

Use of superparamagnetic particles for isolation of cells

Summary

This report describes the preparation and characterization of synthetic ferritin-like particles produced by precipitation of magnetite from a mixture of ferrous and ferric ions in the presence of dextran. The 3-nm diameter particles, containing magnetite cores surrounded by chemisorbed dextran, had a magnetization of 46.7 emu/g of iron with Mössbauer quadrupole splitting of $2\Delta = 0.76$ mm/s. The application of these particles as a laboratory reagent for isolation of *Legionella* from other water bacteria was successfully tested. A 400-fold enrichment for *Legionella* was obtained.

Key words: Magnetophoresis; magnetite; antibody; cell separation; paramagnetism; microspheres.

Introduction

Iron oxide hydrates form hydrosols from solutions containing solutes which complex with iron. These solutes seem to limit the growth of the hydrate crystals. Hydrosol forming agents previously investigated with hydrated iron oxides were citrate [1], dodecanoic acid [2], and dextran [3]. The presence of citrate in polymerizing ferric oxide hydrate solutions resulted in spherical particles of uniform diameter, about 7 nm, similar to cores of the protein ferritin [1]. Particles polymerized from preparations with dextran were 3 nm in diameter and had superparamagnetic properties like ferritin, but their magnetization was quite low [3]. High concentrations of dodecanoic acid present during precipitation of magnetite from a mixture of ferrous and ferric ions produced non-uniform particles below 10 nm diameter. These particles remained as a magnetic hydrosol, termed 'ferrofluid' [2].

The protein ferritin is essentially a ferric oxide hydrate hydrosol stabilized by apoferritin. If the inorganic cores could be partially reduced to magnetite, the product would then be a magnetic hydrosol or ferrofluid as defined above, but would contain much smaller sized particles. Partial reduction of the cores of ferritin in situ does not produce magnetite, however, because ferritin reduction goes to completion with the formation of soluble ferrous ion instead of magnetite [4]. We describe the preparation, properties and uses of particles resembling ferritin or Imferon [5], but with a magnetization great enough to permit their use for magnetophoretic [6] separations of bacteria.

Materials and Methods

Preparation of magnetite-dextran complex

A 30-ml solution containing 0.18 g of $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 0.14 g of $\text{FeCl}_2 \cdot 4 \text{H}_2\text{O}$ and 0.2 g of dextran T-500 ($M_w = 478\,000$, $M_n = 192\,600$; Pharmacia Fine Chemicals, Piscataway, NJ) was added dropwise, with stirring, to a solution containing 6.4 g of NaOH in 60 ml of deionized water in a beaker that was placed in a boiling water bath. After stirring for 30 min, the precipitate was cooled to 25°C, adjusted to pH 8.5 with 12 N HCl, dialyzed against water, and stored at 4°C.

To determine the stoichiometric equivalence of the iron and dextran, preparations were made in test tubes from 1/3 the above quantities. For these experiments, the amounts and molecular weights of dextran were varied from 4000 to 2 000 000. The magnetite which formed in iron excess settled readily out of solution. The supernatant fluids from the preparations were analyzed for dextran by the colorimetric indole test [7] and for total iron by an atomic absorption spectrometer (Perkin Elmer Corp., Stamford, CT).

Magnetization was determined by the Curie method [8] using 50 μl particle suspensions containing 300 μg of iron. Particle suspensions were placed in capillary tubes and exposed to a non-homogeneous magnetic field generated by an electromagnet. Forces were measured with an electrobalance (Cahn Instrument Co., Paramount, CA). Magnetization was determined with applied fields from 3000 gauss (G) up to the saturation level for the particles, 15 000 G. To determine the relative amounts of ferric and ferrous iron in the oxide complexes, particles were dissolved in concentrated sulfuric acid, adjusted to pH 4 with 2 M acetic acid, and analyzed by absorption spectroscopy at 515 nm (ferrous) and 396 nm (ferrous and ferric isosbestic point) according to a published procedure [9]. Mössbauer spectra were obtained with a custom-built apparatus. Test samples were supported on filter paper. The 5 mCi ^{57}Co gamma radiation source was oscillated at a constant acceleration at 10 Hz by means of a function generator (Model NFG-N-5, Proneida Co., Haifa, Israel) coupled to a driver and transducer (Model NDF-N-5, Proneida). The intensity of different gamma energies transmitted through the samples were monitored by a scintillation detector (Bicron Electronics, Newbury, OH) and accumulated for 10 h on a multichannel analyzer (Proneida). An iron foil containing ^{57}Fe was used to calibrate the apparatus.

Purification of protein A

The procedure was a modification of a published method [10]. *Staphylococcus aureus* Cowan 1 was grown to late exponential phase in 10 l of phytone-yeast extract- K_2HPO_4 (PYK) broth, pH 7.2, containing 0.2% glucose [11]. The culture was harvested by centrifugation at $10000 \times g$ for 20 min, washed twice in 0.85% NaCl, and stored at $-20^\circ C$. Approximately 30 g wet weight of bacteria were thawed, suspended in 500 ml of buffer consisting of 50 mM Tris-HCl, 0.15 M NaCl, and 6 mM $MnSO_4$, pH 7.5, and extracted [10]. Pooled, peak protein fractions eluted from Sephadex G-100 (Pharmacia) were lyophilized, dissolved in buffer to a protein concentration of 4 mg/ml, checked for the presence of protein A by Ouchterlony immunodiffusion with anti-protein A (Sigma Chemical Co., St. Louis, MO), and stored at $-20^\circ C$.

Coupling of protein A to dextran-magnetite

A 10 ml suspension of dextran-magnetite particles containing 0.3 mg/ml of iron was adjusted to pH 11 with 5 M NaOH, cooled to $20^\circ C$, and reacted with two 10-mg additions of cyanogen bromide (Eastman Kodak Co., Rochester, NY) added slowly with stirring over a period of 2 min, while maintaining the pH at 11 with 1 M $NaHCO_3$. The suspension was cooled to $4^\circ C$, 100 mg of purified protein A was added, and the suspension was gently mixed overnight at $4^\circ C$. Glycine was then added to 1 M final concentration and the suspension was gently mixed for 2 h at $4^\circ C$. Free protein A and glycine were removed by elution from a 2×45 cm Sepharose 2B (Pharmacia) column with 20 mM phosphate, 0.5 M NaCl buffer, pH 7.0. The eluate was stored at $4^\circ C$ for subsequent use in magnetophoretic separation experiments.

Preparation of antibody-coated dextran-magnetite

Anti-*Legionella* antibody was produced by immunization of rabbits [12]. The IgG fraction was isolated by elution of 2.8 ml of serum with 20 mM sodium phosphate buffer, pH 7.4, from a 2×30 cm² column of DEAE Sephacel (Pharmacia). Peak IgG fractions were pooled, concentrated by lyophilization, and rehydrated in 1 ml of sterile, deionized water. A 500- μ l suspension of dextran-magnetite containing 0.3 mg/ml of iron was added to 500 μ l of purified anti-*Legionella* antibody with an indirect fluorescent antibody titer of 1:1024 and a protein concentration of 4 mg/ml. After incubation at $37^\circ C$ with mixing for 1 h, the material was placed on a Sepharose 2B column and eluted as described above. Fractions which contained antibody-bound magnetic particles were pooled and stored at $4^\circ C$.

Magnetophoretic separation experiments

Legionella pneumophila, serogroup 1, and *Flavobacterium aquatale*, each suspended in 100 μ l of sterile, deionized water were mixed together with 300 μ l of magnetic particles that had specific anti-*Legionella* antibody covalently coupled to their surface. The suspensions were incubated at $37^\circ C$ for 30 min in small plastic test tubes (#19001B, Denville Scientific Co., Denville, NJ). The tubes were placed in contact with the sharp edge of a pole of a 7500 G permanent magnet for 30 min. The supernatant fluid containing excess dextran-magnetite was removed by aspira-

tion with a sterile Pasteur pipet, spread uniformly on buffered agar medium and incubated for 72 h at 36°C in a moist air atmosphere supplemented with 5% CO₂. The buffered agar (BCYE) [13] contained yeast extract, 10.0 g; activated charcoal (Norit SF, Sigma), 2.0 g; L-cysteine-HCl · H₂O, 0.4 g; ferric pyrophosphate soluble, 0.25 g; agar, 17.0 g; ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid, Calbiochem-Behring, LaJolla, CA), 10 g. The pH of the medium was adjusted with 1 M KOH to produce a pH of 6.9 after autoclaving. The magnetically precipitated material enriched for *Legionella* on the walls of the tubes was suspended in 300 μl of sterile water and cultured similarly. Magnetic particles that did not attach to *Legionella* remained in suspension during the magnetic precipitation step because they were too small to be attracted to the magnet as described by Kronick [14].

Results and Discussion

Preparation and characterization of dextran-magnetite

During this investigation magnetite was prepared in the presence of chelating dextrans. Titration experiments with magnetite and varying amounts of dextran indicated that a specific concentration of dextran completely solubilized the magnetite (Fig. 1). The figure shows the amount of iron in the supernatant fluid as a function of dextran concentration and M_w . This is a measure of the quantity of magnetite solubilized by dextrans with differing molecular weights. Therefore, dextran acted like a detergent under these conditions. That is, single dextran molecules did not solubilize but instead collected on the surfaces of the growing magnetite particles. When iron was in excess, the dextran was exhausted from the solution before the magnetite was solubilized.

The magnetite-dextran in the supernatant fluid was not stoichiometric with respect to glucose residues. The solubilizing concentration of glucose residues

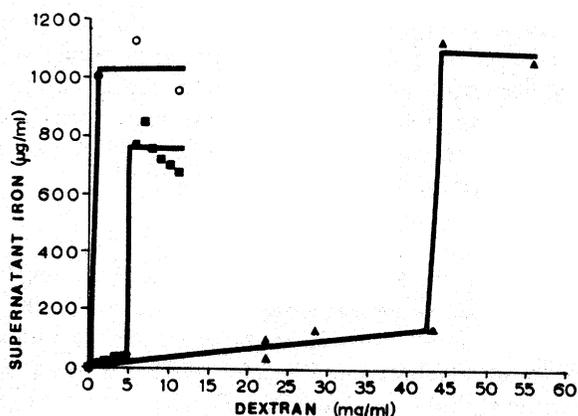


Fig. 1. Titration of magnetite with dextran, following procedure of text. Dextran molecular weights are: (○) 2000000; (■) 478000; and (▲) 4000.

decreased rapidly as the molecular weight of dextran increased from 4000 to 2000000 (Fig. 1). The reciprocal relationship between solubilization and dextran molecular weight indicated that higher molecular weight dextrans bound more strongly to the magnetite surfaces.

The particles which formed initially during these experiments were magnetite, but they slowly oxidized in air to ferric oxide. A typical intermediate composition, aged for 6 days, was 1.0 Fe_3O_4 :0.9 Fe_2O_3 . The magnetization of this product was 46.7 emu/g of Fe. This compares favorably with the theoretical value, 48.3 emu/g of Fe, based on the above composition and the magnetization of commercial magnetite (Wright Industries, Brooklyn, NY), which is 118.3 emu/g of Fe. The magnetite particle sensitivity to oxidation was mostly due to weak cooperative interactions between iron atoms since the magnetite crystals were so small, and partly due to the particles' high surface-to-volume ratio.

This effect of size was evident from the superparamagnetic properties observed in the Mössbauer spectrum of fresh magnetite-dextran samples handled under nitrogen to prevent oxidation (Fig. 2). Mössbauer spectra were resolved by measuring Doppler shifts of gamma radiation from a source of high purity gamma radiation, ^{57}Co , as it moved at a constantly changing velocity. The resolved gamma energies on the abscissa were expressed directly as velocity (mm/s) (Fig. 2). The energies of the spectral lines reflect the difference between the ground and excited nuclear states of ^{57}Fe in the magnetic particle preparation. The location of the centroid of the spectrum is the isotope shift (δ), which is determined by the electron density at the iron nucleus. The electric field gradient at the iron nucleus arising from the anisotropy of the iron crystal field, interacting with the nuclear quadrupole moment of iron, causes the spectral line to be split by an amount of 2Δ . The magnetic field at the iron nucleus did not affect the spectra of our samples because of a rapid thermal alteration of the local magnetic field produced by the particles' superparamagnetism. The spectral quadrupole split of $2\Delta = 0.76$ mm/s and isotope shift $\delta = 0.34$ mm/s for our preparations resembled that of ferritin with $2\Delta = 0.60$ mm/s and $\delta = 0.50$ mm/s [15], or that of Imferon with $2\Delta = 0.62$ mm/s and $\delta = 0.36$ mm/s [3], or those of 3.5-nm diameter magnetite particles with $2\Delta = 0.86$ mm/s prepared by oxidation of ferrocene [16], by precipitation from lignosulfonate solution with

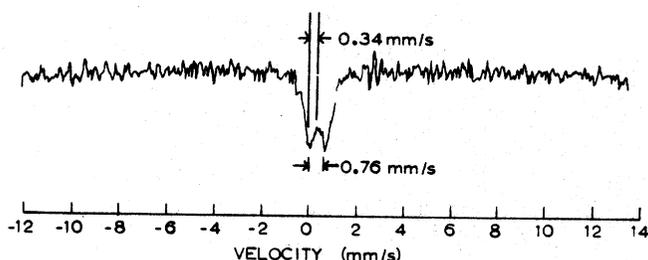


Fig. 2. Mössbauer spectrum of dextran-magnetite complex. Molecular weight 498000. The ordinate is intensity of gamma energy transmitted; the abscissa represents energy of gamma radiation photons.

Fig. 3. Electron micrograph of dextran-magnetite complex. Bar represents 0.1 μm .

$2\Delta = 0.62$ mm/s [17], or by milling [18]. The similarities among these spectra were expected because of the rapid relaxation of the magnetic moment in superparamagnetic particles. However, the large value of magnetization for our preparation indicated that it had ferromagnetic properties, unlike Imferon.

An electron micrograph of magnetite-dextran produced with dextran having a molecular weight of 478 000 is shown in Fig. 3. The particles appear as electron-dense cores separated by transparent sheaths, identical to those observed in micrographs of Imferon [5]. Both magnetite-dextran and Imferon have 3-nm diameter cores. However, the molecular weight of the dextran in Imferon is much lower, 2000 to 8000, than that used in our preparation. Both Imferon and our magnetite-dextran particles appear to have formed by the same mechanism: deposition of dextran on cores of ferric oxide (Imferon) or on cores of magnetite (our preparation), because they reached an equivalent 3-nm particle diameter, Imferon particles could not consist of single dextran molecules of 8000 molecular weight because dextran of this molecular weight does not have a 3 nm diameter. Although our particles were produced with dextran of approximately 5×10^5 molecular weight there was no evidence to suggest that the preparations consisted of single molecules of dextran. It should be noted that the apparent particle clumping observed in Fig. 3 is an artifact of the drying process used to prepare the sample for electron microscopy.

TABLE 1
MAGNETOPHORETIC SEPARATION OF BACTERIA ^a

Culture	Starting suspension	Magnetophoretic	
		supernatant	pellet
<i>L. pneumophila</i>	7.3×10^4	3.3×10^3	5.1×10^4
<i>F. aquatile</i>	3.1×10^4	1.8×10^4	5.0×10^1
Ratio L/F ^b	2.35	0.18	1020.00

^a Total colony forming units (CFU) in the starting suspension and magnetophoretically separated fractions are presented.

^b Ratio of total CFU of *Legionella* divided by total CFU of *Flavobacterium*.

Separation of Legionella from Flavobacterium

Protein A specifically binds the Fc portion of IgG antibody molecules so that any IgG antibody that was needed for a particular experiment could be bound to them. These particles could then be used to isolate particulate antigens that contained antigenic determinants that were recognized by the antibody bound to the surface of the particles. *F. aquatile* and *L. pneumophila* were magnetophoretically separated with dextran-magnetite particles containing anti-*Legionella* IgG antibody bound to their surface (Table 1). This antibody did not crossreact with *Flavobacterium*. After processing, the ratio of *Legionella* to *Flavobacterium* in the magnetic precipitate was 400-fold greater than the ratio in the starting mixture. A 0.1% (v/v) concentration of a non-ionic detergent, Tween 80, was incorporated into the buffer system during this separation procedure to prevent adherence of the bacteria to the walls of the polystyrene test tubes. This concentration of detergent did not affect the viability of the test bacteria. Although the titer, 1:1024, of the anti-*Legionella* antibody used in this investigation was low, *Legionella* were clearly enriched in the magnetophoretic precipitate.

Simplified description of the method and its application

Dextran-magnetite superparamagnetic particles may be useful as laboratory reagents for magnetophoretic cell separation procedures [14]. For example, such a procedure may be useful for isolating specific bacteria and viruses from samples heavily contaminated with normal flora microorganisms. Antibody-coupled pseudomagnetoferritin should also find use as a ferritin-like stain for organelles in electron microscopy, coupled with magnetophoretic isolation of the same organelles. With one exception [6], magnetic particle preparations tend to clump and therefore may entrap and produce high background recoveries of unwanted materials. The particles described in this paper did not clump because they were superparamagnetic and therefore did not attract each other in the absence of an applied magnetic field. These superparamagnetic preparations were synthesized by preparing 30 ml of solution containing 0.18 g of $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 0.14 g of $\text{FeCl}_2 \cdot 4 \text{H}_2\text{O}$ and 0.2 g of dextran T-500. This was added dropwise, with stirring, to a solution containing 6.4 g of NaOH in 60 ml of deionized water in a beaker that was placed in a boiling water bath. After stirring for 30 min, the precipitate was cooled to 25°C, adjusted to pH 8.5 with 12 N HCl, dialyzed against water, and stored at 4°C. Suspensions were stable for several months when stored at 4°C. Although anaerobic storage was optimum, magnetite particles could be regenerated from

the oxidized product by reduction with sodium hydrosulfite. The magnetite-dextran particles were used to separate and isolate bacteria to which a specific antibody was prepared [12] and coupled to particles containing protein A prepared by a published method [10]. The protein A was covalently coupled to the magnetite-dextran particles by taking a 10-ml suspension of dextran-magnetite particles containing 0.3 mg/ml of iron, adjusted to pH 11 with 5 M NaOH, cooling to 20°C, and reacting with two 10-ml additions of cyanogen bromide added slowly with stirring over a period of 2 min, while maintaining the pH at 11 with 1 M NaHCO₄. The suspension was cooled to 4°C, 100 mg of purified protein A was added, and the suspension was gently mixed overnight at 4°C. Glycine was then added to 1 M final concentration and the suspension was gently mixed for 2 h at 4°C. Free protein A and glycine were removed by elution from a 2×45 cm Sepharose 2B column with 20 mM phosphate, 0.5 M NaCl buffer, pH 7.0. The eluate containing the particles was stored at 4°C for subsequent use in magnetophoretic separation experiments. Enrichment for *L. pneumophila* from a mixture containing *L. pneumophila* and *F. aquatile* was accomplished with these paramagnetic particles.

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