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Dynamic constitutional chemistry towards efficient nonviral vectors

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A B S T R A C T

Dynamic constitutional chemistry has been used to design nonviral vectors for gene transfection. Their design has been thought in order to fulfill *ab initio* the main requirements for gene therapy. As building blocks were used hyperbranched PEI as hydrophilic part and benzentrialdehyde and a diamine linear siloxane as hydrophobic part, connected through reversible imine linkages. The obtaining of the envisaged structures has been confirmed by NMR and FTIR spectroscopy. The dynamic synthesized amphiphiles proved to be able to self-assemble in nano-sized spherical entities as was demonstrated by TEM and DLS, characterized by a narrow dimensional polydispersity. Agarose gel electrophoresis proved the ability of the synthesized compounds to bind DNA, while TEM revealed the spherical morphology of the formed polyplexes.

As a proof of the concept, the nonviral vectors promoted an efficient transfection on HeLa cells, demonstrating that dynamic constitutional chemistry can be an important tool in the development of this domain.

1. Introduction

Conceptualized in 1972 and being at this moment at the forefront of medicine, gene therapy is an innovative procedure used in the treatment or for the amelioration of the patients' state of health [1,2]. Gene therapy uses nucleic acids as drugs by their delivery into the patient's pathological cells, aiming to replace the defective gene or to correct a genetic defect or a chronic disease, including cancer [3–5]. The key step in gene therapy is by far the development of an appropriate carrier for DNA, which has to fulfill mainly two major and contradictory requirements: firstly it has to bind strong enough the semirigid chains of the anionic DNA and secondly it has to bind it by reversible interactions in order to allow the release of the DNA into the cell nucleus [6,7]. To skip the barrier from fundamental design to application, they must fulfill many other and equally important requirements: the carrier should be nontoxic, biodegradable, it should facilitate endocytosis and also protect the genetic material from enzymatic degradation and so on. Moreover, the surface properties of a carrier are also highly important, along with its size and shape [8]. To this aim, great effort has been dedicated toward a simple design with efficient transfection. As the viral vectors proved important drawbacks, being even dangerous for the human body, the researchers' attention turned to a nonviral design which presents important advantages such as an easy preparation, high versatility regarding their design and structure, limited immunogenicity and ability to carry larger amounts of DNA [9,10]. The major drawback of the nonviral vectors is their transfection efficiency which is considerable lower than in the case of the viral vectors. This is why, in the last years the obtaining of new nonviral vectors for gene therapy with high transfection efficiency is one of the main targets in medicine related research.

The main candidates used in gene therapy for the nonviral approach are the cationic polymers such as chitosan [11], cationic lipids [12], polyethyleneimines [13,14], polypropyleneimines [15] or polyamidoamines [16].

Among these, polyethyleneimine (PEI) is by far the most frequently used polymer in nonviral vectors preparation, presenting a high ability to complex the genetic material, intrinsic endosomal activity and also a unique buffering capacity known as “proton sponge” effect [17,18]. More than this, studies demonstrated that PEI interacts with DNA with forces lower than 25 pN, forming stable polyplexes in physiological conditions but which are susceptible to be degraded by replisomes [19].

Intensive studies on the transfection ability and on the cytotoxicity of PEI demonstrated a close correlation of these two parameters with its molecular weight, both the transfection efficiency and cytotoxicity increasing with the molecular weight [20–24]. More than this, the transfection efficiency of the polyplexes based on PEI and DNA depends also on the N/P ratio. Thus, the polyplexes formed at an N/P ratio higher than 3 contain an excess of amine groups from PEI, which seems to facilitate the endosomal escape [25], fact which explains the superior transfection efficiency at higher N/P ratios.

In order to increase the transfection efficiency, different structures trying to mimic somehow the viral carriers were designed and obtained, based mainly on the cationic mediated transferring strategies. Therefore, liposomes [26], polymersomes [27], comb-like structures, star shaped structures [28] or dendrimers [29] have been prepared and demonstrated superior transfection efficiencies compared to the simply

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cationic polymers. This superior behavior has been attributed to the obtaining of spherical three-dimensional morphologies, which have a high density charge on the surface promoting the DNA binding and transfecting. Moreover, in the case of the dendrimers, an improved transfection was obtained for higher dendrimer generation [30], especially when hydrophobic units are comprised in each generation [31]. This is closely related to the vectors' ability to form small and stable nanoentities which assure a large distribution of the functionalities and allows further the DNA binding.

In this context, the present paper proposes a new design for nonviral vectors, by using hydrophobic and hydrophilic building blocks linked through reversible imine linkages, by dynamic constitutional chemistry (DCC). DCC is based on the use of reversible bonds at both molecular and supramolecular levels and was involved up to now in the development of many and diverse classes of molecules such as: metallo-cycles, molecular hosts, liquid crystals, hydrogels and many others [32–36]. The use of this synthetic pathway allowed in this case the obtaining of dynamic amphiphiles able to self-assemble into almost monodisperse spherical nanoentities, overcoming the synthesis and purification difficulties encountered in the obtaining of dendrimers or nanoparticles [30,37]. In order to fulfill *ab initio* the main requirements for nonviral vectors among which the ability to bind DNA, to facilitate the endosomal escape and to penetrate cell membrane, PEI was chosen as a hydrophilic part for the amphiphiles, while a linear siloxane was used as the main moiety for the hydrophobic part. The dynamicity at molecular level is conferred by the fact that the building blocks are linked together through reversible imine linkages, while the one at supramolecular level by the establishment of hydrophobic/hydrophobic interactions between the parts of the molecule which contain siloxane.

1.1. Rational design

The vectors' design has been thought to be formed from a hydrophobic core and a hydrophilic shell. As literature data indicate that the nonviral vectors containing siloxane units induced high transfection efficiency [11,38], a siloxane based hydrophobic core has been synthesized. To this aim a siloxane bearing diamine has been reacted with a trialdehyde in 1/1 molar ratio, to create an oligomeric chain with aldehyde functionalities (**A**), capable to bind the hydrophilic shell in multiple sites (Scheme 1). Hyperbranched PEI with low molecular weights of 800 Da and 2000 Da were used as hydrophilic moieties, being known that this polycationic polymer, presents the ability to act as a binder for DNA by electrostatic interactions and also to generate transfection, due to its "proton sponge" effect [13]. To attach the hydrophilic part to the hydrophobic one, the hydrophobic oligomer **A** has been reacted with hydrophilic PEI, considering 1 mol of PEI per 1 mol of structural unit of A, in diluted solution (1%). The reaction conditions were chosen in order to facilitate the yielding of a core-shell hydrophobic/hydrophilic structure with a maximum of PEI units, by imination and transimination reactions [39] toward a spherical stable entity. The idea was to use low molecular weight PEI chains, which are known as nontoxic but which, because of being linked on the same molecular entity, should be able to generate a high transfection efficiency, similar to the one of high molecular weight PEI. On the other hand, in reaching the desired spherical design, an import role is played by the hydrophobic/hydrophilic segregation of the resulted amphiphile in aqueous medium. Therefore, by virtue of the dynamicity at both molecular (reversible imine bonds) and supramolecular level (hydrophobic-hydrophilic forces), along with the natural tendency of energy minimization, the resulted oligomeric species should be able to self-assemble in water, forming spherical nanoentities [40]. In this manner, should result nano-sized nonviral vectors with a hydrophobic core based on the linear siloxane and the aldehyde, and a hydrophilic shell formed by PEI800 or PEI2000. The synthetic strategy has been designed in the Scheme 1.

2. Experimental

2.1. Materials

Benzene-1,3,5-tricarboxaldehyde from Manchester Organics, hyperbranched PEI (MW = 800 Da, and 2000 Da 50 wt% in H_2O), cell culture plates supplied by Corning (New York, USA) were used as received. Siloxane from Sigma-Aldrich Chemie (Germany; alpha-MEM, penicillin-streptomycin-amphotericin B mixture and Trypsin-Versene from Lonza; fetal bovine serum (FBS) from Gibco; HeLa cells from CLS-Cell-Lines-Services-GmbH, Germany; CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit and Bright-Glo(TM) Luciferase Assay System kit from Promega.. Single stranded low molecular wight (almost 250 base pairs), salmon sperm DNA was purchased from Fluka (St. Louis, USA). Plasmids pCS2 + MT-Luc (pLuc) encoding for firefly luciferase and pCS2 + NLS-eGFP (pEGFP) which encodes for enhanced green fluorescent protein were kindly provided as a gift from Prof. Adrian Salic (Harvard University, Boston) and were multiplied in *E. Coli* DH5a (gift supplied by Dr. Anca Gafencu, "Nicolae Simionescu" Institute of Cellular Biology and Pathology, Bucharest), and further purified using E.Z.N.A. Endo-free Plasmid Mini II kit (Omega Bio-Tek, Inc.).

2.2. Synthesis

2.2.1. Synthesis of the hydrophobic core

In the first step, the hydrophobic core has been synthesized by the condensation reaction between benzene 1,3,5-tricarboxaldehyde (0.162 g, 1 mmol) and 1,3-bis-(3-aminopropil)-1,1,3,3-tetramethyl siloxane (0.261 g, 1 mmol) in chloroform (8.46 mL) at a final concentration of the reagents of 5% *w/v*, at 20 °C, overnight (24 h). The compound was obtained as a yellow viscous liquid. The resulted compound will be further noted **A**.

A: yellow viscous liquid, η = 89%

^{1}H NMR (400.13 MHz, $CDCl_3$, ppm): δ 10.2($Ph(C\underline{H}O)_3$); 10.15 (1H, $Ph(C\underline{H}O)_2CH=N$-; 10.05 (1H, $Ph(C\underline{H}O)(CH=N\text{-})_2$); 8.63, 8.48, 8.3, 8.22, 8.16, 8.08, 7.94 (13H, $C\underline{H}=N$ and Ph-$\underline{H}$); 3.6, 3.5 (11H, $H_2N\text{-}C\underline{H}_2$), 1.7 (11H, $H_2N\text{-}CH_2\text{-}C\underline{H}_2$), 0.5 (11H, $H_2N\text{-}CH_2\text{-}CH_2\text{-}C\underline{H}_2$).

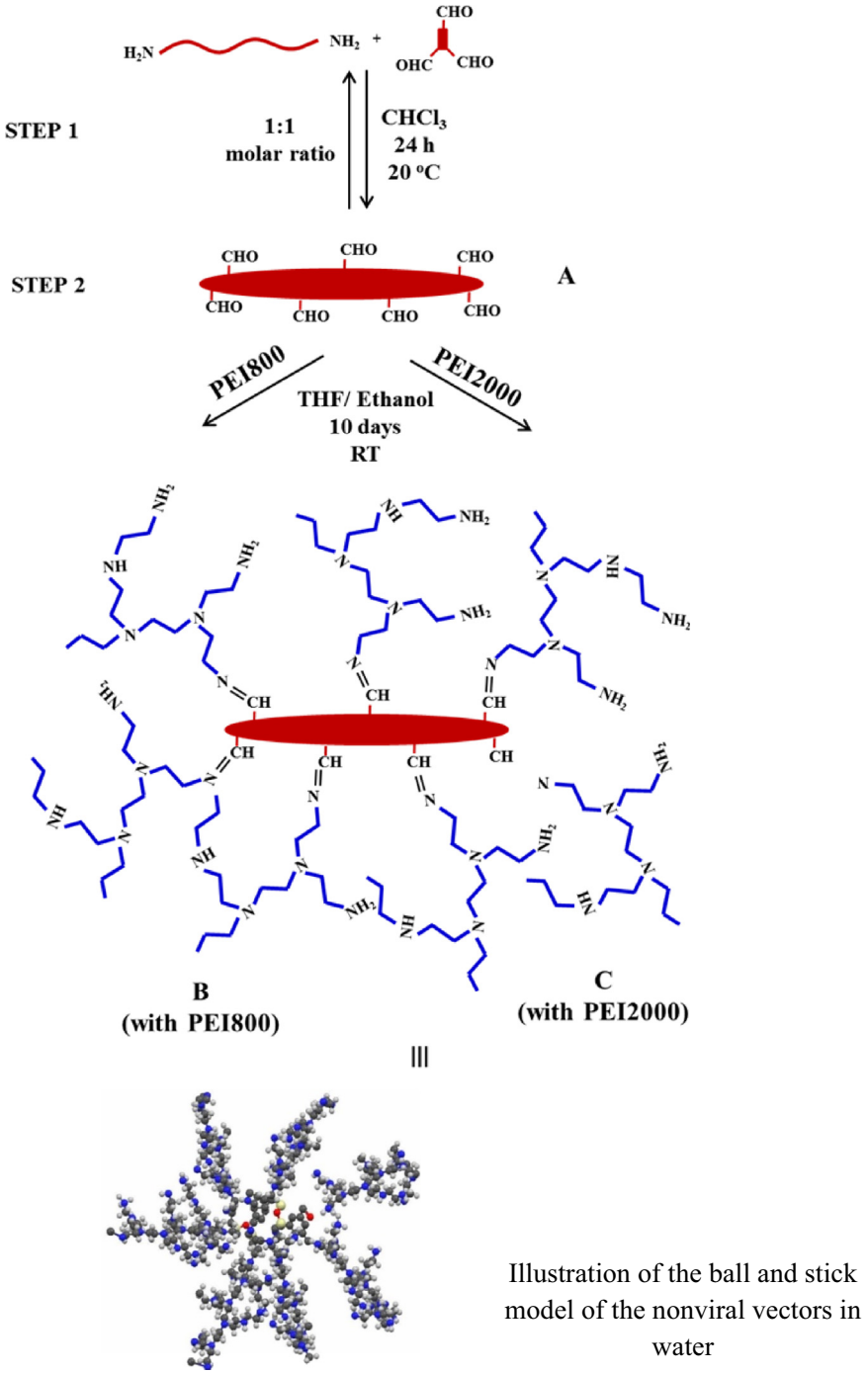
FTIR (KBr, cm^{-1}) 3430 (ν_{NH2}), 2949, 2830 ($\nu_{Si\text{-}C}$, ν_{CH3}, ν_{CH2}), 1705 ($\nu_{C=O}$), 1647 ($\nu_{CH=N}$), 1055 ($\nu_{Si\text{-}O}$), 838, 739 ($\nu_{CH\ aromatic}$).

GPC: M_n = 2558 g/mol; M_w = 2877 g/mol; M_z = 3360 g/mol; M_p = 1757 g/mol; M_w/M_n = 1.125; M_z/M_w = 1.168.

2.2.2. Synthesis of the amphiphiles

In the second step, PEI of two different molecular weights (800 and 2000 Da) has been reacted with the hydrophobic compound A, by a condensation reaction in a 1:1 molar ratio of the structural unit of A to the PEI. For that, the hydrophobic part **A** (0.039 g, 0.1 mmol) has been dissolved in 3.92 mL THF in order to obtain a solution with the concentration of 1% *w/v*, while the hydrophilic PEI with a molecular weight of 800 Da (PEI800) (0.06 g, 0.1 mmol) or of 2000 Da (PEI2000) (0.18 g, 0.1 mmol) was solved in 6 mL and 18 mL, respectively of ethanol, in order to form also a solution of 1% w/v concentration. The reactions were conducted in diluted systems in order to facilitate the imination and transimination processes and to give rise to individual nanoentities by the self-assembling of the resulted amphiphiles [40]. The two diluted solutions have been mixed together and kept under magnetic stirring for 10 days, at room temperature. The reaction mixture was dried under vacuum. From now on, the code **B** stands for the compound obtained using PEI800, while the code **C** for the one using PEI2000. Both compounds were soluble in water.

B: yellowish solid



Scheme 1. The obtaining of the nonviral vectors **B** and **C**.

^{1}H NMR (400.13 MHz, D_2O, ppm): 8.53, 8.08, 8.00 (C<u>H</u>=N and Ph-<u>H</u>); 3.64, 3.48 (<u>H</u>$_2$N-CH_2); 3.39, 3.17 (H_2N-C<u>H</u>$_2$), 2.91, 2.82 (H_2N-CH_2-C<u>H</u>$_2$), 2.74 (H_2N-CH_2-CH_2-C<u>H</u>$_2$).

FTIR (KBr, cm^{-1}) 3390, 3280 (NH_2), 2949 ($\nu_{CH3,}$ ν_{CH2}), 2830 (ν_{Si-C}), 1657 ($\nu_{CH=N}$), 1055 (ν_{Si-O}), 838, 739 ($\nu_{CH\ aromatic}$).

C: yellowish solid

^{1}H NMR (400.13 MHz, D_2O, ppm): 8.53, 8.08, 8.00 (C<u>H</u>=N and Ph-<u>H</u>); 3.64, 3.48 (<u>H</u>$_2$N-CH_2); 3.39, 3.17 (H_2N-C<u>H</u>$_2$), 2.91, 2.82 (H_2N-CH_2-C<u>H</u>$_2$), 2.74 (H_2N-CH_2-CH_2-C<u>H</u>$_2$).

FTIR (KBr, cm^{-1}) 3390, 3280 (NH_2), 2949, 2830 (ν_{Si-C}), 1055 (ν_{Si-O}), 1657 ($\nu_{CH=N}$), 2949, 2832 ($\nu_{CH3,}$ ν_{CH2}), 838, 739 ($\nu_{CH\ aromatic}$).

2.3. Preparation and characterization of the DNA polyplexes

The polyplexes, consisting in the synthesized amphiphilic compounds **B** or **C** and salmon sperm dsDNA or plasmid DNA (pLuc with 4870 pb) have been prepared at different N/P molar ratios, starting from 10 to 400 (N represents the number of nitrogen atom in the synthesized compounds and P represents the number of phosphate anions in the dsDNA or plasmid chain, taking into consideration that 1 μg of dsDNA, regardless of its type, contains 3 nmole of phosphorus) [41].

Considering that no secondary products resulted simultaneously with vectors preparation, the percentage of nitrogen was calculated taking into consideration the amount of the PEI to the entire reaction

mixture and it was established to be equal to 19.7% in the case of **B** and to 26.8% in the case of **C** (see supporting file). The appropriate amounts of the synthesized compounds from a stock solution in PBS have been mixed with a 1 μL water solution containing 1 μg of dsDNA (salmon sperm or plasmid) in order to obtain polyplexes with different N/P ratios. After 60 min of gentle shaking and incubation at room temperature, the polyplexes have been subjected to agarose gel electrophoresis, cytotoxicity and transfection efficiency tests.

2.4. Equipment and methods

The NMR spectra were recorded using a BRUKER Avance DRX 400 MHz spectrometer with a 5 mm direct detection QNP probe with z-gradients. The NMR spectra were recorded at room temperature and the chemical shifts are reported as δ values (ppm) relative to the residual peak of the used solvent.

Transmission electron microscopy (***TEM***) was performed on a Transmission Electron Microscope HT7700 Hitachi, in high resolution mode, at a potential of 100 kV. The samples were prepared by placing 3 μL of aqueous suspension (1% in double distilled water) on a carbon covered copper grid and further drying by solvent evaporation at room temperature, for 48 h.

Gel retardation assay. The polyplexes formation was firstly evaluated by an agarose gel retardation assay. Naked dsDNA and the polyplexes were mixed with phosphate buffer and loaded into agarose gel, 1% for the salmon sperm DNA tests and 0.8% for the plasmid. The experiments have been recorded at a potential of 90 *V*, during 60 min for the salmon dsDNA and respectively 120 min for the plasmid in TAE buffer solution (40 mM Tris-HCl, 1% acetic acid, 1 mM EDTA). The migration of the free and complexed DNA was visualized by illumination under UV lamp, after the gel has been stained using ethidium bromide.

Zeta potential has been determined using Delsa Nano C Submicron Analyzer from Bechman Coulter on solutions with the same concentration as in agarose gel electrophoresis and at room temperature.

UV-Vis spectra were recorded with a LAMBDA 35 UV/Vis Systems (Perkin Elmer Inc., USA).

Cell culture. HeLa cells were cultured as a monolayer of adherent cells in tissue culture flasks with complete cell medium (alpha-MEM supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin B mixture) until sufficient were obtain for transfection and cytotoxicity experiments. All cell cultures were carried out at 37 °C in humidified incubator with 5% CO_2.

In vitro toxicity assay of polyplexes. CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the cytotoxic effect of polyplexes. The CellTiter 96® AQueous One Solution Reagent has a tetrazolium compound (MTS) [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent (phenazine ethosulfate; PES) to enhance the solution stability. The cells with intact mitochondrial function transform MTS into formazan, therefor the quantity of formazan is proportional to the number of living cells treated with MTS.

HeLa cells were seeded in 96 transparent well plates at a density of 10^4 cells/well with 100 uL/well complete medium and allowed to rest and adhere overnight. The next day the medium was replaced with fresh 100 uL/well complete medium containing polyplexes with pLuc DNA. The final concentration of DNA in transfection medium was 5 ng/μL. After 44 h, 20 μL/well CellTiter 96® AQueous One Solution Reagent were added to the cells and the plates were incubated for other 4 h. Finally the absorbance at 490 nm was recorded with EnSight plate reader (PerkinElmer). The cell viability was expressed as percentage of the viability of untreated cells.

Transfection studies. The efficiency of transfection was determined by luciferase assay. For these experiments, HeLa cells were seeded in 96-well white opaque microplates at a density of 10^4 cells/well with 100 uL/well complete medium. The next day the medium was replaced with complete medium containing polyplexes with pLuc plasmid in a final concentration of 5 ng DNA/uL. After 48 h 100 uL/well of Bright-Glo reagent were added to the plates and the luminescence was measured at Ensight multifunctional plate reader.

Fluorescence microscopy. Transfection efficiency of polyplexes was evaluated qualitatively by fluorescence microscopy. The experiment was carried out similarly to cytotoxicity evaluation with the exception that the plasmid used was pEGFP. Treated cells were investigated 48 h post transfection using an inverted microscope, Leica DMI 3000B fitted with GFP filtercube.

Statistical analysis. Each experiment was repeated three times and performed with at least 3 replicates. GraphPad Prism 6.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to analyze data. Results are presented as means ± standard error of the mean (S.E.M.). S.E.M. was chosen to evaluate the accuracy of the mean values. Statistical analysis was performed by one-way analysis of variance (ANOVA), and differences were considered significant when $p < 0.05$.

3. Results and discussions

3.1. Synthesis

Nonviral vectors based on hydrophobic/hydrophilic architectures have been synthesized in two steps. In the first step 1,3,5 benzene trialdehyde was reacted to 1,3-bis-(3-aminopropil)-1,1,3,3-tetramethyl siloxane in a molar ratio of 1:1, in order to form a hydrophobic polyimine A (Scheme 1), containing 7 aldehyde groups as resulted from NMR and GPC data (Fig. 1 s and 2 s). In the second step, PEI 800 or PEI 2000 Da were reacted to **A**, in order to give rise to amphiphilic structures (Scheme 1), noted **B** and **C**, respectively. Both resulted amphiphiles were water soluble.

3.2. Structural characterization

The structural characterization of the nonviral vectors and of the hydrophobic intermediate was performed by ^{1}H NMR and FTIR spectroscopy and GPC measurements.

3.2.1. Characterization by NMR and GPC

In the NMR spectrum of the **A** compound (Fig. S1a) there are signals corresponding to the mono reacted aldehyde around 10.15 ppm and to the bi-reacted aldehyde around 10.10 ppm. The signals appeared splitted, the most probably due to some couplings of the aldehyde protons with imine ones in syn and/or anti-configurations. As expected, the chemical shifting of the protons in the siloxan unit occurred in the aliphatic region, from 3.6 to 0.5 ppm (Fig. S1-a). Between 8 and 9 ppm, appeared superposed peaks which belong to the imine and to the aromatic protons. A raw estimation of the composition of the **A** compound from the integral ratio, indicated approx. 5 imine units, corresponding to an oligoimine containing 7 aldehyde groups, 2 situated on the terminal aromatic rings and five on each aromatic unit in the chain. Further DLS measurements on **A** solution indicated the presence of aggregates, in agreement with not so good quality of the NMR spectra (Fig. S2) [42].

The GPC curve of the hydrophobic imine **A** was unimodal and sharp, with a slight shoulder, indicating a quite narrow polydispersity, measured by the ratio of Mw/Mn as being 1.125 (Fig. S3). This is in accordance with the predominant presence of one chemical specie. The molecular weight was 2877 g/mol, value which agree well with the composition determined by NMR spectroscopy, confirming the preponderant formation of a hydrophobic chain which contains 7 aldehyde groups.

In the NMR spectra of the **B** and **C** it was observed the complete disappearance of the characteristic signals of the aldehyde groups indicating its total consumption in the condensation reaction with the

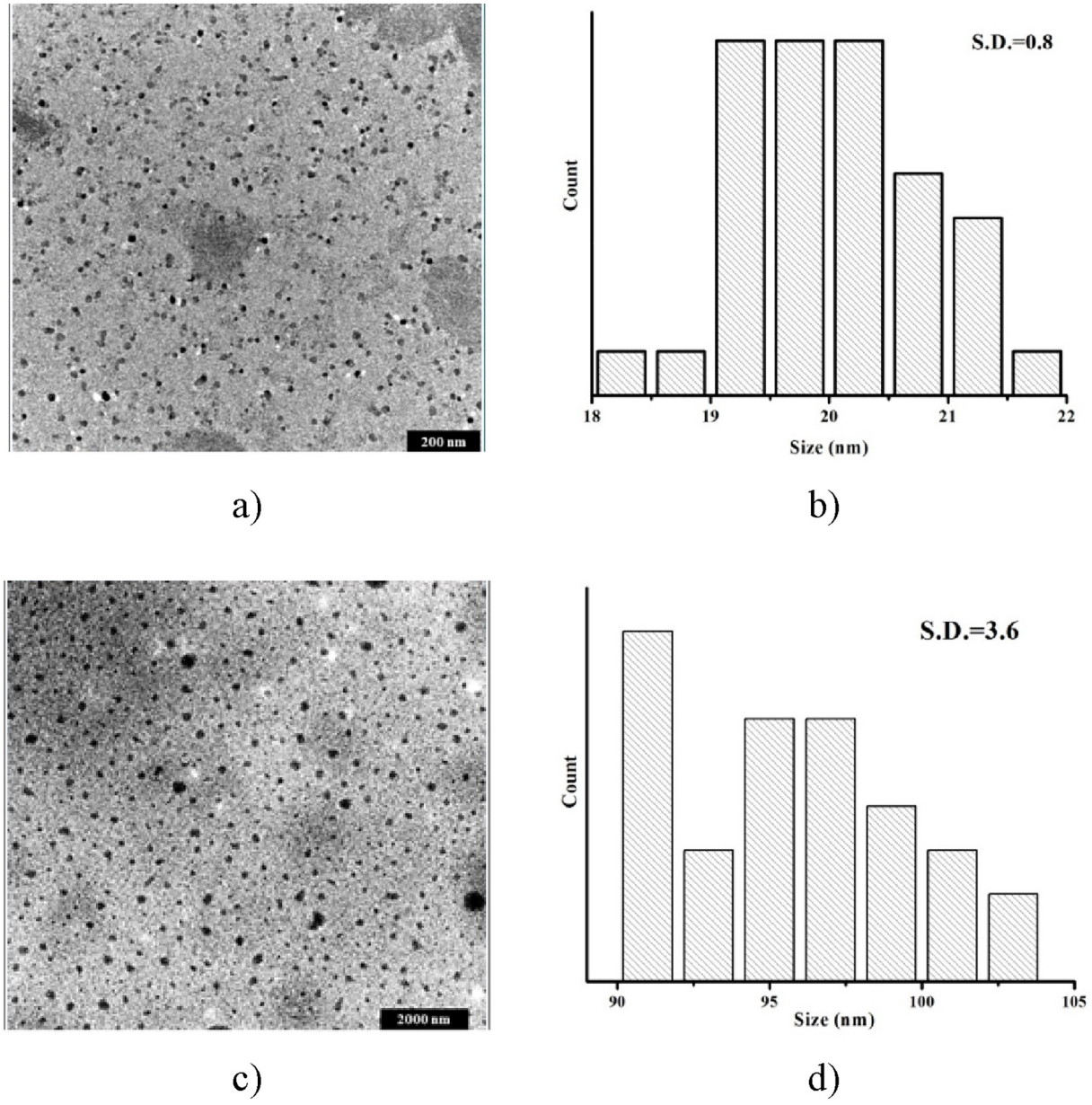


Fig. 1. Transmission electron microscopy images for a) b) **B** and c) d) **C** and the corresponding histograms.

amine groups of PEI800 or PEI2000 molecules. Due to the high density and concentration of the amine groups in PEI structure, the signals corresponding to the aromatic region of the compounds were less intense (Fig. S1-b, c) [40].

3.2.2. Structural characterization by FTIR

In order to evidence the spectral changes due to the imination reaction, FTIR spectra for the hydrophobic moiety **A** and for the amphiphiles were analyzed (Fig. S4). The FTIR spectrum of the hydrophobic **A** revealed all the characteristic absorption bands of the newly formed compound, confirming the NMR data. Thus, in the finger print region, it could be observed a band at 1705 cm^{-1} assigned to the stretching vibrations of C=O group, indicating the formation of a chain with free, unreacted aldehyde groups on it, in agreement with NMR spectrum. The presence of the absorption band at 1647 cm^{-1} indicated the formation of the imine groups by the acid condensation of the aliphatic amine and aldehyde groups of the reagents. The significant shifting of the imine band to higher wavenumbers is in agreement with the lower conjugation of the aliphatic-aromatic imine unit, as well documented in the literature [43–45]. Symmetric and anti-symmetric stretching vibrations of the C–H bond of CH_3 of CH_2 groups are also present in the FTIR spectrum of **A** at 2949, 2832 cm^{-1} and also the corresponding bands to the aromatic CH at 838 and 739 cm^{-1} (Fig. S3). In comparison with the FTIR spectrum of **A**, in the FTIR spectra of **B** and **C**, no absorption bands characteristic to the aldehyde groups could be observed, indicating their complete consumption in the condensation reaction with PEI. Moreover, the appearance in the FTIR spectra of a sharp absorption band at 1657 cm^{-1} confirmed the formation of new imine linkages, which in comparison with the imines in the hydrophobic **A**, is shifted even to higher wavenumbers [44–47].

3.3. The dynamicity of the B and C amphiphiles

Taking into consideration that the synthesized **B** and **C** amphiphiles are formed by hydrophilic and hydrophobic building blocks linked together through reversible imine linkages, they should present dynamicity under certain conditions. Therefore, their dynamic character was investigated by UV-VIS spectroscopy. At first, the UV-VIS spectra of the

benzenetrialdehyde was recorded, presenting a maximum of absorbance at 300 nm, attributed to the π-π* electronic transitions of the CHO group. The carriers **B** and **C** didn't present any absorption maxima in the UV-VIS spectrum, very probably because of the hyperbranched polyethylene imine which shielded the chromophoric groups. By successively adding small amounts of HCl 10^{-5} M, the peak corresponding to the aldehyde appeared when the pH reached the value of 6.1, revealing that the imine linkages which kept together the hydrophobic core and the hydrophilic shell were cleaved by the acid presence (Fig. S5). The same was observed by NMR spectroscopy. The NMR spectra of the two amphiphiles was recorded in deuterium oxide at different time intervals. The NMR spectra didn't change in time, not even 24 h, indicating the stability of the two compounds at neutral pH. By adding a small amount of HCl in the NMR tubes, to decrease the pH at the value of 5.8 the appearance of the chemical shift corresponding to the trialdehyde appeared, suggesting the cleavage of the imine linkages, indicating dynamicity under acidic conditions (Fig. S6).

3.4. Transmission electron microscopy (TEM) studies

The size of the carriers and of their polyplexes is an important parameter which influences in a significant manner the DNA binding ability and also the transfection efficiency [8,46,48].

For this reason and also in order to check the theoretical design, according to which spherical structures should be obtained by the self-assembling of the synthesized amphiphiles, the compounds have been analyzed from the morphological point of view by transmission electron microscopy. In both cases it was observed the formation of spherical structures with sizes in the nano domain (Fig. 1). Thus, the compound based on PEI2000 generated structures with a mean diameter of 95 nm, while the compound based on PEI800 led to smaller entities, with a mean diameter of 20 nm, values generally reported for nonviral vectors with high efficient transfection [45]. It is also worth to remark that both compounds generated spherical nanoentities with a quite narrow dimensional polydispersity as could be observed from the histograms and from the standard deviation values (Fig. 1b, d). This is an important achievement if we take in consideration that dimensional uniformity is a main requirement for nanoentities which are designed for gene therapy, the best example in this sense being the dendrimers, for which significant abilities to bind and transfect DNA were reported [25,29].

3.5. The polyplex formation. Agarose gel electrophoresis

The ability of the synthesized compounds to interact with DNA was evaluated by agarose gel electrophoresis, using at first a salmon sperm commercial dsDNA. The agarose gel electrophoresis tests were performed at different N/P ratios, ranging from 10 to 400. As it can be observed from the obtained images, both compounds (**B** and **C**) presented a very good ability to interact with the salmon sperm DNA, starting at low N/P ratios, equal to 20 (Fig. 2a, b). Moreover, when salmon sperm DNA was used in the case of the **C** sample, at N/P ratios higher than 50, the presence of a light shadow moving to the cathode was noticed, suggesting the fact that the poliplex surface might have a slightly positive charge (Fig. 2). It is very likely that this may happen due to the higher size of the entities formed by **C** in comparison with **B** and further the higher density of the amine groups capable to be protonated and to act as sites for DNA binding [40].

Due to the fact that the DNA used in gene therapy is made by a higher number of base pairs (> 4000 bp) in comparison with the salmon sperm DNA (250 bp), it was also investigated the ability of the synthesized compounds to complex a double stranded DNA with a size of 4870 bp, pEGFP. The same type of DNA was used in the cytotoxicity tests and in determining the transfection ability. As it can be observed in Fig. 2c,d, in comparison with the naked pEGFP, the migration of the plasmid is fully retarded by the **B** compound at an N/P ratio equal to 150 and at an N/P ratio equal to 60 by the **C** compound.

3.6. The stability of the polyplexes under acidic conditions

Previous studies revealed that PEI is able to bind DNA with forces lower than 25 pN, allowing by this the formation of stable polyplexes under physiological conditions [19]. This being known, we decided to evaluate the stability of the polyplexes. With this aim, at first, the UV-VIS spectrum of the plasmid was registered showing a peak at 260 nm. The polyplexes formed by **B** and **C** with the DNA presented in the UV-VIS spectra only a shoulder at higher wavelengths, around 285 nm, which might be attributed to the DNA complexation. By successivelly adding small amounts/portions of HCL 10^{-5} M, the peak corresponding to the naked DNA appeared for pH = 6.3, suggesting that the decomplexation took place (Fig. S7).

3.7. Morphological and dimensional characterization of the polyplexes

TEM was used in order to visualize the morphology, size and polydispersity of the polyplexes based on B and C and the plasmid DNA.

Several studies revealed that good transfection efficiencies were obtained for polyplexes with sizes lower than 250 nm [47–49]. Fig. 3 shows representative images of the polyplexes based on **B** and **C**, at different N/P ratios.

TEM images revealed a strong correlation between the size of the polyplexes and the molecular weight of the used PEI. Therefore, a different behavior of the two synthesized systems was noticed. The compound **B** which generated spherical structures with a diameter of 20 nm, led to larger polyplexes, with higher sizes than the one of the naked compounds (100 nm for N/*P* = 200, 60 nm for N/*P* = 300 and 50 nm for N/*P* = 400), while the other compound **C**, which self-assembled in spherical structures with a mean diameter of 95 nm, generated smaller polyplexes (75 nm for N/P = 30, 60 nm for N/*P* = 60 and 40 nm for N/P = 400).

In the light of the TEM data, the polyplexes formation based on **B** and **C** can be envisaged as following: the compound based on PEI2000 self-assembled in larger spherical structures, which presented on the surface protonated amine groups, many enough such the vector could compensate the negative charge of DNA by its self. On contrary, the compound based on PEI800, led to smaller nanoentities, but the size of the resulted polyplexes is higher. This fact indicates a potential aggregation of the nanoentities generated by **B** in order to be able to compensate the negative charge of DNA (Scheme 3).

3.8. DLS measurements

Previous work stated that particle size is an important parameter which influences nonviral vectors stability and their behavior during cells internalization [8], being known that entities with sizes in the range 50 to several hundreds of nanometers suffer much easier endocytosis [49]. The TEM images of the vectors and of their polyplexes indicated dimensions appropriate for a good transfection. On the other hand, it must be taken into account that the transfection takes place in wet environment and so, the behavior of the polyplexes in biomimetic medium must be evaluated because it can bring important clues related to the factors which influence transfection. In this regard DLS measurements of vectors and polyplexes in PBS were performed. As the DLS measurements require high amounts of samples, and the plasmid is an expensive product, the measurements were done on the polyplexes obtained using the salmon sperm DNA.

DLS measurements confirmed the ability of the synthesized compounds to self-assemble into spherical structures due to their amphiphilic behavior, with a mean diameter of 586.7 nm in the case of **B** and of 871.4 in the case of **C** (Fig. 4), values which are considerably higher than the ones obtained by TEM. As can be observed from the Fig. 4, the distribution curves are quite sharp, indicating a low dimensional polydispersity of the nanoentities formed by the two amphiphiles. In the case of **B**, the hydrodynamic volume of the polyplexes increased in

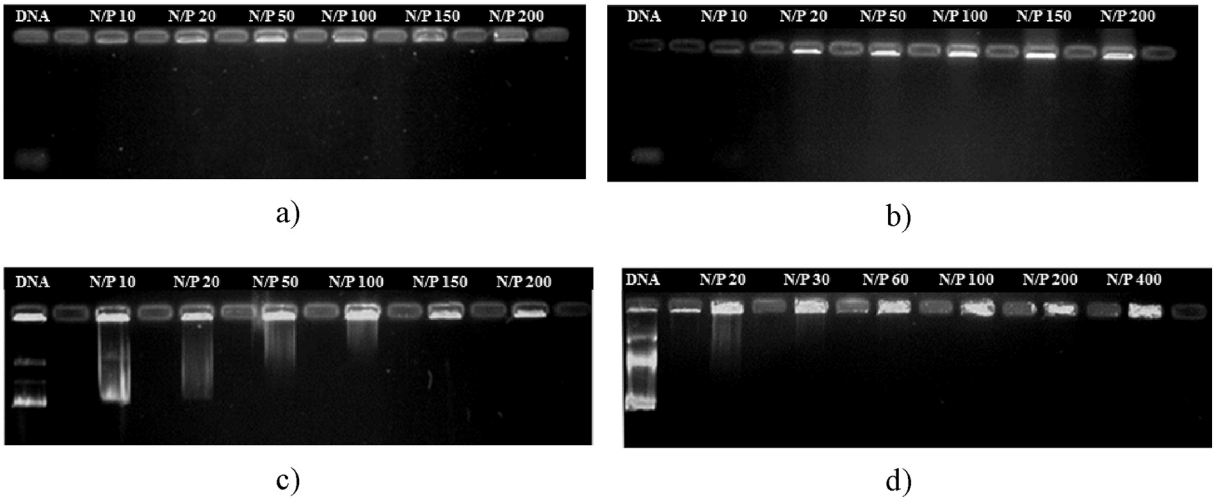


Fig. 2. Agarose gel electrophoresis with salmon sperm DNA for a) **B** and b) **C** and with the plasmid pEGFP for c) **B** and d) **C**. The naked salmon sperm DNA or plasmid p-EGFP were used as control.

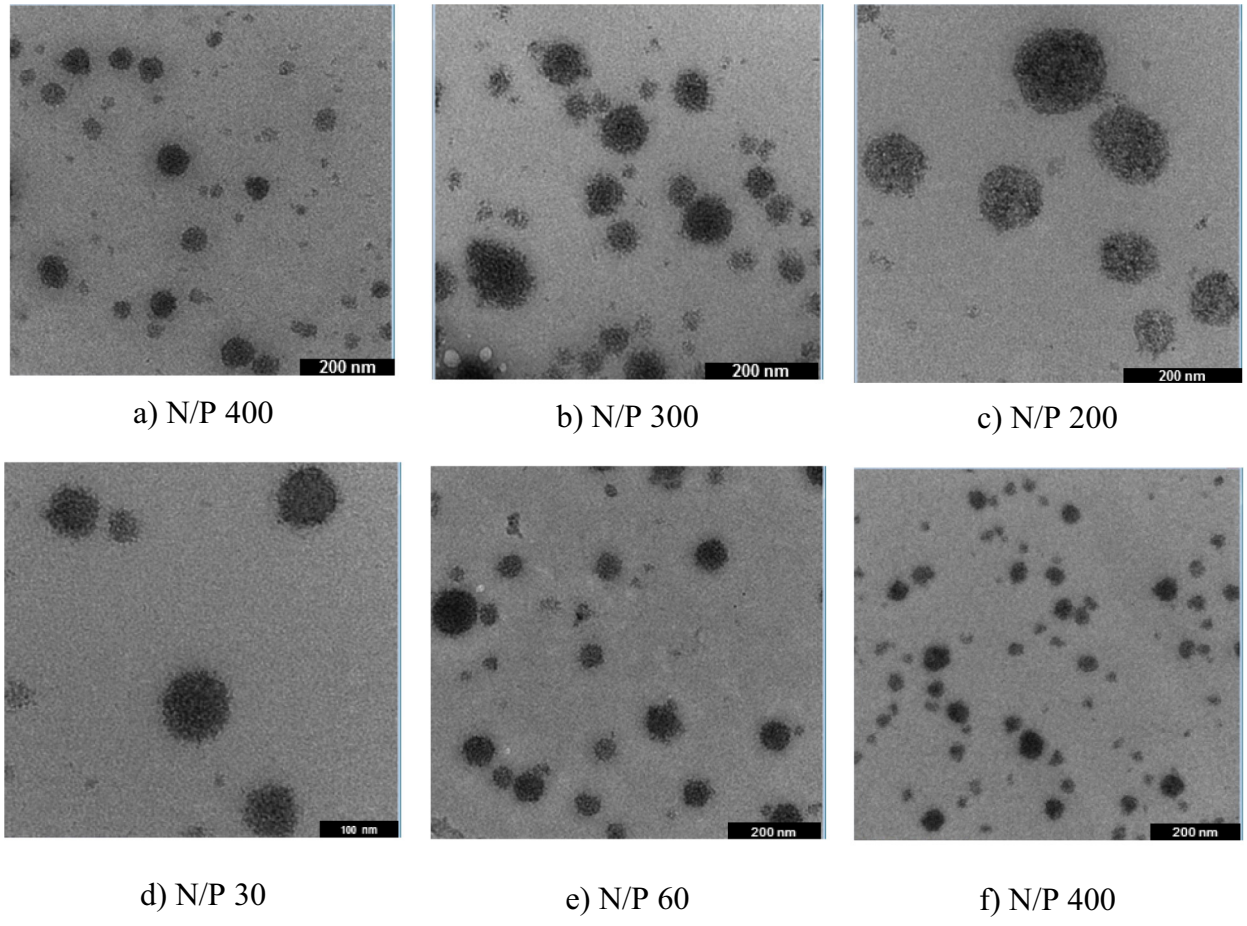
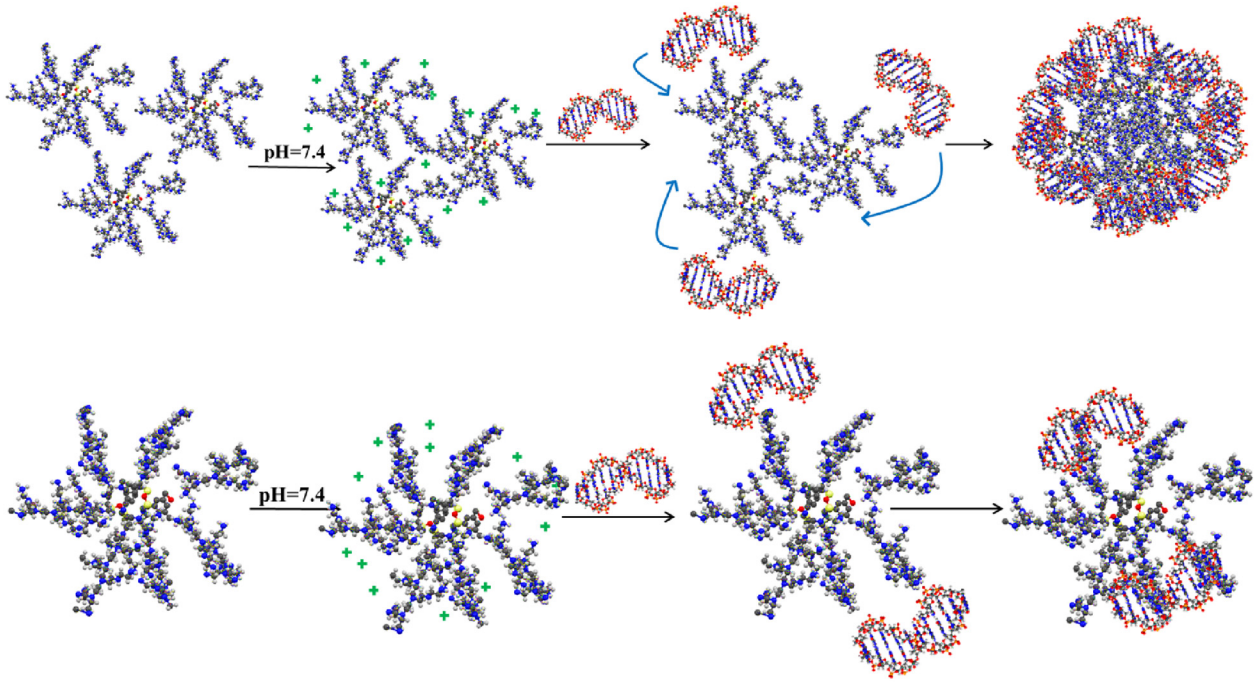


Fig. 3. TEM images of the polyplexes based on **B** (a,b,c) and **C** (d,e,f) at different N/P ratios.

comparison with the one of naked compound, reaching even micrometric sizes with a maximum value of 1.4 μm. This data are in agreement with TEM images, which showed a similar relationship for the polyplexes *vs*. naked compound in dried state. In the case of **C**, the size of the polyplexes is lower than the one of the naked compound and more than this they are characterized by a much narrow dimensional polydispersity in comparison with the polyplexes formed by **B**, confirming once more the TEM images and also the scenario drawn according to TEM.

Although, it has to be underlined that the obtained values of the hydrodynamic diameter of the polyplexes is not the real one, the one which will be encountered in the biological medium, due to the fact that a different kind of DNA is used for transfection – plasmid with 4870 bp, which has the ability to wrap better the compounds, leading by this to smaller sizes [14].



Scheme 3. Schematic representation of the proposed mechanism for the polyplexes formation of **B** and **C**.

3.9. Zeta potential measurements

With the aim of evaluating the stability of both vectors and polyplexes, their Zeta potential was determined by electrophoretic light scattering in phosphate buffer solution (pH = 7.4). Due to the fact that the method requires high amounts of samples, the measurements were done on the polyplexes obtained using the salmon sperm dsDNA. The method confirmed the obtaining of polycations, due to the presence of the hyperbranched PEI on their surface, both vectors presenting positive values of the Zeta potential equal to 18.35 mV – the vector **B** and to 37.59 mV – the vector **C**. The values indicate a better stability of **C** compared to **B**; the higher charge of the surface of **C** promoting stronger electrostatic repulsions, hindering their aggregation (See Supplementary Info).

On the other hand, the lower charge on the surface of **B** molecules could allow their aggregation in the presence of DNA, under the pressure of forming stable polyplexes, as TEM and DLS suggested.

Interesting enough, the maximum value of the Zeta potential reached for both polyplexes is almost equal, suggesting once more the hypothesis that the **B** vectors aggregates in the presence of DNA to give stable polyplexes. Regarding the polyplexes Zeta potential, it was observed that at lower N/P ratios, they are anionic, indicating an incomplete binding of DNA but they become positive with the increase of the N/P ratio (Fig. S8).

3.10. Cytotoxicity

The cytotoxicity of the polyplexes based on **B** and **C** was evaluated on HeLa cells at different N/P ratios by measuring the cell viability in the presence of the polyplexes. For comparison, it was tested also the toxicity of the polyplexes based on PEI800 and PEI2000. The obtained results are presented in Fig. 5a,b.

As it could be observed from the Fig. 5, the cytotoxicity of the polyplexes based on **B** is lower than the one of the **C** polyplexes, the cell viability in the first case remaining over 90% even at N/P ratios higher than 400. More than this, the toxicity of the polyplexes of **B** is lower than the one of the PEI800 precursor, for all the tested N/P ratios. An unacceptable decrease of the cell viability was observed for values of the N/P ratios higher than 400 in the case of the **B** containing polyplexes. On the other side, the polyplexes of **C** presented as expected a higher cytotoxicity, similar to the one of the free PEI2000, in this case the cell viability presenting satisfactory values up to an N/P ratio equal to 100. This data point out the biocompatibility of the designed nonviral vectors, which can be safely used up to an N/P ratio of 400, the one based on **B** and up to 100, the one based on **C**, being appropriate to be used as nonviral vectors for bioapplications.

3.11. The evaluation of the transfection efficiency of the polyplexes

The evaluation of the transfection ability was realized in a first step qualitatively by fluorescence microscopy using the reporter gene GFP. The tests revealed that the transfection efficiency increases with the increase of the N/P ratio, both compounds presenting a superior ability to transfect the HeLa cells in comparison with the free PEI800 and PEI2000, used as reference (Fig. 6 and Fig. S9). This is a consequence of different characteristics of the synthesized vectors, all of them playing an important and equal role. Studies on blood brain barrier, which is one of the most difficult to be crossed biological membranes, demonstrated that lipophilic molecules are able to penetrate it, precisely due to their hydrophobicity [50,51]. Moreover, vectors based on siloxane, which is highly hydrophobic, showed a high transfection ability of the HeLa cells, fact also explained due to the hydrophobicity of the carrier containing siloxane [38]. Therefore, the high transfection ability of the synthesized carriers may be explained taking into consideration the influence of the siloxane building block. On the other hand, the positive values of the Zeta potential of the polyplexes, at high N/P ratios, definitely facilitate transfection, being known that a cationic surface helps in the cellular internalization process [52]. The nanometric size of the polyplexes, along with their spherical morphology also contributes in reaching the high transfection efficiency.

Moreover, the **C** vector, which is based on PEI2000 Da presented a higher ability of transfection in comparison with **B**, fact which is a consequence of both the higher molecular weight of the PEI and also of the smaller size of the polyplexes, as it was revealed by TEM. The higher Zeta potential values registered for the polyplexes based on **C** may also play a role in achieving the superior transfection ability in this case

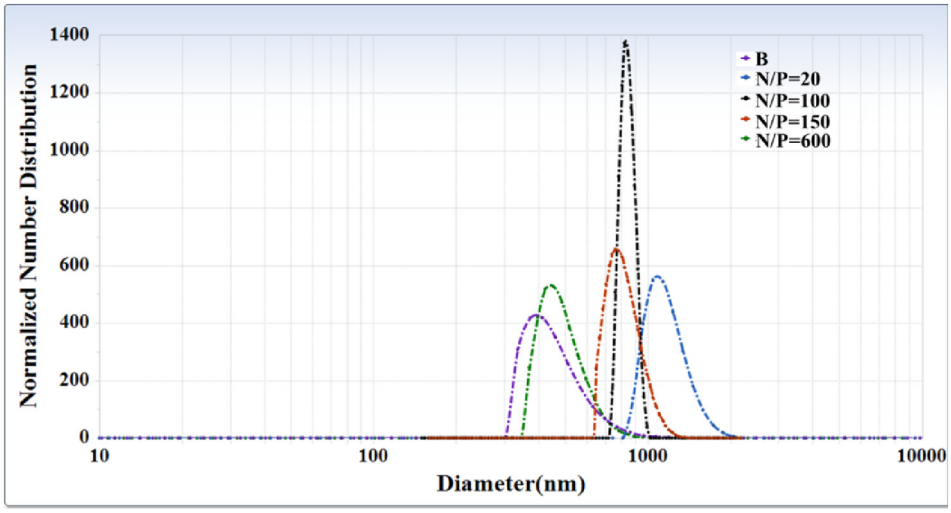


Fig. 4. The numeric size distribution of the synthesized compounds and of some representative polyplexes a) **B** and b) **C** on a logarithmic scale with a base of 10.

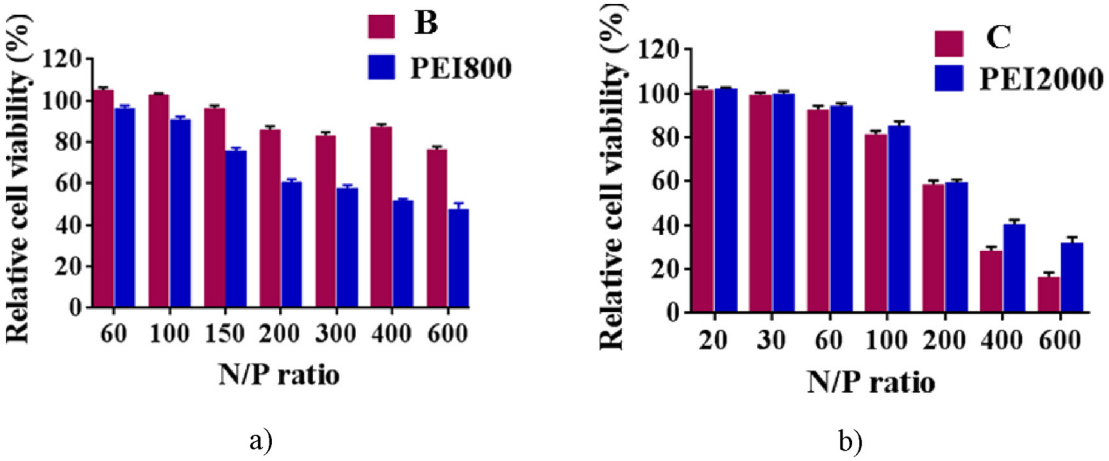


Fig. 5. The viability of the HeLa cells in the presence of the polyplexes obtained using the synthesized compounds or the PEI precursors and the plasmid pEGFP, at different N/P ratios. The concentration of the plasmid DNA in the medium was 5 ng/μL. The results are presented as a mean value ± the standard deviation obtained from three independent experiments in triplicate.

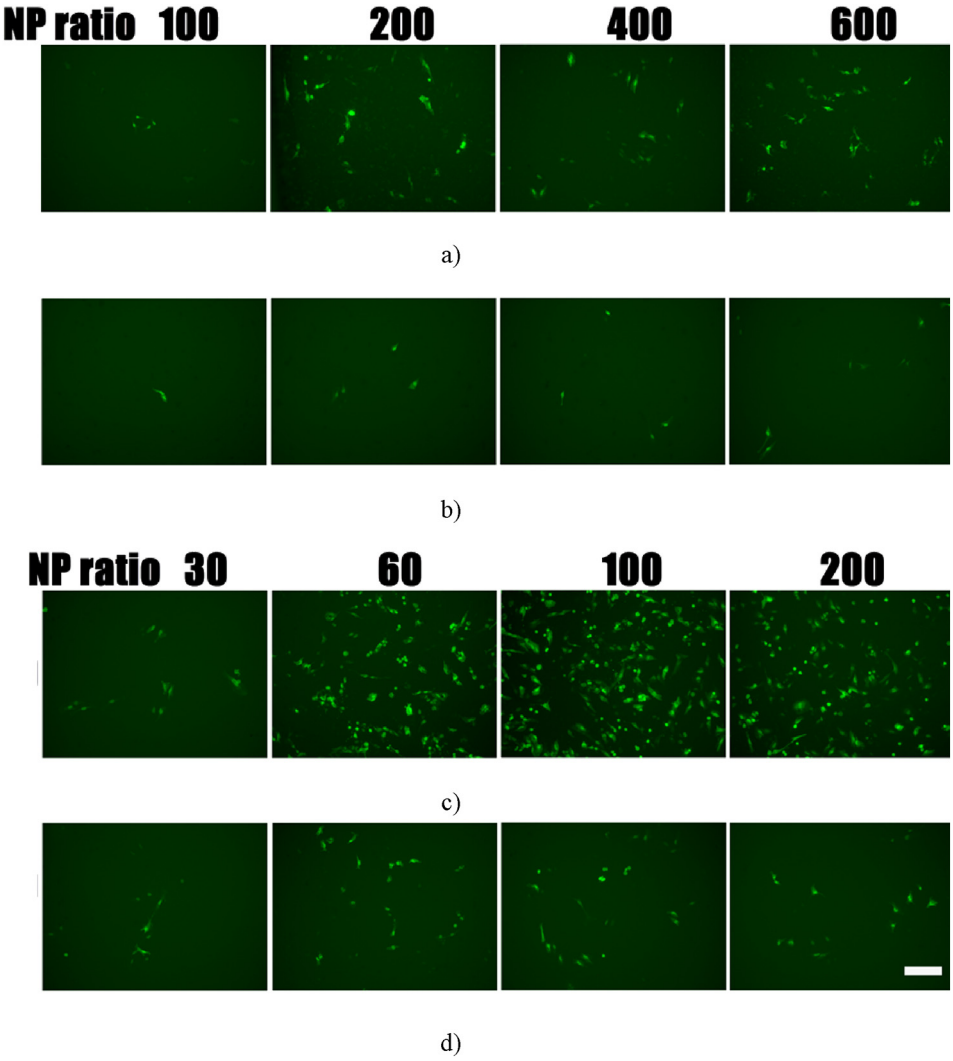


Fig. 6. The visualization of the GFP protein in the transfected HeLa cells with the polyplexes based on a) **B** and b) PEI800 at the following N/P ratios: 100, 200, 400, 600 and respectively c) **C** and d) PEI2000 at the following N/P ratios: 30,60,100,200 Scale bar, 200 μm.

[53–58].

The transfection efficiency was determined further quantitatively by luciferase assay, when it was confirmed once more the superior transfection ability of the **C** compared to **B** (Fig. 7).

Thus, the results show a maximum transfection of 7×10^6 in the case of **C** at an N/P ratio equal to 100, while in the case of **B**, the transfection efficiency reaches a maximum of only 8×10^5 at a much higher value of the N/P ratio, 600. This is a consequence of both Zeta

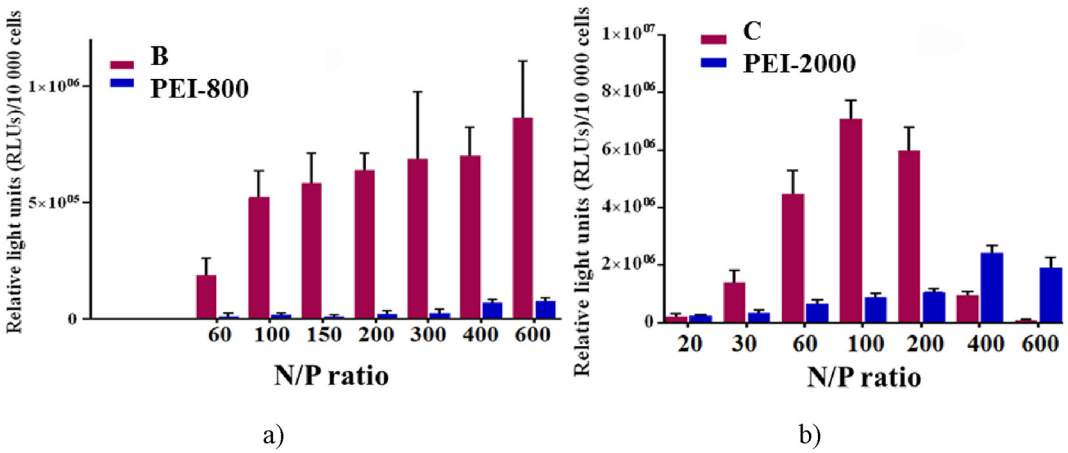


Fig. 7. The efficiency of transfection after 48 h. HeLa cells were transfected with pLuc polyplexes made with a) **B or PEI800** and b) **C or PEI2000**. Data is represented as relative light units (RLUs).

potential and size of the polyplexes at this N/P ratio, being known that an increase in terms of charge and a decrease regarding the size, are both beneficial for cell membrane penetration and further for transfection [46,52,59]. An important parameter which must be taken into consideration is the cytotoxicity of the polyplexes at these N/P ratios. The measurements of the HeLa cells viability showed that **C** polyplexes allowed a viability of the HeLa cells around 83% at an N/P ratio equal to 100, value which generated the maximum transfection yield. On the other side, the **B** containing polyplexes even if they present a lower cytotoxicity in comparison with the **C** based ones, at the N/P ratio equal to 600, which generated the highest transfection, are more cytotoxic, the cell viability remaining only at 75%. Moreover, if we compare the transfection efficiency of the two synthesized compounds for the same N/P ratio, in all cases the transfection efficiency is higher with an order of magnitude in the case of the **C** polyplexes. The importance of the hydrophobic core containing siloxane is revealed also by these tests, for all N/P ratios our vectors being able to transfect the HeLa cells much better than the free PEIs.

4. Conclusions

Nanosized nonviral vectors with spherical morphologies have been obtained exploiting the reversibility of the imine linkage and the natural tendency of minimizing the contact with water of the hydrophobic part of the amphiphiles. The design of the nonviral vectors and the components of the systems were properly chosen in order to create structures which fulfill the main requirements for nonviral vectors *ab initio*: hyperbranched polyethylene imine with two different molecular weights (800 Da or 2000 Da) to assure the DNA binding ability and the endosomal escape and a linear siloxane as part of the hydrophobic core for cell membrane penetration. As expected, the synthesized compounds proved a great ability to bind DNA, forming polyplexes characterized by nanometric sizes and narrow dimensional polydispersity as was revealed by transmission electron microscopy and dynamic light scattering. The high transfection efficiency, demonstrated on HeLa cells, proved the success of the theoretical design enlightening the fact that dynamic constitutional chemistry can be successfully used as a synthetic pathway to obtain nonviral vectors for gene delivery.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2018.10.002.

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