Carbohydrate Polymers 102 (2014) 207–215

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Pullulan–protamine as efficient haemocompatible gene delivery vector: Synthesis and in vitro characterization

S.S. Priya, M.R. Rekha*, Chandra P. Sharma**

Biosurface Technology Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India

ARTICLE INFO

Article history:
Received 8 August 2013
Received in revised form
12 November 2013
Accepted 20 November 2013
Available online 27 November 2013

Keywords:
Cationized pullulan
Protamine
Gene delivery
Non- viral vector
Haemocompatible

ABSTRACT

Biodegradable non-viral vectors with good transfection efficiency is essential for successful gene delivery. The purpose of this study was to design a non-viral vector by conjugating protamine to pullulan and elucidate the potential use of pullulan protamine conjugate (PPA) as an effective, non toxic and haemocompatible gene delivery system. The particle size and surface charge were measured using Nanosizer. Derivatization was confirmed by NMR, FTIR and DSC analyses. Acid base titration revealed the buffering behaviour of the conjugate. The protection of DNA from nuclease enzyme and interaction of plasma components on the stability of nanoplexes were also analysed. The uptake studies confirmed the plasmid delivery into the nucleus and the inhibitor studies determined the uptake mechanism. Transfection experiments revealed the capability of PPA to cellular uptake in C6 cells and facilitate high gene expression. Thus, PPA proves to be a promising non-viral vector.

1. Introduction

Gene therapy is the delivering of exogenous DNA into cells for the purpose of enhancing or suppressing the expression of a specific gene. A major challenge for successful gene therapy is the development of a more secure and effective delivery mechanism. The viral vectors showed promising results, however, significant problems were observed in in vivo studies such as immunogenicity, transient gene expressions and inability to persist in host cells (Reschel, Konak, Oupicky, Seymour, & Ulbrich, 2002). Current studies investigate non-viral vectors since they have the ability to target specific cells, they are safer for repeat-administration, are more stable in storage and are easier to produce in large quantities (Jayakumar et al., 2010). The advantages of gene therapy hold promise and far outweigh the disadvantages.

The advent of nanotechnology offers numerous non-viral vectors suitable for transmitting DNA and other biopharmaceuticals into cells and subcellular compartments. The cationic polymers can cause DNA condensation, and this enhances the half-life of DNA and prevents degradation through protection from plasma nucleases. The size and surface charge (zeta potential) of the DNA complexes is one of the major factors determining vector efficiency. It also plays an important role in biodistribution, cellular internalization and intracellular trafficking (Mintzer & Simanek, 2009).

Protamine is a small arginine-rich cationic nuclear protein, essential for sperm head condensation and DNA stabilization in the later stages of spermatogenesis. Also, reports shows that the role of protamine is to deliver the sperm DNA into the nucleus of the egg following fertilization. This function of protamine will ensure the transport of plasmid DNA from the cytoplasm into the nucleus (Sorgi, Bhattacharya, & Huang, 1997) Therefore, protamine has been chosen as the polycationic polymer of choice for the investigation of a new effective gene delivery system. Cytotoxicity and poor haemocompatibility can limit gene delivery efficacy in cationic polymers, which was solved by the addition of a non-toxic, non-immunogenic, biodegradable and biocompatible material such as pullulan as base material for the transportation of vectors (Kimoto, Shibuya, & Shiobara, 1997). Further more, it is well known that protamines are small peptides of MW around 4000 Da and is very basic in nature due to the high arginine content. However, the tendency of protamine alone to form aggregates with DNA is a limiting factor, hence the conjugation to a polymer may result in a cationic polymer which can be used effectively for gene delivery. With this thought, we developed a conjugate of pullulan–protamine for gene delivery application.

Previous work in our lab also reported the haemocompatibility and efficiency of pullulan conjugates, indicating its success as a gene delivery vector component (Rekha & Sharma, 2009).

* Corresponding author. Tel.: +91 4712520284.
** Corresponding author.
E-mail addresses: rekhamr@sctimst.ac.in, rekharamesan@gmail.com (M.R. Rekha), sharmacp@sctimst.ac.in (C.P. Sharma).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.11.024

This study thus explored the possibility and potential of utilizing pullulan–protamine conjugate as an effective carrier for gene delivery, through the development of pullulan–protamine/pDNA complexes and its interaction with blood components, uptake mechanism and its in vitro transfection efficiency.

2. Materials and methods

2.1. Materials

Pullulan, protamine sulphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMEM, Trypsin, EDTA, Polyethylenimine, PEI (MW 25,000 Da), DNase I, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), 2 iminothiolane were purchased from Sigma–Aldrich Chemicals Co., USA. YOYO iodide, Hoechst 33342 (Invitrogen), p53 control DNA (Clontech, USA), FBS (GIBCO, USA), and Calf thymus DNA (ctDNA) was purchased from Worthington Biochemical Corp. All other reagents were of analytical grade from Merck, India.

2.2. Conjugation of pullulan–protamine (PPA)

Pullulan was dissolved in 20 mM Borax, pH 10 and allowed to react with traut's reagent to introduce thiol group to pullulan. The reaction mixture was incubated for 1 h at 30 °C under stirring. The obtained solution was dialysed against phosphate buffer saline (PBS) containing 1 mM EDTA. Protamine (1 mg/ml) was then activated with a heterobifunctional cross linking agent MBS. This mixture was placed under stirring, and dialysis was carried out in PBS overnight to remove the unbound reactant. Modified pullulan was allowed to react with activated protamine in different ratios (1:1, 2:1, 3:1 and 4:1) under stirring for 30 min at room temperature (RT). Dialysis was carried out in distilled water for 48 h with timely replacements with fresh water.

2.3. Chemical characterization

2.3.1. ^1H NMR studies

The PPA polymer was dissolved in D_2O and used for analysis. The ^1H NMR spectra were obtained using NMR spectrometer (Bruker Avance DPX 300).

2.3.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of pullulan, protamine and the conjugate PPA were recorded with a Thermo Nicolet 5700 spectrophotometer in the spectral range 4000–400 cm^{-1}.

2.3.3. Differential scanning calorimetry

Thermal properties of pullulan, protamine and PPA were studied by DSC (TA Instrument, USA). Samples were weighed 10 mg each and was covered in an aluminium pan and heated at a rate of 10 °C/min to 400 °C. High purity nitrogen was used with a gas flow of 50 ml/min.

2.4. Trinitrobenzenesulphonic acid (TNBS) and copper sulphate ($CuSO_4$) assay

The quantification of free amino group in PPA was determined by both TNBS as well as $CuSO_4$ assay. TNBS assay was carried as described elsewhere (Snyder & Sobocinski, 1975). Analysis was carried out in triplicates. Protamine of varying concentrations was taken as standards. Similarly, $CuSO_4$ method was also used to confirm the amino content of the cationized pullulan (Ungaro, Rosa, Miro, & Quaglia, 2003). Sample was prepared in 1 mg/ml concentration where varying concentration of PEI (25 kDa) was prepared as standards. To which 5 ml of copper sulphate solution (0.145 mg/ml) was added and absorbance recorded at 285 nm.

2.5. Nanoplex formulation

Nanoplexes of weight ratios 0.5:1–60:1 were formed by mixing varying concentration of cationized pullulan to a fixed concentration of ctDNA (10 μg) and diluted to a final volume of 1 ml with distilled water. This was then vortexed for 30 s and incubated at RT for 20 min.

2.6. Particle size and zeta potential determination

Particle size determination was carried out by dynamic laser light scattering measurement using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C. The complexes were prepared in water with increasing DNA/polymer ratio, with a constant concentration of ctDNA (1 mg/ml). The particle size of nanoplexes were expressed as mean diameter (*z* average),obtained by the cumulant analysis of correlation function based on viscosity and refractive index of water. The surface charges of the complexes of varying ratios were also evaluated using the same instrument at 25 °C. All experiments were performed in triplicate.

2.7. Determination of buffering capacity

The buffering capacity of PPA, normal saline and protamine was evaluated by acid base titration over a pH range of 10–5. The samples were dissolved in normal saline (100 μg/ml). The pH of the solution was corrected to 10 using 0.01 N NaOH, and then titrated against 0.01 N HCl. A graph was plotted with pH against the volume of HCl and the buffering capacity was assessed.

2.8. Gel retardation

The ctDNA condensation capability of the conjugate was assessed by agarose gel electrophoresis. Nanoplexes of desired polymer/DNA ratios were prepared (5:1–20:1) with ctDNA. The stability of nanoplexes in the presence of plasma was also analysed to assess its stability under physiological condition. For which, nanoplexes of the same ratios were taken and incubated with 20 μl of plasma for 30 min. Electrophoresis was carried out in Tris–acetate–EDTA (TAE) buffer solution, staining with 2 μl of 10 mg/ml EtBr in a Bio Rad electrophoresis system (Bio-Rad laboratories, CA,USA) at 100 V for 30 min. The DNA bands were visualized and the gel was photographed using Las 4000, Fuji.

2.9. Ethidium bromide (EtBr) displacement assay

DNA condensation was measured by EtBr displacement assay described earlier (Petersen et al., 2002). The fluorescence intensity of the complexes were measured at λ_{ex} 510 nm and λ_{em} at 590 nm using a microplate reader (Tecan, USA). Here, Fluorescence of DNA with EtBr was taken as control. The results were noted as relative fluorescence intensity.

$$\%\text{Relative fluorescence} = \frac{\text{Fluorescence}(\text{DNA} \pm \text{EtBr}) - \text{Fluorescence}(\text{test})}{\text{Fluorescence}(\text{DNA} + \text{EtBr})} \times 100$$

2.10. DNase I protection assay

This was performed to investigate the ability of PPA polymer to protect DNA from endonuclease degradation. PPA/ctDNA

nanoplexes of different ratios (5:1–20:1) were prepared. The complexes containing 2 μg of DNA were incubated with 2.28 μl of 853 U/ml DNase at 37 °C for 10 min. The reaction was then terminated using buffer containing 0.5 M EDTA, 2 M NaOH and 0.5 M NaCl. Naked ctDNA with or without DNase I treatment were used as positive and negative controls respectively. The amount of ctDNA released was evaluated using agarose gel electrophoresis in a Bio-Rad electrophoresis system (Bio-Rad Laboratories, USA).

2.11. Haemocompatibility studies

Blood was drawn from a healthy human donor in tubes containing 3.8% sodium citrate at ratio 9:1 (blood:anticoagulant). The studies were done for PPA and compared to that of normal saline (NS) and PEI.

2.11.1. Haemolysis and RBC aggregation

Blood compatibility of polymer/DNA nanoplexes were evaluated in terms of haemolysis by method described elsewhere (Kainthan et al., 2006). Briefly, RBC was washed thrice with PBS (pH 7.4). PPA of different concentrations (25–100 μg) was incubated with 100 μl of RBC for 2 h at 37 °C. The samples were then centrifuged at 1500 rpm for 5 min, the supernatant was analysed at 541 nm by UV–vis spectrophotometer (Varian). Triton X 100 was used as the positive control (100% lysis) and saline as the negative control (0% lysis). Blood compatibility was also evaluated by RBC aggregation studies (Murthy, Robichaud, Tirrell, Stayton, & Hoffman, 1999). The RBC (100 μl) diluted with PBS (1:8, pH 7.4) was incubated with the PPA (100 μg) for 30 min. The RBCs incubated with saline and PEI was also assessed. Images were captured using LeicaDMI3000 B (Leica, Germany).

2.11.2. WBC and platelet aggregation

WBCs/platelets were separated by centrifuging anticoagulated blood overlaid on Histopaque at 800 rpm for 20 min. 100 μg of the PPA were incubated with 100 μl each of WBCs and platelets for 30 min. PEI and saline incubated with WBC/platelets were considered as positive and negative control respectively. Presence of aggregation, if any, was detected through LeicaDMI3000 B (Leica, Germany).

2.11.3. PAGE studies

Native PAGE analysis was performed to assess the interaction of PPA polymer with plasma proteins. The polymer was incubated with 20 μl plasma for 30′, subjected to centrifugation at 4000 rpm for 5 min. Supernatant was collected and electrophoresis was carried out in a Mini-PROTEAN II electrophoresis cell (Bio-RAD, USA) at 100 V for 90 min. Gel was stained with 0.2% coomassie brilliant blue, destained and digitalized using an image analyser (LAS 4000, Fuji).

2.12. Cytotoxicity

Toxicity of PPA was analysed using MTT assay and performed on C6 glioma cells. The cells were trypsinized using 0.25% trypsin-EDTA. Cells were seeded at a density of 1×10^5 cells/well in a 48 well plate and incubated for 24 h in CO_2 incubator at 37 °C under 5% CO_2 atmosphere. Further, the medium was removed and polymers of different concentrations (25, 50, 75, 100, 200, 400, 600 μg/ml) in triplicates along with positive and negative controls was added and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. After incubation, the samples were removed and 200 μg MTT in PBS was added to each well and incubated for 3 h. The reagent was then removed, and dimethyl suphoxide (DMSO) added to dissolve the MTT formazan crystals. The absorbance was measured in 96 well plates in a microplate reader at 570 nm. Cell viability was expressed as the mean percentage of sample absorbance relative to untreated cells.

$$\text{Cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.13. Cellular uptake studies

Nanoplexes were formed with YOYO tagged p53 plasmid DNA and PPA at ratios 1:15 and 1:20. This was then incubated in C6 glioma cells for 2 h at 37 °C in DMEM/Ham's F12:MEM (1:1) medium with 10% FBS. Nuclear staining was done with Hoechst 33342 by incubation for 30′. The cells were washed with PBS and fixed in 1% formlaldehyde. Nanoplex uptake was visualized and photographed using a fluorescence microscope (Leica DM IRB, Germany).

2.13.1. Inhibition studies

The uptake mechanism of the PPA nanoplexes was studied with inhibitors of the endocytic pathways. The C6 cells were pretreated with chlorpromazine (2 μg/ml), filipin (5 μg/ml) and amiloride (2 μg/ml) to inhibit clathrin, caveolae mediated and macropinocytosis respectively for 30 min at 37 °C. The medium was then replaced with fresh media and the YOYO tagged nanoplex with the ratio 20:1 were added to the cells and incubated for 4 h. The cellular internalization was observed using fluorescence microscopy (Leica DM IRB, Germany).

2.14. Transfection studies

C6 cells were seeded into 4 well plates and incubated in 5% CO_2 at 37 °C. The cells were then incubated with the nanoplexes of PPA/DNA containing 3 μg at ratios 20:1 and 25:1 for 5 h. At the end of the incubation period, the medium was replaced with fresh medium and the cells were then incubated for an additional 43 h. After incubation, the cells were washed thrice with PBS. The Live and Dead assay was performed using the kit protocol. PBS containing 2 μM calcein AM and 4 μM Ethidium homodimer 1 (EthD-1) was prepared and 200 μl added to the cells, which was further incubated for 30 min at 37 °C. The cells were visualized using a fluorescence microscope (Leica DMI3000B, Germany).

3. Results and discussion

Among the various pharmaceutical issues that must be addressed for any therapeutic, the two most important criteria for successful gene delivery systems are safety and efficacy (Morille, Passirani, Vonarbourg, & Clavreul, 2008). While fulfilling these criteria, the delivery system must interact with the therapeutic gene in such a way that it should provide stability to the electrostatically bound gene in the extracellular environment and at the same time release DNA in the intracellular environment (Buddhadev & Jagdish, 2012). The purpose of selecting protamine was to cationize pullulan and thereby improve complexation with DNA and its transfection ability.

3.1. Preparation and characterization of PPA polymer

Pullulan was modified with protamine and the quantification of free amino groups of PPA was determined using TNBS assay, reconfirmed by $CuSO_4$ assays (data not shown). Based on these findings, an optimized ratio of 3:1 was chosen for further studies. From both experimental data, it was clearly observed that, PPA, had a high amount of primary amino groups with an overall positive charge required for the effective binding of polymer to the nucleic acids. The conjugation was confirmed by NMR and FTIR spectroscopies.

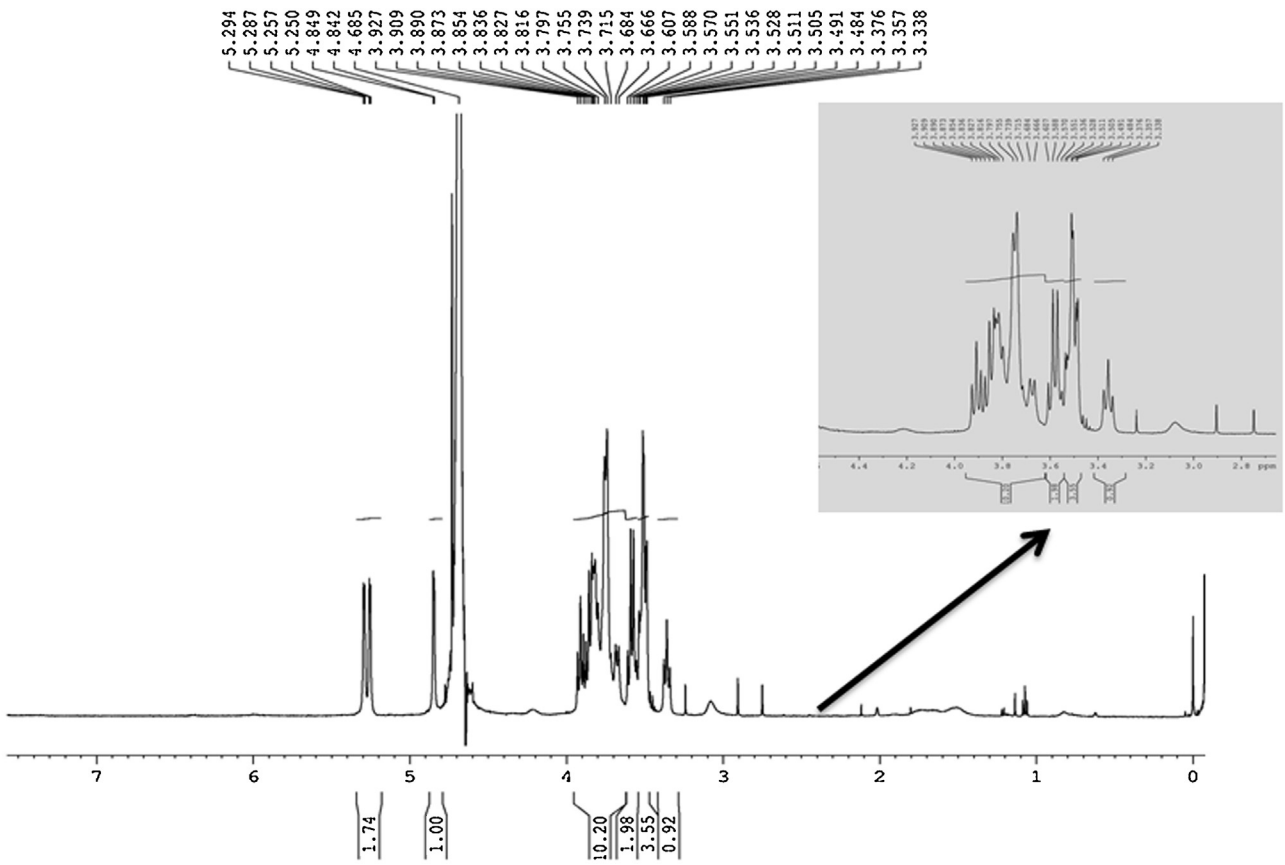

Fig. 1. ^{1}H NMR spectra of pullulan–protamine.

NMR peaks of PPA are given in Fig. 1 and that of pullulan and protamine are given in supplementary figure 1. The NMR peaks between 4.63 and 5.29 ppm corresponds to the hydroxyl proton of pullulan and was found to be reduced in PPA spectrum when comparing with the intensity signals in pullulan. The characteristic peaks of arginine groups in protamine are observed at 2.5–3.3 ppm. This indicates the conjugation of pullulan with protamine. In FTIR, the PPA spectrum showed strong amide absorbance as indicated by new peaks at 1549.6 cm^{-1} and 1422.2 cm^{-1} (supplementary figure 2). The formation of the product was also confirmed by the change in thermal behaviours of the parent compound and its derivatives using DSC (supplementary figure 3). The parent polymer pullulan showed a melting temperature of 167.89 °C and protamine at 133 °C whereas its derivative was found to be 175 °C.

3.2. Biophysical properties (size, zeta potential)

Biophysical properties such as mean diameter and zeta potential of PPA/DNA nanoplexes were determined via DLS and zeta potential measurements. Nanoplexes of nanometer size were developed by the condensation of ctDNA with PPA at various polymer/DNA ratios ranging from 0.5:1 to 60:1. As shown in Fig. 2A, initially the formation of relatively larger nanoparticles was observed between the polymer/DNA ratios of 0.5:1–2:1, which decreased with increasing polymer concentration. The optimum sizes observed at ratios 15:1, 20:1, 25:1 and 50:1 with average particle size ranging from 50 to 100 nm. As the concentration of polymer increases, the extent of interaction with the DNA also increases which resulted in more compact structure and better cellular internalization and transfection efficiency. Larger particle with diameter larger than 200 nm accumulate in the spleen and liver and are processed by the MPS cells. The smaller particle size is also a prerequisite for the in vivo transgene expression. Thus the particle size obtained here are ideal for using as successful gene delivery matrix. The optimum sized nanoparticle has the polydispersity index of ≥0.2. The zeta potentials measured from the surface of PPA/DNA complexes was found to be positive except in the lower ratios such as 0.5:1 and 5:1 where it gives negative values. As in Fig. 2B, with increasing polymer concentration the positive charge increased correspondingly and stabilized. Thus, ratios above 10:1 were chosen for in vitro studies. The positive charges of cationic polymers proved to be advantageous for the adhesion of nanoplex to the negatively charged biological membranes. This can also facilitate in the uptake of nanoparticle in to the cell, where the positive charge induces fluidity and thereby increases cellular uptake. On the other hand, the binding of negatively charged nanoparticles to a lipid bilayer causes local gelation and prevents cellular uptake (Wang, Zhang, Bae, & Granick, 2008). Hence based on the above results PPA/DNA complex with ratios above 10:1 were found suitable for further studies.

3.3. Buffering capacity of PPA

The success of non-viral gene therapy is very much dependent on various extra- and intracellular barriers that affect the efficacy of the delivery systems. Titration studies were conducted to determine the buffering capacity of PPA within the endosomal/lysosomal compartments of the cell. As shown in Fig. 3, PPA possessed good buffering capacity over a pH range of 10–4 and showed good resistance at a pH range of 7–4, which is in agreement with cationic polymers like PEI. PEI showed resistance at the pH range of 7–4 and induces facilitated endosomal escape due to the uptake of protons by the amino groups (Midoux, Kichler, Boutin, Maurizio, & Monsigny, 1998). Similarly, the observed increase in the buffering capacity of PPA was also due to the presence of free amino groups

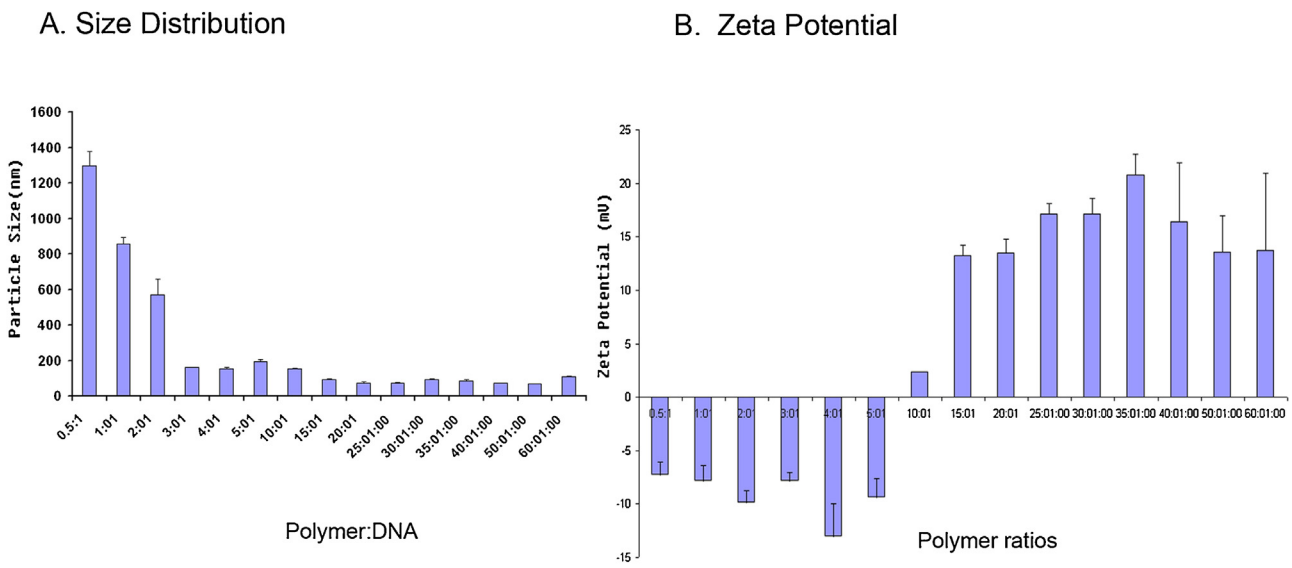

Fig. 2. (A) Particle size and (B) zeta potential of PPA/ctDNA nanoplex.

in the conjugate which in turn is contributed by the conjugation of positively charged protamine to pullulan.

3.4. DNA complexation studies (Gel retardation assay and EtBr displacement assay)

Physical integrity of the DNA after complexation is a prerequisite for biological activity of DNA to mediate successful transfection (Lin et al., 2006). The gel electrophoresis was conducted to assess the polymer/DNA stability in the presence and absence of plasma. The ability of PPA polymer to retain DNA within complexes was compared by observing whether or not free DNA bands were dissociated. Naked DNA was used as the control. ctDNA was found to be totally retained in the well with ratios above 5:1 as is shown in Fig. 4A (lanes 2–4) and similar such observation was noted in the presence of plasma as seen in Fig. 4A (lanes 7–10). These results indicated that the nanoplexes formed were stable and that the negatively charged plasma proteins failed to displace DNA from the complex. The ability of the pullulan conjugate to condense DNA effectively was further evaluated by measuring the reduction in fluorescence intensity of ethidium bromide (EtBr) due to its displacement from DNA (Warning, 1965). A steep decrease in the fluorescence was observed with the increase in the polymer ratio (supplementary figure 4) indicating the capability of the polymer to strongly bind the DNA.

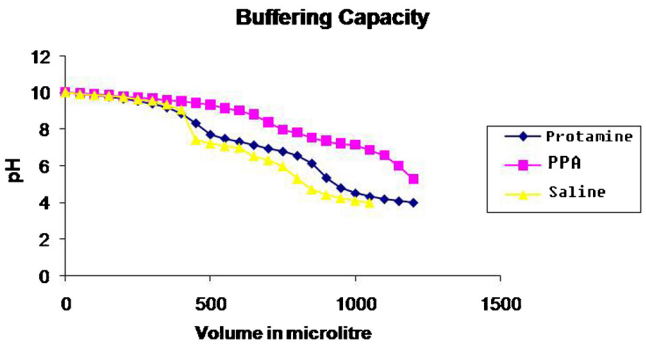

Fig. 3. Buffering capacity of pullulan–protamine conjugate (PPA) in comparison with protamine and normal saline.

3.5. DNase I protection assay

The protection of DNA from nuclease-catalysed degradation may facilitate its cellular internalization and improved efficiency both in vivo and in vitro (Azzam et al., 2002). Therein, DNase I protection assay was employed to investigate the ability of the PPA to protect DNA from extracellular nuclease degradation. The DNase I protection assay showed that PPA polymer was able to form tight complexes with DNA and that even in the presence of DNase I, no release of DNA from the well was seen as shown in Fig. 4B (lanes 2–5). This strongly indicates polymers shielding effect on DNA. This was confirmed by comparing to the naked DNA digested with DNase I (lane 1) where no band was seen. The stability of the nanoplex in the presence of nucleases was thereby confirmed.

3.6. Haemocompatibility studies

Stability in blood is the major hurdle of cationic polymers that limits its application in in vivo. To assess the haemocompatibility of PPA; haemolysis, RBC, WBC, platelet aggregation studies and PAGE studies were carried out.

3.6.1. Haemolysis and RBC/WBC/platelet aggregation

Aggregation and haemolysis are indicators of interaction and incompatibility of cationic polymers with red blood cells. Positive zeta potential of cationic polymers is highly essential for cellular internalization and transgene expression. At the same time, increased intensity of positive charges leads to cationic polymer interaction with the negatively charged RBC membrane to cause aggregation and lysis (Ogris, Brunner, Schüller, Kircheis, & Wagner, 1999). Therefore, evaluation of RBC compatibility with cationic polymers is highly essential under in vitro conditions. The pullulan conjugate, PPA, showed no haemolysis even at higher polymer concentration of 100 μg (data not shown). Thus, PPA proved itself to be non-haemolytic. Another major problem faced during intravenous administration of cationized polymer is the nanoparticle induced aggregation of erythrocyte, WBC and platelet. Aggregation of WBC adversely affects the immune response of the body (Moreau, Domurado, Chapon, Vert, & Domurado, 2002). It is established that aggregation of platelets is crucial for vascular haemostasis and thrombosis (Gorbet & Sefton, 2004). However higher aggregation of platelets give rise to risk factors leading to stroke and cardiovascular diseases (Studzińska, Seroka, Łępicka, Roszek, & Komoszyński,

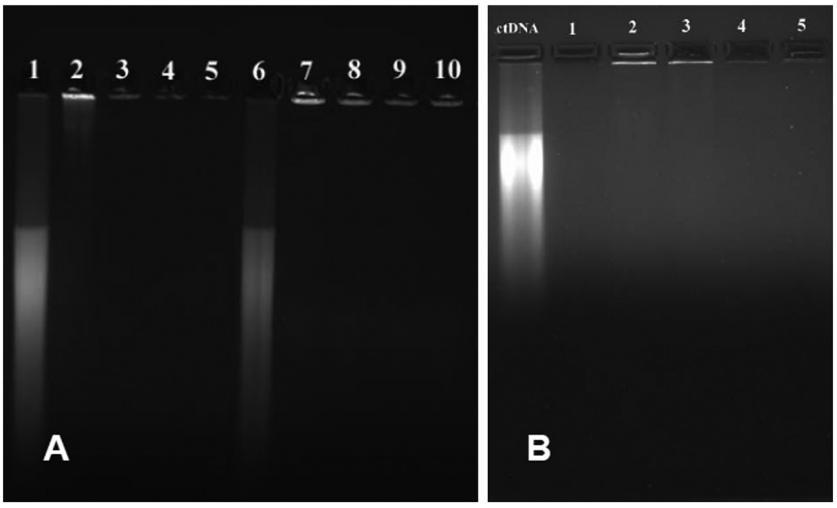

Fig. 4. Agarose gel electrophoresis performed on PPA/DNA nanoplexes of different ratios with or without plasma and also in the presence of DNase I enzyme. (A) Lanes 1 and 6 are the control DNA; Lanes 2–5, nanoplexes of ratios 5:1,10:1,15:1 and 20:1; Lanes 7–10, nanoplexes of ratios 5:1,10:1,15:1,20:1 in the presence of plasma. (B) Lane 1 indicates native DNA in the presence of DNase 1; Lanes 2–4, nanoplexes ratios 5:1,10:1,15:1,20:1 in the presence of DNase 1.

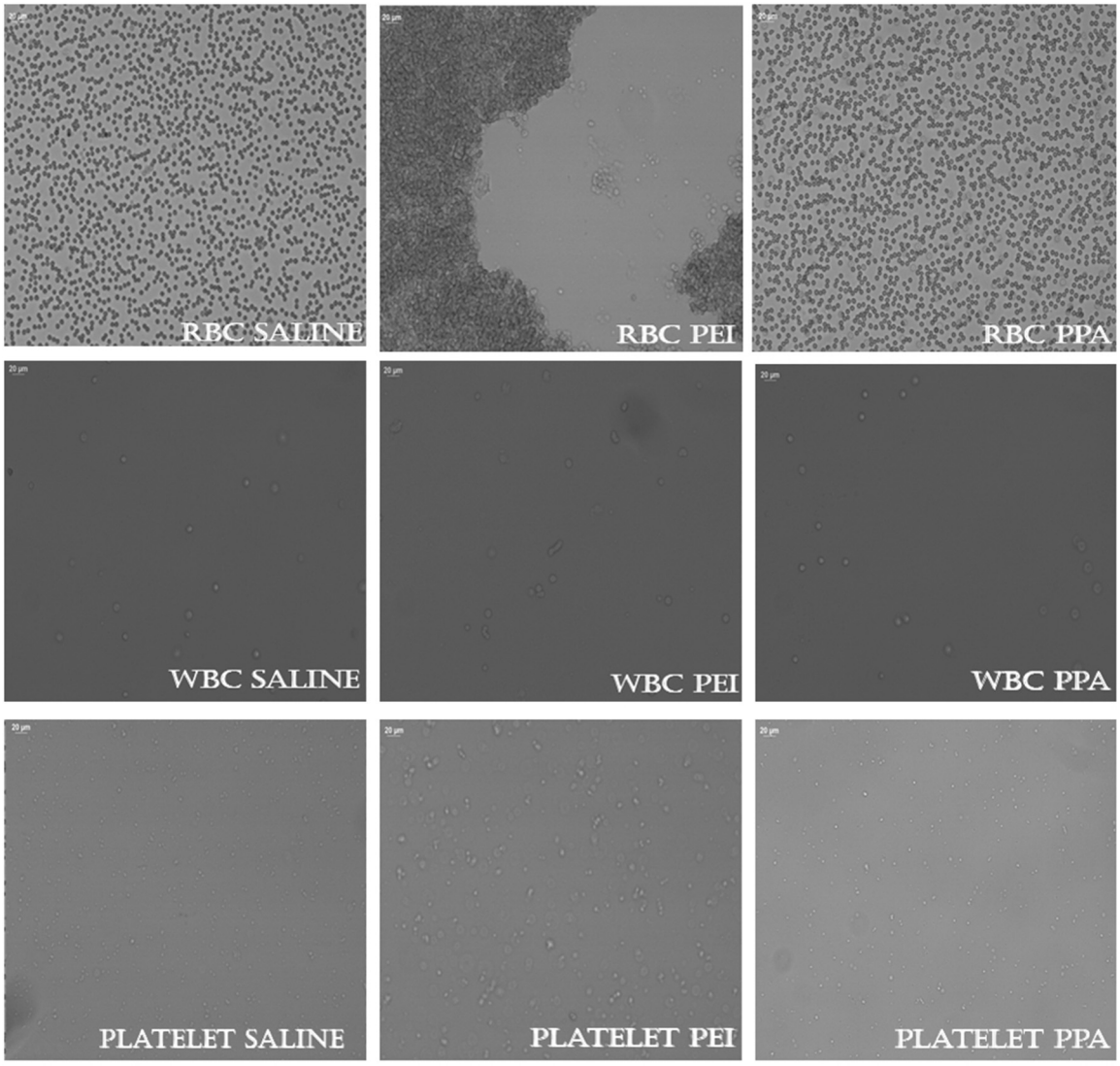

Fig. 5. Micrographs of RBC, WBC and Platelet after 20 min incubation with cationic pullulan derivative, PPA along with normal saline and PEI as negative and positive control respectively.

2010). The cationic PPA induced aggregation of erythrocyte, platelet and WBC were analysed. As shown in Fig. 5, results showed no aggregation with all the cells and morphology of the cells were also found to be similar to that of cells treated with normal saline. This was compared with the cationic polymer PEI which showed extensive aggregation in all. The haemocompatible nature of PPA might be due to the presence of pullulan which protects the cationic sites from interacting with the blood components.

3.6.2. PAGE analysis

Another major hurdle observed in the in vivo gene delivery system is the cationic polymer interaction with the protein. Blood half-life is highest for neutral nanoparticles whereas positively charged nanoparticles are cleared most quickly from the blood. The unequal half-lives of different charges are a result of the interactions of nanoparticles with serum proteins, which leads to, instant metabolism, clearance, and immune response (Cedervall et al., 2007). The longer a nanoparticle remains in circulation, the higher chances of it to enter the target cells in vivo (Lynch & Dawson, 2008). In this line, the plasma protein binding with the PPA polymer was examined by performing native PAGE by incubating samples with plasma. Plasma alone was taken as a negative control whereas PEI interaction with plasma was considered as a positive control. There was no absence of plasma protein bands observed in the gel, and the results were similar to that of negative control, which clearly indicated its stability in the presence of plasma proteins (supplementary fig. 5). However, a marked reduction in the protein bands was observed in the presence of PEI. These properties of PPA, was due to the non toxic, non-immunogenic and biocompatible nature of pullulan (Rekha & Sharma, 2011). These qualities marked PPA as a biocompatible gene transfecting agent.

3.7. In vitro cytotoxicity

Toxicity is a crucial factor in assessing biosafety for biomedical application of cationic polymers. The significant disadvantage of cationic polymer as gene vector is that it shows high cytotoxicity to many kinds of cells (Benns & Kim, 2000). Since low cytotoxicity and high transfection efficiency are two fundamental criteria for all gene delivery systems, viability of cells were assessed on C6 cells by reduction of MTT following exposure to various concentrations of PPA. The cell viability of PPA was comparable to that of normal growth medium. Studies showed that cytotoxicity is mediated by ionic interactions between anionic domains on the cell surface and cationic moieties present on the vector. This is thought to result in polyplex aggregation and accumulation at the cell surface which can severely impair membrane function and ultimately lead to cell death. As shown in Fig. 6, the cell viability was found to increase initially with increasing polymer concentration (up to 100 μg) and then decreased gradually. The cell viability was around 70% for 200–400 μg and toxic at 600 μg/ml concentrations. This might be due to the presence of pullulan which spares minimum positive charge for anionic membrane interaction and thereby reduces the interaction of amine groups with external macromolecules or surfaces. This proves the suitability of PPA as an efficient gene delivery vector.

3.8. Cellular uptake and inhibitor studies

The major factor governing an efficient gene delivery vector is its capability, to successfully deliver the gene into the host cell nucleus and thereby induce gene expression for longer periods of time (Zanta, Belguise-Valladier, & Behr, 1999). The cellular uptake of the plasmid was visualized by using a fluorescence microscope after 3 h of incubation of C6 cells with PPA complexed with DNA tagged using YOYO-I. The uptake of the nanoparticle was evaluated by the

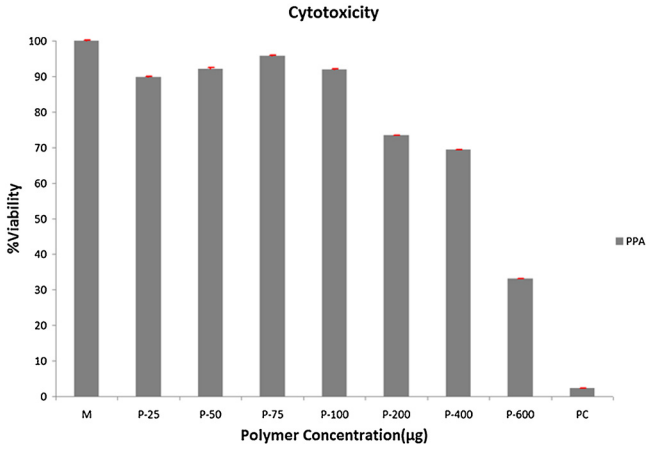

Fig. 6. Cytotoxicity of C6 cells after incubation of PPA of different concentration for 24 h. Cytotoxicity was evaluated by the MTT assay and expressed as % cell viability. M (medium) and PC (positive control for cytotoxicity).

influx of the fluorescently tagged plasmid DNA depicted in green dots. The polymer/DNA ratio 15:1 and 20:1, showed significant uptake in to the cell. Fig. 7A showed the uptake of PPA with the ratio 20:1 and was seen that high fluorescence signals from plasmid DNA were seen localized in the nucleus along with diffused fluorescence in the cytosolic area. This observance is in accordance with the existing literature. It was reported that the arginine rich sequences in protamine functions as nuclear localization signals aiding the DNA entry into nucleus (Reynolds, Weissleder, & Josephson, 2005). Thus, it is observed that conjugation to pullulan is not affecting the protamine's efficiency in delivering the DNA into the nucleus. The intracellular uptake mechanism of PPA nanoplexes was studied using inhibitors of endocytic pathways such as chlorpromazine, filipin and amiloride which inhibits the clathrin, caveolae and macropinocytosis mediated endocytosis respectively. As shown in Fig. 7B–D, PPA nanoplexes are taken up by multiple endocytotic pathways as there is reduction in the uptake in presence of all inhibitors, still higher reduction in uptake was observed with caveolae mediated endocytic pathway.

3.9. Transfection studies

To investigate the in vitro gene transfer capability of the pullulan derivative, transfection studies were performed on C6 cells using p53 gene. The gene transfection activity of the polymers was evaluated in terms of live dead assay. The transfection efficiency is a function of multiple parameters including composition, particle size, and surface charge of the complexes, polymer/DNA ratio and ionic strength of the medium. The nanoplexes chosen for transfection studies were in the ratios 20:1 and 25:1 as it showed good intracellular uptake. The efficacy of the PPA in delivering the p53 into the cell was proved by visualizing the expression of the gene in terms of cell death. The morphology of the transfected cells was observed to investigate the apoptotic behaviour of the cells. As shown in Fig. 8, PPA/DNA ratio 25:1 showed good transfection where live cells were stained green, and dead cells were stained red. It was noteworthy that high gene transfection efficiency was observed in the ratio 25:1 as compared to 20:1 (data not shown) where the cell death was observed to be more than 80% after 48 h of incubation. To ensure comparability, it is good to present the PEI transfection efficiency as 100% (Launay, Caminade, Lahana, & Majoral, 1994). However, with increasing polymer/pDNA ratios, of PEI, resulted in lower transfection efficiency. This opposite adverse trend of transfection efficiency by PEI could be due

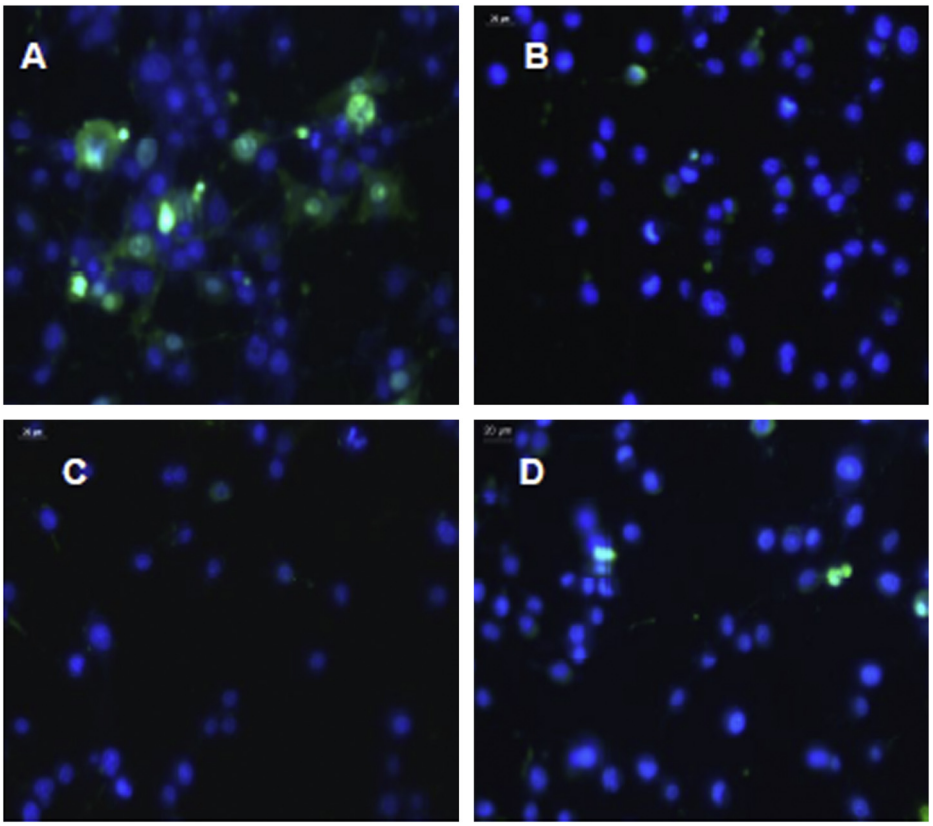

Fig. 7. Fluorescent image of intracellular distribution of PPA/DNA nanoplex of ratio 20:1 comprising of YOYO tagged (green) plasmid where blue indicates Hoechst stained nucleus. (A) Uninhibited cells; (B) chlorpromazine treated cells; (C) filipin treated cells; (D) amyloride treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

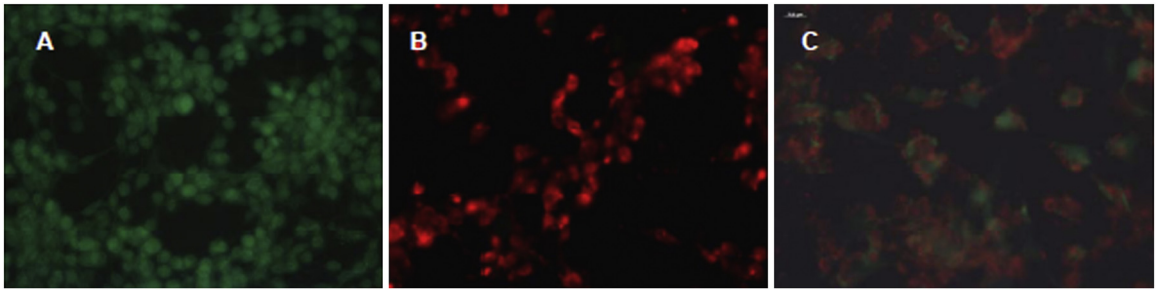

Fig. 8. Live/dead assay of C6 cells transfected with PPA/DNA complexes of ratio 25:1.

to several reasons, including high cytotoxicity of PEI and/or slow release of pDNA from the polymer/pDNA complex. In contrast, protamine having an inherent transfection acceleration property, showed lower cytotoxicity and good transfection efficiency even at higher PPA concentration of 250 μg. Hence it can be considered as a promising vector for in vivo applications.

4. Conclusion

In this work, a biocompatible cationized pullulan was synthesized by conjugating protamine to pullulan. The ^{1}H NMR and FTIR spectra confirmed the conjugation. The DLS measurement as well as AGE studies confirmed the ability of PPA to condense effectively with DNA to form positively charged nanoparticle with the size of less than 100 nm. This polymer also has the ability to protect DNA from DNase degradation and plasma protein interactions. Due to the presence of hydrophilic group in pullulan, PPA was found to have excellent haemocompatiblity and also an improved cell viability of PPA comparable to the medium even at a high polymer concentration of up to 400 μg/ml. Finally, the PPA conjugate showed high cellular uptake and improved transfection efficiency probably due to the unique nuclear localization signals of protamine. This new way of preparing cationic polymers that have no cytotoxicity but bear improved biocompatibility, haemocompatibility and transfecting activity could be very helpful to in vivo gene transfection where a large amount of cationic polymers are required.

Acknowledgements

We express our sincere thanks to The Director, SCTIMST for the facilities provided. Authors are thankful to the financial support from DBT, New Delhi. The authors acknowledge the support by Dr. V. Kallyana Krishnan for providing the facility for FTIR studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.11.024.

References

Azzam, T., Raskin, A., Makovitzki, A., Brem, H., Vierling, P., Lineal, M., et al. (2002). Cationic polysaccharides for gene delivery. *Macromolecules*, *35*, 9947–9953.

Benns, J. M., & Kim, S. W. (2000). Tailoring new gene delivery designs for specific targets. *Journal of Drug Targeting*, *8*, 1–12.

Buddhadev, L., & Jagdish, S. (2012). N-hexanoyl, N-octanoyl and N-decanoyl chitosan: Binding affinity, cell uptake, and transfection. *Carbohydrate Polymers*, *89*, 403–410.

Cedervall, T., Lynch, I., Lindman, S., Berggard, T., Thulin, E., Nilsson, H., et al. (2007). Understanding the nanoparticle protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 2050–2055.

Gorbet, M. B., & Sefton, M. V. (2004). Biomaterial-associated thrombosis: Roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials*, *25*, 5681–5703.

Jayakumar, R., Chennazhi, K. P., Muzzarelli, R. A. A., Tamura, H., Nair, S. V., & Selvamurugan, N. (2010). Chitosan conjugated DNA nanoparticles in gene therapy. *Carbohydrate Polymers*, *79*, 1–8.

Kainthan, R. K., Gnanamani, M., Ganguli, M., Ghosh, T., Brooks, D. E., & Souvik, M. (2006). Blood compatibility of novel water soluble hyperbranched polyglycerol based multivalent cationic polymers and their interaction with DNA. *Biomaterials*, *27*, 77–90.

Kimoto, T., Shibuya, T., & Shiobara, S. (1997). Safety studies of a novel starch, pullulan: Chronic toxicity in rats and bacterial mutagenicity. *Food and Chemical Toxicology*, *35*, 323–329.

Launay, N., Caminade, A. M., Lahana, M., & Majoral, J. P. (1994). A general synthetic strategy for neutral phosphorus-containing dendrimers. *Angewandte Chemie International Edition*, *33*, 1589–1592.

Lin, C., Zhong, Z. Y., Lok, M. C., Jiang, X. L., Hennink, W. E., Feijen, J., et al. (2006). Linear poly (amidoamines) with secondary and tertiary amino groups and variable amounts of disulphide linkages: Synthesis and in vitro gene transfer properties. *Journal of Controlled Release*, *116*, 130–137.

Lynch, I., & Dawson, K. A. (2008). Protein–nanoparticle interactions. *Nano Today*, *104*, 2050–2055.

Midoux, P., Kichler, A., Boutin, V., Maurizot, J. C., & Monsigny, M. (1998). Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chemistry*, *9*, 260–267.

Mintzer, A. M., & Simanek, E. E. (2009). Nonviral vectors for gene delivery. *Chemical Reviews*, *109*, 259–302.

Morille, M., Passirani, C., Vonarbourg, A., & Clavreul, A. (2008). Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. *Biomaterials*, *29*, 3477–3496.

Murthy, N., Robichaud, J. R., Tirrell, D. A., Stayton, P. S., & Hoffman, A. S. (1999). The design and synthesis of polymers for eukaryotic membrane disruption. *Journal of Controlled Release*, *61*, 137–143.

Moreau, E., Domurado, M., Chapon, P., Vert, M., & Domurado, D. (2002). Biocompatibility of polycations: In vitro agglutination and lysis of red blood cells and *in vivo* toxicity. *Journal of Drug Targeting*, *10*, 161–173.

Ogris, M., Brunner, S., Schüller, S., Kircheis, R., & Wagner, E. (1999). PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy*, *6*, 595–605.

Petersen, H., Kunath, K., Martin, A. L., Stolnik, S., Roberts, C. J., Davies, M. C., et al. (2002). Star-shaped poly(ethylene glycol)-block-polyethylenimine copolymers enhance DNA condensation of low molecular weight polyethylenimines. *Biomacromolecules*, *3*, 926–936.

Rekha, M. R., & Sharma, C. P. (2009). Blood compatibility and in vitro transfection studies on cationically modified pullulan for liver cell targeted gene delivery. *Biomaterials*, *30*, 6655–6664.

Rekha, M. R., & Sharma, C. P. (2011). Hemocompatible pullulan–polyethyleneimine conjugates for liver cell genedelivery: In vitro evaluation of cellular uptake, intracellular trafficking and transfection efficiency. *Acta Biomaterialia*, *7*, 370–379.

Reschel, T., Konak, C., Oupicky, D., Seymour, L. W., & Ulbrich, K. (2002). Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations. *Journal of Controlled Release*, *81*, 201–217.

Reynolds, F., Weissleder, R., & Josephson, L. (2005). Protamine as an efficient membrane-translocating peptide. *Bioconjugate Chemistry*, *16*, 1240–1245.

Snyder, S. L., & Sobocinski, P. Z. (1975). An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. *Analytical Biochemistry*, *64*, 284–288.

Sorgi, F. L., Bhattacharya, S., & Huang, L. (1997). Protamine sulfate enhances lipid-mediated gene transfer. *Gene Therapy*, *4*, 961–968.

Studzińska, B., Seroka, A., Łępicka, M., Roszek, K., & Komoszyński, M. (2010). The increase of adenylate kinase activity in the blood can control aggregation of platelets in coronary or peripheral arterial ischemia. *Health*, *2*(3), 246–252.

Ungaro, F., Rosa, G. D., Miro, A., & Quaglia, F. (2003). Spectrophotometric determination of polyethylenimine in the presence of an oligonucleotide for the characterization of controlled release formulations. *Journal of Pharmaceuticals and Biomedical Analysis*, *31*, 143–149.

Wang, B., Zhang, L., Bae, S. C., & Granick, S. (2008). Nanoparticle-induced surface reconstruction of phospholipid membranes. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 18171–18175.

Warning, M. J. (1965). Complex formation between ethidium bromide and nucleic acids. *Journal of Molecular Biology*, *13*, 269–282.

Zanta, M. A., Belguise-Valladier, P., & Behr, J. P. (1999). Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 91–96.