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Aqueous Two-phase Extraction System Using an Assembled Dextran-conjugated Magnetite Prepared by the Dextranucrase Reaction

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Abstract: Dextranucrase reacts with sucrose to produce dextran and fructose. Dextranucrase is known to form a complex with the dextran product at the active site. Using this characteristic, dextran was introduced to the surface of magnetite through the dextranucrase reaction by reaction with sucrose to obtain dextran-conjugated magnetite. This modified magnetite was applied in an aqueous two-phase system for protein purification with the aid of an applied magnetic field. Two sizes of the magnetite used were 320 nm and 5.0 μm . Dextran-conjugated magnetite (320 nm), assembled magnetically, was able to rapidly extract hemoglobin with a high yield because the dextran on the surface of the magnetite has an entangled structure with a high density for capturing hemoglobin.

Key words: dextran, dextranucrase, aqueous two-phase extraction, magnetite

INTRODUCTION

Aqueous two-phase extraction is a technique used for separation of proteins and DNA that uses immiscible solutions of polymers and/or salts at high concentration.^{1,2)} Usually, for immiscible polymer systems, the polymers used are dextran and polyethylene glycol (PEG). The partitioning of the target molecules between the two phases depends upon the concentrations of polymers, as described by corresponding phase diagrams.

Dextran is mainly composed of glucose polymer with α -(1,6) bonding. Other than in aqueous two-phase systems, dextran has been used as a plasma volume expander, and in gel permeation chromatography resins. Dextran is produced by enzymatic reaction with dextranucrase (DSase), reacting with a sucrose substrate. The dextran product forms a complex with DSase at its active site.³⁾ In previous work, we modified the surface of membranes and particles with dextran using the DSase reaction, utilizing these for colloidal particle filtration^{4,5)} and for the regulation of protein adsorption.⁶⁾ The density and the length of the dextran are regulated by the immobilization density and the reaction time with DSase, respectively.^{7,8)}

In an aqueous two-phase extraction system, the formation of the two-phases and the extraction performance are dependent on the concentrations of polymers and, as noted above, dextran is one of the most commonly used polymers. The concentration of dextran in a phase is controllable by movement of a dextran-conjugated particle. Magnetite (ion oxide) was used for this purpose in this study to control the

assembly and dispersion state of dextran. A magnet located far from the magnetite particles, allowed their dispersal, while placing the magnet near the magnetite particles would cause particle assembly. The assembly and dispersion state of dextran-conjugated magnetite was thus controlled along the magnetic field lines formed by the magnet, resulting in changes in the assembled density of dextran on the magnetite. That is, the density of dextran on the magnetite surface is controllable by a magnet. Goto et al. applied a magnetite-dextran fluid in an aqueous two-phase system to promote rapid formation of two-phase polymers.⁹⁾

In this study, the surface of magnetite was modified with dextran produced by DSase for the aqueous two-phase extraction of protein (hemoglobin), as shown in Fig. 1. By applying a magnet to dextran-conjugated magnetite, the dextran density would be increased through aggregation of magnetite, thus enhancing the protein extraction performance. A magnetic fluid containing dextran⁹⁾ and a dextran-magnetite gel have been prepared in previous work.¹⁰⁾ However, in this study, dextran was formed on the surface of the magnetite using an enzymatic reaction and the resultant dextran-conjugated magnetite was applied in an extraction system by changing the dextran density through the application of a magnetic field. This is the first report of dextran formation on magnetite via enzymatic reaction for separation engineering. Magnetite particles with sizes 320 nm and 5.0 μm were used for modification and DSase was immobilized on the surface of the magnetite. Dextran was grown from the active site of DSase by immersing DSase-immobilized magnetite particles in a sucrose solution. The dextran-conjugated magnetite was mixed with a solution of protein and PEG to determine the extraction behavior of hemoglobin.

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Abbreviations: DSase, dextranucrase; PEG, polyethylene glycol; U, activity unit; C_0 , the activity of DSase in the feed solution; C , the activity of DSase in the filtered solution; V , volume of solution; W , weight of magnetite.

Fig. 1. Illustration of novel dextran formation on magnetite by the dextransucrase reaction, to form a high-concentration domain of assembled dextran.

MATERIALS AND METHODS

Materials. Fe (III) $\text{Cl}_3 \cdot 6\text{H}_2\text{O}$ and Fe (II) $\text{Cl}_2 \cdot 4\text{H}_2\text{O}$ were purchased from Wako Pure Chemical Industries (Japan). Dextransucrase (DSase) from *Leuconostoc mesenteroides* (D-9909 Lot No. 128H4026, 10 U-DSase/mg-DSase) was purchased from Sigma Chemical Co. and used without further purification. One gram of powder is equal to 12.81×10^3 U of enzyme activity. Hemoglobin was obtained from Wako Pure Chemical Industries. Polyethylene glycol (M_w 6,000) was obtained from Katayama Chemical Ltd. A doughnut magnet (inner diameter 7 mm, outer diameter 20 mm, height 4 mm) was obtained from BEST Corporation, Japan. Other chemicals used were of analytical grade or higher.

Preparation of magnetite particles with large and small sizes. Two sizes of magnetite particles were prepared using the following methods.¹¹⁾ The concentrations of Fe (III) $\text{Cl}_3 \cdot 6\text{H}_2\text{O}$ and Fe (II) $\text{Cl}_2 \cdot 4\text{H}_2\text{O}$ dissolved in distilled water were 0.2 and 0.1 M, respectively. 10 M NaOH was added to the iron-ion solution with stirring for 30 min. The resultant magnetite-containing solution was then centrifuged (High Speed Refrigerated Micro Centrifuge MX-301, TOMY SEIKO Co. Ltd.) at 5,000 rpm for 10 min to recover the magnetite. After washing the recovered magnetite with water and methanol, the magnetite was dried and sieved. The average size of the magnetite was determined by optical microscopy and scanning electron microscopy (SEM, JSM-5200, JEOL Ltd., Tokyo, Japan) to be 5.0 μm .

PEG (3.0 g) was dissolved in distilled water to obtain a concentration of 100 g/L. The concentrations of Fe (III) and Fe(II) in the above-prepared solution were set at 0.64 and 0.32 mM, with a solution volume of 30 mL. While stirring at 300 rpm, 60 mL of 0.75 M ammonia solution was added dropwise at 318 K into the iron ion solution. After 120 min, the solution was centrifuged, and the magnetite product was washed with water, then redispersed by sonification (Ultra Sonic Cleaner Single Frequency USK-3R, AS ONE Corp., Japan). The size of the magnetite prepared was 320 nm.

One gram of magnetite was dispersed in a water/ethanol solution (1/9 by volume) under stirring. Hexyltrimethoxysilane (1.0 mL) silane coupling agent was added and stirred for 6 hours at ambient temperature. The obtained modified magnetite was recovered by vacuum filtration. The sample

was then dried for 24 h at 333 K and the yield of the sample was 0.92 g. The obtained modified magnetite was referred to as HTMS-magnetite.

Conjugation of dextran to magnetite via DSase reaction. Magnetite (0.2 g) was immersed in DSase solution (5 mL, 0.2 U/mL, 0.2 M acetate buffer, pH 5.5) at 303 K for one hour to immobilize DSase on the magnetite. The amount of DSase immobilized was determined from the residual activity in solution after removal of magnetite by filtration. After DSase immobilization, the solution was centrifuged at 5000 rpm for 10 min. The supernatant was used to determine the residual activity of DSase and the recovered DSase-immobilized magnetite was washed with acetate buffer three times. The activity (U/mL) of DSase was determined by reaction with sucrose. An enzyme activity of 1 U was defined as that required to produce 1 μmol of fructose in 1 min. The DSase activity in the residual solution was determined from the fructose concentration using the Somogyi–Nelson method.^{12,13)}

$$\text{The amount of DSase immobilized on magnetite (U/g)} = (C_0 - C)V/W \quad (1)$$

where C_0 and C are the activities of DSase before and after immobilization, respectively; V is the volume of solution; and W is the mass of magnetite.

To produce dextran with the immobilized DSase, the DSase-immobilized magnetite was immersed in 10 mL of sucrose solution (100 g/L, 0.2 M acetate buffer, pH 5.5) at 303 K for 3 days. The fructose concentration, a product of the DSase reaction, was determined by the Somogyi–Nelson method. The dextran-conjugated magnetite was recovered by centrifugation (5,000 rpm, 10 min). The obtained magnetite was washed with acetate buffer and stored at a concentration of 10 mg-dextran-conjugated magnetite per mL of acetate buffer to prevent drying of the conjugated dextran. The dextran-conjugated magnetite was characterized using FT-IR (JASCO Co., FT/IR-410, Tokyo, Japan) using the KBr method.

Protein extraction using dextran-conjugated magnetite.

Four milliliters of a solution of dextran-conjugated magnetite (in acetate buffer) was mixed with 2 mL of hemoglobin solution (2.0 mg/mL, in water) and 4 mL of 25% (w/w) PEG solution at ambient temperature. The final concentrations of hemoglobin and PEG were 0.4 mg/mL and 10% (w/w), respectively. A sample bottle containing 10 mL of the above

solution was allowed to stand with and without the application of a magnet. The magnet was applied at the bottom of the bottle. After a prescribed time, the concentration of hemoglobin in the supernatant (upper layer) was determined by absorbance at 406 nm by UV-Vis spectroscopy (UV-1800, Shimadzu Corp., Kyoto, Japan). The concentration of hemoglobin was altered by extraction into the introduced dextran or the dextran assembled on the magnetite through the application of magnetic field. Unmodified magnetite was also used in a similar manner as the blank control. The percentage hemoglobin extraction was calculated using the equation

$$\begin{aligned} \text{Extraction percentage (\%)} &= 100\% [(\text{concentration of hemoglobin before extraction} \\ &\quad - \text{(concentration of hemoglobin after extraction)}) \\ &\quad / (\text{concentration of hemoglobin before extraction})] \quad (2) \end{aligned}$$

RESULTS AND DISCUSSION

Characterization of dextran-conjugated magnetite.

An enzymatic reaction with DSase was used to form dextran on the magnetite surface. DSase forms a complex with dextran while reacting with sucrose. Thus, by immobilizing DSase on the particle surface via hydrophobic interaction and reacting with sucrose, it is possible to form dextran on the surface of the magnetite. Dextran was formed on two different sizes of magnetite particle by immersing samples of each size in DSase solution for one hour. Subsequently, DSase-immobilized magnetite was immersed in sucrose solution to produce dextran on the magnetite surface.

The existence of dextran produced by DSase was confirmed spectrophotometrically by FT-IR. The FT-IR spectra of the magnetite and dextran-conjugated magnetite are shown in Fig. 2. Dextran has specific peaks around 1,200–900 cm^{-1} . The peak at 1,050 cm^{-1} is assigned to valent vibrations of the C-O and C-C bonds,¹⁴⁾ indicating that dextran is formed on the magnetite via enzymatic reaction. The stretching peak at 3,450 cm^{-1} also revealed the evidence of dextran on the magnetite. The amount of DSase immobilized, the amount of dextran produced, and the density of dextran are summarized in Table 1. Dextran is produced at the active site of DSase and has a weak interaction with DSase so is formed on the surface of the magnetite. Assuming that all of the immobilized DSase has a uniform dextran production activity, the density of dextran on the magnetite corresponds to that of the immobilized DSase. The density of DSase on the magnetite was calculated using the following equation,

$$\begin{aligned} \text{Density of DSase immobilized (mg/m}^2\text{)} &= (\text{the amount of DSase immobilized}) / (\text{surface area of magnetite}) \quad (3) \end{aligned}$$

Extraction of hemoglobin by dextran-conjugated magnetite.

Magnetically induced assembly of dextran-conjugated magnetite would result in a high protein extraction percentage because of a high local density of dextran. Hemoglobin extraction was performed by the dextran-conjugated magnetite with and without using the magnet. Assembled images of dextran-conjugated magnetite are shown in Fig. 3. Magnetite in the absence of the magnet was dispersed homogeneously throughout the solution, while that in the presence of the magnet was

Fig. 2. FT-IR spectra of dextran-conjugated magnetite.

(a) Dextran modified (large), (b) Dextran modified (small), HTMS magnetite, (d) Dextran and (e) Magnetite.

Table 1. Properties of dextran-conjugated magnetite.

	Small size (320 nm)	Large size (5.0 μm)
Amount of DSase immobilized (U/g-magnetite)	4.9	4.8
Density of DSase (number/m ² -magnetite)	5.5×10^{13}	2.6×10^8
Amount of dextran produced (mg/g-magnetite)	0.60	0.60

Fig. 3. Images of assembled dextran-conjugated magnetite.

Magnetite size 5.0 μm , amount of dextran produced 0.6 mg/g-magnetite.

strongly and heterogeneously assembled. The structure of assembled magnetite was governed by the magnetic field lines induced by the magnet. Without using the magnet (Fig. 3 (a)), magnetite precipitated gradually by gravity. While, with magnet (Fig. 3 (b)), the magnetite was quickly assembled to the bottom of the sample bottle. The macroscopic structure of the assembled magnetite was maintained by the magnetization line, predicting that the dextran conjugated to the magnetite would function as an extraction site of aqueous two-phase system at high speed. Some of the assembled domains composed

Fig. 4. Extraction of hemoglobin by dextran-containing magnetite.

(a) with magnet and (b) without magnet. ○, Small particle; △, large particle; □, unmodified particle (small); ▽, unmodified particle (large).

of magnetite were observable by the naked eye.

Time course curves for hemoglobin extraction in the presence and absence of the magnet are shown in Fig. 4. In Fig. 4 (b), a small amount of hemoglobin was adsorbed on the unmodified magnetite particles. A hydroxyl group exists on the surface of the magnetite, which would adsorb hemoglobin through the protein amino groups via hydrogen bonding. In the presence of the magnet, magnetite became strongly assembled, such that hemoglobin did not reach the magnetite surface for adsorption. Hemoglobin adsorption to the unmodified magnetite was dependent on their sizes. The assembled magnetite by magnetization had the higher adsorption performance, predicting that the adsorption of hemoglobin would occur not only at the surface of the magnetite but between the assembled magnetite particles, especially for small unmodified particle. When applying the magnet to dextran-conjugated magnetite, the hemoglobin extraction percentage was higher for the small than for the large dextran-conjugated magnetite particles. Although assembly caused a high local density of dextran in both cases, in the case of small-size magnetite, assembly promoted the entanglement of dextran between particles, giving a higher extraction percentage on the smaller dextran-conjugated magnetite particles.

Extraction rate by dextran-conjugated magnetite was higher along the magnetic field lines due to the higher density formation of dextran there. It was found that magnetic control of the dextran density on the magnetite enhanced the aqueous two-phase extraction of proteins for kinetic studies.

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