AMINO-FUNCTIONALIZED FERROMAGNETIC NANOPARTICLES FOR THE RAPID SEPARATION AND CONCENTRATION OF PATHOGENIC *Campylobacter jejuni*

Wittawat Srijanboon[a], Wanwisa Poolapdecha[b], Kooranee Tuitemwong[a]*, Pravate Tuitemwong[c]**

[a]*Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[b]*BioScience Program, Department of Microbiology, King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, 10140, Thailand*
[c]*Food Safety Center, Institute for Scientific and Technological Research and Services, ISTRS, KMUTT*

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ABSTRACT

Campylobacter jejuni is the most common zoonotic disease worldwide. The rapid detection is a time - consuming test for high concentration requirement of *C. jejuni*. This study produced ferromagnetic nanoparticles to concentrate, and separate the target pathogen for further detection or visualization. We synthesized amino-functionalized ferromagnetic nanoparticles (amino-MNPs), attached with specific monoclonal antibodies with the aid of glutaraldehyde for rapid concentration and isolation of the target pathogens. Faced centered cubic amino-MNPs were prepared through a polyol technique at a temperature of 121°C, using a laboratory autoclave for 2 h/cycle, 3 consecutive cycles. The desirable structure of amino-MNPs of average 15 to 25 nm in diameter was achieved. They attached readily on the surface of the pathogens. The TEM image clearly showed that the amino-MNPs attached on the cell wall of *C. jejuni*. This method - amino-MNPs coupled with mCCDA plating - had a detection limit of 103 CFU/ml of pure culture trial, with capture efficiency of 37.13%. It resulted in high specificity to *C. jejuni* and did not bind to C. coli nor *E. coli*.

KEYWORDS: *Campylobacter jejuni, nanoparticles, amino-functionalized, ferromagnetic*

* Corresponding authors; e-mail: Kooranee Tuitemwong, fscikrt@ku.ac.th and
** Corresponding authors; e-mail: Pravate Tuitemwong, foodsafety@kmutt.ac.th; ptt59@hotmail.com

INTRODUCTION

Thermophilic Campylobacter spp. is a major food-borne and zoonotic enteric illness worldwide such as in the European Union, with 190,566 cases in 2008. Campylobacter continues to be one of the most commonly reported, contributing up to one third of all human Campylobacteriosis cases. *C. jejuni* is the leading causative agent (95%) in human cases, and a lesser amount (about 5%) is caused by C. coli. In particular, with children under 5 years of age, *C. jejuni*, *C. coli* and other Campylobacter spp. are all associated with enteric disease. Asymptomatic infection with *C. jejuni* and *C. coli* in adults is also alarming.

The rapid detection of this pathogenic *Campylobacter* is needed to insure food safety. The conventional method for the detection of pathogens in foods is a time-consuming and labor-intensive process. The newly developed and more advanced rapid methods, for example, polymerase chain reaction and DNA probes, are likely to be adopted as for their more sensitive and selective techniques. However, these methods still require an enrichment step for detection. Nanotechnology, on the other hand, allows one to increase the signal from a single pathogen without the time dependent primary and secondary enrichments [1,2,3,9]. However, food matrixes cause artifacts that interfere with captured movement and visualization of the particles. Moreover, many enzymatic reactions of the tests are partly or fully inhibited by food matrix and also caused a slow-growing enrichment step. The needs for rapidly separated and concentrated bacteria directly from food samples could reduce this problem and allow rapid growth and/or detection of the pathogen.

Separation and concentration methods help save the pathogen of interest from interfering with food components, as well as increasing the bacterial number to a detectable level.

Separation methods currently available in the market include centrifugation, affinity binding to solid phases, and immuno-magnetic separation. The magnetic nanoparticles currently developed have physical, chemical, and unique biological properties, and are suitable for different applications. Amino functionalized magnetic nanoparticles (amino-MNPs) of face-cubic centered shape synthesizing with a facile single step at a low temperature have the potential to be used for rapid microbial detection and for many biological applications [4].

Ferromagnetic materials also exhibit a long-range ordering phenomenon at the atomic level, causing the unpaired electron spins to line up parallel with each other in a region called a domain. Normally the domains are oriented randomly with respect to each other and are unmagnetized. With small external magnetic fields employed, the magnetic domains can line up to each other and are magnetized. The high degree of magnetization enables the ferromagnetic particles to have a large drag force toward the magnet.

The ferromagnetic nanoparticles are functionalized with amino groups that allow the attachment of antibodies with or without the aids of ligands, and other surface modifications. They could readily and rapidly separate and concentrate bacteria from food samples in less time than required for cultural enrichment, which usually takes 18-24 hours.

Songworawit *et al.* [2] and Tuitemwong *et al*. [4] reported the success of the development and application of ferromagnetic nanoparticles for the separated and concentration target cells, and with combination to silica nanoparticles as signal reporter, rapidly detect entero-haemorhagic *E. coli* O157 (EHEC). Monoclonal antibodies are often useful for specific detection of a wide variety of microbes and their products because they provide an indefinite supply of single antibody with enhanced sensitivity, specificity, reproducibility and reliability.

This paper describes the facile synthesis and preparation of amino-functionalized ferromagnetic nanoparticles, and surface modification with monoclonal antibodies for separating and concentration of the target *Campylobacter jejuni* to enhance the concentration leading to rapid detection of the pathogens.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

Campylobacter jejuni ATCC 33291 and *Campylobacter coli* NCTC 11353 strains were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. The *Campylobacter* strains were resuscitated and selected on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and grown on Blood Agar (BA), supplemented with 5% sheep blood (Danish Veterinarym Institute, Aarhus, Denmark) then incubated at 42 °C in anaerobic jar modification microaerophilic conditions by Campygen Gaspack with no water needed (Oxoid) (6% O_2, 14% CO_2 and 80% N_2).

2. Chemicals

Amino-functionalized magnetic microparticles (amino-MNPS) (approximately 2 mg/ml) were prepared by the polyol technique in a condition at 121 °C for 9 hr (3 cycles) as described by Songvorawit *et al.* (2011). An autoclave (Sanyo Model MLS-3020, Japan) was used as a reactor for amino-MNPs synthesis. The reagents for amino-MNPs synthesis are iron compound, which in this studies used iron (III) chloride hexahydrate ($FeCl_3.6H_2O$; Poch, Poland), sodium hydroxide (NaOH; Rankem, India), sodium acetate (CH_3COONa; Merck, Germany), ethylenediamine (Panreac, EU) and ethylene glycol (Unilab, Australia). Ethanol (95%) and distilled water were used for washing amino-MNPs after the synthesis. The Specific *C. jejuni* monoclonal antibody [BD1717] (IgG1) were purchased from Abcam Inc., UK.

3. Characterization of the ferromagnetic nanoparticles

MNPs were dispersed in distilled water for 15 min with ultrasonicator. The top of the particle suspension was placed on a copper grid. It was air dried for 20 min before the morphology and size of the nanoparticles were examined under TEM. Morphology and size of the nanoparticles were determined by the images from Transmission Electron Microscope (TEM; JEOL Model JEM-1010, Japan). MNPs were ground, spread on carbon tape and attached on holder. The samples were analyzed by using SEM-EDS. The structure of nanoparticles was characterized by X-ray Diffractometer (XRD; Bruker AXS Model D8 Discover, Germany) with target: Cu, 40kV, 40mA, angle 20-80 degree, increment 0.02 degrees/step, scan speed 0.3 s/step. The magnetic properties were characterized by vibrating sample

magnetometer (VSM) developed by Department of Physics at Kasetsart University, Thailand.

4. Preparation of Amino-functionalized magnetic nanoparticle immune-probes

Amino-functionalized magnetic nanoparticles (amino-MNPs) (approximately 2 mg/ml) were prepared by using the polyol technique, as described by Guo *et al*. [6], Songvorawit *et al*. [1] In the modification step, an amine group was attached to the surface of nanoparticles during the synthesis step. Briefly, 2.0 g of $FeCl_3.6H_2O$ was added into 40mL of ethylene glycol and then mixed in the solution until the solution was a clear yellow color. An aliquot of 6.0 g of CH_3COONa, 1.68 g of NaOH and 20mL of ethylenediamine was added to the solution. The mixture was stirred at a low speed for 30 min. The solution then turned a yellowish brown color. The yellowish brown solution was further heated in an autoclave at 105 kPa, 121°C, for 2 hr per cycle for 3 cycles. After the reaction was completed, the amino-MNPs were isolated by magnetic bar, and washed with distilled water and 95% ethanol several times in order to remove the solvent (dark solvent) as shown in Figure 2. The amino-MNPs were dried at 37 °C overnight, ground, and then stored in a dry bottle covered with aluminum foil. In the further surface modification step, glutaraldehyde was used as the conjugating agent before the attachment of mAb. An aliquot of 100 ml of the amino-MNPs solution (2 mg/ml PBS pH 7.4) was activated by reacting with 3 ml of 5% glutaraldehyde [5] for 3 hr on a rotary shaker at a room temperature. The particles were washed three times with phosphate buffered saline (PBS, pH 7.4), re-suspended in 100 ml PBS (pH 7.4), and stored in a glass bottle covered with aluminum foil at a refrigeration temperature (5°C). After the modification step, the amino-MNPs was conjugated by the specific *C. jejuni* monoclonal antibodies (0.1 mg/ml [BD1717] IgG1, Abcam Inc., UK.). Non-covalent binding of IgGs on particles was also studied using untreated MNPs to incubate with IgG solution at the same condition as described above. The amount of protein on the particles was determined by Lowry method [7].

The antibodies were conjugated to the glutaraldehyde activated amino-MNPs by adding 500 µl of specific *C. jejuni* monoclonal antibody (0.1 mg/ml) in 500 µl of the activated amino-MNPs solution, mixed well, and incubated the mixed solution at a room temperature for 24 hr for conjugation, followed by washing three time of PBS, pH 7.4. The particles were finally re-suspended in 1 ml of PBS, and stored at 4°C. It could be kept in refrigerated conditions for 1 month without losing immunological activity [5]. The particles were examined under TEM.

5. Preparation of the antigen

Campylobacter jejuni ATCC 33291 and *Campylobacter coli* NCTC 11353 were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. All strains were resuscitated and selected on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and grown on Blood Agar (BA supplemented with 5% sheep blood, Danish Veterinary Institute, Aarhus, Denmark), then incubated at 42°C in anaerobic jar under modified microaerophilic conditions by Campygen Gaspack (Oxoid) in order to obtain 6% O_2, 14% CO_2 and 80% N_2 condition. Typical colonies were picked up from mCCDA and re-inoculated in 9 ml of Tryptic Soy Broth (TSB), then incubated at 42 °C in an anaerobic jar under microaerophilic conditions for 48 hr. The pure culture in TSB were diluted by PBS pH 7.4 in order to obtain the final *C. jejuni* concentrations of 10^0 to 10^7 CFU/ml PBS.

6. Detection procedure

In this experiment, 200 µl of amino-MNPs coated with specific monoclonal antibodies (MNPs-mAb) of *Campylobacter jejuni* were added to 20 µl of the *C. jejuni* ATCC 33291 culture dilutions (in PBS pH 7.4), and incubated at a room temperature, 30°C, for 30 min, with gentle rotation. *Campylobacter* cells bound to the nanoparticles were collected by a magnetic force. The collected bacterial-nanoparticles were washed five times with 1 ml of washing buffer (10 mM PBS and 0.05% Tween 20) to completely removal of non-specific bound bacteria. Then, the nanoparticles were re-suspended in 1 ml PBS pH 7.4; and 0.5 ml of the bacterial-particles complex which was used for the examination under transmission electron microscope (TEM). The challenge was made with *C. coli* NCTC 11353 and *E. coli* using the same procedure as that of *C. jejuni*.

RESULTS AND DISCUSSION

1. Morphology and Structure of amino-functionalized MNPs (Amino-MNPs)

The amino-MNPs were prepared by polyol technique using $FeCl_3.6H_2O$ which was a precursor of magnetic nanoparticles and amino group on the particles surface from the ethylenediamine. The synthesis of amino-MNPs was conducted in the autoclave which provided a

lower temperature without using any inert gas and oxygen gas. The morphology of amino-MNPs was identified by Transmission Electron Microscope image and X-ray Diffractometer as face centered cubic [1]. Figure 1 showed a black-brown color morphology of amino-MNPs and small particles.

Fig. 1. The amino-MNPs powder prepared through polyol technique at 105 kPa, 121°C, 2 hr per cycle for 3 cycles

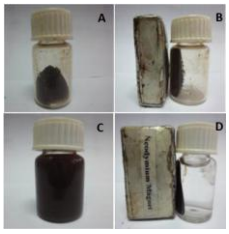

Fig. 2. The amino-MNPs powder (A) was attracted by a magnetic bar (B), dispersed well in water (C), and readily separated from the solution if attracted by a magnetic bar (D)

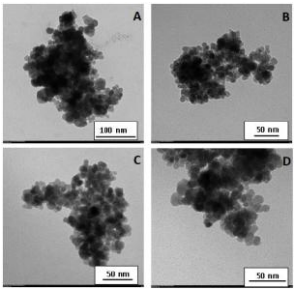

Fig. 3. TEM images showed the morphology of amino-MNPs at 80000x (A) 100000x (B), 120000x (C) and 150000x (D)

Figure 2 illustrates the magnetic properties of amino-MNPs in dry and solution formats. Figure 3 displays the TEM images of the morphology of amino-magnetic nanoparticles with TEM at 80000x (A) 100000x (B), 120000x (C) and 150000x (D). The images of the nanoparticles indicated a spherical shape with an average size of 15 to 25 nm.

2. Magnetic properties

Magnetization curves of amino-MNPs at room temperature indicated that the MNPs were ferromagnetism, but nearly complete superparamagnetism with a saturation magnetization (Ms) of about 48 emu/g, remanence (Mr) of 1.7 emu/g and coercivity (Hc) of 23.5 Oe (Figure 4). The amino-MNPs were well dispersed in water and also can be separated from the solution if attracted by a magnet. Magnetite particles would exhibit complete superparamagtetic properties when their size below 25 nm, which is a critical size of magnetite [8]. At this size, each particle has only one magnetic domain, and when a magnetization curve is plotted, the curve showed intersection at the zero point with no remanence or coercivity. Although, the amino-MNPs produced in this work were not super-paramagnetic because their size was larger than critical size, and low ferromagnetic properties, they were sufficient to be used in any biological applications as they could be well dispersed in aqueous solution and separated by magnet as well [1,2].

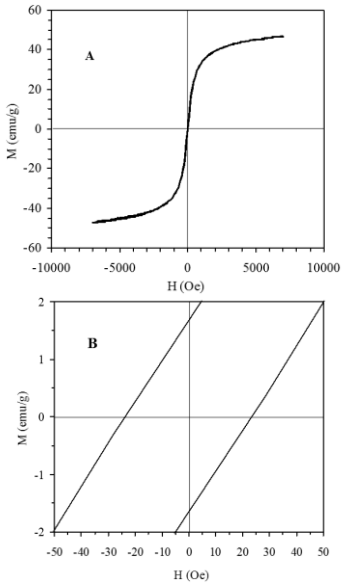

Fig. 4. Magnetization curves of (A) amino-MNPs and (B) hysteresis loop of magnetization curve

3. Conjugation of monoclonal antibodies

The success or failure in this study depended on various factors, such as quality of the capture antibodies, type of monoclonal antibodies, specificity, as well as the properties of nanoparticles, such as size and functional group of the particles surface. It was important to assure the presence of monoclonal antibodies on the nanoparticles surface, as well as their immunological capacity toward target bacterial cell after the conjugation step. To this end, the magnetic nanoparticles coated with specific monoclonal antibodies of *C. jejuni* were analyzed under the TEM as shown in Figure 5.

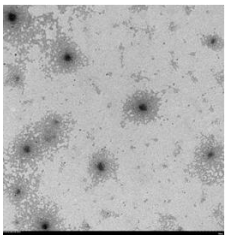

Fig. 5. TEM images of the nanoparticles with diameter of 15-25 nm at 20000x showing morphology of amino-MNPs conjugated with specific monoclonal antibodies of *C. jejuni*

Figure 5 shows morphology of the amino-MNPs conjugated with monoclonal antibodies against *C. jejuni*. The particles were in black color and covered by conjugating agent (glutaradehyde) and specific monoclonal antibodies seen as gray halo surrounding the particles. The average size of the conjugated ones was bigger than that of the amino-MNPs without conjugation. Some appeared to aggregate at the greater extent than the amino-MNPs without conjugation step. Because the conjugating agent and activated amino-MNPs were made to have amino groups on their surface, they facilitated the bioconjugation of the amino-MNPs. Glutaraldehyde, which was well known as a cross linking reagent between two amino groups of protein, was used for IgG conjugation. Two aldehyde groups on both sides of glutaraldehyde molecule are a reactive group that can react with amino group via covalent bonding and allow the IgG to be immobilized on amino-MNPs.

complex of antibody and antigen (target bacteria) could be considered as the first criteria for selecting the most suitable antibody in this step. The efficiency of MNPs to detection *C. jejuni*, was, subsequently, evaluated by their binding capacity with different concentrations of *C. jejuni* and *C. coli* as shown in Table 2. The biological activity of mAb-MNPs was the highest and down to the concentration of 10^3 CFU/ml that the bacterial cell could be detected. The mAb- MNPs was selective to *C. jejuni* and non *C. coli* and was used in subsequent experiments. To evaluate the specificity of the detecting process, the mAb-coated nanoparticles were used to react with various concentrations of different bacterial pathogens (Table 1). Captured bacterial cell on the mAb-MNPs were confirmed by hippurate test (Data not shown). The results indicated that the binding capacity of the mAb-coated nanoparticles was the highest with the target bacterial strain *C. jejuni* without the non-specific adsorptions of *C. coli*. Binding capacity of each concentration of *C. jejuni* in PBS buffer (pH 7.4) was shown as the signal (+) for their growth when spread the particles on CCDA after the capture reaction (antigen-antibodies complexed) and negative (-) for no growth on mCCDA.

Table 1. Comparison of activated amino-conjugated with specific monoclonal antibodies to *C. jejuni* at different concentrations of *C. jejuni*, *C. coli* and *E. coli*

Type of bacteria	**The concentration of bacterial cell(CFU/ml)**							
	10^7	10^6	10^5	10^4	10^3	10^2	10^1	10^0
C. jejuni ATCC 33291	+	+	+	+	+	-	-	-
C. coli NCTC 11353	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-

4. Sensitivity and specificity of the amino-functionalize magnetic nanoparticles for detection C. jejuni

The specificity and the sensitivity of the assay are important for developing the new method for detecting the foodborne pathogens. To activate amino-MNPs conjugated with specific monoclonal antibodies of *C. jejuni* (mab-MNPs), the sensitivity and the specificity depend on the quality and type of the antibody conjugated on the magnetic nanoparticles surface. We, therefore, used *C. jejuni*-specific monoclonal antibodies for detection *C. jejuni*. One goal of mAb-MNPs is to isolate as many as possible of the target bacterial cell from the pure culture or food matrix. For this reason, the immune

Table 1 displays efficiency of monoclonol antibodies conjugated amino-MNPs to capture *C. jejuni* at different concentrations (10^3-10^7 cells/ml) of the target analyses. It was found that the conjugated amino-MNPs captured cells best from 10^3 up. Lower than 10^3 cells/ml, the capture appeared to be ineffective which may be in part due to undetectable with the mCCDA plating. The particles with cells lower than 10^3 cells/ml could not show thebgrowth of *C. jejuni* on mCCDA. The lower detection limit, as low as 10^1 cell/ml, was reported when the more sensitive method of detection such as polymerase chain reaction was used Le *et al.* [5]. The author reported that immuno-magnetic (Dynal bead IMS) coated with monoclonal antibodies and

polyclonal antibodies could separate *C. jejuni* from the suspension at 10^1 to 10^2 and 10^2CFU/ml, respectively, with polymerase chain reaction (PCR) expression. The capturing efficiency relied mainly on quality of antibodies. Monoclonal antibodies appeared to give better results than that of the polyclonal. However, the detection with PCR (10^1 cells/ml) was more effective than the use of mCCDA plating (10^3 cells/ml) of this work.

Table. 2 Comparison of activated amino-MNPs conjugated with specific monoclonal antibodies of *C. jejuni* of the different concentrations of *C. jejuni* in PBS buffer (pH 7.4). The harvested cells were grown on mCCDA for enumeration

C. jejuni (CFU/ml)	**Initial cells (total cells)**	**Capture (%)**
10^7	1.02×10^7	37.13
10^6	1.02×10^6	32.77
10^5	1.02×10^5	12.94
10^4	1.02×10^4	5.78
10^3	1.02×10^3	5.66
10^2	1.02×10^2	-
10^1	1.02×10^1	-
10^0	1.02×10^0	-

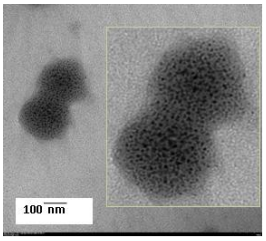

Fig. 6. TEM images (80 kV) at 80000x showing the morphology of amino-MNPs conjugated with specific monoclonal antibodies attached on the cell wall of *C. jejuni*. The bracket was the expansion of the cell showing black dots of MPNs covering the cell.

Capture efficiency (%) of this method was shown in Table 2. Capture efficiency of the single exposure to the particles were 37.7% at 10^7 cells/ml when detected by mCCDA plating. The level of capturing was 10^3 cells/ml. The specificity of the monoclonal antibodies was evidence. No nonspecific binding with *C. coli* and *E. coli* was observed. Figure 6 illustrates *C. jejuni* captured by amino-MNPs conjugated with specific monoclonal antibodies at 80000x magnification (A) and 40000x (B). This electron transmission microscope (TEM) image clearly displayed cells covered with monoclonal antibody conjugated MNPs which can be seen as black dots on cell surface. The attachment enabled the separation of these cells from the matrix with magnetic force.

CONCLUSION

The new concentration and isolation method using amino-MNPs conjugated with specific monoclonal antibodies (mAb-MNPs) for *C. jejuni* from the culture was successfully developed. The cells captured on mAb-MNPs were transferred to a buffer and enumerated on mCCDA. This method provides rapid, simple, nontoxic, highly specific capture and separation that could be coupled with other detection or visualization methods including direct plating, polymerase chain reaction, and real-time PCR for the rapid detection of *C. jejuni* in food or other matrixes.

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