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Synthesis and Characterization of Four-Arm Poly(ethylene glycol)-Based Gene Delivery Vehicles Coupled to Integrin and DNA-Binding Peptides

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Abstract: The goal of this study was to develop a gene delivery vehicle that can specifically target cell surface receptors with low nonspecific protein adsorption and low cytotoxicity. Toward this goal, four-arm poly(ethylene glycol) vehicles were functionalized with DNA-binding peptides (DBPs) and integrin-binding (RGD) peptides. We have previously described a novel PEG-based gene delivery vehicle functionalized with DBPs that successfully transfected Chinese hamster ovary (CHO) cells with low toxicity and low protein adsorption. This work investigated whether incorporating RGD peptides onto PEG-DBP vehicles could target specific cell surface receptors and increase transfection efficiency of HEPG2 cells. DBP and RGD peptides were coupled onto PEG-tetraacrylate (PEG-TA) in three combinations (molar ratios of DBP:RGD of 1:3, 2:2, and 3:1) and characterized by measuring particle size, ζ potential, and transfection efficiency as a function of charge ratio (peptide amine groups:DNA phosphate). Nonspecific protein adsorption and cytotoxicity of PEG-DBP-RGD vehicles were also measured. Dynamic light scattering showed that PEG-DBP-RGD vehicles condensed DNA into particles having mean diameters of 250–300 nm and ζ potentials ranging from -10 to 7 mV. It was found that coupling two RGD peptides to the PEG-DBP_2 vehicle increased the transfection efficiency at a polymer/DNA charge ratio of 5:1 (+/−) and 6:1 (+/−) and that these vehicles had transfection efficiencies similar to those of polyethylenimine (PEI)/DNA particles. However, coupling one or three RGD peptides to PEG-DBP vehicles did not increase the transfection efficiency. Additionally, the PEG-DBP-RGD/DNA particles adsorbed less protein than PEI particles and were less toxic to HEPG2 cells.

Keywords: Gene delivery; integrin; poly(ethylene glycol) vehicles

Introduction

Gene delivery has the potential to treat single gene defect diseases such as hemophilia, sickle cell anemia, and cystic fibrosis.[1,2] Over 600 clinical trials worldwide are currently testing gene therapy as a potential treatment for these diseases.[3] However, the risks associated with the viral vectors that are being used in most clinical trials have limited the applications of these treatments.[4] Recent focus has been placed on the development of safe and efficient nonviral gene delivery vehicles.[5,6] A suitable nonviral gene delivery vehicle should be nontoxic, efficiently deliver therapeutic DNA, and effectively target cell surface receptors while resisting nonspecific protein adsorption in blood.

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Cationic polymers, such as polyethylenimine (PEI) and poly L-lysine (PLL), are frequently used as synthetic gene delivery vehicles. Cationic polymers electrostatically condense plasmid DNA to form particles[7,8] and bind to negatively charged proteoglycans on the cell surface, leading to cellular internalization. However, cationic polymer-based vehicles are limited by nonspecific protein adsorption (which leads to short clearance times) and their inability to target specific cell surface receptors. Previous research has focused on coupling cell targeting ligands, such as RGD peptides, onto cationic polymer vehicles in order to target integrin receptors on the cell surface.[9–12] Integrin receptors, such as $\alpha_V\beta_3$, are favorable targets because they are upregulated in tumors, a common target of gene therapy,[13] and are used by adenoviruses and bacteria for cell binding and internalization.[14,15]

Specifically, conjugating noncyclic RGD peptides to PEI vehicles was found to increase the transfection efficiency by an order of magnitude in HeLa cells, MRC45 cells, and Mewo human carcinoma cells when compared to PEI alone.[10,16] These studies indicate that conjugating RGD peptides to gene delivery vehicles can increase transfection efficiency along with increasing specific receptor binding. However, PEI-RGD conjugate vehicles, because of their high charge density, are limited by nonspecific protein adsorption onto the PEI-DNA particle surface resulting in rapid clearance from circulation.

Conjugating poly(ethylene glycol) (PEG) to the exterior of PEI-based particles has been shown to reduce nonspecific interactions with blood components, such as plasma proteins[17] and complement activation proteins,[18] which in turn extend the circulation time of the PEG-conjugated vehicles. However, conjugation of PEG to the exterior of PEI-RGD particles has been shown to interfere with cell surface binding interactions, causing a decrease in the efficiency of the vehicles.[19] Several studies have incorporated a PEG spacer into the PEI-RGD conjugate to reduce nonspecific protein adsorption, while retaining the function of RGD peptides.[9,12,20] Similarly, these studies found a drastic reduction in PEI-RGD efficiency with the incorporation of PEG spacer. Additionally, there were inconclusive results as to whether the coupling of RGD to PEG-PEI increased transfection efficiency.[16,20,21]

This paper describes a bifunctional gene delivery vehicle consisting of a four-arm PEG backbone coupled to DNA binding peptides (DBPs) to package DNA and RGD peptides to target integrin receptors. DNA-specific binding peptide, such as that derived from Bzip, coupled to PEG diacrylate (PEG-DA) or PEG tetraacrylate (PEG-TA) transfected Chinese Hampster Ovary (CHO) cells successfully with low toxicity and low nonspecific protein adsorption.[22] These PEG-DBP vehicles have not previously been combined with RGD peptides for gene delivery. This study describes the coupling of RGD peptides and DBPs to a PEG-TA backbone. DNA was complexed with PEG-DBP-RGD vehicles and characterized by particle size, ζ potential, and transfection efficiency in both CHO and HEPG2 cells. In addition, the nonspecific protein adsorption and cytotoxicity of PEG-DBP-RGD vehicles were compared to PEI vehicles.

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Materials and Methods

Chemicals and DNA. Branched PEI 50 kD, phenylarsine oxide (PhAsO), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). PEG tetraol (10000 MW) was purchased from Shearwater Polymers (Huntsville, AL) and acrylated as previously described.[23] Plasmid DNA encoding for β-galactosidase (pSV-β-galactosidase, Promega, Madison, WI) and green fluorescent protein (pSv-GFP, Packard Biosciences, Wellesley, MA) were purified from overnight bacterial culture using a Qiagen (Valencia, CA) Megaprep kit.

DBP (Ac-GCGKRKEFLERNRVAASKFRKRK-NH_2) and RGD peptides (Ac-CGCGKGYGGRGDSP-NH_2) were synthesized with an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) using standard solid-phase Fmoc chemistry. Amino acids (Nova Biochem, Madison, WI) were deprotected in piperidine (Sigma), activated with [2(1*H*-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU) (Applied Biosystems), and washed with *N*,*N*-methylpyrrolidone (Applied Biosytems). Rink amide resin (Nova Biochem) was used to initiate growth of peptides. After synthesis, peptides were cleaved from the resin using a cleavage cocktail (9 mL of trifluoroactetic acid (TFA) (Sigma), 0.5 mL of triisopropylsilane (TIPS) (Sigma), 0.5 mL of water per 1 g of peptide). Peptides were precipitated in diethyl ether (Sigma) and dried under vacuum. Matrix-assisted laser desorption ionization−time-of-flight (MALDI−TOF) (Applied Biosystems) mass spectrometry was used to verify peptide masses. Peptides were purified with reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using 0.1% TFA in water and acetonitrile (Fisher, Hampton, NH, HPLC grade) as the mobile phase. Peptides were eluted from a C18 Xterra Prep RP15 column (19 × 150 mm) (Waters, Milford, MA). Purified peptides were lyophilized and stored at −20 °C.

PEG-Based Vehicle Preparation and Purification. Peptides were coupled to PEG-TA using a Michael-type addition.[23] PEG-TA and the peptides were dissolved in 0.1 M phosphate buffer pH 8 and then coupled for 90 min sequentially at desired molar ratios. Peptide coupling to PEG acrylates was monitored using Ellman's reagent (5,5′-dithiabis(2-nitrobenzoic acid), Sigma). Ellman's reagent reacts with free sulfhydryl groups to form 2-nitro-5-thiobenzoic acid (TNB), a yellow substance, which absorbs light at 412 nm.

For some studies, the resulting PEG-DBP vehicles synthesized as described above at the desired molar ratio (i.e., 2:1 for PEG-TA DBP_2 vehicles) were purified with HPLC using a C18 analytical column with an acetontrile/0.1% TFA in water gradient from 20 to 60% over 20 min, and peptides were detected with a UV detector at 210 nm. The identity of each fraction was verified with MALDI−TOF. The concentration of the desired vehicle was determined using standard curves for both PEG-TA and peptide alone. Standard curves (molar concentration vs peak area) for PEG-TA and peptides were determined by direct injection of PEG-TA and peptides at various concentrations into the HPLC C-18 column. The area of the peak detected at 210 nm for each concentration was calculated by integration. The product yield was determined by dividing the moles of desired vehicle by the total moles of all PEG-based vehicles obtained.

Preparation of Particles. Polymer (PEG-DBP, PEG-DBP-RGD, or PEI) and plasmid DNA (40 μg/mL, 5% pSv-GFP, 95% pSV-β-galactosidase) were mixed in 150 mM NaCl and HEPES buffer (pH 7.4, 50 mM stock, 25 mM final) to achieve the desired charge ratios (positive charge of vehicle to negative charge of DNA) and incubated for 1 h at room temperature. Particle size and ζ potential were measured with a 90S Particle Sizer (Brookhaven Instruments, Holtsville, NY) using dynamic light scattering (DLS).

Gel Migration. PEG-DBP-RGD vehicles were complexed with plasmid DNA (40 μg/mL, pSV-β-Galactosidase) at charge ratios of 0.01:1 (+/−) to 10:1. The solutions were incubated for 1 h at room temperature in order for particles to form. Particles (10 μL) at each charge ratio were run on a 1% agrose gel at 80 mV for 1 h in 0.5× Tris-borate EDTA buffer (890 mM Tris-base, 890 mM boric acid, 0.5 M EDTA pH 8) with ethidium bromide (1 μg/ml). Gel was imaged with UV light.

***In Vitro* Transfection.** Particles were added to HEPG2 cells (American type Culture Collection, Manassas, VA), an epithelial cell line grown at 37 °C with 5% CO_2 in complete growth media (American type Culture Collection) supplemented with 1% antibiotic−antimycotic (ABAM) and 10% fetal bovine serum (FBS). Cells were seeded 24 h prior to transfection in a 24-well tissue culture plate at 1.25×10^4 cells/cm^2. DNA/polymer particles (50 μL) were added to each well and incubated for 4 h at 37 °C in serum-free Opti-Mem media (Invitrogen), unless otherwise noted. Media was then replaced with complete growth media after 4 h. After 48 h, cells lysates were collected with 1× cell culture lysis buffer (CCLR) (Promega) and analyzed by Bradford Assay (Biorad, Hercules, CA) and β-galactosidase activity assay (Promega). Transfection efficiency was expressed as a ratio of β-galactosidase activity to total cellular protein (measured by the Bradford assay).

Protein Adsorption. Protein adsorption to the surface of PEI- and PEG-based particles was characterized as previously described.[22] PEI- and PEG-based vehicles were complexed with DNA at the desired charge ratio. Particles were incubated with 1% (w/v) bovine serum albumin (BSA, Sigma) for 3 h at 37 °C. Final DNA and BSA concentrations were 80 μg/mL and 1 mg/mL, respectively. The particle/BSA solutions were centrifuged at 14000*g* for 1 h at 4 °C. The supernatant was removed, and the pellet was washed with deionized (dI) water to remove unbound BSA. The pellet was resuspended in dI water (10 μL) and loading buffer (0.2 M Tris-HCl pH 6.8, 10% w/v sodium dodecyl sulfate (SDS), 20% v/v glycerol, 0.05% w/v bromophenol blue, 10 mM dithiothreitol) (10 μL) and incubated at 90 °C for 5

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min to extract bound BSA from particles. Extracted solutions were separated on a 12% denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was run for 1 h at 20 mA and stained with RAPID Stain (Geno Technology, St. Louis, MO) to visualize BSA that had adsorbed onto particles.

Cell Viability. HEPG2 cells were incubated with PEI, PEG-DBP, or PEG-DBP-RGD vehicles with or without DNA for 24 h. A LIVE/DEAD assay (Molecular Probes, Carlsbad, CA) was used to determine the cell toxicity levels in the presence of the synthetic vehicles. Live cells are distinguished by the presence of intracellular esterase activity, which emits green fluorescence in the presence of Calcein AM. Dead cells were labeled red with ethidium homodimer-1, which entered cells with damaged membranes and underwent a 40-fold fluorescent enhancement upon interacting with intracellular nucleic acid. The cells were then counted (three images per group, ~200 cells/image) for each type of vehicle or particle using fluorescent microscopy (IX70 microscope, Olympus, Center Valley, PA, and MagnaFire 2.1C camera, Optronics, Goleta, CA). The percent viability was determined by the number of live cells over the total number of cells per field.

Statistical Analysis. Statistical analysis was performed using Statistica (version 5.5, Statsoft, Tulsa, OK). Comparative analyses were completed using the Scheffe's F posthoc test by analysis of variance at the 95% confidence level. Mean values and standard deviation are reported, unless otherwise noted.

Results

Synthesis of PEG-DBP-RGD Vehicles. DBPs and RGD peptides were covalently conjugated to a four-arm PEG backbone in order to develop a PEG-based vehicle, which can package DNA and target integrin receptors. Previous research has shown that covalently attaching DBPs to difunctional and tetrafunctional PEG allows DNA packaging for gene delivery.[22] In this study, DBPs and RGD peptides were coupled to acrylate groups on each end of PEG-TA via Michael-type addition with the thiol side chain of a single cysteine residue on each peptide.[23] The coupling reactions were monitored using Ellman's reagent (Figure 1). A reduction in thiol concentration, measured with Ellman's reagent, indicates peptide coupling to PEG-TA or the formation of disulfide bonds between peptides. When DBPs were reacted with PEG-TA at a molar ratio of 2:1, there was an 80% reduction in thiol concentration over 90 min (reaction half-life of 15 min, Table 1). Formation of disulfide bonds between peptides was monitored by incubating DBP or RGD peptide alone at the same concentration (disulfide bond reaction half-life is 360 or 866 min, respectively). Since the half-life of the disulfide bond formation is significantly longer than the half-life of the PEG-peptide reaction, most of the reaction in the presence of PEG is assumed to be PEG-peptide coupling. To determine if RGD peptides couple to PEG-TA, RGD was incubated with PEG-TA at a molar ratio of 2:1. The Ellman's reagent showed that 90% of free thiol was reacted over 90 min (reaction half-life of 9 min). These

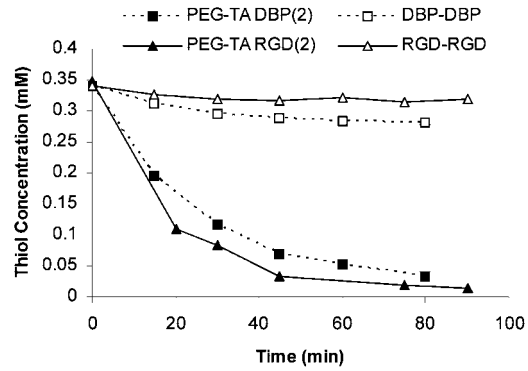

Figure 1. Monitoring of PEG-peptide reaction rates using an Ellman's assay to quantify the thiol concentration in solution which corresponds to uncoupled peptide. The half-life of reaction of both RGD peptide (▲) and DB peptide (■) with the PEG-TA backbone is shorter than that of the DB peptide in solution by itself (□). Over 80% of the free RGD peptide is coupled to the PEG backbone after 80 min.

Table 1. Reaction Half-Lives of PEG−Peptide Reactions[a]

reaction	half-life (min)
DBP-DBP	350.0
PEG-TA DBP(2)	15.7
RGD-RGD	866.0
PEG-TA RGD(2)	9.3

[a] The half-life of reaction of both RGD peptide and DB peptide with the PEG-TA backbone is shorter than that of the DB peptide in solution by itself.

results indicate that the DBP and RGD peptides can be coupled to the PEG-TA backbone. The reaction products were verified with HPLC.

Yield of PEG-DBP-RGD Vehicles. PEG-DBP-RGD vehicles were synthesized using a two-step process. DBPs were reacted with PEG-TA at the desired molar ratio (i.e., 2:1 for PEG-TA DBP_2) and incubated for 90 min. This reaction was monitored using Ellman's reagent (approximately 80% of DBP reacted). The product from this reaction was then reacted with RGD peptide at the desired molar ratio (i.e., 2:1 RGD:PEG-TA DBP for PEG-TA DBP_2 RGD_2). Ellman's reagent was used at 90 min, to determine the percentage of reacted RGD peptide. It was found that 75–100% of the RGD peptide was reacted. The PEG-DBP-RGD vehicles synthesized by the above method were used with no further purification. No difference was seen in the transfection efficiency of unpurified and purified PEG-DBP-RGD vehicles as discussed below.

To determine the yield of different PEG-DBP products, the products of the first step (PEG-DBP reaction) described above were separated using HPLC (Table 2A). Standard curves of both PEG-TA alone and free peptide were used to quantify the amount of each reaction product. When one molar equivalent of DBP was reacted with PEG-TA, the yield of desired product, PEG-TA DBP_1, was 62%. The yield of desired product was similar when 3 molar equiv of DBP was reacted with PEG-TA to form PEG-TA DBP_3 (54%).

Table 2. (A) Product Yields Determined by Purification with HPLC Found after Addition of the DBP to the PEG-TA Backbone. (B) Product Yields of PEG-DBP Vehicles Coupled to RGD Peptides at Desired Ratios

(A) Product Yields of PEG-DBP Vehicles

	product yields (%)				
PEG-TA:DBP molar ratio	PEG-TA	PEG DBP_1	PEG DBP_2	PEG DBP_3	PEG DBP_4
1:1	0.00	61.51	37.25	1.24	0.00
1:2	0.00	19.35	40.30	39.58	0.76
1:3	0.00	0.00	30.08	53.54	16.39

(B) Product Yields of PEG-DBP-RGD Vehicles

		product yields (%)			
vehicle	molar equiv of RGD added	no RGD	+ RGD1	+ RGD2	+ RGD3
PEG-TA DBP_1	3			31.33	68.67
PEG-TA DBP_2	2		54.99	45.01	
PEG-TA DBP_3	1		100.00		

However, when two DBPs were coupled to PEG-TA, the yield of PEG-TA DBP_2 was 40%.

The second step of the reaction involved coupling RGD peptide to PEG-DBP vehicles. To determine the products of these reactions, we coupled RGD peptides to purified PEG-DBP. The yields of these reactions are displayed in Table 2B. Reacting RGD peptide with purified PEG-TA DBP_1 at a 3:1 molar ratio yielded 31% PEG-TA DBP_1 RGD_2 and 68% of the desired product, PEG-TA DBP_1 RGD_3. When RGD peptide was reacted at a 2:1 molar ratio with PEG-TA DBP_2, the yield of PEG-TA DBP_2RGD_2 was 45%. PEG-TA DBP_3 RGD_1 was the only major product of the reaction of RGD and PEG-TA DBP_3 at a molar ratio of 1:1. These results demonstrate the variety of vehicles obtained from the coupling of DBP and RGD peptides to PEG-TA. The reaction yields could be improved by reacting PEG-TA DBP vehicles with excess RGD peptide.

HPLC and MALDI of Purified Vehicles. The synthesis of purified PEG-DBP-RGD vehicles was verified using HPLC and MALDI. A gradient of 5−45% acetonitrile in 0.1% TFA in water was passed through a C-18 column over 25 min. Detection of the reaction products at 210 nm is displayed in Figure 2. PEG-TA alone was eluted from the column after 23 min at approximately 45% acetonitrile. Coupling hydrophilic DBPs to PEG-TA reduced the retention time of the product. PEG-TA DBP_3 vehicles eluted from the column at 12 min. There was an additional reduction in elution time seen when RGD peptides were coupled to PEG-TA DBP_1 and PEG-TA DBP_2 vehicles. The elution times of all PEG-DBP-RGD vehicles were around 12 min. The change in HPLC elution time confirms the synthesis of PEG-TA-based vehicles coupled to DBP and RGD peptides.

The fractions collected from each purified vehicle were verified by MALDI. PEG-TA has an average molecular weight of 10000 Da, which was confirmed (Figure 2E). The MALDI spectrum also shows the size distribution of PEG-TA. Spacing between peaks is 44 Da, the molecular weight of one ethylene oxide unit. Coupling of DBPs (2806 Da) to the PEG-TA backbone was demonstrated by a shift in the vehicle spectra of ~2800 Da for each DBP conjugated to the backbone (Figure 2F−H). These spectra were then shifted again with the addition of RGD peptides (1252 Da) to the PEG backbone. These results demonstrate the synthesis of a four-arm PEG-backbone vehicle with two different peptides at three different peptide configurations.

PEG-Based Particle Characterization. Particle size and ζ potential are critical to the first two steps in the gene delivery pathway, cell binding, and internalization. ζ potential affects the particle's ability to bind to the cell surface. If the particle has a negative charge, it will be electrostatically repelled by the negatively charged cell surface; but, if it has a high positive charge, it will be toxic to the cell. Therefore, a slight positive charge is desired to allow attraction to cell surface without significant toxicity. Particle size regulates endocytosis of the particles. If the particle is too large (>500 nm), it will not be able to enter the cell via endocytosis.[24] Parts A and B of Figure 3 show the mean particle diameter and ζ potential, respectively, at three charge ratios (+/−) for PEG-TA based vehicles, with and without RGD peptides, compared to a common cationic polymer vehicle, PEI. Particle diameters were typically in the range of 250–300 nm, similar to that of PEI particles formed under physiological conditions (150 mM NaCl). It was also noted that coupling RGD peptides to PEG-DBP vehicles did not significantly affect particle diameter.

PEG-based particle formation was monitored over time at a charge ratio of 6:1 and at various DNA concentrations (data not shown). The PEG-based vehicles formed particles of 250–300 nm immediately after mixing with DNA and remained at a constant size for at least 90 min. The concentration of DNA had no significant effect on particle size.

The ζ potentials of PEG-DBP-RGD vehicles ranged between –10 and 7 mV. These values were significantly lower than those of the cationic PEI particles, which had an average ζ potential of +30mV. This indicates that the PEG-TA-based vehicles have a lower charge density on their surface compared to PEI particles. There was no significant difference in ζ potential found between the three combinations of PEG-DBP-RGD vehicles (Figure 3B).

Gel migration studies were performed to confirm the condensation of DNA with the PEG-based vehicles. PEG-based vehicles with RGD peptides were complexed with DNA at charge ratios of 0.01:1 (+/−) to 10:1. The particles incubated for 1 h and then were run on a 1% agrose gel to determine if the DNA was complexed to the PEG vehicle.

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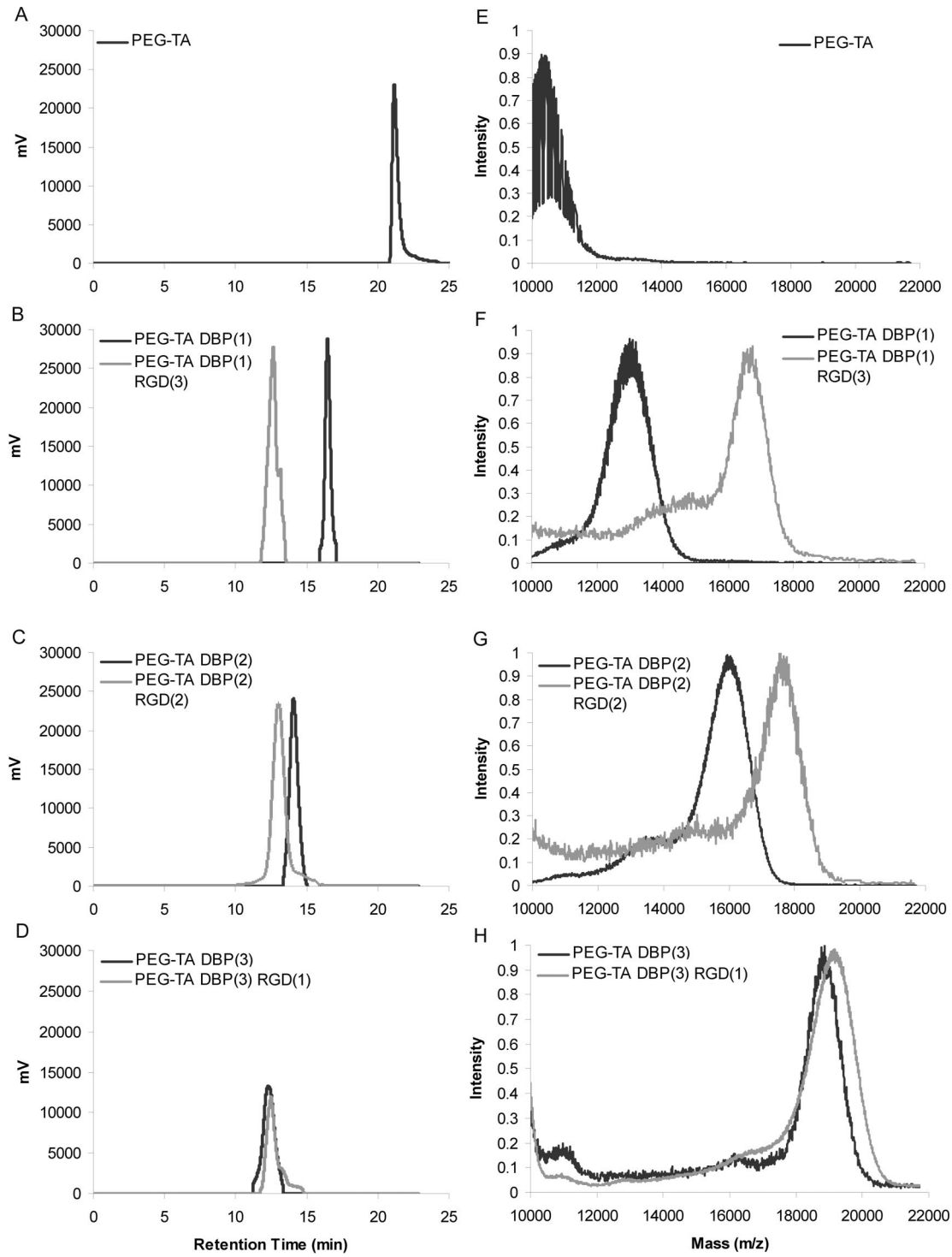

Figure 2. Retention time of PEG-based vehicles coupled to RGD and DB peptides using high pressure liquid chromatography (HPLC) with a gradient of 5−45% acetonitrile over 25 min: (A) PEG-TA alone was retained in the column for 22 min; (B) PEG-TA DBP_1 (black), PEG-TA RGD_3 DBP_1 (gray); (C) PEG-TA DBP_2 (black) PEG-TA RGD_2 DBP_2 (gray); (D) PEG-TA DBP_3 (black) PEG-TA RGD_1 DBP_3 (gray). MALDI−TOF was used to verify the final PEG-based vehicle: (E) PEG-TA alone has an average molecular weight of 10000 Da; (F) PEG-TA coupled to one DBP (MW_{avg} = 12806 Da, black), PEG-TA DBP_1 RGD_3 (MW_{avg} = 16562 Da, gray) (G) PEG-TA DBP_2 (MW_{avg} = 15612 Da, black), PEG-TA DBP_2 RGD_2 (MW_{avg} = 18116 Da, gray); (H) PEG-TA DBP_3 (MW_{avg} = 18418 Da, black), PEG-TA DBP_3 RGD_1 (MW_{avg} = 19670 Da, gray)

At charge ratios of 0.01:1 and 0.1:1, the DNA migrated slightly in the gel, indicating that it may not be fully complexed in the PEG-based vehicle (Figure 3C). However, at higher charge ratios, there was no movement toward the

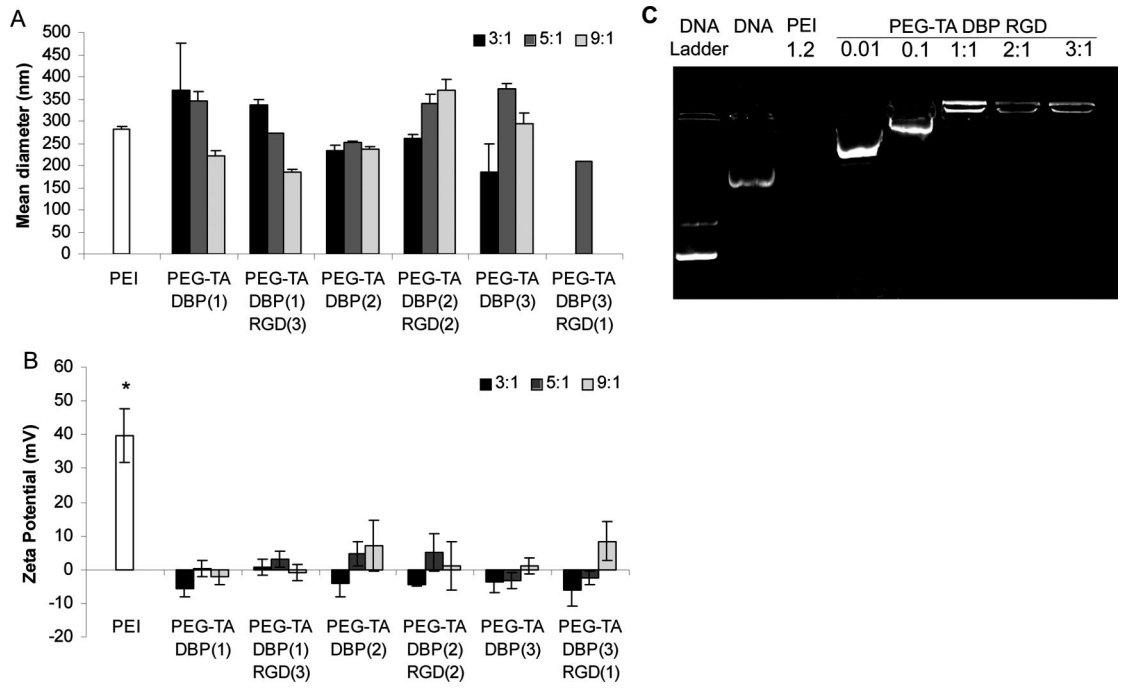

Figure 3. (A) Mean particle diameter of PEG-TA particles with and without RGD peptides at charge ratios 3, 5, and 9:1 (+/−) compared to 1.2:1 PEI particles. (B) Mean ζ potential of PEG-TA particles with and without RGD peptides at charge ratios (3:1, 5:1, and 9:1) compared to 1.2:1 PEI particles. The $*$ indicates the difference between PEG particles with and without RGD peptides and PEI particles. (C) Analysis of DNA particles formation by agrose gel electrophoresis. Above a charge ratio of 1:1 for the PEG-DBP-RGD particle, all of the DNA remains in the well. For the PEI particle, there is no DNA seen due to migration toward the negative electrode immediately.

positive electrode. This indicates that at charge ratios at 1:1 or higher, the DNA movement is retarded by the PEG-based vehicles. There was also no movement of the particles in the negative direction, which either supports the ζ potential results that the particles are neutrally charged or indicates that the size of the particles limited movement in the gel. DNA from the PEI particles was not visible in the gel. This could be explained by movement of PEI particles toward the anode or exclusion of ethidium bromide due to protection of DNA by PEI.

In Vitro **Transfection Results.** To determine transfection efficiency of the PEG-based vehicles, HEPG2 cells were incubated with PEG particles at various charge ratios. HEPG2 cells were used because 99% were found to express integrin α_v receptor, which binds to vitronectin and fibronectin (data not shown).[25] Transfection efficiency was quantified as the β-galactosidase expressed divided by the total cellular protein. The highest transfection efficiency for the PEG-TA DBP_2RGD_2 particle was seen at a charge ratio of 6:1 (+/−) (Figure 4A). This value was not statistically different from the transfection efficiency of PEI ($p > 0.05$). The cationic control vehicle was complexed at the average optimal charge ratio found in literature for HEPG2 cells (1.2:1, equivalent to an N/P ratio of 6).[26–28] Importantly, there was a significant increase in transfection efficiency seen at charge ratios 5:1 and 6:1 for PEG-TA RGD_2 DBP_2 compared to vehicles without RGD (PEG-TA DBP_2). The transfection efficiency increased 3-fold when two RGD peptides were coupled to the PEG backbone. These results demonstrate that coupling RGD peptides to PEG-based vehicles can increase transfection efficiency and can have similar effectiveness to that of PEI for gene delivery to HEPG2 cells.

To determine whether endosomal escape efficiency was limiting the transfection of our PEG-DBP-RGD vehicles in HEPG2 cells, we used chloroquine, a lysosomotropic agent that blocks the acidification of the lysosome. When transfection was performed in the presence of chloroquine with a 6:1 (+/−) charge ratio of PEG-DBP-RGD vehicles, the transgene expression increased more than 4 fold, resulting in β-gal levels of 62% of those observed for PEI with chloroquine in the same experiment (Figure SI-1). Thus, we concluded that endosomal escape is a limiting factor for the transfection efficiency of PEG-DBP-RGD vehicles.

Similar experiments were performed using CHO cells to determine if the increase in transfection efficiency with RGD

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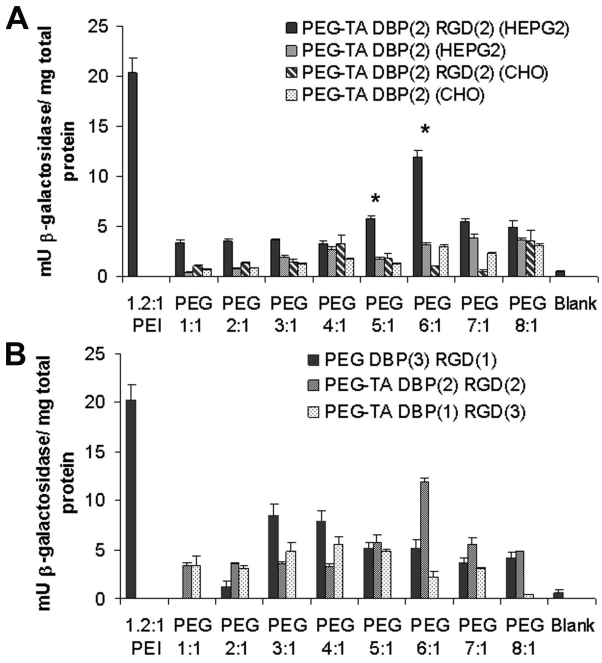

Figure 4. (A) Transfection efficiency of PEG-based particles with and without RGD peptides at various charge ratios (1:1 to 8:1) when transfecting HEPG2 cells and CHO cells. The ∗ indicates the difference between PEG vehicles with and without RGD peptides at that charge ratio ($p < 0.05$). (B) Comparison of transfection efficiencies of PEG-TA vehicles with 1, 2, or 3 RGD peptides. Error bars indicate standard error.

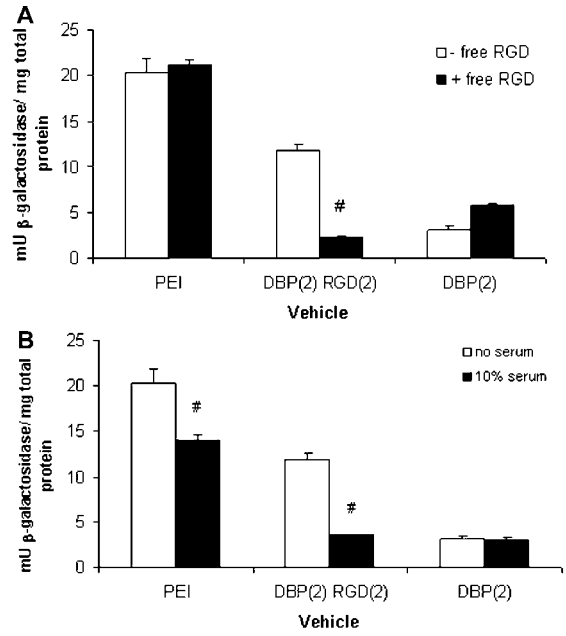

Figure 5. (A) Transfection efficiency of PEG-based vehicles at charge ratios 6:1 compared to PEI when transfecting HEPG2 cells with and without (A) free RGD or (B) 10% FBS. The # indicates the difference in transfection efficiency of vehicles with no free rgd or serum. Error bars indicate standard error.

peptide coupling was cell type dependent. CHO cells were transfected with both PEG-TA DBP_2 RGD_2 particles and PEG-TA DBP_2 particles at charge ratios of 1:1–10:1. The transfection efficiency of PEG-based vehicles with RGD peptide was not different from the transfection efficiency of PEG-based vehicles with only DBPs (Figure 4A). We hypothesize that this is due to low expression of integrin receptors by CHO cells compared to HEPG2 cells or different methods of binding and internalization of PEG-based vehicles.

Transfection efficiencies of PEG-TA DBP_3 RGD_1 and PEG-TA DBP_1 RGD_3 particles in HEPG2 cells were also compared to the transfection efficiency of the same vehicles without RGD (PEG-TA DBP_3 and PEG-TA DBP_1 particles) (data not shown). The highest transfection efficiency for these PEG-based vehicles was seen at charge ratios of 3:1 for PEG-TA DBP_3 RGD_1 particles and 5:1 for PEG-TA DBP_1 RGD_3 particles (Figure 4b). However, both of these vehicles had lower transfection efficiencies than the PEG-TA DBP_2 RGD_2 particles and were not significantly higher than the PEG-DBP particles without RGD peptides.

RGD Competition Assay. To determine if RGD binding to integrin receptors was responsible for the increase in transfection efficiency, an RGD competition assay was performed. HEPG2 cells were transfected with PEI, PEG-TA DBP_2 and PEG-TA DBP_2 RGD_2 with 1 mM free RGD peptide in the transfection media. As expected, the transgene expression by cells transfected with PEG-TA DBP_2RGD_2 particles was reduced in the presence of free RGD, but the transfection efficiency of PEI and PEG-DBP_2 vehicles was not affected (Figure 5A). These results suggest that the increase in transfection efficiency observed with coupling of RGD peptides to PEG-DBP vehicles is mediated through integrin receptors on HEPG2 cells.

Protein Adsorption. Protein adsorption onto gene delivery particles has been shown to limit the efficiency of delivery for *in vivo* applications. Previous research has shown that cationic polymer vehicles adsorb proteins onto their surface leading to aggregation, opsonization, and rapid clearance from the blood.[17,18] To measure protein adsorption onto the surface of PEG-DBP-RGD particles, the particles were incubated with BSA for 3 h. Free BSA was washed from the particle solution with PBS and the particles were collected by centrifugation. BSA bound to the surface of the particles was removed using SDS and separated from the vehicle using SDS-PAGE. The amount of BSA adsorbed onto PEG-based vehicles was compared to the amount of BSA adsorbed onto cationic polymer vehicles, PEI. As a negative control, BSA was added to solution with no particles (blank) to account for BSA adsorption to the microcentrifuge tube.

As shown in Figure 6, the band of the BSA removed from PEI particles, after BSA incubation, is much larger than the band for BSA removed from the PEG-TA DBP_2 RGD_2 particles. There is no BSA band seen in the blank lane, indicating that all BSA seen in the PEI and PEG particles lanes is due to protein adsorption to the particle surface. Note

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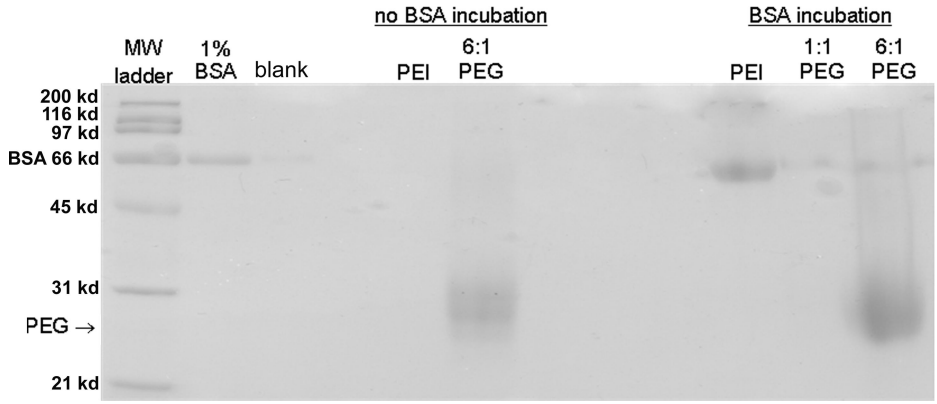

Figure 6. Protein adsorption onto surface of PEG-TA DBP_2 RGD_2 particles compared to protein adsorption onto PEI particles. Particles were incubated with bovine serum albumin (BSA) for 2 h. Unbound BSA was removed by washing and centrifugation. Bound BSA was removed from the particles and run on a SDS-PAGE gel. The BSA removed from the PEI particles has a larger band than the BSA removed from the PEG DBP RGD particles.

that PEG vehicles are visible in gel at ~ 24 kD for the 6:1 charge ratio. This band is not visible for PEG vehicles at a 1:1 charge ratio, which contains a lower concentration of PEG. There is no noticeable difference in BSA adsorption between charge ratios 1:1 and 6:1 for PEG-TA DBP_2 RGD_2 particles. These results demonstrate that PEG-TA DBP_2 RGD_2 particles adsorb less protein than PEI particles.

In Vitro **Transfections with Serum.** In addition to measuring protein adsorption onto the surface of our PEG-based vehicles in comparison with PEI, transfection efficiency was measured in the presence of 10% FBS. HEPG2 cells were transfected with PEI, PEG-TA DBP_2, and PEG-TA DBP_2RGD_2 particles at the most efficient charge ratio determined previously (1.2:1 and 6:1). It was found that transfection efficiency of PEG DBP_2 vehicles was not affected by the presence of serum in the media. This suggests that PEG-based vehicles are resistant to serum adsorption and degradation by serum proteins, both of which limit current cationic polymer gene delivery vehicles. The transfection efficiency of PEI and PEG-TA DBP_2 RGD_2 particles decreased in the presence of serum. Particle sizing of PEG-TA DBP_2 RGD_2/DNA particles after incubation in 10% serum found that the particle sizes did not increase (152 ± 62 nm), suggesting that the particles were not aggregated in solution. It is hypothesized that transfection efficiency of PEG DBP_2 RGD_2 vehicles was reduced to due competition with serum proteins, vitronectin and fibronectin, for binding of integrin receptors, not aggregation of complexes.

Cell Viability. The viability of HEPG2 cells transfected with PEG-DBP-RGD vehicles was compared to the viability of HEPG2 cells transfected with PEI with and without DNA. Cells transfected with combinations of PEG-DBP-RGD vehicles complexed with DNA were 98–99% viable. This was significantly higher than the viability of cells transfected with PEI complexes, ~85% (Figure 7). Similar results were seen when vehicles alone were added to HEPG2 cells for 24 h. This indicates that DNA complexing did not affect cell viability. No decrease in cell viability was seen with higher concentrations of PEG-based vehicles up to a charge ratio of 20:1 (data not shown).

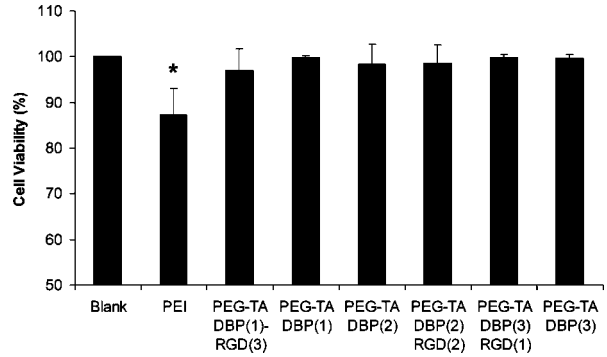

Figure 7. Cell viability of HEPG2 cells transfected with PEG-based particles compared to cell viability of HEPG2 cells transfected with PEI particles after 24 h incubation. Cell viability was measured using a LIVE/DEAD assay kit. The ∗ indicates significant difference in cell viability compared to blank ($p < 0.05$).

Discussion

A key step in nonviral gene delivery is targeting cell surface receptors *in vivo* without triggering opsonization. Increased specificity of nonviral particles is desired while preventing nonspecific binding of polymers to serum proteins.[20] Cationic polymer-based gene delivery vehicles, such as PEI, lack specificity for cell surface receptors. The high positive net charge of PEI/DNA particles leads to nonspecific adsorption of proteins to the particle surface, leading to particle aggregation and rapid blood clearance.[17,18] Additionally, cell surface binding and internalization of PEI/DNA particles is mediated by nonspecific electrostatic interactions.

PEG incorporation in gene delivery vehicles is desirable because PEG can shield highly charged vehicles, reducing nonspecific protein interactions, and increasing blood circulation time. But, there is a significant reduction in transfection efficiency when PEG is coupled to the exterior of PEI-based/DNA vehicles. We have developed a PEG-based gene delivery vehicle using a PEG backbone coupled with functional groups, instead of conjugating PEG to the particle

surface, to reduce nonspecific adsorption without shielding the function of cell targeting peptides.

We have previously demonstrated the transfection of CHO cells using a four-arm PEG backbone coupled to four DBPs.[22] PEG-DBP/DNA particles have a near-neutral ζ potential, low toxicity, and reduced nonspecific protein adsorption compared to PEI/DNA particles. Our PEG-DBP vehicles are also advantageous because they have potential for the addition of other functional groups, such as peptides or proteins to target specific cell receptors. This paper focuses on the synthesis and characterization of bifunctional PEG-based vehicles coupled to DBP and RGD peptides.

RGD peptides were coupled to the PEG backbone to specifically target integrin receptors. Integrin receptors, such as $\alpha_V\beta_3$, are favorable targets because they are upregulated in tumors, a common target of gene therapy,[13] and are also used by adenoviruses and bacteria for cell binding and internalization.[14,15] Previous studies have shown that coupling RGD peptides to PEI vehicles increases transfection efficiency. We were interested in the effect of conjugating RGD peptides to our PEG-DBP vehicles on transfection efficiency and receptor specificity.

To determine if purification of PEG-DBP-RGD vehicles affects transfection results, transfection efficiency of purified and unpurified PEG-DBP-RGD vehicles were compared. Studies found that there was no difference in the transfection efficiency of the purified PEG-DBP-RGD vehicles versus unpurified vehicles. HPLC demonstrated that unpurified products contain a variety of PEG-DBP-RGD vehicles (as seen in Table 2). However, the overall ratio of DBP and RGD peptides to PEG in vehicle solutions is different for the three combinations (1:3, 2:2, and 3:1 DBP:RGD) whether they are purified or unpurified. In addition, during the formation of PEG/DNA particles, multiple PEG-DBP-RGD vehicles are used to condense each plasmid of DNA. We hypothesize that the final molar ratio of RGD and DBP to PEG in these particles is similar whether the vehicles are purified or unpurified which is why no difference in was seen transfection efficiency.

Our results showed that coupling RGD peptides to PEG-DBP vehicles improved transfection efficiency at specific conditions. Coupling two RGD peptides to PEG-TA DBP_2 vehicles led to a significant increase in transfection efficiency at charge ratios (PEG-DBP-RGD amine groups:DNA phosphate) of 5:1 and 6:1 when transfecting HEPG2 cells. At these conditions, PEG-TA DBP_2 RGD_2 particles were similar to PEI particles in the level of reporter gene expression, demonstrating that they are efficient gene delivery vehicles. This is an improvement over PEI-PEG-RGD (linear) vehicles, which in transfection efficiency varied between 4 and 25% of that of PEI alone, depending on the length of PEG.[16,21] The improvement in efficiency indicates that using a multiarm PEG backbone instead of coupling RGD peptides to PEI via a PEG spacer could be more efficient. It also demonstrates that coupling a cell receptor targeting peptide to a PEG-based gene delivery vehicle can increase transfection efficiency.

Previous studies have shown that increased transfection efficiency is a result of increased cell surface binding and internalization.[29] To determine if increased specific binding to integrin receptors led to increased transfection efficiency when RGD peptides were coupled to PEG-TA DBP_2vehicles, HEPG2 cells were transfected with PEG-TA DBP_2 and PEG-TA DBP_2 RGD_2 particles in the presence of free RGD. Short RGD peptides mimic binding of cell adhesion proteins, such as vitronectin and fibronectin, to integrin receptors.[10] The decrease in transfection efficiency of PEG-DBP-RGD particles but not PEG-DBP particles in the presence of free RGD indicates that free RGD peptide competes with PEG-DBP-RGD vehicles for binding to integrin receptors. Similar results were seen for transfections in the presence of serum. These results suggest that PEG-DBP-RGD particles may have had to compete with vitronectin and fibronectin in serum to bind cell surface integrin receptors, which reduced transfection efficiency.

In addition to receptor specificity, the physical characteristics of the PEG-based particles are important for effective gene delivery. Particle size of integrin-bound particles can regulate the method of internalization. Integrin α_v receptors are known to be internalized via a "zippering" mechanism, which can only internalize particles up to 500 nm in diameter.[30] Therefore, particle size of the PEG-based vehicles should not exceed 500 nm. Our previous studies demonstrated that PEG-DBP/DNA particles were around 300 nm similar to PEI/DNA particles. We investigated the effect of coupling RGD to the vehicles on the particle diameter. In this study, dynamic light scattering showed that PEG-DBP-RGD vehicles formed particles in the range of 250–300 nm when complexed with DNA, similar to PEG-DBP vehicles. It was also observed that particles size was independent of the number of RGD peptides coupled to the PEG-based vehicle. This supports previous studies with PEI-based particles, which conclude that PEG and RGD peptides do not increase particle size but help stabilize particles. PEG reduces charge–charge interactions between particles through shielding effects which reduces particle aggregation, leading to smaller PEI particles.[16,17,31]

Surface charge of gene delivery particles is also an important characteristic of gene delivery vehicles. A higher ζ potential indicates more nonspecific electrostatic interactions of particles with serum proteins and cells. The surface charge of PEG-DBP-RGD/DNA particles was around neutral for all charge ratios. A neutral zeta potential suggests that there should be little nonspecific adsorption and cell binding. Coupling RGD to PEG-DBP vehicles did not change the zeta potential of the particles formed. Therefore, the nonspecific

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adsorption and binding of the particles should not change for PEG- DBP vehicles with the addition of RGD peptides. This was confirmed by our protein adsorption studies. The amount of nonspecific BSA adsorption onto the particles surface was similar for PEG-TA DBP_2/DNA particles and PEG-TA DBP_2 RGD_2/DNA particles.

Our results did indicate that the number of DBPs and RGD peptides and the amount of PEG-based vehicle per particle can influence the effect of RGD peptides on transfection efficiency. At high charge ratios or when 1 or 3 RGD peptides were coupled to PEG-DBP vehicles, there was no improvement in transfection efficiency when RGD peptides were coupled to the vehicle. Previous studies have shown that affinity of particles to integrin receptors on the cell surface determines whether an integrin-bound particle is internalized or remains on the cell surface.[32] Low-affinity binding results in low receptor clustering, a prerequisite for signal transductions of integrin receptors and subsequent internalization.[33] High-affinity binding to integrin receptors can result in extracellular adherence and low cellular internalization.[32] Particle affinity for integrin receptors was affected by the number of RGD peptides available for integrin binding on the particle surface. In this study, the number and orientation of RGD peptides on the particle surface were altered by varying the charge ratio and number of RGD peptides per vehicle. Therefore, these two factors influenced the effect of RGD peptides on transfection efficiency of PEG-DBP-RGD vehicles. This effect may also be a function of the number of DBPs per vehicle. However, these results cannot be separated in this study. Future studies will explore time dependent binding and internalization of PEG-based vehicles with and without RGD peptides to give more indication as to how affinity and avidity affects cell surface binding and internalization.

In conclusion, this paper demonstrates that bifunctional PEG-based vehicles, with low cytotoxicity and resistance to protein adsorption, can successfully target and transfect cells expressing target receptors. A significant increase in transfection efficiency was seen when two RGD peptides were coupled to the PEG-TA DBP_2 vehicles at charge ratios 5:1 and 6:1. These vehicles were found to be significantly similar to PEI vehicles based on the level of transgene expression. This indicates that PEG-based vehicles, functionalized with DBP and RGD peptides, can be used for gene delivery. Future experiments will focus on coupling endosomal escape peptides to PEG-TA arms to enhance endosomal escape, another potential barrier to gene delivery.

Supporting Information Available: Figure SI-1. This material is available free of charge via the Internet at http://pubs.acs.org.

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