Guiding Liposomal Therapeutics to Clinical Evaluation - Points to Consider in Formulation and Process Development

Andreas Wagner
A PRIVATE COMPANY
Developing and Manufacturing Biopharmaceuticals
and Liposomal Formulations for Human Application

- CEO: Dr. Dietmar Katinger
- Founded: 1992
- Employees: 55
2011 Polymun has moved to a new site

DONAUSTR. 99, 3400 KLOSTERNEUBURG, AUSTRIA
Core activities

- CONTRACT DEVELOPMENT OF BIOPROCESSES
  for the production of bio-pharmaceuticals for human application with focus on mammalian cell culture products

- CONTRACT MANUFACTURING OF BIOPHARMACEUTICALS
  production license according § 63 of the Austrian pharmaceutical law

- LIPOSOMAL FORMULATION OF DRUGS AND VACCINES
  formulation development and production of GMP-material

- RESEARCH REAGENTS
  manufacturing and distribution, mainly HIV reagents and recombinant trypsin

- OWN R&D PROJECTS
  funded by revenues from contract development and contract manufacturing
# Selected reference projects - Liposomes

<table>
<thead>
<tr>
<th>CUSTOMER/PARTNER</th>
<th>PRODUCT</th>
<th>SCOPE OF POLYMUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mirna Therapeutics Inc., USA</td>
<td>Liposomal miRNA MRX34 with Smarticle technology for cancer treatment</td>
<td>Process development, GMP production</td>
</tr>
<tr>
<td>Wittycell SAS, France</td>
<td>Liposomal adjuvant WTCc</td>
<td>Formulation and process development, GMP production</td>
</tr>
<tr>
<td>AC Immune SA, Switzerland</td>
<td>ACI-24, liposomal Alzheimer’s disease vaccine with MPLA adjuvant</td>
<td>Process development, GMP production</td>
</tr>
<tr>
<td>EuroNeut-41, EU FP7 project coordinated by Sanofi-Pasteur, France</td>
<td>Liposomal formulation of HIV membrane protein gp41 and MPLA as HIV vaccine</td>
<td>Formulation and process development, GMP production</td>
</tr>
<tr>
<td>Signpath Pharma, USA</td>
<td>Liposomal formulation of curcumin for the treatment of cancer</td>
<td>Formulation and process development, GMP production</td>
</tr>
<tr>
<td>ProNAi Inc., USA</td>
<td>Liposomal of DNAi PNT100 with Smarticle technology for cancer treatment</td>
<td>Process development, GMP production</td>
</tr>
<tr>
<td>Dafra Pharma Research and Development bvba, Belgium</td>
<td>OIPC liposomes for the treatment of leishmaniasis</td>
<td>Formulation and process development, GMP production</td>
</tr>
<tr>
<td>Merck Serono, Germany</td>
<td>Survivac, liposomal cancer vaccine</td>
<td>Formulation and process development</td>
</tr>
</tbody>
</table>
Liposomes from a CMC point of view

- Raw Materials
- Formulation Procedure
- Refining Process
Raw materials - Lipids

- Lipid quality
- Analytics: qualitative and quantitative methods
- Chemical, physiological properties
  - Formation of bilayer structure
  - pH sensitivity
  - Fusogenic activity
  - Amphoteric properties
- Chemistry
  - Saturated vs. unsaturated phospholipids
  - Long chain vs. short chain lipids
- Stability:
  - Behavior/Stability upon processing
  - Sterilization behavior
  - Stability upon storage
Lipid Quality: QA & QC expectations

- CoA
  - Purity
  - Impurities: degradation products, byproducts
  - Process related impurities: residual solvents, heavy metals
  - Microbial attributes: endotoxin, bioburden
  - Appearance
- TSE/BSE statement
- Certificate of origin
- Certificate of Compliance
# Lipid Quality: Certificate of Analysis

<table>
<thead>
<tr>
<th>Specification</th>
<th>Results</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl choline (P x 24.54, on an anhydrous weight basis)</td>
<td>98.7 %</td>
<td>TP-PC</td>
</tr>
<tr>
<td>Lysophosphatidyl choline</td>
<td>0.1 %</td>
<td>ADC3</td>
</tr>
<tr>
<td>Concomitant components</td>
<td>1.0 %</td>
<td>ADC3/ADC5</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>4.0 - 4.2 %</td>
<td>TP</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.1 %</td>
<td>ADC5</td>
</tr>
<tr>
<td>Fatty acids composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>50.6 %</td>
<td>GC-FS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>49.3 %</td>
<td>GC-FS</td>
</tr>
<tr>
<td>Water (KF)</td>
<td>0.4 %</td>
<td>USP&lt;921&gt;1a</td>
</tr>
<tr>
<td>Ethanol</td>
<td>151 ppm</td>
<td>GC-LM</td>
</tr>
<tr>
<td>Aceton</td>
<td>1.1 ppm</td>
<td>GC-LM</td>
</tr>
<tr>
<td>Peroxide value</td>
<td>0</td>
<td>POZ</td>
</tr>
<tr>
<td>Jodine value</td>
<td>33</td>
<td>JZ</td>
</tr>
<tr>
<td>Consistency</td>
<td>powder, agglomerates</td>
<td>powder, agglomerates</td>
</tr>
<tr>
<td>Color</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>1.0 ppm</td>
<td>USP&lt;231&gt;11</td>
</tr>
<tr>
<td>Endotoxins (LAL-Test)</td>
<td>1.1 EU/g</td>
<td>EU</td>
</tr>
<tr>
<td>Bacteriological account</td>
<td>n.d.*</td>
<td></td>
</tr>
<tr>
<td>C.F.U. / g</td>
<td>max. 100</td>
<td>n.d.*</td>
</tr>
<tr>
<td>E. Coli / 10g</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

Liposome Technology, Wagner | Page 9
Lipid Quality: Certificate of Origin, TSE/BSE Statement

All types of [removed] synthetic phospholipids are made from purely plant-derived materials, i.e. from natural sn-glycero-3-phosphocholine and vegetable fatty acids or non-animal materials. As such, they are not addressed by the EMEA Guideline 410/01 review 2 on “Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products”.

During receipt, manufacturing, storage and distribution, the products do not come into contact with any animal-derived components that are BSE-TSE risk materials. Hence, there is no risk of BSE / TSE to be considered with our synthetic phospholipids.

We will inform our customers, should there be any change to the statements above.

Statement of Origin

Dear Sir/Madam:

As you requested, I have included the source of the starting materials for the following product(s):

1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (#770557 and 850457)

Glycerophosphocholine (GPC): produced from soybean lecithin isolated from soybeans grown in the [removed]

Palmitic Acid: refined from plant oils (palm oil)

Oleic Acid: refined from plant oils (high oleic sunflower)

This material is manufactured by [removed] facility [removed].

All other materials and excipients used in the synthesis and purification of 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) are not of animal origin. If you have any questions or need additional information, please do not hesitate to contact me.
Lipid Quality: Certificate of Compliance

Certificate of cGMP Compliance

[Company Name] certifies that to our knowledge we comply with the guidelines of the current Good Manufacturing Practice (cGMP) for the manufacture of the product specified below. We also certify that should it be necessary to change the manufacturing process or specifications for this product, [Company Name] will notify the company and the appropriate regulatory agencies prior to implementing the change.

I hereby certify that the above information is authentic and accurate. This batch of product has been manufactured, including packaging and quality control at the above mentioned site in full compliance with the GMP requirements of the local Regulatory Authority and with the specifications in the regulatory filing. The batch processing, packaging and analysis records were reviewed and found to be in compliance with GMP.
Lipid degradation: Oxidation & Hydrolysis

- Poor quality raw materials
- Harsh process conditions
  - pH
  - Temperature
  - Pressure
  - Ionic strength
- Improper sterilization conditions
- Improper storage conditions
Lipid degradation - Oxidation

- Oxidation – appears mainly in unsaturated lipids
  - via free radical chain mechanism

- Oxidation process develops in three stages
  - Conjugation of isolated double bonds
  - Formation of lipid hydroperoxides and cyclic peroxides
  - Aldehyde production and chain scission
  - Result: increased permeability of bilayer

- Oxidation products can be determined by
  - GC analysis of remaining fatty acids
  - UV absorbance method to determine conjugated dienes and trienes at 233 and 270 nm
  - Iodometric methods for hydroperoxides
Lipid degradation - Hydrolysis I

Hydrolysis scheme of a phospholipid

Stability of liposomes upon sterilization and storage

N.J. Zuidam and D.J.A. Crommelin

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands.

*Present address: IDEA GmbH, Frankfurter Ring 193a, 80807 München, Germany.

FIGURE 8: The effect of pH on the hydrolysis of saturated soybean PC. Buffer concentration = 0.05 M; Each point represents the mean of at least two separate determinations. Filled symbols: 40°C; open circles: 70°C. Taken from Grit et al., J. Pharm. Sci. 82, 362-366, 1993.
Lipid degradation - Hydrolysis II

- Hydrolysis of ester glycerophospholipids to:
  - free fatty acids
  - lysolipids
  - glycerophospho compounds

- Effects on liposomal formulation:
  - increase in particle size and polydispersity
  - micelle formation
  - increase in permeability of bilayer – drug release
  - aggregation and fusion - gelation

Fig. 1. Concentrations of (A) DPPC and (B) MPPC in DPPC/DSPE-PEG<sub>2000</sub> liposomes stored at temperatures of 22 °C (solid symbols) or 4 °C (open symbols) in 300 mM citrate buffer at a pH of 2 (■) 4 (○), or 6.5 (▲). Lines in panel A were generated by fitting data to a first-order exponential decay.

Effects of phospholipid hydrolysis on the aggregate structure in DPPC/DSPE-PEG<sub>2000</sub> liposome preparations after gel to liquid crystalline phase transition

Ludger M. Ickenstein<sup>a,b,1</sup>, Maria C. Sandström<sup>c,d</sup>, Lawrence D. Mayer<sup>e</sup>, Katarina Edwards<sup>f</sup>

<sup>a</sup> Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC, Canada V6T 1Z3
<sup>b</sup> Department of Advanced Therapeutics, The British Columbia Cancer Agency Research Centre, 601 West 10th Avenue, Vancouver, BC, Canada V5Z 1L3
<sup>c</sup> Department of Physical Chemistry, Uppsala University, Box 579, 75123 Uppsala, Sweden
<sup>d</sup> CaluCell Technologies Inc., Suite 208, 604 West Broadway, Vancouver, BC, Canada V6Z 1G1

Received 27 October 2005; revised in revised form 31 January 2006; accepted 14 February 2006
Available online 10 March 2006
## Analytical methods for liposomal products

<table>
<thead>
<tr>
<th>Basic characterization assays</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>pH meter</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>Osmometer</td>
</tr>
<tr>
<td>Trapped volume</td>
<td>Measure of intra-liposomal aqueous phase</td>
</tr>
<tr>
<td>Phospholipid concentration</td>
<td>Lipid phosphorus content (modified Bartlett method), HPLC, enzymatic assay</td>
</tr>
<tr>
<td>Phospholipid composition</td>
<td>TLC (combined with the Bartlett method), HPLC</td>
</tr>
<tr>
<td>Phospholipid acyl chain composition</td>
<td>GC</td>
</tr>
<tr>
<td>Cholesterol concentration</td>
<td>Enzymatic assay, HPLC</td>
</tr>
<tr>
<td>Active compound concentration</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Residual organic solvents and heavy metals</td>
<td>NMR, GC, pharmacopeial protocols</td>
</tr>
<tr>
<td>Active compound / phospholipids ratio</td>
<td>Determination of active compound and phospholipids concentrations</td>
</tr>
</tbody>
</table>

### Chemical Stability

<table>
<thead>
<tr>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid hydrolysis</td>
</tr>
<tr>
<td>Non-esterified fatty acid concentration</td>
</tr>
<tr>
<td>Phospholipid acyl chain autoxidation</td>
</tr>
<tr>
<td>Cholesterol autoxidation</td>
</tr>
<tr>
<td>Antioxidant degradation</td>
</tr>
<tr>
<td>Active compound degradation</td>
</tr>
</tbody>
</table>

### Physical characterization

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Pharmacopeial protocols (visual inspection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicle size distribution</td>
<td></td>
</tr>
<tr>
<td>Submicron range</td>
<td>Dynamic light scattering (DLS), static light scattering (SLS), microscopy, gel exclusion chromatography, turbidimetry</td>
</tr>
<tr>
<td>Micron Range</td>
<td>Coulter counter, light microscopy, laser diffraction, SLS and light obscuration</td>
</tr>
<tr>
<td>Electrical surface potential and surface pH</td>
<td>Use of membrane bound electrical field probes and pH-sensitive probes</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>Electrophoretic mobility</td>
</tr>
<tr>
<td>Thermotropic behaviour, phase transition, and phase separation</td>
<td>DSC, NMR, fluorescence methods, FTIR, Raman spectroscopy, ESR, specific turbidimetry</td>
</tr>
<tr>
<td>Percentage of free drug</td>
<td>Gel exclusion chromatography, ion exchange chromatography, precipitation by polyelectrolyte, (ultra)centrifugation</td>
</tr>
</tbody>
</table>

### Microbiological assays

<table>
<thead>
<tr>
<th>Sterility</th>
<th>Pharmacopeia protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogenicity (endotoxin level)</td>
<td>Pharmacopeia protocols</td>
</tr>
</tbody>
</table>

## Methodology

- Basic characterization assays
- Chemical Stability
- Physical characterization
- Microbiological assays
- Analytical methods for liposomal products
DNAi-project using smarticle technology (Novosom – now Marina Biotech)
- degradation of one proprietary lipid compound during processing
- could not be detected with originally established QC - method
- strong impact on processing and storage
DNAi-project using smarticle technology (Novosom – now Marina Biotech)

- degradation of one proprietary lipid compound during processing
- could not be detected with originally established QC - method
- strong impact on processing and storage

QC example 1: Lipid degradation upon processing

![Modified HPLC method](image)
PNT2258 - DNAi in Liposomes

DNAi® - A Differentiated Approach to Nucleic Acid Development

| Targets: Genomic DNA disease loci | Potential Advantage: Multiple mechanisms to trigger apoptosis |
| Chemistry: Unmodified oligonucleotides | Potential Advantage: Improved safety profile |
| Delivery: Liposome encapsulation | Potential Advantage: Enhanced delivery |

20 September 2010

ProNAi Initiates Phase I Cancer Clinical Study of PNT2258

KALAMAZOO, MI, September 20, 2010 -- ProNAi Therapeutics, Inc., a privately held biopharmaceutical company, announced today dosing of its first patient in an open-label, single-arm Phase I dose-escalation study of PNT2258 in patients with advanced solid tumors for which no standard therapy exists. Patients will receive PNT2258 as an intravenous infusion once daily for 5 consecutive days (Days 1-5) of every 21-day cycle (3 weeks). The first-in-human clinical trial of PNT2258 has the primary objective to assess the safety and tolerability. The study will also examine the pharmacokinetics of PNT2258 and explore biomarkers with the goal of identifying doses for subsequent efficacy studies in cancer patients.

ProNAi’s approach capitalizes on a proprietary and differentiated oligonucleotide platform designed to target nuclear DNA. PNT2258 is the Company’s first drug candidate, a 24-base single-stranded oligonucleotide (PNT100), designed to modulate the BCL-2 oncogene, encapsulated within a proprietary delivery technology called SMARTICLES®. This novel encapsulation of an oligonucleotide was chosen to prolong circulation times and protect PNT100.
QC example 2: natural PC vs. POPC

- Issues
  - Quantification of lipid compound in complex liposome formulation
  - Detection of byproducts / degradation products
Established appropriate light and window filters in the formulation lab, analytical lab, and filling suite

Amber glass bottles or stainless steal for all process steps
Analytical methods

- lipid degradation product methods
  - HPLC (ELSD/CAD/MS – detection)
  - Enzymatic assays
  - TLC
  - Free fatty acids by GC

- other stability indicating methods
  - DSC
  - Light scattering techniques
  - Drug release tests
Qualitative and quantidative detection of lysolipids

- HPLC (CAD-Detector)
Qualitative and quantitative detection of lysolipids

- Thin layer chromatography

1: DMPC Std
2: DPPC Std
3: 14:0 Lyso PC Std
4: 16:0 Lyso PC Std
5: DMPC/DPPC-liposomes
6: DMPC/DPPC liposomes after UDF
7: DMPC/DPPC liposomes autocl.
8: DMPC/DPPC-liposomes
9: DMPC/DPPC liposomes after UDF
10: DMPC/DPPC liposomes autocl.
Liposome preparation methods

PREPARATION/PRODUCTION TECHNIQUES

- film technique
- membrane extrusion
- sonication
- homogenization techniques
- detergent removal
- various solvent injection methods

Liposome preparation methods

PREPARATION/PRODUCTION TECHNIQUES

- **Mild methods**
  - various solvent injection methods
  - detergent removal

- **Invasive methods**
  - sonication
  - High pressure homogenization

Lipid degradation
- Oxidation
- Hydrolysis
Invasive techniques

- Sonication
  - tip sonicator or bath sonicator
- Homogenization
  - Ultrathurax
  - French Press
  - Microfluidizer
  - Homogenizer

- Prevention of degradation
  - Use of fully saturated lipids
  - Inert atmosphere
  - Use of antioxidants (Vit. E, Vit. ...
Liposome formulation - non invasive methods

- Film method – most frequently used lab scale liposome formulation technique
  - Powdered Lipids including Lipid-Soluble Drug
  - Organic Solvent
  - Aqueous Solution including Water-Soluble Drug
  - Hydration Agitation
  - Large Multilamellar Vesicles
  - Sonication Extrusion Homogenisation
  - Small Unilamellar Vesicles

- Lab scale ethanol injection method according to Batzri et al.

[Diagram of film method and lab scale ethanol injection]
Non invasive methods - The Polymun Liposome Technology

Process parameters

- lipid concentration in ethanol
- injection pressure/flow rate
- injection hole diameter
- lipid composition
- aqueous phase – ionic strength, pH
- flow rate aqueous phase
- process temperature
- solvent / solvent mixtures
Advantages of the Polymun Technology

- Full scalability
- Aseptic process conditions
- Homogeneous, uniform vesicles
- Single step process
- Excellent batch to batch consistency
- Mild procedure - stability
Liposomal Formulation in the lab scale: 20 mL in 2 seconds
Liposomal formulation at large scale: 250 L in 15 hour
Excellent batch to batch consistency

- The crossflow injection technique is a very precise procedure that allows processing of sensitive drugs. High quality raw materials and precisely controllable process parameters guarantee high batch to batch consistency – essential for pharmaceutical products.
Polymun liposomal products are stable over Years

- The crossflow injection technique is a very mild procedure that allows processing of sensitive drugs. High quality raw materials guarantee long term stability of liposomes even at room temperature.
The production system - aseptic conditions

Closed containment including sterile barriers for all raw material transfer → sterile production plant
According to European Pharmacopoeia and U.S. Pharmacopoeia at least 3 definitions are:

- free from living organism
- a product that meets the requirements as stipulated in a special monograph in a Pharmacopoeia
- If the chance of finding a unit that is contaminated with living microorganisms is less than 1 in $10^6$ sterilized units of the final product

From: Handbook of Nonmedical application of Liposomes; Ed. By Barenholz & Lasic; Chapter 6: Sterilization of liposomes; Zuidam N.J.; Talsma H. and Crommelin J.A.
Steps towards a sterile product

- Controlled process / Process validation
- Control over raw materials
- Effective sterilization process of final (and) intermediate products
  - 0.22 µm filtration, sterile filtration
  - Heat sterilization – Autoclaving
  - High - pressure sterilization
  - γ-irradiation

From: Handbook of Nonmedical application of Liposomes; Ed. By Barenholz & Lasic; Chapter 6: Sterilization of liposomes; Zuidam N.J.; Talsma H. and Crommelin J.A.
Sterilization processes

- 0.22 µm filtration, sterile filtration
  - Size, homogeneity and lipid concentration
- Heat sterilization – Autoclaving
  - Works for empty liposomes @ neutral pH;
- High - pressure sterilization
  - Release of API and degradation of lipids depends on process conditions (time, temperature and pressure) and lipid composition
- γ-irradiation
  - Degradation of lipid compounds can be minimized by treatment of frozen or lyophilized products
  - Headgroups of phospholipids can be oxidized

From: Handbook of Nonmedical application of Liposomes; Ed. By Barenholz & Lasic; Chapter 6: Sterilization of liposomes; Zuidam N.J.; Talsma H. and Crommelin J.A.
Stability upon long term storage

- Stability of liposomal products should meet standards of conventional pharmaceutical products (> 1 year)

- Liquid storage
  - Bilayer compounds - long chain, saturated lipids
  - Surface charge
  - Addition of cholesterol
  - Antioxidants and inert atmosphere
  - Optimized buffer conditions

- Cryo-preservation
  - Addition of cryo-protectants: saccharides, glycerol
  - Process variables: freezing/drying-time, -temperature, cooling/drying rate, rehydration conditions
Summary

- Successful liposomal drug product developments
  - Peptides
  - Proteins
  - Oligonucleotides
  - small molecular weight drugs (incl. antibiotics, anticancer drugs)
- GMP-compliant processes were developed
- Full scalability was proven