

GENERATION OF MAGNETIC RETROVIRAL VECTORS WITH MAGNETIC NANOPARTICLES

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Abstract. We have successfully synthesized iron and gamma ferric oxide magnetic nanoparticles with particle sizes of 4 ± 0.8 nm through a high-yield chemical thermal reflux method. Polyethyleneimine (PEI) was used to enhance biological compatibility and monodispersion of nanoparticles. The PEI coated magnetic nanoparticles have sizes between 100 to 200 nm. More importantly, we formed magnetic retroviral vectors through combining Moloney leukemia virus based retroviral vectors with the particles. An external magnetic field can bring the magnetic retroviral vectors to the targeted cells and significantly raised the gene transduction efficiency. We present physical properties and biological activities of magnetic nanoparticles associated with retroviral vectors.

1. INTRODUCTION

Inorganic nanostructures that interface with biological systems have recently attracted widespread interest in biological and medical applications [1,2]. Hybrid organic/inorganic nanoparticles have promised potential as novel tools for diagnostics and drug delivery. Within all the nanoparticles, nanoparticles with magnetic properties are particular important for these applications. Magnetic nanoparticles (MNPs) can be attracted and guided by an external magnetic field. Since magnetic field is generally harmless to the living creatures for transient exposures, MNPs can be concentrated and targeted to specific organs by external magnet field without adverse effect. In addition, magnetic resonance imaging and some magnetic detection systems can detect the strength and distribution of MNPs in living organisms without invasive procedures. Therefore,

combining MNPs with existing systems can generate new tools for drug delivery and diagnostics [3]. Based on the properties of MNPs, we created viral vectors associated with MNPs for gene transfer and gene therapy applications.

Through recombinant DNA technologies, virologists were able to convert viruses into viral vectors. Most of effective viral vectors for medical applications are developed from animal viruses. These vectors are able to introduce foreign genes to cells *in vitro* and *in vivo*. One of the best examples is the development of Moloney leukemia virus (MLV) into MLV-based vector. MLV is a retrovirus. MLV can cause leukemia in rodents and can efficiently transduce host cells with viral genes. MLV-based vectors, like MLVs, can transduce host cells efficiently. Conversely, they, un-like MLVs, are not able to replicate in host cells or to introduce any viral genes to host cells. Thus, it would not cause any adverse

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effects. Currently, it is considered to be one of the most efficient vehicles for gene transfer to animal cells. More importantly, MLV-based vectors have been used for human gene therapy applications. In many trials, the success of gene therapy by MLV-based vectors is compromised by the low concentration of vectors at the targeted site. Due to biological properties of MLV, this limitation is difficult to overcome by recombinant DNA technology alone. In this study, we sought to overcome this deficiency by developing a magnetic retroviral vector (MRV) system to achieve site-specific delivery. The development of MRV will allow us to enhance viral transduction. Furthermore, with MRVs, it is feasible to use a simple magnet to accurately target delivery of MRVs to specific sites.

2. MATERIALS AND METHODS

MNPs of $\gamma\text{-Fe}_2\text{O}_3$ were synthesized by chemical thermal reflux method. We used (η^4 -6,8-cyclohexadiene) tricarbonyl iron [$(\eta^4\text{-C}_6\text{H}_8)\text{Fe}(\text{CO})_3$] as precursor and octylamine as solvent. After mixing the precursor with solvent in proper concentrations and volumes, we slowly heated the mixture to reflux and kept at fixed temperature of 190 °C under Ar gas of $10^{-2} \sim 10^{-3}$ Torr. The color of solution gradually became black. It indicated that iron MNPs were forming. After refluxing for one hour, the iron MNPs in the solution were oxidized into $\gamma\text{-Fe}_2\text{O}_3$ MNPs in flowing oxygen gas, then the resulted solution was cooled to room temperature. At the end, the magnetic $\gamma\text{-Fe}_2\text{O}_3$ MNPs were precipitated and baking upon adding excess ethanol and centrifuging. The precipitate can be easily redispersed in octane or toluene solvents. The physical properties of $\gamma\text{-Fe}_2\text{O}_3$ MNPs were characterized by low- and high-resolution transmission electron microscopes (HRTEM), electron diffraction, X-ray powder diffraction (XRD), and *dc* magnetometer. These results will be described elsewhere.

We employed two different methods to synthesize PEI coated $\gamma\text{-Fe}_2\text{O}_3$ MNPs. In the first method, we directly injected the PEI solution to the starting mixture during the chemical reaction of $\gamma\text{-Fe}_2\text{O}_3$ MNPs synthesizing. We referred this method as the thermal reflux method. Alternatively, we added the PEI to the synthesized $\gamma\text{-Fe}_2\text{O}_3$ MNPs and created the PEI-coated particles through sonication. The PEI-coated MNPs were also characterized as described for MNPs.

To generate MLV-based vectors, we employed a transient transfection method [4]. The genes, which are needed for MLV-based vector production, are

separated into three expression plasmids. The first plasmid contains a Vesicular Stomatitis Virus glycoprotein (VSV-G) gene, which serves as a surrogate envelope for MLV-based vectors. The second plasmid contains *gag/pol* gene, which can provide all proteins and enzymes that are needed for viral vector structure and transduction. The last plasmid contains the genomic construct, which contains long terminal repeat and packaging sequences and β -galactosidase (β -gal) gene. The β -gal gene is served as a marker gene since X-gal staining can easily detect its expression. The β -gal MLV-based vectors were generated as described previously [4]. Briefly, we combined these three plasmids together and introduced into a human cell line, 293T by calcium phosphate precipitation. The β -gal MLV-based vectors were harvested from the conditioned medium (CM) of the transduced 293T culture. Routinely, we were able to harvest $1 \cdot 10^7$ transforming units of VSV-G pseudotyped β -gal MLV-based vectors in 1 ml of CM.

To prepare MRVs, we first washed the PEI-coated MNPs extensively with phosphate based saline and adjusted with phosphate-based saline to 1 OD₄₆₀ unit per ml. Then, the equal volume of PEI-coated MNPs and β -gal MLV-based vectors were mixed and incubated at room temperature for 20 min to form MRVs. To demonstrate the effect of magnetofection, we added 20 μ l of the MRVs to a 6-wells plate, which has been seeded with $1 \cdot 10^5$ HT1080 cells per well one day before the transduction. Three Nd₂Fe₁₄B-disk magnets with 1 cm in diameter were placed directly under 3 wells of the 6-well plate for 20 min. The magnet supplied a field of $H \sim 3200$ Oe on the central surface of the magnet. Three remaining wells without magnet were served as control. After 20 min of incubation, all the wells were washed with fresh medium and incubation was continued for additional 36 hours. At the end of incubation, the cells in the wells were fixed and stained with X-gal solution as described previously [4,5]. The β -gal MLV-based vectors transduced HT1080 cells became blue after three hours of X-gal staining.

3. RESULTS AND DISCUSSION

The major nanoparticle researches have focused upon semiconductors and noble metals due to their novel optoelectronic properties and the excellent catalysis properties. Therefore, most efforts have been expanded on the patterned media arrays of discrete single domain magnetic nanoparticles. These produced have potential applications on magnetic information storage systems. However, rela-

Table 1. The PEI coating method, solvent, coercivity (coercive force) H_c , saturation magnetization M_s , and remnant magnetization M_r for four sample batches of PEI-coated magnetic nanoparticles.

Batch of MNPs	PEI coating method	Solvent	Mass for measuring MH loop (mg)	H_c (Oe)	M_s (emu/g)	M_r (emu/g)
1	Thermal reflux	Octylamine	3.10	25.7	39.3	1.2
2	Thermal reflux	Octylamine	2.10	39.8	38.0	16.1
3	Thermal reflux	Octylamine	0.35	264	29.0	5.3
4	Sonication	EtOH	29.6	724	100	38.5

tively little work has been done on the fabrication of monodispersed oxide nanoparticles with uniform size distribution. These types of nanoparticles are essential for many promised applications, *e.g.*, in ferrofluids, medical imaging, drug targeting, catalysis and refrigeration systems *etc.* Several magnetic oxide nanoparticles including iron, magnetite and $\gamma\text{-Fe}_2\text{O}_3$ have been synthesized by using microemulsion and other methods. Nevertheless, those methods fabricated comparatively poor uniformity in particle size and poor crystallinity. Here, we have developed a low-cost, simple, and well-control method to prepare crystalline and monodisperse MNPs without a further size selection process.

The stable chemical and physical properties of $\gamma\text{-Fe}_2\text{O}_3$ MNPs make them top choice to synthesize our MRVs. It is easy to fabricate and can become biocompatible by polymer coating. Therefore, we selected monodisperse $\gamma\text{-Fe}_2\text{O}_3$ MNPs for the biological application in this study. As described in the previous section, we synthesized $\gamma\text{-Fe}_2\text{O}_3$ MNPs using a standard airless technique initially under argon and followed by oxygen for oxidation process. The procedures were based on the nucleation and growth control of crystalline iron or/and iron oxide particles in organic matrix through the reaction control of a metallorganic precursor with a combination of the hydrolysis and polymerization below 200 °C. The reaction conditions influence the size and crystallinity of MNPs in the organic matrix. Our method allows us to produce selectable particle size from 4 nm to 20 nm by controlling the reflux time of mixture solution. The high-resolution TEM images showed that monodisperse $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles with an average size of 4 nm \pm 0.8 nm and a narrow distribution in our preparation. The MNPs exhibited a maghemite $\gamma\text{-Fe}_2\text{O}_3$ structure according to the XRD patterns. In this study, we demonstrated the ther-

mal decomposition methods could be used to fabricate monodispersed Fe and $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles without a size selection process.

The inorganic MNPs are not compatible with viruses. Previously studies have shown that PEI can be used as surfactant to coat MNPs and to form MRVs with retroviral vectors [3,6]. Accordingly, we synthesized PEI coated $\gamma\text{-Fe}_2\text{O}_3$ magnetic nanoparticles as our MNPs. We prepared four different batches of PEI-coated MNPs as described in previous section. In each batch, the fabrication methods were altered in PEI coating and the solvent used. Table 1 lists the related experimental conditions and magnetic data of each batch. We found these different conditions would not grossly change the yield and size of the PEI-coated MNPs. The sizes of these PEI-coated MNPs were between 100 to 200 nm as determined by HRTEM. For samples #1 and #2 the reversible M - H loops were obtained and the results are shown in Fig. 1a. The result indicates that both batches of PEI-coated MNPs have no significant hysteresis effect and are belong of soft magnet. In Fig. 1b, however, the irreversibly $M(H)$ loops of samples #3 and #4 show magnetic hysteresis effect in low field range, so they are so-called hard magnets.

To demonstrate the biological effects of MRVs, we designed an *in vitro* experiment to explore the feasibility of targeting by external magnetic field associated with MRVs either with soft or hard magnetic property. We, then, added the MRVs into a well with HT1080 cell. The tissue culture well is 3.6 cm in diameter and with a 1 cm magnet under the well. The scheme of this design is outlined in Fig. 2a. Since X-gal staining can detect the HT1080 cells transduced with the b-gal MRVs the guiding and focusing effects of the magnetic interaction can be easily determined in this experiment.

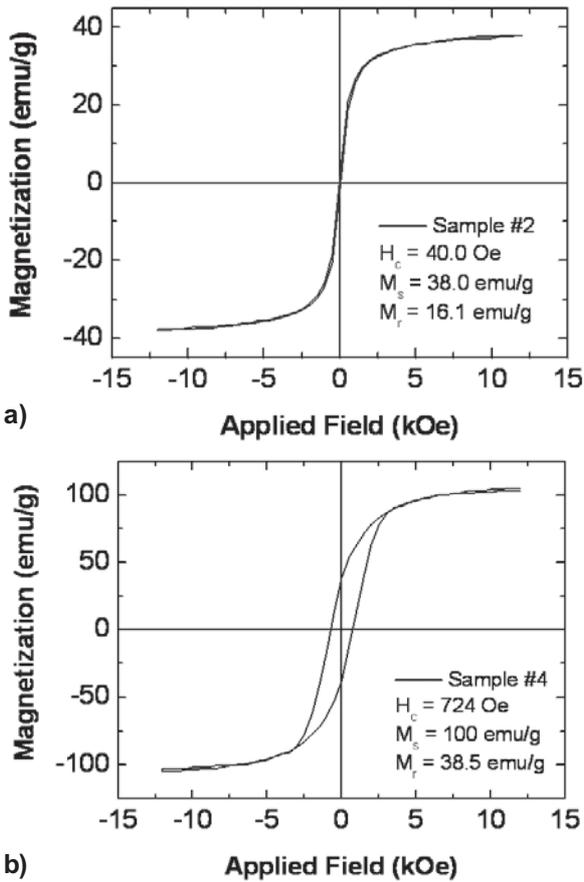
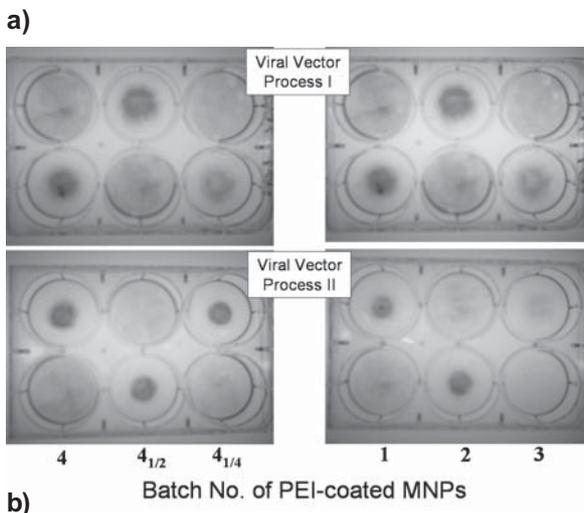
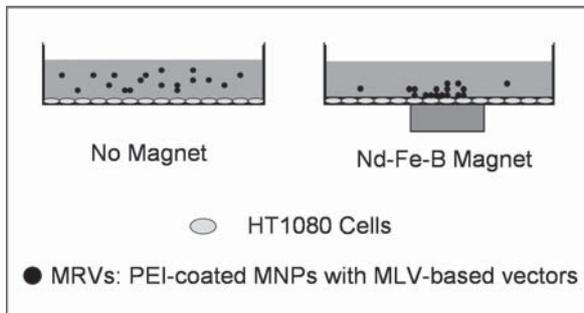


Fig. 1. Magnetic M(H) loops of PEI-coated $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles at room temperature. (a) MNPs sample batches #2 and (b) MNPs sample batches #4.



b)

In this study, we prepared two batches of VSV-G pseudotyped β -gal MLV-based vector independently. Then, the vectors were mixed with soft (sample batches #1 and #2) and hard (sample batches #3 and #4) MNPs to form MRVs before the transduction. Three Nd-Fe-B magnets were placed under the 6-well plate diagonally. Except for sample batch #4, we mixed 1 OD unit of MNPs with the viral vectors. For sample #4, we mixed 1, 1/2 and 1/4 OD units of MNPs with the viral vectors. All the samples were done in duplication and only one well in the duplicated set has the Nd-Fe-B magnet attached under the wells. After X-gal staining, the β -gal vectors transduced cells would turn into blue. We found both soft and hard MRVs can be concentrated and guided by attached permanent magnets. The high concentration of the MRVs at the magnet

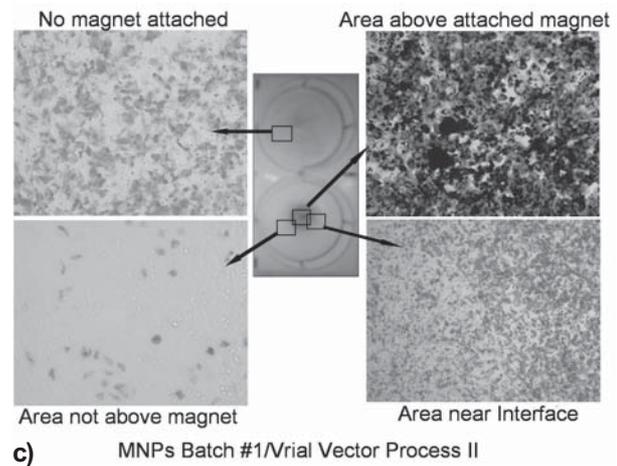


Fig. 2. (a) The scheme of our experimental design. The HT1080 cells (presented as circle in this pictures) were plated in a 6-well tissue culture plate one night before the transduction. The MRVs (presented as dark circles) were added to the wells either with or without a magnet under the well. If the MRVs have the magnetic property, it will attract to the magnet. (b) The biological activity of MRVs was demonstrated by the conversion of β -gal negative cells to β -gal positive cells (as shown in blue color after X-gal staining). The plates were fixed and stained with X-gal solution. The pictures were taken under a regular light box. The blue circles represented position of the magnets. (c) The microscope pictures of sample #1 transduced cells with a magnification of 200X at four different fields. The intensity of blue color in cells represented the intensity of β -gal expression. Blue cells are the HT1080 cells, which have been transduced by β -gal MRVs. The dotted line represents the boundary of the magnet place under the well.

sites did not cause any toxic effect in the HT1080 cells. As shown in Fig. 2b, we demonstrated the magnetofection effect by two independent preparations of viral vectors, I & II, and the four independent preparation batches of PEI coated γ -Fe₂O₃ MNPs. The enhancement of viral transduction by the magnet is shown in Fig. 2c.

In summary, we have shown retroviral vector development can be benefit from magnetic nanoparticles. By creating MRVs, we are not only able to direct the vector transduction in the specific sites, but also able to concentrate vectors. In addition, we have also observed four facts: (1) both soft and hard MNPs can be used to form MRVs, (2) the ratio of PEI-coated MNPs and VSV-G pseudotyped MLV-based vector is not critical, (3) the conjugation of MNPs with VSV-G pseudotyped MLV-based vectors did not interference with vector transduction, and (4) the PEI-coated MNPs is not toxic to the cells, even after concentrated by external magnetic field. Our study is the first demonstration of VSV-G pseudotyped MLV-based vector with PEI-coated MNPs. VSV-G can pseudotype both MLV-based and HIV-based vectors [5]. The pseudotyped vectors

have board host ranges and can be concentrated by centrifugation. Combing the advantages of the VSV-G envelop and the magnetic property of MNPs, we believe the new MRVs will be very useful for gene transfer *in vivo* and may even apply to human gene therapy.

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