A rapid-mixing design for conventional NMR probes

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Abstract

A simple stopped-flow design for rapid mixing of two liquids within the NMR probe is presented. The device uses no switches or relays but exploits instead the torque exerted by the magnetic field on a current-leading-coil to open and close the start and stop valves. Two serially arranged tangential jet mixer blocks provide a homogeneous mixture with, depending on conditions and requirements, a filling time in the 50–100 ms range and a subsequent stabilization time in the range of 10–40 ms as tested by mixing various combinations of liquids and observing their ^{1}H NMR spectrum. Factors influencing the mixing process are analyzed.
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1. Introduction

NMR spectroscopy is extensively used for studying dynamical phenomena in a range of time scales whose width is unparalleled in other spectroscopies. In a typical experiment, NMR is applied to a material in equilibrium. NMR in non-equilibrium systems [1,2] is less frequent, partly because averaging of the typically weak NMR signal is less straightforward in those experimental situations. Even if the signal strength is not a limiting factor, information about the investigated dynamical phenomenon can only be obtained if that modulates a spin coupling on the time scale of the inverse of that particular coupling. Hence, NMR is typically limited to observe non-equilibrium dynamics that is in the order or slower than a millisecond. This lower limit is much higher than that for, e.g., optical spectroscopies. In the $>10^{-3}$ s regime, however, NMR has a great potential which, however, cannot be exploited by an NMR spectrometer with conventional equipment. To initiate a change in a sample and then place that in a probe inside the magnet can hardly be performed faster than a few seconds. The present work is about accessing the 10^{-2}–10 s (and above) time window in liquid materials where kinetics following a change in composition is studied by NMR.

There are several prior examples for probes which allow in situ mixing of two different liquids in the NMR sample space. Among those designs, stopped-flow [3] arrangements dominate and excel [4–11]. In particular, mixing with dead time (the sum of time for liquid transport and homogenization and the overlapping/subsequent time for dissipation of mechanical disturbances) of a few milliseconds has been claimed. Although rapid, many of those designs suffer from several possible shortcomings such as (i) limited adaptability to any conventional NMR probe (ii) limited and sometimes untested compositional homogeneity of the mixture, (iii) the creation of long-lived turbulent regions within the mixed liquid, and (iv) relaxation effects caused by changing the nuclear spin polarization as liquids are pumped between regions with low (initially) and high (in the observation chamber) magnetic field. As concerning adaptability to conventional NMR probes and simplicity, there are other designs [2,12–14] that perform well but sometimes

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at the cost of limited mixing speed or quality. In this paper, we demonstrate our approach to achieve another, for our research goals better, compromise among speed, adaptability, simplicity, mixture homogeneity, and lack of motional and spin polarization artifacts.

2. The design

The stopped-flow design (Fig. 1) presented below is intended for the 50 mm inner diameter room-temperature shim tube of a wide-bore superconducting magnet by Bruker (adaptation to other diameters is straightforward). It is manufactured mainly (most body parts) from Teflon as a plug-in into a spinner body Z which allows for easy manual loading (after filling the component chambers A via outlets M) from the magnet top. There is one electric wire, one pressurized air tube, and one silicon rubber discard tube (hanging on which the setup is loaded into the magnet) that connects through the upward part of the shim tube to the setup. Hence, our design can be used together with any conventional 5–10 mm probe and can be manufactured in any standard workshop. The system is designed to permit contact of the solutions only with quartz glass, ceramics, Teflon, Kel-F, and silicon rubber tube where the latter can be also substituted by Teflon.

In our stopped-flow apparatus, the components are stored before the experiment in two cylindrical chambers (A, made of stress-resistant quartz glass) from
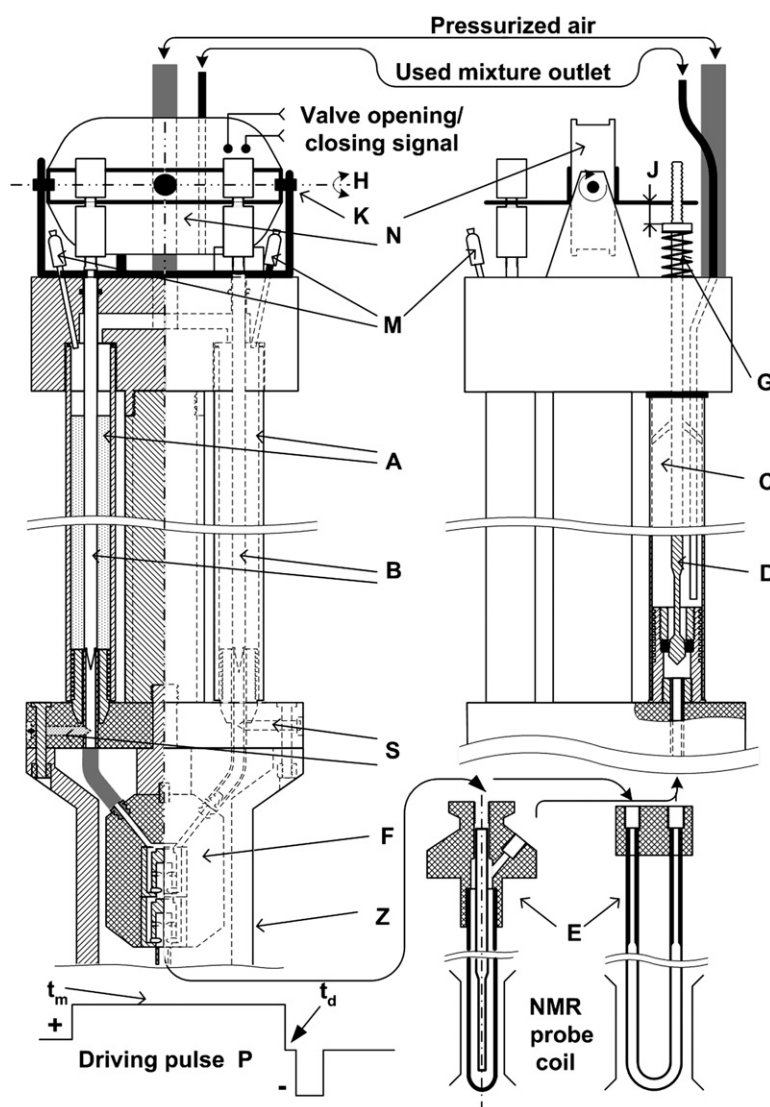

Fig. 1. A schematic diagram of our stopped-flow NMR apparatus. Initially, the component liquids are stored in chambers A with valves B at the bottom. The output chamber C is connected via valve D to the outlet of the observation chamber E to store the discarded mixture. Mounted onto a conventionally shaped spinner Z, the observation chamber E inserts into the sample space of any conventional NMR probe. The observation chamber is either made of a conventional 5 mm NMR tube or from a U-bent 1.5 mm i.d. capillary. For the rest of the design elements, see text.

where they are driven through two serially arranged tangential jet mixer blocks F [15] made from machineable ceramics (Macor). The mixed liquid streams into the observation chamber E located in the sample space of the NMR probe. Thereafter, the mixture may continue into an output chamber (C, also made of quartz glass). As can be seen in Fig. 1, the liquid components are stored in our design directly above the spinner top. Since this region is about 10–15 cm above the sample space, the unmixed liquids are stored (and become polarized) in a magnetic field that is only 5–10% less than that in the sample space. This sets an advantageously low upper limit to spin polarization artifacts. The short transfer line from the component chambers to the observation chamber also suppresses unfavorable oscillations after the abrupt stop of the flow.

Pressurized air (maximum 7 atm, controlled by remote reduction valves, has been tested) that drives the liquids is constantly applied to the open top of the component chambers. The experiment is initiated by feeding a current (∼5 A) pulse P through flexibly connected wires into solenoid N (200 turns of 0.35 mm copper wire yielding approximately 0.1 mH inductance and 0.5 Ω resistance) that rests on a horizontal axis H, kept in place by hinges K. Since the whole apparatus is placed inside a strong magnetic field, solenoid N experiences a strong torque (the t_m-long positive part of the driving pulse P corresponds to clockwise current). Turning solenoid N opens valves B and D (connected to N, the working surfaces of the valves were made of elastic silicon rubber rings) upon which the component liquids are pushed through mixers F into the measuring chamber E. Any liquid initially residing in E moves into the output chamber via valve D. When the positive part of pulse P is finished, spring G (helped by the upward pressure of the liquid on valve D) turns the solenoid backward, valve D closes, and the flow stops. It is important to note that spacer J bridges the distance between the equilibrium height of G and the initial position of N. Hence, at this point valves B remain open and must be actively closed by subsequent short (∼3 ms) negative part of pulse P that completely returns solenoid N to its initial position. The delay t_d (∼1 ms) between the positive and negative parts of P must exceed the time necessary for complete closing of valve D. This requirement ensures that the stream is stopped at the end (D) rather than at the beginning (B) of the liquid column where the latter scenario usually leads to cavitation and irreproducible results [3]. The stopped-flow experiment itself is completed after closing valve D and the progress of any physical or chemical change can be monitored under apparently static conditions. One could note that most conventional stopped-flow designs use plungers instead of valves to stop and drive liquid flow. In contrast to that arrangement, our design does not require precise and low friction syringe-plunger

fitting. On the other hand, the volume of mixed liquid is controlled less precisely (the reproducibility of mixing ratio over separate experiments is in the order of a few percent).

Pulse P can be easily supplied by bipolar current generators for pulsed-field-gradient experiments which are nowadays ubiquitous. An alternative solution is to take any TTL pulse output from the spectrometer such as an external trigger signal and feed that into a home-built DC amplifier that creates the rectangular positive pulse of set length and also forms a subsequent short negative pulse. The amplifier circuit consists of a chain of three monostable multivibrators and several standard push–pull transistor cascades; the scheme can be sent upon request. The lengths of the positive and negative parts of P and t_d are adjusted by the time constants of the RC chains of the multivibrator circuits.

The length of pulse P should exceed the time necessary for reliable transportation of all used mixture through and out of the observation chamber E. This is obviously dependent on the driving pressure. At 6 atm and with the 0.5 ml re-filling volume of the sample/transportation tubes, the proper length of P is approximately 70 ms. That time should be considered as the lower limit of filling dead time of the present apparatus and can be decreased down to ∼50 ms by either increasing the driving pressure to 7 atm or inserting a smaller (1 mm i.d.) U-bent capillary tube as observation chamber. Although this value is about one order of magnitude higher than filling dead times claimed by more sophisticated designs, it is also comparable or faster than dead times obtained by designs of similar simplicity [12,13].

The present construction is primarily designed for 1:1 mixing ratio of the components. However, the mixing ratio can be modified in the 1:1–1:3 range by manually adjusting (by <5% reproducibility) the positions of screws S. The input chambers (A) are initially filled through outlets M by using standard glass pipettes. Since the total volume of the connection lines and the sample tube is ∼0.5 ml and the volume of one input chamber is slightly over 1.5 ml, at least four subsequent stopped-flow experiments can be done without re-filling the apparatus.

3. Design test results and discussion

The compositional homogeneity of the prepared mixtures and eventual motional artifacts were tested by recording conventional ^1H NMR spectra after mixing (i) H_2O and ethanol (96%), (ii) H_2O and ethylene glycol (99%, from Merck), and (iii) aqueous solutions of D-alanine (0.5 M, 99%, from Merck) with pH 6 and 14, respectively. All solutions used in these experiments have prepared from degassed and Millipore-filtered

water, and all spectra were recorded in a Bruker AMX300 spectrometer. In all spectra, spectral broadening caused by magnetic field inhomogeneity was in the order or less than 5 Hz.

The tests were based on spectra of the obtained mixtures that are, in all cases, strongly dependent on the actual mixing ratio of the input components. Thus, the exchange of water and ethanol hydroxyl protons is strongly dependent on the ethanol concentration [16]: at high ethanol content the exchange is slow and the ^{1}H spectrum contains one separate line for each of those two proton species as shown in (Fig. 2A). On the other hand, the exchange is fast at low ethanol concentration yielding one water/hydroxyl line. In the 40–60% concentration range, one experiences intermediate exchange as illustrated in Fig. 2B by the spectrum of a pre-mixed water–ethanol sample. Thereby, it is in this concentration range where the ^{1}H spectrum is most sensitive to the homogeneity of the mixture. Similarly, the shift and the width of the water/hydroxyl line of the water–ethylene glycol mixture depend on the composition [17]. In case of the alanine solution, the shift of the methyl resonance depends on the pH [18].

All stopped-flow experiments were performed with 5 atm driving pressure and a 1.5 mm capillary tube as observation chamber (with $t_m \sim 80$ ms). Since the mixing enthalpies ΔH_{mix} of water–ethanol and water–ethylene glycol mixtures are large [19,20], initial heating (from the mixing enthalpy) and subsequent cooling (by heat loss through the wall of the observation chamber) effects have been compensated for by appropriate pre-heating and thermostating of the observation chamber. In effect, the set temperature difference between chambers A and E corresponds to $\sim \Delta H_{mix}/c_p$, where c_p is the heat capacity of the mixture. This measure has a particularly large effect for the experimental outcome in the water–ethanol mixture where the chemical shift shows a strong temperature dependence.

Selected spectra for the water–ethanol mixture, recorded with delays $t_w = 20$ ms (Fig. 2C) and $t_w = 10$ min (Fig. 2D) after the end of the positive pulse P are shown in Fig. 2. Clearly, there are no signs of either incomplete local mixing (absence of slow or intermediate exchange signatures such as split or significantly broadened water/hydroxyl line in Fig. 2C) or large scale inhomogeneities that would be equilibrated by diffusion over the observation chamber (virtually identical spectra in Figs. 2C–D).

Two series of spectra obtained for mixtures (ii) and (iii) are shown in Figs. 3A and B, with interscan delays of 800 ms for the first eight slices and of 10 s for the last ones. At start, the observation chamber D had only one of the components, pure water for (ii) and alanine solution with pH 6 for (iii). Mixing was initiated so that the second spectra in each series were recorded at $t_w = 20$ ms. Thereby, the first recorded slices are static ^{1}H spectra while the subsequent ones represent the results of mixing as a function of time. Clearly, there is no sign of incomplete mixing (local or large scale) in either case. The slight and roughly exponential increase of the peak intensity observed in Figs. 3A and B as well as the small difference between peak intensities in Figs. 2D–C can be assigned to lower (by less than 10%) nuclear magnetization of the incoming fresh liquids.

The proper minimum waiting time t_w between the driving pulse and the beginning of an NMR experiment can be found experimentally. The small wiggles in Fig. 2C and in the second spectra in Figs. 3A and B are motional artifacts: tested in subsequent stopped-flow experiments in the $t_d = 10$–40 ms range, they are always <5%
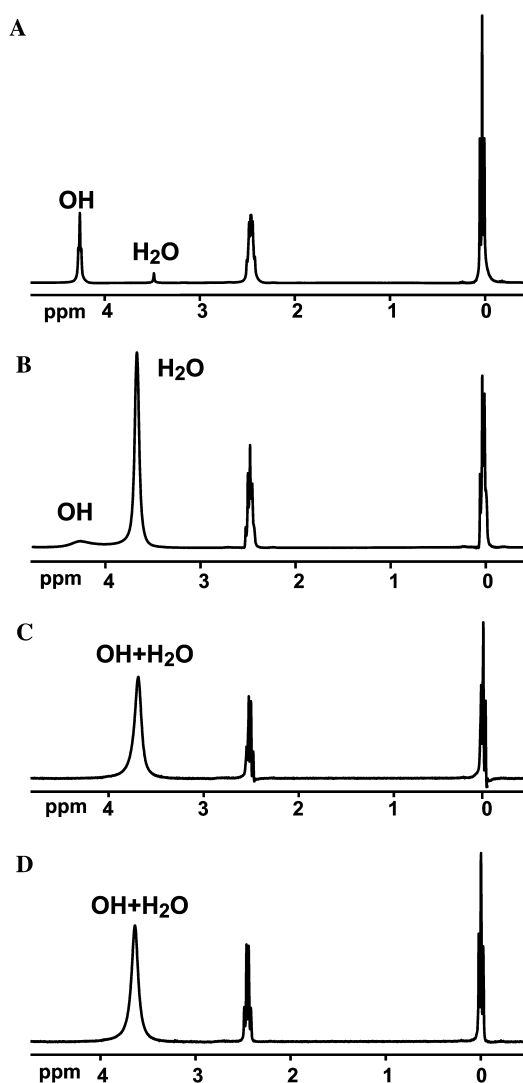

Fig. 2. [1]H spectra of aqueous solutions of ethanol. The spectrum of a 99% ethanol (A) and a pre-mixed 54% ethanol sample (B) can be compared to spectra obtained 20 ms (C) and 10 min (D) after the stopped-flow mixing of a 50% sample. The shift scale is relative to the methyl peak of ethanol.
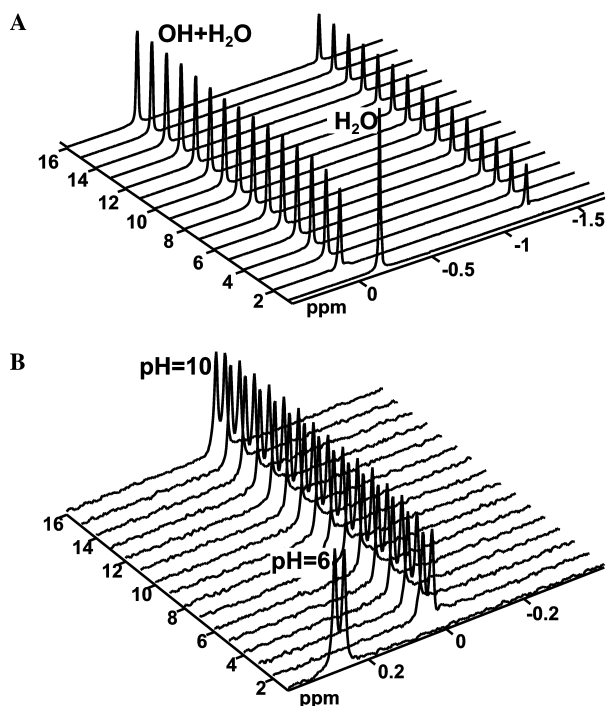
Fig. 3. Sections of ^{1}H spectra of mixtures of water and ethylene glycol (A) and of 0.5 M aqueous solutions of alanine with pH 6 and 14 (B). The chemical shift scale is relative to the water (A) and the methyl (B) peaks after mixing. At start, the observation chamber D had only one of the components, pure water (A) or pH 6 alanine solution (B). Mixing was initiated by driving pulse P at 20 ms before the acquisition started for the second spectra in both series. Further spectra were recorded at interscan delays of 800 ms (first eight slices) and 10 s (last ones).

Fig. 4. The dependence of the total replaced liquid volume V on the filling time t_{m} at driving pressure 4 atm (A) and on the driving pressure p at $t_{\mathrm{m}} = 60$ ms (B), for water (■) and ethanol (●).

of total peak intensities and disappear altogether at waiting times $\geqslant 40$ ms, with no evidence of incomplete mixing in any of those tests.

In a stopped-flow apparatus, the concentration of the obtained mixture depends primarily on the ratio R_{fl} of flow rates of the two input liquids. The R_{fl}-value set by adjusting screw S has to be reproducible and constant over the whole period of mixing. To investigate the factors influencing R_{fl}, additional test experiments that measured the total replaced volume of liquids $V(t_{\mathrm{m}},p)$ for ethanol and for water (in separate experiments, with both input chambers having the same liquid) upon varying t_{m} in the 20–150 ms range and the driving pressure p over 2, 4, 6, and 7 atm were performed (Fig. 4). Although water and ethanol have different viscosities, densities, and surface tensions, the observed linear dependence of $V(t_{\mathrm{m}},p)$ on t_{m} shows that the period of time necessary for the initial acceleration of the pumped liquid is relatively small and is therefore not influencing R_{fl}. Moreover, the insignificance of the acceleration time also means insignificance of the initial inertial forces. Although a complete description of all hydrodynamic processes taking place in the presented setup is obvi-
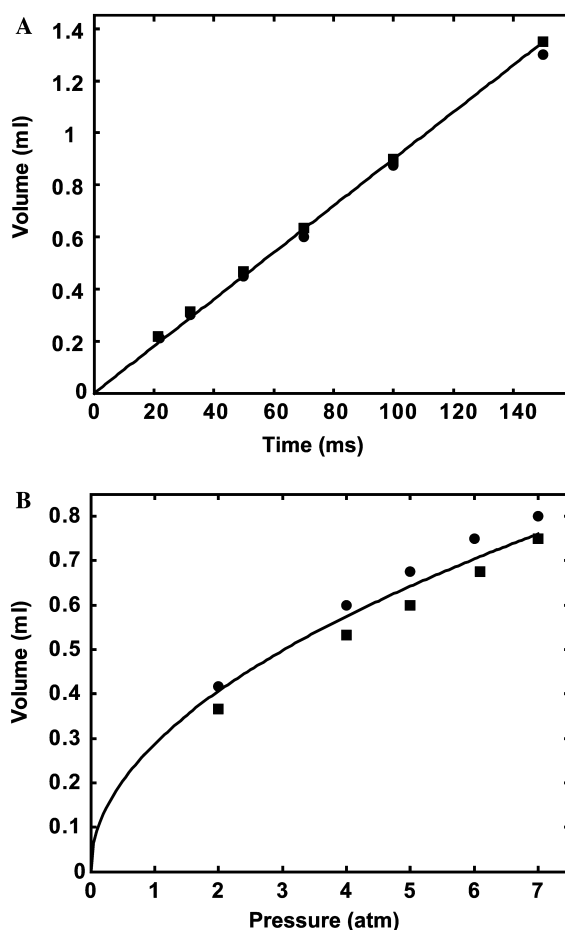
ously complicated, another important conclusion can be drawn from linear extrapolation $V(0,p) \sim 0$ that shows that the effective opening/closing response time is negligibly short and does not influence the performance of presented apparatus.

Theoretically, the character of flow with velocity v depends of the so-called Reynolds number $R = \frac{\rho v d}{\mu}$, where ρ is the density, μ is the (dynamic) viscosity of the liquid, and d is the tube diameter [21]. At low (<2000) Reynolds numbers, the flow is dominated by viscous stress and is therefore laminar with flow velocity $v \sim \Delta p$ where Δp is the pressure drop over the tube length. At high (>4000) R, inertial forces dominate and the flow is completely turbulent with $v \sim \sqrt{\Delta p}$. Our typical Reynolds number values, calculated for all used tube diameters and for the observed ~ 10 ml/s flow rate (at 6 atm driving pressure), were rather high (typically over 4000). Consistently, the experimental dependence of $V(t_{\mathrm{m}},p)$ on p was found for ethanol and for water to be approximately proportional to the square root of the driving pressure (Fig. 3B). Differences between water ($\mu \sim 1$ cP)

and ethanol ($\mu \sim 1.2$ cP) flow behavior are thereby probably insignificant. One should also note that ethylene glycol has a much higher viscosity than the other used liquids. Therefore, its Reynolds number inside the connecting tube is in the order of 10^2 and the character of flow is close to lamellar. However, the flow regime is probably turbulent even for that liquid inside the mixing chambers. The good mixing homogeneity witnessed in Fig. 3B shows that this is sufficient, and our design perform well even in the case of large viscosity differences between the two input liquids.

Since inertial forces dominate the flow, $v \sim \sqrt{\frac{p}{\rho}}$ must approximately prevail. Hence, R_{fl} and thereby the mixing ratio of the presented setup depends merely on the square root of the density ratios of the used liquids. Nevertheless, R_{fl} must be experimentally calibrated by adjusting screw S. Since the effective inertial forces for turbulent flow around valves B may be irreproducible, it is important that the internal diameter of tubes connecting chambers A and mixing chambers F is small enough to create sufficiently high friction for the streaming liquid. If effective friction over the connecting tubes/ screws S dominates, the setup provides a stable R_{fl}.

Note that other factors may also affect R_{fl}. For example, transition from an initial lamellar into a turbulent flow could take significant time, different for different liquids. If so, R_{fl} would vary over t_{m}, with a strongly varying concentration profile of the mixture along the B–D pathway as a result. This effect was found to be negligibly small. Any eventual influence of left-over solution placed after the observation chamber has been suppressed by putting a constricting "neck" just after the observation chamber outlet (shown in Fig. 1 for the capillary version of the observation chamber). Similar neck on the input side suppresses effects from concentration variation originating from sudden closing of valves B. The construction can also be simplified under certain conditions. For instance, the absence of stop valve D does not lead to strong cavitation if degassed liquids are used.

4. Conclusions

Any design intended for in situ mixing of liquids for NMR experiments is marred by compromises as remedies for problems of stability, chemical homogeneity, speed, motional artifacts, bubble formation, etc. often counteract each other. The presence of magnetic field may also exclude certain driving options. In the presented equipment, we turned the magnetic field into an advantage: by exploiting the Lorentz torque liquid storage and pumping can be concentrated just above the sample space of the NMR probe. If any, this design element has been essential to achieve a satisfying compromise, as presented above, among the many conflicting demands on a fast-mixing NMR setup. Our results indicate compositional homogeneity and thereby complete mixing at less than 20 ms after the end of the driving pulse in a wide range of liquid viscosities.

References

[1] R.R. Ernst, G. Bodenhausen, A. Wokaun, Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon Press, Oxford, 1987.

[2] N.A.J. van Nuland, V. Forge, J. Balbach, C.M. Dobson, Real-time NMR studies of protein folding, Acc. Chem. Res. 31 (1998) 773–780.

[3] S.R. Crouch, F.J. Holler, P.K. Notz, P.M. Beckwith, Automated stopped-flow systems for fast reaction-rate methods, Appl. Spectrosc. Rev. 13 (1977) 165–259.

[4] J.L. Sudmeier, J.J. Pesek, Fast kinetics by stopped-flow chlorine-35 nuclear magnetic resonance. Reactions of mercury(II)-bovine serum albumin with various ligands, Inorg. Chem. 10 (1971) 860–863.

[5] J.J. Grimaldi, J. Baldo, C. McMurray, B.D. Sykes, Stopped-flow nuclear magnetic resonance spectroscopy, J. Am. Chem. Soc. 94 (1972) 7641–7645.

[6] J.J. Grimaldi, B.D. Sykes, Design of stopped-flow NMR rapid-mixing cells, Rev. Sci. Instrum. 46 (1975) 1201–1205.

[7] C.A. Fyfe, M. Cocivera, S.W.H. Damji, High resolution nuclear magnetic resonance study of chemical reactions using flowing liquids. Kinetic and thermodynamic intermediates formed by the attack of methoxide ion on 3,5-dinitrocyanobenzene, J. Chem. Soc., Chem. Commun. (1973) 743–744.

[8] D.A. Couch, O.W. Howarth, P. Moore, Kinetic studies by stopped-flow pulse Fourier transform nuclear magnetic resonance, J. Phys. [E] 8 (1975) 831–833.

[9] C.A. Fyfe, M. Cocivera, S.W.H. Damji, Flow and stopped-flow nuclear magnetic resonance investigations of intermediates in chemical reactions, Acc. Chem. Res. 11 (1978) 277–282.

[10] R.O. Kühne, T. Schaffhauser, A. Wokaun, R.R. Ernst, Study of transient chemical reactions by NMR. Fast stopped-flow Fourier transform experiments, J. Magn. Reson. 35 (1979) 39–67.

[11] S. Funahashi, K. Ishihara, S. Aizawa, T. Sugata, M. Ishii, Y. Inada, M. Tanaka, High-pressure stopped-flow nuclear magnetic resonance apparatus for the study of fast reactions in solution, Rev. Sci. Instrum. 64 (1993) 130–134.

[12] M. Hamang, A. Sanson, L. Liagre, V. Forge, P. Berthault, Fast mixing device inside a nuclear magnetic resonance magnet: a tool for observing early steps in protein folding, Rev. Sci. Instrum. 71 (2000) 2180–2183.

[13] K.H. Mok, K. Nagashima, I.J. Day, J.A. Jones, C.J.V. Jones, C.M. Dobson, P.J. Hore, Rapid sample-mixing technique for transient NMR and photo-CIDNP spectroscopy: applications to real-time protein folding, J. Am. Chem. Soc. 125 (2003) 12484–12492.

[14] D.B. Green, J. Lane, R.M. Wing, A standard session stopped-flow NMR tube, Appl. Spectrosc. 41 (1987) 847–851.

[15] Q.H. Gibson, L. Milnes, Apparatus for rapid and sensitive spectrophotometry, Biochem. J. 91 (1964) 161–171.

[16] I. Weinberg, J.R. Zimmerman, Concentration dependence of chemical exchange and NMR multiplet structure in water–ethanol mixtures, J. Chem. Phys. 23 (1955) 748–749.

[17] A.B. Kudryavtsev, V.I. Ermakov, P.A. Zagorets, PMR study of the structure of the water–glycol system, Russ. J. Struct. Chem. 15 (1974) 136–137.

[18] H. Günther, NMR Spectroscopy, Wiley, Chichester, 1995.

[19] D.V. Batov, A.M. Zaichikov, V.P. Slyusar, V.P. Korolev, Enthalpies of mixing and state of components in aqueous-organic mixtures with nets of hydrogen bonds, Russ. J. Gen. Chem. 71 (2001) 1208–1214.

[20] T. Sato, A. Chiba, R. Nozaki, Dynamical aspects of mixing schemes in ethanol–water mixtures in terms of the excess partial molar activation free energy, enthalpy, and entropy of the dielectric relaxation process, J. Chem. Phys. 110 (1999) 2508–2521.

[21] S.B. Pope, Turbulent Flows, Cambridge University Press, Cambridge, 2000.