Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron
Tetrahedron 63 (2007) 12207–12214

The synthesis and characterisation of a novel dendritic system for gene delivery

Daniel J. Coles,[a] Shu Yang,[b] Anna Esposito,[a] Deanne Mitchell,[b] Rodney F. Minchin[b] and Istvan Toth[a,*]

[a]*School of Molecular and Microbial Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia*
[b]*School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia*

Received 6 June 2007; revised 4 September 2007; accepted 20 September 2007
Available online 26 September 2007

Abstract—There has been increasing interest in recent years in gene delivery. We report the synthesis of non-viral delivery systems composed of variations of the cell penetrating peptide TAT, a nuclear localisation signal peptide and dendritic polylysine. The delivery systems were tested for their ability to form complexes with plasmid DNA by utilising gel shift analysis, isothermal titration calorimetry, particle size analysis, zeta potential and transmission electron microscopy. These techniques indicated the successful formation of complexes between the peptide dendrimer and DNA.

1. Introduction

Gene delivery is the process where a gene of interest is delivered to a specific tissue with the aid of a vector.[1] The delivery of naked nucleic acids (NAs) is not effective as they are degraded very quickly by nucleases and they do not passively diffuse across plasma membranes due to their size and negative charge. Therefore, the vector selected is required to neutralise the negative charge and condense the NA to allow transfer across cell membranes. Optimal requirements for gene delivery systems include protection of nucleic acids from nucleases, transportation into cells, the ability to release NAs in the cytoplasm or nucleus, targeting specific cell types and no toxicity.[2] Vectors for gene delivery are classified as viral and non-viral.

Traditionally, viral vectors were used for gene delivery into the host for long-term expression.[3] They are very efficient gene-transfer vectors. However, they often require specific conditions to operate and they may induce an immune response.[4] In one clinical trial in 1999, a participant died from what was believed to be a severe immune response to the adenovirus vector.[5] Due to the immunogenic effects of viral vectors, non-viral delivery systems are now widely studied to enhance gene delivery. They are less immunogenic, easier to manufacture and in some cases cheaper than viral vectors.[6] Many different non-viral gene delivery systems have emerged in recent years.[7] They are designed to show the same efficacy as viral vectors but without the associated problems. Some have already been commercialised. However, these are inefficient for in vivo use due to high toxicity.[8] Most commonly used non-viral delivery systems include liposomes,[9] polymers (PEI)[10] and dendrimers (PAMAM).[11] More recently, cell penetrating peptides (CPPs) have been used for gene delivery. They are short cationic peptides such as TAT, which is derived from the human immunodeficiency virus transcription activating factor (amino acids 58–60).[12] They have the ability to translocate cell membranes and carry with them any cargo they are attached to such as NAs and proteins.[13] Another peptide used in gene delivery is the nuclear localisation signal (NLS) peptide. It is derived from the Simian virus 40 large T antigen and has the ability to translocate the nuclear membrane thereby effectively targeting DNA to the nucleus.[14]

Previously, we have successfully used dendrimers that incorporated lipoamino acids, polylysine and carbohydrates for the delivery of a sense oligonucleotide (ODN-1).[15–18] We have also used polylysine dendrimers ligated to specific ligands to deliver DNA to cells both in vitro and in vivo.[19–21] We have now synthesised new dendrimers incorporating the TAT peptide (GRKKRRQRRRPPQ),[22] an NLS peptide (PKKKRKV)[23] and polylysine. They are shown in Figure 1. Each peptide dendrimer was synthesised to determine which peptides or combinations of peptides are required to be the most effective in gene delivery. However, an important attribute for any DNA carrier is its ability to reversibly bind to and condense DNA into a nanoparticle structure. This is determined by its ability to neutralise the negatively charged DNA molecule. Below, we describe the synthesis of a library of peptide dendrimers and their ability to form complexes with pGL3 DNA. These complexes were characterised by

Keywords: Gene delivery; Dendrimer; TAT; NLS.
* Corresponding author. Tel.: +61 7 33469892; fax: +61 7 33654273; e-mail: i.toth@uq.edu.au

0040–4020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2007.09.048

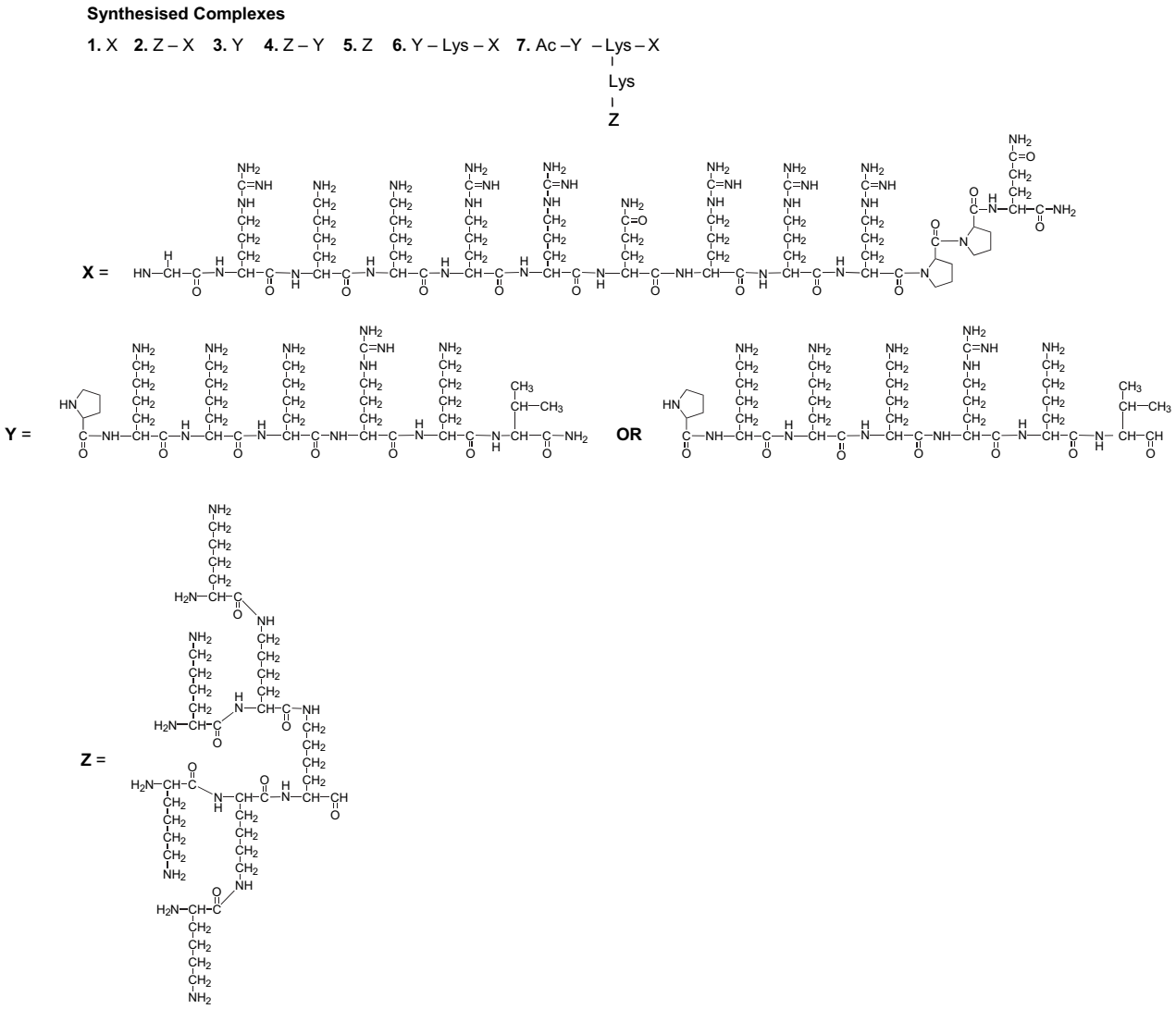

Figure 1. Structures of the synthesised dendrimers. **1**, TAT; **2**, TAT–D; **3**, NLS; **4**, NLS–D; **5**, D; **6**, TAT-Lys-NLS; **7**, TAT-Lys-NLS(Ac)-D (Ac—acetate).

gel shift analysis, isothermal titration calorimetry, particle size analysis, zeta potential and transmission electron microscopy.

2. Results and discussion

2.1. Design and synthesis of the gene delivery complexes

There are many barriers to overcome to have success in gene delivery. Therefore, this requires a delivery system with different functionalities. First of all, DNA is large and negatively charged which give it little hope of passing through the negatively charged plasma membrane unaided. To effectively condense and neutralise the DNA, we have used dendritic polylysine with four terminal lysine residues giving it a charge of 8^+. Previous experiments using the same polylysine were effective in forming complexes with a single stranded oligonucleotide.[16] Stability of the gene complex is essential as nucleases readily digest any gene that is unshielded. The first barrier in the cellular delivery of a gene complex is the plasma membrane. There are numerous ways to cross the membrane such as using lipids for adsorptive endocytosis,[9] carbohydrates for active or facilitated transport,[24] or CPPs, which utilise endocytosis for membrane penetration.[25] Of the many CPPs already under investigation such as penetratin[26] and oligoarginine,[22] we have chosen TAT.[22] It has been widely used in the past and has advantages over viral carriers such as low toxicity and fast cellular uptake.[25] The cellular uptake of TAT has been reported to occur by many different ways including clathrin and caveolae mediated endocytosis and macropinocytosis.[25] Once the gene complex is inside the cell, it is required to enter the nucleus for gene expression to occur. One such peptide used to aid nuclear transport is the NLS peptide. This peptide utilises the nuclear pore complex (NPC), which is responsible for the trafficking of molecules into the nucleus. The interaction between the NPC and the NLS peptide has allowed complexes up to 50 MDa to translocate the nuclear membrane.[27]

Each of the peptides mentioned above were synthesised separately and in different combinations to determine which particular peptide or combination of peptides is required for the most efficient delivery of DNA (Fig. 1). Dendrimer **1** is TAT. Dendrimer **2** is TAT coupled with polylysine.

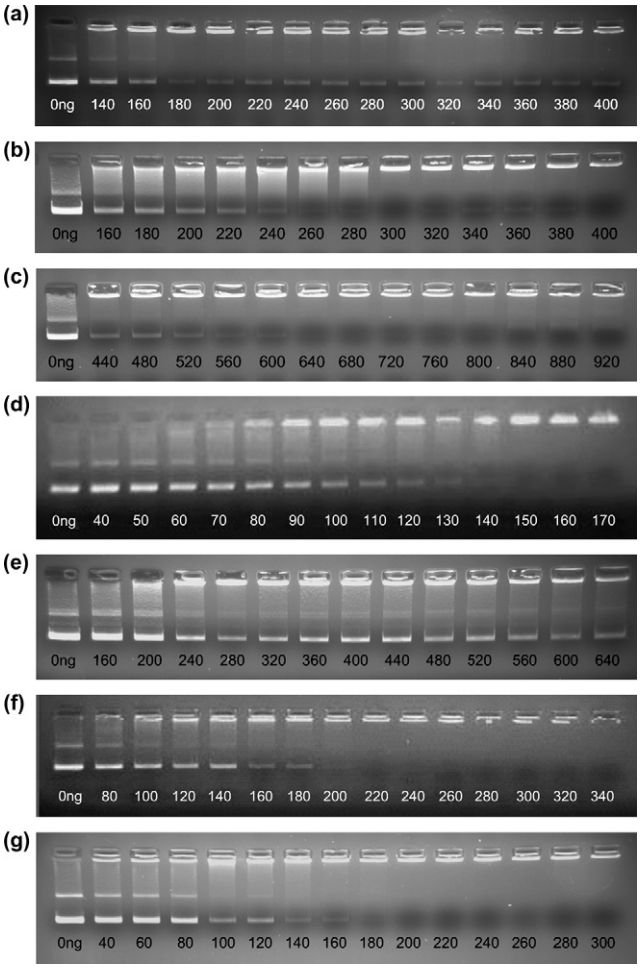

Figure 2. Gel shift assay of the dendrimer/DNA complexes. The first lane is a control with pGL3 DNA only. The following lanes contain complexes with increasing amounts of peptide dendrimer (indicated under each lane). Gels were viewed under UV light. pGL3 DNA complexed with dendrimer: (a) **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5**, (f) **6** and (g) **7**.

Dendrimer **3** is NLS. Dendrimer **4** is NLS coupled with polylysine. Dendrimer **5** is polylysine. Dendrimer **6** is TAT coupled with NLS linked by lysine. Dendrimer **7**, which consists of all three peptide components, required the use of an orthogonal protection group strategy during synthesis. In our case, we used Boc-Lys(Fmoc) to link the TAT and NLS peptide by selectively removing the Boc group. The N-terminus was acetylated. The Fmoc protecting group on the side chain of the lysine was then removed followed by the coupling of Boc-Lys(Fmoc). The Boc protection was selectively removed allowing the sequential coupling of Boc-Lys(Boc) to afford the desired dendritic lysine. The Fmoc from the lysine side chain was removed with the possibility of other moieties to be added (not included in this study). The terminal Boc groups were removed and once the peptide was separated from the resin it was purified by HPLC so that the purity was $\geq$95%.

2.2. Gel shift

Gel shifts have been widely used to report the interaction between polycationic carriers and DNA.[28] To determine the ratio of dendrimer to pGL3 DNA required for condensation, a gel mobility assay was employed (Fig. 2). Lane 1 is a control which shows unbound DNA. The two bands correspond to the supercoiled (lower band) and open circular (upper band) forms of plasmid DNA.[29] The following lanes contain increasing amounts of dendrimer complexed to the DNA. As the amount of peptide dendrimer increases, the intensity of the DNA band decreases and shifts to the wells of the gel. This is due to the dendrimer forming complexes with the DNA that are too large to pass through the gel and also the neutralisation of the DNA. Both forms of DNA are condensed at a similar rate. However, the open circular form has completely shifted prior to the supercoiled form. This may be due to the open circular form forming larger complexes earlier than the supercoiled form. Also, the open circular form is present at a lower concentration than the supercoiled form because the DNA is highly purified. The minimum amount of dendrimer that completely retarded the pGL3 DNA in the well was used to estimate the condensation ratio. In general, this was approximately equivalent to a charge ratio of 1:1. A small amount of unbound DNA was observed (unknown reason) when it was condensed with dendrimer **5** (Fig. 2e).

2.3. Isothermal titration calorimetry

The titration of dendrimers **1**–**7** with pGL3 DNA is exothermic, as heat is released during the interaction of the dendrimer with the DNA. Once this is complete, there may be endothermic or exothermic heats of dilution. The titration of dendrimer **3** only showed exothermic heats even after a large excess had been added. All other dendrimers have a charge ratio around 1:1 when the interaction between the dendrimer and DNA is complete (Table 1). These results correlate well with the gel shift results. While it may be viewed as a simple charge interaction, the complexation between peptide dendrimer and DNA is more complicated. It has been reported that polylysine carriers exhibit cooperative binding to DNA.[30] That is, once one carrier is bound to DNA the following carrier binds more easily. However, our experiments were limited in the amount of DNA used, therefore not suitable to obtain thermodynamic parameters. Our dendrimers contain either a linear peptide (**1**, **3** and **6**)

Table 1. Isothermal titration calorimetry results

Dendrimer	Volume of dendrimer (μL)	Concentration of dendrimer (mM)	Moles of dendrimer	Molar ratio (dendrimer/DNA)	Charge ratio (+/−)
1	24	0.2	4.8E-09	1130/1	0.86/1
2	28	0.1	2.8E-09	659/1	1.00/1
3	—	1.0	—	—	—
4	36	0.1	3.6E-09	848/1	1.05/1
5	35	0.2	7.0E-09	1648/1	1.25/1
6	30	0.1	3.0E-09	706/1	0.94/1
7	20	0.1	2.0E-09	471/1	0.99/1

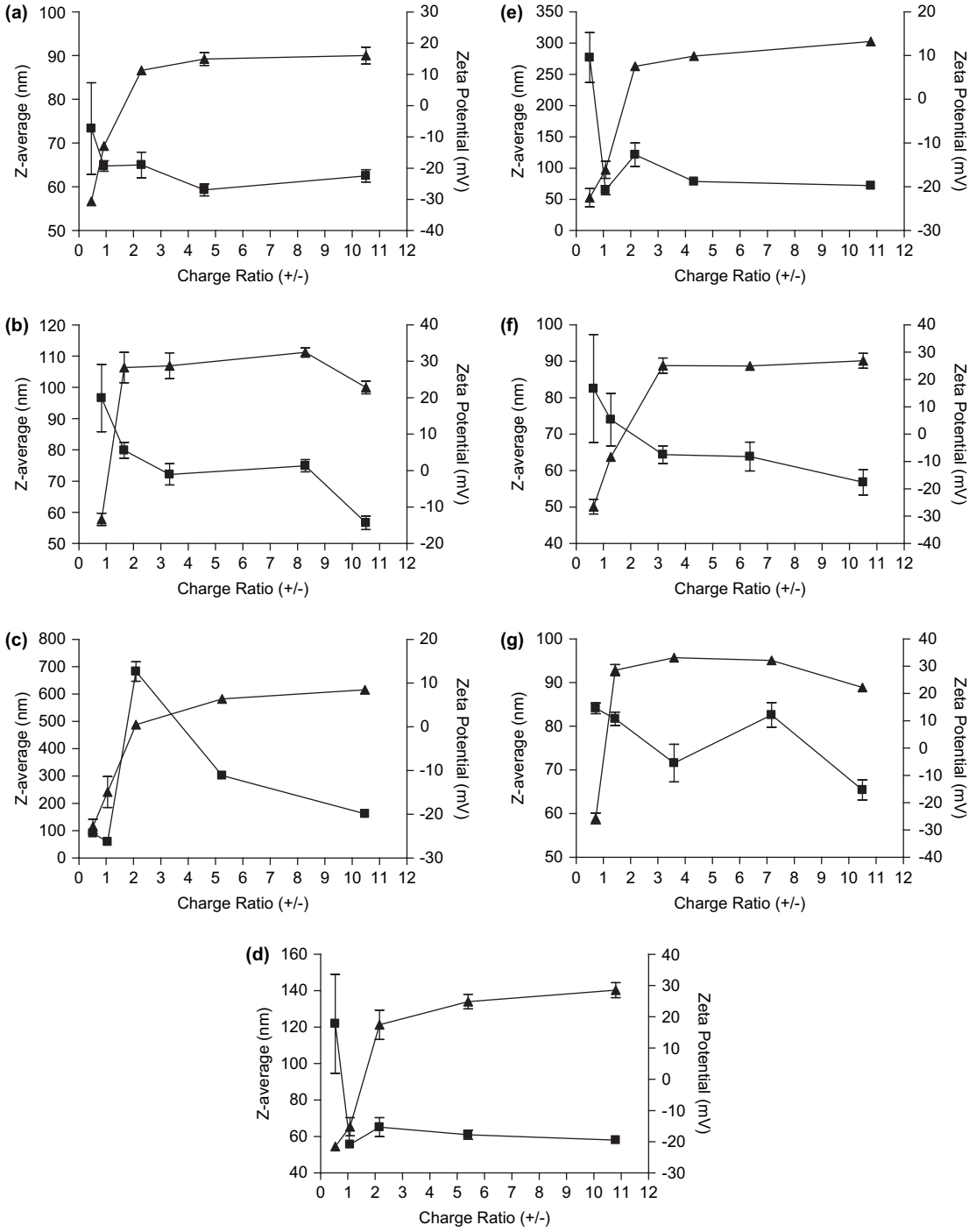

Figure 3. Particle size (z-average) and zeta potential of dendrimer/pGL3 DNA complexes. pGL3 DNA complexed with dendrimer: (a) **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5**, (f) **6** and (g) **7**. Measurements were conducted at five different charge ratios (P:N). Square (■) indicates z-average and triangle (▲) indicates zeta potential. All data presented as mean±SEM, n=3.

a branched peptide (**5**) or both (**2**, **4** and **7**). The differences between the structures of the dendrimers may determine how they bind to the DNA. Dendrimer **3** is a short peptide (seven amino acids) with a charge of 5^+. The inability of dendrimer **3** to interact with DNA in the ITC experiments is unknown. Experiments using higher concentrations of a different DNA resulted in a standard binding curve (data not shown). Dendrimer **3** was shown to condense DNA in the gel shift assay, where the dendrimer and DNA are more concentrated than in the ITC experiments.

2.4. Particle size and zeta potential

The z-average (particle diameter) and zeta potential analysis for the dendrimer/pGL3 DNA complexes are shown in Figure 3. At lower charge ratios (<2) there is a lot more variability between the triplicates analysed compared to the case when a higher charge ratio (>2) is used. The z-average of the dendrimer/pGL3 DNA complexes generally decreases (60–80 nm) as the charge ratio increases. Of the different cell uptake mechanisms that the TAT peptide may utilise,

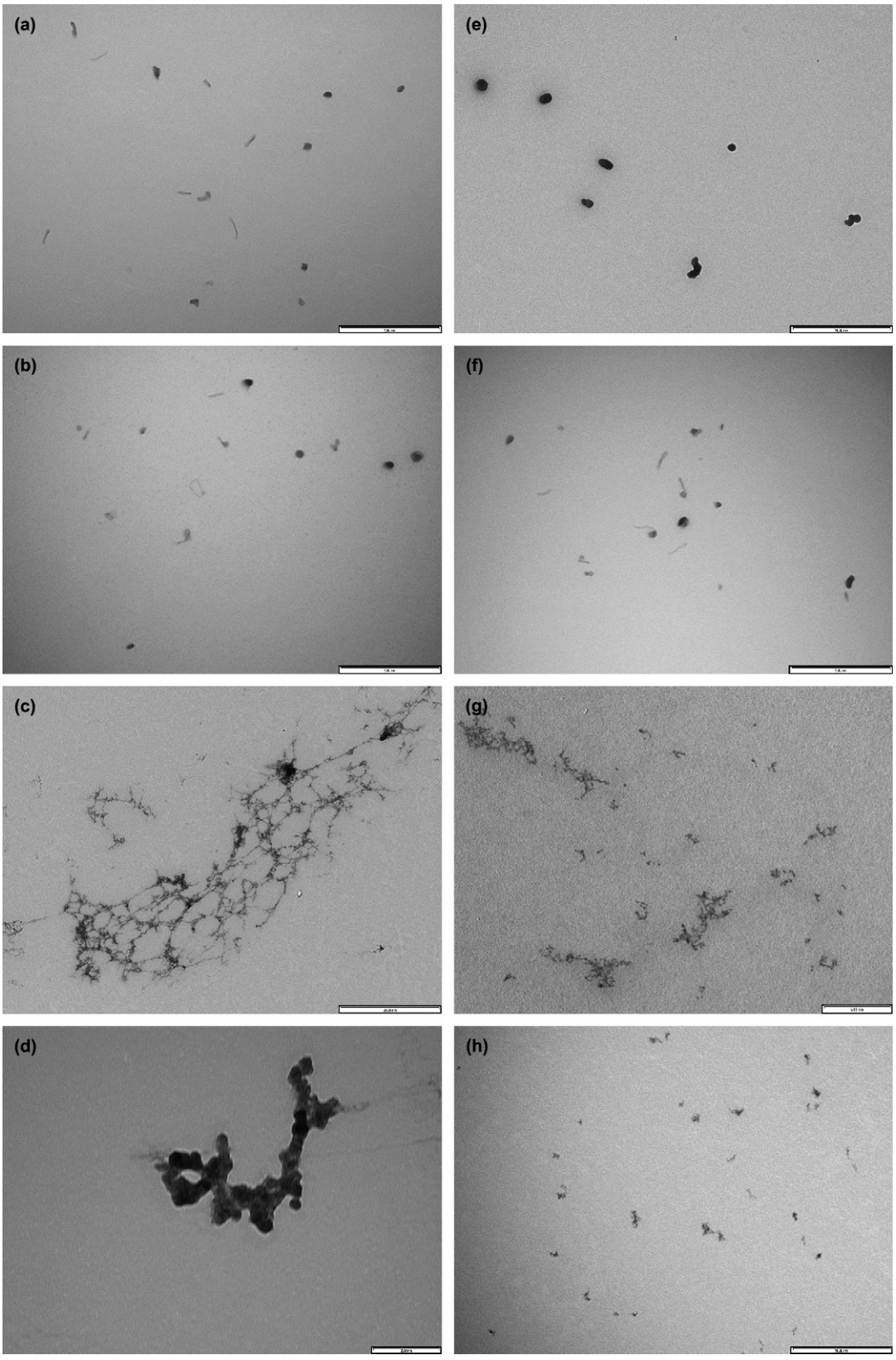

Figure 4. Transmission electron microscopy photographs of pGL3 DNA complexed with dendrimer: (a) **1** (bar 500 nm), (b) **2** (bar 500 nm), (c) **3** (bar 2000 nm), (d) **4** (bar 200 nm), (e) **5** (bar 1000 nm), (f) **6** (bar 500 nm), (g) **7** (bar 500 nm) and (h) DNA only (bar 1000 nm).

clathrin-mediated endocytosis is the most likely mechanism as our complexes are below 100 nm.[31] There are many factors that affect the size of a complex, such as the buffer used and its concentration and the duration between complex formation and measurement. While we have not addressed these issues here, the particle size of polylysine/DNA complexes has been shown to increase as the duration between complex formation and measurement increases.[32] Our

complexes could be regarded as relatively stable because they are very small, even though measurements occurred 1 h after complex formation. Dendrimer **3** (Fig. 3c) shows an interesting result for particle size at a charge ratio of 2. It has a very large *z*-average compared to the other charge ratios. This may be due to the formation of aggregates. The zeta potential, which measures the surface charge of a particle, is negative when there is an excess of DNA (charge ratio $\leq$1). As the amount of dendrimer increases, the zeta potential increases becoming positive and plateaus out. The zeta potential for dendrimers **1**, **3** and **5** reached 10–15 mV compared to dendrimers **2**, **4**, **6** and **7**, which show zeta potentials up to 30 mV. This may be due to the latter group of dendrimers containing more positive charges.

2.5. Transmission electron microscopy

The particle size measurements have shown the average size of the particles but not their structure. Transmission electron microscopy (TEM) experiments of the dendrimer/pGL3 DNA complexes were conducted to determine their physical characteristics. The complexes were found to be rods and spheres as shown in Fig. 4 (DNA alone is shown in Fig. 4h). The average size of a single sphere was measured to be 50 nm in diameter and the length of a rod was 100 nm, which indicates good correlation with the *z*-averages obtained. Aggregates were also present, which included a fibre-like network (Fig. 4c) and a cluster of particles (Fig. 4d). The aggregation of complexes containing dendrimer **3** also correlates with the large *z*-averages obtained when measuring the particle size. The formation of aggregates could occur due to electrostatic and hydrophobic interactions between the dendrimer and DNA.[33] However, these results are not indicative of the size of the particles in solution. The formation of aggregates may be due to the drying of the sample, which was required prior to analysis.

3. Conclusions

We have synthesised dendrimers consisting of TAT, NLS and polylysine and have shown that they form complexes with pGL3 DNA using various methods. These data significantly help for the best preparation of complexes for in vitro studies and in the future design of dendrimers. The optimal dendrimer will be determined from in vitro studies by examining cellular uptake and gene expression, which are reported elsewhere.

3.1. Concluding remarks

The lack of suitable gene delivery vectors necessitates the continued search for appropriate vectors in this area. Our research may provide a non-toxic, generally applicable gene delivery complex suitable for human use.

4. Experimental

4.1. Materials

p-4-Methyl benzhydryl amine (MBHA) resin and Boc-L-amino acids were purchased from NovaBiochem (Switzerland) or Reanal (Budapest, Hungary). Peptide synthesis grade dimethylformamide (DMF), trifluoroacetic acid (TFA) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Auspep (Melbourne, Australia). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand) or Honeywell-Burdick & Jackson (Morristown, NJ). All other reagents were purchased from Sigma–Aldrich (Castle Hill, Australia) at the highest available purity.

4.2. General procedure for the synthesis of dendrimers 1–7

Dendrimers **1**–**7** were synthesised by solid phase peptide synthesis using Boc chemistry.[34] *p*-MBHA resin (100–200 mesh, 0.31 mmol/g loading) was swollen in DMF in a sintered glass peptide synthesis vessel for 1 h. Each Boc-L-amino acid (4 equiv) was activated in a mixture of HBTU (0.5 M in DMF, 4 equiv) and *N*,*N*-diisopropylethylamine (DIPEA) (6 equiv) and then mixed with the resin for 30 min. Coupling efficiency was monitored using the negative ninhydrin reaction (5 min) and showed nearly quantitative coupling ($\geq$99.6%). The Boc group was removed using neat TFA (2$\times$1 min) followed by in situ neutralisation. Side chain protecting groups used include Lys(2-Cl-Z), Lys(Fmoc), Arg(Tos) and Gln(Xan). Between all manipulations, the resin was washed thoroughly with DMF. Dendrimer **7** was synthesised using an orthogonal protection group strategy. Boc-Lys(Fmoc) was coupled after the TAT peptide. N^{α}-Boc was removed, followed by coupling of the NLS peptide in a stepwise fashion. N-terminal acetylation was achieved by treating the resin with acetic anhydride (1 mL), DIPEA (0.5 mL) and DMF (10 mL) twice for 30 min. With the N-terminus protected, Lysine N^{ε}-Fmoc deprotection was performed using 20% piperidine in DMF (5 min and 20 min). Boc-Lys(Fmoc) was then coupled after which the Boc protection was selectively removed followed by the addition of Boc-Lys(Boc) until four terminal lysines are present. Lysine N^{ε}-Fmoc deprotection was again performed allowing other moieties to be added to the dendrimer (not included in this study). Upon completion of the dendrimer formation, terminal Boc groups were removed and the resin washed exhaustively with DMF, dichloromethane and methanol. The resin was dried over KOH under vacuum. The dendrimers were cleaved from the resin using hydrogen fluoride (10 mL/g resin) and *p*-cresol (10%) at 0 °C for 2 h. The cleaved dendrimers were precipitated in diethyl ether and then redissolved in 50% ACN and lyophilised to give a white amorphous powder.

4.3. Purification

The crude dendrimers were analysed using analytical reverse phase-high performance liquid chromatography (RP-HPLC) on a Shimadzu instrument (LC-10AT liquid chromatograph, SCL-10A system controller, SPD-6A UV detector, an SIL-6B auto injector with an SCL-6B system controller and column C18 (Zorbax, 3.5 μm pore size, id=4.6, 150 mm)) to identify the dendrimers' retention time and establish an appropriate gradient for preparative HPLC. Preparative HPLC was performed on a Waters HPLC system (Model 600 controller, 490E UV detector, F

Table 2. Analytical data of the purified dendrimers

Dendrimer	MW	ES-MS (*m*/*z*)	Retention time in acetonitrile (min)	Retention time in methanol (min)
1	1718.03	1718.9 (*z*=1) 860.1 (*z*=2) 574.3 (*z*=3)	9.49	12.56, 13.11
2	2615.24	1309.2 (*z*=2) 873.2 (*z*=3)	11.25	14.34
3	882.15	882.9 (*z*=1) 442.4 (*z*=2)	9.61	10.81
4	1779.36	1781.0 (*z*=1) 891.2 (*z*=2) 594.8 (*z*=3)	10.76	13.21
5	914.24	915.2 (*z*=1) 458.4 (*z*=2)	8.91	9.2
6	2711.32	1357.2 (*z*=2) 905.3 (*z*=3)	11.34	14.63
7	3778.74	1891.3 (*z*=2) 1260.9 (*z*=3) 946.5 (*z*=4)	11.82	15.68

pump and TSK Gel C18 column with 10 μm pore size and 22 mm id) with 100 mg of crude dendrimer. It was separated using a gradient of solvent A (0.1% TFA/H_2O) and solvent B (90% ACN/0.1% TFA/H_2O) and the fractions collected were characterised by electro-spray mass spectroscopy (ES-MS) (Perkin–Elmer API 3000 instrument). The purified dendrimers were analysed by ES-MS and analytical RP-HPLC using solvent A (0.1% TFA/H_2O) and either solvent B1 (90% ACN/0.1% TFA/H_2O) or solvent B2 (90% MeOH/ 0.1% TFA/H_2O) (Table 2).

4.4. Gel shift

A gel shift was used to determine how the peptide dendrimers interact with pGL3 DNA. Various amounts of dendrimer in 20 mM Hepes buffer were mixed with 250 ng pGL3 DNA at room temperature. The complexes were then electrophoresed through a 1% agarose gel in tris acetate EDTA (TAE) buffer containing 1 μg/mL ethidium bromide at 80 V for 45 min. Gels were viewed under UV illumination.

4.5. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC Microcalorimeter (MicroCal Inc, USA) with Origin software and VPViewer 2000. In all titrations the micro-syringe contained the peptide dendrimer solution in 20 mM Hepes buffer, the sample cell pGL3 DNA (2.36 nM) and the reference cell 20 mM Hepes buffer. Before measurements were taken all solutions were degassed. Injections occurred every 4 min and the reaction was kept at a constant temperature of 25 °C. The concentration of pGL3 DNA remained the same in each experiment.

4.6. Particle size and zeta potential

A Zetasizer Nano ZP instrument (Malvern Instruments, UK) with DTS software was used for particle size and zeta potential measurements of the dendrimer/pGL3 DNA complexes. Sizes were analysed using a non-invasive backscatter system and zeta potentials were measured using M3-PALS technique. Measurements were taken at 25 °C with scattering angle of 173° using disposable capillary cuvettes. Complexes were prepared by diluting various amounts of peptide dendrimer with 500 μL of 20 mM Hepes buffer and adding dropwise to 2 μg pGL3 DNA in 500 μL of 20 mM Hepes buffer. The solution was vortexed and left for 60 min prior to analysis. The experiments were performed in triplicate and five different charge ratios (positive:negative, P:N) were used.

4.7. Transmission electron microscopy

Samples were prepared by adding peptide dendrimer to pGL3 DNA (5 μg/mL) at a 5:1 charge ratio (P:N) in 20 mM Hepes buffer. The sample was added to glow discharged carbon coated 200 mesh grids for 3 min and then wicked off with filter paper. Uranyl acetate (1%) was used to negatively stain the grid for 30 s, then wicked off and allowed to dry.[33] Pictures were taken from a JEM-1010 transmission electron microscope (JEOL Ltd., Japan) operated at 80 kV.

Acknowledgements

This project was supported by the NH & MRC of Australia. The authors thank Mr. Makan Khoshnejad for performing TEM experiments.

Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.09.048.

References and notes

1. Liu, F.; Huang, L. *J. Controlled Release* **2002**, *78*, 259–266.
2. Toth, I.; Sakthivel, T.; Wilderspin, A. F.; Bayele, H.; O'Donnell, M.; Perry, D. J.; Pasi, K. J.; Lee, C. A.; Florence, A. T. *S.T.P. Pharma Sci.* **1999**, *9*, 93–99.
3. Steer, C. J.; Kren, B. T. *Atheroscler. Suppl.* **2004**, *5*, 33–42.
4. Pleyer, U.; Ritter, T. *Prog. Retin. Eye Res.* **2003**, *22*, 277–293.
5. Verma, I. M.; Weitzman, M. D. *Annu. Rev. Biochem.* **2005**, *74*, 711–738.
6. Lee, M.; Kim, S. W. *Pharm. Res.* **2005**, *22*, 1–10.
7. Chirila, T. V.; Rakoczy, P. E.; Garrett, K. L.; Lou, X.; Constable, I. J. *Biomaterials* **2002**, *23*, 321–342.
8. Omidi, Y.; Hollins, A. J.; Benboubetra, M.; Drayton, R.; Benter, I. F.; Akhtar, S. *J. Drug Target.* **2003**, *11*, 311–323.
9. Akhtar, S.; Hughes, M. D.; Khan, A.; Bibby, M.; Hussain, M.; Nawaz, Q.; Double, J.; Sayyed, P. *Adv. Drug Deliv. Rev.* **2000**, *44*, 3–21.
10. Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat. Rev. Drug Discov.* **2005**, *4*, 581–593.
11. Eliyahu, H.; Barenholz, Y.; Domb, A. J. *Molecules* **2005**, *10*, 34–64.
12. Kerkis, A.; Hayashi, M. A. F.; Yamane, T.; Kerkis, I. *IUBMB Life* **2006**, *58*, 7–13.
13. Futaki, S. *Biopolymers* **2006**, *84*, 241–249.
14. Vaysse, L.; Gregory, L. G.; Harbottle, R. P.; Perouzel, E.; Tolmachov, O.; Coutelle, C. *J. Gene Med.* **2006**, *8*, 754–763.

15. Wimmer, N.; Marano, R. J.; Kearns, P. S.; Rakoczy, E. P.; Toth, I. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2635–2637.
16. Marano, R. J.; Wimmer, N.; Kearns, P. S.; Thomas, B. G.; Toth, I.; Brankov, M.; Rakoczy, P. E. *Exp. Eye Res.* **2004**, *79*, 525–535.
17. Marano, R. J.; Toth, I.; Wimmer, N.; Brankov, M.; Rakoczy, P. E. *Gene Ther.* **2005**, *12*, 1544–1550.
18. Parekh, H. S.; Marano, R. J.; Rakoczy, E. P.; Blanchfield, J.; Toth, I. *Bioorg. Med. Chem.* **2006**, *14*, 4775–4780.
19. Puls, R. L.; Minchin, R. F. *Gene Ther.* **1999**, *6*, 1774–1778.
20. Edwards, R. J.; Carpenter, D. S.; Minchin, R. F. *Gene Ther.* **1996**, *3*, 937–940.
21. Carpenter, D. S.; Minchin, R. F. *Gene Ther.* **1998**, *5*, 848–854.
22. Futaki, S. *Adv. Drug Deliv. Rev.* **2005**, *57*, 547–558.
23. Bendifallah, N.; Rasmussen, F. W.; Zachar, V.; Ebbesen, P.; Nielsen, P. E.; Koppelhus, U. *Bioconjugate Chem.* **2006**, *17*, 750–758.
24. Blanchfield, J.; Toth, I. *Curr. Med. Chem.* **2004**, *11*, 2375–2382.
25. Jones, S. W.; Christison, R.; Bundell, K.; Voyce, C. J.; Brockbank, S. M. V.; Newham, P.; Lindsay, M. A. *Br. J. Pharmacol.* **2005**, *145*, 1093–1102.
26. Letoha, T.; Gaal, S.; Somlai, C.; Czajlik, A.; Perczel, A.; Penke, B. *J. Mol. Recognit.* **2003**, *16*, 272–279.
27. Lechardeur, D.; Lukacs, G. L. *Hum. Gene Ther.* **2006**, *17*, 882–889.
28. Ohsaki, M.; Okuda, T.; Wada, A.; Hirayama, T.; Niidome, T.; Aoyagi, H. *Bioconjugate. Chem.* **2002**, *13*, 510–517.
29. Singh, R.; Pantarotto, D.; McCarthy, D.; Chaloin, O.; Hoebeke, J.; Partidos, C. D.; Briand, J. P.; Prato, M.; Bianco, A.; Kostarelos, K. *J. Am. Chem. Soc.* **2005**, *127*, 4388–4396.
30. Ehtezazi, T.; Rungsardthong, U.; Stolnik, S. *Langmuir* **2003**, *19*, 9387–9394.
31. Rejman, J.; Conese, M.; Hoekstra, D. *J. Liposome Res.* **2006**, *16*, 237–247.
32. Okuda, T.; Kidoaki, S.; Ohsaki, M.; Koyama, Y.; Yoshikawa, K.; Niidome, T.; Aoyagi, H. *Org. Biomol. Chem.* **2003**, *1*, 1270–1273.
33. Niidome, T.; Ohmori, N.; Ichinose, A.; Wada, A.; Mihara, H.; Hirayama, T.; Aoyagi, H. *J. Biol. Chem.* **1997**, *272*, 15307–15312.
34. Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180–193.