Flow cytometry as a new approach to investigate drug transfer between lipid particles

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Differential scanning calorimetry (DSC)

The physical state of the matrix lipid in the donor lipid nanoparticles was investigated in a Micro DSC III (Setaram, F-Caluire). Approximately 300 µl of the sample were measured at a scan rate of 0.5 °C/min. A measurement consisted of two heating runs and two cooling runs with alternating heating ((1) 25 to 65 °C; (2) -5 to 65 °C) and cooling steps ((1) 65 to -5 °C; (2) 65 to 25 °C). Between heating and cooling an isothermal phase of 5 min was entered. A crucible filled with undecane was used as reference.

Donor emulsions (stored at room temperature) usually did not contain melting peaks of crystalline matrix lipid in the thermograms of the first heating run confirming the liquid state of the lipid. This state was retained as long as the donor emulsions were used for transfer experiments. Only in some cases small amounts of crystalline material were observed with a melting enthalpy value not exceeding 1 % of the melting enthalpy of the second heating run (i.e., less than 1 % of the triglyceride had crystallized in these formulations). Thermograms of donor suspensions (stored at refrigerator temperature) displayed a broad melting transition when heated for the first time confirming the crystalline state of the matrix lipid. The thermograms had not changed when the samples were measured again after some weeks. The broad endothermic peak (peak temperature 52.5 °C) represents the melting transition of the solid trimyristin in the β-modification. A comparable melting event was usually also observed when the nanoparticle dispersions were reheated after crystallization during the first cooling run. As an exception, lipid nanoparticles loaded with 1.5 mg/ml temoporfin displayed indications for the polymorphic behavior of trimyristin: Freshly crystallized samples exhibited an exothermic event (peak top at 24.5 °C) prior to a somewhat narrowed endothermic melting of the trimyristin β-modification. The exotherm indicates the presence of trimyristin α-modification in the freshly crystallized samples. During storage of the recrystallized samples the size of the exotherm peak decreased and had disappeared after 48 hours of storage. Since the suspensions used for the flow cytometric investigations had previously been cold stored (~ 2-8 °C) for at least 3 days it can be assumed that these samples were present in the β-modification at the time of the experiments.

Determination of the lipid content in the donor lipid nanoparticles

Since the concentration of the matrix lipid trimyristin in the donor lipid nanoparticles may change during the preparation process the actual concentration of trimyristin was determined using reversed-phase HPLC and evaporative light scattering detection (Varex MKIII ELSD, Alltech GmbH, D-Unterhaching). The analysis was performed with a 25 cm x 4 mm LiChrocart column packed with LiChrospher 100 RP 18 (Merck KgaA, D-Darmstadt) in a System Gold 126 HPLC (Beckman Coulter Inc., US-Fullerton). Acetonitrile-tetrahydrofurane 55:45 (v/v) was used as mobile phase and the isocratic flow-rate was set at 1 ml/min. The donor suspension particles were dissolved in acetonitrile-tetrahydrofurane 20:80 (v/v) and 20 µl of the solution were injected into the HPLC for analysis. Peaks were integrated and the calculated areas from 6 measurements were averaged.

The determined lipid concentrations are listed in the Supplemental Table I for the donor suspensions. An equal lipid content was assumed for the donor emulsions.

Supplemental Table I.

Concentration of matrix lipid (trimyristin) in the donor lipid nanoparticle formulations determined by HPLC - the lipid content (nominal value 20 mg/ml) was determined for the donor suspensions (n = 6) and an equal content of the donor emulsions is assumed.

	averaged lipid content ± SD [mg/ml]
DoEmu-1/ DoSusp-1	22.92 ± 0.13
DoEmu-2/ DoSusp-2	17.96 ± 0.12
DoEmu-3/ DoSusp-3	22.41 ± 0.09
DoEmu-4/ DoSusp-4	22.20 ± 0.04
DoEmu-5/ DoSusp-5	20.18 ± 0.13

Additional investigations on donor formulations loaded with temoporfin

As during the preparation of the donor liposomes (DoLip-3-Temo) nominally containing 1.5 mg/ml temoporfin a loss of dye was visually detectable, the actual dye content was determined using spectrophotometry. The measurements were done in methanolic solutions at a wavelength of 649 nm and revealed a loss of approximately 13 % temoporfin (actual content 1.3 mg/ml).

In order to verify the complete dissolution of the introduced temoporfin (nominally 1.5 mg/ml) in the donor formulations, microscopic investigations were conducted using a Leica DMRXP microscope (Leica Microsystems GmbH, D-Wetzlar) with a hot stage (Linkam Scientific Intsruments Ltd., UK-Tadworth). The examination of the samples was done at a 100fold magnification in bright field mode with a stage heating rate of 10 °C/min. Temoporfin-free donor formulations and crystals of trimyristin and temoporfin were used as control samples.

Microscopic investigations of the temoporfin-containing donor liposomes did not reveal the presence of crystalline material. In the donor lipid nanoparticles (donor emulsion and suspension) loaded with 1.5 mg/ml temoporfin a low number of small crystals (< 5 µm) of different appearance was, however, detected. Beside agglomerates of small round-shaped crystals larger polygonal or needle-shaped clear crystals were occasionally observed. Trimyristin bulk material investigated as a reference contained agglomerated round crystals which, observed with hot stage microscopy, melted at ~ 57 °C. A few agglomerated crystals melting around 50 to 55 °C were also observed in dye-free nanoparticle dispersions. Reference temoporfin crystals did not melt at up to 80 °C. Neither did the anisometric crystals in the dye loaded donor dispersions melt upon heating up to 80°C. This led to the conclusion that in the nanoparticle dispersions loaded with 1.5 mg/ml some temoporfin crystals may have been present.

Investigation of dye transfer using flow cytometry

Instrument adjustment and method development

An adequate dilution of the acceptor emulsion in water prior to the measurement had to be established first. For this purpose, 5 to 10 µl of the unloaded acceptor emulsion were diluted in 1 ml purified water in a measurement tube and subsequently measured by flow cytometry. The right dilution was achieved when a count rate of approximately 250 events per second with a low sample pressure was reached. After the detection of 10,000 events (corresponding to 10,000 droplet counts, measurement time 30 to 45 seconds) the measurements were stopped.

During the measurement, appropriate instrument settings had to be defined which included adjusting the voltage and the gain of the scattering detectors (detection of forward and sideward scattered laser light) and the photomultiplier tubes (PMT, detection of fluorescence). After these adjustments, the scattering events caused by unloaded acceptor droplets were visible well in the scattering dot plots and the events in the fluorescence histograms occurred at the left side of the histogram (Supplemental Figure I). The established settings for the scattering detectors were kept constant for all experiments. The settings for the respective PMTs usually had to be varied within a small range for a new acceptor emulsion.

The applied flow cytometer has a lower size detection limit of 0.5 µm.[1] As a consequence, only larger particles can cause a scattering signal which is strong enough to be recognized by the scattering detectors. A detection of the donor particles (z-average diameter < 0.2 µm) should thus be impossible. Fluorescence signals, on the other hand, will only be analyzed when they are connected to scattering signals. Thus, fluorescence from the aqueous phase or from the donor formulations cannot be measured.

Nevertheless, some tests were conducted in order to clarify if the donor particles might disturb the measurements. Compared to the acceptor droplets, the donor particles were only present in a very high dilution during the transfer experiments. Measurements of similarly diluted donor particles without addition of acceptor droplets revealed a detection of at most 5 events during a measurement time equal

to the transfer studies. This is in accordance with the usually achieved count rates during cleaning steps conducted between the single measurements. We assume that events during measurements of diluted donor and during cleaning most likely originate from dust or from residual acceptor droplets and that the donor particles do not interfere with the transfer measurements.
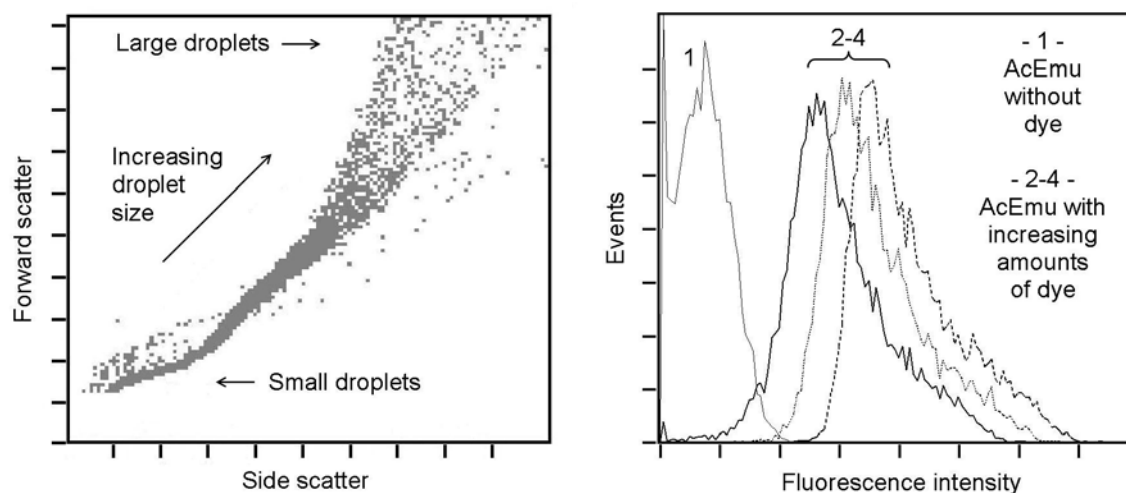
Supplemental Figure I. Graphs displayed by the flow cyotmeter for the acceptor emulsion droplets (AcEmu). Left: scattering dot plot containing all events of forward and side scattered light irrespective of fluorescence. Right: fluorescence intensity histogram – in AcEmu without dye no fluorescence signal is detected (1), with increasing amounts of dye in AcEmu the fluorescence signal shifts to the right due to increasing fluorescence intensity (2-4).

Calibration and control transfer measurements

In order to quantify the measurement results, which were displayed as fluorescence intensity by the flow cytometer, acceptor calibration samples were loaded with different amounts of dye. Approximately 5 to 10 µl of these samples were diluted in purified water and immediately measured at the flow cytometer. A linear correlation between the amount of dye in the acceptor and the detected fluorescence intensity was obtained in any case. The standard curves were usually obtained from 14 to 16 different calibration samples (7 to 8 different dye concentrations were used with each concentration being present twice). Each sample was diluted three times and measured. An example of a resulting calibration curve is given in the Supplemental Figure II.
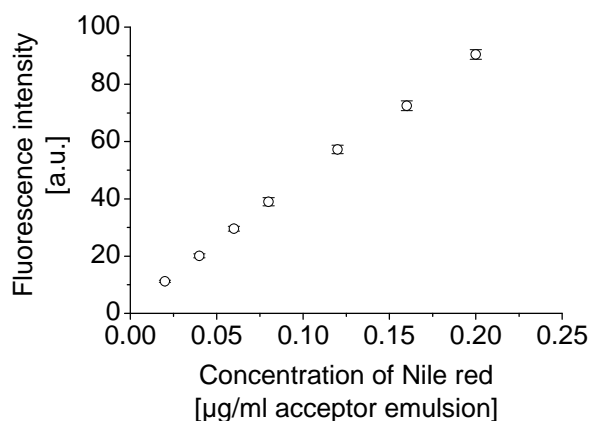
Supplemental Figure II. Calibration curve of Nile red transfer. Acceptor emulsion droplets (AcEmu-1-NR) were loaded with different amounts of Nile red and measured using flow cytometry (n = 6).

Control transfer experiments were conducted with Nile red as a control dye and lipid nanoparticles as control donor formulation. The transfer experiments were conducted at a LMR$_{nom}$ of 1 + 25 without dilution in water. The mixtures were only diluted immediately prior to the measurement at the flow cytometer. Altogether four different donor and acceptor batches were investigated. All showed a transfer (velocity, amount) as presented in Figure 1 (A) of the article. The results presented in this figure are compared with the results of two control experiments in Supplemental Figure III.
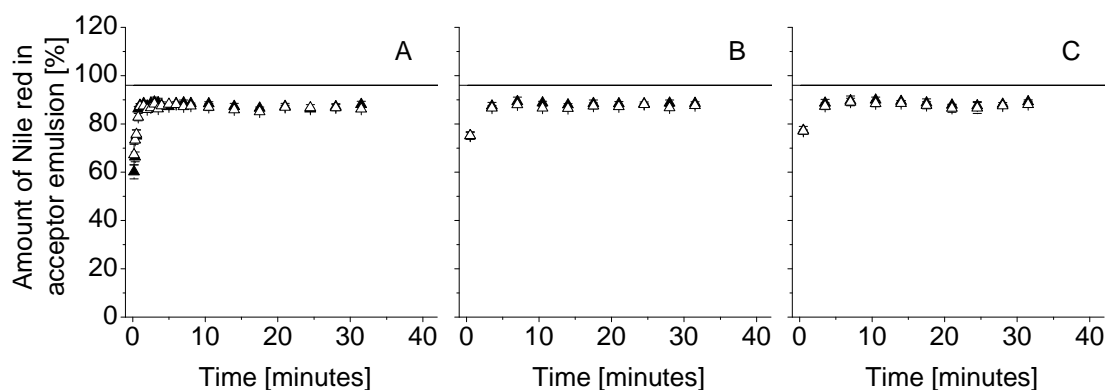

Supplemental Figure III. Transfer of Nile red from donor nanoparticles to acceptor emulsion in a nominal LMR of 1 + 25 for different batches of donor and acceptor particles. 100 % dye in AcEmu correspond to 0.16 µg Nile red, n = 3, ▲ = DoEmu, △ = DoSusp, continuous line = equal distribution of Nile red between donor and acceptor, (A) DoEmu/DoSusp-1-NR to AcEmu-1, (B) Control experiment: DoEmu/DoSusp-1-NR to AcEmu-3, (C) Control experiment: DoEmu/DoSusp-3-NR to AcEmu-4. The results of DoEmu and DoSusp are superimposed in most cases.
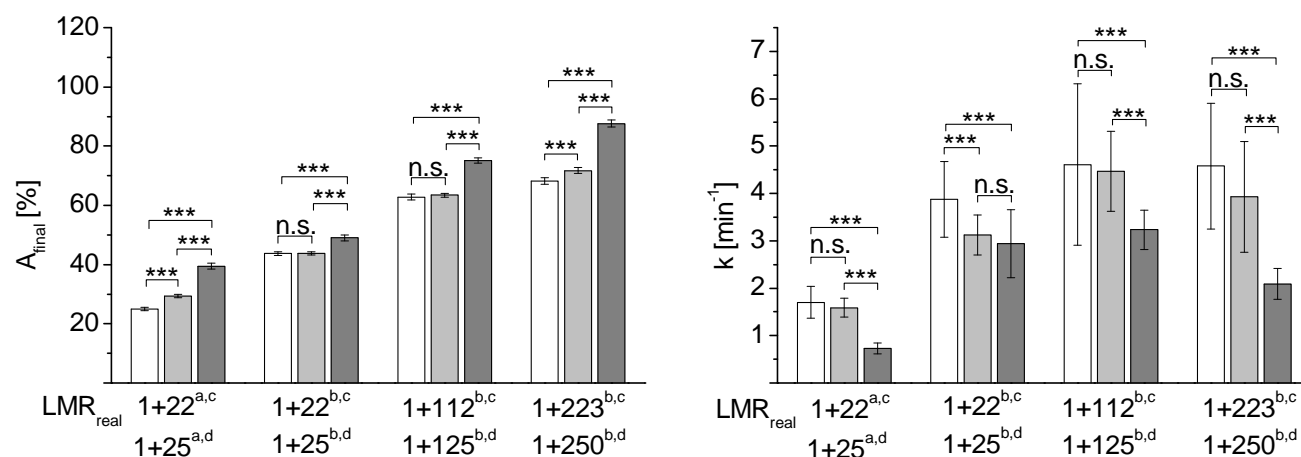
Nile red transfer – statistics
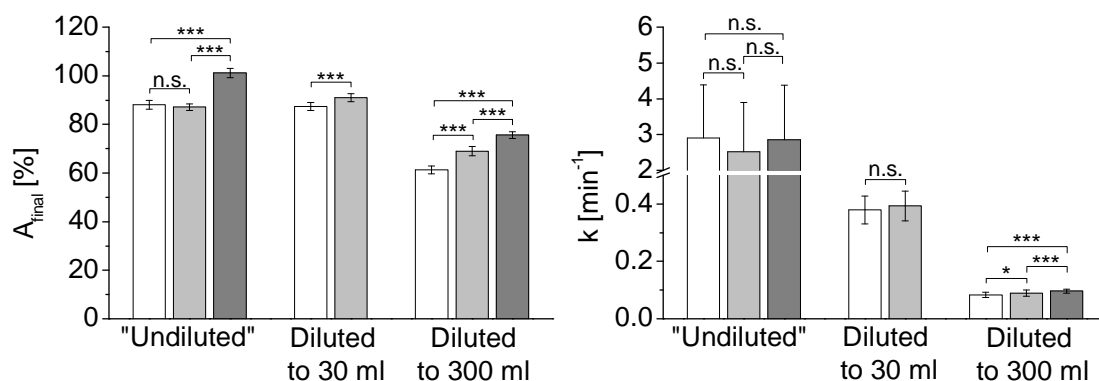
Supplemental Figure IV. Comparison of the finally released percental amount of Nile red (A_{final} – left) and the rate constants (k – right) for Nile red transfer into acceptor emulsions. White = DoEmu, light grey = DoSusp, dark grey = DoLip, n.s. = no statistically significant differences (p = 0.05), * = statistically significant differences (p = 0.05), *** = highly statistically significant differences (p = 0.01). Formulations used can be found in Table 3 of the article.

DiI transfer – statistics
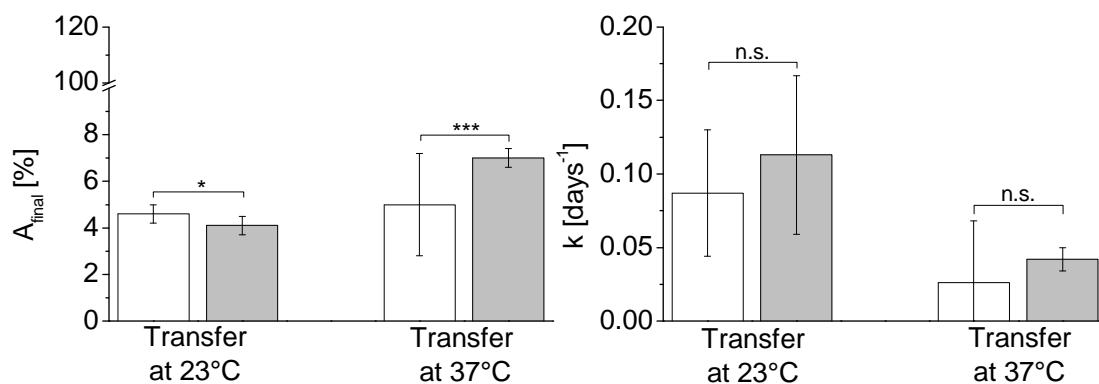
Supplemental Figure V. Comparison of the finally released percental amount of DiI (A_{final} – left) and the rate constants (k – right) for DiI transfer into acceptor emulsions. White = DoEmu, light grey = DoSusp, n.s. = no statistically significant differences (p = 0.05), * = statistically significant differences (p = 0.05), *** = highly statistically significant differences (p = 0.01). Formulations used can be found in Table 4 of the article.

Supplemental Figure VI. Comparison of the finally released percental amount of temoporfin (A_{final} – left side) and the rate constants (k – right side) for temoporfin transfer into acceptor emulsions. White = DoEmu, light grey = DoSusp, dark grey = DoLip, LMR_{real} = actually applied donor-acceptor lipid mass ratio, [a] = low temoporfin concentration (15 µg/ml), [b] = high temoporfin concentration (1.5 mg/ml), [c] = DoEmu/DoSusp, [d] = DoLip, n.s. = no statistical significant differences (p = 0.05), *** = highly statistical significant differences (p = 0.01). Formulations and LMR_{nom} can be found in Table 5 of the article.

Reference

(1) Beckman Coulter Inc. Epics XL and XL MCL Flow Cytometry Systems. http://www.beckmancoulter.com/literature/Bioresearch/DS-10610.pdf (accessed 08/06/02), part of Flow Cytometry. http://www.beckmancoulter.com/ (accessed 08/06/02).