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Chromatography of carbon nanotubes separated albumin from other serum proteins: a method for direct analysis of their interactions

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Chromatography technology was employed to clarify the mechanism of interaction between multi-wall carbon nanotubes (MWCNT) and proteins. A column (16×100 mm) was packed with purified MWCNT, and various proteins were eluted with phosphate buffered saline (PBS) with and without gradient systems. It was found that albumin in bovine serum was eluted immediately from the column without any adsorption to MWCNT. Conversely, the non-albumin proteins, including a protein of 85 kDa molecular mass and a group of proteins with molecular masses higher than 115 kDa, exhibited considerably high affinity towards MWCNT. A sample of pure bovine serum albumin was also eluted immediately from the column, while lysozyme did not elute as a peak with PBS, but eluted with 0.6 M NaCl. Fundamentally, carbon nanotubes are devoid of any electrical charge. Therefore, other forces including the hydrogen bonds, hydrophilic interactions, and van der Waals forces were most probably responsible for the differential elution behaviors. In conclusion, this chromatographic method provided a simple and direct analysis of the interactions between carbon nanotubes and the various proteins.

Keywords: Carbon nanotubes, Proteins, Chromatography

INTRODUCTION

To explore, exploit, and expand the medical, dental, and biological applications of multi-wall carbon nanotubes (MWCNT), it is of paramount importance to have a clear and explicit understanding of the interaction between this novel material and proteins. This is because if and when the MWCNT is introduced into animal tissues or bodies —irrespective of the route of introduction, the first step of interaction is well foreseen to be the encounter between CNT and the cell-surface proteins or protein components of the body fluids. Such an inevitable encounter then raises the alarm of potential harmful effects posed by CNT in its applications. Against this backdrop, several research efforts have been devoted to elucidating the interaction between MWCNT and cultured cells.

Indeed, researchers have identified a strong adhesive interaction between MWCNT and cultured cells, thus suggesting that some cell-surface proteins might interact with CNT. However, which protein(s) is(are) responsible for this strong adhesion remains an unresolved question[1,2]. To compound the intrigue surrounding the interaction between CNT and proteins, there remains a lack of a definitive tool and approach to investigate the interaction mechanism.

A reason for this protracted absence of a definitive investigative tool stems from the fact that MWCNT is essentially an insoluble solid-phase material, whereas most proteins are studied in solutions. Straddled between solid MWCNT and protein solutions, new systems are hence needed to study their interaction mechanisms. To negotiate this standoff between solid and liquid entities, we resorted to the aggregation phenomenon of MWCNT which was triggered by the addition of collagen molecules to a stable suspension of CNT[3]. With an aggregation meter, it was found that native collagen induced distinct aggregation with MWCNT, but not so for denatured collagen[3]. Such clear difference from native collagen was observed for albumin and lysozyme proteins, which also did not cause CNT to aggregate. Findings of our previous study[3] hence heightened the need to develop a more general methodology to clarify the interactions between solid MWCNT and proteins in solution.

In this study, conventional column chromatography was employed to investigate the MWCNT-protein interaction. The aims of this investigation were to: (1) test the efficacy of MWCNT-packed chromatographic columns in separating protein mixtures such as serum; and (2) determine the elution profiles of various standard proteins. Results of this study would then render an objective verdict if chromatography was a simple and direct method and a highly useful tool for comparative studies of individual interactions between various proteins and MWCNT.

MATERIALS AND METHODS

Reagents

Albumin (bovine serum albumin, Sigma-Aldrich, Tokyo, Japan), lysozyme (chicken egg white lysozyme, Sigma-

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Aldrich, Tokyo, Japan), and bovine serum (fetal bovine serum, Cansera International, Toronto, Canada) were commercially purchased and stored at −20°C until use.

Preparation of columns for chromatography

Multi-wall carbon nanotubes (diameter: 20−30 nm; length: 30−50 μm) were generously contributed by CNT Co. Ltd. (Incheon, Korea). They were processed with heat (500°C, 90 minutes) and acid (12 N HCl, 6 hours at room temperature) treatments, each followed with extensive washing using distilled water. To obtain powder particles with sizes ranging roughly between 100 and 400 μm, the heat- and acid-treated carbon nanotubes were ground using an agate mortar and pestle. To obtain a bed volume of 16×100 mm, purified multi-wall carbon nanotubes (3.0 g) were packed into a commercial glass column for chromatography (XK 16/20, GE Healthcare, Tokyo, Japan).

Chromatography elution systems

The column was washed with at least five column volumes (60 ml) of 6 M urea/0.05 M Tris-HCl, pH 7.4, and then with Dulbecco's physiological saline (PBS) at a flow rate of 300 ml/hour using a ceramic pump (VSP-3200W, Eyela, Japan).

To analyze the elution profile of fetal bovine serum (FBS, CCT), a sample of 2 ml was applied to the column. The latter was first eluted with 60 ml of PBS, then with a straight gradient system consisting of PBS and 25 mM NaOH at 150 ml each. In other words, 25 mM NaOH was added to and mixed with PBS at the same rate with which the mixture was run out of the column. Elution was monitored manually using a UV spectrophotometer at 230 nm or by an automatic UV monitor system at 254 nm (AC-5100, Atto Co., Japan). Chromatographic peaks were then analyzed by SDS gel electrophoresis.

To analyze the elution profiles of the standard proteins, albumin and lysozyme, the column was eluted first with PBS, then with 1 M NaCl. For the mixture of albumin and lysozyme, the column was eluted with the gradient system consisting of PBS and 1 M NaCl. Elution was monitored at 254 nm by an automatic UV monitor system. All the chromatographic experiments were confirmed by repeating several times.

SDS gel electrophoresis

Electrophoresis in the presence of 0.1% SDS was carried out on a 7.5% polyacrylamide gel stained with Coomassie Brilliant Blue. Pre-stained protein markers of known molecular masses (Apro Co., Tokushima, Japan) were run on the same gel.

RESULTS

SEM images of MWCNT

MWCNT, used as the chromatographic column packing material in this study, appeared as irregular granules with a porous structure when observed under SEM (Fig. 1A). Particle sizes of the granules ranged from 100 to 400 μm (Fig. 1A). At a higher magnification, each granule was shown to comprise the characteristic fibrous network of MWCNT (Fig. 1B).

Most probably, the porous structure of MWCNT facilitated the rapid flow of elution buffer at a rate of 300 ml/hour with low pressure (less than 50 kPa). This flow rate was selected because same patterns of separation were obtained when compared to those obtained with a flow rate lower than 300 ml/hour.

Elution profile of fetal calf serum

Figure 2 shows an elution profile of fetal calf serum obtained from the column filled with MWCNT, while Fig. 3 shows the electrophoretic patterns of the eluted peaks. Peak A was the breakthrough fraction, which was not adsorbed on MWCNT. The electrophoretic pattern of Peak A was almost the same as the original serum, exhibiting albumin bands as the major

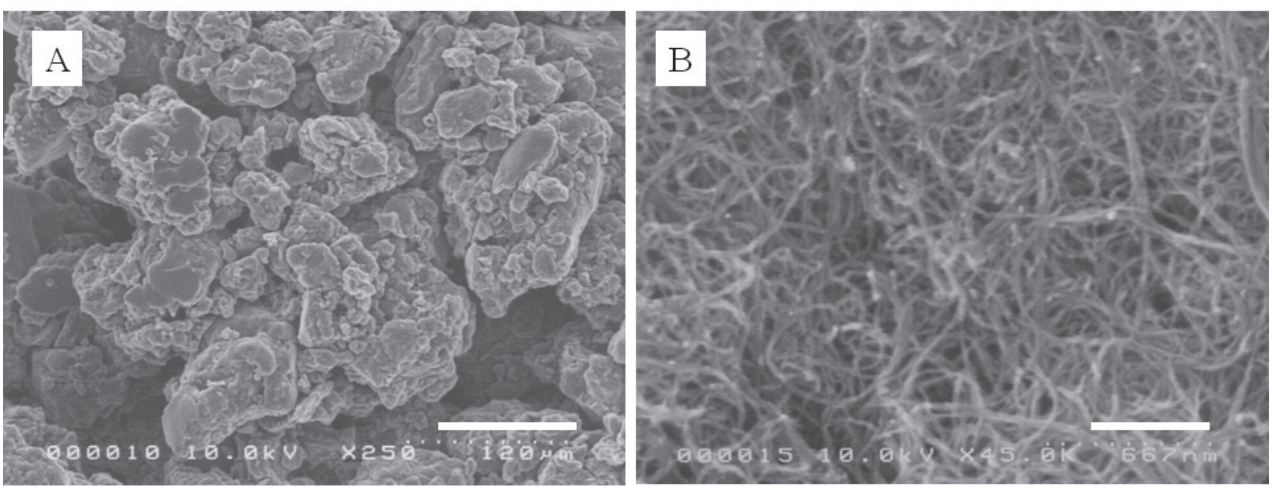

Fig. 1 SEM images of MWCNT used in the column chromatography at a lower magnification of ×250 (A) where the bar indicates 0.1 mm, and at a higher magnification of ×45,000 (B) where the bar indicates 500 nm.

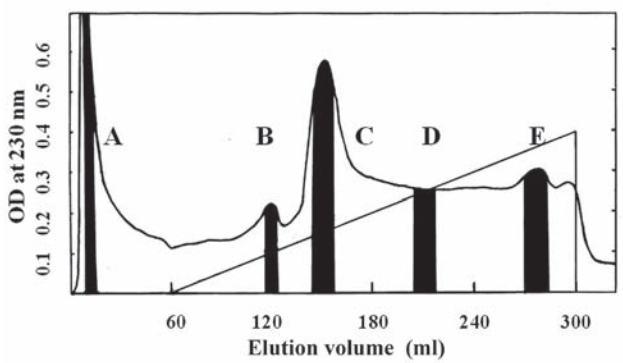

Fig. 2 Elution profile of bovine serum proteins from a chromatographic column packed with MWCNT.

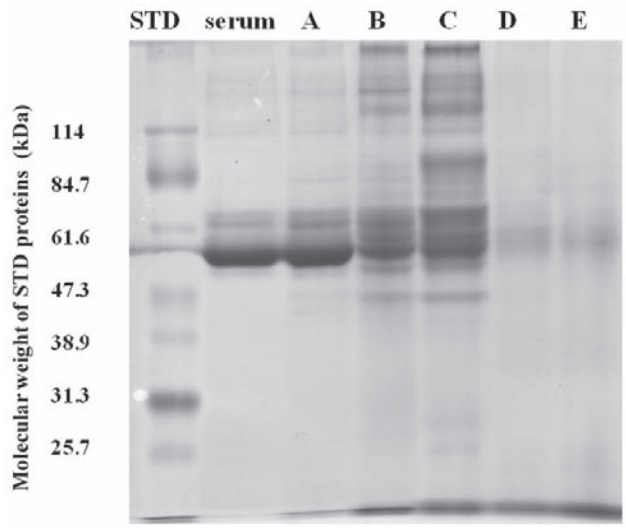

Fig. 3 SDS gel electrophoretic patterns of the chromatographic peaks in Fig. 2.

components.

As for Peaks B and C, their electrophoretic profiles were quite different from those of the original serum and Peak A. Peaks B and C had numerous bands of higher molecular weight components than that of albumin (60 kDa). Most notably, two distinct bands slightly above the 114 kDa molecular mass marker were observed for Peaks B and C, and which were not clear in the electrophoretic pattern of the total serum. In addition, another distinct band corresponding to 87.9 kDa molecular mass marker was observed for Peak C.

Elution profiles of standard proteins

Figure 4 shows the elution profiles of bovine serum albumin (A), chicken egg white lysozyme (B), and their mixture (C). A sample of pure bovine albumin (5 mg) eluted after approximately 1 minute (6 ml), and no

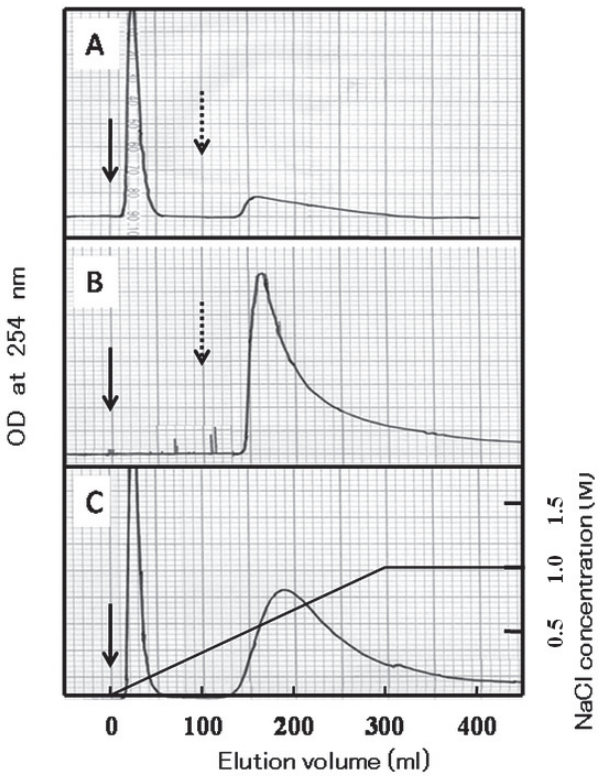

Fig. 4 Elution profiles of pure bovine serum albumin (A), chicken egg white lysozyme (B), and the mixture (C) of albumin (5 mg) and lysozyme (10 mg). Arrows indicate the injection of the sample and dotted arrows the start of 1 M NaCl elution.

protein peak was detected after 1 M NaCl was applied to the column, indicating that albumin did not adsorb to the MWCNT column in this system (A). On the other hand, lysozyme eluted only after 1 M NaCl was applied (B), showing a certain affinity of this protein to MWCNT in this system.

As for the mixture of albumin (5 mg) and lysozyme (10 mg), it was loaded onto the column with a gradient elution system consisting of PBS and 1 M NaCl (100 ml each). Albumin eluted in the breakthrough fraction and lysozyme with 0.6 M NaCl, resulting in a reasonable separation of the two proteins. In a preliminary experiment, bovine myoglobin was loaded onto the same MWCNT column under the same conditions. This protein then eluted in the breakthrough fraction, indicating that there was little or no affinity towards the MWCNT column.

DISCUSSION

Several authors[4-6] have discussed the possible use of MWCNT-packed column in chromatography, but only a few reports have described the actual separation of proteins by MWCNT chromatography. According to our literature survey, MWCNT has been used as an

extraction sorbent for determination of trace copper in water samples[7], or as an absorbent for trapping volatile organic compounds from environmental samples[8,9]. To the best of our knowledge, Du *et al.*[10] were the first to demonstrate the separation of hemoglobin and cytochrome C by means of a tiny column (2.3×5 mm) packed with modified MWCNT. Surface modification of MWCNT was done *via* nitric acid treatment, which was a strong oxidant, to induce a negative charge on the surface of MWCNT[10]. In another study[11], Du *et al.* also demonstrated that PDDA [poly(diallyl dimethyl ammonium chloride)]-modified MWCNT could trap acidic proteins such as bovine serum albumin.

In recent years, rapid advances in nanotechnology have turned the attention on the possible biological applications of MWCNT. In other words, there arises an urgent need for a good knowledge on the interaction between intact (chemically unmodified) MWCNT and proteins, especially cell-surface proteins[1-3]. In this paper, the classical column chromatography method —which entailed the use of a MWCNT-packed column— proved to be useful for the separation of serum proteins.

In the present study, the purity of the as-obtained MWCNT material was 96%, which meant that it might still contain impurities such as Fe and amorphous carbon. Therefore, a recommended purification method using acid and heat treatments was performed. Although treatment in concentrated HCl and heating at 500°C were generally believed to have no effect on MWCNT structure, there remained a slight possibility of the presence of carboxylic acid and hydroxyl groups after the acid treatment. To confirm the presence of these functional groups on the surface of MWCNT after the purification process, further studies need to be carried out in the future.

The electrophoretic profile of Peak A and that of serum in Fig. 3 were very similar, indicating that major serum protein components including albumin had eluted in the breakthrough fraction. Peak A was the breakthrough fraction, which was not adsorbed on MWCNT. Its electrophoretic pattern closely resembled that of the original serum, exhibiting albumin bands as the major components. This was probably the result of an excessive amount of serum proteins over the adsorption capacity of MWCNT column.

As for Peaks B and C, their electrophoretic patterns were clearly different from those of Peak A or the serum, indicating the presence of higher molecular weight components. Peaks B and C contained many bands which corresponded to proteins or peptides with molecular masses higher than 114 kDa. In particular, a distinct band of approximately 84.7 kDa detected in Peak C was not detected in the original serum or Peak A. These results clearly showed that some of the serum proteins had selectively adsorbed on MWCNT until the NaOH concentration reached a certain percentage (approximately 10 mM judging from the slope of the gradient). For albumin, its corresponding band clearly decreased in intensity in Peaks B and C. As for the group of high molecular weight serum proteins adsorbed on MWCNT, further analysis is needed for their identification. Nonetheless, some possible candidates were anticipated to be immunoglobulin (18–19 kDa)[12], transferrin (75 kDa)[13], α2-macroglobulin (720 kDa for tetramer)[14], and fibrinogen (340 kDa)[15].

Chromatograms of pure bovine serum albumin and lysozyme in Fig. 4 showed that albumin was not able to bind to MWCNT under the elution conditions of the present study. Albumin eluted in the breakthrough fraction (A), while lysozyme eluted only after elution with 1 M NaCl (dotted arrow) —which commenced at 12 minutes after the sample was applied (B). Differential elution of these two proteins was further demonstrated by the gradient elution system (C), in which a mixture of albumin and lysozyme was applied (C). Under the gradient elution conditions, albumin eluted in the breakthrough fraction and lysozyme with 0.6 M NaCl.

Albumin has been identified as the major protein component of serum, constituting more than 66% of total serum protein. For this reason, it is oftentimes desirable to remove albumin in order to perform a detailed analysis of the other serum proteins, especially for proteomic analyses for clinical diagnostic purposes. While an MWCNT column might seem to be an attractively simple and practical method to eliminate albumin, there remained two opposing arguments against its use. First, not many publications are available on the application of MWCNT for liquid chromatography of proteins, and hence a critical lack of knowledge and scientific credibility for this application. Moreover, the lack of a sufficient supply of pure MWCNT poses another challenge. However, the latter situation has improved immensely recently.

In general, carbon nanotubes are devoid of electrical charges. Therefore, other intermolecular forces apart from electrostatic forces, such as hydrogen bonding, hydrophilic interactions, and van der Waals forces most probably contributed to the MWCNT-protein interaction mechanism[3], on the assumption that the MWCNT material used in this study was completely pure. However, as afore discussed, there remained a slight possibility of the presence of electric charges on the surface of MWCNT. In a study by Kwon and Park on the retention times of various low molecular weight volatile organic compounds such as 1-butanol, benzene, or 1,4-dioxane in a chromatographic column packed with MWCNT[5], the roles of hydrogen bonding, lipophilicity, and dipolarity of those compounds were discussed in detail to account for their interactions with MWCNT. However, there were no conclusive discussions on the interaction between intact MWCNT and proteins in their report[5] nor in any other published literature.

In this study, lysozyme —a basic protein with a pI value of 11— exhibited an affinity toward MWCNT, whereas albumin —a typical acidic protein with a pI value of 4.9— showed no affinity. In addition,

preliminary experiments showed that no adsorption of bovine myoglobin (pI 7.0) occurred on the MWCNT column. Taken together, these findings seemed to suggest of a probable link between the pI values of proteins and their retention in MWCNT column. However, to confirm this hypothesis, the use of a systematic approach involving various proteins would need to be conducted through further investigations in the laboratory.

On the superiority of MWCNT chromatography over other methods of protein analysis such as electrophoresis, one such advantage lies in the gross separation of albumin from other serum proteins as shown in this study. Another advantage is that the MWCNT-packed column is highly stable for repeated uses, since MWCNT is a stable material chemically and mechanically. Finally, the inherently small size of MWCNT allows for a small MWCNT column, which then renders the system more suitable for small-scale analyses.

CONCLUSION

Results of this study indicated that MWCNT showed definite selectivity in the adsorption of serum protein components. Albumin was not adsorbed on MWCNT, while non-albumin proteins —including a protein of 85 kDa and a group of proteins with molecular masses higher than 115 kDa— exhibited considerably high affinity towards MWCNT.

The phenomenon of selective adsorption of serum proteins on the MWCNT column might pave the way for a new separation system between albumin and other serum proteins. While the aforementioned new system has yet to be realized, this simple chromatography method has nonetheless proven to be a feasible and useful tool for investigating the mechanism of interaction between MWCNT and various proteins.

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