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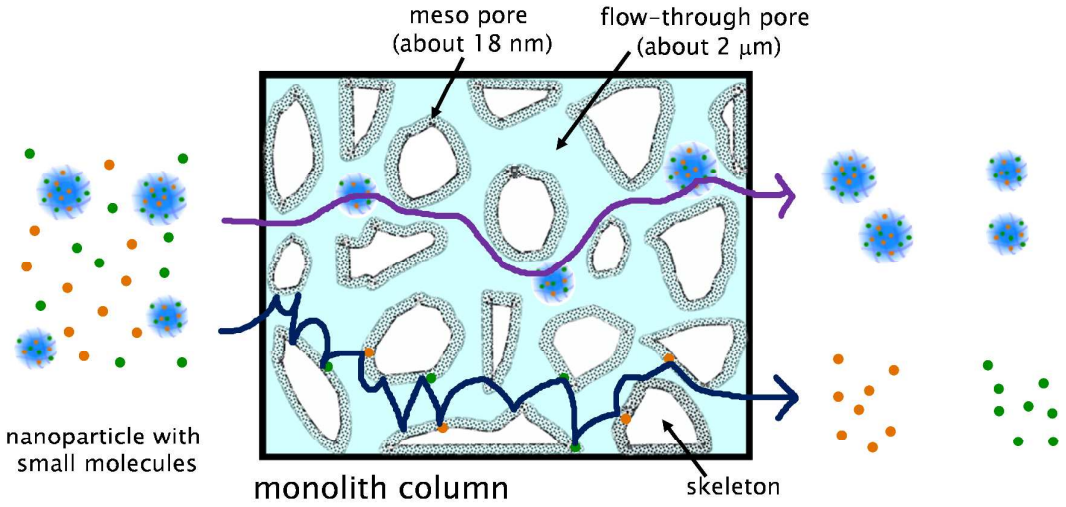
The bimodal structure of the monolithic column is promising for the simultaneous separation, because nanoparticles and small molecules were separated by micrometer-and nanometer-sized pores, respectively.



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Simultaneous Analysis of Nanoparticles and Small Molecules by High-Performance Liquid Chromatography Using a Silica Monolithic Column

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A high-performance liquid chromatography method using a commercially available silica monolithic column for the simultaneous analysis of nanoparticles and small molecules was developed. The method uses the micrometer-sized flow-through pores and nanometer-sized mesopores of the monolithic column for separation: first, size separation of nanoparticles was performed by the micrometer-sized pores using the hydrodynamic mode, and then small molecules are separated by the nanometer-sized pores using the normal-phase mode. The method was used to evaluate and compare three existing methods for purifying nanoparticles and to analyse nanoparticle stability. The bimodal structure of the monolithic column is promising for the simultaneous separation of nanoparticles and small molecules.

New functional nanoparticles are continually being developed for applications in diagnostics, therapeutics, catalysis, tissue engineering, and imaging. Encapsulating functional molecules within nanoparticles is an effective way to add functionality to nanoparticles.[1–3] Encapsulation improves the stability and usability of functional molecules.[4–6] Nanoparticles containing an encapsulated medicine or probe molecule have been developed to deliver the encapsulated molecule to a targeted organ or tissue, and such functional molecule–containing nanoparticles have received much attention as noninvasive medical devices. Encapsulating two or more molecules within nanoparticles can produce a synergistic effect and may permit the fabrication of dual-function devices, such as theranostic devices.[7–9]

Many factors, including nanoparticle size, quantities of nanoparticles and functional molecules, and existence of degradates and impurities, must be evaluated in analysing a dispersed solution of functional molecule–containing nanoparticles. Even for nanoparticles containing a single type of functional molecule, separation of functional molecules from their degradates or impurities is necessary because degradates and impurities sometimes have undesirable effects.[10]

High-performance liquid chromatography (HPLC) is among the most commonly used methods for separation of various compounds, including nanoparticles.[11-13] Size-exclusion chromatography (SEC), which is one of the separation modes of HPLC, can be used to separate nanoparticles on the basis of their size.[14-15] A column is packed with particles with small pores, and nanoparticles are separated by their differing abilities to enter the pores. The pores must be big enough to allow entry of nanoparticles, which have diameters ranging from 10 to 100 nm. However, because the mechanical strength of the packing material particles decreases with increasing pore size, large strong particles must be used for nanoparticle separation. The use of large particles also prevents clogging of the column by the nanoparticles because the size of the gaps between particles increases with increasing particle size. However, columns packed with the large particles necessary for separation of nanoparticles show low separation efficiency. To make matters worse, SECs cannot be used to separate small molecules each other. Therefore, separation of nanoparticles from small molecules usually requires pretreatment by ultrafiltration, dialysis, or ultracentrifugation, after which the nanoparticles and small molecules are analysed separately. The necessity for pretreatment makes analysis of functional molecule–containing nanoparticles laborious and time-consuming.[16]

Recently, monolithic columns with micrometer-sized flow-through pores and smaller diffusive pores have been used for separation of various compounds.[17-21] The large flow-through pores (about 2 μm)[20] prevent nanoparticles from clogging the column, which is an inevitable problem in conventional packed-column analysis of nanoparticles with diameters of more than a few tens of nanometers.[12,13] In addition, nanoparticles rarely collapse under the low back pressure of a monolithic column. Another important feature of monolithic columns is the feasibility of controlling macro- and mesoporous structures independently, which may be useful for

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simultaneous separation of nanoparticles and small molecules.[21] That is, it is possible to design a monolith that has both flow-through pores large enough to allow penetration by nanoparticles and small pores for the separation of small molecules. Although restricted access media (RAM) have received much attention as a pretreatment for small molecule analysis,[22] the conventional RAM column does not have large through pores for the penetration of the nanoparticles. On the other hand, size separation of nanoparticles on silica monolithic columns using the hydrodynamic mode has been reported,[23] and there have been reports of separation of small molecules from other small molecules by means of the large surface area of the mesopores in either normal or reversed-phase mode.[24] However, there have been no reports of the simultaneous separation of nanoparticles and small molecules. Because the flow rate of the mobile phase in the hydrodynamic mode (about 0.05 mL/min) differs substantially from the flow rates in the normal and reversed-phase modes (about 0.5 mL/min), simultaneous separation of both nanoparticle and small molecule must be achieved by optimization of the separation conditions for each type of analyte.

In this study, we used two commercially available monolithic columns for the fast and simple simultaneous separation of nanoparticles and small molecules. We prepared nanoparticles containing the fluorescent compounds rhodamine 110 and fluorescein, measured the quantity and size of the nanoparticles and the quantities of the fluorescent molecules, and determined the stability of the nanoparticles. To evaluate its effectiveness, we used our rapid and efficient method for comparison of three existing methods for purifying nanoparticles.

EXPERIMENTAL METHODS

Chemicals L-Arginine and acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tetraethyl orthosilicate (TEOS) and cyclohexane were obtained from Tokyo Chemical Industry (Tokyo, Japan). Fluorescein sodium and rhodamine 110 were obtained from Sigma-Aldrich (St. Louis, MO). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA).

Preparation of Nanoparticles Containing Fluorescent Molecules Silica nanoparticles containing fluorescent molecules were prepared by means of a reported method.[25,26] Arginine (10 mg), 0.5 μg of rhodamine 110, and 0.5 μg of fluorescein were dissolved in 10 mL of deionized water, and then 0.45 mL of cyclohexane and 0.55 mL of TEOS were added to the solution. The mixture was stirred at 60 °C for 20 h using a Teflon-coated magnetic stirring bar at 300 rpm. The reaction mixture initially consisted of two phases: a water phase containing arginine and an oil phase containing unhydrolyzed TEOS. The size of the nanoparticles increased when the amount of TEOS was increased.

Purification Procedures

(1) Ultrafiltration. The prepared nanoparticle solution (1 mL) was purified by using a Vivaspin concentrator (Sartorius Stedim, Göttingen, Germany) with a MW cutoff of 30,000 Da. After centrifugation at 3000g for 5 min in a Model 7780II centrifuge (Kubota Co., Tokyo, Japan), 150 μL of the supernatant was collected and analysed by HPLC.

(2) Dialysis. The prepared nanoparticle solution (1 mL) was sealed in a Spectra/Por dialysis bag (MW cutoff: 10,000; Spectrum Laboratories, Inc., Compton, CA, USA) and incubated in purified water (30 mL) for 1 day. The incubation solution was changed three times during the dialysis. After dialysis, the solution was analysed by HPLC.

(3) Ultracentrifugation. The prepared nanoparticle solution (500 μL) was placed in a microtube and centrifuged in an Optima TLX ultracentrifuge (Beckman Coulter, Inc., Brea, CA, USA). Two conditions were used for ultracentrifugation: rotation at 14,200*g* for 30 min, and rotation at 9,100*g* for 15 min. The entire bottom layer (100 μL) was collected and analysed by HPLC.

Stability Assay The dialyzed nanoparticle solution was placed in a refrigerator and analysed by HPLC after various storage times.

HPLC Analysis HPLC (Hitachi, Tokyo, Japan) analysis was performed with two L-2160U LaChrom Ultra pumps, an L-2200U LaChrom auto sampler, an L-2455U LaChrom diode array detector, an L-2485U LaChrom fluorescence detector, and an HPLC system organizer. No surface modified and octadecyl modified silica monolithic columns (250 mm × 3 mm, GL Sciences, Tokyo, Japan) were used. Mobile phase A was water/acetonitrile (95:5 v/v), and mobile phase B was acetonitrile. The gradient elution program of the mobile phases was as follows: 100% (A) from 0 to 25 min and then 100–60% (A) from 25 to 35 min. The flow rates were 0.05 mL/min for 0–25 min and 0.5 mL/min after 25 min. The injection volume was 10 μL, and a diode array detector and a fluorescence detector (Ex. 480 nm, Em. 520 nm) were used for detection. All samples were filtered with a Millex-LG syringe filter (pore size 0.2 μm, Millipore) before analysis.

Particle Size Measurement A Nanotrac Wave dynamic light scattering (DLS) instrument (Nikkiso Co., Ltd., Tokyo, Japan) was used to measure the diameters of the nanoparticles. Measurements were carried out at room temperature and used a laser beam at 780 nm. At least three replications were performed for each sample. A sample solution of 20 μL was used for the measurement. Size distribution graphs, which represent dependences of relative intensity of scattered light versus hydrodynamic diameter of nanoparticles, were obtained.

RESULTS AND DISCUSSION

Because the nanoparticles penetrated the flow-through pores and the small molecules could access the interiors of the mesopores (Fig. 1), the nanoparticles eluted faster than the small molecules, and the nanoparticles and small molecules could be analysed simultaneously under different elution conditions. In our method, the nanoparticles were eluted first under suitable conditions, and then the conditions were changed to elute the small molecules.

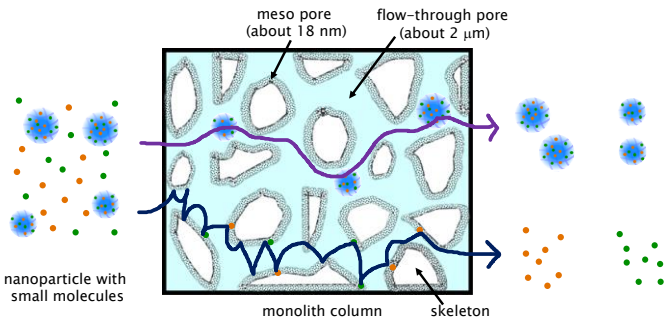

Fig. 1 Schematic diagram of separation of nanoparticles and small molecules with a monolithic column.

Elution of Nanoparticles Using the Hydrodynamic Mode. Elution times of nanoparticles of five different diameters (22, 25, 29, 56, and 87 nm) were determined using the hydrodynamic mode. Because the flow rate of the mobile phase was set to 0.05 mL/min for elution, the back pressure of the column was low (approximately 1.3 MPa). The low back pressure prevented the breaking or collapse of

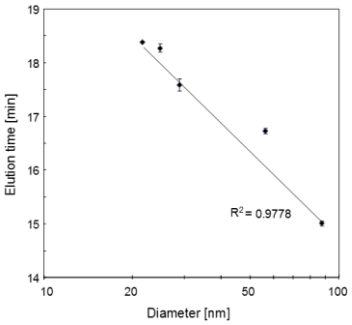

Fig. 2 Calibration curve for elution time versus nanoparticle size.

nanoparticles during analysis. The order of elution corresponded to the diameter of the nanoparticle: the largest nanoparticles (87 nm) eluted at 15.0 min, and the smallest nanoparticles (22 nm) eluted at 18.4 min. The elution times of the nanoparticles were negatively correlated (R^2 = 0.978) with the average diameters of the nanoparticles (Fig. 2). Similar results were obtained using an octadecyl-modified monolithic column (Fig. 3) and in other studies[19,20] using modified monolithic columns. Although the surfaces of the monolithic columns in those studies were modified with different groups, the elution profiles of the nanoparticles were similar. Hence, the modifying groups on the surface of a monolithic column have a negligible effect on the elution of nanoparticles, and the elution profile of the nanoparticles is primarily determined by the skeletal structure of the column. The size of the mesopores of the monolithic column was about 18 nm,[20] smaller than that of the nanoparticles used in this study. Therefore the mesopores did not affect the elution of the nanoparticles. The 250-mm columns were used for the separation of nanoparticles in this time. It is expected that the better separation will be obtained if the length of the column become longer.[23d]

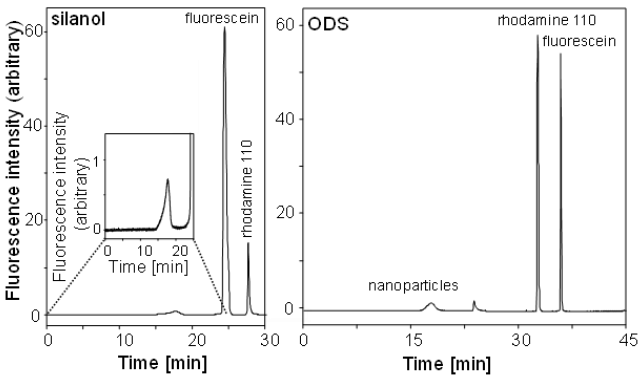

Fig. 3 Typical chromatograms for separation of nanoparticles and small molecules using unmodified (silanol) and ODS-modified monolithic columns.

Simultaneous Analysis of Nanoparticles and Small Molecules. Because the nanoparticles eluted before 20 min, we decided to change the mobile phase conditions after 25 min. We used normal-phase mode and a flow rate of 0.5 mL/min, because small molecules are not likely to break under high pressure and because the total time for analysis is reduced. Two fluorescent small molecules, fluorescein and rhodamine 110, were chosen as test molecules. Because the excitation and emission wavelengths of these molecules are similar (495 and 520 nm, respectively), it was necessary to separate them prior to measurement of their fluorescence intensities. Simultaneous separation of nanoparticles and small molecules was achieved at approximately 30 min with the unmodified column (Fig. 3). Fluorescein and rhodamine 110 eluted at 24 and 28 min, respectively. The relative standard deviation values for measurements of the elution time and peak areas were less than 0.6 and 4%, respectively, and these measured values were acceptable for further analysis. Hence, our method is suitable for the simultaneous analysis of nanoparticles and small molecules within 40 min.

Application of the Method to Evaluate and Compare Three Existing Purification Methods. The method was used to evaluate and compare three existing methods for purifying nanoparticles: (1) ultrafiltration, (2) dialysis, and (3) ultracentrifugation [two conditions: fast rotation (14,200*g*) and slow rotation (9,100*g*)]. Recovery ratios were calculated from the following equation:

recovery ratio = (peak area of compound after purification)/(peak area of compound before purification)

Ultrafiltration gave the highest recovery ratio followed by dialysis, ultracentrifugation at 14,200*g*, and ultracentrifugation at 9,100*g* (Fig. 4). The elution time for the nanoparticles was the same for all purification methods, indicating that the diameters of the eluted nanoparticles were the same.

The recovery ratio for ultrafiltration of the nanoparticles was greater than 90%. In the method, about 0.15 mL (15%) of solution was collected after ultrafiltration of 1 mL of sample solution. Hence, the expected recovery ratio for a compound that is not affected by the filtration film is about 15%. Although the recovery ratio for rhodamine was about 15%, that for fluorescein was about 50%. The high recovery ratio for fluorescein probably derives from electrostatic repulsion between the negatively charged fluorescein and the positively charged polyethersulfone film.

The recovery ratio for dialysis of the nanoparticles was approximately 65%. The principal reasons for the relatively low recovery ratio were (a) decreasing nanoparticle concentration with increasing solution volume during dialysis and (b) adsorption of nanoparticles to the dialysis film. The signals for the two small molecules were very small or below the detection limit after dialysis, indicating that these molecules were removed almost completely by the dialysis. We believe that dialysis is an effective technique for removing small molecules.

In the ultracentrifugation study, 500 μL of the dispersed solution was subjected to ultracentrifugation, and the entire bottom layer (100 μL) was collected; thus the recovery ratio of a compound that was unaffected by ultracentrifugation is 20%. The recovery ratios for the fast (14,200*g*) and slow (9100*g*) rotation conditions were about 55%

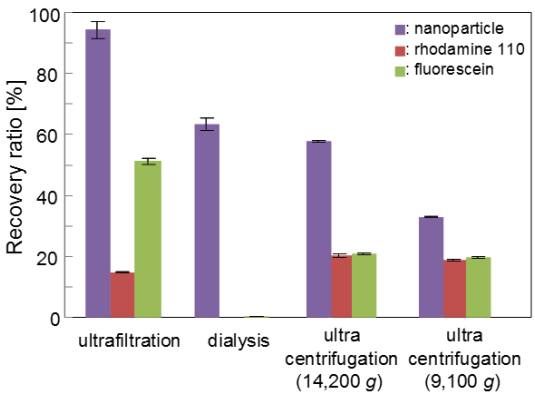

Fig. 4 Recovery ratios of nanoparticles and small molecules by three different purification methods.

and 30%, indicating that the recovery ratio increased as the rotation speed increased. The recovery ratios of both small molecules were 20%, and this value did not change with increasing rotation speed. Therefore, these small molecules were not affected under these rotation conditions.
These results indicate that simultaneous separation of nanoparticles and small molecules is useful for evaluating existing methods for purifying nanoparticles.

Analysis of Nanoparticle Stability. The quantity and size of nanoparticles, and leakage of small molecules from nanoparticles, are important in evaluating the quality of nanoparticles with encapsulated functional molecules. We used our method to evaluate the stability of nanoparticles after dialysis. Fig. 5 a) depicts the chromatograms of the nanoparticles solutions at different storage times. Because no clear differences were observed among the elution time and shape of these peaks, corruption or aggregation of the nanoparticles did not occur for 14 days storage. The analytical results of the dialyzed solution using DLS are shown in Fig. 5 b). These DLS results also indicated the good stability of the nanoparticles.
Peak areas of the nanoparticle and small molecules were compared at different storage times. As shown in Fig. 5c), about 60 and 0.3 % of nanoparticle and fluorescein, respectively, were remained after dialysis and the quantity of rhodamine 110 was below detection limit. The quantity of nanoparticle and rhodamine 110 did not changed during the storage time. Although the quantity of the fluorescein was decreased a little, the additional peak derived from degradates was not detected by diode array detection or fluorescence spectroscopy detection. Therefore leakage of the small molecules was negligible. These result indicated that the nanoparticles and small molecules were stable over that period. We conclude that our method is applicable for evaluating the stability of nanoparticles.

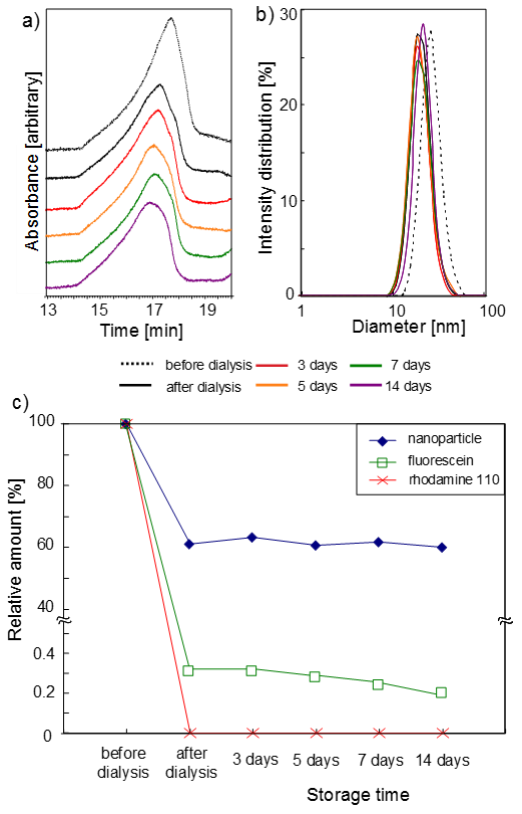

Fig. 5 a) Chromatograms and b) DLS analysis of the dispersed solutions of the nanoparticles with different storage times, and c) stability analysis of the nanoparticles and leakage assay of small molecules from the nanoparticles.

Conclusions

We have developed a simple and fast method for the simultaneous separation and analysis of nanoparticles and small molecules. The method uses the structural properties of a monolithic column for separation: nanoparticles are separated by micrometer-sized flow-through pores using the hydrodynamic mode, and small molecules are separated by mesopores using the normal-phase mode. Because the size and shape of the bimodal structure of the monolithic column, and the surface groups of the monolith, are independently tunable, the monolithic column has great potential for analysis of nanoparticles and will be invaluable in determining the safety and reliability of functional molecule–containing nanoparticles.

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