Analytical Methods

Dynamic light scattering-based method to determine primary particle size of iron oxide nanoparticles in simulated gastrointestinal fluid

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A B S T R A C T

Simple dynamic light scattering (DLS)-based methodologies were developed to determine primary particle size distribution of iron oxide particles in simulated gastrointestinal fluid. Iron oxide particles, which easily agglomerate in aqueous media, were converted into dispersed particles by modification of surface charge using citric acid and sodium citrate. After the modification, zeta-potential value decreased to −40 mV at pH 7. Mean particle diameters in suspensions of iron oxide nano- and microparticles stabilized by the mixture of citric acid and sodium citrate were dramatically decreased to 166 and 358 nm, respectively, which were close to the particle size distributions observed in the micrographs. In simulated gastrointestinal fluid, both iron oxide nano- and microparticles were heavily agglomerated with particle diameters of almost 2600 and 5200 nm, respectively, due to charge shielding on the citrate-modified surface by ions in the media. For determining primary particle size distribution by using DLS-based approach, the iron oxide particles incubated in the simulated gastrointestinal fluid were converted to monodisperse particles by altering the pH to 7 and electrolyte elimination. The simple DLS-based methodologies are well suited to determine primary particle size distribution of mineral nanoparticles at various physical, chemical, and biological conditions.

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1. Introduction

Iron oxide (Fe₂O₃, Iron (III) oxide) nanoparticles have attracted a lot of interest as they are biocompatible and less toxic in biological systems compared to other metals (Corot, Robert, Idee, & Port, 2006). Iron oxide nanoparticles are being used for different applications such as essential mineral supplements, controlled release pharmaceutical and nutraceutical agents, lipstick, and cosmetics (Bulte & Kraitchman, 2004; Weinstein et al., 2009).

The most important and simple parameter for characterization of iron oxide nanoparticles is determination of particle diameter and size distributions. Dynamic light scattering (DLS) is the most popular and common technique that is used to determine the size distribution profile of sub-micron particles in suspension. However, the particle size distribution measurement by DLS may be affected by the concentration of the suspension, composition of solvents, dust and other additives (in most cases, ions or free radicals). More importantly, aggregates or agglomerates of smaller particles are usually identified or counted as one large particle with the DLS method. This method may give inaccurate and unreliable results. Therefore, an appropriate technique preventing particle agglomeration, which allows optimal particle dispersion, is essential to get the accurate and reliable results.

Iron oxide nanoparticles tend to aggregate into large clusters in aqueous conditions. In addition, even well dispersed colloidal particles may show different physical behaviours depending on the media used such as water, stomach- or small intestine-medium, and other biological fluids. Some media may accelerate the destabilisation of colloidal nanoparticles dispersion, which leads to sedimentation (Santra et al., 2001), since they are susceptible to ionic strength, pH, temperature, enzymes, and chemical composition of solvent and contaminants (Rodríguez & Armstrong, 2004).

Accurate measurement of primary particle size distribution of iron oxide nanoparticles is important for a variety of applications, especially for function and safety evaluation of iron oxide nanoparticles in terms of toxicity, absorption in the gastrointestinal...
tract, fate of nanomaterials, and permeability across the cell wall. But, mineral particles like iron oxide nanoparticle has high tendency for agglomeration during digestion and adsorption in the gastrointestinal tract (Frenkel et al., 2005; Zimmermann & Müller, 2001). Here, we hypothesised that the potential factors affecting the particle agglomeration could be pH, enzymes, and/or electrolyte during digestion. Therefore, a key requirement for accurate measurement of primary particle size distribution is to have monodisperse iron oxide particles in the medium. Several techniques for monodisperse suspension preparation have been proposed to overcome the constraints in achieving the monodispersion. Such techniques include surface modification, encapsulation, functionalization, micellization and coating (Bourlinos, Bakandritsos, Georgakilas, & Petridis, 2002; Xu, Wang, Yang, Deng, & Fu, 2004). However, most methods are time consuming, expensive or use toxic reagents. Herein, therefore, we proposed a simple procedure for stabilizing mono-dispersed iron oxide particles in an aqueous solution; the iron oxide nanoparticles were subjected to different conditions by varying ionic strength, pH, and medium thereafter centrifuged, re-dispersed, and ultrasonicated for accurate characterization of primary particle size distribution.

The mixture of citric acid and sodium citrate was used for the modification of surface charge of iron oxide particles in the aqueous medium. In addition, other analytical techniques such as electrophoretic light scattering (ELS) for zeta-potential measurement and scanning electron microscopy (SEM) for the particle morphology determination, and BET nitrogen adsorption for specific surface area measurement were used to understand physical characteristics of the iron oxide nanoparticles (Baer, Gaspar, Nachimuthu, Techane, & Castner, 2010).

The objectives of this study were to establish a sample treatment procedure for DLS-based determination of the primary particle size distribution of iron oxide nanoparticles in aqueous and simulated gastrointestinal fluid conditions, and to understand the effect of these different treatments on their physical properties.

2. Materials and methods

2.1. Materials and reagents

Commercial nano- and micro-sized iron oxide powders were purchased from American Elements (Nominal particle size <100 nm, Los Angeles, CA, USA) and Alfa Aesar (Nominal particle size 44 µm, Ward Hill, MA, USA), respectively. Citric acid anhydrous, sodium citrate dihydrate and other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Preparation of aqueous suspensions of iron oxide particles

Colloidal suspensions of iron oxide nano- and microparticles were prepared by ultrasonication. For the preparation, 0.1 g of each type of Fe₂O₃ powder was mixed with 100 mL of deionized water, followed by blending with the mixture of 0.3 mL of 0.1 mol/l citric acid and 0.5 mL of 0.1 mol/l sodium citrate to form the iron oxide aqueous suspension. The mixture was stirred at 500 rpm for 30 min. The dispersion was treated by using an ultrasonic processor (VCX-750, Sonics & Materials Inc., Newtown, CT, USA) equipped with 6 mm diameter probe for 20 min. Colloidal iron oxide nano- and microsuspensions without a stabilizer were prepared at the same conditions as described earlier to investigate and validate the stabilizing effect of the mixture of citric acid and sodium citrate. The short notations B Fe₂O₃NP, B Fe₂O₃MP, C Fe₂O₃NP and C Fe₂O₃MP indicate the iron oxide nano- and microparticles, and iron oxide nano- and microparticles stabilized by the mixture of citric acid and sodium citrate, respectively.

2.3. Scanning electron microscopy

Particle size and morphological properties were determined by a field emission-scanning electron microscope (FE-SEM, S-4300, Hitachi, Tokyo, Japan).

2.4. Particle size distribution measurement

Mean hydrodynamic particle diameter and size distributions of the colloidal iron oxide suspensions prepared were measured using a particle size analyzer (Delsa Nano C, Beckman Coulter Inc., Fullerton, CA, USA). For the measurement, suspensions were shaken gently, and 3 mL of each suspension was transferred to an optical-grade cell. The data was analysed based on intensity distribution. The measurements were performed at least in triplicate for each sample.

2.5. Zeta-potential measurement

Electrical stability of the iron oxide suspensions was determined based on the ELS method using the same instrument that was used for the particle size analysis. For the analysis, iron oxide suspensions were diluted 10-fold with deionized water and subjected to pH gradient from 2 to 12. Then, 1 mL of each diluted suspension sample was injected into a flow cell and potentials were measured at different cell positions of 0.7, 0.35, 0, –0.35 and –0.7. The measurements were conducted at least in triplicate for each sample.

2.6. Surface area analysis in aqueous suspension

Estimated surface areas of iron oxide particles in an aqueous medium were calculated based on the mean particle diameter data obtained from the particle size analysis and true density. The true density of the iron oxide particles was determined by a pycnometer (AccuPyc II 1340, Micromeritics Instrument Corporation, Norcross, GA, USA). The estimated surface area was calculated by the equation below

\[ ESA = \frac{6}{\rho \times D} \]  

where ESA: estimated surface area (m²/g); \( \rho \): true density (g/cm³); D: mean particle diameter (µm).

2.7. In vitro digestion in simulated gastrointestinal conditions

In order to investigate the fate and primary particle size distribution of iron oxide particles under the simulated gastrointestinal conditions, iron oxide suspensions prepared with the mixture of citric acid and sodium citrate were allowed to distribute in simulated gastric and intestinal conditions. Determination of primary particle size and agglomeration profile was carried out by using the modified in vitro digestion model (Shim & Kwon, 2010). Briefly, 20 mL of each iron oxide suspension sample was diluted with 20 mL of 20 mol/l phosphate buffered saline (PBS, pH 7.4, Sigma–Aldrich, Louis, MO, USA), and 2 mL of pepsin solution (40 mg/mL in 0.1 mol/l HCl) was added. The resultant solution pH was adjusted to 2, followed by incubating at 37 °C in a shaking water bath (Han Yang Scientific Equipment Co., Ltd, Seoul, Korea) with orbital shaking at 150 rpm for 1 h. In order to adjust to the simulated intestinal condition, the pH of the solution was adjusted to 5.3 by using 1 mol/l NaHCO₃. Then, 2 mL of the enzyme mixture...
consisting of 1 mg/mL lipase, 12 mg/mL bile extract, and 2 mg/mL pancreatin in 0.1 mol/l NaHCO₃ solution was added. Finally, the pH of the solution was adjusted to 7 by using NaOH, and incubated at 37 °C in the shaking water bath.

2.8. Particles agglomeration analysis during in vitro digestion in simulated gastrointestinal conditions

For the determination of particle agglomeration profile, the particle size distribution of each sample was measured at time intervals of 0, 30, 60, 90 and 120 min. At the end of each time period, particle size distribution analysis, electron microscopy, and zeta-potential measurement were performed in order to estimate agglomeration.

2.9. Primary particle size distribution analysis during in vitro digestion in simulated gastrointestinal conditions

In order to determine the primary particle diameter and after the simulated gastrointestinal digestion, the potential factors such as pH, enzymes, and/or electrolyte that affect the particles agglomeration was removed by using the treatment procedures described as follows: for removing the pH effect on the particle agglomeration, a portion of resulting digestive medium at the end of the gastric digestion was transferred into a tube and pH was adjusted to 7. The leftover portion was washed with different solutions such as deionized water, hydrochloric acid–potassium chloride buffer, and phosphate buffer, followed by centrifugation at 2000 × g for 5 min thrice for elimination of electrolytes. Finally, another set of in vitro gastrointestinal digestion was performed with heat-denatured enzymes and the diameter of the iron oxide particles was compared with that of enzyme-digested iron oxide particles at the end of the digestion.

3. Results and discussion

3.1. Particle morphology

Particle diameter and morphological features of the iron oxide particles were observed by scanning electron microscopy. As shown in Fig. 1, B₅Fe₃O₇NP exhibited an approximate spherical shape and 100–300 nm in particle size. It is interesting to note that B₅Fe₃O₇MP exhibited nanometer size. It may be due to aggregation of raw iron oxide micro-sized powder. These results of hydrodynamic particle diameter are in agreement with data obtained from surface area measurement (indicated in Section 3.3). The iron oxide microparticles (Fig. 1, both B₅Fe₃O₇MP and C₅Fe₃O₇MP) exhibited smooth surfaces while the nanoparticles (both B₅Fe₃O₇NP and C₅Fe₃O₇NP) showed a somewhat rough surface with cracks. The morphological differences between bare iron oxide particles and those stabilized by the mixture of citric acid and sodium citrate was not clearly distinguishable in terms of the shape and particle size before-and-after the modification.

3.2. Primary particle size distribution of iron oxide particles in aqueous suspensions

In the case of B₅Fe₃O₇MP suspension, most of the particles were precipitated within a minute leaving a clear aqueous supernatant immediately after the preparation. In contrast, B₅Fe₃O₇NP suspension exhibited slow precipitation of iron oxide particles since they agglomerated gradually. Iron oxide particles in the aqueous suspensions of C₅Fe₃O₇NP and C₅Fe₃O₇MP were homogeneous and well dispersed.

In the suspensions of C₅Fe₃O₇NP and C₅Fe₃O₇MP, no chemical interaction between iron oxide particles and the stabilizer (citric acid and sodium citrate) were observed. FT-IR spectra of the citric acid in B₅Fe₃O₇NP and B₅Fe₃O₇MP as well as C₅Fe₃O₇NP and C₅Fe₃O₇MP showed no associated vibration peaks between iron oxide and citric acid or sodium citrate (data are not shown).

This could mean that the mixture of citric acid and sodium citrate does not affect the interior structure or the surface of the iron oxide particles in an aqueous suspension. But, it is believed that the mixture of citric acid and sodium citrate stabilized the iron oxide particles in an aqueous suspension by inducing selective ionic adsorption on the surface.

Mean particle diameter and polydispersity index (PDI) of the aqueous suspensions of iron oxide particle are shown in Table 1. Mean particle diameters of the aqueous suspensions of bare Fe₅O₇NP and Fe₅O₇MP were 677.9 nm and 4.59 μm, respectively, while those of C₅Fe₃O₇NP and C₅Fe₃O₇MP were 166.10 and 358.87 nm, respectively. Unlike the particle diameters observed in the micrographs, B₅Fe₃O₇NP and B₅Fe₃O₇MP showed very large particle diameters in an aqueous suspension. The iron oxide particles are easily agglomerated in an aqueous suspension because water hardly penetrates into the space among the particles, which are highly insoluble. The mean particle diameters of C₅Fe₃O₇NP and C₅Fe₃O₇MP were 4 and 13 times smaller than those of their counterparts B₅Fe₃O₇NP and B₅Fe₃O₇MP, respectively. Given the average particle diameter calculated from specific surface area and true density, the diameters of iron oxide nano- and microparticles should be much less than 100 and 1000 nm, respectively (see Section 3.3). However, the diameters of B₅Fe₃O₇NP and B₅Fe₃O₇MP were much larger than estimated particle diameter. This indicates that the iron oxide particles are easily aggregated in an aqueous suspension in spite of ultrasonication. In general, colloidal particles are stabilized by two main forces, namely Van Der Waals interaction and electrostatic repulsive force. When the attraction force is less than repulsive force, the colloidal particles are dispersed, and if the attraction force exceeds the repulsive force the particles tend to form aggregates (Trau, Saville, & Aksay, 1997). In this study, B₅Fe₃O₇NP and B₅Fe₃O₇MP showed very low zeta-potential at neutral pH. This indicates that the particle has a very low charge density on its surface at neutral pH. On the other hand, C₅Fe₃O₇MP exhibited relatively high zeta-potential, which means they have higher charge which means they have higher charge density on their surface compared to bare iron oxide particles. PDI, which indicates the size distribution of particles of the iron oxide suspension, has shown good agreement with other results. The value was much lower in the suspensions of C₅Fe₃O₇NP and C₅Fe₃O₇MP than those of B₅Fe₃O₇NP and B₅Fe₃O₇MP (Table 1). This indicates that the suspensions of C₅Fe₃O₇NP and C₅Fe₃O₇MP have a uniform particle size and, therefore exhibited narrow particle size distribution.

3.3. Surface characteristics of iron oxide particles in aqueous suspensions

Fig. 2a shows the zeta-potential profiles of the suspensions of the iron oxide particles in the pH gradient from 2 to 12. The zeta-potential values of the aqueous suspensions of B₅Fe₃O₇NP and B₅Fe₃O₇MP were positive at low pH and decreased gradually with increasing pH. The zeta-potential was slightly higher in the suspension of B₅Fe₃O₇NP than B₅Fe₃O₇MP at moderate pH range. In addition, the isoelectric point (pI), which represents the pH where net electrical charge is zero, of both B₅Fe₃O₇NP and B₅Fe₃O₇MP, was found to be 6 and 7, respectively. Electrical charge density (zeta-potential) on the surface of a colloidal particle is a critical factor for determining the stability of colloidal systems. As the electrical charge density increases, the particles tend to acquire more intensive repulsive forces that prevent contact between them.
general, most of the colloidal particles have either positive or negative charges on their surface. Usually, zeta-potential value of ±30 mV renders proper stability for colloid systems (Lin, Wu, & Chen, 2006). While, the value close to zero indicates less stability resulting from the reduced repulsive force between particles. In such case, the colloidal particles are subject to flocculation resulting in precipitation (Anderson, 1985; Yoon & Deng, 2004).

The zeta-potential values of the aqueous suspensions of both C_Fe2O3NP and C_Fe2O3MP were also positive at low pH. Consequently, zeta-potentials decreased sharply in the pH range 4–6, and remained flat above pH 6. The pl values of C_Fe2O3NP and C_Fe2O3MP were close to pH 4 and 5, respectively. The pl values of C_Fe2O3NP and C_Fe2O3MP were shifted towards lower pH compared to both B_Fe2O3NP and B_Fe2O3MP. Modification of the chemical environment of the aqueous solution, by citric acid and sodium citrate, may have altered the charge on the surface of the iron oxide particles. It is interesting to note that the pH of the final products of all iron oxide suspensions were between 6 and 7.
which indicates that bare iron oxide particles would precipitate quickly in an aqueous suspension because the zeta-potential values of the final suspensions were low (less than ±10 mV) for both nano- and microparticles. On the other hand, C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}NP} and C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}MP} were stable in an aqueous suspension because their pI values were shifted somewhat towards lower pH.

Density and specific surface area of the iron oxide powders are listed in Table 1. The iron oxide particles were calculated based on the particle size in an aqueous suspension, which, in turn, was measured by DLS method and true density. As previously stated, the iron oxide particles are easily agglomerated in an aqueous suspension at neutral pH, and therefore, the surface area of the particles could be decreased. We mentioned earlier that the iron oxide particles are easily agglomerated in an aqueous suspension at neutral pH, and therefore, the surface area of the particles could be decreased. As indicated in Table 1, the values of estimated surface area decreased a long with the increase in particle diameter.

In addition, we predicted the particle diameter of raw iron oxide nano- and microparticles using Eq. (1). We assume that the particles are equal-sized and spherical, which is never the case, but allows for a good approximation. So, we can predict that as the estimated particle diameter of the iron oxide suspensions is closer to the mean size of iron oxide particles, the iron oxide suspension can be recognised as a well-dispersed (mono-dispersed) suspension. In particular, the diameters of the suspensions of B\textsubscript{Fe\textsubscript{2}O\textsubscript{3}NP} and B\textsubscript{Fe\textsubscript{2}O\textsubscript{3}MP} were much larger than calculated values. This indicates that B\textsubscript{Fe\textsubscript{2}O\textsubscript{3}NP} and B\textsubscript{Fe\textsubscript{2}O\textsubscript{3}MP} were rarely dispersed in an aqueous suspension, while C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}NP} and C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}MP} were well dispersed with the aid of the mixture of citric acid and sodium citrate in an aqueous suspension.

### 3.4. Particles agglomeration during simulated in vitro gastrointestinal digestion

Morphological features of iron oxide particles after the in vitro gastrointestinal digestion are shown in Fig. 1e and f. Iron oxide nano- and microparticles exhibited somewhat similar surface morphological features to the particles before digestion, and they existed as individual particles without any particle erosion (or fusion). The particles were highly agglomerated, but each particle maintained its shape. Particle agglomeration profile of the suspensions of C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}NP} and C\textsubscript{Fe\textsubscript{2}O\textsubscript{3}MP} during the digestion is shown in Fig. 3. Both nano- and microparticles exhibited rapid agglomeration as soon as the pH was modified for in vitro gastric digestion. Thereafter, the hydrodynamic particle diameter of both nano- and microparticles increased by more than 10 times compared to the values observed for the aqueous suspension. Thereafter, the diameter of the particles gradually increased as time elapsed. As indicated by SEM analysis, it seemed to be heavily agglomerated. This strongly predicts the fate of both nano- and microparticles in GIT after consumption and need for proper surface modification before using them in the marketed products.

Both types of the particles settled down within minutes of gastric digestion commencing. Under the simulated intestinal conditions, the iron oxide particles that precipitated during the gastric digestion were re-suspended and dispersed into individual particles resulting in a stable dispersion. Hydrodynamic particle diameters of both iron oxide nano- and microparticles were less than 600 and 800 nm, respectively, and remained constant during the entire process (Fig. 3). From these results, it can be speculated that the iron oxide particles agglomeration would be the result of pH change. However, the similar zeta-potential values (+20 mV) of both nano- and micro-sized iron oxide particles stabilized by the combination of citric acid and sodium citrate at pH 2 could create significant repulsive forces between particles. Thus, we assume there may be an exist factor responsible for particles agglomeration. Zeta-potentials before digestion across the pH range are shown in Fig. 2. Zeta-potential profiles obtained from in vitro intestinal digestion were similar to that of gastric digestion.
Zeta-potential profile obtained from in vitro intestinal digestion was similar to that of gastric digestion.

3.5. Establishment of DLS-based method to determine primary particle size during in vitro digestion

Iron oxide particles agglomerated to form large particle clusters spontaneously under the simulated gastrointestinal conditions. In particular, the particles were heavily agglomerated under the in vitro gastric conditions. However, it is important to note that the particles were not fused but physically agglomerated. The particles maintained their individual shape and structure within clusters. In such a case, determining primary particle size and size distribution is important since the agglomerated particles in the gastric condition could be re-dispersed into individual ones, as shown in Fig. 3. In the following section, thus, we propose a simple method for determining primary particle size distribution during in vitro digestion under simulated gastrointestinal conditions. The particle diameter and PDI value of the iron oxide particles after a change of pH from 2 to 7, at the end of the in vitro gastric digestion, are shown in Fig. 4. The mean particle diameters after the pH shift were decreased about two-fold compared to those of iron oxide particles at pH 2 for both nano- and microparticles. Furthermore, PDI values were dramatically decreased, which indicates that the iron oxide solution was stable and monodisperse at pH 7. These results support the reason for the decrease in particle diameter observed under the in vitro intestinal digestion. During the electrolytes elimination, the particle diameters varied depending on the solvents used. Particle diameters of the iron oxide particles immersed in different solutions are shown in Fig. 5. In the case of particles that underwent in vitro gastric digestion, particle diameters were decreased almost by two-fold for nanoparticle and more than three-fold for microparticle on washing with deionized water (pH 2). On the other hand, particle diameters of both iron oxide nano- and microparticles that were washed with hydrochloric acid–potassium chloride buffer (pH 2) were substantially increased compared with gastric media. For the intestinal digestion condition, particle diameters of iron oxide particles, which were washed with deionized water (pH 7), decreased almost by two-fold for both nano- and microparticles. However, exactly opposite was observed with phosphate buffer washing. The lowest particle diameters were observed on washing with deionized water for both the nano- and microparticles (348 and 533 nm, respectively) under in vitro intestinal digestion. These results support the conclusion that the iron oxide particles were also affected by ionic strength of the solution. Ionic species present in hydrochloric acid–potassium buffer and phosphate buffer seemed to affect the particle agglomeration. Thus, deionized water without any ionic species was considered the best for eliminating electrolytes and re-dispersing the iron oxide particles. Therefore, it can be summarized that the particle agglomeration and particle growth were the result of synergism between pH alteration and the presence of electrolytes. It can be concluded that low pH and the presence of electrolytes decrease the surface charge density and reduce electric double layers of the iron oxide particles. Based on results from SEM observations, particle size distributions and zeta-potential profile, we can conclude that the agglomeration of the iron oxide particles under simulated gastrointestinal conditions was due to a reduction in repulsive force between particles resulting from synergism between pH change and the presence of electrolyte. Therefore, primary particle size distribution of colloidal particle in biological simulations could be determined through a change of solvent properties, such as ionic strength and pH.

4. Conclusions

In this paper, we propose simple procedures for stabilizing iron oxide nano- and microparticle in an aqueous suspension, and for determining primary particle size distribution during in vitro digestion using a simulated gastrointestinal model. A mixture of citric acid and sodium citrate stabilized the iron oxide to colloidal suspensions. Although the mechanism of action is unknown, it was evident this mixture of citric acid and sodium citrate stabilized the iron oxide particles, and the stabilization was derived from the regulation of selective adsorption of ions on the surface of particles. Thus, it is important to verify the stabilizing effect of the mixture of citric acid and sodium citrate for a variety of applications. For
primary particle size determination, we have identified factors affecting iron oxide particles agglomeration, which includes pH and the presence of electrolytes in simulated biological fluids. Deionized water was found the most suitable solvent for primary particle size determination. The methods proposed in this paper for preparation of stable and well-dispersed nanometer-scale inorganic materials and to determine the primary particle size in biological fluids could be useful in food applications for nanoparticles and other research fields such as toxicology.

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References


