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Electrostatic Ligand Coatings of Nanoparticles Enable Ligand-Specific Gene Delivery to Human Primary Cells

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ABSTRACT

A general method of coating polymer/DNA nanoparticles was developed. Peptide coated nanoparticles were found to have favorable biophysical characteristics including small particle size, near-neutral ζ potential, and stability in serum. At appropriate formulation conditions including near-neutral charge ratio, the coated nanoparticles enabled effective ligand-specific gene delivery to human primary endothelial cells in serum-containing media. As this nanoparticulate drug delivery system has high efficacy, ligand-based specificity, biodegradability, and low cytotoxicity, it may be potentially useful in several clinical applications.

Gene therapy has the potential to treat many diseases, including cancer and cardiovascular disease, but it is currently limited by the inability to deliver genes in a safe and effective manner. Delivering genes specifically to targeted cells and/or tissues is also particularly challenging. Here, we demonstrate a general approach for coating nanoparticles to functionalize them for ligand-specific delivery to human primary cells. Though we have used DNA as the drug cargo in this application, this nanoparticle coating method may be general enough to be used with other payloads and other charged nanoparticles.

Viral gene delivery is currently plagued by multiple problems including acute toxicity, cellular immune response, oncogenicity due to insertional mutagenesis, limited cargo capacity, resistance to repeated infection, and production and quality control issues.[1,2] Nonviral vector nanoparticles have been formed with numerous biomaterials including calcium phosphate, cationic lipids, cationic polymers, dendrimers, and cyclodextrins, but although generally safer than viruses, these methods have much lower transfection efficacy.[3] Recently, a large library of 2350 structurally unique poly(β-amino esters) was developed using high-throughput combinatorial methods.[4] These polymers have shown considerable promise for drug delivery both in vitro and in vivo.[5−9]

A number of methods for functionalizing nanoparticles have been tested. For example, commonly used gene delivery polymers, such as polylysine and polyethylenimine (PEI), have been covalently modified to include poly(ethyleneglycol) (PEG) and/or targeting ligands such as EGF,[10] transferrin,[11] and RGD[12,13] peptide to promote specific uptake. The results of these experiments have been mixed, with some researchers demonstrating dramatically improved targeting and others showing little improvement.[11−15] Peptides containing the amino acid sequence Arg-Gly-Asp (RGD) can be used for specific targeting to integrin receptors, including the vitronectin receptor $\alpha v\beta 3$, which is known to be highly upregulated in certain tumors.[13]

One of the problems with covalently coupling targeting ligands to a polymer is that it can change the biophysical properties of that polymer and the corresponding polymer/DNA nanoparticle. For example, several researchers have found that, as ligand substitution (either targeting or shielding) increases, overall gene delivery can decrease, presumably due to alteration of the original polymer's functionality for DNA condensation and endosomal buffering.[14,16] Furthermore, gene delivery nanoparticles are generally positively charged, and these positively charged nanoparticles are taken up nonspecifically.[15] Cell-specific nanoparticulate delivery requires both specific uptake by the target cell and inhibition of delivery to nonspecific cell types. Coatings that reduce the positive charge of gene delivery nanoparticles could potentially reduce this nonspecific uptake while still enabling receptor-mediated uptake. Gene delivery nanoparticles at

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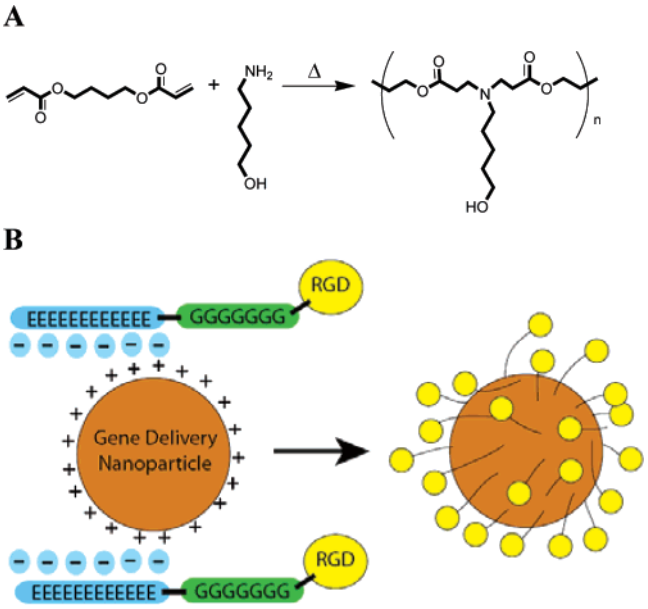

Figure 1. Synthesis schemes. (A) Poly(β-amino ester) C32 structure and synthesis; (B) electrostatic self-assembly of ligand coated nanoparticle.

overall neutral or negative charge may also be desirable to prevent unwanted serum interactions.[17]

Here, we show that electrostatic interactions can drive peptide coating of nanoparticles and enable ligand-specific gene delivery to human primary cells. Our general approach to electrostatically coat gene delivery nanoparticles with ligands provides a simple method of ligand addition as well as a mechanism to neutralize nanoparticle charge. These coatings reduce nonspecific delivery to HUVECs and may similarly affect other cell types. While we use RGD-containing peptide as a model system to investigate nanoparticle coating and ligand-specific delivery to primary endothelial cells, many other peptide ligand sequences, such as those made from antibody fragments, could potentially be readily incorporated into this system as well.

Nanoparticle Formation and Peptide Coating. Polymer C32 is synthesized by the conjugate addition of 5-amino-pentanol to 1,4-butanediol diacrylate. The acrylate and amine monomers used in this experiment and their synthesis scheme can be seen in Figure 1A. Cationic polymeric gene delivery nanoparticles were formed in sodium acetate buffer solution through self-assembly of C32 with plasmid DNA. After a 10 min incubation period, anionic peptide was added to electrostatically coat the cationic nanoparticles, as demonstrated in Figure 1B. As this self-assembly is driven simply by opposing electrostatic charge, virtually any other cationic polymer (polyethylenimine, poly(β-amino ester), or other) could be potentially used to form similarly coated nanoparticles. Additional details on nanoparticle formation can be found in the Materials and Methods section in the Supporting Information. The peptide sequence EEEEEEEEEEE-GGGGGGGRGDS (E12-RGD) was used as a sequence with specific binding to integrin receptors expressed by HUVECs, and the near-identical peptide sequence EEEEEEEEEE-

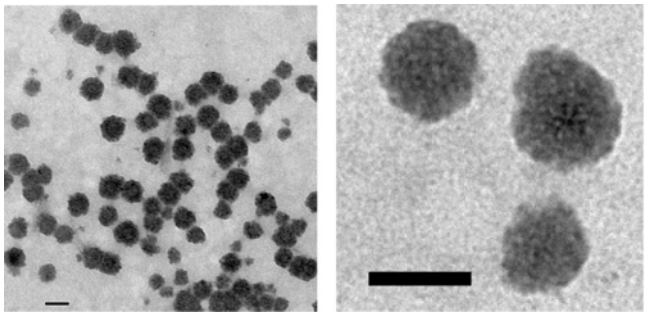

Figure 2. C32 (40 w/w) particles coated with 19 w/w E12RGD in serum media. Scale bar is 100 nm in both figures.

EEGGGGGGGRDGS (E12-RDG) was used as a nonintegrin binding control sequence.[18] As the actual fraction of protonated amines in cationic polymeric gene delivery systems is not typically known, charge ratio is frequently reported as a ratio of amine groups on the cationic polymer (N) to phosphate groups on the anionic plasmid DNA (P). Here, we slightly modify the N/P ratio to also include the carboxylic acid groups on the anionic peptide in a manner analogous to what has been done previously:[19]

$$N/P = \frac{[N]}{[P] + [\mathrm{COO}^-]}$$

We use w/w to refer to the weight ratio balance between the polymer and DNA (μg/μg), whereas N/P refers to the overall charge ratio of the nanoparticles, which can vary with peptide coating composition at a fixed polymer/DNA w/w ratio. The molecular weight of the polymer C32 has been previously optimized[6] and is not varied in these experiments. The molecular weight of the peptide coating is varied by the number of the glutamic acid residues: 8, 12, 16, and 20, with the glycine linker and RGD/RDG ligand remaining fixed.

To verify the formation and particle integrity of the nanoparticles, transmission electron microscopy (TEM) was performed (Figure 2). The nanoparticles were coated with peptide and incubated in media prior to imaging. Dried particles were determined to be ~100 nm in size. To determine DNA encapsulation efficiency, uncoated and coated nanoparticle formulations were tested by gel electrophoresis (Figure 3). For all nanoparticle formulations tested (30, 40, and 50 w/w C32/DNA nanoparticles), no free DNA was detected, indicating 100% encapsulation of DNA. The presence of ligand coatings and/or serum media did not change this DNA encapsulation.

Biophysical Characterization. Previously, we have demonstrated that the size and ζ-potential of gene delivery nanoparticles can change depending on the type of aqueous environment in which they are analyzed. Furthermore, we have also shown that these changes to biophysical properties directly affect transfection efficacy.[20] Thus, in these experiments, the nanoparticles were measured in the actual conditions used during transfection, 12% serum-containing media.

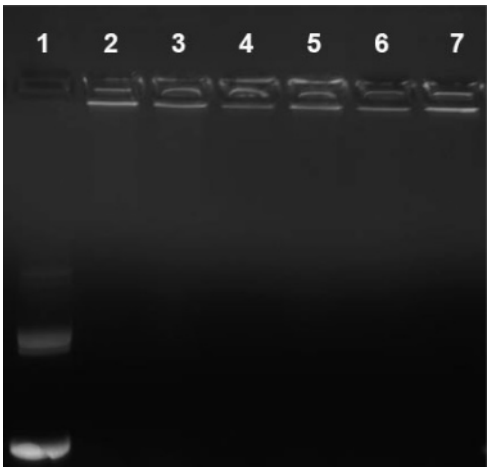

Figure 3. Gel showing full DNA encapsulation of particles. (1) DNA-only control, (2) 30 w/w C32/DNA particles, (3) 40 w/w C32/DNA particles, (4) 50 w/w C32/DNA particles, (5) 40 w/w C32/DNA + 19 w/w RGD coated particles, (6) 40 w/w C32/DNA particles in serum media, (7) C32/DNA + 19 w/w RGD coated particles in serum media.

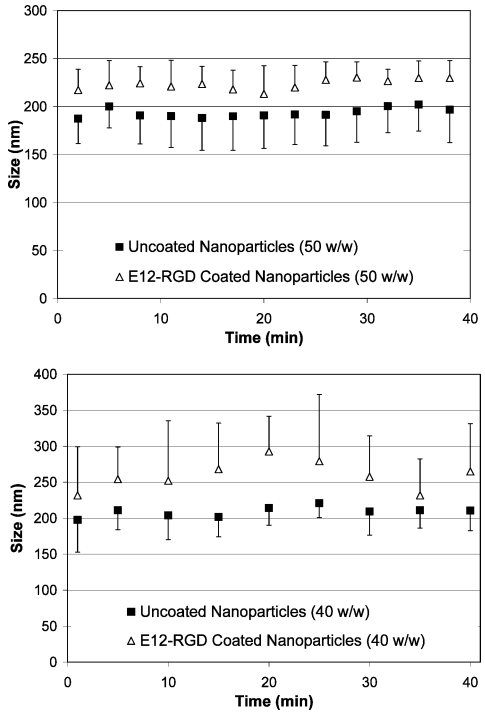

Figure 4. Ligand coated nanoparticles are slightly larger in size than uncoated nanoparticles. Nanoparticles are formed with 50 or 40 w/w C32/DNA and peptide coats with an overall *N*/*P* of 1.55. Coated 50 w/w particles are 27.2−32.8 nm larger than uncoated particles and coated 40 w/w particles are 36.8−60.7 nm larger than uncoated (95% CI). Importantly, coated nanoparticles have a stable, small size in 12% serum-containing media over time. Error bars are standard deviations of independently prepared particle batches.

Figure 4 shows the size and stability of the C32/DNA nanoparticles with and without peptide coating over time. Gene delivery particles are prepared at the same concentrations as they are for typical in vitro bioassays. This figure demonstrates that particles formed at 40 and 50 w/w C32/DNA have a small size of ∼200 nm and are stable in serum over time. The diameter determined by dynamic light scattering in solution is larger than the ∼100 nm size found by TEM of dried samples. This is likely due to the difference in size of the hydrated polymeric nanoparticle versus the dried particle. Coated 50 w/w C32/DNA particles were determined to be 27.2−32.8 nm larger than uncoated particles, and coated 40 w/w C32/DNA particles were 36.8−60.7 nm larger than uncoated particles (95% CI). Importantly, coated nanoparticles have a stable, small size in 12% serum-containing media over time. Thus, E12-RGD coating slightly increases particle size while maintaining serum stability. Small size and serum stability are conditions necessary for many therapeutic in vivo applications.[3] Particle stability is also advantageous for consistent transfections and for storage.

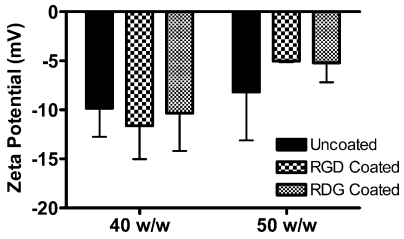

Figure 5. Ligand coated 40 w/w C32/DNA nanoparticles have equivalent negative charge to uncoated nanoparticles while in 12% serum-containing media, whereas 50 w/w C32/DNA nanoparticles are slightly more neutrally charged. Nanoparticles are formed with peptide coats with an overall *N*/*P* of 1.55. RGD ligand coated nanoparticles have equal charge to RDG ligand coated nanoparticles. Error bars are standard deviations of independently prepared particle batches.

Figure 5 shows the ζ-potential of E12-RGD coated and noncoated C32/DNA nanoparticles in 12% serum containing media. The C32/DNA nanoparticles have a negative ζ-potential due to serum interactions with the particles. Ligand coated 40 w/w C32/DNA nanoparticles have equivalent negative charge to uncoated nanoparticles while in 12% serum-containing media, whereas 50 w/w C32/DNA nanoparticles have a more neutral ζ potential. The anionic peptide coating may be able to reduce serum interactions. Reduction of serum interactions may be beneficial for an in vivo application, as serum proteins are known to promote clearance from the blood and to interfere with transfection.[3,17] Ligand coated nanoparticles had equal charge whether RGD ligand peptide coats or RDG control peptide coats were used.

GFP Transfections. Fluorescent activated cell sorting (FACS) and enhanced green fluorescent protein (EGFP) DNA were utilized to determine the efficacy of nanoparticle gene delivery. FACS data was interpreted by using a two-dimensional contour plot that compares the ratio of EGFP channel fluorescence (*x*-axis) to yellow channel autofluorescence (*y*-axis) for greater accuracy than a one-dimensional histogram as previously described.[20] Figure 6 shows the two-dimensional contour plot of representative C32/DNA/E12-RGD and C32/DNA/E12-RDG transfections. These experiments took place under conditions generally seen as difficult for in vitro transfection, but important for an in vivo cardiovascular application: fully confluent, and therefore nondividing, primary cells in the presence of a high concentration of serum proteins.[2] To further ensure appropriate gating and counting of positively transfected cells, 50

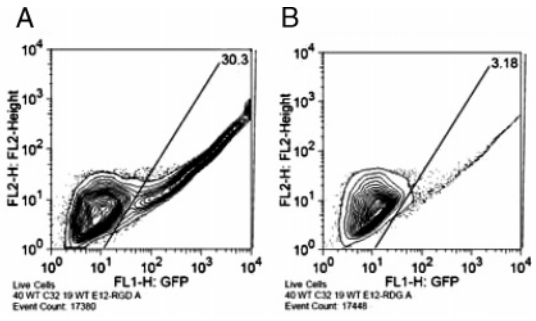

Figure 6. FACS results showing the gating of transfected HUVECs. (A) E12-RGD (ligand) coated nanoparticles significantly transfect HUVECs, whereas (B) E12-RDG (control) coated nanoparticles do not. Nanoparticles are formed with 40 w/w C32 and overall *N*/*P* of 1.55. These results are representative samples from quadruplicate experiments.

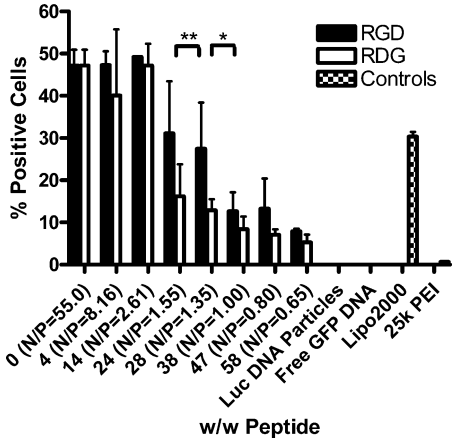

Figure 7. Efficacy and specificity of E12-RGD/E12-RDG ligand coated gene delivery nanoparticles is dependent on w/w peptide and *N*/*P* ratio. Nanoparticles are formed at 50 w/w C32 and are delivered to HUVECs in 12% serum containing media. Error bars are standard deviations and (*) and (**) indicate statistical significance of $p < 0.05$ and $p < 0.01$, respectively.

w/w C32/DNA nanoparticles formed using DNA that encodes luciferase protein (Luc) rather than EGFP was also used as a negative control. Luciferase DNA nanoparticles resulted in 0.0% GFP positive signal (see Supporting Information).

Cell Viability. We have previously shown that poly(*β*-amino ester) nanoparticles are biodegradable and generally noncytotoxic to multiple cell types including HUVECs.[8,20,21] In this study, C32/DNA/E12-RGD and C32/DNA/E12-RDG coated nanoparticles were also found to be noncytotoxic to HUVECs (80−100% cell viability depending on dose, see Materials and Methods for further information).

Glutamic Acid-Based Coats Enable Ligand-Specific Gene Delivery of Poly(*β*-amino ester)/DNA Nanoparticles to Human Primary Cells. Polyglutamic acid-based peptides were used to coat poly(*β*-amino ester)/DNA nanoparticles for ligand-based gene delivery. As Figure 7 shows, at low weight ratios (w/w) of peptide, the nanoparticles have equivalent transfection whether E12-RGD peptide, E12-RDG peptide, or no coating at all is used. However, at higher weight ratios of anionic peptide, the overall charge ratio of the complexes decreases and efficacy changes occur. When the overall *N*/*P* (charge) ratio nears neutrality, E12-RGD coated nanoparticles transfect human endothelial cells significantly better than the same nanoparticles coated with the near- identical E12-RDG scrambled sequence, our negative control. Concurrently, as additional anionic peptide coating is added beyond a threshold, overall transfection decreases. Thus, there is a relatively narrow window where (1) the nanoparticles are ligand-specifically targeted and (2) the nanoparticles maintain high efficacy. The results found using this coating system are consistent with previous findings that showed that polylysine-conjugated EGF gene delivery particles allow specific binding and internalization only at a relatively narrow window of charge.[10]

Figure 7 also demonstrates that C32 ligand coated nanoparticles are 50-fold more effective than the widely used gene delivery polymer, polyethylenimine (PEI), and equivalent to the leading commercially available liposome-based transfection reagent, Lipofectamine 2000. Without polymer, free DNA itself (100% release from polymer) was unable to transfect the HUVECs (0.0% positive). This demonstrates that, for effective gene delivery to at least some human primary cells, the DNA cargo must initially remain encapsulated and be internalized into the cell via an appropriate nanoparticle formulation before later intercellular release.

Polymer Weight Ratio, Overall Charge Ratio, and Peptide Length are Important Parameters for Ligand-Specific Gene Delivery. Nanoparticle and coating parameters including polymer weight ratio, peptide weight ratio, overall charge ratio, and peptide length were analyzed to determine optimal conditions. C32/DNA nanoparticle formulations formed at 30, 40, and 50 w/w polymer to DNA weight ratios showed the same trends: at low amounts of anionic peptide coating, there is no difference in gene delivery between RGD integrin-binding sequence, RDG nonbinding sequence, or uncoated controls, but at near-neutral *N*/*P* ratio, peptide coats formed with RGD integrin-binding sequences delivered DNA much more effectively than the identically formed peptide coated nanoparticles with RDG scrambled sequences. These transfection results of the near-neutrally charged particles can be seen in Figure 8. Interestingly, at 40 w/w C32/DNA and an *N*/*P* ratio of 1.55, integrin-binding E12-RGD coated nanoparticles transfected a 6-fold higher percentage of HUVECs as compared to the scrambled sequence E12-RDG coated control particles, our negative control. On an individual cell basis, the most positive cells express GFP at levels 1000-fold higher than the background. Representative FACS data from these experiments ($n \geq 4$) can be seen in Figure 6. The electrostatic ligand coatings provide a mechanism to neutralize nanoparticle charge and reduce nonspecific cellular uptake, while simultaneously allowing receptor-specific uptake. To confirm integrin receptor-mediated gene delivery of the RGD coated nanoparticles, fibronectin active fragment peptide RGDS, an integrin binding competitor,[22] was added to the cells prior to transfection. RGDS peptide (100 nM) was found to significantly reduce gene delivery of RGD coated nanoparticles ($p = 0.0043$) while not affecting gene delivery of RDG coated nanoparticles ($p = 0.48$), as shown in Figure 9. To test whether free RGDS may have some other

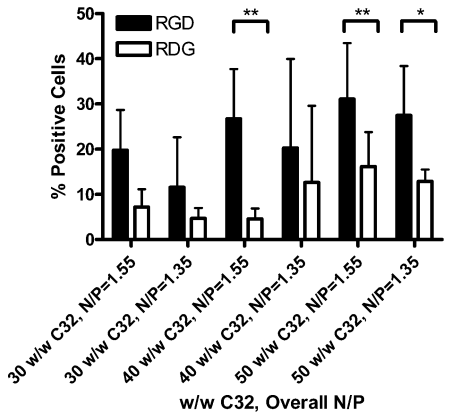

Figure 8. Efficacy and specificity of E12-RGD/E12-RDG ligand coated gene delivery nanoparticles is dependent on w/w C32 and *N*/*P* ratio. Nanoparticles are delivered to HUVECs in 12% serum containing media. Error bars are standard deviations and (*) and (**) indicate statistical significance of $p < 0.05$ and $p < 0.01$, respectively.

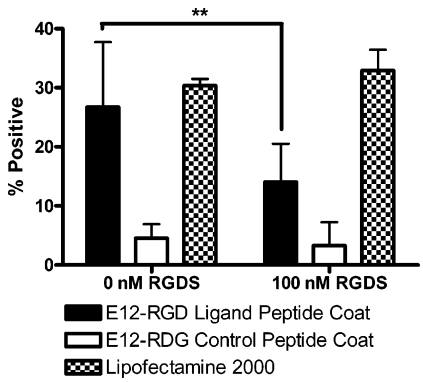

Figure 9. Competitive inhibition experiment with E12-RGD (ligand) and E12-RDG (control) coated gene delivery nanoparticles and free RGDS peptide fragment. Nanoparticles are formed at 40 w/w C32, $N/P = 1.55$, and are delivered to HUVECs in 12% serum containing media. Error bars are standard deviations and (**) indicates statistical significance of $p < 0.01$.

effect on the cells that nonspecifically reduces transfection efficiency, Lipofectamine 2000 was also tested at high RGDS concentration and maintained a constant level of transfection efficiency without regard to the amount of RGDS present.

Peptide length was also determined to be important for effective ligand coatings. Figure 10 demonstrates that, while both intermediate length E12-RGD and E16-RGD coatings allowed for specific delivery, E8-RGD and E20-RGD coatings did not. While the biophysical basis of this trend is unclear, we hypothesize that linker length can affect both accessibility and avidity of ligands to their target receptors. Our data show that it is important to optimize peptide coat lengths in order to both reduce nonspecific delivery and enable ligand-mediated nanoparticle delivery.

Discussion. In these experiments, we used human umbilical vein endothelial cells (HUVECs) as a model primary cell system due to the following: (1) HUVECs are more difficult to transfect than other cell lines[16] and (2) endothelial cells are prime therapeutic targets against cancer (anti-angiogenesis) and cardiovascular disease (therapeutic angiogenesis, prevention of restenosis, etc). We have also recently shown

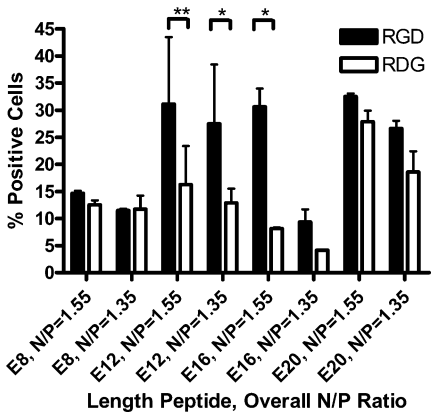

Figure 10. Efficacy and specificity of E12-RGD (ligand) and E12-RDG (control) coated gene delivery nanoparticles is dependent on peptide length and *N*/*P* ratio. Nanoparticles are formed at 50 w/w C32 and are delivered to HUVECs in 12% serum containing media. Error bars are standard deviations and (*) and (**) indicate statistical significance of $p < 0.05$ and $p < 0.01$, respectively.

that poly(β-amino ester) nanoparticles transfect HUVECs significantly better than the leading commercially available nonviral vectors including PEI and Lipofectamine2000 and functionalizing these nanoparticles for targeted delivery would further increase their clinical utility.[20] Here we have shown that these nanoparticles can be coated for not only high efficacy but now also for receptor-mediated delivery.

Previously, electrostatic components have been used with poly(β-amino esters) to construct erodible multilayered films.[23] Here we demonstrate that this concept can be extended to drug delivery nanoparticles to enable single or potentially multilayered coats for functionalized poly(β-amino ester)-based delivery. Poly(acrylic acid) and other polycarboxylic acids have also been recently shown to combine with polyethylenimine to form tertiary-component gene delivery nanoparticles that have reduced serum inhibition and enhanced nonspecific transfection efficacy.[17] As compared to this technique, our technology enables ligand-specific delivery in a biodegradable system. These nanoparticles are composed of biodegradable materials; poly(β-amino esters), such as C32, are readily degradable via the hydrolysis of its ester groups as previously described.[8] Peptides are also known to degrade in vivo.

This nanoparticle peptide coating approach allows for easy incorporation of a potentially wide range of ligands. Though a peptide sequence containing RGD, such as that used here, can be used for targeting to integrin receptors and/or tumors, many other peptide ligand sequences could be incorporated into this system as well.

We have demonstrated a novel method of coating nanoparticles for ligand-based gene delivery. This method is both flexible and general enough for potentially varied nanoparticle applications. This work also highlights the importance of multiple factors including polymer weight ratio, peptide weight ratio, overall charge ratio, and ligand length when developing coated gene delivery nanoparticles. A biphasic efficacy relationship was found for peptide weight ratio, overall charge ratio, and ligand length, with intermediate values of coating being optimal. This finding demonstrates

that a balance is required when seeking to design the nanoparticles to have reduced nonspecific uptake but increased ligand-specific uptake. As this nanoparticulate drug delivery system has high efficacy, ligand-based specificity, biodegradability, low cytotoxicity, and certain safety advantages over viruses, ligand coated poly(β-amino ester) gene delivery nanoparticles may be potentially useful in several clinical applications.

Statistics. Statistical calculations were carried out using GraphPad Prism 4.0 for Windows. Results are reported as mean ± standard deviation. For comparison of gene delivery vectors, statistical significance was obtained by using unpaired, two-tailed, Student's *t*-tests with 95% confidence. For comparisons of coated vs uncoated particle size over time, paired, two-tailed, Student's *t*-tests with 95% confidence were used.

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Supporting Information Available: Detailed description of the materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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