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**PEGlatyon-SPION surface functionalization with folic acid for magnetic hyperthermia applications**

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**Abstract**

Superparamagnetic iron oxide nanoparticles (SPION) are of great interest for application in magnetic fluid hyperthermia (MFH) due to their heat generation capability in an external alternating magnetic field, besides biocompatibility, and surface properties. MFH has emerged as a promisor therapeutic approach for cancer treatment and is based in controlled heating tumor tissue through the accumulation of SPIONs within cancer cells. This work describes a new route for the preparation of folate-conjugated PEGylated SPIONs, which involves the attachment of such molecules at the surface through polycondensation reactions, without the need for coupling agents or prior modification on the species involved. The size of iron oxide cores obtained by transmission electron microscopy was about 12 nm. The conjugation of folate onto SPIONs was confirmed by FTIR spectroscopy. The folate conjugated nanoparticles were colloidal stable in PBS, presenting a hydrodynamic diameter of 109 ± 1 nm and PDI 0.148. The obtained folate-targeted PEGylated SPIONs showed super-paramagnetic behavior with a saturation magnetization of 73.1 emu·g⁻¹ at 300 K. Their specific absorption rate (SAR) ranged from 32.8 to 15.0 W·g⁻¹ in an alternating magnetic field of 10–16 kA·m⁻¹ and frequency of 420–203 kHz. The heat generated was sufficient to raise the sample temperature to the therapeutic range used in MFH establishing this system as promising candidates for use in MFH treatment.

**1. Introduction**

The biomedical interest in superparamagnetic iron oxide nanoparticles (SPION) is due to their biocompatibility, surface engineering capabilities and enhanced magnetic properties [1–3]. This material has been applied in drug delivery systems, magnetic fluid hyperthermia (MFH) [4, 5] and contrast agent for magnetic resonance imaging [6]. Theranostics systems, whose diagnosis and treatment are carried out on a single platform, has also been investigating [7, 8].

Magnetic hyperthermia has emerged as a promising non-invasive therapeutic approach for thermal cancer treatment [9]. SPION release heat when exposed to an alternated magnetic field of appropriated frequency and amplitude, by converting magnetic energy into thermal energy [3, 10]. This therapeutic platform is based on controlled heating of tumor tissue through the accumulation of SPION within cancer cells followed by exposure to an external alternating magnetic field (AMF). The SPION act as nano-heaters by increasing the local temperature in the range of 41–46 °C [11, 12], which triggers cell death mechanisms (apoptosis or and necrosis) while altering the functionality of the protein causing high sensitivity of the cancer cells to traditional treatments (e.g. radiotherapy and chemotherapy) [13]. Sans et al [14] compared the temperature effect on neuroblastoma
SH-SY5Y cells in response to the application of exogenous heating sources with MFH sources. Their findings show that MFH requires 6 °C lower temperatures than exogenous heating sources to induce similar cytotoxic effect.

The ability of SPION to be driven and accumulated in the desired tissue is highly dependent on its surface and magnetic properties. Size and morphology should be finely tuned during the synthesis to increase the heating efficiency of the platform, avoiding the super dosage of magnetic fluid to reach the treatment condition [15–17]. In addition, to the success of MFH treatment, SPION surface must be functionalized to anchor specific ligands to targeting damage cancer cells and improve colloidal stability [9, 18].

In order to ensure the colloidal stability and biodistribution on physiological medium, SPION is frequently functionalized with biocompatible polymers, from a natural source (polysaccharides, such as dextran, chitosan, alginate, etc [19]) to synthetic ones (poly(lactide-co-glycolide) (PLGA) [20], polycaprolactone (PCL) [21] and polyethylene glycol (PEG) [22, 23]). PEG shows high water solubility and low cytotoxicity properties. When grafted on SPION surfaces, PEG extends their systemic circulation time reducing the binding with plasma proteins and macrophage recognition.

Concerning to the selectivity, folate receptors are overexpressed during the disordered growth of tumor cells for the internalization of nutrients required for cell division, such as folic acid [22, 24–26]. Chen et al [27] have described the structural basis of recognition of folic acid by folic receptors. They found that one moiety of the folate acid molecule shows strong bind affinity by the receptors, while another moiety sticks out of the pocket entrance, allowing it to be functionalized with drugs or nanoparticles, without adversely affecting the receptor binding. Bonvin et al [28] report a direct attachment of folic acid molecule to iron oxide surface, which folic acid acts either as coating and targeting agent. They also report the efficiency of specific uptake of composite by prostate cancer cells.

This study reports on the preparation of folate-conjugated PEGylated SPION by polycondensation reaction and the investigation of the structural, morphological, magnetic, and magneto-thermal properties. In contrast to other methodologies for PEGylation and the attachment of folate groups on nanoparticle surface, this methodology is based on de polycondensation reaction of the folate through their carboxylic acid groups with the hydroxyl groups from PEG [23], resulting in a simpler and cleaner route due to the absence of coupling agents or reactants that increase the complexity of chemical routes related in the literature [29, 30].

2. Experimental

2.1. Materials
All chemicals were used as received. Iron(III) chloride hexahydrate (AR, ACS) and sodium hydroxide (AR, ACS) were purchased from Mallinckrodt Chemicals. Iron(II) chloride tetrahydrate (99%, Aldrich), triglyme (99%, Sigma-Aldrich), poly(ethylene glycol) (average Mw 950–1050, Aldrich), citric acid monohydrate, phosphate buffer saline (PBS) (10 mmol L⁻¹ phosphate salts, 0.138 mol L⁻¹ NaCl and 2.7 mmol L⁻¹ KCl) and folic acid (97%, Sigma) were purchased from Sigma-Aldrich (Brazil).

2.2. Synthesis of folate-conjugated PEGylated SPION
Magnetite nanoparticles were synthesized by the chemical co-precipitation method described by Massart [31] with modifications. The PEGylation of the as-obtained SPION was performed following the route described previously by Viali et al [23]. Briefly, 250 ml of Fe³⁺ (0.30 mol L⁻¹) and Fe²⁺ (0.15 mol L⁻¹) aqueous solutions were mixed and purged with argon gas. Then, 100 ml of ammonium hydroxide (25%) aqueous solution was added to the Fe³⁺ and Fe²⁺ solution under vigorous stirring and allowed to react for 30 min. Next, the reactional suspension was heated to boiling for 1 h. The as-synthesised black precipitate (bare-Mag) was collected by magnetic decantation and washed several times with deionized water. For SPION functionalization, the obtained suspension was mixed with citric acid in the proportion of 0.37 mmol of acid per gram of iron oxide at pH 3 and kept under magnetic stirring for 12 h. The SPION functionalized with citrate were separated by magnetic decantation, washed three times with acetone, and dispersed in 120 ml of triglyme. For the PEGylation step, 30 ml of the triglyme-SPION dispersion was added to a 250 ml three-neck round-bottom flask and mixed with 30 ml of PEG. The mixture was sparged with Argon gas and heated up to 120 °C for 3 h. Then, the temperature was raised to 150 °C and kept for 21 h. Afterward, the SPION were precipitated with the addition of acetone, separated with the use of a permanent magnet and washed three times with acetone. The obtained PEGylated SPION (Mag-PEG) were dispersed in 60 ml of triglyme and added to a reaction system as described above. To this dispersion was added 100 mg of folic acid under constant stirring and the system was heated up to 120 °C for 24 h. The folate-conjugated PEGylated SPION (Mag-PEG-FA) were precipitated with acetone, separated by magnetic decantation, and washed several times with deionized water for removal of excess of folic acid. The two resulting PEGylated nanoparticles (Mag-PEG and Mag-PEG-FA) were stored in acetone.
equipped with Cu Kα radiation source in the 2θ range of 20–90°. The size and morphology investigation of the SPION was performed by transmission electron microscopy analysis (TEM) using the Philips CM200 microscope operating at 200 kV. The Fourier Transform Infrared (FTIR) spectroscopy measurements were carried out with samples dispersed in KBr pallets using a PerkinElmer Frontier FT-IR spectrometer; the system resolution was set at 4 cm⁻¹ while performing 32 scans in the range of 4000 to 400 cm⁻¹. Thermogravimetric analysis (TGA) were performed from room temperature up to 800 °C under 50 ml min⁻¹ airflow, and heating rate of 10 °C min⁻¹ using the SDT 2960 system DTA-TGA from TA Instruments. The hydrodynamic diameters of the PEGylated SPION and folate-conjugated SPION were obtained by dynamic light scattering (DLS) measurements using the Zetasizer Nanoseries ZSNano ZEN3600 from Malvern Instruments. DLS measurements were performed with samples dispersed in PBS solution at 25 °C. Hysteresis loops (M–H curves) were recorded in the range of −90 to 90 kOe, at temperatures of 5 and 300 K using the commercial physical property measurement system (PPMS) model 6000 platform with the vibrating sample magnetometer (VSM) module from Quantum Design. The zero-field-cooled (ZFC) and field-cooled (FC) magnetization curves (M–T curves) were recorded in the range of 5–400 K while warming up the samples previously cooled down under zero-field and 50 Oe condition, respectively. The specific absorption rate (SAR) and the intrinsic loss power (ILP) values were obtained by exposing the samples to an AMF in the DM1000 series system, from nanoScale Biomagnetics. Field strength settings ranging from 10.0–16.0 kA m⁻¹ were used in the experiments, while the AMF frequency was varied in the range of 420.0–203.0 kHz. In a typical experiment, the sample was dispersed in a cylindrical vial vessel with 1 ml of SPION in suspension (concentration of 13 mg ml⁻¹). The vessel was placed at the center of AMF coil and the temperature was measured using a fiber optic temperature probe immersed within the suspension at its middle. The temperature versus time slope was determined by Box-Lucas equation parameters.

3. Results and discussion

3.1. Nanoparticle synthesis and characterization

The preparation of folate-conjugated PEGylated SPION followed a three-step approach, comprising the synthesis of the bare SPION, PEGylation of them, and finally their conjugation with folic acid.

The TEM image (figure 1(a)) shows nanoparticles agglomerated nearly spherical in shape. The corresponding selected area diffraction pattern (figure 1(b)) reveals the crystalline iron oxide phase which shows the characteristic d-spacing indexed as (220), (311), (400), (511), and (440) in agreement with cubic ferrite structure. The particle size histogram obtained from the TEM micrographs was curve-fitted using a log-normal distribution function (figure 1(c)). The obtained average particle size ($D_{TEM}$) was 12 ± 3 nm, with a polydispersity index equals to 0.2.

The XRD pattern of the Mag-PEG sample (figure 2) reveals the cubic ferrite structure of the magnetite phase, in agreement with reported protocols used to produce SPION based on the co-precipitation method. The characteristic XRD peaks marked by their Bragg indices (220), (311), (400), (422), (511), (440) and (533) are in correspondence with Fe₃O₄ phase (JCPDF Card No 19–0629). The average x-ray crystallite diameter ($D_{XRD}$), calculated using the Scherrer’s equation [32] was 12 nm. The observed similarity between the $D_{XRD}$ and $D_{TEM}$ is more likely due to the crystallinity improvement of the iron oxide nanoparticles during the PEGylation step [23].
Figure 3 shows the FTIR spectra of the folic acid, Mag-PEG-FA, and Mag-PEG samples. The IR features shown in the Mag-PEG-FA FTIR spectrum (1700–780 cm$^{-1}$ range) confirm the conjugation with folic acid. The bands in the range 1650–1450 cm$^{-1}$ and 1250–780 cm$^{-1}$ were attributed to ring stretching and C-H deformation vibrations, respectively. Bands due to the carboxylate stretching modes were assigned to asymmetrical (1630 cm$^{-1}$) and symmetrical (1390 cm$^{-1}$) vibrations. The strong IR band around 1100 cm$^{-1}$ was assigned to the C–O stretching of the PEG moiety whilst the bands at 640, 550, and 440 cm$^{-1}$ were attributed to Fe–O vibrations [33, 34].

TGA analysis was performed to access information regarding the amount of polymer and folic acid present on the samples Mag-PEG and Mag-PEG-FA. The TGA curves (figure 4) show that mass losses occurred in the temperature range from 50 to 455 °C. The first step of mass loss occurring up to 75 °C and equals to 4.2% and 25.2% for Mag-PEG and Mag-PEG-FA, respectively, was associated with loss of adsorbed acetone and water. The second step, initiated at 200 °C and ended around 455 °C for both samples, was related to the decomposition of the organic coverage (citrate, PEG and folic acid) attached to the nanoparticle’s surface. The mass loss related to organic amount was 15.0% for Mag-PEG and 26.8% for Mag-PEG-FA, which reveals an increase of 11.8% in organic coating after reaction with folic acid, thus suggesting its conjugation onto the nanoparticle’s surface [33, 35].
The colloidal stability of Mag-PEG and Mag-PEG-FA samples was evaluated through DLS measurements. The nanoparticle dispersions in PBS solution at pH 7.4 presented Z-average hydrodynamic diameters (and polydispersity indexes) equal to 94.2 ± 0.2 nm (0.14) for Mag-PEG and 109.7 ± 0.9 nm (0.15) for Mag-PEG-FA. The close values of the hydrodynamic diameters and polydispersity indexes for both samples indicate that the conjugation with folic acid did not affect the colloidal stability of the PEGylated nanoparticles. Figure 5(a) shows the hydrodynamic diameter distribution curves obtained by DLS. For sample Mag-PEG it is observed a hydrodynamic size population centered around 100 nm, which agrees with the Z-average result. However, for the sample Mag-PEG-FA this population appears broadened and it is followed by a second, less intense, particle size population centered at 290 nm. The second population does not correspond to a significant percentage of sample, being not representative when the hydrodynamic diameter is shown by number distribution (figure 5(b)). This bimodal hydrodynamic size distribution contributes to the greater Z-average hydrodynamic diameter for Mag-PEG-FA. In addition, it is important to note from the hydrodynamic size distribution curves...
the absence of aggregates greater than 300 nm for both samples, which is indicative of good colloidal stability. Figure 6 shows the colloidal and magnetic responsiveness of Mag-PEG-FA dispersed in PBS solution. These SPION presented high dispersibility in this media generating a stable brown dispersion (figure 6(a)). When exposed to an external magnetic field the dispersion moves towards it due to the magnetic attraction of the SPION, without present any phase separation (figure 6(b)). These results demonstrate the Mag-PEG-FA SPION present properties that allow its use in protocols of MFH [36].

3.2. Magnetic characterization

Magnetic characteristics of the as-produced SPION were evaluated using magnetic hysteresis loops. The magnetization versus magnetic field curves (figure 7) shows that both samples, Mag-PEG and Mag-PEG-FA present similar magnetic saturation (\(M_s\)) values at 90 kOe, indicating that the folic acid surface-functionalization step did not cause a significant change in the SPION magnetism. The room-temperature \(M_s\) values obtained after normalizing the data with respect to the SPION core (using TGA data) were, 73.1 and 69.2 emu g^{-1} for the Mag-PEG-FA and Mag-PEG samples, respectively. At 5 K the normalized \(M_s\) values were 79.8 and 77.1 emu g^{-1}, respectively. The reduction of the \(M_s\) values of samples Mag-PEG and Mag-PEG-FA (normalized core) with respect to \(M_s\) value of bulk magnetite (92 emu g^{-1}) [37]is consistent with the literature and it is often attributed to the surface contribution of spin canting, surface disorder, stoichiometric deviation, cation distribution and adsorbed species, the latter supported by the TGA data [38, 39]. Moreover, the obtained results suggest that part of the saturation magnetization reduction (~4%) is restored by the folic acid surface-functionalization. The magnetic hysteresis loop data reveal a superparamagnetic behavior at 300 K (figure 7(a)). However, small remanence (\(M_R\)) and coercivity (\(H_C\)) were found at 5 K (figure 7(b)), indicating a thermally blocked state in both samples. The larger coercive field determined for the sample Mag-PEG-FA (\(H_C = 381\) Oe) with respect to sample Mag-PEG (\(H_C = 222\) Oe) seems to be more likely related to the stronger magnetic dipolar interaction among the SPIONs in sample Mag-PEG-FA [40]. On the other hand, ZFC/FC curves of both samples show features of blocked states at low temperature and relaxed states at room temperature are consistent with results determined from hysteresis loops. ZFC/FC curves of sample Mag-PEG show the irreversibility at around 275 K; meanwhile, sample Mag-PEG-FA (figure 7(c)) show the characteristic splitting at around 300 K, indicating that the dipolar particle-particle interactions within agglomerates are stronger in sample Mag-PEG-FA, in agreement with the larger coercive field determined for the latter sample. That scenario can be enhanced by likely the contribution of a small fraction of larger SPION, as observed in TEM micrographs [41].

The heating effect of SPION, when exposed to an AMF, is originated by the conversion of magnetic energy into thermal energy due to delay in relaxation of the magnetic moment [42–44]. The heat generation from hysteresis losses is negligible due to the superparamagnetic behavior of both samples at ambient temperature. Thus, the loss process is governed by the sum of rotational motion (Brownian relaxations) and the internal diffusion magnetic moment (Néel relaxations) [15]. The power dissipation is quantified by measuring the SAR, according to equation (1).
where $c_{\text{liq}}$ and $m_{\text{liq}}$ is the heat capacity and mass of solvent, respectively; $m_{\text{SPION}}$ is the iron oxide content, per gram, in suspension and $\Delta T / \Delta t$ is the initial slope of the time-dependent curve. The heat capacity of SPION is negligible due to the low concentration in fluid, thus the water’s heat capacity ($4.18 \text{ J K}^{-1}$) was considered [45].

$$\text{SAR} = \frac{(c_{\text{liq}}m_{\text{liq}}) \Delta T}{m_{\text{MNP}} \Delta t}$$

Figure 7. Magnetization versus field curves of Mag-PEG and Mag-PEG-FA samples recorded at 300 K (a) and 5 K (b); and (c) FC and ZFC curves of Mag-PEG and Mag-PEG-FA.
The heating efficiency was evaluated for uncoated (bare-Mag) and coated (Mag-PEG-FA) samples under different magnetic field strengths and frequencies. Table 1 summarized the SAR value obtained and figure 8(a) shows the temperature versus time curve for samples with the highest SAR value, 32.8 W g$^{-1}$ and 21.6 W g$^{-1}$ for bare-Mag and Mag-PEG-FA, respectively. The bare-Mag sample reaches the temperature of 42 °C in 80 s, whereas Mag-PEG-FA achieves the same temperature after 222 s after the AMF be turned on. This difference could be attributed to the organic layer comprised of citric acid, PEG and folic acid species that behave as an insulating shell around the SPION’s surface [16, 36]. The SAR parameter is changed if different magnetic strengths and frequencies are carried out to evaluate the heating of the same sample. The heat dissipation shows a linear relationship with the square of magnetic field conduct at f = 420 kHz for Bare-Mag and Mag-PEG-FA samples.

Table 1. SAR and ILP values for magnetic heating at fixed field strength and frequencies.

| Sample      | f (kHz) | $H^b$ (kAm$^{-1}$) | SAR (W g$^{-1}$) | ILP (nHm$^2$ Kg$^{-1}$) | $R^2$ |
|-------------|---------|--------------------|-----------------|-------------------------|-------|
| Bare-Mag    | 203     | 16                 | 14.9 ± 0.5      | 0.39                    | 0.993 |
|             | 280     | 15                 | 22.5 ± 2.4      |                         |       |
| Mag-PEG-FA  | 420     | 14                 | 32.8 ± 7.1      | 0.24                    | 0.999 |
|             | 420     | 10                 | 10.3 ± 3.3      |                         |       |
|             | 203     | 16                 | 13.8 ± 0.7      |                         |       |
|             | 280     | 15                 | 11.7 ± 2.0      |                         |       |

Figure 8. (a) Temperature-time profile for bare-Mag and Mag-PEG-FA samples under AMF 14 kAm$^{-1}$ and 420 kHz; (b) Linear fit of SAR data to square of magnetic field conduct at f = 420 kHz for Bare-Mag and Mag-PEG-FA samples.
during the measurement a new parameter is obtained, in which SAR value is normalizing by frequency and square field amplitude, resulting in ILP parameter [4, 46], according to equation 2.

\[
ILP = \frac{SAR}{fH^2}
\]  

(2)

The linearity of SAR in the function of the square magnetic field was confirmed, as showed in figure 8(b). The correlation coefficient obtained for the Bare-Mag curve fit was 0.98 and for Mag-PEG-FA was 0.99. The ILP values obtained by the slope of SAR versus \(H^2\) curve were 0.34 nHm²-Kg⁻¹ and 0.24 nHm²-Kg⁻¹ for Bare-Mag and Mag-PEG-FA, respectively. This material is an interesting candidate for MFH treatment once achieve in a few minutes the temperature whose cancer cells are more sensitive.

4. Conclusions

The Folate-conjugated PEGylated SPION synthesized in this work proved to be interesting candidates for application in MFH protocols. The SPION dispersion was colloidal stable in PBS and caused a temperature increase in the range of sensitization of cancer cells in a short time interval and at low mass concentration. The presence of PEG and folate species were confirmed by FTIR and TGA analyses, evidencing the efficiency of the synthetic route adopts. The PEG layer can increase the systemic circulation and limits the aggregation minimizing the interaction of the particles avoiding a reduction on SAR values, while the folate moiety is aimed to increase the targeting of SPION for cancer cells.

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