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DC3-Decorated Polyplexes for Targeted Gene Delivery into Dendritic Cells

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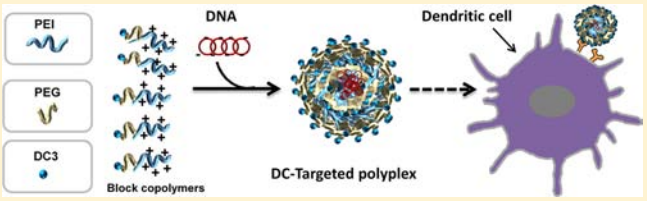
ABSTRACT: Dendritic cells (DCs) are a family of specialized antigen presenting cells (APCs) that detect antigens and initiate a wide spectrum of immune responses against them. These characteristics make them promising candidates for immunotherapy manipulations. In this study we designed and synthesized DC-targeted block copolymers composed of linear polyethylenimine (PEI) conjugated to hydrophilic polyethylene glycol (PEG) installed with a DC-targeting peptide (DC3, primary sequence FYPSYHSTPQRP). Two different conjugation procedures (basic and modified) were employed to synthesize the DC3-PEG-*b*-PEI and the control SCRM-PEG-*b*-PEI (with a scrambled DC3 peptide sequence) by one-pot synthesis, in two steps. In the first, basic conjugation procedure, PEG with *N*-hydroxysuccinimide (NHS) ester and maleimide (MAL) groups (NHS-PEG-MAL, 3.5 kDa) was first coupled to linear PEI (25 kDa) via the NHS group to yield the intermediate MAL-PEG-*b*-PEI, that was then conjugated to N-terminus-cysteine harboring peptides DC3 or SCRM via the MAL double bond to yield the final DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI copolymers, respectively. In the second, modified conjugation procedure, Fmoc-cysteine harboring DC3 or SCRM peptides were first conjugated to NHS-PEG-MAL via the MAL group followed by coupling to linear PEI via the NHS functional group. Fmoc cleavage yielded the same final product as in the basic procedure. The modified conjugation procedure was capable of yielding block copolymers richer with peptides, as determined by ^{1}H NMR analysis. Self-assembly of DC3-PEG-*b*-PEI copolymers and DNA molecules yielded nanosized polyion complexes (polyplexes), with lower surface charge and limited cytotoxicity when compared to the PEI building block. Significant transfection efficiency of the DC-targeted polyplexes by murine dendritic DC2.4 cells was observed only in DC3-PEG-*b*-PEI/DNA polyplexes synthesized by the modified conjugation procedure. These polyplexes, with higher peptide-load, showed greater transfection capability in DC2.4 cells relative to the control nontargeted SCRM-PEG-*b*-PEI/DNA polyplexes, but not in endothelial cells. The transfection efficiency was comparable to or higher than that of the PEI/DNA positive control. The results indicate that PEGylated-PEI polyplexes show significant transfection efficiency into DCs when decorated with DC3 peptide, and are attractive candidates for immunotherapy via DCs.



INTRODUCTION

In the past few decades, a variety of strategies for prevention, detection, and treatment of cancer were developed, nevertheless, cancer is still a major cause of illness and death, mainly because of high relapse rates evoked by minimal residual disease.[1,2] Cancer immunotherapy, the activation of the patient's own immune system against a tumor he carries is, therefore, gaining more interest.[3] It is believed that the immune system components, being constantly spread throughout the body, might be more sensitive than any external therapy in detecting the abandoned cancerous cells that have survived previous treatment, and therefore more efficient in preventing relapse.

Dendritic cells (DCs) are a family of specialized antigen presenting cells (APCs) that can detect tissue damage, pathogen entry, inflammation, and malignantly transformed cells. When recognizing an antigen (Ag), DCs mature and migrate to lymph nodes where they cross-present it to T cells and activate them to initiate a wide spectrum of immune responses.[4] Their ability to detect and cross-present antigens, and to induce an effective immune response, makes DCs an attractive target for immunotherapy manipulations.[5] After loading DCs with a tumor antigen encoding plasmid DNA, the cells will process it to a protein that will be cross-presented on their membrane to T-cells, and thus may activate a tumor antigen-specific immune response.[6,7]

DCs loading with DNA can be accomplished by either ex vivo or in vivo gene delivery methods.[6−8] While ex vivo gene delivery might be easier to perform and evaluate, in vivo gene delivery is less invasive, and does not require the selection and expansion of DCs from the patient. In vivo gene delivery requires a carefully designed carrier that can enable DNA survival in the circulation and high transfection efficiency without increasing toxic or immunogenic responses.[9]

Polyion complexes (polyplexes) are based on cationic polymers that spontaneously complex with the negatively

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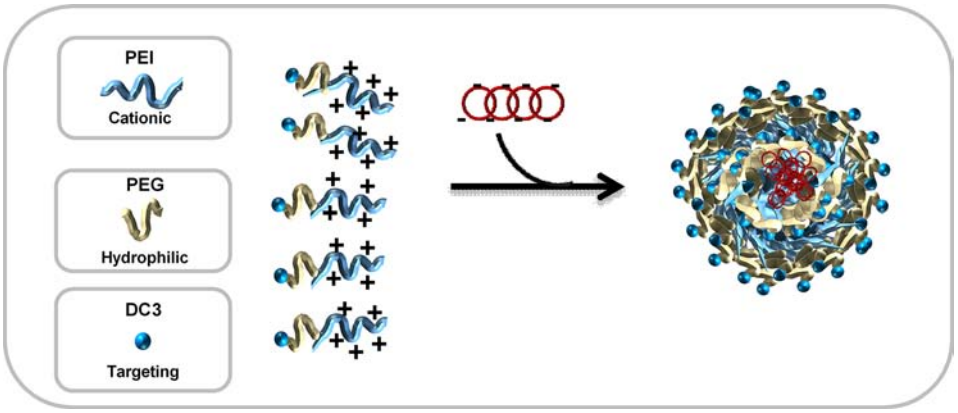
Scheme 1. Self-assembly of DC3-PEG-*b*-PEI/DNA Polyplexes[a]



[a]Positively charged block copolymers bearing DC-targeting ligands (DC3) and negatively charged DNA molecules can self-assemble in an aqueous solution to form DC-targeted polyplexes.

charged oligonucleotides. They are simple to create, and can be easily modified to give desired characteristics.[10,11] Polyethylenimine (PEI) is one of the most useful polycations: it demonstrates high transfection efficiency, DNA binding and condensation activity, and high pH buffering capacity, which is believed to protect DNA from degradation and enable its escape from the endosomal compartment.[12] However, the positive surface charge of PEI/DNA polyplexes result in aggregation, nonspecific adsorption to plasma proteins, recognition by the immune system components, and cytotoxicity.[13−16] Conjugation of a hydrophilic polymer such as polyethylene glycol (PEG) to PEI reduces the positive charge of the polyplex surface, and addresses these issues.[17] Nevertheless, PEG-*b*-PEI/DNA polyplexes show lower transfection efficiency compared to PEI/DNA polyplexes. Together with the fact that DCs compose only 1−3% of cells in peripheral tissues,[18−21] such a system would have a very limited efficiency unless targeted.

DC3 peptide (primary sequence FYPSYHSTPQRP)[22] has been previously reported to bind specifically to monocyte-derived human DCs but not to monocytes, T/B lymphocytes, endothelial cells, or fibroblasts. The cognate ligand for this peptide is unknown. A fusion of DC3 peptide and nona-arginine (9dR) that binds nucleic acids by charge interaction was previously exploited by Subramanya et al. for the specific delivery of siRNA to DCs in vitro and in vivo,[23] and was further used to induce strong human T cell immune responses by silencing immunosuppressive molecules in DCs.[24]

Here we describe the design and synthesis of DC-specific gene delivery system composed of PEI conjugated to PEG and installed with a DC3 peptide as a ligand for targeting DCs (Scheme 1). Two different conjugation procedures (basic and modified) were employed to synthesize the DC3-PEG-*b*-PEI block copolymer. The transfection efficiency and cell specificity of the DC3-PEG-PEI/DNA polyplexes in dendritic and nondendritic (endothelial) cells was tested. To the best of our knowledge, this is the first attempt to implement DC3-PEG-*b*-PEI/DNA for gene delivery into DCs.

RESULTS

We utilized two different approaches for conjugating the hydrophobic DC3 peptide to PEG-*b*-PEI copolymer using one-pot synthesis, as shown in Scheme 2. In the basic conjugation procedure (BP), PEI was first conjugated to MAL-PEG-NHS, and the resulting MAL-PEG-*b*-PEI was then attached to N-terminus-cysteine harboring DC3 or SCRM to yield the final products DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI. In the modified conjugation procedure (MP), Fmoc-cysteine harboring DC3 or SCRM peptides were first conjugated to MAL-PEG-NHS, and the resulting Fmoc-DC3-PEG-NHS or Fmoc-SCRM-PEG-NHS were then attached to PEI. The Fmoc protecting group was then cleaved from the product so that both methods yielded the same final products, DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI. PEG-*b*-PEI was synthesized and used as control.

^{1}H NMR spectra (in D_2O) of block copolymers synthesized by the BP showed the characteristic shifts of PEI (δ2.8−3.1) and PEG (δ3.5−3.7) in PEG-*b*-PEI, DC3-PEG-*b*-PEI, and SCRM-PEG-*b*-PEI (Figure 1A). The proton chemical shift representing the aromatic amino acids of the peptides (2xTyr and Phe) was only present in DC3-PEG-*b*-PEI and SCRM-PEG-*b*-PEI. These results indicate that peptide-harboring block copolymers of the desired composition were obtained by the basic conjugation procedure.

Products of the modified conjugation procedure (MP) were analyzed after Fmoc cleavage (Figure 1B). ^{1}H NMR analysis (in DMSO) of the final products confirmed the presence of all 3 moieties (PEI, PEG, and peptide) in the DC3 or SCRM conjugated PEG-*b*-PEI copolymers. The number of DC3 peptides on each polyplex was determined by BCA protein quantification assay. There were 12.7 peptides on each polyplex synthesized by the BP and 39.1 peptides on each polyplex synthesized in the MP. The ^{1}H NMR spectra of the block copolymer was further analyzed to estimate the content of the conjugated peptide. The content of the peptide in the block copolymer could theoretically be calculated by the ratio between the area of the aromatic amino acid side chains of DC3 (δ6.5−7.5) and PEI (δ2.8−3.1) peaks. Unfortunately the PEI peak was overlapped by the solvent (DMSO) peak, so the ratio could not be defined precisely. However, the fraction of DC3 relative to PEI for each spectrum was at least 2-times higher for block copolymers synthesized in the MP when compared to copolymers prepared by the BP. Furthermore, the water-solubility of the polymer synthesized by the MP was significantly lower than that synthesized by the BP, suggesting that the MP is capable of yielding block copolymers that are richer with the hydrophobic peptides. This is extremely important, as such peptide-rich block copolymers are likely to

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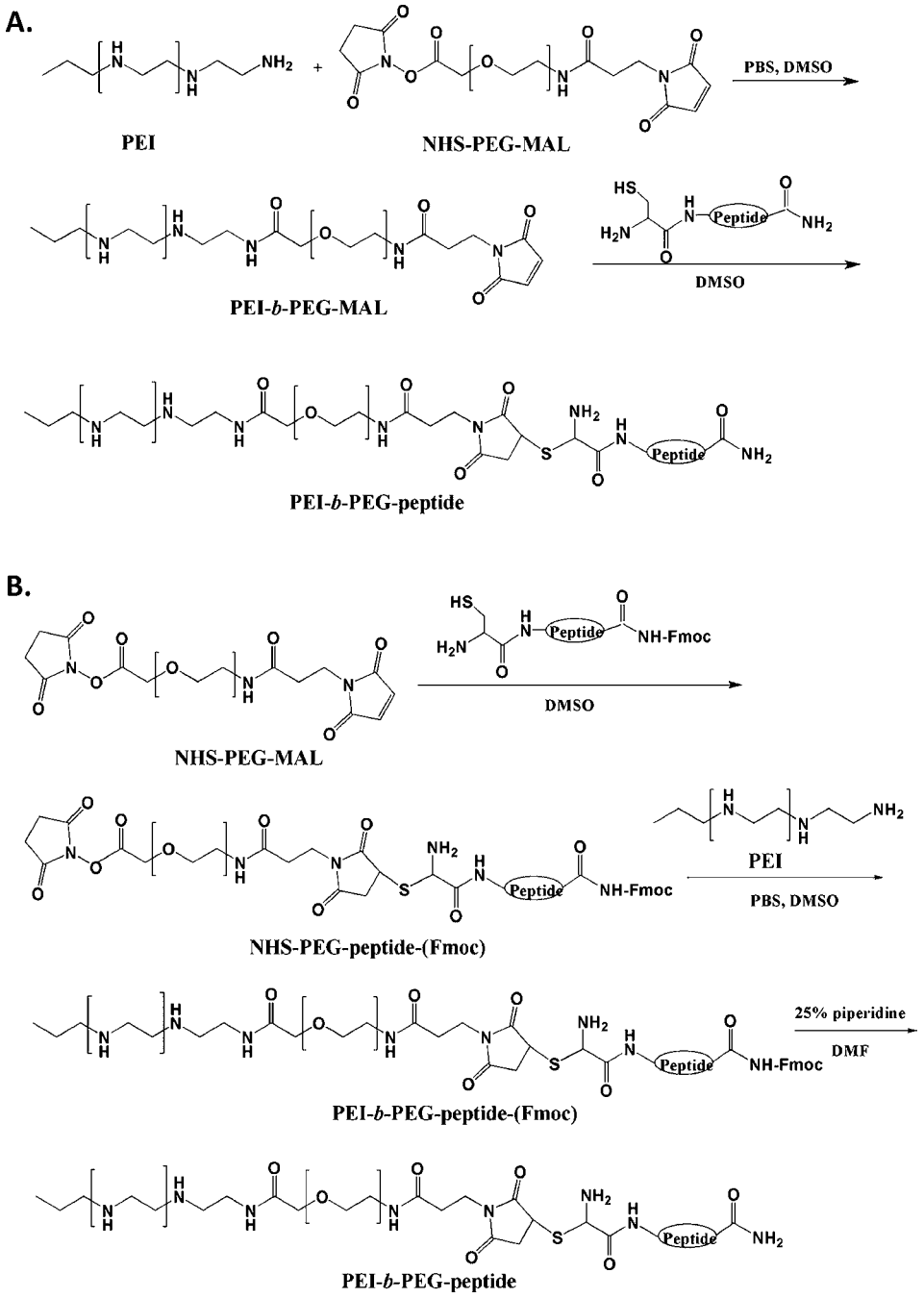
Scheme 2. One-Pot Synthesis DC3-PEG-*b*-PEI[a]



[a]One-pot synthesis DC3-PEG-*b*-PEI by (A) the basic conjugation procedure (BP) or (B) the modified conjugation procedure (MP). (**A**) NHS-PEG-MAL was first coupled to linear PEI and then conjugated to N-terminus-cysteine harboring peptide. (**B**) Fmoc-cysteine harboring peptide was first conjugated to NHS-PEG-MAL followed by coupling to linear PEI. Fmoc cleavage yielded the final block copolymer.

generate polyplexes with higher peptide density on their surface, capable of inducing higher transfection efficiency.

Ideal N/P Ratio Determination. For establishing the minimal N/P ratio needed for maximal complexation, polyplexes at different N/P ratios (between 2 and 20) were loaded on 2% agarose gel and subjected to a gel retardation assay. According to the results depicted in Figure 2, N/P = 8 is the ideal ratio for complexation, as this ratio allowed for a minimal DNA escape from the wells in the smallest expense of block copolymers. Further experiments were therefore performed with polyplexes of N/P = 8.

Particle Size Analysis. DLS measurements were used to determine the mean hydrodynamic size of the polyplexes. PEI/DNA particles were much larger than expected, with a mean diameter exceeding 1.2 μm. This value is not an accurate representation of the size of a single PEI/DNA polyplex, but rather an indication of the aggregation process of PEI/DNA polyplexes in high ionic strength medium (150 mM), as reported in many previous works.[25,26] PEG-*b*-PEI/DNA polyplexes demonstrated a much smaller hydrodynamic size, with a mean diameter of 109.28 ± 5.55 nm. These results are in accordance with previous reports in the literature indicating the

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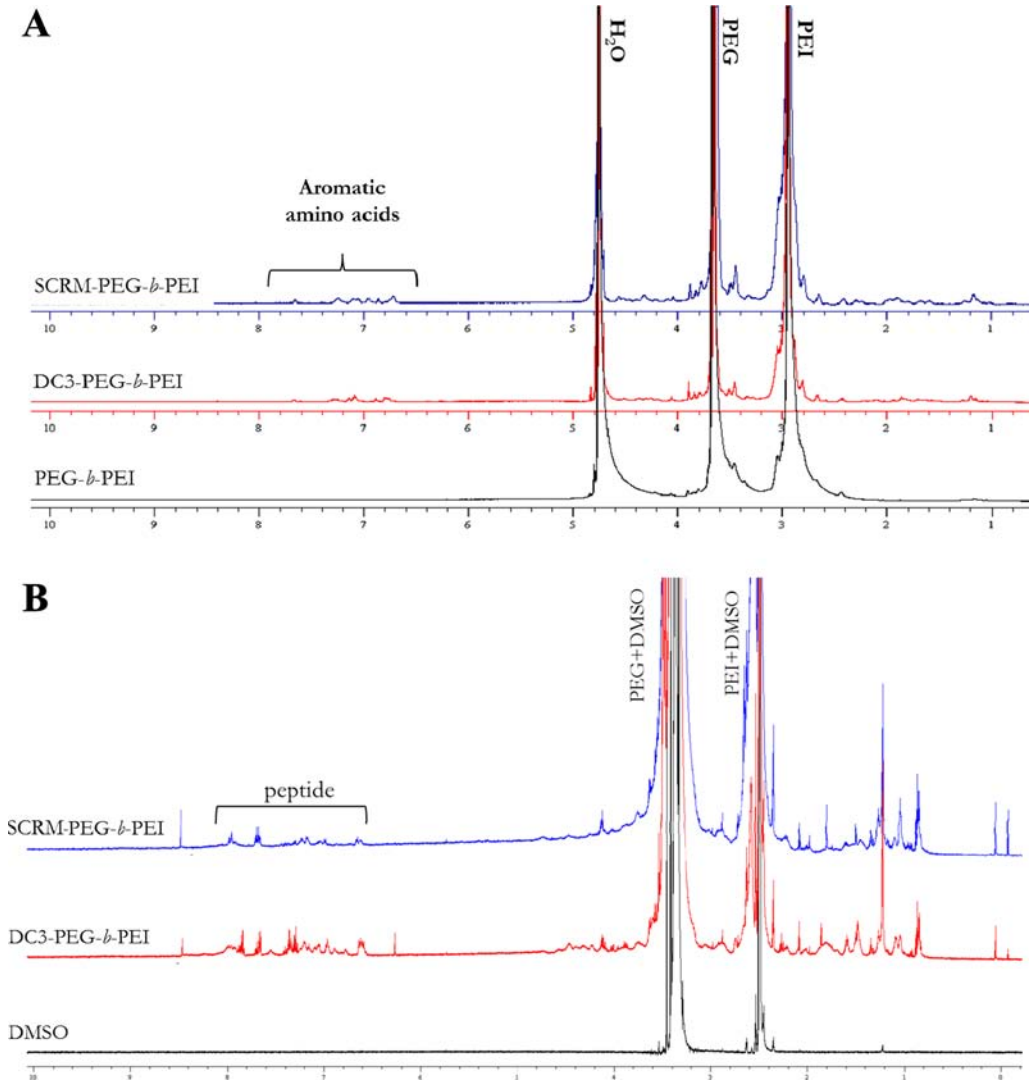

Figure 1. (A) ^{1}H NMR spectra of block copolymers that were synthesized by the BP (A) and the MP (B). (**A**) ^{1}H NMR spectra of PEG-*b*-PEI, DC3-PEG-*b*-PEI, and SCRM-PEG-*b*-PEI in D_2O indicating the chemical shifts of PEI (δ2.8−3.1) and PEG (δ3.5−3.7). The presence of the peptide is indicated by the aromatic proton chemical shifts of phenylalanine and tyrosine (δ 6.5−7.5) in DC3-PEG-*b*-PEI and SCRM-PEG-*b*-PEI, but not in the PEG-*b*-PEI conjugate. (**B**) ^{1}H NMR spectra of DC3-PEG-*b*-PEI and SCRM-PEG-*b*-PEI in DMSO confirm the presence of PEI and PEG (combined with the solvent peak) and peptide in the spectra.

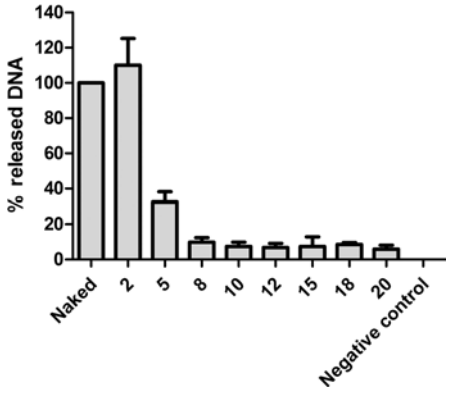

Figure 2. Gel retardation assay of the DC3-PEG-*b*-PEI/DNA polyplexes at different N/P ratios. Quantification of the signal by EZQuant software marked N/P = 8 as the ideal ratio for complexation. Results of 2 independent experiments are presented as % of positive control.

contribution of PEGylation to reduced aggregation and enhanced condensation of PEI/DNA polyplexes.[17,27] The sizes of DC3-PEG-*b*-PEI/DNA and SCRM-PEG-*b*-PEI/DNA synthesized by the BP were comparable to that of PEG-*b*-PEI/DNA, with mean diameters of 122.5 ± 17.25 and 127.6 ± 20.94, respectively. Analysis of DC3-PEG-*b*-PEI/DNA and SCRM-PEG-*b*-PEI/DNA polyplexes synthesized by the MP revealed larger diameters compared to PEG-*b*-PEI/DNA polyplexes (395.48 ± 58.24 and 453.13 ± 39.68 nm, respectively), most probably due to higher density of the hydrophobic peptide that leads to less tightly packed polyplexes, but those were still significantly smaller than PEI/DNA (Table 1) and in the nanometric range. The smaller size of the targeted polyplexes compared to PEI/DNA indicates that conjugation of targeting ligands had limited or no influence on the beneficial effect of PEGylaion. Thus, the particles obtained by complexation of DNA with DC3-targeted block copolymers, present a nanosized diameter suitable for systemic administration.

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Table 1. Physicochemical Characterization of the Polyplexes Synthesized by the Basic and Modified Conjugation Procedure

polyplex	particle size (nm)	surface charge (mV)
PEI/DNA	1226.0 ± 146.4	33.2 ± 1.6
PEG-*b*-PEI/DNA	109.3 ± 5.55	18.7 ± 2.3
BP: DC3-PEG-*b*-PEI/DNA	122.5 ± 17.3	14.7 ± 0.6
BP: SCRM-PEG-*b*-PEI/DNA	127.6 ± 20.9	17.9 ± 1.6
MP: DC3-PEG-*b*-PEI/DNA	395.5 ± 58.24	12.0 ± 2.6
MP: SCRM-PEG-*b*-PEI/DNA	453.1 ± 39.68	14.3 ± 2.3

Surface Charge Measurements. To evaluate the surface charge, polyplexes were subjected to a ζ-potential measurement using a Zetasizer Nano ZS. As expected, PEI/DNA polyplexes demonstrated the highest surface charge (33.2 ± 1.6 mV). The high positive charge observed in the PEI/DNA polyplexes was reduced by approximately half in the PEG-*b*-PEI/DNA and targeted polyplexes (shown in Table 1). A comparable and somewhat lower surface charge was observed for DC3-PEG-*b*-PEI/DNA and SCRM-PEG-*b*-PEI/DNA synthesized via both procedures when compared to PEG-*b*-PEI/DNA. The results suggest that ligand conjugation did not interfere with the desired reduction in particle surface charge induced by PEGylation of the polyplexes.

Evaluation of DC3 Specificity to Dendritic Cells. To confirm the ability of the DC3 peptide to identify cell surface receptor on murine DCs (DC2.4 cell line), DC3 peptide was first attached to a water-soluble and fluorescently labeled *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, to give the DC-targeted copolymer P-(DC3)-FITC, where P represents the HPMA copolymer backbone (Scheme 3). The water-soluble HPMA copolymer is an inert and biocompatible copolymer,[28] and thus can be used to test the specific role of DC3 as targeting ligand. The binding of P-(DC3)-FITC and the control copolymer with a scrambled DC3 sequence P-(SCRM)-FITC to DC2.4 and nondendritic endothelial (cEND) cells was analyzed by flow-cytometry after 4 h at 37 °C (Figure 3). We found that P-(DC3)-FITC can bind significantly and specifically to DC2.4 cells (92.32 ± 4.21% FITC-labeled cells), but not to control cEND cells (4.73 ± 3.58 labeled cells). The binding of the control P-(SCRM)-FITC copolymers to DC2.4 cells was insignificant (5.83 ± 3.14% FITC-labeled cells). The results, illustrated in Figure 3, confirm the specificity of DC3 to DCs as reported by Curiel et al.,[22] and

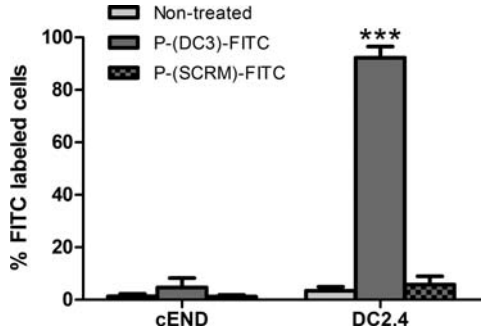


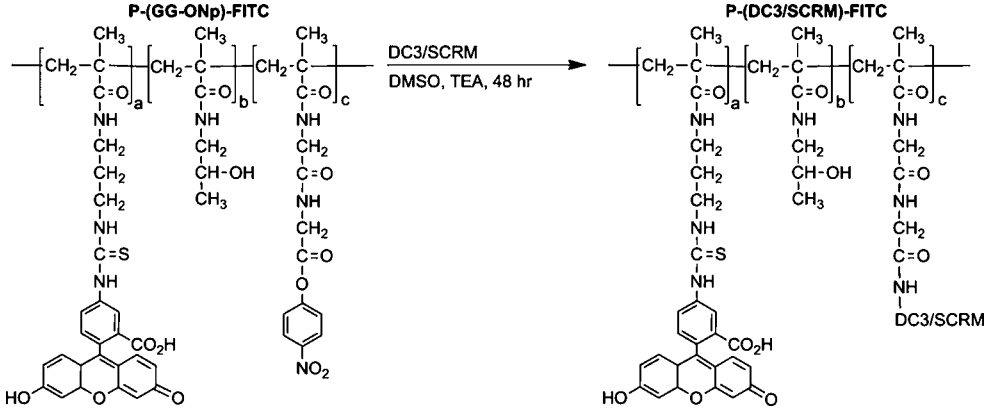
Figure 3. FACS analysis of DC2.4 and cEND cells treated with P-(DC3)-FITC or P-(SCRM)-FITC (50 μg/mL) for 4 h at 37 °C. Values are given as mean ± SD of 3 independent experiments in duplicates. ***$p < 0.001$.

suggest that DC3 may serve as an efficient and specific targeting ligand to DCs.

Cytotoxicity Analysis. The cytotoxicity of the different polyplexes was studied following their growth inhibitory activity against DC2.4 cells 48 h after transfection. The results indicated a relatively high cytotoxicity of PEI/DNA polyplexes (64.91 ± 7.93% viability in PEI/DNA transfected cells), which was in agreement with previous reports in the literature.[13] This cytoxicity is attributed to the positive charge of PEI which cause nonspecific adsorption to cells via electrostatic interactions with the negatively charged cell membrane. The cytotoxicity is thus expected to be reduced in the PEGylated polyplexes, where the hydrophilic PEG was designed and proved by ζ-potential analysis to reduce the positive surface charge. Indeed, all PEGylated polyplexes, targeted and nontargeted, demonstrated reduced cytotoxicity, with 85−90% viability in cells transfected with PEGylated polyplexes (Figure 4). These results suggest that the conjugation of the peptides to the PEG-*b*-PEI copolymers did not interfere with the shielding effect of PEG on PEI/DNA polyplexes, and that the targeted polyplexes possess a very low cytotoxicity, and are likely to be safe for use as gene carriers in vivo.

Serum Stability Evaluation. To evaluate the ability of DC3-PEG-*b*-PEI to protect DNA against the degradation in serum, naked DNA and the DC3-PEG-*b*-PEI/DNA polyplexes were incubated in the presence of 50% fresh human serum for 24 h under shaking at 37 °C. Significant degradation of naked DNA was observed already after 30 minutes of incubation as

Scheme 3. Synthesis of the DC-Targeted HPMA Copolymers, P-(DC3)-FITC, and Control P-(SCRM)-FITC with a Scrambled DC3 Peptide Sequence



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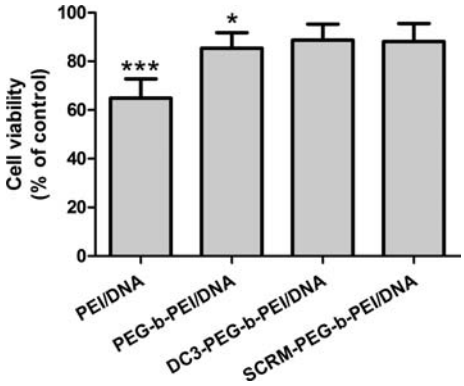

Figure 4. Cytotoxicity of the polyplexes towards DC2.4 cells after 48 h of incubation with PEI/peGFP, PEG-*b*-PEI/peGFP, DC3-PEG-*b*-PEI/peGFP or SCRM-PEG-*b*-PEI/peGFP at N/P = 8. Block copolymers were synthesized by the MP and then complexed with DNA. Cell viability was determined by the MTT assay, relative to non-treated cells. Values are given as mean ± SD of 2 independent experiments in triplicates. $*p < 0.05$, $***p < 0.001$.

shown in Figure 5. Under these conditions, DNA in the polyplexes remained in the loading well for 24 h of incubation,

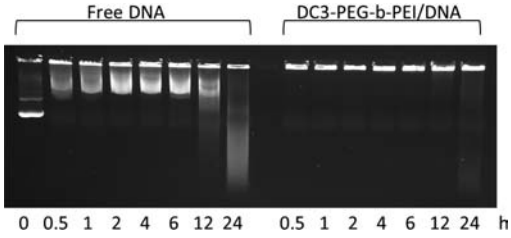

Figure 5. DNA stability of free DNA and DC3-PEG-*b*-PEI/DNA polyplexes. Polyplexes were prepared at N/P ratio of 8, human serum was added to the free DNA and polyplexes at a final concentration of 50% (v/v), and then samples were incubated for 24 h at 37 °C. Protective effects of DC3-PEG-*b*-PEI/DNA against DNA degradation in serum are evident in gel electrophoresis.

suggesting that the block copolymer can protect DNA from degradation in serum under the test conditions.

Transfection Efficiency and Cell Specificity of the DC3-PEG-*b*-PEI/DNA Polyplexes. To assess the transfection efficiency of DC3-targeted polyplexes, targeted and control polyplexes were prepared with either firefly luciferase or GFP-encoding plasmids (pCMVLuc or peGFP, respectively), and used to transfect DC2.4 cells. Total luciferase expression and the percentage of GFP transfected cells were evaluated by luminescence measurements and FACS analysis, respectively, to determine the gene expression induced by the polyplexes as an indication for their transfection efficiency. PEI/DNA polyplexes were used as positive control, as their highly positive surface charge promotes nonspecific adsorption to cells and thus high but nonspecific transfection efficiency. PEG-*b*-PEI/DNA polyplexes were used as negative control, as the hydrophilic PEG shielding, in the absence of an active targeting, has been previously shown to dramatically reduce transfection efficiency. SCRM-PEG-*b*-PEI/DNA polyplexes were used as control for cell-type specificity of the DC3-targeted polyplexes. Finally, the transfection efficiency of the DC3-targeted polyplexes with high peptide loading was also evaluated in endothelial cEND cells to test cell-type specificity.

Transfection efficiency of the DC3-PEG-*b*-PEI/DNA polyplexes synthesized by the BP was first evaluated by luciferase assay. Transfection efficiency was highest for PEI/pCMVLuc polyplexes and 2 orders of magnitude lower for PEG-*b*-PEI/pCMVLuc polyplexes. The transfection efficiency was restored, to a limited extent by DC3-PEG-*b*-PEI/pCMVLuc, but not by SCRM-PEG-*b*-PEI/pCMVLuc polyplexes (Figure 6A). However, transfection rates of DC3-PEG-*b*-PEI/pCMVLUC polyplexes were lower than expected and not comparable to those of the positive control PEI/pCMVLuc. To further explore the transfection efficiency of these polyplexes, the complementary FACS analysis was performed on DC2.4 cells transfected with either DC3-PEG-*b*-PEI/peGFP polyplexes or PEI/peGFP polyplexes. The percentage of transfected cells attained support the luciferase assay results indicating low transfection efficiency of the DC3-PEG-*b*-PEI/peGFP polyplexes (Figure 6B). With the results of those two assays combined, it can be concluded that DC3-PEG-*b*-PEI synthesized by the BP is a nonefficient gene carrier to DCs.

Luciferase assay performed on DC2.4 cells transfected with DC3-PEG-*b*-PEI/pCMVLuc that was synthesized by the MP showed comparable transfection efficiency rates for the positive control PEI/pCMVLuc (Figure 7A). The negative controls PEG-*b*-PEI/pCMVLuc and SCRM-PEG-*b*-PEI/pCMVLuc, however, showed 1−3 orders of magnitude lower transfection efficiency. To further explore the transfection efficiency of

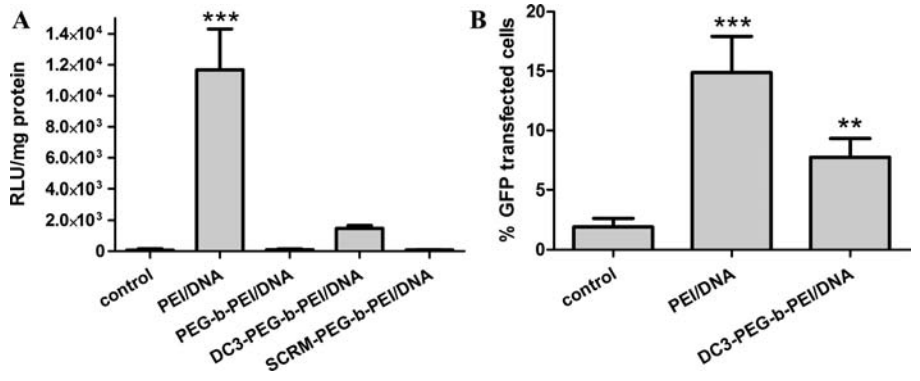

Figure 6. Transfection efficiency of polyplexes synthesized by the basic conjugation procedure (BP) in DC2.4 cells after 24 h of incubation. **A**. PEI, PEG-*b*-PEI DC3-PEG-*b*-PEI, and SCRM-PEG-*b*-PEI were self-assembled with pCMVLuc and the transfection efficiency was determined by luciferase assay. **B**. PEI and DC3-PEG-*b*-PEI were self-assembled with peGFP, and the transfection efficiency was determined by flow cytometry. Values are given as mean ± SD of 2 independent experiments in duplicates. $**p < 0.01$, $***p < 0.001$.

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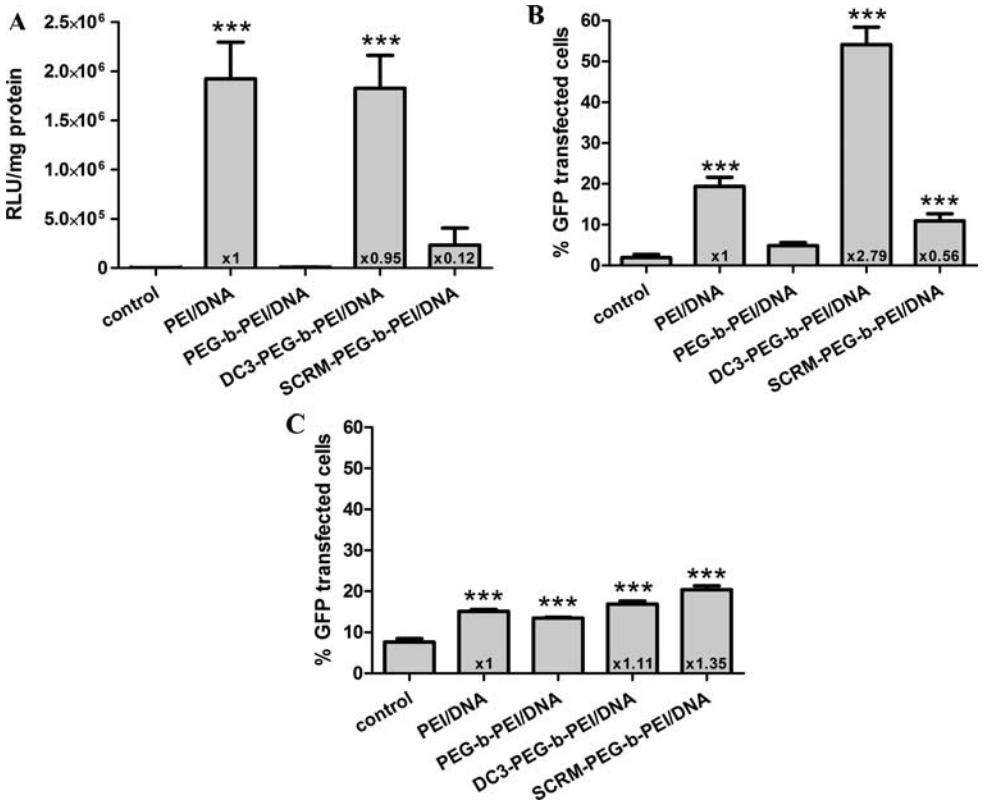

Figure 7. Transfection efficiency of polyplexes synthesized by the modified conjugation procedure (MP) in DC2.4 (A, B) and cEND (C) cells after 24 h of incubation. **A**. PEI, PEG-*b*-PEI, DC3-PEG-*b*-PEI, and SCRM-PEG-*b*-PEI were self-assembled with pCMVLuc and the transfection efficiency was determined by luciferase assay. **B** and **C**. PEI, PEG-*b*-PEI, DC3-PEG-*b*-PEI, and SCRM-PEG-*b*-PEI were self-assembled with peGFP, and the transfection efficiency in DC2.4 (B) or cEND (C) cells was determined by flow cytometry. Results are given as mean ± SD of 4 independent experiments in triplicates. ***$p < 0.001$.

DC3-targeted polyplexes, FACS analysis was performed on DC2.4 cells transfected with DC3-PEG-*b*-PEI/peGFP or control polyplexes. The DC3-PEG-*b*-PEI/peGFP polyplexes demonstrated almost 3- and 5-fold higher transfection efficiency compared to the PEI/peGFP and SCRM-PEG-*b*-PEI/DNA, respectively (Figure 7B). The percent of DC2.4 cells transfected with peGFP was above 50%. SCRM-PEG-*b*-PEI/peGFP and PEG-*b*-PEI/peGFP demonstrated 2- and 4-fold lower transfection efficiency compared to PEI/peGFP, respectively. The transfection efficiency of the targeted DC3–PEG-PEI/peGFP polyplexes with high peptide loading in endothelial cells (cEND) was low and comparable to PEI/peGFP polyplexes, as indicated by FACS analysis (Figure 7C). These results indicate the DC3-PEG-*b*-PEI/DNA polyplexes synthesized by the MP are very effective in transfecting DCs, and exhibit high degree of cell specificity.

■ DISCUSSION

Despite the advances in cancer research and treatment, relapse rates are still very high, mainly due to minimal residual disease. Immunotherapy, the activation of a patient's own immune system against a tumor he carries, may contribute to decrease relapse rates. Where exogenous anticancer agents have failed to produce a strong and effective response against the tumor and induce its regression, endogenous activity of the immune system is hoped to elicit specific antitumor immunity that would result in tumor regression and elimination. Particularly, DC vaccinations are expected to induce immunological memory that would enable recognition and destruction of residual tumor cells that evaded former treatment, thus avoiding recurrence of the disease.[29] Previous studies employing mouse models indicated that the generation of antitumor immunity requires the presentation of tumor-antigens by DC.[30,31] Two unique properties make DCs the most potent APCs: their ability to present exogenous antigens on MHC-I molecules to prime $CD8^+$ T cells (cross presentation), and their capability to initiate, activate, and modulate the various arms of the immune system in a coordinated manner. In vivo targeting of tumor antigens into DCs was shown to elicit a strong tumor-antigen specific $DC4^+$ and $CD8^+$ immune response.[32]

Various in vitro gene transfer attempts have been described to manipulate DC.[33−36] APC are very resistant to transfection due to the specific mechanisms they use to take up and degrade antigens and pathogen-associated molecular patterns, including DNA, which involves various specific recognition sites including toll-like receptors.[37−39] To date, the most efficient gene delivery systems are viral vectors. Using small amounts of DNA they can induce high transfection efficiency and a stable, long-term gene expression. Unfortunately, viral vectors frequently activate immature DCs.[40−42] In addition, viral vectors possess some serious safety issues including toxicity, immunogenicity, and oncogenicity with numerous clinical trials terminated on those grounds.[5,43,44] Reduced immunostimulatory capacity of mature DCs, which has been suggested to be due to either viral immune dominance or to immune regulation by viral proteins,[45] is another important limitation of viral vectors. An alternative strategy is the use of nonviral vectors,

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although up until now these have been relatively inefficient compared to viral vectors. Yet they are simple, easy to manufacture in large scale and can carry unrestricted plasmid sizes. However, the positive charge of synthetic transfection reagents cause some very serious problems, including adsorption to cells and negatively charged blood components or recognition by the immune system components, resulting in rapid clearance from the circulation and cytotoxicity to nontarget cells.[46,47] For these reasons synthetic transfection reagents are usually too toxic for transfection of DCs in vivo.[34]

We therefore focused our attempts at designing a novel gene carrier that can safely and effectively transfect DCs. PEI was utilized as cationic agent for DNA binding and condensation. PEI can further facilitate an escape from the endosomal compartment,[9] an essential step for efficient gene expression, which significantly limits the transfection efficiency of several other transfection agents. In order to reduce its well-known toxicity and potential immunogenicity PEI was coupled to a PEG block.[17] In a recent publication, we reported the successful delivery of plasmid DNA into mannose receptor (CD206)-positive DCs by mannosylated PEG-*b*-PEI block copolymers, both in vitro and in vivo.[48] In this study, we tested whether modification of the PEG-*b*-PEI block copolymer with DC3 targeting ligand can also be used in order to achieve effective gene expression in DCs and minimize off-target gene delivery in bystander cells.

Prior to in vitro experiments with DC3-targeted polyplexes, we wanted to use a simple and well-characterized system to confirm the specificity of DC3 to DCs. In such a system, structure and material composition will not impose complications and the only variant would be DC3 interaction with the cells. For this purpose, we conjugated DC3 or SCRM to the FITC-labeled HPMA copolymer precursor (P-(GG-ONp)-FITC). This polymer precursor is widely used in our lab and was extensively studied.[49] Binding of the resulting P-(DC3)-FITC and P-(SCRM)-FITC to DCs and control cells was assessed using flow cytometry. DC2.4 cells demonstrated significant binding of the P-(DC3)-FITC but not of the copolymer with a scrambled DC3 sequence, suggesting that the dendritic cell targetability of DC3 is derived from an interaction between the precise sequence of amino acids and a unique structure on DC surface, and not merely from hydrophobic interactions between the peptide and the cell membrane. Insignificant binding of P-(DC3)-FITC by control endothelial cells supports the notion.

DC3-PEG-*b*-PEI block copolymers were obtained by two conjugation procedures. Originally, PEI and PEG were first conjugated and the peptide was then attached to the block copolymer. Block copolymer/DNA polyplexes prepared using the products of this synthesis procedure were subjected to all the assays listed above and exhibited characteristics suitable for a gene carrier (nanoscale size, low surface charge, limited cytotoxicity); nevertheless, they demonstrated low and insignificant transfection efficiency in target cells compared to the positive PEI control (Figure 6A and B). As other possible sources for the low transfection efficiency were eliminated, and given the highly significant and specific binding of the P-(DC3)-FITC copolymer demonstrated above (Figure 3), we speculated that insufficient peptide density on the polyplex surface was the cause for the low transfection efficiency observed. We repeated this conjugation procedure using 3-fold higher access of peptide, but this did not increase the content of peptide on the block copolymer (data not shown). We thus assumed that the large PEI blocks (25 kDa) attached to PEG blocks (3.5 kDa) present a steric hindrance, thus reducing peptide conjugation rate. To investigate this assumption, two changes were made in the basic conjugation protocol: First, Fmoc-cysteine harboring peptides were conjugated to PEG prior to PEI conjugation, which was expected to prevent steric hindrance of PEI to peptide conjugation. The presence of Fmoc on the peptides' terminal amine was critical to the procedure as it blocked possible reactions between the peptide and the NHS, and thus the formation of undesired byproducts. Second, a 3-fold higher access of peptide was used compared to the original basic procedure. BCA protein quantification assay and ^{1}H NMR analysis of block copolymers prepared by the improved synthesis procedure revealed more than 2-fold higher peptide content compared to block copolymers prepared by the original, BP. Thus, we expected to see higher peptide density in the resulting polyplexes, capable of inducing higher transfection efficiency.

Polyplexes prepared by the modified conjugation procedure also demonstrated superiority over PEI as a gene delivery carrier for systemic administration, exhibiting smaller (nanoscale) hydrodynamic sizes (Table 1), lower surface charge (Table 1), limited cytotoxicity (Figure 4). Transfection efficiency experiments were performed by both FACS analysis for GFP expression and Luciferase assay. Those two assays are complementary as one reports how many cells took up the polyplexes and were able to produce a protein, while the other reports the total rate of gene expression, i.e., how much protein was produced by a given sample of cells. The total rate of DNA expression in DCs treated with DC3-targeted polyplexes was comparable to the PEI/DNA positive control, as demonstrated by luciferase assay (Figure 7A). The percentage of DCs that were transfected with peGFP using DC3-targeted polyplexes was above 50%, almost 3-fold higher than the PEI/DNA positive control (Figure 7B). This is especially important since relatively few DCs and relatively low doses of antigen are required to elicit high levels of lymphocyte proliferation and differentiation.[50,51] For some applications ∼50% transfection efficiency may be sufficient, since human DCs transfected at <20% efficiency were able to stimulate in vitro expansion and activation of antigen-specific, autologous CD8^{+} T cells for recall antigens, resulting in their lytic activity on target cells in cancer immunization.[36] Furthermore, DC3-PEG-*b*-PEI/DNA polyplexes demonstrated a high degree of target cell specificity (Figure 7C). By combining the results of all assays, it can be concluded that DC3-PEG-*b*-PEI/DNA polyplexes are excellent candidates for in vivo gene delivery applications because of their low cytotoxicity, good stability, and ability to transfect effectively and selectively DCs but no other nontarget endothelial cells.

■ CONCLUSIONS

The delivery of antigens into DCs bears a tremendous potential for immune modulation, specifically for cancer immunotherapy manipulations. Targeting DCs in vivo with a tumor-antigen encoding plasmid DNA can elicit effective and long-lasting tumor-antigen-specific immunity, with minimal inconvenience to the patient. We have presented here a novel gene delivery system based on a PEG-*b*-PEI that was designed to address the many challenges encountered by gene carriers in vivo. This system exhibited the appropriate physicochemical properties required for an efficient gene delivery in vivo with negligible in vitro cytotoxicity. By improving our basic conjugation

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procedure to meet the requirements for a higher peptide density at the polyplex's surface, we have significantly increased the transfection efficiency of the DC-targeted polyplexes. DC3-PEG-*b*-PEI is, therefore, an efficient gene carrier to DCs, suitable for in vivo delivery. In the future, loading this system with a tumor-specific-antigen encoding DNA in combination with an adjuvant to promote DCs maturation shall be tested for its ability to result in antigen processing and presentation on MHC-I molecules in vivo.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma-Aldrich Ltd. (Rehovot, Israel) unless otherwise mentioned. Cell culture media, supplementations, and trypsin were purchased from Biological Industries (Beit Haemek Ltd., Israel). Fmoc-cysteine harboring DC3 (Fmoc-C-FYP-SYHSTPQRP) and SCRM (Fmoc-C-TSQYPRSPFHPY) were purchased from GL Biochem (Shanghai) Ltd. Linear PEI "Max" Mw 40 000 (equivalent to Mw 25 000 in Free Base Form) was purchased from Polyscience, Inc. (Eppenheim, Germany). MAL-PEG-NHS, 3.5 kDa, was purchased from JenKem Technology USA Inc. (Allen, TX). Dialysis tubing with Mw cutoff of 12 000−14 000 g/mol was purchased from Cellu-Sep T4, Membrane Filtration Products, Inc. (Segiun, TX, USA). Plasmid DNA encoding luciferase and GFP (pCMVLuc and peGFP) were a gift from Prof. Moshe Oren (Weismann Institute of Science, Rehovot, Israel). The Micro BCA protein assay kit was purchased from Thermo Scientific (ORNAT, Rehovot, Israel).

Cell Lines and Culture Media. The dendritic cell line DC2.4 was cultured in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 20 IU penicillin, 20 μg/mL streptomycin, and 50 μM β-mercaptoethanol. The brain microvascular endothelial cell line cEND was cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU penicillin, and 100 μg/mL streptomycin. Both cell lines were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air, and passaged every 2−3 days.

Peptide Synthesis and Characterization. The targeting peptide DC3 (C-FYPSYHSTPQRP)[22] and a scrambled control peptide designated SCRM (C-TSQYPRSPFHPY) were synthesized by Solid Phase Peptide Synthesis on Rink Amide MBHA resin using 9-fluorenylmethoxycarbonyl (Fmoc)-α-amine-protected amino acids. Cysteine was added to the original sequence at the N-terminal as means of conjugating the peptides to the block copolymers. The resin was swelled in *N,N*-dimethylformamide (DMF) and deprotected using 25% piperidine in DMF. Each amino acid was activated by *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and coupled in the presence of *N,N*-diisopropylethylamine (DIPEA) for 45−75 min. Completed peptides were washed twice with dichloromethane (DCM) and cold diethyl ether, and dried in a vacuum desiccator. Cleavage from the resin was obtained by stirring the peptides for 135 min in a cleavage mixture containing 94% trifluoroacetic acid (TFA), 2.5% DDW, 1% triisopropylsilane (TIS), and 2.5% ethanedithiol (EDT). TFA was added to the filtered, resin-free peptide solution, and its volume was reduced to a minimum under a stream of air. The peptides were washed with cold diethyl ether, and centrifuged for 15 min at 3000 rpm, repeatedly, to remove thiol traces. Clean peptides were dried in a vacuum desiccator and characterized by MALDI-TOF mass spectrometry (MS). Mw DC3 and SCRM: calculated, 1581.7 g/mol; measured, 1581.6 and 1581.8 g/mol, respectively.

The Fmoc-cysteine harboring DC3 (Fmoc-C-FYP-SYHSTPQRP) and Fmoc-cysteine harboring SCRM (Fmoc-C-TSQYPRSPFHPY) were similar to the synthesized DC3 and SCRM peptides described above, except that their Fmoc on the N-terminus cysteine was not removed at the termination of the synthesis procedure. Peptides were characterized by LC/MS. Mw Fmoc-cysteine protected DC3 and Fmoc-cysteine protected SCRM: calculated, 1804.3 g/mol; measured, 1804.1 g/mol.

Synthesis and Characterization of DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI Block Copolymers. Two different conjugation procedures were employed to yield the final products DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI, the basic and modified procedures, as described below.

Basic Procedure (BP).[52] 3.6 mg (0.001 mmol) of 3.5 kDa PEG with *N*-hydroxysuccinimide (NHS) ester and maleimide (MAL) groups (MAL-PEG-NHS) were dissolved in 50 μL DMSO. Sixteen mg (0.0004 mmol) of 25 kDa linear PEI were dissolved in 1 mL of PBS (pH 7.2) and added to the PEG solution. The reaction was carried out for 1 h at room temperature with shaking. The reaction between PEI's amine and the NHS group yield the intermediate MAL-PEG-*b*-PEI. DC3 or SCRM peptides (10 mg, 0.006 mmol) were dissolved in 200 μL DMSO and then added to MAL-PEG-*b*-PEI in DMSO for 2 h at room temperature with shaking. The reaction between the thiol group on the peptide and the double bond of MAL of PEG-*b*-PEI yielded the final products DC3-PEG-*b*-PEI or SCRM-PEG-*b*-PEI (Scheme 2A). The reaction was terminated by 1 h incubation with access of free cysteine (10 mg) to block unreacted MAL. The final products were dialyzed using 12−14 kDa dialysis tubes (Cellu-Sep T4), lyophilized, and characterized by ^{1}H NMR. Similarly, PEG-*b*-PEI block copolymers (without a targeting peptide) were synthesized as a control.

Modified Procedure MP. Thirty milligrams (0.018 mmol) Fmoc-cysteine harboring DC3 or SCRM peptides were dissolved in 400 μL DMSO and then reacted with 3.6 mg (0.001 mmol) of MAL-PEG-NHS in 50 μL DMSO for 2 h at room temperature with shaking. The presence of Fmoc on the peptides' terminal amine was critical for that step of the procedure as it blocked possible reactions between the peptide and the NHS, and thus the formation of undesirable byproducts. Sixteen milligrams (0.0004 mmol) of PEI were dissolved in 1 mL PBS (pH 7.2) and added to the solution of DC3-PEG-*b*-NHS or SCRM-PEG-*b*-NHS for 1 h at room temperature with shaking, to give the final Fmoc-DC3-PEG-*b*-PEI or Fmoc-SCRM-PEG-*b*-PEI block copolymers. The reaction was terminated by 1 h incubation with free cysteine (10 mg). Products were dialyzed, lyophilized, and incubated with 25% piperidine in DMF (10 mL) for 2 h with stirring to cleave the Fmoc-protecting group and yield the same final products DC3-PEG-*b*-PEI and SCRM-PEG-*b*-PEI (Scheme 2B). The conjugation products were dialyzed, lyophilized, and characterized by ^{1}H NMR (Figure 1). PEG-*b*-PEI block copolymers were synthesized as a control in a similar manner.

Synthesis of DC3-Bearing HPMA Copolymer Conjugate, P-(DC3)-FITC. The FITC-labeled HPMA copolymer precursor having active ester groups (*p*-nitrophenol, ONp) for peptide attachment (designated as P-(GG-ONp)-FITC, where P represents the HPMA copolymer backbone) was synthesized by random radical precipitation copolymerization in a sealed

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vial in acetone/DMSO mixture at 50 °C for 24 h using AIBN as the initiator, as described.[49] DC3 and SCRM were then conjugated to the P-(GG-ONp)-FITC by a native chemical ligation (Scheme 3). Briefly, the polymer precursor P-(GG-ONp)-FITC (5 mg) was dissolved in a solution of 20% benzylmercaptan in anhydrous DMSO (250 μL). After 15 min the peptide (DC3 or SCRM; 8 mg) was added to the solution and reacted for 30 min at 20 °C while stirring. Thiophenol (50 μL) was then added to the mixture which was continuously stirred for 16 h at 20 °C. MOPS buffer (100 mM, pH 7.4) containing guanidinium chloride (200 μL) was added and the mixture was further stirred for 72 h at 20 °C. The reaction mixture was then diluted with PBS (800 μL), purified twice on PD-10 column, and lyophilized. The weight-average molecular weight (Mw), polydispersity (I), and molar percent side chain loading of FITC and peptides, as obtained for the precursor copolymer P-(GG-ONp)-FITC are shown in Table 2.

Table 2. Characteristics of FITC-Labeled DC3-Bearing HPMA Copolymers

HPMA conjugate	MW (Da)[a]	I[a]	% mol FITC[b]	% mol ONp[c]	% mol peptide[d]
P-(GG-ON_p)-FITC	23 000	1.42	1.8	8.3	-
P-(DC3)-FITC	32 500	1.74	1.8	-	5
P-(SCRM)-FITC	31 810	1.67	1.8	-	4.8

[a]Weight-average molecular weight (Mw) and polydispersity (I) of the copolymers were estimated by size-exclusion chromatography (SEC), using Sephacryl 16/60 S-400 (GE Healthcare) with PBS buffer, pH 7.4, calibrated with fractions of known Mw HPMA homopolymers. [b]FITC content was determined spectrophotometrically by measuring the UV absorbance at 492 nm (ε = 82 000 M^1 cm^{-1}). [c]ONp residue content was determined spectrophotometrically by measuring the UV absorbance at 400 nm, (ε = 17 700 M^{-1} cm^{-1}). [d]Targeting moiety content was estimated by ^{1}H NMR at 500 Hz using the proton chemical shifts of phenylalanine and tyrosine (δ 6.5–7.5, m, 13H) for the calculation.

Complexation of Block Copolymers with DNA and Characterization. Block copolymers and peGFP were separately dissolved in 150 mM NaCl, vortexed, and spun-down. Following the addition of block copolymer solution into the DNA solution, the mixture was vortexed for 15 s, spun down, and incubated for 20 min at room temperature for complex formation. Mean hydrodynamic size of the polyplexes was characterized by dynamic light scattering (DLS, CGS-3 LSE-5004, ALV-Laser Vertriebsgesellschaft m-b.H., Langen, Germany) at a scattering angle of 90° through a 400 μm pinhole at room temperature, and surface charge was determined by ζ-potential measurements (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) in 15 mM NaCl. Measurement duration was set to automatic. Average values of the ζ-potential were calculated with the data from 10 measurements. Hydrodynamic size and surface charge of PEI/DNA, PEG-*b*-PEI/DNA, and SCRM-PEG-*b*-PEI/DNA controls were measured in a similar manner. The number of DC3 peptides per polyplex was determined by the micro BCA Protein Assay Kit.

Ideal N/P Ratio Determination. Ideal N/P ratio for complexation (the ratio of amine groups on the PEI to phosphate groups of the DNA) was determined by gel retardation assay as described earlier.[53] Briefly, polyplexes of different N/P ratios (2, 5, 8, 10, 12, 15, 18, and 20) were prepared as described above, loaded on a 2% agarose gel containing ethidium bromide, and electrophoresed for 45 min at 100 V. Naked peGFP was used as a positive control, and block copolymers without DNA were used as negative control. DNA signal outside the wells, representing noncomplexed DNA, was quantified by EZQuant software (EZQuant-Gel 2.11, EZQuant Biology Software Solutions Ltd., Israel) and presented as % of positive control.

Evaluation of DC3 Specificity to DCs. DC2.4 and control cEND cells were seeded in a 24-well plate (2×10^5 cells/well), grown for 24 h, and incubated with 50 μg/mL P-(DC3)-FITC or P-(SCRM)-FITC for 4 h at 37 °C. Cells were then detached from the wells by trypsinization, washed by centrifugation, resuspended in PBS, and analyzed by a flow-cytometer (Guava Easy Cyte mini, Guava Technologies), excitation at 485 nm and emission at 525 nm.

Cytotoxicity Study. To evaluate polyplex cytotoxicity to the cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay was performed according to standard protocols. Briefly, DC2.4 cells were seeded in a 96-well plate (1×10^4 cells/well), allowed to grow for 24 h (37 °C, 5% CO_2), and then transfected with DC3-PEG-*b*-PEI/DNA polyplexes, prepared as described above. PEI/DNA, PEG-*b*-PEI/DNA, and SCRM-PEG-*b*-PEI/DNA polyplexes were used as controls. Following 48 h of incubation, the medium was discarded, and MTT solution (5 mg/mL MTT in PBS) was added into each well to a final concentration of 0.5 mg/mL. Following 4 h incubation, the medium was removed, and 200 μL of DMSO were added to dissolve formazan crystals formed by viable cells. Absorbance at 570 nm was measured by a microplate reader (Infinite M200, TECAN, Switzerland). Cell viability is presented as

$$\frac{\text{Absorbance of transfected cells} \times 100}{\text{Absorbance of nontreated cells}}$$

Serum Stability Test. Serum stability of naked DNA in aqueous solution versus polyplex preparations was characterized using 0.5% agarose gel. Briefly, samples of 12 μg naked DNA or encapsulated DNA in polyplexes were mixed in a 1:1 ratio with fresh human serum (Sigma-Aldrich, St. Louis, MO) to give 50% serum concentration and 6 μg DNA concentration. Samples were then incubated at 37 °C with shaking (175 rpm) for 24 h. 10 μL of aliquots from different incubation times (0, 0.5, 1, 2, 4, 6, 12, 24 h) of each sample were collected and frozen at −80 °C until assayed. Samples were then mixed with 6× sample buffer and loaded onto 0.5% agarose gel containing 0.8 mg/mL ethidium bromide to visualize intact DNA. The gel conditions were performed so that naked DNA would enter the gel and run as a clearly visible band when stained with ethidium bromide. The DNA bands were visualized under a UV transilluminator.

Transfection Efficiency Studies. For transfection experiments 1 or 2 μg DNA were used per well in a 24-well plate. DNA and the appropriate amounts of block copolymers were separately dissolved in 50 μL of 150 mM NaCl, mixed, incubated for 20 min, and the resulting 100 μL complex solution was added to the cells, to a final volume of 1 mL, and a final concentration of 1 or 2 μg DNA/mL. Two complementary assays—Luciferase assay and GFP expression—were employed to evaluate the transfection efficiency of the DC3-targeted polyplexes.

Luciferase Assay. DC2.4 cells were seeded in a 24-well plate (1×10^5 cells/well) and allowed to grow for 24 h (37 °C, 5% CO_2). DC3-PEG-*b*-PEI block copolymers were complexed with

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a luciferase encoding plasmid pCMVLuc as described above, and the resulting DC3-PEG-*b*-PEI/pCMVLuc polyplexes were used to transfect the cells. PEI/pCMVLuc, PEG-*b*-PEI/pCMVLuc, and SCRM-PEG-*b*-PEI/pCMVLuc controls were tested likewise. Following 24 h incubation with polyplexes, cells were washed twice with PBS and lysed by 15 min incubation with Cell Culture Lysis Buffer (Promega), with shaking. Luciferase gene expression was then monitored using the Luciferase 1000 Assay System (Promega), and relative luminescent units (RLU) were measured by a microplate reader (Infinite M200, TECAN, Switzerland). Protein content in the cells was determined by Bradford assay using the Protein Assay Dye Reagent (Bio-Rad) to normalize gene expression measurements. Normalized transfection efficiency values are presented as RLU/mg protein.

GFP Expression. DC2.4 cells were seeded and allowed to grow as described for the luciferase assay. Block copolymers were complexed with the GFP-encoding plasmid peGFP, and the resulting targeted (DC3-PEG-*b*-PEI/peGFP) and control (PEI/peGFP, PEG-*b*-PEI/peGFP and SCRM-PEG-*b*-PEI/peGFP) polyplexes were used to transfect the cells. Following 24 h incubation, cells were washed, trypsinized, resuspended, and analyzed by flow cytometry (Guava Easy Cyte mini, Guava Technologies). Results are expressed as % GFP transfected cells, excitation at 485 nm and emission at 525 nm.

Statistical Analysis. Statistical calculations were carried out using GraphPad Prism v 5 software (GraphPad Software, San Diego, CA). To identify statistically significant differences, one-way ANOVA with Bonferroni's post-test analysis was performed. Differences were considered significant if $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) and are marked accordingly in the figures.

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Notes

The authors declare no competing financial interest.

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