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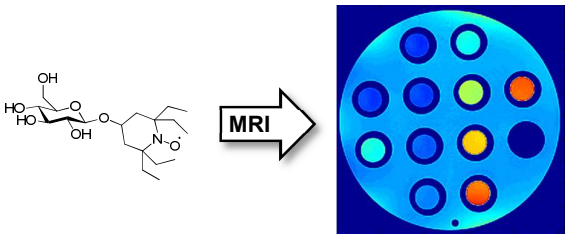

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# Synthesis and *in vitro* phantom NMR and MRI studies of fully organic free radicals, TEEPO-glucose and TEMPO-glucose, potential contrast agents for MRI

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M. Soikkeli,[a] K. Sievänen,[a] J. Peltonen,[b] T. Kaasalainen, [b] M. Timonen,[b] P. Heinonen,[a] S. Rönkkö,[c] V.-P. Lehto, [c] J. S. Kavakka[a] and S. Heikkinen*[a]

**Two organic radical contrast agents, TEMPO-Glc and TEEPO-Glc, were synthesized and their stabilities and contrast enhancing properties were tested with *in vitro* NMR and MRI experiments. Owing to the glucose moieties in the prepared compounds, this study presents potential candidates for a tumor targeting fully organic contrast agents for MRI.**

Noninvasive imaging of living tissue is of vital importance in modern-day medicine. Positron emission tomography (PET) is an imaging technique producing images of accumulating radioactive tracers in tissue pathologies, like tumors.[1] However, it does not produce anatomical images, and thus, PET-data is typically co-registered with computer tomography (CT) images. In PET, the radio-active tracer (often $^{18}$F) is usually covalently attached to a tumor targeting glucose or thymine moiety ($^{18}$F-FDG and $^{18}$F-FLT, respectively).[2] Although PET-CT delivers excellent tracer accumulation images, the imaging facility is very expensive and only available in a few strategic hospitals. Also, due to radioactive decay, the radio tracer is harmful to healthy tissue. Many of these challenges can be overcome with magnetic resonance imaging (MRI). MRI instruments are readily available in most of the hospitals and institutes in developed countries. The superior MRI image quality of soft tissue, together with the harmlessness to the patient, has made MRI one of the most important medical imaging techniques in the world.[3]

Contrast agents are commonly utilized in MRI studies to improve image contrast between diseased and healthy tissue. The majority of FDA-approved MRI contrast agents are based on Gd(III) complexes in addition to few superparamagnetic iron oxide (SPIO) agents.[4] However, free transition metals, such as gadolinium and iron, are highly toxic in human body. Yet, the chelate structure of the contrast agent minimizes the risk of the release of the metal. Nevertheless, gadolinium based contrast agents have been shown to have a tendency to cause nephrogenic systemic fibrosis for patients with renal dysfunctions albeit nowadays the risk is extremely small.[5] Although Gd(III) contrast agents are highly important and have good track record in tumor diagnostics, they are neither specifically targeting to malignant tissue nor they are actually cell permeable.[6]

Fully organic nitroxide radicals have been studied as potential contrast agents for MRI already in the 1980s.[7] However, at that time the research ceased as the used nitroxides showed rapid reduction caused by natural reductants, like ascorbic acid, present in human body. As a result of reduction to corresponding hydroxylamine, the molecule loses its paramagnetic character and hence the ability to act as an MRI contrast agent. The stability of nitroxide radicals can be significantly improved, for instance, by delocalization of the unpaired electron or by steric hindrance. An example of the latter case is the replacement of the methyl substituents of the well-known stable nitroxide, TEMPO, by bulkier ethyl substituents leading to drastically increased resistance towards reduction.[8, 9]

Quite recently, different nitroxides have been successfully utilized in MRI to study for instance the blood-brain-barrier permeability,[10] the tissue redox-state[6, 11] and the contrast enhancement[12, 13]. In addition, they have been applied in EPR imaging.[14] However, to the best of our knowledge, currently there are no tumor targeting nitroxide contrast agents extensively studied or commercially available. These kinds of substances could possibly be achieved by attaching a radical moiety covalently to a targeting unit, analogically to PET and SPECT (single photon emission computed tomography) tracers.

In the current study, two different, potentially tumor targeting nitroxide radical contrast agents (Figure 1) were synthesized. The nitroxide moieties TEMPO and TEEPO were chosen due to their

reported good stability and also due to their relatively small size. Small size of the relaxation providing moiety could be considered as a benefit when attaching it to targeting unit. It could be hypothesized that too bulky radical moiety attached to a targeting unit could interfere the targeting properties as well as cell or blood-brain barrier permeability. Glucose (Glc) was selected as targeting unit based on its successful use in $^{18}$F-FDG PET. Both TEMPO-Glc and TEEPO-Glc were prepared in order to compare their stabilities, the latter assumed as the more stable one.

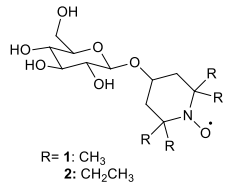
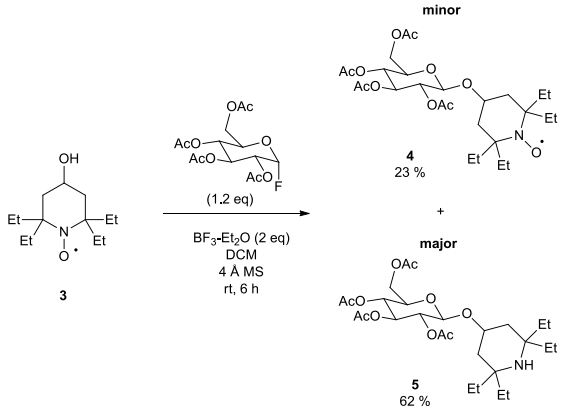



Figure 1. Structures of the radicals TEMPO-Glc (1) and TEEPO-Glc (2)

For the synthesis of product 1 the precursor (4-hydroxy-TEMPO) was readily available from commercial sources. In the case of product 2, the precursor 3 (4-hydroxy-TEEPO) was synthesized as described in literature[15-17] with slight modifications. The glycosylations of the nitroxide precursors were performed according to Sato et al.[18] using fluorinated glucose as the donor and boron trifluoride diethyl etherate as the promoter. The fluorinated glucose compound was prepared by using HF pyridine.[19] The deacetylations of glycosylated nitroxides were performed either by standard Zemplén de-O-acetylation[20] or conveniently by aqueous ammonia solution[21]. In the glycosylation reaction of 4-hydroxy-TEEPO (3), the yield remained low due to futher reduction of 4 to amine 5. The initial idea was to recycle amine 5 back to nitroxide 4 by oxidation. Disappointingly, neither hydrogen peroxide with sodium tungstate dihydrate as catalyst nor *m*-chloro-peroxybenzoic acid produced the desired nitroxide 4. As nitroxides with bulky side chains are resistant towards reduction, consequently the oxidation of the corresponding amine to nitroxide is difficult.




Scheme 1. The product distribution of the glycosylation reaction of 4-hydroxy-TEEPO

As linking nitroxides to glucose seems to have a slight effect on the stability of the compounds[22], radical stabilities of TEMPO-Glc and TEEPO-Glc were studied by $T_1$-measurements as a function of time elapsed from the sample preparation. Human blood plasma was considered to be reasonable matrix to mimic *in vivo* conditions. The measurements were performed at 37 °C using 500 MHz NMR spectrometer. Both compounds were able to prevail several days in blood plasma without losing the capability to shorten water proton relaxation time $T_1$. The results are shown in Supporting Information (Table S1).

More demanding stability tests in presence of a reductant were also performed. Samples with a radical concentration of 5 mM containing 10 µl of $H_2O$ in 600 µl $D_2O$ were prepared. To the samples, a 10-fold excess of ascorbic acid was added and a set of rapid $T_1$-measurements utilizing DESPOT-method (Driven Equilibrium Single Pulse Observation of $T_1$)[23] was immediately initiated. As a result, TEEPO-Glc showed superior stability towards reduction (Figure 2). The relaxing properties of TEEPO-Glc remained effective at least 2-3 hours in 10-fold ascorbic acid excess, whereas TEMPO-Glc was completely reduced after 10 minutes. The results agreed well with the results by Paletta et al.[8], where the ethyl substituted nitroxide, 4-hydroxy-TEEPO, was found to be significantly more stable than the methyl substituted counterpart, 4-hydroxy-TEMPO.

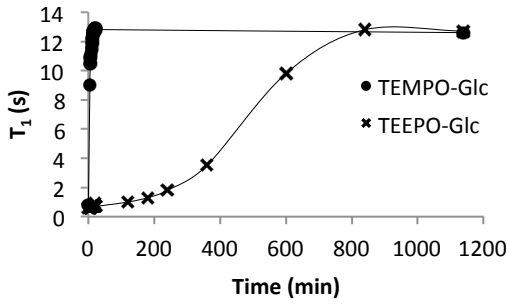



Figure 2. The $T_1$-relaxation times of water in the presence of the nitroxides as a function of time starting from the addition of the 10-fold excess of reductant, ascorbic acid. TEMPO-Glc is completely reduced after 10 min (filled circle), whereas it takes more than 15 hours to completely reduce TEEPO-Glc (cross).

The water proton $T_1$-relaxation time reducing properties of TEEPO-Glc and TEMPO-Glc in blood plasma vs. radical concentration were studied using $^1$H NMR spectroscopy at 11.7 T (1H-frequency 500 MHz, 37 °C). The 11.7 T results presented in Figure 3 show, that both radicals enhance water $R_1$-relaxation rate ($R_1 = 1/T_1$) in blood plasma. As expected, the relaxation rates increase linearly as a function of radical concentration. Linearity suggests the absence of aggregation in the concentration range studied.[12]

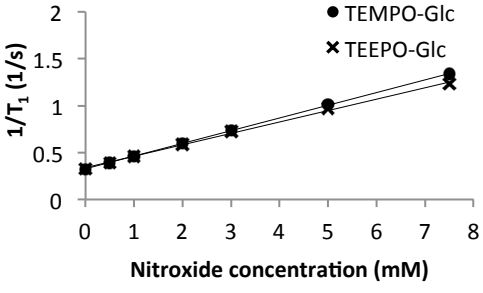



Figure 3. The blood plasma water $R_1$-relaxation rates ($R_1 = 1/T_1$) measured at 11.7 T magnetic field strength ($^1$H-frequency 500 MHz) as a function of nitroxide concentration. The data were measured at 37 °C temperature.

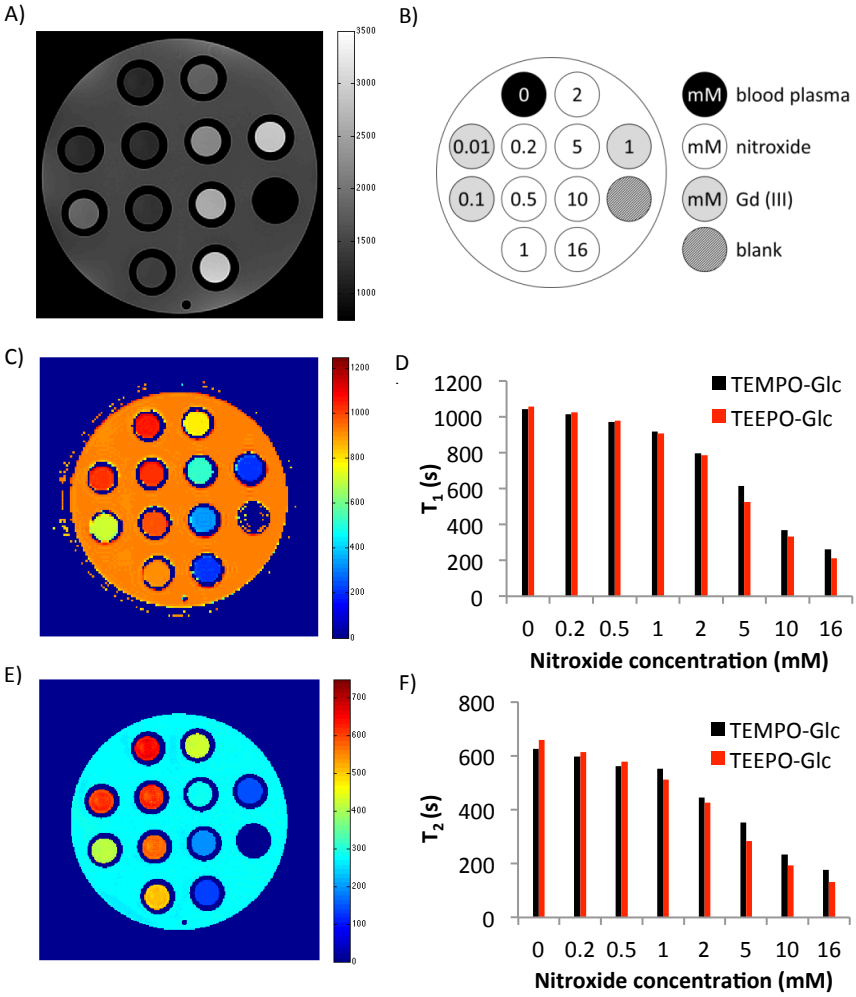



Figure 4. A) 1.5 T $T_1$-weighted gradient echo MR-image of the phantom (90° flip angle, echo time 4.76 ms and repetition time 400 ms) B) The setup and composition of the MRI phantom. The number indicates the concentration (mM) of the contrast agent. C) The 1.5 T $T_1$-map of the TEEPO-Glc phantom. D) The water $T_1$-values of blood plasma vials with varying radical concentrations for both TEMPO-Glc and TEEPO-Glc phantoms (black and red bars, respectively). E) The 1.5 T $T_2$-map of the TEEPO-Glc phantom. F) The water $T_2$-values of blood plasma vials with varying radical concentrations for both TEMPO-Glc and TEEPO-Glc phantoms (black and red bars, respectively).

MRI tests were performed to study the effect of the contrast agents to the relaxation times of water in blood plasma at a clinical MRI magnetic field strength of 1.5 T. Concentrations of 0.2, 0.5, 1, 2, 5, 10 and 16 mM were selected for the phantom. As a reference, three samples of clinically used contrast agent, gadopentetate dimeglumine, were also included. As the relaxation is more effective with agents containing Gd(III) due to the total of seven unpaired electrons in contrast to the one single unpaired electron in the prepared radicals, the concentrations of gadolinium samples were significantly smaller, 0.01, 0.1 and 1 mM. The composition of the phantom, $T_1$-weighted gradient echo MRI, the $T_1$- and $T_2$-maps of the phantoms as well as the $T_1$- and $T_2$-relaxation times at different concentrations are illustrated in Figure 4. In order to emphasize the differences between different concentrations, the $T_1$- and $T_2$-maps are presented in color. It appears, that when the radical concentration exceeds 0.5 mM, the resulting intensity difference in the relaxation time images between undoped and doped vial starts to be visually detectable and at concentration of 1.0 mM the difference is very clear for both $T_1$- and $T_2$-maps. This can also be seen in $T_1$-weighted gradient echo image, especially after 1 mM. This is even more prominent when color scale is used (Electronic Supplementary Information, Figure S1). The obtained results suggest the potentiality of the compound as MRI contrast agent, since similar *in vivo* concentrations have been previously detected for other radical compounds.[24] Furthermore, although the relaxivity of the radical is significantly lower than that of Gd(III)-based contrast agents, it may be hypothesised that the presence of the targeting group might ensure a sufficient radical concentration in the target tissue and thus produce a proper contrast without extreme dosing.

Also, preliminary elucidation of cytotoxicity effects of TEEPO-Glc was performed using HeLa cells. Different concentrations of TEEPO-Glc (0.2, 1 and 10 mM) were incubated for 1, 6 and 24 h and their cytotoxicity was studied using CellTiter-Glo® assay (Figure 5). The assay is based on quantitation of ATP and the amount of ATP is directly proportional to the number of living cells. The results of CellTiter-Glo® assay showed that the very high concentration of TEEPO-Glc (10 mM) and with long incubation time (24 h) decreased significantly cellular viability ($p < 0.05$) when compared to unexposed controls. However, toxicity was not

observed at shorter incubation times (1 h and 6 h). And furthermore, all other tested concentrations of TEEPO-Glc (0.2 mM and 1 mM) with time points at 1, 6 or 24 h did not affect the viability of HeLa cells.

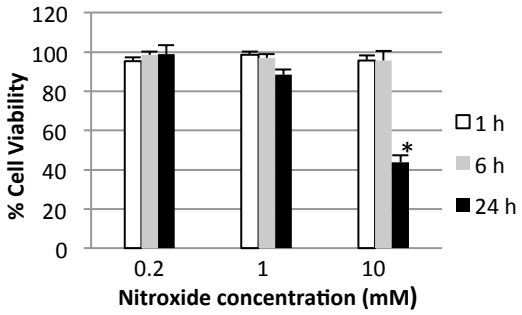



Figure 5. Effect of TEEPO-Glc on HeLa cell viability assayed by CellTiter-Glo® assay kit (ATP measurement). The results (mean ± SD, n = 4) were compared to untreated control cells whose viability was set at 100 %. The level of significance was set at a probability of $p < 0.05$ (*) when compared with untreated control cells (Kruskal-Wallis with Dunnett's test).

## Conclusions

In summary, we have synthesized two nitroxide radicals, TEMPO-Glc and TEEPO-Glc capable of shortening water proton relaxation times significantly at relatively low concentrations. This was demonstrated with phantom studies performed with in vitro NMR and MRI experiments. In addition both of the nitroxides were stable in blood plasma at 37 °C, whereas experiments performed in more harsh conditions using ascorbic acid as a reductant highlighted TEEPO-Glc as the more stable one. This can be acknowledged to the four ethyl substituents providing steric shelter to the unpaired electron in the structure. Additionally, the cytotoxicity study showed that TEEPO-Glc is relatively noncytotoxic and thus further toxicity and *in vivo* studies are relevant and required for the evaluation of performance. Together these features suggest that TEEPO-Glc could be further studied as a potential tumor targeting contrast agent for MRI. Search for alternative synthesis routes with better yields is currently underway. In all, the proposed nitroxide radicals could provide a valuable addition to medical imaging and diagnostics.

## Notes and references

[a] Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014 University of Helsinki, Finland.
[b] HUS Helsinki Medical Imaging Center, P.O. Box 340, FIN-00029 HUS, Helsinki, Finland.
[c] Department of Applied Physics, University of Eastern Finland, P.O. Box 1627, FIN-70211 Kuopio, Finland.

† Electronic Supplementary Information (ESI) available: [Synthesis and characterization of the compounds, description of NMR/MRI parameters used in stability and relaxivity studies and the method for the cell viability study are described]. See DOI: 10.1039/c000000x/

1. M. M. Ter-Pogossian, M. E. Phelps, E. J. Hoffman and N. A. Mullani, *Radiology*, 1975, **114**, 89-98.
2. W. Chen, T. Cloughesy, N. Kamdar, N. Satyamurthy, M. Bergsneider, L. Liau, P. Mischel, J. Czernin, M. E. Phelps and D. H. S. Silverman, *J. Nucl. Med.*, 2005, **46**, 945-952.
3. A. G. Filler, *Internet J. Neurosurg.*, **7**.
4. Molecular Imaging and Contrast Agent Database (MICAD) Internet. Bethesda (MD): National Center for Biotechnology Information (US); 2004-2013. http://www.ncbi.nlm.nih.gov/books/NBK5330/.
5. I. M. Braverman, *F1000 Med. Rep.*, 2010, **2**.
6. K. Matsumoto, F. Hyodo, A. Matsumoto, A. P. Koretsky, A. L. Sowers, J. B. Mitchell and M. C. Krishna, *Clin. Cancer Res.*, 2006, **12**, 2455-2462.
7. V. Afzal, R. C. Brasch, D. E. Nitecki and S. Wolff, *Invest. Radiol.*, 1984, **19**, 549-552.
8. J. T. Paletta, M. Pink, B. Foley, S. Rajca and A. Rajca, *Org. Lett.*, 2012, **14**, 5322-5325.
9. T. Yamasaki, F. Mito, Y. Ito, S. Pandian, Y. Kinoshita, K. Nakano, R. Murugesan, K. Sakai, H. Utsumi and K.-i. Yamada, *J. Org. Chem.*, 2011, **76**, 435-440.
10. Z. Zhelev, R. Bakalova, I. Aoki, K.-i. Matsumoto, V. Gadjeva, K. Anzai and I. Kanno, *Chem. Commun.*, 2009, 53-55.
11. F. Hyodo, R. Murugesan, K.-i. Matsumoto, E. Hyodo, S. Subramanian, J. B. Mitchell and M. C. Krishna, *J. Magn. Reson.*, 2008, **190**, 105-112.
12. A. Rajca, Y. Wang, M. Boska, J. T. Paletta, A. Olankitwanit, M. A. Swanson, D. G. Mitchell, S. S. Eaton, G. R. Eaton and S. Rajca, *J. Am. Chem. Soc.*, 2012, **134**, 15724-15727.
13. M. C. Emoto, K.-i. Yamada, M. Yamato and H. G. Fujii, *Neurosci. Lett.*, 2013, **546**, 11-15.
14. X. Wang, M. Emoto, A. Sugimoto, Y. Miyake, K. Itto, M. Amasaka, S. Xu, H. Hirata, H. Fujii and H. Arimoto, *Tetrahedron Lett.*, 2014, **55**, 2146-2149.
15. T. Schulte, K. O. Siegenthaler, H. Luftmann, M. Letzel and A. Studer, *Macromolecules*, 2005, **38**, 6833-6840.
16. C. Wetter, J. Gierlich, C. A. Knoop, C. Muller, T. Schulte and A. Studer, *Chem-Eur. J.*, 2004, **10**, 1156-1166.
17. K.-i. Yamada, Y. Kinoshita, T. Yamasaki, H. Sadasue, F. Mito, M. Nagai, S. Matsumoto, M. Aso, H. Suemune, K. Sakai and H. Utsumi, *Arch. Pharm.*, 2008, **341**, 548-553.
18. S. Sato, T. Kumazawa, S. Matsuba, J.-i. Onodera, M. Aoyama, H. Obara and H. Kamada, *Carbohyd. Res.*, 2001, **334**, 215-222.
19. A. Steinmann, J. Thimm, M. Matwiejuk and J. Thiem, *Macromolecules*, 2010, **43**, 3606-3612.
20. F. Cinget, D. Gagnaire and P. J. A. Vottero, *J. Carbohyd. Chem.*, 1992, **11**, 921-931.
21. T. Neilson and E. S. Werstiuk, *Can. J. Chem.*, 1971, **49**, 493-499.
22. S. Sato, M. Yamaguchi, A. Nagai, R. Onuma, M. Saito, R. Sugawara, S. Shinohara, E. Okabe, T. Ito and T. Ogata, *Spectrochim. Acta Mol. Biomol. Spectros.*, 2014, **124**, 322-327.
23. J. Homer and M. S. Beevers, *J. Magn. Reson. (1969)*, 1985, **63**, 287-297.
24. F. Hyodo, K.-H. Chuang, A. G. Goloshevsky, A. Sulima, G. L. Griffiths, J. B. Mitchell, A. P. Koretsky and M. C. Krishna, *J. Cereb. Blood F. Met.*, 2008, **28**, 1165-1174.

