Magnetogenic Probes

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Magnetogenesis under Physiological Conditions with Probes that Report on (Bio-)Chemical Stimuli**

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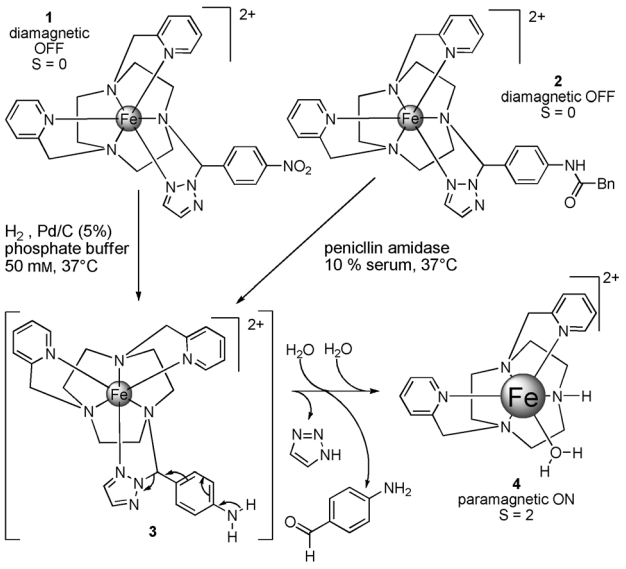
Chemists have long sought to devise particles or molecules that report on a particular chemical or physical stimulus by emitting an exploitable signal.[1] Those species that actively respond to the stimulus by a change in signaling activity have attracted particular attention because they offer better signal-to-background ratios and thus much improved detection sensitivity. Carrying this trend to the extreme would require a reporter molecule that is initially silent. We have introduced the concept that the sleeping electronic spin of a coordination compound may be awakened upon chemical modification of its periphery by a targeted chemical stimulus.[2,3] Iron(II) chelates can be made to adopt a spin of zero or two by only minor modification of their coordination motif from N6 to N5,O1. Such an alteration may be envisaged to occur via the opening of a non-coordinative bond of a chelate ring. The mono-dentate ligand thus created may then relatively easily exchange with the bulk solvent. However, the cleavage of a chelate ring is a thermodynamically highly unfavorable operation, and achieving this at constant environmental conditions constitutes a particular challenge. It is for this reason that the sole attempt to design a *chemo-sensing* (anion-sensing) coordination compound that reports by switching its spin state relied exclusively on the associative interaction of the analyte with the periphery of the respective coordination compound and consequently resulted in a markedly restricted magnetometric effect, not to mention the important limitation to organic and aprotic solvents.[4,5] In contrast, reversible spin switching triggered by physical stimuli has been reported on numerous occasions for the solid state (spin crossover)[6–8] but much less so in solution.[9–11]

To detect chemically transforming analytes, an irreversible concept is required, and we recently reported such a probe responding to a reductive agent in water.[12,13] However, while its robustness and signal-to-background ratio (off–on) were perfectly satisfactory, the probe prototype became paramagnetic only at a pH value lower than 3.5 and responded only under relatively harsh conditions. Water is generally regarded as a challenging medium, but has great relevance for many practical applications. Imposing on this system a particular pH value, especially the generally accepted physiological one of 7.4, puts even more constraints on the practicality of a given probe design. We now present a molecular concept that allows for the conversion of a diamagnetic coordination compound into a paramagnetic one triggered by either a chemical reagent or an enzyme. The two probes **1**[14] and **2** (Scheme 1) are operative at simulated physiological conditions, that is, aqueous, buffered medium at pH 7.4 and 37 °C, and in serum. They can be prepared by a straightforward procedure consisting of a two-step/one-pot multi-component reaction.



Scheme 1. Diamagnetic probes **1** and **2**, the respective (bio-)chemical stimuli to which they respond, and their magnetogenic mechanism.

Probes **1** and **2** are binary ferrous complexes of a macrocyclic hexadentate N6 ligand, including two picolyl pendent arms, which ensures high stability and a low-spin, diamagnetic state. The third pendent arm carries the trigger unit recognized by the targeted chemical stimulus as well as the spacer moiety relaying the event of chemical conversion to the coordination sphere. This process leads to loss of the entire pendent arm by eliminative and hydrolytic processes and turns the remaining chelate irreversibly into a paramagnetic species. The considerable stabilization contributed by metal chelation to an otherwise labile molecular moiety requires departure from familiar concepts of auto-immolative spacers as have been introduced since the late sixties first for the design of prodrugs[15–17] and much more recently for activat-

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able pro-fluorescent probes.[18–21] In fact, a spring-loaded system has to be devised that stores considerable potential energy. This energy can be used to overcome the chelate effect when the chosen stimulus has caused the chemical modification of the periphery of the complex. The challenge consists of obtaining this high-energy content without a) losing the ability to control it with a blocking group (the trigger that reacts with the analyte) and b) losing the possibility to observe an initially robust probe. Targeting aqueous media for any application of such probes requires leaving the familiar realm of solvents such as toluene, dimethylsulfoxide, or acetonitrile and coping with the highly protolytic nature of water.

We are not aware of any report on the deliberate exploitation of the intrinsic fragmentability of an aminal (N/N acetal) moiety, whether comprising an azole moiety[22,23] or not, as a component of a metal chelate ring. We chose to incorporate an azole[24] to benefit from its leaving-group character and give favorable fragmentation rates. Once the target analyte has transformed the trigger, the resulting aniline (**3**) can be regarded as part of a phenylogous N/N/N *ortho*-amide, an unstable moiety. In fact, it is this phenylogy that permits the introduction of a blocking group that can be recognized by a (bio-)chemical stimulus. A previous concept of ours involving a more stable phenylogous hemiaminal (N/O acetal) proved to be inadequate to overcome the chelate effect.[25] As much can be said about a non-phenylogous aminal (N/N acetal) for another coordination compound.[26] To grasp the true challenge of observing fragmentation, it must be remembered that low-spin ferrous complexes are generally taken as kinetically inert, that is, ligand substitution on the central metal atom is slow.[27] The electronic communication between the amino group of **3** generated by the target stimulus and the azole should then allow for the efficient expulsion of the azole (Scheme 1) and subsequent hydrolytic cleavage of the remainder of the pendent arm. The end result is ternary complex **4** which has a water molecule in its first coordination sphere, a situation that has been shown to make the iron center high spin ($S=2$) and paramagnetic.[3]

Monitoring the incubation of probes **1** and **2** with their respective stimuli (catalytic hydrogenation and penicillin amidase) has been carried out by several forms of independent readout: a) direct mass analysis (Supporting Information Figure S7 and S16), b) the change in magnetic susceptibility by $T1$ measurement by an NMR spectrometer (Figure 1),[28] c) the change in magnetic susceptibility by $T1$ measurement this time translated into gray-scale images ($T1$-weighting) generated by an MRI instrument (Figure 2), d) HPLC with UV monitoring (Figure S9), and e) by UV spectral analysis (Figure S8 and S15). A five minute treatment of a sample of **1** in saline phosphate buffer with hydrogen gas in the presence of palladium on carbon (Pd/C), and subsequent filtration, results in total conversion into aniline **3** as borne out by mass analysis (Figure S7). This aniline then converts spontaneously into **4** over the course of 120 min. When the same test is conducted in the NMR spectrometer (at 4 mM), the longitudinal relaxation time of surrounding water hydrogen atoms ($T1$ values, Figure 1) evolves from that of pure buffer (3.5 s, "OFF") to 0.35 s ("ON"), a value measured before in comparable media.[3] This effect amounts to a $T1$ decrease of 80–90%, while the ratio $(T1_{ON}-T1_{media})/(T1_{OFF}-T1_{media})$ approaches infinity in view of the absence of a contribution by intact **1** to the medium. The reaction proceeds with a half time of 25 min under these conditions. Unsurprisingly, the incubation of probe **2** with immobilized penicillin amidase (Waterstone technologies 260 U.I/g) is found to pass through the same intermediate **3**. The response rate is insignificantly smaller than that for **1** with a half time of 60 min (Figure 1).

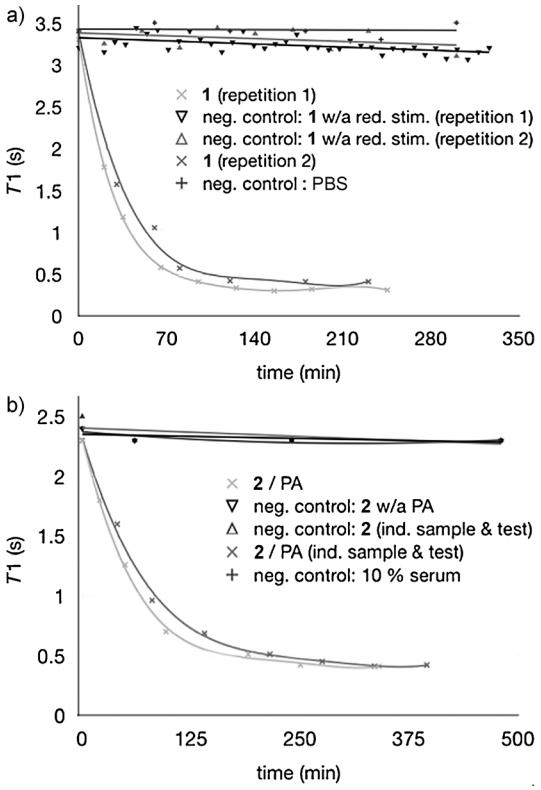


Figure 1. Top: $T1$ monitoring (500 MHz spectrometer, 11.4 Tesla) of time-dependent magnetogenesis in a sample containing **1** (4 mM; 50 mM PBS; 37°C) as initiated by catalytic hydrogenation over 5 min. Bottom: $T1$ monitoring of a sample of **2** (4 mM; 10% serum; 37°C) incubated with immobilized penicillin amidase (PA; The two curves are from two independent sample preparations). $T1$ is the longitudinal relaxation time of the hydrogen nuclei in the surrounding H_2O molecules.

The evolution of magnetic susceptibility of a sample of **1** (test tube) can be advantageously illustrated by preparing gray-scale images with the help of an MRI instrument (Figure 2). Initially, the sample cannot be discriminated (OFF) from a sample of pure buffer that serves as negative control. After 5 min hydrogenation and subsequent filtration the sample quickly undergoes a transformation that results in ever brighter gray scales corresponding to decreasing $T1$ values measured by the MRI instrument (see also Figure S14). The important magnetization of the final sample can be appreciated when comparing to the gray scale generated by a strongly paramagnetic lanthanide complex ([Gd(DOTA)]; at the same concentration) that has seven unpaired electrons in place of the four contained in iron(II) complex **4**.[3] Equally important is the aspect of total

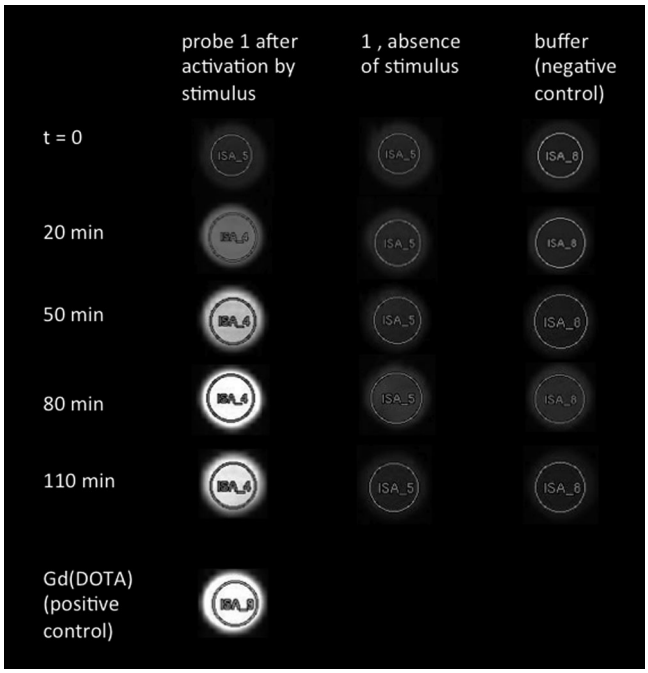
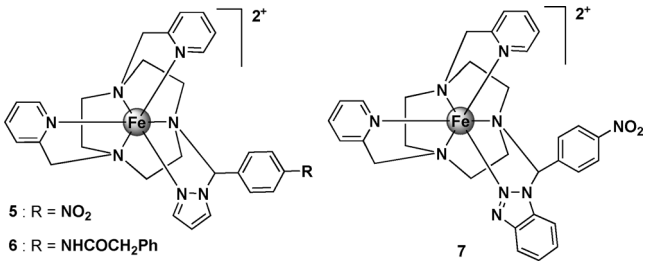


Figure 2. illustrates the time-dependent magnetogenesis in the form of gray-scale levels generated by an MRI device at high field (7 Tesla; *T*1-weighting). Conversion of **1** (4 mM; 50 mM PBS; 37 °C) is initiated by catalytic hydrogenation.

conversion (binary response) of the population of **1** and **2** into **4**. The fragmentation of probe **1** has also been monitored by HPLC/UV analysis (Figure S9) and confirms the direct conversion of **1** via **3** into **4**. Finally, UV/Vis monitoring displays an isosbestic point thus showing the complete conversion of one compound (**1**) into one other (**4**; no change in stoichiometry; Figure S8).

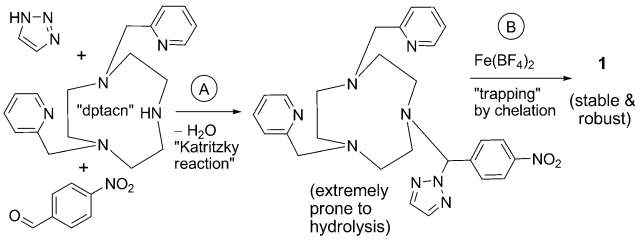
To learn about the structural limits for observing fragmentation, we explored derivatives **5** and **6** comprising a pyrazole unit in place of the triazole. While both are readily



converted by their respective stimuli to the intermediate aniline, this aniline is stable against fragmentation over 15 h at 37 °C, and not even reflux causes it to immolate in the desired manner (Figure S12). This is consistent with the poor leaving-group character of pyrazole that has a pK_a of only 14.2, compared to 9.4 for triazole. On the other hand, derivative **7**, once reduced, fragments readily to **4** in accord with the recognized good leaving-group character of the benzotriazole group (pK_a = 8.2); this happens with an insignificantly slower rate than that for **1** (4 h to completion; Figure S13).

Probes **1** and **2** do not degrade in the absence of their stimulus. This is an important observation. In fact, in the absence of the metal center, the underlying hexadentate ligand that comprises a triazole containing aminal, hydrolyses instantly when exposed to water. Even exposure to traces of water in deuterated chloroform suffices to cause significant losses in material. The fact that **1** and **2** do not degrade in fully aqueous media illustrates the enormous stabilization conferred on the structure by metal chelation. Probe **1** is highly stable at 37 °C for 24 h, be it in 50 mM phosphate buffer (HPLC monitoring; Figure S10 and S11) or in serum. Probe **2** is stable for many hours at 37 °C in 10 % serum or higher (MS; Figure S17). Negative controls for cross reactivity show that **1** does not react with penicillin amidase and **2** does not react with trypsin, a prominent broadband amidase/peptidase (Figure S18).

A crucial aspect to probe design is the ease of synthesis and thus time and cost of preparation. The synthesis of aminals comprising an azole (the "Katritzky" unit) is a three-component convergent reaction and thus straightforward (Scheme 2), if the secondary amine is aliphatic and sterically



Scheme 2. One-pot, two-step, four-component convergent synthesis of **1** (**2** is prepared in the same way).

not too hindered.[24,29] In our case, this is the macrocyclic penta-amine dipicolyl-1,4,7-triazacyclononane ("dptacn").[3,30] The labile nature of the resulting product before metal complexation should not be underestimated. Its extreme proneness to hydrolysis is confirmed by literature reports.[31–33] A breakthrough in synthetic efficiency was achieved when we chose to titrate the initial reaction result with an anhydrous solution of iron(II) tetrafluoroborate, monitored by LCMS to determine the endpoint. Metal chelation greatly stabilizes the Katritzky unit. All five complexes could thus be purified on C18 reversed-phase cartridges with an aqueous mobile phase. The nitro complexes **1**, **5**, and **7** could be reliably recrystallized to obtain pure samples, enabling us to obtain irrefutable characterization data. We obtained complex **1** in batches of 500 mg of pure crystals (Supporting Information), and with a yield of 30 % (3-component reaction followed by one-pot metal complexation). Once the complex formed, its purification does not appear to cause any losses, and its crystallization is nearly quantitative. The synthesis of pyrazole complexes **5** and **6** is highly efficient; even anhydrous conditions are no longer necessary. In contrast, repeated attempts have shown that a tetrazole version is probably impossible to prepare.

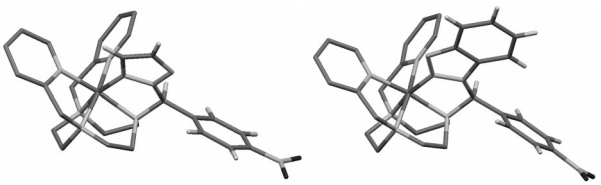


Figure 3. Structure determined by X-ray analysis of single crystals of **1** (left) and **7**; hydrogen atoms of the macrocycle and the two picolyl units omitted for clarity. Black O, light gray N,H, gray C,Fe

Crystallization by diethyl ether diffusion into acetonitrile solutions of **1**, **5**,and **7** yielded crystals suitable for structure determination by X-ray analysis (Figure 3 and ESI). Generally, all three structures show an unperturbed octahedral FeN6 coordination motif with all the Fe–N bonds around 2.0 Å thus showing the low-spin nature of the complexes; a high-spin state would exhibit considerably longer values of 2.2 Å .[12,34] While the structure of **1** revealed a mixed aminal whose triazole is linked by its N2 atom to the central aminal carbon atom, the benzotriazole moiety in complex **7** (the "Katritzky azole") is connected by its N1 atom. In fact, numerous acetal-like compounds involving benzotriazole have been found, they are connected as often through N1 as through N2.[24,33] On the other hand, reports of aminals composed of a simple 1,2,3-triazole and an aliphatic secondary amine are limited to a few examples with formaldehyde. Inspection of the pyrazole structure for **5** (Supporting Information) reveals that no isomerism can occur here; a few examples of the underlying, uncomplexed, aminal structure comprising a pyrazole have been reported in the literature.

Proton NMR spectra for all five complexes were recorded in deuterated acetonitrile (CD_3CN, see Supporting Information) and lie in the 10 ppm window for diamagnetic compounds. Where possible (**1**, **5** and **7**) spectra were obtained from dissolved crystals. The penicillin amidase substrates (**4** and **6**) were characterized from dissolved powders. Spectral resolution varies from one complex to the other, is temperature dependent and reaches a maximum for complex **7** (see Supporting Information). UV and mass spectra complete the characterization of **1**, **4**–**7**. UV spectra (ESI) exhibit the prominent 400 nm band typical of metal-to-ligand charge transfer (MLCT); this band causes the solid complexes to be intensely colored (see Supporting Information for photos) and the corresponding aqueous solutions are light orange (**1**, **2**, **5**, and **6**) to light red (**7** Supporting Information). The mass spectra at low fragmentation voltage offer usually a clean picture of two sets of signals, one stemming from the molecular peak (at *m*/2) and others from the ion pairing with formate, chloride, bicarbonate, or fluoride. This spectral simplicity allowed for efficient monitoring of the activation process of probes **1** and **2** (see Supporting Information).

We have succeeded in the design of two easily accessible molecular probes that react specifically with their respective stimuli and consequently alter their electronic spin from 0 to 2. The synthesis of the probes has been optimized and allows for significant structural variation. Our current results demonstrate how narrow the structural space and the temperature window is in which decent fragmentation rates and sufficient robustness are possible. The "invisibility" of the intact probes, their medium of operation, as well as their molecular size and the associated favorable diffusion rates bode well for their exploitation in already existing applications and in yet to be designed molecular devices.

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