Magnetic restricted access carbon nanotubes for smooth Cu and Zn extraction from Cu, Zn-superoxide dismutase

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Abstract

In this paper we proposed the synthesis, characterization and application of magnetic restricted access carbon nanotubes (M-RACNTs) to smoothly capture Cu and Zn from Cu, Zn superoxide dismutase (Cu,Zn-SOD). Fe_3O_4 magnetic nanoparticles were incorporated on the external surface of carbon nanotubes. The obtained material was encapsulated with a chemically crosslinked bovine serum albumin (BSA) layer, resulting in the M-RACNTs. The BSA capsule acts as a biocompatible barrier that prevents the proteins binding on the M-RACNTs surface. In a pH higher than the isoelectric point of BSA, the proteins from the sample and from the BSA layer, are negatively charged, resulting in an exclusion mechanism by electrostatic repulsion. The characterization tests confirmed the presence of the Fe_3O_4 magnetic nanoparticles in the interior, and the BSA layer on the surface of the M-RACNTs. The kinetic and isotherm tests were carried out for the Cu and Zn sorption by M-RACNTs, and the results confirmed the fast mass transference rate, and the metal retention in monolayers (Sips model, Cu and Zn maximum adsorption capacities of about 65 mg g^{-1}), respectively. M-RACNTs were able to remove 64 and 62% of Cu and Zn, respectively, from a 0.140 mol L^{-1} Cu,Zn-SOD aqueous solution, with only one magnetic dispersive solid phase extraction cycle. During this process, M-RACNTs retained less than 5% of the enzyme, attesting the efficiency of the BSA layer to prevent protein binding. The Cu,Zn-SOD submitted to the extraction lost about 15% of its activity, which was totally recovered when Cu and Zn were reincorporated in the enzyme structure by addition of an aqueous solution of both metals. Thus, we are sure that the M-RACNTs are promising sorbents to obtain apoproteins from metalloproteins in biotechnological applications.

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Graphic abstract

Obtaining Apo-superoxide dismutase from Cu,Zn-superoxide dismutase by extraction with M-RACNTs

Keywords Restricted access materials · Magnetic restricted access carbon nanotubes · Protein exclusion · Copper · Zinc · Cu,Zn-superoxide dismutase · Apoprotein

1 Introduction

Carbon nanotubes (CNTs) are cylindrical and hollow structures formed by a circular layer of carbons bonded between them in sp² configuration [1]. This material has been efficiently used in sample preparation due to its advantageous properties like high surface area, high adsorption capacity, high chemical and physical stability, facility to be chemically modified, among others [2]. In this context, solid phase extractions (SPEs) using CNTs are very efficient to concentrate organic [3, 4] and inorganic analytes [5, 6].

Despite the efficient use of commercial CNTs in conventional SPE, some superficial modifications have been carried out in order to facilitate their use in sample preparation. The association of Fe₃O₄ magnetic nanoparticles with CNTs is a good example. After the modification, the magnetic CNTs (M-CNTs) acquires magnetic susceptibility enough to be used in magnetic dispersive SPE, wherein

a magnet is used to remove the particles from samples/solutions [7–9]. The main advantage of this procedure in comparison with the conventional SPE is the excellent interaction between the sorbent and sample, resulting in higher extraction recoveries. Besides, the use of a magnet to remove the sorbent from the matrix is a very simple and efficient strategy to avoid recurrent problems of cartridges blockage, commonly faced in conventional SPE.

The high adsorption capacity of the commercial CNTs, as well as their low selectivity can result in sorption of matrix components. For example, when CNTs are used in the extraction of analytes from biological samples, the proteins can be retained in high proportion, decreasing the precision and extraction efficiency of the analytes due to the obstruction of the binding sites. To solve this problem, our research group developed restricted access carbon nanotubes (RACNTs) able to retain low molecular weight molecules or ions, excluding, simultaneously, the macromolecules from the sample [10]. These sorbents have been obtained by covering of commercial CNTs with bovine serum albumin (BSA) molecules, crosslinked by glutaraldehyde. This external BSA capsule avoids binding of proteins from biological fluids, when the sample pH is higher than the isoelectric point of proteins. In this case,

both proteins, from the sample and from the BSA layer, are negatively charged, and the exclusion occurs by electrostatic repulsion. At the same time, the analytes penetrate through the BSA layer, being retained in the core of CNTs [10–12]. Due to their recent development, RACNTs were used only in five applications, for inorganic ions [10, 13] and organic compounds [14–16]. In all cases, the sorbent was packed in mini columns and used in online extractions based either in flow injection analysis [10, 13], or in column switching liquid chromatography [14–16]. Moreover, RACNTs have demonstrated high efficiency to retain low molecular weight analytes, with protein exclusion capacities higher than 95%.

Based on the relevant advantages of the M-CNTs and RACNTs in terms of magnetic susceptibility for dispersive SPE and capacity to exclude macromolecules, respectively, we believe that a new hybrid material with both characteristics can be very useful in the extraction of organic and inorganic analytes from complex matrices. In this way, this paper reports the development and characterization of magnetic-restricted access carbon nanotubes (M-RACNTs) and their use in the dispersive SPE for extraction of Cu and Zn from Cu,Zn-superoxide dismutase (Cu,Zn-SOD) to obtain the apoproteic form of this protein. According to Bolster et al. [17], apoprotein is a protein that presents a modification in its constituents, as for example the loss of a complexed metal. Apoproteins have been extensively studied in the last years, given that the modification of metals can result in changes in the protein activity. Gomes et al. [18] emphasize that it is possible to change a metal in a protein in order to modify its physiological function. In this way, apoproteins are very important in biochemistry studies, and some strategies have been used to obtain them by the removing of bound metals, as for example the dialyze with chelating agents, filtration with Sephadex G25 and ion exchange chromatography with Sepharose column [19]. However, all these procedures are slow, complex and expansive. Therefore, alternative procedures to obtain apoproteins are important, like the present study based on dispersive SPE with M-RACNTs.

2 Materials and methods

2.1 Reagents and solutions

Ultra-high purity water (182 Ω m) from a Milli-Q system (Millipore®, Bedford, MA, USA) was used in the preparation of solutions. Multiwalled CNTs (6–9 nm $\times$ 5 μ m), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), BSA, glutaraldehyde (all from Sigma-Aldrich®, Steinheim, Germany) and sodium borohydride (NaBH_4 , Nuclear®, Diadema,

Brazil) were used in the M-RACNT synthesis. HPLC grade methanol and ethanol were purchased from Sigma-Aldrich®. Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid, nitric acid and sodium hydroxide were obtained from Vetec® (Rio de Janeiro, Brazil). Cu,Zn-SOD 30 KU and 1 g L⁻¹ Cu and Zn standard solutions were acquired from Sigma-Aldrich®.

2.2 Instrumentation

The Cu and Zn analyses were performed with a flame atomic absorption spectrometry (FAAS) Shimadzu model AA-6800 (Shimadzu, Tokyo, Japan) equipped with hollow cathode lamps ($I = 8$ mA) for Cu (wavelength = 324.80 nm) and Zn wavelength = 213.19 nm) and with background correction by a deuterium lamp. Acetylene and air were used in the flame at 3.0 and 10.0 L min⁻¹ flow rates, respectively. The protein exclusion tests were carried out in a high performance liquid chromatography system (HPLC) equipped with two LC-20AD pumps (Shimadzu®, Kyoto, Japan), a manual injector type 7725i (Rheodyne®, Waltham, USA), an electronic six-port switching valve model FCV-12AH (Shimadzu®, Tokyo, Japan), and an UV detector model SPD-10AVP (Shimadzu®, Tokyo, Japan). Data acquisition and treatment were performed using the LabSolutions® software (Shimadzu®). An empty cartridge of a guard column (2 cm $\times$ 4 mm, L $\times$ i.d.) was packed with each sorbent to obtain the extraction columns. An orbital shaker model, Vibrax VXR (Ika®, Staufen, Germany), was used in the kinetics and adsorption tests.

2.3 Oxidation process of CNTs and their influence in the Cu and Zn adsorption

Commercial multiwalled CNTs were oxidized using concentrated HNO_3 [10]. A mass of 500 mg of commercial CNTs was added in a flask containing 30 mL of 65% (v/v) HNO_3 . The system was agitated for 2 h, at 100 °C. After, the CNTs were filtrated in PTFE membrane (0.45 μ m) and washed with H_2O until neutral pH.

The adsorption capacities of Cu and Zn were appraised for the oxidized CNTs. A mass of 10 mg of oxidized CNTs was placed into a tube, containing 5 mL of 200 mg L⁻¹ of Cu or Zn in phosphate buffer solutions (50 mmol L⁻¹, pH 6.8). The tube was agitated using a vortex during 20 min and centrifuged at 700 g for 10 min, and the metal concentration was determined in the supernatant by FAAS. The experiment was executed in triplicate for each metal. The same experiment was carried out for commercial (unmodified) CNTs.

2.4 Preparation of the magnetic restricted access carbon nanotubes

M-RACNTs were prepared according to Qu et al. [20]. 1.0 g of the oxidized CNTs (obtained according to the previous section) was added in a flask containing 200 mL of an aqueous solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, at 0.043 and 0.022 mol L^{-1} , respectively. The flask was maintained at 50 °C, in nitrogen atmosphere, under agitation and for 10 min. Then, 10 mL of 8 mol L^{-1} NH_4OH aqueous solution were added into the flask, drop by drop. The obtained precipitant (Fe_3O_4 nanoparticles) was then washed with ethanol and water until neutral pH, and dried under vacuum during 12 h at 60 °C. The obtained material was denominated M-CNTs.

M-CNTs were covered with BSA according to the protocol described by Barbosa et al. [10]. 20 mL of a 1% (m/v) BSA phosphate buffer solution (50 mmol L^{-1} , pH = 5.7) and 5 mL of a 25% (v/v) glutaraldehyde aqueous solution were sequentially flowed through a cartridge containing 200 mg of M-CNTs at 1 mL min^{-1} flow rate. The cartridge was maintained in standby during 5 h. After, 10 mL of 1% (m/v) NaBH_4 aqueous solution were flowed through the cartridge at 1 mL min^{-1} flow rate. The obtained M-RACNTs were then washed with 100 mL of water and dried at 60 °C for 12 h.

2.5 Characterization of the magnetic restricted access carbon nanotubes

Transmission electron microscopy (TEM) images were acquired in a JEOL JEM 2100 microscope, equipped with Gatan ES 500 W and Gatan GIF Tridiem 2 kx2k CCD cameras. The samples were dropped on the copper grid with holey carbon film and analyzed using an acceleration voltage of 200 kV. Infra-red analyses were carried out in a Fourier transform infrared spectrometer—FT-IR (model 8400S, Shimadzu®, Tokyo, Japan), with spectral resolution of 4 cm^{-1} and using a mixture of KBr/sample at 1% (m/m). Thermogravimetric analyses were performed in a thermogravimetric analyzer—TGA (model SDT Q600, TA Instruments, New Castle, USA), at a heating rate of 20 °C min^{-1} from 30 to 1300 °C and under nitrogen atmosphere at 50 mL min^{-1} flow rate.

The zeta potential tests were conducted in a Zeta-sizer Nano ZS apparatus, equipped with MPT-2 Titrator (Malvern, Worcestershire, UK). To perform the titration, aliquots of oxidized CNTs, M-CNTs and M-RACNTs suspensions, at concentration of 1 mg mL^{-1} , were diluted in 10 mL of water. The suspensions were titrated by using 0.25/0.50 mol L^{-1} NaOH and 0.25 mol L^{-1} HCl aqueous solutions. The zeta potential was measured versus pH from 3.0 to 10.0.

2.6 Kinetic and adsorption studies

A volume of 5 mL of 10 mg L^{-1} of a Cu or Zn solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) was placed into 6 different polypropylene tubes containing 10 mg of the sorbent (CNTs, M-CNTs or M-RACNTs). The tubes were agitated in a vortex at 25 °C during 10, 25, 40, 55, 70 and 90 min, respectively for each tube. After, the tubes were immediately centrifuged at 700 g for 10 min, and the metal was determined in each supernatant by FAAS. The experiment was carried out in triplicate. The maximum adsorptive capacity— q_e (mg g^{-1})—was obtained according to Eq. 1 [21], where C_o and C_e (both in mg L^{-1}) are the initial and equilibrium concentrations (analyzed concentration), respectively, V (L) is the volume of solution and m (g) is the mass of the sorbent [15]:

$$q_e = \frac{(C_o - C_e)}{m} \times V. \quad (1)$$

The data were treated according to the kinetic models of pseudo-first order, pseudo-second order and fractional order, taking into consideration the value of the linear correlation coefficient (R^2) and the error function (F_{error}) (Eq. 2) [22], that correlate the theoretical amount of analyte adsorbed by the material with that measured experimentally, considering the number of parameters of the fitted model [23].

$$F_{\text{error}} = \sqrt{\left(\frac{1}{n-p}\right) \sum_i^n (q_{i,\text{exp}} - q_{i,\text{theoretical}})^2} \quad (2)$$

The number of experiments and parameters of the fitted model are n and p , respectively. $q_{i,\text{exp}}$ is each value of q_e measured experimentally and $q_{i,\text{theoretical}}$ is each value of q_e predicted by the fitted model [15].

One adsorption isotherm was built for each metal (Cu and Zn) and for each material (CNTs, M-CNTs or M-RACNTs). A mass of 10 mg of each sorbent was placed into 7 test tubes, containing 5 mL of the metal solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) at the concentrations of 5, 20, 50, 80, 120, 200 or 300 mg L^{-1} . The tubes were agitated using a vortex during 20 min and centrifuged at 700 g for 10 min. The equilibrium concentration (C_e) was obtained by the metal determination in each supernatant by FAAS. The q_e was calculated according to the Eq. 1. All the experiments were executed in triplicate and the data were fitted according to Langmuir [24], Freundlich [25] and Sips [26] models, based on the value of the R^2 and F_{error} .

Fig. 1 Transmission electron micrographs of the M-CNTs (a, c) and M-RACNTs (b, d). Black and white arrows indicate the CNTs and Fe₃O₄ magnetic nanoparticles, respectively

2.7 Protein exclusion test

The proteins exclusion test was carried out according to the protocol described by Moraes et al. [27]. A volume of 500 μL of a 44 g L^{-1} BSA aqueous solution was injected into the chromatographic system, without any columns. The UV detector was operated at 255 nm and phosphate buffer (50 mmol L^{-1} , pH=7.0) was used as mobile phase at a 1 mL min^{-1} flow rate. The obtained signal corresponded to 100% of the BSA (all the injected BSA arrived to the detector). Next, a column (1 $\text{cm} \times 4 \text{ mm}$, L $\times$ i.d.) filled with RACNTs particles was coupled to the HPLC, and the same BSA solution was injected. The peak area obtained, divided by the 100% BSA signal area, was related to the percentage

of the BSA excluded by RACNTs. The same experiment was carried out for CNTs and M-CNTs.

2.8 Use of magnetic restrict access carbon nanotubes to capture Cu and Zn from Cu and Zn superoxide

A Cu,Zn-SOD solution was submitted to the experimental protocols described below:

Protocol 1 (extraction procedure): a volume of 5 mL of a 0.140 mg L^{-1} Cu,Zn-SOD solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) was added into a tube containing 20 mg of M-RACNTs. The tube was agitated during 24 h at 25 $^{\circ}\text{C}$, and the supernatant was

Fig. 2 Infrared spectra of CNTs, M-CNTs and M-RACNTs

Fig. 3 Zeta potential of CNTs, M-CNTs and M-RACNTs

separated from the sorbent using a magnet. The experiment was conducted in quadruplicate.

Protocol 2 (control of the protocol 1): a volume of 5 mL of a 0.140 mg L^{-1} Cu₂Zn-SOD solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) were added into another tube, that was maintained in the same rack of the protocol 1 during 24 h at 25°C . The experiment was conducted in quadruplicate.

The removed metal percentage (*RM%*) from the enzyme (protocol 1) was calculated according to the Eq. 3, where *EC* is the equilibrium concentration of each metal (Cu or Zn) determined by FAAS in the supernatant obtained from the protocol 1, and *TC* is the total concentration of each metal determined by FAAS in the enzymatic solution from the protocol 2.

$$RM\% = \frac{TC - EC}{TC} \times 100 \quad (3)$$

The sorbed enzyme percentage (*SE%*) on the M-RACNTs during the protocol 1 was determined by Eq. 4, where *A*₁ and *A*₂ are the absorbances measured at 280 nm for the supernatants from the protocols 1 and 2, respectively.

$$SE\% = \frac{A_2 - A_1}{A_2} \times 100 \quad (4)$$

Finally, the Cu₂Zn-SOD enzymatic activity of the supernatant from protocols 1 and 2 were spectrophotometrically determined according to the Oyanagui's method, assuming that 1 unit of enzyme is able to produce 50% of inhibition in reaction [28].

In order to appraise the effect of the Cu and Zn addition in the Cu₂Zn-SOD enzymatic activity of the enzyme submitted to the protocol 1, the following experimental protocols were carried out:

Protocol 3: 990 μL of the supernatant from protocol 1 was added in a tube containing 10 μL of a 17 mg L^{-1} Cu and Zn aqueous solution, resulting in a final Cu and Zn solution of $170 \mu\text{g L}^{-1}$. The tube was agitated during 24 h at 25°C , and the enzymatic activity of the solution was determined according to the Oyanagui's method [28]. The same protocol was repeated for final metal concentrations of 300 and $600 \mu\text{g L}^{-1}$. The experiments were carried out in quadruplicate.

Protocol 4 (control of the protocol 3): 990 μL of the supernatant from protocol 1 was added in tube containing 10 μL of water. The tube was agitated during 24 h at 25°C , and the enzymatic activity of the solution was determined according to the Oyanagui's method [28]. The experiments were carried out in quadruplicate.

3 Results and discussions

3.1 Influence of the oxidation process in the adsorption of Cu and Zn on carbon nanotubes

CNTs have been chemically modified to increase their adsorption capacity. For metals, the main modification procedure is the oxidation using nitric acid [18]. Thus, the adsorption capacities for Cu and Zn were evaluated for unmodified and oxidized CNTs. The oxidized material presented adsorption capacities of about 20 and 15% higher than the unmodified CNTs, respectively for Zn and Cu. The best performance of the oxidized CNTs can be attributed to the presence of functional groups containing oxygen,

Table 1 Kinect parameters for the Zn and Cu adsorption in CNTs, M-CNTs and M-RACNTs fitted to the pseudo-first order, pseudo-second order and fractionary order models

Sorbent	Parameter	Pseudo-first order		Pseudo-second order		Fractionary order	
		Zn	Cu	Zn	Cu	Zn	Cu
CNTs	q_e (mg g ⁻¹)	96.103	98.422	96.103	98.422	96.103	98.905
	R^2	0.9991	0.9999	0.9999	0.9999	0.9992	0.8066
	F_{error}	0.4491	0.0812	3.78815×10^{-8}	0.0971	0.4099	0.4375
	K	18.869	18.879	2.85141×10^{35}	2.2988×10^{14}	1.000	1.000
	n_{AV}	—	—	—	—	1.000	1.000
M-CNT	q_e (mg g ⁻¹)	95.218	99.740	95.218	99.470	95.686	99.959
	R^2	0.9999	0.9999	0.9999	0.9999	0.8066	0.8066
	F_{error}	0.1255	0.2421	3.1848×10^{-8}	0.1914	0.4233	0.4422
	K	18.444	0.1280	31814.410	19.351	1.000	1.000
	n_{AV}	—	—	—	—	1.000	1.000
M-RACNT	q_e (mg g ⁻¹)	95.797	99.170	95.797	99.879	95.798	99.342
	R^2	0.9992	0.9996	0.9992	0.9999	0.9993	0.9431
	F_{error}	0.4197	0.3132	0.5913	0.2259	0.3832	0.2444
	K	18.752	19.312	4.7262×10^9	0.0484	1.000	1.000
	n_{AV}	—	—	—	—	1.000	1.000

The equations $q_t = q_e [1 - \exp(-kt)]$, $q_t = (kq_e^2 t) / (1 + Kq_e t)$ and $q_t = q_e \{1 - \exp[-(kt)]^{n_{\text{AV}}}\}$ were used to fit the data to the pseudo-first order, pseudo-second order and fractionary order models, respectively. q_t : amount of analyte adsorbed at time t ; q_e : amount of analyte adsorbed at equilibrium per gram of material; t : time of contact; k : pseudo-first order rate constant, pseudo-second order rate constant or Avrami kinetic constant for pseudo first order, pseudo-second order or fractionary order models, respectively; n_{AV} : fractionary reaction order (Avrami)

like carboxyl, hydroxyl, lactones and phenol [18]. The adsorption mechanism can be attributed to the chemical and electrostatic interactions between the metallic ions and the oxidized CNTs [29].

3.2 Synthesis and characterization of the magnetic restricted access carbon nanotubes

Fe₃O₄ nanoparticles were obtained by the co-precipitation method, due to its simplicity, greater yield and short reaction time. Additionally, the high concentration of iron salts can result in more homogeneous nanoparticle size distribution [30]. After, the Fe₃O₄ nanoparticles were fixed on CNTs surface resulting in the M-CNTs, which were encapsulated with the BSA layer through the bonds between the amine groups of the BSA with glutaraldehyde (cross-linker reagent). The obtained imine groups were reduced to amines with NaBH₄ in order to improve the chemical stability of the M-RACNTs [10]. Figure 1 shows the transmission electron micrographs of the M-CNTs and M-RACNTs. The white arrows indicate the Fe₃O₄ nanoparticle in the M-CNTs and M-RACNTs, and the difference in tonality can be attributed to the BSA layer only in the M-RACNTs. Additionally, the difference in the CNTs diameter and tonality (black arrows) can also attest the presence on the BSA layer only in M-RACNTs surface.

According to the thermogravimetric analyses, CNTs lost about 80% of weight in 632 °C due to their degradation,

whereas about 60% of weight loss for M-CNTs was observed in 405 °C. Probably the incorporation of the magnetic nanoparticle destabilized the CNTs walls, decreasing their thermal resistance. M-RACNTs presented a behavior similar to the M-CNTs, except for the weight loss in 240 °C (about 20%), probably due to the degradation of the BSA layer at this temperature [18].

FT-IR spectra of the CNTs, M-CNTs and M-RACNTs can be observed in Fig. 2. However, few information could be obtained due to the high absorption of the radiation by the black CNTs. The bands of 1750–1631 cm⁻¹ probably correspond to the C=O stretching vibration of –COOH and asymmetric stretching vibration of COO–, and the bands 1079 and 1377 cm⁻¹ can be attributed the stretching vibration C–O–C and C–OH [31]. The presence of the BSA layer in the M-RACNTs can be confirmed by the bands at 1200 and 1650 cm⁻¹, related to the presence of amines [32]. Additionally, subtle bands of iron at 550 cm⁻¹ can be observed in M-CNTs and M-RACNTs spectra, probably due to the presence of Fe₃O₄ nanoparticles.

To verify the efficiency of Fe₃O₄ incorporation in oxidized CNTs and the BSA coating on M-CNTs, a scan of the zeta potentials versus pH, of the three materials (oxidized CNTs, M-CNTs and M-RACNTs), was done. As shown in Fig. 3, the isoelectric points (pI) of the oxidized CNTs, M-CNTs e M-RACNTs were 4.0, 4.5 and 4.8, respectively. The difference in zeta potential between the materials, at the same pH value, can indicates the presence of Fe₃O₄

Fig. 4 Adsorption isotherms of Cu and Zn in the CNTs, M-CNTs and M-RACNTs

nanoparticles and the BSA coating. For the M-RACNTs, the pI value was the same of the BSA ($4.7 < pI < 4.9$ [33]). The zeta potentials were positive and negative for pH s below and above the pI s of all the materials, respectively.

3.3 Kinetic and adsorption studies

Cu and Zn adsorption kinetics in CNTs, M-CNTs and RACNTs were appraised individually at pH 6.8, given that the protein exclusion capacity is better in this condition, according to Gomes et al. [18]. The equilibrium was reached in less than 15 min for all the sorbents and for both Cu and Zn ions. These result was similar to those obtained by Barbosa et al. for the adsorption of Cd in a RACNTs [10]. Moreover, the M-RACNTs presented mass transference speed equal to the unmodified CNTs. This fact attests that the presence of the BSA layer on the CNTs is not a barrier to the Cu and

Zn diffusion. We believe that a few proportion of the ions are retained on the BSA layer, being the high proportion of them is fixed in the CNTs core, according to previous study of Barbosa et al. [10]. The kinetic data were treated according to the pseudo-first order, pseudo-second order and fractionary order models. According to the high and low values of R^2 and F_{error} , the best fits were obtained for the pseudo-second order model for all the sorbents, as it can be seen in Table 1. Thus, it is possible to conclude that the interactions between Cu/Zn and the sorbents are based on the chemical adsorptions, covering all the study range [34].

Figure 4 shows the adsorption isotherms for Cu and Zn. As it can be seen, the adsorption capacities of the CNTs, M-CNTs and M-RACNTs were very similar, demonstrating that the presence of a BSA layer in the M-RACNTs did not compromise the metal adsorptions. Additionally, due to the high concentration of the analytes used in this study, it is possible to see, for all the sorbents, a first fast adsorption step resulted to the high mass transport tax. A slower step can be seen in sequence, probably due to the difficult of the analytes to reach the binding sites of the sorbents, being this step the most important for the equilibrium reaching. In a thirty step, the increase of the metal concentration did not increased its adsorption in the sorbents, attesting the equilibrium was reached [35].

The data were adjusted to the Langmuir, Freundlich and SIPs models, and the obtained parameters are showed in Table 2. Based on the highest value of R^2 and the lowest value of F_{error} , the best fits were obtained for the Sips model for all the sorbents and analytes (Table 2). Thus, probably the surfaces of the materials are energetically heterogeneous [36]. Sips model is a combination between the Freundlich (for low concentrations) and Langmuir (for high concentrations) models [37]. Therefore, the Cu and Zn ions were absorbed in a monolayer (like in the Langmuir model) [38].

3.4 Protein exclusion tests

The abilities of CNTs, M-CNTs and RACNTs to exclude macromolecules were investigated (Fig. 5). As it can be seen, about 66.6% of the proteins injected through the M-CNTs column was retained, whereas the M-RACNTs column was able to exclude about 98.2% of the proteins percolated through it. This good result of the M-RACNTs attests the efficiency of the BSA layer to exclude proteins, probably due to the electrostatic repulsion between the proteins from the solution and from the BSA layers of the RACNTs, both negatively charged in pH 7.0 [18].

Table 2 Isotherm parameters for Cu and Zn adsorption in CNTs, M-CNTs and RACNTs fitted to the Langmuir, Freundlich and Sips models

Sorbent	Model								
	Langmuir			Freundlich			Sips		
	Zn	Cu		Zn	Cu		Zn	Cu	
CNTs									
Q_o	99.62543	88.84195	K _f	17.23771	15.07898	Q_o	90.3041	84.1843	
R^2	0.96872	0.98087	R^2	0.82611	0.87252	R^2	0.97335	0.97748	
F_{error}	7.96266	5.23164	F_{error}	6.67646	5.0009	F_{error}	0.80378	0.80453	
b	0.07111	0.06977	n	0.74937	2.75789	K_s	0.03779	0.0553	
						m_s	1.33809	1.14251	
M-CNT									
Q_o	89.392	94.36448	K _f	22.104	12.74862	Q_o	16.11431	82.2694	
R^2	0.89844	0.96331	R^2	0.94235	0.82304	R^2	0.93678	0.98103	
F_{error}	12.7	8.76097	F_{error}	4.41643	5.4923	F_{error}	15.23231	0.56964	
b	0.10131	0.0503	n	3.31258	2.51468	K_s	0.14351	0.01676	
						m_s	0.44711	1.4978	
M-RACNTs									
Q_o	64.38033	92.14459	K _f	28.25683	9.1806	Q_o	70.1441	78.8221	
R^2	0.95156	0.90732	R^2	0.83978	0.81594	R^2	0.96628	0.89556	
F_{error}	3.69932	16.78641	F_{error}	5.82924	4.75685	F_{error}	0.65117	1.63165	
b	0.69139	0.03177	n	5.1446	2.27342	K_s	0.63877	0.00919	
						m_s	0.68681	1.47898	

The equation $q_e = Q_o b C_e / (1 + b C_e)$, $q_e = k_f C_e^{1/n}$, $q_e = (Q_o K_s C_e^{1/m_s}) / (1 + K_s C_e^{1/m_s})$ were used to fit the data to the Langmuir, Freundlich and Sips, models, respectively. q_e : amount of adsorbed analyte at equilibrium per gram of material, C_e : Analyte concentration at equilibrium, Q_o : theoretical saturation capacity, b: Langmuir affinity constant, K_f and n: constant and exponent of Freundlich model, respectively, K_s and m_s : constant and exponent of Sips model, respectively

Fig. 5 Chromatograms obtained by the injection of 500 μ L of the 44 mg mL^{-1} BSA aqueous solution into the system without column and with the M-CNTs and M-RACNTs columns. a Peak area: 2,356,849; b peak area: 777,409 and c peak area: 2,313,989**Table 3** Concentration of Cu and Zn in a 0.140 mg L^{-1} Cu,Zn-SOD phosphate buffer solution (50 mmol L^{-1} , pH 6.8) before and after the extraction with M-RACNTs

Condition	Concentration/ mg L^{-1}	
	Cu	Zn
Without extraction with M-RACNTs	0.075	0.069
With extraction with M-RACNTs	0.027	0.026
RM% ^a	64	62

^aRemoved metal percentage

3.5 Use of magnetic restrict access carbon nanotubes to capture Cu and Zn from Cu, Zn superoxide

Cu,Zn-SOD is a widely studied metalloprotein, responsible for the protection of cells and tissues against oxidative stress [39], besides being associated with cases of amyotrophic lateral sclerosis [40]. The apoptotic form of

Fig. 6 Enzymatic activity of a 0.140 mg L^{-1} Cu,Zn-SOD solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) before (BE) and after (AE) dispersive SPE with M-RACNTs (supernatants from protocols 2 and 1, respectively). AE + 170, AE + 300 and AE + 600 are the same enzymatic solution after extraction (supernatant from protocol 1, section “Use of magnetic restrict access carbon nanotubes to capture Cu and Zn from Cu and Zn superoxide”) fortified with 170, 300 and $600 \text{ } \mu\text{g L}^{-1}$ of Cu and Zn

Cu,Zn-SOD is very important in studies involving the influence of the metals in its enzymatic activity. Thus, alternative procedures to remove the metals without compromise the structure of the Cu,Zn-SOD are welcome.

Cu and Zn were analyzed in a 0.140 mg L^{-1} Cu,Zn-SOD solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) before (protocol 2) and after (protocol 1) the magnetic dispersive SPE with M-RACNTs, and the obtained concentrations, as well as the *RM%* for Cu and Zn are presented in Table 3. As it can be seen, about 64 and 62% of Cu and Zn (respectively) were removed from the enzyme with one extraction cycle. Additionally, the same 0.140 mg L^{-1} Cu,Zn-SOD solution (prepared in phosphate buffer, 50 mmol L^{-1} , pH 6.8) was spectrophotometrically analyzed at 280 nm before (protocol 2) and after (protocol 1) the magnetic dispersive SPE with M-RACNTs, and the obtained analytical signals were practically equal. Thus, the *SE%* was of about 98%, confirming the ability of the M-RACNTs to avoid the binding of macromolecules on their surface, whereas the metals are captured.

The activities of the Cu,Zn-SOD were determined before (protocol 2) and after (protocol 1) the magnetic dispersive SPE with M-RACNTs and the results (Fig. 6) confirmed that the decreasing in the Cu and Zn concentrations reduces the enzymatic activity. On the other hand, the enzymatic activity was restored when the enzyme was spiked with Cu and Zn aqueous solutions, as it can be seen in Fig. 6. Our results also demonstrated that a high capture of Cu and

Zn did not result in a proportional decreasing in the enzymatic activity, probably because the metals are not totally related to the functionality of the enzyme active center.

We believe the M-RACNTs have a great potential to be used in the obtaining of Apo-SOD in an exhaustive extraction procedures, as well as to obtain other apoproteins.

4 Conclusions

M-RACNTs is a new biocompatible material inspired in the association between RACNTs and M-CNTs. This sorbent presents, simultaneously, magnetic susceptibility to be used in magnetic dispersive SPE and capacity to exclude macromolecules. The conversions of CNTs to M-CNTs and to M-RACNTs were confirmed by TEM, FT-IR and TG. The fast mass transferences as well as the high adsorption capacities of the M-RACNTs were confirmed by the Kinect and isotherm studies, being that the adsorption occurs in monolayers, according to the Sips model.

M-RACNTs were able to remove about 64 and 62% of Cu and Zn from the Cu,Zn-SOD, respectively, with one extraction cycle, whereas less than 2% of the proteins were retained on the material surface. Additionally, it was attested that the enzymatic activity decreased after the Cu and Zn removing, being it restored after the reincorporation of the metals. M-RACNTs are promising sorbents to be used in the extraction of metals from metalloproteins to obtain the apoprotein forms. Commercial strategies for obtaining apoproteins are based on the removal of bound metals by dialysis with chelating agents, filtration with Sephadex G25 and ion exchange chromatography with Sepharose column. All procedures are time consuming and expensive, justifying the importance of efficient and economically viable alternative methods, as in this work. It is important to point out that metal extraction percentages of about 60% were obtained with only one extraction cycle. Therefore, we are sure that further studies can increase the extraction percentages to around 100%. In this scenario, the material can be very promising for commercial applications in Brazil and other countries, especially for research centers and companies interested in obtaining apoprotein from metalloproteins.

Finally, based on the success of RACNTs in SPE of untreated biological samples [13–16], we are sure that the M-RACNTs can also be used in magnetic dispersive SPE, being a good alternative to extract organic compounds and inorganic ions directly from biological samples (e.g. blood, plasma, serum and milk).

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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