NMR relaxation time investigation of highly diluted aqueous solutions of silica-lactose

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Keywords: water; proton NMR relaxation; ultrahigh dilution; silica.

Highlights:

– Investigations into physicochemical properties of ultrahigh dilutions are sparse, but urgently needed
– We measured 20 MHz longitudinal (T1) and transverse (T2) water proton relaxation in highly diluted silica-lactose solutions
– We observed a significant negative correlation of T1 and T2 for both Sil/Lac and LiCl samples, which changed to positive after they were heated

Abstract

The aim of the present investigation was to replicate a study published by Demangeat in 2010 in the Journal of Molecular Liquids. We measured 20 MHz longitudinal (T1) and transverse (T2) proton relaxation of H_2O in highly diluted silica-lactose (Sil/Lac) solutions in 0.15 M LiCl in water (Sil/Lac C6–C24, range 10^{-7} M – 10^{-47} M). The samples were prepared by repetitive succussion and centesimal dilution under rigorously controlled laboratory conditions. Lactose solutions in 0.15 M LiCl in water (Lac C6–C24) were identically and simultaneously treated, as controls. Neither for lactose (Lac) nor for Sil/Lac solutions did T1 and T2 relaxation parameters show a significant correlation with increasing dilution level. A cross-correlation analysis of T1 and T2 revealed a significant negative correlation for both Sil/Lac and LiCl samples, which changed to positive after placing the tubes for 10 minutes in boiling water. These results diverge from those obtained in the study of Demangeat. In our investigation, the process of potentisation (repetitive succussion and dilution) seems to be responsible for the modifications of proton relaxation times, irrespective of the presence of silica. We hypothesise that succussion intensity during dilution is a crucial factor that needs to be investigated in more detail in future investigations.

1. Introduction

For more than a century homeopathy has attracted the curiosity of researchers. This is mainly because homeopathic dilutions, as applied in clinical practice, often exceed the inverse Avogadro's number by several orders of magnitude. Nevertheless, effects of homeopathic preparations have been repeatedly observed in various preclinical and clinical studies[1-10].

Various scientific methods have been applied to investigate a possible mode of action of homeopathic medicines[11, 12]. Predominantly, these methods focus on detecting alterations of the molecular structure and dynamics in water[11]. These efforts aim to explain a transfer of information into water. The presumed long-term storage of any such information in water ('the memory of water')[13] has barely been explored to date[12].

Among physico-chemical research on high dilutions, NMR relaxation time measurements appear to be a promising method to identify any specific molecular properties of homeopathic high dilutions[10]. In particular, a series of NMR relaxation time investigations carried out by the French researcher Jean Louis Demangeat are highly standardised experiments supporting the view that homeopathic preparations exhibit some specific physicochemical properties, which differ from succussed plain solvent. Demangeat stated in a series of publications (starting from 1992) that differences in NMR relaxation time measurements between homeopathically potentised (diluted and succussed) solutions and identically treated solvents are measurable even for dilution levels beyond Avogadro's limit[14-18].

Specifically, in 2010 Demangeat published a 20 MHz water proton NMR relaxation study of aqueous and saline (NaCl and LiCl) high dilutions (C6 – C24 range) of silica-lactose (Sil/Lac) produced in two types of containers (glass, polyethylene)[17]. Sil/Lac preparations showed a slight progressive increase in T1 and decrease in T2 with increasing dilution level, resulting in an increase of the T1/T2 ratio, and showed an inverse correlation between T1 and T2. The modifications were most pronounced in Sil/Lac dilutions prepared in LiCl medium in polyethylene vessels. Proton relaxation times modifications were observed even in the ultramolecular dilution range. After placing the sealed NMR tubes in boiling water for 10 minutes, all relaxation times modifications vanished and the T1/T2 ratio decreased. Furthermore, the inverse correlation between T1 and T2 relaxation times changed to a positive correlation. No significant effects were induced in pure water and salt control solutions, which underwent exactly the same procedure of iterative dilution and agitation.

The author interpreted these findings in terms of a more ordered molecular structure of the highly diluted Sil/Lac preparations. Focused on a hypothesis first put forward by the Nobel prize-winning French virologist Luc Montagnier[19], Demangeat proposed that nanosized superstructures composed of water, ions and nanobubbles are formed. Generated during the mechanical process of succussion, these nanobubbles are thought to nucleate around the solutes, thereby imparting structure to the solvent.

The present study has been designed to investigate the reproducibility of the findings of Demangeat, more precisely, a subset of experiments ("Set 2", published in 2010) [17]. We measured 20 MHz water proton NMR relaxation of high dilutions (C6–24) of silica-lactose, potentised in LiCl in polyethylene vessels, compared to analogously potentised lactose in LiCl dilution medium as controls. Polyethylene vessels were used for potentisation, also to avoid artefacts due to the leaching of silica and other impurities from the walls of the tubes.

2. Methods

2.1. Production of solutions

All samples were prepared with deionized, double distilled and autoclaved water from the same batch (further indicated simply as 'water') under a laminar-flow hood in sterile conditions by means of calibrated Eppendorf pipettes with disposable plastic tips. Tips were discarded after each use. Only new materials were used, previously rinsed three times with deionized water, three times with 70% ethanol and left under laminar flow for one night to dry. All procedures were conducted in Arlesheim (Basel, Switzerland) in the laboratories of the Hiscia Institute in July, August (one systematic negative control series and series 1-3) and November (series 4) 2011.
Sample preparation was adapted from Demangeat[17]. One part of insoluble solid silica (50 mg, p.a., Weleda AG, Schwäbisch Gmünd, Germany, lot n° M137898) was homogenised with 99 parts of lactose (4.95 g, lot 05118) triturating for 10 minutes (three cycles of 3.20 min. trituration and 2 min. scraping out the mortar). 50 mg of the powder obtained was added to 4.95 g of lactose and again homogenised for 10 minutes by trituration. After these two centesimal triturations, 50 mg of the powder (C2) was added to 5 ml of a 0.15 M solution of LiCl (p.a., Merck, lot n° 7447418) to obtain a C3 dilution in a polyethylene tube (sterile polyethylene tubes, VWR, Switzerland, 14 ml, n° 1025). The tube was capped by a plastic cap and then succussed manually (from head to belt high) 100 times with a frequency of 1 Hz. Then 50 µl of the solution obtained was added to 4.95 ml of 0.15M LiCl in the next polyethylene tube to proceed to the C4 potency level. This procedure was repeated 21 times until a C24 dilution was achieved. Seven centesimal dilutions were retained for measurement: C6, C9, C12, C15, C18, C21, C24. The controls were prepared with exactly the same treatment except for the presence of silica at the beginning of the procedure. Each dilution step of the trituration/succussion treatment was carried out alternately between the set of Sil/Lac and the Lac control set. In addition to the laminar-flow sterile conditions, contamination was avoided by using different mortars and pestles for the different sets, and by using new polyethylene tubes and new dropper tips at each step. Four independent series of centesimal dilutions and controls were prepared.
For each retained dilution, 200 µl was dropped in duplicate into two NMR tubes (Duran, 35 x 8 mm, Assistent, Germany). For each sample set, two samples of unpotentised water of the same batch were also prepared with 200 µl. All 30 samples were stoppered (polyethylene stopper, Nn° 8107, Plastik-Haus AG, Arlesheim, Switzerland) but not flame-sealed. SB inserted the NMR tubes in NMR vials (Bruker, Karlsruhe, Germany, NMR Tubes 180x10mm, AR-glass), which were numbered from 1 to 30, with a computer-generated randomised code. All NMR measurements were done by FE with coded (blinded) samples.
All solutions (Sil/Lac dilutions and Lac controls) belonging to one series were prepared within half a day and then kept for 2 days at 4.7 ± 0.1 °C in a refrigerator (Miele, K 3214 S-1, Gütersloh, Germany) before the measurements started. The samples in the refrigerator were arranged in the rack in 3 lines of 10 samples. The temperature of the samples was continuously monitored with an electronic thermometer (Endotherm, Arlesheim, Switzerland).
For use in training and control experiments one series of 30 samples of unsuccussed water was prepared in addition.

Table 1: Differences in sample preparation between the original study of Demangeat[17] and our replication study.

	Demangeat study	Current replication study
Sample succussion	Mechanical	Manual
NMR tube closure	Flame-sealed	Stoppered with polyethylene caps

Concerning preparation of the samples, two differences between the original and our replication study should be outlined (Table 1). In the study of Demangeat a mechanical stroker was used for succussion. The vigorous agitation was achieved by a specific mechanical apparatus ('vertical stroker') manufactured by Boiron (Sainte-Foy-lès-Lyon, France) for industrial applications, which produces 300 violent vertical strokes in 14 s. In our replication study, the probes were manually succussed with a standardised method since the Boiron apparatus was not available. Second, our NMR vials were not flame-sealed but stoppered with plastic caps. Any possible relevance of these differences will be discussed later in this article.

2.2. NMR measurements

We used a minispec mq 20 NMR spectrometer (Bruker optics GMBH, Fällanden, Switzerland, N° ZD025905) for all measurements. The sample temperature in the probe-head was maintained at 4.7 °C using a thermostat (Haake K10, Karlsruhe, Germany, N° 003-4183) through continuous circulation of deionised water. Samples were stored in a refrigerator (set to 4.7 °C). For measurement of a given sample, it was taken out of the refrigerator, the outer walls rapidly dried with laboratory wipes, inserted in the probe-head and left to equilibrate temperature for ten minutes before the T1 measurements were started. After three T1 measurements (about 46 minutes) without pre-delay time, T2 was measured three times with 300 seconds pre-delay time (about 20 minutes). After measurement, samples were put back in the refrigerator.
After all 30 samples of a given series had been measured, the NMR vials were taken out of the refrigerator, and inserted into another rack. The rack was left for 10 minutes in a batch of boiling deionised water. To avoid the expulsion of the plastic caps due to overpressure in the NMR tubes, Duran-glass rods (Assistent, Sondheim, Germany, 35 x 8 mm) were used to keep the plastic caps in place. Afterward the outer walls of the samples were dried and placed in the refrigerator at 4.7 °C for two days, before the series was re-measured under the same technical conditions.
The followings settings were applied for T1 inversion-recovery measurements: scans: 4; recycle delay: 20 s; gain: 77 dB; dummy shots: 0; pulse attenuation: 3 dB; first pulse separation: 50 ms; final pulse separation: 15000 ms; number of data points for fitting: 10; delay sampling window: 0.05 ms; sampling window: 0.02 ms; time for saturation curve display: 3 s; monoexponential curve fitting, phase cycling, pre-delay time for data acquisition: 0 s.
For T2 measurements (Carr–Purcell–Meiboom–Gill sequence): scans: 4; recycle delay: 15 s; gain: 73 dB; dummy shots: 0; pulse attenuation: 3 dB; 90°-180° pulse separation (tau): 1; number of data points for fitting: 7500; number of not fitted echoes: 0; monoexponential curve fitting, phase cycling, pre-delay time for data acquisition: 300 s.

2.3. Statistical analysis

The code of all samples for all series was only disclosed after completion of all experiments. The results of each set of three measurements of T1 and T2 were averaged, and T1/T2 ratios calculated. All statistical calculations and tests were performed with the software Statistica 6.0 (StatSoft, Inc., Tulsa, OK 74104, USA). Linear regression analysis with a significance level (p-value) set to 0.05 was used. Data evaluation followed closely the procedure of Demangeat[17].

3. Results

3.1. Water proton relaxation dependence on the level of dilution

The results obtained are expressed in mean relaxation time values (Table 2) and as a function of the centesimal dilution (Fig. 1A/C/E and Fig. 2A/C/E, respectively).

Table 2: *Mean relaxation times [ms] of all four measurement series (mean ± standard error, SE) and linear regression analysis (Regr. f(CD)) between relaxation time and centesimal dilution level (CD). N.S., non significant (p>0.05).*

	T1		**T2**		**T1/T2**	
	Mean [ms]	Regr. f(CD)	Mean [ms]	Regr. f(CD)	Mean [ms]	Regr. f(CD)
Lactose (controls)	1435 ± 2	N.S.	1266 ± 4	N.S.	1.133 ± 0.004	N.S.
Silica-lactose	1434 ± 2	N.S.	1269 ± 5	N.S.	1.131 ± 0.006	N.S.
Heated lactose (controls)	1449 ± 2	N.S.	1316 ± 7	N.S.	1.102 ± 0.005	N.S.
Heated silica-lactose	1449 ± 3	p=0.033	1308 ± 7	N.S.	1.109 ± 0.007	N.S

No difference was found for the comparison of the overall mean relaxation times of LiCl controls and Sil/Lac solutions. The linear regression analysis did not exhibit significant variations of T1 and T2 relaxation times with increasing dilution, though there was a tendency for an increase of T1 and T1/T2 as a function of dilution level, and a corresponding tendency for a decrease for T2 (Fig. 2 A/C/E). A cross-correlation analysis showed comparable behaviour of Sil/Lac and Lac controls: T1 and T2 were negatively correlated in both controls and in Sil/Lac dilutions (Fig. 3A/B).

3.2 Effect of heating

An increase of the T1 and T2 mean relaxation times was observed after placing the samples in boiling water for 10 minutes (Table 2, Fig. 1B/D/F and Fig. 2B/D/F). The cross-correlation analysis of T1 and T2 demonstrated a reversal into a positive correlation. This was true for both Lac controls and for Sil/Lac dilution (Fig. 3C/D). It is noteworthy that a sample of unpotentised water also showed the same behaviour, exhibiting a positive correlation between T1 and T2 (Fig. 3E).

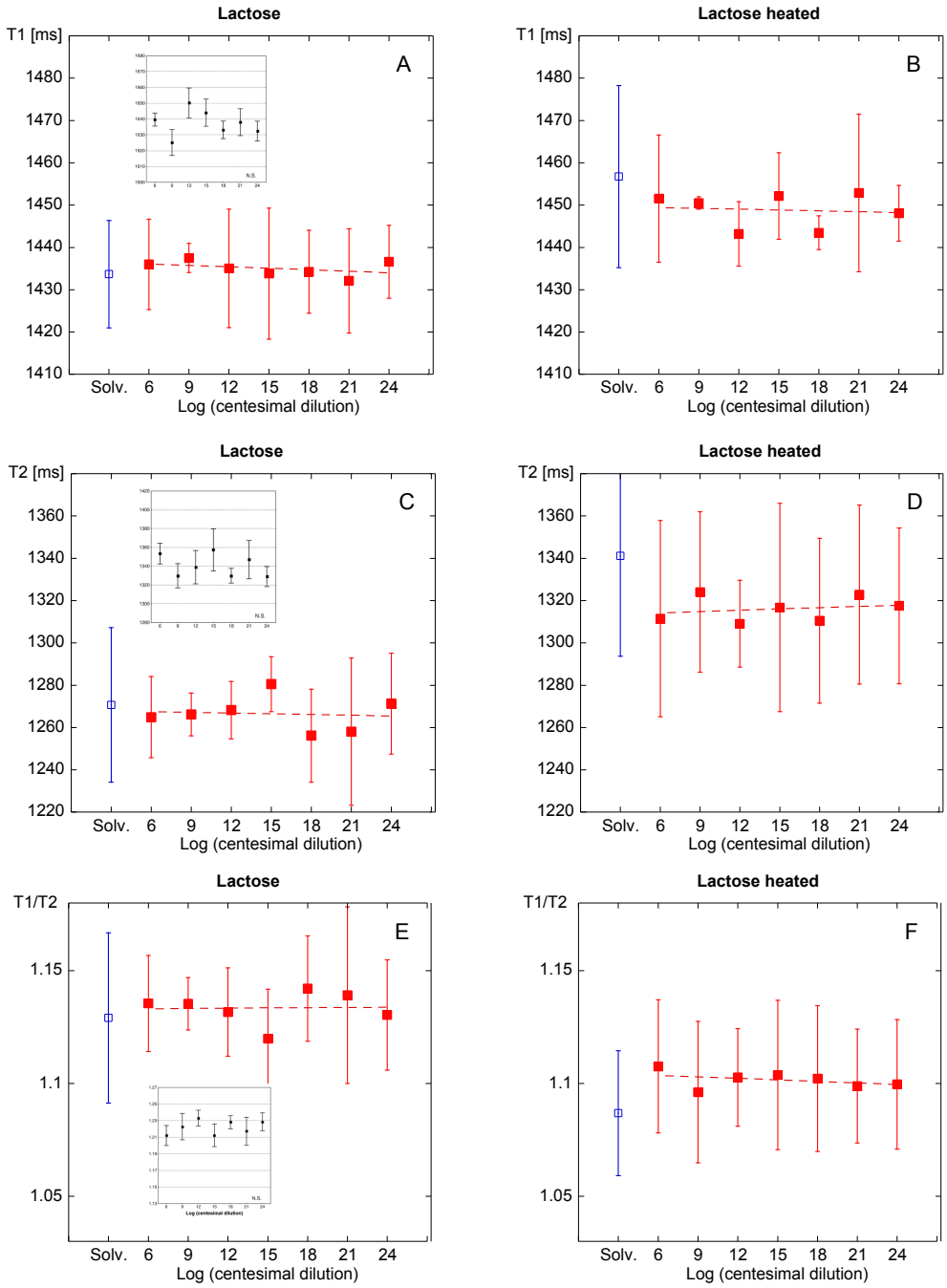

Figure 1: Mean ± SE of T1 (A/B) and T2 (C/D) relaxation times as well as T1/T2 ratio (E/F) as a function of dilution level for lactose controls (A/C/E) and for heated lactose controls (B/D/F). The insets show the analogous graph with the results described in the study of Demangeat, if present in his publication[17] (reprinted with permission from Elsevier). Data from the four measurement series were averaged and fitted by linear regression. Solv. = unpotentised samples (solvent).

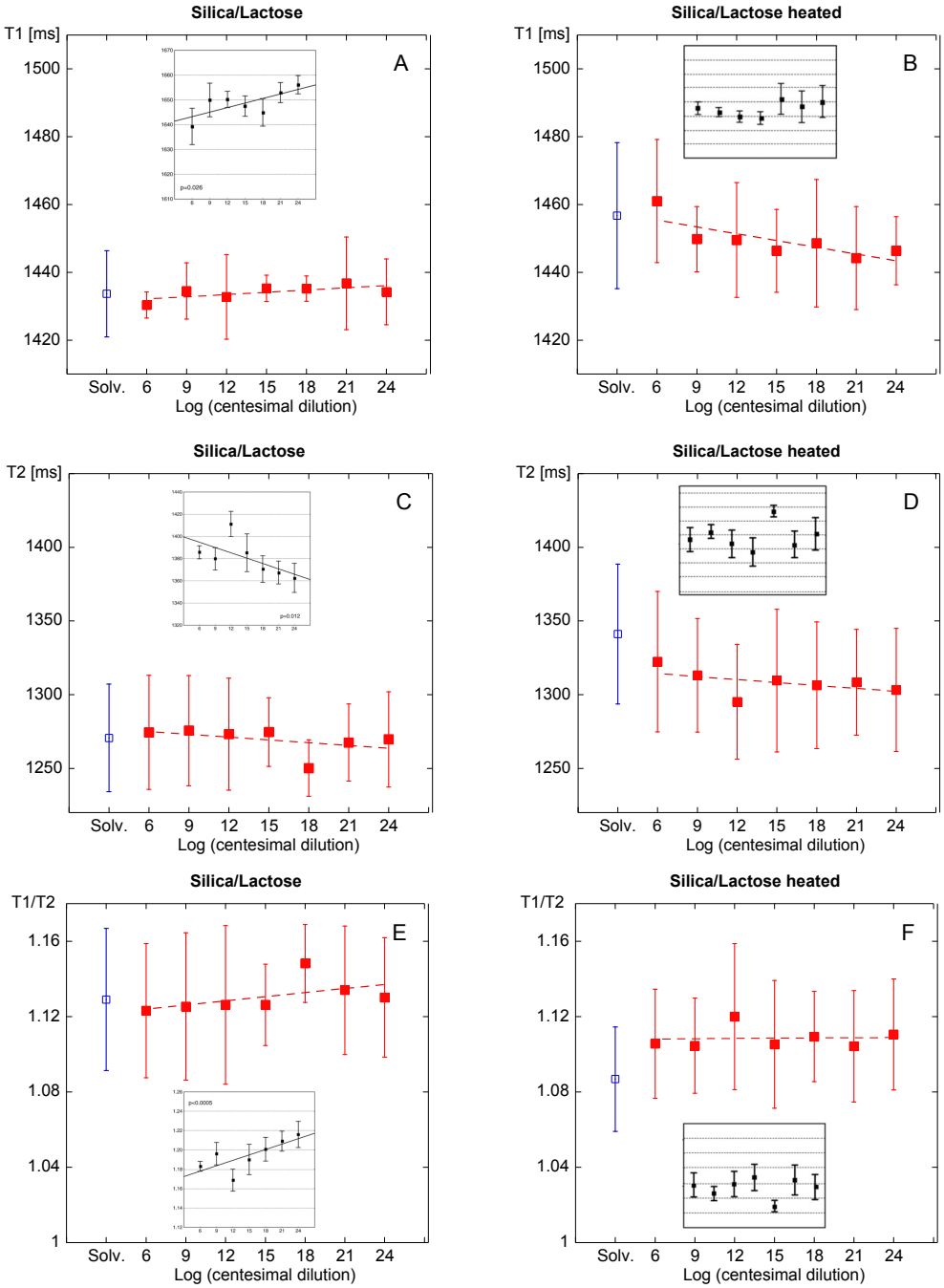

Figure 2: Mean ± SE of T1 (A/B) and T2 (C/D) relaxation times as well as T1/T2 ratio (E/F) as a function of dilution level for silica/lactose samples (A/C/E) and for heated silica/lactose samples (B/D/F). The insets show the analogous graph with the results described in the study of Demangeat[17] (reprinted with permission from Elsevier). Data from the four measurement series were averaged and fitted by linear regression. Solv. = unpotentised samples (solvent).

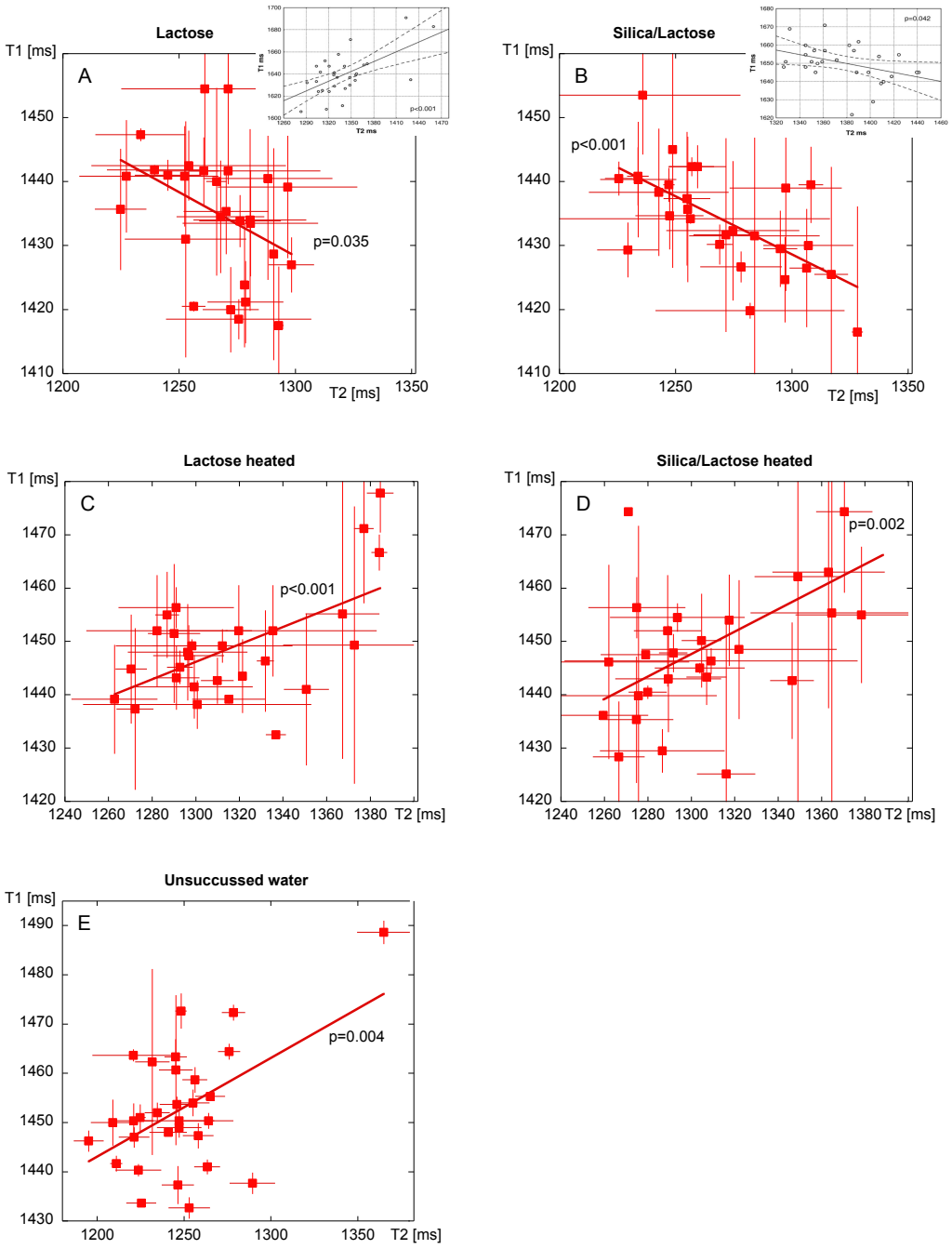

Figure 3: T1 and T2 linear cross-correlation (mean ± SE, p-value for linear regression) of Lac controls (A/C) and Sil/Lac dilutions (B/D) before (A/B) and after (C/D) heating, as well as unpotentised samples of water (E). The insets show the analogous graph with the results described in the study of Demangeat, if present in his publication[17] (reprinted with permission from Elsevier).

4. Discussion

Demangeat's study reported modifications of 20 MHz water proton relaxation time in highly diluted Sil/Lac solutions (C6–C24)[17]. In that study, whereas the lactose control solutions exhibited no obvious variations in relaxation times throughout the agitation/dilution process, significant variations occurred in the Sil/Lac solutions. Sil/Lac in LiCl medium exhibited more pronounced variations, namely a progressive increase in T1 and a progressive decrease in T2 with increasing dilution, resulting in an increase in the T1/T2 ratio for higher dilution levels. This behaviour was highlighted by means of a cross-correlation analysis, demonstrating a positive correlation of T1 and T2 in the lactose controls and a negative correlation in the Sil/Lac dilutions. The modifications remained detectable in the ultrahigh range of dilution. After a 10-min heating/cooling cycle, all these variations completely vanished showing a reversal of the cross-correlation analysis, resulting in a positive T1 and T2 correlation.

These unconventional findings were interpreted in terms of nanosized superstructures that originate stereospecifically around the solute and are composed of water, ions and nanobubbles generated during the vigorous succussion process. According to the interpretation presented in Demangeat's study, the nanosized superstructures reduced the mobility of the water molecules enabling a more ordered structure. More specifically, it was argued that for an isotropic molecular movement that can be described by a unique correlation time τ, a decrease in temperature or the introduction of a solute (which induce molecular organization), τ progressively increases. Consequently, as long as $\omega_0\tau<1$, T1 and T2 both decrease, but T2 does so a little more rapidly (Fig. 4). In that domain, T1 and T2 are positively correlated. For longer correlation times ($\omega_0\tau>1$, e.g. in ice or highly concentrated solutions) T1 and T2 start to diverge and T1 increases again, resulting in a negative T1 and T2 correlation.

In our study a coherent behaviour could be observed in T1 and T2 relaxation times in Lac controls and in Sil/Lac dilutions in the course of the dilution/succussion process. The cross-correlation analysis of T1=f(T2) revealed a negative correlation in controls as well as in Sil/Lac dilutions. The negative correlation in controls and Sil/Lac dilutions were reversed by the heating/cooling process. It is noteworthy that a series of unpotentised samples of water exhibited the same behaviour in a cross-correlation analysis, resulting in a positive correlation. The fact that the lactose controls exhibited a similar behaviour to Sil/Lac dilutions suggests that the dilution/succussion process plays a key role in our study. This is supported by the fact that unpotentised samples and heated samples exhibited the same behaviour in the cross-correlation analysis. We hypothesise that in our study, in both Lac and Sil/Lac samples nanosized superstructures based on nanobubbles were present, which had both been destroyed after heating.

Possible explanations for the divergences from the results of the Demangeat study may be found in the differences in sample preparation (see Table 1). Given that the succussion process enhances the dissolution of atmospheric gases in the solutions, and since the samples were not degassed, air content might be different after mechanical succussion (as in the study of Demangeat) compared to manual succussion (as in the present trial), leading to systematic differences in size and amount of nanobubbles. We furthermore cannot exclude that the use of plastic caps (instead of flame-sealing the samples) allowed an exchange of atmospheric gases during storage of the samples, altering the amount of dissolved air in all our succussed samples (lactose controls and silica-lactose), thus maybe changing the amount of nanobubbles and consequently influencing the formation of nanostructures. Thus, the manual succussion at about 1 Hz (instead of a mechanical one at about 20 Hz) could be one reason for the differences

observed. There is indeed some evidence that different methods of agitation (as well as the type of container) can have a significant influence on homeopathic preparations [20-22]. Correspondingly, future NMR proton relaxation studies on ultrahigh diluted substances should compare different succussion procedures as well as flame-sealed and capped vials.

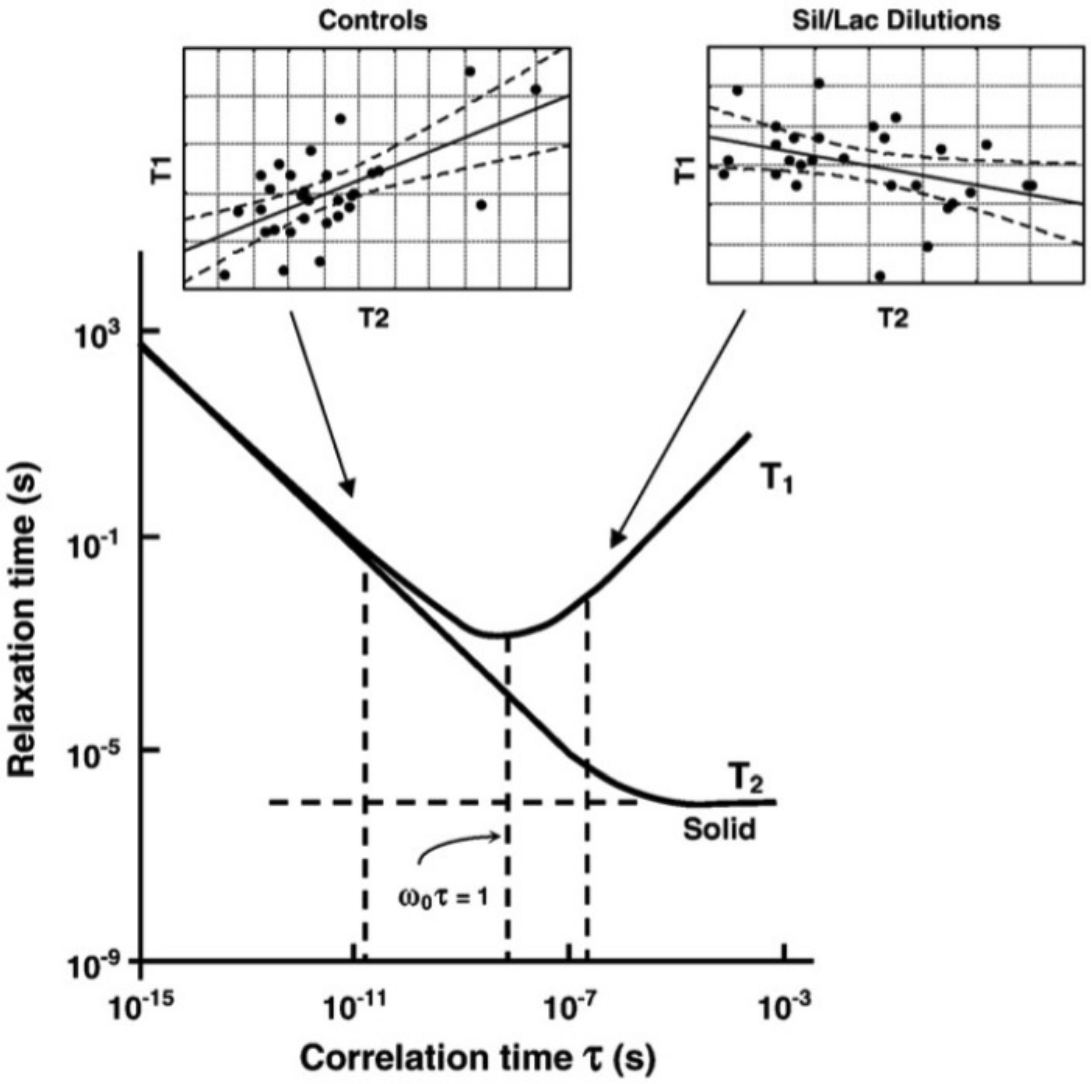

Figure 4: Theoretical behaviour of T1 and T2 at 29 MHz for protons in water as a function of the correlation time (reprinted from Demangeat 2010[17] with permission from Elsevier). In the experiments of the study by Demangeat, carried out at 20 MHz, the minimum of T1 is slightly shifted to the right. Note the inversion of the correlation of T1 and T2 in Sil/Lac dilutions compared to controls for higher correlation times.

A further open question concerns the possible direct influence of paramagnetic molecular O_2 – known as a strong relaxation agent[23,24] – in the present experimental system. Since the succussion process also enhances the dissolution of paramagnetic atmospheric oxygen in the solutions, the oxygen content might be different after mechanical succussion (as in the study of Demangeat) compared to manual succussion (as in the present study), leading to systematic differences in the proton relaxation time. Furthermore, the relaxing effect of dissolved or nanobubble-dispersed O_2 could interfere with the effect of the postulated nano-sized structures on proton relaxation. We therefore propose to investigate proton relaxation as a function of frequency[24,25] to distinguish between the different contributions to proton relaxation. O_2 concentration measurements in succussed samples and consideration in statistical evaluation as recently published[26] are recommended in future investigations. In addition, further O_2 degassing procedures (different from heating, e.g. freezing, vacuum application, etc.) should be investigated in their impact on experimental outcome.

Regarding the measurements, we performed 4 independent series in our study, compared to 6 in the study of Demangeat. Since we observed a significant correlation between T1 and T2 in both Lac controls and Sil/Lac samples (Fig. 3), the reduced number of independent series did not lead to false-negative results due to missing statistical power. Although we observed a tendency for an increase of T1 and T1/T2 as a function of dilution level, and a corresponding tendency for a decrease for T2 (Fig. 2 A/C/E), this effect was considerably smaller compared to the study of Demangeat. Also here, the reduced number of independent series did not lead to false-negative results due to missing statistical power. In our study, samples were coded (blinded), which was not the case in the study of Demangeat. Due to the objective nature of T1 and T2 measurement, we assume that the missing coding (blinding) in the study of Demangeat did not induce any bias in his data set.

NMR relaxation seems to be a promising tool to investigate specific properties of succussed highly diluted preparations. 30 investigations were identified in a recent review[10], with several of them belonging to successfully replicated high-quality studies. The application of sophisticated statistical models as recently used[26,27] adds further promising perspective in this research field.

Since this experiment was completed, a growing body of evidence supporting the formation of nanostructures during the process of potentisation has been published[28–30]. Furthermore, the detection of material in high-diluted solutions challenges the relevance of Avogadro's limit in homeopathic preparations[31,32] though the origin of this material is not yet clear[33,34].

As already mentioned in the introduction, previous research on high dilutions has focused mainly on a tentative change of the water structure throughout the dynamisation process, corresponding to a possible transfer of information into the water. Nonetheless three main questions remain scarcely explored: How might this information be stored over macroscopic timescales? How might it be transmitted to a complex biological system? How might specific responses be induced in such systems[6]? The growing nanobiology developments might offer the technical possibility to further investigate any transmission and effects of high dilutions in biological systems.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

FE, UW, SB: conceptualisation; FE, UW, SB: methodology; FE: sample preparation and data acquisition; UW, SB: supervision; FE: data evaluation; SB: data evaluation validation; FE, SB: visualisation; FE: writing – original draft; UW, SB: writing – review and editing.

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