

ORIGINAL RESEARCH ARTICLE

Preparation and biochemical characterisation of nanoconjugates of functionalized carbon nanotubes and cytochrome c

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ABSTRACT

Objective(s): The present work deals with the preparation of nanobioconjugates based on the immobilization of cytochrome c (cyt c) on functionalized multi-wall carbon nanotubes (f-MWCNTs). The effect of the nanosupport and the immobilization procedure on the biochemical and structural characteristics of the immobilized protein was investigated.

Methods: The MWCNTs were functionalized to provide alkyl chains with different length and terminal functional groups on their surface. The immobilization of cyt c was achieved through physical adsorption and covalent binding. Cyt-c-based nanoconjugates were characterized in terms of peroxidase activity and stability of protein, while UV-visible spectroscopy was used to investigate the structural characteristics of the immobilized protein.

Results: The loading of cyt c on f-MWCNTs was effectively achieved, with immobilization yields reaching up to 77%. The peroxidase activity of cyt c was higher in the case of non covalent immobilization compared to that of covalent procedure. Immobilized cyt c exhibited higher thermal stability than the native protein after 24 h incubation at 40°C, while it preserved up to 100% of its initial activity after incubation in the presence of a denaturing agent such as H₂O₂. No significant changes in the heme microenvironment of cyt c were observed in the presence of f-MWCNTs.

Conclusions: This study has demonstrated that f-MWCNTs are effective supports for the immobilization of cyt c, providing a universally applicable platform for the development of bionanoconjugates with potential use in a wide variety of fields in nanobiocatalysis, biosensing and nanomedicine.

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INTRODUCTION

Nanomedicine, a rapidly growing research field of medicine that applies tools of nanotechnology, involves the use of nanoscale materials for diagnosis, delivery and sensing [1]. Carbon-based nanomaterials have a great potential for biological and nanomedicine applications due to their surface characteristics and good biocompatibility [2,3]. Among these nanomaterials, carbon nanotubes (CNTs) have been widely used in pharmacy and

medicine due to their high surface area that facilitates the conjugation with a variety of therapeutic and diagnostic agents, such as genes, antibodies, drugs and proteins [4]. CNTs are allotropes of carbon, synthesized in cylindrical tubes with nanometer scale in diameter and several millimeters in length. They exhibit excellent mechanical, structural and electrical properties which render from their small size [5]. These characteristics, along with their high surface area, make them potential vehicles for drug

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delivery into cells directly, as well as excellent supports for the loading of other biomolecules with biological function in cells, such as proteins and enzymes [4].

Cytochrome c (cyt c) is a small globule heme protein (~ 12000 Da) found in the inner membrane of mitochondria. It belongs to the cytochrome family containing heme c and participates in electron transport chain, where it carries electrons from cytochrome c reductase (complex III) to cytochrome c oxidase (complex IV) [6]. Moreover, cyt c is a key protein to the initialization of cell apoptosis, as well as a radical scavenger that removes unpaired electrons from superoxide, and thus regenerating molecular oxygen [7]. The structure, simplicity and availability of this redox protein make it an ideal model for understanding the physical electron transfer of proteins, as well as the conformational transitions in an atomic level. Cyt c is among the best characterized proteins due to its spectroscopic characteristics, and presents high peroxidase activity *in vitro*. These characteristics make cyt c an ideal protein for an extensive use in nanomedicine and biocatalysis, providing a better understanding of the molecular mechanisms *in vivo* [8]. Many research groups have investigated the biochemical properties of cyt c, as well as its interactions with various physical and chemical molecules which could lead to functional and structural changes of the protein [9,10]. Moreover, the conjugation of cyt c with biological molecules or synthesized supports, such as nanomaterials, offers the possibility to tailor the catalytic characteristics of the protein [11,12].

In the present study, cyt c from equine heart has been immobilized on functionalized multi-wall carbon nanotubes (f-MWCNTs). The aim of this work has been to investigate the use of these nanomaterials as supports for the immobilization of a key protein in living cells. The effect of f-MWCNTs on the biochemical characteristics of cyt c, such as activity, stability and structure was studied. The results have shown that f-MWCNTs exhibit high ability to form stable conjugations with cyt c, protecting the protein from denaturing conditions, such as temperature and the presence of H₂O₂. The presence of these nanomaterials also maintains the conformational state of the heme pocket of cyt c.

MATERIALS AND METHODS

Materials

Cytochrome c (cyt c) from equine heart was purchased from Sigma-Aldrich (>98%, St. Louis, MO) and used without further purification.

Guaiacol (2-Methoxyphenol) was purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30% w/v, H₂O₂) was obtained from Fluka. N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylpropyl) carbodiimide (EDC) and HEPES were obtained from Sigma. Glutaraldehyde solution 25% for electron Microscopy was purchased from Merck (KGaA Darmstadt, Germany). Multi-wall carbon nanotubes (95 % pure, MWCNTs) were purchased from Aldrich. All other solvents and reagents were of HPLC or analytical grade.

Functionalization of MWCNTs

Functionalized MWCNTs were prepared after the oxidation of the nanomaterials according to previous works. [13,14]. Aminoundecanoic acid, hexamethylenediamine and dodecylamine were used for the addition of terminal carboxyl, amine and alkyl groups, respectively.

Non covalent immobilization of cyt c

In a typical procedure, 3 mg of f-MWCNTs were added in 5.7 mL of phosphate buffer (50 mM, pH 7.0) and sonicated for 30min. Then 1 mL of cyt c in phosphate buffer solution (containing 3 mg of cyt c) was added and the mixture was incubated under stirring for 1 h at 30°C. The f-MWCNTs-cyt c conjugates were separated by centrifugation at 6,000 rpm and washed three times with phosphate buffer solution to remove loosely bound protein. The immobilized cyt c was dried over silica gel and was stored at 4°C until used.

Covalent immobilization of cyt c using glutaraldehyde as cross-linker

3 mg of amine-functionalized MWCNTs were added in 5.7 mL of phosphate buffer (50 mM, pH 7.0) and sonicated for 30 min, in the presence of 110 µL Tween-20. After the dispersion of nanomaterials, 1.76 mL of glutaraldehyde was added and the mixture was incubated under stirring for 1 h at 30°C. The modified nanomaterials were separated by centrifugation at 6,000 rpm and washed three times with phosphate buffer solution to remove extra glutaraldehyde. Then 6 mL of phosphate buffer solution containing cyt c (3 mg of cyt c) were added and the mixture was treated as described for non covalent procedure.

Covalent immobilization of cyt c via diimide-activated amidation

3 mg of carboxyl-functionalized MWCNTs were

added in 5 mL HPLC water and sonicated for 30 min. Then 1 mL of HEPES buffer solution (50 mM, pH 7.0) and 2.3 mL of a 50 mg mL⁻¹ NHS aqueous solution were added to the above suspension and mixed. Under fast stirring, 1.2 mL of a 10 mg mL⁻¹ EDC aqueous solution were added quickly and the mixture was incubated for 30 min at 30°C. The activated nanomaterials were separated by centrifugation at 6,000 rpm and washed three times with HEPES buffer solution to remove extra EDC. The ester-nanomaterials were re-dispersed in 9 mL HEPES buffer solution for 30 min. Then, 1 mL of protein solution in HEPES buffer (containing 3 mg cyt c) was added and the mixture was treated as described for non covalent procedure.

Determination of immobilization yield

The amount of immobilized cyt c was determined by determining the protein concentration in the supernatant after the immobilization procedure according to the Bradford method [15].

FTIR measurements

FTIR spectra were measured with a FTIR-8400 infrared spectrometer (Shimadzu, Tokyo, Japan) equipped with a deuterated triglycine sulphate (DTGS) detector. A total of 64 scans were averaged for each sample with 2 cm⁻¹ resolution, using KBr pellets containing *ca.* 2 wt% sample.

Determination of free and immobilized cyt c peroxidase activity

0.5 mg of free or immobilized cyt c was dispersed in 0.5 mL phosphate buffer (50 mM, pH 7.0) to give a final concentration of 1 mg mL⁻¹. The peroxidase activity of immobilized cyt c was determined using the chromogenic substrate guaiacol, as shown in Scheme 1. Hydrogen peroxide was added to give a final assay mixture (containing 25 µg mL⁻¹ free or immobilized cyt c, 25 mM guaiacol and 10 mM hydrogen

peroxide). The activity of cyt c was monitored by measuring the increase of the absorbance at 470 nm, due to guaiacol oxidation, at 40°C.

The effect of temperature was studied by measuring the activity of both free and immobilized cyt c at different temperatures (ranging from 30 to 75°C).

Stability of free and immobilized cyt c

The stability of immobilized cyt c was investigated and compared to that of the native protein. Phosphate buffer (50 mM, pH 7.0) was pre-incubated at 40°C in the presence of guaiacol (25 mM) or H₂O₂ (10 mM), and a predetermined amount of cyt c was added to give a final concentration of 25 µg mL⁻¹. Samples were withdrawn at regular time intervals in order to measure the remaining activity of cyt c. All experiments were repeated at least 3 times.

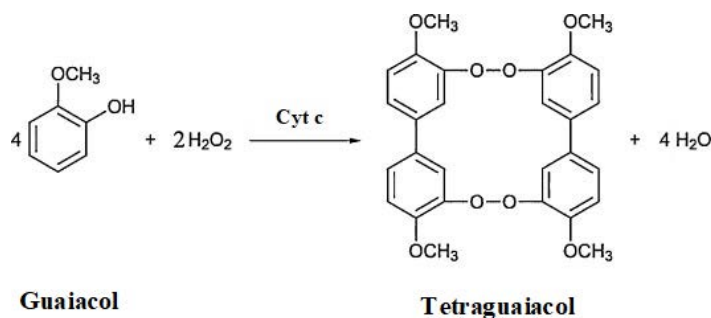
UV-Visible measurements

The conformational changes around the heme microenvironment of cyt c in the presence of f-MWCNTs were determined by recording the protein spectrum at the Soret region. UV-Vis spectra (300–700 nm) of cyt c (25 µg mL⁻¹) in phosphate buffer solution (50 mM, pH 7.0), containing various f-MWCNTs at different concentrations (5–25 µg mL⁻¹) were recorded at room temperature using a UV-1601 Shimadzu spectrophotometer (Tokyo, Japan).

RESULTS AND DISCUSSION

Immobilization of cyt c on f-MWCNTs

In the present work we have investigated the immobilization of cyt c on various f-MWCNTs. The immobilization procedure was carried out under constant experimental conditions (pH 7.0, 30 °C). At this pH, cyt c exhibits high peroxidase activity, while the net charge of the protein is positive, since



Scheme 1. Oxidation of guaiacol by cyt c in the presence of H₂O₂.

its isoelectric point (pI) is 10.5 [13].

The immobilization of cyt c onto f-MWCNTs was accomplished via two different methods: physical adsorption and covalent binding. The physical adsorption of proteins on f-MWCNTs is governed by weak forces such as van der Waals forces, π - π stacking interactions, hydrophobic and electrostatic interactions [2,3,16]. Covalent immobilization was achieved on two different kinds of functionalized carbon nanotubes; those with terminal carboxyl groups and those with terminal amine groups. In the first case, during the immobilization procedure, the carboxyl-functionalized CNTs firstly reacted with EDC to form an amine-reactive O-acilylsouria intermediate which subsequently reacted with an amine group on the surface of the protein to produce a stable amine bond [16]. NHS was added for the stabilization of the O-acilylsouria intermediate by converting it to a semi-stable amine-reactive NHS ester, thus enhancing a more efficient coupling with the protein [17]. Finally, the activated CNTs reacted with the protein. In the case of immobilization on amino-functionalized CNTs, the nanomaterials were firstly activated with the cross-linker glutaraldehyde. In a following step, the free terminal aldehyde groups were cross-linked to amine groups on the enzyme surface through the formation of a Schiff's base [2].

The immobilization efficiency for both covalent and non covalent procedure is presented in Table 1. In all cases, a protein to nanomaterial weight ratio 1:1 was used. The immobilization yield was calculated from the difference in the protein concentration in the aqueous phase before and after the immobilization procedure. As it can be seen, cyt c was successfully immobilized on f-MWCNTs regardless the immobilization procedure, with immobilization yields reaching up to 77%. The successful immobilization of cyt c was also confirmed by FTIR spectroscopy (Supplementary Material, Fig. S1). Efficient immobilization of cyt c on other multi-wall CNTs

and semi-conductive single-wall CNTs has been previously reported [18,19]. Immobilization yields were higher in the case of physical adsorption than in covalent binding, which could be attributed to the limited number of free functional groups on the surface of f-MWCNTs that are available for covalent attachment with protein molecules [20]. The π - π stacking interactions between the sidewalls of CNTs and the aromatic amino acids of cyt c in physical adsorption could also explain the high immobilization yields observed. In the case of physical adsorption, higher immobilization yields were observed when more hydrophobic nanomaterials, such as CNT-C₁₀-COOH and CNT-C₁₁-CH₃, were used as supports. The increase of the hydrophobicity of the nanomaterial (due to the increase of the alkyl chain length) resulted in a more efficient immobilization, suggesting that the main forces during physical adsorption are the hydrophobic interactions developed between the protein molecule and the nanomaterials. In the case of covalent immobilization, cyt c seemed to be more effectively immobilized on carboxyl-functionalized CNTs, indicating that the terminal functional group of nanomaterials also affects the immobilization procedure.

The peroxidase activity of immobilized cyt c was determined by the oxidation of guaiacol in the presence of H₂O₂. As seen from Table 1, the immobilization procedure followed, as well as the chemical characteristics of f-MWCNTs, affected the activity of immobilized cyt c. The peroxidase activity of cyt c was higher in the case of non covalent immobilization compared to that in covalent procedure, indicating that covalent binding may cause conformational changes during the protein grafting, leading to lower catalytic activity [21]. Both in non covalent and covalent immobilization, the activity of cyt c increased with the addition of an alkyl chain on the nanomaterial's surface. The presence of longer alkyl chains could increase the distance between the nanomaterial's

Table 1. Peroxidase activity and immobilization yield (%) of cyt c on f-MWCNTs, calculated as the ratio of protein immobilized on nanomaterials to the initial protein quantity used (standard deviation was less than 2% in all cases).

Nanomaterial	Immobilization yield (%)		Activity (μ M/min mg)	
	Non covalent immobilization	Covalent immobilization	Non covalent immobilization	Covalent immobilization
CNT-COOH	30	32	6.80	5.90
CNT-C ₁₀ -COOH	71	60	13.5	9.80
CNT-C ₆ -NH ₂	59	25	15.4	14.3
CNT-C ₁₁ -CH ₃	77	-	15.0	-

surface and the protein, especially in covalent immobilization, avoiding any substrate diffusion limitations, and thus enhancing the catalytic activity of the protein. Similar results have been reported by our group, when cyt c was immobilized on graphene oxide derivatives functionalized with different alkyl chains and terminal groups [11]. It is interesting to note that, although CNT-C₆-NH₂ exhibited the lowest efficiency as a support for covalent immobilization, the immobilized cyt c on this nanomaterial demonstrated the highest catalytic activity among the other f-MWCNTs studied, indicating that the specific binding of cyt c on CNT-C₆-NH₂ may result in conformational changes in the protein molecule that lead to an enhanced peroxidase activity.

The effect of temperature on the peroxidase activity of free and immobilized cyt c was further investigated at various temperatures ranging from 30 to 75°C (Fig. 1). As seen from Fig. 1, even though no shift of the optimum temperature was observed (70°C for both protein forms), the immobilized cyt c exhibited higher relative activity in the temperature range investigated. This is in accordance to the results previously reported for iso-cytochrome c immobilized on silica nanostructured supports [22]. The optimum temperature was not modified by the enzyme adsorption into the nanomaterials, but the relative activity of the immobilized protein was higher compared to that of the native form. Similar results have been also reported

for immobilized lipase on amino-functionalized multi-wall CNTs [23].

Stability of cyt c

The thermal stability of free and immobilized cyt c was investigated after incubation in buffer solution at 40°C, in the presence of guaiacol. The remaining peroxidase activity was estimated by monitoring the guaiacol oxidation after H₂O₂ addition. As seen in Fig. 2a and 2b, in most cases studied, the stability of the immobilized cyt c was significantly higher than that of the free protein, which is in accordance to the result reported by our group concerning the stabilizing effect of various f-MWCNTs on free cyt c [13]. A similar stabilization effect was observed when functionalized CNTs were used as supports for the immobilization of lipase and chloroperoxidase [14,22]. The covalently immobilized cyt c (Fig. 2b) was in most cases more stable than the non-covalently immobilized protein (Fig. 2a), which is in agreement to the common aspect that the covalent immobilization procedure leads to more stable enzyme-nanomaterial conjugations than the physical absorption [25].

To further investigate the stabilizing effect of f-MWCNTs as immobilization supports, the stability of immobilized cyt c against H₂O₂, an oxidizing agent that deactivates peroxidases, was investigated. The immobilized protein was incubated at 40°C for 30 min and the remaining peroxidase activity was monitored using guaiacol

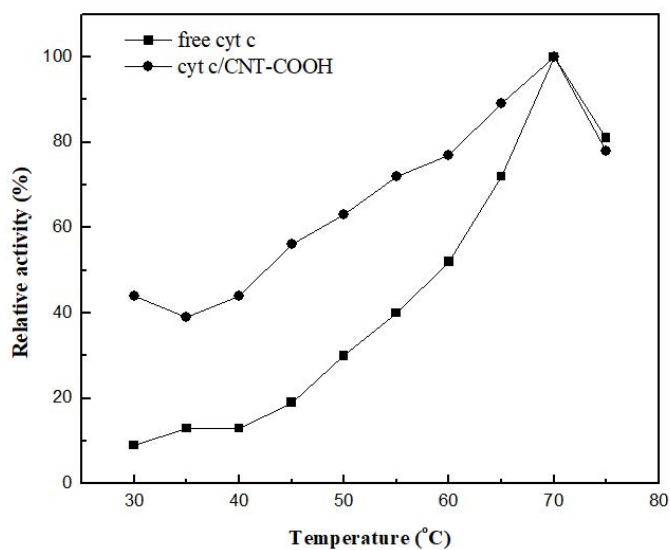


Fig. 1. Effect of temperature on the catalytic activity of free and non covalently immobilized cyt c. As 100% indicated the highest activity exhibited each time, either by free or immobilized cyt c.

as a substrate. As seen in Fig. 3, the remaining activity of immobilized cyt c reached up to 100% after 30 min incubation with H_2O_2 , while the free protein retained only 19% of its initial activity, indicating that the immobilization procedure increases the stability of the protein. Recent reports underline the use of functionalized carbon-based nanomaterials as immobilization supports, offering a more suitable environment for cyt c to maintain its functionality [11]. Comparing the two immobilization methods, the covalently immobilized cyt c appeared more stable than the non-covalently, an effect that could render from the

formation of a stronger bond between the protein and the nanomaterial, leading to a reduction in the protein structure mobility [26]. This trend is in agreement with the findings reported for immobilized lipase on amino-functionalized CNTs [23]. It is interesting to note that in the case of non covalent immobilization, the stability of cyt c increased as the hydrophobicity of the nanomaterial increased. As seen in Fig. 3, when CNT- C_{10} -COOH and CNT- C_{11} - CH_3 were used as immobilization supports, cyt c retained up to 93% of its initial activity, while in the case of CNT- C_6 - NH_2 and CNT-COOH, it retained up to 46%. The

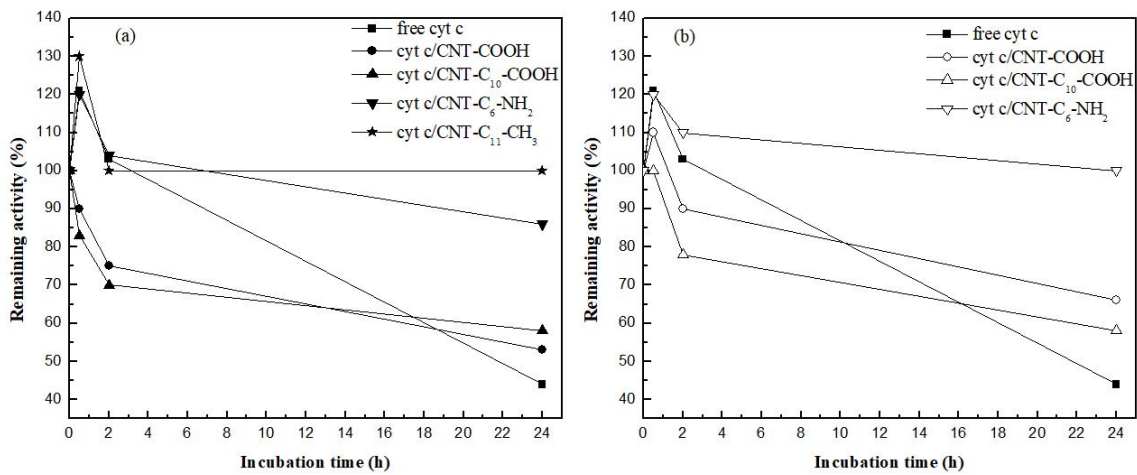


Fig. 2. Stability of free and (a) non covalently and (b) covalently immobilized cyt c on f-MWCNTs, after incubation at 40°C with guaiacol. As 100% is indicated the peroxidase activity of cyt c at t = 0 min.

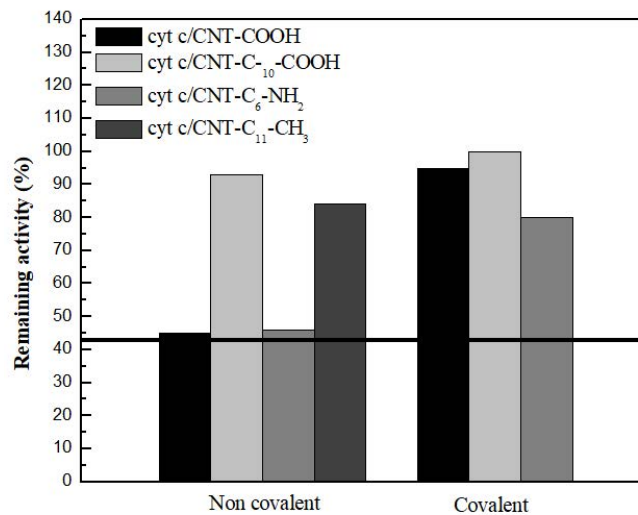


Fig. 3. Stability of free and immobilized cyt c on f-MWCNTs in buffer, after incubation for 30 min with H_2O_2 . As 100% is indicated the peroxidase activity of cyt c at t = 0 min. The line refers to the residual activity of free cyt c.

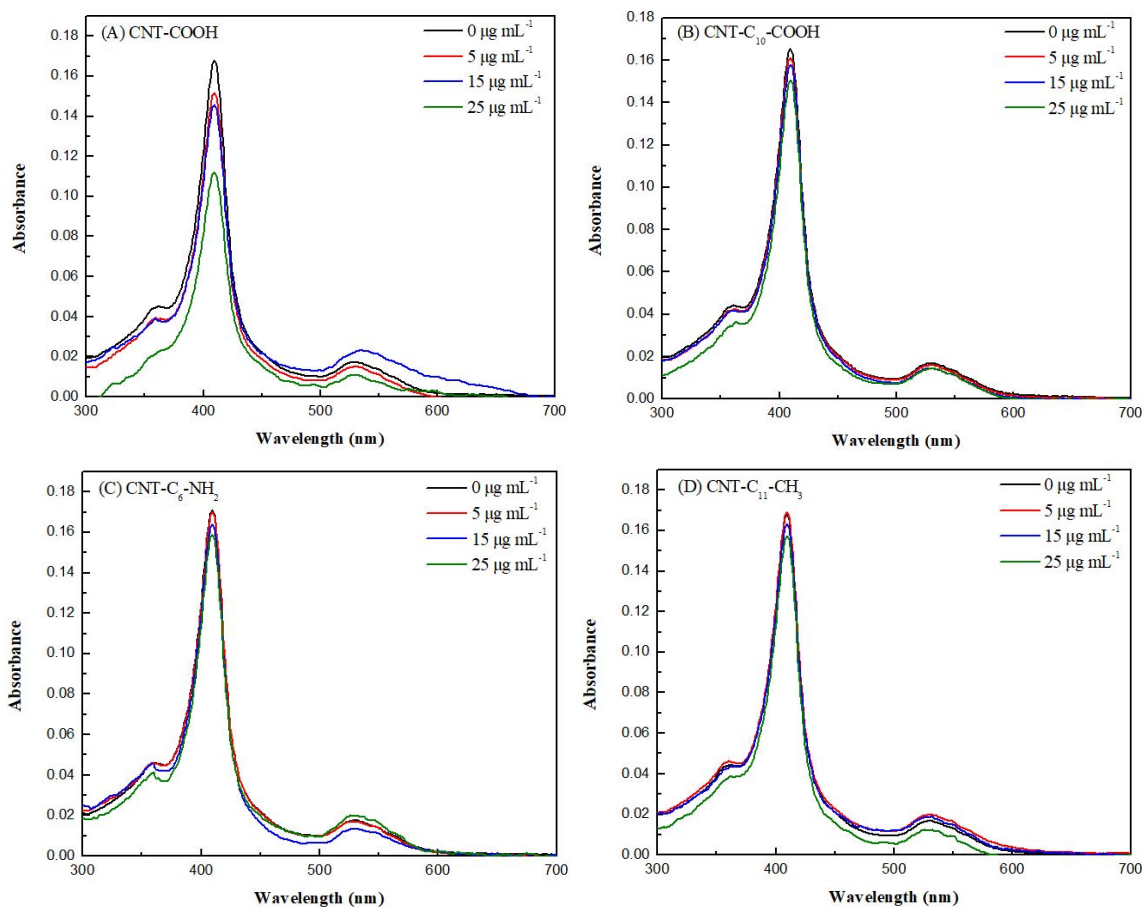


Fig. 4. Absorption spectra of cyt c in the presence of different concentrations of f-MWCNTs.

increase of the alkyl chain of the functionalized CNTs seems to create a more hydrophobic environment around the protein, leading to the limited diffusion of the hydrophilic H_2O_2 , and thus resulting to a lower denaturation effect [11].

Conformational studies of cyt c

The effect of f-MWCNTs on the conformational state of cyt c was investigated by monitoring the absorbance in the Soret region (300-700 nm). Optical absorption spectroscopy is used to monitor changes in the ligation and spin state of the heme iron and hence in the tertiary structure around the heme iron [27]. As seen in Fig. 4, cyt c showed two intense absorption peaks in the visible region: the Soret band at 409 nm and a weaker, broad band at 527 nm, which arise from electronic transitions of the porphyrin chromophore and are characteristic of the low-spin six-coordinated ferric heme [28].

The presence of f-MWCNTs did not remarkably affect the spectrum of cyt c, as indicated in Fig.

4 (B-D). The increased concentrations of the nanomaterials resulted in slight decrease in the absorbance of the Soret band, in most cases, while no shift of the maximum absorbance was observed, indicating that CNTs help cyt c to preserve its tertiary structure, as previously reported after its immobilization on single-wall CNTs and fullerene- TiO_2 gels [29,30]. In the case of CNT-COOH, the changes in cyt c spectrum were more pronounced (Fig. 4A). The presence of this nanomaterial resulted to a sharp reduction in the absorption peak of the Soret band of the protein, which was dependent on the concentration of the nanomaterial. When CNT-COOH was added to a concentration of $25 \mu\text{g mL}^{-1}$, a significant decrease of the absorbance in the Soret peak was observed, which could be correlated to the changes in the microenvironment of the heme of cyt c, that lead to the leach of the Fe(III) ions, as already observed with mesoporous silica nanoparticles [31]. It seems that the non-functionalized CNT-COOH develops

strong interactions with the protein, altering its conformational state, while the presence of longer alkyl chains on the CNTs protects cyt c from structural disruption around the heme moiety.

CONCLUSIONS

Functionalized multi-wall carbon nanotubes were effectively used as supports for the immobilization of cyt c. The immobilization yield and the peroxidase activity of immobilized cyt c were found to depend on the immobilization procedure, as well as on the structural characteristics of the nanomaterials. The increase of the alkyl chain length of the nanomaterials resulted in higher peroxidase activity of the immobilized protein. Furthermore, immobilized cyt c exhibited higher thermal stability than native protein, while it preserved up to 100% of its initial activity after incubation in the presence of H₂O₂, indicating that these nanomaterials offer a protective environment for the protein against denaturing conditions. UV-Vis spectroscopic studies showed that the use of f-MWCNTs preserves the conformational state of the heme prosthetic group of cyt c, which is responsible for the peroxidase activity of the protein. The results indicate that these functionalized CNTs can be promising candidates for their use as immobilization platforms for many biological molecules and their further application in a variety of fields, from biosensing to nanomedicine.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.nanomedicine-rj.com>

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