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Engineered Glycated Amino Dendritic Polymers as Specific Nonviral Gene Delivery Vectors Targeting the Receptor for Advanced Glycation End Products

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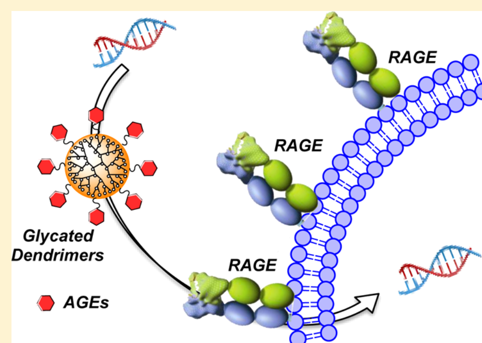
Supporting Information

ABSTRACT: The receptor for advanced glycation end products (RAGE) is involved in diabetes or angiogenesis in tumors. Under pathological conditions, RAGE is overexpressed and upon ligand binding and internalization stimulates signaling pathways that promote cell proliferation. In this work, amino dendritic polymers PEI 25 kDa and alkylated derivatives of PAMAM-G2 were engineered by the nonenzymatic Maillard glycation reaction to generate novel AGE-containing gene delivery vectors targeting the RAGE. The glycated dendritic polymers were easily prepared and retained the capability to bind and protect DNA from endonucleases. Furthermore, while glycation decreased the transfection efficiency of the dendriplexes in CHO-k1 cells which do not express RAGE, glycated dendriplexes acted as efficient transfection reagents in CHO-k1 cells which stably express recombinant RAGE. In addition, preincubation with BSA-AGEs, a natural ligand of the RAGE, or dansyl cadaverine, an inhibitor of the RAGE internalization, blocked transfection, confirming their specificity toward RAGE. The results were confirmed in NRK and RAW264.7 cell lines, which naturally express the receptor. The glycated compounds retain their transfection efficiency in the presence of serum and promote *in vivo* transfection in a mouse model. Accordingly, RAGE is a suitable molecular target for the development of site-directed engineered glycated nonviral gene vectors.



INTRODUCTION

Nonviral vector systems[1,2] are an attractive alternative to viral carriers (retroviruses and adenoviruses) for gene delivery due to their low immunogenicity, carcinogenicity, cytotoxicity, and easy scale-up production.[3] Conventional nonviral vectors are usually based on polycations. These compounds present primary positively charged amino groups and include diverse cationic lipids and polymers which interact with DNA to assist cell uptake by binding or enveloping DNA through charge interactions.[4−6] However, although these vectors work reasonably well *in vitro* and *in vivo*, they lack transfection cell specificity.[7,8] To overcome this problem, a common practice is the conjugation of the polycationic carriers with ligands to provide a receptor-mediated site-directed delivery. A classic example of this so-called "receptor-mediated gene delivery" technology[9,10] is the use of carbohydrates to decorate transfection agents, i.e., galactose to target *in vivo* hepatic cells or mannose to transfect dendritic cells, a transfection strategy termed "glycofection",[11,12] which requires the synthesis of reactive sugar-containing molecules and their covalent attachment to the surface of the transfection agents by means of diverse and eventually sophisticated synthetic glycosylating strategies.

The receptor for advanced glycation end products (RAGE)[13] is a member of the immunoglobulin superfamily of cell surface molecules able to interact with ligands as advanced glycation end products (AGEs), involved in diabetes, or S100/calgranulins, amphoterin, and amyloid fibrils, which play a key role in several pathophysiological processes such as immune/inflammatory disorders, Alzheimer's disease, and tumorigenesis.[14−16]

The AGEs comprise a complex and heterogeneous group of compounds partly responsible for long-term diabetic syndrome.[17−19] They are generated nonenzymatically by the reaction of high concentrations of reducing sugars, such as glucose, with the amino groups present in proteins, lipids, and nucleic acids through a series of reactions, forming Schiff bases and Amadori products. The binding of the AGEs to RAGE induces internalization of the receptor with the concomitant

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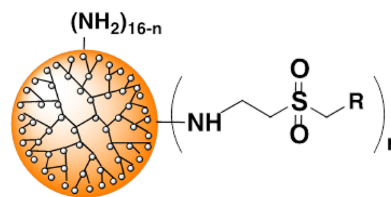
activation of signal transduction pathways, including Ras, Rac/Cdc42 and Jak/Stat pathways.[20] On the basis of these observations, RAGE can be considered as an attractive target for specific gene delivery, having scarcely been explored for the development of new treatments for a diverse group of seemingly unrelated disorders.[21−24] In this general framework and for the first time, we have explored the flexibility of receptor-mediated transfection targeting the RAGE. To this end, we hypothesized that engineering amino dendritic polymers of already proven transfection efficiency by means of their nonenzymatical glycation would constitute a universal and simple synthetic strategy for the preparation of such novel vectors. Derivatized PAMAM-G2 dendrimers[25] and hyperbranched polyethylenimine (PEI)[26] were selected as suitable amino dendritic scaffolds. This choice was based on our previous study on the functionalization of PAMAM-G2 with alkyl sulfonyl chains, which has been shown to be a valuable strategy for the preparation of efficient transfection reagents.[27] Additionally, branched 25k PEI was selected as an efficient transfection reagent due to its higher buffer capacity, called the "proton sponge effect", and its ability to bind and protect DNA from degradation.[28,29] The nonenzymatic glycation of these dendrimers by incubation in the presence of glucose was conceived as a general method for the straightforward preparation of AGE-containing dendrimer-based reagents. The novel vectors were assayed in cell lines that may or may not express a recombinant RAGE,[20] as a model to address their specificity, and were found to be efficient and site-directed nonviral gene delivery vectors for RAGE-containing cells. The results were confirmed in cell lines that naturally express the RAGE. Furthermore, internalization mechanisms, cytotoxicity, and *in vivo* transfection efficiency of these new transfection reagents were fully characterized.

■ RESULTS AND DISCUSSION

Glycation of Amine Dendritic Polymers. The aim of this study was to explore the feasibility of targeting RAGE to develop efficient carrier systems for gene delivery. RAGE is involved in several pathologies such as diabetes, cardiovascular diseases, and tumor progression through enhanced angiogenesis.[18,30,31] While the expression of RAGE takes place at a low level in endothelium, smooth muscle cells, mononuclear phagocytes, neurons, and cardiac myocytes,[32] in pathological processes RAGE expression is increased in response to high circulating AGE levels,[19] and this fact may be exploited in therapy. Thus, the use of a recombinant soluble RAGE to compete with the natural receptor reduces long-term complications in diabetes and antagonists of S100 calgranulins, ligands of the RAGE, prevent tumor growth and metastasis.[33] However, to the best of our knowledge the use of the RAGE for specific gene delivery has yet to be described.

With this aim, amine dendritic polymers were envisaged as suitable scaffolds. In spite of the poor transfection properties usually exhibited by native PAMAM-G2 dendriplexes, amphiphilic PAMAM-G2 dendrimers, namely, PAMAM-G2-a,b (Figure 1), bearing peripheral alkyl sulfone hydrophobic tails were selected on the basis of the synergistic benefits obtained by the vinyl sulfone-based chemistry branching with alkyl chains of different lengths.[27] These functionalized PAMAMs behave as vectors with improved transfection properties when compared to unmodified PAMAM-G2 and commercial transfection reagents such as Lipofectamine 2000 (LP).[27] Besides PAMAM, PEI 25 kDa was also chosen as a suitable aminated



PAMAM-G2-a R = $(CH_2)_{16}Me$
PAMAM-G2-b R = $CH_2O(CH_2)_2NHC(O)(CH_2)_7CH{=}CH(CH_2)_7Me$

Figure 1. Schematic diagram illustrating the chemical structure of the PAMAM-G2 derivatives used for the preparation of the glycated dendrimers.

scaffold given its prominent position among polycationic polymers in the context of nonviral gene carriers.

Glycotargeted versions of PAMAM and PEI have been previously studied as glycotransporters in gene delivery.[34,35] However, despite the versatility and robustness of the well-established synthetic approaches used to generate complex glycoarchitectures, the chemical manipulation of saccharides and their prior transformation into reactive glycosylating reagents is a requisite. *In vitro* glycation of proteins was a source of inspiration that led us to hypothesize that the Maillard reaction may be an appealing alternative that allows a one-pot synthetic strategy. Glycations were designed to attain a controlled partial glycation (50% for PAMAM-G2 derivatives and 10%, 25%, and 50% for PEI) using limiting amounts of glucose to obtain glycated vectors with remaining free primary amine groups for DNA complexation. As expected, the incubation at 37 °C of PAMAM-G2-a,b derivatives or PEI 25K yielded a consumption of glucose (Figure 2A) that was completed after 40 days of reaction. From the glucose consumption data, percentages of modified amino groups of 41% (for gPAMAM-G2-a,b derivatives), and 10%, 25%, and 46% (for gPEI10, gPEI25, and gPEI50), respectively, were obtained. Moreover, these results were confirmed for the glycated PEI (gPEI) derivatives by the assay of free amino groups using the trinitrobenzenesulfonic acid method.[36] In this case the results indicated 11%, 30%, and 51% modification of the amino groups in the PEI derivatives. The resulting compounds showed fluorescent spectra (Figure 2B,C) similar to those reported for glycated proteins.[37]

Glycation of PAMAM-G2, G4, and poly(L-lysine) in the presence of BSA has been previously described as chemical competitors *in vivo* to prevent serum protein glycation in a model of diabetes.[38] However, the efficacy of this technique to prevent long-term complications associated with diabetes is in doubt[39] and the use of glycated compounds to target the RAGE has not been explored. Therefore, the capability of the glycated derivatives described herein to interact with the RAGE was first analyzed in a cell system. CHO-k1 cells were stably transfected with a plasmid pRAGE coding for the native rat RAGE under the control of the CMV promoter. Using this transfected cell line (CHO-RAGE), the effects of incubation with BSA-AGEs, PEI, or gPEI derivatives on the phosphorylation status of ERK1/2 were assayed as a measure of the RAGE mediated signaling.[20] Our results show (Figure 2D) that incubation with gPEI10 and gPEI25 resulted in a large increase in ERK1/2 phosphorylation compared with the natural ligand, BSA-AGEs. Interestingly, the higher degree of glycation in gPEI50 probably prevented recognition by RAGE and, consequently, the increase in phosphorylation of the downstream kinases.

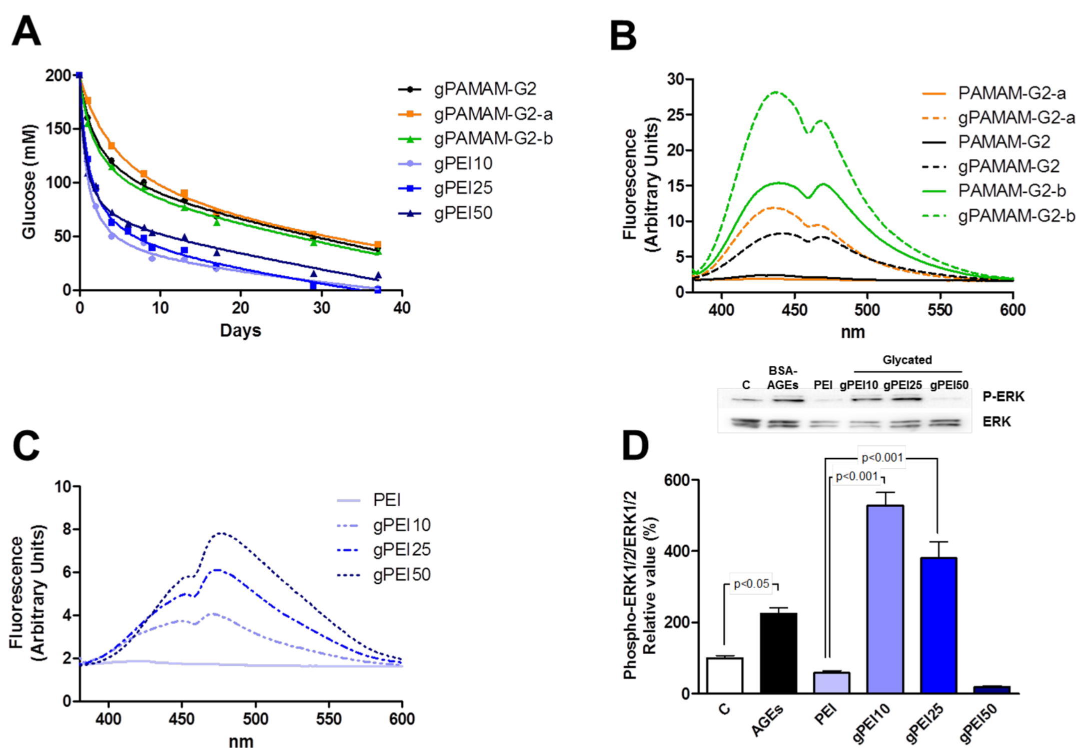


Figure 2. Nonenzymatic glycation of PAMAM-G2 and PEI derivatives. (A) Glycation time course: Glycation reactions were set as described in the Material and Methods section, and at the indicated time points, aliquots were assayed for free glucose concentration using a GOD-POD enzymatic method. Fluorescence emission spectra of glycated PAMAM-G2 (B) and PEI derivatives (C): Dendrimers were dissolved in DMSO:H_2O (1:2 ratio) at a concentration corresponding to a N/P ratio of 10 and emission spectra were recorded with a 370 nm excitation wavelength. (D) ERK1/2 activation mediated by glycated PEI derivatives. CHO-k1 cells expressing RAGE were incubated in the presence of BSA-AGEs or glycated PEI derivatives for 60 min. Cells were lysed and phosphorylation status of ERK1/2 was assayed by Western blot. Results are expressed as means ± SEM (n = 4).

Gene Delivery Capabilities of Glycated Amphiphilic PAMAM-G2 and PEI Derivatives. Any compound susceptible to becoming an effective transfection reagent in vitro should be able to bind and compact plasmid DNA (pDNA) and protect it from DNA degradation. Therefore, the glycated compounds were combined with plasmid pEGFP-N3 which encodes for the green fluorescent protein at N/P ratios ranging from 0.5 to 10 and the resulting dendriplexes were evaluated. The agarose gel shift assay showed that glycation of PAMAM-G2 has a moderate effect on DNA binding capacity and that it is negligible for PEI. Both PAMAM-G2 and amphiphilic PAMAM-G2 (i.e., PAMAM-G2a,b) at N/P ratios of 2 yielded an efficient binding, whereas for the glycated counterparts this was 4 (Figure 3). For PEI a 1:1 ratio is effective regardless of the glycation. The protection of pDNA from endonuclease degradation seems to confirm the influence of the scaffold. DNA protection of PEI was diminished by glycation, whereas glycated PAMAM remained as effective as nonglycated PAMAM (Figure 4). At this point it is important to recall that the N/P ratio assayed for the PAMAM scaffold was 2:1, whereas for the PEI scaffold it was 1:1, since higher ratios prevent the efficient dissociation of the DNA from the complexes after the protection assay.

These results led us to conclude that neither of the two scaffolds could be discarded and their ability to transfect CHO-k1 cells, a common standard cell line that does not express RAGE, used for a variety of transfection agents, was evaluated. Transfections in parallel were carried out in the CHO-RAGE cell line that overexpresses RAGE.[20]

Experiments were performed in the absence of serum and N/P ratios from 2.5 to 20 were screened. Values were normalized to control cells transfected with LP, which were assigned 100% efficiency. As shown in Figure 5A, while nonglycated transfection compounds based on PAMAM-G2 and PEI showed transfection efficiency similar to those of the LP at the preferred N/P ratios, the glycated PAMAM-G2 derivatives had significantly lower transfection efficiency in the CHO-k1 cell line. For the PEIs, the transfection was also blocked by glycation. Hence, glycation of the transfection reagents has a negative effect on transfection efficiency on cell lines nonexpressing RAGE. This could be ascribed to the decrease in pDNA binding (Figure 3A) for the glycated PAMAM-G2 derivatives or to the lower capability to protect pDNA from degradation by endonucleases in the case of gPEIs (Figure 4B), albeit negative steric effects of the AGEs ligands in the binding to the plasma membrane of the CHO-k1 cells could be not precluded.

Next, the transfection efficiency of glycated compounds was measured in CHO-RAGE cells (Figure 5C,D). In these cells, nonglycated compounds showed similar transfection efficiency compared with that of nontransfected CHO-k1 cells. Moreover, gPAMAM-G2-a,b and gPEI10 showed increased transfection efficiency in these cells harboring the RAGE receptor at the plasma membrane. Interestingly, gPEI25, which had a lower capability to stimulate RAGE mediated ERK1/2 phosphorylation compared with gPEI10, showed significantly lower transfection efficiency. gPEI50, which failed to stimulate ERK1/2 phosphorylation, was also unable to produce a significant

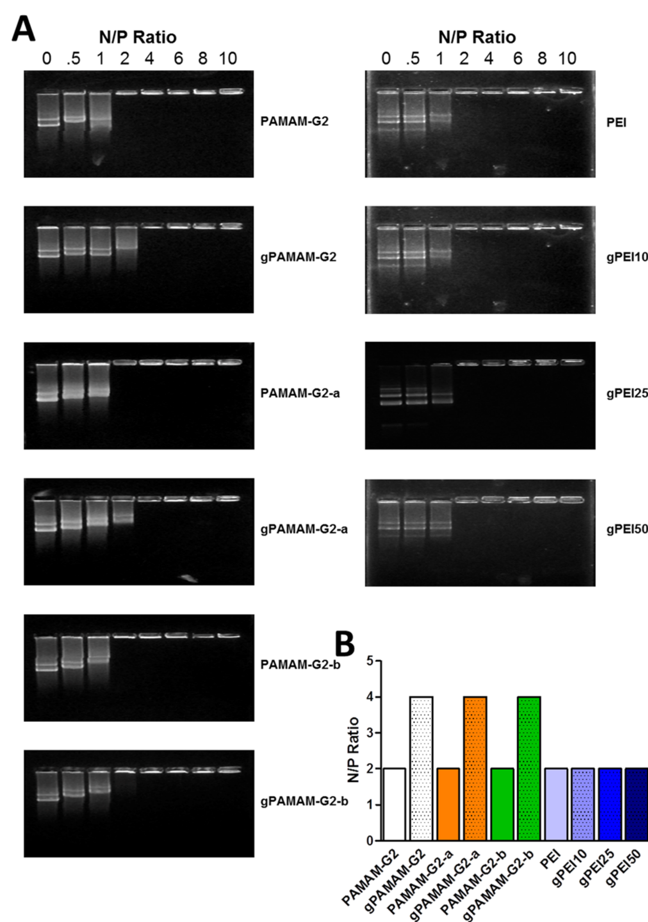


Figure 3. Gel electrophoresis shift assay. (A) Gel shift assays showing PAMAM-G2 or PEI derivatives-pDNA binding at N/P ratios between 0 (pEGFP-N3 only) and 10. Minimum N/P ratio needed to completely inhibit electrophoresis mobility in the shift assay is shown in B.

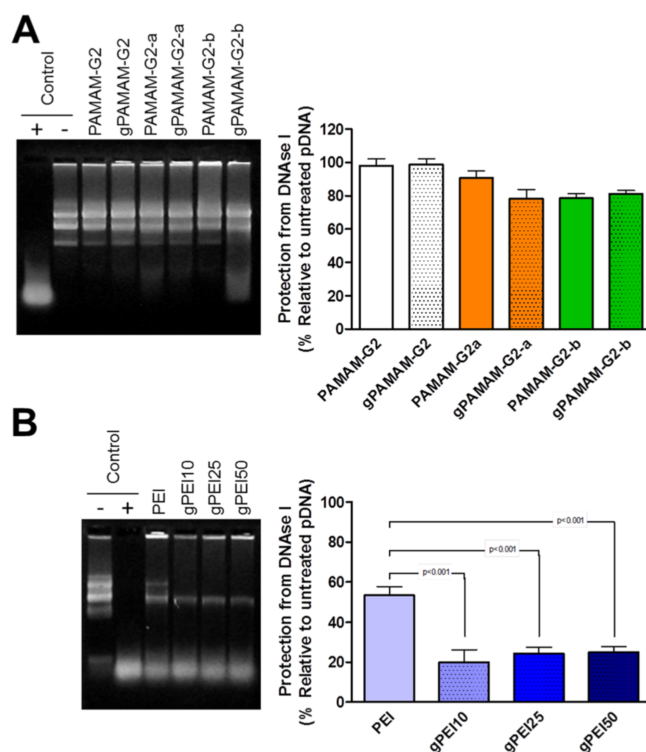


Figure 4. DNase I protection experiments. Representative agarose electrophoresis of samples corresponding to pEGFP-N3 incubated in the absence (−) or presence (+) of DNase I as controls. pEGFP-N3 samples that were complexed with the PAMAM-G2 derivatives at a N/P ratio of 2 (A) or PEI derivatives at a N/P ratio of 1 (B) before the DNase I treatment were run in parallel. Also, quantification of the relative intensity (untreated pEGFP-N3 value equal to 100) of the sum of relaxed and supercoiled electrophoretic plasmid bands corresponding to the pEGFP-N3 samples complexed with PAMAM-G2 or PEI derivatives and treated with DNase I is shown. Results are expressed as means ± SEM (n = 4).

transfection. These results suggest that highly glycated PEI is incapable of a productive interaction with the receptor.

To gain additional insight, the dendriplexes at the N/P ratios which yielded better transfection efficiencies were characterized by dynamic light scattering DLS (Supporting Information Figure 1). In general, glycation produced only a moderate decrease in the value of ζ potential for PEI derivatives and had no effect on PAMAM-G2-based compounds (Supporting Information Figure 1A), confirming that the irreversible glycation of a primary amino group retains a positively charged residue, as in some of the AGEs formed by reaction with Lys residues in proteins.[40] The effect of glycation on the size of the dendriplex is scaffold dependent, remaining basically unaltered for PAMAM-based dendriplexes and decreasing with the degree of glycation for those of PEI (Supporting Information Figure 1B). These data do not support dependence between physical features of the dendriplexes and the biological results with the cell lines, but rather indicate a specific interaction with the RAGE.

Additional confirmation of the RAGE-mediated uptake of the glycated transfection reagents was obtained by a competitive assay. CHO-RAGE cells were preincubated with an excess of BSA-AGEs, a ligand of RAGE, and then transfected with LP, PAMAM-G2, or PEI derivatives. The results (Figure 6) show that while preincubation with BSA-AGEs had no effect on the transfection efficiency of the LP and nonglycated reagents, the addition of AGEs blocked the RAGE-mediated transfection for the gPAMAM-G2-a,b and gPEI10 vectors, confirming their specificity toward RAGE.

Cellular Uptake and Internalization Mechanism Studies for Glycated PAMAM-G2 and PEI Derivatives. Nonviral gene complexes can enter mammalian cells through two different endocytic pathways, clathrin-dependent and caveolae-mediated endocytosis, as a function of the cell type, the nature of the carrier, and their particle size.[41] To investigate the endocytosis pathway of the glycated vectors, chlorpromazine and genistein, which inhibit clathrin-dependent and caveolae-mediated endocytosis, respectively,[42] were used. These inhibitors were not cytotoxic in the CHO-k1 and CHO-RAGE cells (Supporting Information Figure 2). Also, the specificity of these endocytosis inhibitors was confirmed using markers of clathrin-dependent and -independent pathways,[42] as shown in Supporting Information Figure 2. In addition, dansyl cadaverine, a specific inhibitor of receptor internalization,[43] was also added.

The assays revealed (Figure 7A) that in CHO-k1 cells the transfection levels of nonglycated transfection reagents based on PAMAM-G2 or PEI were significantly inhibited in the presence of genistein and increased in the presence of chlorpromazine. These results indicate that the caveolae-mediated pathway is the preferred endocytic pathway for

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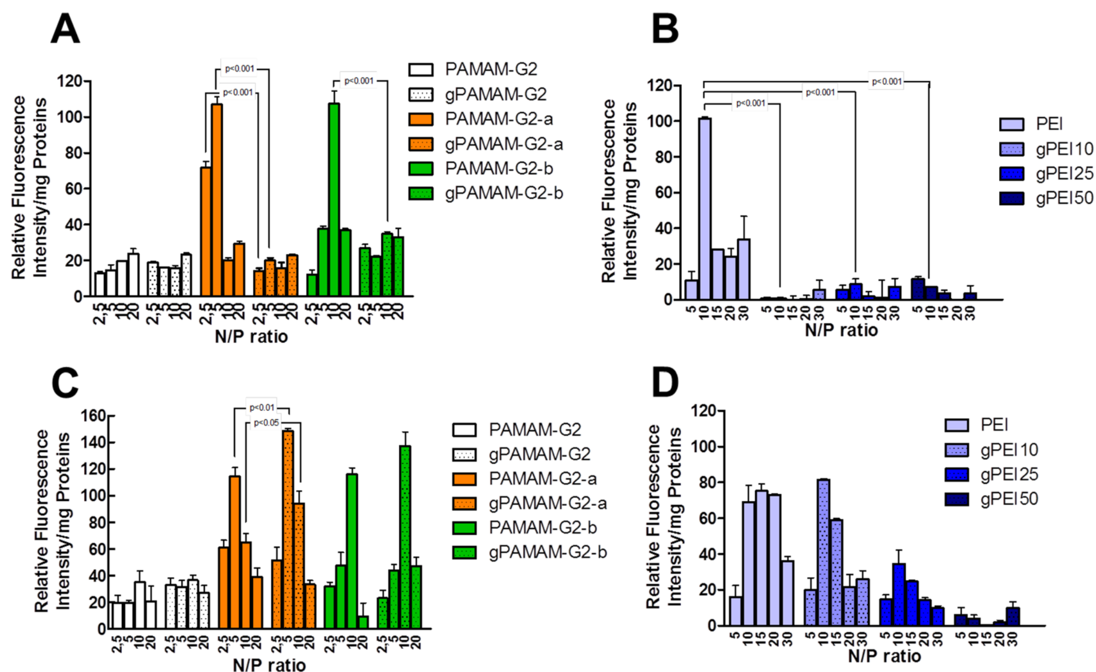

Figure 5. In vitro gene transfection efficiency of the PAMAM-G2 and PEI based dendrimer complexes in CHO-k1 and CHO-RAGE cells. CHO-k1 (A and B) or CHO-RAGE cells (C and D) were transfected with dendriplexes using pEGFP-N3. For each condition, DNA was mixed with the PAMAM-G2 (A and C) or PEI (B and D) derivatives at the indicated N/P ratios. As a positive control, a transfection was performed using LP. The eGFP fluorescence/protein value for the LP transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 8).

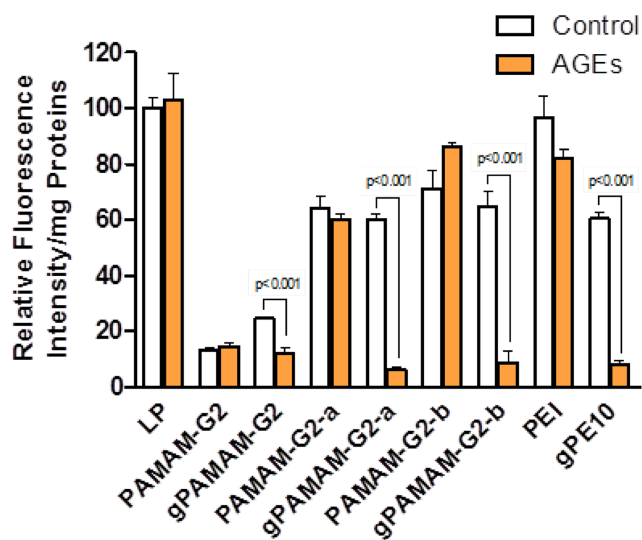

Figure 6. Transfection of CHO-RAGE cells in the presence of BSA-AGEs. RAGE transfected CHO-k1 cells were preincubated with BSA-AGEs (100 μg/mL) for 12 h and were then transfected with the complexes at their most efficient N/P ratio. As control, a transfection was performed using LP. The eGFP fluorescence value for the LP transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 6).

these compounds and are in agreement with our previous finding for the nonglycated PAMAM-G2-based compounds.[27] In addition, it was observed that dansyl cadaverine had no effect on the transfection efficiency of the nonglycated compounds in the CHO-k1 cell line.

Subsequently, the effects of the inhibitors were assayed in the CHO-RAGE cells (Figure 7B). In the case of the nonglycated compounds, the inhibitor profile for chlorpromazine and

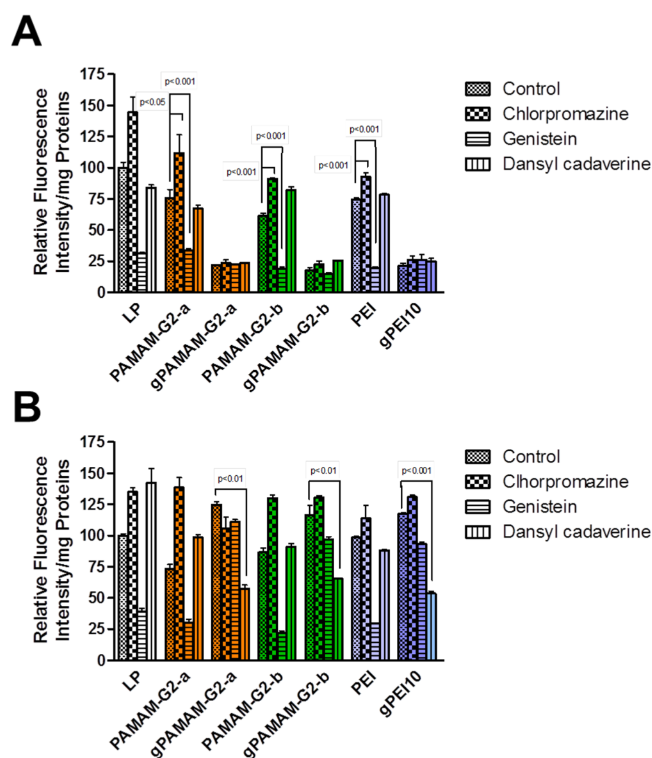

Figure 7. Internalization routes used in the transfection by PAMAM-G2 and PEI derivatives complexes. CHO-k1 (A) or CHO-RAGE (B) cells were pretreated with chlorpromazine (14 μM), genistein (200 μM), or dansyl cadaverine (100 μM) for 30 min before transfection with dendriplexes at their optimal N/P ratio. As control, a transfection was performed using LP. The eGFP fluorescence value for the LP transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 6).

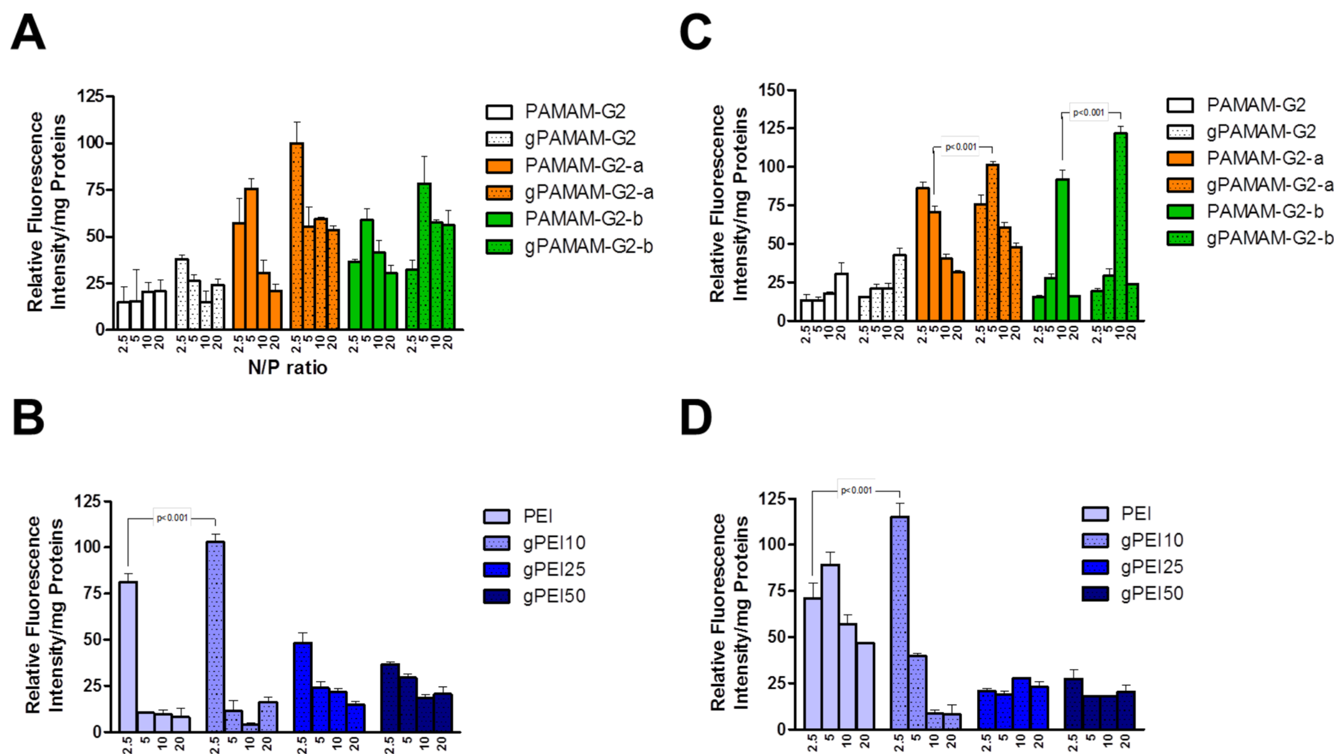

Figure 8. In vitro gene transfection efficiency of the PAMAM-G2 and PEI based dendrimer complexes in NRK and RAW264.7 cell lines. NRK cells (A and B) or RAW264.7 cells (C and D) were transfected with dendriplexes using pEGFP-N3. For each condition, DNA was mixed with the PAMAM-G2 (A and C) or PEI (B and D) derivatives at different N/P ratios. As a positive control, a transfection was performed using LP. The eGFP fluorescence/protein value for the LP transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 8).

genistein remained unchanged, while the transfection mediated by the glycated reagents was significantly decreased upon incubation with dansyl cadaverine. Since we have previously demonstrated that dansyl cadaverine is able to inhibit RAGE internalization[20] and this inhibitor is only able to block transfection for the glycated reagents in the RAGE expressing cells, the results clearly support the entrance of glycated dendritic polymers preferentially via RAGE internalization.

Transfection Assay Using NRK and RAW 264.7 Cell Lines. Although the transfection assays using CHO-k1 cells which may or may not express a recombinant RAGE are a unique setting to establish the specificity of the glycated transfection reagents, we have also evaluated their transfection efficiency in cell lines that naturally express the RAGE.

Normal rat kidney (NRK) and monocyte/macrophage RAW 264.7 cell lines were selected. The kidney is one of the main targets in diabetes for AGE related alterations.[44] In addition, RAW 264.7 cells are attractive because the activation of monocytes to macrophages by AGEs is an indication of the development of atherosclerosis in diabetes.[45] Furthermore, RAW 264.7 is a difficult cell line to transfect.

Normal rat kidney (NRK) (Figure 8A,B) and the monocyte/macrophage cell line RAW 264.7 (Figure 8C,D) were transfected with the glycated and nonglycated dendritic polymers at N/P ratios from 2.5 to 20. In these cell lines, the nonglycated compounds displayed a transfection efficiency similar to nontransfected CHO-k1 cells (Figure 5A,B), while glycated compounds gPAMAM-G2-a,b and gPEI10 showed higher transfection efficiencies than their nonglycated partners, similar to the values obtained in the CHO-RAGE cell line (Figure 5C,D). Therefore, these results validate the specificity of the glycated transfection reagent targeting the RAGE.

In Vivo Transfection Capabilities of the Glycated Dendrimers. Cytotoxicity of the gene delivery systems is an important parameter that has to be evaluated prior to testing them *in vivo*. Cell viability was studied in CHO-k1 cells at the N/P ratio yielding the highest transfection efficiency and revealed that LP, with a moderate toxicity (60% cell viability), is more toxic than the compounds prepared from both PAMAM and PEI scaffold and that glycation has a minor effect on the toxicity of compounds based on PAMAM and no effect on those obtained from PEI (Figure 9).

An additional issue to be addressed prior to *in vivo* analyses is the transfection effectiveness of the novel glycated transfection reagents in the presence of serum. Transfection was first assayed in CHO-k1 (Figure 10A) and CHO-RAGE cells in the presence of serum (Figure 10B). In the case of CHO-RAGE cells, the transfection efficiency of nonglycated PAMAM-G2 derivatives was diminished by incubation in the presence of FBS, while the transfection efficiency of gPAMAM-G2-a,b and gPEI10 was not affected by the presence of serum in the incubation media. This result is consistent with the different mechanism of endocytosis analyzed above, the glycated compounds being internalized via RAGE internalization, and is also evidence that these compounds could be used for *in vivo* transfection experiments.

Since PEI has been extensively used for *in vivo* transfection, we assayed *in vivo* the effects of gPEI10 versus the nonglycated compound. Mice were injected with PEI or gPEI dendriplexes containing a luciferase-expressing plasmid at N/P 10 and transfection was detected by quantifying the resulting fluorescence in the whole animal 24 h after the injection (Figure 10C). The analysis of the fluorescence revealed that PEI produced a moderate signal, compatible with the expected

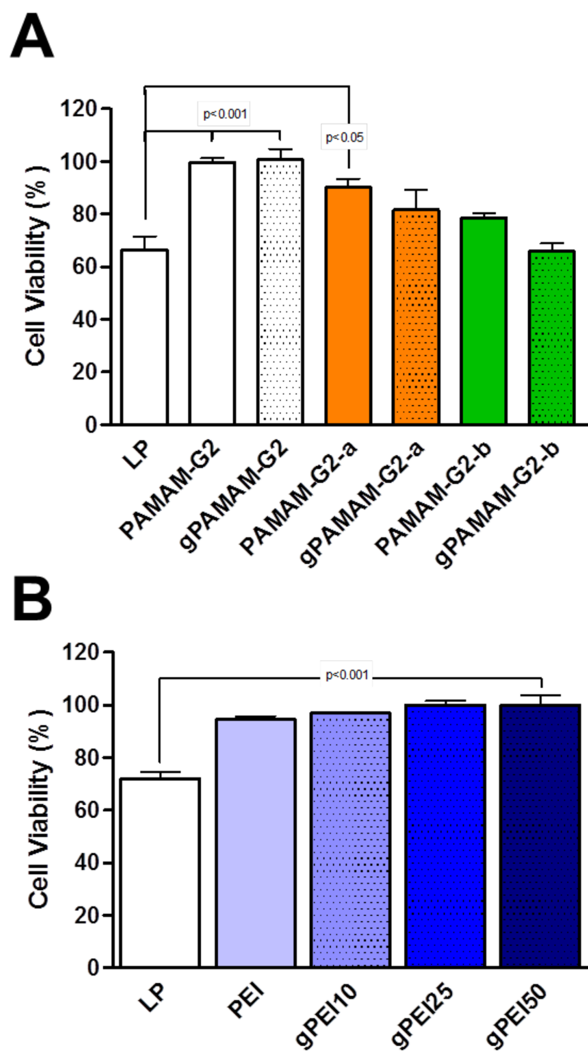

Figure 9. Cell viability of the dendrimer/pDNA complexes. Cell viability of the complexes formed by the PAMAM-G2 (A) and PEI (B) derivatives and pDNA at the most efficient N/P ratio for transfection was evaluated in CHO-k1 cells 24 h after transfection. Results are reported as % viability based on the untreated control cells normalized to 100% viable. Also, cell viability after transfection with Lipofectamine 2000 is shown. Results are expressed as means ± SEM (n = 8).

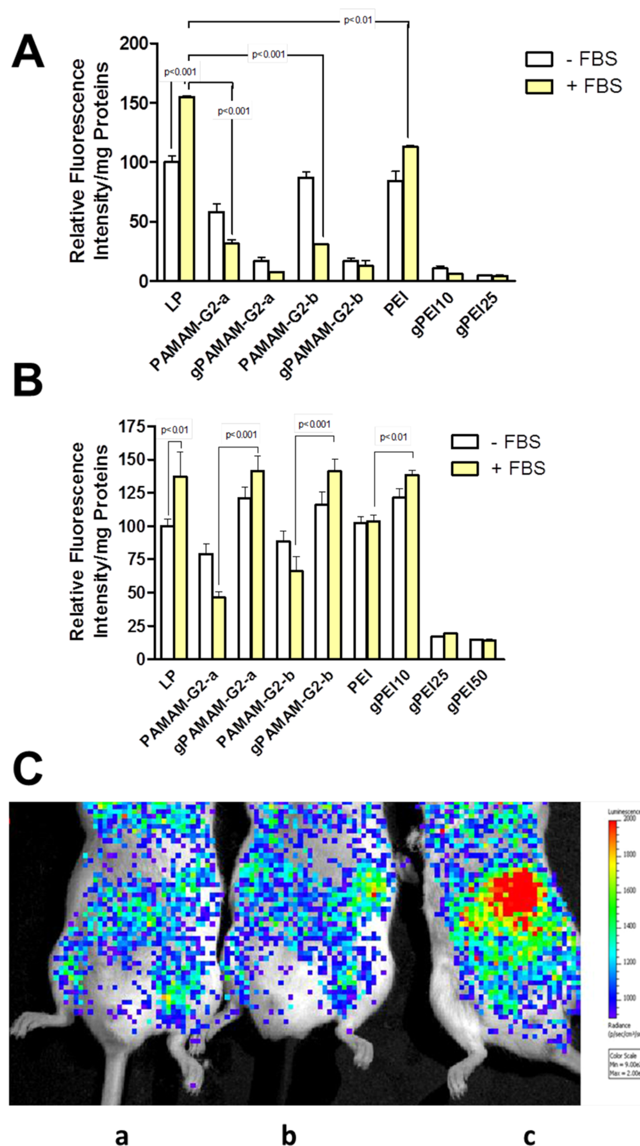

Figure 10. In vivo gene transfection of PAMAM-G2 and PEI based dendrimer complexes. CHO-k1 cells (A) or CHO-k1 cells expressing RAGE (B) were transfected with dendriplexes using pEGFP-N3. For each condition, DNA was mixed with the PAMAM-G2 or PEI derivatives at the indicated N/P ratios. As a positive control, a transfection was performed using LP. Transfection was carried out in the presence or absence of FBS. The eGFP fluorescence/protein value for the LP transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 8). (C) In vivo transfection in mice. Luciferase imaging of DB1 mice 24 h after intravenous tail injection of naked DNA (a), PEI dendriplexes (b), or gPEI10 based dendriplexes (c) containing 50 μg of pGL3-control plasmid at a N/P 10.

targeting of the liver, which was more intense in mice transfected with gPEI10.

■ CONCLUSION

The Maillard reaction has proven to be a straightforward and simple procedure for the preparation of specific glycated transfection reagents toward RAGE. This methodology is valid for scaffolds containing primary amino groups such as PAMAM-G2 or PEI derivatives, which are widely used for the development of transfection reagents. The specificity of the glycated dendrimers has been demonstrated using cells expressing recombinant RAGE. The study of the endocytosis, using specific inhibitors—competitors of the RAGE, demonstrates that the transfection mediated by the glycated reagents is preferentially via RAGE internalization. This feature has been exploited to transfect cell lines in which the expression of RAGE is associated with pathological processes (NRK and RAW 264.7 cell lines). The glycated compounds share features that make them appealing for *in vivo* use: Glycation confers selectivity with a minor effect on the toxicity, and in the presence of serum, the glycated dendriplexes transfect better than their nonglycated counterparts, supporting a different mechanism of endocytosis. Experiments *in vivo* with mice show that glycated PEI are more efficient than PEI. Our experimental results suggest that these compounds have a clear potential for the development of therapeutic agents toward pathological processes that involve RAGE as diabetes or angiogenesis.

■ EXPERIMENTAL PROCEDURES

General Experimental Procedures. Methanolic solution of Starburst PAMAM dendrimer, generation 2, containing 16 surface amino groups (PAMAM-G2) and branched polyethylenimine (PEI, ∼25 kDa) were purchased from Aldrich. LipofectAMINE 2000 (LP) was from Invitrogen (Carlsbad, CA, USA). Dansyl cadaverine, chlorpromazine, and genistein were from Sigma (St. Louis, MO, USA). pEGFP-N3 plasmid (Genbank U57609) was obtained from Clontech Laboratories (Palo Alto, CA). This 4729 bp plasmid encodes for an enhanced red-shifted variant of wild-type GFP (eGFP). pGL3-control vector (Genbank U47296.2) expressing luciferase under the SV40 enhancer and promoter was obtained from Promega (Mannheim, Germany). Eukaryotic plasmid pRAGE coding for the rat RAGE was based on the plasmid pRAGE-EGFP.[20] In pRAGE plasmid the coding sequence for the EGFP gene was removed in order to generate an mRNA coding only for the native rat RAGE under the control of the CMV promoter. Endotoxin-free plasmids were purified from transformed bacteria using the EndoFree Plasmid Maxi Kit from Quiagen (Hilden, Germany). DNA concentration was measured by a fluorimetric method using the Hoechst 33258 dye.[46] Glycated bovine albumin (BSA-AGEs) was obtained in our laboratory using bovine serum albumin (BSA) as described.[20] Human transferrin-Alexa Fluor 594 conjugate and Cholera toxin subunit B-Alexa Fluor 488 were provided by Life Technologies (Paisley, UK).

Synthesis of Alkyl Sulfonyl PAMAM-G2 Derivatives. Functionalization of PAMAM-G2 to give the alkyl sulfonyl derivatives PAMAM-G2-a and PAMAM-G2-b (Figure 1) was performed as described previously.[27] Briefly, the corresponding vinyl sulfone reagents 1-(vinylsulfonyl)octadecane (0.15 mM for PAMAM-G2-a) and (*Z*)-*N*-(2-(2-(vinylsulfonyl)ethoxy)-ethyl)octadec-9-enamide (0.6 mM for PAMAM-G2-b) in THF (15 mL) were added to a solution of PAMAM-G2 (0.3 mM, 1 mg/mL) in Milli-Q-water (15 mL). The solution was magnetically stirred at room temperature for 1 day. Then, the solvent (THF) was evaporated under reduced pressure and the water freeze−dried. The products were directly used in the glycation assays.

Synthesis and Characterization of Glycated PAMAM-G2 and PEI. Nonenzymatic glycation of PAMAM-G2-a,b and PEI was carried out in a sterile PBS buffer containing 200 mM glucose at 37 °C. The amount of dendritic polymers added to this solution was calculated by considering the number of free primary amine groups in these compounds and the degree of theoretical glycation pursued (50% for PAMAM-G2-a,b and 10%, 25%, and 50% for PEI). For these calculations, a 1:1 glucose/amino group stoichiometry was presumed. After 40 days glycation was considered complete.

The degree of glycation of the resulting products (gPAMAM-G2-a, gPAMAM-G2-b, gPEI10, gPEI25, and gPEI50) was established as a function of the glucose consumed in the reaction being enzymatically quantified by the glucose oxidase-peroxidase (GOD-POD) method. The percentage of free amino groups in the glycated dendritic polymers was evaluated using the trinitrobenzenesulfonic acid method.[47] Fluorescence spectra of the glycated compounds were recorded in a JASCO FP-6500 spectrofluorometer at a 370 nm (5 nm bandwidth) excitation and a 380−600 nm (5 nm bandwidth) emission.

To study the effect of glycation on the phosphorylation of extracellular signal-regulated kinases (ERK 1/2), CHO-k1 cells were transfected with plasmid pRAGE using LP. Stably transfected cells (CHO-RAGE) were selected by addition of 700 μg/mL Geneticin to the normal growth medium 48 h after transfection. CHO-RAGE cells were grown to confluence and incubated in the presence of 100 μg/mL BSA−AGEs (as a positive control of activation) and the same quantity of nonglycated and glycated PAMAM and PEI derivatives, for 60 min. Plates were flash-frozen in liquid nitrogen and processed as described previously.[48] The protein concentration of the lysates was measured using the Bio-Rad Protein Assay (Hercules, CA, USA). Proteins were separated by SDS−PAGE by loading 30 μg of total protein per lane and were then transferred to nitrocellulose membranes and immunoblotted with ERK1/2 and phospho-ERK1/2 E10 (Thr202/Tyr204) antibodies (Cell Signaling Technology, Beverly, MA, USA); the immunoblots were developed using an enhanced chemiluminescence detection system.

Preparation of Nonglycated and Glycated PAMAM-G2/DNA and PEI/DNA Complexes. Nonglycated or glycated dendritic polymers (PAMAM-G2, PAMAM-G2-a,b, gPAMAM-G2-a,b, PEI, gPEI10, gPEI25, and gPEI50) were complexed with DNA at several N/P ratios (*N* = number of primary amines or positively charged nitrogen atoms in the conjugate and *P* = number of phosphate groups in the pDNA backbone). For the calculation of the *N*/*P* ratios, we have considered the possibility that the irreversible glycation of primary amino groups may retain a positively charged residue, thus not modifying the *N* value. Plasmid pEGFP-N3 was used for the preparation of the DNA complexes and for transfection assays. Experiments were performed for *N*/*P* 0.5 to 20. The sample was vortexed for a few seconds and incubated for 30 min.

Gel Electrophoresis Shift Assay. The binding capacity of the nonglycated and glycated dendritic polymers for DNA was analyzed by gel electrophoresis. PAMAM-G2 derivatives were diluted in DMSO−H_2O (1:2) solution while PEI derivatives and DNA were diluted in H_2O. pEGFP-N3 DNA (10 μL at 0.1 mg/mL) was mixed with an equal volume of the dendrimer solutions using *N*/*P* ratios 0−10 and incubated for 30 min at room temperature before the addition of loading buffer (2 μL). An aliquot (5 μL) of each sample was subjected to agarose gel electrophoresis (0.8% w/v) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Electrophoresis was carried out at 7 V/cm and gels were stained after electrophoresis with ethidium bromide.

DNase I Protection Assays. pEGFP-N3 DNA (10 μL at 0.1 mg/mL) was mixed with the nonglycated and glycated dendritic polymers (5 μL) to give a final *N*/*P* ratio of 2 for PAMAM-G2 derivatives and 1 for PEI-based compounds, and the resulting mixture incubated for 30 min at room temperature. Next, a solution of DNase I (10 μL, 50 μg/mL in Tris HCl 50 mM pH 8) was added and incubated for 1 h at 37 °C. After the digestion, SDS (2 μL of a 10% solution) was added and the samples incubated for 15 min at 65 °C before the addition of loading buffer (4 μL). Finally, an aliquot (20 μL) of each sample was subjected to agarose gel electrophoresis (0.8% w/v) in TAE buffer. Quantification of the band intensity was performed with the NIH Image Software.[49] A value of 100 was assigned to the intensity of the band corresponding to the control undigested DNA.

Cell Culture and DNA Transfection Assays. Chinese hamster ovary (CHO-k1; ATCC no. CCL-61), RAW 264.7 (ECACC no. 91062702), and normal rat kidney (NRK; ATCC

no. CRL-6509) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine plus 100 U/mL penicillin, and 0.1 mg/mL streptomycin. CHO-RAGE cells were maintained in normal growth medium supplemented with 700 μg/mL Geneticin. All cell lines were incubated at 37 °C in a humidified incubator containing CO_2 (5%) and air (95%). Prior to transfection, cells were seeded in 48 well plates at a density of 1.5×10^4 cells/well and incubated for 24 h to reach a cell confluence of 80−90%. For transfection experiments, pEGFP-N3 plasmid (0.3 μg/well) was mixed with the corresponding PAMAM-G2 and PEI derivatives at N/P ratios of 2.5, 5, 10, and 20 at room temperature for 20 min in a final volume of 20 μL. The mixture was diluted to 0.3 mL with DMEM without serum or supplemented with 10% serum and added to each well. Cells were incubated with the PAMAM and PEI dendriplexes for 5 h, after which, the transfection media was removed and cells were further grown in DMEM plus 10% FBS for an additional period of 24 h. Nontransfected cells and naked pEGFP-N3 were used as negative controls. LP was used as a positive control in gene delivery experiments. LP polyplexes were prepared using 0.6 μL of LP and 0.3 μg of DNA, according to the manufacturer's instructions.

For the assay of the internalization of dendriplexes, cells were preincubated for 30 min with 14 μM chlorpromazine, 200 μM genistein, or 100 μM dansyl cadaverine prior to the addition of the dendriplexes. For the assay of the effects of BSA-AGEs on transfection efficiency, CHO-RAGE cells were preincubated for 12 h with 100 μg/mL BSA-AGEs and then transfected with the different transfection reagents.

Fluorescence and Protein Assay. Transfected cells were washed three times with PBS and lysed with 600 μL of 0.5% Triton X-100 in PBS. eGFP fluorescence was quantified in the cell lysates with a Shimadzu RF-5301PC fluorimeter using an excitation of 480 nm (5 nm) and 510 nm (10 nm) emission wavelengths. In the transfection experiments, fluorescence from nontransfected control cells was subtracted and fluorescence in the positive control using LP polyplexes was assigned a value of 100. Fluorescence results obtained in the transfections with the dendriplexes were normalized to this value. Protein concentration was measured using the Bio-Rad Protein Assay.

Particle Size and ζ Potential Measurements of gPAMAM-G2/DNA and gPEI/DNA Complexes. The particle size and ζ potential were measured on a Zetasizer Nano-ZS90 (Malvern Instruments, Spain) at room temperature. Solutions of dendriplexes were prepared with a pEGFP-N3 concentration of 0.1 mg/mL at the optimum N/P ratio for transfection in each case (N/P = 10 for PAMAM-G2, gPAMAM-G2, PAMAM-G2-b, gPAMAM-G2-b, PEI, gPEI10, gPEI25, and gPEI50; N/P = 5 for PAMAM-G2-a and gPAMAM-G2-a). The resulting dendriplexes were incubated at room temperature for 30 min and then diluted with 150 mM NaCl solution or pure water to 1.0 mL volume prior to measurement. The final sample contained 1 μg of pDNA in 150 mM NaCl for size measurements or in 10 mM NaCl for zeta potential measurements. Zeta potentials were calculated using the Smoluchowsky model, each data point being taken as an average over five independent sample measurements. Size results are given as volume distribution of the major population by the mean diameter.

Cytotoxicity of PAMAM-G2/DNA and PEI/DNA Complexes. Cytotoxicity of the dendriplexes was evaluated 24 h after transfection. Cytotoxicity was assayed by determining the percentage of cell viability (with respect to unexposed cells) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method,[36] which correlates the cellular metabolic activity with the number of viable cells in culture. Results are reported as % viability based on the untreated control cells normalized to 100% viable.

***In Vivo* Transfection Studies.** Female DB1 mice (6−8 weeks of age, 25−30 g weight) were purchased from Harlan Ibérica Laboratories. All animals were studied in accordance with guidelines established by Directive 86/609/EEC and with the approval of the Committee on Animal Research at the University of Granada. Individual mice in groups of four were injected via the tail vein with 200 μL of PEI or gPEI10 dendriplexes containing 50 μg of pGL3 control and prepared at N/P 10. Naked DNA was injected as a negative control. For *in vivo* imaging over time, mice were anesthetized with isofluoran and 150 mg/kg of D-Luciferin (Melford laboratories, Chelsworth, UK) was injected intraperitoneally. Ten minutes later, animals were placed in the dark chamber for light acquisition and images were taken with an IVIS Spectrum (xCaliper Life Sciences, MA, USA) and analyzed with the Living Image v 2.6 software package (Xenogen).

Statistical Analysis. Results are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's test as appropriate. $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

ζ potential and size of the dendriplexes measured by by dynamic light scattering DLS (Figure 1). Cytotoxicity and specificity of the endocytosis inhibitors on the CHO-k1 and CHO-RAGE cell lines (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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