DOI: 10.1002/chem.200700784

The Isolation of Basic Proteins by Solid-Phase Extraction with Multiwalled Carbon Nanotubes

Zhuo Du, Yong-Liang Yu, Xu-Wei Chen, and Jian-Hua Wang*[a]

Abstract: Multiwalled carbon nanotubes (MWCNTs) have been employed for the first time as sorbents for the isolation of basic proteins from other protein species in biological sample matrices by solid-phase extraction (SPE). A microcolumn packed with MWCNTs was incorporated after appropriate pretreatment into a sequential injection system, which facilitates online selective sorption of basic protein species (hemoglobin and cytochrome *c* in this particular case). The retained protein species were afterwards separated from each other by sequential elution from the microcolumn through the employment of appropriate eluents. A 0.025 mol L^{-1} phosphate buffer solution of pH 8.0 facilitated the efficient collection of hemoglobin, while a 0.5 mol L^{-1} NaCl solution ensured the quantitative recovery of the retained cytochrome *c*. With a sample loading volume of 2.0 mL, enrichment factors of 11 and 15 were derived for hemoglobin and cytochrome *c*, along with retention efficiencies of 100% for both species and recovery rates of 98 and 90%, respectively. A sampling frequency of 8 h^{-1} was achieved, and the precisions were 3.0% and 0.8% (RSD) for hemoglobin and cytochrome *c* at a concentration of 5.0 μg mL^{-1}. The practical applicability of this system was demonstrated by processing of human whole blood for isolation of hemoglobin, and satisfactory results were obtained by assay with SDS-PAGE.

Keywords: human blood · nanotubes · proteins · solid-phase extraction

Introduction

During the last decade, various protein analysis schemes and proteomics techniques for understanding of various biological problems associated with proteins have been established.[1] However, in many cases it is difficult to identify the proteins of interest, due either to insufficient sensitivity of detection techniques or to matrix interference. Thus, matrix removal and/or protein isolation/preconcentration are frequently called for. At this juncture, solid-phase extraction is among the widely employed alternatives for protein separation and preconcentration, with its advantages of high efficiency, low running cost, simplicity, ease of automation, and avoidance of the use of toxic organic solvents.[2] However, only a very limited choice of sorbents for protein species is available at present, so the exploitation of novel sorbent materials with satisfactory extraction efficiency and selectivity for protein species is highly desirable.

[a] Z. Du, Y.-L. Yu, Dr. X.-W. Chen, Prof. Dr. J.-H. Wang
Research Center for Analytical Sciences, Box 332
Northeastern University, Shenyang 110004 (China)
Fax: (+86) 24-83676698
E-mail: jianhuajrz@mail.edu.cn

Carbon nanotubes (CNTs), novel and interesting carbon-based materials discovered in the early 1990s, are known either as graphite sheets rolled up into nanoscale tubes (single-walled carbon nanotubes, SWCNTs),[3] or with additional graphite tubes wrapped around the cores of SWCNTs (multiwalled carbon nanotubes, MWCNTs).[4] Since the appearance of these special materials, they have attracted extensive attention, due to their novel electronic, mechanical, and chemical properties, as well as their vast potential in applications as biosensors[5,6] or gas sensors.[7,8] They also constitute promising sorbent materials employed for solid-phase extraction of various species, because of their unique surface properties and large specific surface areas.[9,10] During the last few years, a few comprehensive reviews have been dedicated to CNT-related chemistry.[11,12]

In general, the walls of the CNTs are not active, but on the other hand their fullerene-like tips are well documented to be much more reactive. End-functionalization of CNTs has thus frequently been used to generate various functional groups required for specific purposes (e.g., -COOH, -OH, or -C=O).[13] It can be adopted to attachment of small molecules or biochemically active species onto the surfaces of CNTs, including the immobilization of macrobiomolecules such as DNA[14] and proteins[15] on SWCNTs. Recently, a

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim WILEY InterScience DISCOVER SOMETHING GREAT

number of investigations have been directed towards the employment of CNTs for biological purposes and towards the introduction of CNTs into various biological systems, in which the biocompatible natures of the CNTs were demonstrated.[16–18] In addition, a number of reports concerning the interactions between CNTs and biological molecules such as DNA,[14,19] peptides,[20] and proteins[21,22] can be found in the literature published during the last few years. Furthermore, chemically functionalized carbon nanotubes have recently been exploited as substrates for interfacing with biology (e.g., for cultured neurons or neuronal growth).[23]

CNTs offer large and biocompatible surface areas, which provide not only vast potential for biological applications, but also promising prospects in separation science. At this point in time, a survey of the published literature provides a range of applications of CNTs serving as solid-phase extraction media for the separation/isolation of organic contaminants[24,25] and trace metal species.[26] MWCNTs have also been used as packing materials for the preconcentration of organic compounds by solid-phase extraction followed by chromatographic separation of the isolated organic species by liquid chromatography[27–29] or gas chromatography.[30] Up to now, only a very few investigations of MWCNTs as efficient stationary phases for gas chromatography for the separation of gaseous organic compounds have been demonstrated.[31,32]

So far, no reports about the isolation and preconcentration of basic protein species based on solid-phase extraction on the surfaces of carbon nanotubes and the separation of protein components with different charge properties are to be found. In the work reported here, multiwalled carbon nanotubes were employed as sorbents for the isolation and preconcentration of basic proteins (e.g., cytochrome *c* and hemoglobin (bovine)), followed by their separation from each other. Both species are positively charged and were isolated from the sample matrix by adsorption onto the surface of a microcolumn packed with MWCNTs possessing negatively charged surfaces. Cytochrome *c* and hemoglobin were collected by sequential elution from the column by changing the pH and ionic strength of the eluents. The extraction mechanism is believed to be an electrostatic interaction, in contrast with the hydrophobic interaction discussed previously.[22] The practical applicability of the procedure has been validated by extraction of the protein species of interest from human blood, monitored by SDS-PAGE.

Results and Discussion

Isolation of basic proteins by use of modified carbon nanotubes as sorbents: Our preliminary investigations showed that within an appropriate pH range, the surfaces of carbon nanotubes could effectively retain basic protein species, thus providing a potential medium for isolation of protein species of interest from biological sample matrices. The adsorption of proteins onto solid surfaces is a complex process in which various driving forces might be involved, including hydrophobic interactions, electrostatic interactions, and hydrogen bonding. Generally, hydrophobic and electrostatic interactions tend to dominate the entire process.[33]

The hydrophobic natures of untreated surfaces of carbon nanotubes are well documented.[34,35] At the very beginning it was thus quite reasonable to conclude that the driving force for the adsorption of proteins was attributable to hydrophobic interaction, and that theoretically a maximum retention capacity should be attained at the isoelectric points of the proteins involved, being further improvable by increasing the salt concentration/ionic strength of the sample solution. In order to ascertain whether or not this assumption was indeed valid, bovine serum albumin (BSA, isoelectric pH 4.7) was selected as a model protein to undergo the sorption process at pH 4.7 in a medium consisting of acetate buffer solution ($1.0\,mol\,L^{-1}$). The experimental results revealed no obvious sorption of BSA onto the MWCNTs surface, which was obviously counter to the above assumptions (i.e., hydrophobic interactions were not the main driving force for protein adsorption in this system). This observation led us to consider that the protein adsorption onto carbon nanotubes might be attributable to electrostatic interaction and that an ion-exchange mechanism was involved in the elution/desorption process.

Since peptides, proteins, nucleic acids, and related macrobiomolecules contain ionizable chemical moieties, which render them susceptible to charge enhancement or charge reversion as a function of pH, ion exchange is a widely used technique in bioseparations.[36] When the pH values of sample solutions have been changed, protonation or deprotonation of protein species has been encountered. Depending on the relative ionic charges of the proteins, some components will be retained on the surfaces of MWCNTs, while others will tend to remain in solution. At this point, it could be expected that the pH value of the reaction medium should affect the efficiencies of protein extraction. In addition, through appropriate choice of the elution conditions, sequential elution or separation of protein species from each other might be facilitated through ion exchange in a suitable medium (i.e., their successive stripping from the MWCNT-packed microcolumn in this case).

The surfaces of MWCNTs oxidized with nitric acid are negatively charged at pH 6,[37] whereas hemoglobin (pI 7) and cytochrome *c* (pI 10) are both positively charged. Electrostatic interactions existed between the MWCNT surfaces and the basic proteins, and so their adsorption onto the MWCNT surfaces occurred. Further experiments indicated that pH values of the sample solutions in the pH 5–6 range result in sorption of hemoglobin and cytochrome *c* onto the MWCNT surfaces with favorable retention efficiencies. On the other hand, it has been shown that the electrostatic repulsions of the negatively charged MWCNT surfaces tend strongly to prevent the adsorption of acidic proteins. As an example, BSA is negatively charged at pH 6, and virtually no retention of BSA on the MWCNT-packed microcolumn was observed. This observation provides a potential ap-

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

proach for the separation of acidic and basic protein species simply by adjusting the acidity of the sample solution and the surface properties of the MWCNTs. In this investigation, deionized water of pH 5.8 was employed as a carrier solution to maintain the required pH value for the retention of both hemoglobin and cytochrome *c*.

The various experimental parameters affecting the sorption and elution of basic proteins: Solid-phase extraction has become a very popular separation/purification and preconcentration approach for macrobiomolecules. Generally, the experimental parameters tend to affect the retention efficiencies of the species of interest, the enrichment factors, and the gradient/sequential isolation of similar species. These factors include the nature of the target species, the surface properties of the packing material/sorbent, the acidity of the reaction system, the sampling flow rate, and the appropriate choice of eluents. When employing MWCNTs as a sorbent medium for the separation of proteins from complex matrices of biological origin, it is well worth scrutinizing these parameters.

It is imaginable that a microcolumn packed with very small-sized particles of MWCNTs might readily create flow resistance during the sample loading and elution process. This was demonstrated by use of a microcolumn with a packing length of 20 mm for the sorption of proteins at a sample loading flow rate of 40 $\mu L\,s^{-1}$, when significant flow impedance was encountered. A shorter column length was therefore preferential in this investigation, so a microcolumn length of 5 mm was adopted for the ensuing experiments as detailed in the Experimental Section.

When pretreating macrobiomolecules by solid-phase extraction, it is necessary to keep the ionic strength of the sample medium at a certain level. On the other hand, a higher ionic strength of the buffer solution often tends to hamper the retention performance of the sorbent.[38] The sorption of cytochrome *c* and hemoglobin by the MWCNT-packed microcolumn was investigated in media with ionic strengths of 0.001–0.1 $mol\,L^{-1}$ (phosphate buffer solutions; PBS) at pH 6. The experimental results indicated that fairly good adsorption of both cytochrome *c* and hemoglobin was achieved by maintaining the PBS concentration at levels lower than 0.025 $mol\,L^{-1}$. Thereafter, an increase in the buffer concentration resulted in a significant decline in the retention efficiency of hemoglobin, while no significant variation in the retention of cytochrome *c* on the MWCNT surface was observed with ionic strengths up to 0.05 $mol\,L^{-1}$. Therefore, for further experiments, the ionic strength of the PBS solution was maintained at 0.025 $mol\,L^{-1}$ in order to maintain higher sorption efficiencies for both species.

One of the main purposes of this study was to retain basic proteins effectively on the surface of the MWCNT-packed microcolumn while other proteins were eliminated, followed by separation of the species of interest through their sequential elution from the MWCNT surface. The selection of appropriate eluents is therefore the most critical for this specific requirement.

Our preliminary experiments had demonstrated that hemoglobin could be readily stripped from the MWCNT column by employment of a PBS of a certain pH value and ionic strength, while under the same conditions cytochrome *c* tended to remain on the column surface but could readily be eluted afterwards with a solution of sodium chloride. As favorable retention of both protein species was achieved in the range of pH 5–6, the elution should preferentially be performed at a higher pH value. Figure 1 illustrates

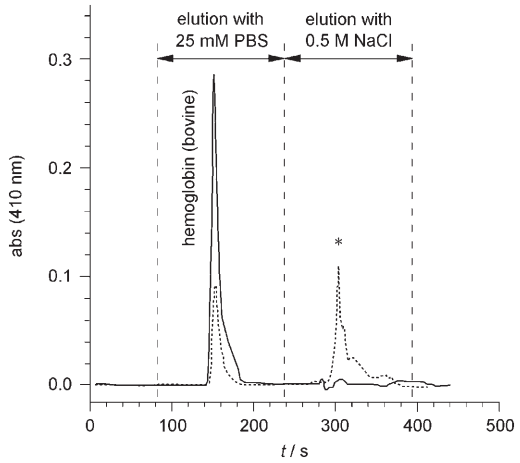

Figure 1. Effect of pH on the elution of hemoglobin from the MWCNT surface: ——: pH 8.0, -----: pH 7.2. The peak marked with an asterisk represents that proportion of the hemoglobin adsorbed onto the MWCNT surface that was not eluted by use of a PBS solution of pH 7.2 (0.025 $mol\,L^{-1}$). Sampling volume: 2 mL. Volumes of eluent 1 and eluent 2: 200 μL. Concentration of hemoglobin: 10 $\mu g\,mL^{-1}$.

the elution of hemoglobin at pH 7.2 and pH 8.0 with use of a 0.025 $mol\,L^{-1}$ PBS solution. It is clear that at pH 7.2 only approximately half of the retained hemoglobin could be collected, but when a higher pH of the PBS (pH 8.0) was adopted, quantitative elution of the retained hemoglobin could readily be achieved. In the meantime, elution of cytochrome *c* was not observed on employment of PBS of an identical concentration with pH values lower than 8. For further experiments, a 0.025 $mol\,L^{-1}$ PBS of pH 8 was employed for the collection of retained hemoglobin from the MWCNT-packed microcolumn.

It has been demonstrated that sodium chloride is an effective stripping reagent for protein species retained on the surface of a MWCNT-packed microcolumn. It was thus employed to recover the adsorbed cytochrome *c* after the complete elution of hemoglobin by use of a PBS. As illustrated in Figure 2, when a flow rate of 10 $\mu L\,s^{-1}$ was employed with 200 μL of eluent as detailed in the Experimental Section, a significant improvement in the elution efficiency or recovery rate was obtained on increasing the concentration of eluent (i.e., NaCl solution, in a range of 0.1–0.5 $mol\,L^{-1}$), and a recovery rate of ca. 90% was achieved at an eluent concentration of 0.5 $mol\,L^{-1}$. Thereafter, further improvement of the elution efficiency was not significant with a further increase in the eluent concentration up to 1.0 $mol\,L^{-1}$. A lower con-

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

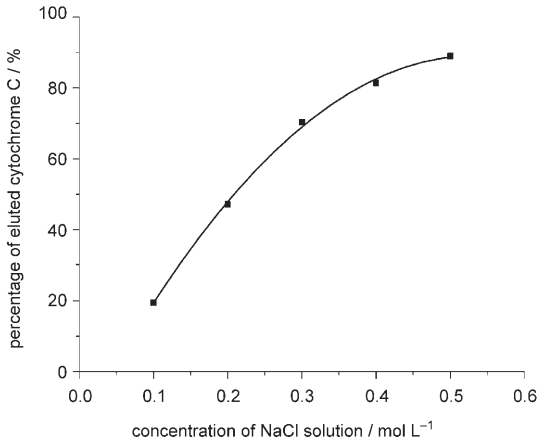

Figure 2. Effect of NaCl concentration on the elution efficiency of cytochrome *c*. Sampling volume: 2 mL. Eluent (NaCl) volume, 200 μL. Concentration of cytochrome *c* in the original solution: 5 μg mL^{-1}.

centration of NaCl in the collected protein solution was preferential in order to minimize its potential influence on the ensuing biological investigations, while at the same time, a higher recovery rate was pursued for this procedure. It was therefore decided that an appropriate (or compromise) concentration of the eluent should be selected. At this point, a NaCl concentration of 0.5 mol L^{-1} was adopted for the subsequent studies.

The volume of eluent employed is obviously an important parameter for the complete stripping of the retained protein species. The experiments had shown that for the two-step elution process, fairly high recovery rates (i.e., 90–100%) could readily be achieved for both hemoglobin and cytochrome *c* by varying the eluent volume within the range of 100–400 μL. In practice, a smaller eluent volume (i.e., 200 μL) was used to produce a relatively higher enrichment factor. It can be seen from Figure 2 that after the elution with 200 μL NaCl solution (0.5 mol L^{-1}), some residual protein (cytochrome *c*) still remained on the MWCNT surface, which results in a carry-over for the next sample processing, and this might be an important error source when the quantification of protein species was pursued. Therefore, after each operation cycle, an additional elution or post-washing was performed with an extra 200 μL NaCl solution to clean the MWCNT-packed microcolumn.

The sample loading flow rate is another critical factor in the solid-phase extraction process. The flow rate not only has a significant effect on the retention efficiency of proteins, but also determines the duration of the entire analytical process. The experimental results indicated that lower sampling flow rates facilitate higher retention efficiencies of hemoglobin and cytochrome *c* on the MWCNT-packed microcolumn, but obviously require longer processing times. On the other hand, when the sampling flow rate was increased to 40 μL s^{-1}, flow impedance was encountered and a significant deterioration in the performance of the MWCNT microcolumnn (i.e., retention efficiency) was observed. As a compromise, a sample loading flow rate of 30 μL s^{-1} was employed for subsequent investigations.

The effect of the elution flow rate on the collection efficiencies of hemoglobin and cytochrome *c* in the 2–15 μL s^{-1} range was investigated. This indicated that much sharper peak shapes were recorded for both protein species with an increase in the eluent flow rate, while the peak areas remained virtually constant and no significant changes in the recovery rates were observed. That is to say, the effect of the elution flow rate is not significant in relation to other experimental parameters. For further investigations, an elution flow rate of 10 μL s^{-1} for both protein species was thus employed. Figure 3 illustrates a typical sample loading and protein isolation procedure followed by sequential elution and protein separation with use of hemoglobin and cytochrome *c* as model proteins.

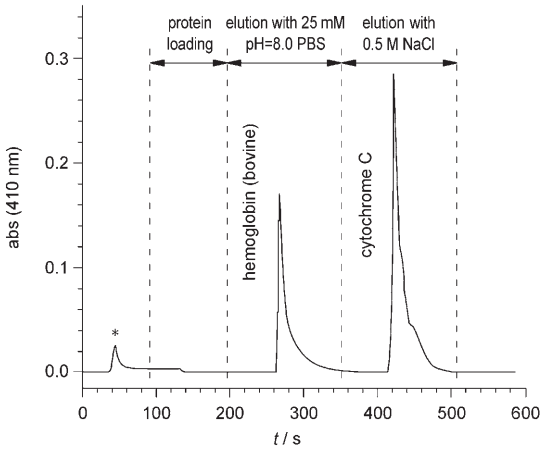

Figure 3. A typical operating process including sample loading and protein isolation from the sample matrix, with sequential elution of proteins by selection of elution conditions with hemoglobin and cytochrome *c* as model proteins. The peak marked with an asterisk is attributable to the original sample solution containing hemoglobin (5 μg mL^{-1}) and cytochrome *c* (5 μg mL^{-1}) without pretreatment by this system. Sampling volume: 2 mL. Volumes of eluent 1 and eluent 2: 200 μL.

Analytical performance of this system and its applications to real world sample pre-treatment: This investigation was focused on the isolation of basic protein species from other proteins and sample matrices in biological samples, followed by effective separation of each basic protein species by sequential elution from the surface of the MWCNT-packed microcolumn. The collected proteins (i.e., hemoglobin and cytochrome *c*) could be used for further biological investigations. In addition, another key point of the procedure is the post-analysis of the two protein species. Thus, appropriate attention should be paid to the characteristic analytical performance data of this system, summarized in Table 1.

It is clear that when a sample volume of 2.0 mL was employed, the enrichment factors obtained for hemoglobin and cytochrome *c* were 11 and 15, together with retention efficiencies of 100% for both species and overall recovery rates of 98% and 90%, respectively. Limits of detection of 0.12

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Characteristic performance data of this SPE system for basic protein separation and preconcentration.

	Hemoglobin	Cytochrome *c*
sampling frequency [h^{-1}]	8	
sampling volume [mL]	2	
eluent volumes [μL]	200	200
linear ranges [$\mu g mL^{-1}$]	0.4–10	0.2–15
sorption capacity [$mg g^{-1}$]	31	24
enrichment factors	11	15
retention efficiencies [%]	100	100
recovery rates [%]	98	90
RSDs (%, 5 $\mu g mL^{-1}$, $n=11$)	3.0	0.8
LODs (3 σ, $\mu g mL^{-1}$)	0.12	0.06

and 0.06 $\mu g mL^{-1}$ were achieved for the two protein species within linear ranges of 0.4–10 $\mu g mL^{-1}$ (hemoglobin) and 0.2–15 $\mu g mL^{-1}$ (cytochrome *c*), along with precisions of 3.0 and 0.8%, respectively, at a concentration level of 5 $\mu g mL^{-1}$. Figure 4 illustrates the recorded peak shapes for 11 repetitive operations, demonstrating well the suitability of the system for long-term operation. This demonstrates potential for its employment in processing real-world biological samples.

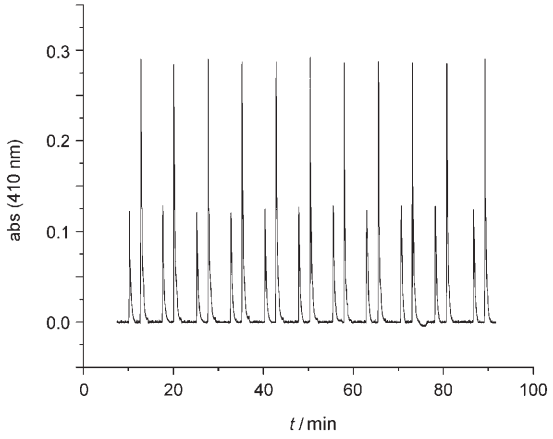

Figure 4. Recorded peak shapes for 11 repetitive sampling runs with this system and 2.0 mL sample solutions containing hemoglobin and cytochrome *c* (5 $\mu g mL^{-1}$).

The feasibility of this system for sorption of basic proteins from biological sample matrices was demonstrated by isolation of hemoglobin from a human whole blood sample.

The human whole blood sample was collected in an additive-free tube from a volunteer and anticoagulated with sodium citrate. The anticoagulated blood was stored in a refrigerator at 5°C. A certain amount of blood (100 μL) was drawn and a 100-fold dilution with deionized water was then carried out. The erythrocytes in the diluted solution were broken, releasing hemoglobin into the supernatant fluid; which and this supernatant fluid was then collected by centrifugation and diluted with deionized water to fit the linear dynamic range of the procedure. Afterwards the extraction process as detailed in the previous section was followed and the recovered hemoglobin was determined. Figure 5A illustrates that the protein was efficiently isolated from the blood sample, as was further demonstrated by SDS-PAGE

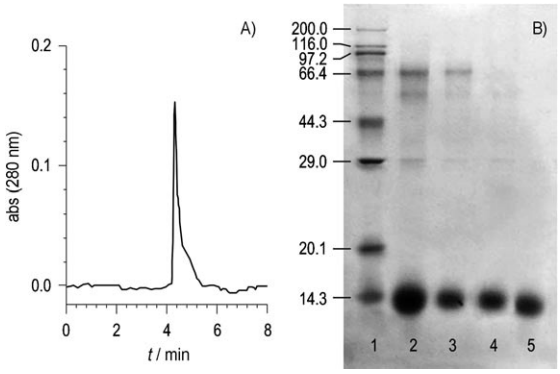

Figure 5. A) Peak recorded for hemoglobin isolated from 5000-fold diluted human whole blood (1.0 mL) by this solid-phase extraction system with MWCNTs as sorbent. B) SDS-PAGE of proteins. The purified proteins were subjected to electrophoresis on an acrylamide gel (15%) at 150 V, with the gel stained with Coomassie brilliant blue G250. Lane 1: the molecular weight standards (Mr in kDa). Lane 2 and lane 3: 200-fold and 500-fold diluted human whole blood without pretreatment with this system. Lane 4: 5000-fold diluted human whole blood after pretreatment by this protocol. Lane 5: a pure hemoglobin solution (500 $\mu g mL^{-1}$).

as shown in Figure 5B. The SDS-PAGE was carried out with 15% polyacrylamide resolving gels, 5% polyacrylamide stacking gels, and standard discontinuous buffer systems as described by Laemmli.[39] It is clear that when 5000-fold diluted human whole blood was pretreated by this procedure, hemoglobin was effectively isolated from the coexisting protein species (e.g., the 66.4 kDa albumin was effectively eliminated). This observation nicely illustrates the practical applicability of this procedure, allowing effective isolation of the basic protein species (hemoglobin) from biological samples matrices.

Conclusion

The use of multiwalled carbon nanotubes (MWCNTs) as a sorbent material for solid-phase extraction of proteins has potential for the effective isolation of basic proteins from other or acidic protein species in biological sample matrices. By simply adjusting/controlling the acidity of the surface of the MWCNTs with neutralized deionized water, the positively charged basic proteins of hemoglobin and cytochrome *c* were readily adsorbed onto the negatively charged MWCNT surface, and could afterwards be eluted by variation of the acidity and ionic strength of the eluent. In this study, the adsorbed hemoglobin and cytochrome *c* were sequentially stripped from the MWCNT surface by use of small amounts of PBS of pH 8 and NaCl solution, respectively. This observation offers promise for the selective separation and/or preconcentration of individual protein species of interest through functionalization of the MWCNT surface

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

(e.g., noncovalent functionalization of the carboxylic groups present in the purified MWCNTs). The successful isolation of hemoglobin from human whole blood by this protocol nicely demonstrated its practical applicability.

Experimental Section

Chemicals: Cytochrome *c* (Sigma, C7752, St. Louis, USA), hemoglobin (bovine, Sigma, H2500, St. Louis, USA) and bovine serum albumin (BSA, Sino-American Biotechnology Company, Beijing, China) were employed as received without further treatment. Other chemicals/reagents used include: potassium phosphate dibasic trihydrate $K_2HPO_4{\cdot}3H_2O$ (Sinopharm Chemical Reagent Co., Shanghai, China-SCRC), sodium dihydrogen phosphate dihydrate $NaH_2PO_4{\cdot}2H_2O$ (SCRC), sodium chloride (SCRC), ethanol (SCRC), nitric acid (SCRC) and hydrochloric acid (SCRC).

The protein molecular weight marker (broad, D532A, TaKaRa Biotechnology Co., Dalian, China) is a mixture of nine purified proteins (Mr in kDa: myosin, 200; β-galactosidase, 116; phosphorylase B, 97.2; serum albumin, 66.4; ovalbumin, 44.3; carbonic anhydrase, 29; trypsin inhibitor, 20.1; lysozyme, 14.3; aprotinin, 6.5). The gel was stained with Coomassie brilliant blue G250 (Fluka) after electrophoresis separation.

All the chemicals involved in this study were at least of analytical-reagent grade and doubly deionized water (18 $M\Omega\,cm^{-1}$) was used throughout.

The physical characteristics of the MWCNTs (Nanotech Port Co., Ltd., Shenzhen, China) employed in this study are summarized in Table 2.

Table 2. Physical characteristics of the MWCNTs used in this study.

Diameter [nm]	Length [μm]	Purity [%]	Ash [%]	Specific surface area [m^2g]	Amorphous carbon [%]
60–100	5–15	≥ 95	≤ 0.2	40–300	<3

The stock solutions of proteins (2.5 $mg\,mL^{-1}$) were prepared by dissolving appropriate amounts of the proteins in deionized water, and working standard solutions were obtained by stepwise dilution of the stock solutions. Phosphate buffers (PBS) of various pH values were prepared by mixing appropriate amounts of the $K_2HPO_4{\cdot}3H_2O$ solution (0.1 $mol\,L^{-1}$) and the $NaH_2PO_4{\cdot}2H_2O$ solution (0.1 $mol\,L^{-1}$) according to the required pH value. A NaCl solution (0.5 $mol\,L^{-1}$) was prepared by dissolving the appropriate amount of NaCl in deionized water.

Apparatus: The UV/Vis spectra were recorded with a 756 PC UV/Vis spectrophotometer (Shanghai Spectrum Instruments, Shanghai, China). A FIAlab-3000 sequential injection (SI) system (FIAlab Instruments, USA) was used for performing online sorption extraction and subsequent elution of the retained proteins. The pH values were measured with an Orion Model 868 pH meter (ThermoElectron). A CQ-6 ultrasonic system (Hucao Ultrasonic Instrument, Shanghai, China) was used for dispersing the MWCNTs in deionized water. The various samples containing protein species were assayed by SDS-PAGE in an electrophoresis cell (DYCZ-28A) with an electrophoresis power supply (DYY-III2, Beijing Liuyi Instruments, China).

Purification and functionalization of the MWCNTs: Before use, the MWCNTs were subjected to the following pretreatment procedures in order to remove graphitic nanoparticles, amorphous carbon, and residual catalyst impurities adhering to the surfaces of the carbon nanotubes.

The MWCNTs (0.1 g) were placed in a beaker with ethanol (50% *v/v*, 10 mL) and stirred for ca. 10 min in order to wet the MWCNTs sufficiently. The MWCNTs was then collected by filtration and rinsed with deionized water, and were afterwards dispersed into HNO_3 (60 mL, 2.2 $mol\,L^{-1}$) with the aid of ultrasonic agitation for 30 min in order to oxidize the coexisting graphitic nanoparticles and amorphous carbon.[40] The admixture was thereafter allowed to stand for 20 h at room temperature, followed by a thorough rinse with deionized water, and collected by filtration. During this process the formation of open-ended and carboxyl-terminated MWCNTs is achieved through oxidation of the defect sites and endings in the MWCNTs with a strong oxidant[41,42] (i.e., nitric acid in this case). Meanwhile, the oxidation of carbon nanotubes induces a negatively charged surface through the ionization of acidic surface groups (i.e., the carboxylic and hydroxyl groups).[43]

After the above pretreatment procedure, the MWCNTs were transferred into an HCl solution (1% *v/v*) and stirred for 3 h in order to remove the residual particulate metallic catalyst on the surface of the MWCNTs. Thereafter, the MWCNTs were thoroughly washed with deionized water until a neutral wash-out solution was obtained. The carbon nanotubes obtained at this stage were usually agglomerated, and they were thus further subjected to sonication for 3 h in order to obtain a well dispersed suspension.[40] The MWCNTs were collected by filtration and were finally equilibrated by impregnation with a PBS solution (pH 6.0, 0.1 $mol\,L^{-1}$) for an extra period of 6 h.

MWCNTs column packing and solid-phase extraction of proteins: The appropriate amount of slurry (ca. 500 μL) containing 8–10 mg of the previously treated MWCNTs was employed to pack a microcolumn in a piece of poly(methyl methacrylate) tubing (2.8 mm in diameter). The equilibrated MWCNT slurry in PBS solution was directed into the column, with the cavity blocked at one end with a small amount of glass wool, while afterwards the other end was blocked similarly to hold the carbon nanotubes in place, giving rise to an effective column length of ca. 5 mm. Before use, the microcolumn was thoroughly rinsed by passing through a sufficient amount of deionized water.

The microcolumn was connected to a sequential injection system for performing online solid-phase extraction of the protein species of interest. The entire system consists of a 5.0 mL syringe pump, a 6-port selection valve, a UV/Vis spectrophotometer, and a holding coil with a capacity of 5.0 mL, as illustrated in Figure 6. All the external channels were made of

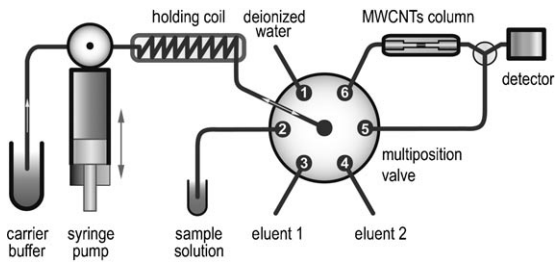

Figure 6. Flow manifold of the sequential injection MWCNTs microcolumn-based solid-phase extraction system for the isolation of basic protein species. Eluent 1: PBS (pH 8.0, 0.025 $mol\,L^{-1}$). Eluent 2: NaCl solution (0.5 $mol\,L^{-1}$).

PTFE tubing (0.8 mm i.d.) connected to the sequential injection system with PEEK nuts/ferrules. As the very tiny MWCNTs particles tend to create flow impedance in such a flow system, so deterioration or tailing of the peak profiles attributable to the concentration gradient along the microcolumn might be encountered during the operation process,[44] a very short column length with a relatively larger column diameter should be preferential in this investigation.

During the solid-phase extraction process, the proteins left in the effluent passing through the microcolumn were quantified by spectrophotometry at 410 nm, in order to evaluate the retention efficiency of proteins on the carbon nanotube-packed column surface. Afterwards, the protein species were eluted from the microcolumn and quantified in the same way. In this case, the solid-phase extraction of proteins with MWCNTs as sorbent includes the following steps:

Before the operation is started, a solution (200 μL) spiked with hemoglobin (5 $\mu g\,mL^{-1}$) and cytochrome *c* (5 $\mu g\,mL^{-1}$) was aspirated into the holding coil (HC) at a flow rate of 50 $\mu L\,s^{-1}$, being directly dispensed

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

through port 5 to flow through the detector at 20 $\mu L s^{-1}$ for measurement of the peaks generated from the proteins without undergoing solid-phase extraction. Afterwards the channel between the selection valve and the detector was rinsed with deionized water (1.5 mL) at 40 $\mu L s^{-1}$ until a stable baseline was obtained.

The surface of the MWCNTs sorbent was adjusted by modifying the pH to facilitate the adsorption of protein species of interest. In this case, this was done by aspiration of carrier buffer (0.025 mol L^{-1}, pH 6.0, 500 μL) into the syringe pump from the carrier reservoir, and this was afterwards dispensed through port 6 at 20 $\mu L s^{-1}$ to precondition the microcolumn. Thereafter, sample solution (2000 μL) was aspirated from port 2 into the HC at a flow rate of 100 $\mu L s^{-1}$, and was then dispensed through port 6 to flow through the microcolumn at 30 $\mu L s^{-1}$ to facilitate the adsorption of the basic proteins of hemoglobin and cytochrome *c* onto the surface of the MWCNT-packed column.

Prior to the elution of the adsorbed proteins, some of the loosely retained interfering components on the column surface were eliminated by a prewashing procedure, by direction of deionized water (500 μL) through the microcolumn. The syringe pump was then set successively to aspirate NaCl solution (0.5 mol L^{-1}, 100 μL), water (2000 μL), eluent 2 (0.5 mol L^{-1} NaCl, 200 μL), water (2000 μL), and eluent 1 (0.025 mol L^{-1} PBS of pH 8.0, 200 μL) into the holding coil. The stacked zones were then dispensed to flow through the microcolumn through port 6, where eluent 1 was used to elute the retained hemoglobin and eluent 2 for recovering cytochrome *c* at the same flow rate of 10 $\mu L s^{-1}$. The eluates were either transferred directly into the detector for quantification or collected for further investigations. The two stacked water zones were employed for washing the column at 20 $\mu L s^{-1}$. Thereafter, the NaCl solution (100 μL) at the end of the zones was employed to clean up the microcolumn and to eliminate any possible carry-over. A final 1000 μL of deionized water was used to precondition the microcolumn for subsequent operating runs.

Finally, a regeneration process of the sorbent surface was included whenever necessary (i.e., after ca. 50 cycles of sorption/elution). The column was equilibrated by impregnating into a PBS solution (pH 6.0, 0.1 mol L^{-1}) for a few hours.

Acknowledgement

The authors are indebted for financial support from the National Natural Science Foundation of China (NSFC-20575010, 20635010) and the 973 program (2007CB714503).

[1] J. Reinders, U. Lewandrowski, J. Moebius, Y. Wagner, A. Sickmann, *Proteomics* **2004**, *4*, 3686–3703.
[2] W. Z. Chen, J. Shen, X. F. Yin, Y. N. Yu, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 35–43.
[3] S. Iijima, *Nature* **1991**, *354*, 56–58.
[4] S. Iijima, T. Lchihashi, *Nature* **1993**, *363*, 603–605.
[5] Y. D. Zhao, Y. H. Bi, W. D. Zhang, Q. M. Luo, *Talanta* **2005**, *65*, 489–494.
[6] K. Maehashi, T. Katsura, K. Kerman, Y. Takamura, K. Matsumoto, E. Tamiya, *Anal. Chem.* **2007**, *79*, 782–787.
[7] L. M. Dai, P. Soundarrajan, T. Kim, *Pure Appl. Chem.* **2002**, *74*, 1753–1772.
[8] Q. F. Pengfei, O. Vermesh, M. Grecu, A. Javey, O. Wang, H. J. Dai, S. Peng, K. J. Cho, *Nano Lett.* **2003**, *3*, 347–351.
[9] G. H. Liu, J. L. Wang, Y. F. Zhu, X. R. Zhang, *Anal. Lett.* **2004**, *37*, 3085–3104.
[10] R. Q. Long, R. T. Yang, *J. Am. Chem. Soc.* **2001**, *123*, 2058–2059.
[11] S. S. Wong, E. Joselevich, A. T. Woolley, C. L. Cheung, C. M. Lieber, *Nature* **1998**, *394*, 52–55.
[12] L. M. Dai, P. G. He, S. N. Li, *Nanotechnology* **2003**, *14*, 1081–1097.
[13] M. Trojanowicz, *Trends Anal. Chem.* **2006**, *26*, 480–489.
[14] Z. J. Guo, P. J. Sadler, S. C. Tsang, *Adv. Mater.* **1998**, *10*, 701–703.
[15] R. J. Chen, Y. G. Zhang, D. W. Wang, H. J. Dai, *J. Am. Chem. Soc.* **2001**, *123*, 3838–3839.
[16] M. Shim, N. W. S. Kam, R. J. Chen, Y. M. Li, H. J. Dai, *Nano Lett.* **2002**, *2*, 285–288.
[17] S. K. Smart, A. I. Cassady, G. Q. Lu, D. J. Martin, *Carbon* **2006**, *44*, 1034–1047.
[18] J. Chlopek, B. Czajkowska, B. Szaraniec, E. Frackowiak, K. Szostak, F. Beguin, *Carbon* **2006**, *44*, 1106–1111.
[19] E. Katz, I. Willner, *ChemPhysChem* **2004**, *5*, 1084–1104.
[20] G. R. Dieckmann, A. B. Dalton, P. A. Johnson, J. Razal, J. Chen, G. M. Giordano, E. Munoz, I. H. Musselman, R. H. Baughman, R. K. Draper, *J. Am. Chem. Soc.* **2003**, *125*, 1770–1777.
[21] F. Balavoine, P. Schultz, C. Richard, V. Mallouh, T. W. Ebbesen, C. Mioskowski, *Angew. Chem.* **1999**, *111*, 2036–2039; *Angew. Chem. Int. Ed.* **1999**, *38*, 1912–1915.
[22] L. E. Valenti, P. A. Fiorito, C. D. Garcia, C. E. Giacomelli, *J. Colloid Interface Sci.* **2007**, *307*, 349–356.
[23] H. Hu, Y. C. Ni, V. Montana, R. C. Haddon, V. Parpura, *Nano Lett.* **2004**, *4*, 507–511.
[24] Q. L. Li, D. X. Yuan, Q. M. Lin, *J. Chromatogr. A* **2004**, *1026*, 283–288.
[25] Y. Q. Cai, G. B. Jiang, J. F. Liu, Q. X. Zhou, *Anal. Chem.* **2003**, *75*, 2517–2521.
[26] P. Liang, Y. Liu, L. Guo, J. Zeng, H. B. Lu, *J. Anal. At. Spectrom.* **2004**, *19*, 1489–1492.
[27] Q. X. Zhou, Y. J. Ding, J. P. Xia, *Chromatographia* **2007**, *65*, 25–30.
[28] Q. X. Zhou, Y. J. Ding, J. P. Xia, *Anal. Bioanal. Chem.* **2006**, *385*, 1520–1525.
[29] Y. Q. Cai, G. B. Jiang, J. F. Liu, Q. X. Zhou, *Anal. Chim. Acta* **2003**, *494*, 149–156.
[30] J. X. Wang, D. Q. Jiang, Z. Y. Gu, X. P. Yan, *J. Chromatogr. A* **2006**, *1137*, 8–14.
[31] M. Karwa, S. Mitra, *Anal. Chem.* **2006**, *78*, 2064–2070.
[32] L. M. Yuan, C. X. Ren, L. Li, P. Ai, Z. H. Yan, M. Zi, Z. Y. Li, *Anal. Chem.* **2006**, *78*, 6384–6390.
[33] A. Wittemann, B. Haupt, M. Ballauff, *Phys. Chem. Chem. Phys.* **2003**, *5*, 1671–1677.
[34] J. H. Walther, R. Jaffe, T. Halicioglu, P. Koumoutsakos, *J. Phys. Chem. B* **2001**, *105*, 9980–9987.
[35] T. Werder, J. H. Walther, R. L. Jaffe, T. Halicioglu, F. Noca, P. Koumoutsakos, *Nano Lett.* **2001**, *1*, 697–702.
[36] P. R. Levison, *J. Chromatogr. B* **2003**, *790*, 17–33.
[37] K. Y. Jiang, A. Eitan, L. S. Schadler, P. M. Ajayan, R. W. Siegel, N. Grobert, M. Mayne, M. Reyes-Reyes, H. Terrones, M. Terrones, *Nano Lett.* **2003**, *3*, 275–277.
[38] S. M. Wheelwright, *Protein purification: design and scale up of downstream processing*, Wiley, New York, **1993**, p. 244.
[39] E. K. Laemmli, *Nature* **1975**, *227*, 680–685.
[40] F. Valentini, A. Amine, S. Orlanducci, M. L. Terranova, G. Palleschi, *Anal. Chem.* **2003**, *75*, 5413–5421.
[41] S. M. Chen, W. M. Shen, G. Z. Wu, D. Y. Chen, M. Jiang, *Chem. Phys. Lett.* **2005**, *402*, 312–317.
[42] Z. F. Liu, Z. Y. Shen, T. Zhu, S. F. Hou, L. Z. Ying, Z. J. Shi, Z. N. Gu, *Langmuir* **2000**, *16*, 3569–3573.
[43] M. S. P. Shaffer, X. Fan, A. H. Windle, *Carbon* **1998**, *36*, 1603–1612.
[44] T. Klose, P. B. Welzel, C. Werner, *Colloids Surf. B* **2006**, *51*, 1–9.

Received: May 24, 2007
Published online: September 13, 2007

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim