kDa) chitosan yielding nanoparticles coated by a rather extended HA corona and high MW chitosan producing nanoparticles with more tightly bound HA. The internalization of both particles in macrophages is CD44 mediated, and better presentation (low MW chitosan) corresponded to both higher affinity and lower capacity/uptake rate. Using nucleic acid payloads, we observed a positive correlation between transfection efficiency and CD44 expression in different cell lines. Interestingly, despite a lower internalization, low molecular weight CS/HA particles provided higher transfection, likely due to a more efficient cytoplasmic delivery.

Conclusion

HA presentation heavily influences the efficiency of CD44 targeting, but intracellular events may be more important in the overall picture of the delivery action.

References:

(1) A. Nasti *et.al.*, *Pharmaceutical Research* **2009**, *26*, 1918-1930.
(2) A. Almalik *et. al.*, *Biomaterials* **2013**, *34*, 5369-80.
(3) A. Almalik *et. al.*, *Macromol Biosci* **2013**, *13*, 1671-80.
(4) A. Almalik *et. al.*, *J Control Release* **2013**, *172*, 1142-50.

11th European Workshop on Particulate Systems in Nanomedicine

March 13-14, 2014

Session 2: Nanoparticles for imaging

SYNTHESIS AND CHARACTERIZATION OF A SELF ASSEMBLING AMPHIPHILIC CHELATING MOLECULE FOR MOLECULAR MRI

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Introduction: Magnetic Resonance Imaging (MRI) is one of the most frequently used non-invasive and non-radioactive technique for medical diagnosis. The high spatial and temporal resolution images obtained is generally due to the use of low molecular weight contrast agents (CAs) carrying a gadolinium (Gd^{3+}) atom in their centre. New designs of contrast agents are needed to enable molecular imaging of diseases. Micelles carrying targeting moieties should enable to reach this goal. These supramolecular constructs constituted of amphiphilic chelates should meet all the requisites: a concentration of gadolinium atoms tightly chelated by a DO3A, a small size enabling enhanced permeation and retention, a delayed opsonisation due to the effect of conjugating poly(ethylene glycol) chains (PEG) at their external surface, and the adjustment of the hydrophilic-hydrophobic balance by the addition of hydrophobic chains of the appropriate length.

Experimental methods: The synthesis relied on the use of a (L)-tyrosine-OH as starting material. The three functional sites of this amino-acid were selectively grafted by a hydrophobic NH-$(C18)_2$ chain, a DO3A chelating agent separated by a benzyl spacer, and a hydrophilic methoxy poly(ethyleneglycol)$_{2000}$ (M-PEG_{2000}).

Results and discussion: After multiple steps of synthesis and purification, the structure of newly synthesised amphiphilic molecule was assessed and confirmed by NMR and mass spectrometry. It had a molecular weight of 3300Da. In water it aggregated as stable micelles at very low concentration (cmc $\approx 10^{-3}$M). A significantly high relaxivity value ($r_1 \approx 22 mM^{-1}.s^{-1}$ at 40MHz and 37°C) was measured for micelles as compared to the traditional contrast agents already available.

Conclusions: We reported the synthesis and characterization of a novel molecular imaging contrast agent able to self-assemble as micelles in aqueous medium. These new particles show promise for the subsequent design of a micellar MRI CA for molecular imaging.

DEVELOPMENT OF A NANOPARTICULAR IMAGING AGENT FOR PANCREATIC AMYLOIDS IN TYPE 2 DIABETES

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[3] Laboratoire de Chimie et Biologie des Métaux (CBM, AFFOND), équipe AFFOND, UMR 5249, CEA Grenoble, France
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Introduction. Type 2 diabetes (T2D) is a pathology in which the pancreas fails meeting the increased needs for insulin, resulting in beta cell loss and hyperglycemia. The reasons of the cellular death are not yet understood. A common feature in most T2D patients is the presence of extra-cellular amyloids within the pancreas. Amyloids are aggregates of proteins which are present in several degenerative diseases, including Alzheimer's and Parkinson's. Our purpose is to develop a contrast agent in order to visualize the pancreatic amyloids *in vivo*. To this end, very small (< 5 nm) polysiloxane-based nanoparticles were synthesized (1). The nanoparticles carry chelated gadolinium for magnetic resonance imaging (MRI) and they are functionalized with targeting moieties. The aim of the present study was to assess the pharmacokinetics and biodistribution of the nanoparticles *in vivo* in healthy animals.

Experimental methods. First, the nanoparticles were injected into healthy male FVB/N mice to assess their pharmacokinetics and biodistribution. Aliquots of blood were withdrawn at predefined time points after the injection and organs have been harvested after sacrifice in order to determine precisely the pharmacokinetics and the fate of the contrast agent.
Imaging experiments were also conducted to assess the efficacy of the particles as a contrast agent and to observe the distribution of the particles in real time.

Results and Discussion. The nanoparticles had a very short plasma half-life (in the order of about 10 minutes) and were eliminated by renal route. There was no accumulation in the spleen, liver, lungs, kidneys, or pancreas.
The MRI contrast in the blood pool was visibly enhanced after the injection of the dispersion of nanoparticles, which means that the nanoparticles are able to provide contrast. The contrast enhancement in the organs was transient; the half-life in the organs corresponded to the one assessed in the blood, meaning that there is no unwanted accumulation of the particles.

Conclusion. The polysiloxane-based nanoparticles investigated in this study have suitable characteristics for an MRI contrast agent, among which a short half-life, elimination by renal route and no undesired accumulation in organs. The efficacy of the targeting will be assessed in transgenic mice, in which pancreatic amyloids are present.

Reference: (1) Lux F. et al, Angew Chem Int Ed Engl, **50**, 12299-12303, 2011.

LOOKING AND LISTENING DYNAMICALLY AT THE TUMOUR EXTRAVASATION OF STERICALLY STABILIZED LIPOSOMES BY MULTISPECTRAL OPTOACOUSTIC TOMOGRAPHY

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Introduction

Multispectral optoacoustic tomography (MSOT) is a powerful imaging technology that allows high-resolution imaging of photo-absorbers deep inside tissue, reaching beyond the classical depth and resolution limitations of conventional optical imaging. However, contrast needs to be significantly complemented by extrinsic contrast agents, such as gold nanoparticles. Here we studied the clinically used PEGylated liposome system that constitutes the chemotherapeutic agent DOXIL, by re-engineering its bilayer with incorporation of indocyanine green (ICG) to form a stable, liposomal agent (LipoICG) to enable high-sensitivity MSOT imaging *in vivo*.

Experimental methods

Liposomal ICG (LipoICG) agents were prepared using lipid-film hydration methodologies combined with freeze-thawing and characterized spectrophotometry, TEM and dynamic light scattering. Tissue distribution in healthy animals following intravenous administration of Lipo-ICG was performed by whole-body NIR fluorescence imaging (IVIS camera) and dynamic MSOT imaging non-invasively under anesthesia. The extravasation of Lipo-ICG through the solid tumor vasculature was investigated using 4T1 and HT29 tumor-bearing animals imaged with two different optoacoustic techniques. Fluorescence imaging of cryosectioned tumor tissue (FCSI) and histology analysis were used to validate the imaging data.

Results and Discussion

The tumor extravasation of LipoICG agents was imaged dynamically over time to offer the high-resolution and high-sensitivity insight on how sterically-stabilised liposomes distribution throughout the entire tumor volume. This data may offer a paradigm shift in the non-invasive assessment of nanoparticle distribution and spatial heterogeneity within the tumor micro-environment.

Conclusion

The engineering of liposomal ICG offers an effective optoacoustic imaging contrast agent by employing clinically approved components in a non-covalent manner to produce a highly adept, versatile and clinically translatable nanoparticle platform.

PRECISE ENGINEERING OF MULTIFUNCTIONAL PEGYLATED POLYESTER NANOPARTICLES FOR CANCER CELL TARGETING AND IMAGING

N. Mackiewicz[1], J. Nicolas[1], T. Blin[1], N. Handké[1], M. Noiray[1], H. R. Lakkireddy[2], D. Bazile[2], P. Couvreur[1]

[1] Institut Galien Paris-Sud, Université Paris-Sud, UMR CNRS 8612, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, F-92296 Châtenay-Malabry cedex, France.

[2] Sanofi Research and Development, Lead Generation to Candidate Realization Platform, 13 quai Jules Guesde, F-94403 Vitry-sur-Seine cedex, France.

Introduction. Spurred by the development of advanced nanoscale systems for drug delivery, the medical application of nanotechnology, usually termed nanomedicine, has recently received tremendous attention and nanoparticles made of polyesters. More specifically, poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA), hold great promise in the field due to their biocompatibility and biodegradability and are Food and Drug Administration-approved polymers. Herein, we report a general strategy deriving from well-established long-circulating PLA-*b*-PEG amphiphilic block copolymer nanoparticles to furnish multifunctional nanoparticles with tunable dual 'click' functionalization with both imaging probes and various targeting ligands for theranostic purposes. Therefore, the nanoparticles comprise: (i) a biodegradable core made of PLA; (ii) a PEG shell for improved colloidal stability and stealth features and (iii) surface-displayed biologically active ligands (with biotin, folic acid or anisamide) for cancer cell targeting, and fluorescent dyes (UV-Vis or near-infrared) for imaging/tracing purposes.

Experimental methods. PLA-*b*-PEG-OMe and PLA-*b*-PEG-N_3 copolymers were obtained by ring-opening polymerization (ROP) of D,L-lactide. PLA-*b*-PEG-ligand and PLA-*b*-PEG fluorescent probe were obtained by copper catalyzed azide-alkyne Huisgen 1,3-dipolar cylcoaddition (CuAAC) between PLA-*b*-PEG-N_3 copolymers and alkynated ligands or fluorescent probes. Nanoparticles formations were realized by nanoprecipitation between copolymers dissolved in AcOEt and an aqueous phase containing 1% w/v Pluronic F68.

Results and Discussion. Well-defined PLA-*b*-PEG-N_3 diblock copolymers were successfully derivatized with a series of: (i) biologically active ligands able to recognize cancer cell receptors (i.e., biotin, folic acid and anisamide) and (ii) fluorescent probes (FP547 and FP682). Multifunctional nanoparticles were then achieved by a simple blending between the different PLA-*b*-PEG copolymers (functional or not) to achieve the desired surface ligand and fluorescent probe densities. Not only the biologically active ligands were efficiently displayed at the surface of the nanoconstructs, as shown by SPR, but their precisely-controlled density allowed optimal binding efficiencies to be determined. *In vitro* cancer cell targeting was successfully demonstrated on different cancer cell lines by flow cytometry and confocal microscopy.

Conclusion. This synthetic strategy, that takes advantage of the orthogonality of the 'click' chemistry coupling, paves the way to the design of various multifunctional PEG-*b*- PLA-based nanoparticles directed towards cancer therapy or against other pathologies, simply by changing the nature of the functional moiety in a Lego-type fashion.

11th European Workshop on Particulate Systems in Nanomedicine

March 13-14, 2014

Session 3: Novel formulations

HYDROGEL NANOPARTICLES FOR DELIVERY OF ANTIMICROBIAL PEPTIDES

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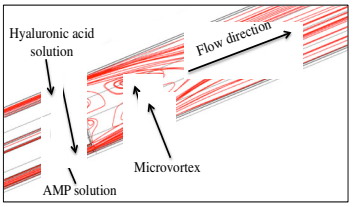

Figure 1. Simulated streamline velocity field

Introduction. Antimicrobial peptides (AMPs) are a new class of potential antibiotics and although the pharmaceutical industry has pursued commercialization of several of these AMPs none has been marketed yet. One of the reasons for this is the high dose requirement for systemic *in vivo* applications. This is associated to systemic toxicity upon administration in many cases due to the effective dose close to the toxic dose of the peptide (Guiliani *et al.* 2007). The use of drug delivery systems could overcome this challenge. This works describes the synthesis of hydrogel nanoparticles based on a hyaluronic acid derivative, an anionic polymer with hydrophobic modifications grafted on the backbone, which is complexed by ionic gelation with a short cationic amphipathic peptide in a microfluidic chip (Figure 1).

Experimental methods. Hydrogel nanoparticles were prepared in a microfluidic device based on hydrodynamic focusing as published by Kim *et al.* (2012) using design of experiments (DoE). Size and zeta potential were measured and morphology was investigated using cryo-TEM. Cell viability was determined using the MTS/PMS assay in HUVEC cells.

Results and discussion. The resulting hydrogel nanoparticle batches from the DoE had a mean size of approximately 100 nm after preparation. The zeta potential however varied between -24 and -57 mV and indicates a change in encapsulation efficiency depending on the formulation and preparation parameters. Cryo-TEM imaging (Figure 2.) showed that the hydrogel nanoparticles have a spherical appearance as often seen for these 'soft' nanoparticles. The cell viability data (Figure 3) showed an improvement of the safety profile of the AMP encapsulated into hydrogels by at least four fold in the HUVEC cells, as compared to the EC_{50} value of 10.5 ± 0.01 μM ($R^2 = 0.995$).

Conclusion. These data show that incorporation of AMP in hydrogel nanoparticles can be a viable strategy to improve the safety profile of these peptides. The peptides can be loaded at high concentration into the hydrogel nanoparticles and can subsequently be released in a sustained manner to exert their therapeutic effect.

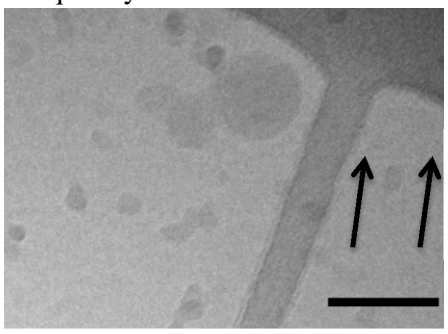

Figure 2. Cryo-TEM image of hydrogel nanoparticles (scale bar = 100 nm).

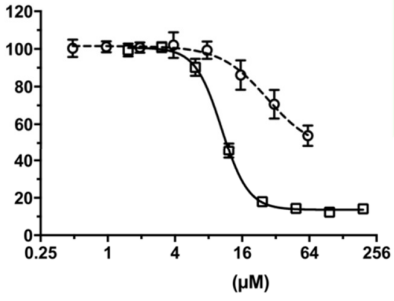

Figure 3. Relative cell viability of HUVEC cells incubated with free AMP (squares) and hydrogel nanoparticles (circles).

CHARACTERIZATION OF DENDRIPLEXES OF CATIONIC PHOSPHOROUS DENDRIMERS AND SIRNA

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Introduction. Cationic polymers are widely used to condense nucleic acids, ensuring stability and efficient cell uptake. Among them, cationic dendrimers are attractive due to their monodispersity and well-defined structures [1]. Poly(amidoamine) (PAMAM) dendrimers are the most studied often showing good gene transfection efficiency and relatively low toxicity [2]. Here, phosphorous containing dendrimers were studied for siRNA delivery. Three generations of the dendrimer (Gen 1, Gen 2, Gen 3) were examined using a model siRNA directed against luciferase.

Experimental methods. The cationic phosphorous dendrimers were synthesized as previously described [3]. Dendriplexes of dendrimers and siRNA were prepared by adding the dendrimer solution to the siRNA solution (both in 10mM Hepes buffered saline) at different amine-to-*phosphate* (*N/P*) *ratios* (5, 10, 20 and 40), charge ratio between cationic dendrimer (N) and anionic siRNA (P). Dendriplex size and polydispersity index (PDI) were determined by dynamic light scattering and confirmed using cryoTEM. Gel electrophoresis was performed using ethidium bromide and siRNA bands were visualized. Isothermal titration calorimetry (ITC) was performed by injecting dendrimer solutions into the siRNA solution and injection heats were evaluated. The cytotoxicity of the dendrimers was assessed using MTT assay.

Results and Discussion. DLS measurements indicated that nanosized dendriplexes (80-180nm) were formed for all of the generations and N/P ratios without the presence of large (> 1μm) aggregates. The size distribution were dependent partly on the mixing procedure and also on the dendrimer generation and N/P ratio. Higher generations resulted in larger complexes with higher polydispersity indices. The cryoTEM images confirmed dendriplexes sizes and their spherical shape. The dendriplexes were stable for more than 2 weeks of storage (4ºC). Gel electrophoresis showed that siRNA was tightly bound to the dendriplexes as free unbound siRNA was not detected. ITC demonstrated concentration dependent endothermic binding contrary to the exothermic binding observed for PAMAM-siRNA dendriplexes[4]. Cell toxicity studies indicated cell viability >50% at 10 μg/ml for Gen 2 and 3 dendrimers and 100% cell viability for Gen 1 dendrimers, similar to other cationic gene transfection agents.

Conclusion. This study demonstrated that phosphorous dendrimers could be suitable delivery vehicles for siRNA. Dendriplexes were formed with strong binding to siRNA and in an appropriate size range for cell uptake.

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SUNITINIB-ELUTING BEADS FOR CHEMOEMBOLIZATION

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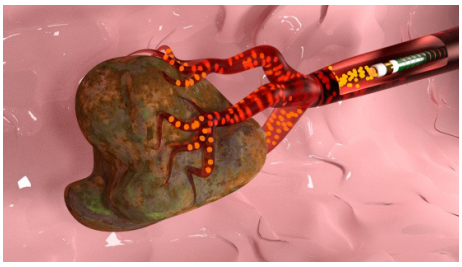
Introduction

Drug-eluting beads are drug carrier systems for local and controlled drug administration. Via a catheter, these beads are introduced into tumor feeding arteries of the liver, a procedure called chemoembolization. We suggest a novel combination of commercially available embolic beads (DC Beads®) approved as medical devices with sunitinib (1), an anti-angiogenic drug that inhibits tumor vessel growth.

Experimental methods

In vitro, sunitinib release from 70-150 µm and 100-300 µm DC Beads® was measured in saline in a pharmacopeia flow-through apparatus and quantified by spectrophotometry. Drug activity after release was confirmed in endothelial and cancer cell lines. For pharmacokinetic and in vivo toxicity evaluation, rabbits received sunitinib either by intra-arterial bead injection or per os. Drug concentrations in the plasma and liver tissue were assessed by LC-MS/MS spectroscopy.



A liver tumor is embolized by beads eluting an anti-angiogenic drug. Figure is courtesy of Adam Wilson.

Results and Discussion

Owing to ionic interactions, sunitinib loading on beads was efficient, close to complete and homogeneous. A total drug release of 80% over approximately 3 hours was measured in saline, with similar fast release profiles for both sphere sizes. After embolization, drug plasma levels remained below the therapeutic threshold, but high concentrations were found in the liver tissue.

Conclusion

Its delayed release and demonstrated activity might qualify the sunitinib-eluting beads as a successful alternative to current standards for the treatment of hypervascular tumors. It suppresses angiogenesis and tumor recurrence triggered by embolization and ischemia (2), while potentially reducing adverse drug effects due to local delivery.

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NOVEL THERAPEUTIC MODALITIES OF SQUALENOYL DOXORUBICIN NANOASSEMBLIES – A UNIQUE LONG CIRCULATING AND NON PEGYLATED ANTICANCER MEDICINE

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Abstract

We identified that the chemical linkage of the anticancer drug doxorubicin onto squalene (SQ-Dox), a natural lipid precursor of the cholesterol's biosynthesis, led to the formation of squalenoyl doxorubicin nanoassemblies of 130-nm mean diameter, with an original "loop-train" structure (**Figure 1**). This unique nanomedicine demonstrates: (i) high drug payload, (ii) reduced cardiac toxicity, (iii) improved therapeutic response, (iv) use of biocompatible transporter material, and (v) ease of preparation, all criteria that are not combined in the currently available nanodrugs.[1]

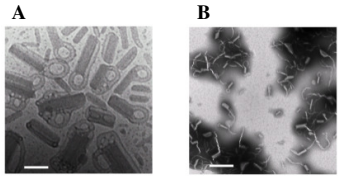

Figure 1. Cryo-TEM (A; scale bar, 100 nm) and TEM (B; scale bar, 500 nm) appearance of the SQ-Dox NAs.

Introduction

Nanotechnology has the potential to revolutionize medicine, as it offers additional therapeutic options, as compared to present conventional therapies.[2] New tailor-made nanodevices can target the diseased organs and tissues at the molecular and cellular levels in a controlled manner.[3] In this context, the present study displays a unique doxorubicin nanomedicine concept with systemic long circulating properties, without the need to use poly(ethylene glycol).

Experimental methods

Squalenoyl doxorubicin nanoassemblies were prepared by the nanoprecipitation method and tested on the MiaPaca-2 tumor bearing mice (**Figure 2**A). All groups of mice received these treatments on days 0, 4, 8, 12, and 16 by intravenous injection in the lateral tail vein. The injected volume was 10 µL/g of the body weight. The mice were monitored regularly for changes in tumor size.

Results and Discussion

As indicated in **Figure 2A**, SQ-Dox NA-treated MiaPaCa-2 pancreatic tumor xenografts in mice decreased by 95% compared with the tumors in the saline-treated mice, which was significantly higher than the 29% reduction achieved by native doxorubicin. To explain the higher anticancer activity and lower toxicity of SQ-Dox NAs vs. free doxorubicin, we performed pharmacokinetics and biodistribution studies in nude mice (**Figure 2B**).[1] As shown in this figure, SQ-Dox NAs induced blood longevity of the parent drug. The prolonged circulation time of SQ-Dox NAs in the bloodstream and their ability to evade clearance mechanisms may be explained by the more elongated morphology of SQ-Dox NAs (**Figure 1B**), compared to the other colloids usually displaying a more spherical shape.

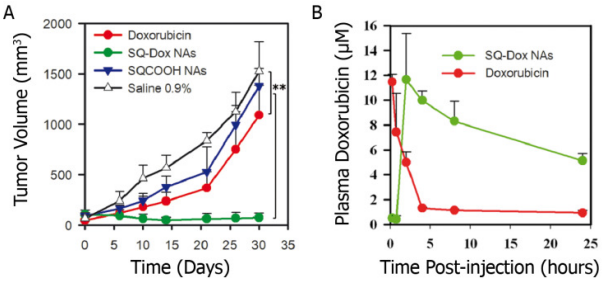

Figure 2. *In vivo* design studies. (A) Tumor growth inhibition by SQ-Dox NAs in mice bearing Miapaca-2 tumors. The mice were treated with doxorubicin (3 mg/kg i.v. injected, MTD), SQ-Dox NAs (15 mg/kg Dox equivalent, MTD), saline or SQCOOH NAs (100 mg/Kg) ($P < 0.01$; $n = 8$). (B) Plasma doxorubicin concentrations resulting from a single injection of SQ-Dox NAs (8 mg/kg Dox equivalent) or free Doxorubicin (8 mg/kg, MTD), as a function of time post injection.

Conclusion

This study opens the way for the clinical application of the squalenoylated doxorubicin nanomedicine in human cancers for which current treatment is limited by doxorubicin's toxicity or insufficient activity.

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Acknowledgments

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11th European Workshop on Particulate Systems in Nanomedicine

March 13-14, 2014

Session 4:
Nanocrystals and inorganic nanoparticles

SYNTHESIS, FUNCTIONALIZATION AND PHOTOOXIDATIVE CAPABILITIES OF TIO_2 NANOPARTICLES

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Introduction

Titanium dioxide (TiO_2) is widely used for applications such as water decontamination, sunscreen, photovoltaics etc. In the dark TiO_2 is characterized by a negligible toxicity under physiological conditions and this biocompatibility renders TiO_2 nanoparticles attractive materials for the transport of e.g. (fluorescence / MRI) imaging agents. On the other hand, TiO_2 also has a photocatalytic activity that gives rise to free radical-based oxidants (ROS) that typically have inflammatory or toxic consequences; this can lead to a selective induction of (photo)toxicity to targeted cells, i.e. to the therapeutic intervention typically known as photodynamic therapy.

Experimental methods

The preparative process employed here is based on two concepts: 1) size control via fine tuning the growth of the nanoparticles through a succession of condensation stages in a non-aqueous "sol-gel" process, and 2) production of "naked" (ligand-free) particles with a fully exchangeable surface in a water environment. Subsequent to their synthesis, the nanoparticles are functionalized, e.g. PEGylated, using catechol- or phosph(on)ate-based ligands, and their photo-oxidative capability was assessed against a model dye (methylene blue) and a macrophage cell line.

Results and Discussion

Size control of the nanoparticles was achieved through two steps: A) the generation of primary (< 4 nm) particles via hydrolysis of alkyl groups in titanium alkoxides and their rapid condensation. B) the controlled thermal agglomeration of primary particles promoting the condensation of less reactive surface groups (yielding alcohols and/or ethers), which produces secondary particles (< 10 nm). After synthesis the nanoparticles were redispersed into an aqueous environment and subsequently PEGylated. Depending on the type and amount of ligand present on their surface, the photo-oxidative capability of the nanoparticles can be controlled. The nanoparticle toxicity (w or w/o light) on macrophages has been conducted.

Conclusion

In essence, presented here is a study linking preparative variables to the photoactivity and the biological performance of TiO_2 nanoparticles.

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LONG-TERM STABILITY OF SMARTCRYSTALS® FROM POORLY SOLUBLE PLANT ACTIVES

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Introduction

The smartCrystal® technology offers the possibility of formulating dermal products containing insoluble plant actives with superior performance compared to those formulated in a traditional way (e.g. incorporation into the oil phase of emulsions). Due to the transfer of active ingredients (drug) micro-sized powder into the nanodimension (<<1,000 nm), skin penetration is enhanced and consequently the bioactivity [1]. However, finely dispersed suspensions with high interfacial energy are critical in long-term stability. In this study, nanocrystals of the citrus flavonoid hesperidin were produced in a large scale process (18 Kg batch) and the physical stability of the obtained nanosuspension was assessed over 1.5 years.

Materials and methods

Hesperidin bulk powder was dispersed in a solution containing Kolliphor® P 188 and Euxyl PE 9010 with a drug content of 18% (all w/w). This suspension was processed by five passages through the bead mill Bühler PML-2 (Bühler AG, Switzerland) with 0.4-0.6 mm yttria oxide stabilized zirconium oxide beads and 2,000 rpm rotator speed. The milled hesperidin nanosuspension was diluted to a final hesperidin concentration of 5% (w/w) and further processed by one cycle high pressure homogenization (HPH) at 500 bar using a homogenizer Avestin C50 (Avestin, Canada). Particle size was measured by photon correlation spectroscopy (PCS) (Zetasizer Nano ZS, Malvern Instruments, UK), laser diffractometry (LD) (Mastersizer 2000, Malvern Instruments, UK) and light microscopy.

Results and discussions

During the bead milling process, the size of hesperidin nanocrystal remarkably decreased as a function of milling passages for the first 3 passages. After 5 passages, the PCS diameter was around 290 nm, polydispersity index (PdI) was 0.237 and LD diameter 90% 1.2 µm. Dilution and processing by 1 cycle HPH further reduced particle size and resulted in a final nanosuspension with PCS diameter of 247 nm, PdI of 0.183 and LD diameter 90% of 0.65 µm. This nanosuspension is available on the market (INCI name: smartCrystal lemon-extract) as 5% (w/w) concentrate for incorporation into cosmetic products and is marketed by Dr. Rimpler GmbH (Wedemark, Germany).

After 1.5 years storage at room temperature, particle size remained practically unchanged. PCS diameter was 242 nm (PdI 0.191) and LD 90% was 0.71 µm. These results prove that this concentrate has a shelf-life of at least 1.5 years and is able to keep its special properties associated to the sub-micron size of the particles, such as increased saturation solubility, dissolution velocity and adhesiveness [1], even after storage over a long period.

Conclusions

- smartCrystal® nanosuspensions possess required shelf-life for commercial use.
- The technology enables the use of poorly soluble plant actives in cosmetic/pharmaceutical products with improved dermal performance compared to standard formulations.

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PROTEIN ADSORPTION PATTERNS ON DIFFERENTLY PRODUCED & SIZED I.V. AMPHOTERICIN B NANOCRYSTALS

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Introduction

The protein adsorption onto i.v. drug carriers is one of the key factors determining their in vivo bio distribution [1]. Opsonins lead to an increased recognition by macrophages (MPS cells) resulting in a fast elimination. The adsorption of dysopsonins (e.g. albumin) is beneficial for injected carries, increasing their ability to avoid MPS uptake ("stealth" properties). Targeting proteins can enhance the uptake in a specific tissue. This was shown for apo E, enabling the carrier to enter the CNS via interaction with the LDL receptor in the blood brain barrier [2]. With this "concept of differential protein adsorption" one can optimize carriers in vitro before going in vivo. In the present study the protein adsorption pattern of i.v. Amphotericin B (AmB) nanocrystals was investigated. Since the mortality of mycoses is high until today [3], there is still an urgent need of optimized formulations for the treatment of systemic fungal infections.

Experimental methods

AmB nanocrystals were produced with high pressure homogenization, H96 process and with the cavi-precipitation method lead to decreasing particle sizes; the sizes were 252 nm, 79 nm and 27 nm.
Subsequently equal amounts of the three different AmB nanocrystals were incubated with human plasma. Unbound proteins were separated by centrifugation and subsequently, desorbed bound proteins were concentrated and purified via ultrafiltration. The total amount of bound protein was determined with the BCA assay and the protein pattern was analyzed with 2D-PAGE.

Results and Discussion

The AmB nanocrystals showed a decreasing amount of total adsorbed protein with decreasing particle size. Furthermore 2D-PAGE analysis indicated a moderate albumin and a low opsonin adsorption for the different nanocrystals, especially for the smallest ones. Additionally all AmB nanocrystals showed a high adsorption of apo A-I. This protein adsorption pattern is beneficial for the AmB nanocrystals. Albumin adsorption, low opsonin adsorption and a low amount of total adsorbed protein especially for the smallest AmB nanocrystals can lead to a reduced MPS uptake. Furthermore besides apo E, also A apolipoproteins, especially apo A-I, are described to play a role in CNS uptake [4].

Conclusion

The results of the investigation of the protein adsorption pattern for the AmB nanocrystals indicated a beneficial distribution behavior after i.v. injection. While a high adsorption of opsonins could exclude a carrier for in vivo studies, these AmB nanocrystals are an interesting candidate for further animal studies.

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11th European Workshop on Particulate Systems in Nanomedicine

March 13-14, 2014

Session 5: Oral applications

INSULIN-LIPID LOADED POLY(LACTIC-CO-GLYCOLIC) ACID PLGA NANOPARTICLES FOR ORAL DELIVERY OF INSULIN

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Introduction

Oral delivery of biopharmaceuticals is one of the major challenges for the pharmaceutical industry nowadays. Polymeric nanoparticles are widely investigated as drug delivery systems, as they may provide a protective shell against the harsh acidic proteolytic environment in the gastrointestinal tract. The hydrophobic nature of many of the investigated polymers hampers effective loading of hydrophilic proteins. Thus, the aim of the present work was to study the use of insulin-lipid complexes to improve loading of insulin into poly(lactic-co-glycolic) acid (PLGA) nanoparticles. This approach further allows easy incorporation of medium chain fatty acids known to enhance drug transport through the intestinal epithelium (1).

Experimental methods

The insulin-lipid complex was prepared by freeze-drying, as previously described (2). PLGA nanoparticles were prepared using the double emulsion-solvent evaporation technique. The insulin-lipid complex was incorporated in the inner aqueous phase and polyvinyl alcohol was used as emulsifier. Nanoparticles were characterized and optimized in terms of size, zeta potential and encapsulation efficacy. The *in vitro* release of insulin in different relevant media was quantified using RP-HPLC.

Results and Discussion

The results showed that PLGA nanoparticles were successfully loaded with insulin. The incorporation of insulin as a lipid complex significantly enhanced the encapsulation efficacy (>80% vs. ~20% without lipids). Further, the resulting nanoparticles had homogeneous size distributions in the range of 200-250 nm. However, the release profile of insulin indicated a very fast burst release occurring within the first hours. Modified PLGA polymers were subsequently tested in order to reduce burst release, increase diffusion and permeability through intestinal mucosa.

Conclusion

Our results demonstrate that the insulin-lipid complex is a good strategy to increase loading of insulin into PLGA-based nanoparticles. The incorporation of absorption enhancers as well as use of modified polymers will be further studied to increase the delivery of insulin across the intestinal barrier.

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DEVELOPMENT OF A NOVEL ORAL COMPLEX FOR IRON DELIVERY: ENCAPSULATION OF HEMIN IN POLYMERIC MICELLES AND ITS *IN-VITRO* ABSORPTION

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Introduction

The aim is to develop a novel micellar complex for oral iron delivery. By encapsulating an analogue of heme iron into micelles composed of a biodegradable and thermosensitive diblock copolymer, we want to achieve increased bioavailability over current iron supplements.

Experimental methods

Hemin which is an iron containing heme analogue, was encapsulated into polymeric micelles consisting of biodegradable thermosensitive diblock copolymer poly(ethylene glycol)-*b*-poly[*N*-(2-hydroxypropyl) methacrylamide-dilactate]; (mPEG-*b*-p(HPMAm-Lac_2)) using different hemin : polymer ratio and concentrations. The hemin-loaded micelles were characterized in terms of loading capacity, encapsulation efficiency, size, cytotoxicity and stability in low and high pH. The micelles with the highest loading capacity were chosen for further *in vitro* absorption testing. *In vitro* absorption of iron was measured after incubation of the hemin-loaded micelles on Caco-2 cells for 24 hours. Iron sulfate, a commercial oral supplement, iron sulfate in combination with ascorbic acid and free hemin were taken along as controls by incubating the same concentrations as the hemin-loaded micelles on the cells. Lysed cells were tested for human ferritin (ELISA), which is the iron storage protein and thus a marker for intracellular iron levels and iron absorption. The ferritin levels were normalized against total protein content of the cells.

Results and Discussion

Hemin was most efficient encapsulated in the micelles with a concentration of 120 µg/ml of hemin in 1,8 mg/ml polymer. This resulted in an encapsulation efficiency of 70% and a loading capacity of 4,5%. The average particle diameter of the hemin-loaded mPEG-*b*-p(HPMAm-Lac_2) micelles was determined by dynamic light scattering and ranged from 75 to 130 nm for concentrations of 40 – 200 µg/ml hemin added to the polymer solution. *In vitro* testing showed that iron sulfate induced maximum ferritin levels of 250 ng/mg cellular protein. The effect of iron sulfate on ferritin reached a plateau at 50 µM. In contrast, hemin-loaded micelles strongly enhanced ferritin expression up to 5000 ng/mg cellular protein, reaching a plateau at 150 µM. Therefore the *in vitro* absorption of Hemin-loaded micelles is 20 times higher than that of iron sulfate. The hemin-loaded micelles also showed to remain stable at pH 2 representing the stomach acidity as also at neutral pH 7.4. Furthermore they also demonstrated superior cell viability compared to iron sulfate and iron sulfate in combination with ascorbic acid.

Conclusion Hemin-loaded micelles with an encapsulation efficiency of 70% and a loading capacity of 4,5% were successfully developed. *In vitro* these micelles drastically increased absorption of iron and therefore ferritin levels, as also give superior cell viability compared to the most often used commercial iron

NANOCRYSTALLINE MAGHEMITE: AN EFFICIENT NOVEL ORAL PHOSPHATE BINDER

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Introduction: Multiple studies have shown a link between dysregulated phosphate homeostasis and increased mortality and morbidity in patients with end stage renal disease (ESRD). Loss of phosphorus homeostasis due to excretion failure results in hyperphosphatemia. This deficiency requires the majority of patients with ESRD to use oral phosphate adsorbers. Of these patients, 40% to 50% remain uncontrolled and a large number are non-adherent to their hyperphosphatemia therapy. The main reasons for low compliance of the current therapies are side effects and high tablet burden. As an approach to a new highly effective, well-tolerated phosphate binder, our group developed the concept of nanocrystalline phosphate-adsorbing maghemite.

Experimental methods: Maghemite (γ-Fe_2O_3) nanoparticles were synthesized by precipitation of $FeCl_2$ and $FeCl_3$ with a sodium hydroxide solution in the presence of D-mannose according to the patent DE102011112898A1. The product thus formed is termed C-PAM-10. The size of maghemite nanoparticles was characterized by transmission electron microscopy (TEM). X-ray diffraction (XRD) and Mössbauer spectroscopy at RT and at 5.4 K were used to characterize the structure of the iron oxide. Multiple in-vitro studies were performed over the range of the gastrointestinal pH to determine the phosphate binding capacity and iron release of C-PAM-10.

Results and discussion: First TEM images revealed particle sizes of around 5 nm. XRD and Mössbauer spectroscopy at RT indicated C-PAM-10 to be an iron oxide but could not distinguish between maghemite and magnetite. Further investigation with the Mössbauer spectroscopy at 5.4 K identified the synthesized product as maghemite. Adsorption in a 0.04 M phosphate solution with adsorber added at a 0.1 M iron concentration resulted in phosphate adsorption per gram iron of 508 mg ± 22 mg (pH 3), 470 mg ± 19 mg (pH 5.5), and 264 mg ± 39 mg (pH 8). Iron release over these pH ranges were < 0.5%. The simulation of a gastrointestinal passage was performed by incubating C-PAM-10 at a 0.1 M iron concentration in a 0.04 M phosphate solution at pH 1.2 followed by a stepwise pH increase to the final pH of 7.5. This revealed a mean phosphate adsorption per gram iron of 596 mg ± 13 mg (pH 1.2) and 658 mg ± 8 mg (pH 2.5), and the adsorption capacity remained relatively consistent as pH 7.5 was reached. Iron release was highest at pH 1.2 (9.2% ± 0.2%) and was not detectable at pH 7.5.

Conclusions: The newly developed nanocrystalline maghemite product combines optimal phosphate-adsorbing properties with minor release of iron ions over the gastrointestinal pH range. These in vitro results make C-PAM-10 a promising candidate as a highly effective oral drug for the treatment of hyperphosphatemia.

AMORPHOUS CapsMorph® WITH HIGH DRUG LOADING CAPACITY FOR ORAL FORMULATION

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Introduction

As a novel technology, CapsMorph® was developed to generate amorphous drug via confining drug in mesopores which do not provide enough space to form crystals. It seems to be a promising strategy to prevent the re-crystallization. Preservation of amorphous state could be proven for a period of > 4.5 years [1]. Another important superiority is the high drug loading capacity, when comparing CapsMorph® to competing approaches, e.g. nanocrystals.

Experimental methods

Model drugs rutin, hesperidin were dissolved in dimethyl sulfoxide (DMSO) (2:11 weight ratio) respectively. 1.3 g solution was added into 1 g porous silica material AEROPERL 300 Pharma dropwisely, under gentle stirring using mortal and pestle. The added DMSO was evaporated at 100 ^{o}C in a compartment dryer. After the fifth addition, the amount of solution added into AEROPERL 300 Pharma was reduced. The samples after the evaporating of DMSO was named CapsMorph®. An x-ray diffractometry (PW1830, Philips, Netherlands) was used to check the amorphous state of drugs after each addition and evaporation to evaluate the maximum drug loading capacity.

Results and discussion

Porous AEROPERL 300 Pharma has a mesopore volume of approx. 1.6 ml/g, coupled with high specific surface area of 300 m^2/g. Therefore, after the fifth addition of 1.3 g solution and the evaporation of DMSO, the amount of added solution was decreased from 1.3 g to 0.65 g. No crystalline halo was observed in rutin and hesperidin CapsMorph® after six times addition of the solution and evaporation of DMSO. The drug loading capacities were more than 50%. The drugs were generated into amorphous form due to the narrow space of the mesopores. There is no sufficient space for the drugs to be arranged into crystal. Some drug is assumed to localize on the surface forming a thin film, which is not able to lead to a crystal formation.

Conclusion

CapsMorph® is a potential future technology for formulating oral poorly soluble drugs in a simple straightforward process. The drug loading capacity is superior to nanocrystals. CapsMorph® appears therefore as attractive alternative delivery solution.

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CYTOTOXICITY AND INFLAMMATORY RESPONSE FOLLOWING EXPOSURE OF POLYMERIC NANOPARTICLES TO A CO-CULTURE OF LUNG CELLS AND MACROPHAGES

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Introduction

The pulmonary route is promising for nanomedicine, allowing local as well as systemic treatments. To model alveolar conditions, we have established and characterized a co-culture of lung alveolar epithelial cells (A549) and macrophages differentiated from human monocytes (THP-1). The co-culture was investigated as an *in vitro* tool to evaluate the cytotoxicity and the inflammatory response after exposure to polymeric biodegradable nanoparticles, relevant as nanocarriers.

Experimental methods

Cell phenotypes in co-culture (CD14, CD11b and CD54) were investigated by flow cytometry. CD14 was used to identify each cell population in co-culture. PLGA nanoparticles were prepared according to an emulsion-evaporation process and stabilized, or not, with polyvinyl alcohol, chitosan or Pluronic® F68. Uptake kinetics (flow cytometry and confocal microscopy), cytotoxicity tests (MTT, trypan blue, selective membrane permeability, LDH release, apoptosis/necrosis) and inflammatory response quantification (MCP-1, IL-6, IL-8 and TNF-α cytokines) were performed after 2, 4, 24 h and 48 h exposure to nanoparticles, on cells in mono and co-culture.

Results and Discussion

All PLGA nanoparticles have a similar size (200-230 m) with different zeta potentials (negative, positive or neutral) and all induce an energy-dependant uptake mechanism, in both cell types. In addition, negatively-charged nanoparticles are internalized in higher rate. In co-culture, macrophages internalized nanoparticles faster and in higher quantity than lung epithelial cells, regardless of their surface properties. PLGA nanoparticles were shown to cause low membrane damages and the observed death was only necrosis. The cytotoxicity of PLGA nanoparticles stabilized with chitosan was consistent with the cytotoxicity of chitosan only. In contrast, the cytotoxicity of other PLGA nanoparticles was attributed to the nanoparticle form. Cytokine secretion levels after exposure to LPS or nanoparticles were higher in co-culture than on single cell lines, confirming the interest for this co-culture to study the inflammatory response. Nanoparticles were found to induce mild but significant cytokine secretions, in a concentration-dependent manner.

Conclusion

The co-culture of epithelial lung cells and macrophages is a sensitive model to detect the inflammatory response to nanoparticles, thanks to synergistic effects compared to single cell lines, and allows the study of selective uptake of nanoparticles. Differences observed among PLGA nanoparticles in terms of cytotoxicity, uptake and inflammatory response highlight the importance of the coating of nanocarriers relevant for drug delivery (1).

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THE ACYL CHAIN LENGTH OF TREHALOSE-6,6'DIBEHENATE ANALOGUES (TDX) EFFECTS THE PHYSICOCHEMICAL PROPERTIES AND IMMUNOLOGICAL PROFILE OF DDA/TDX LIPOSOMAL ADJUVANTS

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Introduction

The cationic liposomal adjuvant based on the surfactant dimethyldioctadecylammonium (DDA) bromide and the glycolipid trehalose-6,6′-dibehenate (TDB) is currently undergoing clinical development. It facilitates antigen delivery and induces a mixed Th1/Th17 response against co-administered antigens via interaction with the Mincle receptor [1,2]. The overall hypothesis of this study was that the structural characteristics of TDB are decisive for the immunopotentiating effect and the physicochemical properties of the adjuvant system. Establishment of structure-activity relationships is important in order to further develop this type of adjuvants. The aim was to study how the acyl chain length in TDB analogues (denoted TDX) affects the physicochemical and immunostimulatory properties of the DDA/TDX formulations.

Experimental Methods

DDA/ TDX liposomes were prepared at a molar ratio of 89:11 by the thin film method (TDX : TDB C_{22} chains, TDA C_{20} chains, TDS C_{18} chains, TDP C_{16} chains, TDMyr C_{14} chains and TDL C_{12} chains). The physicochemical properties of the DDA/TDX formulations were investigated in terms of colloidal stability, thermodynamic behavior and lipid-lipid interactions by dynamic light scattering (DLS), differential scanning calorimetry (DSC) and the Langmuir technique respectively. An immunization experiment in mice was conducted to investigate the immunostimulatory properties of the DDA/TDX formulations.

Results and Discussion

Incorporating the TDX component into DDA liposomes resulted in a high colloidal long-term stability, and the size of these monodisperse formulations was approximately 125-200nm over a 17 week period. DSC data showed a narrow phase transition of the DDA/TDS and DDA/TDP formulations and incorporation of the TDS and TDP component in a DDA monolayer resulted in a tight lipid packing. Testing the adjuvant formulations DDA/TDS and DDA/TDP combined with an antigen in an immunization experiment resulted in a significantly increased induction of the Th1/Th17 response and formation of both memory and effector $CD4^+$ T cells when the adjuvant formulation consisted of DDA/TDP.

Conclusion

This supports the hypothesis that the structure of the glycolipid component is of great importance for the physicochemical properties and the immunological profile of the adjuvant system.

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FORMULATION OF A CATIONIC LIPOSOMAL ADJUVANT WITH DIFFERENT DDA-ANALOGUES AFFECTS THE CD8$^+$ T-CELL RESPONSE

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Introduction

A robust CD8$^+$ T-cell response is very important in the immune response against intracellular pathogens, e.g. viruses. Induction of a CD8$^+$ T-cell response following vaccination requires cross-presentation by lymph node-resident CD8-α$^+$ dendritic cells, which can be targeted by freely draining vaccine particles (1).
The hypothesis is that the free drainage of a vaccine is dependent on the particle size distribution and surface charge of the adjuvant particles, where smaller and less charged particles display better drainage abilities (1, 2).

Experimental methods

The cationic, liposomal adjuvant CAF09 based on DDA, and the immunostimulator MMG and the synthetic TLR3-ligand poly(I:C). CAF09 was prepared by rehydrating the lipid film with either heating and intermittent vortexing or constant heating and high shear mixing. Furthermore, DDA (C18) was replaced with one of the analogues DHDA (C16) or DDDA (C12). The formulations were characterized by size and membrane fluidity, and the adjuvant effect was tested with the model antigen Ovalbumin by s.c. injection of mice 3 times with two-week intervals.

Results and Discussion

The use of high shear mixing reduced the particle size distribution significantly compared to vortexing, and created a more monodisperse formulation, probably due to the increased amount of energy introduced into the system. Replacing DDA with the analogues further reduced the particle size distribution as well as increased the membrane fluidity. The CD8$^+$ T-cell response was increased when using the formulations made with high shear mixing, indicating that a reduced particle size had a positive effect. However, when the membrane was fluid at physiological temperature, as was seen with the DDDA-containing formulation, the CD8$^+$ T-cell response was abrogated despite the small average particle size.

Conclusion

The results of the *in vivo* experiments indicate that the particle sizes of the CAF09 formulations inversely affect the CD8$^+$ T-cell response. Furthermore, the fluidity of the lipid membrane also played a role in the induction of an immune response, as it was decreased with increasing fluidity.

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TOWARDS NANOFORMULATIONS OF NATURAL ANTI-INFLAMMATORY COMPOUNDS

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Introduction

Chronic inflammation is not only involved in immune disorders but is also fundamental to diseases such as cancer and cardiovascular disease [1]. This makes inflammation an important target to overcome the burden these diseases carry. The current treatments of inflammation and inflammatory diseases is hugely dominated by steroidal drugs, which are the strongest anti-inflammatory agents known. However, they possess a broad range of side-effects, patients vary in response and glucocorticoid resistance may occur [2]. The need for alternative treatments is substantial and could probably be found in the imminent field of natural compounds.
In this study we built an assay with which we can easily determine the cytokine production pattern of macrophages in response to an inflammatory stimulus in the presence and absence of compounds. This allows us to assess the anti-inflammatory profile of selected natural compounds compared to prednisolone disodium phosphate (PLP) as golden standard. We aim to select powerful natural compounds as alternatives for steroidal drugs that possess an attractive cytokine inhibition/stimulation profile. These compounds can additionally be encapsulated in delivery systems to enhance their activity.

Experimental Methods

Our assay was performed with RAW 264.7 murine macrophages. For the assay, RAW cells were seeded in 24-well-plates at a 500,000 cells/mL concentration 6-8 h prior to incubation to allow the cells to settle. Subsequently, cells were incubated (n=6) for 2 h with medium containing 50 μg/mL PLP (positive control), medium (negative control) or the selected compounds. After incubation, half of the wells (n=3) were stimulated for 12 h with 250 ng LPS/mL whereas the other half was not stimulated and served as a control. The anti-inflammatory profiles of the compounds were established by screening a range of pro- and anti-inflammatory cytokines (such as IL-6, TNF-α and IL-10) using ELISA and/or RT-PCR. Profiles were then compared to the robust anti-inflammatory profile of PLP.

Results and Discussion

Stimulated control macrophages secreted IL-6, showing that the cells were activated. Treatment with 50 μg PLP/mL significantly reduced IL-6 secretion with 72-87% (two-tailed t-test: $p=0.00668$ and $p=0.00001$). At a 50 μg/mL concentration, pterostilbene phosphate, a derivative of the well-known polyphenol resveratrol found in red wine, reduced IL-6 secretion with only 10% ($p=0.58$). And 50 μg epicatechin gallate/mL, an antioxidant in green tea, significantly increased the IL-6 secretion with 112% ($p=0.002$). Interestingly, 50 μg/mL of berberine chloride (BB), a quarternary alkaloid from *B. vulgaris*, significantly reduced the IL-6 concentration with 47-60% ($p=0.00074$ and $p=0.012$), while incubation with 100 μg BB/mL was equipotent to PLP (reduction of 83%, $p=0.00026$).
Our assay provides a quick screening method to assess the anti-inflammatory profile of natural compounds; we have identified berberine chloride as a potent compound that can now be formulated for optimal delivery to and inhibition of inflammation.

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Posters

COMPARISION OF METHODS TO PRODUCE DERMAL AZITHROMYCIN NANOSUSPENSIONS TO PREVENT LYME BORRELIOSIS INFECTION

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Introduction

Lyme disease, a common tick-borne infection in both Europe and North America, can be prevented by treatment with azithromycin. It has a shorter therapy, quicker onset time and lower dosage than other antibiotics. However, using antibiotics systematically may cause side effects, like bacterial resistance to administered antibiotic and malaise or toxicity. Thus the dermal application of azithromycin is a novel and good strategy to prevent Lyme Borreliosis after tick bites. Considering the poor solubility of azithromycin, we choose three different production methods without any organic solvents to reduce its particle size, consequently increasing its saturation solubility and dissolution velocity. Tocopheryl polyethylene glycol succinate (TPGS) was selected as surfactant due to its safety, permeation enhancement and drug retention profile [1].

Experimental methods

Production of nanosuspensions by high pressure homogenization (HPH)
Nanosuspensions, which contained 10% (w/w) coarse azithromycin dihydrate powder in 1% (w/w) aqueous surfactant solution, were produced by Micron LAB40 applying 15 cycles and 1500 bar, after pre-mixing by Ultra Turrax for 1 min at 8000 rpm.
Production of nanosuspensions by pearl milling (PM)
Nanosuspensions, which had the same composition, were prepared by pearl milling using a PML 2 (Bühler; pearl size 0.1 mm) for 10 min, after analogous pre-mixing.
Production of nanosuspensions by combination technology (CT)
The pearl milled samples were subsequently treated with HPH (1 cycle, 300 bar).
Characterization of the nanosuspensions
Mean particle size (z-ave) and poldispersity index (PI) of the three nanosuspensions were measured by photon correlation spectroscopy (PCS) at day 0 and after 1 month. Zeta potential (ZP) was analyzed by laser Doppler anemometry.

Results and Discussion

PM proved to be the most suitable method providing smallest, highly adhesive nanocrystals among the three methods, taking PCS z-ave (189 nm), PI (0.194), ZP (-31.1 mV), process time (10 minute) and short term stability (1 month) as index. Subsequent processing by HPH in the combination technology did neither lead to further size reduction nor to a distinct improvement in the homogeneity of the nanosuspensions. Very favorable was the short production time in PM.

Conclusion

Azithromycin nanosuspensions with particle size less than 200 nm were fabricated within 10 minutes by PM, exhibiting good physical stability by now. In future studies the nanocrystals will be processed into a gel formulation for dermal use and the penetration behavior will be investigated (pig ear skin test).

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NANO LIPID CARRIERS (NLC) & NANOCRYSTAL FORMULATION FOR COUPEROSIS TREATMENT

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Introduction

Couperosis is one of the most common dermatosis in adulthood with 45 million affected people worldwide (2010). At the beginning, it is characterized by long-lasting facial redness, especially on the nose and the cheek area, caused by the weakness of the binding tissue and the low elasticity of smaller capillaries. When untreated, the instable vessels get damaged, leading to subcutaneous bleeding, visible as permanent violet lines. Therefore, a timely treatment with effective agents is essential to get the disease controlled for a better looking and feeling skin.

Technical considerations

With regards to the selection of active agents, the main focus is on skin soothing properties, a stabilization of the superficial blood vessels, antimicrobial prevention as well as regenerating supporting effects, which all should be integrated into one final formulation. Flavonoids are known for their capillary stabilizing action by reducing the permeability of blood vessels. Comparing the permeability reducing activity of different flavonoids, rutin is the most effective one with the highest anti-permeability-factor (APF) of 8.5 [1]. The problem of poor solubility can be circumvented by producing drug nanocrystal with a size range of 500 to 700 nm. In addition to rutin, vitamin A_1 also seems to be a suitable active agent for the final formulation against couperosis, since it promotes the regeneration of the skin and the neoformation of collagen. Commonly, Vitamin A_1 is used in combination with vitamin K_1 in a ration of 3:10, due to the proven synergistic effects against subcutaneous bleeding [2], caused by vascular stabilizing effects. The limited chemical stability of both vitamins can be a problem for use as a dermal active. Thus, NLC are an optimal delivery system as the solid lipid is able to protect incorporated active agents against chemical degradation.

Results and Discussion

For use as active agents, high physicochemical stable rutin nanocrystal and vitamin A_1 and K_1 NLC formulations could be successfully developed and were incorporated into a hydroxypropyl cellulose gel base containing glycerol as an antimicrobial and penetration promoting adjuvant.

Conclusion

The final formulation could be produced successfully and is now available for testing on patients with couperosis.

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COMPARISON OF THE NANONIZATION POTENTIAL OF HIGH SPEED STIRRING AND PEARL MILLING

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Introduction

ARTcrystal® technology is a combination process for the low-cost production of nanocrystals. The process contains of a premilling part, carried out by a high speed rotor stator system, and is followed by a quick high pressure homogenization process at reduces pressure (2 cycles at 300 bar) [1].
This study's aim was the comparison of the nanonization potential of the high speed rotor-stator system as a standalone system for nanocrystal production to nanonization process by pearl milling.

Experimental methods

Macrosuspensions of coarse and jet milled rutin powder (5% (w/w)), stabilized by Plantacare® 2000, were produced and processed by either 10 minutes high speed stirring in an ART MICCRA D27 system (ART Prozess- und Labortechnik, Müllheim, Germany) at 24,000 rpm or up to 30 minutes of pearl milling at 1100 rpm in cycles of 1 minute milling and 5 minutes of cooling in a Pulverisette 7 premium line pearl mill (FRITSCH GmbH, Idar-Oberstein, Germany). 1 mm ZrO pearls were used for milling. The D27 system was cooled by an Alpha RA12 thermostat at -10°C (Lauda, Lauda-Königshofen, Germany).
Particle size characterization was performed by laser diffraction (Mastersizer 2000), dynamic light scattering (Zetasizer Nano, both Malvern Instruments, Malvern, UK) and light microscopy (DM1000, Leica Microsystems, Wetzlar, Germany).

Results and Discussion

While the D27 system could obtain a nanosuspension from jet milled material with needle-shaped crystals (657 nm), coarse material experienced a distinct reduction in size but was not transformed into nanoscale (1817 nm). The pearl mill exposed a much greater potential in diminuting coarse rutin material, but the Pulverisette 7 misses the ability of cooling, thus reaching process temperatures around 55 °C, measured after 15 and 30 minutes of milling, leading to agglomeration of the needle-shaped nanocrystals obtained from jet milled rutin (1447 nm). Processing coarse material lead to a submicron-suspension (957 nm), with microscale crystals present.

Conclusion

The D27 system is superior to the Pulverisette 7 pearl mill regarding the processing of jet milled material concerning production time and costs, while the D27 lacks the possibility to diminute coarse material into a nanosuspension. To obtain this goal, additional high pressure homogenization at low pressures must be applied.

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ULTRAFINE GELATIN NANOPARTICLES FOR DERMAL DELIVERY OF PEPTIDES & ENZYMES: PRODUCTION AND CHARACTERIZATION

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Introduction

Application of peptides and enzymes in skin care products is increasingly a focal point of interest in cosmetic industry. The attractive advantages of gelatin nanoparticles (GNPs) as delivery system of hydrophilic drugs have been confirmed by many investigations [1]. Smaller particle size leads to increased interface area and adhesive force to the skin. Therefore, the aim of present work was to produce enzyme loaded ultrafine GNPs below 100 nm by using lysozyme as model active.

Experimental methods

A modified two-step desolvation technique was used to produce lysozyme loaded ultrafine GNPs [2]. The particle size analysis was performed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK). The drug loading efficiency was evaluated by HPLC. *In vitro* drug release test was performed by using static Franz diffusion cells at 32°C over 36 hours. The lysozyme concentration was analyzed using HPLC and the cumulative percentage drug release was calculated. The remained biological activity of lysozyme after incorporation was estimated also by HPLC**.**

Results and Discussion

Produced ultrafine GNPs possessed a smallest mean particle size of 58 nm. Incorporation of lysozyme only slightly increased the particle size to 75 nm. The polydispersity index was lower than 0.20, indicating a homogeneous size distribution. A drug loading efficiency of 90% was obtained by HPLC. The loading capacity of ultrafine GNPs was calculated to be 0.12 g lysozyme per gram GNPs. *In vitro* release study demonstrated that approximately 60% in 12 hours, and 70% of the loaded lysozyme could be released from GNPs over a period of 36 hours. The biological activity of the incorporated lysozyme was proven to be 93% as estimated by HPLC.

Conclusion

Enzyme loaded GNPs with particle size below 100 nm could be produced. The activity of the model enzyme remained due to the gentle production conditions. This successful technique could also be transferred to other peptides or enzymes for dermal delivery. By further optimization nanoparticles even smaller than 50 nm appear feasible.

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RISPERIDONE LOADED PLGA MICROSPHERES: FORMULATION DEVELOPMENT AND DEGRADATION STUDY

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Introduction

Medication non-adherence is a major obstacle in the proper treatment of schizophrenia and bi-polar depression. Therefore, a prolonged acting formulation to overcome non-compliance is valuable. A PLGA-based intramuscular (IM) microsphere (MS) of risperidone (Risperdal Consta®) has been approved by the FDA to address the need for a long-acting formulation of antipsychotic medication. Risperdal Consta® sustains the effective plasma drug concentration for 2 weeks after IM administration. However, this marketed formulation has limitations of a long lag phase (~3 weeks) in which the formulation is not therapeutically active. Moreover, this formulation does also not exhibit zero-order release kinetics and this is been suggested to be the cause of observed side effects [1]. To overcome these problems, risperidone -loaded PLGA MS with zero order release kinetics (intended for 5 weeks) were developed and investigated for drug distribution in the PLGA matrix drug release and degradation kinetics *in vitro* as a step-up for later *in vivo* application.

Experimental Methods

Risperidone-loaded MS were prepared by solvent evaporation method by using capped and uncapped PLGA 50:50 and characterized for drug loading and *in vitro* drug release by HPLC, particle size distribution by laser diffractometry, surface morphology by SEM and drug distribution in the particle matrixes by DSC. Particle degradation profiles during the release period were estimated by change in glass transition temperature (Tg in °C) and polymer molecular weight (Mw) using DSC and gel permeation chromatography (GPC).

Results and Discussion

Capped (RMS) and uncapped (A-RMS) PLGA MS show a high risperidone loading and entrapment capacity (>27% and >82%). DSC study illustrated that risperidone is dissolved in the particle matric but also present in crystalline form. Yet, dissolved risperidone shows crystallization in capped PLGA during heating above Tg. RMS showed a burst release phase (about 10%). A-RMS showed continuous release approaching zero order release kinetics over the period of 5 weeks. Degradation studies showed that risperidone accelerated the degradation of capped PLGA only. Drug release from both MS formulations could be shown to be governed by both drug diffusion and polymer degradation.

Conclusion

Prolonged zero order release risperidone MS were developed and characterized successfully. In vivo (PK/PD) evaluation studies are underway.

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MEMBRANE FUSION WITH COILED-COIL LIPOSOMES

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Introduction

Biological membranes are the main barrier for intracellular delivery. Despite their similarity, liposomal membranes do not readily fuse with cellular membranes. However, in Nature there are many processes that involve rapid and complete membrane fusion and these are mostly regulated by proteins that contain large coiled-coil domains. In this project, we have functionalized two populations of liposomes with complementary coiled-coil forming peptides.

Experimental methods

The peptide sequences that we use here are heptad repeats of lysine-rich peptides (K3) and glutamic acid-rich peptides (E3) that are anchored in the lipid bilayer with a cholesterol anchor and a short PEG-spacer. The liposomes consist of a DOPC/DOPE/Cholesterol mixture (50/25/25 ratio) and are approximately 150nm in size. Two populations of liposomes are made that are functionalized with the K or the E peptides. When these populations fuse, an increase in size can be measured by DLS.
To prove that there is also lipid exchange between the liposomes, the CPK containing liposomes are labeled with 0.5% NBD-PE and 0.5% Lissamine Rhodamine-PE (LR)
NBD and LR form a FRET pair and when they are together in the bilayer, NBD is quenched by LR. When two populations of liposomes fuse, the probes are 'diluted' over the other membrane and the FRET distance increases, resulting in dequenching of NBD over time.

Results and Discussion

When CPK and CPE containing liposomes are mixed together, an increase in particle size is measured. This increase is peptide mediated, since control experiments where CPK or CPE containing liposomes are mixed with unfunctionalized liposomes did not show an increase in size. This size increase could indicate fusion, but it does not rule out the possibility that the complementary peptides just make the liposomes aggregate. The lipid mixing assay does prove that there is indeed an exchange of lipids between the two populations, that is again only seen when the K and E peptides are mixed together and not in the control experiments.

Conclusion

Complementary coiled-coil forming peptides can induce liposome-liposome fusion. This is shown by an increase in size, but a lipid mixing assay shows that there is also exchange of lipids between liposomes, indicating actual fusion of the bilayers.

DEVELOPMENT OF METHODOLOGY FOR THE SEPARATION AND QUANTIFICATION OF LIPOSOME-BOUND AND FREE DOXORUBICIN IN PLASMA

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Introduction

Magnetic Resonance Imaging - Guided High Intensity Focused Ultrasound (MR-HIFU) is an emerging technology that holds great potential for improving the efficacy of chemotherapy through the triggered local release of anticancer agents from targeted nanomedicines. In the HIFU-CHEM project, the clinical application of MR-HIFU in combination with a thermosensitive liposomal doxorubicin formulation is addressed. Thermosensitive liposomal doxorubicin (ThermoDox©) is a novel nanomedicine formulation that is stable at body temperature (37°C) but rapidly releases its cytostatic content at temperatures raised slightly above body temperature (42°C). For the pharmacokinetic part of the clinical study, we report here on a bioanalytical method that has been developed successfully for the determination of liposome-encapsulated and free doxorubicin in blood samples of patients.

Experimental methods

A solid phase extraction (SPE) method was developed and optimized to achieve an efficient separation of the liposome-encapsulated and free doxorubicin fractions in media of Hepes buffer (HBS), fetal bovine serum (FBS) and human plasma. Initial experiments were performed on samples that contained either only liposomal doxorubicin (ThermoDox©) or only free doxorubicin to measure recovery percentage after SPE method application. Following these experiments, mixtures that contain both liposomal and free doxorubicin (in different ratios) were analyzed to test the separation efficiency of the SPE method.

Results and Discussion

The developed SPE method provided good recovery of encapsulated and free doxorubicin alone, in all media. Further experiments where mixtures of these fractions were used also revealed efficient separation and recovery of the fractions, also in protein-rich media such as human plasma.

Conclusion

We hereby conclude that the SPE method that was developed enables the physical separation and recovery of encapsulated and free fractions of doxorubicin from buffer, serum and plasma samples. This will allow a robust measurement of the stability/release profiles of the liposomes in both in-vitro and in-vivo experiments.

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FORMULATION OF IMATINIB INTO PLGA MICROSPHERES AS A PLATFORM FOR INTRATUMORAL DELIVERY OF KINASE INHIBITORS

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Introduction

There is an urgent need for drug delivery devices that can localize the activity of antitumor agents, to achieve long-term tumor inhibition with less detrimental effects. Poly (lactic-co-glycolic acid) (PLGA) microspheres are an excellent carrier system for sustained drug release, and such depots can be injected locally in close proximity to the tumor. Imatinib is a tyrosine kinase inhibitor that is used as molecularly targeted therapy in different types of cancer (1). Imatinib is an ionizable amphiphilic compound with a logP around 3. It is known that the formulation of this kind of drugs using emulsion solvent evaporation, the most common method, is challenging due to drug transfer into the water phase during preparation and consequently low encapsulation efficiency. In this study we present an efficient encapsulation protocol and obtained microspheres with a high loading efficiency which release imatinib during a tow-week period.

Experimental methods

Imatinib-PLGA microspheres were prepared by a double emulsion solvent evaporation method in which imatinib was dissolved in water with different concentrations. The inner water phase (250 µl imatinib solution) was then emulsified into 0.5 ml of PLGA solution (23 w/w% in dichloromethane) at high speed, the primary emulsion was further emulsified in 1% PVA containing buffer of different pHs to form the final particles. The release kinetics of the PLGA imatinib microspheres was studied in phosphate buffer pH 7.4 at 37°C, under sink conditions.

Results and Discussion

By increasing the pH of the external water phase, encapsulation efficiency was improved from 10% at pH5 to 90% at pH9. The average size of particles ranged from 20 µm for low drug loading (0.5% w/w) to 8 µm for microspheres with high drug loading (11% w/w). A plausible explanation for this difference in size is that imatinib acts as an emulsifier which leads to the reduction in particles size.

PLGA microspheres release imatinib in a diffusion controlled manner After a small burst (around 10% of the loading) about 70% of imatinib was released in a sustained manner over a period of 14 days. Differential scanning calorimetry (DSC) showed that the drug was molecularly dispersed in the polymer matrix, which is in agreement with the observed release profile.

Conclusion

We have developed an efficient procedure for microencapsulation of an amphiphilic drug (imatinib). The resulting imatinib PLGA microspheres showed a two-week sustained release profile. Further studies will address the therapeutic benefit of imatinib microspheres as drug depot in cancer treatment.

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MULTIMODAL IN VIVO AND EX VIVO OPTICAL IMAGING TO STUDY BIODISTRIBUTION AND INTRATUMORAL PENETRATION OF DUAL-LABELED CORE-CROSSLINKED POLYMERIC MICELLES

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Introduction. Multi-modal imaging based on combined imaging techniques, such as CT-FMT, has shown to be able to more accurately quantify the biodistribution of fluorophore-labeled polymeric nanocarriers.[1] The aim of this work was to study the biodistribution of core-crosslinked polymeric micelles (CCPM).

Experimental methods. mPEG-b-p(HPMAm-Lac-co-ANHS) was synthesized by free radical polymerization and subsequently labeled with Dy-488 and Dy-676 via the NHS-activated carboxylic acid groups of the block copolymer. The micelles were prepared by heating the polymer solution in PBS 7.4 at 50 ^{o}C for one minute, and crosslinked by cystamine. The CCPM were i.v. injected into mice bearing EPR-prone CT26 tumors, and their biodistribution and tumor accumulation were monitored using novel 3D CT-FMT, which was compared to ex vivo conventional 2D FRI, to analyze the biodistribution of the CCPM. Tumors were analyzed by 3D two-photon laser scanning microscopy (TPLSM) to reveal the intratumoral distribution of the CCPM.

Results and Discussion. The micelles showed a time-dependent increase in tumor accumulation (~4% of the injected dose (ID) at 24 h post i.v. injection). The liver uptake of the CCPM was found to be ~20% at 48 h p.i., which results from clearance via the mononuclear phagocytic system (MPS). A relatively prominent kidney accumulation was observed (~20% of the injection dose at 1 h, and increasing to ~30% at later time points). At early time points, also localization in bladder could be detected (~10% ID at 1 h), indicating that part of the kidney signal reflected renal excretion. FRI imaging also indicated that the CCPM most prominently accumulated in kidney, liver and tumor. TPLSM image in which second harmonic generation (SHG) imaging was employed to visualize collagen fibers, indicated that the CCPM penetrate relatively deeply into the tumor interstitium, and are distributed relatively homogenously within tumors.

Conclusion. In vivo imaging using 3D CT-FMT and 2D FRI showed that the micelles accumulated reasonably efficiently in tumors, and prominently in kidney and liver. Ex vivo imaging by 3D TPLSM confirmed the extravasation of the micelles out of tumor blood vessels, as well as their penetration relatively deep into the tumor interstitium.

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AGGREGATION AND GELATION BEHAVIOR OF STEREOCOMPLEXED FOUR-ARM PLA-PEG COPOLYMERS CONTAINING NEUTRAL OR CATIONIC LINKERS

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Introduction

Self-assembly of poly(lactide)-Poly(ethelyne glycol) (PLA)-(PEG) block copolymers into hydrogels, micelles, wormlike micelles, vesicles or polymersomes in water is well-known. The obtained structure is dependent on the, amount dissolved, molecular weight of the blocks, the hydrophilic/hydrophobic ratio and copolymer architecture. In recent years, cationic pH responsive polymers have become of interest due to their ability to form ionic complexes with anionically charged biomacromolecules. Here we describe the preparation of 4-arm $(PEG\text{-}PLA)_2$-R-$(PLA\text{-}PEG)_2$ block copolymers with a central aliphatic linker (R) or when the linker (R) contains secondary amine groups and investigate their self-assembly behavior by analysis of the micelles and obtained hydrogels.

Results and Discussion

Preparation of 4-arm $(PEG\text{-}PLA)_2$-R-$(PLA\text{-}PEG)_2$ block copolymers (ABA type) with a heptane linker (R) afford thermo-reversible hydrogels. These copolymers are present in solution as core shell type aggregates, which form hydrogels by entanglements of PEG chains at lower temperatures.

In contrast, when the linker (R) contains secondary amine groups no hydrogels were formed. In this case micelles are formed with a random distribution of hydrophobic and hydrophilic domains in which the positive charges are shielded by the PEG blocks. This hinders the formation of hydrogels through PEG entanglements when the temperature of the copolymer solutions is lowered. More importantly, in this mechanism the core shell structure of the micelle is disrupted slightly and hydrophobic groups are present at the in the hydrophilic PEG shell. This makes the 4-arm $(PEG\text{-}PLA)_2$-R-$(PLA\text{-}PEG)_2$ with a linker (R) that contains secondary amine groups an interesting candidate for stereocomplexation.

To this end we studied the time dependent assembly of the enantiomeric copolymers upon mixing and stereocomplexation. When R is a heptane linker a thermo-reversible gel is obtained. Gelation is driven by the formation of PEG entanglements at lower temperatures.

Incorporating cationic moieties in the central linker (R) enantiomeric mixtures of these copolymers afforded at relatively high concentrations stereocomplexed gels which are thermo-irreversible. The gelation mechanism of these copolymers is related to the formation of stereocomplexed domains randomly dispersed in the gel, which act as crosslinks.