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Poly(amino ester)s-Based Polymeric Gene Carriers in Cancer Gene Therapy

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1. Introduction

Gene therapy is a novel approach that broadly defined as the transfer of genetic material into a cell, tissue, or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient [1]. Gene therapy refers to local or systemic administration of a nucleic acid construct that can prevent, treat and even cure diseases by changing the expression of genes that are responsible for the pathological condition [2]. As a form of molecular medicine, gene therapy hold great promises to provide new treatments for a large number of inherited and acquired diseases, such as cancer. It has also been considered as suitable substitute for conventional protein therapy, since it can overcome inherent problems associated with administration of protein drugs in terms of bioavailability, systemic toxicity and manufacturing cost [3].

There are two essential components in current gene therapy: an effective therapeutic genetic agent and the gene delivery system [4, 5]. The most extensively studied approach involves the delivery of plasmid DNA (pDNA) for expressing therapeutic transgenes. Considerable efforts have been made in plasmid design. This includes removal of extraneous CG dinucleotides, incorporation of scaffold/matrix attached region sequences to prolong expression, promoter selection for gene expression, and improving plasmid entry into the nucleus [6]. The recently emerged RNA interference (RNAi) has also become recognized as pivotal cellular regulator of genetic events and a useful tool in elucidating pathways during stages of development, pathogenesis and senescent cell regulation [7]. RNAi encompasses the range of endogenous or synthetic short double or single stranded oligonucleotides, including microRNAs (miRNAs), small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), piwi interacting RNAs (piRNAs) and antisense oligonucleotides (ASOs) [8]. The intracellular delivery of genetic

agents for RNAi such as siRNA takes benefits from existing expertise in pDNA transfer, as they share common properties. However, they face distinct challenges due to apparent differences in size, stability of the formed nucleic acid complexes, the location and mechanism of action [9].

Naked genetic therapeutics is vulnerable to enzyme degradation, rapid clearance by renal filtration, poor cellular uptake due to anionic charges of the phosphate backbone, inefficiently escape from endosome into cytosol. Therefore, the development of gene vectors for effectively carrying genes into cells has made a great deal of progress in recent years [5, 10]. Vectors as gene delivery system that have been developed fall into two broad categories: viral and non-viral vectors. Vectors based upon many different viral systems, including retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAV) (Table 1), currently offer the best choice for efficient gene delivery [11, 12]. They are all highly efficient in specific circumstances, but the potential risks of undesired immune response and the risk of insertional mutagenesis following long term viral gene transfer and toxic side reactions have raised concerns [13-15].

Virus	Genome	Size	Advantages	Disadvantages
Retrovirus	ssRNA	7-10 kb	Long-term expression	Application is limited to replicating cells Possibility of insertional mutagenesis
Adenovirus	dsDNA	36 kb	Capable of very efficient episomal gene transfer in a wide range of cells and tissues Easy to grow in large amounts	The host response to the virus appears to limit the duration of expression and the ability to repeat dosing
AAV	ssDNA	5 kb	Structurally simple Provoke less of a host-cell response	Extremely difficult to grow in large amounts

Table 1. Viral vector delivery systems [12].

Although viral vector has the advantages in terms of gene transfer efficiency, non-viral gene therapy has the advantage over viral vector therapies with its ability to target specific cells, being less immunogenic and non-integrating into the host genome, low production cost, scalability despite most studies showing less sustained gene expression [16-18]. Non-viral vectors have been investigated even more aggressively since the death of a patient in a virus-based gene therapy trial [19] and the occurrence of leukemia following gene therapy of children with X-linked severe combined immune deficiency using a retroviral gene therapy vector [20]. Previous efforts focused primarily on cationic lipid/DNA complexes frequently composed of combinations of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [21], dioleoyl phosphatidylethanolamine (DOPE) [22], and dimethylaminoethane-carbamoyl cholesterol (DC-Chol) [23]. These complexes stabilize incorporated DNA against physical and enzymatic damage. On the other hand, numerous agents poly(L-lysine), poly-

thylenimine (PEI), chitosan, dendrimers etc. have now been extensively investigated as polymer-based non-viral vector gene delivery systems [24, 25]. The success of these agents is directly correlated with their ability to overcome issues of low efficiency and inconsistent preparation that have plagued previous non-viral vector delivery systems.

Among polymeric vectors, the widely used PEI with appropriate molecular weight can electrostatically interact with negatively charged phosphate groups of genetic agents to form particulate polyelectrolyte complexes, which exhibit superior transfection efficiency due to high buffering capacity [26]. However, high molecular weight of PEI shows high cytotoxicity, and when further decreasing the molecular weight, both cellular toxicity and transfection efficiency are decreased [27]. Polyethylene glycol (PEG) was conjugated to PEI to ameliorate its cell cytotoxicity and develop functionality which limit its clinical application [28-32]. However, free PEI is not easily biodegradable in cellular space, which may remain additional safety concerns. Therefore, it calls for the development of functional biocompatible materials with favorable gene transfer efficiency to substitute for PEI [33, 34]. Poly(amino ester)s-based polymers are a promising class of polymeric gene vector due to their biocompatibility [35-37]. Poly(amino ester)s can be synthesized by Michael addition reaction using functional amines including a primary and a secondary amine to diacrylate ester [38]. The ease in synthesis and lack of byproducts make them even more favorable gene vector candidates with biocompatibility and biodegradability properties [38-43].

Here, we are focused on recent progress of different strategies of functionalization of synthetic biocompatible poly(amino ester)s and the applications of these. The characterization of physicochemical properties, degradation kinetics, transfection efficiency and toxicity in vitro and in vivo were covered in this chapter.

2. Poly(amino ester)s-based gene therapy

Poly(amino ester)s are promising and efficient gene delivery vectors due to their high transfection efficiency and biocompatibility, which were first synthesized by Langer et al. [44-46].

2.1. Poly(amino ester)s synthesis and degradation kinetics

2.1.1. Linear poly(amino ester)s

Varieties of linear poly(amino ester)s are synthesized by Michael addition reaction of small molecular weight monomers and diacrylate monomers. The Langer group initially investigated the synthesis of poly(β -amino ester)s via the addition of N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine to 1,4-butanediol diacrylate as shown in Fig. 1(A). They reported that addition of secondary amines to diacrylate moieties results into tertiary amines which do not participate in subsequent addition reaction, that otherwise leads to polymer branching or cross linking.

One of the major merit of poly(β -amino ester)s is degradation. Due to the hydrolysis of the ester bonds in the polymer backbones, poly(β -amino ester)s can easily degraded. The degra-

dation of poly(β -amino ester)s presents a particularly attractive basis for the development of new polymeric gene carriers for two reasons: firstly, poly(β -amino ester)s degrade into nontoxic byproducts to increase the safety of gene carrier; secondary, degradation of poly(β -amino ester)s will increase the transfection efficiency. While complexation of DNA with cationic polymers is required to compact and protect DNA during early events in the transfection process, DNA and polymer must ultimately decomplex to allow efficient transcription [44]. In view of this requirement, degradable poly(β -amino ester)s could play an important role in “vector unpackaging” events in the cells [44, 47]. As shown in Fig. 1(C), the polymers degraded more slowly at pH 5.1 than at pH 7.4, and at pH 5.1 each polymer having a half-life of approximately 7-8 h. In contrast, polymers 1 and 3 [Fig. 1(B)] were completely degraded in less than 5 h at pH 7.4. These results are consistent with the pH-degradation profiles of other amine-containing polyesters, such as poly(4-hydroxy-L-proline ester), in which pendant amine functionalities are hypothesized to act as intramolecular nucleophilic catalysts and contribute to more rapid degradation at higher pH [44]. The degradation of polymer 2 occurred more slowly at pH 7.4 than at pH 5.1 due to the incomplete solubility of polymer 2 at pH 7.4 and the resulting heterogeneous nature of the degradation milieu [44].

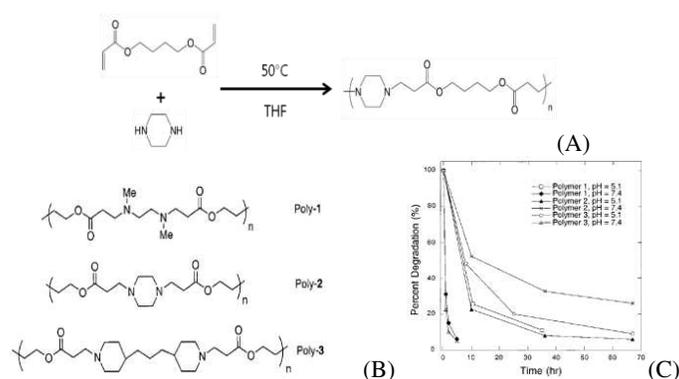


Figure 1. The synthesis of poly(β -amino ester)s from butanediol diacrylate and piperazine (A) and degradation of polymer 1-3 at 37 °C at pH 5.1 and 7.4 (B and C). [Source from Ref. [44]].

After that, the same group reported a parallel approach suitable for synthesis of hundreds to thousands of structurally unique poly(amino ester)s and application of these libraries to rapid and high throughput identification of new gene delivery agents and structure-function trends although they did not report the degradation profiles of poly(amino ester)s in this study [38]. The high throughput method indicated that synthesis of poly(β -amino ester)s are easy to controlable. The advantage of combinatorial chemistry and automated high throughput synthesis is that it has revolutionized modern drug discovery by rapid synthesis and evaluation with greater precision. As shown in Fig. 2, 140 different poly(β -amino ester)s were synthesized from the 7 diacrylate monomers and 20 amine-based monomers as a screening library. Polymerization reactions were conducted simultaneously as an array of individually labeled vials and the reactions were performed in methylene chloride at 45 °C for 5 days.

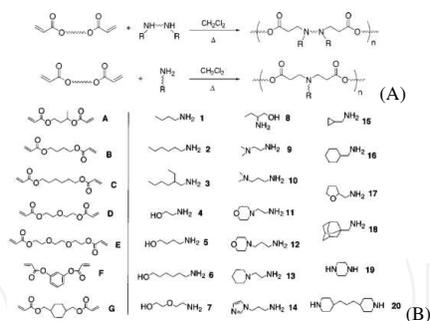


Figure 2. Synthesis of poly(β-amino ester)s. Poly(β-amino ester)s were synthesized by the conjugate addition of primary or bis(secondary amines) to diacrylates using methylene chloride solvent (A) and diacrylate (A-G 7 set) and amine (1-20) monomers chosen for the synthesis of an initial screening library (B). [Source from Ref. [38]].

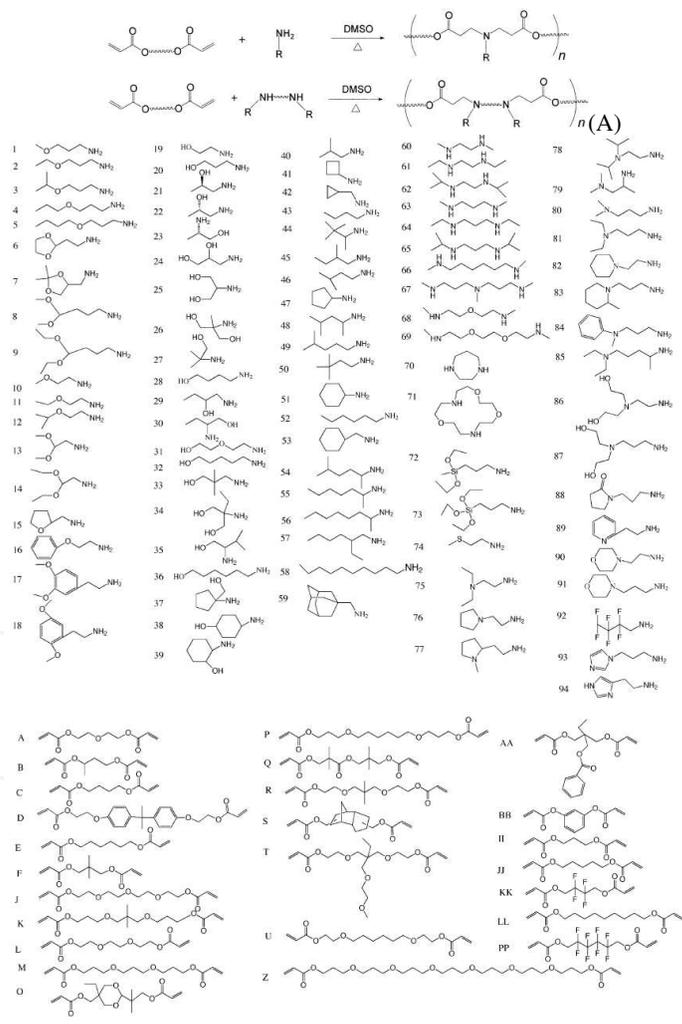


Figure 3. Synthesis of poly(β-amino ester)s. Poly(β-amino ester)s were synthesized by the conjugate addition of primary or bis(secondary amines) to diacrylates using DMSO solvent (A). Amino (numbers) and diacrylate (letters) monomers (B). [Source from Ref. [48]].

Based high throughput methods, in 2003, Anderson synthesized over 2,350 poly(β -amino ester)s as shown in Fig. 3 [48]. Polymerization reactions were performed in 1.6M DMSO at 56 °C for 5 days. Anderson et al. observed that reaction conditions such as optimum temperature and solvent play an important role during the synthesis of poly(β -amino ester)s. Even though maximizing monomer concentration in reaction is desirable to obtain high molecular weight poly(β -amino ester)s and it leads to insoluble gel formation [49].

Park et al. reported the synthesis of linear poly(amino ester)s from three different molecular weights of PEG diacrylate and low molecular weight PEI As shown in Fig. 4 [37].

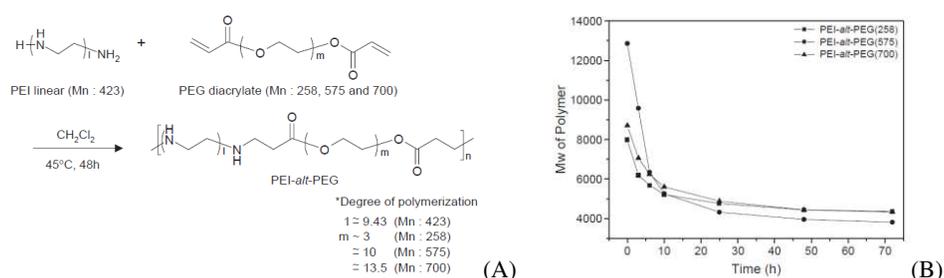


Figure 4. Proposed reaction scheme for copolymer formation (A) and degradation of copolymers (B). [Source from Ref. [37]].

It was found that molecular weights of poly(amino ester)s were maintained relatively constant at about 4kDa during the degradation after 72 h regardless of molecular weight of PEG diacrylate. However, half life was observed depending on molecular weight of PEG diacrylate. Poly(β -amino ester)s composed of PEG diacrylate (Mn: 575) showed an half life of 8 h while that of 25 h for poly(β -amino ester)s with PEG diacrylate (Mn: 700). This rapid degradation in case of linear poly(β -amino ester)s is plausible as even few cleavages may reduce chain length rapidly with quick drop in molecular weight [37].

2.1.2. Branched poly(amino ester)s

Liu et al. synthesized the branched poly(amino ester)s by the polymerization of 1-(2-aminoethyl)piperazine (AEPZ) with 1,4-butanediol diacrylate (BDA), which was carried out by adding BDA dropwise to an equimolar solution of AEPZ in chloroform at 45 °C as shown in Fig. 5 [50]. After the polymerization was performed for around 72 h, a water-soluble polymer, BDA-AEPZ, was obtained by precipitating the solution into acetone containing HCl. The molecular weight of BDA-AEPZ was around 5126 with a polydispersity index of 1.52 as determined by GPC.

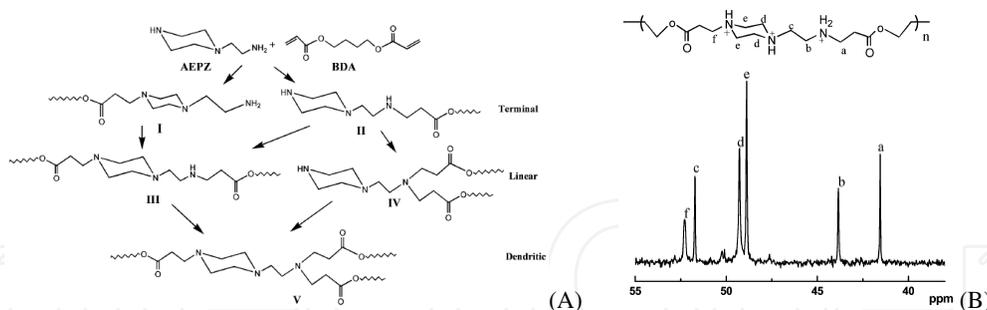


Figure 5. Possible routes of the Michael addition polymerization of trifunctional amine monomers and diacrylates (A) and the structure of poly(amino ester)s obtained and enlarged ^{13}C -NMR (INVGATE) spectrum of methylene carbons attached to the hydrochloride salts of amines in BDA-AEPZ (B). [Source from Ref. [50]].

The polymerization of AEPZ with diacrylate monomers was reported but branched poly(amino ester)s with primary, secondary and tertiary amines were supposed to be formed based on un-solidified experimental conditions, suggesting that secondary amines are more reactive than primary ones in case of trifunctional amines [50]. Wu et al. also synthesized protonated hyperbranched poly(amino ester)s and characterized as gene delivery carriers as shown in Fig. 6(A) [51]. It was found that all these hyperbranched poly(amino ester)s degraded in a controlled manner within 50 days and it was speculated that this phenomenon may be due to the lesser water accessibility of the ester groups in hyperbranched structures [Fig. 6(B)].

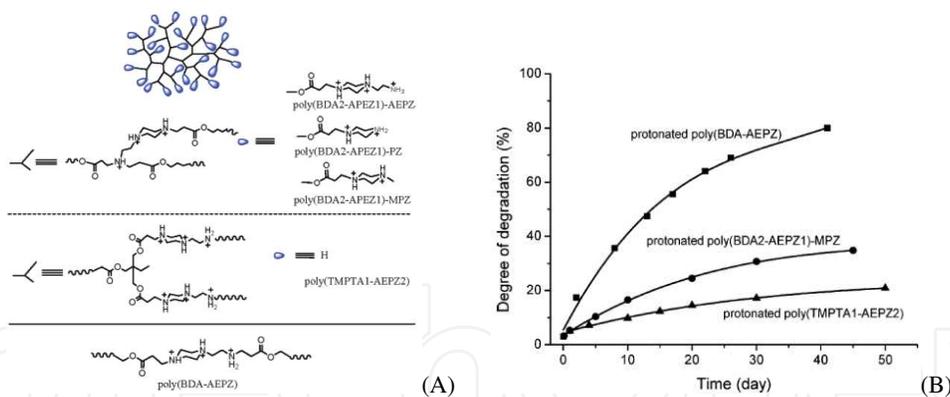


Figure 6. Structure of linear and hyperbranched poly(amino ester)s obtained via Michael addition polymerization of trifunctional amines with diacrylates and triacrylates (A) and comparison of the hydrolysis profiles of protonated hyperbranched poly(BDA2-AEPZ1)-MPZ, hyperbranched poly(TMPTA1-AEPZ2), and linear poly(BDA-AEPZ) in aqueous solutions (B). [Source from Ref. [51]].

Cho's group also reported the synthesis of branch poly(amino ester)s by Michael addition, based on hydrophobic polycaprolactone diacrylate and low molecular weight PEI [Fig. 7(A)] [40]. It was simply an indication of application of ester linkage which supports the easy degradation leaving nontoxic building blocks, thereby increased transfection efficiency and reduced cytotoxicity. The branched poly(amino ester)s showed controlled degradation with the half life of 4-4.5 days as shown in Fig. 7(B).

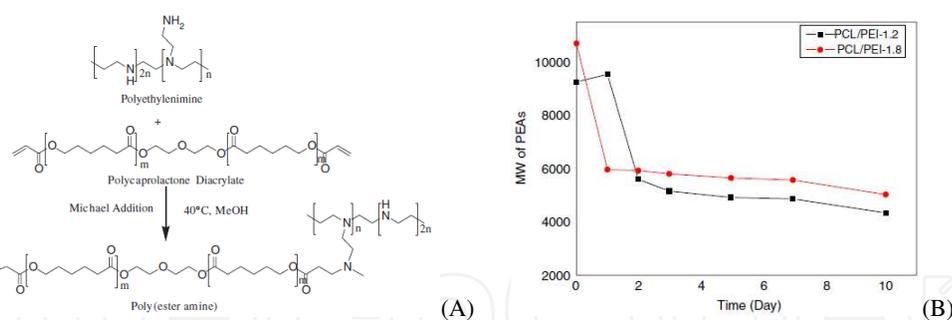


Figure 7. The synthetic scheme of PEAs by Michael addition (A) and degradation of PEAs (PCL/PEI-1.2 and PCL/PEI-1.8) (B). [Source from Ref. [40]].

Same group also reported another degradable branched poly(amino ester)s based on poloxamer diacrylate and low molecular weight PEI [52]. These hyperbranched poly(amino ester)s can be easily synthesized by Michael type addition reaction between poloxamer diacrylate and low molecular weight PEI [Fig. 8(A)] and the hyperbranched poly(amino ester)s showed slow degradation at physiological conditions which was greatly dependent on hydrophilicity of poloxamer [Fig. 8(B)].

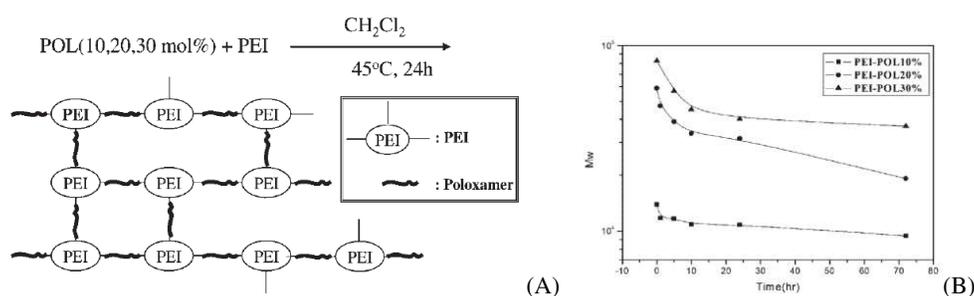


Figure 8. Synthetic scheme of PEA by Michael addition reaction (A) and degradation of PEAs (B). PEAs were dissolved in 0.1 M PBS, and incubated at 37 °C with 100 rpm. [Source from Ref. [52]].

All together, poly(amino ester)s can be easily synthesized by Michael type addition reaction and showed good degradation profiles due to the hydrolysis of the ester bonds in the polymer backbones.

2.2. Characterization of poly(amino ester)s/DNA complexes

2.2.1. DNA condensation and protection

One prerequisite of a polymeric gene carrier is DNA condensation [53]. Polycation-mediated gene delivery is based on the electrostatic interactions between the positive charged polycation and negatively charged phosphate groups of DNA [32]. As shown in Fig. 9(A), retardation of DNA migration begins at poly(β -amino ester)s/DNA ratios as low as 0.1:1 (w/w) and migration is completely retarded at poly(β -amino ester)s/DNA ratios above 5:1 (w/w) [34]. Condensation protects the DNA from degradation by nucleases, and the compact particles can be taken up

by cells via natural processes such as adsorptive endocytosis, pinocytosis and phagocytosis [32]. DNA in the complexes was protected from nuclease attack whereas the naked DNA was degraded. This result suggests that intact DNA could be delivered by poly(β -amino ester)s into cells without degradation [Fig. 9(B)] [34].

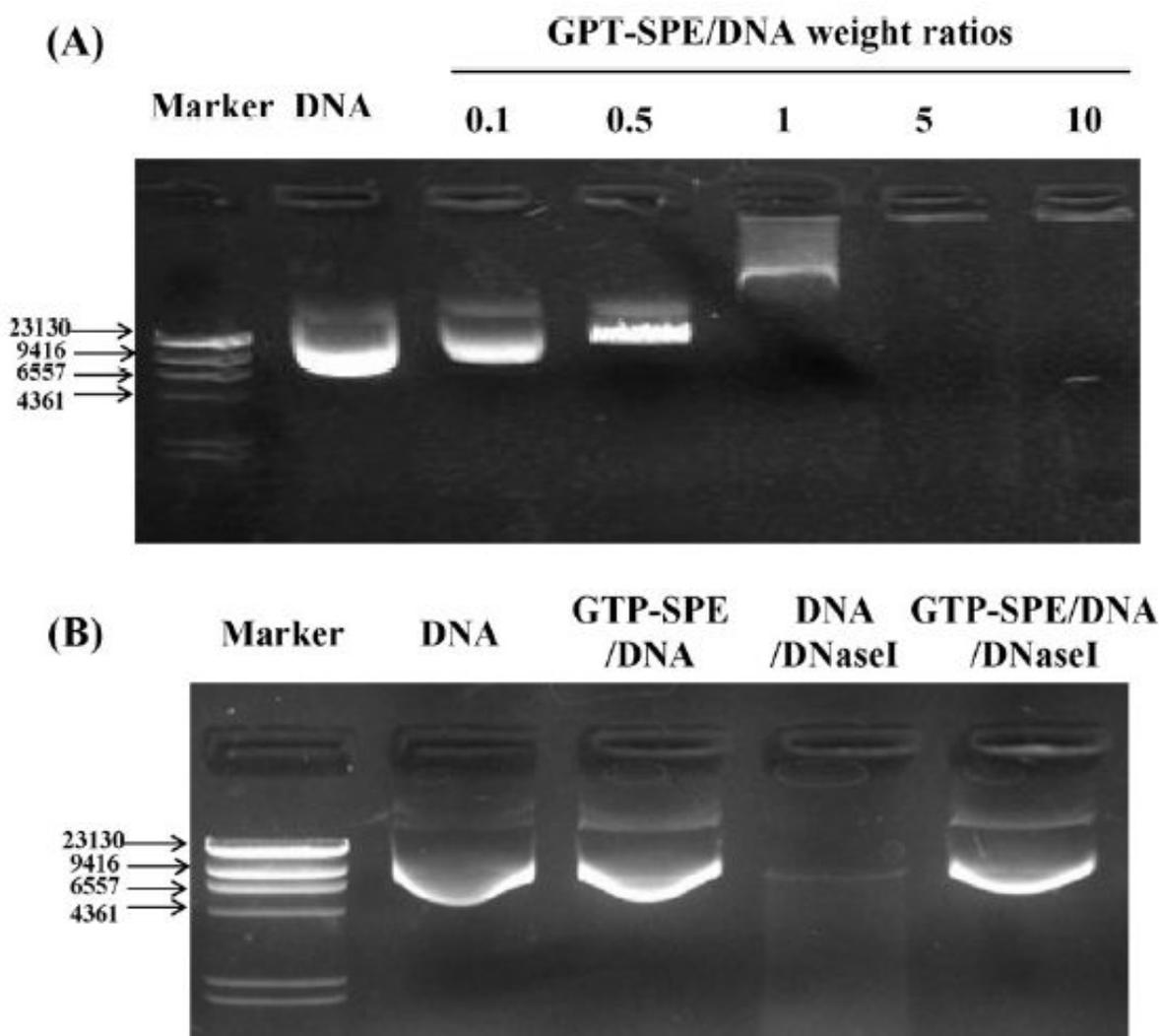


Figure 9. DNA condensation and protection study. Agarose gel electrophoresis of poly(β -amino ester)s (GPT-SPE)/DNA complexes at various weight ratios (A) and DNA protection and release assay (B). [Source from Ref. [34]].

2.2.2. Particle sizes and surface charges of poly(amino ester)s/DNA complexes

Surface properties, such as particle size and surface charge of the complex, are necessary to assure its uptake by cells [53]. In particular, the particle size of a complex is an important factor that influences the access and passage of the complex through the targeting site. Successful gene carrier depends on its ability to condense negatively charged DNA into nanosized particles with positive charges so as to enter into the cells [54]. Compact particles of small size are usually obtained only at higher N/P ratios, resulting in complexes with a strong positive

net charge. For most cell types, the poly(amino ester)s/DNA complexes size requirement is on the order of 200 nm or less [55]. As shown in Fig. 10(A), poly(β -amino ester)s formed complexes with diameters in the range of 50-150 nm at DNA/polymer ratios above 1:2. A positive surface charge of polyplexes is necessary for binding to anionic cell surfaces, which consequently facilitates uptake by the cell [30, 56]. The surface charge of poly(β -amino ester)s/DNA complexes has been examined in terms of ζ -potential. The ζ -potentials for complexes were on the order of +10 to +15 mV at DNA/polymer ratios above 1:1, and the complexes did not aggregate extensively over an 18h period as shown in Fig. 10(B).

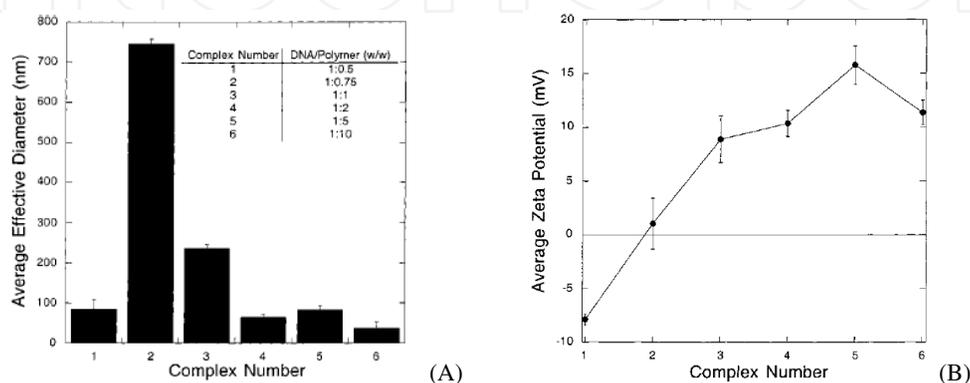


Figure 10. Average effective diameters (A) and ζ -potentials (B) of DNA/polymer complexes formed from pCMV-Luc plasmid and poly(β -amino ester)s (polymer 3) ($M_n = 31000$) as a function of polymer concentration. [Source from Ref. [44]].

2.3. Toxicity and transfection considerations of poly(amino ester)s/DNA complexes *in vitro*

Safe and efficient delivery of genes is critical for the successful application of gene therapy. In fact, it is the only major obstacle in the expansion of gene therapy from bench to bedside. Many vectors with high transfection efficiency show high toxicity while vectors with low toxicity are poor in transfecting cells. Optimum balance between these two parameters is a key to the success in gene therapy [57-59]. As biodegradable polymers are designed to contain a combination of various functional components, it is likely that engineered systems for non-viral gene delivery, especially with the application of biodegradable ester linkage will eventually be constructed. This biodegradable linkage approach to vector development is giving way to a safety profile where low molecular weight amine contain monomers are couples with acylate linkers to yield high molecular weight poly(amino ester)s with reduced toxicity and enhanced transfection efficiency.

Jere et al. evaluated cytotoxicity of mini-library of poly(amino ester)s in 293T and HeLa cells by MTS assay [59]. In order to measure maximum possible cytotoxicity, poly(amino ester)s were administered in increasing concentrations to 293T cells as shown in Figs. 11(A) and (B). In both cell lines, poly(amino ester)s obtained from R106 to R113 exhibited very high cytotoxicity which further increased with increase in weight ratios, while poly(amino ester)s obtained from R114, R115 and R116 showed good cell viability at lower ratios but significant cytotoxicity at higher weight ratios. Excellent cell viability and uniform transfection pattern were observed

with poly(amino ester)s obtained from R117 to R121. Slight cytotoxicity was observed at higher mass ratios (90:1 and 110:1) (viability above 80% in all ratios) indicating that cytotoxicity was highly sensitive to monomer ratio and varied drastically even with small change in monomer concentration. It was reported that the cytotoxicity of cationic polymers is probably caused by polymer aggregation on cell surfaces, impairing important membrane functions. Also, the cationic polymers may interfere with critical intracellular processes of cells: in particular, the primary amine was reported to disrupt PKC function through disturbance of protein kinase activity [60, 61]. On the other hands, in 293T cells, poly(amino ester)s obtained from R106 to R113 showed some transfection at lower weight ratios but it was suddenly decreased with increased weight ratios which may be because of low cell viability at these ratios [Fig. 11(C)]. Poly(amino ester)s obtained from R114 to R119 showed intermediate transfection while poly(amino ester)s obtained from R120 and R121 gave good transfection. However, in HeLa cells slightly different transfection pattern was observed as shown in Fig. 11(D)]. Poly(amino ester)s obtained from R106 to R115 failed to give significant transfection. On the other hand, poly(amino ester)s obtained from R116 to R119 showed intermediate transfection which was slowly increased from R116 to R119. Transfection was highest with poly(amino ester)s obtained from R120 and R121 and it was increased with increasing weight ratios till 90:1 after that it again decreased due to increased cytotoxicity. It was also reported that in addition to factors such as chemical structure and polymer molecular weight, either amine or acrylate terminated also plays a significant role in determining transfection efficiency of poly(amino ester)s [46]. Excess of amine monomers results into amine terminated polymer which effectively binds with cell membrane and promotes its uptake whereas acrylate terminated polymers has poor cellular entry and transfection efficiency.

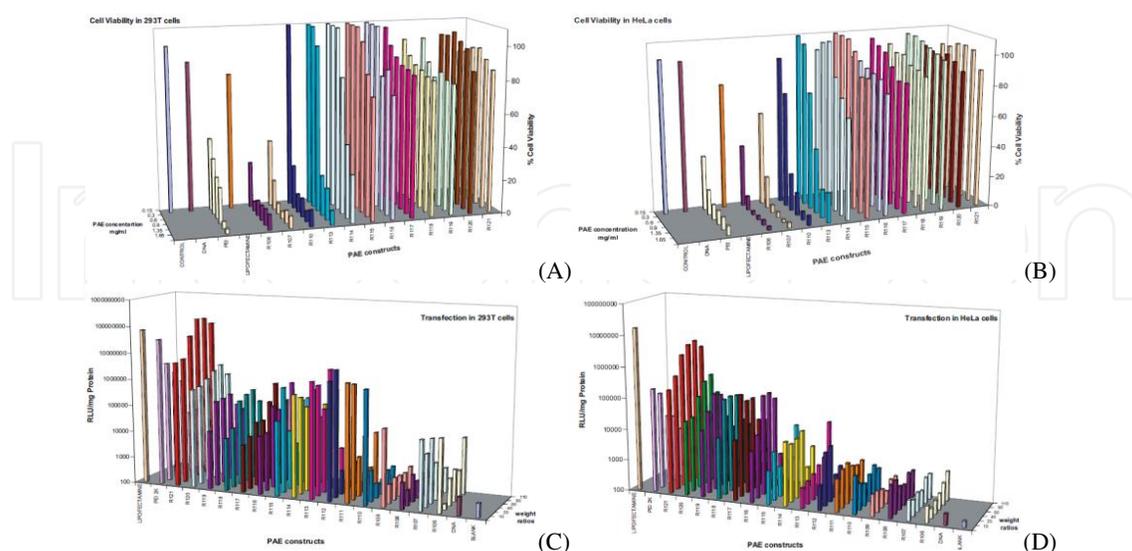


Figure 11. Cytotoxicity of PAEs at various concentrations in 293T cell line (A) and HeLa cell line (B); and transfection efficiency of PAE/DNA complexes in serum free-media at various mass ratios in 293T cells (C) and HeLa cells (D). [Source from Ref. [59]].

2.4. Toxicity and transfection considerations of poly(amino ester)s/DNA complexes *in vivo*

Intravenous administration as one of the most commonly used methods in gene therapy area even most gene therapy vectors, as well as other biomolecules and potential engineered drugs, has short elimination half-lives due to the serum proteins in the blood stream. *In vivo* transfection efficiency of the poly(amino ester)s was studied after intravenous administration into mice [62]. As shown in Fig. 12, the quantity of luciferase was determined in lung, liver, spleen, kidney and heart after 24 h intravenous administration of polymer/DNA complexes. Fig. 12 shows luciferase gene expression in various mouse organs after intravenous administration of the polymer/DNA complexes via the tail vein. As shown in Fig. 12, injection of polymer/DNA complexes resulted in transfection primarily in the lung which is in agreement with previous results [63, 64]. Verbaan et al. suggested two mechanisms regarding this phenomenon of predominant gene expression in the lung; firstly, because the lung is the first organ encountered by polyplexes after tail vein injection, the positively charged polyplexes may electrostatically interact with the negatively charged membranes of the endothelial cells in the lung, secondly, the physical trapping of large aggregates formed by the interaction of polyplexes with blood components like serum proteins and erythrocytes [63, 64]. Also, the poly(amino ester)s/DNA complexes showed the highest transfection activity in the lung regardless of N/P ratio. This may be caused by the positive charge of the poly(amino ester)s/DNA complexes like PEI 25K/DNA complexes. In contrast to PEI 25K/DNA complexes, the poly(amino ester)s/DNA complexes had high transfection in the liver because the liver is the main organ for gene accumulation and subsequent degradation [62]. Plank et al. reported that opsonization of the polyplexes led to a rapid clearance by the mononuclear phagocytic system (MPS) [65]. Uptake by the MPS would be in agreement with the observed liver and spleen accumulations. In addition, the presence of discontinuous or fenestrated endothelia in the vascularization of the liver and spleen may facilitate the gene accumulation in these tissues [66]. The poly(amino ester)s/DNA complexes showed higher transfection efficiency than golden standard PEI 25K/DNA ones, and the luciferase activity was increased in all organs except kidney with increase of N/P ratio indicating that poly(amino ester)s/DNA complexes function efficiently after intravenous administration.

Implantable infusion pumps have been developed as an one of therapy methods for a number of diseases, and there has been remarkable progress in endoscopic and laparoscopic surgical techniques. This progress in surgical techniques and devices could make intraperitoneal administration a conventional and feasible approach for future clinical applications [67]. Intraperitoneal gene delivery may provide a strategy for the treatment of a variety of diseases, including cancer. Zugates et al. synthesized parallel end-modification of poly(β -amino ester)s by the conjugate addition of amines to diacrylate monomers as shown in Fig. 13 [68].

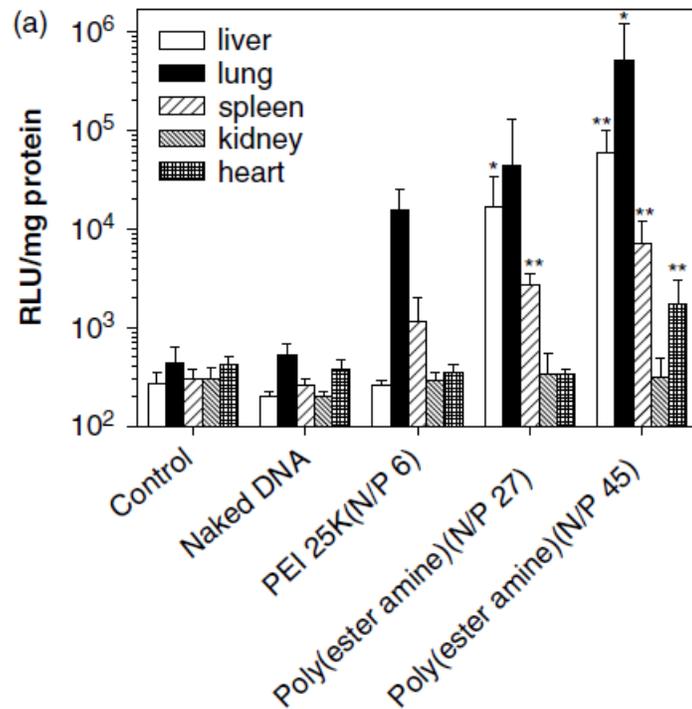


Figure 12. Tissue distribution of poly(amino ester)s/DNA (gWIZ-Luc) complexes administered by intravenous injection and inhalation at various N/P ratios. (* $p < 0.1$; ** $p < 0.05$, Student's t-test, two-tailed). [Source from Ref. [62]].

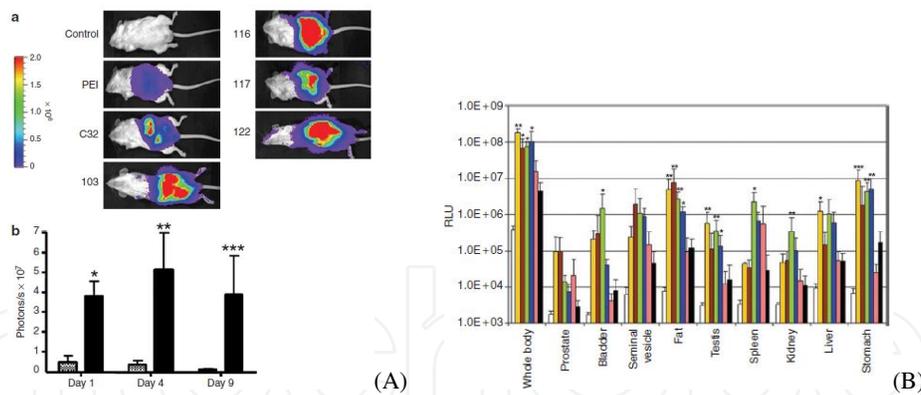


Figure 13. Intraperitoneal gene delivery in mice (A). (a) Whole-body optical images of luciferase expression in FVB/J mice 6 hours after intraperitoneal injection of polymer/DNA complexes. Images show the highest expression obtained for each polymer. The control mouse was injected with 120 μ l of 50 mM NaAc buffer, pH 5.2. Pseudocolor images representing emitted bioluminescence are superimposed over grayscale images. Relative light units (RLUs)/pixel are indicated in the color scale bar on the left. (b) Quantification of whole-body luciferase expression at various times after intraperitoneal injection of C32- (hatched) and C32-117-delivered (solid) DNA. Statistically significant differences between C32 and C32-117 at a given time point are indicated. $n = 4$ for each treatment group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Organ distribution of gene expression (B). Quantification of luciferase expression in whole body and individual organs 6 hours after intraperitoneal injection of polymer/DNA complexes in FVB/J mice. Results are expressed as mean transfection levels (\pm SD) for a buffer control (white), C32-103 (yellow), C32-116 (red), C32-117 (green), C32-122 (blue), C32 (pink), and jet-polyethylenimine (jet-PEI) (black). $n \geq 3$ for each treatment group. [Source from Ref. [68]].

High transfection levels were observed after intraperitoneal injection of polymer/DNA complexes. End-modified polymers resulted in whole-body reporter protein expression more than an order-of magnitude higher than that for jet-PEI. They also outperformed the best poly(β -amino ester)s synthesized to date, C32, with overall expression levels 4- to 12-fold higher. They found that sustained expression past 1 week both with modified C32 and with C32, but modified C32 was expressed at significantly higher levels, reflecting its enhanced delivery capabilities. This effect was most evident between the C32-116 and C32-117 polymers, where the latter displayed 5- to 65-fold higher delivery to the bladder, spleen, liver, and kidney. The only difference between these two diaminopropane end-capping reagents is the ethyl versus dimethyl branching.

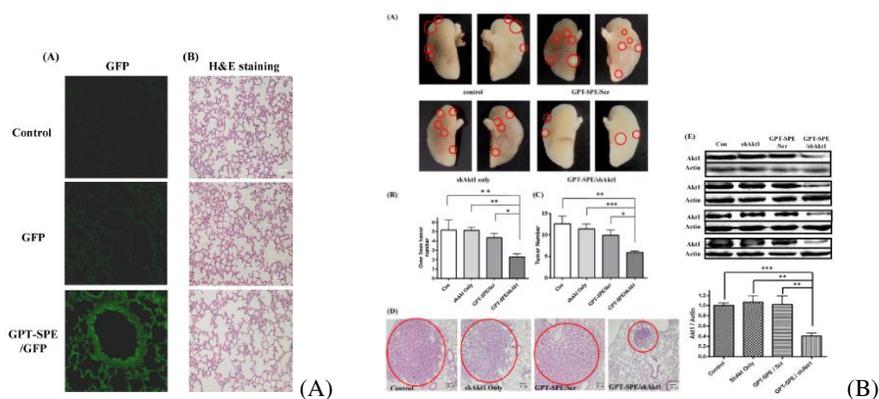


Figure 14. A) In vivo analysis after aerosol administration to lungs. Two days after exposure, mice were sacrificed and lungs were collected for the detection of GFP signal and Hematoxylin & Eosin staining. (A) Transfection efficiency study: GFP expression analysis (magnification: 200 \times). B) Lung histopathology study: Hematoxylin & Eosin staining (magnification: 200 \times , scale bar represents 50 μ m). (B) Therapeutic efficiency of GPT-SPE as aerosol gene delivery carrier in lung tumor bearing *K-ras*^{LA1} mice: aerosol delivery of GPT-SPE/Akt1 shRNA significantly inhibited lung tumor numbers: (A) Lungs showing numerous visible lesions (red circle represents tumor tissues). (B) Total tumor numbers ($n = 4$, * $p < 0.05$, ** $p < 0.01$). (C) Tumor size over 1 mm tumor numbers ($n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Aerosol delivery of GPT-SPE/Akt1 shRNA significantly suppressed lung tumor progression through the Akt signaling pathway. (D) Histopathological characteristics. Red circle indicates the incidence in the lungs (magnification: 200 \times , scale bar represents 50 μ m). (E) Western blot analysis of Akt1 protein expression in the lungs and bands-of-interest were further analyzed by densitometer ($n = 4$, ** $p < 0.01$, *** $p < 0.001$).

One of the most non-invasive approaches to drug/gene delivery is via inhalation. Gene therapy to the lung can potentially be exploited for the treatment of both genetic and acquired diseases. However, any therapeutic approach for the respiratory tract must take into account the heterogeneity of the cellular targets in the lung: epithelial cells, alveolar cells, vascular cells, serous cells in the sub-mucosal glands and a number of other cell types [69]. Our group developed spermine-based biocompatible poly(β -amino ester)s as an aerosol delivery gene carrier [34]. As shown in Fig. 14 (A-A), GFP signal was dominant in the lungs with GPT-SPE/GFP complexes-exposed group compared to the control and naked GFP-exposed groups. No necrosis, degeneration, metaplasia, anaplasia in pneumocytes, atelectasis, or emphysema were detected [Fig. 14 (A-B)]. These results indicate that GPT-SPE functions safely and efficiently in aerosol delivery system. Significant anticancer effects of GPT-SPE/Akt1

shRNA complexes in the lungs through aerosol inhalation were observed in lung tumor bearing *K-ras*^{LA1} mice [Fig. 14 (B)] without toxicity [Table 2]. These result indicating that poly(β -amino ester)s (GPT-SPE) could be a safe and efficient gene carrier in aerosol-administered lung cancer gene therapy.

	Con	GPT-SPE
(A) Routine CBC		
WBC ($\times 10^3$ cells/ μ L)	6.71 \pm 2.06	5.41 \pm 0.97
RBC ($\times 10^6$ cells/ μ L)	9.89 \pm 0.66	10.26 \pm 0.55
HGB (g/dL)	13.37 \pm 1.30	13.47 \pm 1.54
HCT (%)	46.50 \pm 3.68	48.83 \pm 2.63
MCV (fL)	47.43 \pm 0.51	47.60 \pm 0.26
MCH (pg)	13.50 \pm 0.61	13.47 \pm 0.38
MCHC (g/dL)	28.73 \pm 0.64	28.40 \pm 0.44
CHCM (g/dL)	27.80 \pm 0.66	26.70 \pm 0.66
RDW (%)	16.33 \pm 1.37	16.43 \pm 0.40
HDW (g/dL)	1.97 \pm 0.31	1.87 \pm 0.04
PLT ($\times 10^3$ cells/ μ L)	2084.00 \pm 250.32	1778.33 \pm 637.72
MPV (fL)	8.70 \pm 0.30	8.60 \pm 0.10
(B) Platelet parameters		
PDW (%)	60.27 \pm 3.98	52.46 \pm 0.36
PCT (%)	1.57 \pm 0.30	1.39 \pm 0.55
MPC (g/dL)	18.90 \pm 0.36	16.36 \pm 0.70
MPM (pg)	1.39 \pm 0.02	1.20 \pm 0.04
Large Pit ($\times 10^3$ cells/ μ L)	47.43 \pm 0.51	41.63 \pm 0.26

CBC, complete blood count; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; CHCM, mean cell hemoglobin concentration; RDW, red cell distribution width; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; MPC, mean platelet component; MPM, mean platelet mass; Large Pit, large platelets.

Table 2. Toxicological analysis. Blood samples were collected for routine examination and to assess the potential toxicity of GTP-SPE. [Source from Ref. [34]].

2.5. Targeting considerations

Targeting confers another important criterion in gene delivery. To increase specificity and safety of gene therapy further, the expression of the therapeutic gene needs to be tightly controlled within the target tissue. Targeted gene expression has been analyzed using tissue-specific promoters (breast-, prostate-, and melanoma-specific promoters) and disease-specific promoters (carcinoembryonic antigen, HER-2/neu, Myc-Max response elements, DF3/MUC). Alternatively, expression could be regulated externally with the use of radiation-induced promoters or tetracycline-responsive elements [70]. Recently, Arote et al. coupled folic acid moiety to the poly(amino ester)s backbone using PEG (MW: 5000 Da) as a linker for targeting of folate receptor, a tumor associated glycosylphosphatidylinositol anchored protein [71]. As shown in Fig. 15, folate-PEG-poly(amino ester)s (FP-PAEs) showed marked anti-tumor activity against folate receptor-positive human KB tumors in nude mice with no evidence of toxicity

during and after therapy using the TAM67 gene. Anti-tumor activity with PAEs without folic acid moiety (PEGylated-PAEs, P-PAEs) proved ineffective against a xenograft mice model than that with FP-PAEs at the same dose, suggesting that FP-PAEs is a highly effective gene carrier capable of producing a therapeutic benefit in a xenograft mice model without any signs of toxicity.

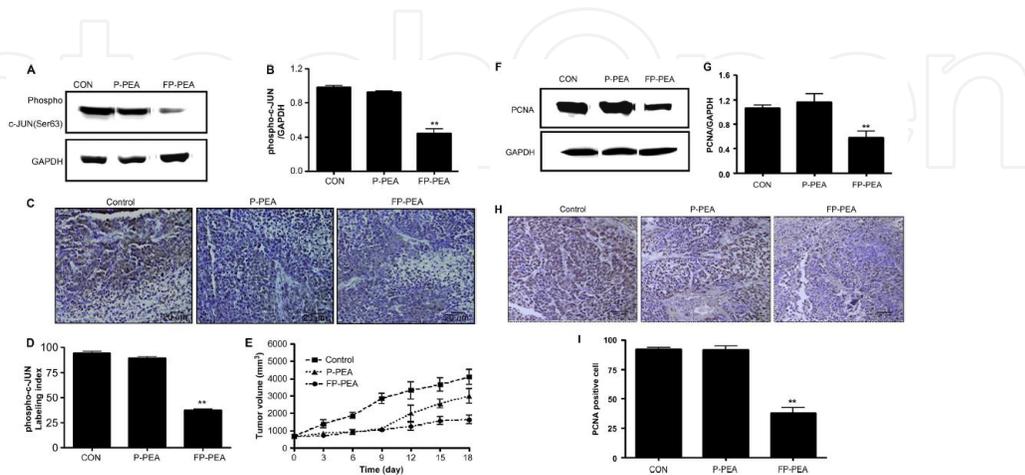


Figure 15. Effect of FP-PEAs/TAM67 complexes on tumor growth. The tumor volume in BALB/c mice bearing KB cells was recorded every 3 d. Tumor tissue homogenates were subjected to western blot analysis. Blots were probed with antibodies as indicated. (A) Expression level of phospho-c-Jun. (B) The bands-of-interest were further analyzed by densitometer. (C) Immunohistochemical analysis of phospho-c-Jun in the tumors. Dark brown color indicates the phospho-c-Jun expression (magnification, X 400; bar = 20 μ m). (D) Comparison of phospho-c-Jun labeling index in tumors. phospho-c-Jun positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB positive cell per 100 cells at X 400 magnification. (E) Suppression of tumor growth by FP-PEAs/TAM67 complexes (F) Expression level of PCNA. (G) The bands-of-interest were further analyzed by densitometer. (H) Immunohistochemical analysis of PCNA in the tumors. Dark brown color indicates the PCNA expression (magnification, X 400; bar = 20 μ m). (I) Comparison of PCNA labeling index in tumors. PCNA positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB positive cell per 100 cells at X 400 magnification. (*, $P < 0.05$; **, $P < 0.01$ compared with control; #, $P < 0.05$; ##, $p < 0.01$ compared with vector control; $n = 4$). [Source from Ref. [71]].

3. Conclusion

Gene therapy shows tremendous promise for a broad spectrum of clinical applications. Development of a safe and efficient gene delivery system is one of the main challenges to be solved before this strategy can be adopted for routine use in clinical trials. As a degradable cationic polymeric gene carrier, poly(amino ester)s comprise many desirable properties in the context of gene delivery, including condensation of DNA into nanoscale-size particles and protects DNA from endogenous nucleases and efficiently deliver DNA with low toxicity. The need for clinical application of poly(amino ester)s, more comprehensive preclinical investigations such as exact quality control (QC) of polymer, pharmacokinetics and toxicological studies should be performed.

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