Bioconjugate Chemistry

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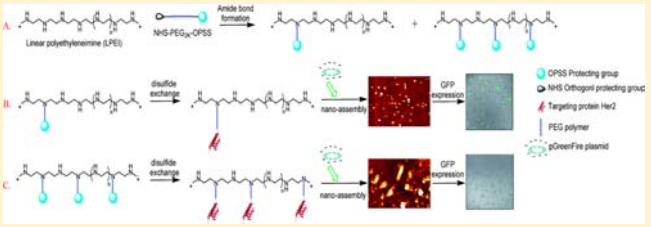
Optimization of Liganded Polyethylenimine Polyethylene Glycol Vector for Nucleic Acid Delivery

Salim Joubran,[§] Maya Zigler,[§] Neta Pessah,[§] Shoshana Klein,[§] Alexei Shir,[§] Nufar Edinger,[§] Anna Sagalov,[§] Yair Razvag,[‡] Meital Reches,[‡] and Alexander Levitzki*,[§]

[§]Unit of Cellular Signaling, Department of Biological Chemistry and [‡]Institute of Chemistry and The Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel

Supporting Information

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



INTRODUCTION

One of the hurdles facing molecular medicine is the targeted delivery of therapeutic agents such as DNA or RNA molecules. An emerging strategy is the construction of nonviral vectors, such as cationic polymers and cationic lipids, which bind and condense nucleic acids. These nonviral cationic vectors possess many advantages over viral gene vectors, as they are non-immunogenic, non-oncogenic, and easy to synthesize.[1−4] Currently, several synthetic polycationic polymers are being developed for nucleic acid delivery.[5] Among these, polyethylenimines (PEIs) are considered promising agents for gene delivery.[6,7]

PEIs are water-soluble, organic macromolecules that are available as both linear and branched structures.[8] PEIs change their degree of ionization over a broad range of pH, since every third atom in their backbone chain is an amino-nitrogen, which can be protonated. Approximately 55% of the nitrogens in PEIs are protonated at physiological pH.[9] They possess high cationic charge density, and are therefore capable of forming non-covalent complexes with nucleic acids. Furthermore, their physicochemical and biological properties can be altered by various chemical modifications.[10] PEI-based complexes (also known as polyplexes) can be endocytosed by many cell types.[11] Following internalization of the polyplexes, endosome release and high efficiency gene transfer are driven by the "proton sponge effect".[12] The ability of PEI to condense DNA, generating small particles, appears to be an important factor in delivering large DNA constructs into many cell types. A major concern in the utilization of PEIs as delivery carriers is toxicity, due to their high positive surface charge, which may lead to nonspecific binding.[13] PEI cytotoxicity is the major barrier to successful in vivo transfection, due to its intrinsic, nonspecific cytotoxicity at high concentrations and because it can interact with blood proteins and may activate the complement system.[14] Recent attempts have been made to improve the selectivity and biocompatibility of nonviral vectors. This has led to the modification of PEI molecules with polyethylene glycol (PEG), in order to shield the PEI particle.[15] The conjugation of heterobifunctional PEG groups to PEI facilitates coupling of the PEI to a targeting ligand, which provides efficient gene delivery into cells harboring the cognate receptor.[15]

The primary aim of this study was to develop and characterize cationic polymers that target Her-2 overexpressing tumor cells. We previously generated EGF-receptor targeting vectors consisting of branched PEI (brPEI-EGF) or linear PEI (LPEI) tethered to EGF.[16] Although the advantage of LPEI-EGF over brPEI-EGF was noted, the full characterization of the

Received: June 8, 2014
Revised: July 27, 2014
Published: August 5, 2014

   dx.doi.org/10.1021/bc500252a | *Bioconjugate Chem.* 2014, 25, 1644−1654

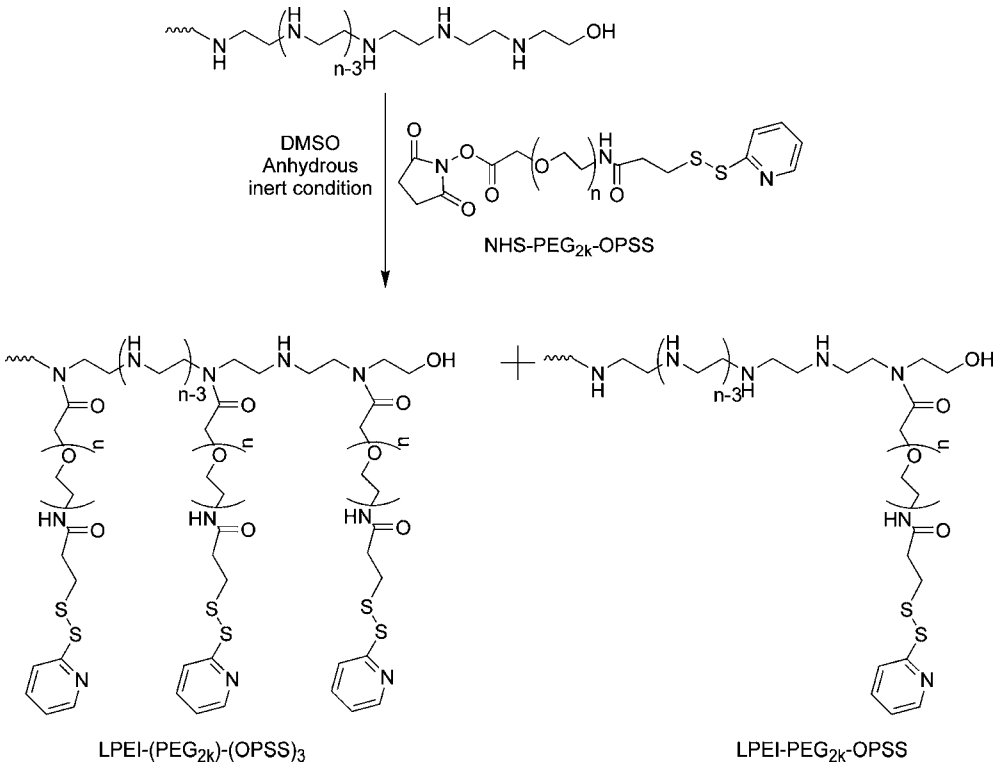


Figure 1. Conjugation of LPEI (~22 kDa) with NHS-PEG-OPSS (~2 kDa) yielded mainly two copolymeric networks that differ in the degree of PEGylation. The copolymer LPEI-$(PEG_{2k})_3$-$(OPSS)_3$ ("diconjugate 1:3") consisted on average of 1 mol of LPEI and 3 mol of PEG, whereas copolymer LPEI-PEG_{2k}-OPSS ("diconjugate 1:1") consisted on average of 1 mol of LPEI and 1 mol of PEG. (Ratios of LPEI:PEG were determined by ^{1}H NMR analysis.)

derivatized LPEI was not presented. Herein we describe two differentially PEGylated copolymers (LPEI-PEG_{2k}) that are conjugated to a Her-2 affibody, which functions as a Her-2 targeting agent. Affibody molecules are small, soluble, and robust affinity ligands, which were developed from the B domain of staphylococcal protein A (SPA).[17] Affibodies have low molecular weight (58 amino acids) and bind the extracellular domain of Her-2 with high affinity and selectivity, both in vivo and in vitro.[18] We provide detailed biophysical characterization of the conjugates, and evaluate their efficacy and specificity in gene delivery to Her-2 overexpressing breast cancer cells.

RESULTS

Synthesis of Thiol Reactive Copolymers. Previous studies have demonstrated the PEGylation of LPEI and its conjugation to an EGFR targeting moiety.[16] However, the extent of PEGylation on a single LPEI chain has not been fully characterized. To generate differentially PEGylated copolymers, the secondary amines on LPEI were conjugated to the terminal NHS ester orthogonal protecting group on PEG. The *N*-hydroxysuccinimide (NHS) ester spontaneously reacts with the secondary amines of the LPEI backbone, providing efficient PEGylation of LPEI. Furthermore, the reaction of the NHS-PEG-OPSS with the amines of PEI results in the formation of stable, irreversible amide bonds (Figure 1).

The PEGylation products were purified by cation exchange chromatography. Two peaks were eluted at high concentrations of NaCl, one at ~120 mS/cm, and the other at ~132 mS/cm (Supporting Information (SI) Figure S1). The purity of the eluted fractions of the diconjugates was assessed using reverse phase HPLC (SI Figures S2, S3). The two products were presumed to differ in their ratios of LPEI:PEG and consequently in their net positive charges. ^{1}H NMR spectra were analyzed using the relative integral values of the hydrogen atoms on PEG ($-CH_2-CH_2-O-$) (Figure 3a) and the integral values of the hydrogen atoms on LPEI ($-CH_2-CH_2-NH-$) (Figure 3b). This analysis indicated that the material eluted in the first peak consisted of a copolymer in which each mol of LPEI was conjugated to approximately 3 mol of PEG. This product was named LPEI-$(PEG_{2k})_3$-$(OPSS)_3$ ("diconjugate 1:3"). The second peak consisted of a copolymer in which equal numbers of moles of PEG and LPEI were conjugated, and was named LPEI-PEG_{2k}-OPSS ("diconjugate 1:1") (Figure 2).

Synthesis of the Triconjugates, LPEI-PEG_{2k}-Her2 Affibody (Triconjugate 1:1) and LPEI-(PEG_{2k})-$(Her2)_3$ Affibody (Triconjugate 1:3). The primary aim of this study was to develop a cationic polymer that would target Her-2 overexpressing tumor cells. Since Her-2 is an "orphan receptor" and has no known ligand, affibody molecules targeting the Her-2 receptor were used to generate Her-2 targeting triconjugates. We expressed and purified Her-2 affibody with a Cys residue at the C-terminal to allow further conjugation (SI Figure S4). The thiol reactive copolymers, diconjugate 1:1 and 1:3, were conjugated to Her-2 affibody through its terminal Cys residue, generating triconjugates 1:1 and 1:3, respectively (Figure 3). In order to generate the triconjugates, the reaction had to be performed with low concentration of affibody (to prevent aggregation) and in the presence of 10% acetonitrile (ACN) as an organic polar solvent for increased solubility. The reaction mix was purified by cation exchange (SI Figure S5). The yields for both triconjugate reactions were approximately 33% as determined by copper assay. To confirm the conjugation of the

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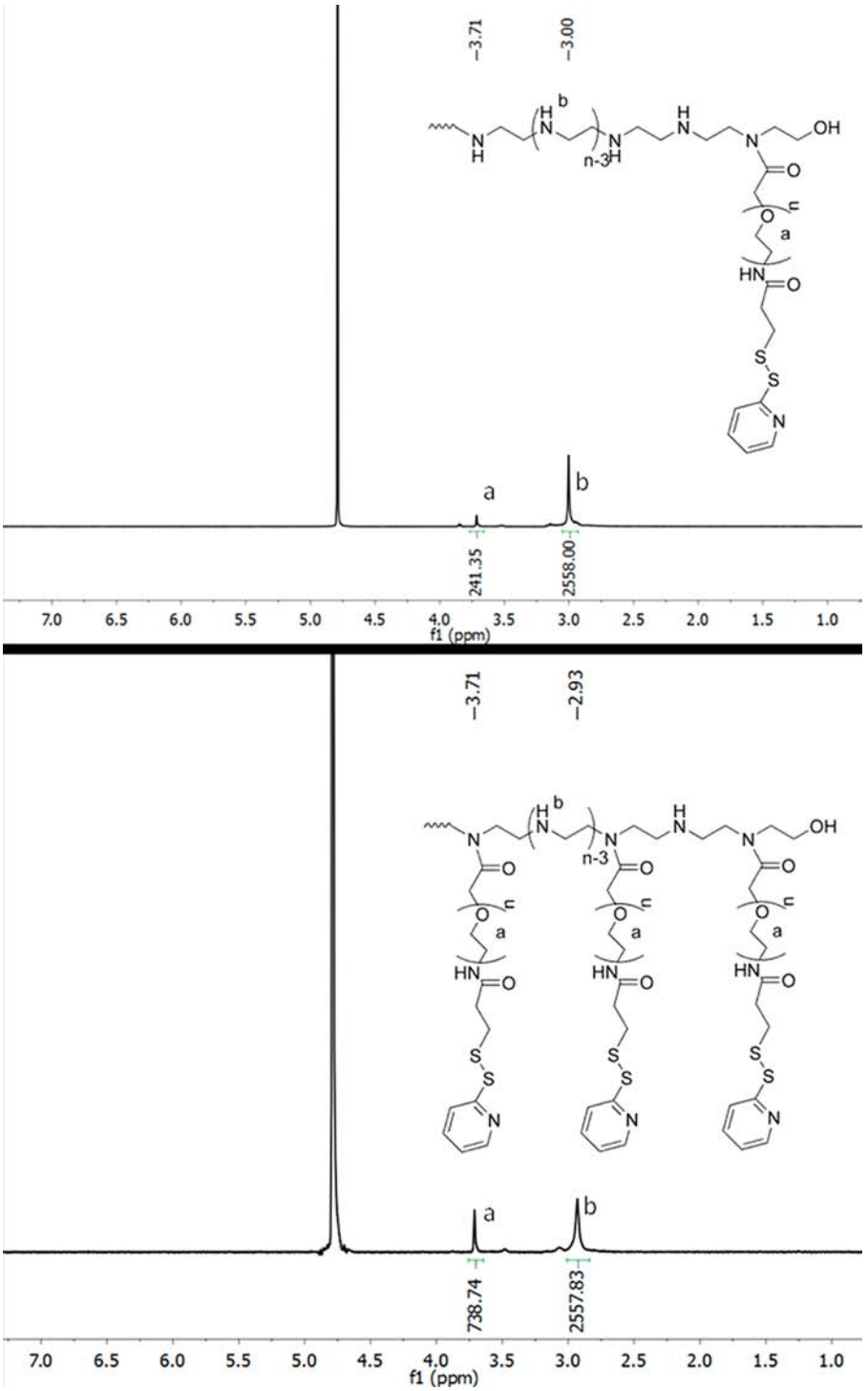

Figure 2. ^{1}H NMR analysis of the two diconjugates, LPEI-PEG_{2k}-OPSS (diconjugate 1:1) and LPEI-$(PEG_{2k})_3$-$(OPSS)_3$ (diconjugate 1:3). The coupling of PEG groups to LPEI was indicated by the presence of the chemical shifts that correlate to ethylene glycol hydrogens (a) at 3.7 ppm and ethylenimine 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.

affibody to the diconjugate, the triconjugate products were reduced with DTT and separated on SDS-PAGE. InstantBlue staining confirmed that the reduced triconjugate released the affibody (Figure 4). The amount of Her-2 affibody present in the triconjugates was determined by measuring A_{280}. Using copper assay, we quantified the LPEI. As described above, ^{1}H NMR analysis showed that the ratios of LPEI:PEG in the purified diconjugates were 1:1 or 1:3. Comparing the molar ratios of Her-2 affibody and LPEI, we determined that the average ratio of Her-2 affibody to LPEI in triconjugate 1:1 was 1:1, and in triconjugate 1:3 the average ratio was 3:1. Thus, we conclude that nearly complete conjugation of affibody to LPEI-PEG was achieved.

To generate polyplexes, the pure diconjugates and triconjugates (SI Figure S6) were complexed with plasmid DNA, as described in the Materials and Methods.

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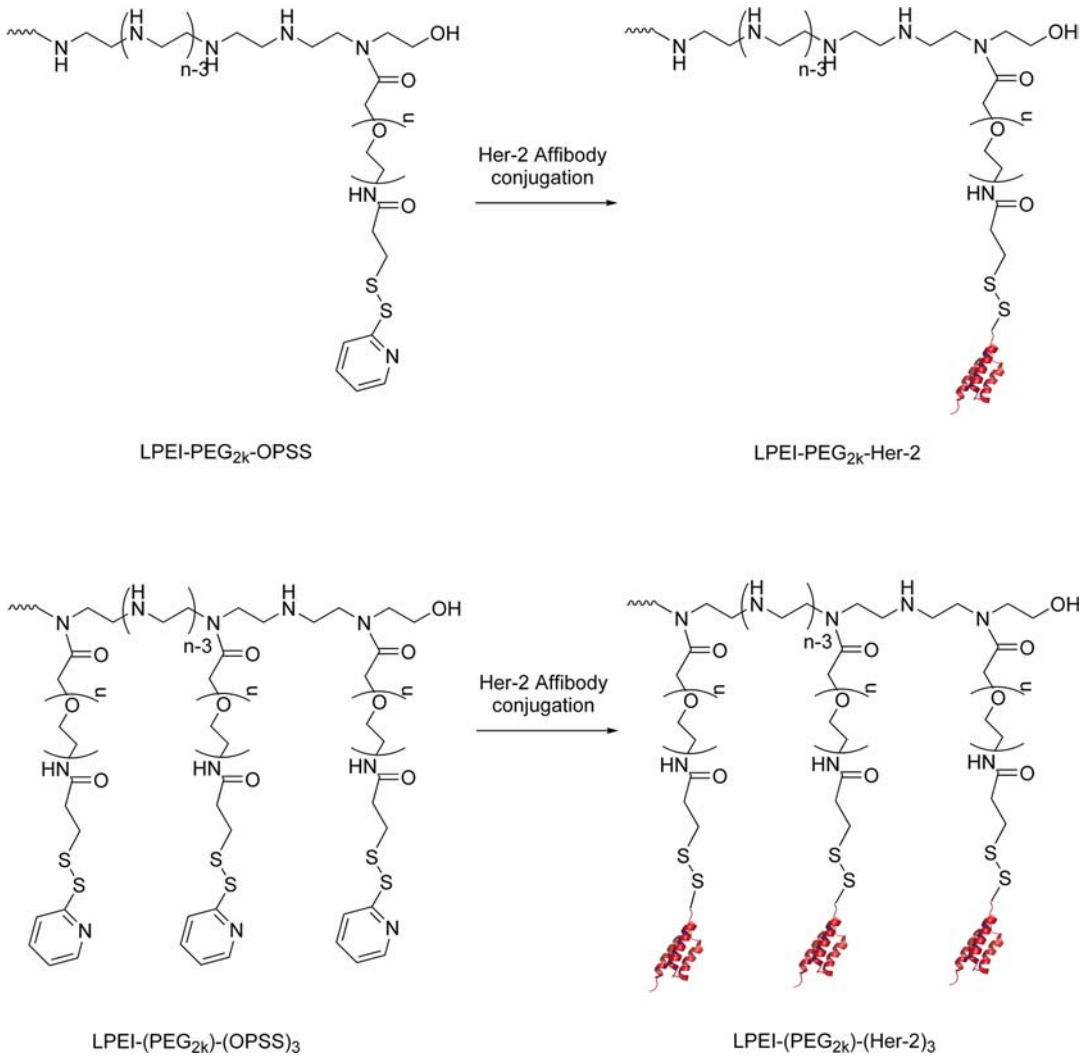

Figure 3. Conjugation of the thiol reactive copolymers (diconjugate 1:1 and diconjugate 1:3) to the affibody ("Her-2") through disulfide exchange reaction, resulting in the generation of two differently PEGylated triconjugates.[19]

ζ Potential and Sizing of Polyplexes. We next characterized the polyplexes, with respect to size and surface charge, using dynamic light scattering (DLS). Polyplexes were formed with pGreenFire1 with a ratio of nitrogen/phosphate (N/P) of 6. Previous studies showed that complexation of positively charged conjugate with plasmid at a ratio of N/P = 6 produces small, very stable polyplexes, which do not aggregate and which exhibit efficient plasmid transfection.[20,21] The size of a polyplex has a significant impact on its delivery properties.[22] In order to investigate the effect of adding the targeting ligand on the size of a polyplex, we compared the sizes of the diconjugates and the triconjugates following complexation of each with plasmid DNA (polyplexes). Diconjugate 1:1 polyplex had an average particle size of 115.2 ± 8.2 nm and diconjugate 1:3 had an average particle size of 253.1 ± 9.5 nm. The polyplex generated from triconjugate 1:1 with plasmid gave an average particle size of 141 ± 5.8 nm, whereas triconjugate 1:3 complexed with plasmid had an average particle size of 256 ± 24.2 nm (Figure 5). The smallest particles (73.9 ± 3.0 nm) were obtained in polyplexes generated by complexing the plasmid with LPEI alone. The conjugation of the affibody to the diconjugates had only a minor effect on the particle size. The number of PEG groups, however, did affect the particle size, suggesting that the PEG groups caused steric hindrance, interfering with plasmid condensation.

A positive surface charge facilitates polyplex binding to the negatively charged cell surface, but excessive positive charge can lead to nonspecific binding and significant toxicity.[15] The ζ potentials of the various complexes, presented in Figure 6, are in agreement with previous studies, which showed a decrease in ζ potential with increased number of PEG units.[16] To assess the effect of PEG groups on the surface charge of our chemical vectors we measured the ζ potentials of polyplexes formed by complexation of plasmid DNA with the precursors, diconjugates 1:1 and 1:3, and with the triconjugates 1:1 and 1:3. Diconjugate polyplex 1:1 had an average ζ potential of 27.0 ± 0.1 mV and diconjugate polyplex 1:3 had an average ζ potential of 20.0 ± 1.0 mV. Triconjugate polyplex 1:1 showed ζ potential with an average of 17.1 ± 0.7 mV, whereas triconjugate polyplex 1:3 showed an average of 10.2 ± 0.44 mV (Figure 6). Unlike the sizes, the ζ potentials of the polyplexes were affected by both the number of PEG groups and the conjugation of the Her-2 affibody. Although the smallest, most positively charged polyplexes were obtained with naked LPEI, these particles can be extremely toxic when used at high concentration in vivo, due to nonspecific interactions.[23] We expected that the addition of

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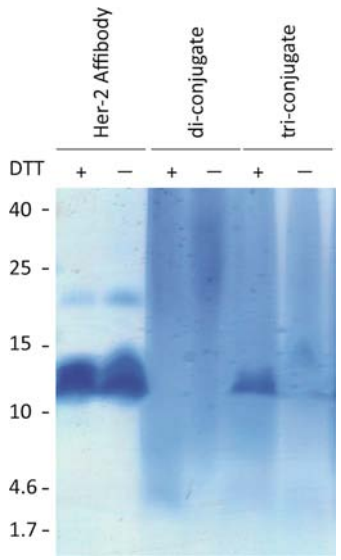

Figure 4. Conjugation of the affibody to the diconjugate, to produce the triconjugate (data presented for triconjugate 1:1 only), was confirmed by SDS/PAGE of purified affibody, diconjugate, and triconjugate, in the absence and in the presence of DTT. In the presence of DTT, the affibody was released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

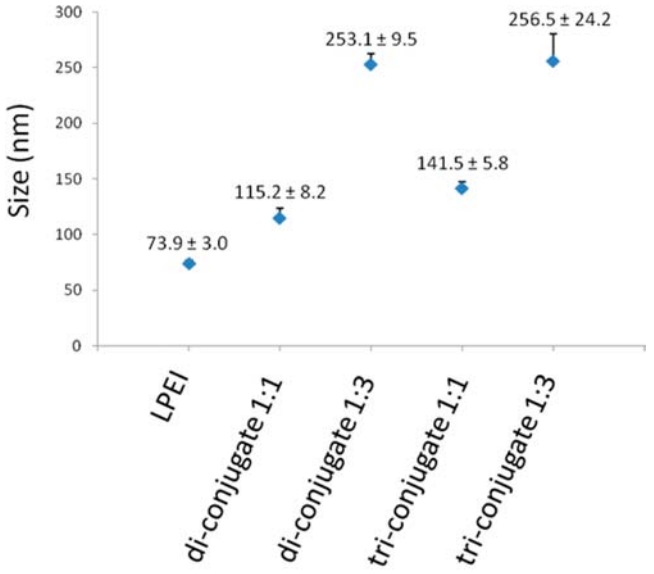

Figure 5. Particle sizing using DLS measurements of LPEI, the diconjugates, and the triconjugates complexed with plasmid pGreenfire 1 in HBG buffer pH 7.4.

PEG groups and a targeting moiety would diminish toxicity, but because the polyplexes were still relatively small in size, we hoped that their efficiency as nucleic acid delivery vectors would not be compromised.

Assessment of Polyplex Shape Using AFM. The importance of particle shape and its influence on delivery properties is gaining recognition.[24] We analyzed the morphology of the polyplexes obtained with the triconjugates in solution using AFM. The diameters of triconjugates 1:1 and 1:3 polyplexes were both in the nanosize range (Figure 7). This is in agreement with the results obtained by DLS. The triconjugate 1:1 polyplex displayed spherical and elliptical particles. Most particles ranged in diameter from 101 to 178

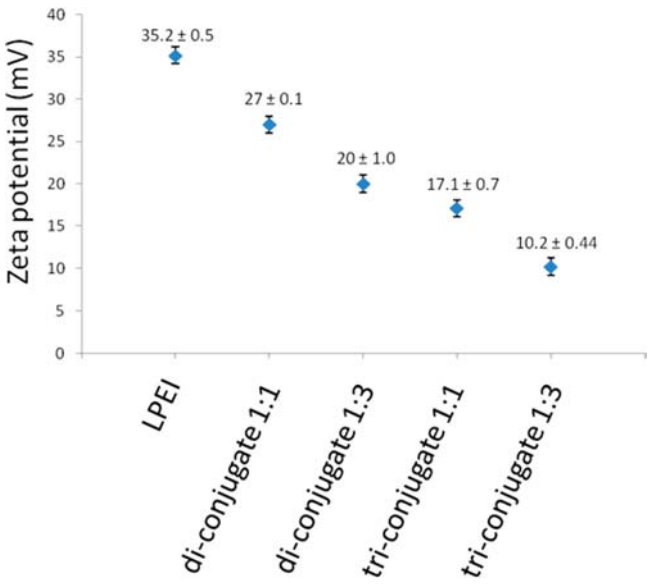

Figure 6. ζ potential distributions of LPEI, diconjugates and triconjugates complexed with plasmid pGreenfire1. The zeta potentials were measured by DLS and calculated by the Smoluchowski equation.

nm, with an average particle diameter of 142 ± 35.3 nm (based on 31 particles). Few particles were exceptionally large, with some even reaching >250 nm (Figure 7A). Triconjugate 1:3 polyplex was more heterogenic in shape and, moreover, yielded large aggregates with undefined particle shape (Figure 7B). These ranged in length from 150 to 650 nm, with an average particle length of 312 nm; they ranged in width from 85 to 400 nm, with an average width of 175 nm (based on 23 particles).

DNase Protection Assay. Successful in vivo gene delivery depends on efficient protection from nucleases. To determine the ability of the triconjugates to protect plasmids from degradation and enable efficient gene delivery, the polyplexes were treated with DNase I and analyzed using gel electrophoresis. As shown in Figure 8, naked plasmid pGreenFire1 DNA was fully degraded following 10 min of incubation with 2 units of DNase I. In contrast, when polyplexes were generated by mixing plasmid with the triconjugates, the plasmid was protected from degradation by DNase I. Complete protection of the plasmid was observed for triconjugate polyplex 1:3, while some nicking did occur for triconjugate polyplex 1:1, as shown by the shift from the supercoiled (s.c.) to the open circular (o.c.) form of the plasmid. The stronger protection from DNase I conferred by triconjugate polyplex 1:3 may be attributed to the increased steric hindrance provided by the additional PEG−protein units in these complexes. Indeed, previous studies have shown that PEGylation of PEI can stabilize polyplexes and increase their circulation in the blood, by impeding their interactions with enzymes and serum factors.[25,26] Thus, the ability of both triconjugates 1:1 and 1:3 to protect the plasmid from DNase I degradation suggests that the complexes will be stable upon systemic application, facilitating efficient gene delivery.

Biological Activity of the Targeting Triconjugate Polyplexes 1:1 and 1:3. Polyplex size and ζ potential influence the efficiency of targeted DNA delivery and gene expression, but the effect of size appears to be dependent on the particular conjugate.[2,22] To evaluate the specificity and the efficiency of transfection of the triconjugate polyplexes 1:1 and 1:3, two breast cancer cell lines that differentially express Her-2

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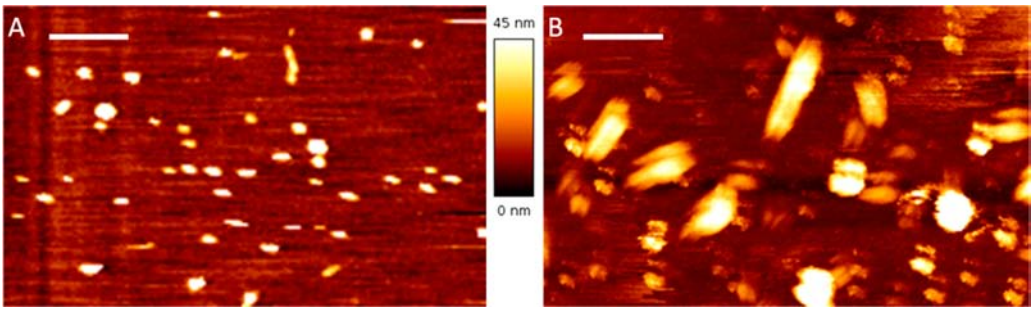

Figure 7. AFM measurements were performed in HBG buffer pH 7.4 for both polyplexes: (A) triconjugate 1:1 Polyplex; (B) triconjugate 1:3 Polyplex. Scale bar is 1 μm.

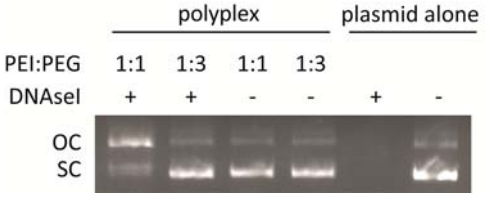

Figure 8. Differentially PEGylated polyplexes protect plasmid pGreenFire1 from DNase I degradation. 1 μg plasmid (pGreenFire1) alone or triconjugate polyplexes 1:1 and 1:3 were treated with or without DNase I (2 IU). Supercoiled plasmid (s.c.), open circular plasmid (o.c.).

were utilized. Polyplexes of the triconjugates 1:1 and 1:3 were formed with pGreenFire1 and transfected into MDA-MB-231 cells (expressing approximately 9×10^3 Her-2 receptors/cell[27]) and BT474 cells (expressing approximately 1×10^6 Her-2 receptors/cell) (SI Figure S7).[28] Differential luciferase activity was observed 48 h after transfection. Both triconjugate polyplexes 1:1 and 1:3 led to more than 300-fold higher luciferase activity in BT474 cells than in MDA-MB-231 (* $p < 0.001$) (Figure 9A). More efficient gene delivery to BT474 was confirmed by GFP expression, as seen by confocal microscopy (Figure 9B). These results show that polyplex selectivity is dependent on Her-2 expression.

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

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

DISCUSSION

The purpose of the present study was to explore in-depth the biophysical properties and biological activity of differentially PEGylated polyplexes that were designed to deliver nucleic acids into Her-2 overexpressing cells. Gene delivery by polyplexes is strongly affected by the biophysical properties of the polyplexes, including size, shape, and surface charge.[15]

Polycations, especially PEI, have been intensively investigated as agents for gene transfection.[29,30] Optimal transfection efficacies are obtained when the polymeric nanoparticle complexes possess an overall positive charge, which allows them to bind to the negatively charged heparin sulfate proteoglycans on the cell surface.[31] Previous studies showed that linear PEI (LPEI) is more effective in gene transfection than branched PEI (brPEI),[32−36] but LPEI has higher positive charge and hence is more toxic. Various shielding entities, such as PEG,[15] poly(ethylene oxide)-poly(propylene oxide)-poly-(ethylene oxide) (PEO-PPO-PEO),[37,38] polyethylene glycol-poly ε-caprolactone (PEG-PCL)[39] and poly(ethylene oxide),[40] have been conjugated to cationic polymers, in an attempt to lower the positive charge and the consequent toxicity. Indeed, shielding of PEIs with PEG groups of varying lengths significantly lowered toxicity while maintaining transfection efficiency.[15,16,41]

In the current study, the conjugation of LPEI with PEG_{2k} yielded diconjugate copolymers comprising various ratios of LPEI to PEG_{2K}. These diconjugates could be separated from one another using cation exchange chromatography, due to differences in charge, which reflect the different numbers of PEG_{2k} groups conjugated. ^{1}H NMR analysis confirmed that the diconjugates differed from one another in the average number of PEG_{2k} units per LPEI unit, where diconjugate 1:1 had an LPEI: PEG_{2k} ratio of 1:1 and diconjugate 1:3 had an LPEI: PEG_{2k} ratio of 1:3. The conjugation of the Her-2 targeting affibody to each of the purified diconjugates yielded a triconjugate product of the appropriate molecular weight, i.e., from diconjugate 1:1 we obtained "triconjugate 1:1" with LPEI:PEG_{2k}:Her-2 equal to 1:1:1 and from diconjugate 1:3 we obtained "triconjugate 1:3″, with ratio 1:3:3. This protocol enabled us to obtain homogeneous products, with nearly complete conjugation of targeting affibody to LPEI-PEG, in a reproducible manner.

We observed that PEGylation strongly affects the size of the polyplex particles obtained upon complexation of the diconjugates or triconjugates with plasmid DNA. Both diconjugate 1:3 and triconjugate 1:3 polyplexes had average particle sizes larger than diconjugate 1:1 and triconjugate 1:1 polyplexes. We believe that increasing the amount of PEGylation on a single cationic chain leads to steric hindrance, which prevents the polymeric chain from condensing the plasmid to a smaller particle. This is consistent with the finding that the naked LPEI polyplex had the smallest particles. Moreover, while both triconjugates 1:1 and 1:3 protected complexed plasmid from DNase I, the triconjugate 1:3 polyplex

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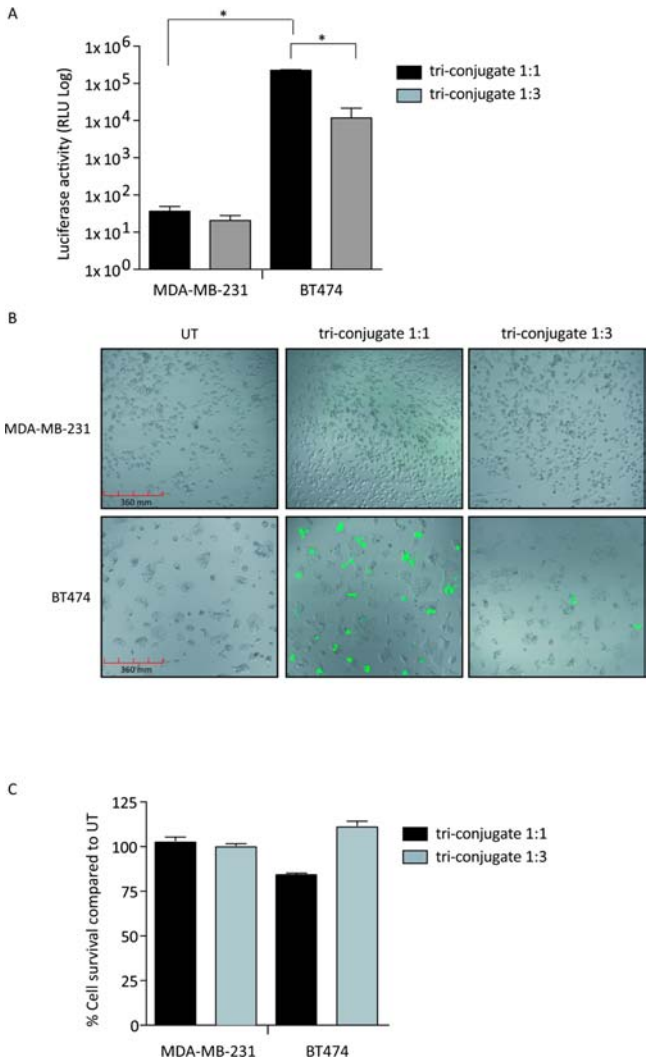

Figure 9. Her-2 mediated gene transfer of pGFP-LUC using the triconjugate 1:1 polyplex and the triconjugate 1:3 polyplex containing LPEI:PEG ratios of 1:1 and 1:3, respectively. BT474 and MDA-MB-231 breast cancer (10 000 cells/well) were treated for 48 h with triconjugates 1:1 and 1:3 complexed with pGFP-LUC (1 μg/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.

provided better protection, possibly due to the increased steric hindrance.

Previous studies suggested that increasing the molecular weight of the PEG units conjugated to cationic polymers leads to decreased surface charge of the polyplexes obtained upon complexation with nucleic acids.[16] Our data show that increasing the number of PEG groups of similar molecular weights leads to decreased surface charge, as defined by ζ potential distribution. Indeed, the highest surface charge was shown by naked LPEI complexed with plasmid. These results support the idea that the more neutral entities present in a chemical vector, the lower the surface charge. Surprisingly, the triconjugate polyplexes had lower surface charge than the diconjugate polyplexes, showing that the Her-2 affibody (which itself has slight positive charge) also reduced the surface charge of the particles. We suspect that Her-2 affibody changes the topography of the particle, with more targeting moieties masking the charge on the surface, leading to a decrease in surface charge.

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

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

Previous studies have shown that high levels of PEGylation can result in reduced gene transfection.[44] These results are in accordance with our observation that the highly PEGylated triconjugate 1:3 polyplex showed a significant reduction in gene delivery, as compared to the less PEGylated triconjugate 1:1 polyplex, as shown by luciferase activity and GFP expression. The increased gene delivery by the lesser PEGylated triconjugate 1:1 polyplex was accompanied by slight cellular toxicity, most likely due to its higher surface charge.

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 speculated that triconjugate 1:3, which has 3 mol of Her-2 afffibody molecules conjugated per mol of LPEI, would show increased receptor-mediated particle internalization. However, the triconjugate 1:3 polyplexes showed lower ζ potential, larger particle size, and heterogeneous, nonspherical shape, all of 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 heterogeneity, and that these properties had profound effects on targeted gene delivery. Our simplified synthesis allows purification of homogeneous products in a reproducible fashion, which can now be expanded to generate different triconjugates, using a variety of targeting moieties.

MATERIALS AND METHODS

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

Synthesis of ∼22 kDa LPEI (Free Base Form). The cationic polymer linear polyethylenimine (LPEI) was synthesized as described previously[45] with modifications. Briefly, 8.0 g (0.16 mmol) of poly(2-ethyl-2-oxazoline) were hydrolyzed with 100 mL of concentrated HCl (37%) and refluxed for 48 h, yielding a white precipitate. The solid was filtered by vacuum through a sinter glass and washed several times with water. The resulting LPEI hydrochloride salt was air-dried overnight, dissolved in 50 mL of water, and freeze−dried (5 g, 78%, ^{1}H NMR, D_2O, 400 MHz: singlet 3.5 ppm). The resulting LPEI salt (4.5 g) was made alkaline by adding aqueous NaOH (3 M) and the resulting white precipitate was filtered and washed with water until neutral. The solid was then dissolved in water and further lyophilized to give a white solid (2 g, 81%).

Synthesis of LPEI-PEG$_{2k}$-OPSS Diconjugates (Diconjugate 1:1 and 1:3). 174 mg (8 μmol) of LPEI were dissolved in 2.7 mL of absolute EtOH and agitated at room temperature for 15 min. A 5-fold molar excess of OPPS-PEG$_{2k}$-CONHS (79 mg, 39.5 μmol) was dissolved in 500 μL of anhydrous DMSO and introduced in small portions into the LPEI mixture. The reaction mix was agitated at ∼800 rpm on a vortex stirrer at ambient temperature for 3 h. Different PEG-substituted LPEIs were separated by cation-exchange chromatography, using an HR10/10 column filled with MacroPrep High S resin (BioRad). The purity of the eluted fractions of the diconjugates was assessed using reverse phase HPLC equipped with analytical Vydac C-8 monomeric 5 μm column (300 Å, 4.6 × 150 mm), using a linear gradient of 5−95% acetonitrile over 25 min at 1 mL/min flow. Fractions with 95% purity or higher were combined. The combined fractions were further dialyzed against 20 mM HEPES pH 7.4. The ratio of PEG$_{2k}$ groups conjugated to LPEI in the diconjugates was determined by ^{1}H NMR. The integral values of the hydrogens from the polyethylene -(CH$_2$−CH$_2$−O)- and from the LPEI -(CH$_2$−CH$_2$−NH)- were used to determine the ratio between the two conjugated copolymers. Of the various products obtained from the cation-exchange, two products, LPEI-PEG$_{2k}$-OPSS (diconjugate 1:1, with molar ration of LPEI to PEG ∼1:1) and LPEI-(PEG$_{2k}$)$_3$-(OPSS)$_3$ (diconjugate 1:3, with molar ratio of ∼1:3), were chosen for the generation of triconjugates. A copper assay was used to evaluate the copolymer concentration.[46] Briefly, the copolymers were incubated with $CuSO_4$ (23 mg dissolved in 100 mL of acetate buffer) for 20 min and their absorbance at 285 nm was measured.

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

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

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

Verification and Purity of Chemical Vectors Conjugated to Targeting Protein. The triconjugates were electrophoresed on SDS-PAGE, and stained with InstantBlue, to confirm the conjugation of the affibody to LPEI-PEG$_{2k}$. The purity of the triconjugates was confirmed by reverse phase HPLC, using an analytical Vydac C-8 monomeric 5 μm column (300 Å, 4.6 × 150 mm) at 1 mL/min while monitoring at 220 nm. A gradient elution with acetonitrile, 5−95% in 25 min with triple distilled water (TDW) containing 0.1% TFA as mobile phase, was used for the HPLC analysis.

dx.doi.org/10.1021/bc500252a | *Bioconjugate Chem.* 2014, 25, 1644−1654

Polyplex Formation. Plasmid pGreenFire1, encoding Firefly Luciferase and GFP (System Biosciences, Inc.), was amplified in *E. coli* and purified by Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The triconjugate 1:1 or triconjugate 1:3 was complexed with plasmid at a ratio of N/P = 6 (where N = nitrogen from LPEI and P = phosphate from DNA) in HEPES-buffered glucose (HBG: 20 mM HEPES, pH 7.4, 5% glucose, w/v), generating two polyplexes. To allow complete formation of the polyplex particles, the samples were incubated for 30 min at room temperature. The final plasmid concentration in the polyplex samples was 100 μg/mL, whereas for DNase protection assay and luciferase assay, the final concentration of the plasmid was 10 μg/mL in HEPES-buffered saline (HBS: 20 mM HEPES, 150 mM NaCl, pH 7.4).

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

Atomic Force Microscopy. For AFM measurements, polyplexes were placed on freshly cleaved Mica disks (V1 12 mm, Ted Pella USA). Imaging was carried out in HBG buffer at 25 °C, using commercial AFM, a NanoWizard 3 (JPK instrument, Berlin, Germany). Si_3N_4 (MSNL-10 series, Bruker) cantilevers with spring constants ranging from 10 to 30 pN nm^{-1} were calibrated by the thermal fluctuation method (included in the AFM software) with an absolute uncertainty of approximately 10%.

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

Cell Culture. Her-2 overexpressing BT474 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 10^4 U/L penicillin, and 10 mg/L streptomycin at 37 °C in 5% CO_2. MDA-MB-231 human breast carcinoma cells were cultured in Leibovitz L-15 medium with 10% FBS, 10^4 U/L penicillin, and 10 mg/L streptomycin at 37 °C without CO_2. Cell lines were from the ATCC and cell culture reagents were from Biological Industries, Bet Ha'emek, Israel.

Luciferase Assay and Confocal Microscopy. 10 000 BT474 and MDA-MB-231 cells were plated in triplicate in 96-well plates. Cells were treated with triconjugate 1:1 and triconjugate 1:3 complexed with plasmid. 48 h following treatment, cells were washed with PBS and lysed with 30 μL of cell lysis buffer (Promega, Mannheim, Germany) per well. Luciferase activity was measured in 25 μL samples of the lysates, using the Luciferase Assay system (Promega) according to manufacturer's recommendations. Measurements were performed using a Luminoskan Ascent Microplate Luminometer (Thermo Scientific). Values, in relative light units (RLU), are presented as the mean and standard deviation of luciferase activity from the triplicate samples. Confocal microscopy (FV-1200 Olympus) was used to visualize the GFP, which was taken to reflect the internalization of plasmid pGreenFire1. Pictures were taken at ×10 magnification.

Quantification of Cell Viability. Cell viability was measured by means of a colorimetric assay using methylene blue, as described previously. Briefly, 10 000 BT474 and MDA-MB-231 cells were plated in triplicate in 96-well plates. The cells were treated with polyplexes 1:1 and 1:3 containing 1 μg/mL pGreenFire1. 48 h following treatment, the cells were fixed with 1% formaldehyde in PBS (pH 7.4), washed with DDW, and then stained with a 1% (wt/vol) solution of methylene blue in borate buffer for 1 h. Thereafter, the stain was extracted with 0.1 M HCl and the optical density of the stain solution was read at 630 nm in a microplate reader (ELx800 BIO-TEX instruments Inc.).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: alex.levitzki@mail.huji.ac.il. Phone: 972 2 6585 404. Fax: 972 2 6512 958.

Author Contributions

Salim Joubran and Maya Zigler contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. M. Lebendiker for his guidance and help with protein and conjugate purification. We thank Dr. S. Magdassi and Dr. R. Abu Reziq for providing the Zetasizer for zeta potential measurements and particle sizing. M.R. acknowledges the support of Marie Currie Reintegration Grant. We would also like to thank Dr Naomi Melamed-Book for assistance with confocal microscopy (Confocal Microscope Unit at HUJI). This work was supported by ISF Grant No. 1556/13 (A.L.), ERC Advanced Grant No. 249898 (A.L.) and the Lady Davis Post-Doctoral Fellowship Trust (M.Z.).

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