Bacterial Inactivation by Applying an Alternating Electromagnetic Field Using PAMAM Dendron-modified Magnetic Nanoparticles

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ABSTRACT

In this study, a method involving polyamidoamine dendron-modified magnetic nanoparticles (PAMAM-MNPs) along with application of an alternating magnetic field (AMF) was developed for inactivation of bacteria in water samples. The PAMAM-MNPs efficiently bound to Escherichia coli cells, resulting in magnetic recovery of cells from aqueous solutions. By applying the AMF (5 kW, 250 kHz) to the cell suspension, E. coli cells were successfully inactivated within 10 min in the presence of the MNPs, while no effect was observed in the absence of the MNPs. The use of PAMAM-MNPs could increase the inactivation rate of E. coli under the applied AMF. E. coli cells with PAMAM-MNPs stained by propidium iodide (PI) exhibited apparent fluorescence after exposure to the AMF, suggesting the occurrence of membrane damage in the cells because of direct heat transfer from the PAMAM-MNPs. Our technique can be used to address bacterial contamination with wide varieties of microorganisms in water samples.

Keywords : Magnetic Nanoparticles (MNPs), Alternating Magnetic Field (AMF), Bacterial Inactivation, PAMAM-MNPs

1. Introduction

Bacterial cell inactivation in aqueous solutions is a crucial issue in relation to the supply of good-quality drinking water and manufacturing processes in food, cosmetic, and pharmaceutical industries.1-3 Bactericides and antibiotics are often used as bacterial inactivation agents in water although there is concern about their toxicity and effect on manufactured products.4-6 Cost-effective approaches, such as those involving ozone, ultraviolet light, or electrochemical methods, have been used because they do not lead to the formation of lethal amounts of toxic compounds in the treated water.7,8 Localized heat generation using magnetic nanoparticles (MNPs) and an alternating magnetic field (AMF) has been proposed as a novel technique for the inactivation of microorganisms.9,10

The MNPs can generate heat by transforming electromagnetic energy from an external AMF.11 The AMF heating method has advantages in remote actuation and local heating at a specific site.12 Thus, magnetic hyperthermia has been well recognized as a powerful tool for cancer therapy.9,10 Recently, these methodologies have been used for inactivation of bacteria, for example, Pseudomonas aeruginosa in biofilms13 and Staphylococcus aureus cells in mouse tissue,14 which was previously difficult to implement using conventional methods.

We previously developed polyamidoamine (PAMAM) dendron-modified MNPs as a magnetic carrier for DNA extraction; this modification provided large net positive charges on the surface.15,16 The PAMAM-MNPs can interact with negatively charged DNA molecules, which enables their use for highly efficient DNA extraction.17,18 Since the bacterial cell surface is also negatively charged, the PAMAM-MNPs can be used for cell recovery. The use of MNPs as both heating agents and magnetic carriers will expand the utility of localized heat generation by the AMF.

In this study, the PAMAM-MNPs were used as a heating agent for bacterial inactivation and as a magnetic carrier for cell recovery. Escherichia coli was used as a model microorganism. Prior to the cell inactivation, the heating capability of the MNP was investigated using different sizes of MNPs. Then, the effect of the applied AMF on cell viability was investigated to determine the effectiveness of this system in sterilization and disinfection methods used for bacterial contamination in water samples.

2. Experimental

2.1 Evaluation of cell binding of PAMAM dendron-modified MNPs

PAMAM-MNPs were prepared as described in previous studies.15,16 The amine number on the MNP surface was estimated to be $8 \times 10^5$ amines per particle, which was approximately 50% of the theoretical coverage of PAMAM-MNPs. The ability of PAMAM-MNPs to bind to E. coli (ATCC 11775) cells was evaluated as follows: PAMAM-MNPs (5 mg mL$^{-1}$) and E. coli cells ($5 \times 10^7$ CFU mL$^{-1}$) were suspended in 0.2 mL of phosphate buffered saline (PBS, pH 7.0) and incubated for 30 min. After incubation, the cells bound to the MNPs were magnetically separated. After the magnetically separated cells were re-suspended in PBS, the cells were plated and cultured in lysogeny broth (LB) at 37°C. The cells remaining in the supernatants were also plated and cultured. The cell recovery ratio by magnetic separation was calculated based on colony formation using Eq. (1):

$$\text{Cell recovery ratio} (\%) = \frac{\text{colony numbers from magnetically separated fraction}}{\text{colony numbers from supernatant}} \times 100$$

(1)

2.2 Bacterial cell inactivation by using the AMF

In order to evaluate inactivation of E. coli cells by applying the AMF, the cells ($5 \times 10^7$ CFU mL$^{-1}$) and PAMAM-MNPs (5 mg mL$^{-1}$) were mixed with PBS (0.2 mL) in a polypropylene tube and incubated for 30 min (Fig. 1a). The tube was then placed at the center of the AMF coil (Fig. 1b). The tube was immersed in a 10 mL glass vial filled with water to shield it from the heat generated because of the coil. After AMF irradiation, the cell suspensions were...
 plated on LB agar plates and incubated overnight at 37°C to estimate cell viability. A 5 kW induction heating power supply (EASYHEAT; Ameritherm Inc., Scottsville, NY) was used with a remote heating station and custom-made coils. A two-turn, 30-mm outer diameter coil resonating at 250 kHz (5 kW, 620 A) was used. During the experiments, cooling water (10–16°C) was circulated through the coil. All the experiments were performed three times. The temperature changes in the suspensions containing MNPs were monitored using a thermometer (FL-2000; Anritsu Meter Co., Ltd., Tokyo, Japan) equipped with a fiber optic sensor (FS-100; Anritsu Meter Co., Ltd., Tokyo, Japan). The cell survival ratio was evaluated based on the colony formation.

\[
\text{Survival ratio (\%)} = \frac{\text{colony numbers with AMF application}}{\text{colony numbers without AMF application}} \times 100 \tag{2}
\]

2.3 Fluorescent microscopy

LIVE/DEAD® BacLight™ Bacterial Viability Kits (Invitrogen, CA, USA) were used to evaluate damage to the cell membrane of E. coli cells. After applying the AMF for 3 min as described above, the cells were sampled from a tube and stained with SYTO 9 and propidium iodide (PI) for 15 min at room temperature. The cells were observed using a fluorescent microscope.

3. Results and Discussion

3.1 Evaluation of heating capacity of MNPs by applying AMF

The change in temperature of MNP suspensions induced by applying the AMF was monitored to estimate the heating capacities of MNPs (Fig. 2). The heat generation mechanisms for superparamagnetic and ferromagnetic MNPs are different, and are mainly derived from relaxation and hysteresis losses for superparamagnetic and ferromagnetic MNPs, respectively.19 Thus, we investigated superparamagnetic, ferromagnetic single-domain, and ferromagnetic multi-domain MNPs with sizes of 10, 40, and 250 nm, respectively.

![Figure 2. Time course of temperature in MNP suspension on applying the AMF. MNP concentration: 5 mg mL\(^{-1}\).](image2)

The temperatures increased with time and reached plateaus after 10 min, at approximately 43°C for 10 nm, 73°C for 40 nm, and 52°C for 230 nm. The MNPs having a diameter of 40 nm were the most effective agent for heat generation. The temperature increase for ferromagnetic MNPs corresponded to their coercivities (9.5 kA m\(^{-1}\) for 40 nm and 4.6 kA m\(^{-1}\) for 250 nm), suggesting that the observed differences in the temperature increase were mainly derived from the hysteresis losses of MNPs.19 Thus, the MNPs having a diameter of 40 nm were used for the subsequent investigations.

3.2 Evaluation of electrostatic binding of PAMAM-MNPs to E. coli cells

PAMAM-MNPs (40 nm diameter) were mixed with the cell suspension to estimate the binding ability to E. coli cells. Since bacterial cell concentrations in drinking waters are observed in the range of 10\(^3\) to 10\(^5\) CFU mL\(^{-1}\),20,21 we used E. coli cell at the concentration of 5 x 10\(^3\) CFU mL\(^{-1}\); MNP concentration: 5 mg mL\(^{-1}\). The amount corresponds to 2.9 x 10\(^12\) particles, which is sufficient against the cell number in the solution. After 30 min incubation, the MNPs attaching to E. coli cell surface was observed for both non-treated MNPs and PAMAM-MNPs by TEM analysis (Fig. 3). To evaluate the binding efficiency, the cell recovery ratio from the suspension (PBS, pH 7.0) by magnetic separation was measured and compared. The recovery ratios were 94.4 ± 2.8% for PAMAM-MNPs and 4.2% ± 1.4% for non-treated MNPs at pH 7.0. Furthermore, efficient cell recovery using the PAMAM-MNPs was observed between pH 5.0 and pH 8.0. This phenomenon was in good agreement with the surface charges of the PAMAM-MNPs; i.e., PAMAM-MNPs are positively charged at pH 5.0 to 8.0, almost neutral at pH 9.0, and negatively charged at pH 10.0.22 The surfaces of majority of bacterial cells including E. coli are negatively charged.

![Figure 3. Transmission electron micrographs of E. coli cells after mixing with non-treated MNPs (a) or PAMAM-MNPs (b) for 30 min. Cell concentration: 5 x 10\(^3\) CFU mL\(^{-1}\); MNP concentration: 5 mg mL\(^{-1}\).](image3)
3.3 Inactivation of \textit{E. coli} cells by applying AMF

Inactivation of \textit{E. coli} cells was investigated by applying the AMF in the presence of MNPs. The survival ratios decreased according to the application time of the AMF. The decrease in survival ratios in the case of non-treated MNPs could be due to the increase in temperature of aqueous solutions. After an application time of 3 min, the survival ratios were 77.0\% for non-treated MNPs and 21.6\% for PAMAM-MNPs (Fig. 4). The survival ratios for both non-treated MNPs and PAMAM-MNPs decreased to zero after 10-min application time of the AMF. There were clear differences between the survival ratios for non-treated and PAMAM-MNPs. These differences indicate that the tight binding of MNPs with the cells enables efficient heat transfer from the MNPs to the bacterial cells, thereby causing significant damage to these cells. Inactivation of \textit{E. coli} cells was not observed in the absence of MNPs. Thus, the observed bacterial inactivation was attributable to the localized heat generated from the MNPs by applying the AMF.

3.4 Microscopic analysis of membrane damage of \textit{E. coli} cells

To examine the membrane damage to \textit{E. coli}, the cells were stained with PI after treatment with the AMF and PAMAM-MNPs. One of the main causes of cell inactivation is commonly acknowledged to be membrane damage,\textsuperscript{8,13} damage to the cell membrane has been evaluated to observe the increased uptake of PI into the cells. All the cells were visualized by SYTO 9 staining (Fig. 5a and 5b). \textit{E. coli} cells stained by PI exhibited no fluorescence before exposure to the AMF (Fig. 5c), while most of the cells stained with PI exhibited apparent fluorescence after exposure to the AMF (Fig. 5d). The damage to the cell membrane could be attributable to the effect of localized heating of cells on PAMAM-MNPs. These results indicate that the membrane damage induced by the AMF treatment is the main cause of inactivation of \textit{E. coli} cells.

4. Conclusions

This study demonstrated efficient cell inactivation by localized heat generation on the PAPAM-MNPs by applying the AMF. The

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure.png}
\caption{Time course of the survival ratios of \textit{E. coli} on applying the AMF using MNPs. Cell concentration: 5 \times 10^3 CFU mL\textsuperscript{-1}; MNP concentration: 5 mg mL\textsuperscript{-1}. AMF: 5 kW (250 kHz).}
\end{figure}

\begin{figure}[h]
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\caption{(Color online) Fluorescent microscopic images of \textit{E. coli} cells in the presence of the PAMAM-MNPs. Before (a, c) and after (b, d) applying AMF (5 kW, 250 kHz). The cells were stained with SYTO 9 (a, b) and PI (c, d).}
\end{figure}

PAMAM-MNPs efficiently bound to \textit{E. coli} cells, and the cells were recovered from aqueous suspensions. The use of the PAMAM-MNPs could increase the inactivation rate of \textit{E. coli} under the applied AMF. The membrane damage could be the main cause of the inactivation of \textit{E. coli} cells. Since our proposed technique enables removal of bacterial cells from aqueous solutions and their inactivation using MNPs, it is proposed to be a novel sterilization or disinfection method for addressing contamination with wide varieties of microorganisms in water samples.

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