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Cellular delivery of doxorubicin mediated by disulfide reduction of a peptide-dendrimer bioconjugate

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ABSTRACT

In this study, we developed a peptide-dendrimer-drug conjugate system for the pH-triggered direct cytosolic delivery of the cancer chemotherapeutic doxorubicin (DOX) using the pH Low Insertion Peptide (pHLIP). We synthesized a pHLIP-dendrimer-DOX conjugate in which a single copy of pHLIP displayed a generation three dendrimer bearing multiple copies of DOX *via* disulfide linkages. Biophysical analysis showed that both the dendrimer and a single DOX conjugate inserted into membrane bilayers in a pH-dependent manner. Time-resolved confocal microscopy indicate the single DOX conjugate may undergo a faster rate of membrane translocation, due to greater nuclear localization of DOX at 24 h and 48 h post delivery. At 72 h, however, the levels of DOX nuclear accumulation for both constructs were identical. Cytotoxicity assays revealed that both constructs mediated ~80% inhibition of cellular proliferation at 10 μM, the dendrimer complex exhibited a 17% greater cytotoxic effect at lower concentrations and greater than three-fold improvement in IC_{50} over free DOX. Our findings show proof of concept that the dendrimeric display of DOX on the pHLIP carrier (1) facilitates the pH-dependent and temporally-controlled release of DOX to the cytosol, (2) eliminates the endosomal sequestration of the drug cargo, and (3) augments DOX cytotoxicity relative to the free drug.

1. Introduction

The targeted delivery of drugs to diseased tissues is fundamental to the development of effective therapies, especially tumor-specific anticancer systems. The primary challenges here have been the targeting of the therapeutic to desired sites combined with its efficient delivery across the plasma membrane to the cellular cytosol; many therapeutics are not sufficiently lipophilic to cross the membrane bilayer (Dinca et al., 2016; Lundberg and Langel, 2003; Sun et al., 2004; Tsutsumi and Neckers, 2007; Wang et al., 2000; Wijesinghe et al., 2011). To address this, various drug-carrying vehicles capable of overcoming the plasma membrane barrier while increasing the effective intracellular concentration of the therapeutic have been developed. These drug carriers include various nanoparticles (NPs), liposomal structures, and peptides (Din et al., 2017; Zaidi et al., 2017). While many of these systems have shown the ability to (1) facilitate large drug loading capacity, (2) protect against drug degradation, (3) enable controlled drug release, and (4) improve drug pharmacokinetics, the specific cellular targeting of these drug carriers and their subsequent sequestration within the endolysosomal pathway remain critical roadblocks.

Peptide-mediated drug delivery represents one promising avenue for the targeted cellular delivery of therapeutic drugs. Compared to NP-based drug vehicles, which are typically on the order of 50–100 nm, peptides are considerably smaller (~3–5 nm) and can circulate and penetrate tissue efficiently (Acar et al., 2017). Most of the cell delivery peptides described in the literature fall under the general classification of "cell penetrating peptide" (CPP). The canonical CPP, derived from the HIV-1 transactivator of transcription (TAT), is a positively-charged (arginine- and lysine-rich) peptide that mediates the cellular uptake of attached cargos through initial electrostatic interactions with the negatively-charged cell surface followed by uptake into the endocytic pathway (Richard et al., 2005; Vives, 2003). Indeed, for the majority of CPPs described to date, the general consensus is that endocytosis is the primary means of internalization of the CPP, which necessitates strategies for the subsequent endosomal escape of the peptide-appended drug cargo (Kauffman et al., 2015). This can lead to poor bioavailability and overall decreased effective concentrations of the delivered drug (Deshayes et al., 2004; Dinca et al., 2016; Duchardt et al., 2007; Guidotti et al., 2017; Henriques et al., 2005; Richard et al., 2003). Thus, nonspecificity and endosomal sequestration remain two critical challenges in the implementation of CPPs as vehicles for therapeutic drug delivery.

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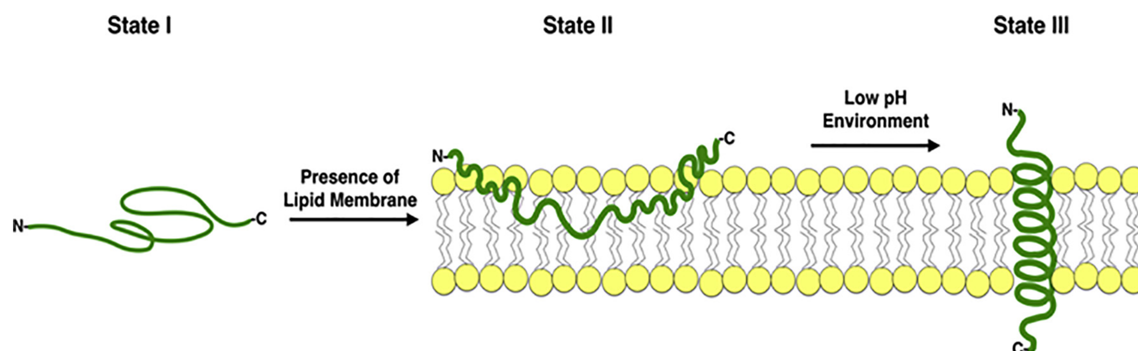

Fig. 1. pHLIP exists in three different states depending on its environment. At physiological pH in aqueous environments, pHLIP is an unstructured, soluble peptide (State I). In the presence of a lipid bilayer, pHLIP remains unstructured but associates with the membrane (State II). In an acidic environment pHLIP adopts an alpha helical structure and inserts across the lipid bilayer with the C-terminus in the cytosol (State III).

The pH (Low) Insertion Peptide (pHLIP), a peptide that exhibits specific targeting/insertion into cancer cells mediated by the acidic microenvironment of many tumor tissues, is a promising alternative peptidyl motif for the delivery of drugs directly to the cellular cytosol. The low pH environment associated with cancer cells arises from the "Warburg effect", as cancer cells rapidly undergo aerobic glycolysis, resulting in the acidification of the immediate extracellular environment (Gatenby and Gillies, 2004; Gogvadze et al., 2008; Kim and Dang, 2006; Ristow, 2006; Semenza et al., 2001; Seyfried and Mukherjee, 2005; Seyfried and Shelton, 2010; Warburg, 1956). The pHLIP peptide exhibits three distinct states depending on its local environment (Fig. 1). At physiological pH in aqueous (extracellular) environments, pHLIP is an unstructured, soluble peptide (State I). In the presence of a lipid bilayer (*e.g.*, the cell's plasma membrane), pHLIP remains unstructured but becomes preferentially associated with the membrane (State II) due to the thermodynamically favorable burying of hydrophobic residues into the lipid bilayer (Reshetnyak et al., 2008). Upon lowering of extracellular pH, the membrane-associated pHLIP adopts an alpha helical structure and inserts across the lipid bilayer (State III) with the peptide's C-terminus presented to the cytosol (Hunt et al., 1997; Reshetnyak et al., 2006). pHLIP has been shown to translocate a myriad of cargos across the plasma membrane, including fluorescent molecules, peptide nucleic acids, and antimicrobial peptides (An et al., 2010; Burns et al., 2016; Burns and Thevenin, 2015; Cheng et al., 2015; Moshnikova et al., 2013; Onyango et al., 2015; Reshetnyak et al., 2006; Wijesinghe et al., 2011), pHLIP has also been shown to translocate clinically relevant chemotherapeutics, including doxorubicin (DOX) and a variety of NPs in a concentration- and pH-dependent manner (Antosh et al., 2015; Burns et al., 2017; Burns et al., 2015; Davies et al., 2012; Han et al., 2013; Kyrychenko, 2015; Song et al., 2016; Tian et al., 2017; Wei et al., 2017; Wijesinghe et al., 2013; Yao et al., 2013a; Yao et al., 2013b; Yu et al., 2016; Zeiderman et al., 2016; Zhao et al., 2013).

These attributes of pHLIP served as motivation for us to develop a pHLIP-based NP bioconjugate system for multivalent drug display coupled with pH-dependent control over drug release to the cytosol. To date, studies demonstrating the use of pHLIP-NP conjugate systems for the multivalent display of drugs combined with improved drug efficacy remain scant. In a study aimed solely at imaging, Janic et al. reported the use of pHLIP for the translocation a generation five polyamidoamine (PAMAM) dendrimer decorated with ~44 Gd-DOTA-4AmP contrast agent chelates and showed pH-dependent delivery to breast cancer and glioblastoma cells using magnetic resonance imaging (Janic et al., 2016). More recently, Zhao et al. reported the synthesis of DOX-loaded mesoporous silica NPs (MSNs) conjugated to multiple copies of pHLIP *via* disulfide linkages (Zhao et al., 2013). In that study, the ensemble pHLIP-MSN conjugates were rather large (140 nm diameter) and were bulk loaded with DOX in the MSN core. Thus, they displayed minimal temporal control of DOX release as evidenced by the significant inhibition of cellular proliferation in cells exposed to the conjugates at neutral pH (non-triggered state). Further, the conjugates did not mediate any improvement in the IC_{50} of DOX compared to the free drug.

To address these shortcomings, here we present a pHLIP-dendrimer-drug conjugate system for the polyvalent display and cytosolic/nuclear delivery of DOX. We chose DOX as the model drug cargo as it is a prominent anticancer drug that exerts it cytotoxic effect by intercalating between DNA base pairs and inhibiting topoisomerase II, resulting in DNA damage and the induction of apoptosis (Ai et al., 2011; Song et al., 2016; Soudy et al., 2013). The clinical application of DOX, however, is hampered by its off-target cardiotoxicity, mediated by the generation of reactive oxygen species (Danz et al., 2009). Thus, there is significant interest in improved strategies for the specific cellular delivery of DOX and the controlled augmentation of its toxicity (Maksimenko et al., 2014; Tacar et al., 2013). To this end, we synthesized a conjugate system comprised of a single copy of pHLIP covalently attached to a generation three PAMAM dendrimer (~3.5 nm diameter) that displayed four copies of DOX per dendrimer. The DOX moieties were attached to the dendrimer surface *via* disulfide linkages such that insertion of the dendrimer into the cytosol resulted in release of multiple DOX molecules per pHLIP. As a control, we synthesized pHLIP-DOX conjugates that displayed a single copy of DOX attached through either a disulfide linkage or a non-cleavable thioether bond.

Biophysical characterization showed that all three conjugates exhibited the characteristic membrane insertion and translocation behavior of pHLIP at low pH. While both disulfide-containing conjugates efficiently delivered DOX to the nucleus of HeLa cells, we noted distinct differences between the two conjugate systems in their rate of nuclear accumulation of DOX and eventual cytotoxicity. The pHLIP construct displaying a single disulfide-linked DOX exhibited slightly faster DOX nuclear localization kinetics compared to the multiple DOX-displaying dendrimer form. Based on time-resolved confocal fluorescence microscopy, we attributed this to the slower membrane translocation and cytosolic release of DOX in the pHLIP-PAMAM-DOX conjugate system. Still, both constructs facilitated significant cytotoxicity (~80% inhibition of cellular proliferation) at high peptide concentration (10 μM). At lower peptide concentrations (< 0.63 μM), however, the polyvalent pHLIP-PAMAM-DOX conjugate exhibited as much as a ~17% greater anti-proliferative effect compared to the single DOX displaying conjugate and a greater than three-fold improvement in IC_{50} over free DOX. We attribute this to the slower membrane translocation of the dendrimer coupled with the sustained intracellular release of multiple DOX from the dendrimer surface. Cumulatively, our results demonstrate the utility of the pHLIP-dendrimer system for the facile and robust multivalent display and pH-dependent cellular delivery of drug cargos that is independent of the endocytic pathway. Further, our findings point to the exciting possibility of the use of this conjugate system for the simultaneous cellular delivery of multiple disparate drug and imaging agent cargos.

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