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## Dextran-conjugated tetrathiatriarylmethyl radicals as biocompatible spin probes for EPR spectroscopy and imaging

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### Abstract

Tetrathiatriarylmethyl (TAM) radicals represent soluble paramagnetic probes for biomedical electron paramagnetic resonance (EPR)-based spectroscopy and imaging. There is an increasing demand in the development of multifunctional, biocompatible and targeted trityl probes hampered by the difficulties in derivatization of the TAM structure. We proposed a new straightforward synthetic strategy using click chemistry for the covalent conjugation of the TAM radical with a water-soluble biocompatible carrier exemplified here by dextran. A set of dextran-grafted probes varied in the degrees of Finland trityl radical loading and dextran modification by polyethylene glycol has been synthesized. The EPR spectrum of the optimized macromolecular probe exhibits a single narrow line with high sensitivity to oxygen and has advantages over the unbound Finland trityl of being insensitive to interactions with albumin. *In vivo* EPR imaging of tissue oxygenation performed in breast tumor-bearing mouse using dextran-grafted probe demonstrates its utility for preclinical oximetric applications.

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Synthesis of the first organic free radical, triphenylmethyl, was reported by Gomberg in 1900<sup>1</sup>. By the late 1990s, these compounds with sterically protected trivalent carbon regained attention as the core structural fragment for the synthesis of stable organic radicals. Nycomed Innovation designed the sterically crowded trityl radicals, TAMs (tetrathiatriarylmethyl), in order to avoid hydrogen hyperfine coupling and enhance radical stability and water solubility<sup>2-3</sup>. Currently TAMs represent one of the major classes of soluble paramagnetic probes characterized by extraordinary stability toward tissue redox processes, long relaxation time and narrow line width making them particularly attractive for electron paramagnetic resonance (EPR)-based spectroscopy and imaging applications<sup>3-7</sup>. Figure 1 shows the most popular structures of TAM oximetric probes, Finland trityl (FTr)<sup>8-9</sup> and OX063<sup>4</sup>, their deuterated derivatives<sup>5, 10-12</sup>, and the recently synthesized multifunctional monophosphonated probe, HOPE<sup>13-14</sup> (sensor for pH, Oxygen and

Phosphate in Extracellular microenvironment). Wide application of the highly hydrophilic TAM probes, Ox063<sup>4</sup> and its deuterated analog, Ox071<sup>12</sup>, is hampered by the lack of affordable large-scale syntheses and difficulties in functional derivatization of the core structure for extended multifunctional applications.

The recent progress in the development of multifunctional<sup>6, 15–17</sup> (e.g., HOPE, Figure 1) and targeted trityl probes<sup>18–21</sup> is mostly associated with the use of the Finland trityl core structure allowing for a large range of synthetic modifications. However, the relatively lipophilic nature of the aryl core of the Finland trityl is responsible for its hydrophobic interaction with bio-macromolecules such as albumin<sup>22</sup>, resulting in a signal loss and preventing systemic delivery of the corresponding spin probes.

Here we report a proof-of-concept of a new strategy for the development of multifunctional, biocompatible and targeted TAM structures based on covalent conjugation of the deuterated Finland radical core (dFTr) with a water-soluble biocompatible carrier. Scheme 1 illustrates this strategy utilized in the current work for the dextran polymer grafted with dFTr and polyethelene glycol (PEG), the latter allowing for the enhancing aqueous solubility of the probe. Previously, dextran has been widely explored as biocompatible carrier for probe and drug conjugation<sup>23</sup>.

The dextran biopolymer with an average molecular weight of 20 kDa was functionalized through the etherification of the alcohol groups of the polysaccharide with 1-azido-2,3-epoxypropane (see Scheme 1)<sup>24</sup>. According to quantitative <sup>13</sup>C NMR spectral analysis illustrated in Figure 2, about 8% of 125 sugar units of 20 kDa dextran chain were modified with 1-azido-2-hydroxypropyl chains, therefore resulting in total in 10 azide groups per dextran molecule.

Dextran grafting by the dFTr radical was achieved by a copper-catalyzed azide-alkyne cycloaddition (CuAAC) of a TAM mono propargyl ester **1** followed by addition of excess of commercially available alkyne-PEG (1 kDa, see Scheme 1 and SI). The reaction crude was easily purified by dialysis, and the completion of the reaction was confirmed by the disappearance of the azide peak on the IR spectrum (2110 cm<sup>-1</sup>). The TAM mono propargyl ester **1** was synthesized by esterification of dFTr using a deuterated propargyl tosylate **2** as depicted in Scheme 2 (see SI for the details).

Taking into account that all ten azide groups of azidified dextran were modified with 1 kDa dFTr or by 1 kDa PEG, the resulting grafted dextran has a molecular weight of approximately 30 kDa and its composition is described by the general formula Dextran-TAM<sub>x</sub>PEG<sub>10-x</sub>. To study the structure-function relationship of new macromolecular spin probes, we synthesized a set of the Dextran-TAM<sub>x</sub>PEG<sub>10-x</sub> that range from low to high trityl radical loading (see Table 1 and SI for the details).

An average number of TAM radicals bound to one dextran molecule, *x*, was measured using UV absorbance of Dextran-TAM<sub>x</sub>PEG<sub>10-x</sub> at 490 nm (characteristic for trityl radical, 16000 cm<sup>-1</sup>M<sup>-1</sup>) and supposing molecular weight of the grafted dextran equal to 30 kDa based on the initial molecular weight of 20 kDa and additional 10 azide sites modified by 1 kDa substitutes, dFTr or PEG. Figure 3 shows reverse phase HPLC chromatograms and UV

spectra of the integrated peak of TAM mono-propargyl ester **1** and Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> sample (low TAM loading).

Figure 4 shows the L-band EPR spectra of two Dextran-TAM<sub>x</sub>PEG<sub>10-x</sub> probes with low and intermediate TAM loading. An increase in spin density from  $x=3.6$  (Figure 4A) to  $x=6.5$  (Figure 4B) did not significantly affect linewidth but resulted in an increase of signal intensity of about 1.6 times. Further increase in spin density in the Dextran-TAM<sub>8,1</sub>PEG<sub>1,9</sub> probe resulted in the strong broadening effect of its L-band EPR spectrum apparently due to intramolecular spin-spin interaction, therefore we excluded this probe from the following studies. EPR spectra of both Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> and Dextran-TAM<sub>6,5</sub>PEG<sub>3,5</sub> demonstrated similar linear dependences on oxygen concentration (Figure 4C and 4D) making them useful oxygen-sensitive spin probes. Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> demonstrated an important advantage of lacking an interaction with albumin (cf. red and black EPR spectra in Figures 4A) compared with significant albumin-induced line broadening for TAM<sub>6,5</sub>PEG<sub>3,5</sub> (Figure 4B) apparently due to a larger number of hydrophilic PEG chains. Therefore, Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> probe has been selected for testing its ability for tissue oxygen mapping, *in vivo*.

Figure 5A shows the oxygen distribution in a breast cancer tumor measured in PyMT tumor-bearing mouse using rapid scan 800 MHz EPR imager after intratissue injection of the Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> probe. The histogram of  $pO_2$  distribution (Figure 5B) clearly shows the presence of normoxic and hypoxic areas characteristic for highly heterogeneous tumor microenvironment<sup>5, 25-26</sup>. Interestingly, the intensity of the EPR signal was not significantly decreased over more than 1 hour, suggesting comparatively slow probe clearance from the tumor tissue of the anesthetized animal.

In summary, we synthesized a series of new dextran-conjugated trityl probes varied in the degree of Finland trityl radical loading and dextran modification by polyethylene glycol using a straightforward click chemistry approach. An optimized dextran-conjugated trityl probe demonstrated advantage over free Finland trityl in biocompatibility being more hydrophilic and lacking interaction with albumin. The probe exhibits an oxygen-sensitive narrow EPR spectral line allowing for *in vivo* oxygen mapping. The proposed strategy based on easy covalent conjugation of the deuterated Finland radical core with water-soluble biocompatible carriers such as dextran or chitosan, may result in the development of multifunctional<sup>6</sup>, biocompatible and targeted TAM structures with potentially favorable/tunable pharmacokinetics. It can be easily extended toward incorporation into the biopolymer structure of multifunctional trityls<sup>6</sup>, targeting moieties<sup>19</sup> and/or therapeutic agents for the synthesis of theranostic probes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

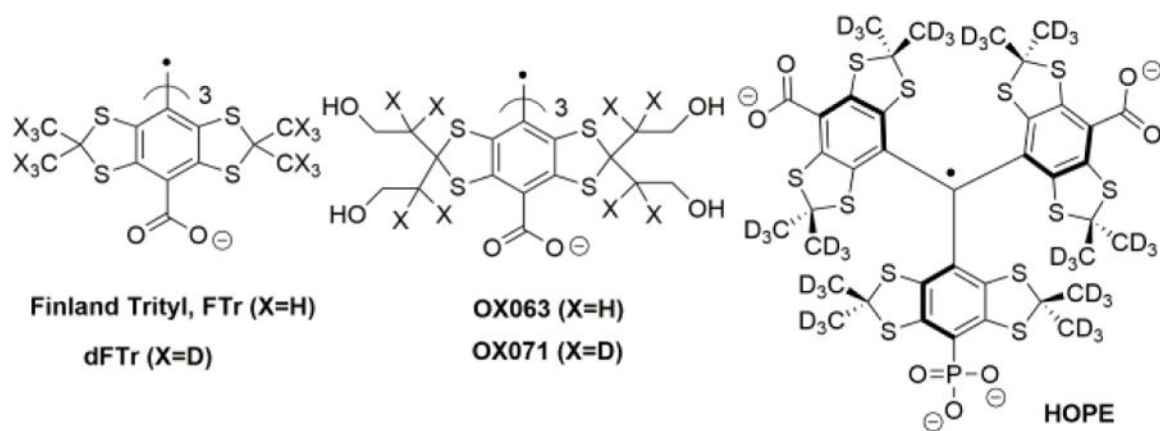
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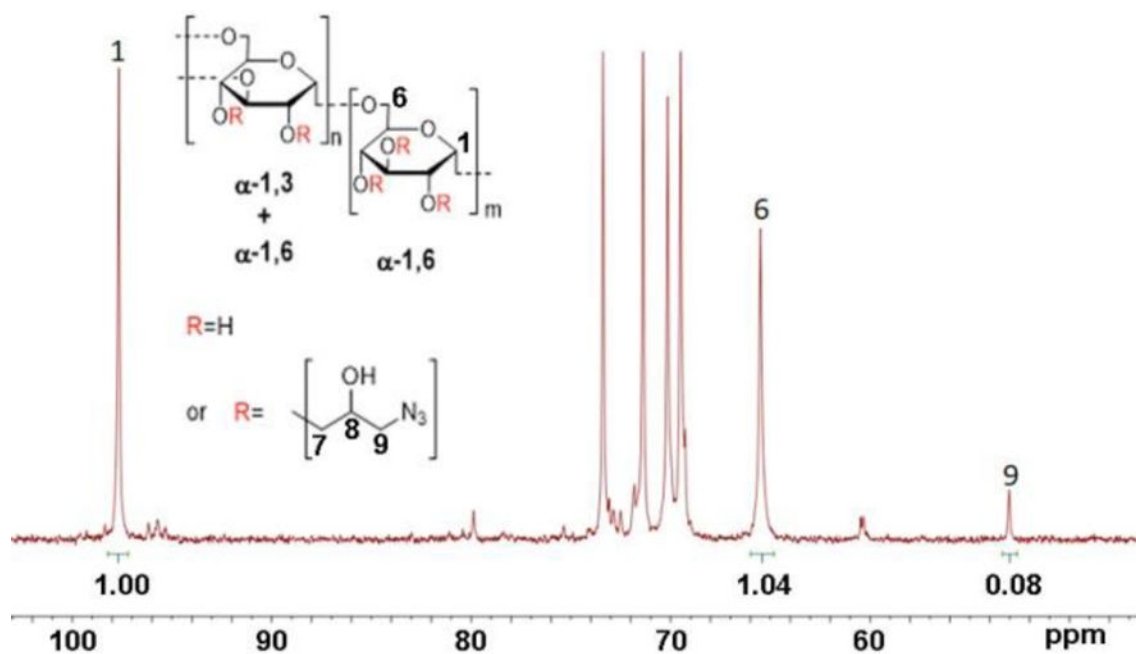
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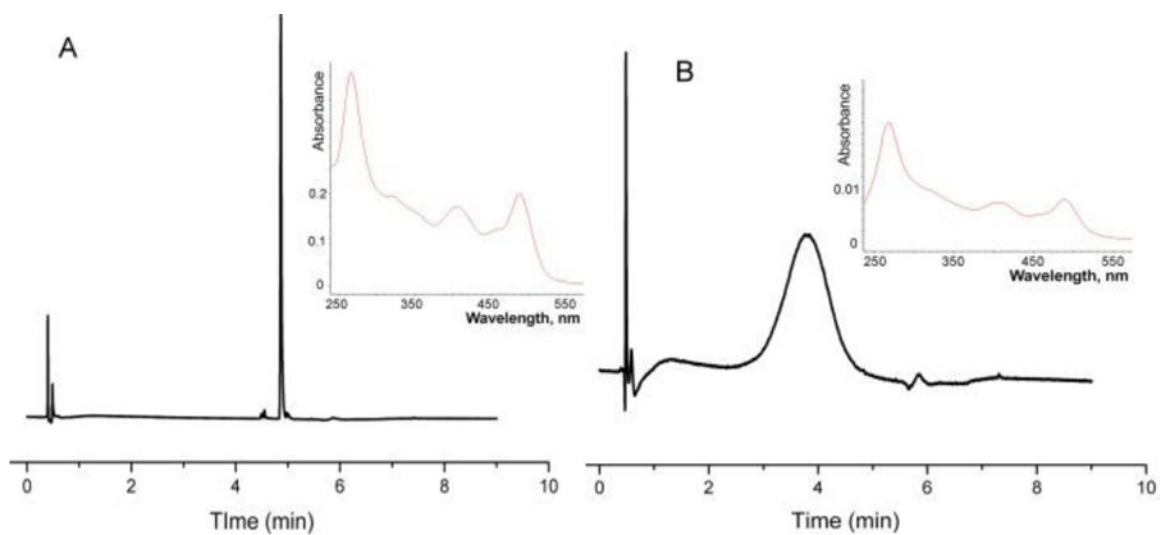


**Figure 1.**  
Representative structures of TAM radicals.



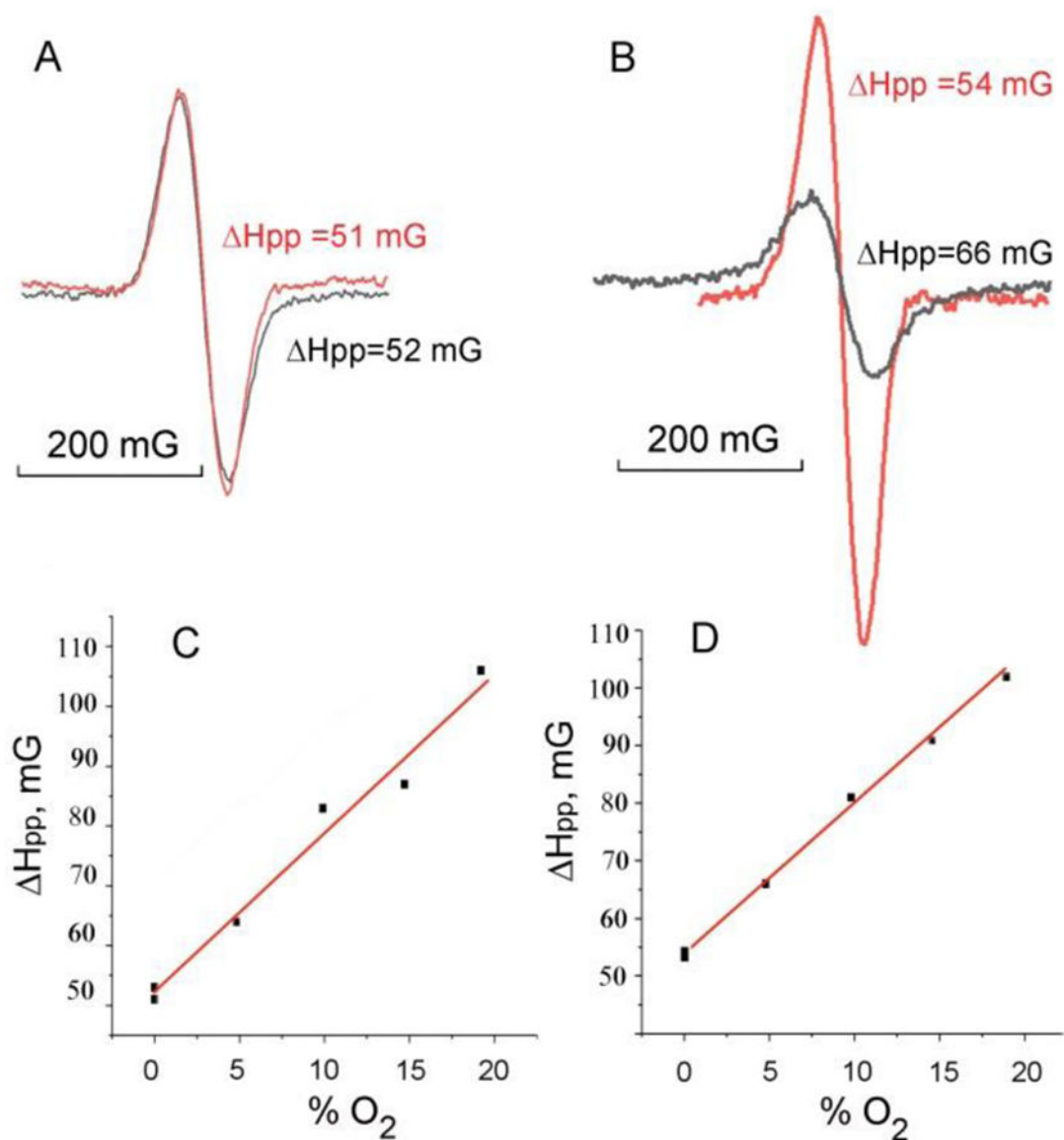
**Figure 2.**

$^{13}\text{C}$  NMR spectrum of azidified dextran. Ratio of integrals of the peaks 9 and 1 gives the fraction of modification of sugar units by azide groups. Acquisition parameters: 100 MHz,  $\text{D}_2\text{O}$ , time delay=15 s, 8000 scans. *Insert:* schematic structure of azidified dextran showing  $\alpha$ -1,6 glycosidic linkages between glucose monomers with branches from  $\alpha$ -1,3 linkages. The numbering of the carbon in the dextran structure corresponds to the corresponding peak numbering in the NMR spectrum.



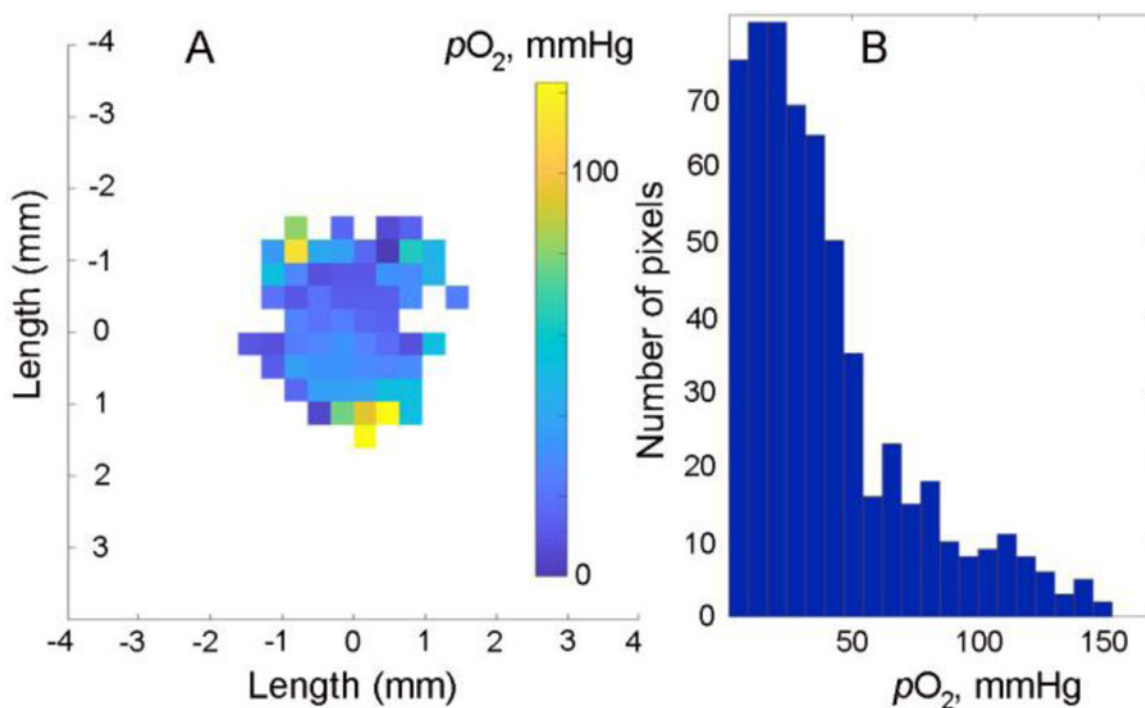
**Figure 3.** HPLC chromatograms of (A) TAM mono-propargyl ester radical and (B) dextran grafted with TAM radicals and PEG chains. *Inserts:* the UV spectrum of the main peak for each chromatogram (See SI for details).





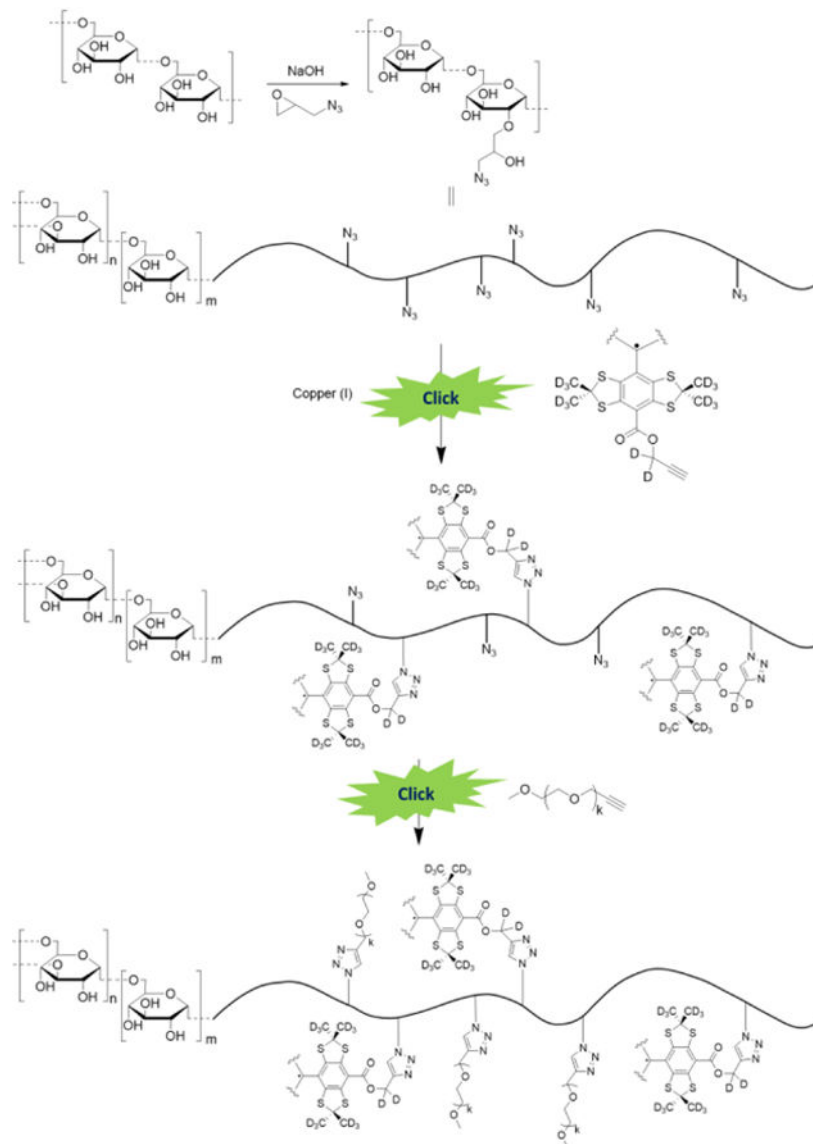
**Figure 4.**

L-band (1.2 GHz) EPR spectra of Dextran-TAM<sub>3,6</sub>PEG<sub>6,4</sub> (A) and Dextran-TAM<sub>6,5</sub>PEG<sub>3,5</sub> (B) spin probes, and the corresponding dependences of their linewidth on oxygen concentration, (C) and (D). The spectra were measured for 0.5 ml samples of 65  $\mu$ M solutions of the probes in deoxygenated 100 mM phosphate buffer in the absence (red lines) and presence (black lines) of 500  $\mu$ M bovine serum albumin. The values of the peak-to-peak linewidth,  $H_{pp}$ , are shown near the spectra. The linear fits of the linewidth dependences on oxygen yield the spectral sensitivities to oxygen being equal to 2.7 mG/(% O<sub>2</sub>) (C) and 2.6 mG/(% O<sub>2</sub>) (D). The spectrometer settings were as follows: sweep time, 30 sec, sweep width, 480 mG; modulation amplitude, 30 mG; modulation frequency, 100 kHz; power attenuation, 15 dB.



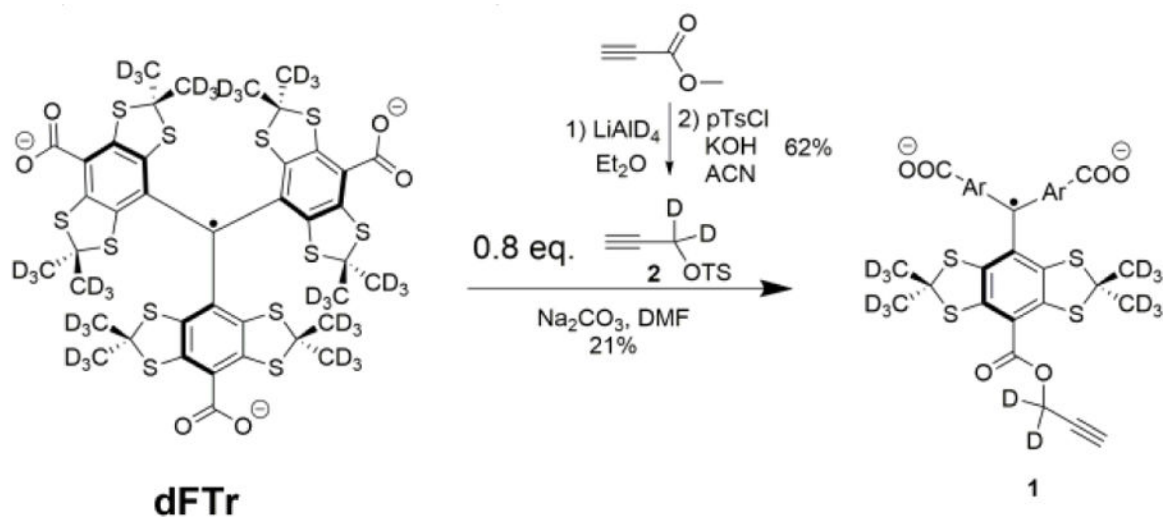
**Figure 5.**

*In vivo* 4D (1-spectral-3-spatial) rapid scan 800 MHz EPR image of oxygen distribution in a breast tumor of a PyMT tumor-bearing mouse. An image acquisition was started 25 minutes after intratissue injection of 20  $\mu$ l solution of 0.75 mM probe in 10 mM phosphate buffered saline; tumor volume, 214  $\text{mm}^3$ . Data acquisition parameters were as follows: acquisition time, 16.5 minutes; number of projections, 2546; rapid scan frequency, 9.4 kHz; and maximum gradient, 3G/cm. An integral intensity threshold of 30% was implemented to remove low signal-to-noise data. **A.** A two-dimensional slice (xz-plane) of the image is shown. **B.** A histogram of  $pO_2$  distribution within the entire image.



**Scheme 1.**

Schematic representation of the synthesis of dextran-conjugated TAM radicals using a click chemistry approach. The dextran grafting by the dFTr radical was achieved by copper-catalyzed azide-alkyne cycloaddition (CuAAC) of a TAM mono-propargyl ester followed by addition of excess of alkyne-PEG. Varying the trityl radical/dextran ratio allows for the tuning of the radical loading onto the polymer. Incorporation of PEG chains enhances the solubility of the grafted dextran and prevents spin-spin interactions between trityl radicals.



**Table 1.**

Experimental conditions and final trityl radical loading, x, for a series of grafted dextran probes, Dextran-TAM<sub>x</sub>PEG<sub>10-x</sub>\*.

Loading	TAM	Dextran-N <sub>3</sub>	PEG	x
Low	3 mg	20 mg	60 mg	3.6
Medium	6 mg	20 mg	60 mg	6.5
High	12 mg	20 mg	60 mg	8.1

\* synthesized according to the Scheme 1 (see SI for details).

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