Peptide Mini-Vectors for Gene Delivery**

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It is widely anticipated that gene therapy will represent one of the key medical technologies of the next century. However, a great deal of progress still needs to be achieved for this to be possible. In general terms, gene therapy may be defined as "the delivery of nucleic acids (with a vector) to patients for some therapeutic purpose".[1a] Hence a major preoccupation of current gene therapy research has been the design of appropriate vectors for nucleic acid delivery. This is a sphere in which the chemist has a clear role to play,[1] and indeed we ourselves have been working on the development of synthetic cationic liposome vector systems for the delivery of nucleic acids in vivo.[2] Here we describe an alternative, peptide-based system which represents one of the smallest and simplest vector systems yet reported for the delivery of nucleic acids.[1]

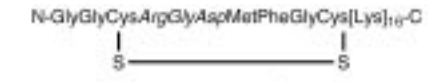
The peptide-based system described here originated from our initial discovery that synthesized peptide **1**, containing a cyclic N-terminal moiety and a hexadeca(L-lysine) DNA-binding moiety, could mediate gene delivery in vitro.[3] The cyclic N-terminal moiety of **1** contains an Arg-Gly-Asp (RGD) peptide motif of a type shown to interact with high avidity to integrins such as $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$,[4, 5] proteins which are found routinely at many cell-surface membranes.[5, 6] We had anticipated that **1** would act by binding to nucleic acids by means of the hexadeca(L-lysine) moiety and then enter cells by a two-step process involving cell-surface integrin binding followed by receptor-mediated endocytosis. Such an integrin-mediated cell entry process is widely used by several pathogens including the adenovirus,[7] which has itself been the subject of numerous gene delivery experiments in recent times.[1] Therefore, we were in effect anticipating that **1** would operate like a minimalist adenovirus gene delivery vector.

Gratifyingly, peptide **1** did indeed behave in the anticipated way,[3] but it proved very difficult to synthesize. A second-generation RGD peptide vector was then prepared in the form of peptide **2** in which the hexadeca(L-lysine) moiety was relocated to the N terminus. Whilst **2** outperformed **1** as a gene delivery vector, it also proved difficult to prepare.[8] There are known to be "difficult sequences" in solid-phase peptide synthesis which form secondary structures (such as α helix and β sheet) on the solid-phase resin as they are being synthesized. These secondary structures are believed to reduce amino acid coupling efficiencies and lower the recovered yields of peptide products.[9, 10] Such difficult sequences may be identified by the equation of Milton et al.[11] [Eq. (1)], where $\langle P_C^* \rangle$ is the average coil parameter

$$\langle P_c^* \rangle = \Sigma P_{c\,i-n}^* / n \qquad (1)$$

for a developing protected peptide chain of total length n residues, and $P_{C\,i-n}^*$ are the individual refined coil parameters for each amino acid in the protected peptide chain at each individual position i. Typically, when the sequence of a given protected peptide chain is analyzed with Equation (1), a high $\langle P_C^* \rangle$ value (1.0–1.4) suggests that the chain will have a tendency to form random coil. This implies that the next amino acid residue to be coupled will be incorporated with ease (amino acid coupling efficiency $>99.8\,\%$). A low $\langle P_C^* \rangle$ value (0.7–0.9) suggests the opposite. When a complete analysis was made of the protected peptide chains leading to the synthesis of **1**, $\langle P_C^* \rangle$ values of approximately 0.9 were obtained throughout. Similar results were also obtained for **2**. Therefore, both **1** and **2** appeared to be uniformly comprised of difficult sequences.

Given the potential importance of **1** and **2** for nucleic acid delivery, we were concerned to develop an alternative solid-phase peptide synthesis strategy which would overcome the problems presented by difficult sequences and also allow for alternative functional peptide moieties to be attached to the hexadeca(L-lysine) moiety. This would widen the utility and potential applicability of peptide-based vector systems for the delivery of nucleic acids. In developing this alternative strategy, we chose to prepare two new nucleic acid delivery peptides **3** and **4**. Peptide **3** is a variant of **1** and represents our third generation of RGD peptide vectors. In contrast, peptide **4** is made up of an alternative fusogenic peptide moiety



RGD[K]$_{16}$ **1**

[K]$_{16}$RGD **2**

RGD[K]$_{16}^{+}$ **3**

JTS-1[K]$_{16}$ **4**

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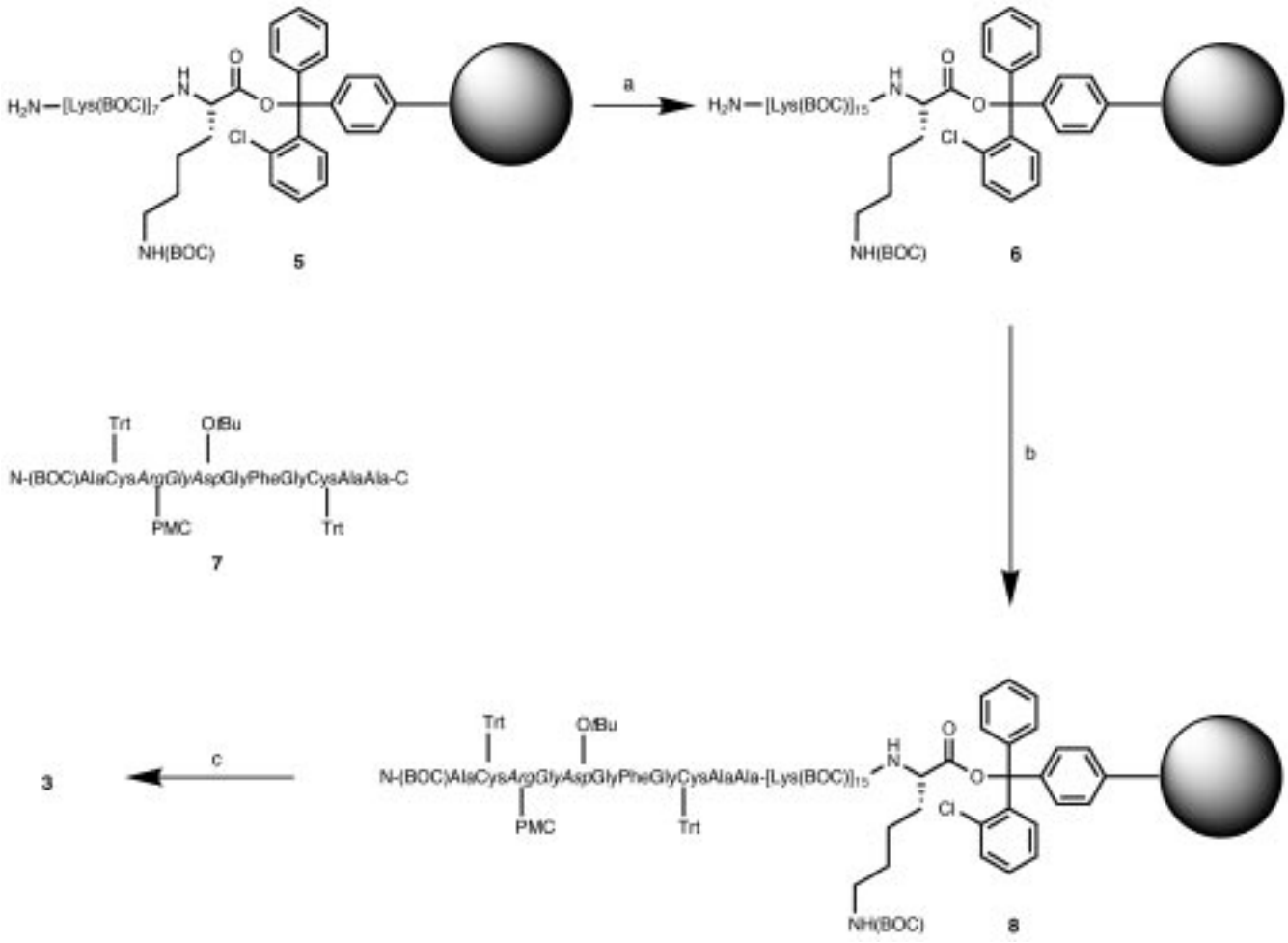
attached at the N terminus of the hexadeca(L-lysine) DNA-binding moiety. This fusogenic peptide moiety, christened JTS-1, had been previously shown to have potent endosomolytic properties.[12] Therefore, we anticipated that if **3** and **4** were used in combination, the resulting peptide-based vector system would be able to bind electrostatically to nucleic acids, enter cells by integrin-binding mediated endocytosis by virtue of the RGD peptide motif of **3**, and then promote the escape of electrostatically associated nucleic acids from endosome compartments through the endosomolytic activity of the N-terminal fusogenic peptide moiety of **4**. Endosome escape is well known to be a serious problem for non-viral nucleic acid delivery systems.[1] Hence the action of **4** was expected to synergize with the action of **3** and result in a more efficacious peptide-based vector system for the delivery of nucleic acids.

The solid-phase synthesis strategy for the preparation of **3** and **4** was as follows. A convergent segment condensation approach was adopted in which resin-bound octa(L-lysine) **5** was initially prepared on a super acid-labile 2-chlorotrityl resin and then coupled to FMOC-[L-Lys(BOC)]$_8$-OH (**5a**) with benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) to give the resin-bound hexadeca(L-lysine) **6** (Scheme 1).[13] Oligo(L-lysine) peptides such as **6** would normally be difficult to synthesize in a direct linear manner, especially when more than eight amino acid residues are involved ($\langle P_C^* \rangle$ values 0.8 – 0.9). Therefore, this particular segment condensation was carried out to avert this complication and optimize the yield of full-length **6**. One portion of **6** was then coupled to a fully protected RGD motif peptide **7** with PyBOP to give **8**, which was then cleaved from the resin and deprotected to give **3** in an overall yield of 3% (Scheme 1).

Another portion of **6** was coupled to fully protected fusogenic peptide **9** with dicyclohexylcarbodiimide/ hydroxybenzotriazole (DCC/HOBt) to give **10**. This too was released from the resin and deprotected to give **4**, also in an overall yield of 3% (Scheme 2). The synthesis of the intermediate RGD motif peptide **7** was performed by linear solid-phase peptide synthesis without incident. By contrast, the synthesis of intermediate fusogenic peptide **9** proved more difficult ($\langle P_C^* \rangle$ values approx. 0.8 throughout synthesis), and was only accomplished with the extensive use of extended coupling times and double coupling throughout. Overall, the yields of purified **3** and **4** were judged to be satisfactory, especially given the fact that the syntheses of both by normal linear



Scheme 1. Reagents and conditions: a) 1. FMOC-[L-Lys(BOC)]$_8$-OH (**5a**), PyBOP (2 equiv), *i*Pr$_2$EtN (3 equiv), DMSO/DMF (1/3), RT, 72 h; 2. piperidine in DMF (20% v/v); b) **7**, PyBOP (10 equiv), *i*Pr$_2$EtN (10 equiv), DMSO/DMF (1/3), RT, 24 h; c) 1. phenol (7% w/v), ethanedithiol (2% v/v), thioanisole (4% v/v), water (4% v/v) in TFA, RT, 5 h; 2. MTBE (overall yield 3%). BOC = *tert*-butoxycarbonyl, *t*Bu = *tert*-butyl, FMOC = (9-fluorenyl)methoxycarbonyl, MTBE = methyl *tert*-butyl ether, PMC = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, TFA = trifluoroacetic acid, Trt = trityl.

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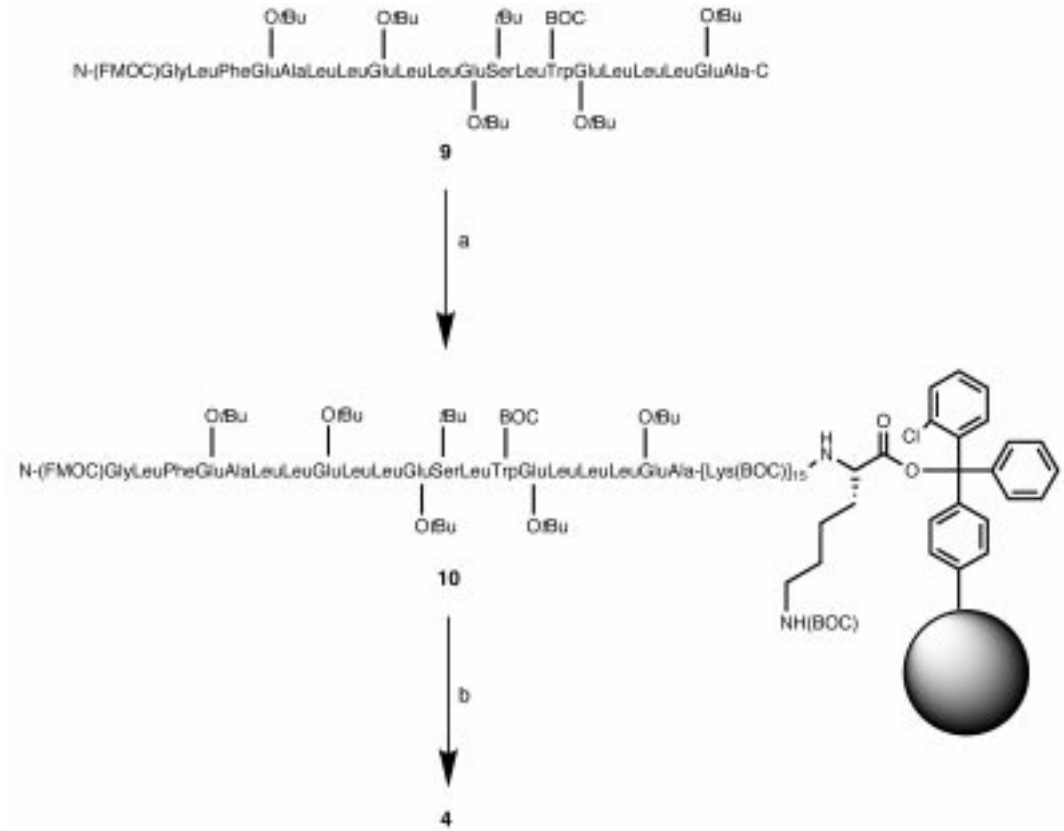

Scheme 2. Reagents and conditions: a) **6**, DCC (40 equiv), HOBt (40 equiv), DMSO/DMF (1/4), RT, 24 h; b) 1. piperidine in DMF (20 % v/v), RT, 45 min; 2. ethanedithiol (2.5 % v/v), water (2.5 % v/v) in TFA, RT, 3 h; 3. MTBE (overall yield 3 %).

synthetic strategies were judged to be essentially impossible according to an analysis made with Equation (1).

Initially, the efficacy of **3** was evaluated in comparison to **2** as a vector for the in vitro delivery of the pGL3 plasmid into CaCo2 (human colonic epithelial) cells. The pGL3 plasmid expresses the American firefly (*Photinus pyralis*) luciferase reporter gene, leading to an accumulation of luciferase enzyme activity in CaCo2 cells following plasmid gene delivery. The level of enzyme activity then found in cells is directly proportional to the efficacy of the gene delivery process,[8] so that the higher the enzyme activity is, the more effective is the gene delivery process. With the pGL3 delivery assay, peptides **2** and **3** were found to be indistinguishable as plasmid gene delivery vectors (results not shown). In contrast, our third-generation RGD-peptide vector **3** was able to deliver the pGL3 plasmid between four and five times more effectively than **4**, the endosomolytic vector (Figure 1). Gratifyingly, when a combination of **3** and **4** was used, the gene delivery efficiency of the combination was found to be almost ten times the efficiency of **3** alone (Figure 1). This showed that **3** and **4** were indeed functioning synergistically as we had hoped, most likely by the mechanism suggested above. The combined gene delivery effects of **3** and **4** is beginning to rival the in vitro gene delivery efficiency of LipofectAMINE cationic liposomes (Figure 1). Therefore, we are confident that the combination peptide system described here could supersede the use of cationic liposomes in vitro, given further optimization studies. However, a more fruitful route forward

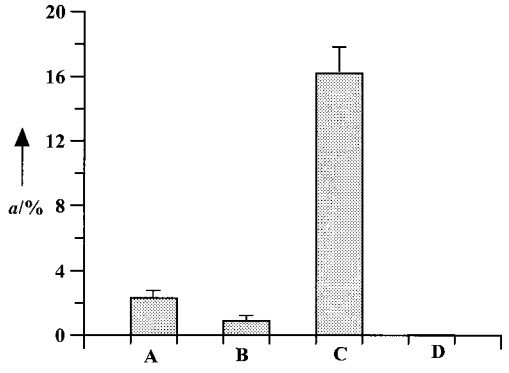

Figure 1. Luciferase enzyme activity *a* in cell-free extracts of CaCo2 cells 48 h after peptide mini-vector mediated delivery of the pGL3 plasmid which expresses the luciferase gene. Plasmid gene delivery was effected by treating cells with plasmid pGL3 condensed with either **3** and/or **4**, as indicated. In each transfection performed, the optimal plasmid/total peptide ratio was 1 μg/2.5 μg (1/2.5 w/w). The plasmid/peptide mixtures were pGL3 + **3** (A), pGL3 + **4** (B), pGL3 + **3** and **4** (C), and pGL3 alone (D). Luciferase enzyme activity is expressed as a percentage (%) of the activity found in the cell-free extracts of CaCo2 cells following control cationic liposome mediated plasmid gene delivery experiments, performed concurrently with LipofectAMINE (GIBCO BRL) cationic liposomes and pGL3 plasmid (24 μg/1 μg, 24/1 w/w).

may be to adopt an additional combination strategy where both peptides and cationic liposomes are used together to effect nucleic acid delivery. Indeed, we have just recently discovered that our second-generation RGD peptide vector **2** will enhance the in vitro gene delivery efficacy of Lipofect-

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AMINE cationic liposomes,[1] by at least an order of magnitude.[14] Such an enhancement of cationic liposome gene delivery efficacy could have important implications for in vivo cationic liposome-mediated gene delivery.

In conclusion, we have devised a convergent synthesis for peptide mini-vectors, which were demonstrated to be able to mediate plasmid gene delivery in vitro. To the best of our knowledge, these are some of the smallest delivery vectors for nucleic acids yet reported and could be very useful tools for future gene therapy studies.

Experimental Section

All peptides were synthesized on an ABI 431A solid-phase batch peptide synthesizer. Unless otherwise stated above, syntheses were conducted on an 0.1-mmol scale with a fivefold excess of FMOC-protected L-amino acids (Novabiochem, Nottingham, UK) and FastMoc reagents 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/hydroxybenzotriazole (HBTU/HOBt) (Alexis Corporation, Laufelfingen, Switzerland and SMPE Ltd, Croydon, UK) as the amide coupling agent. Coupling steps were carried out in *N*-methylpyrrolidinone (NMP) and dichloromethane (Rathburn, Walker-burn, UK) on super acid-labile 2-chlorotrityl resins (200–400 mesh; Novabiochem, Nottingham, UK) as the solid support. After synthesis, peptides were cleaved from the resin by trifluoroacetic acid (TFA) in dichloromethane (1% v/v), followed by treatment with pyridine (2.5 equiv) in methanol. Desalting was performed on a P2 biogel column (2×28 cm; Bio-Rad Laboratories, Herts., UK) attached to an FPLC system (Amersham Pharmacia Biotech UK, Bucks., UK) eluting with 0.1% aqueous TFA (0.75 ml min^{-1}; monitoring at 214 or 280 nm). Reverse-phase HPLC purification was usually carried out with a Vydac column (C18, 5μm, 2×25 cm; Hichrom Ltd, Berks., UK) attached to a Gilson HPLC system (Anachem, Beds., UK). Peptides were eluted with a gradient of acetonitrile in 0.1% aqueous TFA (5 ml min^{-1}; monitoring at 220–230 nm). Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was performed on a LaserMat 2000 (Thermobioanalysis Ltd, Herts., UK) with a matrix of α-cyano-4-hydroxycinnamic acid (α-CMC) (33 mM) in acetonitrile/methanol (Hewlett-Packard, Cheshire, UK).

Peptide **3** was synthesized as described above (Scheme 1). After final desalting, HPLC purification (eluting with acetonitrile at 53.5%, v/v) and lyophilization, **3** was obtained as a white powder. Overall yield: 8 mg (2.6 μmol, 3%); MS (MALDI-TOF) *m/z* calcd for $C_{136}H_{255}N_{46}O_{30}S_2$: 3078.9 $[M+H]^+$; found 3078.1. The sequence was further confirmed by amino acid composition and sequence analyses; homogeneity was judged to be greater than 95% by HPLC analysis.

Peptide **4** was synthesized as described above (Scheme 2). After final HPLC purification (eluting with acetonitrile at 96.5% v/v) and lyophilization, **4** was obtained as a white powder. Overall yield: 12 mg (2.7 μmol,3%); MS (MALDI-TOF) *m/z* calcd for $C_{206}H_{366}N_{53}O_{48}$: 4353.5 $[M+H]^+$, found 4353.8. The sequence was further confirmed by amino acid composition and sequence analyses; homogeneity was judged to be greater than 95% by HPLC analysis.

Peptides **3** and **4** (100 μg mL^{-1}) were dissolved in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.3, containing 150 mM NaCl (HEPES buffered saline). The solution of **3** was then stirred for 16 h at room temperature in order to form an intramolecular disulfide bond. Bond formation was monitored by Ellman assay.[15] Finally, the gene delivery efficacies of oxidized **3** and/or **4** were tested as described previously.[8]

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Metathesis of Alkanes: Evidence for Degenerate Metathesis of Ethane over a Silica-Supported Tantalum Hydride Prepared by Surface Organometallic Chemistry**

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Here we report on evidence for a catalytic process in which ethane molecules mutually exchange a methyl group with one another on the silica-supported tantalum(III) hydride catalyst[1] ($\equiv SiO)_2Ta\text{-}H$ ($[Ta]_s\text{-}H$) (**1**) under mild conditions. We recently reported that the surface complex **1** catalyzes the metathesis of linear and branched alkanes and effectively

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