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Research paper

Arginine-tagging of polymeric nanoparticles via histidine to improve cellular uptake

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ABSTRACT

Polyarginine, a cell-penetrating peptide, has been shown to aid cellular penetration of bioactives into cells. We utilized a novel approach of using a histidine linker to produce poly(ethyl-cyanoacrylate) (PECA) nanoparticles tagged with oligoarginine and investigated cellular uptake. MALDI TOF/TOF (tandem) analysis revealed that di-arginine-histidine (RRH) covalently bound to PECA nanoparticles to form cationic particles (+18 mV), while longer oligoarginine peptides did not co-polymerize with PECA nanoparticles. Although RRH-tagged nanoparticles had similar size and FITC-dextran entrapment efficiency compared to unmodified nanoparticles, RRH-tagging of nanoparticles resulted in a greater release of FITC-dextran. As the nanoparticles were found to aggregate in Hanks Balanced Salt Solution (HBSS), the effect of phosphate on the zeta-potential of nanoparticles was studied. Treating the nanoparticles with poloxamer-407 prevented aggregation. RRH-tagged PECA nanoparticles increased cellular uptake by a further 30% compared to unmodified PECA nanoparticles and was concentration dependent. We suggest that enhanced cell uptake can be achieved using a di-arginine-histidine construct as opposed to the previously published findings that a minimum of hexa-arginine is necessary. Further, the cationic zeta-potential of the cell-penetrating peptide may not be needed to enhance uptake.

1. Introduction

Cell-penetrating peptides (CPPs) are short cationic peptides of 10–30 amino acids that consist of mainly basic residues, such as arginine and lysine, and can be either derived from natural sources or produced synthetically [1,2]. These peptides are able to improve cellular uptake [3] and enhance permeation [4] with minimal toxicity [5], thus making them potentially valuable tools for drug delivery. CPPs have been investigated for the delivery of therapeutic proteins [6], nucleic acids [7], and small molecules [8]. An example of a widely studied CPP is oligoarginine, which has been documented to improve insulin absorption across the gut in rats [9]. Although the greatest improvement in cell uptake has been shown with 15 arginine residues, octa-arginine (R8) is more extensively studied due to the cost-effectiveness in oligoarginine synthesis [10].

CPPs have to be associated with bioactive molecules, either covalently or non-covalently, to enhance cell penetration [11]. Current covalent conjugation methods involve fusion proteins produced synthetically in bacteria or attachment to the bioactive through covalent side-chain linkages, such as a peptide bond or a disulfide bridge. Non-covalent linkages include simple electrostatic interaction and "piggy-back" attachment, where the CPP is attached covalently to a smaller molecule that is linked non-covalently to the bioactive [11]. The major limitation of direct covalent linkage is alteration of the activity and/or efficacy of the bioactive [3], whereas non-covalent linking requires a substantially higher amount of CPP for effective electrostatic association with the bioactive [9].

An alternative strategy to overcome the disadvantages of both covalent and non-covalent attachment in linking CPPs to a bioactive molecule is to covalently associate CPPs to nanoparticles containing the bioactive, thus leaving the encapsulated bioactive molecule in its native form. Also, colloidal carriers, such as polymeric nanoparticles, could increase the oral bioavailability of the bioactive by protecting the encapsulated bioactive from the hostile gastrointestinal environment [12]. Polymeric nanoparticles, such as poly(alkyl-cyanoacrylate) (PACA) nanoparticles, prepared using water-in-oil microemulsions can offer additional advantages. These polymeric nanoparticles do not require purification and

Abbreviations: PECA, poly(ethyl-cyanoacrylate); ECA, ethyl-2-cyanoacrylate; FACS, flourescence-associated cell sorting; R, arginine; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; FITC, fluorescein isothiocyanate; HEPES, 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid; HBSS, Hanks Balanced Salt Solution; TFA, trifluoroacetic acid.

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can be administered orally in the microemulsion template as the template is biodegradable, biocompatible [13] and exhibits permeation enhancing effects [14].

CPPs have been conjugated to polymeric carriers through epoxy conjugation [15] and cross-linking with dextran [16–18]. However, both techniques are time-consuming and require multiple steps. Therefore, a simple and fast conjugation method is desirable. Kafka et al. [19,20] discovered that the histidine residue in the gonadotropin-releasing hormone analogue D-Lys^6-GnRH covalently conjugates to poly(ethyl-cyanoacrylate) (PECA) nanoparticles both during and after nanoparticle formation. These authors suggested that the unprotonated histidine side chain (pK_a 6.04) in D-Lys^6-GnRH acts as a nucleophile [20], instead of hydroxyl ions, to initiate the anionic interfacial polymerization of ethyl-cyanoacrylate (ECA) subunits [21]. However, the usefulness of histidine as a linker for deliberate covalent attachment of functional peptides such as oligoarginine to PECA nanoparticles in a single step polymerization has yet to be evaluated.

The aim of this study was to prepare arginine-tagged (R-tagged) PECA nanoparticles via histidine anchoring, to characterize these particles and to evaluate the cellular uptake of PECA nanoparticles with arginine-tagging.

2. Experimental sections

2.1. Reagents

Arginine-histidine (RH), di-arginine-histidine (RRH), tetra-arginine-aminocaproic acid-histidine (R4-aca-H), octa-arginine-histidine (R8H), octa-arginine-di-histidine (R8H2) and octa-arginine (R8) (all ⩾95% purity) from GLS (Shanghai, China) were used as supplied. For preparation of the microemulsion, ethyloleate (GPR™) was supplied by BDH Laboratory Supplies (Poole, England), while the surfactants sorbitan monolaurate (Crill 1) and ethoxy 20 sorbitan mono-oleate (Crillet 4) were kindly provided by BTB chemicals (Auckland, New Zealand). The monomer ethyl-2-cyanoacrylate (ECA), fluorescein isothiocyanate (FITC)-dextran (MW = 70 kDa, 500 kDa and 2000 kDa), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (⩾99.5% purity), sodium bicarbonate (⩾99.5% purity), Hanks Balanced Salt Solution (HBSS; without sodium bicarbonate and phenol) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Poloxamer-407 (Lutrol-F127®) was purchased from BASF (Ludwigshafen, Germany). Methanol (HPLC grade), acetonitrile (HPLC grade), chloroform were supplied by BDH Laboratory Supplies (Poole, England) and hydrochloric acid (fuming 37%, for analysis) and D(+)-glucose were sourced from Merck (Darmstadt, Germany). Sodium chloride (NaCl), di-sodium-hydrogen phosphate (Na_2HPO_4) and sodium-di-hydrogen phosphate (NaH_2PO_4) were purchased from Univar, Asia Pacific Specialty Chemicals Limited (Sydney, Australia) and absolute ethanol was supplied by Anchor Ethanol (Auckland, New Zealand). Distilled, ultra-pure water was produced using a Milli-Q® water Millipore Purification System™ (Billerica, USA). BD Pharmingen™ Propidium iodide 50 μg/mL was purchased from BD Biosciences (California, USA).

2.2. Preparation of PECA nanoparticles from a microemulsion template

The microemulsion was prepared based on the method described by Watnasirichaikul et al. [22]. Briefly, a mixture of Crill 1 and Crillet 4 was prepared in a weight ratio of 4:6. Then the surfactant mix, ethyloleate oil and water were mixed together at a weight ratio of 5.4:3.6:1, respectively, to produce the microemulsion template [20]. ECA monomer (200 μL ECA dissolved in 600 μL of chloroform) was added drop-wise to 10 g microemulsion template under constant stirring at 700 rpm at 4 °C. The polymerization process was allowed to progress for a minimum of 4 h.

2.3. R-tagging and FITC-dextran encapsulation

To produce R-tagged nanoparticles, either RH (10 μmol), RRH (10 μmol), R4-aca-H (5.6 μmol), R8H (4 μmol), R8H2 (5.2 μmol) or R8 (4 μmol) was added to the aqueous phase of the microemulsion, prior to polymerization. To load the nanoparticles with FITC-dextran, 1 mg of FITC attached to dextran of either MW 70 kDa, 500 kDa and 2000 kDa was dissolved in the aqueous phase, prior to polymerization.

2.4. Isolation of nanoparticles from microemulsion

Either 0.1 or 0.2 g microemulsion containing the nanoparticles was gently mixed with 600 μL of dilute HCl (pH 2.5) and 600 μL of methanol 80% (v/v). Then the sample was centrifuged at 20,800*g* (Eppendorf Centrifuge 5417C) for 30 min to isolate the nanoparticles from the microemulsion. The pellet of nanoparticles was re-suspended via brief sonication in absolute ethanol to wash the isolated nanoparticles and then spun at 20,800*g* for 30 min. This washing procedure was repeated twice. Bulk isolation of 1 or 2 g microemulsion was performed by mixing with 6 mL of dilute HCl (pH 2.5) and 6 mL of methanol 80% (v/v). The sample was centrifuged at 40,100*g* at 25 °C for 35 min in Beckman Optima™ L-80 Ultracentrifuge. The pellet of nanoparticles was re-suspended via brief sonication in absolute ethanol to wash the isolated nanoparticles and then spun at 40,100*g* for 30 min. This cleaning procedure was repeated twice.

2.5. Characterization of nanoparticles

2.5.1. Screening for covalent association using MALDI-TOF mass spectrometry

Detailed sample preparation and operation protocols used were as described by Kafka et al. [20]. Briefly, the nanoparticles were isolated from 0.1 g microemulsion and dissolved with sonication in 20–30 μL acetonitrile. An aliquot (0.5 μL) of the dissolved nanoparticle suspension was added to 9.5 μL matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in aqueous acetonitrile 60% (v/v) with TFA 0.1% (v/v)). An aliquot (0.8 μL) was then spotted onto the MALDI-plate (Opti-TOF 384 well plate, Applied Biosystems, Framingham, MA, USA) and air-dried prior to analysis. All mass spectrometry (MS) spectra were obtained in positive-ion reflector mode with 1000 laser pulses per sample spot. Relevant precursor ions were chosen for collision induced dissociation tandem mass spectrometry (CID-MS/MS). Laser pulses were then set at 2000–4000 per selected precursor using 2 kV mode and air as the collision gas at a pressure of 1×10^{-6} Torr. Nanoparticles with oligoarginine covalently bound to the PECA polymer were selected for further characterization.

2.5.2. Size and zeta-potential

Isolated nanoparticles from 0.1 g microemulsion were either re-suspended in absolute ethanol (containing 0.2% polysorbate 80) for size measurements, or 1 mM NaCl solution for zeta-potential measurements (Zetasizer ZEN3600, Malvern instruments Ltd., UK). The arginine-tagged nanoparticle formulation that bore the highest zeta-potential was selected for *in vitro* release studies.

2.5.3. Entrapment efficiency

Isolated nanoparticles from 0.1 g microemulsion were dissolved in 1 mL aqueous acetonitrile 80% (v/v) and the FITC-dextran released was determined using Hitachi F-7000 Spectrofluorometer (excitation 485 nm and emission 516 nm). Entrapment efficiency