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L-histidine functionalized multi-walled carbon nanotubes for on-line affinity separation and purification of immunoglobulin G in serum

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ABSTRACT

In this work, the multi-walled carbon nanotubes were covalently functionalized with L-histidine (His–MWNTs) as online pseudospecific affinity adsorbent for immunoglobulin G (IgG) separation and purification with a simple surface modification method, using 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimde (NHS). The affinity of the His–MWNTs toward IgG was investigated in a microcolumn incorporated into a sequential injection system, which also involves an UV spectrometer with a flow cell for online real-time detection. The incorporation of histidine as affinity groups noticeably increased the selectivity and binding capacity of MWNTs for IgG and the His–MWNTs exhibited high retention and recovery rate of nearly 100% under optimized conditions. This separation and enrichment process made it possible to determine a lower concentration range of IgG in serum from 1.0–33 μg/mL with a detection limit of 0.3 μg/mL with a sampling volume of 4.0 mL. The static and dynamic adsorption capacities obtained were 267 mg of IgG/g His–MWNTs and 35 mg/g in aqueous solution, respectively, which are among the highest reported results in literatures employing affinity separation methods. Desorption of IgG from His–MWNTs could be accomplished by lowering the pH to 1.5 with glycine–HCl buffer. The practical application of His–MWNTs for separation of IgG in serum was evaluated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis which confirmed that the purity of recovered IgG from human serum was over 85% and better than a commercial product.

1. Introduction

Since their discovery, carbon nanotubes (CNTs) with remarkable electrical, structural and mechanical properties have become one of the most exciting nanomaterials for diversified applications [1–3]. In recent years, functionalization of carbon nanotubes is an attractive and challenging work to increase their dispensability and biocompatibility, which are very important properties for their applications of binding and purification of biomacromoleculars in physiological solution [4]. CNTs' high surface area, ability to establish π–π interactions, excellent chemical, mechanical and thermal stability, etc. make them very attractive as solid phase materials for separation and detection of either nonpolar or polar compounds [5]. Recently, the biological applications of CNTs have arisen and biomolecule modified CNTs have been proven as new types of adsorption materials, although the non-specific hydrophobic interaction could pose a serious drawback for the use of CNTs in biomolecule purification. Some previous works showed that appropriate functionalized MWNTs can be used as adsorbents for the purification of some proteins, [6,7] but the specificity and selectivity was not satisfactory for their application on adsorbing of special interested proteins.

Among various schemes, affinity separation has been widely used as a powerful technique for purifying proteins and biomolecules. This technology is based on the specific interaction between a target biomolecule and its corresponding ligand, often immobilized on a solid support. Therefore, the quality of the solid matrix plays an essential role in the separation scheme. Ideally, solid supports/matrices should be chemically and physically stable, have good mechanical strength and high surface area, and exhibit specific adsorption or interaction for target biomolecules while minimizing their non-specific binding to contaminants. However, from a detailed revision of the literature, it is also clear that most of works have used pristine or oxidized CNTs as solid support for organic and inorganic analysis, and the main driving forces for these adsorbents are hydrophobic or electrostatic interactions [8]. It has not attracted enough attention that CNTs with acquired carboxyl group resulting from the acid treatment can be covalently coupled with affinity ligands to interact with proteins and other biomolecules and be eluted in mild elution conditions to preserve their bioactivity. In order to

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utilize CNTs as affinity adsorbent, the functionalization of CNTs is one of the essential steps in satisfying requirements such as high adsorption capacity and good selectivity of target separation.

Immunoglobulin G (IgG), a major class of serum glycoproteins, constitutes an important therapeutic protein for a number of malignancies and is also required for immunodiagnostic and immunochromatographic purposes. Therefore, there is a high demand for high purity IgG, free from other serum proteins [9]. The proteins A and G are the most prominent biological specific ligands used in affinity adsorption for IgG, since they allow the direct and most efficient capture of IgG from serum or cell culture supernatant. Hence, these media require a reduced number of pre-operational steps such as desalting, buffer changing and pH adjustments, which are laborious and inconvenient. However, their obvious drawbacks are the chemical and enzymatic instability, high cost, degradation and leakage of affinity ligands on the adsorbent surface, which might lead subsequently to decreased binding capacities for the target proteins and cause the pollution of final products. In contrast to protein A or G, the pseudobio-specific ligand displayed not only reduced production costs at large scale, but also increased resistance to physical, chemical or microbial degradation [10]. Several amino acids were already reported as potential antibody capture materials, especially histidine, tryptophan and phenylalanine [11]. Histidine as a pseudo-specific ligand increases resistance to biochemical actions and has higher biochemical stability. This facilitates handling and storage of the cartridge and increases its lifetime considerably. Another advantage is that even if ligand leakage was to occur, this would be much less problematic than with protein A or G if the purified IgG is destined for clinical or analytical application [12]. With all these advantages, L-histidine has been loaded on various carriers for the separation of IgG, such as beads, hollow fiber membranes, monoliths, sepharose and bentonite [13–15]. These composites were tested offline or online, and for offline methods the processing time for one sample could be up to 180 min, while online methods fastened the processing rate with very limited adsorption capacities. In this work, we combined the specificity of the ligand histidine with MWNTs to create a powerful affinity adsorbent for online rapid and selective solid-phase extraction (SPE) of IgG in serum. Compared to the traditional batch affinity binding, the online separation method can increase the recovery rate of targeted analytes and reduce the operation time by facilitating the interaction of IgG and MWNTs, which are important for keeping the bioactivity of IgG. The extraction conditions, analytical efficiency and practical applicability of His–MWNTs for purification of IgG in serum are presented and discussed in detail.

2. Experimental section

2.1. Chemicals and biologicals

Multi-walled carbon nanotubes (MWNTs, Timestub^{TM}, purity $>90\%$, outer diameter: 30–60 nm, inner diameter: 20–50 nm, length: 1–10 μm, special surface area $>200\ m^2/g$, ash $<5\%$) were purchased from Chengdu Organic Chemicals Co. Ltd. of Chinese Academy of Sciences and pretreated before use as described in experimental section. Human immunoglobulin G, bovine serum albumin, human serum, newborn calf serum, glycine, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 2-(N-morpholino) ethane sulfonic acid (MES) were purchased from Beijing Solarbio Science & Biotechnology Co., Ltd., China. Rabbit immunoglobulin G was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin Reagent Corporation (Shanghai, China). Other chemicals employed include sodium chloride, ethanol, nitric acid and hydrochloric acid were at least of analytical reagent grade and purchased from Guangzhou Chemical Reagent Factory, China.

The prestained protein molecular weight marker (PM0032, Beijing Dingguo Changsheng Biotechnology Co. Ltd., China) was a mixture of 5 native proteins ranging in size from 25 to 105 kDa. The gel was stained with Coomassie brilliant blue G250 (Fluka) after electrophoresis separation. Purified water (SYSU Yixianquan, Guangzhou, China) was used throughout.

2.2. Equipments and instruments

A MicroCSP3000 sequential injection pump and a C25-3188EMH multiposition valve (FIAlab Instruments, USA) were used for on-line separation and preconcentration of proteins. A T6 UV/Vis spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China) was used in protein quantification. Fourier transform infrared (FTIR) spectra of the MWNTs after functionalization process were recorded in the range of 400–4000 cm^{-1} on a NICOLET AVATAR 330 Fourier transform infrared spectrometer. Powder X-ray diffraction (XRD) was done on a D8 Advance diffractometer (Bruker AXS, Germany) with Cu Kα radiation at 40 kV, 40 mA at the scan speed of 6°/min. The elemental analysis was conducted with an automated Elemental Analyzer (Vario ELIII, Elementar Analysensysteme GmbH, Hanau, Germany). The pH values were measured with a S40K SevenMulti^{TM} pH meter (Mettler-Toledo Inc., Switzerland). The samples containing proteins were assayed by SDS-PAGE in a BG-verMINI electrophoresis cell with a BG-Power600i electrophoresis power supply (Baygene Biotech Co. Ltd., China). A CQ-6 ultrasonic system (Hucao Ultrasonic Instrument, Shanghai, China) was utilized for dispersing MWNTs. A C-MAG HS7 digital magnetic stirrer (IKA-Werke GmbH & Co. KG, Germany) was employed for stirring solutions.

2.3. Preparation of histidine-modified carbon nanotubes

MWNTs were pretreated as described in the literature with minor adjustment [16]. 1.0 g of MWNTs were dispersed into concentrated HNO_3 (30 mL, 2.2 mol/L) in an ultrasonic bath for 30 min and afterwards stirred at 50 °C for 2 h in order to purify and oxidize the surface of MWNTs sufficiently. Then the MWNTs were collected by filtration and rinsed with purified water at least 3 times until the effluent was neutral. Finally, the collected solid was dried overnight at 65 °C.

The activation buffer (100 mL, 0.1 mol/L pH 5.5–6.0 MES, 0.5 mol/L NaCl) was prepared by mixing 2.13 g of MES and 2.93 g of NaCl in 50 mL water and the pH value was adjusted to 6.0 by adding appropriate amounts of Tris solution and brought to volume by purified water. The coupling buffer (100 mL, 0.1 mol/L pH 7.2–7.5 phosphate-buffered saline, 0.15 mol/L NaCl) was prepared by mixing appropriate amounts of Na_2HPO_4 solution (0.05 mol/L) and NaH_2PO_4 solution (0.10 mol/L) and 0.88 g NaCl to obtain the required pH value and ionic strength. 0.15 g of oxidized MWNTs was dispersed in 10 mL of activation buffer with the aid of ultrasonic agitation for 20 min. Then EDC (60 mg) and NHS (90 mg) were added into the solution and allowed to react with MWNTs at room temperature for 15 min to activate the carboxylic acid groups on the MWNTs surface. The sediment was collected by centrifugation at 3000 rpm and washed with coupling buffer at least five times to remove the unreacted EDC. Subsequently, the collected pellet was suspended in 10 mL coupling buffer in an ultrasonic bath followed by the addition of 0.15 g histidine. This reaction was carried out for 2 h at room temperature on a vibrator and terminated with 20 mmol/L (0.024 g) Tris. The histidine modified MWNTs were recovered by

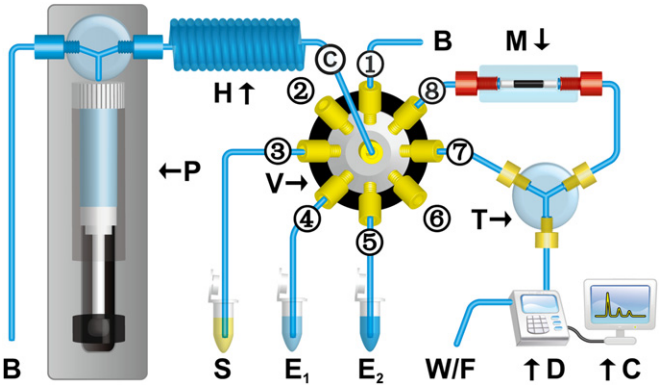

Fig. 1. Flow manifold of the sequential injection system incorporating a microcolumn for on-line SPE of IgG. P, sequential injection pump; H, holding coil; V, 8-port multi-position valve; M, microcolumn; T, 3-way connector; D, UV/vis spectrometer; C, computer; B, loading buffer/purified water; S, sample; E1, weak eluent; E2, strong eluent; W/F, waste or fractions.

centrifugation at 3000 rpm, washed with purified water and then dried overnight at 65 °C.

2.4. Configuration of an on-line SPE system

The entire on-line solid-phase extraction (SPE) system consists of a 2.5 mL sequential injection pump, an 8-port multi-position valve, a SPE microcolumn, an UV/vis spectrophotometer, and a holding coil with inner capacity of 2.5 mL, as illustrated in Fig. 1. All the external channels are PTFE tubing (outer diameter: 1.59 mm, inner diameter: 0.75 mm) and connected to the sequential injection system with flangeless nuts and ferrules. The solid-phase extraction microcolumn was made of poly(methyl methacrylate). The column length, effective column length, outer diameter and inner diameter are 50, 28, 20 and 2.5 mm, respectively. The appropriate amount of His–MWNTs (ca. 13 mg) was packed into the microcolumn and blocked with quartz wool at both ends. The length of adsorbent was about 13 mm. Two sets of flangeless nuts and ferrules were used to hold the quartz wool in place and connect the microcolumn to the sequential injection system for on-line SPE of IgG.

2.5. On-line SPE of proteins

The protein stock solutions (1.0 mg/mL) were prepared by dissolving appropriate amount of proteins in purified water and the working standard solutions were obtained by stepwise dilution of the stock solutions with corresponding buffer solutions. During the solid-phase extraction process, the effluent containing proteins was quantified in a flow cell with an UV spectrophotometer at 280 nm, in order to evaluate the retention and recovery rate of proteins on the His–MWNTs. The amount of proteins flowed through or eluted from the adsorbent was determined using commercial proteins as standards in corresponding buffer solutions with similar ionic strength and pH to minimize the matrix effects.

In a typical SPE process, the SPE of proteins with His–MWNTs as absorbent includes the following steps: before starting the extraction operation, all tubing was rinsed with purified water at 20 μL/s until a stable baseline was obtained and then different solutions were aspired into the relevant tube. Thereafter, 500 μL of loading buffer and 2.0 mL of sample solution were aspirated from port 3 into the holding coil at a flow rate of 100 μL/s, and were then dispensed through port 8 to flow through the microcolumn at 20 μL/s to facilitate the adsorption of proteins onto the His–MWNTs surface. Prior to the elution of the adsorbed proteins, some of the loosely retained interfering components on the column surface were eliminated by a washing procedure, by flushing the microcolumn with 2.0 mL of carrier buffer and 100 μL of weak eluent. The syringe pump was then set to successively aspirate 2.0 mL of loading buffer and 100 μL of strong eluent into the holding coil and then dispense the stacked zones into the microcolumn through port 8 at 20 μL/s to elute the retained proteins. The eluate were then either transferred directly into the detector for quantification or collected for further investigations. Finally, 2.0 mL of loading buffer and 200 μL of NaCl solution (2.0 mol/L) were employed to clean up the microcolumn and eliminate any possible carry-over and precondition the microcolumn for next operating run.

2.6. Dynamic binding study of proteins on His–MWNTs

The proteins solution of 25 μg/mL was continuously directed to flow through a microcolumn packed with His–MWNTs (13 mg) with a peristaltic pump at a flow rate of 0.5 mL/min (8.3 μL/s). The dynamic breakthrough curve of proteins on His–MWNTs was recorded in a flow cell located in a UV spectrometer at 280 nm until 100% breakthrough was reached. The amount of protein $m_{protein}$ adsorbed onto the adsorbent surface is equivalent to the shadow area in the curve, i.e.,

$$m_{protein} = vc_p(t_e - t_0) - v\int_{t_0}^{t_e} c(t)dt$$

$$q_m = \frac{m_{protein}}{m_{adsorbent}}$$

where v is the sampling flow rate, c_p is the concentration of protein, t_0 is the dead time, t_e is the time when break-through point is reached, and q_m is the dynamic sorption capacity.

2.7. Static binding study of IgG on His–MWNTs

The static adsorption and desorption behaviors of IgG on His–MWNTs were investigated using the batch equilibration method. The static binding capacity measurement was performed in a series of standard IgG solutions of different concentrations (125, 250, 500 and 1000 μg/mL) prepared in purified water. An amount of 2.0 mL of the IgG solution was added into 5.0 mg of His–MWNTs. The supernatant was separated with the solid after vibrated for 10 min by centrifugation at 2000 rpm and filtration with a syringe filter, and the UV absorbance of the supernatant was then detected at 280 nm. Desorption of retained IgG was performed by agitating the adsorbent for 10–120 min in 1.0 mL of glycine–HCl buffer (0.2 mol/L, pH 1.5) or 2 mol/L NaCl. The eluate was also measured at 280 nm.

3. Results and discussion

3.1. Chemical modification process and characterization of MWNTs bonded by histidine

Fig. 2 is a schematic diagram of the functionalization of MWNTs with histidine. When the MWNTs were immersed in EDC/NHS/MES solution, the C=O groups on the MWNTs were activated as C–OOH with the help of EDC. When the activated MWNTs were added to the histidine containing PBS solution, the C–OOH was then directly bound with $-NH_2$ groups on histidine covalently via the peptide bond.

The FTIR spectra of MWNTs after each modification step are shown in Fig. 3A. No significant difference is observed in the spectra of MWNTs and oxidized MWNTs treated with HNO_3. The absorbance of the –C=C– functional groups on MWNTs at 1634–1628 cm^{-1} was not affected by the histidine binding after the

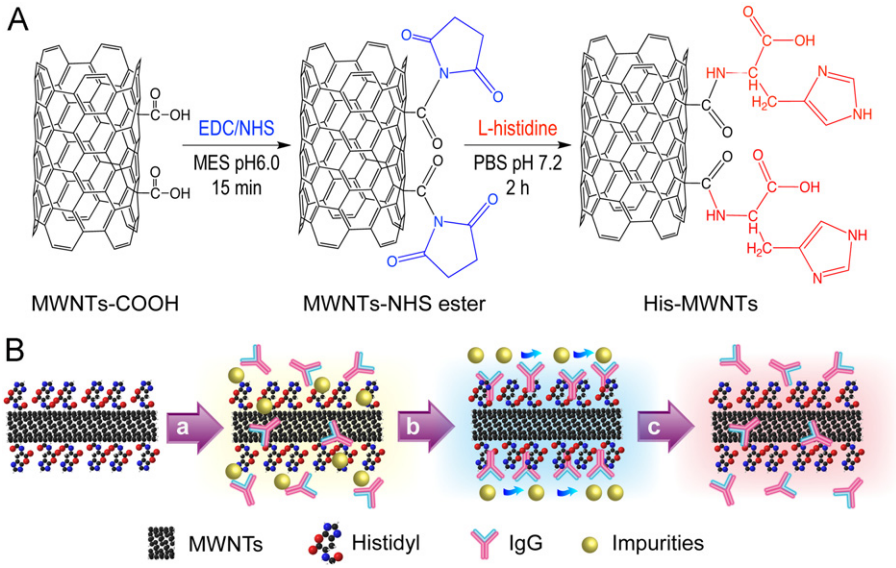

Fig. 2. (A) Schematic for preparing the histidine modified carbon nanotubes (His–MWNTs). (B) On-line affinity extraction process of IgG in serum with His–MWNTs. (a) serum solution (mainly containing albumin and IgG) passed though the microcolumn; (b) weak eluent and sufficient loading buffer, IgG was retained on the surface of His–MWNTs and separated with impurities; (c) strong eluent, IgG was eluted with glycine–HCl buffer and collected.

functionalization process [17]. The characteristic absorbance of –NH_2/=NH group in histidine at 3411 cm^{-1} in Fig. 3A-3 could not be observed in the spectrum of His–MWNTs (Fig. 3A-2) due to the broad adsorption bands of O–H stretching vibrations at around 3428 cm^{-1}. In Fig. 3A-3, the peak at 1499 cm^{-1} was assigned to –C=N– group in histidine, which shifted to 1470 cm^{-1} and greatly weakened after binding with MWNTs, which shows successful functionalization of MWNTs with histidine.

The degree of surface functionalization for the MWNTs with L-histidine is a crucial factor governing the adsorption selectivity and capacity of the His–MWNTs in SPE process. It was believed that because of entanglement and bundling of the CNTs, it is necessary to disperse them well in solution to facilitate the surface modification of MWNTs and to introduce abundant functional groups on surface of MWNTs. For this purpose, two batches of His–MWNTs were synthesized with or without ultrasonic dispersion during the oxidization and modification process, respectively. Instead of dispersing the MWNTs in solution with 20 min of ultrasonic in each step for His-MWNTs, the control His-MWNTs′ was only synthesized under gentle stirring. The XRD experiments were performed on MWNTs–COOH, His-MWNTs, His-MWNTs′ and L-histidine samples, and the patterns are shown in Fig. 3B. As shown in Fig. 3B-1, the diffraction peaks of MWNTs–COOH appears at around 26°, 43°, 54° and 78°, which could be assigned to the (002), (100)/(101), (004) and (006) planes, respectively [18], while the two main diffraction peaks of histidine appears around 11.4° and 23.1° (Fig. 3B-3). After the functionalization of MWNTs with histidine, the intensity of the MWNTs peaks becomes weaker and some new peaks appear at around 12°, 23°, 32°, 46°, 56°, 66° and 75° (Fig. 3B-2 and 2′). Compared to the XRD pattern of His-MWNTs′ (Fig. 3B-2′), the intensity of these new peaks of His-MWNTs (Fig. 3B-2) becomes stronger, while the peak high of diffraction peaks of MWNTs–COOH at 26° and 43° is lower, which suggested that the ultrasonic dispersion greatly affected the covalent modification of the histidyl groups on the surface of MWNTs.

The amount of immobilized L-histidine on MWNTs was determined by measuring the initial and final concentrations of amino acid within the coupling buffer at 295 nm. Although the L-histidine has the maximum absorbance at 280 nm, this wavelength was not used for L-histidine quantification because the crosslinker also has strong absorbance at this region, and trace quantities of the retained reagents could greatly interrupt the detection of histidine. A calibration curve constructed with L-histidine solution of known concentration was used in the calculation of amino acid in the solutions. An amount of 250 mg of activated MWNTs was accurately weighted and well dispersed in 10 mL of coupling buffer in an ultrasonic bath and afterwards equally dispensed into five centrifuge tubes, and a certain amount of L-histidine was then added into each tube. After a 2 h reaction on a vibrator, the concentrations of histidine left in the coupling buffer were detected after centrifugation in an orderly manner. This was followed by cleanup of possibly retained histidine on MWNTs with 2 mL of coupling buffer. The amount of histidyl groups in the structure was also measured with elemental analyses (EA). The results were shown in Table 1. The original MWNTs contain about 97.48 wt% C and 0.00 wt% N, while the as-prepared His–MWNTs contain about 80.99 wt% C and 0.87 wt% N at most from the results of elemental analyses and the binding amount increased with the increment of the feeding concentration of histidine. The results detected by UV and EA are in agreement and the maximum binding density of histidyl groups was found to be about 210 μmol/g composite using nitrogen stoichiometry when the feeding concentration was 50 mg/mL. These results are much higher compared to previously published results [19]. The extraordinary binding density of histidyl groups built a solid foundation for good adsorption capacity of IgG and low non-specific interaction with impurities.

Combining with the above-mentioned results, it is believed that histidine functional groups was chemically attached to the surface of MWNTs and suggesting the degree of functionalization can be easily controlled by the simple adjustment of the parameters such as oxidation time, oxidation temperature, histidine concentration and dispersion degree.

3.2. Sorption and separation mechanism of IgG on His–MWNTs

Affinity purification involves specific noncovalent binding interactions between ligand and target molecules, which allow the target molecules to bind to the immobilized ligand molecules by their specific affinity. A typical on-line affinity extraction process of IgG in serum with His–MWNTs was shown in Fig. 2B.

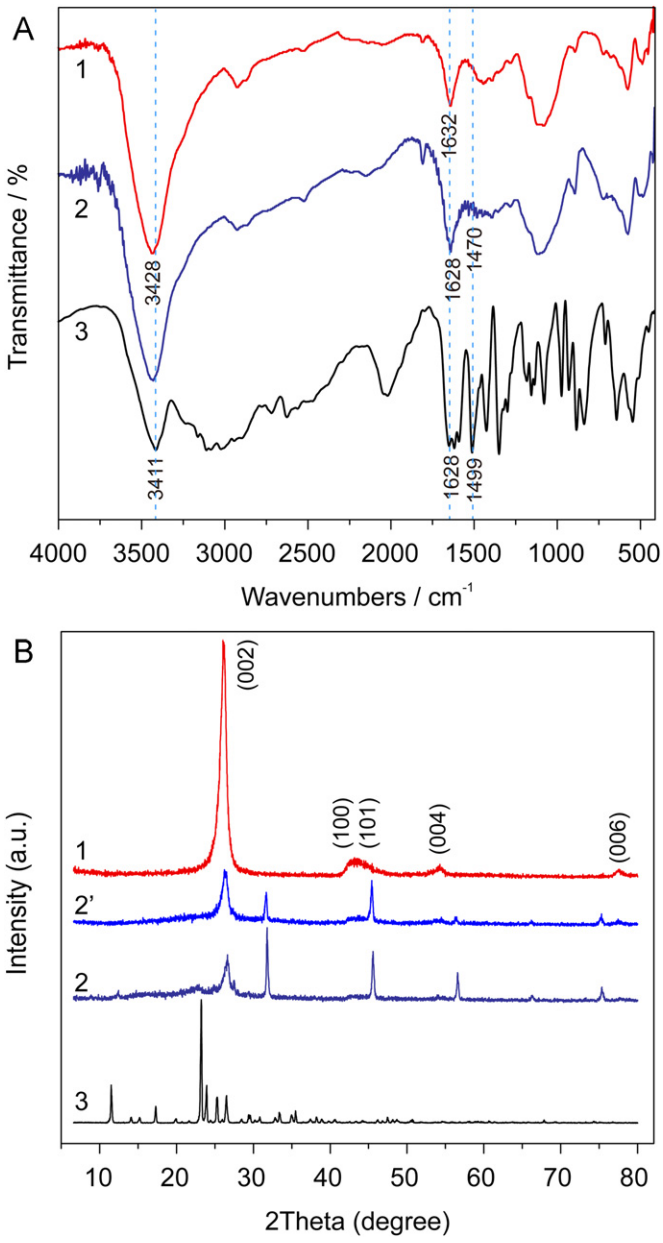

Fig. 3. FTIR spectra (A) and XRD powder diffraction patterns (B) of (a) oxidized MWNTs, (b) His–MWNTs-b, (2′) His–MWNTs-2′ and (c) L-histidine.

Table 1
Effect of initial L-histidine amount on binding density of histidyl group on MWNTs.

Feeding of L-histidine (mg/mL)	MWNTs mass (mg)	Binding mass of L-histidine (mg)	Histidyl ratio on His–MWNTs (wt%)	N (%)	Histidyl density on His–MWNTs (μmol/g)
5.0	5.0	0.19	3.66%	0.45	108.6
10.0	4.8	0.21	4.19%	0.50	120.7
15.0	5.1	0.25	4.67%	0.55	132.8
25.0	5.1	0.27	5.03%	0.72	173.8
50.0	4.8	0.30	5.88%	0.87	210.0

The sample or serum was properly diluted with loading buffer to form a solution, mainly containing albumin and IgG. When the MWNTs were added into this solution and interact with the target molecules. After that, weak eluent and sufficient loading buffer were applied on the absorbent to wash away the loosely retained impurities. The IgG on the surface of His–MWNTs was eluted with strong eluent and collected for further investigation. With respect to the sorption and separation mechanism, it was reported that the interaction between IgG and histidine occurs because of hydrogen bonding, electrostatic and hydrophobic interactions [20]. However, El-Kak et al. suggested it is an ion-pairing mechanism affected by pH, salt and temperature, rather than net charge on protein, but with some localized complementary charges recognizing the unprotonated imidazole nitrogen. The interaction between the ligand and IgG also involved hydrogen bonding and low affinity to the Fc fragment of IgG, which is similar to the N-terminus of protein A [21]. Denizli and Hacettepe reviewed the interaction between IgG and histidine which include hydrogen bonding, electrostatic and hydrophobic interactions and he also found the affinity of histidine to IgG exists because of its capacity to transfer charge on its imidazole ring [22]. In order to understand the interaction between IgG and histidyl groups on His–MWNTs, a series of experiments were performed to evaluate the affinities of this adsorbent, and some important factors tending to govern the binding and recovery rate of IgG on the His–MWNTs were also investigated.

3.3. Selection of sample medium

Firstly, it is necessary to control the ionic strength and pH value of the sample medium at appropriate levels for the sorption of IgG onto the His–MWNTs composites. Fig. 4 illustrates the dependence of retention efficiency of IgG and BSA on the ionic strength and the pH value of the sample medium. The ionic strength was determined by adjusting the concentration of NaCl in solution and the pH value was controlled by glycine–HCl (pH 1.5–5.0) and Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.0–11.0). Fig. 4A illustrates that high sorption efficiency of IgG was readily achieved in an aqueous medium with an ionic strength lower than 0.01 mol/L. Then the nearly linear decreasing of sorption efficiencies happened with the increasing of the ionic strength from 0.01–0.10 mol/L. When the ionic strength was up to 0.10 mol/L, there was only 15% of IgG could be adsorbed. Fig. 4B shows that stable sorption efficiency of IgG varied between 86% and 99% when the pH value of the sample medium was increased from 3 to 11, which indicates the quantitative adsorption could readily be achieved in a wide pH range. Therefore, the adsorption of IgG was mainly affected by ionic strength of sample medium, and the appropriate dilution of real sample in aqueous buffers is required for achieving the best adsorption of IgG, while the pH range for adsorption could be varied by 5.0–9.0 for subsequent investigations.

3.4. Selection of eluent

After washing away unbound components of the complex mixture, the captured target molecules are released and recovered (i.e., eluted) from the ligand molecules using buffer conditions that disrupt the affinity interaction. Appropriate buffer conditions for binding and elution steps in affinity purification are as varied as the types of molecules concerned and their chemical binding properties. Unique binding/elution conditions exist for certain affinity systems, such as between lectins and sugars, antibody and antigen, chelated divalent metals and histidine tags, and substrates and enzymes. Usually, raising or lowering the pH or altering the ionic state to disrupt the binding interaction often can accomplish elution. Any bound protein that remains on an affinity column would lower the column's binding capacity for subsequent rounds of purification, thus complete elution is desirable.

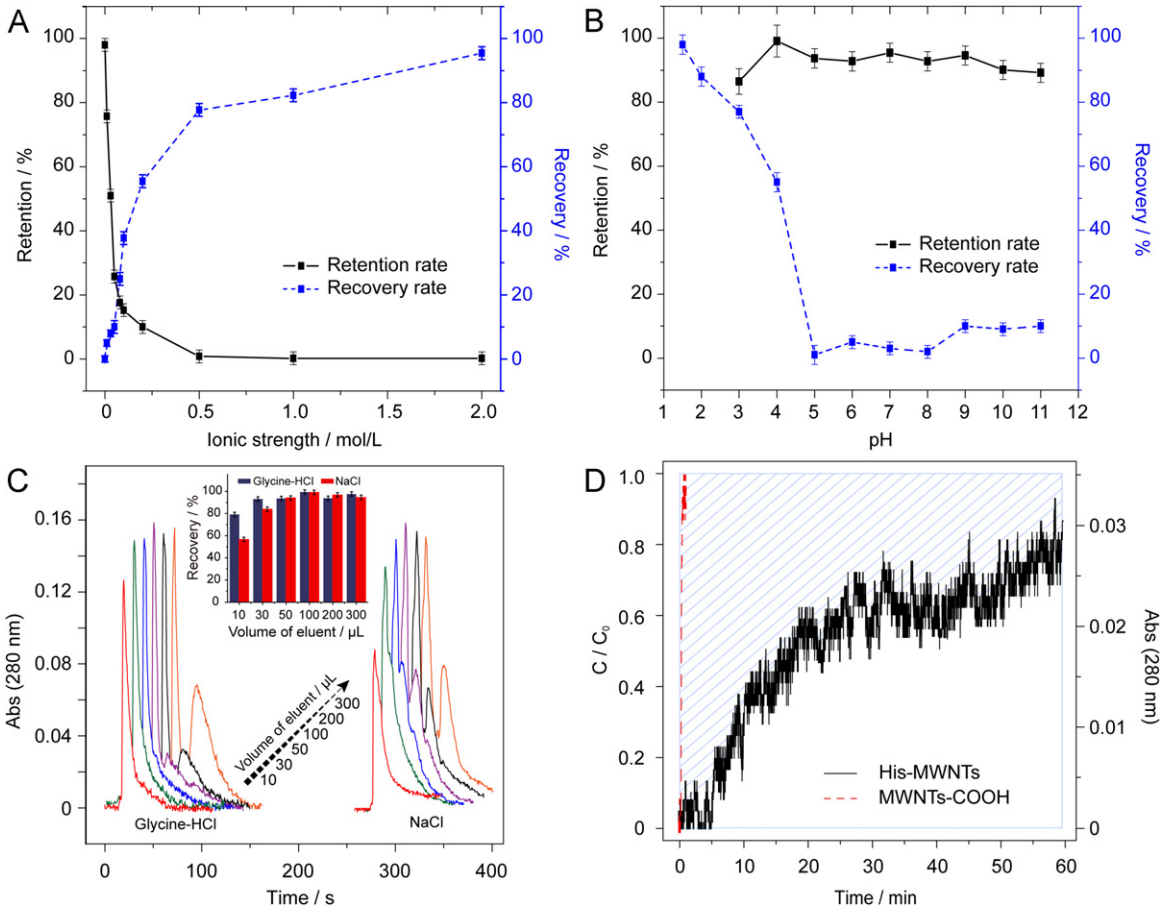

Fig. 4. Effect of ionic strength (A) and pH value (B) on the retention and recovery rate of the IgG on His–MWNTs. Experimental conditions: 2.0 mL of 100 μg/mL IgG solution, flow rate: 20 μL/s, sampling volume: 2 mL, (a) pH of sample: 7.0, eluent: 50 μL NaCl, (b) ionic strength of sample: 10 mmol/L, ionic strength of eluent: 0.2 mol/L; (C) Effect of eluent and its volume on eluate peak shape and recovery of IgG; (D) Dynamic breakthrough curve of rabbit IgG on MWNTs–COOH (red dashed line) and His–MWNTs (black line). Original concentration of IgG solution: 25 μg/mL, flow rate: 0.5 mL/min (8.3 μL/s), adsorbent mass: 13 mg, temperature: 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Different types of eluents were employed to recover the IgG retained on the surface of His–MWNTs after the adsorption process and the recovery rates were compared according the peak high of the eluate at 280 nm. NaCl is the most commonly used eluent, but for affinity purification of proteins, the most widely used elution buffer is glycine–HCl buffer (0.1–0.2 mol/L, pH 2.5–3.0). This glycine–HCl buffer effectively dissociates most protein–protein and antibody–antigen binding interactions without permanently affecting protein structure. The effect of different eluents on IgG desorption was studied here by using glycine–HCl buffer, phosphate buffer, and NaCl solution covering a pH range from 1.5 to 11 and ionic strength range from 0.01 to 2.0. Fig. 4 shows the effect of the nature of the buffer, ionic strength and pH on desorption of IgG on His–MWNTs, which seemed to depend on the combination of the nature, pH and ionic strength of the eluent.

As shown in Fig. 4A, the recovery rate increased from 0% to 95% when the concentration of the NaCl solution increased from 0.01 to 2.0 mol/L, which means the complete elution can only be done with 2.0 mol/L NaCl solution, but it will harm the downstream process and required one more step for desalting. While the pH displayed a more significant impact on the effective desorption of IgG with a maximum recovery of 98% at pH 1.5, which is lower than usually used pH value of eluent in affinity separation and showing a much stronger interaction between the histidine modified MWNTs and IgG. Above this pH, the recovery was greatly reduced and virtually no desorption occurred at pH 5.0 (Fig. 4B). Therefore, 0.2 mol/L pH 1.5 glycine–HCl buffer was used as the elution buffer. In addition, a weak eluent with an ionic strength of 0.2 mol/L and a pH of 5.0 was employed to minimize the unwanted retention of other proteins.

3.5. Effect of flow rate and volume on IgG enrichment

The sampling flow rate and volume were also studied because they are two critical parameters for the performance of an on-line sorption system. The experimental results indicated that changing the sampling flow rate from 5 to 30 μL/s resulted in observable reduction in the sorption efficiency of IgG on His–MWNTs. In addition, slower flow rate is good for prolonging the lifetime of the column. Therefore, a relatively low sampling flow rate of 20 μL/s was employed. At this flow rate, the operation time of a His–MWNTs packed microcolumn could last for about 100 h. The effect of the elution flow rate on the elution process was also studied. The results showed that the recorded peak shape was much sharper and well-shaped as the elution flow rate increased from 5 to 30 μL/s. Considering the lifetime of the packed column and processing time, an elution flow rate of 20 μL/s was employed for further investigations.

Generally, the volume of eluent governs not only the recovery efficiency of the retained species but also the enrichment factor. Fig. 4**C** illustrates the dependence of the recovery rate on the volume of 0.2 mol/L glycine–HCl buffer solution (pH 1.5) and 1.0 mol/L NaCl solution. It shows that recovery of 80%–98% for IgG

could be achieved when varying the volume of glycine–HCl buffer solution within the range of 10–300 μL and a fairly high recovery rate of over 80% was obtained by using only 10 μL of glycine–HCl buffer. In contrast, the stable recovery of IgG could be done when the volume of NaCl solution was up to 100 μL. Comparing the peak shape of the eluates, the peaks eluted with NaCl usually have serious trailing and bifurcation, which are harmful for protein quantification, while the peaks eluted with glycine–HCl buffer were much smoother when the eluent volume is fewer than 200 μL. Thus, an eluent volume of 100 μL of 0.2 mol/L glycine–HCl buffer solution (pH 1.5) was used as strong eluent for further investigations to guarantee complete desorption of IgG and better peak shape.

3.6. Dynamic binding study of IgG on His–MWNTs

Fig. 4D illustrated the dynamic breakthrough curve of IgG adsorption onto the His–MWNTs. If the absorbent is saturated by proteins, the concentration of IgG in the effluent would be equal to that in the original solution, i.e., dynamic sorption–desorption equilibrium was established. The adsorbent of MWNTs–COOH was packed into another microcolumn as a negative control. It is obvious that the sorption of IgG by the His–MWNTs packed microcolumn is a very fast process, characterized by quantitative retention of IgG at the concentration level of 25 μg/mL at the first 5 min, as shown in Fig. 4D. Compared with the dynamic breakthrough curve of IgG on MWNTs–COOH, the curve on His–MWNTs was much broader and could not achieve the dynamic sorption–desorption equilibrium after 1 h, which indicated the potential tremendous adsorption capacity but relatively poor accessibility and interaction of IgG to the aggregated and heterogeneous absorbent—always a problem in the case of small home-packed columns. The much sharper breakthrough curve of the BSA also shown in Fig. 4D, indicates that it is not the column efficiency but rather some heterogeneity of the interaction, which is at fault. This indicates that mass transfer effects also influence the chromatographic behavior. Further calculations derived the mass and sorption capacity of IgG adsorbed on the His–MWNTs as 450 μg and 35 mg/g (His–MWNTs), while the sorption capacity of IgG on the MWNTs–COOH was only 0.5 mg/g.

3.7. Static binding capacities

The static binding capacity results of IgG on His–MWNTs are shown in Table 2, which indicated that the static binding capacity increased with the original concentration of IgG increasing and could be up to 267.8 mg/g. The retained IgG in supernatant was detected after 10, 20, 30 and 60 min, and the measured absorbance values showed negligible difference, indicating an equilibration time of 10 min was sufficient for IgG absorbed onto His–MWNTs. Afterwards, the desorption of retained IgG was performed by agitating the adsorbent for 10–120 min in 1.0 mL of glycine–HCl buffer (0.2 mol/L, pH 1.5) or 2 mol/L NaCl. The absorbance values of the eluates were also measured at 280 nm. However, the recovery of retained IgG was very low even if the elution time was up to 120 min. Summing up these observations, the strong interaction between His–MWNTs and IgG not only facilitated the fast adsorption and high retention rate, but also caused very low recovery in this batch equilibration method, in other word, the desorption could only be performed in flow-based online methods.

A comparison of dynamic and static adsorption capacities with previous published results is listed in Table 3, in which the His–MWNTs show greatly higher binding capacities with comparable

Table 2
Static binding capacities of IgG on His–MWNTs.

Feeding of IgG (μg/mL)	MWNTs mass (mg)	Abs of the supernatant at 280 nm	Retained IgG in supernatant (μg/mL)	Binding mass of IgG (μg)	Static binding capacities of IgG on His–MWNTs (mg/g)
125	5.0	0.045	18.0	214.0	42.8
250	5.0	0.051	20.4	459.2	91.8
500	5.0	0.197	78.8	842.4	168.5
1000	5.0	0.826	330.4	1339.2	267.8

Table 3
Comparison of the characteristics of histidine based affinity adsorbents for purification of IgG.

	Adsorbent	Adsorption condition	Elution condition	Time (min)	Equilibrium mode	Adsorption capacity (mg/g)	Ref.
1	MWNTs	10 mM PBS (pH 7.0) or 0.2 M glycine–HCl buffer (pH 5.0)	0.2 mol/L Glycine–HCl buffer (pH 1.5)	<10	Dynamic	35	This work
			––	10	Static	268	
2	Poly(GMA-co-EDMA) Monolithic Column	25 mM Tris or Hepes buffer (pH 7.4)	0.4 M NaCl in 25 mM Tris or Hepes buffer (pH 7.4)	10	Dynamic	11	[19]
3	Sepharose	30 mM MOPS (pH 7.2)	0.3–1 M NaCl in 30 mM MOPS[a] (pH 7.2)	100	Dynamic	0.5	[23]
4	Bentonite	0.1 M NaCl in 25 mM PBS (pH 7.4)	2.0 M NaCl	>180	Static	17～100	[13]
5	PHEMA[a] beads	–	1 M NaCl in 25 mM acetate buffer, pH 4.0	–	Dynamic	3.5～44.8	[24]
6	PEVA[a] hollow fiber membrane	25 mM MOPS (pH 6.5)	0.2 M NaCl in 25 mM Tris–HCl buffer (pH 7.4)	100	Dynamic	–	[25]
7	PEVA hollow fiber membranes	25 mM Tris–HCl (pH 7.4)	0.1 M NaCl	10	Static	80	[12]

The values of adsorption capacity in literatures were converted from mg/mL to mg/g by considering the gel density as 1.3 g/mL.

[a] MOPS: 3-(N-morpholino)propanesulfonic acid, PHEMA: Poly(2-hydroxyethyl methacrylate), PEVA: poly(ethylene vinyl alcohol).

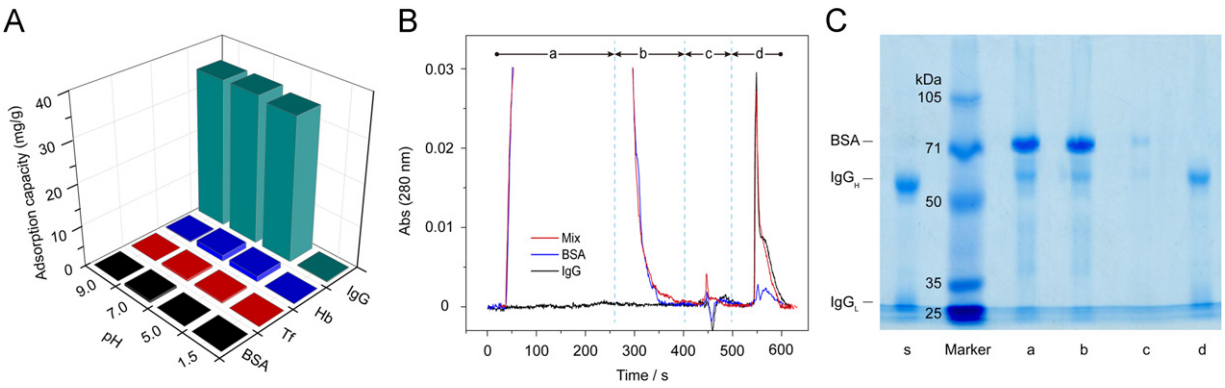

Fig. 5. (A) Comparison of the adsorption capacities of BSA, transferrin (Tf), hemoglobin (Hb) and IgG on the surfaces of His–MWNTs. (B) Comparison of the sorption and desorption behaviors of BSA (500 μg/mL), IgG (25 μg/mL) and their mixing solution of same concentration on the surfaces of His–MWNTs: (a) sampling, (b) washing, (c) elution with 0.2 mol/L glycine–HCl buffer at pH 5.0, (d) elution with 0.2 mol/L glycine–HCl buffer at pH 1.5. (C) SDS-PAGE electrophoretogram of the various purification fractions of mix solution of BSA (500 μg/mL) and IgG (25 μg/mL). Lane s: commercial rabbit IgG (500 μg/mL), lane a–d: effluent in relative step of mixing solution separation.

Table 4
Characteristic analytical performance of the present system for extraction and detection of rabbit IgG on a His–MWNTs packed microcolumn with different sample volumes.

Sample volume (mL)	Eluent volume (mL)	Time (min)	Linear range (μg/mL)	EFs	Recovery (%)	RSDs (%, 25 μg/mL IgG)		LODs (μg/mL, n-11)
						One column (n-7)	Column to column (n-3)	
10.0	0.1	13	0.4–13	75 ± 5	96	4.0 ± 0.5	10.0 ± 1.0	0.1
4.0	0.1	8	1.0–33	30 ± 2	98	3.5 ± 0.5	10.0 ± 1.0	0.3
0.1	0	0.2	6.0–1000	–	–	1.5 ± 0.2		2.0

or shorter processing time. Although due to the aggregation of the MWNTs in online SPE microcolumn, the dynamic adsorption capacity is not as excellent as we expected, the unusually static adsorption capacity shows its great potential for further investigation and application.

3.8. Affinities of proteins on His–MWNTs

The affinities of IgG, BSA, transferrin (Tf) and hemoglobin (Hb) on MWNTs–COOH and His–MWNTs were investigated in detail. Fig. 5A shows the non-specific and specific adsorption of several kinds of model proteins on His–MWNTs. The selected model proteins are high abundance of blood proteins and have different isoelectric status and molecular size. The dynamic adsorption experiments were performed at different pH values and the results showed that the non-specific adsorption of the model proteins on His–MWNTs was rather low and the adsorption of BSA and Tf onto the His–MWNTs could not be achieved no matter what the ionic strength and pH was. The non-specific Hb adsorption on the His–MWNTs was slightly higher than other model proteins and its adsorption was interrupted by raising pH, which might be due to the residual carboxyl groups on MWNTs. While a much higher adsorption capacity up to 35 mg IgG/g was obtained with the His–MWNTs. This proved that the hydrophobic groups on absorbent has been removed after the functionalization of MWNTs and the weak electrostatic interaction still exists on the His–MWNTs composites but is not the main driving force for the sorption of IgG. The adsorption behavior of IgG and Hb on MWNTs–COOH was also studied as a comparison. There was no observable retaining of IgG on MWNTs–COOH, while the Hb could be absorbed quantificationally. All these fact indicated that the His–MWNTs are effective affinity adsorbent for separation of IgG in complex real sample. Further study showed that the adsorption capacity of IgG increased with increasing IgG initial concentration in the loading solution at the given concentration range. However, His–MWNTs as pseudospecific affinity adsorbent for IgG, the tested IgG obtained from rabbit, calf and human serum showed no obvious difference on adsorption and desorption behavior.

A standard mix solution of rabbit IgG (25 μg/mL) and BSA (500 μg/mL) was prepared and evaluated with the same SPE process as the BSA and IgG simplex solution to compare their sorption and desorption behaviors in the present extraction system. As shown in Fig. 5B, it is obvious that the complete sorption of IgG in the loading buffer could be achieved on His–MWNTs and a high desorption efficiency was obtained by using glycine–HCl buffer at pH 1.5, while the adsorption of BSA was negligible. There was slight loss of IgG when eluted at pH 5.0, but the recovery was still more than 90%. The effluent in relative step of mixing solution separation was assayed with SDS-PAGE eluate (Fig. 5C), which shows the IgG was enriched and highly purified in the final eluate.

3.9. Analytical performance of the present system

The present investigation aims at isolating IgG from complex real sample matrices with better analytical performance. The enrichment factors (EFs), adsorption capacity, one-column repeatability and column-to-column reproducibility of IgG on His–MWNTs were performed under the conditions optimized above and the analytical performance data of this system were summarized in Table 4. Under the optimized conditions, when employing 4.0 mL of IgG sample volume and 100 μL of eluent volume, an enrichment factor of 30 was achieved, along with a retention rate of nearly 100% and an overall recovery rate of 98%. A limit of detection of 0.3 μg/mL was derived within a linear range of 1.0–33 μg/mL. The RSD were 3.5% for one column and 10.0% among columns obtained at a concentration level of 25 μg/mL. No obvious change in adsorption capacity was observed in 10 days of storage, indicating acceptable stability.

3.10. Method validation and application to real serum samples

To evaluate the practical applicability of this His–MWNTs packed microcolumn, two real samples of newborn calf serum and human serum were tested here, and the major protein components in these samples include serum albumin (pI=4.6), transferrin (pI=6.7–7.2), immunoglobulin G (pI=5.8–7.3) and so on. The serum samples were centrifuged at 3000 rpm for 1 min and then the supernatant fluid was 50-fold diluted with loading buffer (0.01 mol/L pH 7.0 PBS) and reserved at 4 °C for further use. The SPE process was performed as described in the Experimental Section. Usually the purified water or loading buffer solution was used for driving and isolating the fluid zones, but in order to maintain the volume and concentration of collected fractions, air was used here for dispensing the fluid flow and emptying the tubing and microcolumn. The effluent was collected with several 500 μL of centrifuge tubes in an orderly manner instead of being dispensed into the detector. The proteins in those collected fractions were then assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fig. 6 shows the protein composition of the various purification fractions. Part of those fractions (10 μL) from each step of the purification was electrophoresed on a 12% SDS–acrylamide gel. After the electrophoresis, the protein bands were visualized by Coomassie staining. IgG are large molecules of about 150 kDa composed of four peptide chains. It contains two identical class γ heavy chains of about 50 kDa (IgG_H) and two identical light chains of about 25 kDa (IgG_L). For the newborn calf serum (lane C1), there is a broad band at 50 kDa seems strong, but the IgG_L band could barely be seen at 25 kDa, which means the original amount of IgG in the newborn calf serum is relatively low. After the extraction process, the bands at 64 kDa and 75 kDa were effectively removed and two IgG bands could be seen in the eluate II (lane C5). In contrast, the IgG_H and IgG_L bands of human serum are very clear in lane H1. The lane H4 shows several bands of impurity proteins removed with weak eluent at pH 5.0. In lane H5, the clear IgG bands in eluate II could be seen, and the purity of this collected IgG is over 85% (calculated with Gel-Pro analyzer), which is higher than the commercial human IgG (76%, lane S). The dynamic adsorption capacity obtained is 60 mg of IgG/g His–MWNTs in 50-diluted serum. This observation well illustrated the practical applicability of present purification procedure for the effective separation and extraction of IgG from biological samples with complex matrix components.

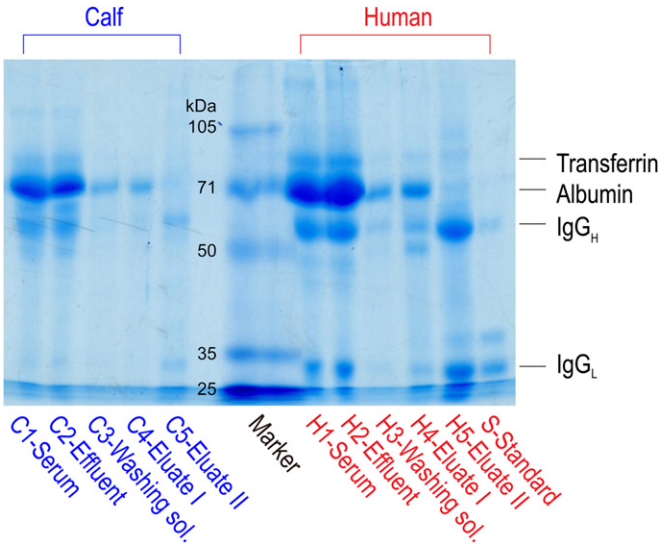

Fig. 6. SDS-PAGE electrophoretogram. Lane C1: 50-fold diluted newborn calf serum solution; lane C2: effluent passed through the microcolumn; lane C3: effluent from the washing procedure; lane C4: eluate eluted with 0.2 mol/L pH 5.0 glycine–HCl buffer; lane C5: eluate eluted with 0.2 mol/L pH 1.5 glycine–HCl buffer; lane H1: 50-fold diluted human serum solution; lane H2: effluent passed through the microcolumn; lane H3: effluent from the washing procedure; lane H4: eluate eluted with 0.2 mol/L pH 5.0 glycine–HCl buffer; lane H5: eluate eluted with 0.2 mol/L pH 1.5 glycine–HCl buffer, lane S: commercial human IgG (500 μg/mL).

4. Conclusion

In brief, the application of L-histidine covalently functionalized carbon nanotubes (His–MWNTs) in affinity separation of IgG in serum has been demonstrated. The incorporation of histidine as affinity groups noticeably increased the selectivity and binding capacity of MWNTs for IgG during the course of affinity separation. An additional advantage of this functionalization is its capability to reduce the non-specific interaction between CNTs with hydrophobic domains of biomolecules and the His–MWNTs also exhibited high retention and recovery rate of nearly 100% under optimized conditions. This separation and enrichment process made it possible to determine a lower concentration range of IgG in serum from 1.0 to 33 μg/mL with a detection limit of 0.3 μg/mL with a sampling volume of 4.0 mL. The dynamic adsorption capacity obtained is 35 mg of IgG/g His–MWNTs in aqueous solution and 60 mg/g in serum, which is among the highest reported in the literature employing online methods. Desorption of IgG from His–MWNTs could be accomplished by lowering the pH with glycine–HCl buffer. Together with the fast processing time (a few minutes) and high recovery, the MWNTs based SPE adsorbent shows great potential in the affinity separation of proteins in complex matrix.

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