International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 5 No. 3 Mar. 2014, pp. 192-199

http://www.issr-journals.org/ijias/

In-vitro cytotoxicity of Polyethyleneimine on HeLa and Vero Cells

Edet Uwem Okon, Gihan Hammed, Poussy Abu El Wafa, Opetiti Abraham, Nicole Case, and Elecia Henry

Department of Biotechnology, School of Science at Medway, University of Greenwich,
London, United Kingdom

ABSTRACT: The success of gene therapy depends on the choice of a suitable vector that is biocompatible and efficient in delivering therapeutic DNA into disease cells. After more than two decades, such an ideal vector is still a wish. Viral vectors though naturally evolved to transfect cells are immunogenic. As alternatives, non-viral vectors such as polyethyleneimine have been exploited. We decided to investigate the *in-vitro* cytotoxicity of branched polyethyleneimine 800D, 25kD and linear 20kD on HeLa and Vero cells. At exponential phase, cells were exposed to polymers at concentration range of 0.5 to 1000mg/ml. Cells were MTT assayed after 24, 48 and 72hours for viability (IC_{50}). Linear PEI was less toxic than the branched PEI in both cells. The IC_{50} (mg/ml) values (Mean±SEM, n=6) post 72hours of PEI800D, 25kD and PEI20kD on HeLa cells were 2.42±0.22, 2.92±0.59, and 3.03±0.11 and for Vero cells, 7.42±0.29, 7.26±0.12, and 6.89±0.53 respectively. Two tailed t-test ($P<0.05$) of each polymer on both cells 72hours post dosing gave a significant P value of <0.0001. The results indicate that branched PEI800D, PEI25kD and linear PEI20kD are differently apoptotic to HeLa and Vero cells. The toxicity also time, cell line and concentration dependent. More research aimed at improving biocompatibility and transfection efficiency is needed.

KEYWORDS: HeLa, Vero, Polyethyleneimine, Poly L-lysine, Cytotoxicity, Dextran.

1 INTRODUCTION

The success of gene therapy depends on the choice of a suitable vector and there is no perfect vector. There are basically two privileged groups of vector commonly used in gene therapy clinical trials: viral and non-viral vectors [1]. Viral vectors have evolved over millions of years the capability to naturally infect all kinds of cells [3]. Despite the successes of viral vectors as seen recently with Glybera in 2012, there still exist limitations to the use of viral vectors in gene therapy. These include immunogenicity, insertional mutagenesis, safety profile of the vector in humans, difficult to produce viral titres, oncogenicity, and fear of germline line alteration which could be inheritable, low insert size and high cost [1], [2], [3], [4]. These ethical and safety concerns have led some investigators to consider non-viral vectors as alternative nano-delivery system.

1.1 NON-VIRAL VECTORS

Commonly used non-viral vectors are cationic lipids and polymers. Their use is based on their ability to interact with negatively charged DNA through electrostatic interaction. This leads to the formation of lipoplexes and polyplexes, respectively with a net positive charge which allows them to interact with the negative charge on the target membranes [1], [4]. They have outstanding ability to condense plasmid DNA and interact with cells [5]. Unlike their viral counterparts, they are easier to produce on a large scale, lower immunogenicity, larger insert up to 52 kilobases, possibility of selected modification to enhance vector carrying capacity, reduce toxicity, less ethical issues and acceptable cost[1], [4], [6]. Other classes include inorganic particles such as calcium phosphate, silica, gold particles and also physical methods such as electropororation and magnetofection [7].

Corresponding Author: Edet Uwem Okon (eu207@greenwich.ac.uk)

1.2 POLYETHYLENEIMINE

First introduced in 1995, polyethyleneimine (PEI) is a versatile non-viral vehicle that is widely studied [6]. It has a privilege place in gene therapy when compared to other polymers due to its potential for endosomal escape by proton sponge effect and higher transfection efficiency in a broad range of cell types [8]. PEI is a polymer of ethylene imine monomers that exists in mainly two forms: linear and branched molecule [6]. It can be synthesized in different lengths and also undergo functional group addition or substitution. Its polycationic nature enables it to complex DNA and form polyplexes at the appropriate nitrogen to phosphate ratios [6], [8]. However, it is also toxic like other cationic polymers. Its condensation with plasmid DNA or RNA is also believed to be majorly by electrostatic interaction between the negative phosphates on nucleic acids and positive groups of the amines [4], [6], [8].

Many factors have been shown to affect their degree of transfection and toxicity and they include degree of branching, molecular weight, ionic strength, zeta potential, concentration used and particle size [9], [10], [11]. Low molecular weight and moderately branched PEI has been shown by studies to be less toxic to cells and also have good transfection [12], [13]. Two types of toxicities have been reported in the use of PEI-mediated transfection: immediate toxicity associated with free PEI and delayed cytotoxicity associated with the PEI/DNA complexes [14]. Generally, it is assumed that the backbone linkages (carbon-carbon or carbon-amide bonds) are non-degradable at physiological pH and are resistant to systemic clearance, and accumulate in cells leading to further toxicity [15]. PEI has shown relatively low cytotoxicity when complexed with DNA and also higher transfection efficiencies significantly better than those observed with PLL and naked DNA [13].

The aim of this study was to establish the *in-vitro* cytotoxicity of branched polyethyleneimine (Br-PEI) molecular weight 800D and 25kD and linear polyethyleneimine (L-PEI) molecular weight 20kD on HeLa and Vero cells using poly L-lysine (PLL) and dextran as positive and negative controls respectively.

2 MATERIAL AND METHODS

2.1 MATERIALS

Multiskan EK plate reader and HERA cell 150i CO_2 incubator were supplied by Thermo Scientific Loughborough UK. Class II microbiological safety cabinet supplied by Envair, Lancashire UK. Sterile and non-pyrogenic tissue culture multi-well plates and 75cm^2 culture flasks obtained from Corning Incorporated, New York USA. Minimum Essential Media Eagle (MEM) and Dulbecco Modified eagle Medium (DMEM) were purchased from Life technologies Paisley, UK. Both media were supplemented by adding 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin and glutamine (P/S/G). The HeLa (NR1 CRL-13011™) and Vero (CCL-81™) cells were obtained from ATCC, Middlesex, UK. Thiazoyl blue tetrazolium with 98.5% bromide with CAS 298-93-1 were supplied by Sigma Aldrich USA. Dimethyl sulfoxide with CAS 6768-5 was supplied by Fisher Scientific Loughborough UK. PEI branched MW 25,000 with CAS 9002-98-6, PEI branched MW 800 with CAS 25987-06-8 and PEI linear MW 20,000 with CAS 764965-1G, Dextran molecular weight 35,000-45,000 with CAS 9004-54-0, and PLL MW > 30,000 with CAS 26124-78-7 were equally obtained from Sigma Aldrich, St. Louis USA. Industrial Methylated Spirit was obtained from Fisher Scientific, Loughborough, UK.

2.2 CELL CULTURE AND SEEDING OF PLATES

The HeLa and Vero cells were cultured using corning culture flasks with vented caps. The HeLa cells were grown on MEM and the Vero cells on DMEM both supplemented with 10%FBS and 1% penicillin, streptomycin and glutamine. The culturing and seeding were aseptically done in a class II safety cabinet. The flasks were kept at 37^{o}C in 5% CO_2 and the cells passaged every 2 to 3 days in order to keep the cells alive.

2.3 DOSING OF CELLS

Following the seeding of the well plates with both HeLa and Vero cell at density of 1x10^4, the plates were incubated at 37^{o}C in 5% CO_2 overnight. Serial dilutions of concentrations 1mg/ml, 0.5mg/ml, 0.1mg/ml, 0.05mg/ml, 0.01mg/ml, 0.005mg/ml, 0.001mg/ml, and 0.0005mg/ml were prepared with Br-PEI 800D, Br-25kD and L-PEI20kD, PLL and dextran respectively. The cell lines were then incubated with these dilutions for 24, 48 and 72hours for branched PEI 25kDa and dextran. While with Br-PEI800, L-PEI 20kDa and PLL, the cells were incubated for 24 and 48hours. Post 24, 48 and 72hours of dosing, MTT assay as briefly described below was then carried out.

2.4 MTT ASSAY

The MTT toxicity assay was then carried out on both HeLa and Vero cells after 24hours for testing the toxicity of PLL, dextran and the PEI both linear and branched. The first and last wells on each labelled columns and rows were left out since they are labile to evaporation. To each of the remaining 6 wells, 10□l of MTT was added and the mixture mixed by gently tapping. This was repeated to each of the culture well plates. The plates were then incubated at 37^{o}C and 5% CO_2 for 4 hours. After incubation, the MTT and the media were aspirated out completely, and 100□l of DSMO was added to the same wells to ensure the dissolution of the formed insoluble formazan crystals, and incubated for 30 minutes. After incubation, absorbance (OD_{540}) were taken and recorded.

2.5 STATISTICAL ANALYSIS

All readings for the toxicity were converted to cell viability and expressed as a percentage using an excel template. The cell viability graphs and analysis of IC_{50} values were done using Graphpad Prism6.0.

3 RESULTS

The toxicity recorded for both cells were plotted and analysed as shown in figures 1 -4 and tables 1-3. As shown in the graphs, our positive control dextran did not result in any significant toxicity to our cell lines. However, abnormal spikes were seen in figures 2, 4 and 5 for positive control dextran, and also in figures 1, 4 and 5 for the test polymers. We included them in our graphs because F-test did not result in any significance. The error bars in the graphs correspond to Mean±SEM. Generally, concentrations ≥ 2mg/ml of both negative control and our study test polymers were toxic to the cells.

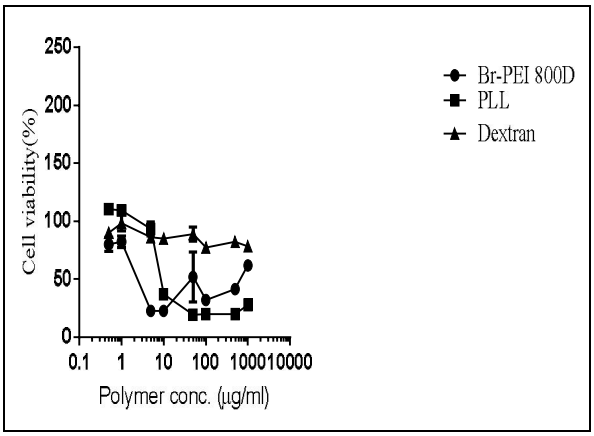

Fig. 1. HeLa cell viability after 72hours with Br- PEI 800D, dextran and PLL

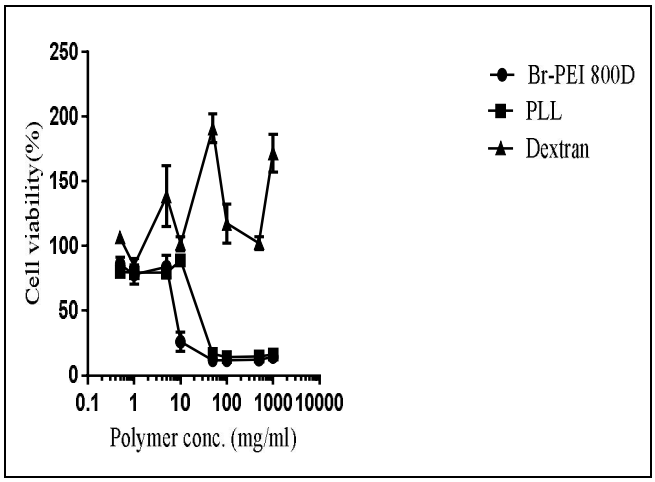

Fig. 2. Vero cell viability after 72 hours with Br- PEI 800D, dextran and PLL

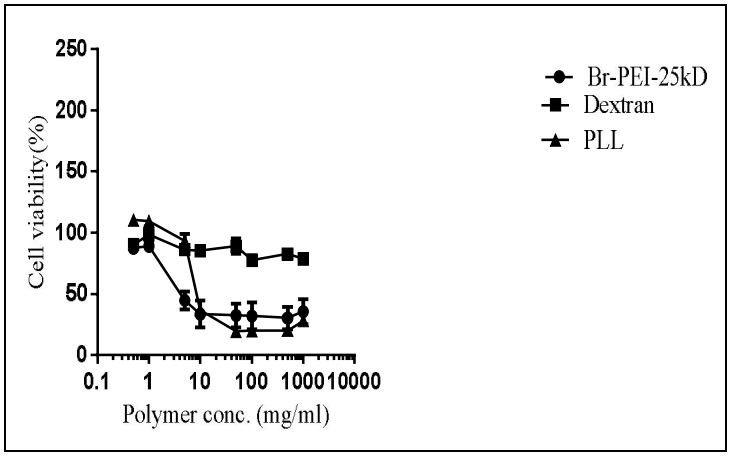

Fig. 3. HeLa cell viability after 72 hours with Br- PEI 25kD, dextran and PLL

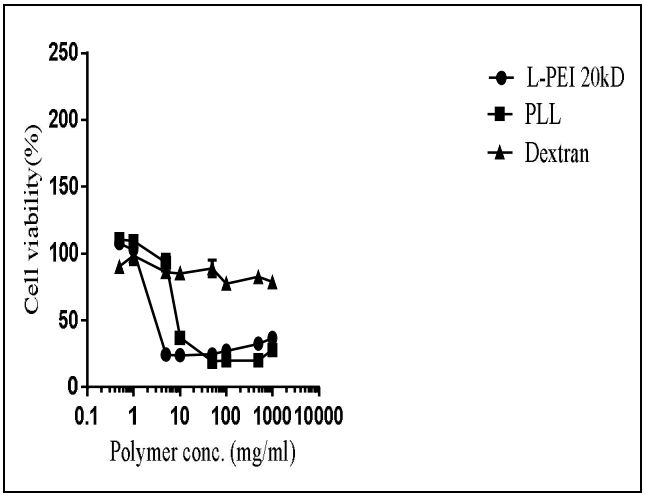

Fig. 4. HeLa cell viability after 72 hours with L-PEI 20kD, dextran and PLL

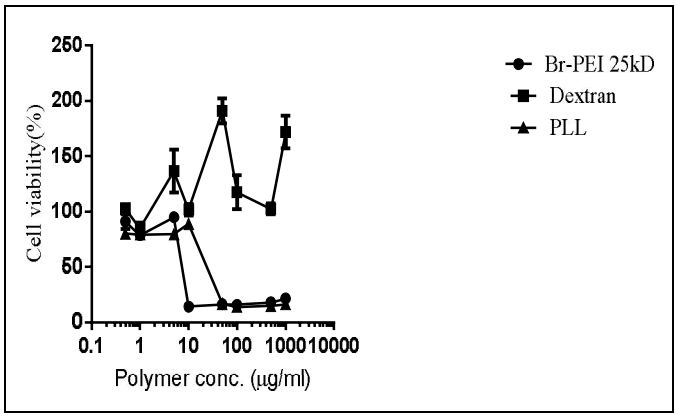

Fig. 5. Vero cell viability after 72 hours with Br- PEI 25kD, dextran and PLL

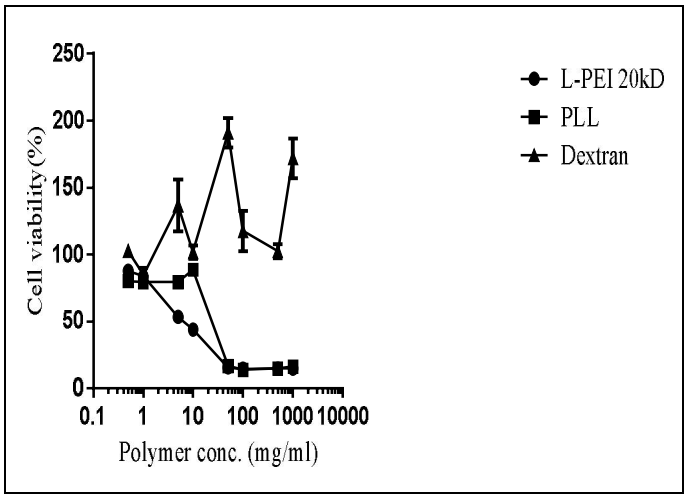

Fig. 6. Vero cell viability after 72 hours with L-PEI 20kDa, dextran and PLL

Table 1. T-test analysis of IC_{50} values for test and control polymers at various incubation times on HeLa and Vero cells

Cell line	Br-PEI800D	L-PEI20kD	Br-PEI25kD	Dextran	PLL
HeLa 24h	*3.957±0.219	29.70±1.910	9.930±0.416	> 1000	29.400±6.981
HeLa 48h	-	-	1.993±0.281	> 1000	-
HeLa 72h	2.422±0.216	3.033±0.110	2.917±0.590	> 1000	8.780±0.206
Vero 24h	2.762±0.157	66.830±3.707	26.850±9.763	> 1000	29.230±2.116
Vero 48h	-	-	7.325±0.2646	> 1000	-
Vero 72h	7.425±0.290	6.887±0.530	7.258±0.122	> 1000	24.350±0.917

**Cytotoxicity is expressed as an IC_{50} (g/ml) Mean±SEM, n=6.*

Table 2. T- test analysis of test polymers IC_{50} (µg/ml) at various incubation times on Vero and HeLa cells

Type of Polymer /Incubation time	Cell type	Difference of Mean±SEM	Significance At P<0.05	R^2	P Value
L-PEI 20KD 24 Vs 72hours	Vero	-59.950±3.745	Yes	0.962	<0.0001
L-PEI 20KD 24 Vs 72 hours	HeLa	-26.670±1.914	Yes	0.951	<0.0001
Br-PEI 800D 24 Vs 72hours	Vero	4.663±0.290	Yes	0.963	<0.0001
Br-PEI 800D 24 Vs 72hours	HeLa	-1.535±0.308	Yes	0.977	0.0006
Br-PEI 25kD 48 Vs 72hours	Vero	-0.067±0.291	No	0.005	0.8236
Br-PEI 25KD 24 Vs 72hours	Vero	19.590±9.764	No	0.287	0.0735
Br-PEI 25KD 24 Vs 48hours	Vero	19.530±9.767	No	0.286	0.0735
Br-PEI 25KD 24 Vs 72hours	HeLa	-7.013±0.719	Yes	0.905	<0.0001
Br-PEI 25KD 48 Vs 72hours	HeLa	0.9242±0.650	No	0.168	0.185
Br-PEI 25KD 24 Vs 48hours	HeLa	7.938±0.5017	Yes	0.967	<0.0001

Table 3. T- test analysis of test polymers on Vero and HeLa cells after 72hours of incubation

Types of Polymers	Cell types	Incubation time(hours)	R^2	*Diff in Mean±SEM	Significance at P<0.05	P Value
L-PEI20kD	HeLa Vs Vero	72	0.840	-3.853 ±0.539	Yes	< 0.0001
Br-PEI 800D	HeLa Vs Vero	72	0.960	5.003 ± 0.326	Yes	< 0.0001
Br-PEI 25kD	HeLa Vs Vero	72	0.840	-4.342 ± 0.599	Yes	< 0.0001

*n=6, degree of freedom = 10. * Represents the resulting IC_{50} (Difference in Mean±SEM) values in (µg/ml).*

Table 1 shows the IC_{50} values of the polymers for HeLa and Vero cells post 24, 48 and 72hours post dosing for test polymers, positive and negative controls. Dextrans gave a high IC_{50} values as our negative control. All three of our test polymers showed toxicity values much lower than that the negative control PLL in both cell lines meaning the test polymers were more toxic to than the positive and negative controls at concentrations and dosing duration used for this study. In table 2, t-test analysis of IC_{50} values (24 and 72 hours) after dosing for linear PEI, was significant (P<0.0001) with R^2 values of 0.962 and 0.951, respectively for Vero and HeLa cells. In the same vein, the branched PEI800D was also significant (P values = <0.0001, 0.0006) and (R^2 values = 0.963, 0.977) for Vero and HeLa cells respectively. On the other hand, our branched PEI25kD was not significant (P= 0.0735, R^2= 0.287) for Vero cell but was significant for HeLa cell (P<0.0001, R^2=0.905). For PEI800 also gave a significant value (P<0.0001, R^2= 0.967) for 48 and 72 hours post dosing, while others did not (see table 2).

A comparison of the IC_{50} values for both cells using only 72hours post dosing gave an significant value (P<0.0001, R^2 = 0.840, 0.960, and 0.840) for our PEI 20kD, 800D and 25kD respectively. This indicates that 72hours post dosing was consistently toxic to both cells as opposed to 24 and 48hours.

4 DISCUSSION

4.1 IN VITRO CYTOTOXICITY OF BRANCHED AND LINEAR PEI

Gene therapy it is thought will make the world a better place by helping treat and cure diseases as far as they have a molecular basis [16]. It has even been named as medicine of the future [17]. Since the characteristic of the coding and non-coding sequences of the human genome is different, there is no single effective vector that can be used to deliver all therapeutic DNAs into cells and tissues [1].

Immunological, safety and toxicity concerns limits the use of viral vectors despite their high natural transfection abilities in various cells [18], [19], [20], [21]. On this backdrop, safer alternatives have been investigated including PEI, PLL, and poly amidoamine and so on [22], [23]. PEI is one of the most promising non-viral vectors that have been widely studied since first introduced in 1995 by Boussif [6]. The main challenge still remains its high toxicity to cells [6], [23], [24], [25].

Branched PEI 25kD has been regarded as the gold standard but its transfection efficiency is marred by high toxicity to different cell lines [5], [18], [24], [25]. Linear PEI has been shown to be less toxic to cell but less efficient in delivering DNA to cells when compared to their branched counterparts [13], [18]. Studies have confirmed that the high charge density in the methylene ($-CH_2(CH_2)N(X)-$) backbone of branched PEI is proportional to their toxicity [26], [27]. Studies have also shown that PEI25kD branched is more toxic than linear PEI25kD in epidermal cell A431 [18] and this conforms to our values as represented in table 2 even though our linear PEI was 20kD and incubation time 72hours, the toxicity was roughly similar. PEI800 branched have been shown to cause unacceptable massive necrosis compared to linear 25kD, branched PEI1.8, PEI2 and PEI11 which showed more acceptable toxicity [28].

Our study confirms that Br-PEI800D, L-PEI20kD and Br-PEI25kD are indeed apoptotic agents as previously reported [6], [29]. The toxicity was however time, dose and cell line dependent. Br-PEI 800D and 25kD gave an IC_{50} values lower than the linear PEI 20kD in our study in both HeLa and Vero cells and this was consistent with that know fact that PEI toxicity depends on cell type and degree of branching[13], [18]. Omidi *et al* 2011 reported IC_{50} values of 37□g and 74□g for PEI25kD branched and linear on A431 cells. These were higher than our findings of post 24, 48 and 72 hours of dosing as shown in table 1. Roughly same IC_{50} values of 10□g have been reported for HeLa and 293T cells by Huang *et al*, 2010. The difference in toxicity seen in both cells in this study is probably due to the difference in physiology of both cells. HeLa cells are derived from

human cervical cancer cell and Vero gotten from normal kidney cell of African Green Monkey. The difference in toxicity in both cell lines and in different polymers indicates a high significance post 72hours for each polymer for both cells with $P<0.0001$ (table 3)

The toxicity of L-PEI20kD, Br-PEI 800D and 25kD is also dependent on time. For HeLa cell, all the polymers gave a significant increased toxicity post 24 and 72 hours incubation with polymers. On the other hand, Vero cells did show increased but insignificant toxicity with PEI25kD, significant increased toxicity with PEI800D and a significant reduced toxicity with linear PEI20kD.

5 CONCLUSION

The success of gene therapy is highly dependent on the choice of a suitable vector. An ideal vector should have high transfection and no toxicity to the target cells. So far these essential requirements have not been meet in both viral and non-viral vectors studied thus far. There may never be an ideal vector for shuttling all genes by gene therapy given the different biological behaviour of viruses and physicochemical properties of non-viral vectors. Our studies have shown that polyethyleneimine branched 800D and 25kD, and linear polyethyleneimine 20kD are indeed cytotoxic to HeLa and Vero cell lines. This toxicity was seen to be cell type, concentration, polymer and time dependent. Post doing time of 72hours appeared to be the most toxic duration with significant difference in both cell lines and all three polymers employed for this study. All three polymers appeared to be more toxic to HeLa than Vero cells with time.

Given the higher cytotoxicity seen in our study for Br-PEI800 and 25KD and the lower toxicity for L-PEI 20kD, their transfection efficiencies also needs to be studied properly in not just HeLa and Vero cell but also other clinically important cell lines. Studies have shown that polyethyleneimine can be made more biocompatibility with copolymers. Therefore, it is equally important to look at the toxicity profiles and transfection efficiencies with such copolymers such as poly ethylene glycol, chitosan and dextran.

ACKNOWLEDGEMENT

We sincerely acknowledge the unrelenting supervisory role played by Dr Simon Richardson S. W. during the entire duration of the project. This piece would not be possible without him. In the same vein, our sincere gratitude also goes to Fred Momoh, Dongchu, and Susan Shooters for their selfless commitment to our research work and encouragement during the project.

REFERENCES

[1] S. N. Uddin, Cationic lipids in non-viral gene delivery systems. *Biotechnology and Molecular Biology Review*, 2(3): 058-067, 2007.

[2] Panno, J. (2010). Viruses: Cornerstones of Gene Therapy. Gene Therapy, In: Gene Therapy Treatments and Cures for Genetic Disease. Rev. Ed. Bang Printing USA. pp. 55-71, 2010.

[3] T. W. Writh and Y. H. Seppo, History of gene therapy. *Journal of Gene***,** 525(2):162-169, 2013.

[4] M. Morille, C. Passirani, A, Vonarbourg, A. Clavreul and J. P. Benoit, Progress in developing cationic vectors for non-viral system gene therapy against cancer, *Biomaterials,* 29:3477-3496, 2008.

[5] F. W. Huang, H. Y. Wang, C. Li, H. F., Wang, Y. X. Sun and J. Feng, PEGylated PEI-based biodegradable polymers as non-viral gene vector, *Acta Biomaterialia,* 6:4285-4295, 2010.

[6] H. Lv, S. Zhang, B. Wang, S. Cui, and J, Yan, Toxicity of cationic lipids and cationic polymers in gene delivery, *Journal of Control Release*, 114:100-109, 2006.

[7] Gascón, Pozo-Rodríguez and Solinís,2013 Non-Viral Delivery Systems in Gene Therapy, Gene Therapy - Tools and Potential Applications, F. Martin (Ed.), ISBN: 978-953-51-1014-9, InTech, DOI: 10.5772/52704. Available: http://www.intechopen.com/books/gene-therapy-tools-and-potential-applications/non-viral-delivery-systems-in-gene-therapy, (July 7, 2013).

[8] C. Tros de Ilarduya, Y. Sun, and N. Duzgunes, Gene delivery by lipoplexes and polyplexes. *European Journal of Pharmaceutical Sciences*. 40:159-170.

[9] K. Kunath, D. von Harpe, H. Fisher, U. Petersen, K. Bickel, T. Voight and T. Kissel, Low molecular weight polyethyleneimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular polyethyleneimine, *Journal of Control Release*, 89:113-125, 2003.

[10] M. Lungwitz, M. Breunig, T. Blunk, and A. Go-pferich, Polyethylene mine based non-viral gene delivery systems, *Eur. J. Pharm. Bio pharm*, 60:247-266, 2005.
[11] Y. H. Kim, J. H. Park, M. Lee, Y. H. Kim, T. G. Park, and S. W. Kim, Polyethyleneimine with acid-labile linkages as a biodegradable gene carries, *Journal of Control Release,* 103:209-219, 2005.
[12] M. Breunig, U. Lungwitz, R. Liebl, C. Fontanari, J. Klar, and Kurtz A, et al,. Gene delivery with low molecular weight linear polyethyleneimine, *J. Gene Med*, 7(10):1287-98, 2005.
[13] W. T. Godbey, K. K. Wu, A.G. Mikos and G. J. Hirasaki, Improved packing of polyethyleneimine/DNA complexes increases transfection efficiency, *Gene Therapy,* 6:1380-1388, 1999.
[14] W. T. Godbey, K. K. Wu and A.G. Mikos, Size matter: molecular weight affects the efficiency of polyethyleneimine as a gene delivery vehicle, *J. Biomed. Mater.*R*es,* 45:268-275, 2001
[15] M. R. Park, K. O. Han, I. K. Han, M .H. Cho, J. W. Nah, Y. J. Choi, and C. S Cho, Degradable polyethyleneimine-alt-poly (ethylene glycol) copolymers as novel gene carriers, *Journal of Controlled Release,* 105:367-380, 2005.
[16] A. F. Vargas, The Human Genome Project and its importance in clinical medicine, *International Congress Series,* 1237: 3 –13, 2002.
[17] K. Lundstrom and T. Boulikas, Viral and Non-viral Vectors in Gene Therapy: Technology Development and Clinical Trials, *Technology in Cancer Research and Treatment*, 2(5): 471-485, 2003.
[18] O. Kafil and Y. Omidi, Cytotoxic Impact of Linear and Branched Polyethleneimine Nanostructures in A431 cells, *Bioimpacts,* 1(1):23-30, 2011.
[19] J. R. Goss, W. F. Goins, D. Lacomis, M. Mata, J. C. Gloriso, and D. J. Fink, Herpes simplex-mediated gene transfer of the nerve growth factor protects against peripheral neuropathy in streptozotocin-induced diabetes in the mouse, *Diabetes*, 51:2227-2232, 2002.
[20] M. Giacca and S. Zacchigna, Virus-mediated gene delivery for human gene therapy, *Journal of Controlled Release*, 161**:**377-388, 2012.
[21] S. Yla-Herttuala, Endgame: Glybera finally recommended for approval as the first therapy drug in the European Union, *Molecular Therapy,* 20: 1831-1832, 2012
[22] S. C.W. Richardosn, N. G. Pattrick, N. Lavignac, P, Ferruti and R. Ducan, Intracellular fate of bioresponsive poly(amidoamine) *in vitro* and *in vivo, Journal of Controlled Release*, 142**:**78-88, 2010.
[23] Y. J. Choi, S. J. Kang, Y. J. Kim, Y.B. Lim and H.W. Chung, Comparative studies on the genotoxicity and cytotoxicity of polymeric gene carriers polyethyleneimine (PEI) and polyamidoamine (PAMAM) dendrimers in Jurkat T-cells, *Drug and Chemical Toxicology*, 33(4):357-366, 2010.
[24] B.I. Florea, C. Meaney, H. E. Junginger, and G. Borchard, Transfection efficiency and toxicity of polyethyleneimine in differentiated Calu-3 and non-differentiated COS-1 cell culture, *AAPS Pharm. Sci.*, 4(3):E12, 2002.
[25] M. Turk, S. Dincer, I.G. Yulug and E. Piskin, In vitro transfection of HeLa cells with temperature sensitive polycationic copolymers, *Journal of Controlled Release,* 96:325-340, 2004.
[26] J. H. Jeong, S. H. Song, D.W. Lim, H. Lee, and T.G. Park, DNA transfection using linear poly(ethyleneimine) prepared by concentration acid hydrolysis of poly(2-ethyl-2-oxazoline), *Journal of controlled Release,* 73:391-399, 2001.
[27] M.P. Xiong, M.L. Forrest, G. Ton, A. Zhao, N.M. Davies and G.S, Kwon, ,Poly(aspartate-g-PEI800), a polyethyleneimine analogue of low toxicity and high transfection efficiency for gene delivery, *Biomaterials,* 28(32): 4889-4990,2007
[28] D. Fischer, T. Bieber, Y. Li, and H.P. Elasser, (1999). A novel non-viral vector for DNA delivery for DNA delivery based on low molecular weight, branched polyethyleneimine: effect of molecular weight on transfection efficiency and cytotoxicity, *Pharma. Res*., 16:1273-1279, 1999
[29] A.C. Hunter, Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv. Drug Del. Rev.,* 58:1523-1531, 2006.