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Optimisation of vectorisation property: A comparative study for a secondary amphipathic peptide

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ABSTRACT

RNA interference provides a powerful technology for specific gene silencing. Therapeutic applications of small interfering RNA (siRNA) however require efficient vehicles for stable complexation and intracellular delivery. In order to enhance their cell delivery, short amphipathic peptides called cell-penetrating peptides (CPPs) have been intensively developed for the last two decades. In this context, the secondary amphipathic peptide CADY has shown to form stable siRNA complexes and to improve their cellular uptake independent of the endosomal pathway. In the present work, we have described the parameters influencing CADY nanoparticle formation (buffers, excipients, presence of serum, etc.), and have followed in details the CPP:siRNA self-assembly. Once optimal conditions were determined, we have compared the ability of seven different CADY analogues to form siRNA-loaded nanoparticles compared to CADY:siRNA. First of all, we were able to show by biophysical methods that structural polymorphism (α-helix) is an important prerequisite for stable nanoparticle formation independently of occurring sequence mutations. Luciferase assays revealed that siRNA complexed to CADY-K (shorter version) shows better knock-down efficiency on Neuro2a-Luc[+] and B16-F10-Luc^{+} cells compared to CADY:siRNA. Altogether, CADY-K is an ideal candidate for further application especially with regards to *ex vivo* or *in vivo* applications.

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1. Introduction

Since the first demonstration of the potent gene silencing effect through injection of double stranded RNA in 1998, short interfering RNAs (siRNAs) have been widely considered as powerful tools to specifically control protein activation and/or gene expression post-transcriptionally (Fire et al., 1998), and thus have important therapeutic potential in several diseases, including cancer (Castanotto and Rossi, 2009; Pecot et al., 2011). However, biological barriers remain the main obstacle for the delivery of macromolecular pharmaceuticals (de Fougerolles et al., 2007; Whitehead et al., 2009). Several technologies have been proposed to improve the cellular uptake of therapeutic molecules including lipids, polycationic polymers, nanoparticles and peptide-based formulations, but only a subset of them are efficiently applied in a clinical context (Heitz et al., 2009; Zahid et al., 2010). Cell penetrating peptides (CPPs) are one of the most promising non-viral strategies to overcome both extracellular and intracellular limitations of various large biomolecules and to improve their intracellular route (Dietz and Bähr, 2004; Heitz et al., 2009; Järver et al., 2010). CPPs are polybasic and/or amphipathic, usually short (up to 30 amino acids) peptides and can be either derived from naturally occurring protein transduction domains [e.g. TAT (48–60) (Vivès et al., 1997)] or rationally designed [e.g. TP10 (Pooga et al., 1998)].

Two strategies of CPP development have been described resulting in either a chemical link between the CPP and its cargo (covalent strategy), or in electrostatic and hydrophobic interactions between both moieties (non-covalent strategy) (Crombez et al., 2009; Hoyer and Neundorf, 2012; Järver et al., 2010). The

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non-covalent strategy has the advantage of avoiding laborious chemical conjugation between the CPP with its cargo and of protecting CPP:cargo complexes from degradation (Margus et al., 2012). The non-covalent complexes usually involve a one-step process consisting of a simple mixing of both partners, for example a CPP and an oligonucleotide (ON) (Heitz et al., 2009). In general, the formulation of peptide-based nanoparticles (PBN) reduces the ON concentration required to achieve a specific biological response (Deshayes et al., 2008; Lehto et al., 2010).

The notion of peptide:cargo complexes was first described in 1997 with the design of the MPG peptide-carrier for the non-covalent delivery of short nucleic acids and plasmids (Morris et al., 1999, 1997). Then in 2001, the primary amphipathic Pep-1 carrier was developed for non-covalent cellular delivery of proteins and peptides (Morris et al., 2001). More recently, the CADY peptide was especially designed for the cellular internalisation of small oligonucleotides and siRNA (Crombez et al., 2008). The sequence of CADY peptide was derived from ppTG1 (Rittner et al., 2002), a chimeric peptide carrier derived from the fusion peptide JTS1 (Gottschalk et al., 1996) in which some residues were mutated. The resulting distribution of hydrophilic, aromatic and hydrophobic residues enabled the formation of a secondary amphipathic helical structure (Konate et al., 2010; Rydström et al., 2011) when complexed with different partners. Though originally based on amphipathic peptides, the non-covalent approach has been extended to other classes of cell penetrating peptides such as polyarginine and peptidic analogues that are able to self-assemble with ONs to form stable CPP:ON complexes (Crombez et al., 2008) and several of them have been reported to improve ON delivery into mammalian cells (Crombez et al., 2008; Margus et al., 2012).

Nowadays, the effectiveness of CPPs is clearly established even if the mechanism of their entry is still widely debated. Cellular uptakes of cell penetrating peptides have long been thought to be independent of endocytosis, requiring no energy. However, Richard and colleagues highlighted some experimental artefacts in earlier studies and proposed a mechanism associated with the endosomal pathway (Richard et al., 2003). CPP mediated transport into the cell occurs via the endocytic pathway, which is the most common mode regardless of strategies used, covalent or not (Ezzat et al., 2011). But, this mode of entry leads to an endosomal entrapment which seriously reduces the bioavailability of the cargo (Hansen et al., 2009). In contrast, several amphipathic CPPs such as CADY or Penetratin can enter cells by different ways including a direct translocation process (Morris et al., 2008; Räägel et al., 2010). Generally, this mechanism implies strong interactions with phospholipids which induce reorganisation of the membrane bilayer allowing CPP uptake (Eiríksdóttir et al., 2010).

Secondary structure, structural polymorphism as well as the dynamics of CPPs play a major role in cellular uptake of CPP:cargo complexes especially by direct translocation (Deshayes et al., 2010, 2008; Konate et al., 2010). Binding to siRNA or phospholipids triggers a conformational transition of CADY from an unfolded state to an α-helical structure, thereby stabilizing the CADY:siRNA complex and improving its interaction with the cell membrane (Konate et al., 2010). Interactions of CADY with the cellular membrane are driven by its structural polymorphism which enables stabilisation of both electrostatic and hydrophobic contacts with surface membrane proteoglycan and phospholipids. Recently, direct translocation through the membrane and not the endosomal pathway was shown to be the major route of CADY: siRNA cellular uptake (Rydström et al., 2011). Indeed, CADY:siRNA complexes do not co-localise with most endosomal markers and remain fully active in the presence of endosomal pathway inhibitors. Furthermore, we have shown that CADY:siRNA complexes clearly induce a transient cell membrane permeabilisation, which is rapidly restored by cell membrane fluidity.

In recent years, electrostatic interactions of cell-penetrating peptides with nucleic acids have prevailed over covalent conjugates. However, it is crucial that the size of the resulting CPP: cargo nanoparticles are thoroughly adjusted. Ideally, the diameter should not exceed 100–300 nm for efficient uptake (Litzinger et al., 1994; Zhou et al., 2014). Care should also be taken with respect to fine-tuning of the stability of the complexes by varying the molar ratio. Dissociation of the nucleic acids from the carrier inside the cell could be hindered, if not prevented, when electrostatic interactions are too strong.

In the present work, we have combined several physicochemical technologies (circular dichroism, fluorescence spectroscopy, dynamic light scattering etc.) in order to characterise the optimal parameters of CADY:siRNA formulation. Then, to elucidate the role of amino acid composition of CADY's sequence, mutations were introduced to analyse their influences on siRNA association, PBN assembly and transfection efficiency. We demonstrate that in most of the cases structural polymorphism of the CPP plays an important role for the nanoparticle formation and that specific amino-acid modifications of the CADY sequence induce only in minor cases the loss of the siRNA self-assembling property. Altogether, a shorter version, CADY-K (without C-terminal cysteamide and alanine residues), could be considered as a novel CPP for efficient siRNA delivery *in cellulo* in different future applications.

2. Materials and methods

2.1. Materials

Dioleylphosphatidylglycerol (DOPG) and dioleylphosphatidylcholine (DOPC) phospholipids, and sphingomyelin (SM) were purchased from Avanti Polar Lipids and cholesterol (Chol) from Sigma–Aldrich. Peptides were purchased from PolyPeptide (France), LifeTein (US) or were synthesized in house (sequences in Table 1). Unlabelled siRNA and siRNA-Cy3B were obtained from Eurogentec (France) and from BioSynthesis (US), respectively. The different siRNA sequences are for anti-Luciferase: 5′-CUU-ACG-CUG-AGU-ACU-UCG-AdTdT (sense strand) and anti-GAPDH: 5′-CAU-CAU-CCC-UGC-CUC-UAC-UdTdT-3′ (sense strand) used as control. The stock solution of siRNA was prepared in RNase-free water.

DMF, Piperidine, TFA and acetonitril (HPLC grade) were purchased from SDS (France). Fmoc-amino acids were obtained from SENN Chemicals (Switzerland). HATU, DIEA were from Applied Biosystems (US), phenol, thioanisol, ethanedithiol and triisopropylsilane were from Sigma-Aldrich (Switzerland) ant the Expansin^{TM} resin was purchased from Société Expansia (France). Atto633 maleimide was obtained from ATTO-Tec GmbH (Germany).

Table 1
Designed peptides based on CADY's sequence.

Peptide	Nb a.a.	Sequences	Changes
CADY	20	GLWRALWRLLRSLWRLLWKA-Cya	–
CADY-K	19	GLWRALWRLLRSLWRLLWK	−[A + Cya]
s-CADY	15	G-WRALWRLWRSLWR−–A	short
CADY-H	20	GLW**H**ALW**H**LL**H**SLW**H**LLW**H**A	all H
PSW	20	GLWRALWRL**W**RSLWRLLWKA	L −> W
PSR	20	GLWRALWRLLRSLWRLW**R**KA	+R
PG09	21	GLWRALWR**A**L**W**RSLWRL**KR**K**V**	multiple
PG16	20	GLWRALWR**G**LRSLWRLLWK**V**	multiple

Nb a.a. = number of amino acids.

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