Preparation of a transferrin-targeted M13-based gene nanocarrier and evaluation of its efficacy for gene delivery and expression in eukaryote cells

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Abstract: Bacteriophages are appropriate gene carriers that might be targeted toward target cells using different strategies. Here we prepared a transferrin-targeted M13-based gene nanocarrier (Tf-targeted M13-GFP) and examined its gene delivery and expression efficacy in the AGS cell line. M13 phagemid particles bearing a GFP expression cassette (M13-GFP) were obtained from a recombinant lambda phage through an in vivo excision procedure. Chemical coupling of human holotransferrin molecules (Tf) to the surface of these phagemid particles resulted in Tf-targeted M13-GFP formation, which was then characterized by Phage-ELISA and Cell-ELISA experiments. Immunocytochemistry (ICC) and fluorescence-activated cell sorting (FACS) analysis were used for internalization assay and examination of gene delivery/expression efficacies in the human AGS cell line. The ELISA experiments revealed high-density attachment of Tf molecules to the surface of M13-GFP particles and ICC confirmed highly efficient internalization of the Tf-targeted M13-GFP particles into the AGS cells. Moreover, FACS analysis showed significant increase of GFP-positive cell counts in the samples treated with Tf-targeted M13-GFP (8.09%) in comparison with the samples treated with wild M13-GFP (1.2%). We conclude that this strategy might improve phage-mediated gene delivery and expression in eukaryote cells.

Key words: Chemical coupling, phage-mediated gene transfer, gene delivery, transferrin-targeted delivery

1. Introduction
One of the most important issues of gene therapy is to achieve efficient, safe, and targeted gene carriers. Bacteriophages might be suitable candidates for this purpose due to characteristics including protection of transgenes by coat proteins, having no tropism for eukaryote cells, ease of targeting by phage display and/or chemical coupling approaches, low-cost and large-scale production, physical stability, ease of genetic manipulation, safety, and relative immunotolerance (Poul and Marks, 1999). These characteristics have made bacteriophages attractive gene carriers. These particles can especially be adopted for cancer therapy, either with suicide gene or anticancer drug delivery to the cancerous tissues/cells (Urbanelli et al., 2001; Li et al., 2010; Stephanopoulos et al., 2010; Huang et al., 2011; Shoae-Hassani et al., 2013a, 2013b). Two main approaches that can be exploited for targeted delivery of bacteriophages into eukaryote cells are phage display and chemical coupling strategies. Chemical coupling might be achieved by crosslinking an active group from a targeting molecule to another active group (such as –COOH and –NH3) present on the phage coat proteins. Thus, the targeting of gene carriers such as bacteriophages can be achieved directly through simple and various chemical reactions without need for complex genetic manipulations (Khalaj-Kondori et al., 2011).

Transferrin is one of the appropriate targeting molecules for drug and gene delivery to cancer cells and tissues because cancer cells overexpress transferrin receptors on their surfaces for acquiring the iron needed for their rapid growth and metabolism (Qian et al., 2002). Transferrin has been successfully applied for targeted delivery of various nanodrugs and nanoparticles. For example, targeting of liposome nanoparticles with transferrin resulted in specific entering of liposomes into the cancer cells in vitro and solid tumor tissues in vivo (Ishida et al., 2001). Moreover, an artemisinin-transferrin conjugate was used to inhibit the growth of prostate cancer cells (Nakase et al., 2009). In another work, transferrin molecules were coupled to the surface of bacteriophage HK97 particles that were already labeled with fluorescein molecules. This strategy showed efficient uptake of HK97 particles by different cell lines, which resulted in high-density fluorescent signals in the cells (Huang et al., 2011).
This study was aimed to formulate a transferrin-targeted M13-based gene nanocarrier using a chemical coupling strategy and to evaluate its potential for transgene delivery and expression in eukaryote cells. Human holotransferrin molecules were coupled on the surface of recombinant bacteriophage M13 particles bearing a GFP transgene (M13-GFP), which resulted in formulation of a transferrin-targeted M13-GFP gene nanocarrier. Finally, the carrier was characterized and evaluated using the human gastric adenocarcinoma AGS cell line (ATCC CRL-1739).

2. Materials and methods

2.1. Preparation of M13-GFP phage particles

We have already reported the construction of a recombinant lambda phage bearing a GFP encoding sequence (λ-GFP) using the lambda ZAP-CMV XR (Stratagene, USA) vector (Khalaj-Kondori et al., 2010). The lambda ZAP-CMV XR vector has been designed in such a way that after cloning it permits any inserted sequence to be obtained as a pCMV-Script phagemid particle by an in vivo excision mechanism. Therefore, we used the recombinant λ-GFP phage for preparation of M13-GFP phage particles. *E. coli* XL1-Blue MRF’ cells (Stratagene) grown in LB broth supplemented with 20% (w/v) maltose and 10 mM MgSO₄ were infected with the λ-GFP phage. The culture was then titered and the plates were incubated for 12 h at 37 °C. The appeared plaques were confirmed by plaque-PCR using GFP-specific primers. One of the positive plaques was amplified and used for obtaining the pCMV-Script-GFP phagemid construct by in vivo excision protocol. For in vivo excision, 50 mL of LB broth supplemented with 20% (w/v) maltose and 10 mM MgSO₄ was inoculated with the λ-GFP phage. The culture was then incubated for 60 min on ice and the precipitant was incubated for 1 h at 37 °C, and then 10 µL of it was plated on LB agar with 50 µg/mL kanamycin (Sigma-Aldrich, Germany) and the plate was incubated for 16 h at 37 °C. The appeared colonies were analyzed by colony-PCR using GFP-specific primers. One of the positive colonies was inoculated into 5 mL of 2X YT broth containing 50 µg/mL kanamycin and R408 helper phage (Stratagene) (multiplicity of infection (MOI): ~10) and incubated for 8 h. The culture was then transferred into 200 mL of fresh 2X YT broth containing 50 µg/mL kanamycin and incubated for 18 h at 37 °C. The culture was heated to 70 °C for 20 min and cell debris was collected by centrifugation at 5000 rpm for 10 min. The supernatant was transferred into fresh Falcon tubes and polyethylene glycol 8000 and NaCl were added in final concentrations of 10% and 1 M, respectively. The phage M13-GFP particles were then precipitated by incubation for 60 min on ice and the precipitant was collected by centrifugation at 17,000 × g and 4 °C for 20 min. The pellet was resuspended in SM buffer and titered. The titer of M13-GFP particles was calculated by the following formula (Mount et al., 2004):

\[ \text{virions/mL} = A_{269} \times 6 \times 10^{16} / \text{number of nucleotides in the genome}, \]

where \( A_{269} \) is the absorbance of M13-GFP solution at 269 nm.

M13-GFP phage particles (5 mL of 1 × 10ⁱ¹ cfu/mL) in SM buffer were purified and concentrated with 150K MWCO concentrator (Thermo Scientific Pierce, USA) and its buffer was exchanged with PBS (0.1 M sodium phosphate, pH 7.2). The final volume was adjusted to 5 mL and its absorbance at 269 nm was measured, and the number of phage particles was determined by the above formula.

2.2. Confirmation, amplification, and preparation of M13-GFP phage particles for chemical coupling reaction

*E. coli* XLOLR (500 µL; Stratagene) grown in LB broth was inoculated into 5 mL of 2X YT broth containing 50 µg/mL kanamycin and R408 helper phage (Stratagene) (multiplicity of infection (MOI): ~10) and incubated for 8 h. The culture was then transferred into 200 mL of fresh 2X YT broth containing 50 µg/mL kanamycin and incubated for 18 h at 37 °C. The culture was heated to 70 °C for 20 min and cell debris was collected by centrifugation at 5000 rpm for 10 min. The supernatant was transferred into fresh Falcon tubes and polyethylene glycol 8000 and NaCl were added in final concentrations of 10% and 1 M, respectively. The phage M13-GFP particles were then precipitated by incubation for 60 min on ice and the precipitant was collected by centrifugation at 17,000 × g and 4 °C for 20 min. The pellet was resuspended in SM buffer and titered. The titer of M13-GFP particles was calculated by the following formula (Mount et al., 2004):
2.4. Chemical coupling reaction
For coupling reaction we mixed 2 mL of oxidized holotransferrin (0.125 mg/mL in PBS buffer; 0.1 M sodium phosphate, pH 7.2) with 5 mL of M13-GFP phages in PBS (0.1 M sodium phosphate, pH 7.2; ~5 × 10^{11} phage particles). Then 70 µL of 5 M NaCNBH₃ (Sigma-Aldrich; final concentration 50 mM) was added to the solution and incubated overnight at 4 °C. The reaction was then quenched by adding 200 µL of Tris-HCl (pH 7.4). The coupling reaction product was purified and concentrated with 150K MWCO concentrator. This solution, which now contained holotransferrin-coupled M13-GFP particles (Tf-targeted M13-GFP gene nanocarrier), was diluted in 5 mL of PBS buffer (pH 7.4) and its absorbances at 260, 269, and 280 nm were measured. The concentration of Tf-targeted M13-GFP particles was calculated by the abovementioned formula.

2.5. Phage-ELISA
Phage-ELISA was done according to our previous report (Khalaj-Kondori et al., 2011). First, 100 µL of PBS containing 100 µg/mL rabbit M13 phage coat protein antibody (Thermo Scientific) was coated into the wells of an ELISA plate (Nunc, Denmark). The coating solution was discarded and the wells were washed with washing buffer (0.05% Tween 20 in PBS). The coated wells and the purified wild M13-GFP phage solution were blocked with blocking buffer (3% milk in PBS) at room temperature for 30 min. The wells were then read by an ELISA reader (Bio-Rad Microplate Reader). The absorbances of the holotransferrin-coated wells were used to obtain a standard curve, which used for estimation of the approximate milligrams of transferrin present in the wells coated with the Tf-targeted M13-GFP particles. The number of transferrin molecules coupled per phage particle was estimated considering the molecular weight of transferrin.

2.6. Quantification of coupled transferrin molecules
Four serial dilutions of holotransferrin in PBS with known concentration were coated into the wells of a 96-well plate, each with two replicates. Aliquots (100 µL) of a purified Tf-targeted M13 GFP solution in PBS, each with about 10^9 particles, were coated into the wells in triplicate format. The plate was incubated overnight at 4 °C. The solutions were collected from wells and their absorbances were measured to subtract transferrin molecules or Tf-targeted M13-GFP particles that had not attached to the wells. The wells were washed (0.05% Tween 20 in PBS) and blocked (3% milk in PBS) at room temperature for 60 min. The wells were then poured off and rinsed, and 100 µL of antihuman transferrin IgG-HRP conjugate (Alpha Diagnostic International Inc.) (1:1000 dilutions in blocking buffer) was added to each well and incubated at room temperature for 60 min. After discarding the solution, wells were washed three times and 100 µL of TMB (Thermo Scientific Pierce) was added to each well and incubated at room temperature for 60 min. After discarding the blocking solution, wells were washed with washing buffer and incubated with 100 µL of holotransferrin (1:1000 dilutions of 1 mg/mL) in blocking buffer at room temperature for 60 min. The solution was poured off and wells were rinsed with washing buffer and incubated with 100 µL of antihuman transferrin IgG-HRP conjugate (Alpha Diagnostic International Inc.) (1:1000 dilutions in blocking buffer) at room temperature for 60 min. The solution was discarded and wells were washed with washing buffer, and then 100 µL of TMB was added to each well. After 30 min the reaction was stopped by 10 µL of 0.5 M sulfuric acid. The absorbances at 450 nm were then read by an ELISA reader (Bio-Rad Microplate Reader).

2.7. Cell-ELISA
About 20,000 AGS cells/well were seeded in a 96-well plate and incubated at 37 °C until 90% confluency. Cells were washed with PBS and fixed by paraformaldehyde 4% (v/v) in RPMI at 4 °C for 10 min. Wells were rinsed with cold PBS and blocked with 3% milk in PBS at room temperature for 60 min. After discarding the blocking solution, wells were washed with washing buffer and incubated with 100 µL of holotransferrin (1:1000 dilutions of 1 mg/mL) in blocking buffer at room temperature for 60 min. The solution was poured off and wells were rinsed with washing buffer and incubated with 100 µL of antihuman transferrin IgG-HRP conjugate (Alpha Diagnostic International Inc.) (1:1000 dilutions in blocking buffer) at room temperature for 60 min. The solution was discarded and wells were washed with washing buffer, and then 100 µL of TMB was added to each well. After 30 min the reaction was stopped by 10 µL of 0.5 M sulfuric acid. The absorbances at 450 nm were then read by an ELISA reader (Bio-Rad Microplate Reader).

2.8. Internalization assay
Internalization assay was done according to our previous report (Khalaj-Kondori et al., 2011). About 10^5 AGS cells per well were seeded on coverslips in 6-well plates 24 h before treatments. The cells were treated with either Tf-targeted M13-GFP or wild M13-GFP at MOI of 10^7 and incubated for 3 h at 37 °C. Cells with no phage treatment were used as a negative control. The medium was removed and the coverslips were washed with PBS. Phage particles bound to the cell membrane were eluted by glycine buffer (50 mM glycine, pH 2.8, 500 mM NaCl). The cells were washed with PBS and fixed with 3.7% paraformaldehyde for 5 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS, washed, and blocked with 3% BSA in PBS. They were then read by an ELISA reader (Bio-Rad Microplate Reader).
secondary antibody Alexa-555 (Molecular Probes, the Netherlands; diluted 1/300 in PBS and 1% BSA) at room temperature for 45 min. A drop of DAPI stain was placed on each glass slide and after washing of the coverslips they were inverted onto the DAPI spots. The sides of coverslips were sealed with nail polish and analyzed with a Zeiss fluorescence microscope.

2.9. Transfection of AGS cells by Tf-targeted M13-GFP
AGS cells (1 × 10^5 cells/well) were seeded into 6-well plates 24 h prior to transfection. Cells were treated with either 10^9 Tf-targeted M13-GFP or 10^9 wild-type M13-GFP or, for competition assay, 10^9 Tf-targeted M13-GFP particles plus 1 µg of holotransferrin. Wells with no phages were used as a negative control. After incubation for 4 h at 37 °C, the media were changed with fresh complete medium and incubated for 48 h. Cells were then collected and fixed with 5% paraformaldehyde in PBS at room temperature for 20 min. The cells were washed with PBS, resuspended in 500 µL of PBS containing 0.01% sodium azide, and analyzed by fluorescence-activated cell sorting (FACS; Becton Dickinson Biosciences, USA). The percentage of GFP-positive cells per total counted cells was reported as transfection efficiency. Transfections were done in duplicate and performed three times.

3. Results
3.1. Preparation of M13-GFP phage particles
The recombinant lambda phage bearing the GFP cassette (λ-GFP) (Khalaj-Kondori et al., 2010) was amplified and confirmed by plaque-PCR. One of the confirmed λ-GFP plaques was used for in vivo excision of the pCMV-Script-GFP phagemid. The in vivo excision procedure resulted in packaging of the pCMV-Script-GFP construct as M13-GFP phage particles, which were then used for infection of E. coli XLOLR cells. XLOLR colonies containing the pCMV-Script-GFP construct were confirmed by colony-PCR (Figure 1).

3.2. Evaluation of transferrin receptor expression on the AGS cell line
Transferrin receptor (Tfr) expression on the surface of AGS cells was evaluated by Cell-ELISA. Holotransferrin molecules that bonded to the Tfr(s) on the cell membrane of fixed AGS cells in Cell-ELISA experiments were detected by antitransferrin HRP conjugate. The results showed that AGS cells expressed Tfr on their surface and it was suitable for transferrin-targeted delivery (Figure 2).

3.3. Preparation and characterization of Tf-targeted M13-GFP gene nanocarrier
3.3.1. Chemical coupling
For preparation of the Tf-targeted M13-GFP gene nanocarrier, human holotransferrin molecules were coupled chemically to the surface of M13-GFP phage particles. The overall reaction is depicted in Figure 3. To do this, holotransferrin was treated with sodium metaperiodate for oxidation of its sugar chains. It was reported that sodium metaperiodate causes oxidative damage to tyrosine residues and hence the tyrosine residue at the iron binding site of transferrin might be partially oxidized (Lai et al., 2005; Khalaj-Kondori et al., 2011). Oxidation of tyrosine at the iron binding site results in release of iron ions, which in turn may reduce the binding affinity of transferrin to its receptors on the cell surface (Lai et al., 2005). To reveal the effect of oxidation on the iron content of holotransferrin, the absorbances...
of the holotransferrin solution before and after oxidation reaction were measured at 470 and 280 nm. The $A_{470}/A_{280}$ ratios for holotransferrin and oxidized transferrin were 0.058 ± 0.004 and 0.037 ± 0.007, respectively. These results showed that the iron content of the oxidized transferrin in comparison to nonoxidized holotransferrin was approximately 64%.

3.3.2. Detection of coupled transferrin molecules
Phage-ELISA was used to reveal the coupled transferrin molecules to the surface of phage particles. Phage particles with or without coupled transferrin molecules were trapped by anti-M13 antibody, which had been coated in the wells. The coupled transferrin molecules to the phage surface were then detected by HRP-conjugated antihuman transferrin. Phage-ELISA experiments confirmed the successful attachment of transferrin to the surface of phage particles and hence obtaining of the Tf-targeted M13-GFP particles (Figure 4).

3.3.3. Quantitation of transferrin molecules coupled per phage
To reveal the average number of transferrin molecules coupled per phage particle, ELISA was used. Either serial dilutions of holotransferrin or purified Tf-targeted M13-GFP particles were coated in the wells. The absorbances obtained from holotransferrin-coated wells were utilized to make a standard curve (Figure 5), the formula of which was then used for obtaining the nanograms of transferrin present in the wells coated with Tf-targeted M13-GFP particles. The Table shows a summary of the calculations. Knowing the number of Tf-targeted M13-GFP particles coated in each well and the molecular weight of transferrin (78 kDa), we calculated the number of transferrin molecules coupled per phage particle. The results showed that, on average, 397 transferrin molecules had been coupled to each phage particle.
3.3.4. Evaluation of binding capacity and functionality
To find out whether transferrin molecules coupled to the surface of phage particles retained their functionality/ability for binding to the transferrin receptors on the cell surface, Cell-ELISA was performed. AGS cells were seeded, fixed, and blocked in a 96-well plate. Cells were then treated with Tf-targeted M13-GFP, holotransferrin, M13-GFP phages, or nothing. The trapped transferrin molecules or Tf-targeted M13-GFP on the surface of cells was detected with HRP-conjugated antihuman transferrin. The results showed that the absorbance value for wells treated with Tf-targeted M13-GFP was lower than that of the wells treated with holotransferrin, while it was significantly higher than that of the wells treated with wild-type M13-GFP (data not shown). These results imply that the transferrin molecules that coupled to the surface of M13-GFP phages were active and could interact with cell surface transferrin receptors.

3.4. Internalization of Tf-targeted M13-GFP into AGS cells
Internalization of the Tf-targeted M13-GFP particles with AGS cells was studied with immunocytochemical (ICC) staining. Figure 6 shows that the cells treated with Tf-targeted GFP-M13 and permeabilized with Triton X-100 efficiently internalized the gene nanocarrier and produced more and brighter fluorescence signals due to interactions between Tf-targeted M13-GFP/anti-M13/Alexa-555 labeled secondary antibodies. Those cells permeabilized and treated with wild M13-GFP phages showed faint fluorescence signals. These observations reveal that Tf-targeted M13-GFP particles internalized with AGS cells via specific interactions between the transferrin molecules attached to the M13-GFP phage particles and the transferrin receptors on the AGS cells. Moreover, no signals were observed in the nonpermeabilized cells, which in turn confirmed that the observed signals were due to immunoreaction with the internalized particles rather than particles adhering to the cells surface.

3.5. Transfection of AGS cells with Tf-targeted M13-GFP
To reveal Tf-targeted M13-GFP-mediated transgene delivery and expression in AGS cells, FACS analysis was used. AGS cells were seeded in 6-well plates and treated with Tf-targeted M13-GFP or M13-GFP particles at MOI of 10^4. After 48 h, cells were collected and analyzed by FACS. The results showed 8.09% GFP-positive cells for the wells treated with Tf-targeted M13-GFP, whereas it was 1.2% for the wells treated with naïve M13-GFP particles (Figures 7A and 7B). Moreover, addition of 1 µg of holotransferrin together with 10^9 Tf-targeted M13-GFP particles could efficiently prevent transfection of the AGS cells and resulted in 1.55% GFP-positive cells (Figure 7C). FACS allows not only the counting of the number of positive transfected cells but also measurement of the intensity

**Table.** Calculating the number of transferrin molecules coupled per phage.

<table>
<thead>
<tr>
<th>Equation</th>
<th>( y = 0.01505x + 0.143 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>y: mean of absorbances</td>
<td>0.711</td>
</tr>
<tr>
<td>x: ~nanograms of transferrin per well</td>
<td>37.74 ng</td>
</tr>
<tr>
<td>1 ng Tf</td>
<td>( 7.8 \times 10^9 ) molecules</td>
</tr>
<tr>
<td>Number of Tf molecules in the well</td>
<td>( 294.4 \times 10^9 ) molecules</td>
</tr>
<tr>
<td>Number of Tf-targeted M13-GFP particles used for coating per well</td>
<td>( 1 \times 10^9 )</td>
</tr>
<tr>
<td>Number of uncoated Tf-targeted M13-GFP particles per well</td>
<td>( 2.6 \times 10^9 )</td>
</tr>
<tr>
<td>Number of Tf-targeted M13-GFP particles coated per well</td>
<td>( 7.4 \times 10^9 )</td>
</tr>
<tr>
<td>Number of Tf molecules coupled per phage (( 294.4 \times 10^9 / 7.4 \times 10^9 ))</td>
<td>397 molecules</td>
</tr>
</tbody>
</table>
Figure 6. Immunocytochemical staining for internalization assay; AGS cells were seeded on coverslips in 6-well plates and incubated for 24 h. Either Tf-targeted M13-GFP or wild M13-GFP particles were added to the wells at a MOI of $10^5$ and incubated for 3 h at 37 °C. Cells with no phage treatment were used as a negative control. The wells were then washed with PBS and cell membrane-bound phage particles were eluted by glycine buffer. The cells were fixed and permeabilized (left) or not permeabilized (right) with Triton X-100 and treated with the rabbit anti-M13 antibody. Coverslips were incubated with the secondary antibody Alexa-555, washed, and inverted onto a drop of DAPI stain on glass slides. The coverslips were analyzed using a Zeiss fluorescence microscope.

Figure 7. Left: FACS analysis of AGS cells treated with $10^9$ Tf-targeted M13-GFP (A), $10^9$ wild M13-GFP (B), $10^9$ Tf-targeted M13-GFP particles plus 1 µg holotransferrin (C), or no phages (D). Transfections were done with a MOI of $10^4$. Right: Mean fluorescence intensity (MFI) of the GFP-positive cells for the same treatments.
of fluorescent signals of individual GFP-positive cells. Results showed that mean fluorescence intensity (MFI) of the cells transfected with Tf-targeted M13-GFP was about eight-fold more than the MFI of the cells transfected with naive M13-GFP particles (Figure 7). These results revealed that coupling of transferrin molecules to the surface of M13-GFP particles might result in improvements not only in the number of transfected cells but in the transgene expression as well.

4. Discussion
Targeting of cancerous, infected, or malfunctioning cells with therapeutic genes is very important for treatment of patients. However, there are some obstacles including safety, efficiency, and specificity of gene carriers. Animal viral vectors are the most efficient gene delivery systems, but they suffer from risk of intrinsic toxicity as well as the high cost for their production (Chung et al., 2008). One alternative that has attracted researchers is bacteriophages. These bacterial viruses can be targeted toward eukaryote cells/tissues by displaying cell/tissue type-specific targeting molecules such as small peptides, receptor-specific ligand molecules, and antibodies on their surfaces (Khalaj-Kondori et al., 2011; Shoae-Hassani et al., 2013b). Targeting molecules can be displayed on the surface of phage particles either via common phage display technology (Poul and Marks, 1999; Urbanelli et al., 2001; Mount et al., 2004) or with chemical coupling strategies (Li et al., 2010; Huang et al., 2011; Khalaj-Kondori et al., 2011). Chemical coupling has been applied as a straightforward strategy for linking targeting molecules and/or drugs with nanoparticles/nanobioparticles. In particular, this strategy has achieved some insights in imaging (Singh et al., 2006; Li et al., 2010; Huang et al., 2011) and drug/gene delivery (Stephanopoulos et al., 2010; Khalaj-Kondori et al., 2011; Shoae-Hassani et al., 2013a, 2013b; Bakhshinejad and Sadeghizadeh, 2014; Bakhshinejad et al., 2014) using targeted bacteriophage particles.

In the present study we used the chemical coupling strategy for formulation of a transferrin-targeted bacteriophage M13-based (Tf-targeted M13-GFP) gene nanocarrier. The M13-GFP phage particles were obtained with an in vivo excision procedure from a recombinant λ-GFP phage. Subsequently, human holotransferrin molecules were coupled to the surface of the M13-GFP phage particles, which resulted in the formulation of the Tf-targeted M13-GFP gene nanocarrier. For coupling, the carbohydrate chains of human holotransferrin were oxidized. Oxidation might damage tyrosine residues present in the iron binding site of holotransferrin, which in turn might lead to the release of iron molecules and reduction of its interaction with cell surface transferrin receptors (Lai et al., 2005, 2009). Monitoring of the A470/

A280 ratios of the solution before and after oxidation showed 36% reduction in the iron content of holotransferrin, which was in the range of reports by others (Mount et al., 2004; Lai et al., 2005; Khalaj-Kondori et al., 2011). Characterization of the Tf-targeted M13-GFP gene nanocarrier revealed that an average of 397 transferrin molecules were coupled per phage particle. This achievement is valuable, because it was reported that the copy number of displayed/coupled targeting molecules on the phage/nanoparticle surface dictates the efficiency of internalization of them by cells (Ivanenkov et al., 1999a; Mount et al., 2004; Li et al., 2005). Moreover, transferrin is a large and complex glycoprotein and displaying of it in high copy numbers on the phage surface with the common phage display approach seems to be impossible. Assembling of phage particles in the case of using coat proteins fused with bigger and complex proteins would be insufficient (Ivanenkov et al., 1999b; Gupta et al., 2003; Hodyra and Dąbrowska, 2015).

Internalization of the Tf-targeted M13-GFP particles with AGS cells was studied by ICC. The ICC experiments showed that the targeted particles had been internalized with very high efficiency by the cells (Figure 6). This achievement might be due to the high copy number of transferrin molecules attached to the phage particles. In contrast to the very highly efficient internalization, the count of GFP-positive cells obtained with FACS was about 8.09%. This implies that the internalization is not sufficient by itself and there are some barriers on the way to the nucleus that need to be removed. One of the possible determinants might be the conformation/structure of the phage genome (Abedheydari et al., 2014). There are some strategies for getting rid of the barriers. For example, Volcy and Dewhurst (2009) studied the effect of inhibitors of proteasome as well as lysosomal proteases. They showed that these inhibitors strongly enhanced the phage-mediated transgene expression, suggesting that these pathways contribute to the destruction of intracellular phage particles. One alternative might be displaying the nucleus localization signal (NLS) peptide on the phage surface. In this way, Eguchi et al. (2005) improved the nuclear transport of lambda phage particles displaying peptides containing the minimum NLS of SV40 T antigen on their surface. Moreover, the targeting molecules displayed on the phage surface dictate their internalization and intracellular trafficking route. Kim et al. showed that 3D8 VL-M13 phages (M13 displaying a cell-penetrating light chain variable domain 3D8 VL transbody) routed to the cytosol and remained stable for more than 18 h without further trafficking to other subcellular compartments, whereas TAT-M13 phages (M13 displaying TAT peptide) routed to several subcellular compartments before being degraded in lysosomes even after 2 h of internalization (Kim et al., 2012). These studies emphasize that it would
be possible to increase the phage-mediated gene transfer either by recruiting a proper targeting molecule or with combined targeting molecules/strategies.

In conclusion, the results showed that the Tf-targeted M13-GFP gene nanocarrier was able to interact efficiently with the transferrin receptors on AGS cells, be internalized with them, and deliver encapsulated DNA into the cells, leading to enhancement of the transgene expression in AGS cells. Moreover, our results further confirmed that the chemical coupling approach might be considered as a straightforward strategy for attaching more targeting ligands to the surface of phage particles with the aim of improving phage-mediated transgene delivery to eukaryote cells.

Acknowledgment
This work was supported by the Iran National Science Foundation, Science Deputy of Presidency, Islamic Republic of Iran.

References


