Palmitic Acid-Modified Poly-L-Lysine for Non-Viral Delivery of Plasmid DNA to Skin Fibroblasts

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Palmitic acid conjugates of poly-L-lysine (PLL-PA) were prepared, and their ability to deliver plasmid DNA into human skin fibroblasts was evaluated *in vitro*. The conjugates were capable of condensing a 4.7 kb plasmid DNA into 50−200 nm particles (mean ± SD = 112 ± 34 nm), which were slightly smaller than the particles formed by PLL (mean ± SD = 126 ± 51 nm). Both PLL and PLL-PA were readily taken up by the cells, but PLL-PA delivered the plasmid DNA into a higher proportion of cells. DNA delivery was found to be reduced by endocytosis inhibitor Brefeldin A, suggesting an active mechanism of particle uptake. Using enhanced green fluorescent protein (EGFP) as a reporter gene, PLL-PA was found to give the highest number of EGFP-positive cells among several carriers tested, including polyethyleneimine, Lipofectamine-2000, and an adenovirus. Although some carriers gave a higher percentage of EGFP-positive cells than PLL-PA, they were also associated with higher toxicities. We conclude that PLL-PA is a promising gene carrier for non-viral modification of human fibroblasts.

Gene therapy aims to reconstitute a missing or defective gene with a correct copy in a host genome.[1] This is possible when one utilizes biocompatible gene carriers to transport desired genes into target cells.[2] Viral carriers have been preferred by clinicians for this purpose because viruses have evolved to effectively transfect mammalian cells and to facilitate intracellular trafficking of the genetic material for sustained gene expression. The viral carriers are, however, associated with important drawbacks. They have the potential to give rise to uncontrolled proliferation of modified cells,[3] cause inflammation at the site of administration, and raise immunological reactions against the modified cells.[4] These concerns have limited the application of gene therapy only to a select set of disorders, most notably cancers, whose potential benefits of the therapy clearly justify the possible adverse effects associated with viral gene delivery. Synthetic biomaterials capable of delivering an exogenous DNA into mammalian cells have been pursued as a clinically safer alternative to viral carriers.[5] Polymeric biomaterials with cationic charges can effectively condense a DNA molecule into nanosize particles, which facilitates the intracellular uptake of DNA. The polymers are more compatible with physiological systems because they cause minimal host reactions, but their low effectiveness has hampered their utility in a clinical setting. More effective carriers capable of transferring exogenous DNA into target cells will greatly facilitate the application of gene therapy.

This study was conducted to engineer effective non-viral carriers for modification of clinically useful cells. The non-viral vectors were designed by combining a prototypical cationic polymer (poly-L-lysine, PLL), which displays a strong DNA binding property, with an endogenous lipid, palmitic acid (PA; $HOOC\text{-}(CH_2)_{14}\text{-}CH_3$). PA is utilized by mammalian cells for intracellular protein trafficking; by introducing a hydrophobic moiety to hydrophilic (i.e., water-soluble) proteins during post-translational modification, proteins transport is facilitated through lipid-based sub-cellular membranes on their way to the site of activity.[6] We reasoned that incorporating this natural lipid molecule into a cationic polymer should result in a biomimetic mechanism for facilitated transport of exogenous DNA into cells. The feasibility of this approach was explored by using a model plasmid encoding enhanced green fluorescent protein (pEGFP-N2) and skin fibroblasts as a clinically useful cell target. These cells are actively pursued in human gene therapy protocols[7] because they are readily harvested from patients, expanded *in vitro*, and grafted into a host without systemic immunosuppressants. Skin fibroblasts can be manipulated *ex vivo* to construct functional tissues for tissue replacement[8] or used for constitutive delivery of systemic gene products.[9] As with other cells, gene delivery into fibroblasts has been primarily achieved with viral vectors, but in this study, we show that palmitylated PLL (PLL-PA) is capable of delivering an effective dose of exogenous DNA into cells, is compatible with cellular growth in culture, and can sustain transgene expression in a significant (10−50%) fraction of fibroblasts.

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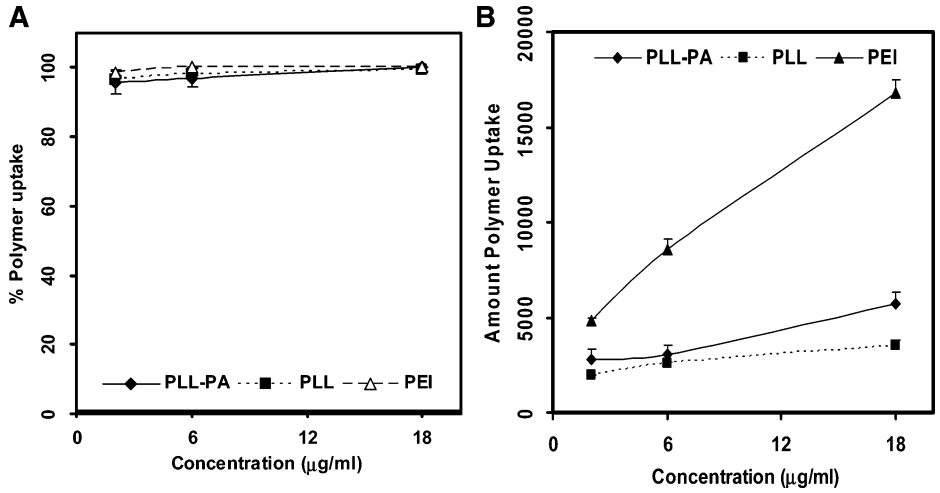
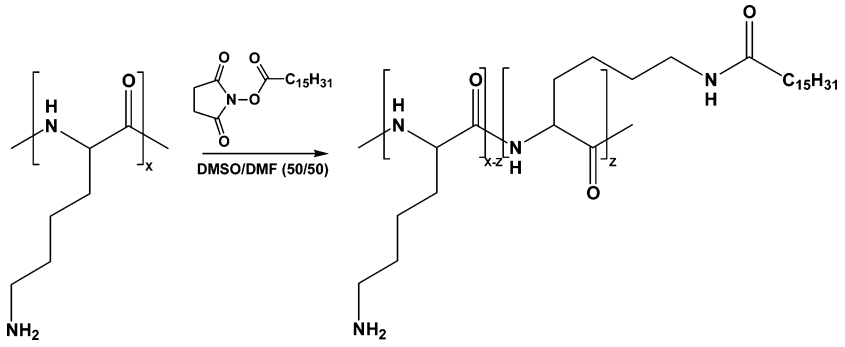


Figure 1. (A) Cellular association of FITC-labeled polymers (mean ± SD; $n = 3$). All polymers gave >95% FITC-positive cells irrespective of the concentration tested. (B) Absolute polymer (in arbitrary fluorescence units) associated with FITC-positive cells. A direct relationship between the cell-associated polymer amount and the polymer concentration incubated with the cells was evident.

Scheme 1



PA was grafted to 25 kDa of PLL (25 mg) by reacting the polymer with the *N*-hydroxysuccinimide ester of PA (<10% amine equivalent) in 50:50 DMF/DMSO (2 mL) for 2 h (Scheme 1; see ref 10[10] for details of the synthesis and characterization). Excess ethyl ether was added to precipitate the product, which was washed with ethyl ether and dried under vacuum at room temperature. The composition of the final product was determined by ^{1}H NMR with D_2O as the solvent. The proton shifts specific for PA (δ ~0.8 ppm; −C*H*$_3$) and PLL (δ ~4.3 ppm; −NH-C*H*-CO) were integrated and normalized for the Hs in each peak to obtain the extent of PA substitution. The PA substitution was effectively controlled by the amount of *N*-hydroxy-succinimide ester of PA in the reaction mixture.[10] PLL-PA, up to 16.2 PA/PLL (mol/mol), was soluble in aqueous buffers so that this polymer was chosen for further investigation in this study.

To determine the cellular uptake of polymers, human skin fibroblasts were cultured in DMEM containing 10% FBS, 100 U/mL Penicillin, and 100 μg/mL Streptomycin (see Supporting Information for detailed procedures). PLL and PLL-PA were labeled with fluorescein isothiocyanide (FITC), according to the manufacturer's instructions (PIERCE), along with another cationic polymer, polyethyleneimine (PEI). PEI, a relatively effective but toxic carrier, was used for the comparison to PLL-based polymers. The fibroblasts were incubated with 2−18 μg/mL of the FITC-labeled polymers, and the percentage of cells displaying polymer uptake were quantified by flow cytometry. Flow cytometry was calibrated so that the cells not exposed to polymers displayed ~1% uptake as a background. Almost all cells in the population (>90%) displayed significant polymer-associated fluorescence at all polymer concentrations (Figure 1A). This was indicative of the homogeneity of the fibroblast population with respect to polymer binding. As expected, the extent of polymer binding, given by the average fluorescence for the FITC-positive cells, was proportional to the polymer concentration in medium (Figure 1B). Although polymers appeared to display differential propensity for binding (given by levels of intracellular fluorescence, Figure 1B), this data cannot be used as a relative measure of uptake efficiency because of the differences in the FITC-labeling efficiency of individual polymers (data not shown). It was important to note that the uptake study was conducted in the presence of a protein-rich medium (10% serum), and the proteins did not appear to impede polymer binding/uptake even at low polymer concentration (2 μg/mL).

A critical requirement for cationic polymers for effective gene delivery is their ability to condense string-like DNA molecules into compact nanosize particles suitable for cellular uptake. Atomic force microscopy (AFM) was used to investigate the complexation between the polymers and the plasmid DNA. A 4.7 kb plasmid (pEGFP-N2) incorporating an EGFP and a kanamycin resistance gene was replicated in DH5-α *E. coli* strain for this purpose.[11] Freshly cleaved mica surfaces were used to deposit polymer/DNA complexes, which were prepared by complexing equal amounts of plasmid and polymer for 30 min. Particles smaller than ~20 nm were ignored in this analysis because these were also visualized with 3 mM NaCl solution alone and were likely to represent NaCl crystals formed under analysis conditions. An equivalent diameter for the larger

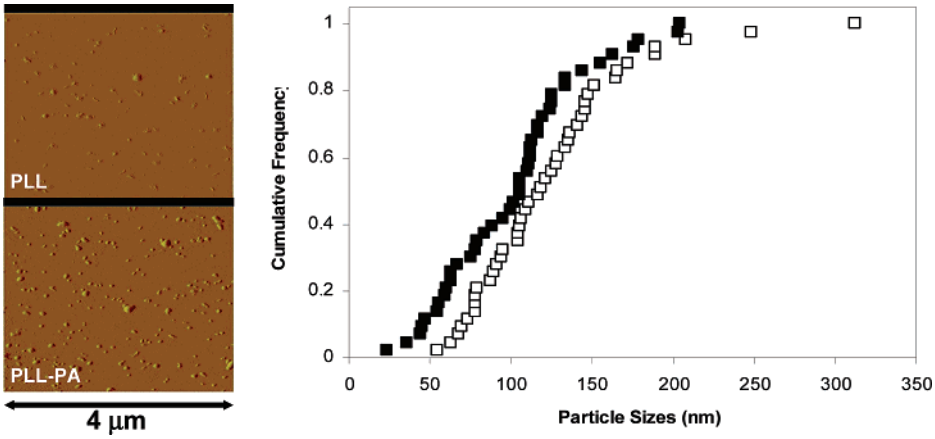


Figure 2. Size of particles formed by the condensation of pEGFP-N2 with PLL and PLL-PA. Representative images for PLL- and PLL-PA-complexed pEGFP-N2 particles are shown on the left, along with the cumulative distribution of particle sizes formed by these polymers. Sample preparations were performed in triplicate.

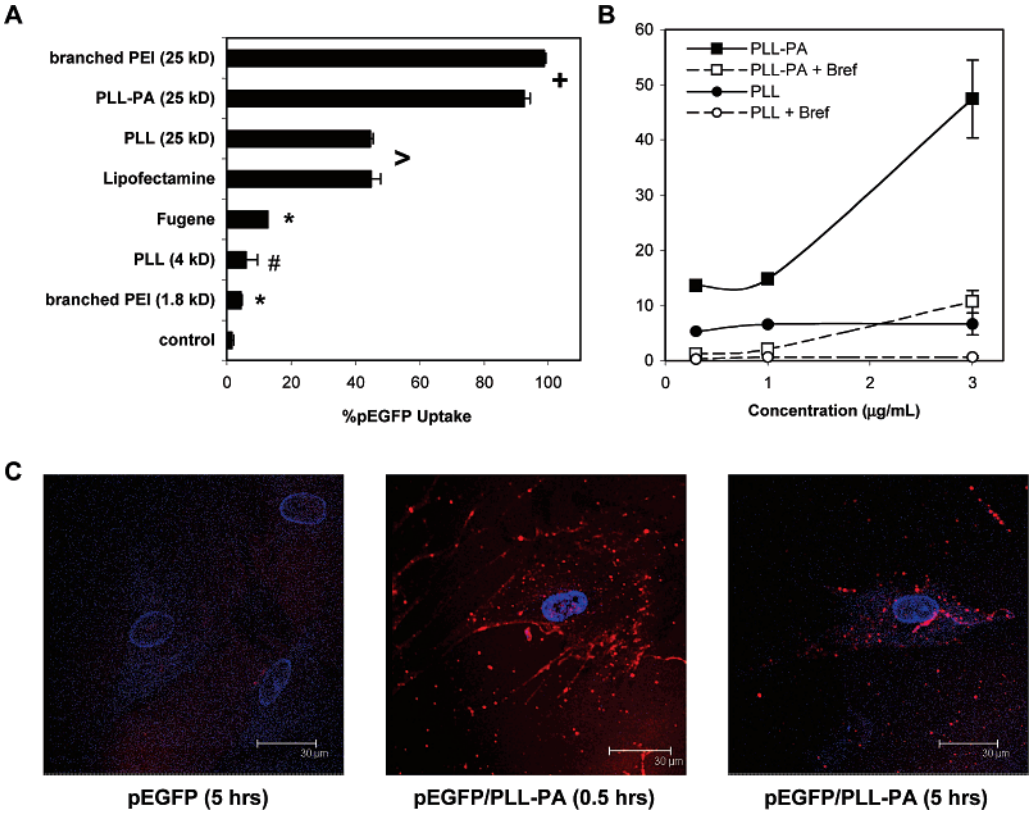


Figure 3. (A) Cellular delivery of Cy5.5-labeled pEGFP-N2 by non-viral carriers (mean ± SD). Significant variations among the chosen carriers were evident, with PEI and PLL-PA giving the most cell-associated plasmid. (#) $p > 0.2$. (*) $p < 0.05$. (>) no significant difference between PLL (25 kDa) and Lipofectamine-2000. (+) PEI vs PLL-PA, $p < 0.05$. (B) Effect of Brefeldin A on cell-associated pEGFP-N2, as investigated by flow cytometry. Note that for both PLL and PLL-PA, the endocytosis inhibitor Brefeldin A (3.7 μM) reduced the percentage of cells positive for pEGFP-N2 after 8 h of incubation. (C) Confocal microscopy images of fibroblasts exhibiting pEGFP-N2 uptake (cell nuclei, blue; polyplexes, red). (Left) Fibroblasts incubated with pEGFP-N2 (5 h) without any carriers. (Middle) Fibroblasts incubated with pEGFP-N2/PLL-PA complexes for 0.5 h. (Right) Fibroblasts incubated with pEGFP-N2/PLL-PA complexes for 5 h.

particles was measured assuming a spherical geometry. The particles were in the size range of 55−312 nm (mean ± SD = 126 ± 51 nm) for PLL and 50−200 nm (mean ± SD = 112 ± 34 nm) for PLL-PA (Figure 2; $p < 0.01$, Student's *t*-test), suggesting that PA modification led to more compact particles. The distribution of the particles was uniform, with no preferential size range. A greater degree of heterogeneity was reported by Chan et al.[12] for PLL/plasmid particles, ranging from ~20 nm to as large as 800 nm (depending on the N/P ratio). Miyata et al.,[13] however, reported PLL/DNA particles in the 80−300 nm range, similar to our results. Unlike tursoid-like particles reported in some studies, our particles were all spherical and compact. Under similar conditions, PEI gave particles ranging in size from 94 to 499 nm (mean ± SD: 270 ± 64 nm), and the naked plasmid without any polymer displayed the expected string-like structure (>1000 nm in length) with no visible particle formation (not shown). These particle sizes were consistent with the sizes found suitable for cellular uptake.[14]

The capability of the polymers to deliver a plasmid cargo into the cells was then evaluated. After labeling pEGFP-N2 with Cy5.5 according to the manufacturer's directions (Amersham), the plasmid was formulated with the carriers in 150 mM NaCl, incubated with the cells for 24 h, and the percentage of cells positive for the labeled plasmid was determined by flow

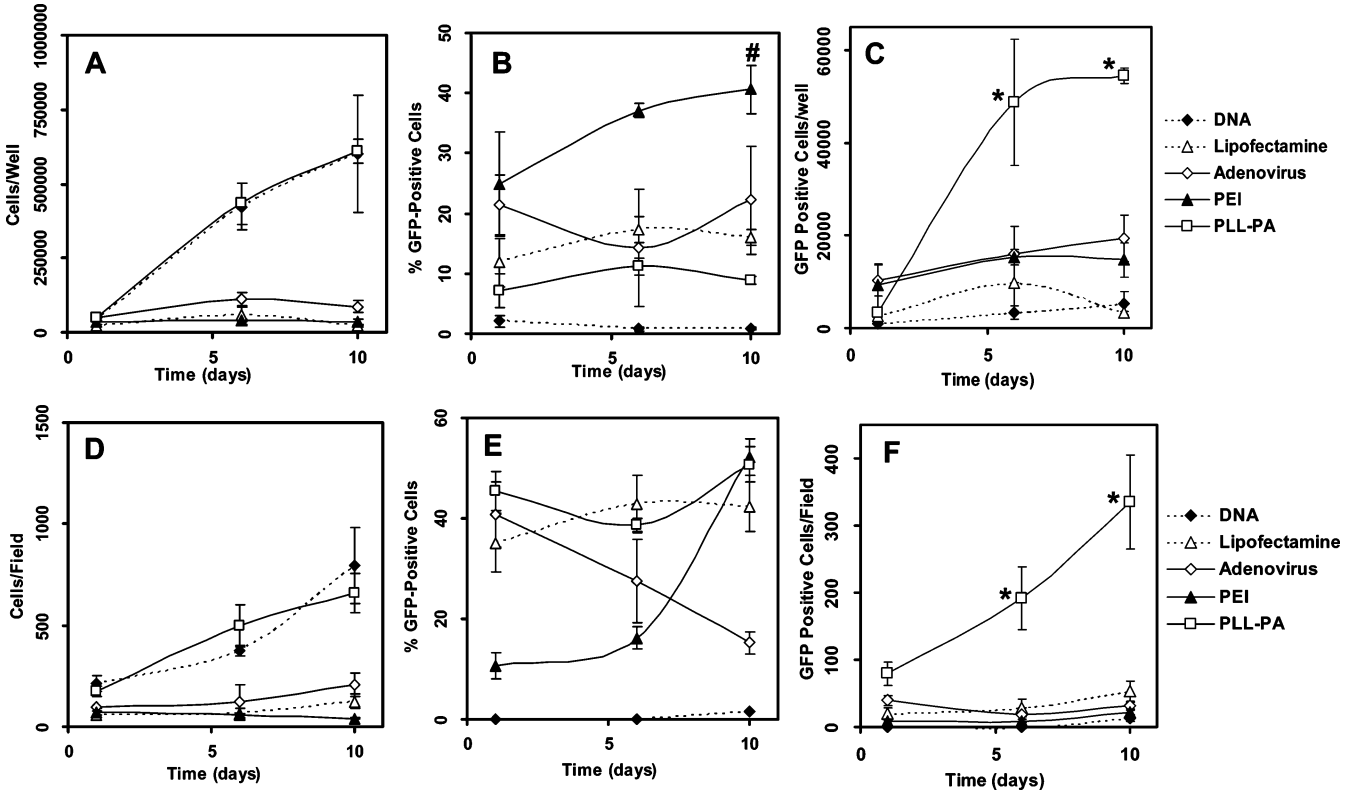


Figure 4. Transfection of fibroblasts with pEGFP-N2/polymer complexes, as assessed by flow cytometry (A, B, and C) and epifluorescent microscopy (D, E, and F). Note that cells exposed to pEGFP-N2 alone and the pEGFP-N2/PLL-PA complex exhibited robust growth with no significant difference between the two groups (A and D). The number of cells recovered from these two groups was significantly higher than that from the other groups. The percentage of EGFP-positive cells was variable among the carriers (B and E), but the total number of cells positive for EGFP (based on direct cell numbers per well or number of cells in a standard microscopic field) was highest for the PLL-PA carrier (C and F). (#) $p < 0.05$ vs Adenovirus, $p < 0.001$ vs Lipofectamine-2000 on day 10 (B). (*) $p < 0.02$ vs Adenovirus on day 6 and 10 in C, and $p < 0.02$ vs Lipofectamine-2000 on day 6 and 10 in F.

cytometry. The flow cytometry was calibrated so that only ~1% of the cells displayed pEGFP-N2 association in the absence of any carrier (equivalent to cells incubated with no plasmid). pEGFP-N2 alone was not expected to penetrate into the cells because of its non-compact nature and the anionic charge of both the cell surface and the plasmid DNA. Among the carriers tested, PEI and PLL-PA were most effective in delivering the plasmid cargo to the cells (Figure 3A). This was followed by PLL (25 kDa) and Lipofectamine-2000, a cationic lipid formulation most widely used as a commercial transfection agent. Other carriers tested, including short-branched PEI (1.8 kDa), PLL (4 kDa), and Fugene (a cationic lipid formulation), were relatively ineffective as carriers. The most effective carriers PEI and PLL-PA were able to modify >90% of the cells, again indicating a relatively uniform delivery of the plasmid cargo to the cells. PEI and PLL-PA displayed effective polymer uptake as well as a good ability to carry a plasmid payload into the cells. However, this was not the case for PLL because the extent of plasmid delivery seemed to be less than the extent of polymer delivery. To determine if cell-associated fluorescence actually represented internalized pEGFP-N2, Cy5.5-labeled pEGFP-N2 was complexed with PLL and PLL-PA and incubated with the cells in the presence of Brefeldin A, a known inhibitor of cellular endocytosis at the trans-Golgi network.[15] After 8 h of incubation, a significant reduction in cell-associated pEGFP-N2 was observed with both carriers as a result of Brefeldin A treatment (Figure 3B), suggesting internalization of the complexes to be a significant reason for cell-associated fluorescence from flow cytometry. Confocal microscopy was additionally used to investigate the presence of pEGFP-N2 in the fibroblasts. Using AlexaFluor 546-labeled pEGFP-N2, there was no cell-associated pEGFP-N2 after 0.5 h (not shown) and 5 h of incubation (Figure 3C) of the plasmid in the absence of any carriers. pEGFP-N2 complexes with the PLL-PA were visualized as red-fluorescent particles associated with the cells at both 0.5 and 5 h time points (Figure 3C). Whereas the pEGFP-N2/PLL-PA particles were present at the cell periphery at the initial 0.5 h time point, internalized particles adjacent to the cellular nuclei were clearly evident after 5 h of incubation. Unlike the initially observed distinct particles, a significant fraction of the polyplexes inside the cell was aggregated after 5 h of incubation. Taken together, these observations indicated a significant internalization of the polyplexes formed by PLL-PA in skin fibroblasts.

The capability of PLL-PA for EGFP expression in fibroblasts was evaluated next. The pEGFP-N2/PLL-PA formulation was compared to Lipofectamine-2000, PEI and an adenoviral system expressing GFP. Two complementary methods, one based on flow cytometry (cells grown in 6-well plates) and another based on epifluorescent microscopy (cells grown on glass coverslips), were utilized for the detection of EGFP-positive cells. Gene expression was followed during a 10-day period, and cell growth was quantified during this period on the basis of either direct cell counts with a hemocytometer (for cells used in flow cytometry) or the number of cells visualized in the microscopic field of coverslips (for cells used for microscopy). Control cells exposed to pEGFP-N2 alone exhibited ~20-fold increase in cell numbers under the experimental conditions (Figure 4A), indicating robust cell proliferation without any adverse effect of the plasmid DNA. The growth of the cells exposed to pEGFP-N2/PLL-PA was not altered as compared to cells treated with only pEGFP-N2. Fibroblasts treated with PEI, Lipofectamine-2000, and the adenovirus all displayed a lack of growth during the

study period (Figure 4A). PEI was most effective in inducing EGFP expression, on the basis of the percentage of cells that were positive for EGFP, whereas other carriers induced a similar level of gene expression (Figure 4B). The total number of GFP-positive cells was calculated on the basis of the total cell numbers counted (Figure 4A) and the percent expression levels (Figure 4B). On the basis of this analysis, PLL-PA was the most effective carrier among all carriers (Figure 4C); although PEI was promising in having the highest percentage of GFP-positive cells, its toxic effect in long-term culture was detrimental. This high toxicity of PEI was consistent with the findings of others, who attempted to modify PEI to improve its cell compatibility.[16] Finally, PLL was not effective, giving a level of gene expression equivalent to that of pEGFP-N2 alone (not shown).

Similar observations were obtained when EGFP expression was followed by epifluorescent microscopy. Cell growth, based on the number of cells counted in a standard microscopic field, was unaltered in PLL-PA-exposed cells (Figure 4D), and gene expression was most effective with Lipofectamine-2000 and PLL-PA during the 10-day study period (Figure 4E). PEI gave relatively low transfection efficiencies at early time points, which increased gradually during the study period (similar to flow cytometry results). An analysis based on the total number of cells positive for EGFP (= %GFP-positive cells × number of cells/field) also indicated PLL-PA to be the most effective carrier (Figure 4F), consistent with flow cytometry results. Note that the assessment of EGFP-positive cells by microscopy indicated some differences from the flow cytometric assessment, for example, Lipofectamine-2000 and PEI gave higher and lower %EGFP-positive cells under epifluorescent microscopy as compared to flow cytometry, respectively. There might be several reasons for such discrepancies: (i) differences in the sensitivity of each assessment technique, (ii) the need to utilize trypsinization for flow cytometry, unlike epifluorescent microscopy, retaining the cells in their native state, and (iii) the need to grow the cells on glass cover slips for microscopy, but not flow cytometry, where substrate-dependent changes might influence the extent of transgene expression. Irrespective of quantitative differences between the two techniques, both techniques indicated PLL-PA to be a superior carrier (on the basis of the total number of GFP-positive cells as the outcome measure) as compared to other vectors, ultimately yielding 3−8 fold higher numbers of EGFP-positive cells at the end of the 10-day cell growth (Figure 4C and F).

Our collective results indicated palmitylation of the cationic polymer PLL to be a promising lead for designing improved gene carriers; under conditions that allowed robust cell growth, PLL-PA provided the highest number of cells with exogenous gene expression. The level of transgene expression, based on the percentage of cells positive for EGFP, was generally comparable to the efficiencies of adenoviral (30−50%[17]) and retroviral carriers (∼60%[18]), both of which were utilized for the expression of GFP in human fibroblasts. The non-viral vectors typically yield transient gene expression, and the maximal duration of expression with PA-modified PLL remains to be explored at this stage. A 10-day expression period, as observed in this study, might be sufficient for applications that require transient expression, for example, when one needs to express proteins that initiate tissue repair.[19] Other genetic disorders might require constitutive gene expression such as systemic Factor VIII deficiency,[20] and more studies will be needed to determine the maximal duration of gene expression with palmitylated polymeric vectors. We pursued palmitylation *a priori* for improved gene delivery because palmitic acid has the appropriate fatty acid chain length (C16) to interact with lipid membranes in a metastable fashion, enabling transient associations with lipid bilayers during entry and discharge from a bilayer. Other lipids, such as saturated myristic acid (C14), unsaturated geranylgeranyl moiety (C20), and polycyclic cholesterol, are also utilized by cells for intracellular trafficking of proteins.[21] Attempts to incorporate the latter lipid into PEI have yielded a promising PEI-based carrier by an independent group,[22] and future studies to identify the optimal lipid-polymer combination will be indispensable for the design of more effective non-viral carriers.

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Supporting Information Available. Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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