Transmittance Measurements in Non-alternating Magnetic Field as Reliable Method for Determining of Heating Properties of Phosphate and Phosphonate Coated Fe₃O₄ Magnetic Nanoparticles

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Received: 23 April 2021 / Accepted: 14 June 2021 / Published online: 17 June 2021
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Abstract
Different phosphates and phosphonates have shown excellent coating ability toward magnetic nanoparticles, improving their stability and biocompatibility which enables their biomedical application. The magnetic hyperthermia efficiency of phosphates (IDP and IHP) and phosphonates (MDP and HEDP) coated Fe₃O₄ magnetic nanoparticles (MNPs) were evaluated in an alternating magnetic field. For a deeper understanding of hyperthermia, the behavior of investigated MNPs in the non-alternating magnetic field was monitored by measuring the transparency of the sample. To investigate their theranostic potential coated Fe₃O₄-MNPs were radiolabeled with radionuclide ¹⁷⁷Lu. Phosphate coated MNPs were radiolabeled in high radiolabeling yield (> 99%) while phosphonate coated MNPs reached maximum radiolabeling yield of 78%. Regardless lower radiolabeling yield both radiolabeled phosphate MNPs may be further purified reaching radiochemical purity of more than 95%. In vitro stable radiolabeled nanoparticles in saline and HSA were obtained. The high heating ability of phosphates and phosphonates coated MNPs as sine qua non for efficient in vivo hyperthermia treatment and satisfactory radiolabeling yield justifies their further research in order to develop new theranostic agents.

Keywords Magnetic nanoparticles · Hyperthermia · Laser transmittance · Non-alternating magnetic field · ¹⁷⁷Lu labeling

1 Introduction
In the past few decades magnetic nanoparticles (MNPs) have attracted far-reaching attention due to the expeditiously growing possibilities for their applications such as nanomaterial-based catalysis, biomedicine and tissue specific targeting, magnetic resonance imaging, magnetic particle imaging, data storage, environmental remediation, etc. [1, 2]. Due to their extraordinary physico-chemical qualities, MNPs are especially used in the biomedicine. The MNPs may be similar to cell, virus, protein or gene size, but also they can easily enter a variety of cell structures. The heating of MNPs by changing the orientation of their magnetic domains in an alternating external magnetic field is substantial for various medical applications such as hyperthermia and triggered drug delivery to the targeted region of the body by induced self-heating [3, 4]. However, surface functionalization of MNPs is pivotal for the fruitful application in medicine. Coating of MNPs increases the colloidal stability, prevents aggregation and agglomeration of the particles and provides nontoxicity in physiological conditions. It is noteworthy that phosphates and phosphonates are water soluble and biocompatible and allow convenient stability and reduced cytotoxicity of MNPs for medical application [5]. Furthermore, they have a large binding affinity to the bone tissue which allows application in various medical conditions such as osteoporosis and bone tumors [6, 7]. In addition, phosphates and phosphonates are highly efficient chelators. Bisphosphonate-based coordination complexes with ⁹⁹mTc have been widely used for bone scintigraphy, due to their high sensitivity, specificity, and accuracy for

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detecting skeletal metastatic diseases [8]. The binding of phosphates and phosphonates to the surface of MNPs and labeling with different radionuclides make them appropriate for combined radionuclide-hyperthermia therapy or SPECT/PET-MRI diagnostics [5, 9]. 177Lu has become favored in recent years in the therapy of neuroendocrine and prostate tumors, due to very suitable physicochemical characteristics ($T_{1/2} = 6.7$ days, $E_{\beta_{\max}}$ of 497 keV (78.6%)) [10]. An additional advantage of using 177Lu is γ-ray emission ($E_\gamma$ of 208 keV (11%) and 113 keV (6.6%)) during its decay which allows the monitoring of therapy. There are a small number of studies that refer to the potential use of 177Lu labeled MNPs in cancer radionuclide therapy [11].

In the present study heating capacity of Fe₃O₄ based MNPs coated with inositol hexaphosphate (IHP), imidodiphosphate (IDP), hydroxyethylidene diphosphonic acid (HEDP), and methanediylbis(phosphonic acid) (MDP) has been investigated for possible hyperthermia treatment. In order to analyze and explain the heating capacity of synthesized samples minutely, we monitored the behavior of MNPs in a non-alternating magnetic field by measuring laser transparency and correlated obtained data with hyperthermia measurements. Further, phosphate and phosphonate functionalized MNPs were labeled with 177Lu with the aim to investigate their potential for use in diagnosis or hyperthermia cancer therapy.

2 Experimental

Methylene diphosphonate (medronic acid, MDP), 1-hydroxyethane-1,1-diphosphonate (etidronic acid, HEDP), imidodiphosphate tetrasodium salt (IDP), inositol hexaphosphoric acid sodium salt (IHP), iron (III) chloride hexahydrate (FeCl₃ˑ6H₂O), iron (II) sulfate heptahydrate (FeSO₄ˑ7H₂O) and aqueous ammonia solution (25% w/w) were purchased from Sigma–Aldrich and were analytical grade reagents used without further purification. The human serum was obtained from the National Blood Transfusion Institute (Belgrade, Serbia). Water used in all experiments was purified using the Milli–Q system (Millipore Co., Billerica, MA, USA). Lutetium-177 was obtained from Polatom, Poland (in the form of 177LuCl₃, specific activity > 500 GBq/mg).

2.1 MNPs Synthesis

Synthesis and characterization of Fe₃O₄-MDP, Fe₃O₄-HEDP, Fe₃O₄-IDP, and Fe₃O₄-IHP MNPs were previously reported [12, 13]. Briefly, after synthesis of Fe₃O₄ MNPs by coprecipitation method, a water solution of phosphate or phosphonate ligand (MDP, HEDP, IDP, IHP) at the ratio Fe₃O₄:coating ligand = 1:1 was added, and the functionalization reaction was carried out overnight at room temperature. The excess of unreacted coating ligand was eliminated by dialysis against deionized water for one day.

2.2 Measurements in an Alternating and Non-alternating Magnetic Field

The magnetic hyperthermia efficiency of Fe₃O₄-MPD, Fe₃O₄-HEDP, Fe₃O₄-IDP, and Fe₃O₄-IHP MNPs was analyzed by using Commercial AC applicator (model DM100, nB nanoscale Biomagnetics). The heat generation under alternating magnetic field (30 mT) and the resonant frequency of 397 kHz was measured directly on the samples dispersed in water. The heating ability of MNPs (2 mg/ml), defined as specific power absorption (SPA), calculated according to the following formula: $SPA = \frac{(C_p \cdot m_w / m_m) \cdot (\Delta T/\Delta t)}$, where $C_p$ is the specific heat capacity of the medium ($C_p \approx C_{water} = 4.18 \text{ Jg}^{-1} \text{ K}^{-1}$), $m_w$ and $m_m$ are the masses of the medium (water) and the magnetic nanoparticles, and $\Delta T/\Delta t$ is the initial slope of the time dependent temperature curve [14].

For the analysis of the sample in non-alternating external MF, device shaped in our laboratory was used [15]. Sanyo laser diode DL5147-040 in the single mode regime at wavelength $\lambda = 655$ nm was applied. Transmitted laser light was measured with a photodiode.

2.3 Labeling of Coated MNPs with 177Lu

177Lu-labeling of phosphates and phosphonates coated Fe₃O₄ MNPs was obtained using the method previously described [11, 16]. Briefly, 177LuCl₃ solution (approximately 185 MBq in 5 µl) was added to an aqueous suspension of coated Fe₃O₄ MNPs (5 mg/ml at pH 4.5) and incubated at room temperature with continual stirring for 1 h. To quantify the radiolabeling yield of 177Lu-labeled MNPs and their radiochemical purity after purification by magnetic decantation, ITLC was performed on SG sheets with 0.1 M acetate buffer as the mobile phase. In this system, 177Lu-labeled MNPs remained at the origin ($R_f = 0.0–0.1$), while the unbound 177Lu³⁺ migrated with the solvent front ($R_f = 0.8–0.9$).

2.4 In Vitro Stability of 177Lu-MNPs

The in vitro stability of purified 177Lu-labeled coated MNPs was determined in saline or human serum solution (total volume of 2 ml) by measuring the free, unbound 177Lu in relation to the 177Lu-labeled coated MNPs (broad 177Lu) during incubation at 37 °C for 96 h. Small amounts of sample (50 µl) were taken at different time points (1, 24, 48, and 96 h) and analyzed by ITLC (SG plates) using 0.1 M acetate buffer as the mobile phase.
3 Results and Discussion

3.1 Measurements in an Alternating and Non-alternating Magnetic Field

Characterization for Fe₃O₄-MDP, Fe₃O₄-HEDP, Fe₃O₄-IDP, and Fe₃O₄-IHP MNPs including X-ray powder diffraction, Fourier transform infrared spectroscopy and transmission electron microscopy are presented in Supplementary Material (Figs. S1–S4).

In order to apply magnetic hyperthermia, it is necessary to take into account physiological limitations. Due to the eddy currents, MFs of high frequencies can cause local heating also in the sections of the tissue where no magnetic particles have been found. Along with clinical restrictions, technical issues should be also taken into account, since most studies on biological samples encompass a tight frequency range. The applied frequencies, together with the amplitude of the alternating field, are mainly based on literature data [17, 18]. The temperature increase of Fe₃O₄-MDP, Fe₃O₄-HEDP, Fe₃O₄-IDP, and Fe₃O₄-IHP MNPs (5 mg/ml) as a function of the time was evaluated under a frequency of 397 kHz and magnetic field strength of 30 mT (Fig. 1).

Although all specimens under investigation have shown substantial heating capacity, it is obvious that Fe₃O₄-HEDP MNPs achieved the highest temperature values. In order to explain obtained trends, we employed the analysis of MNPs behavior in a non-alternating magnetic field, by measuring laser transmittance [19–23]. Previously this method gave satisfactory valuable data [15, 24]. The analysis was carried out at 30 mT and 400 mT and the results are depicted in Fig. 2. At the beginning of the measuring, the MF is switched off, and the specimens showed initial transparency. By employing the MF, the transparency of the samples abruptly decreases. The magnitude of this decrease depends on the specimen’s type and field strength. Regardless of the field used Fe₃O₄-MDP and Fe₃O₄-HEDP MNPs showed a lesser decline of transparency in comparison to Fe₃O₄-IHP and Fe₃O₄-IDP MNPs. After some time (app 60 s) sudden increase of relative transmittance has been observed, due to the zippering of magnetic chains. It is noteworthy that external MF arranges MNPs along the field lines, like miniature magnetic needles [15, 20, 23]. Such ordering of magnetic domains causes strong mutual attraction and subsequent formation of magnetic chains. In addition, the
magnetic chains are arranged in space thereby building a quasi-lattice made of parallel lined magnetic threads. The source of laser is positioned in such a manner that light propagates through the quasi-lattice parallel with lines of non-alternating MF. Contrary to the case when nanoparticles are chaotically distributed, magnetic chains encounter a much lesser number of scattering centers. In other words, the quasi-lattice possesses far smaller cross section for scattering compared to randomly distributed particles [15, 20, 23]. As a consequence, the intensity of the transmitted light rises. The slope of the increase is very steep when the field of 400 mT was employed, whereas for the lower fields the increase is gradual. Previous study has shown that depth and width of the well depend on MF strength and kind of ferrofluid sample [15, 25]. Comparing the results from Figs. 1 and 2 it is obvious that the heating capacity of the sample (Fig. 1) stands in correlation with depth and width of the well (Fig. 2). The specimens displaying the highest heating capacity in alternating MF, Fe₃O₄-MPD, and Fe₃O₄-HEDP MNPs, also show the shallower and narrower wells in non-alternating MF. Fe₃O₄-MPD and Fe₃O₄-HEDP MNPs reach again the initial transparency in the shorter time interval in comparison to Fe₃O₄-IHP and Fe₃O₄-IDP MNPs. By employing the field of 30 mT Fe₃O₄-IHP and Fe₃O₄-IDP MNPs have not reached the initial transparency even after 300 s. Furthermore at the point of saturation Fe₃O₄-MPD and Fe₃O₄-HEDP MNPs showed significantly higher values of transparency.

By comparing the amount of precipitate formed in the time regime when the field was operative (Fig. 3, samples 1b, 2b, 3b, 4b) and also at the saturation point (Fig. 3, samples 1c, 2c, 3c, 4c) it can be observed that the highest precipitation occurs in the case of Fe₃O₄-MDP and Fe₃O₄-HEDP MNPs. Fe₃O₄-IDP and Fe₃O₄-IHP MNPs samples have shown less tendency to precipitate, which has been noted by the amount of precipitate at saturation point.

In addition to the practiced MF strength and frequency, other factors such as particle size and shape along with the concentration of the sample significantly influence the behavior of MNPs in non-alternating and alternating MF [26]. Generally, more concentrated dispersions of MNPs broaden the application, therefore various electric and magnetic fields can be used. By diminishing the concentration of the sample, SPA values decline, and below 2 mg/ml heating effect could not be detected (Fig. 4).

The behavior of Fe₃O₄-HEDP MNPs in non-alternating MF (400 mT) for different concentrations is depicted in Fig. 5.

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**Fig. 3** Fe₃O₄-MPD MNPs (1a, 1b, 1c), Fe₃O₄-HEDP MNPs (2a, 2b, 2c), Fe₃O₄-IDP MNPs (3a, 3b, 3c), and Fe₃O₄-IHP MNPs (4a, 4b, 4c) precipitate formed in non-alternating magnetic field.
The field of 400 mT has been used in order to compare the well depth at different concentrations of Fe₃O₄-HEDP MNPs, since at 30 mT the wells are not distinct, and effect is very poor. At the highest tested concentration (8 mg/ml) the initial transparency, when the MF is switched off, is the lowest. By decreasing the concentration the initial transparency of the sample rises. The depth of the well increases with the dilution until the concentration of 1 mg/ml. This concentration for this particular sample is turning point, from which the decrease of the well depth starts (the effect is getting weaker). At the lowest concentrations (0.1 and 0.2 mg/ml), the well could not be observed and the increase of transparency is very small reaching only the initial value (when the MF was switched-off). Observations are in correlation with hyperthermia studies, i.e. the SPA values decrease with a dilution of the sample till the concentration

Fig. 4  SPA values of Fe₃O₄-HEDP MNPs at different concentrations (397 kHz, 30 mT)

![Graph showing SPA values of Fe₃O₄-HEDP MNPs at different concentrations.](image)

Fig. 5  Time dependence of transmitted diode laser light (λ = 655 nm) for different concentrations of the Fe₃O₄-HEDP MNPs at magnetic field strength of 400 mT

![Graph showing time dependence of transmitted diode laser light for different concentrations.](image)
of 1 mg/ml, when the loss of heating capacity has been observed (Fig. 4.). Since the shape of the well depends on the magnetic properties and the applied concentration of the sample, by finding a turning point with a sharp decrease in the depth of the well, it is possible to detect the concentration at which there is a loss of heating capacity.

3.2 Radiolabeling and In Vitro Stability of $^{177}$Lu-MNPs

The aim was to optimize the $^{177}$Lu radiolabeling of coated MNPs toward their further in vivo applications. $^{177}$Lu as trivalent metal usually easily make complexes at room temperature with MNPs that possess available phosphates and phosphonates functional groups on the surface. The radiolabeling yield of $^{177}$Lu-MNPs as well as their radiochemical purity after purification by magnetic decantation was determined using ITLC-SG chromatography with 0.1 M acetate buffer as the mobile phase (Table 1). Radiolabeling yield after 30 min of reaction time was over 99% for $^{177}$Lu-IDP-MNPs and $^{177}$Lu-IHP-MNPs while the radiolabelling yield of $^{177}$Lu-MDP-MNPs and $^{177}$Lu-HEDP-MNPs was 78.7 ± 0.7% and 75.0 ± 1.1% respectively. Both radiolabeled phosphate-coated MNPs can be further purified from free $^{177}$Lu using magnetic purification reaching radiochemical purity of over 95%. The in vitro stability of $^{177}$Lu-MNPs was tested by examining their radiochemical purity at different time intervals after storage of the samples at 37 °C in saline and human serum up to 96 h. In vitro stability of $^{177}$Lu-MNPs is shown in Fig. 6. $^{177}$Lu-IDP-MNPs and $^{177}$Lu-IHP-MNPs were quite stable, showing the minimal release of $^{177}$Lu after 96 h, confirming the strong metal binding to Fe$_3$O$_4$-IDP and Fe$_3$O$_4$-IHP MNPs. $^{177}$Lu-MDP-MNPs and $^{177}$Lu-HEDP-MNPs show about 10% of released $^{177}$Lu after 96 h indicating their less desirable in vivo behavior in relation to phosphate-coated MNPs.

4 Conclusions

The results given, point out that the MDP, HEDP, IDP, and IHP-coated MNPs could achieve significant heating capacity at 397 kHz and 30 mT frequencies and thus show potential for biomedical applications. Based on the interaction of Fe$_3$O$_4$-MDP, Fe$_3$O$_4$-HEDP, Fe$_3$O$_4$-IDP, and Fe$_3$O$_4$-IHP MNPs with non-alternating magnetic field and by continual measuring of laser transmittance through those samples, a more comprehensive understanding of hyperthermia can be achieved. The obtained curves of laser transmittance showing that Fe$_3$O$_4$-MDP and Fe$_3$O$_4$-HEDP MNPs have higher magnetic activity than Fe$_3$O$_4$-IDP and Fe$_3$O$_4$-IHP MNPs, which is in accordance with hyperthermia measurements.

| $^{177}$Lu-MNPs          | Radiolabeling yield (%) | Radiochemical purity after purification using magnetic decantation (%) |
|-------------------------|-------------------------|---------------------------------------------------------------------|
| $^{177}$Lu-MDP-MNPs     | 78.7 ± 0.7              | 95.4 ± 0.5                                                          |
| $^{177}$Lu-HEDP-MNPs    | 75.0 ± 1.1              | 95.9 ± 0.6                                                          |
| $^{177}$Lu-IDP-MNPs     | 99.1 ± 1.3              | 99.7 ± 1.2                                                          |
| $^{177}$Lu-IHP-MNPs     | 99.0 ± 0.9              | 99.9 ± 0.7                                                          |

Fig. 6  In vitro stability of $^{177}$Lu-MDP-MNPs, $^{177}$Lu-HEDP-MNPs, $^{177}$Lu-IDP-MNPs, and $^{177}$Lu-IHP-MNPs in (A) saline and (B) human serum after incubation at 37 °C over 96 h
The dilution of the sample leads to a lowering of heating capacity, and below 2 mg/cm³ heating effect could not be detected. Additionally, substantial changes in the laser transmittance curve profile have been recorded below the concentration of 2 mg/cm³. Based on the present study continual measurement of laser transmittance through the MNPs samples in the non-alternating magnetic field has shown as a good method for assessment of MNPs heating capability. Furthermore, phosphate and phosphonate coated MNPs are chosen to be labeled with ¹⁷⁷Lu. Future studies should investigate the potential of ¹⁷⁷Lu-MNPs as theranostic agents, for diagnostic imaging and therapeutic hyperthermia as well.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1007/s10904-021-02059-1.

Funding
The research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (contract numbers 451-03-9/2021-14/200017, 451-03-9/2021-14/200168, 451-03-68/2020-14) and through funding the VINCENT Center of Excellence.

Availability of Data and Material
The data that support the findings of this study are available on request from the corresponding author.

Declarations
Conflict of interest
No potential conflict of interest was reported by the authors.

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