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Utilization of the dry impact blending method to prepare irregularly shaped particles for high-performance liquid chromatographic column packings

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Abstract

The use of the dry impact blending method to prepare various materials for HPLC column packings that might otherwise be unsuitable is proposed. Crystalline hydroxyapatite (HA) was adopted as a model of a useful but fragile, irregularly shaped material and was embedded on the surface of polyethylene beads by impact blending. The resulting HA composites were evaluated for the crystallinity of the HA, the ability to adsorb and desorb proteins and performance as an HPLC column packing for proteins. Non-specific irreversible adsorption of proteins was observed. This adsorption could be saturated, however, and subsequently the HA composites performed satisfactorily in the HPLC of proteins.

1. Introduction

In high-performance liquid chromatographic (HPLC) separations, for good results it is necessary that the particles of the column packings have regular shapes, for example, spherical with uniform size in the range 3–10 μm in diameter [1]. Methods for the preparation of particles that satisfy these conditions have been established for silica gel and some organic polymers. For that reason, a number of packings have been prepared by chemically modifying the surface of spherical silica gel with alkyl groups, ion-exchange groups, etc. They demonstrate high performance, and a number of solutes have been determined with their use with good results. Silica gel has disadvantages, however; its uncoated surface is dissolved by alkaline solution and bonds to alkyl groups tend to be cleaved by acidic solution. Further, the unique adsorption characteristics of some other materials offer advantages for the separation of substances with which satisfactory results have not yet been obtained with silica-based materials. Examples of such alternative materials include titania [2–4], zirconia [2–7] and polypeptides [8]. Only a few such materials have been used as HPLC column packings because it is difficult to shape

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many of them into spheres which are rigid and have a proper uniform size.

The preparation of composite particles by combinations of diverse particles has been developed in the pharmaceutical field [9–11]. The technique is called dry impact blending. Powder particles are suspended in a high-speed air stream and vigorously blended. The impact forces that attend the collision between the particles or between the particles and striking pins in a machine makes the diverse particles adhere. For example, when 5–200 μm polymer particles and inorganic ultramicroparticles under 1 μm are blended with the impact method, we can obtain composite particles that have the inorganic ultramicroparticles densely packed on the surface of the polymer particles. It has been confirmed experimentally that small particles are fixed on large particle surfaces when the ratio of the diameter of the large particle to that of the small particle is larger than 10:1.

We have previously prepared composite particles consisting of 0.3–0.9-μm silica ultramicrospheres (with an ODS coating) and 5–10-μm polyethylene microspheres for HPLC column packings [12]. The particles were useful for separating proteins of high molecular mass. The study indicated that the ultramicrospheres, which could not be utilized directly for HPLC column packings, could be used as a composite as a stationary phase in HPLC with acceptable flow-rates of the mobile phase.

In this paper, we describe the preparation and utilization of composite particles prepared from irregularly shaped particles as HPLC column packings. We selected crystalline hydroxyapatite as a model of an irregularly shaped particle. It exhibits characteristic adsorption of proteins and has well known chromatographic properties. Although crystalline hydroxyapatite has frequently been employed for the purification of antibodies in column chromatography, it must be processed by sintering or by being packed with a special method when it is used as an HPLC column packing [13–18], since the intact crystal is so fragile that it cannot resist high pressures. We anticipated that forming crystalline hydroxyapatite into composite particles would solve this problem.

2. Experimental

2.1. Chemicals and reagents

Low-density spherical polyethylene (PE) beads (average diameter 10 μm) as core particles of the composite were supplied by Sumitomo Seika (Osaka, Japan). Crystalline hydroxyapatite (HA) was prepared from Na_2HPO_4 and $CaCl_2$ according to the Tiselius method [19]. Na_2HPO_4, $CaCl_2$, NaOH, KH_2PO_4, K_2HPO_4, trifluoroacetic acid (TFA), Triton X-100 [polyoxyethylene(10)octyl phenyl ether] and acetonitrile of analytical-reagent grade were purchased from Wako (Osaka, Japan). Albumin (from bovine serum), cytochrome *c* (type III, from horse heart), lysozyme (grade I, from chicken egg white), ribonuclease A (type III, from bovine pancreas) and myoglobin (from horse skeletal muscle) were purchased from Sigma (St. Louis, MO, USA). For the determination of proteins, a Simpack CLC-ODS column (150 mm × 6.0 mm I.D.), purchased from Shimadzu (Kyoto, Japan), was used. Empty columns (50 mm × 2.0 mm and 4.6 mm I.D.) were purchased from Nihon Chromato (Tokyo, Japan). A spherical HA-packed column (KB column, 135 mm × 7.8 mm I.D.) from Koken (Tokyo, Japan) was used for comparison with the HA composite column.

2.2. Apparatus

For the preparation of HA composite particles, an O.M. Dizer and a Hybridizer (NHS-0; Nara Machinery, Tokyo, Japan) were used. HA and HA composite particles were observed by scanning electron microscopy (SEM) (JSM-T220; JEOL, Tokyo, Japan) after being sputter coated with gold with an ion sputtering apparatus (JFC-1100; JEOL).

All chromatographic tests were performed on an LC-6A gradient system with a Rheodyne

Model 7125 injection valve, connected to an SPD-6A UV spectrophotometric detector and a Chromatopac C-R6A integrator (Shimadzu).

2.3. Dry impact blending method

Details of the machines and the method were described in previous papers [9–12]. PE and HA powders were blended (1400 rpm for 10 min) with the O.M. Dizer; three kinds of mixtures, which contained 30, 40 or 50% (w/w) of HA, were prepared and the total amount was fixed at 15 g. The resulting mixtures were treated by the dry impact blending method using the Hybridizer, with a rotational speed of 16 000 rpm and a treatment time of 10 min. The vessel was cooled by circulation of water through the jacket during the treatment.

2.4. Evaluation of HA composite particles by X-ray diffractometry

To evaluate HA, PE and HA composite particles containing 30% HA (30% HA composite), X-ray powder diffraction patterns were measured using an MXP^3 System computer-controlled X-ray diffractometer (Mac Science, Tokyo, Japan). CuKα radiation patterns were recorded from 5 to 90° (2θ) in steps of 0.020°.

2.5. Measurement of maximum amounts of protein adsorbed

A 10-mg amount of HA or a 20 mg amount of HA composite particles was suspended in 500 μl of 1 mM potassium phosphate buffer [an equimolar mixture of K_2HPO_4 and KH_2PO_4 (pH ≈ 6.8) (KPB)]. A 500-μl volume of the BSA solution which contained 2 or 1 mg/ml of BSA in 1 mM KPB was added. After mixing, the mixture was allowed to stand for 30 min at room temperature. A 200-μl volume of the supernatant was withdrawn and its protein content was determined by RP-HPLC. A 800-μl volume of 400 mM KPB was added to the sedimented HA composite; after mixing, the mixture was allowed to stand for 30 min at room temperature. The protein content of the supernatant was determined by RP-HPLC. The sample volume was 20 μl and Shim-pack CLC-ODS was used as an analytical column. Elution was carried out with a linear gradient in 15 min from 25% to 50% of acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min and the eluate was monitored at 220 nm. The maximum amount of BSA adsorbed on HA and HA composite particles from 1 mM KPB solution was calculated from the protein contents of both supernatants.

2.6. Adsorption–desorption behaviour of HA and HA composite particles

A 20-mg amount of HA or HA composite particles was suspended in 500 μl of 5 mM KPB. A 20-μl volume of a protein mixture was added, containing 1 mg/ml each of BSA, cytochrome c, lysozyme and ribonuclease A. After mixing, the mixture was allowed to stand for 30 min at room temperature for adsorption. A 500-μl volume of KPB of the appropriate strength was added to adjust the KPB concentration of the suspensions to 5, 25, 50, 100, 150 and 200 mM. After mixing, the mixtures were allowed to stand for 30 min at room temperature for desorption. The protein contents of the supernatants were determined by RP-HPLC. The sample volume was 200 μl and analysis was performed in a manner similar to that described above.

2.7. Separation of proteins

Composites with 30% or 50% HA were slurry-packed into stainless-steel columns with 5 mM KPB. A volume of 10 μl or 20 μl of the protein mixture solution, which contained 1 mg/ml of each protein, was applied to the columns. Linear gradient elution was employed with KPB from 5 to 202.5 mM at a flow-rate of 0.5 ml/min and the eluate was monitored at 220 nm.

3. Results and discussion

3.1. Observation of HA composite particles

Typical SEM photographs of HA and HA composite particles are shown in Figs. 1 and 2, respectively. As can be seen in Fig. 1, HA consisted of plate-like flakes. In HA composite particles, large HA particles are absent and PE coated with superfine fragments was observed. We consider that HA was fractured into many superfine fragments by the strong impact forces which arose from mutual collisions between different particles or between particles and the striking pins attached to the rotor.

When the physical appearance of the surface was compared for HA composite particles with different HA contents, 30% HA had a relatively smooth surface which was densely coated with rounded fragments of HA having a uniform size of ca. 0.1 μm. As the HA content increased, particularly with 50% HA, several larger sized unfractured fragments were clearly observed, and thus the fragment sizes became varied. As a result, 30% and 40% HA had a homogeneous coating layer with fine HA fragments, whereas 50% HA was rough because of the partially overlaid layer of HA fragments. Further, 50% HA was also observed to contain many irregularly shaped particles of about 1 μm, which were considered to be aggregates of excess HA.

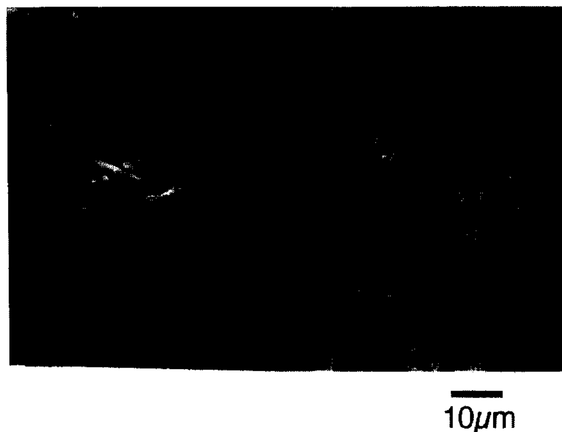

Fig. 1. Typical SEM photograph of HA.

These observations suggested that the process of forming the HA coating layer was as follows. When a small amount of HA was added, HA was well fractured and fixed on the PE surface immediately through dry impact blending. However, if a larger amount of HA than the required amount was added, it was difficult to fix all the HA on PE homogeneously. Repeated collisions could also lead to enlarged HA aggregates which subsequently might be fixed on the HA layer of composite particles. The result was irregularly shaped particles and partial overlaid layers at 50% HA.

When HA fragments are assumed to be spheres of 0.1 μm, the amount of HA required to cover the whole surfaces of PE beads is calculated to be about 11% of the total mass (see Appendix). In 30% HA, almost all HA particles were regarded as adhering to PE surfaces because an excess of HA which was not fixed on HA composite particles was hardly observed. Therefore, it is considered that HA fragments, although they seemed to form a monolayer coating, were fixed on PE with about a double-layer thickness, that is, the HA coating layer was about 0.2 μm thick.

3.2. Evaluation of crystallinity of HA and HA composite particles

The following mechanism has been proposed for the separation of proteins on HA [13]. Two types of vertical main surface appear on an HA, called the a (or b) surface and the c surface. The a or b surface has C sites, which adsorb carboxyl groups, whereas the c surface has P sites, which adsorb basic groups. Protein molecules can be adsorbed using a number of different local molecular surfaces, each of which can face the crystal surface and can orient in different directions on the crystal surface. Hence a specific chromatographic separation can be obtained on the HA column based on subtle differences in the geometrical arrangement of the adsorption groups on a protein.

Mechanical effects such as trituration, friction and compression change the physico-chemical properties of some crystals, such as crystal form

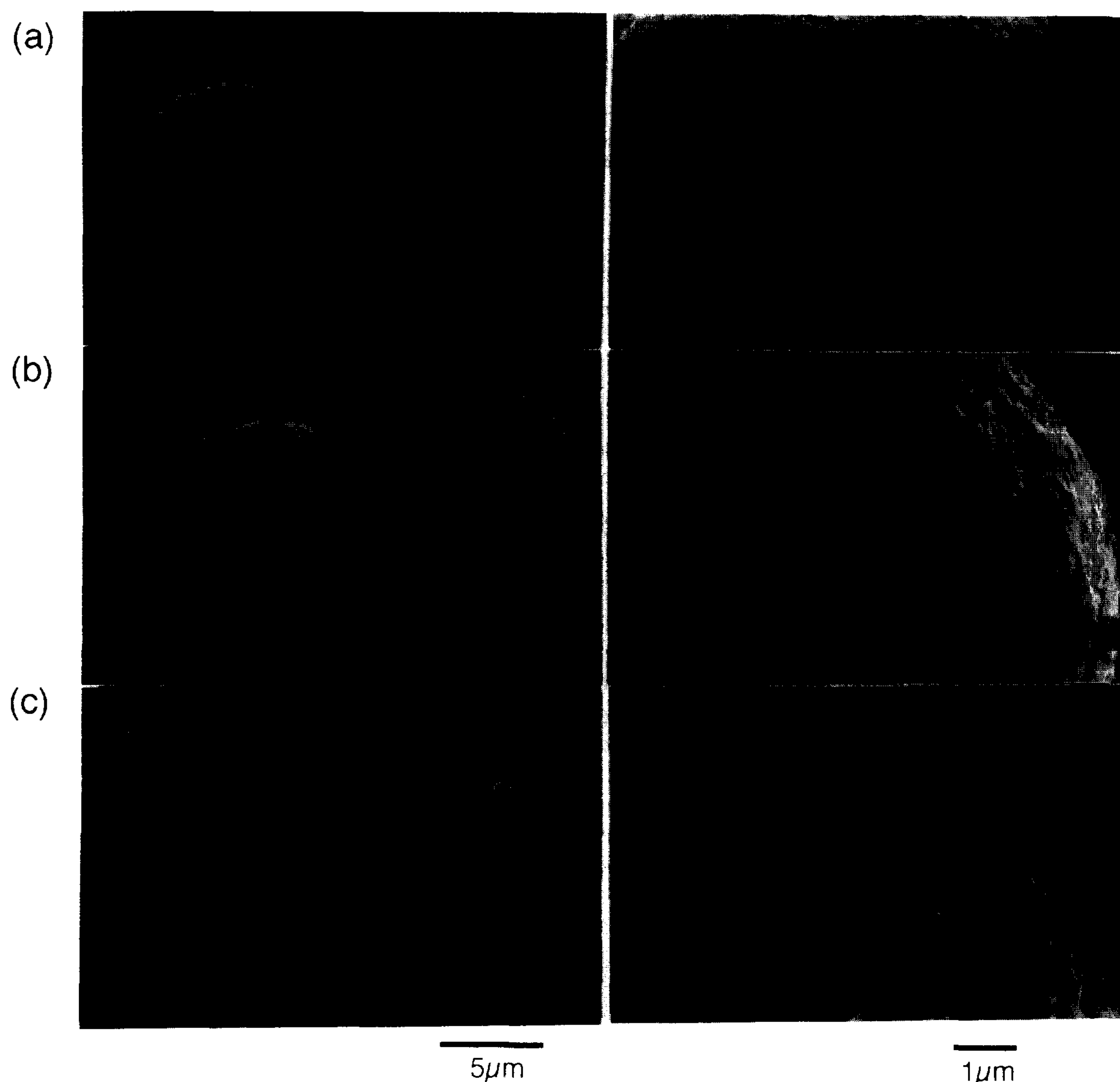

Fig. 2. Typical SEM photographs of HA composite particles: (a) 30% HA; (b) 40% HA; (c) 50% HA.

and reactivity. This phenomenon is termed mechanochemistry. It has been reported that some powders changed their forms from crystalline to amorphous when they were fixed on large particles by dry impact blending [20,21]. The changes were regarded as mechanochemistry. In the work cited, the change was utilized to improve the dissolution properties of slightly soluble drugs. In this study, however, if the crystalline form of HA is changed by dry impact blending, then the chromatographic properties of HA composite particles may differ from those of intact HA.

The crystallinity of HA composite particles was compared with that of HA by powder X-ray diffractometry. The diffraction patterns of HA, PE and 30% HA are shown in Fig. 3. The diffraction pattern of HA matches the pattern of

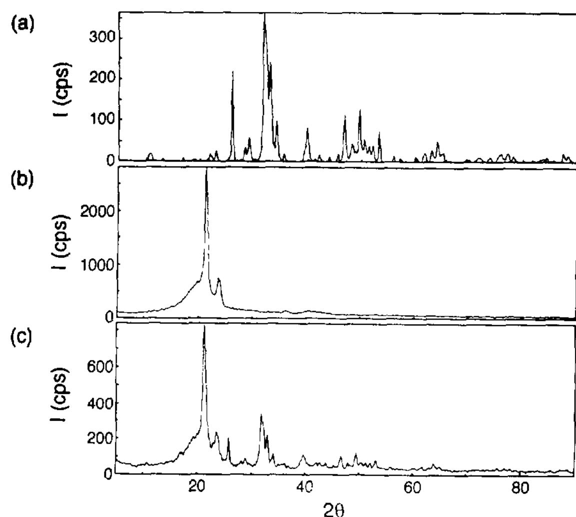

Fig. 3. X-ray powder diffractograms of (a) HA, (b) PE and (c) 30% HA composite particles.

synthetic hydroxyapatite [$Ca_5(PO_4)_3(OH)$] (diffraction data from the Joint Committee in Powder Diffraction Standards). When the diffraction pattern of the 30% HA was compared with that of HA, the main peaks of both patterns agreed completely, except for some broad peaks on HA-composite particles which were distributed around $2\theta = 21°$ derived from PE. This indicates that the crystalline form of HA is maintained after dry impact blending.

3.3. Maximum amounts of protein adsorbed

The maximum amount of BSA adsorbed was independent of the HA content of the HA composite particles, the maximum amounts adsorbed in 1 m*M* KPB being 68.6, 5.70, 5.67 and 6.09 mg/g for HA and 30%, 40% and 50% HA adsorbents, respectively. The results suggest that the specific surface area of HA on all the HA composite particles is approximately equal. Hence, when the maximum amount of BSA adsorbed on HA composite particles is compared with that of HA, it is considerably smaller than the values that are expected from the HA content. Therefore, an increase in the content of HA is not effective in increasing the maximum amount of proteins adsorbed. Roughly estimated, it is considered that more than half of the HA surface area is inaccessible because HA fragments are embedded in PE, adhere to PE or aggregate with each other.

3.4. Adsorption–desorption properties of proteins

The adsorption–desorption properties of proteins on HA composite particles in KPB solution were evaluated and the results are shown in Fig. 4. With intact HA, all proteins were adsorbed in 5 m*M* KPB, then desorbed between 25 and 200 m*M*, except for cytochrome *c*. Cytochrome *c* was not desorbed completely below 400 m*M* KPB (data not shown). This indicates that a certain amount of cytochrome *c* is adsorbed by HA extremely strongly.

Next, adsorption–desorption properties of proteins on HA composite particles were compared with those on HA (Fig. 4). In 5 m*M* KPB, every HA composite particle adsorbed almost all proteins. In contrast with HA, some adsorbed proteins were not desorbed completely from HA

Fig. 4. Adsorption–desorption behaviour in KPB for (a) BSA, (b) cytochrome *c*, (c) lysozyme and (d) ribonuclease A of (○) HA and (●) 30%, (▲) 40% and (◆) 50% HA composite particles.

composites, in spite of using KPB concentrations up to 200 m*M*. Although the desorption curves for different HA composites showed similar patterns for individual proteins, there was no simple relationship between the degree of desorption with 200 m*M* KPB and the HA content or the kind of protein. This seems to indicate that with HA composites, protein adsorption is due in part to forces other than ionic interactions.

An explanation for the incomplete desorption is that HA was fractured into fine fragments during dry impact blending. Crystal sections that hardly appeared on the surface of large crystals are thereby abundantly exposed, and this plane might adsorb proteins more strongly. To investigate the influence of fracture, the fractured HA that was obtained by the dry impact blending treatment with only HA was applied to the adsorption–desorption test. Irreversible adsorption was not increased compared with intact HA (Fig. 5), and therefore fracturing HA did not influence its adsorption of proteins.

As an alternative explanation, the incomplete desorption is due to the hydrophobic interaction of proteins with exposed surfaces of the PE used as the core particle for the composite. In an attempt to prevent any hydrophobic interaction, KPB containing 0.1% (v/v) Triton X-100 was used as an adsorption–desorption medium. The desorption curves showed similar patterns to crystalline HA for individual proteins, and further, almost all proteins were desorbed from HA composite particles below 200 m*M* KPB (Fig. 6). The results suggest that the hydrophobic interaction of proteins is responsible for incomplete desorption from HA composites. Also, it is avoidable by adding Triton X-100 without losing the ionic interaction with proteins.

We further investigated in the adsorption of proteins by HA composites by hydrophobic interaction how the adsorption influenced the adsorption–desorption behaviour by ionic interactions. The degree of adsorption and desorption of proteins was measured again with HA composite particles that had been used for the adsorption–desorption test once and had already adsorbed a certain amount of proteins hydrophobically. The used HA composite particles were suspended in 500 μl of 5 m*M* KPB after they had been washed twice with 400 and 5 m*M* KPB, respectively. The correlation between protein desorption and KPB concentration is shown in Fig. 7. Almost all proteins were desorbed from HA composite particles below 200 m*M* KPB. The irreversible adsorption of proteins seems to have reached saturation during the first

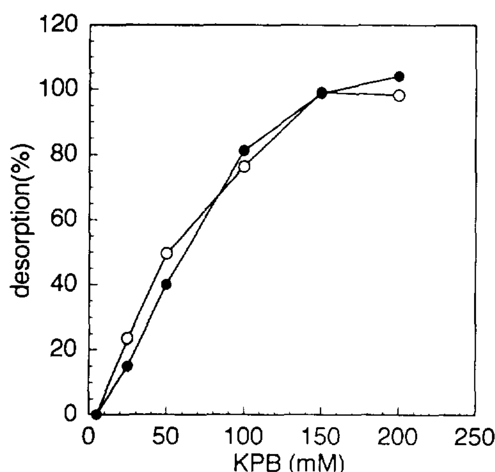

Fig. 5. Adsorption–desorption behaviour in KPB for total proteins of (○) HA and (●) crushed HA.

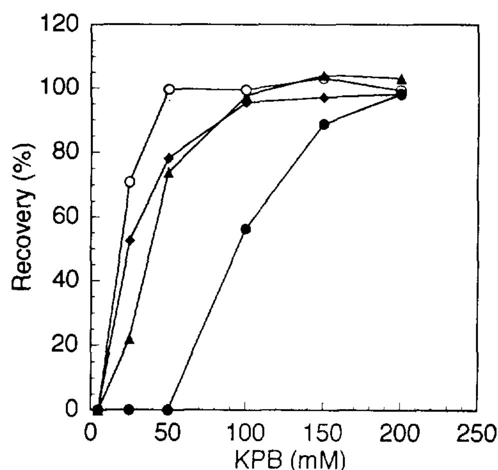

Fig. 6. Adsorption–desorption behaviour in KPB containing 0.1% (v/v) Triton X-100 for (○) BSA, (●) cytochrome *c*, (▲) lysozyme and (◆) ribonuclease A of 30% HA.

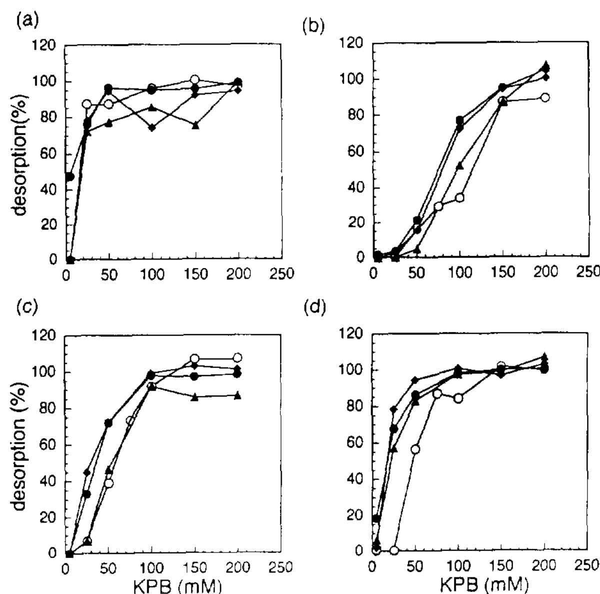

Fig. 7. Adsorption–desorption behaviour in KPB for (a) BSA, (b) cytochrome *c*, (c) lysozyme and (d) ribonuclease A of (○) HA and (●) 30%, (▲) 40% and (◆) 50% HA composite particles, which for the adsorption-desorption test once.

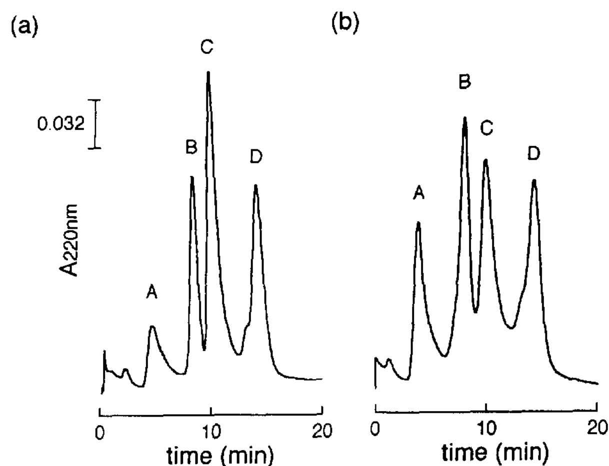

Fig. 8. Separation of standard proteins on the columns of (a) 30% and (b) 50% HA composite particles. Conditions: column, 50 mm × 2.0 mm I.D.; elution, 15-min linear gradient from 5 to 202.5 m*M* KPB; flow-rate, 0.5 ml/min; detection, UV at 220 nm. Peaks: A = BSA; B = ribonuclease A; C = lysozyme; D = cytochrome *c*.

test, and further irreversible adsorption did not occur. Each of the proteins that were adsorbed on each HA composite particle in 5 m*M* KPB now showed a characteristic desorption behaviour that was dependent on KPB concentration.

Consequently, HA composite particles demonstrated adsorption–desorption properties based on ionic interactions similar to crystalline HA.

3.5. *Protein separation*

The 30% and 50% HA composites were packed into columns (50 mm × 2.0 mm I.D.), which were used for the separation of a protein standard mixture. The back-pressures of the columns were 2.94 and 5.49 MPa for 30% and 50% HA, respectively, when 5 m*M* KPB was used as the mobile phase at a flow-rate of 0.5 ml/min. From the observations of HA composite particles by SEM, 50% HA contains a number of irregularly shaped small particles that resemble aggregates of HA fragments. We speculate that the back-pressure was higher than that for 30% HA because the flow path in the 50% HA column was plugged with the small particles.

Fig. 8 the results of protein separation. Both of the columns were able to separate model proteins with a linear gradient of KPB concentration. The 50% HA column exhibited relatively broad peaks and could not separate each peak to the baseline.

The 30% HA column exhibited a relatively favourable separation under the same conditions. When the column bed volume was increased, five proteins could be separated (Fig. 9). When the two chromatograms obtained from the 30% HA column (Fig. 9) and a conventional spherical HA-packed column (Fig. 10) were compared, they agreed in the elution order of proteins, and cytochrome *c* was resolved into two peaks, corresponding to the oxidized and the photoreduced forms [14] on both columns. Therefore, it is indicated that these proteins were recognized by the 30% HA column similarly to the conventional spherical HA-packed column. Although the 30% HA column totally eluted proteins at a lower concentration of KPB than the spherical HA-packed column, this is thought to be due to the feature of the crystalline

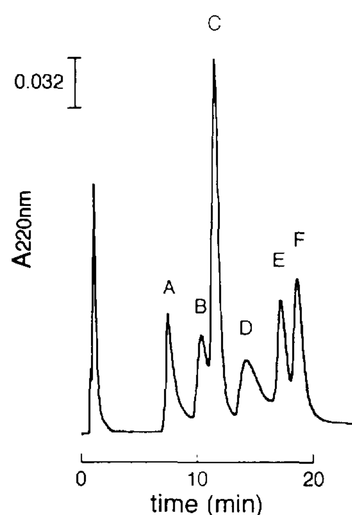

Fig. 9. Separation of standard proteins on the 30% HA column. Conditions: column, 50 mm × 4.6 mm I.D.; elution, 15-min linear gradient from 5 to 202.5 m*M* KPB; flow-rate, 0.5 ml/min; detection, UV at 220 nm. Peaks: A = BSA; B = myoglobin; C = ribonuclease A; D = lysozyme; E = cytochrome *c* (reduced); F = cytochrome *c* (oxidized).

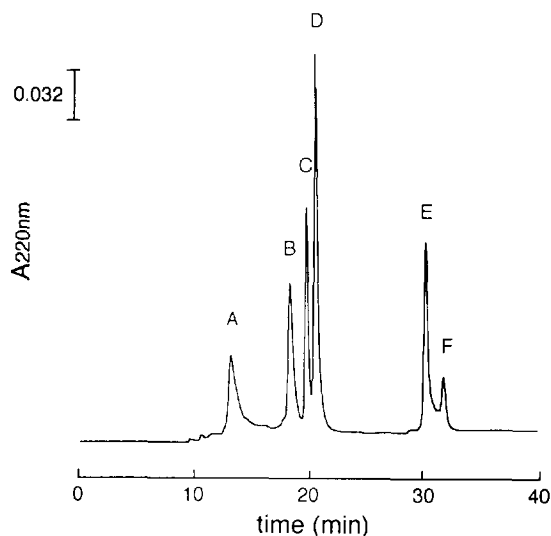

Fig. 10. Separation of standard proteins on a conventional spherical HA-packed column. Conditions: column, 135 mm × 7.8 mm I.D.; elution, 30-min linear gradient from 5 to 300 m*M* KPB; flow-rate, 1.0 ml/min; detection, UV at 220 nm. Peaks: A = BSA; B = myoglobin; C = ribonuclease A; D = lysozyme; E = cytochrome *c* (reduced); F = cytochrome *c* (oxidized).

HA used as the material, because the HA composite particles demonstrated adsorption–desorption abilities similar to those of the crystalline HA.

Irreversible adsorption of proteins on the HA composite columns was also observed in chromatographic usage with the first injection of the protein mixture. However, after 100 μg of protein had been applied, the irreversible adsorption disappeared. The irreversible adsorption could also be avoided in the presence of Triton X-100 (Fig. 11). The chromatogram of KPB containing Triton X-100 was well matched with the chromatogram without Triton X-100 in the KPB elution concentration of proteins.

The performance of the HA composite column (30% HA was employed) was evaluated by different types of examination.

Plots of plate height H vs. linear flow velocity u for tryptophan ($k' = 0.45$) with isocratic chromatography are shown in Fig. 12. The H values increase linearly with increasing velocity. When the relationship is applied to the Knox equation ($h = Av^{1/3} + B/v + Cv$; $h = H/d_p$, $v = ud_p/D$;

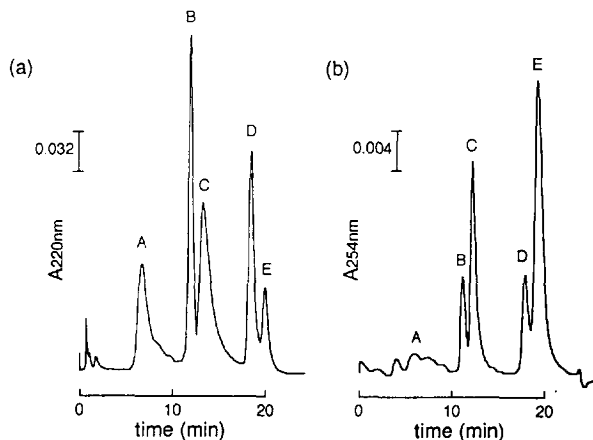

Fig. 11. Separation of standard proteins on the 30% HA column in the absence or presence of triton X-100. (a) Conditions: column, 50 mm × 4.6 mm I.D.; elution, 19-min linear gradient from 5 to 202.5 m*M* KPB; flow-rate, 0.5 ml/min; detection, UV at 220 nm. (b) Conditions: elution, 19-min linear gradient from 5 to 202.5 m*M* KPB containing 0.1% (v/v) Triton X-100; detection UV at 254 nm (a longer wavelength was employed because the absorbance by Triton X-100 was very strong at 220 nm); other conditions as in (a). Peaks: A = BSA; B = ribonuclease A; C = lysozyme; D = cytochrome *c* (reduced); E = cytochrome *c* (oxidized).

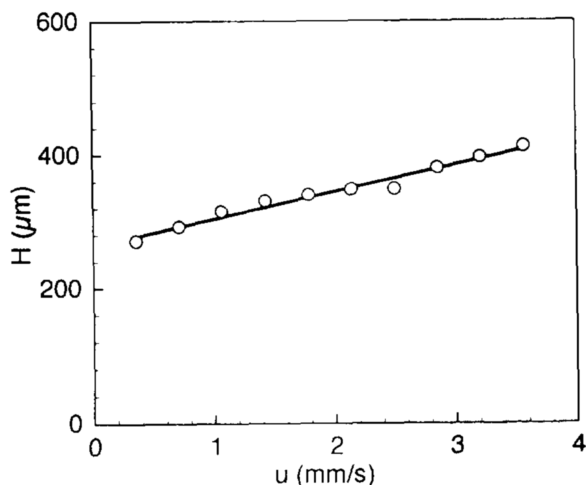

Fig. 12. Plots of plate height for tryptophan (k' = 0.45) on the 30% HA column as a function of the mobile phase velocity. Conditions as in Fig. 9.

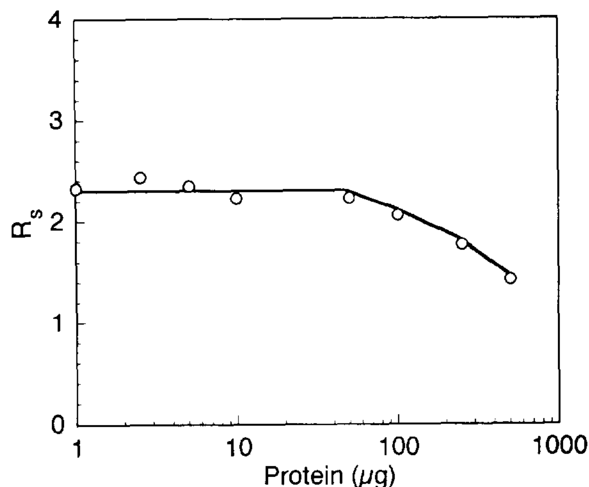

Fig. 13. Dependence of the peak resolution on sample loading of the 30% HA column. Conditions as in Fig. 9.

d_p = particle diameter of the packing, D = diffusion coefficient of solute), it is clear that coefficients A and C, that is, diffusion at inter-particles and dispersion from slow mass transfer of solute molecules, are the main reasons for band spreading on the column. As the size of the particles is relatively large, this may be responsible for the diffusion and dispersion.

The effect of sample loading was evaluated from the peak resolution under linear gradient conditions. Various concentrations of the protein solutions that contained BSA and myoglobin in the ratio 5:2, in order to be equal in peak height, were separated on the column, then total protein amount vs. resolutions (R_s) were plotted (Fig. 13). R_s remained constant at loadings up to 50 μg and then decreased with further increase in the sample loading. Accordingly, the maximum sample load resulting in the highest resolution with closed peaks is 50 μg as total amount.

The reproducibility of the HA composite particles was examined by comparing R_s on each of the columns packed with particles from five separate batches. These batches were derived from one batch of PE and two batches of crystalline HA. The R_s of BSA and myoglobin peaks was measured five times for each column using the conditions indicated in Fig. 13 and the average values obtained were 2.26, 2.24, 2.21, 2.29 and 2.49. The HA composite particles prepared by the treatment thus demonstrated good reproducibility.

After usage as an HPLC column packing for 3 weeks continuously, the particles were removed from the column and observed with SEM (Fig. 14). Elimination of the HA layer is hardly observed and the surface appearance of the used particles is the same as that of the particles before use. This confirms that HA composite particles are stable when employed continuously as HPLC column packings for at least 3 weeks.

In conclusion, the HA composite column could separate proteins using a gradient of KPB concentration, and then the reproducibility and the mechanical stability of the HPLC column packings has been demonstrated.

4. Conclusions

This study demonstrated that the dry impact blending method could produce an HPLC column packing from irregularly shaped materials too fragile for direct use in HPLC. Dry impact blending is also potentially capable of producing HPLC packings reproducibly on a large scale.

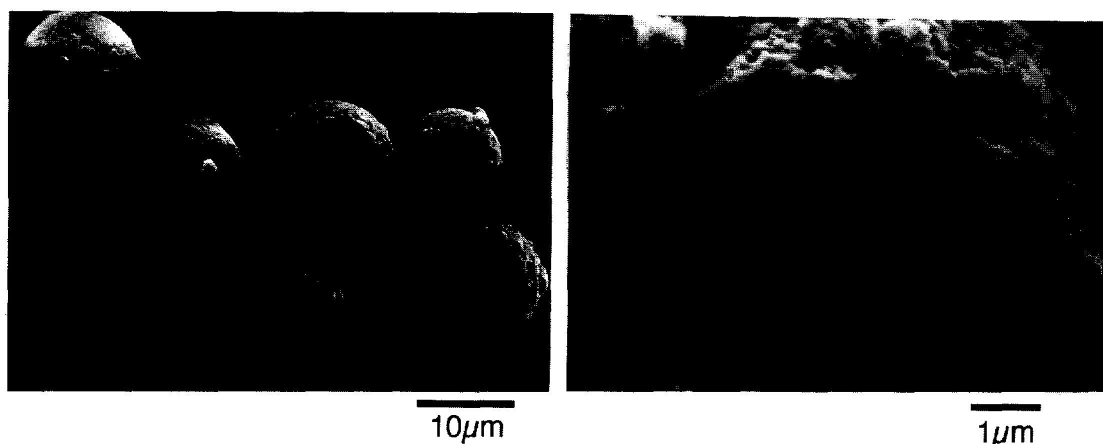

Fig. 14. Typical SEM photographs of HA composite particles after use as an HPLC column packing for 3 weeks continuously.

Appendix

If the shapes of a core and a wall particle are spherical, the optimum mixing ratio for coating the core particles with a dense monolayer of the wall particles is calculated as follows.

The maximum number of the wall particles that are arranged on the core particle can be approximated by small circles which are placed on a unit sphere in a closest-packing array. Therefore, if the radii of the core and the wall particle are R and r, respectively, the number of wall particles for coating the core particle (A) is calculated as follows [12]:

$$A \approx \frac{4\pi[R + r(1-2k)]^2}{\pi r^2} \cdot \frac{\pi}{\sqrt{12}}$$

where k is the ratio of embedded depth of the wall particle to its diameter and $\pi/\sqrt{12}$ is the approximation of the maximum coating ratio of the sphere surface due to the circles. The approximation applies when the number of fixed circles is significantly large [22]. The optimum mixing ratio of the wall particle (M) is then calculated as follows:

$$M\ (\%) = \frac{Ar^3\rho_r}{R^3\rho_R + Ar^3\rho_r} \cdot 100$$

where ρ_R and ρ_r are the specific gravity of the core particle and the wall particle, respectively.

As the densities of PE and HA are 0.92 and 3.16 g/cm^3, respectively, M for covering the 10-μm PE beads with 0.1-μm spherical HA with $k=0$ is calculated to be ca. 11%.

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