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Fast microwave-assisted conjugation of magnetic nanoparticles with carboxylates of biological interest†

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A new technique of surface modification of nanoparticles (Fe_3O_4-NP) with carboxylates of biological interest has been developed with a microwave-assisted heating method. This two-step technique reduces the reaction time to 15 min (for each step) and increases the number of substituents to 2300 molecules compared to traditional methods with a maximum of 440 molecules.

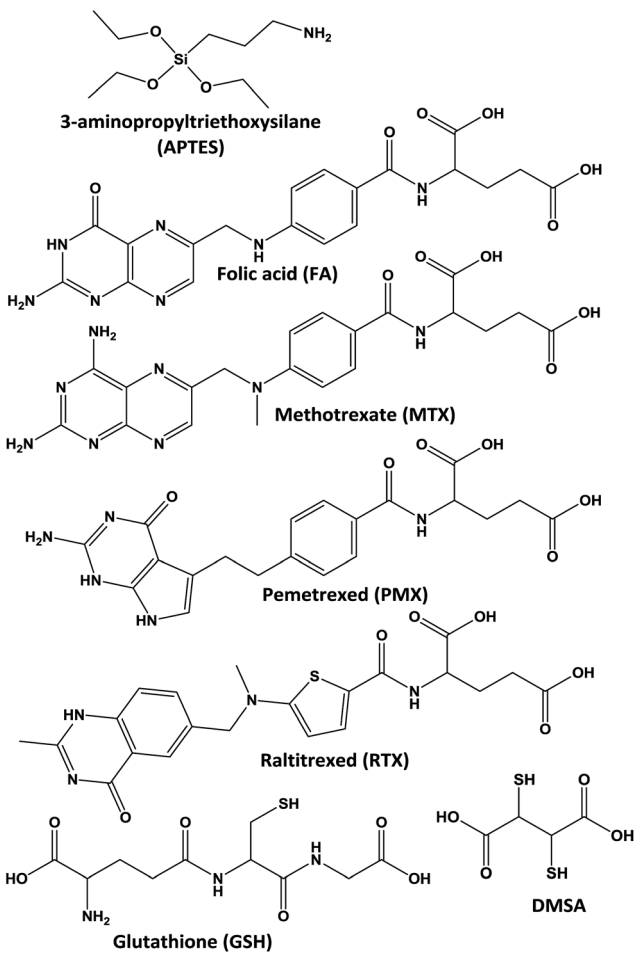
Introduction

In the search of new functional materials, it is important to consider magnetic nanoparticles (Fe_3O_4-NP) as a valuable support for biological applications, such as magnetic fluid hyperthermia applications, magnetic resonance imaging (MRI), immunoassays, labelling for cells catalysis, and targeted drug delivery.

In most cases Fe_3O_4-NP are synthesized in the presence of surfactants, like oleic acid, to prevent aggregation among them. In order to improve the stability of the nanoparticles, the ligand molecules on the surface can be exchanged by others that can possibly provide new properties or functionality to the particles, with the incoming ligand molecule binding more strongly to the inorganic nanoparticle surface. Nanoparticles with better stability are those with strong covalent interactions with their ligands.[1–4]

An excellent proposal as a bifunctional linker is 3-aminopropyltriethoxysilane (APTES) which is able to make covalent binding at both sites, the first with inorganic nanoparticle surface and the second with organic molecules *via* amidation.[5,6] It is important to conjugate the largest number of molecules on Fe_3O_4-NP surface to improve efficiency of materials. For that reason we propose a fast method assisted by microwave heating. Also, in this work we tested different carboxylates, namely, folic acid (FA), methotrexate (MTX), pemetrexed (PMX), raltitrexed (RTX), glutathione (GSH) and dimercaptosuccinic acid (DMSA) (Scheme 1), with biological applications like drugs for treatment and prevention of cancer and heavy metals chelation.[2,5,7–10]

In previous works,[5–7,11–13] conjugation of Fe_3O_4-NP with APTES and similar linkers takes long reaction times and poor yields which does not allow Fe_3O_4-NP to be functionalized beyond 300–500 molecules per each nanoparticle (1.2 molecules per nm^2).[3,5] In general microwave processes are energy and time



Scheme 1 Linker and biological molecules used in this work to functionalize Fe_3O_4-NP.

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saving, reduce thermal gradient effects, thus leading to better results, and also can provide cleaner reactions.

In the present research, we exploit the potential of the microwave technique, on one hand, to improve the coupling processes with carboxylates on the Fe_3O_4-NP surface and, on the other hand, to reduce the reaction time from hours to few minutes.[14] The amount of molecules loaded on magnetic nanoparticles was estimated by UV-Vis and TGA, and the results have been compared with those reported in the literature.

Results and discussion

Synthesis of Fe_3O_4-NP *via* thermal decomposition

Among different kinds of methodologies to synthesize Fe_3O_4-NP,[15] the one that controls the NP size is the best option to get monodisperse Fe_3O_4-NP with 3 to 20 nm in diameter. The uniform shape and size distribution of Fe_3O_4-NP was analyzed by transmission electron microscopy (TEM) and showed an iron oxide core of about 8 nm in diameter on average. Fig. 1 shows FTIR with typical $-CH_2-CH_2-$ absorption band at 2920 cm^{-1} and $-CH_2-CH_3$ at 2850 cm^{-1}, C=O bond at 1590 cm^{-1}, and Fe–O bond for magnetic nanoparticles at 630 cm^{-1}.

Functionalization of Fe_3O_4-NP-APTES *via* microwave and characterization

In the search of better hybrid nanomaterials, the key is a correct functionalization of the particle surface. As the first step to improve traditional methods it is important to choose the appropriate solvent for microwave heating, to increase temperatures and decrease reaction times. For instance, in previous works,[5] the reaction is undertaken in toluene anhydride, sonicating for 4 hours at 60 °C and finally washed with EtOH. According to the bibliography, we used the same reagents proportions and tested different organic solvents to enhance the insertion of APTES on Fe_3O_4-NP surface. Water, EtOH and MeOH were not used because they can hydrolyze APTES. The final product was magnetically separated and washed with EtOH to remove rests of APTES in the reaction medium. From the results of these reactions gathered in Table 1 it can be inferred that toluene is the best option. Accordingly, we determined the best conditions for microwave heating at 120 °C, 2 bar, and 15 minutes ($P = 400$ W) with toluene as solvent. Other solvents (acetone, acetonitrile) reached higher pressures under the same reaction conditions, but were not good candidates because they are not able to promote the exchange of substituents. In other cases, solvents with higher boiling points than toluene make difficult to clean the final product. APTES coating was confirmed by FTIR (Fig. 1) with typical Si–O bond at 1000 cm^{-1}. There are also bands at 628 and 446 cm^{-1} which are typical for Fe–O. By inspecting the FTIR results, obtained from 1 mg of dried product and 100 mg of KBr (in both cases), a high functionalization for the microwave-assisted process is observed.

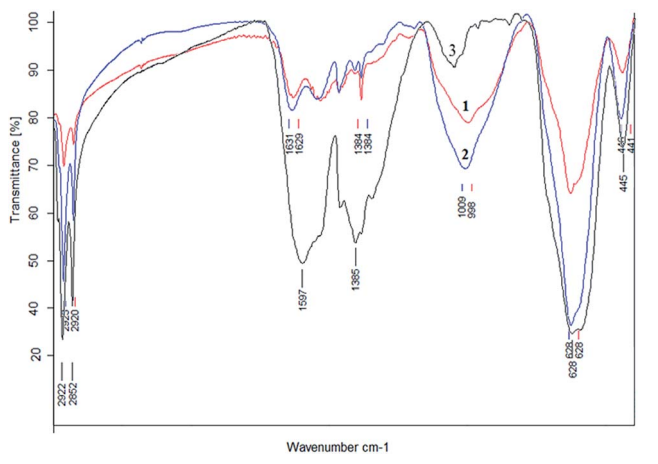


Fig. 1 FTIR of Fe_3O_4-NP-APTES (1) traditional reaction, (2) microwave reaction and (3) Fe_3O_4-NP-oleic acid.

Amidation on Fe_3O_4-NP surface and characterization

In order to study fast covalent bonding of antifolates and other organic molecules, we prepared six different hybrid nanoparticles: Fe_3O_4-NP-APTES-FA, Fe_3O_4-NP-APTES-MTX, Fe_3O_4-NP-APTES-PMX, Fe_3O_4-NP-APTES-RTX, Fe_3O_4-NP-APTES-GSH, and Fe_3O_4-NP-APTES-DMSA. Analogously to the previous chapter, the microwave treatment and the classical heating mode for the Fe_3O_4-NP functionalization were compared. The free $-NH_2$ groups present on APTES react with antifolates *via* activation of the carboxylic groups by the carbodiimide/*N*-hydroxysuccinimide method using the microwave technique. Traditional processes[7,16] perform amidation reaction on the Fe_3O_4-NP surface at 37 °C overnight with DMSO as solvent. Here we propose a fast method, adapted from the literature,[17–19] using microwave heating ($P = 400$ W) and MeOH/H_2O as solvent at 120 °C. All experiments reached 4 bar of pressure in 15 minutes of reaction. Size distribution of different Fe_3O_4-NP was analyzed by TEM (Fig. 2) showing particles of 8 nm in average. For a better view, TEM complete microphotographs are shown in the ESI (Fig. S12 and S13†).

FTIR shows the correct functionalization of Fe_3O_4-NP in all cases. The presence of C=O stretching bands at 1630 cm^{-1} are typical of monosubstituted amide groups, confirmed with the presence of an N–H band at 1550 cm^{-1}; Si–O bond is still present at 1000 cm^{-1}, and Fe–O bond at 630 cm^{-1} (Fig. 3). The general procedure of modification *via* microwave is shown in

Table 1 List of results of different solvents used for the Fe_3O_4-NP-APTES functionalization

Solvent	Boiling point (°C)	Pressure (bar)	Results
Acetone	56.0	4	No reaction
Acetonitrile	82.0	3	No reaction
CH_2Cl_2	39.6	7	Precipitate
DMF	153.0	0	No reaction
DMSO	189.0	0	No reaction
Hexane	68.0	2	Precipitate
Toluene	110.6	2	Precipitate
Xylene	137.0	1	Precipitate

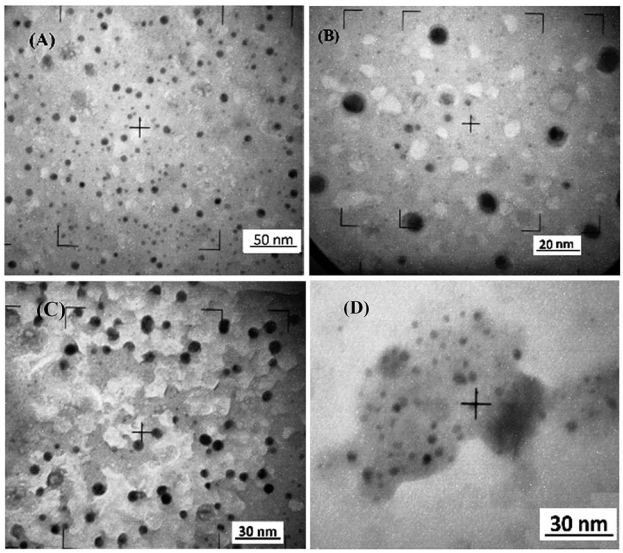


Fig. 2 TEM microphotographs (A) Fe_3O_4-NP-oleic acid. (B) Fe_3O_4-NP-APTES-. (C) Fe_3O_4-NP-APTES-GSH. (D) Fe_3O_4-NP-APTES-FA.

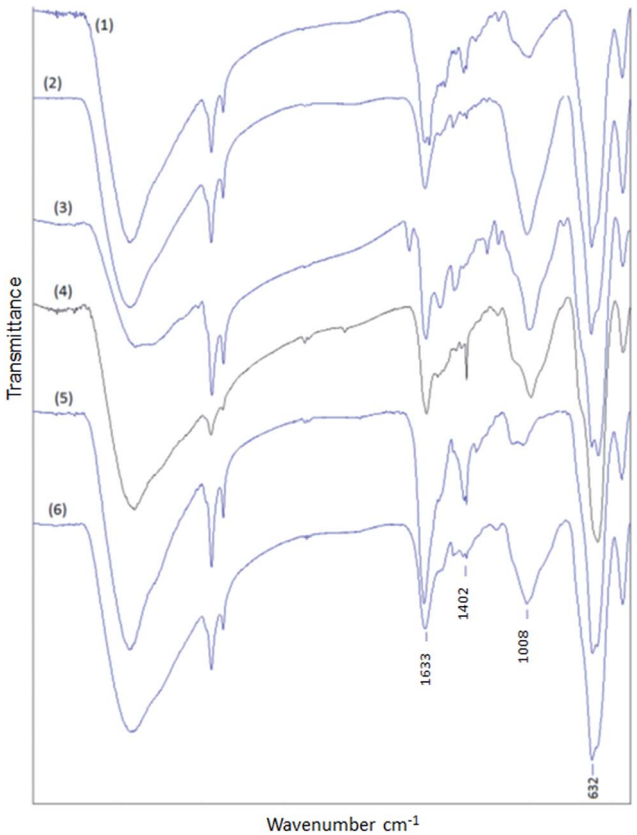


Fig. 3 FTIR of Fe_3O_4-NP-APTES- (1) FA, (2) MTX, (3) PMX, (4) RTX, (5) GSH and (6) DMSA.

Scheme 2. The FTIR spectra performed for the free carboxylates are shown in the ESI (Fig. S1–S5†). In addition, the MS spectrum data are included in the Experimental section. These results confirm that the reaction was successfully performed.

Determination of number of molecules on Fe_3O_4-NP surface

It is important to have a precise control on the number and orientation of the immobilized molecules on nanoparticles because carboxylates coordination occurs *via* APTES free amine groups. In previous works, the concentration of APTES and carboxylates (MTX, PMX and RTX) covalently bounded on the Fe_3O_4-NP surface was estimated by the standard addition method by ^{1}H-NMR and UV-VIS through the residues of the reaction applying the following equations:

$$N = \frac{\pi}{6}\frac{D^3\rho}{\mathrm{mw}}\frac{1}{N} = \frac{\text{nanoparticles}}{\text{mol } Fe_3O_4}$$

where $1/N$ refers to the number of Fe_3O_4-NP for each mol of Fe_3O_4. D is the average diameter of Fe_3O_4-NP in cm (provided by TEM microphotographs), ρ is Fe_3O_4 density (5.196 g cm^{-3}) and mw is the molecular weight of Fe_3O_4 (231.53 g mol^{-1}).[20] In order to improve and clarify the number of substituents, we chose TGA method to determine the conjugation rate by measuring the mass loss due to decomposition. Experiments were performed with a constant heating rate of 10 °C min^{-1} from room temperature (25 °C) to 1000 °C. The mass lost from 25 °C to 100 °C has not been taken into account in the calculations, because it corresponds to the loss of water and/or solvents that may contain the sample. The TGA curves show weight loss in both cases (Fig. 4). Using weight loss percentage values it is possible to quantify the number of molecules on Fe_3O_4-NP surface by applying previous equations. Our main results together with bibliographic data are summarized in Table 2.

The microwave-assisted functionalization and conjugation reported herein clearly shows a substantial improvement in most cases over other reported methods. For APTES conjugation, the density of molecules per nm^3 (ξ) is doubled, thereby increasing the number of $-NH_2$ groups available for further amidation. The same result is observed for the amidation process, which suggest two ideas; first, a low number of linkers results in a low concentration of carboxylates and, second, the reaction conditions for the amidation reaction reported in the literature produces very low reaction yields if compared with our results.

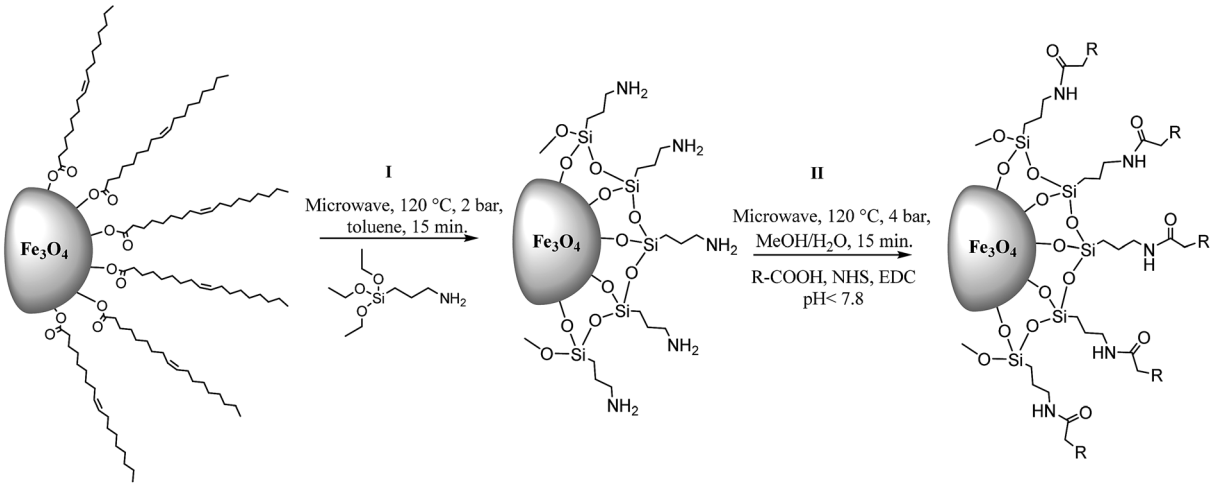
Experimental section

Materials

All commercially available reagents: oleylamine (≥98% (primary amine)), oleic acid (≥99% (GC)), dibenzyl ether (purum, ≥98.0% (GC)), were supplied by Sigma-Aldrich. All the solvents were purchased from Scharlau (all solvents have superior to 98% purity). Ferric acetylacetonate (purum, ≥97.0% (RT)) was supplied by Fluka.

Instrumentation

Infrared (IR) was obtained on Bruker Tensor 27 instrument in solid state. Ultraviolet-visible (UV-Vis) was obtained on a Shimadzu UV-2401PC Spectrometer. Matrix-assisted laser desorption/ionization mass spectra (MALDI), was recorded with a Autoflex III MALDI TOF/TOF mass spectrometer provided with



Scheme 2 General procedure for Fe_3O_4-NP functionalization.

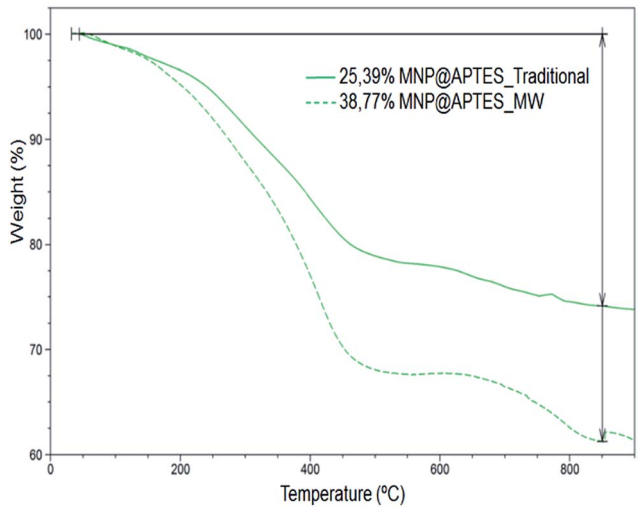


Fig. 4 Thermogravimetric analysis for Fe_3O_4-NP-APTES.

an Smart beam Laser at 200 Hz. Functionalization of Fe_3O_4-NP was performed on a Biotage Initiator Classic Microwave Synthesizer at 400 W. TGA were performed on a SDT Q-600 instrument.

Methods

FTIR. For IR experiments, 100 mg of anhydrous KBr were mixed with 1 mg of each product (perfectly dried) and grounded in a mortar to make a tablet.

TGA. To perform the analysis, all the samples were perfectly dried with vacuum before weighing them.

TEM. The samples of nanoparticles were dissolved in ethanol to support them in grids. The micrographics were prepared in ultrathin carbon type A400 mesh (pkg/50) grids.

Mass analysis. All the samples of nanoparticles were prepared with 1 mg of dried sample, digested for 1 hour with HCl (37%) filtered, vacuum dried and redissolved in 1 mL of Milli-Q water.

Synthesis and characterization of Fe_3O_4-NP

For the synthesis of Fe_3O_4 nanoparticles, an adapted procedure of S. Sun *et al.*[21] was used. $Fe(acac)_3$ (2 mmol), oleic acid (6 mmol), oleylamine (6 mmol) were mixed into 20 mL of dibenzyl ether and magnetically stirred under a flow of argon. The solution was heated firstly at 200 °C for 1 h and then at 300 °C for 2 h with vigorous magnetic stirring with reflux under Ar atmosphere.

Table 2 Thermogravimetric and particle size values of Fe_3O_4-NP functionalized by the two methods

	Technique	Analysis method	θ (nm)	Weight loss (%)	Molecules/Fe_3O_4-NP	ξ (molecules per nm^3)
Fe_3O_4-NP-oleic acid	—	TGA	8	42.01	2142	4.18
Fe_3O_4-NP-APTES	Traditional	TGA	8	25.39	1284	2.51
Fe_3O_4-NP-APTES	Microwave	TGA	8	38.72	2380	4.65
Fe_3O_4-NP-APTES[a]	Traditional	^{31}P NMR titration	10	—	1200	1.20
Fe_3O_4-NP-APTES-MTX[b]	Traditional	Standard addition	10	—	419	0.42
Fe_3O_4-NP-APTES-MTX	Microwave	TGA	8	52.69	2046	4.00
Fe_3O_4-NP-APTES-RTX[c]	Traditional	Standard addition	6	—	363	1.70
Fe_3O_4-NPAPTES-RTX	Traditional	TGA	8	9.39	1142	0.54
Fe_3O_4-NPAPTES-RTX	Microwave	TGA	8	38.55	1142	2.23
Fe_3O_4-NP-APTES-PMX[c]	Traditional	Standard addition	6	—	164	0.85
Fe_3O_4-NP-APTES-PMX	Microwave	Standard addition	8	—	2113	4.13

[a] F. Benyettou *et al.*[3] [b] N. Kohler *et al.*[7] [c] K. López *et al.*[5]

The solution was cooled at room temperature. Nanoparticles were precipitated by adding 25 mL of ethanol to the mixture. The precipitated was collected by centrifugation at 8000 rpm, washed with ethanol (3 mL × 15 mL) and redispersed in 25 mL of hexane or acetonitrile (depending on the type of reaction that will take place later), to a final concentration of 0.025 mmol mL^{-1}. FTIR (KBr): 3406, 2921, 2851, 1591, 1384, and 632 cm^{-1}.

Functionalization of Fe_3O_4-NP with APTES *via* microwave

For APTES functionalization procedure has been adapted from Zhang *et al.*[7,11] to microwave reactor. 0.1 mL of APTES was added to 1.7 mL of Fe_3O_4-NP-oleic acid colloidal suspension in a 5 mL flask with 3 mL of toluene and hermetically closed. The mixture was heated in the microwave reactor under magnetic stirring for 15 min at 120 °C and 2 bar. Fe_3O_4-NP-APTES was magnetically precipitated and washed with EtOH (3× 5 mL) and finally dispersed in MeOH for the next step. FTIR (KBr): 3384, 2920, 2651, 1629, 1384, 998 and 628 cm^{-1}.

Covalent conjugation *via* microwave: amidation reaction

Amidation reaction procedure has been adapted from bibliography[3,11,22,23] to microwave reactor. 0.025 mmol of carboxylic acid (FA = 11.04 mg, MTX = 11.36 mg, PMX = 10.69, RTX = 11.46 mg, GSH = 7.68 mg, DMSA = 4.56 mg) is dissolved in distillated water. Moreover 34.8 mg of EDC and 5.81 mg of NHS was dissolved in 1 mL of distillated water, both dissolutions were mixed and pH was adjusted to 8, finally Fe_3O_4-NP-APTES dispersed in MeOH is added to the mixture, hermetically closed and heated in the microwave reactor under magnetic stirring for 15 min at 120 °C and 2 bar. Fe_3O_4-NP-APTES-R was magnetically precipitated and washed with distillated H_2O (3 × 5 mL). To preserve the product, Fe_3O_4-NP-APTES-R was dissolved on a NH_4OH solution of 1%.

FTIR (KBr): Fe_3O_4-NP-APTES-FA. 3385, 2921, 2852, 1631, 1385, 993, 630, 590 and 445 cm^{-1}. Fe_3O_4-NP-APTES-MTX: 3385, 2921, 2852, 1625, 1555, 1446, 1384, 1199, 990, 590 and 442 cm^{-1}. Fe_3O_4-NP-APTES-PMX: 3380, 2921, 2851, 1729, 1628, 1546, 1459, 1260, 1011, 631 and 591 cm^{-1}. Fe_3O_4-NP-APTES-RTX: 3385, 2912, 2854, 1626, 1558, 1448, 1384, 997, 596 and 444 cm^{-1}. Fe_3O_4-NP-APTES-GHS: 3419, 2922, 2852, 1635, 1556, 1385, 998, 59 and 443 cm^{-1}. Fe_3O_4-NP-APTES-DMSA: 3385, 2921, 2852, 1631, 1385, 993, 630, 590 and 445 cm^{-1}.

MALDI. APTES-FA: found *m*/*z* 624.844 [M], [$C_{25}H_{33}N_8NaO_8Si$] requires 624.209. APTES-MTX (+): found *m*/*z* 701.501 $[M]^+$, [$C_{25}H_{33}FeN_9Na_2O_7Si^+$] requires 701.502. APTES-PMX: found *m*/*z* 475.342 $[M]^+$, [$C_{20}H_{25}N_6Na_2O_5^+$] requires 475.168. APTES-RTX: found *m*/*z* 440.950 $[M]^-$, [$C_{21}H_{21}N_4O_5S^-$] requires 441.123. APTES-GSH: found *m*/*z* 510.880 [M], [$C_{15}H_{31}FeN_4O_8SSi$] requires 511.098. APTES-DMSA found *m*/*z* 383.967 [M], [$C_9H_{18}FeNO_6S_2Si$] requires 383.968.

Conclusions

In summary, we have developed a new and fast microwave-assisted technique for the functionalization of Fe_3O_4-NP with a high yield of conjugation and short reaction times, and demonstrated that the grafting rate was increased by using the microwave technique. A significant difference was encountered when comparing the nanoparticles functionalized *via* microwave with those obtained in a traditional way.

The number of molecules on Fe_3O_4-NP surface has been determined by thermogravimetric analysis and compared with standard addition method, the former showing better results. All the carboxylates used in this work showed similar results for the amidation reaction.

Acknowledgements

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