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New environmental nontoxic agents for the preparation of core-shell magnetic nanoparticles

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Coal humic acids are successfully tested for the first time as new nontoxic agents for the preparation of core-shell iron oxide magnetic nanoparticles.

Modified iron oxide (MIO) magnetic nanoparticles are of great interest in modern fields of nanomedicine such as hyperthermia, targeted delivery of drugs, protein separation, biological objects labeling and tomography.[1–4] Various methods of MIO preparation *via* coprecipitation,[5] microemulsion synthesis,[6] hydrothermal treatment[7] and aerosol spray pyrolysis[8] are developed. However, important problems remain including MIO stabilization and also key-lock behaviour in body fluids. MIO suspensions still demand the search for new effective surfactants and protective shells providing biocompatibility of the modified MIO and enhancing effectiveness of their practical applications.[9] We developed for the first time a preparation route of a new nontoxic magnetic nanocomposite material composed of environmental humic acids (HAs) and MIO nanoparticles.

Humic substances (HSs) are inexpensive natural surfactants, which can be used as stabilizing agents.[10] Contrary to artificial dendrimers with a regular structure already considered as perspective objects for nanomedicine,[11] HSs possess highly developed branches with irregularly located organic functional groups originating from biochemical and microbiological transformations with organic materials under environmental conditions. It provides a readily available source of a whole spectrum of naturally produced highly branched dendrite-like surfactants potentially suitable to serve as protective/stabilizing shells for nanoparticles.

Naked MIO were prepared by the ASP method described elsewhere.[12] Humic acids of leonardite were isolated as described previously[13] from commercial potassium humate (Powhumus, Humintech Ltd., Germany). For this purpose, a known portion of potassium humate was dissolved in distilled water and centrifuged to separate insoluble mineral components. The supernatant was then acidified to pH 2 with concentrated HCl and centrifuged. The precipitate of HAs was collected, washed with distilled water, desalted using electrodialysis, evaporated at 60 °C and stored in a desiccator over P_2O_5. After that, a desired amount of the NaCl–γ-Fe_2O_3 composite was added to the solution of HA to obtain a stable colloid. The iron content of the solutions was determined using photometric analysis with *o*-phenanthroline and, independently, on a Clarus 600 series mass spectrometer (Perkin–Elmer) after oxidative digestion of organic materials using $K_2S_2O_8$ and Fe_2O_3 dilution with a citric acid. The obtained results have shown that iron concentration in the solutions varied in the range of 2–30 mg dm^{-3}.

A simple visual test of magnetic properties of the suspension was performed by placement of a Sm–Co permanent magnet near the wall of a glass with the stable colloid that resulted in attraction of floating magnetic nanoparticles and appearing for 3–5 min a large reddish spot on the inner part of the wall adjacent to the magnet. Magnetic properties of composite were studied using a SQUID magnetometer (Quantum Instruments) at room temperature in magnetic fields of 2 T. The EPR measurements were performed using a Varian E-4 X-band (9.2 GGz) spectrometer equipped with a liquid-nitrogen cooling system. Powder-like samples (5–10 mg) for EPR measurements were placed into quartz tubes. A test for magnetocaloric effect was performed using a specially designed microcalorimeter working in constant magnetic fields of 0–2 T. Pure water was used as a test sample and showed no changes in temperature after switching on the magnetic field. Before the measurements of magnetic suspensions, calibration was performed to neglect effects of heat transfer at initial and final stages of the experiment.

In order to separate large particles and keep fine fractions of the modified particles in the solution, different suspensions were centrifuged at 9000–16000 g for 3–10 min at 4–25 °C dependent on the purpose. The size and morphology of magnetic microspheres were observed by transmission electron microscopy (TEM, Hitachi 8100), while dynamic light scattering (DLS) (ALV CGS-6010 instrument and a 632.8 nm helium–neon laser as the light source) was used to measure directly the particle size distribution in colloidal solutions. The supernatant droplets containing different fractions of modified or original nanoparticles were placed onto mica supports, dried at room temperature and then analyzed by AFM (Solver, NT MDT) for detection of the average particle size.

Cytotoxicity was analyzed using NCTC clone L929 cells grown in DMEM/F12 (1:1) medium containing 5% (v/v) fetal bovine serum (FBS) in 5% CO_2 and 90% humidified air at 37 °C. NCTC cells were plated onto 48-well culture plates for cell viability assay at a density of 40000 cell cm^{-2}. After 24 h, the medium was replaced by MIO suspensions (modified or

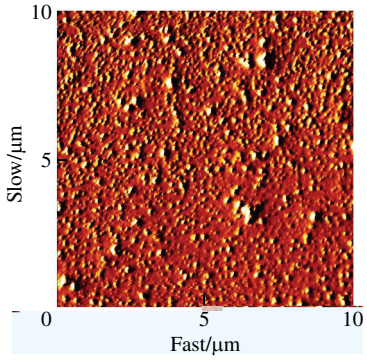

Figure 1 AFM image of associates of original iron oxide magnetic nanoparticles dried from a solution droplet.

unmodified) diluted with DMEM/F12 by a factor of 10, 30 or 100 (1:10, 1:30 or 1:100), as well as by DMEM/F12 supplemented with corresponding quantities of HA. DMEM/F12 supplemented with 5% FBS or without serum were used for the preparation of divorced suspensions. Accordingly DMEM/F12 supplemented with 5% FBS and serum serum-free DMEM/F12 was used as the controls in the appropriate experiments. The viability of NCTC cells was evaluated in 24 h after the beginning of experiment by measuring the reduction of a colourless salt of tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) by mitochondrial and cytoplasmic dehydrogenases of living metabolically active cells with the formation of water-insoluble purple blue intracellular formasan crystals. Cells were treated with MTT (0.25 mg cm^{-3}) at 37 °C for 3 h. The medium

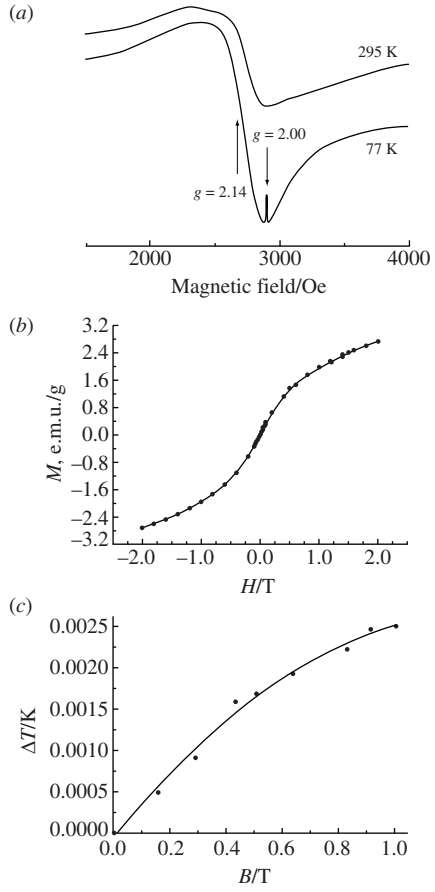

Figure 2 Magnetic properties of original iron oxide magnetic nanoparticles. (*a*) EPR spectra of the dried sample at room and liquid nitrogen temperatures; narrow line, reference free-radical signal ($g = 2.00$). (*b*) Magnetization of the original ASP-produced sample. (*c*) Dependence of the magnetocaloric effect of MIO modified with humic acids on applied field at 298 K (sample weight, 1.1455 g or *ca.* 4.5 μg of Fe^{3+} in superparamagnetic nanoparticles linked with HAs).

was then removed and the reduction product, formasan, was solubilized with dimethyl sulfoxide (DMSO). Absorption of each sample solution was measured at 540 nm using a Biorad plate spectrophotometer Model 680 (Biorad, USA). The data are expressed in terms of the activity in relation to the control group. Each value is a mean of three separate experiments. The statistically significant difference between groups was estimated by the Student's t-test. The value of $p < 0.05$ was considered to be a significant difference. The viability of NCTC cells was also tested by staining with trypan blue. Cells were exposed to 0.1% (w/v) trypan blue solution at room temperature for 10 min and then examined under a microscope.

Figure 1 represents AFM data of a typical precursor sample of naked MIO encapsulated in NaCl microgranules obtained by the ASP method at the temperature of furnace hot zone of 650 °C, dissolved in water and then dried. It is evident that the untreated sample consists of a wide range of nanoparticles not exceeding 500 nm in size. Perhaps the largest objects are not single particles but their residual or just formed associates. This seems to be a simple consequence of a very common problem of strong agglomeration of nanoparticles, which reduces a lot the stability of their suspensions and demands for the search of stabilizing agents.

Figure 2 shows the low temperature spectra of the sample MIO encapsulated in NaCl microgranules, after removal of salt microgranules by dilution in water and drying. Characteristic hyperfine parameters of Mössbauer spectra are presented in Table 1. The ultrafine particles of maghemite show superparamagnetic behaviour. The isomer shift of doublet is typical of Fe^{3+} cations, which are located in octahedral oxygen surroundings at room temperature. At decreasing temperature the 'paramagnetic' signals from Fe^{3+} ion (doublet and monoline at 300 K) turn into two sets of hyperfine structure lines (HFS) describing two-dimension γ-Fe_2O_3 nanoclusters in a state of magnetic order ($16 < T < 180$ K).

Magnetization curves of the samples at room temperature, the EPR signals and magnetocaloric effect found for the particles are shown in Figure 2. The samples demonstrate clearly a behaviour typical of superparamagnetic nanoparticles. The EPR signal both at room and liquid nitrogen temperatures has an asymmetrical shape [Figure 2(*a*)]. Effective linewidth and *g*-value, determined by the peak-to-peak method, are 500 Oe and 2.14, respectively. Since the EPR signal has not changed markedly after sample cooling from 295 to 77 K (Figure 2), it should be concluded that nanoparticles remain superparamagnetic even at liquid nitrogen temperature. The resonance line is relatively narrow in comparison with that for magnetite nanoparticles[14] and insignificantly shifted to a low magnetic field region. This is quite typical of γ-Fe_2O_3 nanoparticles.[15] According to the SQUID data, the nanoparticles are magnetic but have a reduced value of magnetization of about 2.5 emu g^{-1} [Figure 2(*b*)]. Finally, the nanoparticles in a water solution demonstrate a magnetocaloric effect under constant magnetic field conditions [Figure 2(*c*)]. Thus, it is reasonable to assume that the suspension consists of γ-Fe_2O_3 nanoparticles having an average size below

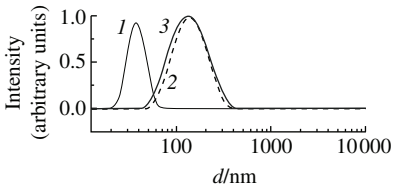

Figure 3 DLS data for different samples: (*1*) as-dissolved MIO after separation of largest nanoparticles by centrifuging and then used for the preparation of nanocomosite, $d = 40 \pm 15$ nm, (*2*) pure HA solution used as a surfactant for the preparation of nanocomposite, $d = 146 \pm 54$ nm, (*3*) a stable suspension of the MIO–HA nanocomposite, $d = 145 \pm 60$ nm.

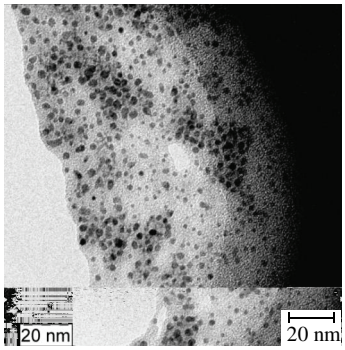

Figure 4 TEM image of modified MIO nanoparticles.

several dozens of nanometers and this is in a complete agreement with our previous results.[12]

Preparation of modified MIO nanoparticles using HAs as surfactants results in increasing the average particle size found in the suspension by DLS just up to the size of initial HA (Figure 3). Most probably it means incorporation of the nanoparticles in the existing branched structure of HA and linking them within the randomized polymer. Indeed, the particle distribution peak of the MIO–HA nanocomposite is single and contains no shoulder in the smaller particle size area (Figure 3) while it would be mandatory in the case of non-interacting MIO nanoparticles and HA. At the same time, MIO–HA nanocomposites demonstrate either a relatively high iron content according to the chemical analysis data or increased magnetization evident even from the simple visual test with a permanent magnet described above. TEM data confirm the hypothesis that MIO nanoparticles penetrate between the HA branches. Simply, this gives a picture of slightly associated groups of MIO nanoparticles shown in Figure 4. The average particle size in the groups is close to 20–30 nm as agreed well with the data of DLS (Figure 3).

Thus, dissolution of the nanocomposite in water and addition of specially prepared HA species gives a highly stable water suspension of magnetic nanoparticles. This suspension was diluted by cultural medium by a factor of 10, 30 or 100 and tested for citotoxicity.

In general, the cultivation without serum is not optimal for the long-term survival of cells. However, the presence of serum in the culture medium may distort the results of testing the cytotoxicity because of possible interaction of nanoparticles with the proteins of serum. We have tested the viability of NCTC cells in the presence of MIO suspensions in the serum supplemented and the serum-free media and have found no significant difference with the control in the level of MTT reduction between all tested samples in both cases, although the level of MTT reduction in suspensions diluted with serum-free DMEM/F12 was slightly lower than in the presence of serum [Figure 5(*a*),(*b*)]. At the same time, the level of MTT reduction by cells in the presence of 20% DMSO was significantly lower than in control to demonstrate cell death in the presence of toxic reagent.

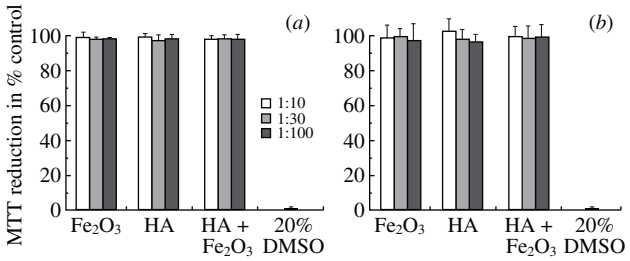

Figure 5 MTT reduction by NCTC cells (MTT viability test) in the presence of MIO suspensions in the cultural medium: (*a*) DMEM/F12 supplemented with 5% FBS and (*b*) the serum-free medium DMEM/F12.

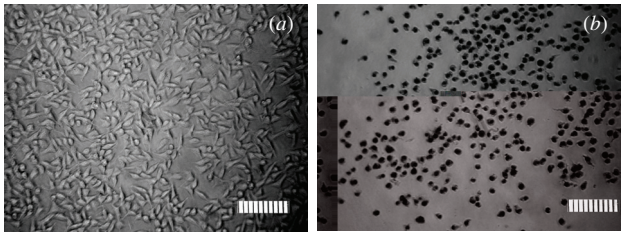

Figure 6 Trypan blue staining NCTC cells in the presence of (*a*) 10% of MIO suspensions and (*b*) 20% DMSO in the serum-free medium DMEM/F12.

Therefore, to determine whether the reduction in the amount of formazan produced by cells is due to cytotoxicity, the experiment was repeated under the same conditions except that trypan blue staining was performed to determine cell viability instead of the MTT test. The number of stained (dead) cells was less than 1% in all the samples, even in control, indicating that the reduction in MTT was not due to the cytotoxic properties of MIO suspensions [Figure 6(*a*)]. At the same time, the cell death have arrived 100% in the presence of 20% DMSO [Figure 6(*b*)]. Thus, MIO suspensions are nontoxic for cells.

In conclusion, we have demonstrated that environmental HAs have a high application potential as new nontoxic agents for the preparation of stable suspensions of magnetic nanoparticles for future medical applications.

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