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Evaluation of hydroxyapatite microspheres made from a borate glass to separate protein mixtures

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Abstract A hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), transformed from a calcium-containing borate glass, has been investigated for its protein adsorption and chromatographic characteristics. Microspheres of the borate glass were transformed into HA by reacting them with a 0.25 M phosphate (K_2HPO_4) solution for 24 h at 37 °C (pH 9.0). The HA microspheres with a diameter of 45–90 μm were hand packed into a steel column (4.6 mm × 80 mm) and used to separate a binary protein mixture of bovine serum albumin (BSA) and lysozyme. HA microspheres, with a diameter <45 μm, were used for separating a protein mixture of BSA, myoglobin, and lysozyme. These microspheres had a diameter that was 20–30 times larger than commercial HA column packing spherical particles, 2–3 μm, but these microspheres had a six times larger surface area and a more uniform spherical shape. These advantages compensated for their larger size and the separation results were comparable to those commercially available HA columns in the separation of the proteins studied. These unique HA microspheres, made from microspheres of a borate glass, are considered to be useful as packing materials for protein separation in chromatography.

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Introduction

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is a major component of human bones. It belongs to the hexagonal system, with a space group, $P6_3/m$. This space group is characterized by a sixfold *c*-axis perpendicular to three equivalent axes at angles 120° to each other. The apatite structure is very hospitable, allowing substitutions of many other ions, such as $(CO_3)^{2-}$ and F^-. The surface chemistry of HA depends on the composition and pH of the solution in the environment. An acid environment will cause partial dissolution of the surface, enriching the population of Ca^{2+}, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, H^+, OH^- and ion pairs such as $CaH_2PO_4^+$ and $CaOH^+$ in a hydrated layer [1].

HA is most well known as an important biomaterial due to its biocompatibility and ability to bond to bone [2]. HA also finds applications in drug delivery [3, 4], ion filtration [5], medical imaging [6, 7], and protein separation and purification [8].

HA can adsorb proteins present in body fluids. It has excellent bioactivity and biocompatibility, provides attachment sites for bone cells, and can induce and activate osteoblasts at the interface between body fluids and HA [9, 10]. The mechanism of protein adsorption and desorption on HA is very useful for protein separation and purification in liquid chromatography.

The concept of using HA as a column packing material for liquid chromatography was first introduced by Tiselius in 1956 [11], and systematic studies had been undertaken by Bernardi [12–14]. With the development of high performance liquid chromatography (HPLC), HA columns have become commercially available and been successfully applied to a wide range of protein and nucleic acid separation, but some problems remain to be solved. For example, the resolution is not as high as that obtained by other modes of HPLC [8].

The primary objective of this study was to conduct a preliminary performance evaluation of protein adsorption and separation using HA microspheres that have been produced by transforming microspheres of a calcium-containing borate glass. The transformed HA microspheres have a large surface area and uniform spherical shape, which should be advantages compared to commercial HA packing particles. Microspheres of the borate glass were transformed into HA by reacting them with a 0.25 M phosphate solution (K_2HPO_4). Two protein samples, a binary and a ternary mixture, were used in the HPLC experiments.

Experimental

Glass preparation and transformation

Two calcium sodium borate glasses were made whose molar ratio composition is $1CaO \cdot 2Na_2O \cdot 6B_2O_3$ and $2CaO \cdot 2Na_2O \cdot 6B_2O_3$. These glasses are hereafter designated as 1-2-6 and 2-2-6 glass, respectively. The weight and mole percent composition of each are shown in Table 1. The glasses were made by melting a homogeneous mixture of reagent grade calcium carbonate ($CaCO_3$), sodium carbonate (Na_2CO_3) and boric acid (H_3BO_3) powders at 1,000 °C for 1 h and a platinum crucible was used to avoid contamination.

Each melt was quenched into a thin plate between two cold stainless steel plates to prevent crystallization. The quenched glass was crushed to irregular particles with a steel mortar and pestle and sieved to size $\leq$90 μm. Part of the irregular glass particles were passed through a propane flame, where they melted, became spherical and were collected in a stainless steel cylinder. The collected microspheres were sieved into two sizes, microspheres between 45 and 90 μm, and microspheres smaller than 45 μm.

Both irregular glass particles and microspheres were reacted in a 2 liter beaker filled with a 0.25 M phosphate (K_2HPO_4) solution (pH 9.0 ± 0.05) at 37 °C to form HA. The beaker was placed onto a heated sand bath while the solution was stirred vigorously to prevent the particles or microspheres from clumping together or settling. After 24 h at 37 ± 1 °C, the phosphate solution was decanted and the transformed glass particles or microspheres were rinsed first with distilled water and then with alcohol followed by drying in air at room temperature for 12 h before being stored in a desiccator.

Table 1 1-2-6 and 2-2-6 glass weight and mole percent composition (%)

Glass	Weight percent			Mole percent		
	CaO	Na_2O	B_2O_3	CaO	Na_2O	B_2O_3
1-2-6	9.3	20.7	70.0	11.1	22.2	66.7
2-2-6	17.1	18.9	64.0	20.0	20.0	60.0

Protein adsorption

Chicken egg lysozyme (Sigma Co., Cat. #L-6876) was used to evaluate the protein adsorption of the reacted glass particles and microspheres. Reacted irregular particles of the 1-2-6 and 2-2-6 glass and microspheres of the 2-2-6 glass in the size range of 45–90 μm, were used for the lysozyme adsorption experiment. The adsorption experiments were performed at room temperature. Three 10 mL glass vials were filled with 5 mL of the lysozyme solution (0.5 mg/mL). About 0.2 g of the transformed 1-2-6 glass particles, 2-2-6 glass particles and 2-2-6 glass microspheres were added to each vial, respectively, after which the glass vials were capped. After 16 h, 0.5 mL of the upper clear solution was removed from each vial. Its absorbance was measured and converted to lysozyme concentration.

The surface area of the 45–90 μm transformed 1-2-6 and 2-2-6 glass particles and 2-2-6 glass microspheres was measured by the Braunauer–Emmett–Teller (BET) procedure. As-reacted glass particles/microspheres were outgassed for 6 h at 125 °C to remove all adsorbed water before the specific surface area was measured.

Protein separation

Elution chromatography was carried out using a phosphate eluent on a sequence-programmable HP series 1050 HPLC instrument. A 5 μL sample of the protein mixture was injected into the HPLC system and the sample elution was monitored by measuring the UV absorption at a wavelength of 280 nm. The chromatograms were recorded with an automatic HP 3396 series 2 integrator.

Protein mixture sample

The proteins used in the present work were bovine serum albumin (BSA, Sigma Co., Cat. #A-7906), chicken egg lysozyme (Sigma Co., Cat. #L-6876) and horse heart myoglobin (Nutrational Biochemical Co., Cat. #2920).

A binary protein mixture of BSA and lysozyme and a ternary protein mixture of BSA, lysozyme and myoglobin were prepared by dissolving approximately equal amounts of protein in deionized water. The initial concentration of each protein was 10 mg/mL (stock solution) which was diluted to 0.5 mg/mL (working solution).

Eluent (solvent) preparation

Phosphate solutions were made of equal mole percent of Na_2HPO_4 and NaH_2PO_4 at molarities of 0.01 (A), 0.1 (B), and 0.5 M (C) by dissolving appropriate amounts of phosphates in deionized water. For the binary protein mixture separation, eluents were 0.01 M (A) phosphate and 0.1 M (B) phosphate solutions. Ternary protein mixture elution was done with 0.01 M (A) and 0.5 M (C) phosphate solutions. All solutions were filtered through a millipore filter (0.45 μm) under vacuum to remove gases as well as suspended matter. The pH of each phosphate solution was 6.8 ± 0.1.

Column packing

Transformed 2-2-6 microspheres (45–90 μm) were packed into a steel column used for the binary protein mixture separation and microspheres smaller than 45 μm were used for the ternary protein mixture separation. The HA microspheres (45–90 μm) shown in Fig. 1 are typical of those used for column packing. Figure 2 shows the appearance of the external surface of the HA microspheres, which was composed of nano-sized (30–50 nm) grains of HA.

A steel column, with an inside diameter of 4.6 mm and a length of 80 mm, was packed by hand with the transformed microspheres. A metal filter was placed at each end of the column to avoid contamination. Both the column and the filters were washed in an ultrasonic water bath before packing. Approximately 1.5 g of dry transformed microspheres were poured into the column using a small funnel. The side wall of the column was tapped gently during filling to help the microspheres pack evenly.

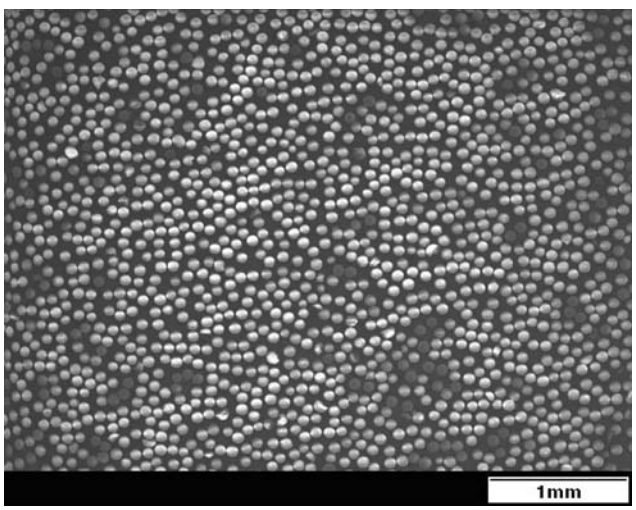

Fig. 1 SEM image of the transformed microspheres (45–90 μm) used in HPLC column

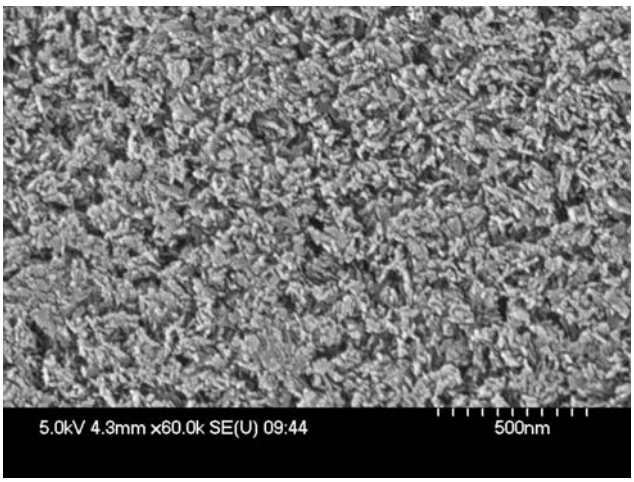

Fig. 2 The appearance of the external surface of the transformed HA microsphere (45–90 μm) used in HPLC column

Elution procedure

The eluent phosphate concentration was controlled by mixing the two phosphate solutions, A and B for the binary protein mixture and A and C for the ternary protein mixture separation. The volume percent of, A + B or A + C, was always 100% at any time. For example, eluent of 30% B solution means that 30% of the B solution and 70% of the A solution, in volume percent, were mixed together before entering the HPLC tubing system.

The packed column was connected to the HPLC system and washed overnight with deionized water at a flow rate of only 0.01 mL/min. This was done to avoid uneven distribution of the microspheres in the column, which could cause local blocking and consequently loss of pressure control. The eluent was then changed to the initial phosphate concentration used for each elution and the flow rate was slowly increased to 1.0 mL/min in steps of 0.1 mL/20 min. When the flow rate reached 1.0 mL/min (at 200 min), the system was ready to receive the protein mixture sample. The system pressure was maintained at around 700 ± 30 psi for the column packed with 45–90 μm microspheres and at 1000 ± 50 psi for the column packed with ≤45 μm microspheres.

Results

Protein adsorption

The purpose of the protein adsorption experiment was to determine if the HA transformed from the borate glass can adsorb protein and if there was a difference in the adsorption for irregular particles and microspheres

(dependence on shape). Lysozyme was used in the adsorption experiment as it is a common protein.

The results of the lysozyme adsorption experiments with HA are given in Table 2 for irregular particles and microspheres. As shown by the percent concentration change in Table 2, the 2-2-6 microspheres adsorbed more lysozyme than irregular particles of either glass even though the particles and microspheres were in the same size range. This can be explained, at least partly, by the specific surface areas listed at the bottom line in Table 2. The specific surface area of the HA microspheres transformed from the 2-2-6 glass was three times larger than that of the irregular particles. The larger specific surface area made more interaction possible between the lysozyme and the transformed 2-2-6 microspheres, and thus the microspheres had a larger adsorption ability. The surface inaccessibility may be the reason why the increase in capacity is not proportional to the large increase in surface area. The spheridization, heat treatment, is a likely explanation for why the microspheres had a three times larger surface area than the irregular particles, but the exact aspect of the spheridization process that maybe responsible for the higher surface area is not known. It is speculated that the smooth spherical surface of glass microspheres favors nano-sized grain formation of HA in the presence of the phosphate solution while the rough surface on irregular glass particles favors larger grain formation. The nano-sized grain may be responsible for the surface area increase.

Protein separation

Binary protein mixture separation

Figure 3 shows the isocratic elution chromatograms for the binary mixture of BSA and lysozyme at a flow rate of

Table 2 Lysozyme adsorption to the transformed 1-2-6, 2-2-6 irregular glass particles and 2-2-6 glass microspheres in the size range of 45–90 μm and their corresponding specific surface areas

HA transformed	1-2-6 particles	2-2-6 particles	2-2-6 microspheres
Starting lysozyme concentration (mg/mL, ±0.02)	0.5	0.5	0.5
Final lysozyme concentration after adsorption (mg/mL, ±0.02)	0.27	0.26	0.20
Concentration change (mg/mL, ±0.02)	0.23	0.24	0.30
Percent concentration change	46%	48%	60%
Specific surface (m^2/g)	52.3	68.7	190.4

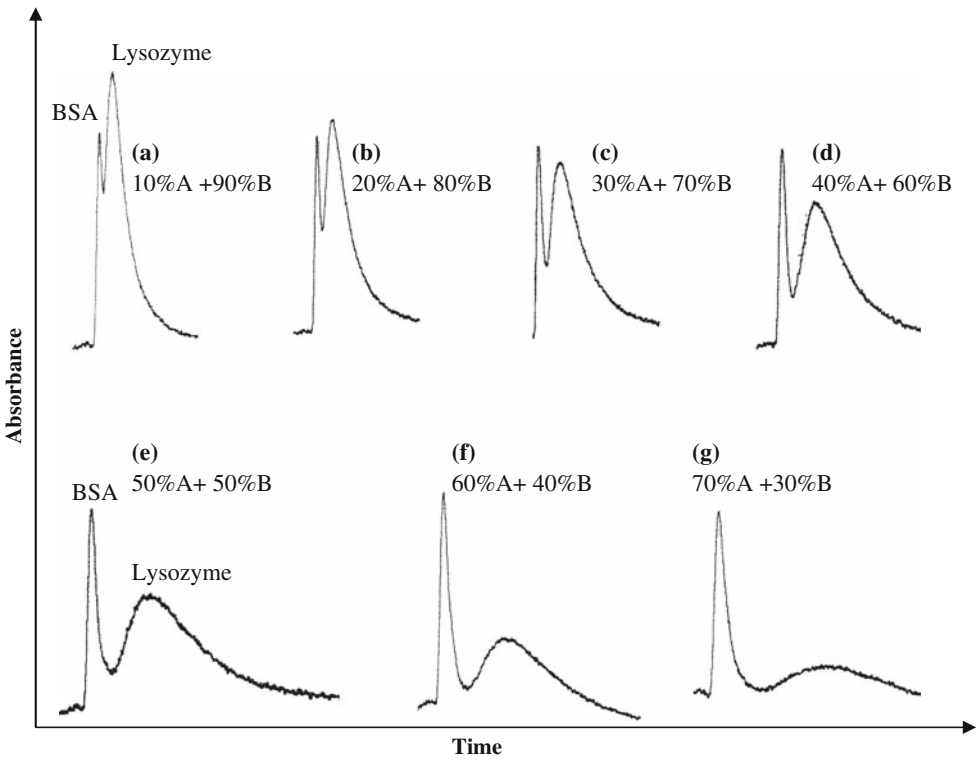

Fig. 3 Isocratic elution chromatograms for the binary protein mixture, composed of equal parts of BSA and lysozyme, 0.5 mg/mL, dissolved in deionized water, using a HA column packed with the transformed 2-2-6 glass microspheres (45–90 μm). Eluent phosphate compositions are as shown. The 0.01 M phosphate solution A and 0.1 M phosphate solution B were made of equal moles of Na_2HPO_4 and NaH_2PO_4

Table 3 Retention times, selectivity factors and resolutions of the BSA and lysozyme peak for the isocratic elution of the binary protein mixture (fixed volume percent of a 0.01 M phosphate solution (A) and a 0.1 M phosphate solution (B))

B Solution percent	90%	80%	70%	60%	50%	40%	30%
BSA retention time (min)	0.81	0.80	0.80	0.81	0.83	0.90	0.94
Lysozyme retention time (min)	1.18	1.23	1.36	1.53	1.84	2.47	–
Selectivity factor	3.47	4.07	5.00	5.80	6.94	7.54	–
Resolution	0.52	0.54	0.58	0.64	0.71	0.76	–

1.0 mL/min. The chromatograms are arranged in order of the descending phosphate concentration in the eluent.

At the high phosphate concentration end (high percentage of B solution), Fig. 3a, two sharp peaks are evident, but they overlap each other significantly. As the eluent phosphate concentration was lowered, namely a decrease in the percentage of the B solution, the two peaks became more separated and distinct as indicated by the increase in selectivity factors and resolutions between the peaks listed in Table 3.

In all chromatograms, the BSA peak was always sharper than the peak for lysozyme. However, both peaks became broader at lower phosphate concentrations, especially the peak for lysozyme. The peak for BSA remained sharp down to 30% B solution, whereas, the lysozyme peak became broader and at 30% B solution, the lysozyme peak was barely detectable. The small, broad peak in Fig. 3g indicated that the eluent phosphate concentration was too low to remove the lysozyme from the HA microspheres. When the B solution changed from 90 to 40%, the retention time for lysozyme changed by 1 min compared with only 0.09 min for BSA. It was clear that the phosphate concentration had a greater effect on the lysozyme peak than on the BSA peak.

On the basis of resolutions, the best results to date are shown in Fig. 3e and f, but the separation was still not from the baseline. The information from these chromatograms in Fig. 3 was the basis for gradient elution experiments, which often enhance separation performance. BSA and lysozyme have different sensitivity to the change of phosphate concentration in the eluent, which made gradient elution possible.

On the basis of the results for the isocratic elution, a gradient elution was designed. According to Fig. 3, an initial phosphate concentration of 25–30% B solution seemed to have the potential to make a complete separation—a lower phosphate concentration can be used to first remove BSA and then increase the phosphate concentration to remove lysozyme from the column.

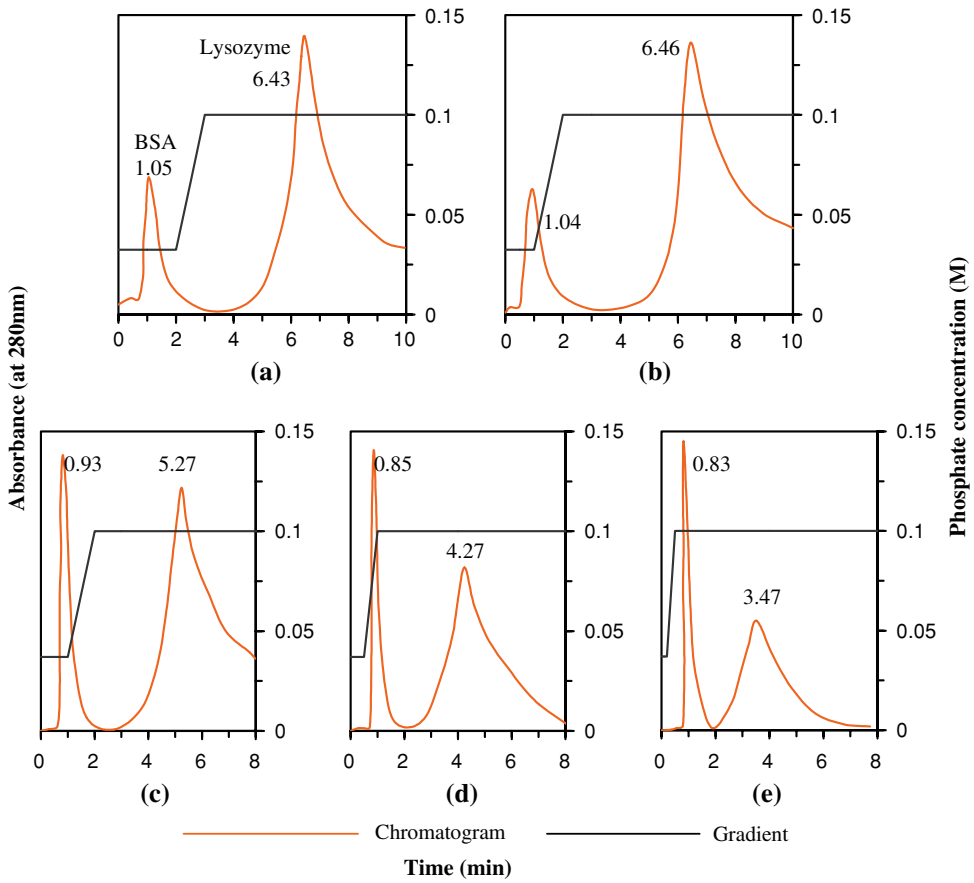

Fig. 4 Gradient elution chromatograms for the binary protein mixture, BSA and lysozyme, gradients shown in black. (**a**) Gradient 1, (**b**) Gradient 2, (**c**) Gradient 3, (**d**) Gradient 4, and (**e**) Gradient 5

Table 4 Selectivity factors and resolutions between the BSA and lysozyme peak for the gradient elution of the binary protein mixture

Gradient no.	Selectivity factor	Resolution
Gradient 1	14.8	2.5
Gradient 2	15.3	2.6
Gradient 3	17.1	2.3
Gradient 4	19.0	1.8
Gradient 5	16.5	1.6

The five gradients in Fig. 4 were tested. As shown in Fig. 4a, gradient 1, beginning with the 25% B solution for 2 min and then increasing the concentration of B solution linearly from 25 to 100% from 2 to 3 min, completely separated BSA and lysozyme with a resolution of 2.5.

To increase column performance further, experiments were carried out using gradient 2, 3, 4 and 5, by either changing the initial phosphate concentration or accelerating the increase in concentration, or both. The corresponding chromatograms are shown in Fig. 4b–e. Table 4 lists the corresponding selectivity factors and resolutions between the two peaks for each gradient elution.

The gradient elution results demonstrated that the binary protein mixture, BSA and lysozyme, could be separated by the HA column in about 6 min with a resolution of 1.6.

When the flow rate was increased to 1.5 mL/min, both BSA and lysozyme came out of the column faster than at 1.0 mL/min flow rate. At the same time, as shown in Fig. 5, the 2 components were separated within 5 min. This indicates that a higher flow rate improves the performance

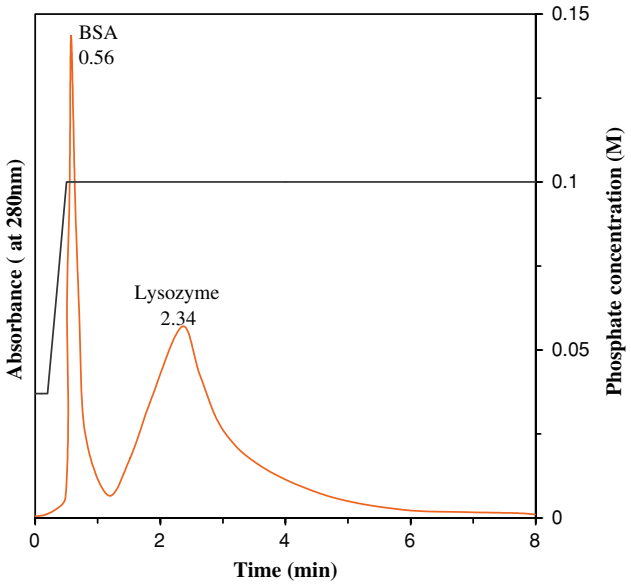

Fig. 5 Chromatogram of the binary protein mixture, BSA and lysozyme. The elution was done using gradient 5 at a flow rate of 1.5 mL/min

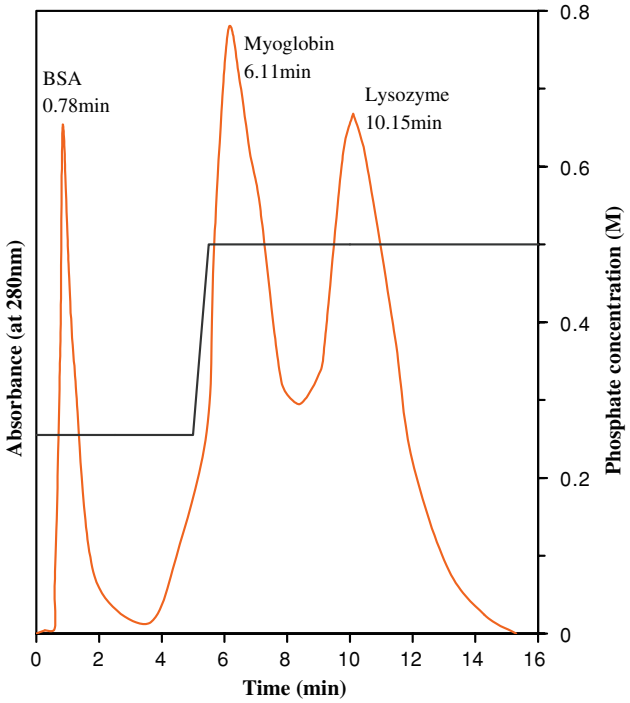

Fig. 6 Chromatogram of the ternary protein mixture, BSA, lysozyme and myoglobin at a flow rate of 1.0 mL/min

of the HA column while maintaining a complete separation.

Ternary protein mixture separation

The chromatogram for the ternary protein mixture is shown in Fig. 6. Similar to the binary mixture, BSA exited the column first, followed by myoglobin and then lysozyme. With a microsphere diameter smaller than 45 μm, the retention time for lysozyme increased to more than 10 min. However, the myoglobin and lysozyme peaks were not completely separated. The increase in lysozyme retention time as the microsphere diameter decreased made it possible for multiple protein separation. In this case, the time between BSA and lysozyme was filled by the peak for myoglobin.

Discussion

Separation mechanism

The HA crystal unit cell is characterized by the following crystallographic parameters: $a = b = 942$ pm, $c = 688$ pm, $\alpha = \beta = 90°$, $\gamma = 120°$. It can be deduced from the crystal structure that two main types of surface appear on a HA crystal. One is called the *a* or *b* surface, parallel to the *bc* or *ac*

plane, where positively charged adsorbing sites, C, each formed by two calcium ions, are arranged in a rectangle manner with an interdistance in the *a* and *b* directions of 942 and 344 pm in the *c* direction, half the *c*-axis unit cell length. The other is called the *c* surface, parallel to the *ab* surface, where negatively charged adsorbing sites, P, each formed by six oxygen ions belonging to three phosphate ions (PO_4^{3-}), are arranged in a hexagonal manner with the minimal interdistance in both the *a* and *b* direction of 942 pm [15]. Thus, the surface of HA presents a mosaic of positive and negative sites.

Adsorption of proteins to HA results primarily from nonspecific electrostatic interactions. In the process of HA chromatography, competition occurs between acidic proteins and anions and between basic proteins and cations for adsorption onto the C and P sites, respectively [15, 16]. BSA has an isoeletric point of 4.7, which is negatively charged at a pH of 6.8 and interacts primarily with the C sites. It comes off the column at a low phosphate molarity. For basic proteins, like lysozyme, the higher the isoelectric point of a protein, the stronger its adsorption onto HA because of the stronger binding between the positive charge of the proteins and P sites so a high phosphate molarity is needed to remove them from the HA column.

Comparison with commercially available HA columns

Kadoya et al. [17] studied a commercially available HA column (8.0 mm i.d. × 100 mm) from Toa Nenryo Co.-Asahi Optical Co., which was packed with 2–3 μm "spherical" particles of HA (surface area not available). With these particles, the selectivity factor of BSA and lysozyme is 1.3 and that of myoglobin and lysozyme is 1 at a flow rate of 1.0 mL/min (phosphate eluent pH 6.8). In comparison, our selectivity factor of BSA and lysozyme is around 15 and that of myoglobin and lysozyme is around 1.7, which are better.

Kato et al. [7] studied a HA column (7.5 mm i.d. × 75 mm), trade name TSK gel HA-1000 (Toyo Soda, Tokyo, Japan), that was packed with 5 μm diameter spherical HA having a surface area of 30 m^2/g. The separation of BSA and lysozyme from this column took 15 min and a complete separation of myoglobin and lysozyme was achieved at a flow rate of 1.0 mL/min (phosphate eluent pH 6.8). Again, our separation of BSA and lysozyme was three times faster, but the myoglobin and lysozyme peaks overlapped, which is not as good as their result.

In both cases, the operational parameters, column length, column inside diameter, flow rate, and eluent type and pH, were comparable with our experiments. Our results are comparable with the TSK gel HA-1000 column and superior to the column studied by Kadoya.

Data on other proteins is lacking, so a more comprehensive comparison is not possible. As far as these three proteins are concerned, however, we achieved comparable separation with a column packed with HA microspheres transformed from the calcium-containing borate glass, whose diameter was 20–30 times larger than the commercially available HA.

The advantage of a larger particle size is the ease of manufacturing. Normally, the particles size used for HA column is about 3–5 μm. To the authors' knowledge, a spray process [18] is a common method for manufacturing spherical particles of this size, but this process is expensive. The low temperature transformation process used to produce the HA microspheres used in the present study has a characteristic feature of retaining the geometric shape and size of the initial glass microspheres. The complexity of producing highly spherical HA microspheres, see Fig. 7c, is avoided by spheridizing the starting glass particles, which are easy to shape.

Normally, the efficiency of an HPLC column is measured quantitatively by theoretical plate number. This

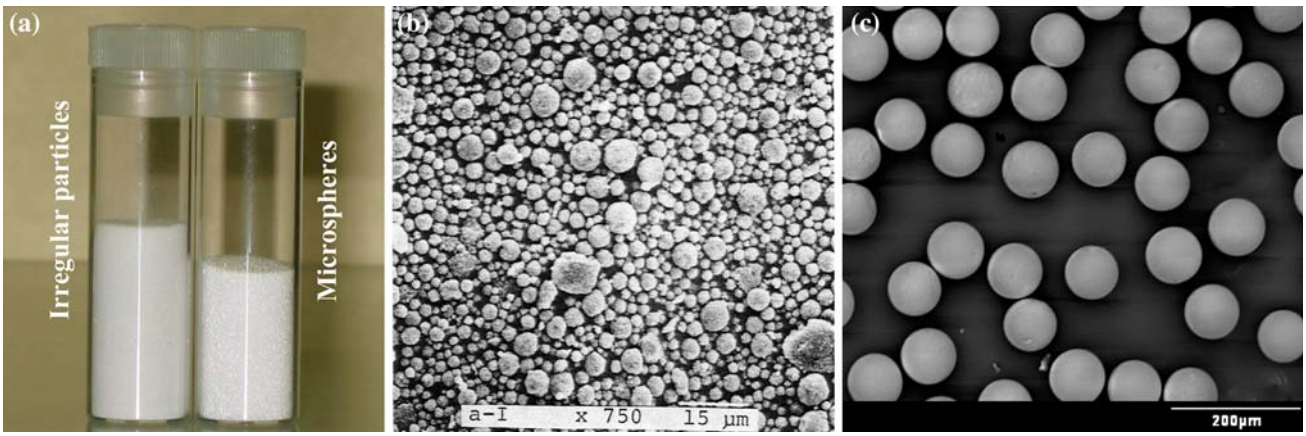

Fig. 7 (**a**) Packing efficiency comparison between irregular particles and microspheres. (**b**) SEM image of HA spherical particles from Toa Nenryo Co.-Asahi Optical Co. [19]. (**c**) SEM image of HA microspheres transformed from the 2-2-6 glass

number is closely related to the packing particle size. As the particle size decreases, the efficiency is expected to improve dramatically.

Our packing particle size was much larger, up to 20–30 times, than the commercially available HA column, but we achieved comparable separation performance for the proteins studied. A likely reason is that the surface area of the transformed microspheres was 6 times larger than the TSK gel HA-1000 packing particles. This indicates that the surface area of the packing particles may be also important to improve HA column performance. A larger surface area provides more interaction between the sample and stationary phase and thus compensates for a larger particle size.

Moreover, microspheres also pack more tightly in a column than irregular-shaped particles. Figure 7a shows that for the same kind of particles differing only in shape, spherical particles occupy less volume and pack more efficiently. The theoretical plate number for spherical HA particles is ten times larger than for irregularly shaped HA particles for the same size range of 2–3 μm according to Kadoya [17].

An uniform spherical shape is also expected to contribute to good column packing and increased separation performance. Figure 7b and c shows the comparison of spherical HA particles from Toa Nenryo Co.-Asahi Optical Co. [19] and the HA microspheres made from the borate glass. The spherical particles in Fig. 7b are not as spherical, nor as uniform in size, as the microspheres in Fig. 7c which have a nearly perfect spherical shape.

Conclusion

The successful use of HA microspheres transformed from a $2CaO \cdot 2Na_2O \cdot 6B_2O_3$ glass in protein adsorption and separation has been demonstrated. Its performance is comparable to commercially available HA column in separating BSA, myoglobin and lysozyme, although the microspheres were as large as 90 μm, which is 20–30 times larger than the "spherical" HA column particles which have been used in HPLC columns.

The advantages of the glass-derived HA microspheres are the large specific surface area (190 m^2/g), highly spherical shape, and uniform size and ease of manufacturing. A larger surface area enhances the interaction between HA and proteins and the spherical shape and uniform size contribute to good column packing, both of which are desirable for chromatographic columns. Ease of manufacturing is also an important economic and practical issue.

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