Quantitative separation of bacteria in saline solution using lanthanide Er(III) and a magnetic field

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A trivalent lanthanide ion, erbium (Er³⁺), has been used in combination with a magnetic separation technique to isolate seven bacterial species from suspensions in 0.9% saline. Erbium has an exceptionally high atomic magnetic moment of 9.3 Bohr magnetons, and following addition as ErCl₃ (final concentration 5 mM) to bacterial suspensions, it imparts the magnetic moment to the bacterial cells by ionic binding to the cell surface. Strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Enterococcus faecalis* were obtained from the Quality Control Depository of The Cleveland Clinic Foundation, Cleveland, Ohio, USA as suspensions in 0.9% NaCl, in concentrations ranging from 10² to 10⁸ c.f.u. ml⁻¹. Bacteria were separated from solution inside a capillary flow cell exposed to a highly non-homogeneous magnetic field (maximum field intensity was 0.4 T) and quantified by a light scattering method. The quantity of cellular deposition in the magnetic field was correlated with the initial concentration of cells in the suspension, expressed in c.f.u. ml⁻¹, and sample volume (1.5 and 3.0 ml), sample pH (prior to ErCl₃ addition), affinity to Gram stain (negative vs positive) and species. Magnetic deposition was observed for concentrations as low as 10² and 10³ c.f.u. ml⁻¹, and a significant correlation between average scattered light intensity and initial cell concentration (correlation coefficient ≥ 0.98) was established in the range 10⁴ to 10⁸ c.f.u. ml⁻¹ for all but one species (*P. aeruginosa*). Magnetic deposition increased with increasing pH from 7.0 to 10.0 which is consistent with the prevailing view on the mechanism of the lanthanide ion binding to bacterial cells (ionic, to the cell surface). No significant difference in magnetic deposition was observed between individual strains, or between Gram-positive and -negative bacteria. Magnetic isolation of cells may find application in rapid total cell count determination, such as rapid urine screening.

Introduction

At the present time there are several commercial sources of magnetic reagents suitable for use in the magnetic separation of cells. The reagent which has become most widely reported, as used in the laboratory as well as the clinical purification of cells, is a suspension of uniformly-sized polymeric beads doped with magnetite and surface-covered with different functional groups, such as -COOH (Bangs Laboratories) suitable for binding to target cells.

Beads conjugated to a selection of antibodies, such as sheep anti-mouse IgG (Dynabeads, Dynal) (Kemshead *et al.*, 1990) or anti-human leukocyte (Collaborative Research) are also available. The available sizes of the beads range from submicron to mm.

In this study we have used ErCl₃ which forms trivalent erbium cations in aqueous solution and preserves its

exceptionally high atomic magnetic dipole moment (9.3 Bohr magnetons) in various chemical structures (Bozorth, 1968). The published data demonstrate that lanthanides, such as Er, have a high affinity for the external cell surface of micro-organisms (Evans, 1990). In previous studies by other authors, erbium, dysprosium, holmium, thulium, and gadolinium were evaluated for efficacy in imparting magnetic susceptibility to polymeric particles and their subsequent removal from flowing fluid by a non-homogeneous magnetic field (Ahlberg & Walley, 1982; Evans & Tew, 1983; Russell *et al.*, 1987). Erbium was used for magnetization of osseous and cartilaginous wear particles in specimens of synovial fluid followed by magnetic separation and morphological analysis (Evans, 1983). Preliminary results on magnetic separation of erbium-treated bacteria and blood cells have also been reported (Russell *et al.*, 1983; Zborowski *et al.*, 1991).

Fig. 1. Elements of the magnetic separation process. A sample of bacterial suspension with 5 mM- ErCl_3 was transferred by a peristaltic pump to a capillary flow glass chamber where the magnetized cells separate from the flowing medium and concentrate at the bottom of the flow chamber in the area of the inter-polar gap of a strong permanent magnet assembly. The effluent is absorbed by a wick mounted at the fluid exit end of the flow chamber whereby the magnetically concentrated cells are dry-fixed inside the chamber. Following the magnetic concentration, the wick with the absorbed fluid is discarded and the flow chamber with the concentrated cells is transferred to a microscope (not shown) set in a dark background mode and coupled to a light detector meter and recorder. The scattered light intensity, proportional to the number of cells in the microscope field of view, is recorded as a function of distance along the flow chamber's longitudinal axis of symmetry.

The magnetic separation technique and the evaluation of the magnetically concentrated cellular mass was adapted from an industrial instrument, the ferrograph, used for screening of wear particles in engine lubricants (Anderson, 1983). The particular questions posed in this study were: (1) is magnetic separation of bacteria capable of producing a quantitative measure of the cell concentration in the sample, (2) what is the sensitivity of the method for evaluating cell concentration in 0.9% saline suspensions of typical bacterial species found in urine and (3) are there any differences in the separation of Gram-positive and -negative species? The results of this study were compared with the results of bacterial growth and colony counts on solid media.

Methods

Bacterial suspensions. All seven species of bacteria which were used in this study, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Enterococcus faecalis*, were obtained as suspensions in 0.9% saline from the Quality Control strains (stored at -20°C) of the Microbiology Department of the Cleveland Clinic Foundation, Cleveland, Ohio, USA. These bacterial suspensions were diluted to about 1.0×10^8 c.f.u. ml^{-1} as evaluated by McFarland standards of BaCl_2 (aq) turbidity in filtered normal saline (0.9% NaCl, $0.2\ \mu\text{m}$ sterile filter, Gelman Sciences). The original bacterial suspensions, whose pH values were between 6.5 and 7.0, were then sequentially diluted tenfold with filtered normal saline and adjusted to a specified pH value of 4.0, 6.0, 7.0, 8.0, 9.0 or 10.0 using 0.5 M-NaOH or HCl solutions previously filtered with $0.2\ \mu\text{m}$ filters. The concentration of the bacterial suspensions was also evaluated by colony counting after 24 h incubation at 35°C .

Magnetizing agent. ErCl_3 (99.99% purity, A. Johnson Matthey) was prepared in ready-to-use vials. This was done by dissolving solid ErCl_3

in twice-distilled water to give a stock solution with a concentration of 150 mM. The stock solution was filtered through a $0.2\ \mu\text{m}$ filter and dispensed in $50\ \mu\text{l}$ volumes to each sample vial (1.5 ml, Tekmar Co.). The vials were placed in a convective heating cabinet at 60°C for 6 h to evaporate off the water. The concentration of ErCl_3 in the vials was 5 mM after addition of 1.5 ml bacterial suspension.

Bacterial deposition substrate. The bacterial deposition substrate consisted of two sections. One section was the flow chamber made from modified microscope cover slides, and the other section was a wick made from filter paper, as illustrated in Fig. 1. The wick served the purpose of collecting the effluent from the flow chamber. The flow chamber was made of two glass slides, 60 mm long $\times$ 25 mm wide $\times$ 0.2 mm thick, with a painted Teflon pattern forming a 5 mm wide fluid channel. These slides were provided by the Standard Oil Engineered Materials Co. and were prepared for use in their Slide Ferrograph Analyzer (Zborowski *et al.*, 1991). Three layers of Scotch double-coated tape (#665, 3M Co.) separated the bottom slide from top slide by 0.2 mm.

Magnet assembly. A pair of neodymium-iron-boron magnets (maximum energy product 28 MGOe, dimensions $5 \times 5 \times 1.3$ cm; Permag Co.) were used. The magnets were connected by a large bottom plate and two pole pieces of soft iron with tapered ends for amplification of magnetic flux lines in the area of the inter-polar gap in which the magnetic separation took place. An aluminium plate of 1.4 mm thickness maintained a uniform distance between the pole pieces of the magnet and formed a high field-intensity, high field-gradient magnetic separation area (Fig. 1).

Experimental procedure. The investigational plan consisted of optimization of the magnetic separation parameters at low concentrations of a single bacterial species in saline suspensions, and determination of differences in magnetic separation between Gram-negative vs Gram-positive species mixtures.

Bacterial suspensions with preset concentrations of 10^2 , 10^3 , 10^4 , 10^5 , 10^6 or 10^8 c.f.u. ml^{-1} at volumes of 3.0 ml were added to the ErCl_3 vials and incubated at 37°C for 3 min. The solutions were then pumped through Tygon tubing (i.d. $\frac{1}{8}$ in, o.d. $\frac{1}{4}$ in, length 1.5 ft; Fisher Scientific Co.) into the separation chamber placed onto the magnet at a flow rate of $1\ \text{ml min}^{-1}$ using a peristaltic pump (Perista, Atto). After pumping

the sample, the flow channel was dried using the Microduster (Texwipe) to dry the specimen.

The solid deposition of bacterial cell mass in the interpolar gap region of the separation chamber was quantified by measurement of the intensity of light scattered by the deposition using a photographic exposure meter attached to a microscope (BH-2, Olympus). The microscope was adjusted for dark background viewing. The intensity of light scattered by the individual bacterial cells present in the flow chamber was recorded as a function of position of the microscope stage using an x - y recorder (Plotamatic type 815M, MFE Corporation). The y input was connected to the exposure meter and the x input was connected to a 10-revolution potentiometer linked to a longitudinal stage position knob supplied with 12 V DC. The area under the plotted peak of the scattered light intensity as a function of distance y vs x , within the range of twice the width of the interpolar gap and centred about the midline of the gap, was measured with a planimeter (Keuffel & Esser Co.). The value obtained is referred to as an average scattered light intensity I_{av} , in relative units.

The values of I_{av} were correlated with the values of initial bacterial concentrations, c , and the pH values of the initial bacterial suspension.

The interference due to dust particles and other possible artefacts ('noise') were evaluated using 0.9% saline in 3.0 ml vials with ErCl_3 and processed in the same way as the bacterial samples.

The results were analysed by Statgraphics (Version 2.6, STSC) on an IBM AT personal computer using regression analysis (correlation coefficient, intercept, slope, line of 95% confidence limit) and hypothesis testing by Student's t test (a difference was taken as significant if $P < 0.05$).

Results

The optimization of magnetic separation parameters was done using *E. coli*. The magnetic separator response to two different volumes of the sample, 1.5 and 3 ml, of 10^5 c.f.u. *E. coli* ml^{-1} is presented in Fig. 2. The number of magnetically collected cells rose in direct proportion to the volume of the sample.

The effects of pH on the number of magnetically collected cells, as measured by the light scattering technique, is presented in Fig. 3 showing data obtained for *E. coli*, *P. aeruginosa* and *Ent. faecalis*. These studies were performed with cell concentrations adjusted to 10^5 c.f.u. ml^{-1} . A significant correlation between pH and the strength of the scattered light signal was observed in the range pH 7.0 to 10.0 for all three species. The correlation was weak, but still noticeable, in the range pH 4.0 to 7.0. In the protocol used for subsequent evaluation of the sensitivity of the magnetic separation technique the samples were adjusted to pH 8.6 prior to mixing with ErCl_3 .

Fig. 4 shows the correlation between the number of magnetically collected cells, as measured by the scattered light intensity, I_{av} , and their initial concentration, c , as evaluated by colony counting, for all seven species. Control samples, comprising only 0.9% NaCl and 5 mM- ErCl_3 , showed $\ln I_{av} < 0$. The correlation coefficient of a regression line between mean $\ln I_{av}$ and $\log c$ was equal to or higher than 0.98 for concentrations ranging from

Fig. 2. Variation of cellular deposition density in the magnetic field, quantified by scattered light measurements, with 1.5 (■) or 3.0 ml (□) sample pumped through the flow chamber (at constant cell concentration) and with varying cell concentration (at constant sample volume). Cell type: *E. coli* incubated for 3 min with 5 mM- ErCl_3 in physiological saline at 37 °C; initial pH of cell suspension, 6.0.

Fig. 3. Variation of cellular deposition density in the magnetic field, quantified by scattered light measurements, with varying initial pH of the cell suspension prior to incubation with 5 mM- ErCl_3 (sample volume 3 ml). Bacteria: *E. coli* (■), *P. aeruginosa* (▤) and *Ent. faecalis* (□).

$3 \leq \log c \leq 8$ for four of the seven species, *Prot. mirabilis*, *S. epidermidis*, *S. saprophyticus* and *Ent. faecalis*. Interestingly, slopes of the $\ln$ - $\log$ regression lines fall within a rather narrow range of 0.42 (*P. aeruginosa*) to 0.60 (*S. epidermidis*). The presence of magnetic deposition was detected even at lower concentrations than 10^4 or 10^3 c.f.u. ml^{-1} ; however, the intensity of the optical signal did not correlate with the cell concentration as evaluated by colony counting in this low concentration range, especially for *P. aeruginosa*. A possible explanation of this effect is an inherent inability of the colony counting method to account for dead cells although these bind erbium (Evans, 1990) and can be detected by magnetic

Fig. 4. Correlation of magnetic deposition density, quantified by scattered light measurement, of a single strain of bacteria, to cell concentration, c , in physiological saline suspensions, presented as a stack of $\ln$ -log charts. Values on each chart represent means of repeated experiments on a single bacterial strain. Bacterial species are identified by numbers 0–6 and are described below. For each species a regression analysis was performed in the concentration ranges in which scattered light intensity varied (almost) linearly: $\ln I_{av} = m \log c + b$, as described below.

(0) *E. coli*

$$m = 0.58, b = -1.66, 4 \leq \log c \leq 8, n = 20, r = 0.979$$

(1) *K. pneumoniae*

$$m = 0.55, b = -1.38, 4 \leq \log c \leq 8, n = 20, r = 0.985$$

(2) *Prot. mirabilis*

$$m = 0.56, b = -1.32, 3 \leq \log c \leq 8, n = 25, r = 0.979$$

(3) *S. epidermidis*

$$m = 0.60, b = -1.44, 3 \leq \log c \leq 8, n = 25, r = 0.947$$

(4) *S. saprophyticus*

$$m = 0.51, b = -0.99, 3 \leq \log c \leq 8, n = 25, r = 0.986$$

(5) *P. aeruginosa*

$$m = 0.42, b = -0.48, 3 \leq \log c \leq 8, n = 25, r = 0.935$$

(6) *Ent. faecalis*

$$m = 0.45, b = -0.48, 3 \leq \log c \leq 8, n = 25, r = 0.988$$

separation at low c.f.u. ml⁻¹. Moreover, cellular debris and cell culture substrate fragments could also bind erbium and concentrate on the microscope slide in the magnetic field, producing a false positive signal at low cell concentration.

Additional variability in the intensity of the optical signal around its mean value is attributed to the

Fig. 5. Summary of results of magnetic cell deposition evaluation using scattered light measurement, as a function of cell concentration in suspension, grouped for Gram-negative (a) and Gram-positive species (b). Solid lines represent the regression line, broken lines represent 95% confidence limits.

limitations of the light scattering technique at its present stage of development. The area viewed by the optical sensor through the 100 $\times$ magnification objective covered only about one-tenth of the total width of the sample pathway and therefore was subject to interferences from random cell aggregations. This drawback of the deposition scanning could be overcome in future studies by matching the flow channel width with the light sensor viewing area diameter.

Extrapolation of the regression line to the level of $\ln I_{av} = 0$, representing signal noise, intersects the abscissa at cell concentrations of about 10² c.f.u. ml⁻¹ for all of the bacterial species evaluated, which represents the lowest cell concentration detectable by this method in the ideal situation of absence of interference from cellular debris and non-viable cells.

Comparison of summary data for Gram-positive and

-negative organisms is given in Fig. 5. At concentrations of 10^4 c.f.u. ml⁻¹ and higher, Gram-positive organisms apparently showed a higher scattered light signal than Gram-negative organisms; however, this observation could not be supported statistically due to high variability of the optical signal.

Discussion

Use of a paramagnetic lanthanide salt, ErCl₃ at 5 mM concentration in 0.9% saline, allowed us to magnetically tag bacterial cells and isolate them from a flowing solution in a flow chamber exposed to a high-gradient magnetic field. The effect of magnetic interaction was observed for both Gram-positive and -negative organisms. A correlation better than 0.98 between the initial cell concentration in suspension and the cellular mass in the magnetically separated material, as measured by scattered visible light densitometry, was observed for cell concentration ranges from 10^4 (for some species 10^3) to 10^8 c.f.u. ml⁻¹, except for one species, *P. aeruginosa*. No explanation of the exceptional behaviour of *P. aeruginosa* can be given at this time; possibly, it was related to the limitations of the light scattering technique employed. Magnetic separation effects, not correlated to the cell concentration, were observed at 10^2 and 10^3 c.f.u. ml⁻¹.

Erbium in aqueous solutions in the pH range tolerated by bacterial cells, pH 6–8, exists predominantly in the form of a trivalent cation (Evans, 1990). The mechanism of Er³⁺ binding to the cell surface is mostly ionic, with many different Er³⁺ binding sites, such as carboxyl groups of glycoproteins, differing in affinity and binding capacity (the biochemistry of lanthanides is extensively reviewed in Evans, 1990). The other well recognized lanthanide binding sites are the Ca²⁺ receptor sites on the cell wall (Lansman, 1990). Published observations which are typical of the cell surface binding mechanism of lanthanides include uptake of ¹⁴⁰La³⁺ by *Ent. faecalis* independent of temperature, accumulation of ¹⁴⁴Ce in about equal amount in live and dead cells, and the high reaction rate (equilibrium is attained in 1 min) (Evans, 1990).

Erbium has a strong complexing character and thus interacts strongly with the most commonly used buffers containing phosphate or bicarbonate. Adequate results have been reported with the use of HEPES (Graham & Selvin, 1982), and with Krebs' and Ringer's solutions in which phosphate buffer had been replaced by Tris buffer. Erbium generally has a lower reaction rate constant than Ca²⁺ or Mg²⁺ and other metal ions in binding to the external cell surface, and therefore the presence of other metal ions in solution interferes little with the Er³⁺ reaction with bacterial cells (Evans, 1990).

The observed increase in the magnetic deposition with increasing pH in the range pH 6.0 to 10.00, at a constant cell concentration, could be explained on the grounds of the ionic binding of Er³⁺ to carboxyl groups on the cell membrane surface. These groups contribute to the isoelectric point of about 6 (pI 5.32 with other functional groups blocked) (Sherbet & Lakshmi, 1973). Other studies of the effect of extracellular pH on metal ion binding to bacterial cells also show increased metal ion binding with increased pH (Hoyle & Beveridge, 1983). The contribution of the Ca²⁺ binding sites to the overall Er³⁺ binding was not evaluated in this study. A considerable variability in the optical signal precluded demonstrating a significant difference in the magnetic deposition between the Gram-positive and -negative bacteria treated with ErCl₃ although higher binding of several metals to Gram-positive bacteria has been reported (Mullen *et al.*, 1989).

The use of a physically dissolved ionic paramagnetic marker, such as Er³⁺, in this study, instead of a suspension of magnetic microbeads avoided interference from a free magnetic probe with the magnetically separated cells. Indeed, in the control samples in this study, in which only 5 mM-ErCl₃ solution in 0.9% saline was processed in the magnetic separator, no deposition was observed which could be directly related to the presence of ErCl₃ itself. In other words, the magnetic field did not perceptibly precipitate ErCl₃ from the solution unless Er³⁺ had been bound to the particulate elements of the suspension.

The novel magnetic separation apparatus we have described is rather simple in operation. Assuming that it could be adapted to the specialized requirements of a particular specimen, such as urine, one may hope to obtain a quantitative response of the instrument to bacteria in a specimen in about 15 min. The magnetically induced deposition of dry cellular mass on the glass slide is suitable for storage and further analysis, such as staining for specific pathogen identification and microscopic examination. Studies on urine specimens are needed to evaluate possible interferences from other urine components such as inorganic phosphate and proteins (Pezzlo, 1988).

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