Fully water-soluble polyphosphorhydrazone-based radical dendrimers functionalized with Tyr-PROXYL radicals as metal-free MRI T_1 contrast agents

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ABSTRACT The finding of alternative imaging probes to Gadolinium (Gd) and other metal based contrast agents (CA) is crucial to overcome their established toxicity. Herein we describe the synthesis and characterization of an entirely organic metal-free magnetic resonance imaging (MRI) contrast agent based on polyphosphorhydrazone (PPH) dendrimers, fully functionalized with up to 48 organic nitroxide radical units. We propose an innovative synthetic procedure based on the use of an amino acid linker (Tyr) coupled to each dendrimer's branch that permits the anchoring of the radicals and at the same time makes possible the control over their water solubility. We demonstrate that the negatively charged resulting PPH Gn-Tyr-PROXYL (n=0-3) radical dendrimers are excellent candidates to be used as MRI contrast agents, suited for biomedical applications as they show high water solubility, no aggregation

problems and low cytotoxicity, as well as good stability in highly reducing environments. It was achieved a remarkable r_1 relaxivity *ca.* four times higher ($13 \text{ mM}^{-1}\text{s}^{-1}$) than the gold-standard Gd-DTPA, used in clinics. Furthermore, the r_1 and r_2 relaxivity per unit of radical showed an increase with the increase in generation of dendrimers.

INTRODUCTION

Magnetic resonance imaging (MRI) is a commonly used clinical imaging procedure that provides images of soft tissue anatomy in excellent detail. This method is largely used for non-invasive diagnosis and characterization of tumors and related pathological conditions, as well as for choosing the appropriate cancer treatment approaches. Gadolinium (Gd) based contrast agents (CA) are the most widely used in magnetic resonance imaging. They are categorized as mainly T_1 CA and present high relaxivities due to the high spin of paramagnetic Gd (III) ion (spin 7/2).¹ These CAs have historically been considered as safe, but for more than a decade, a linear relationship between their administration and the development of nephrogenic systemic fibrosis has been recognized in patients with severe renal impairment.²⁻⁴ Recent reports have emerged regarding the accumulation of residual toxic Gd ions in the brain, bones, skin, liver and kidneys of patients with normal renal function and intact blood–brain barrier, following the administration of these Gd-based CAs.⁵⁻⁶ Since the use of CAs in MRI is of vital importance to gain lifesaving clinical information, it is critical to find alternative imaging probes that own the same or even better paramagnetic properties of current Gd-based CAs. Stable organic free radicals, such as nitroxides, have been investigated as T_1 CAs for MRI.⁷⁻¹⁶ They were shown minimal *in vivo* toxicity, in comparison with Gd-based CAs.¹⁷⁻²⁰ Since in the case of nitroxides, MRI contrast is achieved via paramagnetic water relaxation, they could, in principle, be immediately translated to clinical applications. Despite all of these, the widespread application of nitroxides as MRI CAs has been rather limited, due to their inherent low paramagnetic relaxivity and rapid bio-reduction.^{9,21} One strategy to achieve higher molecular relaxivity is through the incorporation of many nitroxyl units to a conventional linear polymer or a hyperbranched

polymer, where the relatively low relaxivity per nitroxide is multiplied by the number of bounded nitroxides.²²⁻²⁵ Moreover, a protective shield effect could be provided to the radicals, by restricting the fast access of reducing agents in such polymers.²⁶ When discussing about hyperbranched polymers it is inevitable to think about dendrimers. Despite their architectural resemblance at first sight, dendrimers differ by the strict control over their structure, making them nearly perfect monodisperse macromolecules. Their tunable size and multifunctionality make them excellent candidates for theranostic applications²⁷ and dual modality bio-imaging.²⁸ Indeed, dendrimers have attracted much interest for the development of new MRI CAs,²⁹ most of them based on the incorporation of Gd chelates in their periphery.³⁰ On the contrary, only few reports describe their functionalization with organic radicals for MRI CA applications,³¹⁻⁴⁰ being most of these works devoted to study their electronic properties.^{31-32,35,37,39-40} In those few reports, PAMAM and PPI dendrimers were functionalized with 2,2,5,5-tetramethylpyrrolidin-1-oxyl (PROXYL) radical units,³⁴ or nitronyl nitroxides (NIT) radicals³⁶ resulting in high paramagnetic systems with a high molecular relaxivity due to the large number of radical units. However, for higher generations (Gn), the relaxivity per radical unit decreases. This fact can be due to aggregation issues promoted by the lower water solubility of larger Gn, what makes radical units inaccessible by water molecules.³³ Indeed, PAMAM and PPI dendrimers have tertiary amines that are incompletely protonated in physiologic pH.

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Aware of the potential of radical dendrimers as an entirely organic metal-free MRI CA alternative, we describe herein the synthesis and structural characterization of polyphosphorhydrazone (PPH) dendrimers peripherally functionalized with PROXYL radical units. Two challenges had to be overcome at the same time, the anchoring of the radical units and confer high water solubility to the systems. Water solubility is a crucial issue usually difficult to solve, especially in large generations, that is sometimes overcome by PEGylation or by having free polar end groups, among others. Here we propose an innovative procedure consisting in the use of an amino acid (Tyrosine, Tyr) as linker between the PPH branches and the radicals. Tyr is coupled to the dendrimers' branches via the phenolic hydroxyl group, providing an available amino group for radical coupling, and a methyl ester group to provide negative charges via their hydrolysis,

which confer high solubility at physiological pH and low toxicity for intravenously administration (Gn-Tyr-PROXYL dendrimers, Figure 1). This synthetic procedure it is also valuable for the anchoring of other kind of groups/molecules and permits to obtain the same molecular structure either soluble in water or not depending on the methyl ester hydrolysis. To summarize, by one hand, the dendrimer scaffold allows a better control over the number of radical units compared with other polymer-based systems, and, on the other hand, the negative charges provided by this new method permits to have at the same time high water-soluble systems. It is worth saying that there are very few reports of PPH dendrimers fully functionalized with radicals, almost all published by the authors, but to our best knowledge, this is the first time that PPH radical dendrimers are being designed for MRI applications. Their magnetic properties were investigated by EPR, followed by the evaluation of their relaxivity, resistance against chemical reduction, cytotoxicity and *ex vivo* assays demonstrating their potential as MRI CAs.

Figure 1. Structures of Gn-Tyr-PROXYL dendrimers with n= 0-3.

RESULTS AND DISCUSSION

Synthesis. The synthetic procedure for Gn-Tyr-PROXYL (with n=0,1,2,3) dendrimers is shown in Scheme 1. The starting point of their synthesis was the hexachlorocyclotriphosphazene (Gn-Cl, n=0) and the P(S)Cl₂ terminated PPH dendrimers (Gn-Cl, n=1,2,3), containing a hexafunctional N₃P₃ core. G1-Cl

dendrimer was obtained and characterized as previously reported,⁴³⁻⁴⁴ while G2-Cl and G3-Cl dendrimers were obtained from Biodendrimers International. Terminal chlorine groups attached to the phosphorus atoms were replaced by units of N-Boc-protected tyrosine methyl ester (Boc-Tyr-OMe), via phenolic groups, in basic conditions using THF as a solvent, to afford Gn-Boc-Tyr-OMe dendrimers. The full functionalization of the dendrimers with Boc-Tyr-OMe groups was verified by ¹H NMR spectroscopy and their purity monitored by size exclusion chromatography (SEC). Subsequently, the amine groups of tyrosine were deprotected with trifluoroacetic acid and coupled with 3-carboxy-PROXYL radicals using HATU as coupling reagent and DIPEA as base in CH₂Cl₂ to obtain Gn-(Tyr-OMe)-PROXYL dendrimers, only soluble in organic solvents. Finally, the methyl esters were hydrolyzed with LiOH in THF:H₂O (2:1), resulting in the water-soluble Gn-Tyr-PROXYL (with n= 0-3) dendrimers, containing theoretically 6, 12, 24 and 48 terminal Tyr-PROXYL radical units and molecular weights ranging from 2261 to 26013 g·mol⁻¹. Gn-Boc-Tyr-OMe dendrimers were purified by precipitation with THF/n-pentane. For G2 and G3, their purification was followed by column chromatography and ultrafiltration, respectively. Ultrafiltration was also used to purify Gn-(Tyr-OMe)-PROXYL and Gn-Tyr-PROXYL dendrimers, with the exception of G1-(Tyr-OMe)-PROXYL dendrimers that were purified by column chromatography. Due to the presence of paramagnetic PROXYL units, the full functionalization of radical dendrimers was verified by EPR and their purity monitored by SEC. Unfortunately, it was not possible to use MALDI-TOF for the validation of the dendrimers' purity due to their high level of fragmentation. Only after different conditions tested, it was possible to observe the quasi-molecular ion for G0-Boc-Tyr-OMe and G0-(Tyr-OMe)-PROXYL dendrimers (Figure S12 and S13 in the Supporting Information). Indeed, as previously reported, MALDI-TOF does not give reliable results in the case of PPH dendrimers.⁴⁵ Fragmentations and rearrangements, in particular at the level of the hydrazone linkages are observed due to the relatively strong UV absorption of these dendrimers at 337 nm (UV MALDI conditions).

Scheme 1. Synthesis of Gn-Tyr-PROXYL dendrimers ($n = 0-3$).

EPR, SEC, DLS, Z-potential and TEM characterization. Considering our goal to obtain dendrimers fully functionalized with nitroxyl radicals, it was crucial to quantify their functionalization by EPR since the presence of paramagnetic units make impossible a quantitative evaluation by NMR. The EPR spectra of 0.21 mM solutions of 3-carboxy-PROXYL and Gn-(Tyr-OMe)-PROXYL dendrimers (CH_2Cl_2 , 300 K) are presented in Figure 2a.

Figure 2. a) X-band EPR spectra (CH_2Cl_2 , 300K) of 0.21 mM solutions of 3-carboxy-PROXYL and Gn-(Tyr-OMe)-PROXYL ($n=0-3$) dendrimers obtained under identical conditions. b) Double integral of the EPR spectra vs number of PROXYL units.

The spectrum of free 3-carboxy-PROXYL presents three resolved sharp lines centered at $g = 2.0062$, with similar intensities with a hyperfine constant of $a_N \approx 14.5 \text{ G}$, due to the coupling between the unpaired electron and the ^{14}N nucleus. In contrast, in the EPR spectra of Gn-(Tyr-OMe)-PROXYL dendrimers appear a broad single line overlapped with the three lines whose

intensity increases as the generations rise until dominate completely the spectral appearance. These broad lines result from the spin exchange and dipole-dipole interactions between neighboring PROXYL radical units.^{31,35,40} The increasing lack of spectral resolution is consistent with the increasing number of nitroxyl end groups that are interacting with each other, with several hyperfine transitions (decrease of the splitting value a_N/n and an increase of the number of lines $2n+1$, where n is the number of interacting radicals).

The calculated double integral of all spectra shown in Figure 2b, carried out at the same concentration and EPR's acquisition parameters, reflects the increase in the number of radical units as generations rises. This correlation resulted in a good linearity with the theoretical number of pendant PROXYL units. The integrals increase nearly the double with the increase of G_n and the integral of G_0 is nearly six times higher than that of 3-carboxy-PROXYL. Considering that the small deviations in the calculated integrals are explainable by the common sources of error in quantitative EPR,⁴⁶ it can be viewed as a practical full functionalization of the dendrimers for all generations, at this synthetic step.

The next preparation stage was the hydrolysis of the methyl ester group of G_n -(Tyr-OMe)-PROXYL dendrimers to obtain the negatively charged water-soluble G_n -Tyr-PROXYL dendrimers. The EPR spectra of 0.21 mM solutions of G_n -Tyr-PROXYL dendrimers in 100 mM phosphate buffer (PB) pH 7.4 at 300 K are presented in Figure 3a. For all generations, the EPR spectra are dominated by a broad single intense line overlapped with the three lines typical of non-interacting nitroxide units.

Figure 3. *a)* X-band EPR spectra (100 mM phosphate buffer pH 7.4, 300K) of 0.21 mM solutions of Gn-Tyr-PROXYL (n= 0-3) dendrimers obtained under identical conditions. *b)* Double integral of the EPR spectra of Gn-Tyr-PROXYL dendrimers (0.21 mM, 100 mM PB pH 7.4, 180 K) vs number of PROXYL units.

The double integral of the EPR spectra was calculated from the experiment at 180 K (Figure 3b and S25) due to the higher signal-to-noise ratio (no interference of the dielectric absorption of water). The very good correlation observed between the number of radical units and the double integral corroborates the complete functionalization of the branches of the dendrimer with the PROXYL radical units. Thus, the hydrolysis step did not affect the content of nitroxyl radicals at the periphery. SEC chromatography confirmed the purity of such radical dendrimers (Figure 4).

Figure 4. Normalized SEC chromatograms of Gn-Tyr-PROXYL (n= 0-3) dendrimers in water.

All generations of both families of radical dendrimers, Gn-(Tyr-OMe)-PROXYL and Gn-Tyr-PROXYL, presented an EPR $|\Delta m_s| = 2$ transition at half-field in frozen conditions. This signal is characteristic of dipolar coupled spins and gives a direct evidence of the presence of a high-spin. As it was observed in diluted conditions, its origin is due to intramolecular dipolar interactions, as expected for the proximity of the radical units. The intensity of such a signal increases generation by generation by a factor of 2, observing also a very good direct proportionality between the $|\Delta m_s| = 2$ signal intensity and the number of radicals of each generation (Figure S26 in the Supporting Information).

The average hydrodynamic diameter of Gn-Tyr-PROXYL dendrimers, determined by DLS in similar conditions (Table 1 and Figures S27) ranged from 2.8 nm for G0-, to 4.8 nm for G3-Tyr-PROXYL, being similar to other water-soluble dendrimers in the same range of molecular weight.⁴⁷⁻⁴⁸ It is worth saying that none of the Gn-Tyr-PROXYL dendrimer generations showed significant aggregation at physiological pH (< 0.2 % by volume). This is also in accordance with the Z-potential data obtained (Table 1) which reflects the good stability in solution of such species. We also obtained TEM images of G3-Tyr-PROXYL using the negative stain technique with

Uranyl acetate (see the SI), observing a very homogeneous particle size distribution with an average diameter of $d = 4.86 \pm 0.05$ nm, which is consistent with the data obtained by DLS.

Figure 5. TEM image of G3-Tyr-PROXYL dendrimers using the negative stain technique with Uranyl acetate, $d = 4.86 \pm 0.05$ nm.

Table 1. Hydrodynamic diameters of Gn-Tyr-PROXYL dendrimers (100 mM phosphate buffer (PB) pH 7.4 at 300 K) shown in the Z-average with standard deviation values (N = 5) and their corresponding Z-potential in water.

Compound	Hydrodynamic diameters (nm)	Z-potential (mV)
G0-Tyr-PROXYL	2.81 ± 0.58	-57,6
G1-Tyr-PROXYL	3.44 ± 0.75	-68,7
G2-Tyr-PROXYL	4.23 ± 1.30	-84,1
G3-Tyr-PROXYL	4.78 ± 1.23	-59,2

Rate of PROXYL reduction. The effect of the dendritic scaffold on nitroxyl rate of reduction was investigated under pseudo-first-order conditions, using a 10-fold excess of sodium ascorbate per PROXYL unit in 100 mM PB pH 7.4 at 300 K. The reduction was monitored by EPR

spectroscopy following the decrease of the area under the spectra as a function of time (Figure 6a) until 7.5 h. As reference, the rate of reduction of 3-carboxy-PROXYL was determined under identical conditions. The reduction of PROXYL groups did not show simple first-order kinetics.⁴⁹⁻⁵⁰ It was possible to identify different stages of reduction, with different kinetics (Table S1). The first stage, where the reduction of more than 50% of the available PROXYL units (half-life) happened, presented a faster kinetics. Interestingly, the half-life time of PROXYL units increased progressively with the increase in generation number, as observed in Figure 6b, being in the case of G3-Tyr-PROXYL a remarkable four times higher than that of free 3-carboxy-PROXYL radical. This impressive increase of resistance against chemical reduction could be explained by conformation changes with the increase of generation. It is known that dendrimers progressively change from an open to a more globular architecture, due to the possibility of back-folding of the terminal groups. As a result, peripheral nitroxyl groups in larger generations are less easily accessible by the ascorbate ions, making their chemical reduction more difficult.

Figure 6. a) Reduction kinetics of 2 mM of PROXYL unit with 20 mM of ascorbate, of 3-carboxy-PROXYL and Gn-Tyr-PROXYL dendrimers in 100 mM phosphate buffer (pH 7.4) at 300 K, measured by EPR during the first 20 min. (the lines are guide to the eyes). b) Half-life times calculated from the first stage of reduction (30 min – 1 h of reduction).

Relaxivity measurements. The r_1 and r_2 relaxivity constants per nitroxyl radical units were obtained at 7 T in 100 mM phosphate buffer pH 7.4 and 300K (Table 2). The plots of proton relaxation rates $^1\text{H } R_1$ and $^1\text{H } R_2$ ($R_n = 1/T_n$) of water molecules versus the nitroxyl units' concentration were linear for all the agents, over the studied concentration range (0.5-10 mM) (Figure S28). Interestingly, on the contrary to the works reported previously based on PPI and PAMAM nitroxyl terminated dendrimers,^{34,36} we did not observe a decrease in r_1 and r_2 up to G3 (48 terminal nitroxyl groups) but an increase (r_1 0.27 $\text{mM}^{-1}\text{s}^{-1}$ for G3 vs 0.20 $\text{mM}^{-1}\text{s}^{-1}$ for free PROXYL radical). This improvement in relaxivity is most probably due to the higher solubility of the negatively charged Gn-Tyr-PROXYL dendrimers at physiological pH. In all generations, the ratio r_1/r_2 was near 1, what makes favorable their use as T_1 imaging CA. The presence of a high number of paramagnetic nitroxide units in the dendrimer's periphery resulted in high molecular relaxivities (Figure 7). While G1-Tyr-PROXYL dendrimer presents a similar relaxivity (2.9 $\text{s}^{-1}\text{mM}^{-1}$) than the most widely used GdCA in clinics Magnevist (Gd-DTPA, 3.2 $\text{s}^{-1}\text{mM}^{-1}$ r.t. 7T), one molecule of G3-Tyr-PROXYL dendrimer presents a relaxivity of 13 $\text{s}^{-1}\text{mM}^{-1}$ that means a remarkably 4 times higher value than Gd-DTPA. Furthermore, the control over the size of the dendrimers, opens the opportunity to modulate their distribution profile in the body, which is impossible in the case of Gd-DTPA.

Table 2. Longitudinal r_1 and transverse r_2 relaxivity constants per nitroxyl radical unit determined at 7T in 100 mM phosphate buffer pH 7.4, 300K.

Compound	r_1^{7T} ($\text{mM}^{-1}\text{s}^{-1}$)	r_2^{7T} ($\text{mM}^{-1}\text{s}^{-1}$)
3-carboxy PROXYL	0.20±0.014	0.23±0.06
G0-Tyr-PROXYL	0.23±0.014	0.24±0.08

G1-Tyr-PROXYL	0.24±0.007	0.29±0.05
G2-Tyr-PROXYL	0.24±0.008	0.29±0.03
G3-Tyr-PROXYL	0.27±0.009	0.33±0.09

Figure 7. Longitudinal (r_1) and transverse (r_2) relaxivity per molecule of Gn-Tyr-PROXYL dendrimers determined at 7T in 100 mM phosphate buffer pH 7.4, 300K.

Cytotoxicity and *ex vivo* MRI analysis. To assess the toxicity of Gn-Tyr-PROXYL dendrimers, *in vitro* cell viability assays were conducted with a fetus normal lung tissue cell line (MRC-5). In these assays, the cells were incubated with Gn-Tyr-PROXYL dendrimers (n=0-3) at different concentrations ranging from 0.016 to 2mM per radical unit (Figure 8) for 24 and 48 h. Cell viability was determined by the MTT assay that measure cell activity produced by the mitochondrial compartment. As a result, Gn-Tyr-PROXYL dendrimers (n=0-3) did not show any cytotoxicity *in vitro*, in the tested concentration range (see Figure 8).

Figure 8. *In vitro* cell viability assays conducted with the fetus normal lung tissue cell line (MRC-5) incubated with Gn-Tyr-PROXYL dendrimers (n=0-3) in a concentration of 2mM per radical unit for 24 and 48 h.

It is known that nitroxides on their own are not considered cytotoxic¹⁷⁻²⁰ and negatively charged dendrimers do not interact with biological environment, since they are repelled by the negatively charged cell membrane.⁵¹ The combination of these two attributes is confirmed by our results, giving biocompatible dendrimers better suited for clinical applications.

In view of the excellent *in vitro* MRI results obtained with PPH Gn-Tyr-PROXYL dendrimers, we proceeded to perform post mortem *ex vivo* MRI analysis studies in mice following an *ex vivo* method developed to select CAs with good potential for *in vivo* efficiency.⁵² G3-Tyr-PROXYL that presented the best *in vitro* relaxivity was the dendrimer of choice to make such evaluation, and it was compared with the gold-standard Gd-DTPA. When G3-Tyr-PROXYL was locally injected in the brain parenchyma there was a clear relative contrast enhancement (RCE) in that area, in such environment (see Figure S29) and it was needed 4 times less amount of G3-Tyr-PROXYL dendrimers than Gd-DTPA (Magnevist®) to produce the same calculated RCE (see

Table S2). This result was in agreement with the *in vitro* MRI results obtained (relaxivity r_1 of G3 4 times higher than Gd-DTPA).

Conclusions. Four novel generations of polyphosphorhydrazone (PPH) dendrimers fully functionalized with 6, 12, 24 and 48 pendant PROXYL radical units were prepared via an innovative synthetic procedure. The use of a Tyrosine amino acid (Tyr) in each PPH branch allowed us at the same time the anchoring of the radical units and the control over their water solubility. By one hand, we obtained four radical dendrimers generations only soluble in organic solvents (Gn-(Tyr-OMe)-PROXYL, $n = 0-3$) while after their methyl ester hydrolyzation the resulting negatively charged Gn-Tyr-PROXYL radical dendrimers ($n = 0-3$) were highly water-soluble. EPR was the technique of choice to verify the complete functionalization of the dendrimers with nitroxyl radicals. NMR was used to characterize all the prior synthetic steps. Dendrimer purity was monitored by SEC. The entirely organic metal-free water-soluble PPH Gn-Tyr-PROXYL dendrimers did not show significant aggregation (< 0.2 % by volume in DLS) at physiological pH, what we believe is the main reason for not observing a decrease in relaxivity per unit of radical with the increase of generation, as was previously described in literature. G3-Tyr-PROXYL dendrimers showed a high r_1 relaxivity, *ca.* four times larger in comparison to Gd-DTPA, used in clinics. Similar results were obtained in the *ex vivo* MRI studies.

Our water-soluble PPH based Gn-Tyr-PROXYL dendrimers offered to the pendant radicals a higher stability (four times higher half-life for G3-Tyr-PROXYL) against reduction as shown by the EPR chemical reduction study with ascorbate ions, a higher paramagnetic relaxivity and low cytotoxicity, demonstrating they are excellent candidates to be used as MRI contrast agents suited for biomedical applications. Furthermore, the control over the structure of these dendrimers, opens

the window of opportunity for a future modulation of their biodistribution profiles which are in progress.

ASSOCIATED CONTENT

Supporting Information. Materials and methods, synthesis and characterization of all radical dendrimers, additional EPR spectra, SEC chromatograms, DLS analysis, rates of nitroxide reduction with ascorbate and relaxivity plots. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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