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In vitro and *in vivo* complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers

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ABSTRACT

Complement activation by polymeric gene and drug delivery systems has been overlooked in the past. As more reports appear in the literature concerning immunogenicity of polymers and their impact on gene expression patterns, it is important to address possible immune side effects of polymers, namely complement activation. Therefore, in this study the activity of low and high molecular weight poly(ethylene imine) and two PEGylated derivatives to induce complement activation were investigated in human serum. These *in vitro* results revealed that PEI 25 kDa caused significant and concentration dependent complement activation, whereas none of the other polymers induced such effects at their IC₅₀ concentrations determined by MTT-assays. To verify these *in vitro* results, additionally, studies were carried out in a swine model after intravenous administration, showing complement activation-related pseudoallergy (CARPA), reflected in symptoms of transient cardiopulmonary distress. Injections of PEI 25 kDa or PEI(25k)–PEG(2k)₁₀ at a dose of 0.05 and 0.1 mg/kg caused strong reactivity, while PEI 5 kDa and with PEI(25k)–PEG(20k)₁ were also reactogenic at 0.1 mg/kg. It was found that PEI 25 kDa caused both self- and cross-tolerance, whereas the PEG–PEIs were neither self- nor cross-reactively tachyphylactic. As a result of this study, it was shown that PEGylation of polycations with PEG of 20 kDa or higher molecular weight may be favorable. However, potential safety concerns in the development of PEI-based polymeric carriers for drugs and nucleic acids and their translation from bench to bedside need to be taken into consideration for human application.

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1. Introduction

Complement-activating properties of liposomes are well known under *in vitro* [1] and *in vivo* [2] conditions. Concerning polymeric vectors for delivery of nucleic acids, however, more than a decade after the first report of *in vitro* complement activation by the

polyamine poly(ethylene imine) (PEI) [3], still no *in vivo* data can be found in the literature. PEI enjoys great popularity for both *in vitro* and *in vivo* transfections [4], despite of an increasing amount of mechanistic studies on toxicity of polymers [5–9] indicating that non-viral vector systems can no longer be considered as inert substances. Complement (C) activation, one of the so far underestimated adverse effects of PEI, represents an important barrier for *in vivo* application, especially after systemic administration. To date, complement activation has only been investigated *in vitro* [3]. It was shown that free PEI polymer, in contrast to poly(amido amine) (PAMAM) dendrimers and poly(L-lysine) (PLL), exerted comparable complement activation to PEI complexes formed with plasmid DNA [3]. Extensive PEGylation, on the other hand, significantly reduced consumption of complement factors *in vitro* [10]. In

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a different study, surfaces with hydroxyl- and methyl-groups were shown to be more potent complement activators through the alternative pathway than surfaces bearing nucleophilic amino groups [11]. This was attributed to the ability of PEI to bind negatively charged serum proteins, which shielded the surface and made it unavailable for C3b access. PEGylation is generally believed to sterically protect nano objects against recognition by the immune system leading to long circulating devices [12]. The shielding mechanism of PEG is explained by the hindrance of protein adsorption onto the surface and subsequent interaction with macrophages [13–15]. However, PEG was recently reported to cause accelerated blood clearance (ABC) after repeated applications in rats and rhesus monkeys [16] due to IgM induction [17] and subsequent C3-mediated C activation [18]. These results indicated, however, that PEGylated liposomes did only activate the complement system due to IgM recognition but not in the first injection, whereas in other studies negatively charged, PEGylated liposomes, including DoxilTM were found to be potent C activators [19,20].

Since C activation can lead to transient cardiopulmonary derangement in experimental animals and men, it is one of the most severe adverse immune effects of such drug carriers. Measurable effects involve pulmonary hypertension, systemic hypotension and blood cell changes such as leukopenia with subsequent leukocytosis. As the potential *in vivo* C activation by liposomes is well known, the goal of the present study was to explore and correlate C activation by PEI-based PEG-free and PEGylated polymers both under *in vitro* and *in vivo* conditions. The formation of the C terminal complex (SC5b-9) in human serum was used as a marker of *in vitro* activation, and cardiopulmonary changes in pigs after intravenous injection served as endpoints of C activation-related *in vivo* toxicity. The effects of two PEI polymers, a high molecular weight (25 kDa) and a low molecular weight (5 kDa) construct, were tested. In addition, two derivatives of PEI 25 kDa with varying poly(ethylene glycol) (PEG) chain length (2 kDa and 20 kDa) and different grafting degrees (10 and 1, respectively) but constant PEI content were investigated. Structure-activity relationships were examined concerning polymer architecture and reactive surface groups.

2. Materials and Methods

2.1. Polymers

Branched poly(ethylene imine) with a molecular weight of 25 kDa (PEI 25 kDa, PolymintTM) and a molecular weight of 5 kDa (PEI 5 kDa) were gifts from BASF (Ludwigshafen, Germany). The block copolymers poly(ethylene glycol)-graft-poly(ethylene imine) PEI(25k)-PEG(20k)₁ and PEI(25k)-PEG(2k)₁₀ were synthesized as described earlier [21]. Polymer solutions were prepared in endotoxin-free water and plastic ware (Pyroquant Diagnostik GmbH, Mörfelden-Walldorf, Germany). Schematic structures of the tested polymers and block copolymers are shown in Fig. 1A.

2.2. USP bacterial endotoxins test

The Limulus Amoebocyte Lysate (LAL) test kit PyrotellTM, Control Standard Endotoxin (CSE) and USP Sterile Water for Injection or Irrigation (WFI) were a gift from Pyroquant Diagnostik GmbH, Mörfelden-Walldorf, Germany. The assay was performed according to the manufacturer's protocol. Briefly, polymer solutions were diluted to 10 mg/ml with WFI of which 100 µl was mixed with 100 µl reconstituted Pyrotell in the designated glass test tubes. A positive control of 2λ Control Standard Endotoxin (CSE) and a negative control of WFI were treated like the polymer samples. The mixtures were incubated for 60 min at 37° C before the test tubes were flipped upside down and checked for gel retention at the bottom of the test tube.

2.3. *In vitro* complement assay

The ELISA-based method for quantification of serum S-protein-bound C terminal complex (SC5b-9) and levels of the catalytic subunit of Complement factor B (Bb) was performed as described earlier [22,23]. In brief, the test polymers, at the concentration determined to be the IC₅₀ value (see Supporting Information), and control compounds were incubated with different human sera for 45 min at 37° C.

After terminating the reaction by adding chilled specimen diluent (provided in the ELISA kits) at a 20-fold volume, samples were tested for SC5b-9 and Bb levels using the respective ELISA kits (TCC and Bb kits, Quidel Co, San Diego, CA), following the manufacturer's instructions. All reactions were tested in duplicates.

2.4. *In vivo* tests of complement-mediated cardiopulmonary reaction

Experiments using Yorkshire swines of both genders (5 male and 8 female, 24–29 kg) were performed in accordance with the guidelines of the Ethical Committee of Semmelweis University, Budapest, Hungary. After premedication with Ketamine (500 mg i.m.), the animals were anesthetized with sodium pentobarbital (25 mg/kg i.v.) and then maintained under anesthesia with 5–10 mg/kg/h. Intubation was carried out with a 6.5 Fr tracheal tube to maintain free airways, and to enable controlled ventilation if needed. A catheter was introduced into the left femoral artery for systemic arterial pressure (SAP) measurement. An intravenous line was established at the left femoral vein for fluid supplementation with saline and administration of subsequent doses of the anesthetics as necessary. To measure pulmonary arterial blood pressure (PAP), a Swan-Ganz catheter was introduced into the right external jugular vein and led into the pulmonary artery via the right atrium and ventricle. Test materials at doses of 0.05–0.5 mg/kg were injected through the Swan-Ganz catheter into the pulmonary circulation as a 5 ml bolus. Between injections of test and/or reference material, a resting period of at least 20 min was maintained. SAP, PAP and standard ECG leads I, II, and III were recorded continuously with an ADInstruments PowerLab Recorder at a sampling rate of 2000 Hz and were evaluated using LabChartTM Pro v.6 software (ADInstruments, Bella Vista, NSW, Australia).

3. Results and discussion

Based on literature data reporting that positively charged liposomes were able to activate the alternative pathway of the complement system [1], and PEI consumed complement factors under *in vitro* conditions [3], it was hypothesized that colloidal, xenotropic, positively charged polymers could be potent C activators as well depending on their structure and degree of PEGylation. Positively charged PEI complexes are known to bind bovine serum albumin [24] and complement factors [3,10] which in turn can trigger the activation of complement cascades. Subsequent opsonization [3] and the lack of stability of PEI- and PEG-PEI-complexes with nucleic acids upon intravenous application supposedly explained their low and organ-specific transfection efficiency *in vivo* [24,25]. Due to their electrostatic mechanism of assembly, (PEG-)PEI complexes with nucleic acids are always present as equilibrium of free polymer, free nucleic acids, and associated polymer-nucleic acid-complex [26]. This equilibrium is rearranged upon changes in ionic strength and causes disassembly of polyplexes in presence of serum [24]. The presence of free polymer in serum-containing samples may also explain why no differences between PEI polymer and PEI complexes were found in the *in vitro* complement assay described by Plank et al. [3]. Therefore, in this study, the behavior of free polymers was investigated as “worst-case-scenario”.

3.1. Measurement of endotoxins

LPS contamination as cause of *in vitro* C activation was ruled out on the basis that none of the polymer samples induced formation of stable gels in the LAL assay. The validity of the test was corroborated by positive control samples. The concentration of endotoxins in all polymer samples was below the minimum detection limit of 0.03 Endotoxin-Units (EU) per ml.

3.2. *In vitro* complement assay

To determine *in vitro* C activation by the polymers, ELISA measurement of SC5b-9 formation were performed in 6 different healthy normal human sera (NHS). In 3 of these sera, 80 µg/ml unmodified PEI 25 kDa caused significant elevation of SC5b-9 over PBS baseline, while the other polymers did not exhibit activity

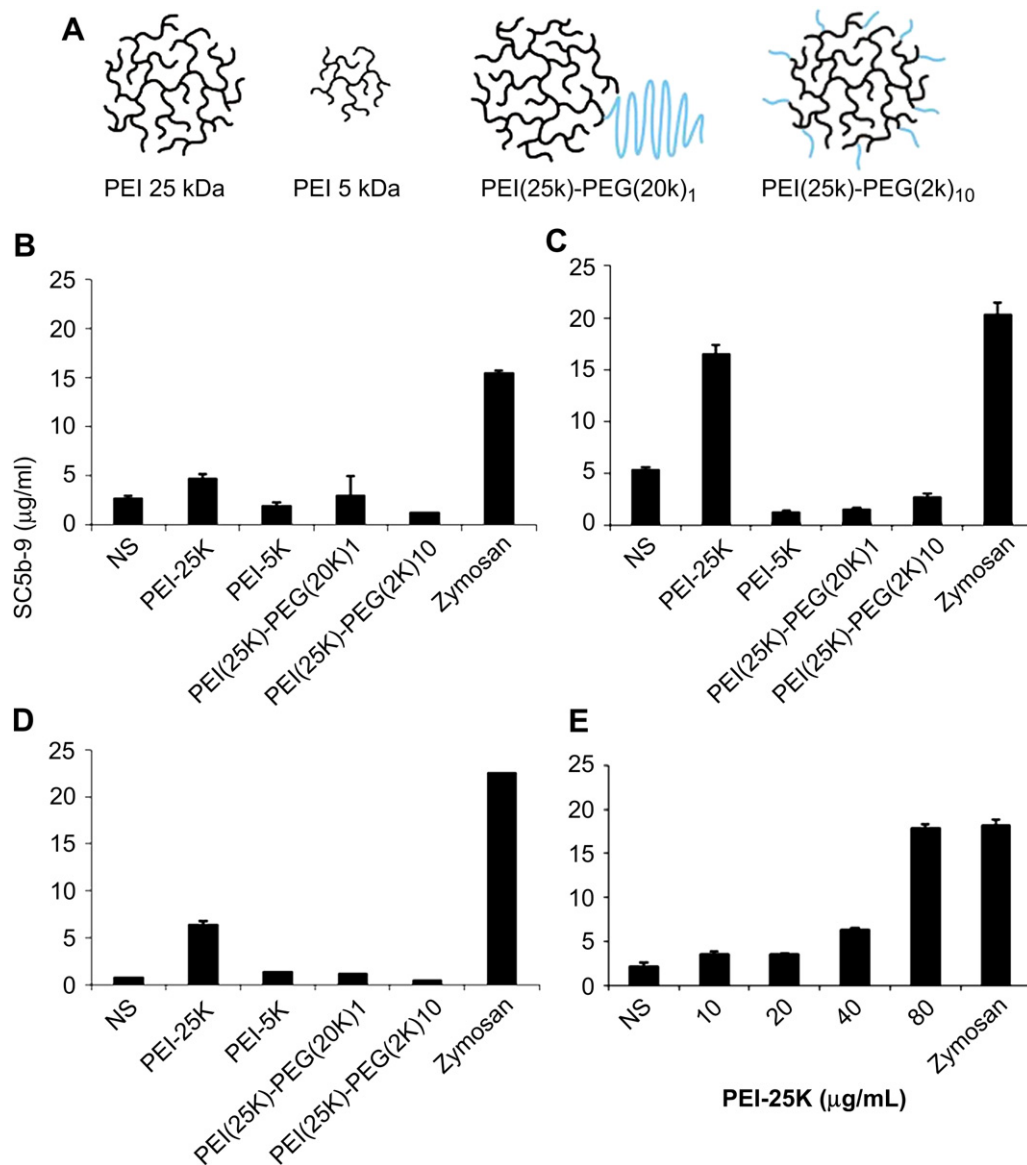


Fig. 1. A) Schematic composition of the polymers tested. B–E) *In vitro* complement activation through the alternative pathway by the IC₅₀ concentrations of the above polymers clearly differed depending on the serum. Unmodified PEI 25 kDa, however, elicited the highest activity in all sera while decrease in molecular weight and PEGylation caused a decrease in immunogenicity.

(Fig. 1B–D). Based on these measurements, the individual variation of SC5b-9 increase ranged approximately between 2 and 10-fold. As shown in Fig. 1E, the effect of PEI 25 kDa was dose-dependent in the range between 20 and 80 μg/ml. In one out of 6 sera, only PEI 5 kDa caused significant C activation (3-fold increase compared to PBS) at a 30-fold higher dose than PEI 25 kDa (2.5 mg/ml, data not shown), indicating that PEI 5 kDa can be reactogenic as well, but at a much higher dose. In 2 out of 6 sera, none of the polymers caused significant C activation (data not shown), although the positive zymosan control was active. These results indicate that first of all, PEI 25 kDa and PEI 5 kDa can activate C in certain normal human sera, while their PEGylated derivatives were inactive, at least in the number of subjects tested. These findings confirm results from an *in vitro* assay based on antibody-sensitized sheep erythrocytes, where dense PEGylation of PEI resulted in decreased complement activation [10]. Secondly, PEI 25 kDa had a greater activity than PEI 5 kDa, and thirdly, there is significant individual variation of reactivity toward these polymers. This substantial individual variation

is consistent with previous data on liposomal complement activation in multiple normal human sera [19,27]. In analogy to PEGylated “stealth” liposomes, PEG–PEIs were developed to decrease the self-aggregation and interaction of PEI with proteins in biological fluids and to increase salt and serum stability by steric stabilization [28]. Additionally, PEGylated and transferrin-modified polyplexes were both claimed to circulate longer than unmodified polyplexes [29,30] based on shielding effects of transferrin and PEG. These shielding effects were thought to prevent the opsonization of nanoparticles by complement components, resulting in lower recognition and phagocytotic capture by cells of the reticulo endothelial system (RES), and hence reduced clearance from the circulation [31]. By contrast, polyplexes of DNA [25,29] or siRNA [24] with PEG–PEI of low graft density (up to 10 PEG chains per PEI) did not display elevated blood levels compared to unmodified PEI which was explained by instability of the complexes. However, complement activation *in vivo* has so far not been investigated experimentally.

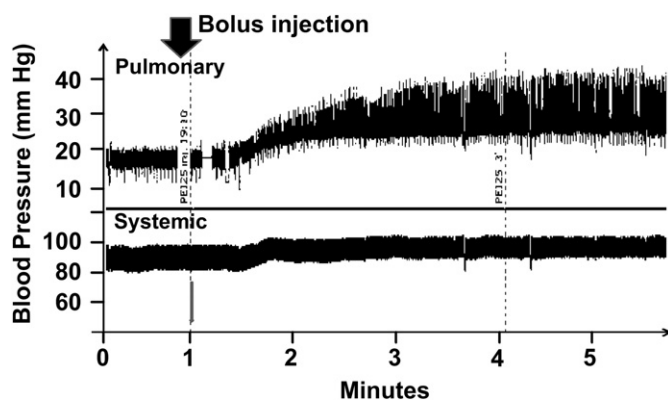


Fig. 2. Blood pressure changes following the administration of 0.05 mg/kg PEI 25 kDa. The systolic pressure increased more strongly than the diastolic, resulting in a significant rise of both the mean PAP and the pulmonary pulse pressure. The systemic blood pressure showed only a minor change in this case.

3.3. Polymer-induced hemodynamic changes in pigs

To explore the occurrence and *in vivo* consequences of C activation by the polymers tested in this study, hemodynamic alterations in pigs were evaluated that were previously observed following i.v. administration of C activator substances, such as liposomes [23,32–34]. Experiments in 13 pigs revealed that all polymers were active in terms of causing significant hemodynamic changes compared to baseline, including a rise of PAP, rise or decline of SAP, rise of heart rate and major ECG alterations. Fig. 2 illustrates the first minutes of pulmonary blood pressure changes following systemic administration of 0.05 mg/kg PEI 25 kDa, wherein the PAP rose within a minute to the 2-fold of its original value. The systolic pressure increased more strongly than the diastolic, resulting in a significant rise of both the mean PAP and the pulmonary pulse pressure. The systemic blood pressure showed only a minor change in this case. Fig. 3 shows details of a stronger reaction to 0.1 mg/kg PEI 25 kDa, wherein the PAP rose up to 300% of the initial mean PAP, the SAP decreased by 70%, and the heart rate increased by 50%, reaching a peak value 5–10 min after the injection and returned to the baseline 30 min after treatment. The initial biphasic changes of PAP, a typical phenomenon in the case of strong hemodynamic response, can be explained by the shortly delayed temporary decrease of the SAP. The figure also shows that repetition of the

same bolus injection after regaining the baseline of PAP and SAP values led to significantly less effects compared to the first bolus, indicating tachyphylactic response.

Table 1 summarizes the PAP and SAP changes after the first bolus injection of different polymers. Similar to *in vitro* conditions, there were substantial individual differences in cardiovascular responses. Therefore, the differences among the maximal rise of PAP and rise or fall of SAP caused by the four different polymers at 0.1 mg/kg dose were not significant (one-way analysis of variance, $p = 0.59$). Table 1 also shows that in 2 animals in which 0.05 mg/kg injections of PEI 25 kDa or PEI(25k)–PEG(2k)₁₀ was tested mild, yet clear reactions were observed, suggesting that the minimal effective dose is even below 0.1 mg/kg. The variability of PAP responses to different polymers is consistent with the substantial variation in the clinical picture of human hypersensitivity responses to allergens. Its underlying factors are not known at present. It limits our capability to draw fine qualitative comparisons of the reactions of different polymers and correlation with *in vitro* complement activation, however, clear distinction among non-reactivity and minor and severe reactivity are possible, which represents the information of value in this study.

In addition to the PAP and SAP changes shown in Figs. 2 and 3 and Table 1, tachycardia (up to 50%) and ECG changes were observed, mainly ST-T elevations and extrasystoles, as further manifestations of hypersensitivity reaction (data not shown). Furthermore, as shown in Fig. 4, at the end of each *in vivo* experiment, in a final injection, the effects of zymosan (0.5 mg/kg) as positive control were tested. Without exception, this treatment caused major reactions (up to 300% rise in PAP, 60–80% change of SAP), proving the functional integrity of the effector arm of immunologically triggered cardiovascular reactions. Bolus “vehicle” injections of the solvent for polymers (PBS) were also tested at the beginning of the experiments and did not cause significant changes in blood pressure in any of the animals (data not shown).

3.4. Tachyphylaxis and cross-tolerance of hemodynamic changes caused by different polymers

As illustrated in Fig. 3, despite all treatment remaining the same, the second bolus of PEI 25 kDa caused less hemodynamic changes

Table 1
Blood pressure changes in pigs following i.v. administration of PEI polymers.

Polymer	No	Dose (mg/kg)	PAP			SAP		
			Max. Change (%)	Mean	SD	Max. Change (%)	Mean	SD
PEI 25 kDa	1	0.05	123			106		
	2	0.1	328			15		
	3	0.1	292			111		
				248	109		77	54
PEI 5 kDa	4	0.1	110			103		
	5	0.1	598			53		
	6	0.1	263			114		
				324	249		90	32
PEI(25k)–PEG(20k) ₁	7	0.1	157			108		
	8	0.1	233			130		
	9	0.1	140			109		
	10	0.1	125			107		
				164	48		115	13
PEI(25k)–PEG(2k) ₁₀	11	0.05	132			125		
	12	0.1	95			91		
	13	0.1	392			120		
				206	162		112	18

Maximum changes expressed as % relative to baseline. PAP, pulmonary arterial pressure; SAP, systemic arterial pressure, both measured as mm Hg, relative to baseline. ANOVA showed no significant differences among the four treatments. Injection of the vehicle alone caused <5% changes in blood pressures.

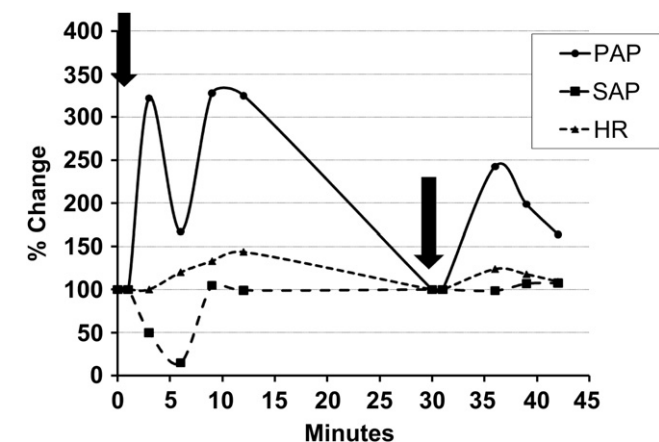


Fig. 3. Normalized changes in pulmonary arterial pressure (PAP), systemic arterial pressure (SAP), and heart rate (HR) in % following sequential injections of 0.1 mg/kg PEI 25 kDa.

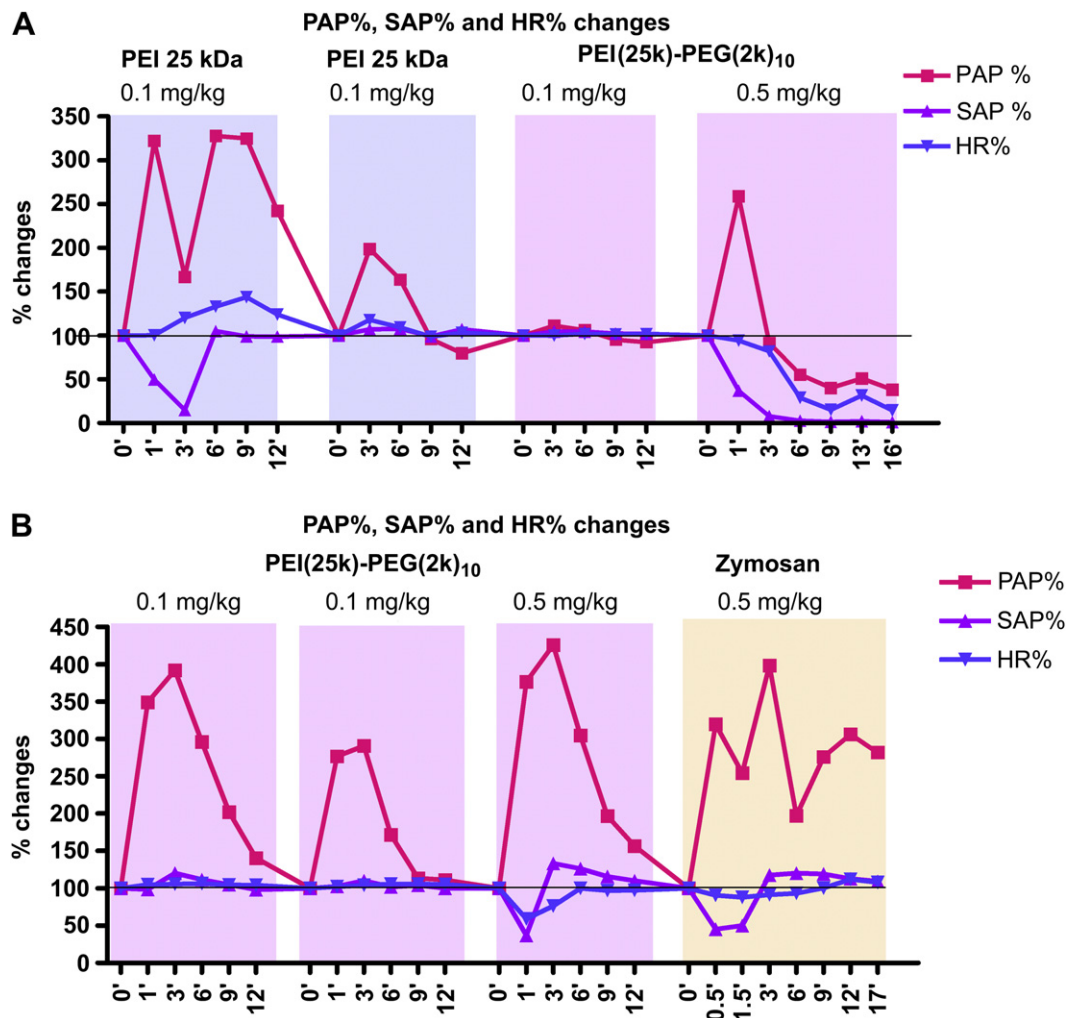


Fig. 4. Changes of pulmonary arterial pressure (PAP), the systemic arterial pressure (SAP), and heart rate (HR) after serial injection of PEI 25 kDa, PEI(25k)–(PEG2k)₁₀, and Zymosan. The percent changes of mean values are plotted following the copolymer injections (time in minutes).

than the first, implying tachyphylaxis. Fig. 4 shows further features of tolerance induction by PEI polymers, i.e., cross-tolerance between unmodified and PEGylated polymers (Fig. 4A) and breaking the tolerance by increasing the dose (Fig. 4B). Interestingly, as shown in Fig. 4A, the highly reactogenic first dose of 0.1 mg/kg PEI 25 kDa caused a tolerating effect of the animal, but not only toward a second similar dose of PEI 25 kDa but also toward PEI(25k)–PEG(2k)₁₀. This observation is in contrast to the non-tachyphylactic effect of PEI(25k)–PEG(2k)₁₀ itself, as shown in Fig. 4B, and suggests that PEGylation has no extra reactivity on top of PEI 25 kDa, but tolerance of any of the PEI-based polymers involves dysfunction of PEI-recognition at some, yet unknown step in the reaction chain. The breaking of the tolerance by a five-fold-increased dose of PEI(25k)–PEG(2k)₁₀ in Fig. 4B indicates that the induction of tolerance is most importantly a strongly dose-dependent phenomenon.

Fig. 5 summarizes the information gained on the tachyphylactic effect of different polymers. In the case of non-PEGylated PEI (Fig. 5 A, B), the pulmonary hypertensive effects were tachyphylactic in 4 of 5 animals when a dose of 0.1 mg/kg was repeatedly administered. The molecular weight of PEI was not critical, as tachyphylaxis was seen both with PEI 25 kDa (Fig. 5A) and PEI 5 kDa (Fig. 5B). In the case of PEGylated derivatives, tachyphylaxis was not seen in any of 3 animals treated with a dose of 0.1 mg/kg PEI(25k)–PEG(20k)₁

(Fig. 5C), while tachyphylaxis of PEI(25k)–PEG(2k)₁₀ was present in 1 of 3 animals tested (Fig. 4B and Fig. 5D). These observations suggest that PEGylation with long polymer chains (20 kDa PEG) might possibly modify the tolerance of PEI polymers, while short polymer chains (2 kDa PEG) at a higher graft density show less influence in this regard. Overall, the available data pertaining to the tachyphylactic nature of PEI-induced hemodynamic changes suggest that:

- (1) tachyphylaxis is induced by unmodified PEI independent of the molecular weight,
- (2) PEGylation seems to influence tachyphylaxis to some degree depending on graft density and molecular weight of PEG,
- (3) tachyphylaxis is dose-dependent and may be overcome by doses exceeding the inducing dose.

The fact that PEGylation did not prevent reactivity of PEI polymers in pigs was surprising in the light of earlier reports which described reduced complement activation upon increased PEGylation under *in vitro* conditions [10]. However, these strong porcine CARPA reactions in pigs injected with PEGylated PEI are in line with their strong immunogenicity and capability to activate macrophages and induce inflammation [7]. As observed here and earlier [7,35], both PEG–PEIs were able to elicit immune responses on

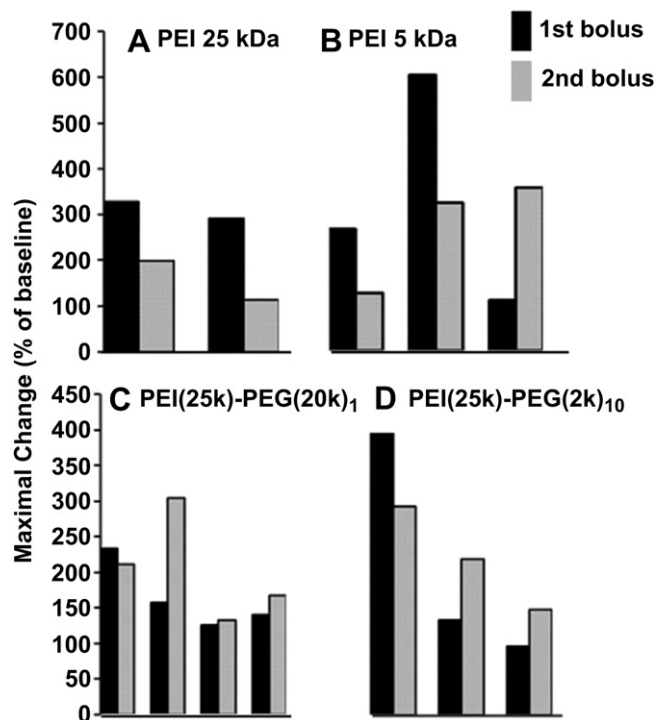


Fig. 5. Reactions of identical repetitive i.v. boluses of different polymers in pigs. Bars represent the maximal change of PAP relative to baseline in individual animals injected with 0.1 mg/kg of the polymers defined above the bars, with black and gray shades showing the 1st and 2nd boluses, respectively. Values above 100% show reactivity, and a decrease of the second (gray) bar relative to the first (black) indicates tachyphylaxis, i.e., tolerance caused by the first bolus. Further details are described in the Methods.

various levels. While PEI(25k)–PEG(20k)₁ caused comparably low reactivity with the least variability (Fig. S2), PEI(25k)–PEG(2k)₁₀ was highly reactogenic at very low concentrations with lack of tachyphylaxis (Fig. 4B).

While anti-PEG IgM induction did not play an important role in the experiments described above due to the short time period in between injections and the need of at least 3 days for B cells to produce antibodies [17], it should however be kept in mind as another serious concern in repeated application of PEG–PEI.

These results confirm C activation by PEI polymers *in vitro*, and show immediate, major cardiopulmonary changes in pigs that are typical of complement activation. This activity in the porcine CARPA model implies that PEI polymers, even if PEGylated, may potentially induce anaphylactic reaction in an occasional hypersensitive individual. This conclusion is based on the remarkable similarity between the symptoms of liposome-induced hypersensitivity reactions in humans and cardiopulmonary changes in pigs [33]. They are manifested in a small fraction of humans (up to 10%) and practically in all pigs. Although the physiological effects of PEGylated polymers and liposomes are obviously not directly comparable, the identity of symptoms suggests that the effector arm of the reactions is the same for liposomes and PEI polymers. In both cases, they involve the release of thromboxanes, leukotrienes and a range of further potent vasoactive substances from mast cells and basophil leukocytes upon binding of C3a and C5a to their respective receptors on these cells [33]. Interestingly, elevated levels of prostaglandin E, another product in the eicosanoid synthesis, were found *in vitro* in macrophages treated with PEI(25k)–PEG(20k)₁ [7].

Since the strongest *in vitro* C activation was caused by PEI 25 kDa both in terms of liberated SC5b-9 and ratio of reactive sera (50%), amino groups may play a key role in C activation. The fact that *in vitro* C activation was not observed with the PEGylated forms

corroborates previous studies showing reduced complement activation as a consequence of PEGylation [10]. In light of the finding that only PEI 25 kDa showed major C activation *in vitro*, yet all tested polymers showed cardiopulmonary reactions, it is obvious that the porcine CARPA model is more sensitive than the *in vitro* ELISA assay. Additionally, immune responses are multiplied by various cascades in the living individual but only to a certain extent in isolated serum. The underlying cause of this high sensitivity of the pig model has not been clarified to date. It may be related to the presence of intravascular macrophages (PIM cells) in the microcirculation of porcine lungs. As it has been outlined previously, the major hemodynamic changes entailing complement activation are most likely due to the release of thromboxanes, other eicosanoids and leukotrienes, histamine and a range of further potent vasoactive substances from mast cells and basophil leukocytes upon binding of C3a and C5a to their respective receptors on these cells [33].

Regarding the human relevance of this oversensitivity, it was pointed out earlier that the dose of Doxil that causes reactions in pigs corresponds to the human dose that reaches the circulation within a few seconds in reactive patients [34]. Therefore, the porcine CARPA test represents a model of hypersensitive humans.

4. Conclusion

We show here that *in vitro* C activation was prominently caused by PEI 25 kDa, whereas low molecular weight PEI was less reactogenic and the PEGylated versions of PEI 25 kDa showed no significant activity. However, *in vivo* cardiopulmonary reactions of all PEI-based polymers were found in pigs, depending on the concentration. This confirms that “CARPA”, as earlier described for pseudoallergic reactions toward liposomes, may be a major cause for the cardiopulmonary distress caused by i.v. injected polymers. The fact that PEGylated polymers, with no apparent *in vitro* C activation still caused reactions in pigs, suggest that the porcine CARPA test endpoints provide a more sensitive biomarker for anaphylactic reactions than the *in vitro* C tests. Since the porcine CARPA test represents a model of hypersensitive humans, the present study highlights a high risk for all PEI polymers to trigger anaphylactic reactions in a few percentages of hypersensitive individuals, regardless of PEGylation. Based on the tendency observed, PEGylation with long PEG chains of >20 kDa may be favorable. These observations of immune reactivity of polyethylenimine-graft-poly(ethylene glycol) block copolymers applied for drug or nucleic acid delivery therefore highlight the need for relevant *in vivo* biocompatibility tests in the development of safer drug delivery systems.

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Appendix Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.03.035.

References

- [1] Chonn A, Cullis PR, Devine DV. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J Immunol* 1991;146(12):4234–41.

- [2] Barron LG, Meyer KB, Szoka Jr FC. Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. *Hum Gene Ther* 1998;9(3):315–23.
- [3] Plank C, Mechtler K, Szoka Jr FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther* 1996;7(12):1437–46.
- [4] Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J Gene Med* 2005;7(8):992–1009.
- [5] Akhtar S, Benter I. Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. *Adv Drug Deliv Rev* 2007;59(2–3):164–82.
- [6] Beyerle A, Irmeler M, Beckers J, Kissel T, Stoeger T. Toxicity pathway focused gene expression profiling of PEI-based polymers for pulmonary applications. *Mol Pharm* 2010;7(3):727–37.
- [7] Beyerle A, Merkel O, Stoeger T, Kissel T. Pegylation affects cytotoxicity and cell-compatibility of poly(ethylene imine) for lung application: structure-function relationships. *Toxicol Appl Pharmacol* 2010;242(2):146–54.
- [8] Kabanov AV, Batrakova EV, Sridibhatla S, Yang Z, Kelly DL, Alakov VY. Polymer genomics: shifting the gene and drug delivery paradigms. *J Control Release* 2005;101(1–3):259–71.
- [9] Merkel OM, Beyerle A, Beckmann BM, Zheng M, Hartmann RK, Stoeger T, et al. Polymer-related off-target effects in non-viral siRNA delivery. *Biomaterials* 2011;32(9):2388–98.
- [10] Brus C, Petersen H, Aigner A, Czubyko F, Kissel T. Physicochemical and biological characterization of poly(ethyleneimine)-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes. *Bioconjug Chem* 2004;15(4):677–84.
- [11] Toda M, Kitazawa T, Hirata I, Hirano Y, Iwata H. Complement activation on surfaces carrying amino groups. *Biomaterials* 2008;29(4):407–17.
- [12] Allen TM, Hansen CB, de Menezes DEL. Pharmacokinetics of long-circulating liposomes. *Adv Drug Deliv Rev* 1995;16(2–3):267–84.
- [13] Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. *Biochim Biophys Acta* 1991;1070(1):187–92.
- [14] Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim Biophys Acta* 1991;1062(1):77–82.
- [15] Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov Jr AA, Trubetskoy VS, Herron JN, et al. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim Biophys Acta* 1994;1195(1):11–20.
- [16] Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, et al. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J Pharmacol Exp Ther* 2000;292(3):1071–9.
- [17] Ishida T, Ichihara M, Wang X, Kiwada H. Spleen plays an important role in the induction of accelerated blood clearance of pegylated liposomes. *J Control Release* 2006;115(3):243–50.
- [18] Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, et al. Injection of pegylated liposomes in rats elicits peg-specific IgM, which is responsible for rapid elimination of a second dose of pegylated liposomes. *J Control Release* 2006;112(1):15–25.
- [19] Szebeni J, Baranyi L, Savay S, Lutz HU, Jelezarova E, Bunger R, et al. The role of complement activation in hypersensitivity to pegylated liposomal doxorubicin (doxil®). *J Liposome Res* 2000;10(4):467–81.
- [20] Szebeni J, Baranyi L, Savay S, Milosevits J, Bunger R, Laverman P, et al. Role of complement activation in hypersensitivity reactions to doxil and hynic peg liposomes: experimental and clinical studies. *J Liposome Res* 2002;12(1–2):165–72.
- [21] Petersen H, Fechner PM, Fischer D, Kissel T. Synthesis, characterization, and biocompatibility of poly(ethyleneimine)-graft-poly(ethylene glycol) block copolymers. *Macromolecules* 2002;35(18):6867–74.
- [22] Szebeni J, Muggia FM, Alving CR. Complement activation by cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J Natl Cancer Inst* 1998;90(4):300–6.
- [23] Szebeni J, Baranyi L, Savay S, Milosevits J, Bodo M, Bunger R, et al. The interaction of liposomes with the complement system: in vitro and in vivo assays. *Methods Enzymol* 2003;373:136–54.
- [24] Merkel OM, Librizzi D, Pfestroff A, Schurra T, Buyens K, Sanders NN, et al. Stability of siRNA polyplexes from poly(ethyleneimine) and poly(ethyleneimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by fluorescence fluctuation spectroscopy and single photon emission computed tomography (SPECT) imaging. *J Control Release* 2009;138(2):148–59.
- [25] Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, et al. Pegylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. *Bioconjug Chem* 2005;16(4):785–92.
- [26] Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. Purification of poly(ethyleneimine) polyplexes highlights the role of free poly-cations in gene transfer. *J Gene Med* 2004;6(10):1102–11.
- [27] Chanan-Khan A, Szebeni J, Savay S, Liebes L, Rafique NM, Alving CR, et al. Complement activation following first exposure to pegylated liposomal doxorubicin (doxil(r)): possible role in hypersensitivity reactions. *Ann Oncol* 2003;14(9):1430–7.
- [28] Petersen H, Fechner PM, Martin AL, Kunath K, Stolnik S, Roberts CJ, et al. Poly(ethyleneimine)-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjug Chem* 2002;13(4):845–54.
- [29] Kunath K, von Harpe A, Petersen H, Fischer D, Voigt K, Kissel T, et al. The structure of peg-modified poly(ethylene imine)s influences biodistribution and pharmacokinetics of their complexes with NF- κ B decoy in mice. *Pharm Res* 2002;19(6):810–7.
- [30] Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. Pegylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* 1999;6(4):595–605.
- [31] Lim HW. The complement system. Activation, modulation, and clinical relevance. *Dermatol Clin* 1990;8(4):609–18.
- [32] Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, et al. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble C1 and anti-C5a antibody. *Circulation* 1999;99(17):2302–9.
- [33] Szebeni J, Baranyi L, Savay S, Bodo M, Milosevits J, Alving CR, et al. Complement activation-related cardiac anaphylaxis in pigs: role of C5a anaphylatoxin and adenosine in liposome-induced abnormalities in ECG and heart function. *Am J Physiol Heart Circ Physiol* 2006;290(3):H1050–8.
- [34] Szebeni J, Baranyi L, Savay S, Bodo M, Morse DS, Basta M, et al. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. *Am J Physiol Heart Circ Physiol* 2000;279(3):H1319–28.
- [35] Merkel OM, Beyerle A, Librizzi D, Pfestroff A, Behr TM, Sproat B, et al. Nonviral siRNA delivery to the lung: investigation of peg-PEI polyplexes and their in vivo performance. *Mol Pharm* 2009;6(4):1246–60.

***In vitro* and *in vivo* Complement Activation and Related Anaphylactic Effects Associated with Polyethylenimine and Polyethylenimine-graft-poly(ethylene glycol) Block Copolymers**

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Supporting Information:

MTT assays:

Cell viability after treatment with polymers was investigated in L929 cells according to the US pharmacopoeia. L929 cells were seeded with 8000 cells per well in 96-well plates 24 h before they were treated with increasing concentrations of polymer solutions. Another 24 h later, medium was changed; MTT solution was added into fresh serum-free medium and incubated with the cells for 4 h. Remaining mitochondrial enzyme activity compared to untreated cells was determined photometrically after cell lysis in 200 μ l DMSO by measuring absorbance of enzymatically formed formazane at 580 nm with 690 nm background corrections. IC₅₀ values were calculated by non-linear fitting of normalized data in a sigmoidal dose-response model with variable slope using GraphPad Prism 4.03 (Graph Pad Software, La Jolla, USA). Results are given as mean values of a replicate of four $\pm$ SD.

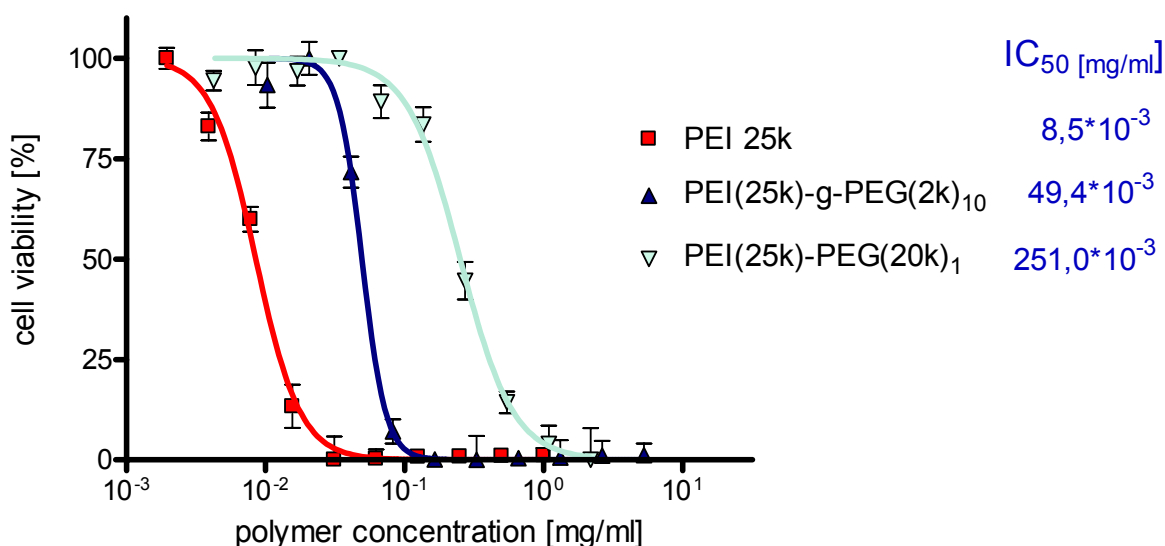


Figure S1: Cell viability as determined by MTT assays clearly not only depends on the percentage of PEI content but also on the grafting degree and PEG chain length. Toxicity of unmodified siRNA is decreased by PEGylation by a factor 5.8 to 29.5 – depending on the PEG chain length. PEI(25k)-PEG(20k)₁-treated cells still showed > 80% cell viability at 0.14 mg/ml, whereas PEI(25k)-PEG(2k)₁₀-treated cells only exhibited 71 % cell viability at 0.04 mg/ml. The IC₅₀ values derived from this assay were used for *in vitro* determination of complement activation.

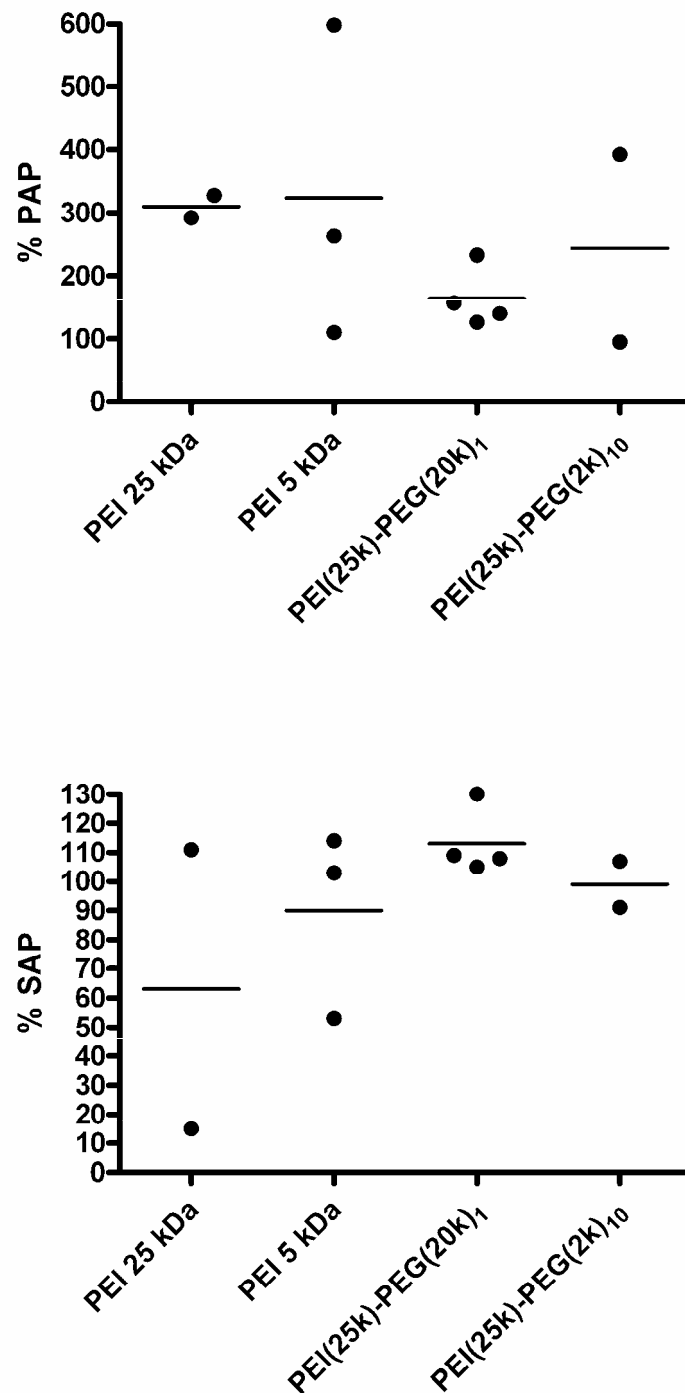


Figure S2: Individual normalized changes in pulmonary arterial pressure (PAP) and systemic arterial pressure (SAP) of pigs treated with i.v. boluses of 0.01 mg/kg different polymers in pigs. Of all polymers, PEI(25k)-PEG(20k)₁ caused the lowest reactions with the least variability.