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Nuclear singlet multimers (NUSIMERS) with long-lived singlet states†

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Magnetic resonance (NMR) is a powerful tool in chemical analysis, structure determination and in medical diagnostics. Developing novel biological sensors for this field holds promise to better investigate protein structures or target diseases more efficiently. Herein, we explore nuclear spin singlet states in dendritic macromolecules as a platform molecule to develop stimuli responsive probes. We have developed a nuclear singlet multimer (NUSIMER) based on a generation 5 poly(amidoamine) dendrimer (PAMAM) which contains on average about 90 accessible nuclear spin singlet states with lifetimes up to 10-fold longer than the T_1 relaxation times (up to 10 seconds T_s vs. $T_1 < 0.5$ seconds) in a single molecule. We demonstrate little influence on the singlet lifetime in phosphate buffer (H_2O) and a high viscosity gel environment in the presence of paramagnetic oxygen. Additionally, we demonstrate an increase in singlet lifetime upon the release of a protective chemical moiety from the NUSIMER following a stimulus, whereby no change in longitudinal relaxation time is observed. The robustness and change in singlet lifetime of the NUSIMER holds promise for the development of a novel type of biosensors.

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Introduction

Nuclear spin singlet states are spin states that are not directly observable in NMR. However, observable magnetization can be transformed and stored in a singlet state and retrieved back at a later time.^{1–31} In a singlet state, two given $I = 1/2$ nuclei are brought into a state in which they couple into an effective spin 0 state. Spins in such a state are immune to intramolecular dipole–dipole relaxation, which is one of the main nuclear spin relaxation mechanisms.³¹ This can lead to long equilibration times between singlet and triplet state which we refer to as singlet lifetimes T_s . They can be significantly longer than the corresponding spin-lattice relaxation times T_1 . Long T_s can be extremely useful in hyperpolarized NMR, in which nuclear spin polarization is dramatically enhanced and more than four orders of magnitude in signal-to-noise ratio can be gained.³² Singlet states were used to observe slow processes on timescales longer than it was normally accessible by standard NMR methods, including the diffusion processes of certain compounds in solution or changes in the close surroundings of the observed compound.^{6,10,16} Although the singlet lifetime depends on the rotational correlation time of the molecule under study (and therefore small molecules are typically

investigated), it was shown that singlet states between proton pairs can be populated in proteins.^{7,33} This is probably due to the local degree of freedom in motion. First steps have been made to develop nuclear spin singlet states into biological probes.^{16,34,35} In order to develop a bio-probe, sensitivity needs to be high enough that the singlet states can be observed at the location of interest in an organism. Hyperpolarization may be used to boost the NMR signal, and so far minute long singlet lifetimes were observed in organic solvent or in the absence of oxygen *in vitro*.^{8,13,23,25} Observing protein binding has so far been the main biological-oriented application of singlets but due to the short T_s of protons and fluorine nuclei used in these experiments, long traceability *in vivo* may not be possible.^{16,17,29} In order to circumvent sensitivity issues we have designed macromolecules based on a dendrimer in which an average of 90 pairs of nearly equivalent protons per molecule are present. Our specific goal here is not to hyperpolarize the compound but rather rely on thermal polarization and potentially increase the detections sensitivity by averaging. Hyperpolarization may however become an option in the future upon the discovery of *e.g.* molecules with hour-long singlet states that can be attached to the here proposed structures. The proton pairs in the dendrimers can be simultaneously toggled between magnetization and singlet states, allowing for the detection of a double digit μM concentration of the macromolecules in a two-scan experiment. Proton pairs were chosen since they have a proven versatility and a high gyromagnetic ratio for increased NMR sensitivity.³⁶ The robustness of the proton singlet state was investigated by exposing them to paramagnetic oxygen in air, non-deuterated solvents/buffers and to a high viscosity

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agarose/PBS gel, the T_s times are longer than T_1 . The relaxation times in agarose are comparable to those measured in PBS solution, which is true for the NUSIMERS as well as for the free tripeptides (Table 1). The measured relaxation times for the free tripeptides at 37 °C are longer than the ones of the respective NUSIMERS. In D_2O solution, the singlet lifetime for the free tripeptide **1** was $T_s = 13$ s whereas **2** showed a $T_s = 10.2$ s. The T_1 values are significantly shorter, $T_1 = 0.90$ s for **1** and $T_1 = 0.43$ s for **2**, respectively. The T_s in the two different NUSIMERS are influenced quite significantly by the presence of the Boc protection group. The removal of this group decreases the singlet life-time from $T_s = 10.2$ s for **2** to $T_s = 6.9$ s for **4** whereas the T_1 values are not influenced, see Table 1. Such behavior is accentuated in PBS solution. The singlet state in the NUSIMER containing the Boc protection group **2** is not accessible anymore in aqueous PBS. This accessibility tuning by ions is not observed in compound **4**. Upon cleavage of the Boc group in non-degassed PBS solution, the singlet state becomes accessible again and a $T_s = 1.81$ s was obtained whereas the longitudinal relaxation changes within 20% ($T_1 = 0.45$ s and 0.35 s). The influence of the agarose gel environment on T_1 and T_s is within 20% as well (Table 1). Furthermore we observed that T_s measurement without decoupling in **4** did not change and was $T_s = 6.89$ s. This has been shown to not be the case in weakly coupled systems.³¹

A stimulus responsive probe

These observations lead us to propose a stimulus responsive bio-probe based on the design and experiments shown in Fig. 3. Here a protection group is cleaved in presence of a stimulus, triggering the NUSIMER accessibility and modifying singlet lifetimes. In the herein investigated case, detection of an acidic

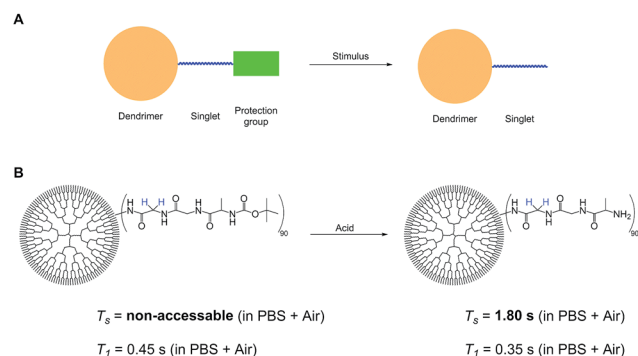


Fig. 3 (A) Removal of the Protection group with a stimulus frees the singlet state containing chain thus making the "hidden" singlet state accessible. (B) Treatment of **2** with acid removes the protection group to form **4**. The singlet state becomes accessible while the T_1 changes within 20%.

environment is possible by T_s time measurements. Other possible stimuli include interaction with certain proteins as well as ion detection by accessibility of certain singlet states. Further investigations are currently conducted in our group. With a view on potential translatability in the future, MRI experiments in mouse models have been performed by an intravenous injection of 4 mg of PAMAM to acquire good images.⁵⁰ Toxicity studies have shown that in particular hydroxyl terminated dendrimers are non-toxic in mice even in doses over 400 mg kg^{-1} .⁵¹ This amount of substrate is much higher than investigated here. Although *in vivo* singlet-MRI is still a long path to go, our presented dendrimers promise a large step towards this direction.

Conclusions

In summary, we have shown that by adding a glycine-glycine-alanine sequence on the terminal groups of a G5-PAMAM dendrimer, conditions are met that allow for populating on average 90 long lived nuclear spin singlet states per molecule at the same time. The NUSIMER long-lived states remain stable in non-degassed PBS environment as well as in an agarose gel used to simulate brain tissue, suggesting potential applications in biomedical imaging. The significant change in specifically singlet lifetimes upon the cleavage of the Boc protection group makes NUSIMERS highly interesting as possible stimuli-responsive biosensors. This effect cannot be observed in the longitudinal relaxation. The chemical shift proximity of the proton pair signal makes decoupling for singlet maintenance unnecessary. These properties, along with detectability in μM concentrations, make the NUSIMERS possible candidates for biological applications. Here, we have demonstrated the concept of NUSIMER in a group of highly versatile macromolecular compounds with conceivable biological application in the future.

Conflicts of interest

There are no conflicts to declare.

Table 1 Singlet state lifetimes T_s and relaxation times T_1 of different samples in different solvents. Solvents have not been degassed prior to measurements

Compound ^a	Solvent	T_1 [s] ^b	T_s [s] ^c
1	D_2O	0.90 ± 0.04	13 ± 2
1	H_2O (PBS)	0.70 ± 0.07	3.87 ± 0.06
1	Agarose (PBS)	0.6 ± 0.1	3.1 ± 0.2
2	D_2O	0.43 ± 0.01	10.2 ± 0.9
2	H_2O (PBS)	0.45 ± 0.01	n.a.
2	Agarose (PBS)	0.38 ± 0.26	n.a.
3	D_2O	1.60 ± 0.02	30 ± 3
3	H_2O (PBS)	1.54 ± 0.17	7.8 ± 0.6
3	Agarose (PBS)	1.14 ± 0.09	8.59 ± 0.06
4	D_2O	0.49 ± 0.01	6.9 ± 0.6
4	D_2O	0.49 ± 0.01	6.9 ± 0.5^d
4	H_2O (PBS)	0.35 ± 0.01	1.8 ± 0.2
4	Agarose (PBS)	0.43 ± 0.08	1.5 ± 0.1

^a Concentrations of the free tripeptides are 12.8 mM. Concentrations for the NUSIMERS are 100 μM . ^b T_1 relaxation times have been determined using the inversion recovery experiment. Two samples have been measured for each compound and the average is represented here. ^c T_s have been determined using the SLIC experiment⁴⁷ with additional phase cycling.⁴⁸ The average over two samples is represented in the table. ^d Measured values without applying a spin-locking field.



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