Dextran-magnetite complex: conformation of dextran chains and stability of solution

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Three kinds of dextran-magnetite (DM) complexes were prepared using alkali-treated dextrans with molecular weights of 1900, 4200 and 9600, respectively. The number of dextrans binding to a magnetite core was determined. The number was proportional to the area of core surface and the area occupied by a dextran was 2.5 nm^2 for molecular weight of 1900, 2.8 for 4200 and 3.8 for 9600. The binding of dextrans to core may be conditioned by the conformation of dextran chains in water (possibly by the steric hindrance between dextrans covering core). Stability of the DM solution was examined at 80 °C. Aggregation and/or precipitation of DM particles were observed within two weeks. The stability of DM solution was found to increase with increasing molecular weight of dextran. The dissociation of dextran from the core may cause the aggregation and subsequent precipitation of DM particles (the dissociation constant at 20 °C, 3.7×10^{-6} for a molecular weight of 1900 and 5.4×10^{-7} for 9600).

1. Introduction

The dextran-magnetite (DM) complex was developed as a new biocompatible magnetic fluid applicable to the medical field for magnetic resonance imaging (MRI) and hyperthermia [1] etc. We and other groups already reported the physical and chemical properties [2, 3], safety for living body [2], nuclear magnetic resonance (NMR) relaxivity [4, 5] and uptake and metabolic behaviors in rat organs [6–13]. From these data, it has been suggested that DM complex is useful as an MRI contrast agent of tumors of the reticuloendothelial system, particularly of the liver [10–13]. However, little is known about the structure of DM particle in water, such as the conformation of dextran chains covering magnetite core.

How many dextrans bind to a core? This information is important for considering the conformation of dextrans, even if the number of dextrans may depend on the molecular weight (chain length), core size and condition of core surface. Previously, we prepared a homologous series of DM complex using magnetite particles of 8 nm diameter and six kinds of dextran with molecular weights from 1800 to 27 000 and reported the following results [14]. (i) The number of dextrans binding to a core decreases with increasing molecular weight of dextran. (ii) The conformation of dextrans possibly changes from a fully-extended state to a random-coiled state with the molecular weight from 1800 to 27 000.

However, no data has yet been obtained for the relation between the number of dextrans and core size. If the number is proportional to the area of core surface, the binding of dextrans to core may be conditioned by the conformation of dextrans. While if the number is not proportional, the binding of dextrans may be influenced by the condition of the core surface. To solve this problem is the purpose of the present study.

Next, the stability of DM solution is important for application to medical field. Although DM particle itself has a low toxicity, the aggregation of DM particles in blood is very dangerous, since it gives rise to thrombosis [13]. For only magnetite cores, the cores easily aggregate and instantly precipitate, even if they are very fine particles [2]. For DM particle in water, the stability may be due to the repulsion force between DM particles and the force is attributable to the dextran chains covering core. In this study, the stability of DM solutions has been examined through a storage test at 80 °C.

If some dextrans dissociate from the core, the possibility of aggregation of DM particles may increase, since the dextran chain dissociated once seems not to bind again with core. If this theory is correct, the dextran with a low dissociation constant may be preferable for medical applications. To reveal the aggregation mechanism of DM particle, we have determined the dissociation constant of dextran binding to core.

2. Materials and methods

2.1. Materials

Three kinds of DM complexes were prepared using alkali-treated dextrans with the number average molecular weight of 1900, 4200 and 9600. The average molecular weights of the dextran were 4000, 7000 and 20 000, respectively. We refer to the DM samples as DM–1, DM–2 and DM–3 in the order of molecular weight. The alkali-treated dextran has a carboxyl group at the reduction end [14]. The dextran chain binds to a magnetite core by linkage between the carboxyl group and an iron atom on the core surface (Fig. 1).

In this study, DM complex was directly synthesized from alkali-treated dextran and a solution of iron chlorides ($FeCl_3, FeCl_2$). We call this the ''one step method'', in distinction from ''two step method'' (magnetite core is prepared in advance) reported previously [14]. The procedure of the one step method is as follows. The iron salt solution was added into 20% (w/v) solution of dextran under N_2 atmosphere. The ratio of dextran and Fe in the mixture was about 5 in weight. The mixture was adjusted to pH 11 with a drop of NaOH solution. The carboxyl group of dextran binds to the iron atom at pH 11. The value of pH decreased to 7 by HCl and then the mixture was refluxed for 1 h at 100 °C. The DM particles may be formed in the reflux process. After cooling to room temperature, CH_3OH was added to 41–48% (w/v) to precipitate the DM particles. After centrifugation ($2000 \times g$ for 5 min), the precipitate was dialyzed against running water overnight. The DM solution thus obtained was adjusted to pH 8 with a drop of NaOH solution and was then concentrated to 1 M in Fe base using an evaporator. The Fe yield of the DM samples prepared in this study was 80–85%.

2.2. Methods

Gel chromatography of DM sample was carried out using Sephadex G-200 (Pharmacia). Sephacryl S-300 HR (Pharmacia) was not useful, since the gel absorbed DM particles. We used a column of downward-flow type (Shoei Work, SD-1000) of 3 cm diameter and 1 m length. The height of the gel bed was 90 cm and the operating pressure was 20–25 cm in water height. The buffer was 0.1 M sodium phosphate (pH 7.0). The condensed solution of DM (1 M in Fe base) was charged by 2.5 ml on top of the gel bed. The flow rate was $\sim$ 30 ml/h and the volume of fraction was 6 ml. The void volume of the gel was checked using blue dextran (Pharmacia) with molecular weight greater than 2×10^6.

X-ray diffraction (XRD) of the powdered DM sample which was prepared by lyophilization was measured using a conventional diffractometer (Fe-filtered CoKα, $\lambda = 0.179$ nm. The pattern was attributable to magnetite crystal (Fe_3O_4), though all the diffraction peaks were very broad because of ultrafine particles. The shape and size of the magnetite cores were examined using a transmission electron microscope (TEM) (Hitachi H-8100). The sample used in the TEM observation was prepared by dropping dilute DM solution on a microgrid coated with carbon film. The distribution of core size was obtained from the TEM photographs using an image analyzer (Nippon Regulator, Luzex-500).

Dynamic (quasielastic) light scattering from the DM particles was measured using a light scattering instrument with He-Ne laser (Union Giken, SLS-600). The DM solution ($\sim$ 10 mM in Fe base) without unreacted (free) dextran was used in the light scattering experiment. We analyzed the scattering data by the time-integral method [15] and determined the whole size of the DM particles.

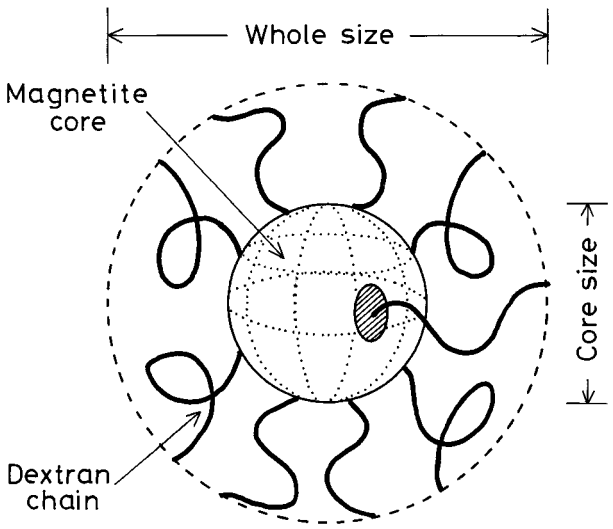

Figure 1 Illustration of a DM complex in water. The size of magnetite core is exaggerated. The hatched area represents the area of core surface occupied by a dextran chain.

3. Results

3.1. Shape and size of magnetite core

Fig. 2 shows a TEM photograph of a DM-3 sample. The electron diffraction pattern of a dark spot (indicated by the arrow) is attributable to magnetite crystal. No dark spot was observed for only dextran, since the dextran molecule consists of light elements. Thus, all the dark spots in Fig. 2 are due to the magnetite cores in DM particles. The shape of the cores is roughly spherical and the core size is less than 10 nm.

Fig. 3 shows the number and weight distributions of core size which are obtained from the TEM photographs.

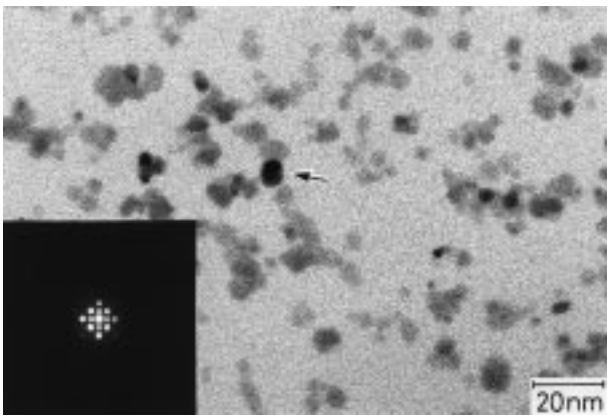

Figure 2 TEM of DM particles prepared using dextran with a molecular weight of 9600 (DM-3 sample). The electron diffraction pattern at the left corner was obtained from the black spot indicated by an arrow.

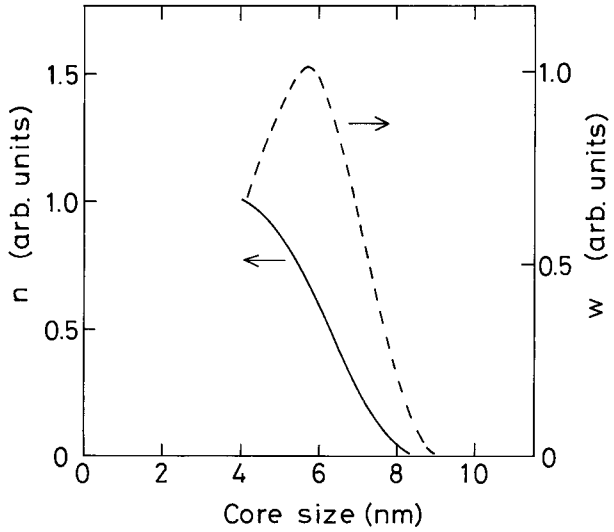

Figure 3 Number and weight distributions of core size for DM-3 sample. The solid and dashed curves represent the number (n) and weight (w) distributions obtained from TEM, respectively. The data of core size less than 4 nm are omitted due to size of error.

The data from core size less than 4 nm are omitted, since the errors are significantly large. The weight distribution is estimated from the number distribution, assuming that all the cores are spherical and have the same density. The number average size is not clear but the weight average size can be estimated to be 5.7 nm from the peak position of the dashed curve. The weight average core size was also estimated from the most intense (311) peak in the powder XRD pattern, using Scherrer's Equation [16]. The size (5 ± 2 nm) is consistent with that obtained by TEM observation. This suggests that most of the cores consist of a single domain of magnetite crystal.

3.2. Gel filtration

Fig. 4 shows the elution pattern of DM-3 sample by Sephadex G-200. The solid curve represents the dextran concentration which was measured using the H_2SO_4-Anthrone method. The dashed curve represents the iron concentration which was measured by the O-phenanthroline method [17]. For the solid curve, a large peak appears at around 170 ml and subsequently a broad peak appears from 300 to 500 ml. The large peak is due to the elution of DM particles, since a peak of the iron concentration is observed at the same position. On the other hand, the broad peak is due to the elution of free dextrans, since no iron is detected in the fractions. Thus, this gel filtration technique is useful for separation of DM particle and free dextran.

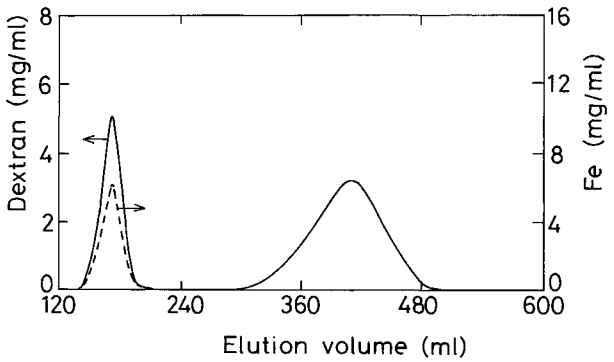

Figure 4 Elution pattern of DM-3 sample by gel chromatography of Sephadex G-200. The solid and dashed curves represent the dextran and iron concentrations, respectively.

The blue dextran was eluted at around 120 ml, suggesting that the void volume of gel corresponds to 120 ml. Since the elution of DM particles starts from 150 ml, the volume of DM particles is smaller than the void volume.

Fig. 5 shows the relationship between iron and dextran concentrations for the fractions containing DM particles. The numbers near the data points represent the fraction numbers. The inset shows the positions of the respective fractions on the elution pattern of iron. The trace from No. 26 to No. 31 is counterclockwise as indicated by arrows. The counterclockwise trace was observed for all the DM samples of DM-1 to DM-3. If the number of dextrans binding to a core is proportional to core volume, the trace should be a straight line, since the dextran concentration is proportional to the number of dextrans and the Fe concentration is proportional to the core volume.

Why does the trace show a counterclockwise rotation? In the case of downward-flow type, it seems to be a problem whether or not the gel filtration of DM particles is influenced by gravity, though it is known that the effect of gravity on gel filtration is usually negligible [18]. However, the DM particle is a special complex, since the density of core is five times larger than that of dextran [14]. To check the effect of gravity, we carried out gel chromatography experiments of upward-flow type in the same condition as for the downward-flow type. However, the elution pattern was similar to that of downward-flow and the trace of upward-flow was also counterclockwise. This suggests that the gel filtration of DM particles is not influenced by gravity.

3.3. Concentration of core in fraction

The number of Fe atoms in a core can be obtained from the core volume, density and Fe content. The core

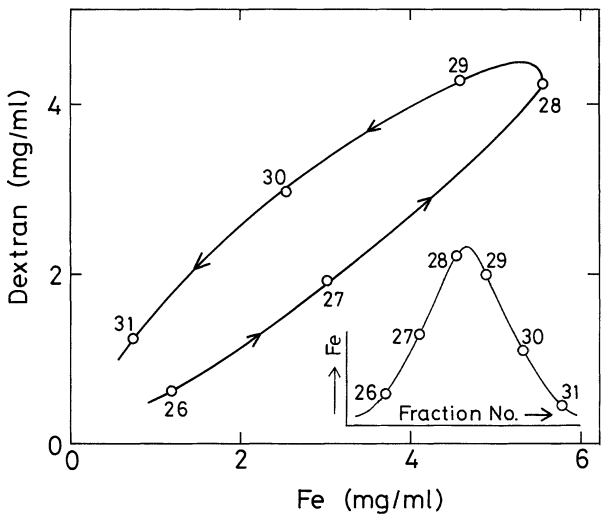

Figure 5 Relation between iron and dextran concentrations for fractions containing DM particles (DM-3 sample). The fraction numbers are indicated near the data points. In the inset, the positions of the fractions are shown on the elution pattern of iron.

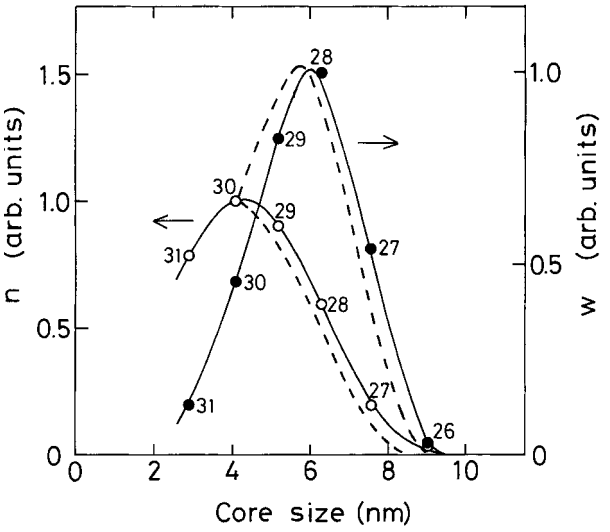

Figure 6 Core size of fractionated DM particles (DM-3 sample). The open and solid circles represent the number (n) and weight (w) distributions, respectively. The numbers near the data points are the fraction numbers. The dashed curves are the data of Fig. 3.

volume is given by core size, assuming spherical shape. For the density and Fe content, we used the values [4.0 g/cm^3, 62% (w/w)] obtained from a chemical analysis of the magnetite core itself, although the data of magnetite crystals is 5.2 g/cm^3, 72% (w/w) [19]. Then, the concentration of core in fraction (number of cores) can be estimated from the number of Fe atoms in a core, since the Fe concentration of fraction is obtained in advance.

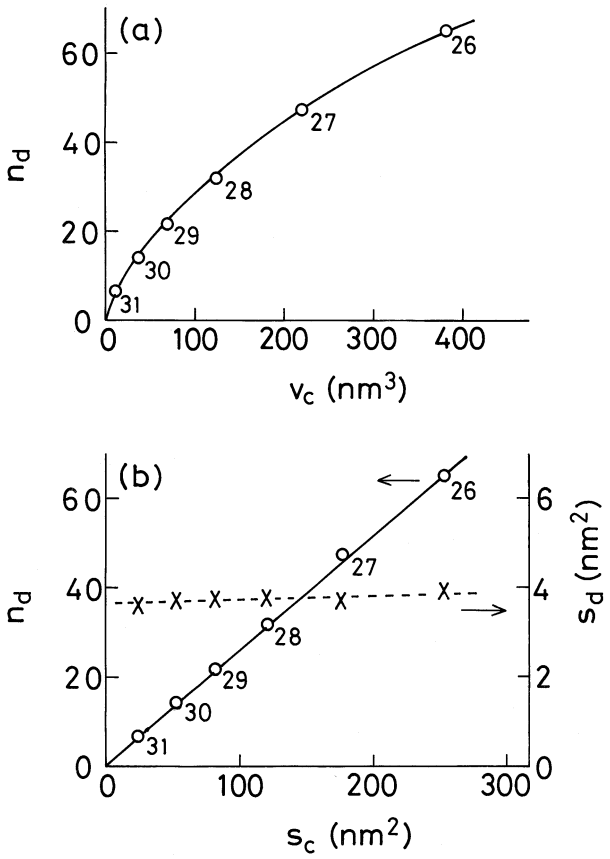

Figure 7 (a) Number of dextrans binding to a core (N_d) plotted against core volume (V_c) for DM-3 sample. (b) The number plotted against area of core surface (S_c). The numbers near the data points are the fraction numbers. The cross points in (b) represent the area of core surface occupied by a dextran chain ($S_d = S_c/N_d$).

Fig. 6 shows the concentration of core versus core size for the fractions from No. 26 to No. 31. The dashed curves represent the data before gel filtration (Fig. 3), which are superimposed by a suitable scaling. Both number and weight distributions are consistent with those before gel filtration. This suggests that the gel chromatography by Sephadex G-200 is useful for fractionation of DM particles with different core sizes.

3.4. Number of dextrans binding to a core

The number of dextrans binding to a core (N_d) can be estimated from both concentrations of dextran and core in fraction. In Fig. 7(a), N_d is plotted as a function of core volume (V_c). The value of N_d increases with an increase in V_c but the curve is parabolic. Fig. 7(b) shows N_d as a function of area of the core surface (S_c). The relationship between N_d and S_c is represented by a straight line. This indicates that N_d increases in proportion to S_c. In Fig. 7(b), the area of the core surface occupied by a dextran chain ($S_d = S_c/N_d$) is also plotted against S_c. The value of S_d is almost constant ($3.8 \pm 0.2\,\text{nm}^2$).

3.5. Storage test of DM solution

The stability of fractionated DM solution was examined at 80 °C using an incubator, since no change was observed for at least six months at 20 °C. For the storage test at 80 °C, the DM solutions (2 mg/ml in Fe weight) exhibited aggregation and/or precipitation of DM particles within two weeks. Table I shows the time from a start of the storage test up to the aggregation and/or precipitation. The time is in the order of DM-1 < DM-2 < DM-3. This suggests that the stability of DM solution increases with molecular weight of dextran.

No change in the stability by addition of NaCl (0.15 M) was observed. While the addition of free dextran was effective for the stability of fractionated DM solution without free dextran. When the concentration of free dextran was the same as that of dextran binding to core, the stability was improved by 1.5–2 times for all the DM samples. This suggests that the dissociation of dextran binding to the core may be suppressed by the existence of free dextran.

3.6. Dissociation constant of dextran

In general, when an equilibrium relation ($AB \leftrightarrow A + B$) holds in water, the dissociation constant K of A or B is defined as $K = [A]\,[B]/[AB]$, where the brackets represent concentrations of the respective components [20]. If the same relation can be applied to DM solution in an equilibrium state, the dissociation constant of dextran binding to the core is given by $K = [A]\,[B]/[AB]$, where $[A]$ is free dextran, $[B]$ is open binding site (Fe atom) on core surface and $[AB]$ is dextran binding to core.

The DM samples prepared in this study contain many free dextrans, since a sufficient amount of dextran was used against core materials. In this case, it can be assumed that $[A] > [AB]$ and $[B] = 0$. On the other hand, the free dextran can be completely removed by gel chromatography of Sephadex G-200, as mentioned in

TABLE I Stability of dextran-magnetite (DM) complex solution and dissociation constant of dextran binding to magnetite core

Sample	M	t(day)	K
DM-1	1900	3–4	3.7×10^{-6}
DM-2	4200	7–9	1.2×10^{-6}
DM-3	9600	10–12	5.4×10^{-7}

M, number average molecular weight of dextran. t, time from start of storage test at 80 °C up to aggregation and/or precipitation of DM particles. K, dissociation constant of dextran binding to core at 20 °C.

Section 3.2. This suggests that $[A] = 0$ for the fractionated DM solution. Since $[A] = 0$, $[AB]$ can be obtained from the dextran concentration of the fractionated DM solution.

The fractionated DM solution was stored at 20 °C for at least five days till it attained an equilibrium state. Some dextrans binding to core gradually dissociate into water (become free dextrans) in the process towards the equilibrium state. The dissociation constant of dextran in the equilibrium state is given by

$$K = [A]^*[B]^*/[AB]^* \quad (1)$$

where $[A]$*, $[B]$* and $[AB]$* are the concentrations of free dextran, open binding site and dextran binding to core in the state, respectively. Since $[A] = 0$ and $[B] = 0$ at the initial stage, it can be assumed that $[A]^* = [B]^*$ and $[AB]^* = [AB] - [A]^*$. Thus, Equation 1 is rewritten as

$$K = ([A]^*)^2/([AB] - [A]^*) \quad (2)$$

If the value of $[A]$* is obtained, we can determine K from Equation 2, since $[AB]$ is known in advance.

In this study, $[A]$* was obtained by the ultrafiltration technique shown in Fig. 8. The membrane filter (Toyo-Roshi) can easily pass free dextran of molecular weight less than 50 000. The fractionated DM solution of 3–5 mg/ml in Fe weight was used in this experiment. The DM solution (50 ml) was poured into the vessel and stirred constantly under a pressure of 1 kg/cm^2 by Ar gas. After 5–7 days, the dextran solution passed the membrane filter and was corrected by 5 ml; then the dextran concentration was measured by chemical analysis. The concentration was used as $[A]$*, assuming that the equilibrium state did not change during ultrafiltration. Table I shows the dissociation constant K thus obtained. The value of K is in the order of DM-3 < DM-2 < DM-1.

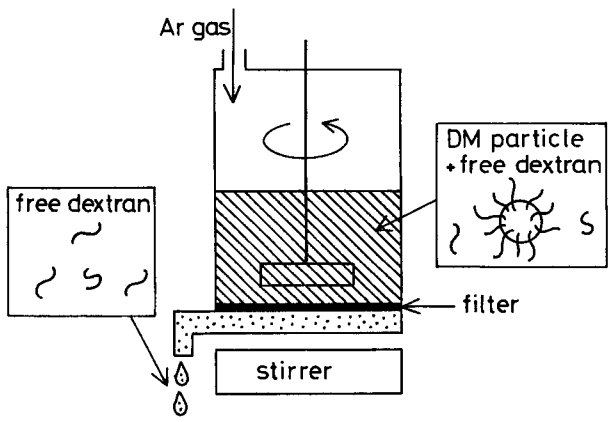

Figure 8 Schematic illustration for ultrafiltration of DM solution.

3.7. Electric charge of DM particle

If DM particle is positively or negatively charged in water, the charge has some effect against aggregation of DM particles. We examined the electric charge of DM particle by electrophoresis on filter paper. All the DM samples moved toward the negative electrode and the movement distances were similar. This suggests that DM particle is positively charged in water. Since the dextran binding to core has no charge [14], the positive charge of DM particle may be due to the Fe ions on the core surface. At the present stage, however, the effect of charging on aggregation of DM particles is not clear.

4. Discussion

4.1. Interpretation of counterclockwise trace

As mentioned in Section 3.4, the number of dextrans binding to a core is proportional to the area of core surface ($N_d \propto S_c$). In this case, the counterclockwise trace in Fig. 5 can be explained as follows. Here, we consider straight lines between the data points and origin, though they are not drawn in Fig. 5. The slope of the straight line represents the ratio of the dextran concentration and Fe concentration for a fraction. If the ratio (dextran/Fe) increases with fraction number, the trace shows a counterclockwise rotation, since both dextran and Fe concentrations become maximal in the middle of the trace (between No. 28 and No. 29).

The Fe concentration is proportional to the third power of core size, since the Fe concentration is proportional to core volume. On the other hand, the dextran concentration is proportional to N_d. In the case that $N_d \propto S_c$, the dextran concentration is proportional to S_c, i.e. the second power of core size. Thus, the ratio (dextran/Fe) is inverse to the core size. This means that the ratio increases with a decrease in core size. The core size decreases with the fraction number, as shown in Fig. 6. Thus, the trace in Fig. 5 shows the counterclockwise rotation. If N_d is proportional to core volume ($N_d \propto V_c$), the ratio of dextran/Fe is constant for all the fractions. In this case, the trace should become a straight line.

4.2. Structural model of DM particle in water

Table II shows the structural parameters of three DM complexes prepared in this study. The core sizes are similar but the whole size of the DM particle increases with molecular weight of dextran. The number of dextrans binding to a core (N_d) decreases and the area of core surface occupied by a dextran chain (S_d) increases with the molecular weight of dextran. Fig. 9 shows the structural models proposed for the DM particles on the basis of the data in Table II. The

TABLE II Structural data of DM complex in water

Sample	D_c (nm)	D_p (nm)	N_d	S_d(nm^2)
DM-1	6.2	27	48	2.5
DM-2	7.7	42	67	2.8
DM-3	5.7	53	27	3.8

D_c, average size of magnetite core in diameter. D_p, average size of DM particle in diameter. N_d, number of dextran chains binding to a core. S_d, area of core surface occupied by a dextran chain.

conformation of dextran for DM-1 is close to a fully-extended state and the conformation for DM-3 is close to a random-coiled state. The conformation for DM-2 is considered to lie between DM-1 and DM-3.

For DM-3, the number of Fe atoms on the surface of a core can be estimated as ~ 10 per S_d (~ 4 for A site, ~ 6 for B site in spinel structure) from the average core size. The number is much larger than unity. Thus, the binding of dextrans to core is not conditioned by Fe atoms on the core surface. Since the value of S_d is constant irrespective of core size, the binding of dextrans to the core may be controlled by the steric hindrance between dextrans covering core. This idea does not contradict the result that S_d increases with molecular weight of dextran.

4.3. Stability of DM solution

If the dissociation of dextran binding to core causes aggregation of DM particles, the aggregation may easily occur at a higher temperature, since the dissociation is due to thermal agitation. This agrees with the result that all the DM solutions are stable at 20 °C but become unstable at 80 °C.

The stability of DM solution increases with the molecular weight of dextran. This is consistent with the result that the dissociation constant of dextran decreases with the molecular weight of dextran. The bond strength between dextran and core may be similar irrespective of the molecular weight of dextran, since a COO^- terminal of dextran binds to an Fe atom on the core surface [14]. Thus, the dissociation constant of dextran may be closely related to the conformation of dextrans covering the core. The high dissociation constant for DM-1 is possibly due to the fully-extended conformation and the low constant for DM-3 is due to the random-coiled conformation.

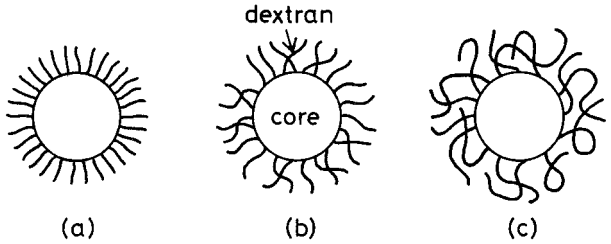

Figure 9 Structural models of DM particles in water: (a) DM-1 (molecular weight of dextran, 1900); (b) DM-2 (4200); (c) DM-3 (9600). The core size is exaggerated.

4.4. Utility of DM complex as an MRI contrast agent

Gd^{3+}-DTPA (diethylene-triamine-pentaacetic acid) is a conventional MRI contrast agent [21]. The dissociation constant of Gd^{3+} (10^{-22} to 10^{-23} [21]) is lower than that of dextran (10^{-6} to 10^{-7}). However, LD_{50} of Gd^{3+}-DTPA for rat (10 mM/kg [21]) is comparable to that of DM complex (20–30 mM/kg in Fe weight [2]), where LD_{50} represents the amount of dose which kills possibly half of the administrated animals. This suggests that Gd^{3+}-DTPA is safe but the Gd ion is highly toxic. In contrast, Fe ion has a low toxicity, since a little amount of Fe ion is necessary for the living body. Thus, LD_{50} of DM complex may be associated with the formation of thrombosis by aggregation of DM particles.

On the other hand, NMR relaxivity of DM complex is much larger than that of Gd^{3+}-DTPA (5×10^3 times for T_1, 5×10^4 times for T_2), since DM complex has super-paramagnetic properties [2]. Here, T_1 is the spin-lattice relaxation time of proton (^1H) in NMR and T_2 is the spin-spin relaxation time. Thus, in the case of DM complex, a very little amount or very low concentration is sufficient for the MRI diagnosis. From these data, it can be said that DM complex is a useful MRI contrast agent.

5. Conclusions

Three kinds of dextran-magnetite (DM) complexes were prepared by a one step method. The DM particles with different core sizes could be fractionated by gel chromatography of Sephadex G-200. The following conclusions can be reached from this study.

1. The number of dextrans binding to a core is proportional to the area of the core surface (the area occupied by a dextran is constant irrespective of core size). This suggests that the binding of dextrans to core may be conditioned by the conformation of dextran chains (possibly by the steric hindrance between dextrans covering the core).
2. The DM solutions were stable at 20 °C for at least 6 months but at 80 °C, aggregation and/or precipitation of DM particles occurred within two weeks. The stability of DM solution increased with the molecular weight of dextran. The dissociation of dextran from the core may cause the aggregation of DM particles, since the dissociation constant decreased with molecular weight of dextran.
3. All the DM particles are positively charged in water.

Acknowledgment

The authors thank Mrs Y. Nagaoka and H. Shoji for technical assistance.

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Received 2 December 1998
and accepted 18 November 1999