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Functionalization of Fe_3O_4 magnetic nanoparticles with RGD peptide derivatives

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Derivatives of RGD peptide, such as N^{ω}-Pbf-L-Arg-Gly-L-Asp(OAlk)$_2$ (Alk = Me or Bu^t), which contain glutaric acid moiety as a linker, were synthesized and immobilized to Fe_3O_4 magnetic nanoparticles obtained by gas condensation method.

At present, linear and cyclic peptides containing the amino acid sequence, L-arginine–glycine–L-aspartic acid,[1] the so-called RGD peptides are used for design of new imaging agents for cancer diagnostics, as well as pharmaceutical systems for drug delivery, including those based on magnetic nanoparticles (MNPs).[2,3]

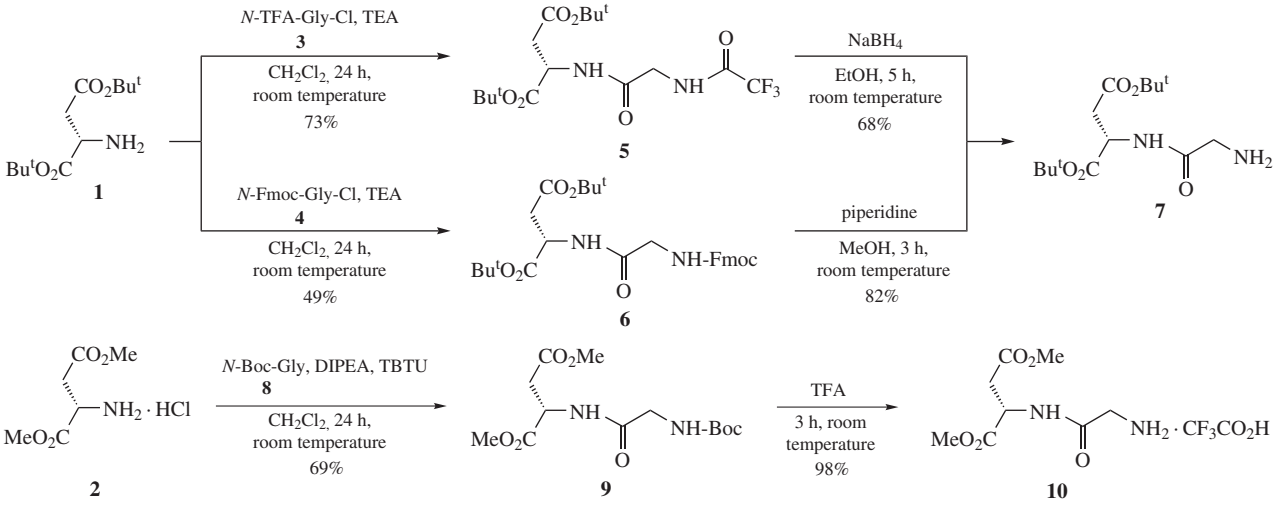
The methods for condensation of RGD peptide with the residues of polyethylene glycols or other polymers, as well as fatty acids can be found in the literature. Such derivatives are used for the preparation of micellar or liposomal forms of nanocomposites for medical purposes.[4–6] The use of short linkers for covalent binding of RGD peptides directly to the MNP surface was not described. The aim of this work was to synthesize new derivatives of RGD peptide and to demonstrate the ability of their covalent immobilization to Fe_3O_4 nanocrystalline powders obtained by gas condensation method.[7] Previously, for biomedical, analytical and catalytic purposes MNPs used were obtained by chemical methods.[8–11] However, the surface properties of MNPs prepared by precipitation from an aqueous solution and by gas condensation method differ significantly (*e.g.*, in the latter case the MNP surface is more hydrophobic). We believe that the study of new materials based on MNPs prepared by gas condensation will reveal new opportunities for their use in biology and medicine.

Synthesis of the required peptides is outlined in Schemes 1 and 2. RGD peptide derivatives **16** and **17** containing the glutaric acid fragment as a linker that is attached to the α-amino group of L-Arg were obtained by successive coupling of the properly protected L-Asp, Gly, and L-Arg similar to the previously published synthesis of RGD peptides.[12] We used glutaric acid as dicarboxylic compound for direct binding of the L-Arg α-amino group to the amino group of (3-aminopropyl)silane-modified (APS-modified) MNPs.[13] Compounds **16** and **17** were obtained starting from di-*tert*-butyl and dimethyl esters of L-Asp (compounds **1** and **2**, respectively). Di-*tert*-butyl ester of *N*-Gly-L-Asp **7** was synthesized by coupling of *N*-TFA- or *N*-Fmoc-glycine acyl chlorides **3** and **4** to ester **1** followed by removal of N-protection from dipeptides **5** and **6** (Scheme 1).

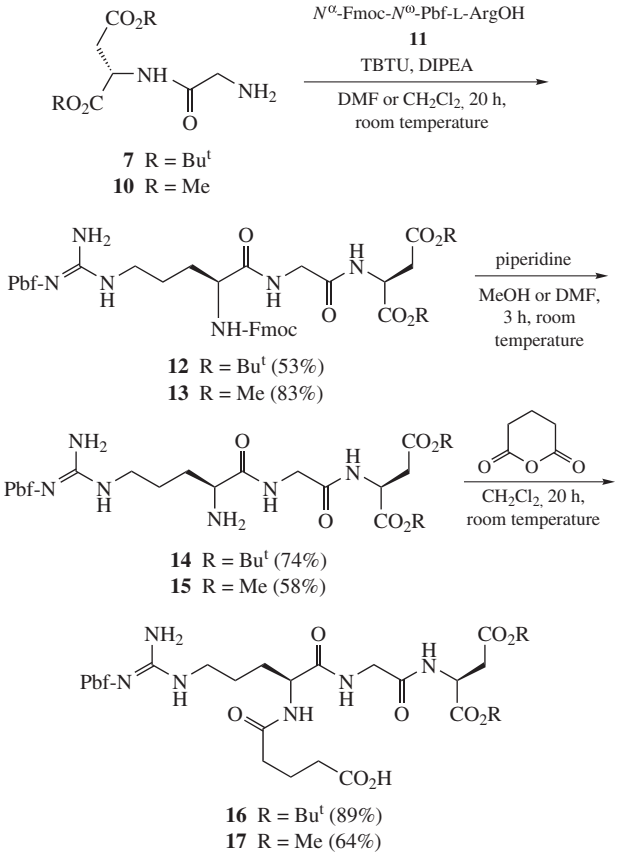
Coupling of ester hydrochloride **2** to *N*-Boc-Gly **8** in the presence of *N*,*N*-diisopropylethylamine (DIPEA) and *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as a coupling agent followed by deprotection of amino group afforded dipeptide trifluoroacetate **10**.

Protected tripeptides N^{α}-Fmoc-N^{ω}-Pbf-L-Arg-Gly-L-Asp(OBu^t)$_2$ **14** and N^{α}-Fmoc-N^{ω}-Pbf-L-Arg-Gly-L-Asp(OMe)$_2$ **15** were synthesized by successive coupling of dipeptides **7** and **10** to N^{α}-Fmoc-N^{ω}-Pbf-L-Arg-OH **11**. The Fmoc protection from tripeptides **12** and **13** was selectively removed by treatment with piperidine in MeOH (see Scheme 2).

To minimize possible steric hindrances in the further coupling of protected RGD peptides **14** and **15** and to more efficiently attach them to the MNPs surface, we chose glutaric acid moiety



Scheme 1



Scheme 2

as the linker. For this purpose, peptides **14** and **15** were reacted with glutaric anhydride (at a 1:1 molar ratio of the reactants) to yield RGD derivatives **16** and **17** (see Scheme 2). The target products were purified by crystallization and flash chromatography. The structure of the compounds obtained was confirmed by ^{1}H NMR and IR spectral data and elemental analysis (see Online Supplementary Materials).

Blocking of functional groups of RGD peptides was carried out in such a way as to ensure their attachment to the MNP surface only by amino group of L-Arg, and to leave unaffected the other functional groups. It is necessary to preserve the specificity of peptide binding to surface receptors of tumor cells (in particular, to $\alpha_v\beta_3$ integrins),[14,15] to which the RGD-modified MNPs should subsequently be bound in order to implement their diagnostic properties in magnetic resonance imaging (MRI).

In this study we used APS-modified MNPs obtained according to the described procedure.[13] Previously, we have shown that attachment of organic compounds to the surface amino groups most efficiently proceeds in acetonitrile in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a coupling agent. In this work, for immobilization of peptides we used the similar conditions (Scheme 3).[13,16] RGD peptide derivative **16** (or **17**) and EDC were added successively to a suspension of APS-modified MNPs in acetonitrile (10 mg MNPs per 5 ml MeCN) under sonification and stirring. After 20 h, the nanocomposites obtained were precipitated from suspension under the action of an external magnetic field and then washed with acetonitrile and acetone (four portions) to remove unreacted compounds and byproducts.

The methods for qualitative and quantitative evaluation of the degree of immobilization of RGD peptides to the MNP surface have been developed based on the IR spectral and elemental analysis data.

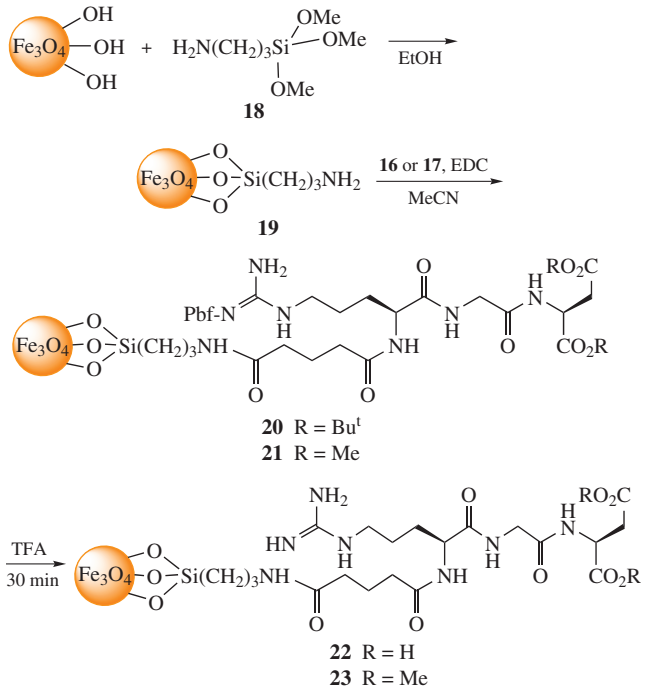
In the FT-IR spectra of the modified MNPs, we observed the absorption bands characteristic of the initial reaction components, but they were shifted, which indicates the formation of a covalent bond between RGD peptides and amino groups of MNPs. The most characteristic absorption bands of nanocomposites modified with RGD peptide derivatives are the bands at 1727–1730 cm^{-1} that correspond to vibrations of the ester groups (C=O) and the bands in the range of 1650–1540 cm^{-1} attributed to vibrations of amide bonds (NH–C=O) (see Figure S1, Online Supplementary Materials).

The removal of protecting groups in L-Arg and L-Asp was carried out in concentrated trifluoroacetic acid (TFA). As a result, we obtained MNPs modified with RGD peptide with free functional groups that can take part in the specific binding to the surface receptors of different tumor cells.

As an example (see Figure S1), in the FT-IR spectrum of MNPs **22** modified with L-Arg-Gly-L-Asp tripeptide after treatment with TFA the absence of the absorption band at 1729 cm^{-1} corresponding to the stretching vibrations of the C=O bond of *tert*-butyl ester of the parent peptide **16** as well as the bands at 1369 and 1091 cm^{-1} relating to the vibrations of the S=O bond of Pbf group indicates the complete deprotection of tripeptide immobilized to the surface of MNPs.

When MNPs **21** modified with N^{ω}-Pbf-L-Arg-Gly-L-Asp(OMe)$_2$ tripeptide were treated with TFA, only Pbf protecting group was removed. In the FT-IR spectrum of MNPs **23**, we did not observe the absorption band relating to the vibrations of S=O bond of Pbf group. The absorption band relating to stretching vibrations of the C=O bond of methyl ester did not disappear, but it was shifted to 1719 cm^{-1}. We managed to hydrolyze methyl ester by treatment with 0.01 N NaOH. However, a significant desorption of the peptide derivative from the MNPs surface accompanied by the rupture of Si–O–Fe bond occurred, as it was evidenced by the decreased intensity of vibration bands characteristic of the original RGD peptide and APS in the FT-IR spectra.

The amounts of immobilized RGD peptide were calculated based on the elemental analysis data by subtraction of the carbon mass fraction of the original APS-modified MNPs from the carbon mass fraction of MNPs modified with RGD peptide. The removal of protecting groups from the RGD peptide fragment resulted in the MNPs containing up to 0.1 mmol of RGD peptide per gram of nanoparticles.



Scheme 3

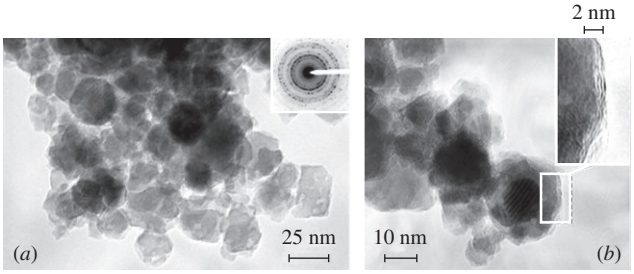


Figure 1 TEM images and electron diffraction patterns (inset boxes) of MNPs **22** modified with RGD peptide.

Electronic diffractogram [see box in Figure 1(*a*)] of this area is typical of magnetite and demonstrates that spinel Fe_3O_4 phase state remains unchanged after the chemical treatment.

According to the transmission electron microscopy (TEM), there were no significant changes in the morphology of the particles after functionalization; the average particle diameter was about 20 nm (Figure 1).† Electron diffraction pattern [see inset box in Figure 1(*a*)] of this area is typical of magnetite and testifies that spinel Fe_3O_4 phase state remains unchanged after the chemical treatment. A surface fragment, on which the low-contrast surface layer (1–2-nm thick) was observed, is shown at a greater magnification in Figure 1(*b*). This layer corresponds to the preliminary applied organosilicon coating obtained as a result of silanization, and probably to the immobilized RGD peptide which presence was confirmed by IR spectroscopy (Figure S1, spectrum *3*).

Evaluation of the hydrodynamic diameter and ζ-potential of MNPs aqueous suspensions (pH 6.5) after each step of modification (see Scheme 3) was carried out by dynamic light scattering (DLS).‡ For example, the hydrodynamic diameter and ζ-potential of APS-modified MNPs **19** were 144 nm and 37 mV, respectively. As a result of their modification with peptide **16**, MNPs **20** with the parameters being 177 nm and 37 mV were prepared. After treatment of MNPs **20** with TFA (for the removal of protecting groups), MNPs **22** with hydrodynamic diameter of 123 nm and ζ-potential of 39 mV were obtained. This may point to a slightly greater stabilization of the RGD-modified MNPs **22** suspension due to the appearance of a larger number of basic groups on the surface as compared to the initial MNPs **19**. The nanocomposites obtained after functionalization exhibit high magnetic properties (specific magnetization of 59 emu g^{-1}), which are slightly lower than those for the initial MNPs (70 emu g^{-1}) (Figure 2).§ Probably,

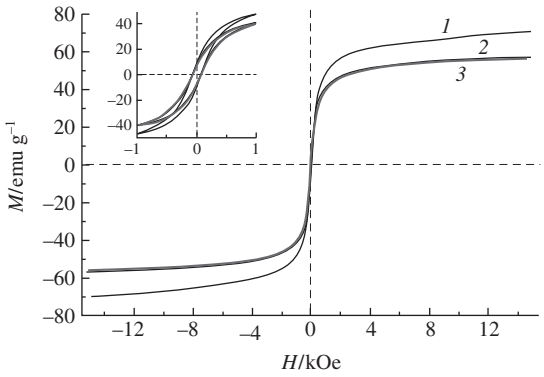


Figure 2 Hysteresis loops of (*1*) APS-modified MNPs **19**, (*2*) MNPs **20** modified with RGD peptide with protected groups, and (*3*) MNPs **22** modified with RGD peptide with free groups.

† Morphological studies of particles were performed using a Philips CM30 transmission electron microscope (TEM).

‡ DLS measurements were carried out using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.).

this can be explained by the presence of the non-magnetic RGD peptide and APS on the surface of the nanoparticles. The magnetization curves of the samples before and after treatment can be described by superparamagnetic behavior, which may make it possible to visualize them using MRI. The existence of hysteresis and coercive force of about 120 Oe indicates that a small proportion of the MNPs in all the samples studied are in the ferromagnetic single-domain state.

Thus, we have proposed the synthetic routes for the selectively protected derivatives of RGD peptide and the methods for their immobilization to Fe_3O_4 MNPs followed by removal of protecting groups. The obtained MNPs contain up to 0.1 mmol RGD peptide per gram of nanoparticles. We believe that such functionalization of MNPs will make it possible to develop new promising contrast agents for MRI cancer diagnostics.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2013.12.006.

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§ The specific magnetization of the powders of modified MNPs was measured at room temperature using a vibration magnetometer in a magnetic field up to 15 kOe.