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# Targeting of polyamidoamine–DNA nanoparticles using the Staudinger ligation: Attachment of an RGD motif either before or after complexation

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ABSTRACT

Two new methods for the modular synthesis of targeted gene delivery systems are reported. The PEGylated polyamidoamine DMEDA-PEG-DMEDA-(MBA-DMEDA)$_{n+1}$-PEG-DMEDA **3** was sequentially modified to contain an integrin-binding peptide ligand via the Staudinger ligation. The conjugation of the ligand was achieved either before particle complexation (precomplexation) or after particle complexation (postcomplexation). Comparison of the two systems showed that postcomplexation strategy led to small and discrete toroidal nanoparticles whilst the precomplexation particles showed loose complexes. The targeted particles showed an increased uptake into cells compared to unmodified complexes however no significant increase in transfection was seen.

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## 1. Introduction

Polymer based non-viral gene delivery systems have been studied as a potential way of delivering genes to faulty cells. Several polymer based delivery systems have been studied including poly-L-lysine,[1] polyethyleneimines,[2] polyamidoamine dendrimers[3] and linear polyamidoamines,[4] all of which have had some success as suitable delivery vehicles. The efficiency of non-viral systems could be markedly improved by attachment of ligands recognising specific receptors on cell surfaces. Ligand targeting can enhance both specificity and uptake. Some examples of this include polysaccharides and peptides.[1a,2b,4c]

Poly(ethylene glycol)-functionalised (PEGylated) polyamidoamine:*N,N′*-methylenebisacrylamide-alt-*N,N′*-dimethylethylenediamine has been developed for gene delivery[5,6] and by targeting this construct it should be possible to increase the efficiency of uptake and therefore expression of the gene. There has been extensive success using RGD-based ligand as targeted delivery via the integrin receptors,[7] both for drugs and other gene delivery systems.[8] The 'RGD' motif is known to bind to several members of the integrin receptor family. Binding of the ligand then triggers receptor mediated endocytosis. The 'RGD'–integrin interactions are responsible for diverse cellular functions, including signalling, growth, differentiation, adhesion and angiogenesis.[7] The decapeptide (RGDSPASSKP) containing the 'RGD' motif comprising residues 1615–1624 of fibronectin[7] was chosen as the ligand for these studies. The 10-residue sequence ensures a native spacer chain between the integrin-binding 'RGD' motif and the chemical ligation site. This sequence has previously been used as a competitive inhibitor of fibronectin[7b] and it binds well with integrin receptors.

Amongst the numerous methodologies for ligand attachment, we considered that the ligation based on the Staudinger reaction[9] is one of the most robust and chemospecific. The 'Staudinger ligation' is a reaction between an appropriately modified phosphine and an azide, in which an amidophosphonium intermediate is hydrolyzed in water to form an amide bond. It was independently developed by Bertozzi[10] and Raines[11] and has many applications in chemical biology.[12] A particular advantage of using the Staudinger ligation is that the ligand can be readily attached either before assembly of the complexes (precomplexation) or to the assembled nanoparticle (postcomplexation). This will enable us to explore which of these strategies is most advantageous in terms of both the chemistry and the assembly of complexes to produce the most effective delivery vehicles.

In our strategy, the linear block polymer, a PEGylated polyamidoamine has been *N*-acylated with 2-azidoacetic acid, which effectively acts as a masked glycine residue. The 10-residue native peptide chain can then be ligated to the modified linear polymer. Postcomplexation ligation of the peptide should guarantee that the targeting ligand is on the outside and minimize the disruption of the particles. Precomplexation ensures that the ligation is easy to monitor and that the products are well defined. However, the latter route has the adverse possibilities for the ligand to be sequestered inside the complex and thus unavailable for binding, and also for the ligand to affect the complexation between the

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polymer and DNA. Furthermore, this paper describes for the first time the successful application of Staudinger ligation between a phosphinothioester peptide and an azido-bearing block polymer–DNA complex.

## 2. Results

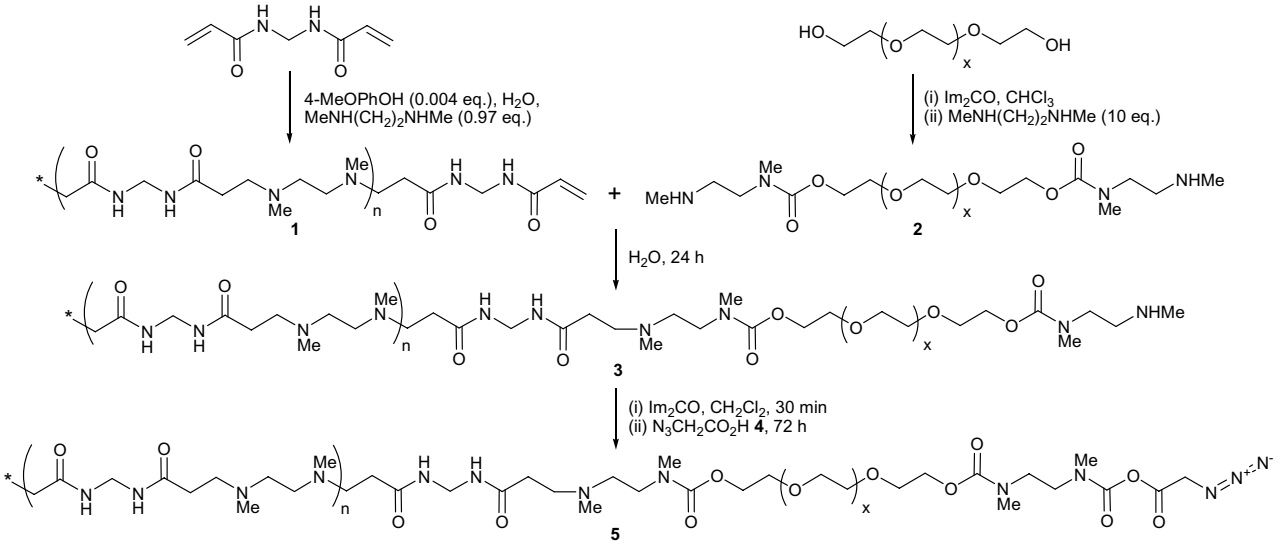
Firstly, the readily prepared polyamidoamine $(MBA\text{-}DMEDA)_n$ **1** and amine terminated DMEDA-PEG-DMEDA **2** were coupled together to give the PEGylated polyamidoamine DMEDA-PEG-DMEDA-$(MBA\text{-}DMEDA)_{n+1}$-PEG-DMEDA **3** (Scheme 1). A large excess (>10-fold) of the amine component DMEDA-PEG-DMEDA **2** was used to ensure the formation of desired triblock polymer product and to minimise uncontrolled multiblock polymerisation. Next, 2-azidoacetic acid **4** was prepared in excellent yield (89%), by reacting bromoacetic acid with sodium azide. The bis-DMEDA terminated PEGylated polyamidoamine **3** was then reacted with carbonyldiimidazole to give a bis(1-imidazolyl carboxamido) intermediate, which on exposure to excess 2-azidoacetic acid afforded the required azide terminated PEGylated polyamidoamine **5**. Here, the conjugation is via a carbamic anhydride bond. The azido-terminated polymer **5** was then blended with varying ratios of $(MBA\text{-}DMEDA)_n$ **1** followed by complexation with the plasmid DNA gWIZLuc in order to form small, discrete particles as described by Rackstraw et al.[5] Following a series of preliminary experiments, we established that the **5**:**1** blend was best achieved at a ratio of 1.75:1 and complexed with gWIZLuc at a polyamidoamine repeating unit:DNA nucleotide ratio of 2:1. This formulation gave small toroid like structure as seen by TEM (Fig. 1).

The peptide ligands were synthesised using standard Fmoc solid-phase synthesis on 2-chlorotrityl poly(styrene) resin (Scheme 2).[13] The decapeptide RGDSPASSKP was chosen as the targeting sequence,[7] and RGRSPASSKP as the non-binding sequence as it has no known binding affinity with integrin receptors. The assembled protected decapeptides were efficiently cleaved from the polymer support using a mild acidolytic mixture containing 1% TFA, and stored in their protected forms prior to thioesterification with (diphenylphosphino)methanethiol **6**. The phosphinothiol reagent **6** was readily prepared in five steps from thioacetic acid using a refined method recently developed by Raines and co-workers.[11c] The synthetic strategy (Scheme 2) involved the preparation of an air stable borane–diphenylphosphine complex, which was *P*,*S*-deprotected immediately prior to condensation with the synthesised decapeptides. Although the overall yield of the phosphothiol reagent **6** was low (7%), each of the chemical transformations were achieved in modest to good yields (40–69%). In the final step, best yields were obtained when the saponification was carried out using $N_2$-purged reagents, as well as the use of $N_2$-purged EtOAc–hexane for column chromatography. Thus, (diphenylphosphino)methanethiol **6** was coupled to the protected decapeptides using carbodiimide-mediated esterification. In order to simplify the work-up procedure, either the polymer-bound or water-soluble carbodiimide reagents were used. Following acidolytic treatment with a 90% TFA mixture, the thioester peptides **7** and **8** were obtained as white solids in good yields (ca. 90%). Unexpectedly, HPLC and MS analysis revealed that racemisation, most likely epimerisation of the C-terminal amino acid residue (Pro) occurred prior to the formation of the thioester. Fortuitously, the crucial 'RGD' motif is located at the N-terminus, some seven residues from the C-terminal and is therefore unlikely to affect binding to integrin. In light of this observation, it is recommended that in future experiments an achiral spacer, for example, glycine or β-alanine, should be installed at the C-terminus.

The RGDSPASSKP-(diphenylphosphanyl)methyl thiolate **7** and azidoacetyl functionalised polyamidoamine **5** were successfully ligated by stirring in phosphate buffer for 24 h to afford the 'RGD'-peptide targeted polymer **9** (precomplexation ligation). The overall strategy is summarised in Scheme 2. It is worth noting that following Staudinger ligation, the azidoacetyl moiety is revealed as a Gly residue. The linear polymer was then purified by ultrafiltration and the amount of peptide bound was determined by amino acid analysis (Table 1). This ligation strategy was then repeated for the 'non-binding' RGRPASSKP-(diphenylphosphanyl)methyl thiolate **8** to afford the 'RGR'-peptidyl polymer **10**.

Using similar conditions, but in distinctively modular manner, postcomplexation (i.e., using pre-formed polymer blend–gWIZLuc particle) ligation was successfully performed, and the modified particle products were purified by dialysis. Amino acid analysis (Table 1) confirmed the presence of the peptide ligand on both the precomplexation and the postcomplexation particles with 9% and 46% substitution for the 'RGD'-peptidyl products.

TEM (Fig. 1) and DLS (Table 1) studies were carried out on the precomplexation **9** and **10** formulated gWIZLuc-complexed particles, and the postcomplexation **11** and **12** particles in order to as-




**Scheme 1.** Synthesis of the 2-azidoacetyl-modified linear block polymer **5**.

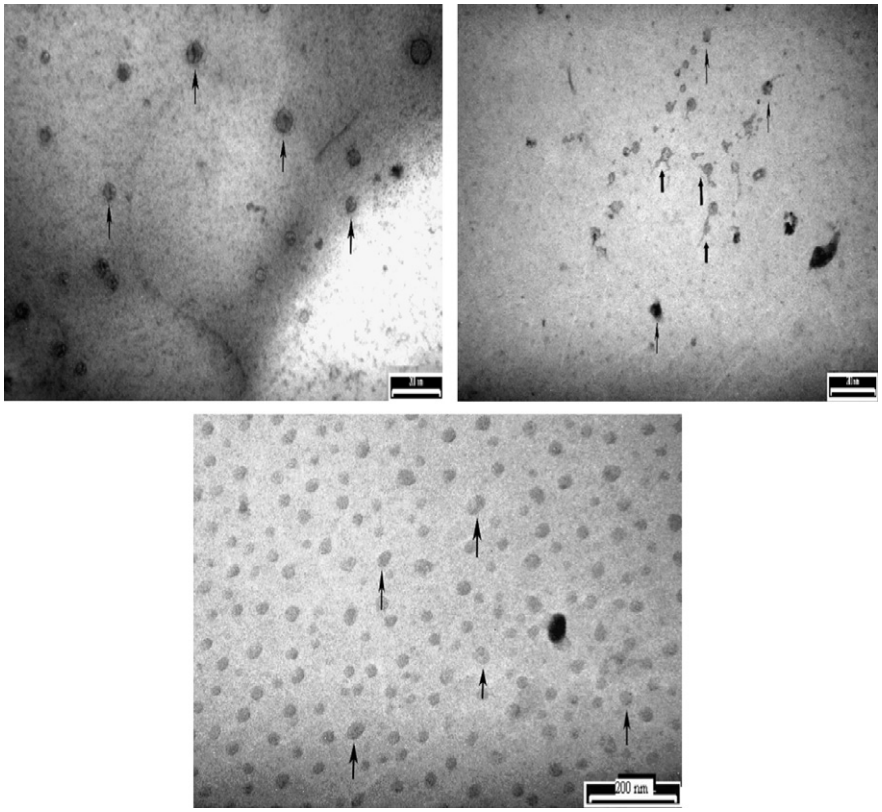



**Figure 1.** TEM image of 'naked' and 'RGD'-ligated particles. (Top left) Azido-modified PEGylated polyamidoamine **5**:(MBA-DMEDA)$_n$ **1** (1.75:1) polymer blend/gWIZLuc (2:1) complex. Magnification: 100,000; scale bar: 200 nm. The arrows point to individual particles. (Top right) Precomplexation-ligation derived particles following ultrafiltration. Magnification 40,000×; scale bar 1000 nm. Diameter 55 nm. Small arrows indicate particles, large arrows indicate loose complex. (Bottom) Postcomplexation ligated particles with purification by dialysis. Magnification 100,000×; scale bar 200 nm. Diameter 53 nm. Arrows showed discrete spherical particles.

sess their physicochemical characteristics. In all cases, small and discrete particles were produced. For each particle the Relative Standard Deviation is given as a measure of peak polydispersity, where a figure of <20% is considered as monodisperse (i.e., equivalent to a polydispersity index of <0.1).

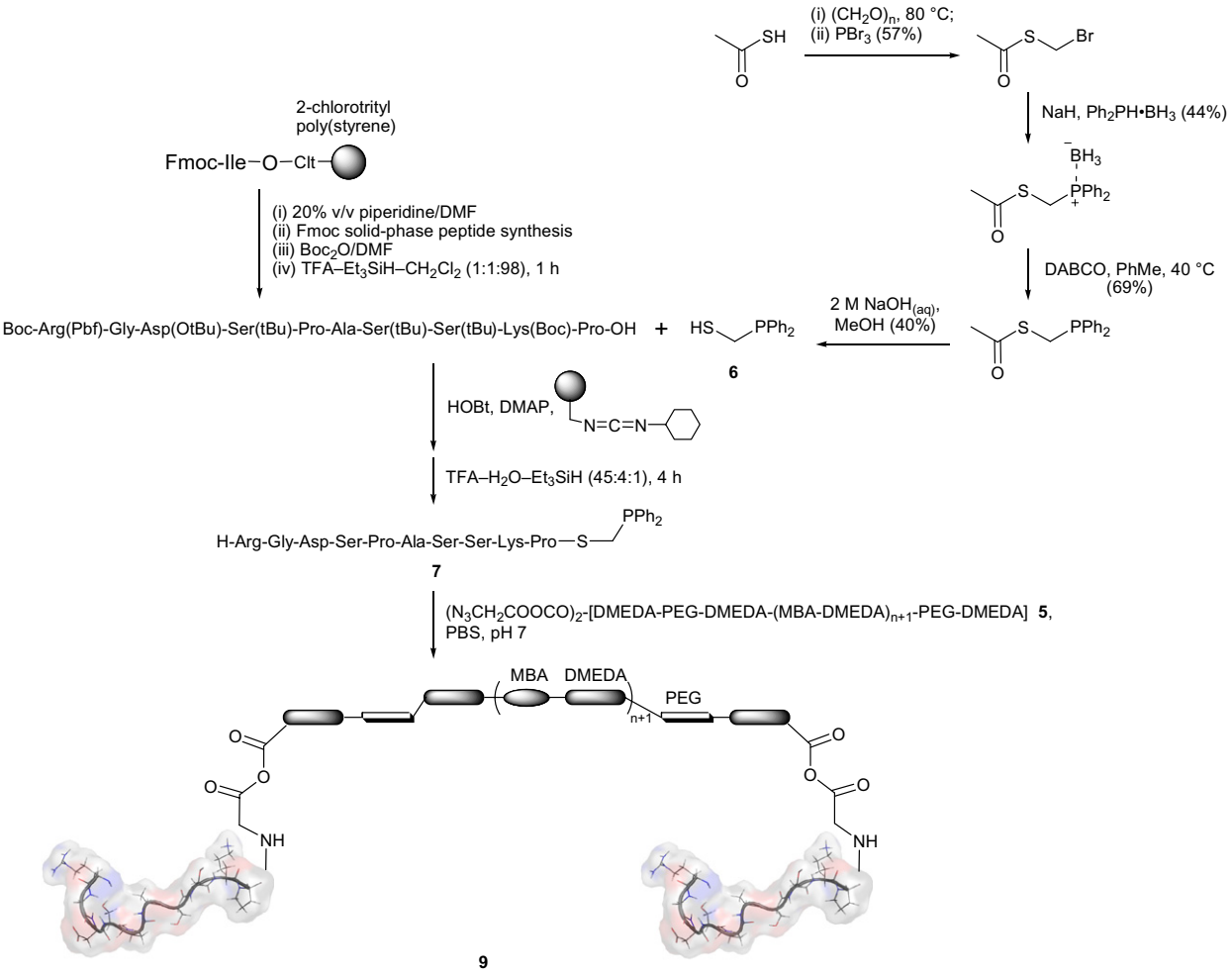
Binding and uptake studies were carried out for the precomplexation **8** and **9** formulated particles, and postcomplexation ligated **10** and **11** particles using 3T3 and A549 cells (Fig. 2). Fluorescein-labelled RGDPASSKP was used as a binding standard. YOYO-labelled DNA was used to form and visualise all of the particles used in the binding/uptake studies. Mono-intercalant dyes cannot be used in these assays, as the polymer–DNA binding excludes these dyes from the complex, however, the binding of bis-intercalant dyes such as YOYO is sufficiently strong to maintain a high level of labelling. The initial binding and uptake was low, however after 4 h the fluorescence was high indicating that significant levels of uptake had taken place in both cell lines. The difference in fluorescence between 10 min and 4 h was greater in 3T3 than in A549 cells, but no significant differences between the 'RGD' targeted and 'RGR' non-targeted complexes were seen. There was also no significant difference between the precomplexed ligated and the postcomplexed ligated systems.

Finally, the complexes were transfected into 3T3 and A549 cells (Fig. 3). LipofectAMINE™ was used as a positive control, and (MBA-DMEDA)$_n$–gWIZLuc (5:1) and **5**:**1** blend–gWIZLuc (2:1) were used as standards. The overall levels of transfection were low and the 3T3 cells which should have a higher receptor density than the A549 cells showed lower transfection levels than expected. The transfection studies showed that targeting with an integrin-binding peptide ligand did not restore or improve on the transfection levels of (MBA-DMEDA)$_n$ complexes or PEGylated (MBA-DMEDA) **5** complexes.

## 3. Discussions

The PEGylated polyamidoamine DMEDA-PEG-DMEDA-(MBA-DMEDA)$_{n+1}$-PEG-DMEDA **3** was synthesised using methods previously described.[5,6] This was then blended with (MBA-DMEDA)$_n$ **1** followed by complexation with the plasmid gWIZLuc to produce well formed discrete nanoparticles, with average size of 75 nm determined by TEM and 100–200 nm by DLS. The PEGylated particles were generally round in shape with some toroidal characteristics (Fig. 1). The original publication by Rackstraw et al.[5] demonstrates the robustness of this methodology. Unless the formulation is correct, compact toroids are not obtained, and the only possible formulation under the optimum conditions is one in which the PEG chains are arranged on the outside of the complex where DNA is tightly bound at the core.

An azide group was required to perform the Staudinger ligation between the ligand and the PEG modules. The azidoacetyl moiety was introduced to the terminal amines of **3** through a carbamic anhydride, which is envisaged as a biologically acid labile linker to aid the release of the particle, and in the further development of the nanoparticle. Carbamic anhydrides have been shown to be stable in a wide range of solvents[14] and have been used as stable intermediates for the synthesis of leukotriene antagonists[15] and X-ray contrast agents.[16] No change was seen in the size or the morphology of the particles when 2-azidoacetic acid was attached to the N-termini of DMEDA-PEG-DMEDA-(MBA-DMEDA)$_{n+1}$-PEG-DMEDA **3** and the complexes were formed (data not shown).




**Scheme 2.** Synthesis of the 'RGD'-containing decapeptidyl (diphenylphosphanyl)methyl thiolate **7** and the Staudinger ligation product peptidylglycyl PEGylated polyamidoamine block polymer **9**.

**Table 1**
Dynamic light scattering, TEM and amino acid substitution for postcomplexation and precomplexation ligated DNA-complexed particles

| | | DLS | Polydispersity (relative standard deviation) | TEM | Amino acid substitution |
|---|---|---|---|---|---|
| Precomplexation ligation | RGD (**8**) | 118 nm | 13.1 | 55 nm | 9% |
| | RGR (**9**) | 103 nm | 22.6 | 71 nm | 0.4% |
| Postcomplexation ligation | RGD (**10**) | 146 nm | 5.2 | 53 nm | 46% |
| | RGR (**11**) | 94 nm | 6.4 | n.a. | n.a. |

The (diphenylphosphino)methanethiol **6** was synthesised using the method developed by Raines[11] and gave comparable results. The reagent was coupled to the 'RGD' and 'RGR' containing peptides so that the targeting ligand could be ligated to the azide terminated polymer. The Staudinger ligation was carried out under aqueous conditions, for both the precomplexation and the postcomplexation ligated systems. After 24 h the reaction products were purified to remove any unreacted peptides and by-products. This was achieved by ultrafiltration for the precomplexation ligation and dialysis for the postcomplexation ligation, due the small sample volume. Attachment of the peptide was assessed by amino acid analysis which showed the peptide had successfully ligated to the polymer in both the precomplexation and the postcomplexation ligation (Table 1). The 9% ligation was good for the precomplexation as the azide was in a relatively low concentration and the efficiency of the reaction is typically considered to be lower in water compared to a THF–water mix.[11b] Surprisingly, the ligation was nearly five times higher, 46%, when assembled complexes were used. We speculate that this is due to the azide functionality being more accessible on the surface of the complexes allowing the ligation to occur more efficiently. Precomplexation ligation using the 'RGR'-containing thioester peptide **8** was of unexpectedly low efficiency (typically 0.4–2%), which is likely to be a result of reaction variability and no attempts were made to optimise reaction conditions. However, it is worth noting that, compared to the 'RGD'-containing peptide **7**, the peptide **8** is anticipated to be highly cationic which may indeed negatively affect its ligation efficiency with the cationic polyamidoamine block polymer.

DLS showed that small particles were formed, in which the postcomplexation ligation particles were observed to be slightly larger than the precomplexation particles. This may be due the addition of the peptide on the surface affecting the hydration of

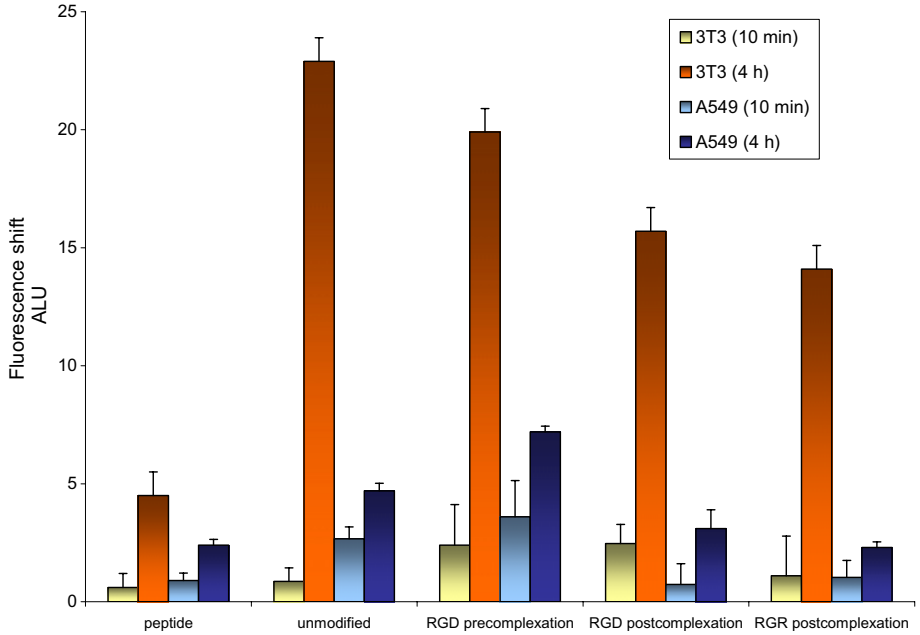



**Figure 2.** Uptake studies using A549 and 3T3 cells. The fluorescence shift is used as an indication of the amount of complex taken up by the cells.

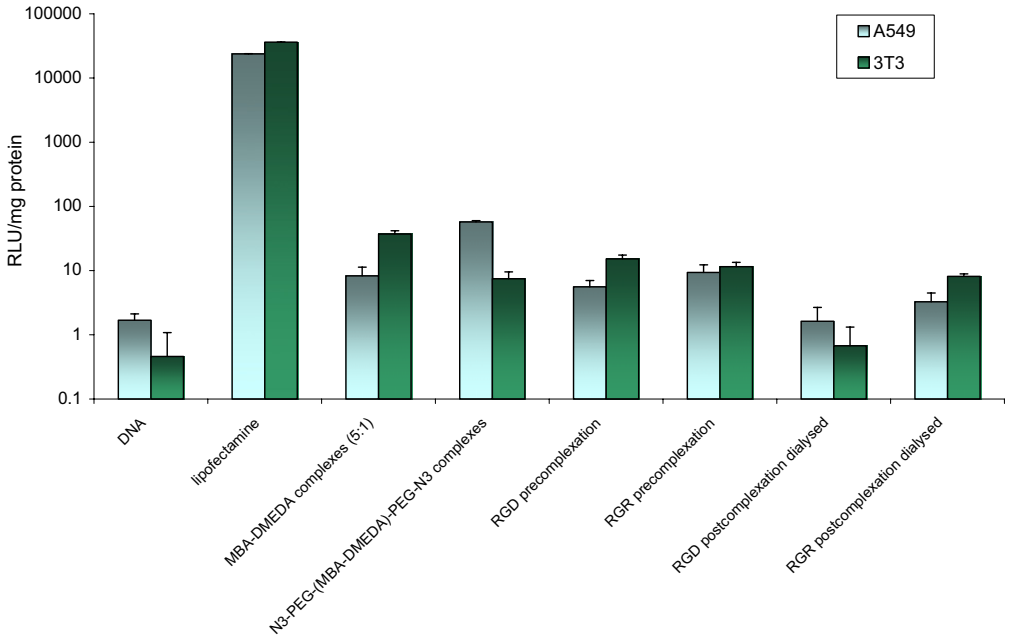



**Figure 3.** Transfection of A549 cells ($n = 6$) and NIH 3T3 cells ($n = 6$) using ligated block-polyamidoamine–gWIZLuc plasmid (2:1) complexes.

the particles or the thickness of the sterically stabilised PEG layer. However, there was no significant difference in size when the particles were measured by TEM (Table 1). TEM showed that the precomplexation ligated particles appeared to display some loose complexation and were not toroidal or spherical as seen with the 'naked' PEGylated polyamidoamine–plasmid complex (Fig. 1). This implied that the presence of the peptide was disrupting the condensation between the DNA and the MBA-DMEDA backbone, leading to loosely formed nanoparticles. Adjusting the formulation may lead to better condensed and well formed particles. The postcomplexation ligated particles formed small particles with toroidal characteristics similar to the PEGylated nanoparticles with a similar diameter determined by DLS.

For the cell uptake studies, fluorescein-labelled RGDSPASSKP, in which the 5-carboxyfluorescein was installed at the Lys-ε-amino group, was synthesised and used to gain an idea of how many receptors were present on the cell. The integrin receptor expression was found to be low. However, the level of fluorescence is in the expected range for the density of receptors reported for the cell type used.[17] The precomplexation and the postcomplexation particles, prepared using YOYO-labelled gWIZLuc, were incubated with the cells for 10 min and 4 h in order to gauge the binding and uptake properties of the complexes, respectively (Fig. 2). The unmodified complexes, which have no targeting moiety, gave an indication of non-specific uptake. With the 3T3 cells, both the RGD precomplexation and postcomplexation ligated particles showed greater up-

take than the 'naked' unmodified or the RGR-ligated complexes after 10 min. This implied that receptor binding is occurring.

In contrast, after 4 h incubation there was a substantial increase in the binding and uptake of the complexes compared to the short incubation time. Only the precomplexation ligated particles, in the A549 cell line showed a higher uptake than the unmodified complex. The 3T3 cell uptake and the postcomplexation ligated A549 cell uptake was less than unmodified complex uptake. This implied that, following prolonged exposure, non-specific uptake is likely the main method of internalisation of complexes.

Transfections of the polymer blend–gWIZLuc complexes into A549 cells and 3T3 cells showed no significant change in the transfection levels compared to the parent azido PEGylated polyamidoamine **5** and did not restore the transfection levels seen by $(MBA\text{-}DMEDA)_n$. The small increase seen in the 3T3 cells is due to increased non-specific interaction caused by the peptide on the surface of the particles.[18] The observed good uptake but poor transfection could be the result of the particles entering a recycling pathway and not the lysosomal pathway, hence the plasmid is not delivered effectively to the cell interior. Good uptake but poor transfection has been reported previously by folate targeted DNA particles and was attributed to uptake by a different pathway, resulting in the plasmid not being delivered effectively to the cell interior.[19] Another explanation is that the azido PEGylated polyamidoamine **5** particles show higher transfection than has been seen previously, and is comparable with the $(MBA\text{-}DMEDA)_n$ complexes.[5,6] These particles have been optimised to form neat, toroidal and spherical structures. The well formed particles and the addition of the peptide ligand may have inhibited the release of the complexed plasmid. A similar result has been reported by Clements et al.,[20] where the use of RGD ligand did not increase the transfection levels of their complex, They suggested that this may be due to inhibited binding, however our results suggest poor release of the plasmid DNA, as we have shown both good particle formation and binding of the targeted complexes to A549 and 3T3 cells.

## 4. Conclusions

The azido-terminated PEGylated polyamidoamine **5** polymer and the **5**:**1** blend–DNA nanoparticles have been successfully conjugated with a peptide ligand via the Staudinger reaction. Formulation has led to well formed, discrete nanoparticles with a small size. The postcomplexation Staudinger ligation, demonstrated for the first time in such a complex chemical environment, was superior to the precomplexation ligation both for efficiency of ligand conjugation and in terms of ensuring surface localisation of ligand. While a low level of binding of complexes to cells and uptake into cells were seen, we could demonstrate neither specificity nor increased transfection activity of the RGD decorated complexes. Despite these disappointing results with the RDG ligand, this modular method of ligand attachment could be used to target other receptors in order to improve the transfection efficiency of non-viral gene delivery systems.

## 5. Experimental

### 5.1. Instrumentation

NMR spectra were recorded on a Bruker Ultrashield 400 spectrometer. The $^{13}C$ spectra were proton decoupled. Chemical shifts are reported in parts per million using a residual protic solvent as an internal standard and coupling constants ($J$) are in Hz. Mass spectra were recorded on a Micromass LCT. UV spectra were recorded using WPA lightwave UV machine and glass cuvettes with a path length of 1.0 cm.

Flash column chromatography was carried out using silica gel 60 (40–63 μm) from Merck. Analytical TLC was performed using precoated silica gel 60 ($F_{254}$) aluminium sheets from Merck and examined at 254 nm or by permanganate stain. HPLC was carried out using Waters 510 pumps, Waters 484 detector and Kromasil $C_8$ analytical column (150 × 4.6 mm, 5 μm) at a flow rate of 1.0 ml $min^{-1}$ and the effluent was monitored at 220 nm. Gradient elution was from 10% to 50% B in 23 min, and the solvents used were solvent A (0.06% v/v TFA in Milli-Q water) and solvent B (0.06% v/v TFA in MeCN–Milli-Q water, 9:1 v/v).

Peptide synthesis was carried out in a 1 × 10 cm column (Kinesis) using a LKB NovaSyn Gem manual peptide synthesizer at a typical flow rate of 2.2 ml $min^{-1}$ with post-column UV monitoring at 290 nm using a LKB Biochrom Ultraspec 4050 spectrometer. Acylation reactions were performed at ambient temperature and the mixtures were intermittently stirred.

Luminescence was measured on a Turner TD-20E luminometer. Protein concentration was measured at 595 nm using a Beckman DU-640 UV spectrophotometer and analysed using Kineticalc version 2.7. Dynamic light scattering was measured using either a Malvern 4700 photo correlation spectrometer or Viscotek DLS with Omnisize analytical software. Flow cytofluorimetry was carried out using a Beckman coulter cytometer and analysed using EXPO 3.2 software.

### 5.2. Chemicals

Chemicals, biochemical reagents and solvents were purchased from Sigma–Aldrich (Gillingham, UK) and Fischer Scientific UK Ltd (Loughborough, UK). Fmoc amino acids, resin support and coupling reagents were purchased from Novabiochem Merck Biosciences Ltd (Nottingham, UK). Peptide synthesis grade DMF was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). The plasmid gWIZLuc, containing the firefly luciferase gene was purchased from Aldevron (South Fargo, USA) as a 1 mg $ml^{-1}$ solution.

### 5.3. Synthetic procedures

#### 5.3.1. Polyamidoamine:(*N*,*N*′-dimethylethylenediamine-alt-*N*,*N*′-methylenebisacrylamide)-*N*-propionamidomethylacrylamide, $(MBA\text{-}DMEDA)_n$ (1)

To a suspension of methylenebisacrylamide (3.10 g, 20.1 mmol) and 4-methoxyphenol (0.010 g, 0.08 mmol) in distilled $H_2O$ (10 ml) at 0 °C, *N*,*N*′-dimethylethylenediamine (2.08 ml, 19.6 mmol) was added with vigorous stirring under $N_2$ atmosphere. The mixture was brought up to 27 °C and stirred for 30 min before being left to react in the dark for 2 days. The viscous solution was diluted with water and acidified to pH 2 with 1.0 M $HCl_{(aq)}$. The solutions were stored at 4 °C before being purified by ultrafiltration and lyophilised to afford a white plastic like solid (4.5 g). GPC: number average molecular weight, $M_n$ 5854, weight average molecular weight, $M_W$ 32,037, most frequent mass, $M_P$ 22,027, polydispersity index, PDI 5.47; $\delta_H$ (400 MHz, $D_2O$) 2.73, 2.87 (s, $NCH_3$), 3.44, 3.65, 4.50 (s, $NCH_2N$); $\delta_C$ (100 MHz, $D_2O$) 29.09, 40.51, 44.22, 50.22, 52.70, 171.56.

#### 5.3.2. Poly(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate), DMEDA-PEG-DMEDA (2)

Poly(ethylene glycol) 2000 (3.5 g, 1.75 mmol) and carbonyldiimidazole (1.16 g, 7.1 mmol) was dissolved in $CHCl_3$ (20 ml) and stirred for 20 min and *N*,*N*′-dimethylethylenediamine (1.86 ml, 17.5 mmol) was added. The solution was stirred overnight before being concentrated and triturated with ether to produce a white precipitate. This was collected by filtration and dried under vacuum to give the title product (2.75 g, 70%) as a white powder. $\delta_H$ (400 MHz, $D_2O$) 2.11 (s, $NCH_3$), 3.59 (s, $OCH_2CH_2O$), 4.70 (s, $NCH_2CH_2$).

#### 5.3.3. Poly(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate)-block-polyamidoamine:(*N*,*N*′-dimethylethylenediamine-alt-*N*,*N*′-methylenebisacrylamide)-*N*-propionamidomethyl-acrylamide-block-poly(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate), DMEDA-PEG-DMEDA-(MBA-DMEDA)$_{n+1}$-PEG-DMEDA (3)

The (MBA-DMEDA)$_n$ **1** (0.95 g, 0.029 mmol, $M_W$ 32,037) was dissolved in distilled $H_2O$ and DMEDA-PEG-DMEDA **2** (0.77 g, 0.35 mmol) was added. The solution was stirred for 24 h at room temperature before the pH was adjusted to pH 2 using 1 M $HCl_{(aq)}$. The triblock polymer was then purified by ultrafiltration and lyophilised to yield the title polymer (0.797 g). GPC: $M_n$ 10,707, $M_W$ 39,849, $M_P$ 28,436, PDI 3.72; $\delta_H$ (400 MHz, $D_2O$) 2.73, 2.87, 3.44, 3.58, 3.65, 4.50; $\delta_C$ (100 MHz, $D_2O$) 29.09, 40.51, 44.22, 50.22, 52.70, 69.54, 171.56.

#### 5.3.4. 2-Azidoacetic acid (4)

Sodium azide (0.98 g, 15 mmol) was dissolved in water (10 ml) and cooled on an ice bath. Bromoacetic acid (1.04 g, 7.5 mmol) was added to the mixture and was stirred at 5 °C for 2 h before being brought up to room temperature and allowed to react for 24 h. The mixture was acidified to pH 5 with 1 M $HCl_{(aq)}$, extracted into diethyl ether (3× 30 ml). The ethereal extract was dried and evaporated to dryness to give 2-azidoacetic acid (0.730 g, 89%) as a pale yellow oil. $\nu_{max}$ ($cm^{-1}$) 1662s, 2110w; $\delta_H$ (400 MHz, $CDCl_3$) 2.51 (s, $CH_2$); $\delta_C$ 49.99, 174.44; *m*/*z* ($ES^+$, HRMS) found $MH^+$ 102.1008, Calcd for $C_2H_4N_3O_2^+$ 102.0298.

#### 5.3.5. *N*-2-Azidoacetyl-poly(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate)-block-polyamidoamine: (*N*,*N*′-dimethylethylenediamine-alt-*N*,*N*′-methylenebisacrylamide)-*N*-propionamidomethyl-acrylamide-block-*N*-2-azidoacetyl-poly-(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate) (5)

DMEDA-PEG-DMEDA-(MBA-DMEDA)$_{n+1}$-PEG-DMEDA **3** (100 mg, 0.0025 mmol) was dissolved in $CH_2Cl_2$ and carbonyldiimidazole (0.5 mg, 0.0030 mol) was added. The solution was stirred for 30 min, after which azidoacetic acid **4** (2.5 mg, 0.025 mmol) was then added and the resultant solution was stirred for a further 72 h. The solution was concentrated, diluted with water (10 ml), washed with chloroform (3× 20 ml). The aqueous extract was then evaporated to dryness, the residual material triturated with diethyl ether, and finally redissolved in distilled water (20 ml). The solution was lyophilised to give the title compound as a white solid (82 mg). $\nu_{max}$ ($cm^{-1}$) 1662s, 2110w.

#### 5.3.6. *N*-9-Fluorenylmethoxycarbonyl-prolyl-oxychlorotrityl poly(styrene)

The 2-chlorotrityl chloride poly(styrene) resin (0.429 g, 0.60 mmol, 1.4 mmol $g^{-1}$, 100–200 mesh, 1% DVB)[13] and Fmoc-Pro-OH (0.202 g, 0.6 mmol) were placed in a round bottom flask and dried in vacuo for 1 h. The solvent $CH_2Cl_2$ (10 ml) was added followed by *N*,*N*-diisopropylethylamine (154 μl, 0.90 mmol, DIEA), and the resultant mixture was stirred for 2 h. MeOH (1.0 ml) was added and the mixture stirred for a further 10 min. The resin was collected, washed with DMF (10× 1 ml), $CH_2Cl_2$ (10× 1 ml) and hexane (10× 1 ml), dried in vacuo to give the resin product (0.606 g) and stored at 4 °C. The amino acid loading, determined by Fmoc substitution level[13b] was found to be 0.78 mmol $g^{-1}$.

#### 5.3.7. Boc-Arg(Pbf)-Gly-Asp(OtBu)-Ser(tBu)-Pro-Ala-Ser(tBu)-Ser(tBu)-Lys(Boc)-Pro-OH

Fmoc-Pro-oxychlorotrityl poly(styrene) (0.385 g, 0.30 mmol, 0.78 mmol $g^{-1}$) was used for the construction of the protected decapeptide by standard solid-phase peptide synthesis (SPPS) methodology.[13] The amino acid residues were installed using *N*-Fmoc-protected amino acids, and activated using TBTU/HOBt/DIEA (1:1:2 equiv) in DMF; the activated amino acids were used in five-fold excess and the coupling reactions were typically allowed to proceed for 2–3 h. Repetitive Fmoc-deprotection was effected by 20% v/v piperidine in DMF for 10–15 min. The N-terminal capping was achieved using di-*tert*-butyl dicarbonate (0.655 g, 3.0 mmol) in DMF for 2 h. The protected peptidyl resin was collected and dried in vacuo, followed by treatment with TFA/$Et_3SiH$/$CH_2Cl_2$ (1:1:98 v/v, 10 ml) for 1 h at ambient temperature. The mixture was filtered in a solution of pyridine (150 μl) in MeOH (7.5 ml). The filtrate was evaporated to dryness in vacuo, triturated with water and the residual material was collected to give the protected peptide (0.236 g, 47%) as a white solid. A portion of the protected peptide (5 mg) was treated with TFA/$H_2O$/$Et_3SiH$ (45:4:1 v/v, 2.5 ml) for 2 h, followed by evaporation to dryness and trituration with diethyl ether to afford H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-OH (2 mg) as a white solid. HPLC: $t_R$ 3.66 min; *m*/*z* ($ES^+$, HRMS) found $MH^+$ 1001.5825 Calcd for $C_{40}H_{69}N_{14}O_{16}^+$ 1001.5011.

#### 5.3.8. Boc-Arg(Pbf)-Gly-Arg(Pmc)-Ser(tBu)-Pro-Ala-Ser(tBu)-Ser(tBu)-Lys(Boc)-Pro-OH

Fmoc-Pro-oxychlorotrityl poly(styrene) (0.282 g, 0.20 mmol, 0.706 mmol $g^{-1}$) was used for the construction of the protected 'RGR'-decapeptide by SPPS methodology[13] outlined above. The protected peptidyl resin was collected, dried in vacuo and treated with TFA/$Et_3SiH$/$CH_2Cl_2$ (1:1:98 v/v, 10 ml) for 1 h at ambient temperature. The mixture was filtered in a solution of pyridine (150 μl) in MeOH (7.5 ml). The filtrate was evaporated to dryness in vacuo, triturated with water and the residual material was collected to give the title protected peptide (0.270 g, 70%) as a white solid. A portion of the protected peptide (5 mg) was treated with TFA/$H_2O$/$Et_3SiH$ (45:4:1 v/v, 2.5 ml) for 2 h, evaporated to dryness and triturated with diethyl ether to afford H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-OH (2.4 mg) as a white solid. HPLC: $t_R$ 3.74 min; *m*/*z* ($ES^+$, HRMS) found $MH^+$ 1042.4474 Calcd for $C_{42}H_{76}N_{17}O_{14}^+$ 1042.5752.

#### 5.3.9. H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys(fluorescein-5-carbonyl)-Pro-OH

Fmoc-Pro-oxychlorotrityl poly(styrene) (0.144 g, 0.10 mmol, 0.686 mmol $g^{-1}$) was used for the construction of above fluorescein-labelled 'RGD'-peptide. The Lys residue was installed using the pseudo-orthogonally protected Fmoc-Lys(ivDde)-OH.[21] Following assembly and *N*-capping, the *N*-ivDde group was removed using 3–5% v/v $NH_2NH_2{\cdot}H_2O$ in DMF for 10 min. The peptidyl resin was then treated with 5-carboxyfluorescein (94 mg, 0.25 mmol)–7-aza-1-hydroxybentriazole, HOAt[22] (34 mg, 0.25 mmol)–*N*,*N*′-diisopropylcarbodiimide (0.046 ml, 0.3 mmol) in DMF (2 ml) and stirred gently overnight. The resin product was then washed with 20% v/v piperidine in DMF (2 ml $min^{-1}$) for 10–15 min, washed with DMF, collected and dried in vacuo. A sample of the peptidyl resin (103 mg) was treated with TFA/$H_2O$/$Et_3SiH$ (45:4:1 v/v, 10 ml) for 2 h, followed by evaporation to dryness and trituration with diethyl ether to afford the titled fluorescein-labelled 'RGD'-peptide (31 mg) as a bright yellow solid. HPLC: $t_R$ 10.83 min; *m*/*z* ($ES^+$, HRMS) found $MH^+$ 1359.4891 Calcd for $C_{61}H_{79}N_{14}O_{22}^+$ 1359.5488.

#### 5.3.10. *S*-Bromomethyl ethanethiolate

A mixture of thioacetic acid (4.00 g, 58 mmol) and paraformaldehyde (1.78 g) heated at 80 °C overnight. The mixture was then filtered by gravity and the resultant yellow oil was cooled in an ice bath under nitrogen. Phosphorus tribromide (10.70 g, 39.5 mmol) was added slowly keeping the temperature <5 °C. The solution was stirred at 0 °C for 10 min before being warmed to room temperature. The mixture was then poured into ice water (10 ml) and ex-

tracted into diethyl ether (3× 20 ml). The organic extract was washed with water (2× 50 ml), dried, concentrated and purified by column chromatography (silica, ether) to give pure *S*-bromomethyl ethanethiolate (5.57 g, 57%) as a yellow oil. The product was stored in a nitrogen atmosphere below 4 °C. $\delta_H$ (400 MHz, $CDCl_3$) 2.42 (3H, s, $CH_3$) 4.75 (2H, s, $CH_2$) TLC: $R_f$ 0.775 (ether).

#### 5.3.11. Borane–*S*-(diphenylphosphanyl)methyl ethanethiolate complex

NaH (0.754 g, 22.4 mmol, 70% dispersion in oil) was placed in a two-necked round bottom flask connected to a nitrogen bubbler. The NaH was washed three times with hexane, dissolved in DMF (10 ml) and the mixture was cooled to 0 °C. Borane diphenylphosphine complex (4.5 g, 22.4 mmol) in DMF (3 ml) was added slowly keeping the mixture below 5 °C. The *S*-bromomethyl ethanethiolate (3.74 g, 22.4 mmol) was then added and the mixture stirred at 0 °C for 10 min before being allowed to warm to room temperature. The mixture was stirred for a further 24 h, concentrated, redissolved in EtOAc (70 ml), filtered and evaporated to dryness. The crude product was purified by column chromatography (silica, EtOAc/hexane 1:10), to give required borane–thiolacetic acid ester complex[11c] (2.854 g, 44%) as a clear oil. $\delta_H$ (400 MHz, $CDCl_3$, P decoupled) 0.51–1.68 (3H, br m, $BH_3$), 2.28 (3H, s, $CH_3$), 3.74 (2H, d, $J$ = 6 Hz, $CH_2$), 7.47–7.53 (6H, m, Ph) 7.73–7.74 (4H, m, Ph); $\delta_C$ (100 MHz, $CDCl_3$) 24.15 (d, $J$ = 35.5 Hz, 30.46, 127.97 (d, $J$ = 55.2 Hz), 129.28 (d, $J$ = 10.2 Hz), 132.19 (d, $J$ = 2.6 Hz), 132.85 (d, $J$ = 9.3 Hz), 193.64; $m/z$ ($ES^+$, HRMS) found $MH^+$ 289.0509 Calcd for $C_{15}H_{19}BOPS^+$ 289.0981.

#### 5.3.12. *S*-(Diphenylphosphanyl)methyl ethanethiolate

The above borane–phosphanylmethyl ethanethiolate complex (182 mg, 0.633 mmol) was dissolved in toluene (3 ml) and stirred under $N_2$. DABCO in toluene (3 ml) was added and the mixture was stirred at 40 °C for 4 h. The solution was then evaporated to dryness and redissolved in chloroform (20 ml). This solution was then washed with 1 M HCl (2× 10 ml) and brine (10 ml), dried, evaporated to yield (diphenylphosphanyl)methyl thiolacetate[11c] (120 mg, 69%) as a clear viscous oil, and was used without further purification. $\delta_H$ (400 MHz, $CDCl_3$, P decoupled) 2.30 (3H, s, $CH_3$), 3.52 (2H, s, $CH_2$), 7.35–7.36 (6H, m, Ph ) 7.42–7.47 (4H, m, Ph); $\delta_C$ (100 MHz, $CDCl_3$) 26.38 (d, $J$ = 23.2 Hz), 30.85, 129.33 (d, $J$ = 7.2 Hz), 129.69, 133.28 (d, $J$ = 18.9 Hz), 137.33 (d, $J$ = 13.7 Hz), 195.22; TLC: $R_f$ 0.15 (EtOAc/hexane 10:1). HPLC: $R_f$ 8.84 min; $m/z$ ($ES^+$, HRMS) found $MH^+$ 275.0062 Calcd for $C_{15}H_{16}OPS^+$ 275.0654.

#### 5.3.13. (Diphenylphosphino)methanethiol (6)

The above (diphenylphosphanyl)methyl thiolacetate (119 mg, 0.43 mmol) was dissolved in degassed MeOH (3 ml) and stirred under nitrogen. A solution of aqueous NaOH (5 M in degassed water, 2 ml) was added and the solution was stirred for 2 h before being concentrated and redissolved in chloroform (20 ml). The organic solution was washed with a mixture 1 M $HCl_{(aq)}$ and brine (3× 10 ml), dried over $MgSO_4$ and evaporated to dryness. The crude product was then rapidly purified by column chromatography (silica, degassed EtOAc/hexane 3:1) to give the title compound[11b] (40 mg, 40%) as a colourless viscous oil. $\delta_H$ (400 MHz, $CDCl_3$, P decoupled) 1.40 (1H, t, $J$ = 8.0 Hz, SH), 3.06 (2H, d, $J$ = 7.5 Hz, $CH_2$), 7.36–7.38 (6H, m, Ph) 7.42–7.45 (4H, m, Ph); $\delta_C$ (100 MHz, $CDCl_3$) 20.86 (d, $J$ = 23.7 Hz), 128.59, 128.65, 129.15, 132.79 (d, $J$ = 18.5 Hz); TLC: $R_f$ 0.15 (EtOAc/hexane 1:10), $R_f$ 0.66 (EtOAc/hexane 1:3); $m/z$ ($ES^+$, HRMS) found $MH^+$ 233.0522 Calcd for $C_{13}H_{14}PS^+$ 233.0548.

#### 5.3.14. H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-(diphenylphosphanyl)methyl thiolate (7)

To a solution of Boc-Arg(Pbf)-Gly-Asp(tBu)-Ser(tBu)-Pro-Ala-Ser(tBu)-Ser(tBu)-Lys(Boc)-Pro-OH (65 mg, 0.039 mmol) in chloroform was added *N*-poly(styrene)methyl-*N*′-cyclohexylcarbodiimide (76 mg, 0.13 mmol; 1.7 mmol $g^{-1}$, 200–400 mesh, 2% DVB), HOBt (9.6 mg, 0.071 mmol) and 4-(*N*,*N*-dimethylamino)pyridine, DMAP (1.0 mg, 0.008 mmol). The mixture was stirred for 30 min, after which (diphenylphosphino)methanethiol **6** (45 mg, 0.19 mmol) was added, and the mixture was stirred for a further 48 h. The mixture was filtered, the filtrate evaporated to dryness, and the residual material triturated with water. The resulting solid was dissolved in the mixture TFA/$H_2O$/$Et_3SiH$ (45:4:1 v/v, 10 ml) and left for 4 h. The mixture was evaporated to dryness and the residual material was triturated with diethyl ether to give an off-white solid. The solid was dissolved in water and lyophilised to yield H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ (42 mg, 88%). HPLC: $t_R$ 16.76, 17.34 min (2:1); $m/z$ ($ES^+$, HRMS) found $MH^+$ 1215.5190 and 1215.5458 Calcd for $C_{53}H_{80}N_{14}O_{15}PS^+$ 1215.5380.

#### 5.3.15. H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-(diphenylphosphanyl)methyl thiolate (8)

A solution of Boc-Arg(Pbf)-Gly-Arg(Pmc)-Ser(tBu)-Pro-Ala-Ser(tBu)-Ser(tBu)-Lys(Boc)-Pro-OH (15 mg, 0.008 mmol) in chloroform (4 ml) was added *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide·HCl (4 mg, 0.021 mmol), HOBt (2.25 mg, 0.016 mmol) and DMAP (0.3 mg, 0.0024 mmol), and resultant mixture was allowed stirred for 30 min. (Diphenylphosphino)-methanethiol **6** (10 mg, 0.043 mmol) was then added and the mixture was stirred for 48 h. The solution was evaporated to dryness and the residual material was triturated with water. The resulting solid was dissolved in the mixture TFA/$H_2O$/$Et_3SiH$ (45:4:1 v/v, 10 ml) and left for 4 h. The solution was evaporated to dryness and the residual material was triturated with diethyl ether to give an off-white solid. The solid was dissolved in water and lyophilised to afford the peptide thioester H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ (9.8 mg, 98%). HPLC: $t_R$ 14.6 min; $m/z$ ($ES^+$, HRMS) found $MH^+$ 1254.5745 Calcd for $C_{55}H_{87}N_{17}O_{13}PS^+$ 1256.6122.

#### 5.3.16. *N*-(H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Gly)-poly-(ethylene glycol)bis(*N*-methyl-2-methylamino)ethyl carbamate)-block-polyamidoamine:(*N*,*N*′-dimethylethylenediamine-alt-*N*,*N*′-methylenebisacrylamide)-*N*-propionamidomethyl-acrylamide-block-*N*-(H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Gly)-poly-(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate) (9)

The azidoacetyl-modified polymer **5** (39 mg) and H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ **7** (5.0 mg, 0.004 mmol) were dissolved in phosphate buffer saline (2 ml) and allowed to react overnight. The resulting solution was then purified by ultrafiltration at 5000 rpm (3 × 10 min). The solution was then lyophised to yield a white plastic like solid (28 mg, 71% w/w). Amino acid analysis: 9% substitution.

#### 5.3.17. *N*-(H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Gly)-poly-(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate)-block-polyamidoamine:(*N*,*N*′-dimethylethylenediamine-alt-*N*,*N*′-methylenebisacrylamide)-*N*-propionamidomethyl-acrylamide-block-*N*-(H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Gly)-poly-(ethylene glycol)bis(*N*-methyl-2-(methylamino)ethyl carbamate) (10)

The azidoacetyl-modified polymer **5** (25 mg) and H-Arg-Gly-Arg-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ **8** (5.0 mg, 0.004 mmol) were dissolved in phosphate buffer saline (2 ml) and allowed to react overnight. The resulting solution was then purified by ultrafiltration at 5000 rpm (3 × 10 min). The solution was then lyophised to yield a white plastic like solid (23 mg, 92% w/w). Amino acid analysis: 0.4% substitution.

### 5.4. Fluorescent tagging of DNA

gWIZLuc (100 μl, 5 mg $ml^{-1}$) was mixed with YOYO the solution (20 μl, 1 mM in DMSO) by vortex and incubated at 4 °C for 24 h. The labelled DNA was stored at 4 °C for future use.

### 5.5. Gel permeation chromatography

Tris/NaCl solution was prepared from Tris acetate (12.1 g $l^{-1}$, 0.1 M) and sodium chloride (11.7 g $l^{-1}$, 0.2 M) and filtered through 0.22 μm filter and purged with $N_2$. TSK G3000 and TSK G4000 pw columns were attached to the pump in series and equilibrated with Tris/NaCl at 0.6 ml $min^{-1}$. Solutions of PEG 8600, PEG 23000, PEG 40000 (1 mg $ml^{-1}$) were prepared using Tris/NaCl buffer, and were used to generate a linear calibration curve. The polyamidoamine samples were then analyzed to determine the molecular weight and polydispersity.

### 5.6. Polymer:gWIZLuc complex preparation

Unless otherwise stated the complexes where prepared by adding a solution of the polymer to a dilute solution of the DNA in the required amounts to produce the complexes required at the correct repeating unit (RU):DNA nucleotide ratio. This was calculated from the following equation:

$$\text{Amount of polymer} = [\text{RU MW}_{\text{polymer}}/\text{RU MW}_{\text{DNA}}] \times \text{ratio} \times \text{Amount of DNA}$$

The RU $MW_{DNA}$ is taken as 308 (mean molecular weight of the nucleotides) and RU $MW_{polymer}$ for MBA-DMEDA as 315.

### 5.7. Gel retardation electrophoresis

Complexes were produced at a range of ratios by adding the required amount of polymer to gWIZLuc (2 μl) and tris acetate buffer (TAE, 16 μl). The solution was mixed by vortex and allowed to form for 30 min before gel loading buffer (3 μl) was added. The complexes (10 μl) were loaded into 0.8% agarose gel (1.6 g in 250 ml TAE) containing 1% ethidium bromide. The gel was developed at 100 V for 60 min using TAE as the running buffer. The gels were then visualised using a UV transilluminator to visualise the DNA before being stained with Brilliant blue R250 (0.5 g in methanol/glacial acetic acid/distilled water; 250:50:50 ml) for 1 h and destained (methanol/glacial acetic acid/distilled water; 1:1:1 v/v) overnight.

### 5.8. Formulation

Polymer blends were prepared using the equation:

$$V_{\text{pol 1}} = [\text{MW}_{\text{pol 1}}/\text{MW}_{\text{pol 2}}] \times \text{ratio} \times V_{\text{pol 2}}$$

where $V$ is volume of polymer required and MW is the RU molecular weight.

The individual polymers were in 1 mg $ml^{-1}$ stock solutions, and a single portion of azido-terminated polymer **5** was aliquotted into polyamidoamine $(MBA\text{-}DMEDA)_n$ **1**.

### 5.9. Staudinger ligation to postassembled complexes

#### 5.9.1. Postcomplexation RGD-modified particles (11)

The **5**:**1**–DNA complexes were prepared and left for 30 min. The decapeptidyl thioester H-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ **7** (ca. threefold excess) was added to the solution and allowed to react overnight. The resulting solution was then purified by ultrafiltration 5000 rpm (3 × 10 min) or by dialysis overnight. Amino acid analysis: 48% substitution.

#### 5.9.2. Postcomplexation RGR-modified particles (12)

The **5**:**1**–DNA complexes were prepared and left for 30 min. The decapeptidyl thioester H-Arg-Gly-Agr-Ser-Pro-Ala-Ser-Ser-Lys-Pro-$SCH_2PPh_2$ **8** (ca. threefold excess) was added to the solution and allowed to react overnight. The resulting solution was then purified by ultrafiltration 5000 rpm (3 × 10 min) or by dialysis overnight.

### 5.10. Particle size analysis

Single aliquots of the polymer was added to gWIZLuc (4 μl) and 1/10 PBS (50 μl) in a Uvette® and gently mixed. The size and polydispersity was then determined by dynamic light scattering using a DLS.

#### 5.10.1. Transmission electron microscopy

Complexes were prepared by adding polymer to gWIZLuc (4 μl) and 1/10 PBS (500 μl) and incubated for 30 min. A drop of the solution was placed a copper grid coated with Pioloform resin, left for 1 min, the excess liquid was removed and the process repeated. After air drying, the grid was inverted in uranyl acetate for 20 min. The grid was washed in 50% $ethanol_{(aq)}$ twice followed by water. The excess water was removed by blotting and the grids were air dried. The grids were examined using a JEOL JEM-1010 transmission electron microscope. Images were taken between 20,000 and 120,000× using a Kodak megaplus digital camera and analysed using Analysis 3.0 software.

### 5.11. Biological evaluation

#### 5.11.1. Cell lines and routine subculture

A549 cells (ECACC No. 86012804) and NIH 3T3 cells (ECACC No. 93061524) were routinely cultured in DMEM supplemented with 10% heat inactivated foetal calf serum, antibiotics and antimicrobials, 3T3 cells were also supplemented with L-glutamine, at 37 °C and 5% $CO_2$. The cell lines were subcultured every seven days at a ratio of 1:20, using trypsin EDTA to remove cells from flask surface.

#### 5.11.2. Cell fixation

A549 and 3T3 cells were grown to confluence in T75 flasks. The cells were detached using trypsin/EDTA and ultrafitration at 5000 rpm for 5 min. The cells were washed with PBS and suspended in 4% $paraformaldehyde_{(aq)}$ before being incubated at 37 °C for 10 min followed by 4 °C for 10 min. The cells were ultrafiltered at 5000 rpm for min and washed twice with PBS and stored at 4 °C.

#### 5.11.3. Fluorescence microscopy

3T3 and A549 cells were seeded into a 6-well plate at a density of $10^5$ cells per well, and grown for 24 h. Complexes were prepared as previously described, 300 μl of the complex was added to DMEM (3 ml). The media was removed for the cells, washed with PBS and then replaced with the complexes (1 ml × 2 wells). The cells were incubated for 4 h then examined using a Nikon microscope and a mercury lamp at 500× magnification.

#### 5.11.4. Cell binding studies

A549 were grown in a T75 flask cells were fixed using paraformaldehyde and resuspended in 5 ml of PBS. 500 μl aliquots were treated with native **3**:**1**-derived particles, unmodified **5**:**1**-derived particles, RGD precomplex modified particles, RGD postcomplex modified particles, RGR postcomplex modified particles and RGD fluorescein-labelled peptide for 10 min. The cells were ultrafiltered at 5000 rpm for 5 min in an MSE Centaur centrifuge, washed twice with PBS and resuspended in 300 μl of PBS. The cells were read in a

cytofluorometer and analysed using EXPO software. The same process was repeated using 3T3 cells.

### 5.11.5. Uptake experiments

A549 cells were seeded into 6-well plates at 250,000 cells per well and incubated at 37 °C for 24 h. The native **3**:**1**-derived particles, unmodified **5**:**1**-derived particles, RGD precomplex modified particles, RGD postcomplex modified particles, RGR postcomplex modified particles and the fluorescein-labelled 'RGD'-peptide (1 mg $ml^{-1}$) were prepared and diluted in 1.0 ml DMEM. The complexes were added to the cells and incubated at 37 °C for 10 min. The cells were washed twice with PBS before being loosened with trypsin/EDTA (300 μl). The cells were centrifuged at 5000 rpm for 5 min, washed with PBS, resuspended in 4% paraformaldehyde$_{(aq)}$ (600 μl) and incubated at 37 °C for 10 min followed by 4 °C for 10 min. The cells were centrifuged at 5000 rpm for 5 min washed twice with PBS and resuspended in PBS (300 μl). The cells were read by cytofluorometry and analysed using EXPO software. The method was repeated incubating the cells for 4 h and using 3T3 cells at both 10 min and 4 h.

### 5.11.6. Transfections

A549 or 3T3 cells were seeded into 6-well plates at a density of 50,000 cells per well and grown for 24 h to reach 60–70% confluence. Complexes were prepared as previously described in 300 μl using 4 μg of gWIZLuc and were added to OptiMEM buffer (3 ml). Complexes of LipofectAMINE™ were prepared using 4 μg gWIZLuc according to the manufacturer's protocol. The medium was aspirated from the cells and rinsed with PBS (1.0 ml). The complexes were added to the wells (1.0 ml × 3 wells) and incubated for 4 h at 37 °C. The media was then aspirated off the cells, was replaced with fresh DMEM (2.0 ml) and the cells were then incubated for a further 48 h. Concentrated (5×) cell lysis buffer (Promega) was diluted with distilled water to give a '1×' solution. The media was removed from the cells and the cells washed with PBS (1.0 ml) before cell culture lysis buffer was added (400 μl) and left for 1 min. The cell lysate was collected into centrifuge vials and centrifuged at 13,000 rpm for 5 min using a bench top microcentrifuge and the supernatant was collected.

**5.11.6.1. Luciferase activity.** The luciferase activity was measured by mixing the cell lysate (20 μl) with Luciferin reagent (100 μl) in a scintillation vial and measured using a luminometer calibrated with luciferin reagent (100 μl). Each sample was measured three times and the average value used in analysis.

**5.11.6.2. Bradford assay.** A solution of lysis buffer (1:10, 10.0 ml) was prepared and used to make bovine serum albumin standards of 0.3, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.003125 mg $ml^{-1}$ from a stock solution (1 mg $ml^{-1}$). Solutions of all the samples were prepared (3× 200 μl) using the distilled $H_2O$ so that they were diluted by a factor of 10. Samples and standards (10 μl) were transferred into a 96-well plate and Bradford assay reagent (200 μl) was added, the solutions were incubated in the dark for 15 min before being read at 595 nm using a plate reader.

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