# Bioconjugate Chemistry

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# <sup>1</sup> Optimization of Liganded Polyethylenimine Polyethylene Glycol <sup>2</sup> Vector for Nucleic Acid Delivery

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S Supporting Information



ABSTRACT: The delivery of nucleic acids into cells is an attractive approach for cancer therapy. Polyethylenimine (PEI) is 8 among the most efficient nonviral carriers. Recent studies have demonstrated that PEI can be conjugated to targeting ligands, 9 such as epidermal growth factor (EGF) and transferrin (Schaffert et al., 2011; Abourbeh et al., 2012; Ogris et al., 1999). Herein 10 we present a simplified protocol for producing homogeneous preparations of PEGylated linear PEI: LPEI-PEG<sub>2k</sub>. We generated 11 two well-characterized copolymers, with ratios of LPEI to PEG of 1:1 and 1:3. These copolymers were further conjugated 12 through disulfide bonds to a Her-2 targeting moiety, Her-2 affibody. This reaction yielded two triconjugates that target Her-2 13 overexpressing tumors. Polyplexes were formed by complexing plasmid DNA with the triconjugates. We characterized the 14 biophysical properties of the conjugates, and found that the triconjugate 1:3 polyplex had lower  $\zeta$  potential, larger particle size, 15 and more heterogeneous shape than the triconjugate 1:1 polyplex. Triconjugate 1:1 and triconjugate 1:3 polyplexes were highly 16 selective toward cells that overexpress Her-2 receptors, but triconjugate 1:1 polyplex was more efficient at gene delivery. Our 17 studies show that the biophysical and biological properties of the conjugates can be profoundly affected by the ratio of 18 19 LPEI:PEG<sub>2k</sub>:ligand. The procedure described here can be adapted to generate a variety of triconjugates, simply by changing the 20 targeting moiety.

# 21 INTRODUCTION

22 One of the hurdles facing molecular medicine is the targeted 23 delivery of therapeutic agents such as DNA or RNA molecules. 24 An emerging strategy is the construction of nonviral vectors, 25 such as cationic polymers and cationic lipids, which bind and 26 condense nucleic acids. These nonviral cationic vectors possess 27 many advantages over viral gene vectors, as they are non-28 immunogenic, non-oncogenic, and easy to synthesize.<sup>1-4</sup> 29 Currently, several synthetic polycationic polymers are being 30 developed for nucleic acid delivery.<sup>5</sup> Among these, poly-31 ethylenimines (PEIs) are considered promising agents for gene 32 delivery.<sup>6,7</sup>

PEIs are water-soluble, organic macromolecules that are 33 34 available as both linear and branched structures.<sup>8</sup> PEIs change 35 their degree of ionization over a broad range of pH, since every 36 third atom in their backbone chain is an amino-nitrogen, which 37 can be protonated. Approximately 55% of the nitrogens in PEIs 38 are protonated at physiological pH.<sup>9</sup> They possess high cationic

charge density, and are therefore capable of forming non- 39 covalent complexes with nucleic acids. Furthermore, their 40 physicochemical and biological properties can be altered by 41 various chemical modifications.<sup>10</sup> PEI-based complexes (also 42 known as polyplexes) can be endocytosed by many cell types.<sup>11</sup> 43 Following internalization of the polyplexes, endosome release 44 and high efficiency gene transfer are driven by the "proton 45 sponge effect".<sup>12</sup> The ability of PEI to condense DNA, 46 generating small particles, appears to be an important factor 47 in delivering large DNA constructs into many cell types. A 48 major concern in the utilization of PEIs as delivery carriers is 49 toxicity, due to their high positive surface charge, which may 50 lead to nonspecific binding.<sup>13</sup> PEI cytotoxicity is the major 51 barrier to successful in vivo transfection, due to its intrinsic, 52

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**Figure 1.** Conjugation of LPEI (~22 kDa) with NHS-PEG-OPSS (~2 kDa) yielded mainly two copolymeric networks that differ in the degree of PEGylation. The copolymer LPEI-(PEG<sub>2k</sub>)<sub>3</sub>-(OPSS)<sub>3</sub> ("di-conjugate 1:3") consisted on average of 1 mol of LPEI and 3 mol of PEG, whereas copolymer LPEI-PEG<sub>2k</sub>-OPSS ("di-conjugate 1:1") consisted on average of 1 mol of LPEI and 1 mol of PEG. (Ratios of LPEI:PEG were determined by <sup>1</sup>H NMR analysis.)

<sup>53</sup> nonspecific cytotoxicity at high concentrations and because it <sup>54</sup> can interact with blood proteins and may activate the <sup>55</sup> complement system.<sup>14</sup> Recent attempts have been made to <sup>56</sup> improve the selectivity and biocompatibility of nonviral vectors. <sup>57</sup> This has led to the modification of PEI molecules with <sup>58</sup> polyethylene glycol (PEG), in order to shield the PEI particle.<sup>15</sup> <sup>59</sup> The conjugation of heterobifunctional PEG groups to PEI <sup>60</sup> facilitates coupling of the PEI to a targeting ligand, which <sup>61</sup> provides efficient gene delivery into cells harboring the cognate <sup>62</sup> receptor.<sup>15</sup>

The primary aim of this study was to develop and 63 characterize cationic polymers that target Her-2 overexpressing 64 65 tumor cells. We previously generated EGF-receptor targeting 66 vectors consisting of branched PEI (brPEI-EGF) or linear PEI (LPEI) tethered to EGF.<sup>16</sup> Although the advantage of LPEI-67 68 EGF over brPEI-EGF was noted, the full characterization of the 69 derivatized LPEI was not presented. Herein we describe two 70 differentially PEGylated copolymers (LPEI-PEG<sub>2k</sub>) that are 71 conjugated to a Her-2 affibody, which functions as a Her-2 72 targeting agent. Affibody molecules are small, soluble, and 73 robust affinity ligands, which were developed from the B domain of staphylococcal protein A (SPA).<sup>17</sup> Affibodies have 74 low molecular weight (58 amino acids) and bind the 75 76 extracellular domain of Her-2 with high affinity and selectivity, 77 both in vivo and in vitro.<sup>18</sup> We provide detailed biophysical 78 characterization of the conjugates, and evaluate their efficacy 79 and specificity in gene delivery to Her-2 overexpressing breast 80 cancer cells.

#### RESULTS

**Synthesis of Thiol Reactive Copolymers.** Previous <sup>82</sup> studies have demonstrated the PEGylation of LPEI and its <sup>83</sup> conjugation to an EGFR targeting moiety.<sup>16</sup> However, the <sup>84</sup> extent of PEGylation on a single LPEI chain has not been fully <sup>85</sup> characterized. To generate differentially PEGylated copolymers, <sup>86</sup> the secondary amines on LPEI were conjugated to the terminal <sup>87</sup> NHS ester orthogonal protecting group on PEG. The *N*- <sup>88</sup> hydroxysuccinimide (NHS) ester is spontaneously reactive with <sup>89</sup> the secondary backbone amines of LPEI, providing efficient <sup>90</sup> PEGylation of LPEI. Furthermore, the reaction of the NHS- <sup>91</sup> PEG-OPSS with the amines of PEI results in the formation of <sup>92</sup> stable, irreversible amide bonds (Figure 1). <sup>93</sup> fi

The PEGylation products were purified by cation exchange 94 chromatography. Two peaks were eluted at high concentrations 95 of NaCl, one at ~120 mS/cm, and the other at ~132 mS/cm 96 (Supporting Information (SI) Figure S1). The purity of the 97 eluted fractions of the diconjugates was assessed using reverse 98 phase HPLC (SI Figures S2, S3). The two products were 99 presumed to differ in their ratios of LPEI:PEG and 100 consequently in their net positive charges. <sup>1</sup>H NMR spectra 101 were analyzed using the relative integral values of the hydrogen 102 atoms on PEG (-CH2-CH2-O-) (Figure 3a) and the 103 integral values of the hydrogen atoms on LPEI (-CH2-CH2- 104 NH–) (Figure 3b). This analysis indicated that the material  $_{105}$ eluted in the first peak consisted of a copolymer in which each 106 mole of LPEI was conjugated to approximately 3 mol of PEG. 107 This product was named LPEI-(PEG<sub>2k</sub>)<sub>3</sub>-(OPSS)<sub>3</sub> ("di- 108 conjugate 1:3"). The second peak consisted of a copolymer 109 in which equal numbers of moles of PEG and LPEI were 110

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**Figure 2.** <sup>1</sup>H NMR analysis of the two diconjugates, LPEI-PEG<sub>2k</sub>-OPSS (diconjugate 1:1) and LPEI-(PEG<sub>2k</sub>)<sub>3</sub>-(OPSS)<sub>3</sub> (diconjugate 1:3). The coupling of PEG groups to LPEI was indicated by the presence of the chemical shifts that correlate to ethylene glycol hydrogens (a) at 3.7 ppm and ethylenimine hydrogens at ~3.0 ppm (b). The integral values of these peaks provide molar ratios of PEG to LPEI, from which the illustrated structures of diconjugate 1:1 (A) and diconjugate 1:3 (B) were deduced.

111 conjugated, and was named LPEI-PEG<sub>2k</sub>-OPSS ("di-conjugate
 112 1:1") (Figure 2).

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Synthesis of the Triconjugates, LPEI-PEG<sub>2k</sub>-Her2 Affi-114 body (Triconjugate 1:1) and LPEI-(PEG<sub>2k</sub>)-(Her2)<sub>3</sub> Affi-115 body (Triconjugate 1:3). The primary aim of this study was 116 to develop a cationic polymer that would target Her-2 117 overexpressing tumor cells. Since Her-2 is an "orphan receptor" 118 and has no known ligand, affibody molecules targeting the Her2 receptor were used to generate Her-2 targeting triconjugates. <sup>119</sup> We expressed and purified Her-2 affibody with a Cys residue at <sup>120</sup> the C-terminal end to allow further conjugation (SI Figure S4). <sup>121</sup> The thiol reactive copolymers, diconjugate 1:1 and 1:3, were <sup>122</sup> conjugated to Her-2 affibody through its terminal Cys residue, <sup>123</sup> generating triconjugates 1:1 and 1:3, respectively (Figure 3). In <sup>124</sup> f3 order to generate the triconjugates, the reaction had to be <sup>125</sup> performed with low concentration of affibody (to prevent <sup>126</sup>

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Figure 3. Conjugation of the thiol reactive copolymers (diconjugate 1:1 and diconjugate 1:3) to the affibody ("Her-2") through disulfide exchange reaction, resulting in the generation of two differently PEGylated triconjugates.<sup>19</sup>

127 aggregation) and in the presence of 10% acetonitrile (ACN) as an organic polar solvent for increased solubility. The reaction 128 mix was purified by cation exchange (SI Figure S5). The yields 129 130 for both triconjugate reactions were approximately 33% as determined by copper assay. To confirm the conjugation of the 131 affibody to the diconjugate, the triconjugate products were 132 reduced with DTT and separated on SDS-PAGE. InstantBlue 133 134 staining confirmed that the reduced triconjugate released the 135 affibody (Figure 4). The amount of Her-2 affibody present in 136 the triconjugates was determined by measuring  $A_{280}$ . Using copper assay, we quantified the LPEI. As described above, <sup>1</sup>H 137 NMR analysis showed that the ratios of LPEI:PEG in the 138 purified diconjugates were 1:1 or 1:3. Comparing the molar 139 140 ratios of Her-2 affibody and LPEI, we determined that the average ratio of Her-2 affibody to LPEI in triconjugate 1:1 was 141 1:1, and in triconjugate 1:3 the average ratio was 3:1. Thus, we 142 conclude that nearly complete conjugation of affibody to LPEI-143 144 PEG was achieved.

<sup>145</sup> To generate polyplexes, the pure diconjugates and <sup>146</sup> triconjugates (SI Figure S6) were complexed with plasmid <sup>147</sup> DNA, as described in the Materials and Methods.

<sup>148</sup>  $\zeta$  Potential and Sizing of Polyplexes. We next <sup>149</sup> characterized the polyplexes, with respect to size and surface

charge, using dynamic light scattering (DLS). Polyplexes were 150 formed with pGreenFire1 with a ratio of nitrogen/phosphate 151 (N/P) of 6. Previous studies showed that complexation of  $_{152}$ positively charged conjugate with plasmid at a ratio of  $N/P = 6_{153}$ produces small (lack of aggregate formation), very stable 154 polyplexes, which do not aggregate and which exhibit efficient 155 plasmid transfection.<sup>20,21</sup> The size of a polyplex has a significant  $_{156}$ impact on its delivery properties.<sup>22</sup> In order to investigate the 157 effect of adding the targeting ligand on the size of a polyplex, 158 we compared the sizes of the diconjugates and the triconjugates 159 following complexation of each with plasmid DNA (poly-160 plexes). Diconjugate 1:1 polyplex had an average particle size of 161 115.2  $\pm$  8.2 nm and diconjugate 1:3 had an average particle size <sub>162</sub> of 253.1  $\pm$  9.5 nm. The polyplex generated from triconjugate 163 1:1 with plasmid gave an average particle size of  $141 \pm 5.8$  nm,  $_{164}$ whereas triconjugate 1:3 complexed with plasmid had an 165 average particle size of 256  $\pm$  24.2 nm (Figure 5). The smallest 166 fs particles (73.9  $\pm$  3.0 nm) were obtained in polyplexes 167 generated by complexing the plasmid with LPEI alone. The 168 conjugation of the affibody to the diconjugates had only a 169 minor effect on the particle size. The number of PEG groups, 170 however, did affect the particle size, suggesting that the PEG 171

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**Figure 4.** Conjugation of the affibody to the diconjugate, to produce the triconjugate (data presented for triconjugate 1:1 only), was confirmed by SDS/PAGE of purified affibody, diconjugate, and triconjugate, in the absence and in the presence of DTT. In the presence of DTT, the affibody was released from the triconjugate, and migrated alongside purified affibody (slightly above 10 kDa).



**Figure 5.** Particle sizing using DLS measurements of LPEI, the diconjugates, and the triconjugates complexed with plasmid pGreenfire 1 in HBG buffer pH 7.4.

172 groups caused steric hindrance, interfering with plasmid 173 condensation.

A positive surface charge facilitates polyplex binding to the 174 175 negatively charged cell surface, but excessive positive charge can lead to nonspecific binding and significant toxicity.<sup>15</sup> The  $\zeta$ 176 potentials of the various complexes, presented in Figure 6, are 177 in agreement with previous studies, which showed a decrease in 178 potential with increased number of PEG units.<sup>16</sup> To assess 179 L 180 the effect of PEG groups on the surface charge of our chemical 181 vectors we measured the  $\zeta$  potentials of polyplexes formed by 182 complexation of plasmid DNA with the precursors, diconju-183 gates 1:1 and 1:3, and with the triconjugates 1:1 and 1:3. 184 Diconjugate polyplex 1:1 had an average  $\zeta$  potential of 27.0  $\pm$ 

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**Figure 6.**  $\zeta$  potential distributions of LPEI, diconjugates and triconjugates complexed with plasmid pGreenfire1. The zeta potentials were measured by DLS and calculated by the Smoluchowski equation.

0.1 mV and diconjugate polyplex 1:3 had an average  $\zeta$  potential 185 of 20.0 ± 1.0 mV. Triconjugate polyplex 1:1 showed  $\zeta$  potential 186 with an average of 17.1 ± 0.7 mV, whereas triconjugate 187 polyplex 1:3 showed an average of 10.2 ± 0.44 mV (Figure 6). 188 Unlike the sizes, the  $\zeta$  potentials of the polyplexes were affected 189 by both the number of PEG groups and the conjugation of the 190 Her-2 affibody. Although the smallest, most positively charged 191 polyplexes were obtained with naked LPEI, these particles can 192 be extremely toxic when used at high concentration in vivo, due 193 to nonspecific interactions.<sup>23</sup> We expected that the addition of 194 PEG groups and a targeting moiety would diminish toxicity, but 195 because the polyplexes were still relatively small in size, we 196 hoped that their efficiency as nucleic acid delivery vectors 197 would not be compromised.

Assessment of Polyplex Shape Using AFM. The 199 importance of particle shape and its influence on delivery 200 properties is gaining recognition.<sup>24</sup> We analyzed the morphol- 201 ogy of the polyplexes obtained with the triconjugates in 202 solution using AFM. The diameters of triconjugates 1:1 and 1:3 203 polyplexes were both in the nanosize range (Figure 7). This is 204 f7 in agreement with the results obtained by DLS. The 205 triconjugate 1:1 polyplex displayed spherical and elliptical 206 particles. Most particles ranged in diameter from 101 to 178 207 nm, with an average particle diameter of  $142 \pm 35.3$  nm (based 208 on 31 particles). A few particles were exceptionally large, with 209 some even reaching >250 nm (Figure 7A). Triconjugate 1:3 210 polyplex was more heterogenic in shape and, moreover, yielded 211 large aggregates with undefined particle shape (Figure 7B). 212 These ranged in length from 150 to 650 nm, with an average 213 particle length of 312 nm; they ranged in width from 85 to 400 214 nm, with an average width of 175 nm (based on 23 particles). 215

**DNase Protection Assay.** Successful in vivo gene delivery 216 depends on efficient protection from nucleases. To determine 217 the ability of the triconjugates to protect plasmids from 218 degradation and enable efficient gene delivery, the polyplexes 219 were treated with DNase I and analyzed using gel electro- 220 phoresis. As shown in Figure 8, naked plasmid pGreenFire1 221 f8 DNA was fully degraded following 10 min of incubation with 2 222 units of DNase I. In contrast, when polyplexes were generated 223

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Figure 7. AFM measurements were performed in HBG buffer pH 7.4 for both polyplexes: (A) triconjugate 1:1 Polyplex; (B) triconjugate 1:3 Polyplex. Scale bar is 1  $\mu$ m.



**Figure 8.** Differentially PEGylated polyplexes protect plasmid pGreenFire1 from DNase I degradation. 1  $\mu$ g plasmid (pGreenFire1) alone or triconjugate polyplexes 1:1 and 1:3 were treated with or without DNase I (2 IU). Supercoiled plasmid (s.c.), open circular plasmid (o.c.).

224 by mixing plasmid with the triconjugates, the plasmid was 225 protected from degradation by DNase I. Complete protection 226 of the plasmid was observed for triconjugate polyplex 1:3, while 227 some nicking did occur for triconjugate polyplex 1:1, as shown 228 by the shift from the supercoiled (s.c.) to the open circular 229 (o.c.) form of the plasmid. The stronger protection from 230 DNase I conferred by triconjugate polyplex 1:3 may be 231 attributed to the increased steric hindrance provided by the 232 additional PEG-protein units in these complexes. Indeed, 233 previous studies have shown that PEGylation of PEI can 234 stabilize polyplexes and increase their circulation in the blood, 235 by impeding their interactions with enzymes and serum 236 factors.<sup>25,26</sup> Thus, the ability of both triconjugates 1:1 and 1:3 237 to protect the plasmid from DNase I degradation suggests that 238 the complexes will be stable upon systemic application, 239 facilitating efficient gene delivery.

Biological Activity of the Targeting Triconjugate 240 <sup>241</sup> Polyplexes 1:1 and 1:3. Polyplex size and  $\zeta$  potential 242 influence the efficiency of targeted DNA delivery and gene 243 expression, but the effect of size appears to be dependent on the particular conjugate.<sup>2,22</sup> To evaluate the specificity and the 244 efficiency of transfection of the triconjugate polyplexes 1:1 and 245 246 1:3, two breast cancer cell lines that differentially express Her-2 were utilized. Polyplexes of the triconjugates 1:1 and 1:3 were 247 formed with pGreenFire1 and transfected into MDA-MB-231 248 cells (expressing approximately  $9 \times 10^3$  Her-2 receptors/cell<sup>27</sup>) 249 <sup>250</sup> and BT474 cells (expressing approximately  $1 \times 10^{6}$  Her-2 <sup>251</sup> receptors/cell) (SI Figure S7).<sup>28</sup> Differential luciferase activity was observed 48 h after transfection. Both triconjugate 2.52 polyplexes 1:1 and 1:3 led to more than 300-fold higher 253 254 luciferase activity in BT474 cells than in MDA-MB-231 (\* p < 255 0.001) (Figure 9A). More efficient gene delivery to BT474 was 256 confirmed by GFP expression, as seen by confocal microscopy 257 (Figure 9B). These results show that polyplex selectivity is 258 dependent on Her-2 expression.

Targeted delivery to BT474 cells by triconjugate polyplex 1:1 259 was 10-fold more efficient than delivery by triconjugate 260 polyplex 1:3 (Figure 9A,B), even though triconjugate 1:3 has 261 more targeting moieties. This may reflect the higher  $\zeta$  potential 262 and lower size of triconjugate polyplex 1:1. 263

We next tested the survival of MDA-MB-231 and BT474 264 cells following treatment with triconjugate polyplexes 1:1 and 265 1:3. Neither polyplex showed cytotoxic effects in MDA-MB-231 266 cells, in a methylene blue assay. Similar results were observed in 267 BT474 cells treated with triconjugate polyplex 1:3. However, a 268 slight increase in cell cytoxicity was observed in BT474 treated 269 with triconjugate polyplex 1:1 (Figure 9C). Altogether, these 270 results indicate that the small size and higher  $\zeta$  potential of 271 triconjugate polyplex 1:1 confer efficient targeted delivery 272 properties, with only a slight increase in toxicity. Thus, the 273 polyplex of the triconjugate polyplex 1:1 is superior in gene delivery to 274 the more shielded triconjugate polyplex 1:3.

## DISCUSSION

The purpose of the present study was to explore in-depth the 277 biophysical properties and biological activity of differentially 278 PEGylated polyplexes that were designed to deliver nucleic 279 acids into Her-2 overexpressing cells. Gene delivery by 280 polyplexes is strongly affected by the biophysical properties of 281 the polyplexes, including size, shape, and surface charge.<sup>15</sup> 282

Polycations, especially PEI, have been intensively investigated 283 as agents for gene transfection.<sup>29,30</sup> Optimal transfection 284 efficacies are obtained when the polymeric nanoparticle 285 complexes possess an overall positive charge, which allows 286 them to bind to the negatively charged heparin sulfate 287 proteoglycans on the cell surface.<sup>31</sup> Previous studies showed 288 that linear PEI (LPEI) is more effective in gene transfection 289 than branched PEI (brPEI),<sup>32–36</sup> but LPEI has higher positive 290 charge and hence is more toxic. Various shielding entities, such 291 as PEG,<sup>15</sup> poly(ethylene oxide)-poly(propylene oxide)-poly- 292 (ethylene oxide) (PEO-PPO-PEO),<sup>37,38</sup> polyethylene glycol- 293 poly  $\varepsilon$ -caprolactone (PEG-PCL)<sup>39</sup> and poly(ethylene oxide),<sup>40</sup> 294 have been conjugated to cationic polymers, in an attempt to 295 lower the positive charge and the consequent toxicity. Indeed, 296 shielding of PEIs with PEG groups of varying lengths 297 significantly lowered toxicity while maintaining transfection 298 efficiency.<sup>15,16,41</sup>

In the current study, the conjugation of LPEI with  $PEG_{2k}$  300 yielded diconjugate copolymers comprising various ratios of 301 LPEI to  $PEG_{2K}$ . These diconjugates could be separated from 302 one another using cation exchange chromatography, due to 303 differences in charge, which reflect the different numbers of 304  $PEG_{2k}$  groups conjugated. <sup>1</sup>H NMR analysis confirmed that the 305 diconjugates differed from one another in the average number 306

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Figure 9. Her-2 mediated gene transfer of pGFP-LUC using the triconjugate 1:1 polyplex and the triconjugate 1:3 polyplex containing LPEI:PEG ratios of 1:1 and 1:3, respectively. BT474 and MDA-MB-231 breast cancer (10 000 cells/well) were treated for 48 h with triconjugates 1:1 and 1:3 complexed with pGFP-LUC (1  $\mu$ g/mL) to generate the two polyplexes, at PEI nitrogen/DNA phosphate ratio of 6 (N/P = 6) in HBS. (A) Measurements of luciferase activity demonstrate significantly less pGreenFire1 delivery to MDA-MB-231 cells than to BT474, and reduced gene delivery mediated by triconjugate 1:3 polyplexes as compared to triconjugate 1:1 (\* p < 0.001). Luciferase activity was measured in triplicates after 48 h. Relative luciferase units (RLU) are shown (mean + S.D.). (B) Fluorescent images of cells treated with polyplexes. Images are shown at X10 magnification and are representative of three experiments performed. (C) Methylene blue assay depicts percent cell survival compared to untreated (UT) cells.

307 of  $PEG_{2k}$  units per LPEI unit, where diconjugate 1:1 had an 308 LPEI:  $PEG_{2k}$  ratio of 1:1 and diconjugate 1:3 had an LPEI: 309  $PEG_{2k}$  ratio of 1:3. The conjugation of the Her-2 targeting 310 affibody to each of the purified diconjugates yielded a 311 triconjugate product of the appropriate molecular weight, i.e., 312 from diconjugate 1:1 we obtained "tri-conjugate 1:1" with 313 LPEI:PEG<sub>2k</sub>:Her-2 equal to 1:1:1 and from diconjugate 1:3 we 314 obtained "tri-conjugate 1:3", with ratio 1:3:3. This protocol 315 enabled us to obtain homogeneous products, with nearly 316 complete conjugation of targeting affibody to LPEI-PEG, in a 317 reproducible manner. We observed that PEGylation strongly affects the size of the 318 polyplex particles obtained upon complexation of the 319 diconjugates or triconjugates with plasmid DNA. Both 320 diconjugate 1:3 and triconjugate 1:3 polyplexes had average 321 particle sizes larger than diconjugate 1:1 and triconjugate 1:1 322 polyplexes. We believe that increasing the amount of 323 PEGylation on a single cationic chain leads to steric hindrance, 324 which prevents the polymeric chain from condensing the 325 plasmid to a smaller particle. This is consistent with the finding 326 that the naked LPEI polyplex had the smallest particles. 327 Moreover, while both triconjugates 1:1 and 1:3 protected 328 complexed plasmid from DNase I, the triconjugate 1:3 polyplex 329 provided better protection, possibly due to the increased steric 330 hindrance. 331

Previous studies suggested that increasing the molecular 332 weight of the PEG units conjugated to cationic polymers leads 333 to decreased surface charge of the polyplexes obtained upon 334 complexation with nucleic acids.<sup>16</sup> Our data show that 335 increasing the number of PEG groups of similar molecular 336 weights leads to decreased surface charge, as defined by  $\zeta$  337 potential distribution. Indeed, the highest surface charge was 338 shown by naked LPEI complexed with plasmid. These results 339 support the idea that the more neutral entities present in a 340 chemical vector, the lower the surface charge. Surprisingly, the 341 triconjugate polyplexes had lower surface charge than the 342 diconjugate polyplexes, showing that the Her-2 affibody (which 343 itself has slight positive charge) also reduced the surface charge 344 of the particles. We suspect that Her-2 affibody changes the 345 topography of the particle, with more targeting moieties 346 masking the charge on the surface, leading to a decrease in 347 surface charge. 348

The shape of a polyplex has a significant effect on its 349 performance as a drug delivery candidate,<sup>42,43</sup> although it is not 350 yet known which polyplex shapes are desirable for effective 351 drug delivery. The effect of PEGylation on polyplex shape has 352 not been investigated, to our knowledge, until now. In AFM 353 pictures, the triconjugate 1:1 polyplex—which was more 354 effective in gene delivery—presented shape homogeneity, 355 while the triconjugate 1:3 was more heterogenic, with many 356 asymmetrical, undefined shape particles. Moreover, unlike the 357 polyplex 1:1, the polyplex 1:3 seemed to form large aggregates. 358

Selective gene transfer using cationic polymers remains a 359 major challenge. Previous studies have shown that targeting of 360 LPEI and LPEI-PEG conjugates, with EGF or transferrin, 361 increased their selectivity and decreased nonspecific inter- 362 actions both in vitro and in vivo.<sup>36,37</sup> To examine the selectivity 363 of our Her-2 targeting triconjugates 1:1 and 1:3 polyplexes, we 364 utilized two breast cancer cell lines that differentially express 365 Her-2. Gene delivery, as shown by luciferase activity and GFP 366 expression, was significantly higher in BT474 cells, which highly 367 overexpress the Her-2 receptor, than in MDA-MB-231 cells, 368 which express 100-fold fewer Her-2 receptors on the cell 369 surface. Thus, the data demonstrate that both triconjugates 1:1 370 and 1:3 are highly selective for Her-2 overexpressing cells. 371

Previous studies have shown that high levels of PEGylation 372 can result in reduced gene transfection.<sup>44</sup> These results are in 373 accordance with our observation that the highly PEGylated 374 triconjugate 1:3 polyplex showed a significant reduction in gene 375 delivery, as compared to the less PEGylated triconjugate 1:1 376 polyplex, as shown by luciferase activity and GFP expression. 377 The increased gene delivery by the lesser PEGylated 378 triconjugate 1:1 polyplex was accompanied by slight cellular 379 toxicity, most likely due to its higher surface charge. 380 Our working hypothesis before engaging in this study was that increasing the number of targeting moieties per LPEI unit would lead to improved gene delivery and/or selectivity. We seculated that triconjugate 1:3, which has 3 mol of Her-2 sis afffibody molecules conjugated per mole of LPEI, would show increased receptor-mediated particle internalization. However, the triconjugate 1:3 polyplexes showed lower  $\zeta$  potential, larger which might contribute to the decreased transfection which might contribute to the decreased transfection efficiencies actually observed. Our results show that the less PEGylated triconjugate 1:1 is superior to the more PEGylated triconjugate 1:3 in mediating selective and efficient gene delivery into Her-2 overexpressing cells.

This study shows that adding PEG and an efficient targeting moiety to LPEI-based polyplexes led to decreased surface charge, increased polyplex size, and increased shape heterogreater and that these properties had profound effects on targeted gene delivery. Our simplified synthesis allows purification of homogeneous products in a reproducible for fashion, which can now be expanded to generate different triconjugates, using a variety of targeting moieties.

#### 402 MATERIALS AND METHODS

403 **Chemicals.** NHS-PEG-OPSS (ortho-pyridyldisulfide-poly-404 ethyene glycol-*N*-hydroxylsuccinimide ester), also named PDP-405 PEG-NHS (PDP: pyridyl dithio propionate), with molecular 406 weight of ~2 kDa, was purchased from Creative PEGworks 407 (Winston, USA). Poly(2-ethyl-2-oxazoline), average molecular 408 weight ( $M_n$ ) ~50 kDa, and anhydrous dimethyl sulfoxide 409 (DMSO) were purchased from Sigma-Aldrich (Israel). 410 Absolute ethanol was purchased from Romical (Israel). All 411 solvents were used without further purification.

<sup>412</sup> Synthesis of ~22 kDa LPEI (Free Base Form). The cationic <sup>413</sup> polymer linear polyethylenimine (LPEI) was synthesized as <sup>414</sup> described previously<sup>45</sup> with modifications. Briefly, 8.0 g (0.16 <sup>415</sup> mmol) of poly(2-ethyl-2-oxazoline) were hydrolyzed with 100 <sup>416</sup> mL of concentrated HCl (37%) and refluxed for 48 h, yielding <sup>417</sup> a white precipitate. The solid was filtered by vacuum through a <sup>418</sup> sinter glass and washed several times with water. The resulting <sup>419</sup> LPEI hydrochloride salt was air-dried overnight, dissolved in 50 <sup>420</sup> mL of water, and freeze-dried (5 g, 78%, <sup>1</sup>H NMR, D<sub>2</sub>O, 400 <sup>421</sup> MHz: singlet 3.5 ppm). The resulting LPEI salt (4.5 g) was <sup>422</sup> made alkaline by adding aqueous NaOH (3 M) and the <sup>423</sup> resulting white precipitate was filtered and washed with water <sup>424</sup> until neutral. The solid was then dissolved in water and further <sup>425</sup> lyophilized to give a white solid (2 g, 81%).

Synthesis of LPEI-PEG<sub>2k</sub>-OPSS Diconjugates (Diconjugate 426 427 1:1 and 1:3). 174 mg (8  $\mu$ mol) of LPEI were dissolved in 2.7 428 mL of absolute EtOH and agitated at room temperature for 15 429 min. A 5-fold molar excess of OPPS-PEG<sub>2k</sub>-CONHS (79 mg, 39.5  $\mu$ mol) was dissolved in 500  $\mu$ L of anhydrous DMSO and 430 431 introduced in small portions into the LPEI mixture. The 432 reaction mix was agitated at ~800 rpm on a vortex stirrer at 433 ambient temperature for 3 h. Different PEG-substituted LPEIs were separated by cation-exchange chromatography, using an 434 435 HR10/10 column filled with MacroPrep High S resin 436 (BioRad). The purity of the eluted fractions of the diconjugates was assessed using reverse phase HPLC equipped with 437 438 analytical Vydac C-8 monomeric 5  $\mu$ m column (300 Å, 4.6  $\times$ 439 150 mm), using a linear gradient of 5-95% acetonitrile over 25 440 min at 1 mL/min flow. Fractions with 95% purity or higher 441 were combined. The combined fractions were further dialyzed 442 against 20 mM HEPES pH 7.4. The ratio of PEG<sub>2k</sub> groups

conjugated to LPEI in the diconjugates was determined by <sup>1</sup>H 443 NMR. The integral values of the hydrogens from the 444 polyethylene -(CH<sub>2</sub>--CH<sub>2</sub>--O)- and from the LPEI -(CH<sub>2</sub>- 445 CH<sub>2</sub>--NH)- were used to determine the ratio between the two 446 conjugated copolymers. Of the various products obtained from 447 the cation-exchange, two products, LPEI-PEG<sub>2k</sub>-OPSS (dicon- 448 jugate 1:1, with molar ration of LPEI to PEG ~1:1) and LPEI- 449 (PEG<sub>2k</sub>)<sub>3</sub>-(OPSS)<sub>3</sub> (diconjugate 1:3, with molar ratio of ~1:3), 450 were chosen for the generation of triconjugates. A copper assay 451 was used to evaluate the copolymer concentration.<sup>46</sup> Briefly, the 452 copolymers were incubated with CuSO<sub>4</sub> (23 mg dissolved in 453 100 mL of acetate buffer) for 20 min and their absorbance at 454 285 nm was measured.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 456 of Proteins. Samples (30  $\mu$ L) were diluted in SDS protein 457 sample buffer with or without 100 mM DTT and then applied 458 to Tricine gel (13% polyacrylamide). Electrophoresis was 459 performed using cathode buffer (0.1 M Tris, 0.1 M Tricine, and 460 0.1% SDS pH 8.25) and anode buffer (0.21 M Tris pH 8.9) and 461 protein bands were visualized by staining with InstantBlue 462 (Expedeon, UK). 463

Affibody Expression and Purification. A plasmid 464 encoding Her-2 affibody Z:2891 was a gift from Donna 465 Arndt-Jovin. The Her-2 affibody sequence was cloned by PCR 466 into plasmid pET28a, generating a vector encoding Z:2891 467 affibody fused to an N-terminal hexahistidyl (His6) tag and a 468 C-terminal Cys residue. The affibody was expressed in E. coli 469 BL21 (DE3) as follows: The cells were grown at 37 °C to 470  $OD_{600} \sim 0.7$ . IPTG was added to a final concentration of 0.5 471 mM, followed by incubation at 30 °C for 4 h. The cell pellet 472 was stored at -80 °C. The affibody was purified at the Protein 473 Purification Facility, Wolfson Center for Applied Structural 474 Biology, The Hebrew University of Jerusalem, as follows: The 475 cell pellet was resuspended in buffer A (20 mM HEPES pH 7.4, 476 500 mM NaCl, 10% glycerol, 10 mM imidazole, and 2 mM  $\beta$ - 477 mercaptoethanol) to which were added lysozyme (3 mg/mL), 478 DNase I (20 units/mL), and protease inhibitors. The bacteria 479 were disrupted using a Microfluidizer Processor M-110EHI 480 according to the manufacturer's instructions. The soluble 481 fraction was recovered by centrifugation at 12 000×g for 10 min 482 at 4 °C. The resulting fraction was loaded onto a Ni affinity 483 column (Clontech, Mountain View, CA). The column was 484 washed with buffer A (14 column volumes (cv)). Thereafter, a 485 step gradient was applied, using increasing concentrations of 486 buffer B (20 mM HEPES pH 7.4, 500 mM NaCl, 10% glycerol, 487 500 mM Imidazole, and 2 mM  $\beta$ -Mercaptoethanol): 6% buffer 488 B (5 cv), 10% buffer B (1.5 cv), 30% buffer B (2 cv). The 489 bound protein was eluted with 100% buffer B (5cv). The eluted 490 fractions were then concentrated with an Amicon filter (3 kDa 491 cutoff) and loaded onto a gel filtration column comprising 492 Superdex 30 prep grade (120 mL) (GE Healthcare). The 493 purified protein was further analyzed by SDS-PAGE, and its 494 identity confirmed using Western blot analysis with anti- 495 affibody antibody (Abcam). The purity was further assessed by 496 reverse phase HPLC (Merck-Hitachi model L-7100). 497

Synthesis of PEI–PEG–ligand Affibody (Triconjugate 1:1 498 and 1:3). 4.97 mg ( $2 \times 10^{-4}$  mmol) of each diconjugate (1:1 499 and 1:3) were dissolved in 940  $\mu$ L of 20 mM HEPES pH 7.4. 500 Then, 3.4 mg ( $3.8 \times 10^{-4}$  mmol, ~2 equiv) of Her-2 affibody in 501 HBS was added dropwise to the reaction. Four milliliters of 20 502 mM HEPES plus 700  $\mu$ L of acetonitrile (HPLC grade) was 503 introduced into the reaction mix for increased solubility. The 504 reaction was further vortexed (800 rpm) in the dark at room 505 506 temperature until  $A_{343}$  indicated complete turnover. The 507 resulting triconjugates were purified by cation exchange 508 chromatography on a HR10/10 column filled with MacroPrep 509 High S resin (BioRad) (using a three-step gradient elution of 510 20 mM HEPES pH 7.4 to 20 mM HEPES containing 3 M 511 NaCl). The eluted fractions were introduced to analytical RP-512 HPLC to assess the purity of the triconjugates. Fractions with 513 95% purity and higher were combined and were kept at -80 514 °C. The concentration of the triconjugate was determined by 515 copper assay (as above). The amount of conjugated protein was 516 determined by  $A_{280}$  using Nano-Drop 2000.

S17 Verification and Purity of Chemical Vectors Con-S18 jugated to Targeting Protein. The triconjugates were S19 electrophoresed on SDS-PAGE, and stained with InstantBlue, S20 to confirm the conjugation of the affibody to LPEI-PEG<sub>2k</sub>. The S21 purity of the triconjugates was confirmed by reverse phase S22 HPLC, using an analytical Vydac C-8 monomeric 5  $\mu$ m column S23 (300 Å, 4.6 × 150 mm) at 1 mL/min while monitoring at 220 S24 nm. A gradient elution with acetonitrile, 5–95% in 25 min with S25 triple distilled water (TDW) containing 0.1% TFA as mobile S26 phase, was used for the HPLC analysis.

Polyplex Formation. Plasmid pGreenFire1, encoding 527 528 Firefly Luciferase and GFP (System Biosciences, Inc.), was 529 amplified in E. coli and purified by Qiagen Plasmid Maxi Kit 530 (Qiagen, Valencia, CA, USA) according to the manufacturer's 531 protocol. The triconjugate 1:1 or triconjugate 1:3 was s32 complexed with plasmid at a ratio of N/P = 6 (where N = <sup>533</sup> nitrogen from LPEI and P = phosphate from DNA) in HEPES-534 buffered glucose (HBG: 20 mM HEPES, pH 7.4, 5% glucose,  $_{535}$  w/v), generating two polyplexes. To allow complete formation 536 of the polyplex particles, the samples were incubated for 30 min 537 at room temperature. The final plasmid concentration in the 538 polyplex samples was 100  $\mu$ g/mL, whereas for DNase 539 protection assay and luciferase assay, the final concentration 540 of the plasmid was 10  $\mu$ g/mL in HEPES-buffered saline (HBS: 541 20 mM HEPES, 150 mM NaCl, pH 7.4).

542 **ζ-Potential and Sizing Measurements.** The sizes of the 543 polyplex particles obtained after dispersal in HBG buffer were 544 measured at 25 °C, by dynamic light scattering using a Nano-545 ZS Zetasizer (Malvern, UK), using volume distribution 546 calculation. The instrument is equipped with a 633 nm laser, 547 and light scattering is detected at 173° by back scattering 548 technology (NIBS, Non-Invasive Back-Scatter). Each sample 549 was run in triplicate.  $\zeta$  potential measurements were also 550 performed at 25 °C using a Nano-ZS Zetasizer (Malvern, UK). 551 The  $\zeta$  potential was evaluated after incubation of polyplexes in 552 HBG buffer (pH 7.4). Light scattering from the moving 553 particles was detected at 17°, and the Smoluchowski Model was 554 used to determine the value of the Henry's function.

Atomic Force Microscopy. For AFM measurements, s56 polyplexes were placed on freshly cleaved Mica disks (V1 12 s57 mm, Ted Pella USA). Imaging was carried out in HBG buffer at s58 25 °C, using commercial AFM, a NanoWizard 3 (JPK s59 instrument, Berlin, Germany) with QI mode. Si3N4 (MSNLs60 10 series, Bruker) cantilevers with spring constants ranging s61 from 10 to 30 pN nm<sup>-1</sup> were calibrated by the thermal s62 fluctuation method (included in the AFM software) with an s63 absolute uncertainty of approximately 10%. QI settings were as s64 follows: Z-length: 0.1  $\mu$ m; applied force: 0.5 nN; speed: 50 s65  $\mu$ m/s.

**DNase Protection Assay.** DNase I protection assays were s67 conducted as described previously. Briefly, 1  $\mu$ g of pGreenFire1 s68 DNA alone, with polyplex 1:1, or with polyplex 1:3 was mixed in a final volume of 50  $\mu$ L in HBS solution. Following 30 min 569 incubation at room temperature, 2  $\mu$ L of DNase I (2 units, 570 Roche) or PBS was added to 10  $\mu$ L of each sample and 571 incubated for 15 min at 37 °C. DNase I activity was terminated 572 by the addition of 5  $\mu$ L of 100 mM EDTA for 10 min at room 573 temperature. To dissociate the plasmid from the triconjugates, 574 10  $\mu$ L of 5 mg/mL heparin (Sigma, St. Louis, MO) was added, 575 and the tubes were incubated for 2 h at RT. Samples were 576 electrophoresed on an 0.8% agarose gel and stained with 577 ethidium bromide. Images were acquired using a Gel Doc EZ 578 Imager (Bio Rad Laboratories, Inc.). 579

**Cell Culture.** Her-2 overexpressing BT474 cells were 580 cultured in RPMI medium supplemented with 10% fetal bovine 581 serum (FBS),  $10^4$  U/L penicillin, and 10 mg/L streptomycin at 582 37 °C in 5% CO<sub>2</sub>. MDA-MB-231 human breast carcinoma cells 583 were cultured in Leibovitz L-15 medium with 10% FBS,  $10^4$  U/ 584 L penicillin, and 10 mg/L streptomycin at 37 °C without CO<sub>2</sub>. 585 Cell lines were from the ATCC and cell culture reagents were 586 from Biological Industries, Bet Ha'emek, Israel. 587

Luciferase Assay and Confocal Microscopy. 10000 588 BT474 and MDA-MB-231 cells were plated in triplicate in 96- 589 well plates. Cells were treated with triconjugate 1:1 and 590 triconjugate 1:3 complexed with plasmid. 48 h following 591 treatment, cells were washed with PBS and lysed with 30  $\mu$ L of 592 cell lysis buffer (Promega, Mannheim, Germany) per well. 593 Luciferase activity was measured in 25  $\mu$ L samples of the 594 lysates, using the Luciferase Assay system (Promega) according 595 to manufacturer's recommendations. Measurements were 596 performed using a Luminoskan Ascent Microplate Lumin- 597 ometer (Thermo Scientific). Values, in relative light units 598 (RLU), are presented as the mean and standard deviation of 599 luciferase activity from the triplicate samples. Confocal 600 microscopy (FV-1200 Olympus) was used to visualize the 601 GFP, which was taken to reflect the internalization of plasmid 602 pGreenFire1. Pictures were taken at ×10 magnification. 603

**Quantification of Cell Viability.** Cell viability was 604 measured by means of a colorimetric assay using methylene 605 blue, as described previously. Briefly, 10000 BT474 and MDA- 606 MB-231 cells were plated in triplicate in 96-well plates. The 607 cells were treated with polyplexes 1:1 and 1:3 containing 1  $\mu$ g/ 608 mL pGreenFire1. 48 h following treatment, the cells were fixed 609 with 1% formaldehyde in PBS (pH 7.4), washed with DDW, 610 and then stained with a 1% (wt/vol) solution of methylene blue 611 in borate buffer for 1 h. Thereafter, the stain was extracted with 612 0.1 M HCl and the optical density of the stain solution was read 613 at 630 nm in a microplate reader (ELx800 BIO-TEX 614 instruments Inc.).

# ASSOCIATED CONTENT 616

#### **Supporting Information**

Experimental methods involving the purification and separation 618 of the diconjugate 1:1 and 1:3 (HPLC chromatograms and 619 cation exchange chromatogram). Experimental methods for the 620 efficacy and toxicity of LPEI, diconjugate 1:1 and 1:3, 621 triconjugate 1:1, and 1:3 polyplexes. This material is available 622 free of charge via the Internet at http://pubs.acs.org. 623

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629 Salim Joubran and Maya Zigler contributed equally to this 630 work.

#### 631 Notes

632 The authors declare no competing financial interest.

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