31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Degradable Polymers for Gene Delivery

Joel Sunshine, Nupura Bhise, and Jordan J. Green, *EMBS Affiliate*

Abstract—**Degradable polymers were synthesized that self-assemble with DNA to form particles that are effective for gene delivery. Small changes to polymer synthesis conditions, particle formulation conditions, and polymer structure led to significant changes to efficacy in a cell-type dependent manner. Polymers presented here are more effective than Lipofectamine 2000 or polyethylenimine for gene delivery to cancerous fibroblasts or human primary fibroblasts. These materials may be useful for cancer therapeutics and regenerative medicine.**

I. Introduction

GENE delivery has great potential, both as a therapeutic to treat disease on the genetic level and as a technology to enable regenerative medicine. The central challenge is finding a safe and effective delivery system. As viral gene therapy can have serious safety concerns, many recent efforts have focused on non-viral methods that utilize biomaterials. Many materials have been shown effective for delivering genes *in vitro* including cationic lipids, sugars, peptides, and polymers [1-3].

One of the lead delivery polymers is polyethylenimine (PEI), which due its cationic structure, can be very effective for binding DNA and forming gene delivery particles [4]. PEI is also particularly effective at promoting endosomal escape of PEI/DNA particles through the proton sponge mechanism [5, 6]. This is critical to prevent lysosomal degradation of the DNA and to enable efficient delivery of the DNA to the cytoplasm. This endosomal escape mechanism has been used in the design of other synthetic gene delivery polymers, including polylysine-based polymers that contain an imidazole group in the side chain [7]. Although PEI shows promise compared to other biomaterials, it also leads to significant cytotoxicity [8] and has lower effectiveness than viral methods.

One newer group of polymers used for gene delivery are poly(beta-amino ester)s [9]. They are useful due to their ability to bind DNA, promote cellular uptake, facilitate escape from the endosome, and allow for DNA release in the cytoplasm [10-12]. Unlike PEI, they are readily biodegradable due to their ester linkages, which reduces cytotoxicity [10, 13]. It has been shown that within this class, acrylate-terminated polymers have low gene delivery, whereas amine monomer-terminated polymers have higher delivery [14]. Recently, end-modification with diamine monomers has shown that some of these polymers can rival adenovirus for gene delivery *in vitro* and are also effective *in vivo* [15, 16].

Another interesting approach to increase gene delivery effectiveness while reducing cytotoxicity is adding bioreducible linkages to polymers. Disulfide linkages have been added to PEI to produce bioreducible versions with lower cytotoxicity than high molecular weight versions of the parent polymer [17, 18]. Other researchers have shown that bioreducible poly(amido amines) can have higher efficacy than PEI while also having reduced cytotoxicity [19, 20].

It was recently demonstrated that IMR-90 human primary fibroblasts can be reprogrammed to induced pluripotent stem cells with integrating viruses [21]. Reprogramming human differentiated cells into undifferentiated, pluripotent cells could potentially enable a patient to receive a customized cell therapy that is a perfect genetic match.

In this report we investigate how small modifications to the monomer ratio used during polymerization in combination with modifications to end-capping group chemical structure used post-polymerization affects gene delivery. We compare how chemical structure tunes gene delivery efficacy between a cancerous fibroblast line and human primary fibroblasts. In particular, we highlight that small changes to the molecular weight or changes to just the ends of the polymer, while leaving the main chain of the polymer the same, significantly enhances or decreases the overall delivery of the polymer.

II. Materials and Methods

A. Polymer Synthesis

1,4-butanediol diacrylate (Alfa Aesar), 5-amino-1-pentanol (Alfa Aesar), 1-(3-aminopropyl)-4-methylpiperazine (Lancaster), 1-(3-aminopropyl)pyrrolidine (Acros Organics), 4-aminophenyl disulfide (Sigma-Aldrich), dimethyl sulfoxide (Sigma-Aldrich), 25 kDa polyethylenimine (Sigma-Aldrich), and Lipofectamine 2000 (Invitrogen) were used as received. Polymers were synthesized using a two-step procedure that is described in Figure 1. Acrylate-terminated poly(1,4-butanediol diacrylate-co-5-amino-1-pentanol) was first synthesized at two different acrylate monomer to amine monomer molar ratios, 1.05:1 and 1.2:1. For the 1.05:1 ratio, 3532 mg of 1,4-butanediol diacrylate (17.8 mmol) was added to 1754 mg of 5-amino-1-pentanol (17.0 mmol) and for the 1.2:1 ratio, 3532 mg of 1,4-butanediol diacrylate (17.8 mmol) with 1533 mg of 5-amino-1-pentanol (14.8 mmol).

J. Sunshine, N. Bhise, and J.J. Green are with the Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205 USA (corresponding author J. J. Green: 410-614-9113; fax: 477-287-6298; e-mail: green@jhu.edu).

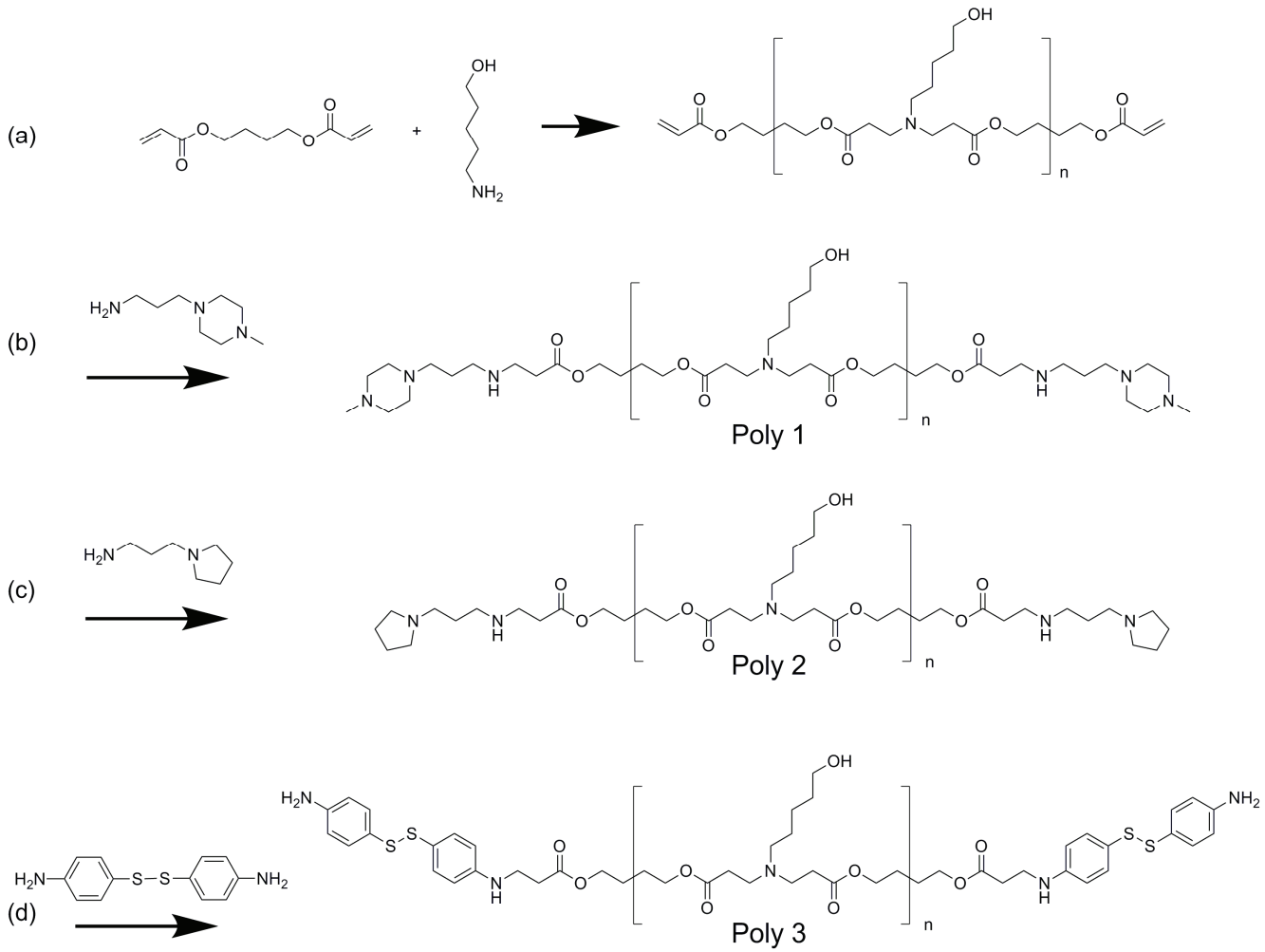

Fig. 1. Synthesis of degradable gene delivery polymers. (a) 1,4-butanediol diacrylate reacts with 1-amino-5-pentanol to form the acrylate-terminated poly(beta-amino ester) precursor. This react with (b) 1-(3-aminopropyl)-4-methylpiperazine to form Poly 1, (c) 1-(3-aminopropyl)pyrrolidine to form Poly 2, or (d) 4-aminophenyl disulfide to form Poly 3.

Reactions took place in DMSO (500 mg/mL) in glass vials in the dark under magnetic stirring for 48 hrs at 40°C. As a second step, three amine-containing small molecules were individually conjugated to the ends of each polymer post-polymerization. Reactions were performed by mixing 321 mg of poly(1,4-butanediol diacrylate-co-5-amino-1-pentanol) in DMSO (500 mg/mL) with 800 µL of 0.25 M amine solution. Excess amine is used to fully end-modify the base polymer. Reactions were performed in 1.5 mL tubes in a multi-tube vortexer with constant agitation for 24 hours at room temperature. Polymers were stored at -20°C with desiccant until use. Polymers were analyzed by gel permeation chromatography using a Waters Breeze System and 3 Styragel Columns (7.8 x 300mm) in series: HR 1, HR 3, and HR 4. The eluent was 95% THF/5% DMSO/0.1M piperidine and ran at 1 mL/min.

B. Cell Culture

COS-7 and IMR-90 cells (ATCC, Manassas, VA) were grown following ATCC recommended protocols and reagents. COS-7s were grown in Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (ATCC) and 100 units/mL of penicillin and streptomycin (Invitrogen). IMR-90s were grown in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (ATCC) and 100 units/mL of penicillin and streptomycin. Cells were subcultured upon confluence and IMR-90s were used prior to passage eight.

C. Gene Delivery Assays

Cells were plated in white 96-well plates at 15,000 cells in 100 µL per well and allowed to adhere overnight. CMV-Luc

DNA (Elim Biopharmaceuticals, Hayward, CA) was diluted in 25 mM sodium acetate (pH=5) to 0.06 mg/mL. Polymers at 100 mg/ml in DMSO were diluted in 25 mM sodium acetate buffer to concentrations that generate the varying polymer to DNA weight ratios (20, 40, 60, 100). One hundred microliters of diluted polymer solution was mixed vigorously with 100 µL of DNA solution in a 96-well plate using a multichannel pipette. After 10 minutes wait time, 20 µL of each formulation was added to the cells that

contained 100 µL of complete media per well. Particles were incubated with the cells for four hours and then removed with a 12-channel aspirator wand. Warm, complete media was added to the cells (100 µL/well) and they were allowed to grow for two days at 37°C and 5% CO_2. Polyethylenimine/DNA particles were formed in a similar manner to the other polymers, except that they were formed at a w/w ratio of 1 (N/P~8) in 150 mM NaCl solution as has been previously described [4, 22]. Lipofectamine 2000 was used following the manufacturer instructions. Forty-eight hours post transfection, gene expression was measured using Bright-Glo luminescence assay kits (Promega), a Synergy 2 multilabel plate reader (Biotek), and a one second read time per well. Protein content per well was measured using the BCA protein assay kit (Pierce) and the Synergy 2 plate reader to measure absorbance at 562 nm.

III. RESULTS AND DISCUSSION

Base acrylate-terminated polymers were synthesized via the conjugate addition of 5-amino-1-pentanol to an excess of 1,4-butanediol diacrylate in a manner similar to that previously described, but at a lower temperature and for a longer reaction time while being dissolved in DMSO [15, 16]. Polymerizations were performed at molar ratios of 1.05:1 and 1.2:1 at 40 °C for 48 hrs. Subsequently, the polymers were end-modified by conjugate addition of 1-(3-aminopropyl)-4-methylpiperazine (Poly 1), 1-(3-aminopropyl)pyrrolidine (Poly 2), or 4-aminophenyl disulfide (Poly 3) to the base polymers at room temperature for 24 hrs (Figure 1). Polymers were analyzed by gel permeation chromatography as shown in Table 1. For the 1.2:1 molar ratio, polymers had M_w ~ 6 kDa. At a 1.05:1 molar ratio, the molecular weight was higher, M_w ~ 6.5-8.5 kDa. Gene delivery particles were formed in buffer through self-assembly between the cationic polymers and anionic DNA.

TABLE I
POLYMER MOLECULAR WEIGHT

	Ratio	M_n	M_w	PDI
Poly 1	1.05:1	5415	7696	1.42
	1.2:1	4580	6124	1.34
Poly 2	1.05:1	4249	6459	1.52
	1.2:1	3943	5714	1.45
Poly 3	1.05:1	5680	8521	1.50
	1.2:1	3547	5448	1.54

At lower weight ratios (20 and 40 w/w), polymeric particles formed with polymers synthesized at 1.05:1 were generally more efficient for gene delivery than the same polymers formed at 1.2:1. This is likely due to the higher MW of these polymers. In some cases, these changes were dramatic. For example, for Poly 1 at 20 w/w, the 1.05:1 ratio is more than 10-fold as effective as the 1.2:1 ratio with COS-7 cells and 400-fold more effective for IMR-90s. Interestingly, the difference in MW between these two polymers is only ~ 1.5 kDa. However, at higher weight ratios (60 and 100 w/w), this trend is not seen and the effectiveness of both polymer synthesis conditions is comparable. The one exception to this trend is Poly 2, which has comparable delivery between the two synthesis ratios at 60 and 100 w/w in COS-7 cells, but in IMR-90 cells, only the 1.05:1 ratio polymer is effective at any weight ratio tested. Thus small changes to MW may be able to tune delivery properties to alter cell-type specificity.

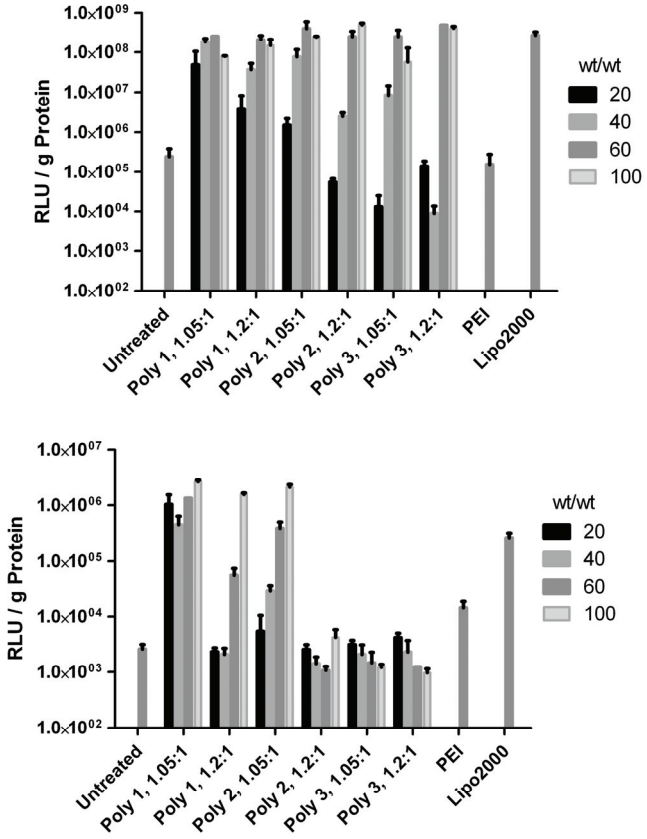

Fig. 2. Gene delivery efficacy of Poly 1, Poly 2, Poly 3, and commercial reagents PEI and Lipofectamine 2000 to COS-7 cells (above) and IMR-90 cells (below). Luciferase-encoding DNA is delivered and expression is measured as relative light units per gram protein. Ratio 1.05:1 and 1.2:1 refer to polymerization conditions and wt/wt is the weight ratio of polymer to DNA. Graphs show mean+SD, n=4.

Certain end-modifying groups also appeared to show cell-type specificity. For example, Poly 3 at 60 w/w or 100 w/w has very high gene delivery to the COS-7 cancerous fibroblasts, but very poor delivery to the IMR-90 human primary fibroblasts. In comparison to Lipofectamine 2000, Poly 3 (1.2:1 ratio and 60 w/w) enables twice the gene expression in COS-7 cells, but over 200-fold less expression in IMR-90s. For transfection of IMR-90s, Poly 1 or Poly 2 at a polymerization ratio of 1.05:1 and polymer to DNA weight ratio of 100 were the most effective. These

conditions enabled transfection of human primary fibroblasts in serum-containing media at 8-10 fold the levels of Lipofectamine 2000. Lipofectamine 2000 is a leading-commercially available lipid-based transfection reagent and the polymers presented here can achieve comparable or higher delivery to both cancerous and primary cell types. Compared to 25 kDa branched polyethylenimine, a leading of-the-shelf gene delivery polymer, the polymers presented here are up to 2-3 orders of magnitude more effective. In all cases, cells remained viable and comparable to untreated controls as determined by visual inspection and relative levels of protein content per well through the BCA assay.

Small molecule amine containing end-groups were chosen to increase DNA binding affinity compared to acrylate terminated polymers and to potentially improve endosomal release of the particles (Poly 1 and Poly 2) or bioreducible release of the DNA (Poly 3). As these small structural modifications also lead to a significant change in cell-type specificity, the end-group may also play an important role in directing particle uptake. We are currently quantifying these mechanisms to better understand structure/function relationships of these materials. Further exploration of polymer structures may lead not only to safe and effective transfection reagents, but to materials with a natural proclivity to targeting certain types of cells.

REFERENCES

[1] T. G. Park, J. H. Jeong, and S. W. Kim, "Current status of polymeric gene delivery systems," *Advanced Drug Delivery Reviews,* vol. 58, pp. 467-486, JUL 7 2006.

[2] D. W. Pack, A. S. Hoffman, S. Pun, and P. S. Stayton, "Design and development of polymers for gene delivery," *Nature Reviews Drug Discovery,* vol. 4, pp. 581-593, JUL 2005.

[3] M. C. Pedroso de Lima, S. Simoes, P. Pires, H. Faneca, and N. Duzgunes, "Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications," *Advanced Drug Delivery Reviews,* vol. 47, pp. 277-94, Apr 25 2001.

[4] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J. P. Behr, "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine," *Proc Natl Acad Sci U S A,* vol. 92, pp. 7297-301, Aug 1 1995.

[5] N. D. Sonawane, F. C. Szoka, and A. S. Verkman, "Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes," *Journal of Biological Chemistry,* vol. 278, pp. 44826-44831, NOV 7 2003.

[6] A. Akinc, M. Thomas, A. M. Klibanov, and R. Langer, "Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis," *Journal of Gene Medicine,* vol. 7, pp. 657-663, MAY 2005.

[7] D. Putnam, C. A. Gentry, D. W. Pack, and R. Langer, "Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini," *Proc Natl Acad Sci U S A,* vol. 98, pp. 1200-5, Jan 30 2001.

[8] S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, and A. Szewczyk, "A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy," *Mol Ther,* vol. 11, pp. 990-5, Jun 2005.

[9] J. J. Green, D. G. Anderson, and R. Langer, "A combinatorial polymer library yields insights into the field of non-viral gene delivery," *Accounts of Chemical Research,* vol. 41, pp. 749-759, 2007.

[10] D. M. Lynn and R. Langer, "Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA.," *J Am Chem Soc,* vol. 122, pp. 10761-10768, 2000.

[11] D. G. Anderson, A. Akinc, N. Hossain, and R. Langer, "Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters)," *Molecular Therapy,* vol. 11, pp. 426-34, Mar 2005.

[12] A. Akinc, D. M. Lynn, D. G. Anderson, and R. Langer, "Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery," *Journal of the American Chemical Society,* vol. 125, pp. 5316-23, May 7 2003.

[13] J. J. Green, J. Shi, E. Chiu, E. S. Leshchiner, R. Langer, and D. G. Anderson, "Biodegradable polymeric vectors for gene delivery to human endothelial cells," *Bioconjugate Chemistry,* vol. 17, pp. 1162-1169, 2006.

[14] A. Akinc, D. G. Anderson, D. M. Lynn, and R. Langer, "Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery," *Bioconjugate Chemistry,* vol. 14, pp. 979-988, SEP-OCT 2003.

[15] J. J. Green, G. T. Zugates, N. C. Tedford, Y. Huang, L. G. Griffith, D. A. Lauffenburger, J. A. Sawicki, R. Langer, and D. G. Anderson, "Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus," *Advanced Materials,* vol. 19, pp. 2836-2842, 2007.

[16] G. T. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y. H. Huang, R. Langer, J. A. Sawicki, and D. G. Anderson, "Rapid Optimization of Gene Delivery by Parallel End-modification of Poly(beta-amino ester)s," *Mol Ther,* vol. 15, pp. 1306-1312, 2007.

[17] M. A. Gosselin, W. J. Guo, and R. J. Lee, "Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine," *Bioconjugate Chemistry,* vol. 12, pp. 989-994, NOV-DEC 2001.

[18] M. L. Forrest, J. T. Koerber, and D. W. Pack, "A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery," *Bioconjug Chem,* vol. 14, pp. 934-40, Sep-Oct 2003.

[19] L. V. Christensen, C. W. Chang, W. J. Kim, S. W. Kim, Z. Zhong, C. Lin, J. F. Engbersen, and J. Feijen, "Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery," *Bioconjugate Chemistry,* vol. 17, pp. 1233-40, Sep-Oct 2006.

[20] C. Lin, C. J. Blaauboer, M. M. Timoneda, M. C. Lok, M. van Steenbergen, W. E. Hennink, Z. Zhong, J. Feijen, and J. F. Engbersen, "Bioreducible poly(amido amine)s with oligoamine side chains: synthesis, characterization, and structural effects on gene delivery," *Journal of Controlled Release,* vol. 126, pp. 166-74, Mar 3 2008.

[21] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, and J. A. Thomson, "Induced pluripotent stem cell lines derived from human somatic cells," *Science,* vol. 318, pp. 1917-20, Dec 21 2007.

[22] M. M. O. Sullivan, J. J. Green, and T. M. Przybycien, "Development of a novel gene delivery scaffold utilizing colloidal gold-polyethylenimine conjugates for DNA condensation," *Gene Therapy,* vol. 10, pp. 1882-1890, OCT 2003.