Gene Therapy (2005) 12, S139–S145
© 2005 Nature Publishing Group All rights reserved 0969-7128/05 $30.00
www.nature.com/gt

CONFERENCE PAPER

Intelligent polymers as nonviral vectors

S Dinçer[1,2], M Türk[1,2] and E Pişkin[1,2]
[1]*Chemical Engineering Department and Bioengineering Division, Hacettepe University, Ankara, Turkey; and*
[2]*TÜBİTAK-ÜSAM-Biyomedtek, Hacettepe University, Ankara, Turkey*

The successful gene therapy largely depends on the vector type that allows a selective and efficient gene delivery to target cells with minimal toxicity. Nonviral vectors are much safer and cheaper, can be produced easily in large quantities, and have higher genetic material carrying capacity. However, they are generally less efficient in delivering DNA and initiating gene expression as compared to viral vectors, particularly when used in vivo. As nonviral vectors, polycations may work well for efficient cell uptake and endosomal escape, because they do form compact and smaller complexes with plasmid DNA and carry amine groups, which give positive charge and buffering ability that allows safe escape from endosome/lysosome. However, this is a disadvantage in the following step, which is releasing the plasmid DNA within the cytosol. In order to initiate transcription and enhance gene expression, the polymer/plasmid complex should dissociate after releasing from endosome safely and effectively. There are also other limitations with some of the polycationic carriers, for example, aggregation, toxicity, etc. Intelligent polymers, also called as 'stimuli responsive polymers', have a great potential as nonviral vectors to obtain site-, timing-, and duration period-specific gene expression, which is already exhibited in recent studies that are briefly summarized here.
Gene Therapy (2005) **12,** S139–S145. doi:10.1038/sj.gt.3302628

Keywords: *nonviral vectors; intelligent polymers*

Introduction

Today, there is a strong tendency in modern therapies in which several biological molecules including nucleic acids (plasmid DNA, antisense oligonucleotides, therapeutic RNAs, siRNAs, etc) and proteins (monoclonal antibodies, growth factors, hormones, therapeutic enzymes, synthetic oligopeptides, etc) are being used as highly specific pharmaceutical agents. Gene therapy aims to treat diseases by intracellular delivery of nucleic acids that alter gene expression within a specific cell population thereby manipulating cellular processes and responses. Although it was originally devised for the treatment of inherited genetic disorders, recent work has expanded the applications of gene therapy to develop strategies for treatment of a wide range of metabolic, infectious, and inflammatory diseases.[1–3]

All these novel therapeutics including plasmid DNA carrying genetic information are 'fragile', in other terms they are faced with biodegradation within the body before they reach their target. A 'carrier vehicle' or a 'vector' is needed that allows targeted and intracellular delivery. There are many major barriers for these vehicles to reach the target cells and for intracellular trafficking. For *ex vivo* gene delivery, mechanical techniques including gene gun, hydrostatic pressure, electroporation, continuous infusion, and sonication can be used. They provide a relatively high gene transfer efficiency *ex vivo*; however, these procedures are costly and may not be appropriate for all situations. Application of these techniques *in vivo* is certainly difficult. In most of the cases it is not possible, or even if it is possible is required surgery, which is of course not very desirable. Reaching the target cells especially *in vivo* gene delivery is an unmet need today. There are a number of approaches under investigation, and the most important and futuristic one is using targeting molecules (eg, bioligands) that are attached onto the carrier vehicle and allow it to be directed to the target cells via several body compartments. Oligopeptides are among the most attractive bioligands having very specific biorecognition ability to the specific receptors that exist (or may be created) on the target cell population. These targeting peptides can be selected from the peptide libraries by novel techniques like phage display or maybe some other more futuristic techniques (eg use of some novel 'cell-chips'), and are synthesized synthetically. They may not only direct the vehicles to the target cells but also bring about their effective and specific uptake by the cells.

Viruses are quite effective gene delivery vectors. Some types have the ability to find some specific cell populations within the body, or even some targeting bioligands can be attached on their surfaces for better and more specific delivery. They have evolved a specific machinery to deliver DNA into cells and even into the nucleus. There are also several recurring issues that have led to a reconsideration of their use in human clinical trials. These include the ability of some viral vectors to integrate their DNA with the host genome and permanently alter its genetic structure, which may also be a random integration into the host chromosome. This could lead to an activation of oncogenes or an inactivation of tumour suppressor genes, in other terms insertional mutagenesis that has already been observed in

Correspondence: Professor E Pişkin, Chemical Engineering Department and Bioengineering Division, Hacettepe University, Beytepe, 06800 Ankara, Turkey and TÜBİTAK-ÜSAM-Biyomedtek, Beytepe, 06800 Ankara, Turkey

some applications. It should also be noted that there are still considerable immunological problems with viral vectors. Another current drawback is the carrying capacity of the viral vectors, in other words the limitation on the amount of genomic information that can be introduced into these vectors. It is believed that all the existing viral vectors need to be re-evaluated and further modified with novel targeting molecules to generate a safer, more specific and efficient viral gene delivery, and most importantly with sustained expression.

Nonviral vectors

Nonviral vectors made of lipids, peptides, and polymers have been receiving increasing attention, since they are much safer and cheaper, can be produced easily in large quantities, and have higher genetic material carrying capacity. However, they are generally less efficient in delivering DNA and initiating gene expression as compared to viral vectors, particularly when used *in vivo*. There is a great interest in developing these vehicles for more effective gene delivery.

Polycations have also attracted a lot of attention as nonviral vectors. Polycations used for gene therapy studies to date include poly(L-lysine), poly(L-ornithine), both linear and branched polyethyleneimine) (PEL), diethylaminoethyl-dextran, poly(amidoamine) dendrimers, and poly(dimethylaminoethyl methacrylate).[1–6] They usually carry protonable amine groups, which gives positive charge, and therefore form particulated complexes (or 'condensates') with negatively charged DNA enabling its effective transport through the negatively charged cell membrane, usually by endocytosis. In addition, the amine groups exhibit a buffering effect (also called the 'proton sponge' effect) in the endosome in which as a result of the pH-mediated influx of chloride ions, osmotic swelling and rupture lysosome/endosome occur, which in turn allows the vector and its cargo to be safely released in the cytosol.[7]

The properties of DNA condensates formed by polycations depend on some parameters such as the type of the polycation and N/P ratio of the complex that are resulting condensates having different shapes (eg, toroidal, spheroidal, or even amorphous shapes) and size and different surface charges from about −40 to +40 mV (as 'zeta potential'). Note that polycation and DNA may form larger condensates, which include more than one polymer chain and/or plasmid DNA. These condensates do aggregate as their concentration increases, and are quickly precipitated above their critical flocculation concentration. This causes an important problem since nonviral gene delivery systems at therapeutic doses require high concentrations of polymer-plasmid DNA complexes. Here, the preparation protocol and conditions, such as pH, concentration of salt (ionic strength) and type (usually NaCl), and temperature, gains importance to overcome this problem. Thus, the way of adding the polycation solution to the DNA solution (or *vice versa*), the DNA and salt upon mixing, diluting the complexes after their preparation, all influence the aggregation. The stabilization of condensates is necessary for extended circulation times that are required to target particular cell types. In order to circumvent the aggregation problem, hydrophilic polymers like polyethylene glycol (PEG) have been used to create PEGylated particles to provide steric stabilization by decreasing self and nonself, nonspecific interactions.[8,9] However, it is not easy to make a generalization for the prevention of aggregation since there is no optimal strategy to do this. Therefore, a related procedure should be evaluated for each system seperately and applied for appropriate situations.

One of the limitations of nonviral gene delivery systems is their toxicity, and therefore much current work is involved in preparing carriers that have lower toxicity. For example, recent evidence shows that low molecular weight preparations of polycations such as chitosan, polyethyleneimine (PEL) and β-cyclodextrin-containing polymers are significantly less toxic than high molecular weight polycations both in cultured cells and in animals.[10–12] Additionally, the distance between charge centres along the backbone of a polycation has been shown to affect the toxicity.[12] Thus, the molecular architecture of the nonviral delivery system can modulate the toxicity, and these data suggest that the toxicity should be controllable.

Vectors should also release safely their content (eg, plasmid DNA) within the cytosol, better to carry it into the final destination, which is in nucleus in the case of gene therapy. As nonviral vectors, polycations may work well for efficient cell uptake and endosomal escape, because they do squeeze the plasmid DNA and enhance even further the effectiveness of these steps as mentioned before due to more compact and smaller complex formation and amine groups that they carry, which gives positive charge and buffering ability. However, this is a disadvantage in the following step, which is releasing the plasmid DNA within the cytosol. In order to initiate the transcription and enhance gene expression, the polymer/plasmid complex should dissociate after releasing from the endosome safely and effectively. As also discussed by Yokoyama in his recent review, it seems almost impossible to fulfill these two opposing phenomena: tight complex formation and ease of complex dissociation.[13] In other words, an 'intermediate' tightness is not possible with conventional cationic gene carrier systems and this situation can be overcome by using intelligent vectors that can possess two contrary functions simultaneously, a tight complex can be formed to ensure evading DNA degradation and high cellular uptake, but at the later step, by introducing a stimulus that can maximize by complex dissociation for high transcription, as described in the examples given below.

Intelligent polymers

Polymers that exhibit large, sometimes discontinuous changes in their physical state or properties to small changes in environmental stimuli are often called 'intelligent' or 'smart' polymers. They are also known as 'stimuli-responsive', or 'environmentally sensitive' polymers. These polymers may be dissolved in aqueous solution, adsorbed or grafted on aqueous–solid interfaces, or crosslinked in the form of hydrogels.[14] Many different stimuli such as changes in temperature, pH, or ionic strength, using solvents, applying electrical field, magnetic field, or radiation result in a response that may cause changes in phase, size/shape, reactivity,

permeability, surface wettability, etc. For instance, change of temperature may result in precipitation of the intelligent polymer from its solution; the surface-adsorbed intelligent polymer may collapse, which consequently changes surface wettability; or hydrogels made of intelligent polymers may shrink. It should be noted that most of these changes are reversible; therefore, they return to their original state when the stimulus is removed.

pH- and temperature-responsive polymers are the two most popular members of the intelligent polymer systems. Polymers having pH responsivity character are generally consist of a the hydrophobic monomer and an ionizable comonomer having more hydrophilic nature. Change in pH and therefore in the net charge causes the phase change depending on hydrophobic and hydrophilic balance of the copolymer. Typical examples are the copolymers of methylmethacrylate (MMA) with methacrylic acid (MAc) or dimethylaminoethyl methacrylate (DMAEMA). MMA is the hydrophobic section while MAc is the hydrophilic part of the chains. MAc is hydrophilic at high pH when COOH groups are deprotonated, but becomes more hydrophobic when -COOH groups are protonated. The phase change occurs around the p*K* value of -COOH groups, which is around 4.5–5.5. The copolymers of MMA with DMAEMA, which is hydrophilic at low pH, when amino groups are protonated but more hydrophobic when amino groups are deprotonated. These copolymers are soluble at low pH but precipitate at slightly alkaline conditions.

Temperature-sensitive polymers undergo a temperature-induced precipitation. Temperature sensitivity of these polymers depends on the interaction (H-bonding) between polymer molecules with water. An increase in temperature reduces the efficiency of hydrogen bonding, and phase separation of the polymer takes place when the efficiency of hydrogen bonding becomes insufficient for the solubility of the polymer. Once the temperature goes beyond a certain critical temperature, which is known as the 'lower critical solution temperature' (LCST), also referred as 'cloud point', phase separation takes place, and the polymer chains change from water-soluble coils to water-insoluble globules. Poly(*N*-isopropylacrylamide) (poly(NIPA)), is the most extensively studied polymer among those exhibiting temperature-induced phase seperation. Poly(NIPA) is soluble in water below its LCST value (32°C), as favorable interactions via hydrogen bonding between amide groups of polymer and water molecules lead to dissolution of polymer chains. Increasing temperature above this value causes breaking of H-bonds and a tendency of polymer molecules to leave the solution depending on the water molecules expelled from polymer chains. The LCST value of poly(NIPA) can be adjusted by copolymerizing it with monomers having different hydrophilicity. If NIPA copolymerizes with more hydrophilic monomers such as acrylamide, then the LCST increases and may even disappear. If NIPA monomer is copolymerized with more hydrophobic monomers, such as *n*-butyl acrylamide, the LCST decreases. Adding hydrophilic and charged comonomers (eg, acrylic acid (AAc) or DMAEMA) into the polymeric structure can make the resulting copolymer both temperature and pH responsive.

Light-sensitive systems have been used in diverse applications for the development of novel intelligent materials and systems. Here the idea is the change in the conformation of the molecules induced by the photo-promoted or thermally promoted isomerization which enables it to tailor the physical and chemical properties, including viscosity, refractive index, conductivity, pH, solubility, wettability, mechanical properties, polymer morphology, etc. Following this general idea, chemists have developed photo-responsive polymers possessing very attractive characteristics. These kinds of polymers include a photosensitive moiety as a side chain or in the main chain like azobenzene chromophore group. Azobenzene is very a popular molecule that undergoes an isomerization as response to UV light. This phenomenon is reversible and it turns back to the original *trans* isomer state upon irradiation with visible light; therefore, it has been used in many light-responsive polymeric systems.[15]

Intelligent polymeric vectors in gene therapy

As mentioned above, intelligent polymeric vectors have a great potential to obtain site-, timing-, and duration period-specific gene expression, which is already exhibited in the recent publications of different groups including the author's, and are very promising, briefly summarized below. Note that in these studies, pH-, temperature-, and light-sensitive groups have been introduced (incorporated) into polycationic carriers for controlling both intracellular uptake and release of the plasmid DNA within the cytosol for more effective gene delivery.

Hinrichs *et al*[16] have synthesized thermo-responsive copolymers of NIPA (for temperature sensitivity) and DMAEMA (for positive charge/amino groups) and investigated the transfection efficiency in ovarian cancer cells. They prepared a series of copolymers with various monomer ratios and molecular weights by free radical polymerization in organic or aqueous media. Both homo- and copolymer/plasmid DNA complexes were prepared, and effects of complexation on LCST value, particle size, and zeta potential were evaluated. They reported that below a certain polymer/plasmid DNA ratio, the complexes formed aggregates, and the temperature dependency was lost because of this aggregation. Appropriate complexation having temperature sensitivity was achieved above a certain polymer/plasmid DNA ratio. However, transfection efficiency was decreased with an increase in polymer/plasmid DNA ratio because toxicity of polycation chains remained unoccupied. As an expected tendency, reducing zeta potential caused a decrease not only in toxicity but also in transfection. They also observed an interesting effect of NIPA, that is, a masking of toxicity of DMAEMA. They stated that low cell uptake caused by decreased zeta potential depending on NIPA content can be an interesting approach for the design of targeted systems. Although change in molecular weight affected the a size of the complex, molecular weight of polymers did not affect transfection efficiency as long as stable complexes was produced. It was estimated that complexes with size of around 200 nm was prerequisited for efficient transfection. Note that the complete covering of plasmid DNA by polymer chains is required for maximal transfection. They concluded that high molecular weight copolymers were efficient at compacting DNA into

particles that transfected ovarian cancer cells, although no direct correlation between transfection efficiency and polymer LCST was found.

Kurisawa *et al*[17] developed a terpolymeric gene carrier system composed of a thermo-responsive unit (NIPA), a cationic unit (DMAEMA), and a hydrophobic unit (BMA), and evaluated its transfection efficiency at different incubation temperatures in COS1 cells *in vitro*. Random copolymers were synthesized by radical polymerization with different compositions. Here, the solubility of terpolymer/DNA complexes is probably regulated by both ionic and hydrophobic interactions, due to the existing DMAEMA and BMA, respectively. They investigated the effect of complexation on LCST value, and estimated that a terpolymer containing 8 mol% of DMAEMA and 11 mol% of BMA had a phase transition temperature of 21°C, which was found to be the same after complex formation with DNA. They showed the partial dissociation of terpolymer/DNA complexes below LCST but not above this temperature, suggesting that the formation/dissociation of the complexes was also modulated by temperature. Transfection experiments were performed at the following two different conditions: (1) 47 h at 37°C and (2) 20 h at 37°C+3 h at 20°C+24 h at 37°C. While the transfection efficiency of poly(DMAEMA) homopolymer decreased with decreasing temperature, poly(IP-9DA) (91 mol% NIPA, 9 mol% DMAEMA) showed only a few transfected cells in both cases. Furthermore, poly(IP-8DA-11BM), (81 mol% NIPA 8 mol% DMAEMA, 11 mol% BMA) showed considerable numbers of transfected cells indicating that the hydrophobic unit (BMA) contributed to the transfection. The positive effect of temperature on transfection efficiency was explained as owing to complex dissociation that occured at the temperature, which was very close to the LCST of the terpolymer (21°C). In short, the transfection efficiencies of terpolymer/DNA complexes incubated at lower temperatures were much higher than for those incubated at higher temperatures even for longer times. In their later study, they also obtained two more compositions of the carrier polymers showing temperature-controlled gene expression, and investigated the effect of complex preparation temperature on gene expression of COS1 cells *in vitro*.[18] They reported that the complexes prepared at higher temperatures (37 and 45°C) than the phase transition temperature (21°C) resulted in higher enhancement by lowering of cell incubation temperature than the complexes prepared at room temperature near LCST. The authors concluded that a new concept for gene delivery, gene expression control by temperature, is very important for the future of gene therapy.

Twaites *et al*[19] have prepared a range of cationic polymers including derivatives of branched PEI containing short hydrophobic side chains (ie, octanamide), copolymers of PEI and poly(NIPA), and polymers containing different amounts of NIPA, DMAEMA, and (HA) (hexylacrylate). All these polymers were soluble at pH 7.4 in PBS, while only PEI-poly(NIPA) copolymers and poly(NIPA/DMAEMA/HA) terpolymers exhibited temperature sensitivity with somewhat broader phase transitions in which the LCST values were in between 22 and 50°C. Fluorescence spectroscopy, gel retardation assays, dynamic light scattering, and atomic force microscopy were used to characterize the binding of plasmid DNA (a double strand plasmid pX61 with 6144 base pairs) to these materials. Ethidium bromide displacement assay showed the enhanced affinity of PEI-octanamide (comparing to PEI) to DNA in the complex as a result of hydrophobic interactions of the alkyl side chains with the charge neutralized DNA and competition with the dye for hydrophobic intercalation sites. Variable temperature assays indicated almost no change in affinity to DNA of PEI and PEI-octanamide, whereas the other copolymers containing NIPA appeared at a slightly reduced extent in complexation at temperatures above their LCST. In summary, however, they observed no large differences in the complexation tendency of polymer with DNA as a result of changes in polymer phase behaviour. The thermoresponsive polymers also exhibited changes in particle morphology across the same temperature ranges with polymer–DNA complexes prepared at N/P ratios of 2:1 generating spherical particles varying in radius between 30–70 nm at 25°C and 60–100 nm at 40–45°C. More stable complexes were achieved at temperatures below the LCST (for the complexes formed with PNDHA1). Although this, the complexes were differed in terms of size-to-charge ratio when run at a higher temperature. That means polymer phase transitions taking place after the complexation event were still able to alter the structures of the complexes. All the responsive polymer complexes increased in diameter at the polymer LCSTs and then contracted above the temperature by which the polymer would be expected to complete an LCST transition. Preliminary transfection experiments indicated that all the polymers in this study were effective in transporting plasmid DNA to cell nuclei, while thermoresponsive polymers also achieved low levels of protein expression in mouse muscle C2C12 cells. The results suggested that synthesis of thermoresponsive polymers with the appropriate functionality and molecular architectures may allow the compression of plasmid DNA reversibly into particles that can be rapidly taken up by cells and which may ultimately also exhibit enhanced properties as gene delivery vehicles.

Nagasaki *et al*[20] synthesized a novel water-soluble polyazobenzene dendrimer modified with L-lysine at the periphery and investigated interactions of this polycation with plasmid DNA that is photoregulated by radiation. The light-scattering and gel filtration chromatography studies that showed the particle size is controllable by UV and visible light irradiation. The affinity of this cationic dendrimer toward DNA was photo-controllable due to changes of zeta potential as a result of alteration in the dendrimer's surface amine groups (coming from L-lysin residues). In the *in vitro* transfection studies with COS1 cells, interestingly, UV light irradiation after the polymer/plasmid condensate was taken up in the cells caused about 50% increase in the transfection efficiency, which was explained by the UV radiation promoted dissociation of the complex in the cytoplasm. They concluded that light is superior in terms of temporal and spatial manipulability as stimulation source and if light irradiation can destabilize endocytic vesicle membranes to improve escape of an agent from the vesicles and delivery of the agent into the cytosol, an ideal intracellular delivery system could be developed.

Nagasaki *et al*[21] have also studied cationic lipids having a photoisomerizable azobenzene structure

('KAON12') as gene delivery vectors. This lipid base carrier has a lysine residue (hydrophilic and carriers amino groups) and a didodecylamide structure (hydrophobic region). The observations with TEM showed that the carrier is in the form of small unilameller vesicles (SUVs), approximately 20 nm in size. However, the azobenzene structure undergoes *trans* to *cis* isomerization, and lamellar structures appear due to membrane fusion by applying UV irradiation. Their transfection studies that were performed *in vitro* with COS1 cells showed that even without UV radiation, transfection efficiency of their liposomes was about twice than those observed with Lipofectin® (a commercially available cationic lipid gene transfecting agent) that they have also used in their parallel studies. UV irradiation further improved the transfection efficiency of KAON12. Finally, they confirmed that the transfection efficiency of this novel photoresponsive cationic lipid having an azobenzene structure can be controlled by UV irradiation in which the liposomic and cationic nature of poly(lysine) was used for internalization through the cell membrane. *Trans* to *cis* isomerization of the azobenzene structure caused destabilization the endosome membrane since it increased in size.

Another approach for light-induced enhancement of gene transfection is based on the use of photosensitizing compounds, which localize in the membranes of endocytic vesicles and, upon activation by light, induce photochemical reactions, leading to the permeabilization of the vesicular membranes and cytosolic release of the vesicular content, for example, transfecting DNA.[22,23] It was claimed that cytosolic delivery facilitated by the photochemical treatment not only helps the transfecting DNA to avoid the degradative enzymes present in endocytic vesicles, but should also increase the chances for nuclear entry, since it increases the amount of cytosolic DNA available for nuclear transport. It was demonstrated that photochemical treatment, inducing permeabilization of the endocytic vesicles and liberation of the entrapped transfecting genes, can substantially increase the efficiency of gene transfection mediated by nonviral vectors.[24,25] However, in spite of significant increase, they were not able to transfect the entire population of cells compared to the results obtained for photochemically enhanced adenovirus-mediated gene delivery.[26] By relying on the idea that the effectiveness of nonviral transfection systems depends on the cell cycle status,[27] they investigated the role of the cell cycle status in photochemical transfection mediated by two types of synthetic transfection agents: a cationic polypeptide polylysine (polyfection) and a cationic lipid formulation *N*-(2-amino ethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidyl ethanol amine.[28] The effect of photochemical treatment on EGFP expression in cells illuminated in different cell cycle phases was compared. In conclusion, in human colon carcinoma HCT 116 cells, photochemical treatment stimulated gene transfection, especially polyfection. They estimated that the photochemical transfection mediated by the cationic polypeptide was dependent on the cell cycle phase when illumination was performed. The cell cycle dependence of photochemical transfection mediated by the cationic lipids was very low. It was gathered that illumination performed during the G2/M phase led to the highest level of transfection. They hypothesized that the transgene, liberated into the cytosol by light during or close to mitosis, has the highest opportunity to enter the nucleus and be expressed. However, it was found that photochemical treatment affected different cellular processes, which might limit the efficiency of photochemical transfection as well as influence the relationship between photochemical transfection and the cell cycle.

Recently, a series of water-soluble poly(*N*-isopropylacrylamide-block-polyethyleneimine) copolymers have been synthesized and applied in *ex vivo* transfection of both HeLa cell lines and primary cells in the author's laboratories, which are briefly given here.[29–31] Poly(NIPA)PEI block copolymers were synthesized by using carboxyl-ended poly(NIPA) and PEI (both branched 'B' and linear 'L' and with different molecular weights) in the presence of an activating agent (EDAC). Copolymerization of poly(NIPA) chains with more hydrophilic PEI chains caused observable increases in the LCST of the homopolymer from 31°C to around body temperature (36–39°C). The relative size of the blocks on the copolymer chains was reported as an important parameter in the resultant hydrophobicity of the copolymers, and therefore their LCST values. PEGFP-N2, which carries a gene that expresses a green fluorescent protein, was used as model the plasmid DNA and was condensed with the block copolymers due to the interaction of the positively charged PEI blocks in the copolymer and negatively charged plasmid DNA. Sizes and zeta potentials of the homo- and copolymers, plasmid and copolymer–plasmid DNA complexes were measured at 25 and 37°C using a Zetamaster HSA3000 (Malvern Instrument, France). Plasmid DNA was negatively charged with a zeta potential of −21 mV. Zeta potential values of the copolymers increased with both increase in the chain length of PEI used and also with branching. The zeta potentials of the copolymer/plasmid DNA were between −3.1 and +21.3 mV. Higher values were observed for the complexes prepared with branched and higher molecular weight PEIs. Particle sizes of the polymer/plasmid complexes were in the range of 190–992 nm. The smallest complexes were obtained with the copolymer prepared with branched PEI with 25 kDa molecular weight. Copolymers were able to squeeze plasmid DNA more at the body temperature due to phase transition of the copolymer.

Human cervix epithelioid carcinoma cell line (HeLa) and two primary cells namely human umbilical vein endothelial cell and rabbit aortic smooth muscle cells, were used *in vitro* cell culture and transfection studies. Cytotoxicity of the homo- and copolymers used in these studies were investigated, and it was found that branched PEIs especially with higher molecular weights were more cytotoxic. Copolymerization reduced the cytotoxicities. Primary cells (especially endothelial cells) were more sensitive to the polymers compared to the cell line. The naked plasmid DNA molecules were not able to enter the cells as expected, while transfection efficiencies in the range of 5–70% were observed with polymer/plasmid complexes that we have used. Transfections with linear PEI homopolymer (molecular weight 2 kDa) were around 10–20%. Much higher transfections were reached with the complexes prepared with the copolymers with higher molecular weights (25 kDa), especially

in the case where the branched PEI was used. From this part of the study, it was concluded that complexes prepared with copolymers with about 25 kDa PEI blocks are highly effective for transfection of cells *in vitro* with high efficiencies. A positive charge around 10–14 mV and complex size in the range of 200–300 nm seem optimal to reach high transfection efficiencies. The complexes prepared with the linear PEI were less effective compared to the ones prepared with the branched PEI. However, the latter one exhibits higher cytotoxicity; therefore, the linear one seems the best.

The GFP expression efficiencies were in the range of 5–35% with polymer/plasmid complexes. It means that the complexes can enter into the cells, but the plasmid cannot find the host DNA and integrate with it for GFP expression. Rather low transfection efficiencies were observed with the copolymer prepared with low molecular weight PEI (poly(NIPA)/PEI2B) in parallel to low transfection efficiencies. The most successful gene expressions were achieved with the poly(NIPA)/PEI25L; about 35% expression (the maximum) was observed at a polymer/plasmid ratio of 6. Note that this is almost half of the transfection efficiency (around 60%) observed with the same complex and with the same polymer/plasmid ratio. However, surprisingly, gene expressions reached with the high molecular weight branched copolymer were around 20%, compared to about 70% transfections (which was the maximum) observed with the complexes prepared with this copolymer. Gene expressions were lower with the primary cells, especially with endothelial cells. According to these results, it was concluded that the complexes prepared with copolymers with linear PEI with a molecular weight of 25 kDa are highly effective for transfection of cells *in vitro* with high efficiencies. A positive charge around 10–14 mV and a complex size in the range of 200–300 nm seems optimal to reach high transfection efficiencies. In spite of the lower gene expression (comparing to corresponding transfection efficiencies), the complexes prepared with a poly(NIPA)/PEI copolymer with a polymer/plasmid ratio of 6 seem to be a safe (low cytoxicities) and therefore a quite satisfactory potential polycationic nonviral vector system to the alternative existing ones.

As presented above, the copolymer has a temperature sensitivity because of NIPA, and the incorporation of PEI blocks into poly(NIPA) increased the LCST temperature from 31 to 37°C. It means that at 37°C the copolymer is in a more compact state, nicely condenses the plasmid DNA and therefore allows high cell uptake. However, the gene expression efficiency achieved is rather low (around 30%) as the copolymer does not allow DNA for an efficient gene expression. Here, it is necessary to release DNA from this compact copolymer structure. In order to use the benefits of intelligence of the copolymers synthesized, after 3 h of incubation at 37°C, the cell culture medium temperature was reduced to 28°C and held for about 45 min (which was decided after several trials) at this lower temperature for dissociation of the complexes. Note that at this temperature the copolymer chains became more soluble and reached in an extended form; therefore, dissociation of the gene expression plasmid in the cytoplasm occurred, which in turn resulted in better expression. A very significant increase in the GFP expression efficiency from 30 to 50% was observed.

Conclusion and future perspectives

Gene therapy is receiving great attention not only as a treatment of inherited genetic disorders, but also to develop strategies for treatment of a wide range of metabolic, infectious, and inflammatory diseases. A 'vector' is certainly needed, which allows targeted and intracellular delivery of the plasmid DNA that carries the gene of interest, that is certainly one of the most important and exciting futuristic challenges. Mechanical techniques can be used in *ex vivo* transfection of cells with high gene transfer efficiency; however, these procedures are costly and may not be appropriate for all situations, especially *in vivo* transfections. Reaching the target cells especially *in vivo* gene delivery is an unmet need today. There are a number of approaches under investigation, and the most important and futuristic one is using targeting molecules (eg, bioligands) that are attached onto the carrier vehicle and allow it to be directed to the target cells via several body compartments. Viruses are quite effective gene delivery vectors. Some types have the ability to find some specific cell populations within the body. However, they do have important drawbacks, including unsafe and limited gene carrying capacity. There is certainly great interest for developing new generations of viral vectors, that will not only find the target cells, but will also put the gene in the correct position on the host genome. We may be pioneers of designing much safer and effective viral vectors in the coming years.

One of the most important advantages of nonviral vectors over viral vectors is their great potential for producing large quantities with rather controllable structures at required GMP conditions, since they are simply polymers. Different types of monomers can be selected to synthesize polymers with quite defined chemical structures, with optimum and required hyrdophilicities, therefore solubilities (which also describes their three-dimesional molecular confirmations), in aqueous environment, and with positive charges for both effective condensation with negatively charged plasmid DNA and transfer it to the host cells. If the polymerizations are conducted at controlled conditions, it is possible to produce polymers with desired chemical structures and molecular weights/polydispersity indexes. Polymer technology has been highly developed to produce polymers with almost any quantitity in large-scale polymerization reactors. Purification of these polymers is much more easy and inexpensive compared to the purification of the viruses even for direct clinical applications.

Nonviral vectors are much safer to use, but less effective than viral ones today. There is great interest in developing these vehicles for more effective gene delivery. Besides the targeting strategies mentioned above for reaching the target cells, they have to be specifically takenup by these cells, which is mainly by endocytosis. The targeting ligands that are attached on to the vectors may allow or trigger this specific uptake. In some cases, such as polycationic carriers, a positive charge may cause or enhance the uptake. Endosomal escape is necessary before lysosomal activity, which causes a lots (degradation) of the gene (nucleic acid). Avoiding lysosomal activity is another important problem in intracellular delivery that needs to be solved,

in order to increase gene delivery, especially by using nonviral vectors. As nonviral vectors, polycations may work well for efficient cell uptake and endosomal escape, because they form compact and smaller complexes with plasmid DNA and carry amine groups, which give positive charge and buffering ability that allows safe escape from endosome/lysosome. However, for effective transfection, the vector should release the plasmid DNA in the cytosol. Due to tight complex formation, dissociation is difficult and should be enchanced. Intelligent polymeric carriers produced by introducing pH, temperature, and light sensitive groups onto the polymer chains may have a critical role in targeted delivery and intracellular trafficking of plasmid DNA even at this releasing stage and are very promising, as has already been exhibited in some recent studies and also briefly reviewed above. Further developments are certainly needed in this direction, especially on local control of the environmental conditions (pH, temperature, light intensity, etc), which will allow to use the ability of the intelligent polymers, or in other terms their intelligency, which will certainly appear in the coming years.

Acknowledgements

Professor Erhan Pişkin is supported by the Turkish Academy of Sciences as a full member.

References

1 Choi YHF *et al*. *In vivo* delivery to tumors of DNA complexed with linear poliethylenimine. *Hum Gene Ther* 1999; **10**: 2657–2665.
2 Bielinska AU, Kukowskalatallo JF, Baker JR. Regulation of gene expression with double-stranded phosphothioate oligoncleotides. *Biochim Biophys Acta* 1997; **1353**: 180–190.
3 Boussif O, Zanta MA, Behr JP. Optimized galenics improve *in vitro* gene transfer with cationic molecules up to 1000-fold. *Gene Therapy* 1996; **3**: 1074–1080.
4 Erbacher P *et al*. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. *Pharm Res* 1998; **15**: 1332–1339.
5 Ferrari S *et al*. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells *in vitro* and *in vivo*. *Gene Therapy* 1997; **4**: 1100–1106.
6 Smith LC *et al*. Humoral and cellular immune responses of dogs immunized with a nucleic acid vaccine encoding human carcinoembryonic antigen. *Adv Drug Deliv Rev* 1998; **30**: 115–131.
7 Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci USA* 1999; **96**: 5177–5181.
8 Ogris M *et al*. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy* 1999; **6**: 4595–4605.
9 Kwoh DY *et al*. Stabilization of poly-L-lysine /DNA polyplexes for *in vivo* gene delivery to the liver. *Biochim Biophys Acta* 1999; **1444**: 171–190.
10 Richardson SCW, Kolbe HVJ, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. *Int J Pharm* 1999; **178**: 231–243.
11 Fischer D *et al*. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res* 1999; **16**: 1273–1279.
12 Hwang S, Bellocq N, Davis ME. Effects of structure of β-cyclodextrin-containing polymers on gene delivery. *Bioconjugate Chem* 2001; **12**: 280–290.
13 Yokoyama M. Gene delivery using temperature responsive polymeric carriers. *Drug Discovery Today* 2002; **7**: 426–432.
14 Hoffman AS. Bioconjugates of intelligent polymers and recognition proteins for use in diagnostics and affinity separations. *Clin Chem* 2000; **46**: 1478–1486.
15 Shimoboji T *et al*. Photoresponsive polymer-enzyme switches. *Proc Natl Acad Sci USA* 2002; **99**: 16592–16596.
16 Hinrichs WLJ *et al*. Thermosensitive polymers as carriers for DNA delivery. *J Control Release* 1999; **60**: 249–259.
17 Kurisawa M, Yokoyama M, Okano T. Gene expression control by temperature with thermo-responsive polymeric gene carriers. *J Control Release* 2000; **69**: 127–137.
18 Yokoyama M, Kurisawa M, Okano T. Influential factors on temperature-controlled gene expression using thermoresponsive polymeric gene carriers. *J Artif Organs* 2001; **4**: 138–145.
19 Twaites B *et al*. Thermo and pH responsive polymers as gene delivery vectors:effect of polymer architecture on DNA complexation *in vitro*. *J Control Release* 2004; **97**: 551–566.
20 Nagasaki T *et al*. Synthesis of a novel water-soluble poliazobenzene dendrimer and photoregulation of affinity toward DNA. *Mol Cryst Liq Cryst* 2000; **345**: 227–232.
21 Nagasaki T, Wada K, Tamagaki S. Photo-enhancement of transfection efficiency with a novel azobenzene-based cationic lipid. *Chem Lett* 2003; **32**: 88–89.
22 Berg K *et al*. Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. *Cancer Res* 1999; **59**: 1180–1183.
23 Hogset A *et al*. Photochemical transfection: a new technology for light-induced, site-directed gene delivery. *Hum Gene Ther* 2000; **11**: 869–880.
24 Prasmickaite L *et al*. Role of endosomes in gene transfection mediated by photochemical internalisation(PCI). *J Gene Med* 2000; **2**: 477–488.
25 Prasmickaite L, Hogset A, Berg K. Evaluation of different photosensitizers for use in photochemical gene transfection. *Photochem Photobiol* 2001; **73**: 388–395.
26 Hogset A *et al*. Light-induced adenovirus gene transfer, an efficient and specific gene delivery technology for cancer gene therapy. *Cancer Gene Ther* 2002; **9**: 365–371.
27 Brunner S *et al*. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Therapy* 2000; **7**: 401–407.
28 Prasmickaitea L, Hogset A, Berg K. The role of the cell cycle on the efficiency of photochemical gene transfection. *Biochim Biophys Acta* 2002; **1570**: 210–218.
29 Dinçer S, Tuncel A, Piskin E. Potential gene delivery vector: *N*-isopropylacrylamide-ethyleneimine block copolymers. *Macromol Chem Phys* 2002; **203**: 1460–1465.
30 Türk M *et al*. *In vitro* transfection of HeLa cells with temperature sensitive polycationic copolymers. *J Control Release* 2004; **96**: 325–340.
31 Türk M, Dinçer S, Pişkin E. *In vitro* transfection of smooth muscle cells with smart polycationic macromolecules. *J Biomat Sci:* Polym Ed, submitted, 2005.