Thermo-Responsive Stationary Phases for High Performance Liquid Chromatography (HPLC)

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In classical liquid chromatography, proteins are generally separated by employing RP-18 columns in the presence of acetonitrile or other organic solvents at low pH buffers as mobile phase. However, the presence of organic solvents often leads to the total denaturation of proteins, while acidic environments are often destructive to the activity of many enzymes. One possible way to prevent the denaturation of proteins in liquid chromatography is to use purely aqueous mobile phases. However, RP columns do not function well under such conditions.

Recently a novel method has been developed by Kanazawa *et al*, in which the stationary phase changes its surface properties from hydrophilic to hydrophobic as a response to temperature [1]. The main advantage of these temperature sensitive stationary phases is that they can separate mixtures of biomolecules (large peptides, proteins) in a pure aqueous environment under isocratic conditions [2]. Thus, the harsh conditions normally associated with protein separation and denaturation by employing RP-18 columns in the presence of organic solvents gradients can then be avoided.

Termoresponsive stationary phases are produced by attaching thermo-responsive polymers onto a pre-formed chromatographic supports such as silica or polymeric beads [3] or monoliths [4]. Thermo-responsive polymers are polymers that at certain temperature, called lowest critical solution temperature (LCST), are changing their properties. The most studied thermo-responsive polymer is poly N-isopropylacrylamide (PNIPAAm), which has a sharp phase transition at 32°C (LCST) [5]. Below the LCST, the polymer chains show an expanded conformation in water due to strong hydration. Above the LCST the PNIPAAm changes to compact forms by dehydration and intermolecular hydrogen bonding between the carbonyl and NH groups.

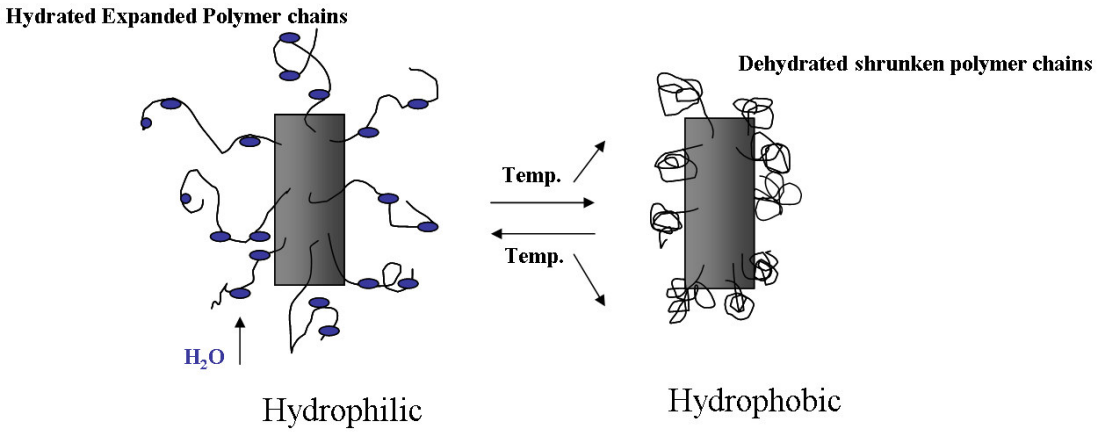

Figure 1: Schematic of a thermo-responsive polymer on a solid surface below and above the LCST

By using these features, PNIPAAm and related polymers have been used to generate temperature-sensitive stationary phases for size exclusion [6], hydrophobic [7], ionic [8] and affinity chromatography [9]. The synthesis of such thermo-responsive stationary phases by grafting PNIPAAm has been previously reported on silica beads and monoliths and was used for the separation of steroids [9], [10] amino acids [7], [11] or nucleotides [12].

Figure 2 shows the effect of temperature on the performance of such thermo-responsive monolithic column in terms of the separation of a mixture of five steroids, employing PNIPAAm as the grafted thermo-responsive polymer under isocratic conditions in an aqueous mobile phase.

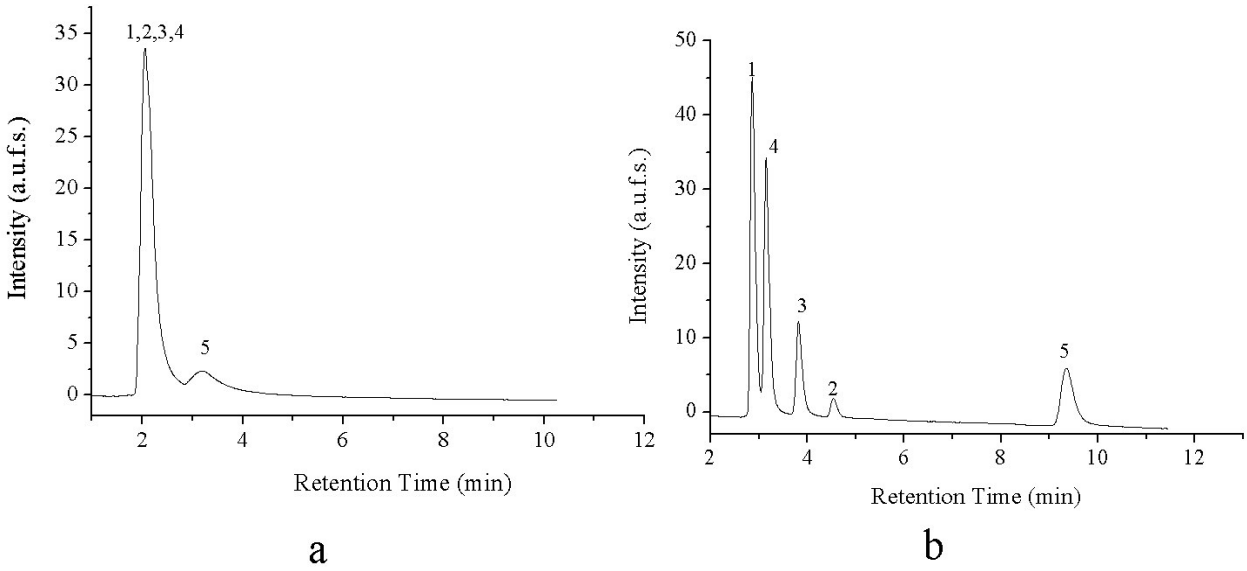

Figure 2: Elution profile of an aqueous mixture of five steroids at a) 5 °C, b) 55°C on the monolithic columns packed with thermo-responsive polymer chains with the number average molecular weight of 8500 g/mol. 1)Hydrocortisone, 2)Hydrocortisone acetate, 3) Dexamethasone, 4) Prednisolone, 5) Testestorone, flow rate:1 ml/min, mobile phase: water

Below 30℃ the PNIPAAm chains are in the expanded conformation in the aqueous mobile phase. The surface of the monolith is hydrophilic; therefore the separation of the steroids is poor (c.f. Figure 2 a). At 55℃, the steroids can already be baseline separated because the surface behaviour of the monolithic stationary phase changes from hydrophilic into hydrophobic. In this state, PNIPAAm has an adjustable hydrophobic character due to the isopropyl groups located on the collapsed chains. Thus, the separation of the mixture of steroids is assumed to take place mainly *via* hydrophobic-hydrophobic interactions between the collapsed PNIPAAm chains and the analytes. Hydrophobic interactions and partitioning between analytes and the PNIPAAm grafted silica stationary phase are driving forces for the separation.

Except PNIPAAm, our group works with other alternative co-polymers exhibiting similar LCST behaviour such as poly (isopropyl oxazoline-N-propyloxazoline) (PIPOX-PNPOX) [13] and poly-(2-(2-Methoxyethoxy)-ethylmethacrylate-oligo(ethyleneglycol)methacrylate) (PMEO2MA-POEGMA) [14]. A huge advantage of these co-polymers is the possibility of tuning LCST in water over a wide

temperature range based on varying hydrophobic-hydrophilic monomer composition *via* a well-defined gradient. Thus, by lowering the LCST, it is possible to work in an aqueous mobile phase at room temperature.

The advantage of employing the thermo-responsive stationary phases over the RP columns due to aqueous mobile phases operating conditions is a persuasive motivation to study further and improve the performance of such thermo-responsive columns.

This analytical system is based on nonspecific adsorption by the reversible transition of a hydrophilichydrophobic PNIPAAm-grafted surface. The ability of the proposed thermoresponsive polymer-modified stationary phase to separate the solutes without the use of an organic solvent is advantageous from the point of view of maintaining the biological activity. The electrostatic and hydrophobic interactions could be modulated simultaneously with the temperature in an aqueous mobile phase, thus the separation system would have potential applications in the separation of biomolecules.

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