Review

Structural polymorphism of non-covalent peptide-based delivery systems: Highway to cellular uptake

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ABSTRACT

During the last two decades, delivery has become a major challenge for the development of new therapeutic molecules for the clinic. Although, several strategies either viral or non viral have been proposed to favor cellular uptake and targeting of therapeutics, only few of them have reach preclinical evaluation. Amongst them, cell-penetrating peptide (CPP) constitutes one of the most promising strategy and has applied for systemic in vivo delivery of a variety of therapeutic molecules. Two CPP-strategies have been described; using peptide carriers either covalently-linked to the cargo or forming non-covalent stable complexes with cargo. Peptide-based nanoparticle delivery system corresponds to small amphipathic peptides able to form stable nanoparticles with either proteins/peptides or nucleic acids and to enter the cell independently of the endosomal pathway. Three families of peptide-based nanoparticle systems; MPG, Pep and CADY have been successfully used for the delivery of various biologically active cargoes both ex vivo and in vivo in several animal models. This review will focus on the mechanism of the peptide-based nanoparticles; Pep, MPG and CADY in a structural and biophysical context. It will also highlight the major parameters associated to particle formation/stabilization and the impact of the carrier structural polymorphism in triggering cellular uptake.

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1. Introduction to cell-penetrating peptides

The recent deciphering of the human genome has provided new insights for the development of novel therapeutic approaches. Understanding genes and related proteins associated to their function and their mechanism of regulation has allowed the design of new potent molecules more specific for their targets. However, biological membranes constitute impermeable barriers for this new generation of bioactive molecules and although they exhibit potent activities, their cellular internalization still remains a major challenge. In order to overcome these limitations, several viral and non viral strategies have been developed to improve cellular delivery of therapeutics.

Carrier peptides or cell-penetrating peptides (CPPs) represent a promising approach opening new perspectives for drug delivery and have undergone a real increase of interest during the last decade [1]. Although CPPs’ definition is constantly evolving, they can be described as short peptides of less than 30 amino acids which are usually amphipathic, possess a positive net charge and are able to penetrate biological membrane and to transfer cargos into cells [2]. So far numerous CPPs have been reported to favor the delivery of a large panel of cargos from small particles to peptides, proteins and nucleic acids into a wide variety of cell types and in vivo models [3,4]. Since the first definition of CPP and the groundwork established by Langel et al. in 2002 [5], the field of CPPs has significantly evolved and major rules and parameters have been defined to classify a peptide as a CPP: including peptide-secondary structure, uptake mechanism and the cargo-related biological activity.

Although, the efficiency of CPPs is not questionable, the mechanism through which they allow the translocation of active cargo across plasma membrane is still a matter of controversy and it remains difficult to establish general rule for their uptake mechanism. The use of live cells together with the identification of endocytotic pathways by combining new inhibitors has allowed a better characterization of the CPP-initialization process and a re-evaluation of their cellular uptake mechanism and of their direct translocation ability [6]. Several cellular uptake mechanisms have been proposed for CPP: from direct translocation [7–10] to endocytosis [11–13] as well as the combination of several pathways [14]. Considering that several parameters have been implicated to control the cellular uptake pathway of CPP: their structure, ability to interact with cell membrane components, the nature of the cargo and the cell type, and mechanism should be deciphered on a case-by-case basis [6,15–17].

CPP-uptake process is more complicated than it was expected originally as peptide/lipid interactions have been shown not to be sufficient for the cellular uptake. The presence of negatively charged residues in the peptide sequence seems to be essential for translocation by interacting with cell surface proteoglycan, receptor proteins as well as with membrane potential. Therefore, a number of CPPs do not directly interact with phospholipids and present a cellular uptake mediated by endocytosis which is triggered by electrostatic interactions with cell surface. Other CPPs have been demonstrated to actually cross the cell membrane. In the latter case, evidence have been reported that the CPP/phospholipid interactions and the peptide-secondary structure play a major role in the cellular uptake mechanism and the orientation of cell entry pathway [9,18]. From a structural point of view, the importance of specific conformation of the peptide, in membrane insertion originally proposed such as amphipathic helical structure, seems now more associated to the concept of conformational versatility or structural polymorphism [18–22].

CPPs can be subdivided into two main classes, the first requires chemical linkage with the cargo [23–26] and the second involves the formation of stable, non-covalent complexes [26,29] both of them were successfully used at preclinical or clinical levels[1,3,30]. The non-covalent strategy is based on short amphipathic peptides that form stable nanoparticles with cargoes without requiring any cross-linking or chemical modifications [24,29,31]. This strategy has been shown to constitute a potent alternative for drug delivery and offers several advantages, including no requirement for chemical cleavage which favor a better release of the cargo inside the targeted cells, and facilitates modifications in order to increase specificity for the cargo and/or the target.

The first non-covalent CPP, MPG was proposed in 1997 [32] for the delivery of short nucleic acids, then the strategy was extended to protein and peptide non-covalent cellular delivery by the development of Pep-1 [33]. To date, three families of peptide-based-nanoparticle (PBN) delivery system: MPG, Pep and CADY have been developed [29]. MPG and Pep are primary amphipathic peptides, combining hydrophobic domain together with a hydrophobic amino acid rich motif [34]. CADY is a secondary amphipathic peptide which adopts an α-helical conformation in the presence of lipids or siRNA, thereby distributing the residues all along the helix that leads to form distinct aromatic, hydrophobic and hydrophilic domains [21–35]. Cellular uptake mechanism of PBN is controlled by their structural polymorphism, their ability to form stable nanoparticles with the cargoes and to interact with membrane components. PBN mainly enters the cell independently of the endosomal pathway and efficiently deliver cargos into a large variety of cell lines as well as in animal models [25,29]. Non-covalent strategies for protein and oligonucleotide delivery have been recently extended to other CPPs, including TAT [28–36], poly-arginine [37,38], Transportan- [39], Penetratin- [40] or TP10-derived peptides [41].

The present paper will be focused on the biophysical features of peptide-based nanoparticle delivery systems and will review the main common characteristics of MPG, Pep-1 and CADY carrier peptides from the structural features to their ability to form nanoparticles with different therapeutic cargoes. The impact of the structural polymorphism on the cellular mechanism of CPP will be discussed.

2. Peptide-based nanoparticle families

Three different families of PBN have been developed, based on either primary or secondary amphipathic peptides: MPG, Pep and CADY. MPG and Pep-1 are primary amphipathic peptides consisting of two distinct hydrophilic and hydrophobic domains (Table 1). The C-terminal hydrophilic domain of both carriers is a lysine rich motif which is derived from the nuclear localization sequence (NLS) of SV40 large T antigen (KKKKR). This part is generally required for the interactions with nucleic acids, intracellular trafficking of the cargo and to improve solubility of the carrier peptide. The N-terminal hydrophobic domain differs between MPG and Pep-1. The hydrophobic motif of MPG is derived from the fusion sequence of the HIV protein gp41 (GALFLGFLGAAGSTMGA) [32–35,42] and is required for efficient interactions with the cell membrane and cellular uptake. For Pep-1, the hydrophobic part involves a tryptophan-rich motif derived from the reverse transcriptase of HIV-1 (KETWWETWWTE), which is also involved for efficiently targeting cell membrane and for hydrophobic interactions with peptide or protein cargoes [33]. The hydrophilic and hydrophobic parts are associated via a short linker (WSQP) with a proline residue that keeps the flexibility and the integrity of both domains. CADY is a secondary amphipathic peptide (GLWRLAWRLLRSLWRLLWKA) derived from the PPTG1 peptide [43], a variant of the fusogenic peptide JTS1 [44]. CADY adopts a helical

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>aa</th>
<th>MW [Da]</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPG</td>
<td>Ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-Cya</td>
<td>27</td>
<td>2908.5</td>
<td>[22,23]</td>
</tr>
<tr>
<td>Pep-1</td>
<td>Ac-KETWWETWWTEWSQPKKKRKV-Cya</td>
<td>21</td>
<td>2907.4</td>
<td>[24]</td>
</tr>
<tr>
<td>CADY</td>
<td>Ac-GLWRLAWRLLRSLWRLLWKA-Cya</td>
<td>20</td>
<td>2653.0</td>
<td>[25]</td>
</tr>
</tbody>
</table>
conformation allowing a distribution of residues in three domains; an aromatic, a hydrophilic and a hydrophobic [35]. All peptide sequences are acetylated at their N-terminus and bear a cysteamide group at their C-terminus, both of which are essential for the stability of the peptides and their transduction mechanism [45]. The cysteamide function has been introduced into several peptide carriers as it offers the advantage of being compatible with Fmoc synthesis and avoids the use of cysteine, thereby protecting its side chain [32,42,46]. The presence of cysteamide group at the C-terminus of the peptide stabilizes both peptide/cargo and peptide/phospholipid interactions and favors the formation of homogenous nanoparticles throughout peptide disulfide bridges. Several variants of MPG and Pep-1 peptides have been proposed to either favor interaction with specific cargoes and/or to improve in vivo cargo delivery and selective targeting [35,47].

3. Carrier/cargo interactions and formation of peptide-based nanoparticles

MPG, Pep-1 and CADY were designed to transfer different cargoes (nucleic acids, plasmid DNA, short oligonucleotides, siRNA, proteins and peptides) into cells in a non-covalent manner. MPG strongly interacts with charged molecules whereas Pep-1 is more appropriate for protein/peptide and neutral DNA mimic molecules [27,48]. CADY is less selective for one type of cargo and forms stable particles with both charged and uncharged molecules [35,49]. These peptides exhibit high affinity in the nanomolar range and associate rapidly in solution with their respective cargoes through non-covalent electrostatic or hydrophobic interactions. They all form stable non-covalent nanocomplexes with their cargo that can be used both in cultured cells and in vivo [25]. Thus understanding the nature and the parameters which control the carrier/cargo interactions is essential to standardize formulations and generally required to combine several biochemical and biophysical technologies.

3.1. Formation of MPG/DNA complexes

MPG promotes the internalization of different types of nucleic acids from plasmid DNA to small oligonucleotide (antisense, siRNA) [32,41,45,50]. The formation of MPG/DNA nanocomplex involves mainly electrostatic interactions between phosphates of nucleic acids and charged residues of the peptide (arginine and lysine). It has been characterized by gel shift assays and by fluorescence spectroscopy, monitoring changes either in the tryptophan intrinsic MPG-fluorescence in the presence of nucleic acid or in fluorescently labeled-oligonucleotide upon binding of MPG [48]. As reported in Fig. 1A, titration of a fluorescently labeled-antisense oligonucleotide (18-mer) by increasing concentration of MPG results in an important quenching of fluorescence, with saturation occurring for a ratio of about 7 peptides per oligonucleotide which corresponds to a positive/negative charge ratio of 2 [48]. Determination of the equilibrium constants from both experiments indicates that MPG exhibits high affinity for oligonucleotides (Kd of about 6-5 nM). Similar results were obtained by gel shift assays, showing that over a peptide/oligonucleotide molar ratio of 7/1 and a peptide/plasmid DNA charge ratio of +2, no free form of the nucleic acid is detected and the amount of complex unable to enter the gel is significantly increased. In addition anisotropy measurements led to the same results and identical molar and charge ratio, as fluorescence anisotropy is directly correlated to changes in the size of the particles (Fig. 1A). All together, these data indicated a strong interaction between MPG and the oligonucleotide cargo to form non-covalent stable MPG/oligonucleotide complexes. Further characterization of these complexes by both light scattering measurement and scanning electron microscopy has revealed the presence of stable homogenous nanoparticles of 100 nm diameter [50].

3.2. Formation of CADY/siRNA complexes

CADY forms stable nanoparticles with siRNA, that mainly involved electrostatic interactions between Arg/Lys of CADY and phosphate group of the siRNA [21]. The ability of CADY to interact with siRNA was investigated by agarose gel shift assays and fluorescence spectroscopy. A siRNA (21-mer) targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been used as reference for both in vitro and in cellulo measurements. Results demonstrated that complex formation is initiated at a low molar ratio of CADY/siRNA (1/1), and that siRNA molecules are entirely associated with CADY at a molar ratio of 15/1. However, that higher molar ratio ≥40/1 is required to obtain a strong biological response in cultured cell suggests that CADY is able to form high-molecular weight complexes or aggregates, as previously described for MPG. The formation of non-covalent complexes was also studied by steady-state fluorescence spectroscopy and fluorescence anisotropy. The four Trp residues of CADY constitute excellent internal probes to monitor the interaction with siRNA. The binding of siRNA to CADY induced 42% quenching of fluorescence, together with a 7-nm blue shift of the emission spectrum from 345 to 338 nm. Binding saturation was achieved at molar ratio ranging between 5/1 and 10/1 and CADY dissociation constant for siRNA was estimated at 15 nM, revealing that several molecules of CADY interact with high affinity with one siRNA molecule [35]. CADY/siRNA interaction has also been followed using a FITC-labeled siRNA. Binding of CADY to siRNA induced 80% quenching of extrinsic fluorescence, with saturation achieved for a peptide/siRNA molar ratio of about 10/1 [21]. In addition, fluorescence polarization of the FITC-siRNA clearly increased with peptide concentration, revealing a direct correlation between changes in fluorescence and the formation of CADY/siRNA particles. Taken together, these data are consistent with the tight interaction between CADY and siRNA and the formation of a particle of peptides surrounding the siRNA [21].

3.3. Formation of Pep-1/protein complexes

Pep-1 carrier was mainly designed to improve translocation of proteins and peptides through the plasma membrane [33]. Pep-1 is able to form nanocomplexes with different cargos by a combination of hydrophobic and hydrophilic interactions. The ability of Pep-1 to interact with several cargoes has been investigated by fluorescence spectroscopy and size exclusion chromatography. The binding of a 32-mer peptide (P-A) cargo to Pep-1 induced a marked quenching of the intrinsic tryptophan fluorescence (36%), associated with a 11 nm blue shift of the fluorescence emission maximum (from 350 to 339 nm), suggesting that the Trp residues of Pep-1 interact directly with the cargo. Data analysis has revealed that more than one molecule of Pep-1 strongly interacts with P-A with a dissociation constant in the range of 120–150 nM. That the interaction between Pep-1 and P-A remains stable at high salt concentrations (300 mM NaCl), revealing that Pep-1 associated rapidly with cargo into non-covalent stable complexes mainly through hydrophobic interactions. Similar results were obtained with several distinct cargoes indicating that Pep-1 binding is not specific for a given peptide [33,51]. Finally analysis of the nanoparticles complexes obtained with Pep-1 by both light scattering and scanning electron microscopy has revealed that Pep-1/cargo complexes consist in globular nanoparticles with size in the range of 100–200 nm (Fig. 1C and D) [52].

4. Structural features of MPG, Pep-1 and CADY

Although, the structural behavior of CPPs is well documented, the impact of their conformation on the ability to enter the cell and to deliver drug remains unclear. For both primary and secondary amphipathic peptides, conformational versatility has been reported to be crucial for cellular uptake efficiency. MPG, Pep-1 and CADY
4.1. Conformation of carrier peptides in solution

The structural plasticity of MPG, Pep-1 and CADY was analyzed by a combination of different spectroscopic methods. Circular dichroism (CD) investigation has revealed that these carriers share a common mainly disordered conformation at low concentration in solution (Fig. 2A). In water or phosphate buffer, MPG exhibits a CD spectrum with a single minimum at 198 nm, a characteristic of a disordered structure, independent of the concentration of peptide [48]. CD profiles of CADY obtained in water are composed of a single minimum at 203 nm, indicative of mainly random coil conformations whatever the concentration of peptide used [21]. However, the higher position of the single minimum (203 nm instead of 198 nm) associated to a slight deviation around 220 nm suggests the contribution of residual helical secondary structure to the CD spectrum (Fig. 2A). Pep-1 displays a slightly similar structural behavior than CADY in water. However, while no concentration effects occurred for MPG and CADY structure, increasing Pep-1 concentration up to 3 mg/ml induces a high propensity of the peptide to fold into an α-helix. Moreover, the folding or Pep-1 into an α-helix at high concentration was confirmed by FTIR and NMR spectroscopy analyses [51].

4.2. Membrane mimicking environment triggers peptide folding

Structural studies of the peptides in the presence of membrane mimicking environment have been investigated by combining several spectroscopic methods, using different types of solvents or liposomes. In the presence of phospholipid vesicles, peptide-based-nanoparticles undergo a structural transition from a disordered state to a specific secondary structure. Although MPG, Pep-1 and CADY seem to have a similar versatility, their conformational states strongly differ within membranes. In the presence of phospholipid vesicles or SDS, MPG has a net tendency to fold into a β-sheet structure [48]. The existence of a sheet structure as the major structural component of MPG was confirmed by FTIR with the existence of a major Amide I band around 1625 cm\(^{-1}\) associated with a broad shoulder at 1655 cm\(^{-1}\), in the presence of phospholipids [53]. FTIR and CD experiments have pointed out an α-helical conformation for Pep-1 in the presence of lipids. CD spectra exhibit two minima at 206 and 222 nm and one maximum around 190 nm, indicating that helical structure is the
hydrophilic C-terminal domain; i.e. the nuclear localization sequence (NLS) domain is disordered and has no specific conformation with phospholipids [54]. Thus, both MPG and Pep-1 adopt a specific secondary structure only in their N-terminal domain in the presence of liposomes, whereas CADY is able to fold into a helix throughout the sequence [21].

4.3. Conformational analyses in the presence of respective cargoes

MPG, Pep-1 and CADY form stable nanoparticles with their respective cargoes (oligonucleotides, proteins, peptides and siRNA...). These complexes are stabilized by a combination of electrostatic and hydrophobic interactions between the carrier and the cargo and also by cargo-mediated secondary structure changes of the peptide. Analysis of the influence of the respective cargoes on the structural behavior of MPG and CADY have revealed that both peptides are able to adopt a specific secondary structure in the presence of oligonucleotide and siRNA, respectively [48,51]. The addition of an oligonucleotide to a solution of MPG for a peptide/cargo ratio of 20/1 promotes structural transition of the peptide from random coil to β-sheet structure, which is further increased by the addition of lipid vesicles [48]. Binding of CADY to siRNA at a peptide/cargo ratio of 20/1 results in a change in the CD spectrum, indicative of an increase in α-helical contributions combined to a decrease in the amount of unfolded peptide, then as observed for MPG, intensity of the helical spectral contribution is enhanced in the presence of lipids [21]. In contrast, to MPG and CADY, no modification of Pep-1 secondary structure was observed in the presence of different peptide cargoes and only the presence of lipid vesicles induced changes in the secondary structure of Pep-1 engaged in the complex [51].

5. Interactions of PBN with membrane and membranes components

Cellular membrane constitutes by its physico-chemical properties one of the main barriers to the use of large therapeutic molecules in the cultured cells and in vivo. Therefore, understanding the parameters that control and trigger molecule/CPP/membrane interactions is a major question to address in order to optimize their cellular uptake. Several technologies can be combined to investigate molecule/membrane and CPP/membrane interactions [55,56]. Although artificial membranes cannot be considered as physiological membranes, they constituted potent tools to investigate the mechanism of membrane insertion of CPPs, together with their ability to form stable complexes with phospholipids. In the last decade, membrane technology has significantly evolved and offers now the possibility of using mixture of different phospholipids with composition mimicking physiological membranes and the choice of constituting lipid or lipid/protein membrane micro domains or rafts. Therefore, the use of artificial membrane has a major impact on understanding CPP cellular uptake when the mechanism required direct interaction with membrane phospholipids. Intrinsic amphipathic feature of peptides and their insertion into phospholipid monolayers can be analyzed by surface physic methods using monolayer approaches. The ability of peptides to interact with liposomes can be monitored following changes of their intrinsic tryptophan fluorescence in the presence of small unilamellar vesicles. By combining these different methods, we have identified intrinsic properties of the amphipathic MPG, Pep-1 and CADY peptides in membrane mimicking condition.

5.1. Adsorption at the air/water interface: amphipathicity

The amphipathicity of MPG, Pep-1 and CADY was evaluated by surface physic experiments using the monolayer approach [57,58]. The surface pressure induced by adsorption of peptides at the air/water interface was recorded in function of the concentration of peptides injected in the subphase. Then saturating surface pressure...
was identified by plotting surface pressure ($\pi$) in function of the concentration of peptide. This study revealed that CADY is more amphipathic than MPG and Pep-1, the latter displaying a poor affinity for the interface (Fig. 3A). Comparison of the saturating pressure ($\pi_{\text{sat}}$) indicated that CADY is by far the most amphipathic peptide ($\pi_{\text{sat}} = 30 \, \text{mN/m}$) while Pep-1 is the less one ($\pi_{\text{sat}} = 5 \, \text{mN/m}$) [21,51,53]. In addition, MPG and CADY displayed a similar critical micellar concentrations (CMCs) with values of 250 nM and 230 nM, respectively, while that of Pep-1 is slightly higher (500 nM). Taken together, these results emphasize that Pep-1 has a weaker amphipathic character than CADY and MPG, which is not surprising considering that the N-terminus tryptophan-rich domain of Pep-1 does not correspond to a classical hydrophobic segment.

5.2. Insertion into phospholipid monolayers

The ability of MPG, Pep-1 and CADY to insert into phospholipid monolayers spread at the air/water interface was monitored by measuring variations in surface pressure ($\Delta \pi$) using different lipid films with distinct initial surface pressures ($\pi_i$) and by injecting a given peptide concentration in the subphase [59]. The penetration curve, corresponding to the variations of surface pressure ($\Delta \pi$) in function of the initial surface pressure ($\pi_i$), allows the extrapolation of the critical pressure of insertion (CPI) ($\pi_i$ for $\Delta \pi = 0$) reflecting the insertion of peptides into phospholipid monolayers [59]. Penetration experiments were carried out using various phospholipids with headgroups of different natures (zwitterionic or negatively charged) and in different physical state (liquid expanded or liquid condensed). The examination of the variation of surface pressure measured for an air/water interface at low lipid content ($\Delta \pi$ for $\pi_i = 0$) provides information on the interactions occurring between the peptide and the phospholipids. That CPI values are higher than those obtained for the pure peptides at saturation ($\pi_{\text{sat}}$) indicating that CADY/MPG/Pep-1 peptides strongly interact with phospholipids in membrane monolayers and a spontaneous insertion of the peptide into natural membrane [53,57]. CPI value for MPG increases from 13 mN/m to 30 and 38 mN/m for DPPC and DPPG, respectively. In the case of liquid expanded monolayers, CPI values of 48 mN/m and 33 mN/m were obtained for DOPG and DOPC, suggesting a better uptake of MPG by negatively charged phospholipids and that MPG insertion depends on both the initial conformational state of the peptide and the nature of the phospholipid headgroups. Pep-1 induces a strong increase in the surface pressure of liquid expanded monolayers. Both DOPC and DOPG yielded identical CPI (45 mN/m) and extrapolation at zero

![Fig. 3](image-url)
initial surface pressure was (16 and 32 mN/m for DOPC and DOPG, respectively) significantly higher than that measured in the absence of lipid (πsat). For the liquid condensed monolayers (DPPC and DPPG), the CPI values are also relatively high whatever the nature of the head groups (33 mN/m), suggesting that Pep-1 strongly interacts with all phospholipids and spontaneously inserts into biological membranes [51].

Similarly, CADY exhibits a strong affinity for both air/water interface and phospholipids. The presence of phospholipids in the liquid expanded states (DOPC and DOPG) at low initial pressure significantly decreases the CMC of CADY from 230 nM to 180 nM for DOPC and to 100 nM for DOPG, highlighting strong interactions between CADY and phospholipids [21]. A similar CPI value of 40–42 mN/m was obtained for DOPC, DOPG and DPPG revealing a spontaneous insertion of CADY in phospholipid monolayers irrespective of the nature of head groups (Fig. 3B). Moreover, extrapolated surface pressure for zero initial pressure (Δπ = 40 mN/m for m = 0) are higher than those observed for the free peptide at saturation (πsat = 30 mN/m) (Fig. 3B), confirming tight interactions between CADY and the different phospholipids. In the case of the liquid condensed DPPC, CADY inserts with a high CPI value (~36 mN/m) but the value of surface pressure at low initial pressure (24 mN/m) indicates moderate CADY/DPPC interactions.

5.3. Insertion into phospholipid bilayers

The insertion of MPG, Pep-1 and CADY into phospholipid bilayers has been investigated by fluorescence spectroscopy. MPG, Pep-1 and CADY contain one, five and four Trp residues in their sequences, respectively, which constitute sensitive probes to monitor interaction with phospholipids and changes in the peptide environment upon penetration into phospholipid bilayers. Binding of MPG, CADY or Pep-1 peptides to phospholipids induced a drastic modification of their fluorescence emission associated to a blue shift of the maximum of the Trp-fluorescence emission from 348/350 nm to 328/330 nm depending on the peptides. The shift of fluorescence emission reveals a switch in the Trp residue environment from polar to apolar in the presence of phospholipid vesicles and indicates that the tryptophan domain of the peptide is embedded in the phospholipid bilayer (Fig. 3C). The change in MPGemission wavelength is associated to an increase in the fluorescence intensity, whereas for Pep-1 it is accompanied by quenching of fluorescence [51]. These observations suggest that Pep-1 tryptophans are embedded in the lipidic core and that fluorescence energy transfer occurs between Trp residues organized in clusters when Pep-1 adopts a helical structure (Fig. 3D). The interaction between CADY and phospholipid vesicles also results in a marked change in the environment of Trp residues from polar to nonpolar, characterized by a sphere−fluorescence emission blue shift of 17 nm and 13 nm, associated to a 1.8 and 2.0 fold enhancement of fluorescence intensity, in the presence of DOPC and DOPG vesicles, respectively. Both DOPC and DOPG liposomes induced fluorescence emission shift with a saturation occurring for a similar lipid/peptide molar ratio (ca. 3), which confirmed that the nature of phospholipid head groups has no influence on the ability of CADY to insert into phospholipid mono- and bilayers [21].

5.4. Interaction with cell surface proteoglycan

Before reaching the phospholipid part of the plasma membrane, the carrier/cargo complex (PBN/cargo) needs to deal with components of the extracellular matrix such as proteoglycans, which are playing a critical role in the internalization of external molecules [60]. It is now well admitted than the first step of the CPP mechanism corresponds to electrostatic interactions with cell surface proteoglycan (GAGs), resulting in a clustering at the cell surface which triggers activation of intracellular signals/actin remodeling and cell entry throughout numerous distinct pathways involving either direct translocation or endocytotic processes [61–63]. Therefore investigation of the interaction between CPPs and heparan sulfate is also a major piece of the puzzle to consider. The ability of MPG and CADY to interact with proteoglycan has been investigated by gel shift experiments. In order to study the impact of GAG on the stability of MPG/DNA and CADY/siRNA complexes, MPG was associated, in water, with an 18-mer oligonucleotide at a charge ratio of 5, and CADY with a siRNA at different charge ratios at which the complexes have been shown to be highly stable [21,48,64]. Complexes were then incubated in the presence of increasing amounts of several soluble GAGs, including heparin, dextran sulfate and hyaluronic acid, which have different numbers of sulfate groups per disaccharide unit and both complex integrity and stability were analyzed by electrophoresis on agarose gels (Fig. 4A). The presence of heparin or dextran sulfate, both sugars bearing a high density of negative charges, induced a GAG concentration-dependent dissociation of the complexes resulting in migration of free DNA or siRNA into the gel. In contrast, hyaluronic acid, which has a low charge density and no sulfate group, had no effect on complex stability. The results strengthened the major implication of electrostatic interactions within the MPG/ or CADY/ oligonucleotide nanoparticles. Complex dissociation is dependent on the concentration of GAG but also on the MPG/DNA or CADY/siRNA molar ratio. CADY/siRNA complexes at molar ratio of 40/1, which corresponds to complex used on cultured cells and in vivo, remained highly stable in the presence of high concentrations of GAG [21]. These results indicate that MPG and CADY peptides can interact with negatively charged GAG, a major component of proteoglycans of the cell surface and extracellular matrix, interactions which probably, as for many other delivery systems, allow the binding of the complex and its accumulation at the cell surface. Then peptides or complexes which exhibit a high affinity for phospholipids will be able to dive into the cell membrane. The fact that high concentration of negatively charged GAG affects the stability of the MPG/cargo and CADY/siRNA complexes, pointed out the importance of the integrity of the PBN for cellular and in vivo applications [64].

5.5. Membrane potential plays a major role in PBN cellular uptake

The membrane potential (or transmembrane potential) plays a major role in cellular membrane integrity and can modulate or control the internalization of a molecule through the plasma membrane. Membrane potential corresponds to the electrical potential difference between the cytoplasm of the cell and the culture medium. Moreover membrane potential has also been reported to control endosomal escape [65], thus the influence of the membrane potential and its associated voltage dependence are additional parameters to consider as they have already been reported to affect cellular uptake of several CPPs [66,67]. These parameters were studied for primary amphipathic peptides MPG and Pep-1 using either Xenopus oocytes or artificial lipid bilayers.

The application of a solution of free peptides to voltage-clamped oocytes induced marked increases in membrane conductance. Increases were detected from the variation in membrane currents recorded during voltage ramps applied from −80 mV (the usual holding potential) to +80 mV and from −40 mV to +40 mV as shown for MPG in Fig. 4B. The reversal potential for the peptide induced current was close to −8 mV and −12 mV for Pep-1 and MPG respectively, values which are similar to what has already been found for other pore-forming peptides selective for monovalent cations [68]. With regard to the cargoes (oligonucleotide for MPG and peptide Pep-A for Pep-1), the variations in membrane conductance induced by the nanoparticle are slightly different. While the addition of free peptide Pep-A and free oligonucleotide has no effect on the voltage ramps (Fig. 4B and C), the association with Pep-1 and MPG did not affect the reversal potential (−8 mV and −12 mV) but appears to reduce the
current amplitude (Fig. 4C). However, it remains difficult to conclude whether this decrease in the current arises from an artificial decrease of the peptide concentration because it is engaged in the complexes, or to modification of the transmembrane current characteristics. Both hypotheses will explain the fact that complex formation significantly reduced and even totally abolish the toxicity of the peptide carrier. In order to better understand the membrane potential dependence of the peptide cellular uptake, artificial lipid bilayers were used to detect macroscopic currents and single channel events. Peptides were incorporated into azolectine planar lipid bilayers according to the Montal–Mueller technique [69]. Macroscopic recording revealed net I–V curves suggesting voltage dependence (Fig. 4D). In addition, single channel events were identified for lower concentrations than those used for macroscopic experiments (Fig. 4D). Taken together, these results suggested the formation of small but significant voltage-dependent pore-like structures [9] and that the permeabilizing property of MPG and Pep-1 is associated to the formation of membrane transient ion channels [9,48,51]. Finally, it should be mentioned that such a condition, which gives rise to transmembrane currents, does not generate propidium iodide cellular internalization confirming the lack of cytotoxicity associated to MPG or Pep-1 cellular uptake.

6. Model for the translocation process of carrier/cargo complexes

The development of new carrier peptides is often limited by a poor understanding of the features required for their efficiency. Although the use of several approaches allows the identification of specific properties, it is still hard to know how to combine most of them to conceive a clear model for the translocation process [34]. The PBN; Pep-1, MPG and CADY have been shown to significantly improve the delivery of different types of cargoes both in cultured cells and in vivo.
It was clearly reported that cellular uptake mechanism leading to a cargo-mediated active biological response is independent of the endosomal pathway. We demonstrated that PBN cellular uptake and cargo-mediated biological response are not affected by the presence of inhibitors of the endosomal pathways as well as by heparin or trypsin treatments [21,29,45,64]. PBN tightly binds phospholipids, induces cell membrane alteration and remodeling of the actin network [21,64]. However, we cannot exclude that binding to the cell surface of PBN–cargo complexes induces the formation of vesicles that are rapidly destabilized thanks to the amphipathic property of the PBN. By combining cellular observation together with structural and biophysical investigations of the different peptides we can propose the following model for the cellular internalization mechanism of non-covalent MPG/Pep-1/ or CADY/cargo complexes. The carrier peptides MPG, Pep-1 and CADY shared a number of common properties that favored and drove the cellular uptake pathway.

- The peptides form highly stable nanoparticles with their respective cargoes and strongly interact with phospholipid monolayers or bilayers, mainly throughout electrostatic interactions.
- CADY/Pep-1 and MPG exhibit a marked structural polymorphism. Their free forms are mainly unfolded in solution, whereas they all adopt a specific secondary structure, folding into a β-sheet (MPG) or an α-helix (Pep-1 and CADY) upon interaction with phospholipids. Similarly, in the presence of cargo, they partially fold into a β-sheet (MPG) or an α-helix (CADY), structures which are enhanced when peptide/cargo complexes interact with phospholipids.
- The carrier peptides whether associated or not with a cargo, induce a voltage-dependent membrane permeability (Pep-1 and MPG).

According to both structural and biophysical investigations, the combination of the different properties identified for MPG, Pep-1 and CADY led to a six-step mechanism (Fig. 5). This model involves first electrostatic and hydrophobic interactions between carrier and cargo which induce a partial folding of peptide followed by the formation of the core of the complex (1). Carrier/cargo complexes are stabilized by peptide/peptide interactions that constitute the surface carrier-peripheral (2). Electrostatic interactions of carrier/cargo complexes with cell surface proteoglycan (3) initiate the interaction with phospholipids (4) and then insertion into cell membrane mediated by the carrier-peripheral (5). Finally, insertion of the complexes into the membrane is associated to both peptide conformational transition and membrane potential, which induces membrane structure perturbations and leads to the release of the core complex into the cytoplasmic side, followed by the nuclear targeting or cytosolic release depending on the carrier used (6).

Fig. 5. Model for the formation and cellular internalization of MPG/oligonucleotide, Pep-1/protein and CADY/siRNA nanoparticles. The model consists of six steps corresponding to (1) electrostatic and hydrophobic interactions between carrier and cargo initiate a partial helical or β-strand folding of peptide and form the core of the complex; (2) carrier/cargo complex are stabilized by peptide/peptide interactions, corresponding to carrier-peripheral; (3) electrostatic interaction of carrier/cargo complexes with cell surface proteoglycan and then (4) interaction with phospholipids; (5) insertion into cell membrane mediated by the carrier-peripheral and (6) release into the cytoplasmic side of the core complex.
7. Conclusions and perspectives

There is still a long way to go, before understanding fully the mechanism by which CPPs cross the plasma membrane and deliver several drugs to their specific targets. However, during the last two decades, we have witnessed a net increase in the number of approaches to decipher the cellular uptake of CPPs and the combination of a large set of biophysical studies had rendered possible the identification of crucial features required for the peptide carrier activity. We have proposed a new generation of delivery system, the peptide-based-nanoparticles, that offers an interesting alternative for the delivery of different cargoes throughout a non-covalent strategy [29]. The leader peptides Pep-1, MPEG and CADY, have been shown to mainly enter cells independently of the endosomal pathway and to efficiently deliver cargos into cells as well as in vivo. The biophysical characterization of these three carriers and of their interaction with cargos has revealed that they share common properties leading to a model for the mechanism of internalization of the carrier/cargo particles. Although this model merge most of conformational and interaction parameters, some points still remain to be clarified. Other CPPs covalently used, have also been reported entering the cell by a non-endosomal mechanism, but only when high concentrations of CPP-cargo are applied to the cell surface [70]. In contrast, the organization of PBN-cargo in nanoparticle exposes several peptides at their surface creating, even at a low concentration of particle, a high affination of the membrane which will control the cellular uptake mechanism [71].

In comparison to other CPPs, PBN-cargo particles need to be sufficiently stable in physiological conditions in order to cross the plasma membrane and reach the cytosol but, at the opposite; these particles should also be able to disassemble inside the cell in order to release the therapeutic cargo. Indeed the cytosolic release or decaying process of the particles needs more investigation and parameters such as the association/dissociation constants and thus the particle stability should be considered. Both size and homogeneity of the nanoparticles are dependent on the carrier/cargo ratio and optimal protocols for transduction have been described for carrier/cargo ratio of about 10/1 to 15/1 depending on the nature of the cargo. One of the advantages of non-covalent strategy resides in the possibility of modification of the surface peptide layer of the particle in order to stabilize the particle for systemic in vivo administration or for targeting that will open interesting perspective to improve cargo trafficking in cellulo and in vivo.

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