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Novel endosomolytic peptides for enhancing gene delivery in nanoparticles

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ABSTRACT

Trapping in the endosomes is currently believed to represent the main barrier for transfection. Peptides, which allow endosomal escape have been demonstrated to overcome this barrier, similarly to the entry of viruses. However, the design principles of such endosomolytic peptides remain unclear. We characterized three analogs derived from membrane disrupting antimicrobial peptides (AMP), viz. LL-37, melittin, and bombolitin V, with glutamic acid substituting for all basic residues. These analogs are pH-sensitive and cause negligible membrane permeabilization and insignificant cytotoxicity at pH 7.4. However, at pH 5.0, prevailing in endosomes, membrane binding and hemolysis of human erythrocytes become evident. We first condensed the emerald green fluorescent protein (emGFP) containing plasmid by protamine, yielding 115 nm diameter soluble nanoplexes. For coating of the nanoplex surface with a lipid bilayer we introduced a hydrophobic tether, stearyl-octa-arginine (SR8). The indicated peptides were dissolved in methanol and combined with lipid mixtures in chloroform, followed by drying at RT under a nitrogen flow. The dry residues were hydrated with nanoplexes in Hepes, pH 7.4 yielding after a 30 min incubation at RT, rather monodisperse nanoparticles having an average diameter of 150–300 nm, measured by DLS and cryo-TEM. Studies with cell cultures showed the above peptides to yield expression levels comparable to those obtained using Lipofectamine 2000. However, unlike the polydisperse aggregates formed upon mixing Lipofectamine 2000 and plasmid, the procedure described yields soluble, and reasonably monodisperse nanoparticles, which can be expected to be suitable for gene delivery in vivo, using intravenous injection.

1. Introduction

In order to provide a real therapeutic utility, gene therapy requires an efficient uptake and insertion of genes into individual cells [1]. Because of the cytotoxicity and immunogenicity occasionally associated with viral gene delivery, non-viral vectors continue to be actively developed. Two currently studied non-viral approaches are i) polyplexes, i.e. complexes of plasmids and polymers, and ii) lipoplexes, composed of plasmids and cationic lipids [2,3]. However, the transfection levels achieved so far have been inferior to those obtained using viruses. Endocytosis is believed to represent the major cellular route for the uptake of these vectors [4,5], however, after internalization the majority of plasmids in the above complexes remain within endocytic vesicles [5–7]. Accordingly, there is a need to develop means to avoid the entrapment and degradation of plasmids in this compartment [6]. For this utility additives such as membrane disruptive pH-sensitive peptides, fusogenic lipids, lysosomotropic agents, 'synthetic' viruses, toxins, and different polymers have been incorporated into gene delivery systems [2,6,8–17]. Among these, peptides are gaining increasing attention because of the ease of their synthesis and introduction into different gene delivery systems [9,18,19]. Moreover, peptides are biodegradable, of small size, low cytotoxicity and immunogenicity, yet able to promote the escape of plasmids from endosomes, similarly to viruses [9,19–23]. Features such as DNA condensation, membrane translocation together with cellular and nuclear targeting further emphasize the potential use of peptides in gene delivery systems [9,17,20].

Several approaches have been used to optimize the amino acid sequences of cell penetrating peptides to enhance gene delivery by

Abbreviations: aBom, acidic bombolitin V; aLL-37, acidic LL-37; aMel, acidic melittin; AMP, antimicrobial peptides; caAM, calcein acetoxymethylester; Chol, cholesterol; cnDNA, condensed DNA; cryo-TEM, cryo-transmission electron microscopy; DAPI, 4′, 6-diamidino-2-phenylindole; DIC, differential interference contrast; DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle's medium; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; eggPC, egg phosphatidylcholine; emGFP, emerald green fluorescent protein; emGFP-Math1, emerald green fluorescent protein and *Math1* gene construct; EtBr, ethidium bromide; FCS, fetal calf serum; Glu, glutamic acid; HPLC, high-performance liquid chromatography; hRBC, human red blood cells; MTT, (4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; PBS, phosphate buffered saline; PDI, polydispersity index; pDNA, plasmid DNA; pSEP, pH sensitive endosomolytic peptides; SR8, stearyl-octa-arginine; Z_{av}, average hydrodynamic diameter

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Table 1
Amino acid sequences of the wild type and modified AMP.

Peptides	Sequences
LL-37	L L G D F F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S
aLL-37	10 20 30 C L L G D F F **E E** S **E** E **E** I G **E** E F **E E** I V Q **E** I **E** D F L **E** N L V P **E** T E S 10 20 30
Melittin	G I G A V L K V L T T G L P A L I S W I K R K R Q Q
aMel	10 20 C G I G A V L **E** V L T T G L P A L I S W I **E E E E** Q Q 10 20
Bombolitin V	I N V L G I L G L L G K A L S H L
aBom	10 C I N V L G I L G L L G **E** A L S **E** L 10

Below each sequence is shown the modified sequence with the introduced substitutions by Glu underlined and bold.

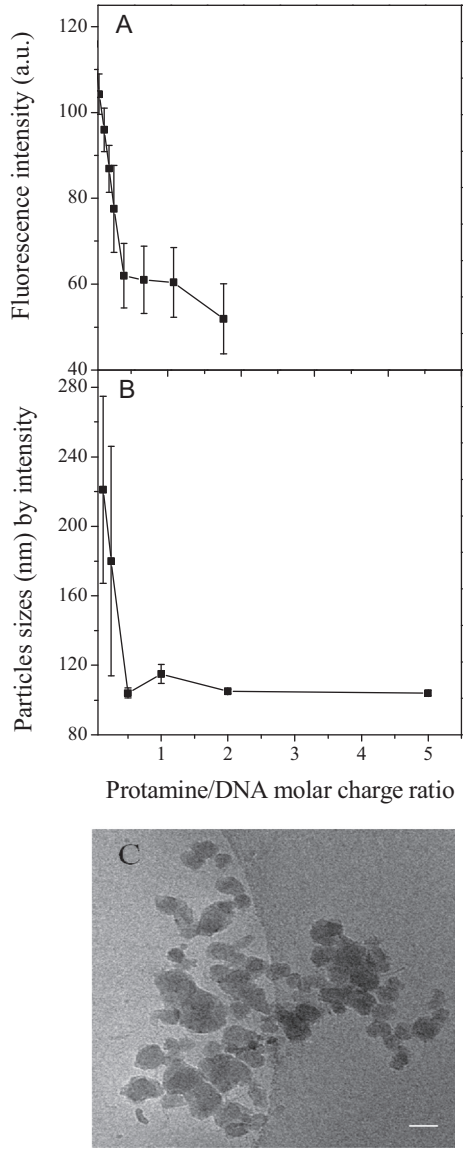

Fig. 1. Panel A: Condensation by protamine of the emGFP-Math1 plasmid monitored by the EtBr displacement assay. Panel B: Sizes of protamine–DNA complexes determined by DLS with increasing concentrations of protamine sulfate. Both panels depict mean and standard error for three independent experiments. Panel C: Cryo-TEM images of protamine–DNA complexes prepared at 1:1 charge ratio (7.4 μg protamine sulfate and 10 μg DNA) (15.15 nmol in base-pairs). Scale bar = 100 nm.

Table 2
Compositions of the nanoplexes given as molar ratios of the indicated peptides and lipids.

Nanoplexes	aLL-37	aMel	aBom	SR8	DOPE	eggPC	Chol
A				0.1	0.4	0.3	0.2
B	0.015			0.1	0.4	0.285	0.2
C	0.03		0.1	0.4	0.27	0.2	
D		0.03		0.1	0.4	0.27	0.2
E			0.03	0.1	0.4	0.27	0.2

facilitating endosomal escape [17,20,24–32]. pH-sensitive peptides were first developed and utilized, but their toxicity at physiological pH 7.4 is restricting their use [30]. A limited number of endosomolytic peptides have been described in the literature [6,9,24], mostly derived from viral proteins such as HA2, HA2-TAT, INF7, HGP, H5WYG, GALA, KALA, and E5WYG [6,8–12,15,18,23,25], in some cases resulting in a remarkable enhancement of gene expression. Peptides such as listeriolysin O, diphtheria toxin, exotoxin A, shiga toxin, cholera toxin, ricin, saporin, gelonin, and melittin were derived from bacteria, plants, and animals [6,8,13,26]. Also histidine-rich endosomolytic peptides and imadazole containing biopolymers have been made and characterized [20,27–29] for this purpose. However, principles for the design of endosomolytic peptides and their role in gene expression remain incompletely understood.

We here report the characterization of endosomolytic peptides derived from three membrane active antimicrobial peptides (AMP). To understand the molecular basis of their pH-sensitive cellular toxicity, we studied their hemolytic activity by the integrity of human red blood cells (hRBC) assessed by the release of hemoglobin and the calcein acetoxymethylester (caAM) assay. Transfection of 3T3 cells by condensed plasmid incorporating nanoparticles containing these peptides was studied and compared to transfection by the commercial reagent Lipofectamine 2000.

2. Materials and methods

2.1. Materials

Protamine sulfate and caAM were from Sigma. Stearoyl octaarginine (SR8) was from Storkbio (Tallin, Estonia). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), egg phosphatidylcholine (eggPC), and cholesterol (Chol), were from Avanti Polar Lipids (Alabaster, AL). The purity of lipids was checked by thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with a chloroform/methanol/water mixture (65:25:4, v/v/v). Examination of the plates after iodine staining revealed no impurities. Lipid concentrations were determined gravimetrically with a high precision electrobalance [33]. The emGFP-math1 plasmid pcDNA6.2/C-EmGFP-Math1 [34] was used as a model for complex preparation and transfection experiments. Other chemicals were of analytical grade and from standard sources.

Table 3
Particle sizes and polydispersities by DLS for the plasmid DNA (pDNA) nanoparticles before and after the addition of indicated lipids and the peptides (Table 2). The content of the peptides is expressed in terms of mol% of the total amount of lipids and SR8.

Nanoplexes (See Table 2)	Peptide added (mol%)	Z_{av} diameter	PDI
A	None, control	194 ± 42.65	0.37 ± 0.12
B	aLL-37 (1.5 mol%)	198 ± 10.25	0.39 ± 0.04
C	aLL-37 (3 mol%)	927.47 ± 191.9	0.82 ± 0.14
D	aMel (3 mol%)	179.9 ± 17.46	0.27 ± 0.003
E	aBom (3 mol%)	186.23 ± 42.98	0.4 ± 0.16