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Stability of Hydrophilic Magnetic Nanoparticles Under Biologically Relevant Conditions

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Hydrophilic 2,3-dimercaptosuccinnic acid (HOOC-CH(SH)-CH(SH)-COOH, DMSA) coated monodisperse magnetic nanoparticles (Fe_3O_4) were dispersed in water, RPMI-1640 with 10% (v/v) fetal calf serum, RPMI-1640, PBS and MES, respectively, to investigate their stability under biologically relevant conditions. The Photon Correlation Spectroscopy (PCS) results showed that DMSA-Fe_3O_4 nanoparticles existed as aggregate under biological conditions. UV-vis, MRI and AFM results indicated that DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640, PBS and MES presented poor stability, whereas those dispersed in RPMI-1640 with fetal calf serum exhibited excellent stability, which was due to their adsorption from fetal calf serum, as confirmed by zeta potential and IR results. Additionally, *in vitro* cell experiments showed that DMSA-Fe_3O_4 nanoparticles with adsorption from fetal calf serum had higher intracellular uptake than those without adsorption from serum, indicating that fetal calf serum could play a great role in intracellular uptake of nanoparticles.

Keywords: Magnetic Nanoparticles, Stability, Adsorption, Serum, Intracellular Uptake.

1. INTRODUCTION

Magnetic nanoparticles have attracted great interests for both *in vitro* and *in vivo* applications, such as magnetic resonance image (MRI),[1,2] magnetic separation,[3] DNA detection[4] and magnetic hyperthermia.[5,6] All above-mentioned biological applications require nanoparticles to keep stable under biologically relevant conditions. As we know, magnetic nanoparticles dispersed in aqueous solution keep stable through electrostatic or steric repulsion. However, they are ready to form aggregates due to attractive van der Waals or magnetic dipole–dipole interactions, and their stability can change with pH values and salt concentrations under physiological conditions. Therefore, stability of magnetic nanoparticles under biological conditions is one of the important parameters to consider for biological applications. However, up to day, stability investigation of magnetic nanoparticles dispersed in biological media has not been well-established.

In this work, monodisperse Fe_3O_4 nanoparticles were synthesized by thermal decomposition of iron-oleate, and DMSA-modified magnetic nanoparticles (DMSA-Fe_3O_4) were obtained via surface exchange with 2,3-dimercaptosuccinnic acid (DMSA),[7] and dispersed in water, RPMI-1640 with 10% (v/v) fetal calf serum, RPMI-1640, PBS and MES, respectively, to investigate their stability. We found that DMSA-Fe_3O_4 nanoparticles existed as aggregate under biologically relevant conditions, and DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640 with fetal calf serum exhibited excellent stability, compared to the other biological media, due to their adsorption from fetal calf serum (abbreviated as serum-DMSA-Fe_3O_4). Similar phenomena have been observed by Chithtani et al.,[8] Zhu et al.[9] and Casey et al.[10] using Ag, muti-walled nanotubes and single walled carbon nanotubes, respectively. Furthermore, Casey et al. suggested that the presence of serum be seen to aid the stability of carbon nanotubes. Here, we further focused on the effects of serum on surface property of nanoparticles, and resultant effects on intracellular uptake. In *in vitro* cell experiments, DMSA-Fe_3O_4 and serum-DMSA-Fe_3O_4 nanoparticles were incubated with KB and SMMC-7721 cells for 12 hours, respectively, in RPMI-1640 without 10% (v/v) fetal calf serum, to compare their intracellular uptake. The results showed that serum-DMSA-Fe_3O_4 nanoparticles had higher intracellular uptake, which suggested that the effects of fetal calf serum in *in vitro* cell experiments should be considered, because they could affect stability and surface properties of magnetic nanoparticles, and eventually affect the interactions between nanoparticles with cells.

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2. EXPERIMENTAL DETAILS

2.1. Materials

1-octadecene was purchased from Alfa Aesar. 4-morpholineethanesulfonic acid (MES) was purchased from Pierce and RPMI-1640 was purchased from Gibco. The other chemicals were analytical reagents and purchased from Shanghai Chemical Reagent Corporation, China. All chemicals were used as received. Deionized water was used for all the experiments.

2.2. Synthesis of Hydrophilic Monodisperse Magnetic Nanoparticles

2.2.1. Synthesis of Monodisperse Fe_3O_4 Nanoparticles

Synthesis of monodisperse Fe_3O_4 nanoparticles was based on reported work.[11] In a typical experiment, 1.08 g of $FeCl_3 \cdot 6H_2O$ and 3.65 g of sodium-oleate was dissolved in a mixture solvent containing 6 ml water, 8 ml ethanol and 14 ml hexane. The resulting solution was stirred for four hours at 70 °C. And then, the upper organic layer containing iron-oleate was washed three times with 3 ml water in a separatory funnel. After water and hexane was evaporated off under vacuum, solid iron-oleate was collected. The obtained 2.8 g iron-oleate and 0.5 ml oleic acid was dissolved in 17 ml 1-octadecene, and the mixture was heated to 320 °C with a constant heating rate of 3.3 °C/min and then kept at that temperature for 30 min. After that, the resulting solution was cooled and precipitated by addition of excess ethanol and centrifugation. And then, the precipitate containing Fe_3O_4 nanoparticles was washed 4–5 times with ethanol. Finally, Fe_3O_4 nanoparticles were stored for next surface modification.

2.2.2. Synthesis of Hydrophilic Fe_3O_4 Nanoparticles Modified by DMSA

Synthesis of hydrophilic Fe_3O_4 nanoparticles modified by DMSA via surface double-exchange was according to our previous work.[7] In a typical experiment, 200 mg as-made Fe_3O_4 nanoparticles was dissolved in 20 mL chloroform followed by addition of 100 μL triethylamine and a solution containing 100 mg and 20 mL dimethyl sulfoxide (DMSO). The resulting solution was vortexed at 60 °C for 24 hours. The initial solution became turbid, and the black precipitate was observed. The black precipitant was separated by centrifugation and washed three times with ethanol. After that, the obtained precipitate was dissolved in 200 ml ethanol to repeat reaction according to above-described method. The final product was obtained by centrifugation and washed with ethanol. Finally, DMSA-Fe_3O_4 nanoparticles were transferred into water for stability investigation.

2.3. Cell Culture and Intracellular Uptake of Nanoparticles

Human epithelial mouth carcinoma cells (KB) and human hepatoma cells (SMMC-7721) were used in cell experiments. Cells were cultured at 37 °C in a 5% CO_2 atmosphere, in a 24-well culture plate containing 0.6 ml RPMI-1640 medium supplemented with 10% fetal calf serum. And then, the cells were washed twice with PBS to eliminate fetal calf serum, and incubated with nanoparticles in RPMI-1640 without fetal calf serum for 12 hours. To quantitate intracellular uptake of the nanoparticles, iron concentration was quantified based on reported work.[12] Three replicates were measured and the results were averaged with standard deviation.

2.4. Characterization

The size and morphology of the particles were determined by transmission electronic microscopy (TEM, JEOL, JEM-200EX) operating at 120.0 kV. Samples were dropped, either from chloroform or water, onto a carbon-coated copper grid and dried under room temperature. The size of aggregates under biological conditions was determined on an atomic force microscope (AFM, PicoPlus, Agilent). To prepare samples for AFM, a silica substrate was placed on the bottom of a 25×25 weighing bottle containing nanoparticles dispersion solution for two days, and then nanoparticles precipitated on the substrate were washed several times with water to get rid of residual salts and biological molecular that biological medium itself contained. Furthermore, Photon Correlation Spectroscopy (PCS) was used to determine hydrodynamic sizes of aggregates using a Submicron Particle Analyzer (N4 Plus, Beckman Coulter). Three replicates were measured and the results were averaged. Surface charge measurements were performed with a Zeta Potential Analyzer (Delsa 440SX, Beckman Coulter). Magnetic resonance imaging (MRI) measurements were conducted using a 1.5 T MR spectrometer (Marconi Elipse). UV-visible absorbance spectra were recorded using a U-4100 spectrophotometer. IR spectra were recorded on a Nicolet Nexus 870 FT-IR spectrometer and powder samples were dried at 100 °C under vacuum for 24 h prior to fabrication of the KBr pellet. Spectra were recorded with a resolution of 2 cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. Stability of DMSA-Fe_3O_4 Nanoparticles Under Biological Conditions

The success of transferring monodisperse Fe_3O_4 nanoparticles from organic to aqueous phase via surface double-exchange method has been confirmed in our previous work.[7] DMSA is used to exchange with long-chain oleic acid on the surface of Fe_3O_4 nanoparticles

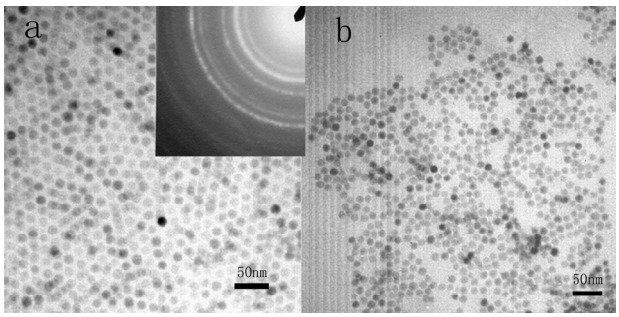

Fig. 1. TEM images of Fe_3O_4 nanoparticles (a) and DMSA-Fe_3O_4 nanoparticles (b) dispersed in chloroform and water, respectively. Insert is ED pattern of Fe_3O_4 nanoparticles.

synthesized by thermal decomposition of iron-oleate. DMSA first forms a stable coating through its carboxylic chelating bonding and further stability is obtained through intermolecular disulfide cross-linking between DMSA. The remaining carboxylates ensure surface charges and can be used for conjugating with biological molecules and further applications. Figure 1 showed that Fe_3O_4 nanoparticles synthesized by thermal decomposition were monodisperse and kept monodispersity after surface modification with DMSA. Afterwards, we chose representative water, RPMI-1640 with 10% (v/v) fetal calf serum, RPMI-1640, PBS and MES as media in which DMSA-Fe_3O_4 nanoparticles were dispersed, to systematically investigate their stability under biological conditions.

To quantify stability of DMSA-Fe_3O_4 nanoparticles dispersed in water, RPMI-1640 with 10% (v/v) fetal calf serum, RPMI-1640, PBS and MES, respectively, UV-visible absorbance spectra were used to monitor the absorbances of the corresponding dispersion solution containing DMSA-Fe_3O_4 nanoparticles at a fixed wavelength (450 nm).[13, 14] If nanoparticles are not stable and sedimentate rapidly, they can be monitored by a decreased absorbance as a function of time. Figure 2 shows that DMSA-Fe_3O_4 nanoparticles dispersed in water and RPMI-1640 with fetal calf serum present excellent stability, whereas those dispersed in RPMI-1640 without fetal calf serum, PBS and MES exhibit poor stability, because the loss of the former is less than 20% after five days, whereas the latter is larger than 80%. This result suggests that DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640, PBS and MES aggregate rapidly, which is confirmed by PCS result. PCS results (Table I) show that DMSA-Fe_3O_4 nanoparticles dispersed in all media exist as aggregates which make them unstable, however, their aggregating degree and stability is different. For DMSA-Fe_3O_4 nanoparticles dispersed in water and RPMI-1640 with fetal calf serum, the sizes of aggregates are about 312.4 and 68.4 nm, respectively, while for those dispersed in RPMI-1640, PBS and MES, the sizes are about 513.8, 488.7 and 677.3 nm, respectively. Furthermore, the sizes of aggregates of DMSA-Fe_3O_4 nanoparticles dispersed in water and RPMI-1640 with fetal calf serum almost don't change with time, whereas sizes of aggregates of those dispersed in RPMI-1640, PBS and MES change observably. Based on above results, we conclude that DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640 with fetal calf serum are the most stable because its size of aggregates is the smallest and almost doesn't change with time. In contrast, DMSA-Fe_3O_4 nanoparticles dispersed in MES are the most unstable.

Table I. Average hydrodynamic sizes of aggregates of DMSA-Fe_3O_4 nanoparticles dispersed in different media. Note that no hydrodynamic size of the RPMI-1640 with 10% (v/v) fetal calf serum itself is observed in PCS measurement.

Medium	Time		
	0 day	1 day	2 days
Water	312.4 ± 7.1 nm	315.4 ± 6.2 nm	319.8 ± 8.6 nm
RPMI-1640 with fetal calf serum	68.3 ± 1.2 nm	71.1 ± 1.1 nm	72.5 ± 1.7 nm
RPMI-1640	513.8 ± 12.6 nm	678.4 ± 13.5 nm	965.7 ± 20.6 nm
PBS	488.7 ± 8.3 nm	549.9 ± 9.6 nm	607.6 ± 16.4 nm
MES	677.3 ± 11.2 nm	1197.2 ± 30	1541.3 ± 84.3 nm

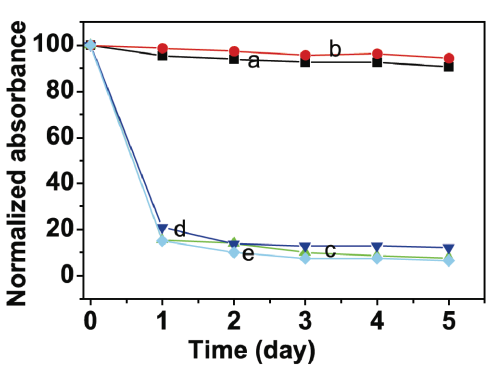

Fig. 2. Normalized UV-Vis absorbance of DMSA-Fe_3O_4 nanoparticles dispersed in (a) water, (b) RPMI-1640 with 10% (v/v) fetal calf serum, (c) RPMI-1640, (d) PBS and (e) MES, respectively, as a function of time.

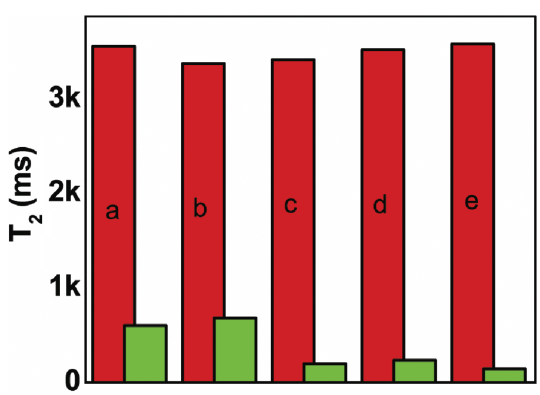

Fig. 3. T_2 relaxation times of media and corresponding dispersion solutions containing DMSA-Fe_3O_4 nanoparticles. The media are (a) water, (b) RPMI-1640 with 10% (v/v) fetal calf serum, (c) RPMI-1640, (d) PBS and (e) MES, respectively.

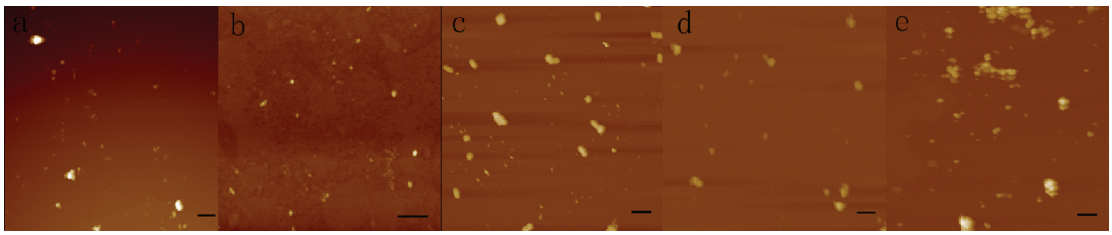

Fig. 4. AFM images of DMSA-Fe_3O_4 nanoparticles dispersed in (a) water, (b) RPMI-1640 with 10% (v/v) fetal calf serum, (c) RPMI-1640, (d) PBS and (e) MES, respectively. The bars are 1 μm.

Magnetic resonance image technology (MRI) is also a powerful tool to investigate aggregation of nanoparticles. As described in some work,[15, 16] the difference in aggregation state of nanoparticles could result in remarkably large concomitant changes in T_2 contrast. For example, aggregation of nanoparticles can reduce T_2 value, resulting in a darker signal. So we compare aggregates of DMSA-Fe_3O_4 nanoparticles dispersed in different media through T_2 changes observed by MRI technology. Figure 3 shows T_2 values of medium itself and corresponding dispersion solution containing DMSA-Fe_3O_4 nanoparticles. Their interaction is described as follows:

$$\Delta T = T_{2N}/T_{2P}$$

where T_{2P} is T_2 of medium and T_{2N} is T_2 of corresponding dispersion solution containing DMSA-Fe_3O_4 nanoparticles. We find that, for water, RPMI-1640 with fetal calf serum, RPMI-1640, PBS and MES, ΔT values are 15.9 ± 0.21, 20.2 ± 0.26, 5.1 ± 0.18, 6.7 ± 0.12 and 4.1 ± 0.22, respectively. The smaller ΔT is, the better T_2 contrast effect is, indicating a darker image. And it can be also observed that ΔT values of RPMI-1640 with fetal calf serum solution are 3–4 times more than that of RPMI-1640 and MES solution. Since the nanoparticles and their concentrations used in MRI measurements are the same, this result may be attributed to the difference of the sizes of aggregates dispersed in different media, which is in accordance with PCS results.

The above UV-vis, PCS and MRI measurements were used to investigate aggregates, and then AFM measurements were performed to observe the size of aggregates. Figure 4 presents the sizes of aggregates of DMSA-Fe_3O_4 nanoparticles dispersed in water, RPMI-1640 with fetal calf serum, RPMI-1640, PBS and MES, respectively. And the relation of the sizes of aggregates among them is that: MES > RPMI-1640 > PBS > water > RPMI-1640 with fetal calf serum, which is consistent with previous results.

3.2. Effects of Fetal Calf Serum on Surface Property and Stability of DMSA-Fe_3O_4 Nanoparticle

Casey et al. found that single walled carbon nanotubes could be well-dispersed in medium (F12K) with 5% (v/v) foetal bovine serum (FBS), resulting from that both components of F12K and FBS interacted with carbon nanotubes likely through a physisorption, observed by UV-vis absorption spectroscopy, fluorescence spectroscopy and Raman spectroscopy.[10] And they found that although the nanotubes were dispersed by the medium, they remained as larger diameter bundled aggregates rather than individual nanotubes. Beside the effect of serum on stability mentioned above, we wonder whether serum can have a great role in surface property of nanoparticles, because surface property is crucial to the applications of nanoparticles.

For this purpose, zeta potential measurements were firstly carried out to estimate the effect of fetal calf serum on the surface charge of DMSA-Fe_3O_4 nanoparticle. Figure 5 is a plot of zeta potential versus pH for DMSA-Fe_3O_4 nanoparticles and DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 with fetal calf serum (Note that zeta potential measurements were performed in water). For DMSA-Fe_3O_4 nanoparticles, no isoelectric point (IEP) is observed, indicating that deprotonated carboxylates (COO^-) are on the surface of nanoparticles and thus make them negatively charged in the range of pH = 2–12. However, for DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 with fetal calf serum, an IEP is observed at pH = 4.5, suggesting that the surface charges of nanoparticles after precipitating from RPMI-1640 with fetal calf serum change remarkably in comparison with DMSA-Fe_3O_4 nanoparticles. Then, IR spectra were recorded to confirm that the change of surface charges does not result from salts in RPMI-1640, but fetal calf serum. Figure 6 indicates IR spectra of DMSA-Fe_3O_4 nanoparticles and

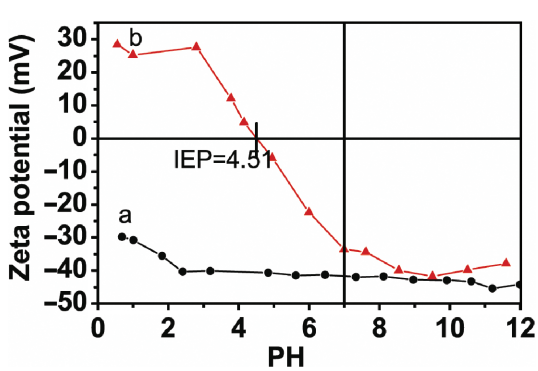

Fig. 5. Zeta potentials of (a) DMSA-Fe_3O_4 and (b) DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 with fetal calf serum.

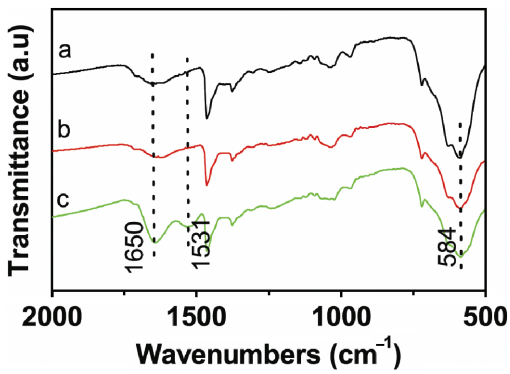

Fig. 6. IR spectra of (a) DMSA-Fe_3O_4 nanoparticles, (b) DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 without fetal calf serum and (c) DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 with fetal calf serum, respectively.

DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 and RPMI-1640 with fetal calf serum, respectively. We find that IR spectra of DMSA-Fe_3O_4 nanoparticles and DMSA-Fe_3O_4 nanoparticles precipitated from RPMI-1640 are almost the same except for two characteristic bands of amide at 1650 and 1531 cm^{-1}, which are also observed by Mikhaylova et al.,[17] indicating that amides-containing molecules in fetal calf serum are adsorbed onto the surface of DMSA-Fe_3O_4 nanoparticles. Afterwards, we compared the stability of DMSA-Fe_3O_4 and DMSA-Fe_3O_4 nanoparticles with adsorbed amides-containing molecules from fetal calf serum (serum-DMSA-Fe_3O_4) under varying pH and increased salts concentrations (NaCl). Like Figure 2, Figure 7 illustrates that serum-DMSA-Fe_3O_4 nanoparticles exhibit their excellent stability to varying pH and increased NaCl concentrations in comparison with DMSA-Fe_3O_4 nanoparticles, which is attributed to adsorption from fetal calf serum. This result gives the reason that DMSA-Fe_3O_4 nanoparticles can keep stable in RPMI-1640 with fetal calf serum. Meanwhile, it indicates that DMSA-Fe_3O_4 nanoparticles keep stable mainly due to electrostatic repulsion; however, serum-DMSA-Fe_3O_4 nanoparticles keep stable through steric repulsion. Based on above results of zeta potentials, IR and stability contrast, we consider that, DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640 with fetal calf serum can readily adsorb some molecules in fetal calf serum; the molecules

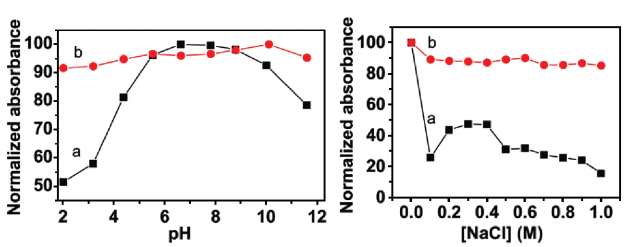

Fig. 7. Stability of (a) DMSA-Fe_3O_4 and (b) serum-DMSA-Fe_3O_4 nanoparticles to varying pH and increased salts concentrations.

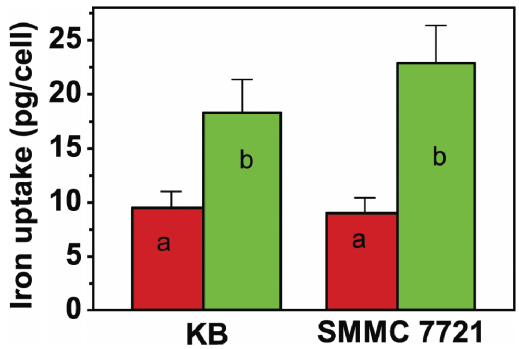

Fig. 8. Intracellular uptake of (a) DMSA-Fe_3O_4 and (b) serum-DMSA-Fe_3O_4 nanoparticles to KB and SMMC-7721 cells.

adsorbed greatly affects the surface property of DMSA-Fe_3O_4 nanoparticles and thus improves their stability.

3.3. Intracellular Uptake of Nanoparticles

It is well-known that intracellular uptake of nanoparticles is dependent on not only cell species,[18] but also the size[8] and surface properties of nanoparticles.[19–22] Among these factors, surface properties of nanoparticles may be a key parameter. In *in vitro* cell experiments, RPMI-1640 with 10% (v/v) fetal calf serum is a common medium, where nanoparticles are usually added and incubated with cells. As mentioned above, fetal calf serum can affect surface property and stability of nanoparticles. We thus infer that fetal calf serum may play a role in intracellular uptake of nanoparticles. For testifying it, we add DMSA-Fe_3O_4 and serum-DMSA-Fe_3O_4 nanoparticles, respectively, into RPMI-1640 without fetal calf serum, and incubate nanoparticles with cells for 12 hours. Figure 8 shows intracellular uptake of DMSA-Fe_3O_4 and serum-DMSA-Fe_3O_4 nanoparticles by KB and SMMC-7721 cells. It is found that intracellular uptake of serum-DMSA-Fe_3O_4 nanoparticles by KB and SMMC-7721 cells increases greatly in comparison with that of DMSA-Fe_3O_4 nanoparticles, indicating that fetal calf serum should be a considerable factor to intracellular uptake of nanoparticles.

4. CONCLUSION

We investigated stability of monodisperse DMSA-Fe_3O_4 nanoparticles under biologically relevant conditions, such as water, RPMI-1640 with 10% (v/v) fetal calf serum, RPMI-1640, PBS and MES. We found that, although DMSA-Fe_3O_4 nanoparticles could not keep stable in RPMI-1640, PBS and MES, DMSA-Fe_3O_4 nanoparticles dispersed in RPMI-1640 with fetal calf serum presented excellent stability, deriving from adsorption from fetal calf serum onto the surface of DMSA-Fe_3O_4 nanoparticles. The adsorption from fetal calf serum not only affected surface property of DMSA-Fe_3O_4 and made them stable, but also improved their intracellular uptake by KB and

SMMC-7721 cells. So in the investigation of the interactions between nanoparticles and cells in *in vitro* cell experiments, fetal calf serum was a factor that should be concerned on.

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