Multiplex Sorting of Foodborne Pathogens by on-chip Free-Flow Magnetophoresis

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Abstract

This study reports multiplex sorting of *Salmonella typhimurium* and *Escherichia coli* 0157, from broth cultures and from pathogen-spiked skinned chicken breast enrichment broths by employing microfluidic free-flow magnetophoresis. Magnetic beads of different sizes and magnetite content, namely Dynabeads® anti-salmonella and Hyglos-Streptavidin beads together with the corresponding pathogen-specific biotinylated recombinant phages, were utilised as affinity solid phases for the capture and concentration of viable *S. typhimurium* and *E. coli* 0157. Following optimisation, the protocol was used to demonstrate continuous magnetophoretic sorting of the two pathogen-bound magnetic bead populations from mixed cultures and from pathogen-spiked chicken pre-enrichment broths under the influence of a Halbach magnet array. For example, in the latter case, a pure population of *S. typhimurium*-bound Dynabeads (72% recovery) was sorted from a 100 µL mixture containing *E. coli* 0157 bound Hyglos beads (67% recovery) within 1.2 min in the presence of 0.1% Tween 20. This proof-of-principle study demonstrates how more than one pathogen types can simultaneously be isolated/enriched from a single food pre-enrichment broth (e.g. Universal food enrichment broth).

Keywords: Multiplex sorting; *Salmonella & E.coli* 0157; Food enrichment broth; free-flow magnetophoresis; Affinity magnetic separation

1. Introduction

A key control measure of safety and quality of food is the analysis of food products for presence of pathogenic and spoilage microorganisms, respectively. Development of rapid detection and identification methods for pathogens is important to ensure compliance with the existing strict safety standards and managing risk during food production, processing and distribution [1]. Traditional analytical methods for foodborne pathogens comprise cultural growth enrichment, plating on selective and/or differential agar media, followed by biochemical/serological/genomic confirmation. Such methods, although highly sensitive, are labour- and material-intensive, time-consuming and highly cumbersome [1-3].

Numerous alternative methodologies exist for the rapid analysis of foodborne pathogens, including immunoassays (e.g. automated Vidas [4, 5] and lateral flow immunochromatography [6-8]), and quantitative PCR (qPCR) (e.g. Bax™ from DuPont [9] and 3M™ Molecular Detection System [10, 11]). These allow analytical results to be obtained within 24 h, as opposed to the conventional methods (4-6 days) [12]. Food producers are often striving for even quicker and simpler methods that are cost-effective and reliable, that will allow faster release of the manufactured products. For this purpose, physicochemical and affinity-based technologies for the capture, concentration and detection of pathogens are becoming increasingly popular [13-19]. Immunomagnetic separation (IMS) is one such example, where antibody or other affinity reagents (lytic phages and recombinant phage proteins) are coupled with magnetic beads (micro- and nano-beads) to produce functionalized magnetic beads. These have been used successfully in a batch mode to capture, concentrate and detect pathogens such as *Salmonella* and *Escherichia coli* 0157 from food matrices [13, 17, 18].

Magnetophoresis is a microfluidic process for resolving mixtures of magnetically susceptible materials based on the application of external magnetic fields [20, 21]. Unlike the conventional IMS process which does not allow different pathogens to be sorted simultaneously, the sorting of magnetic beads by free-flow magnetophoresis occurs due to the differences in their sizes and magnetic content that result in varying magnetically induced deflection from the direction of laminar flow. Free-flow magnetophoresis (FFM) has been widely reported as a means for continuous sorting of mixed magnetic beads from each other, as well as from non-magnetic materials [22-26]. Continuous separation and purification of magnetically-labelled cells or DNA have been demonstrated using on-chip FFM, allowing analysis of markers on cell surfaces and further investigations in medical diagnostics [27-30]. The value of this technology for foodborne pathogen sorting and analysis has not yet been determined, although there are potential advantages for the future, including multiple sorting of pathogens cultured in Universal enrichment broths. As

opposed to the tube-based assay, magnetophoresis can potentially be automated and integrated with an *in-situ* detector (e.g. optical imaging) to allow rapid sorting and detection of various pathogens.

The present study aims to assess the potential of FFM, in combination with IMS, for the rapid sorting and detection of the viable foodborne pathogens, *S. typhimurium* and *E.coli* 0157 from mixed cultures and from food pre-enrichment broths. The enrichment of pathogens from the background particulates and flora present in food sample is achieved by IMS with the benefit of multiplex sorting of FFM.

1.1 FFM for multiplex sorting of foodborne pathogens

FFM is a process for the continuous sorting of magnetic beads under the influence of a magnetic field (Fig. 1). The separation chamber for magnetophoresis is commonly a few mm in width and length, and tens of μm in depth, with a number of inlets and outlets for the sample and buffer solutions. The laminar flow regimes, predominant in microfluidic systems, result in the liquids flowing straight through the chamber in the *x*-direction, including any magnetic particles contained in the sample. When an inhomogeneous magnetic field is applied perpendicular (*y*-direction) to the direction of the flow, the magnetically susceptible particles are moved towards the magnet and thus, deflected from their direction of flow. The magnetically induced velocity, $\mathbf{u}_{\mathrm{mag}}$, on the magnetic particles depends on the volume of magnetic material (V_m) and the overall particle radius (r) [31]:

$$\mathbf{u}_{\mathrm{mag}} \propto \frac{V_m}{r} \qquad (1)$$

Under the influence of a given magnetic field, different bead types can be sorted from each other according to their size and the volume of magnetic material they contain. This principle is implemented here for the multiplex sorting of *S. typhimurium* and *E. coli* 0157 from food pre-enrichment broths using two different commercially available magnetic beads (Dynabeads® anti-salmonella) and Hyglos-Streptavidin beads (Fig. 1). The pathogen-bound bead mixture was introduced into the separation chamber together with the buffer. Under the influence of a Halbach array magnet, the magnetic beads experience differential magnetic force that leads to resolution of the two bead types (weakly magnetic Dynabead-bound *S. typhimurium* and strongly magnetic Hyglos bead-bound *E. coli* 0157).

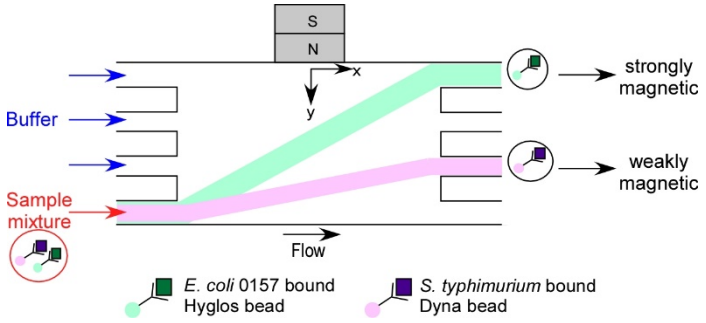

Figure 1.

2. Materials and Methods

2.1 Materials

S. typhimurium (NCTC 12023/ATCC® 14028) and *E.coli* 0157 (NCTC 12900/ATCC® 700728) were purchased from Pro-lab Diagnostics (UK). Phosphate buffered saline (PBS) and Tween 20 were supplied by Sigma-Aldrich (USA). Buffered peptone water (BPW), trypticase soy agar (TSA), tryptic soy broth (TSB), chromID®_Salmonella and chromID®_0157:H7 were supplied by bioMérieux (France). Dynabeads® anti-salmonella or Dynabeads® M270-Streptavidin were purchased from Invitrogen Dynal AS (Norway). The Hyglos-Streptavidin beads were obtained from Hyglos GmbH (Germany). Skinned chicken breast was purchased from a local supermarket. Deionised water used in the study was processed through the Simplicity® water purification system (EMD Millipore Corp., Billerica, MA, USA) with 18 MΩ cm resistivity.

2.2 Instrumentation

The design of the PMMA chip used for magnetophoresis is shown in Fig.2. The separation chamber (3 mm wide, 10 mm long, 100 µm deep) was supported by 7 round posts (200 µm diameter). The buffer inlet channel (1 mm) branched to three inlets with a similar width (0.4 mm). The sample inlet and outlet channels were also 0.4 mm wide. This chip design was different from the glass chips reported previously [32]. A deeper channel (100 µm as opposed to 70 µm [32]) was employed to achieve higher volume throughput (1.4 times), whilst the laminar flow regime was still maintained (Reynolds number (*Re*) = 0.028 at a maximum total flow rate operated (1.55 mL h^{-1})). The chip was sealed with BOPP tape (bioMérieux, France) and fitted onto the bottom part of a custom-made chip holder, whilst the top part of the holder enabled the interfacing of tubing to the chip access holes via nuts and ferrules that screwed into the holder (Fig. 3). Fused silica capillaries (150 µm ID, 363 µm OD, Polymicro Technologies, LLC, UK) were inserted into the PMMA chips inlet (length = 20 cm) and outlet (length = 5 cm) holes through the chip holder. Tygon tubing (0.254 mm ID, 0.762 mm OD, Cole-Palmer) was attached to each of the outlet capillaries and fed into an Eppendorf tube to collect the sample for further analysis.

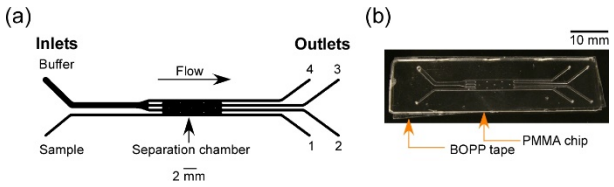

Figure 2.

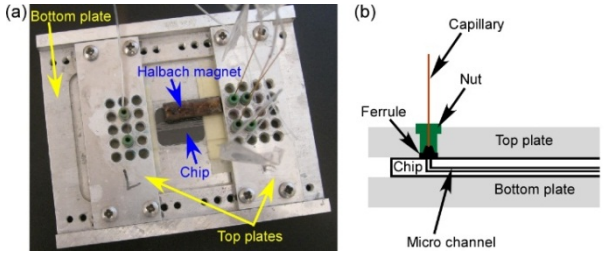

Figure 3.

The magnetic field was generated by an assembly of four NdFeB block magnets, each 4 mm x 5 mm x 5 mm arranged as a Halbach array (N50M Block set, Magnet Sales, UK) placed on the chip at the edge of the separation chamber (Fig. 3a). The advantages (i.e. concentration of the magnetic flux and high uniform spatial distributions of magnetic forces) of using of a Halbach array configuration over conventional magnets for magnetic sorting has been reported previously [33-35]. The array consists of an arrangement of magnets, each possessing uniform polarity throughout its volume. The direction of polarity is varied by precise angles between successive magnet units to provide reinforced magnetic field lines on one side of the array, whilst a negligible field is observed on the opposite side. The magnetic flux produced by the Halbach array over the separation chamber was simulated in two dimensions (*x* and *y*) employing FEMM-freeware (Fig. 4). The magnetic flux density at the magnet surface was 1833 mT and reduced to 371 mT at the sample inlet. The flux density of a single 4 mm x 5 mm x 5 mm NdFeB magnet at the surface was 1245 mT and reduced to 159.7 mT at the sample inlet (result not shown).

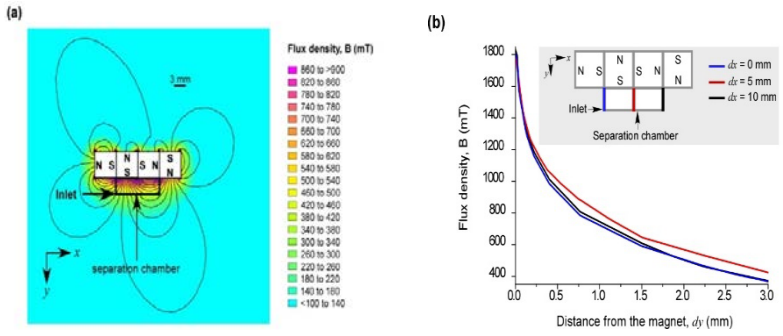

Figure 4.

2.3 Sample preparation

Preparation of unbound magnetic beads

A portion (25 µL) of Dynabeads® anti-salmonella or Hyglos-Streptavidin was washed (x3) and re-suspended in 1 mL PBST before use.

Preparation of pathogen-bound magnetic beads using pure cultures

Fresh culture isolates of *S. typhimurium* or *E.coli* 0157 were prepared on TSA plates using the standard streak plate method. An isolated colony was sub-cultured in 9 mL TSB and incubated at 37 °C for 18 h. A portion (0.2 mL) of the broth was diluted in 9 mL BPW and the absorbance (600 nm; CECIL 1011, 1000 Series) was measured and adjusted, if necessary, to ~ 0.07 corresponding to 0.5 McFarland (≡ $1.5x10^8$ CFU mL^{-1}). To confirm the actual levels in the broth (control inoculum), serial decimal dilutions were prepared (d-1 to d-5, corresponding to ~10^7 to ~10^3 CFU mL^{-1}, respectively) and a portion (100 µL) of d-5 was streaked onto TSA plate (in duplicate) and incubated overnight at 37 °C The number of colonies on the plates were enumerated, from which the actual levels were determined.

Affinity magnetic separation (AMS) of S. typhimurium and E. coli 0157 from broth cultures

For the AMS experiments, broths containing approximately 10^6 CFU mL^{-1} of *S. typhimurium* and *E.coli* 0157 were utilized with the corresponding Dynabeads® anti-salmonella and Hyglos-Streptavidin-coupled phage protein. Briefly, 25 µL of unbound beads were dispersed in 1 mL of the cultures and incubated on a rotator (Stuart Rotator SB3, Bibby Scientific Limited, UK) at ambient temperature for 20 min. The pathogen-bound magnetic beads were separated using an external NdFeB magnet (Magnet Sales, UK). A portion (100 µL) of the remaining supernatants were serially diluted in 9 mL BPW (d-1 to d-2) and 100 µL of d-1 were plated (x2) onto the chromID®_Salmonella and chromID®_0157:H7 plates. The plates were incubated overnight at 37 °C. The resulting typical colonies were enumerated and the percentage pathogen capture by IMS was calculated by noting the difference in the cell levels in the broths before and after the IMS process, as shown in the following equation.

$$\text{Capture efficiency (\%)} = 100 \times (Nt - No)/Nt \qquad (2)$$

where Nt = number of total bacteria in the original sample; No = number of un-captured pathogens in the supernatant.

or the magnetophoresis experiments, the pathogen-bound magnetic beads were washed (2 x 1 mL) with PBS buffer containing Tween 20 (0.05%; PBST) and re-suspended in 1 mL PBST before use.

AMS of Salmonella and E. coli 0157 from spiked chicken pre-enrichment broths

Skinned chicken breast samples (1 g) were each mixed with 9 mL BPW in a plastic filter bag (Tempo, bioMérieux) and homogenized for 30 s in a Stomacher (IUL Instruments, Germany). The bag was incubated at 37°C for 4 h before adding 0.1 mL of ~10^8 CFU mL^{-1} *S. typhimurium* or *E.coli* 0157, yielding a final cell concentration of ~10^6 CFU mL^{-1}. Aliquots (1 mL) of the pre-enrichment broths were treated with the pathogen-specific magnetic beads and prepared for the magnetophoresis experiments as described in the previous paragraph.

2.4 Magnetophoresis experiments

Each of the experiment described below was undertaken on a single-use PMMA chip. Before replacing with a new chip, the interface and connectors (Fig. 3.) were cleaned sequentially by flushing with water, ethanol and PBST (1 mL each) prior to re-assembling.

Deflection of native magnetic beads

Deflection of the native Dynabeads® anti-salmonella (or Dynabeads® M270-Streptavidin coupled phage protein) and Hyglos-Streptavidin-coupled phage protein beads were carried out separately at various flow rates (bead suspensions: 400 – 700 µL h^{-1}, PBST buffer: 12 – 21 mL h^{-1}). The flow rate ratio of the bead suspensions and PBST buffer was kept constant at 1:30. The bead suspensions were introduced into the microfluidic chip via 100 µL glass syringes (SGE Analytical Science, UK) and the PBST buffer via a 5 mL glass syringe (SGE Analytical Science, UK), placed in two separate syringe pumps (Harvard Apparatus, Pump 11 Elite, USA). The bead resolution was visualized with a CCD (PV10, Olympus, Japan) connected to an inverted microscope (Nikon Eclipse, TE2000-U, UK).

Deflection of pathogen-boundmagnetic beads

In preliminary experiments, 0.1 mL of the pathogen-bound beads were used in separate experiments to determine the extent of pathogen-bead deflection by quantifying the level of viable pathogens in the samples (collected in 1.5 mL Eppendorf tubes positioned at each outlet) on their respective selective media. The extent of the pathogen-bound beads that remained strongly immobilized to the separation chamber was determined as follows: (i) the Halbach magnet was removed; (ii) the syringe delivering the pathogen bound beads was replaced with 1 mL PBST and the chip flushed. The whole volume exiting the chip was collected from all the outlets and appropriate dilutions plated onto the respective selective media. This fraction was referred to as “R” (Retained). The experimental controls comprised passage of the beads through the chip and analysis of samples exiting the chip in the absence of the Halback magnet (referred to as ‘Ref’).

In the case of a mixture of two types of pathogen-bound beads, whether in pure cultures or from the chicken pre-enrichment broths, 0.5 mL of each bead suspension was premixed and an aliquot (100 µL) taken for the deflection experiments. The bead suspensions were applied to the chip at a flow rate of 5 mL h^{-1}, whereas the PBST flow rate was 150 mL h^{-1}. The extent of bead deflection and, hence, sorting of the viable pathogens was quantified in the samples (collected in 1.5 mL Eppendorf tubes positioned at each outlet) by enumerating on their respective selective media. After the deflection experiments, the extent of the pathogen-bound beads that remained strongly immobilized to the separation chamber was determined as described in the previous paragraph.

For magnetophoresis involving chicken pre-enrichment broths, a range of concentrations of Tween 20 in PBS buffer (0.05 - 0.10%) was assessed in order to determine the effect of the surfactant on the extent of bead aggregation.

3. Results and Discussion

3.1 Deflection characteristics of Dynabeads® anti-salmonella and Hyglos-Streptavidin beads

The Dynabeads (2.8 µm) are uniform in size, whilst the Hyglos beads vary from 0.7 to 2.3 µm (Figs. 5a & b, respectively). The reported iron oxide content of the Hyglos beads (50-60 wt %) is up to 4 times higher than that of Dynabeads (15 wt %).

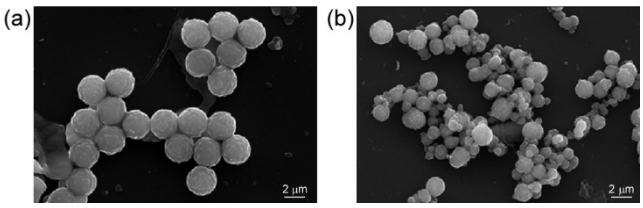

Figure 5.

Figure 6 illustrates the trajectories of the Dynabeads® anti-salmonella and Hyglos-Streptavidin in the presence of the Halbach magnet. At the bead flow rates of 400-600 µL h^{-1} (equivalent to a flow velocity of 0.11 – 0.17 mm s^{-1} in the separation chamber), both the bead types showed a strong attraction to the magnetic field and were largely trapped in the separation chamber adjacent to the magnet. By increasing the flow rate to 700 µL h^{-1} (flow velocity = 0.19 mm s^{-1}), the beads were attracted less and flowed through the chamber exiting the outlets. At this flow rate, in general, the Hyglos beads were recovered at the outlet 4, whilst the majority of the Dynabeads was retrieved at the

outlets 2 and 3. As expected, the Dynabeads deflected less than the Hyglos beads, due to their lower iron oxide content (as per equation 1).

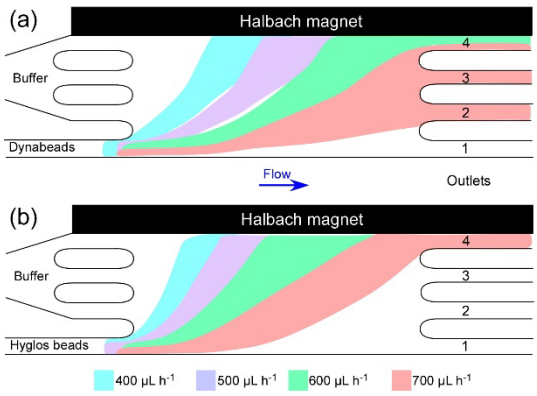

Figure 6.

3.2 AMS and magnetophoresis of pathogen-bound beads

The gold standard microbiological culturing technique were employed to characterize the efficiency of our platform. It should be emphasized that this method is not envisaged to be employed in the automated integrated version of the device. The IMS process is not 100% specific for the capture of the target pathogen, especially when applied to mixed microbial populations in the chicken enrichment broths. In the latter case, the beads are also likely to non-specifically bind to some background flora [36,37]. The beads used for the IMS process are normally for the purpose of enrichment of the target pathogens (i.e. increase the signal-to-noise ratio) and not 100% purification of the target pathogens from the background flora [38]. It should also be noted that selective media are not 100% selective for the isolation of the target pathogen, especially when present in a mixed microbial population. Some background organisms can grow in the selective media too [39]. Thus, for characterization of the magnetophoresis process applied to mixed beads (previously subjected to IMS in food enrichment broths), the use of selective media is necessary in order to detect/enumerate the 'typical' colored colonies produced by the test pathogens.

The binding efficiency of pure cultures of *S. typhimurium* and *E. coli* 0157 to the corresponding affinity Dynabeads and Hyglos beads by the AMS process was found to be excellent (Table 1; 97.5% and 100% for *S. typhimurium* and *E. coli* 0157, respectively.

Table 1. Binding efficiency of pure cultures to the magnetic beads by the AMS process.

Pathogen	Starting concentration (CFU mL^{-1})	Concentration after AMS process (CFU mL^{-1})	Binding efficiency (%)
S. typhimurium	$1.40x10^6$	$1.37x10^6$	97.5
E. coli 0157	$1.05x10^6$	$1.05x10^6$	100

Figure 7 shows the magnetophoretic deflection of *S. typhimurium* and *E. coli* 0157 specifically immobilized to the affinity Dynabeads and Hyglos beads, respectively, under the influence of the Halbach magnet array. The deflection characteristics of the pathogen-bound beads was notably different to that of the native beads (Fig. 6). Higher flow rates (up to 5 mL h^{-1} as opposed to ≤ 0.7 mL h^{-1} for the native beads, Fig. 6) were needed in order to pass the beads through the microfluidic chip because of bead agglomeration noticed with both the bead types, resulting in strong attraction to the magnet (Figs. 7 a, b). The bead aggregation is attributed to the formation of immune complexes between the cells and the affinity beads. Interestingly, this is advantageous as it allows a higher volume throughput of the beads. As expected, the strongly magnetic *E. coli* bound-Hyglos beads were deflected to a greater extent, a fraction of which exited the outlet 4, whilst the majority were retained in the chamber (Fig. 7b). In contrast, the *Salmonella* bound-Dynabead population exited all the four outlets, as well as a proportion being retained in the chamber (Fig. 7a).

Fig. 7c shows the results of deflection of a mixture of the two pathogen-bound bead populations. The majority of *E. coli* 0157- Hyglos beads exited the outlet 4, with smaller fractions also emerging from the outlets 1 and 3, unlike the observation in Fig. 7b with non-mixed bead population. This shows that interaction between different pathogens and beads can lead to aggregates of varying sizes (and magnetic contents) that create overlaps in the magnetophoretic deflection of mixed bead populations. Despite this observation, it was possible to resolve a significant proportion of a mixture of *E. coli* 0157-Hyglos beads (outlet 4) and *S. typhimurium*-Dynabeads (outlet 2); starting from a sample of the mixed beads. Although, the resolution of both the pathogen-bound bead populations was not perfect, and needs to be improved further, the sorting was achieved within 1.2 min indicating potential for rapid multiplex sorting of the foodborne pathogens.

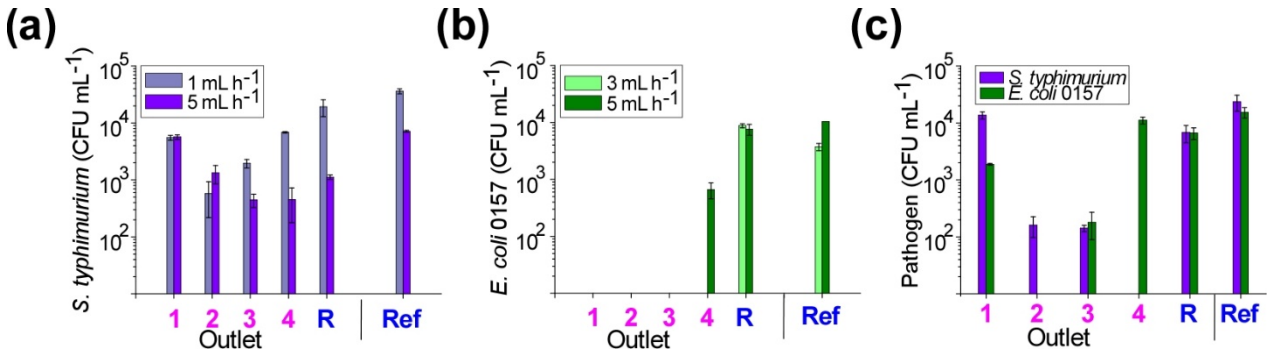

Figure 7.

3.3 AMS and magnetophoretic sorting of *S. typhimurium* and *E. coli* 0157 from spiked chicken pre-enrichment broth (n=2)

The AMS capture rates of the pathogens from pure broth cultures was much higher (97.5 - 100%) compared with the pathogens isolated from the spiked chicken pre-enrichment broths (40% and 82% for *S. typhimurium* and *E. coli* 0157, respectively, Table 2). This is attributed to background components (e.g. natural microbial flora, fat and proteins) in the chicken pre-enrichment broth that can interfere with the specific binding of the pathogens to the affinity magnetic beads. Similar observations have been reported previously (e.g. *E. coli* 0157 from pre-enriched ground beef [37, 40]).

Table 2. AMS binding efficiency from the spiked chicken pre-enrichment broths.

Pathogen	Starting concentration (CFU mL^{-1})	Concentration after AMS process (CFU mL^{-1})	Binding efficiency (%)
S. typhimurium	$9.35x10^5$	$3.74x10^5$	40
E. coli 0157	$9.30x10^5$	$7.63x10^5$	82

The deflection behaviour of the mixed *S. typhimurium*-Dynabeads and *E. coli* 0157-Hyglos beads prepared from the spiked chicken pre-enrichment broths containing varying concentrations of Tween 20 is shown in Fig. 8. Depending on the concentration of Tween 20 used (Fig. 8b), there is a fraction (~5- 35%) of *S. typhimurium* recovered along with *E. coli* 0157 (~25-90%) from the outlets 4. This differs from the observation for the pathogen-bead mixture prepared using pure cultures, where no *S. typhimurium* was recovered from the outlet 4 (Fig. 7c). It is

possible that the background food components/organisms may cause an increase in the bead aggregation and, hence, the magnetic content of the agglomerate leading to a fraction of the *S. typhimurium*-beads emerging in the outlet 4.

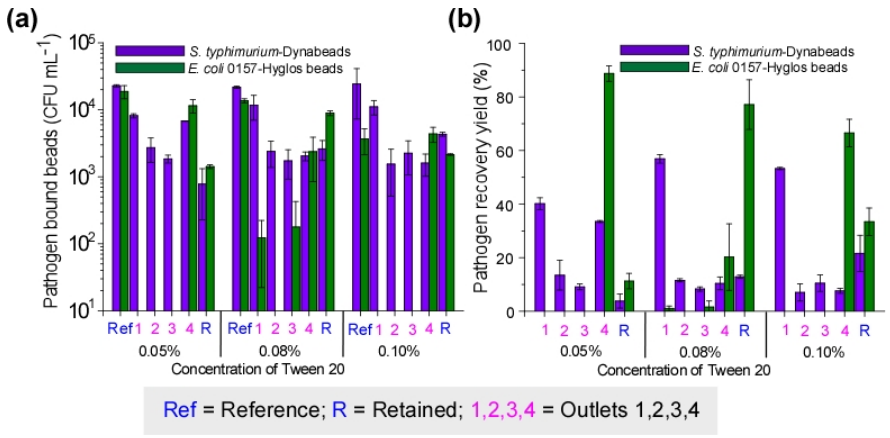

Figure 8.

Despite this, a pure population of *S. typhimurium*-Dynabeads was recovered from the outlets 1 to 3 (total 62%), whilst 90% of *E. coli* 0157-Hyglos beads (and a lower proportion (34%) of *S. typhimurium*-Dynabeads) were retrieved at the outlet 4 in the presence of 0.05% Tween 20 (Fig. 8b). Although the sorting of the pathogen-bound bead mixture is not absolute, in this preliminary study the magnetophoresis process clearly allows separation of each of the two types of pathogen-bound beads. The sorting process may be improved further, e.g. by reducing the extent of particulates before the AMS step, inclusion of biomaterials that prevent/reduce the bead aggregation and non-specific binding in the chips during magnetophoresis and varying the chip configurations.

At 0.08% Tween 20, approximately 77% of the *S. typhimurium*-Dynabeads (and 1.5% *E. coli* 0157-Hyglos beads) were retrieved from the outlets 1-3, conversely 78% of *E. coli* 0157-Hyglos and 13% *S. typhimurium*-Dynabeads) were recovered after flushing the chamber with PBST (Fig. 8b). At 0.1% Tween 20, a pure population of *S. typhimurium*-Dynabeads was recovered (total 72 %) from the outlets 1-3, whilst a mixed population of *S. typhimurium*-Dynabeads (6 %) and *E. coli* 0157-Hyglos beads (67 %) was recovered from the outlet 4. In this experiment, the recoveries of *E. coli* 0157-Hyglos and *S. typhimurium*-Dynabeads after flushing the chamber were ~33% and ~20%, respectively (Fig. 8b). These findings are also considered of practical value for the sorting of each of the two types of pathogen-bound beads. Several parameters are expected to improve the bead resolution and

recovery by magnetophoresis, including the use of alternative surfactants at sub-lethal concentrations, and manipulation of the deflection flow rates, temperature [31] and the medium viscosity.

The current workflow comprised: a food pre-enrichment step; 40 min AMS process; 5 min microfluidic device set-up; fast magnetophoresis (~ 1.2 min) to sort/enrich different pathogen-bound beads from bead mixtures; and plating of the resolved beads onto the pathogen selective culture media for subsequent detection of the typical colonies. Magnetophoretic resolution of foodborne pathogens grown in a Universal primary food enrichment broth is foreseen to present several advantages, including: (i) enhanced detection of the resolved pathogen on conventional selective cultural media attributed to reduced interference from the background flora; (ii) potential to enhance performance of the current qPCR methods through reduction of interference from the background PCR inhibitors and high concentration of the non-specific microbial sequences; and (iii) potential to integrate the magnetophoretic sorting with enhanced detection, (e.g. quantum dot and time-resolved fluorescence [41-43] and surface enhanced Raman spectroscopy (SERS, [44, 45]) directly into the chip.

4. Conclusions

To the author's knowledge, this is the first time continuous on-chip magnetophoresis has been demonstrated for the fast multiplex sorting/enrichment of two foodborne pathogens (*S. typhimurium* and *E. coli* 0157), pre-captured and concentrated on different magnetic beads by AMS of the pathogens from a primary food enrichment broth. The study systematically describes the characterisation and optimisation of the magnetophoretic sorting parameters for two different size and magnetic content beads, the AMS of the foodborne pathogens and their magnetophoretic resolution from mixed bead populations. The results are discussed in detail and the potential future implications of the findings considered.

5. Acknowledgements

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Figure captions

Figure 1. Principle of multiplex sorting of two different types of pathogen-bound magnetic beads by free-flow magnetophoresis.

Figure 2. (a) Schematic design of the microfluidic. (b) The final fabricated PMMA chip.

Figure 3. (a) A photograph of the chip holder fabricated in-house, consisting of a bottom plate with a viewing section and two top plates with multiple screw holes to connect to the bottom plate. (b) Schematic illustrating the interfacing of an inlet/outlet capillary to the microfluidic device placed between the top and bottom plates of the chip holder. The capillary was held by a ferrule, which covered the chip inlet hole and sealed against the chip when the PEEK nut was tightly screwed into the chip holder.

Figure 4. Two-dimensional simulation of the magnetic field generated by the assembly of four small NdFeB magnets in a Halbach array over the separation chamber, employing FEMM freeware (http://femm.foster-miller.net). (b) Comparison of the different magnetic flux densities (B) at various distances (*dy*) across the separation chamber (3 mm wide, 10 mm long) where *dx* refers to the distance along the separation chamber (= 0 mm and 10 mm at the inlet and outlet, respectively).

Figure 5. SEM micrographs: (a) Dynabeads® anti-salmonella and (b) Hyglos-Streptavidin beads used for the AMS of *S. typhimurium* and *E. coli* 0157, respectively.

Figure 6. Deflection of (a) Dynabeads® anti-salmonella and (b) Hyglos-Streptavidin beads as a function of the bead flow rates. Experiments were performed in duplicate (n=2).

Figure 7. Deflection behaviour of (a) *S. typhimurium*-Dynabeads and (b) *E.coli* 0157-Hyglos beads at flow rates of 1 – 5 mL h^{-1}, and (c) a mixed sample containing *S. typhimurium*-Dynabeads and *E.coli* 0157-Hyglos beads prepared from pure cultures at 5 mL h^{-1}. “Ref” refers to the experimental controls comprising passage of the beads through the chip and analysis of the samples exiting the chip in the absence of the Halbach magnet. “R” refers to the recovery of the pathogen-bound beads that remained strongly immobilized to the separation chamber (n=2).

Figure 8. Effect of varying concentrations of Tween 20 on the deflection characteristics, expressed as CFU mL^{-1} (a) and % pathogen recovery (b), of mixed *S. typhimurium*-Dynabeads and *E.coli* 0157-Hyglos bead suspensions prepared from chicken pre-enrichment broth (flow rate: 5 mL h^{-1}; n=2).