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ARTICLE

Time efficient detection of protein–ligand interactions with the polarization optimized PO-WaterLOGSY NMR experiment

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Abstract The identification of compounds that bind to a protein of interest is of central importance in contemporary drug research. For screening of compound libraries, NMR techniques are widely used, in particular the Water-Ligand Observed via Gradient SpectroscopY (WaterLOGSY) experiment. Here we present an optimized experiment, the polarization optimized WaterLOGSY (PO-WaterLOGSY). Based on a water flip-back strategy in conjunction with model calculations and numerical simulations, the PO-WaterLOGSY is optimized for water polarization recovery. Compared to a standard setup with the conventional WaterLOGSY, time consuming relaxation delays have been considerably shortened and can even be omitted through this approach. Furthermore, the robustness of the pulse sequence in an industrial setup was increased by the use of hard pulse trains for selective water excitation and water suppression. The PO-WaterLOGSY thus yields increased time efficiency by factor of 3–5 when compared with previously published schemes. These time savings have a substantial impact in drug discovery, since significantly larger compound libraries can be tested in screening campaigns.

Keywords Drug design · NMR screening · Protein–ligand interactions · WaterLOGSY · Water handling

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Introduction

NMR-based screening is a well established technology for the identification of small molecules interacting with a protein drug target (Shuker et al. 1996). NMR can be applied either in protein- or ligand-observed manner. Protein-observed spectroscopy allows to detect ligands with dissociation constants (K_D) ranging from nanomolar up to millimolar and can yield information about the precise location of the interaction on the protein. However, protein-observed NMR spectroscopy requires larger amounts of protein, which in many cases needs to be isotope-labeled, and is restricted to proteins smaller than 30 kDa for standard applications. The size range can be expanded to 100 kDa or even 800 kDa by combined use of deuteration and transverse relaxation optimized spectroscopy (Fernández et al. 2001; Fiaux et al. 2002; LeMaster 1994; Pervushin et al. 1997). Nevertheless, ligand-observed NMR experiments are favored over protein-observed experiments in screening campaigns, where hundreds to thousands of compounds are tested for binding, since there is virtually no size limitation of the target protein, the protein does not need to be isotope labeled and comparatively less protein amounts are needed. Although NMR suffers from intrinsic low sensitivity and therefore requires more protein material than other screening methods, it is commonly used for compound binding screening owing to its inherent versatility, robustness and ability to detect weakly binding ligands.

Water-Ligand Observed via Gradient SpectroscopY (WaterLOGSY) is a widely applied ligand-observation technique for detection of protein–ligand interactions (Dalvit et al. 2000, 2001). Its standard application is in primary screening of weak ligands with dissociation constants in the μM to mM range and in validation of hits from screening by other methods. Competition binding and

titration experiments permit to extend the applicability to strong binders and make possible the determination of dissociation constants (Dalvit et al. 2001). WaterLOGSY has recently been used to determine the orientation of a ligand bound to a protein by mapping its solvent accessibility (Ludwig et al. 2008).

A major issue of this experiment is however its limited sensitivity. A 1D WaterLOGSY experiment takes about 20–30 min measuring time on a conventional probe head under standard experimental conditions used for NMR screening (e.g. protein concentration in the low micromolar range and compound concentration typically about 10–20 times higher). This limits the size of compound libraries that can be tested and therefore the number of potential hits. Furthermore, the low sensitivity of the experiment also hinders the routine application in titration experiments for K_D determination and represents a significant hurdle in the effort of reducing protein consumption and minimizing sample volumes i.e. for micro-coil NMR (Dalvit et al. 2001; Hopson and Peti 2008).

In the WaterLOGSY experiment, bulk water magnetization is transferred during the mixing time via the protein–ligand complex to the free ligand, which is present in a saturated state at the onset of the mixing time (Dalvit et al. 2000, 2001). Water magnetization that is transferred through a slowly tumbling protein–ligand complex yields an NOE on the ligand of the same sign as the water polarization. Magnetization transfer from water to unbound ligand gives raise to NOEs with the opposite sign. Therefore binders and non-binders can easily be identified. Furthermore, the signals of non-binding compounds like chemical shift referencing compounds can serve as an internal control for e.g. sample aggregation.

The magnitude of the water polarization during the mixing time is crucial for the sensitivity of the WaterLOGSY experiment. In the conventional experiment, however, the water polarization is destroyed in each scan in order to avoid receiver overflow (Dalvit et al. 2000). A 20–30% gain in sensitivity over the conventional experiment was reported with a WaterLOGSY sequence, where part of the water polarization was conserved by means of selective water flip-back pulses prior to detection (Dalvit et al. 2001). Here in this communication we further exploited the special advantages offered by water flip-back schemes to design the polarization optimized WaterLOGSY (PO-WaterLOGSY). Theoretical considerations and simulations were used to assist the optimization of the lengths of the mixing time and the recovery delay. This resulted in a 1.8 and 1.4-fold sensitivity increase over the conventional (Dalvit et al. 2000) and the flip-back version (Dalvit et al. 2001) of the WaterLOGSY, respectively. Furthermore, the robustness of the sequence was increased by replacing the selective water excitation and water suppression schemes by hard pulse WATERGATE sequences (Liu et al. 1998; Sklenar et al. 1993), leading to an additional, sample dependent, increase in sensitivity. In an industrial setup the implementation of the PO-WaterLOGSY yields an improved time efficiency by factor of 3–5 and a corresponding increase in the number of potential hits.

Materials and methods

NMR experiments

All NMR spectra were recorded at 296 K on a Bruker DRX600 NMR spectrometer equipped with a 5 mm $^{1}H\{^{13}C,^{15}N\}$-triple resonance probe head with shielded xyz-gradient coils. The data were processed and analyzed with the software TOPSPIN 1.3 (Bruker BioSpin, Switzerland).

NMR samples

The NMR experiments were performed with protein samples in the presence of low molecular weight compound mixtures, among which validated ligands are known, either from internal drug discovery programs or from the literature. In all cases, the protein concentration was 10 μM and the ligand concentration was 200 μM. Reference 1D proton and 1D WaterLOGSY spectra were measured in all cases for the ligands in the absence of protein.

Water handling

The flip-back pulse power and its phase correction were determined interactively in a separate experiment consisting of a hard 90° pulse followed by a selective 90° pulse of inverted phase on the water resonance and an acquisition command (Hiller et al. 2005). The longitudinal ^{1}H relaxation rate T_1 of water was determined by an inversion recovery experiment (Ernst et al. 1987), where a weak gradient was applied to avoid radiation damping effects. The equilibrium water magnetization was measured with a "tap"-pulse of 0.1 μs duration (Hiller et al. 2005).

The efficiencies f_{ab} and f_{cd} of the pulse sequence were measured by inserting a strong magnetic field gradient along the z-axis followed by a tap pulse and an acquisition command at time points b and d of the PO-WaterLOGSY pulse-sequence (Fig. 1). For measurements of the steady-state water polarization at a given time point during the PO-WaterLOGSY pulse sequence the same scheme was used. The water polarization was read out by applying the above scheme after 32 repetitions of the unaltered PO-WaterLOGSY pulse-sequence.

Numerical simulations

Simulations were carried out in the program Microsoft Excel with the Solver tool (Fylstra et al. 1998).

Results

As the PO-WaterLOGSY experiment starts from water magnetization and its performance strongly depends on the state of the water magnetization, special emphasis was made on optimization of the water handling throughout the entire pulse sequence (Fig. 1). To this end, a water selective flip-back pulse (Grzesiek and Bax 1993) (ϕ_3 in Fig. 1) is applied at the end of the mixing time, after the read-out 90° pulse (ϕ_2 in Fig. 1). This water flip-back pulse always acts as a flip-"up" pulse, e.g. to flip water coherence from the xy-plane to the +z axis, in order to consistently have the same effect from radiation damping during the pulse.

The optimized experiment is run as pairs of consecutive scans, where the water magnetization during the mixing time is along the +z and the −z axis in the first and the second scan, respectively. In this scheme the main part of the water polarization is conserved along the +z axis after a scan in which the water polarization is along the +z axis during the mixing time. Therefore, there is no need for a recovery delay after such a scan, since the experiment only relies on the water magnetization and not on that of the observed nuclei in the sample. Thus, a recovery delay is only needed after every second scan, which significantly reduces the total measurement time. Because a fraction of the water magnetization can also be conserved in the second scan by the water selective flip-back pulse, this recovery delay can be shorter than in the standard experiment, where the water is saturated in each scan.

In order to fully exploit the advantage of the PO-WaterLOGSY experiment, the duration of the mixing time and the recovery delay have to be adjusted. The optimal delays can be determined either experimentally or approached by simulations. We opted for simulations as a first step, in order to have a general solution, and to test the predicted values experimentally with different samples.

Assessment of optimal delay duration with simulations

The sensitivity, *S/N*, of the PO-WaterLOGSY experiment depends on the NOE buildup during the mixing time, the magnitude of the water polarization available during this mixing time and the number of scans that can be recorded per time unit. Based on the assumption that the PO-WaterLOGSY signal intensity is proportional to the magnitude of the water magnetization at the beginning of the mixing time, Eq. 1 was formulated.

$$S/N \propto I(\tau_m) \cdot \overline{\left|M^{b,ss}_{z,\mathrm{water}}(\tau_m, t_r)\right|} \cdot \sqrt{n_t(\tau_m, t_r)} \qquad (1)$$

$I(\tau_m)$ is the empirically determined relative intensity of the PO-WaterLOGSY NOE signal as a function of the mixing time τ_m (Fig. 2), M_z is the fractional steady state water magnetization at the beginning of the mixing time as calculated below and $\sqrt{n_t}$ is the number of recordings per unit time. n_t is calculated from the average time per recording, which includes the mixing time τ_m, the recovery delay t_r

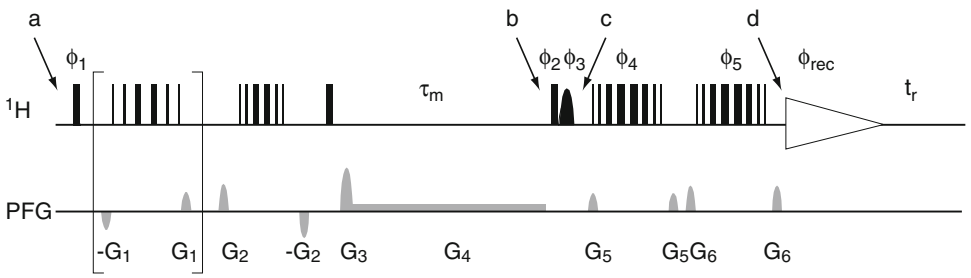

Fig. 1 Pulse sequence of the PO-WaterLOGSY experiment. *Solid bars* represent 90° radio frequency (rf) pulses applied at maximum power. The pulse trains are W3 and W5 WATERGATE schemes. For selective excitation of the water resonance a single W3 with an inter-pulse delay of 280 μs or two W5 with inter-pulse delays of 560 and 280 μs are used. For water suppression a double W5 with an inter-pulse delay of 280 μs is used in an excitation sculpting scheme. The selective water flip-back pulse has a duration of 3.2 ms and has a Gaussian shape with 5% truncation. The shaped pulse und the delays are optimized for a static field of 600 MHz. The mixing time, τ_m, is 0.8 ms and the recovery delay, t_r, is 0 and 3 s in subsequent scans. The rf pulse phases are x unless otherwise indicated above the pulses. The phase cycling is: $\phi_1 = \{-x, x\}$, $\phi_2 = \{x, x, -x, -x\}$, $\phi_3 = \{-x, x, x -x\}$, $\phi_4 = \{x\}$, $\phi_5 = \{y\}$, $\phi_{rec} = \{x, -x, -x, x\}$. For better water suppression the phase cycle of ϕ_4 and ϕ_5 can be extended. For the WATEGATE pulse trains only the phases of the first half of the pulses are given, the phase of the second part is inverted. On the line marked PFG, curved shapes indicate sine bell-shaped pulsed magnetic field gradients along the z-axis, with the following duration and strengths: G_1: 0.8 ms, 6.0 G/cm; G_2: 0.8 ms, 14.25 G/cm; G_3: 1.0 ms, 20 G/cm; G_4: length = τ_m, 0.05 G/cm; G_5: 0.8 ms, 4.5 G/cm; G_6: 0.8 ms, 10.5 G/cm. A delay of 1 ms was inserted after gradient 4 to allow the system to equilibrate. The letters a–d mark positions in the pulse sequence, which are referred to in the text

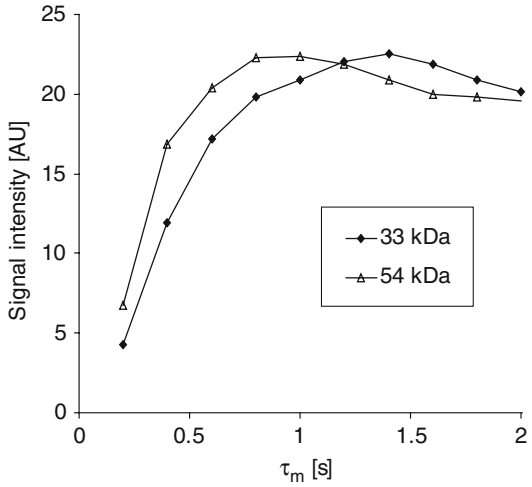

Fig. 2 Ligand signal intensity as a function of the mixing time (τ_m) in the WaterLOGSY experiment. The average of the two curves was used for $I(\tau_m)$ in Eq. 1. The standard pulse sequence (Dalvit et al. 2000) was used with a recovery delay of 10 s. The molecular weight of the two different proteins used in these experiments is indicated. The intensity is given in arbitrary units, as the ligands are not directly comparable. The concentrations of the protein and the ligand were, respectively, 10 and 200 μM

the acquisition time, and the sum of all pulses and remaining delays in the pulse sequence.

Model calculation of the water polarization

The water polarization M_z as a fraction of the equilibrium water polarization M_0 was calculated at different time points in the experiment (a–d in Fig. 1) (Hiller et al. 2005). During the pulse sequence the water magnetization is affected by pulses (i.e. a^i-f_{ab}-b^i and c^i-f_{cd}-d^i, where i is the running number of scans) and delays (i.e. a^i-τ_m-b^i and d^i-t_r-a^{i+1}). The effect of the pulse schemes on the water magnetization can be summarized as either inversion or conservation with an efficiency f. In the case of the standard sequence, saturation can be described with $f = 0$.

$$M_z^{b,i} = \pm f_{ab} \cdot M_z^{a,i} \tag{2}$$

The equations describing the water relaxation during the mixing time and the recovery delay have the following form:

$$M_z^{c,i} = M_0 - \left(M_0 - M_z^{b,i}\right) \cdot \exp\left(\frac{t}{T_1}\right) \tag{3}$$

M_0 is the equilibrium water polarization, T_1 is the longitudinal relaxation rate of water protons, and t stands for the mixing time τ_m or the recovery delay t_r. T_1, f_{ab} and f_{cd} were determined experimentally as described in the experimental section, and were found to be 3.0 s, 96% and 96%, respectively.

The values for the steady-state water polarization, M_z^{ss}, were calculated iteratively with $i = 32$. The calculated values are in close agreement with the experimentally determined values (Fig. 3). With this reliable calculation of the water magnetization, the expected sensitivity of the PO-WaterLOGSY experiment could be approximated with Eq. 1 for different values of the mixing time and the recovery delay.

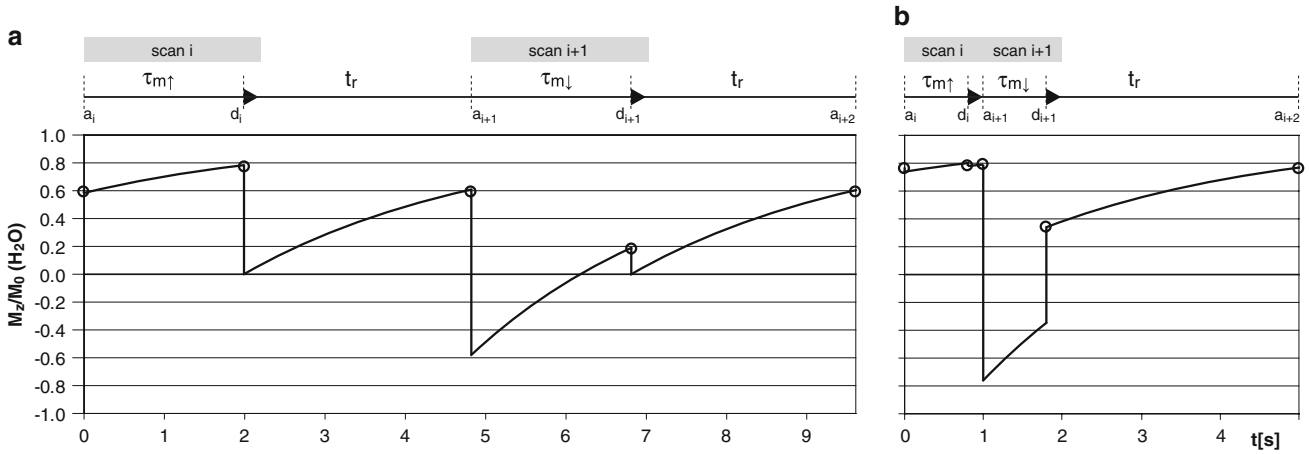

Fig. 3 Time evolution of the water polarization, M_z/M_0, in the standard WaterLOGSY (Dalvit et al. 2000) (**a**) and the PO-WaterLOGSY (**b**) experiment, after steady state has been reached. The basic unit of the experiment is shown, consisting of two consecutive scans, with the water polarization during the mixing time along the +z-axis ($\tau_m\uparrow$) and the −z-axis ($\tau_m\downarrow$). A simplified scheme of the experiment is depicted on top, showing the mixing times (τ_m), the acquisition time (triangles) and the recovery delay (t_r) on the time axis with the individual scans represented by grey boxes. The letters a_i and d_i refer to the time points in the pulse-sequence in Fig. 1, and i indicates the running number of scans. *Circles* and *lines* represent experimental data and results from the model calculation based on Eqs. 2 and 3, respectively. In **a** $\tau_m = 2.0$ s, $t_{acq} = 0.2$ s, $t_r = 2.6$ s were used, in **b** $\tau_m = 0.8$ s, $t_{acq} = 0.2$ s, $t_{r,i} = 0$ s and $t_{r,i+1} = 3.0$ s

Evaluation of the calculated values for the recovery delay and the mixing time

The values of the mixing time (τ_m) and the recovery delay (t_r) were numerically optimized for maximal sensitivity. For the PO-WaterLOGSY described here (Fig. 1), values of $\tau_m = 0.8$ s and $t_r = 0$ s (odd scan numbers) and 3 s (even scan numbers) were obtained from this simulation. The sensitivity of the previously published standard version of the WaterLOGSY experiment with $\tau_m = 2.0$ s and $t_r = 2.6$ s as described by Dalvit was also simulated using $f_{cd} = 0$ (Dalvit et al. 2000, 2001). The improvement in sensitivity compared to that experiment was calculated to be 1.8. The simulated sensitivity improvement is in good agreement with the experimental value of 1.76. Also the optimal combination of τ_m and t_r was found to agree well between simulation and experiments. The sensitivity maximum is relatively shallow: values for τ_m and t_r between 0.7–1 and 2–3.5 s, respectively, yielded values within 85% of the maximum sensitivity.

The validity of the simulation was further confirmed by numerically optimizing the duration of the delays for the standard version of the experiment. This yielded optimal values for $\tau_m = 1.0$ s and $t_r = 4.0$ s and a sensitivity improvement of 1.35 compared to $\tau_m = 2.0$ s and $t_r = 2.6$ s. This corresponds well with the experimentally determined sensitivity improvement of 1.33. Moreover, the flip-back version of the standard WaterLOGSY was compared to the one presented here (Dalvit et al. 2001). The experimental sensitivity gain when using these optimized values of τ_m and t_r was 1.4.

Further improvements to the pulse sequence

The robustness of the PO-WaterLOGSY pulse sequence was further increased by the use of gradient flanked WATERGATE hard pulse trains for selective water excitation and water suppression (Liu et al. 1998; Sklenar et al. 1993). For the selective excitation of the water resonance, either a W3 WATERGATE or a combination of two WATERGATE sequences of different excitation bandwidth can be used. The single W3 scheme can lead to artifacts close to the water line in the processed spectrum when used in combination with a narrow water suppression bandwidth. The alternative double W3 or W5 schemes come at a cost of a few percent signal loss due to the larger number of pulses and delays applied during the pulse sequence. For compounds with signals close to the water line, unwanted spurious signals were observed arising from imperfections of the water flip-back pulse.

Evaluation of the final pulse sequence

In the evaluation of the PO-WaterLOGSY pulse sequence with proteins of 17, 33 and 54 kDa molecular weight and several ligands we found that the same signal to noise ratio was obtained after only 6 min of measurement time compared to 20–30 min with the conventional WaterLOGSY (Dalvit et al. 2000). We attribute the additional sensitivity gain from the calculated values to the better robustness of the hard pulse schemes used in the experiment proposed here. Particularly the use of hard pulses for selective water excitation and water suppression instead of shaped pulses, as implemented in previously published versions of the experiment, makes an important difference, which is most pronounced when measuring salt containing samples on cryogenic probe heads.

Discussion and conclusions

In the optimized PO-WaterLOGSY scheme presented here, the sensitivity of the WaterLOGSY experiment was increased through full exploitation of the special advantages of the water flip-back strategy. Since a large fraction of the equilibrium water magnetization can be conserved during signal acquisition of the nuclei of interest, the recovery delay can be shortened or even omitted in every second scan. In scans where the water polarization is along the +z-axis during the mixing time, longitudinal relaxation leads to an increased water polarization at the end of the scan. Since the selective water flip-back scheme conserves the major part of the water polarization, there is no need for a recovery delay after such a scan, i.e. the mixing time adopts the function of the recovery delay. In contrast, for scans where the water magnetization is along the −z-axis, the water polarization decreases during the mixing time and a recovery delay is still needed before the next scan. Therefore, the experiment is recorded as pairs of scans with a subsequent recovery delay.

The two different types of scans lead to slightly different amounts of water polarization at the start of the mixing time. This, however, represents no issue in terms of artifact suppression. At the start of every scan all but the water magnetization is dephased by a strong gradient. Artifacts that build up during the scan are cancelled out, as the relevant delays during the experiment are identical in both types of scans. Overall, as shown in Fig. 3, the time course of the water polarization during the mixing times of two consecutive scans is more comparable in the PO-WaterLOGSY than in the original WaterLOGSY experiment.

The amount of steady-state water polarization and hence the sensitivity of the PO-WaterLOGSY experiment can be accurately estimated with model calculations (Fig. 3). Here, we simulated the sensitivity of the experiment based on the assumption that the empirically determined PO-WaterLOGSY signal at different mixing times is directly proportional to the amount of the available water polarization. The duration of the mixing time and the recovery delay were then numerically optimized for maximal sensitivity. For samples in which the relevant parameters used in the simulation strongly differ from the ones chosen here, thus influencing the longitudinal water relaxation time or the NOE buildup, the calculations to determine optimal values for τ_m and t_r may need to be carried out with modified parameters. However, we consistently obtained comparable values using parameters from different samples or from the literature (Dalvit et al. 2001). Overall, the experimentally determined sensitivities correspond well with the calculated values.

The PO-WaterLOGSY experiment with the numerically optimized delays has a significantly increased time efficiency and, at the same time, yields a higher value of the steady-state water polarization at the beginning of the mixing time when compared to the standard experiment (Dalvit et al. 2001). This allows collecting a signal of comparable intensity in half of the time. Moreover, we attribute an important part of the signal gain to the shortening of the mixing time. In scans with the water polarization along the −z-axis, its magnitude decreases rapidly and ends up along the +z-axis for long mixing times (Fig. 3), thereby annihilating part of the net magnetization transfer.

In addition to the main sensitivity gain emerging from the improved water handling and delay optimization described above, we found that replacement of shaped pulses for selective water excitation and water suppression by WATERGATE hard pulse trains increased the robustness of the experiment for industrial applications. This often results in additional sensitivity gain, since routine, accurate calibration of the crucial 40 ms water-selective shaped pulse, as implemented in previously published sequences, is time demanding, error prone and partially saturates the water. Particularly, when measuring salt containing samples on cryogenic probe heads we could see sensitivity improvements of more than a factor of two when using the WATERGATE sequence instead of the scheme with water excitation by a shape pulse. The WATERGATE scheme as employed here may be also be preferred in other experiments that rely on selective excitation of the water resonance like CLEANEX (Hwang et al. 1997). Moreover, the water flip-back pulse (ϕ_3 in Fig. 1) has been designed now in a way that it always acts as a flip-up pulse, which makes calibration of a corresponding flip-down pulse unnecessary.

Generally, negative signals, like the signal of DSS at 0 ppm in Fig. 4, are stronger in the PO-WaterLOGSY than in the conventional WaterLOGSY (Dalvit et al. 2000). The strong signal can serve as a valuable internal control for aggregation in the sample, since aggregation represents a major source of false positive results in ligand observed binding experiments. Obviously, the clear signal also simplifies calibration of the spectra.

In our hands, the PO-WaterLOGSY experiment presented here allows us to reduce the measurement time from typically 30 to 6 min per sample (Fig. 4). Therefore, significantly larger numbers of compounds can be screened in the same amount of time, thereby increasing the potential number of hits. We expect that the main impact of the PO-WaterLOGSY on drug discovery programs is the ability of screening larger libraries by NMR, but it also opens new possibilities on hit validation. The achieved reduction of the measurement time with the PO-WaterLOGSY allows performing previously time consuming measurements, including K_D determinations, on a day-to-day basis, or to include protein detected experiments in NMR screens in order to detect ligands with slow off rates. Alternatively, protein consumption can be significantly reduced owing to the increased sensitivity of the experiment either by reducing the concentration of the samples or

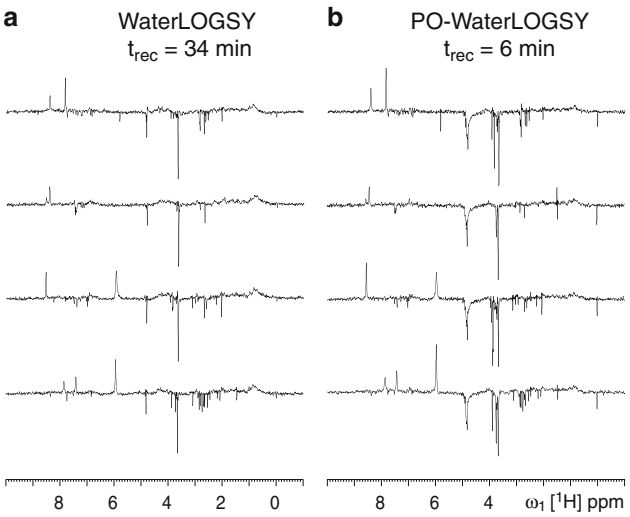

Fig. 4 1D ^{1}H spectra of different compound mixtures with a target protein (33 kDa) recorded in 34 min (**a**) and 6 min (**b**), using the standard WaterLOGSY pulse sequence (Dalvit et al. 2000) and the PO-WaterLOGSY presented here, respectively. The spectra were selected from a standard NMR screen in an industrial setup, where pulses were optimized for the first sample in a series. The signal to noise ratio of spectra (**a**) and (**b**) as calculated with TOPSPIN 1.3 was found to be equivalent on average. The samples contained 10 μM protein with a mixture of eight compounds each at 200 μM in 25 mM d-Tris pH 8.0, 125 mM NaCl, 0.5 mM d-TCEP, 1 mM DSS, 0.4% d-DMSO and 5% D_2O

by opening the possibility to perform measurements with micro-coil probe heads.

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