Non-covalent interactions between poly(*N*-isopropylacrylamide) and small aromatic probe molecules studied by NMR spectroscopy

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Abstract:

One of the most important goals in drug delivery is to carry drug molecules to their target as selectively and efficiently as possible. To accomplish this goal it is crucial to understand the interactions of carriers and their loading. The interactions between a thermoresponsive potential drug carrier polymer, poly(*N*-isopropylacrylamide) (PNIPA) and small aromatic probe molecules: phenol, dopamine and indole derivatives including tryptophan were studied by using solution-state NMR spectroscopy. These substances represent structural elements often found in pharmaceutically relevant compounds. The indole ring is an important part of biologically active natural products, it can be found in several plants and animals. To investigate the effect of temperature on binding and the significance of coil-to-globule transition, ^{1}H T_1 and T_2 relaxation times, ^{1}H one- and two-dimensional nuclear Overhauser effect spectroscopy (NOESY) and diffusion ordered spectroscopy (DOSY) measurements were carried out in D_2O and organic solvents. In the case of phenol and indole derivatives a strong interaction was observed above the lower critical solution temperature (LCST), for it to be much weaker below. According to relaxation measurements only the aromatic ring of tryptophan is bound to the polymer. No interaction was observed between dopamine and the polymer.

Keywords:

NMR spectroscopy

poly-(*N*-isopropylacrylamide) (PNIPA)

Lower Critical Solution Temperature (LCST),

Intermolecular interaction

Indole derivatives

Article:

1. Introduction

In drug delivery there is a rapidly growing interest in carriers that are capable of controlled release. Compared to traditional medicine, materials with stimuli-responsive quality offer the opportunity to create systems which among other features can be used for controlled release of drugs at exact doses [1].

Due to its thermoresponsive characteristics, poly(*N*-isopropylacrylamide) (PNIPA) has potential applications in drug delivery [2], bioengineering [3] and selective separation [4]. PNIPA (Fig. 1.) is biocompatible [5] and non-toxic [6, 7]. Since it was first described 50 years ago, many papers have been published involving PNIPA and its copolymers in application as gels and surface layers [8]. PNIPA has a lower critical solution temperature (LCST) of *ca.* 32 °C, close to the temperature of the human body [4]. At this temperature, the reorientation of polymer chains occurs induced by a shift in the balance of entropic and enthalpic forces that govern the interaction of water with the hydrophobic (isopropyl, polymer backbone) and hydrophilic (amide group) sections [9-13].

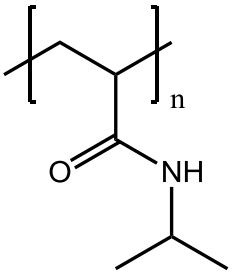

Fig. 1. Linear PNIPA

The exact value of the LCST depends on several factors, *e.g*. molecular weight, end groups and can be tuned via copolymerization [14]. Studies have shown that linear PNIPA and lightly crosslinked gels show similar thermoresponsive characteristics [4]. The gel has a volume phase transition (VPT) at around the LCST of a single chain (volume phase transition

temperature – VPTT). At the VPTT the degree of swelling decreases drastically. Drug uptake and release of the gel are determined by several factors, such as the rate and degree of swelling of the gel, the polymer-guest interactions, and the solubility and size of the guest molecule [15-18]. Thus intermolecular interactions have a crucial importance on determining the drug release kinetics [16, 19-23]. Despite their importance, drug-polymer interactions are not widely investigated. The following papers report ionic, hydrophilic (H-bonding) and hydrophobic interactions between loaded molecules and the polymer: [16, 19, 20, 23-34]

Several additives were found to influence the phase transition temperature of PNIPA [19, 24-26], indicating the presence of host-guest interaction. The binding ability of benzoates to PNIPA hydrogel and the corresponding linear polymer was investigated using differential scanning calorimetry (DSC), X-ray powder diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR) measurements [16]. Hydrophobic interactions were observed in case of various benzoates between their aromatic ring/ester side chain and the hydrophobic groups of PNIPA. Interactions between benzaldehyde derivatives and PNIPA were studied using ^{1}H NMR relaxation measurements [20]. A correlation was found between the shift of the LCST to the lower temperature and the incorporation of the drug with the polymer. The binding of hydrophilic amino acids is based on hydrogen bonding [19, 28]. Phenol is bound to the polymer by two ways: the hydrophobic interaction between the phenyl group and the polymer's isopropyl group, and the formation of hydrogen bonds between hydroxyl group and the polymer's amide group [24-26]. The different effects of ortho-, para- and meta-substitution patterns revealed the importance of hydrophilic/hydrophobic interactions in the conformational changes of PNIPA. The interaction is determined by the number and steric accessibility of the hydroxyl and the aldehyde groups [29]. Interaction of phenol/dopamine and PNIPA was investigated via solid-state ^{1}H CRAMPS NMR method at room temperature [33, 34, 35]. Phenol was found to be strongly interacting with the polymer, while no

interaction was observed between dopamine and PNIPA. Density functional theory (DFT) calculations were used to define the exact conformation of the phenol-PNIPA complex.

NMR investigations of phase transition in aqueous polymer solutions and gels have been reviewed by Spěváček [36]. Phase separation in aqueous polymer solutions or the volume phase transition in hydrogels affects NMR spectra as well as NMR relaxation times and diffusion coefficients. In spectra measured above LCST, a severe broadening of polymer signals occur caused by the decrease in mobility of polymer chains. Spin-lattice relaxation times T_1 showed a typical liquid-like behavior below the LCST, however above the phase transition T_1 values of the broad signal were decreasing with increasing temperature; identifying a solid-like state behavior [37]. In another study discontinuity in T_1 values and a minimum of the diffusion coefficient D was observed during the phase transition [38].

The goal of this paper was to study and understand the interaction present between PNIPA and several small molecules, namely: phenol, dopamine, indole, 5-aminoindole, 5-hydroxyindole and tryptophan in solution. Phenol and its derivatives are precursors and intermediate of pharmaceuticals, most notably aspirin [39], thus they are widely used as model molecules for several small aromatic drugs [26]. Dopamine is a neurotransmitter present in the brain and the peripheral nervous system [40]. The interaction of phenol and dopamine with the polymer is known in swollen gels, the temperature dependence of the interaction has however not yet been studied. Indole derivatives occur widely in natural products, existing in different kinds of plants, animals and marine organisms. The indole core is a near-ubiquitous component of biologically active natural products [41]. The interaction of indole and the polymer has not been studied. With these small molecules the influence of functionalities on the interaction with PNIPA can be understood. Since their interactions with the hydrogel and the linear polymer are similar, the latter can be used as a model for the former. Solution-state NMR methods, such as spin-lattice (T_1) and spin-spin (T_2) relaxation

times, nuclear Overhauser effect spectroscopy (NOESY) and diffusion ordered spectroscopy (DOSY) measurements were carried out on phenol-PNIPA and dopamine-PNIPA systems. Measurement of T_1 and NOESY were used to investigate non-covalent interactions present between PNIPA and indole derivatives. Changes in LCST in PNIPA – small molecule systems were studied via ultraviolet-visible (UV-VIS) spectroscopy. Differential scanning calorimetry (DSC) was used to determine changes in temperature of VPT of PNIPA hydrogel swollen in the presence of small molecules.

2. Experimental

2.1. Synthesis of linear and crosslinked PNIPA

N-isopropylacrylamide (NIPA, 97%, Sigma-Aldrich) was recrystallized twice from hexane. 1-propanethiol (99%, Sigma-Aldrich) was used without further purification. 2,2′Azoisobutyronitrile (AIBN, 98%, Sigma-Aldrich) was recrystallized twice from methanol. Tetrahydrofuran (THF, >99%, Molar Chemicals) and 1,4-dioxane (>99%, Molar Chemicals) was refluxed from $LiAlH_4$ and was used after distillation under nitrogen. Diethyl ether (>99% Molar Chemicals) was used without further purification. Poly(*N*-isopropylacrylamide) PNIPA was sythesized by chain transfer radical polymerization (CTRP). NIPA and the AIBN were added in a round button flask and were degassed by bubbling argon for 20 min. Deoxygenated dioxane and 1-propanethiol (CTA) were added to the system. The NIPA:CTA molar ratio was 50:1. The reaction mixture was heated to 65 ºC and after 6 hours the polymer was terminated with air and cooled to RT, then precipitated twice by addition of diethyl ether and filtered. The precipitation and filtration process was repeated. After that the product was dried in vacuum at 60 ºC until constant mass was obtained. The yield of the reaction was 74.8 % with a number average molecular weight (M_n) of 5.5 kg mol^{-1} and polydispersity a (PDI), M_w/M_n of 1.7, measured by a gel permeation chromatographic (GPC) equipped with a Waters 515 HPLC pump, and Mixed C separation columns (see SI, Fig. S1.). THF was used as eluent with a flow rate of 1 ml min^{-1}. The molecular weight was determined on the basis of calibration with polystyrene standards of narrow molecular weight distribution.

The PNIPA polymer gel was synthesized from *N*-isopropylacrylamide (NIPA) monomer (>98%, Tokyo Chemical Industry, Japan) and *N,N'*-methylenebisacrylamide (BA) cross-linker (99%, Sigma Aldrich) in aqueous medium at 20 °C by free radical polymerization. The reaction was initiated by ammonium persulphate (>98% APS, Sigma Aldrich) and *N,N,N',N'*-tetramethylethylenediamine (~99%, TEMED, Fluka). For the

synthesis of a pure PNIPA gel with a cross-linking ratio [NIPA]/[BA] = 150, 18.75 ml of a 1 M aqueous solution of NIPA and 1.225 ml of a 0.1 M solution of BA were mixed with 4.9 ml of water and 25 ml of TEMED. Finally, 1.25 ml of a 10 w/w% solution of APS was added to the mixture, and polymerization took place at 20 °C. Films of 2 mm thickness were prepared. All chemicals were used as received, except NIPA, which was recrystallized from a toluene–hexane mixture. Doubly distilled water was used for the synthesis and purification.

2.2. Materials

Phenol, dopamine, indole and tryptophan were purchased from Sigma-Aldrich, 5-aminoindole, and 5-hydroxyindole were purchased from Apollo, their purity was ≥ 98%.

Deuterium oxide and (dimethyl sulfoxide)-d_6 were purchased from Sigma Aldrich, they contained 99.9 atom% D. Methanol-d_4 was purchased from Cambridge Isotope Laboratories containing 99.96% D.

2.3. NMR instrumentation and experimental details

For relaxation measurements of phenol and dopamine a narrow bore Varian NMR SYSTEM spectrometer operating at 400 MHz (Larmor frequency of ^{1}H) was used, equipped with an inverse two channel HX probe. For relaxation measurements of indole and its derivatives, and for all one and two-dimensional NOESY and DOSY measurement a Varian NMR SYSTEM spectrometer operating at 600 MHz (Larmor frequency of ^{1}H) was used, equipped with an inverse three channel HCX probe. The spectrometer was equipped with a Z direction gradient of 65 Gauss/cm.

Pulse sequences from Varian's VnmrJ 4.0 software were used without modification. Inversion recovery (Invrec) for spin-lattice relaxation, Carr-Purcell-Meiboom-Gill (CPMGT2) for spin-spin relaxation, double pulsed-field-gradient echo (DPFGE) NOESY (NOESY1D) for selective one-dimensional NOE, NOESY for two-dimensional NOE, convection compensated bipolar pulse pair stimulated echo, Dbppste_cc for DOSY measurements. The

sample was temperature-controlled by heated airflow with an accuracy of 0.1 K. The samples were equilibrated at all temperatures for at least 30 minutes before the measurements. All temperature dependent measurements were performed starting from the lowest temperature to avoid differences in LCST because of hysteresis [42]. Details of NMR pulse sequences can be found in Supporting Information (SI).

2.4. Determination of LCST via UV-VIS spectroscopy

UV-VIS transmittance of the PNIPA-containing systems at 488 and 532 nm were measured with a Jasco V-650 spectrometer equipped with Jasco MCB-110 mini Circulation Bath and Peltier thermostat (See SI, Fig. S2.). The value of LCST was determined as the average of the inflection point of the measured curves at the two wavelengths. Measurements were taken between 15 and 45 °C, incrementing the temperature 0.2 °C steps at a rate of 0.2 °C min^{-1}. There was a 5 minute delay before every acquisition.

2.5. DSC measurements

Differential scanning microcalorimetry (MicroDSC) measurements were made on ground samples using a MicroDSCIII apparatus (SETARAM, France). About 10 mg of dry gel sample were placed in contact with 500 ml of Millipore water, and the solutions of small molecules (concentrations are in Table 1.) and kept at the initial temperature for 2 hours to allow the gels to equilibrate in the swollen state and to obtain a stable baseline. The samples were heated with a 0.03 ˚C min^{-1} scanning rate. The measurements were performed in the 10 to 50 ˚C temperature range. Determination of the peak position of the volume phase transition were performed using instrument software.

3. Results and discussion

3.1. LCST and VPTT values measured by UV-VIS spectroscopy and DSC

LCST values of PNIPA with and without the presence of small molecules were determined by UV-VIS spectroscopy, presented in Table 1. The polymer solution was additionally measured without the probe molecules in H_2O and D_2O. A *ca.* 1 °C difference was observed between the transition temperatures of the two systems. Transmittance of every PNIPA-small molcule system was measured in H_2O, however NMR measurements were taken in D_2O, thus it can be assumed that the coil-to-globule transition occured *ca.* 1 °C higher within the NMR samples. The results show that dopamine and tryptophan have no effect on the LCST of PNIPA; 5-aminoindole presented a slight decrease, while phenol, indole and 5-hydroxyindole significantly decreased the LCST of PNIPA, indicating the presence of strong intermolecular interactions. DSC measurements of the hydrogel swollen in H_2O with and without the presence of indoles (Table 2.) show similar tendencies, however changes of VPTT are less drastic. Thus NMR measurements of the linear PNIPA in solution can be used as a model for the hydrogel to study the present interactions with the small molecules, which can help to predict the release kinetics of drugs in future application.

System	$\rho_{polymer}$ (mg/ml)	$c_{small\ molecule}$ (μmol/ml)	$n_{small\ molecule}$: $n_{polymer}$	LCST (°C)
PNIPA - D_2O	4.3	-		34.1
PNIPA - H_2O	4.3	-		33.4
PNIPA - indole	4.3	20	1:2	22.5
PNIPA - 5-aminoindole	4.3	20	1:2	31.6
PNIPA - 5-hydroxyindole	4.3	20	1:2	23.7
PNIPA - tryptophan	4.3	20	1:2	33.4
PNIPA - phenol	14.3	30	1:4	25.5
PNIPA - phenol	2.9	30	1:1	27.5
PNIPA - dopamine	14.3	30	1:4	34.0

Table 1. LCST value of the polymer in the different systems measured by UV-VIS spectroscopy. Concentration and molar ratio of the small molecules and PNIPA

System	T_{VPT} (°C)
PNIPA gel	33.9
PNIPA gel - indole	29.8
PNIPA gel - 5-aminoindole	32.2
PNIPA gel - 5-hydroxyindole	28.2
PNIPA gel - tryptophan	34.0

Table 2. Temperature of the volume phase transition (VPT) of PNIPA gel ([NIPA]/[BA] = 150) measured by DSC

3.2. Temperature dependence of binding of phenol and dopamine to PNIPA

One of the advantages of NMR spectroscopy is that the dynamics of the model molecule and the polymer can be observed separately [43, 44]. The purpose of the following NMR measurements was to study the effect of temperature and coil-to-globule transition on the strength and quality of the interaction between phenol/dopamine and PNIPA.

The fraction (p) of phase separated units of PNIPA can be determined from the relation [45]:

$$p = 1 - (I/I_0) \quad (1)$$

where I and I_0 are the integrated intensities of the polymer CH_3 signal below (25 °C) and above (45 °C) LCST, respectively. According to ^{1}H onepulse spectra p = 0.79 (referenced to dopamine) in this case, meaning that 21 % of the polymer do not participate in the transition and can be directly detected above the LCST in high-resolution NMR spectra.

Relaxation times are related to motional correlation times (τ_c), thus they provide information about the dynamics of small molecules. NMR relaxation measurements show all NMR active nuclei – all ^{1}H in this case – separately, thus the binding of the small molecules can be described in more detail. The timescale of molecular events that can be monitored by the longitudinal relaxation time (T_1) are in the order of the reciprocal of the resonance frequency $(2\pi\gamma)^{-1}$, and with present day spectrometers include times around nanoseconds, while CPMG sequence (T_2) gives information in the μs-ms range. Relaxation measurements

were focusing on the small molecules in this study. Differences in T_1 and T_2 relaxation time of the model molecules in polymer-free and polymer-containing solutions can be a proof of the interaction. The measured relaxation rate ($T_{1\,O}^{-1}$) is the weighted average of the relaxation rates of free ($T_{1\,F}^{-1}$) and bound ($T_{1\,B}^{-1}$) guest molecule [46]:

$$T_{1\,O}^{-1} = X_{\mathrm{F}} \cdot T_{1\,F}^{-1} + X_{\mathrm{B}} \cdot T_{1\,B}^{-1} \qquad (2)$$

where X_{F} and X_B are the mole fractions of the free and bound form, respectively. Temperature dependence of T_1 relaxation times of phenol in D_2O and polymer-containing solutions are shown in Fig. 2.

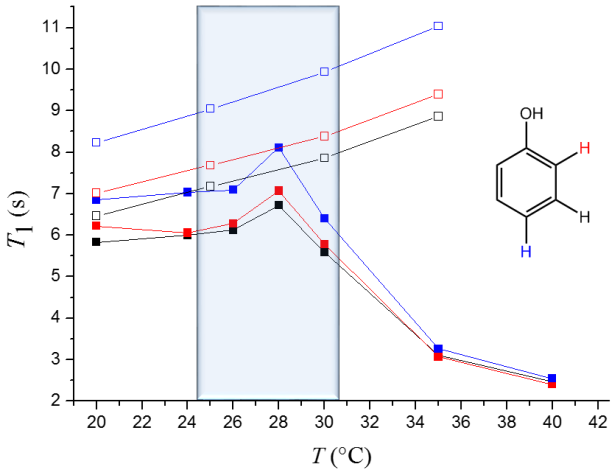

Fig. 2. Temperature dependence of T_1 relaxation time of the three ^{1}H nuclei of phenol between 20 and 40 °C, (-□- phenol in D_2O, -■- phenol in PNIPA-containing solution). The range between the maximum and minimum transmittance measured by UV-VIS spectroscopy is highlighted in blue. The measured intensities show monoexponential decay. The fitting errors of T_1 relaxation times are less than ± 0.06 s.

The transition temperature of the polymer is *ca.*26.5 °C in the presence of phenol. Below the LCST, there is a slight decrease of phenol's T_1 in the polymer-containing solution compared to phenol in D_2O. This difference is the least exactly at the LCST. However, above the LCST the difference is more significant. The T_1 of phenol in the presence of the polymer further

decreases with increasing temperature. This can be attributed to the increasing amount of interacting phenol. Because of the different timescale of motions responsible for spin-spin (T_2) relaxation, fitting a single exponential decay to the experimental data gave systematic errors. Thus biexponential was fitted giving a smaller and a higher T_2 value of phenol in polymer-containing system. The shorter one being within the range of the T_2 of the polymer, while the longer one between the polymer's and free phenol's (Fig. 3.).

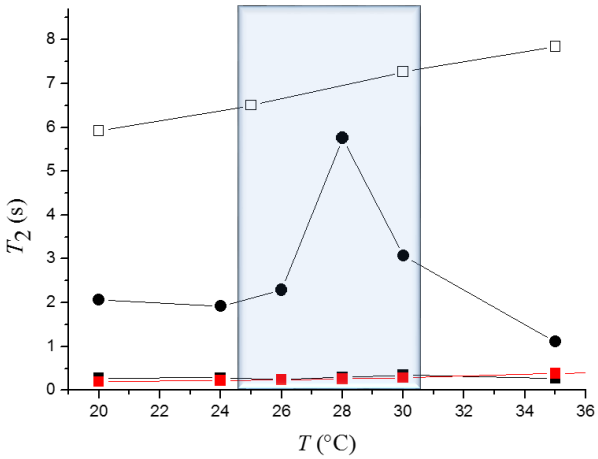

Fig. 3. Temperature dependence of T_2 relaxation time of ^{1}H nuclei of phenol. Biexponential was fitted to the experimental data of phenol-PNIPA solution. -●- longer T_2 of phenol (from biexponential fit) in PNIPA-containing solution, -■- shorter T_2 of phenol (from biexponential fit) in PNIPA-containing solution, -□- phenol in D_2O, -■- PNIPA in D_2O (at higher temperatures T_2 values of PNIPA correspond only to the fraction which is not involved in the transition). The fitting errors of T_2 relaxation times are less than ± 0.12 s.

Hofmann *et al.* [20] distinguished three possible sites of salicylaldehide in presence of PNIPA: completely incorporated, completely free and loosely bound. The latter is in fast exchange with the completely free species. Relaxation measurements show that in case of phenol the same three sites exist. T_2 which is in the range the polymer's T_2 can be attributed to completely bound site, which presents a slight increase from 7 to 14 % between 20 and 40 °C. The higher T_2 value is the weighted average of completely free and loosely bound sites. From

these results it also can be concluded, that the amount of loosely bound phenol decrease at the coil-to-globule transition and increases above it with rising temperature. Based on relaxation measurements phenol is weakly bound below the LCST – the interaction is weakest at the LCST – and more strongly above it with increasing temperature. Temperature dependence of T_1 relaxation time of dopamine in D_2O, with and without the presence of the polymer can be seen in Fig. 4. There is no difference in T_1 relaxation times of dopamine in the two systems, showing the absence of interaction in the temperature range of measurements. Both T_1 and T_2 relaxation times depend on viscosity. Measurements were taken in dilute solutions (their concentrations can be seen in Table 1.) where the molar ratio of the water/monomer units was *ca.* 400. According to relaxation times of dopamine in polymer-containing and polymer-free solution, precipitation of the polymer above the LCST does not alter the viscosity.

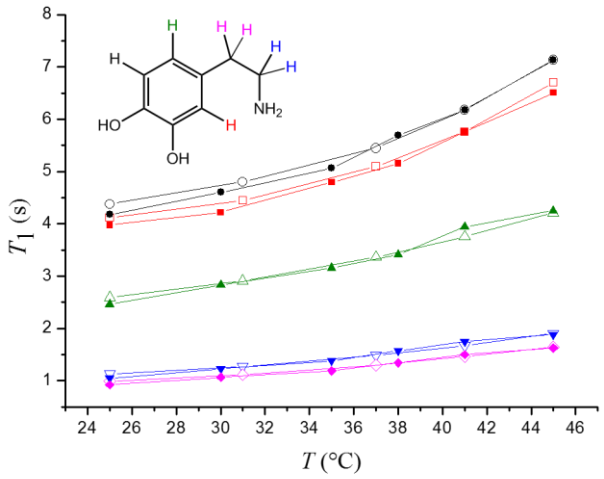

Fig. 4. Temperature dependence of T_1 relaxation times of the ^{1}H nuclei of dopamine in D_2O with (filled squares) and without (empty squares) the presence of the polymer

DOSY measurements were taken at several temperatures between 20 and 45 °C. The relationship between the measured diffusion coefficient (D), and the size (r – radius of a spherical particle) can be given by the Einsten-Stokes equation:

$$D = \frac{k_b T}{6\eta\pi r} \qquad (3)$$

where k_b is the Boltzmann constant, T is the temperature and η is the dynamic viscosity. Similarly to relaxation rates, the observed D is the weighted average of free and bound molecules:

$$D_{observed} = X_{free} \cdot D_{free} + X_{bound} \cdot D_{bound} \quad (4)$$

Changes in the size of the small molecule after adding PNIPA to the system can only be attributed to its interaction with the polymer, as viscosity does not change significantly. There is a decrease of D_{phenol} after adding PNIPA to the system (monomer:phenol 1:1), while D_{dopamine} does not differ in the polymer-free and polymer containing systems (Fig. 5.). D_{phenol} increases with increasing temperature in both systems (D and T are directly proportional, eq. 3). However, the slope of the line is smaller in case of the polymer-containing system because of the change of r, which proves the interaction. This correlates well with the result of relaxation measurements; more phenol is bound to the polymer with increasing temperature, while dopamine does not interact with PNIPA in the measured temperature range.

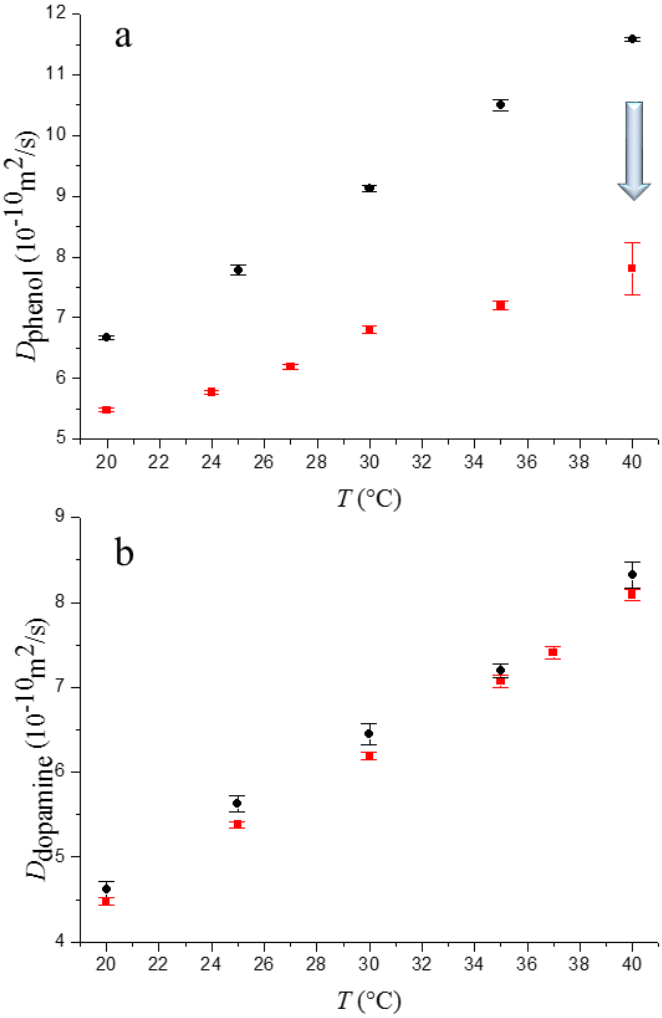

Fig. 5. Results of ^{1}H DOSY measurements: temperature dependence of *D* of a, phenol; b, dopamine in polymer-free (-●-) and polymer-containing (-■-) systems. Increase in size is indicated by the blue arrow. Fitting errors are shown by error bars.

Two-dimensional NOESY spectra were measured below and above the LCST (20 and 35 °C). Above the transition temperature cross peaks appear between the three protons of the phenol ring and the isopropyl protons of the polymer, which indicates the two nuclei are close in space (Fig. 6.). Below and around the transition temperature no cross peaks appear, likely due to the small concentration of bound phenol.

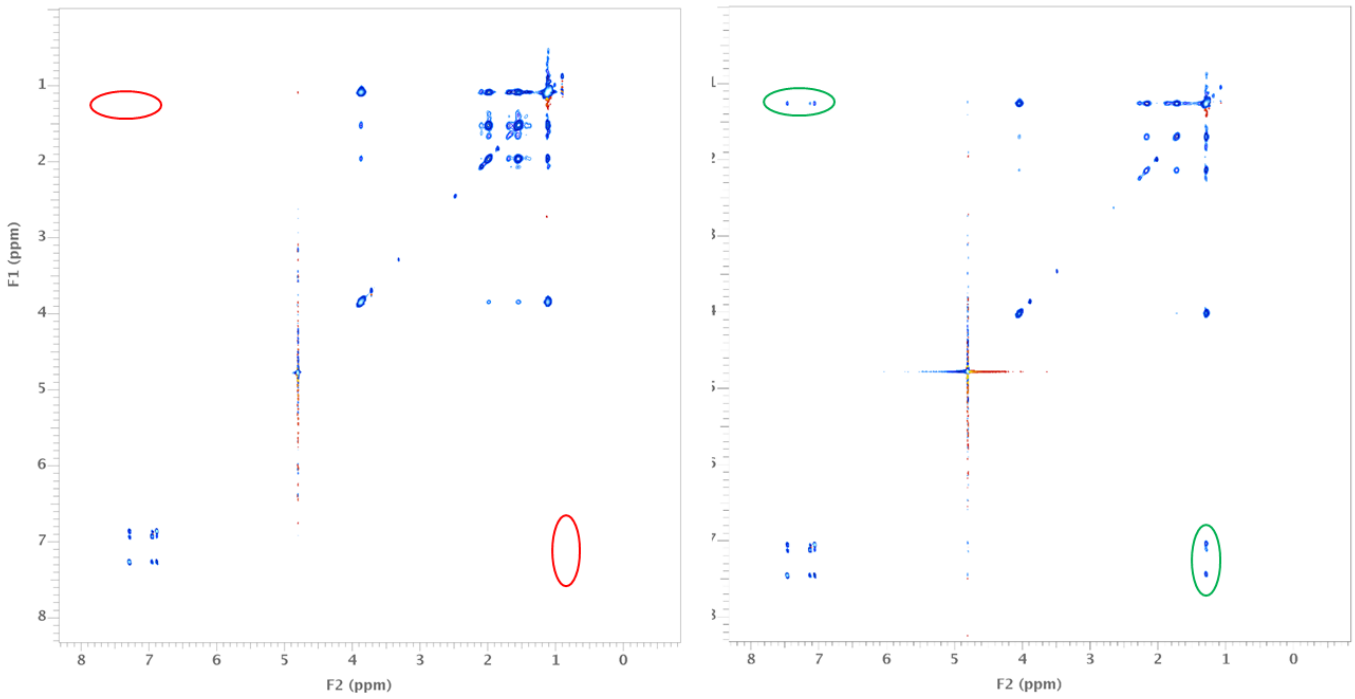

Fig. 6.: ^{1}H NOESY spectra of phenol in D_2O in the presence of PNIPA at 20 °C (left) and 35 °C (right). Red circles indicate the absence, green circles the presence of intermolecular cross peaks. NOESY spectrum at 27 °C is identical to the one at 20 °C. At 35 °C NOESY cross peaks (green) correspond only to the fraction of the polymer which is not precipitated.

3.3. Binding of indole and its derivatives to PNIPA below and above LCST

Based on measurements of phenol and dopamine in the presence of PNIPA it was concluded that measuring spin-lattice relaxation time is the most suitable technique to study non-covalent interaction in these systems, since it is fast and sensitive. The binding of indoles to the polymer below and above the coil-to-globule transition were qualitatively studied at 20 and 40 °C respectively. The interactions between indoles and PNIPA were also investigated via one dimensional selective NOESY measurements at the same temperatures to corroborate the results with an independent method.

In case of indole there is no difference in T_1 relaxation time of the ^{1}H nuclei in polymer-free and in polymer-containing solution within error at 20 °C (Fig. 7.), showing the absence of intermolecular interactions (^{1}H attached to the nitrogen cannot be seen in D_2O). Above the LCST there is however a significant decrease in the spin-lattice relaxation time in the presence of PNIPA (Fig. 7.). This indicates that indole is strongly bound to the polymer. Selectively exciting the methyl group of the polymer below the transition temperature, no signal of indole in NOESY spectrum can be seen (Fig. 8.). In case of the two substituted indoles (5-aminoindole and 5-hydroxyindole) even below the transition temperature, a small decrease occurred in T_1 in the presence of the polymer compared to the polymer-free solution (Fig. 7.), above the LCST show similar behavior to indole, both were strongly bound to the polymer, similarly to indole. Despite their different influence on the LCST value of PNIPA shown by UV-VIS and DSC investigations, similar strength of host-guest interaction have been found in case of the amino and hydroxyl derivatives of indole according to NMR relaxation measurements. Adding a hydroxyl or amino group to the indole core makes the interaction with the polymer stronger below the coil-to-globule transition resulting in a lower LCST than of the pure PNIPA. The transition temperature is higher than in the case of PNIPA-indole system which can be related to the presence of H-bond acceptor groups. Signal of the two substituted indoles appear in selective 1D NOESY spectrum below the transition temperature (Fig. 8.), as was expected based on relaxation measurements. In case of the amino acid tryptophan there are no change in T_1 relaxation time in polymer-containing system compared to the polymer-free one under the LCST and only small decrease above it, attributed to the weak tryptophan-polymer interaction. Only the aromatic protons present a decreased T_1 above LCST, indicating an interaction between the polymer and the indole ring. T_1 of the aliphatic protons behaved in a similar manner to the PNIPA-free system as they did not change while the indole core interacted with the polymer. Selectively irradiating the

methyl protons of the polymer, no intermolecular NOE signals of tryptophan protons can be seen below the transition temperature (Fig. 8.). Above the LCST all four indoles gave intermolecular NOE signals.

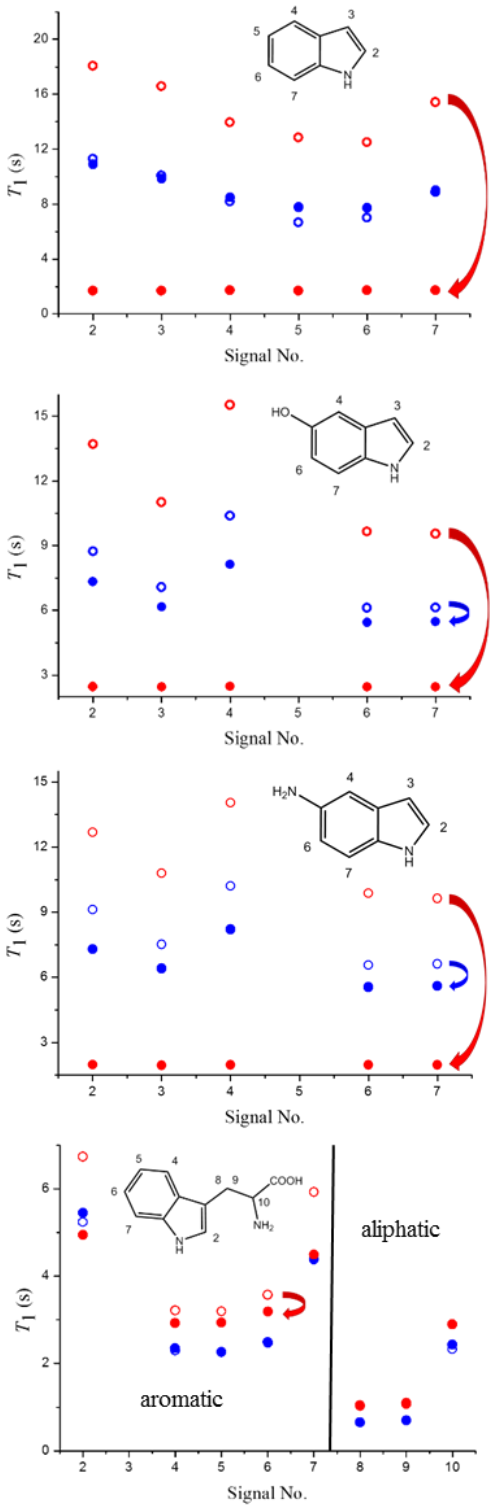

Fig 7. T_1 relaxation times of the ^{1}H nuclei of indole, 5-hydroxyindole, 5-aminoindole and tryptophan in D_2O (empty circles) and in PNIPA-containing solutions (filled circles), below (blue) and above (red) the LCST

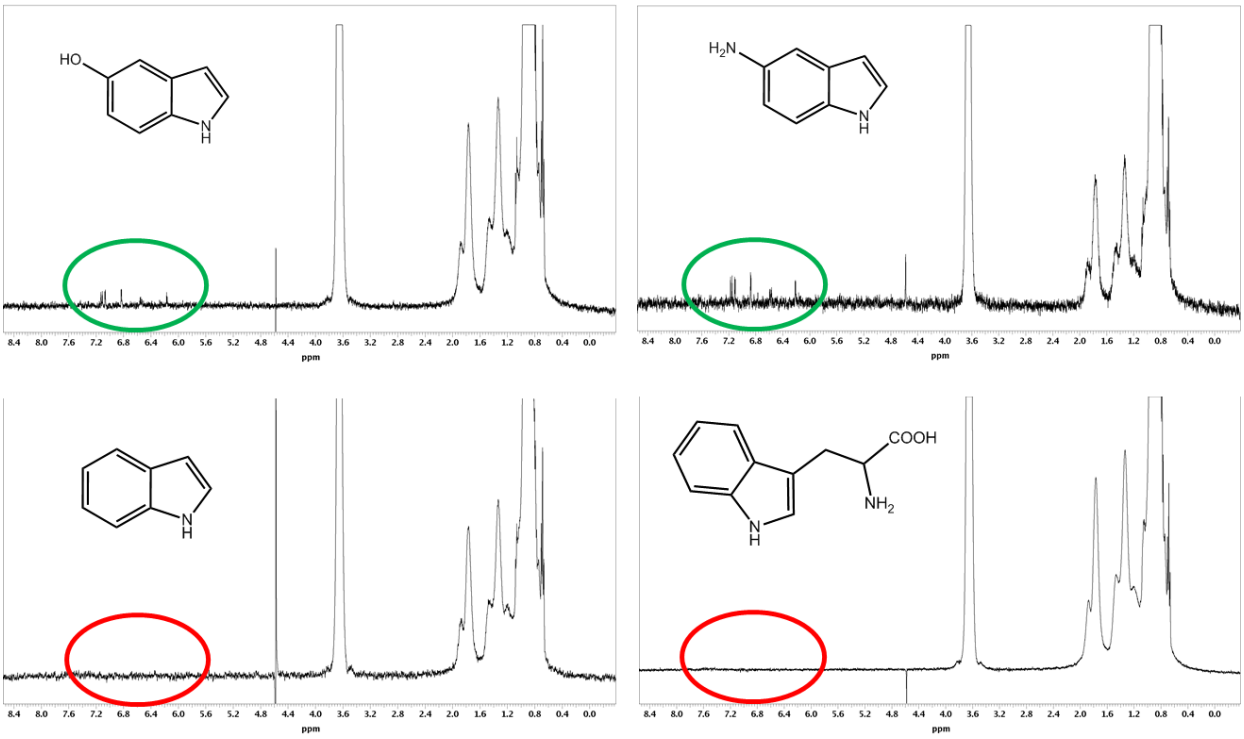

Fig. 8.: Selective one-dimensional DPFGSE NOESY spectra of polymer-containing 5-hydroxyindole, 5-aminoindole, indole and tryptophan solution at 20 °C (below the LCST). Irradiating the methyl group of the polymer at 1 ppm, intermolecular NOE peaks can be detected in the case of 5-amino- and 5-hydroxyindole (indicated with green circles), however there are no such peaks in the case of indole and tryptophan (indicated with red circles).

Interaction between PNIPA and tryptophan were also studied in two organic solvents: (dimethyl sulfoxide)-d_6 and methanol-*d4*. Spectra were recorded at the same temperature as in D_2O, except in (dimethyl sulfoxide)-d_6 (25 °C instead of 20 °C). In the two organic solvents where the phenomenon of coil-to-globule transition does not exist, no difference can be seen between chemical shifts and T_1, and T_2 relaxation times of tryptophan in polymer-containing and polymer- free solutions, which implies the role of water in the binding process. Irradiating the methyl signal of PNIPA side chains, no intermolecular NOE peaks of tryptophan can be seen, identifying the absence of interactions between the polymer and tryptophan in non-aqueous solutions.

4. Conclusions:

^{1}H spin-lattice (T_1) and spin-spin (T_2) relaxation, NOESY and DOSY NMR methods were used to determine and characterize interactions between small molecules: phenol, dopamine, indole, 5-hydroxyindole, 5-aminoindole, tryptophan and a thermoresponsive polymer, PNIPA. The LCST values of the polymer in the presence of these guest molecules were determined by UV-VIS spectroscopy. Significant decrease in the transition temperature in the case of phenol, indole, 5-hydroxyindole and moderate in the case of 5-aminoindole implied the presence of host-guest interaction with the polymer.

The results of relaxation and NOESY measurements consequently revealed a strong interaction between the polymer and phenol above the LCST. Temperature dependence of T_1 and T_2 relaxation times identified the amount of the interacting phenol, which was minimum at the LCST and increased with increasing temperature above it. Three sites were found for phenol molecules: i) strongly bound *ca.* 7-14 %, slightly increasing with increasing temperature, ii) loosely bound and iii) completely free sites. Dopamine showed no interaction with PNIPA at the measured temperature range. Value and temperature dependence of diffusion coefficients (D) of phenol and dopamine confirms the results of relaxation measurements. The different behavior of the two guest molecules can be attributed to the fact that dopamine has two hydroxyl and one amino group capable of hydrogen bonding compared to phenol which only has one. Thus dopamine-dopamine interactions are more favorable than dopamine-PNIPA, while phenol-PNIPA is strong particularly above the LCST.

Indole interacts with the polymer only above the temperature of the coil-to-globule transition, which is shifted to lower temperature (22.5 °C instead of 33.4 °C). The two substituted indoles, 5-hydroxyindole and 5-aminoindole showed similar behavior to indole, however there was a weak interaction with the polymer even below the transition temperature. The LCST was shifted to lower temperature in the presence of the two substituted indoles too,

but it was higher than in the case of the PNIPA-indole system. Although the two indoles showed different behavior according to UV-VIS measurements, they behaved similarly at 20 and 40 °C shown by NMR relaxation studies. Amino and hydroxyl groups differently change the hydrophilic – hydrophobic character of the polymer when bound, resulting in different LCST values, however the strength and quality of the interaction of the indole core and the polymer is similar in case of both molecules at these two temperatures. Adding an H-bond donor and acceptor group such as hydroxyl or amino, thus making the small molecule more hydrophilic promoting an interaction with the polymer below the transition temperature. Tryptophan was interacting weakly with the polymer and only above the LCST. Spin-lattice relaxation times (T_1) showed that only the indole ring interacts with the polymer, while the aliphatic amino acid part behaved similarly to polymer-free solutions. The absence of interaction between the amino acid part and the polymer explains the smaller effect of tryptophan on the LCST of PNIPA. More specific knowledge is provided by NMR relaxation measurements about the nature of host-guest interactions and the role of functional groups in case of indole derivatives.

VPTT of the hydrogel in the presence of indole derivatives showed similar tendencies to the LCST of the linear polymer, confirming that the latter is a useful model of the former. In this way the explored interactions in the solution state can be used to predict release kinetics of the hydrogel, which is important in future applications.

Funding: This work was supported by the (National Research, Development and Innovation Office, Hungary [NKFIH K115939]. A.D. acknowledges the support of the Bolyai Fellowship.

Supplementary material

The supplementary material contains the details of the applied NMR pulse sequences, the gel permeation chromatogram of the synthesized linear PNIPA and the measured UV-VIS transmittance curves of all small molecule-PNIPA systems.

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