Thin thermo-responsive polymer films onto the pore system of chromatographic beads *via* reversible addition–fragmentation chain transfer polymerization†

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Mesoporous silica beads modified with an azo initiator were used for grafting of a thermo-responsive polymer (poly-*N*-isopropylacrylamide, PNIPAAM) through reversible addition–fragmentation chain transfer (RAFT) mediated polymerization. The RAFT mediation allowed an efficient control of the grafting process and led to suppression of the solution propagation preventing any visible gel formation. The resulting composites were characterized by Fourier transform infrared spectroscopy, nitrogen adsorption analysis, elemental analysis, transmission and scanning electron microscopy and as thermo-responsive stationary phases in chromatography. The resulting material proved to be efficient for the separation of a mixture of five hydrophobic steroids and the retention time and efficiency of separation improved with increasing the column temperature above the lowest critical solution temperature of PNIPAAM.

Introduction

In recent decades, stimuli responsive polymers have been intensively used in a variety of fields of engineering and medicine.[1–3] This type of polymers can change their structural and physiochemical properties in response to external signals. There are many external factors such as pH[4] or electric field[5] that can trigger the above mentioned changes but it seems that temperature is one of the most feasible tools. Such thermo-responsive polymers have been widely utilised for drug delivery systems[6–8] cell culture substrates[9] and bioconjugates.[10]

One of the most commonly used thermo-responsive polymers is poly-*N*-isopropylacrylamide (PNIPAAM) due to its sharp phase transition at 32 °C.[11] At this temperature, known as the lower critical solution temperature (LCST), PNIPAAM exhibits reversible soluble–insoluble changes in aqueous solution. Due to this temperature dependent behaviour, PNIPAAM grafted surfaces exhibit temperature-responsive hydrophilic–hydrophobic surface alterations. By using these features, PNIPAAM and related polymers have been previously also used to generate temperature-sensitive stationary phases for chromatography.[12–14] These thermo-responsive stationary phases function by only changing the column temperature, in stark contrast to changing the mobile phase composition as in the case of standard gradient chromatography. Thus, these temperature sensitive stationary phases have the advantage that they could separate mixtures of biomolecules in a pure aqueous environment and isocratic conditions thus avoiding the denaturation of peptides and proteins.

So far, the thermo-responsive stationary phases were mainly synthesized using standard ester–amine coupling "grafting to" methods.[15] This methodology had the disadvantage that the grafting density of the polymer chains due to sterically restricted reactivity with the surface functional groups is limited. A "grafting from" method using a surface-immobilized azo-initiator and conventional radical polymerization has been also employed to produce thermo-responsive composites.[16] However, because of only one-point attachment of the initiator, solution polymerization and poor control over grafting density and chain length could not be avoided. This limited the usefulness of the method.

Here, we report on using chain transfer agents to control the azo-initiated PNIPAAM grafting. The use of dithioesters has been proven particularly versatile in this regard.[17–19] They allow the polymerization to proceed *via* a reversible addition fragmentation chain transfer mechanism (RAFT). This features a fast capping of the majority of the propagating chains by the RAFT agent followed by the establishment of a dynamic equilibrium between growing and dormant chains, resulting thus in a low radical concentration near the surface, less termination by radical recombination, slower kinetics and linear time–conversion curves. Furthermore, interchain equilibration reduces chain length dispersity and heterogeneity of the grafts. The focus of this work is the synthesis of thermo-responsive composites using the "grafting from" method *via* RAFT polymerization and their use as stationary phases in chromatography is proved here by the separation of hydrophobic steroids.

Results and discussion

Characterization of PNIPAAM grafted silica beads

We have synthesised ACPA modified Si-100 by consecutive coupling of APS to rehydroxylated silica followed by coupling

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† Electronic supplementary information (ESI) available: Fig. S1: Chromatograms of the steroids mixture and benzene on the pure Si column at four different temperatures. Table S1: Plate numbers of steroids at four different temperatures. Table S2: Symmetry factors of steroids at four different temperatures. Table S3: log *P* values of the steroids. See DOI: 10.1039/b800851e

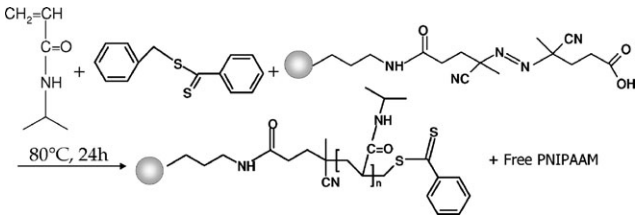

Fig. 1 Scheme depicting the grafting of PNIPAAM polymer film from porous silica support controlled by addition of the RAFT agent.

of ACPA. The initiator density was tuned to be 0.5 μmol m^{-2}. The ACPA modified supports were subsequently used for grafting of a thermo-responsive polymer film in the presence of the RAFT agent; benzyl dithiobenzoate (DBD) (Fig. 1).

The SEM micrographs show uniform polymer coatings due to the controlled character of the polymerization using the RAFT agent (Fig. 2(B)). It is known that due to one-point attachment of the azo-initiator, uncontrolled solution polymerization is most likely to occur leading to thicker and inhomogeneous polymer grafts. This can be here minimized due to the reversible addition–fragmentation chain transfer mechanism.

The polymer graft density was calculated from elemental analysis data as shown in the experimental part. The amount of grafted PNIPAAM was 0.425 (chains nm^{-2}) The molecular weight of the free PNIPAAM in solution and also the grafted PNIPAAM from the surface of silica was determined using GPC. The grafted polymers were cleaved from the surface using the trifluoroacetic acid method (TFA).[20] The RAFT polymer cleaved from the silica surface had a polydispersity index higher than 1 (Table 1), which is presumably due to the porous geometry of the silica bead and the fact that the polymer chains propagate first both inside and outside the pores. After a while, due to pore blockage the polymerization takes place mainly at the outer surface resulting thus in different length polymer chains. Also the molecular weight of the cleaved polymer was smaller than that of the free polymer in solution, which may be due to the large size of the silica particles in comparison to small monomers, which leads to an increase in steric hindrance. In this case the rate of polymerization in solution would be higher than on the surface of silica beads.[21]

Fig. 3 shows the FT-IR spectra of the material containing the pure silica, the silica containing immobilized azo initiator together with the resulting composite. The ACPA functionalization of the silica surface is confirmed by the appearance of the stretching vibration of the amide and carboxylic acid groups at about 1650 and 1700 cm^{-1}. After polymerization of NIPAAM, intensity increase of the two adsorption peaks at around 2900 cm^{-1} (ν_{C-H}) is observed. The increase in intensity of the bands at 1650 and 1700 cm^{-1} corresponding to the amide band together with the appearance of a new band at 1540 cm^{-1} corresponding to the NH amide of the PNIPAAM

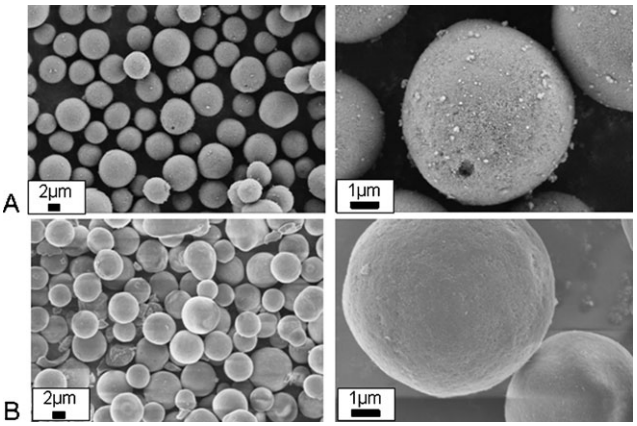

Fig. 2 Scanning electron micrographs of (A) pure silica Si-100 and (B) Si-PNIPAAM composite prepared using RAFT polymerisation.

chains confirms the presence of grafted polymers on the silica beads.

For chromatographic applications, in order to facilitate mass transfer it is very important that some of the mesopores in the initial silica material remain accessible after the polymer grafting. Table 2 shows the pore structural parameters of the initial and composite materials. The adsorption–desorption isotherms of the original Si-100 material and the resulting composite are given in Fig. 4. It can be observed that about half of the mesopore volume is filled upon PNIPAAM polymerization, the rest being still accessible for analyte transport. The surface areas decrease from 300 m^2 g^{-1} in the original silica materials to 170 m^2 g^{-1} in the composite, which corresponds essentially to the weight gain without adding additional pores, *i.e.* is exactly along the theoretical expectation. The polymerization process in the presence of RAFT agents therefore occurs in a controlled manner, both on the outer and inner surface of the silica particles, resulting in a homogenous polymer film that leads to a decrease in pore volume and surface area compared to the starting material.

The porosity data are also supported by the microtomed transmission electron micrographs (Fig. 5). Thus a clear difference can be observed between the pore system of the pure silica (A) and the resulting composite which shows a homogenous pore filling and the remaining of about half of the existing mesopores (B).

Chromatographic characterization of the thermo-responsive stationary phases

The resulting composites were slurry packed into HPLC columns (120 × 4.5 mm) and evaluated for their ability to separate a mixture of steroids in pure aqueous mobile phase. This allowed us to investigate the thermo-responsivity of the as-prepared composites by examining the temperature-

Table 1 Characterization of the amino, initiator or PNIPAAM grafted silica beads

Material	%C	%N	Immobilized groups/μmol m^{-2}	Grafted polymer/mg m^{-2}	M_n (soln.)/ g mol^{-1}	(M_w/M_n) (soln.)	M_n (cleaved)/ g mol^{-1}	(M_w/M_n) (cleaved)	Grafted chain density/nm^{-2}
Si-APS	4.7	2.0	1.45	—	—	—	—	—	—
Si-ACPA	8.47	3.0	0.57	—	—	—	—	—	—
Si-PNIPAAM	19	4.3	—	1.32	18 461	1.27	1866	5.44	0.425

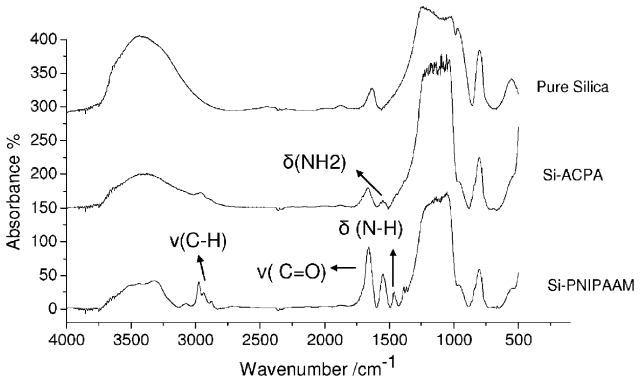

Fig. 3 FT-IR of starting silica material, immobilized initiator and the PNIPAAM composite.

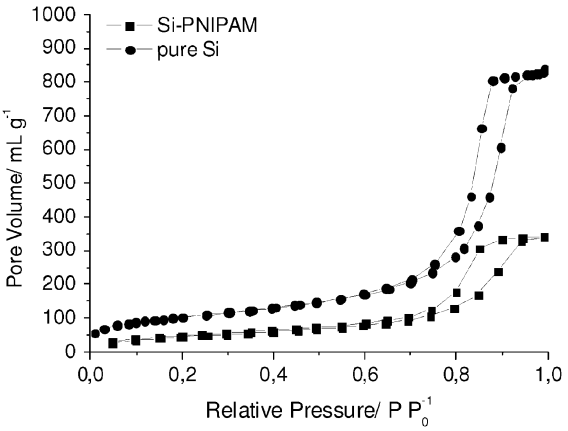

Fig. 4 Adsorption–desorption isotherms of the starting silica material and the PNIPAAM composite.

dependent analyte interactions. Thus, we examined the temperature-dependent elution profiles of steroid molecules with different hydrophobicity. The back pressure at 5 °C was 120 bar and this decreased with increasing the temperature due to collapsing of the polymer chains, so that at 60 °C we had a back pressure of 75 bar. The column efficiencies determined from the void marker (benzene) were *ca.* 15 000 number m^{-1} indicating that the columns were properly packed.

Fig. 6 shows the elution profiles of a mixture of five steroids at different temperatures in a pure aqueous environment together with their temperature-dependent retention time. Because of the phase transition (32 °C) from extended chains to the collapsed state, the behaviour of the PNIPAAM changes with increasing temperature from hydrophilic to hydrophobic. Therefore it can be noticed that below 32 °C, the separation of the five steroids is poorer, especially for hydrocortisone acetate and dexamethasone which have very similar hydrophobicity. Thus, the separation factor between hydrocortisone acetate and dexamethasone increases from 1 at 5 °C to 1.66 at 65 °C.

Once we increase the temperature the surface becomes hydrophobic and interaction with hydrophobic solutes increases. Thus at 65 °C a base line separation of all five steroids was accomplished. The retention factors at four different temperatures are given in Table 3.

The data in Table 3 and Table S3 (ESI†) indicate that the main driving force for retention in this system is the hydrophobic interactions between the solute molecules and the collapsed polymer chains on the surface, although more rapid kinetics at higher temperatures may also allow the steroids to penetrate deeper into the polymer film leading to an increased retention. In case of hydrophobic–hydrophobic interactions one might expect a dramatic change in retention factor with temperature as well as a shift in the elution order between some of the compounds. On the other hand, PNIPAAM in its collapsed state is not very hydrophobic, its hydrophobicity being due to the isopropyl group only. Furthermore, as given in ESI† (Table S3), the calculated log *P* values of the steroids are not as different from one to another to produce a shift in elution. In some additional experiments (to be published) we did the same experiments with smaller molecules that have a higher difference in their log *P* and in some cases a change in elution order was observed.

At temperatures higher than LCST, the PNIPAAM grafted surface exhibited a hydrophobic character, and the sensitivity to hydrophobicity of the solutes is increased. It can be observed that the more hydrophobic the steroids are the more they are retained. The retention time of each steroid increases with increasing temperature as seen from Fig. 7, but the increase is more pronounced the more hydrophobic the steroid is. Testosterone (log P = 3.32), being the most hydrophobic has the strongest increase in the retention time proving thus that hydrophobic interactions play an important role in the retention mechanism.

For a better understanding of the chromatographic properties of the column, the plate numbers and the symmetry factors of each steroid were calculated at all the previously mentioned temperatures, according to the following equations:

$$P = \frac{5.55(T_r/W_{0.5})^2}{L}$$

Table 2 Pore structural characterization of the pure and PNIPAAM modified silica beads

Material	Surface area/m^2 g^{-1}	Pore volume/ml g^{-1}	Pore size/nm
Pure silica	300	1	10
Si-PNIPAAM	170	0.5	6.3

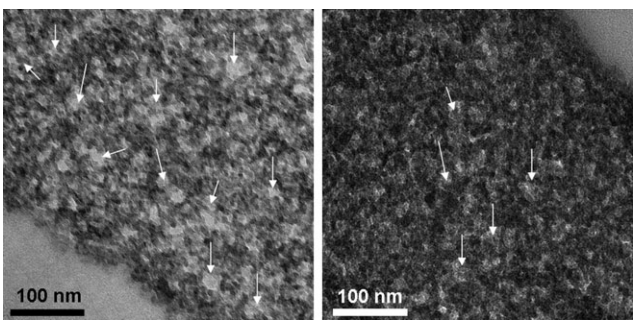

Fig. 5 Microtomed transmission electron micrographs of (A) pure silica Si-100 and (B) silica-PNIPAAM composite prepared using RAFT polymerisation.

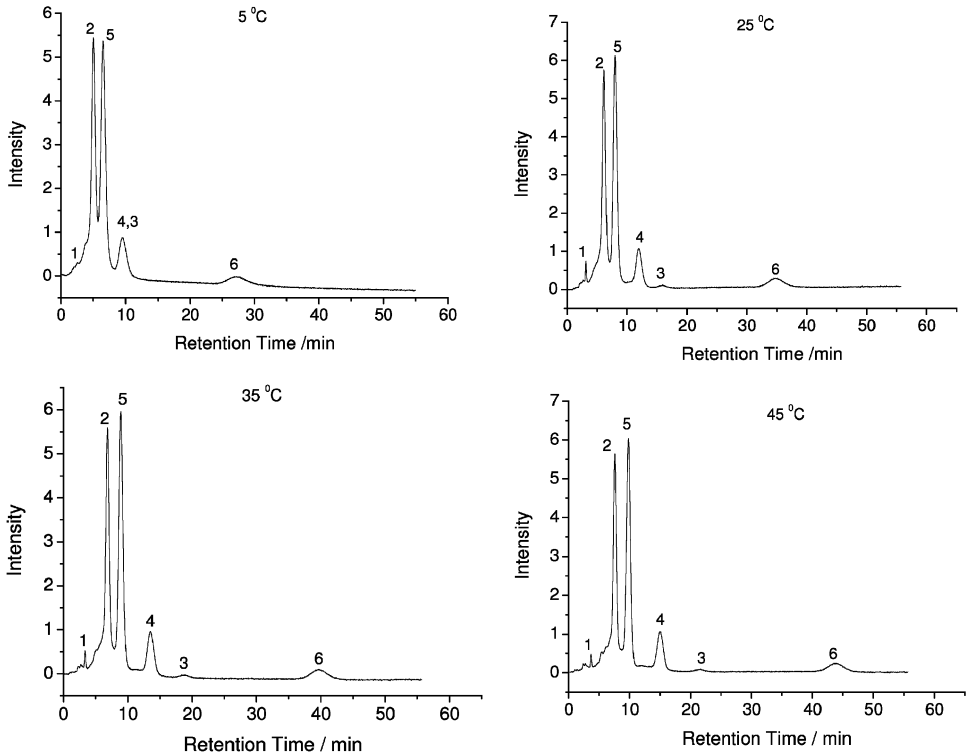

Fig. 6 Chromatograms of the steroid mixture and benzene on the Si-PNIPAAM column at four different temperatures: 1: benzene, 2: hydrocortisone, 3: hydrocortisone acetate, 4: dexamethasone, 5: prednisolone, 6: testosterone.

where P = is the plate number; T_r = is the retention time of the analyte in min and $W_{0.5}$ is the peak width at half height in min and L is the length of the column.

$$S = \frac{W_{0.05}}{2f}$$

where S = is the symmetry factor; $W_{0.05}$ = is the peak width at 5% peak height and f is the distance from the peak maximum to the leading edge of the peak. The distance being measured is at a point 5% of the peak height from the baseline.

For all the steroids the plate number increased with increasing the temperature (Table S1, ESI†). As it can be seen from the above chromatograms the change in retention time is more pronounced than the change in the peak width. Furthermore, with increasing the temperature, the grafted polymer changes from a swollen state into a collapsed state, leading to a slight decrease of the overall particle size which might contribute to an increase of plate number. It was observed that the symmetry factor of the analytes increases slightly by increasing the temperature (Table S2, ESI†) due to the slight increase of the peak width which is due on one hand to the more difficult diffusion of steroid molecules into the NIPAAM shrunken layer and on the other hand to the enhanced interaction causing retarded steroid elution.

As a reference experiment, we also analyzed the chromatographic behaviour of the starting silica before the PNIPAAM modification. No separation was accomplished at any of the previously mentioned temperatures (Fig. S1, ESI†). This clearly suggests that the successful separation of the steroid mixture is only due to the presence of polymer onto the pore surfaces. The driving force for the separation of hydrophobic

Table 3 Retention factors calculated form the retention times of the five steroids at different temperatures

	Retention factor (k)			
Analyte	5 °C	25 °C	45 °C	65 °C
Hydrocortisone	0.78	1.01	1.12	1.15
Hydrocortisone acetate	2.28	4.46	5.15	5.28
Dexamethasone	2.28	3.06	3.27	3.18
Prednisolone	1.2	1.67	1.77	1.71
Testosterone	8.32	9.96	11.16	10.82

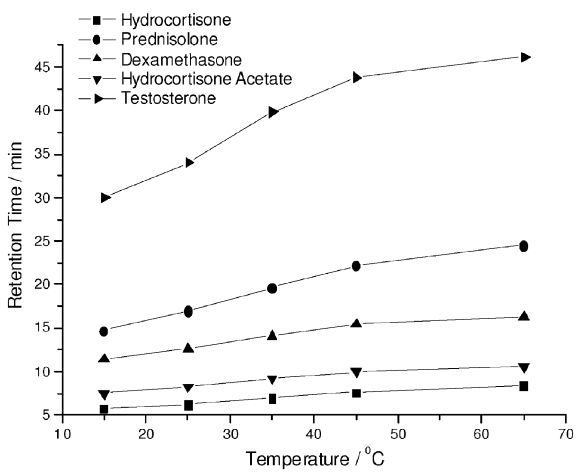

Fig. 7 Temperature-dependent retention time changes of steroids on the AZO–RAFT–PNIPAAM composite column.

steroids is presumably the partition into the PNIPAAM swollen layer below the LCST and hydrophobic interactions above the LCST.

Conclusions

We have synthesised a thermo-responsive composite by immobilization of an azo-initiator on the surface of a mesoporous silica gel and subsequent grafting of NIPAAM in the presence of a chain transfer agent. The composite showed a homogenous grafted polymer layer which has led to the successful packing into an HPLC column and separation of five steroids using the thermo-responsive surface property alterations in an aqueous media. This shows that the RAFT polymerization is a convenient methodology for the synthesis of thermo-responsive stationary phases, thus avoiding the problems associated with inhomogeneous polymer layers and inherent packing difficulties associated with conventional grafting from techniques. Furthermore, due to the living properties of RAFT polymers the method allows further attachment of different co-polymers in order to better tune the interactions in chromatography. The RAFT technique is a versatile tool for a wide range of monomers, therefore a lot of different temperature-responsive stationary phases combining other functions such as pH responsive or molecularly imprinted can be created. Thus, this method represents a rather general platform for chromatography *via* grafted polymers.

Experimental

Materials

Si-100 was kindly provided by Merck. (3-Aminopropyl)-triethoxysilane (APS) and 1,1,3,3 hexamethyledisilazane was obtained from Aldrich. 4,4′-Azobis(4-cyanopentanoic acid) (ACPA), ethyl chloroformate and triethylamine were obtained from Fluka. *N*-Isopropyl acrylamide and 2,2-azobisisobutyronitrile (AIBN) were purchased from Acros and were recrystalized from *n*-hexane and methanol, respectively. The Raft agent (benzyldithiobenzoate [DBD]) was synthesized according to the literature.[22] The steroids hydrocortisone, hydrocortisone acetate, dexamethasone, prednisolone and testosterone, and the void marker benzene were purchased from Sigma-Aldrich.

De-ionized water was taken from a Seral purification system (PURELAB Plus) with a conductivity of 0.06 μS cm^{-1}.

Immobilization of ACPA

The immobilization of the initiator was performed in four consecutive steps. First, the silica surface was rehydroxylated by reflux in HCl (17%). After activation, APS was coupled to the silica surface by suspending 2 g rehydroxylated silica in 9 mL dry toluene followed by dropwise addition of 0.3 ml (3-aminopropyl)trimethoxysilane in 2 ml dry toluene under inert conditions. The mixture was refluxed overnight, the product was isolated by filtration, washed with toluene, MeOH and water, and dried at 80 °C overnight under vacuum. In order not to interfere in our chromatographic separation, the remaining silanol groups were end-capped with 1,1,3,3-hexamethyldisilazane by reacting the Si-APS (2 g) with 1 ml of this reagent in DCM (10 ml) at room temperature overnight followed by reflux for 3 h. The end-capped product was filtered, washed with DCM, EtOH and water and dried under vacuum at 80 °C. For the attachment of ACPA, first 10 ml dry THF were cooled with a mixture of liquid nitrogen and ethanol. To this solution, under inert Ar atmosphere, 1 mmol (280.3 mg) of ACPA, 1 mmol (110 mg) of ethyl chloroformate and 1 mmol (105 mg) triethylamine were added. After stirring at −78 °C for 30 min, 2 g of end-capped silica were introduced into the mixture and stirred for 3 h at −78 °C and for 4 additional h at −10 °C. The initiator-modified silica particles were washed with THF, MeOH, water and dried at 80 °C under vacuum.

Grafting of PNIPAAM on silica

1.7 g of azo-initiator modified silica particles were suspended in a polymerization mixture containing 25.5 mmol (2.9 g) of *N*-isopropyl acrylamide, 25.5 μmol (7.3 mg) RAFT agent (BDB) and 1 mg AIBN as an initiator in 10 ml dry DMF. After three freeze–thaw cycles, the mixture was heated to 80 °C for 24 h. The composite was then washed with DMF, MeOH and water and dried under the vacuum at 60 °C overnight.

HPLC Measurements

The composite materials were slurry packed into stainless steel columns (120 × 4.5 mm) using MeOH–H_2O (80 : 20, v/v) as the pushing solvent, and evaluated chromatographically using water as mobile phase. The flow rate was 1 ml min^{-1} and 10 μl aliquots of 1 mg ml^{-1}solutions of steroids and a mixture of them was injected. The elution was monitored at 254 nm. The retention factors (k) and separation factors (α') were calculated using the following formulas: $k = (t_R - t_0)/t_0$ and $\alpha' = k_1/k_2$ where t_R = retention time of the analyte and t_0 = retention time of the void marker, benzene.

Calculation of grafting density

The amount of immobilized ACPA initiator and PNIPAAM grafted on the silica beads was calculated using the following equations:

$$\text{Immobilized initiator} = \frac{\%C_i}{\%C_{i(\text{theory})}[1 - (\%C_i/\%C_{(\text{theory})})]S}$$

$$\text{Grafted PNIPAAM} = \frac{10^3 \%C_p}{\%C_{p(\text{theory})}[1 - (\%C_p/\%C_{p(\text{theory})}) - (\%C_i/\%C_{i(\text{theory})})]S}$$

where $\%C_i$ ($\%C_p$) = increase in carbon percent from elemental analysis; $\%C_{i(\text{theory})}$ ($\%C_{p(\text{theory})}$) = calculated weight percent of carbon in initiator or monomer (or polymer); S = specific surface area (m^2 g^{-1}); 10^3 = is a factor for converting (g) to (mg):

$$\text{Graft density of PNIPAAM} = \frac{m_p N_A}{M_n}$$

where m_p = amount of grafted polymer on the silica beads (g m^{-2}); N_A = Avogadro's number; M_n = number average molecular weight of grafted PNIPAAM.

Determination of PNIPAAM number-average molecular weight

In order to determine the number-average molecular weight and the polydispersity index, the grafted PNIPAAM was cleaved from the silica surface using TFA and analyzed using GPC. 0.3 g of PNIPAAM-Si composite was treated with 3 ml of TFA solution for 3 h. After, the solution was neutralized with sodium carbonate, filtered and dialyzed against MilliQ water using a dialysis membrane (molecular weight cutoff: 1000) for three days while changing the water every day. The polymers was recovered by freeze-drying and analyzed by GPC.

Characterization methods

Scanning electron microscopy (SEM) measurements were performed by the high resolution scanning electron microscope, Gemini 1550 (120 kV, Carl Zeiss, Oberkochen). The samples were dried on a carbon sample holder for SEM.

The transmission electron microscopy (TEM) of the samples were measured using a Zeiss Omega 912 (100 kV, Carl Zeiss, Oberkochen). The diluted samples were applied as drops on 400 Mesh Copper grids that were vaporized with carbon film. The solvent was evaporated under normal pressure.

Molar mass distributions were provided by GPC measurements that were carried out with Thermo Separation Products with a UV detector: UV 1000 (λ = 260) and Shedox Refractive index detector: RI-71. The samples were dissolved in NMP solvent and went through the column packing of MZ-Gel CD plus 10 E 3, 10 E 5, 10 E 6 at a temperature of 30 °C.

The FT-IR spectra were recorded on an FTS 6000 spectrometer (Bio-Rad).

(C, H, N, S) Elemental analyis were performed on a Vario EL Elementar (Elementar Analysen-System, Hanau, Germany).

The nitrogen adsorption was measured using a QUADROSORB SI, equipped with Automated surface area and pore size analyzer. Before analyzing the samples, they were degassed at 100 °C for 20 h using a Masterprep degasser (Model: QUADRASORB).

The HPLC measurements were performed using an Agilent technology equipped with 3D-Quaternary pump with degasser and a Diodenarray detector. The mobile phase was Milli-Q water with a flow rate of 1 ml min^{-1}. The elution behaviour was monitored by UV (254 nm).

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