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A quantitative study of the intracellular fate of pH-responsive doxorubicin-polypeptide nanoparticles

Jing Wang, Jayanta Bhattacharyya, Eric Mastria, Ashutosh Chilkoti*

Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, United States

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ABSTRACT

Nanoscale carriers with an acid-labile linker between the carrier and drug are commonly used for drug delivery. However, their efficacy is potentially limited by inefficient linker cleavage, and lysosomal entrapment of drugs. To address these critical issues, we developed a new imaging method that spatially overlays the location of a nanoparticle and the released drug from the nanoparticle, on a map of the local intracellular pH that delineates individual endosomes and lysosomes, and the therapeutic intracellular target of the drug—the nucleus. We used this method to quantitatively map the intracellular fate of micelles of a recombinant polypeptide conjugated with doxorubicin *via* an acid-labile hydrazone linker as a function of local pH and time within live cells. We found that hydrolysis of the acid-labile linker is incomplete because the pH range of 4–7 in the endosomes and lysosomes does not provide complete cleavage of the drug from the nanoparticle, but that once cleaved, the drug escapes the acidic endo-lysosomal compartment into the cytosol and traffics to its therapeutic destination—the nucleus. This study also demonstrated that unlike free drug, which enters the cytosol directly through the cell membrane and then traffics into the nucleus, the nanoparticle-loaded drug almost exclusively traffics into endosomes and lysosomes upon intracellular uptake, and only reaches the nucleus after acid-triggered drug release in the endo-lysosomes. This methodology provides a better and more quantitative understanding of the intracellular behavior of drug-loaded nanoparticles, and provides insights for the design of the next-generation of nanoscale drug delivery systems.

1. Introduction

Nanoscale drug delivery systems are a common strategy to enhance the delivery of anti-cancer drugs to tumors [1,2]. These systems—typically involving a carrier such as a polymeric nanocarrier [3] or an inorganic nanoparticle [4]—often incorporate acid-labile linkers between the drug and the carrier to enable intracellular controlled drug release in endo-lysosomes [5–8]. However, two critical issues related to the intracellular fate of pH-responsive drug delivery systems have not been quantitatively investigated in live cells. Because most drugs act in locations outside endo-lysosomes in cells and the susceptibility of various acid-labile linkers to acid-sensitive hydrolysis is different, the first question relates to the cleavage efficiency of acid-labile linkers within endo-lysosomes [9,10]. A second and related issue is the mechanism and kinetics of transport of the drug within the cell and especially out of the endo-lysosomal compartment into the cytosol [11,12]. This is because nanoparticle-delivered drugs usually enter cells by endocytosis and are then trafficked to acidic endosomes and lysosomes [13], where the conjugated drug may be trapped. The issue of lysosomal entrapment is an even greater concern with weakly basic drugs, such as doxorubicin, as they are ionized in the acidic endosomes and lysosomes, and studies have shown that the ionized form of the drug cannot diffuse across the endo-lysosomal membrane to the cytosol as freely as the non-ionized form of the drug [14,15]. Furthermore, the pH of endosomes and lysosomes has a broad distribution, so that it is critical to investigate drug release from nanoparticles as a function of pH within live cells at a spatial resolution that captures the dynamics of the intracellular trafficking of the drug-loaded nanoparticles and release of the drug from nanoparticles at the level of individual endosomes and lysosomes. Answering these questions is critical for the rational design of nanoparticle based drug delivery systems, and provides the motivation for this study.

Previous studies have attempted to address these issues using various fluorescence techniques, such as fluorescence resonance energy transfer (FRET) [16], fluorescence-lifetime imaging microscopy (FLIM) [17] and fluorescence de-quenching [18,19]. These studies arrived at the general conclusions that acid-labile linkers can be successfully cleaved in acidic endo-lysosomes, and that the released drug

* Corresponding author.
E-mail address: chilkoti@duke.edu (A. Chilkoti).

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can escape from endo-lysosomes to the cytosol. However, none of these studies quantified the cleavage of acid-labile linkers across the pH range that exists in endo-lysosomes nor did they quantitatively spatially map the intracellular trafficking and distribution of drugs and carriers associated with local pH.

To address these questions quantitatively, we have developed a new method to quantify the intracellular distribution of drug-loaded pH-responsive nanoparticles in endosomes and lysosomes associated with local pH in live cells. This method involves pixel-by-pixel analysis of multi-color fluorescence images of: (1) ratiometric fluorescence imaging of a pH-sensitive dye to map the intracellular pH [20], (2) fluorescence imaging of a nanoparticle carrier, and (3) fluorescence dequenching of a fluorescent drug—doxorubicin—to spatially map the intracellular distribution of drug-loaded nanoparticles, and release of the drug from the nanoparticle as a function of pH and time within live cells. Although each technique used in this paper has been reported separately in previous papers [21–23], these techniques have, to the best of our knowledge, never been combined to spatially and—quantitatively—map the intracellular fate of pH-responsive drug-loaded nanoparticles.

We used this methodology to track the spatial distribution, trafficking and drug release in live cells of a well-characterized, drug-loaded nanoparticle with an acid-triggered drug release mechanism. The nanoparticles are spherical micelles of a chimeric polypeptide (CP)-doxorubicin (Dox) conjugate. The CP consists of a hydrophilic elastin-like polypeptide (ELP) and a short peptide trailer that contains multiple cysteine residues to which Dox is attached by an acid-labile hydrazone linker. CP-Dox self-assembles into nanoscale spherical micelles in aqueous solution. Compared with the free drug, pH-sensitive CP-Dox micelles have significantly longer plasma half-life, enhanced tumor accumulation and higher therapeutic efficacy *in vivo*, though the precise mechanism of drug release within cells has not been elucidated [24–26]. Because this nanoparticle is a precise molecularly engineered delivery system with demonstrated *in vivo* efficacy across multiple tumor models and multiple drugs such as doxorubicin and more recently paclitaxel [27], it provides a well-characterized system to examine the intracellular fate of drugs that are covalently conjugated and loaded into nanoparticles through acid-labile linkers.

We successfully quantified the uptake and intracellular trafficking of doxorubicin-loaded polypeptide nanoparticles and showed that unlike free drug, which can enter the cytosol directly through the cell membrane and then traffic into the nucleus, Dox-loaded nanoparticles are almost exclusively trafficked into endosomes and lysosomes upon intracellular entry. We quantified the kinetics of drug release in endo-lysosomes as a function of pH in live cells and demonstrated the pH-dependent cleavage of acid-labile hydrazone linker below pH 6 in endo-lysosomes. We found that the weakly basic drug released from the nanoparticle can successfully escape the acidic endo-lysosomes, and then enters the cytosol and traffics to the nucleus. This more circuitous route retards the kinetics of cytosolic and nuclear accumulation of the drug that is delivered by the nanoparticle compared to delivery of free drug, but does not change the final intracellular distribution of the drug between different organelles compared with free drug. Our study also points out that an efficient mechanism of acid-triggered drug release with a pH optimum between pH 4 and 7 is ideal to enable maximum release of sequestered drug from the nanoparticle.

2. Materials and methods

2.1. Materials

Lysosensor yellow/blue DND-160 and Alexa Fluor 488 NHS ester were purchased from Life Technology. BHQ2 amine was from Biosearch Technology. Dox-SMCC was from MedKoo Biosciences. TCEP, BMPH, sulfo-SMCC were from Thermo Scientific. Chloroquine and all other reagents were from Sigma.

2.2. Cell culture

Human pharynx squamous cell carcinoma (FaDu) cells were grown in Eagle' s Minimum Essential Medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C with 5% CO_2.

2.3. Chemical conjugation and micelle preparation

The CP consists of two segments: the first segment is an elastin-like polypeptide (ELP). ELPs are a class of artificial peptide polymers composed of a XGVPG pentapeptide repeat derived from human tropoelastin, where the guest residue, X, can be any amino acid except proline [28]. The specific ELP we used herein consists of 160 repeats of XGVPG, where X = V: A: G [1: 8: 7]. The second—drug attachment segment—is a WPC$(GGC)_7$ peptide that is embedded at the C-terminus of the CP, where the cysteine residues provide a unique site for conjugation of drugs. In this study, a CP with the amino acid sequence—SKGPG$(XGVPG)_{160}$WPC$(GGC)_7$—where X = V: A: G [1: 8: 7]) was synthesized and purified as previously described [24].

2.3.1. CP-hyd-Dox

The pH-sensitive CP-hyd-Dox conjugate (hyd: hydrazone linker) (Fig.S1A) was synthesized by conjugating Dox to the cysteine residues in the CP using an N-[β-maleimidopropionic acid] hydrazide (BMPH) crosslinker, as described previously [24]. First, the hydrazide group in BMPH was reacted with the ketone group of Dox to form an acid-labile hydrazone bond, and then the maleimide moiety of the product was reacted with the free thiol of the cysteine residues in the CP to form a thioether bond. Briefly, 400 μmol Dox was first reacted with 360 μmol BMPH in anhydrous methanol (containing 100 μl TFA) for 16 h at room temperature. The activated Dox was then coupled to 9 μmol TCEP-reduced CP, which corresponds to 72 μmol free cysteine residues, by continuously stirring at room temperature for another 16 h. The CP-hyd-Dox conjugate was then separated from unreacted Dox by ultracentrifugation (Amicon Ultra-15 Centrifugal Filter Units, 10 K MWCO, Millipore) using 30% acetonitrile and 70% PBS. The purity of the CP-hyd-Dox conjugate was verified by size exclusion high pressure liquid chromatography (SE-HPLC) (HPLC: Shimadzu; column: Shodex SB-804HQ, Phenomenex) using 30% acetonitrile and 70% PBS as the elution buffer. The HPLC profile of purified CP-hyd-Dox is shown in Fig.S1B. CP and Dox concentrations were calculated by the following equations: $C_{(CP,\ M)} = A_{280\ nm} - 0.918 \times A_{494\ nm} / 5690$, where 5690 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the CP; $C_{(Dox,\ M)} = A_{494\ nm} / 8030$, where 8030 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the Dox. The conjugation ratio of CP-hyd-Dox was ~3.2 Dox molecules per CP.

2.3.2. CP-ami-Dox

The pH-insensitive CP-ami-Dox conjugate (ami: amide linker) (Fig.S2A) was synthesized by conjugating Dox-SMCC to the cysteine residues in the CP to form a thioether bond. 24 μmol Dox-SMCC was reacted with 1 μmol TCEP-reduced CP, which corresponds to 8 μmol cysteine residues, in 75% methanol and 25% PBS by continuously stirring for 16 h at room temperature. Purification and purity evaluation of the CP-ami-Dox conjugate was carried out identically to the CP-hyd-Dox conjugate. The HPLC profile of purified CP-ami-Dox is shown in Fig.S2B. The conjugation ratio of CP-ami-Dox was ~3.2 Dox molecules per CP.

2.3.3. CP-BHQ2

The pH-insensitive CP-BHQ2 conjugate (Fig.S3A) was synthesized by conjugating BHQ2 to the cysteine residues in the CP using a sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) crosslinker. The NHS ester in sulfo-SMCC was reacted with the