Liposome characterization by asymmetrical flow field-flow fractionation

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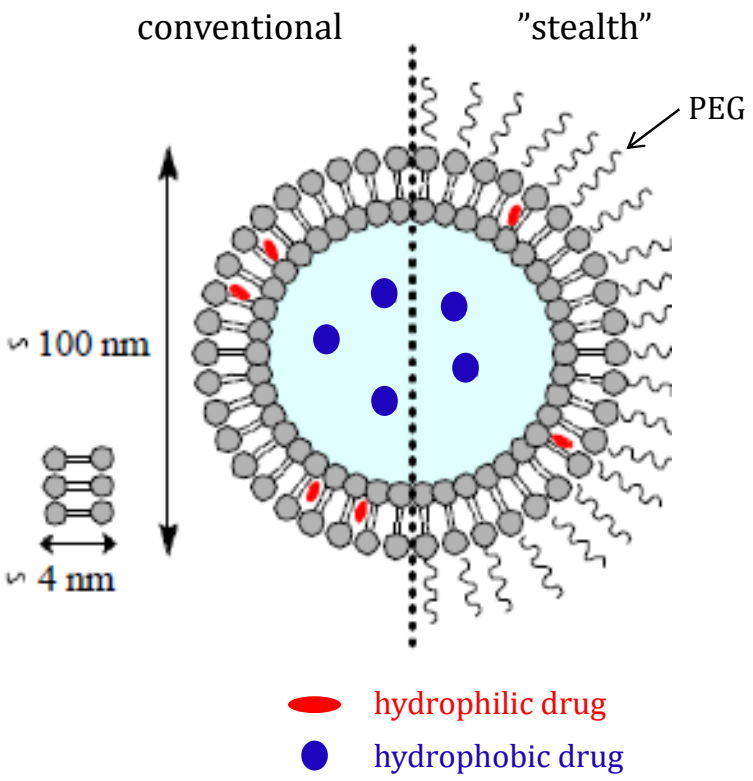
Outline

- Liposomes as drug carriers – characterization issues
- Introduction into AF4 and MALS
- Examples for characterization of liposomes
  - Size determination
  - Drug release and transfer
- Summary and conclusion

Liposomes as drug carrier

- Phospholipid vesicles (physiological lipids such as phosphatidylcholine)
- Small (d ≤ 100 nm) and unilamellar liposomes (i.v. administration)
- Possible surface modification
  - Stealth liposomes (prolongation of circulation time)
  - Specific ligands (active targeting)
- Carrier for both hydrophilic (liposome aqueous core) and hydrophobic (liposome membrane) drugs



Liposomes as drug carrier

Administration routes:

- parenteral (i.v.)
- dermal
- oral
- …

Drug solubilization and absorption enhancement

Drug targeting (i.v.)

"magic bullet concept" (Paul Ehrlich, 1897)

Passive targeting

Enhanced permeation and retention (EPR)

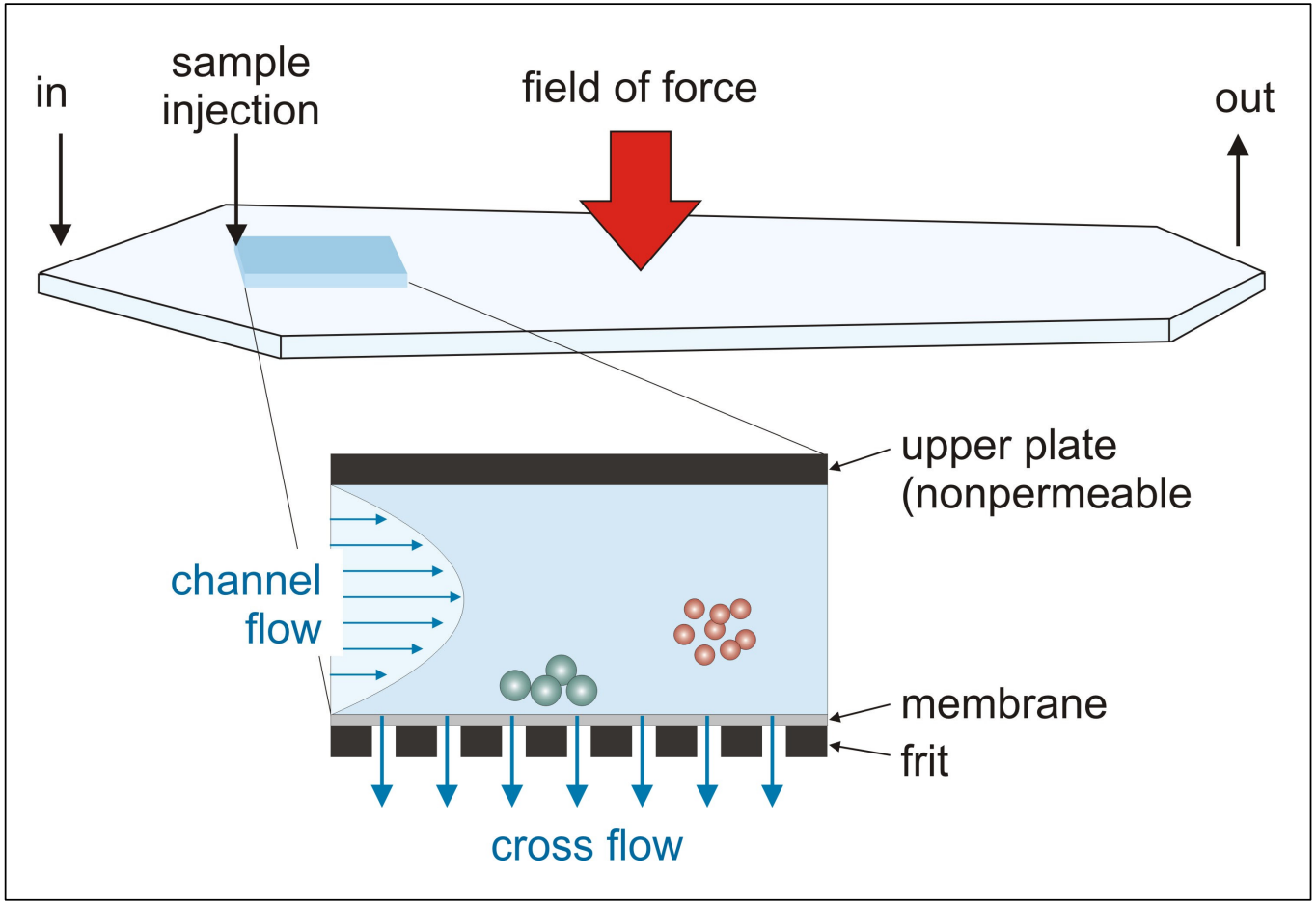
Active targeting

Specific ligand-receptor binding

- **Size, morphology** and **homogeneity**
- **Stability** of the carrier in physiological media (e.g. serum)
- Efficient **drug retention** within the carrier but **drug release** at the target side (drug targeting)

➡ **AF4/MALS**

Flow field-flow fractionation – Separation principle



- Separation in dependence on hydrodynamic size
- Smaller particles elute faster than larger ones

Alternative separation method to size exclusion chromatography (SEC)

AF4/MALS: Instrumentation

Isocratic pump and degasser (Agilent)

Variable wavelength detector (VWD, Agilent)

Temperature-controlled autosampler (Agilent)

Multi-angle light scattering (MALS) detector (Wyatt)

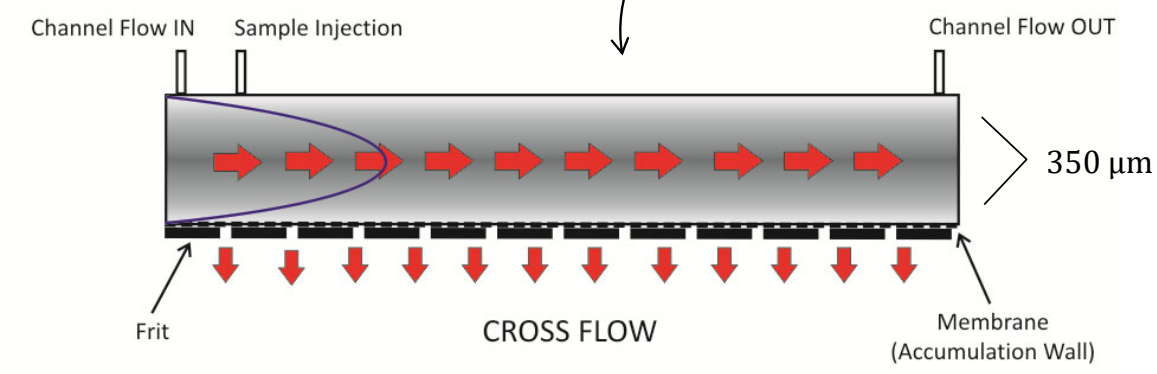
Differential RI (dRI) detector (Wyatt)

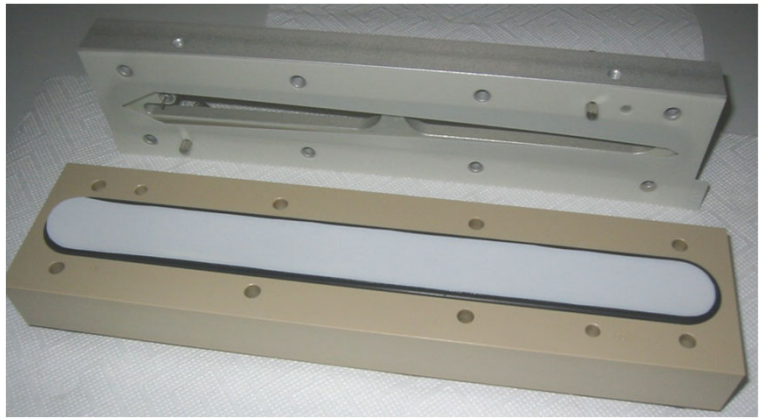
AF4 (Wyatt)

Separation channel

The AF4 channel

Closed on top → Asymmetrical f-FFF (AF4)





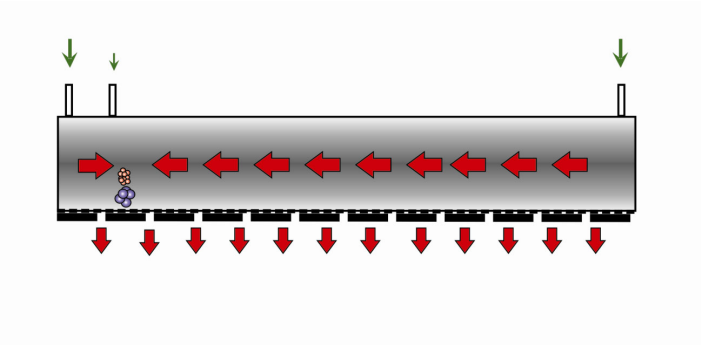
Cover plate (closed)

Spacer (defines channel geometry)

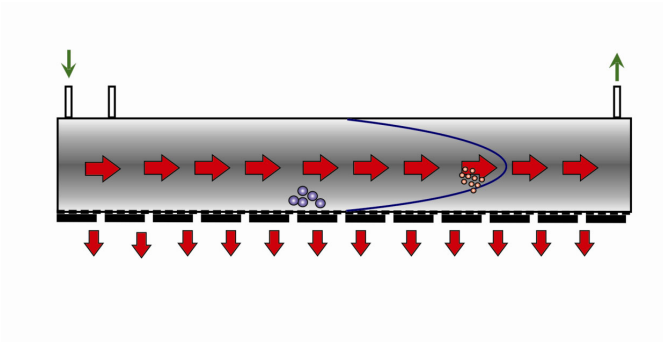
Membrane (accumulation wall)
→ usually PES or RC, MWCO 5-10 kDa

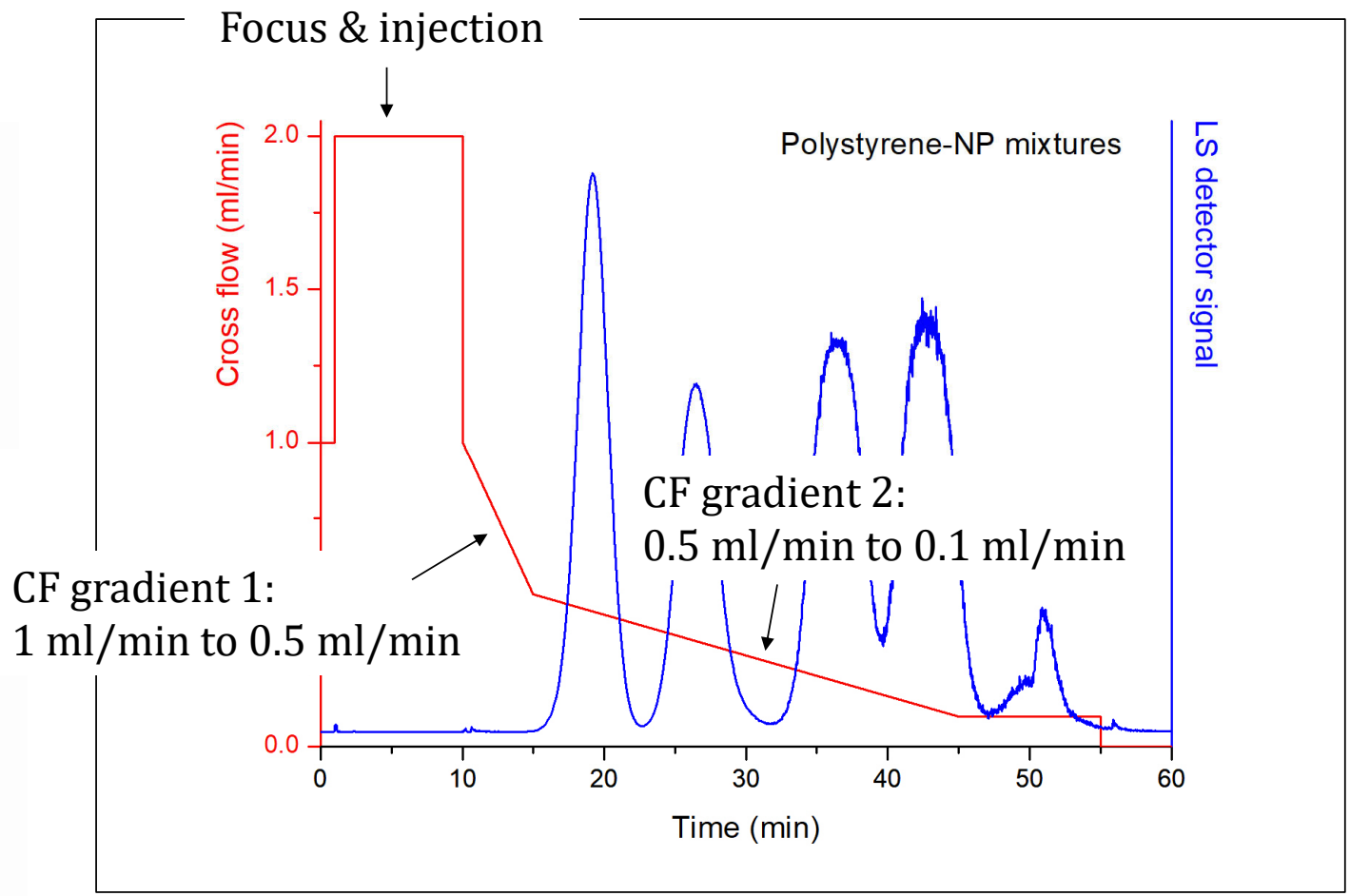
Steps of sample fractionation

Focus & injection



Elution



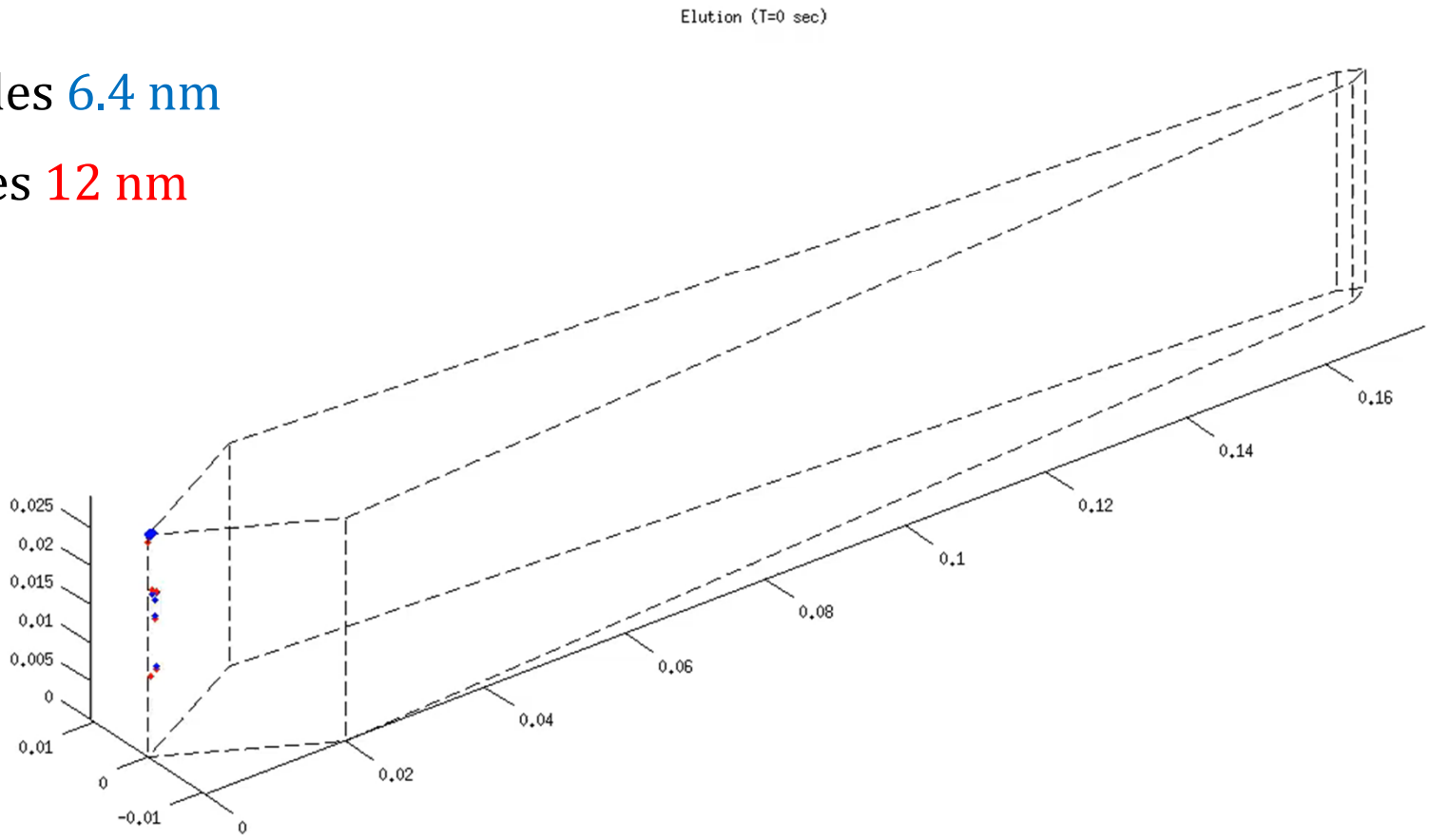


Simulation of focusing and elution

Diameters

blue particles 6.4 nm

red particles 12 nm



Simulation video from Wyatt Technology (Fraunhofer ITWM)

Size determination

Retention time (AF4 theory)

- Requires calibration (membrane swelling)
- For size distributions – sample concentration must be known

diameter

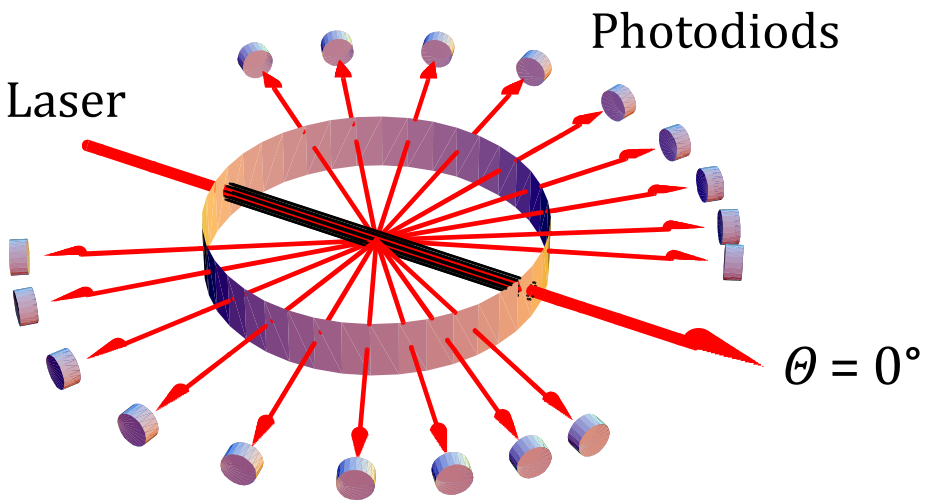
channel height (thickness)

$$t_R = \frac{1}{2} \cdot \frac{\pi \cdot \eta \cdot d}{k \cdot T} \cdot \frac{t_o \cdot V_x \cdot w^2}{V_c}$$

Multi-angle light scattering (MALS)

- Absolute measurement (no calibration)
- Analyzing the angle-dependent light scattering profile
- Homogeneous sample fraction required

MALS detector



Source: Wyatt Ligth Scattering Seminar.

Fitting the light scattering data

Analysis of the angle-dependent light scattering profile

e.g. Debye $\frac{R(\theta)}{K} \Rightarrow \sin^2(\frac{\theta}{2})$

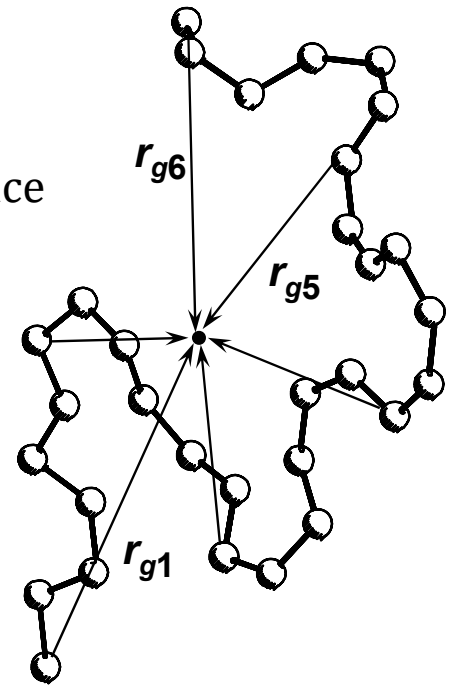
Radius of gyration or Root Mean Square (RMS) radius

If the structure of the particles is known, the geometric size can be calculated:

Compact spheres (emulsion): $\boldsymbol{r}_{geometric} = \sqrt{\frac{5}{3}} \cdot RMS$

Spherical Shell (Liposomes): $\boldsymbol{r}_{geometric} = RMS$

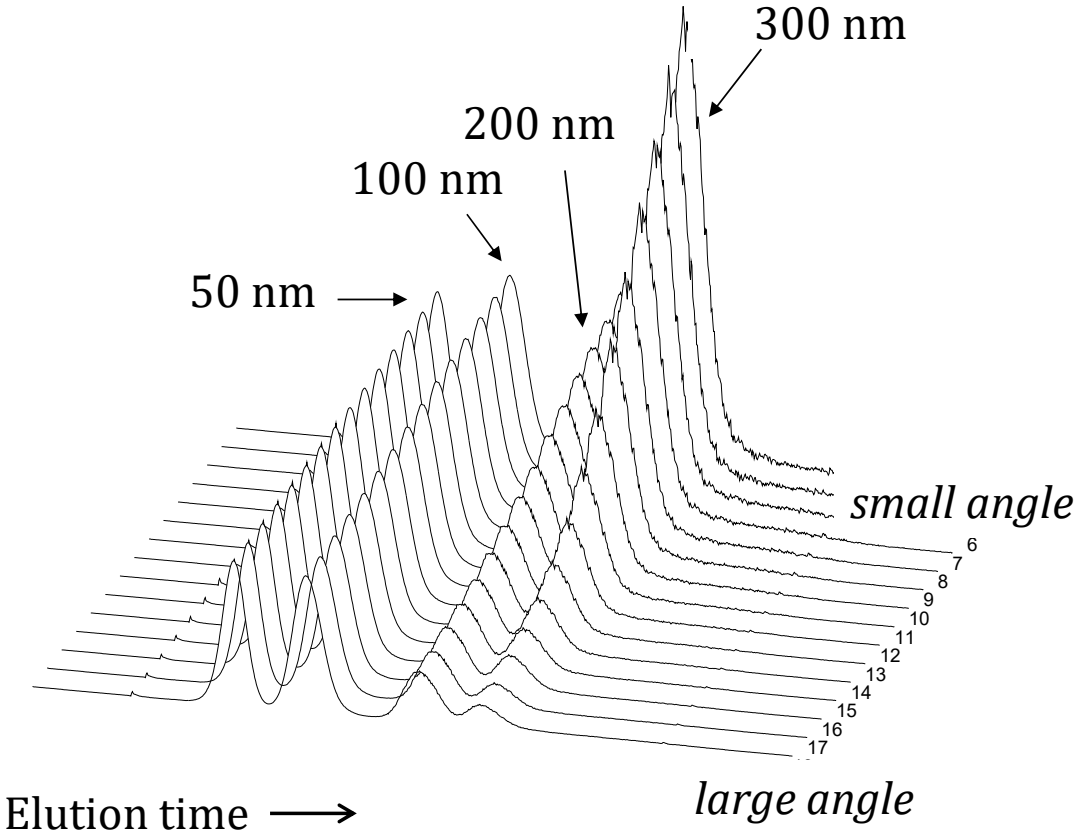
Distribution of distance to center of gravity.



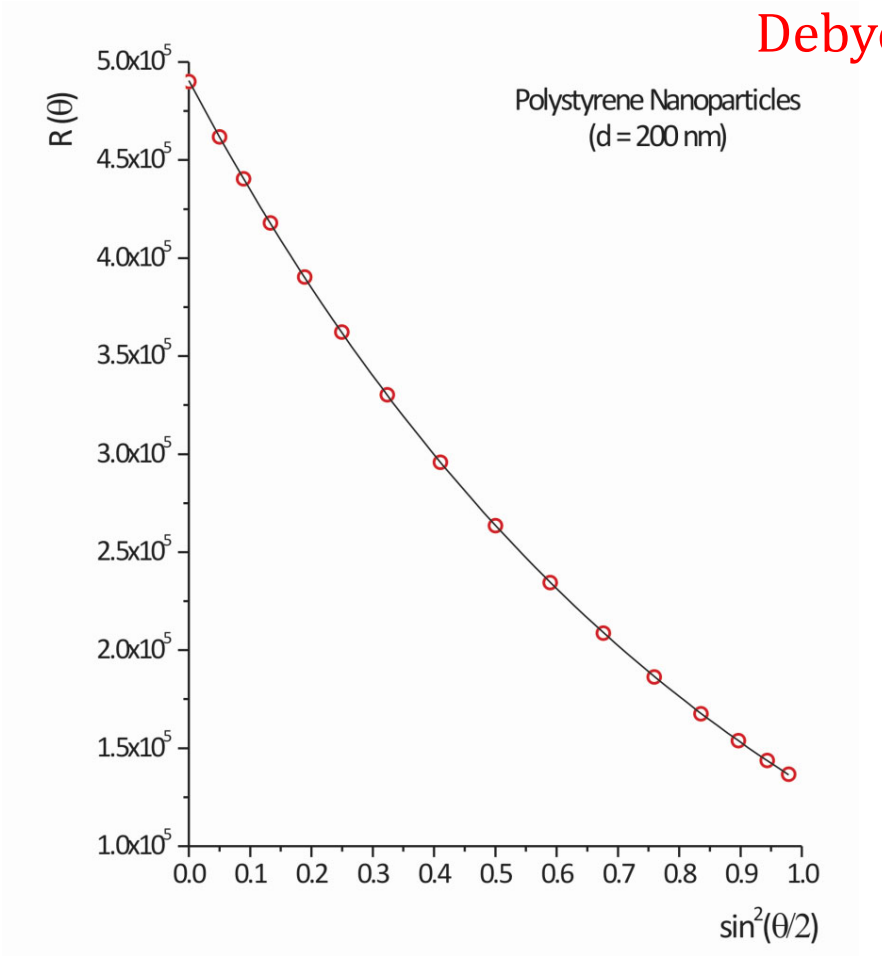
Source: Wyatt Ligth Scattering Seminar.

Size determination by MALS (Mixture of polystyrene NP standards)

3D-plot

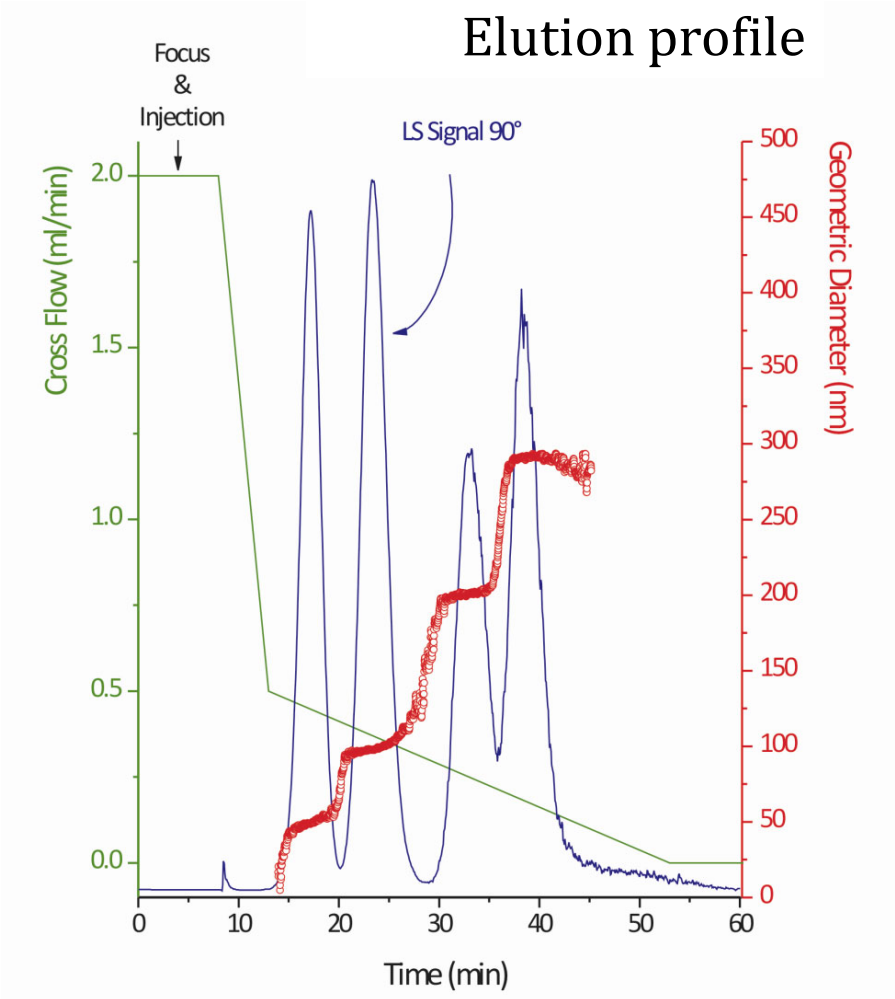


Debye Plot

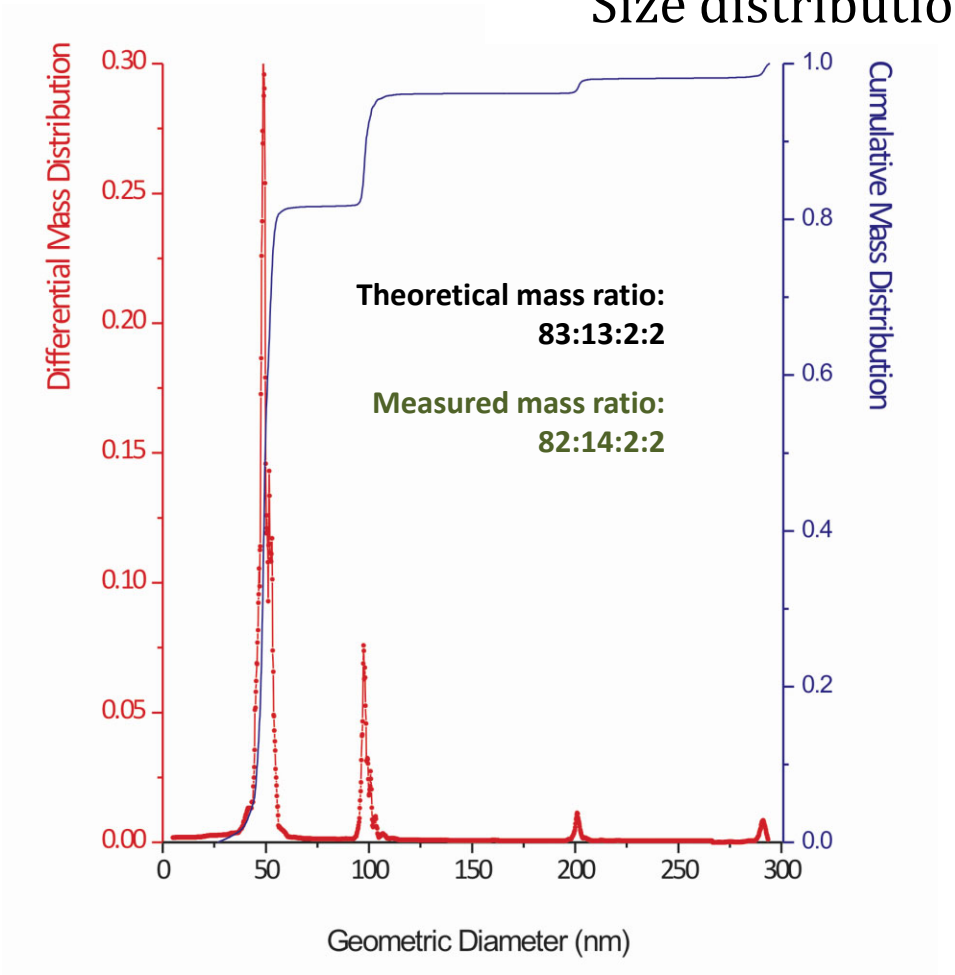


Size determination by MALS

Elution profile

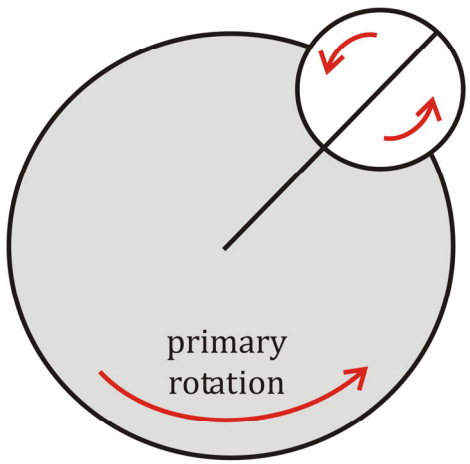


Size distribution

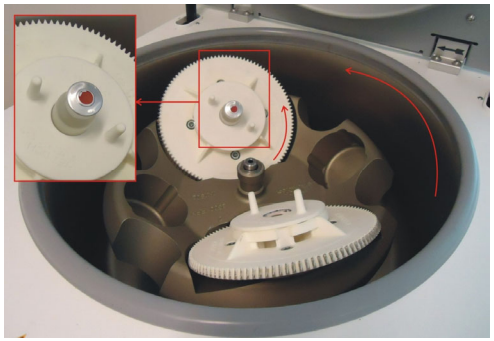


Size determination of liposomes

Liposome preparation by Dual centrifugation



ratio primary to secondary rotation: 1:3
(Zentrimix, Hettich)



- Very efficient mixing
- Preparation of highly concentrated dispersions (emulsions, liposomes, …)
- ”Single-pot-preparation”

2 g lecithin (Lipoid E80S)
3 g 10 mM Tris buffer pH 7.4
5 or 10 g glass beads
(Ø 0.3, 2 or 3 mm)

↓

Dual centrifugation (DC)
Zentrimix (Hettich)
1500-2500 rpm at 5, 25 or 40 °C
Plastic vials (15 or 60 ml)

↓

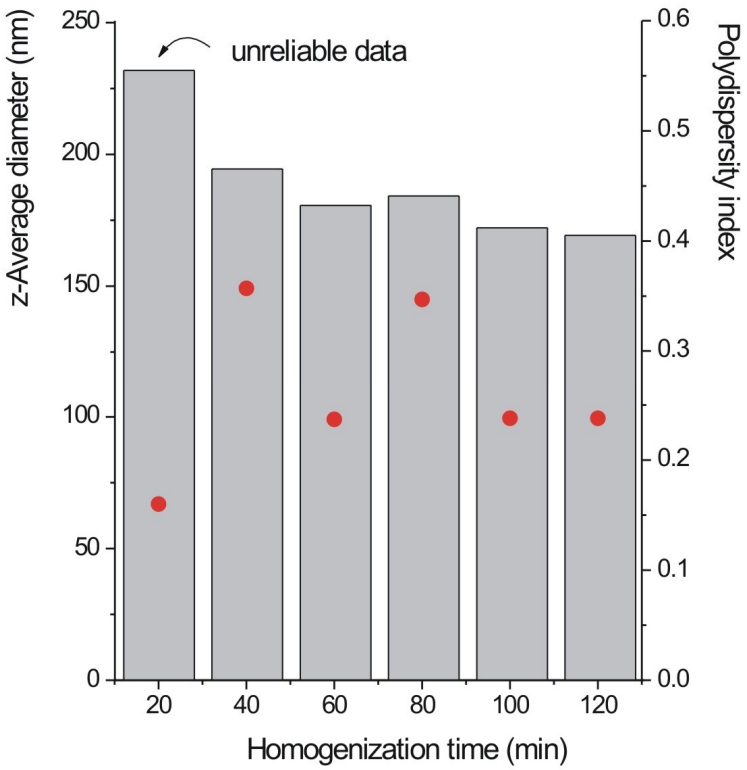
Sample dilution & vortexing
(stock 40 mg/ml)

↓

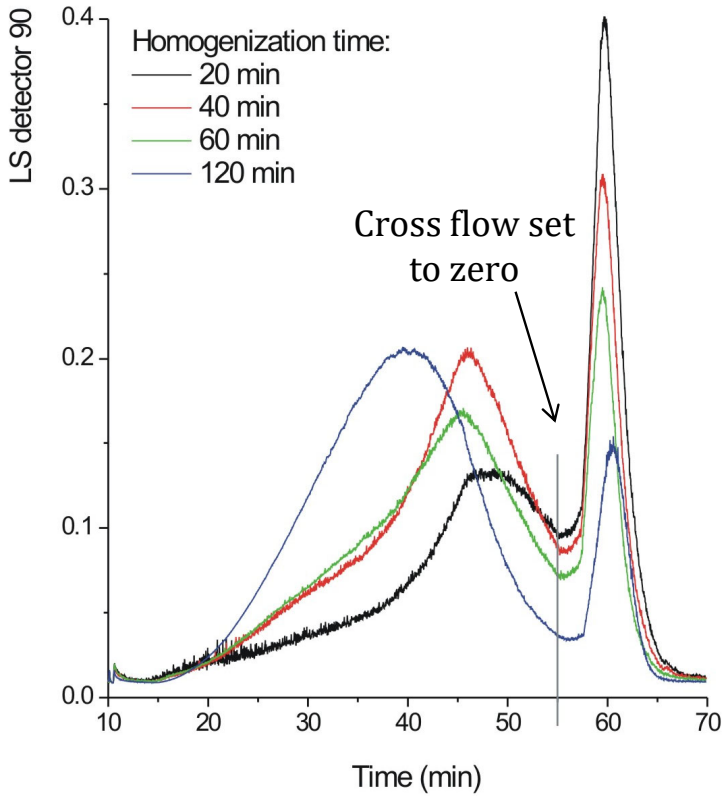
DLS (size)	AF4/MALS (size)	NMR (lamellarity)

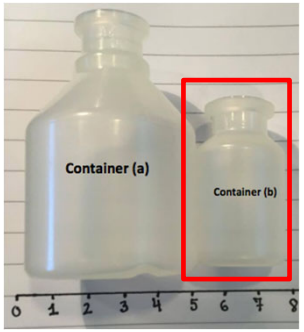
Size reduction by dual centrifugation (DC)

DLS



AF4/MALS (elution profiles)

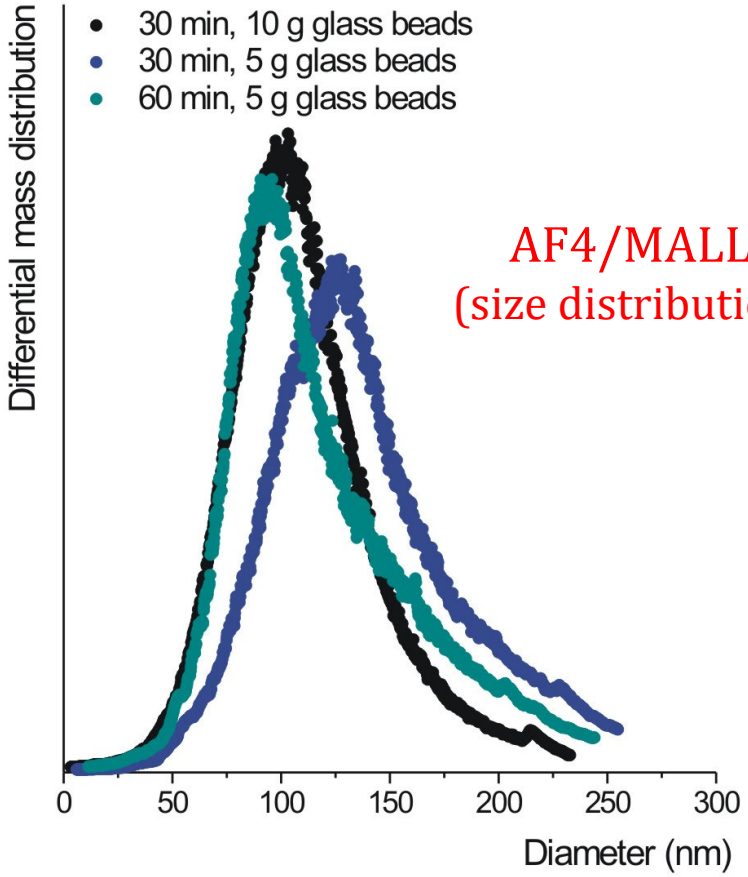




Small sample vials (15 ml)
5 g glass beads (Ø 2 mm),

- Decrease of liposome size with time
- Fraction of large-sized liposomes
- Broad size distribution even after 120 min

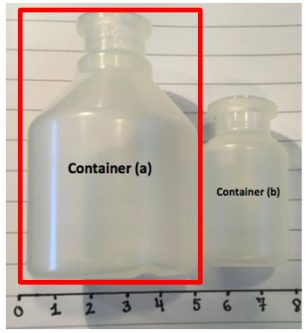
Optimization (DC)



AF4/MALLS
(size distributions)

DLS

Z-average	PDI
130 nm	0.072
160 nm	0.119
138 nm	0.107



large sample vials (15 ml)
5 or 10 g glass beads(Ø 3 mm),

- More efficient size reduction in larger sample vials (larger surface)
- Higher amount of glass beads increases efficiency (shorter processing times)

Drug release and redistribution

Drug release and redistribution

Challenges:

- Separation of the carrier particles from the release medium and other acceptor phases
- Analytical challenges (e.g. drug quantification)

Methods:

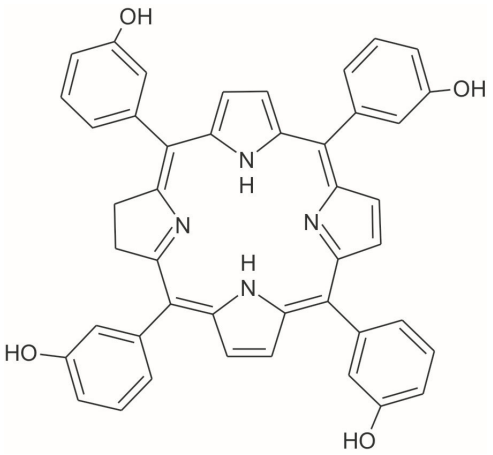
- (Dialysis – **in most cases not applicable** to study release hydrophobic drugs under sink conditions)
- Centrifugation
- Ultracentrifugation/ultrafiltration

Limited applicability to study drug redistribution in physiological media such as serum → AF4

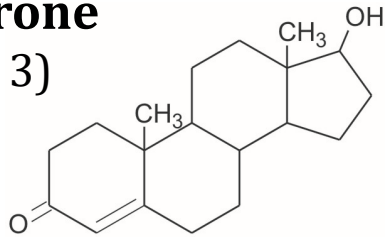
Lipid and drug recovery

- Radioactively labeled liposomes (lipid ^{3}H and drug ^{14}C)
- Preparation by thin-lipid film and extrusion
- 20 mg/ml DPPC/DPPG, drug load 8 mol%
- Liposome diameter (DLS) ~ 110-120 nm
- AF4 – Lipid and drug recovery

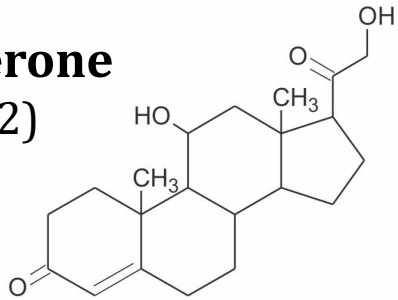
Temoporfin (mTHPC)
(log P ~ 9)

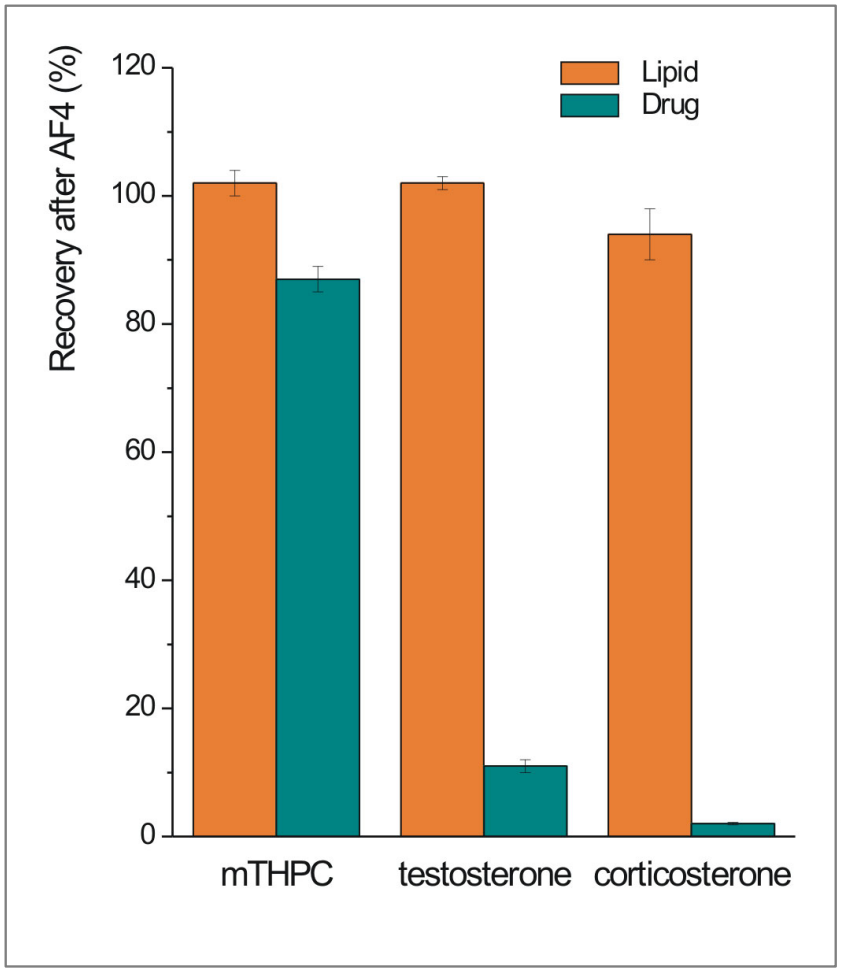


Testosterone
(log P ~ 3)



Corticosterone
(logP ~ 2)





Kuntsche et al., J. Sep. Sci. 35 (2012).

Drug release and redistribution - Setup

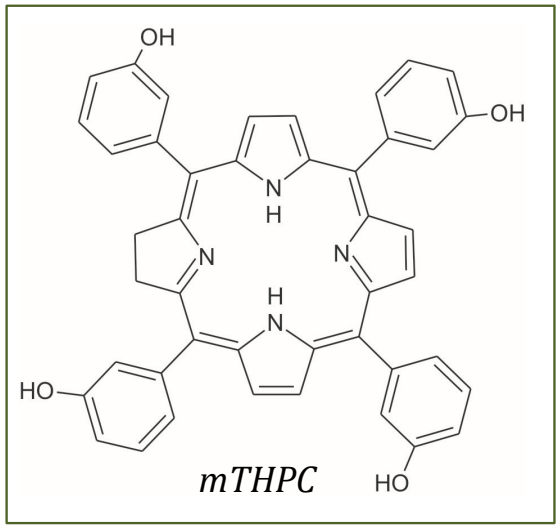
Donor
pTHPP-loaded liposomes

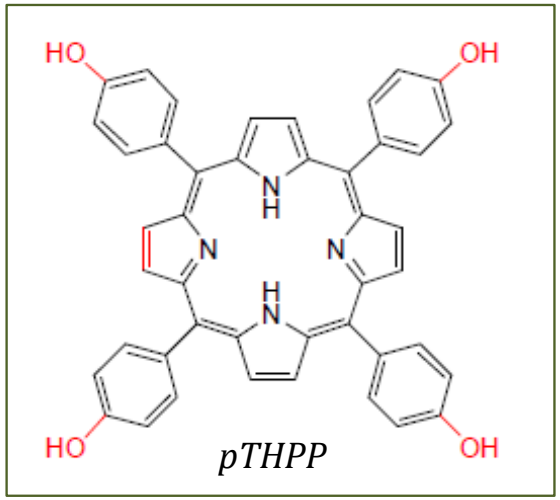
Acceptor
Liposomes, micelles serum

Egg lecithin
(Lipoid E80S)
5 mol% pTHPP

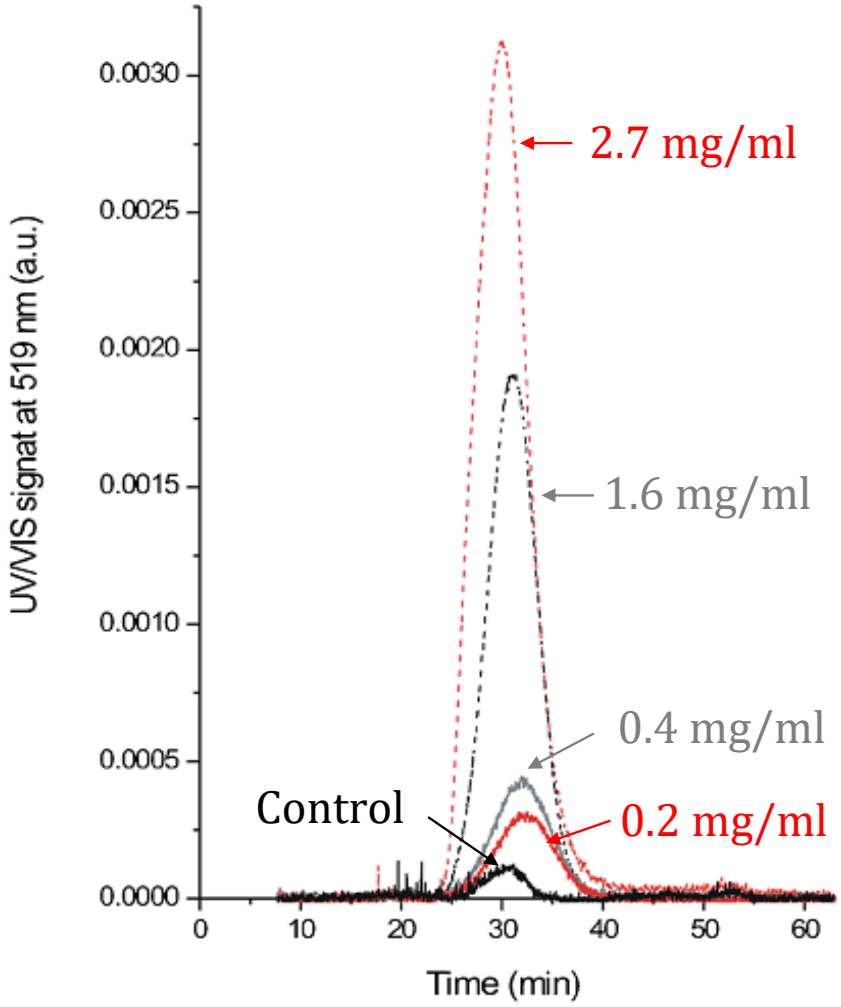
INCUBATION
37 °C, stirring

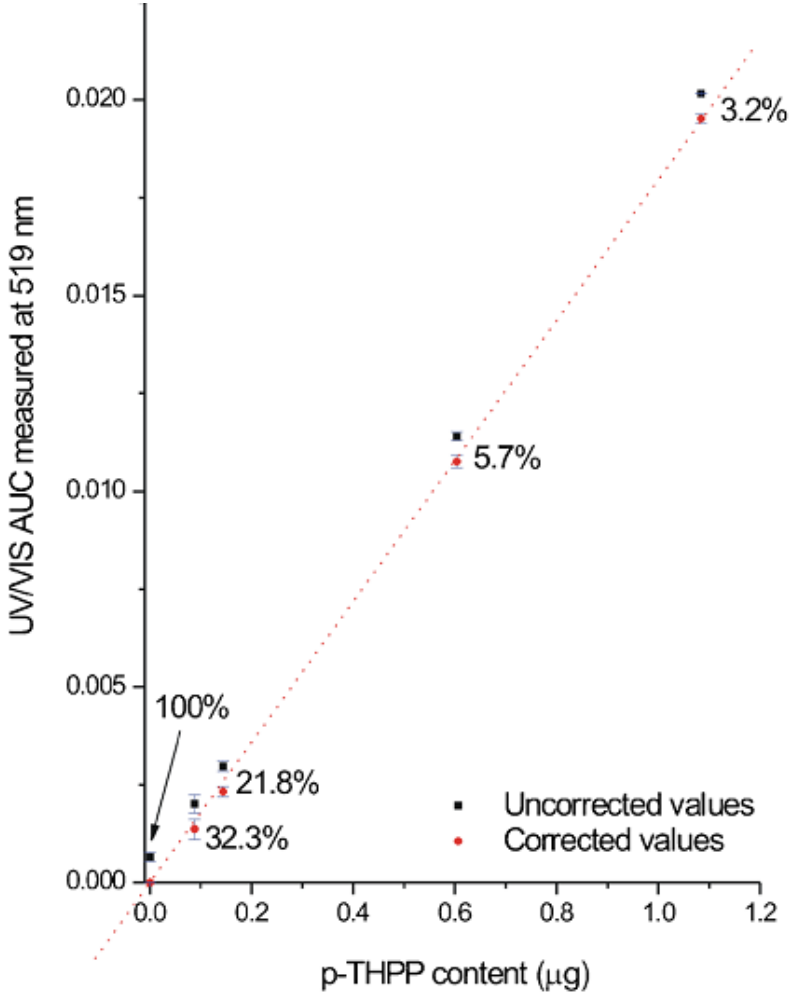
Fractionation (**AF4**) and quantification of pTHPP in sample fractions





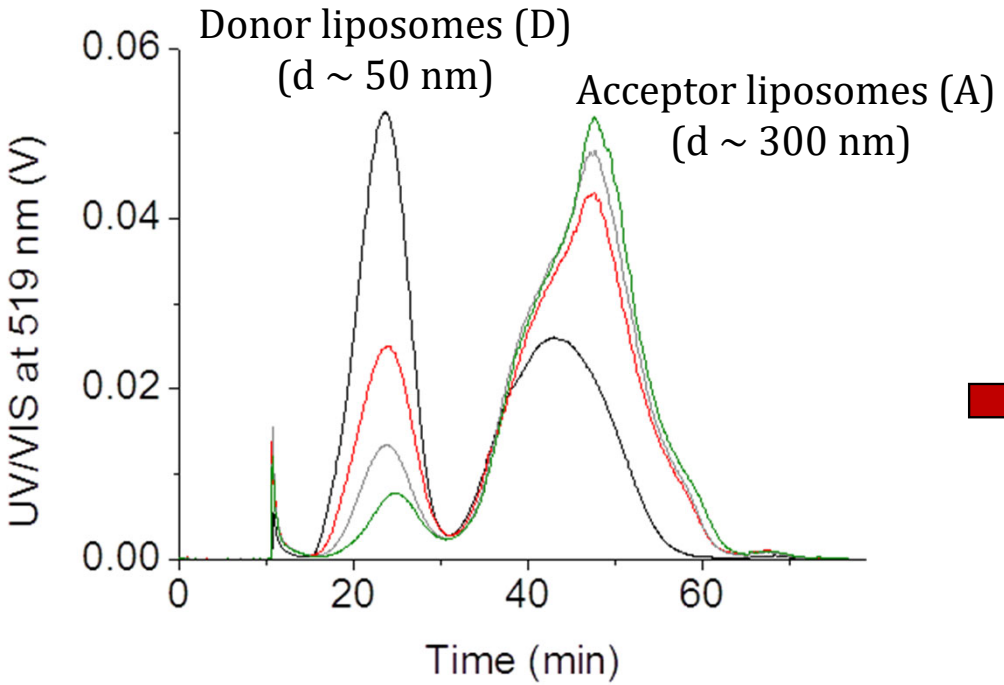
Online-quantification: Liposomes with different drug loads

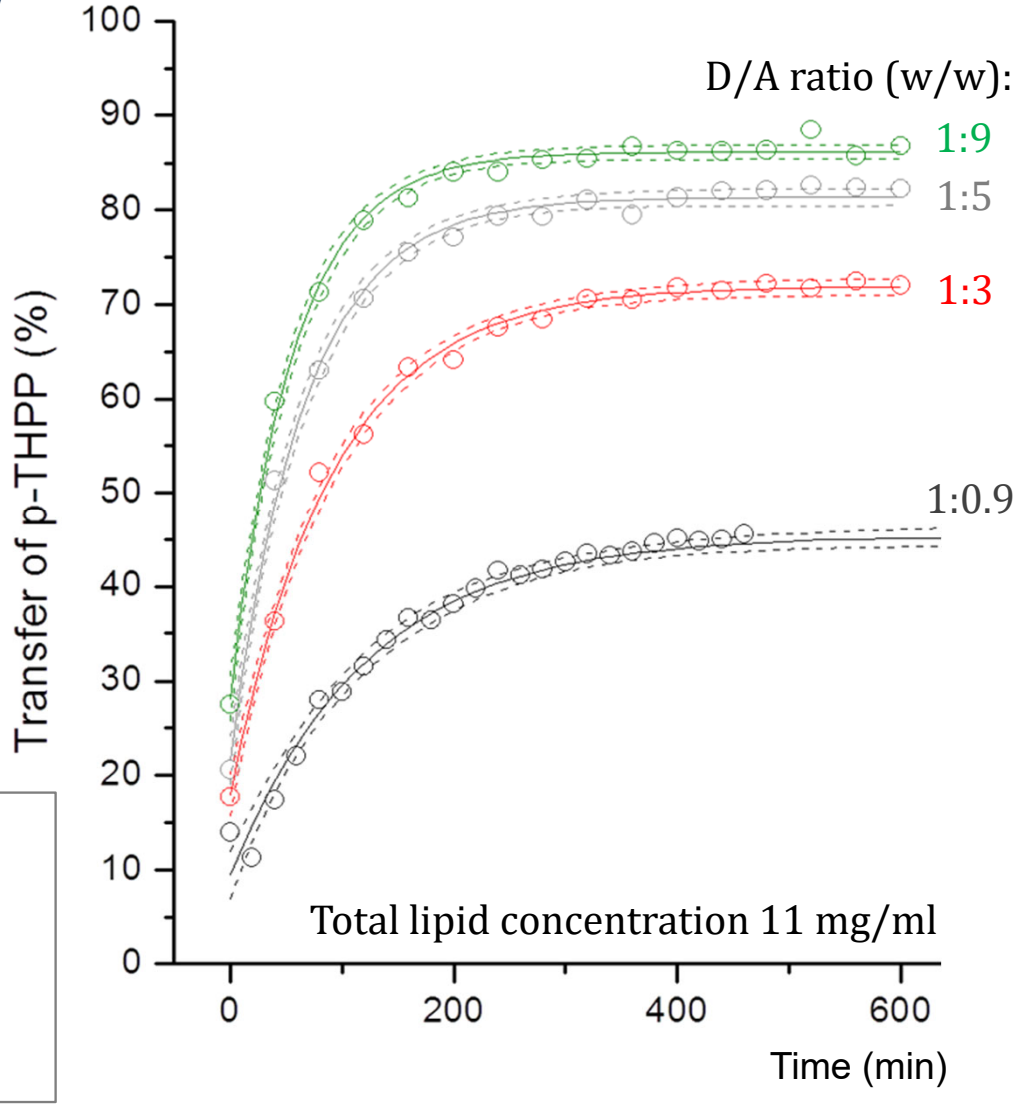




Hinna et al., Analy. Bioanal. Chem. 406 (2014).

pTHPP redistribution between liposomes

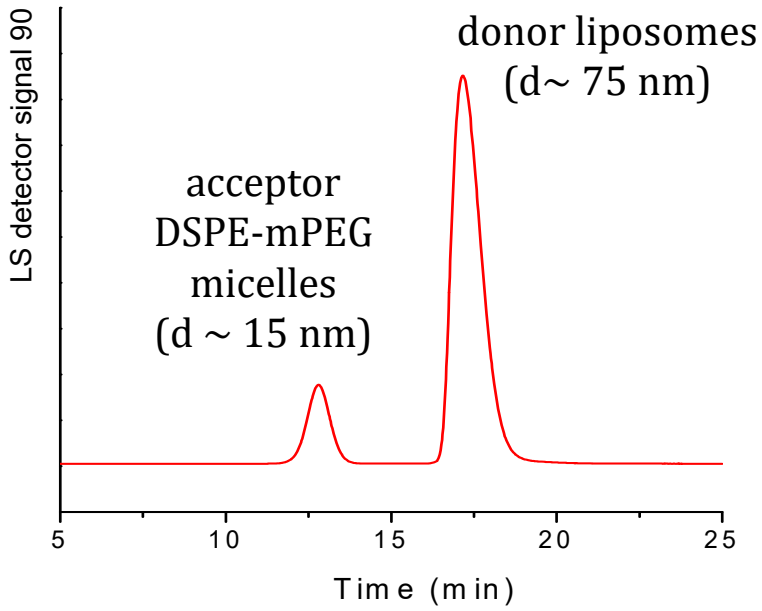


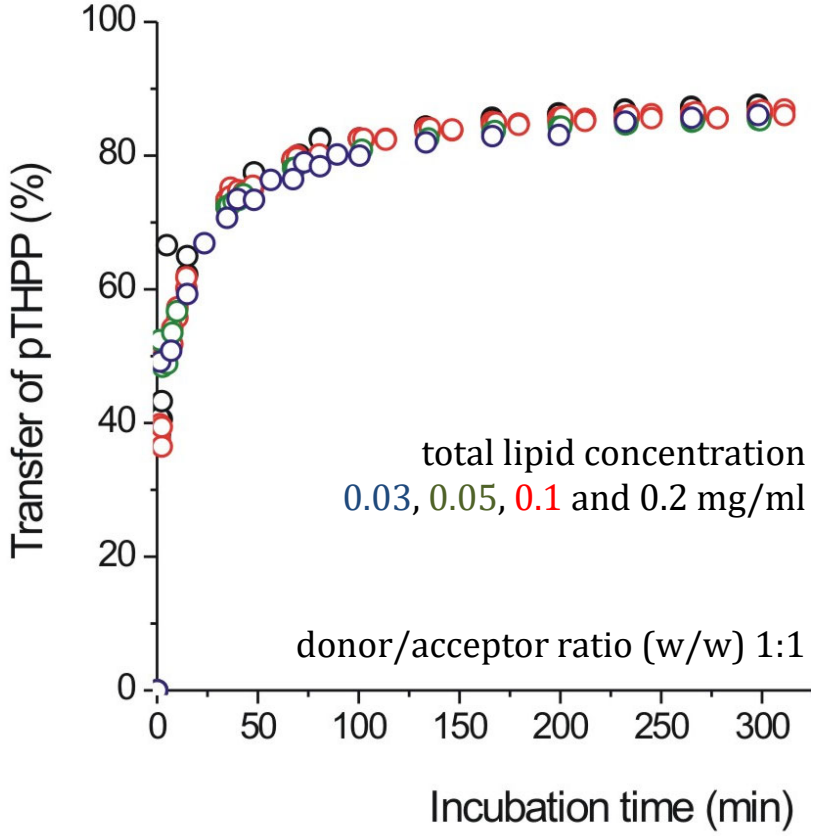


- Increasing transfer rate with increasing acceptor concentration
- Redistribution according to the D/A lipid ratio in equilibrium

Hinna et al., J. Pharm. Biomed. Anal. 124 (2016) & J. Control. Rel. 232 (2016).

Kinetics of pTHPP redistribution between liposomes and micelles

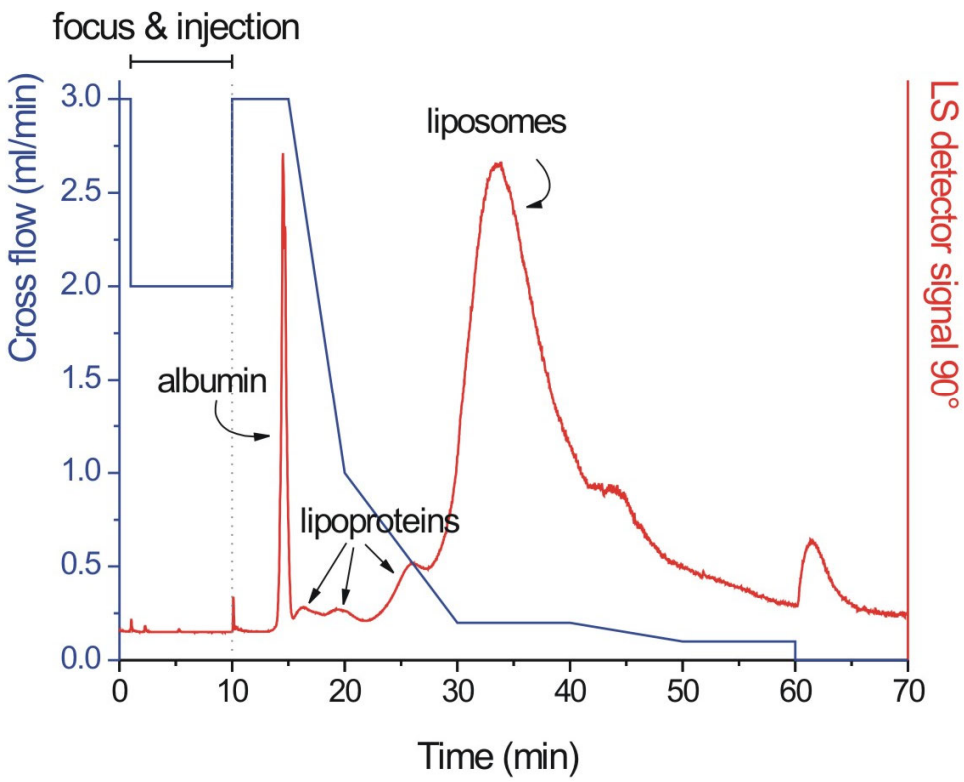


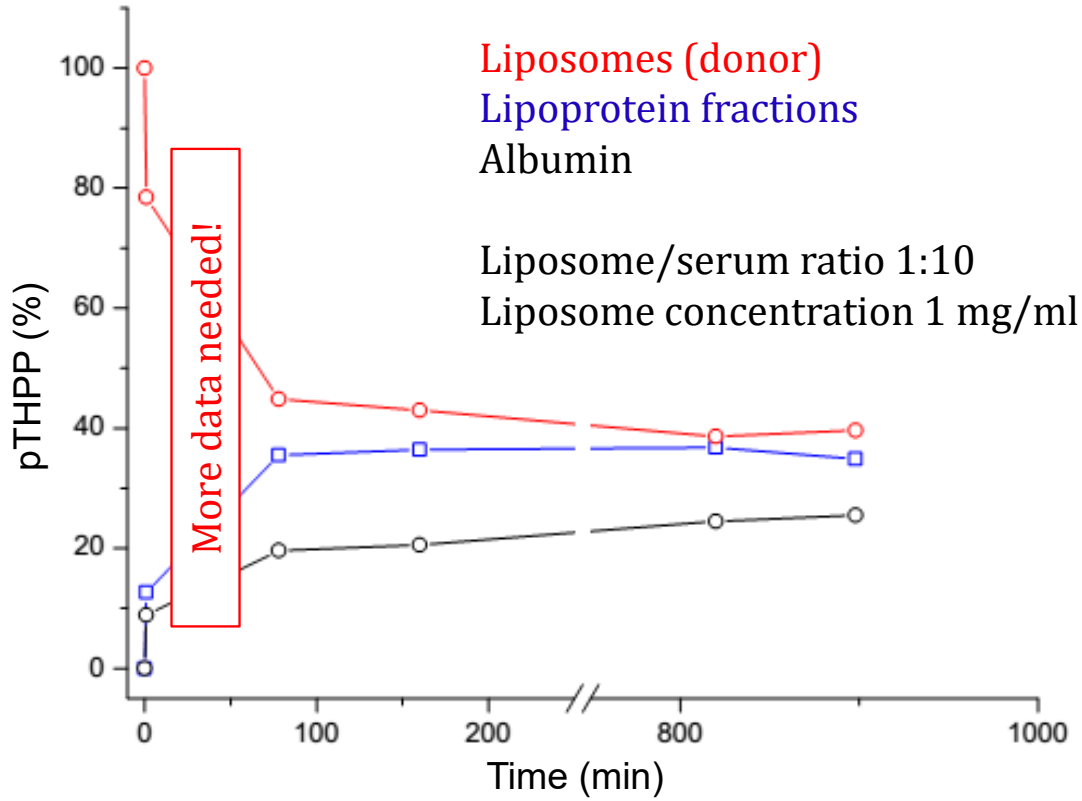


- Very fast transfer of pTHPP to acceptor micelles (same kinetics for all concentrations)
- High accumulation of pTHPP in micelles at equilibrium

Kristensen, Master thesis, SDU 2016.

pTHPP redistribution to serum components

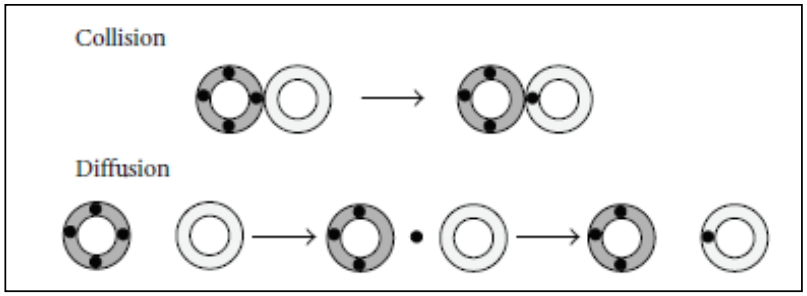




- Baseline not reached at the end of analysis, but acceptable results in subsequent fractionations
- Rapid redistribution of pTHPP from liposomes to serum components

Summary: Drug release and redistribution

- Release of incorporated drug with moderate logP upon AF4 ("ultrafiltration")
- No alteration of liposome or micelle integrity (size) during time period of incubation and fractionation
- Changes of liposome size at longer incubation times in serum
- Rapid redistribution of pTHPP (practically insoluble in water, logP ~ 9) to lipophilic acceptor phases
- Particle size (total surface area and number of acceptor particles) plays an important role for transfer kinetics (transfer mainly by collision)



Fahr et al., J. Liposome Res. 16 (2006)
Loew et al., J. Drug Del., article ID 376548 (2011).

Summary and conclusions

Advantages of AF4/MALS

- ✓ Large range of application for the characterization and analysis of colloids
- ✓ High versatility in separation conditions → can be adjusted to the needs of the samples
- ✓ Broad separation range over the whole colloidal size range
- ✓ Robust and reproducible separations, no stationary phase
- ✓ Monitoring of changes in size during, e.g., drug release/transfer studies

Challenges

- Requirements on sample (size in the nm-range, physical stability)
- Potential sample-membrane interactions and artifacts due to sample dilution
- Loss of sample (e.g. drug "release") upon fractionation
- Relatively time-consuming (normally ~ between 45 and 70 min)

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