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Immobilization of a pH-low insertion peptide onto SiO_2/aminosilane-coated magnetite nanoparticles

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Experimental

1. Materials

Iron (III) chloride (Sigma, 97%), oleic acid (Roth, ≥99%), 1-octadecene (Sigma, ≥95%), ethanol (Aldrich, ≥95%), hexane (Sigma Tec, A.G.), cetyltrimetylammonium bromide (CTAB; Sigma, ≥98%), tetraethyl orthosilicate (Sigma Tec, A.G.), (3-aminopropyl)triethoxysilane (Alfa Aesar, England), ethyl acetate (Sigma Aldrich, ≥99.8%), sodium hydroxide (Sigma Aldrich, ≥97.0%), butanol-1(Sigma, ≥99,4%), chloroform (Sigma, ≥99%), phosphate-buffered saline (PBS, Sigma Aldrich), 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS; Fluka), pHLIP with ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT structure (pHLIP; Bachem, > 93%) were used. All other chemicals were purchased from commercial suppliers.

2. MNPs synthesis

The preparation of two sorts of Fe_3O_4 MNPs with narrow size distribution and average diameters of 10 and 20 nm and step-by-step coated with SiO_2, 3-aminopropylsilane and pHLIP was carried out in accordance with Scheme 1.

1. Synthesis of iron(III) oleate complex

Initially, 50 mmol of sodium oleate and 17 mmol of anhydrous iron(III) chloride were dissolved in mixture of 33 ml of ethanol, 25 ml of water and 60 ml of hexane under vigorous stirring. The obtained solution was heated to 70 ^oC for 4 hours. The brown phase was collected and solvent was evaporated until waxy substance of iron oleate complex was obtained.

2. Synthesis of MNPs with average core size of 10 nm (MNPs-1) and 20 nm (MNPs-2)

Initial Fe_3O_4 MNPs were synthesized via thermal decomposition method according to Ref. [S1] where 9-12 nm nanoparticles were obtained. In this work the protocols for preparation of two sorts of MNPs with average diameters of 10 and 20 nm were developed.

For synthesis of MNPs with average core size of 10 nm (MNPs-1) 2.2 mmol of iron(III) oleate was dissolved in 10 ml of 1-octadecene. Then the mixture was heated up to reflux under argon flow, intensive stirring and with 3.3 °C/min heating rate. The system was kept at the temperature for one hour followed by cooling to ambient temperature. The nanoparticles collected by magnetic decantation in butanol-1. The precipitate was redispersed in chloroform. The MNPs with average core size of 20 nm (MNPs-2) were synthesized according to the same protocol but extra 12 mmol of oleic acid was added.

The synthesized MNPs had pronounced hydrophobic properties. So, for further synthesis MNPs-1 and MNPs-2 were hydrophylized via protocol described in Ref. [S2]: 5 ml of 50 mg/ml MNPs solution in chloroform was sonicated intensively with 15 ml of 0.2 M CTAB solution in water until grey emulsion is formed. Then the chloroform was evaporated via continuously sonication in ultrasonic bath. The final solution was used for further aminosilation.

3. Synthesis of 3-aminopropylsilane-modified MNPs (MNPs-1-APS and MNPs-2-APS)

The chemistry of functionalization of MNPs with silica (SiO_2) and 3-aminopropylsilane molecules is well studied.[S2-S8] In this work TEOS and APTES were used for modify of nanoparticles surface to obtain stable shell layer with functional amine-groups on its surface.

2.4 ml of MNPs-1 solution in water was diluted with the mixture of 198 ml of distilled water and 1.14 ml of 2M NaOH. Then the mixture was heated up to 70 °C under vigorous stirring and 0.457 mL of TEOS in 5.5 mL of ethyl acetate was added to the reaction solution. After 10 min, 0.366 mL of APTES was added and the solution was stirred for 3 h. The same protocol of aminosilylation for MNPs-2 was used. The synthesized core-shells Fe_3O_4 MNPs were collected via centrifugation and redispersed in distilled water.

4. CTAB removal

As CTAB possess a toxic effect arising from its ability to induce protein denaturation and bind to DNA molecules, it was removed before the peptide immobilization in according to Ref. [S2]. Solution of 1 N HCl was added to 20 mL of MNPs-1-APS and MNP-2-APS colloidal solutions in water (2 mg/mL) up to pH 1.4. The obtained colloidal solutions of MNPs-1 and MNP-2 were kept for 3 h at 60 °C under stirring. After that, the MNPs were precipitated using a centrifuge (19 rpm) for 15 minutes. After decanting, the MNPs were washed with water (5 times) up to pH 7 and acetonitrile (1 time). The resulting MNPs were dispersed in 20 mL of acetonitrile (2 mg/mL).

5. Synthesis of EMCS-modified MNPs (MNPs-1-EMCS and MNPs-2-EMCS)

EMCS-functionalization of MNPs-1 and MNPs-2 was carried out according to the recently developed procedures[S9,S10]. A solution of 0.023 mmol EMCS in 2 mL of acetonitrile was added to 20 mL of MNP solution (2 mg/mL) in 20 mL of acetonitrile and stirring during 4 h. The obtained MNPs were precipitated by a magnet, washed with acetonitrile (3 times) and diluted with 20 mL of phosphate buffer solution (PBS, pH 7.4).

6. Synthesis of MNP conjugates with pHLIP (MNPs-1-pHLIP and MNPs-2-pHLIP)

To conjugation of MNPs with pHLIP at 20 mL solution of MNPs-1 and MNPs-2 in PBS (2 mg/mL) 0.73 μmol pHLIP in 1.5 mL PBS was added and the reaction mixture was stirred for 16 h. Ar was bubbled through the suspension of MNPs to remove O_2 and prevent the formation of pHLIP-pHLIP dimeric side-products as a result of disulphide-condensation. pHLIP-modified nanoparticles were collected by a magnet and washed with PBS (3 times).

3. MNPs characterization

Transmission electron microscopy (TEM) images of the MNPs were obtained on a JEOL JEM-1400 (JEOL, Japan) transmission electron microscope (120 kV). The crystal structure was investigated by DRON-4 diffractometer from 20° to 120° of 2θ diffraction angles diapason with 0.1° increment and 3 sec. exposition time (Co Kα-radiation at λ = 0.179 nm, tube voltage – 40 kV, current – 30 mA). The IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Electron Corporation) by the attenuated total reflectance method on a diamond crystal in the range of 4000−400 cm^{-1} with 192 scans and at 4 cm^{-1} resolution. The mass fraction of carbon was determined on a Perkin Elmer PE 2400, series II CHNS-O EA 1108 automatic analyzer (PerkinElmer Inc., Waltham, MA, USA). Thermogravimetric analysis (TGA) was performed on a TGA/DSC1 thermogravimetric analyzer (Mettler Toledo, Columbus, OH, USA) with a heating rate of 10 °C/min and a temperature range of 30−900 °C under compressed Ar with flow rate 60 mL/min). The specific magnetization of the powders of modified MNPs was measured at room temperature using a vibration magnetometer in a magnetic field 2.2 MA/m.

4 Calculation of the amount of pHLIP immobilized on MNPs

The content of immobilized pHLIP in MNPs-pHLIP was calculated by subtracting the carbon mass fraction calculated from the elemental analysis data for the EMCS-modified MNPs (ω_1) from the carbon mass fraction of MNPs modified with pHLIP (ω_2). Based on the obtained

value of the carbon mass fraction in the sample, the amount of pHLIP was calculated according to the formula (1) (**S-Table 1**) alike[S9-S12]:

$$c = \frac{(\omega_2 - \omega_1) \times 1g}{\omega_3 \times M}, \qquad (1)$$

where c is the amount of pHLIP on the surface of nanoparticles, mol/1 g MNPs;

ω_1 is the carbon mass fraction of the sample of MNPs-EMCS calculated from the elemental analysis data;

ω_2 is the carbon mass fraction of the sample of MNPs-pHLIP calculated from the elemental analysis data;

ω_3 is the calculated carbon mass fraction in the residue of pHLIP (0.5579);

M is the molecular weight of the residue of pHLIP (4111.69 g/mol).

Table S1 The elemental analysis data of EMCS- and pHLIP-modified MNPs.

	MNPs-1-EMCS	**MNPs-1-pHLIP**	**MNPs-2-EMCS**	**MNPs-2-pHLIP**
ω_1	0.0539		0.0371	
ω_2		0.0645		0.0463
c, μmol/1g MNPs		**4.62**		**4.01**

5. *In vitro* cytotoxicity evaluation

To evaluate cytotoxicity mouse 4T1 murine mammary carcinoma cell («N.N. Blokhin National Medical Research Center of Oncology» of the Ministry of Health of the Russian Federation), 3T3-L1 fibroblasts (State Research Center of Virology and Biotechnology VECTOR, Russian Federation), MDA-MB231 human breast adenocarcinoma cell lines (Institute of Cytology and Genetic SB RAS, Russian Federation) were used. The cells were grown in DME/F12 cultural medium supplemented with 10% fetal bovine serum (HyClone), L-glutamine and gentamicin. The cell viability was measured using thiazolyl blue tetrazolium bromide (MTT; Sigma) according standard protocol. In brief, 10^4 cells were seeded in 96-well plate and incubated overnight in humidified atmosphere with 5% CO_2. MNPs suspended in PBS were added to a final concentration 10-100 mg/mL (6 replicates for each concentration) and incubated for 24 h. After than supernatant was discarded, cells were washed twice with PBS and 150 μL of MTT-reagent[S13] dissolved in sterile PBS buffer (0.5 mg/mL) was added to each well. Cells were incubated 4-6 h at 37 °C for formazan granules formation. After incubation supernatant was carefully discarded and formazan granules are dissolved in 200 μL of DMSO. Solution was transferred in 96 U-well plates, centrifuged at 3000 g for 10 min, than 150 μL of supernatant was transferred to 96-flat bottom 96-well plate and optical density was measured at 540 nm on

microplate reader (Sunrise, Tecan). Cells incubated with PBS were used as control and cell viability was expressed as percentage of control. Triton X100 treated cells were used as positive control of cell death.

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