Journal of Controlled Release 315 (2019) 23–30

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Predicting drug release kinetics from nanocarriers inside dialysis bags

Minzhi Yu[a,b,1], Wenmin Yuan[a,b,1], Dan Li[a,b,2], Anna Schwendeman[a,b], Steven P. Schwendeman[a,b,c,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, 428 Church St, Ann Arbor, MI, 48109, United States
[b] Biointerfaces Institute, NCRC, 2800 Plymouth Rd, Ann Arbor, MI, 48109, United States
[c] Department of Biomedical Engineering, 2200 Bonisteel Blvd, Ann Arbor, MI, 48109, United States

ARTICLE INFO

Keywords:
Dialysis
Drug release
Nanoparticles
Mathematical model

ABSTRACT

Dialysis methods are frequently used to determine the *in vitro* drug release kinetics of nanoparticle drug delivery systems. However, the need for the released drug to diffuse through the dialysis membrane delays its appearance in the sampling compartment. Thus, the apparent drug release data outside the dialysis bag typically does not match the desired release kinetics inside the bag adjacent to the nanocarriers. To address this issue, here we describe a simple approach to determine the actual drug release kinetics from nano drug carriers inside the dialysis bag from the experimental data measured from the sampling compartment. First, a calibration experiment is carried out to determine the diffusion barrier properties of the dialysis membranes. The apparent drug release profile of the nanocarrier is then determined using the dialysis method, and a mathematical model is applied to determine the actual drug release kinetics from the experimental data. The model was tested on DOXIL® (doxorubicin liposomes), and an excellent agreement was found between the predicted and measured drug concentration inside the dialysis membranes. By taking the barrier effects of dialysis membranes into consideration, our model independent of drug carrier not only enables the proper interpretation of the data from dialysis studies but also helps to evaluate the dialysis methodology applied to *in vitro* drug release assays.

1. Introduction

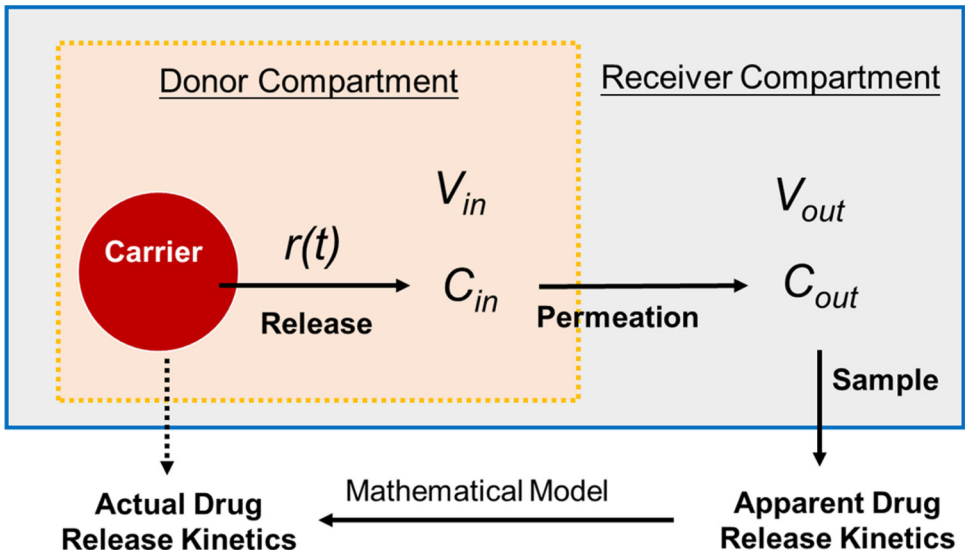
Dialysis methods are frequently used to determine the *in vitro* drug release profiles of nanoparticle drug delivery systems. The drug release profile generated from dialysis-based assays has been widely used to guide formulation development, facilitate quality control and regulatory filing, and, in the best-case scenario, establish the *in vitro-in vivo* correlation (IVIVC) of the nanoparticle formulation [1–3].

However, the validity of the dialysis method as well as the reliability of the release data generated from dialysis assays have long been questioned [4–6]. For conventional dialysis settings (Scheme 1), the drug that is released from the nanocarriers first enters the solution inside the dialysis bag (donor compartment) and then permeates through the dialysis membrane to reach the bulk solution outside the dialysis bag (receiver compartment). Thus, the apparent drug release kinetics, which is measured by sampling the receiver compartment, is determined by both the actual drug release kinetics and drug permeation kinetics. In practice, it is usually assumed that the membrane permeation process is rapid (i.e., not rate limiting) and can be neglected. As a result, the apparent drug release kinetics is often interpreted as the actual drug release kinetics without further processing. However, accumulating evidence suggests that dialysis membranes can significantly delay the translocation of the released drug, in which case the apparent drug release profile will not properly describe the actual drug release kinetics [5,7–10]. For example, Zambito et al. [4] compared the drug release data of diclofenac-loaded nanoparticles using dialysis and an ultracentrifugation method. It was found that in the dialysis method, the membrane permeation process, instead of the actual drug release kinetics, dominated the rate by which the drug appeared in the receiver compartment where samples are taken [4]. Under this circumstance, even though the drug is rapidly released from the nanocarrier, the apparent drug release rate remains low, which may be wrongly attributed to the sustained-release of the carriers if not carefully interpreted.

Some studies that discuss the pitfalls of dialysis methods conducted a parallel release assay using different methods such as

* Corresponding author at: Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, 428 Church St, Ann Arbor, MI, 48109, United States.
E-mail address: schwende@umich.edu (S.P. Schwendeman).
[1] The authors contributed equally to this work.
[2] Current address: Merck & Co Inc, North Wales, Pennsylvania, 19454.

https://doi.org/10.1016/j.jconrel.2019.09.016
Received 19 May 2019; Received in revised form 23 September 2019; Accepted 25 September 2019
Available online 16 October 2019
0168-3659/



Scheme 1. The relationship between apparent and actual release kinetics occurring during release testing by the dialysis method. The drug molecules are released from the carrier with the actual rate of *r(t)* in the solution inside the dialysis membrane (the donor compartment). Then the released drug diffuses through the dialysis membrane to reach the bulk solution outside the dialysis membranes (the receiver compartment). The samples are taken from the receiver compartment to calculate the apparent drug release fraction from the carrier. V_{in} is the volume inside the dialysis donor compartment, C_{in} is the concentration of the released drug in the donor compartment, V_{out} is the volume of the receiver compartment, and C_{out} is the concentration of the released drug in the receiver compartment.

ultracentrifugation, ultrafiltration, and the drug-selective electrode to determine the actual drug release kinetics for comparison [4,11]. However, the external forces used in alternative methods such as centrifugation force may also alter the drug release profile, leading to an improper comparison. Also, such strategies typically only allow for the qualitative, but not quantitative, methodological analysis of dialysis methods. Thus, simple mathematical methods which differentiate the actual drug release kinetics and the effects of dialysis membranes from the apparent drug release data could be very useful for the proper data interpretation and methodology evaluation.

Several mathematical methodologies have been described to determine the exact drug release kinetics by taking the effects of dialysis membranes into consideration [6,11–13]. For example, Anderson et al. developed a series of mechanism-based mathematic models to determine the drug release constant of liposomes using the apparent release profiles derived from the dialysis assays [11,12]. However, most models have focused on certain drug delivery systems and required drug-specific parameters such as solubility, pKa, particle sizes and distribution coefficients, which complicates their general application in determining actual drug release kinetics for drug delivery systems. In the present study, we do not seek to explain the release of a specific nanocarrier nor the effect of the release set-up on drug release kinetics. Instead, we propose a general approach, which determines the actual drug release kinetics inside the dialysis bag and enables quantitative methodological analysis. In the approach, calibration experiments are performed first to determine the barrier property of dialysis membranes. The apparent drug release kinetic data is then collected by the conventional dialysis assay while determining any volume changes in the bag. The apparent release data outside the bag is suitably fit to allow an estimation of its time derivative before a mathematical model is applied to calculate the actual drug release kinetics. The free drug concentration inside the dialysis bag can also be predicted and analyzed for methodology evaluation purposes. The proposed model, which is independent of the drug carrier, not only enables the proper interpretation of the data of dialysis assays, but also could become a valuable tool to evaluate the appropriateness of the dialysis methodology used for *in vitro* drug release testing of nanoparticle drug delivery carriers.

2. Methods

2.1. Materials

DOXIL® (doxorubicin HCl liposome injection) was purchased from the University of Michigan Hospital Pharmacy. The mean particle size of DOXIL® liposomes is reported to be 87 nm by our previous manuscript [14]. Doxorubicin hydrochloride (DOX•HCl) was purchased from SHJNJ Pharmatech (Shanghai, China). 2-Hydroxypropyl-beta-cyclodextrin (HP-CD) was purchased from SHJNJ Pharmatech (Shanghai, China). Sucrose was purchased from Fisher Chemical. Dialysis bags from regenerated cellulose (RC) membranes with molecular weight cut-off (MWCO) of 10 kD and 20 kD and Float-A-lyzer® cellulose ester (CE) dialysis tubes with MWCO of 8–10 kD, 20 kD, 50 kD, 100 kD and 300 kD were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Zeba™ spin desalting columns (0.5 mL, 7 kD MWCO) were purchased from Thermo Fisher Scientific. All other reagents were of analytical grade and purchased from Sigma.

2.2. In vitro drug release kinetic experiments

The USP-4 (flow-through cell apparatus) drug dissolution method, where the nanoparticle formulation is trapped in dialysis bags and dialyzed against the circulating release media flowing through the cells, is selected to represent the typical experiment set-up of dialysis-based nanoparticle drug release assays. A rapid, discriminatory, and rubust USP-4 drug release assay for DOXIL® was developed previously in our lab and used to perform the calibration and drug release kinetics experiments [14]. Briefly, 0.4 mL DOX•HCl stock solution (2 mg/mL, in 10 mM Histidine•HCl, 10% sucrose (w/v), pH 6.5) or 0.4 mL DOXIL® formulation (2 mg/mL in DOX•HCl) was added to the dialysis bags for calibration experiments or drug release experiments respectively, together with 1.2 mL release media composed of 100 mM NH_4HCO_3, 5% sucrose (w/v), 75 mM MES, 5% HP-CD (w/v), and 0.02% NaN_3 (pH 6.0). The dialysis tubes were inserted into the flow-through cells of the USP-4 apparatus. 78.4 mL release media was added into the media reservoir to achieve the total volume of 80 mL. The experiment temperature was set to 45 °C to achieve the accelerated release of DOXIL®. The flow rate was 16 mL/min [15]. The absorbance of the released doxorubicin was detected using Specord 200 plus Spectrophotometer at 480 nm. A linear relationship between free doxorubicin concentration and UV absorbance ($R^2 > 0.999$) was confirmed by the standard curve. To determine the UV absorbance of doxorubicin once completely released from the carriers, a free DOX control was set by directly placing 0.4 mL DOX•HCl stock solution and 79.6 mL release media in the media reservoir. The apparent cumulative drug release fraction (f_{app}) is calculated with the following formula:

$$f_{app} = \frac{A_{(Released\ DOX)} - A_{(Background)}}{A_{(Free\ DOX\ control)} - A_{(Background)}} \bullet 100\%$$

Where $A_{(Released\ DOX)}$ is the UV absorbance of the released DOX in bulk solution outside the dialysis membrane, $A_{(Free\ DOX\ control)}$ is the UV absorbance of the control DOX solution, and $A_{(background)}$ is the UV absorbance of blank release media.

2.3. Quantification of doxorubicin

At 0 h, 1 h, 3 h, 6 h, and 20 h after the start of the release assay, the experiment was stopped, and the dialysis devices (bags or tubes) were removed from the USP-4 apparatus. The solution inside the dialysis bags or tubes was carefully collected after gentle centrifuge (500 *g* ×2 min, room temperature), and the volume of the solution was determined. Zeba™ spin desalting columns (7 kD MWCO) were used to separate liposomal doxorubicin and released free doxorubicin. The validation of the method as well as the determination of the recovery of free DOX and DOX-loaded liposomes are detailed in Supplementary Materials. For sample processing, 100 μL solution was passed through the desalting columns at 1500 *g* for 1 min at the room temperature. The volume of the collected liposome was adjusted to 1 mL using 1% Triton X-100 water solution for liposomal DOX concentration quantification. To determine the total amount of doxorubicin, another 100 μL sample was withdrawn from the dialysis bags and diluted with 1% Triton X-100 to the final volume of 1 mL. The DOX concentrations were quantified at UV 480 nm via microplate-reader. The free DOX concentration in the sample was calculated with the following formula:

$$C_{free} = C_{total} - \frac{C_{liposmal}}{Liposomal\ Recovery\ \%}$$

where C_{free} is the concentration of free DOX in the sample, C_{total} is the concentration of the total DOX in the sample, $C_{liposomal}$ is the concentration of liposomal encapsulated DOX after passing through the desalting columns, and *Liposomal Recovery %* is the recovery of DOX liposomes from the desalting columns.

2.4. Model development

As seen in Scheme 1, we denote the volume of the donor compartment as V_{in}, the volume of the receiver compartment as V_{out}, and the concentration of the released drug in the donor compartment and the receiver compartment as C_{in} and C_{out}, respectively. A is the surface area of the dialysis bags where diffusion occurs, and P is the permeability or mass transfer coefficient of the drug passing through the dialysis membranes. To generate the mathematical model considering mass transport during dialysis, we assume that the drug concentration in each compartment is homogeneous for each of the models below.

2.4.1. Model 1: Dialysis with large receiver reservoir with constant volumes

For simplification, in this model, we assume that V_{in} and V_{out} are maintained constant during both the calibration and drug release experiments. We also assume that the volume of the receiver reservoir is much larger than that inside the bag, i.e., $V_{out} > > V_{in}$, and in most cases $C_{in} > > C_{out}$, holds during the dialysis process. Thus C_{out} is neglected in the driving force for diffusion across the dialysis membrane.

First, we consider the drug transport during the calibration experiment where drug translocates through the dialysis membranes in the absence of any drug carrier. A simple mass balance about the donor compartment accounting for the drug loss due to diffusion may be written as follows:

$$V_{in}\frac{dC_{in}}{dt} = -APC_{in} \quad (1)$$

Integrating (1) between times 0 and t gives:

$$C_{in} = C_0 e^{-\frac{A}{V_{in}}Pt} \quad (2)$$

where C_0 is the initial drug concentration in the dialysis bags. Therefore, there is a simple first-order decay of drug expected as the drug leaves the bag by simple diffusion.

In practice, it is most convenient to sample outside the bag. Therefore, the second mass balance is about drug transport to the receiver compartment, which may be written as follows:

$$V_{out}\frac{dC_{out}}{dt} = APC_{in} \quad (3)$$

Inserting (2) into (3), gives

$$V_{out}\frac{dC_{out}}{dt} = APC_0 e^{-\frac{A}{V_{in}}Pt} \quad (4)$$

Multiplying by dt/V_{out} on both sides, and integrating (4) from $t = 0$ to time t gives

$$C_{out} = \frac{C_0 V_{in}}{V_{out}}(1 - e^{-\frac{A}{V_{in}}Pt}) \quad (5)$$

Since $V_{out} > > V_{in}$ (in our experiment, V_{out} = 49 x V_{in}), the final drug concentration in the receiver compartment (C_f) can be written as:

$$C_f = C_0 V_{in}/V_{out} \quad (6)$$

Inserting (6) into (5) gives the drug fraction released into the receiver compartment (f_{app}) as follows:

$$f_{app} = \frac{C_{out}}{C_f} = 1 - e^{-\frac{A}{V_{in}}Pt} \quad (7)$$

If the calibration and the drug release experiments are performed following the same protocol, which is the case for our study, A and V_{in} are kept the same in the two experiments, making AP/V_{in} as a constant denoted as k_{cal}. Thus,

$$f_{app} = \frac{C_{out}}{C_f} = 1 - e^{-k_{cal}\cdot t} \quad (8)$$

where k_{cal} is a calibration constant for a specific drug/bag combination determined by the slope of the linear decay of $ln(1\text{-}f_{app})$ vs. time.

Then, we consider the scenario where the nanocarriers are placed in the dialysis bag and the dialysis assay is performed under the same conditions as the calibration experiment. A mass balance around the solution in the bag may be written as follows:

$$V_{in}\frac{dC_{in}}{dt} = r(t) - APC_{in} \quad (9)$$

where $r(t)$ is the mass release rate of the drug from the nanoparticle dosage form as a function of time. Rearranging and integrating (9) from $t = 0$ to time t gives:

$$\int_0^t r(t)dt = V_{in}\int_0^{C_{in}} dC_{in} + AP\int_0^t C_{in}dt \quad (10)$$

Normalizing the released mass of drug ($\int_0^t r(t)dt$) by the total drug mass, M_0, gives and initial expression for the actual drug release fraction from the formulation in the bag:

$$f = \frac{\int_0^t r(t)dt}{M_0} = \frac{V_{in}C_{in}}{M_0} + \frac{AP}{M_0}\int_0^t C_{in}dt \quad (11)$$

Next, we consider the mass balance in the receiver compartment. As is the case without the nanocarrier, the mass balance in the receiver compartment is from (3). Rearranging (3) gives C_{in} as a function of the rate of change in concentration with time in the receiver compartment as follows:

$$C_{in} = \frac{V_{out}}{AP}\bullet\frac{dC_{out}}{dt} \quad (12)$$

Integrating (12) from $t = 0$ to time t gives

$$\frac{C_{out}V_{out}}{AP} = \int_0^t C_{in}dt \tag{13}$$

Inserting (12) and (13) into (11):

$$f = \frac{\int_0^t r(t)dt}{M_0} = \frac{V_{in}V_{out}}{APM_0}\frac{dC_{out}}{dt} + \frac{C_{out}V_{out}}{M_0} \tag{14}$$

Noting the apparent fraction drug released (f_{app}) assuming the negligible resistance of the dialysis bag is

$$f_{app} = \frac{C_{out}V_{out}}{M_0} \tag{15}$$

Taking the derivative of both side of Eq. (15) with respect to t gives:

$$\frac{df_{app}}{dt} = \frac{V_{out}}{M_0}\frac{dC_{out}}{dt} \tag{16}$$

Then (14) may be written as a function of f_{app} in a simple form after using (15) and (16)

$$f = \frac{V_{in}}{AP}\frac{df_{app}}{dt} + f_{app} \tag{17}$$

Finally noting the definition for k_{cal} ($\equiv AP/V_{in}$), (17) becomes:

$$f = \frac{1}{k_{cal}}\frac{df_{app}}{dt} + f_{app} \tag{18}$$

and from (12), (16) and the expression for k_{cal}, the free drug concentration in the donor compartment is

$$C_{in} = \frac{M_0}{V_{in}k_{cal}}\bullet\frac{df_{app}}{dt} \tag{19}$$

Hence, from the calibration constant, k_{cal}, and the measured and fitted apparent fraction release kinetics f_{app}, the actual release kinetics of the formulation (i.e., f vs. t) may be readily determined from (18). Moreover, the value of C_{in}, the concentration of drug inside the bag, may be also predicted from (19).

2.4.2. Model 2: Dialysis under conditions with constant volumes and where the concentration of drug in the receiver media cannot be ignored

In the derivation of the Model 1, we assume that the $C_{in} > > C_{out}$ during the entire release experiment. However, depending on the setup of the dialysis assays, this assumption may not always be met. To address this limitation, we revise the Model 1 by considering the presence of C_{out} in the driving force for diffusion. The volume of the donor compartment is still assumed to keep constant during dialysis.

In the calibration experiment, no drug carrier is present, and all drug molecules are dissolved in the media inside the dialysis bag. Since $V_{in} < < V_{out}$, C_{out} may still be negligible in the early phase of the translocation. For example, V_{out} is 49-fold larger than V_{in} in our experimental setup. In this case, C_{in} remains ten times higher than C_{out} before 83% drug is translocated in the receiver compartment. Thus, Eq. (1)–(8) still holds true in the early stages of the calibration experiments.

For drug release assays with a nanocarrier, under the assumptions of Model 2, the mass balance in the donor compartment is revised from Model 1 as follows:

$$V_{in}\frac{dC_{in}}{dt} = r(t) - AP(C_{in} - C_{out}) \tag{20}$$

The new mass balance in the receiver compartment is:

$$V_{out}\frac{dC_{out}}{dt} = AP(C_{in} - C_{out}) \tag{21}$$

Solving (20) and (21) yields the actual drug release fraction as follows (the complete derivation process is provided in the Supplementary Materials):

$$f = \frac{V_{in}C_{out}}{M_0} + \frac{1}{k_{cal}}\bullet\frac{df_{app}}{dt} + f_{app} \tag{22}$$

With the time-dependent C_{in} as

$$C_{in} = C_{out} + \frac{M_0}{V_{in}k_{cal}}\bullet\frac{df_{app}}{dt} \tag{23}$$

2.4.3. Model 3: Dialysis with volume changes and appreciable receiver drug concentration

During the dialysis process, the volume of the donor compartment and the receiver compartment may be subject to change due to osmotic pressure and membrane swelling. To account for the volume change of the donor compartment, the mass balance in the donor and the receiver compartment can be rewritten as:

$$\frac{d(C_{in}V_{in})}{dt} = r(t) - AP(C_{in} - C_{out}) \tag{24}$$

$$\frac{d(C_{out}V_{out})}{dt} = AP(C_{in} - C_{out}) \tag{25}$$

Note that for conventional dialysis bags, the surface area A typically remains the same when V_{in} changes. For dialysis tubes such as the Float-A-Lyzer apparatus, since the bottom area is fixed, A changes with V_{in}, with the fixed ratio of $A/V_{in} = 2/R$, where the R is the radius of dialysis tubes. Thus, the two conditions should be solved respectively (see Supplementary Materials), which summarized below.

For conventional dialysis bags:

$$f = f_{app} + F_V\left(\frac{V_{in(0)}C_{out}}{M_0} + \frac{1}{k_{cal}}\bullet\frac{df_{app}}{dt}\right) \tag{26}$$

$$C_{in} = C_{out} + \frac{M_0}{V_{in(0)}}\bullet\frac{1}{k_{cal}}\bullet\frac{df_{app}}{dt} \tag{27}$$

For dialysis tubes with fixed bottom areas:

$$f = f_{app} + \frac{1}{k_{cal}}\bullet\frac{df_{app}}{dt} + F_V\frac{V_{in(0)}C_{out}}{M_0} \tag{28}$$

$$C_{in} = C_{out} + \frac{1}{F_V}\bullet\frac{M_0}{V_{in(0)}}\bullet\frac{1}{k_{cal}}\bullet\frac{df_{app}}{dt} \tag{29}$$

where the volume correction factor $F_V = \frac{V_{in}}{V_{in(0)}}$, $V_{in(0)}$ is the initial volume of the donor compartment and V_{in} is the time-dependent volume of the donor compartment.

2.5. Data analysis

For the calibration experiment, three parallel runs were made for each dialysis membrane and the apparent drug release fraction (f_{app}) was recorded. For each run, ln(*1-f*$_{app}$) was plotted against time (t) using the data of the first 4 h. Simple linear regression was used to fit each scatter plot. The negative slope of the linear regression was used for k_{cal} of this dialysis membrane according to Eq. (8).

For the drug release experiment, three parallel runs were made for each dialysis membrane. The time-dependent apparent drug release fraction (f_{app}) was recorded as described in Section 2.2 and plotted against time (t). The f_{app} vs t scatter plots were fitted with the Weibull function

$$f_{app} = 1 - e^{-at^b} \tag{30}$$

where a is the scale parameter while b is the shape parameter. Specifically, when Weibull function could not fit the data well, which was the case for 20 kD CE release data (owing to constrained diffusion), the data were fitted by a linear function ($f_{app} = ct + d$). After the parameters were determined using nonlinear fitting in R (version 3.4.3), the time derivative function df_{app}/dt was calculated. Then, the fraction of drug

release from nanocarriers (f) and drug concentration within the bag (C_{in}) were calculated by the equations derived by Model 1, 2 or 3.

For data simulation, based on the assumption that all parameters are normally distributed, at each defined time points from time zero to 20 h, 100 values of parameters including a, b, and k_{cal} were simulated by random sampling from the respective normal distribution with mean and standard error estimated from previous model fitting. Fraction of drug release from nanocarriers (f) and drug concentration within the bag (C_{in}) were calculated together with mean and 95% confidence interval by the equations derived by Model 3. To simplify the simulation process, a fixed value of V_{in}, which was the average value of V_{in} at 24 h time point of all runs, was used when needed.

3. Results and discussion

3.1. Calibration of doxorubicin diffusion through dialysis membranes

In the calibration experiments, doxorubicin solution was placed in different dialysis bags to determine the membrane permeation kinetics. Only data during the first 4 h were fitted, since the assumption of $C_{in} > > C_{out}$ only holds true when less than 85% drug has been translocated to the receiver compartment. As seen in Fig. 1, a linear relationship was found between $ln(1\text{-}f_{app})$ and time for every dialysis membrane, confirming first-order diffusion kinetics of free doxorubicin molecules through dialysis membranes as described by Eq. (8). The calibration constant (k_{cal}), which is the negative of the slope of these kinetic curves, was calculated and listed in Table 1. As expected, for the dialysis membranes of the same type, k_{cal} increased with the increase in MWCO, indicating that the barrier effects of dialysis membranes decreased as MWCO increased. When comparing dialysis membranes of different materials, CE membranes have significantly lower k_{cal} compared to RC membranes with the same MWCO, indicating that CE membranes have a higher resistance to doxorubicin diffusion compared to RC membranes. Such results suggested that both MWCO and the membrane type affect the barrier properties of the dialysis membranes,

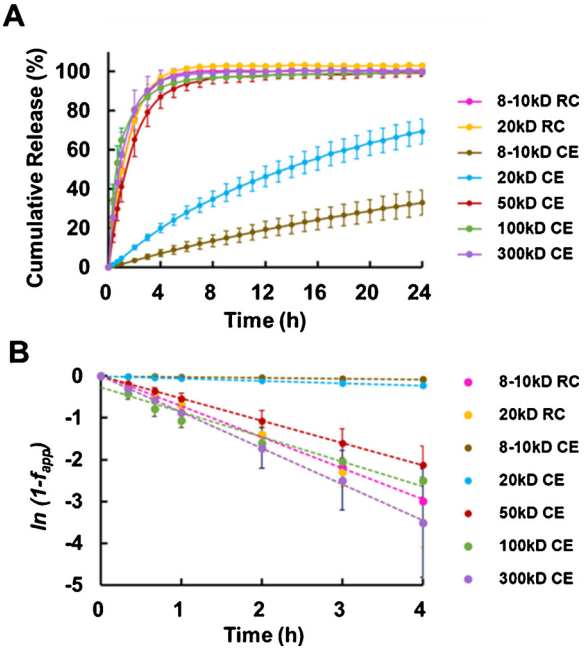


Fig. 1. DOX diffusion kinetics through different dialysis membranes. (A) Cumulative release of DOX from nanocarrier-free drug solution through different dialysis membranes (lines drawn through data). (B) The k_{cal} was calculated from the linear regression of the first 4 h free DOX release of the plot according to Eq. (8). Dashed lines are least squares linear regression lines with k_{cal} listed in Table 1.

Table 1
Calibration constant k_{cal} of different membranes for free DOX from Fig. 1B.

Membrane	k_{cal} (h^{-1})[a]
8-10 kD CE	0.019 ± 0.003
20 kD CE	0.055 ± 0.004
50 kD CE	0.534 ± 0.067
100 kD CE	0.690 ± 0.013
300 kD CE	0.862 ± 0.164
8-10 kD RC	0.733 ± 0.009
20 kD RC	0.812 ± 0.074

[a] The results are presented as mean ± SEM (n = 3). Least squares linear regression resulted in $r^2 > 0.98$.

and choosing dialysis membranes solely based on MWCO may not be reliable. For example, it has been suggested that the dialysis membrane for drug release assays should have an MWCO which is at least 100 times higher than the molecular weight of the drug molecule [15]. The molecular weight of DOX is 543.5 g/mol, requiring dialysis membranes to have an MWCO of > 50 kD to meet the criteria. However, the CE membrane with 50 kD exhibited medium permeation, whereas the RC membrane with MWCO of 20 kD was associated with a faster translocation of DOX. The different permeation kinetics of membranes made by different materials may be related to the porosity of the dialysis membranes or the interactions between drug molecules and membrane materials. However, few research articles have been published so far to address the impact of the dialysis material on translocation rate of drugs.

Based on the k_{cal} determined in this section, two dialysis membranes with rapid and medium permeation rates to DOX, the RC membrane with MWCO 20 kD and the CE membrane with MWCO 50 kD, were selected for evaluation of DOXIL® release and evaluation of the mathematical methods to determine release on the nanoparticle side of the membrane.

3.2. Fitting apparent release data

In order to obtain the time derivative of the apparent drug release fraction curve (df_{app}/dt), the f_{app} vs. time curve were fitted by Weibull function except for 20 kD CE membranes where a linear regression is used. These fits are displyed in Fig S2. It is worth noting that while the Weibull function has been commonly used to fit drug release kinetics data [16], the choice of the function used for model fitting is made on the case-by-case basis. The following criteria are applied to choose the proper function to fit the apparent drug release data. First, there must be a good agreement between the experimental data and the fitted line. When such agreement can be achieved by several functions, the function with fewer parameters and least standard errors for the estimates of the parameters is more favorable for the simulation process. For example, when developing our approach we initially fitted the data with 4^{th} and 5^{th} order polynomial functions. However, use of the polynomial fits resulted in poor predictions of the initial release data, which resulted in inaccurate predictions of C_{in} in early time points. Furthermore, we observed large standard errors of the parameter estimates when fitting data with polynomial functions, which, when introduced to the simulation process, produced a wide confidence interval of the predicted values and weakened the usefulness of the model. On the other hand, the Weibull functions fits did not have these issues and were therefore preferred.

3.3. Applying mathematical models to predict actual DOXIL® release kinetics

In dialysis assays, the actual fraction of drug released (f) occurring inside the dialysis bag can be written as follows:

$$f = f_{app} + \frac{M_{in}}{M_0}$$

As seen above, f_{app} represents the apparent fraction of drug released (i.e., the fraction of free drug that has accumulated in the receiver compartment), M_{in} is the amount of free drug in the donor compartment, and M_0 is the total amount of drug in the formulation. In practice, M_{in} /M_0 is often neglected due to difficulty in monitoring the free drug concentration in the dialysis bags and the assumption that the released drug is rapidly transported to the receiver compartment and/or the amount of drug in the bag is small compared to that outside the bag. However, determining M_{in} is important if it is desired to know accurately the actual drug release fraction f.

By applying the k_{cal} determined in the calibration experiments and fitting the f_{app} release kinetics to the Weibull function, three models developed with different assumptions were used to calculate the time-dependent free drug concentration in the donor compartment. The calculated values are compared with the experimental data. As seen in Fig. 3, Model 1 predicted drug concentration inside the bag with a good agreement to the experimental data in the first few hours, but failed to predict well the data at later stages of the release. This discrepancy can be attributed to the fact that the assumption $C_{in} > > C_{out}$ is compromised at the late stage of the release. When taking the diminishing concentration gradient into consideration, Model 2 presented better C_{in} predictions of the experimental data. The Model 2 was further revised to account for the volume change in dialysis tubes during the experiment. As seen in Fig. 2, there was a small yet significant decrease in the volume inside the dialysis bags during the first 3 h of dialysis, after which the volume kept constant. While it is possible to mathematically describe the volume change and incorporate it into the general model, for simplification purposes, we used the equilibrium volume to calculate the volume correction factor F_v in our calculation. The kinetics of C_{in} predicted by Model 3 demonstrated the closest agreement with the experimental data.

The Model 3 was then applied to calculate the actual drug release kinetics from the apparent drug release data. As seen in Fig. 4, while the apparent drug release data significantly underestimated the actual drug release fraction, a good agreement between the predicted value and the experimental value of actual drug release fraction was found in both assays performed in 20 kD RC membranes and 50 kD CE membranes. Thus, Model 3 served as a reliable tool to determine the actual drug release kinetics from apparent drug release data of dialysis methods, allowing for a more proper data interpretation.

3.4. The impact of dialysis membrane on drug release

The Model 2 was further used to calculate the free drug concentration in the donor compartment (C_{in}) as well as the actual drug release kinetics (f) from the apparent drug release profiles derived from dialysis assays using different membranes. As seen in Fig. 5B, increased C_{in} was observed in the early few hours of dialysis in each dialysis membrane. The accumulation of the free drug in the dialysis bags

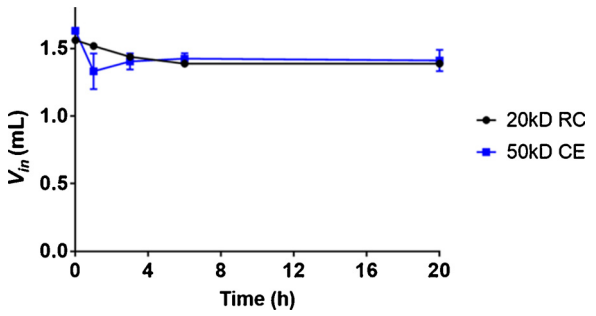


Fig. 2. Volumes of donor compartments (V_{in}) during the release process. Lines drawn through data. The results are presented as mean ± SD (n = 3).

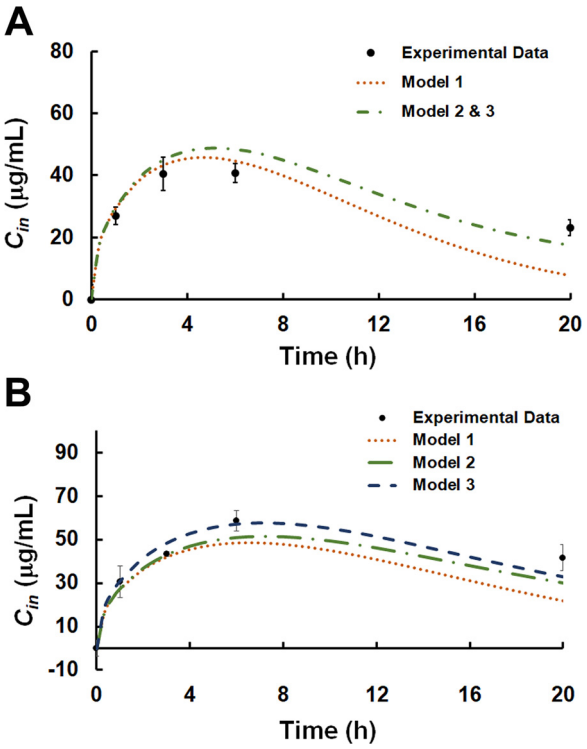


Fig. 3. Predicted and observed free DOX concentrations in the donor compartment (C_{in}) during release from DOXIL® in (A) 20 kD RC membrane dialysis bags and (B) 50 kD CE membrane dialysis tubes. The experimental data are presented as mean ± SD (n = 3). Error bars not shown when smaller than symbols.

indicates that the drug release rate from the liposomes exceeded the drug permeation rate through the dialysis membranes. Over this period, dialysis membrane diffusion kinetics, rather than the actual drug release kinetics from the nanocarrier, was the largest determinant of the drug translocation to the receiver compartment (that is, the apparent drug release kinetics). For dialysis membranes with higher barrier effects like the MWCO 20 kD CE membrane, the membrane diffusion remained as the rate-limiting factor throughout the dialysis process, as evidenced by the high free drug concentration in the donor compartment. In addition, we noticed that there was a large variance in apparent drug release in 3 repeated runs using the 20 kD CE tubes, which led to a more scattered simulation result compared to those of other membranes. Since the apparent drug release profile is predominately controlled by membrane diffusion, such large variance can be attributed to the variability between different 20 kD CE dialysis tubes. For dialysis membranes with faster drug permeation rates such as the MWCO 20 kD RC membrane and the MWCO 300 kD CE membrane, while they have a shorter membrane-dominating phase and a lower drug accumulation inside the dialysis bags, the membrane diffusion was still the largest rate-limiting factor in the first 4 h.

The actual drug release kinetics was also predicted for additional dialysis bags. As shown in Fig. 5A, the actual drug release rate inside the dialysis bags was positively correlated with the k_{cal} of the membrane. This can be attributed to the accumulation of free drug inside the dialysis bags, which violates an effective sink conditions for the drug releasing liposome. In dialysis settings, the sink condition is assumed to be achieved by having a large volume of release media in the receiver compartment which is 10–20 times higher than the volume required for the saturated drug solution [17]. The underlying assumption of this practice is that the free drug can quickly equilibrate across the dialysis membrane, thus maintaining a low free drug concentration inside the dialysis bag. However, this assumption may not always be true due to the barrier effects of dialysis membranes. As seen in Fig. 5B, the free

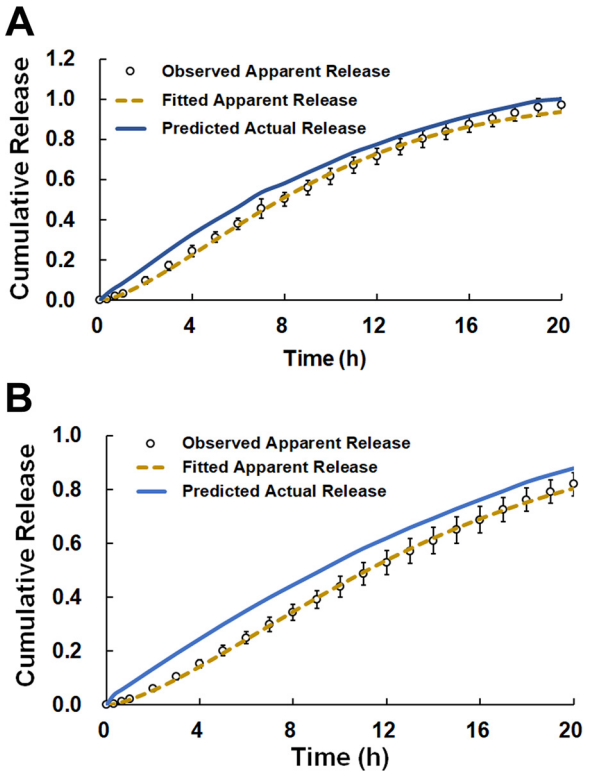


Fig. 4. Predicted actual (f) and apparent (f_{app}) cumulative release kinetics of DOX from DOXIL®. Analysis and experiments were performed using (A) 20 kD RC membrane dialysis bags and (B) 50 kD CE membrane dialysis tubes. The predicted actual release was performed with Model 3 and fitted apparent release curve were performed with Weibull functions. The results are presented as mean ± SD (n = 3).

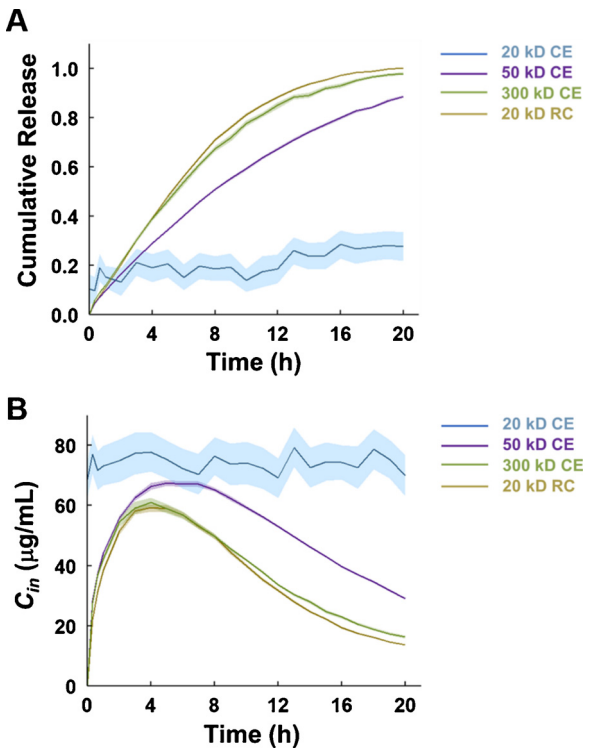


Fig. 5. (A) Predicted liposome cumulative release kinetics of DOX from DOXIL® from different dialysis devices and (B) Predicted free drug concentration in the donor compartment (C_{in}). The shade indicates 95% confidence interval (CI) generated by data simulation.

drug concentration during the dialysis was 2–8 times higher than the final equilibrium concentration of doxorubicin outside the bag, which was 10 μg/ml. This effect is more prominent for dialysis membranes with low permeability coefficients. For example, for the CE membrane with MWCO of 20 kD, the actual drug release was much slower compared to other dialysis membranes, and approximately followed zero-order kinetics. In this case, the barrier effects of the dialysis membrane not only delayed the apparent drug release profile but also hindered the 'actual' drug release, indicating the membrane was unsuitable to evaluate drug release kinetics under these conditions. Analyzing the free drug concentration inside the dialysis bags using the models developed in the present paper may serve as a useful tool for the methodological evaluation of dialysis assays.

4. Conclusion

Despite the common use of dialysis methods for *in vitro* drug release kinetics studies, several common issues associated with their use such as barrier effects of dialysis membranes and violated sink conditions decrease assay accuracy. These limitations often lead to underestimated drug release rate, which may be wrongly attributed to the sustained release of drug from the nanoparticles without proper methodology evaluation and data interpretation. In the present study, a straightforward strategy to manage these effects is proposed independent of the drug carrier in the bag. First, the dialysis membrane is calibrated by a diffusion experiment across the dialysis membrane without the nanocarrier. After fitting a suitable mathematical expression to the apparent drug release data from the nanocarrier obtained from the conventional dialysis assays, a mathematical model can be used to predict that actual drug release kinetics. In the cases where the volume changes in the dialysis bags, a volume correction factor can be added to the mathematical model to enable a better prediction. An excellent agreement is observed between the predicted actual release kinetics and concentration in the bag and the experimentally determined values. Our model not only enables the proper interpretation of the data from dialysis studies but also helps to evaluate the dialysis methodology applied to *in vitro* drug release assays.

Acknowledgment

The research was supported by a grant from the US-Israel Binational Science Foundation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jconrel.2019.09.016.

References

[1] D. Solomon, N. Gupta, N.S. Mulla, S. Shukla, Y.A. Guerrero, V. Gupta, Role of in vitro release methods in liposomal formulation development: challenges and regulatory perspective, AAPS J. 19 (6) (2017) 1669–1681.
[2] M.A. Shetab Boushehri, A. Lamprecht, Nanoparticles as drug carriers: current issues with in vitro testing, Nanomedicine (Lond.) 10 (21) (2015) 3213–3230.
[3] J. Shen, D.J. Burgess, In vitro dissolution testing strategies for nanoparticulate drug delivery systems: recent developments and challenges, Drug Deliv. Transl. Res. 3 (5) (2013) 409–415.
[4] Y. Zambito, E. Pedreschi, G. Di Colo, Is dialysis a reliable method for studying drug release from nanoparticulate systems?-A case study, Int. J. Pharm. 434 (1–2) (2012) 28–34.
[5] G. Moreno-Bautista, K.C. Tam, Evaluation of dialysis membrane process for quantifying the in vitro drug-release from colloidal drug carriers, Colloids Surf. A Physicochem. Eng. Asp. 389 (1–3) (2011) 299–303.
[6] C. Washington, Evaluation of non-sink dialysis methods for the measurement of drug release from colloids - effects of drug partition, Int. J. Pharm. 56 (1) (1989) 71–74.
[7] S.J. Wallace, J. Li, R.L. Nation, B.J. Boyd, Drug release from nanomedicines: selection of appropriate encapsulation and release methodology, Drug Deliv. Transl. Res. 2 (4) (2012) 284–292.

[8] N.S.S. Magalhaes, H. Fessi, F. Puisieux, S. Benita, M. Seiller, An in-vitro release kinetic examination and comparative-evaluation between submicron emulsion and polylactic acid nanocapsules of clofibride, J. Microencapsul. 12 (2) (1995) 195–205.
[9] M.Y. Levy, S. Benita, Drug release from submicronized O/W emulsion - a new in-vitro kinetic evaluation model, Int. J. Pharm. 66 (1–3) (1990) 29–37.
[10] S.A. Abouelmagd, B. Sun, A.C. Chang, Y.J. Ku, Y. Yeo, Release kinetics study of poorly water-soluble drugs from nanoparticles: are we doing it right? Mol. Pharm. 12 (3) (2015) 997–1003.
[11] S. Modi, B.D. Anderson, Determination of drug release kinetics from nanoparticles: overcoming pitfalls of the dynamic Dialysis method, Mol. Pharm. 10 (8) (2013) 3076–3089.
[12] K.D. Fugit, B.D. Anderson, Dynamic, Nonsink method for the simultaneous determination of drug permeability and binding coefficients in liposomes, Mol. Pharm. 11 (4) (2014) 1314–1325.
[13] R. Schwarzl, F. Du, R. Haag, R.R. Netz, General method for the quantification of drug loading and release kinetics of nanocarriers, Eur. J. Pharm. Biopharm. 116 (2017) 131–137.
[14] W. Yuan, R. Kuai, Z. Dai, Y. Yuan, N. Zheng, W. Jiang, C. Noble, M. Hayes, F.C. Szoka, A. Schwendeman, Development of a flow-through USP-4 apparatus drug release assay to evaluate doxorubicin liposomes, AAPS J. 19 (1) (2017) 150–160.
[15] X. Xu, M.A. Khan, D.J. Burgess, A two-stage reverse dialysis in vitro dissolution testing method for passive targeted liposomes, Int. J. Pharm. 426 (1–2) (2012) 211–218.
[16] V. Papadopoulou, K. Kosmidis, M. Vlachou, P. Macheras, On the use of the Weibull function for the discernment of drug release mechanisms, Int. J. Pharm. 309 (1–2) (2006) 44–50.
[17] C. Washington, Drug release from microdisperse systems - a critical-review, Int. J. Pharm. 58 (1) (1990) 1–12.