



Article

Synthesis, Characterization, and Magnetic Properties of Fe(BIP)₃, a Novel Paramagnetic Relaxation Agent

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Abstract

Background/Objectives: First row transition metal ions have recently regained attention in coordination chemistry as alternatives to gadolinium-based paramagnetic contrast agents, motivated by emerging safety concerns associated with certain Gd³⁺-based contrast agents. In this study, we report the development of a novel homoleptic diketonate Fe³⁺ complex functionalized with biocompatible indole moieties. We investigate its potential as a paramagnetic relaxation agent by evaluating its ability to modulate the T₁ and T₂ relaxation times of water proton. **Methods:** Iron(III) tris-1,3-(1-methylindol-3-yl)propanedionate [Fe(BIP)₃] was synthesized via a thermal method from bis(1-methylindol-3-yl)-1,3-propanedione (HBIP) using Fe(ClO₄)₃·6 H₂O as the metal source. The complex was characterized by UV-Vis, IR and NMR spectroscopy, differential scanning calorimetry–thermogravimetric analysis, and single-crystal X-ray diffraction. Fe(BIP)₃ aggregation behavior in aqueous environment, including size and morphology of aggregates, was investigated using dynamic light scattering and scanning electron microscopy. Incorporation of the aggregates into phospholipid vesicles was evaluated by fluorescence resonance energy transfer and fluorescence correlation spectroscopy. The paramagnetic properties of monomeric Fe(BIP)₃ were probed in solution by nuclear magnetic resonance recurring to the Evans bulk magnetization method. **Results:** The designed synthetic procedure successfully afforded Fe(BIP)₃, which was fully characterized by UV-Vis and IR spectroscopy, as well as single-crystal X-ray diffraction. Aqueous solutions of Fe(BIP)₃ spontaneously formed rice-grain-shaped nanoscale aggregates of hydrodynamic radius ≈ 30 nm. Incorporation of these aggregates into phospholipid vesicles enhanced their stability. The longitudinal r₁ and transverse r₂ relaxivities of Fe(BIP)₃ aggregates were assessed to be 1.92 and 52.3 mM^{−1}s^{−1}, respectively, revealing their potential as paramagnetic relaxation agents. **Conclusions:** Fe(BIP)₃ aggregates, stabilized through incorporation into phospholipid vesicles, demonstrate promising potential as novel paramagnetic relaxation agents in aqueous environments.



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Keywords: iron coordination complexes; paramagnetic relaxation agent; relaxivity; aggregation; dynamic light scattering; fluorescence spectroscopy; nuclear magnetic resonance

1. Introduction

Iron is a key element in life: from hemoglobin in red blood cells to the ferredoxin and cytochrome protein families, it is a ubiquitous element in all living beings [1–3]. The human body has a high tolerance to it, up to 20 mg/Kg [4], and it is able to store iron as ferric hydroxides in ferritin, a protein found especially in the liver [5]. Iron insufficiency can cause severe health effects, from leaves yellowing in plants [6] to anemia in mammals [7]. Conversely, its overload can pose significant danger as well [8].

From a chemistry point of view, iron ions are regarded as highly oxophilic Lewis acids; thus, they tend to form strong bonds with oxygen and oxygen-based compounds like hydroxyls, carboxylates, catechols, and carbonyls. The most common oxidation states are Fe^{2+} and Fe^{3+} , the latter displaying high stability in an oxidating environment. With 5 electrons in its 3d orbitals, Fe^{3+} is paramagnetic in both its low spin state ($S = 1/2$) and its high spin state ($S = 5/2$). Paramagnetic ions shorten the observed longitudinal ($T_{1,\text{obs}}$) and/or transverse ($T_{2,\text{obs}}$) relaxation times of any given NMR-active nuclei in their vicinity: these compounds are referred to as paramagnetic relaxation agents (PRAs) and may be employed as contrast agents in magnetic resonance imaging (MRI). The reciprocal value of T_i ($i = 1, 2$) is expressed as relaxivity r_i ($i = 1, 2$).

The best relaxivity performances are nowadays obtained with Gd^{3+} -based contrast agents (GBCAs) due to the fact that this rare-earth element ion has seven unpaired electrons ($S = 7/2$): most MRI contrast agents contain a Gd^{3+} core, like gadopentetic acid $[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ ($^{\text{®}}$ Magnevist) and gadodiamide Gd-DTPA-BMA ($^{\text{®}}$ Omniscan) [9]. However, Gd toxicity to kidneys (nephrogenic systemic fibrosis) and accumulation in the human brain and other organs have been demonstrated [10–12], raising concerns in the medical community and prompting certain regulatory bodies to partially restrict Gd^{3+} use [13,14]. Another point to consider is that the commerce of rare-earth elements can be influenced by opposing parties competing for resources and governments promoting executing stricter environmental regulations, thus resulting in unpremeditated shifts and imbalances in the face of ever-growing demand. In fact, over recent years, tariffs and royalties on rare element exports have become a crucial point of discussion and factor of political leverage on an international level [15,16].

All these emerging issues, both medical and political–economic, have induced researchers to look for alternatives [17] and Fe^{3+} -based contrast agents are enjoying renewed attention [18–20], after an initial interest in the late 1980s and early 1990s [21–23] and subsequent abandonment in favor of Mn^{2+} derivatives [24]. Fe^{3+} -containing supramolecular structures, spanning from nanoparticulate formulations like superparamagnetic iron oxide nanoparticles (SPION and USPIO) [25–27] to repurposed iron chelator compounds such as Deferasirox and the like [28] and complexes that feature simple aminopolycarboxylic acids (APCAs) as ligands [29,30], can become valid contrast agents as an alternative to GBCAs, without potentially suffering from the usual disadvantages encountered with Gd [26,27,31–37]. Namely, since Fe^{3+} is inexpensive and abundant, it can be manufactured on a large scale economically. Furthermore, it can be administered in larger quantities without severe side-effects, because Fe^{3+} is an endogenous ion. Finally, as a matter of fact, the same formulations display potential in other medical applications, most notably including the magnetothermal treatment of cancers [38,39].

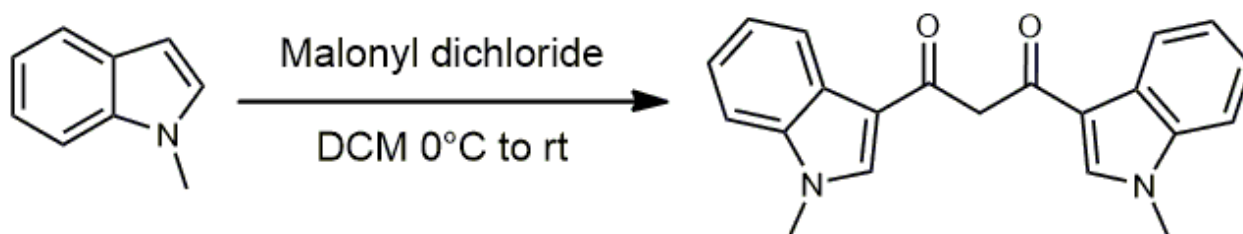
The engineering of new ligands capable of caging Fe^{3+} ions within stable and biocompatible coordination compounds has a pivotal role in the development of new contrast agents based on Fe^{3+} paramagnetic response. As previously mentioned, Fe^{3+} forms highly stable coordination compounds with oxygen-based ligands like carboxylates [40,41], hydroxamates [42], catecholates [43,44], and 1,3-diketones [45–47]. Iron(III) β -diketonates have already demonstrated the ability to modify the relaxivity of the NMR-active nucleus ^{19}F : notable examples are the works of Kislukhin et al. [48], Jahromi et al. [49], and Wang et al. [50].

Our group has long-dating expertise in the synthesis and characterization of 1,3-diketones and their complexes [51,52]. In the present work, we report on the synthesis and characterization of a novel paramagnetic relaxation agent based on an Fe^{3+} core and exploiting as a ligand bis(1-methylindol-3-yl)-1,3-propanedione (HBIP), which is able to coordinate through its 1,3-diketone oxygen atoms as enolate BIP^- and bears two highly biocompatible indole moieties, $\text{Fe}(\text{BIP})_3$. We further demonstrate the ability of this compound to spontaneously aggregate into morphologically homogeneous rice-grain-shaped, nanometric-sized particles when percolated in an aqueous environment. The latter can be stabilized by incorporation within phospholipid vesicles. Both bare and phospholipid vesicle-incorporated $\text{Fe}(\text{BIP})_3$ aggregates effectively quench water protons T_1 and T_2 in a 9:1 water/DMSO mixture.

2. Results and Discussion

2.1. Synthesis

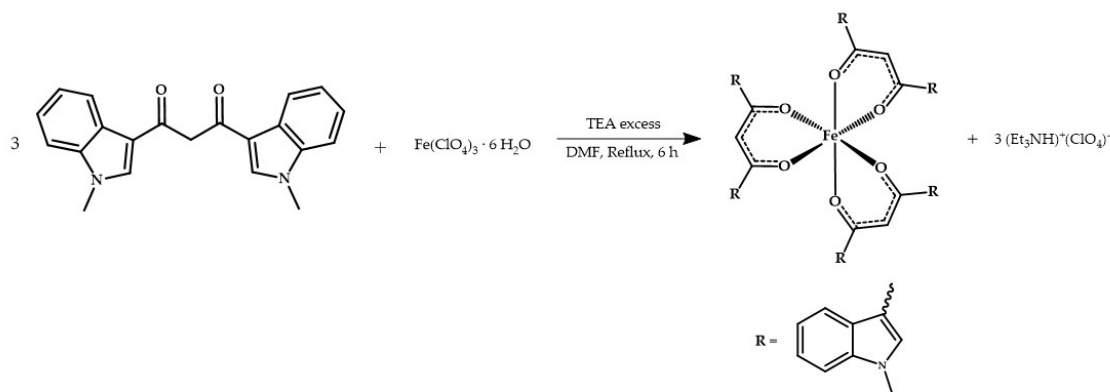
The synthesis of the ligand bis(1-methylindol-3-yl)-1,3-propanedione HBIP followed a classic Friedel–Crafts acylation of commercially available 1-methylindole with malonyl dichloride (Scheme 1). Details of this procedure are reported elsewhere [53–56] and in the Supplementary Materials. The obtained yellow solid was then further purified by Soxhlet extraction using methanol to isolate the clean product as an off-white powder.



Scheme 1. Synthesis of ligand HBIP.

HBIP was then used for the synthesis of the Fe^{3+} coordination compound by the thermal method, using $\text{Fe}(\text{ClO}_4)_3 \cdot 6 \text{H}_2\text{O}$ as the metal source, *N,N*-dimethylformamide (DMF) as the solvent, and triethylamine (TEA) as the base (Scheme 2). The perchlorate anion was chosen due to its very low coordination ability, to potentially avoid unwanted incorporation of species other than the metal ion and the diketonate ligand in the complex. DMF was preferred over other polar aprotic solvents due to its poor coordinating ability, following the rationale employed for the metal anion choice. The complex $\text{Fe}(\text{BIP})_3$ precipitated from the reaction mixture and was isolated by filtration, purely, since the only co-product, triethylammonium perchlorate, was soluble in DMF. Following a drying process at 100 °C under vacuum for a period of 3 h, the coordination compound was isolated, yielding a total of 43%. The product appeared as a dark powder with a violet hue and was poorly soluble in most organic solvents (toluene, solubility < 100 μM ; acetone, acetonitrile, methanol, ethanol, visible deposits already at 1 mg/mL) and water (turbidity is apparent even at

micromolar concentrations, *vide infra*). Detailed synthesis and purification procedures are reported in the Supplementary Materials.



Scheme 2. Synthesis of $\text{Fe}(\text{BIP})_3$ by the thermal method. The chemical structure of the final compound is not shown for clarity reasons.

2.2. Characterization of $\text{Fe}(\text{BIP})_3$

The synthesized compound was subjected to comprehensive characterization, including NMR, IR and UV-Vis spectroscopy, as well as single-crystal X-Ray diffraction and DSC-TGA analysis.

FTIR-ATR spectroscopic analysis of solid-state $\text{Fe}(\text{BIP})_3$ provided preliminary structural insights (Figure S4 in the Supplementary Materials). Weak bands at 3050 and 2912 cm^{-1} were assigned to aromatic and aliphatic C-H stretching modes, respectively; a very strong band at 1531 cm^{-1} , attributed to the out-of-phase C=O carbonyl stretching, showed a strong red shift of 87 cm^{-1} with respect to the corresponding C=O stretching of HBIP ligand, at 1618 cm^{-1} . This red shift is consistent with coordination of the ligand to the Fe^{3+} center through its enolate form [57]. By comparing the C=O stretching in $\text{Fe}(\text{acac})_3$ ($\tilde{\nu}_{\text{C=O}} = 1570\text{ cm}^{-1}$) with that in $\text{Fe}(\text{BIP})_3$ ($\tilde{\nu}_{\text{C=O}} = 1531\text{ cm}^{-1}$), a red shift of 40 cm^{-1} can be noticed, ascribable to a heteroaromatic resonance effect, very similar to the case reported for Cu^{2+} complexes of phenylacetylacetonates [58]. The intense shoulder peak located at 1521 cm^{-1} can be assigned to the asymmetric $\text{C}=\text{C}_\alpha=\text{C}$ stretching of the 1,3-diketone carbons of the ligands. Moreover, the other intense, strong band centered at 1498 cm^{-1} , absent in the FTIR-ATR spectrum of the ligand, can be attributed to the 19a vibrational mode (Wilson notation) of aromatic and heteroaromatic rings [59] (see Figure 1).

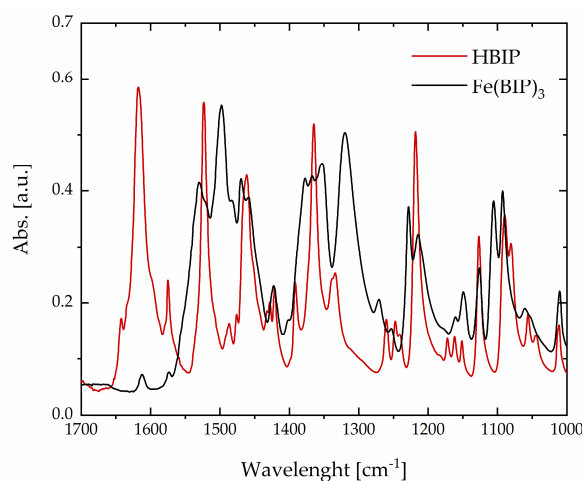


Figure 1. Superposition of IR spectra of $\text{Fe}(\text{BIP})_3$ (black line) and HBip (red line) between 1700 and 1000 cm^{-1} .

The electronic properties of $\text{Fe}(\text{BIP})_3$ were investigated by UV-Vis spectroscopy in DMSO (Figure 2). UV-Vis characterization of the HBIP ligand was previously reported by us [52] and will not be further discussed here. The spectrum of $\text{Fe}(\text{BIP})_3$ shows four main absorption peaks in the 260–600 nm range, with the absorbance maximum positioned at 389 nm. Our attribution, reported in Table 1 (peak index in Figure 2), is based on the work by Lintvedt and Kernitsky, who studied the properties of various iron(III)-1,3-diketonate compounds with different substituents on the ligand backbone and assembled a proper spectrochemical series for these metal chelates [60].

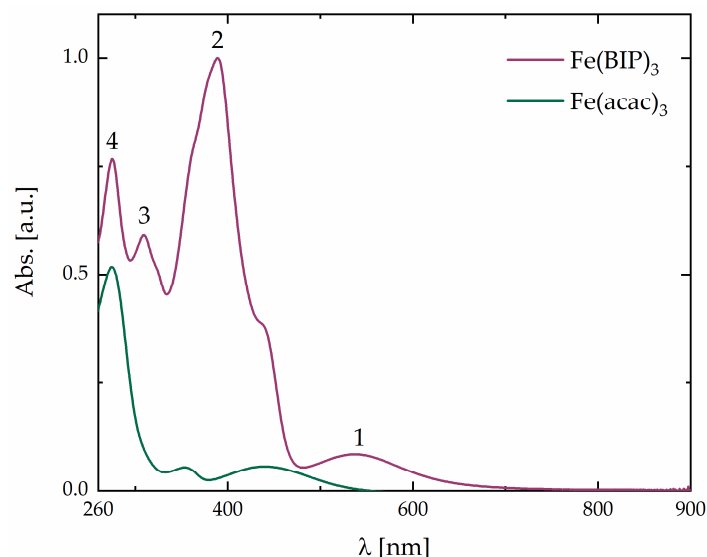


Figure 2. Superimposition of UV-Vis absorption spectra of $\text{Fe}(\text{BIP})_3$ (purple line) and $\text{Fe}(\text{acac})_3$ (green line).

Table 1. $\text{Fe}(\text{BIP})_3$ UV-Vis absorption peak assignment.

Entry	Wavelength (nm)	Transition ¹	Redshift (nm) ²
1	539	LMCT	99
2	389	LLCT	115
3	310	LLCT	-
4	275	LLCT	-

¹ Ligand-to-metal charge transfer (LMCT), ligand-to-ligand charge transfer (LLCT). ² Values calculated by subtraction of $\lambda_{\text{Fe}(\text{BIP})_3} - \lambda_{\text{Fe}(\text{acac})_3}$.

The 539 nm broad band (Peak 1) was attributed by Lintvedt and Kernitsky [60] to a ligand–metal charge transfer (LMCT) transition from the ligand π orbital to the e_g antibonding level. The maximum absorption peak (Peak 2) was ascribed to an intra-ligand π – π^* transition. Furthermore, transitions at higher energy (Peak 3 and 4) were attributed to LLCT.

In Figure 2, the spectrum of the complex is compared with that of $\text{Fe}(\text{acac})_3$. Namely, two equally concentrated (40 mM) solutions of the two complexes were used to record the spectra, which were then normalized to the major peak (Peak 2) of the $\text{Fe}(\text{BIP})_3$ spectrum. By bare visual inspection, a general redshift of the absorption peaks and higher overall values of absorbance can be observed. The former phenomenon is coherent with the reports of Lintvedt [60] and Conradie [61], since the substitution with aromatic moieties, indole in our case, at the 1 and 3 positions of the diketone ligand backbone is predicted to lead to an extensive bathochromic shift, as reported in Table 1, column 4. Higher absorbance values can be explained by considering a light harvesting effect, generated by the increase in conjugation brought by the *N*-methylindole substituents. The maximum absorbance peak

displays two shoulders; the one at 361 nm can be assigned to an intra-ligand transition, as found by Conradie [61], whilst the one at 438 nm can possibly be derived from a merge between two different transitions caused by the general redshift, i.e., a transition related to the ligand [52] and the band ascribable to the MLCT transition [60]. UV-Vis spectroscopy can be also exploited to derive relevant information about the spin configuration of the complex. Namely, in the spectrochemical series of Fe^{3+} tris diketonates made by Lintvedt, $\text{Fe}(\text{acac})_3$ possesses the highest Δ_{oh} value (separation between t_{2g} and e_g orbitals) among all the compounds screened, and it is in a high spin state ($S = 5/2$); aromatic diketonates display lower Δ_{oh} , so these should have a high spin configuration too. Based on these premises, since indole is an aromatic compound, we expect, and actually observe, a similar effect on Δ_{oh} also for $\text{Fe}(\text{BIP})_3$, which suggests that the complex is in a high spin state. This hypothesis is also confirmed by the absence of a d-d transition band in the UV-Vis absorption spectrum, which is forbidden by both Laporte and spin multiplicity rules in the case that the electronic configuration of the metal core consists of five unpaired electrons.

Direct evidence of such spin multiplicity is independently and unequivocally provided by bulk magnetic susceptibility measurement of $\text{Fe}(\text{BIP})_3$, which yields μ_{eff} values of 6.46 effective Bohr magnetons, which is consistent with high-spin Fe^{3+} (theoretical μ_{eff} value of 5.9 Bohr magnetons) [62,63] (see Section S4 in the Supplementary Materials for a more detailed discussion).

Unfortunately, due to the paramagnetic nature of the Fe^{3+} complex in high spin, NMR spectroscopy is not a useful technique for structural investigation. In fact, the ^1H spectrum of the complex recorded in DMSO (400 MHz, 300 K,) shows a broad signal in the 7.5–9.0 ppm region only, ascribable to the indole aromatic's protons resonance (Figure S5 in the Supplementary Materials). ^{13}C -NMR analysis of the complex was also unsuccessful. DSC-TGA data display high thermal stability of the complex up to 300 °C without decomposition or phase change (Figure S6 in the Supplementary Materials). EPR measurements, which could not be afforded due to the lack of suitable instrumentation, would be beneficial to support the bulk magnetic susceptibility results.

2.3. X-Ray Diffraction Analysis

X-Ray diffraction on a single crystal of $\text{Fe}(\text{BIP})_3$ obtained by slow cooling of a saturated solution of the complex in *N,N*-dimethylacetamide (DMAc) finally confirmed our initial hypothesis. Together with the octahedral iron complex, one water and two DMAc are co-crystallized. In the asymmetric unit, there is half of the complex, the other is symmetry-generated, and there is one molecule each of DMAc and water. The complex geometry is distorted-octahedral with O–Fe–O *cis* angles ranging from 85.27° to 98.71° and O–Fe–O *trans* angles ranging from 167.60° to 173.45° (Figure 3). In the asymmetric unit, the tris-chelate complex was refined as Λ isomer, but in the unit cell through symmetry generation, there are two Λ isomers and two Δ isomers present, i.e., a racemic mixture. A Mogul analysis [64] of the complex's core shows that the vast majority of the observed metrical parameters are normal, with only one exception. The O3–C22–C24 angle is particularly acute at 115.01°. In four complexes with relatively closely related ligands that are deposited in the database, analogous angles range from 115.41° to 118.63° [65–67]; i.e., they are all wider than the one observed here. In contrast to the other two ligands, the ligand exhibiting this acute angle is only refined as half a molecule and the complete ligand is generated by symmetry operation (two-fold rotation axis). As a consequence, the atoms of the C–C(=O)–CH–C(=O)–C structural motif together with the iron(III) center are essentially all planar. The other ligands (one refined, the other generated by symmetry operation from the refined one; i.e., they are identical) can bend a little bit away from the center, giving them leverage for a more relaxed geometry altogether and specifically the

adaptation of wider angles. This is apparent when comparing the angles between the planes going through the iron and coordinating oxygen atoms and the plane going through the coordinating oxygen atoms and the three carbon atoms bridging them (C(=O)–C–C(=O)). The respective inter-planar angle is only 0.91° in the case of the geometrically more strained ligand (exhibiting the acute O3–C22–C24 angle) and 25.33° for the more relaxed ligands.

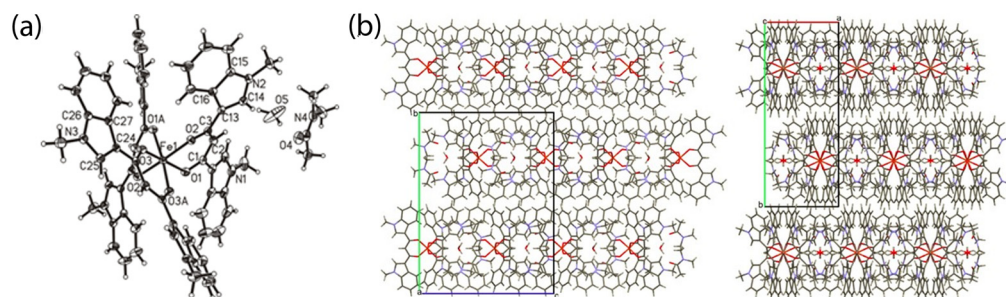


Figure 3. (a): The molecular structure of $\text{Fe}(\text{BIP})_3$. Ellipsoids are shown at the 50% probability level. For the disordered water hydrogen atoms, only one of two orientations are shown. (b) The packing diagrams for the crystal of the title compound $\text{Fe}(\text{Bip})_3$ viewed along the crystallographic *a*- (left) and *c*-axes (right), showing that the molecules are stacked essentially in offset layers in the *a/c* plane. The solvent molecules adopt positions within the layers in voids decorated with the ligands' backbones and with hydrogen bonding in between the three molecules, forming a set of solvents per void (two DMAc and one disordered water) plus H-bonding contacts between DMAc and the complex that together form columns along the crystallographic *a*-axis based on intermolecular interactions.

Only one of the three refined (six in total) coordinated oxygen atoms (O2) is engaged in non-classical hydrogen bonding as an acceptor with a DMAc molecule (Table 2). The water molecule (O5) bridges two adjacent DMAc molecules (O4), which are then engaged again in the C–H...O2 intermolecular contact between DMAc and the complex so that indefinite $-(\text{water-DMAc-complex-DMAc-water-DMAc-complex})_x-$ chains are formed, which present themselves as thick columns protruding through the crystal along the crystallographic *a*-axis. In between the columns, no notable strong interactions are observed but there may be some C–H... π contact in between the *N*-methyl substituent and a benzene ring of the adjacent column. Considering this interaction as sufficiently strong, sheets are formed in the *a/c* plane, which fill the unit cell up to half of its *b*-dimension. These planes are essentially isolated from sheets below and above (i.e., in the *b*-direction), but likely some weak H...H interactions further support the three-dimensional supramolecular arrangement. The cells are very tightly packed with no void space whatsoever. Please see Appendix A for further details on the crystallographic analysis.

Table 2. Hydrogen bond geometry ($\text{\AA}$, $^\circ$) for $\text{Fe}(\text{BIP})_3$.

D—H...A	D—H	H...A	D...A	D—H...A
C14—H14...O5	0.95	2.29	3.196 (4)	160
O5—H5O...O4i	0.96 (1)	2.27 (14)	2.741 (3)	109 (10)
O5—H5P...O4	0.96 (1)	1.82 (4)	2.741 (3)	161 (11)
C35—H35C...O2ii	0.98	2.59	3.476 (4)	151

Symmetry codes: (i) $x + 3/2, y + 1/2, z$; (ii) $x + 3/2, y, z + 1/2$.

2.4. Preparation, Characterization, and Stabilization of the Aggregates

2.4.1. Preparation and Characterization

Aggregates were reproducibly produced by simply percolating a 1 mM DMSO stock solution of $\text{Fe}(\text{BIP})_3$ in distilled water (MilliQ grade) in *v/v* concentrations in the range 0.2–10%. Percolation was executed at room temperature. Gentle stirring by a magnet at

200 rpm favored formation of less polydisperse aggregates. The size and polydispersity index of the aggregates were evaluated systematically at 2% *v/v* concentration of the stock by recurring to dynamic light scattering (DLS) analysis, showing that a transient equilibrium was reached as fast as the first measurement was performed after percolation (i.e., few minutes), in which lowly dispersed (polydispersity index, PDI, of ≈ 0.15) aggregates of hydrodynamic radius 28 ± 5 nm were formed. This value is the average of $N = 87$ correlation functions, acquired on different samples. Different batches of $\text{Fe}(\text{BIP})_3$ were used to prepare the 1 mM stock solutions in DMSO. The reported uncertainty is the pertaining standard error. Figure 4a shows an exemplary correlation function, together with the best fit to a bimodal Gaussian distribution (details on the fitting are reported in Section 3.4.2). The pertaining fitting parameters are summarized in the figure caption.

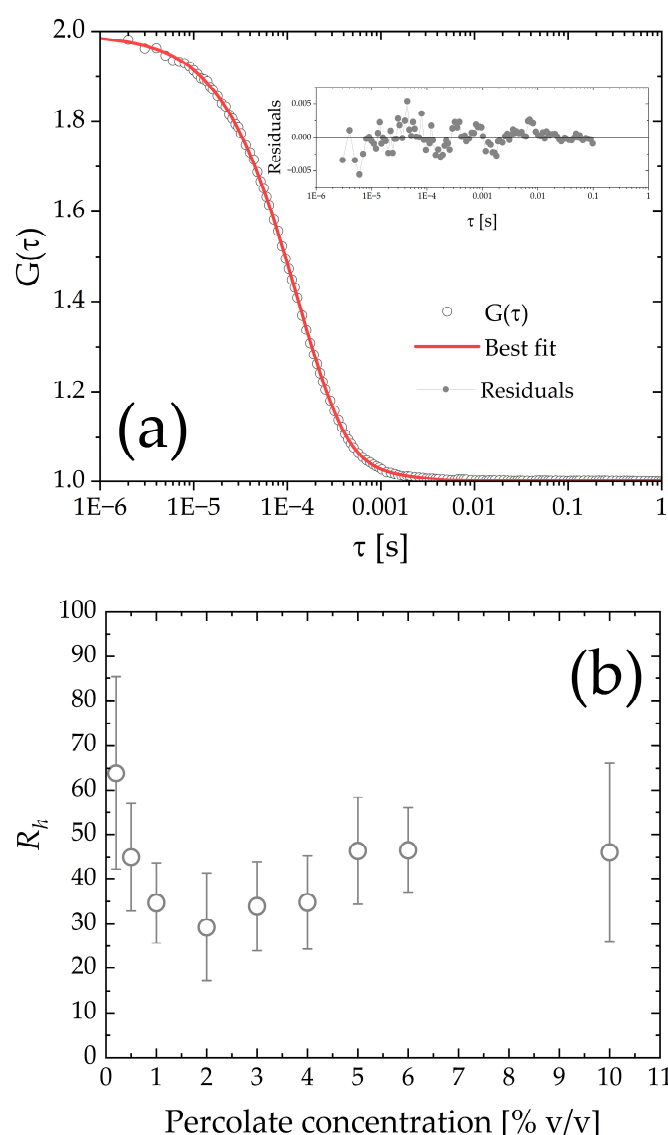


Figure 4. (a) Typical intensity correlation function of the $\text{Fe}(\text{BIP})_3$ aggregates. Points are the evaluated $G(\tau)$, while the line is the best fit according the model introduced in Equation (2) with $a_1 = 0.82$, $a_2 = 0.18$, $\tau_1 = 2.58 \times 10^{-4}$, $\sigma_{\tau_1} = 1.08 \times 10^{-4}$, and $\tau_2 = 3.15 \times 10^{-3}$. The relative residuals of the fit are shown in the Figure inset. (b) Percolate concentration dependence of the average hydrodynamic radius of aggregates. The error bars represent fitting errors as evaluated by the program on a single autocorrelation curve per datapoint. Note that they are much higher than those reported in the text for the average of different parallels.

A slight dependence of particle size on percolate concentration was observed. This effect has been investigated over eight different concentrations within the above-quoted range. The results are shown in Figure 4b. Time lapse measurements (Figure S8) provide evidence that this transient equilibrium lasts essentially unaltered for several hours (>3 h at the higher concentration). However, further clustering in grains of larger size (>1 μm) and higher polydispersity takes place on longer time scales and eventually leads to sedimentation in 12–48 h (depending on the concentration).

In order to elucidate the morphological features of the aggregates, scanning electron microscopy (SEM) images were collected. Namely, drops of the solutions at 1%, 2%, and 3% *v/v* concentration of the stock were withdrawn after 1 h from percolation and deposited onto glass coverslips. The water was evaporated under a gentle nitrogen flux. The 0.2% and 0.5% solutions were not characterized by SEM as too low a number of aggregates remained adhered to the glass surface after fluxing nitrogen, while with the solutions above 4% concentration, the aggregates tended to cluster when the specimen was desiccated. In the left panel of Figure 5, we show a typical SEM image. The aggregates are rather similar to each other, with a characteristic rice-grain shape. The average dimensions of the aggregates were evaluated over ≈ 300 particles of different images, exploiting the software Image-J 1.54f. The average length of the semi-minor axis was found to be 26 nm, while the semi-major axis was on average 47 nm long, with an average PDI of the two axes of 0.09 and an aspect ratio of 1.9. The frequency distributions of semi-minor and semi-major axes are shown in Figure S9 in the Supplementary Materials. The radius of the sphere of equal volume is about 32 nm, in good agreement with the hydrodynamic radius determined through DLS. The slight difference in polydispersity can be attributed to the reduced sampling of the SEM measurements compared to DLS. However, after 6 h from percolation of the DMSO stock in water, the rice-grain-shaped aggregates have already undergone substantial clustering to form much bigger structures, as can be seen from the right panel of Figure 5.

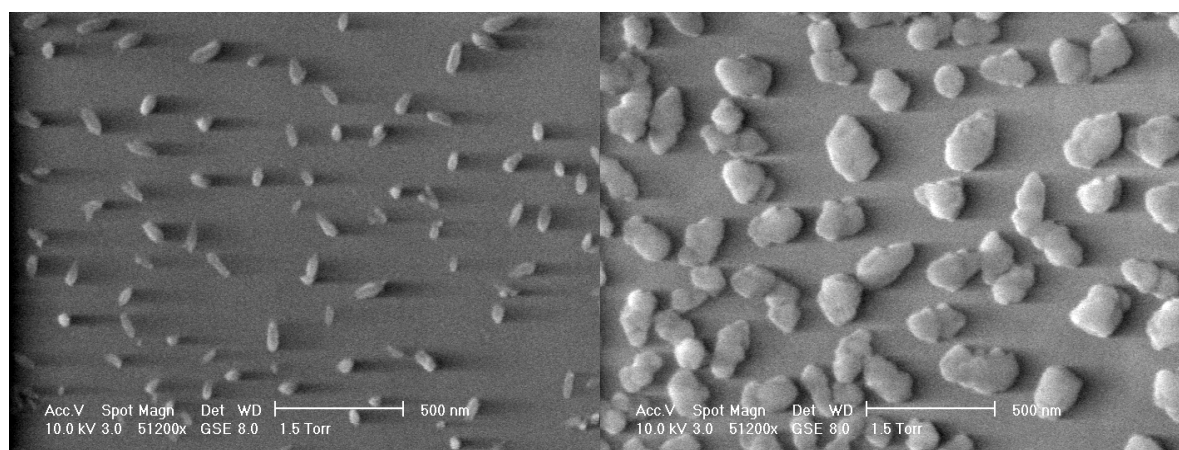


Figure 5. (Left): A typical SEM image of the $\text{Fe}(\text{BIP})_3$ rice-grain-shaped aggregates; the sample was deposited 1 h after percolation. (Right): The same sample deposited 6 h after percolation.

2.4.2. Stabilization

In order to stabilize the aggregates in their metastable rice-grain form, we tested their incorporation within phospholipid vesicles. To this aim, as a first feasibility study, we synthesized soybean lecithin liposomes and added them to aqueous solutions in which the aggregates were previously generated according to the percolation protocol described above. Liposomes were obtained following two different procedures, namely, direct mixing of lipids in aqueous solution and resuspension of thin films. The detailed procedures to

obtain and extrude the liposomes according to both methods are described in Section 3.1. Once again, the quality of the synthesis was evaluated in terms of hydrodynamic radius and polydispersity index assessments through DLS measurements. The samples produced by means of the two alternative protocols did not show any relevant differences. Accordingly, we chose to adopt the direct mixing procedure for the measurements reported hereafter, as it is simpler and more easily controllable. This allows us to obtain with high repeatability liposomes with a hydrodynamic radius of 134 ± 12 nm with a PDI of 0.15. Similarly to the case of $\text{Fe}(\text{BIP})_3$ aggregates, the correlation function was fitted with a size distribution consisting of two species: a Gaussian, with free parameters for mean value and width, to describe the aggregates, and a single-species component to account for possible macro-aggregates or residual impurities in the sample. The details of the fitting procedure are reported in Section 3.4.2. Monitoring several batches of liposomes obtained from independent syntheses through acquisition of DLS autocorrelation functions every 3 days over one month allowed us to demonstrate that the latter were stable for weeks if stored at $4\text{--}8^\circ\text{C}$.

The morphology of liposomes was also evaluated by SEM experiments. With this aim, because both spontaneous evaporation and desiccation under nitrogen flux induced aggregation of the lipidic component of the specimen and formation of an amorphous film on the glass surface, we proceeded to lyophilize the liposomes at 10^{-6} Pa and -40°C . As shown in Figure 6, through this procedure, we managed to make the liposomes adhere to the glass, appreciate their regular spherical shape, and estimate their size, resulting in good agreement with those determined through DLS. Namely, the geometric radius of the deposited liposomes was estimated, by exploiting Image-J on ≈ 200 particles of 22 different images, to be 150 ± 15 nm, with a PDI of 0.18. The pertaining size distribution is shown in Figure S10 in the Supplementary Materials.

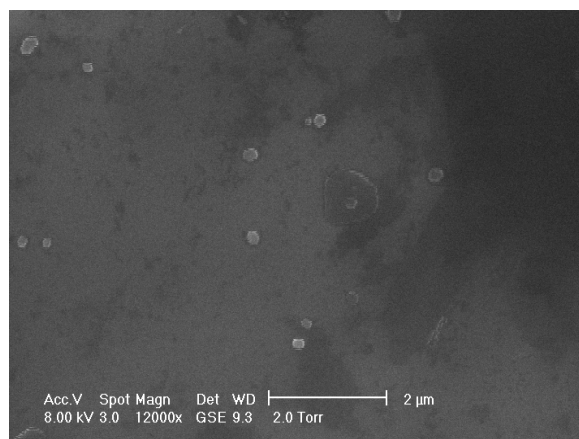


Figure 6. Typical SEM image of lyophilized liposomes.

The idea underlying the stabilization of $\text{Fe}(\text{BIP})_3$ aggregates through incorporation within phospholipid vesicles is that interaction with the aggregates causes preformed ordered lipid structures to undergo a spontaneous phase transition driven by hydrophobic forces, encompassing disruption of the liposomes in favor of arrangement of the phospholipids around the highly hydrophobic aggregate surfaces in the form of lamellar vesicles exposing the aliphatic tails to the aggregate and the hydrophilic polar heads to the solvent. The occurrence of such a transition may in principle be evidenced by DLS as a gradual reduction in the observed hydrodynamic radius. We indeed expect that the aggregates coated with a lipidic monolayer have a hydrodynamic radius approximately equal to the hydrodynamic radius of the uncoated aggregates (≈ 30 nm, vide supra) added with the length of the aliphatic chain of the lecithin phospholipid (≈ 8 nm; see datasheet). However,

the DLS signal scales as the sixth power of the hydrodynamic radius of the scattering centers. Thus, light diffusion by the few undisrupted liposomes surviving the interaction with aggregates dominated the autocorrelation function in case of comparable initial concentrations of lipid and $\text{Fe}(\text{BIP})_3$ or, even worse, an excess of lipids. Conversely, in an excess of $\text{Fe}(\text{BIP})_3$, because the scattering efficiency depends not only on the size of scattering centers but also on the refractive index mismatch of the latter with respect to the solvent, we did not manage to discriminate the diffusive component of the coated aggregates from the one of the bare aggregates in the autocorrelation function, as the coating with lipids corresponds to a strong reduction in the refractive index mismatch.

To overcome this issue and manage to directly observe the liposome-to-vesicle transition, we fluorochromized the liposomes. Namely, 50 μL of a 0.1 mM concentrated stock solution of phosphatidylethanolamine labelled with fluorescein isothiocyanate (FITC-PEA) in methanol (see Section 3 for details) was added to the solution used for the liposomes' synthesis. Fluorescence correlation spectroscopy (FCS) measurements were carried out to determine hydrodynamic radii. The FCS technique [68] is based on the analysis of temporal fluctuations in the fluorescence intensity signal emitted by a small number of particles diffusing by Brownian motion through a tiny (<1 fL) observation volume. The latter is selected within a wider sample exploiting the confocal excitation–collection geometry. Because the fluorophores freely diffuse throughout the whole specimen, the number of those found within the observation volume stochastically varies over time around an average value dictated by the bulk sample concentration. The temporal correlation of the fluorescence intensity signal allows the determination of its persistence time, which in turn scales proportionally to the Stokes–Einstein diffusion coefficient. Therefore, upon suitable calibration of the setup in order to determine the proportionality coefficient linking the persistence time to the diffusion coefficient, FCS can be used as a valuable alternative to DLS to measure hydrodynamic radii. Application of this technique yielded a typical size of (101 ± 8) nm for our liposomes. It is worth noting that the amplitude of fluorescence fluctuations is not plagued by any systematic dependence on the hydrodynamic radius of the fluorophore. Furthermore, because $\text{Fe}(\text{BIP})_3$ cannot be excited at the wavelength of the laser utilized to elicit the fluorescence of FITC-PEA and in any case does not emit sizeable fluorescence in the same band, bare aggregates do not contribute to the FCS signal.

Accordingly, it has been possible to preliminarily characterize via FCS FITC-PEA-containing fluorescent liposomes, add pre-aggregated $\text{Fe}(\text{BIP})_3$ in high excess, and observe a progressive reduction in the persistence time of the fluorescence signal (i.e., of the mean size of the particles diffusing throughout the observation volume). The mean size reaches a stable value of (44 ± 6) nm within 45 min from the addition of the aggregates to the liposome solution. In Figure 7, two autocorrelation curves are reported, recorded, respectively, before (blue line) and 60 min after (red line) the addition of the aggregates.

To further support the hypothesis that the observed reduction in size is due to the formation of phospholipidic films coating the aggregates, we performed an independent fluorescence resonance energy transfer (FRET) experiment. FRET is a quantum resonance phenomenon occurring between the transition dipole moments of two chromophores [69]. The emission spectrum of one of them, named the donor, partially overlaps with the absorption spectrum of the other, called the acceptor. If a donor molecule comes in close proximity (<1 nm) to an acceptor molecule while in the excited state, the excitation energy can be transferred to the acceptor. Therefore, if the acceptor is also a fluorophore, it is possible to observe fluorescence in the acceptor's emission band upon excitation in the donor's absorption band. $\text{Fe}(\text{BIP})_3$ aggregates absorb radiation in a band peaked at 389 nm (vide supra), in which fluorescein has negligible absorbance, and emit faint fluorescence in a structured band with peaks at ≈ 450 nm and ≈ 470 nm, within the main absorption

band of fluorescein (peaked at 488 nm). We thus prepared a specimen in which a small quantity of the 1 mM stock of $\text{Fe}(\text{BIP})_3$ in DMSO (0.5% *v/v*) was made to percolate in water. An excess of FITC-PEA containing preformed liposomes was added after 20 min from percolation. After 60 min from the addition of liposomes, we measured the emission spectrum upon excitation at 389 nm.

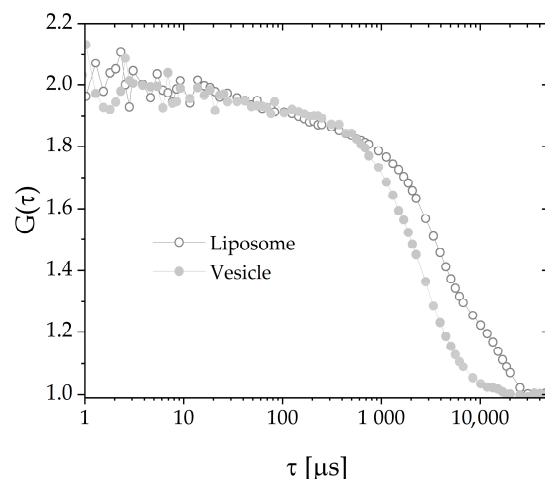


Figure 7. Exemplary fluorescence cross-correlation curves acquired for a sample of FITC-PEA-containing liposomes (empty circles) and FITC-PEA-containing vesicles surrounding the $\text{Fe}(\text{BIP})_3$ aggregates (full dots).

As shown in Figure 8a, besides the peak characteristic of the aggregate emissions, a sizeable fluorescence peak at 515 nm (i.e., at the fluorescein emission peak) was observed, which testifies that in the specimen, $\text{Fe}(\text{BIP})_3$ aggregates are complexed to FITC-PEA molecules and strongly suggests the formation of phospholipid films around the aggregates. We then performed a time lapse experiment in which a sample of aggregates was illuminated at 389 nm and fluorescence at 515 nm was detected at each second for 2.5 h. The results are reported in Figure 8b and show the progressive onset of FRET-elicited FITC-PEA fluorescence due to phospholipid films around the $\text{Fe}(\text{BIP})_3$ aggregates.

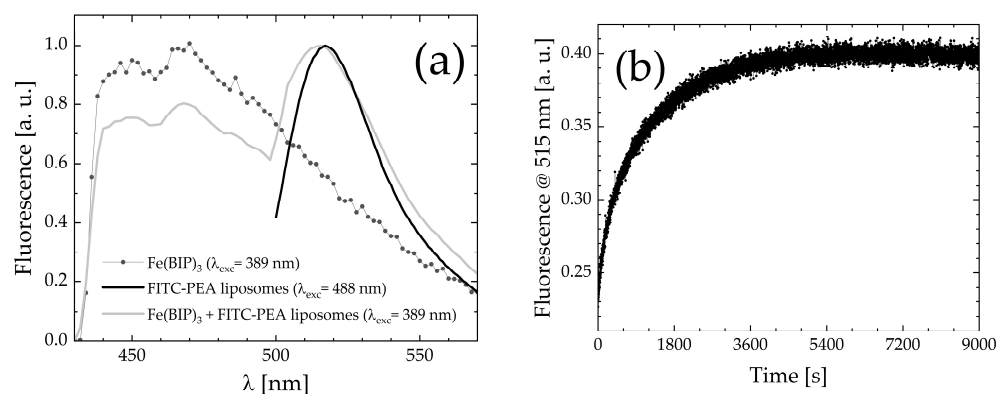


Figure 8. (a) Fluorescence emission spectra of a suspension of $\text{Fe}(\text{BIP})_3$ aggregates acquired 15 min after percolation of the DMSO stock in water (dotted line), an FITC-PEA liposome suspension (black solid line), and the same $\text{Fe}(\text{BIP})_3$ aggregate suspension added (20 min after percolation) with preformed liposomes (gray line). This latter spectrum was recorded 60 min after liposome addition. The spectra are peak-normalized for clarity. (b) Time lapse measurement showing the fluorescence increase at 515 nm (i.e., the FITC-PEA emission peak wavelength). The acquisition was started immediately after addition of liposomes to an $\text{Fe}(\text{BIP})_3$ aggregate sample and lasted for 2.5 h. The plateau is reached ≈ 1 h after liposome addition.

2.5. Relaxivity Measurements

Relaxivities of $\text{Fe}(\text{BIP})_3$ aggregates, bare and incorporated into phospholipid vesicles, were performed at 9.4 T at 300 K in 9:1 water/DMSO solutions. First, we measured the spin–lattice and spin–spin relaxation times of the $\text{Fe}(\text{BIP})_3$ aggregates, $T_{1,\text{obs}}$ and $T_{2,\text{obs}}$, respectively. r_1 and r_2 values were then determined by measuring relaxation rate values versus iron concentrations. Results are reported in Figure 9.

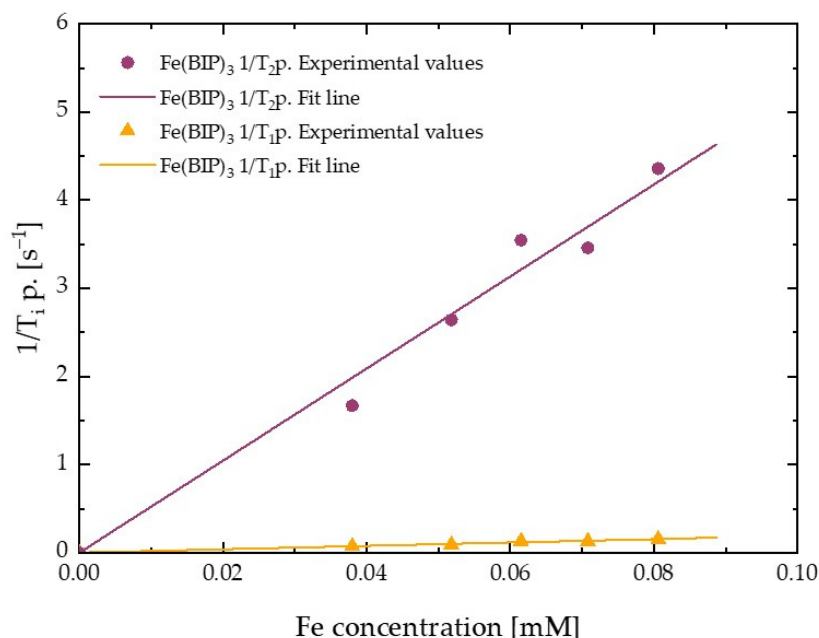


Figure 9. Paramagnetic contributions to water proton relaxation rates $\frac{1}{T_{ip}}$ ($i = 1, 2$) vs. $\text{Fe}(\text{BIP})_3$ concentration (mM). Color code: magenta line and dots, $\frac{1}{T_{2p}}$; yellow line and triangles, $\frac{1}{T_{1p}}$.

The corresponding r_1 and r_2 values and r_2/r_1 ratio for the bare aggregates are reported in Table 3.

Table 3. Values of longitudinal (r_1) and transverse (r_2) relaxivity for $\text{Fe}(\text{BIP})_3$ aggregates at 9.4 T and 300 K.

r_1 (mM ⁻¹ s ⁻¹)	r_2 (mM ⁻¹ s ⁻¹)	r_2/r_1
1.92	52.3	27.2

The spin–spin/spin–lattice relaxivity ratio r_2/r_1 was found to be 27.2 at 300 K. This finding is consistent with quite a long electronic relaxation rate $\tau_S = 2.12 \times 10^{-9}$ s and supports the use of $\text{Fe}(\text{BIP})_3$ aggregates as T_2 paramagnetic relaxation reagents. The r_2 value we found for the aggregates is in line with the ones found for similar iron oxide bimetallic nanoparticulate-coated systems recorded in the same conditions [70].

The intrinsic and predictable instability of the aggregates over long times in the 9:1 water/DMSO stocks (see SEM data in Figure 5 and DLS kinetics in Figure S8 in the Supplementary Materials) led us to stabilize them through their incorporation within vesicles of soy lecithin (vide supra). As for the analysis carried out for the bare aggregates, we also investigated the quenching ability for water T_2 relaxation of the ones incorporated in vesicles. The r_2 value found, $r_2 = 14.5 \text{ mM}^{-1} \text{ s}^{-1}$, is smaller than the r_2 value determined for the bare aggregates ($52.3 \text{ mM}^{-1} \text{ s}^{-1}$), as reported in Figure 10. This loss in quenching efficacy can be explained if we consider the increased distance between the $\text{Fe}(\text{BIP})_3$ core inside the vesicles and the bulk water. Taking into account the interactions of water molecules with a PRA, relaxivity, r_i , can be described as the sum of an inner sphere

contribution, r_i^{IS} (water molecules directly coordinated to the paramagnetic metal center), and an outer sphere contribution, r_i^{OS} (water molecules interacting with polar functional groups on the ligand or bulk water), as shown in Equation (1) [71].

$$r_i = r_i^{IS} + r_i^{OS} \quad i = 1, 2 \quad (1)$$

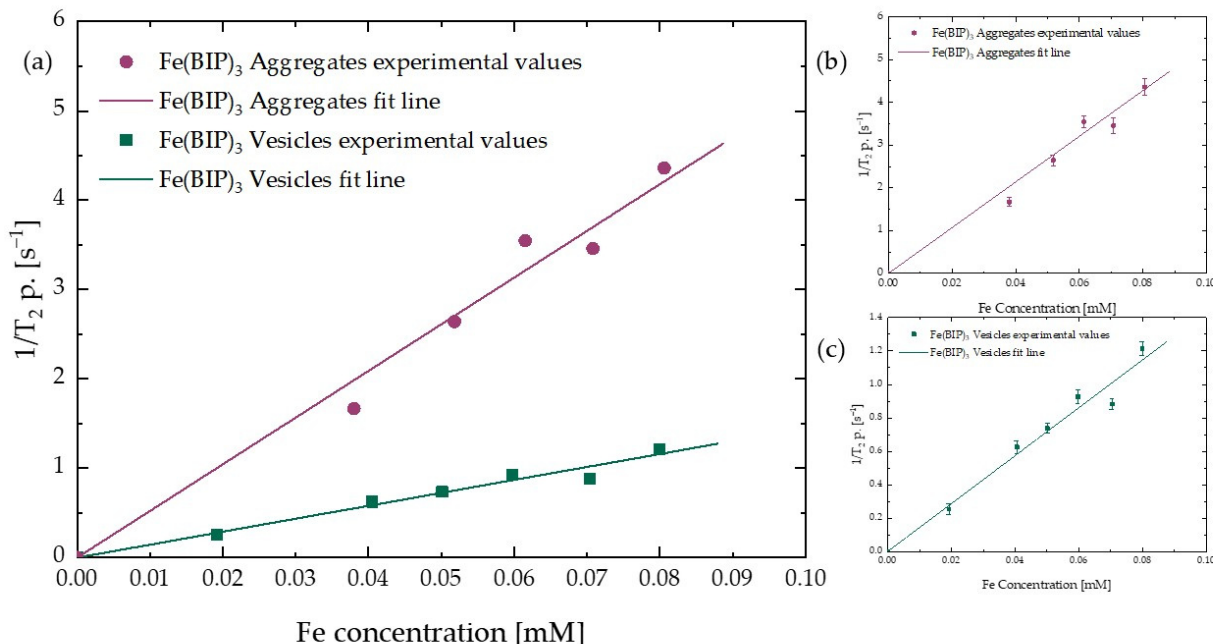


Figure 10. (a) Paramagnetic contribution to the transverse water proton relaxation rate $\frac{1}{T_{2p}}$ versus Fe(BIP)₃ concentration (mM). Color code: magenta line and dots, bare aggregates; green line and squares, aggregates in vesicles. (b) Aggregates: $r = 52.30 \pm 1.75$ and $R^2 = 0.977$. (c) Aggregates in vesicles: $r = 14.50 \pm 0.49$ and $R^2 = 0.973$.

As we can see from the crystal structure of Fe(BIP)₃, the Fe³⁺ metal center is coordinatively saturated by the three 1,3-dikeonate ligands, which strongly bind to it: it is highly unlikely that a water molecule could directly coordinate to the Fe³⁺ ion, so that the overall relaxivity is mainly influenced by outer sphere contributions. Nonetheless, a significant quenching efficacy is also conserved upon incorporation of the aggregates within lecithin vesicles, which on the other hand makes the formulation stable over several weeks.

3. Materials and Methods

3.1. Fe(BIP)₃ Synthesis and Characterization

All reactions were performed under a normal atmosphere using oven-dried glassware. Reagents and solvents were purchased from TCI (Tokyo, Japan) or Merck (Darmstadt, Germany) and used without further purification. Where specified, reactions were monitored by thin layer chromatography (TLC), using the POLYGRAM Xtra SIL G/UV254 (0.2 mm thick layer, Macherey–Nagel); TLC spots were observed under a UV lamp (365 and/or 254 nm, respectively). Gravimetric column chromatography was performed using silica gel as a stationary phase (0.63–2 mm particle size). IR spectra were acquired by the ATR method with a Nicolet iS10 instrument, over the range of 4000–600 cm⁻¹; hereafter, intensities are denoted as follows: vs = very strong, s = strong, m = medium, w = weak, and vw = very weak. ¹H and ¹³C(APT) solution NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker Avance I 400 spectrometer. ¹H and ¹³C data are reported as follows: chemical shifts, integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), and coupling constants (in Hz). The deuterated solvent for NMR measure-

ments, DMSO- d_6 , was used as received from Eurisotop. T_1 and T_2 relaxation values were obtained by NMR data using Dynamics Data Analysis software.

The bulk magnetic susceptibility of Fe(BIP) $_3$ was measured at 300 K by the Evans' NMR method. Elemental composition analyses were performed with a CHN Analyser 2400 Series II instrument (Perkin Elmer, Waltham, MA, USA). Thermal analyses were performed with a STA 409 PC Luxx instrument (Netzsch, Waldkraiburg, Germany; temperature range RT–600 °C, rate of 10 °C/min, N $_2$ atmosphere). UV-Vis absorption spectra were recorded on a UV-2600i spectrophotometer (Shimadzu, Kyoto, Japan) in the wavelength range 260–900 nm at 0.5 nm resolution using 1 cm quartz cuvettes.

The iron concentration title of the 1 mM Fe(BIP) $_3$ complex stock solutions in DMSO was determined by ICP-MS using a i-CAP Q instrument (ThermoScientific, Waltham, MA, USA). Solutions containing the iron complex were mixed in a 1:10 ratio with concentrated HNO $_3$ and heated in an autoclave at 150 °C overnight, yielding a solution of Fe $^{3+}$ aqua ion.

3.2. X-Ray Structural Analysis

The single-crystal X-ray diffraction (SCXRD) data were recorded at 100 K on an XtaLAB Synergy diffractometer from Rigaku (Neu-Isenburg, Germany), with mirror-monochromated Cu-K $_{\alpha}$ -radiation (λ = 1.54184 Å) and a hybrid pixel array detector (HyPix). The sample was mounted on a LithoLoop produced by Molecular Dimensions (Sheffield, UK) and fixed on a pin produced by Hampton Research (Aliso Viejo, CA, USA). An absorption correction was performed using CrysAlisPro version 1.171.42.61a (Rigaku OD, 2022, Tokyo, Japan). The structure was solved by dual methods with SHELXT-2018 and refined by full-matrix least-squares techniques using the SHELXL-2019 executables and the WingX GUI [72–74]. All non-hydrogen atoms were refined with anisotropic displacement parameters. General crystallographic, crystal, and refinement data are provided in Table 2. Crystallographic data were deposited with the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB21EZ, UK. These data can be obtained free of charge upon providing the depository number CCDC 2483872 by email (deposit@ccdc.cam.ac.uk) or their web interface (at <http://www.ccdc.cam.ac.uk>).

The crystal cracked in the cryostream during measurement, which is the reason for sub-optimal R-values. Together with the octahedral iron complex, one water and two DMAc are co-crystallized. In the asymmetric unit, there is half of the complex, the other is symmetry-generated, and there is one molecule each of DMAc and water, the latter in a special position. The water oxygen atom is located on a two-fold rotation axis and, based on the symmetry, water is slightly disordered. The disorder is modelled for the two hydrogen atoms appearing in four positions but not for the oxygen, which has a somehow elongated ellipsoid. The respective O–H and H–H distances are restrained (DFIX). Also, the displacement parameters U_{iso} of the H are constrained to that of the parent oxygen atom (1.5 times U_{eq}). Occupancies are set to 50/50.

3.3. Liposome Preparation

The liposomes were prepared according to two different protocols, i.e., the direct mixing and the thin film resuspension method, slightly modifying protocols found in the literature [75–78] to achieve optimized yield of the obtained lipid nanoparticles in terms of monodispersity and stability. Both procedures are described hereafter. Because no substantial differences were detected in terms of liposome size and polydispersity according to DLS measurements, we adopted the simpler and more controllable direct mixing procedure to prepare the liposomes used in the experiments described above.

3.3.1. Direct Mixing Protocol

Liposomes were prepared following a direct mixing approach. Briefly, 50 mg of lecithin (abcr, Karlsruhe, Germany; CAS No. 8002-43-5) was dissolved in 50 mL of Milli-Q water. The solution was heated to 70 °C (above the lecithin aggregate melting temperature) under magnetic stirring at 200 rpm and maintained at this temperature for 5 min. The solution was then allowed to cool slowly to room temperature while continuing mild stirring (200 rpm), which was maintained for 48 h to promote liposome formation. The resulting liposomes were extruded through a cascade of PTFE syringe filters (pore sizes 1 µm and 400 nm), with each solution undergoing three successive filtrations.

3.3.2. Thin Film Protocol

Liposomes were prepared using a thin film hydration approach. Briefly, 50 mg of lecithin was dissolved in 50 mL of a 4:1 (v/v) mixture of chloroform and methanol. The solution was transferred to a round-bottom flask and heated at 70 °C for 5 min using a rotary evaporator. The solvent was then removed under vacuum with gradual temperature reduction and continuous rotation, allowing the lipid to form a thin film on the bottom of the flask. The thin film was kept under vacuum overnight to ensure complete solvent removal. The lipid film was subsequently hydrated with 50 mL of Milli-Q water and sonicated for 60 min to resuspend the liposomes. Finally, the liposomes were extruded through PTFE syringe filters following the same procedure described for the direct mixing protocol.

3.4. Characterization of $\text{Fe}(\text{BIP})_3$ Aggregates and Liposomes

3.4.1. Fluorescence Spectroscopy

Fluorescence spectra were recorded and FRET experiments were performed using a PTI Fluorescence Master System (Photon Technology International, Birmingham, NJ, USA) apparatus. Samples were held in 1 cm path length quartz cuvettes (Hellma, Müllheim, Germany). Spectra were automatically corrected for the excitation source spectral radiance and the detector quantum efficiency using Felix2000 (V.1.) acquisition software.

3.4.2. Dynamic Light Scattering

DLS measurements were performed using a home-assembled DLS setup, described in detail elsewhere [79,80]. Briefly, the sample, contained in a 1 cm² quartz cuvette with temperature controlled by a circulating bath and continuously monitored by two thermistors, was irradiated by a collimated laser beam (Integrated Optics, Vilnius, Lithuania, mod. 0638L-25A-NI-AT-NF, $\lambda = 637.8\text{nm}$, $P = 50\text{ mW}$). The scattered light, collected at 90° from the incident laser beam using a collimator coupled to a single-mode fiber, was directed to an ALV/SO-SIPD single-photon pseudo cross-correlation detector (ALV GmbH, Langen, Germany). Data was acquired via a fast counter board (PCIe-6612, Nuclear Instruments) and processed with a dedicated software correlator implemented in LabVIEW [81] in real time or offline, allowing raw data storage, post-processing, and tailored reduction for dust removal. The intensity correlation function was fitted to a model in which the contribution of the particles (either liposomes or $\text{Fe}(\text{BIP})_3$ aggregates) was described by a Gaussian intensity distribution $G_{\tau_1, \sigma_{\tau_1}}$, characterized by an average decay time τ_1 and a standard deviation σ_{τ_1} , while contributions from impurities were accounted for by a single exponential term with decay time $\tau_2 \gg \tau_1$:

$$G(q, \tau) = B + \left[a_1 \int_0^\infty G_{\tau_1, \sigma_{\tau_1}}(\tau') e^{-\frac{\tau}{\tau_1}} d\tau' + a_2 e^{-\frac{\tau}{\tau_2}} \right]^2 \quad (2)$$

In the non-linear fitting procedure, implemented using the Levenberg–Marquardt method by exploiting a home-written LabVIEW routine, the parameters B , a_1 , a_2 , τ_1 , σ_{τ_1} , and τ_2 were treated as free variables. The parameter τ_1 represents the average field decay time and is related to the mean hydrodynamic radius of the diffusing particles, R_h , by the relation

$$R_h = \frac{k_B T}{6\pi\eta} q^2 \tau_1 \quad (3)$$

where η is the sample viscosity, T the absolute temperature, and $q = (4\pi/\lambda)n\sin(\theta/2)$, where θ is the scattering angle, λ the laser wavelength in vacuum, and n the refractive index of the solvent.

The PDI has been evaluated as the square of the ratio between the standard deviation and the average value of the R_h distribution.

3.4.3. Scanning Electron Microscopy

Scanning electron microscopy images were collected using an FEI XL30 ESEM-FEG microscope (Philips, Eindhoven, The Netherlands). Glass microscope specimens with $\text{Fe}(\text{BIP})_3$ and liposomes were fixed over a large aluminum stub using a copper tape. SEM images were collected in low-vacuum mode, with an in-chamber water vapor pressure of 1.5 Torr for $\text{Fe}(\text{BIP})_3$ and 2.0 Torr for liposomes, respectively. The accelerating voltage was optimized for both $\text{Fe}(\text{BIP})_3$ and liposomes (12 and 8 kV, respectively). In all cases, images were collected using the in-chamber gas secondary electron detector. Other parameters are reported in the databar of every reported image.

The SEM images were processed in ImageJ to detect the particles. A bandpass filter was first applied to eliminate the speckle noise inherent to image acquisition, as well as the low-frequency variations caused by uneven illumination, making it possible to threshold the images. The particles were subsequently identified using either the TrackMate plugin (threshold-based detection) [82], with additional filtering steps and minor manual adjustments carried out to refine the results, or the Analyze particle tool. For the $\text{Fe}(\text{BIP})_3$ aggregates, the semi-minor and semi-major axis were determined from the best fitting ellipse to each spot.

3.4.4. Fluorescence Correlation Spectroscopy

The experimental setup is fully described elsewhere [83–85]. For the present experiments, the Cobolt Blues laser beam was attenuated through a 2 OD neutral-density filter. A 50 μm pinhole was used. The ellipticity parameter, s , was determined by performing a global fit over multiple fluorescence cross-correlation functions, obtained using 5 nM fluorescein as the diffuser, to the 3D Brownian diffusion model, Equation (4):

$$G_{3D}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{s^2 \tau_D}\right)^{-\frac{1}{2}} + 1 \quad (4)$$

In the above equation, N is the average number of particles in the observation volume and τ_D the persistence time of the fluorescence signal, which in this model coincides with the average time needed for a fluorophore to cross the observation volume. The ellipticity parameter indicates the ratio between the axial and radial dimensions of the observation volume, here approximated to a rotational ellipsoid with 3D Gaussian intensity profile. It depends on the setup alignment and must be determined operatively. In our case, its value was found to be $s = 5.0 \pm 0.3$.

Besides calibrating the setup, the measurements on fluorescein allowed us also to determine the proportionality coefficient linking the diffusion time τ_D to the hydrodynamic

radius, R_h , which is tabulated in the literature for this widely characterized dye. Namely, we adopted the value $R_{h,\text{Fluo}} = 0.56 \text{ nm}$ [86] and used Equation (5),

$$R_h = \frac{2k_B T \tau_D}{3\pi\eta w_0^2} = K\tau_D \quad (5)$$

where w_0 is the beam waist of the observation volume, to obtain the value $K = 8.14 \times 10^{-12} \text{ m/s}$. From this calibration, we were able to estimate R_h values also for the liposomes and lipid-film-coated $\text{Fe}(\text{BIP})_3$ aggregates starting from the measured diffusion times. However, it must be mentioned that the 3D Brownian diffusion model does not provide an accurate fit for the cross-correlation functions measured for these species, since their size is comparable with the radial dimension of the observation volume, while the model assumes diffusion of material points. Moreover, these large and heavy particles have a relatively long diffusion time, which makes the measurement very sensitive to drifts or convection inside the sample.

Accordingly, we tried two other models to interpolate experimental data. The first model considers a steady flow in the sample [87], to account for drifts or convection, while the second incorporates also the finite size of the diffusing particles [88]. For the sake of consistence with the fluorescein calibration data, in the Results section, we report the R_h data determined through fit to Equation (4). However, R_h values obtained by fitting to the three models coincide within $\pm 5\%$ for the liposomes and $\pm 20\%$ for the nanoparticles, and higher-quality fits are obtained with these two alternative models. Furthermore, a good agreement with the above strategies is achieved by estimating the τ_D value simply as the abscissa of the point in which the experimental cross-correlation functions, normalized at unit initial amplitude, cross the value $G(\tau) = 1/2 G(0)$ (i.e., the value assumed by the correlation function $G(\tau)$ at $\tau = \tau_D$ in the 3D diffusion model). A comparison of the R_h values retrieved by the three alternative procedures on an exemplary cross-correlation curve are reported in the Supplementary Materials (Table S2). The relative quality of the data fitting according to the three models can be appreciated from Figure S11 in the Supplementary Materials.

3.5. Relaxivity Measurements

Longitudinal ($\frac{1}{T_{1,obs}}$) and transverse ($\frac{1}{T_{2,obs}}$) relaxation rates were measured using inversion recovery (IR) and Carr–Purcell–Meiboom–Gill (CPMG) sequences, respectively, on a Bruker Avance I spectrometer operating at 9.4 T and 300 K. Relaxation rates are expressed as the sum of a paramagnetic contribution $\frac{1}{T_{i,p}}$ and a diamagnetic contribution $\frac{1}{T_{i,d}}$, as shown in Equation (6).

$$\frac{1}{T_{i,obs}} = \frac{1}{T_{i,d}} + \frac{1}{T_{i,p}} \quad i = 1, 2 \quad (6)$$

The diamagnetic term is the relaxation rate of water protons in the absence of the PRA, while the paramagnetic term is the relaxation rate enhancement caused by the presence of the paramagnetic relaxation agent. The paramagnetic contribution is directly proportional to the PRA concentration, following Equation (7).

$$\frac{1}{T_{i,obs}} = \frac{1}{T_{i,d}} + r_i[\text{PRA}] \quad i = 1, 2 \quad (7)$$

If $\frac{1}{T_{1,obs}}$ and $\frac{1}{T_{2,obs}}$ are considered and the concentration of the paramagnetic relaxation agent is given in molarity, then the parameters r_1 and r_2 are called the longitudinal and transverse relaxivities, respectively. Henceforth, relaxivity r_i ($i = 1, 2$) can be expressed as the slope of the linear plot of $\frac{1}{T_{i,obs}}$ versus the molar concentration of the PRA, while

the intercept represents the diamagnetic contribution $\frac{1}{T_{i,d}}$. In this study, such plots were obtained by performing the above experiments in triplicate. The datapoints reported in Figures 9 and 10 are the averages over the three parallels. The error bars are the corresponding standard deviations.

Usually, r_i values are dominated by the exchange of directly coordinated water molecules. In the case of $\text{Fe}(\text{BIP})_3$, lacking directly coordinated water molecules, water proton relaxation is accelerated by diffusion of water molecules near the paramagnetic center (outer sphere relaxation). For ions characterized by null orbital angular momentum ($L = 0$), such as high-spin $\text{Fe}(\text{III})$ complexes, the outer sphere contribution is described by Equations (8) and (9) [89] according to the Hwang–Freed model [90] to account for the diffusion-controlled dipole–dipole interaction from a distance of closest approach d to infinity.

$$\left(\frac{1}{T_{1,p}}\right)^{\text{OS}} = \frac{32\pi}{405} \left(\frac{\mu_0}{4\pi}\right)^2 \frac{N_A C}{dD} \gamma_I^2 g^2 \mu_B^2 S(S+1) [7J(\omega_S) + 3J(\omega_I)] \quad (8)$$

$$\left(\frac{1}{T_{2,p}}\right)^{\text{OS}} = \frac{16\pi}{405} \left(\frac{\mu_0}{4\pi}\right)^2 \frac{N_A C}{dD} \gamma_I^2 g^2 \mu_B^2 S(S+1) [13J(\omega_S) + 3J(\omega_I) + 4J(0)] \quad (9)$$

where γ_I is the gyromagnetic ratio of the proton, g and μ_B are the electronic Landé factor and the Bohr magneton, respectively, d is the distance between the water protons and the metal ion, and ω_I and ω_S are the proton and electron Larmor frequencies, respectively. At 9.4 T, $\omega_I = 2.51 \times 10^9 \text{ s}^{-1}$ and $\omega_S = 1.65 \times 10^{12} \text{ s}^{-1}$.

$J(\omega)$ represents non-Lorentzian spectral density functions described in Equation (10).

$$J(\omega) = \frac{1 + \frac{5}{8}z + \frac{1}{8}z^2}{1 + z + \frac{1}{2}z^2 + \frac{1}{6}z^3 + \frac{4}{81}z^4 + \frac{1}{81}z^5 + \frac{1}{648}z^6} \quad (10)$$

where

$$z = 2\sqrt{\omega\tau_D + \frac{\tau_D}{\tau_S}}. \quad (11)$$

Here, τ_D is the diffusion time described above and τ_S is the electron relaxation time. Accordingly, the r_2/r_1 ratio can be obtained by Equation (12).

$$\frac{r_2}{r_1} = \frac{1}{2} \frac{13J(\omega_S) + 3J(\omega_I) + 4J(0)}{7J(\omega_S) + 3J(\omega_I)} \quad (12)$$

4. Conclusions

In this work, we have successfully synthesized and characterized $\text{Fe}(\text{BIP})_3$, a novel homoleptic Fe^{3+} complex functionalized with biocompatible indole moieties. The main findings of this study can be summarized as follows:

- $\text{Fe}(\text{BIP})_3$ was fully characterized by UV-Vis and FTIR spectroscopy, single-crystal X-ray diffraction, and magnetic susceptibility measurements, confirming a high-spin configuration ($S = 5/2$) with an effective magnetic moment of 6.4 Bohr magnetons.
- The complex spontaneously aggregates into morphologically homogeneous, rice-grain-shaped nanoparticles with an average hydrodynamic radius of $\sim 30 \text{ nm}$ when percolated in aqueous solution.
- These metastable aggregates can be stabilized through incorporation into soybean lecithin vesicles, as demonstrated by combined DLS, FCS and FRET experiments.
- Relaxivity measurements at 9.4 T yielded $r_1 = 1.92 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 52.3 \text{ mM}^{-1}\text{s}^{-1}$, with an r_2/r_1 ratio of 27.2, consistent with T_2 contrast agent behavior.

The high r_2/r_1 ratio markedly differs from gadolinium-based contrast agents (GBCAs), which typically exhibit ratios of 1–2, and places $\text{Fe}(\text{BIP})_3$ aggregates in the same class as superparamagnetic iron oxide nanoparticles (SPIONs).

While the endogenous nature of Fe^{3+} and the biocompatible indole ligands represent intrinsic advantages over GBCAs, several in vivo challenges remain to be addressed, including the stability of phospholipid-coated aggregates in biological fluids, protein corona formation, and the metabolic fate of released BIP ligands. Future studies should assess relaxivities in biological media at clinically relevant field strengths (1.5 T and 3 T). The coordinatively saturated Fe^{3+} center implies predominantly outer-sphere relaxation, inherently limiting achievable r_1 values. Future research directions include systematic NMRD studies, ligand modification for improved bioavailability or targeting, comprehensive cytotoxicity and biodistribution studies, and exploration of alternative stabilization strategies such as PEGylation to improve colloidal stability for potential in vivo applications.

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Appendix A

Table A1. Crystal, measurement, and refinement data for the X-ray analysis of $\text{Fe}(\text{BIP})_3$.

	$\text{Fe}(\text{BIP})_3$
Chemical formula	$\text{C}_{63}\text{H}_{51}\text{FeN}_6\text{O}_6 \cdot 2(\text{C}_4\text{H}_9\text{NO}) \cdot \text{H}_2\text{O}$
CCDC number	2483872
M_r	1236.20
Crystal system, space group	Orthorhombic, <i>Pccn</i>
Temperature (K)	100
a, b, c (Å)	11.0479 (2), 27.6020 (6), 20.5711 (3)

Table A1. Cont.

Fe(BIP) ₃	
V (Å ³)	6273.0 (2)
Z	4
Radiation type	Cu K α
μ (mm ^{−1})	2.46
Crystal size (mm)	0.14 × 0.07 × 0.04
Diffractometer	XtaLAB Synergy, single source at home/near, HyPix
Absorption correction	Numerical/face-indexed
$T_{\min}$, $T_{\max}$	0.79, 1.00
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	49455, 5519, 4790
R_{int}	0.146
$(\sin \theta / \lambda)_{\text{max}}$ (Å ^{−1})	0.595
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.069, 0.198, 1.04
No. of reflections	5519
No. of parameters	415
No. of restraints	3
H atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta \rho_{\text{max}}$, $\Delta \rho_{\text{min}}$ (e Å ^{−3})	1.01, −0.66

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