Ahmad, Aqeel; Rilla, Kirsi; Zou, Jing; Zhang, Weikai; Pyykkö, Ilmari; Kinnunen, Paavo; Ranjan, Sanjeev

Enhanced gene expression by a novel designed leucine zipper endosomolytic peptide

Published in:
International Journal of Pharmaceutics

DOI:
10.1016/j.ijpharm.2021.120556

Published: 15/05/2021

Published under the following license:
CC BY-NC-ND

Please cite the original version:
Enhanced gene expression by a novel designed leucine zipper endosomolytic peptide

Aqeel Ahmad a,b, Kirsi Rilla c, Jing Zou d,e, Weikai Zhang d,f, Ilmari Pyykkö d, Paavo Kinnunen a,1, Sanjeev Ranjan, Ph.D. a,d,e,

a Helsinki Biophysics and Biomembrane Group, Department of Biomedical Engineering and Computational Sciences, Aalto University, Espoo, Finland
b Department of Medical Biochemistry, College of Medicine, Shaqra University, Shaqra, 11961, Saudi Arabia
c Institute of Biomedicine, University of Eastern Finland, 70211 Kuopio, Finland
d Hearing and Balance Research Unit, Field of Otolaryngology, School of Medicine, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
e Department of Otolaryngology, Head and Neck Surgery, Center for Otolaryngology-Head & Neck Surgery of Chinese PLA, Shanghai Hospital, Second Military Medical University, Shanghai, China
f Department of Orthopaedic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

ARTICLE INFO

Keywords:
Endosomolytic peptides
Leucine zipper
Endosomal escape
Gene delivery
Gene expression
Liposome nanoparticles.

ABSTRACT

An endosomal trap is a major barrier in gene therapy. We have designed an endosomolytic peptide based on the leucine zipper sequence and characterized it both structurally and functionally. The results illustrated that leucine zipper endosomolytic peptide (LZEP) exhibited appreciable hemolysis of human red blood cells (hRBCs) at pH 5.0, but negligible hemolysis at pH 7.4. Calcein release experiment indicated that only at pH 5.0 but not at pH 7.4, LZEP was able to permeabilize hRBCs. LZEP revealed significant self-assembly as well as peptide induced α-helical structure at pH 5.0. Unlike at pH 5.0, LZEP failed to self-assemble and showed a random coil structure at pH 7.4. Transfection data depicted that lipoplexes modified with LZEP resulted in significantly higher gene expression as compared to lipoplexes without LZEP. Interestingly, the transfection efficacy of LZEP modified lipid nanoparticles reached the levels of Lipofectamine 2000 (LF 2000), without any cellular toxicity as observed by MTT assay. The results suggest a novel approach for designing endosomolytic peptides by employing the leucine zipper sequence and simultaneously the designed peptides could be utilized for enhancing gene delivery into mammalian cells.

1. Introduction

To develop an efficient nonviral delivery vector for therapeutic applications is a challenge in gene therapy (Friedmann and Roblin, 1972; Guo and Huang, 2012). Lipid-based nanoparticles embody a hopeful approach for gene therapy (Balazs and Godbey, 2011; Karmali and Chaudhuri, 2007; Zhang et al., 2011b). The endocytic pathway is the major cellular uptake mechanism of lipid-based nanoparticles including several other particles such as DNA, protein, and siRNA (Khalil et al., 2017; de Ilarduya et al., 2010; Martin and Rice, 2007; Tjelle et al., 1996). Therefore, from the last few years, it is a matter of debate to design and explore potent endosomolytic agents. These agents would be crucial for the development of an effective delivery system for the endosomal release of the nanoparticles to prevent lysosome mediated digestion (Coles and Toth, 2009; Guo and Huang, 2012; Morris et al., 2000; Pack et al., 2005).

Many endosomolytic agents such as polymers, lipids, and peptides have been utilized in the gene delivery systems for endosomal escape (Chen et al., 2017; de Ilarduya et al., 2010; Martin and Rice, 2007;

ABBREVIATIONS: ANS, 8-anilinonaphthalene-1-sulfonate; bZips, basic zipper proteins; caAM, Calcein-AM; CD, circular dichroism; Chol, Cholesterol; DAPI, 6-Diamidino-2-phenylindole; DMSO, Dimethyl sulfoxide; DOPE, 1, 2-distearoyl-sn-glycerol-3-phosphoethanolamine; Egg PC, egg phosphatidylcholine; FCS, fetal calf serum; FITC, Fluorescein isothiocyanate; GFP, Green fluorescent protein; hRBCs, human red blood cells; LZEP, leucine zipper endosomolytic peptides; S.D., standard deviation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LF 2000, Lipofectamine 2000; PDI, polydispersity index.

* Corresponding author at: Institute of Biomedicine, University of Eastern Finland, Yliopistonranta 1E, 70211 Kuopio, Finland.

E-mail addresses: sanjeev.ranjan@uef.fi, sanjanroy@gmail.com (S. Ranjan).

https://doi.org/10.1016/j.ijpharm.2021.120556
Received 24 August 2020; Received in revised form 12 March 2021; Accepted 29 March 2021
Available online 30 March 2021
0378-5173/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license
light scattering and 8-anilinonaphthalene-1-sulfonate (ANS) binding experiments. In addition, circular dichroism (CD) experiments have been executed to explore the pH-induced secondary structure of this peptide. Also, pH-induced conformation changes in the peptide have been studied by using different bioinformatics software. We have incorporated our peptide in the lipoplexes to find out the function of this peptide in endosomal escape. Transfection efficiency of lipoplexes containing this peptide was determined in 3 T3 cells and compared to the transfection efficiency of the commercially available reagent LF 2000.

2. Materials and methods

2.1. Materials

Protamine sulphate and caAM were from Sigma, 1, 2-distearyl-sn-glycerol-3-phosphoethanolamine (DOPE), egg phosphatidylcholine (egg PC), and cholesterol (Chol), were from Avanti Polar Lipids (Alabaster, AL). The consistency of lipids was inspected by thin-layer chromatography using silicic acid-coated plates. (Merck, Darmstadt, Germany) set up with a chloroform/methanol/water infusing (65:25:4, v/v/v). Assessing the plates following iodine staining exhibited no contaminants. Lipid concentrations were determined gravimetrically with a high precision electrobalance Super G (Kibron, Helsinki, Finland) as described (Tejera-Garcia et al., 2012). The Id2.3 plasmid was used as a model for the complex preparation and transfection experiments (Ahmad et al., 2015). Numerous different chemical compounds were of analytical grades and from standard sources.

2.2. Peptide synthesis and purification

Peptides [Fig. 1] were purchased from Genescript, USA and were synthesized by utilizing standard Fmoc chemistry and purified by HPLC to >97% purity. Mass spectrometry devices were utilized to certify their weight and pureness.

2.3. Assay for hemolysis

The hemolytic activity of designed LZEP was assayed against fresh hRBCs that were collected in the presence of an anticoagulant from a healthy volunteer and washed 3–4 times with PBS (Ahmad et al., 2009b; Ahmad et al., 2015). The hRBCs were then added. Subsequently, after a 30 min incubation at 37 ◦C, the supernatant was assessed by quantifying the supernatant absorbance at 535 nm in a 96-well microplate reader. (SpectraFluor plus, Tecan Gmbh, Salzburg, Austria) fitted with a 535 nm filter (10 nm bandpass). For negative and positive controls, hRBCs were incubated with 1% Triton X-100 (Atriton) and in 0.2% (v/v, final concentration) Triton X-100 (Atriton) were used, respectively. The hemolysis percentage was estimated as specified (Ahmad et al., 2006) from:

% hemolysis = [(A_{sample} - A_{blank}) / (A_{triton} - A_{blank})] × 100.

2.4. Confocal microscopy

Cells transfected with the plasmid were observed and imaged using a confocal microscope, as described before (Zhang et al., 2011a; Zhang et al., 2011b) Cells expressing GFP were counted as positive cells and cells with DAPI counterstained nuclei were defined as a total number of cells (10⁶ cells were counted) using Image J (http://rsb.info.nih.gov/ij/image). Transfection efficiencies were calculated as the ratio of GFP
positive cells with respect to the total number of cells, observed by Olympus IX70 microscope installed with ANDOR IQ camera, with excitation at 488 nm (blue) and 568 nm (green), and an Ar-Kr laser as the source. The relating emission filters (FITC) were 525/50. DAPI was excited employing a 340–380 nm filter and observed utilizing a 500 LP filter.

2.5. Release of calcein

Calcein release experiments were performed as reported earlier with slight modification (Ahmad et al., 2015; Ahmad et al., 2006; Bratosin et al., 2005). Calcein-AM (caAM) is a non-fluorescent and cell-permeable dye, which is and in live cells hydrolyzed in live cells by intracellular esterases yielding a strongly fluorescent calcein anion retained in the cytoplasm. Accordingly, caAM can be used for determining peptide-induced membrane permeability. In our experiments, hRBCs (0.6%, v/v in the indicated buffer) were incubated with 8 μM caAM in PBS for 1 h to allow caAM to produce fluorescent calcein in the cytosol, whereas free caAM and calcein were removed by washing several times with PBS (Ahmad et al., 2015; Boeckle et al., 2006). Afterward, hRBCs were resuspended in either 20 mM Hepes, 150 mM NaCl, pH 7.4 or 15 mM citric acid, 150 mM NaCl, pH 5.0. The indicated peptides were then added, followed by a 30 min incubation at 37 °C. Fluorescence intensities were measured with a Perkin Elmer spectrofluorometer in the time drive mode at an excitation wavelength of 490 nm and an emission wavelength of 515 nm. The calcein release percentage was ascertained from:

\[
\text{% Calcein release} = \left(\frac{F_p - F_c}{F_t - F_c}\right) \times 100
\]

Where \(F_p\) = fluorescence of sample containing peptide.
\(F_c\) = fluorescence of sample without peptide,
\(F_t\) = fluorescence of sample containing triton X-100 (0.01% final concentration).

2.6. 90° Light scattering experiments

90° light scattering experiments of LZEP in either pH 5.0 or 7.4 buffers were performed as described previously (Code et al., 2010). The excitation and emission wavelengths were picked at 400 nm. The excitation and emission slits were remedied at 5 nm. The increasing concentration of peptides were added in the buffer at pH = 5 or pH 7.4 and the relative change in 90-degree light scattering was measured.

2.7. ANS (8-anilinonaphthalene-1-sulfonate)–peptide binding experiment

ANS is an environment-sensitive fluorescence probe that binds on hydrophobic patches (of peptides/proteins) and therefore, used to detect the aggregation properties of peptides/proteins (Ahmad et al., 2009b). ANS has low fluorescence in an aqueous environment and high fluorescence in a hydrophobic environment. Aggregation of LZEP (~20 μM) was determined at pH 5.0 and 7.4. The concentration of ANS was ~20 μM in each experiment. The excitation wavelength and emission wavelength range for ANS were set at 365 and 410–600 nm, respectively. The slits of excitation and emission were 5 nm.

2.8. Circular dichroism (CD) experiments

CD spectra of LZEP at pH 5.0 and pH 7.4 were recorded using Olis RSM CD (Ahmad et al., 2009b; Ahmad et al., 2006). The samples were...
scanned at room temperature in the wavelength range of 195–250 nm. The fractional helicities (Fh) of LZEP peptide were calculated with the help of mean residue ellipticity values by using the following equation as already reported (Ahmad et al., 2006)

\[
F_{h} = \frac{\theta_{222} - \theta_{0}}{\theta_{222} - \theta_{0}} / \theta_{222}
\]

Where \[\theta_{222}\] = Mean residue ellipticity at 222 nm.

\[\theta_{222}\] and \[\theta_{0}\] correspond to 100 and 0% helix contents were considered to have mean residue ellipticity values of –32,000 and –2000, respectively, at 222 nm.

2.9. Plasmid condensation and assembly with lipids

Lipoplexes were constructed as previously described (Ahmad et al., 2015; El-Sayed et al., 2009). In brief, DOPE: EggPC: Chol: Stearylated-R8 (in a molar ratio of 4:0.3:0.2:0.1) with or without LZEP were dissolved in chloroform and the solvent was removed using a gentle stream of nitrogen. The dry lipid film was secured under low pressure for so many hours to expel decipher quantities of chloroform. The plasmid (Id2.3) (10 μg) compacted by protamine sulfate (7.4 μg) was compiled at a molar charge ratio of 1:1 in 10 mM Hepes, pH 7.4, and was preferred for lipid film hydration for 30 min at room temperature. In this manner, it was exposed to bath sonicator for 1 min. Id2.3 plasmid was used for the preparation of these lipoplexes (El-Sayed et al., 2009). The dynamic light scattering at 25°C was exploited to evaluate the apparent hydrodynamic diameter (Zav) and polydispersity index (PDI) for lipoplexes (Table-1) (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) as reported elsewhere (Ahmad et al., 2015; Zhang et al., 2011b).

2.10. Lipoplexes modified with LZEP mediated gene transfection

NIH 3 T3 (ATCC, Boras, Sweden) was blossomed with 10% Fetal Calf Serum (FCS) (Invitrogen, USA) and 4 mM L-glutamine (Sigma Aldrich, USA) in Dulbecco’s Eagle reconfigured medium (DMEM, Sigma, St Louis, MO, USA) (Zhang et al., 2011a; Zhang et al., 2011b). The cells were trypsinised and replaced one day before transfection in a 4-well chamber slide with 1.5 × 10⁶ cells in each well in 1 ml of FCS-glutamine-DMEM medium (Lab-Tek II, Nalge Nunc International, Naperville, USA). NIH 3 T3 cells were nurtured in DMEM medium, without serum and incubated with 0.1 mM LZEP adapted lipoplexis for 5 h, scrapping the transfection medium with fresh complete medium and implanting the cells for another 36 h (Zhang et al., 2011a; Zhang et al., 2011b). Control cells were also transfected with Id2.3 plasmids according to the instructions provided with the commercially accessible Lipofectamine 2000 (Invitrogen, USA). The cells were flushed with PBS three times for 1 min after 36 h post-transfection and resolved for 30 min with 4% paraformaldehyde. The 4’, 6-Diamidino-2-phenylindole dye (DAPI, 10 ng/ml, Sigma-Aldrich, USA) was utilized for counter-staining the nuclei for 10 min and cells were then mounted with Fluoromount (Sigma, USA) for confocal fluorescence microscopy.

2.11. Assay for cell viability

The survival of NIH 3 T3 cells was ascertained to monitor the potential toxicity of lipoplexes(Ahmad et al., 2009b; Ahmad et al., 2015; Meyer et al., 2007). 10⁴ cells per well had already been studded in 96-well plates and reared overnight in a CO2 incubator for adherence. Complete media were dumped from the plate, and 200 μL of incomplete media per well were introduced. After that, LZEP modified lipoplexes (0.1 mM total lipid) were added and the plates were incubated for 5 h. The medium was subsequently substituted with new complete medium, and the cells implanted for the next 36 h. In each well 20 μL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-i phenyltetrazolium Bromide) (5 mg / mL) solution was appended, and the plates were reared again. The media in 96-well plates were omitted and 200 μL of DMSO was inserted into each well after a three-hour incubation to abolish the MTT crystal. The wells with no sample applied were assumed to be 100% viable. The plates were assessed with a microplate reader for absorption at 550 nm.

3. Results

3.1. Design principles for LZEP

Leucine zipper is a special kind of heptad repeat in which a and d positions are occupied mostly by hydrophobic amino acids such as leucine, isoleucine, or phenylalanine (Ahmad et al., 2009a; Ahmad et al., 2009b; Ahmad et al., 2006; Blackwood and Eisenman, 1991; Grigoryan and Keating, 2008; Landschulz et al., 1988; Liu et al., 2006). Generally, leucine zipper sequence contains a basic region followed by a hydrophobic region which forms coiled coil structure (Ahmad et al., 2009b; Ahmad et al., 2006; Blackwood and Eisenman, 1991; Ciani et al., 2010; Landschulz et al., 1988; Liu et al., 2006; O’Neil et al., 1990). Self-assembly of peptide containing this motif plays a pivotal role in cell toxicity (Ahmad et al., 2011; Ahmad et al., 2009b; Ahmad et al., 2006). Accumulating data as well as our previous reports suggested that acidic amino acid containing peptides display significant membrane destabilizing activity at lower pH (Ahmad et al., 2015; Boeckle et al., 2006; Madhakir and Harashima, 2009; O’Neil et al., 1990). We assumed that it could be possible to design a leucine zipper peptide containing an acidic region followed by a hydrophobic region which will self-assemble at low pH and subsequently form pores in mammalian cells. Therefore, a leucine zipper containing peptide was designed which contain leucine at a or d position and other positions contains either negatively charged amino acids glutamic acid or leucine. As shown in Fig. 1A, positions a, c, d, and g contain hydrophobic amino acids leucine whilst b, e, and f contain negatively charged amino acids glutamic acids. As shown in Fig. 1, in this designed peptide, amino acids are arranged in such a way that one face of peptide contains a hydrophobic region, and another face a hydrophilic region. This peptide contains 3 heptad repeat of 7 amino acids sequence LEELLE. Cysteine was included at their N terminus for coupling the maleimide group containing fluorophores. Fig. 1A, 1B, and 1C depict the amino acid sequences, helical wheel projections and Schiffer, and Edmundson wheel projections of newly designed LZEP. Fig. 1D is showing the hydrophobic interactions between a and a’ or d and d’ positions of a parallel coiled coil leucine zipper motif which is an important factor for oligomerization of this kind of peptide.

3.2. LZEP showed appreciable endosomolytic activity

To determine the endosomolytic activity of designed LZEP, dose-dependent hemolytic activity assay was performed against human red blood cells (hRBC) as shown in Fig. 2. The data are expressed as means ± S.D. for three independent experiments.

![Fig. 2. Determination of dose-dependent hemolysis of hRBC by the designed LZEP at pH 5.0 and 7.4](image-url)
blood cells (hRBCs) at pH 5.0 as well as at 7.4. Interestingly, LZEP exhibited remarkable hemolysis against hRBCs at pH 5.0 (Fig. 2). However, at pH 7.4, the negligible hemolysis of hRBCs was observed. As shown in Fig. 2, at ~20 μM, LZEP showed ~40% hRBCs hemolysis at pH 5.0 whilst only ~2.5% at pH 7.4 with the same peptide concentration. Taken together, the results suggested that the designed LZEP show significant and negligible hemolytic activity at pH 5.0 and 7.4, respectively.

3.3. LZEP exhibited membrane permeabilizing activity at endosomal pH 5.0

To check the pore-forming ability of LZEP, the release of calcein from calcein-entrapped hRBCs in the presence of these peptides was measured at pH 7.4 and 5.0. At pH 5.0, LZEP induced the release of calcein from calcein-entrapped hRBCs, as shown by the dose-dependent increase in the percentage of calcein release (Fig. 3). However, at pH 7.4, LZEP failed to induce the release of calcein from calcein-entrapped hRBCs at the same peptide concentration. As shown in Fig. 3, LZEP showed ~90% calcein release at a peptide concentration of ~10 μM at pH 5.0. On the other hand, at pH 7.4, LZEP showed almost negligible calcein release at the same peptide concentration.

3.4. Detection of aggregation properties of LZEP

The tendency of self-assembly of designed LZEP at pH 5.0 and 7.4 was analyzed by using TANGO software (Fernandez-Escamilla et al., 2004; Mahalka and Kinnunen, 2009). The prediction results suggested that LZEP had more aggregation tendency at pH 5.0 as compared to pH 7.4 (Supplementary Fig. 1). To confirm the prediction result, self-assembly of LZEP was determined by 90° light scattering experiments at pH 5.0 and 7.4. Clearly, LZEP did not show any substantial increase in light scattering with an increase in peptide concentration (Fig. 4A) at pH 7.4. On the other hand, at pH 5.0, LZEP showed a remarkable increase in light scattering on increasing the peptide concentration, indicating the self-association of these peptide molecules. To further support this observation, an ANS-binding experiment was performed at pH 5.0 and 7.4. As shown in Fig. 4B, at pH 7.4, in the presence of peptide ANS showed negligible fluorescence with emission maxima at ~525 nm which was almost similar to free ANS emission maxima at pH 5.0 (~530 nm) as well as at pH 7.4 (~525 nm). However, at pH 5.0, in the presence of LZEP, the fluorescence of ANS increased significantly with an emission maximum at a shorter wavelength at ~460 nm, suggesting of binding of the ANS to the hydrophobic region of peptide which probably resulted from the self-association of the peptide.

![Fig. 3. Detection of pore-forming activity of the designed LZEP as tested by the calcein release from hRBCs at pH 5.0 and 7.4. Panel A represents the profile of calcein release from hRBCs at pH 5.0 and 7.4 induced by designed LZEP. The concentration of LZEP in each experiment was 10 μM. Panel B represents the plots of the percentage of calcein release from hRBC induced by the increasing concentration of designed LZEP at pH 5.0 and pH 7.4. In each experiment CaAM concentration was 8 μM. The data are expressed as means ± SD for 2–3 independent experiments.](image)

![Fig. 4. Detection of aggregation properties of designed LZEP at pH 5.0 and 7.4. (A) Relative 90° light scattering experiment to determine the aggregation tendency of LZEP. (B) ANS-binding experiment to detect LZEP aggregation. The peptide concentration for each peptide was ~20 μM and the ANS concentration was ~20 μM. Symbols: Solid Square, LZEP at pH 5.0; Solid circle, LZEP at pH 7.4; Open Square, Free ANS at pH 5.0; open circle, free ANS at pH 7.4.](image)
3.5. Unlike at pH 7.4, LZEP adopted significant α-helical structure at pH 5.0

The tendency of the α-helical forming propensity of designed LZEP at pH 5.0 and 7.4 was analyzed by using Agadir software (At pH 5.0 or 7.4, ionic strength = 0.1, temperature = 25 °C) (Munoz and Serrano, 1994). The predictions results suggested that LZEP had a more α-helical forming propensity at pH 5.0 as compared to pH 7.4 (Supplementary Fig. 2). To support this result, the structure of the designed LZEP was also predicted. The model of this peptide was generated by using protein structure predication software; LOMETS (MUSTER) (Zheng et al., 2019) . Further, to validate this model, the model of this peptide was also produced by using PEP-FOLD program (Shen et al., 2014) . Afterward, the structure was generated by UCSF chimera software (Fig. 5A and supplementary Fig. 3). To further confirm this observation, CD experiments were performed to determine the secondary structures of LZEP at pH 5.0 and 7.4. At pH 5.0, LZEP adopted a significant α-helical structure (65%), which is evident from the shape of the spectra (Fig. 5B). In contrast, at pH 7.4, LZEP adopted a random coil structure. Taken together the results suggested that LZEP failed to adopt a secondary structure at pH 7.4 and formed an appreciable secondary structure at pH 5.0.

3.6. Characterisation of LZEP modified lipoplexes

Liposomes used in this study contain stearylated-R8 cationic peptide which allows binding with the anionic part of designed LZEP. Different concentrations of LZEP were used to prepare different types of lipoplexes. The sizes of lipoplexes were determined by dynamic light scattering. As shown in table 1, the size of lipoplexes containing 1–2 mol % of the peptide was 200–300 nm whilst 3 mol% containing lipoplexes were 300–400 nm in diameter. However, lipoplexes modified with 5 mol % of peptides formed large aggregates sized > 1000 nm.

<table>
<thead>
<tr>
<th>Lipoplexes</th>
<th>Peptide modification (mol%)</th>
<th>Zav</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPX-1</td>
<td>0</td>
<td>267.5</td>
<td>0.504</td>
</tr>
<tr>
<td>LPX-2</td>
<td>1</td>
<td>230.2</td>
<td>0.252</td>
</tr>
<tr>
<td>LPX-3</td>
<td>2</td>
<td>224.9</td>
<td>0.269</td>
</tr>
<tr>
<td>LPX-4</td>
<td>3</td>
<td>317.5</td>
<td>0.435</td>
</tr>
<tr>
<td>LPX-5</td>
<td>5</td>
<td>1254</td>
<td>0.848</td>
</tr>
</tbody>
</table>

3.7. Transfection efficiency of LZEP modified lipoplexes

Gene delivery with lipoplexes has been described in many papers, but transfection efficiency in most of these reports is poor. Accumulating data suggests that endosomal escape is a major factor responsible for low gene expression of these types of nanoparticles. Therefore, we had modified the lipoplexes with our designed LZEP which could escape from endosomes by rupture the endosomal membrane and checked their transfection efficiency in 3 T3 cells. The transfection efficiency of these lipoplexes was concentration dependent. As shown in Fig. 6, at low peptide concentration (1 and 2 mol%) these lipoplexes showed efficient gene expression. At higher peptide concentration (3 and 5 mol%) their transfection efficiency decreases dramatically because of the aggregation of these particles. Lipoplexes without peptide had ~6% transfected cells; however, LZEP modified lipoplexes depicted ~ 23.5% (Fig. 7) transfected cells which almost reach to the level of LF 2000 (~26% transfected cells).

3.8. Cell viability of NIH 3 T3 cells

The viability of 3 T3 cells was examined using MTT assay with lipoplexes in the absence or presence of designed LZEP. As shown in Fig. 8, lipoplexes (0.1 mM) used in this study showed no remarkable decrease in cell viability.

4. Discussion

We have utilized an amphipathic leucine zipper sequence to generate unique pH-sensitive endosomolytic peptides which depicted remarkable toxic activity at pH 5.0 and simultaneously non-toxic at pH 7.4. The formidable endosomolytic activity of implemented LZEP was recognized by hemolytic and calcium release experiments (Figs. 2 and 3). There are a plethora of findings including our previously reported evidence that additament of acidic amino acids in a peptide sequence makes the peptide active at lower pH (Ahmad et al., 2015; Martin and Rice, 2007; Smith et al., 2018; Wagner, 1999). Furthermore, several anionic peptides are extremely lytic at a lower pH and assist in rupturing the endosomal membrane in line with our current findings (Ahmad et al., 2015; Boeckle et al., 2006; Cupic et al., 2019; El-Sayed et al., 2009; Haas and Murphy, 2004; Meyer et al., 2007; Mudhakir and Harashima, 2009; Varkouhi et al., 2011; Yu et al., 1994). Previously, in our lab three naturally occurring antimicrobial peptides namely Melittin, LL-37 and Bomolitin V were engineered to generate many pH-sensitive endosomolytic peptides, by substituting every basic amino acid by Glutamine (Ahmad et al., 2015). These designed peptides had asserted high hemolytic activity with a pH = 5.0, but had also been observed to have a negligible toxic activity at a pH = 7.4 (Ahmad et al., 2015).

Self-assembly/aggregation of peptides/proteins portrays a vital part in a toxic activity. Quite few peptides are self-assembled in water solution or membrane or membrane mimetic environment and formed pores in the membrane (Ahmad et al., 2011; Ahmad et al., 2009b; Ahmad et al., 2006). Moreover, certain viruses contain proteins that become aggregated at lower pH, and these aggregated particles in the endosomal membrane are extremely toxic and formed pores and thus in this way, they escape from endosomes (Ahmad et al., 2019; Ahmad et al., 2015;
Mudhakir and Harashima, 2009; Varkouhi et al., 2011). Such viral proteins restrain the sequence of acidic amino acids (Ahmad et al., 2019; Li et al., 2004; Mudhakir and Harashima, 2009; Plank et al., 1994). Research achievements mandated that amino acids are partially positive charged at lower pH due to the protonation and de-protonation process of carboxylic groups of these amino acids (Ahmad et al., 2019; Ahmad et al., 2015; Boeckle et al., 2006; Meyer et al., 2007; Plank et al., 1994; Smith et al., 2018). We have recently crafted many peptides that indicated high lytic activity in lower pH like several other anionic peptides available in the literature to date (Ahmad et al., 2019; Ahmad et al., 2015; Boeckle et al., 2006; El-Sayed et al., 2009; Li et al., 2004). Likewise, our designed LZEP becomes membrane-active at lower pH because of the protonation of the carboxylic group. As indicated in Fig. 4, LZEP aggregates at lower pH and form pore in the membrane and this result is reliable with many previously published results (Ahmad et al., 2011; Ahmad et al., 2019; Ahmad et al., 2015; Boeckle et al., 2006; Meyer et al., 2007; Varkouhi et al., 2011). On the other hand, these peptides were not aggregated at pH 7.4 and thus did not form pores. The aggregation state of these peptides was estimated by TANGO software. This program postulated that LZEP had a greater propensity to aggregate at pH 5.0 as compared to pH 7.4 which qualitatively correlated well with our experimental data (Supplementary Fig. 1).

It is very nice documented from Contents in literature that the formation of α-helical structure is a very indispensable parameter for determining the biological activities of several peptides. Many pH-sensitive endosomolytic peptides undergo conformational change on lowering the pH, resulting in the formation of a α-helical structure that

Fig. 6. Detection of GFP expression in NIH 3 T3 cells by confocal microscopy. Cells were transfected using LZEP modified lipoplexes. (LPX-1) Lipoplexes composed of DOPE: EggPC: Chol: Stearylated-R8 (molar ratio, 0.4:0.3:0.2:0.1) and Id2.3 plasmid. (LPX-2) Above lipoplexes with 1 mol% designed LZEP. (LPX-3) Lipoplexes with 2 mol% LZEP. (LPX-4) Lipoplexes with 4 mol% LZEP. (LPX-5) Lipoplexes with 5 mol% LZEP. (LF 2000) For comparison of transfection efficiencies, the cells were also transfected with commercial transfection reagent LF 2000. Scale bar = 100 μm.

Fig. 7. Determination of the percentage of cells transfected by indicated lipoplexes in NIH 3 T3 cells. GFP expressions of transfected NIH 3 T3 cells were quantified by Image J software. The data are expressed as means ± SD for three independent experiments.

Fig. 8. Determination of cell viability of NIH 3 T3 cells using MTT assay in the presence of indicated lipoplexes. The concentration of lipoplexes in each experiment was 0.1 mM (total lipid). The viability of cells (without any additions) cultured in media was considered as 100%. The data are expressed as means ± SD for five independent experiments.
induces lysis of mammalian cells (Cupic et al., 2019; Li et al., 2004; Wagner, 1999). One of the well-studied peptides, GALA is a random coil at pH 7.4 and forms a significant α-helical structure at pH 5.0 (Haas and Murphy, 2004; Li et al., 2004). Similarly, LZEP also formed a significant α-helical structure at pH 5.0 and was a random coil at pH 7.4 (Fig. 5). The secondary structure of this peptide was also determined by Agadir software at pH 5.0 as well as at 7.4. The prediction results indicated that the α-helical forming propensity of this peptide was more at pH 5.0 compared to pH 7.4 which was qualitatively matching with our CD results (Supplementary Fig. 2). Probably, the formation of a significant helical structure at pH 5.0 in LZEP was accountable for membrane destabilizing activity of this peptide such as other pH-sensitive peptides available at hand.

We know that lipopolices are one of the safest and most promising approaches for gene therapy (Ahmad et al., 2019; Balazs and Godbey, 2011; de Ilardiyua et al., 2010; Karmali and Chaudhuri, 2007; Zhang et al., 2011b; Zou et al., 2009). It is very well established that most of the lipopolices are degraded within the endosomes of cells during gene delivery (Ahmad et al., 2019; Balazs and Godbey, 2011; de Ilardiyua et al., 2010; Tiele et al., 1996; Varkouhi et al., 2011; Zhang et al., 2011b). Because of this, the lipid-based transfection system has very low gene expression (Ahmad et al., 2019; de Ilardiyua et al., 2010; Zhang et al., 2011b). In this paper, our goal was to design a potent endosomolytic peptide and incorporate it into the lipipeptide delivery system to escape from endosomes or lysosomes. Such a system would have high gene expression because of endosomal escape similar to our previous earlier results (Ahmad et al., 2015). We have incorporated our designed LZEP in liposome nanoparticles and studied their transfection efficiency in 3 T3 cells (Figs. 6 and 7). Lipopolices without LZEP indicated very low gene expression. However, lipopolices containing an appropriate amount of LZEP displayed very high gene expression hereby comparable with LF 2000. Lipopolices containing a higher amount of LZEP depicted a low level of expression because of aggregation at higher concentrations. Previously, we have also found that a higher concentration of negatively charged endosomolytic peptides causes aggregation of lipopolices and reduced the gene expression (Ahmad et al., 2015).

The viability of cells incubated with LZEP modified lipopolices was examined using the MTT assay. Interestingly, as highlighted in Fig. 8, LZEP-modified lipopolices point out no significant toxicity to cells. However, at higher peptide concentration, for example, LPS-5 exhibited very little toxicity against 3 T3 cells. Although here we were able to develop an efficient lipid-based gene delivery system, further work is necessary to empathize with the intra-cellular trafficking and endosomal escape mechanism of these peptide-based delivery vectors.

5. Conclusion

To the finest of our comprehension, this is the first study on the design of endosomolytic peptides based on the leucine zipper sequence. Our previous and present findings altogether suggested that one could also design potent smaller size endosomolytic peptides based on these approaches. This peptide could be utilized to manufacture peptide-modified lipopolices which are demanding candidates for industrial, research, or in the nanomedicine field. Detail works on peptides based gene therapy are underway in our lab with a strong hope that our data, data available in the literature, and further research would help in developing efficient gene therapy for treating diseases in upcoming years.

Author contribution

Aqeel Ahmad: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. Kirsi Rilla: Resources, Funding acquisition. Jing Zou and Weikai Zang: Cell culture experiments. Ilmari Pyykkö: Resources, Funding acquisition. Paavo Kinnunen: Resources, Funding acquisition, Supervision. Sanjeev Ranjan: Conceptualization, Methodology, Writing - review & editing, and Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This paper is dedicated to the memory of the late Prof. Paavo Kinnunen.

This study was conducted in the European Community 6th framework program; contract NMP4-CT-2006-026556, NANOEAR, aimed at targeted drug & gene delivery into the cells of the inner ear. This work was also financially supported by the KR personal grant (project number 64814), which was financed by the Jane and Aatos Erkko Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120556.

References