The effect of γ-Fe$_2$O$_3$ nanoparticles on *Escherichia coli* genome

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**Abstract**

Extensive production and application of γ-Fe$_2$O$_3$ magnetic nanoparticles (MNPs) has increased their potential risk on environment and human health. This report illustrates a genetic impact of γ-Fe$_2$O$_3$ magnetic nanoparticles (MNPs) on *Escherichia coli* (*E. coli*). After 3000-generation incubation with MNPs addition, obvious genomic variations were revealed by using repetitive extragenic palindromic PCR (rep-PCR) DNA fingerprint technique. The physicochemical interactions between MNPs and bacteria could be responsible for such genomic responses. It was revealed that Fe$^{3+}$ concentration increased in the medium. Transmission electronic microscopy (TEM) and flow cytometry (FCM) analysis consistently demonstrated the occurrences of adsorption and membranes-internalization of MNPs outside and inside cells. Both increased Fe$^{3+}$ ion and the uptake of MNPs facilitated Fe binding with proteins and DNA strands, resulting in enhancing the mutation frequency of *E. coli*. Our results would be of great help to assessing the potential impact of MNPs on human and environment.

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1. Introduction

The iron oxide magnetic nanoparticles (MNPs) are one of the most widely studied nanomaterials. Due to their novel properties, such as enhanced surface-to-volume ratio, superparamagnetism and inherent biocompatibility (Perez et al., 2002; Sjogren et al., 1997), MNPs are being widely used for various biomedical and environmental applications such as magnetic resonance imaging (MRI), targeted delivery of drugs, targeted destruction of tumor tissue, magnetic bioseparations and pollution remediation etc (Kawashita et al., 2005; Prashant et al., 2010; Song et al., 2006; Telling et al., 2009; Veiseh et al., 2005; Wang and Irudayaraj, 2010). With continually increased applications, the exposure of such nanoparticles to human and environment is inevitable through the intentional releases as well as the unintentional discharges from production facilities at the end of their life circles (Lee et al., 2010). The exposure ultimately affects the health of ecosystem and human population. Although the potential benefits of MNPs are fully considerable, there is a distinct need to identify any potential negative responses of ecosystem and human. Unfortunately, the safety of MNPs is not clearly elucidated yet. Most investigations are focused on studying effects of MNPs on cellular viability, morphology and metabolism (Auffan et al., 2008; Xia et al., 2006). We reasoned that these physiological changes are indeed extrinsic exhibitions of genomic mutations induced by nanoparticles. However, thorough studies about their long-term impact on genomes are very limited.

Herein, we examined *Escherichia coli* (*E. coli*) genomic responses to γ-Fe$_2$O$_3$ MNPs at the molecular level by repetitive extragenic palindromic PCR (rep-PCR). The rep-PCR DNA fingerprinting technique has proven to be a valuable tool to identify, track and examine genotypic diversity among medically and environmentally important microorganisms (Ishii and Sadowsky, 2009). It is a simple and rapid method that has the necessary resolving power for microbial identification at subspecies or strain level. *E. coli*, a Gram negative rod-shaped bacterium, is one of the best-studied prokaryotic model organisms. It is commonly found in the environment and lower intestine of human. Most *E. coli* strains do not cause disease under normal circumstances but participate in maintaining human health, however, certain strains of *E. coli* mutated can produce potentially lethal toxins. Although, the interaction of metal oxide nanoparticles with bacteria has already been demonstrated (Auffan et al., 2008; Brayner et al., 2006; Dehner et al., 2011; Kasemets et al., 2009; Pelletier et al., 2010; Stoiimenov et al., 2002), very few studies have been done to assess the mutagenic potential impact of nanoparticles on bacteria. Thus, it is important and urgent to study the effect of MNPs on *E. coli* genome, because genetic changes in *E. coli* may eventually induce some negative impacts on environment and human health. The impact of bulk (micro-scale) iron oxide particles was also assessed for comparative purposes.
2. Materials and methods

2.1. Chemicals and materials

FeCl₃·6H₂O, FeSO₄·7H₂O and (CH₃)₂NOH were obtained from Chemical Reagents Research Institute (Shanghai, China). Meso-2, 3-dimercaptopropanesulfonic acid (DMSA) was from Sigma. Bacterial DNA kit was from Omega. DNA polymerase was from TaKaRa. Other reagents and chemicals were at least analytical reagent grade. Micro-sized iron oxide particles were purchased from Shanghai Demo Chemical Technology Co., China. Standard strain of Micrococcus luteus ATCC 25922 was used as test microorganism in this study. The bacteria were grown aerobically in a vial at 37 °C with shaking (200 rpm) in Luria-Bertani nutrient medium (LB) (5 g L⁻¹ NaCl, 10 g L⁻¹ tryptone powder, and 5 g L⁻¹ beef extract powder, pH 7.2). The iron oxide nanoparticles were administered in the LB nutrient medium at exposure concentration of 0.1 mg/ml (Chen et al., 2011; Gu et al., 2009). E. coli strain subcultured every 12–16 h until 3000 generations. The extracted DNA was eluted and stored at −80 °C until it was analyzed. Equal amount of DNA samples (ca. 20 ng/µl) were subjected to rep-PCR amplification using primer BOX A1R (5’-CTACGGCAAGGCGACGCTGACG-3’) which is less than 0.05.

2.2. Synthesis of γ-Fe₂O₃ MNPs

Iron oxide nanoparticles were synthesized by chemical coprecipitation of Molybdenum. Typically, a solution of FeCl₃ and FeSO₄ mixture (molar ratio 2:1) was prepared under N₂ protecting, followed by the slow addition of enough ammonia aqueous solution with vigorous stirring for 30 min. The black Fe₃O₄ precipitates were obtained and washed immediately with distilled water for five times by magnetic separation. The final precipitates were dispersed in distilled water with concentration of 0.128 M and pH 3.0, and oxidized into more stable γ-Fe₂O₃ by air at the temperature of 90 °C. During this step, the initial black slurry turning into brown could be observed. The prepared γ-Fe₂O₃ nanoparticles were dispersed in distilled water, and the pH of the solution was adjusted to 2.7. Then, DMSA dissolved in DMSO was added to the dispersion with continuous stirring. After the reaction for 5 h at room temperature, the products were collected with a magnet and were dispersed in (CH₃)₂NOH solution, and the pH of the solution was adjusted to 10. The stable magnetic fluids were obtained after the pH of the solution was adjusted to neutral. Coated nanoparticles were washed for five times with distilled water by magnetic separation to remove chemical residues. Then MNPs were resuspended in distilled water and sterilized through a 0.22 µm sterilized filter and stored at 4 °C for use.

2.3. Bacteria strain and incubation with particles

E. coli strain ATCC 25922 was used as test microorganism in this study. The bacteria were grown aerobically in a vial at 37 °C with shaking (200 rpm) in Luria-Bertani nutrient medium (LB) (5 g L⁻¹ NaCl, 10 g L⁻¹ tryptone powder, and 5 g L⁻¹ beef extract powder, pH 7.2). The γ-Fe₂O₃ nanoparticles were administered in the LB nutrient medium at exposure concentration of 0.1 mg/ml (Chen et al., 2011; Gu et al., 2009). E. coli subcultured every 12–16 h until 3000 generations. The extracted DNA was eluted and stored at −80 °C until it was analyzed. Equal amount of DNA samples (ca. 20 ng/µl) were subjected to rep-PCR amplification using primer BOX A1R (5’-CTACGGCAAGGCGACGCTGACG-3’) which is less than 0.05.

2.4. DNA extraction and rep-PCR amplification

Genomic DNA was subsequently extracted using bacterial DNA kit (Omega), following the specifications of the manufacturer. The extracted DNA was eluted and stored at −80 °C until it was analyzed. Equal amount of DNA samples (ca. 20 ng/µl) were subjected to rep-PCR amplification using primer BOX A1R (5’-CTACGGCAAGGCGACGCTGACG-3’). PCR was carried out in 25-µl reaction mixtures containing 1× Taq DNA polymerase (TaKaRa, Japan), and 50 ng of DNA. The temperature profile was as follows: initial denaturation at 95 °C for 2 min; 35 cycles of 94 °C for 3 s, 52 °C for 30 s, 72 °C for 1 min, and 65 °C for 8 min; and final extension at 65 °C for 8 min. PCR products were separated by electrophoresis on 1.5% agarose gels at 70 V and 4 °C for 19 h. After the agarose gels were stained with ethidium bromide, images of the gels were digitized by using a Gel Doc™ EQ imager combined with Quantity one 4.4.0 (Bio-Rad). Cluster analysis of similarity matrices was performed by an unweighted pair group using mathematical averages (UPGMA).

2.5. Iron ion concentration measurement in suspension

The solubility of MNPs in the LB medium was quantified. The particles suspension was centrifuged at 10,000 g for 10 min. The supernatant was carefully collected and filtered through a 0.22 µm filter. The iron ion concentration was measured via an inductively coupled plasma optical emission spectrometer (ICP-OES).

2.6. Characterization of MNPs

The size and morphology of MNPs were determined by transmission electronic microscopy (TEM) (JEDOL, JEM-2000Ex). 5 µl of the MNPs solution was placed onto the carbon-coated copper grids and allowing the solvent to evaporate in the air. MNPs diameter distribution was determined with image software by analyzing more than 400 particles. Zeta potential (ζ) of MNPs in LB medium was performed with a zeta potential analyzer (BECKMAN, Delta 440DX).

2.7. Ultrathin section electron microscopy measurement

Characterization of the interaction of MNPs with bacteria was also done. Thin sections of samples embedded in a resin were prepared for TEM measurement. Control and MNPs-treated bacterial samples were fixed for 3 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After washing with cacodylate buffer, they were further fixed with 1% osmic acid for 2 h and washed with PBS for three times. The cells were then dehydrated through graded ethanol solutions with 30, 50, 70, 90, 95, 100% (v/v in water), and 50% ethanol in acetone for 10 min each time. The samples were finally dehydrated with pure acetone twice for 15 min. Ultrathin sections of 60–80 nm were cut using an ultramicrotome and mounted on a carbon-coated copper TEM grid. The sections were stained with 2% uranyl acetate in distilled water for 10 min and lead staining solution for 5 min prior to TEM analysis. The surface topography of E. coli and MNPs-treated E. coli were by scanning electron microscopy (SEM, JEDOL, JSM-5610LV).

2.8. Detection of nanoparticles uptake through flow cytometry

Flow cytometry (FCM) was also performed for analyzing the uptake of MNPs in the cells. Generally, it is considered that the side scatter (SSC) provides information on internal structures and organelles and the forward scatter (FSC) reflects the size of a cell. We demonstrated here the effects that MNPs interacting with cells on SSC and FSC signal obtained by flow cytometry at 488 nm argon-ion laser (BD FACSCa-libur, BD Biosciences, San Jose, CA, USA). For FCM detection, cells were thoroughly washed with PBS to remove nanoparticles, harvested and prepared into single cell suspension, and then loaded to flow cytometry. At least 10⁵ cells were tested for each sample. Data analysis, the value of SSC intensity was divided into four regions: M1 (0–200), M2 (200–400), M3 (400–600), and M4 (600–1000). Data were analyzed using CellQuest™ software.

2.9. Toxicity assessment

The experiments were designed to check whether the MNPs are able to alter cell growth. The concentration of E. coli grown with and without MNPs after incubation for a certain time was determined using UV–vis spectroscopy at 600 nm. Each treatment was conducted with three replicates, and the results were presented as mean SD (standard deviation). The statistical assessment of experimental data utilized the Student’s t-test. Each of the experimental values was compared to its corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) is less than 0.05.

3. Results and discussion

3.1. MNPs induced changes of E. coli genotypes

Fig. 1a shows a representative TEM of the MNPs generated by coprecipitation. The particles were quasi-spherical in shape. The statistical analysis of MNPs diameters indicates the size distribution of particles and that the main were around 10 nm (Fig. 1b). It was also shown that nanoparticles modified by DMSA via surface double-exchange kept well dispersed with a little aggregation in LB medium (Fig. 1a). The underlying mechanism is due to the negative value ζ of around −52 mV, which makes particles repel each other. Such well-dispersed MNPs can keep stable in aqueous solution at 4 °C at least for one year. The effect of MNPs on E. coli genotypes at a molecular level was investigated by rep-PCR genomic DNA fingerprinting. Typical images of results of rep-PCR can be seen in Fig. 2. It is considered that genotypes having relative similarity values greater than 80% are representatives of a single strain (Oda et al., 2002). Based on this criterion, several different genotypes of E. coli, which are marked by different colors respectively, were observed when treated with MNPs. rep-PCR fingerprinting patterns of the in vitro amplification of DNA extracted from cells treated with MNPs formed different clusters in the dendrogram (Fig. 2). Only 6% of the bacteria were found to have genotypes that were identical to those in the control (shown in point A), 50% of strains had about 70% similarity with those in control (shown in point B). Moreover, 18.7% had only 47% similarity with those in control (shown in point C). In contrast,
the in vitro amplification of DNA extracted from cells under control showed the almost identical genomic fingerprinting patterns (>93%). The genotypes in bulk-Fe₂O₃ particles treatment had also no obvious difference (>84%, Fig. S2). Thus, the appearance of different genotypes in γ-Fe₂O₃ MNPs treatments indicates that MNPs induce mutations of E. coli.

3.2. Physicochemical interaction of MNPs with E. coli

Physicochemical interferences of MNPs on E. coli are suggested to be of the critical factors for bacterial mutations as shown in scheme (Fig. 3). Nano metal oxide particles have been found to be capable of penetrating cell wall and entering bacterial cells (Bryner et al., 2006; Dehner et al., 2011). Consistently, γ-Fe₂O₃ MNPs was found to adsorb and enter bacteria cell in this report. Subsequently the MNPs may interact with the cellular macromolecules such as protein and chromosome.

The uptake of MNPs by E. coli cells was demonstrated using FCM. SSC signal reflects the density and granularity of a cell, higher density and more granules with stronger SSC signal. It has been observed that SSC was increased upon cellular uptake of the nanoparticles in human cells using FCM (Zucker et al., 2010). In this study, after the incubation with MNPs, SSC intensity increased evidently as compared to the control (as shown in Fig. 4). The percentage of cells in the region of M1 (low signal intensity) decreased significantly, resulting in corresponding increase of cells in the regions of M2 and M3 (high signal intensity). For cells incubated with MNPs, the mean intensity of SSC was 306, showing significant increase compared to 186 of control cells. The forward scatter (FSC) reflects the size of a cell. The mean intensity of FSC almost did not change compared with control cells, showing that cells could keep their sizes during the absorption of nanoparticles (histograms not shown).
SEM and TEM were used to directly visualize locations of MNPs and changes in cell morphology. Fig. 5a and b show SEM images of cells in LB medium with and without MNPs. Normally, morphologies of cells are of bacilliform shape with a moderate size (1.5 μm × 0.5 μm). When exposed to MNPs, the sizes of cells were similar to those in control, which is consistent with the results of FSC. However, a layer of MNPs are observed to be absorbed at membranes of cell wall. The possible mechanism would be due to bacterial wall constitution. Carboxyl, amide, phosphate, hydroxyl groups and carbohydrate-related moieties in bacterial cell wall may provide sites for binding with MNPs (Lovern et al., 2007; Omoike and Chorover, 2004).

TEM revealed that MNPs located not only at cell wall and outer membrane, but also at cytoplasm membrane and in cytoplasm (Fig. 5d). The cytoplasm shows unanimous electron density when without MNPs (Fig. 5c). In the presence of MNPs, the cytoplasm of *E. coli* clearly displays an uneven electronic density (Fig. 5d), which might be attributed to the internalization of MNPs. It is well known that prokaryotic cells utilize membrane anchored protein channels for uptakes of large molecules (Schulz, 2002). Molecules with a maximum size range 1.8–2 nm are allowed for non-specific uptake in gram-negative cells (Trias and Benz, 1994). Therefore the possible reason for the entry of nanoparticles (average 10 nm) into a bacterial cell is due to damages of cell wall. As shown in Fig. 5d, several sites of cell membranes in *E. coli* are damaged, which could lead to the entry and internalization of MNPs.

Cytoplasm is filled with ribosome and nuclear material. The entering of nanoparticles facilitates their binding with proteins and DNA strands. TEM graphs of superthin slices stained with uranyl acetate and lead citrate can clearly discern bindings of MNPs to ribosome and chromosomes (Fig. 5e and f). Electron-dense area indicates ribosome, while electron-light region indicates nuclear material (Someya et al., 1979; Zhao et al., 2010). The binding of MNPs and ribosome as well as chromosomes would subsequently affect conformations of DNA strands and proteins. As shown in scheme, *γ*-Fe2O3 nanoparticles interact with DNA assembling as a nanocomposite (Dutta et al., 2006). Formation of DNA-*γ*-Fe2O3 nanocomposite affects gene semi-conservative replication. The four bases, adenine, guanine, thymine, and cytosine (A, G, T, and C), contain carbonyl groups that display high affinities to nanoparticles (Kouassi and Irudayaraj, 2006). As a result, bacterial DNA conformation could be transformed. Similarly, nanoparticles have high affinity with proteins (Dyal et al., 2003). Enzymes involved in DNA replication, hence, could bind MNPs, such as helicases, DNA ligase, DNA synthase etc. Both above processes would raise DNA mismatch during semi-conservative replication, further increase the mutation frequency and finally produce mutants of *E. coli*.

Another crucial factor which may affect *E. coli* is the release of metal ions from nanoparticles. It has been shown previously that the toxicity of nano-ZnO is directly related to particle dissolution and release of toxic Zn2+ in the cell culture medium as well as the uptake of the particle remnants in the cells (Nel et al., 2008). We studied the dissolution of iron oxide nanoparticles in LB medium. Using ICP-OES, the maximal dissolved Fe3+ of nanoparticles in LB medium was 18.3 μg/ml (Fig. 6). As a result, the dissolved Fe3+ might enter the cytoplasm. Fe3+ cation shows stronger interaction

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**Fig. 4.** Side scatter signal of *E. coli* cells detected by flow cytometry (488 nm Argon laser). (a) Control cells, (b) cells incubation with *γ*-Fe2O3 MNPs.

**Fig. 5.** Location of *γ*-Fe2O3 MNPs in *E. coli*. a, b: SEM of bacteria cultivated in LB medium without and with MNPs; c, d: TEM of superthin slices of *E. coli* cultivated in LB medium without and with MNPs; e: TEM of stained superthin slices of *E. coli* indicates that MNPs bind to ribosomes and chromosomes; f: ED analysis of the area enclosed with a black circle. In Fig. 3d and e, Nu – nuclear material, R – ribosome, Ch – chromosome.

**Fig. 6.** The dissolution of Fe3+ ions in LB medium during *E. coli* cultivation. Each data point is the average of three replicates, with error bars denoting the standard deviation.
with bases and backbone phosphate groups of DNA (Ouameur et al., 2005). This process is prone to make DNA mutation during replication.

3.3. Cytotoxicity of MNPs

Surprisingly, MNPs amendment didn’t inhibit growths of E. coli strains when measured following the protocol described in the cytotoxicity assays section. By contrast, cell densities were even higher in MNPs treatments than in control (Fig. 7), indicating that bacterial growth is indeed promoted by MNPs. The underlying mechanism could be that the presence of iron in environments can facilitate both growth and cultivability of E. coli, because iron can serve as both a nutrient and an electron acceptor (Appenzeller et al., 2005).

Overall, our work showed that E. coli cells suffered genomic mutations under MNPs treatments. Both increased Fe$^{3+}$ ion and the uptake of MNPs facilitated Fe binding with proteins and DNA strands, resulting in enhancing the mutation frequency of E. coli. The results also showed that MNPs might promote bacterial growth by offering iron element. Future work is necessary to apply pyrosequencing approach to elucidate variation in genome of E. coli in detail and to combine its genotype and phenotype to deeply figure out the characteristics of mutants of E. coli under MNPs treatment.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2011.08.024.

References


