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Mini review

Octaarginine-modified liposomes: Enhanced cellular uptake and controlled intracellular trafficking

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Abstract

Gene therapy is a promising new approach for treating a variety of genetic and acquired diseases. While viral vectors are highly efficient for gene therapy, their use is associated with high toxicity and immunogenicity. Synthetic or nonviral vectors are attractive alternatives to viral vectors because of their low immunogenicity and low acute toxicity. The main disadvantage of the nonviral vectors is the low transfection efficiency compared to viral vectors. Novel functional devices to enhance the transfection activities of nonviral vectors are needed. In this review, we discuss the modification of liposomal drug carriers with a novel functional device, the octaarginine (R8) peptide, for drug and gene delivery. Decoration of liposomes with R8 enhanced their cellular uptake. In addition, by optimizing the density of the peptide as well as its topology, the liposomes could be internalized via clathrin-independent pathways, which improved the intracellular trafficking through avoiding lysosomal degradation. A special emphasis is given to the need for optimizing the conditions of using the peptide to not only enhance the cellular uptake but also to improve the intracellular trafficking of its cargos. In addition, the use of R8-modified liposomes and nano-particles in gene delivery is discussed.

Keywords: Liposomes; Octaarginine; Endocytosis; Intracellular trafficking; Gene delivery

1. Introduction

Liposomes are self-closed colloidal particles in which bilayer membrane(s) composed from self-aggregated lipid molecules encapsulate(s) a fraction of the medium in which they are suspended into their interior (Lasic and Papahadjopoulos, 1996; Lasic and Templeton, 1996; Lasic, 1998). Their similarity to cell membranes makes them a useful model, tool and reagent in biophysics and biochemistry, as well as in several applications, such as a carrier or sustained release system for the encapsulated (macro)molecules. Liposomes are relatively easily prepared and have a rich selection of physicochemical properties which have made them attractive drug carrier systems. In terms of drug delivery, liposomes have several advantages such as: (i) their biocompatibility, biodegradability, low toxicity and immunogenicity, (ii) they can incorporate various molecules at high concentrations, (iii) the biologically active molecules are protected by the lipid bilayer of the liposome from damage by chemicals and enzymes after injection into the blood stream, (iv) the host cells are protected from the toxicity of the entrapped molecules, and (v) they are very versatile in that their surfaces can be easily modified with a variety of functional devices such as targeting ligands, polyethylene glycol (PEG) molecules and specific devices for optimization.

Despite the high promise of liposomes as drug carriers, the cellular uptake of conventional or non-specific liposomes is very limited (Lasic and Templeton, 1996). High doses and long incubation times are required to achieve a significant cellular uptake. Enhancing the cellular uptake of liposomes can be achieved by including cationic lipids to interact with the negative cell surface constituents or by attaching specific targeting ligands to the liposomal surface to specifically interact with certain cell surface receptors (Lasic and Templeton, 1996). Although the uptake is enhanced, it occurs mainly through classical endocy-

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tosis and ends up in lysosomes for degradation (Elouahabi and Ruysschaert, 2005; Khalil et al., 2006a). In addition, cationic lipids may produce some toxicity for the cells, especially when used in high amounts such as what is usually used in gene delivery (Torchilin et al., 2003). For these reasons, a novel ligand to enhance the cellular uptake and escape from lysosomal degradation while producing no cytotoxicity or immunogenicity is required.

It is not surprising that the discovery of short peptides, which can translocate through cell membranes and transfer large biologically active molecules to inside the cells, has attracted a lot of attention in the field of drug and gene delivery (Derossi et al., 1998; Schwarze et al., 1999, 2000; Lindgren et al., 2000; Dietz and Bahr, 2004; Snyder and Dowdy, 2004; Trehin and Merkle, 2004; Gupta et al., 2005). These peptides are generally known as cell penetrating peptides (CPPs), membrane translocation sequences (MTSs), "Trojan peptides" or protein transduction domains (PTDs). These peptides generally consist of 7–30 amino acids and have a net positive charge. The importance of these peptides in drug discovery lies mainly in their ability to carry massive cargos, several times larger than their own size, to inside the cells. Other advantages include their general inertness, low toxicity, and low immunogenicity (Torchilin et al., 2003; Jarver and Langel, 2004). In addition, these peptides can cause efficient intracellular, and even intranuclear, delivery of different cargos in almost 100% of exposed cells within minutes (Barka et al., 2000). More interestingly, the TAT peptide was able to transport macromolecules across the blood–brain barrier (Schwarze et al., 1999). Collectively, these results indicate that these peptides are promising transport systems for efficiently delivering macromolecules to the inside of the cells.

The PTD peptides share the presence of several arginine residues in their sequences and these arginine residues were shown to be responsible for the activities (Vives et al., 1997; Wender et al., 2000; Futaki et al., 2001a). Peptides consisting of merely arginine residues were similar to the PTD peptides in terms of efficiency (Futaki et al., 2001a; Suzuki et al., 2002). Among oligoarginines, the optimum number of arginine was shown to be around 8. Therefore, the octaarginine (R8) peptide can be regarded as a simplified or a proto-type of the PTD peptides.

The class of PTD peptides seems ideal as efficient and safe functional devices for liposomal drug delivery. In this review, we introduce octaarginine-modified liposomes (R8-Lip) as a potential drug and gene delivery system. Discussion extends to cover several aspects including cellular uptake, uptake mechanism, intracellular trafficking and the use of R8-Lip in gene delivery. Decoration of liposomes with the R8 peptide, not only enhanced the cellular uptake, but also improved the intracellular trafficking towards more efficient escape from lysosomal degradation. R8-modified nano-particles proved to be efficient gene delivery systems with minimum cytotoxicity.

2. Oligoarginines as tools for drug delivery

The most commonly used PTD is the transcriptional activator of transcription (TAT) of HIV-1. TAT-derived peptides, largely studied in the literature, are truncated versions of the TAT protein. Penetratin is another commonly used PTD. Penetratin is the name of the Antennapedia homeodomain 43–58 peptide. The third commonly used PTD is VP22. It is a transcription factor of Herpes simplex virus type 1 (HSV-1) which possesses a remarkable property of transport between cells. Various arginine-rich peptides (RRPs), such as the arginine-substituted TAT (48–60) analogs, the RNA-binding peptides derived from virus proteins, such as HIV-1 Rev and flock house virus coat proteins; and the DNA-binding segments of leucine zipper proteins, such as cancer-related proteins c-Fos and c-Jun, and the yeast transcription factor GCN4, demonstrated the potential to translocate across the membranes (Futaki et al., 2001a). These peptides share only the presence of arginine residues and peptides consisting of only arginine resides were highly active in terms of membrane translocation (Futaki et al., 2001a). The optimum number of arginine residues was shown to be around 8 (Futaki et al., 2001a; Suzuki et al., 2002). Longer or shorter oligoarginines showed reduced activities.

The PTD peptides have been used for transporting a wide variety of small and macromolecules, which cannot traverse the plasma membrane, into the cytosol and the nucleus. From small hydrophobic molecules, such as fluorescent probes, up to large liposomes of 200 nm diameter could be efficiently delivered intracellularly through modification with various PTDs. PTD-mediated delivery has been extensively reviewed in several articles (Dietz and Bahr, 2004; Snyder and Dowdy, 2004; Trehin and Merkle, 2004; Gupta et al., 2005). Focusing mainly on oligoarginines, they could efficiently internalize a wide variety of cargos into cells. GFP conjugates with oligoarginines have been efficiently internalized (Caron et al., 2001). Similarly, the R8 peptide was able to bring exogenous proteins (enhanced green fluorescence protein, eGFP) into cells (Futaki et al., 2004). Delivery of an apoptotic-inducing peptide was accomplished by R8 conjugation and the conjugates were shown to induce apoptosis in HeLa cells (Futaki et al., 2004). Biologically active p16 peptide was efficiently delivered intracellularly using oligoarginines (Zaro and Shen, 2005).

Oligoarginines were also used in the delivery of nucleic acid therapeutics. R8 was able to transfect cells with a luciferase-coding plasmid as efficiently as polyarginine and polylysine (Futaki et al., 2001b). Furthermore, the N-terminal stearylation of R8 significantly improved the transfection efficiency. Stearyleted-R8 was as efficient as Lipofectamine as a transfection agent (Futaki et al., 2001b). Modification of lipid vesicles containing plasmid DNA (pDNA) by R8 increased gene expression significantly (Kogure et al., 2004). These results collectively indicate that oligoarginines, particularly R8, is highly efficient in enhancing cellular uptake of membrane-impermeable molecules.

3. Octaarginine-modified liposomes: enhanced cellular uptake

The attaching of a PTD peptide to liposomes was first reported by Torchilin et al. (2001). They reported that the attachment of TAT directly to the liposome surface or the presence of high

molecular weight PEG molecules prevented the peptide cell surface interaction through steric hindrances. The only preparation succeeded was the one containing the TAT peptide on the tip of short PEG spacers, which allowed more freedom for the TAT peptide to interact with the cell surface. The authors concluded that the direct contact between the TAT peptide and the cell surface in a free form is required for internalization. More recently, Tseng at al. prepared TAT- and penetratin-modified liposomes (Tseng et al., 2002). Fretz et al. as well as Marty et al. successfully prepared TAT-liposomes (Fretz et al., 2004; Marty et al., 2004).

We used a simple and a versatile strategy to attach the R8 to the liposome surface (Khalil et al., 2006b). First, the N-terminal of the R8 was attached to a stearyl group to form stearylated-R8 (STR-R8). The STR-R8 peptide was then mixed with other lipids during forming the liposomes. The hydrophobic stearyl group is expected to be anchored on the lipid bilayer of the liposomes thus leaving the R8 freely attached to the surface (Fig. 1). The incorporation of even small amounts of STR-R8 peptide increased the positive charge of the liposomes, indicating that the R8 peptide was successfully attached to the liposome surface (Khalil et al., 2006b). At around 5 mole% STR-R8, the liposomal surface was saturated with the peptide since no further increase in the positive charge was observed above this concentration.

Although it is simple, this novel strategy proved to be highly efficient. In this strategy, there is no complicated chemical interaction step, which may waste the peptides significantly. Other methods were rather non-specific and probably produced unordered attachment to the liposome surface and this probably explained why a PEG spacer was needed for the internalization in an early report (Torchilin et al., 2001). In the method used here, no spacer was needed and even relatively low STR-R8 concentrations were enough to internalize the liposomes as it will be explained later. The R8-Lip were around 100–150 nm in diameter and all were positively charged. Furthermore, different liposomes containing different model drugs and modified with the R8 peptide were successfully prepared. It is worth mentioning that the STR-R8 peptide proved to be highly efficient by itself as a transfection agent through direct mixing with plasmid DNA (Futaki et al., 2001b), and we previously clarified the mechanism of the improved transfection by the stearylation of the R8 peptide (Khalil et al., 2004).

The modification of liposomes with low or high density of the R8 peptide significantly enhanced the cellular association of a model drug encapsulated in the liposomal aqueous phase compared to the use of free drug or drug encapsulated in conventional liposomes lacking the R8 peptide (unpublished data). Even a relatively low amount of the R8 peptide was enough to enhance the cellular association of the liposomes while increasing the peptide amount increased the affinity of cellular association. The internalization of the R8-Lip was further confirmed using confocal laser scanning microscopy (CLSM). Liposomes modified with low or high peptide densities and carrying either a model hydrophilic or hydrophobic drug were efficiently internalized within the 1-h incubation time tested (Fig. 1 and unpublished data). This validates the use of R8-Lip in enhancing the cellular uptake of drugs and shows that the liposomes can be used to efficiently internalize both hydrophilic and hydrophobic drugs. When double-labeled liposomes were used, high intracellular fluorescence could be observed and both liposomal labels (aqueous and lipid markers) were colocalized on the plasma membrane or in the cytosol of cells (Khalil et al., 2006b). The intracellular colocalization of the markers excludes the possibility of fusion between the liposomal membrane and the cell membrane. In addition, the intracellular fluorescence in both cases appeared as punctuated signals in the cytosol indicating a possible contribution of endocytosis, a finding that is further supported by the strong inhibition of uptake after energy depletion (Khalil et al., 2006b).

Compared with other PTD-liposomes prepared, Torchilin et al. reported that high TAT amount was required for efficient internalization (Torchilin et al., 2001). Similarly, Marty et al. found that liposomes decorated with small amounts of the TAT or Antp peptides were not taken up efficiently (Marty et al., 2004). Liposomes prepared by Fretz et al. and Cryan et al. also needed high PTD concentrations for cellular uptake (Fretz et al., 2004; Cryan et al., 2006). In contrast, the R8-Lip were efficiently taken up even when modified with a relatively small amount of the R8 peptide. Fretz et al. observed only a surface bound liposome after 1 h incubation (Fretz et al., 2004), while others could observe high cellular uptake within 1 h (Marty et al., 2004; Cryan et al., 2006). Consistent with the latter case, both R8-Lip were highly internalized within 1 h. Consistent with all PTD-liposomes reported, there was no intranuclear or nuclear bound

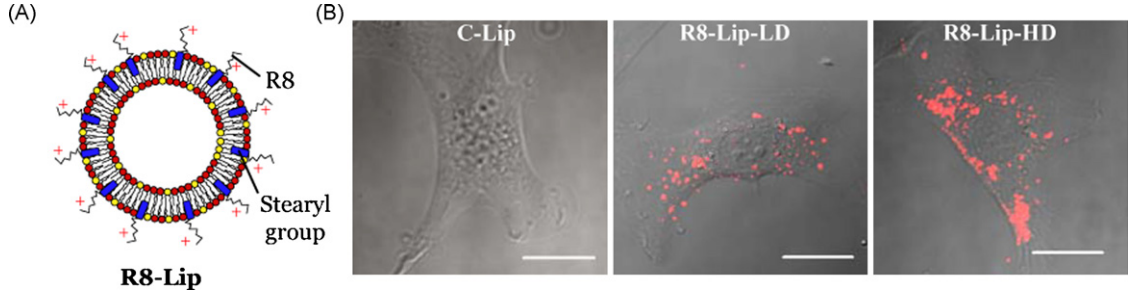

Fig. 1. Cellular uptake of R8-modified liposomes (R8-Lip). (A) A schematic diagram of the R8-Lip. The stearyl group is attached to the N-terminal of the R8 peptide. It acts as an anchor to the lipid surface of the liposomes, leaving the R8 freely attached. (B) Cellular uptake of R8-Lip containing rhodamine aqueous phase. Representative images of NIH3T3 cells incubated with conventional liposomes (C-Lip) or R8-Lip modified with low density (LD) or high density (HD) of STR-R8 in serum-free medium for 1 h. Scale bars represent 20 μm.

liposomes after 1 h incubation. Free PTD peptides usually can be found in the nucleus after 1 h (Futaki et al., 2001a). The difference in the diameter between the free peptides and the liposomal attached peptides may retard the nuclear migration of the larger liposomes and may restrict their delivery to the nucleus. As many recent reports have shown a detrimental effect of fixation on cellular membranes leading to a re-distribution of surface bound fluorescence (Richard et al., 2003; Fretz et al., 2004), live cells were used in the R8-Lip experiments presented here.

4. Octaarginine-modified liposomes: uptake mechanism

Understanding the uptake mechanism and intracellular trafficking of drug carriers is an essential step to optimize these systems to produce maximum effect. While few groups have previously reported the successful use of PTD-modified liposomes for enhancing the cellular uptake (Fretz et al., 2004; Marty et al., 2004; Cryan et al., 2006), no reports are available regarding the factors affecting the uptake mechanism of PTD-modified liposomes. We examined how the R8 peptide mediates the uptake and the intracellular trafficking of the liposomes. More importantly, what are the factors that should be considered to direct the peptide to choose an entrance pathway to achieve improved cellular availability of the liposomal encapsulated drugs.

It has been observed that externally added soluble heparin inhibited the cellular uptake of TAT and TAT-cargos (Tyagi et al., 2001). Treatment with heparinase III to specifically deplete the cell surface heparan sulfate proteoglycans (HSPGs) also inhibited the uptake of TAT peptides. In addition, TAT peptide is unable to bind to cells genetically defective in the biosynthesis of fully sulfated HSPGs (Tyagi et al., 2001). Collectively, this raised the possibility that cell surface proteoglycans are involved in the cellular binding and internalization of the PTDs and their cargos. The presence of heparin almost completely inhibited the binding and uptake of R8-Lip (Khalil et al., 2006b). This indicated that the HSPGs are involved in the uptake of R8-Lip, similar to other PTDs and their cargos. HSPGs probably act as non-specific receptors for the binding of the PTD peptides, which may explain the efficiency of binding to most cell lines since HSPGs exist in almost all cells (Belting, 2003; Yoneda and Couchman, 2003). Although the Antp peptide was shown to be internalized through not only HSPGs (Drin et al., 2001), this peptide is amphipathic and can bind to the lipids of the cell membrane, which is not the case of only basic peptides such as TAT or R8 (Futaki, 2005). HSPGs were shown to mediate the uptake of polyamines, viruses, and polyplexes (Yoneda and Couchman, 2003). In addition, several reports have previously shown a role for cell surface proteoglycans in lipoplexes mediated transfection (Ruponen et al., 1999; Mounkes et al., 1998). However, Zuhorn et al. has recently demonstrated that transfection of epithelial cells with lipoplexes is almost exclusively mediated by the beta1 integrin cell surface receptors which serve as "natural" receptors for lipoplexes (Zuhorn et al., 2007). A possible contribution of similar receptors in the uptake of PTDs and their cargos is yet to be confirmed.

The mechanism responsible for the uptake of PTDs and their wide variety of cargos is a highly controversial issue. Arguments can be made for endocytic and non-endocytic pathways. Recent data using live cells emphasized the endocytic uptake of different PTD-liposomes. Marty et al. suggested an endocytic uptake for TAT and Antp-liposomes through binding to HSPGs (Marty et al., 2004). Fretz et al. showed an endocytic uptake for TAT-liposomes since the uptake was inhibited by low temperature, cytochalasin D and iodoacetamide (Fretz et al., 2004). Cryan et al. suggested an endocytic uptake of TAT-, Antp-, and R8-liposomes, although the uptake was not completely blocked at low temperature (Cryan et al., 2006).

R8-Lip failed to penetrate giant liposomes (unpublished data), which were used to mimic cells, suggesting that the liposomes may not have the ability to directly penetrate the plasma membrane. This should not be generalized for other PTD-mediated delivery since there is a big difference in diameter between the liposomes (∼100 nm) and smaller cargos such as peptide-protein conjugates (∼30 kDa). It is possible that the mechanism of uptake of smaller cargos may be different from larger cargos such as liposomes. The CLSM of cells incubated with double-labeled R8-Lip with a non-fusogenic lipid layer consisting of egg phosphatidylcholine (EPC) and cholesterol (Chol) showed that the intracellular fluorescence of the lipid and the aqueous phase markers of the liposomes were colocalized (Khalil et al., 2006b). This indicated that R8-Lip lacking fusogenic lipids were internalized as intact vesicles and excluded the possibility of fusion between the liposomal and the plasma membranes. This was further confirmed by the minor inhibition of the internalization of the aqueous phase marker in the presence of a fusion inhibitor. Therefore, R8 alone did not cause a significant fusion between the liposomal and the plasma membranes. The contribution of endocytosis was proven to be a major one in the case of R8-Lip since a mixture of metabolic inhibitors dramatically inhibited the internalization (Khalil et al., 2006b). Although the uptake of the low density R8-Lip was inhibited at low temperature, the significant uptake of the high density R8-Lip at low temperature was somewhat surprising (Iwasa et al., 2006). This possibly reflects that while the uptake at 37 °C occurs mainly via endocytosis, a yet unknown mechanism can still work when endocytosis is blocked by low temperature. This unknown mechanism seems to require a high R8 density. A possible candidate for explaining this uptake is potocytosis, a temperature-insensitive type of caveolar uptake (Anderson et al., 1992). Although a model involving an inverted micelle formation was suggested for the uptake of different PTDs and their cargos (Derossi et al., 1996), this model is generally difficult to imagine in the case of liposomes since the liposome diameter is several folds larger than the thickness of the plasma membrane.

We further examined the contribution of different endocytic pathways in the uptake of R8-Lip using flow cytometry and the results were confirmed using confocal microscopy of living cells. The contribution of different endocytic pathways was previously examined for the uptake of different PTD peptides and most of their cargos (Eguchi et al., 2001; Ferrari et al., 2003; Fittipaldi et al., 2003; Nakase et al., 2004; Saalik et al., 2004; Wadia et al., 2004; Foerg et al., 2005; Kaplan et al., 2005);

however, this was not examined in all the reports available for PTD-liposomes (Torchilin et al., 2001, 2003; Tseng et al., 2002; Fretz et al., 2004; Marty et al., 2004; Cryan et al., 2006). The only available data were reported by Marty et al. who showed that the uptake of TAT- and Antp-liposomes did not occur through clathrin-mediated endocytosis (CME) since the internalized liposomes were not colocalized with transferrin (Tf) (Marty et al., 2004). This group also showed that control cys-liposomes were colocalized with Tf and suggested different internalization mechanisms for PTD- and conventional liposomes. Fretz at al. reported that cytochalasin D significantly inhibited the uptake of TAT-liposomes (Fretz et al., 2004). Although they concluded an endocytic uptake, they did not comment about a specific endocytic pathway. Since cytochalasin D only slightly inhibits the uptake via CME (Lamaze and Schmid, 1995), their results probably indicate a clathrin-independent endocytosis.

If we extend the comparison to cargos other than liposomes, several reports suggested different endocytic pathways for PTD peptides and their cargos. Richard et al. suggested that the uptake of TAT and other arginine-rich peptides occurs via CME (Richard et al., 2003, 2005). Another group suggested a caveolae-mediated uptake for TAT peptides and TAT-fusion proteins (Ferrari et al., 2003; Fittipaldi et al., 2003). Other groups suggested a macropinocytosis-mediated uptake for TAT-fusion protein, TAT and R8 peptides (Nakase et al., 2004; Wadia et al., 2004; Kaplan et al., 2005). Furthermore, Kaplan et al. suggested that the arginine content is essential for the internalization via macropinocytosis (Kaplan et al., 2005). The diversity of results regarding the uptake mechanism of arginine-rich peptides suggests that some factors may affect the entry mechanism. These factors include: the type of peptide, its mode of exposure to the cell surface, the nature of the cargo, and the chemical linkage between the peptide and the cargo (Brooks et al., 2005). For example, we have previously shown that the R8 peptide and its complexes with DNA were taken up by different mechanisms, suggesting that the nature of the interaction between the peptide and the cell surface (i.e. the peptide in free or complexed state) affects the uptake mechanism (Khalil et al., 2004). Another possible factor that has been less studied is the effect of peptide density on the internalization mechanism. It was previously shown that a single TAT peptide was sufficient to allow the cellular delivery of an unfolded fusion construct of the same protein (Schwarze et al., 1999). Other studies showed that several TAT peptides attached to the surface of the cargo were required to permit efficient cellular delivery (Eguchi et al., 2001). However, no direct comparison to show the effect of peptide density on the uptake mechanism was conducted. Liposomes are good tools for use in such a comparison since their surface can be easily modified with different densities of peptide and they can provide localized areas of high peptide density that are available to interact with the cell membrane.

We hypothesized that the uptake mechanism of PTD-cargos depends on the peptide density attached to the cargo surface. The mechanism of uptake of liposomes modified with a low R8 density (R8-Lip-LD) shifted from clathrin-mediated endocytosis to macropinocytosis when the density of R8 was increased (Khalil et al., 2006b). The uptake of the R8-Lip-LD was strongly inhibited by a hypertonic treatment but only partially inhibited by caveolae inhibitors and macropinocytosis inhibitors (Khalil et al., 2006b). In contrast, the uptake of liposomes modified with a high peptide density (R8-Lip-HD) was strongly inhibited by different macropinocytosis inhibitors including amiloride, cytochalasin D, methyl-β-cyclodextrin, nystatin and genestein (Khalil et al., 2006b and unpublished data). Although it was recently shown that the internalization of the TAT-Cre fusion protein as well as that of R8 and TAT peptides occurs mainly through macropinocytosis (Nakase et al., 2004; Wadia et al., 2004; Kaplan et al., 2005), our results show that large drug carriers, such as liposomes that are ∼100 nm in diameter can also be internalized by macropinocytosis. Furthermore, a high density of peptide on the liposomal surface was required for internalization by this pathway. These results were further confirmed by a colocalization study with different endocytosis markers (Khalil et al., 2006b). This result points out to a possible explanation of the diversity of results regarding the uptake mechanism of PTDs and their cargos since different reports used different peptide densities. In addition, our results suggest that a high peptide density is required to effectively internalize the particles away from the classical endocytosis, a finding that had high implications when designing an efficient nonviral gene delivery system.

The uptake via macropinocytosis is not highly efficient in most cell types unless the cells are stimulated by mitogenic factors (Swanson and Watts, 1995). We hypothesized that the R8 peptide can act as a mitogenic factor to initiate ruffle formation and stimulate macropinocytosis and a high peptide density is required for this stimulation (Khalil et al., 2006b). Kaplan et al. showed that the TAT peptide could stimulate the uptake via macropinocytosis (Kaplan et al., 2005). In addition, Nakase et al. showed that the R8 peptide induced a significant rearrangement in the cytoskeleton, similar to the stimulation of macropinocytosis by epidermal growth factor (Nakase et al., 2004). The data we obtained were consistent with these reports. R8-Lip-HD were able to stimulate the uptake of neutral dextran, a known fluid phase endocytosis marker (Khalil et al., 2006b). In addition, the R8-Lip-HD could stimulate the macropinocytosis-mediated uptake of the R8-Lip-LD.

We propose that the cell surface HSPGs have a regulatory role in the uptake of R8-Lip. Liposomes modified with a high R8 density may strongly bind to the cell surface through ionic interactions with the cell surface HSPGs. This strong and multiple binding in a localized area may promote proteoglycans (PGs) multimerization. PGs clustering initiates phosphorylation of their cytoplasmic domains leading to a significant rearrangement in the cytoskeleton to stimulate macropinocytosis. Clustering of the PGs under the influence of cationic particles and the cytoskeleton rearrangement in the presence of R8 were previously reported (Belting, 2003; Nakase et al., 2004).

We have previously reported that the uptake of complexes formed directly between R8 or STR-R8 and DNA are mainly internalized by clathrin-mediated endocytosis (Khalil et al., 2004) and are mainly trapped in endosomes (Akita et al., 2004). Meanwhile, the uptake of high density R8 liposomes occurs mainly via macropinocytosis. The difference between peptides complexed with DNA or attached to liposome surface indicates

that the nature of the interaction between the peptide and the cell surface is important in stimulating macropinocytosis. The peptide should be in a free state, i.e. not complexed, not bound, and freely exposed to the cell surface in a high concentration, to trigger uptake via clathrin-independent pathways. This also demonstrates the importance of the lipid envelope in controlling the topology of the peptide to determine the entrance route, since the ability to stimulate macropinocytosis is higher for the R8 peptide on the liposome surface compared to the R8 complexed with DNA. Our data suggest that complexing the peptide with DNA may deteriorate the peptide ability to act as a mitogenic factor to stimulate clathrin-independent uptake. We point out that the peptide should be properly presented on the carrier for such stimulation to occur. Therefore, the peptide density and topology are two important factors among other factors, which control the uptake mechanism of PTD-cargos and these factors collectively should be carefully taken into consideration while designing a drug delivery system based on the PTD peptides.

5. Octaarginine-modified liposomes: controlled intracellular trafficking

It was important to examine the intracellular trafficking of R8-Lip, not only to define its intracellular pass but also to use this information to optimize the system for maximum activities. An important issue is the intracellular fate of particles internalized via CME or macropinocytosis. The particles internalized via CME eventually degraded in lysosomes unless a specific functional device to enhance the cytosolic delivery is included (Schmid, 1997; Khalil et al., 2006a). In contrast, macropinosomes in most cell types fuse only with other macropinosomes and do not extensively fuse with lysosomes (Swanson and Watts, 1995). This is expected to enhance the survivals of the particles inside the cells. In accordance, the intracellular fluorescence in the case of R8-Lip-LD containing a rhodamine aqueous phase was dramatically reduced after a chase time of 6 h (Khalil et al., 2006b). In the case of R8-Lip-HD containing a rhodamine aqueous phase, a high punctuated intracellular fluorescence could be observed after a 6 h chase time, indicating that the liposomes remain intact during that time. However, this result could not exactly determine the extent of escape from macropinosomes. The detected fluorescence may represent liposomes in the cytosol or trapped in macropinosomes. In either case, the liposomes seem to be resisting degradation. In accordance with the hypothesis that macropinocytosis is responsible for the enhanced survival of R8-Lip-HD, the intracellular behavior of R8-Lip-LD was changed when the internalization was shifted to macropinocytosis using non-labeled R8-Lip-HD (Khalil et al., 2006b). In this case, the behavior was similar to the R8-Lip-HD since a high intracellular fluorescence could be observed after a 6-h chase time. This confirmed that the uptake via macropinocytosis enabled the particles to resist in the cells for a longer time.

To confirm the avoidance of lysosomal degradation in the case of macropinocytosis, we examined the colocalization between different R8-Lip and LysoSensor, which stains acidic compartments like late endosomes and lysosomes. R8-Lip-LD were highly colocalized with lysosomes, supporting the hypothesis that their degradation occurs in lysosomes (Khalil et al., 2006b). This also indicated that these liposomes do not have an efficient ability to escape from the endocytic compartment. Meanwhile, R8-Lip-HD were only partially colocalized with lysosomes (Khalil et al., 2006b). The fraction colocalized with lysosomes may reflect the fraction that is internalized through CME (∼35%). The fraction that is not colocalized with lysosomes, which is the dominant fraction, may represent liposomes either in macropinosomes or escaped to the cytosol. The low colocalization between R8-Lip-HD and lysosomes is consistent with other reports showing low colocalization between TAT, Antp, R8 peptides or TAT–GFP fusion protein with lysosomes (Ferrari et al., 2003; Caron et al., 2004; Fischer et al., 2004).

Wadia et al. showed that the TAT-Cre fusion protein could escape from macropinosomes since the removal of a stop region from the construct and the detection of GFP could be observed, which requires the nuclear delivery of the construct (Wadia et al., 2004). The authors suggested that this might occur due to the inherently leaky properties of the macropinosomes compared to other intracellular vesicles (Meier et al., 2002). The enhanced activity of the fusion protein in the presence of a pH-sensitive fusogenic peptide suggested that the particles existed in an acidic compartment and also indicated that the escape from macropinosomes was an inefficient process. In the case of R8-Lip-HD, at least a fraction could escape from macropinosomes, migrate towards and bind to the nucleus (unpublished data). Although we could not observe a clear nuclear delivery of R8-Lip, the existence of the liposomes near the nucleus is useful, since they will be more easily internalized when the cell divides. For example, microinjecting DNA far from the nucleus showed much lower gene expression than when DNA is microinjected near the nucleus (Elouahabi and Ruysschaert, 2005). Torchilin et al. reported a similar slow migration of TAT-liposomes and even TAT-liposomes/DNA complexes to the perinuclear region (Torchilin et al., 2001, 2003). The escape from macropinosomes can be explained either by the leakiness of macropinosomes, or by the existence of an unknown mechanism by which the R8 peptide could mediate the escape. It was suggested that adenovirus triggers macropinosome formation and leakage and this is instrumental for viral escape from macropinosomes for infection (Meier et al., 2002). It is not confirmed whether the R8 peptide may act in the same way to increase the leakiness of the macropinosomes or not. Collectively, the R8 peptide may be a useful tool for enhancing the nuclear migration and binding of conjugated molecules.

6. Octaarginine-modified nano-particles for gene delivery

The use of PTD peptides to improve gene delivery through different strategies was reported earlier. Direct complexation of TAT or R8 peptides to DNA was shown to produce complexes capable of transfection (Rudolph et al., 2003). Contrary to this, another report did not observe any activities through direct complexation with PTD peptides (Hyndman et al., 2004). The addition of hydrophobic moieties to short PTD peptides

was shown to enhance the activities (Futaki et al., 2001b). TAT peptide facilitated gene transfer in combination with cationic liposomes (Hyndman et al., 2004). The activities of Lipofectin lipoplexes were improved by simple mixing of the liposomes and DNA with the TAT peptide. Although the mechanism of this enhancement was not clarified, the uptake was shown to occur by endocytosis and the activities were improved by chloroquine. Torchilin et al. reported an efficient gene delivery in vitro and in vivo and reduced cytotoxicity of complexes formed by TAT-liposomes and DNA (Torchilin et al., 2003).

To investigate the potential of R8-Lip in gene delivery, we adopted a technology based on forming a core–shell structure, which resembles an envelope-type virus (Kogure et al., 2004). The DNA was condensed and coated with a lipid envelope modified with the R8 peptide. The encapsulation of DNA inside the liposomes is more advantageous than the method of direct complexation of the cationic liposomes with DNA, since it provides more protection for the DNA and the lipid envelope can control the topology of the functional devices to exert their activities. The DNA is separated from the outer R8 peptide by the lipid envelope. Thus the peptide remains free and available to interact with the plasma membrane. Since how the R8 is incorporated into complexes or particles determines the uptake mechanism as explained earlier, the topology of R8 should be important in exerting its function. Similar results showing the importance of the topology of peptides have also been reported for the fusogenic peptide GALA (Kakudo et al., 2004). These results clearly show the importance of the lipid envelope in controlling the topology of the equipped devices to exert their function.

Both high and low density R8-Lip with a lipid envelope consisting of EPC and Chol efficiently internalized the DNA encapsulated in their cores. However, the internalized R8-Lip-LD did not show any significant gene expression (Khalil et al., 2006b). The reason of this low transfection is probably the inadequate intracellular trafficking rather than the lack of cellular uptake. This was confirmed by the high transfection activities of the same particles in the presence of the endosome disrupting drug chloroquine (unpublished data). This is on line with the intracellular trafficking study of the R8-Lip-LD, which showed that the particles are extensively degraded in lysosomes. The R8-Lip-HD produced remarkable gene expression even in the absence of any device to enhance the endosomal destabilization, indicating that the particles can escape from macropinosomes by their own (Khalil et al., 2006b). The difference in gene expression levels between DNA-containing R8-Lip-LD and R8-Lip-HD was ~3 orders of magnitude while the difference in the internalization was ~7-fold. This confirms that the intracellular fate of the particles is improved by increasing the peptide density. In the presence of chloroquine, the difference in gene expression between R8-Lip-LD and R8-Lip-HD was around ~8 folds (unpublished data), which can be explained by the difference in the amount internalized in each case.

It is generally believed that the intracellular trafficking is closely related to the entry pathway. The results presented before indicated that the uptake via macropinocytosis is more productive in terms of gene expression than the classical CME since it avoids lysosomal degradation. The contents of macropinosomes may be easily released to the cytosol since they are assumed to be leaky compared to other endocytic vesicles (Meier et al., 2002). In accordance, blocking internalization of R8-modified nanoparticles through macropinocytosis caused a ~95% inhibition in gene expression while blocking CME caused ~35% inhibition (Khalil et al., 2006b). These data confirm that the uptake via macropinocytosis is the main contributor to the efficient gene expression, indicating the significance of macropinocytosis as a potential entrance pathway for improving transfection.

We tested the transfection activities of R8-Lip-HD encapsulating plasmid DNA in NIH3T3 cells. The lipid envelope was composed mainly of the non-fusogenic lipids EPC and Chol or with the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS). In the presence of DOPE, the transfection activities were increased by more than one order of magnitude (Khalil et al., 2006b). The role of DOPE in enhancing endosomal escape has been reported in several reports (Farhood et al., 1995; Zuhorn et al., 2005; Wasungu and Hoekstra, 2006). Therefore, the enhanced activities of the fusogenic R8-Lip may occur due to the enhanced endosomal escape of the fraction of the particles, which was internalized

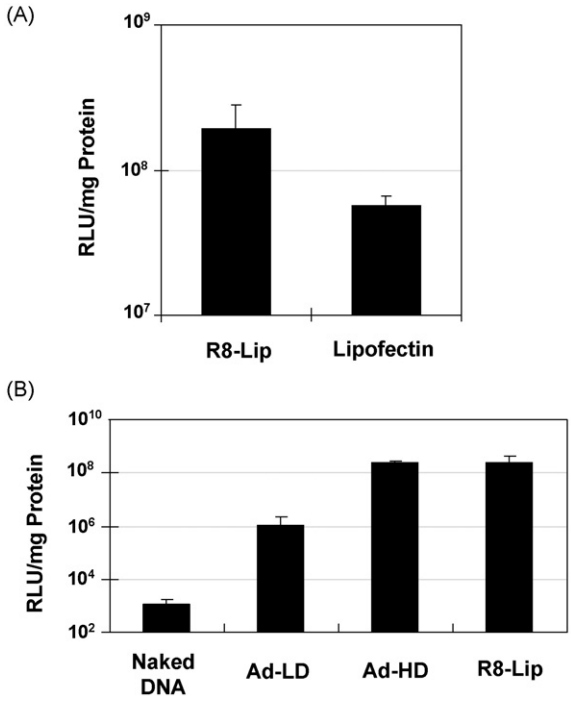

Fig. 2. Transfection activities of R8-Lip encapsulating plasmid DNA. (A) Comparing R8-Lip and Lipofectin. NIH3T3 cells were transfected with R8-Lip encapsulating a luciferase reporter gene (lipid envelope: DOPE/CHEMS/STR-R8) or with complexes formed between plasmid DNA and Lipofectin. Luciferase activities were measured 24 h after transfection and expressed as relative light unit (RLU) per milligram of protein. Data represent the mean and S.D. of three different determinations performed in triplicate. (B) Comparing R8-Lip and Adenovirus. HeLa cells were transfected with naked plasmid DNA, with plasmid DNA encapsulated in R8-Lip (DOPE/CHEMS/STR-R8) or with low (LD) or high dose (HD) of Adenovirus (5×10^3 or 1×10^5 particles per cells). Luciferase activities were measured 48 h after transfection and expressed as relative light unit (RLU) per milligram of protein. Data represent the mean and S.D. of three different determinations performed in triplicate.

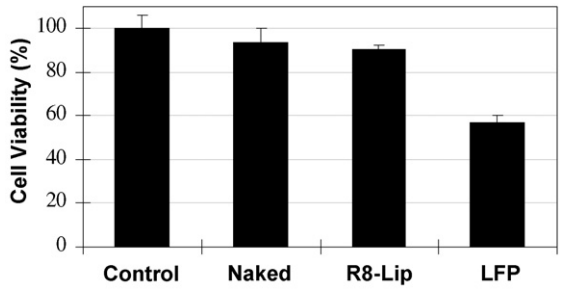

Fig. 3. Cytotoxicity of R8-liposomal systems evaluated by MTT assay. Cells were treated with serum-free medium containing naked plasmid DNA, R8-Lip encapsulating a luciferase reporter gene or Lipofectamine-Plus/DNA complexes (LFP) for 3 h (dose of DNA was 0.4 μg/well). Medium containing serum was then added and incubation was continued for 45 h. The medium was then removed and the cells were subjected to MTT assay. Cell viability was expressed as percent of the viability of the non-treated cells (control).

by clathrin-mediated endocytosis. Another possibility is that the fusogenic lipid can further enhance the release of the particles from macropinosomes. In either case, the presence of devices that can enhance endosomal escape appears to be essential for achieving high transfection activity of particles modified with the R8 peptide. Similar results showing an improvement of the nuclear delivery of TAT–Cre fusion protein in the presence of a pH-sensitive fusogenic peptide were reported (Wadia et al., 2004).

As a result, the rationally designed R8-modified nanoparticles produced high transfection activities in vitro. The activities were comparable to Adenovirus, an efficient viral vector, and were superior to the common lipofection reagent, Lipofectin (Khalil et al., 2007 and Fig. 2). In addition, the system produced minimum cytotoxicity and it was safer than both Adenovirus and Lipofectamine Plus reagent as judged by measuring the protein content and by an MTT assay of cell viability (Khalil et al., 2007 and Fig. 3). It is reported elsewhere that the protein transduction domains are non-toxic both in vitro and in vivo (Jarver and Langel, 2004), while the cationic lipids used in most of the commercially available reagents show high toxicity when used at high doses. Thus the low cytotoxicity of the R8-Lip is reasonable. The enhanced activity and reduced toxicity compared to available methods were achieved based on a clear strategy, which represents a breakthrough in the field of gene delivery. Other advantages of the R8-liposomes include the possibility of further modification of the lipid envelope by other devices and the reasonable diameter of the particles, which makes it more suitable for in vivo use.

7. Conclusion

The R8-Lip presented in this review are highly promising as drug and gene delivery systems. They represent an initial step towards achieving an efficient and safe gene therapy. The R8 peptide proved to be a promising smart functional device for manipulation of gene vectors. It has the ability to not only enhance the cellular uptake, but also control the intracellular trafficking. Its inert properties and biodegradability make it an important tool to increase the safety profile. The proper manipulation of the PTD peptides is important to extract their maximum therapeutic benefit.

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