Magnetic immobilisation as a promising approach against bacteriophage infection

Seyedeh-Masoumeh Taghizadeh¹, Shima Jafari¹, Tahereh Ahmad-Kiadaliri¹, Mohamad Ali Mobasher¹, Neha Lal¹, Mohammad Javad Raei¹, Aydin Berenjian³, Younes Ghasemi¹ and Alireza Ebrahimimizhad²

¹ Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
² Department of Medical Nanotechnology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran
³ School of Engineering, Faculty of Science and Engineering, University of Waikato, Hamilton 3240, New Zealand

E-mail: a_ebrahimi@sums.ac.ir

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Abstract

Bacteriophage infection is a major concern in industrial fermentation, as phage infections can lyse industrially valuable cells, which consequently disturbs the production process. In the present study, L-lysine functionalised magnetic nanoparticles were synthesised through a co-precipitation reaction. The physicochemical properties of the prepared particles were evaluated using several methods such as scanning electron microscopy (SEM), x-ray powder diffractometry (XRD), Fourier transform infrared (FTIR) spectroscopy, and a vibrating sample magnetometer (VSM). Escherichia coli was used as a prokaryotic model, and the cells were decorated with magnetic nanoparticles in various concentrations. The resistance of the magnetically immobilised cells to phage infection was investigated using a double-layer plaque assay, against a head and tail phage. It was observed that magnetic nanoparticles provided a concentration dependent protection against phage infection. An increase in the magnetic nanoparticle concentration up to 250 μg ml⁻¹ resulted in a significant reduction in the incidence of phage infection.

1. Introduction

Magnetic nanoparticles are one of the most applied nanostructures. As a result of their unique physicochemical properties, these particles have several uses in various fields of science and technology [1–6]. The ability to respond to a magnetic field is a unique property, which enables magnetic nanoparticles to be employed in a vast range of biological and biomedical applications such as magnetic resonance imaging (MRI), hyperthermia treatments, drug and gene delivery, immunoassays, enzyme and protein immobilisation, cell labelling, cell separation, cell therapy, and cell immobilisation [7–18]. These particles also have a wide range of applications in the industrial setting and in environmental remediation [19–23]. Recently, magnetic nanoparticles have been used for cell separation and immobilisation in biotechnological processes, and this technique is commonly referred to as magnetic immobilisation [24, 25]. This approach is based on non-specific interactions (such as hydrophobic, electrostatic, and van der Waals forces) of magnetic nanoparticles with the microbial cell wall. Decoration of bacterial cells with magnetic nanoparticles enables them to respond to a magnetic field, thus an external magnetic field could potentially be used to harvest and separate these cells (as illustrated in figure 1).

Decoration of cells with magnetic nanoparticles can also increase the selective permeability of the cytoplasmic membrane, which consequently increases the rate of mass transfer, and thus the metabolic yield [26]. Magnetic immobilisation has also been shown to increase the specific yield of biotechnological products, which provides another interesting advantage for biotechnological processes [24, 25, 27, 28]. Biofilm formation is one of the major concerns in biotechnological industries, as it results in the corrosion of equipment, intensive periodic
cleaning, steady contamination, and high survival competence. It has also been demonstrated that magnetic immobilisation can significantly reduce the total biofilm biomass without affecting the cell viability [29].

Bacteriophage (phage) infection is another concern in industrial biotechnology processes. A phage is a virus that infects and replicates within the cells of prokaryotic bacteria and archaea. These viruses are often found in environments populated with bacterial cells [30]. The occurrence of a phage infection during a fermentation process results in the complete or partial lysis of the industrially valuable cells, which disturbs the production process and reduces the product yield. Currently, several measures have been introduced to eliminate or reduce the risk of phage propagation in fermentation processes, these include the implementation of phage resistant strains, phage-protected sterile operation, alternative cultures, the use of phage inhibitors, and cell immobilisation [31]. Thus, a suitable immobilisation technique is likely to provide protection against phage infection. Consequently, it is necessary to evaluate the various cell and magnetic immobilisation techniques to determine the most favourable option.

Recent experiments have shown that iron-based nanostructures can influence the rate of phage infections. For example, bacterial cells treated with iron-doped apatite nanoparticles prior to phage exposure resulted in increased phage infection and bacterial death [32, 33]. Whereas, another study has demonstrated the ability of iron nanoparticles to reduce the incidence of phage infection. It has also been determined that iron ions released from iron nanoparticles can inactivate bacteriophages, and the direct interaction of phage particles with iron nanoparticles results in the destruction of phage capsids, leading to their inactivation [34]. Therefore, due to the inconsistency in the literature, the aim of this investigation was to determine the impact of magnetic immobilisation on the incidence of phage infection, using *E. coli* as a bacterial cell model.

2. Materials and methods

2.1. Materials
Chemicals including iron salts (FeSO₄·4H₂O and FeCl₃·6H₂O), CaCl₂·2H₂O, MgSO₄·7H₂O, ammonium hydroxide, and L-lysine monohydrochloride were purchased from Merck (Darmstadt, Hessen, Germany), and used without any further treatment. Luria-Bertani media and agar-agar were purchased from Himedia (Mumbai, India). The Whatman 6752–2504 Nylon Puradisc 25 Syringe Filter, 0.45 Micron and Whatman GD/X Sterile Syringe Filter, 25 mm, 0.2 Micron, PVDF Filtration Medium were purchased from Whatman Ltd (Whatman, Maidstone, Kent, UK).

2.2. Synthesis of magnetic nanoparticles
L-lysine caped magnetic nanoparticles were synthesised through the co-precipitation of ferrous and ferric ions in the presence of L-lysine [35, 36]. The reaction was conducted in an aqueous nitrogen protected environment. As part of this process, 0.6 g of ferrous sulphate tetrahydrate (FeSO₄·4H₂O) and 1.17 g of ferric chloride hexahydrate (FeCl₃·6H₂O) were dissolved in deionised water. The L-lysine solution (5 ml, 32%) was added after 30 min while stirring at 70 °C, and stirring was continued for another 30 min. The reaction was initiated by the addition of 6.5 ml ammonium hydroxide (25%) to the solution, and the reaction progress was monitored for two hours. The dark black precipitate formed from the reaction was separated and washed three times with hot water, before drying in an oven at 50 °C.
2.3. Characterisation of nanoparticles

The visual appearance of the prepared nanoparticles was evaluated using scanning electron microscopy (SEM, MIRA3 TESCAN, HV: 15.0 kV). Particle size analysis was done using ImageJ software version 1.47v, an image analysis software developed by the NIH [http://imagej.nih.gov/ij/][1, 37]. The crystal structure of the nanoparticles was analysed through x-ray powder diffraclometry (XRD, Siemens D5000) using a fine powder of prepared nanoparticles. Fourier transform infrared (FTIR, Bruker, Vertex 70) spectroscopy via KBr pellets was used to evaluate the chemical structure and functional groups present on the iron nanoparticles. The magnetic properties of the particles were measured at room temperature using a vibrating sample magnetometer (VSM) [38].

2.4. Bacteriophage isolation and purification

The aim of investigation was to provide basic data, thus E. coli was used as a model organism, as it is a well-known bacterial cell, and is susceptible to infection by several types of phage particles. Wastewater was used as a source of bacteriophages [39]. Wastewater samples were centrifuged (5000 rpm, 5 min) to remove dust and large particles. The supernatant was first filtered through a Whatman 6752–2504 Nylon Puradisc 25 Syringe Filter, 0.45 Micron and then through a Whatman GD/X Sterile Syringe Filter, 25 mm, 0.2 Micron, PVDF Filtration Medium to eliminate all microbial cells. The filtered sample (10 ml) was mixed with an equal volume of E. coli cells cultured in Luria–Bertani (LB) broth with a 0.5 McFarland turbidity. The mixture was then inoculated in 100 ml of LB broth supplemented with 0.1 M calcium chloride (CaCl2·2H2O, Merck, 102382) and 0.1 M magnesium sulphate (MgSO4·7H2O, Merck, 105886). The broth was incubated at 37 °C and 150 rpm in a shaker incubator. After an incubation period of 16 h, the broth was centrifuged (5000 rpm, 5 min) and the supernatant was filtered through a 0.45 and a 0.2 μm syringe filter. The filtered fluid obtained was a concentrated phage suspension, hence a serial dilution (up to 10^-8) was performed to achieve an appropriate phage concentration.

Single phage plaques were obtained using the double-layer plaque technique. As part of this procedure, 200 μl of each phage serial dilution was mixed with 500 μl of fresh bacterial culture. The samples were then incubated at 37 °C for 20 min, before mixing with soft agar (0.7% agar in LB medium supplemented with 0.1 M magnesium sulphate). The mixture was poured onto previously prepared solid agar plates (1.5% agar in LB) and incubated at 37 °C. Phage plaques were observed on the surface of the plates after 18 h. A single plaque was removed using a Pasteur pipette from both the superficial and deep agar layers. The plaque was dissolved in a suspention medium (SM) solution (1.5 ml) and mixed with 500 ml of LB broth. The solution was then inoculated with 10 ml of fresh bacterial culture and incubated at 37 °C. After 18 h, the mixture was centrifuged at 5000 rpm for 7 min and the supernatant was passed through a 0.45 and a 0.22 μm filter. This process was repeated four times to ensure that the single plaques obtained were adequately purified [39].

2.5. Phage visualisation and identification

A polyethylene glycol (PEG 6000) solution (40% w/v) was used to harvest and concentrate the purified phage particles. The PEG solution was mixed with the phage suspension (1:4) and left on ice for 1 h. The mixture was then centrifuged (9000 rpm for 15 min at 4 °C) and the pellet obtained was suspended in SM solution [39]. A drop of the concentrated phage suspension was placed on a carbon coated copper grid and dried at ambient temperature. The grid was stained with uranyl acetate (1%) and the negatively stained phage particles were visualised using TEM microscopy (Philips, CM-10) [39]. The isolated phage particles were identified based on their morphology and structure [40, 41].

2.6. Magnetic immobilisation of bacterial cells

The procedures outlined in previous reports were implemented to magnetically immobilise the bacterial cells [27]. E. coli cells were harvested in the logarithmic phase of growth and washed three times with saline. The bacterial cells were then dispersed in saline (0.5 McFarland) and mixed with an equal volume of the magnetic nanoparticle suspension in various concentrations (ranging from 0 to 2 mg ml^-1). The mixture was then incubated in a shaker incubator (150 rpm) for 15 min at 37 °C, to aid the attachment of nanoparticles to the surface of the bacterial cells. A scanning electron microscope was used to visualise the bacterial cells decorated with the magnetic nanoparticles [24]. The immobilised cells were then exposed to a magnetic field (250 gauss) and the rate of cell harvesting (immobilisation efficiency) was recorded over time, based on equation (1).

\[
E_i = \frac{CFU_{t_0} - CFU_{t_1}}{CFU_{t_0}} \times 100
\]

Where \(E_i\) is the immobilisation efficiency, \(CFU_{t_0}\) is the initial colony forming unit, and \(CFU_{t_1}\) is the colony forming units of non-harvested cells after applying the magnetic field for a certain period of time.
2.7. Effect of magnetic immobilisation on phage infection

The infectivity of the phage particles, with respect to the magnetically immobilised bacterial cells was evaluated using a double-layer plaque assay [39]. As part of this procedure, 200 μl of the purified phage suspension with a concentration of approximately 250 plaque forming units (PFU)/ml, was mixed with 500 μl of bacterial suspension immobilised using various concentrations of magnetic nanoparticles. The suspension was incubated at 37 °C for 20 min, before mixing with soft agar (0.7% agar in Luria Bertani medium supplemented with 0.1 M magnesium sulphate). The mixture was then poured onto previously prepared solid agar plates (1.5% agar in Luria Bertani) and incubated at 37 °C. The phage plaques were counted after an incubation period of 18 h. The experiments were performed in triplicates and the data was reported as mean ± standard deviation (SD). The data was evaluated statistically using the analysis of variance (ANOVA) test and significance was accepted at p < 0.05.

3. Results

3.1. Synthesis and characterisation of magnetic nanoparticles

In a typical co-precipitation reaction, magnetite cores are formed at a pH of 9–11 using ammonia solution. A sudden colour change from light yellow-orange to dark brown or black is observed as the reaction proceeds, following ammonium hydroxide injection. Nanoparticles were produced from magnetite cores using the co-precipitation reaction, which proceeded for two hours. The prepared particles were visualised using SEM analysis, as shown in figure 2(a). The particles were found to have a uniform spherical shape and a size ranging from 8-24 nm.
from 8 nm to 22 nm, with a mean particle size of 14 nm (figure 2(b)) [42]. The crystal structure of the particles was evaluated using XRD analysis with 2 theta angles in the range of 10 to 90 degrees. The characteristic features of the magnetite crystals were recorded with intensity peaks expressed at 30, 35.5, 43, 57, and 63 degrees (figure 3(a)). The FTIR spectra of the particles are depicted in figure 3(b). Iron oxide nanoparticles produce a characteristic peak at about 630 cm$^{-1}$, which is due to the Fe–O bonds, and is usually split into two peaks [43, 44]. Since the co-precipitation reaction is conducted in an aqueous medium, unsaturated Fe atoms on the surface of the synthesised nanoparticles interact with hydroxyl ions or water molecules. The stretching vibration of this OH group produces a peak at 3390.0 cm$^{-1}$, which overlaps with the IR absorption of amine groups in the L-lysine coating. The OH deforming vibration at 1621.6 cm$^{-1}$ also overlaps with the C=O group from L-lysine. The peak at 1398.8 cm$^{-1}$ is due to the presence of C–O groups in L-lysine. The small peak intensity may also represent the interaction between L-lysine and the hydroxyl groups on the nanoparticles surface [45]. The magnetic response of the nanoparticles was evaluated at room temperature, and the results are provided in figure 4. Based on the results, the prepared particles exhibit superparamagnetic behaviour with no hysteresis or remnant.
3.2. Isolation and identification of phage particles
The appearance of the bacteriophage plaques on the surface of the LB agar plates is illustrated in figure 5(a). A micrograph of the isolated and purified phage particle is shown in figure 5(b). Evaluation of the phage morphology indicated that the purified phage was a tailed phage. The phage morphology comprises an icosahedral capsid that is 75 nm in length with a long, flexible, and non-contractile tail that has a length and width of about 290 and 15 nm, respectively. These features enabled the classification of the isolated phage in the order *Caudovirales* and the family *Siphoviridae* [40, 46].

3.3. Magnetic immobilisation of bacterial cells
As depicted in figure 6, the exposure of the bacterial cells to the magnetic nanoparticles resulted in the successful decoration of the cell surface. The attachment of the magnetic nanoparticles to the bacterial cell is understood to occur through non-specific interactions [4, 26, 47–50]. The response of the immobilised cells to the magnetic field was observed to be dependent on the concentration of nanoparticles used for cell immobilisation and the time of exposure to the magnetic field. Bacterial cells that were immobilised with 250 and 500 μg ml⁻¹ of nanoparticles were completely harvested (immobilisation efficiency was calculated to be 100%), following exposure to the magnetic field for 15 and 5 min, respectively. The strength of the applied magnetic field is likely to be another important parameter in determining the rate and efficiency of cell removal. As previously outlined in the experimental procedure, a neodymium magnet with a gauss rating of 250 was used in this investigation.

3.4. Effect of magnetic immobilisation on the phage infection rate
The bacterial cells were exposed to various concentrations of magnetic nanoparticles to evaluate the ability of magnetic immobilisation to protect against phage infection. As depicted in figure 7, the exposure of bacterial
cells to the magnetic nanoparticles (30 $\mu$g ml$^{-1}$), even at low concentrations, provided some protection against phage infection. An increase in the magnetic nanoparticle concentration up to 125 $\mu$g ml$^{-1}$ resulted in a significant reduction in the phage infectivity. However, a further increase in the nanoparticle concentration (above 250 $\mu$g ml$^{-1}$) did not provide any additional protection against phage infection. It must also be noted that high concentrations of magnetic nanoparticles have a negative effect on bacterial cell viability, therefore moderate concentrations are preferred for magnetic immobilisation purposes [24, 25, 27]. Interestingly, 250 $\mu$g ml$^{-1}$ of nanoparticles is sufficient to provide considerable protection against phage infection.

4. Discussion

The ability of magnetic nanoparticles to decorate microbial cells has been exploited in various applications such as to increase the metabolic capacity of microbial cells [26], water decontamination [49], and more recently for the magnetic immobilisation of cells in biotechnological processes [24]. These particles can also reduce the

Figure 6. E. coli cells before (a) and after (b) the exposure to magnetic nanoparticles, the nanoparticles decorate the cell surface through nonspecific forces.
pathogenicity of microbial cells. It has been shown that the decoration of pathogenic bacteria with magnetic nanoparticles results in a significant reduction of its pathogenicity [51]. It has also been demonstrated that the decoration of *Listeria monocytogenes* with magnetic nanoparticles considerably reduces its infectivity. Magnetic nanoparticles can influence the adhesive properties of intracellular bacteria, thereby reducing their invasion power [51]. Also, the decoration of extracellular pathogens such as *Staphylococcus aureus* and *Candida albicans* with magnetic nanoparticles significantly reduces their ability to adhere to the cellular substrates of the host [52].

It is believed that magnetic nanoparticles interact with and decorate the surface of bacterial cells via nonspecific forces. These nanoparticles can act as a physical barrier, reducing the access of microbial cells to host cells and substrates. Furthermore, the surface of microbial cells are equipped with adhesion factors, which enable their attachment to various surfaces. Thus, these adhesion factors are also likely to be suitable sites for the attachment of magnetic nanoparticles [51, 52].

This investigation demonstrates that magnetic nanoparticles can act as a protective shell, which reduces the incidence of phage infection. As aforementioned, the decoration of bacterial cells with nanoparticles can protect the cells from phage infection by (a) acting as a physical barrier, thus reducing phage access to the bacterial cells, and (b) by blocking phage receptors on the surface of bacterial cells, thereby decreasing the phage infection power. Additionally, the release of iron ions from iron based nanoparticles under aerobic conditions, appeared to have virucidal activity against coliphages [34]. However, it must be appreciated that not all nanoparticles have the ability to protect against phage infections, as certain types of nanoparticles such as apatite, iron-doped apatite, silver, and zinc oxide have been shown to increase the rate of phage infection [32, 33, 53].

5. Conclusions

Overall, magnetic immobilisation was introduced as a novel approach for cell immobilisation. From the perspective of mass transfer, this revolutionary approach eliminates the issues encountered with other immobilisation techniques that attempt to combine the advantages of submerged and immobilised cell fermentation. This cell entrapment and harvesting technique also provides additional advantages for industrial processes. Magnetically immobilised cells are more metabolically efficient than free floating cells, and hence enable a greater production yield, while reducing biofilm formation. This investigation also revealed another advantage of magnetic immobilisation, as it was found that L-lysine functionalised magnetic nanoparticles were able to protect industrially valuable strains such as *E. coli* from phage infection. Hence, this technology is a promising novel approach, which offers several valuable advantages for the intensification of industrial processes.

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ORCID iDs

Alireza Ebrahiminezhad  @ https://orcid.org/0000-0002-1219-5816

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