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SPECIAL ISSUE: Biomaterial Foundations of Therapeutic Delivery

Hydrophobe-substituted *b*PEI derivatives: boosting transfection on primary vascular cells

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ABSTRACT Gene therapy targeted to vascular cells represents a promising approach for prevention and treatment of pathological conditions such as intimal hyperplasia, in-stent and post-angioplasty restenosis. In this context, polymeric non-viral gene delivery systems are a safe alternative to viral vectors but a further improvement in efficiency and cytocompatibility is needed to improve their clinical success. Herein, a library of 24 branched polyethylenimine (*b*PEI) derivatives modified with hydrophobic moieties was synthesised, characterised and tested *in vitro* on primary vascular cells, aiming to identify delivery agents with superior transfection efficiency and low cytotoxicity. Low molecular weight PEIs (0.6, 1.2 and 2 kDa) were grafted with long (C18) and short (C3) aliphatic chains, featuring different unsaturation degrees and degrees of substitution. 0.6 kDa *b*PEI-based derivatives were generally ineffective in transfection on vascular smooth muscle cells (VSMCs), while among the other derivatives some promising vectors were identified. Forcing polyplexes on the cell surface by means of centrifugation invariably boosted transfection levels but increased cytotoxicity as well. Of note, a propionyl-substituted derivative (PEI2-PrA1, C3:0) was the most effective on both VSMCs and endothelial cells (ECs), with higher and more sustained gene expression in combination with markedly lower cytotoxicity with respect to the gold standard 25 kDa *b*PEI. In addition, a linoleoyl-substituted derivative (PEI1.2-LA6, C18:2) owing to its high efficiency in VSMCs and relative inefficacy in ECs, combined with tolerable cytotoxicity was proposed as a vector for specific VSMCs targeting.

Keywords: non-viral gene delivery, polyethylenimine, hydrophobic substitution, smooth muscle cells, endothelial cells, transfection

INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide [1]. Consequently, vascular interventions represent one of the most common surgical procedures, whose numbers are predicted to further increase owing to the growth of the elderly demographic of the population [2]. Proliferation, migration and phenotype switch of vascular smooth muscle cells (VSMCs) play pivotal roles in several pathological vascular conditions such as intimal hyperplasia and are crucial events leading to in-stent and post-angioplasty restenosis [3]. In addition, endothelial cells (ECs) are strongly involved in these phenomena since they are responsible for endothelial regeneration and can regulate VSMC proliferation and phenotypic switch [4]. In this context, gene therapy targeted to VSMCs and ECs is a promising therapeutic approach for the targeted inhibition of mechanisms leading to the development of vascular pathologies or to post-intervention complications [5]. Furthermore, *in vitro* gene delivery to primary VSMCs and ECs can be employed for tissue engineering and regeneration applications [6], and in the study of gene function and of the general physiology of vascular tissues [7].

Gene therapy involves the therapeutic introduction of DNA or RNA into cells with the goal of modifying their protein expression profile and halting disease progression. However, the efficient and safe delivery of nucleic acids to cells is still a key challenge towards the clinical application of gene therapy. Traditional gene delivery techniques used in laboratory are in fact difficult to effectively translate to *in vivo* [8]. Viral vectors are generally considered to be

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the most efficient gene delivery systems, but their use is limited by the immune response exerted by the body and other major safety concerns. Chemical non-viral vectors represent a safer alternative, but further improvement of their efficiency, especially in primary cells, is still a key point to clinical success. Among non-viral vectors, cationic polymers display distinct advantages in terms of reproducibility, high degree of molecular diversity and of easiness of chemical functionalization that allows fine-tuning of their physicochemical properties and performance [9]. When exposed to polyanionic DNA, cationic polymers self-assemble into positively charged polymer/DNA complexes, named polyplexes, that protect nucleic acids from degradation and mediate their cellular uptake. Polyethyleneimine (PEI) is recognised as the gold standard polymeric gene carrier; it features a high charge density and a strong buffering capacity at acidic pH that elicits endosomal escape of PEI-based polyplexes via a process named "proton sponge effect" [10]. It is worth to note that high molecular weight (MW) PEIs display not only higher transfection efficiency but also greater cytotoxicity with respect to their low MW counterparts. To improve its efficacy and cytocompatibility, PEI has been chemically tailored with a number of different substituents [9,11,12], among which, hydrophobic modification has been proven as a valuable approach. In fact, the grafting of hydrophobic chains on PEI results in increased cellular uptake via endocytosis and improved interactions between polyplexes and cell membranes [13]. These strategies mainly rely on the functionalization of cytocompatible low MW PEI with hydrophobic moieties to improve transfection efficiency, while retaining limited cytotoxicity. A number of different substitutes have been successfully grafted to PEI thus far: cholesterol [14], cholic acid, long-chain dodecyl and hexadecyl moieties [15], lipoic acid [16], saturated and unsaturated lipids of different lengths such as palmitic acid, stearic acid, oleic acid, and linoleic acid [13,17,18] and, recently, the small hydrophobic propionic acid [19].

In light of the promising results about hydrophobic moiety-substituted PEI and given the importance of developing safe and effective vectors for the transfection of VSMCs and ECs, in this study we explored and compared the potential of a library of hydrophobe-substituted branched PEI (*b*PEI) derivatives to transfect these two cell types. Low MW *b*PEIs (0.6, 1.2 and 2 kDa) were employed as backbone for the synthesis of the derivatives and five hydrophobic substituents were grafted at different grafting degrees aiming to finally identify the best transfection reagents and possibly draw structure-activity relationships.

EXPERIMENTAL SECTION

Materials

The plasmid DNA (pDNA) pGL3-Control Vector (5.2 kb), encoding for the modified firefly luciferase and the Luciferase Assay System were purchased from Promega (Madison, WI, USA); the pDNA pGLuc-Basic 2 Vector, encoding for the secreted *Gaussia* luciferase and the BioLux *Gaussia* Luciferase Assay Kit were from New England Biolabs (Ipswich, MA, USA). Bicinchoninic acid (BCA) protein assay kit, Dulbecco's modified Eagle's medium (DMEM), M199 medium, Gibco Opti-MEM I Reduced Serum Medium, fetal bovine serum (FBS), penicillin/streptomycin solution (100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin), phosphate-buffered saline solution (PBS), AlamarBlue Cell Viability Reagent, were from Thermo Fisher Scientific (Waltham, MA, USA). 2 kDa *b*PEI (M_n: 1.8 kDa, M_w: 2 kDa), 25 kDa *b*PEI (M_n: 10 kDa, M_w: 25 kDa), dimethyl sulfoxide (DMSO), stearoyl chloride (St), propionic acid (PrA) and acryloyl chloride (AoCl), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), chloroform ($CHCl_3$), methanol (MeOH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 0.6 kDa *b*PEI and 1.2 kDa *b*PEI were obtained from the Polysciences Inc. (Warrington, PA, USA). Linoleyl chloride (LA) and α-linoleoyl chloride (αLA) were obtained from NU-CHEK PREP (Elysian, MN, USA). Fluorimetric, spectrophotometric and luminescence analyses were performed using the multi-mode microplate reader system SpectraMax i3x (Molecular Devices, Sunnyvale, CA, USA).

Polymer synthesis and characterization

Hydrophobe-modified *b*PEI derivatives were prepared by grafting hydrophobic molecules onto *b*PEIs via *N*-acetylation as described earlier [17]. In a typical experiment, a lipid chloride (2 mmol L^{-1}) and PEI (1 mmol L^{-1}) were dissolved separately in ice-cold anhydrous chloroform for 30 min. Triethylamine (100 μL) was added to the PEI solution and cooled for another 30 min in ice bath. The lipid chloride solution was added dropwise to the PEI solution while being kept on ice bath under stirring. Reaction mixture was then left stirring overnight (~12 h) at room temperature. Derivatives with propionic acid (PrA) and acryloyl chloride (AoCl) were prepared using EDC/NHS activation method as described in our earlier publication [19]. The crude product was precipitated 3 times in ice-cold diethylether and dried under vacuum for 2 days. The structural composition of the polymers

was analyzed by ^{1}H-NMR spectroscopy (Bruker 300MHz, Billerica, MA, USA) using tetramethylsilane as an internal standard in D_2O to calculate the lipid substitution levels, which are summarized in Table 1.

Preparation of polyplexes

Polyplexes were prepared at room temperature by adding an aqueous solution of pDNA (0.4 μg μL^{-1}) to the *b*PEI derivatives diluted in serum-free medium (Opti-MEM) at the desired concentration (polymer solution), to obtain different polymer/pDNA *w*/*w* ratios. The final pDNA concentration in the polyplex suspensions was 25 ng μL^{-1}. Polyplex suspensions were incubated for 30 min at room temperature prior to use in transfection studies.

DNA binding by polymers

The ability of *b*PEI derivatives to bind and complex DNA was evaluated by a fluorophore-exclusion assay using SYBR Green I (λ_{ex} = 497 nm; λ_{em} = 520 nm) as DNA dye [20]. Polyplexes (400 ng of pDNA per condition) were prepared in serum-free medium at different *w*/*w* ratios as described above. Polyplexes were next diluted to 100 μL in 10 mmol L^{-1} HEPES (pH 7.0) containing 2× DNA dye and, after further 20 min incubation at room temperature, fluorescence was measured in a black 96-well microplate. The fluorescence of the samples was normalized over the signal of free pDNA. For each polymer, fluorescence % was plotted against polymer/pDNA *w*/*w* ratio and fitted to a sigmoidal curve that was used to calculate the BC_{50} (*w*/*w* ratio requi-

Table 1 Properties of the library of *b*PEI derivatives and of unmodified *b*PEIs investigated in this study. The table summarizes the type of substitute, the lipid:PEI feed ratio (mol/mol) used during the reaction, the degree of substitution calculated from ^{1}H NMR analysis and the *w*/*w* ratio required for 50% pDNA binding during complexation (BC_{50}), evaluated by SYBR Green I fluorophore-exclusion assay.

Polymer	Substitute	Feed ratio (mol/mol)	Degree of substitution (mol/mol)	BC_{50}
PEI2-St6	Stearic acid	6.0	2.14	0.678
PEI2-St12	Stearic acid	12.0	4.53	8.088
PEI0.6-LA4	Linoleic acid	4.0	1.09	0.364
PEI1.2-LA4	Linoleic acid	4.0	1.84	0.761
PEI1.2-LA6	Linoleic acid	6.0	2.55	0.686
PEI2-LA6	Linoleic acid	6.0	2.31	0.785
PEI2-LA9	Linoleic acid	9.0	3.20	3.609
PEI0.6-αLA2	α-linoleic acid	2.0	0.80	0.320
PEI0.6-αLA4	α-linoleic acid	4.0	2.30	1.269
PEI1.2-αLA2	α-linoleic acid	2.0	0.94	0.289
PEI1.2-αLA4	α-linoleic acid	4.0	2.45	0.297
PEI1.2-αLA6	α-linoleic acid	6.0	3.17	0.578
PEI2-αLA2	α-linoleic acid	2.0	1.37	0.681
PEI2-αLA4	α-linoleic acid	4.0	2.72	1.015
PEI2-αLA8	α-linoleic acid	8.0	3.68	3.899
PEI0.6-PrA1	Propionic acid	1.0	0.62	0.298
PEI1.2-PrA0.5	Propionic acid	0.5	0.28	0.316
PEI1.2-PrA1	Propionic acid	1.0	0.76	0.310
PEI2-PrA0.5	Propionic acid	0.5	0.15	0.304
PEI2-PrA1	Propionic acid	1.0	0.53	0.367
PEI1.2-AcA1	Acrylic acid	1.0	0.65	0.343
PEI1.2-AcA2	Acrylic acid	2.0	1.21	0.430
PEI2-AcA1	Acrylic acid	1.0	0.51	0.355
PEI2-AcA2	Acrylic acid	2.0	0.86	0.643
0.6 kDa *b*PEI	/	/	/	0.278
1.2 kDa *b*PEI	/	/	/	0.215
2 kDa *b*PEI	/	/	/	0.213
25 kDa *b*PEI	/	/	/	0.274

red for 50% pDNA binding).

Size and ζ-potential of polyplexes

The hydrodynamic diameter (D_H) and the ζ-potential (ζ_P) of *b*PEI derivatives/pDNA complexes were measured through dynamic light scattering (DLS) and laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) equipped with He-Ne laser and operated at 10 mW. Polyplexes were prepared at different *w*/*w* as described above and measured after equilibration at 25°C.

Cells and cell culture

Primary Porcine Aortic SMCs (PAoSMCs) were derived from tunica media of healthy, fibrous plaque-free porcine arteries. Briefly, PAoSMCs were isolated from 1 cm wide aortic rings cut from the aorta of a pig. Aortic segments were collected in PBS, placed in M199 medium supplemented with 1% penicillin/streptomycin solution and immediately dissected. The adventitia tissue and the first external third of the media were carefully removed and the resulting rings were cut in small pieces of approximately 1 mm × 5 mm using a scalpel and moved into a Petri dish containing M199 medium supplemented with 10% FBS and 1% penicillin/streptomycin solution (complete M199, hereafter referred to as cM199). Medium was changed every other day. The explants were removed after one week when a sufficient amount of SMCs was present on the surface of the Petri dish. PAoSMCs were then cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution (complete DMEM, hereafter referred to as cDMEM) at 37°C in a humidified atmosphere of 5% CO_2.

Primary Human Umbilical Artery SMCs (HUASMCs) were purchased from Lonza (CC-2579, Walkersville, MD, USA) and cultured at 37°C in a humidified atmosphere under constant supply of 5% CO_2, in cDMEM enriched with 5 μg mL^{-1} human insulin (Santa Cruz Biotechnology, Dallas, TX), 2 ng mL^{-1} fibroblast growth factor-basic (FGFb, Gibco by Life Technologies, Grand Island, NY, USA), and 0.5 ng mL^{-1} epidermal growth factor (EGF, Invitrogen by Life Technologies), hereafter referred to as cDMEM+.

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from ~15 cm-long umbilical cord pieces obtained from normal-term pregnancies with the consent of the donor mothers. Briefly, the vein was rinsed with PBS, filled with 10× trypsin-EDTA solution (Gibco by Life Technologies) and incubated for 15 min at 37°C. Then the trypsin-EDTA solution containing cells was collected, PBS was added to wash the lumen of the vein, collected and added to the cell-containing suspension. The resulting cell suspension was centrifuged at 1,000 rpm for 5 min, the supernatant was removed and cells were resuspended in cM199 and seeded in a T75 flask. Culture medium was changed after 24 h and then every 48 h until confluence. ECs were cultured in cM199 supplemented with 2 ng mL^{-1} FGFb, 0.5 ng mL^{-1} EGF, 1 μg mL^{-1} L-ascorbic acid (Sigma-Aldrich), 5 μg mL^{-1} human insulin, 1 μg mL^{-1} hydrocortisone (Sigma-Aldrich) and 90 μg mL^{-1} porcine heparin sodium salt Grade I-A (Sigma-Aldrich), hereafter referred to as cM199+.

The protocols for the isolation of PAoSMCs and HUVECs were approved by the Ethics Committee of CHU de Québec Research Centre. For all the experiments cells were used at passage between 6 and 10.

***In vitro* transfection experiments**

Twenty-four hours before transfection experiments PAoSMCs, HUASMCs and HUVECs were seeded in 96-well cell culture plates at a density of 2.0×10^4 cells/cm^2 in the corresponding culture medium (cDMEM, cDMEM+ and cM199+, respectively). Twenty-four hours post-seeding, 160 ng/well (PAoSMCs) or 80 ng/well of pDNA (HUASMCs and HUVECs) were complexed with *b*PEI derivatives at the desired *w*/*w* and added to cells in fresh complete medium. The final pDNA concentration in the culture medium was 1 μg mL^{-1}. Four hours after the addition of polyplexes, the transfection medium was replaced with 100 μL of fresh culture medium. When indicated, cells were centrifuged at 500 ×g for 5 min immediately after the addition of polyplexes.

Evaluation of cytotoxicity and transfection efficiency

The cytotoxicity of polyplexes was assessed by AlamarBlue cell viability assay. The medium was harvested and replaced with 100 μL of complete medium containing 10% AlamarBlue solution. Cells were incubated in standard culture conditions for 3 h and the fluorescence of the medium (λ_{ex} = 560 nm; λ_{em} = 590 nm) was read using the SpectraMax i3x microplate reader. Viability of non-treated control cells was assigned to as 100% and cytotoxicity was determined as follows:

$$\text{Cytotoxicity [\%]} = 100\% - \text{Viability [\%]}.$$

The expression of firefly luciferase (pGL3 plasmid) was evaluated 48 h post-transfection by measuring the luciferase activity using the Luciferase Assay System as previously described [21]. Briefly, cells were washed with PBS, lysed with 0.25× Cell Culture Lysis Reagent (Promega) and

freeze-thawed once. Then, 20 μL of cell lysate were mixed with 50 μL of Luciferase Assay Reagent and luminescence was immediately recorded by the microplate reader. The chemiluminescence signal of the samples was normalized to their corresponding protein content, determined by BCA assay. Data were finally expressed as relative light units per mg of proteins (RLU/mg of proteins).

The expression of *Gaussia* luciferase (pGLuc plasmid), secreted in the culture medium, was assessed at different time points by BioLux Gaussia Luciferase Assay Kit, according to manufacturer's instructions. Culture medium was harvested 24 h post-transfection or 24 h after every medium change and 20 μL of medium were mixed with 50 μL of GLuc assay solution and luminescence was promptly measured by the microplate reader.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analyses were carried out by means of GraphPad Prism v 6.01 (GraphPad software, La Jolla, CA, USA). Comparisons among groups were performed by one-way analysis of variance (ANOVA) with Sidak-Bonferroni adjustment for multiple testing. Correlations were evaluated by Pearson correlation test. Significance was retained when $p < 0.05$.

RESULTS AND DISCUSSION

Primary vascular cells, SMCs and ECs, as many other primary cells, are recognised difficult-to-transfect cell types [22–24]. In this study we investigated the efficiency of hydrophobe-substituted *b*PEI derivatives in the transfection of these cells. Different fatty acid-based substitutes have been investigated, namely stearic acid (St, C18 chain), linoleic acid (LA, C18:2 (6, 9) chain), and propionic acid (PrA, C3 chain), already reported to improve the transfection activity of *b*PEI on other cell types such as human embryonic kidney cells (293T) [17], rat bone marrow stromal cells (rBMSC) [25] and human breast cancer cells (MDA-231 and MCF-7) [19]. Besides, two new families of *b*PEI derivatives, carrying α-linoleic acid (αLA, C18:3 (3, 6, 9) chain) and acrylic acid (AcA, C3:1 (1) chain), were synthesised and studied, aiming to evaluate whether an increase in the level of unsaturation in the aliphatic chain of linoleoyl- and propionyl-based substitutes (i.e., an additional double bond, in position 3 and 1, respectively) could lead to a further enhancement in the transfection efficiency of *b*PEI derivatives on vascular cells. Of note, to find out the optimal chemical structures and to draw overall structure-activity relationships, different *b*PEI backbone MWs and degrees of substitution were tested.

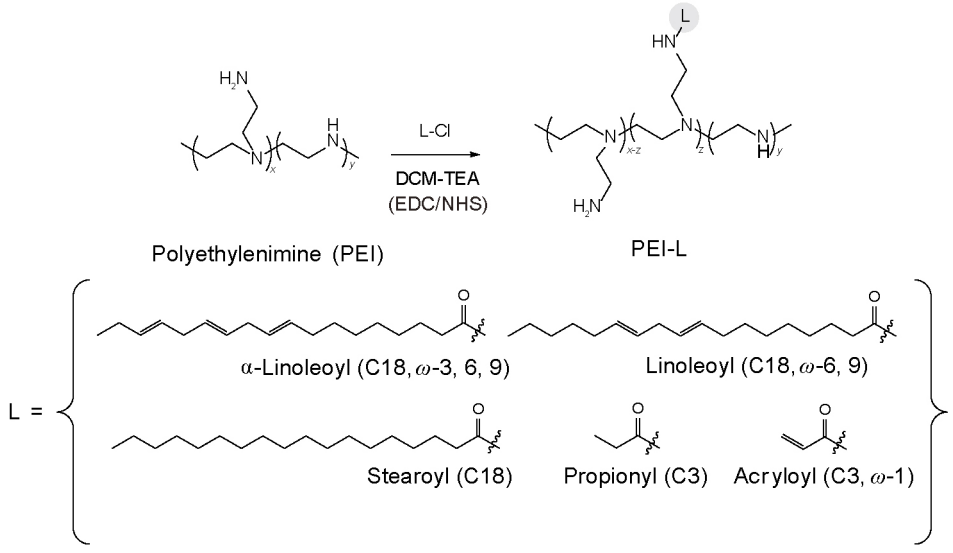
Synthesis and characterization of hydrophobe-substituted *b*PEI derivatives

Chemical strategies previously optimised for lipid substitution on *b*PEI were herein employed for the derivatization of 0.6, 1.2 and 2 kDa *b*PEI with St, LA, αLA, PrA and AcA (Scheme 1) [17,19]; the reaction feed ratio and the resulting degree of substitution, as measured by ^{1}H-NMR, of the resulting polymers are reported in Table 1. As expected, the degree of substitution increased by increasing the feed ratio for every lipid, to give an average yield between 0.54 and 0.56 for all the substituents except St and LA that displayed a significantly lower average yield of 0.37 and 0.38 respectively. Every *b*PEI derivative was water soluble.

DNA complexation ability of *b*PEI derivatives and polyplex characterization

The capacity of every polymer to bind and complex pDNA was evaluated by SYBR Green I-exclusion assay. SYBR Green I is a non-specific intercalating DNA dye with high fluorescence quantum yield and a strong fluorescence enhancement when bound to DNA that has been commonly used to investigate DNA complexation [20]. Representative complexation curves as a function of polymer:DNA ratio (*w*/*w*) are shown in Fig. 1a (please see Supplementary information, Fig. S1 for the complexation curves of all the *b*PEI derivatives) and Table 1 shows the *w*/*w* required for 50% pDNA binding (BC_{50}).

All the complexation curves shared a similar sigmoidal decrease in fluorescence as the polymer/pDNA weight ratios were increased. Generally speaking, the complexation ability of *b*PEI derivatives decreased while increasing the degree of substitution, as indicated by the higher BC_{50} that reached values higher than 2.5 for PEI2-St12, PEI2-LA9 and PEI2-αLA8. In addition, a general positive and statistically significant correlation was found between BC_{50} and the degree of substitution for the three *b*PEI backbones (Fig. 1b, Pearson correlation, $p < 0.05$). This could be explained by the loss of amines available for interaction with negatively charged nucleic acids, owing to amine substitution and to the steric hindrance of the grafted molecules [17]. Dispersity in BC_{50} values was noted at higher substitutions (> 2 hydrophobes per PEI) for 1.2 kDa *b*PEI-based derivatives. It is likely that the nature of the hydrophobic moieties became important in this region, differentially affecting nucleic acid binding due to steric hindrance.



Scheme 1 Synthesis of hydrophobe-substituted *b*PEI derivatives.

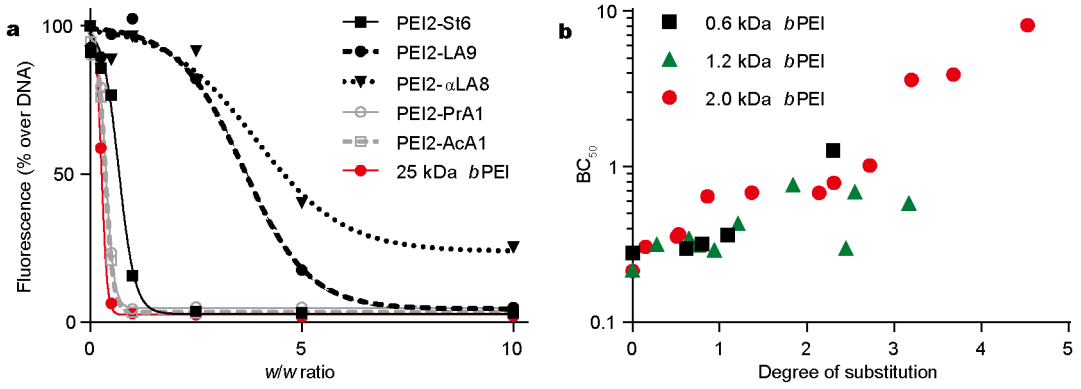


Figure 1 pDNA complexation ability of *b*PEI derivatives as a function of polymer:DNA ratio (*w*/*w*). (a) The complexation curves for PEI2-St6, PEI2-LA9, PEI2-αLA8, PEI2-PrA1 and PEI2-AcA1 are reported as representative examples and compared with 25 kDa *b*PEI. (b) BC_{50} values as a function of the degree of substitution for 0.6 (black squares), 1.2 (green triangles) and 2.0 kDa *b*PEI-based derivatives (red dots). A positive correlation was observed between the BC_{50} and degree of substitution, according to Pearson correlation ($r = 0.91$, $p < 0.05$; $r = 0.67$, $p < 0.05$; $r = 0.84$, $p < 0.05$ respectively for 0.6, 1.2 and 2.0 kDa *b*PEI-based derivatives).

As the physicochemical properties of polyplexes are expected to strongly affect their activity [26], the ζ_P and D_H of polymer/pDNA complexes were measured at *w*/*w* of 5 and 10, conditions that led to significant activity in transfection experiments; the results for the selected derivatives (the most efficient ones in transfection experiments) are shown in Table 2, while those relative to every *b*PEI derivative are reported in the Supplementary information (Table S1). For all the *b*PEI derivatives, nanometric sizes were observed, with D_H always smaller than 300 nm and in general comparable to native *b*PEIs, except for 0.6 kDa *b*PEI that yielded relatively large particles bigger than 1 μm. The only exception was PEI2-St12 that already showed the lowest ability to complex DNA ($BC_{50} = 8.088$) and formed particles larger than 1 μm at *w*/*w* 5 and of ca. 700 μm at *w*/*w* 10. ζ_P was always positive and between +10 mV and +30 mV at both *w*/*w* tested, except for PEI1.2-LA6 that showed an almost neutral surface charge at *w*/*w* 5. In contrast to BC_{50}, no significant correlation with the degree of substitution was observed for both size and ζ_P (Pearson correlation, $p > 0.05$).

Transfection of VSMCs by *b*PEI derivatives

While the ability to efficiently complex DNA is necessary for gene delivery vectors, it does not guarantee effectiveness in transfection. Therefore, we tested the transfection efficiency and the cytotoxicity of polyplexes prepared at different *w*/*w* with *b* PEI derivatives on primary VSMCs

Table 2 Hydrodynamic diameter (D_H), polydispersity index (PDI) and ζ-potential (ζ_P) of the polyplexes prepared using PEI1.2-LA6, PEI1.2-αLA2, PEI2-PrA2 and PEI1.2-AcA2 derivatives and of native *b*PEIs (0.6, 1.2, 2 and 25 kDa) at *w*/*w* 5 and 10 and measured by DLS and laser Doppler micro-electrophoresis

Polymer	*w*/*w*	D_H (nm)	St. Dev. D_H (nm)	PDI	St. Dev. PDI	ζ_P (mV)	St. Dev. ζ_P (mV)
PEI1.2-LA6	5	101	16	0.30	0.11	-1.4	1.8
	10	135	53	0.40	0.15	15.3	4.0
PEI1.2-αLA2	5	97	25	0.33	0.09	21.0	1.5
	10	229	10	0.54	0.01	23.1	0.8
PEI2-PrA1	5	98	3	0.31	0.02	32.2	0.6
	10	112	53	0.41	0.23	27.5	3.7
PEI1.2-AcA2	5	104	3	0.36	0.04	26.0	6.1
	10	94	7	0.21	0.12	22.8	4.5
0.6 kDa *b*PEI	5	1381	281	1.00	0.00	14.4	5.2
	10	1885	1715	0.87	0.13	15.6	0.2
1.2 kDa *b*PEI	5	139	7	0.45	0.03	28.9	0.6
	10	88	0	0.03	0.01	14.9	2.1
2 kDa *b*PEI	5	115	13	0.27	0.05	29.1	5.4
	10	179	152	0.39	0.23	22.0	8.0
25 kDa *b*PEI	5	112	20	0.35	0.05	32.8	0.7
	10	104	16	0.29	0.09	28.2	0.9

obtained from the human umbilical artery (HUASMCs) and from the porcine aorta (PAoSMCs), since pigs are commonly used in vascular research as a model of human disease [27]. The presence of serum in culture medium is necessary for long-term cell cultures and it is commonly employed to evaluate resistance to serum of gene delivery vectors prior to *in vivo* tests [28], consequently, transfection experiments were carried out in cDMEM, containing 10% FBS. Three *w*/*w* ratios (2.5, 5 and 10) were tested, given the recognised dependence of transfection activity on this parameter. Culture medium containing polyplexes was replaced with fresh new cDMEM 4 h after transfection and 44 h later, transfection efficiency and cytotoxicity were evaluated. Firefly luciferase was used as a luciferase reporter system (pGL3), enabling a highly sensitive evaluation of transgene expression. It is worth to note that transfection experiments on HUASMCs were performed with a lower dose of polyplexes (80 *vs.*160 ng of pDNA/well) to avoid massive cytotoxicity.

Transfection outcomes on primary PAoSMCs and HUASMCs are shown in Figs 2 and 3 respectively. As expected, a strong dependence on polymer/DNA *w*/*w* ratio was observed. A low *w*/*w* of 2.5 always led to non-relevant transgene expression (data not shown) while *w*/*w* 10 was usually the most effective ratio for *b*PEI derivatives. The *b*PEI derivatives synthesised with 0.6 kDa *b*PEI backbone were generally ineffective in transfecting VSMCs in comparison to their counterparts synthesised from 1.2 or 2 kDa *b*PEI, suggesting that the substitution with hydrophobic moieties of *b*PEI featuring too small polymeric backbone (0.6 kDa) was ineffective in producing efficient gene vectors, even if such polymers did complex DNA effectively. No obvious general differences between the 1.2 and 2.0 kDa *b*PEI backbones could be observed both in terms of transfection efficiency and cytotoxicity, suggesting that both polymers were suitable substrates for hydrophobic substitution.

As expected from DNA complexation results, and as often reported for *b*PEI derivatives [29–31], the degree of substitution strongly influenced the transfection properties. Very high substitution degrees led to low efficiency (PEI2-St12, PEI2-LA9, PEI1.2-αLA6) both in PAoSMCs and HUASMCs. Surprisingly, despite its low DNA complexation ability (BC_{50} = 3.9), PEI2-αLA8 displayed high transfection levels on PAoSMCs, comparable to its counterparts featuring a lower substitution degree, but negligible activity on HUASMCs. However, no significant direct correlation between transfection efficiency and the extent of lipid substitution was observed for the three *b*PEI backbones (Fig. S2, Pearson correlation, $p > 0.05$). This was quite expected since, as already observed in similar systems [16,19], increasing hydrophobic-moiety degree of substitution initially improves transfection until the attainment of an optimum after which efficiency decreases

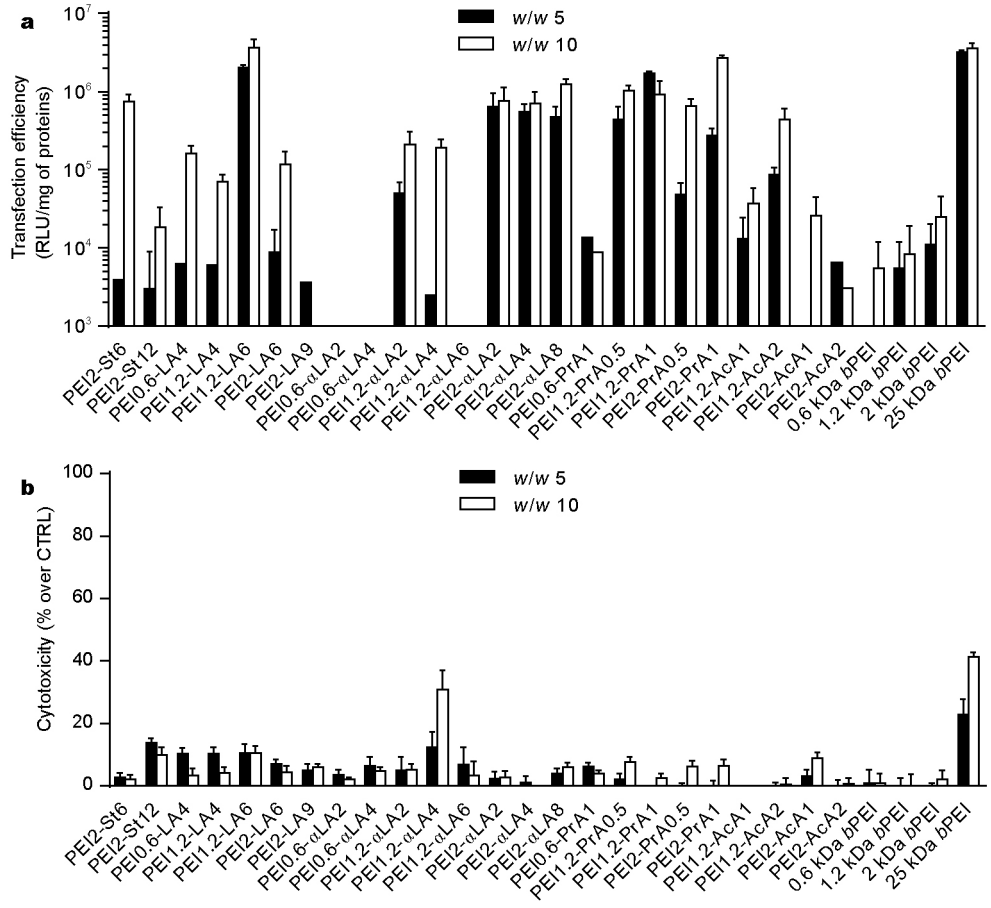


Figure 2 (a) Transfection efficiency and (b) cytotoxicity of *b*PEI derivatives at *w*/*w* 5 and 10 on PAoSMCs. Cells were transfected with pGL3 and transfection efficiency was evaluated 48 h post-transfection and expressed as relative luminescence units (RLU) normalized over the total protein content in every cell lysate. Cytotoxicity was evaluated by AlamarBlue assay and expressed as percent viability loss with respect to untreated control cells. Missing bars in (a) indicate that no significant luciferase activity was detected. Results are shown as mean ± standard deviation ($n \geq 4$).

due to the excessive reduction of the number of amines available for DNA condensation.

The cytotoxicity of the polymers was generally lower than 20% on PAoSMCs, and lower than that of 25 kDa *b*PEI ($p < 0.05$) for all the derivatives tested, except for PEI1.2-αLA4, which was as toxic as 25 kDa *b*PEI. In contrast, HUASMCs were much more sensitive to the overall cytotoxicity of transfectants and we thus reduced the polyplex dose delivered to avoid extensive cell death. As often observed in literature [32], derivatives with negligible activity exerted the lowest cytotoxicity and higher transfection efficiency levels were often associated with increased toxicity. A significant positive correlation between transfection efficiency and cytotoxicity was observed on PAoSMCs (Fig. S3a, Pearson correlation $r = 0.49$, $p < 0.05$) but not on HUASMC (Fig. S3b, Pearson correlation $r = 0.15$, $p > 0.05$) even if both cytotoxicity (Fig. S4a, Pearson correlation $r = 0.72$, $p < 0.001$) and transfection efficiency (Fig. S4b, Pearson correlation $r = 0.58$, $p < 0.01$) significantly correlated between PAoSMCs and HUASMCs.

Even though St-substituted *b*PEIs have been previously described as good transfection agents for siRNA delivery [13], our results showed that PEI-St polymers herein investigated were not so effective on both the VSMC types. PEI1.2-LA6 used at *w*/*w* 10 was the most effective PEI-LA derivative, as efficient as ($p > 0.05$) but much less cytotoxic (cytotoxicity: 10.4 ± 2.4% *vs.* 22.9 ± 4.7%, PEI1.2-LA6 *w*/*w* 10 *vs.* 25 kDa bPEI *w*/*w* 5, $p < 0.05$) than the gold standard polymeric transfectant 25 kDa *b*PEI on PAoSMCs. PEI1.2-LA6 was still the best LA-based derivative on HUASMCs but it was less efficient than 25 kDa *b*PEI ($p < 0.05$) even if less cytotoxic (cytotoxicity: 40.8 ± 3.1% *vs.* 50.2 ± 2.9%, PEI1.2-LA6 *w*/*w* 10 *vs.* 25 kDa *b*PEI *w*/*w* 5, $p < 0.05$). Interestingly, LA-substituted 2 kDa *b*PEI, already reported as one of the best *b*PEI-lipid derivatives on 293T cells and rBMSC [17], did not show high transfection levels on

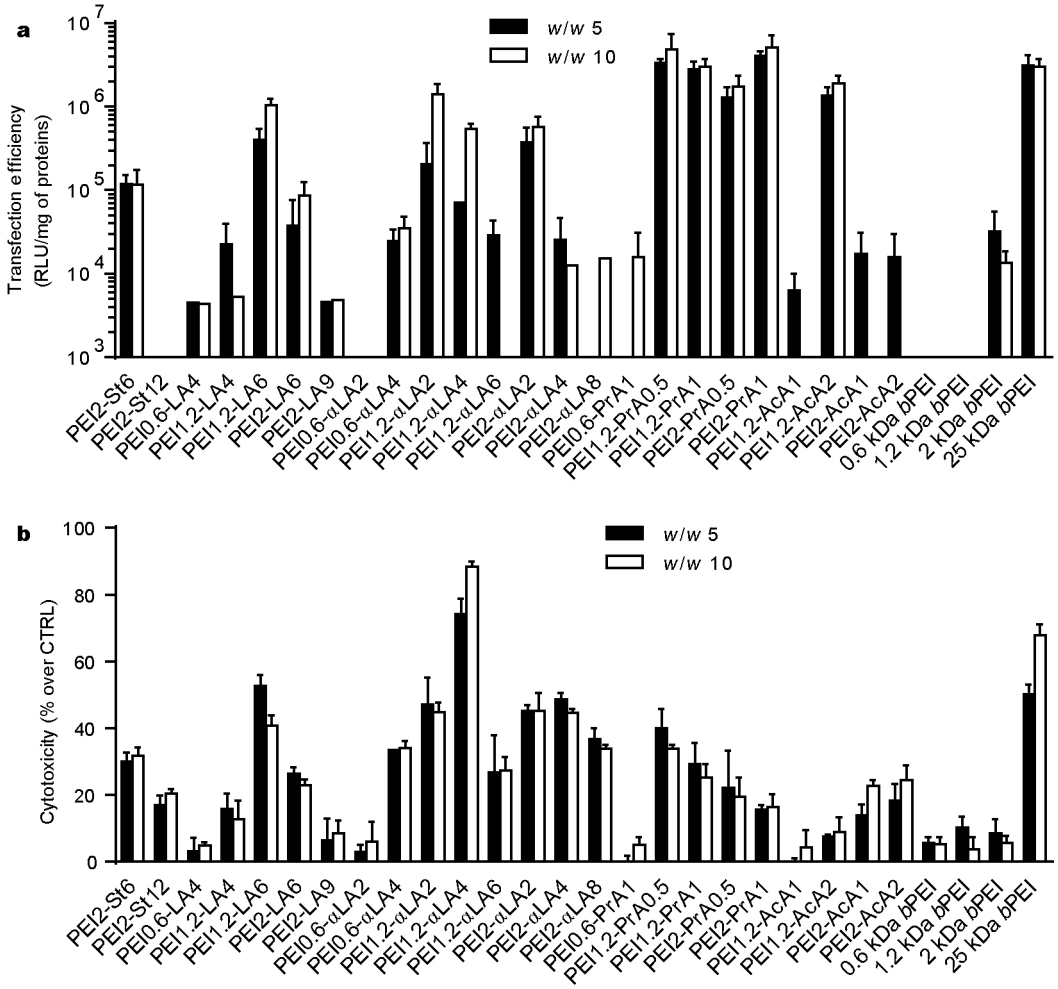


Figure 3 (a) Transfection efficiency and (b) cytotoxicity of *b*PEI derivatives at *w*/*w* 5 and 10 on HUASMCs. Cells were transfected with pGL3 and transfection efficiency was measured 48 h post-transfection and expressed as RLU normalized over the total protein content in cell lysate. Cytotoxicity was evaluated by AlamarBlue assay and expressed as percent viability loss with respect to untreated control cells. Missing bars in (a) indicate that no significant luciferase activity was detected. Results are shown as mean ± standard deviation ($n \geq 4$).

VSMCs. These results suggest that the simultaneous fine tuning of the polymeric backbone MW and of the degree of substitution is a very important cell-specific determinant but that the optimized chemical properties are not universal for all cell types.

In line with the results previously reported on MDA-231 and MCF-7 human breast cancer cells [19], PEI-PrA derivatives displayed high transgene expression especially on HUASMCs. Among them, PEI2-PrA1 was the most effective because the best compromise between high efficiency and low toxicity. In fact, PEI2-PrA1 derivative was as efficient as ($p > 0.05$) but much less cytotoxic than 25 kDa *b*PEI (cytotoxicity: 6.3 ± 2.0 % *vs.* 22.9 ± 4.7 %, PEI2-PrA *w*/*w* 10 *vs.* 25 kDa *b*PEI *w*/*w* 5, $p < 0.05$) on PAoSMCs. It was also the most effective among the tested derivatives on HUASMCs, with transfection levels more than twice higher than 25 kDa *b*PEI ($p < 0.05$) and with significantly lower toxicity (cytotoxicity: 16.4 ± 3.8 % *vs.* 50.2 ± 2.9 %, PEI2-PrA1 *w*/*w* 10 *vs.* 25 kDa bPEI *w*/*w* 5, $p < 0.05$).

It is worthy of note that an increase in the unsaturation level of the substitute (αLA *vs.* LA, and AcA *vs.* PrA) did not lead to an improvement of the transfection activity of the polymers. PEI-αLA and PEI-AcA were invariably less efficient than PEI1.2-LA6 and PEI2-PrA1, respectively, on PAoSMCs ($p < 0.05$). Only PEI1.2-αLA2 displayed levels of transfection comparable to PEI1.2-LA6 ($p > 0.05$) on HUASMC, whilst PEI1.2-AcA2, which was by far the most efficient reagent belonging to the PEI-AcA family, showed a three-time lower luciferase expression as compared to PEI2-PrA1. Noteworthy, considering that oleic acid-sub-

stituted PEI (degree of unsaturation = 1) already demonstrated a much lower efficiency as nuclei acid delivery agent with respect to PEI-LA [33] and taking into account the results on PEI-St (saturated), PEI-LA (degree of unsaturation = 2) and PEI-αLA (degree of unsaturation = 3), it can be speculated that a degree of unsaturation of 2 is optimal for the substitution of low MW PEIs with the C18 lipids.

In light of these results, PEI1.2-LA6, PEI1.2-αLA2, PEI2-PrA2, and PEI1.2-AcA2 polymers were selected for further investigation.

Effect of centrifugation on the transfection activity of *b*PEI derivatives on VSMCs

Centrifugation of polyplexes is a simple way to accelerate their settling onto the cell surface, thus immediately increasing their concentration in the proximity of the cells. This approach can improve cellular uptake and the final transfection efficiency [34]. With this in mind, we challenged VSMCs with every selected *b*PEI derivative subjected to a 5 min-centrifugation at 500 ×g immediately after the addition of polyplexes to the cells. Based on the results reported hereinabove, *b*PEI derivatives were used at *w*/*w* 10, while 25 kDa *b*PEI at *w*/*w* 5. As expected, centrifugation led to a substantial increase of the transfection levels, at least 10 folds higher than non-centrifuged controls (Fig. 4). The enhancement due to centrifugation was polymer-specific in the case of PAoSMCs, but not for HUASMCs for reasons to be elucidated. Unfortunately, higher cytotoxicity was also associated to the increase in transfection efficiency ($p < 0.05$, centrifuged *vs.* non-centrifuged, for every polymer), however, the cytotoxicity of *b*PEI derivatives was still significantly lower than that of 25 kDa *b*PEI. These results highlight the importance of sedimentation of gene delivery complexes on the cell surface to optimize transfection, but also strengthen the almost unavoidable relationship existing between increase in gene delivery activity and cytotoxicity.

Transgene expression kinetics of *b*PEI derivatives in VSMCs

The kinetics of transgene expression for the selected *b*PEI derivatives was investigated to evaluate the medium- and long-term transfection efficiency on VSMCs. In accordance with the above results, polyplexes were centrifuged on the cells in order to maximise transfection efficiency. The plasmid encoding for the secreted *Gaussia* luciferase (pGLuc) was used for these studies. This enzyme endowed with chemiluminescent activity is constitutively secreted into the cell culture media that is harvested, allowing for real-time cell monitoring of the reporter gene expression and making easy the measurement of the expression kinetics.

As shown in Fig. 5a and b, a much more sustained transgene expression was generally observed on PAoSMCs with respect to HUASMCs. In the former, 25 kDa *b*PEI showed a peak of expression within the first 48 h and then abruptly declined to levels nearly two orders of magnitude lower at day 10. However, for the *b*PEI derivatives, GLuc expression was stable or even increased over the first 4 days post-transfection, thereafter gradually decreasing by one order of magnitude. PEI1.2-LA6 and PEI2-PrA1 were the best performing derivatives. PEI2-PrA1 showed transfection levels comparable to 25 kDa *b*PEI within the first 4 days and more than 5-fold greater for longer times ($p < 0.05$, PEI2-PrA1 *w*/*w* 10 *vs.* 25 kDa *b*PEI *w*/*w* 5 at day 10). The improved transfection efficiency over the long run was presumably due to the higher compatibility of low MW PEIs

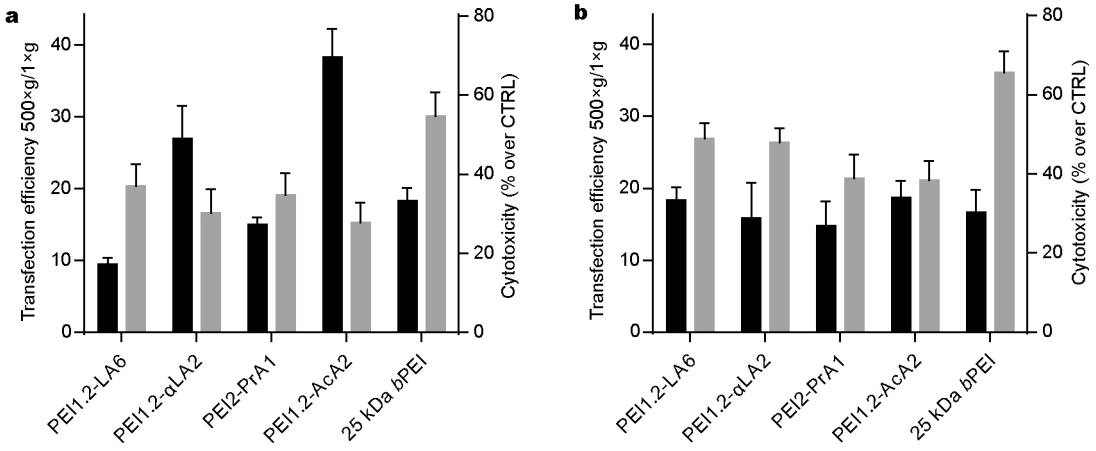


Figure 4 Effect of centrifugation on transfection by *b*PEI derivatives on (a) PAoSMCs and (b) HUASMCs. The ratio between transfection efficiency obtained with (500 ×g) and without (1 ×g) centrifugation is reported (black bars). Cytotoxicity of centrifuged polyplexes (grey bars) is expressed as toxicity percent relative to untreated control cells. Results are shown as mean ± standard deviation ($n \geq 4$).

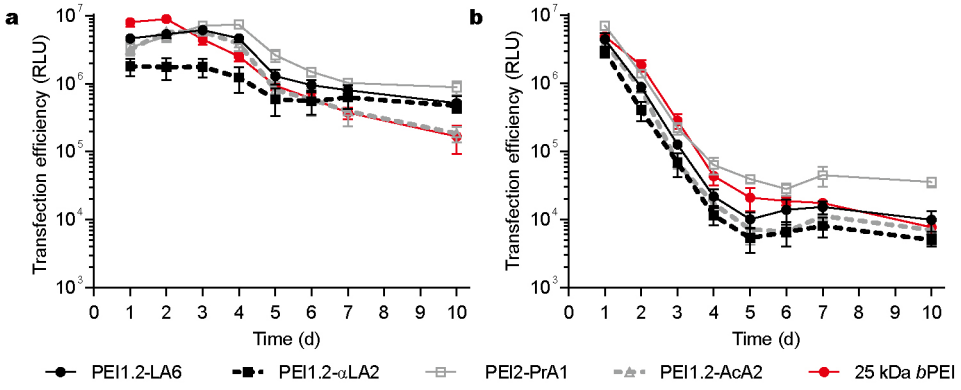


Figure 5 Kinetics of transfection efficiency of selected *b*PEI derivatives on (a) PAoSMCs and (b) HUASMCs. Cells were transfected with pGLuc (*w*/*w* 10 for *b*PEI derivatives, *w*/*w* 5 for 25 kDa *b*PEI) and transfection efficiency was measured at different time points and expressed as RLU. Results are shown as mean ± standard deviation ($n \geq 4$).

(i.e., the higher percentage of modified cells remaining viable over a long period). In fact, the higher cytotoxicity of 25 kDa *b*PEI is expected to negatively affect the long-term survival of the pool of cells that were actually transfected, leading to a more prominent decrease of exogenous transgene expression.

A common behaviour was observed for every polymer tested on HUASMC (Fig. 5b), with the expression maximum occurring at 24 h and a sharp decrease leading to quite steady levels of expression (at least two order of magnitude lower than day 1) after day 4. Of note, PEI2-PrA1 was again the most promising transfectant: transfection efficiency was comparable to that of 25 kDa *b*PEI within the first 72 h, but superior over the long term (4.6 fold higher values at day 10; $p < 0.05$, PEI2-PrA1 *w*/*w* 10 *vs.* 25 kDa *b*PEI *w*/*w* 5 at day 10). Altogether, the superior gene expression profiles combined with the lower cytotoxicity with respect to the gold standard 25 kDa *b*PEI make PEI2-PrA1 a very promising candidate for the transfection of VSMCs. These transfection results are extremely important because they were obtained in the presence of serum and upon centrifugation of the polyplexes over the cells, mimicking the high concentration at the cell surface that could be found in cardiovascular devices such as gene-eluting stents [35] or catheter balloons [36,37].

Cytotoxicity and transgene expression kinetics in HUVECs

VSMCs are not the sole cell type playing a role in in-stent and post-angioplasty restenosis. ECs are present on the luminal surface of arteries and represent a suitable therapeutic target to prevent intima hyperplasia by speeding up the formation of a functional endothelium and preventing VSMCs migration and proliferation [38,39]. With this in mind, the two most promising *b*PEI derivatives PEI1.2-LA6 and PEI2-PrA1, were used to transfect primary HUVECs and compared to 25 kDa *b*PEI. The optimised transfection conditions identified with VSMCs were employed and polyplexes were forced to sediment onto cells by centrifugation. A common behaviour in the transgene expression kinetics for every transfectant was apparent, with a maximum expression at day 1, followed by a sharp decrease over time (Fig. 6a). In line with the results obtained on VSMCs, PEI2-PrA1 displayed an improved and long-lasting transfection in combination with cytotoxicity levels once again lower than 25 kDa *b*PEI (cytotoxicity: 30.6 ± 6.8% *vs.* 48.0 ± 9.6%, PEI2-PrA1 *w*/*w* 10 *vs.* 25 kDa bPEI *w*/*w* 5, $p < 0.05$) and it can be thus considered as the most promising hydrophobe-*b*PEI derivative for the transfection of vascular cells. It is worth noting that, even if PEI1.2-LA6 showed high efficiency on VSMC, it was much less effective on HUVECs compared to both PEI2-PrA1 and 25 kDa *b*PEI. Due to its specificity towards VSMCs and in consideration of its quite low cytotoxicity, PEI1.2-LA6 could therefore be proposed for the specific targeting of VSMCs in the vascular wall.

CONCLUSIONS

Herein, a library of hydrophobe-substituted low MW *b*PEI derivatives with different *b*PEI backbone MWs, degrees of substitution and types of hydrophobic substitutes were investigated as gene delivery vectors for primary VSMCs and ECs. The developed *b*PEI derivatives were found effective in complexing DNA and, of note, the complexation ability was inversely correlated with the degree of substitution. Such polymers were generally less cytotoxic than the gold standard polymeric transfection reagent 25 kDa *b*PEI and some of them displayed an equal or even higher transfection efficiency. Very small polymers (i.e., 0.6 kDa *b*PEI-

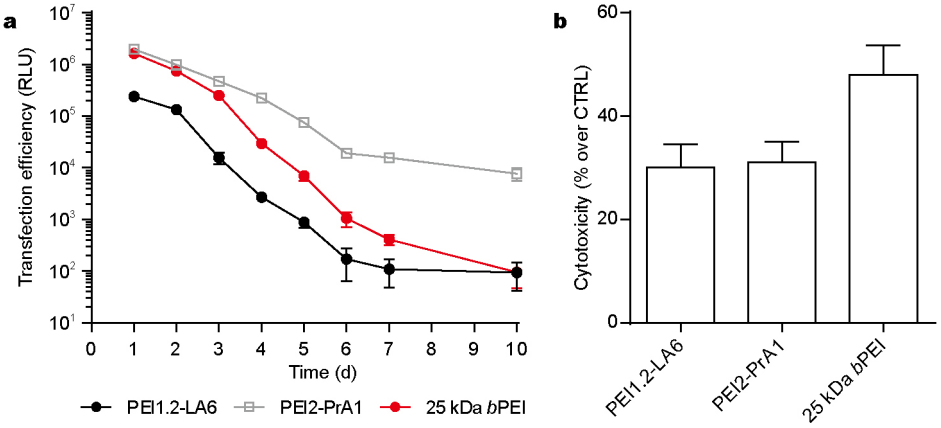


Figure 6 (a) Kinetics of transfection efficiency of selected *b*PEI derivatives on HUVECs. Cells were transfected with pGLuc (*w*/*w* 10 for *b*PEI derivatives, *w*/*w* 5 for 25 kDa *b*PEI) and transfection efficiency was measured at different time points and expressed as RLU. (b) Cytotoxicity of *b*PEI derivatives on HUVECs. Cytotoxicity was evaluated by AlamarBlue assay 48 h post-transfection and expressed as percent toxicity relative to untreated cells. Results are shown as mean ± standard deviation (n ≥ 4).

based derivatives) were ineffective in transfection, while among 1.2 and 2 kDa *b*PEI-derivatives, the propionic acid-substituted polymer PEI2-PrA1 was the most promising compound for VSMCs transfection. Due to the increase of polyplex sedimentation on the cell surface, centrifugation of polyplexes over the cells greatly improved transfection efficiency. It is worth noting that in this experimental setup, mimicking the high polyplex concentration occurring in cardiovascular devices such as gene-eluting stents or catheter balloons, the activity of PEI2-PrA1 was higher and more sustained than 25 kDa *b*PEI on both VSMCs and ECs, while maintaining significantly lower cytotoxicity levels. Interestingly, the linoleic acid-substituted polymer PEI1.2-LA6 was at least as effective as and less cytotoxic than 25 kDa *b*PEI on VSMCs but exerted low transfection on ECs, suggesting that this polymer could be used for the selective gene delivery to VSMCs in blood vessels. Altogether, in this work, hydrophobic moiety-substituted low MW *b*PEI derivatives with superior transfection properties on vascular cells were identified, with possible applications in non-viral gene therapy approaches aimed at the targeted inhibition of vascular pathologies such as intimal hyperplasia and in-stent/post-angioplasty restenosis.

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Author contributions Pezzoli D, Uludağ H, Mantovani D and Candiani G conceived the idea and designed the experiments; Pezzoli D, Tsekoura EK and Bahadur KC R performed the experiments; Pezzoli D and Uludağ H analysed the data and wrote the manuscript with support from Candiani G and Mantovani D. All authors contributed to the general discussion.

Conflict of interest Bahadur KCR and Uludağ H hold ownership position in RJH Biosciences Inc. intended to commercialise the described polymers.

Supplementary information Supplementary data are available in the online version of the paper.

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疏水基取代的分枝状聚乙酰亚胺衍生物：促进原发性血管细胞中的转染

Daniele Pezzoli[1*], Eleni K. Tsekoura[2], Remant Bahadur K.C.[2], Gabriele Candiani[3], Diego Mantovani[1], Hasan Uludağ[2*]

摘要 针对血管细胞的基因治疗代表了一种有望用于预防和治疗内膜增生、血管支架狭窄和血管成形术后狭窄等病理状态的方法. 聚合物非病毒载体的基因传递系统可以安全替代病毒载体, 但是为了提高临床效果, 它们的治疗效率及细胞相容性还需要进一步改善. 本文合成了一系列24种被疏水基团修饰的分枝状聚乙酰亚胺衍生物(*b*PEI), 并进行了表征及在体外原发性血管细胞内的测试, 旨在筛选出具有优异的转染效率和低细胞毒性的传递剂. 低分子量的聚乙酰亚胺(0.6, 1.2 and 2 kDa)以不同取代程度接枝上了不同饱和度的长(C18)和短(C3)的不饱和脂肪链. 丙酰取代衍生物(PEI2-PrA1, C3:0)在血管平滑肌细胞和内皮细胞转染中是最有效的, 与著名的黄金标准25 kDa *b*PEI相比, 具有更优异、更持久的基因表达, 且毒性更低. 此外, 亚油酰基取代衍生物(PEI1.2-LA6, C18:2) 由于在血管平滑肌细胞转染过程中效率高, 而在内皮细胞中相对无效, 且其具有可容忍的细胞毒性, 可作为特定靶向于血管平滑肌细胞的载体.