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A two-stage reverse dialysis *in vitro* dissolution testing method for passive targeted liposomes

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ABSTRACT

A novel two-stage reverse dialysis method has been developed for *in vitro* release testing of liposomal drug product with passive targeting characteristics. The first stage of the test is to mimic the circulation of liposomes in the body, whereas the second stage is to imitate the drug release process at the target. Buffer and surfactant solution were used during the first and second stages, respectively. For formulations containing high phase transition temperature lipids and high cholesterol content, no drug leakage was observed during the first stage of test. In the second stage, however, formulations with different compositions showed significant differences in terms of drug release rate, and discriminatory ability of the method was demonstrated. On comparing two different membrane diffusion techniques, dialysis and reverse dialysis methods, the reverse dialysis method showed significantly lower variation, and therefore is the preferred method. The developed *in vitro* release testing method should help to distinguish formulations with varied compositions for quality control testing purposes. This two-stage reverse dialysis method may pave the way to the development of more bio-relevant release testing methods for liposomal drug products.

1. Introduction

The last few decades have witnessed a rapid development in novel drug delivery systems such as microspheres, liposomes, nano-suspensions, and microemulsions (Kostarelos, 2003). In particular, liposomal drug delivery systems have gained significant interest. Owing to their unique biological and physicochemical properties, liposomes are considered a promising system to deliver a wide range of actives (water soluble and water insoluble small molecules, nucleic acids, as well as large proteins). Liposomes have been formulated to alter the intrinsic distribution of drugs resulting in enhanced therapeutic efficacy and reduced toxicity. To date, twelve liposome formulations have already been approved by the United States Food and Drug administration. With the advances in protein and gene therapeutics (Torchilin, 2005; Torchilin and Lukyanov, 2003) the number of such products is likely to continue to increase.

While these novel parenteral formulations can be very beneficial to the patients, disastrous effects can occur if there is an unanticipated change in product quality or performance. Correspondingly, understanding the factors influencing drug release, from both an *in vivo* and *in vitro* perspective, is essential for the development of meaningful *in vitro* release tests and performance specifications.

The first challenge in developing an *in vitro* release testing method for liposomal drug products is to ensure that it is relevant to the actual *in vivo* release profile. Liposomal drug delivery systems have been used in various application areas, including: tumor targeting, antiviral and antifungal therapeutics, sustained delivery (Kim et al., 1996), as well as non-viral vector for gene delivery (Xu and Burgess, 2011). Different applications require different release testing methods. For sustained delivery purposes, a demonstration of slow drug release over time may be sufficient. For example, a dialysis adapter was successfully used in combination with a standardized flow through cell methods (USP apparatus 4) to demonstrate the different release profiles of various dexamethasone liposomes (Bhardwaj and Burgess, 2010). However, for targeted drug delivery applications, additional demonstration of the absence of drug release prior to reaching the target should also be very helpful. To date, no such type of release testing method has been reported.

To address this problem, in the current study Tenofovir liposomes were selected as a model system to represent passive

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targeted delivery systems. Tenofovir belongs to a class of nucleoside reverse transcriptase inhibitor (NRTI). The encapsulation of this drug into liposomes is a very attractive approach for controlling the progression of AIDS since macrophages play a central role in AIDS pathogenesis by acting as reservoirs and propagators of HIV throughout the immune system (Embretson et al., 1993). Using the naturally occurring macrophage uptake as well as the endocytosis pathway, the encapsulated Tenofovir molecules can be intracellularly delivered directly to the site of action. For this type of the drug delivery, it is important that the liposomes retain the drug molecules in the interior during circulation. This can not only protect the drug from degradation in the serum but also protect the host from experiencing unwanted toxicity due to non-specific drug distribution. However, once the liposomes carrying the drug reach the target the drug should be liberated from the liposomes to achieve the desired therapeutic effect. This kind of delivery strategy is the basis of the *in vitro* release testing method reported here, which can mimic these two stages of the *in vivo* delivery process: (1) there is minimal drug leakage during the initial period (mimicking the phase when the liposomes are circulating in the body prior to uptake at the target); and (2) triggered drug release (mimicking liposome breakdown at the target site).

Another challenge in developing an appropriate *in vitro* release testing method for liposome formulations (or any kind of colloidal system) is to efficiently and accurately separates the released drug content from the carrier for analysis. Currently used techniques can be broadly divided into two categories: (1) sample and separation methods (Kokkona et al., 2000; Vemuri et al., 1991; Xiao et al., 2004); and (2) membrane diffusion methods (such as dialysis sac (Glavas-Dodov et al., 2002; Ruozi et al., 2005; Sezer et al., 2004), reverse dialysis sac (Chidambaram and Burgess, 1999), micro-dialysis (Hitzman et al., 2005), and Franz cells). Methods from the first category suffer greatly from incomplete drug release due to sample loss during sampling and erroneous release profiles are frequently reported if the time scale of drug release is close to the sampling intervals. For this reason, the current study focuses on membrane diffusion based separation techniques (dialysis and reverse dialysis methods).

Additionally, in the current study, various formulations were selected to represent different types of liposome characteristics. This will not only allow demonstration of the discriminatory ability of the release method but also will provide a better understanding of the drug release process from liposomes.

2. Materials and methods

2.1. Materials

Tenofovir was purchased from Resource Technique Corporation (Laramie, Wyoming). Sodium dodecyl sulfate (SDS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salts, Triton X-100, and stearylamine (SA) were purchased from Sigma–Aldrich (St. Louis, MO). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt) (DPTAP) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform, acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Float-A-Lyzer (1 ml, 50 kDa MWCO cellulose ester) were purchased from Spectrum Laboratories. PD-10 Desalting columns (Sephadex™ G-25) were purchased from GE Healthcare (Piscataway, NJ). Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Experimental methods

2.2.1. Preparation of Tenofovir liposomes

All the liposome formulations were prepared using a modified thin-film hydration method (Xu et al., 2011). Briefly, the desired amount of lipids were weighed into a 50 ml pear-shaped flask and ~2 ml of chloroform were added to dissolve the lipids. Chloroform was then evaporated under vacuum at room temperature for 2 h, after which the flask was kept under vacuum overnight to completely remove any residual solvent. Encapsulation of Tenofovir into liposomes was accomplished during the hydration step where dry lipids were hydrated with 10 mM pH 7.4 HEPES buffer (containing the desired amount of drug) at 65 °C for 2 h (vortexed for 30 s every 30 min). After hydration, 1 min of sonication (80 W) was applied to break down any larger particles. Then the samples underwent several freeze–thaw cycles (10 min at −196 °C and 10 min at 65 °C) to facilitate encapsulation of the drug (30 s vortexing between cycles). Subsequently, the samples were put into a LIPEX™ extruder (Northern Lipids Inc., Canada) and passed through a stack of polycarbonate membranes with 200 nm pore sizes to obtain liposomes with the desired particle size. Finally, the samples were purified with two PD-10 columns used in a series configuration.

2.2.2. Tenofovir analysis

Tenofovir was analyzed using an HPLC method as described previously (Xu et al., 2011). In brief, HPLC was performed on a Symmetry C8 column (3.5 μm, 4.6 mm × 100 mm, Waters Corporation, USA) protected with a Symmetry C8 guard column (3.5 μm, 2.1 mm × 10 mm). The mobile phase consisted of acetonitrile–10 mM sodium dihydrogen phosphate (adjusted to pH 6.5 with 1 N sodium hydroxide) at a ratio of (2.5:97.5, v/v). The flow-rate was set at 1 ml/min and the injection volume was 10 μL. Tenofovir was detected at 260 nm using a Perkin-Elmer 785 UV–vis detector, and the retention time was 5.2 min.

2.2.3. Particle size and zeta-potential analysis

Particle size and zeta-potential analysis were conducted using a Malvern ZS90 zeta-sizer. Prepared liposome formulations were diluted at least 50 times to obtain a suspension that was below 0.5 mg/ml. All measurements were conducted at 25 °C and in triplicate, and were reported as mean ± SD.

2.2.4. Diffusion kinetics of Tenofovir through dialysis membranes

A 1 mg/ml Tenofovir standard solution was prepared by dissolving the desired amount of Tenofovir in release medium (10 mM pH 7.4 HEPES buffer), and 0.5 ml of this standard solution was withdrawn and put into each of the dialysis tubes (50 kDa cellulose ester membrane). These dialysis tubes were then put into screw cap glass tubes (200 mm × 25 mm) and dialyzed against 50 ml release medium at 37 °C and 100 rpm. At pre-determined time intervals (10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 540 min after starting the experiment), 1 ml samples were withdrawn from the outside of the dialysis tube and analyzed by HPLC to determine the amount of drug released. The dialysis tubes (total of 6) were divided into three groups to evaluate the effect of surfactants on the diffusion properties of the dialysis membranes. This was necessary since in the later studies surfactants were required to disrupt the liposomes in order to release their contents. Group 1 was tested without any pre-exposure to the surfactants ($n = 2$). Group 2 was treated (24 h at 37 °C) with 1% (w/v) SDS one time ($n = 2$), and Group 3 was treated with 1% (w/v) SDS in combination with 1% (v/v) Triton X-100 for at least two times ($n = 2$). For simplicity, throughout the current manuscript the solution inside the dialysis tubes is referred to as "interior solution" and the solution outside the dialysis tubes is referred to as "exterior solution".

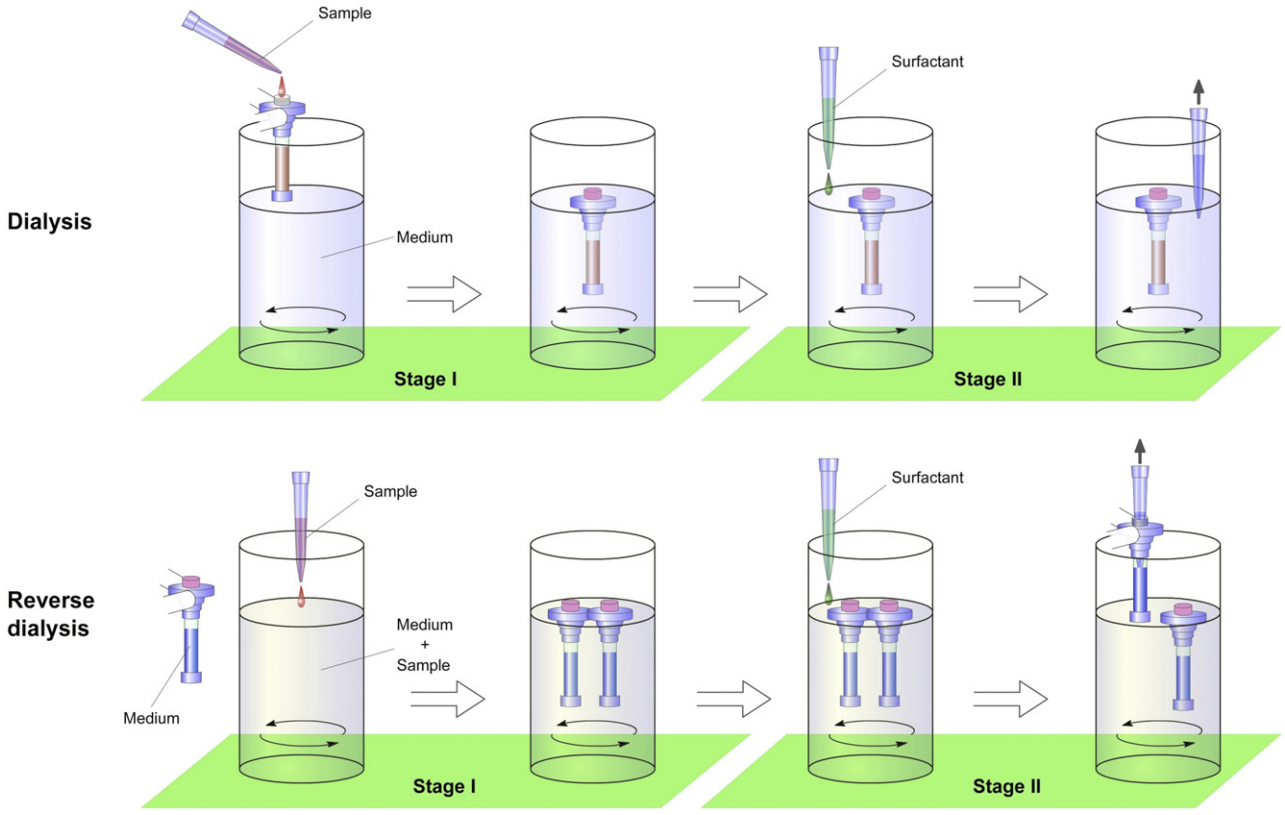

Fig. 1. Comparison of dialysis and reverse dialysis method.

2.2.5. Dialysis method

For the dialysis tube method, liposome dispersions were put into dialysis tubes which were then placed inside screw cap glass tubes filled with release media agitated at 100 rpm and 37 °C in a shaking water bath. At appropriate time intervals, samples were withdrawn from the exterior solution to estimate the percentage of drug released. Fig. 1 demonstrates the different setups for the dialysis and reverse dialysis method. To mimic the *in vivo* release characteristics of the liposomes, a two-stage release test was used.

Stage 1: 0.3 ml of liposome sample was put inside each dialysis tube, which was then put inside a glass tube containing 30 ml of release medium. At 2 and 24 h, 1 ml samples were taken from the exterior solution to determine the drug concentration. After each sample was taken, 1 ml of fresh medium was added back to the exterior.

Stage 2: At 24 h, 0.3 ml of HEPES buffer containing 2% (w/v) SDS and 2% (v/v) Triton X-100 were added inside each dialysis tube to obtain a mixture of 0.6 ml solution containing 1% (w/v) SDS and 1% (v/v) Triton X-100. At the same time, 30 ml of HEPES buffer containing 2% SDS and Triton X-100 were added to the exterior to obtain a 60 ml release medium containing 1% SDS and Triton X-100. At 26, 28, 32, 36, 48 and 72 h, 1 ml samples were taken from the exterior solution to determine the drug concentration. After each sample was taken, 1 ml of fresh medium was added back to the exterior.

2.2.6. Reverse dialysis method

In the reverse dialysis tube method, two identical dialysis tubes each filled with 1 ml of release media and were placed in a screw cap glass tube containing 70 ml of release medium which was agitated at 100 rpm and 37 °C. Liposomes were added to the exterior solution. At predetermined time intervals, 1 ml samples were withdrawn from the interior of alternate dialysis tubes to estimate the percentage of drug released. To mimic the *in vivo* release characteristics of the liposomes, the following two-stage release test was used.

Stage 1: 0.4 ml of liposomes was added to the exterior solution (70 ml). At 4 and 24 h, 1 ml samples were taken from the interior of alternate dialysis tubes to determine the drug concentration. After each sample was taken, 1 ml of fresh release medium was added back to the dialysis tube.

Stage 2: At 24 h, 10 ml of HEPES buffer containing 4% (v/v) Triton X-100 was added to the exterior solution to obtain an 80 ml solution containing 0.5% (v/v) Triton X-100. At 26, 28, 32, 36, 48 and 72 h, 1 ml samples were taken from alternate dialysis tubes to determine the drug concentration. After each sample was taken, 1 ml of fresh release medium was added back to the dialysis tube.

2.2.7. Determination of the rate constant (k)

To be able to compare the release profile of various formulations during the second stage of the release process, the data was normalized to 0% at 24 h (immediately after addition of surfactants) and to 100% after 72 h (or until a plateau was reached). This allows comparison of formulations with different free drug percentages. After normalization, the following model was used to fit the experimental data using non-linear regression. The model was derived based on the assumption that during drug release and the subsequent diffusion processes Fick's law was obeyed. Accordingly, the remaining drug content followed an exponential decay, and using the mass balance the released drug content can be calculated as shown in Eq. (1).

$$Q = 100 \times (1 - e^{-kt}) \quad (1)$$

where Q is the cumulative release percentage, k is the release rate constant and t is the time (h) after addition of surfactant. With known k, T_{99}, T_{90} and T_{50} can then be calculated, which are the times required to release 99%, 90% and 50% of the drug, respectively.

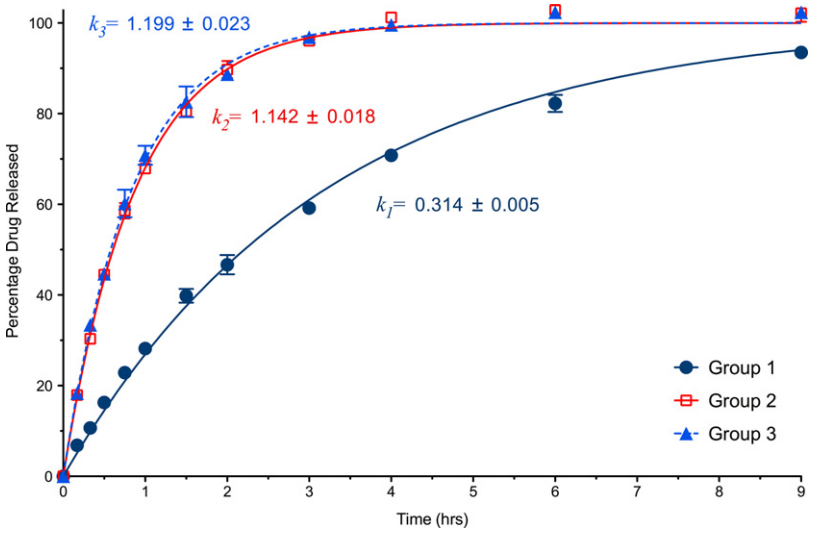

Fig. 2. Diffusion profile of pure drug through dialysis membranes and the effect of surfactant exposures (dialysis membrane MWCO 50 kDa cellulose ester, 10 mM pH 7.4 HEPES buffer, 37 °C, 100 rpm). Group 1: without any pre-exposure to surfactants (n = 2); Group 2: treated (24 h at 37 °C) with 1% (w/v) SDS one time (n = 2); and Group 3: treated with 1% (w/v) SDS in combination with 1% (v/v) Triton X-100 twice (n = 2).

Table 1
Comparison of diffusion rate constant, T_{90} and T_{50} for dialysis membranes with different treatments.

	k (h^{-1})	T_{50} (h)	T_{90} (h)	T_{99} (h)	r^2
Group 1	0.314 ± 0.005	2.21 ± 0.04	7.34 ± 0.12	14.68 ± 0.24	0.996
Group 2	1.142 ± 0.018	0.61 ± 0.01	2.02 ± 0.03	4.03 ± 0.06	0.997
Group 3	1.199 ± 0.023	0.58 ± 0.01	1.92 ± 0.04	3.84 ± 0.07	0.996

3. Results

3.1. Diffusion kinetics of Tenofovir through dialysis membrane

As shown in Fig. 2, without surfactant treatment it took 7 h for 90% of the drug to diffuse through the dialysis membranes (Table 1). However, for membranes treated with surfactants (either one time with SDS or repeatedly with a combination of SDS and Triton X-100) much faster diffusion rates were achieved. As can be seen in Fig. 2, there was no difference between single treatment (Group 2) and multiple treatments (Group 3) (p > 0.01), and hence for all future *in vitro* release studies the dialysis membranes were treated with surfactant at least once prior to the first use (incubated for 24 h at 37 °C). With regard to the effect of the dialysis membrane molecular weight cut-off (MWCO), it was determined that the 20 kDa membrane was not suitable for release testing of Tenofovir as it has a significantly lower diffusion rate constant compared to two other higher MWCO membranes as shown in Fig. 3. Comparable diffusion rate constants were obtained for 25 kDa and 50 kDa membranes. Accordingly, 50 kDa membranes were selected for all future release tests.

3.2. Dialysis method

As shown in Fig. 4, a small portion of the drug (~3%) was detected at the end of the first stage of the release test (24 h), and this portion of the drug was considered to be free drug. In the second stage shortly after addition of the surfactant to the exterior solution, drug started to release from the interior of the dialysis tubes. However, as can be seen in Fig. 4, very large variations

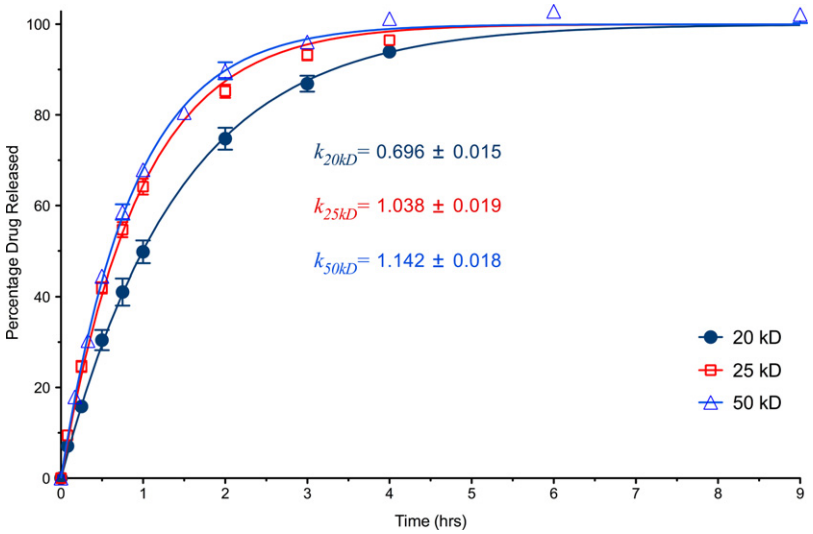

Fig. 3. Comparison of Tenofovir diffusion profiles using different MWCO dialysis membranes (10 mM pH 7.4 HEPES buffer, 37 °C, 100 rpm, each membrane was treated with 1% TX100 and rinsed fully prior to use).

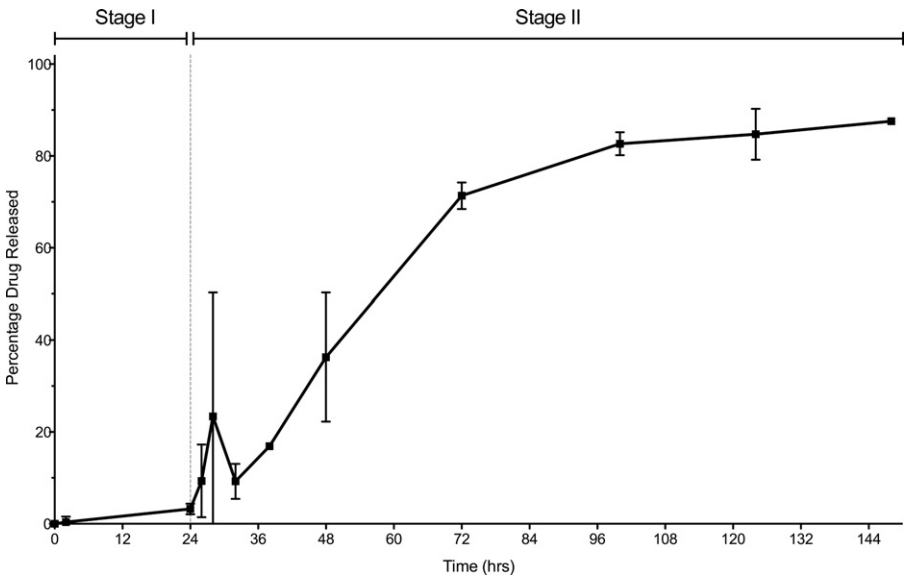

Fig. 4. Release profile of Tenofovir liposomes using a two-stage dialysis method (dialysis membrane MWCO 50 kDa cellulose ester, 10 mM pH 7.4 HEPES buffer, 37 °C, 100 rpm, $n = 3$).

occurred at the initial time points after addition of surfactants. Moreover, it took a very long time (~5 days) for 90% of the drug to release from the liposomes. The high variability and long testing duration makes the dialysis method unsuitable for this type of testing where addition of the surfactant is necessary to trigger drug release.

3.3. Reverse dialysis method

As shown in Fig. 5, during the first stage of the release test, ~4% of the drug was detected after 4 h and remained almost constant until 24 h. This portion of the drug was considered to be free drug. As a general rule, in this study as long as the free drug percentage was below 5% the formulation was considered acceptable (in terms of purity). If the free drug percentage was >5%, as determined during the first stage of the release test, then another purification process was required to remove excess free drug before further release testing.

During the second stage of the release test, immediately after addition of surfactant, the liposomes started to release the drug and this was completed at approximately 48 h later. To be able to compare various formulations with different free drug percentage during the second stage of the release test, the data was normalized to 0% at 24 h (immediately after addition of surfactants) and to 100% after 72 h (or until a plateau was reached). As can be seen from Fig. 5, the variation in the data was very small in both stages. It is worth noting that two identical dialysis tubes were put into each glass tube, and at each time interval samples are taken from alternate dialysis tubes to ensure that there is enough time for drug to reach equilibrium across the dialysis membranes (90% equilibration in 2 h or 99% in 4 h as shown in Table 1).

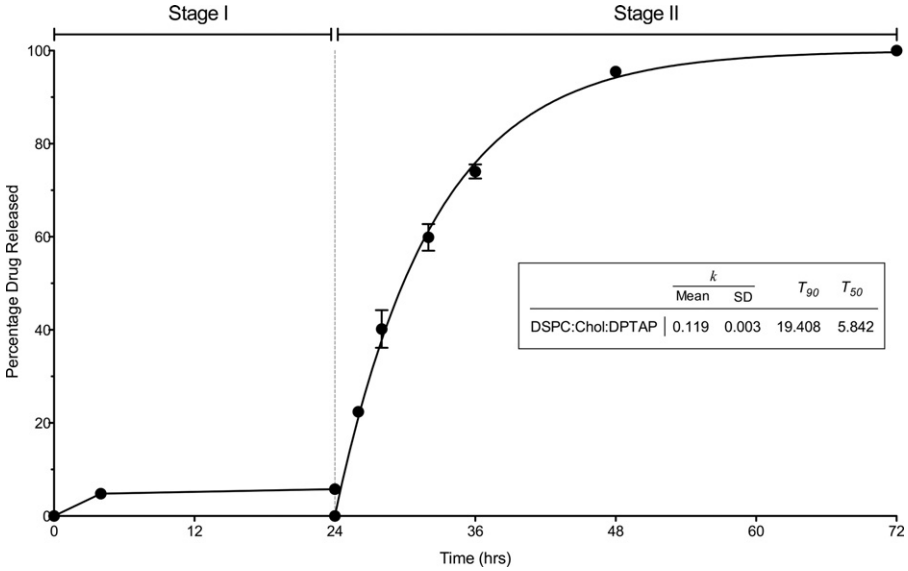

Fig. 5. Release profile of Tenofovir liposomes using reverse dialysis method (dialysis membrane MWCO 50 kDa cellulose ester, 10 mM pH 7.4 HEPES buffer, 37 °C, 100 rpm, $n = 3$).

Table 2
Comparison of the release constants for three liposome formulations ($n = 3$). The particle size data were reported as mean and distribution width.

ID	Formulation composition	Lipid conc. (mg/ml)	Mole fraction (%)	Particle size (nm)	Zeta potential (mV)	k (hr^{-1})	r^2	T_{90} (h)	T_{99} (h)
1	DMPC:cholesterol:DPTAP	50	55:27:18	166.8 (18.1)	71.11 ± 5.72	0.472 ± 0.028	0.994	4.88	9.76
2	DPPC:cholesterol:DPTAP	50	55:27:18	158.1 (32.0)	62.50 ± 2.64	0.395 ± 0.018	0.996	5.82	11.65
3	DSPC:cholesterol:DPTAP	50	55:27:18	159.0 (35.5)	59.76 ± 2.49	0.119 ± 0.033	0.998	19.41	38.82
4	DSPC:cholesterol:SA	50	60:30:10	158.5 (34.7)	31.54 ± 1.90	0.082 ± 0.005	0.989	28.13	56.26

3.4. Comparison of the in vitro release profiles for different formulations

Four liposome formulations, containing DMPC, DPPC, DSPC, and SA, were selected to represent four different types of liposomes with deferent release characteristics. This would not only allow the demonstration of the discriminatory ability of the release method, but also help to understand differences in the release characteristics of the liposomes tested. Detailed formulation conditions are listed in Table 2.

As shown in Fig. 6, all four formulations had less then 5% free drug (data till 4 h). Out of the four formulations, Formulation 1 was least stable and about 4% of the content was released between 4 and 24 h. During the second stage of the release test, Formulations 1 and 2 quickly released the majority of the drug content (>99% within 12 h), while it took nearly 39 h for Formulation 3 to reach 99% release. Formulation 4 (containing a higher percentage of DSPC and SA instead of DPTAP) took even longer (~56 h to reach 99% release). As shown in Table 2, the release constant progressively decreases (p-values for DMPC *vs.* DPPC, and DPPC *vs.* DSPC are both smaller than 0.01) as the phase transition temperature of the main lipid component increases (DMPC: 25 °C, DPPC: 41 °C and DSPC: 55 °C), indicating slower drug diffusion as the liposome membrane became more and more rigid. The developed reverse dialysis method could successfully distinguish formulations containing different main lipids (Formulations 1–3) as well as different percentages of the main lipids (Formulations 3 and 4).

4. Discussion

In the current study, dialysis membranes were used in order to separate the released drug content from liposomes for analysis. The observed overall drug release is therefore a result of two parallel mass transport processes: (1) the "true" drug release from liposomes, and (2) the diffusion of released drug across the dialysis membrane. Correspondingly, it is critical to determine the diffusion kinetics of pure drug through the dialysis membrane in order to understand the "true" drug release characteristics. This will provide a necessary reference when estimating the rate of drug release from various formulations. For example, any formulation presenting a similar "release" profile to the pure drug will be considered to either contain only free drug or that all of the drug is released from the carrier immediately (burst-release). It should be noted that before comparison the "released drug percentage" should be normalized to 0% (at time zero) and to 100% (after reaching plateau) to account for differences in the plateau values due to variation in the free drug percentage.

Ideally, the diffusion of drug through dialysis membrane should be kept at the maximum rate to prevent the dialysis membrane from limiting the drug release. This means the size of the dialysis membrane (in terms of MWCO) should be above a certain cut-off value to allow free diffusion of the drug molecules. Based on our experience, this cut-off value for dialysis membrane is about 100 times the size of the drug, or around 29 kDa MWCO for Tenofovir (M.W. 287.2 Da). As shown in Fig. 3, below the desired MWCO a small increase in the membrane pore size (from 20 kDa to 25 kDa) resulted in significantly faster Tenofovir diffusion. But further increase above the cut-off value (from 25 kDa to 50 kDa) had only a marginal effect on the drug diffusion rate. For this reason, 50 kDa membranes were selected.

In addition to the MWCO, the hydration state of the dialysis membrane can also affect the rate of drug diffusion. To achieve maximum diffusion rate, it is recommended to pretreat the dialysis membrane with surfactant solution and rinse fully prior to the first use. This is because the newly received dialysis membranes normally contain trace amount of glycerin (maybe some other

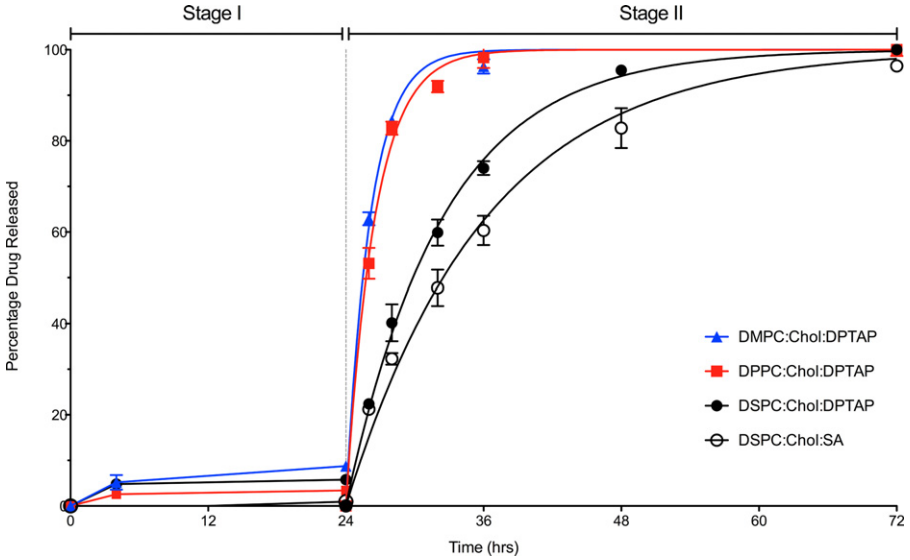

Fig. 6. Comparison of four liposome formulations with different lipid phase transition temperatures (DMPC: 25 °C, DPPC: 41 °C and DSPC: 55 °C) (dialysis membrane MWCO 50 kDa cellulose ester, 10 mM pH 7.4 HEPES buffer, 37 °C, 100 rpm, $n = 3$).

ingredient depending on the vendor) to protect the membranes from cracking. However, this limits drug diffusion (due to a slower membrane hydration) as can be seen in Fig. 2. With just one treatment using surfactant solution, the impurities can be removed and the dialysis membrane can become fully hydrated to allow maximum drug diffusion. As shown in Table 1, pure drug diffusion can reach completion (99%) in about 4 h, and reach 90% in just 2 h. To our knowledge, this is the maximum diffusion rate that can be achieved. For this reason, any formulation that has a drug release duration of less than two hours should not be evaluated using membrane diffusion techniques, as the membrane will become rate-limiting. This may result in incorrect interpretation of the drug release data.

As mentioned earlier, for targeting drug delivery purposes the desired *in vitro* release test should be able to not only demonstrate the release profile of the drug at the targets, but also show the absence of drug release during circulation. For this reason, a two stage *in vitro* release test strategy was developed.

4.1. Drug retention during the first stage

In order to carry the drug directly to the target, the liposomes should have reasonable *in vivo* stability (drug retention) during circulation. To mimic this scenario, in the first stage of the *in vitro* release test, liposome formulations were dialyzed against buffer (10 mM pH 7.4 HEPES buffer) and at body temperature (37 °C). To achieve a high degree drug retention in the liposomes, the following two factors should be considered: (1) the partitioning coefficient of the drug, and (2) the permeability (or rigidity) of the liposome bilayer. Hydrophilic drugs, such as Tenofovir, are encapsulated in the internal aqueous compartment of liposome and are separated from the exterior medium by a hydrophobic bilayer. Due to its high polarity (Log$P$ = −1.7), the partitioning and subsequent diffusion of Tenofovir molecules in the lipid bilayer is prohibited. For this reason, dilution of liposomes in the release medium (100 times as shown in Fig. 1) had no effect on drug retention inside liposomes. This translates to a stable formulation during the first stage of the test. In comparison, hydrophobic drugs have relatively high Log$P$ and hence are embedded inside the lipid bilayer. As a result, the partitioning and subsequent diffusion of the drug can be greatly affected by the dilution conditions (Bhardwaj and Burgess, 2010). In addition to the Log$P$ of drug, the lipid composition also has a great impact on drug retention. For example, in the current study to maximize the stability of the Tenofovir liposomes, relatively high melting point saturated PC lipids, such as DPPC, or DSPC, were used in combination with a high percentage of cholesterol. This resulted in very rigid lipid bilayers, which greatly reduced the diffusion of drug molecules. As shown in Fig. 6, for both DPPC and DSPC liposomes, no drug leakages were observed during the first 24 h.

It should be noted that in this study the targets of the drug are the macrophage and the lymphatic tissue, and hence no modifications were made to the liposome surfaces. However, in cases where targeting to tissues other than reticuloendothelial system (RES) is required, then approximately 5% of PEGylated lipid can be added into the liposome formulation to prevent RES uptake.

4.2. Drug release during the second stage

In the body, after the liposomes enter the target cells *via* endocytosis, it is expected that they will be disrupted in the endosomes due to pH-induced hydrolysis, and the content of the liposomes will be released. *In vitro* to mimic this type of the drug release characteristics, surfactant solutions were added at the end of the first stage test to simulate triggered drug release from the liposomes. Two types of surfactants may be used for this purpose, ionic (such as sodium lauryl sulfate) and nonionic (such as Triton X-100). They trigger drug release by disturbing the stability of the liposome bilayers. Correspondingly, the drug diffusion through the lipid bilayer is enhanced.

4.3. Dialysis vs. reverse dialysis

The dialysis method and the reverse dialysis method setups are shown in Fig. 1. In the case of the dialysis method, liposomes were added to the interior of the dialysis tube and samples were taken from the exterior solution. The issue for this setup is that high variation was observed at the beginning of the second stage and much longer duration was required to finish the test (Fig. 4). Several possible reasons may have caused this: (1) there was insufficient agitation inside the dialysis tubes; (2) high viscosity inside the dialysis tubes formed a barrier slowing down the diffusion; and (3) diffusion of surfactant through the dialysis membrane became a rate-limiting step, causing variability in the rate of disruption of the liposomes and hence subsequent drug release.

On the other hand, for the reverse dialysis method, liposomes were added to the exterior solution and sampling was done from the interior solution. This provided two advantages over the dialysis method: (1) the samples are diluted, making it easier to homogenize the samples (simple water-bath shaking is sufficient); (2) surfactants are added to the exterior solution, where they have direct contact with liposomes thus avoiding the trans-membrane diffusion process prior to liposome disruption, which is believed to be a rate-limiting step for drug release.

4.4. Discriminatory ability of the method

For product development and quality control, an *in vitro* method should be able to discriminate between different formulation variants. As shown in Fig. 6, four formulations had different release profiles. Compared with DPPC and DSPC liposomes, DMPC liposomes were not stable during the first stage of the test and about 4% of the content was released between 4 and 24 h. This could be explained by the phase transition temperature (T_m) differences between DMPC and the other two lipids. DMPC had the lowest T_m (25 °C), and under the testing conditions (37 °C) this lipid was in the liquid crystalline phase. In this state the lipid bilayer is more permeable, and hence it is easier for the drug to diffuse through. The phase transition temperatures of the other two lipids (DPPC and DSPC) were 42 °C and 55 °C, respectively. Therefore at 37 °C, these lipids are in the lamellar gel phase where the lipid bilayer is more rigid and hence much less permeable to the drug. During the second stage, distinctly different release profiles were observed for the four formulations. As shown in Table 2, the release constant progressively decreases as the T_m of the main lipid component increases, indicating slower drug diffusion as the liposome membrane became more and more rigid. Note that even the testing temperature (37 °C) was lower than the DPPC T_m, it was still above its pre-transition temperature (35–37 °C), and hence the DPPC liposomes showed very similar release profile as compared to DMPC. In summary, the developed reverse dialysis method could successfully distinguish formulations containing different main lipids (Formulations 1–3) as well as different percentages of the main lipids (Formulations 3 and 4) and can be used as a quality control testing method.

5. Conclusions

The current study has demonstrated the feasibility of a two-stage release testing method mimicking the two distinct *in vivo* drug disposition processes of targeted delivery parenteral liposomes: no drug release during circulation and triggered release at

the target site. *In vitro*, these two stages were successfully mimicked using a combination of pH 7.4 HEPES buffer (Stage 1) and a 1% TX100 solution in HEPES buffer (Stage 2) both maintained at 37 °C. Most notably, the use of the reverse dialysis membrane resulted in a significant reduction in the variability of the dissolution data compared to the normal dialysis membrane method. The developed method can successfully discriminate formulations with different compositions and can serve as a quality control testing method. Characterization and understanding of the membrane diffusion properties elucidated the relationship between drug release from liposomes and drug diffusion through the dialysis membranes. This will facilitate understanding of the release characteristics of drugs from liposomes in general. The two-stage dissolution methodology developed in this study can be broadly applicable to complex parenteral drug delivery systems.

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