Potential of Functionalized Magnetite (Fe₃O₄) in Decontamination of Pathogenic Bacteria from Milk

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Summary: Magnetite (Fe₃O₄) is getting popular due to its super-paramagnetic properties, high biocompatibility and lack of toxicity to humans. Magnetite (Fe₃O₄) nanoparticles have high surface energy thus these nanoparticles aggregate quickly. This aggregation strongly affects the efficiency of these nanoparticles. So these magnetite nanoparticles are coated with organic or inorganic substance to prevent aggregation. These coatings not only stabilize magnetic nanoparticles but can also be used for further functionalization. The aim of this study was to evaluate the efficiency of functionalized magnetite to remove pathogenic bacteria (*E.coli and B.cereus*) from milk considering binding capability of magnetite with bacterial cell wall. Magnetite (Fe₃O₄) was prepared by co-precipitation method and subsequently functionalized with oleic acid (OA) and ethylene diamine (EDA). In present study role of magnetite (Fe₃O₄) and functionalized magnetite (EDA-Fe₃O₄, OA-Fe₃O₄) in removal of pathogenic bacteria (*E.coli and B.cereus*) from milk was investigated. The morphology of functionalized magnetite was determined by Scanning Electron microscopy (SEM). Their removal efficiency was studied based on time (10, 20 and 30 minutes). Concentration of uncoated magnetite (Fe₃O₄) and coated magnetite (EDA-Fe₃O₄, OA-Fe₃O₄) was fixed at 4mg/50mL. Magnetite was successfully synthesized in range of ±3nm. Highest capturing efficiency (74.45%) of oleic acid magnetite (OA-Fe₃O₄) was observed for *Bacillus cereus* at 30 minutes. However for *Escherichia coli,* both ethylene-diamine magnetite (EDA-Fe₃O₄) and oleic acid magnetite (OA-Fe₃O₄) showed maximum capturing efficiency (61.65% and 63.91% respectively). It was concluded from the study that magnetite coated with oleic acid and ethylenediamine removed pathogenic bacteria from milk efficiently. However, more research is required to study the effect of these magnetic nanoparticles on nutritional composition of milk.

Key Words: Potential, Magnetite, Functionalization, Pathogenic Bacteria, Milk.

Introduction

Global concerns regarding food safety demand considerable attention of researchers to employ novel technologies to ensure the provision of safe food to people. Despite strict compliance with safety regulations of food borne pathogens, toxins, and other contaminants that pose serious threat to human health resulting in higher incidence of food borne illnesses [13]. Recently, nanotechnology has emerged as a promising solution for food safety issues in terms of removing biological hazards to extend the shelf life of foods [16] . Antimicrobial nano-sensors and controlled release technology of nano-particles aids in ensuring food safety by detecting microbes and improving microbial decontamination [4] . This new, rapidly developing technology impacts every aspect of the food system from production to processing, packaging, transportation and bioavailability [5].

"Nano-science" has a remarkable power to exploit atoms and molecules on the nano-scale (1–100 nm), and their controlled manipulation results in unique properties of nanostructures and nano-materials – such as a large surface area, high activity, and small size [3]. Recent research has proved that nano-particles are highly effective in binding and removing microbes [17]. Bacterial binding to nano-particle is advocated through hydrogen bonding and ligand exchange. The iron oxide nano-particles binds to lipoteichoic acid and lipopolysaccharides of Gram positive and Gram negative bacterial cells [9]. The most important effect after interaction is the shortening of log phase of bacterial growth. The probable effect is micro biostatic outcome [2]. Production of reactive oxygen species; super oxide radicals, hydroxide radicals and singlet oxygen by nano-particles appears to inhibit growth of most of the bacteria [12].

Microbial load of raw milk affects the shelf life of commercial milk and its products. Particularly in low-income countries, current microbial status of milk hinders the provision of safe and hygienic milk to the consumers and exists as a constant problem for

dairy industries [15] .The research work is being carried out to explore an efficient and effective way to reduce microbial contamination in milk [8]. Considering the tremendous potential of nano-particles, the humble effort was made to develop iron based nanoparticles (magnetite) and to evaluate the capturing efficiency of magnetite with two different surface coatings OA and EDA at three different times 10, 20 and 30 minutes for *Bacillus cereus* (ATCC 11778), and *Escherichia coli* (ATCC 25922). Magnetite nanoparticles have been utilized because iron has no toxicity and iron is a fundamental element in human body [1]. It can be added to the bodies iron reserves after particle degradation. The research work was carried out to validate the potential of magnetite nanoparticles to reduce microbial contamination in milk.

Experimental

Nano-particle Synthesis

Synthesis of magnetite was performed by using method described by khan et al., (2011) with slight modifications [10]. Magnetite was synthesized by dissolving 9.939 grams of Ferrous Chloride (FeCl$_2$.4H$_2$O) (Daejung, S. korea) in 25 mL distilled water. Another 25 mL of Ferric Chloride (FeCl$_3$.6H$_2$O) was prepared by dissolving 6.7575 grams of Ferric Chloride in distilled water. A 250 mL of 0.9 M NaOH solution was also made for co-precipitation protocol. In a three neck round bottom flask, 16.5mL of FeCl$_3$.6H$_2$O and 4.1 mL of FeCl$_2$.4H$_2$O solutions were mixed. The 0.9 M NaOH was added drop wise at a constant rate (4mL/min) and continuous mixing at 80°C. The stirring was done till a black color precipitates appear. Solution was heated for 30 minute at the same temperature to transform the iron hydroxide into magnetite. Surface modification was performed with Ethylene diamine (EDA) and Oleic acid (OA) separately (Daejung, S. Korea). Precipitates were cooled at room temperature. Then the particles were washed with ethanol (AnalaR grade BDH) till pH turned to 7. Nano-particles were dried at 50°C in an oven (Memmert, Germany) for 20 h [10].

Ethylene diamine was coated to the surface of Fe$_3$O$_4$ magnetic nano-particles by dispersing magnetic nano-particles with 66 nmol solutions for 8 h. Particles were separated by magnet field, and supernatant was discarded. Later, coated particles were washed with distilled water three times [14] . In the other preparation 1 mL Oleic Acid (OA) was mixed with 0.6 g of magnetic iron oxide nano-particles in flask for overnight in orbital shaker at 10 rpm. OA-coated magnetic iron oxide particles were washed with 1 mL of 0.1 M sodium carbonate by vortex for 10 min to obtain a stable colloidal dispersion [18].

Sample Preparation

Milk samples were collected from different street vendors of Multan region (Pakistan). The samples were stored in refrigerator at 4°C till needed.

Capturing Efficiency Determination

The pH of the milk was maintained at 7.4 with phosphate buffer solution (8g NaCl. 0.2g KCl. 1.44g of Na$_2$HPO$_4$. 0.25g of KH$_2$PO$_4$/1000mL). The milk pH was determined with pH meter and dilutions made in phosphate buffer saline solution (PBS) (8g NaCl. 0.2g KCl. 1.44g of Na$_2$HPO$_4$. 0.25g of KH$_2$PO$_4$/1000mL) at pH=7.4. All samples were 10 folded serially diluted and were spread on to Plate count agar platesto check initial CFU log of *Bacillus cereus* (ATCC 11778) and *Escherichia coli* (ATCC 25922). Then 50mL of each milk sample wasspiked with *Bacillus cereus* (ATCC 11778) and *Escherichia coli* (ATCC 25922).

Removal efficiency of uncoated and coated magnetite (oleic acid and ethylene-diamine) was determined with various types of spiked milk samples. Concentration of magnetite was also optimized to 4 mg/mL. After 10, 20, 30 minutes, milk samples were serially diluted and 0.1 mL of diluted, spiked milk was spread on solid medium (plate count agar). These petri plates were kept for 24 h in incubator (Memmert, Germany) at 37°C. Removal efficiency of bacterial concentration was calculated by standard plate count technique [14]. Data in triplicate obtained were analyzed by applying analysis of variance with two factor factorial design. Means and standard error of means were calculated [11].

SEM (Scanning Electron Microscopy) of Synthesized Magnetite

To analyze the size of magnetite and its interaction with bacteria, slides of magnetite particles were made in nutrient broth that contained inoculums of tested strains *Bacillus cereus* (ATCC 11778) and *Escherichia coli* (ATCC 25922) and SEM at (500X at 25 KV) was performed at Food Microbiology Laboratory, Pakistan Council of Scientific and Industrial Research, Karachi.

Results and Discussion

Characterization of Nano-Particles (NP)

Texture and color of uncoated and coated magnetic nano-particles was shown in Fig. 1. Magnetite nano-particles were prepared by co-precipitation method.Color of uncoated magnetite was found brown. Color of ethylene-diamine coated magnetite was shiny black andOleic acid coated magnetite nanoparticles were dark brown in color.
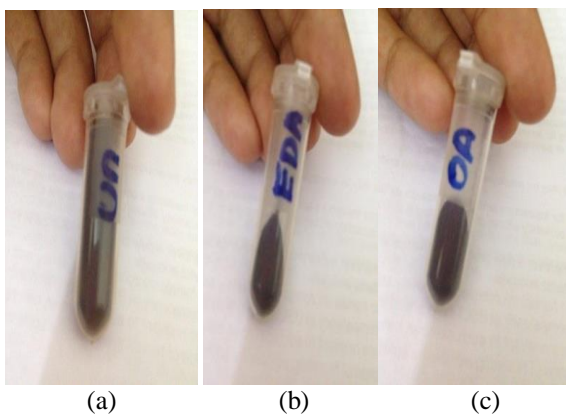

(a)   (b)   (c)

Fig. 1: a) Color of uncoated magnetite was brown, b) Ethylene-diamine coated magnetites were shiny black c) Oleic acid coated magnetic nano-particles were dark brown in color.

Scanning Electron Microscopy of Magnetite (Fe₃O₄)

Scanning Electron Microscopy of Magnetite (Fe_3O_4)

Size of magnetite was determined by Scanning Electron Microscopy (SEM). These micrographs of bacteria bound to NP obtained by SEM were shown in Fig. 2. These micrographs showed that size of magnetite was 3nm and they bind with bacteria.
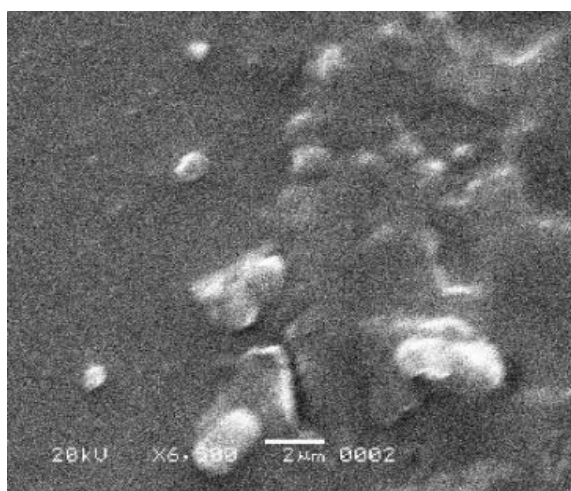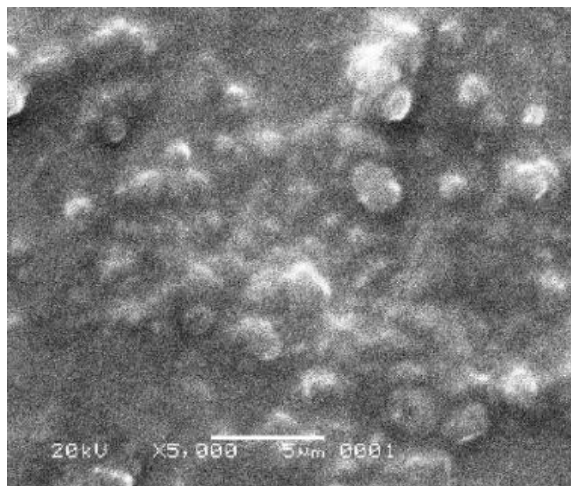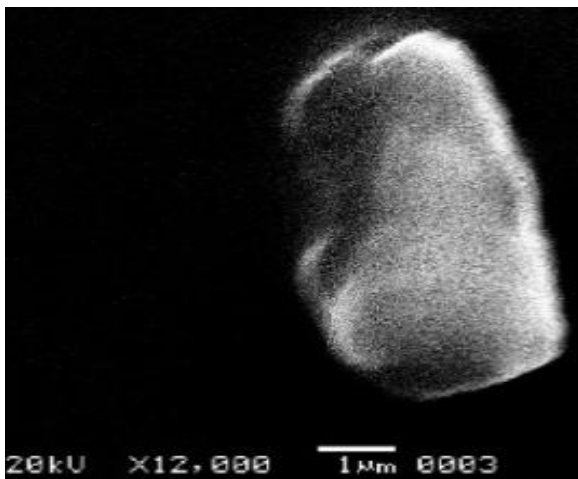

Fig. 2: SEM (500X at 25 KV) images of functionalized magnetite showing binding of modified magnetite with bacteria.

Capturing Efficiency with Magnetite (Fe_3O_4) NP

Magnetic iron oxide nano-particles functionality was assessed for *Bacillus cereus* (ATCC 11778), and *Escherichia coli* (ATCC 25922) by counting the colony forming units (CFU) in milk sample before and after addition of NP (nano-particles) to spiked milk. Removal efficiency of uncoated magnetite, ethylene diamine (EDA) and oleic acid (OA) coated magnetite and for *Bacillus cereus*were described in Table-1.

In present study we tested two bacterial strains *Bacillus cereus* (ATCC 117780) and *Escherichia coli* (ATCC 25922) as a model to check their affinity towards oleic acid and ethylene-diamine coated magnetite at different time periods (10, 20, 30min). Statistical results indicated the significant

(p<0.05) capturing efficiency values of coated magnetite with OA-Fe$_3$O$_4$ (Coating I) and EDA-Fe$_3$O$_4$ (Coating II) as compared to uncoated magnetite (Uncoated Fe$_3$O$_4$) at 10, 20 and 30 minutes in milk samples spiked with *Bacillus cereus* (ATCC 11778). Maximum capturing efficiency (74.45%) was observed with OA-Fe$_3$O$_4$ (Coating II) at 30 minutes as compared to EDA-Fe$_3$O$_4$(Coating II) (53.7%) and uncoated Fe$_3$O$_4$ (52.16%). Lowest efficiency (37.20%) was noted with EDA-Fe$_3$O$_4$ at 10 minutes among all treatments. While considering capturing efficiency of coated and uncoated magnetite at 10 and 20 minutes, OA-Fe3O4 coated magnetite showed high capturing efficiency (54.43% and 64.35% respectively) as compared to others. Oleic acid coating was considered best for decontamination of *Bacillus cereus* at 30 minutes.

Table-1: Efficiency (%) of uncoated, ethylene-diamine (EDA) and oleic acid (OA) coated magnetites for *Bacillus cereus* (ATCC 11778) at different time periods

Time (Min)	Uncoated	Coating I	Coating II	Overall Mean
10	41.10^{ef}± 6.0	37.20^f±9.1	54.43^c±7.2	44.25^c±7.4
20	48.34^d±4.4	43.59^e±10.0	64.35^b±3.9	52.09^b±6.1
30	52.16^{cd}±4.7	53.7^c±6.4	74.45^a±6.4	60.10^a±5.8
Overall Mean	44.83^b±5.0	47.20^b±8.5	64.41^a±5.8	

Values are mean ± standard deviation (SD)
Mean followed by different letters in the same columns and rows represent significant difference (p<0.05)
Uncoated: Uncoated magnetite (Fe$_3$O$_4$)
Coating I: Ethylene-diamine coated magnetite (EDA-Fe$_3$O$_4$)
Coating II: Oleic Acid coated magnetite (OA-Fe$_3$O$_4$)

Capturing efficiency is based on surface charge and local environment charge of magnetite [6]. Milk pH ranges from 6.4 to 6.7, therefore charge on uncoated magnetite was slightly negative and it showed affinity towards *Bacillus cereus*. Surface charge was altered by functionalization of magnetite with ethylene-diamine and oleic acid. Oleic acid provides the magnetite with negative charge in acidic and basic environment due to the presence of carboxylic acid while EDA coated magnetite has positive surface charge due to the presence of amine group NH_2^+on the surface [14] .Oleic acid coated magnetite (OA-Fe$_3$O$_4$) showed higher efficiency value (74.45%) for *Bacillus cereus* (ATCC 117780) because of its negative surface charge. This is due to electrostatic interaction between oleic acid coated magnetite (OA-Fe$_3$O$_4$) and *Bacillus cereus*. While ethylene-diamine coated magnetite (EDA- Fe$_3$O$_4$) showed efficiency value 54% for Gram positive bacteria.According to study conducted byReddy et al., (2012) polyacrylic acid coated magnetite (PAA-Fe$_3$O$_4$) showed capturing efficiency value 20%at pH 7 for *Staphylococcus epidermidis*andpoly-ethyleneimine coated magnetite (PEI- Fe$_3$O$_4$) showed

capturing efficiency value 96% at pH 7for *Staphylococcus epidermidis* in water sample [14] .

It was revealed from the results (Table-2) that stay time significantly(p<0.05) affect the capturing efficiency of uncoated magnetite (Fe$_3$O$_4$) , ethylene-diamine coated magnetite(EDA-Fe$_3$O$_4$) and oleic acid coated magnetite (OA-Fe$_3$O$_4$) towards *Escherichia coli.* Statistically result indicated that maximum capturing efficiency of uncoated (60.72%), Coating I (EDA-Fe$_3$O$_4$) (63.91%) and Coating II (OA-Fe$_3$O$_4$) (61.65%) was observed at 30 minutes. Lowest valueswere observed with Coating I (EDA-Fe$_3$O$_4$) (47.07%) and Coating II (OA-Fe$_3$O$_4$) (46.52%) at 10 minutes of stay time.

Table-2: Efficiency (%) of uncoated, ethylene-diamine (EDA) and oleic acid (OA) coated magnetites for *Escherichia coli (*ATCC 25922) at different time periods.

Time(Min)	Uncoated	Coating I	Coating II	Overall Mean
10	51.66^d ± 5.34	47.07^e ±4.4	46.52^e±5.7	48.42^c±5.1
20	56.03^b ± 4.1	52.28^{cd}±6.6	55.82^{bc}±5.9	54.71^b±5.5
30	60.72^a ± 5.0	61.65^a±8.6	63.91^a±4.5	62.09^a±6.0
Overall Mean	56.14^a±4.8	55.42^{ab}±6.5	53.66^b±5.3	

Values are mean ± standard deviation (SD)
Mean followed by different letters in the same columns and rows represent significant difference (p<0.05)
Uncoated: Uncoated magnetite (Fe$_3$O$_4$)
Coating I: Ethylene-diamine coated magnetite (EDA-Fe$_3$O$_4$)
Coating II: Oleic Acid coated magnetite (OA-Fe$_3$O$_4$)

Current study results are slightly different from the values (88.5%-99.1%) reported by Huanget al., (2010) in their study, it is supposed that low efficiency values found in current study than reported values are because of change in medium [7].

Limited research work was done so far on capturing efficiency of functionalized magnetite with EDA and OA by using milk as medium. We have tried to explore binding affinity of magnetite for *Bacillus cereus* and *Escherichia coli* spiked in milk. The recent study was a humble effort towards this direction digging out some striking features for the researchers to focus on this dairy related aspect of nanotechnology.

Commercialization of functionalized magnetite based milk decontamination coupled with traditional heat treatments can increase the shelf life of milk and help to reduce nutritional losses that occur due to extensive heat treatment of milk. Future belongs to study the interaction of modified magnetite with the goal to evaluate the nutritional profile of target medium.

Conclusions

The research results showed that magnetite nano-particles modified with oleic acid and ethylene-diamine can be useful in removing pathogenic bacteria (*Bacillus cereus* and *Escherichia coli*) from milk. At 30 minutes, the maximum capturing efficiency (74.45%) of oleic acid (OA-Fe$_3$O$_4$) was noted for *Bacillus cereus* while for *Escherichia coli, both* ethylene-diamine (EDA-Fe$_3$O$_4$) and oleic acid (OA-Fe$_3$O$_4$) showed highest capturing efficiency (61.65% and 63.91% respectively). It was concluded that functionality and electrostatic force of attraction plays an important role in removing bacteria from milk. The recent work was, however, a different approach since not much work has been done on magnetite exploitation for decontamination by using milk as medium. More effort is still required to explore the use of functionalized magnetite and their interaction with components of target medium in food sector. Moreover, safety of nanomaterial with perspective of their use in food industry should be investigated to provide a uniform international regulatory framework for nanotechnology in food.

Acknowledgement

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