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# Tunable temperature responsive liquid chromatography through thiolactone-based immobilization of poly(*N*-isopropylacrylamide)

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ABSTRACT

A straightforward and efficient functionalization of aminopropylsilica with polymeric structures is described for the development of temperature responsive stationary phases applicable in purely aqueous liquid chromatography. The immobilization of the thermoresponsive polymers involves a thiolactone-based ring opening using the primary amines in aminopropylsilica, with a simultaneous one-pot, thiol-ene functionalization with an acrylate of choice. This mild, straightforward and modular grafting process results in high polymer coupling yields. By variation of the acrylate for the thiol-ene reaction, different stationary phases can be readily obtained. Two stationary phases as a result of the modular modification of aminopropylsilica were evaluated with test mixtures of hydrophobic analytes and a mixture of di- and tripeptides. Analyses using the 5 μm material packed in 10 cm × 4.6 mm columns revealed high hydrophobic retention, which proved adaptable as a function of the temperature in aqueous mobile phases. High versus low retention were obtained at temperatures above and below the lower critical solution temperature of the polymer, respectively. Moreover, the columns depict potential for diastereoisomeric peptide separation. Finally, the lower retention, observed when using PEGylated silica, illustrates the potential of the approach for modular stationary phase tuning.



## 1. Introduction

Quantitative immobilization of polymers on chromatographic stationary phases is a process that can be limited by a variety of parameters. These include the diffusion characteristics of the polymer, the number of the functional groups and their corresponding location in the polymer or on the packing material, the particle size, shape, pore dimensions and area to weight ratio of the packing material etc. Although the yields can be improved by the adapting the solvent, temperature, and by the incorporation of spacer molecules, the inherent reactivity of the functional groups involved is crucial for effective coupling. In many cases, this is paired with the necessity of anhydrous reaction conditions. Macromolecules can for example be coupled to HPLC silica packing materials through direct silylation procedures [1,2], epoxide ring opening processes of 3-glycidoxypropyltrimethoxysilane derivatised silica [3] or by amidation or reductive amination on aminopropyl-silica, respectively [4–6]. Synthetic polymers are also sometimes polymerized on the supporting material via *grafting from* types of approaches [7]. However, since the introduction of the concept of 'click' chemistry (coined as such by Sharpless in 2001 [8,9]), the potential of this strategy has also enjoyed increasing appreciation in terms of reactivity, speed and versatility, when targeting stationary phase modifications for chromatographic purposes via the immobilization of specific (macro)molecules [10]. The more established click reactions, such as copper catalyzed alkyne-azide cycloaddition (CuAAC), thiol-ene, thiol-yne, and Diels–Alder based chemistry, have to some extent been used in the last few years for the immobilization of small molecules on stationary phases [10,11]. However, whereas all these reactions comply with most of the criteria of a click reaction [8], some limitations for the immobilization on silica became apparent in the light of chromatographic applications. In this way, the use of CuAAC is limited due to the difficulty to remove toxic residual Cu (I) species exhaustively from the silica matrix and due to the explosive nature of low-molecular-weight azides. As

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thiol-ene or thiol-yne based click chemistry still require the accompanying presence of thermally of photolytically cleavable initiator, and as Diels Alder reactions (involving maleimide and furan moieties) display temperature-induced reversibility [12], the availability of metal- and trigger-free chemistries would offer desired, robust additional immobilization strategies for chromatographic applications. Moreover, methods enabling both efficient immobilization of (macro)molecules, accompanied by the introduction of a second residue in a single process, allow finer adjustment of tunable stationary phases.

In this regard, the goal of the present contribution is the evaluation of the nucleophilic amine-thiol-ene conjugation, involving a thiolactone unit, a primary amine and an acrylate, for the preparation of unique types of multimodal stationary phases. This one-pot site-specific double modification chemistry, schematically presented in Fig. 1, has proven useful for the synthesis and post-polymerization modification of linear polymers [13]. The aminolysis of thiolactones, introduced as side-chain functionalities, with the primary amines present in aminopropylsilica, followed by thiol functionalization with an acrylate, appears as an attractive and powerful approach for the immobilization of polymers on stationary phases. Therefore, in the framework of the development of improved temperature responsive stationary phases, the suitability of the nucleophilic amine-thiol-ene conjugation for the facile immobilization of poly(*N*-isopropylacrylamide) (PNIPAAm) on aminopropyl silica with concomitant incorporation of additional functionalities via the thiol-ene step, has been the aim of this research effort.

Although over the last two decades the development of HPLC columns containing immobilized temperature responsive phases has been explored [2,4,14–21], the performance of these columns is still insufficient to effectively compete with conventional reversed phase chromatography as the most used separation mode in LC. Polymers such as PNIPAAm or polyvinylcaprolactam (PVCL) exhibit a conversion from hydrophilic to hydrophobic properties when exceeding the lower critical solubility temperature (LCST) in aqueous environments [22,23]. Although depending on the molecular weight, the cloud point temperature of PNIPAAm is fairly well centered around 32 °C [4] whereby PVCL displays a broader transition zone between 32 and 37 °C.

The benefit of temperature responsive liquid chromatography (TRLC) is that it allows obtaining comparable elution orders as in reversed phase liquid chromatography while only requiring water as mobile phase whereby the retention is only modulated via the temperature. High and low retentions are thereby obtained at temperatures above and below the LCST, respectively, as the stationary phase becomes more hydrophobic. The methodology contrasts with other purely aqueous HPLC approaches such as high temperature HPLC, whereby temperatures up to 150 °C or higher are used with solute and column stability issues as a consequence [24]. On the other hand, ion exchange chromatography (IEC) is also a purely aqueous approach, but this technique is inherently limited to the separation of charged solutes [25,26].

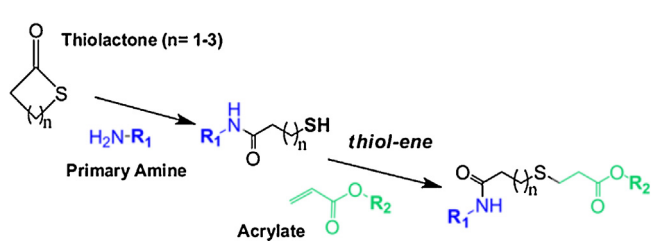


**Fig. 1.** General concept of the amine-thiol-ene one-pot double modification. The thiol is released by nucleophilic ring-opening of the cyclic thiolester and subsequently a thiol-ene can occur, incorporating $R_1$ and $R_2$ residues.

In principle, TRLC has the potential to offer the same generic applicability as in reversed phase LC while avoiding the need of organic solvents providing the solutes are soluble in water. However, the approach is currently still limited in the way that only a limited number of biologically relevant water soluble molecules have been retained thus far, whereby other mechanisms are inevitably exploited next to hydrophobic retention such as ionic interactions with copolymers including temperature responsive units [27,28]. As it can be foreseen that progress in TRLC goes hand in hand with the development of more retentive phases operating in the reversed phase mode, the availability of chemistries allowing the grafting of large amounts of this type of polymers in an easier way could pave the way for more generic implementation of TRLC. The followed reasoning is thereby that the biomolecules typically display insufficiently hydrophobic retention to allow for satisfactory resolution. This can be improved by increasing the polymer loading and therefore the ensuing hydrophobicity at elevated temperatures. Note that use of PNIPAAm co-polymers including more hydrophobic moieties does not necessarily offer a solution as this affects (lowers) the LCST and therefore the tunability of the retention [2]. Although it can be expected that by the grafting of the polymers on increasingly smaller particles, significantly increased column efficiencies will be obtained, solving the retention problem in this technique is essential for broader implementation of TRLC. Therefore, the possibilities offered by the thiolactone-based ring opening process, with concomitant thiol-ene functionalization using an acrylate, are studied here in order to manufacture improved TRLC columns in an efficient and user-friendly way. Two modified aminopropyl-silica phases are prepared in a similar manner by variation of the nature of the acrylate (resp. benzyl acrylate and PEG acrylate) and the separation potential of the corresponding LC columns is assessed with conventional test solutes to illustrate the high retention on the one hand but also with peptides to illustrate the capability to retain and separate peptide diastereoisomers, considered as some of the more challenging separations in RPLC, on the other hand.

## 2. Experimental

### 2.1. Reagents and materials

2-{[(Butylsulfanyl)-carbonothioyl]sulfanyl}propanoic acid was used as RAFT agent. It was synthesized according to a recipe described elsewhere [29]. Thiolactone acrylamide was synthesized as reported before [30]. *N*-Isopropyl acrylamide (NIPAAm) (Aldrich, 97%) was recrystallized from a mixture of toluene/*n*-hexane (1:1 v/v). PEG-acrylate (number average molecular weight, $M_n$ = 480 Da) (Sigma-Aldrich), trinitrobenzene sulfonic acid (TNBS, from Thermo Scientific), methanol, pyridine, tetrahydrofurane (THF), *N*,*N*-dimethylacetamide (DMA) chloroform, methyl-, ethyl-, propyl- and butyl-paraben, hydrocortisone, prednisolone, methyl-prednisolone, cortexolone and testosterone were obtained from Sigma-Aldrich (Bornem, Belgium), benzyl acrylate from ABCR, 1,4-dioxane from Janssen Chimica, dichloromethane from Fisher (Loughborough, UK), acetic anhydride from Riedel-deHaen (Bornem, Belgium), 2-2′-azobis(isobutyronitrile) (AIBN) from Acros, Geel, Belgium, acetronitrile from Fisher Chemical (UK). Aminopropylsilica (Chromatorex NH2 SPS 100 Ä, 5 μm) was purchased from Fuji Silysia Chemical S.A. (Lausanne, Switzerland). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). 1000 μg/ml stock solutions of the parabens and of the steroids were prepared in acetonitrile. The di- and tripeptides were synthesized in-house via standard solid phase peptide chemistry and dissolved in acetonitrile/water (80/20) at

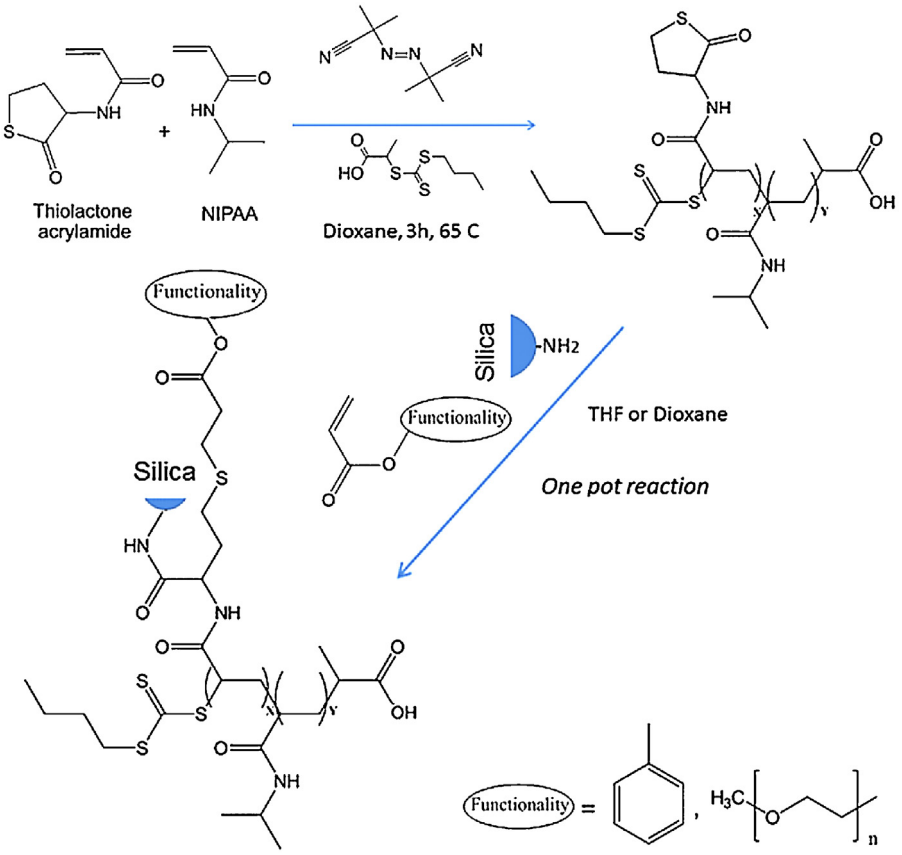


**Fig. 2.** RAFT copolymerization of NIPAAm and thiolactone-acrylamide, followed by a one-pot immobilization on aminopropylsilica based on the thiolactone ring opening process with concomitant modification using an acrylate via thiol-ene chemistry.

1 mg/ml [31]. Stock solutions were diluted with water to their final concentrations mentioned in the results and discussion section.

## 2.2. Methods

### 2.2.1. Stationary phase synthesis

The functionalized PNIPAAm with thiolactone groups was obtained via RAFT polymerization according to a prior described procedure [30]. In brief, 7.5 g of NIPAAm (66.28 mmol) was dissolved along with 0.6534 g of thiolactone-acrylamide (3.31 mmol) in 39 ml of dioxane containing 0.316 g of RAFT agent (1.33 mmol) and 0.024 g of AIBN (0.146 mmol). The solution was degassed through three freeze-pump-thaw cycles, backfilled with nitrogen, before stirring for 3 h at 65 °C. The reaction was allowed to proceed up to when no further increase in molecular weight was observed (as assessed by SEC). The obtained polymer was precipitated three times in cold diethylether, and filtered through a millipore-vacuum system, before drying under vacuum.

Size exclusion chromatography (SEC) was performed on a Waters instrument with a Waters 2414 Refractive Index Detector (RI), equipped with 3 PSS serial columns (GRAM Analytical 30 and 1000 Å, 10 μm particle size) at 35 °C. Poly(methyl methacrylate) standards were used for calibration and DMA containing LiBr (0.42 g/l) was used as an eluent at a flow rate of 1 ml/min. Molecular weights and polydispersities (PD) were determined using the Empower software. $^1$H NMR spectra were recorded in $CDCl_3$ (Eurisotop) on a Bruker AM500 spectrometer at 500 MHz or on a Bruker Avance 300 at 300 MHz. Chemical shifts are presented in parts per million relative to $CDCl_3$ (7.26 ppm in $^1$H NMR) as the internal standard.

The grafting of the stationary phase with the functionalized polymer was obtained by dissolving 1.51 g (0.25 mmol) of the latter with 0.28 g (1.73 mmol) of an acrylate in 45 ml of THF containing, 4.5 g of the aminopropylsilica. The suspension was gently shaken overnight at room temperature. Subsequently, the excess of reagents were filtered off through a Millipore filter under vacuum and the grafted silica was rinsed and cleaned first with 400 ml water and 150 ml methanol. The dried grafted silica was then treated with 4.6 ml (48.7 mmol) of acetic anhydride and 1.315 ml (16.3 mmol) of dried pyridine in 60 ml of $CHCl_3$ for 20 h in order to end-cap residual amino groups, followed by rising and filtration with 600 ml of water and 250 ml MeOH, and drying. Around 5.5 g of grafted product was obtained in this way. TGA analysis were performed in nitrogen in a temperature range between 25 and 800 °C at 10 °C/min. 1.6 g of the silica was subsequently slurried in 15 ml of water in an ultrasonic bath followed by packing at 450 bar in 10 cm × 4.6 mm ID stainless steel column with a Haskel air driven pump (Burbank, CA, USA).

### 2.2.2. Chromatographic conditions

HPLC experiments were performed on a Waters Alliance 2690 system equipped with a variable wavelength UV absorbance detector. All experiments were performed in quadruplicate. The mobile phase, composed of 100% Milli-Q water was percolated through the columns at a flow rate of 1 ml/min, injection volumes were set at 10 μl and the column temperature was controlled with a Julabo F10-VC water bath (Seelbach, Germany) allowing 0.1 °C accuracy. Columns were equilibrated for 10 min at the various temperatures used (5 to 50 °C) prior to each run. Paraben, steroid and peptide detection were performed at 210, 254 and 280 nm, respectively. Log *Ko/w* values were calculated by means of WSKOWIN software (Environmental Protection Agency, Washington DC, USA).

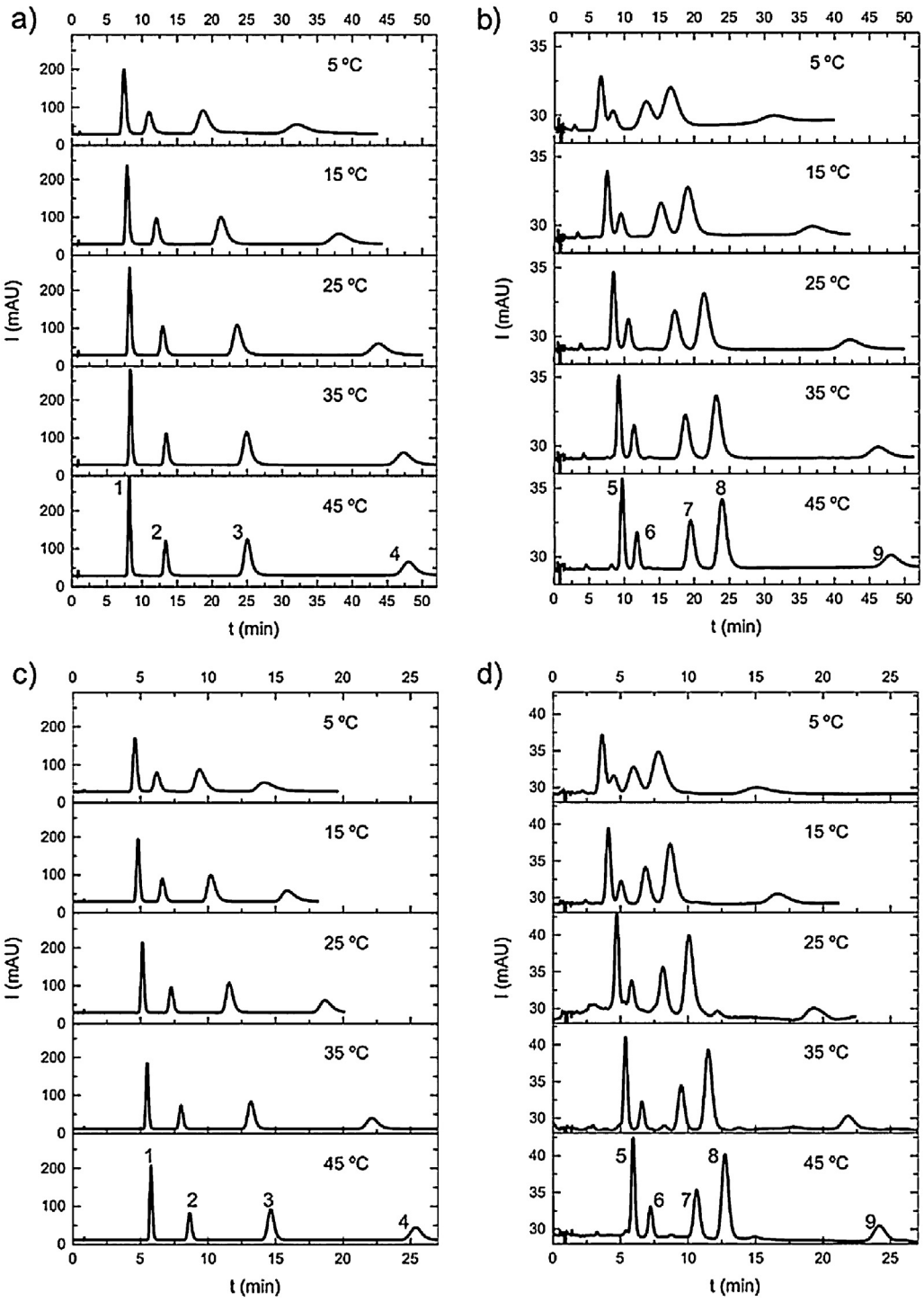


**Fig. 3.** TRLC analysis performed at various temperatures of the paraben and steroid mixture on the benzyl acrylate (a,b) and on the PEG-acrylate (c,d) terminated PNIPAAm column, respectively. Peak identification: (**1**) methyl-paraben (20 μg/ml), (**2**) ethyl-paraben (20 μg/ml), (**3**) propyl-paraben (20 μg/ml), (**4**) butyl-paraben (20 μg/ml), (**5**) hydrocortisone (2 μg/ml), (**6**) prednisolone (14 μg/ml), (**7**) methyl-prednisolone (13 μg/ml), (**8**) cortexolone (8 μg/ml) and (**9**) testosterone (14 μg/ml).

## 3. Results and discussion

In order to obtain highly retaining TRLC columns for improved analysis of more polar analytes, the performance of an immobilized thiolactone-PNIPAAm copolymer on aminopropyl silica materials was investigated in terms of retention and separation. The RAFT copolymerization procedure, involving NIPAAm and a thiolactone-containing acrylamide as monomers, allowed for a narrow dispersity of 1.3 and an $M_n$ of 6 kDa as observed by SEC (cf. Fig. 2 and supplementary Fig. S-1).

The synthesis of the copolymers was optimized in order to produce relatively low molecular weights, facilitating maximal introduction of the polymers on the silica material. NMR, FTIR and elemental analysis of the polymer depicted incorporation of about 2 thiolactone groups per polymer chain (Figs. S-2 and S-3). It should be noted that polymerization conditions were also chosen to obtain

a rather low, but tunable thiolactone content. The introduction of more or less thiolactone groups would either affect the flexibility of the polymeric chains on the silica surface after immobilization and therefore the modulation of the retention time, or alternatively not allow for satisfactory coupling of the polymers on the aminopropylsilica, respectively.

A unique characteristic of the formed copolymer, mainly consisting of a PNIPAAm backbone as a thermoresponsive material and bearing some thiolactone units along the chain, is the facility by which it couples to aminopropylsilica by aminolysis, while being unreactive to water. Moreover, in the presence of an acrylate, the ring opening process is occurring simultaneously with the thiol-ene reaction leading to the coupling of an acrylate at the anchoring point of each PNIPAAm chain (see Fig. 2). The acrylate can be of any type, which can be used to protect the silica against hydrolysis, control the LCST of the temperature responsive polymer or influence the selectivity or the retention of the analytes.

To elaborate the proof of principle of the approach, the grafting was executed by immobilizing the copolymer onto aminopropylsilica in the presence of benzyl acrylate or PEG acrylate in THF (Fig. 2). The silica material was suspended in this solution during 12 h at ambient conditions and followed by end-capping of the unreacted amine functions. TGA of the treated silica revealed the presence of over 20% m/m carbon load on the stationary phase (Fig. S4). While this carbon load is elevated but not necessarily exceptional for modified silica, the facility by which the immobilization of such amounts of polymer in a controlled way was obtained, should be stressed. Earlier work on the immobilization of PNIPAAm via the more conventional amide bonding chemistry on aminopropylsilica revealed that even after performing the coupling procedure in triplicate, lower carbon loads are obtained [32]. Residual amine groups were successfully end-capped with acetic anhydride, as assessed through a negative color test with trinitrobenzene sulfonic acid [33].

The ensuing HPLC columns were subsequently evaluated with a mixture of parabens and steroids such as to assess retention, efficiency, peak symmetry and the temperature responsive characteristics of the column. As can be seen in Fig. 3, similar elution patterns are observed as described before on PNIPAAm based columns, confirming the reversed phase mechanism. When elevating the column temperature, significant increases in retention are observed while this simultaneously leads to increased peak symmetry and plate numbers.

The efficiency of all four parabens in Fig. 3a, measured at 45 °C, spanned 2977–3139 plates, corresponding to plate heights of 32–34 μm. Although further improvements in column efficiency can be obtained by reducing the flow rate of the mobile phase or the particle size and mass of the polymer chains, and possibly also by further improving packing strategies, these results illustrate that achieving efficient chromatograms is viable with this approach. The chromatograms in Fig. 3a and b also illustrate a higher hydrophobic retention in comparison to more conventionally grafted PNIPAAm columns [32]. In Fig. 4a, the retention factors of the parabens are plotted versus their hydrophobicity in order to allow comparison with earlier PNIPAAm based work (whereby retention factors exceeding 25 were not obtained). It can be seen that butylparaben reaches retention factors above 60 at the more elevated temperatures. Comparable behavior was obtained for all other solutes. Reducing the column temperature leads to 35% reductions in retention. These changes in retention are smaller compared to when linear PNIPAAm chains are conventionally anchored to silica. This is most probably related to unchangeable hydrophobicity introduced by the benzyl acrylate groups. The chemistry of both groups is, however, easily adjusted. In Fig. 5a, the corresponding Van't Hoff plots have been constructed. The non-linearity thereof illustrates the changing retention mechanisms as a function of temperature. Note

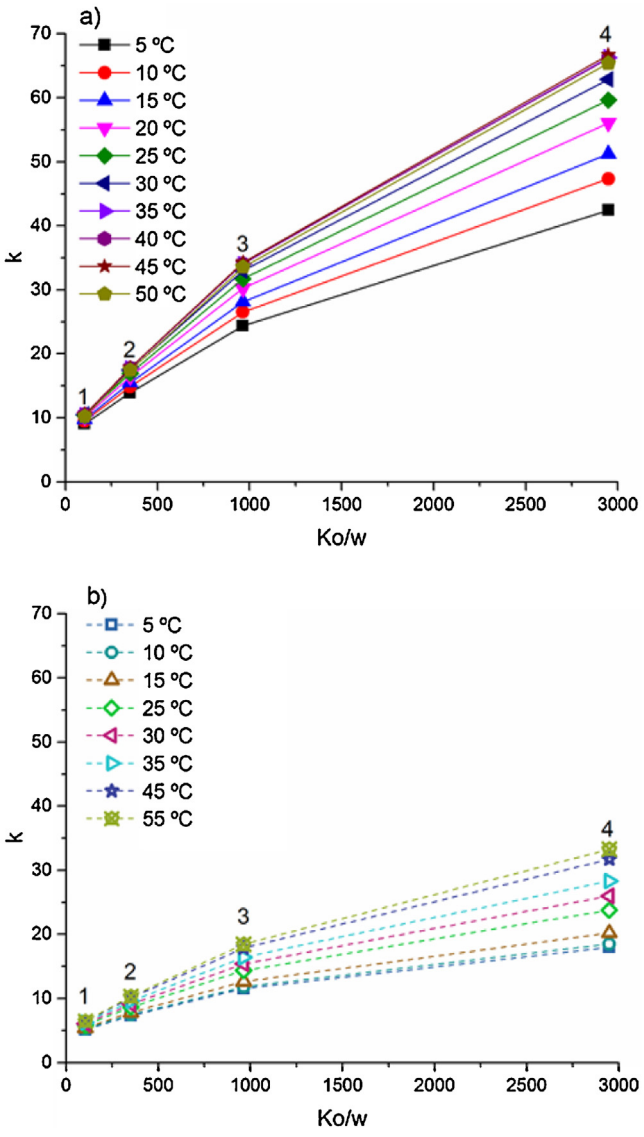


**Fig. 4.** Representation of the measured retention factors (k) versus the calculated octanol water partitioning coefficient of the 4 parabens on (a) the PNIPAAm/benzyl acrylate grafted column and on the (b) PNIPAAm/PEG acrylate grafted column.

that the slope of these plots is the inverse from the observations in conventional reversed phase LC, whereby in the vast majority of cases retention decreases linearly as a function of the temperature. The increase in retention with temperature is caused by the gradual evolution of the separation mechanism toward the increasingly accessible hydrophobic moieties on the stationary phase above the cloud point of the polymer [19,32]. This leads to a significant increase of the negative (exothermic) enthalpy term of the interaction, such as to exceed the, according to the Van't Hoff equation, expected loss of retention with increasing temperature [34].

It is worthwhile noting that repetitive injections of the different test mixtures on the columns in combination with the use of the various temperature cycles did not result in observable losses of column performance. Inter-day precision measurements on the paraben mixture (at 35 °C) over a period of 6 weeks showed retention time variations below 1% RSD for all solutes, depicting excellent column stability.

As high retention of the more hydrophobic solutes was obtained, and as retention of biomolecules solely based on hydrophobicity is not trivial, subsequently peptide samples were investigated. In Fig. 6, the analysis of a dipeptide and of two diastereoisomeric

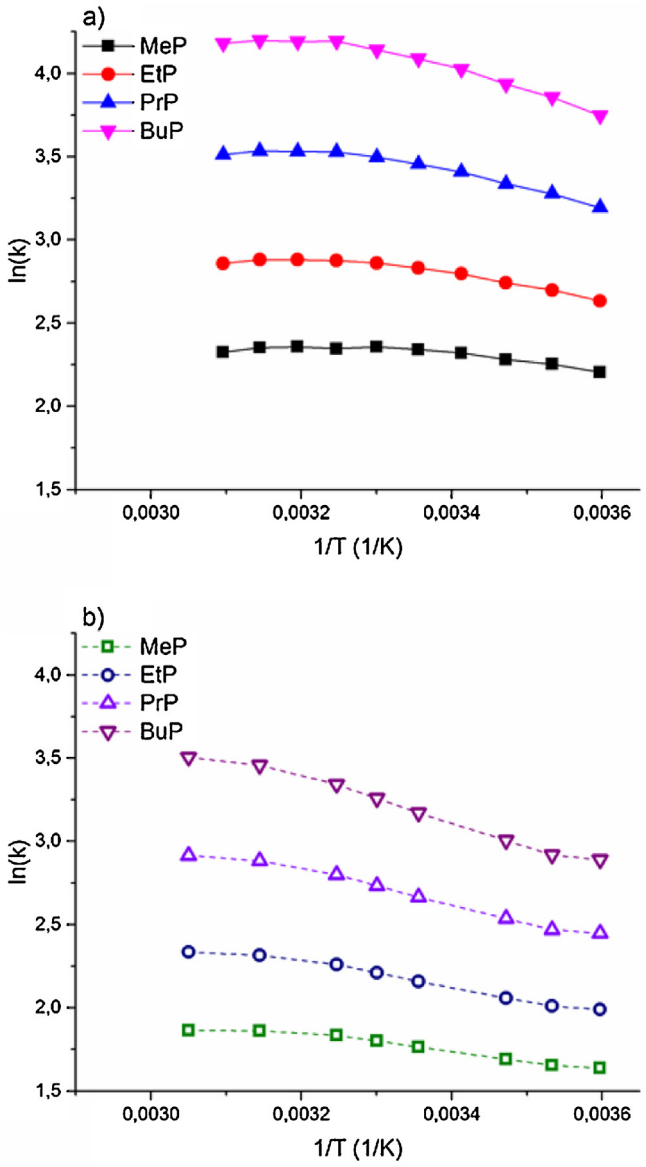


**Fig. 5.** Representation of Van't Hoff plots measured with the 4 parabens on the (a) PNIPAAm/benzyl acrylate and on the (b) PNIPAAm/PEG acrylate grafted column, respectively.

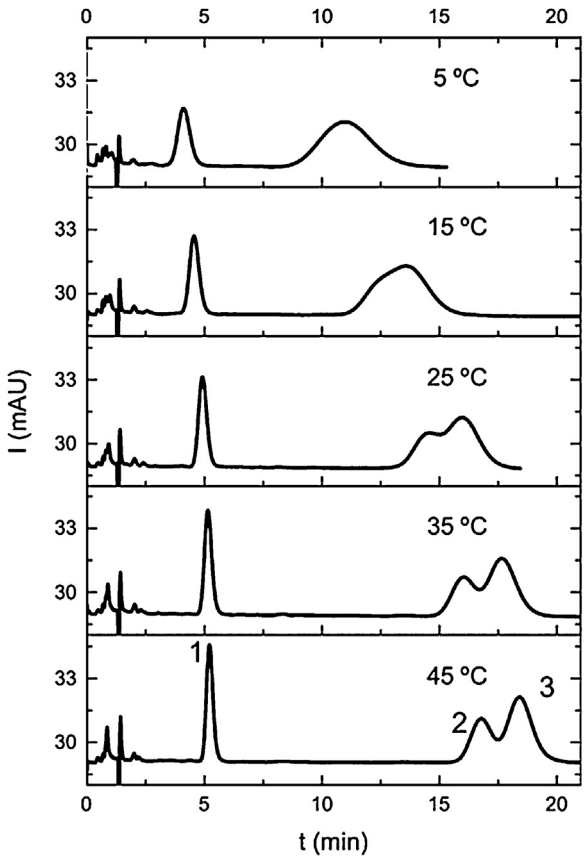


**Fig. 6.** Analysis of a dipeptide and of two diastereoisomeric tripeptides on the modified PNIPAAm column at various temperatures. Peak identification: (**1**) AcNH-L-Tyr-D-Phe-$CONH_2$ (72 μg/ml), (**2**) AcNH-L-Tyr-D-Phe-D-Phe-$CONH_2$ (175 μg/ml) and (**3**) AcNH-D-Tyr-L-Phe-D-Phe-$CONH_2$ (160 μg/ml).

tripeptides is represented at various temperatures. Also for these solutes high retention is obtained.

Especially at the lower temperatures, broader signals can be observed due to slower diffusion of this type of solutes in the stationary phase. The most interesting observation here is that improved separation of the diastereoisomeric pair is obtained at the higher temperatures. Note that the latter is non-trivial and that in conventional reversed phase LC, these peptides tend to co-elute very close to each other and require high column efficiencies and application of slow gradients to obtain separation. As the used column packed with 5 μm particles can in principle not offer more than 10,000 plates ($H = 2$ dp), which is 2.5× lower compared to more traditional 25 cm columns, the obtained separation is promising for analysis of small biologically related molecules such as peptides. Due to the racemic nature of the thiolactone-acrylamide and of the polymerization process, the chiral centers in the polymers can in principle not assist the separation of the diastereoisomers. Although only slightly higher retention and otherwise identical results were obtained when adding 0.1% formic acid to the mobile phase, the successful addition thereof without stationary phase deterioration also illustrates the distinct possibility for sensitive ESI-MS detection in combination with TRLC.

As mentioned in the introduction, the thiolactone ring opening process can essentially occur with a simultaneous one-pot functionalization with an acrylate of choice. Therefore, alternative to the modification with benzyl acrylate, the silicagel was also treated with an acrylate group anchored to a hydrophilic polyethylene glycol oligomer (480 Da) [35]. TGA of the modified silica revealed comparable carbon loads on the PNIPAAm based stationary phase co-functionalized with benzyl acrylate compared to the PEG (Fig. S-4). Chromatographic analysis, performed under identical conditions as before for the analysis of a number of parabens and steroids, illustrated that the elution order of the solutes was not at all affected by the incorporation of the PEG groups, but that this resulted in about a halving of the retention factors (Figs. 3c,d and 4b). Although these observations match with the expectations, the degree of the reduction in retention is still striking as it is only based on the incorporation of short PEG groups at the amino-propyl anchoring points. Also note that the Van't Hoff plots display a curve inflection which couldn't be observed with the other column, most probable related to an induced shift in the cloud point of the PEG functionalized system (Fig. 5b). These results illustrate that the incorporation of selected moieties next to the temperature responsive polymers has a strong influence on the retention behavior, allowing tuning of the properties of the phase.

## 4. Conclusions

A novel approach to functionalize aminopropylsilica with macromolecular structures was developed. The procedure consisted of a one-pot reaction between thiolactone containing PNIPAAm, a selected acrylate and the aminopropyl-silicagel at room temperature. The reaction involves the thiolactone ring opening by the amine group whereby the thiol group is swiftly trapped by an acrylate of choice. Adaptation of the latter leads to easy introduction of variability in the developed columns. HPLC analyses using only water as mobile phase allowed to ascertain the high hydrophobic retention of the developed phases, while symmetric peaks were obtained and satisfactory column efficiencies were measured. Thermal responsivity of the columns was observed whereby low and high retention were observed at temperatures below and above the LCST of the polymer, respectively. The benzyl acrylate terminated column showed satisfactory retention of peptides while diastereoisomeric separation was observed. An expected, yet dramatic drop in retention was observed when using polyethylene glycol grafted columns, illustrating the potential of the approach for straightforward stationary phase tuning.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.11.063.

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