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Research Article

Home-made *in vitro* magnetic nanoparticle-based gene delivery system

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ABSTRACT

Introduction: Biotechnology applications of Magnetic Nanoparticles (MNPs) have been rapidly investigated and are becoming the leading trend in the field. Although making an appearance almost 20 years ago, gene delivery using magnetic nanoparticles has not achieved much significant success. This study aims to establish promising and simple MNP-based protocol for gene delivery initially. Specifically, we aim to evaluate whether sodium chloride (NaCl) could enhance gene transfer with naked plasmid DNA without the need to combine liposomes or polymersomes. **Methods:** In this work, our "homemade" MNPs were mixed with plasmid DNA (phEF1-hKO – which expresses orange fluorescence) under various treatments of NaCl to enhance the formation of MNP-DNA complex for transfection. Then, these complexes were added to cultured cells. Delivery efficiency was represented by fluorescent expressing cells. **Results:** NaCl facilitated the magnetofection process in a dose and treatment duration co-dependent manner. Initial data showed that the optimal condition was 150mM NaCl with a 40-minute duration. **Conclusion:** Our homemade gene delivery system based on magnetic nanoparticles was established successfully. The viability of cells in magnetofection was maintained. Magnetofection without liposomes and polymersomes is certainly possible, but further researches have to be carried out in order to tell the definite optimal conditions.

Key words: Gene delivery, magnetic nanoparticles, magnetofection, transfection

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INTRODUCTION

Gene delivery refers to the process of transferring foreign DNA or RNA to host cells for applications such as genetic research or gene therapy. Successful gene delivery requires the foreign genetic material to remain stable within the host cell and can either integrate into the genome or replicate independently of it. It is often considered one of the most important and the most challenging steps in the whole process due to the lack of optimization. Thus, there is still much room for improvement. To name a few, the choice of vectors has always been a problem. The trade-off between cost and efficiency of different delivery systems has also been an issue, and the saturation of new technology applications in the field is unjustified. Take, for example, the problem of choosing vectors. Currently, there are viral vectors and non-viral vectors. Viral vectors are most effective, but their immunogenicity, oncogenicity limit their application, and the small size of the DNA they can transport. Non-viral vectors are safer, lower-cost, reproducible, and do not present DNA size limits. The main limitation of non-viral systems is their low transfection efficiency, although different strategies have improved it and the efforts are still ongoing[1]. Therefore, optimizing gene delivery is a matter of internal individual lab adjusting, achieve by trial and error. On the other hand, since most of the time, optimizing was not the main target of the whole project, every little change and renovation were cast aside by the grand process and went unnoticed in the academic field.

There are many methods, as well as slightly alternative versions of them, available for gene delivery. They could be divided into groups based on the fundamental principle: physical-based or chemical-based. The two most well-known as well as most popular methods are lipofection and nucleofection.

The first one is chemical-based transfection that focuses on combining DNA with vesicles. These vesicles are either liposomes or polymersomes. The resulting DNA/lipid complexes fuse with the anionic cytoplasmic membrane and/or are introduced to the cells via an endocytic pathway[2]. Although it is generally not suitable for most primary isolated cells, it is the most widely used method nowadays, which is to say its importance[3]. However, this method requires changing of buffers frequently and is known for average toxicity.

The other system is nucleofection. It is the leading technology in physical-based transfection. This

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method involves applying brief electric pulses to cells or tissues to increase the permeability of cells to macromolecules[4]. However, the tendency for a high level of damaged cells is a problem as well.

Another approach to the current situation is gene delivery by the use of magnetic forces, so-called magnetofection[5]. Gene delivery is targeted by the application of a magnetic field and is believed to have the potential application on targeting of gene expression into the desired organ and tissue *in vivo*[1,6,7]. Magnetic nanoparticles (MNPs) are a class of nanoparticles that can be manipulated using magnetic fields. Generally, there are oxides and metals in terms of magnetic material. However, their fundamental characteristic relies on the superparamagnetic characteristic, exhibiting magnetic behavior only when an external magnetic field is applied. Although, the magnetic plate is a one-time investment and reusable, magnetofection reagents are consumable and expensive. In past research, it would be used in combination with lipofection as means for protecting and helping vectors enter cells' membranes, further increase the efficiency of lipofection[8]. However, this combination will additionally increase the cost of transfection process.

Previous literature had shown that the association between vector (DNA) and MNPs resembles the aggregation of nanomaterials[5]. Furthermore, that aggregation is induced by salt. However, most magnetofectin included in those researches was mixed with liposomes[5] or polymersomes[9]. By removing such vesicles from magnetofection, the efficiency would be reduced in theory, but with the benefit of much less toxicity and simpler to control with fewer factors.

The primary purpose of this study is to investigate magnetofection in terms of efficiency when using independently of poliplexes and with the help of a collection of ideas. Those ideas serve two main points of interest: the forming of DNA – magnetic nanoparticles complex (magnetofectins) and the inducing capability of buffers used in transfection into the cell. Thus, we aimed to initially investigate the optimal condition in which magnetofection can be used independently with naked plasmid DNA. The data from this study will eventually provide suggestions for further follow-up researches and effective methods for drug delivery in the future.

MATERIALS AND METHODS

Plasmid DNA purification

Plasmid DNA (phEF1-hKO-IRES-Puro^R) was used as material for gene delivery by taking advantage of fluorescent signal (hKO). This plasmid was constructed and provided by the Laboratory of Gene Regulation, Tsukuba University, Japan. These plasmids were transformed into *E. coli* DH5α to multiply the number of plasmids. Then, plasmid DNA was extracted by Wizard® *Plus* Minipreps DNA Purification System (Promega, Cat. #7100), according to the manufacturer's instruction. The quantity and quality of plasmid DNA were assessed by combining NanoDrop One machine (ThermoFisher Scientific) and the conventional agarose gel electrophoresis UV exposure. The extracted DNA was stored in a -80^{o}C freezer for further usage.

Cell culture preparation

HEK293T cells (RIKEN Bioresource Center, RBRC-RCB:2202:293T) were cultivated in a humidified incubator at 37^{o}C with 5% CO_2, with a 3-day interval between changing media (Dulbecco's modified Eagle's medium (DMEM) (4.5g/L Glucose, 11995-05, Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (16000-044, Gibco) and 1% Penicillin-Streptomycin (Sigma 13752-5G-F, Sigma 59137-25G, Merck KGaA, Darmstadt, Germany). Before transfection, 2x10^4 cells in a 50μl culture medium were transferred into each well of 96-well plate. Cells were cultured 16-18 hours before transfection.

A mixture of Magnetic Nanoparticles (MNPs) and Plasmid DNA

The MNPs are basically Fe_3O_4 nanoparticles and were coated with silica (MNP@SiO2). They were prepared and gifted by Dr. Pham Xuan Hung (Konkuk University, Korea). MNPs and plasmid DNA were mixed together with the ratio of 1:1 in terms of weight (100ng per well), combined with an appropriate buffer (NaCl). The final volume was adjusted to 50 μL using DMEM without FBS. The DNA + MNPs mixture (magnetofectins) sat undisturbed for 20 minutes, then transferred directly into the prepared cell culture. The ratio of MNPs and DNA, as well as the mixture duration, has followed the instruction of a commercial kit - Magnetofection^{TM} (Guideline is available online through http://www.interchim.fr/ft/B/BC3012.pdf).

Transfection of cultured cells

Magnetofectins were added directly to the prepared cell culture, made up a total of 100μl per well, necessarily diluting the salt concentration by half. The 96-well plate was put on a magnetic plate[10]. The system containing a 96-well plate and magnetic plate was kept in a humidified incubator (37^{o}C with 5% CO_2).

The magnet plate is home-made with strong permanent magnets, which are 5x2-mm nickel-coating magnets. Transfections with 20-minute, 40-minute duration were tested. After a tested time, the magnetic plate was detached, the solution was taken out, replaced by fresh 70μl DMEM containing 10% FBS for each well. The transfection efficiency and cell viability were also investigated by treating not changing medium after short periods (all media were subject to be changed after 24 hours).

On the second day of transfection, transfection efficiency could be inspected. We collected cells in each well and spread the specific number of cells into each new well. Let cells attach to the bottom of the well and observed cells under a Nikon Eclipse N*i* microscope with specialized excitation filters (550nm filter for Kusabira Orange fluorescence). For positive controls, Lipofectamine® 2000 (Thermo Fisher Scientific Inc.) was used according to the manufacturer's instruction with the same amount of DNA (100ng in each well).

Gene delivery efficiency

The transfection efficiency was represented by the number of detectable fluorescents expressing cells under a 550nm excitation filter. The captures from microscopic viewing were processed and quantified using ImageJ. The process of highlighting signal and contrast value is described in Figure 1 for easier and more precise counting.

Statistical analysis

The data were obtained from at least three independent replications and analyzed by Student's *t-test* to perform statistical analyses. Data are presented as mean $\pm$ standard error (SE).

RESULTS

Setting up the magnetofection system

To deliver DNA efficiently to cells, the formation of DNA–MNP complex was mandatory and a major point of interest in this research. The term magnetofectin referred to the complex that fundamentally could be broken down into magnetic interaction agent and DNA plasmid, with or without some form of protection to the whole complex, to endure endocytosis[7]. As mentioned above, salt ions improve the aggregation of MNP–DNA particles. Sodium chloride (NaCl) appeared continuously in previous successful magnetofection[5,7]. For the nucleic acid purification method, silica-containing spin column is a common use. In the presence of chaotropic sodium salt, DNA is bound to the silica membrane to separate them from other components. Taking advantage of materials, MNPs in our hands are coated by silica. The choice of NaCl to facilitate the aggregation of DNA and MNPs is theoretically applicable. In the optimal condition of sodium salt, the DNA would bind to solid silica particles which coated MNPs. They would form a DNA-MNPs complex, as illustrated in Figure 2A. These complexes, consequently, would increase their magnetic ability. Additionally, we made a magnetic plate to enhance magnetic force to concentrate DNAs carrying by magnetofectin complexes onto the target cells. Moreover, NaCl has inducing effects on increasing DNA and polyethylenimine complexes, hence, enhancing transfection efficiency[11]. Therefore, in our initial establishment, we would test NaCl salt as an inducing buffer for magnetofection.

In the attempt to set up a novel gene delivery system by MNPs, we built a protocol (Figure 2B) which was a customized version of much related previous literature[5,8] and instruction from a costly commercial system (Magnetofection™). Essentially, we could adjust some inputs to optimize magnetofection: salt concentrations and duration of the transfection process. Based on the cell types, seeding density, and the amount of DNA used, these inputs could be varied to achieve higher expression and lower toxicity. To minimize the workload, we first customized some inputs to optimize magnetofection: salt concentration and transfection duration.

NaCl — Inducing buffer for magnetofection without the need for liposomes

We hypothesized that NaCl could act as an inducing buffer in terms of DNA+MNPs formation as well as endocytosis independently of liposomes. To test that, we first performed magnetofection followed up the mentioned protocol in Figure 2B. In the research of Plank and his colleagues, without the presence of 150mM NaCl, there was no binding association between opposite charge DNA and particles and much less association between similar charge DNA-particle[5]. The 20-minute duration was the norm in most previous magnetofection literature[5,8]. In our initial study, the concentrations of NaCl and the duration for magnetofection were first inspected at 150mM and 20 minutes, respectively, as following previous literature.

As shown in Figure 3A, the lower panel is representative photos of cells after two days of transfection with MNPs – DNA/NaCl. The orange fluorescent signal indicated that the fluorescent coding gene

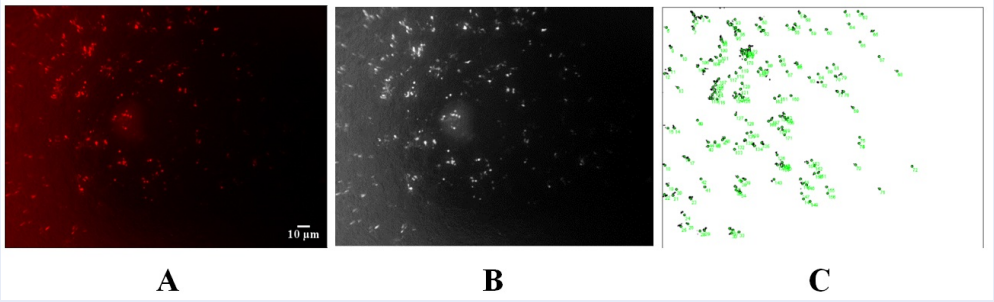

Figure 1: Capture processed by ImageJ. (A) Original (B) RGB value adjusted (C) Black/White threshold adjusted and counted manually.

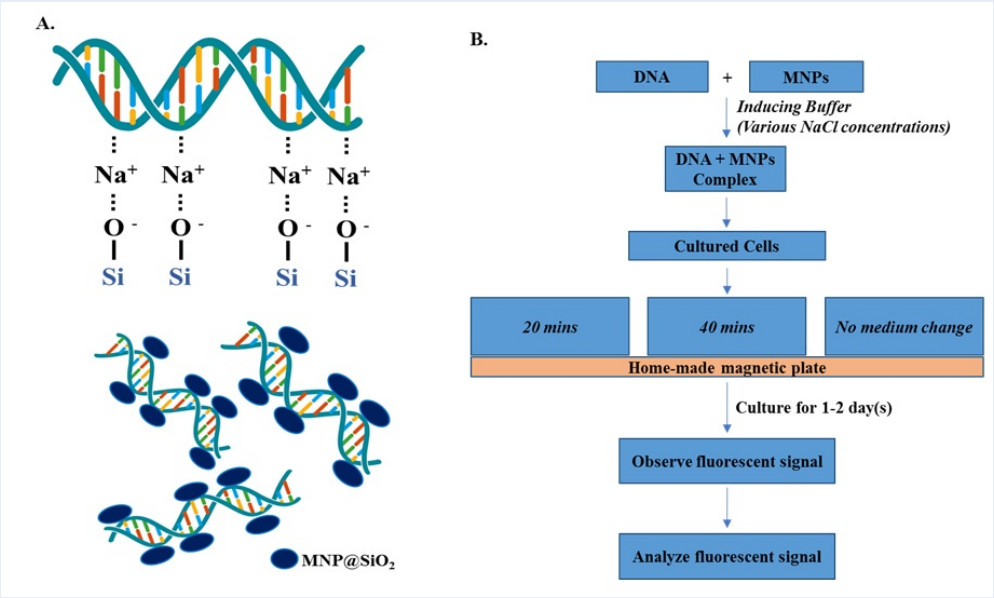

Figure 2: **Scheme for setting up MNP – based gene delivery system.** (A) Illustration for sodium-induced DNA-MNP aggregation (B) Experimental procedure for magnetofection. Italic letters stand for examined factors in this study.

(DNA/ phEF1-hKO) was successfully transferred into the cells. In the group treated with only MNPs and DNA, no fluorescent signal was observed. in other words, no gene delivery without 150mM NaCl. This result suggested that 150mM NaCl could act as an inducing buffer for magnetofection, which is independent of liposomes.

In previous research, Plank and his colleagues suggested the usage of 150mM NaCl for aggregation of DNA and magnetic particles for magnetofection[5]. However, no other concentration of NaCl was tested. Following up on our success in using 150mM, we tested different increasing concentrations of NaCl with our expectation in enhancing transfection efficiency. The duration for magnetofection was also inspected at 20 minutes.

Right after transfection, cells were in healthy condition in groups treated with 150 and 300mM. We observed cell shrinkage in the treated groups with NaCl concentration over 300mM (figure not shown). Consequently, the cells died. It could be explained that NaCl at high concentration created osmotic stress to the cells. It suggested that, in our tested system, the upper limitation for cell tolerance is 300mM.

In 150mM and 300mM treated groups, after two days of transfection, we observed fluorescent signals. As expected, the transfection efficiency leaned toward 300mM NaCl buffer with more than double cells flu-

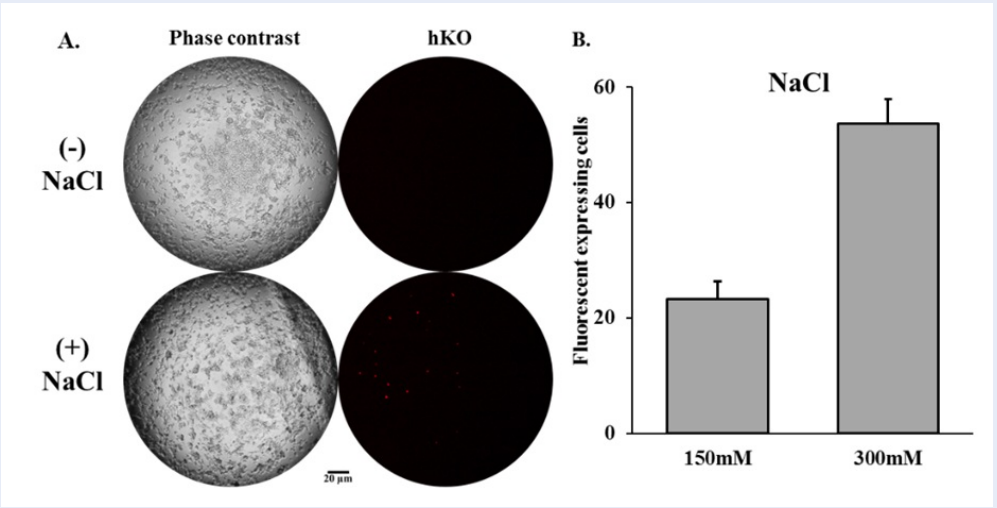

Figure 3: **Transfection result of 20-min duration magnetofection by NaCl.** (A) Morphology and fluorescent signal of cells treated without and with NaCl under phase contrast and hKO filter, respectively; (B) Diagram of transfection efficiency (fluorescent signal counts) of MNP treated groups. (n = 3) (Scale bar 20 μm).

orescent count (55 to 24) (Figure 3B). From this result, the role of NaCl in magnetofection was further confirmed. The achieved efficiency resulted from 20-minute transfection; it raised up the question whether lengthen transfection duration could help us in increasing transfection efficiency or not.

150mM NaCl and 40-minute duration is potential for magnetofection

Although we achieved some preliminary results on magnetofection by utilizing NaCl only, to further optimize our magnetofection system, different durations for magnetofection were set at 20 minutes, 40 minutes, and 1440 minutes. To make it clear, 1440-minute duration (24-hour duration or not changing medium at all) means we aimed to test the effect of longtime transfection (magnetic plate was still attached to the cell plate and no medium change within 24 hours). After 24 hours, all treatments' nutrient culture would often be replaced to maintain fresh conditions for healthy cells.

Overall, cells were healthy after 40 minutes of magnetofection. Despite having some result at NaCl 150mM and 300mM, not changing medium (roughly 24-hour duration transfection or medium change after 24 hours) after transfection affected the cells negatively. This might have resulted from the combination of lack of nutrition and toxicity presented.

In 150mM NaCl treated group, as shown in Figure 4A, the fluorescent signals emitted from transfected cells increased when lengthening transfection duration. 40-minute duration magnetofection yielded the highest efficiency. After that time, the transfection efficiency decreased. Although the number of fluorescents expressing cells was lower than the positive control (Lipofectamine® 2000), the efficacy of 40-minute duration could compare with that of the positive control. The result also showed that magnetofection conditions have the potential to compete with lipofection in terms of efficiency.

In contrast to our expectation, at 300mM, elongating transfection time did not result in higher efficiency. The highest yield for magnetofection is a 20-minute duration (Figure 4B). However, the efficiency is still lower than expected, especially compared to NaCl 150mM with a 40-minute duration.

Our experiments found that 300mM NaCl showed better result than 150mM in 20 mins but not in 40 mins. In the research of Sang and his colleagues in 2015, they tested the effect of 0, 150, 300, 600mM NaCl on the particle size of PEI-DNA. The increasing concentration of NaCl led to increasing PEI-DNA aggregation. Therefore, in our initial experiment with 20-min duration of transfection, the aggregation of DNA and MNPs might be more facilitated by 300mM than 150mM NaCl. Consequently, more magnetofectins were concentrated on the surface of targeted cells which led to better transfection efficiency.

On the other hand, the increasing concentration of NaCl also led to an increase the particle size of PEI-DNA. The particle size is one of the important factors that affect cellular uptakes of particles through

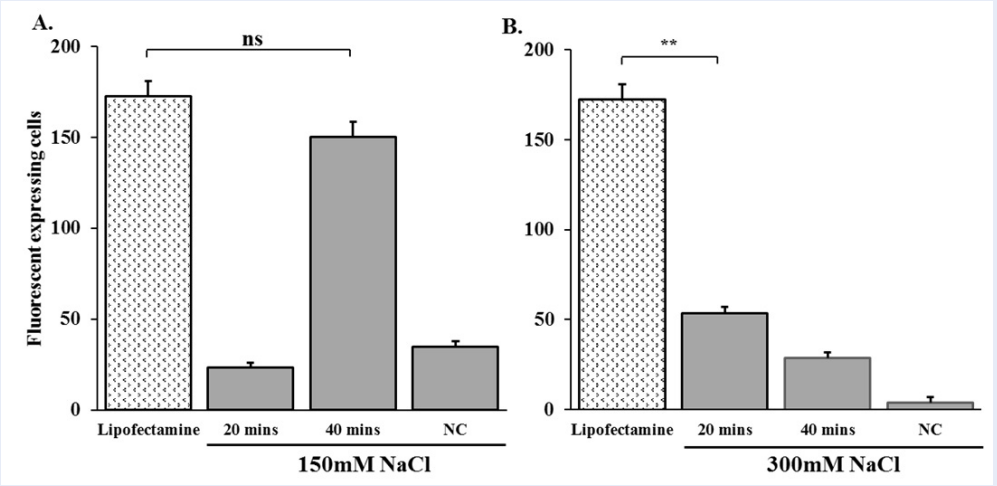

Figure 4: **Transfection efficiency (fluorescent expressing cell counts) at different treatments by NaCl.** (A) 150mM NaCl, (B) 300mM NaCl (NC – Not changing medium) (n = 3, ^{ns} – not significant, ** p < 0.01)

the cytoplasmic membrane. The larger the DNA complexes are, the lower the transfection efficiency. In our research, although NaCl concentrations were diluted while adding magnetofectin mixture into cell culture, prolonging duration for magnetofection (20 mins to 40 mins) might also increase the particle size of magnetofectins. In our experiments, we have not performed any measurement on magnetofectin sizes yet to conclude on this point exactly. They might be too large for cellular uptakes. Hence, 40-min duration led to decrease transfection efficiency in the group treated with 300mM NaCl.

From our general observation, this result might suggest a co-dependent relationship between doses and durations in terms of genetic delivery, with peaks reached either low concentration and high duration or vice versa.

Although our in-hand data should proceed with further confirmation and further evaluation on other cell lines, our initial findings provide promising conditions for magnetofection, particularly for adherent cells. Based on the data we obtained ultimately, we suggest a potential process regarding magnetofection with naked DNA, as shown in Figure 5.

DISCUSSION

In this study, we attempted to establish a simple protocol to enable the delivery of naked plasmid DNA with the MNPs. We succeeded in establishing an economical and simple procedure for gene transfer by utilizing MNPs although the efficacy is evaluated on adherent cells, HEK293T cells particularly. The protocol should have been tested on other cell types to give a further evaluation on the establishment system.

On the other hand, the efficacy of magnetofection is tightly controlled to magnetic force between MNPs and magnetic plates. We could not control to the maximum extent was the magnetic plate. As was pointed out by Plank *et al.*[5], magnetic plates for magnetofection could be home-made with strong permanent magnets. However, the complete regulation of how strong the gradient field has to beis left unclear in terms of physical force. To add up, the fields of individual magnets nearby will influence each other, making it difficult to achieve an even distribution of magnetically sedimented vectors on the cells. We did achieve positive results; however, with NaCl as the only buffer, this proves the magnetic plate in use had effects. With that being said, using the commercially available product can be much potential to achieve a better result.

In previous researches, Calcium chloride ($CaCl_2$) was proven to be a key element in Calcium phosphate transfection – a commercially available protocol widely offered by many big brands such as ThermoFisher Scientific, with relatively low cost and low toxicity[12,13]. Furthermore, $CaCl_2$ also appeared to have the ability to enhance lipofection in very recent research[9]. Moreover, the MNP we had in hand are silica-coated, and Guanidinium chloride (GuHCl) has been used as a binding inducing factor for plasmid DNA and silica material in DNA separation silica adsorption, albeit lack of concrete confirmation[14]. Taken together, we hypothesize that $CaCl_2$ and GuHCl can affect the forming of magnetofectin,

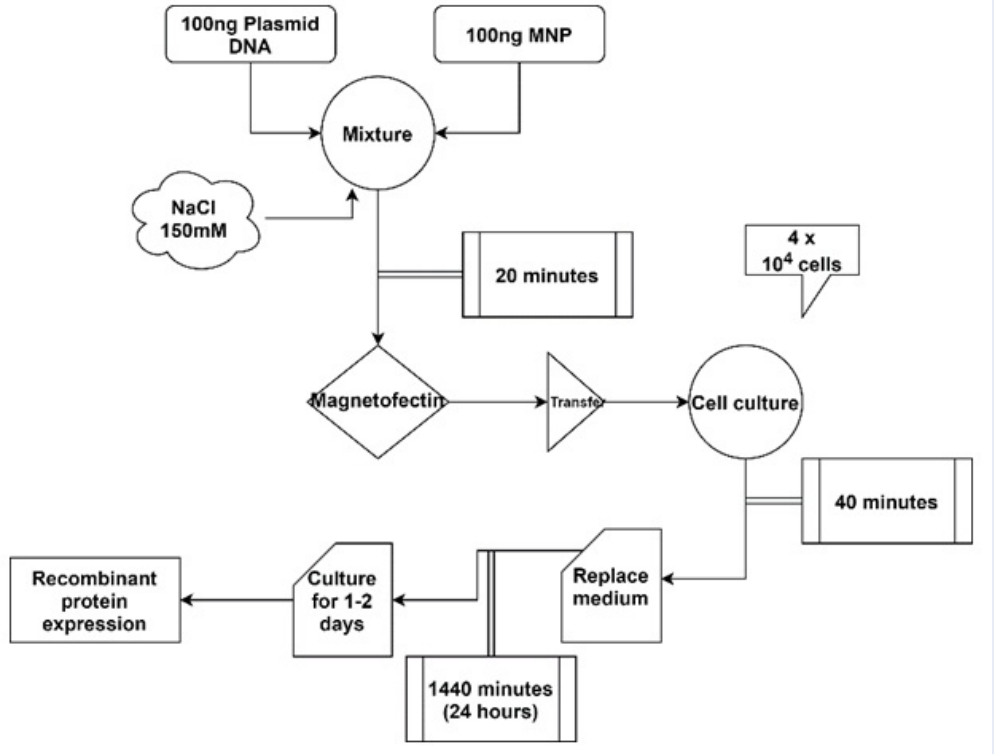

Figure 5: The recommended procedure for magnetofection of adherent cells.

as well as enhance the subsequent delivery of plasmid. For further investigation, we will focus on the effect of the two salts on magnetofection.

CONCLUSIONS

We can conclude that our homemade *in vitro* magnetic nanoparticle-based gene delivery system is established. Magnetofection without liposomes or polymersomes is certainly possible. The optimal condition in NaCl treatment was 150mM with a 40-minute duration.

LIST OF ABBREVIATIONS

$CaCl_2$ Calcium chloride
GuHCl Guanidinium chloride
hKO Humanised Kusabira Orange
MNPs Magnetic Nanoparticles
MNP@SiO_2 Silica-coated iron oxide magnetic nanoparticles
NaCl Sodium chloride

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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AUTHORS CONTRIBUTION

Le Minh Thong suggested study idea. Tran Thi Hai Yen and Pham Tuan Huy contributed to the study conception and design. Tran Thi Hai Yen, Pham Tuan Huy, Nguyen Pham Quynh Anh and Tong Thi Hang performed research, collected, and analyzed data. The first draft of the manuscript was written by Pham Tuan Huy, reviewed, and edited by Tran Thi Hai Yen. Nguyen Hoang Khue Tu, Le Minh Thong and Nguyen Van Thuan commented on the previous version of manuscript. All authors read and approved the final manuscript.

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