ASSEMBLY AND *IN VITRO* BIOLOGICAL BEHAVIOR OF PEI/DNA POLYPLEXES

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Small individual stable aggregates are produced by the condensation of Deoxyribonucleic acid (DNA) by Polyethyleneimine (PEI) through electrostatic bidding. These polyplexes facilitate the delivery of the genetic material payload into cells. We synthesized polyplexes using both forms of PEI; linear and branched at different N/P rations. The resultant polyplexes become stable when N/P ≥ 3 and had optimal diameter and polydispersity and remained stable for 48h at least. Furthermore, we investigated the behavior of these particles on epithelial-like cells (NCTC), human dermal fibroblasts (HDF), and HeLa cells. The results of the MTT, LDH, and Live/dead assays on the cell lines suggest a high tolerance to the PEI/DNA polyplexes reflected by a low toxicity and high proliferation profiles.

Keywords: Polyethyleneimine, PEI/DNA polyplexes, Self-assembly, Gene therapy

1. Introduction

Gene therapy is a promising treatment approach that involves introducing genetic material into a patient's cells to correct faulty genes, treat diseases, or modify the clinical course of conditions [1, 2].

In a general manner, gene delivery systems can be divided into two main categories: viral and non-viral. Numerous non-viral gene transfer vectors have been investigated and developed in response to limitations associated with viral gene delivery systems such as genomic integration, immunogenic responses, and liver toxicity [3-5]. One of the most promising and investigated synthetic non-viral vectors for both *in vitro* and *in vivo* applications is polyethyleneimine (PEI) [6-8].

Polyethyleneimine is a cationic polymer that proved to be highly efficient in intracellular gene delivery [9, 10]. PEI forms individual stable aggregates commonly called polyplexes through the condensation of deoxyribonucleic acid (DNA), and this *via* electrostatic binding between DNA's anionic phosphate groups

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and PEI's cationic amino groups [11, 12]. Moreover, the high-density amine groups of the PEI can act as a buffer at various pH values, which gives these vectors the ability to escape endosomes and transfer the gene payload to the cytoplasm [13, 14].

PEI comes in two distinguished topologies, linear and branched. While linear PEI (L-PEI) contains only secondary amines and one unique primary amine end, branched PEI's (B-PEI) structure is made of primary, secondary, and tertiary amines [15]. Both PEI forms were the focus of a myriad of gene delivery and transfection studies [16, 17].

Regarding the clinical uses of formulations based on PEI, PEI-based systems of different molecular weights and structures were assessed for cancer treatment [18], immunotherapy where DNA/PEI polyplexes induced immune responses against hepatitis B virus [19], HIV [20], and others. Alongside these applications, other pathologies were reviewed, like myocardial infarction [21], hepatitis [22], and neurological disorders [23].

The effectiveness of PEI based polyplex transfection has been found to be influenced by a wide range of factors, including complex size, degree of branching, N/P ratio, and molecular weight [13]. The molecular weight of PEI was observed to be directly proportional to the condensation ability and surface charge of the polyplexes and inversely proportional to their size [24]; furthermore, the same study showed that, compared to PEI 2k and PEI 5k, PEI 25k produced smaller polyplexes and had better transfection efficiencies in both HeLa and MCF7 cells [25]. In another paper, the focus was directed to the concentration of PEI and DNA, where the study showed that it is possible to regulate the charge and size of gene delivery carriers and, possibly even, increase the efficacy of gene transfection by varying the concentration of DNA and PEI [26]. In addition, it has been shown that, for a given topology, a high molar mass PEI is more efficient in condensing DNA and that, for a given molar mass, linear chains are more effective than their branching counterparts in neutralizing DNA [11].

The main focus of the current study consists in the formulation of polyplexes based on two types of PEI and the investigation of hydrodynamic characteristics using dynamic light scattering (DLS), their stability and morphology, as well as the biological behavior on three different cell lines: epithelial-like cells (NCTC), human dermal fibroblasts (HDF), and HeLa cells by means of MTT, LDH, and Live/Dead assays.

2. Material

L-PEI, with a molecular weight of Mw = 10000 g/mol and B-PEI with Mw = 25000 g/mol, DNA with 2000 bp from salmon testes, and Trizma base with Mw

= 121.14 g/mol from Sigma-Aldrich, USA. All chemicals were used without further purification, and the experiments were performed using pure water.

3. Methods

3.1. Preparation of PEI/DNA complexes

The PEI/DNA polyplexes were prepared at different N/P ratios by dropping under vortex mixing, the necessary amount of PEI solution (1 mg/mL, 10 mM Trizma buffer, pH 7.4) to a constant DNA solution (0.05 mg/mL, 10 mM Trizma buffer, pH 7.4). Then the resulted solutions were incubated for 20 minutes at room temperature, to allow the assembly of DNA in the presence of polycation, respectively, the generation of PEI/DNA polyplexes. Freshly assembled polyplexes were used in further experiments.

3.2. Hydrodynamic characterization, zeta potential, and morphology

The hydrodynamic diameter (d, nm) and polydispersity (PdI) of polyplexes were determined by DLS (Zetasizer Nano ZS, Malvern Instrument). The samples were transferred in a cuvette, and three measurements with 15 successive cycles at 25 °C were run.

The zeta potential (ζ) measurements were performed using the principle of laser Doppler velocimetry of Zetasizer Nano ZS, Malvern Instrument. The samples were transferred in special cuvette for zeta potential measurement, and the measurements were done at 25 °C, and the reported ζ -potentials are the averaged results of three measurements with 30 successive cycles. For stability studies, the evolution of hydrodynamic characteristics of assembled polyplexes at selected time points during 48 h was monitored (t = 15 min, t = 6 h, t = 24 h, and t = 48 h).

3.3. Cytocompatibility studies

The cytocompatibility of polyplexes was studied on three different cell lines: NCTC, HDF, and HeLa cells, through three methods (MTT, LIVE/DEAD, and LDH). The biological experiments were done in triplicate.

The cells were cultured at a cell density of 1×10^5/mL cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 atmosphere for 24 h to allow the cells attachment.

Cell viability and proliferation potential of polyplexes were analyzed after 24 h and 72 h, using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) metabolic assay. The samples were incubated with 1 mg/mL MTT solution for 4 h at 37 °C and the absorbance at 550 nm was measured by a FlexStation 3 spectrophotometer.

The cytotoxic potential was evaluated using the LDH assay, by mixing the samples with the components of the Tox-7-KT kit according to the manufacturer's instructions and incubating for 20 min in darkness, then the samples were spectrophotometrically measured at 490 nm, using unstimulated cells as control. Further, the qualitative evaluation of the cytocompatibility was achieved using the Live/Dead kit (ThermoFisher Scientific, Foster City, CA, USA) after staining the live and dead cells from the media with fluorescein diacetate (FDA). Cell morphology was studied using a confocal microscope (Carl Zeiss LSM 710, Jena, Germany), and the images were processed by means of Zeiss Zen 2010 software.

4. Results and discussion

The present work involves the formulation of self-assembled PEI/DNA polyplexes with optimal hydrodynamic properties, biocompatibility, and stability based on N/P ratios with the insight of applying these systems in future transfection studies. The obtained results concord with the conclusions drawn from previous research [27].

4.1. DLS characterization of the PEI-DNA particles

The assembly of PEI/DNA polyplexes can spontaneously occur by means of the electrostatic interactions between positively charged PEI (amino groups) and negatively charged DNA (phosphate functionalities). Given that electrostatic interaction drives the creation of the polyplexes, the ratio of cationic PEI to anionic DNA (N/P), mostly determines the complex's size, distribution, and charge [28, 29].

The variation of the hydrodynamic parameters and zeta potential of PEI/DNA polyplexes based on two types of PEI (L-PEI, B-PEI) in function of the N/P molar ratio at fixed DNA concentration (0.05 mg/mL) was investigated by dynamic light scattering, and the results are shown in Fig 1 (a, b, c, d).

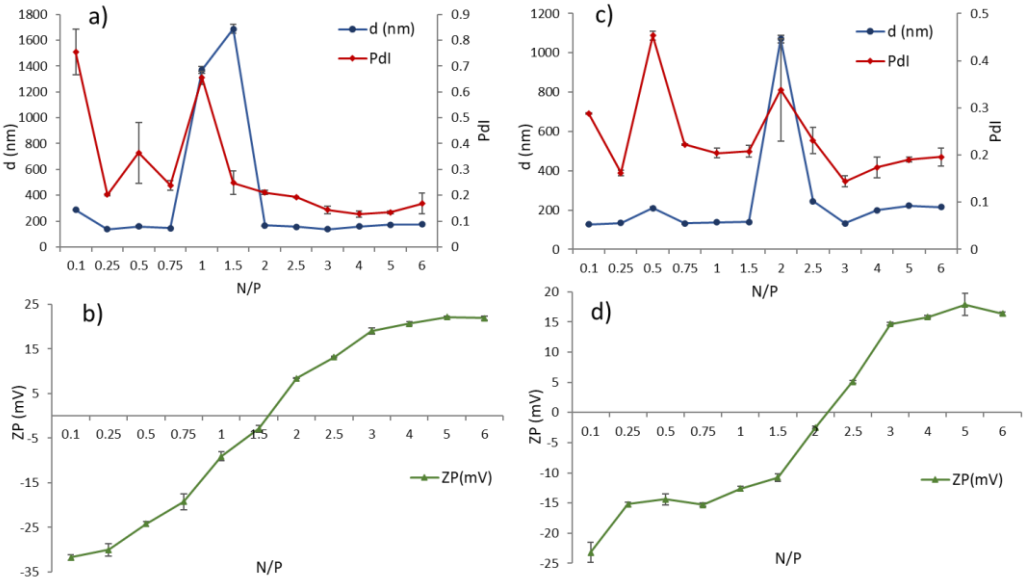

Fig 1. Evolution of hydrodynamic parameters and zeta potential of a, b) L-PEI/DNA and c, d) B-PEI/DNA polyplexes formulated at different N/P molar ratios. Negative zeta potential shows insufficient DNA stabilization, while positive zeta value suggests the formation of stable polyplexes, in corroboration with hydrodynamic measurements.

Generally, there are no significant differences in the mean diameter and size distribution of polyplexes, regardless of the structure of the cationic polymer (L-PEI, B-PEI)-mediated the complexation reaction.

As shown in Fig 1, mass aggregation occurred at N/P molar rations between 0.75 and 2 when L-PEI mediated the complexation, respectively 1.5 and 2.5 when B-PEI was used. The process of aggregation occurs in close proximity to the zone of electroneutrality. At this point, the system's positive and negative charges are mutually neutralized. Further increasing the amount of cationic polymer (up to N/P = 2.5 for L-PEI and 3 for B-PEI) polyplexes with an average diameter of 150–200 nm and a suitable size distribution (PdI <0.3) were formed. Previous research demonstrated that monomolecular DNA-based nanoparticles should have a mean size of between 30 and 40 nm [30, 31]; each colloid most likely included multiple copies of DNA.

Then, zeta potential of polyplexes (Fig 1, b, d) was negative at low N/P molar ratios due to an excess of negative charges produced by the unbounded DNA in the solution. The increase of the N/P molar ratio led to a positive reversion of the zeta potential, which has the cross point in the electroneutrality region, and above this region (N/P~3) the surface charge of polyplexes was no longer affected by the addition of the polycation. This fact suggests that a part of the PEI chains was free

in the solution mixture and might play an important role in promoting gene transfection [32].

4.2. Stability studies

Studying the stability of PEI-DNA polyplexes over time is essential for understanding their behavior, optimizing their design, and, more importantly, improving gene delivery efficiency. In this respect, we studied the stability of polyplexes based on two types of PEI (L-PEI and B-PEI), formulated above the electroneutrality zone (N/P ratios from 2 to 6) by monitoring the variation of the hydrodynamic parameters over a period of 48 h (Fig 2, a, b, c, d).

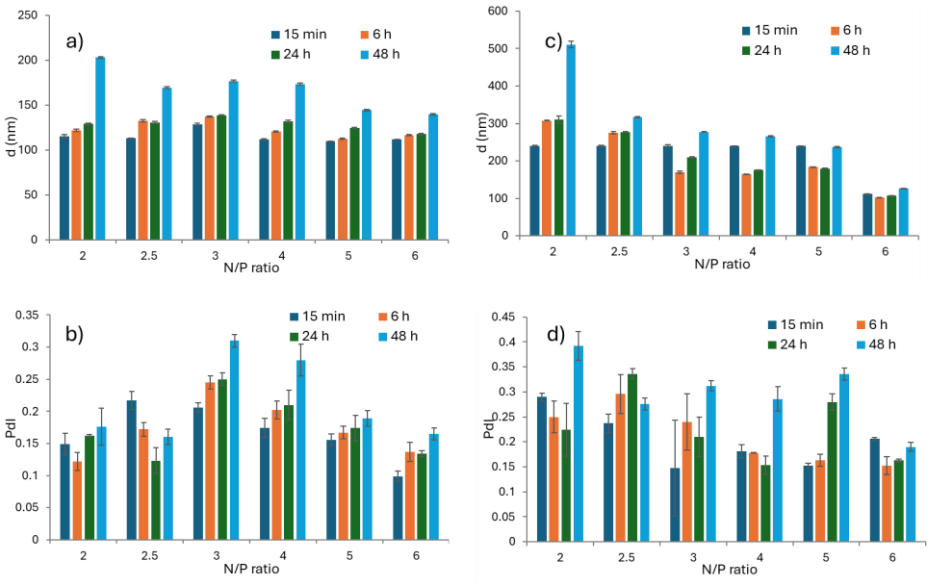

Fig 2 Variation of the mean hydrodynamic size (d, nm) and polydispersity (PdI) of a, b) L-PEI/DNA and c, d) B-PEI/DNA polyplexes studied during 48 h (15 min, 6 h, 24 h, 48 h)

Generally, the stability of polyplexes was impacted by both the type of polycation used in the assembly of polyplexes and the N/P molar ratio. The polyplexes (L-PEI/DNA or B-PEI/DNA) obtained at N/P molar ration between 2-3 presented a reduced stability as significant variations in their polydispersity and mean diameter at measured time points was noted. This may be correlated with the insufficient amount of positive charges to stabilize the anionic compound and form stable polyplexes (in agreement with the DLS measurements). The stability of polyplexes formulated at N/P > 3 was improved, while L-PEI/DNA polyplexes exhibited a good stability profile during the study, as no significant variations in the mean diameter and PdI index can be observed over the investigated period of time

(Fig 2, a, b), in contrast with those based on B-PEI for which the variations of these parameters were more evident (Fig 2, c, d).

In conclusion, this study showed that complexing DNA by linear PEI produces particles with enhanced in-time stability when compared to the ones obtained by branched PEI.

4.3. Biological studies

Cellular biocompatibility and cytotoxicity represent important characteristics in designing efficient and safe biomaterials for biomedical applications. Therefore, in light of the stability studies, L-PEI/DNA and B-PEI/DNA polyplexes obtained at N/P = 3 and N/P = 4 were selected for the biological investigations. In order to validate the safety of the formulated polyplexes, the biocompatibility and proliferation potential, along with the cytotoxic potential were evaluated on three different cell lines: NCTC, HDF, and HeLa through both quantitative (MTT, LDH) and qualitative (LIVE/DEAD) methods, after 24 and 72 hours of exposure in standard culture conditions. The results of the biocompatibility and capability of polyplexes to support and promote the development of the selected cell lines assessed by the MTT assay as well as the cytotoxicity potential examined by the LDH test were presented in Fig 3.

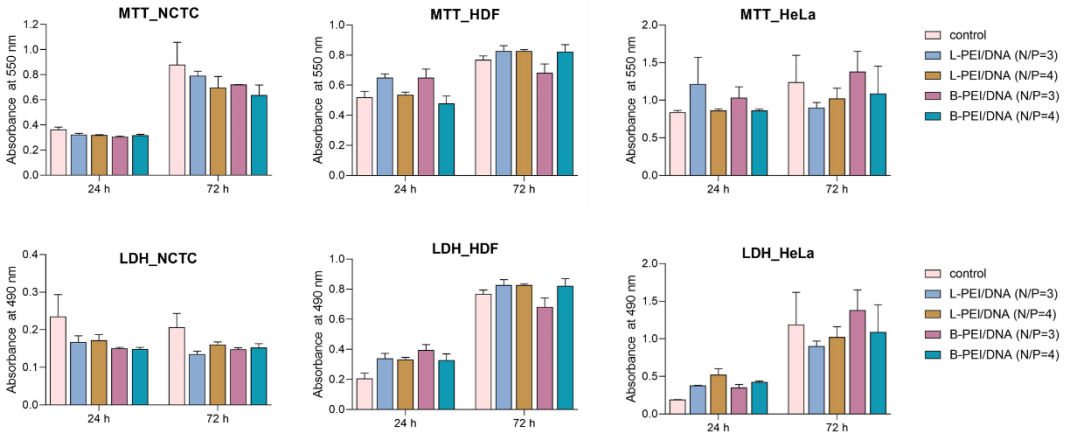

Fig 3**.** Biocompatibility and cytotoxic response of NCTC, HDF, and HeLa cells after 24 h and 72 h of incubation with investigated polyplexes based on two types of PEI obtained at N/P = 3 and N/P = 4 molar ratios.

Generally, the results of the MTT assay showed a good cellular response on the investigated samples after 24 h of incubation; the cellular viability is comparable with the control samples, in the case of NCTC and HeLa cell lines, while in the case of HDF, an improved cellular response was noted (the cellular

viability slightly exceeded that of the control sample). After 72 h, visible improvement in biocompatibility and proliferation potency was noted in the case of polyplexes incubated in the presence of NCTC and HDF cell cultures, while no significant differences were registered for samples incubated with HeLa cells, as compared to results registered after 24 h. The different behavior of cells in the presence of polyplexes may be correlated with the morphological and biological particularities of the used cellular line [33, 34]. Further, the cytotoxic potential of selected polyplexes was studied by determining the amount of dead cells indicated by the amount of LDH release in the culture media, using standard cellular culture as a control. The LDH assay confirmed the good biocompatibility of polyplexes on NCTC cells; the cellular response was significantly lower as compared to control samples, regardless of the investigated time point. At the same time, the level of LDH released in the media in the case of HDF and HeLa cell cultures was comparable to that of controls, suggesting a low cytotoxic potential.

Subsequently, the confocal microscopy images of qualitative evaluation of the *in vitro* cellular viability through the LIVE/DEAD assay (Fig 4) showed a good cellular viability; the general number of dead cells was insignificant next to the living cells, regardless of the type of cellular line (NCTC, HDF, and HeLa) and the investigated time points (24 h and 72 h), supporting the conclusions of the MTT and LDH analyses. An increasing trend in cellular proliferation and development, comparable to control samples was noted after 72 h of incubation. Furthermore, the cells presented an elongated morphology, which suggested their adhesion to the substrate and proliferation in the seeded media [35].

Overall, the results confirmed the good biocompatibility and proliferation potential of investigated polyplexes based on both types of PEI.

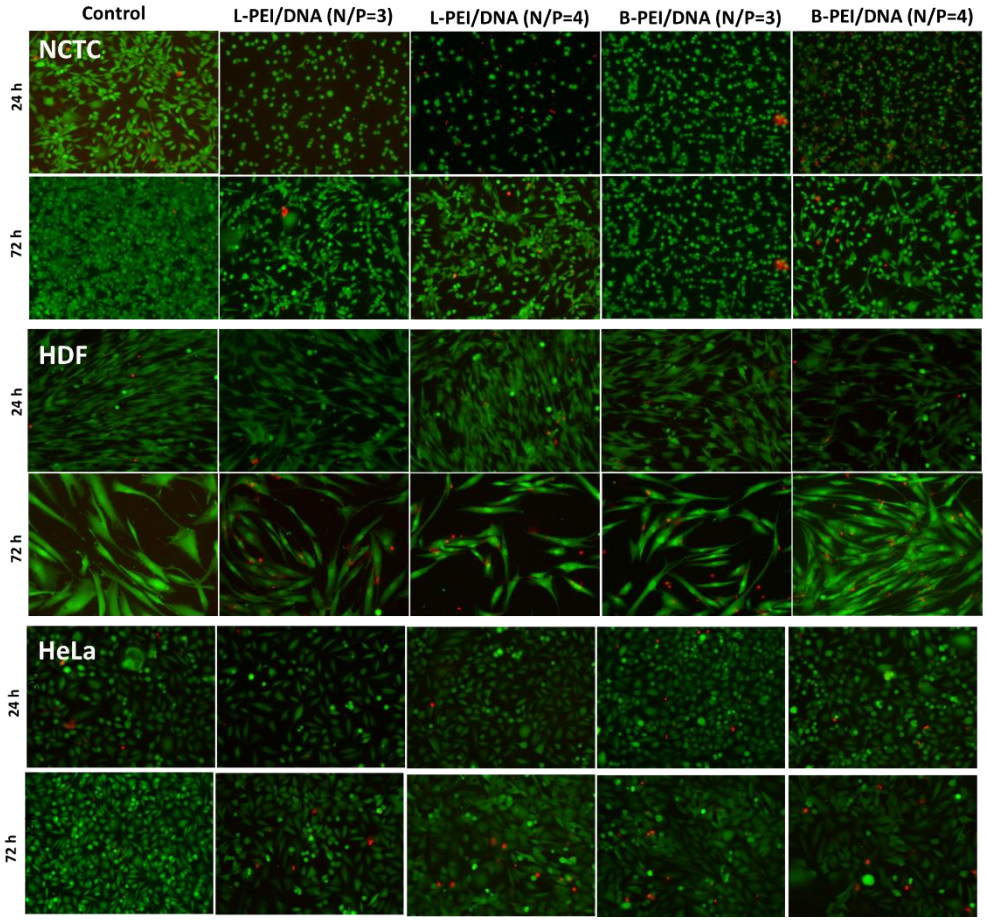

Fig 4. LIVE/DEAD cells viability assay of NCTC, HDF, and HeLa cells after 24 h and 72 h of incubation with polyplexes based on two types of PEI obtained at N/P = 3 and N/P = 4 molar ratios

5. Conclusion

Small aggregates, or polyplexes, are produced when DNA and PEI are mixed. To achieve high gene transfection, these polyplexes must have optimal hydrodynamic characteristics. In this paper, we formulated and investigated the distinctive characteristics of PEI/DNA complexes engineered at varying N/P ratios, including size, polydispersity, and zeta potential.

In general, stable polyplexes are obtained at around N/P ~ 3, and the mean diameter and size distribution did not exhibit substantial variations, irrespective of the cationic polymer structure (L-PEI, B-PEI) used in the complexation reaction.

The stability of polyplexes during time was impacted by both the type of polycation used in the assembly of polyplexes and the N/P molar ratio. The

polyplexes made of L-PEI and formulated at N/P >3 showed better stability compared to the other type of polyplexes, which was evidenced by the slight variation in the mean diameter and size distribution.

Finally, to investigate the safety of the formulated polyplexes, three different cells populations (NCTC cells, HDF cells, and Hela cells) were exposed to the two types of polyplexes. Then safety was assessed through both quantitative (MTT, LDH) and qualitative (LIVE/DEAD) methods. Overall, the results confirmed the good biocompatibility and proliferation potential of investigated polyplexes based on both types of PEI, with a slight advantage observed in favor of L-PEI polyplexes after 72 hours, which could be explained by their superior stability profile over time in comparison to their counterparts.

REFERENCES

[1] *S. Indu, V. Ramesh, N. Oza, K. V. Prashad,* Gene therapy: An overview, Journal of Orofacial Sciences, **5** (2013), No. 2, 83-87, 10.4103/0975-8844.124249.

[2] *E. L. Scheller, P. H. Krebsbach, Gene therapy: design and prospects for craniofacial regeneration,* J Dent Res, **88** (2009), No. 7, 585-96, 10.1177/0022034509337480.

[3] *C. E. Thomas, A. Ehrhardt , Kay MA,* Progress and problems with the use of viral vectors for gene therapy*,* Nat Rev Genet, **4** (2003), No. 5, 10.1038/nrg1066

[4] *X. Li, Y. Le, Z. Zhang, X. Nian, B. Liu, X. Yang,* Viral Vector-Based Gene Therapy, Int J Mol Sci, **24** (2023), No. 9,7736, 10.3390/ijms24097736.

[5] *E. Capra, A. Godfrey, A. Loche, J. Smith,* Gene-therapy innovation: Unlocking the promise of viral vectors, McKinsey & company, (2021).

[6] A. *Hall, U. Lächelt, J. Bartek, E. Wagner, S. M. Moghimi,* Polyplex Evolution: Understanding Biology, Optimizing Performance, Mol Ther, **25** (2017), No. 7, 1476-1490, 10.1016/j.ymthe.2017.01.024.

[7] *B. Schwarz, O.M. Merkel,* Functionalized PEI and its role in gene therapy, Material Matters, **12** (2017), No. 2, 63-67.

[8] *K. C. Bahadur, & H. Uludağ,* PEI and its derivatives for gene therapy (2016), 10.1016/B978-0-08-100520-0.00002-3.

[9] *C. M. Varga, N. C. Tedford, M. Thomas, A.M. Klibanov, L. G. Griffith, D. A. Lauffenburger*, Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes, Gene Ther, **12** (2005),No. 13, 1023-32, doi: 10.1038/sj.gt.3302495.

[10] *J. Casper, S. H. Schenk, E. Parhizkar, P. Detampel, A. Dehshahri, J. Huwyler*, Polyethylenimine (PEI) in gene therapy: Current status and clinical applications, Journal of Controlled Release, **362** (2023), 667-691. doi: 10.1016/j.jconrel.2023.09.001.

[11] *Z. Dai, & I. Wu I, How Does DNA Complex with Polyethylenimine with Different Chain Lengths and Topologies in Their Aqueous Solution Mixtures?*, Macromolecules, **45** (2012), No. 10, 4346–4353, doi: 10.1021/ma2027963.

[12] *K. Utsuno, H. Kono, E. Tanaka, N. Jouna, Y. Kojima, H. Uludağ,* Low Molecular Weight Branched PEI Binding to Linear DNA, Chem Pharm Bull, **64** (2016), No. 10, 1484-1491, doi: 10.1248/cpb.c16-00454.

[13] *A. Rodriguez, A. del, M. Angeles,* Non-Viral Delivery Systems in Gene Therapy [Internet]. Gene Therapy - Tools and Potential Applications, InTech; 2013. Available from: http://dx.doi.org/10.5772/52704

[14] *B. Singh, S. Maharjan, T. E. Park, T. Jiang, S. K. Kang, Y. J. Choi, C. S. Cho*, Tuning the buffering capacity of polyethylenimine with glycerol molecules for efficient gene delivery: Staying out of the endosomes, Macromol Biosci, **15** (2015), No. 5, 622-35, doi: 10.1002/mabi.201400463.

[15] *C. N. Lungu, M. V. Diudea, M. V. Putz, I. P. Grudziński*, Linear and branched PEIs (Polyethylenimines) and their property space, Int. J. Mol. Sci, **17** (2016), No. 4,555, doi: 10.3390/ijms17040555.

[16] *S. Lu, V. B. Morris, V. Labhasetwar*, Codelivery of DNA and siRNA via arginine-rich PEI-based polyplexes, Mol Pharm. **12** (2015), No. 2, 621-9, doi: 10.1021/mp5006883.

[17] *L. Parhamifar, A. K. Larsen, A. C. Hunter, T. L. Andresen, S. M. Moghimi*, Polycation cytotoxicity: a delicate matter for nucleic acid therapy—focus on polyethylenimine, Soft Matter, **16** (2010), 4001-4009, doi: 10.1039/C000190B.

[18] *H. L. Jiang, M. A. Islam, L. Xing, J. Firdous, W. Cao, Y. J. He, Y. Zhu, K. H. Cho, H. S. Li, C. S. Cho*, Degradable Polyethylenimine-based gene carriers for cancer therapy, Top. Curr. Chem, **375** (2017), No. 2, doi: 10.1007/s41061-017-0124-9.

[19] *X. Zhou, B. Liu, X. Yu, X. Zha, X. Zhang, Y. Chen, X. Wang, Y. Jin, Y. Wu, Y. Chen, Y. Shan, Y. Chen, J. Liu, W. Kong, J. Shen,* Controlled release of PEI/DNA complexes from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine, J. Control, **121** (2007), No. 3, 200-7, doi: 10.1016/j.jconrel.2007.05.018.

[20] *J. Lisziewicz, D. I . Gabrilovich, G. Varga, J. Xu, P.D. Greenberg, S.K. Arya, M. Bosch, J. P. Behr, F. Lori, I*nduction of potent human immunodeficiency virus type 1-specific T-cell-restricted immunity by genetically modified dendritic cells, J. Virol, **75** (2001), No. 16, 7621-8, doi: 10.1128/JVI.75.16.7621-7628.2001.

[21] *K. Zhu, M. Wu, H. Lai, C. Guo, J. Li, Y. Wang, Y. Chen, C. Wang, J. Shi,* Nanoparticle-enhanced generation of gene-transfected mesenchymal stem cells for in vivo cardiac repair, Biomaterials, **74** (2016), doi: 10.1016/j.biomaterials.2015.10.010.

[22] *H. Sasaki, S. Yoshida, T. Kitahara, T. Yoshioka, H. Nakagawa, T. Nakamura, N. Ichikawa, K. Nishida, J. Nakamura, M. Nakashima,* Influence of disease stage on polyethylenimine-mediated plasmid DNA delivery in murine hepatitis, Int. J. Pharm, **318** (2006), No. 1-2, 139-45. doi: 10.1016/j.ijpharm.2006.03.027.

[23] *H.P. Song, J.Y. Yang, S.L. Lo, Y. Wang, W.M. Fan, X.S. Tang, J.M. Xue, S. Wang,* Gene transfer using self-assembled ternary complexes of cationic magnetic nanoparticles, plasmid DNA and cell-penetrating tat peptide,**31** (2010), No. 4, 769-78, doi: 10.1016/j.biomaterials.2009.09.085.

[24] *W.T. Godbey, K.K. Wu, A.G. Mikos,* Size matters: Molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle, J Biomed Mater Res, **45** (1999), No. 3, 268-75, doi: 10.1002/(sici)1097-4636(19990605)45:3<268::aid-jbm15>3.0.co;2-q.

[25] *M. M. Mady, W.A. Mohammed, N. M. El-Guendy, A. A. Elsayed*, Effect of polymer molecular weight on the DNA/PEI polyplexes properties, ROMANIAN J. BIOPHYS. **21** (2011), No. 2, 151-165.

[26] *S. Hou, N. Ziebacz, S.A. Wieczorek, E. Kalwarczyk, V. Sashuk, T. Kalwarczyk, T. S. Kaminskia, R. Holyst***,** Formation and structure of PEI/DNA complexes: quantitative analysis, Soft Matter, **7** (2011), 6967-6972.

[27] *M. Ogris, P. Steinlein, M. Kursa, K. Mechtler, R. Kircheis, E. Wagner*, The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells, Gene Ther, **5** (1998), No. 5, 1425-33. doi: 10.1038/sj.gt.3300745.

[28] *J. D. Ziebarth, D. R. Kennetz, N. J. Walker, Y. Wang,* Structural comparisons of PEI/DNA and PEI/siRNA complexes revealed with molecular dynamics simulations, Journal of Physical Chemistry B, **121** (2017), No. 8, 1941-1952, doi: 10.1021/acs.jpcb.6b10775.

[29] *S. Mahajan, & T. Tang,* Polyethylenimine-DNA Ratio Strongly Affects Their Nanoparticle Formation: A Large-Scale Coarse-Grained Molecular Dynamics Study, Journal of Physical Chemistry B, **123** (2019), No. 45, 9629–9640, doi: 10.1021/acs.jpcb.9b07031.

[30] *C. Iliescu, C. Marculescu, S. Venkataraman, B. Languille, H. Yu, G. Tresset,* On-Chip Controlled Surfactant−DNA Coil−Globule Transition by Rapid Solvent Exchange Using Hydrodynamic Flow Focusing, American Chemical Society, **30** (2014), No. 44, 13125-36, doi: 10.1021/la5035382.

[31] *G. Tresset, C. Iliescu,* Microfluidics-Directed Self-Assembly of DNA-Based Nanoparticles, Journal of Microelectronics, Electronic Components and Materials, **46** (2016), No. 4, 183 – 189.

[32] *Y. Yue, F. Jin, R. Deng, J. Cai, Y. Chen, M. C. Lin, H. F. Kung, C. Wu,* Revisit complexation between DNA and polyethylenimine - Effect of uncomplexed chains free in the solution mixture on gene transfection, J Control Release, **155** (2011), No. 1, 67-76, doi: 10.1016/j.jconrel.2010.10.028.

[33] *E. Haladjova, S. Panseri, M. Montesi, A. Rossi, A. Skandalis, S. Pispas, S. Rangelov,* Influence of DNA Type on the Physicochemical and Biological Properties of Polyplexes Based on Star Polymers Bearing Different Amino Functionalities, Polymers, **15** (2023), No. 4, 894-894, doi: 10.3390/polym15040894.

[34] *E. Haladjova, S. Halacheva, D. Momekova, V. Moskova-Doumanova, T. Topouzova-Hristova, K. Mladenova, J. Doumanov, M. Petrova, S. Rangelov,* Polyplex Particles Based on Comb-Like Polyethylenimine/Poly(2-ethyl-2-oxazoline) Copolymers: Relating Biological Performance with Morphology and Structure**.** s.l, Macromolecular Bioscience, **18** (2018), No. 4, doi: 10.1002/MABI.201700349.

[35] *E. Cojocaru, J. Ghitman, G. G. Pircalabioru, C. Stavarache, A. Serafim, E. Vasile, H. Iovu,* Electrospun nanofibrous membranes based on citric acid-foncionalized chitosan containing rGO-TEPA with potential application in wound dressings, Polymers, **14** (2022), 2, doi: 10.3390/polym14020294.

[36] *R. Kircheis, L. Wightman, E. Wagner***,** Design and gene delivery activity of modified polyethylenimines, Advanced Drug Delivery Reviews, **53** (2001), No. 3, 341-58, doi: 10.1016/s0169-409x(01)00202-2.