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Application of polymeric macroporous supports for temperature-responsive chromatography of pharmaceuticals

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ABSTRACT

A macroporous particulate support prepared previously by reactive gelation under shear and functionalized with poly(*N*-isopropylacrylamide), PNIPAM, brushes of variable length is applied for temperature-responsive chromatography, whereby temperature modulates hydrophobic interactions. Several different analytes, including small pharmaceuticals, peptides, proteins and monoclonal antibodies are employed. Contrary to the most commonly observed behavior in conventional chromatography, increasing retention is observed at elevated temperatures. Peak broadening is quantified using the peak standard deviation, which depends on both the polymer chain conformation and analyte adsorptivity. The favorable effect of grafted polymer thickness on retention becomes progressively less pronounced for thicker grafted PNIPAM layers. The effect of eluent composition on solute–sorbent interactions was investigated by introducing NaCl, methanol, dioxane and by varying the pH. Salt or organic solvent addition affects apart from the analytes solution properties, the hydrophobicity of the stationary phase itself. Frontal analyses performed at different temperatures to determine dynamic binding capacities, indicate small mass transfer resistances imposed by this novel packing material.

1. Introduction

High-performance liquid chromatography (HPLC) is pivotal to the purification of high-value compounds, from small molecules up to biomacromolecules [1]. Most commonly employed chromatographic modes are reversed phase (RPC), hydrophobic interaction (HIC) and ion-exchange chromatography (IEC), as well as their combinations, which all typically operate under an organic solvent or salt gradient [2]. Apart from environmental stress, this practice is associated with reduced product bioactivity and increased costs [3,4], particularly in the pharmaceutical and biotechnological sector, where target-components are sensitive and purification costs represent a major part of the overall production costs [1,5]. Temperature-responsive chromatography has been proposed as an alternative method to overcome above mentioned limitations [3,4,6–9].

While in conventional chromatography temperature is not usually control parameter [10], in temperature-responsive chromatography it is used to alter the stationary phase properties and thus modulate solute interaction. To this end, the surface of the stationary phase is functionalized with a temperature-responsive polymer, which undergoes a reversible transition around its lowest critical solution temperature (LCST). The most prominent example is poly(*N*-isopropylacrylamide), PNIPAM, with LCST ~32 °C in aqueous solution [11], which has been utilized in numerous other applications so far, including for instance drug delivery [12,13],

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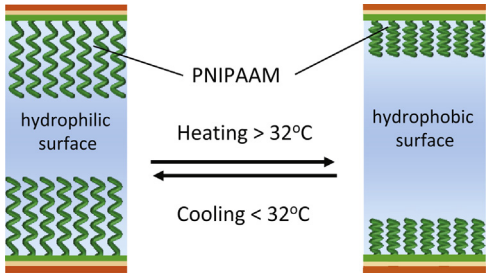

Fig. 1. Temperature-responsive functionality of PNIPAM brushes grafted from a pore surface.

bioconjugates [14], microfluidics [15], cell culture substrates for regenerative medicine [16,17] and sensors [18]. At temperatures below LCST, PNIPAM chains grafted from a surface are in an extended conformation, owing to their extensive hydration. In fact, H-bonding takes place between H_2O and imide groups, as well as orientation of H_2O molecules around their hydrophobic parts, namely the isopropyl groups and the chain backbone. Above LCST the binding and ordering of these H_2O molecules are disrupted, while formation of inter- and intra-molecular hydrogen and hydrophobic bonds is favored instead, forcing PNIPAM chains to collapse [11,19,20]. At this conformation the dehydrated PNIPAM is relatively hydrophobic, thus favoring hydrophobic interactions between its isopropyl groups and apolar solutes [13,21]. This behavior is qualitatively sketched on Fig. 1.

Regarding temperature-responsive separation applications, PNIPAM-bearing supports have been applied for capillary electrophoresis [22], microcapillary chromatography [23], affinity chromatography [24,25], as well as Inverse-Size Exclusion Chromatography (ISEC) [26]. Temperature-responsive adsorption chromatography has actually attracted the largest interest. There, apart from PNIPAM homopolymer [7,27–34], copolymers of NIPAM with more hydrophobic comonomers have been applied in order to increase the hydrophobic interactions [35,36], as well as copolymers with ionic comonomers in order to introduce pH-responsiveness [25,37], or with both types of comonomers [38–42]. With such functional copolymers several classes of analytes have been targeted, from steroids [7,25,28–32,34,35,37] to proteins [36,39,41,42] and others compounds [6,8,9,25,30,33,35,37,38,40,43].

So far, the vast majority of literature reports have focused on functional stationary phases suitable for analytical applications. Most of them concern silica-based supports [7,25,28–32,34–38,40,41,43] which however have the disadvantage of instability at high pH values [3,5] while traditional polymeric supports, such as agarose [39,42], suffer from compressibility issues at high flow rates [1,2]. Polymeric supports, with good chemical and mechanical stability [33,39] have not yet been exploited much for temperature-responsive chromatography, especially macroporous supports [6,27], which are well-suited for preparative applications, where good throughput, i.e. speed of separation, is necessitated [2,5]. In this regard, large and well-interconnected pores would provide high bed permeability, which allows operation at high flow rates [44]. If at the same time these large pores are perfusive, i.e. able to maintain a fraction of convective flow, then the separation efficiency is decoupled from the increase in flow rate [45].

A porogen-free method for the preparation of mechanically rigid, macroporous polymeric particles, which relies on the aggregation of latex nanoparticles into fractal clusters as a pore-generating mechanism and is thus named reactive gelation under shear, has recently been developed [46]. Chromatographic columns packed with polystyrene-based particles, produced via this novel approach exhibited perfusive behavior [47]. PNIPAM brushes of varying length were further grafted from the surface of such packing material, following a flow-through approach [48] for surface-initiated atom-transfer radical polymerization (ATRP) [49]. Evaluation of the grafting characteristics indicated that the grafted chains were in the brush regime, i.e. dense and long enough to stretch, up to a certain extent, away from the surface [50,51]. These brushes were actually found to respond to thermal stimuli [48], which is a crucial function for the chromatographic performance of such columns. Herein, we demonstrate the ability of these PNIPAM-functionalized columns to operate effectively under temperature-responsive mode. Several potential analytes have been tested, including small molecules, peptides, proteins and monoclonal antibodies. Frontal analyses, i.e. loading experiments, have been performed at different temperatures, while the effects of grafted PNIPAM amount on retention, as well as that of salt (NaCl), pH and organic solvent content (methanol and dioxane) in the mobile phase have also been investigated.

2. Experimental

2.1. Materials

All chemicals were of minimum 97% purity, from commercial sources and used as received, with the exception of three monoclonal antibodies mAb03, mAb10 and mAb14, which were supplied by industrial partners and had molecular weight ~150 kDa and isoelectric point (pI) between 7.35 and 8.15. Deionized water was further treated by a Millipore Simpack®2 purification device. All eluents were filtered with 0.45 μm cut-off Durapore® membrane filters (Millipore).

The macroporous polystyrene-based particulate material was prepared by reactive gelation under shear and characterized following procedures communicated previously [46,52]. The resulting microclusters were irregularly shaped, with average radius of gyration 40 μm and compact internal structure, as indicated by the large fractal dimension of $d_f = 2.7$. They exhibited BET surface area of 11.2 m^2/g (by N_2 sorption porosimetry), average pore diameter around 1 μm and intraparticle porosity of 70% (measured both by Hg intrusion porosimetry). The packing of such macroporous microparticles into chromatographic columns and their subsequent functionalization with PNIPAM brushes by surface-initiated ATRP has also been described previously [48]. Their grafting characteristics, such as grafting density and chain length, their porosity and pore size distributions have been determined as well. Thus, four columns with grafting density of $\sigma_P = 0.187$ PNIPAM chains/m^2 and varying PNIPAM chain lengths were prepared, hereafter referred to as PNP_13.0, PNP_16.7, PNP_22.9 and PNP_46.3, where the numbers indicate the grafted amounts in mg of PNIPAM per m^2. The resins essentially retained their macroporous character after functionalization.

2.2. Operation under temperature-responsive chromatographic mode

An Agilent 1100 Series HPLC system, equipped with gradient quaternary pump, column oven and multiple-wavelength UV detector was used. The eluent composition was itself one of the main parameters to investigate, while the detection wavelength was selected according to the absorbance of the individual analytes. Pulse experiments were performed at mobile phase flow rate of 1.0 ml/min. Comparisons of the analytes' retention were done using the dimensionless peak retention factor [1]:

$$k' = \frac{\mu_1 - \mu_{1,t}}{\mu_{1,t}} \quad (1)$$