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Nano-polyplex as a non-viral gene carrier for the expression of bone morphogenetic protein in osteoblastic cells

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ABSTRACT

The nano-polyplexes based gene delivery using the combination of water-soluble chitosan and polyethyleneimine (PEI) previously showed synergistic effect on gene transfection in a variety of human cell lines. This system simply facilitates gene therapy via a non-viral gene delivery system. Here, we have started to apply this system for therapeutic gene delivery into the osteoblastic-like cell line, MG-63. The transfection efficiency and cytotoxicity in MG-63 were investigated, intracellular uptake of nano-polyplexe was confirmed through confocal laser scanning microscope. Finally, bone morphogenesis protein (BMP) gene delivery was performed using our developed transfection system. The expression level of BMP-2 therapeutic gene was measured via reverse transcriptase polymerase chain reaction (RT-PCR). Comparing with commercial available system, lipofectamine, the expression of BMP were prolonged. The chitosan-PEI blend polyplex studied here showed the advantages in the aspects of high gene transfection with low cytoxicity into MG-63 cells. This study proposes the non-viral vector as a promising approach for bone regeneration though gene therapy.

1. Introduction

Nanotechnology has become the model approach in biomedical application including the delivery of therapeutic molecules to cell, tissues or targeted organs (Shi, Votruba, Farokhzad, & Langer, 2010). Nano-based gene delivery is one of the promising approaches for treating genetic defective diseases. To date, viral-based vectors for gene delivery have long been shown to be effective. However, the viral-based delivery system is subject to concern about biological safety, which has limited the clinical application of this vector. Recently, non-viral gene delivery has gained much attention for use as an alternative choice in gene delivery (Wong, Pelet & Putnam, 2007). Non-viral vectors, especially polymeric-based vectors, have several advantages especially in terms of safety, biocompatibility, simplicity of use and scaling up of transfections (Anderson, 1998; Li & Huang, 2000). Therefore, several on-going studies related to non-viral vectors have been intensively studied in order to enhance gene delivery whilst minimizing the safety concerns.

Previously, we synthesized a methylated *N*-(4-pyridinylmethyl) chitosan chloride (MPyMeChC) with degree of quaternization 80% and molecular weight 12 kDa. MPyMeChC was synthesized to improve water solubility and gene transfection efficiency of original chitosan. The study revealed that MPyMeChC has the potential to be used as a gene carrier by forming a polymeric complex with DNA via electrostatic interaction resulting in a nano-sized polyplex (Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009). Furthermore, we then improve gene transfection efficiency of MPyMeChC using polyethyleneimine (PEI) blended-MPyMeChC delivery system. This blended-vector was developed based on the knowledge that chitosan is generally low in cytoxicity but not a high efficiency transfection reagent (Lee, Kwon, Kim, Jo, & Jeong 1998) whereas PEI strongly promotes nucleic acid condensation and facilitates endosomal release (Godbey, Wu, & Mikos, 1999). The synergistic effect of PEI/MPyMeChC blend was found in HeLa cells showing the potential of the system to be used as an alternative carrier for gene therapy (Rakkhithawatthana et al., 2010). Therefore, to verify the value of the PEI/MPyMeChC blended nano-polyplex as a non-viral gene delivery system, we studied this carrier for the delivery of therapeutic genes, focusing on the bone morphogenetic protein (BMP)-encoding gene to accelerate immature bone cell (osteoblast) maturation.

So far, recombinant BMP-2 has been cloned (Wang, Rosen, D'Assandro, Bauduy, & Cordes, 1990) and applied in medical uses by exogenous protein delivery (Liu, Huse, de Groot, Buser, & Hunziker, 2007; Saito, Okada, Horiuchi, Ota, & Takahashi, 2003). For post-natal bone regeneration, there are several clinical approaches

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Table 1
PCR primers used for semi-quantitative assay of β-actin and BMP-2 gene expressions.

Gene		Sequence	PCR product (base pairs)
β-Actin	Sense	5′-ACG GGT CAC CCA CAC TGT GC-3′	656
	Anti-sense	5′-CTA GAA GCA TTT GCG GTG GAC GAT G-3′	
BMP-2	Sense	5′-TTC CTG CAG TTG TAC GTG GAC TTC AGT GAC-3′	334
	Anti-sense	5′-AAC AGG ATC CCT AGC GAC ACC CAC AAC C-3′	

involved in both allograft and xenograft (Blokhuis & Lindner, 2008; Wang, Zou, Yuan, Wang, & Chen, 2009). The BMP can be directly delivered as recombinant proteins or expressed by genetically modified cells to induce bone formation (Kirker-Head, 2000). Therefore, gene delivery can be the alternative approach to deliver recombinant tissue inductive protein especially BMP. Previous experiments revealed the advantage of BMP gene delivery over BMP protein delivery since a smaller amount of protein is required to induce bone healing (Dragoo et al., 2003; Leach & Mooney, 2004). Another important advantage of BMP gene delivery is that the strategy can continuously feed BMP and prolongs its biological activity in a bone defective site.

In this study, the potential of a non-viral gene carrier consisting of blended-cationic polymers, PEI/MPyMeChC, was evaluated in osteoblastic cells, MG-63. The BMP-2-containing plasmid encoding BMP-2 protein was employed as the therapeutic gene to induce bone formation. The transfection efficiency and cell cytotoxicity were evaluated. Finally, the amounts of mRNA transcribed from BMP gene were measured to confirm osteoblast maturation. Here, we show the promising polymeric gene delivery system to deliver a therapeutic gene, BMP-2. This study may be beneficial for the treatment of bone defective conditions and in tissue engineering.

2. Materials and methods

2.1. Chemicals

Chitosan (Mw of 276 kDa) was purchased from Seafresh Chitosan Lab, Thailand. Methylated *N*-(4-pyridinylmethyl) chitosan chloride (MPyMeChC) was synthesized as previously described (Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009). The branched polyethylenimine, PEI (Mw of 25 kDa) was purchased from Aldrich, USA. The Lipofectamine 2000™ was purchased from Invitrogen, USA. Plasmid pGL3-basic containing CMV promoter/enhancer was used to monitor transfection efficiency (Tencomnao, Rakkhitawatthana, & Sukhontasing, 2008). Plasmid pBacBH2 containing human BMP-2 gene (pBacBH2-hBMP2) was kindly provided by Prof. Y. Tabata (Hosseinkhani et al., 2006). The plasmids were propagated in *Escherichia coli* which were grown in LB broth (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl) supplemented with ampicillin under shaking condition 250 rounds per minute at 37 °C. The plasmid was extracted using the PureLink™ Hipure Plasmid DNA Purification kit (Invitrogen, USA). The extracted plasmid was observed by electrophoresis on a 1.0% agarose gel. Plasmid purity and concentration were determined by measuring light absorbance at 260 and 280 nm using SpectraMax M2 microplate reader (MDS Inc., Canada). Primers for reverse transcriptase polymerase chain reaction (RT-PCR) of β-actin and BMP-2 genes were listed in Table 1.

2.2. Preparation of nano-polyplex; MPyMeChC/DNA, and PEI/MPyMeChC/DNA

To prepare nano-polyplexes, MPyMeChC and PEI were separately dispersed into sterile distilled water to prepare a stock solution with a final concentration of 1 mg/ml each. Different sets of polyplexes were prepared based on amount of polymer used. For complexes of MPyMeChC/DNA, w/w ratios of polymer per DNA were 1/1, 5/1, 10/1 and 20/1. The mixtures of MPyMeChC and DNA were induced to form polyplexes through a self-assembly mechanism after pipetting and subsequently incubated at room temperature for 15 min before use. For a polyplex combined with both MPyMeChC and PEI, polyplexes were prepared by constantly using 1 μg of MPyMeChC, weight ratios of PEI/MPyMeChC/DNA were 0.5/1/1, 1/1/1, 5/1/1 and 10/1/1. MPyMeChC and DNA were mixed together as above and followed by adding of PEI to coat on the MPyMeChC/DNA surface by pipetting and incubated at room temperature for 15 min.

2.3. Size and zeta potential measurement

The Z-average hydrodynamic diameter, polydispersity index (PDI), and surface charge of methylated Ch derivatives/DNA complexes were determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at room temperature. The complexes were prepared and made up to be 1 ml with DI water. All samples were measured in triplicate.

2.4. Cell culture

The MG-63 cell line was grown in Eagle's Minimum Essential Medium (EMEM) (Gibco, USA) containing 10% fetal bovine serum. After transfection, cell differentiation was induced in differentiation medium supplementary with 0.2 mM L-ascorbic acid 2-phosphate, 0.1 μM dexamethasone and 10 mM β-glycerophophate cells (Saito et al., 2003; Yamazaki et al., 2000). Cells were incubated at 37 °C and 5% CO_2-air for 24 h and periodically subculture until prior to transfection.

2.5. In vitro gene transfection and luciferase assay

For *in vitro* transfections, osteoblastic cells, MG-63, were seeded in 96-well plate at density of 1×10^5 cells/well in 96-well plates. Prior to transfection, the media were removed and the cells were rinsed with PBS twice then plated and incubated with serum-free EMEM. Cells were incubated with polyplex at 37 °C for 4 h in serum-free media then replaced with growth media. Twenty-four hours after transfection, luciferase activity in each well was determined according to manufacture's recommendations (Promega, USA). Luciferase activity was quantified as relative light units (RLU) using luciferase assay system (Promega, USA). Luciferase activity was normalized for protein concentration using the Bradford assay was measured to be controlled. Lipofectamine 2000™ (Invitrogen, USA) was used as a control carrier for gene transfection.

2.6. Evaluation of cytotoxicity

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate cell viability after treating with polyplex. All cells were seeded as the same density applied for transfection. Cells were incubated overnight at 37 °C in a humidified air of 5% CO_2 atmosphere. The assay was performed 24 h after transfection according to manufacturer's

recommendations. Percentage cell viability was related to the untreated control cells.

2.7. Assessment of polyplex internalization into cell

Plasmid DNA, pGL3-basic containing CMV promoter/enhancer was labeled with RITC using the Mirus labeling kit (Mirus Bio Corporation, USA). The labeled-DNA then formed a complex with PEI and/or MPyMeCh yielding PEI/DNA (0.5/1, w/w), MPyMeChC/DNA (1/1, w/w), PEI/MPyMeChC/DNA (0.5/1, w/w) and lipofectamine (according to the manufacture's recommends). Transfection experiments on MG-63 cells were performed by seeding the cells at density of 1×10^5 cells on cover slip in a 24-well plate. The transfected cells were stained with acridinine orange. After washing in PBS buffer, cells were visualized under the confocal laser scanning microscopy (Carl Zeiss, Inc., Germany). Z-stack images were captured to localize polyplex in the cells.

2.8. Transcriptional analysis of exogenous BMP-2 by reverse transcriptase-polymerase chain reaction

To isolate total RNA, MG-63 cells were seeded in 6-well plates at a density of 1×10^5 cells/well. RNA extraction with TRIzol (Invitrogen, USA) was performed according to manufacturer's recommendations. The quantity and integrity of the RNA obtained were evaluated by spectrophotometer and gel electrophoresis stained with ethidium bromide. RNA were then treated with Deoxyribonuclease I (DNase I), Amplification Grade (Promega, USA) to eliminate contaminated DNA at 37 °C for 30 min. Two steps of RT-PCR were carried out using Impromt II™ reverse transcription (Promaga, USA) to synthesize first-strand cDNA, and Taq polymerase (NEB, UK) was used for PCR under the following conditions: 95 °C for 5 min, followed by 35 cycles of denaturation (95.0 °C 30 s), annealing (58 °C for 30 s for β-actin gene and 65 °C for 30 s for BMP-2 gene), and extension (72 °C for 45 s), and finally a single extension at 72 °C for 10 min. A control negative RT-PCR was performed in the absence of reverse transcriptase to check the DNA contamination in the RNA preparation. Each BMP expression was normalized against the expression of the β-actin gene to eliminate the effect of cell population. Each relative BMP expression was then compared to that obtained from control normal cells.

2.9. Statistical analysis

Experiments were carried out with triplicates. One-way ANOVA with Turkey's *post hoc* test was applied for statistical analysis with $P < 0.05$ considered as a statistically significant difference.

3. Results and discussion

3.1. Phycochemical analysis and polyplexes formation

The polyplexes formed at different weight ratios of DNA, MPyMeChC and/or PEI were previously characterized by gel retardation, polyplex formations, sizes and zeta potentials (Rakkhithawatthana et al., 2010). Physicochemical characteristics of the PEI-blended MPyMeChC polyplexes used in this research were summarized in Table 2. At the weight ratio of MPyMeChC/DNA 1/1, the data revealed that polyplexes were formed in nano-size (277.00 nm) but their total surface charges remained negative (−16.90 mV). A more positive charge was expected to promote the interaction of polyplexes and cells. In order to induce positive charge on the surfaces of polyplexes, we added the highly positively charged polymer, PEI, into the system. The result showed that the surface charges of polyplex PEI/MPyMeChC/DNA were significantly improved after addition of PEI. The positive charges on the surfaces depend on the amount of PEI supplied, varying from 0.5 to 40 μg. Morphological study under atomic force microscopy (AFM) indicated that particle formations occurred in spherical shape as showed in Fig. 1. The different amounts of PEI added (0.5 and 1 μg) have an affect on particle condensation. It seems that adding the more PEI resulted in more compact polyplexes. Besides, the use of excess PEI, yields high polydispersity polyplexes, it is very harmful the cell as well. In our system, therefore, addition of PEI up to 5 μg provided nanoparticle with the appropriate size that was applicable for cell transfection.

Table 2
Particle sizes and zeta potential values of MPyMeChC/DNA and PEI/MPyMeChC/DNA polyplexes at different weight ratios.

Polyplex	Weight ratio	Mean diameter (nm) ± SD	Zeta potential (mV) ± SD
MPyMeChC/DNA	0.5/1	266.00 ± 7.07	−32.70 ± 2.40
	1/1	277.00 ± 8.49	−16.90 ± 0.14
	5/1	191.00 ± 5.57	34.00 ± 2.65
	10/1	234.00 ± 17.58	33.67 ± 1.15
	20/1	237.33 ± 14.98	46.00 ± 1.00
	40/1	221.50 ± 7.78	43.75 ± 2.62
PEI/MPyMeChC/DNA	0.5/1/1	675.50 ± 26.16	9.00 ± 0.40
	1/1/1	294.00 ± 4.24	24.50 ± 0.71
	5/1/1	355.00 ± 2.83	30.60 ± 1.27
	10/1/1	3665.00 ± 35.36	47.05 ± 5.16
	20/1/1	4805.00 ± 148.49	51.67 ± 6.70
	40/1/1	4977.00 ± 263.53	58.03 ± 2.72

3.2. Transfection efficiency and synergistic effect of combined polymers on gene delivery into MG-63

Our previous study found that blending of MPyMeChC and PEI could diminish the drawbacks of each polymer for transfection into HeLa and A549 cells (Rakkhithawatthana et al., 2010). The study has presented the potential of PEI/MPyMeChC polyplex for gene delivery in mammalian cells. Therefore, we applied the system to deliver the BMP-2 therapeutic gene into MG-63, the osteoblast-like cell. To evaluate the capability of the system, the gene transfection was

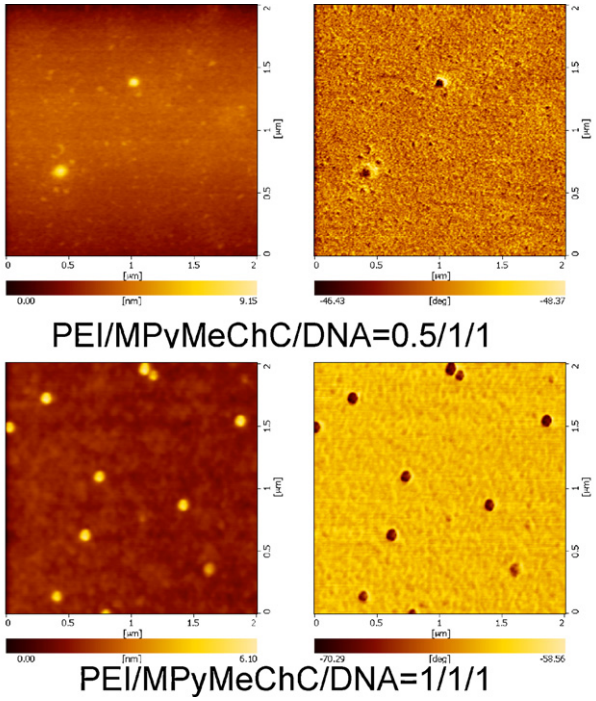

Fig. 1. AFM image of MPyMeChC-PEI blends at PEI 0.5 and PEI 1 μg.

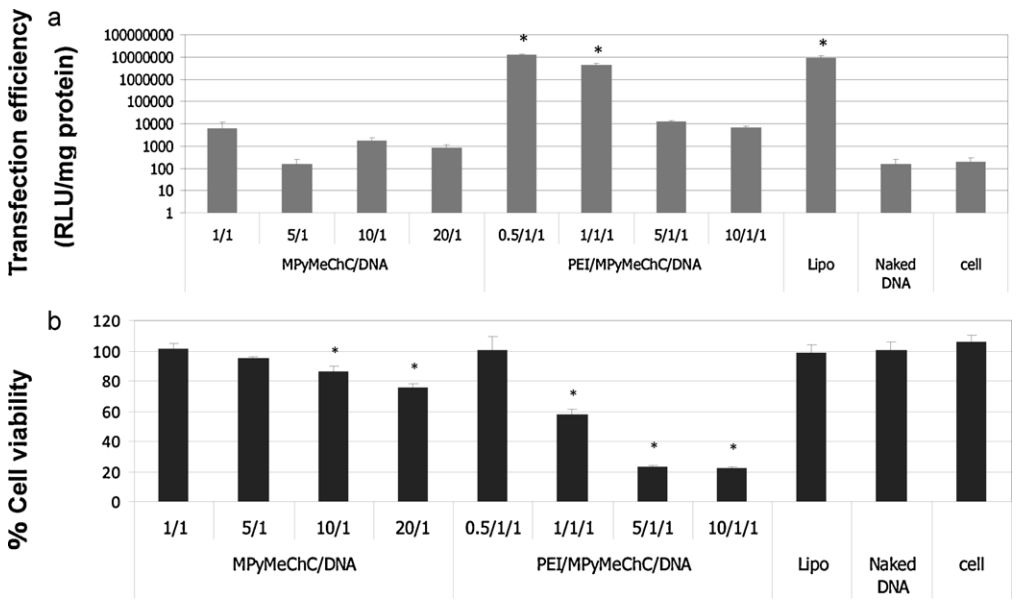

Fig. 2. Transfection efficiency (a) and cytotoxicity assay (b) in MG-63 cell line. Asterisks indicate significant differences between control cells and other treatments ($P < 0.05$).

preliminarily performed to quantitatively investigate luciferase expression in MG-63. The result showed that the luciferase expression levels of cells transfected with MPyMeChC/DNA were not significantly different with the expression level of free-cell and free-cell transfected with naked DNA (Fig. 2(a)). It is implied that increasing the concentration of MPyMeChC failed to promote the transfection efficiency. This result indicates the limitation of using MPyMeChC as a gene delivery vector in MG-63. Supplementary PEI to the MPyMeChC/DNA complex in the range of 0.5 and 1 μg significantly enhanced gene transfection efficiency about 10^3 times compared to polyplex without PEI. The obtained expression level was almost identical to that of lipofectamine, the widely used commercial reagent. This study demonstrated a simple method to improve gene transfection in MG-63, which are not easily transfected with chitosan-DNA polyplexes (Corsi, Chellat, Yahia, & Fernandes, 2003).

3.3. Evaluation of cytotoxity

The effect of polyplexes on MG63 cells was evaluated to determine cell viability and biocompatibility after transfection using the MTT assay. Cells were treated with polyplexes under the same conditions identical to transfection procedures. Cytotoxicity of both MPyMeChC and PEI are polymer dose-dependent (Fig. 2(b)). MPyMeChC exhibited approximately 50% survival effect at weight ratio 10/1 and survival rate was decreased along with the amount of added compound. In the group of cell exposed with PEI/MPyMeChC/DNA, the toxicity was significantly higher than that of MPyMeChC/DNA. This cytotoxic effect corresponded with the amount of added PEI, a high cationic charge polymer, which introduces cell leaking and apoptosis, which are finally the cause of cell death (Hunter & Moghimi, 2010). Gene transfection efficiency and cytotoxicity revealed the feasible ratios that were favorable for delivery BMP-2 gene. We, therefore, tentatively selected PEI/MPyMeChC/DNA at 0.5/1/1 and 1/1/1 for further investigation.

3.4. Cellular internalization of polyplex

Confocal microscopy verified the cellular uptake of DNA mediated nano-polyplexes. PEI/DNA (0.5/1, w/w), MPyMeChC/DNA (1/1, w/w) and PEI/MPyMeChC/DNA (0.5/1, w/w) were transfected into the MG-63 cell line. At 6 and 24 h after transfection, the transfected cells were observed under confocal florescence microscope. The image revealed the intracellular distribution of polyplexes ensuring the achievement of our delivery system in MG-63 (Fig. 3). At 6 h post transfection, the complexes have already entered into cells. Meanwhile the distribution of complexes in the cytoplasm and nucleus compartment remained observed at 24 h post transfection, which is the time luciferase has been shown to be expressed. Taken together with the data suggested from confolcal Z-stack images scanning (Fig. 4), we presumed that nano-polyplex is a practical DNA delivery vehicle for therapeutic gene delivery into osteoblast-like cells.

3.5. PEI/MPyMeChC/DNA polyplex as the BMP gene carrier

Taking into account the results of size, zeta, luciferase transfection and cytotoxicity assays, we supposed that the uses of PEI/MPyMeChC/DNA at weight ratio 0.5/1/1 and 1/1/1 would be effective for BMP gene delivery since the obtained polyplexes are the nanoparticle with positively surface charges. However, negative RT-PCR results revealed that the strands of DNA were not easily released from the complex at the ratio 1/1/1. This phenomenon will subsequently cause interfere in the BMP production assay. Furthermore, the PEI/MPyMeChC/DNA polyplex at the weight ratio of 1/1/1 is also the cause of higher cell dead as well. Therefore, we decided to study the transfection using PEI/MPyMeChC blending system at the weight ratios of PEI/MPyMeChC/DNA at 0.5/1/1 to introduce BMP-2 gene into MG-63. After transfection with the polyplex carrying pBacBH2-hBMP2, the transfected cells were continually incubated for 1, 3, 7, 10 and 14 days. The cells were then lysed for RNA isolation as described above. The RNA samples were then treated with DNase I to eliminate contaminated DNA. Negative RT-PCR from the different incubation times were carried out to investigate DNA contamination in each samples and was performed in triplicate. The expression of BMP gene was followed up using RT-PCR. PCR products from the house keeping gene, β-actin, were used to normalize gene expression values. The differences of expression levels of BMP between free cells and transfected cells were detected in all time intervals at days 1, 3, 7, 10 and 14. In the group of cells transfected with lipofectamine, the BMP expres-

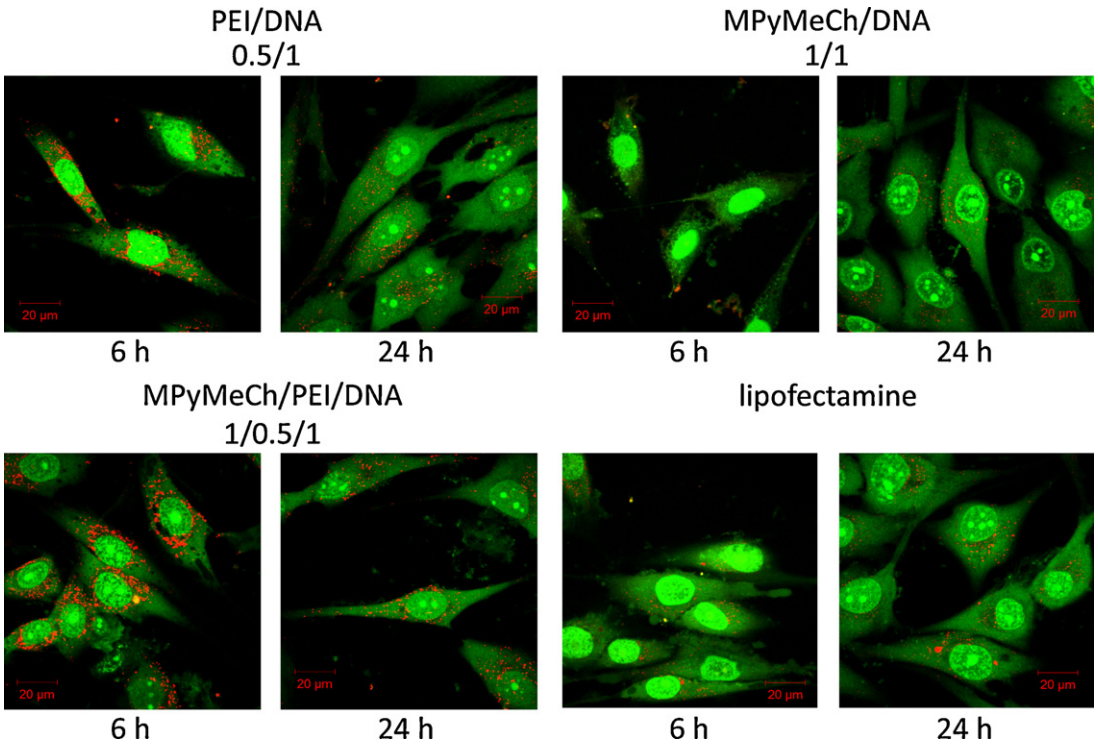

Fig. 3. Confocal image of cells after transfection at 6 and 24 h (green = acridine orange stained live cell, red = TRITC stained DNA).

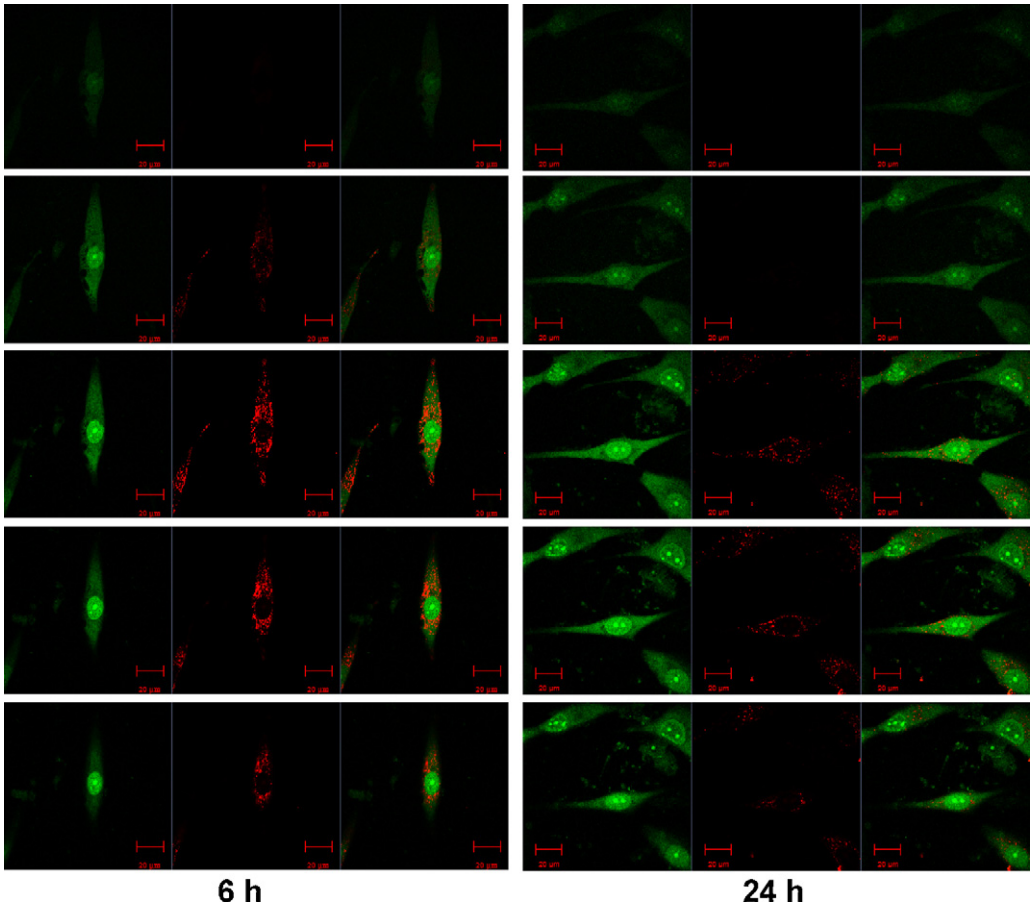

Fig. 4. Merged Z-stack confocal image confirmed the cellular internalization of PEI/MPyMeChC/DNA polyplex (0.5/1/1).

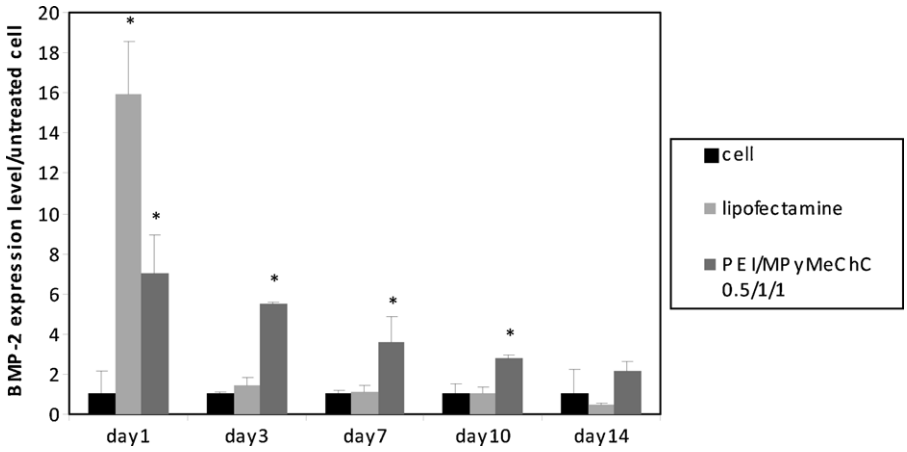

Fig. 5. Semi-quantitative PCR result shows the expression of BMP gene in MG-63 at days 1, 3, 7, 10 and 14 after transfection by MPyMeChC-PEI blends. Asterisks indicate significant differences of BMP expression levels between control cells and other treatments ($P < 0.05$).

sion signal was drastically high in day 1 which was about 14 times higher than that of free cells (Fig. 5). The signals then lowered to levels equivalent to that of the control in days 3, 7, 10 and 14. As the groups of cells transfected with PEI/MPyMeChC/DNA polyplex, the expression was about 6 times higher than control at day 1 and continuously detected with a slight decrease in amount at 5, 3, 2, and 1 times higher that control at days 3, 7, 10, and 14, respectively. Apparently, the decreasing of BMP expression is time-dependent, which might correspond to the release of DNA from the positively charged polyplexes inside the cells. Using this delivery system, BMP gene therapy might become the preferable approach to the release of ontogenesis-stimulating factors at a bone-healing site.

4. Conclusion

In this study, we demonstrated the use of combined cationic polymer, chitosan and PEI, in a bone formation application. The transfection with luciferase, the general gene marker, indicated the potential of this system in transfection in osteoblastic cells at weight ratio of PEI/MPyMeChC/DNA at 0.5/1/1. To our knowledge, there are very few non-viral carriers aiming to be applied in gene delivery of osteoblast-like cells (Corsi et al., 2003; Oliveria, Ferraz, Monteiro, & Simõeset, 2009). BMP-encoding plasmid was successfully introduced into MG-63 cell line. This result emphasized the potential of our established gene carrier for delivery of the existed therapeutic gene. *Ex vivo* BMP gene transfection based on chitosan-PEI blend polyplex can be applied for bone repairing. This study provides a novel and simple strategy for gene transfection in osteoblastic cells.

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