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Exploring intracellular fate of drug nanocrystals with crystal-integrated and environment-sensitive fluorophores

Wei Gao, Deborah Lee, Zhengjie Meng, Tonglei Li*

Department of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, IN 47907, United States

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ABSTRACT

Formulating a poorly water-soluble drug substance into nanocrystals offers many advantages. Understanding of the *in vivo* fate of drug nanocrystals is however very limited. In this study, we utilized the hybrid nanocrystal concept and studied the kinetic process of dissolution in cancer cells. By taking advantage of aggregation-induced emission (AIE), hybrid paclitaxel (PTX) nanocrystals integrated with tetraphenylethene (TPE) enabled a novel way for estimating the intracellular dissolution process of the nanocrystals. When TPE is entrapped in a nanocrystal, fluorescence is emitted when the nanocrystal is optically excited. When an entrapped TPE molecule is released to a liquid medium due to the dissolution of the nanocrystal, its fluorescence is quenched. By monitoring the change in fluorescence, it is possible to quantify the dissolution of nanocrystals in a biological environment. Cellular uptake studies of hybrid nanocrystals were conducted with KB and HT-29 cell lines and characterized by confocal microscopy, flow cytometry, and HPLC. The results suggest that drug nanocrystals were taken up directly by the cells, and subsequently dissolved in the cytoplasm. The extent to which the drug nanocrystal dissolved was estimated according to the fluorescence measurement. The cellular uptake and intracellular dissolution could be influenced by drug concentration, incubation time, and surface coating, as well as the type of cell line.

1. Introduction

Formulating a poorly water-soluble drug into nanosized, carrier-free, crystalline particles offers many advantages over conventional approaches [1–4], including high efficiency of drug loading, increased physicochemical stability, reduced toxicity, feasible scale-up of production, relatively low cost of preparation, and applicability to various administration routes, which span oral [5], parenteral [6], ocular [7], and pulmonary delivery [8]. We have studied nanocrystals (NCs) of several poorly soluble anticancer compounds and learned that the carrier-free drug nanocrystals could achieve better treatment efficiency with significantly reduced side effects [9–15]. Importantly, because drug nanocrystals are in the crystalline state and thus physically stable, the *in vivo* drug release is expected to be consistent and predicable [10,15].

Little is known, however, about the *in vivo* fate of drug nanocrystals such as dissolution kinetics and biodistribution. We could measure the total drug concentration in an organ but we had few or no means to discern nanocrystals and dissolved molecules. It remains unclear how drug nanocrystals exhibit anticancer effect against cancer cells, for example, whether through drug molecules diffusing into the cells after being released outside, or though molecules released intracellularly from nanocrystals being endocytosed. While our previous study suggests that drug nanocrystals can be taken up directly by cancer cells [16], no information is available regarding the kinetic process of nanocrystal dissolution and molecule release inside cells. Without garnering a comprehensive picture of how drug nanocrystals behave kinetically at the pharmacokinetic and intracellular levels, it is difficult to know and subsequently design what properties of nanocrystal-based drug delivery systems, such as particle size and morphology, can lead to an optimal treatment outcome. There is no report to date, however, that studies the dynamic dissolution process of drug nanocrystals *in vivo*. Although electron microscopy has been shown to be able to image drug nanocrystals in tissues [3], the method is generally limited to sampling size and *ex vivo* tissue samples. Lacking of reliable analytical approaches is obviously blamed for the missing information about the pharmacokinetics of drug nanocrystals in the body. To address the challenge, the purpose of our current study is to test the concept of a novel way for exploring nanocrystal dissolution kinetics inside cancer cells, which is expected to be applicable for eventually studying the *in*

* Corresponding author at: Department of Industrial & Physical Pharmacy, College of Pharmacy, Purdue University, RHPH Building, Room 124B, 575 Stadium Mall Drive, West Lafayette, IN 47907-2091, United States.
E-mail address: tonglei@purdue.edu (T. Li).

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vivo transport and drug release of drug nanocrystals.

Over last a few years, we have tested the hybrid nanocrystal concept for drug delivery and bioimaging [9,13–15]. By physically integrating an insignificant amount of imaging agents inside drug nanocrystals, we could achieve both anticancer therapy and whole-body imaging. Nonetheless, the fluorescent probes used in our studies were indiscriminate in signifying nanocrystal dissolution. The limitation may be circumvented if the probe is sensitive to its immediate environment and entrapped in nanocrystals. For this purpose, we resorted to aggregation-induced emission (AIE) and developed hybrid nanocrystals that integrated with AIE fluorophores in order to characterize the dissolution kinetics of the nanocrystals inside cells and eventually in animal models. Our first system, reported here as a proof of concept, was PTX NCs that were doped physically with tetraphenylethene (TPE). The probe has four aromatic rings conjugated together *via* the rigid ethylene and shows excellent AIE [17–19]. When the molecule is restricted in motion, such as in a crystalline state, dissipation of the excited state is accomplished by photon emission; when the aromatic groups of the molecule are free to rotate, such as in a solution, the energy is commonly decayed thermally [20–23]. Studies are reported in the literature to chemically conjugate TPE to micelles and utilize its AIE property for tracing the delivery systems in cells [24–27]. It is thus expected that TPE-integrated PTX NCs emit fluorescence when being excited and the intensity should diminish when the nanocrystals dissolve, releasing both drug and dye molecules to the medium. We also investigated the surface treatment of PTX/TPE NCs with PEG-based surfactants and characterized how the PEGylation could influence the cellular uptake and intracellular dissolution kinetics. In our previous study of PTX NCs coated with PEG-PPG-PEG block polymers (Pluronic® F68), significant enhancement of systemic circulation in tumor-bearing mice was observed [15]. Apparently, more could be learned about the polymer coating on the crystal surface in the body with the new concept of TPE-integrated nanocrystals. The same method of surface treatment was used in this study.

2. Methodology

2.1. Materials

Paclitaxel (> 99.5% purity) was purchased from LC Laboratories (Woburn, MA, USA). 1,1,2,2-tetraphenylethylene (TPE) (≥ 98% purity) and F68 were bought from Sigma-Aldrich (St. Louis, MO, USA). Gibco® folate free RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). McCoy's 5A medium was provided by Corning® (Corning, NY, USA).

2.2. Nanocrystal preparation

Pure TPE nanocrystals (TPE NCs) and hybrid PTX nanocrystals integrated with TPE (PTX/TPE NCs) were prepared by the anti-solvent method described in previous studies [13,15]. Briefly, 1 mL of TPE (0.1 mg/mL), or paclitaxel (2.5 mg/mL) and TPE (0.05 mg/mL) ethanol solution, was introduced to 20 mL of deionized water under high speed stirring in a 3-neck flask placed in a sonication water bath (Thermofisher, Waltham, MA, USA). Upon crystallization, the suspension was filtered through a 50-nm polycarbonate filter and re-suspended in deionized water by a high-sheer homogenizer (Thermofisher, Waltham, MA, USA). Pure PTX NCs were similarly prepared for comparison.

To treat the nanocrystals with F68, 0.1 mg of TPE nanocrystals or 2.5 mg of PTX hybrid nanocrystals were re-suspended into 20 mL of 0.4% F68 solution sonicated for 10 min in an ice-water bath. The mixture was kept for 3 h at 4 °C, followed by triplicate centrifugation-washing-resuspension cycles to remove any loosely bound surfactants. This method led to about 3% surface coating of the polymer compared with the whole delivery system [15].

2.3. Nanocrystal characterization

Particle size and size distribution of the nanocrystals were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS instrument (Zetasizer 3000HS, Malvern, Worcestershire, UK) at 25 °C. Each group of nanocrystals was diluted with 100 mmol/L phosphate buffer at pH 7.0 by 1:1 volume ratio to measure the *zeta* potential in triplicate. In addition, nanocrystals were examined by a scanning electron microscope (SEM) (Nova Nano SEM, FEI, Hillsboro, OR, USA). The samples were completely dried by filtration at room temperature and were sputter-coated with layers of gold palladium (Au/Pd) for 1 min, prior to the imaging.

2.4. Fluorescence measurement

Fluorescence of prepared TPE NCs and PTX/TPE NCs was measured in various mixtures of ethanol and water. The emission and excitation spectra were collected using a FluoroMax-3 Spectrofluorimeter (Horiba, Ltd., NJ, USA).

2.5. Cell culture

Two human cancer cell lines, KB (nasopharyngeal epidermal carcinoma) and HT-29 (human colon adenocarcinoma), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). KB cells were cultured in RPMI-1640 medium and HT-29 cells in McCoy's 5A at 37 °C in a humidified atmosphere containing 5% CO_2. Each medium was supplemented with 10% FBS and 1% penicillin streptomycin.

2.6. Drug uptake and confocal imaging

KB and HT-29 cells were respectively seeded in confocal petri dishes at a density of 3×10^5 cells/well and cultured for 24 h. Upon adhering to dish walls, the cells were treated with TPE NCs, PTX/TPE NCs, F68-coated TPE NCs and PTX/TPE NCs, respectively, with equivalent 50 μM of TPE in each group for various durations (1, 2, 3 and 7 h). At the end of treatment, cells were gently washed with cold PBS for three times. DiI (red) was added at the concentration of 6 μM and the cells were further incubated at 37 °C for 20 min. After being collected and washed, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, and then washed with cold PBS three times. The cells were imaged by a laser scanning confocal microscope (Nikon A1, Nikon Co. Ltd., Tokyo, Japan). In addition, cells that were treated for 3 h were gently washed with cold PBS three times, replenished with 2 mL of medium, and further incubated for additional durations (1, 2, 4, 8 and 24 h). At the end of incubation, the cells were gently washed with cold PBS three times, prior to DiI staining. The cells were imaged by the laser scanning confocal microscope.

2.7. Cellular uptake and flow cytometry measurement

KB and HT-29 cells were respectively seeded in 6-well plates (Corning, NY, USA) at a density of 2×10^5 cells/well. After incubation for 24 h, the medium in each well was replaced with 1 mL of fresh cell medium without FBS containing each of four treatment groups, TPE NCs, PTX/TPE NCs, F68/TPE NCs, and F68/PTX/TPE NCs for various durations (0.5, 1, 2, and 3 h). The concentrations of the nanocrystals varied (25, 50, and 100 μg/mL of PTX/TPE NCs; 0.5, 1.1, and 2.2 μg/mL of TPE NCs). After incubation, the cells were washed three times with cold PBS (0.1 M, pH 7.4), detached with 0.05% trypsin, and washed for additional three times with cold PBS. Finally, the cells were re-suspended in PBS and measured by a flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells without treatment were used as a control to correct for auto-fluorescence. Moreover, cells that were treated for 3 h were gently washed with cold PBS three times, and replenished with 1 mL of medium, and incubated for an additional 1 or 2 h. The cells