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Mathematical Modeling and Experimental Validation of Nanoemulsion-Based Drug Transport Across Cellular Barriers

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Abstract

Purpose—Nanoemulsions have shown potential in delivering drug across epithelial and endothelial cell barriers, which express efflux transporters. However, their transport mechanisms are not entirely understood. Our goal was to investigate the cellular permeability of nanoemulsion-encapsulated drugs and apply mathematical modeling to elucidate transport mechanisms and sensitive nanoemulsion attributes.

Methods—Transport studies were performed in Caco-2 cells, using fish oil nanoemulsions and a model substrate, rhodamine-123. Permeability data was modeled using a semi-mechanistic approach, capturing the following cellular processes: endocytotic uptake of the nanoemulsion, release of rhodamine-123 from the nanoemulsion, efflux and passive permeability of rhodamine-123 in aqueous solution.

Results—Nanoemulsions not only improved the permeability of rhodamine-123, but were also less sensitive to efflux transporters. The model captured bidirectional permeability results and identified sensitive processes, such as the release of the nanoemulsion-encapsulated drug and cellular uptake of the nanoemulsion.

Conclusions—Mathematical description of cellular processes, improved our understanding of transport mechanisms, such as nanoemulsions don't inhibit efflux to improve drug permeability. Instead, their endocytotic uptake, results in higher intracellular drug concentrations, thereby increasing the concentration gradient and transcellular permeability across biological barriers. Modeling results indicated optimizing nanoemulsion attributes like the droplet size and intracellular drug release rate, may further improve drug permeability.

Keywords

Oil-in-water nanoemulsion; cellular transport mathematical model; rhodamine-123

1. INTRODUCTION

Vascular endothelial and epithelial cells on mucosal surfaces form selectively permeable biological barriers that, safeguard various tissues of the body; but while these barriers are

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protective, they pose a formidable challenge for drug delivery because they hinder the efficient transport of drug molecules to the target site (1, 2). Biological barriers, such as the blood brain barriers (BBB), intestinal mucosa, nasal and pulmonary epithelium etc. have long remained the focus of drug delivery research (3–6). Nanoparticulate systems have been studied extensively to facilitate drug delivery across tough-to-penetrate biological barriers, most of which express efflux transporters. (4, 7, 8). Depending on their size and composition, nanoparticles interact with cellular membranes to enter the cells via clathrin and caveolin dependent or independent endocytotic and pinocytotic processes (9). As an example of nanoparticulate drug delivery system, nanoemulsions, which are oil-in-water (O/W) or water-in-oil (W/O) formulations consisting of edible oils, surface-active agents, and water, where the diameter of inner phase is reduced to nanometer length scale (10, 11), have shown great potential for increasing drug permeation across various biological barriers. Selected examples establishing the utility of nanoemulsions include: studies performed by Tiwari et al, demonstrating that pine nut oil nanoemulsions improved bioavailability of a hydrophobic drug paclitaxel following oral dosing in mice (12) and results published by Shah et al, showing improved CNS exposures and analgesic effects of a neuro-active peptide, following encapsulation in a fish oil nanoemulsion and systemic dosing in mice (13, 14). Additionally, several reports have been published which specifically investigate the effectiveness of nanoemulsions as delivery vehicles for transporting drugs which are efflux substrates across cellular barriers which express efflux transporters. Such studies have been performed with or without the concomitant administration of an efflux inhibitor. For example, Ganta et al, demonstrated that coadministration of curcumin and paclitaxel in flax-seed nanoemulsions, significantly enhanced cytotoxicity in drug resistant SKOV3TR ovarian adenocarcinoma cells (15). The authors suggested that the improved cytotoxicity resulted from the downregulation of efflux transporters and enhanced intracellular drug delivery following nanoemulsion treatment. Similarly, Prabhakar et al, published that lipid nanoemulsions formulated with Tween 80, provide an effective means to deliver Indinavir, a protease inhibitor indicated for HIV treatment to the CNS (16). The authors attributed it to lipid-mediated endocytosis of the nanoemulsion droplet and efflux inhibition by Tween 80 at the BBB cells.

Despite several proof-of-concept experiments that illustrate the usefulness of nanoemulsions as drug delivery vehicles in an in-vivo setting, the transport mechanisms responsible for nanoemulsion-encapsulated drugs crossing various biological barriers in the presence of efflux transporters are not well explained; and the kinetics of sensitive transport processes, plus the attributes of formulation that result in enhanced drug delivery following in-vivo dosing of such nanoemulsion-based formulations, are unidentified. Overall, in fact, formulation optimization for nanoemulsions remains an empirically driven process. A better understanding of the kinetics and mechanisms of various cellular-level transport processes of nanoemulsion-encapsulated drugs will inform and rationalize formulation optimization efforts in this field. The work described in this paper, focused on understanding the transport mechanism of nanoemulsions at a cellular level. Through carefully designed in-vitro experiments, we investigated, not only the effectiveness of nanoemulsions in improving drug delivery across cellular barriers, but also transport processes responsible for the same.

Generally, in the absence of any mechanistic understanding of sensitive transport processes, formulation optimization is based on a trial and error approach. Mathematical modeling offers a valuable tool for formulation optimization, by facilitating an integrated and quantitative assessment of the outcome of various formulation attributes on the response parameter(s) of interest, without the need to experimentally test each individual scenario. Studies by Zainol et al, illustrate the utility of the Response Surface Methodology in optimizing Levodopa Palm-based nanoemulsions (17): these authors were able to successfully predict the effects of varying the formulation composition and manufacturing process on three nanoemulsion variables: particle size, zeta potential and polydispersity index. Although this process is empirical, it nonetheless highlights the benefits of applying mathematical modeling to formulation optimization efforts. To further explore the rationale around formulation design of nanoemulsions, we built a mathematical model to describe the transport kinetics of the nanoemulsion-encapsulated and free drug across the cellular barrier. Model simulations of drug permeability were performed to identify critical transport mechanisms and key formulation variables which are most likely to affect these transport processes.

The goal of this study was to integrate experimental data from proof-of-concept experiments and perform mathematical modeling to maximize insights obtained about the cellular transport mechanism associated with nanoemulsions, and ultimately performs simulations to identify sensitive kinetic processes associated with nanoemulsion transport.

2. EXPERIMENTAL METHODS

Preparation of dosing formulations

Permeability experiments were performed with two different dosing formulations; rhodamine-123 aqueous solution and rhodamine-123 in oil-in-water nanoemulsion. The oil-in-water nanoemulsion formulation was prepared by sonication method according to a well established protocol (15). The oil phase consisted of fish oil, which has high concentrations of omega-3 rich polyunsaturated fatty acids, and the model drug, rhodamine-123. The aqueous phase was prepared by suspending egg phosphatidylcholine (Lipoid E80®) and polysorbate-80 (1% w/v) in water. Subsequently, both phases were independently heated to 70°C (in a water bath). The oil phase (1 ml) was gradually added to the aqueous phase (4 ml) with constant stirring. The resultant mixture was stirred using a silverson homogenizer (Model: L4RT-A, Silverson Machines, East Longmeadow, MA) at 6,000 rpm for 2 minutes, followed by sonication using the Vibra Cell VC 505 probe sonicator (Sonics and Material Inc., Newtown, CT, USA) for 10 minutes. The probe sonicator was adjusted at 22% amplitude and 50% duty cycle. The resulting stable dispersion was a uniform, milky-white formulation and was stored at 4°C until use. Extra pure grade fish oil was provided as a gift by Jedwards International (Quincy, MA, US). Lipoid E80® was purchased from Lipoid GMBH, (Ludwigshafen, Germany). Tween 80® was purchased from Sigma Chemicals, Inc. (St. Louis, MO). Rhodamine-123 was purchased from Life Technologies, Invitrogen, (Grand Island, NY). All other chemicals were procured from Fisher Scientific, (Fair Lawn, NJ) and were used as received.

Characterization of rhodamine-123 oil-in-water nanoemulsion

Nanoemulsion prepared by sonication was characterized for particle size, morphology, surface charge, and stability. Particle size measurements were performed using dynamic light scattering, on Brookhaven Instrument's 90Plus ZetaPALS particle size analyzer, (Holtsville, NY), at a 90° fixed angle, and at 25°C. The average oil droplet hydrodynamic diameter and the polydispersity index (PDI) were determined. ZetaPALS instrument was used to measure the surface charge (zeta potential) of nanoemulsion. The refractive index of nanoemulsion was set at 1.33, and the viscosity at 1.0 cps, to mimic the values for pure water. Zeta potential values were determined from the electrophoretic mobility of the oil droplets, utilizing a built-in software that uses Helmholtz-Smoluchowski equation. The morphology of oil droplets in the nanoemulsion formulation was visualized with TEM analysis. Nanoemulsion was placed on formvar-coated copper grids, EM Sciences, (Hatfield, PA, USA), and negatively-stained for 10 minutes with 50 µL of 1.5% phosphotungstic dye at room temperature. Excess liquid was drained off with filter paper, and the grid containing dry film of nanoemulsion sample was observed with a JEOL 100-X transmission electron microscope (Peabody, MA). Nanoemulsion loading and encapsulation efficiency were measured as previously described (13).

Transporter profiling and efflux modulation in Caco-2 cells

Caco-2 cells, a gift from X. Lin (Novartis Institutes for Biomedical Research, Inc. Cambridge, MA), were cultured in DMEM-Glutamax® medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1% Pen-Strep, 1% non-essential amino acids, and 1% sodium pyruvate. RT-PCR was used to confirm the presence of efflux transporters (MDR1, MRP1, BCRP, and MRP2) in Caco-2 cells cultured in our laboratory. Briefly, RNA was extracted from the Caco-2 cells using an RNA isolation kit, Roche Applied Sc, (Indianapolis, IN). cDNA was synthesized using the Superscript III® kit from Invitrogen. cDNA was amplified by RT-PCR with primers from the Eurofin library, Eurofins MWG Operon, (Huntsville, AL). The PCR product was loaded on precast E-gels®, Invitrogen, (Grand Island, NY) and bands were separated by gel electrophoresis. ImageS software was used to quantify the band intensity.

Efflux modulation in Caco-2 cells was performed using curcumin, which is known to have inhibitory effects on efflux transporters (18). Briefly, cells were treated with curcumin nanoemulsion or solution (concentration of 20 µM) for 1 or 4 hours, following which, the cells were washed and lysed. RT-PCR as described above was performed to measure transporter expression following curcumin pre-treatment.

Measurement of rhodamine-123 release from nanoemulsion

The rate of rhodamine-123 release from nanoemulsion was estimated using a modified dialysis method (19). The rhodamine-123 nanoemulsion was filled in a dialysis cassette (Spectrum Float-a-Lyser®, MWCO 3500-5000) of 1 ml capacity. The dialysis cassette was then immersed in 150 ml HBSS media containing 1% polysorbate 80 in a beaker that served as the receiver fluid maintaining sink conditions. A stir bar was used at a mixing speed of 100 rpm to enable mixing inside the beaker, and a hot plate was used to maintain the temperature of the media at 37 °C. Aliquots (1 ml) were taken at various time intervals for

20 hours from the receiver compartment. Samples were analyzed using fluorescent plate reader at excitation of 485 nm and emission 528 nm. A similar process was also repeated for the rhodamine-123 aqueous solution.

Measurement of uptake of rhodamine-123 dosed in solution versus a nanoemulsion based formulation

Before performing bidirectional permeability experiments, the intracellular uptake of rhodamine-123 in Caco-2 cells was experimentally investigated. Caco-2 cells were treated with rhodamine-123 (5 μ M), dosed either in an aqueous solution or fish oil nanoemulsion. The difference in the intracellular concentrations of rhodamine-123, resulting following dosing in an aqueous solution versus a fish oil nanoemulsion, would be indicative of differences in uptake of rhodamine-123, resulting from passive permeability of the free drug versus endocytosis-mediated uptake of the fish oil encapsulated rhodamine-123, respectively. Briefly, Caco-2 cells were cultured on flat-bottomed 12-well plates for 7 days. After removal of media and washing with buffer, cells were incubated with 5 μ M rhodamine-123 aqueous solution or fish oil nanoemulsion at 37°C. At pre-determined time points, the cells were washed and lysed to measure intracellular rhodamine-123 concentration. The intracellular concentration of rhodamine-123 was measured using fluorescence at a wavelength of 485 nm (excitation)/528 nm (emission) and was normalized to the protein concentration of the cells, determined using a BCA Protein Assay Kit (Thermo Scientific™).

Permeability of rhodamine-123 across the Caco-2 monolayers

In vitro drug permeability experiments were performed using Caco-2 cells, grown on 1.0 μ m pore size 12-well Transwell® insert plates for 21-days (TEER: 1000 Ω /cm²). Cells were washed and maintained in Hank's buffered salt solution (HBSS) supplemented with 10 mM of buffering agent HEPES prior to permeation measurement. Rhodamine-123 is a well-known substrate for different efflux transporters (20, 21). Therefore it was used as test drug, to investigate the permeability differences between a solution and nanoemulsion-based formulation, in the presence and downregulation of efflux transporters. Efflux downregulation was achieved by treating the cells with curcumin as described in the transporter profiling and efflux modulation section. Rhodamine-123 (5 μ M) solution or nanoemulsion was used to assess apical-to-basolateral permeation (A:B) and basal-to-apical permeation (B:A) across Caco-2 monolayers in separate experiments. Briefly, apical-to-basal permeability (A:B) permeability experiment was performed by dosing rhodamine-123 (5 μ M) solution or nanoemulsion in the apical compartment and measuring the concentration of rhodamine-123 in the basal compartment. For basal-to-apical (B:A) permeability experiment, rhodamine-123 (5 μ M) solution or nanoemulsion was dosed in the basal compartment and the concentration of rhodamine-123 was measured in the apical compartment. The extent of efflux was indicated by the ratio of B:A to A:B permeability. Permeability experiments were conducted over a 4-hour interval and rhodamine-123 concentration in the apical or basal compartment was measured using fluorescence at a wavelength of 485 nm (excitation)/528 nm (emission).

Mathematical modeling of release and permeability data

Release of rhodamine-123 from the nanoemulsion, was described by a first order rate constant Krel. Kinetics of rhodamine-123 release and transport in the dialysis cassette were described using equations 1 and 2.

$$\frac{d(\text{Rh123}_{\text{donor amount}})}{dt} = \text{Krel} * (\text{NE Rh123}_{\text{donor amount}}) - Q * (\text{Rh123}_{\text{donor conc}} - \text{Rh123}_{\text{receiver conc}}) \quad 1$$

$$\frac{d(\text{Rh123}_{\text{receiver amount}})}{dt} = Q * (\text{Rh123}_{\text{donor conc}} - \text{Rh123}_{\text{receiver conc}}) \quad 2$$

Krel and Q were estimated by fitting release data from rhodamine-123 nanoemulsion and aqueous solution simultaneously.

Transport of rhodamine-123 solution and nanoemulsion across the Caco-2 monolayer, was modeled using a semi-mechanistic/compartamental approach (Figure 1). The experimental system was represented using three compartments: apical, cellular and basal. The kinetic processes considered in the model were: 1) Endocytosis-mediated uptake of the nanoemulsion droplet from the apical to cellular layer, described by a first order rate constant Kendo 2) rhodamine-123 release from the nanoemulsion (either in the apical or cellular layer), described by Krel, as estimated from the release experiments 3) Saturable efflux of rhodamine-123 from the cellular to apical compartment, described by two Michaelis–Menten parameters Vmax and Km 4) Passive permeability of rhodamine-123 across the apical/cellular or cellular/apical compartment described by a linear parameter P. In addition to the above listed parameters, the apical, basal and cellular compartmental volumes were fixed to values relevant to the dimensions of 1.0 µm pore size 12-well Transwell® insert plates. Variables used to describe rhodamine-123 transport in the semi-mechanistic model are listed in Table I. Kinetic expressions used for modeling the transport of rhodamine-123 solution or nanoemulsion across the Caco-2 monolayer are outlined below.

Transport mechanisms for rhodamine-123 solution:

$$\frac{d(\text{Rh123}_{\text{apical amount}})}{dt} = P * (-\text{Rh123}_{\text{apical conc}} + \text{Rh123}_{\text{cellular conc}}) + \frac{V_{\text{max}} * (\text{Rh123}_{\text{cellular conc}})}{\text{Rh123}_{\text{cellular conc}} + K_m} \quad 3$$

$$\frac{d(\text{Rh123}_{\text{cellular amount}})}{dt} = P * (\text{Rh123}_{\text{apical conc}} - \text{Rh123}_{\text{cellular conc}}) - \frac{V_{\text{max}} * (\text{Rh123}_{\text{cellular conc}})}{\text{Rh123}_{\text{cellular conc}} + K_m} + P * (\text{Rh123}_{\text{basal conc}} - \text{Rh123}_{\text{cellular conc}}) \quad 4$$

$$\frac{d(\text{Rh123}_{\text{basal amount}})}{dt} = P * (-\text{Rh123}_{\text{basal conc}} + \text{Rh123}_{\text{cellular conc}}) \quad 5$$

Transport mechanisms for rhodamine-123 nanoemulsion:

$$\frac{d(\text{NE Rh123}_{\text{apical amount}})}{dt} = -K_{\text{endo}} * (\text{NE Rh123}_{\text{apical amount}}) - K_{\text{rel}} * (\text{NE Rh123}_{\text{apical amount}}) \quad 6$$

$$\frac{d(\text{Rh123}_{\text{apical amount}})}{dt} = K_{\text{rel}} * (\text{NE Rh123}_{\text{apical amount}}) + P * (-\text{Rh123}_{\text{apical conc}} + \text{Rh123}_{\text{cellular conc}}) + \frac{V_{\text{max}} * (\text{Rh123}_{\text{cellular conc}})}{\text{Rh123}_{\text{cellular conc}} + K_m} \quad 7$$

$$\frac{d(\text{NE Rh123}_{\text{cellular amount}})}{dt} = -K_{\text{endo}} * (\text{NE Rh123}_{\text{apical amount}}) - K_{\text{rel}} * (\text{NE Rh123}_{\text{cellular amount}}) \quad 8$$

$$\frac{d(\text{Rh123}_{\text{cellular amount}})}{dt} = K_{\text{rel}} * (\text{NE Rh123}_{\text{cellular amount}}) + P * (\text{Rh123}_{\text{apical conc}} - \text{Rh123}_{\text{cellular conc}}) + \frac{V_{\text{max}} * (\text{Rh123}_{\text{cellular conc}})}{\text{Rh123}_{\text{cellular conc}} + K_m} + P * (\text{Rh123}_{\text{basal conc}} - \text{Rh123}_{\text{cellular conc}}) \quad 9$$

$$\frac{d(\text{Rh123}_{\text{basal amount}})}{dt} = P * (-\text{Rh123}_{\text{basal conc}} + \text{Rh123}_{\text{cellular conc}}) \quad 10$$

The SimBiology® application in MATLAB® was used for model fitting and parameter estimation. Simultaneous estimation of all the model parameters was performed by fitting apical-to-basal (A:B) and basal-to-apical (B:A) permeability data, following dosing with the rhodamine-123 solution and nanoemulsion, in the presence and absence of curcumin, in a single step.

Parameter scans to identify sensitive formulation parameters

Parameter scans were performed to identify sensitive drug (passive permeability of rhodamine-123 in solution) or formulation related variables (drug release rate from the nanoemulsion and endocytosis-mediated uptake rate for the nanoemulsion). The effect of varying each parameter on the apical-to-basal permeability (A:B) of rhodamine-123, following the dosing of the nanoemulsion, was evaluated individually. Simulations were performed by varying K_{rel} , K_{endo} and P over a four-fold range.

3. RESULTS

Figure 2 describes the experimental workflow and modeling and simulation approach used for this work. Briefly, Caco-2 cells were used as the *in vitro* cellular model for mimicking a biological barrier to drug permeation. Though primarily used to represent the intestinal barrier (22), Caco-2 cells have also been used as surrogate BBB penetration models in the past (23). With their efflux transporters expressing properties and high TEER values and utility in predicting oral absorption, Caco-2 cells provided a universal framework to study drug permeation across biological barriers. As the test drug we used rhodamine-123 in our

experiments. As the test drug we used rhodamine-123 in our experiments. Rhodamine-123 is a well-known efflux substrate and has been used extensively as a tracer dye to study transport mechanism across cellular barriers (20, 21). In addition, rhodamine-123 is sparingly soluble in water, making it a suitable candidate for encapsulation in the oil core of the nanoemulsion. Therefore, we used rhodamine-123 as a tool drug to study the application of nanoemulsions in enhancing drug delivery in the presence of efflux transporters. Though the experiments described in this paper, are limited to rhodamine-123, this approach can be strengthened in the future by using drugs other than rhodamine-123. Further we used curcumin to downregulate the expression of efflux transporters in Caco-2 cells (18) and generate permeability data representing both the passive permeability and efflux mediated reduction in permeability of the free and nanoemulsion-encapsulated rhodamine-123.

Rhodamine-123 nanoemulsion characterization

The average oil droplet hydrodynamic diameter of the drug free nanoemulsion and rhodamine-123-loaded nanoemulsions was 180 ± 9 nm and 220 ± 9 nm respectively, and the polydispersity index (PDI) was less than 0.2 for both the formulations. The placebo nanoemulsion formulations had a zeta potential of -50 ± 6 mV. Rhodamine-123 loaded nanoemulsion had zeta potential of -18.2 ± 0.8 mV, suggesting a change in surface charge after rhodamine-123 loading. The morphology and size of the nanoemulsion were confirmed using TEM. The particles were spherical and the size ranged from 180–220 nm. We also confirmed by size and surface charge measurements that the nanoemulsion formulation was stable, with no change in appearance, size and surface charge for up to at least 6 months on refrigeration storage. Both the loading and encapsulation efficiency were estimated as $> 98\%$, suggesting that the administered dose of rhodamine-123 following nanoemulsion treatment, was mostly present in the oil droplet.

Transporter profiling and efflux modulation in Caco-2 cells

The results of RT-PCR on untreated and curcumin treated Caco-2 cells are summarized in Figure 3. The raw band intensity following gel electrophoresis was quantified by Image S. RT-PCR on untreated cells, confirmed the expression of efflux transporters: MRP1, MRP2, BCRP and MDR1 by Caco-2 cells. On treatment with the curcumin solution and nanoemulsion, MRP2 expression remained unaltered. However, curcumin treatment ($20 \mu\text{M}$) significantly reduced the expression of MRP1, BCRP and MDR1 by Caco-2 cells. Based on these results, $20 \mu\text{M}$ concentration of curcumin, administered either in a solution or nanoemulsion-based formulation, was used to downregulate efflux in Caco-2 cells during permeability experiments.

Measurement of uptake of rhodamine-123 dosed in solution versus a nanoemulsion based formulation

Table II summarizes the intracellular concentrations of rhodamine-123, resulting from the $5 \mu\text{M}$ rhodamine-123 aqueous solution and fish oil nanoemulsion. Considering the high encapsulation efficiency of the rhodamine-123 nanoemulsions and the short time-course of this experiment, the kinetics of endocytosis-mediated uptake of the nanoemulsion droplet by Caco-2 cells are indirectly reflected in the intracellular concentration-time course of rhodamine-123, following dosing in the nanoemulsion-based formulation. At all the time

points up to 1.5 hours, except at the 0.25 hour time point, the intracellular concentration of rhodamine-123 in Caco-2 cells was significantly greater when rhodamine-123 was administered in a nanoemulsion-based formulation as compared to the aqueous solution (Table II). The results of this experiment confirmed that endocytosis mediated uptake of rhodamine-123 encapsulated in the nanoemulsion oil droplet results in more efficient uptake of rhodamine-123 as compared to that resulting from passive diffusion of the free drug alone.

Mathematical modeling results for rhodamine-123 release kinetics from the nanoemulsion

The open circles and triangles in Figure 4 represent the concentrations of rhodamine-123 in the receiver compartment of the dialysis cassette at various time-points, following treatment with the nanoemulsion and solution-based formulation; respectively. Because the solution-based formulation does not involve a release step, as expected, rhodamine-123 appeared at a faster rate in the receiver compartment from the solution-based formulation as compared to the nanoemulsion-based formulation. The above described data was modeled using equations 1 and 2. The modeling results are also depicted in Figure 4. The solid-line represent the model fits for the nanoemulsion-based formulation, whereas the dash-line represents the model fits for the solution-based rhodamine-123 formulation. The parameter estimate for the release rate constant (K_{rel}) of rhodamine-123 from the nanoemulsion, is listed in Table III.

Mathematical modeling results for rhodamine-123 permeability in Caco-2 cells following treatment with the solution or nanoemulsion-based formulation

The apical-to-basal permeability (A:B) and basal-to-apical (B:A) permeability of rhodamine-123 was measured in four separate assays: 1) rhodamine-123 nanoemulsion in curcumin treated Caco-2 cells 2) rhodamine-123 nanoemulsion in untreated Caco-2 cells 3) rhodamine-123 solution in curcumin treated Caco-2 cells 4) rhodamine-123 solution in untreated Caco-2 cells. For the same formulation, permeability data from treated vs. untreated cells captured the effect of efflux transporters, whereas for a given condition (curcumin treated or untreated) of Caco-2 cells, permeability data from the nanoemulsion vs. solution, captured the permeability difference associated with free rhodamine-123 and nanoemulsion encapsulated rhodamine-123 droplet. Figure 5a and 5b represent permeability results following dosing with Rh123 solution in curcumin untreated and treated cells, respectively and Figure 5c and 5d represent permeability results following dosing with Rh123 nanoemulsion in curcumin untreated and treated cells, respectively. Two key trends were observed in the permeability experiments: 1) the reduction in efflux ratio following curcumin treatment was more pronounced for rhodamine-123 solution as compared to rhodamine-123 nanoemulsion, and 2) regardless of the presence or absence of curcumin, dosing rhodamine-123 in a nanoemulsion-based formulation always resulted in increased basal concentrations i.e. apical-to-basal permeability (A:B) of rhodamine-123 nanoemulsion was always higher than that of rhodamine-123 solution. These results suggested that uptake of rhodamine-123 following dosing with a nanoemulsion-based formulation, was less sensitive to the presence of efflux transporters and the permeability of a nanoemulsion droplet in Caco-2 cells may be superior than that of free rhodamine-123.

A semi-mechanistic model schematically represented in Figure 1 and mathematically described by equations 2–10, was fitted to the above described permeability data. The modeling fits are depicted in Figure 6; the open circle represent observed rhodamine-123 concentrations in the apical or basal compartment and the solid line represent the model fits. Overall, the model performed reasonably well in capturing the permeability of rhodamine-123 under different assay conditions. Table III summarizes the model-estimates for the different kinetic parameters.

Parameter-scan results to identify sensitive formulation parameters

The developed model enabled prediction of the effect of changes in kinetic parameters on drug permeation across the cellular monolayer. In addition, by varying each parameter individually, it is possible to observe which of the transport processes (i.e. drug release vs drug permeability vs nanoemulsion uptake) has the biggest influence on the permeability of free drug across the cells. *K_{rel}*, *K_{endo}* and *P* were varied over a fourfold range and the apical-to-basal permeability (A:B) of rhodamine-123 nanoemulsion was simulated. Simulation results in Figure 7 suggest that, *K_{rel}* and *K_{endo}* are more sensitive than *P*, with respect to their effect on rhodamine-123 permeability across Caco-2 cells. Following the same fold variation in parameter values, the extent in change in permeability associated with *P* was less than that associated with *K_{rel}* and *K_{endo}*.

4. DISCUSSION

In this work, we have used an integrated approach combining experimental data and mathematical modeling to describe the permeability of rhodamine-123, which is an efflux substrate, across Caco-2 cells, either from a solution-based or a nanoemulsion-based formulation, both in the absence and presence of efflux transporters. There were two key objectives associated with this work; first, to illustrate the utility of nanoemulsions as drug delivery system, to enhance cellular permeability of drug molecules in the presence of efflux transporters and second, using mathematical modeling we wanted to identify sensitive kinetic processes, which may be optimized in the future to further increase drug permeability from a nanoemulsion formulation.

During permeability studies of rhodamine-123, curcumin was used to downregulate the expression of efflux transporters in Caco-2 cells. Both in the absence and presence of curcumin, i.e. with and without efflux activity in Caco-2 cells, the nanoemulsion-based formulation resulted in increased apical-to-basal permeability (A:B) of rhodamine-123, as compared to the solution-based formulation. Also following curcumin treatment, the reduction in efflux for rhodamine-123 was less noticeable from the nanoemulsion based formulation as compared to the solution-based formulation. The results of permeability experiments in Caco-2 cells suggested that, though dosing a drug in nanoemulsion may not prevent efflux of the free drug from the cellular to the apical compartment, nanoemulsions still may result in increased apical-to-basal permeability (A:B) permeability of the free drug. A possible explanation for this observation may be that the free drug from a solution-based formulation enters the cells via bidirectional passive diffusion, while the nanoemulsion encapsulated drug can enter the cells by unidirectional endocytosis-mediated cellular uptake,

therefore the nanoemulsion-based formulation may result in increased intracellular concentrations of rhodamine-123 as compared to the solution-based formulation. Higher intracellular concentrations of rhodamine-123, may result in an increased concentration gradient across the cellular and basal compartment, thereby resulting in higher apical-to-basal permeability (A:B) of rhodamine-123 in Caco-2 cells.

Following the generation of experimental results, we developed a semi-mechanistic compartmental model to describe the kinetics of passive and active transport of rhodamine-123 in Caco-2 cells. The model parameters were estimated by fitting the apical-to-basal permeability (A:B) and basal-to-apical permeability (B:A) data for the nanoemulsion-based and solution-based formulation of rhodamine-123 in Caco-2 cells, simultaneously. Our model structure provided a holistic framework to capture the transport of rhodamine-123 in Caco-2 cell, as a free drug or as a nanoemulsion encapsulated drug both under conditions of passive or active transport. Though the model was developed using rhodamine-123 and Caco-2 cells, a similar approach may be used for any efflux substrate and cells expressing efflux transporters.

An important assumption of the mathematical model described above was that following endocytosis of the nanoemulsion droplet into the cells, only the free drug can diffuse out of the cell into the apical or basal compartment. This assumption is supported by publications which propose that, most nanoparticles enter the cells through pinocytosis, following which the endocytosed cargo is enclosed within early endosomes or caveosomes and then directed to lysosomes via the late endosomes (24, 25). The assumption is supported by an understanding that nanoemulsions are not rigid colloidal structures and are less likely to remain intact in the acidic environment of the lysosomes in the presence of proteases and other degradative enzymes. To the best of our knowledge, there are no reports where the intracellular integrity of nanoemulsions has been demonstrated. Further, due to bioanalytical limitations, we cannot distinguish between the free and nanoemulsion-encapsulated rhodamine 123. Therefore, for the purpose of modeling this data, we assumed that the transcytosis or exocytosis of intact nanoemulsion droplets was insignificant and the entire payload content must be released intracellularly before the payload can diffuse out of the cell. In Figure 6f and 6h, a slight discrepancy was noted between the observed data and model predictions. The model over predicted the apical concentrations resulting following B:A dosing in untreated Caco-2 cells, suggesting that the model structure overestimates the efficiency of efflux, when rhodamine-123 diffuses out of the cellular compartment into the apical compartment. This may be explained by differences in accessibility to efflux transporters of a drug substrate present on the luminal side of the cellular membrane as compared to a drug substrate present intracellularly. The model described in this paper assumes that efflux transporters are equally accessible from the apical and cellular compartment as there was limited data to differentiate and estimate these efflux processes separately.

Any mathematical model has two important applications in an early discovery; firstly to test various mechanistic hypotheses about the underlying system and secondly to identify sensitive parameters or processes of the system. We used our model for the second application. Using the model structure described in this paper and parameter estimates listed

in Table III, we simulated the effect of varying the passive permeability of the free drug, the release rate of the free drug from the nanoemulsion and the cellular uptake rate of the nanoemulsion droplet, on the apical-to-basal permeability (A:B) of rhodamine-123 in Caco-2 cells, following dosing with the rhodamine-123 nanoemulsion. The simulation results following the parameter scans identified the release of rhodamine-123 from the nanoemulsion droplet and uptake of the nanoemulsion droplet in the cells as the most sensitive kinetic processes. On the other hand, increasing the passive permeability of the free drug had a lesser influence of the apical-to-basal permeability (A:B) of rhodamine-123 following dosing with the nanoemulsion-based formulation. The results of the parameter scans enhanced our understanding of key transport mechanism. When a drug is encapsulated in a nanoemulsion droplet, cellular concentrations of the free drug mainly result from the uptake of the nanoemulsion droplet rather than passive permeability of the free drug. Further increasing the passive permeability of the free drug will increase drug transport, not only across the interface of the cellular and basal compartments but also the interface of the apical and cellular compartments. Therefore, modifying passive permeability has a smaller effect in increasing on the apical-to-basal permeability (A:B) of nanoemulsion encapsulated rhodamine-123 in Caco-2 cells.

Parameter scans also identified the release of free drug from the nanoemulsion droplet as a dominant kinetic process; in order to increase the permeability of nanoemulsion encapsulated rhodamine-123 across Caco-2 cells. However, these results must be interpreted with caution. One limitation of our model is that, it does not differentiate the rate of rhodamine-123 release from the nanoemulsion, in the apical compartment from that in the cellular compartment. The release rate constant in both the apical and cellular compartments is fixed to the parameter estimate value, obtained from the release experiment in the dialysis cassette. Based on the differences in the content and pH of the cellular environment vs. the extracellular environment, one would expect the release rate constant to vary in the apical and cellular compartments. Due to lack of measurements in the cellular compartment and the inability to distinguish fluorescence from the nanoemulsion encapsulated and free rhodamine-123, our modeling approach was unable to resolve the differences in release rate in the cellular and apical compartment. Ideally a formulation should be stable in circulation and only release the free drug at the intended site of drug delivery. Therefore, the influence of the release rate of the free drug from the nanoemulsion droplet should only be interpreted in the context of rhodamine-123 release in the cellular compartment and not in the apical compartment. Any attempts to modify the formulation to increase the release rate, should aim at developing formulations which quickly release the free drug only after cellular uptake and not in the systemic circulation. Though not addressed in this paper, several strategies have been employed in the past to enhance the rate of intracellular release of free drug from a drug delivery system, using triggers such as change in pH and high enzyme levels present specifically in a cellular environment (26–28). Lastly, the uptake rate of the nanoemulsion droplet from the apical to cellular compartment was also identified as a sensitive parameter to increase rhodamine-123 permeability across Caco-2 cells. As the next step, our lab plans to experimentally test the effect of different uptake rates of the nanoemulsion droplet on the permeability of the free drug across Caco-2 cells. Several publications have suggested that, the particle size greatly influences the rate of endocytosis of the nanoparticles across the cell

membrane (29, 30). Therefore, we plan to test the effect of particle size on the permeability of nanoemulsion encapsulated drugs in cells expressing efflux transporters.

CONCLUSIONS

Using Caco-2 cells which express efflux transporters and rhodamine-123 which is an efflux substrate, we showed that nanoemulsions may provide a formulation approach to enhance the transcellular permeability of drug molecules. A semi-mechanistic model was developed, that described the transport kinetics of rhodamine-123 dosed in a nanoemulsion, across Caco-2 cells in-vitro. This model was used to identify sensitive formulation variables such as the size of the emulsion droplet and the intracellular release rate of the free drug, optimizing which, may further enhance the permeability of the drug molecule. Modeling and simulation results suggested that, nanoemulsions may not improve drug permeability by reducing or inhibiting efflux of the free drug from the cellular compartment to the apical compartment, because efflux of the release free drug is unavoidable. However, the most beneficial effect of nanoemulsions is their ability to deliver high concentrations of the free drug intracellularly, as compared to that obtained by passive diffusion of the free drug only. Higher intracellular concentrations result in an increased concentration gradient between the intracellular and extracellular environment, thereby resulting in an overall increase in the cellular to basal permeability of the drug molecules, while the apical to cellular permeability is mainly driven by the endocytosis mediated uptake of the nanoemulsion.

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ABBREVIATIONS

A:B	Apical-to-basal
B:A	Basal-to-apical
BBB	blood brain barrier
BCRP	Breast Cancer resistance protein
Conc	Concentration
CUR	curcumin
hr	hour
Kendo	first order endocytosis-mediated uptake rate constant for the nanoemulsion droplet
Krel	first order release rate constant for rhodamine 123 from the nanoemulsion
L	liter
MDR1	Multi-Drug Resistance Gene

MRP1	Multi-drug resistance associated protein 1
MRP2	Multi-drug resistance associated protein 2
mV	millivolts
NE	nanoemulsion
nm	nanometer
O/W	Oil-in-water
P	passive permeability mediated transport of rhodamine 123
PDI	polydispersity index
Pgp	P-glycoprotein
Rh123	rhodamine-123
RT-PCR	reverse transcription polymerase chain reaction
S	solution
SD	standard deviation
SE	standard error
TEER	trans-epithelial electrical resistance
TEM	transmission electron microscopy
W/O	Water-in-oil

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Figure 1.
Schematic representation of the workflow of experiments and analyses used to compare drug delivery from rhodamine-123 nanoemulsion vs. solution.

Figure 2.

Schematic of mathematical modeling structure illustrating key transport mechanisms for the free drug and nanoemulsion (NE) in the Caco-2 monolayer.

1. Endocytosis of the NE droplet from the apical to the cellular compartment
2. First order release of the free drug from the NE droplet
3. Efflux of the free drug from the cellular to the apical layer
4. Passive permeability of the free drug across the Caco-2 monolayer

Figure 3.

Transporter expression in Caco-2 cells was confirmed using RT-PCR. In Figure 3a, bottom to top bands represent BCRP, MDR1, MRP2, MRP1 and β -actin, respectively. (NE: nanoemulsion, S: aqueous solution, CUR: curcumin pre-treated, U: untreated). In Figure 3b, semi-quantitative results of band quantification using NIH ImageJ software are presented. As compared to the untreated arm, treatment with 20 μ M CUR either as a NE or S resulted in significant downregulation of BCRP, MDR1 and MRP1 transporters. MRP2 transporter expression was unaltered. ** = $p < 0.05$ (Student's t-test)

Figure 4.

In vitro release of rhodamine-123 from nanoemulsion (NE) and aqueous solution (S). Markers represent the observed data and lines represents the model predicted profile. Rhodamine-123 concentration was measured in the receiver fluid outside the dialysis membrane.

Figure 5. Time-dependent permeability results for rhodamine-123 in Caco-2 cells. Figure 5a and 5b represent permeability results following dosing with rhodamine-123 in aqueous solution in curcumin untreated and treated cells, respectively. Figure 5c and 5d represent permeability results following dosing with rhodamine-123 in nanoemulsion formulation in curcumin untreated and treated cells, respectively. The presence of curcumin and/or treatment with nanoemulsion reduces the efflux ratio for rhodamine-123.

Figure 6.

Semi-mechanistic modeling of nanoemulsion-based cellular transport. X-axis: time (h), Y-axis: rhodamine-123 concentration (μM). Circles represent observed data and solid line represents model predictions. 6a-6d: Y-axis: Basal concentration (μM) following dosing the nanoemulsion (NE) (Figure 6a,6b) and solution (S) (Figure 6c,6d) in the apical compartment. 6a and 6c represent results in curcumin treated cells (Only passive transport present) and 6b and 6d represent results in curcumin untreated cells (Both passive and efflux transport present). 6e-6h: Y-axis: Apical concentration (μM) following dosing the nanoemulsion (NE) (Figure 6e,6f) and solution (S) (Figure 6g,6h) in the basal compartment. 6e and 6g represent results in curcumin treated cells (Only passive transport present) and Figures 6f and 6h represent results in curcumin untreated cells (Both passive and efflux transport present).

Figure 7.

Effect of variations in kinetic constants on rhodamine-123 A:B permeability across Caco-2 monolayers dosed within nanoemulsion (NE) formulation. Figure 7a represents the effect of varying the NE endocytosis rate constant over a 4 fold range (X: Model estimated parameter value for the NE endocytosis rate constant). Figure 7b represents the effect of varying the free drug release rate constant from the NE over a 4-fold range (X: Model estimated parameter value for the free drug release rate constant from the NE). Figure 7c represents the effect of varying the free drug permeability a 4-fold range (X: Model estimated parameter value for free drug permeability).

List of variables used to describe rhodamine-123 transport in the semi-mechanistic model

Table I

Variables	Units	Process
Rh123 _{donor/receiver amount}	<i>umoles</i>	Amount of free rhodamine-123 in the donor or receiver compartment in the dialysis cassette
Rh123 _{donor/receiver conc}	<i>uM</i>	Concentration of free rhodamine-123 in the donor or receiver compartment in the dialysis cassette
NE Rh123 _{donor amount}	<i>umoles</i>	Amount of rhodamine-123 encapsulated in the nanoemulsion in the donor compartment of the dialysis cassette
Rh123 _{apical/basal/cellular amount}	<i>umoles</i>	Amount of free rhodamine-123 in the apical, basal or cellular compartment of the Transwell plate
Rh123 _{apical/basal/cellular conc}	<i>uM</i>	Concentration of free rhodamine-123 in the apical, basal or cellular compartment of the Transwell plate
NE Rh123 _{apical/basal/cellular amount}	<i>umoles</i>	Amount of rhodamine-123 encapsulated in the nanoemulsion in the apical or cellular compartment of the Transwell plate

Rhodamine-123 concentration in Caco-2 cells (normalized to cellular protein content) following uptake studies with rhodamine-123 (5 μ M) aqueous solution (S) and nanoemulsion (NE)-based formulation

Table II

Time (hr)	Rh123 conc (S) (μ M/mg)	SD	Rh123 conc (NE) (μ M/mg)	SD	P-value Unpaired t-test (n=3)
0.083	0.0216	0.0013	0.036	0.0023	0.0007
0.25	0.0389	0.0008	0.04	0.008	0.77
0.5	0.0537	0.0023	0.077	0.0083	0.0098
1	0.0901	0.0019	0.166	0.0009	<0.0001
1.5	0.1111	0.008	0.208	0.009	0.0002

Table III

List of model parameters and estimated values

Parameter	Units	Process	Estimate	SE
K _{real}	$\frac{1}{hr}$	First order release rate constant for rhodamine-123 from the nanoemulsion	7.26E-02	4.60E-03
Q	$\frac{L}{hr}$	Inter-compartmental clearance of free rhodamine-123 across the donor and receiver chamber	1.37E-05	2.60E-07
K _{endo}	$\frac{1}{hr}$	First order endocytosis-mediated uptake rate constant for the nanoemulsion droplet from the apical to cellular compartment	2.00E-01	4.82E-06
V _{max}	$\frac{\mu moles}{hr}$	Zero order efflux rate of rhodamine-123 from the cellular to apical compartment	9.60E-05	1.00E-08
K _m	$\frac{1}{hr}$	Cellular concentration of rhodamine-123, which reaches half its maximum value	5.90E-01	4.63E-01
P	$\frac{L}{hr}$	Passive permeability mediated transport of rhodamine-123 across the apical/cellular and cellular/basal compartments	7.60E-06	1.16E-06
V _{apical} , V _{basal}	L	Volume of the apical & basal compartment, respectively (Based on the volume of dosing/receiver solution)	1.00E-03, 2.00E-03	Fixed
V _{cellular}	L	Volume of the cellular compartment (Based on the cell monolayer thickness (0.003 cm) and cell monolayer growth area (0.9 cm ²))	2.70E-06	Fixed