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Preparation of poly(vinyltetrazole) chain-grafted poly(glycidymethacrylate-co-ethylenedimethacrylate) beads by surface-initiated atom transfer radical polymerization for the use in weak cation exchange and hydrophilic interaction chromatography

Junxia Hao[a], Fuqiang Wang[a], Xiaojun Dai[a], Bolin Gong[a,*], Yinmao Wei[b,**]

[a] Key Laboratory of Energy & Chemical Engineering, Ningxia University, Yinchuan 750021, PR China
[b] Department of Chemistry, Northwest University, Xi' an 710069, PR China

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ABSTRACT

A novel stationary phase for weak cation exchange (WCX) and hydrophilic interaction chromatography (HILIC) was prepared with surface-initiated atom transfer radical polymerization (SI-ATRP). Vinyltetrazole was grafted onto the surface of the beads in water medium with the polyglycidylmethacrylate beads ($P_{GMA/EDMA}$) previously modified with 2-bromoisobutryl bromide as the macromolecule initiators and CuCl as catalyst. The poly(vinyltetrazole)-grafted beads obtained with different atom transfer radical polymerization (ATRP) formulations were tried as chromatographic packings in ion-exchange chromatography. The results showed that the prepared columns could separate the tested proteins with high efficiency and high capacity, and the retention time of protein had a positive relationship with increasing the chain lengths of the grafted poly(vinyltetrazole) (PVT). The prepared column was also found to be able to separate nucleosides by hydrophilic interaction chromatographic mode.

1. Introduction

The conventional chromatographic stationary phases are obtained by the surface derivation methods involving the use of activation agents or free radical polymerization techniques by the covalent attachment of ligands onto the silica or polymer particles [1]. However, the radical polymerization methods offer little control over the surface graft density and the polymer chain length. This drawback could be overcome using the surface-initiated atom transfer radical polymerization (SI-ATRP) techniques where the graft density is controlled by the surface density of the initiator, and the chain length by the ratio of monomer to initiator [2]. The first example of SI-ATRP in the preparation of stationary phase was to prepare polymer-based strong cation exchange HPLC support by using 3-sulfopropyl methacrylate as monomer and transition-metal complexes (CuCl and 2,2-bipyridyl) as catalyst [1]. Since then, numbers of new packings have been prepared [3–6], and some of them showed special selectivity due to high density of ligands [7–9]. So, SI-ATRP is expected to play an important role in the preparation of functional materials in the field of separation science.

With emerging targets in fields as diverse as environmental studies, proteomics, metabolomics, and biotechnology, HPLC faces many new challenges. Most of these new areas require highly efficient separations of very complex mixtures, in which currently available columns may not perform adequately. Therefore, it is necessary to develop new separation media. Nevertheless, exploring new ligands or new preparation methods are the desire way to obtain new media.

Tetrazole has similar aqueous pKa value as carboxylic acid, and thus it has become a widely known alternative to carboxylate moiety. So, tetrazole group might be used as the ligands of weak cation exchanger [3]. In this paper, we prepared polyvinyltetrazole (PVT)-grafted stationary phase with SI-ATRP, and further investigated the application of PVT-grafted packings in the separation of proteins and nucleosides.

2. Materials and methods

2.1. Reagents and materials

Glycidylmethacrylate (GMA) (Aldrich, USA) was distilled under vacuum. Poly(vinylpyrrolidone, k-30) (PVP, k-30) was purchased

* Corresponding author. Tel.: +86 951 2062300; fax: +86 951 2062860.
** Corresponding author. Tel.: +86 29 88302604; fax: +86 29 88302604.
E-mail addresses: gongbl@nxu.edu.cn, gongbolin@163.com (B. Gong), ymwei@nwu.edu.cn (Y. Wei).

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from Aldrich (USA). α,α-Azobisisobutyronitrile (AIBN) and cyclohexanol were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). GMA was distilled under vacuum prior to use. Copper (I) chloride (CuCl) was purified by washing in glacial acetic acid overnight, then it was filtered and washed with absolute ethanol and ether, and then dried under vacuum overnight and stored under argon.

Cytochrome C (horse heart, Cyt-c), ribonuclease (bovine pancreatic, RNase), lysozyme (chicken egg white, Lys), α-chymotrypsinogen A (bovine pancreatic, α-Chy-A), bovine serum albumin (bovine serum, BSA), ovalbumins (egg white, OVA) and trypsin inhibitor (soybean, STI) were purchased from Sigma (St. Louis, USA). Uracil, uridine, cytimidine, cytidine, adenine, adenosine and guanosine were purchased from Aladdin Reagent Inc. (Shanghai, China).

2.2. Synthesis of 5-vinyltetrazole (5-VT)

The monomer, 5-vinyltetrazole (5-VT), was prepared according to the reference [10]. Briefly, 5.3 g (0.1 mol) of acrylonitrile, 13.3 g (0.1 mol) of anhydrous aluminum chloride and 29 g (0.445 mol) of sodium azide were added into 200 mL tetrahydrofuran (THF), and was refluxed for 24 h under nitrogen. The reaction mixture was acidified by addition of 150 mL of 15% hydrochloric acid, and the excess hydrazoic acid was removed by an aspirator. The organic layer was separated from the water layer. The water layer was extracted with 50 mL of ethyl acetate for four portions. Evaporation of all the combined extractions gave 3.6 g of crude product, and the crude was purified by crystallization to produce 3.3 g of 5-VT. Melting point: 126–127 °C; IR (KBr), ν (cm^{-1}): 3444 (N–H, stretch), 2250–3250 (N–H, broad and strong band, tetrazole), 3115 and 3000 (stretch $=CH_2$ and $=CH$); 1200–1600 (tetrazole stretch and bend, C–N, N–N, C=N, N=N) [11]. Mass, highest *m/e* 96; ^1H NMR, δ: 6.882–5.814 (1H, 1H, 1H, corresponding to 3H of the vinyl).

2.3. Preparation of 5.0 μm PGMA/EDMA beads

The monodisperse, 5.0 μm hydrophilic macroporous poly(glycidymethacrylate) beads were prepared by using a single-step swelling and polymerization method [12]. About 0.3 g of 1.8 μm dispersed linear poly(glycidylmethacrylate) seed beads and 20 mL of 0.1% sodium dodecyl sulfonate (SDS) solution were placed into a 250 mL flask, and the mixture was stirred slowly by a mechanical stirrer. Then 8.0 mL of the mixture consisting of 2.0 mL GMA, 2.0 mL ethylene dimethacrylate (EDMA), 2.0 mL cyclohexanol, 2.0 mL toluene and 3.0% (w/w) benzyl peroxide (BPO) initiator in terms of the total monomers were added into 75 mL of 0.1% and 1.0% polyvinyl alcohol (PVA) solution and then emulsified under ultrasonic condition. The emulsion was subsequently added into the dispersion solution of the seed beads. The mixture was stirred for 10–15 h at room temperature. Then it was degassed by purging with nitrogen for 20 min. The polymerization was carried out at 70 °C for 24 h under stirring. The beads obtained were washed with hot water and methanol, and then extracted with THF for 48 h to remove the porogens, cyclohexanol and toluene. The obtained beads were washed with methanol again and dried in vacuum at 50 °C, affording 3.6 g of monodisperse beads. The scanning electron micrograph illustrated that the prepared beads are uniform with the size of 5.0 μm (Fig. 1). The medium pore diameter of the prepared $P_{GMA/EDMA}$ beads was measured to be 500 nm, and the surface area calculated from the BET isotherms was 11.2 $m^2\,g^{-1}$. A duplicated experiment was done, and 3.5 g of the monodisperse beads was obtained.

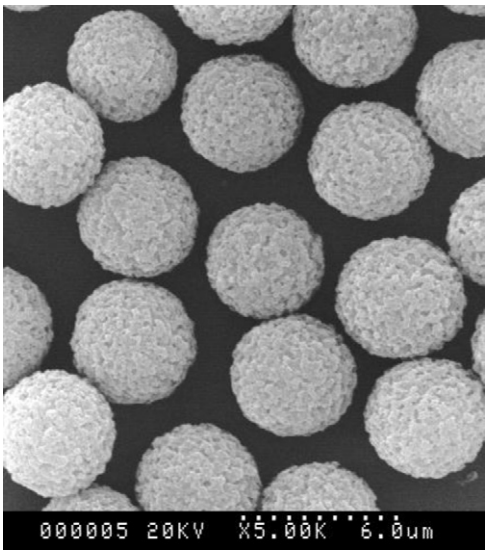

Fig. 1. Scanning electron micrograph of the monosized porous beads.

2.4. Preparation of initiator carrying PGMA/EDMA beads [13]

Firstly, 2.3 g of $P_{GMA/EDMA}$ beads were hydrolyzed in 100 mL of 0.1 mol L^{-1} sulfuric acid at 60 °C under stirring for 10 h. The hydrolyzed beads were then filtered, washed with water until neutral, and dried under vacuum. Secondly, 2.0 g of the hydrolyzed beads and 17 mL of dry THF were added into a round-bottomed flask, and stirred for 1 h. After cooling to 0 °C, 1.5 mL of triethylamine and 1.0 mL of 2-bromoisobutrylbromide was added in dropwise. The mixture was stirred at 0 °C for 0.5 h, then at room temperature for 24 h. The beads were filtered, washed with THF, water, ethanol and acetone for several times and dried in vacuum oven at 30 °C, so the solid initiators, $P_{GMA/EDMA}$-Br beads, were prepared. Two duplicated experiments were done, and 2.0 g of the solid initiators, $P_{GMA/EDMA}$-Br beads were obtained.

2.5. Synthesis of PVT-grafted packings by SI-ATRP

In a typical synthesis, 5-vinyltetrazole (1.8 g), NaBr (3.0 g) and deionized water (15 mL) were added into a round-bottomed flask equipped with a magnetic bar, and the solution was adjusted to pH 8.5 with NaOH solution, and then 2.0 g of $P_{GMA/EDMA}$-Br beads was added. The mixture was degassed by four freeze-pump-thaw cycle. CuCl/2, 2′-bipyridyl (BPy) (50 mg/125 mg) solution was rapidly injected under protection of N_2 flow in the ice-salt bath (NaCl:NH_4Cl:smash ice, 40:20:100, w/w). The flask was then subjected to two additional freeze–pump–thaw cycle, and placed under stirring at ambient temperature for 24 h. The flask was exposed to air to terminate the polymerization. The resulting PVT-grafted beads were filtered, washed with chloroform, ethyldiamine tetraacetate (EDTA), water and ethanol for several times, and dried in vacuum oven at 30 °C.

The amounts of the grafted PVT on $P_{GMA/EDMA}$ beads were calculated based on the elemental analysis data according to the following Eq. (1) [14].

$$\text{Grafted PVT} = \frac{\%N(10^3)}{\%Np(\text{calcd.})[1 - \%Np/\%Np(\text{calcd.}) - \%Ni/\%Ni(\text{calcd.})]S} \quad (1)$$

where, $\%N_p$ is the percent nitrogen increase over that of the initiator-immobilized beads, $\%N_p$ (calcd.) is the calculated weight percent of nitrogen in vinyltetrazole, $\%N_i$ is the nitrogen composition of the initiator, $P_{GMA/EDMA}$-Br beads, and $\%N_i$ (calcd.) is the calculated weight percent of nitrogen in initiator unit, *S* is the

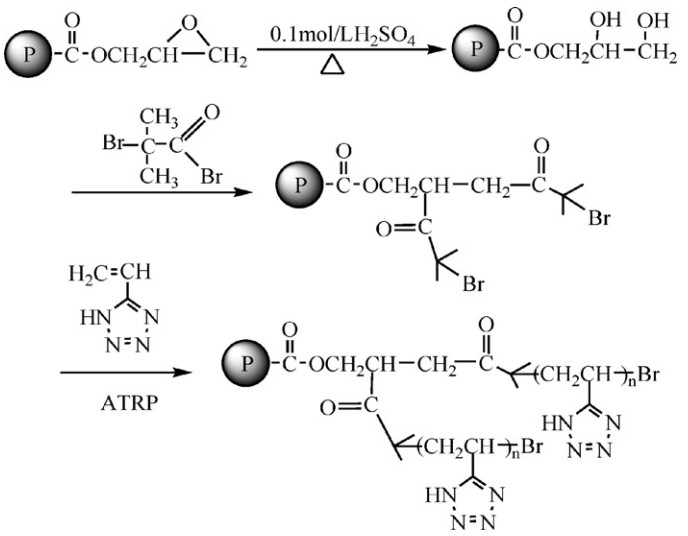

Fig. 2. Synthesis scheme for preparation of PVT-grafted chromatographic stationary phase.

specific surface area of the original $P_{GMA/EDMA}$ bead support in 11.2 $m^2\,g^{-1}$ (measured by nitrogen gas sorption).

2.6. Purification of lysozyme from egg white

Egg white was obtained from fresh egg and diluted four fold by 20 mmol L^{-1} sodium phosphate buffer (pH 7.0). The 10.0 cm × 0.46 cm I.D. column was used for chromatographic purification experiments. The egg white sample was loaded after equilibrating the column with equilibrium buffer (20 mmol L^{-1} sodium phosphate buffer, pH 7.0). Then the column was eluted with a salt gradient and the fractions were collected and assayed.

2.7. Chromatographic experiment

The PVT-grafted beads were packed into a stainless steel column (10 cm × 0.46 cm I.D.) by using distilled water as slurry solvent under 30 MPa. In weak cation exchange chromatographic mode, the mobile phase was obtained by mixing the solution A (20 mmol L^{-1} phosphate, pH 7.0) and the solution B (20 mmol L^{-1} phosphate + 1.0 mol L^{-1} NaCl, pH 7.0), and it was filtered through a 0.45 μm membrane prior to use. Detection wavelength was set at 280 nm. In HILIC mode, the mobile phase was prepared by mixing the solution A (10 mmol L^{-1} ammonium formate, pH 6.5) and the solution B (acetonitrile). Flow rate of the mobile phase: 1.0 mL min^{-1}; UV detection: 254 nm. All the column temperatures were at room temperatures.

3. Results and discussion

3.1. Preparation of PVT-grafted chromatographic packings

The preparation procedure for PVT-grafted packings was given in Fig. 2. The first and second steps were easy to carry out. The third was to graft PVT onto the surface of the $P_{GMA/EDMA}$ beads with SI-ATRP. As well known, ATRP is a powerful tool for exactly controlling the molecular weight and the molecular weight distribution of polymer not only in solution but also in many heterogeneous ATRP systems initiated with the particles carrying immobilized initiators [15–18]. In order to investigate the effect of the grafted polymer chain on the separation, three stationary phases with different chain lengths of the grafted PVT were tried by manipulating the polymeric conditions. Because

Table 1
Properties of the PVT-grafted packings obtained with different ATRP formulations.

Samples	Elemental composition [%, w/w][a]			Amount of grafted PVT (mg m^{-2})
	C	H	N	
$P_{GMA/EDMA}$-Br	45.77	6.411	0.031	–
ATRP 1	46.39	6.011	1.171	1.80
ATRP 2	44.59	5.855	2.045	3.20
ATRP 3	42.59	5.755	2.504	3.80

[a] Measured by elemental analysis of C, H and N.

the length of the grafted polymer chain is depended on the concentration of the monomer in polymeric system and polymerization time, so in this work, the concentrations of 5-VT were let to change but the polymerization time kept the same for the three PVT-grafted beads. For the first one named as ATRP1, [5-VT]/[initiator]/[CuCl]/[BiPy] ratios were selected as 30:1:1:2, the second (ATRP2), [5-VT]/[initiator]/[CuCl]/[BiPy] ratios as 50:1:1:2 and the third (ATRP3), [5-VT]/[initiator]/[CuCl]/[BiPy] ratios as 100:1:2:4.

Elemental analyses of C, H and N for the PVT-grafted $P_{GMA/EDMA}$ beads were summarized in Table 1. Obviously, N content had a significant increase after the reaction of $P_{GMA/EDMA}$-Br beads with 5-VT, and the shortest PVT-grafted chains on the particles corresponded to the lower monomer/initiator ratios in polymerization system, for which the amount of PVT was calculated to be 1.80 mg m^{-2}. Higher PVT contents were achieved for ATRP2 and ATRP3 because of the higher monomer/initiator ratios with respect to ATRP1. Thus, the length of the PVT-grafted chains on $P_{GMA/EDMA}$ beads would be increased with increasing vinyltetrazole initial concentration [19].

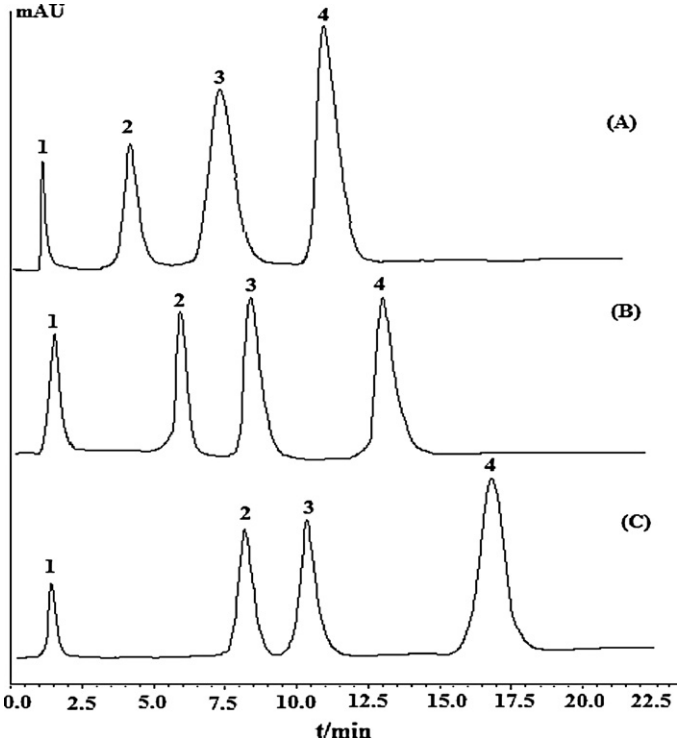

Fig. 3. Chromatogram four protein mixtures separated by the PVT-grafted columns. Columns: (A) ATRP1; (B) ATRP2; (C) ATRP3; column: 10 cm × 0.46 cm I.D. The linear gradient elution was from 100% solution A (20 mmol L^{-1} of phosphate, pH 7.0) to 100% solution B (20 mmol L^{-1} of phosphate + 1.0 mol L^{-1} NaCl, pH 7.0) for 20 min. Flow rate: 1.0 mL min^{-1}. AUFS: 0.08, UV detection at 280 nm. Proteins: (1) solvent + Myo; (2) RNase; (3) Cyt-C; and (4) Lys.

3.2. Chromatographic separation on the PVT-grafted packings in WCX mode

As stated above, tetrazoles-functionalized particles might be regarded as weak cation exchanger. In order to identify the chromatographic performance of the PVT-grafted stationary phase, a mixture of basic proteins consisting of Myo (p*I* 7.3), RNase (p*I* 8.8), Cyt-C (p*I* 9.4) and Lys (p*I* 11.0) were separated on three PVT-grafted stationary phases in mobile phase at pH 5.0–8.5 (p*I* is theisoelectric point of the protein). As shown in Fig. 3, successful separation of four basic proteins were achieved on the three PVT columns, respectively. The elution order of the basic proteins accorded with their isoelectric points (p*Is*), while the acid protein mixtures consisting of BSA (p*I* 4.9), OVA (p*I* 4.7) and STI (p*I* 4.5) were not retained. At pH 7.0, all the acidic proteins have negative charges, as a result no interaction with the stationary phase happened because tetrazole groups also deprotonated to form the anions. However, basic proteins have positive charge, and were retained on the columns by the static electric interaction with tetrazole groups. Therefore, the elution orders of the proteins were depended on their p*Is*, indicating the retention mechanism to be of cation exchange.

In comparison with the three columns, the retention time of the same protein was found to gradually increase from ATRP1, ATRP2 to ATRP3, indicating that the retention time of protein had a positive trend along with the number of grafted tetrazole groups on the stationary phase. In ion exchange chromatography, the higher the density of the charge on ion-exchanger, the longer the retention time of solute [20,21]. As tetrazole served as ion-exchange group, the longer length of the PVT chains reflected the higher density of the charge, so the retention times were increased with the length of the grafted PVT.

Table 2
Adsorption capacity, *q*, with different lysozyme concentrations on the column packed with the PVT-grafted beads.[a]

Concentration (mg mL^{-1})	Capacity (mg)
0.5	11.70
0.6	16.74
0.7	22.37
0.8	27.17
0.9	32.57
1.0	38.57

[a] Column (ATRP1): 5.0 cm × 0.46 cm I.D.; equilibrium solution: 0.02 mol L^{-1} NaH_2PO_4, pH 7.0; the loading buffer: 0.02 mol L^{-1} NaH_2PO_4 + 1.0 mg mL^{-1} Lys; flow rate: 0.3 mL min^{-1}. The dead time calculated was in solution A (0.02 mol L^{-1} NaH_2PO_4) adding $NaNO_2$.

3.3. Chromatographic properties of WCX

3.3.1. Effect of flow-rate on proteins separation

Fig. 4 shows the chromatograms of protein at different flow-rate. At a flow rate of 3.0 mL min^{-1}, the four standard proteins could be separated in less than 5.0 min while the column efficiency is almost the same as that at the flow-rate of 1.0 mL min^{-1}. This indicated that the PVT-grafted column had the advantages of high performance and fast mass transfer, and thus it could be used for fast separation of biological products.

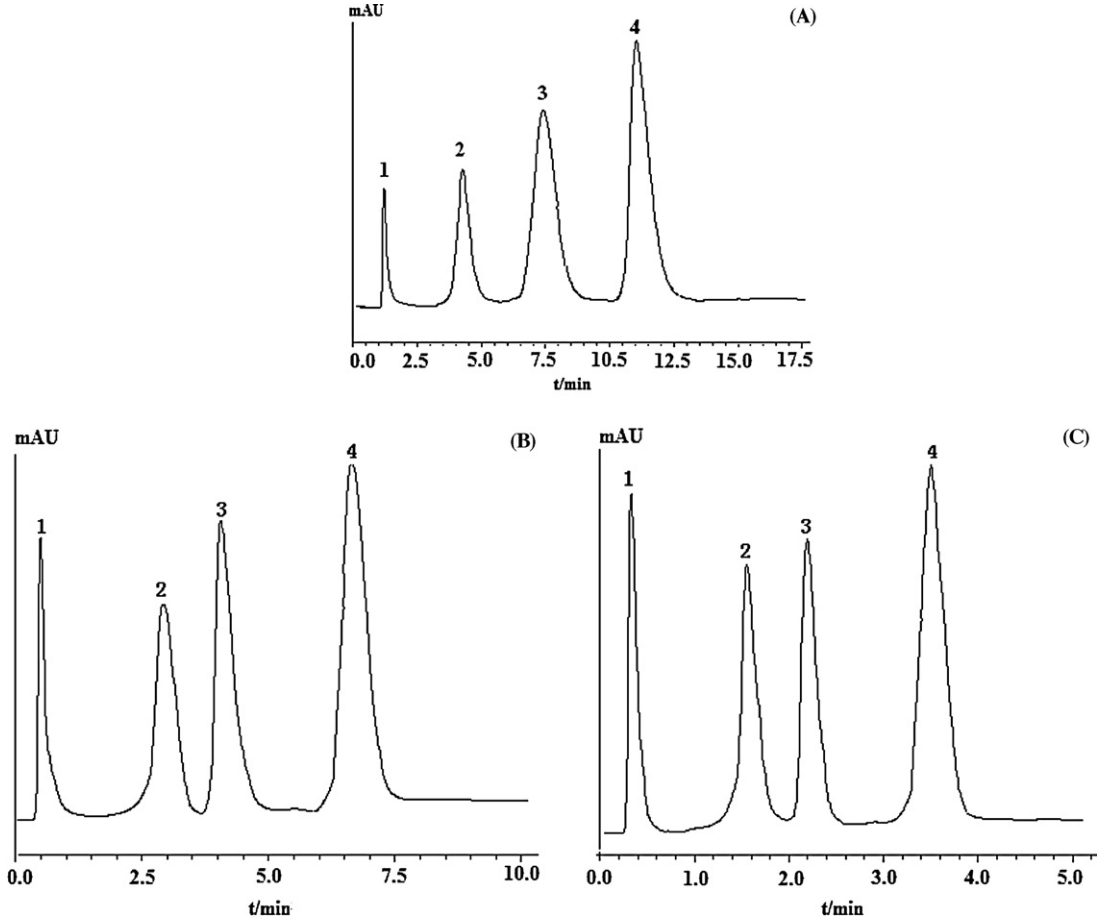

Fig. 4. Effect of mobile phase of flow rate on the retention of proteins. (A) 1.0 mL min^{-1}, (B) 2.0 mL min^{-1}, (C) 3.0 mL min^{-1}, Conditions: The linear gradient elution was from 100% solution A to 100% solution B. Proteins: (1) solvent + Myo; (2) RNase; (3) Cyt-C; and (4) Lys.

3.3.2. Dynamic capacity of the PVT-grafted WCX packings

Breakthrough curves provide valuable information for the evaluation of the dynamic capacity of the separation medium. The adsorption capacity of Lys on the prepared packings (ATRP1) was determined by the frontal analysis [22,23]. The Langmuir isotherm model, originally constructed for gas adsorption on homogeneous glass surface, has been frequently used for predicting the adsorption of solutes on various types of adsorbents due to its simplicity [24,25]. Based on the Langmuir isotherm model in Eq. (2), the dynamic capacity from $Q_{50\%}$ to $Q_{100\%}$ break-through consecutively was presented.

$$q* = \frac{qmC*}{Kd + C*} \quad (2)$$

(c^*:the equilibrium concentration of adsorbate; q^*: equilibrium adsorbate; q_m: the largest single molecular adsorption layer adsorption on particle surface; k_d:constant of langmuir).

The adsorption amount, q, at the midpoint of the break-through curve was obtained as show in Table 2. The q value was found to increase as the protein concentrations were increased from 0.5 to 1.0 mg mL^{-1}, and at 1.0 mg mL^{-1}, the q value reached a maximal of 38.1 mg mL^{-1}. This value was the so-called dynamic capacity at 50% break-through ($Q_{50\%}$) for the column [26,27]. The dynamic capacity of the prepared ATRP1 column was higher compared with silica-based weak cation exchanger [3].

3.4. Separation of nucleosides on the synthesized packings in HILIC mode

Hydrophilic interaction chromatography (HILIC) as a viable separation technique has found an increasing number of applications in analyzing a wide variety of polar compounds in various matrixes, such as foods, drugs and biological fluids [28–32]. HILIC is an alternative HPLC mode for separation of polar compounds with polar stationary phases as that in normal-phase liquid chromatography (NPLC), but aqueous mobile phases are similar to reversed-phase liquid chromatography (RPLC) [33]. In contrast to RPLC, the retention increases with increasing polarity of the stationary phase and decreasing polarity of the mobile phase and polar solutes are more strongly retained than non-polar ones.

Tetrazole containing heterocycles has strong dipole and strong ability of hydrogen bonding acceptor, so it may be used as HILIC separation medium. Several polar compounds were used to investigate the separation properties of ATRP1 column and the hydrolyzed $P_{GMA/EDMA}$ beads column in HILIC mode. As shown in Fig. 5(A), four nucleosides could be baseline separated in less than 12 min by using acetonitrile and aqueous ammonium formate solution as mobile phase on the ATRP1 column. Contrary to ATRP1 column, the four nucleosides were not separated completely on the hydrolyzed $P_{GMA/EDMA}$ beads column (Fig. 5(B)), and the retention times of nucleosides were longer than ATRP1 column. The elution order was the same as that observed on silica-based commercial HILIC column [34]. This demonstrates that the PVT-grafted packings were also used as HILIC separation medium, whose separation ability was better than the hydrolyzed $P_{GMA/EDMA}$ beads column in the HILIC mode.

3.5. Stability of the PVT-grafted column

The stability of the column is an important criterion for the evaluation of HPLC columns. The stability of the columns was investigated over a period of 4 months. Over 400 runs were carried out using the mobile phase varied from pH 5.0 to 8.5 during this period, the same proteins were found to be separated with the similar retention times as those obtained initially, indicating that the column efficiency was kept well during the successive use. Therefore, the PVT-grafted column had long-term stability for the separation of proteins and nucleosides.

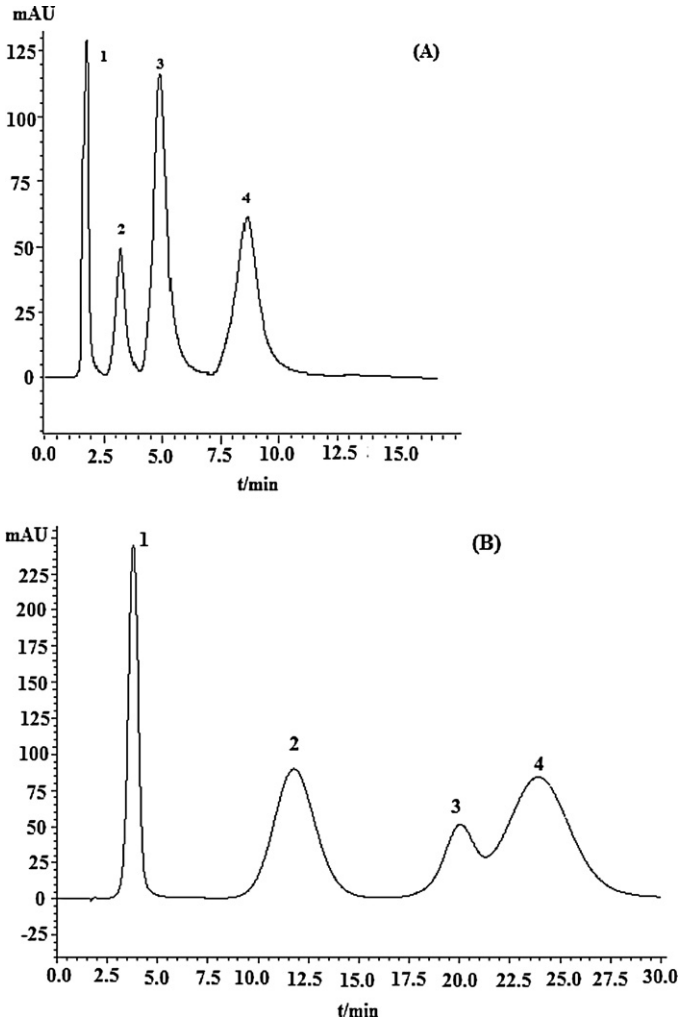

Fig. 5. Separation of four nucleosides on ATRP1 column and the hydrolyzed $P_{GMA/EDMA}$ beads column. Flow rate: 1.0 mL min^{-1}; Ambient temperature; Mobile phase: solution A, 10 mmol L^{-1} ammonium formate aqueous solution pH 6.5; solution B, acetonitrile (A:B = 5:95, V/V) 15 min isocratic; UV detection: 254 nm. Compounds: (1) solvent + uracil; (2) adenosine; (3) cytosine; and (4) cytidine.

3.6. Separation and purification of lysozyme from egg white by the the PVT-grafted column

There exist many proteins in the egg white, ovalbumin, ovomucoid, globulins and conalbumin are the major components occupying 54, 11, 10 and 13% of the whole egg white, respectively, while Lys is only a small portion of the egg white, about 3.5% [35]. Therefore, egg white is an ideal feed stock for the separation of multiprotein. In this study, the 10.0 cm × 0.46 cm I.D. PVT-grafted column (ATRP1) was used for the separation of Lys from the egg white by a single-step (Fig. 6). Egg white Lys has a p*I* of 11.0, it possesses net positive charge under the experiment conditions, and thus was retained on the column, whereas ovomucoid (p*I* 4.0), ovalbumin (p*I* 4.6), globulin (p*I* 5.5–5.8) and conalbumin (p*I* 6.6) possess net negative charge under the given conditions, so they were not retained on the synthesized column. In Fig. 6, the first band is miscellaneous proteins, the band marked by an asterisk (*) is Lys, which is compared to standard proteins. It can be seen from the chromatogram, Lys was completely separated from other miscellaneous proteins, and its purity is more than 95%.

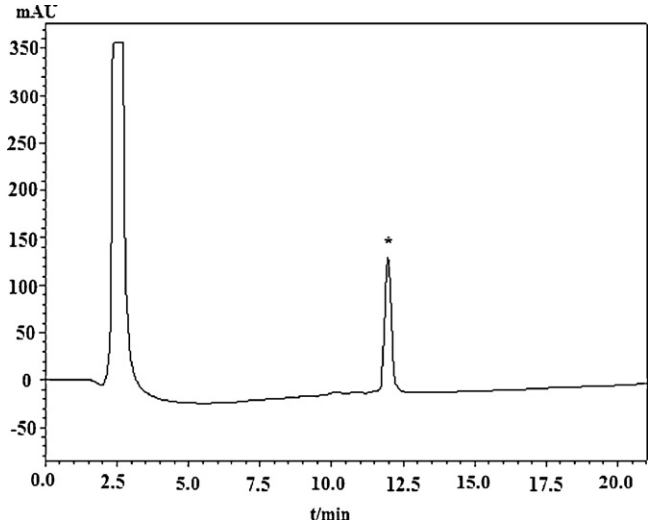

Fig. 6. Separation and purification of lysozyme from egg white by the PVT-grafted column. Column: (A) ATRP1. Column: 10 cm × 0.46 cm I.D. The linear gradient elution was from 100% solution A (20 mmol L^{-1} of phosphate, pH 7.0) to 100% solution B (20 mmol L^{-1} of phosphate + 1.0 mol L^{-1} NaCl, pH 7.0) for 20 min. Flow rate: 1.0 mL min^{-1}. AUFS: 0.08, UV detection at 280 nm. *Purified lysozyme.

4. Conclusion

A new method of SI-ATRP has been described to design PVT-grafted chromatographic packings based on monodisperse $P_{GMA/EDMA}$ beads. The different lengths of the PVT grafted chains on the surface of the $P_{GMA/EDMA}$ particles were prepared by changing the ratios of initiator/monomer in polymeric system. The different chain length of 5-VT grafted beads showed different chromatographic properties for separation of proteins in WCX mode. The PVT-grafted packings were developed for separation of nucleosides in HILIC mode. The dynamic protein loading capacity of the synthesized ATRP1 packing for Lys was up to 38.1 mg mL^{-1}, it has a higher column loading, a comparable the silica-based packings [3]. The PVT-grafted column was also used for purification of Lys from egg white with only one step to obtain a satisfactory results.

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