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Supporting Information

Switching Enzyme Activity by a Temperature Responsive Inhibitor Modified Polymer

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Materials & Methods

Synthesis

N-Isopropylacrylamide was commercially purchased and recrystallized from ethanol prior to its use. 4,4′-Azobis(4-cyanovaleric acid) (ABCVA) (Sigma Aldrich, ≥ 98 %), tacrine (Enamine Ltd., 95 %), 1,4-dioxane (fischer scientific, ≥ 99.5 %, unstabilized), chloroform (Sigma Aldrich, ≥ 99.5 %), ethanol (chemsolute, ≥ 99.5 %), and diethyl ether (chemsolute, ≥ 99.5 %) were also commercially purchased, but used as received. The chain transfer agent 2-butylsulfanyl-thiocarbonylsulfanyl-propionic acid was kindly provided by Bruno de Geest from Ghent University.[1] The co-monomer TlaAm and the polymeric platform PNIPAAm-*co*-TlaAm was synthesized according to literature method[2] with slight adaptions: TlaAm was recrystallized from ethanol instead of dichloromethane. For the synthesis of PNIPAAm-*co*-TlaAm ABCVA was used as the radical initiator, therefore the reaction temperature had to be increased to 92 °C.

AChE Assays

Acetylcholinesterase (EC 3.1.1.7) from *electric eel* was purchased from Sigma Aldrich (Type VI-S, lyophilized powder, 500 U vial). Phosphate buffer saline (PBS) was prepared single-handedly with the following concentrations: NaCl 8.02 g/L, KCl 0.2 g/L, $NaHPO_4 \cdot 2H_2O$ 1.45 g/L, KH_2PO_4 0.24 g/L. Acetylthiochinoline chloride (Sigma Aldrich, ≥ 99 %) was used as the substrate and Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Aldrich, ≥ 98 %) as the dye in order to monitor the generation of thiol groups due to consumption of substrate by AChE. Stabilizing agents bovine serum albumin (BSA; ≥ 98 %) and ethylenediamine tetraacetic acid (EDTA; ≥ 99 %) were both purchased from Roth.

For the AChE assays the following three pipetting solutions were prepared: *AChE*, *reaction mixture* (containing both substrate and dye) and *inhibitor*. The *AChE* pipetting solution had an activity of 50 $mU \cdot mL^{-1}$, which was freshly prepared on each day of measurement from a freezed (-18 °C) 50 $U \cdot mL^{-1}$ AChE stock solution. The stock solution was prepared by adding 10 mL of an 0.1 wt% BSA in PBS solution into the 500 U vial resulting in an activity of 50 $U \cdot mL^{-1}$, of which several aliquots were kept in a freezer at -18 °C. The PBS based pipetting solution *reaction mixture* (*RM*) exhibited 1 $mmol \cdot L^{-1}$ acetylthiochinoline chloride, 0.25 $mmol \cdot L^{-1}$ Ellman's reagent DTNB, and 5 $\mu mol \cdot L^{-1}$ EDTA. The third assay component *inhibitor* had varying concentrations of the respective inhibitor. For the positive as well as the blank sample PBS was used instead of an inhibitor containing solution; the pipetting scheme is summarized in **Table SI 1**. Of each pipetting solution 50 µL were put into a well with *AChE* being the last one, resulting in a total volume of 150 µL.

Table SI 1 Herein used pipetting scheme for the blank, positive, and inhibition samples. Each x represents 50 µL of the pipetting solution named in the upper column.

	RM	*AChE*	*inhibitor*	*PBS*
blank	x			xx
positive	x	x		x
inhibition	x	x	x	

For the determination of inhibition constants IC_{50} and K_i, and relative AChE activity the readily pipetted assays were incubated in the dark for 15 minutes at room temperature. Finally, the absorption at 410 nm was measured as a measure for substrate conversion: The hydrolysis of acetylthiochinoline releases a thiol, which then reduces the Ellman's reagent DTNB. The appearance of the reduced Ellman's reagent can be monitored by measuring the absorption at 410 nm (**Fig. SI 1**).

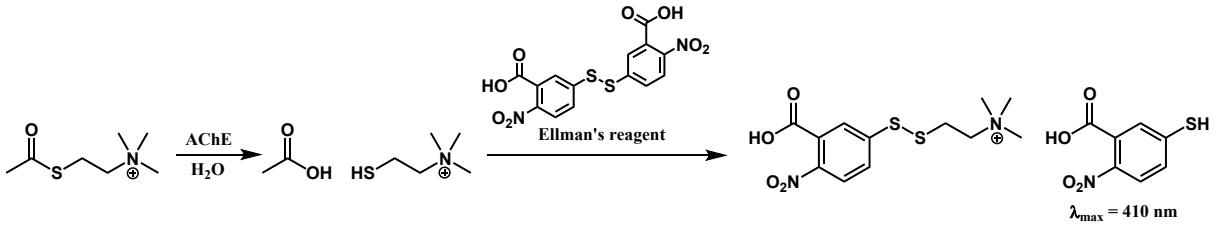


Fig. SI 1 The reaction cascade occurring within herein executed AChE assays is displayed. After the hydrolysis of acetylthiochinoline the resulting thiol reduces Ellman's reagent DTNB, which then releases 2-nitro-5-thiobenzoat that exhibits an absorption maximum λ_{max} at 410 nm.

IC_{50} was determined by examining concentration series of inhibitors (tacrine, PNIPAAm-tacrine). The collected absorption got subtracted by the blank's absorption. Relative AChE activity was determined by dividing the absorption of the inhibited samples by the absorption of the positive sample (eq. 1).

$$rel.\,AChE\,act. = \frac{(A_{410\,nm}^{Inhibitor} - A_{410\,nm}^{blank})}{(A_{410\,nm}^{positive} - A_{410\,nm}^{blank})} \quad \text{(eq. 1)}$$

The $c_{\text{inhibitor}}$ dependent AChE activity values were fitted in a sigmoidal mode *via* the hill equation by OriginPro 9.1G 64Bit. The resulting equation was solved for $y = 0.5$ by OriginPro in order to obtain IC_{50}. Inhibitory constant K_i was determined *via* the CHENG-PRUSOFF equation[3] that relates IC_{50} and K_i (eq. 2), additionally taken substrate concentration [*S*] (0.333 mmol·L^{-1}) and the MICHAELIS-MENTEN constant K_M (0.08 mmol·L^{-1})[4] into account.

$$K_i = \frac{IC_{50}}{(1 + [S])/K_M} \quad \text{(eq. 2)}$$

All measurements for the determination of inhibition constants were run in triplicates if not mentioned differently in the description of the respective figure.

In order to estimate the influence of heating-cooling cycles towards AChE stability the following experiment was performed: Four AChE solutions (25 mU·mL^{-1}) were prepared, one of which contained AChE only, while the others contained additionally PNIPAAm, P(NIPAAm-*co*-TlaAm) and PNIPAAm-tacrine (each at a concentration of 37.5 µg·mL^{-1}), respectively. These sample solutions were repeatedly heated to 32 °C for 5 minutes and subsequently cooled to room temperature. After 5, 10 and 20 cycles 100 µL of each sample solution were given into a well plate. After addition of 50 µL of *RM* the resulting solution were incubated in the dark for 15 minutes before the absorption at 410 nm was measured. After subtraction of the blank's absorption relative AChE activities were calculated according to eq. 1.

For the evaluation of the effect that precipitation of PNIPAAm and P(NIPAAm-*co*-TlaAm) domains has on AChE activity, the following experiment was performed: A standard positive sample (17 mU·mL^{-1} AChE, reaction mixture) and a sample additionally containing P(NIPAAm-*co*-TlaAm) (25 µg·m L^{-1}) were heated up to 30 °C (above the cloud point). Within three-minute-intervals 100 µL portions were taken out and $A_{410\ nm}$ was measured at room temperature. This measurement was run in duplicates in order to ensure correctness of observed trends.

Other Materials

Syringes were purchased from Carl Roth (NORM-JECT® Luer Solo 5 mL) as well as the used syringe filters (Rotilabo®, PTFE, unsterilized, pore size 0.20 µm).

Instruments

NMR spectra were obtained by a Bruker Avance at 300 MHz in $CDCl_3$ (Eurisotop, 99.5 D), of which the remaining portion of $CHCl_3$ served as an internal standard[5].

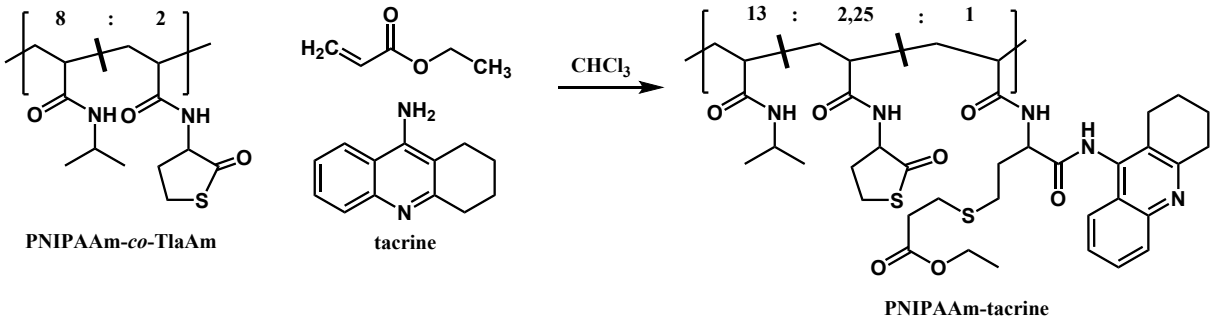
Gel permeation chromatography (GPC) was performed in order to determine M_n and M_w of the investigated polymer using DMF as eluent and a combination of the columns PSS GRAM Guard (8 mm × 50 mm), PSS GRAM 1000 Å (300 mm × 7.5 mm) and PSS GRAM 30 Å (300 mm × 7.5 mm) with a flow rate of 1.0 mL/min. For detection the refractometer SEC-3010 (WGE Dr. Bures) was used. Analysis was performed using the software ParSEC 5.62 (Brookhaven Instruments).

Temperature dependent turbidity measurements for the determination of cloud points were performed with a Malvern Zetasizer Nano Series + Titrator.

Absorption measurements (AChE assays) were executed by a Tecan Infinite M200 PRO using transparent flat-bottom 96-well plates (Paul Boettger GmbH & Co. KG).

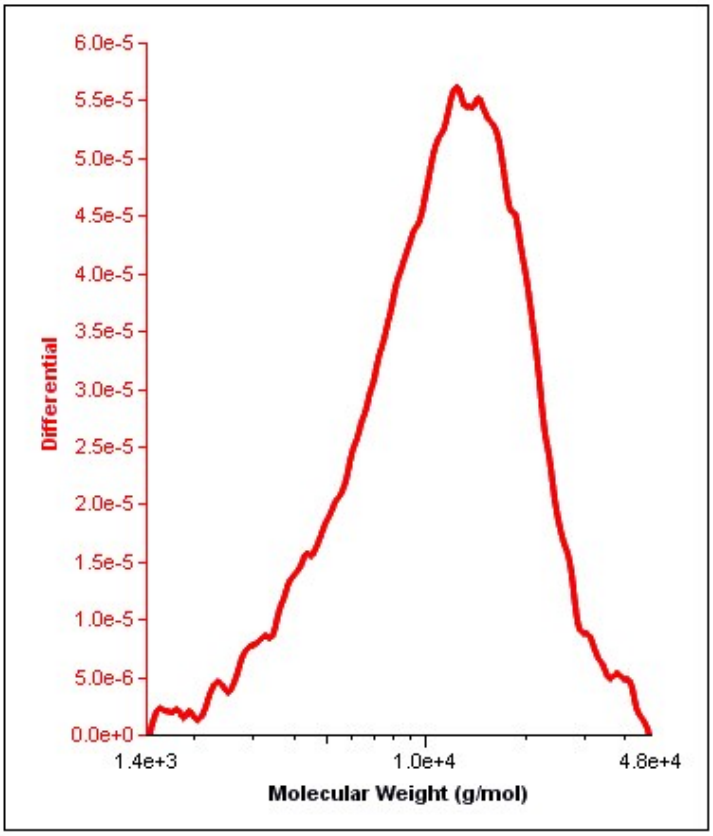
For **centrifugation** of precipitated polymer a Sigma 3K30 was used at 8000 rpm/min.

Synthesis



PNIPAAm-tacrine was synthesized according to literature[2] by adding tacrine (318 mg, 1.6 mmol) to a mixture consisting of $CHCl_3$ (2 mL), PNIPAAm-*co*-TlaAm (200 mg, 0.32 mmol of TlaAm) and ethyl acrylate (175 µL, 1.6 mmol). After stirring the reaction

mixture for three hours at room temperature it was slowly added to Et_2O (20 mL). The resulting suspension was centrifuged, the supernatant got decanted and the obtained product was dissolved in chloroform again. This procedure was repeated three times providing the polymeric product, which was finally dried *in vacuo* (10 mbar) and 42 °C.



Results

M_n	9.188e+03	g/mol
M_w	1.305e+04	g/mol
M_z	1.703e+04	g/mol
M_p	1.235e+04	g/mol
M_w/M_n	1.421	
M_z/M_w	1.304	
$<M_{10}$	7.522e+03	g/mol
$>M_{50}$	2.776e+04	g/mol
V_p	28.33	mL
$Area_{RI}$	6.430e-02	V*mL
Concentration	undetermined	mg/mL
dn/dc	0.0733	mL/g
Sample Recovery	undetermined	%
[η]	undetermined	mL/g
Mark-Houwink: k	undetermined	
Mark-Houwink: alpha	undetermined	
$<Rg>_n$	undetermined	nm
$<Rg>_w$	undetermined	nm
$<Rg>_z$	undetermined	nm

Fig. SI 2 SEC trace of PNIPAAm-tacrine including the software's output.

SEC analysis revealed an M_n of 9190 g·mol^{-1} and an M_w of 13050 g·mol^{-1} resulting in a PDI of 1.42 (**Fig. SI 2**).

NMR Evaluation

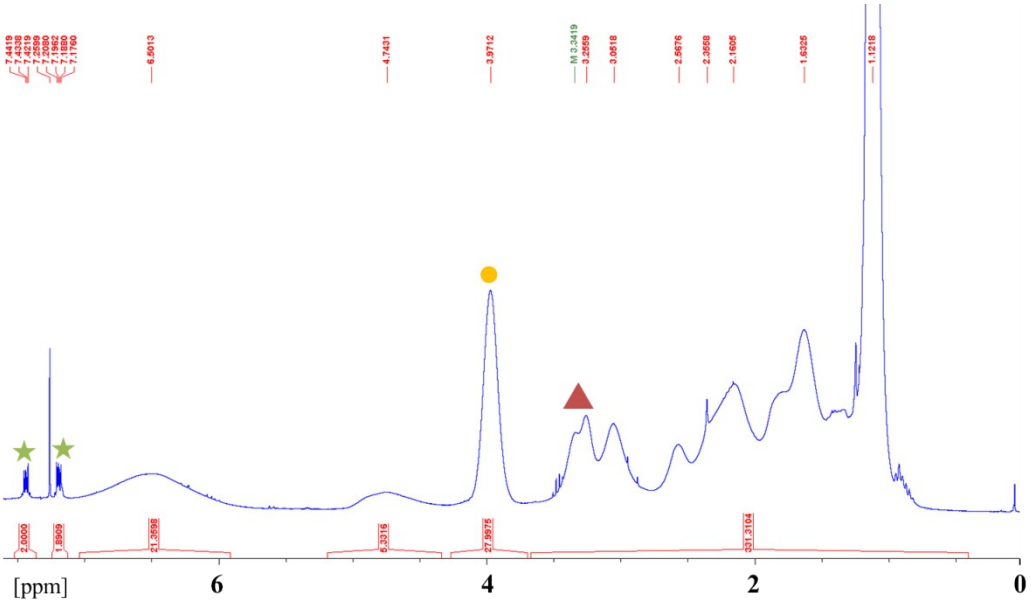


Fig. SI 3 ^{1}H NMR spectrum of PNIPAAm-tacrine recorded in $CDCl_3$. Undeuterated chloroform was used as an internal standard with δ = 7.26 ppm. The green stars (➢) represent the four aromatic protons of the tacrine moiety. The yellow circle (ꝏ) corresponds to the NIPAAm proton C*H*$(CH_3)_2$ and the red triangle (◻) marks the double peak given by the two C*H*$_2$ groups of the thiolactone ring.

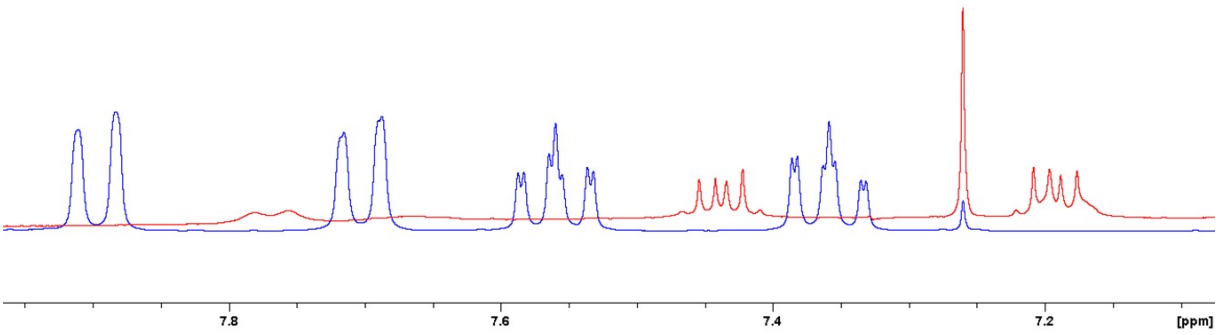


Fig. SI 4 Aromatic region of 1H NMRs of PNIPAAm-tacrine (red line) and molecular tacrine (blue line) recorded in $CDCl_3$.

NMR evaluation revealed that the thiolactone ring was not completely converted as the typical thiolactone double peak at 3.3 ppm is still visible (red triangle in **Fig. SI 3**). Additionally, the aromatic multiplets at 7.1 ppm and 7.4 ppm correspond to the four aromatic protons of tacrine and can be used for the calculation of the conversion of thiolactone rings. The precursor polymer PNIPAAm-*co*-TlaAm had a molar ratio of 8:2 (NIPAAm:TlaAm). In the final polymer-inhibitor conjugate a ratio of 13:1 for NIPAAm:tacrine is found (see **Fig. SI 3**). Accordingly around one third of thiolactone rings was converted resulting in an overall ratio for NIPAAm vs unreacted TlaAm vs TlaAm-tacrine of 13 : 2.25 : 1. By comparing the 1H NMR spectra of bare tacrine with that of PNIPAAm-tacrine it is also proven that tacrine is indeed bound to the polymeric backbone as the aromatic signals are clearly shifted (see **Fig. SI 4**).

Determination of Cloud Point

For PNIPAAm-tacrine the cloud point was determined at a concentration of 50 µg·mL^{-1} in PBS, which translates to a tacrine concentration of 21 µmol·L^{-1}. For that purpose 1.18 mg of PNIPAAm-tacrine was dissolved in 23.6 mL PBS, while cooling with ice. The clear, ice-cold solution was filtered *via* a syringe filter with a pore size of 0.22 µm and then filled into a polystyrene cuvette. The turbidity measurement was executed starting at 10 °C up to 45 °C with 1 °C steps.

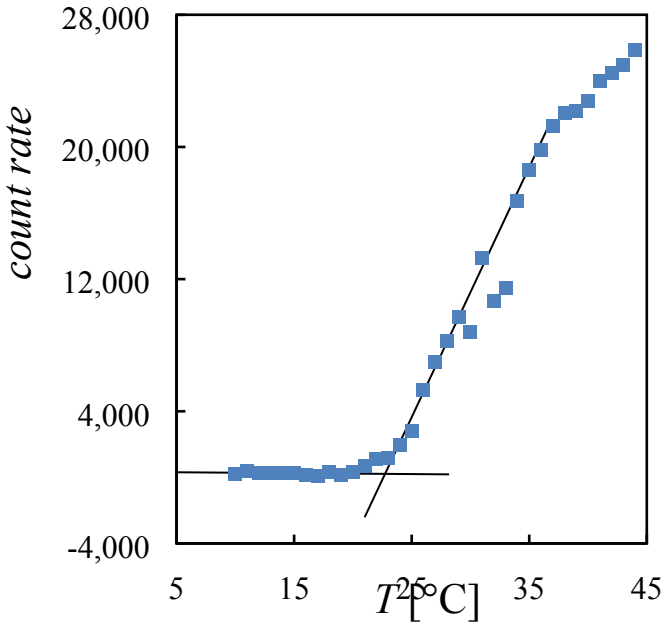


Fig. SI 5 Temperature dependent turbidity measurement of PNIPAAm-tacrine in PBS recorded at $c_{PNIPAAm\text{-}tacrine}$ = 50 µg·mL^{-1}.

The temperature dependent turbidity measurements resulted in a cloud point of 22.7 °C for the investigated concentration of 50 µg·mL^{-1}. For the onset fitting curve the first ten data points (10 °C – 20 °C) were used as the count rate can be considered as almost constant within that reason, which is also the cause of the poor R^2 value for the linear fit. For the offset fitting curve the corresponding data points of 23 °C to 29 °C were used. The intersection of the two obtained fitting curves is regarded as the cloud point of PNIPAAm-tacrine at 50 µg·mL^{-1}.

AChE Assays

Determination of Inhibitory Constants

With regard to the inhibition potential of PNIPAAm-tacrine the precursor P(NIPAAm-*co*-TlaAm) as well as pure PNIPAAm were also investigated. Neither PNIPAAm, nor P(NIPAAm-*co*-TlaAm) cause a change in enzymatic activity. Interestingly, an increased stability of AChE is noted instead (**Fig. SI 6**).

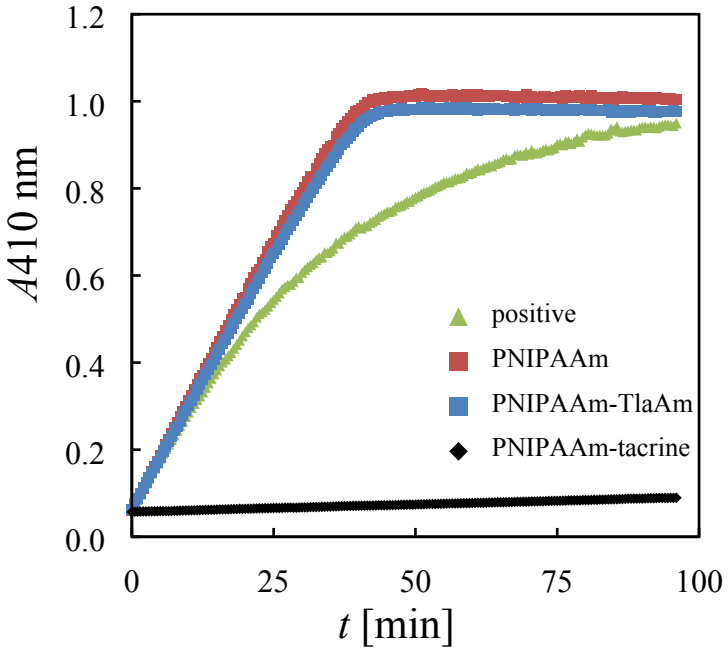


Fig. SI 6 Absorption at 410 nm as a function of time for different AChE containing samples: positive sample (green) and samples containing additionally PNIPAAm-tacrine (black), PNIPAAm (red), and P(NIPAAm-*co*-TlaAm) (blue) (each 25 µg/mL). The assay here is performed in an online-monitoring fashion instead of performing end-point detection in triplicates.

In the following tables **SI 2** + **3** the raw data of the inhibition measurements and their examination according to the methods described above and eq. 1 and eq. 2 is shown. The shown data is the basis of **figure 4** in the main manuscript. In determined IC_{50} and K_i values of tacrine and PNIPAAm-tacrine are summarized. The obtained K_i value of tacrine fits well to those determined by other research groups.[6]

Table SI 2 Absorption values at 410 nm (*A1* – *A3*) of the inhibition series with varying tacrine concentrations. Relative AChE activity is determined according to eq. 1. Standard deviation is abbreviated with *SD*.

tacrine									
$c_{tacrine}$ [µmol/L]	log($c_{tacrine}$)	*A1*	*A2*	*A3*	A_{mean}	*SD*	A_{mean} - A_{mean_blank}	*rel. AChE act.*	*SD*
100,878	2,00	0,0492	0,0493	0,0511	0,0499	0,0009	-0,0047	-0,0140	0,0026
10,088	1,00	0,0565	0,0570	0,0586	0,0574	0,0009	0,0028	0,0083	0,0027
1,009	0,00	0,0639	0,0643	0,0668	0,0650	0,0013	0,0104	0,0310	0,0038
0,101	-1,00	0,1602	0,1596	0,1579	0,1592	0,0010	0,1047	0,3110	0,0029
0,01	-2,00	0,3353	0,3352	0,3251	0,3319	0,0048	0,2773	0,8239	0,0142
0,001	-3,00	0,3959	0,4029	0,3938	0,3975	0,0039	0,3430	1,0190	0,0116
0,0001	-4,00	0,4000	0,3999	0,3729	0,3909	0,0128	0,3364	0,9994	0,0379
0,00001	-5,00	0,3939	0,4156	0,4083	0,4059	0,0090	0,3514	1,0440	0,0268
0 (positiv)	-	0,3978	0,3880	0,3876	0,3911	0,0047	0,3366	1,0000	0,0140
0 (blank)	-	0,0563	0,0568	0,0506	0,0546	0,0028	0,0000	0,0000	0,0084

Table SI 3 Absorption values at 410 nm (*A1 – A3*) of the inhibition series with varying PNIPAAm-tacrine concentrations. Relative AChE activity is determined according to eq. 1. Standard deviation is abbreviated with *SD*.

PNIPAAm-tacrine										
c_{P_012} [µg/mL]	$c_{tacrine}$ [µmol/L]	log($c_{tacrine}$)	*A1*	*A2*	*A3*	A_{mean}	*SD*	$A_{mean} - A_{mean\_blank}$	*rel. AChE act.*	*SD*
23,34	9,9409	1,00	0,0711	0,0709	0,0704	0,0708	0,0003	0,0188	0,0313	0,0005
2,33	0,9941	0,00	0,1469	0,1487	0,1511	0,1489	0,0017	0,0969	0,1609	0,0029
0,73	0,3124	-0,51	0,2716	0,2716	0,2610	0,2681	0,0050	0,2161	0,3587	0,0083
0,23	0,0994	-1,00	0,4551	0,4627	0,4423	0,4534	0,0084	0,4014	0,6663	0,0140
0,02	0,0099	-2,00	0,6124	0,6265	0,6071	0,6153	0,0082	0,5634	0,9351	0,0136
0,002	0,0010	-3,00	0,6682	0,6505	0,6176	0,6454	0,0210	0,5935	0,9851	0,0348
0,0002	0,0001	-4,00	0,6553	0,6605	0,65780001	0,6579	0,0026	0,6059	1,0058	0,0043
0,00002	0,00001	-5,00	0,6689	0,6598	0,6177	0,6488	0,0223	0,5968	0,9906	0,0370
0 (positiv)	0 (positiv)	-	0,6528	0,6733	0,6372	0,6544	0,0148	0,6025	1,0000	0,0245
0 (blank)	0 (blank)	-	0,0530	0,0542	0,0487	0,0520	0,0024	0,0000	0,0000	0,0039

Table SI 4 Summary of determined IC_{50} and K_i values of tacrine and PNIPAAm-tacrine obtained from AChE inhibition assays.

	tacrine	PNIPAAm-tacrine
IC_{50} [nmol·L^{-1}]	46.1 ± 9.5	180.9 ± 17.6
K_i [nmol·L^{-1}]	11.1 ± 2.3	43.5 ± 4.2

Stability towards an Extended Number of Heating-Cooling Cycles

For the reactivation of AChE, heating above the cloud point of PNIPAAm-tacrine is essential, as it is demonstrated in the main manuscript for three cycles. Nevertheless, frequent repetition of the heating-cooling procedure might have an impact on the structural integrity of AChE resulting in a decreased activity. Therefore, an enhanced number of heating-cooling cycles was performed for samples containing no polymer, PNIPAAm, P(NIPAAm-*co*-TlaAm) and PNIPAAm-tacrine. Their activities were compared to those of their untreated analogs (**Fig. SI 7**).

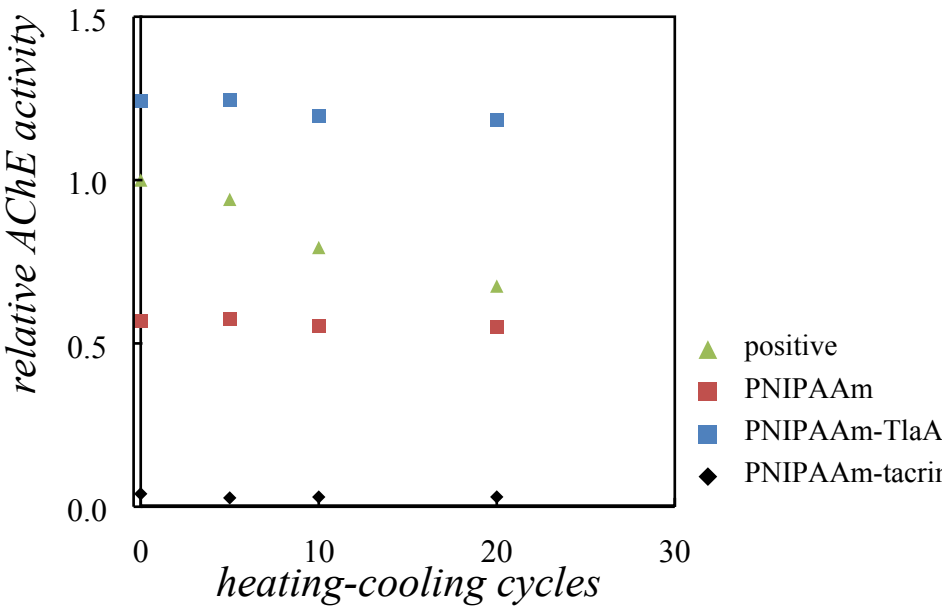


Fig. SI 7 Relative AChE activities of different AChE solutions additionally containing PNIPAAm (■), P(NIPAAm-*co*-TlaAm) (■), PNIPAAm-tacrine (◆) compared to a AChE only solution (□ - positive sample). AChE activities were derived from $A_{410\,nm}$ values recorded after 15 minutes of incubation according to equation 2.

From **Fig. SI 7** a steady decrease of AChE activity can be observed for the positive sample with an increasing number of heating-cooling cycles, leading to the conclusion that AChE stability is negatively affected by the cycles. Interestingly, the activities of the AChE solution containing PNIPAAm or P(NIPAAm-*co*-TlaAm) remain constant, not showing distinct activity losses. This finding again indicates a stabilizing effect of the PNIPAAm domain towards AChE. As expected, no activity is detectable for the inhibited sample (PNIPAAm-tacrine).

Influence of precipitation towards AChE activity

The influence of PNIPAAm and P(NIPAAm-*co*-TlaAm) towards AChE activity at room temperature (in a dissolved state) has been elucidated already and a stabilizing effect could be revealed. However, by heating above the cloud point of the respective polymers the interactions between enzyme and polymer backbone might change drastically, resulting in lower or even higher activity. Especially the latter would question the whole herein described reactivation mechanism. In order to ensure that the reactivation of AChE above the cloud point of PNIPAAm-tacrine is not a consequence of the changing interactions between AChE and the NIPAAm and/or TlaAm domains, the influence of precipitation of P(NIPAAm-*co*-TlaAm) towards AChE activity was elucidated by recording the absorption of a positive sample and a sample additionally containing P(NIPAAm-*co*-TlaAm). Both have been heated above the respective cloud point (30 °C) for specific time intervals before cooling down and recording a measuring point.

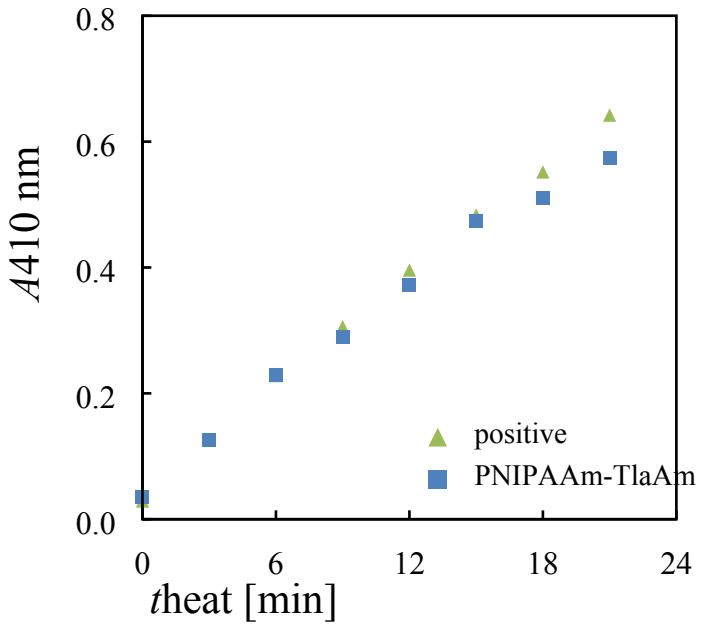


Fig. SI 8 Absorption at 410 nm of AChE/P(NIPAAm-*co*-TlaAm) (17 mU·mL^{-1} and 25 µg·mL^{-1}, respectively; blue squares) and a positive sample containing AChE only (17 mU·mL^{-1}; green triangles) recorded at room temperature after different heating times (30 °C).

With regard to **Fig. SI 8** no effect of the precipitation of P(NIPAAm-*co*-TlaAm) towards AChE activity is detectable. Both samples exhibit almost identical AChE activities. This finding underlines that the reactivation of AChE at elevated temperatures is indeed only a result of the decreasing tacrine concentration in solution that occurs upon precipitation of the inhibitor bearing polymer.

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