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Use of Primary and Secondary Polyvinylamines for Efficient Gene Transfection

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TOC

ABSTRACT

Gene transfection with polymeric carrier remains a challenge, particularly high transfection levels combined with low toxicity are hard to achieve. We herein revisit polyvinylamines, an old and neglected family of cationic polymers. They can be readily obtained by controlled hydrolysis of polyvinylamides prepared through (controlled) radical polymerization. A series of tailor-made and well-defined polyvinylamines bearing primary amino groups, and poly(*N*-methylvinylamine) bearing secondary amines, were evaluated for the transfection of cells with pDNA as a function of their molar mass, molar mass distribution and degree of deacetylation. Unexpected high transfection levels, in combination with low cytotoxicity were recorded for both series. Surprisingly, a great impact of the molar mass was observed for the primary amine polyvinylamine series, whereas the results were mostly independent on molar mass or dispersity for the polymer bearing secondary amine. It was further established that a certain percentage of acetamide groups increased the transfection level, while maintaining

low cytotoxicity. These results highlight for the first time the real potential of polyvinylamines as gene carriers, and make these polymers very attractive for further development in gene therapy.

1. Introduction

Gene delivery aims at the introduction of genetic materials (DNA and RNA) in cells in order to modulate the cell activity, with a therapeutic objective in most cases.¹ Efficient delivery is achieved through the use of vectors that prevent polynucleotides from degradation in physiological medium.^{2, 3} Viral vectors allow very efficient transfection and represent the leading strategy in the field.^{3, 4} However, various drawbacks have been revealed, such as immune response, inflammation, random genomic integration and the limited size of the genetic material that can be transported.⁴⁻⁶ Synthetic carriers have been considered in order to circumvent these limits. Among them, cationic polymers represent an important class of vectors that fulfil most requirements for gene delivery application.⁷ They allow to (i) easily complex nucleic acids into small particles thanks to electrostatic interactions, (ii) protect DNA or RNA against degradation by nucleases, and (iii) release them into the cell.⁸ Moreover, further advantages can be quoted such as the unlimited size of the genetic material that can be delivered, their easy production following the good manufacturing practice (GMP), and their low price production. Branched or linear polyethylenimine (bPEI or lPEI) is known as gold standard, thanks to its transfection efficiency.^{7, 9, 10} However, it suffers from a certain degree of cytotoxicity. Chemical modifications of the backbone have been largely investigated to improve both its cell viability and/or transfection efficiency. In this respect, the grafting of cyclodextrins,^{11, 12} *N*-ethylamine or *N*-dimethylethylamine,¹³ alkylcarboxylate,¹⁴

carboxyalkylate,¹⁵ sugars such as galactose¹⁶ or amino acids such as histidine¹⁷ to the amine functions was considered. The introduction of hydrophobic moieties was also studied through the partial acetylation of the amine function¹⁸⁻²⁰ or the grafting of methylcarboxytrimethylene carbonate (MTC).²¹ Significant improvement of the biocompatibility of these vectors was reported, but the transfection efficiency remains low as compared to that reported for viruses. Following the development of PEI as potential gene carrier,²² various other polymer families have been tested such as polyamidoamine (PAMAM),²³ chitosan,²⁴ poly(2-*N*-dimethylaminoethyl)methacrylate (PDMAEMA)²⁵ and poly(β -amino ester)s^{26,27}. Surprisingly, the transfection ability of polyvinylamine (PVAm), an isomer of PEI, has been poorly studied so far.²⁸ In 1999, PVAm with different molar masses and rather broad molar mass distributions were prepared via free radical polymerization (FRP) of *N*-vinylformamide (NVF) and subsequent alkaline hydrolysis of the amide functions. Condensation of a 6 kb plasmid by PVAm was observed to form nanoparticles (polyplexes) but no significant transfection was ensured when applied to Human Embryonic Kidney 293 cells (HEK 293). Since then, the use of PVAm as transfecting agent has been completely disregarded.²⁶

Figure 1. Synthesis of primary and secondary polyvinylamines.

Given the importance of the macromolecular parameters for the transfection efficiency, which was demonstrated for several polymeric vectors,²⁹⁻³³ recent developments in controlled radical polymerization (CRP), particularly in the synthesis of well-defined polyvinylamines (Figure 1), could relaunch the research on PVAm for gene transfection. Primary PVAm with precise molar mass and low dispersity were notably obtained by reversible Addition-Fragmentation Transfer Polymerization (RAFT) of *N*-vinylphthalimide (NVPi) followed by the complete hydrazinolysis of the phthalimide groups (Figure 1, route A).³⁴ On the other hand, the Organometallic Mediated Radical Polymerization (OMRP) of *N*-vinylacetamide (NVA) and *N*-methylvinylacetamide (NMVA) and subsequent mastered acidic hydrolysis of the pendant amide functions led to the controlled synthesis of, respectively, primary and secondary polyvinylamines bearing a defined amount of remaining pendant amides (Figure 1, route B).³⁵

In the present work, we take benefit from the latest progress in CRP for further exploring the gene transfer performance of polyvinylamine derivatives. A large library of primary and secondary polyvinylamines, PVAm and PMVAm, respectively, with various molar masses and dispersities was prepared via FRP and CRP and tested for pDNA transfection. The cell viability was also systematically evaluated. Most of the controlled syntheses were achieved by OMRP, which gives access to primary and secondary polyvinylamines. Nevertheless, important information on the structure/activity relationship was also collected from polymers prepared by RAFT.

2. Experimental part

2.1. Materials

N-vinylacetamide (NVA) (> 98 %, TCI), *N*-vinylphthalimide (NVPi) (99 %, Acros Organics), cobalt(II) acetylacetonate (Co(acac)₂) (97 %, Aldrich), 2,2'-azobis(4-methoxy-2,4-dimethyl valeronitrile) (V-70, $t_{1/2}$ = 10 h at 30°C) (> 98 %, Wako), 2,2'-azobis[2-methyl-*N*-(2-hydroxyethyl)propioamide] (VA-086, $t_{1/2}$ = 10 h at 86°C) (> 98 %, Wako), 2,2'-Azobis(2-methylpropionitrile) (AIBN, 98 %, Aldrich), potassium ethyl xanthogenate (96 %, Aldrich), ethyl 2-bromopropanoate (99 %, Aldrich) and hydrazine monohydrate (98 %, Aldrich) were used as received. *N*-methylvinylacetamide (NMVA) (> 98 % Aldrich) and vinyl acetate (> 99 %, Aldrich) were purified by distillation under reduced pressure and degassed by freeze-drying cycles under vacuum. Propanethiol (PrSH) (99 %, Aldrich), dichloromethane (CH₂Cl₂) (p.a.), ethanol (p.a.), dimethylformamide (DMF) (p.a.) and methanol (MeOH) (p.a.) were degassed by bubbling argon for 30min. The alkyl-cobalt(III) adduct initiator ([Co(acac)₂-(CH(OAc)-CH₂)₄R₀]; R₀ being the primary radical generated by 2,2'-azo-bis(4-methoxy-2,4-dimethyl valeronitrile) (V-70, Wako)) was prepared as described in the literature³⁶ and stored as a CH₂Cl₂ solution at -20°C under argon. Deuterated methanol (MeOD) and deuterated water (D₂O) used for NMR characterization were ordered from Eurisotop. Dialysis membranes (cut-off, 1 kDa) were purchased from VWR. UV irradiation was performed with a Laboratory-UV-Reactor system 2 purchased from UV-Consulting Peschl. The photo-reactor is composed of a medium pressure mercury lamp TQ 150 (power input: 150 W), a quartz immersion tube, a quartz cooling tube, power supply, reactor vessel with converter tube, discharge cock and sealing cap. The Acid Digestion Vessel (23 mL) used for hydrolysis reactions was purchased from Parr. Linear polyethyleneimine (IPEI) of M_w =22 kDa was prepared as described.¹⁸

2.2.Characterization

SEC. Number-average and weight-average molar masses (M_n and M_w) and molar mass distribution (D), of the polyvinylamides were determined by size exclusion chromatography (SEC) in dimethylformamide (DMF) containing LiBr (0.025 M) at 55°C (flow rate: 1 mL/min) with a Waters chromatograph equipped with three columns (Waters Styragel PSS gram 1000 Å (x 2), 30 Å), a dual λ absorbance detector (Waters 2487) and a refractive index detector (Waters 2414). Multi-angle laser light scattering (MALLS) measurements were performed with a Dawn Heleos apparatus from Wyatt Technology to determine the absolute molar masses (M_n^{LS}). Data were processed with Astra V Software (Wyatt technology). For each polymer, the dn/dc value was measured by refractometry using a Wyatt OptilabREX refractive index detector ($\lambda = 658$ nm). A polystyrene calibration was also used to evaluate the molar mass distribution. After hydrolysis, the resulting PVAm, PMVAm were analyzed by SEC in water containing NaCl (0.1 M) and TFA (0.1 %v) at 30°C (flow rate: 1 mL/min) using a Waters SEC equipped with a pre-column (PSS NOVEMA Max analytical 10 micron 8.0 x 50 mm) and a linear column (PSS NOVEMA Max analytical linear S micron 8.0 x 300 mm). Poly(2-vinylpyridine) (P2VP) standards were used to generate the calibration plot.

NMR. 1H NMR spectra were recorded in D_2O (solvent) at 298K with Bruker spectrometer (400 MHz) and treated with MestraNova software.

EA. Elementary analyses were carried out by the Service de Microanalyse ICSN– CNRS (Gif sur Yvette, France) in order to determine the C/N ratios in the copolymers before and after acidic treatment in order to evaluate the degree of deacetylation of the amide functions.

ICP. Inductively coupled plasma (ICP) analyses were carried out by the Institut Malvoz de la Province de Liège (Liège, Belgium) in order to determine the quantity of residual cobalt polymers after acidic treatment ($[Co] < 0.4$ ppm).

Titration. Aqueous solutions of polymers (50 mg in 10 mL of 1N HCl, PN255-Cs and PM140-Fh, Table 1, entries 3 and 8) were titrated between pH 1 and 12 using an automatic titrator (TIM 900, Radiometer Analytical) controlled by the TitrMaster 85 software. The polymer was first dissolved in 1N HCl and 0.5N NaOH was added progressively; each addition was performed after stabilization of the pH (SI Figure S1A). The method described by Colombani *et al.*³⁷ was used to determine the protonation rate of these polyelectrolytes versus pH. (SI Figure S1C). At physiological pH, the protonation ratio of PVAm and PMVAm were around 0.74 and 0.79, respectively. The apparent pKas of the same polymers were then determined following Borukhov *et al.*³⁸ and found to be 10.6 and 12.4, respectively (SI Figure S1D). The buffering capacity is the proton quantity required to decrease the pH by one unit. It can be determined from the slope of the titration curves between pH = 6.5 to 7.5, the relevant pH range of endosome acidification in the cell. The buffer capacity determined as the reverse of the slope of the titration curves in Figure S1B (SI) was 0.13 mL and 0.09 mL for PVAm and PMVAm, respectively.

2.3.Synthesis of PNVA by FRP.

Following a procedure adapted from reference³⁹, NVA (4.0 g, 47 mmol) and V70 (320 mg, 1.0 mmol) were placed under argon and dissolved in degassed methanol (6 mL). The reaction was heated at 30°C for 45 min (total monomer conversion measured by ¹H NMR in D₂O by integrating the signals of the methine proton of the monomer (at 6.78 ppm, *1H*) and the methyl protons (1.9 to 2.0 ppm, *3H*) for both the monomer and the polymer, conv. = 35 %). The polymer was purified by precipitation in acetone, filtrated and dried under vacuum before dissolution in water and dialysis (membrane cut off 1 kDa) against pure water for two days with regular water exchanges.⁴⁰ After lyophilization, PNVA was collected as a white solid and characterized by ¹H NMR in D₂O (*cf.* SI, Figure S2), and SEC-MALLS in DMF (dn/dc

PNVA in DMF = 0.060, $M_{n,SEC\ DMF\ MALLS} = 58.3\ \text{kg/mol}$, $M_{n,SEC\ DMFcal\ PS} = 31.3\ \text{kg/mol}$, $D_{SEC\ DMFcal\ PS} = 1.69$). (Table 1, entry 1)

2.4. Synthesis of PNVA by photo-initiated OMRP.

Following a procedure adapted from reference ³⁵, Co(acac)₂ (204 mg, 0.79 mmol), VA-086 (460 mg, 1.6 mmol) and NVA (15.0 g, 176 mmol) were added in a reactor designed for photochemistry (Laboratory-UV-Reactor system 2 purchased from UV-Consulting Peschl), an immersion-well reactor in which the lamp is surrounded by the solution in a quartz tube. The medium is degassed by three vacuum/argon cycles before addition of degassed MeOH (15 mL). The reactor was maintained at 0°C in an ice-bath and the mixture was irradiated with an UV lamp (TQ 150 with a power input at 150 W) for 4 h at 0°C. After the irradiation was stopped, the medium was allowed to return to room temperature (~20°C) and the polymerization proceeded for 7 h 30. A sample was picked out of the reactor, quenched by TEMPO, and used for determining the conversion by ¹H NMR (23 %) and the molar mass of the polymer by SEC analyses. In order to quench the reaction as reported elsewhere,⁴⁰ degassed 1-propanethiol (1.40 mL, 15.8 mmol) was added with a syringe under inert atmosphere to the reaction medium which became instantaneously black. The medium was stirred overnight at room temperature. The reaction medium was eluted through a celite pad allowing removing black residues. The polymer was then recovered by several precipitations in acetone and dried under vacuum. The polymer was further purified by dialysis against water for two days. After lyophilisation, an off-white solid was obtained and characterized by SEC, NMR and ICP. ($dn/dc_{PNVA\ in\ DMF} = 0.060$, $M_{n,SEC\ DMF\ MALLS} = 56.3\ \text{kg/mol}$, $M_{n,SEC\ DMF\ cal\ PS} = 48.1\ \text{kg/mol}$, $D_{SEC\ DMF\ cal\ PS} = 1.18$, $M_{n,SEC\ H_2O\ cal\ P2VP} = 31.0\ \text{kg/mol}$, $D_{SEC\ H_2O\ cal\ P2VP} = 1.27$) (Table 1, entry 4). Similar experiments were carried out varying the [NVA]/[VA-86]/[Co(acac)₂] ratio and the reaction time, as reported in Table 1.

2.5. Synthesis of PNMVA by FRP.

Following a procedure adapted from reference³⁹, V70 (416 mg, 1.35 mmol) was placed under argon in a flask, dissolved in degassed methanol (14 mL) followed by addition with a syringe of distilled and degassed NMVA (14.0 mL, 13.4 g, 135 mmol). The reaction mixture was then heated at 30°C for 4 h (total monomer conversion measured by ¹H NMR in D₂O by integrating the signals of the methine proton of the monomer (at 7.04 and 7.35 ppm, 1H) and the *N*-methyl protons region (2.8 to 3.0 ppm, 3H) for both the monomer and the polymer, conv. = 14 %). The polymer was then purified by precipitation in diethyl ether, filtrated and dried under vacuum before dissolution in water and dialysis (membrane cut off 1 kDa) against water for two days. After lyophilization, PNMVA was collected as a white solid and characterized by ¹H NMR in D₂O (*cf.* SI, Figure S2), and SEC-MALLS in DMF (dn/dc_{PNMVA} in DMF = 0.071, $M_{n,\text{SEC DMFMALLS}} = 13.7 \text{ k g/mol}$, $M_{n,\text{SEC DMFcal PS}} = 17.8 \text{ kg/mol}$, $D_{\text{SEC DMF cal PS}} = 1.79$). (Table 1, entry 8). Similar experiments were carried out varying the [NMVA]/[70] ratio and the reaction time, as reported in Table 1.

2.6. Synthesis of PNMVA by OMRP.

Following a procedure adapted from reference³⁵, a solution of alkyl-cobalt (III) initiator ($[\text{Co}(\text{acac})_2-(\text{CH}(\text{OAc})-\text{CH}_2)_4\text{R}_0]$ in CH_2Cl_2) (1.30 mL of a 0.15 M stock solution, 0.195 mmol) was introduced in a flask under argon and evaporated to dryness under reduced pressure at room temperature. Distilled NMVA (6.0 mL, 58 mmol) was added under argon into the flask and the polymerization mixture was stirred at 40°C. After 16 h, the polymerization was stopped by addition of 1-propanethiol (0.35 mL, 3.9 mmol) and the medium became instantaneously dark black. Stirring was maintained overnight at room temperature. After elution of the polymer solution through a celite pad for removing the black matter, the polymer was precipitated in diethylether, filtered and dried. A second precipitation

was performed in Et₂O after solubilizing the polymer in MeOH. Finally, the polymer was purified by dialysis against water for two days and was collected as an off-white solid after lyophilisation. The resulting PNMVA was analyzed by SEC and ¹H NMR. (dn/dc PNMVA in DMF = 0.071, $M_{n,SEC\ DMF\ MALLS}$ = 30.8 kg/mol, $M_{n,SEC\ DMF\ cal\ PS}$ = 21.6 kg/mol, $D_{SEC\ DMF\ cal\ PS}$ = 1.12, $M_{n\ SEC\ H_2O\ cal\ P2VP}$ = 3.5 kg/mol, $D_{SEC\ H_2O\ cal\ P2VP}$ = 1.8, Table 1, entry 13). Similar experiments were carried out varying the initial [NMVA]/[RCo(acac)₂] molar ratio and the reaction time in order to produced PNMVA of different molar masses as reported in Table 1.

2.7. Synthesis of the polyvinylamine derivatives by hydrolysis of the polyvinylacetamide precursors.

Following a procedure adapted from reference³⁹, 400 mg of poly(*N*-methylvinylacetamide) (PNMVA) were dissolved in HCl 6 N (5.5 mL). The resulting polymer solution (7 % w/v) was then placed in an Acid Digestion Parr Vessel and heated at 120°C for 64 h (*harsh* conditions). After cooling, the solution was dialyzed against pure distilled water for one night, and water was exchanged twice. During the last two hours of dialysis, the pH was maintained at 7 (pH controlled with pH meter and adjusted by addition of NaOH 0.1 N or HCl 1 N). The polymer was recovered upon lyophilization. The polymer was analyzed by ¹H NMR in D₂O (*cf.* SI, Figure S2), IR and EA. In addition, the hydrolysis of PNVA was performed under milder conditions (5 % w/v, HCl 2 N, 120°C, 14 h) according to reference.³⁹ Finally, partially deacetylated PMVAm, P(MVAm-*co*-NMVA), were obtained using HCl 3 N at 100°C for different periods according to kinetics performed by Buys.⁴¹ The determination of the percentage of hydrolysis for each polymer was achieved using the elementary analysis (EA). The method was based on the C/N ratio in the polymer before and after acidic treatment rather than on absolute mass percentage of C and N in order to prevent mistakes coming from the hydroscopic character of the polymers (SI Table A1).

2.8. Synthesis of *O*-ethyl-*S*-(1-ethoxycarbonyl) ethyl dithiocarbonate (CTA-X1).

The synthesis of *O*-ethyl-*S*-(1-ethoxycarbonyl) ethyl dithiocarbonate was performed according to reference⁴² by mixing ethyl 2-bromopropionate (2.0 mL, 2.8 g, 15 mmol) in ethanol (18 mL) and adding portion-wise potassium ethyl xanthogenate (2.03 g, 12.7 mmol) at 0°C. Then the reaction took place at room temperature (~20°C) overnight. After complete conversion, the product was extracted by Et₂O/ pentane (2/1), washed with H₂O and dried over MgSO₄. The solvents were removed under reduced pressure in order to obtain the desired product as an orange liquid. The product was analyzed by ¹H NMR (CDCl₃, 300 MHz): δ 1.28 ppm (t, 3H, *J* = 7.1 Hz), 1.41 ppm (t, 3H, *J* = 7.1 Hz), 1.57 ppm (d, 3H, *J* = 7.3 Hz), 4.20 ppm (q, 2H, *J* = 7.1 Hz), 4.38 ppm (q, 1H, *J* = 7.3 Hz), 4.64 ppm (q, 2H, *J* = 7.1 Hz), 97% yield, > 98 % pure.

2.9. Synthesis of PNVPi by RAFT polymerization.

Following a procedure adapted from references^{34, 43, 44}, NVPi (5.00 g, 28.9 mmol) was dissolved in a solution of DMF (14 mL), containing AIBN (23.5 mg, 0.143 mmol) and CTA-X1 (132 mg, 0.594 mmol) (Table 3, entry 2). The mixture was placed in a flask equipped with a magnetic stirring bar and the solution was degassed by Argon bubbling for 30 min. Thereafter the polymerization was performed at 60°C for 24 h. After cooling, the polymer was centrifuged to remove a black precipitate, precipitated in Et₂O, filtrated and dried under vacuum. The resulting PNVPi was analyzed by SEC and ¹H NMR, dn/dc_{PNVPi in DMF} = 0.13, *M*_n SEC DMF LS = 12.9 kg/mol, *M*_n SEC DMF cal PMMA = 4.6 kg/mol, *D*_{SEC DMF cal PS} = 1.6). Similar experiments were carried out varying the initial [NVPi]/[AIBN]/[CTA-X1] molar ratio and the reaction time in order to prepare PNVPi of different molar masses (SI, Table S2).

2.10. Hydrazinolysis of PNVPI.

Following a procedure adapted from references^{34, 43, 44}, 4.5 g of PNVPI (26 mmol of NVPI) was dissolved in 1,4-dioxane/MeOH 1/2 (100 mL) and hydrazine monohydrate (31.0 mL, 0.636 mol) was added under inert atmosphere. The reaction was performed at 65°C overnight under stirring. After cooling, the solvents were removed under reduced pressure. In order to obtain the desired polyvinylamine, the resulting solution was treated with acid (HCl 6 N). The final polymer was dialysed against pure water for two days, maintaining the final water at pH 7. The aqueous solution was lyophilized. The resulting PVAm was analyzed by ¹H NMR in D₂O and EA. The ¹H NMR spectra showed the absence of residual 2,3-dihydro-1,4-phthalazinedione, a side product of the hydrazinolysis. Hydrazinolysis degrees were determined by EA using the C/N content ratio in the polymer before and after acidic treatment (SI Table S1, entries 7 to 9).

2.11. Acetylation of PVAm.

Following a procedure adapted from reference⁴⁵, PVAm (PN170-R, $M_n = 7.2$ kg/mol, $DP_n = 167$, 0.10 g, 2.3 mmol of VAm, *cf.* Table S2), prepared by hydrazinolysis of PNVPI, was dissolved in 0.40 mL of distilled water, and triethylamine (0.40 mL, 2.9 mmol) and then methyl acetate (0.1 mL, 1.3 mmol) were added under inert atmosphere. The reaction was performed at 30°C for one night. The polymer was dialyzed against pure water for two days, maintaining the final water at pH 7, and then lyophilized. The resulting PVAm was analyzed by ¹H NMR in D₂O. A degree of acetylation of 50% was determined by elemental analysis (EA). The method was based on the determination of the C/N ratio in the polymer before and after acidic treatment rather than on absolute mass percentage of C and N to take in account the hygroscopic character of the polymers that may alter the deacetylation degree determination.

2.12. Plasmids.

pCMV-luc (pTG11033, 9514 bp, Transgene S.A., Strasbourg, France) was a plasmid DNA encoding the firefly luciferase (luc) gene under the control of the human cytomegalovirus (CMV) promoter. pCMV-EGFP (5130 bp) was a plasmid DNA encoding the jellyfish *Aequorea victoria* enhanced green fluorescent protein (EGFP) under the control of the human cytomegalovirus (CMV). Supercoiled plasmid was isolated from *Escherichia coli* DH5a super competent bacteria (Invitrogen, Cergy Pontoise, France) by alkali lysis and purification with QIAGEN Mega Kit Endotoxin free Plasmid (Qiagen, Courta-boeuf, France).

2.13. Polyplexes.

Polyplexes were prepared at various polymer/DNA weight ratios (WR) in HEPES 10 mM, pH 7.4. The proper amount of polymers was added to the pDNA solution (2.5 µg in 32.5 µL of HEPES, 1 mg/mL) and vortexed for 4s. Typically, for a WR of 3, 15 µL of a 0.5 mg/mL solution of the polymer in HEPES (corresponding to 7.5 µg of polymer) was added to 32.5 µL (2.5 µg) of the pDNA solution (at a concentration of 1 mg/mL). After 30 min at room temperature, the solutions containing polyplexes were adjusted to a total volume of 500 µL using culture medium supplemented with 10 % FBS (MEM for HeLa and 16HBE, DMEM for C2C12 and RPMI 1600 for DC2.4).

2.14. Size and ζ potential measurements of polyplexes.

Polyplexes were prepared as above with 2 µg pDNA in a total volume of 200 µL of 10 mM HEPES, pH 7.4. The size of polyplexes measured by dynamic light scattering (DLS) and ζ -potential measurements were performed by using SZ-100 Nanopartica (Horiba, Les Ulis,

France). Samples were illuminated with a 633 nm laser, and the intensities of scattered light at an angle of 173° and 15° were measured using an avalanche photodiode, respectively for size measurement and zeta potential determination. The z-average hydrodynamic diameter (D_h) of the samples was calculated automatically.

2.15. Gel retardation assay.

Polyplexes were freshly prepared at various polymer/pDNA weight ratios (WR) in a solution of HEPES 10 mM pH 7.4. Samples of 20 μ L were loaded onto 0.9% agarose gel containing EtBr (0.3 mg/mL) in a Tris-borate-EDTA buffer, pH 8.6 (95 mM Tris, 89 mM boric acid, 2.5 mM EDTA and 10 mM DTT, pH 8.6) and ran at 100 V for 45 min. The gels were analyzed using the AzureTM Biosystem c600 to visualize pDNA.

2.16. Determination of the phosphate/nitrogen N/P molar ratio.

The mole number of protonable nitrogen atoms at pH 7.4 per g polymer (N^{N+} in mol/g) was calculated taking into account the deacetylation degree of the polymers, reported in Tables 1 and 2), the molar mass of each monomer unit and the molar fractions of the monomer units containing nitrogen atoms protonable at pH 7.4, *i.e.* VAm and MVAm (eq. 1).

$$N^{N+} = \frac{(F_{VAm} + F_{MVAm})}{\sum M_i F_i} \quad (\text{eq. 1})$$

, where M_i and F_i correspond to the molar mass and the molar fraction of the comonomer units in the copolymer, *i.e.* VAm and MVAm.

The mole number of charged phosphate per g of pDNA (N^{P-} in mol/g) was determined according to equation 2.

$$N^{P-} = \frac{1}{MM_{bp}/2} = 3.058 \times 10^{-3} \text{ mol/g} \quad (\text{eq. 2})$$

, where MM_{bp} corresponds to the mean molar mass of a base pair ($MM_{bp} = 654 \text{ g/mol}$)

The N/P ratio is calculated based on equation 3 and takes into account the polymer/pDNA weight ratio (WR, reported in Tables 4 and 5).

$$\frac{N}{P} = \frac{N^{N+}}{N^{P+}} \times WR \quad (\text{eq. 3})$$

2.17. Cell culture.

Human epithelioid cervix carcinoma cells (HeLa cells; CRL1772, C2C12, Rockville, MD, USA) were cultured in MEM medium supplemented with 10 % FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 % non-essential amino acid, and 1 % GlutaMAX™. Cells were maintained at 37°C in a humidified 5 % CO₂ atmosphere. Cells were checked for mycoplasma presence using the MycoAlert1 Mycoplasma Detection Kit (Lonza, Levallois Perret, France).

2.18. Transfection and luciferase gene expression measurement.

One day before transfection, cells were seeded in a 24-well plate at a density of 1.5×10^5 cells per well in 1 mL in culture medium. After removing the culture medium, they were incubated with freshly made polyplexes (500 µL, 2.5 µg DNA) for 4h at 37°C. Then, the medium was removed and replaced with fresh complete medium. The Luciferase activity was measured 48h after transfection by using a luminometer (LUMAT LB 9507). Then the Luciferase activity was normalized to total cell protein using a BCA protein assay kit (Uptima, Interchim

SA, Montluçon, France), and expressed as relative light units (RLU) per mg of protein. Data are presented as means and standard deviation (s.d.) based on three independent experiments, each in duplicate for the determination of the RLU and in triplicate for protein quantification.

2.19. Cytotoxicity.

MTT assays were performed to determine the toxicity of polyplexes as well as polymers. 48 h after transfection, 50 μ L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/ml in PBS) was added to each well and the cells were incubated for 4 h at 37°C. The medium was taken off and the cells were washed with PBS (500 μ L). Then acidified isopropanol (1 mL) and a solution at SDS 3 % (200 μ L) were added. MTT was thus converted in water-soluble formazan. The absorbance was measured at 560 nm with a Victor I spectrophotometer and expressed as a percentage of the absorbance of non-transfected cells. Data are presented as mean and standard deviation (s.d.) based on two independent experiments, each in triplicate.

2.20. Flow cytometry experiments.

After 48 h, transfected cells were washed two times with PBS, harvested by trypsin, centrifuged (1500 rpm for 5 min at 4°C) and suspended in PBS. The cell-associated fluorescence intensity was measured by flow cytometry (LSR, Becton Dickinson). The cell-associated fluorescence was recorded at 520 nm after excitation at 488 nm and was expressed as the mean fluorescence intensity (MFI) of 1×10^4 cells.

2.21. Confocal laser scanning microscopy (CLSM).

HeLa cells were seeded two days prior to the experiments, at a density of 1.4×10^4 cells in 0.5 mL culture medium per well in a 4-well Lab-Teck chambered coverglass (Nunc, Dutsher

S.A., Brumath, France). Cells were transfected with polyplexes containing pCMV-EGFP. After 48h, confocal microscopy analysis was performed using Zeiss Axiovert 200M inverted microscope (equipped with a Plan-Apochromat 63x objective, NA = 1.4) coupled with Zeiss LSM 510 scanning confocal head (Carl Zeiss Co., Ltd., Jena, Germany).

3. Results and discussion

3.1.Synthesis of primary and secondary polyvinylamines

Following recently reported procedures,^{35, 46} a series of polyvinylamines (PVAm) and poly(*N*-methylvinylamines) (PMVAm) was prepared by FRP or OMRP of *N*-vinylacetamide (NVA) or *N*-methylvinylacetamide (NMVA) followed by the acidic hydrolysis of the pendant amide functions of the poly(vinylacetamide)s (Figure 1, route B). The synthesis of these primary and secondary polyvinylamine derivatives is commented below and their macromolecular characteristics are presented in Tables 1 and 2. For the sake of clarity, an abbreviated polymer terminology was used. PN and PM stand for PVAm and PMVAm, respectively. The following number refers to the number-average degree of polymerization (DP_n). Behind it, the polymerization method is designated by F and C, for free and controlled cobalt-mediated radical polymerization, respectively. The final letter provides information on the hydrolysis conditions: s for *soft* (HCl 2 N), m for *moderate* (HCl 3 N) and h for *harsh* (HCl 6 N). For more precision, the deacetylation degree of some polymers is indicated at the end of the abbreviated name. For instance, PM155-Fm23 designates a PMVAm with a degree of polymerization of 155 prepared by free radical polymerization and subsequent hydrolysis using HCl 3 N with 23 % of hydrolysis.

Synthesis of poly(vinylacetamide) precursors: Poly(vinylacetamide) (PNVA) was first synthesized by FRP of NVA initiated by V70 at 30°C. The resulting polymer was

characterized by a molar mass of 58 kg/mol ($DP_n = 685$) and a quite broad molar mass distribution ($\bar{D} = 1.69$) (Table 1, entry 1). Note that shorter polymers could not be obtained by FRP even when very high amounts of V70 were used probably due to the high reactivity of NVA.³⁹ On the other hand, we prepared a series of PNVAs with various molar masses and a narrow molar mass distribution via photoinitiated OMRP of NVA using VA-086 as initiator and Co(acac)₂ as controlling agent, as reported elsewhere.³⁵ By varying the [NVA]/[VA-086]/[Co(acac)₂] ratio and the reaction time, five PNVAs were obtained with molar masses ranging from 13 kg/mol ($DP_n = 153$) to 129 kg/mol ($DP_n = 1510$) (Table 1, entries 2-6). The molar mass dispersity was low, *i.e.* between 1.06 and 1.27, except for the PNVA with the highest molar mass ($M_n = 129$ kg/mol, $DP_n = 1510$, and $\bar{D} = 1.56$). Poly(*N*-methylvinylacetamide)s (PNMVAs) were also prepared by FRP and OMRP. A set of four PNMVAs with a molar mass ranging from 9 kg/mol to 28 kg/mol and a molar mass distribution around 2 was obtained by FRP using different NMVA/V70 molar ratios (Table 1, entries 7-10). In addition, as previously reported,⁴⁶ the controlled polymerization of NMVA was performed using an alkylcobalt complex as OMRP initiator at 40°C leading to well-defined PNMVAs with molar masses ranging from 11 kg/mol to 67 kg/mol (Table 1, entries 11-14). Again, except for the sample with a highest degree of polymerization ($DP_n = 677$), the molar mass distribution of the PNMVA samples were below 1.2 (Table 1, entry 14).

Table 1. Characteristics of poly(*N*-vinylacetamides) (PNVA) and poly(*N*-methylvinylacetamides) (PNMVA) and the corresponding poly(*N*-vinylamines) (PVAm) and poly(*N*-methylvinylamines) (PMVAm) synthesized by FRP and OMRP followed by amide hydrolysis.

Entry	Name	Polymer	Poly(vinylacetamide)s				Polyvinylamines			
			Synthesis	M_n^{LS} (kg/mol) ^g	DP_n^h	$\bar{D}^i$	% of hydrolysis ^k	Composition		M_n (kg/mol) ^m
								DP_n amine (n') ^l	DP_n acetamide (n) ^l	
1	PN700-Fs	PVAm ^{a,e}	FRP	58	685	1.69	94	644	41	31.2
2	PN150-Cs	PVAm ^{b,e}	OMRP	13	153	1.06	91	139	14	7.2
3	PN255-Cs			22	256	1.14	88	233	23	12.0
4	PN660-Cs			56	662	1.18	90	596	66	31.2
5	PN940-Cs			80	940	1.27	87	818	122	45.5
6	PN1510-Cs			129	1510	1.56	92	1389	121	70.0
7	PM100-Fh	PMVAm ^{c,f}	FRP	9	93	2.02 ^j	78	73	20	6.2
8	PM140-Fh			14	138	1.79	81	112	26	9.0
9	PM165-Fh			16	164	1.99 ^j	88	144	20	10.2
10	PM285-Fh			28	284	2.11 ^j	71	202	82	19.6
11	PM110-Ch	PMVAm ^{d,f}	OMRP	11	107	1.08	82	88	19	6.9
12	PM265-Ch			26	265	1.14	87	230	35	16.6
13	PM310-Ch			31	313	1.12	93	291	22	18.7
14	PM680-Ch			67	677	2.02	91	616	61	41.1

^a Free Radical Polymerization of NVA, conditions [NVA]/[V70] = 47, in MeOH, NVA/MeOH = 4/6 w/v %, 30°C for 45 min. ^b Organometallic Mediated Radical Polymerization of NVA with photo-initiation at 0°C for 4 h then polymerization at rt. Conditions are respectively for PN150-Cs, PN255-Cs, PN660-Cs, PN940-Cs and PN1510-Cs, [NVA]/[VA086]/[Co(acac)₂] = 110/2/1 for 2 h and 4 h, [NVA]/[VA086]/[Co(acac)₂] = 220/2/1 for 7.5 h and 6 h and [NVA]/[VA086]/[Co(acac)₂] = 440/2/1 for 7.5 h. ^c Free Radical Polymerization of NMVA. Conditions for PM100-Fh, PM140-Fh, PM165-Fh and PM285-Fh are respectively [NMVA]/[V70] = 25, [NMVA]/[MeOH] = 1/1 v/v %, at 30°C for 23 h, [NMVA]/[V70] = 100, [NMVA]/[MeOH] = 1/1 v/v %, at 30°C for 4 h, [NMVA]/[V70] = 51, [NMVA]/[MeOH] = 1/1 v/v %, at 30°C for 14.5 h and [NMVA]/[V70] = 100, [NMVA]/[MeOH] = 1/1 v/v %, at 30°C for 22 h. ^d Cobalt Mediated Radical Polymerization of NMVA. Conditions for PM110-Ch, PM265-Ch, PM310-Ch and PM680-Ch are respectively [NMVA]/[RCO] = 113, 302, 297 and 538, in bulk at 40°C for 5 h, 20.5 h, 16 h and 48 h. ^e Hydrolysis performed with HCl 2 N at 120°C, for 14 h. ^f Hydrolysis performed with HCl 6 N at 120°C for 64 h. ^g Determined by SEC in DMF equipped with a MALLS detector, dn/dc_{PNVA} = 0.060 and dn/dc_{PNMVA}

= 0.071. LS = light scattering. ^h Calculated using the following formula: $DP_n = M_n^{LS}/MM_{\text{monomer}}$. ⁱ Determined by SEC in DMF using a PS calibration. ^j Determined by SEC in DMF at 60°C using a PMMA calibration using two PSS GRAM 1000Å columns (8x300 mm; separation limits: 1-1000 kg/mol) and one PSS GRAM 30Å (8x300 mm; separation limits: 0.1-10 kg/mol). ^k Determined by elemental analysis (EA) (SI Table S1 for crude EA results and calculations). ^l n and n' stand for the number of amides and amines after hydrolysis in the polymer backbone (cf. structure in Figure 1B). ^m Number-average molar mass calculated by the molar mass of the polyamide precursor and the level of hydrolysis of the amide moieties.

Amide hydrolysis: The above prepared poly(vinylacetamide)s were then converted into the corresponding polyvinylamines by acidic hydrolysis of the acetamido groups (Table 1). The percentage of deacetylation was determined by comparing the C/N ratio measured by EA in the polymer before and after acidic treatment (SI, Table S1).³⁹ The PNVA samples were hydrolyzed using conditions described by Akashi *et al.*,^{39, 47} *i.e.* HCl 2 N at 120°C for 14 h, whereas harsher conditions were necessary for hydrolyzing the PNMVAs (HCl 6 N at 120°C for 64 h).³⁹ Under these conditions, the percentage of deacetylation was 87-94 % and 70-94 % for PNVA and PNMVA, respectively. Typical ¹H NMR spectra of the resulting PVAm and PMVAm are provided in Figure S2 and confirm the low amount of residual acetamido groups along the backbone. A third hydrolysis condition, reported by Buys *et al* ⁴¹ (HCl 3 N at 100°C), was applied to a PNMVA sample prepared by FRP (DP_n 155) in order to prepare a series of P(MVAm-*co*-NMVA) containing different amounts of residual acetamido groups (Table 2). The acidic treatment was stopped at different reaction times and 23 % to 94 % hydrolysis was reached. As discussed later, these compounds allowed us to assess the possible effect of the residual acetyl groups on the transfection efficiency. Because these polymers are dedicated to a biomedical application, it is important to mention that cobalt was carefully removed from all polymers synthesized by OMRP. Indeed, after purification, less than 0.4 ppm of Co was found in the polyvinylamines according to the inductively coupled plasma analysis.³⁵

Table 2. Characteristics of P(NMVA-*co*-MVAm) copolymers obtained from PM155-F through partial deacetylation

Entry	Polymer	% of hydrolysis ^a	Composition of P(NMVA-co-MVAm)		M_n (kg/mol) _b
			DP_n amine (n')	DP_n acetamide (n)	
1	PM155-Fm23	23	36	119	27.7
2	PM155-Fm37	37	57	98	13.9
3	PM155-Fm44	44	68	87	12.2
4	PM155-Fm54	54	84	71	11.8
5	PM155-Fm64	64	99	56	11.2
6	PM155-Fm76	76	120	35	10.3
7	PM155-Fm94	94	146	9	9.2

The PM155-F precursor was obtained by Free Radical Polymerization of NMVA. Conditions: [NMVA]/[V70] = 51, in bulk, at 30°C for 19 h. M_n^{LS} = 15500, D = 1.55. ^a Hydrolysis conditions performed with HCl 3 N at 100°C, for different times. ^b Number-average molar mass calculated by the molar mass of the precursor and the level of hydrolysis of the amide moieties.

3.2. Polyplexes formation and characterization

Polyplexes were formed in 10 mM HEPES buffer, pH 7.4. The amount of polymer necessary to allow complete pDNA condensation (corresponding to ratio 1 in the following Figures, and to the lowest ratio reported in Table 3) was determined by agarose gel retardation assays upon electrophoresis of polyplexes made with increasing amounts of polymer (Figure S3). In contrast to free pDNA, no migration was observed when pDNA was condensed with the polyvinylamine derivatives, *e.g.* PN255-Cs, PN700-Fs, PM310-Ch or PM155-Fm76 (in Figure S3), suggesting the proper complexation of the nucleic acid.

Table 3 reports for each polymer the lowest polymer/pDNA weight ratios (WR) (ratio 1) allowing complete pDNA condensation and a second one (ratio 2) made with twofold amount of

polymer. For both types of polyvinylamines (the primary and secondary one) the ratio 1 was generally 1 and 3, excepted for the shortest PVAm (PN-C150) for which higher amounts of polymer were needed (WR=4 and 8). The polymer/pDNA *weight* ratios (WR) can be translated into the nitrogen/phosphate (N/P) *molar* ratio, which relates the number of protonable nitrogen atoms (N) needed to complex efficiently one phosphate group (P) of the pDNA (see experimental section for calculations). In all cases, an excess of protonable amines was necessary to achieve the pDNA condensation. Moreover, less protonable nitrogens were necessary to reach the complete condensation of pDNA with PMVAm (N/P charge ratio from 4 to 9) as compared to PVAm (6 to 25). This suggests that secondary amines are more efficient to condense the pDNA than primary amine, at least for this class of cationic polymer. It is well known that a small size and a positive charge are essential parameters for an efficient gene carrier: positively charged polyplexes interact easily with the negatively charged cell membranes through electrostatic interactions, and the uptake by the cells is favoured for small polyplexes.⁴⁸⁻⁵⁷ Most of the PVAm or PMVAm-based polyplexes had a hydrodynamic diameter ranging from 100 to 300 nm and ζ -potentials ranging from + 15 to + 55 mV (Table 3).

Table 3. Characterization of PVAm and PMVAm polyplexes.

Entry	Polyplexes	polymer/pDN		D_h^c (nm)	ζ^d (mV)
		A WR ^a	N/P ^b		
1	PN700-Fs-plex	1	7	310	+26
		3	21	200	+42
2	PN150-Cs-plex	4	25	140	+50
		8	50	190	+42
3	PN255-Cs-plex	1	6	180	+48
		3	18	190	+48
4	PN660-Cs-plex	1	6	99	+26
		3	18	200	+41
5	PN940-Cs-plex	1	6	260	+50
		3	18	190	+42
6	PN1510-Cs-plex	1	6	230	+54
		3	20	260	+52
7	PM100-Fh-plex	1	4	150	+32
		3	11	160	+17
8	PM140-Fh-plex	1 ^e	4	1521	+32
		3	12	230	+42
		6	24	132	+23
9	PM165-Fh-plex	1	4	140	+45
		3	14	100	+32
10	PM285-Fh-plex	2	7	130	+32
		4	13	150	+33
11	PM110-Ch-plex	1	4	190	+36
		3	12	150	+39
12	PM265-Ch-plex	2	9	130	+47
		4	18	130	+47
13	PM310-Ch-plex	1 ^e	5	170	+42
		3	15	220	+32
		6	30	140	+42
14	PM680-Ch-plex	1	5	136	+22
		3	15	140	+32
15	IPEI	2	1	130	+48
		3	2	120	+42

^a WR = polymer/pDNA weight ratio. ^b amine/phosphate molar ratio calculated as described in experimental part.

^c Hydrodynamic diameters D_h of the polyplexes at 298K in HEPES 10 mM, pH 7.4. ^d ζ potential of polyplexes at 298K in HEPES 10 mM, pH 7.4. ^e Transfection efficiency and cytotoxicity not evaluated at this WR.

Polyplexes were also prepared by complexing pDNA with PMVAm obtained by partial hydrolysis of PNMVA ($DP_n = 155$, PM155-F) thus containing different amounts of residual

acetamido groups (Table 4). Gel retardation assays showed that higher quantities of polymer were necessary to condense pDNA with poorly deacetylated polymers, as expected (Figure S3, for PM155-Fm76 and PM155-Fm23). In all cases, positively charged complexes were again formed. However, at constant degree of polymerization, we observed a dependency of the polyplexes size on the polymer hydrolysis rate. Indeed, polymers with lower amine content, *i.e.* resulting from lower deacetylation degrees, usually produced bigger complexes. For example, the formation of large aggregates with a diameter superior to 5 μm was notably observed for PM155-Fm23, PM155-Fm37 and PM155-Fm54.

Table 4. Characteristics of polyplexes prepared with increasingly hydrolysed PNMVA.

Entry	Polyplexes	polymer/pDNA WR ^a	N/P ^b	D _h ^c (nm)	ζ ^d (mV)
1	PM155-Fm23- plex	3	2.5	879	+9
		6	5	6316	+1
2	PM155-Fm37- plex	1	1.5	^e	+16
		3	4	675	+22
3	PM155-Fm44- plex	2	5	1197	+21
		4	7	564	+27
4	PM155-Fm54- plex	1	2.3	5434	+25
		3	7	130	+19
5	PM155-Fm64- plex	1	3	237	+23
		3	8	136	+23
6	PM155-Fm76- plex	1	4	194	+27
		3	11	145	+25
7	PM155-Fm94- plex	1	5	391	+31
		3	15	148	+30

^a polymer/pDNA weight ratio. ^b amine/phosphate molar ratio calculated as described in experimental part. ^c hydrodynamic diameters D_h of the polyplexes at 298K in HEPES 10 mM, pH 7.4. ^d ζ potential of polyplexes at 298K in HEPES 10 mM, pH 7.4. ^e Out of range.

On the other hand, the hydrodynamic diameter of almost all polyplexes formed with the less acetylated polymers, PM155-Fm64, PM155-Fm76, PM155-Fm94, was roughly between 150 and 250 nm. Increasing the amount of amino groups along the backbone improves the polyplexes size reduction capacity for this polymer family. Because the transfection efficiency of a polyplex is notably affected by its size, it will be important to remember the differences in size when the performance of the polyvinylamine vectors with different amounts of residual acetamido groups will be evaluated in the next section.

3.3. Polyvinyl amines as gene carriers

Primary vs. secondary polyvinylamines as gene carrier: The transfection efficiency and cytotoxicity of the polyvinylamines-based polyplexes were evaluated on HeLa cells at two WR (Figures 2 and 3). Linear polyethylenimine (IPEI) was used as reference. Transfection was carried out with pDNA (pTG11033) encoding the luciferase gene and the luciferase activity was evaluated 48 h post-transfection.

In agreement with Wolfert *et al.*,²⁸ the primary polyvinylamine PN700-Fs ($\bar{D} = 1.69$) prepared by FRP showed low transfection efficiency, *i.e.* 10^4 RLU/mg proteins (Figure 2A). Moreover, a similarly low efficiency was found for PN660-Cs, a PVAm of comparable molar mass but prepared by OMRP and possessing therefore a much lower molar mass distribution ($\bar{D} = 1.18$). Consequently, the dispersity of PVAm is not a crucial parameter in contrast to the molar mass of the carrier. In contrast, for PVAm of lower molar masses obtained by OMRP, *i.e.* PN255-Cs and PN150-Cs much higher transfection efficiencies were measured. For example, the level of transfection achieved by PN150-Cs was almost comparable to the one recorded for IPEI (10^8 vs.

10^9 RLU/mg proteins, respectively). The dependency of the transfection performances on the degree of polymerization of PVAm is even clearer when comparing PN150-Cs to PN1510-Cs. The transfection increased by four order of magnitude (from 10^4 to 10^8 RLU/mg proteins) when the chain length of the polymer was divided by ten.

Figure 2. Transfection efficiency of HeLa cells. Cells were transfected with (A) PVAm and (B) with PMVAm polyplexes at two polymer/pDNA ratios (ratio 1 and ratio 2 correspond to the ratios reported in Table 3 using less and more polymer respectively). The luciferase activity was measured 48 h after the transfection and expressed as RLU/mg of protein.

This trend was somehow unexpected regarding the data reported by Wolfert *et al.*²⁸ In this latter study, PVAm of three different molar masses (3, 8 and 60 kg/mol) were produced by alkaline hydrolysis of polyvinylformamide (PNVF).^{28, 58} They showed poor transfection efficiencies for pDNA encoding for β -galactosidase (three order of magnitude lower than formulations using poly(L-)lysine). In their study, the effect of the molar mass on gene transfection was strictly

opposite to our observations, *i.e.* best performances were reported for the highest molar mass polymers. Although it is not easy to fully rationalize this discrepancy, some hypotheses can be formulated. First, Wolfert used a different plasmid (pDNA of 6 kb coding for β -galactosidase) and a N/P molar ratio of 2 leading to large and almost neutral polyplexes ($\zeta = -1$ or -2 mV). In our study, we used a N/P molar ratio superior to 6, meaning that higher amounts of polymer were involved in the polyplex formation, which results in polyplexes with a positive surface charge ($\zeta = +26$ to $+54$ mV) and hydrodynamic diameter between 150 and 260 nm (Table 2). Secondly, the PVAmS were prepared by different routes, *i.e.* by alkaline hydrolysis of PNVF^{28, 58} and acidic hydrolysis of PNVA (in this work) respectively. Consequently, the sets of PVAm differ by the nature and amount of residual amides along their backbones, and the effect of the latter on the transfection is unknown. *A priori*, no formamide groups remain after alkaline hydrolysis of PNVF⁵⁸ whereas 5 to 9 % of residual acetamido groups are present in the PVAmS investigated here (Table 1).

The transfection efficiency of secondary polyvinylamines (PMVAm) was also evaluated (Figure 2B). In contrast to the PVAm polyplexes, no significant influence of the polymer molar mass was noticed with PMVAm. Indeed, almost all PMVAm-based polyplexes reached remarkable transfection efficiency, between 10^8 and 10^9 RLU/mg proteins whatever the PMVAm's degree of polymerization. In this series, three candidates slightly outperformed IPEI with a transfection efficiency above 10^9 RLU/mg proteins, namely PM140-Fh, PM165-Fh and PM310-Ch. No difference in transfection was noticed for PMVAmS produced by FRP (PM100/140/165/285-Fh) and OMRP (PM110/265/310/680-Ch). This is easily conceivable considering the low impact of the PMVAm's molar mass on transfection and the fact that polymers prepared by FRP and

OMRP essentially differs by their molar mass dispersity ($\mathcal{D} < 1.3$ and ~ 2 for OMRP and FRP, respectively).

Comparison of the transfection performance of PVAm and PMVAm possessing similar degrees of polymerization ($DP_n \sim 150$) and around 90 % of hydrolysis, for example PN150-Cs and PM165-Fh, emphasizes the beneficial effect of the methyl substitution of the nitrogen in the repeating units, *i.e.* the presence of pendant *secondary* amino groups instead of *primary* amino groups along the polymer backbone. In the case of the *secondary* polyvinylamines, PMVAm, the transfection was actually improved by a factor of ten. The difference of transfection efficiency observed for primary and secondary polyvinylamines could result from differences in basicity and protonation rate at physiological pH. Indeed, titration of PVAm and PMVAm samples (SI Figure S1A) allowed us to estimate their apparent pKa values to 10.6 and 12.4, respectively, which reveals a slightly stronger basicity of the secondary polyvinylamine compared to the primary polyvinylamine. For both polymers, quite high protonation degrees (0.74 for PVAm and 0.79 for PMVAm) were calculated at pH 7.4 (SI Figure S1C). Note that the buffering capacity, that is the proton quantity required to decrease the pH by one unit between pH 6.5 and pH 7.5, was surprisingly lower for PMVAm (0.09 mL) compared to PVAm (0.13 mL) for comparable polymer weight concentration (SI Figure S1B). The endosomal release of polyplexes involving the PVAm may then be favoured, compared to polyplexes formulated with PMVAm. However, endosomal escape of the polyplexes may not be the limiting step in gene transfection using polyvinylamines. Other factors, such as the internal structure of the polyplexes or the strength of the complexes may affect the release of the pDNA in the cytosol, thus the efficiency of the gene delivery.

In addition to high transfection efficiency, gene carriers should exhibit high viability. The cytotoxicity was determined by using the MTT assay after 48 h of transfection (Figure 3). The lower molar mass PVAmS exhibiting the best transfecting performances also presented the highest cell viabilities (Figure 3A). For example, cell viability as high as 95 % was measured for PN150-Cs which is much higher than that of IPEI for which cell viability barely reached 50 %. However, lower viabilities around 45-60 % were recorded for less performant PVAmS with higher molar masses, *e.g.* PN940-Cs and PN1510-Cs.

Figure 3. Viability of transfected HeLa cells. Transfection was performed with (A) PVAm polyplexes and (B) PMVAm polyplexes at two polymer/pDNA ratios (ratio 1 and ratio 2: lower and higher amount of polymer, Table 3). The cell viability was evaluated by MTT assay 48 h after transfection and expressed as percentage relative to untreated cells.

On the other hand, all polyplexes formed with PMVAm showed higher cell viabilities (between 55 and 100 %) than with LPEI (Figure 3B). In this series, however, PMVAm with the best transfection capacities (PM140-Fh, PM165-Fh and PM310-Ch) presented the lowest cell viabilities (between 55 % and 70 %). Nevertheless, the other PMVAm polyplexes (*e.g.* PM285-Fs, PM110-Ch and PM265-Ch), whose transfection abilities are still valuable, showed excellent cell viabilities between 90 and 100 %.

Finally, it should be noted that no significant differences in toxicity for polymers synthesized by either free radical polymerization or Co-mediated OMRP were observed; meaning that, after purification, the amount of Co (determined to be < 0.4 ppm) in the polymers is below the critical limit of toxicity.

Importance of the percentage of hydrolysis and of residual acetamido groups: The presence of acetamido groups in the polymer may play a role in both the transfection efficiency and cell viability. Indeed, it has been reported that the introduction of functional pendant moieties to a cationic polymer can modify the internal structure of the polyplex from an organized supramolecular structure to a loose amorphous organization with a weaker binding energy between the partners, facilitating the pDNA release in the cell and the transfection efficacy. Such an effect has already been described for LPEI and its histidinylated derivative.⁵⁹ For this reason, we explored the gene carrier performances of the series of PMVAm containing different amounts of residual acetamido groups prepared from partial hydrolysis of PNMVA (from 23 % to 94 % of hydrolysis). Figure 4A clearly shows that acetamido-rich polymers, obtained after short

hydrolysis times (degrees of hydrolysis < 50 %), namely PM155-Fm23, PM155-Fm37 and PM155-Fm44, led to low transfection levels ($\sim 10^5$ RLU/mg proteins).

Figure 4. (A) Transfection efficiency and (B) cell viability of HeLa cells. Transfection was performed with increasingly hydrolyzed PMVAm polyplexes at two polymer/pDNA ratios (ratio 1 and ratio 2: lower and higher amount of polymer, cf. Table 4, WR). The luciferase activity was measured 48 h after the transfection and expressed as RLU/mg of protein. The cell viability was evaluated by MTT assay 48 h after transfection and expressed as percentage relative to untreated cells.

In contrast, amino-rich polymers, containing more than 70 % of amines, like PM155-Fm76 and PM155-Fm94, presented high transfection efficiencies ($\sim 10^9$ RLU/mg proteins). One can thus conclude that the residual acetamido groups within PMVAm should not exceed 30 % for preserving the transfection capacities of the carrier. Note that the loss of efficiency of the

acetamido-rich polymer was somewhat expected considering the very large size of the resulting polyplexes and the formation of large aggregates ($D_h > 1 \mu\text{m}$) (Table 4). Finally, Figure 4B indicates that the cell viability was not affected by the composition of the amount of the residual acetamido groups.

Even if we demonstrated that the system tolerates between 6 and 30 % of residual acetamido groups without loss of transfection efficiency, it remains still unclear whether the latter are beneficial or not. To answer this question, polyvinylamine free of acetyl groups should be prepared and tested. Such PMVAm are not accessible because they are exclusively obtained from PNMVA (*cf.* Figure 1, route B) and complete deacetylation of the amide functions has never been achieved so far. However, well-defined acetyl-free PVAm can be prepared via RAFT polymerization of *N*-vinylphthalimide followed by the complete hydrazinolysis of the phthalimide groups of the PNVPi precursor (Figure 1, route A).²⁷ Following this approach, we synthesized three PVAm with various degrees of polymerization, *i.e.* 55, 170 and 200 (SI, Table S2). Hydrazinolysis was successfully achieved leading to polymers exclusively composed of pendant amines. Positively charged polyplexes were then prepared with these compounds (SI Table S3) and tested in the pDNA transfection of and cell viability of HeLa cells (SI Figure S4).

Compared to IPEI the transfection efficiency of the acetyl-free PVAm was lower in all cases. Moreover, in spite of their similar degree of polymerization, the level of transfection observed for PN170-R obtained by RAFT (10^6 - 10^7 RLU/mg proteins, depending on the WR, Figure 5A) was significantly lower than with PN150-Cs synthesized by OMRP which possessed 9 % of residual acetamides (10^8 RLU/mg proteins, Figure 2A). Similar to OMRP derived PVAm series, excellent cell viabilities were measured with PVAm prepared by RAFT (between 80 to 100 %) (much better than with IPEI). These observations highlight the importance and especially the

positive impact of the residual acetamido groups present in polyvinylamines obtained by deacetylation of a poly(vinylacetamides).

As a final confirmation of the beneficial effect of the pendant amides along the polyvinylamine backbone, PN170-R was acetylated at 50 % (named PN170-R-OAc, Table S3, entry 2') and tested. Interestingly, the transfection efficiency was improved by more than one order of magnitude (till 10^9 RLU/mg proteins) (SI Figure S4), close to that of IPEI.

Figure 5. (A) Transfection efficiency and (B) cell viability of HeLa cells. Transfection was performed with PVAm (made by RAFT) polyplexes at two polymer/pDNA ratios (ratio 1 and ratio 2: lower and higher amount of polymer, Table S3). The luciferase activity was measured 48 h after the transfection and expressed as RLU/mg of protein. The cell viability was evaluated by MTT assay 48 h after transfection and expressed as percentage relative to untreated cells.

3.4. Transfection efficiency and cell viability on other cell lines

Polyvinylamines showing the best transfection efficiency on Hela cells, *i.e.* the secondary PM310-Ch and PM140-Fh, were tested on other cell lines (Figure 6). Skeletal muscle (H2K2B4 and C2C12), pulmonary (16HBE) and dendritic (DC2.4) cells were selected for their interest in gene transfer in relevant cellular models for Duchenne Muscular Dystrophy (DMD), cystic fibrosis and vaccination, respectively. Both PM310-Ch and PM140-Fh transfected the H2K2B4 cells less efficiently than IPEI (10^6 vs. 10^8 RLU/mg proteins). The transfection efficiency was also lower for 16HBE cells when using PM140-Fh instead of IPEI. However, for all other cases, PMVams and IPEI showed quite similar efficiencies (Figure 6A). On the other hand, the cell viability with IPEI polyplexes was dramatically low: 50 %, 30 %, 30 % and 10 % for H2K2B4, C2C12, 16HBE and DC2.4 cells, respectively (Figure 6B).^{19, 60} In this respect, PM310-Ch and PM140-Fh offered much higher cell viability than IPEI, making them attractive alternative gene carriers. Noteworthy, PM140-Fh polyplexes demonstrated excellent constant cell viability close to 100 % on all cell lines.

Figure 6. (A) Transfection efficiency and (B) cell viability of different cells lines. H2K2B4, C2C12, 16HBE and DC2.4 cells were transfected with the polyplexes made with PM310-Ch (N/P = 6), PM140-Fh (N/P = 3) and IPEI (N/P = 3). The luciferase activity was measured 48 h after the transfection and expressed as RLU/mg of protein. The cell viability was evaluated by MTT assay 48 h after transfection and expressed as percent to untreated cells.

Finally, PM140-Fh was further investigated for transfecting HeLa, C2C12, DC2.4 cells and fibroblasts with a plasmid DNA encoding for EGFP (SI Figure S5). Phase contrast images showed nice regular shape, whatever the cell line. No cytotoxicity was observed. The transfection of EGFP in the various cell lines was evidenced by the green fluorescence of the cell cytoplasm, which is the visible sign of the successful gene expression. Furthermore, the fluorescence/phase contrasts image overlay tends to demonstrate that the transfection occurs without any damage to the cells. No effect of the WR ratio could be noticed by this experiment.

4. Conclusion

Motivated by the recent advances in controlled radical polymerization giving access to well-defined polyvinylamine derivatives, we explored in details the potential of this class of disregarded polymers in gene transfection. For this purpose, a library of primary polyvinylamines (PVAm) and secondary poly(*N*-methylvinylamines) (PMVAm) with various molar masses and dispersities was prepared by free radical polymerization and organometallic-

mediated radical polymerization of the corresponding *N*-vinylacetamides followed by the acidic hydrolysis of the pendant amides. The transfection efficiency of pDNA/PVAm polyplexes on HeLa cells was found strongly dependent on the molar mass of PVAm. Only low molar mass PVAm synthesized by OMRP showed good transfection efficiencies, approaching the performance of IPEI (10^9 RLU/mg proteins). In contrast, all PMVAm samples presented high transfection efficiencies (10^8 - 10^9 RLU/mg proteins) whatever the synthesis route (FRP or OMRP) and the degree of polymerization, at least for DP_n comprised between 100 and 700. While OMRP appeared to be a necessary tool for designing PVAm with low molar masses ensuring considerable transfection levels, FRP, which is much easier to implement than OMRP, should be privileged for the preparation of the PMVAm carriers. Interestingly, in most cases, the cell viabilities were higher with both primary and secondary polyvinylamines compared to IPEI, which constitutes a major advantage over the latter. The beneficial effect of a few percent of residual acetamido groups along the polyvinylamine backbone, resulting from the partial deacetylation upon hydrolysis, was also clearly demonstrated. Nevertheless, a majority of pendant amines (> 70 % in the case of PMVAm) was found necessary for the proper complexation of pDNA and transfection of HeLa cells. Finally, some PMVAm were successfully tested for the transfection of other cell lines including skeletal muscle, pulmonary and dendritic cells.

Overall, this work highlights for the first time the real potential of polyvinylamines as gene carriers. Their good transfection efficiency and low toxicity could certainly be further improved in the future by fine adjustment of their structure to make them even more attractive DNA carriers.

ASSOCIATED CONTENT

Elemental analyses, titration curves, NMR spectra, electrophoresis retardation assays, detailed conditions synthesis of polyvinylamines, polyplexes characterization, transfection efficiency and cell viability tests, fluorescent confocal microscopy, are available free of charge on the ACS Publications website.

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REFERENCES

- (1) Friedmann, T.; Roblin, R. *Science* **1972**, *175*, 949-955.
- (2) Bertrand, N.; Leroux, J.-C. *Journal of Controlled Release* **2012**, *161*, 152-163.
- (3) Ibraheem, D.; Elaissari, A.; Fessi, H., Gene therapy and DNA delivery systems. *International Journal of Pharmaceutics* **2014**, *459*, 70-83.
- (4) Günzburg, W. H.; Salmons, B. *Molecular Medicine Today* **1995**, *1*, 410-417.

- (5) Bessis, N.; Garcia-Cozar, F. J.; Boissier, M. C. *Gene Therapy* **2004**, *11*, S10-S17.
- (6) Nagasaki, T.; Shinkai, S. *Journal of Inclusion Phenomena and Macrocyclic Chemistry* **2007**, *58*, 205-219.
- (7) Nguyen, D. N.; Green, J. J.; Chan, J. M.; Langer, R.; Anderson, D. G. *Advanced Materials* **2009**, *21*, 847-867.
- (8) Labhasetwar, V. *Current Opinion in Biotechnology* **2005**, *16*, 674-680.
- (9) Kundu, P. P.; Sharma, V. *Current Opinion in Solid State and Materials Science* **2008**, *12*, 89-102.
- (10) von Harpe, A.; Petersen, H.; Li, Y.; Kissel, T. *Journal of Controlled Release* **2000**, *69*, 309-322.
- (11) Forrest, M. L.; Gabrielson, N.; Pack, D. W. *Biotechnology and Bioengineering* **2005**, *89*, 416-423.
- (12) Pun, S. H.; Bellocq, N. C.; Liu, A.; Jensen, G.; Machemer, T.; Quijano, E.; Schluep, T.; Wen, S.; Engler, H.; Heidel, J.; Davis, M. E. *Bioconjugate Chemistry* **2004**, *15*, 831-840.
- (13) Brissault, B.; Leborgne, C.; Guis, C.; Danos, O.; Cheradame, H.; Kichler, A. *Bioconjugate Chemistry* **2006**, *17*, 759-765.
- (14) Oskuee, R. K.; Dehshahri, A.; Shier, W. T.; Ramezani, M. *The Journal of Gene Medicine* **2009**, *11*, 921-932.

- (15) Oskuee, R. K.; Philipp, A.; Dehshahri, A.; Wagner, E.; Ramezani, M. *The Journal of Gene Medicine* **2010**, *12*, 729-738.
- (16) Kunath, K.; von Harpe, A.; Fischer, D.; Kissel, T. *Journal of Controlled Release* **2003**, *88*, 159-172.
- (17) Bertrand, E.; Goncalves, C.; Billiet, L.; Gomez, J. P.; Pichon, C.; Cheradame, H.; Midoux, P.; Guégan, P. *Chemical Communications* **2011**, *47*, 12547-12549.
- (18) Gabrielson, N. P.; Pack, D. W. *Biomacromolecules* **2006**, *7*, 2427-2435.
- (19) Brissault, B.; Kichler, A.; Guis, C.; Leborgne, C.; Danos, O.; Cheradame, H. *Bioconjugate Chemistry* **2003**, *14*, 581-587.
- (20) Nimesh, S.; Aggarwal, A.; Kumar, P.; Singh, Y.; Gupta, K. C.; Chandra, R. *International Journal of Pharmaceutics* **2007**, *337*, 265-274.
- (21) Teo, P. Y.; Yang, C.; Hedrick, J. L.; Engler, A. C.; Coady, D. J.; Ghaem-Maghami, S.; George, A. J. T.; Yang, Y. Y. *Biomaterials* **2013**, *34*, 7971-7979.
- (22) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proceedings of the National Academy of Sciences* **1995**, *92*, 7297-7301.
- (23) Dufès, C.; Uchegbu, I. F.; Schätzlein, A. G. *Advanced Drug Delivery Reviews* **2005**, *57*, 2177-2202.
- (24) Borchard, G. *Advanced Drug Delivery Reviews* **2001**, *52*, 145-150.

- (25) Chu, D. S. H.; Schellinger, J. G.; Shi, J.; Convertine, A. J.; Stayton, P. S.; Pun, S. H. *Accounts of Chemical Research* **2012**, *45*, 1089-1099.
- (26) Gao, Y.; Huang, J.-Y.; O’Keeffe Ahern, J.; Cutlar, L.; Zhou, D.; Lin, F.-H.; Wang, W. *Biomacromolecules* **2016**, *17*, 3640-3647.
- (27) Liu, S.; Gao, Y.; A, S.; Zhou, D.; Greiser, U.; Guo, T.; Guo, R.; Wang, W. *ACS Biomaterials Science & Engineering* **2016**.
- (28) Wolfert, M. A.; Dash, P. R.; Nazarova, O.; Oupicky, D.; Seymour, L. W.; Smart, S.; Strohm, J.; Ulbrich, K. *Bioconjugate Chemistry* **1999**, *10*, 993-1004.
- (29) Liu, L.; Yang, Y.-L.; Wang, C.; Yao, Y.; Ma, Y.-Z.; Hou, S.; Feng, X.-Z. *Colloids and Surfaces B: Biointerfaces* **2010**, *75*, 230-238.
- (30) Zhong, Z.; Feijen, J.; Lok, M. C.; Hennink, W. E.; Christensen, L. V.; Yockman, J. W.; Kim, Y.-H.; Kim, S. W. *Biomacromolecules* **2005**, *6*, 3440-3448.
- (31) Newland, B.; Tai, H.; Zheng, Y.; Velasco, D.; Di Luca, A.; Howdle, S. M.; Alexander, C.; Wang, W.; Pandit, A. *Chemical Communications* **2010**, *46*, 4698-4700.
- (32) Prevette, L. E.; Kodger, T. E.; Reineke, T. M.; Lynch, M. L. *Langmuir* **2007**, *23*, 9773-9784.
- (33) Popielarski, S. R.; Mishra, S.; Davis, M. E. *Bioconjugate Chemistry* **2003**, *14*, 672-678.
- (34) Maki, Y.; Mori, H.; Endo, T. *Macromolecular Chemistry and Physics* **2007**, *208*, 2589-2599.

- (35) Dréan, M.; Guégan, P.; Detrembleur, C.; Jérôme, C.; Rieger, J.; Debuigne, A. *Macromolecules* **2016**, *49*, 4817-4827.
- (36) Debuigne, A.; Champouret, Y.; Jérôme, R.; Poli, R.; Detrembleur, C. *Chemistry – A European Journal* **2008**, *14*, 4046-4059.
- (37) Colombani, O.; Lejeune, E.; Charbonneau, C.; Chassenieux, C.; Nicolai, T. *The Journal of Physical Chemistry B* **2012**, *116*, 7560-7565.
- (38) Borukhov, I.; Andelman, D.; Borrega, R.; Cloitre, M.; Leibler, L.; Orland, H. *The Journal of Physical Chemistry B* **2000**, *104*, 11027-11034.
- (39) Drean, M.; Guégan, P.; Jerome, C.; Rieger, J.; Debuigne, A. *Polymer Chemistry* **2016**, *7*, 69-78.
- (40) Debuigne, A.; Caille, J.-R.; Jérôme, R. *Macromolecules* **2005**, *38*, 5452-5458.
- (41) Buys, H. C. W. M.; Vercauteren, F. F.; van Elven, A.; Tinnemans, A. H. A. *Recueil des Travaux Chimiques des Pays-Bas* **1989**, *108*, 123-127.
- (42) Destarac, M.; Brochon, C.; Catala, J.-M.; Wilczewska, A.; Zard, S. Z. *Macromolecular Chemistry and Physics* **2002**, *203*, 2281-2289.
- (43) Chikhaoui-Grioune, D.; Aqil, A.; Zalfen, A. M.; Benaboura, A.; Jérôme, C. *Journal of Applied Polymer Science* **2010**, *117*, 1005-1012.
- (44) Maki, Y.; Mori, H.; Endo, T. *Macromolecular Chemistry and Physics* **2010**, *211*, 45-56.

- (45) Kolbe, H. V. J.; Boussif, O.; Delair, T.; Veron, L. Transgene S.A., patent: WO 1999005183 A1
- (46) Debuigne, A.; Morin, A. N.; Kermagoret, A.; Piette, Y.; Detrembleur, C.; Jérôme, C.; Poli, R. *Chemistry – A European Journal* **2012**, *18*, 12834-12844.
- (47) Akashi, M.; Nakano, S.; Kishida, A. *Journal of Polymer Science Part A: Polymer Chemistry* **1996**, *34*, 301-303.
- (48) Burke, P. A.; Pun, S. H.; Reineke, T. M. *ACS Macro Letters* **2013**, *2*, 928-934.
- (49) Kwon, Y. J. *Accounts of Chemical Research* **2012**, *45*, 1077-1088.
- (50) Haussecker, D. *Journal of Controlled Release*.
- (51) Dean, D. A.; Gasiorowski, J. Z. *Cold Spring Harbor Protocols* **2011**, *2011*, top101.
- (52) Thomas, M.; Klibanov, A. M. *Appl Microbiol Biotechnol* **2003**, *62*, 27-34.
- (53) Jones, C. H.; Chen, C.-K.; Ravikrishnan, A.; Rane, S.; Pfeifer, B. A. *Molecular Pharmaceutics* **2013**, *10*, 4082-4098.
- (54) Adil, M. M.; Erdman, Z. S.; Kokkoli, E. *Langmuir* **2014**, *30*, 3802-3810.
- (55) Wyrwal, M.; Leduc, C.; Sarna, M.; Goncalves, C.; Kepczynski, M.; Midoux, P.; Nowakowska, M.; Pichon, C. *International Journal of Pharmaceutics* **2015**, *478*, 372-382.
- (56) Díaz-Moscoso, A.; Vercauteren, D.; Rejman, J.; Benito, J. M.; Ortiz Mellet, C.; De Smedt, S. C.; Fernández, J. M. G. *Journal of Controlled Release* **2010**, *143*, 318-325.

- (57) Hess, G. T.; Humphries Iv, W. H.; Fay, N. C.; Payne, C. K. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **2007**, *1773*, 1583-1588.
- (58) Gu, L.; Zhu, S.; Hrymak, A. N. *Journal of Applied Polymer Science* **2002**, *86*, 3412-3419.
- (59) Maury, B.; Gonçalves, C.; Tresset, G.; Zeghal, M.; Cheradame, H.; Guégan, P.; Pichon, C.; Midoux, P. *Biomaterials* **2014**, *35*, 5977-5985.
- (60) Saegusa, T.; Yamada, A.; Taoda, H.; Kobayashi, S. *Macromolecules* **1978**, *11*, 435-436.