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Research paper

A dialysis-based *in vitro* drug release assay to study dynamics of the drug-protein transfer of temoporfin liposomes

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ABSTRACT

Today, a growing number of nanotherapeutics is utilized to deliver poorly soluble compounds using the intravenous route of administration. The drug release and the direct transfer of the active pharmaceutical ingredient to serum proteins plays an important role in bioavailability and accumulation of the drug at the target site. It is closely related to the formation of a protein corona as well as the plasma protein binding of the compound. In the present study, two in vitro drug release methods, the flow-through cell and the dispersion releaser technology, were evaluated with regards to their capability to measure a time-resolved profile of the serum protein binding. In this context, the photosensitizer temoporfin and temoporfin-loaded liposomes were tested. While in the fine capillaries of the flow-through cell a rapid agglomeration of proteins occurred, the dispersion releaser technology in combination with the four-step model enabled the measurement of the transfer of drugs from liposomes to proteins. In presence of 10% of fetal calf serum approximately 20% of the model compound temoporfin were bound to serum proteins within the first 3 h. At higher serum concentration this binding remained stable for approximately 10 h.

1. Introduction

For many years, nanocarrier technology has been applied to deliver poorly soluble compounds using the intravenous route of administration [1]. The drug product Foscan® was approved for palliative second-line therapy of patients with head and neck cancer by the European Union [2–4] and a liposomal formulation was evaluated in a phase I clinical trial under the trade name of Foslip® [5]. Both products contain the poorly soluble photosensitizer *meso*-tetrakis(3-hydroxyphenyl) chlorin (temoporfin, mTHPC).

Upon administration, drug and carrier are exposed to high concentrations of serum proteins. Over time, the major fraction of temoporfin is distributed into deeper compartments of the human body, however, between 85 and 87% of the drug are bound to plasma proteins according to the European Public Assessment Report [6]. Within 24 h the drug is redistributed to lipoproteins with approximately 73% bound to high density lipoprotein, 8% bound to low-density lipoprotein, and 3% bound to very low density protein [7].

Giving a focus to nanocarrier formulations, the formation of a protein corona involves direct interactions of serum proteins with the particle surface [8–11] allowing a transfer of drug molecules to serum proteins [12]. Additionally, proteins can act as a diffusion barrier to drug release affecting the *in vivo* performance of the drug delivery system [13].

Currently, a wide range of methods is applied to test the drug release from nanosized carriers [14]. More often, sample filtration

Abbreviations: A4D, adapter for dialysis; ß-CD, methyl-ß-cyclodextrin; CE, cellulose ester; DR, dispersion releaser; DLS, dynamic light scattering; FCS, fetal calf serum; HPLC, high performance liquid chromatography; temoporfin/mTHPC, *meso*-tetrakis(3-hydroxyphenyl)chlorin; MWCO, molecular weight cut-off; MLV, multilamellar vesicles; PDI, polydispersity index; PBS, phosphate buffered saline; PEEK, polyether ether ketone; Penstrep, penicillin streptomycin; PTFE, Polytetrafluoroethylene; rpm, revolutions per minute; SD, standard deviation; USP, United States pharmacopoeia; %EE, encapsulation efficiency

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requires high mechanical pressure which is limiting sensitivity of the method [15]. When testing drug formulations such as liposomes or polymeric nanocarriers, dialysis-based approaches are preferred [14,16,17].

Among other techniques, two methods were successfully evaluated. Burgess and co-workers used a flow-through cell apparatus 4 of the United States Pharmacopoeia (USP) in combination with the 'adapter for dialysis' (A4D) [18,19]. It is mounted into a 22.6 mm cell and was used to investigate drug release from dexamethasone-loaded liposome formulations [18,19]. This setup was also applied to investigate the release of vitamin E acetate from nanoemulsions [20]. Alternatively, the dispersion releaser (DR) technology is a dialysis-based adapter comprising a sample holder cell for dispersed dosage forms [21]. It is mounted into USP apparatus 2 and equipped with a dialysis membrane [17].

In the present investigation, A4D and DR technology were evaluated to study protein interactions of Foscan® and temoporfin-loaded liposomes. For this purpose, permeation experiments were conducted using dialysis membranes with a molecular-weight cut-off (MWCO) of 50 and 300 kDa, respectively. The smaller membrane pore size leads to an effective retention of serum proteins [17]. To exclude the influence of the lowered surface area on drug diffusion, the release profiles were normalized using the *four-step model* [17,22]. Applying the DR technology, a time-resolved profile of free and the protein-bound temoporfin released from the carrier was obtained. Being aware that only a small fraction of the rather lipophilic drug substance reaches blood circulation in an unbound state [23], the analytical method holds great potential for future investigations.

2. Materials and methods

2.1. Materials

Temoporfin was kindly provided by biolitec research GmbH (Jena, Germany). The DR was fabricated from polyether ether ketone (PEEK) at the facilities of Goethe University (Frankfurt am Main, Germany). Spectra/Por cellulose ester (CE) dialysis tubing with a molecular weight cut-off (MWCO) of 50 kDa or 300 kDa were purchased from Spectrum Labs (Rancho Dominguez, USA). Methyl-ß-cyclodextrin (ß-CD) and penicillin streptomycin (Penstrep®) solution were purchased from Sigma Aldrich (Steinheim, Germany). FCS was purchased at PAA Laboratories GmbH (Egelsbach, Germany). For liposome preparation, the lipid Lipoid S 100 was purchased from Lipoid GmbH (Ludwigshafen, Germany). All organic solvents were gradient grade for liquid chromatography.

2.2. Determination of drug solubility in release media

Prior to the release experiments the thermodynamic solubility of temoporfin in release media was tested. For this purpose, an excess of the drug was added to 3 mL of each medium and incubated at 37 °C for 24 h. The release medium was composed of a 10 mM phosphate buffered saline (pH 7.4) supplemented with 0.1% of ß-CD. An amount of 0%, 10% and 50% (v/v) of FCS was added and 1% (v/v) of PenStrep® to avoid microbial growth. The supernatants were filtered through a 0.45 µm PTFE syringe filter and drug concentration was quantified by HPLC analysis.

2.3. Quantification of temoporfin by high performance liquid chromatography

The quantification of temoporfin was conducted using two different high performance liquid chromatography (HPLC) systems. All samples obtained from the permeation experiments conducted with the flow-through cell were analyzed in a Waters Alliance (Milford, USA) separation module (no. 2695) equipped with a photodiode array detector (no. 996).

All other samples were measured using a Hitachi Chromaster HPLC system (Tokyo, Japan) equipped with a photodiode array detector (no. 5430) and fluorescence detector (no. 5440), an auto sampler (no. 5260), a column oven (no. 5310) and a pump (no. 5160). A similar analytical method was used on both systems as described previously [24]. All samples were diluted with mobile phase comprising acetonitrile and 0.1% (v/v) trifluoroacetic acid in purified water (57.5:42.5, m/m) before the injection into a 5 µm Gemini NX-C18 reversed phase column (Phenomenex Inc., Aschaffenburg, Germany). The samples comprising FCS were additionally homogenized for 30 min in a thermomixer (Thermomixer comfort, Eppendorf AG, Hamburg, Germany) at 18 °C and 750 revolutions per minute (rpm) and afterwards centrifuged at 18 °C and 13,994 rpm for 10 min (Centrifuge 5430 R with rotor FA-45-30-11, Eppendorf AG, Hamburg, Germany). The flow rate was adjusted to 1.0 mL/min and the temperature was kept constant at 30 °C. For the photodiode array detector, a detection wavelength of 420 nm was used. The fluorescence detector was operated at excitation and emission wavelength of 410 nm and 653 nm, respectively.

2.4. Preparation of temoporfin liposomes

Lipoid S100 and approximately 4 mg of temoporfin were dissolved in a volume of 3 mL of methanol. The organic solvent was removed under vacuum using a rotary evaporator (Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Schweiz) and the resulting thin lipid film was then hydrated with phosphate buffered saline (PBS) at a pH 7.4. The extrusion was performed on multilamellar vesicles (MLV) using a LiposoFast-Basic Extruder from Avestin, Inc. (Ottawa, Canada) with a 100 nm pore sized polycarbonate membrane. For this purpose, liposomes (1 mg/mL) were repeatedly cycled through the membrane (40 times). Un-entrapped drug was removed by a centrifugation step at 10,000 rpm for 15 min (Centrifuge 5430 R with rotor FA-45-30-11, Eppendorf AG, Hamburg, Germany).

2.5. Liposome characterization

The intensity mean diameter (z-average) and polydispersity index (PDI) of the liposomal formulation were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Panalytical Ltd, Malvern, UK) with a backscatter detector at an angle of 173°. Liposomes were diluted 400-fold with PBS at a pH 7.4 and all measurements were between 6 and 9 attenuation.

2.6. Quantification of encapsulated temoporfin

The HPLC method described in Section 2.3 was used to quantify the amount of temoporfin entrapped into the liposomes. The analysis was performed on a Hitachi Chromaster HPLC system (Tokyo, Japan) equipped with a fluorescence detector (no. 5440), an auto sampler (no. 5260), a column oven (no. 5310) and a pump (no. 5160). Encapsulation Efficiency (%EE) was calculated using following Eq. (1):

$$\%EE = \frac{W_{entrapped}}{W_{total}} * 100 \quad (1)$$

$W_{entrapped}$ denotes the amount of temoporfin entrapped into the liposomes and W_{total} denotes the total amount of drug added during preparation.

2.7. In vitro permeation and release experiments

Two different dialysis-based setups were used for the *in vitro* permeation and release experiments.

An A4D dialysis adapter was used in conjunction with a 22.6 mm sample cell for a USP dissolution apparatus 4 (Sotax AG, Switzerland). A dialysis membrane was mounted around this housing (CE membrane,

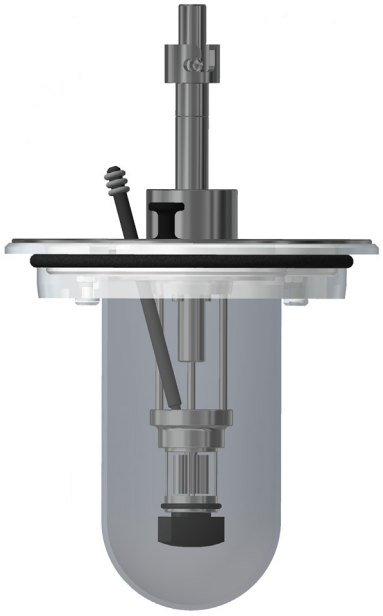

Fig. 1. 3D schematic of the dispersion releaser system.

MWCO 50 kDa). A ruby bead (5 mm diameter) was placed at the base of the 22.6 mm sample cell and 4 g of 1 mm diameter glass beads were added to fill the bottom conical part of the sample cell. Temoporfin was added to the donor compartment and the opening was sealed with an O-ring. The system was operated at 37 °C at a flow rate of 16 mL/min. The release medium was composed of phosphate buffered saline (PBS) at a pH of 7.4 supplemented with 0.1% of ß-CD and different concentrations of FCS depending on the experiment. 1% [v/v] of a PenStrep® solution was added to avoid microbial growth. For de-aeration of the media, the method suggested in the USP was applied. The drug permeation coefficient (k_M) was determined using a medium comprising the same amount of ß-CD but in absence of serum proteins.

The DR setup (see Fig. 1) was used in combination with a USP apparatus 2 equipped with a mini-vessel configuration (Pharma Test Apparatebau AG, Hainburg, Germany). A CE dialysis membrane with a MWCO of 50 kDa or 300 kDa was mounted around the donor compartment of the DR and fixed with O-rings. Afterwards the donor chamber was partly filled with release medium. The medium in the donor and the acceptor compartments was composed of phosphate buffered saline (PBS) at a pH of 7.4 supplemented with either 0.1% or 0.05% of ß-CD and different concentrations of FCS depending on the experiment. An amount of 1% [v/v] of a PenStrep® solution was added to avoid microbial growth. For de-aeration of the media, the method suggested in the USP was applied. The drug permeation coefficient (k_M) was determined using a medium comprising the same amount of ß-CD but in absence of serum proteins.

A total weight of 140 g of the medium was filled into each vessel. Prior to the experiment, the dialysis membrane was treated according to the instructions of the manufacturer. Foscan® as well as the liposomal formulation were filled into the donor compartments with a syringe and a blunt needle (Sterican® MIX, 1.2 × 40 mm, B.Braun, Melsungen, Germany). The amount of drug was adjusted to 336 µg for Foscan® and 404 µg for the liposomes in each vessel to ensure sink conditions.

At predetermined time points (1, 2, 3, 4, 6, 8, 10 h for the permeation experiment with the A4D; 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10 h for the permeation experiment with the DR and two additional time points after 24 and 48 h for the release experiment with the liposomal formulation) samples of 2 mL in case of flow-through cell experiments and 500 µL for the experiments with the DR were collected from the acceptor compartment and replaced with fresh medium. These experiments were conducted at a temperature of 37 ± 0.5 °C and at a stirring rate of 75 rpm.

2.8. Calculation of drug permeation coefficient and normalized drug permeation profiles

The normalized permeation profiles as well as the drug permeation coefficient (k_M) were calculated using the reference experiments conducted in absence of FCS with the help of the four-step model [22]. The initial amount of temoporfin (Q_0) remains constant throughout the experiment allowing the calculation of the concentration in the donor compartment (Cd) (see Eq. (2)):

$$C_d(t) = \frac{[Q_0 - C_a(t) \cdot V_a]}{V_d} \tag{2}$$

The concentration profile in the acceptor compartment was assumed to follow Fick's law of diffusion (see Eq. (3)):

$$\frac{dC_a}{dt} = \left[\frac{k_m \cdot A}{h \cdot V_a}\right] \cdot [C_d(t) - C_a(t)] \tag{3}$$

The technical parameters defined by the experimental setup of the *in vitro* test are the membrane surface area (A), the thickness of the dialysis membrane (h), the volume of donor (Vd) and acceptor compartment (Va). These two equations were summarized as follows (see Eq. (4)):

$$\boldsymbol{\frac{dC_a}{dt} = \left(\frac{k_m \cdot A}{h \cdot V_a}\right) \cdot \left[\frac{Q_0 - C_a(t) \cdot V_a}{V_d} - C_a(t)\right]} \tag{4}$$

In a closed dialysis system, $\frac{Q_0}{V_a + V_d}$ represents the final concentration of temoporfin (see Eq. (5)):

$$C_a(t) = \left[\frac{Q_0}{V_a + V_d}\right] \left\{1 - e^{\frac{A \cdot k_m \cdot t \cdot (V_a + V_d)}{h \cdot V_a \cdot V_d}}\right\} \tag{5}$$

The drug permeation coefficient (k_M) was educed using non-linear regression fitting this equation. The coefficient was further used to calculate the slope $\frac{dC_a}{dt}$ by linear regression of three following data points from the permeation profiles. From these experiments, the normalized permeation of temoporfin in the donor compartment was calculated according to Eq. (6):

$$C_d(t) = \left(\frac{\Delta C_a}{\Delta t}\right) * \frac{h * V_a}{k_m * A} + C_a(t) \tag{6}$$

2.9. Statistical and graphical analysis

All data are expressed as the mean value ± standard deviation (SD). All experiments were performed with n = 3 and samples of the permeation and release experiments were analyzed using HPLC. The mean value and standard deviation were calculated by using Microsoft Excel (Microsoft, Redmond, USA). The permeation profiles of temoporfin were analyzed and illustrated using SigmaPlot 14.0 (Systat Software GmbH, Erkrath, Germany). The drug permeation coefficient (k_M) as well as the normalized profiles were calculated using R (www.r-project.org). The log P value ± standard deviation was calculated using ChemSketch (Advanced Chemistry Development, Inc., Toronto, Canada).

3. Results and discussion

The protein corona can affect biodistribution but also the release behavior of nanocarrier formulations [13]. In this context, the protein bound fraction of the drug exhibits a rather different biodistribution pattern compared to the free compound [13,25]. Against this background, the time-resolved analysis of the drug protein transfer at the nanocarrier surface provides a valuable tool for formulation development.

To study the protein interaction of temoporfin, drug permeation of the photosensitizer was tested in presence and in absence of serum proteins. Two different dialysis-based setups commonly used for drug release studies were compared. Both systems have been applied for testing the drug release from liposomal parenterals previously [18,26]. Further experiments were conducted with the DR setup investigating the drug release and how it was affected by the presence of serum proteins.

3.1. Determination of drug solubility in release media

When administered intravenously, drug formulations enter a complex physiological environment and undergo rapid dilution in the human blood stream. To simulate this elevated diffusion pressure and to expose the drug to serum proteins, a phosphate buffered saline was adjusted to pH 7.4 and supplemented with 0.1% of ß-CD. To determine the impact of serum proteins 10% and 50% of FCS were added. Surprisingly, the solubility of temoporfin decreased when adding FCS to the medium. In absence of serum proteins, a solubility of 94.6 ± 1.8 µg/mL was observed. Increasing the amount to 10% (v/v) FCS resulted in a solubility of 54.5 ± 1.7 µg/mL while, in presence of 50% (v/v) of FCS, a solubility of 40.3 ± 3.9 µg/mL was achieved. This was also confirmed by a second solubility study using centrifugation to separate the particulate fraction of temoporfin (data now shown).

Looking at the current literature, there is a certain probability that, under the conditions of the solubility experiment, temoporfin assembles into larger drug-protein complexes [12] leading to a retention of the drug when using a separation method based on particle size and sedimentation behavior. Kurkov and co-workers [27] reported that high concentrations of cyclodextrins are required to affect the drug protein binding. Even though, due to a higher mobility of molecules, the initial dissolution is likely driven by cyclodextrins, after 24 h most of the drug is redistributed to serum proteins. This also confirms a general observation that, depending on the experimental set-up, the conditions used for measuring drug solubility often do not translate to the methods applied for *in vitro* dissolution testing [28]. Under the selected experimental conditions sink conditions were maintained in the donor compartment as well as in the total volume of the permeation or release test.

3.2. Comparison of drug permeation using USP4 method with A4D and USP2 with dispersion releaser

Two different dialysis-based techniques for testing drug release from nanocarrier formulations were evaluated to study the protein interactions of temoporfin [17,18]. The photosensitizer is a poorly soluble compound with a calculated log P value of 9.17 ± 1.53. The drug exhibits a plasma protein binding of approximately 85 to 87% according to the European Public Assessment Report [6].

In absence of FCS the DR technology led to a more rapid permeation of temoporfin compared to the A4D system when using standard configuration (see Fig. 2A). After 10 h a permeation of approximately 80% was reached in the flow-through cell and 100% in the DR system. This was in line with earlier observations reporting a quicker membrane transport of the DR due to the elevated membrane pressure [17]. Surprisingly, supplementing the release media with 10% of FCS led to an increase of membrane permeation in the A4D. Both permeation experiments, in A4D and DR, were comparable in terms of the membrane transport (see Fig. 2B). For the A4D the release rate was even higher than in absence of proteins. At the highest protein concentration (50% of FCS), the A4D was releasing the drug even more rapidly (see Fig. 2C). While the drug permeation in the DR decreased to approximately 80% after 10 h, the A4D setup resulted in a final release of 100%.

This outcome was explained by a strong aggregation of serum proteins occurring during the release test in the flow-through cell. After the experiment, precipitates of the protein were observed in the sample cell as well as in the capillaries of the USP apparatus 4. Using an FCS concentration of 10% (v/v) this precipitation was even more pronounced compared to higher concentrations. It is likely that this precipitation led to a lowered protein concentration but also altered the hydrodynamics inside the sample cell. Against this background, the release rates detected with the flow-through system do not necessarily reflect the conditions defined by the composition of release media and equipment.

For all permeation experiments, the drug permeation coefficient (k_M) was calculated. The four step model provides a single value comparison of membrane permeation relative to the reference experiment [17,22]. Generally speaking, the detected k_M values of the A4D method were lower compared to the DR system. This was attributed to the slow membrane transport achieved in the reference experiment (see Table 1).

These observations were difficult to explain assuming a diffusion-based permeation mechanism and a relatively high protein binding. Previous studies have confirmed a delayed membrane transport of proteins through the 50 kDa membrane system in the DR [17]. Albumin, one of the most abundant serum proteins with a molecular weight of approximately 65 kDa, is responsible for the high protein binding of temoporfin and was expected to delay membrane permeation significantly.

Further investigations were conducted using the DR setup avoiding serum protein aggregation. These findings also suggest careful use of the flow-through system when testing drug release in presence of biorelevant media comprising proteins. The liquid flow inside capillaries, pumping device and the sample chamber are likely to result in significant shear stress.

3.3. Normalized permeation in presence of serum proteins

To investigate the protein-related retention of temoporfin, the normalized permeation profiles were calculated including the mean k_M value, k_M + SD and k_M − SD value [22]. For normalization procedure, a reference experiment in absence of proteins was used (see Section 2.8). The presence of 10% of FCS led to a slightly delayed membrane transport during the first 3 h. After this time period, there was no further effect on membrane permeation observed. At the higher concentration of 50% of FCS approximately 80% of the drug permeated through the membrane. A fraction of 20% was redistributed to serum proteins (see Fig. 3) and remained within the donor compartment over the time of the experiment.

This delay in the membrane transport was also reflected by a slight reduction of the membrane permeation constant (k_M) from $1.133 * 10^{-3}$ to $9.79 * 10^{-4}$ in presence of 10% of FCS to $6.86 * 10^{-4}$ in presence of 50% of FCS, respectively. Since the release medium contains a considerable amount of ß-CD, the membrane transport is not exclusively driven by protein interaction. However, the effect was reliably quantified by the release method. To further analyze the influence of the reference experiment on the sensitivity of the assay, two more profiles were calculated including k_M + SD and k_M − SD (see Fig. 3A and C). There was no significant difference between the three normalized profiles observed, highlighting the suitability of this method to provide a time-resolved profile of the plasma protein binding.

3.4. Comparison of permeation profiles using different membrane pore sizes

As highlighted by the normalized permeation profiles (see Section 3.3), the MWCO of 50 kDa leads to a significant retention of protein-bound temoporfin and minimizes the free exchange of serum proteins between the inner and the outer compartment [17].

To confirm sensitivity of the method with regards to protein-related permeation, permeation through a 50 and a 300 kDa membrane were compared. The permeation profile measured with a 300 kDa membrane

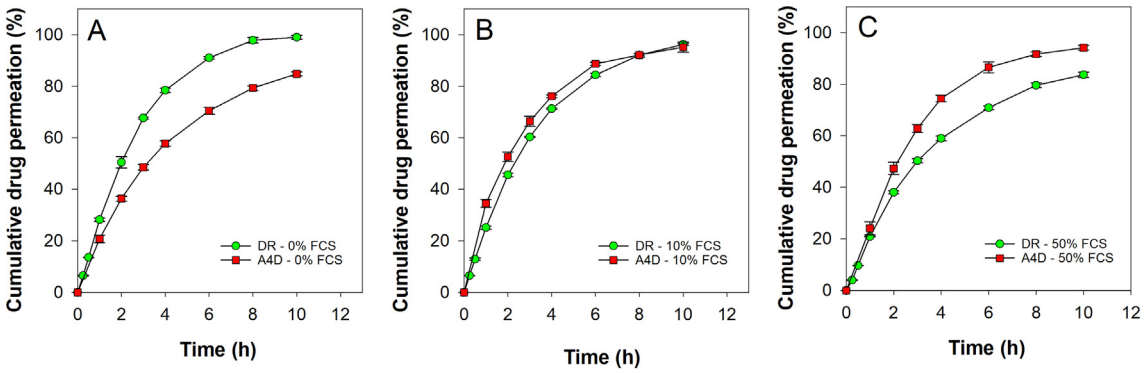

Fig. 2. Cumulative drug permeation profiles observed using the flow-through cell (red square) and DR (green circle) with a 50 kDa membrane in presence of: 0% of FCS (A) show a faster permeation of temoporfin when using the dispersion releaser technology, 10% of FCS (B) present almost identical release profiles and 50% (v/v) of FCS (C) demonstrate a more rapidly drug release in case of the flow-through cell as described in the text, release medium comprised 0.1% of ß-CD, average ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
k_M values determined at different concentrations of FCS and using a 50 kDa membrane and two different dialysis techniques.

	Dispersion releaser k_M value [cm^2/h] ± SD	USP apparatus 4 + A4D k_M value [cm^2/h] ± SD
0% FCS	$1.133 \cdot 10^{-3} \pm 2.22 \cdot 10^{-5}$	$2.11 \cdot 10^{-4} \pm 6.11 \cdot 10^{-6}$
10% FCS	$9.79 \cdot 10^{-4} \pm 3.77 \cdot 10^{-5}$	$3.92 \cdot 10^{-4} \pm 1.58 \cdot 10^{-5}$
50% FCS	$6.86 \cdot 10^{-4} \pm 2.84 \cdot 10^{-5}$	$3.32 \cdot 10^{-4} \pm 1.83 \cdot 10^{-5}$

in presence of 10% of FCS was almost identical to the permeation profile measured with a 50 kDa membrane in absence of serum proteins (see Fig. 4A). This confirms the absence of a delayed drug transport as a result of protein-membrane interaction (see Fig. 4A).

As a next step, a 50 kDa membrane and a 300 kDa membrane were used to measure temoporfin retention in presence of 10% of FCS resulting in a considerably slower membrane permeation (see Fig. 4B). This was even more pronounced when reducing the concentration of ß-CD and, by that, altering the ratio between serum protein and the solubilizer (see Fig. 4C). It is likely, that this effect was due to a reduced competition between the two complexation reactions of ß-CD and serum proteins.

3.5. Preparation of a liposomal formulation of temoporfin

The liposomes were prepared by the well-known film hydration method [29]. The colloidal drug delivery system exhibited a mean diameter of 141 ± 2 nm and a PDI of 0.054 ± 0.007. An amount of % EE 82.1% of the drug was encapsulated into the liposomes.

3.6. Time resolved measurement of the drug-protein transfer from liposomes

To provide evidence for the drug protein transfer from the liposomes, the normalized release profiles were calculated. In the cumulative drug release profile, release kinetics is affected by both, the serum protein binding and the membrane kinetics. Using the four step model enables a direct comparison of the release profiles obtained from different membrane pore sizes [22].

Corresponding to the %EE of 82.1%, approximately 20% of the drug were released within the first hours with an almost identical release profile detected with both membrane pore sizes. After this initial burst, release kinetics differed significantly, due to the retention of protein-bound temoporfin (see Fig. 5).

This suggests an effective time-resolved separation of the fractions of temoporfin bound to liposomes and plasma proteins from the free drug. Each of these fractions is characterized by different physicochemical characteristics and, consequently, *in vivo* distribution behavior [30]. In the context of drug therapy, this finding becomes even more valuable as the free fraction is known to be the most representative for the pharmacologically active concentration at the target site [31,32]. In particular, to maximize the effect in photodynamic therapy, temoporfin requires diffusion into deeper compartments such as the skin. This highlights the advantages of dialysis-based separation methods in determining the kinetic processes involved in drug release.

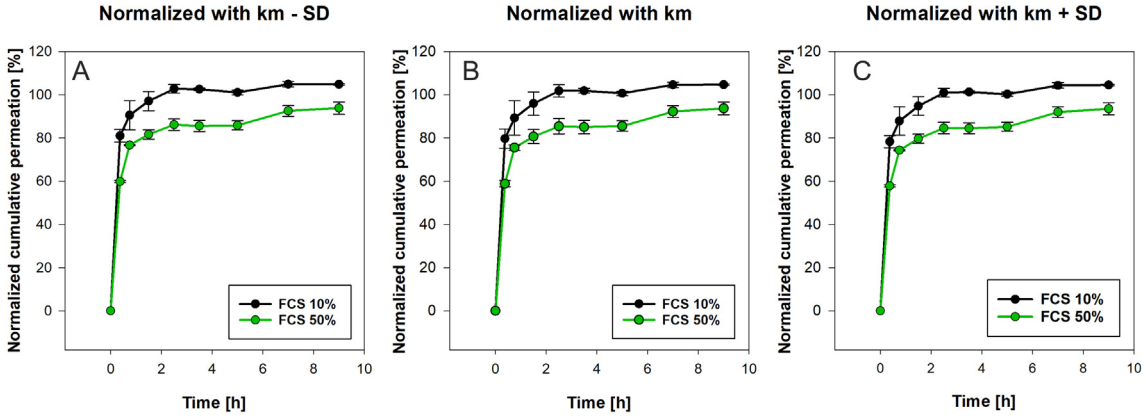

Fig. 3. Normalized permeation profiles with k_M – SD value (A), mean k_M value (B) and k_M + SD value (C) using the DR with a 50 kDa membrane in presence of 10 (in black) and 50% of FCS (in green) and 0.1% of ß-CD show identical release profiles as described in the text, average ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

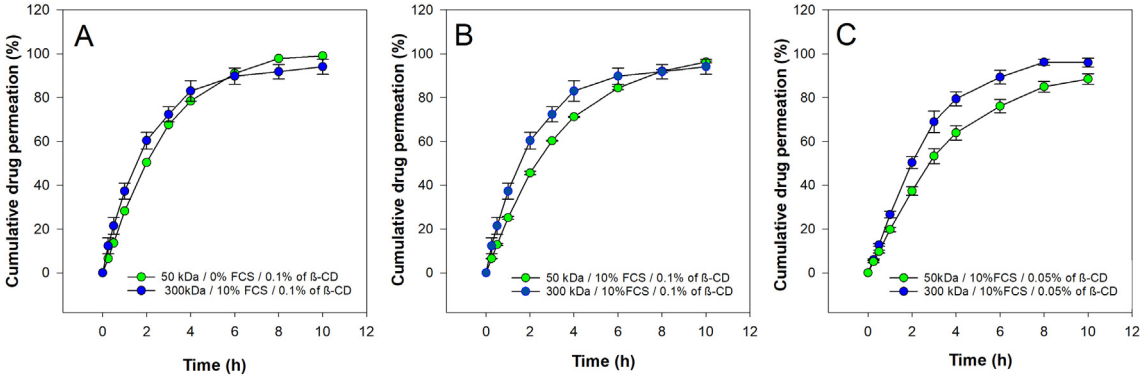

Fig. 4. Comparison of drug permeation profiles of temoporfin when using a 50 kDa membrane (green circle) in absence of FCS and a 300 kDa membrane (blue circle) in presence of 10% FCS (A) indicates no influence of protein-membrane interaction on delayed membrane transport, when using a 50 kDa membrane and a 300 kDa membrane in presence of FCS and 0.1% of ß-CD (B) and when using a 50 kDa membrane and a 300 kDa membrane in presence of FCS and 0.05% of ß-CD (C) demonstrate temoporfin retention in presence of 10% of FCS as described in the text, average ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

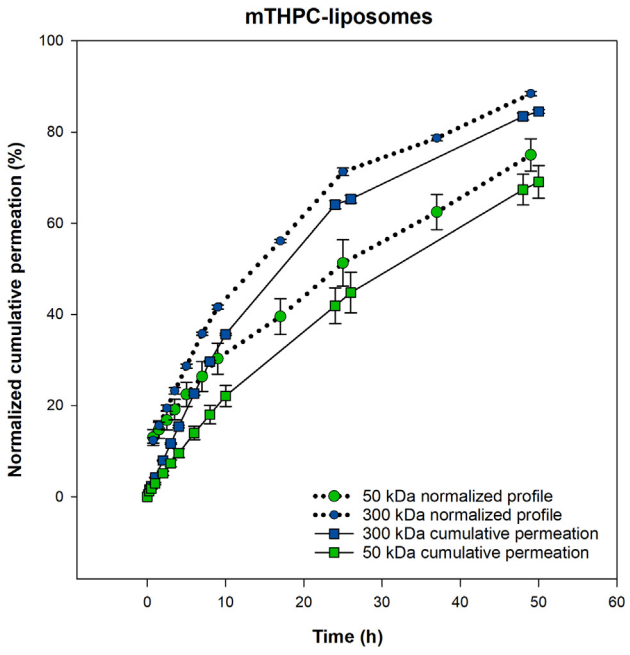

Fig. 5. Comparison of normalized permeation profiles of temoporfin-liposomes using a 50 kDa membrane (green circle) and a 300 kDa membrane (blue circle) in presence of 10% FCS and 0.05% of ß-CD shows significant differences in release kinetics as described in the text. The normalized permeation profiles are plotted with a dotted line, average ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The interaction with serum proteins strongly affects the *in vivo* distribution of drugs but also the surface structure of intravenously administered nanocarrier formulations such as nanoparticles or liposomes [13]. As a dialysis-based technique, the DR technology was capable to sensitively discriminate between different size fractions in the lower nanometer range. Consequently, the *in vitro* release assay reveals the fraction of the drug available for passive diffusion into deeper compartments. For temoporfin, as for most other compounds, this unbound fraction is a major driver of the pharmacological effect at the target site.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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