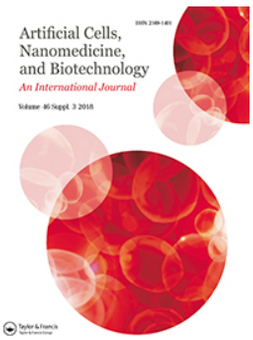

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Development of novel self-assembled polymeric micelles from partially hydrolysed poly(2-ethyl-2-oxazoline)-*co*-PEI-b-PCL block copolymer as non-viral vectors for plasmid DNA *in vitro* transfection

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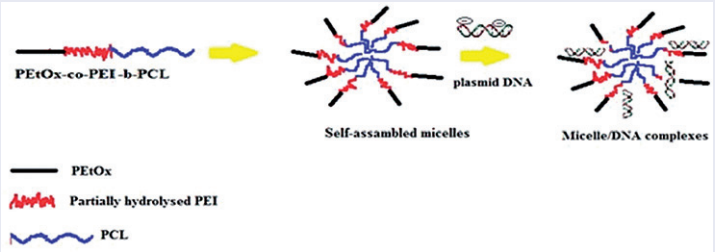
ABSTRACT

A new efficient, non-viral gene delivery cationic polymeric micellar system was developed by partial hydrolysis of poly(2-ethyl-2-oxazoline) (PEtOx) with two different hydrolysis percentages of PEtOx (30% and 60%) to reduce the disadvantages of the PEI. These self-assemble amphiphilic cationic micelles prepared from poly(2-ethyl-2-oxazoline)$_{30\%}$-*co*-poly(ethyleneimine)-block-poly(ε-caprolactone) (PEtOx$_{30\%}$-*co*-PEI-*b*-PCL) (PPP30) and poly(2-ethyl-2-oxazoline) $_{60\%}$-*co*-poly(ethyleneimine)-block-poly(ε-caprolactone) (PEtOx$_{60\%}$-*co*-PEI-*b*-PCL) (PPP60) block copolymers were successfully condensed with pEGFP-C3 plasmid DNA via electrostatic interactions to form micelle/DNA complexes with desirable particle sizes. All formulations showed low critical micelle concentration (CMC) values that means highly stable in serum containing medium. Polymeric micelles were also evaluated for their stability in the presence of serum and nuclease as well as cytotoxicity and transfection efficiency. All our results proved that our novel polymeric micellar system prepared by PPP60 block copolymer offer to be an efficient promising carrier for gene delivery applications. Moreover, these findings contribute to design and development of novel gene vectors with tunable and functionality features and also to reduce the cytotoxicity of PEI by partial hydrolysis of PEtOx an alternative synthesis method to produce linear PEI.



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Polymeric micelle; partial hydrolysis; PEtOx; non-viral gene delivery

Introduction

Gene therapy is a technique to transfer the genes into the nuclei of target cells to replace a mutated gene with a healthy copy. To perform the efficient gene delivery, it is necessary to develop specific gene carriers that can be safely used in humans and protect the DNA from nucleases. There are two approaches to deliver gene effectively to the target site [1]. In spite of high transfection efficiency of viral vectors, because of several intrinsic drawbacks including inherent immunogenicity, difficulties in repeated administrations, alternative nonviral gene delivery systems have become attractive interest [2–4]. Among them, polymeric polycationic systems have recently attracted more attention with special role in various biomedical applications. Recently, polymeric micelles derived from block copolymers have gained interest as promising drug and gene delivery vehicles because of unique morphological behaviour and prolonged circulation [5]. Block copolymer micelles with hydrophobic block, hydrophilic block and cationic block can form self-assembly into a core–shell structure [6]. This structure serves complexation of negatively charged DNA with the cationic block to form complexes [7]. PEI is a widely studied polymer with ability to condense

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plasmid DNA (pDNA) to form nano-sized polyplexes [8]. Also, PEI can protect DNA from degradation by nucleases and show superior transfection efficiency. Despite PEI being a gold standard, high polycationic nature causes higher cytotoxicity. Thus, reducing the cytotoxicity of PEI and increasing gene transfer efficiency, the partial hydrolysis of poly(2-ethyl-2-oxazoline) (PEtOx) to obtain linear PEI is the most used method for its preparation [9,10]. Partial hydrolysis of the amide chain of PEtOx reduces the PEI high cationic charge [11]. In recent years, PEtOx has attracted interest in biological and biomedical applications with stealth characteristics that provide a steric barrier similar to PEG. PEtOx has easily controlled features with end group functionalization and shown low polydispersity index (PDI) [12–14].

In this study, to reduce the PEI-derived cytotoxic effect of block copolymers and to increase the gene transfection efficiency, we aimed to prepare novel polymeric micelle-based gene delivery system with partial hydrolysis of PEtOx. PEtOx was used as hydrophilic block as an alternative to PEG and evaluated as nonviral gene carrier. Self-assembled polymeric micelles were prepared by using poly(2-ethyl-2-oxazoline)-*co*-poly(ethyleneimine)-block-poly(ε-caprolactone) (PEtOx-*co*-PEI-*b*-PCL) with two different hydrolysis percentage degree of PEtOx (30% and 60%). The resulting micelle was condensed with pDNA and micellar system was characterized for its various physicochemical properties, complexation ability, stability, cytotoxicity and transfection efficiency in two different breast cancer cell lines (MCF-7 and MDA-MB-468 cells).

Materials and methods

Materials

PEtOx$_{\%30}$-*co*-PEI-*b*-PCL and PEtOx$_{\%60}$-*co*-PEI-*b*-PCL of the amphiphilic polymers were synthesized, purified and characterized by the research group of TUBITAK MAM Materials Institute. MCF-7 and MDA-MB-468 human breast adenocarcinoma cell lines, which were previously purchased from the American Type Culture Collection (ATCC, LGC Promochem, Rockville, MD) were provided by Dr. Esendağlı, Hacettepe University (Ankara, Turkey). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), 0.25% trypsin –ethylenediaminetetraacetic acid (EDTA) solution, antibiotics (penicillin and streptomycin) were obtained from Gibco, ThermoFisher (Grand Island, NY). pEGFP-C3 was provided from Dr. Telci, Yeditepe University (Istanbul, Turkey). DRAQ7 cell viability dye was purchased from Biolegend (San Diego, CA). All other chemicals were of reagent grade and solvents were of HPLC grade.

Synthesis of partially hydrolysed PEtOx-co-PEI-b-PCL amphiphilic block copolymers

The synthesis of well-defined amphiphilic block copolymers was performed by copper-catalysed azide-alkyne cycloaddition (CuAAC) click chemistry. Poly(2-ethyl-2-oxazoline) as hydrophilic block and poly(ε-caprolactone) as hydrophobic block were produced and then partial acidic hydrolysis of PEtOx was carried out. The precursors, ω-azido-functionalized poly(2-ethyl-2-oxazoline)-*co*-poly(ethyleneimine) and α-alkyne-functionalized poly(ε-caprolactone) were combined by utilizing copper sulphate/sodium ascorbate catalyst system under warm conditions. The structures of precursors and amphiphilic block copolymers were enlightened by using spectroscopic and chromatographic analyses.

First, to obtain PEtOx-N_3, the living CROP of the monomer 2-ethyl-2-oxazoline (EtOx) and with the methyl p-toluenesulfonate initiator (MeTos) dissolved in acetonitrile (ACN) was carried out under an inert atmosphere at 130 °C, and then the polymerization was quenched with sodium azide (NaN_3). In the second step, the partial acidic hydrolysis of obtained PEtOx-N_3 was performed under reflux conditions that the polymer was hydrolysed at 100 °C in a flask with a stirring bar during different hydrolysis times. Thus, the azide functionalized copolymers of PEtOx$_{\%30}$-*co*-PEI and PEtOx$_{\%60}$-*co*-PEI possessing different two hydrolysis degrees were formed and analysed for FT-IR and ^{1}H NMR spectroscopy [15,16]. ^{1}H NMR (600 MHz, CD_3OD): δ3.80–3.28 (4H, –CH_2–CH_2–N–), 3.00–2.65 (4H, –NH–CH_2–CH_2–), 2.45–2.13 (2H; –NCOCH_2–CH_3), 2.20–2.00 (2H, CH_3CH_2COOH), 1.20–0.85 (3H, –NCOCH_2CH_3, 3H, CH_3CH_2COOH). FT-IR (ATR): ν (cm^{-1}) 2100 (azide), 1630 (carbonyl) and 1580 (N–H bend). The degree of hydrolysis was calculated according to the equation below.

$$\text{Conversation } (\%) = \frac{\text{backbone PEI}}{\text{backbone PEI} + \text{backbone PEtOx}} \times 100$$

On the other hand, to obtain PCL-alkyne, the ROP of the monomer epsilon-caprolactone with the Sn(Oct)$_2$ catalyst and the propargyl alcohol initiator dissolved in toluene was conducted under an inert atmosphere at 120 °C [17,18]. Finally, the precursors, ω-azido-functionalized poly(2-ethyl-2-oxazoline)-*co*-poly(ethyleneimine)s and α-alkyne-functionalized poly(ε-caprolactone) were combined by using copper sulphate/sodium ascorbate catalyst system under warm conditions. In this respect, copper catalysed azide – alkyne cycloaddition of ω-azido-functionalized PEtOx$_{\%30}$-*co*-PEI or PEtOx$_{\%60}$-*co*-PEI and α-alkyne-functionalized PCL with the catalyst system of copper sulphate/sodium ascorbate dissolved in dimethylformamide (DMF) was followed [17,19,20].

Preparation of blank micelle and micelle/DNA complexes

Blank micelles were prepared by using thin film hydration method [21]. PPP30 and PPP60 block copolymers were dissolved in 2 mL of dichloromethane (DCM). Then, the solvent was removed by rotary evaporation to form a thin film. Subsequently, by adding ultrapure water heated to 60 °C, the film was hydrated, vortexed and filtered. Micelle/DNA complexes were prepared by using various molar ratios ranging from 0.5 to 30 (ratio of N/P) between amine groups of PEI and DNA phosphate groups. Therefore, blank micelles were diluted to different concentrations for desired ratios of N/P and DNA solution (including 4 μg pEGFP-C3) was added dropwise onto micelle solution with equal volumes. Then, the mixture was vortexed and left for complex formation.

Determination of critical micelle concentration (CMC)

The CMC of the block copolymers containing PEI in two different hydrolysis percentages were determined by standard pyrene method [22]. Various dilutions were prepared and added to pyrene to obtain pyrene to be 6.0×10^{-7} M in final concentrations. All samples were allowed for magnetic stirring overnight and analysed in SpectraMax spectrofluorometer (Molecular Devices, Sunnyvale, CA). CMC was calculated from pyrene fluorescence peaks ($\lambda_{ex} = 333$ nm and $\lambda_{ex} = 336$ nm; $\lambda_{em} = 390$ nm) by using the peak 1:peak 3 ratio.

Physicochemical characterization of blank micelles and micelle/DNA complexes

The hydrodynamic diameter, PDI and zeta potential measurements of freshly prepared blank micelles and micelle/DNA complexes were characterized by dynamic light scattering (DLS) method by using ZetaSizer Nano ZS (Malvern Instruments, Worcestershine, UK). The measurement of each formulation was made in three replicates and expressed as mean.

Morphological characterization of blank micelles and micelle/DNA complexes

Morphological characterization of freshly prepared blank micelles and selected micelle/DNA complexes was analysed by transmission electron microscopy (TEM). Sample was dropped onto a carbon-coated copper grid and the solution was evaporated [5]. TEM images were performed using Tecnai G^2 Spirit Biotwin 20-120 kV (FEI Company, Eindhoven, Netherlands).

Gel retardation analysis

Micelle/DNA complex formation was determined by gel retardation assay. The complexes with various ratios of N/P (0.5–30) were loaded to 0.8% agarose gel and electrophoresis was carried out in 1× Tris acetic acid EDTA (TAE) buffer at 100 mV for 45 min with Bio-Rad Subcell Electrophoresis System (Bio-Rad Lab. Inc., Hercules, CA) and photographed in Kodak Gel Logic 200 Imaging System (Kodak, Frederick, MD).

Stability studies

DNase-I nuclease stability

The nuclease stability of PPP30 and PPP60 micelle/DNA complexes with selected two different ratios of N/P (20 and 30) was tested for DNase-I nuclease assay. Micelles were complexed with 1 μg pDNA and complexes were incubated with 1 U DNase-I for 10 min at 37 °C. 0.5 M EDTA added to reaction to inactivate the DNase-I. One microgram naked pEGFP-C3 pDNA was digested with DNase-I and non-treated pDNA was used as control. All samples were analysed on 0.8% agarose gel for 45 min.

Serum stability

PPP30 micelle/pDNA and PPP60 micelle/pDNA complexes with selected N/P ratios were incubated with the equal volume of cell culture medium supplemented with 10% FBS at 37 °C at certain time points. Naked pDNA was treated in the same manner as control. At the end of the time points, a sample was removed and 0.5 M EDTA was added and incubated at 60 °C. Afterwards, heparin was added to mixture to release the pDNA from micelle. The samples were analysed by gel electrophoresis.

Cell culture

MCF-7 and MDA-MB-468 cells were grown in DMEM that was supplemented with 10% FBS and antibiotics (100 μg/mL of streptomycin and 100 unit/mL of penicillin) at 37 °C in a sterilized humidified incubator with 5% CO_2. Cells were sub-cultivated with trypsin–EDTA solution (0.05%) when reached to 80% confluency.

Flow cytometric cell viability assay for blank micelles and micelle/DNA complexes

The influence of blank, PPP30/DNA and PPP60/DNA complexes with various N/P ratios (8, 10, 20 and 30) on MCF-7 and MDA-MB-468 breast cancer cells' viability was determined by flow cytometry using DRAQ7 exclusion assay. The cells (1×10^5/well) were incubated in completed medium (FBS, 10%) for overnight. The medium was removed and replaced with fresh serum-free medium. Then, 50 μL micelle/DNA complexes containing pEGFP-C3 plasmid (4 μg) or blank micelles were added into each well. After 4 h-long incubation, the serum-free media were supplemented with FBS and antibiotics in order to reduce the unfavourable effects of transfection procedure on the cell viability. Following 48 h post-transfection, the cells were collected as single cell suspension and incubated with DRAQ7 for 5 min at room temperature. The cells which were not labelled with DRAQ7 were gated as viable subpopulation [23]. The analyses were performed (633 nm excitation, 660 nm emission) on a flow cytometer (FACSAria II, Becton-Dickinson, San Jose, CA). At least 1×10^4 cells were read and untreated cells served as control. All assays were carried out in triplicates and data were expressed as mean ± standard deviation (SD).

Analysis of transfection efficiency and transgene expression by flow cytometry and fluorescence microscopy

Transfection of pEGFP-C3 plasmid, which carries the enhanced green fluorescence protein (EGFP) as a reporter gene, into MCF-7 and MDA-MB-468 cells was performed at selected N/P ratios of both PPP30 and PPP60 micelle/DNA complexes, according to the procedures detailed above. Transfection efficiency was determined according to the cells percentage positive for EGFP determined by flow cytometry (488 nm excitation, 525 nm emission) 48 h post-transfection.

In order to visualize EGFP expression, MCF-7 and MDA-MB-468 cells were seeded into six-well plates (5×10^4 cells/well) and transfected with PPP60 micelle/DNA complexes with selected N/P ratios. After 48 h, the medium was

removed and the cells' nuclei were stained with DAPI according to manufacturer protocol. The micrographs showing EGFP-positive cells was taken on an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Untransfected cells were used as negative control.

Statistical analysis

The data were presented as mean ± standard deviation (SD). Student's *t*-test was used to compare two groups. The differences between groups were considered statistically by GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). $p < .05$ was considered to be statistically significant.

Results and discussion

Synthesis of partially hydrolysed PEtOx-co-PEI-b-PCL amphiphilic block copolymers

PEtOx-*co*-PEI-*b*-PCL amphiphilic block copolymers containing PEI in two different hydrolysis percentages (30% and 60%) were synthesized by several step reactions (Figure 1).

The synthesis of well-defined amphiphilic block copolymers was carried out by CuAAC click chemistry. Initially, PEtOx-N_3 was synthesized by living cationic ring opening polymerization (ROP) of 2-ethyl-2-oxazoline (EtOx) monomer with methyl p-toluene-sulphonate (MeTos) initiator and terminated with sodium azide to obtain an azide-terminated polymer. Then, linear PEI with two different hydrolysis percentages was prepared by partial hydrolysis of PEtOx with a certain hydrolysis time (Table 1).

Also PCL-alkyne was prepared by ROP of ε-caprolactone using tin (II) 2-ethylhexanoate ($Sn(Oct)_2$) as catalyst and propargyl alcohol as initiator. Finally, PEtOx-*co*-PEI-*b*-PCL amphiphilic block copolymers containing PEtOx as hydrophilic segment and poly(ε-caprolactone) as hydrophobic segment were synthesized by combination of coordination-insertion ROP and living CROP (cationic ROP) with CuAAC (copper catalysed azide-alkyne cycloaddition) click chemistry [17,19,20]. These reactions provided the easy functionalization of PEtOx block copolymers showed uniform molecular weight distribution (PDI = 1.35). Figure 2(A,C) shows the ^{1}H NMR spectrum of PPP30 and PPP60 block copolymers. The two different hydrolysis percentage of linear PEI was determined by ^{1}H NMR by comparing the integration of resonances 2.77 ppm (–$NHCH_2$–) and 3.43 ppm (–$N(C{=}O)CH_2$–). The ratios of the peak areas were evaluated to determine the ethyleneimine unit and hydrolysis degree on polymer backbone.

As a result, to reduce cytotoxicity of PEI, amphiphilic copolymers containing PEI with different hydrolysis were successfully synthesized by partial hydrolysis of PEtOx. Hydrolysis degree was adjusted by changing the hydrolysis time and the results revealed that it was substantially time dependent. Also these results revealed the importance of using PEtOx for preparing efficient gene delivery vector.

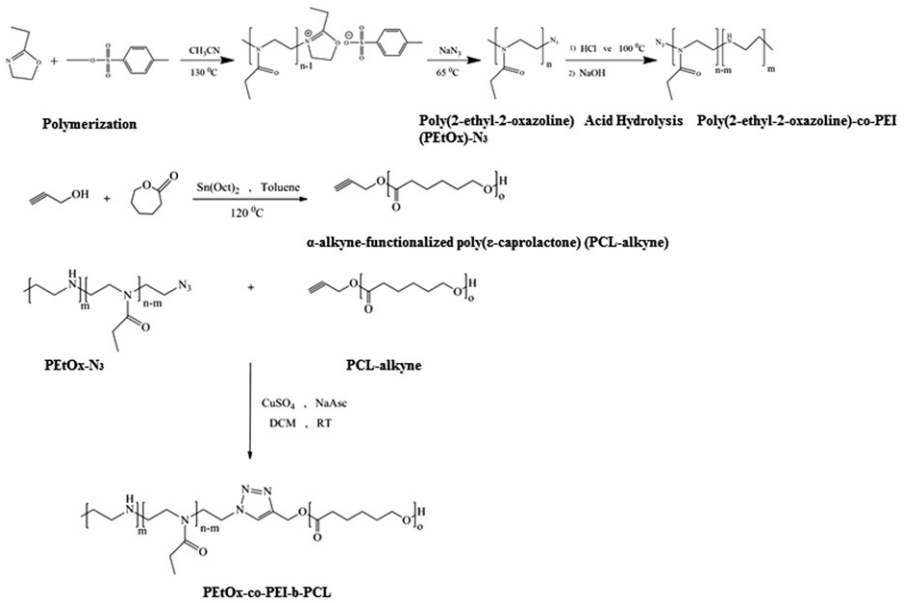

Figure 1. The synthesis reactions of PEtOx-*co*-PEI-*b*-PCL block copolymer.

Table 1. Hydrolysis conditions of block copolymers and characterization of the resulting copolymers.

	Mn (Da)				PDI		Hydrolysis	
	PEtOx							
Code	Before hydrolysis	After hydrolysis	PEI segment in block copolymer	PCL segment in block copolymer	PetOx	PCL	Hydrolysis degree (%)	Hydrolysis duration (h)
PPP30	10,700	7500	1400	2100	1.14	1.35	30	8.5
PPP60	10,700	4300	2800	2100	1.14	1.35	60	17

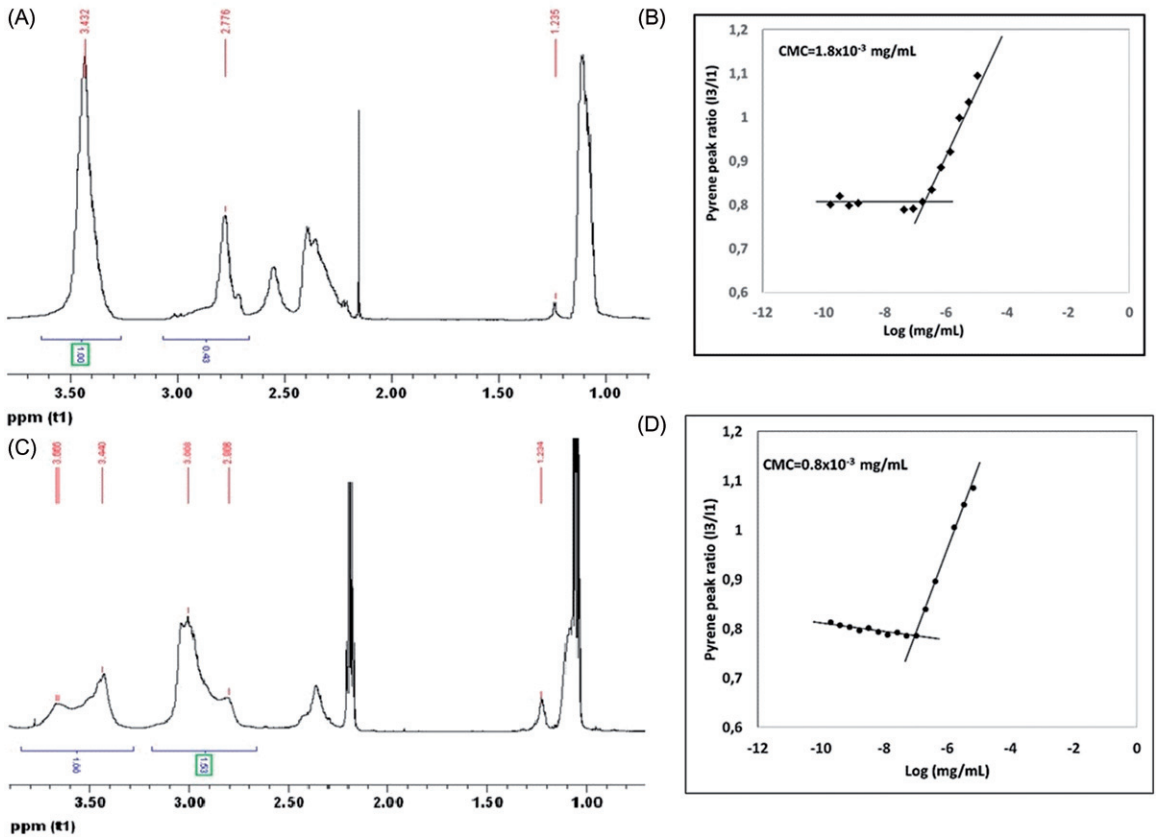

Figure 2. [1]H NMR spectrum and critical micelle concentration analysis of PPP30 (A,B) and (C,D) PPP60 block copolymer, respectively (solvent: D_2O).

Table 2. Size, polydispersity index (PDI) and zeta potential results of blank micelles prepared from PPP30 and PPP60 block copolymers (the data represent mean ± SD of triple experiments).

Micelle formulations (blank)	Size (nm)	PDI	Zeta potential (mV)
$PEtOx_{\%30}$-*co*-PEI-*b*-PCL (PPP30)	81.6 ± 0.5	0.2 ± 0.0	21.8 ± 0.2
$PEtOx_{\%60}$-*co*-PEI-*b*-PCL (PPP60)	118.6 ± 1.0	0.2 ± 0.0	36 ± 1.2

Determination of the critical micelle concentration

The CMC value of PPP30 was 1.8×10^{-3} mg/mL, however PPP60 was 0.8×10^{-3} mg/mL (Figure 2(B,D)). The results revealed that both block copolymers were self-assembled into micelles even at low concentrations. The lower CMC of PPP60 micelles was associated with the higher PEI units that caused the increase in the hydrophobicity of block copolymer to form micelles at lower concentrations [22]. Consequently, the low CMC observations showed that these block copolymers extremely can preserve their stability in blood stream.

Physicochemical characterization of blank micelles and micelle/DNA complexes

Dynamic light scattering results indicated that blank micelles prepared from PPP30 copolymer had significant small particle size compared to PPP60 micelles. Also micelles had highly positive charge and low PDI as given in Table 2. These results indicated that increase in hydrolysis and molecular weight of PEI caused increase in particle size and zeta potential.

To evaluate the ionic strength of media, micelle/DNA complexes were prepared in two different media. As shown in Figure 3(A), ratios of N/P (8–30) indicated complex forming and the particle size of PPP30 micelle/DNA complexes were around in the range of 165–217 nm in HEPES buffer while 115–144 nm in ultrapure water. The particle size of PPP60 micelle/DNA complexes were around in the range of 169–182 nm in HEPES buffer at N/P = 8 to 30 ratios while 142–151 nm in ultrapure water (Figure 3(E)). Although PPP60 copolymer contains more PEI segment, PPP60 micelle/DNA complexes exhibited lower particle size. Also both of micelle formulations could efficiently condense the pDNA and complexes were highly compacted at highest N/P ratio. These findings indicated that particle size of micelle/DNA complexes decreased gradually when the N/P ratio increased. Our results are compatible that increasing amount of cationic polymer caused more tight condensation with DNA that leads to small particles [1,24]. The largest particle size was observed at N/P = 4 for PPP30 micelle/DNA complexes and at N/P = 2 for PPP60 micelle/DNA complexes. It is because, above these ratios, full condensation occurred and zeta potential being neutral. The neutral surface charge distinctly caused the aggregation of complexes because of the decreasing the repulsion between the particles that concluded formation of large particles [9].

Zeta potential of PPP30 micelle/DNA complexes was negative until N/P = 4, when the N/P ratio reached above, positive

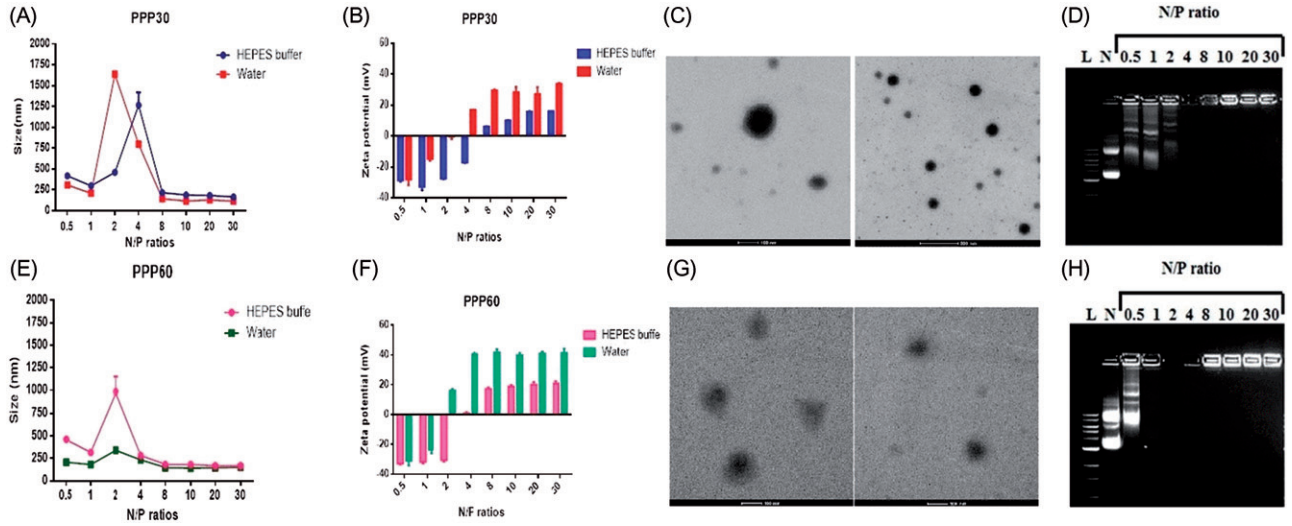

Figure 3. The analysis of particle size (A) and zeta potential (B) of PPP30/DNA complexes and (E,F) PPP60/DNA complexes at various N/P (0.5–30) in ultrapure water and HEPES buffer. (C,D) respectively represent TEM images (right for N/P = 30 and left for N/P = 20) and electrophoresis images of PPP30/DNA complexes. (G,H) represent for PPP60/DNA complexes L: ladder (1 kb), N: naked pDNA (1 μg), lanes 3–10, N/P = 0.5–30 of micelle/DNA complexes (the data represents mean ± SD of triple experiments).

charge was gradually increased (Figure 3(B)). Also zeta potential of PPP60 micelle/DNA complexes was negative among the ratio of N/P = 0.5 to 2. Above this, surface charge became positive and gradually increased when the N/P ratio increased (Figure 3(F)). These positive charged micelles can facilitate the interaction of negative charged cellular membranes and can facilitate the cellular uptake. The negative zeta potential values suggested that micelles did not keep the DNA effectively. Also surface charges of all N/P ratios of complexes were evaluated in ultrapure water. The results exhibited that both PPP30 and PPP60 micelle/DNA complexes had excess positive charge at highest N/P ratio (Figure 3(B,F)). These results supported the observations of particle size that excess cationic charge leading to small particle size due to high repulsion of particles causes to prevent the aggregation [9,25].

Hence, all micelle/pDNA complexes were prepared in HEPES buffer due to the low ionic strength of buffer for further experiments. Also our results compatible with the Jafari et al. [25] indicated that the media were considerably important on the particle size and charge. Moreover, these results indicated that both PPP30 and PPP60 micelle/DNA complexes had desirable size for biological applications and could be passively targeted by EPR effect [26,27].

Morphological characterization of blank micelles and micelle/DNA complexes

Morphological characterization of PPP30 and PPP60 micelle/pDNA complexes was performed by TEM. N/P ratios of 20 and 30 were evaluated due to the lower particle size and relatively higher zeta potential. TEM observations revealed that these all formulations showed typical morphologies as follows, well-defined homogenous spherical, rigid shape and optimum size (almost 100–200 nm). No obvious aggregation was observed (Figure 3(C,G)). These findings were highly consistent with our DLS measurements.

Gel retardation analysis

The complexation ability of pDNA with two different micelle formulations (PPP30 and PPP60) at various N/P ratios was analysed by gel retardation assay [28]. As expected increased N/P ratio caused the reduction of DNA migration on gel. The complete retardation of PPP30 micelles with pDNA occurred at N/P = 4 (Figure 3(D)), however, PPP60 micelles started to form complexes at N/P = 2 (Figure 3(H)). Depending on the increase in concentration of cationic polymer, pDNA was gradually bound and the mobility of pDNA was reduced due to formation of micelle/DNA complexes [2]. The reason is that the higher hydrolysis percentage of ethyleneimine units dominate more free amine groups to condense the pDNA and it causes more neutralization [1,9].

Stability studies

DNase-I nuclease stability

Gel images revealed that naked DNA was completely degraded by DNase-I that any band was monitored on the gel (Figure 4(A,B)). Despite, the band was not detected for PPP30 micelle/DNA, PPP60 micelle/DNA complexes with the N/P = 20, showed little patterns with predominantly protected bands observed on gel. The N/P = 30 for PPP60/DNA complex was completely stable with the prominent pDNA bands whereas PPP30/DNA complex showed some degradation because of the presence of visible smaller DNA fragments. These results indicated that PPP60 micelle/DNA complexes with N/P = 30 were seen as more stable and effective formulation with high resistance.

Serum stability

Serum stability assay was performed with selected N/P ratios of micelle/DNA complexes by serum component as 10% FBS at predetermined time intervals at 37 °C. pDNA fragments seen in the gel images may detect due to presence of

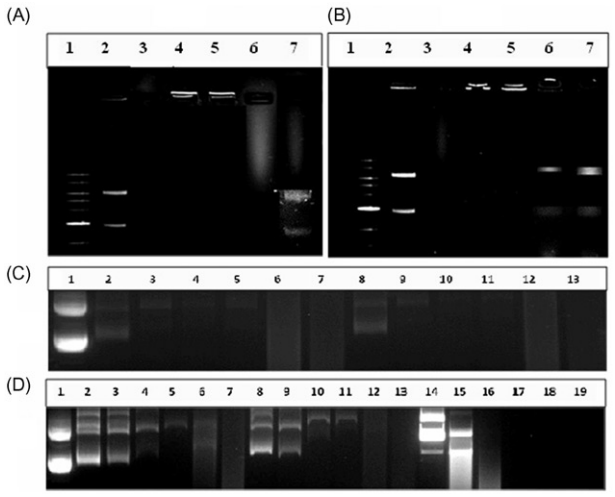

Figure 4. Stability analysis of PPP30/DNA complexes and PPP60/DNA complexes. DNase-I stability image (a) of PPP30/DNA complexes, (b) PPP60/DNA complexes. (1) Ladder, (2) naked pDNA (1 μg) (not treated with DNase-I), (3) naked DNA (treated), (4) blank micelle concentration of N/P = 20, (5) blank micelle concentration of N/P = 30, (6) micelle/DNA complexes at N/P = 20, (7) micelle/DNA complexes at N/P = 30. (c, d) Serum stability analysis of PPP30/DNA complexes (c) and PPP60/DNA complexes (d) at predetermined time intervals. Lane 1. 1 μg naked DNA (nontreated), lanes 2–7. PPP60/DNA complexes for N/P = 30 incubated for 30 min to 48 h, respectively, lanes 8–13. PPP60/DNA complexes for N/P = 20 ratio incubated for 30 min to 48 h, respectively, lanes 14–19. Naked DNA incubated for 30 min to 48 h, respectively.

heparin agent that caused to release pDNA from micelle because of the highly negative charge features that induced the destabilization of micelle/pDNA complexes. The gel observation indicated that naked pDNA was completely degraded at 37 °C after 1 h. In contrast, detectable bands were obtained for both selected PPP30 and PPP60 micelle/pDNA complexes even large amounts of pDNA still exist in the loading well. PPP 30 micelle/pDNA complexes with N/P = 20 and 30 showed similar band brightness revealed N/P = 30 formulations were able to protect DNA for 4 h (Figure 4(C)). However, PPP60/pDNA complexes more stable in active serum with those much brighter bands were detected even after 24 h for N/P = 30 (Figure 4(D)). The findings suggested that PPP60 micelle/DNA complexes (N/P ratio of 30) had more effective formulations with higher stability that it could protect the pDNA from serum degradation.

Effect of blank micelles and micelle/DNA complexes on MCF-7 and MDA-MB-468 cell viability

The viability of MCF-7 and MDA-MB-468 cells following 48 h-long treatment with blank micelles or micelle/DNA complexes was tested by flow cytometric DRAQ7 exclusion test and compared that to untreated controls. The viability of both MCF-7 and MDA-MB-468 cells was not hampered (all of the cells were DRAQ-negative) when they were treated with any N/P ratios of PPP 30 micelle/DNA complexes. On the other hand, PPP60 micelle/DNA complexes exhibited robust cytotoxicity. This might be due to the lower PEI hydrolysis degree induced enough to condense DNA and positive charge of PEI was hidden by pDNA [29]. With the PPP60 micelle/DNA complexes at the ratio N/P = 30, the viability of MCF-7 cells was 67% (Figure 5(A)). MCF-7 cells were more sensitive compared to MDA-MB-468. Accordingly, in the MDA-MB-468 cells that was treated with the PPP60 micelle/DNA complexes, the viability was reduced from 89% to 78% as the N/P ratio was increased from 8 to 30 (Figure 5(B)). Therefore, the decrease in cell viability was correlated with the increase in N/P ratios. These data indicated the negative effect of high hydrolysis degree of PEtOx and higher molecular weight of PEI in the polymer chain on cell viability.

No significant toxicity was observed with PPP30 and PPP60 blank micelles on both cell lines. Incubation with PPP60 blank micelles resulted in 87.7% cell viability which was comparable to that obtained with PPP30 blank micelles (89.1%) at the highest N/P ratio on MCF-7 (Figure 5(C)). Additionally, PPP60 blank micelles had low toxicity, which might be because of the charge density that was strong enough to condense the pDNA.

The PPP30 blank micelles were almost non-toxic at all N/P ratios (Figure 5(D)) whereas for the PPP60 micelles the increased N/P ratio depending on the increase in polymer concentration caused a decrease in the cell viability in MDA-MB-468 cells. The reason can be quite clear since increases in hydrolysis degree would eventually exert toxic effects on cell viability with some variations. In recent years, many studies showed the favourable effects of low molecular weight of PEI. Therefore, in our study, we synthesized PEI by partial hydrolysis of PEtOx to reduce many limitations of PEI with quite low molecular weight as 1400 and 2800. In accordance with the literature, our cytotoxicity data verified the extent of PEtOx hydrolysis as a critical factor influencing cell viability [15].

Transfection efficiency of PPP30 and PPP60 micelle/pDNA complexes and fluorescence microscopy

Since a pDNA construct encoding EGFP gene was used in PPP30 and PPP60 micelle/pDNA complexes, EGFP expression which was obtained by the transfection of MCF-7 and MDA-MB-468 cells with pEGFP-C3 plasmid was used as a reporter for the *in vitro* transfection efficiency. The cells that were incubated with naked DNA or the untreated cells were used as controls. Expectedly, MCF-7 and MDA-MB-468 cells displayed differential capacities as gene delivery hosts; moreover, the N/P ratio and the percentage of PEI hydrolysis were critical for a successive transfection.

EGFP-positive cells were maximum 6% even at the highest N/P ratio for the PPP30 micelle/DNA complexes. Nevertheless, the PPP60 micelle/pDNA complexes demonstrated improved efficiency when the N/P ratio was increased (Figure 5(E)). The highest N/P ratio gave the highest percentage of EGFP + cells (45%). Correspondingly, this highest N/P ratio for PPP60 complex contained relatively high PEI content on polymer backbone which could more effectively condense DNA. The percentages of EGFP + MCF-7 cells following the transfections with N/P = 20 and N/P = 30 for PPP30 and PPP60 micelle/DNA complexes were statistically significant ($p < .05$). The results obtained with MDA-MB-468 cells and N/P = 8 were compatible with those of the naked pDNA for PPP30 complexes ($p > .05$); however, the PPP60 micelle/DNA complexes

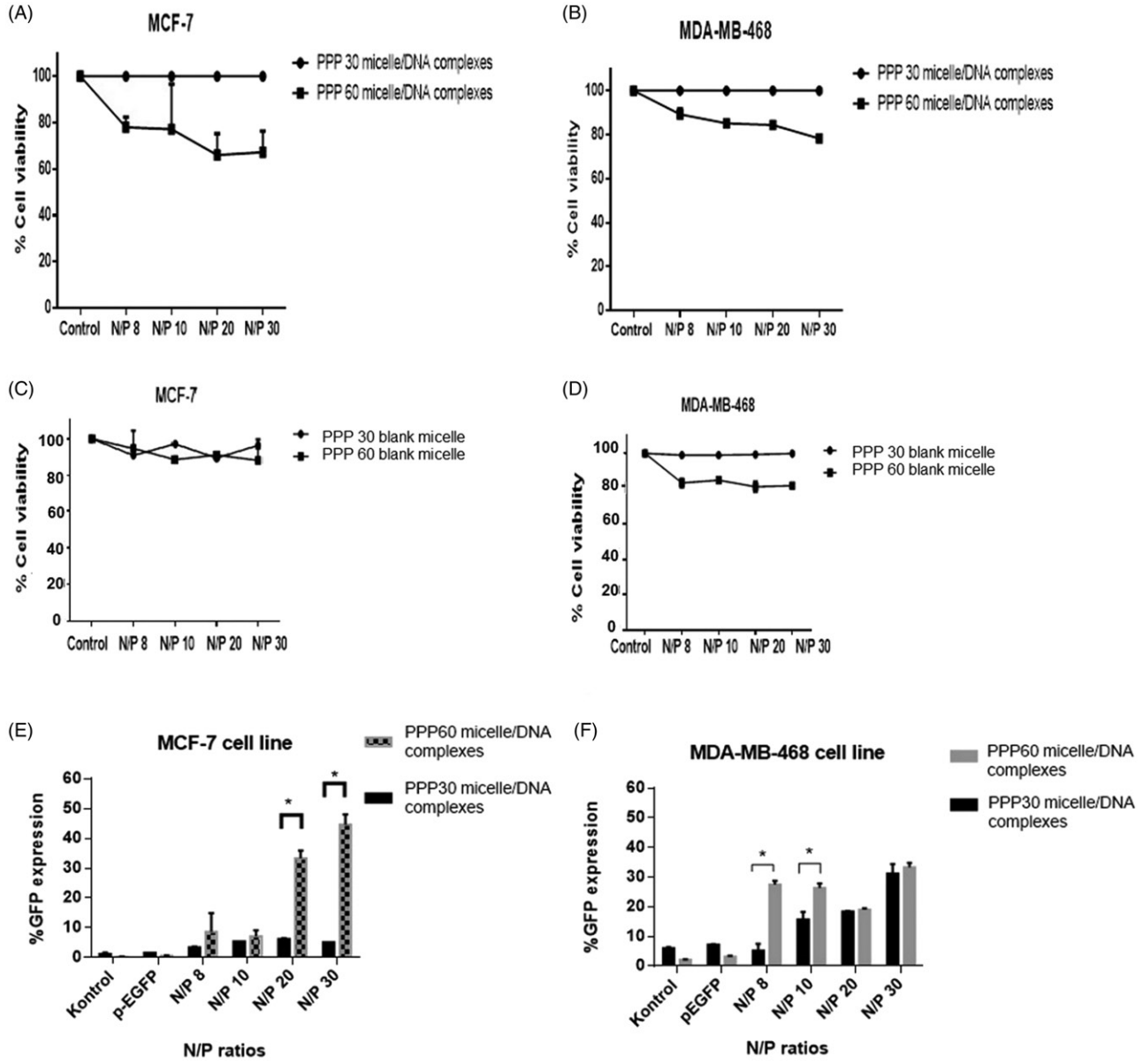

Figure 5. The cell viability and transfection efficiency analysis of complexes and blank micelles. The cell viability of PPP 30 and PPP60/DNA complexes with various N/P ratios on (A) MCF-7, (B) MDA-MB-468 cells for 48 h and PPP 30 and PPP60 blank micelles with various N/P ratios on (C) MCF-7 and (D) MDA-MB-468 cells for 48 h. Transfection efficiency of PPP30 and PPP60 micelle/DNA complexes on (E) MCF-7 cells and (F) MDA-MB-468 cells with various N/P ratios. Data represent mean ± SD and statistical significance was determined by multiple Student's *t*-test (*$p < .05$).

had high transfection efficiency even at N/P = 8 (EGFP+, 27.5%). Both complexes at N/P 30 showed the highest percentages of EGFP expression as compared to that obtained with the naked DNA (EGFP+, ~32%) (Figure 5(F)).

As the most efficient transfection was obtained with PPP60 micelle/DNA complexes, the data were supported by fluorescence microscopy for $EGFP^+$ cells. The PPP60 micelle/DNA complexes at N/P = 30 showed the strongest green fluorescent intensity in MCF-7 cells compared to N/P = 20 (Figure 6). Naked pEGFP-C3 alone did not result in EGFP positivity. Even though MCF-7 cells were more compatible for transfection, these observations also confirmed with MDA-MB-468 cell line wherein N/P = 30 was identified as a better ratio for transfection (Figure 7). Hence, the fluorescence intensity was enhanced when the N/P ratio was increased from 20 to 30.

The PPP60 micelle/DNA complexes showed improved transfection efficiency both in MCF-7 and MDA-MB-468 cells, especially at N/P = 30. PEI content of block copolymer was important for successful gene transfection. Collectively, *in vitro* transfection studies indicated the ability of PEI, which was produced by partial hydrolysis, to successfully deliver DNA. In the light of these findings, PPP60 micelle/DNA complex could be a favourable promising gene delivery platform for *in vitro* and potentially for *in vivo* applications.

Conclusions

To sum up, in our study, we have prepared novel self-assembled block copolymer micelles by partial hydrolysis of PEtOx with two hydrolysis percentages. It was built in conjunction with PEI to impart a positive charge for DNA

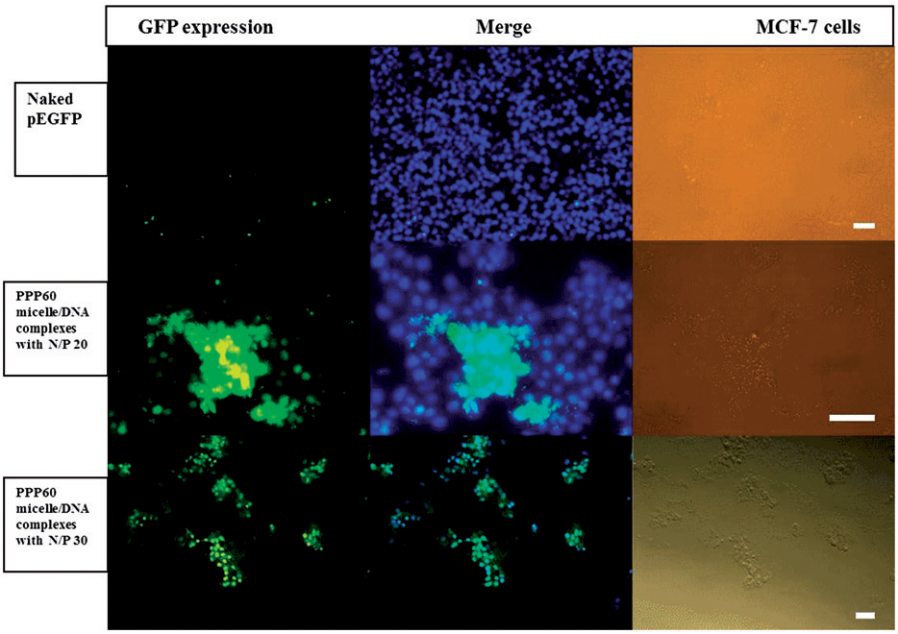

Figure 6. Fluorescence image of GFP expression in MCF-7 cells transfected with PPP60 micelle/pEGFP-C3 complexes with N/P = 20 and 30. Scale bar is 50 μm.

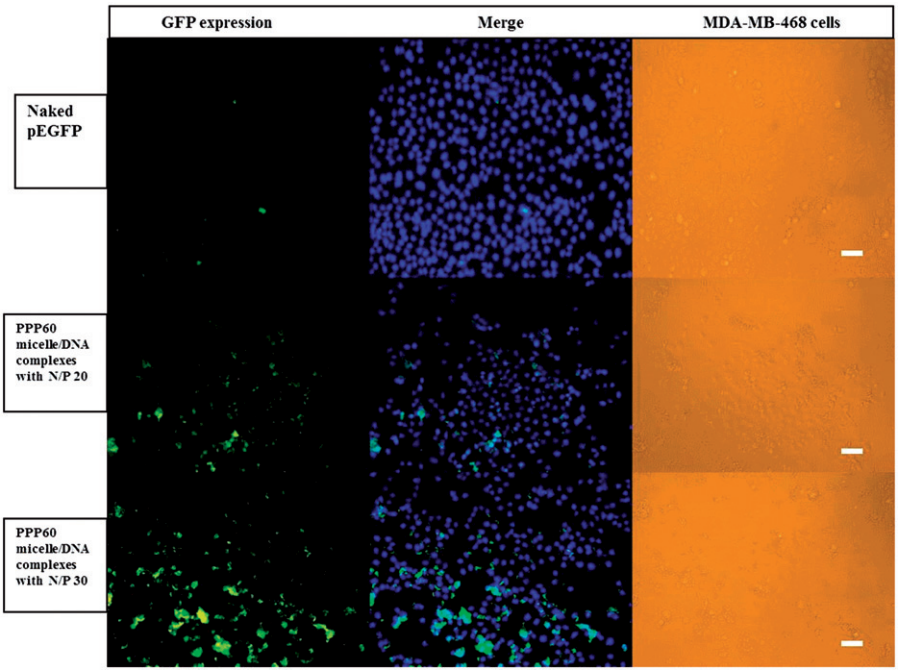

Figure 7. Fluorescence image of GFP expression in MDA-MB-468 cells transfected with PPP60 micelle/pEGFP-C3 complexes with N/P = 20 and 30. Scale bar is 50 μm.

complexation. This is the first study about synthesizing PEI containing copolymer micelles by partial hydrolysis of PEtOx and to evaluate the effect of hydrolysis degree on micelle characteristics and gene transfection studies. The results revealed that both micelle complexes could self-assemble in aqueous solution even at low concentrations with appropriate particle size. Even both PPP30 and PPP60 micelles could condense pDNA, PPP60 micelles showed highest transfection efficiency with high stability. Also cell viability studies indicated that cytotoxicity of micelle/DNA complexes is substantially dependent on hydrolysis percentage of PEI in chain of block copolymer. Based on findings, it was considered that our novel polymeric micelles would be an innovative approach in future for treatment of cancer by gene therapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

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