Electronic Supplementary Information (ESI)

pHLIP-Modified Magnetic Nanoparticles for Targeting Acidic Diseased Tissues

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Experimental

1. Materials

(3-aminopropyl)trimethoxysilane (APTMS, 97%; Alfa Aesar, England), 6-maleimidohexanoic acid N-hydroxysuccinimide ester (EMCS; Fluka, USA), pH-low insertion peptide with ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT structure (pHLIP; Bachem, USA) were applied. All other chemicals of analytical grade were purchased from commercial suppliers and used as received.

2. Synthesis

2.1 Preparation of Fe_3O_4 MNPs

$FeSO_4 \cdot 7H_2O$ (1.0 mmol) and $FeCl_3 \cdot 6H_2O$ (2.0 mmol) were dissolved in water (20 mL) and heated to 40 °C. The saturated aqueous ammonia (3 mL) was added to the resulting solution up to pH 11 under stirring in an ultrasound bath. The reaction mixture was stirred for 30 min; MNPs were precipitated by a magnet, washed with deionised water to neutral pH, and resuspended in EtOH.

2.2 Preparation of APS-modified MNPs (MNP-APS)

A solution of APTMS in EtOH (3 mmol APTMS per 1 g MNPs) was added to a suspension of MNPs (2 mg mL^{-1}) in 75% EtOH under stirring. The reaction mixture was agitated at room temperature for 24 h. The resulting MNP-APS were precipitated by a magnet, washed with EtOH and resuspended in MeCN.

2.3 Immobilization of pHLIP on MNPs (MNP-pHLIP)

EMCS (1.2 mole equivalent of the number of amino groups in MNP-APS) was added to a suspension of MNP-APS in MeCN. The reaction mixture was stirred at room temperature

for 3.5 h. The resulting MNP-EMCS particles were centrifuged at 18,000 rpm for 15 min, washed with MeCN and twice with PBS.

A suspension of MNP-EMCS in PBS was saturated with argon to prevent pHLIP dimerization due to the formation of disulfide derivatives. The pHLIP solution (taken in a 15:1 molar ratio of amino groups in MNP-APS and pHLIP) in PBS was added under continuous stirring and the reaction mixture was stirred for 16 h. After that, MNP-pHLIP was precipitated by a magnet, washed three times with PBS and stored in PBS.

3. Characterization

3.1 FTIR analysis of MNP-pHLIP

IR spectra were recorded on a Nicolet 6700 Thermo FTIR-spectrometer by the attenuated total reflection (ATR) method on the diamond crystal in the range of 4000–400 cm^{-1} with 128 scans and at 4 cm^{-1} resolution.

According to obtained IR data the quantitative analysis of 3-aminopropylsilane on the surface of MNP-APS was carried out.[S1] The FTIR spectra of the analysed samples were recorded in five repeats. The formula (1) was used to calculate the amount of APS (c) on MNP surface (mmol per g of MNPs).

$$c = 1.038 \times x^{-1.279}, \quad (1)$$

where c is the amount of APS on the surface of nanoparticles, mmol per 1 g of MNPs;

x is the ratio of integrated intensities of Fe–O (in the range of 790–1180 cm^{-1}) and Si–O (in the range of 480–755 cm^{-1}) adsorption bands in the FTIR spectra.

3.2 TEM of MNP-pHLIP

Transmission electron microscopy (TEM) images of the MNPs and MNP-pHLIP were received on a Philips CM30 instrument at 200 kV.

3.3 SEM and EDS analysis of MNP-pHLIP

Examination of the MNP-pHLIP by scanning electron microscopy (SEM) and X-ray microanalysis (energy dispersive spectroscopy, EDS) was carried out using Merlin scanning electron microscope (Carl Zeiss, Germany) equipped with Gemini column with Schottky field emission cathode as an electron source. The beam stability of the electron source was about 0.2% per hour and 0.4% per day. Spatial resolution for the electron column: 1.0 nm at 15 kV, 1.9 nm at 1 kV. The range of accelerating voltages was 0.1 – 30 kV. Surface morphology was visualized with a resolution of 1.5 nm using secondary electron detectors: Everhart Thornley detector and in-lens semiconductor detector. Elemental analysis by EDS was carried out using an Inca Energy 350X-MAX energy dispersive X-ray spectrometer (Oxford Instruments, UK) with a spatial resolution about 1 μm and a spectral resolution 125 eV at Mn K_α line. The data processing was performed using SmartSEM (Carl Zeiss, Germany) and IncaEnergy (Oxford, UK) software.

3.4 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed on a TGA/DSC1 instrument (Mettler Toledo) with a heating rate of 10 °C min^{-1} over a temperature range of 30–900 °C under compressed Ar.

3.5 Investigation MNP and MNP-pHLIP of magnetic properties

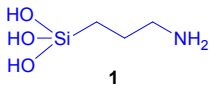
The specific magnetization of the modified MNPs powders was measured at room temperature using a vibration magnetometer in a magnetic field up to 2.2 MA m^{-1}.

3.6 Elemental analysis

The mass fraction of carbon was measured using a CHN PE 2400 II automatic analyser (Perkin Elmer).

Calculation of the amount of APS residues immobilized on MNP-APS. The amount of APS residues immobilized on the MNPs surface was calculated according to the formula (2):

$$c = \frac{\omega_1 \times 1g}{\omega_2 \times M}, \qquad (2)$$



1

where c is the amount of APS on the surface of nanoparticles, mol per 1 g of MNPs;

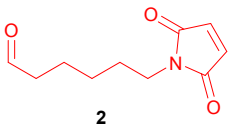
ω_1 is the carbon mass fraction of the sample of initial MNP-APS;

ω_2 is the calculated carbon mass fraction in the residue of 3-aminopropylsilane **1**;

M is the molecular weight of the residue of 3-aminopropylsilane **1** (137.22 g mol^{-1}).

Calculation of the amount of EMCS immobilized on MNPs. The content of immobilized EMCS on MNP-APS was calculated by subtracting of the carbon mass fraction (calculated from the elemental analysis data) in the initial APS-modified MNPs (ω_1) from the carbon mass fraction of MNPs modified with EMCS (ω_3). Based on the obtained value of the carbon mass fraction in the sample, the amount of EMCS was calculated according to the formula (3) (**S-Table 1**):

$$c = \frac{(\omega_3 - \omega_1) \times 1g}{\omega_4 \times M}, \qquad (3)$$



2

where c is the amount of EMCS on the surface of nanoparticles, mol per 1 g of MNPs;

ω_1 is the carbon mass fraction of the sample of initial MNP-APS;

ω_3 is the carbon mass fraction of the sample of MNP-EMCS;

ω_4 is the calculated carbon mass fraction in the residue of 6-maleimidohexanoic acid **2**;

M is the molecular weight of the residue of 6-maleimidohexanoic acid **2** (195.22 g mol^{-1}).

Calculation of the amount of pHLIP immobilized on MNPs. The content of immobilized pHLIP in MNP-pHLIP was calculated by subtracting the carbon mass fraction (calculated from the elemental analysis data) for the EMCS-modified MNPs (ω_3) from the carbon mass fraction of MNPs modified with pHLIP (ω_5). Based on the obtained value of the carbon mass fraction in the sample, the amount of pHLIP was calculated according to the formula (4):

$$c = \frac{(\omega_5 - \omega_3) \times 1g}{\omega_6 \times M}, \qquad (4)$$

H_2N-ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT-COOH

3

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

ω_3 is the carbon mass fraction of the sample of MNP-EMCS;

ω_5 is the carbon mass fraction of the sample of MNP-pHLIP;

ω_6 is the calculated carbon mass fraction in the residue of pHLIP **3**;

M is the molecular weight of the residue of pHLIP **3** (4111.69 g mol^{-1}).

3.7 The hydrodynamic properties characterization

Zeta potential and dynamic light scattering (DLS) characterization of MNP-pHLIP suspensions in aqueous media were carried out using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd.).

3.8 MRI experiment

All ^{1}H-MRI experiments were performed on a Biospec 117/16 USR horizontal MRI-scanner with a magnetic field intensity of 11.7 T (Bruker, Germany) equipped with transmitter volume (500.3 MHz, diameter of 72/89 mm, Bruker) and receiver surface (500.3 MHz, size of 123 × 64 × 31 mm, Bruker) ^{1}H coils. To characterize the MRI-contrast properties of nanoconjugates, T1 and T2 relaxation maps of ferrofluid phantoms were recorded. The MR image (slice thickness, 0.5 mm, field of view 4.0 × 4.0 cm, matrix, 256 × 256 dots, 90°-τ-180°-τ-90°) was obtained by a T2 spin echo sequence RARE (rapid with relaxation enhancement) with the pulse sequence parameters TR = 12.5–5425 ms and TE = 10–80 ms. The signal intensities of each ROI (region-of-interest) in the T2 map were measured for each concentration. T1 and T2 relaxation time data of test solutions were calculated automatically by Paravision 5.0.[S2]

3.9 MNP-pHLIP binding to the cells *in vitro*

The HTC cells (rat hepatoma, Institute of Cytology of the Russian Academy of Sciences, Russia) were grown in 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS). The medium with pH 6.0 was prepared by addition of MES hydrate up to a concentration of 25 mM. MNP-

pHLIP at a concentration of 10 and 40 μg mL^{-1} were added to 10^5 HTC cells and incubated at pH 7.4 or 6.0. After 1 h, cells were washed twice with PBS (pH 6.0/7.4), detached by trypsin-EDTA solution and lysed using 0.1 M NaOH. The iron concentration was determined using colorimetric ferrozine-based assay[S3] with initial dissolution of nanoparticles in 5 M HCl at 60 °C for 4 h.

3.10 *In vitro* cytotoxicity evaluation

Cytotoxicity assay was performed using HTC cell line. MNP-pHLIP at five various concentrations (5–100 μg mL^{-1}) were incubated with cells for 2 and 24 hours. The cell viability was measured by the MTT assay and expressed as a fraction of viable cells normalized to the cells without co-incubation with MNP-pHLIP.[S4]

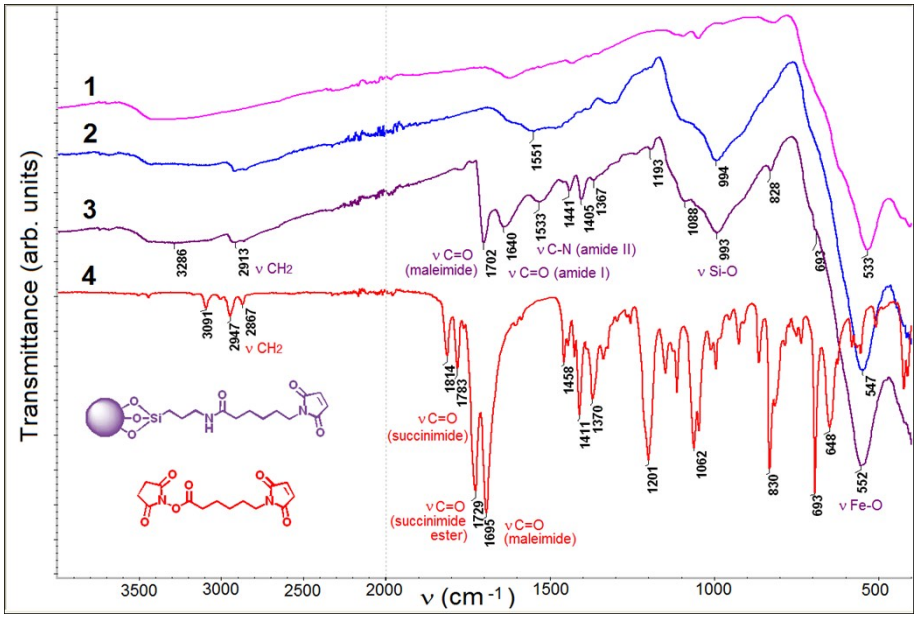
3.11 *In vivo* experiments

Male C57BL/6 mice, aged 8–10 weeks and weighted 25-28 g, were purchased from Laboratory of Experimental Biological Modeling, Institute of Pharmacology (Tomsk, Russia). Lewis lung carcinoma (LLC) cells were obtained from the Blokhin Russian Cancer Research Centre (Moscow, Russia). Mice were kept three in each cage under conventional condition and fed at standard diet and tap water ad libitum. All experiments and the maintenance of experimental animals were performed according to the guidelines of local Ethics Committee of the Siberian State Medical University. LLC cells were injected into the right hind leg muscles of mice ($3x10^6$ cells) (n=9). Seven days after 200 μL MNP-pHLIP (n=3) or MNP-APS (n=3) in a dose of 0.25 mg L^{-1} in PBS buffer were administrated in tail vein of animals. The tumour-bearing mice without administration were used as control. Mice were sacrificed in 40 h after nanoconjugate administration. The liver and tumour were extracted and placed into 10% neutral buffered formalin. Tissue sections were cut into 4-5 μm-thick slices. Standard haematoxylin and eosin and Prussian blue staining protocols were used. The histological microsections were analysed by optical microscope Axiostar plus (Carl Zeiss).

3.12 Statistical analysis

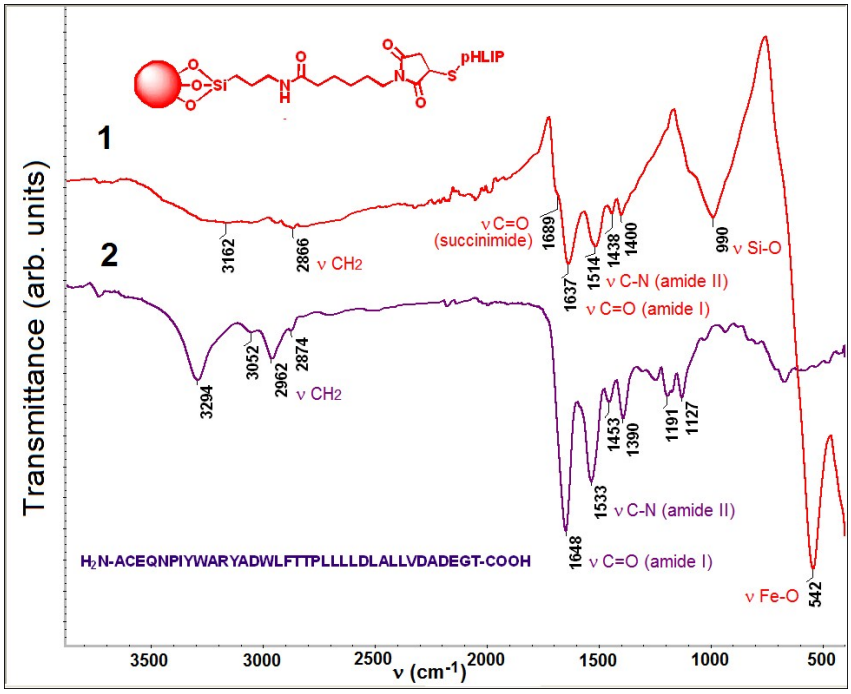
The obtained data were statistically analyzed using the software package GraphPad Prism version 5.0 for Windows. Data were presented as the mean±SD. The differences between data groups were calculated using Student's t-test. The P values below 0.05 were considered significant.

S1-1 FTIR spectra of initial MNPs (**1**), MNP-APS (**2**), MNP-EMCS (**3**) and EMCS (**4**).



In the FTIR spectrum of EMCS (**S-Fig. 1**, curve **4**), the most characteristic absorption bands were observed at ν 1814 and 1783 (stretching vibrations of the succinimide C=O group), 1729 (stretching vibrations of the ester C=O) and 1695 cm^{-1} (stretching vibrations of the maleimide C=O group). In the FTIR spectrum of MNP-EMCS (curve **3**), in addition to the absorption bands observed in the spectrum of the initial APS-modified MNPs (curve **2**: ν 993 (Si-O, Si–O–Si, Fe–O–Si) and 547 cm^{-1} (Fe–O)), absorption bands at ν 1702 cm^{-1} (stretching vibrations of the maleimide C=O group), as well as absorption bands at ν 1640 and 1533 cm^{-1} corresponding to the stretching vibrations of C=O (amide I) and C–N (amide II) groups were presented. The characteristic bands of the EMCS hydroxysuccinimide fragment were absent. Thus, based on the FTIR spectral data, formation of an amide bond between the APS amino group and N-hydroxysuccinimide ester of EMCS was supposed.

S1-2 FTIR spectra of MNP-pHLIP (**1**) and pHLIP (**2**).



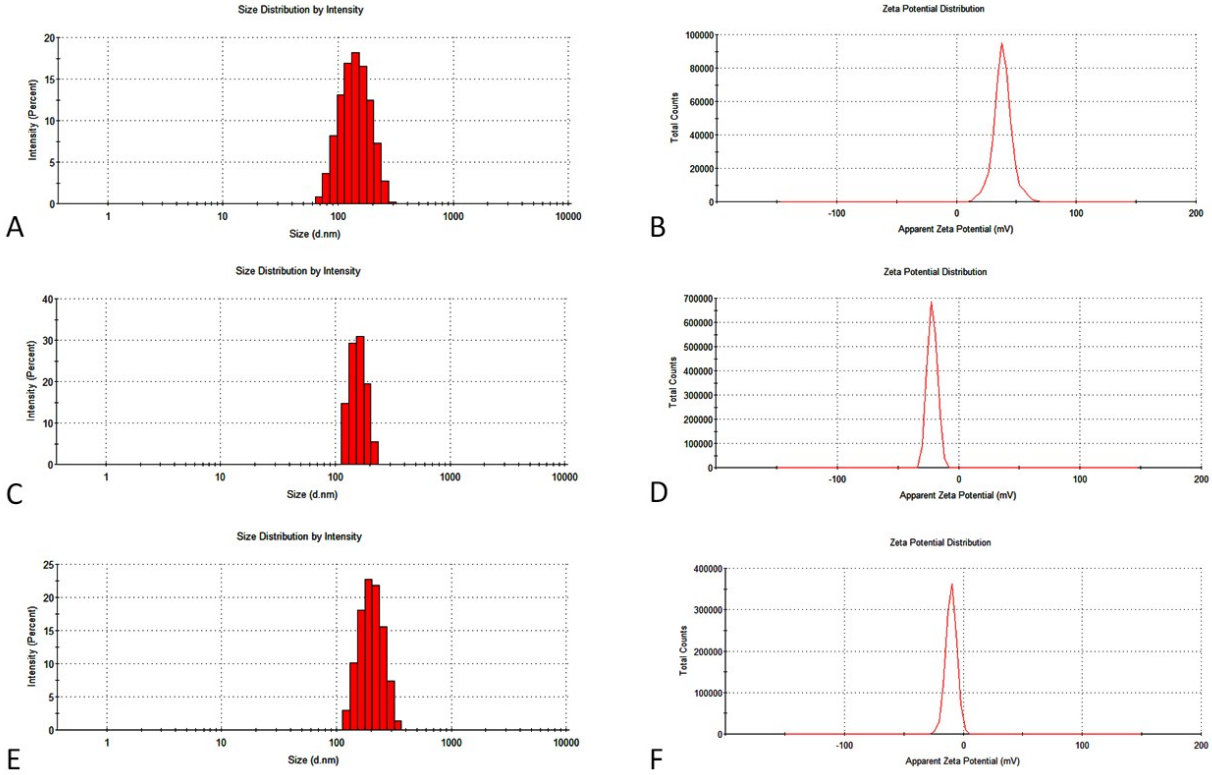
In the FTIR spectrum of MNP-pHLIP nanoconjugate (curve **1**) the characteristic absorption bands of APS-modified MNPs (ν 547 (Fe–O) and 994 cm^{-1} (Si–O)) as well as of pHLIP (ν 1648 (C=O, amide I) and 1533 cm^{-1} (C–N, amide II)) slightly shifted to ν 1637 and 1514 cm^{-1} (amide I and amide II, respectively) indicated the binding of pHLIP to MNPs surface. pHLIP binds to MNPs due to the nucleophilic addition of the thiol group of L-Cys (the second amino acid from N-terminus of pHLIP) to the double bond of the maleimide fragment, which results in the formation of stable 3-thiosuccinimidyl ester. A shoulder at ν 1689 cm^{-1}, attributed to the stretching vibrations of the succinimide C=O group, due to which pHLIP binds to the MNPs, was indicated in FTIR spectrum of MNP-pHLIP.

S2 Table. The elemental analysis data of MNP-APS, MNP-EMCS and MNP-pHLIP.

	MNP-APS	**MNP-EMCS**	**MNP-pHLIP**
C, %	2.69	4.69	6.23
ω_1	0.0268		
ω_2	0.263		
ω_3		0.0469	
ω_4		0.615	
ω_5			0.0623
ω_6			0.558
M	137.22	195.22	4111.69
c, mmol g^{-1} MNPs	**0.747***	**0.167**	**0.00671**
m, mg g^{-1} MNPs	102.0	32.6	27.6

*The amount of APS residues immobilized on the MNPs surface calculated according FTIR data was 0.792 mmol g^{-1}. The value was close to the result obtained by elemental analysis.

S3 Hydrodynamic diameter and zeta potential of MNP-APS (**A**, **B**) in H_2O, MNP-pHLIP (**C**, **D**) in H_2O and MNP-pHLIP (**E**, **F**) in DMEM with 10% fetal bovine serum.

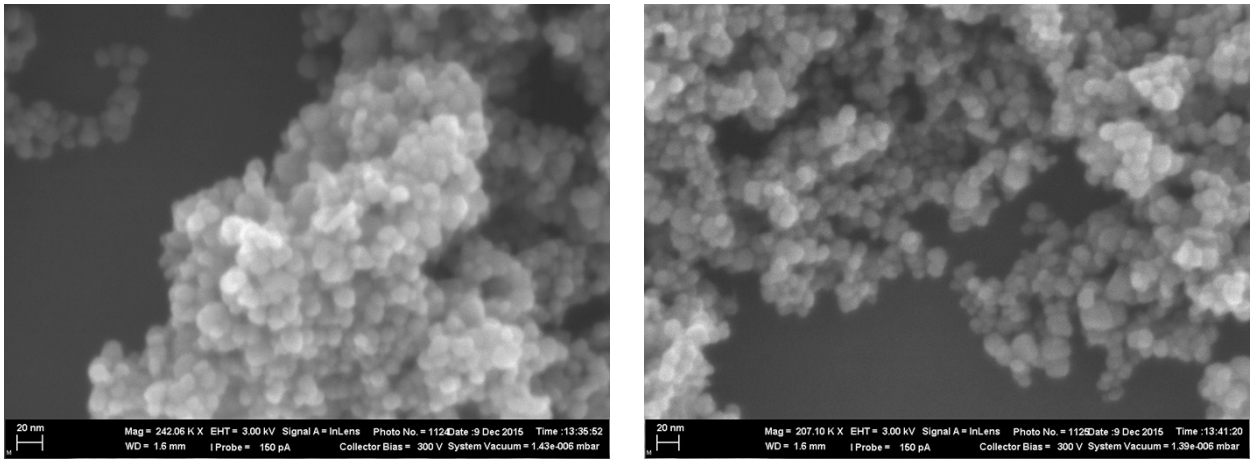


It has been shown that the obtained nanoconjugate existed in stable suspension in water and in DMEM with 10% fetal bovine serum. The surface charge of the particles was changed from positive in MNP-APS (due to the presence of amino groups on the surface of MNPs) to negative in MNP-pHLIP (due to the presence of peptide with free carboxyl groups on the surface).

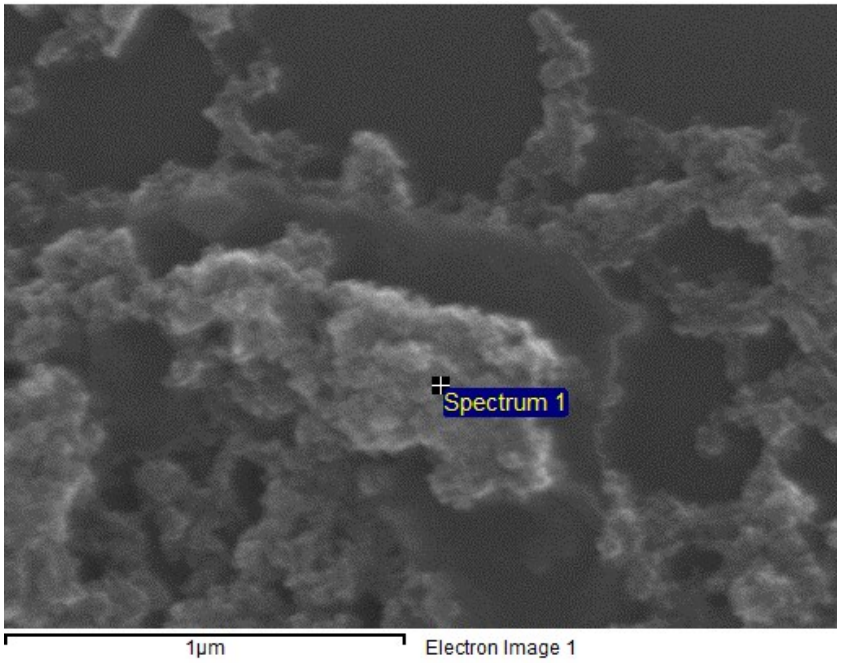
Table. Hydrodynamic characteristics of MNP-APS and MNP-pHLIP suspensions.

MNPs	water			DMEM with 10% fetal bovine serum		
	D_h, nm	ζ, mV	PdI	D_h, nm	ζ, mV	PdI
MNP-APS	131	+38	0.14	-	-	-
MNP-pHLIP	155	–22	0.09	180	–10	0.20

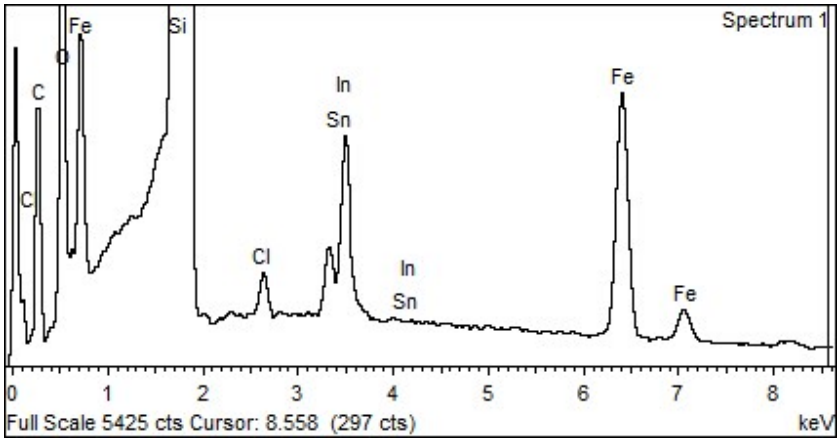
S4-1 SEM images of MNP-pHLIP.



S4-2 (**A**) Electron image and (**B**) EDS spectrum of MNP-pHLIP.



A



B

S5 Hysteresis loops of (**1**) initial MNPs, (**2**) MNP-APS and (**3**) MNP-pHLIP.

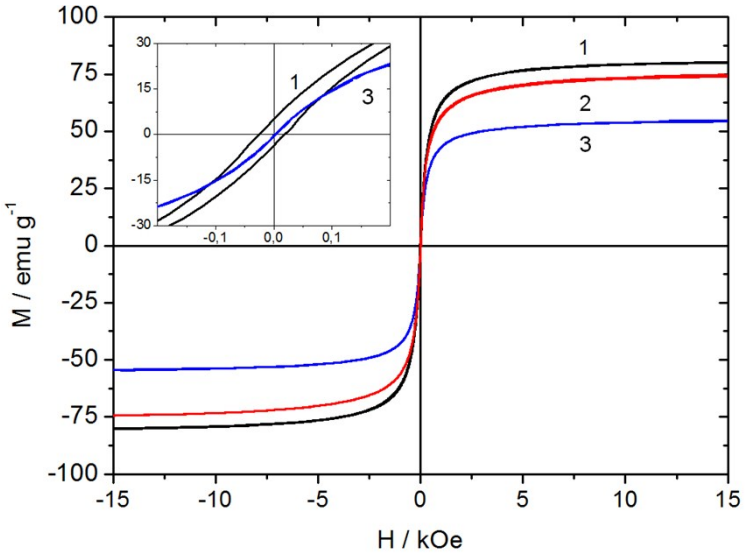
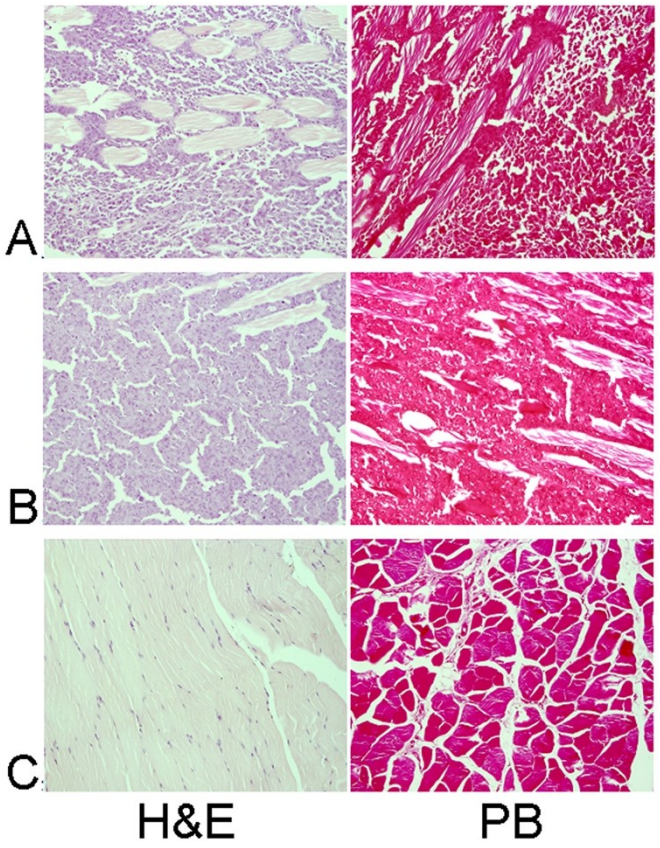


Table. Specific magnetization of the initial MNPs, MNP-APS and MNP-pHLIP.*

MNP	Specific magnetization, emu g^{-1}
MNP	81
MNP-APS	76
MNP-pHLIP	52

*The specific magnetization of MNP-pHLIP was lower than the magnetization of initial MNPs in view of the presence of silane coating and peptide on the surface of MNPs. The magnetization curves of MNP-pHLIP had zero remanence and coercivity.

S6 Haematoxylin and eosin (H&E) and Prussian blue (PB) staining (×200) of (**A**) LLC-tumour tissue extracted from mouse without any administration; (**B**) LLC-tumour tissue extracted from mouse 40 hours after MNP-APS administration in dose of 2 mg kg^{-1} and (**C**) right hind leg muscles of healthy mouse 40 hours after MNP-pHLIP administration in dose of 2 mg kg^{-1}.



LLC tumour consisted of atypical multimorph cells with prominent nucleoli and abundant basophilic cytoplasm. Tumor tissue invaded adjacent muscular tissue. There was no pathological change in muscle tissue of healthy hamster after nanoconjugate administration.

References

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