Springer

Dear Author

Here are the proofs of your article.

- You can submit your corrections **online** or by **fax**.
- For **online** submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.
- For **fax** submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.
- Together with the proof please return the cover sheet (including the *Copyright Transfer Statement)* and the *Offprint Order Form.* They can either be scanned and sent electronically or sent by fax.
- Remember to note the journal title, article number, and your name when sending your response via e-mail, fax or regular mail.
- **Check** the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.
- **Check** the questions that may have arisen during copy editing and insert your answers/corrections.
- **Check** that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the *Edited manuscript*.
- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.
- Please **do not** make changes that involve only matters of style. We have generally introduced forms that follow the journal's style.
  Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.
- If we do not receive your corrections **within 48 hours**, we will send you a reminder.

Please note

Your article will be published **Online First** approximately one week after receipt of your corrected proofs. This is the **official first publication** citable with the DOI. **Further changes are, therefore, not possible.**

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL:

`http://dx.doi.org/10.1007/s12010-013-0224-0`

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information, go to: http://www.springerlink.com.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us, if you would like to have these documents returned.

The **printed version** will follow in a forthcoming issue.

Metadata of the article that will be visualized in OnlineFirst

Please note: Images will appear in color online but will be printed in black and white.

1	Article Title	**Dynamics of PEGylated–Dextran–Spermine Nanoparticles for Gene Delivery to Leukemic Cells**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer Science+Business Media New York 2013 (This will be the copyright line in the final PDF)**	
4	Journal Name	Applied Biochemistry and Biotechnology	
5	Corresponding Author	Family Name	**Rosli**
6		Particle	
7		Given Name	**R.**
8		Suffix	
9		Organization	Universiti Putra Malaysia
10		Division	UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience
11		Address	Serdang 43400 UPM, Selangor, Malaysia
12		Organization	Universiti Putra Malaysia
13		Division	Medical Genetics Laboratory, Faculty of Medicine and Health Sciences
14		Address	Serdang 43400 UPM, Selangor, Malaysia
15		e-mail	rozita@medic.upm.edu.my
16	Author	Family Name	**Amini**
17		Particle	
18		Given Name	**R.**
19		Suffix	
20		Organization	Universiti Putra Malaysia
21		Division	Medical Genetics Laboratory, Faculty of Medicine and Health Sciences
22		Address	Serdang 43400 UPM, Selangor, Malaysia
23		Organization	Ilam University of Medical Sciences
24		Division	Clinical Microbiology Research Center
25		Address	Ilam 69391 IUMS, Iran
26		e-mail	
27	Author	Family Name	**Jalilian**
28		Particle	

29		Given Name	**F. Azizi**
30		Suffix	
31		Organization	Ilam University of Medical Sciences
32		Division	Department of Medical Microbiology, Faculty of Medicine
33		Address	Ilam 69316 IUMS, Iran
34		e-mail	
35		Family Name	**Abdullah**
36		Particle	
37		Given Name	**S.**
38		Suffix	
39		Organization	Universiti Putra Malaysia
40	Author	Division	Medical Genetics Laboratory, Faculty of Medicine and Health Sciences
41		Address	Serdang 43400 UPM, Selangor, Malaysia
42		Organization	Universiti Putra Malaysia
43		Division	UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience
44		Address	Serdang 43400 UPM, Selangor, Malaysia
45		e-mail	
46		Family Name	**Veerakumarasivam**
47		Particle	
48		Given Name	**A.**
49		Suffix	
50		Organization	Universiti Putra Malaysia
51		Division	Medical Genetics Laboratory, Faculty of Medicine and Health Sciences
52	Author	Address	Serdang 43400 UPM, Selangor, Malaysia
53		Organization	Universiti Putra Malaysia
54		Division	UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience
55		Address	Serdang 43400 UPM, Selangor, Malaysia
56		Organization	Perdana University
57		Division	Perdana University Graduate School of Medicine
58		Address	Serdang 43400, Selangor Darul Ehsan, Malaysia
59		e-mail	
60		Family Name	**Hosseinkhani**
61	Author	Particle	
62		Given Name	**H.**

63		Suffix	
64		Organization	National Yang Ming University
65		Division	Department of Biomedical Engineering
66		Address	No 155, Sec.2, LiNong St., Taipei, Taiwan
67		e-mail	
68	Author	Family Name	**Abdulamir**
69		Particle	
70		Given Name	**A. S.**
71		Suffix	
72		Organization	Al-Nahrain University
73		Division	Department of Microbiology, College of Medicine
74		Address	Baghdad, Iraq
75		e-mail	
76	Author	Family Name	**Domb**
77		Particle	
78		Given Name	**A. J.**
79		Suffix	
80		Organization	Hebrew University-Hadassah Medical School
81		Division	Department of Medicinal Chemistry and Natural Products, School of Pharmacy
82		Address	Jerusalem 91120, Israel
83		e-mail	
84	Author	Family Name	**Ickowicz**
85		Particle	
86		Given Name	**D.**
87		Suffix	
88		Organization	Hebrew University of Jerusalem
89		Division	Institute for Drug Research, School of Pharmacy
90		Address	Jerusalem, Israel
91		e-mail	
92	Schedule	Received	12 August 2011
93		Revised	
94		Accepted	7 April 2013

95 Abstract Leukemic cells are hard-to-transfect cell lines. Many transfection reagents which can provide high gene transfer efficiency in common adherent cell lines are not effective to transfect established blood cell lines or primary leukemic cells. This study aims to examine a new class of cationic polymer non-viral vector, PEGylated–dextranu–spermine (PEG-D-SPM), to determine its

		ability to transfect the leukemic cells. Here, the optimal conditions of the complex preparation (PEG-D-SPM/plasmid DNA (pDNA)) were examined. Different weight-mixing (*w/w*) ratios of PEG-D-SPM/pDNA complex were prepared to obtain an ideal mixing ratio to protect encapsulated pDNA from DNase degradation and to determine the optimal transfection efficiency of the complex. Strong complexation between polymer and pDNA in agarose gel electrophoresis and protection of pDNA from DNase were detected at ratios from 25 to 15. Highest gene expression was detected at *w/w* ratio of 18 in HL60 and K562 cells. However, gene expression from both leukemic cell lines was lower than the control MCF-7 cells. The cytotoxicity of PEG-D-SPM/pDNA complex at the most optimal mixing ratios was tested in HL60 and K562 cells using MTS assay and the results showed that the PEG-D-SPM/pDNA complex had no cytotoxic effect on these cell lines. Spherical shape and nano-nature of PEG-D-SPM/pDNA complex at ratio 18 was observed using transmission electron microscopy. As PEG-D-SPM showed modest transfection efficiency in the leukemic cell lines, we conclude that further work is needed to improve the delivery efficiency of the PEG-D-SPM.
96	Keywords separated by ' - '	Non-viral - Gene delivery - Dextran–spermine - Nanoparticles - Cationic polymer
97	Foot note information	

Appl Biochem Biotechnol
DOI 10.1007/s12010-013-0224-0

Dynamics of PEGylated–Dextran–Spermine Nanoparticles for Gene Delivery to Leukemic Cells

R. Amini · F. Azizi Jalilian · S. Abdullah · A. Veerakumarasivam · H. Hosseinkhani · A. S. Abdulamir · A. J. Domb · D. Ickowicz · R. Rosli

Received: 12 August 2011 / Accepted: 7 April 2013

Abstract Leukemic cells are hard-to-transfect cell lines. Many transfection reagents which can provide high gene transfer efficiency in common adherent cell lines are not effective to transfect established blood cell lines or primary leukemic cells. This study aims to examine a new class of cationic polymer non-viral vector, PEGylated–

R. Amini · S. Abdullah · A. Veerakumarasivam · R. Rosli
Medical Genetics Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

R. Amini
Clinical Microbiology Research Center, Ilam University of Medical Sciences, 69391 IUMS Ilam, Iran

F. A. Jalilian
Department of Medical Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, 69316 IUMS Ilam, Iran

S. Abdullah · A. Veerakumarasivam · R. Rosli (✉)
UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
e-mail: rozita@medic.upm.edu.my

A. Veerakumarasivam
Perdana University Graduate School of Medicine, Perdana University, Serdang 43400, Selangor Darul Ehsan, Malaysia

H. Hosseinkhani
Department of Biomedical Engineering, National Yang Ming University, No 155, Sec.2, LiNong St., Taipei, Taiwan

A. S. Abdulamir
Department of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

A. J. Domb
Department of Medicinal Chemistry and Natural Products, School of Pharmacy, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

D. Ickowicz
Institute for Drug Research, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem, Israel

dextranu–spermine (PEG-D-SPM), to determine its ability to transfect the leukemic cells. Here, the optimal conditions of the complex preparation (PEG-D-SPM/plasmid DNA (pDNA)) were examined. Different weight-mixing (*w/w*) ratios of PEG-D-SPM/pDNA complex were prepared to obtain an ideal mixing ratio to protect encapsulated pDNA from DNase degradation and to determine the optimal transfection efficiency of the complex. Strong complexation between polymer and pDNA in agarose gel electrophoresis and protection of pDNA from DNase were detected at ratios from 25 to 15. Highest gene expression was detected at *w/w* ratio of 18 in HL60 and K562 cells. However, gene expression from both leukemic cell lines was lower than the control MCF-7 cells. The cytotoxicity of PEG-D-SPM/pDNA complex at the most optimal mixing ratios was tested in HL60 and K562 cells using MTS assay and the results showed that the PEG-D-SPM/pDNA complex had no cytotoxic effect on these cell lines. Spherical shape and nano-nature of PEG-D-SPM/pDNA complex at ratio 18 was observed using transmission electron microscopy. As PEG-D-SPM showed modest transfection efficiency in the leukemic cell lines, we conclude that further work is needed to improve the delivery efficiency of the PEG-D-SPM.

Keywords Non-viral · Gene delivery · Dextran–spermine · Nanoparticles · Cationic polymer

Introduction

Hematological malignancies are of interest for gene transfer approaches due to several factors such as: (1) The neoplastic cells circulate in the blood, thus large numbers of tumor cells can be harvested and sorted for ex vivo manipulation, and (2) the efficiency of gene transfer and transgene effects can be monitored from direct analysis of the blood. Gene transfer approaches in malignant blood cells needs an appropriate vector to achieve high gene transfer efficiency, without major cytotoxicity. Many transfection reagents which show high gene transfer efficiency in common adherent cell lines are not effective to transfect established blood cell lines or primary leukemia cells from patients [1].

An essential factor for a successful gene therapy for hematological disease is the development of a vehicle that can selectively and efficiently deliver gene to target cells. Early efforts in vector design have focused primarily on genetically engineered viruses [2–4]. Nevertheless, intrinsic limitations related to the viral vectors, such as safety concerns, have limited their use in the clinic [5]. Non-viral synthetic vectors are, consequently, being designed as the substitutes to the viral vectors [6]. Non-viral vectors are easy to produce since they are chemically based materials and freely designed. In addition, they exhibit low toxicity with minimal possibility of genetic integration into the host's genome [7, 8]. Unfortunately, the non-viral vectors show lower efficacy of gene transfer compared with the viral systems [9, 10]. Two classes of the synthetic gene delivery systems being mostly investigated are cationic lipids and cationic polymers [11].

Polycationic vectors neutralize the negative charge of DNA and decrease the electrostatic repulsion between DNA and cells. They also protect DNA from enzymatic digestion by nucleases in serum and extracellular fluids [12]. Polycations commonly used in gene delivery are polyethylenimine (PEI) [13], poly(L-lysine) [14], poly-brene [15], gelatin [16], and cationic polysaccharides [17]. Most polycations are toxic to cells and nonbiodegradable. The high cationic charge of polycations mediates DNA condensing and buffering capacities, therefore, the requirement for the addition of endosomolytic agents will decrease [18, 19]. The activity of polycation is related to their molecular weight, polymer type, polymer–DNA mixing

ratio, and molecular structure. We have previously reported on the use of polycation in combination with principal of tissue engineering to enhance in vitro gene transfection [20–24].

Cationic polysaccharides are known to be one of the most attractive candidates among the various polycations for transfection. They are natural, non-toxic, biodegradable, and biocompatible materials and can be easily modified for improved physicochemical properties [25, 26]. Our research team has constructed a new type of biodegradable polycation, dextran–spermine (D-SPM), based on grafted oligoamine residues on natural polysaccharides. They are effective in delivering plasmids for a high biological effect. As these carriers are water-soluble, they can be readily transported to cells by known biological processes and perform as effective vehicles for transporting agents complexed to them [27]. D-SPM polycations are prepared by the reductive amination synthesis between oxidized dextran (dialdehyde derivatives) and the naturally occurring tetramine spermine [28]. The spermine residues in D-SPM polycations play a crucial role in cell transfection. Therefore, D-SPM conjugates are active in transfecting a wide range of cell lines [28–30].

Conjugation of polycation with polyethylene glycol (PEG) reduces particle aggregation in buffers, specifically in the aqueous medium and maintains them aggregation-free after lyophilization. PEG modification of the polycation can increase circulation time and significantly reduces plasma protein adsorption in vivo [31]. It was reported that D-SPM conjugated with PEG showed high level of gene expression in the liver after intravenous injection when compared with D-SPM, which showed no expression in all organs. Generally, PEGylation of D-SPM showed remarkable increase in the complex stability and transfection efficiency in serum-rich media [9, 28].

To date, no study has been performed to determine the transfection efficiency of PEGylated-D-SPM polycation in leukemic cells. Here, we determined the optimal conditions for gene expression in leukemic cell lines via the transfection of PEG-D-SPM/plasmid DNA (pDNA) complexes. The complexation and stability of PEG-D-SPM was also investigated. In vitro cytotoxicity of the polymer was evaluated, and transfection efficiency of PEG-D-SPM/pDNA complexes in suspension leukemic cells and adherent cells was compared. The application of PEG-D-SPM in this study is to improve and maintain the physicochemical stability of D-SPM in transfection media and especially in the blood stream for subsequent in vivo study.

Materials and Methods

Preparation of Plasmid DNA

Plasmid pcDNA3.1$^+$ carrying green fluorescent protein (GFP) reporter gene was prepared by isolation of hMGFP gene in the *Hind*III–*Not*I restriction sites of phMGFP plasmid (Promega, Madison, WI, USA) and insertion in the same restriction sites of pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA, U.S.A). The constructed plasmid is under the control of CMV promoter and enhancer. The length of the expected product was 1,023 bp and was confirmed by gel electrophoresis analysis. Following digestion, ligation, and transformation of JM109 cells (Invitrogen Co., USA), positive colonies were selected and confirmed by endonuclease digestion. Positive colonies were then sequenced for final verification. Finally, pCDNA3.1/hMGFP plasmid was purified using the EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA). The purity and concentration of constructed plasmid was determined using Nanodrop (Thermo, Wilmington, DE, USA).

Preparation of PEG-D-SPM/pDNA Complex

Complexation of PEG-D-SPM with the pDNA was performed by mixing the two materials at various weight-mixing (*w/w*) ratios. Briefly, 150 μl 0.1 M deionized phosphate-buffered saline solution (DPBS, pH 7.4) containing 100, 90, 80, 70, 60, 50, 40, and 30 μg of PEG-D-SPM was added to the same volume of PBS containing 4 μg of pDNA, respectively. The final solution was gently agitated at room temperature (RT) for 30 min to form PEG-D-SPM/pDNA complexes.

Gel Retardation Assay

To identify DNA condensation ability of the PEG-D-SPM, agarose gel electrophoresis was performed. The cationic PEG-D-SPM/pDNA complexes with different weight-mixing ratios (25, 22.5, 20, 17.5, 15, 12.5, 10, and 7.5) in DPBS buffer were prepared and incubated for 30 min at RT. Next, 10 μL of complex suspension containing 4 μg of DNA was electrophoresed on the 0.75 % (*w/v*) agarose gel in 1× of Tris–acetate–EDTA (TAE) running buffer at 80 V for 40 min. Then, the gel was stained and visualized under Alpha-Innotech gel documentation system (San Leandro, CA).

Complex Sensitivity to DNAse I Digestion

DNase I protection assay was performed on several weight-mixing ratios of PEG-D-SPM/pDNA complexes as in the gel retardation assay. Briefly, 8 μl of each complex was incubated with 2 units of DNase I (Promega, USA) at 37 °C for 30 min in a final volume of 10 μL. The reaction was inactivated by the addition of stop solution following incubation at 65 °C for 10 min. The integrity of pDNA released from the complexes was assessed by agarose gel electrophoresis as described above.

Cell Culture

In vitro assays were performed on K562 (human erythromyeloblastoid leukemia cell line) and HL60 (human promyelocytic leukemia cell line) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI with 10 % fetal bovine serum (FBS) (Hyclone, Austria). MCF-7 cells (human breast adenocarcinoma cell line) (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and were used as the control. The cells were incubated at 37 °C in humidified 5 % CO_2.

Cytotoxicity Assay

The CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay (MTS) was used to evaluate the effects of PEG-D-SPM/pDNA on HL60 and K562 cells viability by measuring the uptake and reduction of MTS compound into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture using ELISA microplate reader (Tecan, Austria). Different weight-mixing ratios of PEG-D-SPM/pDNA were prepared [10, 12, 14, 16, 18, 20, 22, 24], with a fixed concentration of pDNA (0.5 μg) scaling up to a total of 200 μl OptiMEM® I medium (Invitrogen Co., USA). The samples were mixed for 5 min and incubated at room temperature for 30 min. Then,

JrnlID 12010_ArtID 224_Proof# 1 - 15/04/2013

100 μl of each ratio was added in the 96-well plates containing 1×10^4 cells/well. The plates were incubated in 37 °C in humidified 5 % CO_2 atmosphere for 72 h. The cell viability was calculated by the following formula: cell viability (%)=optical density (OD) of the treated cells/OD of the non-treated cells.

Transfection of Cells Grown In Vitro

MCF-7 cells were seeded at an initial density of 1×10^5 cells/well on a 24-well plate and grown in 500 μl DMEM and 10 % FBS. HL60 and K562 cells were seeded at density of 5×10^5 cells/well in 500 μl RPMI and 10 % FBS on a 24-well plate and incubated in 37 °C for 24 h prior to transfection. The PEG-D-SPM/pDNA complexes were formed at different weight-mixing ratios (10, 12, 14, 16, 18, and 20 *w/w*). However, each formulation of complexes contained 0.5 μg of pcDNA3.1 according to the conditions describe above. At time of transfection, cell growth medium was aspirated and replaced with 200 μl of the transfection mixture. At 4 h post-transfection, 500 μl complete growth medium was added to each well, and cells were incubated for 72 h. Branched 25 kDa PEI (Sigma-Aldrich Co., USA) and lipofectamine 2000 (Invitrogen Co., USA) were used as the positive controls. Briefly, PEI/pDNA (10: 1 N/P ratio) in a total of 420 μL Opti-MEM®I was added to each well and incubated for 24 h. Then, the transfection media were exchanged with complete growth media and further incubated for another 48 h. The lipofectamine 2000/pDNA (2 μl/0.5 μg) complex in a total of 100 μl OptiMEM®I was added to each well and incubated for 4 h at 37 °C in a CO_2 incubator. The transfection media was replaced with fresh complete media, followed by incubation at 37 °C for another 72 h.

Reporter Gene Expression Analysis

To determine the transfection efficiency, cells were harvested and washed with 1× DPBS and then resuspended in 1× DPBS/2%FBS in a FACS tube (BD Biosciences, San Jose, USA). An aliquot of suspension were analyzed for GFP expression using flow cytometry (BD Biosciences, USA). Side and forward scatter signals were applied to limit the analysis to viable cells. The data were analyzed with the aid of CellQuest Software (Becton Dickinson). The K562 and MCF-7 cells transfected with the same condition mentioned above were also used for observation of GFP expression under inverted fluorescent microscope (Olympus 1X 51, Olympus Corporation, Japan).

Transmission Electron Microscope (TEM)

The PEGylated-D-SPM/pDNA complexes were prepared to a fixed weight-mixing ratio of 18 in nuclease-free water. The aqueous solution was transferred to the transmission electron microscopy (TEM)-gold grids to determine the shape and average diameter of the particles. TEM was performed by the Microscopy Unit, Institute of Bioscience, Universiti Putra Malaysia.

Statistical Analysis

All analyses were performed using SPSS 16.0 software. Before analysis, all data were subjected to Kolmogorov–Smirnov test for normality. To show the differences among groups, ANOVA was applied. Mean comparison was done using Duncan multiple range test. The comparison of results between or among the groups was analyzed by paired sample

*t*test. Test results were considered statistically significant if the probability was less than 0.05.

Results

Physiochemical Properties of the PEG-D-SPM/pDNA

Determination of Optimal Weight-Mixing Ratio of PEG-D-SPM/pDNA

The strength of complexation of PEG-D-SPM with pDNA was determined by performing gel retardation assay (0.75 % agarose gel). The weight-mixing ratio was calculated considering that a fixed amount of pDNA (micrograms) corresponds to different concentration (micrograms) of PEG-D-SPM. From Fig. 1, it was observed that the electrostatic interaction between the polymer and pDNA completely hampered the free movement of the pDNA at ratios 25–15 (lanes 1–5). The higher intensity of fluorescence was observed at lanes 1–4, and it gradually diffused as the weight ratio decreased. As the polymer concentration decreased (ratios 12.5 to 7.5), the PEG-D-SPM gradually lost its ability to hamper pDNA mobility (lanes 6–8).

Stability of PEG-D-SPM Complex in Presence of DNase

Different weight-mixing ratio of PEG-D-SPM/pDNA complexes (as described above) and free pDNA were treated with DNase I and subsequently incubated for 30 min at 37 °C. After incubation, stop solution was added to each mixture to end the reaction. The protection assay was determined by the detection of the intensity of fluorescence from the DNA bands. Figure 2 shows the DNase I challenge study on agarose gel. The intense fluorescence was seen in some loading wells (lanes 1–5) whereas diffused fluorescence was detected in other wells (lanes 6–8). The PEG-D-SPM/pDNA at ratios 17.5 and 15 presented maximum protection as the higher intensity of fluorescence was observed in the well (lanes 4 and 5). The uncomplexed pDNA was rapidly degraded by DNase I (lane 9).

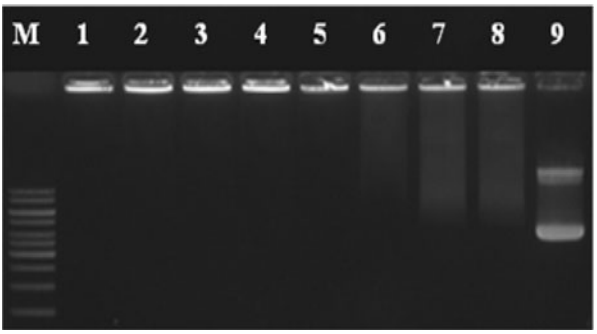


Fig. 1 Agarose gel electrophoresis of PEG-D-SPM/pDNA complexes at different weight ratios. *Lane M*, 1 kb DNA ladder; *lanes 1–8*, PEG-D-SPM/pDNA complexes at ratios 25, 22.5, 20, 17.5, 15, 12.5, 10, 7.5; *lane 9*, free pDNA

JrnlID 12010_ArtID 224_Proof# 1 - 15/04/2013

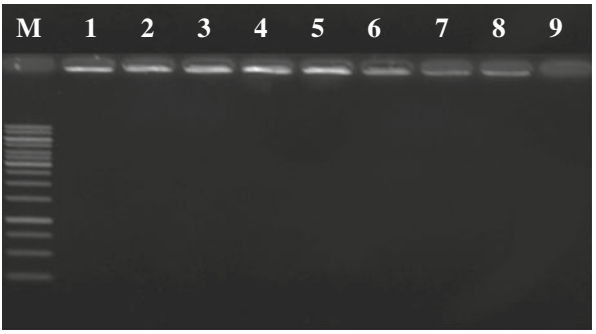


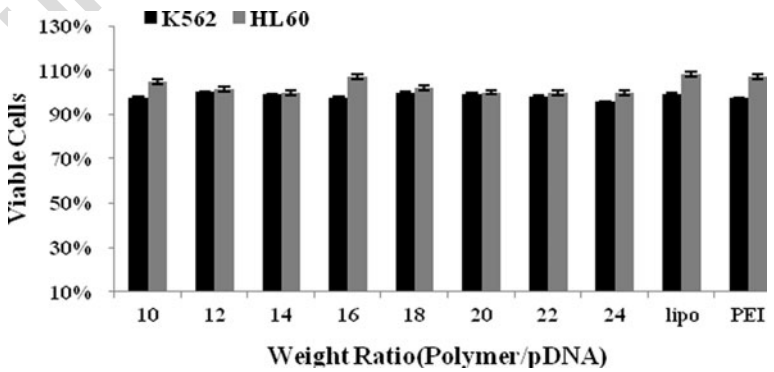
Fig. 2 Agarose gel electrophoresis of PEG-D-SPM/pDNA complexes following DNase I digestion. *Lane M*, 1 kb DNA ladder; *lanes 1–8*, DNase I-treated PEG-D-SPM/pDNA complexes at weight ratios 25, 22.5, 20, 17.5, 15, 12.5, 10, 7.5; *lane 9*, DNase I-treated naked DNA

In Vitro Cytotoxicity

In cytotoxicity assay, the different concentrations of PEG-D-SPM ranging from 10 to 24 μg were added to a fixed amount of plasmid DNA (0.5 μg). The readings from PEG-D-SPM/pDNA complexes along with PEI/pDNA and lipofectamine/pDNA were taken in triplicates. The treated cells were normalized against the untreated cells. The percent viability of cells was compared in Fig. 3. The quantitative evaluation of cytotoxicity by the MTS assay of cells after contact with the complexes showed more than 90 % metabolically active cells for each gene carrier. No significant difference between the viability of K562 and HL60 cells by all three gene carriers was observed.

In Vitro Transfection Activity

The transfection efficiency of PEG-D-SPM/pDNA (carrying hMGFP reporter gene) was assessed in MCF-7, HL60, and K562 cells at different weight-mixing ratios (*w/w*) from 10–20. After 72 h, GFP expression was evaluated using flow cytometry in transfected cells to



Q3 **Fig. 3** Cytotoxicity effect of PEG-D-SPM/pDNA complexes at different weight ratios, PEI, and lipofectamine 2000. The cell viability was evaluated by the MTS assay. The values represent the percentage of viable cells following 72 h of treatment compared with untreated cells. Each data point is presented as mean± SEM ($p<0.05$)

determine the ideal weight-mixing ratio that would generate the optimal gene expression. The delivery of PEG-D-SPM/pDNA was compared with those of the commercial transfection reagents, PEI (cationic polycation) and lipofectamine 2000 (cationic liposome), in terms of delivery efficiency of each reagent in suspension (K562 and HL60) and adherent (MCF-7) cells. As shown in Fig. 4, among various ratios of PEG-D-SPM/pDNA, the highest level of GFP expression was detected at ratio 18 in the three cell lines. On the other hand, comparing three cell lines, MCF-7 revealed significantly higher GFP level at this ratio. The results of the gene delivery using PEI showed a low level of GFP expression in all cell lines, whereas lipofectamine exhibited a significantly high GFP expression in the transfected cells compared with the rest, especially in MCF-7 cells. In addition, by visualizing the transfected K562 and MCF-7 cells under inverted fluorescent microscope, similar trend of GFP expression was observed in all cells transfected by the three gene carriers (Fig. 5). As shown by the figure, higher level of GFP expression in K562 and MCF-7 cells was observed by lipofectamine compared with PEG-D-SPM and PEI. Also, higher intensity of the green fluorescence was observed in MCF-7 cells compared with K562 cells using lipofectamine.

Morphological Characterization of Nanoparticles

TEM analysis was performed when the aqueous solution of PEG-D-SPM/pDNA at ratio of 18 was prepared and transferred to the TEM-gold grids. The representative TEM image of PEG-D-SPM/pDNA is shown in Fig. 6. The findings revealed the nano-nature of the particles and inferred that the particles are mostly spherical in shape. Analyzed particles covered a narrow diameter of size range between 35 and 61 nm when complexes are formed with pDNA. The TEM image also showed that the nanoparticle complexes are uniform in shape.

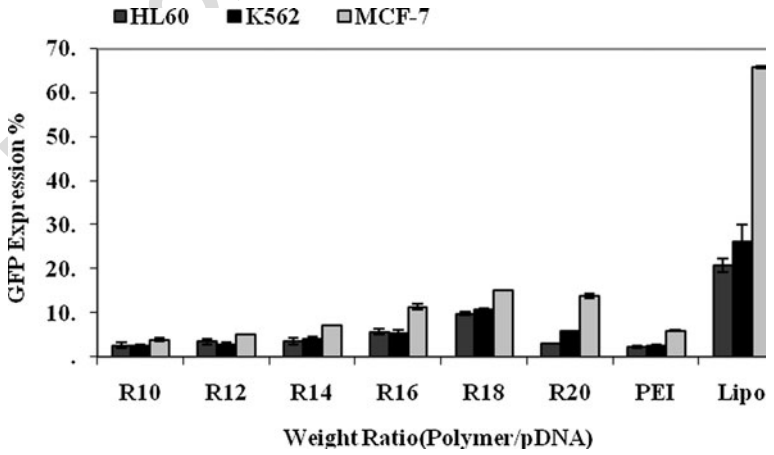


Fig. 4 Transfection efficiency of PEG-D-SPM/pDNA complexes at various weight ratios. PEI and lipofectamine 2000 were used as the positive controls. GFP expression was measured 72 h post-transfection. The fluorescence intensity of GFP-positive cells was detected using flow cytometry. The GFP expression in treated cells was normalized against untreated cells. Each data point is presented as mean±SEM (n=3). The significance level of GFP expression among the ratios in each group was determined according to Duncan's multiple test (p<0.05)

JrnlID 12010_ArtID 224_Proof# 1 - 15/04/2013

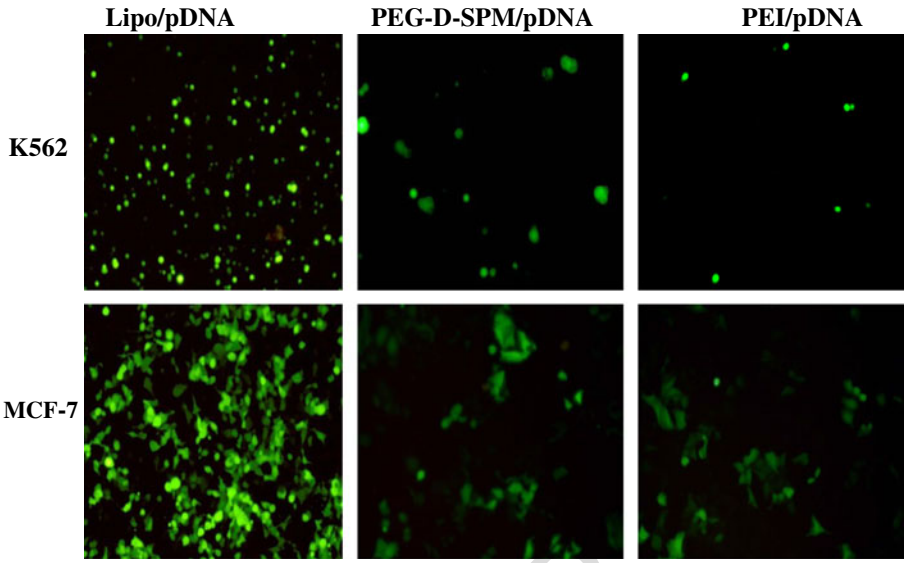


Fig. 5 Fluorescence microscopy of K562 and MCF-7 cells transfected with gene carriers. Fluorescent cells were visualized at 72 h post-transfection. Lipofectamine/pDNA, PEG-DSPM/pDNA (ratio 18), and PEI/pDNA (×100 total magnification)

Discussion

One of the most important factors for achieving an optimal transfection efficiency of polycation-based nanoparticles is the weight-mixing (*w/w*) ratio of polycation to pDNA [28]. The formation of strong complexation between polymer and pDNA limits the mobility of the DNA due to the electrostatic interaction between the pDNA and polymer [32]. In this study, the complexation ability of PEG-D-SPM was investigated at different *w/w* ratios of polymer/pDNA complexes using agarose gel electrophoresis. As the *w/w* ratio of PEG-D-SPM/pDNA decreased, the pDNA gradually started to move toward the anode due to the decrease in the shielding effect of PEG-D-SPM over pDNA. Hosseinkhani et al. reported that the migration of pDNA in different N/P molar ratio of D-SPM/pDNA [1–7] was

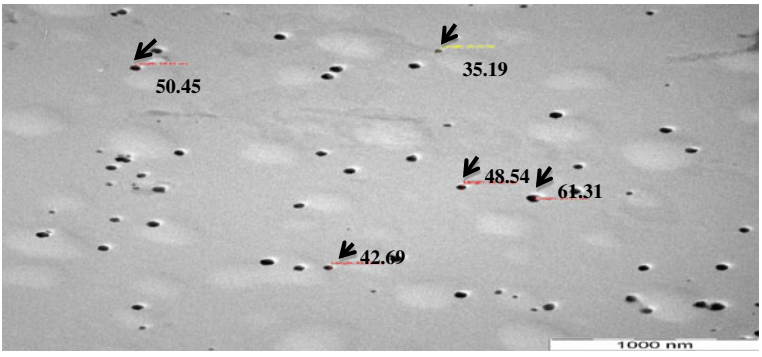


Fig. 6 Transmission electron microscopy (TEM) images of PEG-D-SPM/pDNA complex. PEG-D-SPM/pDNA (ratio 18) with diameters of 35 to 61 nm, are highlighted by *arrows* (magnification of ×30,000)

retarded by increasing the N/P molar ratio (N/P=3–7) [9]. In the study by Syahril et al. gel retardation assays demonstrated the DNA immobility of D-SPM/pDNA complexes at ratios of 14, 16, and 18 [33]. In our study, the electrophoretic movement of DNA toward the anode was observed at weight ratio of less than 15. To explain, the ideal weight-mixing (*w/w*) ratio of polycation to pDNA is different from polymer to polymer [28]. It was also reported that the percentage of PEG introduced to dextran–spermine can alter the electrophoresis migration pattern of plasmid DNA [9]. Degradation of DNA by nucleases is another factor that can impede the success of gene delivery [34]. In this study, the fragmentation of unprotected pDNA by endonuclease activity of DNase I was analyzed to evaluate the encapsulation strength of pDNA by PEG-D-SPM. Protection of pDNA from nuclease digestion by PEG-D-SPM was determined by the observation of the fluorescence in the well compared with the naked DNA where no band was seen. The identification of intense fluorescence at ratio 15 to 25, confirmed the strong association between PEG-D-SPM and pDNA. It also showed the entrapment of the DNA surrounded by the polymer nanoparticles, while diffused fluorescence at ratio 7.5 to 12.5 was due to the degradation of some amounts of DNA from the complex. In another study, the partial protection of pDNA by D-SPM was observed at ratio 14, 16, and 18 [33]. The higher protection of pDNA from DNase activity by PEG-D-SPM compared with D-SPM can be due to the effect of PEG introduction in D-SPM. It was reported that PEG can protect the pDNA complex from interaction with enzymatic degradation and biological substances [9].

Toxicity is still one of the barriers to the use of non-viral vectors in gene delivery [35]. In the current study, in vitro cytotoxicity analysis of PEG-D-SPM/pDNA complexes was performed at several *w/w* ratios ranging from 24 to 10. The PEG-D-SPM /pDNA complexes were found to be safe for both HL60 and K562 cells. Besides, two commercial reagents which were used in our study as the control for transfection efficiency showed no toxicity on two leukemic cell lines, as the viability of cells was more than 90 % in the MTS assay. Our finding is consistent with that of Hosseinkhani et al. who reported that cationized dextran, with or without PEG introduction, had no cytotoxic effect on mouse fibroblast L929 cells [9]. In addition, by using MTT assay, Kim et al. demonstrated that the D-SPM/pDNA was not toxic on HepG2 and HeLa cell lines [36]. Therefore, as with other studies, here we showed that the PEG-D-SPM/pDNA complex was safe to be used on the K562 and HL60 cells.

Following previous findings using gel retardation and DNA stability assays, identification of the optimal weight ratio of PEG-D-SPM/pDNA was continued by performing in vitro transfection assay. The transfection was performed using PEG-D-SPM/pDNA (carrying GFP reporter gene) at ratio 10 to 20 on HL-60, K562, and MCF-7 cell lines. Flow cytometry analysis revealed the highest expression of GFP at ratio 18 in HL-60, K562, and MCF-7 cells (9.88 %, 10.84 %, and 15.18 %, respectively). This can be caused by the robust complexation of PEG-D-SPM with DNA at ratio 18. The ratio of polymer/DNA complex is critical factor for transfection efficiency. The polymer/DNA ratio represents the charge ratio between the positively charged polymer and the negatively charged DNA resulting in strong complexation of polymer/DNA. Therefore, the optimal ratio of polymer/DNA affects a number of critical transfection properties, such as the stability, cytotoxicity, and cellular uptake level of the complex [37, 38].

From this quantitative analysis, we found that the GFP expression in all three cell lines was proportional to the increasing *w/w* ratios of PEG-D-SPM/pDNA, with a significant decrease at ratio 20. This reduction could be due to the loss of the protecting effect of polymer over DNA once the complexation reaches its maximum packaging ability [39]. This pattern of expression was similar in all the three tested cell lines. However, there was a

JrnlID 12010_ArtID 224_Proof# 1 - 15/04/2013

significant difference in GFP expression in MCF-7 cells, where a higher GFP intensity was detected in all ratios, compared with the two other cell lines, HL60 and K562.

In this study, PEI, a commercially available cationic polyamine, was applied as a control to compare its transfection efficiency in all three cell lines. Surprisingly, the GFP expression levels in all three cell lines transfected using the PEI/pDNA complex were significantly lower than the levels expressed by cells treated with PEG-D-SPM at the ratio of 18. As transfection activity with PEI provided low levels of GFP expression, especially in adherent cells (MCF-7), therefore in addition to PEI, we used a cationic lipid (lipofectamine 2000) with an effective delivery profile to check the delivery conditions in our experiments. Results of transfection with lipofectamine 2000 showed considerable level of GFP expression compared with PEI and PEG-D-SPM (65.8 %, 26.18 %, and 20.85 for MCF-7, K562, and HL60, respectively). Besides, the fluorescence microscopy observation supported the flow cytometry results. The transfection efficiency of D-SPM in COS-7 cells was evaluated by Syahril et al. where it was reported that the highest GFP expression was at ratio of 12 with level of expression approximately similar to our results, especially that of the MCF-7 cells. Previous studies of D-SPM, with or without PEG modification, reported a high and effective transfection activity with about 50 % reporter gene expression in some adherent cells [10, 28]. The lower level of GFP expression in the leukemic suspension cells, K562 and HL60 cells, is supported by some studies which emphasized that leukemic cells are hard-to-transfect [40]. It was claimed that low transfection efficiency by non-viral vectors is a considerable issue in gene-transferring approaches in the treatment of hematologic malignancies. Also, it was reported that non-viral carriers usually fail in the efficient transfection of primary cells [41]. Several non-viral methods have been tested in human primary hematopoietic CD-34+ cells, including liposome-mediated transfection, particle-mediated gene transfer by gene gun, and electrotransfection. All studies showed low transfection efficiencies for these types of cells [42–44].

The physiochemical characteristics of nanoparticle complex, including particle size, shape, and morphology, are very important factors affecting delivery efficiency [45, 46]. Complex size can affect the mechanism of internalization. Trafficking of molecules between the cytoplasm and nucleus is size-dependent and occurs through passive diffusion or as a controlled process. Molecules larger than approximately 40–60 kDa need a nuclear localization signal to actively be transported through nuclear pores with 9–10 nm diameter [47]. Transmission electron microscopy evaluation of PEG-D-SPM/pDNA at ratio of 18, which showed the highest level of GFP expression, illustrated the nano-nature of the particles (less than 100 nm) with narrow size range of 35–61 nm. Also, the particles showed a uniform spherical shape which has high probability of entering the cell [48]. The observed particle size and shape of complex seem to be favorable for transfection as the complexation of DNA into small particle improves cell entry [49].

Conclusion

Further optimizations to discover the most optimal conditions of PEG-D-SPM for high level gene expression in leukemic cells is needed. Modifications on the physiochemical properties of the PEG-D-SPM, such as increasing the polymer length or masking the anionic charges of the complex, should be done before the GFP gene can be substituted with the gene of interest to target leukemic cells.

Q4 **References**

1. Zelphati, O., Wang, Y., Kitada, S., Reed, J. C., Felgner, P. L., & Corbeil, J. (2001). *Journal of Biological Chemistry, 276*(37), 35103.
2. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., & Lieber, A. (2000). *Journal of virology, 74*(6), 2567.
3. Stripecke, R., Cardoso, A. A., Pepper, K. A., Skelton, D. C., Yu, X. J., Mascarenhas, L., et al. (2000). *Blood, 96*(4), 1317.
4. Nienhuis, A. W. (2008). *Blood, 111*(9), 4431.
5. Cusack, J. C., & Tanabe, K. K. (2002). *Surgical Oncology Clinics of North America, 11*(3), 497–519.
6. Liu, F., & Huang, L. (2002). *Journal of Controlled Release, 78*(1-3), 259–266.
7. Kim, W. J., Yockman, J. W., Lee, M., Jeong, J. H., Kim, Y. H., & Kim, S. W. (2005). *Journal of Controlled Release, 106*(1-2), 224–234.
8. Choi, S., & Lee, K. D. (2008). *Journal of Controlled Release, 131*(1), 70–76.
9. Hosseinkhani, H., Tabata, Y. (2005). *Controlled Release,* 540–556. Q1
10. Azzam, T., Eliyahu, H., Makovitzki, A., Linial, M., & Domb, A. J. (2004). *Journal of Controlled Release, 96*(2), 309–323.
11. Li, S., & Huang, L. (2000). *Gene Therapy, 7*(1), 31–34.
12. Putnam, D., Gentry, C., Pack, D., & Langer, R. (2001). *Proceedings of the National Academy of Sciences of the United States of America, 98*(3), 1200.
13. Remy, J., Abdallah, B., Zanta, M., Boussif, O., Behr, J., & Demeneix, B. (1998). *Advanced Drug Delivery Reviews, 30*(1-3), 85–95.
14. Wu, G., & Wu, C. (1987). *Journal of Biological Chemistry, 262*(10), 4429.
15. Mumper, R., Duguid, J., Anwer, K., Barron, M., Nitta, H., & Rolland, A. (1996). *Pharmaceutical Research, 13*(5), 701–709.
16. Leong, K., Mao, H., Truong, L. V., Roy, K., Walsh, S., & August, J. (1998). *Journal of Controlled Release, 53*(1-3), 183–193.
17. Azzam, T., Raskin, A., Makovitzki, A., Brem, H., Vierling, P., Lineal, M., et al. (2002). *Macromolecules, 35*(27), 9947–9953.
18. Behr, J. (1997). *Chimia International Journal for Chemistry, 1*(2), 34–36.
19. Boussif, O., Lezoualc'h, F., Zanta, M., Mergny, M., Scherman, D., Demeneix, B., et al. (1995). *Proceedings of the National Academy of Sciences of the United States of America, 92*(16), 7297.
20. Hosseinkhani, H., Khademhosseini, A., Gabrielson, N. P., Pack, D. W., & Kobayashi, H. (2008). *Journal Biomedical Materials Research Part A, 85*, 47–60.
21. Hosseinkhani, H., Inatsugu, Y., Hiraoka, Y., Inoue, S., Shimokawa, H., & Tabata, Y. (2006). *Biomaterials, 27*, 1387–1398.
22. Hosseinkhani, H., Kobayashi, H., Hiraoka, Y., Shimokawa, H., Domb, A. J., & Tabata, Y. (2006). *Biomaterials, 27*, 4269–4278.
23. Hosseinkhani, H., Hiraoka, Y., Inoue, S., Shimokawa, H., & Tabata, Y. (2005). *Tissue Engineering, 11*, 1459–1475.
24. Hosseinkhani, H., Inoue, S., Hiraoka, Y., & Tabata, Y. (2005). *Tissue Engineering, 11*, 1476–1488.
25. Berscht, P., Nies, B., Liebendörfer, A., & Kreuter, J. (1995). *Journal of Materials Science: Materials in Medicine, 6*(4), 201–205.
26. Carreño-Gómez, B., & Duncan, R. (1997). *International Journal of Pharmaceutics, 148*(2), 231–240.
27. Azzam, T., Eliyahu, H., Makovitzki, A., & Domb, A. (2003). *Wiley Online Library, 247–262*.
28. Hosseinkhani, H., Azzam, T., Tabata, Y., & Domb, A. (2004). *Gene Therapy, 11*(2), 194–203.
29. Azzam, T., Shapira, L., Linial, M., Barenholz, Y., & Domb, A. J. (2002). *J. Med. Chem Biol, 45*, 1817–1823.
30. Azzam, T., Makovitzki, A., Brem, H., Vierling, P., & Lineal, M. (2002). *Macromolecules, 35*, 9947–9953.
31. Mao, H. Q., Roy, K., Troung-Le, V. L., Janes, K. A., Lin, K. Y., Wang, Y., et al. (2001). *Journal of Controlled Release, 70*(3), 399–421.
32. Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O., & Seymour, L. W. (1996). *Human Gene Therapy, 7*(17), 2123–2133.
33. Syahril, A., Wai, Y., Hossienkhani, H., Mohsen, H., Ehab, M., Rajesh, R., et al. (2010). *Journal of Biomedicine and Biotechnology.* Q2
34. Hashida, M., Mahato, R. I., Kawabata, K., Miyao, T., Nishikawa, M., & Takakura, Y. (1996). *Journal of Controlled Release, 41*(1-2), 91–97.
35. Morille, M., Passirani, C., Vonarbourg, A., Clavreul, A., & Benoit, J. P. (2008). *Biomaterials, 29*(24-25), 3477–3496.
36. Kim, S. H., Jeong, J. H., Kim, T. I., Kim, S. W., & Bull, D. A. (2009). *Mol Pharm, 6*(3), 718–726.

37. Schaffer, D. V., Fidelman, N. A., Dan, N., & Lauffenburger, D. A. (2000). *Biotechnology and Bioengineering, 67*(5), 598–606.
38. Anderson, D. G., Akinc, A., Hossain, N., & Langer, R. (2005). *Molecular Therapy, 11*(3), 426–434.
39. Thomas, J. J., Rekha, M. R., & Sharma, C. P. (2010). *International Journal of Pharmaceutics, 389*(1-2), 195–206.
40. Zumbansen, M., Altrogge, L. M., Spottke, N. U., Spicker, S., Offizier, S. M., Domzalski, S. B., et al. (2009). *J RNAi Gene Silencing, 29*(6, 1), 354–360.
41. Hamm, A., Krott, N., Breibach, I., Blindt, R., & Bosserhoff, A. K. (2002). *Tissue Engineering, 8*(2), 235–245.
42. Marit, G., Cao, Y., Froussard, P., Ripoche, J., Dupouy, M., Elandaloussi, A., et al. (2000). *European Journal of Haematology, 64*(1), 22–31.
43. Ye, Z. Q., Qiu, P., Burkholder, J. K., Turner, J., Culp, J., Roberts, T., et al. (1998). *Human Gene Therapy, 9*(15), 2197–2205.
44. Toneguzzo, F., & Keating, A. (1986). *Proceedings of the National Academy of Sciences of the United States of America, 83*(10), 3496.
45. He, R., Qian, X., Yin, J., & Zhu, Z. (2002). *Journal of Materials Chemistry, 12*(12), 3783–3786.
46. Wang, X., Zhuang, J., Peng, Q., & Li, Y. (2005). *Nature, 437*(7055), 121–124.
47. Brunner, S., Sauer, T., Carotta, S., Cotton, M., Saltik, M., & Wagner, E. (2000). *Gene Therapy, 7*(5), 401–407.
48. Chithrani, B. D., Ghazani, A. A., & Chan, W. C. W. (2006). *Nano Letters, 6*(4), 662–668.
49. Lin, C., Zhong, Z., Lok, M. C., Jiang, X., Hennink, W. E., Feijen, J., et al. (2006). *Journal of Controlled Release, 116*(2), 130–137.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Please provide an update on the contribution title and volume no. reference “Hosseinkhani and Tabata (2005).
Q2. Please provide an update on “Syahril et al.”
Q3. Figures 3 & 4 contains poor quality text. Please provide replacement. Otherwise, please advise if okay to proceed with the figure/s as is.
Q4. Reference 28 and 40 based on original manuscript we received were identical. Hence, the latter was deleted and reference lists and citations were adjusted. Please check if appropriate.