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***In vitro* pharmacokinetic cell culture system that simulates physiologic nanoparticle exposure to macrophages**

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Abstract

Purpose—An *in vitro* dynamic pharmacokinetic (PK) cell culture system was developed to more precisely simulate physiologic nanoparticle/drug exposure.

Methods—A dynamic PK cell culture system was developed to more closely reflect physiologic nanoparticle/drug concentrations that are changing with time. Macrophages were cultured in standard static and PK cell culture systems with rifampin (RIF; 5 µg/ml) or β-glucan, chitosan coated, poly(lactic-co-glycolic) acid (GLU-CS-PLGA) nanoparticles (RIF equivalent 5 µg/ml) for 6 h. Intracellular RIF concentrations were measured by UPLC/MS. Antimicrobial activity against *M. smegmatis* was tested in both PK and static systems.

Results—The dynamic PK cell culture system mimics a one-compartment elimination pharmacokinetic profile to properly mimic *in vivo* extracellular exposure. GLU-CS-PLGA nanoparticles increased intracellular RIF concentration by 37% compared to free drug in the dynamic cell culture system. GLU-CS-PLGA nanoparticles decreased *M. smegmatis* colony forming units compared to free drug in the dynamic cell culture system.

Conclusions—The PK cell culture system developed herein enables more precise simulation of human PK exposure (i.e., drug dosing and drug elimination curves) based on previously obtained PK parameters.

Keywords

Cell culture; macrophage; nanoparticles; pharmacokinetic

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Supplementary material

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ESM 1 (DOCX 1276 kb)

Introduction

Nanomedicine, medicine coupled with nanotechnology, is an emerging field for the development of advanced drug delivery formulations. Nanomedicines have higher stability and increased circulation time, and improve targeted cargo delivery thereby resulting in enhanced efficacy while limiting drug toxicity (1). Numerous types of nanoparticles, including alginate (2), PLGA (3, 4, 5, 6, 7, 8), silver (9, 10), silver and zinc (11, 12), polymeric (13), gallium (III) (14), chitosan (15, 16), lipid (17), and silica (18) have been reported as drug delivery vehicles for therapeutics that target infectious diseases. For example, acrylate nanoparticles increased the intracellular concentration of anti-tuberculosis chemotherapeutics to macrophages compared to free drugs (19). In addition, poloxamer nanoformulations have increased the concentrations of HIV antiretroviral drugs in macrophages (20). These studies have focused primarily on improving drug loading and intracellular drug bioavailability. Overall, very few nanoparticles have moved beyond preclinical testing most likely due to *in vivo* failure such as systemic toxicity or ineffective cellular targeting (14, 17, 21, 22, 23). In particular, very few cell-targeted nanoparticles for infectious disease have moved beyond preclinical testing most likely due to *in vivo* failures.

Preclinical *in vivo* testing is primarily based on previous *in vitro* cell culture findings. However, current *in vitro* standard static cell culture systems do not mimic physiologic drug exposures, making it a relatively poor model for *in vivo* conditions. The major drawbacks to the standard static cell culture model are that cells are exposed to a constant drug dose as well as biological waste products and metabolites accumulate for the entire duration of the study. These limitations may seriously compromise the reliability and significance of data for translation of drug dosages to *in vivo* studies (21, 22, 23). Therefore, an *in vitro* dynamic pharmacokinetic (PK) cell culture system that more precisely simulates physiologic cell drug exposure based on dosing and elimination pharmacokinetic parameters was developed. We used rifampin (RIF), a model antibiotic with a short half-life used for the treatment of tuberculosis (TB) (24, 25), to explore the advantages of a dynamic PK cell culture system to study intracellular drug concentrations for nanoparticle and free drug compared to standard cell culture approaches.

Materials and Methods

Materials

Poly(lactic-co-glycolic acid) (PLGA) (lactide:glycolide = 50:50, molecular weight (MW) = 38–54 kDa), poly(vinyl alcohol) (PVA) (Mowiol®4–88, MW = 31 kDa), chitosan oligosaccharide lactate (CS) (MW = 5 kDa), β -glucan (1,3 beta-glucan) (GLU), and rifapentine (RifP) were purchased from Sigma Aldrich (St. Louis, MO). Rifampin (RIF) was purchased from Tokyo Chemical Industry (TCI) America (Portland, OR). All other reagents were used as received without further purification.

Preparation and Characterization of Nanoparticles

Nanoparticles were fabricated using a water-oil-water (w/o/w) emulsion with solvent evaporation (7, 26). RIF (8 mg/ml) and PLGA (40 mg/mL) were dissolved in

dichloromethane (DCM) (500 μ l) and added to a vial containing 100 μ l water. The vial was probe sonicated for 60 s in a 4°C water bath. To the resulting emulsion, 500 μ l of a 20 mg/ml PVA, 1.2 mg/ml CS, 20 μ M sodium acetate buffer pH 4.4 was added and probe sonicated again for 60 s in a 4°C water bath to obtain the double emulsion. The resulting w/o/w emulsion was stirred overnight to remove DCM. The nanoparticle suspension was washed once with water by centrifugation (16,100 x g, 10 min) and resuspended in water. One-hundred μ l of β -glucan (5 μ g/l suspended in water) was added to the nanoparticle suspension and incubated for 5 min before centrifugation (16,100 x g, 10 min) and resuspended in water. To visualize cellular uptake of nanoparticles by microscopy, 2 μ l of Nile red (0.5 mg/ml in acetone) was added to the DCM phase during preparation. Nanoparticle size and polydispersity index were determined by dynamic light scattering (DLS) (Brookhaven 90 plus Particle Analyzer, Brookhaven Instruments, Holtsville, NY) or transmission electron microscopy (TEM). Surface morphology was also analyzed by TEM by staining with 0.1% w/v phosphotungstic acid. Zeta potential was determined by Zeta PALS (Brookhaven Instruments).

PK Cell Culture Approach

A PK cell culture system was developed that simulates the elimination profile of a drug's plasma concentration by controlling the rate of fresh media addition to the central reservoir (culture vessel with cells) while simultaneously controlling the rate of media removed from the central reservoir. A simplified schematic is shown in Fig. 1a. **(a)** Fresh media containing no drug or nanoparticles in the diluent reservoir was pumped into the central reservoir (T25 flask containing cells and 20 ml of media) at a controlled rate (76.8 μ l/min) using a calibrated peristaltic pump (Ismatec® Reglo ICC 4 channel pump, Cole-Parmer, Inc., Vernon Hills, IL). **(b)** Concurrently, drug containing media (“waste media”) was pumped out of the central reservoir at a controlled rate (77.2 μ l/min). **(c)** Drug concentration in the waste media was continuously monitored by pumping waste media through a flow cell cuvette (Type 583.4, Starna Cells, Inc., Atascadero, CA) in a spectrophotometer. **(d)** Waste media was then collected in a separate reservoir to be disposed of properly. All components were connected with polyethylene lined, light resistant tubing (Smiths Medical, Dublin, OH). T25 flasks were sealed with a septa pierced by 3 18G needles [inlet, outlet, and gas exchange, see Supplementary Fig. S1] and placed on a rocker plate mixer to insure adequate mixing of fresh diluent. In addition to the dynamic PK cell culture system, a closed-loop PK cell culture system that continuously monitored UV-VIS absorbance was used as a control to account for any changes to drug concentration caused by drug degradation or absorbance of free drug or nanoparticles to tubing walls and/or the luer connections (Fig. 1b). **(a)** Media was pumped out of the central reservoir (T25 flask containing cells) at a controlled rate (77 μ l/min) using the calibrated peristaltic pump. **(b)** Drug concentration was continuously monitored by pumping media through a flow cell cuvette in a spectrophotometer. **(c)** Media was returned to central reservoir (T25 flask containing cells).

The inflow and outflow rates were determined by performing least squares regression of Eq. 1 to simulated data from a one compartment elimination model (Eq. 2) using MATLAB (v. 2017b, MathWorks Inc., Natick, MA).

$$C = A_o \left((r_{in} - r_{out}) * t + V_o \right)^{\frac{-r_{in}}{r_{in} - r_{out}}} * V_o^{\frac{r_{out}}{r_{in} - r_{out}}} \quad (1)$$

where C is the concentration in solution (mg/ml), A_o is the initial amount of RIF (mg) in the central reservoir, V_o is the initial volume of the central reservoir (ml), r_{in} is the rate of inflow (ml/h), r_{out} is the rate of outflow (ml/h). Note: values used were $A_o = 100$ mg and $V_o = 20$ ml to match the simulated data generated as described in Eq. 2.

$$C = \frac{A_o}{V_o} * e^{-k * t} \quad (2)$$

where $k = \ln(2)/t_{1/2}$, RIF $t_{1/2} = 3$ h, $A_o = 100$ mg and $V_o = 20$ ml (24, 25). Note: the value of A_o does not affect the calculation of r_{in} and r_{out} , and is chosen as 100 to assume 100% as the starting condition.

Using the above values, the calculated flow rates are: $r_{in} = 4.6103$ ml/h [76.8 μ l/min] and $r_{out} = 4.6298$ ml/h [77.2 μ l/min].

UV-VIS spectra using a spectrophotometer (8453A controlled by ChemStation with the Biochemical Analysis package v.B.05.02, Agilent Technologies, Inc., Santa Clara, CA) with an 8-position multicell transport attachment (G1120A, Agilent Technologies, Inc.) were taken every 5 min for the first hour, and then the time was increased by 10% between each subsequent sampling point until 6 h. Samples were blanked prior to each measurement. A blank cuvette was used to avoid undue variation caused from the lamp power changing over time, as the 8453A spectrophotometer is a single beam spectrophotometer.

Cell Type and Exposure

THP-1 macrophages (1×10^6 cells/ml) were differentiated from THP-1 monocytes in the presence of phorbol 12-myristate-13-acetate (100 nM, 4 days) in T25 flasks (27). THP-1 macrophages were exposed to RIF (5 μ g/ml) or the GLU-CS-PLGA nanoparticles (equivalent RIF dose [5 μ g/ml]) for 6 h (2 half-lives) in static and PK cell culture systems. After 6 h supernatant was collected and cells were lysed with 0.05% SDS. Supernatant and cell lysates were analyzed using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

UPLC-MS/MS

Sample (e.g., lysed cell pellet, cell culture supernatant) (100 μ l) was added to 300 μ l of acetonitrile:methanol (1:1) containing 0.5 mg/ml ascorbic acid and 1 μ g/ml of rifapentine (RifP, internal standard). Samples were vortexed and centrifuged at 13,000 x g for 15 min. The supernatant was transferred to separate vial for analysis. RIF concentrations were determined in a UPLC-MS/MS system consisting of an Acquity sampler manager, UPLC binary pump, heated column oven, and an Acquity TQD triple quadrupole mass spectrometer.

(Waters Corporation, Milford, MA). Reverse phase chromatography separated RIF on an Acquity BEH Phenyl (2.1×100 mm, $1.7 \mu\text{m}$, Waters Corporation) with an Acquity BEH Phenyl (2.1×5 mm, $1.7 \mu\text{m}$, Waters Corporation) guard column and detected using a positive electrospray ionization MS/MS method. Chromatographic separation was performed using an isocratic mobile phase of 60:40 0.05% formic acid pH 5:acetonitrile filtered through a $0.22 \mu\text{m}$ membrane filter (Millipore, Milford, MA). The mobile phase flow rate was 0.4 ml/min, column temperature set to 30°C , and injection volume was $2 \mu\text{l}$. The selected reaction monitoring scheme followed transitions of the $[\text{M} + \text{H}]^+$ precursor to selected product ions with the following values: m/z 823.56 $\rightarrow$ 791.37 for RIF, m/z 877.67 $\rightarrow$ 845.40 for RifP. Standard curve ranged from 0.2 to 100 $\mu\text{g/ml}$ for RIF. Data were collected and processed using Waters Empower Chromatography Software v3.

Intracellular Imaging

THP-1 macrophages were cultured in static and PK cell culture systems. Cells were incubated with equal concentrations of GLU-CS-PLGA nanoparticles containing Nile red, a hydrophobic dye. After 6 h, cells were fixed with 4% paraformaldehyde at 37°C for 10 min. Cells were subsequently incubated with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). THP-1 macrophages were imaged using an EVOS[®] FL Cell Imaging System (Thermo Fisher Scientific, Carlsbad, CA) at 20x and 40x magnification. The light cube used to visualize DAPI staining had an excitation of 357/44 nm and emission of 447/60 nm. The light cube use to visualize Nile red had an excitation of 534/40 nm and emission of 593/29 nm.

Mycobacterium Smegmatis (*M. smegmatis*) Intracellular Survival

M. smegmatis was cultured in Middlebrook 7H9 liquid medium supplemented with 10% oleic acid, albumin, dextrose, catalase, and 5% glycerol. *M. smegmatis* was harvested by centrifugation, washed, and resuspended in complete medium to a concentration of 5×10^7 CFU/ml. THP-1 macrophages (1×10^6 cells/ml) were infected with a multiplicity of infection (MOI) of 5 for 3 h, were then washed with PBS to remove extracellular bacteria, and were then incubated with fresh medium (28). Twelve hours later, THP-1 macrophages were exposed to RIF (5 $\mu\text{g/ml}$) or GLU-CS-PLGA nanoparticles (equivalent RIF dose [5 $\mu\text{g/ml}$]) for 6 h (2 half-lives) in static and PK cell culture systems. Cells were lysed in sterile distilled water and the lysates were diluted and plated separately on LB agar plates and incubated at 37°C . Five days post plating, colony counts (colony forming units (CFU)) were performed. Control cells were infected with *M. smegmatis* at a multiplicity of infection (MOI) of 5 for 3 h, were then washed with PBS to remove extracellular bacteria, and were then incubated with fresh medium (28). Twelve hours later these control THP-1 macrophages were used in both static and PK cell culture systems in the absence of drug or nanoparticles. There was no significant difference in CFU in the control cells in either the static or PK cell culture systems, therefore we averaged the CFUs.

Statistics

Statistical significance was determined using either Student's t-test or one-way ANOVA followed by a post-hoc Tukey test (Prism v.7.0, GraphPad Software, La Jolla, CA).

Results

Nanoparticle Characterization

Nanoparticles with a PLGA core (encapsulating RIF) and a CS shell with a surface adsorbed ligand GLU (GLU-CS-PLGA) have an average hydrodynamic diameter (size) of 235 nm as determined by dynamic light scattering (DLS) and TEM (Fig. 2a), with a zeta potential of +12.47 mV. GLU-CS-PLGA nanoparticles provide controlled release of RIF in a pH 7.4, 10 mM phosphate buffer with 80% released after 6 h (Fig. 2b).

Cell Culture System Comparisons

A) Half-life: THP-1 macrophages were cultured with RIF or the RIF loaded GLU-CS-PLGA nanoparticles in both static and PK cell culture systems for 6 h (2 half-lives). Raw spectrophotometric data were fit using Eq. 2 in GraphPad Prism and the resulting half-life was compared to the expected (3 h) (Fig. 3a, Supplementary Fig. S3). The one compartment model-fit half-life of RIF was 2.74 h (95% CI 2.511 to 3.017) and of NP was 3.10 h (95% CI 2.657 to 3.700), indicating that the PK cell culture system is capable of mimicking the predicted *in vivo* exposure. In addition, Supplementary Fig. S2 demonstrates that Eq. 1, which was used to calculate the pump flow parameters, approximates Eq. 2, a standard one-compartment model, through 6 h, and the AUC_{0-6h} of Eq. 1 is only 0.036% less than that of Eq. 2.

B) Cellular Uptake: Image analysis of THP-1 macrophages following a 6 h incubation with GLU-CS-PLGA nanoparticles encapsulated with Nile red (a hydrophobic dye, pseudocolored red in image), demonstrates that intracellular uptake is higher in the static cell culture system compared to the PK cell culture system (Fig. 3b).

C) Intracellular RIF concentrations: Intracellular concentration of RIF in macrophage were higher in the static system than in the PK cell culture system (Fig. 3c). Furthermore, when comparing free drug to RIF loaded GLU-CS-PLGA nanoparticles, GLU-CS-PLGA nanoparticles increased intracellular RIF concentration by 37% compared to free drug in the dynamic PK cell culture system.

M. smegmatis Intracellular Survival

Following infection of THP-1 macrophages with *M. smegmatis*, macrophages in both static and PK cell culture systems were treated as described previously. There was no significant difference in CFUs in the control cells in either the static or PK cell culture systems, therefore data was averaged and labeled as control in Fig. 4. RIF alone and RIF loaded GLU-CS-PLGA nanoparticles significantly reduced CFU compared to control in both the static and PK systems (Fig. 4). In the static system, RIF loaded GLU-CS-PLGA nanoparticles had significantly less CFU than RIF alone. In the PK system, RIF loaded GLU-CS-PLGA nanoparticles had significantly less CFU than RIF alone. However, both had more CFU than in the static system.

Discussion

There are other well-established cell culture systems that consist of cells cultured on the outside of semi-permeable molecular weight cut-off hollow fibers, that enable the transport of therapeutics, nutrients, and waste products in and out of the bulk media, which are often used to culture suspension cell culture lines or bacteria in bioreactors (29). During the development of this unique dynamic PK cell culture system, our laboratory attempted to use a commercial hollow fiber membrane cell culture system, and realized that the addition of the semi-permeable membrane in the system further complicates the exposure of cells to drugs. The need to separate cells from the fresh media was unwarranted as these THP-1 macrophages are an adherent cell line. Therefore, we developed an alternative dynamic PK cell culture system to mimic the PK exposure of nanoparticles and free RIF to macrophages. In addition, the dynamic PK cell culture system described enables continuous, real-time monitoring of drug concentrations, without the need for sampling at discrete time points. Another advantage of the PK cell culture system is that it does not require an extensive set up or proprietary membrane fiber cartridges that may be cost prohibitive and require large media volumes to operate. One can use a calibrated peristaltic pump or even two syringe pumps, which are common lab equipment, with common cell culturing techniques.

The PK elimination profile of RIF *in vivo* is best described by a one-compartment model with a half-life of 3 h (24,25). Therefore, we designed the *in vitro* PK cell culture system to mimic a one compartment model (first-order model) in comparison to a traditional static cell culture system that is described by a zero-order model. The major difference between the static and dynamic models is that the exposure (i.e., area under the curve (AUC)) in the static model will always be higher than the PK cell culture system. This is extremely important for compounds with 'short' half-lives, being studied at 'longer' time-points. For example, determining the intracellular drug concentration of a drug with a 3 h half-life (e.g., RIF) after 2 half-lives (i.e., measurement at 6 h) the calculated AUC of the static cell culture system is 1.85-fold higher than the AUC of the PK cell culture system. Based on these AUC calculations, the intracellular uptake and concentration in the static cell culture system was higher compared to the dynamic PK cell culture system. In addition, intracellular RIF concentrations were significantly higher when delivered by the nanoparticles than free drug in both cell culture systems. A similar finding was seen for anti-bacterial activity against *M. smegmatis*, our model pathogen to mimic *M. tuberculosis*. Overall activity against *M. smegmatis* was higher in the static system compared to the PK system. Again, in both cell culture systems, RIF loaded GLU-CS-PLGA nanoparticles reduced CFU more than free RIF. These data show the intrinsic differences in cellular uptake and antimicrobial activity that occurs when cells are exposed to a constant concentration (i.e., static cell culture system) compared to when cells are exposed to a dynamic concentration (i.e., PK cell culture system). These data demonstrate that when translating *in vitro* data to *in vivo* experiments, there is significant potential to underestimate the *in vivo* dosage based on data obtained from a traditional static cell culture system. These data demonstrate the need for a paradigm shift from traditional static culture approaches in short half-life drugs, to better represent physiologic exposures.

Limitations

A major limitation of this study is that the half-life of the nanoparticles is unknown. In order to make the data ‘more’ interpretable, we made a gross assumption that the PK of the free drug and nanoparticle were the same. Without first performing *in vivo* experiments to assess the PK of the nanoparticle formulation, it is impossible to accurately mimic the *in vivo* PK in the dynamic PK cell culture system.

Conclusion

This newly developed PK cell culture system has the potential to change how efficacy and toxicity of free drug or nanoparticles are characterized *in vitro* and provide a complementary *in vitro* evaluation system to improve dosing strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments and Disclosures

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Abbreviations

BAL	Bronchoalveolar lavage
CFU	Colony forming units
CS	Chitosan oligosaccharide lactate
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DLS	Dynamic light scattering
HIV	Human immunodeficiency virus
PK	Pharmacokinetic
PLGA	Poly(lactic-co-glycolic) acid
PVA	Poly(vinyl alcohol)
RIF	Rifampin
RifP	Rifapentine

TB	Tuberculosis
TEM	Transmission electron microscope
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry

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Fig. 1. Schematics of cell culture systems.

(a) PK cell culture system; (b) closed-loop PK cell culture system.

Fig. 2. Nanoparticle characterization.

(a) Size of GLU-CS-PLGA nanoparticles determined by DLS. Inset: TEM image; (b) RIF release profile from GLU-CS-PLGA nanoparticles ($n = 4$).

Fig. 3. Cell culture systems.

(a) Media RIF concentration versus time. PK flow parameters set for 3 h half-life. RIF concentration was continuously measured using a UV-VIS spectrophotometer. (b) Intracellular uptake of Nile red labeled nanoparticles by macrophages 6 h post-incubation. a) control, b) static cell culture system, c) PK cell culture system. The red fluorescence arises from Nile red encapsulated within the nanoparticles, and the blue fluorescence arises from DAPI staining of nuclei. (c) Intracellular RIF concentrations in THP-1 macrophages after exposure ($n = 4$) to RIF [$5 \mu\text{g}/\text{mL}$] or the RIF loaded GLU-CS-PLGA nanoparticles (equivalent RIF dose [$5 \mu\text{g}/\text{mL}$]) RIF for 6 h (2 half-lives), * $p < 0.05$ (Student's t-test). RIF = rifampin; NPs = RIF loaded GLU-CS-PLGA nanoparticles.

Fig. 4. Antibacterial activity.

Colony forming units (CFU) after THP-1 macrophage infected with *M. smegmatis* were exposed to RIF [5 µg/ml] or the RIF loaded GLU-CS-PLGA nanoparticles (equivalent RIF dose [5 µg/ml]) for 6 h (2 half-lives)), ($n = 7$); * $p < 0.05$ compared to static free RIF; # $p = 0.004$ compared to PK free RIF) (ANOVA). Control cells were infected with *M. smegmatis* as described above, then used in the static or PK cell culture system in the absence of RIF or GLU-CS-PLGA nanoparticles. There was no significant difference in CFUs in the control cells in either the static or PK cell culture systems, therefore the data was averaged. RIF = rifampin; NPs = RIF loaded GLU-CS-PLGA nanoparticles.