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In vitro stability analyses as a model for metabolism of ferromagnetic particles (Clariscan™), a contrast agent for magnetic resonance imaging

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Abstract

Clariscan™ is a new contrast agent for magnetic resonance imaging. It is an aqueous suspension of ferromagnetic particles injected for blood pool and liver imaging. Previous experiments showed that particles made of ^{59}Fe were taken up by the mononuclear phagocytic system and then solubilised. The present work aims at explaining a possible mechanism for the dissolution of these ferromagnetic particles in the body. The particles were diluted in 10-mM citrate or 10-mM acetate buffers at pH 4.5, 5.0 and 5.5 and incubated at 37 °C for up to 22 days, protected from light. The mixtures were analysed at different times during this incubation period using photon correlation spectroscopy, magnetic relaxation, visible spectroscopy and reactivity of the iron with the chelator, bathophenanthroline disulphonic acid. The data obtained with these techniques showed that the particles were almost completely solubilised within 4–7 days when incubated in 10 mM citrate, pH 4.5. Incubation in 10 mM citrate buffer, pH 5.0 revealed a slower solubilisation of the particles, as the changes observed after 72 h of incubation at pH 5.0 were 43–71% of the changes observed at pH 4.5. Incubation in 10 mM citrate, pH 5.5 revealed an even slower solubilisation of the particles, as the changes observed after 72 h of incubation at pH 5.5 were 12–34% of those observed at pH 4.5. Incubation of the particles in 10 mM acetate at pH 4.5, 5.0 and 5.5, as well as incubation of the particles in water pH adjusted to pH 5.1, resulted in only minor or no solubilisation of the particles. The results indicate that the low pH of endosomes and lysosomes, as well as endogenous iron-complexing substances, may be important for the solubilisation of these ferromagnetic particles following i.v. injection of Clariscan™.

Keywords: Ferromagnetic particles; Metabolism; Chelators; pH; Magnetic resonance imaging

1. Introduction

Most of the contrast agents for magnetic resonance imaging are low molecular weight Gd^{3+}-containing chelates, which are rapidly excreted in urine without being metabolised. However, these

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agents distribute rapidly into the extravascular space, thus reducing the vessel-to-background contrast. Accordingly, several approaches have been tried to make contrast agents with increased retention time in blood and reduced extravasation. These agents, often referred to as blood pool agents, include Gd^{3+}-containing macromolecules [1,2], as well as small iron oxide particles with different types of coatings to increase solubility and stability [3–8]. Such particulate agents may also be used for liver imaging [9] as they are taken up by the Kupffer cells, i.e. the resident liver macrophages that are directly exposed to the blood stream.

Clariscan™ (NC100150) is a new contrast agent under development for magnetic resonance imaging of the blood vessels and liver [10]. It consists of an iron oxide core, with a diameter of 6.4 nm and a water penetrable coating of oxidised oligomeric starch fragments which extends the hydrodynamic diameter to ≈ 12 nm [8]. The Fe(II) content of the iron oxide core is only a small fraction of the total iron present, such that this core can be considered as essentially Fe(III) maghemite [8]. Experiments performed using particles made of ^{59}Fe showed that most of the particles were taken up by the mononuclear phagocytic system (reticuloendothelial system). Thus, 8 h following injection of 10 mg Fe/kg of these particles in rats, ≈ 73, 18 and 5% of the radioactivity was recovered in liver, bone marrow and spleen, respectively. The particles then became solubilised, as shown by a 42% recovery of ^{59}Fe in blood cells 51 days post injection (Nycomed Imaging, unpublished results).

As it is unlikely that enzymes are involved in solubilisation of the iron core of these particles, we therefore considered whether the low pH of intracellular vesicles might be of importance for the solubilisation of the iron core. It is well known that endocytosed particles will follow an intracellular transport pathway through endosomes to lysosomes. During this transport, the particles will be present in vesicles that contain solutions with a pH gradually decreasing to 4.5–5.0 [11,12]. It may then be speculated that the low pH within these vesicles may be of importance for the solubilisation of these iron oxide particles. It should be noted that the cellular uptake of Fe proceeds through receptor mediated endocytosis of the Fe(III)-transferrin complex, followed by dissociation of Fe(III) from transferrin in the endosomes due to the low pH within these vesicles. It is not known how the released Fe is transported from the endosomes into cytosol and then to mitochondria, where it is used for heme synthesis. It is, however, likely that transport of the Fe from endosomes to cytosol involves binding to low molecular weight substances or transmembrane proteins. It may be speculated that such endogenous Fe(III) chelating substances in the endosomes may also promote dissolution of the iron oxide particles by binding to solubilised Fe atoms. It is known that several low molecular weight compounds in the cell—such as phosphate, nucleotides and dicarboxylic acids, i.e. citrate and isocitrate—may form complexes with Fe. The concentration of these or other Fe-complexing molecules within endosomes and lysosomes is unknown, so their contribution to the solubilisation process of the iron particles is speculative at present.

In this article, we show that the iron oxide particles of Clariscan™ are solubilised when incubated at pH 4.5–5.0 in the presence of the dicarboxylic acid citrate, which forms stable complexes with Fe(III), but not in the presence of the monocarboxylic acid acetate, which forms less stable complexes with Fe(III). The data presented indicate that the low pH of endosomes/lysosomes and some endogenous Fe-complexing substances may be responsible for the solubilisation of these ferromagnetic particles.

2. Experimental

2.1. Materials

Clariscan™ was from Nycomed Imaging AS, Norway. Bathophenanthroline disulfonic acid (BPS; 4,7-diphenyl-1,10-phenanthroline disulfonic acid) was from Sigma Chemical Co., MO; Fe(III)citrate was from Aldrich-Chemie GmbH, Germany (Cat. No. 22897-4). Water was

purified by using reverse osmosis, ion exchange and filtration through a 0.45 μm filter using a Milli-Q system (Millipore, MA). All other chemicals were of analytical grade quality. Sterile 0.2 μm filters (Acrodisc®) was obtained from Gelman Sciences Inc., MI and sterile 0.1 μm filters (Anotop 25 Plus) was obtained from Whatman Int. Ltd., UK.

2.2. Incubations

Seven incubation mixtures, each containing 30 μg Fe/ml, were made by the addition of 0.2 ml Clariscan™ (30 mg Fe/ml; pH 7.3) to 200 ml of buffer/water, previously passed through 0.2 μm sterile Acrodisc® filters. Three of these mixtures were obtained by diluting Clariscan™ with sodium citrate buffers (10 mM; pH 5.5, 5.0, 4.5) and three additional mixtures were obtained by diluting with sodium acetate buffers (10 mM; pH 5.5, 5.0, 4.5). The last mixture was obtained by dilution in water followed by the addition of HCl to give a final pH of 5.1; this mixture is referred to as 'water, pH 5.1'. These seven mixtures were incubated at 37 °C up to 22 days protected from light. Analyses of the solutions were carried out as detailed below.

2.3. Photon correlation spectroscopy

The scattering of light incident on a dilute suspension of particles smaller than ≈ 50 nm (I_s) is proportional to the mass concentration of particles (C) and their diameter (D) to the 3rd power [13]:

$$I_s \sim C \cdot D^3 \quad (1)$$

Monitoring the scattering intensity from a sample therefore provides a sensitive method for detecting dissolution and disintegration of suspended particles. For determination of particle size in such samples, photon correlation spectroscopy (PCS) is a convenient technique [10].

The particle size and the intensity of light scattered from Clariscan™ in the seven incubation mixtures was measured with a Malvern 4700 PS/MW spectrophotometer (Malvern Instruments Ltd., UK). The instrument was set up with a 7032 Multi 8, 128 channel, correlator and a Cyonics 70 mW Argon ion (488 nm) laser (Cyonics Corp., USA). Light output power was set to 70 mW, scattering angle was 90° and the aperture opening in front of the photomultiplier detector was 200 μm. Samples were filtered through 0.1 μm sterile, single-use filters directly into the measuring cuvettes and incubated at 37 °C between measurements. The samples were analysed immediately after preparation and after incubation for 6, 24, 48 and 72 h and 7, 9, 14 and 22 days; the mixture called 'water, pH 5.1' was measured immediately after preparation and after 22 days incubation only. Scattering intensities were recorded immediately after sample preparation and at various time-points. To evaluate the scattering from the solute (excess scattering), the background scattering from the pure diluent was also measured. This value was subtracted from the values measured for the suspension samples. In addition to intensity measurements, the particle size was determined by PCS on the same instrument and the intensity weighted average (z-average) diameter was calculated.

2.4. Relaxation measurements

The longitudinal relaxation time (T_1) was determined using a Minispec pulse NMR spectrometer (Bruker), operating at 0.47 Tesla (20 Mhz) and 37 °C. The T_1 values were then obtained using an inversion recovery pulse sequence with 12 different recovery times. The resulting protein signal intensity (volts) versus time (ms) was fitted to an exponential recovery curve using a three-parameter non-linear regression model, giving the T_1 relaxation time. The data are presented as $R_1 = 1/T_1$ (U: s^{-1}) as the decrease in this parameter is proportional to the decrease in the concentration of super paramagnetic particles in aqueous solutions [10]. This analysis was performed immediately after preparation of the mixtures and on the mixtures incubated for 6, 24, 48, 72 and 96 h and 22 days. This analysis was not performed on the samples incubated for 7 and 14 days (as for all other analyses).

2.5. Absorbance spectroscopy and iron chelation measurements using BPS

All absorbance measurements were obtained using a Cary 3E UV-Vis spectrophotometer and cuvettes with a light-path of 10 mm. The absorbance measurements at 378 nm were performed using the incubation mixtures without further dilution. Samples were analysed immediately after preparation of the incubation mixtures and after incubation for 6, 24, 48, 72 and 96 h and 7, 9, 14 and 22 days.

The amount of soluble Fe was estimated using the chelator BPS, which forms a highly coloured complex with Fe(II) but not with Fe(III) [14]. These analyses were performed on incubation mixtures that were diluted five times (addition of four volumes of the buffer/water used for that mixture). To 1.0 ml of this diluted solution, 40 μl of BPS (4.95 mM) was added and the concentration of Fe(II) estimated based on the absorbance at 535 nm obtained 90 s after the addition of BPS. Ascorbate (20 μl of 100 mM solution freshly made every day and kept on ice) was then added to reduce the soluble Fe(III) present in the mixture. A new reading of the absorbance at 535 nm was performed 8 min after the addition of ascorbate (the time needed to reach a constant absorbance level) and the difference between this value and the value obtained before the addition of ascorbate was used to estimate the amount of free or solubilised Fe(III). The iron content was calculated by using a standard curve made from solutions of Fe(III) citrate of which the Fe content was analysed using ICP-MS (Sciex Elan 5000 from Perkin Elmer, CT). The samples were analysed immediately after preparation of the mixtures and after incubation for 24, 48, 72 and 96 h and 7, 9, 14 and 22 days.

2.6. Data handling

The calculations were performed using Microsoft® Excel 97 SR-2 (Microsoft Corp.) and the graphical presentations were produced using GraphPad Prizm v. 2.00 (GraphPad Software Inc.)

3. Results and discussion

3.1. Light scattering analysis using PCS

Immediately after preparation, all samples gave an excess scattering intensity in the range of 6–8 k photons per second, with a particle size in the range of 8–11 nm. The results for the samples suspended in citrate buffer are shown in Fig. 1(A). As can be seen from these results, the scattering intensity decreased with time at a rate that was dependent on pH. At pH 4.5, the intensity dropped off rapidly, reaching the background level of pure diluent after ≈ 10 days. At pH 5.0, the rate was significantly slower, reaching zero excess scattering after 22 days. At pH 5.5, the decrease was still slower and the sample displayed a drop in scattering intensity of $\approx 60\%$ after 22 days. These results demonstrate that the particles suspended in citrate buffer dissolve completely, with a rate dependent on pH.

As the scattering intensity drops the results for particle size become less reliable. Hence, values for this parameter were calculated only for samples giving an intensity > 5 k photons per second. The data obtained with this method indicated that the particles diluted in citrate buffer did not display significant variations from the value immediately after sample preparation for any of the samples (data not shown). Although these data should be interpreted with caution due to the low signal intensity of the samples, these data indicate that most of the particles had the same size, but some of the particles rapidly decreased in size. These results then indicate that the particles with the greatest initial rate of solubilisation seem to be completely solubilised within a relatively short time frame.

The particles suspended in acetate buffer displayed some increasing aggregation with time. As the scattering intensity is proportional to the 3rd power of the diameter (Eq. (1)), this intensity was observed to increase strongly with time, so the results for these samples are not reported. Only minor changes were observed in the mixture containing the particles in 'water, pH 5.1' (Fig. 1A).

3.2. Magnetic relaxation measurements

The R_1 data are shown in Fig. 1(B). As observed with the light scattering technique, these data show that the particles incubated in citrate buffer were solubilised faster when the pH of the mixtures decreased from 5.5 to 4.5. These data indicate an almost complete solubilisation of the particles after 4 days of incubation in 10 mM citrate, pH 4.5. Incubation of the iron particles in the acetate buffers revealed only very minor changes in the R_1 values during the first 4 days of incubation and there were no significant differences between the three acetate buffers of pH 4.5, 5.0 and 5.5. No changes were observed in the R_1 values of the mixtures containing the particles in 'water, pH 5.1'.

3.3. Absorbance spectra

Pilot experiments showed that during the solubilisation of the particles the solution changed colour and became more yellow. Absorbance spectra obtained in the region 300–700 nm showed that the maximum spectral difference between solutions containing intact or solubilised particles was obtained at 378 nm (data not shown). As pilot experiments also showed that the particles were not solubilised when stored at 4 °C (data not shown), the absorbance at 378 nm for the solutions stored at 37 °C was measured using the same mixture stored at 4 °C in the reference cuvette.

The spectral changes observed at 378 nm as a function of incubation time are shown in Fig.

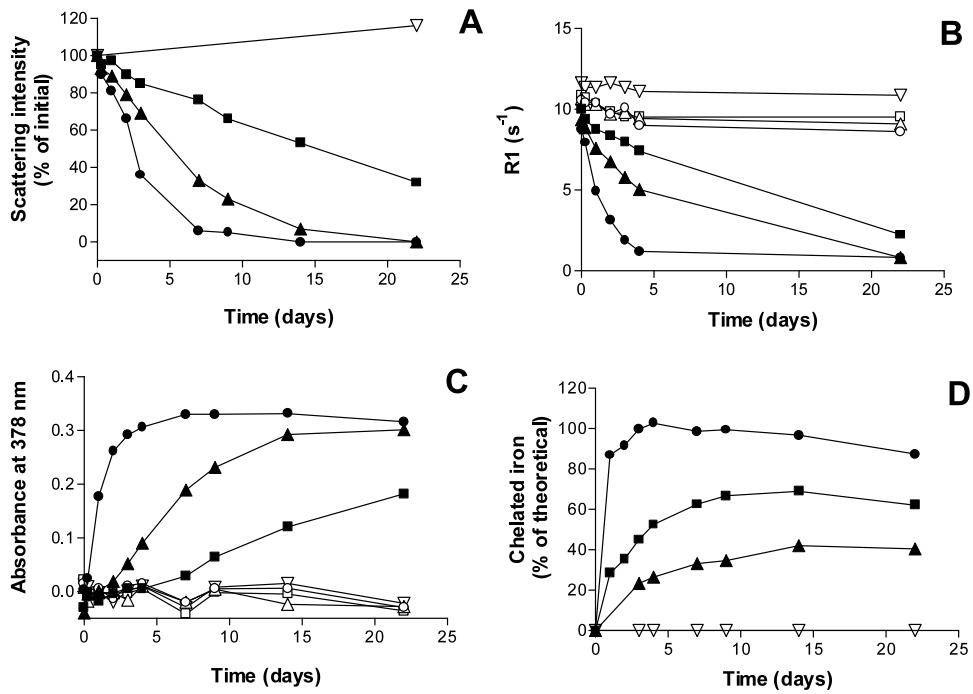

Fig. 1. Changes observed with solutions containing iron oxide particles from Clariscan™ as a function of incubation time at 37 °C protected from light. The symbols indicate that the particles have been diluted in citrate buffer, pH 4.5 (●); citrate buffer, pH 5.0 (▲); citrate buffer, pH 5.5 (■); acetate buffer, pH 4.5 (○); acetate buffer, pH 5.0 (△); acetate buffer, pH 5.5 (□); 'water, pH 5.1' (▽). (A) The scattering intensities (shown as percent of the initial value) are shown as a function of incubation time for the three mixtures in citrate buffers and for 'water, pH 5.1'. (B) The magnetic relaxation data (shown as R_1 values) of all mixtures are shown as a function of incubation time. (C) The spectral changes observed at 378 nm are shown as a function of incubation time for all mixtures. (D) The percent of iron in the mixtures that can be chelated by BPS in the presence of ascorbate are shown as a function of incubation time for the three mixtures in citrate buffers and for 'water, pH 5.1'.

1(C). The data show a very similar kinetics for the solubilisation of the particles diluted in the three citrate buffers compared to that observed for these mixtures with light scattering (Fig. 1A) and magnetic relaxation (Fig. 1B). The solutions containing particles in the three acetate buffers, as well as in 'water, pH 5.1', did not show any changes in the absorbance spectra (Fig. 1C), in good agreement with the data obtained using magnetic relaxation measurements (Fig. 1B).

3.4. Iron able to form complexes with BPS

These analyses were performed using the incubation mixtures in which the particles were diluted in the three citrate buffers and in 'water, pH 5.1'. A very low reactivity was observed before the addition of ascorbate, i.e. all samples then showed an absorbance at 535 nm below 0.05, indicating that $<4\%$ of the iron in these mixtures was present as Fe(II) in a form that could bind to the chelator. The data obtained after the addition of ascorbate, i.e. the amount of chelator reactive Fe(III) in the mixtures, are shown in Fig. 1(D). As practically no reaction was observed immediately after preparation of the mixtures, the curves in Fig. 1(D) show that the solubilisation of the iron core increased with decreasing pH from 5.5 to 4.5. The maximum reactivity was observed in the citrate buffer at pH 4.5 after 4 days of incubation. The amount of iron able to react with BPS at this time point corresponds to 103% of that theoretically present in the mixture. At this time point, $\approx 53\%$ of the iron in the incubation mixtures with citrate buffer at pH 5.0 was in a chelator-reactive form, whereas $\approx 26\%$ was in a chelator-reactive form in the incubation mixture with citrate buffer at pH 5.5. For the particles incubated in 'water, pH 5.1' $<1\%$ of the iron was chelated with BPS at all time points. The curves obtained for the mixtures with citrate buffers in Fig. 1(D) show some lower iron reactivity in the samples stored for many days. Thus, the data obtained after 22 days of incubation showed 87% reactivity in 10 mM citrate, pH 4.5, i.e. 16% less than that obtained after 4 days of incubation. This effect is most probably due to aggregation/precipitation of some of the Fe(III) present during the long incubation time under these in vitro conditions in the presence of oxygen in air.

Table 1
Relative rate of solubilisation of the ferromagnetic particles diluted in 10 mM citrate at pH 4.5, 5.0 and 5.5

Analytical method	pH 5.0 (%)	pH 5.5 (%)
Photon correlation spectroscopy	71	26
Magnetic relaxation measurements	58	34
Absorbance spectroscopy	43	12
Chelator reactive iron	45	23

The changes measured in the mixtures with pH 5.0 and 5.5 are given as a percentage of that measured in the mixture with pH 4.5 during the first 72 h of incubation at 37 °C.

4. Overall discussion and conclusion

The data obtained with the four different analyses all show a more rapid solubilisation of the ferromagnetic particles in citrate buffers than in acetate buffers, indicating that low molecular weight iron-complexing substances may be important for the solubilisation of these particles. Furthermore, the rate of solubilisation was much faster in 10 mM citrate, pH 4.5 than at pH 5.0, which in turn was much faster than at pH 5.5. The relative rates of solubilisation at the three different pH-values are indicated in Table 1. These data show that after 72 h of incubation, the changes observed in the mixture at pH 5.0 were in the range of 43–71% of the changes observed at pH 4.5, whereas the changes observed at pH 5.5 were in the range of 12–34% of that observed at pH 4.5. In conclusion, the results indicate that the low pH in endosomes/lysosomes, as well as endogenous iron-complexing substances, may be important for the solubilisation of these ferromagnetic particles following i.v. injection.

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