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Short communication

Porous ceramic/agarose composite adsorbents for fast protein liquid chromatography

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ABSTRACT

Porous ceramic/agarose composite adsorbents were designed and prepared with silica ceramic beads and 4% agarose gel, and then functionalized with a special ligand carboxymethyl. A novel method was introduced to fabricating of the porous silica ceramic beads. The morphology of SEM shows a spherical shape and a porous structure of the ceramic beads. Nitrogen adsorption–desorption analysis gives an average pore size of 287.5 Å, a BET surface area of 29.33 m^2/g and a porosity of 41.8%, respectively. Additionally, X-ray diffraction pattern indicates that the amorphous silica has been transformed into two crystal phases of quartz and cristobalite, leading to a porous and rigid skeleton and ensuring the application of the composite beads at high flow velocities. Lysozyme of hen egg-white with the activity of 12,700 U/mg was purified by the composite ion-exchanger in one step and the recovery and purification factor reaches 95.2% and 7.9, respectively.

1. Introduction

Fast protein liquid chromatography (FPLC) is a commonly used liquid chromatography for biomacromolecules in bioseparation process with low pressure and high capacity. The adsorbents, composed with gel matrix and functional ligand, are one of the most important aspects for the chromatography.

Polysaccharide gels such as agarose, cellulose and dextran are firstly and widely chosen as the materials of FPLC adsorbents to keep the bioactivity of target. The most well-known commercial one is Sepharose Fast-Flow introduced by Amersham Biosciences (now GE Healthcares) which is fabricated by crosslinked 6% or 4% agarose with a size of 45–165 μm. However, this kind of gel is non-rigid framework, so it was easy to be deformable at a relatively high flow velocity and more time of packing a column is needed for the low density.

Porous silica is the most popular mineral chromatographic solid phase for high performance liquid chromatography (HPLC) in a very long history [1], directly due to its high mechanical resistance, high surface area and porosity, adjustable polarity and easy surface modification [2]. It is usually prepared through sol–gel process described by Nawrocki et al. [3], and several methods [4–7] have been used to refine pore size and structure. However, following these methods, the silica beads are usually unstable in alkaline conditions [8] with a size of below 10 μm.

As reported in many literatures, the components being responsible for the interaction with the proteins to be separated had been polymerized into a porous shell made of polyacrylamide [9], celite [10], silica [11], mineral oxide [12]. The rigid skeleton provides mechanical strength and the insert gel provides adsorption sites. Therefore, these adsorbents for protein purification could be operated at extremely high velocity without loosing binding capacity.

Based on the virtue of the rigid framework of porous silica beads and the biocompatibility of polysaccharide gels, a porous silica ceramic/agarose composite adsorbent was prepared for biomacromolecules chromatography. To avoid the limitations in sol–gel method, the porous silica beads were fabricated by sintering from conglutinated nanometer silica powders to be a kind of ceramic. An ingenious method for agarose gel composite was presented. In addition, carboxymethyl (CM) was coupled onto this composite beads and the chromatographic performance was briefly evaluated.

2. Experimental

2.1. Materials

Silica powder with a mean particle diameter of 500 nm was ordered from Wanjing New Material Co., Ltd. (Hangzhou, China).

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High Vacuum oil HFV-100 was purchased from Huifeng Petroleum & Chemical Co., Ltd. (Shanghai, China). Polyethylene glycol 10000 and sodium alginate were from Sinopharm Chemical Regent Co., Ltd. (Shanghai, China). Fresh eggs were from local market. Other reagents were of analytical reagent grade from the local store.

2.2. Preparation of porous ceramic/agarose composite adsorbents

2.2.1. Porous silica ceramic beads

The preparation process is shown in Fig. 1. Generally, a mixture of 2.4% (w/v) sodium alginate, 0.5% (w/v) polyethylene glycol 10000 and 20% (w/v) SiO_2 powder in a 250 mL beaker was heated to boil in a microwave oven and gently stired by a magnetic stirrer. The suspension was then dispersed in 250 mL vacuum oil phase with the agitation by the method of water-in-oil suspension. Then the mixed beads were solidified by adding saturated calcium chloride solution. The beads were washed with ethanol and water for five times, and dried at 60 °C in a drying oven overnight. After that, the dried beads were sintered at 1000 °C for 1 h to form the porous silica ceramic beads. For the heating rate, 0–500 °C, 10 °C/min; 500–800 °C, 5 °C/min; 800 to 1000 °C, 2 °C/min.

2.2.2. Compositing with agarose gel

The 4% agarose gel was filled into the pore space and onto the surface of silica ceramic beads as shown in Fig. 1. An agarose solution was obtained by dispersing 2 g of agarose powder in 50 mL deionized water by heating to boil in a microwave oven. Such a solution was transferred into a 200 mL beaker and then mixed with 20 g porous silica beads obtained as above-mentioned way. The mixture was kept at 105 °C in an autoclave for 1 h, latter progressively cooled down to room temperature. Finally, the sedimentary beads were mildly grinded with water in a mortar, and the superfluous gels were removed by elutriation in a fluidized bed. By screened with standard sieves, the composite beads with a particle size range of 50–75 μm were obtained.

2.2.3. Coupling with carboxymethyl to be ion-exchanger

The composite beads were ideal support for liquid chromatography by chemical modifying in classical ways. The beads were cross-linked with glycol diglycidyl ether and epichlorohydrin as reported by Pernemalm et al. [13]. Then, carboxymethyl (CM) groups suitable for cation exchanger were coupled to the composite beads as reported by Ghetie et al. [14].

2.3. Measurement of physical properties

The shapes and structures of porous ceramic particles were observed by an environmental scanning electron microscope (ESEM), Quanta-200 (FEI, The Netherlands). Pore properties including specific surface area, pore volume and pore size distribution were determined by nitrogen adsorption–desorption analysis on a Micromeritics ASAP-2020 apparatus (Micromeritics, USA). X-ray diffraction (XRD) patterns were taken on a D 8 advance X-ray diffractometer (Bruker AXS, Germany) using Cu Kα radiation. The size range and average size of composite beads were determined with the laser particle size analyzer, Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Chromatographic performance, expressed as the height equivalent to a theoretical plate (*HETP*) and back pressure in column, determined by the ÄKTA Purifier system (GE Healthcare, USA). The *HETP* calculated by the half-peak height method and acetone as tracers was determined as follows:

$$HETP = \frac{L}{N} \quad (1)$$

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \quad (2)$$

where H is the bed height, N is the theoretical plates number, t_R is the residence time and $W_{1/2}$ is the half peak width of *RTD* response.

2.4. Purification of lysozyme with composite ion-exchanger

The sample of lysozyme of hen egg-white was prepared by mixing egg white and equivoluminal 20 mM disodium hydrogen phosphate–citric acid buffer (pH 6.0). The chromatographic process was conducted with ÄKTA purifier system from GE Healthcares (Uppsala, Sweden). 3.5 mL adsorbents was packed in a glass column (20 cm × 1 cm). The flow rate was 1 mL/min, the injected sample was 0.5 mL and 10 mM disodium hydrogen phosphate–citric acid (pH 6.0) was used as the start buffer. The elution was performed using a linear gradient from 0 to 1 M sodium chloride in 8 CV. Lysozyme activity was determined by shugar method as described by Stellmach [15]. The protein was analyzed by SDS-PAGE following the method of Laemmli [16] and Coomassie brilliant blue R-250 staining method [17].

3. Results and discussion

3.1. Fabrication of porous silica ceramic/agarose composite beads

Combination of ceramic pulp with silica powder, sodium alginate, PEG 10000 and water is the first and important work for preparing the spherical porous silica particles. Sodium alginate, a sodium salt of polyuronic acid containing (1 → 4)-linked β-D-mannuronic acid and α-L-guluronic acid [18], is selected as binder for the formation of insoluble hydrogel by chelate bonds when contacted with calcium and multivalent cations [19]. PEG with long chains and good water-solubility at low temperature [20] could increase the viscosity of ceramic pulp and help to enhance the porosity of silica beads. Typically, for one batch of elemental silica particles prepared by the method of oil emulsion and crosslinked with calcium chloride which is shown in step 1 in Fig. 1, about 40 mL elemental silica particles are obtained, equal to about 80% of total material added.

In step 2, the water and the organics of alginate and PEG 10000 in mixed silica particles are eliminated at 60 and 600 °C, respectively. And then at about 1000 °C, the types of silica crystalloid is changed and the porous silica ceramic beads are obtained. Fig. 2 (SEM images of a–d) gives the SEM images of ceramic beads. The beads show good sphericity and smooth surfaces with a diameter below 75 μm. Mesopores distribute densely and irregularly and form a network in the beads. However, due to the remove of water and organics and the transformation of silica crystal form, the shrinkage of beads accompanies with the sintering procedure and about 25% of volume decreases at last.

For the composite process in step 3, agarose gel should be filled uniformly into porous silica ceramic beads. Moreover, the gel outside of the beads should be controlled in a thin layer to keep the rigid structure in the column. An autoclave is used to provide a high temperature and pressure for composite of agarose gel and porous beads. Based on the high mechanical strength of the ceramic beads, the particle shape of composite beads is recovered by an ingenious polish method of mildly grinded in a mortar. As show in Fig. 2e and f reveal the agarose layer on the composite beads. The thickness on most silica ceramic beads lies between 0.5 and 1 μm, but regions with much thinner coverage or no apparent cover.

Finally, the porous silica ceramic/agarose composite adsorbents are obtained by functionalizing the composite beads with CM.

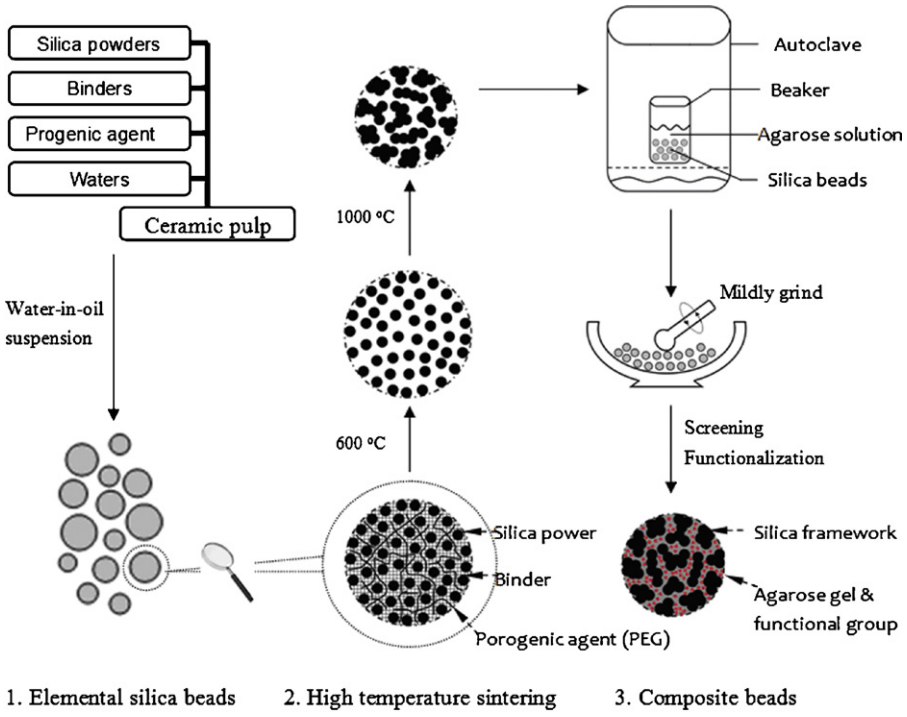

Fig. 1. Preparation of porous ceramic/agarose composite adsorbents.

3.2. XRD analysis of silica ceramic beads

The XRD patterns of the silica beads obtained by sintering at different temperatures are shown in Fig. 3. Judging from their X-ray diffraction bread-shape peak curve, silica beads sintered at 500 and 800 °C are amorphous, but silica beads sintered at 1000 °C exhibit various diffraction peaks with two crystal phases of quartz and cristobalite. The result is similar to the literatures [21,22] that amorphous silica could be transformed into cristobalite phase by heating at 900–1300 °C. Generally, the silica evolves into cristobalite between 1470 and 1700 °C up to the melting point [23]. Whereas, the Ostwald's rule of stages [24] can be used to explain the phenomenon that the amorphous silica could be changed into cristobalite at a low temperature. During the amorphous silica evolving into thermodynamically stable phase (tridymite and cristobalite), the cristobalite low phase emerges firstly and then changes into quartz and tridymite [25]. Yet, the tridymite does not exist without adscititious chemical composition [26]. So, the silica crystal forms are only available in quartz and cristobalite at normal atmosphere. The used silica powders obtained by sol–gel method at about 100 °C contain abundant unlinked hydroxyl (—Si—OH) [27] which also contributes to reduce the temperature for forming cristobalite phase [28]. In addition, the small mean size of the silica powders increases surface energy, which benefits for the transformation process. The crystallographic transformation definitely improves the strength and physical and chemical properties of ultimate porous silica beads.

3.3. Physical and chemical properties

3.3.1. Pore properties of silica ceramic beads

Several important parameters including specific surface area, pore volume, porosity, mean pore diameter and pore size distributions calculated by nitrogen adsorption–desorption isotherms are used to characterize the pore properties of porous silica beads. The nitrogen adsorption–desorption isotherms and the corresponding BJH (Barrett–Joyner–Halenda model with Halsey equation) [29] pore size distribution curves of the silica beads obtained at 1000 °C are shown in Fig. 4. The sample exhibits type III isotherm with a hysteresis loop, and its corresponding pore size distribution curves shows a single peak at about 800 Å. As calculated by Halsey equation, the average pore diameter is 287.5 Å. In addition, the silica ceramic beads indicate excellent porous structure with a BET (Brunauer–Emmett–Teller) surface area of 29.33 m^2/g, a pore volume of 0.246 cm^3/g and a porosity of 41.8%. The results demonstrate that the silica beads have a high porosity and large pore size which benefit for chromatographic performance.

3.3.2. Composite ion-exchanger

The physical and chemical properties of composite ion-exchanger are also measured in this work and the results are shown in Table 1. When compositing porous silica ceramics with 4% agarose gel, the wet density of composite particles reaches 1.70 g/mL, obviously larger than traditional agarose adsorbents such as Sepharose series. The high density could enhance the settling velocity, which is in favour of column packing process. The size range is controlled in a narrow range of 48.6–76.3 μm by screening with standard sieves and the average particle size is about 64.1 μm. Because of the 58.2% volume taken up by the ceramic skeleton, the total ionic capacity achieves only 60 μmol/mL after conventional activation and coupling. This would be resolved in our later work by using a new coupling method for a higher capacity. Most of all, the rigid framework results in a lower back pressure and a high *HETP* in column at high flow velocity. At the flow velocity of 770 cm/h, the data of back pressure and *HETP* are 0.18 MPa and 0.50 mm, respectively. In an additional investigation on flow hydrodynamic, the back pressure in the column was 0.06, 0.08, 0.11, 0.13, 0.16, and 0.18 MPa at flow rate of 385, 462, 539, 616, 693, and 770 cm/h (fitting equation: $y = 0.0003x - 0.0643$, $R^2 = 0.997$), respectively. The fitted line with an intercept of −0.0643 does not cross the origin of coordinate axis, which means a little compression has been already occurred upon the thin outer agarose layer at the flow rate below 385 cm/h. However, the straight line relationship between back pressure and flow rate clearly demonstrates that almost none

Table 1
Physical and chemical properties of composite ion-exchanger.

BET surface area,[c] m^2/g	Pore volume,[c] cm^3/g	Porosity[c]	Average pore diameter,[c] Å	Wet density, g/mL	Size range, μm	Average particle size, μm	Total ionic capacity, μmol/mL	Back pressure, MPa	HETP, mm
29.33	0.246	41.8%	287.5	1.70	48.6–76.3	64.1	60	0.06[a] 0.18[b]	0.41[a] 0.50[b]

[a] The flow velocity used in this experiment: 385 cm/h.
[b] The flow velocity used in this experiment: 770 cm/h.
[c] The data determined with porous silica ceramics beads.

of compression is shown in the porous silica beads at the high velocity of 385–770 cm/h. That means, the weak compression of thin agarose layer is unable to influence the performance, and the composite beads could still operate at high velocity with low back pressure. These results indicate that the composite adsorbents are suitable for fast chromatography.

3.4. Purification of lysozyme with composite ion-exchanger

Lysozyme of hen egg-white is selected to determine the function of composite ion-exchange. Lysozyme with molecule of 14.4 kDa and *pI* of 11.2 is a kind of hydrolase which acts on cell wall of microbes. Due to the main proteins in egg-white are acidic proteins whose *pI* is almost below 5 (Ovalbumin, 4.6–4.7; α-ovomucoid, 3.8–4.4), one step purification procedure is designed in this work. As shown in Fig. 5, most proteins are broken through the column at pH of 6.0, while lysozyme is eluted in a single peak. The image of SDS-PAGE indicates that a high purity of lysozyme could be obtained after eluting at 0.4–0.8 M NaCl. At last, the activity of purified lysozyme reaches 12,700 U/mg, the data of recovery and purification factor are 95.2% and 7.9, respectively.

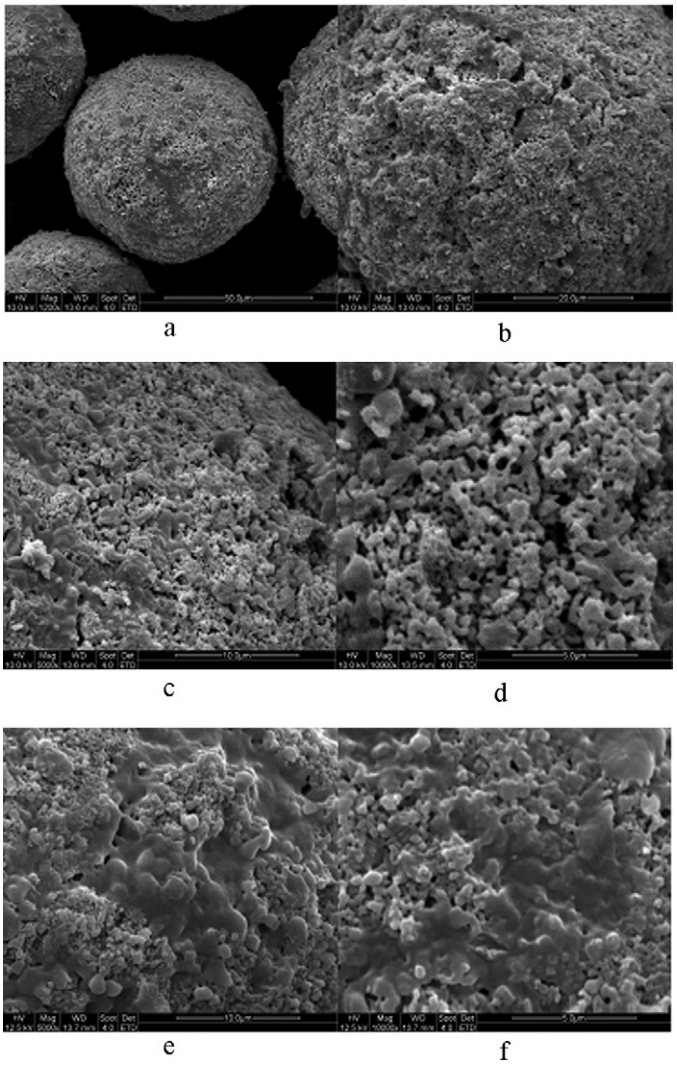

Fig. 2. SEM images of porous silica ceramic beads.

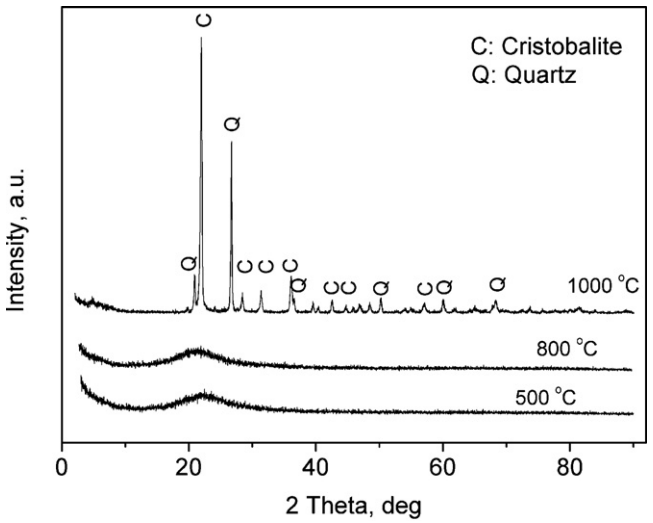

Fig. 3. XRD patterns of the silica beads obtained by sintering at different temperatures.

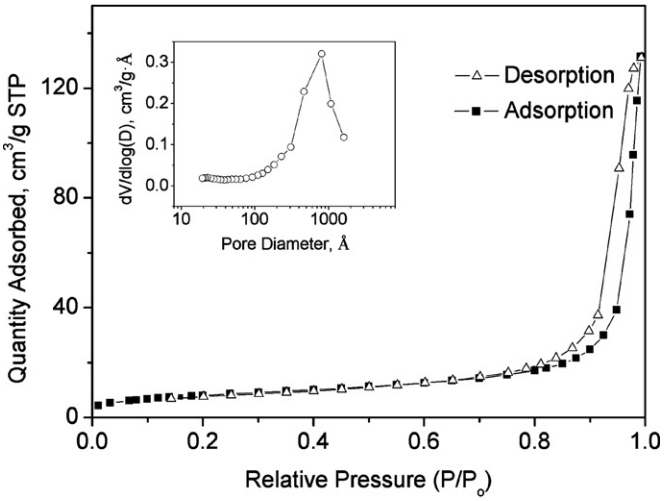

Fig. 4. Nitrogen adsorption–desorption isotherm of silica ceramic beads. The corresponding pore size distribution (inset) was obtained from the desorption branch of the isotherm using the Barrett–Joyner–Halenda (BJH) method.

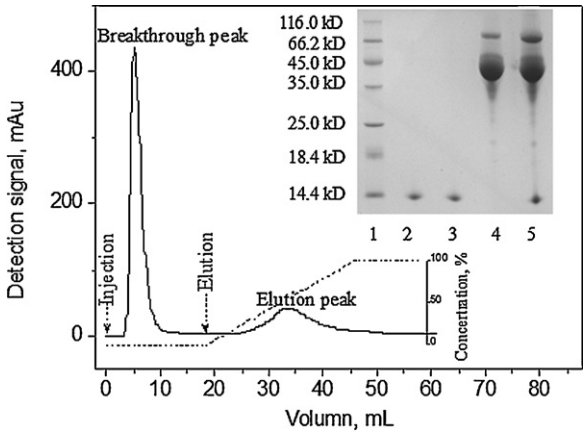

Fig. 5. Chromatographic purification of lysozyme and SDS-PAGE analysis of the peaks. Lanes: 1, marker; 2, standard lysozyme; 3, elution; 4, breakthrough; 5, sample.

4. Conclusion

In this study, porous ceramic/agarose composite adsorbents were designed and prepared through three steps, fabrication of porous silica ceramic beads from nano silica powders, compositing the porous beads with agarose gel and functionalizing the composite particles with a special ligand. A novel method using sodium alginate as binder and PEG as porogenic agent was introduced in this work. The morphology, pore property and physical properties were indicated by X-ray diffraction, scanning electron microscopy and nitrogen adsorption–desorption analysis, etc. The application of composite beads functionalized by cation-exchange ligand CM for purification of lysozyme of hen egg-white shown that the porous ceramic/agarose composite adsorbents could be suitable for FPLC.

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