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(54) **Title**: METHOD OF THE SYNTHESIS OF HYBRIDE NANOPARTICLES FROM AGGLOMERATES OF MULTICOMPONENT COMPLEX METALLIC OXIDES NANOPARTICLES

(57) **Abstract**: Hybride nanoparticles of the multicomponent complex metallic oxides are created by grains of the magnetic oxides. The grains are provided with a continuous layer of hydrated silica oxide ensuring due to the biocompatible surface their harmlessness in the organism. Utilizable in medicine for diagnostic and therapeutic applications for magnetic resonance imaging and magnetic fluid hyperthermia.

Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles

Technical field

The invention concerns to the synthesis of biocompatible hybride nanoparticles for medical applications, above all of magnetic fluid hyperthemia and magnetic resonance imaging.

Background art

Today the used hybride magnetic nanoparticles for diagnostic and therapeutic purposes in medicine are based on the magnetic cores of magnetite and maghemite. A considerable disadvantage of these materials is a restricted possibility of a modification of their magnetic properties for specific applications. Their use for magnetic fluid hyperthermia is made difficult partly because of inconveniencies to achieve a required heating efficiency and partly because of high values of the Curie temperature of the transition from the ferromagnetic to paramagnetic state, namely T_c (Fe_3O_4) = 477 °C and T_c (γ-Fe_2O_3) = 585 °C, where a danger of overheating and from that following necrosis of the health tissue cannot be excluded.

They are applied into an organism in a form of the water suspensions stabilized by low molecular stabilizers like various surfactants or hydrofilic polymers, most frequently dextran. A weak adhesion to magnetic cores decreases stability of such water suspension and consequently leads to the formation of agglomerates. Analogous difficulties happen also in an application of these materials as contrast agents for magnetic resonance, where beside dextran the use of others polysacharides, like arabinogalaktan or proteins like albumin or synthetic polymers is described. At the same time polymeric coating increases substantially size of the particles which affect adversely their penetration into cells and a rate of the metabolic clearing in the body. Uneasy modifiability of the coating makes difficult a specific uptake in particular cells of target tissues or linking of drugs or ligands targeted on specific types of cells.

With respect to MR imaging magnetite and maghemite nanoparticles possess indeed a sufficiently high T2-relaxivity considerably increasing the contrast in MR image but in

practice can be used only for MR imaging, perhaps even detection of marked cells. These nanoparticles appears to be usually superparamagnetic and thus does not allow MR navigated and controllable magnetic hyperthermia.

Disclosure of the invention

Solution based on an employing of hybride nanoparticles of complex multicomponents metallic oxides, with an advantage grains of the perovskite phase $La_{1-x}Sr_xMnO_3$, provided with a coherent layer of hydrated silica oxide eliminates mentioned difficulties. The used synthesis procedure of the grains of magnetic oxides is based on a sol-gel citrate method where the prepared precursor is subsequently thermal treated usually at temperatures in the range of 650 °C - 900 °C. Under these conditions a tendency to sintering manifested by formation of connecting bridges between the arising grains of magnetic oxides grains leads to their agglomeration. Agglomeration of grains makes surface modification, overlaying by a continuous layer of hydrated silica oxide and thus their use in medicine considerably difficult. Grains of a high quality of coating have insulated cores whereby their exposition against organism is restricted. Therefore a mechanical treatment based on a combination of rolling and milling allowing a disruption of the synthesized agglomerates on the individual grains should be used. Links among the formed grains are broken by rolling in the first step, final separation of the individual grains and their dispersion in a liquid is achieved in the second step. A possibility to vary composition and size of the grains and adjust thus exactly magnetic properties critical for a given medical application is an expressive benefit of the described and proved procedure.

Magnetic cores covered by a coherent layer of the hydrated silica oxide generates in a water medium of pH > 4 suspensions of a high stability. The formed silica shell restricts exposure of the organism against the magnetic core and an harmlessness of the nanoparticles is achieved. Surface of the synthesized grains is activated in an acidic medium and suspension is consequently stabilized by amonium citrate. Overlaying by the hydrated silica oxide is carried out in a medium of water, ethanol and ammonia under an increased temperature, employing substituted alkoxysilanes, like tetraethoxysilane. Hybrid nanoparticles carrying aminoalkyl chains and thus providing suitable groups for further derivatization can be obtained by the addition of an aminoalkylalkoxysilane to the encapsulation procedure. Therefore it is possible to attach on the surface of hybride nanoparticles additional molecules or entities and prepare complex nanoparticles with

functional elements intended for a specific use like targeting to selected tissues or a combined therapy mediated by bonded molecules.

Hydrophile coating of hydrated silica oxide provides a satisfactory biocompability thereby availibility for medical applications. Its thickness at least in the range of 5 nm – 50 nm can be controlled by a selection of the reaction conditions, like temperature, time, composition of the reaction mixture.

Heating efficiency up to 300 W/g_{Mn} can be achieved by a suitable adjustment of the chemical composition of the magnetic cores ($0.2 \leq x \leq 0,5$) and their size (20 nm – 60 nm). Simultaneously the transition from the ferromagnetic to paramagnetic state can be set up in the temperature range of 40 °C – 60 °C, i.e. just above the temperature of healing. Self-controlled mechanisms of the magnetic heating restricting an overheating of the health tissue thus is applied.

T2-relaxivity in the field of 0.5 T (i.e. ability to shorten relaxation time of water and thus increase the contrast of the magnetic resonance imaging) achieves for these hybride nanoparticles of the size 20 nm – 60 nm values of ~ 600 s^{-1}/mM_{Mn}, i.e. markedly higher than the value of ~ 170 s^{-1}/mM_{Fe} of the dextran coated superparamagnetic iron oxides particles.

Viability of cells in a medium under a presence of the hybrid nanoparticles (0.11 mM_{Mn}) achieves approximately 95 %. The particles thus can be used also for the cells marking and due to their higher relaxivity better results can be achieved for substantially lower concentrations than for standardly used contrast agent Endorem based on iron oxides . While the relaxation ratio of the cell suspension 2.1 s^{-1} (related to 10^6 cells/ml) at concentration of 1.1 mM_{Fe} in media (Endorem contrast agent) is achieved marking of the cells by hybride $La_{1-x}Sr_xMnO_3$ nanoparticles gives the value of the relaxation ratio 2.9 s^{-1} (related to 10^6 cells/ml) at concentration ten times lower, of 0.11 mM_{Mn}.

Examples

Example 1

Nanoparticles of the ferromagnetic phase of the composition $La_{0,75}Sr_{0.,25}MnO_3$ were synthesized by a two stage procedure, preparation of the precursor by a sol - gel citrate method and subsequent thermal treatment.

The starting compounds La_2O_3, $SrCO_3$ and $MnCO_3$ of the contents of the cationic components determined by the chemical analysis were separately dissolved in 1:1 diluted nitric acid and mixed with citric acid in the ratio of $(0,75[La^{3+}]+0,25[Sr^{2+}]+[Mn^{2+}])/1,5$[citric

acid] /2,25[ethylenglycol] and pH was adjusted to 9 by an addition of NH_4OH. Precursor of an amorphous character confirmed by the X-ray diffraction powder analysis was prepared by the evaporation of water at 80 °C – 90 °C and drying at 160 °C. The precursor was calcinated for 6 hours at 400 °C in air and then heated in air for 3 hours at temperature of 700 °C. Single phase product of the mean size of the grains 30 nm was obtained. The synthesized material was subjected to a combined mechanical treatment by rolling and milling. Vertical arrangement of the rollers of the diameter 54 mm from the hardened steel was applied for the rolling. Rotation speed of the rollers was 9 rev/min, process was three times repeated and the distance betwen the rollers was gradually decreased on less than 0.03 mm. Vibrational mill with milling can of the volume 25 ml and one grinding ball of the diameter 20 mm, both from the stainless steel were employed for the subsequent milling. Milling parameters: weight of the sample 0.5 g, volume of the liquid (ethanol) 10 ml, milling time 60 min, vibration frequency 30 vib/sec.

Stability of the agglomerates is disrupted by rolling when the bridges connecting the grains, arising as a consequence of a tendency to sintering during the thermal treatment, are broken. The grains are then dispersed by vibrating milling in the liquid media.

Efficiency of the used procedure is obvious from the observation of the nanoparticles microstructure by the transmission electron microscopy. The mean size of the grains determined by X-ray diffraction before and after mechanical treatment remains unchanged while hydrodynamic size of the particles markedly, approximately three times decreases. Therefore it confirms a distinct suppression of the tendency of grains to agglomeration.

In the next step nanoparticles (130 mg) were treated by 1 M ice-cold nitric acid (20 ml) in an ultrasound bath. After the removal of the nitric acid by centrifugation the nanoparticles were redispersed in the ice-cold 0.1 M citric acid (20 ml) using ultrasound homogenization for 15 min. Then the particles were separated by centrifugation from the solution of citric acid and a redundant amount of the citric acid was eliminated in one cycle by washing (20 ml of water) and centrifugation. Subsequently the particles were redispersed in water alkalized by a small amount of ammonia (5 drops) in order to transfer the residual citric acid, fixed on the surface of the nanoparticles, to ammonium citrate, stabilizing nanoparticles in the water suspension. The suspension was exposed to ultrasound irradiation and dispersed for 30 min. Then the suspension was added dropwise under ultrasound and mechanical stirring into a flask located in an ultrasound thermostatic bath warmed on 40 °C, containing a mixture of ethanol (96 % azeotropic mixture), water (70 ml) and ammonia (25% water solution) in the ratio 15 : 4 :1 (400 ml). Amount of tetraethoxysilan

corresponding to the required thickness of the envelope layer (2670 μl for 25 nm) was added and the mixture was left in the thermostatic ultrasound bath for 24 hours under mechanical stirring.

When the encapsulation of the nanoparticles by a layer of the hydrated silica oxide was terminated the required fraction was isolated from the reaction mixture. This procedure included collection of the supernatant by centrifugation in angular rotor at 3000 rev./min for 15 min, separation of the nanoparticles from the supernatant by the centrifugation at 8000 rev./min for 40 min. The separated nanoparticles were than purified by two washing cycles in ethanol and four cycles in water (always 60 ml of the wash liquid). After the last cycle the sediment of nanoparticles was filled up to 20 ml and redispersed by ultrasonification. In order to eliminate residual traces of ethanol the suspension was warmed in a vacuum dryer at temperature of 35 °C and pressure of ~ 1 Pa for 1 hour. Measurement of the hydrodynamic size of the coated particles evidenced a narrow distribution function described by the values 134 ± 18 nm at the 80% probability level. Transmission electron microscopy confirmed presence of the envelope layer of the approximate thickness of 25 nm. Its chemical character was confirmed by the infrared spectroscopy. Measurement of the zeta potential in the range of pH 1 – 13 affirmed stability of the suspensions of the coated nanoparticles in the water media in the range of pH needed for medical applications.

Example 2

Nanoparticles of the ferromagnetic perovskite phase of the composition $La_{0,75}Sr_{0.,25}MnO_3$ and mean size of the grains 30 nm were prepared according to the procedure described in the example 1. Charge of the nanoparticles (200 mg) was treated by 1 M ice-cold nitric acid (20 ml) in an ultrasound bath for 15 min. Removing of the residual nitric acid was carried out in three washing cycles (always 25 ml of water). Then the nanoparticles were dispersed by ultrasound probe in water (50 ml) for 1 hour. Then the suspension was cooled down in an ice bath and added dropwise into the prepared solution of polyvinylpyrrolidon (PVP) (350 ml) of the mean molecular weight M_r = 24 000. Its solution had been treated by the ultrasonification for one hour in the ultrasound bath in order to uncoil the polymer chains properly before the nanoparticles were added. Composition of the mixture entering into the reaction was selected as follows: 400 ml suspension with the concentration of the perovskite phase 0.5 mg/ml, containing total amount 3.67 g of PVP, corresponding to the ratio of 15 molecules of the PVP polymer per 1 nm^2 of the surface of the nanoparticles with the diameter of 30 nm. The suspension was homogenized in the thermostatic ultrasound bath at 25 °C for

24 hours. During ultrasonification the adsorption equilibrium of PVP on the surface of nanoparticles was established. Stabilized nanoparticles were separated by centrifugation and the redundant PVP was eliminated by one washing cycle in ethanol (20 ml). The solid residue was transferred into a flask (500 ml) and filled up by ethanol (400 ml). The suspension was dispersed in a thermostatic bath at 25 °C for 5 min under simultaneous acting of ultrasound and mechanical stirring. Subsequent mixing of the mixture was carried out only by mechanical stirring. Then tetraethoxysilan in the amount, which according to the mentioned spheric approximation, corresponds to the formation of an envelope layer of the thickness of 4 nm (236 µl), presently aminopropyltriethoxysilan in an amount four times lower (59 µl) and finally after a short mixing 64 ml of ammonia (25 % water solution) which catalyses basic hydrolysis of alkoxysilanes were added into the flask. The mixture was left in the thermostatic ultrasound bath for 24 hours.

When the encapsulation was terminated all nanoparticles were separated by centrifugation, whereas the solid residue was washed by three washing cycles in ethanol (always 60 ml). Subsequently the required fraction of the coated nanoparticles was isolated. This procedure included ultrasound dispersion of the product in ethanol (200 ml), collection of the supernatant by centrifugation in an angular rotor at 3000 rev./min for 15 min, separation of the nanoparticles from the supernatant by the centrifugation at 8000 rev./min for 40 min. The separated nanoparticles were then redispersed in ethanol (50 ml) by the ultrasound homogenization. Transmission electron microscopy confirmed presence of an envelope layer of a thickness lower than 10 nm. Presence of Si-O-Si a Si-O-H bonds was confirmed by the infrared spectroscopy. The concentration of surface accessible amino-groups (0,23 µmol(NH_2R, povrch)/mg(Mn)) was determined by spectrophotometric analysis of p-nitrobenzaldehyde, that was covallently coupled to the nanoparticles via imine bond formation at first and then it was again hydrolysed.

Industrial applicability

Hybride nanoparticles of the multicomponent complex metallic oxides are utilizable in medicine, e.g. as contrast agents for diagnostic magnetic resonance imaging and for therapy by magnetic fluid hyperthermia and for combine therapy mediated by molecules fixed on amino groups on the surface of complex silica layers.

CLAIMS

1. Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles characterized in that the agglomerates are disrupted in individual grains, their surface is activated in an acid media, grains are dispersed in water and give rise to suspension stabilized by amonium citrate, grains are dispersed in media of ethanol, water and ammonia and a substituted alkoxysilan in a quantity of 10 – 50 μl on each 1 m^2 of the surface of grains and each 1 nm of the thickness to create a layer of hydrated silica oxide is added.

2. Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles according to claim 1 characterized in that tetraethoxysilan is used as a substituted alkoxysilan.

3. Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles according to claim 1 characterized in that aminoalkyltriethoxysilan is used as a substituted alkoxysilan.

4. Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles according to claim 1 characterized in that tetraethoxysilan and aminoalkyltriethoxysilan together are used as a substituted alkoxysilan.

5. Method of the synthesis of hybride nanoparticles from agglomerates of multicomponent complex metallic oxides nanoparticles according to claim 1 characterized in that nitric acid is used as acid media.

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
INV. C01G1/02 C01G45/00 H01F1/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C01G C09C H01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VASSEUR S ET AL: "Lanthanum manganese perovskite nanoparticles as possible in vivo mediators for magnetic hyperthermia" JOURNAL OF MAGNETISM AND MAGNETIC MATERIALS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 302, no. 2, 1 July 2006 (2006-07-01), pages 315-320, XP024984479 ISSN: 0304-8853 [retrieved on 2006-07-01] page 316, paragraph 2.1 - page 317, paragraph 2.2 ----- -/--	1-5

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YU LU, YADONG YIN, BRIAN T. MAYERS, YOUNAN XIA: "modifying the surface properties of supermagnetic iron oxide nanoparticles through a sol-gel approach" NANO LETTERS, vol. 2, no. 3, 2002, pages 183-186, XP002556765 the whole document	1-5
Y	VAN DER BLAADEREN A ET AL: "Synthesis and Characterization of Colloidal Dispersions of Fluorescent, Monodisperse Silica Spheres" LANGMUIR, ACS, WASHINGTON, DC, US, vol. 8, 1 January 1992 (1992-01-01), pages 2921-2931, XP002486298 ISSN: 0743-7463 page 2923, paragraph III	1-5
Y	A. VAN BLAADEREN, A. VRIJ: "Synthesis and Characterisation of monodisperse colloidal organo-silica spheres" JOURNAL OF COLLOID AND INTERFACE SCIENCE, vol. 156, 1993, pages 1-18, XP002556766 page 4, paragraph III; figure 1	1-5