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BRIEF ARTICLE

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Poly(ethylene imine) Nanocarriers Do Not Induce Mutations nor Oxidative DNA Damage in Vitro in MutaMouse FE1 Cells

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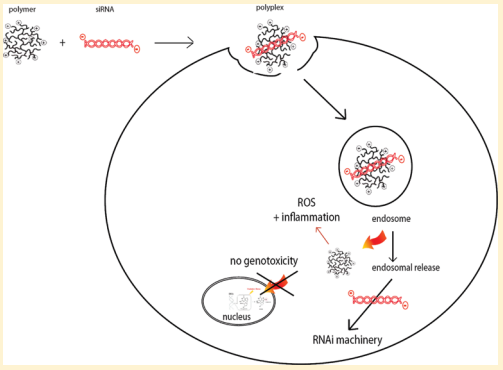
ABSTRACT: Genotoxicity information on polymers used for gene delivery is scant, but of great concern, especially when developing polymeric nanocarriers as nonviral vector systems for cancer treatment. The genotoxicity of some engineered nanomaterials, e.g., metal oxides like ZnO, TiO_2, and CuO but also carbon based materials like carbon black or nanotubes, has commonly been related to oxidative stress, and subsequent inflammation. Recent studies of poly(ethylene imine) (PEI)-based polymers, important nonviral vector systems for pDNA and siRNA, might raise concerns because of their toxic effects dominated by cellular oxidative stress and inflammatory responses, similar to the mentioned effects of engineered nanoparticles. In this study, we employed a FE1-MutaMouse lung epithelial cell line based mutation assay to determine the genotoxicity of three PEI-based polymers and nanosized zinc oxide particles (NZO), all of which have previously been shown to trigger oxidative stress and inflammation. In addition, oxidative DNA damage (8-OH-dG) in FE1 cells was assessed by ELISA. The well-known carcinogen benzo[*a*]pyrene (B[a]P) was used as positive control. FE1 lung epithelial cells were exposed for eight sequential 72 h incubations, and reporter-gene mutation frequency or 8-OH-dG formation was determined to assess mutagenicity and oxidative DNA damage, respectively. No cytotoxic effects were detected at the exposure levels examined, which are representative of PEI concentrations normally used in *in vitro* transfection studies. In contrast to B[a]P, neither PEI-polymers nor NZO showed any significant mutagenic activity or oxidative DNA damage in the exposed cells, although PEI-based polymers have been shown to generate significant levels of cellular stress and inflammatory responses. We suggest that the lack of any detectable mutagenic/genotoxic activity of the PEI-based polymers studied here is a crucial step toward a safe use of such nanocarriers in clinical trials.



KEYWORDS: poly(ethylene imine) (PEI), genotoxicity, oxidative stress, inflammation, FE1, MutaMouse

INTRODUCTION

Poly(ethylene imine) (PEI) has widely been used as nonviral gene carrier due to its capability to form stable nanocomplexes by electrostatic interactions with nucleic acids. The major drawback of PEI is its high toxicity due to aggregation of huge PEI clusters on the cell membrane, and its interaction with blood components.[1,2] To reduce the cytotoxicity of PEI, several studies have introduced modified PEIs such as block or graft copolymers containing cationic and hydrophilic nonionic components.[3,4] Copolymers of PEI and hydrophilic poly(ethylene glycol) (PEG) with various molecular weights and graft densities were shown to possess improved cytotoxicity and potential for DNA and siRNA delivery.[5−7] To the best of our knowledge, and despite the strong interaction of PEI with nucleic acids, no information is available about any mutagenicity or genotoxicity resulting from treatment with PEI polymers.

Nonviral vectors based on PEI usually contain an excess of free PEI that is not complexed with nucleic acids.[8] Therefore, it is of great interest to assess the ability of PEI polymers to induce DNA damage and gene mutations. In previous studies, we comprehensively analyzed the safety and biocompatibility of different PEGylated PEI polymers to be used for pulmonary applications in murine epithelial-like type II cells and alveolar macrophages.[9,10] We found that the degree of PEGylation correlated with cytotoxicity and the oxidative stress response, but not with proinflammatory effects. High doses of AB type copolymers with long PEG blocks caused severe membrane damage and significantly decreased the metabolic activity of lung cells. Moreover, AB type PEI copolymers with long PEG blocks significantly increased the release of oxidative stress related lipid mediators such as 8-isoprostanes (8-IP) and prostaglandin E2 (PGE_2) in a dose-dependent manner. In addition, cytokine profiling indicated high level releases of acute-phase cytokines such as TNF, IL-6, and G-CSF, but without any obvious structure−function relationship.[10]

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Recently, the potential genotoxicity of a different class of drug delivery vehicles, namely, that of solid nanoparticles, attracted more attention, since exposure to some of these unique nanomaterials has been associated with adverse health effects.[11] The limited information that is slowly emerging indicates oxidative stress as a major pathway driving the toxicity of many nanomaterials, finally leading to inflammatory responses, cytotoxicity or even genotoxicity[12]

We have recently shown that subtoxic doses of different PEI polymers induced oxidative stress in lung epithelial cells,[10] and this raises concerns since oxidative DNA damage has been implicated in induction of mutations and increased cancer risk.[13,14] In the present study we hypothesized that cellular stress caused by PEI treatment could alter the level of oxidized nucleosides and reporter gene mutation frequency in MutaMouse epithelial lung cells exposed *in vitro*.

More specifically, three different previously described PEI-based polymers which represent two specimens characterized by high oxidative potency, PEI(25) and PEI(25)-PEG(0.55)30, and one with comparable low oxidative potency PEI(25)-PEG-(2)10,[9,10] were investigated in the MutaMouse FE1 pulmonary epithelial cell line to determine their ability to elicit (i) *lacZ* transgene mutations and (ii) formation of 8-hydroxy-2′-desoxyguanosine (8-OH-dG), one of the most abundant oxidized nucleosides of DNA. Accumulation of 8-OH-dG could lead to increased genomic instability that in turn may result in the malignant phenotypic behavior of tumors (i.e., cancer).[15] The presence of oxidative 8-OH-dG DNA adducts can cause G:C to T:A transversions, or even cell death, unless the adduct is repaired before DNA replication, which would mainly occur by base excision repair. Nanosized zinc oxide (NZO) particles were included as reference material since they have previously been shown in related studies to induce oxidative stress.[9,10]

EXPERIMENTAL SECTION

Particles and Polymer Solutions. Benzo[*a*]pyrene (B[a]P) and nanosized zinc oxide (NZO) were purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Branched poly(ethylene imine) (PEI) with a molecular weight of 25 kDa (Polymin, water-free, 99%) was a gift of BASF (Ludwigshafen). The copolymers poly(ethylene imine)-*graft*-poly(ethylene glycol) (PEI-PEG) with a PEG content of approximately 50% (w/w) were synthesized as previously described[4,7] by grafting linear PEG of 0.55, 2 kDa onto branched PEI 25 kDa. These graft copolymers were designated using the following nomenclature: PEI(25k)-*g*-PEG(x)$_n$. The number in parentheses (25k or x, where x = 0.55 k, 2 k) represents the molecular weight of the PEI or PEG block in Da, and the index n is the average number of PEG blocks per PEI molecule. This number was calculated on the basis of ^{1}H NMR spectra as described previously.[4] Polymer dilutions were prepared in sterile, sodium chloride solution (150 mM) to obtain a final concentration of 0.5 μg/mL PEI.

FE1 MutaMouse Lung (MML) Cell Line. The development and characterization of the FE1MML epithelial cell line has been described previously.[16] The cells were cultured in DMEM/F12 (1:1) medium containing L-glutamine (Invitrogen, Gibco, Germany) supplemented with 2% FBS (Biochrom AG, Germany), 100 U/mL penicillin G (Biochrom AG, Germany), 100 mg/mL streptomycin (Biochrom AG, Germany), and 1 ng/mL human epidermal growth factor (Roche, Germany).

Cell Incubations for Analysis of Mutant Frequency (Repeated Dosing Scheme). The exposure setup was carried out as described by Jacobsen et al.[17] Briefly, 300,000 FE1MML cells were seeded in 10 mL of growth medium in 90 mm dishes (Corning, Sigma-Aldrich, Germany) and incubated at 37 °C and 5% CO_2 for 24 h before exposure. The negative controls were incubated in unaltered exposure medium, whereas the positive controls received 0.1 μg/mL B[a]P. The cells were incubated for 72 h, washed with phosphate-buffered saline (PBS), trypsinized, and centrifuged at 1200*g* for 5 min at 4 °C. After resuspension in medium, cells were counted, and 300,000 reseeded in a new dish. After 24 h, fresh serum-free exposure medium containing DMEM/F12 (1:1) supplemented with L-glutamine and human epidermal growth factor, with or without the test substance, was added. Following a 6 h treatment, the exposure medium was replaced by fresh growth medium. Cells were exposed for eight rounds to NZO or polymers, resulting in a total exposure time of (8 × 72 h) 576 h. Accordingly, the cumulative concentration added was 40 μg (8 × 0.5 μg × 10) for PEI polymers and 80 μg (8 × 1 μg × 10) for nanosized ZnO and 5 μg (5 × 0.1 μg × 10) for B[a]P. For each exposure round 0.5 μg of PEI, 0.1 μg of B[a]P and 1 μg of NZO was used, thus the average concentrations were 4 μg of (8 × 0.5 μg of) for PEI polymers and 8 μg of (8 × 1 μg) for NZO and 0.5 μg (5 × 0.1 μg) for B[a]P. B[a]P exposure was only repeated five times since at that time the progressive decline in cell growth yielded only 25% confluence compared to approximately 80% for all other samples. Therefore, these samples received fresh growth medium for the remaining three exposure rounds. After the eighth treatment, dishes were washed thoroughly with PBS to remove the excess of particles/polymers, trypsinized and reseeded without test substance for 72 h to allow for mutation fixation.

Isolation of High Molecular Weight DNA for Mutant Analysis. Cells were digested overnight in lysis buffer containing 10 mM Tris pH 7.6, 10 mM EDTA, 10 mM NaCl, 1 mg/mL proteinase K and 1% SDS. DNA was extracted and purified using chloroform/phenol extraction, and precipitation in ethanol as previously described.[16] Freshly isolated DNA was dissolved in TE buffer (10 mM Tris (pH 7.6), 1 mM EDTA) and stored at 4 °C for further analysis.

LacZ Mutation Analysis. Transgene mutant frequency was determined using the P-gal (phenyl-β-D-galactopyranoside) positive selection assay.[18] Briefly, *gal*E$^-$ host bacterium was used to facilitate isolation and enumeration of mutant copies of the *lacZ* transgene.[19] Briefly, λgt10*lac*Z DNA copies were rescued from genomic MutaMouse DNA using the Transpack lambda packaging system (Stratagene, La Jolla, CA). Packaged phage particles were mixed with the host bacterium (*Escherichia coli* Δ*lac*Z, *gal*E$^-$, *rec*A$^-$, pAA119 with *gal*T and *gal*K,[19,20] plated on minimal agar with 0.3% w/v P-gal (Sigma-Aldrich), and incubated overnight at 37 °C. Concurrent titers on nonselective minimal agar without P-gal were employed to enumerate total plaque-forming units (pfu). Mutant frequency was expressed as the ratio of the mutant plaques to total pfu.

8-OH-dG Enzyme-Linked Immunosorbent Assay. 8-OH-dG was determined using 8-OH-dG ELISA kit obtained from the Japan Institute for the Control of Aging (NIKKEN SEIL Co., Ltd.). DNA samples were directly used without further dilution, and the 8-OH-dG detection was carried out following the manufacturer's instructions.

Statistical Analysis. All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA 92037, USA),

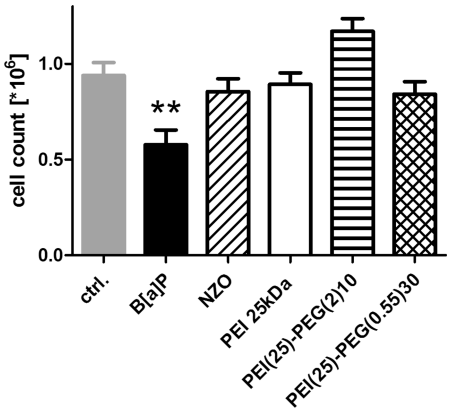

Figure 1. The effect on cell number of each test particle. Cell counts of cumulative exposures to B[a]P = 5 μg, NZO = 80 μg, and PEI polymers = 40 μg are shown. Values are mean ± SD (n = 10) taken following all 8 exposure rounds, plus a postexposure wash, dose is given as cumulative dose, and asterisks show statistical significance with $^{**}p < 0.01$ compared to negative control (ctrl).

except for mutant frequency (MF) data, which were analyzed by Poisson regression using SAS version 9.1 (SAS Institute, Cary, NC, USA). *LacZ* mutant frequency results were fit to the model $\log(E(Y_i)) = \log t_i + \beta \mathbf{x}_i$, where $E(Y_i)$ is the expected value for the ith observation, β is the vector of regression coefficients, $\mathbf{x}_i$ is a vector of covariates for the ith observation, and t_i is the offset variable used to account for differences in observation count period (i.e., pfu). The offset (i.e., natural log of pfu) was given a constant coefficient of 1.0 for each observation, and log−linear relationships between mutant count and test article concentration were specified by a natural log link function. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment. 8-OH-dG values are presented as mean ± standard deviation (SD) of at least three independent experiments, and significant differences between groups were evaluated using the Student's t test or one-way ANOVA, followed by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Several transgenic rodent models have been introduced to assess the mutagenic activity of selected test agents. The MutaMouse system, which contains 80 copies of a stably integrated $\lambda gt10lacZ$ shuttle vector in the mouse genome, is a straightforward and effective *in vivo* mutation assay system.[21] In this study we used the FE1MML cell line derived from pulmonary epithelium of the MutaMouse[16] to assess the mutagenic activity of NZO and PEI polymers *in vitro*. Compared to previous studies that employed single exposures, low particle/polymer concentration regimes were applied here, to ensure minor cytotoxicity and sufficient cell proliferation despite the substantial cumulative exposure.[10]

The morphology of the exposed FE1 cells was monitored using phase-contrast microscopy (data not shown), and cell counts were measured after each exposure over all treatment (Figure 1).

According to earlier studies, at low density, directly after reseeding and 6 h postexposure, the cells appeared well distributed with distinct subcellular inclusions, while at higher density (after 72 h exposure) the cells displayed less cytoplasm and formed a tight, uniform monolayer.[16] No difference in cell morphology was observed comparing treated and control cells.

Table 1 summarizes the *lacZ* mutant frequency for NZO and PEI-based polymers together with the positive control B[a]P. Whereas B[a]P exposure dramatically increased the mutant frequency (i.e., more than 40-fold increase vs negative control), exposure of FE1 cells to the PEI-based polymers and NZO did not elicit any statistically significant increase in *lacZ* mutant frequency.

Since the ability to generate cellular stress, like increased levels of proinflammatory cytokines, induction of oxidative stress genes and high levels of 8-isoprostane, has been described for PEI-based polymers as well as for NZO[9,10] (Table 2), we checked the effect of polymer treatment on DNA oxidation and 8-OH-dG formation using an ELISA technique. 8-OH-dG serves as pivotal marker for measuring the effect of endogenous oxidative damage to DNA. LC−MS/MS method is considered as the gold standard for determining 8-OH-dG. Nevertheless, we have used here the commercial 8-OH-dG ELISA which is considered as an improved version in terms of specificity, due to capacity reasons. In addition, overestimations of 8-OH-dG levels have been described as significant limitations of the ELISA method. This might however be of less concern here, since within our comparison only the positive control B[a]P, but neither any of the PEI polymers nor NZO showed significantly increased 8-OH-dG levels (Figure 2). In contrast the lower detection limit (0.125 ng/mL) might be seen as a weak point, which will not allow us to exclude the generation of low levels of 8-OH-dG by the polymer treatment.

In summary, we have evaluated for the first time the mutagenic properties of PEI-based polymers in a representative lung epithelial *in vitro* model. Since we have recently described proinflammatory and oxidative properties of these polymers, DNA modifications and mutations caused by subsequent oxidative stress could be expected (Table 2).[9,10] However, our results showed no indication that PEI polymers induce oxidative DNA damage or mutation in cultured murine cells. This outcome may be related to the relatively low single dosing applied here. But since the effects on cell viability and proliferation have to be considered as well, significantly higher doses would preclude the ability to carry out the required multiple rounds of exposure, which is needed to induce DNA damage. Nevertheless, the cumulative concentrations used in this experiment are in the same range of those normally applied for *in vitro* transfection experiments using PEI-based nonviral vectors. With regard to *in vivo* application of PEI-based nonviral vector systems, the undesired side effects identified *in vitro,* like acute inflammation and induction of oxidative stress, should be kept in mind due to possible secondary genotoxicity. Further improvement of these PEI-based carriers is needed to avoid the inflammatory and oxidative stress response and following secondary effects. In regard to therapy or animal experiments, the single dose of 0.5 μg/mL, used in the here described *in vitro* experiment, corresponds to a 0.25 μg/cm^2 dose, which relates to 0.06 mg/lung in small animals like mice,[22] which is in the range of therapeutically applied PEI doses with N/P ratios up to 20 when siRNA is administered in a range of 2−5 nmol.

Our results show that, despite the ability of PEI polymer treatment to cause distinct cellular stress responses like lipid peroxidation and respective gene expression in pulmonary epithelial cells,[9,10] the nucleus of these cells with its most vulnerable genetic information might still be guarded from oxidative DNA damage.

Table 1. The Effect of PEI-Based Nanocarriers on *lacZ* Mutant Frequency

replicate	total mutants	total plaques[a]	MF ($\times 10^5$)[b]	mean MF ($\times 10^5$)	SEM	fold change[c]
			Negative Control			
1	356	518785.2	68.5	67.1	1.8	N/A
2	387	599216.3	64.5			
3	179	250570.8	71.2			
4	354	573206.7	61.7			
5	416	570556.0	72.8			
6	269	396274.7	67.9			
7	389	512904.0	75.8			
8	247	360490.7	68.5			
9	251	404889.3	62.0			
10	248	430070.7	57.7			
			Positive Control (B[a]P)[d]			
1	7059	351627.5	2007.5	2709.5	360.0	**40.4**
2	9106	509259.3	1788.1			
3	3602	212301.8	1696.4			
4	2713	161607.8	1678.8			
5	3267	168483.0	1938.8			
6	4256	127894.7	3327.7			
7	4488	108677.3	4129.7			
8	4096	108014.7	3792.1			
9	5416	134521.3	4026.1			
			NZO 1 μg/mL[e]			
1	419	604103.5	69.3	60.4	2.4	0.9
2	401	589939.0	67.9			
3	311	550510.3	56.4			
4	424	659022.0	64.3			
5	426	662583.8	64.3			
6	101	208243.0	48.5			
7	200	418805.3	47.8			
8	324	505614.7	64.1			
9	334	561941.3	59.4			
10	323	522844.0	61.8			
			PEI(25) 0.5 μg/mL[f]			
1	423	571384.3	73.9	64.8	3.6	1.0
2	117	219922.5	53.2			
3	413	541730.0	76.1			
4	448	560450.3	79.9			
5	295	542475.5	54.4			
6	286	511578.7	55.9			
7	332	556640.0	59.6			
8	315	553989.3	56.9			
9	357	489048.0	73.0			
			PEI(25)-PEG(2)10 0.5 μg/mL[g]			
1	535	498739.5	107.3	74.0	4.6	1.1
2	369	476457.3	77.4			
3	372	479936.3	77.5			
4	375	559539.2	67.0			
5	434	543800.8	79.7			
6	448	579833.3	77.3			
7	240	382358.7	62.8			
8	258	355189.3	72.6			
9	318	473144.0	67.2			

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Table 1. Continued

replicate	total mutants	total plaques[a]	MF ($\times 10^5$)[b]	mean MF ($\times 10^5$)	SEM	fold change[c]
10	297	585797.3	50.7			
			PEI(25)-PEG(0.55)30 0.5 μg/mL[h]			
1	391	534192.2	73.1	74.6	5.3	1.1
2	232	454920.7	51.0			
3	391	382441.5	102.1			
4	363	480102.0	75.6			
5	387	376974.5	102.7			
6	343	552001.3	62.1			
7	243	409859.3	59.3			
8	387	556640.0	69.5			
9	342	453264.0	75.5			
10	363	481758.7	75.3			

[a] The total number of pfus screened. [b] *lacZ* mutant frequency. [c] Bold values represent statistically significant increases relative to the negative control ($p < 0.05$). [d] Poisson regression chi-square for test article effect = 124.1, $p < 0.0001$. [e] Poisson regression chi-square for test article effect = 3.62, $p = 0.06$. [f] Poisson regression chi-square for test article effect = 0.12, $p = 0.73$. [g] Poisson regression chi-square for test article effect = 1.85, $p = 0.17$. [h] Poisson regression chi-square for test article effect = 1.56, $p = 0.21$.

Table 2. Summary of Cellular Stress and Genotoxicity of NZO and PEI-Based Polymers[a]

	inflammation[9,10]	OxStress[9]	lipid peroxidation[10]	genotoxicity	
	cytokines	genes	8 IP	mutagenicity	8-OH-dG
NZO	CCL2, CXCL10	↑↑↑	↑↑	–	–
PEI(25)	IL1a, IL-6	↑↑↑	↑↑	–	–
PEI(25)-PEG(2)10	IL-6, CXCL1, −10, GM-CSF, TNF	–	–	–	–
PEI(25)-PEG(0.55)30	IL6, CXCL10	↑↑	↑	–	–

[a] The three PEI polymers represent two specimens characterized by high oxidative potency (indicated by one to three arrows), PEI(25) and PEI(25)-PEG(0.55)30, and one with comparable low oxidative potency PEI(25)-PEG(2)10 (indicated by "–"). Oxidative potency assessed by oxidative stress related gene expression[9] and 8-isoprostane formation.[10] All polymers induce inflammatory cytokine production in murine lung epithelial cells (LA4).[9,10] Nanosized zinc oxide (NZO) was used as positive control causing high levels of oxidative stress (indicated by arrows) and inflammation in murine lung epithelial cells (LA4). Neither NZO nor PEI treatment caused genotoxicity in FE1 pulmonary epithelial cells as indicated by "–".

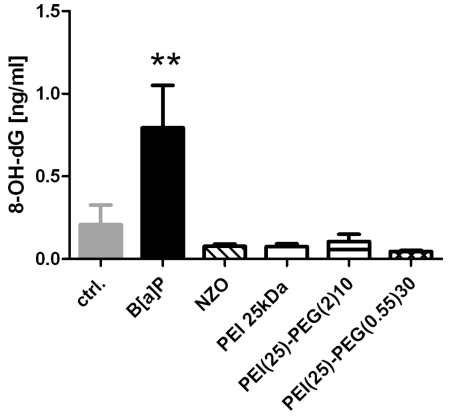

Figure 2. 8-OH-dG ELISA. DNA damage was analyzed using the 8-OH-dG ELISA. Values shown are mean ± SD ($n = 10$), asterisks showing statistical significance with $^{**}p < 0.01$ compared to negative control (ctrl).

Up to a certain degree, protection of the genome can be accomplished by constantly active DNA repair processes. In this context it might be noteworthy that induced expression of several DNA damage-inducible transcripts like *Ddit3*, *E2f1*, *Atm*, and *Ercc4* has been detected in our previous profiling study.[9] Similar to what is known for irradiation-induced DNA damage, also nanoparticle-driven oxidative DNA damage might be effectively counteracted by an endogenous collection of repair processes to prevent carcinogenesis pathways as far as possible.[23] However at high doses the repair system may be overburdened and fail. In contrast to the study from Sharma and colleagues,[24] who employed the comet assay to detect an increased DNA damaging potential for NZO at concentrations greater than 0.8 μg/mL in the human epidermal carcinoma cell line A431, in our settings NZO did not elicit any detectable genotoxicity.

According to earlier studies performed in different representative murine lung cell lines, the concentrations used in this study ruled out any cytotoxic effects.[9,10] Moreover, due to the repeated dosing scheme in this study, the cumulative concentrations obtained over the entire exposure rounds are within ranges for PEI-based nanocarriers and NZO that do not normally elicit any cytotoxic effects.[9,10] It was shown that modifying the PEI 25 kDa backbone reduces polymer cytotoxicity, but appears to be at the cost of its proinflammatory activity and increased oxidative stress responses, such as enhanced lipid peroxidation.[10]

Even though our results cannot definitively rule out the mutagenic activity of PEI-based polymers in general, we have provided clear evidence that even modified PEI polymers, with distinct pro-oxidative and proinflammatory potency, are not likely to constitute a genotoxic hazard.

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ABBREVIATIONS USED

B[a]P, benzo[*a*]pyrene; 8-OH-dG, 8-hydroxy-2′-desoxyguanosine; NZO, nanosized zinc oxide particles; pDNA, plasmid DNA; PEI, poly(ethylene imine); PEG, poly(ethylene glycol); siRNA, small interfering RNA

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