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Drug release studies from lipid nanoparticles in physiological media by a new DSC method

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ABSTRACT

Lipid nanoparticles are an interesting parenteral delivery system for poorly water-soluble drugs. In order to approach physiological conditions when conducting release studies from such systems the release media should preferentially contain lipophilic acceptor compartments such as lipoproteins or other colloidal lipophilic components. In practice, drug release studies under such close to physiological conditions may be complicated by the small size of lipid nanoparticles, which is in the same range as that of the potential acceptor particles. This study describes a novel differential scanning calorimetry (DSC) method for drug release measurements which works without separation of donor and acceptor particles. The technique is based on measuring the crystallization temperature of trimyristin nanoparticles by DSC. The crystallization temperature of the nanoparticles decreases proportionally with the amount of active ingredient incorporated and thus increases as a result of drug release. Liquid trimyristin nanoparticles loaded with fenofibrate, orlistat, tocopherol acetate and ubidecarenone were studied in three different release media with increasing complexity and comparability to physiological conditions: a rapeseed oil nanoemulsion, porcine serum and porcine blood. Using the new method, a correlation between release behavior and drug lipophilicity was observed: the higher the logP value of the drug, the slower the release. The extent of drug release was influenced by partition equilibrium as indicated by increased drug release in the rapeseed oil nanoemulsion compared to porcine serum and blood.

1. Introduction

Lipid nanoparticles are colloids based on a lipid matrix surrounded by an emulsifier layer. They are being investigated for the formulation of poorly water-soluble drugs and are especially interesting as parenteral drug delivery systems due to their small particle size [1]. To assess the potential of these systems for parenteral applications, it is important to evaluate their drug release behavior.

Release studies are usually performed in vitro. A correlation of the results obtained in vitro to the in vivo situation is only feasible if the chosen release analysis setup is adequately adapted to physiological conditions. With regard to parenteral, especially intravenous administration, a general limitation of many in vitro drug release methods is the use of simple aqueous release media, e.g. buffer solutions. These media do not reflect the physiological situation in the bloodstream, where (lipo)proteins and cellular components act as potential lipophilic acceptors for released drug. Many drugs employed in drug release studies with lipid carrier systems are very lipophilic. Their distribution into the aqueous medium can be thus limited by their aqueous solubility or by partition phenomena, which would in turn lead to incorrect release results. Consequently, especially for release investigations with poorly water-soluble drugs, the release media should be adapted to in vivo conditions and, therefore, should contain lipophilic components as acceptors. To improve the solubility of the investigated drug, the aqueous release media can be supplemented with albumin, surfactant or organic solvent [2–6]. To approach the physiological situation as closely as possible the use of physiological acceptor media would be desirable, e.g. plasma, serum or blood should be used.

Release studies on colloidal drug delivery systems in complex acceptor media are often complicated by a similar size of the donor particles and lipophilic structures in the acceptor media. This makes separation of the drug-containing acceptor medium from the donor particles difficult. Different approaches to this problem can be found in the literature: The investigation of drug transfer from colloidal, drug-loaded particles into a lipophilic bulk phase of liquid triglycerides allowed a simple separation of donor and acceptor compartment [7]. Since the acceptor lipid was not dispersed, the interfacial area available for drug transfer was, however, limited and the diffusion pathways were very long, which caused an unrealistically slow transfer of drug [8]. By adding large multilamellar liposomes to the release media,

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which could be separated from the donor system by centrifugation, it was attempted to imitate the physiological conditions better, since these dispersed lipophilic acceptor compartments exhibit a higher specific surface area [9]. Drug transfer was also studied to small unilamellar liposomes as lipophilic components. Acceptor and donor particles were separated by an ion exchange column technique in this case [10]. Another approach focused on the incorporation of lipophilic acceptor nanoparticles into Ca-alginate microparticles [11]. This way, the advantages of small acceptor particles with a large surface area were combined with simple separation from the donor particles by filtration of the Ca-alginate microparticles. In particular for larger Ca-alginate microparticles drug transfer to the enclosed acceptor nanoparticles can, however, be influenced by the diffusion barrier of the hydrogel matrix of the microparticles. Another newly developed method, based on flow cytometry, does not require a separation step between donor and acceptor particles [8]. Using this method the transfer of model drugs from lipid donor nanoparticles into micron-sized oil-in-water emulsion droplets was investigated by fluorescence detection. However, for surface-active substances the release seemed to be influenced by the size of the acceptor particles due to surface area limitations. Furthermore, this method is not universally applicable since it can only be used with fluorescent substances.

As an even closer approach to the in vivo situation, several studies investigated drug transfer into plasma and serum as release media [12–16]. For example, Decker et al. analyzed the transfer properties of temoporfin from liposomes to lipoproteins in human plasma with a gel chromatographic method for separating the individual lipoprotein fractions [15]. Another possibility is the separation of small donor particles from larger acceptor liposomes with online drug quantification by asymmetrical flow field-flow fractionation [16]. However, this method is not easy to carry out and requires a high degree of expertise.

The nature of the lipophilic acceptor in release studies is very important as it influences the drug release in various ways. The challenge is to find a release investigation method which is close to physiological conditions, allows the use of blood or similar physiological media, is applicable to a wide range of drugs and achieves a high time resolution ideally without the necessity to separate donor and acceptor particles.

The aim of this study was to evaluate a new method for drug release measurements which works without separation of donor and acceptor particles and which can be applied in physiological media such as serum or blood as well as in corresponding model release media. The method is based on previous observations that the crystallization temperature of drug-containing triglyceride nanoparticles decreases proportionally with the amount of incorporated active ingredient [17]. It was thus assumed that the crystallization temperature should increase again when drug is released from the triglyceride particles. The crystallization temperature can be easily determined by differential scanning calorimetry (DSC). Provided that only the triglyceride donor particles undergo a phase transition in the temperature region of interest, no separation of donor and acceptor particles should be necessary. This would make release studies feasible also in complex physiological acceptor media such as serum and blood.

The solid triglyceride trimyristin (bulk melting point 56 °C) exhibits strong supercooling in the nanoparticulate state. Therefore, trimyristin nanoparticles remain liquid after preparation by melt-homogenization and crystallize only below room temperature [18]. On this basis, a DSC-based method for drug transfer studies was developed and the release of different drugs from such trimyristin nanoparticles was investigated. Three different media with increasing complexity and comparability to physiological conditions were chosen as acceptor: As a first approach, drug transfer into the droplets of a rapeseed oil nanoemulsion, intended to mimic the lipophilic components in the blood, was investigated. At later stages, porcine serum and porcine blood were used as release media. All transfer studies were carried out at two donor/acceptor mixing ratios to investigate the influence of the partition equilibrium on drug transfer [8]. In order to study the effect of drug lipophilicity on the release behavior, four drugs with different lipophilicity were chosen. According to the logP value (Table 1), fenofibrate (FB) is least lipophilic, followed by orlistat (OL) and α-D-tocopherol acetate (TPA), while ubidecarenone (Q10) has the highest lipophilicity.

Table 1
Information on loaded drugs.

Drug	Abbreviation	logP of drug[a]	Concentration [%] related to trimyristin
Fenofibrate	FB	5.8	3.0
Orlistat	OL	7.0	10.0
α-D-Tocopherol acetate	TPA	10.7	16.6
Ubidecarenone	Q10	19.1	20.7

[a] Source: SciFinder.

2. Materials and methods

2.1. Materials

The triglyceride trimyristin (Dynasan® 114) was a kind gift of Sasol GmbH, Witten, Germany, the surfactant poloxamer 407 (pol 407) (Kolliphor® P127) was donated by BASF AG, Ludwigshafen, Germany. Sodium azide, glycerol and refined rapeseed oil were obtained from Roth, Karlsruhe, Germany. The drugs fenofibrate, α-D-tocopherol acetate and ubidecarenone were purchased from Sigma-Aldrich, Steinheim, Germany, while orlistat was a kind gift from Formosa Laboratories Inc., Taoyuan, Taiwan. Porcine serum was from Biochrom GmbH, Berlin, Germany and porcine blood (with sodium citrate added as coagulation inhibitor) was obtained from a local slaughterhouse. All materials were used as received. Used water was bidistilled quality.

2.2. Preparation of donor lipid nanoparticles and rapeseed oil nanoemulsion as acceptor

The donor lipid nanoparticles consisted of trimyristin which was dispersed in a concentration of 10% in the aqueous phase. The respective poorly water-soluble drug was dissolved in the melted triglyceride in a concentration according to Table 1 (concentration for the particles used in the release studies, for calibration experiments varying amounts of drug were loaded into the particles). The drugs were loaded at concentrations below their respective solubility limit (as determined in separate studies). The emulsion contained 5% poloxamer 407 as surfactant dissolved in the aqueous phase, 2.25% glycerol as isotonizing agent and was preserved with 0.05% sodium azide. All concentrations are given related to the total weight of the emulsions (w/w). The nanoemulsion employed as acceptor medium contained 10% refined rapeseed oil as lipid phase, while the aqueous phase was equal to that of the donor emulsion. The lipid and the aqueous phases were blended for 4 min with an Ultra-Turrax (T25 digital, IKA, Staufen, Germany) at 6000 rpm. Afterwards this premix was submitted to high-pressure homogenization (Micofluidizer M110-PS, interaction chamber type F12Y DIXC, Microfluidics, Newton, USA) for 10 cycles at 350 bar. With trimyristin-containing emulsions, all steps were carried out at 75 °C, while rapeseed oil-emulsions were produced at room temperature. For control measurements, unloaded trimyristin nanoemulsions were prepared as described above.

After homogenization, the emulsions were filtered through a polyvinylidene fluoride filter with 0.45 µm pore size (Rotilabo®, Karlsruhe, Germany) and stored in glass vials at 20 °C, so that the trimyristin nanoparticles remained in a supercooled liquid state.

2.3. Particle size analysis

The intensity weighted mean diameter (z-average diameter) and polydispersity index (PdI) of the lipid nanoemulsions were measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK) at an angle of 173° backscatter. Prior to the measurements, each sample was diluted with purified and filtered water to obtain an appropriate scattering intensity. Following an equilibration time of 300 s, three measurements of 300 s each were run at 25 °C. z-Average and PdI were calculated as means of the three runs.

2.4. Differential scanning calorimetry measurements

Differential scanning calorimetry (DSC) measurements were carried out using a Mettler Toledo DSC 1 STAR^e system with FRS 5 sensor, which had been calibrated with zinc and indium. Approximately 20 mg of the samples were weighed into 40 μl aluminium pans (Mettler Toledo, Gießen, Germany) and cold welded. An empty pan was used as reference. The emulsion droplets were crystallized by cooling from 25 °C to 0 °C using a scan rate of 2.5 K/min. The onset value of the crystallization signal was determined as indicator for the crystallization temperature (T_{cryst}).

2.4.1. Influence of drug load on crystallization characteristics

Trimyristin nanoparticles were loaded with different concentrations of the respective drug directly during production and the change in crystallization temperature depending on the concentration of incorporated drug was determined by DSC.

2.4.2. Transfer experiments

The drug-loaded donor particles were mixed 1 + 9 or 1 + 99 with the respective acceptor system (1 ml in total), i.e. rapeseed oil emulsion, porcine serum or porcine blood in 2 ml glass vials and incubated at 25 °C on a horizontal shaker with 200 rpm. At various time points samples were taken with an Eppendorf pipette directly from the transfer vial and measured by DSC (Fig. 1). Upon cooling in the DSC, only the trimyristin particles crystallize while the rapeseed oil particles, the porcine serum and blood ingredients do not display a phase transition as confirmed by control experiments. Based on the crystallization temperature of the trimyristin nanoparticles, the change in drug content due to release was investigated. Unloaded trimyristin particles were diluted with the acceptor systems in the same ratios and measured at the same time points as control.

The release studies were performed in multiple batches, indicated in the following as n = number of independent release studies. The results are shown as the fraction of transferred drug (normalized to 100%) against the incubation time (hours). Drug transfer from the trimyristin nanoparticles to the lipophilic acceptor was calculated by the change in crystallization temperature which increased upon drug transfer. A correlation between the amount of incorporated drug and the resulting crystallization temperature of the trimyristin nanoparticles was obtained by preparing nanoparticles with various drug concentrations. These particles were produced and analyzed in the DSC as described above.

2.4.3. Determination of $T_{cryst.}$ fluctuations by dilution in acceptor media

Because the change in $T_{cryst.}$ was used to determine the drug transfer, the reproducibility of each measurement had to be examined in the various media, to be able to assess the measuring fluctuation compared to the change in crystallization temperature induced by drug transfer. To do this, the unloaded trimyristin particles were mixed in a ratio of 1 + 9 and 1 + 99 with every acceptor medium used in the release studies. The samples were prepared three times and each was measured three times directly after mixing.

2.4.4. Stability studies of trimyristin nanoparticles in serum

The unloaded trimyristin particles were diluted with porcine serum 1 + 9 and incubated at 25 °C and at 37 °C for 24 h. Samples were taken at the same time points as in the release studies and the droplets were crystallized in the DSC by cooling from 25 °C to 0 °C using a scan rate of 2.5 K/min.

3. Results

3.1. Characteristics of donor and acceptor particles

3.1.1. Particle size

After preparation, the size of the loaded and unloaded trimyristin nanoparticles and the rapeseed oil nanoparticles was measured by PCS. All z-average diameters were between 150 and 160 nm with PdIs between 0.08 and 0.13. During the transfer studies, there were no remarkable changes in the particle size (at most ± 3 nm) and PdIs (at most ± 0.02) in the transfer mixtures.

3.1.2. Crystallization characteristics

Prior studies suggested a linear correlation between crystallization temperature ($T_{cryst.}$) and drug content of Q10-loaded triglyceride particles [17]. If this phenomenon could also be observed for other drugs, this could be used as a basis for drug determination in release studies. To check the influence of drug load on crystallization, trimyristin nanoparticles were loaded with different concentrations of the respective drug and the change in crystallization temperature was determined by DSC. All chosen drugs led to a linear decrease in crystallization temperature ($\Delta T_{cryst.}$) of the trimyristin nanoparticles with increasing drug concentration (Fig. 2). These correlations provided the calculation basis for drug release quantification. The drug concentration chosen for the release studies was below the maximum loading capacity of the donor nanoparticles, but still high enough to obtain a sufficiently large difference in the crystallization temperatures between loaded and unloaded particles. The higher the temperature difference, the more pronounced is the effect of a change in drug

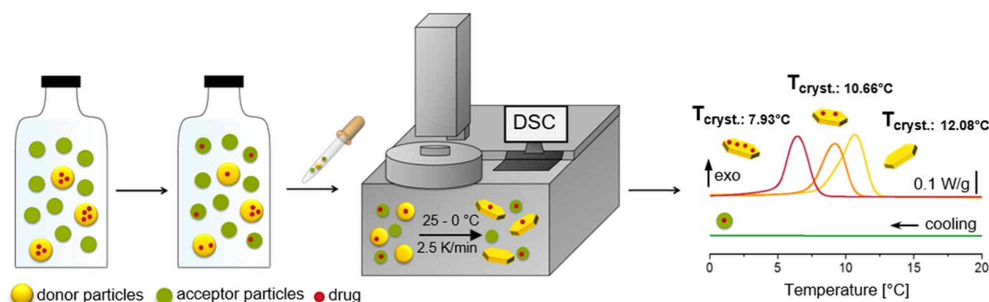

Fig. 1. Experimental approach: drug-loaded donor particles were mixed with the respective acceptor system and the change in crystallization temperature was monitored by DSC. Decreasing drug load of the trimyristin nanoparticles led to an increase in crystallization temperature.

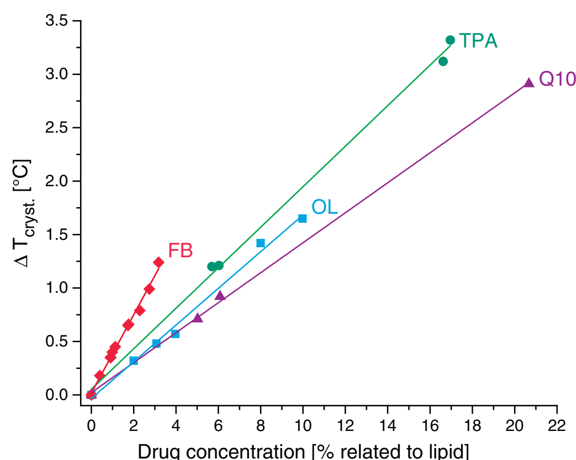

Fig. 2. Correlation between the decrease in crystallization temperature and amount of drug incorporated in trimyristin donor particles (n = 3 DSC measurements, maximum standard deviation ± 0.05 °C).

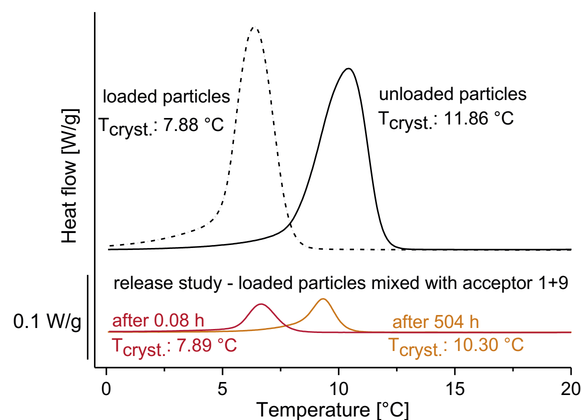

Fig. 4. DSC crystallization curves and corresponding $T_{cryst.}$ values for an example release study of tocopherol acetate-loaded trimyristin nanoparticles in rapseseed oil nanoemulsion (donor + acceptor: 1 + 9). The black lines mark the crystallization peaks of the undiluted trimyristin particles (…… loaded, —— unloaded) the colored ones those during the release experiment.

concentration and the more accurate is the quantification of the fraction of transferred drug. Overall, the differences in $T_{cryst.}$ to be measured were very small and represented a challenge for the DSC method. Therefore, it was necessary to conduct a variety of control measurements to evaluate possible influences on the signal.

3.2. Investigation of drug transfer using DSC

3.2.1. General considerations

Before starting the release studies, a decrease in $T_{cryst.}$ was observed in all drug-containing systems compared to the unloaded trimyristin particles, which were used as control. The control particles were reproducibly prepared in three batches with similar values for the crystallization temperature (12.08 °C, 12.10 °C, 12.04 °C).

To induce drug release, the drug loaded-trimyristin donor emulsions were mixed with the appropriate acceptor medium in two different ratios: 1 + 9 and 1 + 99 (donor + acceptor). These mixing ratios were chosen to investigate the influence of the donor-acceptor ratio on the drug release behavior and to simulate the dilution of the donor particles in the bloodstream after intravenous administration. For a realistic simulation of the in vivo situation, the mixing ratio would have to be higher in most cases. The trimyristin crystallization peaks could, however, not be properly evaluated at dilutions higher than 1 + 99, due to low heat flow. At the chosen mixing ratios, the $T_{cryst.}$ measurements showed good reproducibility (Fig. 3), demonstrated by the low deviations. For the higher dilutions, the standard deviations were higher due to the smaller size of the crystallization signal, which was more difficult to evaluate. The mixing with release media led to a slight $T_{cryst.}$ reduction in all dilutions compared to the undiluted particles.

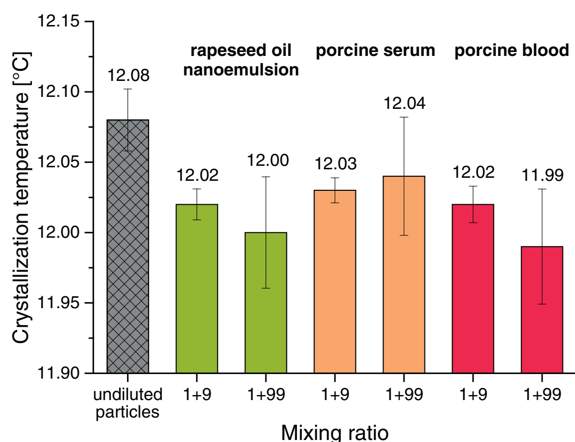

Fig. 3. $T_{cryst.}$ values and their reproducibility in dependence on the dilution with acceptor media (n = 6 DSC measurements).

For release studies, the chosen time points of sampling were defined by the duration of the single DSC runs. A cooling run from 25 to 0 °C at a rate of 2.5 K/min took 10 min, so that the resulting time resolution was lower than in other studies [8,11]. However, the time resolution was regarded as still sufficient considering how much time the nanoparticles would need to reach a target via the bloodstream, e.g. tumor tissue [19,20]. The first measurable time point in the release studies was after approximately 5 min of incubation in acceptor medium due to sample preparation and cooling time in the DSC until reaching the crystallization point.

3.2.2. Calculation of released drug fraction

Because the $T_{cryst.}$ of the trimyristin nanoparticles decreased by loading with drug, the drug transfer from the donor nanoparticles to the lipophilic acceptor particles could be investigated via the change in $T_{cryst.}$, which increased upon drug release. In the following, an example calculation is given for drug transfer evaluation by changing $T_{cryst.}$ (Fig. 4). For this example, a release study of tocopherol acetate-loaded particles in rapeseed oil nanoemulsion was chosen due to the large difference in $\Delta T_{cryst.}$. The values were obtained on a different batch of particles than for the other studies, resulting in a different $T_{cryst.}$ compared to the actual release experiments. The unloaded trimyristin nanoparticles, used as control, crystallized at 11.86 °C which corresponded to 0% drug content in the particles. Upon drug loading the $T_{cryst.}$ decreased to 7.88 °C. $\Delta T_{cryst.}$ was thus 3.98 °C, which corresponded to 16.6% absolute drug content related to the lipid and this was normalized to 100% drug concentration in the donor particles. In the transfer studies, the donor and acceptor particles (rapeseed oil nanoemulsion) were mixed 1 + 9, so that the heat integral decreased by 90%. The $T_{cryst.}$ of the loaded particles increased from 7.89 °C to 10.30 °C within 500 h upon drug release out of the trimyristin into the lipophilic rapeseed oil particles. This temperature increase of 2.41 °C corresponded to 39.4% of drug remaining in the donor particles, whereas 60.6% of drug was transferred. As a control, unloaded particles were mixed with the rapeseed oil particles and were also measured at each time point. Fluctuations in the crystallization temperature compared to the unloaded control particles, which were not diluted with acceptor medium, were thus identified and subtracted from the change in crystallization temperature of the sample, because this was not caused by drug transfer but by the measurement method.

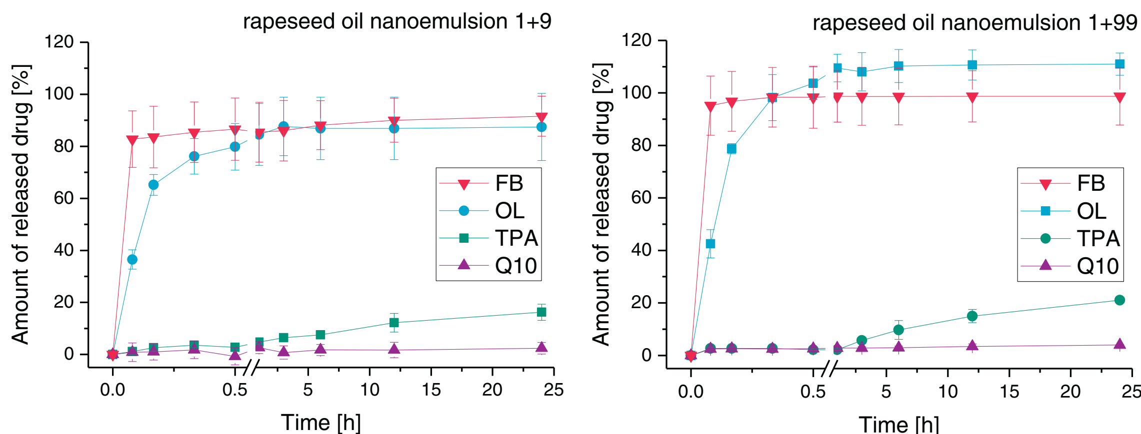

Fig. 5. Drug release in rapeseed oil nanoemulsion at two different mixing ratios (± SD, $n = 3$ release experiments performed at 25 °C).

3.3. Drug release studies in rapeseed oil nanoemulsion

First, the release studies were carried out with refined rapeseed oil nanoparticles as acceptor system. After mixing the respective drug-loaded donor particles with the rapeseed oil nanoemulsion, the crystallization temperatures were measured over 24 h. The unloaded control particles, diluted with the acceptor medium, showed no significant change in $T_{cryst.}$ for 24 h (around 0.03 °C) compared to the particles without acceptor. Fig. 5 presents the transfer results in the rapeseed oil nanoemulsion. After mixing the donor and acceptor systems, the $T_{cryst.}$ of the fenofibrate-loaded trimyristin particles increased rapidly, which is equivalent to a rapid increase in the amount of released drug. Within the first 10 min, a maximum value was reached and the transfer seemed to be completed. The transfer extent obtained at the mixing ratio 1 + 9 was lower than that observed at the higher mixing ratio 1 + 99. Assuming an equal distribution of the fenofibrate between the lipid matrices of donor and acceptor, about 90% and 99% of the drug would be expected within the acceptor medium after completion of drug transfer. The actual plateaus were approximately 88% for the 1 + 9 ratio and approximately 98% for the 1 + 99 ratio, confirming the assumption of an equal distribution. Obviously, the amount of released drug is determined by a distribution equilibrium: the more lipophilic acceptor available, the more drug is released. The loading with fenofibrate resulted only in a slight reduction of the $T_{cryst.}$ (approximately 0.54 °C for 3% FB), therefore the difference between unloaded and loaded particles was small. Consequently, minor fluctuations led to a relatively high standard deviation.

Orlistat-loaded particles displayed a slower increase in $T_{cryst.}$, indicating a more delayed release, which stopped when a distribution equilibrium was reached between donor and acceptor particles (at approximately 85% drug transfer). Consequently, the $T_{cryst.}$ of the unloaded particles was not reached in the 1 + 9 dilution. At the higher mixing ratio 1 + 99, however, more drug was released (apparently > 100% drug transfer), which suggested an equilibrium adjustment also in this case. The apparent release of > 100% was caused by an increase in $T_{cryst.}$ of the samples above the estimated $T_{cryst.}$ of the unloaded particles. The $T_{cryst.}$ of the empty particles after complete drug release is not accessible, which is why we used the $T_{cryst.}$ of the unloaded control particles for calculation. For this reason $T_{cryst.}$ of empty particles is only an approximation of the true value and might vary a bit. However, the control and the drug-loaded particles were produced exactly under the same conditions and had almost the same particle size (at most ± 4 nm), so that the crystallization temperatures should be approximately equal.

The $T_{cryst.}$ of tocopherol acetate-loaded particles increased very slowly in both mixing ratios, indicating a slow drug transfer which was not completed within 24 h (amount of released drug < 20%). The ubidecarenone-loaded sample did not exhibit any increase in the $T_{cryst.}$ when mixed with acceptor medium. No drug transfer between acceptor and donor particles occurred here within the time frame of the experiment.

After extension of the transfer examination time for the TPA- and Q10- loaded particles in the rapeseed oil nanoemulsion with a mixing ratio of 1 + 9, no drug release was observed for Q10 even after > 400

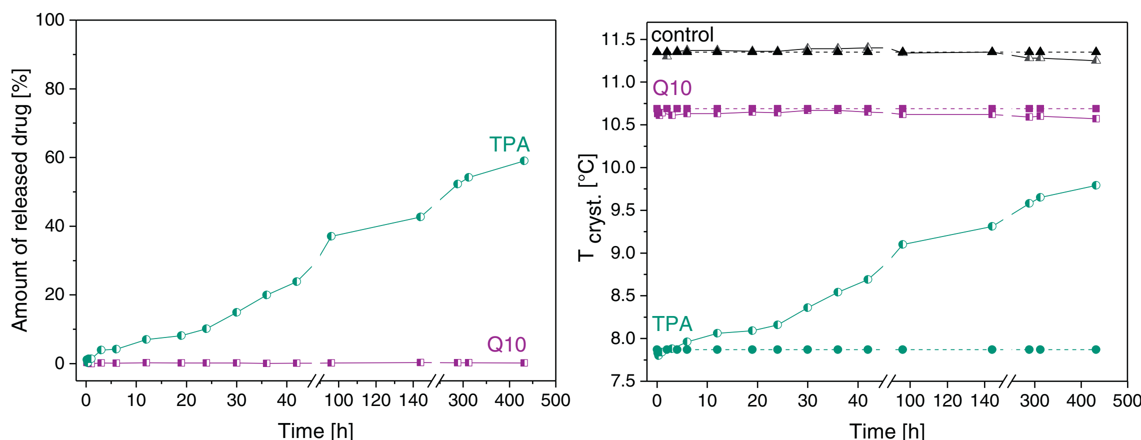

Fig. 6. Release of TPA- and Q10 from trimyristin nanoparticles in rapeseed oil nanoemulsion (1 + 9) over 432 h at 25 °C. Left: calculated extent of released drug, right: changes of $T_{cryst.}$ over time, (––––––– and full symbols = particles without acceptor medium, —— and half-full symbols = particles with acceptor medium, actual sample) ($n = 1$ release experiment). This experiment was performed with a different batch of nanoparticles, resulting in a different $T_{cryst.}$ compared to the other release experiments.

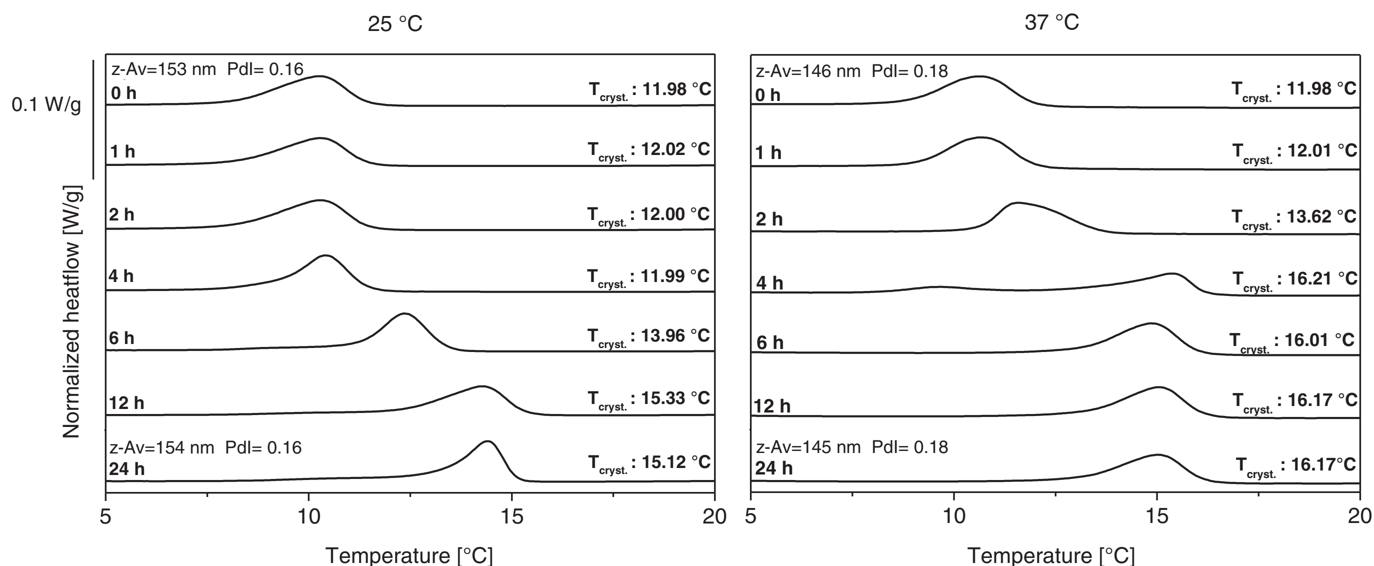

Fig. 7. DSC crystallization curves and $T_{cryst.}$ of trimyristin nanoparticles incubated in porcine serum at 25 °C and at 37 °C over time. Results of PCS measurements at the beginning and the end of the experiment are also given (z-Av = z-average, PdI = polydispersity index).

h (Fig. 6). For TPA, the transfer continued, but was not complete after the end of the experiment. The control measurements still revealed no significant change in $T_{cryst.}$, indicating the suitability of the DSC method even for prolonged release studies.

3.4. Drug release studies in porcine serum

3.4.1. Control measurements

To adjust the method to more realistic conditions, studies were carried out in porcine serum at two different incubation temperatures (25 °C and 37 °C). First, control measurements were performed with unloaded particles incubated in serum to examine possible alterations in crystallization behavior due to interaction with serum ingredients. The results indicated that release investigations in serum at 25 °C are feasible only within the first 4 h (Fig. 7). During this time the $T_{cryst.}$ remained constant. At later time points, the crystallization behavior of the trimyristin nanoparticles was affected as indicated by changing $T_{cryst.}$ and crystallization peak geometry. $T_{cryst.}$ was shifted significantly to higher temperatures, which would make a correct evaluation of drug transfer impossible. When incubated at 37 °C, the crystallization event was affected considerably already after 2 h. Presumably, the lipid nanoparticles were degraded by serum lipases [21,22] and the resulting surface-active free fatty acids influenced the crystallization behavior [23]. The lipase activity increased with increasing temperature, resulting in a more rapid degradation of the trimyristin nanoparticles. The particle sizes and particle size distributions did not change over the time frame of the experiments indicating that DSC is a more sensitive method than PCS to detect degradation processes of such particles.

3.4.2. Drug release studies

With regard to the released amount, the tendencies remained the same as in the rapeseed oil nanoemulsion: fenofibrate-loaded particles showed a rapid release and more drug was released at higher mixing ratio (Fig. 8). However, the transfer curves ended at significantly lower plateau levels than in the release study with rapeseed oil nanoemulsion. These results suggested a distribution equilibrium-controlled process. Orlistat was released from the donor particles more slowly and in the 1 + 99 dilution with acceptor substantially more drug was released. However, the transfer was not complete in the feasible time frame of 4 h. For tocopherol acetate- and ubidecarenone-loaded particles there were no significant changes in $T_{cryst.}$ and therefore, there was no drug transfer within 4 h.

3.5. Drug release studies in porcine blood

In an approach to optimize the DSC method regarding realistic and close to physiological release conditions, the transfer studies were also carried out with porcine blood as acceptor medium (Fig. 9). Compared to serum, in whole blood there are additional lipophilic components such as erythrocytes and other cell components, which can act as

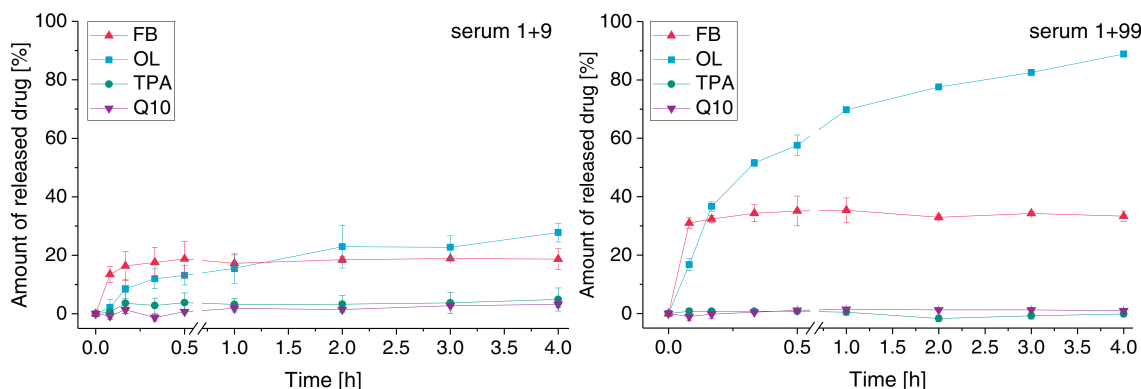

Fig. 8. Drug release in porcine serum (± SD, n = 3 release experiments, performed at 25 °C).

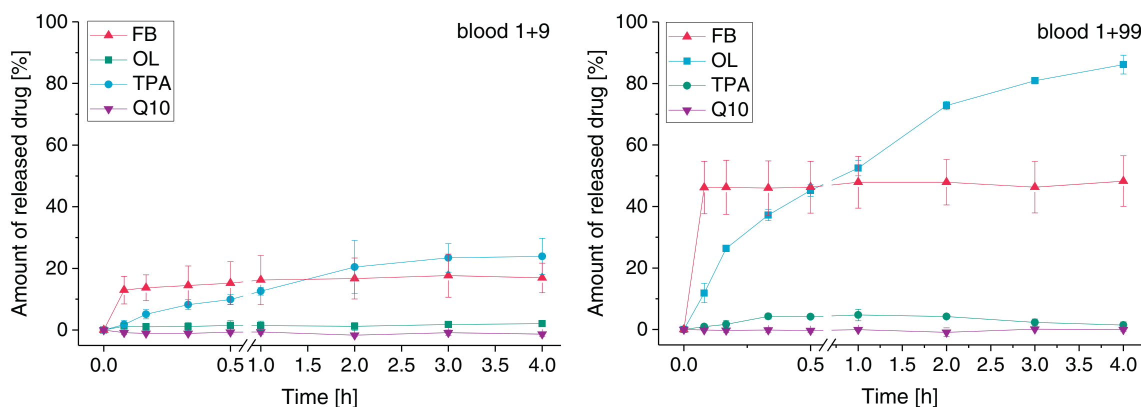

Fig. 9. Drug release in porcine blood (± SD, n = 3 release experiments, performed at 25 °C).

acceptors for released drug. The observed transfer behaviors were, however, comparable to those observed for release in porcine serum (Fig. 8). A slightly higher transfer of fenofibrate was found in porcine blood than in porcine serum, while no differences were observed for orlistat, tocopherol acetate and ubidecarenone compared to serum. The control particles showed no changes in crystallization behavior over the time frame of the experiments. However, a degradation of the control particles was again observed at later time points, similar to the situation in serum (data not shown).

Overall, in the release studies with porcine serum and porcine blood, significantly less drug was released compared to the investigations in which rapeseed oil nanoemulsion was used as acceptor. This suggests either that not enough lipophilic acceptors were available for the transfer of lipophilic drug in serum and blood, or that the transfer was hampered by serum or blood components, e.g. proteins due to formation of a corona around the donor particles [24,25]. In order to elucidate the underlying phenomenon rapeseed oil nanoemulsion was added to the mixture of fenofibrate loaded-donor particles and porcine blood (1 + 9) after 4 h. Before the addition of emulsion, the fenofibrate release was < 20%. After adding rapeseed oil nanoemulsion to the sample (1 + 1), the transferred amount increased very rapidly, reaching a maximum value of nearly 100% release (Fig. S1 in Supporting Information). If the release had been hampered by e.g. a protein shell surrounding the donor particles, the transfer extent should not increase by adding more lipophilic components. Instead, the process seems again to be distribution equilibrium-controlled.

4. Discussion

4.1. Use of DSC for drug release studies

The present study examined the transfer of poorly water-soluble drugs from colloidal nanocarriers into various acceptor media under close to physiological conditions. The newly developed DSC method is promising for drug release studies under certain preconditions; it is very simple, easy to handle and not limited to drugs with specific properties such as fluorescent or radiolabeled substances. A major advantage is that no separation of acceptor and donor particles is required. Under the chosen conditions only the trimyristin donor particles crystallize, while the ingredients of the acceptor media like rapeseed oil nanoemulsion droplets or blood components exhibit no phase transition and there is thus no overlay of thermal events. The applicability of a variety of acceptor systems is an advantage to the setup of Castelli et al., who investigated the drug uptake into unilamellar vesicles as a biomembrane model via the change in transition temperature of the liposomal phospholipids [26,27]. The achievable time resolution in our DSC release investigations is lower than with other approaches [8,11] due to the duration of each crystallization measurement (10 min). Thus, a very fast transfer cannot be monitored in real time. It is, however, possible to differentiate between dispersions with rapid and with sustained release. Colloidal drug carriers are interesting especially for drug targeting strategies, where it is essential that the drug is not released immediately after administration. Instead, the drug should circulate with its carrier over a certain period of time in the bloodstream until reaching the target. The achievable time resolution of this DSC method should be sufficient to decide whether a drug is retained long enough in the carrier to have a realistic chance to be transported to the target site.

As a drawback, the applicability of this method is limited to supercooled trimyristin donor particles and similar systems, e.g. trilaurin or hard fat nanoparticles [18,28]. For this reason, the method cannot be used universally for release studies. It can, however, be employed as a comparative technique for the validation of other newly developed release methods and to obtain fundamental knowledge on the factors governing the release behavior of colloidal carrier systems. Especially for lower drug loads, the sensitivity of the change in crystallization temperature might become a critical factor, as the method is very delicate due to the very small signal strength. Such DSC investigations cannot be regarded as routine measurements. To obtain reliable results, many controls are required. Uncertainty is added by the use of the $T_{cryst.}$ of the unloaded in comparison to that of the loaded particles for calculating the amount of released drug. The $T_{cryst.}$ of the control particles is used to represent complete drug release, but if the loaded and unloaded particles are produced in different batches it is only an estimation of the real $T_{cryst.}$ after complete release. This may cause an apparent release of > 100% as observed for orlistat in our studies. A remedy for this inaccuracy could be passive drug loading [29,30] for which unloaded trimyristin particles are produced that are subsequently loaded with drugs. This ensures that loaded and unloaded particles are from the same batch and have the same output crystallization temperature, making it easier to identify when the particles are empty. However, passive loading does not work with all substances, especially not with very lipophilic drugs like ubidecarenone [29].

Unfortunately, the transfer studies in physiological media as performed in our study are limited to a period of about 4 h due changes in $T_{cryst.}$ at later time points which prohibit quantitative analysis. The change in $T_{cryst.}$ is probably caused by a degradation of the lipid donor particles by lipase. At the physiological temperature of 37 °C an even faster degradation of donor particles was observed due to increased lipase activity ruling out to use this temperature for release experiments. Theoretically, with the concurrent control measurements it should still be possible to evaluate the transfer by subtracting the changes in $T_{cryst.}$ caused by interaction with serum components from the $T_{cryst.}$ of the samples. However, the respective mode of action of the drug under investigation has to be considered, as the released drug might affect lipase activity (as in the case of orlistat and fenofibrate) and lead to uncontrollable temperature variations. A suppression of the enzyme activity by e.g. lipase inhibitors or heat treatment might be a possibility to extend the useful period of study.

4.2. Transfer results

Various factors affected the drug transfer behavior from the donor particles to the acceptor systems. Lipophilicity, estimated according the logP value of the drugs, was one of the determining factors. Fenofibrate was chosen as drug with the lowest lipophilicity (logP 5.7) and exhibited the fastest transfer. Orlistat had a slightly higher logP value (7.0) and was released in a bit more sustained manner. Tocopherol acetate (logP 10.7) was more lipophilic and a very slow transfer could be observed for this compound. Ubidecarenone had the highest lipophilicity of all investigated compounds (logP 19.1) and practically no transfer could be detected within the time frame of the experiments. These results suggest a correlation between rate of drug release and logP value: the higher the logP, the slower the release. The results are in agreement with the conclusion by Takino et al., who postulated that a logP value of about 9 was required to accomplish sustained drug release from colloidal lipid emulsion droplets [31].

The transfer behavior seems also to depend on the structure and the mass ratio of the lipophilic acceptor system. In the rapeseed oil nanoemulsion, fenofibrate was released nearly completely. In contrast, in porcine blood and serum only a comparatively small amount of fenofibrate could be released, limited by the distribution equilibrium as a result of the lower amount of available lipophilic acceptors. The results with two different mixing ratios also suggest a dependence of the transferred drug fraction on the ratio between donor and available acceptor. With an increasing ratio of acceptor compared to donor, the amount of transferred drug increased. This was also observed in previous studies: drug transfer was an equilibrium controlled process [8,11]. The increased transfer upon addition of further lipophilic acceptor (rapeseed oil nanoemulsion to fenofibrate-loaded particles in porcine blood) supports this assumption. The postulated obstruction of drug release by the formation of a protein corona surrounding the donor particles [24] does not seem to play a crucial role in our release studies with fenofibrate.

A rapeseed oil nanoemulsion was used as a lipophilic acceptor system as a very simple approach to the physiological blood composition. This model-blood system, however, did not lead to the same transferred amount of drug as serum or blood in the release investigations, especially for fenofibrate. More lipophilic acceptors seem to be available in the rapeseed oil nanoemulsion than in the physiological media, resulting in a higher drug transfer. Therefore, the lipid mass ratio needs to be adjusted to achieve comparable results. On the other hand, the higher amount of lipid in the rapeseed oil nanoemulsion might offer an opportunity to compensate one disadvantage of the DSC method: The dilution of the donor particles is limited to about 1 + 99 by difficulties in evaluating very small crystallization peaks. To achieve a realistic dilution with regard to administration in humans, release studies would have to be carried out with significantly higher mixing ratios, e.g. 1 + 999. At this higher mixing ratio, much more lipophilic acceptors are available, which would also lead to a higher extent of transfer due to the partition equilibrium. By using a rapeseed oil nanoemulsion-based system as acceptor, an approximation to the real lipophilic conditions upon administration into the bloodstream might be achievable with mixing ratios that are still evaluable in the DSC method. In this regard, the use of mixtures of rapeseed oil nanoemulsion with physiological media would be conceivable.

5. Conclusion

The developed DSC method could successfully be applied to investigate the transfer of different poorly water soluble drugs from lipid nanoparticles into physiological acceptor media, like serum and blood. Compared to commonly applied release methods, the major advantage of this method is that no separation step of acceptor and donor particles is required. That way, it is possible to carry out drug release studies with a broad variety of acceptor systems, especially realistic media. This allows a better approach to the in vivo conditions in blood, e.g. after iv injection. Based on this method, it is possible to investigate moderately fast, slow and very slow transfer processes from lipid emulsion systems. There are no specific requirements concerning the properties of the drugs, e.g. fluorescence. However, the applicability of this method is limited to supercooled trimyristin donor nanoparticles and similar systems.

By using realistic acceptor media such as blood and serum, several factors influencing the release of poorly water soluble drugs could be identified. A correlation between transfer behavior and drug lipophilicity in the order fenofibrate (low lipophilicity, fast and pronounced transfer), orlistat (medium lipophilicity, slightly sustained, but also pronounced transfer), α-D-tocopherol acetate (slightly higher lipophilicity, slow and minor transfer) and ubidecareone (high lipophilicity, no detectable transfer) was observed. Furthermore, the transfer in the rapeseed oil nanoemulsion used as model-blood system was more pronounced than in the physiological media, suggesting an influence on the amount released by partition equilibrium.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.04.032.

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