PREPARATION OF CHROMATOGRAHPY MATRICES HAVING THERMORESPONSIVE POLYMER BRUSH STRUCTURE

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Introduction

We have prepared thermoresponsive chromatographic matrices by introducing poly(*N*-isopropylacrylamide) chains onto silica beads for separation of bioactive compound in aqueous media [1]. This system is highly useful to control function and properties of the stationary phase for high performance liquid chromatography (HPLC) by only change in column temperature, and has advantages in maintaining biological activity for peptides and proteins and reduced pollution by organic solvents. We have investigated the preparation and characterization of a series of thermoresponsive polymer modified surfaces as the chromatographic stationary phase. Properties of thermoresponsive modifier on the silica surfaces influence the separation efficiency. In addition, polymer grafting method has also significant importance, because graft conformation of PIPAAm on silica surfaces, determined by grafting method, greatly influences the temperature-responsive wettability changes and elution behavior of solutes [2,3]. In the present paper, we prepared high-density PIPAAm brushes on silica beads surfaces using surface-immobilized ATRP initiator in order to characterized the densely PIPAAm grafted silica beads as a chromatographic stationary phase (Figure 1). Property of densely PIPAAm grafted silica as a novel stationary phase is investigated with elution behavior of steroids and peptides.

Methods

Preparation of Densely PIPAAm Grafted Silica Beads

Silica beads were washed in concentrated hydrochloric acid for 3h at 90°C, then rinsed with large amounts of distilled water repeatedly, followed by thorough drying under vacuum at 110°C for 18h. Silica beads (11.5g) was placed into round-bottomed flask and dried under vacuum at 3h at 150°C. To this was added 2-(*m*/*p*-chloromethylphenyl)ethyltrichlororsilane (3.43g, 11.9mmol) in 50mL of dry toluene under nitrogen gas atmosphere. Reaction proceeded overnight at 120°C under continuous stirring. ATRP initiator-immobilized silica beads were collected by filtration and extensively rinsed with toluene, methanol, dichloromethane and acetone, respectively, and dried at 110 °C for 1h.

IPAAm (4.86g, 429mmol), CuCl (85mg, 0.86mmol) and Me_6TREN (0.20g, 0.86mmol) were dissolved in 42.8 mL of distilled DMF. Monomer solution was degassed by triplicate freeze-thaw cycles and sealed under reduced pressure with a stopcock. ATRP initiator-immobilized silica beads (1.0g) was placed into 50mL of glass vessel. Both monomer solution and the silica beads were placed into a glove bag, inside of the bag were purged with dry nitrogen gas by repeated vacuum and nitrogen flush three

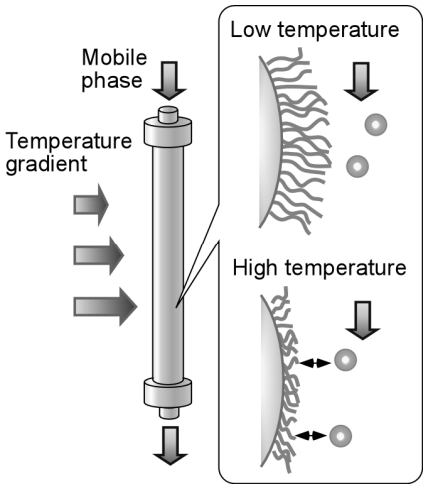

Fig.1 Temperature responsive chromatography using densely PIPAAm grafted silica beads as a chromatographic stationary phase

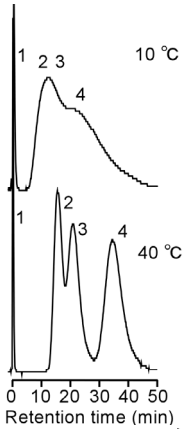

Fig.2 Chromatograms of steroids on column using densely PIPAAm grafted silica column at 10°C and 40°C. Mobile phase is Milli-Q water. Peaks: 1, adenosine; 2, hydrocortisone; 3, prednisolone; 4, dexamethasone.

times to remove oxygen. The monomer solution was then poured into the silica beads containing glass vessel, which was sealed under nitrogen. The ATRP reaction proceeded for predetermined time period at 25 °C under continuous shaking. After the reaction, the PIPAAm modified silica beads was washed by repeated sequential centrifugation and resuspension in DMF, 50mM EDTA solution and finally with Milli-Q water until no catalyst was detected in the supernatant. Modified silica beads were filtered and rinsed with Milli-Q water and acetone, and dried in high vacuum oven at 50°C for 5h.

Temperature Responsive Elution of steroids and peptides

PIPAAm-grafted silica beads were packed into a stainless steel column (50 mm x 4.6 mm i.d.) from a slurry of beads in water/methanol mixture solvents (1:1) using a column packer at 350 kg/cm^2 for 1h, followed by equilibration with Milli-Q water for at least 12h. PIPAAm-grafted beads-packed columns were connected to an HPLC system controlled by a personal computer. Hydrophobic steroids, hydrocortisone, prednisolone, dexamethasone, and hydrophilic adenosine used as an internal standard were dissolved in Milli-Q water as samples to produce chromatograms. Concentration for each sample is: 0.25mg/mL for hydrocortisone, 0.25mg/mL for prednisolone, 0.1mg/mL for dexamethasone, and 0.2mg/mL for adenosine, respectively. Each sample solution was mixed together to produce the chromatograms. Milli-Q water was used as a mobile phase. Thermoresponsive elution behavior for these compounds was monitored at 254nm with a flow rate of 0.5mL/min. Column temperature was thermostated within a deviation of ±0.1°C using a thermostated water bath.

Results and Discussion

Separation of Steroids Using Temperature-Responsive Surfaces

To investigate surface property of PIPAAm-grafted silica beads prepared by ATRP, we examined temperature-dependent elution profiles of steroid molecules having different partition coefficient and water solubility as indexes for temperature-responsive hydrophobic interactions. Since we observed that the more significant amount of PIPAAm is immobilized onto the surfaces with ATRP

than conventional radical polymerization methods by elemental analysis and SEM observation, strong hydrophobic interaction with hydrophobic solutes is supposed to be affected by PIPAAm-modified surfaces. Figure 2 shows chromatograms of steroids at 10°C and 40°C on densely PIPAAm grafted silica beads column using Milli-Q water as a mobile phase. Retention time of steroids at 40°C was longer than that at 10°C. This is because grafted PIPAAm on the silica beads surfaces dehydrated at 40°C, and the hydrophobic interaction between the surface and steroids occurs.

Although column lengths used in this study was shorter than those used in the previous studies [4], relatively longer retention times of the steroids were observed. This is probably due to the densely grafted PIPAAm onto silica bead surfaces, affecting to steroid penetration within the PIPAAm brush layers. Hydration of PIPAAm chains in the dense PIPAAm brush layers may be different from sparsely grafted PIPAAm chains on the surfaces, and this also shows stronger interactions with steroids. Thus, densely PIPAAm grafted chains enables controlling strong hydrophobic interaction with steroids by changing temperature.

Conclusions

We prepared chromatographic stationary phase surfaces modified with dense PIPAAm brush layers prepared by ATRP to investigate temperature dependent elution behavior of bioactive compounds in aqueous mobile phase. Retention times of steroids using the column are increased with increasing temperature. In addition retention times of steroids are longer than for the previously developed PIPAAm modified columns. This is probably due to the grafted polymer amounts and the different molecular conformation of the PIPAAm brushes prepared by ATRP. In conclusion, densely packed PIPAAm brushes grafted onto silica bead surfaces were prepared by ATRP, and prepared surfaces showed relatively strong hydrophobic interaction with solutes. Thus, high density PIPAAm brush surfaces may be one of the effective stationary phases for thermoresponsive chromatography.

References

1. Kikuchi, Akihiko and Teruo Okano (2002) “Intelligent thermoresponsive polymeric stationary phase for aqueous chromatography of biological compounds” *Prog. Polym. Sci.*, *207*, 1165-1193.
2. Kanazawa, Hideko; Kazuo Yamamoto; Yoshikazu Matsushima; Nobuhiro Takai; Akihiko Kikuchi; Yasuhisa Sakurai and Teruo Okano (1996) “Temperature-responsive chromatography using poly(*N*-isopropylacrylamide)-modified silica” *Anal. Chem*, *68*, 100-105.
3. Yakushiji, Taiji; Kiyotaka Sakai; Akihiko Kikuchi; Takao Aoyagi; Yasuhisa Sakurai and Teruo Okano (1998) “Graft architecture effects on thermoresponsive wetability change of poly(*N*-isopropylacrylamide)-modified surfaces” *Langmuir, 14*, 4657-4662
4 Yakushiji, Taiji; Kiyotaka Sakai; Akihiko Kikuchi; Takao Aoyagi; Yasuhisa Sakurai and Teruo Okano (1999) “Effects of cross-linked structure on temperature-responsive hydrophobic interaction of poly(*N*-isopropylacrylamide) hydrogel-modified surfaces with steroids” *Anal. Chem*, *71*, 1125-1130.