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In vitro Labeling of Neural Stem Cells with Poly-L-Lysine Coated Super Paramagnetic Nanoparticles for Green Fluorescent Protein Transfection

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ABSTRACT

Background: The magnetic nanoparticle-based transfection method is a relatively new technique for delivery of functional genes to target tissues. We aimed to evaluate the transfection efficiency of rat neural stem cell (NSC) using poly-L-lysine hydrobromide (PLL)-coated super paramagnetic iron oxide nanoparticles (SPION). **Methods:** The SPION was prepared and coated with PLL as transfection agent and the transfection efficiency was evaluated in rat NSC using enhanced green fluorescent protein-N1 plasmid containing GFP as a reporter gene. NSC was incubated for 24 h in cell culture media containing 25 µg/ml SPION and in different concentrations of PLL (0.25, 0.50, 0.75, 1 and 2 µg/ml). Cell viability was determined before and after transfection for every concentration using Trypan blue assay. Characterization of prepared uncoated (SPION) and coated (SPION-PLL) complexes were evaluated by a transmission electron microscope and the zeta potential. Results: PLL at 0.75 µg/ml showed optimal results with 25 µg/ml SPION concentration compared with other PLL concentrations (0.25, 0.50, 1 and 2 µg/ml). The 18% efficiency of the transfected cells showed green fluorescence. **Conclusion:** Transfection with SPION is an efficient, non-viral gene transfere method. *Iran. Biomed. J. 17 (2): 71-76, 2013*

Keywords: Neural Stem cells (NSC), Nanoparticles, Transfection

INTRODUCTION

Neural stem cells (NSC) are undifferentiated cells that are able to self-renew as well as to generate three other major cell types of the central nervous system: neurons, astrocytes, and oligodendrocytes. NSC can be used to replace dead tissues in patients suffering from Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, stroke and spinal cord injury [1-3].

Super paramagnetic iron oxide nanoparticles (SPION) are widely used *in vitro* and *in vivo* biomedical applications. The new development of nanoscience and magnetic nanoparticles may play an important role in cell technology and expression of genes in cells [4, 5]. *In vitro* SPION applications usually include cell, microbe and hormone diagnosis [6-8]. Due to their low toxicity in animal tissues [9], their applications also include the detection of oligonucleotides, DNA, proteins, and immunoassays [9-12]. *In vivo* SPION applications focus on magnetic resonance imaging, target drug delivery and tissue engineering [13, 14]. SPION are also used in gene therapy and gene delivery to target cells and tissues [15].

To reduce the aggregation of SPION within the body, biopolymers were added to make it more biocompatible. Biocompatible polymers attached to the surface of nanoparticles improved and increased their intracellular uptake for cell tracking [16, 17]. Most SPION are usually used with dextran coating; however, it shows limited or insufficient cellular uptake to enable cell tracking [18]. Recent studies have shown that ferumoxides or SPION using poly-L-lysine hydrobromide (PLL) are suitable for transfecting human stem cells. Its advantages are high biosafety and biocompatibility, high flexibility and easy synthesis [19, 20].

PLL is a polycation widely used as a non-viral transfection agent for gene delivery and DNA complexing because of the presence of NH_2 groups that promote cell adhesion [20]. Several research groups have independently developed magnet-based transfection methods [21, 22]. This method of

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transfection was inspired by the concept of magnetic drug targeting [15]. Nanoparticles without a polymer coating have a distinct biodistribution and metabolic clearance profile than conventional polymer-coated particles [23]. However, it is unclear whether these particles can be taken up by cells. The cationic polymer PLL coated with SPION can be complexed to plasmid DNA. Apart from suitable magnetic nanoparticles, magnet-based nanofection also requires suitable magnetic fields, which are provided by the Super Magnetic Plate SHM04 (Sigma-Aldrich Co., USA) especially designed for magnet-based nanofection. This plate produces a pattern of higher or lower densities of transfected cells according to the geometry of the magnetic field lines. The use of magnetic nanoparticles at optimal concentration coupled with gene vectors incubated at statistic magnetic field resulted in dramatic increase of transfection efficiency [24, 25].

In this study, we aimed to evaluate the transfection efficiency of rat NSC using PLL and SPION in different concentrations.

MATERIALS AND METHODS

$FeCl_3 \cdot 6H_2O$ 99%, $FeCl_2 \cdot 4H_2O$, 99%, NH_3 25%, tetramethylammonium hydroxide 25% were purchased from Fluka Chemicals, Germany. Distilled-deionized water was used as solvent for reagent preparation for preparation of SPION. Briefly, 4.0 ml of 1 M $FeCl_3$ and 1.0 ml of 2 M $FeCl_2$ were added in 100 ml beaker under vigorous stirring. An aqueous solution of NH_3 (50 ml and 1.0 M) was then slowly added over a period of 5 minutes using the co-precipitation method, which is the common method of SPION use for biomedical applications [25]. Then, the precipitate was treated with tetra methylammonium hydroxide 25% and gently stirred with a glass rod for 2-3 minutes to suspend the solid in the liquid. For dispersion method, the powder was accurately weighted, transferred to a flask and dissolved with ultrapure water by ultra-sonification for 5 min (Model B-12 Ultrasonic bath, Wise Clean Co., Shelton, Conn., USA).

Cell harvesting and culture. Our experiments were approved by the Shefa Labs in the Neuroscience Research Center, Khatam-Al-Anbia Hospital, Tehran, Iran. Surgical procedures were done according to the guidelines of Ethics Committee of Tarbiat Modares University, Tehran. Bone marrow was collected under sterile conditions from the rat femur and tibia of 8-10-week-old adult Sprague-Dawley rats (200-250 g). The rats were obtained from the Razi Vaccine and Serum Research Institute, Tehran. The rats were sacrificed and their tibia and femurs were dissected. The ends of each bone were cut aseptically and the bone marrow was aspirated with 5 ml culture media containing 500 units of heparin, using a 5-ml syringe and a 21-guage needle. The contents of the rat femurs and tibias were transferred to a flask and expanded. They were then isolated from bone marrow stem cells using centrifugation and cultured in DMEM supplemented with 20% heated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B, and 2 mM L-glutamine. The harvested cells were seeded in a 75-cm^2 flask (Nunc, Denmark) and maintained in a humidified atmosphere at 37°C with 5% CO_2. The culture medium was replaced every 2-3 days, and all non-adherent floating cells including hematopoietic cells and debris were removed by changing the medium. When the cells were confluent, the adherent bone marrow stem cells were trypsinized and passaged into an approximately 1:3 split and after passage 3, the medium was replaced with fresh completed NSC media.

Neurosphere culture. Neurosphere cultures were prepared in DMEM:F12 medium containing 2% B-27 supplement, 50 U/ml penicillin, 50 mg/ml streptomycin, 4 ng/ml heparin, 20 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor. Cell culture reagents were purchased from Gibco UK. Culture dishes (non-treated surface) and tissue culture-grade plastics were purchased from TPP Techno Plastic Products AG (Switzerland).

SPION-PLL complex and transfection. PLL (molecular weight of 300 kDa, culture grade, catalog no. P1524 [Sigma, St. Louis, Mo, USA]) was used as the transfection agent. A stock solution of PLL (1.5 mg/ml) was added to the culture media at different dilutions and mixed with SPION (50 µg/ml) on a rotating shaker (600 rpm) at room temperature for 1 h. These culture media containing the SPION-PLL complex were added to the cells. The final concentration of SPION was 25 µg/mL and the concentrations of PLL were 0.25, 0.50, 0.75, 1, and 2 µg/ml, respectively. The cultures were maintained at 5% CO_2 37°C overnight and the complex solution was then removed and replaced with NSC medium containing 10% FBS [16]. The final concentration of SPION was 25 µg/ml and the final dilution of PLL was 0.75 µg/ml culture media. For transfection of GFP, NSC cells at passages 3 were plated in a 6-well plate and placed on the super magnetic plate and cultured for 20 minutes. GFP expression was assayed after 24 h.

Statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA). Statistical analyses were performed using SPSS software (version 17) and data were expressed as standard error of the mean (mean ± SEM). And statistical comparisons were

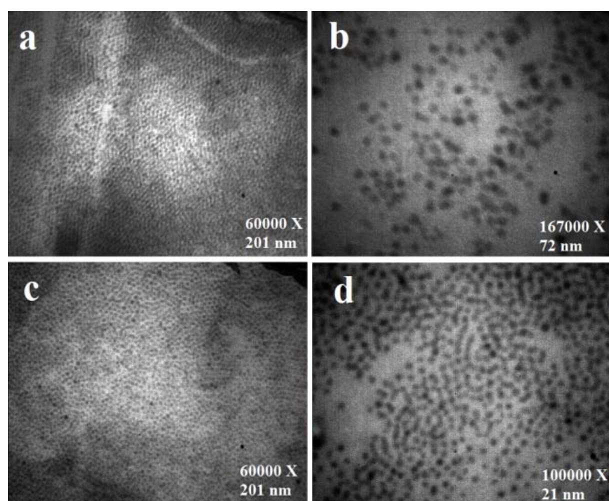

Fig. 1. The ultrastructural morphology of the super paramagnetic iron oxide nanoparicles. The electron micrograph shows the morphology of uncoated (**a** and **b**) and coated (**c** and **d**) magnetic nanoparticles.

performed using Student's-*t* test. $P<0.05$ was considered as statistically significant.

RESULTS

The SPION were synthesized using the co-precipitation of ferric chloride and ferrous chloride. The characteristics of the SPION were determined using the transmission electron microscopy (LEO 906, Germany). The SPION were about 10 nm in diameter with a uniform distribution as shown in Figure 1. The hydrodynamic size and zeta potential were evaluated in the Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The zeta potential indicated that the PLL-coated SPION had positive surface charges of about ξ+16.9 mV compared with SPION alone (ξ-31.3 mV). The fluorescent microscope showed that NSC has been successfully transfected after being treated with SPION-PLL complex with GFP expressions in different concentrations of PLL-coated SPION (Fig. 2).

The viability of NSC in the culture before and after labeling, as determined by Trypan blue stain, was greater than 94%. As shown in Figure 2, the viability of NSC was not affected by PLL labeling with different concentrations, especially at a concentration of 0.75 µg/ml of PLL-coated SPION.

Transfection efficiency. Transfection efficiency of GFP was evaluated at different concentrations of PLL-coated SPION (0.25, 0.50, 0.75, 1 and 2 µg/ml, respectively). After 24 h of transfection, 1 and 2 µg/ml of PLL-coated SPION showed no expression, while other concentrations (0.25, 0.50 and 0.75) showed GFP expression. This expression was particularly considerable at a concentration of 0.75 µg/ml (Fig. 3). The results of different experiments at different concentrations of PLL were significant. The student's *t*-test results are shown in Tables 2, 3, 4, and 5. $P<0.005$ showed significant difference.

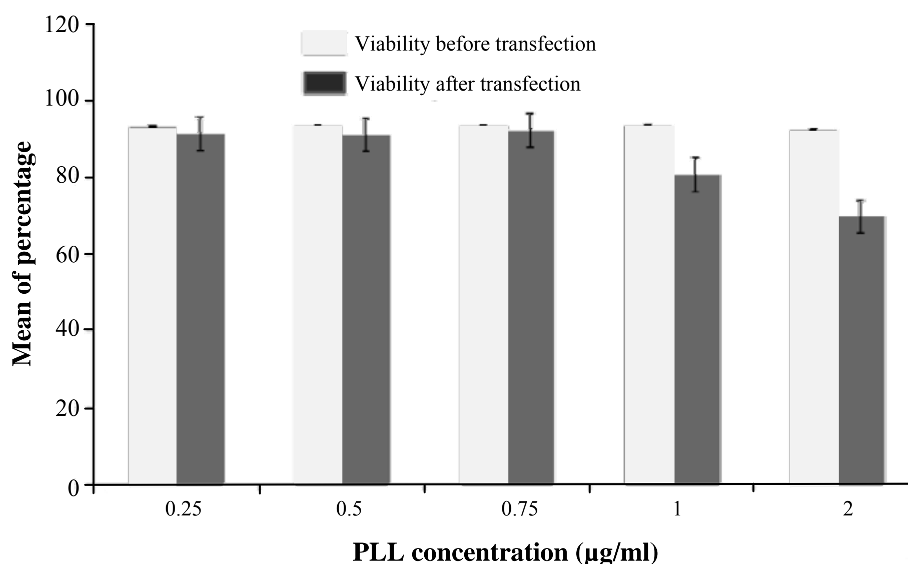

Fig. 2. The dose response of poly-L-lysine (PLL)-coated super paramagnetic iron oxide nanoparicles. The viability was used to evaluate the toxicity of different concentrations of PLL (0.25, 0.5, 0.75, 1 and 2 µg/ml), incubated with the neural stem cells before (light gray columns) and after (dark gray columns) transfection.

Table 1 . The viability of neural stem cells (NSC) before and after transfection with different concentrations of poly-L-lysine-coated super paramagnetic iron oxide nanoparicles

Comparison before and after cell loading (µg/ml)	Paired differences								
	mean	Std. deviation	Std. Error Mean	95% Confidence interval of the difference		t	df	Sig. (2 tailed)	*P* value
				lower	upper				
0.25	1.95252	0.29622	0.17102	1.21666	2.68838	11.417	2	0.008	0.008
0.50	2.31837	0.07293	0.04210	2.13721	2.49953	55.062	2	0.000	0.000
0.75	1.10107	0.01254	0.00724	1.06991	1.13222	152.071	2	0.000	0.000
1	12.87384	0.65595	0.37871	11.24438	14.50330	33.994	2	0.001	0.001
2	22.64618	0.48928	0.28249	21.43074	23.86162	80.167	2	0.000	0.000

The result shows no significant difference between the viability of NSC before and after the transfection. Std. deviation, mean standard deviation; Std. error mean, standard error of the mean; t, t statistics; df, degree of freedom and Sig., significant

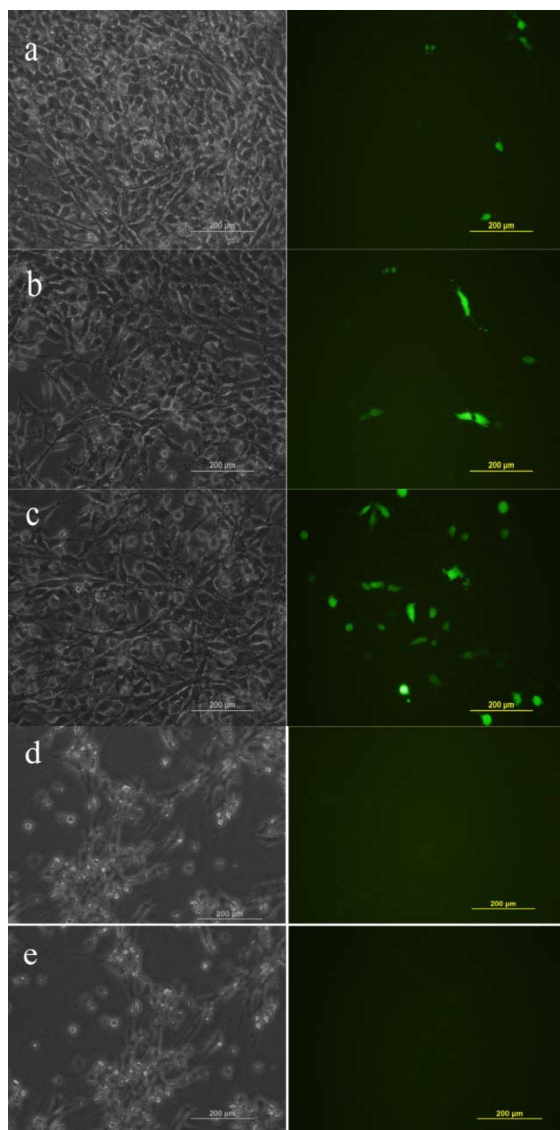

Fig. 3. The morphology of the transfected neural stem cells (NSC) incubated with super paramagnetic iron oxide nano-particles coated with different concentration of poly-L-lysine (PLL: 0.25, 0.50, 0.75, 1 and 2 µg/ml). The left panel represents the phase contrast images of transfected NSC with enhanced green fluorescent protein vector at the above concentrations of PLL (**a**, **b**, **c**, **d** and **e**, respectively).

Cell labeling and histological analysis

Prussian blue staining. The fixed NSC were washed three times with Prussian blue staining, incubated for 30 min with 5% potassium ferrocyanide (Merck, Germany) in 5% hydrochloric acid, rewashed and then counterstained with safranin stain (Merck, Germany). A light microscope was used to detect the intracellular iron oxide in labeled NSC (Fig. 4). Therefore, the NSC cells were labeled with SPION stained with Prussian blue and counterstained with safranin stain. Prussian blue staining of labeled stem cells revealed abundant uptake of most SPION in the cytoplasm. Labeling efficiency was reproducible at approximately 98% of NSC.

DISCUSSION

In order to prepare stable magnetite colloidal, it is important to remove the impurities remaining after the synthesis of the magnetite and its oxidation accurately. Purification was achieved using magnetic separation in water and washing with ultrapure water. In clinical studies, especially for tracking stem cells after transplantation, knowledge about stem cell labeling using magnetic nanoparticles stem cells is essential. Neural cells can be successfully transfected using SPION and are enhanceable using the magnetofection method [26, 27].

Transmission electron microscopy of labeled NSC showed the hydrodynamic size of coated particles with an average size of about 10 nm, which seemed to be increased with the PLL/iron oxide due to a thickening of the shell (Fig. 3). The stability of the SPION was revealed to the negative charges of the uncoated magnetic nanoparticles, as shown by their low zeta potential (ξ-31.3 mV). PLL coated SPION documented an increase in zeta potential (ξ+16.9 mV). Previous studies showed that transfection agents could enhance the labeling efficiency of ferumoxides to stem cells and to other mammalian cells [13, 28-30].

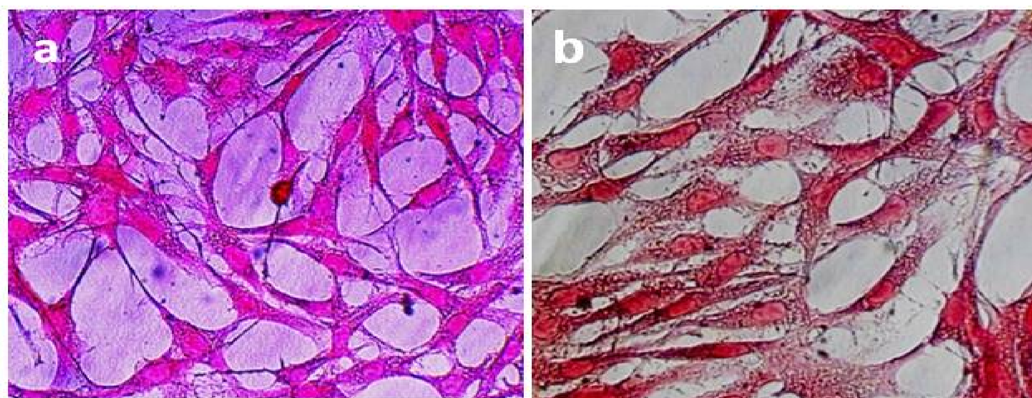

Fig. 4. The morphology of neural stem cell (NSC) loaded with superparamagnetic iron oxide nanoparticles (SPION). NSC were incubated with SPION only (uncoated) under static magnetic field and fixed with 4% parafolmaldehyde, stained with Prussian blue stain and counterstaind with safranin. **(a)** Intracellular blue color dots of the SPION stained with Prussian blue and **(b)** NSC unlabelled with SPION (negative control) (magnification 1000×).

Polycationic transfection agents such as PLL-SPION complexes are made through electrostatic interactions and are efficient and effective for incorporating the SPION within endosomes [28].

Our results are consistent with other previous studies which show that transfection agents improve the labeling efficiency of SPION to stem cells and to other mammalian cells [29, 30]. The major finding of our study is that the labeling of NSC with SPION at a concentration of 25 µg/ml combined with the transfection reagent PLL at a concentration of 0.75 µg/ml did not have toxic effects on the viability of NSC.

We observed that the optimal concentration of PLL is 0.75 µg/ml for labeling of NSC with SPION at 25µg/ml in order to prevent toxic effects on the viability of NSC.

NSC transfection efficiency can be significantly enhanced by SPION/PLL complex at optimal conditions. Labeling efficiency was reproducible in approximately 95% of the labeled NSC, using 25 µg/ml concentration of iron oxide. In our study, 0.75 µg/ml PLL in combination with 25 µg/ml SPION showed no adverse effect on the cell proliferations and viabilities. Therefore, NSC can be safely and efficiently labeled for future studies using the transfection agent PLL.

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