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Polyelectrolyte Complexes of Low Molecular Weight PEI and Citric Acid as Efficient and Nontoxic Vectors for in Vitro and in Vivo Gene Delivery

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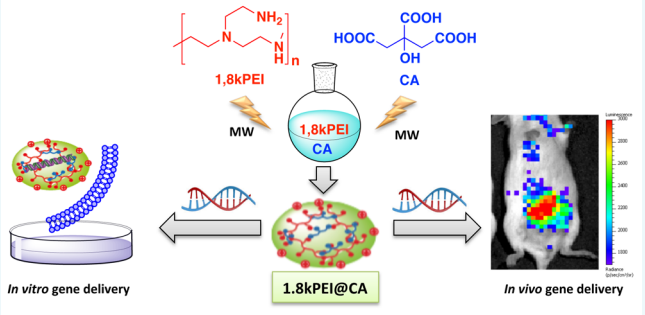
Supporting Information

ABSTRACT: Gene transfection mediated by the cationic polymer polyethylenimine (PEI) is considered a standard methodology. However, while highly branched PEIs form smaller polyplexes with DNA that exhibit high transfection efficiencies, they have significant cell toxicity. Conversely, low molecular weight PEIs (LMW-PEIs) with favorable cytotoxicity profiles display minimum transfection activities as a result of inadequate DNA complexation and protection. To solve this paradox, a novel polyelectrolyte complex was prepared by the ionic cross-linking of branched 1.8 kDa PEI with citric acid (CA). This system synergistically exploits the good cytotoxicity profile exhibited by LMW-PEI with the high transfection efficiencies shown by highly branched and high molecular weight PEIs. The polyectrolyte complex (1.8 kDa-PEI@CA) was obtained by a simple synthetic protocol based on the microwave irradiation of a solution of 1.8 kDa PEI and CA. Upon complexation with DNA, intrinsic properties of the resulting particles (size and surface charge) were measured and their ability to form stable polyplexes was determined. Compared with unmodified PEIs the new complexes behave as efficient gene vectors and showed enhanced DNA binding capability associated with facilitated intracellular DNA release and enhanced DNA protection from endonuclease degradation. In addition, while transfection values for LMW-PEIs are almost null, transfection efficiencies of the new reagent range from 2.5- to 3.8-fold to those of Lipofectamine 2000 and 25 kDa PEI in several cell lines in culture such as CHO-k1, FTO2B hepatomas, L6 myoblasts, or NRK cells, simultaneously showing a negligible toxicity. Furthermore, the 1.8 kDa-PEI@CA polyelectrolyte complexes retained the capability to transfect eukaryotic cells in the presence of serum and exhibited the capability to promote in vivo transfection in mouse (as an animal model) with an enhanced efficiency compared to 25 kDa PEI. Results support the polyelectrolyte complex of LMW-PEI and CA as promising generic nonviral gene carriers.



INTRODUCTION

Gene transfection, an important technology in molecular and cell biology and gene therapy,[1] relies on the development of efficient and safe gene vectors to facilitate extracellular and intracellular trafficking of gene material (plasmid DNA or small interfering RNA, siRNA). With this aim, gene transfection mediated by cationic polymers with different structures has been shown to be an adequate methodology, also referred to as polyfection, useful under in vitro or in vivo conditions.[2−4] Among the different synthetic polycations available nowadays, polyethylenimine (PEI)[5] is one of the most intensively studied systems due to its high transfection efficiency.[4,6−12] These polyelectrolytes are well suited for producing formulations with nucleic acids, since they self-assemble mainly by electrostatic interactions, forming nanosized particles, namely, polyplexes, which are efficient gene delivery systems.[13−15] They provide a unique combination of high charge density due to the aminated polymers and an inherent buffering capacity that triggers escape from lysosomes (the so-called proton sponge effect).[16] However, the transfection efficiency and the cytotoxicity of PEI/DNA formulations are highly dependent on the physicochemical properties of PEI such as branching ratio and molecular weight.[17,18] Highly branched PEIs (HMW-PEI) form smaller polyplexes achieving higher transfection efficiencies but simultaneously possess higher toxicity, due to their high molecular weight, excessive positive charge, and lack of biodegradability. Conversely, low molecular weight PEIs (LMW-PEIs) with favorable cytotoxicity profiles display

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minimum transfection activities as a result of inadequate DNA complexation and protection.

With the aim of obtaining PEI-based vectors with improved gene delivery capabilities with respect to those of unmodified PEIs, the structure of PEI has been the focus of extensive modifications.[19] A compromise between efficiency and toxicity as well as enhancement of in vivo cell and tissue targeting are the rationale behind the synthetic design and structural diversity of the second generation of PEI-related gene vectors. In particular, the chemical modification of nontoxic LMW-PEIs has been undertaken by a variety of strategies based on the covalent modification of their amino groups. The reported methods are categorized in alkylation, acylation, and copolymerization of PEIs by a variety of reactions that allow the tuning of the key parameters that determine the structure−activity relationship such as the amine content, the functionalization, the hydro-lipophilicity balance, or the conjugation with bioactive molecules. An appealing concept is the design of degradable PEIs, and in this context, stimuli-responsive degradable PEIs and ligand-conjugated degradable PEIs that consist of LMW-PEIs and cross-linking reagents containing biodegradable bridges have been reported.[20−23] The subjacent principle of such novel vectors is the synergistic exploitation of a higher stability in extracellular conditions, maximum DNA payload, and transfection efficiency displayed by high molecular weight PEI carriers combined with a minimal cytotoxicity due to the presence of bioreversible linkages that break down the gene vectors into less toxic low molecular weight structures after cellular uptake. The incorporation of reducible (disulfide)[24,25] and hydrolyzable (amides, esters, imines, carbamates, and ketals)[20] groups into the coupling reagents used for the preparation of biodegradable PEI polymers and networks has demonstrated the potential of those gene vectors. However, the synthesis of those degradable PEI-based vectors usually involves complex coupling reactions, which yield ill-defined macromolecular structures as well as broad molecular weight distributions.

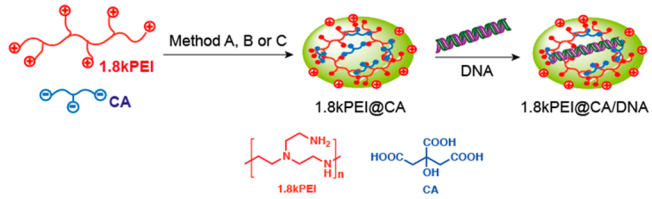
In the search for new gene vectors that exploit the favorable cytotoxicity profile of LMW-PEIs such as branched 1.8 kDa PEI (1.8kPEI) but with improved DNA condensation and transfection abilities, we hypothesized that a poly(carboxylic acid) such as citric acid (CA) would be adequate for the preparation of PEI-based polyelectrolyte complexes that will behave as bioreversible gene vectors. CA is a nontoxic compound that can be considered a green chemical, as it can be derived from fermentation, and it is also cost effective because it is readily abundant and inexpensive. Noncovalent association of LMW-PEI with CA would provide a simple assembly methodology for the construction of high molecular weight complexes composed of ionic cross-linked 1.8kPEI that would yield transfection efficiencies comparable to or even higher than those obtained with HMW-PEI. Furthermore, after transfection it is expected that the exposure to the cytoplasm environment would reverse of the high molecular weight complexes back to their low molecular weight PEI components, leading to a lower toxicity and the intracellular release of the DNA and its translocation to the nucleus.

Herein, we disclose the synthesis of branched 1.8 kDa PEI and CA complexes (1.8kPEI@CA) and we describe the DNA condensation abilities, colloidal properties (size and zeta potential), and cytotoxicity profiles of the corresponding polyplexes as well as their in vitro and in vivo gene transfection efficiencies. The reported results are relevant because the synthetic methodology is simple, avoiding complicated synthesis/separation steps that are normally involved in the covalent modification of PEI-based gene vectors, and it yields a nonviral gene carrier with higher gene condensation affinity, better gene transfection efficiency, and lower cellular toxicity when compared to branched 25 kDa PEI (25kPEI) or Lipofectamine 2000 (LP2000). These results demonstrate the suitability of LMW-PEI in the construction of safe, nontoxic, and highly efficient gene vectors.

RESULTS AND DISCUSSION

Synthesis, Optimization, and Characterization of 1.8kPEI@CA Complexes. The rationale behind the design of novel LMW-PEI-based gene vectors was to maximize the transfection efficiency of these polycationic polymers while maintaining low cytotoxicity through incorporation into nanstructures of higher molecular weight by means of reversible ionic interactions. To this aim, 1.8kPEI was selected as an archetypal LMW-PEI and CA as a constitutive element possessing multipoint attachment sites with a dual action: ionic cross-linking and reduction of charge density. Preparation of branched 25kPEI-based nanoparticles via the formation of noncovalent complexes through ionic cross-linking using bioreducible 3,3′-dithiodipropionic acid has recently been described by a more complex synthetic strategy.[26] Nevertheless, the reported compounds exhibited a moderate increase in the transfection efficiency respecting to pristine 25kPEI. On the basis of this result, simple mixing of the commercial reagents in water as solvent at room temperature followed after 10 min by dialysis and lyophilization was assayed as a novel preparative protocol (Scheme 1, Method A). The validity of the approach

Scheme 1. Preparative Strategies for the Synthesis of 1.8kPEI@CA Complexes and the Corresponding 1.8kPEI@CA/DNA Polyplexes[a]



[a]Treatment of a 1.8kPEI and CA mixture in H_2O; Method A: room temperature, 10 min; Method B: reflux for 10 min; Method C: MW irradiation at 800 W, 105 °C, 10 min.

was put to the test by evaluation of the transfection efficiency of the resulting ionic complex, referred to as 1.8kPEI@CA-RT, in CHO-k1 cells, a common standard cell line, using the plasmid pEGFP-N3 encoding for the green fluorescent protein by measuring the fluorescence of the transfected cells. The assays were performed in the absence of serum at ratios ranging from 5 to 35 μg reagent/μg DNA weight (w/w) (Figure 1A) and using as a positive control (100% value) LP2000, a commercial reagent described as a high efficiency vector for the transfection of many cell types. The as-prepared DNA complexes showed transfection efficiencies similar to native 1.8kPEI.

Considering the poorness of this initial assay, the application of energy to the mixture of reagents was envisioned to favor the interaction between PEI and CA. Two sources of energy were assayed: thermal heating and microwave irradiation (MW). The rationale behind the use of MW was based on our previous

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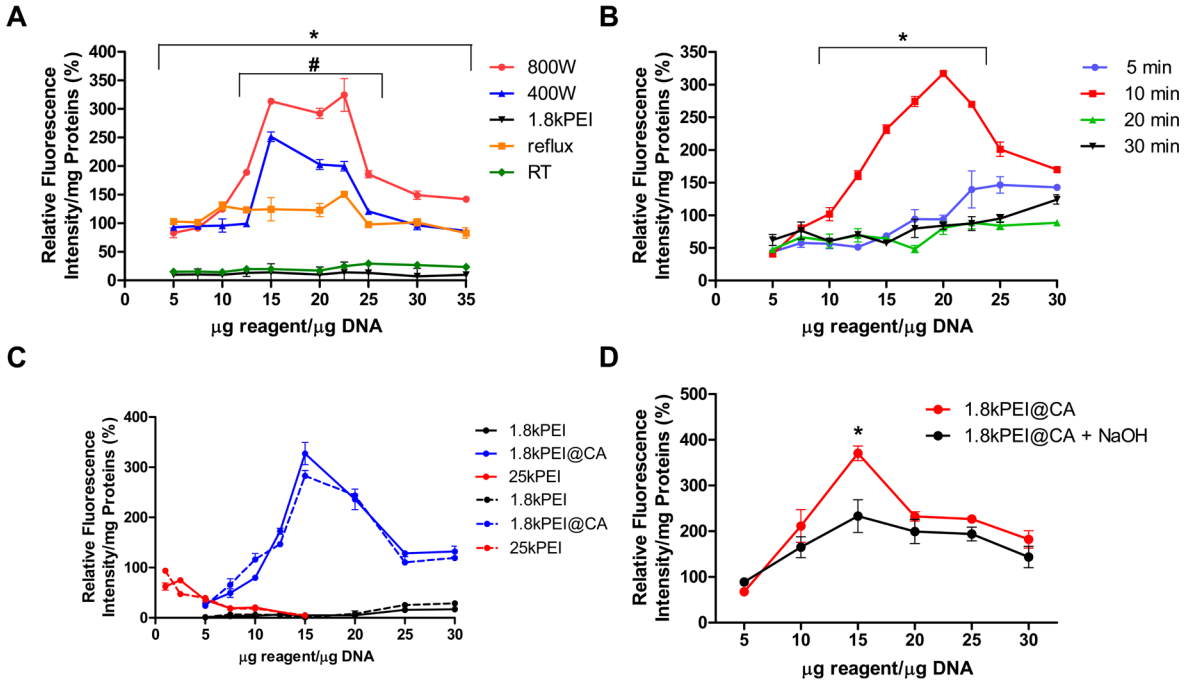

Figure 1. Optimization of the preparative conditions of 1.8kPEI@CAs complexes. Transfection of CHO-k1 cells with polyplexes of 1.8kPEI@CAs and pEGFP-N3 at w/w ratios ranging from 5 to 35 using transfection with LP2000 as a positive control. An arbitrary value of 100% was assigned to the eGFP fluorescence/protein for the LP2000 transfection and the other samples were normalized to that of the LP2000. Results are expressed as means ± SEM (n = 8). (A) Influence of the energy supply: room temperature (RT), reflux or MW irradiation (power output: 800 or 400 W) *p < 0.05 vs 1.8kPEI and RT; #p < 0.05 vs reflux and 400 W. (B) Influence of the MW irradiation time: 5 to 30 min, 105 °C and 800 W. *p < 0.05 vs other incubation times. (C) Influence of the incubation time in complete medium after transfection: 24 h (solid lines) vs 48 h (dotted lines). (D) Influence of the purification conditions on the transfection efficiency of 1.8kPEI@CA-MW (105 °C, 800 W): dialysis under neutral vs moderate basic conditions (10 mM NaOH). *p < 0.05 vs basic conditions.

experience about the benefits reported by this methodology on the preparation of CA-based carbon nanodots (CDs) passivated with PEI.[27] Thus, 1.8kPEI@CA-reflux and 1.8kPEI@CA-MW complexes were respectively obtained by reflux or MW irradiation (Scheme 1, Methods B and C, respectively). Preliminary assays revealed that 10 min of energy supply and a temperature of 105 °C in the case of the MW protocol were adequate conditions. In this last protocol, a standard 800 W power output was initially arbitrarily chosen. The manipulation of the crude materials obtained was identical to that described for the assay at room temperature: dialysis against water and subsequent lyophilization. This handling easily afforded foamy or solid materials that can be stored at low temperatures for unlimited time prior to the preparation of the DNA polyplexes and the transfection assays (Scheme 1).

Quantification of the transfection efficiencies was determined for 1.8kPEI@CA-reflux and 1.8kPEI@CA-MW complexes in CHO-k1 cells following the protocol described above. Different variables were evaluated to elucidate their influence on the performance as gene vectors of the as-prepared complexes to ascertain the more optimal preparative conditions. First, the energy supply method was analyzed (Figure 1A). It is known that the MW-assisted methodology is effective in the so-called bottom-up approach for preparing carbon nanodots (CDs) and other nanoparticles, and also that CA can be used as a carbon source in such processes.[28] According to the literature, promotion of MW pyrolysis seems to be determined by the lack of water as solvent.[29] Our conditions (the presence of water and moderate temperature) should ensure the absence of MW pyrolysis (see below). On the basis of this premise and in contrast to the results observed for complexation at room temperature, the thermally heated 1.8kPEI@CA-reflux complexes exhibited moderate transfection capability compared to the unmodified 1.8kPEI and similar to that of LP2000. Despite the limited applicability of this finding, the result is remarkable taking into account that unmodified 1.8kPEI lacks transfection capabilities. However, the results evidence that MW irradiation is an appropriate methodology to achieve more efficient 1.8kPEI@CA transfection reagents. Additionally, the MW irradiation power output was evaluated by preparing the 1.8kPEI@CA-MW complexes using also a 400 W power output. According to the results, 800 W is more optimal to get complexes with a better performance as gene vectors. Notably, the optimized protocol (MW, 800 W, 105 °C) renders transfections reagents with transfection efficiencies significantly higher than that of LP2000 (3−3.5-fold). This finding evidences the validity of the working hypothesis. Once the optimal energy supply was established, the influence of the MW irradiation time was the next variable investigated (Figure 1B) using periods ranging from 5 to 30 min. Results confirm a better performance for the 1.8kPEI@CA-MW obtained after 10 min of MW irradiation and indicate that the amount of energy supplied is a significant parameter.

The third variable investigated was the incubation time (Figure 1C). One drawback when using nonviral vectors in gene therapy is their short duration because only a continued gene expression at a therapeutic dose is meaningful for clinical applications. Therefore, we decided to evaluate the transfection efficiency determined at two different incubation times after transfection: 24 and 48 h. Determination of fluorescence/proteins reveals a similar performance at the two periods of time assayed confirming a sustained expression of the EGFP in

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our in vitro model. Accordingly, an incubation time of 24 h was considered optimal and selected to later analyze the transfection efficiencies of the reagents and the effects of inhibitors on the internalization by confocal analysis (see below).

As stated in our working hypothesis, the rationale behind the design of 1.8kPEI@CA complexes is the generation of nanoparticles constituted by PEI-based ionic cross-linked polyelectrolytes, Coulumbic interactions that would translate in a bioreversibility inside the cells. To prove that ionic interactions are operative in the 1.8kPEI@CA complexes a series of assays were performed including spectroscopic characterization of the set of prepared complexes (1.8kPEI@CA-RT, 1.8kPEI@CA-reflux, and 1.8kPEI@CA-MW), studies in a model system and the influence of the purification protocol in the transfection efficiencies.

Concerning the spectroscopic characterization, the Fourier transform infrared (FT-IR) spectra for the 1.8kPEI@CA complexes are almost identical (Supporting Information, Figures S1−6). A comparison with the starting materials (1.8kPEI and CA) reveals the disappearance of the characteristic symmetric (C═O) bands of carboxylic acid at 1780 and 1720 cm^{-1} for CA and the appearance of two bands resulting from the asymmetric and symmetric stretching modes of the carboxylate group at around 1560 and 1400 cm^{-1}. These findings confirm the formation of the ionic interactions between PEI and CA.[30,31] Additionally, the broad absorption peak around 3300 cm^{-1}, a hallmark of the numerous primary and secondary amines present in PEI, decreases significantly after the formation of the 1.8kPEI@CA complexes. Finally, characteristic absorption for the C═O stretching bond frequency of amide groups at around 1600 cm^{-1} was not observed, disregarding the formation of covalent linkages between PEI and CA through amidation reactions.

NMR experiments (Supporting Information, Figures S7−14) showed identical ^{1}H and ^{13}C NMR spectra for the 1.8kPEI@CA complexes. Particularly, the ^{1}H NMR spectra clearly indicate the ammonium salt formation of the surface amine groups of PEI due to the presence of CA and also the PEI characteristic signals that exhibit a downfield shift with respect to those corresponding to native PEIs. These observations are in concordance with previously reported protonation of PEI by carboxylic acid derivatives.[31] Furthermore, the characteristic peak of the methylene groups of CA at around 3.0 ppm experiences an upfield displacement (2.3−2.4 ppm). In the same way, ^{13}C NMR experiments reveal the deprotonation of the carboxylic acid because of the changes observed for the signal corresponding to the methylene group in the acid from 44 ppm in CA to 39.1 and 37.3 ppm in the 1.8kPEI@CA complex. In addition, characteristic peaks for the carboxylate groups of citrate are observed at 174 and 178 ppm in the 1.8kPEI@CA complex showing a downfield shift with respect to CA (181.8 and 179.0 ppm) and disregarding again the presence of amide bonds that usually exhibit a peak at around 172 ppm.[32]

To further confirm that the ionic interactions are the constitutive binding forces in the 1.8kPEI@CA complexes, a model complex was prepared by MW irradiation of a mixture of CA and ethanolamine (EA) in the optimized conditions (105 °C, 800 W, 10 min). In fact, formation and characterization of the tris(2-hiydroxyethyl)ammonium citrate salt resulting from this complexation by the simple mixing of the reagents in water at room temperature has been previously described in a contribution of Giannelis et al.[32] In our case, MW irradiation of CA and EA renders the same citrate salt as confirmed by their spectroscopic data that were identical to those reported (data not shown).

To get additional experimental evidence for the purification procedure for 1.8kPEI@CA complexes, it was thought that dialysis against a moderately basic solution (10 mM NaOH) instead of distillated water upon the preparation of 1.8kPEI@CA complexes using the MW optimized protocol should partially break the ionic interactions, rendering a compound that should exhibit a different transfection efficiency. This modification of the purification protocol (Figure 1D) produced, as expected, complexes with a lower transfection efficiency with respect to those observed for the materials obtained by the dialysis against distilled water, pointing out the relevance of the ionic interactions, and corroborating the initial protocol as optimal.

As mentioned above, MW-assisted technique is an effective route in the so-called bottom-up approach for gaining carbon nanodots (CDs) and other nanoparticles.[28] The technique exhibits appellant synthetic advantages such as the rapid and uniform heating of the reaction medium, efficiency, time-savings, cost-effectiveness, environmental friendliness, and narrow size distribution of the CDs. CA has been used, although not exclusively, as a carbon source of choice in numerous reported CD preparations including MW-assisted pyrolysis of this material in the presence of a variety of passivation agents. The most popular protocols use nitrogen-containing compounds for passivation with a structural variety ranging from simple amines to nitrogenated polymers such as PEI or PAMAM.[33,34] Although the preparative procedures of CA-based CDs differ in the use of water as solvent or the direct heating of a neat mixture of reagents, the absence of solvent has been demonstrated to be a prerequisite for the pyrolitic formation of CDs in a time dependent manner.[29] In addition, the demonstrated stability of PEI under MW irradiation[35] ensures no side reactions involving this product in the preparation of PEI-based CDs using this technique and guarantees the functional abilities of PEI, including their role as a polyelectrolyte to condense DNA. In this respect and to the best of our knowledge, the report of Pierrat et al.[36] is the sole case in which CDs were obtained by a MW assisted synthesis starting from 25kPEI and CA and their capabilities as transfection reagents studied. However, these CDs showed only transfections efficiencies similar to the unmodified PEI. Remarkably, our results indicate that significantly higher transfection is achieved when the thermal pyrolysis of PEI is avoided by using shorter MW irradiation times, limiting the reaction temperature and refluxing to circumvent solvent evaporation and ensure a constant reaction volume. Our data supports the exclusive formation of ionic complexes and that longer irradiation times (20 and 30 min) render fluorescent compounds (data not shown), which exhibit significantly lower transfection efficiencies, leading us to conclude that the molecular bases of the transfection by 1.8kPEI@CA are different from those reported for PEI-based CDs.

Finally, the proton buffer capacity of the 1.8kPEI@CA complexes was characterized by considering the accepted relation between the buffering capacity of gene nanocarriers and their endosome scope. Compared to pristine 1.8kPEI, the 1.8PEI@CA complexes are less alkaline. The titration graph (Supporting Information, Figure S15) shows that the preparative protocol has a clear influence on the buffering capacity of the 1.8PEI@CA composites, with the behavior of

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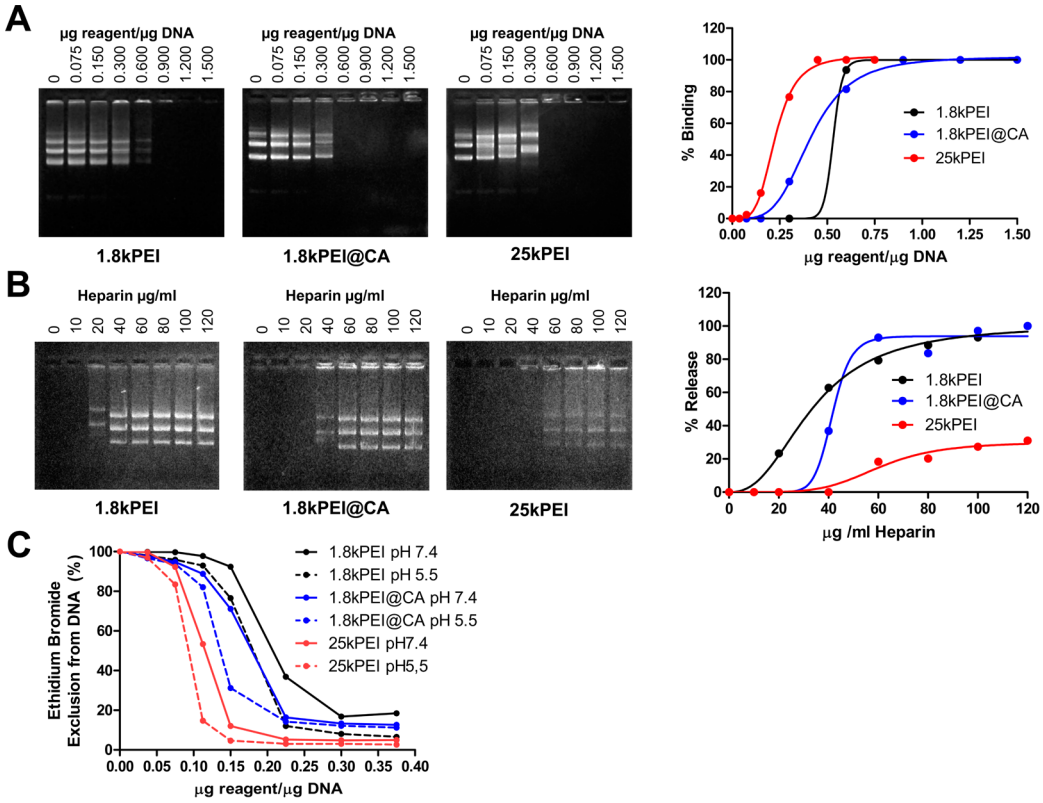

Figure 2. 1.8kPEI@CA/DNA polyplex binding and release assays: (A) Representative gel shift assays showing pDNA binding to the PEI derivatives. (B) Heparin competition assay. The experiments were performed at ratios between 0 (only pEGFP-N3) and 1.5 w/w for the gel shift assay and a 0.75 w/w ratio for the heparin competition assay. The relative percentage of binding and release has been calculated by quantification of the intensity of the plasmid bands corresponding to the pEGFP-N3 samples complexed with PEI derivatives. The mean values of three independent experiments are shown. (C) Ethidium bromide exclusion experiments: Ethidium bromide exclusion from DNA due to the binding to PEI derivatives was assayed by the decrease in fluorescence at 592 nm as described in the Experimental Procedures at pH 7.4 and pH 5.5. The % of exclusion was calculated as fluorescence of the sample/fluorescence of the control × 100.

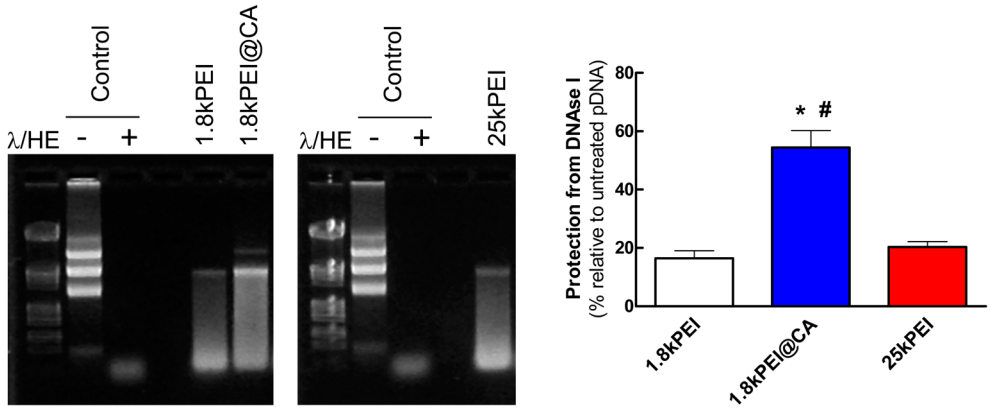

Figure 3. DNase I protection experiments. Representative agarose electrophoresis of samples corresponding to pEGFP-N3 incubated in the absence (−) or presence (+) of DNase I as controls. pEGFP-N3 samples complexed with the PEI derivatives at a ratio of 0.375 w/w before DNase I treatment were run in parallel. 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 PEI derivatives and treated with DNase I is shown. Results are expressed as means ± SEM ($n = 6$). *$p < 0.05$ vs 1.8kPEI; #$p < 0.05$ vs 25kPEI. A value of 100 was assigned to untreated pEGFP-N3.

1.8PEI@CA-reflux being close to that of pristine 1.8kPEI. Results showed that the buffering capacity for a pH interval ranging from 7 to 4 for 1.8PEI@CA-RT and 1.8PEI@CA-MW is 42% and 64%, respectively, of that for pristine 1.8kPEI. (Supporting Information, Table S1).

DNA Binding and Protection Capabilities of 1,8kPEI@CA Complexes. Once the optimal preparative conditions for the generation of 1.8kPEI@CA-based gene vectors were established, their capability to bind and compact pEGFP-N3 plasmid DNA (pDNA) was evaluated. Unmodified 1.8kPEI and 25kPEI were included in the analysis as controls. Polyplexes

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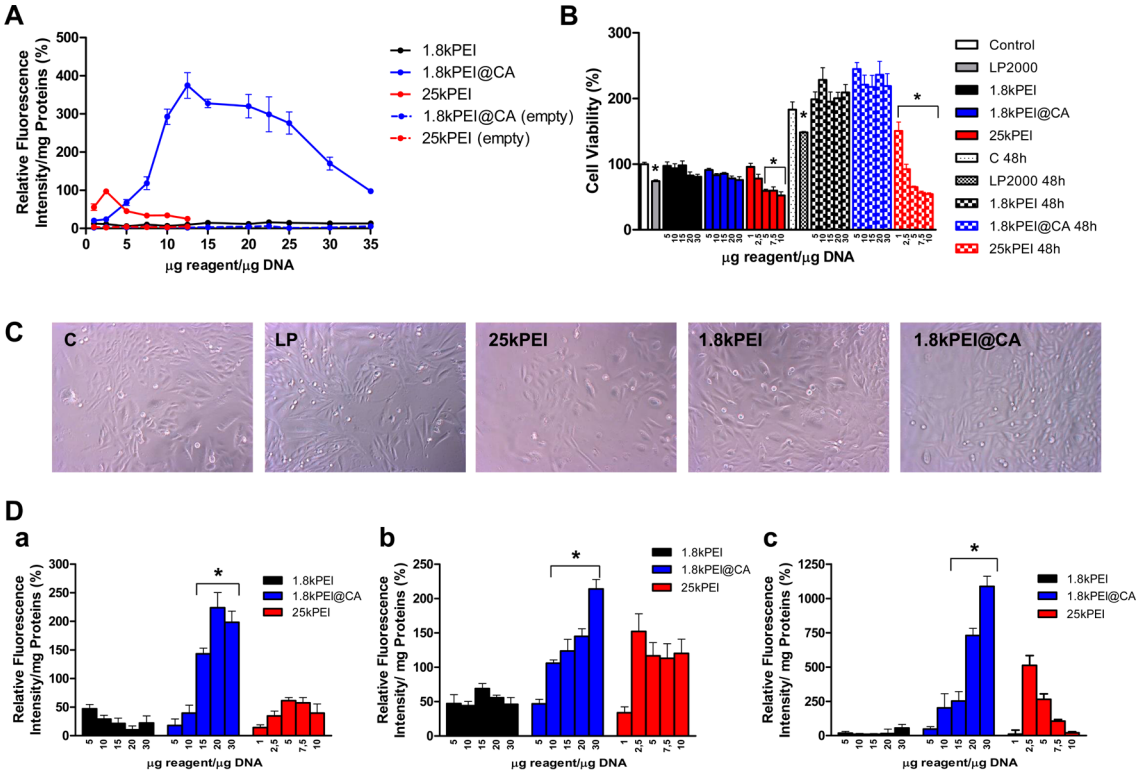

Figure 4. In vitro gene transfection efficiency and cell toxicity of the 1.8PEI@CA/DNA polyplexes. (A) Transfection efficiency in CHO-k1 cells transfected with pEGFP-N3 polyplexes or empty particles. For each condition, pDNA was mixed with the PEI derivatives at ratios from 0 to 35 w/w. Transfection using LP2000 was included in the analysis as a positive control. The eGFP fluorescence/protein value for the LP2000 transfection was assigned an arbitrary value of 100 and the ability of the other reagents to transfect was normalized to the efficiency of LP2000 (absolute values of fluorescence for the LP2000 transfection in CHO-k1 cells is 46.280 ± 4056.2 RFU/mg proteins). (B) Cell viability of CHO-k1 cells treated with 1.8PEI@CA/DNA polyplexes and evaluated 24 and 48 h after transfection. Results are reported as % viability based on the untreated control cells at 24 h normalized to 100% viable. Also, cell viability after transfection with LP2000 is shown. *$p < 0.05$ vs control cells. (C) Cell morphology images taken 48 h post-transfection. (D) Transfection efficiency in other cell lines. FTO2B (a), L6 myoblasts (b), or NRK cells (c) were transfected with complexes using pEGFP-N3 as described in (A). Absolute values of fluorescence for the LP2000 transfection in FTO2B are 21.715 ± 1025.74, in L6 are 11.895 ± 499.39, and in NRK are 12.230 ± 812.72 RFU/mg proteins. Results are expressed in all cases as means ± SEM ($n = 8$). *$p < 0.05$ vs 1.8kPEI; #$p < 0.05$ vs lower amounts (5 and 10 w/w ratios) of 1.8kPEI@CAs.

were prepared by simple mixing of pDNA and the PEI derivatives at several w/w ratios (0 to 1.5) and gel electrophoresis shift assays performed as they reflect how a vector is able to compact pDNA. Results revealed an enhanced binding capability for the 1.8kPEI@CA and 25kPEI vectors while 1.8kPEI clearly exhibited a lower efficiency compared to compact DNA. (Figure 2A). In addition, the assays were also performed in the presence of heparin to gain additional insight into the DNA release from the complexes and the ability of intracellular DNA unpacking (Figure 2B). In this competitive assay, 25kPEI/DNA dissociated poorly from DNA while the DNA was released by 1.8kPEI@CA/DNA and 1.8kPEI/DNA polyplexes in a similar manner. These results led us to conclude that 1.8kPEI@CA complexes showed a behavior that combines a high capability to compact DNA together with a high efficiency for intracellular release of DNA. Furthermore, an ethidium bromide exclusion experiment at pH 7.4 and 5.5 was carried out showing that at both values 1.8kPEI@CA has an intermediate capability to exclude ethidium bromide compared with 25kPEI and 1.8kPEI. Remarkably, 1.8kPEI@CA retained the capability to bind DNA and therefore protect it from degradation at pH 5.5, which is close to the pH described for endosomes.

An important feature for any transfection reagent is the ability to protect pDNA from endonuclease degradation. This parameter was evaluated for the different prepared PEI/DNA polyplexes. The assays were performed using a 0.375 w/w ratio (Figure 3). At this value, unmodified PEIs (1.8k and 25k) showed a limited capability to protect DNA from degradation in concordance with previous reported cases.[37] However, the corresponding CA-based polyelectrolyte complexes of 1.8kPEI showed a significant comparative increase in their ability to protect DNA from degradation that is statistically significant (i.e., $p < 0.05$).

From the complete set of experiments described above, it can be concluded that the CA cross-linking and complexation wit LMW-PEI render compounds that showed an enhanced DNA binding capability associated with a facilitated intracellular DNA release and a higher DNA protection from endonuclease degradation.

Transfection Efficiency for 1.8kPEI@CA Complexes. Transfection efficiency of 1.8kPEI@CA complexes compared with the unmodified PEIs was next assayed in CHO-k1 cells using pEGFP-N3 plasmid (Figure 4A). Transfecting cells with empty nanoparticles without DNA to detect any increase in fluorescence not due to the eGFP expression validated the essay. In these conditions, no increase in fluorescence compared with the nontransfected cells has been detected. Transfection values for unmodified 1.8kPEI and 25kPEI were in agreement with results previously described in the literature:

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almost null for 1.8kPEI at the entire range of ratios assayed and approximately 80−90% of the LP2000 transfection value at the optimal rate of 2.5 w/w for 25kPEI. Interestingly, the results obtained with 1.8kPEI@CA complexes showed approximately 3.8-fold higher transfection efficiencies compared to LP2000 at 12.5−15 w/w ratios. This result is more remarkable when compared with the practically null transfection efficiency exhibited for unmodified 1.8kPEI.

Although 25kPEI and LP2000 are considered efficient transfection reagents, cellular toxicity during transfection events is considered a main drawback for these compounds, particularly in the case of 25kPEI. Conversely, LMW-PEI derivatives such as 1.8kPEI exhibit very low cell toxicity but concomitantly small transfection efficiency. These cytotoxicity profiles have been confirmed in the present work when assayed in the CHO-k1 cell line 24 and 48 h after transfection (Figure 4B). The cytotoxicity produced by the transfection reagents analyzed revealed a similar profile at 24 and 48 h. As expected, LP2000 showed a significant toxicity (ca. 20−25% decrease in cell viability at its optimal transfection ratio). In addition, the toxicity of 25kPEI is low while maintained at low concentrations but significant at ≥5 w/w ratio, toxicity that increased 48 h post-transfection. As expected, 1.8kPEI has no effect on cell viability at any transfection ratio. Notably, 1.8kPEI@CA complexes showed a moderate toxicity at its optimal transfection rate (12.5 and 15 μg reagent/μg DNA). Additionally, cell morphology images taken at 48 h provide visual evidence that correlated perfectly with the measurement of cytotoxicity (Figure 4C). Transfection efficiency experiments have been expanded to FTO2B hepatomas, L6 myoblasts, and Normal Rat Kidney (NRK) cells as representative examples of cell lines recalcitrant to transfection (Figure 4D). Having assigned to LP2000 transfected cells a value of 100%, unmodified 1.8kPEI and 25kPEI showed very small and moderate transfection efficiencies, respectively, whereas 1.8kPEI@CA complexes showed at least a 2-fold increase in transfection efficiency compared with LP2000. These results are especially relevant in the NRK cell line, where 1.8kPEI@CA showed a 10-fold increase in transfection efficiency compared with LP2000.

Size and ζ Potential Measurements of 1.8kPEI@CA/DNA Polyplexes. The average hydrodynamic diameter (Z-average) and polydispersity index (PI) exhibited in HEPES buffer by the polyplexes that showed the best transfection results (1.8kPEI@CA and unmodified 1.8kPEI at 15 w/w ratio and 25kPEI at 2.5 w/w ratio) were measured indicating that they were suitable for application in gene delivery (Table 1).

Concerning the intensity size distributions for the analyzed polyplexes (Figure 5A) only a significant peak was found for 1.8kPEI based polyplexes, and two peaks for 25kPEI ones. In this case, the system is more polydisperse and particles with lower sizes are present.

With respect to the average ζ potentials for the polyplexes analyzed, positive values were obtained in all cases (Table 1). The average ζ potential gives no useful information in the case of samples presenting different peaks in the ζ potential distribution (Figure 5B). When 1.8kPEI was used as the reagent, lower ζ potential values than those corresponding to the other PEI-based compounds were found in the distribution. This could be due to the similar conformation of the 25kPEI and the networks formed by the 1.8kPEI@CA complexes around the circular DNA plasmid that leads to more positive particles.

For the three types of polyplexes under study, the size distribution obtained in HEPES buffer 20 h after polyplex formation was compared with the size distributions corresponding to those systems but diluted in HEPES buffer or diluted in acetate buffer (Figure 5C). In all cases there was an initial effect of dilution on the size distribution (data not shown) due to polymer desorption, irrespective of the pH. However, once the equilibrium was reestablished (20 h), a different behavior was observed depending on the polyplex system. In the case of 1.8kPEI@CA/DNA (Figure 5Ca), the distributions for the diluted and nondiluted system in HEPES were similar; hence, no dilution effect was observed.

However, by changing the pH of the system from 7.4 to 5.6, the size distribution became wider, showing particles with larger sizes also. Therefore, for the 1.8kPEI@CA the polyplexes presented a more relaxed structure at acid pH. This change in the particle size distribution was not observed for 1.8kPEI/DNA and 25kPEI/DNA polyplexes upon pH change (Figure 5Cb,c, respectively). This is remarkable since the pH shift from 7.4 to 5.6 resembles the changes of pH described between the cytosol and the endosomes,[38] and relaxation of the structure is more pronounced for the 1.8kPEI@CA/DNA. Size and ζ potential distributions of 1.8kPEI@CA/DNA at 15 w/w were measured up to 4 days after polyplex formation (Supporting Information, Figure S7) showing that the system remained stable at least for that time.

These data, together with the values of DNA release in the presence of heparin, suggest for these polyplexes a unique behavior related to its polycationic nature that probably facilitates DNA escape from endosomes and justifies its higher transfection efficiency compared to 25kPEI. Furthermore, compared with 1.8kPEI, 1.8kPEI@CA complexes form more compact particles even upon dilution at pH 7.4, which could facilitate the entrance across the cell membrane.

Internalization Mechanism for 1.8kPEI@CA Complexes. Nonviral gene complexes can enter into mammalian cells through two different main endocytic pathways, clathrin-dependent and caveolae-mediated endocytosis,[39] albeit other entrance routes are possible. To investigate the endocytosis pathway of the 1.8kPEI@CA complexes, chlorpromazine and genistein, which inhibit clathrin-dependent and caveolae-mediated endocytosis, respectively,[40] were assayed in CHO-k1 cells. At the concentrations used, these inhibitors are not cytotoxic and the specificity of these endocytosis inhibitors has been confirmed using markers of clathrin-dependent and -independent pathways in this cell line.[37] The assays revealed that in CHO-k1 cells the transfection levels of 1.8PEI@CA/DNA and 25kPEI/DNA complexes were significantly inhibited in the presence of genistein and increased in the presence of chlorpromazine (Figure 6A). These results indicate that the

Table 1. Colloidal Properties of 1.8kPEI/DNA Polyplexes Showing the Best Transfection Results[a]

Parameter	25kPEI/DNA w/w = 2.5	1.8kPEI/DNA w/w = 15	1.8kPEI@CA/DNA w/w = 15
Average hydrodynamic diameter (nm)	47.0 ± 0.6	91.4 ± 0.7	105 ± 0.7
Polydispersity index	0.476 ± 0.05	0.207 ± 0.018	0.209 ± 0.009
Mean zeta potential (mV)	31.20 ± 0.14	30.4 ± 0.5	30.7 ± 0.5

[a]Errors correspond to the standard deviations of three independent sample measurements.

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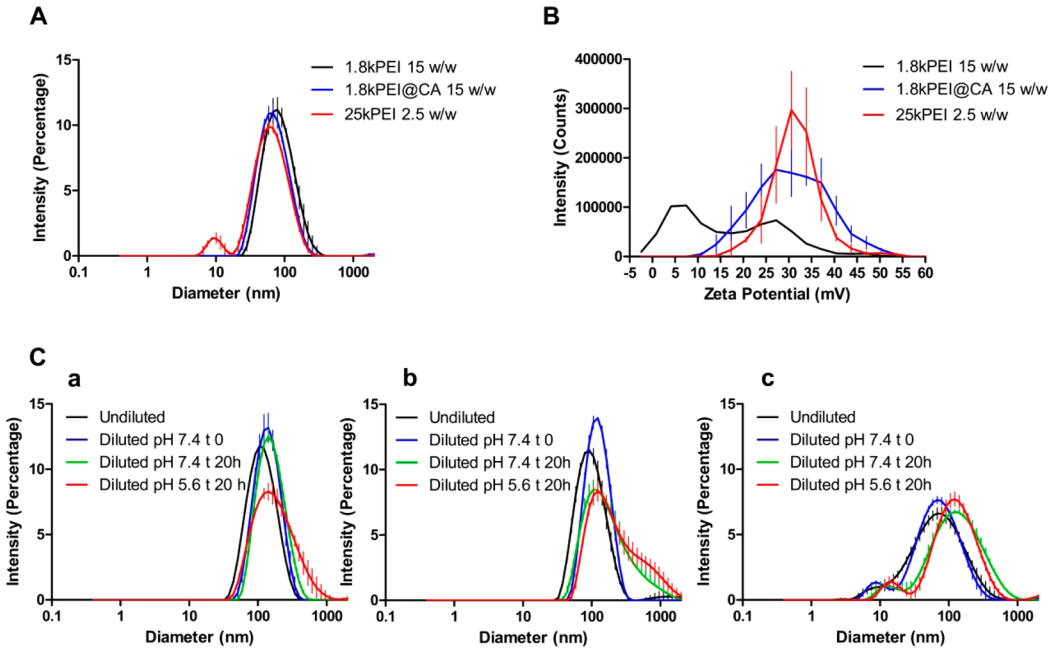

Figure 5. Size and zeta (ζ) potential distributions of modified 1.8kPEI-based polyplexes showing the best transfection results (15 w/w) and those formed with unmodified 1.8kPEI and 25kPEI at 15 and 2.5 w/w ratios, respectively. Errors correspond to the standard deviations of three independent sample measurements. (A) Hydrodynamic diameter distributions of polyplexes by intensity in HEPES buffer. (B) Zeta potential distribution of polyplexes by intensity in HEPES buffer. (C) Hydrodynamic diameter distributions of polyplexes by intensity 20 h after polyplex formation in HEPES, diluted in HEPES, and diluted in acetate buffer: a. 1.8kPEI@CA; b. 1.8kPEI; c. 25kPEI.

caveolae-mediated pathway is the preferred productive endocytic pathway for these compounds in agreement with our previous finding for other PEI-based compounds.[37] However, the clathrin-dependent route that leads to inefficient transfection events is also used by the 1.8PEI@CA supporting the chlorpromazine positive effect. In addition, the effects of bafilomycin A on transfection efficiency were assayed (Figure 6B). Bafilomycin A is an inhibitor of proton pump ATPase that is involved in endosome acidification. Inhibition of the proton pump leads to a decrease in transfection efficiency for all the assayed compounds, being especially significant for the 1.8kPEI@CA. These results, together with the ethidium bromide exclusion experiments at pH 5.5 commented above, support the idea that low endosomal pH plays a role in the increase in transfection activity mediated by 1.8kPEI@CA.

To confirm the use of the caveolae- or chlatrin-mediated endocytosis pathway for the internalization of 1.8PEI@CA, confocal experiments were carried out where CHO-k1 cells were first preincubated in the presence of Cholera toxin subunit B-Alexa Fluor 488 as a marker of clathrin-independent endocytosis or human transferrin−Alexa Fluor 594 conjugate as a marker of clathrin-dependent endocytosis. These cells were then transfected with 1.8PEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes. After 2 h, confocal images of the transfected cells were acquired (Figure 7A) showing that Alexa Fluor 488, Alexa 594, and Cy5 colocalize inside the cells. These findings support the use of both endocytic routes as the internalization pathway for 1.8PEI@CA complexes. To further confirm the use of the productive clathrin independent pathway, 1.8PEI/CA was directly labeled with Rhodamine and confocal experiments were carried out (Figure 7B). CHO-k1 cells were first preincubated in the presence of Cholera toxin subunit B-Alexa Fluor 488 as a marker of clathrin-independent endocytosis and then transfected with Rhodamine-labeled 1.8PEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes. After 2 h, confocal images of the transfected cells were acquired (Figure 7B) showing that Alexa Fluor 488, Cy5, and Rhodamine labels colocalize inside the cells. These findings support the use of the caveolae-mediated endocytosis as the preferred but not unique internalization pathway for 1.8PEI@CA complexes and allow us to conclude that these compounds use the same internalization routes than 1.8kPEI and 25kPEI. Therefore, its enhanced efficiency is not based on the internalization process.

In Vivo Transfection Capabilities of 1.8kPEI@CA Complexes. The ultimate goal in the design of a gene delivery system is its implementation in vivo. However, many nucleic acid carriers that are efficient to transfect cells in vitro fail to operate in vivo. In addition, prior to testing in vivo the presence of serum is an important issue that needs to be evaluated. The transfection efficiency of the 1.8kPEI@CA complexes was studied in CHO-k1 cells at the ratios that yield the highest transfection efficiency in the presence of two serum concentrations (Figure 8A). As expected, these assays revealed that LP2000 increased its transfection efficiency, probably due to its liposome nature, but unmodified PEIs (1.8k and 25k) and 1.8PEI@CA complexes showed reduced transfection efficiency. However, the fact that in the presence of serum the remaining transfection efficiency for 1.8PEI@CA complexes was similar to that of LP2000 encouraged us to assay 1.8PEI@CA in vivo.

Mice were injected with naked DNA (negative control), LP2000/DNA and 25kPEI/DNA (standards for in vivo transfection), or 1.8PEI@CA/DNA complexes containing a luciferase-expressing plasmid at a 1, 5, or 12.5 w/w ratio, respectively, and transfection was detected by quantifying the resulting luminiscence in the whole animal 24 and 48 h after the injection (Figure 8B). As expected, naked DNA failed to transfect and 25kPEI yielded a very moderate signal in the

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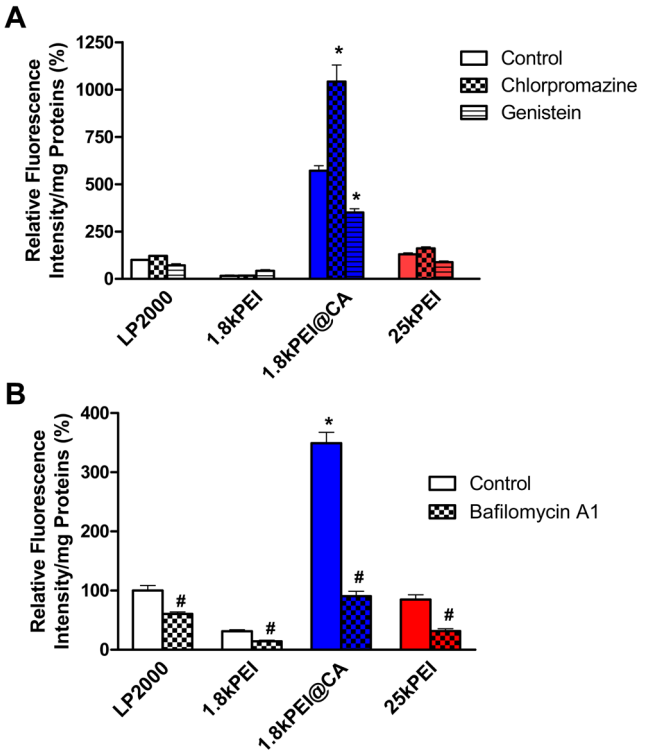

Figure 6. Effects of internalization route inhibitors in the transfection by 1.8PEI@CA/DNA polyplexes. CHO-k1 cells were pretreated with (A) chlorpromazine (14 μM) or genistein (200 μM) or (B) Bafilomycin A1 (100 nM) for 30 min before transfection with PEI-based polyplexes at their optimal ratio. As control, a transfection was performed using LP2000. The eGFP fluorescence value for the LP2000 transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 6). *p < 0.05 vs untreated cells.

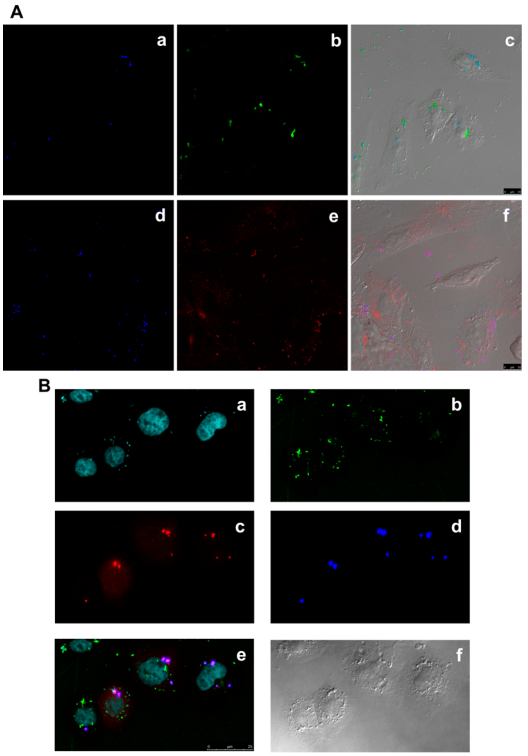

Figure 7. Internalization routes in the transfection of 1.8PEI@CA/DNA polyplexes. (A) CHO-K1 cells were seeded onto coverslips and were incubated for 20 min in the presence of Cholera toxin subunit B-Alexa Fluor 488 as a marker of clathrin-independent endocytosis (a, b, c) or human transferrin−Alexa Fluor 594 conjugate as a marker of clathrin-dependent endocytosis (d, e, f). Then, 1.8kPEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes at 12.5 w/w ratio were added, and after 2 h, cells were fixed and analyzed by confocal microscopy. (a,d) Cy5 signals; (b,e) Alexa Fluor 488 and Alexa Fluor 594 signals, respectively; (c,f) overlaid images. (B) CHO-K1 cells were seeded onto coverslips and were incubated for 20 min in the presence of Cholera toxin subunit B-Alexa Fluor 488. Then, cells were transfected with Rhodamine labeled-1.8kPEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes at 12.5 w/w ratio. After 2 h of transfection, cells were fixed and confocal microscopy was performed to detect nuclei stained with Hoechst 33258 dye (a, gray), Cholera toxin subunit B-Alexa Fluor 488 (b, green), Rhodamine labeled-1.8kPEI@CA (c, red) or Cy5 labeled pEGFP DNA (d, blue). A merged image (e) as well as and differential interference contrast (Nomarski) image (f) are shown.

transfected animal. LP2000, a more efficient in vivo transfection reagent, produced at 24 and 48 h a significantly stronger signal. It is noteworthy that animals transfected with 1.8PEI@CA polyplexes showed at 24 h an enhanced luminescence when compared to the 25kPEI signal and similar to the obtained with LP2000. At 48 h, the signals resulting from the transfection events with 1.8PEI@CA polyplexes not only remained but were significantly higher than those from LP2000 and those obtained at 24 h. These preliminary results show the potential of 1.8PEI@CA polyelectrolyte complexes as in vivo transfection agents.

CONCLUSIONS

In conclusion, the results presented herein demonstrate that the simple modification of low molecular weight 1.8kPEI by ionic cross-linking with CA, an archetype of a poly(carboxylic acid), yields nanoparticles (1.8kPEI@CA) that behave as efficient and nontoxic nonviral gene carriers. This approach combines the intrinsic good cytotoxicity profile exhibited by low molecular weight 1.8kPEI with the high transfection efficiencies of branched and high molecular weight PEIs in a novel material. The synthetic preparative protocol is green, inexpensive, and straightforward involving the microwave irradiation of a mixture of commercially 1.8kPEI and CA. The new 1.8kPEI@CA complexes showed enhanced DNA binding capability associated with higher facilitated intracellular DNA release and enhanced DNA protection from endonuclease degradation compared with unmodified PEIs. In addition, while transfection values for LMW-PEIs are almost null, transfection efficiencies of the new reagent range from 2.5- to 3.8-fold to those of LP2000 and 25kPEI. The fact that the novel vectors show a negligibly toxicity makes them particularly attractive for the development of safe gene delivery vehicles. Furthermore, the 1.8kPEI@CA polyelectrolyte complexes retained the capability to transfect eukaryotic cells in the presence of serum and exhibited the capability to promote in vivo transfection in mouse (as an animal model) with an enhanced efficiency compared to 25kPEI.

EXPERIMENTAL PROCEDURES

General Experimental Procedures. Branched polyethylenimine 1.8 kDa (50 wt % sol. in water, 1.8kPEI), branched polyethylenimine 25 kDa (25kPEI), citric acid (CA), heparin sulfate, HEPES, chlorpromazine, genistein, bafilomycin A1, and

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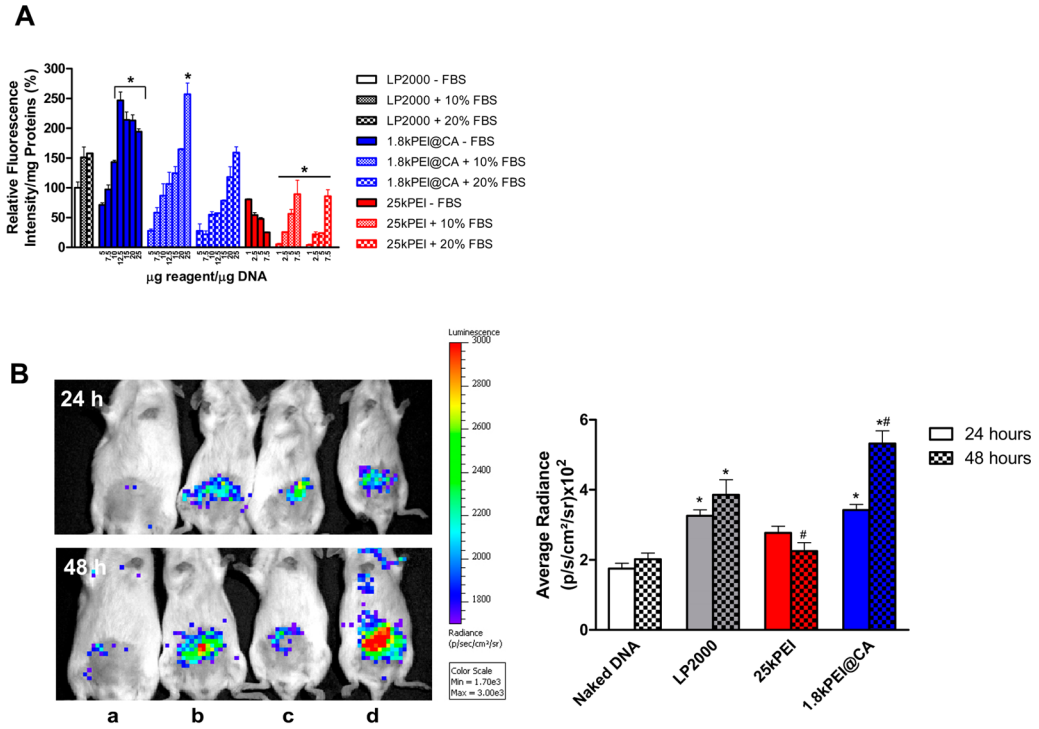

Figure 8. Cell transfection in the presence of serum and in vivo gene transfection of 1.8PEI@CA/DNA polyplexes. (A) CHO-k1 cells were transfected with polyplexes using pEGFP-N3. For each condition, DNA was mixed with the PEI derivatives at the best w/w ratios. As a positive control, a transfection was performed using LP2000. Transfection was carried out in the absence or presence of 10% and 20% FBS. The eGFP fluorescence/protein value for the LP2000 transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 8). *p < 0.05 vs LP2000 transfected cells for each serum concentration. (B) In vivo transfection in mice. Representative luciferase imaging of DB1 mice 24 and 48 h after intravenous tail injection of 50 μg of pGL3-control plasmid as naked DNA (a), LP2000/DNA poliplexes at 1 w/w ratio (b), 25kPEI/DNA polyplexes at 2.5 w/w ratio (c), or 1.8kPEI@CA/DNA polyplexes at 12.5 w/w ratio (d) and average radiance of the abdominal area of the in vivo transfected mice at 24 and 48 h is plotted. Data is shown as means ± SEM (n = 4). *p < 0.05 vs naked DNA transfected mice at 24 h; #p < 0.05 vs LP2000 transfected mice at 48 h.

dialysis membranes (12000 D cutoff) were obtained from Sigma (St Louis, MO, USA). Lipofectamine 2000 (LP2000) was purchased from Invitrogen (Carlsbad, CA, 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). 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.[41] Cholera toxin subunit B-Alexa Fluor 488 was provided by Life Technologies (Paisley, UK).

Optimized Synthesis of 1.8kPEI@CA Complexes. *Method A* - A solution of 1.8kPEI (24 mL) and CA (2 g) in water (16 mL) was prepared and maintained at room temperature for 10 min. After this time the solution was extensively dialyzed against distilled water and lyophilized to yield the 1.8kPEI@CA-RT complexes. *Method B* - Thermal synthesis: A solution of 1.8kPEI (24 mL) and CA (2 g) in water (16 mL) was refluxed for 10 min in an oil bath heated at 105 °C. After cooling the crude was extensively dialyzed against distilled water and lyophilized to yield the 1.8kPEI@CA-reflux complexes. *Method C* - MW-assisted synthesis: A solution of 1.8kPEI (24 mL) and CA (2 g) in water (16 mL) was placed in a 100 mL round-bottom flask. The mixture was irradiated at 400 or 800 W and 105 °C for 10 min in a MW apparatus (MycroSYNTH − Milestone Inc.) with a condenser capping the bottom-flask. After cooling, the crude was extensively dialyzed against distilled water and lyophilized to yield the 1.8kPEI@CA complexes.

Gel Electrophoresis Shift Assay. Unmodified PEI (1.8 kDa and 25 kDa) or 1.8kPEI@CA complexes and DNA were first diluted in HEPES 20 mM pH 7.4. pEGFP-N3 DNA (10 μL at 0.1 mg/mL) was mixed with an equal volume of the PEI-based solutions previously prepared using μg reagent/μg DNA weight (w/w) ratios 0−1.5 and incubated for 30 min at room temperature before the addition of loading buffer (2 μL). An aliquot (5 μL) of each sample of the resulting polyplexes 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. Quantification of the band intensity was performed with the NIH Image Software.[42] A value of 100 was assigned to the intensity of the band corresponding to the control DNA and % binding was calculated as 100 minus % intensity of the nonretarded DNA bands of each sample with respect to the control band. The DNA releaseability from the polyplexes was measured by a heparin replacement assay. Polyplexes were generated as above at a 0.75 w/w ratio and after 30 min of incubation they were mixed with increasing concentrations of heparin sulfate to yield a final concentration that ranges from 10 to 200 μg/mL. Samples were further incubated for 10 min at room temperature and then analyzed by gel electrophoresis as

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described above. A value of 100 was assigned to the intensity of the band corresponding to control DNA and % release was calculated as % intensity of the nonretarded DNA bands of each sample with respect to the control band.

Buffering Capacity of 1.8kPEI@CA Complexes. The polymer solutions were prepared in a 50 mL flask (0.5 mg/mL, 30 mL) and lyophilized. Commercial 1.8kPEI was used as a control. The pH of the resulting solutions was 9.4 for the 1.8kPEI@CA complexes and 10.2 for the control. Without adjusting the initial pH, the solutions were titrated by addition of 5 μL increments of 0.1 M HCl while measuring the pH response with a pH electrode until pH below 3.6. The buffering capacity (β) was calculated according to $\beta = d[HCl]/dpH$ from the plot [HCl] vs pH in the range from 7 to 4.

Ethidium Bromide Exclusion Experiments. Solutions containing 50 μg/mL of pEGFP-N3 plasmid, 0.4 μg/mL ethidium bromide in either 20 mM Na HEPES buffer pH 7.4 or 20 mM Na acetate buffer pH 5.5 were incubated in the presence of increasing amounts of PEI derivatives (0−0.375 μg reagent/μg DNA) for 30 min at room temperature and the fluorescence was quantified with a Shimadzu RF-5301PC fluorimeter using 526 nm (5 nm) and 592 nm (5 nm) as excitation and emission wavelengths, respectively. The percentage of ethidium bromide exclusion from DNA due to the binding to PEI derivatives was calculated as fluorescence of the sample/fluorescence of the control × 100.

DNase I Protection Assays. pEGFP-N3 DNA (10 μL at 0.1 mg/mL) was mixed with the PEI derivatives to give a 0.375 w/w ratio and the resulting mixture was 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 were 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.[42] 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 cells (CHO-k1; ATCC no. CCL-61), rat hepatoma cells FT02B (Darville et al., 1992), L6.C11 rat skeletal muscle myoblasts (ECACC No. 92102119), and normal rat kidney cells (NRK; ATCC no. CRL-6509) were grown at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine plus 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Prior to transfection, cells were seeded in 48 well plates at a density of 1.5×10^4 cells/well 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 PEI derivatives at 2.5−35 w/w ratios at room temperature for 30 min in a final volume of 20 μL. Next, the mixture was diluted to 0.3 mL with DMEM without serum or supplemented with 10% or 20% FBS and added to each well. Cells were incubated with the polyplexes for 5 h. The transfection media was then 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. LP2000 was used as a positive control in gene delivery experiments. LP2000 polyplexes were prepared using 0.8 μL of LP2000 and 0.3 μg of DNA, according to the manufacturer's instructions.

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 450 nm (5 nm) and 510 nm (10 nm) as excitation and emission wavelengths, respectively. In the transfection experiments, fluorescence from nontransfected control cells was subtracted and fluorescence in the positive control using LP2000 polyplexes was assigned a value of 100. Fluorescence results obtained in the transfections with the polyplexes were normalized to this value. Protein concentration was measured using the Bio-Rad Protein Assay.

Cytotoxicity of 1.8kPEI@CA/DNA Polyplexes. Cytotoxicity of the 1.8kPEI@CA/DNA polyplexes was evaluated 24 and 48 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[43] 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 at 24 h normalized to 100% viable.

Cell Internalization Assays and Confocal Microscopy. For the assay of the internalization of 1.8kPEI@CA/DNA polyplexes, CHO-k1 cells were preincubated for 30 min with 14 μM chlorpromazine or 200 μM genistein or 100 nM bafilomycin A1 prior to the addition of the polyplexes. Transfection experiments with the prepared polyplexes were carried out as described above. For the confocal analysis of the internalization routes, CHO-k1 cells were seeded onto coverslips and incubated for 20 min in the presence of Cholera toxin subunit B-Alexa Fluor 488 (10 μg/mL) as a marker of clathrin-independent endocytosis or human transferrin-Alexa Fluor 594 conjugate (20 μg/mL) as a marker of clathrin-dependent endocytosis. 1.8kPEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes at 12.5 w/w ratio were then added, and after 2 h cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and coverslips were mounted on glass slides using Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA). Alternatively, 1.8kPEI@CA was labeled with Rhodamine B isothiocyanate mixed isomers (Sigma-Aldrich) at a 90:0.3 mg ratio following manufactured instructions. Rhodamine-labeled 1.8kPEI@CA was extensively dialyzed against distilled water and lyophilized. CHO-k1 cells were seeded onto coverslips and incubated for 20 min in the presence of Cholera toxin subunit B-Alexa Fluor 488 (10 μg/mL) as a marker of clathrin-independent endocytosis. Then, cells were transfected with Rhodamine labeled-1.8kPEI@CA and Cy5 labeled pEGFP-N3 plasmid DNA complexes at 12.5 w/w ratio. After 2 h, cells were fixed as described above. Coverslips were mounted on glass slides using Vectashield with Hoechst 33258 dye. Confocal microscopy was performed on a Leica TCS-SP5 II multiphoton confocal microscope. To prevent cross-talk of Alexa Fluor 488, Alexa Fluor 594, Rhodamine, Hoechst 33258, or Cy5 chromophores, distinct excitation laser lanes and non-overlapping detection channels were selected for their detection. Under these conditions, no signal overspill among the individual fluorescence channels was observed. For color analysis, images were collected separately in single channel mode using a sequential acquisition mode. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 1024 × 1024. Series were acquired in the *xyz* mode. Data was processed using Leica AF software package.

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Particle Size and Zeta (ζ) Potential Measurements of 1.8kPEI@CA/DNA Polyplexes. Polyplexes were prepared using a DNA solution at 20 μg/mL in HEPES 20 mM pH 7.4. Working solutions of unmodified PEI (1.8k and 25k) and 1.8kPEI@CAs complexes were prepared in HEPES 20 mM pH 7.4 at 2-fold the final concentration in the measurement cuvette. Equal volumes of DNA and polymer solutions (400 μL) were mixed at room temperature, and the sample was vortexed for a few seconds and incubated for 30 min at room temperature. The final DNA concentration was 10 μg/mL and the best transfection w/w ratios were analyzed.

The polyplex hydrodynamic diameter and ζ potential were determined by using a Zetasizer NanoZeta ZS device (Malvern Instrument Ltd., U.K.) working at 25 °C with a He−Ne laser of 633 nm and a scattering angle of 173°. Each data point is taken as an average over three independent sample measurements. For data analysis, dispersant viscosity and refractive index are considered the same as those for pure water at 25 °C (0.8905 mPa/s and 1.333, respectively). The size characterization of the polyplexes was carried out from Dynamic Light Scattering (DLS). For each polyplex, the average hydrodynamic diameter (*Z*-average or cumulant mean) and the polydispersity index (PI) were obtained. These parameters are calculated through a cumulant analysis of the data, which is applicable for narrow monomodal size distributions.[44] With the aim of performing a more complete size characterization of the samples, we also obtained the intensity size distribution from an algorithm present in the Zetasizer software (General Purpose). Such an algorithm uses the non-negative least-squares (NNLS) analysis, which is appropriate for broad monomodal or multimodal size distributions. The intensity size distribution is useful to evaluate mean values and width of the peaks. However, in order to estimate relative amounts of material in separate peaks, number size distribution should be considered. We used the Zetasizer software to convert intensity size distributions into number size distributions by applying Mie theory and using a particle refractive index of 1.45 and a particle absorption at 632 nm of 0.001, the same as for proteins.[45]

Zeta (ζ) potentials were calculated by determining the electrophoretic mobility (by the technique of Laser Doppler Electrophoresis) and then applying the Helmholtz-Smoluchowsky equation.[46] A zeta (ζ) potential distribution as well as an average zeta potential were obtained for each sample.

Size and zeta (ζ) potential measurements were repeated 1 day and 4 days after polyplex formation in order to gather information on the colloidal stability of the system. The prepared polyplexes were stored at 4 °C.

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 2012/707/UE and with the approval of the Committee on Animal Research at the University of Granada (13−1−15−181). Individual mice (n = 4) were injected via the tail vein with 200 μL of LP2000, 25kPEI, or 1.8kPEI@CA/DNA polyplexes containing 50 μg of pGL3 control and prepared at 1, 5, and 12.5 w/w ratios, respectively. 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. After 10 min, 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 2.6 software package (Xenogen). For display, the luminescent image (pseudocolor) was overlaid on a photographic image. For each animal a similar region of interest (ROI) encompassing the abdomen was selected and the average radiance (p/s/cm^2/sr) from that ROI was used as measurement for analysis 24 and 48 h after transfection.

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

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00576.

FT-IR, ^{1}H NMR, and ^{13}C NMR spectra for CA, PEI, and 1.8kPEI@CA. Buffering capacity and colloidal stability versus time of 1.8kPEI@CA/DNA polyplexes. (PDF)

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Notes

The authors declare no competing financial interest.

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