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Probing label-free intracellular quantification of free peptide by MALDI-ToF mass spectrometry

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ABSTRACT

Cell-penetrating peptides are promising reagents for gene and drug delivery. They can efficiently traverse the plasma membrane and deliver various cargo materials ranging from genes to nanoparticles. The functional efficiency of cargo often depends on the completeness of intracellular peptide uptake, which can be measured, but its quantification remains largely inconclusive. Existing approaches rely on the use of radioactive and fluorescent labels or tags which allow colorimetric, fluorescent or spectrometric detection, but lack the ability to detect free peptide. Herein we describe a generic label- and tag-free method to measure the concentration of internalised peptide by matrix-assisted laser desorption/ionisation time of flight mass spectrometry. Quantification is preceded by two-dimensional chromatography and is performed at benign temperatures for the lysates of human dermal fibroblasts transfected with cell penetrating peptides in free form. Isotopically labelled peptides of the same structure are used as internal standards to enable accurate determination of concentration of the recovered free peptide. The method offers a minimalistic approach for intracellular quantification, which can be used as a correlative measure for fluorescence-based imaging methods.

1. Introduction

Molecular therapy offers imminent therapeutic control over major diseases including cardiovascular, neurodegenerative and genetic disorders and cancers [1]. Various genetic therapies are available. However, the lack of systemic use hampers commercialisation [2]. Current research and development trends are built upon the recognition of that more efficient and quantitative approaches for intracellular delivery are necessary, which also conforms to the critical need to harmonise legislation and suitable standards for gene therapy products [3]. Synthetic non-viral formulations that are being used to suppress or modulate genetic reactions appear to have overcome the problems of excretion and uptake by phagocytes, release from endosomes and entry into the nucleus [1–4]. The formulations are typically nanoparticulate systems based on metal colloids, polymersomesor liposomes [5]. However, many of these lack surface chemistry and are associated with levels of heterogeneity in size or composition which compromises their use as human drug carriers [6]. Priority is given to those systems that can encapsulate therapeutic cargo and can allow for surface modifications and tagging, which stimulates the development of macromolecular delivery vectors [5,6]. The majority of such vectors comprise several components, each having a specialised function including cell targeting, endosomal escape and nucleic acid complexation [7,8]. Single-component systems, which incorporate all these functions into one macromolecule, can provide a more efficient alternative [9].

Recently we have described a de novo cell penetrating peptide, which can translocate across human cell membranes and promote an active uptake of nucleic acids into the cytoplasm enabling the expression of a target protein [10]. The design, dubbed a gene transporter (GeT), adopts an amphipathic helical structure – separate clustering of hydrophobic and cationic amino-acid residues – upon binding to acidified membranes. This feature is common for membrane-active peptides including those with antimicrobial and cell-penetrating properties [11–13]. It is also characteristic for α-helical domains of capsid proteins of non-enveloped viruses that use such domains to lyse endosomal membranes thereby promoting the egress of the viral material into the cytoplasm [14]. It can be concluded therefore that de novo amphipathic domains can mimic natural approaches for gene delivery and can provide efficient single-component systems.

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However, as is the case for other systems, it is essential to quantitatively probe intracellular peptide uptake before considering drug development [15].

Current methods use fluorescent or radioactive reporter probes that are monitored by fluorescence microscopy and mass spectrometry [13,15,16]. These approaches appear to facilitate in the quantification of the uptake. However, controversies remain to be addressed regarding discordant results between different studies and the need for artificial labelling, which continue questioning the general applicability of these methods [17]. To progress with quantification any further necessitates the development of detection protocols that will enable direct measurements with a minimal use of specialist materials. A notable example is quantification by MALDI-ToF mass spectrometry [18]. This protocol makes use of an internal standard labelled with a stable isotope. The standard and the detected analyte are both functionalized with biotin which ensures peptide capture by streptavidin-coated magnetic beads and subsequent pre-concentration and purification of both peptides before mass spectrometry analysis. By quantifying the area of the $[M+H]^+$ signals of the analyte [^{1}H] and the internal standard [^{2}H] it was possible to measure internalised intact peptide at 0.5–50 pmol for 10^6 CHO-K1 cells incubated over an hour with 7.5 μM peptide, which corresponds to total intracellular concentrations of 0.35–35 μM (at a total intracellular volume of 1.5 μL) [15,18]. With typical variations of <20% the protocol has no upper limits of detection which can also be adjusted by the amount of the internal standard added. A critical stage in these measurements is the addition of the standard into cells before cell lysis which is performed at elevated temperatures. Heating helps prevent peptide degradation by proteases, which are released during cell lysis but become denatured upon heating. The following capture by streptavidin-coated magnetic beads should be equally efficient for the standard and analyte that are prone to the same type of side interactions, i.e. with nucleic acids [18]. However, elevated temperatures can cause aggregation that may hamper the capture and analysis of the internalised peptides, whereas the transfection efficiency of a tagged peptide may be different from that of its free form. These points are important to consider in the development of quantitative protocols to be sufficiently generic for intracellular drugs including those that are thermally labile or for which the use of a tag is not acceptable [19]. Unless the total peptide recovered from cell lysates can be estimated it remains difficult to benchmark tag-based methods. Surprisingly however, scarce information is available with regard to the intracellular quantification of free intact peptides from cell lysates.

Herein we describe a strategy which intends to fill this gap. Specifically, we probe the intracellular quantification of two cell-penetrating peptides, the engineered GeT and a transduction sequence of the naturally occurring HIV Trans-Activator of Transcription (Tat) protein. The aim of this study is to quantify free peptide concentrations recovered from cell lysates by mass spectrometry without the use of labels, tags or specialist reagents. A protocol designed to achieve this is schematically shown in Fig. 1.

2. Materials and methods

2.1. Peptide preparation

2.1.1. Synthesis and characterisation

All peptides were chemically assembled on a Rink amide-4-methylbenzhydrylamine resin using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid-phase protocols and HCTU/DIPEA as

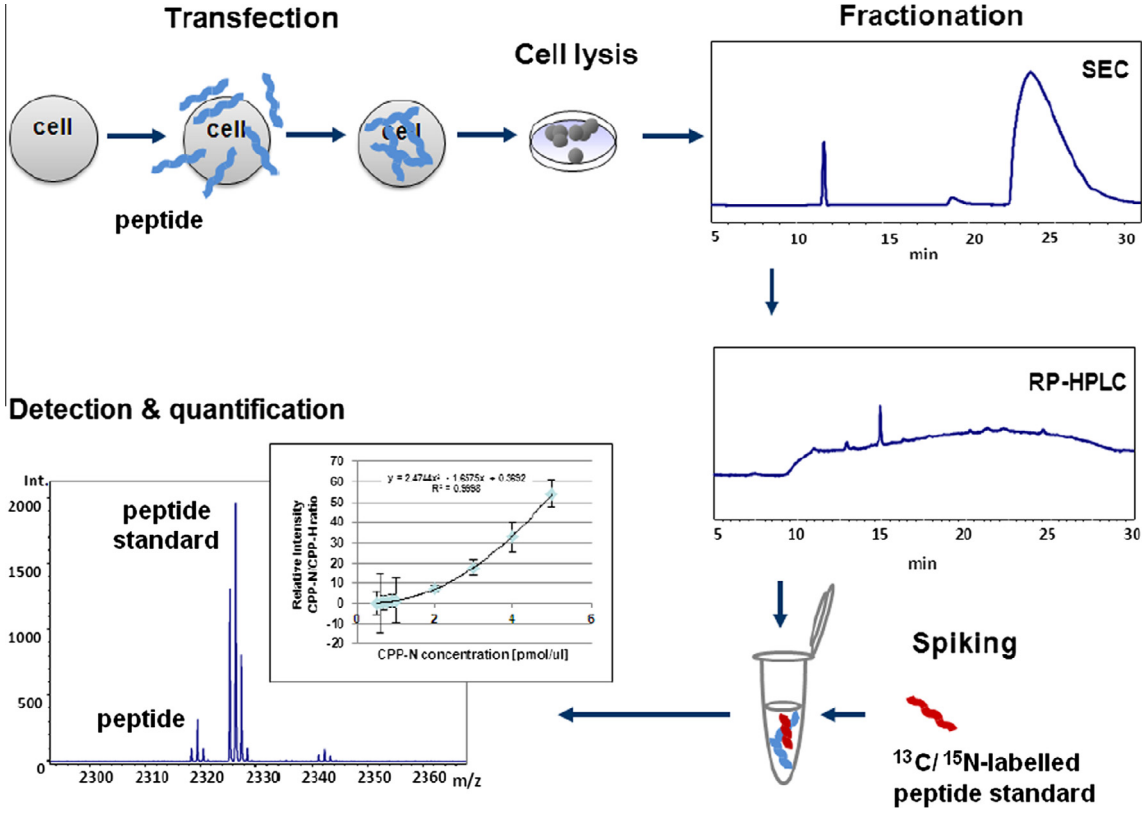

Fig. 1. Label-three approach for the quantification of intracellular peptide delivery. The method is carried out in three main stages. Adherent cells are peptide transfected, washed, trypsin treated and washed to remove surface-bound peptide. This is followed by cell lysis at benign temperatures and peptide is recovered from cell lysates first by size exclusion chromatography and then by reversed phase high performance liquid chromatography. The recovered peptide fractions are spiked with internal standards (corresponding peptide with a ^{13}C/^{15}N-labelled residue) which are then analysed by MALDI-ToF mass spectrometry and peptide concentrations are back-calculated from isotope dilution standard curves.