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Molecular binding of self-assembling peptide EAK16-II with anticancer agent EPT and its implication in cancer cell inhibition

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ABSTRACT

The current drug delivery techniques involve encapsulation, targeting and controlled release of the drug with various molecules or nanoparticles, but rarely has the drug molecular state or form been investigated. It is necessary to deliver a drug with a prescribed molecular state in order to maximize drug therapeutic effects. Here we present two facile methods to characterize molecular states of the anticancer drug ellipticine (EPT) encapsulated in the self-assembling peptide EAK, and relate the different molecular states of EPT to their respective cancer inhibition efficacies. The first method is UV-based, where drug loading capacity of a particular molecular state was determined. The experimental data corroborated a molecular binding model, where peptide–drug interaction was assumed to be electrostatic in nature. The developed model could elucidate a unique pH effect on protonated EPT loading capacity. The second method is based on fluorescence characteristics of EPT, which could differentiate the two molecular states: protonated and crystalline of EPT *in situ*. The inner filter effect was, however, found with this method, presenting an ineluctable obstacle in quantitative analysis of fluorescence data. A correction method for the inner filter effect was thus developed. With this approach, concentrations of EPT at different molecular states in its peptide complex solutions were determined. *In vitro* cytotoxicity assay was applied to evaluate the efficacy of the two molecular states of EPT, showing that protonated EPT was more efficient at killing cancer cells than crystalline EPT. The molecular binding model and two characterization methods for EAK–EPT complexation could be extended to other carrier-drug systems.

1. Introduction

Self-assembling peptides are emerging as promising nano-biomaterials in the field of bio-nanotechnology because of their favorable properties, such as biocompatibility, less-immunogenicity and the ability to be biodegraded to non-toxic amino acids. Combined with flexible design approaches and advancements in synthetic tools, peptides are becoming an attractive class of building blocks of nano-biomaterials with various engineering and biomedical applications, such as tissue scaffolding, regenerative medicine and drug delivery [1–4]. Specifically, the unique properties of many peptides for cell targeting and penetration [5–7] make them promising biomaterials for drug delivery. As a result, many novel peptide-mediated drug delivery systems have been developed, including cancer targeting delivery [5,8,9] and cell penetrating delivery for gene/siRNA therapeutic agents [10–12].

Among the self-assembling peptides, ionic complementary peptides are a special class, discovered in the early 1990s from a yeast Z-DNA binding protein [13]. These peptides feature a unique amphiphilic structure consisting of alternating hydrophobic and hydrophilic amino acids in the sequence. The positive and negative charges are alternatively arranged, resulting in a complementary ionic structure. These peptides are reportedly able to self-assemble into unusually stable nanostructures, which can withstand extreme pH values, high temperatures, digestive enzymes and denaturation agents [13,14]. EAK is a typical self-assembling, ionic complementary peptide, composed of 16 amino acids with alternating pairs of negative (E, glutamic acid) and positive (K, lysine) charges, separated by a hydrophobic amino acid (A, alanine). It self-assembles into β-sheet rich nanostructures with separated hydrophobic and hydrophilic faces [13,15,16]. The hydrophilic face is usually exposed to aqueous solution while the hydrophobic face forms a hydrophobic interior. This spontaneously organized nanostructure may provide a protected and stable environment for a hydrophobic cargo. EAK exhibits good biocompatibility with cultured mammalian cells [15] and no detectable immune responses were reported when injected into animals [13]. These properties make EAK a very promising biomaterial to construct nanocarriers for hydrophobic drug delivery.

In our previous studies, EAK was shown to suspend pyrene microcrystals in aqueous solution. The controlled release of encapsulated

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pyrene from the peptide-pyrene complex into egg phosphatidylcholine can be achieved by changing the peptide-to-pyrene ratio during the encapsulation [17]. In a more recent work, this peptide was reported to be able to stabilize the hydrophobic anticancer drug ellipticine (EPT) in aqueous solution [18] at concentrations significantly higher than the drug's solubility in water (~0.62 μM at neutral pH) [19]. With different mass ratios of EAK to EPT, two different molecular states of EPT (protonated and crystalline) were observed. The effect of different molecular states on the release kinetics of EPT from the peptide–EPT complex to EPC vesicles was also investigated, which showed that protonated EPT had a faster transfer rate than that of crystalline EPT. *In vitro* studies have also shown that the EAK–EPT complex might exhibit high anticancer activity [20].

The current drug delivery techniques involve encapsulation, targeting and controlled release of the drug with various molecules and nanoparticles, but rarely has the drug molecular state or form been investigated. Different molecular states would most likely possess different drug efficacies in therapy. It is necessary to deliver a drug with a prescribed molecular state in order to maximize drug therapeutic effects. Although we had studied extensively EAK self-assemblies and EAK–EPT complexes, the therapeutic efficacies of the two states of EPT still have not been reported. One of the main difficulties is related to the determination of the molecular states of encapsulated drugs, which is intrinsically related to the molecular interaction/binding between carrier and drug molecules, i.e., EAK and EPT in the present case.

In this work, we proposed a molecular binding model with electrostatic interaction as the primary intermolecular force between peptide and protonated EPT. This model could elucidate pH effect on the binding of protonated EPT and predict its loading capacity when complexed with EAK. Two characterization methods were developed for quantifying the amount of each of the two molecular states of the drug. A UV-based quantitative analysis was developed and the results were used to assess the validity of the molecular binding model. The second method is another commonly accessible physicochemical tool—fluorescence spectrometry. The fluorescence technique is non-invasive and has been a primary tool to characterize different forms of molecules, especially in their complexes. However when using it for relatively high fluorophore concentrations, interference can occur because of the so called inner filter effect. We demonstrated the existence of the inner filter effect in fluorescence characterization of the EAK–EPT complexation. Considering that dilution, which can avoid the inner filter effect, would likely change the composition of complexes and hence the molecular state of the drug, the inner filter effect became the main obstacle on quantitative analysis by fluorescence measurement. We therefore developed a correction method for the inner filter effect, with which we were able to quantify the concentrations of both protonated and crystalline EPT. In order to relate the different molecular states of EPT to their respective cancer inhibition efficacies, *in vitro* study was performed with peptide–EPT complexes containing different molecular forms of EPT against two cancer cell lines, A549 and MCF-7.

2. Materials and methods

2.1. Materials

EAK16-II (Mw = 1657 g/mol, crude, 84% purity) was obtained from CanPeptide Inc. (Pointe-Claire, Quebec, Canada). The N-terminus and C-terminus of the peptide were protected by acetyl and amino groups, respectively. The anticancer agent EPT (99.8% pure) was purchased from Sigma-Aldrich (Oakville, ON, Canada) and used as received. Tetrahydrofuran (THF, reagent grade 99%) and dimethyl sulfoxide (DMSO, spectral grade 99%) were from Calendon Laboratories Ltd. (Georgetown, ON, Canada) and Sigma-Aldrich (Oakville, ON, Canada), respectively. Cell culture reagents including Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and trypsin–ETDA were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Phosphate buffer saline (PBS) and penicillin streptomycin (p/s, 10,000 U) were obtained from MP Biomedicals Inc. (Solon, OH, USA).

2.2. Sample preparation

Appropriate amounts of the peptide powder were first dissolved in pure water (18 MΩ; Millipore Milli-Q system) to obtain fresh peptide solutions at concentrations of 3, 2, 1, 0.5, 0.2 and 0.1 mg/ml ("crude" peptide concentration as weighted). The solution was then immersed in a bath sonicator (Branson, model 2510) for 10 min. The EAK–EPT complexes were prepared by adding the freshly prepared peptide solution into a glass vial containing a thin film of EPT at the bottom, followed by mechanical stirring at 900 rpm for 24 h; the EAK–EPT complexes made by 0.1 mg/ml EAK solution in the following parts were sonicated before stirring, in order to suspend all the EPT and keep the total concentration of EPT to be 0.1 mg/ml. To make a thin film of EPT at the bottom of the vials, calculated volumes of EPT stock solution in THF were transferred to the vials, and dried in a gentle stream of filtered air (0.22 mm pore size filter) for ~10 min. Pure water, instead of peptide solution, was also added to another vial to make a control sample. The purpose of using an EPT concentration of 0.1 mg/ml in *in vitro* study was to obtain distinguishable cellular toxicity of the complexes and the control sample. All the vials and solvents were sterilized, and the samples were prepared in a biological safety cabinet to avoid possible contamination. For dynamic light scattering (DLS) measurements, the solvents were filtered, and all samples were prepared in the biosafety cabinet to eliminate potential dust contamination. The complexes were characterized with several techniques, see later, to determine complex dimensions and the molecular states of the EPT in the complexes.

2.3. EPT loading capacity measurement

The amount of EPT stabilized in EAK solution was determined by EPT UV-absorption. A calibration curve was obtained from the linear fitting of EPT absorption at a wavelength of 294 nm as a function of EPT concentration (1×10^{-4}–5×10^{-4} mg/ml) prepared in a mixture of 95% DMSO and 5% water. The oversaturated EAK–EPT suspension was centrifuged at 4000 rpm for 20 min and the supernatant, which only contained protonated EPT (which could be verified by fluorescence, see below), was diluted in DMSO until within the range of the calibration curve. 80 μl of the solution was then transferred to a quartz microcell with a 1 cm light path and tested on a UV–vis spectrophotometer (Biochrom Ultraspec 4300 Pro, Cambridge, England). The EPT loading capacity was determined by the EPT concentration on the calibration curve taking into account the dilution factor. The value of EPT loading capacity was averaged from 3 measurements.

2.4. Atomic Force Microscopy (AFM)

The EAK self-assembled nanostructures and EAK–EPT complexes were imaged on a PicoScan™ AFM (Molecular Imaging, Phoenix, AZ). The samples were prepared with the following procedure: 10 μl of 0.5 mg/ml EAK-16II solution or 0.5 mg/ml:0.1 mg/ml EAK–EPT complex solution was put on a freshly cleaved mica surface. The sample was then incubated for 10 min under ambient condition before being washed with approximately 100 ml of pure water (18 MΩ; Millipore Milli-Q system) to remove unattached samples. After air drying for 3 h, AFM imaging was performed at room temperature using tapping mode. All images were acquired using a 225 mm silicon single-crystal cantilever (type NCL, Molecular Imaging, Phoenix, AZ,