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Physicochemical and biological study of selected hydrophobic polyethylenimine-based polycationic liposomes and their complexes with DNA

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Abstract—Non-viral gene therapy is based on the development of efficient and safe gene carrier systems able to transfer DNA into cells. Polyethylenimine (PEI), the most promising non-viral vector, with its high cationic-charge-density potential is able (1) to compact DNA in complexes (polyplexes) smaller than those formed by liposomes (lipoplexes) and (2) to destabilize the endosomal membrane by a 'proton sponge' effect. Several PEI's hydrophobic modifications were reported in the last several years but in some cases a reduced transfection efficiency was observed. The mechanism underlying this phenomenon is not well understood so far. In order to extensively investigate these mechanisms, we reported a physicochemical and biological study of selected hydrophobic PEI's derivatives grafted with chains of different length and percentages of substitution able to form vesicles (polycationic liposomes) and to bind DNA. Their properties were studied by means of dynamic light scattering, freeze-fracture microscopy, potentiometric titrations, gel retardation assays, polyanion exchange reactions, toxicity assays, in vitro transfections, and fluorescence microscopy. Our results indicate that even if polyplexes are able to pass through the cellular membrane, the stability of PEI's hydrophobic polyplexes likely explain their different transfection efficiency in vitro.

1. Introduction

Gene therapy is essentially based on the development of efficient and safe gene carrier systems able to transfer DNA into cell.[1–4] Viral systems are the most widely used owing to their high gene transfer efficiency. Their main disadvantage is the ability to elicit immunogenic responses.[5,6] Consequently, different non-viral systems were developed in the last years, especially cationic liposomes[7–10] able to complex DNA and deliver it into the cell. The mechanisms of gene transduction are supposed to pass through the formation of cell endosomes after endocytosis of liposome/DNA complex (lipoplex).[11,12] However, one of the major disadvantages of cationic liposomes is their low transfection efficiency due to: (1) DNA degradation in lysosomes, (2) strong cytotoxicity.[13]

Thus, much effort is devoted to improve non-viral techniques and delivery systems,[14] and several cationic polymers have been tested: polylysine and its conjugates,[15–17] DEAE–dextran,[18] dextran–spermine polycations,[19] chitosan,[20] polyamidoamine dendrimers,[21] lipopolyamines,[22–24] and polyethyleneimine (PEI).[25] These molecules differ in chemical composition as well as in the architecture of the polymer backbone which may be linear, randomly branched, dendrimeric, grafted or block-copolymer, and in the number of repeating units

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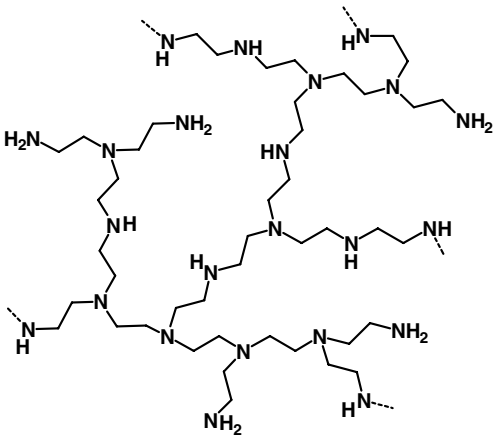
(monomers). As a result, a wide range of different polyplexes can be synthesized and evaluated in order to determine the most efficient systems (Scheme 1).

PEI is an organic polymer with a high cationic charge-density potential able to compact DNA more efficiently than liposomes, leading to small complexes. Once penetrated into cells, PEI destabilizes the endosomal membrane due to the protonation of its numerous amino groups ('proton sponge' effect) releasing DNA in the cytoplasm. Since every third atom is one protonable amino nitrogen, PEI retains a substantial buffering capacity at virtually any pH. The influx of protons and counterions into endosomes leads to membrane lysis and to the release of their internal contents.

Polyethylenimine acid–base properties,[26] DNA complex (polyplex) formation, and ability in gene delivery were reviewed in recent years.[27] It has been reported that the higher is the molecular weight of the polymer used to form complexes, the higher its transfection efficiency but also its cytotoxicity. Therefore, increasing efficiency of transgene expression improving bioavailability of the polyplex and decreasing its toxicity is a major goal.[28] A good compromise between efficiency and toxicity was found for branched PEI with a molecular weight of 25 kDa. Indeed, several authors efficiently employed this polymer for in vitro and in vivo experiments.[25,29] Grafting low molecular weight PEIs with hydrophobic moieties (i.e., cholesteryl moieties) remarkably enhances the transfection efficiency of the otherwise ineffective polymer reducing also its toxicity.[30,31] On the contrary, hydrophobic high molecular weight PEI's derivatives have a reduced transfection efficiency,[32] even if this phenomenon was not completely unraveled so far.

To investigate the reasons of this behavior, and to suggest new synthetic strategies for designing novel non-viral vectors, we prepared several hydrophobic PEI (25 kDa) derivatives grafting the polymer with three different hydrophobic chains (C12, C14, and C16) and three fatty acid residues (lauroyl-, myristoyl-, and palmitoyl-) at different percentages of substitution. A physicochemical and biological study of these molecules and their complexes was carried out by means of dynamic light scattering, freeze-fracture microscopy, gel retardation assays, polyanion exchange reactions, toxicity assays, in vitro transfections, and potentiometric titrations. Our results indicate that the relative stability of hydrophobic PEI's polyplexes is likely correlated with their transfection efficiency in vitro.



Scheme 1. Schematic representation of branched PEI.

2. Results

2.1. Potentiometric titrations

Acid–base properties of PEI's derivatives were examined by means of potentiometric measurements. Taking into account the **P25-C14** series compounds (Fig. 1) 1.54 ml of titrant are required to reach pH 11 when titrating the more basic compounds **P25-C14-3**, 1.43 ml for **P25-C14-6**, 1.23 ml for **P25-C14-15**, while **P25-C14-24**, the less basic compound, required only 1.03 ml. A similar behavior was also observed for **P25-C12** and **P25-C16** series (Fig. 1B) and for **P25-CO12**, **P25-CO14**, and **P25-CO16** (Fig. 1C) ones. No significant differences were reported for samples with the same substitution across different series. Moreover, acid–base properties of PEI grafted with different hydrophobic chains at the lowest percentage of substitution were found to be very close to that of PEI itself suggesting that these systems should interact with DNA in a similar way. Therefore, these data point out that chemical grafting with hydrophobic or fatty acid chains has a marginal role in determining significant variations of polymer acid–base properties at least at the same percentage of substitution.

2.2. Gel electrophoresis

The ability of PEI's derivatives to bind DNA was studied by gel retardation assays (0.8% agarose gel) varying the N/P ratio. The N/P ratio generally indicates the ionic balance between PEI's amino groups (N) and DNA phosphate groups (P) of their complexes, and were calculated considering that 1 μg DNA corresponds to 3 nmol of phosphate, and 1 μl of PEI (or PEI's derivatives) 10 mM (monomer) solution to 10 nmol of amine nitrogen. After DNA complexation by PEI and PEI's derivatives, a fluorescence quenching of the intercalating dye ethidium bromide was observed either by varying the percentage of substitution, the N/P ratios, the chain length or the grafting molecule (data not shown). In order to overcome this problem and study the interaction properties of PEI and PEI's derivatives with DNA, Hoechst 33258 was dissolved in agarose gel instead of ethidium bromide. **P25-C14** series derivatives at different percentages of substitution (3%, 6%, 15%, and 20%) were mixed with DNA at a N/P ratio of 15.5 and run on agarose gel. All derivatives are able to bind DNA without displaying significant differences during complexation (Fig. 2A). Moreover, no significant differences were obtained by varying the N/P ratio (Fig. 2B) or the grafting molecule (Fig. 2C). Following this alternative

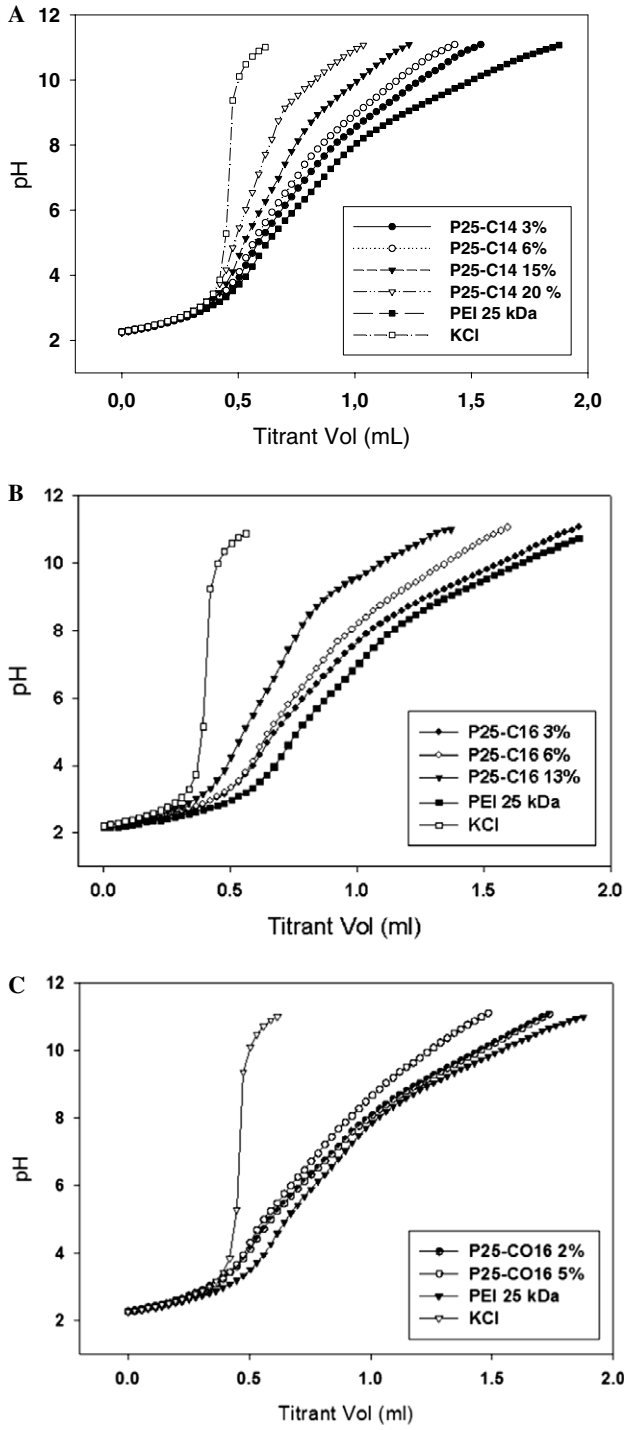


Figure 1. Titration data of (A) **P25-C14**, (B) **P25-C16**, and (C) **P25-CO16** derivatives at 25 ° C in KCl 0.1 M. [HCl] = 0.1 M was used as titrant solution.

staining procedure we were able to report that PEI and PEI's derivatives strongly bind DNA (the binding constant of PEI 50 kDa to DNA was estimated to be $1.2 \times 10^5\ M^{-1}$)[33]. Our findings allowed us to point out that even a small amount of PEI or PEI's derivative is able to completely and strongly bind DNA. In our hands, the N/P = 15.5 ratio maximized the transfection, while a higher ratio induced a greater cytotoxicity. This is in good agreement with what was previously reported by O. Boussif et al.[25] that the maximum transfection activity of PEI was observed at a N/P ratio of 13.5. Moreover, we found that at N/P = 15.5 no free DNA is present in solution due to a complete binding by polycationic molecules. We previously reported that cationic liposomes bearing amino groups on their lipid heads efficiently interact with DNA, leading to hindered adducts unable to migrate on agarose gel.[33] Moreover, due to the efficient complexation by these polycations, free DNA is no more present in solution and the correspondent band was no longer visible on the gel. It has been reported that when similar polycations (ExGen 500, Superfect, other branched PEIs) bind to plasmid DNA, neutralization of negative charges in the phosphate backbone of DNA takes place, and the migration of these large electroneutralized complexes through the gel has not been observed.[34] Even if the loaded complexes bear a net positive charge due to their high N/P ratios, a migration to the cathode was not observed. We tested all of the complexes formed with PEI and PEI's derivatives at nitrogen to phosphate (N/P) ratios of 3.87, 7.75, and 15.5, and we observed that all the molecules form DNA complexes.

2.3. Light-scattering and freeze-fracture measurements

With respect to PEI, hydrophobic and fatty acid derivatives spontaneously aggregate in buffered (pH 7) solutions, giving rise to quite homogeneous spherical particles measurable by dynamic light-scattering technique. The freeze-fracture technique allowed us to extensively investigate the inner structure of these aggregates. By examining the **P25-C14**-series derivatives it was possible to emphasize the liposome-like assembly of such molecules, leading to vesicles that allowed us to classify these systems as 'polycationic liposomes' (Fig. 3). Similar results were also obtained with all of the other synthesized series leading us to hypothesize that this phenomenon is general for these kinds of hydrophobic derivatives.

Examining the dimension of these systems a quite uniform behavior between different derivatives was observed. Taking into account the mean diameters of **P25-C12-3**, **P25-C14-3**, and **P25-C16-3** it was not possible to derive a particular trend as a function of the chain length, and the particle size ranged from 235 up to 480 nm. On the contrary, taking into account the effect of hydrophobic grafting on these molecules, **P25-C14-3**, **P25-C14-6**, **P25-C14-15**, and **P25-C14-20** having an increasing percentage of substitution displayed also different properties. In particular, **P25-C14-3** particles have a mean diameter of 135 nm (±18 nm), **P25-C14-6** of 225 nm (±12 nm), **P25-C14-15** of 218 nm (±15 nm), and **P25-C14-20** of 370 nm (±20 nm). This increasing trend is however much less evident for the fatty acid series, leading us to consider the hydrophobic grafting a determinant in the induction of polyplexes with different dimensions.

Dynamic light scattering allowed us also to measure the DNA control sample (plasmid DNA in DMEM without polycations), used for the preparation of

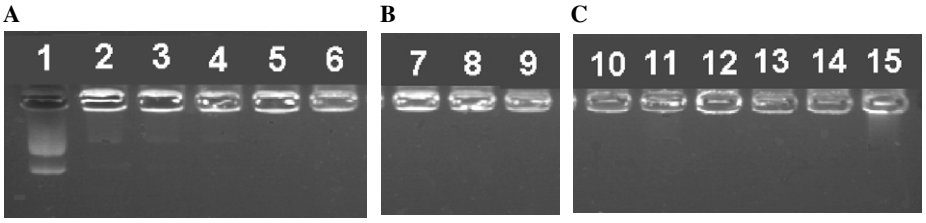


Figure 2. Gel retardation assays (agarose 0.8%, TBE) of selected PEI's derivatives stained with Hoechst 33258. All complexes were formed at N/P ratio = 15.5 unless otherwise specified. (A) Lanes: (1) reference DNA, (2) PEI/DNA, (3) **P25-C14-3**/DNA, (4) **P25-C14-6**/DNA, (5) **P25-C14-15**/DNA, (6) **P25-C14-20**/DNA. (B) Lanes: (7) **P25-C14-3**/DNA (N/P ratio = 15.5), (8) **P25-C14-3**/DNA (N/P ratio = 7.75), (9) **P25-C14-3**/DNA (N/P ratio = 3.87). (C) Lanes: (10) **P25-C12-3**/DNA, (11) **P25-CO12-4**/DNA, (12) **P25-C14-3**/DNA, (13) **P25-CO14-3**/DNA, (14) **P25-C16-3**/DNA, (15) **P25-CO16-2**/DNA.

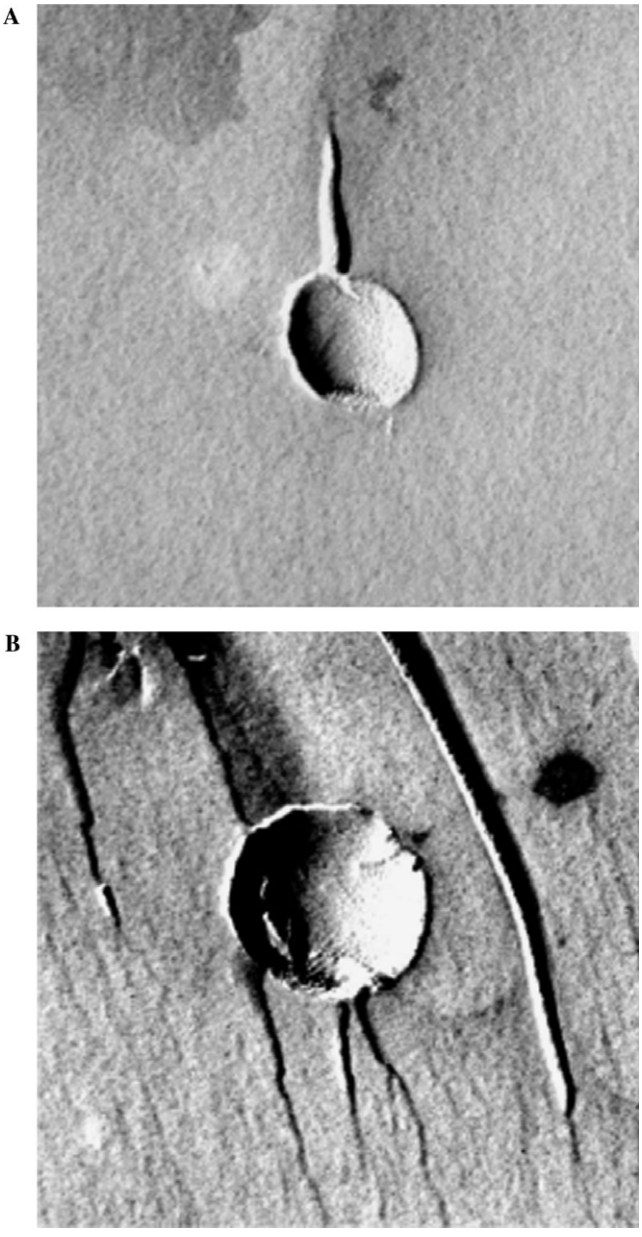


Figure 3. Freeze fracture images of **P25-C14-3** (A) and **P25-C14-6** (B) derivatives outlying the polycation assembly into vesicles.

polyplexes. This sample exhibited only small particles (15–30 nm) that we found to be a result of the serum component used in DMEM, and most likely had no relationship to DNA macromolecules present in the solution.

The size distribution of polyplexes was examined mixing complexes in DMEM at various N/P ratios (3.87, 7.75, 11.6, and 15.5). Only the **P25-C12-3** derivative is reported here but similar results were also obtained with the other series. PEI/DNA polyplex particles have an effective diameter of about 550–580 nm, while **P25-C12-3**/DNA complexes display a different diameter as a function of the N/P ratio. Examining the size distribution of **P25-C12-3**/DNA complexes as a function of different N/P ratios, it was reported that particles at N/P ratio = 15.5 are centered around a maximum of about 597 nm, very similar to that of PEI's, at a N/P ratio of 11.6 are centered at 637 nm, at N/P ratio of 7.75 are centered at 1480 nm, while at a N/P ratio of 3.87 the complex is even larger and centered at 1780 nm (Table 1 and Fig. 4). All polyplexes showed a quite limited size distribution, with a polydispersity index lower than 0.25. These data emphasize that the N/P ratio is an important parameter to take into account when a polyplex of a well-determined size should be used for further experiments.

2.4. Biological assays

In order to verify the toxicity of PEI's derivatives and to evaluate transfection efficiency of PEI–DNA complexes, transient transfection using NIH3T3 fibroblasts was performed and the efficiency was evaluated. PEI did not display a marked toxicity toward this cell line at the concentration used for this assay and we therefore

Table 1. Dynamic light-scattering parameters of **P25-C12-3**/DNA complexes at different N/P ratios: (a) N/P = 15.5, (b) N/P = 11.6, (c) N/P = 7.75, and (d) N/P = 3.87

d (nm)	*G* (*d*)			
	(a)	(b)	(c)	(d)
243.48	46			
374.95	46	11	86	
577.39	100	100	90	58
889.14	34	35	95	95
1369.21	24		100	100
2108.48			95	95
3246.91			90	90
5000			7	10

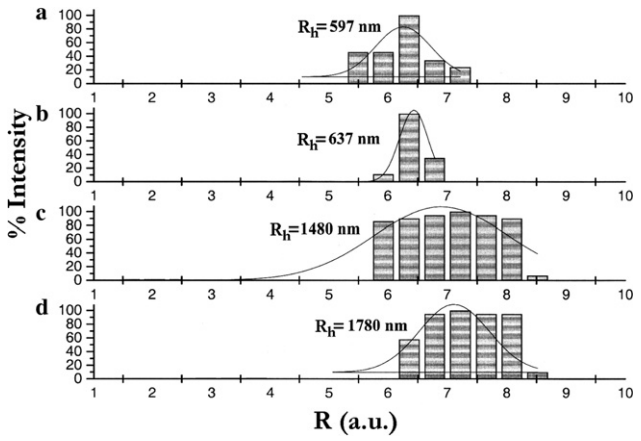


Figure 4. Dynamic light-scattering measurements of **P25-C12-3**/DNA complexes at different N/P ratios: (a) N/P = 15.5, (b) N/P = 11.6, (c) N/P = 7.75, and (d) N/P = 3.87.

considered it as the reference compound. Toxicity of the other compounds (expressed as cell viability) is comparable to that of PEI itself indicating that these compounds are relatively non-toxic at the concentration used (Fig. 5). The translocation of these compounds was also examined fixing NIH3T3 cells after transfection and visualizing DNA with the intercalating dye Hoechst 33258. The presence of DNA complexes to the cell was revealed as fluorescent bodies into the cytoplasm (Fig. 6) quite distinct from genomic DNA. No appreciable differences in translocation ability were observed using PEI or PEI's derivatives.

However, transfection efficiency of PEI's derivatives was lower than that of PEI (Fig. 7). An increasing trend from **P25-C12-3** to **P25-CO12-4** and from **P25-C14-3** to **P25-CO14-3** was observed. These data seem to indicate that fatty acid residues slightly increase transfection efficiency with a maximum observed for **P25-CO14-3**. Taking into account the hydrocarbon series, **P25-C16-3** is the most efficient compound leading us to hypothesize that the chain length is another important parameter that should be carefully considered in order to increase transfection efficiency.

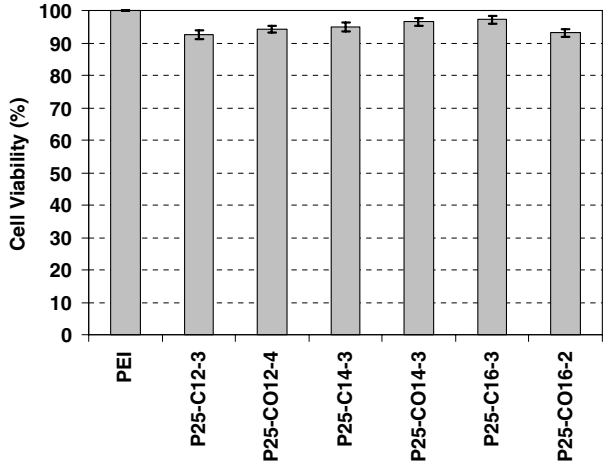


Figure 5. NIH3T3 cell viability (%) of some selected PEI's derivatives.

2.5. Polyelectrolyte exchange reactions

The stability of polyplexes against polyelectrolyte exchange reactions was evaluated incubating PEI, **P25-C12-3**, and **P25-CO14-3** polyplexes (N/P = 15.5) with poly-L-aspartic acid (PAA) at 37 °C. Aliquots were collected at regular intervals (1, 2, 3, 4, 18, and 24 h) and run on 0.8% agarose gel. After 3 h from the addition of PAA, PEI/DNA polyplexes were no longer visible in the well while, on the contrary, the fluorescence of **P25-C12-3**/DNA complexes is still present (Fig. 8A). This behavior is more evident at 18 or 24 h when the signal of **P25-C12-3**/DNA complex is still very intense (Fig. 9). Similar results were also obtained for **P25-CO14-3**/DNA polyplex (Fig. 10). In order to exclude that some enzymatic degradation processes (i.e., DNAses from solvents or PAA itself) might have determined the disappearance of PEI's polyplex fluorescence signal, DNA solutions (in PBS) with and without PAA in the absence of polycations were loaded on agarose gel. Figure 8B shows that DNA remained unaltered even after 6 h both in the presence or absence of PAA emphasizing that the lack of fluorescence observed in Figures 8A and 9 is somehow ascribable to the interaction of PAA with the polyplex leading to DNA exchange.

Moreover, some inference on the kinetic of DNA polyplex formation in the presence of PAA may be done. After 10 min from the addition of PAA, PEI has already formed the complex with DNA and no residual free DNA was present (lane 2 in Fig. 8A). On the contrary, in the presence of PAA **P25-C12-3** completely forms the polyplex after 1 h from the addition of DNA (lane 8 in Fig. 8A), while for **P25-CO14-3**/DNA polyplex (Fig. 10) a complete DNA binding occurs after 3 h from the addition of PAA.

3. Discussion

Previously reported studies focusing on differential scanning calorimetry and electron microscopy demonstrated that hydrophobic modification of PEI leads to a stronger interaction with cell phospholipids with respect of the unmodified polymer.[35] The presence of long lipophilic chains on PEI moiety can therefore be expected to improve and strengthen the interaction of polyplexes with the cell surface. Despite numerous efforts toward the design and development of novel non-viral vectors, a careful investigation about the relationships between structural properties and biological efficiency was not reported so far. With our physicochemical and biological study we tried to assess not only the role of hydrophobic grafting and how this may determine the final polymer structure, but also the influence these modifications may have in DNA complexation and transport into the cell.

Taking into account the acid–base behavior of a series of hydrophobic **P25** derivatives (**P25-C14**, **P25-C16**,

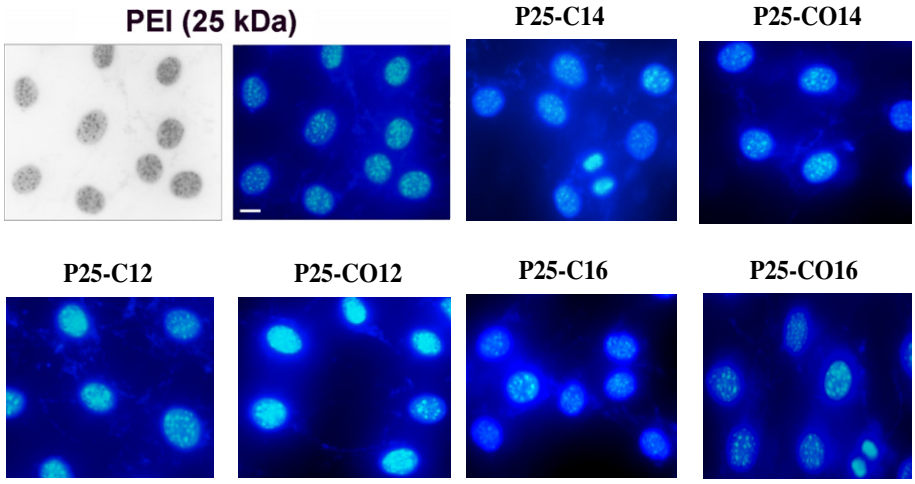


Figure 6. Visible and fluorescence microscopy of NIH3T3 cells transfected with PEI. Fluorescence microscopy of selected PEI's derivatives (**P25-C12**, **P25-CO12**, **P25-C14**, **P25-CO14**, **P25-C16**, **P25-CO16**) polyplexes stained with Hoechst 33258. White scale bar represents 10 μm.

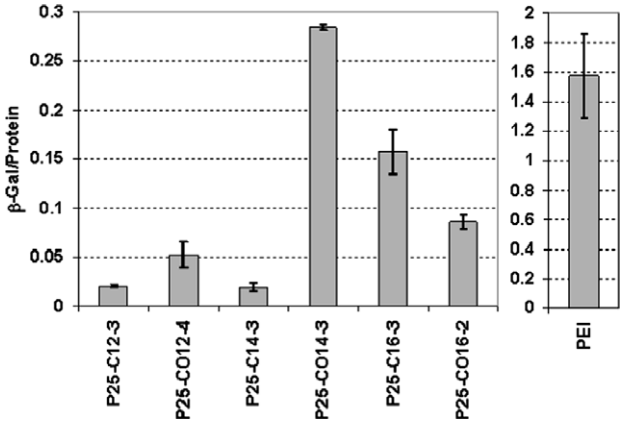


Figure 7. β-Gal expression efficiency of NIH3T3 cell (measured as OD after 24 h) transfected with some selected PEI's derivatives.

and **P25CO16**), it was possible to derive a general trend valid for all the derivatives: the higher is the percentage of substitution the minor is the basicity of the resulting molecule, due to conversion of some amino groups from primary to secondary. The reduced basicity is also inferred taking into account the volume of titrant added to PEI's derivative solutions to reach the same pH value (Fig. 1). However, the reduced basicity of these compounds due to conversion of amino groups from primary to secondary (and likely from secondary to tertiary in the highly substituted ones) does not interfere with the polyplex formation process (Fig. 2B) and this is likely due to the strong base nature of PEI ('proton sponge'). Hence, the acid–base properties suggest that once the DNA/PEI or DNA/PEI's derivatives complexes penetrate into cells and the endosome is swelled, the DNA release mechanism should be quite similar.

The structure and dimensions of PEI, PEI's derivatives, and their DNA polyplexes were studied in order to verify if a different kind of chemical substitution may account for some structural changes. Dynamic light-scattering and freeze-fracture techniques allowed us to report that all PEI's derivatives form vesicular struc-

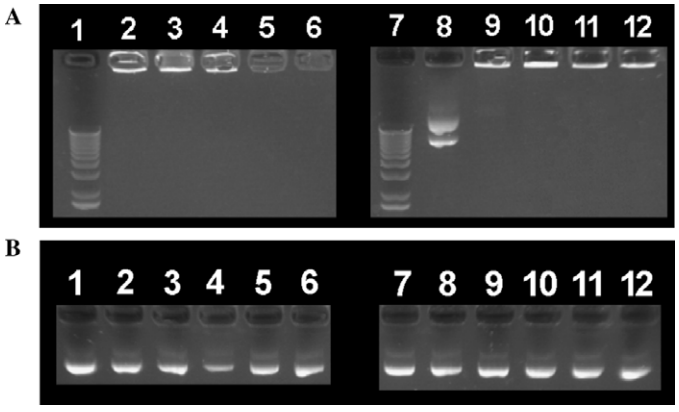


Figure 8. (A) Gel retardation assays (agarose 0.8%, TBE) of PEI and **P25-C12-3** polyplexes prepared at N/P ratio = 15.5 after the addition of PAA. Lanes: (1) DNA ladder (1 kb), (2) PEI/DNA (t = 10 min), (3) PEI/DNA (t = 1 h), (4) PEI/DNA (t = 2 h), (5) PEI/DNA (t = 3 h), (6) PEI/DNA (t = 4 h), (7) DNA ladder (1 kb), (8) **P25-C12-3**/DNA (t = 10 min), (9) **P25-C12-3**/DNA (t = 1 h), (10) **P25-C12-3**/DNA (t = 2 h), (11) **P25-C12-3**/DNA (t = 3 h), and (12) **P25-C12-3**/DNA (t = 4 h). (B) Gel retardation assays (agarose 0.8%, TBE) of DNA (in PBS) with (lanes 1–6) and without (lanes 7–12) the addition of PAA. (1) **DNA/PAA** (t = 1 h), (2) (t = 2 h), (3) (t = 3 h), (4) (t = 4 h), (5) (t = 5 h), (6) (t = 6 h), (7) **DNA** (t = 1 h), (8) (t = 2 h), (9) (t = 3 h), (10) (t = 4 h), (11) (t = 5 h) and (12) (t = 6 h).

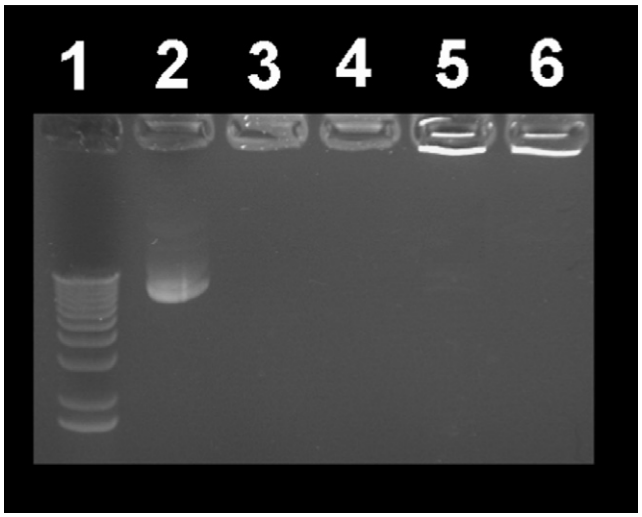


Figure 9. Gel retardation assays (agarose 0.8%, TBE) of PEI and **P25-C12-3** polyplexes prepared at N/P ratio = 15.5 after the addition of PAA. Lanes: (1) DNA ladder (1 kb), (2) reference DNA, (3) PEI/DNA (t = 18 h), (4) PEI/DNA (t = 24 h), (5) **P25-C12-3**/DNA (t = 18 h), and (6) **P25-C12-3**/DNA (t = 24 h).

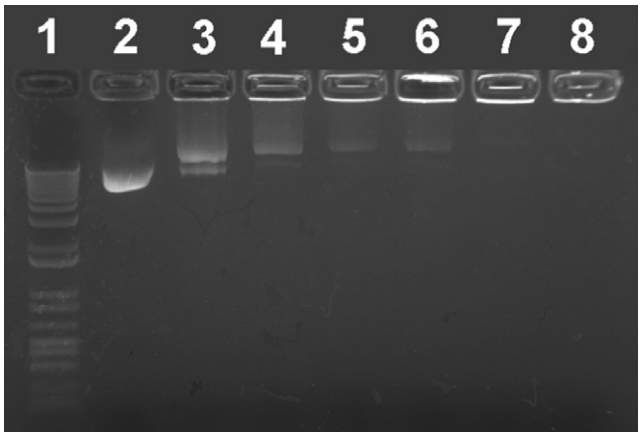


Figure 10. Gel retardation assays (agarose 0.8%, TBE) of **P25-CO14-3** polyplexes prepared at N/P ratio = 15.5 after the addition of PAA. Lanes: (1) DNA ladder (1 kb), (2) reference DNA, (3) **P25-CO14-3**/DNA (t = 10 min), (4) **P25-CO14-3**/DNA (t = 1 h), (5) **P25-CO14-3**/DNA (t = 2 h) (6) **P25-CO14-3**/DNA (t = 3 h) (7) **P25-CO14-3**/DNA (t = 18 h), and (6) **P25-CO14-3**/DNA (t = 24 h).

tures, the so-called 'polycationic liposomes,' and that in the case of **P25-C14** series these structural assemblies (Fig. 3) resemble the structure of a cationic polylysine hydrophobic derivative (also called 'dendron') observed by means of transmission electron microscopy (TEM).[36] This hydrophobic polylysine derivative self-assembles in water forming unusual small vesicular structures, that the authors called 'dendrisomes,' whose chemical structure is very close to those of PEI's derivatives examined by us. These dendrisomes, having a diameter of about 311 nm and a double layer structure ranging from 6.6 to 10 nm, show a very close correlation with the structural features obtained with our derivatives.

Examining the dimensional changes occurring by varying the N/P ratios during PEI and PEI's derivatives DNA-polyplex formation (Fig. 4), we observed that PEI's derivatives are able to form smaller complexes when the N/P ratio increases. All PEI's derivatives displayed an analogous trend and the complex size seems to depend only on the N/P ratio. Moreover, in good agreement with the literature, our biological assays outlined that the optimal N/P ratio for transfections is 15.5, where the smallest polyplex particles were reported to occur. These data indicate that polyplex dimensions and their N/P ratios should be carefully considered in order to maximize transfection efficiency. In order to verify the differences during DNA complexation existing between PEI and PEI's derivatives, we carried out some gel retardation assays on 0.8% agarose gel. Unexpectedly, all polyplexes displayed a complete fluorescence quenching of ethidium bromide (EtBr) used to intercalate DNA. In agreement with our data, recent findings reported that when the adduct DNA/EtBr interacts with PEI, almost complete quenching of dye fluorescence was observed when PEI is added at N/P > 3.[37] This quenching effect is reduced by 50% when a minor groove dye (i.e., Hoechst 33258) was used instead of EtBr. Unfortunately, this phenomenon was not discussed in details and a reasonable explanation of this behavior was not provided. We may hypothesize that a greater binding constant is required in order to avoid the displacement (or quenching) of dye molecules by PEI considering that the reported DNA association constant with EtBr is estimated to be $2.5(0.1) \times 10^6$ M^{-1}, while that with Hoechst 33258 is $1.3(0.1) \times 10^8$ M^{-1}.[38] Moreover, it has been reported that this quenching effect may be justified considering that the interaction of PEI with DNA leads to an alteration of the B-type structure of DNA and induces a transition to the Z-type hence preventing a further intercalation of dye molecules.[39] Unfortunately, such a transition was not reported so far with Hoechst 33258 and no conclusions may be drawn up in this case.

For these reasons we set up a modified agarose gel retardation assay in which we dissolved Hoechst 33258 instead of ethidium bromide. As expected, we were able to observe a residual fluorescence signal due to DNA intercalation by Hoechst 33258 that led us to monitor the complexation behavior of the synthesized derivatives (Fig. 2). All derivatives efficiently bind DNA even at low N/P ratios and no significant differences with respect to PEI were observed, as expected from their similar structures and acid–base properties.

Toxicity (Fig. 5), translocation ability (Fig. 6), and transfection efficiency (Fig. 7) of selected hydrophobic PEI's derivatives were also investigated. According to the literature, we confirmed that the higher substituted PEI's derivatives displayed similar biological properties (toxicity, translocation ability, and transfection efficiency) with respect to the lowest ones.[32] Therefore, we decided to focus our studies and discussion only on those PEI's derivatives with the lowest degree of substitution: these compounds displayed the major differences with respect to PEI, being however the minor substituted ones. It was clear that even a small degree of substitution, namely 2% or 3%, was enough to significantly alter some physicochemical properties and, as a

consequence, also biological ones. PEI and PEI's derivatives displayed a comparable low toxicity and the internalization ability of these compounds was examined following transfection experiments of NIH3T3 cells with fluorescence microscopy. DNA was visualized and revealed as fluorescent bodies into the cytoplasm (Fig. 6) after intercalating complexes with Hoechst 33258. We found that hydrophobic chemical grafting, regardless of the percentage of substitution, does not alter the polyplex's ability to migrate (translocate) into the cell.

Interestingly, different transfection efficiencies were measured for some selected PEI's derivatives with PEI being the most effective. A slight increasing trend was reported going from **P25-C12-3** to **P25-CO12-4** and from **P25-C14-3** to **P25-CO14-3** even if an opposite trend was observed for **P25-C16-3** and **P25-CO16-2**. These data indicate that even a little percentage of substitution (namely 3%) is enough to decrease the transfection efficiency of these compounds.

In order to explain the reasons of such decreased efficiency, not ascribable to polyplex dimensions, toxicity or translocation ability, we investigated the mechanism and the kinetic of DNA release from polyplexes by means of a polyanion exchange reaction assay on agarose gel (Figs. 8 and 9). This in vitro assay was designed to obtain additional information on polyplexes relative stability. Poly-L-aspartic acid (PAA) is a polyanion able to form electrostatic interactions with PEI and PEI's derivatives. Hence, a competitive DNA exchange reaction should be expected once comparable amounts of PAA are incubated with polyplexes for a determined period of time. If this exchange takes place effectively, after loading samples on agarose gel a band of the exchanged DNA (free DNA) should be observable. Unfortunately, no free-DNA signal was visible on the gel leading us to hypothesize that a different reaction between these two species should occur. In particular, in order to explain this strange phenomenon, we considered that PEI's polyplexes at a N/P ratio = 15.5 have an overall net positive charge and that PAA may interact with PEI's polyplex without determining a DNA exchange but inducing only a binding rearrangement between these four species (PEI, DNA, fluorescent dye, and PAA). Due to the complexity of the binding pattern, it was difficult to predict how these species interact with each other in the formation of the final adduct. Nevertheless, polyelectrolyte exchange assays allowed us to underline the different behavior of PEI's and PEI's derivatives polyplexes. In particular, incubating PEI's DNA-polyplex with PAA up to a period of 24 h, we reported the progressive formation of a non-fluorescent supramolecular adduct indicating that a rapid (3 h) rearrangement has occurred. On the contrary, PAA has a negligible (or null) effect on **P25-C12-3**/DNA complex incubated for the same period (Figs. 8 and 9) and the fluorescence due to the DNA-polyplex persisted up to 24 h. Similar results were also obtained with **P25-CO14-3**/DNA polyplex (Fig. 10) pointing out that hydrocarbon or fatty acid chains likely impart a similar effect.

Finally, these findings suggest that hydrophobic grafting confers to PEI's polyplexes a great stability against competitive exchange reactions by polyanions. It is reasonable to think that the kinetic inertness of PEI's derivatives polyplexes from being displaced by a polyanion may reflect a greater stability with respect to PEI's complexes and, as a consequence, a reduced propensity to release DNA once penetrated into the cell. This behavior could hence reasonably explain the reduced transfection efficiency displayed by hydrophobic PEI's derivatives.

4. Conclusions

PEI's derivatives (hydrophobic and fatty acids ones) reported in this work display low toxicities and form complexes with DNA. The acid–base properties of these compounds are dependent on the degree of substitution but in general they behave quite similarly to PEI itself showing only a slightly reduced basicity. All of the PEI's derivatives reported in this work are able to form vesicles ('polycationic liposomes') of definite dimensions and measurable by dynamic light-scattering and freeze-fracture techniques. Moreover, their complexes with DNA may have different sizes as a function of N/P ratio even if at certain values (i.e., N/P = 15.5) the mean DNA-complex diameter is comparable with that of PEI's polyplex. Independently from the degree of substitution, the reported PEI's derivatives are able to migrate into the cell, as demonstrated by fluorescence assays but showed different transfection efficiencies. Polyanion exchange assay was found to be a useful tool to investigate the mechanism of DNA release from polyplexes and to give a reasonable interpretation of transfection efficiency results in vitro.

In the near future, taking advantage of the ability of these derivatives to form vesicles and efficiently penetrate into the cell, we will devote a closer look to their application as useful polycationic liposomes for the delivery of some therapeutic molecules (i.e., antisense oligos, siRNA, etc.) or contrast agents[40,41] (for diagnostic and therapeutic purposes).

5. Experimental

5.1. General

All reagents and solvents were used as received without further purification. High molecular weight branched polyethylenimine (PEI 25 kDa), cellulose dialysis tubing, lauryl bromide (97%), myristyl bromide (97%), cetyl bromide (97%), lauroyl chloride (97%), myristoyl chloride (98%), palmitoyl chloride (98%), and poly-L-aspartic acid (PAA, 15–50 kDa) were purchased from Sigma–Aldrich.

^{1}H and ^{13}C NMR spectra were recorded on a 300-MHz Varian instrument. PEI and PEI's derivatives were used as 45 mM monomer aqueous solution, neutralized with HCl, and filtered through a Millipore 0.2 μm membrane.

5.2. Potentiometric titrations

PEI and PEI's derivatives (45 mM monomer aqueous solution) were titrated in a jacketed cell at 25 °C at 0.1 M ionic strength (KCl) using a Crison Titrino MicroTT to control the addition of acid or base. The concentration of HCl and NaOH solutions was standardized following reported procedures.[42] Data were analyzed and plotted with Sigma Plot Ver.8.0.

5.3. Gel retardation assay

Agarose gel retardation assays were prepared following standard procedures. Mixtures containing an appropriate amount of PEI or PEI's derivatives solutions (2 mg/mL) and plasmid DNA (β-Gal) were incubated at 37° C for 5 min, mixed with loading buffer, loaded into 0.8% agarose gel (in TBE buffer), and run for 45 min at 100 V. A stock solution (10 mg/ml) of the intercalating dye Hoechst 33258 was prepared dissolving the pure solid in DMSO. DNA bands were visualized by UV illumination. PEI's derivatives and the corresponding complexes are indicated in the text with the same acronyms.

5.4. Polyanion exchange reaction

Agarose gel electrophoresis was also performed loading mixtures of 2 μg of poly-L-aspartic acid (PAA) for each microgram of DNA, to the PEI/DNA and PEI's derivatives/DNA complexes 10 min after their formation. Lanes were loaded with solutions collected after 1, 2, 3, 4, 18, and 24 h from PAA addition.

5.5. Particle size measurement

Light-scattering experiments were performed by means of a classic set-up with a correlator (BI9000AT, Brookhaven) operating with logarithmic sample time spacing. The light source was a 10-mW He–Ne laser (wavelength 632.8 nm) and the data were acquired at a scattering angle of 90°. In the dynamic light-scattering technique, the normalized time autocorrelation of the intensity of the scattered light is measured. For a dilute suspension of monodisperse particles it decays exponentially with a decay rate $G = 2q^{2D}$, where q is the magnitude of the scattering wave vector and D the diffusion coefficient. The hydrodynamic radius of the diffusing particles is obtained through the Stokes–Einstein relationship. For a polydisperse system, when the single exponential decay is no longer valid, the size and size distribution of the diffusing particles were found using the standard data analysis program CONTIN, in terms of a continuous distribution of exponential decay times.

PEI and PEI's derivatives solutions were prepared by thin-film method as previously reported.[43,44] Briefly, polymers were dissolved in 20 ml CH_3Cl/CH_3OH (1:1) and the solvent was removed by evaporation. Distilled water was added to the lipid film in order to have a final polymer concentration of 2 mg/ml. Polyplex solutions with N/P ratio of 3.87, 7.75, 11.6, and 15.5 were prepared by mixing the appropriate amount of PEI or PEI's derivatives solution (2 mg/ml) to 2 μg of plasmid DNA (β-Gal) in 400 μl of Dulbecco's modified Eagle's medium (DMEM). After 15 min of incubation, polyplex solutions were analyzed.

5.6. Freeze-fracture microscopy

Selected PEI's derivatives were examined by means of freeze-fracture microscopy: samples were soaked with 30% glycerol, frozen into partially solidified Freon 22, fractured in a freeze-fracture device (−105 °C, 10^{-6} mmHg), and replicated by evaporation from a platinum/carbon gun. The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids, and examined with a Philips CM 10 transmission electron microscope.

5.7. Cell culture, plasmids, and transfections

Mouse NIH3T3 fibroblasts were cultured at 37 °C in DMEM (Invitrogen) supplemented with 10% DONOR calf serum (Clontech). Transient transfections were performed as follows. In 24-well plates, 2×10^5 cells were transfected with 1 μg of pEGFP (Clontech) and 1 μg of CMV β-Gal plasmid, as control of transfection efficiency, plus 2 μg of PEI or equivalent amounts of different PEI's derivatives, in 500 μl medium total volume. Briefly, DNA was added to PEI, or PEI's derivatives, suspended in 100 μl of medium without serum, incubated for 10 min and then added to cells. After 3 h, medium was changed. Cells were harvested 48 h after transfection, GFP fluorescence examined to check that transfection occurred, and the β-Gal activity was assayed on whole cell extracts in accord to manufacturer's protocol (Promega) and to literature.

Cytotoxicity assay was performed plating 1×10^4 cells in 96-multi-well plates and transfected with 1/5 of volumes used for transfections in 24-well plates. Viability was assayed spectrophotometrically with the Cell Titer Blue kit (Promega) according to manufacturer's protocol.

5.8. Fluorescence microscopy

Internalization assays were performed transfecting cells with a reporter plasmid. After 24 h from transfection, cells were fixed and stained with Hoechst 33258 (1 μg/ml in PBS) that marks all DNA double stranded molecules. Cells were examined with a ZEISS Axioskop fluorescence microscope provided with a Zeiss Axiocam. The magnitude used was 40×.

5.9. Synthesis

5.9.1. P25-C12-3. A solution of lauryl bromide, obtained after the addition of 1-bromododecane (0.178 g, 0.72 mmol) to dichloromethane (50 cm^3), was added over a period of 3 h to a solution of PEI (1 g, 0.04 mmol) in dichloromethane (100 cm^3). The resulting mixture was stirred at rt for 1 day, and the solvent was then concentrated up to a volume of ca.15–20 cm^3 under reduced pressure. The product was first dialyzed against an ethanol/water (1:1) solution (4 L for 5 times), then against

pure water for 1 day. The mixture was concentrated and lyophilized affording **P25-C12-3** as a white solid. Yield 1.02 g (91%). Integration of the proton magnetic resonance (^{1}H NMR) spectrum of the product in D_2O indicated 3 mol % of lauryl groups ($C_{12}H_{25}$; 1–1.6 ppm) per residue mol of ethylenimine unit (C_2H_4N; 2.2–3.2 ppm) in the polymer. The substituted polymer may be represented by the stoichiometric formula $(C_2H_4N)_m(C_{12}H_{25})_{0.03m}$, $m = 595$. Anal. Calcd: C, 60.15; H, 10.16; N, 29.69. Found: C, 60.41; H, 10.20; N, 30.03. ^{1}H NMR (D_2O): δ (ppm) 0.92 (b, CH_3), 1.30 (b, –$(CH_2)_8$–), 2.90 (b, –CH_2CH_2N–). ^{13}C NMR (D_2O): δ (ppm) 14.4, 23.1, 29.9, 30.2, 32.3, 37.8, 39.5, 44.0, 45.9, 47.7, 49.3, 51.4, 52.0, 53.1, 54.2, 69.2, 75.8.

5.9.2. P25-C12-6. 1-Bromododecane (0.356 g, 1.43 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.25 g (92%). $(C_2H_4N)_m(C_{12}H_{25})_{0.06m}$, $m = 595$. Anal. Calcd: C, 62.60; H, 10.62; N, 26.78. Found: C, 62.52; H, 10.40; N, 25.93. ^{1}H NMR (D_2O): δ (ppm) 0.95 (b, CH_3), 1.32 (b, –$(CH_2)_8$–), 2.97 (b, –CH_2CH_2N–). ^{13}C NMR (D_2O): δ (ppm) 14.7, 23.4, 30.0, 30.2, 32.1, 37.5, 39.6, 44.2, 45.8, 47.8, 49.4, 51.6, 52.2, 53.0, 54.1, 69.1, 75.6.

5.9.3. P25-C12-24. 1-Bromododecane (1.423 g, 5.71 mmol) was added to a solution of PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.28 g (90%). $(C_2H_4N)_m(C_{12}H_{25})_{0.24m}$, $m = 595$. Anal. Calcd: C, 70.89; H, 12.19; N, 16.93. Found: C, 71.12; H, 12.40; N, 17.33. ^{1}H NMR (D_2O): δ (ppm) 0.94 (b, CH_3), 1.31 (b, –$(CH_2)_8$–), 2.98 (b, –CH_2CH_2N–). ^{13}C NMR (D_2O): δ (ppm) 14.8, 23.3, 30.1, 30.3, 32.4, 37.3, 39.4, 44.0, 45.6, 47.5, 49.6, 51.5, 52.1, 53.2, 54.2, 69.1, 75.5.

5.9.4. P25-C14-3. 1-Bromotetradecane (0.198 g, 0.72 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.10 g (92%). $(C_2H_4N)_m(C_{14}H_{29})_{0.03m}$, $m = 595$. Anal. Calcd: C, 60.60; H, 10.23; N, 29.16. Found: C, 61.09; H, 10.2; N, 29.31. ^{1}H NMR (D_2O): δ (ppm) 0.89 (b, CH_3), 1.29 (b, –$(CH_2)_{10}$–), 1.84 (b, –(CH_2)–), 2.84 (b, –CH_2CH_2N–), 3.32 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 14.8, 23.2, 30.2, 30.3, 32.5, 37.2, 39.6, 44.2, 45.6, 47.5, 49.4, 51.4, 52.2, 53.2, 54.4, 69.2, 75.5.

5.9.5. P25-C14-6. 1-Bromotetradecane (0.396 g, 1.43 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.27 g (91%). $(C_2H_4N)_m(C_{14}H_{29})_{0.06m}$, $m = 595$. Anal. Calcd: C, 63.32; H, 10.74; N, 25.94. Found: C, 63.69; H, 11.01; N, 26.11. ^{1}H NMR (D_2O): δ (ppm) 0.91 (b, CH_3), 1.31 (b, –$(CH_2)_{10}$–), 1.85 (b, –(CH_2)–), 2.93 (b, –CH_2CH_2N–), 3.30 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 14.5, 23.0, 30.4, 32.3, 37.6, 39.8, 44.3, 45.3, 47.2, 49.6, 51.0, 52.1, 53.2, 54.2, 69.2, 75.2.

5.9.6. P25-C14-15. 1-Bromotetradecane (0.990 g, 3.57 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.73 g (87%). $(C_2H_4N)_m(C_{14}H_{29})_{0.15m}$, $m = 595$. Anal. Calcd: C, 68.69; H, 11.74; N, 19.57. Found: C, 68.54; H, 11.05; N, 19.31. ^{1}H NMR (D_2O): δ (ppm) 0.93 (b, CH_3), 1.30 (b, –$(CH_2)_{10}$–), 1.83 (b, –(CH_2)–), 2.96 (b, –CH_2CH_2N–), 3.35 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 14.0, 23.2, 30.2, 32.3, 37.5, 39.3, 44.0, 45.0, 47.5, 49.5, 51.7, 52.3, 53.2, 54.3, 69.3, 75.6.

5.9.7. P25-C14-20. 1-Bromotetradecane (1.3 g, 4.76 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.98 g (85%). $(C_2H_4N)_m(C_{14}H_{29})_{0.20m}$, $m = 595$. Anal. Calcd: C, 70.71; H, 12.11; N, 17.18. Found: C, 70.64; H, 11.95; N, 17.31. ^{1}H NMR (D_2O): δ (ppm) 0.92 (b, CH_3), 1.32 (b, –$(CH_2)_{10}$–), 1.80 (b, –(CH_2)–), 2.94 (b, –CH_2CH_2N–), 3.34 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 14.3, 23.5, 30.0, 32.4, 37.2, 39.7, 44.2, 45.0, 47.3, 49.4, 51.5, 52.3, 53.0, 54.3, 69.2, 75.5.

5.9.8. P25-C16-3. 1-Bromohexadecane (0.220 g, 0.72 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.08 g (88%). $(C_2H_4N)_m(C_{16}H_{33})_{0.03m}$, $m = 595$. Anal. Calcd: C, 61.04; H, 10.31; N, 28.66. Found: C, 60.84; H, 10.75; N, 27.91. ^{1}H NMR (D_2O): δ (ppm) 0.9 (b, CH_3), 1.29 (b, –$(CH_2)_{12}$–), 2.66 (b, –CH_2CH_2N–), 3.46 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 13.9, 23.0, 30.5, 32.8, 37.4, 39.3, 44.7, 45.5, 47.4, 49.7, 51.5, 52.4, 53.2, 54.3, 69.5, 75.6.

5.9.9. P25-C16-6. 1-Bromohexadecane (0.436 g, 1.43 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.21 g (84%). $(C_2H_4N)_m(C_{16}H_{33})_{0.06m}$, $m = 595$. Anal. Calcd: C, 64.0; H, 10.85; N, 25.15. Found: C, 64.24; H, 10.75; N, 25.91. ^{1}H NMR (D_2O): δ (ppm) 0.87 (b, CH_3), 1.23 (b, –$(CH_2)_{12}$–), 2.60 (b, –CH_2CH_2N–), 3.43 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 14.2, 23.8, 30.9, 33.2, 37.8, 39.5, 44.9, 45.7, 47.5, 50.1, 51.3, 52.4, 53.1, 54.5, 69.8, 75.9.

5.9.10. P25-C16-13. 1-Bromohexadecane (0.945 g, 3.09 mmol) was added to PEI (1 g, 0.04 mmol). Yield 1.54 g (79%). $(C_2H_4N)_m(C_{16}H_{33})_{0.13m}$, $m = 595$. Anal. Calcd: C, 68.63; H, 11.70; N, 19.66. Found: C, 68.94; H, 10.95; N, 20.01. ^{1}H NMR (D_2O): δ (ppm) 0.92 (b, CH_3), 1.34 (b, –$(CH_2)_{12}$–), 2.7 (b, –CH_2CH_2N–), 3.5 (b, –(CH_2)–). ^{13}C NMR (D_2O): δ (ppm) 13.4, 23.6, 30.7, 32.4, 37.0, 39.5, 44.5, 45.5, 47.5, 49.5, 51.0, 52.0, 53.2, 54.0, 69.6, 75.8.

5.9.11. P25-CO12-4. For the synthesis of this compound a slightly different procedure was followed as the correspondent acyl chloride was used instead of the alkyl bromide. Glassware was oven-dried prior to use and reactions were carried out under an inert atmosphere. A solution of lauroyl chloride (0.208 g, 0.95 mmol) in dichloromethane (50 cm^3) was added dropwise, over a period of 3 h, to a solution of PEI (1 g, 0.04 mmol) in dichloromethane (100 cm^3). The resulting mixture was stirred at rt for 2 h, and the solvent was then concentrated up to a volume of ca. 15–20 cm^3 under reduced pressure. The product was first dialyzed against an ethanol/water (1:1) solution (4 L for 5 times), then against pure water for 1 day. The mixture was concentrated and

lyophilized affording **P25-CO12-4** as a white solid. Yield 1.16 g (96%). Integration of the proton magnetic resonance (^{1}H NMR) spectrum of the product in CD_3OD indicated 4 mol % of lauroyl groups ($C_{11}H_{23}$; 1–1.6 ppm) per residue mol of ethylenimine unit (C_2H_4N; 2.2–3.2 ppm) in the polymer. The substituted polymer may be represented by the stoichiometric formula $(C_2H_4N)_m(C_{12}H_{23}O)_{0.04m}$, $m = 595$. Anal. Calcd: C, 60.33; H, 10.04; N, 28.32. Found: C, 61.04; H, 10.86; N, 29.01. ^{1}H NMR (CD_3OD): δ (ppm) 0.81 (b, CH_3), 1.21 (b, –$(CH_2)_8$–), 1.51 (b, –(CH_2)–), 2.59 (b, –CH_2CH_2N–), 3.37 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.6, 23.8, 27.1, 30.6, 30.8, 33.1, 37.3, 38.6, 39.5, 41.1, 47.9, 50.6, 51.2, 53.3, 54.3, 70.8, 77.6, 176.5.

5.9.12. P25-CO12-8. Lauroyl chloride (0.416 g, 1.90 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.33 g (90%). $(C_2H_4N)_m(C_{12}H_{23}O)_{0.08m}$, $m = 595$. Anal. Calcd: C, 62.71; H, 10.38; N, 24.64. Found: C, 62.92; H, 10.82; N, 24.41. ^{1}H NMR (CD_3OD): δ (ppm) 0.85 (b, CH_3), 1.23 (b, –$(CH_2)_8$–), 1.52 (b, –(CH_2)–), 2.60 (b, –CH_2CH_2N–), 3.39 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.9, 24.0, 27.3, 30.3, 30.5, 33.0, 37.3, 38.5, 39.5, 41.3, 47.7, 50.7, 51.2, 53.0, 54.8, 71.0, 77.9, 177.2.

5.9.13. P25-CO12-16. Lauroyl chloride (0.833 g, 3.81 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.7 g (93%). $(C_2H_4N)_m(C_{12}H_{23}O)_{0.16m}$, $m = 595$. Anal. Calcd: C, 65.94; H, 10.84; N, 19.64. Found: C, 66.22; H, 11.12; N, 19.21. ^{1}H NMR (CD_3OD): δ (ppm) 0.82 (b, CH_3), 1.32 (b, –$(CH_2)_8$–), 1.61 (b, –(CH_2)–), 2.54 (b, –CH_2CH_2N–), 3.30 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 15.2, 24.6, 28.0, 30.0, 30.7, 33.9, 37.0, 38.8, 39.5, 41.5, 47.0, 50.4, 51.5, 53.0, 54.5, 71.3, 80.3, 178.3.

5.9.14. P25-CO12-24. Lauroyl chloride (1.25 g, 5.71 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 2.0 g (89%). $(C_2H_4N)_m(C_{12}H_{23}O)_{0.24m}$, $m = 595$. Anal. Calcd: C, 68.12; H, 11.15; N, 16.26. Found: C, 68.03; H, 10.91; N, 16.0. ^{1}H NMR (CD_3OD): δ (ppm) 0.83 (b, CH_3), 1.30 (b, –$(CH_2)_8$–), 1.60 (b, –(CH_2)–), 2.55 (b, –CH_2CH_2N–), 3.31 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 15.0, 24.5, 28.3, 30.2, 30.4, 33.6, 37.4, 38.8, 39.4, 41.2, 47.3, 50.5, 51.5, 53.5, 54.9, 71.5, 80.7, 178.0.

5.9.15. P25-CO14-3. Myristoyl chloride (0.176 g, 0.71 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.11 g (94%). $(C_2H_4N)_m(C_{14}H_{27}O)_{0.03m}$, $m = 595$. Anal. Calcd: C, 60.07; H, 10.02; N, 28.91. Found: C, 60.36; H, 10.29; N, 28.34. ^{1}H NMR (CD_3OD): δ (ppm) 0.82 (b, CH_3), 1.22 (b, –$(CH_2)_{10}$–), 1.53 (b, –(CH_2)–), 2.60 (b, –CH_2CH_2N–), 3.38 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.7, 23.8, 27.1, 30.9, 33.2, 37.3, 39.6, 41.2, 45.4, 47.9, 50.9, 51.4, 53.4, 54.5, 55.6, 70.9, 77.6, 176.1.

5.9.16. P25-CO14-4. Myristoyl chloride (0.235 g, 0.95 mmol) was added to a solution of PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.14 g (92%). $(C_2H_4N)_m(C_{14}H_{27}O)_{0.04m}$, $m = 595$. Anal. Calcd: C, 60.90; H, 10.14; N, 27.69. Found: C, 61.03; H, 10.25; N, 27.94. ^{1}H NMR (CD_3OD): δ (ppm) 0.80 (b, CH_3), 1.29 (b, –$(CH_2)_{10}$–), 1.55 (b, –(CH_2)–), 2.61 (b, –CH_2CH_2N–), 3.40 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.9, 23.9, 27.1, 31.3, 33.0, 37.2, 39.6, 41.4, 45.8, 48.0, 50.4, 51.5, 53.4, 54.6, 55.6, 71.3, 77.3, 176.3.

5.9.17. P25-CO14-8. Myristoyl chloride (0.470 g, 1.90 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.34 g (91%). $(C_2H_4N)_m(C_{14}H_{27}O)_{0.08m}$, $m = 595$. Anal. Calcd: C, 63.59; H, 10.53; N, 23.70. Found: C, 63.05; H, 10.27; N, 23.54. ^{1}H NMR (CD_3OD): δ (ppm) 0.81 (b, CH_3), 1.25 (b, –$(CH_2)_{10}$–), 1.50 (b, –(CH_2)–), 2.62 (b, –CH_2CH_2N–), 3.35 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 15, 24.1, 27.4, 31.2, 33.0, 37.3, 39.7, 41.2, 45.2, 48.4, 51.0, 51.4, 53.0, 54.7, 55.6, 70.2, 77.0, 175.6

5.9.18. P25-CO14-18. Myristoyl chloride (1.06 g, 4.29 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.85 g (90%). $(C_2H_4N)_m(C_{14}H_{27}O)_{0.18m}$, $m = 595$. Anal. Calcd: C, 67.77; H, 11.15; N, 17.49. Found: C, 67.27; H, 11.02; N, 17.34. ^{1}H NMR (CD_3OD): δ (ppm) 0.83 (b, CH_3), 1.23 (b, –$(CH_2)_{10}$–), 1.53 (b, –(CH_2)–), 2.61 (b, –CH_2CH_2N–), 3.33 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.2, 23.4, 27.1, 30.5, 33.4, 37.4, 39.6, 41.0, 45.4, 47.3, 50.4, 51.3, 53.6, 54.8, 56.0, 70.4, 77.0, 176.5.

5.9.19. P25-CO16-2. Palmitoyl chloride (0.130 g, 0.47 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 0.96 g (85%). $(C_2H_4N)_m(C_{16}H_{31}O)_{0.02m}$, $m = 595$. Anal. Calcd: C, 59.50; H, 9.94; N, 29.87. Found: C, 59.42; H, 9.27; N, 29.53. ^{1}H NMR (CD_3OD): δ (ppm) 0.80 (b, CH_3), 1.19 (b, –$(CH_2)_{12}$–), 1.51 (b, –(CH_2)–), 2.56 (b, –CH_2CH_2N–), 3.35 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 14.4, 23.2, 26.8, 30.4, 32.9, 37.0, 39.3, 41.0, 45.0, 47.4, 50.5, 51.4, 53.0, 54.3, 55.4, 70.6, 77.5, 175.9.

5.9.20. P25-CO16-5. Palmitoyl chloride (0.330 g, 1.20 mmol) was added to PEI (1 g, 0.04 mmol) following the previously reported procedure. Yield 1.10 g (83%). $(C_2H_4N)_m(C_{16}H_{31}O)_{0.05m}$, $m = 595$. Anal. Calcd: C, 62.28; H, 10.36; N, 25.88. Found: C, 62.09; H, 9.97; N, 25.75. ^{1}H NMR (CD_3OD): δ (ppm) 0.81 (b, CH_3), 1.23 (b, –$(CH_2)_{12}$–), 1.53 (b, –(CH_2)–), 2.62 (b, –CH_2CH_2N–), 3.35 (b, –(CH_2)–). ^{13}C NMR (CD_3OD): δ (ppm) 15.0, 24.1, 27.8, 31.5, 33.7, 37.4, 40.2, 41.0, 45.4, 47.6, 51.4, 51.8, 53.0, 54.5, 55.4, 71.2, 77.3, 177.3.

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