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The Effect of Fibronectin Coating on Protein Corona Structure and Cellular Uptake of Single-Walled Carbon Nanotubes

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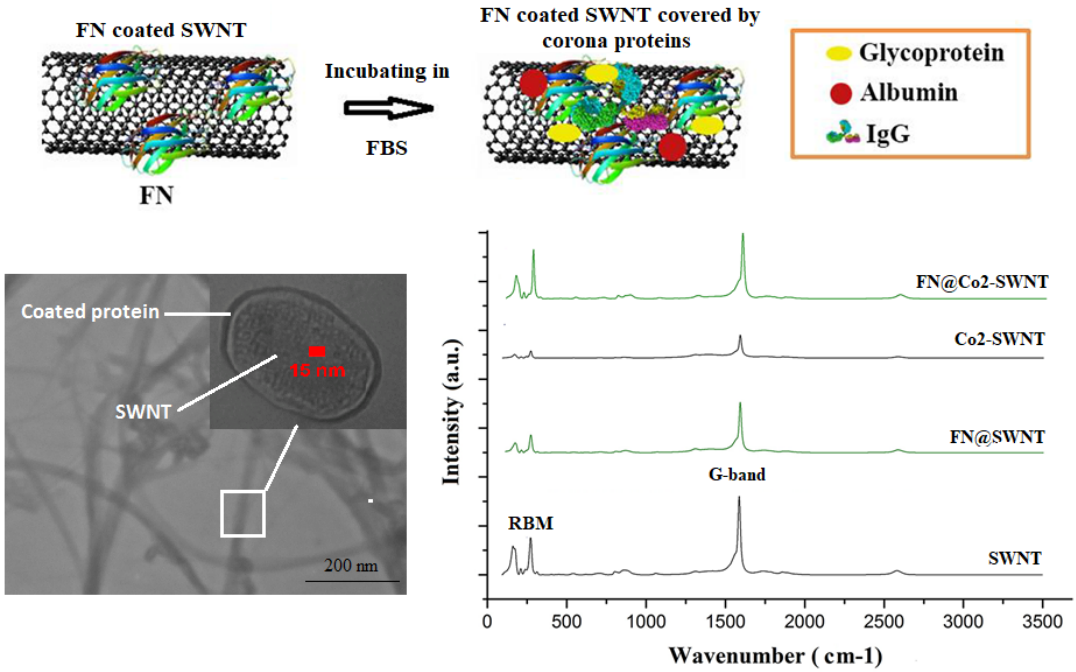
Graphical Abstract



Abstract

Protein coating, as an outstanding surface modification strategy, influence the organization of biomolecules in the interface of nanomaterials. In the present study, fibronectin (FN) was used to modify the surface chemistry of single-walled carbon nanotubes (SWNTs) and carboxylated SWNTs (CO_2-SWNTs) to analyze its effects on the protein corona composition and cellular uptake. At first, the successful coating of FN on the surface of both SWNTs was confirmed by transmission electron microscopy (TEM) and Raman spectroscopy. The results showed that the biomolecular organization of SWNTs and CO_2-SWNTs coronas was changed after FN coating based on the evidence obtained from the surface plasmon intensity of the samples. Moreover, the MTT assay and confocal microscopy imaging revealed less cytotoxicity and cellular uptake of SWNTs coronas in comparison to bulk samples, respectively. It is suggested that protein coating of SWNTs can modify the corona pattern and consequently the biological behavior of carbon nanotubes.

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Keywords: Protein coating, Fibronectin, Single-walled carbon nanotube, SWCNT, Corona.

Purpose and rationale

The purpose of this study is to pre-coat two different types of single walled carbon nanotubes (SWNT) including pristine SWNT and Co_2-functionalized SWNT with fibronectin (FN) to analyze the effect of protein coating on corona composition and biological behavior of SWNTs. It is hypothesized that FN coating not only reduces the toxicity of SWNTs but also decrease their internalization into the cells because it affects the affinity of corona proteins to nanotubes and change their normal pattern of toxicity and cellular uptake

Introduction

Single-walled carbon nanotubes (SWNTs) are cylindrical carbon-based nanomaterials that have been established their reputation as promising nanostructures in the field of nanomedicine [1]. The unique features of SWNTs such as suitable biocompatibility, extraordinary physical and chemical properties, and exceptional biological activities have been broadened their application in targeted drug delivery, tissue engineering, bio-imaging, etc. [2-7]. Recently, great attention has been paid to the effects of surface chemistry of nanomaterials on the organization of biomolecules in their bio-interface [4, 8-12]. SWNTs can interact with different biomolecules such as proteins, nucleic acids, etc. that affect their biological behavior in terms of cytotoxicity, bioavailability, and cellular uptake. It is suggested that the surface properties of SWNTs can dictate their interaction with different biomolecules in vivo. Upon SWNTs entrance to the biological fluids, protein corona will cover their surface. Therefore, it seems that the surface chemistry of SWNTs can change the composition of protein corona that can consequently define the cellular responses against SWNTs [5, 6, 13-19]. Protein corona composition mainly depends on the features of nanoparticles including size, charge, and colloidal stability. Moreover, types of proteins existed in blood, plasma or serum, and environmental conditions such as temperature and incubation time define the protein corona composition [18, 20-25]. In spite of many advances in the field of protein corona, the relationship between the structure of protein corona and surface chemistry of nanoparticles is less understood. Therefore, in the present study, we attempted to pre-coat the surface of SWNTs and carboxylated SWNTs (CO_2-SWNTs) with fibronectin (FN) to assess the changes in the composition of SWNTs protein coronas and its effects on their biological activities. Some studies have evaluated the effect of surface properties of nanoparticles on protein corona composition. For example, Selvador-Morales et al. reported that fibrinogen, apolipoprotein, and C1q are the most important blood proteins that can be absorbed by bulk SWNTs and double-walled CNTs (DWNTs) [26]. Zhang et al. found that functionalized CNTs specifically bind to α-chymotrypsin's catalytic site and regulate its enzymatic function [27]. It was also reported that classical (C1q) and alternative (C3b) complement pathways could be activated by albumin coated SWNTs [28]. In another study, it was shown that coating SWNTs with amino-PEG and methoxy-PEG improved the ability of these nanomaterials in increasing the levels of C4d and SC5b-9 (two complement products) which consequently activated the complement system [29]. Accordingly, changing the surface properties of CNTs not only improves their interaction with biomolecules but also could affect their cytotoxicity [30]. We showed in our previous study that electrostatic bonds between carboxylated SWNTs and FN improve the biological properties of SWNTs in terms of hydrophilicity and biocompatibility [31]. Here, w coated SWNTs and CO_2-SWNTs with FN to evaluate its effects on the cytotoxicity and corona composition of both SWNTs. We proposed that FN, as an important extracellular matrix (ECM) protein, may influence the protein corona composition of SWNTs and their toxicity and cellular internalization. To the best of our knowledge, some studies have described the CNTs protein corona; however, the effect of protein coating on the composition of SWNTs protein corona has not yet been reported. In the present study, a comparison was made between the corona composition of FN coated SWNTs, whether functionalized or non-functionalized. Moreover, the impact of

protein coating on cytotoxicity and cellular uptake of SWNTs were also assessed.

Materials and Methods

2.1 Sample preparation

First, 0.01g of SWNT and CO_2-SWNT (Neutrino Company, Iran) were added to 1% w/w sodium tetrachloroaurate (III) trihydrate (Sigma-Aldrich) and sonicated for 1 hour (amplitude: 60 Hz; 5 min on, 5 min off) to prepare homogenous solutions. Afterward, 0.3 mg/mL of FN (from human plasma, MW: 450 kDa, Sigma-Aldrich) was incubated with each SWNT solution for 24 hours at 4°C. We showed in our previous study that 0.3 mg/mL was the best concentration of FN that can increase the biological responses of SWNT [2]. Therefore, four different groups were prepared as follow: bulk SWNT (SWNT), FN coated SWNT (FN@SWNT), CO_2-SWNT (CO_2-SWNT), and FN coated CO_2-SWNT (FN@CO_2-SWNT),

2.2 Hard corona formation

For hard corona formation, all the samples were incubated with 100% fetal bovine serum (FBS; Gibco) in 37ºC for 1 hour. The incubated samples were then centrifuged in 14000 rpm for 20 minutes, washed and re-suspended in fresh phosphate buffer saline (PBS; Sigma-Aldrich). The centrifugation step was repeated three times and the remaining suspensions were used for further analysis.

2.3 Validating the FN coating on the surface of SWNTs

TEM was performed to analyze the successful coating of FN on the surface of SWNTs. For this, the suspended samples were dried on TEM grid and evaluated under TEM (Zeiss, EM900). Moreover, Raman spectroscopy (Almega Thermo Nicolet Dispersive Raman Spectrometer; Germany) was done in the spectral range of 100 cm^{-1} to 4200 cm^{-1} with a resolution of 4 cm^{-1} to monitor the structural changes of functionalized and non-functionalized SWNTs before and after FN coting.

2.4 Characterization

2.4.1 Evaluating the surface plasmon resonance of protein coated SWNTs

Localized surface plasmon resonance (LSPR; Nanomabna Iranian; NMBCLSPR; 920225) was used as nanobiosensor for detecting the interactions between labeled serum proteins with gold nanoparticles (AuNPs; Size: 10 nm; Nanomabna Iranian Company) and the samples. The critical aspect of using this method was to identify whether AuNPs-labeled serum proteins could directly interact with SWNTs or were indirectly absorbed by FN on the surface of coated SWNTs. Moreover, the effect of surface modification on corona composition of SWNTs was also evaluated. The surface chemistry of AuNPs, as a sensing agent, was reported to be dominated by negatively charged functionalities like OH and O which account for electrostatic interaction with serum proteins. Consequently, AuNPs were incubated with FBS and sputtered on sterile coverslips and FBS/AuNPs were considered as sensing agents. All the samples were then loaded on FBS/AuNPs sensor, separately. Finally, LSPR was conducted in transmission mode to analyze the interaction between AuNPs-labeled serum proteins and the samples.

2.4.2 Evaluation of hard corona composition

Absorption of serum proteins on the surface of nanomaterials plays an important role in their interaction with living matter. In the present study, SDS-PAGE was performed for characterizing the protein composition of formed hard corona around the coated and un-coated SWNTs. For this, the samples were incubated with FBS for 1 hour and the protein bonded complexes were separated from excess plasma by centrifugation and extensive washing was performed to remove the unbound proteins. The bound fractions were then resolved by SDS-PAGE. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was further performed to quantify the amount of most relevant proteins in the corona structure of SWNTs (bulk and carboxylated). The normalized percentage of spectral counts SpC) was measured using the following equation (1):

$$(SpC)k = \left(\frac{SpC}{(Mw)k}\right) \Big/ \sum_{i=1}^{n}\left(\frac{SpC}{(Mw)i}\right) \times 100$$

Equation (1)

2.4.3 Cytotoxicity evaluation

MTT assay was performed for quantification the cellular viability before and after incubating the samples with FBS. After sterilizing the sample with ultraviolet (UV) light for 45 minutes, they were placed in a 12-well culture plate and 2×10^4 U373-MG cells (National cell bank of Iran) were seeded on each sample and kept in an incubator at 37ºC and 5% CO_2 for 24 hours. Afterward, the culture media (DMEM/10%FBS) were removed and the cells were stained with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL) solution for 4 hours. The formed formazan was solubilized with isopropanol for 15 minutes and the absorbance was read at 570 nm. The tissue culture polystyrene was used as a negative control group.

2.4.4 Cellular uptake

Confocal fluorescence scanning microscopy (CFSM) was used to assess the cellular uptake of coated and un-coated SWNTs. Briefly, U373-MG cells were cultured in DMEM/10%FBS at 5% CO_2, 95% humidity, and 37 °C. The cells were then allowed to adhere to a glass coverslip a in 6-well plate for 24 hours. The medium was removed and the cells were incubated with 100 µg/mL of coumarin for 4 hours. Afterward, the samples were washed 3 times with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes to stain the nuclei and rewashed 3 times with PBS. The cells were then fixed with 4% formaldehyde for 10 minutes at 4 °C and analyzed by CFSM.

2.4.5 Quantification the cellular uptake

For quantifying the cellular uptake of SWNTs, U373-MG cells were cultured under optimal conditions. For this, SWNTs were labeled with coumarin and incubated with cells for 4 hours. The samples were then subjected to flow cytometry (BD LSR II analyzer with 575/26 bandpass filter).

2.5 Statistical analysis

Statistical comparisons were performed using one-way ANOVA with SPSS 16.0 (SPSS, USA) for evaluating the statistical variations. *P* values of less than 0.05 were considered statistically significant.

Results

3.1 Validating the FN coating on the surface of SWNTs

TEM micrographs exhibited the bundles of SWNTs with uniform structure. As it is observed, a dark core of SWNTs is coated with a light shell of FN protein indicating the successful coating of FN on the surface of SWNTs (Figure 1a). Raman spectroscopy also defined the structural changes of functionalized and non-functionalized SWNTs before and after FN coating. The spectra show that the intensity of G-band and RBM band, as characteristic peaks of SWNTs, was somehow decreased in non-functionalized SWNTs after FN coating. In contrast, a completely different trend was observed in the spectra of functionalized SWNTs after protein coating indicating an increase in the intensity of RBM and G-band (Figure 1b)

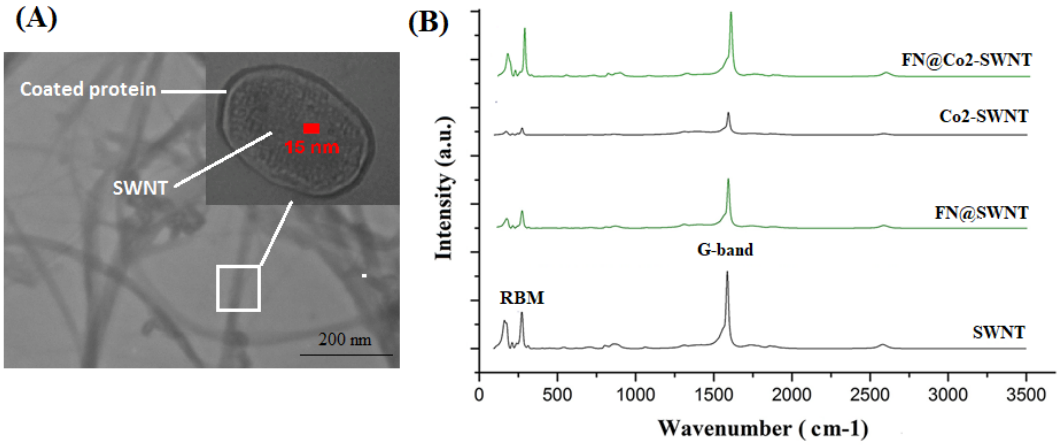

Figure 1. A) TEM micrograph indicating the core-shell structure of FN coated SWNTs, B) Raman spectra of functionalized and non-functionalized SWNTs before and after FN coating.

3.1 Surface plasmon resonance of protein coated SWNTs

LSPR revealed some valuable information about the interaction between labeled serum proteins with SWNTs. A general comparison between the plasmon intensity of SWNTs and CO_2-SWNTs revealed that non-functionalized SWNTs, whether pre-coated with FN or not, had less plasmon intensity as a result of more interaction with AuNPs-labeled serum proteins (Figure 2).

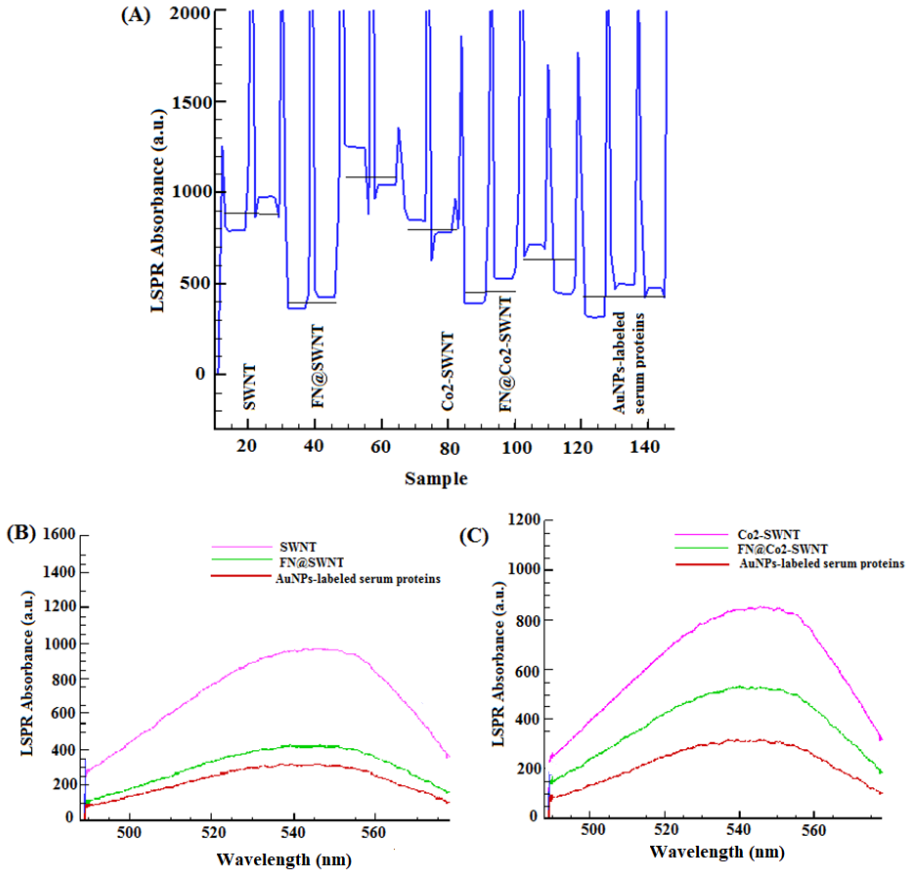

Figure 2. *The LSPR spectra of the sensor chip of Au NPs-labeled FBS in the presence of SWNTs.*

FN@SWNT and FN@CO2-SWNT showed less plasmon intensity indicating the higher interaction of FN with AuNPs-labeled serum proteins. These observations suggest that FN as a high molecular weight molecule had less plasmon intensity with more free AuNPs the presence of which may limit the interaction of labeled serum proteins with SWNTs. This means that the interaction of serum proteins in mostly with coated FN rather than SWNT in FN@SWNT.

3.3 Hard corona composition

SDS-PAGE was used for evaluating the binding affinity of serum proteins with functionalized and non-functionalized SWNTs. As it is clearly shown in figure 3A, albumin (~70 KDa) was the most abundant protein that had an affinity to all the samples with a little variation. LC/MS/MS analysis also revealed that IgG has less affinity for functionalized and non-functionalized SWNTs. Other serum proteins such as plasma protease inhibitor c, transthyretin, and apolipoprotein alpha-1 had a similar affinity all the samples and no significant differences were observed between different groups. Keratin, alpha-1-antitrypsin, type-1- cytoskeletal, fibrinogen-beta-chain, and elongation factor 1-alpha had also more affinity to functionalized samples. Additionally, LC/MS/MS was performed to quantify the hard corona composition of the samples. Table 1 shows the normalized percentage (SpC%) of proteins identified by LCMS/MS in the hard coronas of functionalized SWNTs before and after FN coating. The SpC% of the most abundant proteins in the hard coronas of CO_2-SWNT based samples are also presented in figure 3B.

It should be noticed that LC/MS/MS was only performed for samples containing functionalized SWNTs because the most differences in the affinity of serum protein with samples were observed in these samples based on the results obtained from SDS-PAGE

Table 1. *List of normalized percentage (SpC%) of each protein identified by LCMS/MS in the hard coronas on the Co_2-SWNT before and after coating with FN.*

MW [kDa]	Protein identity	CO_2-SWNT NSpC	FN@ CO_2-SWNT NSpC
69.321	Serum albumin	0.15	0.04
262.46	Fibronectin	0.04	0.01
65.999	Keratin, type II cytoskeletal	0.09	0.02
36.083	Ig gamma-1 chain C region	0.05	0.01
62.027	Keratin, type I cytoskeletal	0.03	0.008
46.707	Alpha-1-antitrypsin	0.08	0.08
187.03	Complement C3	0.01	0.07
41.26	Ig gamma-3 chain C region	0.02	0.01
123.722	Vinculin	0.008	0.08
55.892	Fibrinogen-beta-chain	0.01	0.02

3.4 Cytotoxicity

MTT assay revealed the cytotoxicity of SWNTs and CO_2-SWNTs. The proliferation rate of U373-MG cells on bulk SWNTs was significantly lower (P<0.05) than control and other samples.

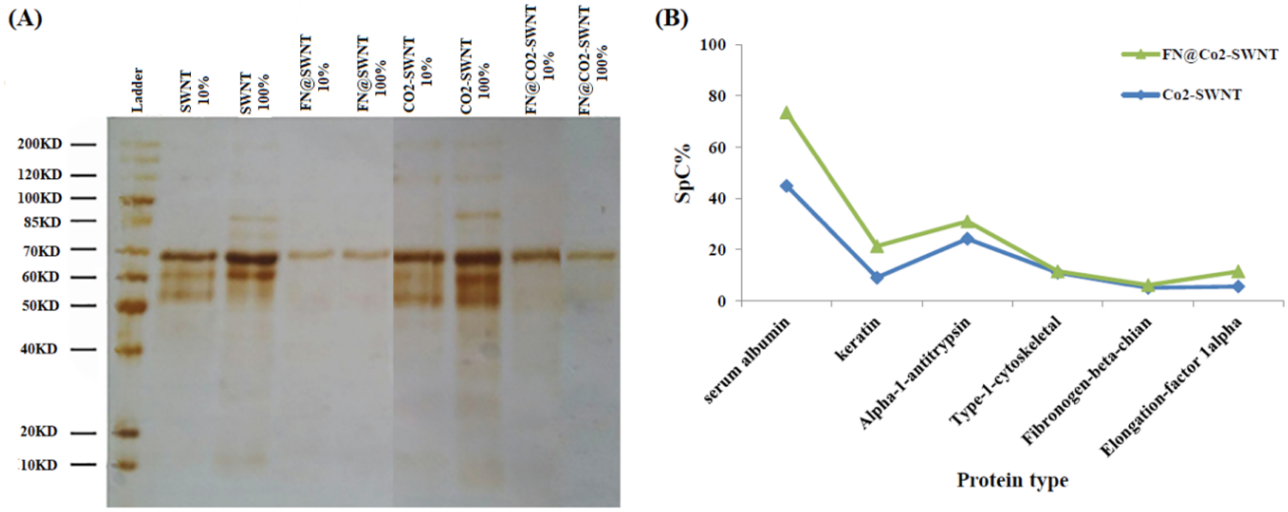

Figure 3. *A) SDS-PAGE of bulk and functionalized SWNTs incubated in 10% and 100% FBS before and after coating with FN, B) Mass analysis of CO_2-SWNTs before and after FN coating indicating the effect of surface modification on the absorption of proteins existed in serum.*

A comparison between FN coated and un-coated SWNTs showed that FN@SWNT had less cytotoxicity compared with bulk SWNT while no significant differences were observed between FN coated and un-coated CO_2-SWNTs. However, the proliferation rate of U373-MG cells on FN@CO_2-SWNT was higher than in other groups (Figure 4A). The proliferation of U373-MG cells on SWNTs coronas and CO_2-SWNTs coronas was almost equal to the control group. Moreover, it seems that FN coating improved the proliferation rate of cells grown on CO_2-SWNT (Figure 4B).

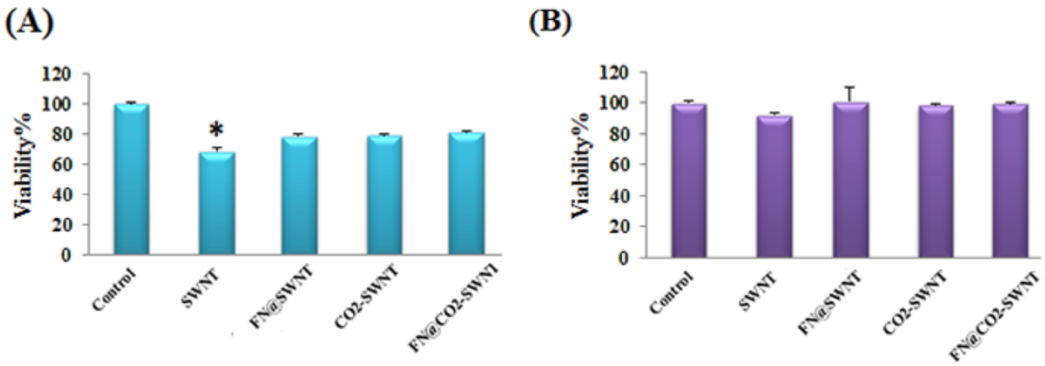

Figure 4. *Cell viability of samples: A) before corona formation, B) after corona formation. Although FN coating somehow increased cellular proliferation but it had no significant effect on the growth of cells compared with control groups.*

3.5 Confocal microscopy

To evaluate the uptake of samples into the cells, confocal microscopy was used. In this regard, CO_2-SWNTs showed more endocytosis into the cells in comparison to non-functionalized SWNTs (Figure 5).

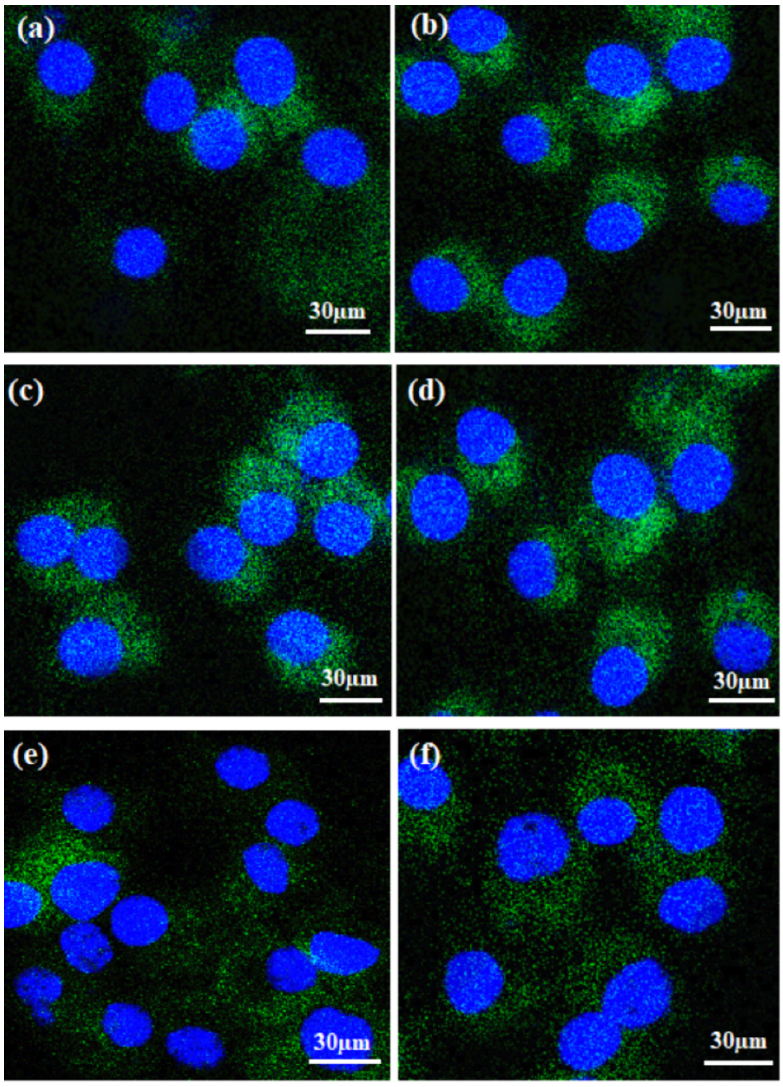

Figure 5. *Cellular uptake of samples: A) SWNT, B) CO_2-SWNT C) FN@SWNT, D) FN@ CO_2-SWNT, E) FN@SWNT corona, F) FN@ CO_2-SWNT corona.*

FN coated SWNTs also exhibited more cellular internalization compared with control groups (un-coated samples) (Figure 5). However, the FN coated SWNTs and CO_2-SWNTs showed less uptake into the cells after corona formation.

3.6 Flow cytometry

It is known that coating nanomaterials with different molecules such as proteins could decrease the uptake of nanomaterial coronas. The flow cytometric analysis showed that the cellular internalization of SWNTs and Co2-SWNTs was not significantly affected by corona because the uptake of these nanomaterials was almost equal before and after corona formation. However, the FN coating decreased the cellular uptake of both SWNTs indicating the effect of protein coating on corona composition and biological behavior of carbon nanotubes (Figure 6).

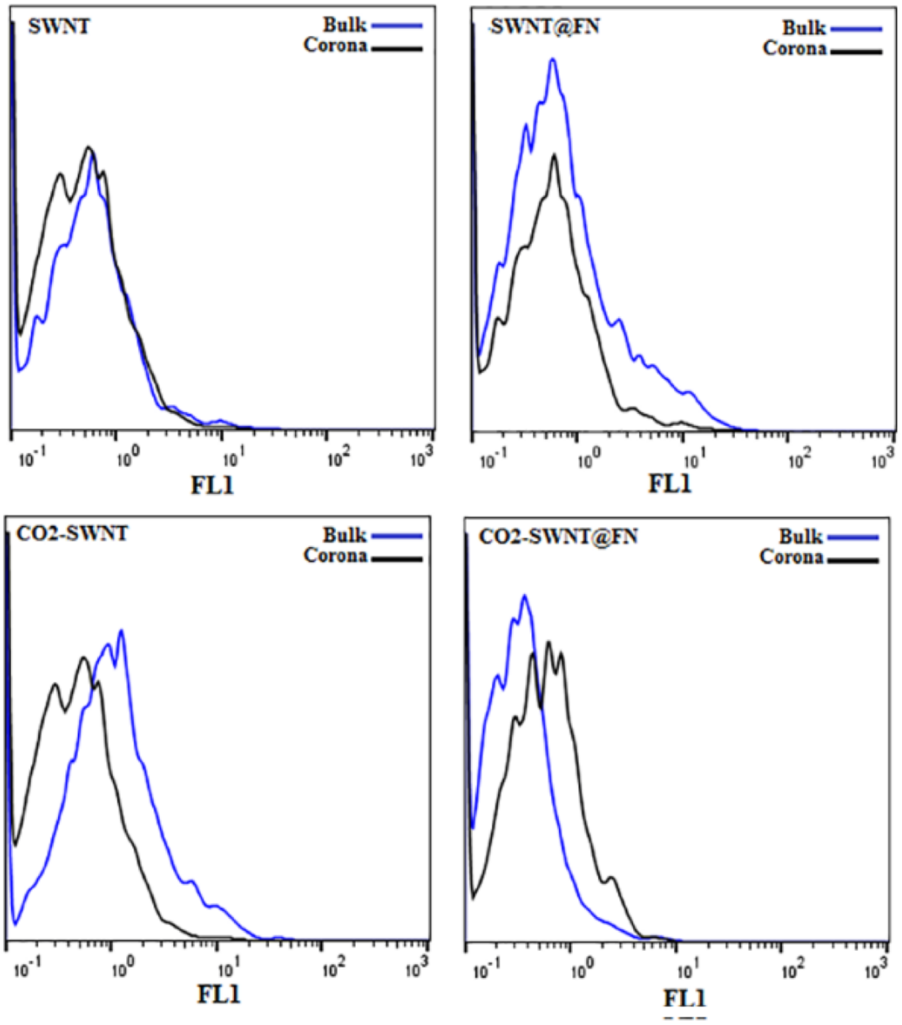

Figure 6. *Flow cytometric quantification of cellular uptake.*

Discussion

Unique characteristics of SWNTs make them ideal candidates for many biomedical applications. However, some features such as composition, surface properties, hydrophobicity, and cytotoxicity restricted their clinical applications. It is confirmed that modifying the surface properties of SWNTs with different biomolecules such as proteins could affect their biomolecular interface organization and protein corona composition [32-37]. For this, in the present study, the strategy was designed based on coating the surface of two different types of SWNTs, bulk and carboxylated, with FN to evaluate its effect on biological properties and protein corona composition of SWNTs. Generally, the interaction between nanomaterial and biomolecules is a critical step that triggers

many biological processes. Many factors affect the interaction of SWNTs with other biomolecules, including homogenic/non-homogenic dispersion, competitive adsorption/desorption processes, experimental conditions, and surface modification and structural composition [3, 38]. Accordingly, the ability of different SWNT types to interact with serum proteins was characterized by different methodologies. It seems that the interaction of FN was mostly with CO_2-SWNTs in comparison to bulk SWNTs. It has been reported in other studies that functionalization of SWNTs with amine or carboxyl groups reduced its cytotoxicity while it could enhance their interaction with other biomolecules [39, 40]. Therefore, functionalized SWNTs are more potent for binding to serum proteins before and after surface modification.

LSPR is a powerful method that can precisely detect the interaction between nanomaterials and biomolecules [41, 42]. Here, for detecting the interaction between bulk and functionalized SWNTs with serum proteins, a novel biosensing system was optimized with LSPR. AuNPs, as a sensing agent were conjugated to proteins, existed in FBS; their binding with SWNTs and CO_2-SWNTs were assessed by detecting the changes in mass concentration at molecular interfaces. Based on the obtained results, the surface plasmon resonance of SWNTs was higher than CO_2-SWNTs that suggested the higher affinity of serum proteins for CO_2-SWNTs, as predicted. Functionalized SWNTs had more potential for binding to different biomolecules. In both groups, whether functionalized or non-functionalized, the lower surface plasmon resonance was related to FN coated SWNTs, because FN was able to densely cover the surfaces of both SWNTs. Therefore, it affected the direct interaction of labeled serum proteins involved in the formation of the corona with SWNTs, as a sensor. A similar study was conducted by Lee et al. They used SPR biosensing for detecting the interaction between CNT and human erythropoietin (EPO) and human granulocyte macrophage colony-stimulating factor (GM–CSF). They claimed that using SPR could provide a powerful sensing system for multiple proteins in a wide dynamic range (0.1–1000 ng/ml) and introduced SPR as a forceful analysis tool for developing biopharmaceutical production [43]. Cedervall et al. had also used SPR for determining the rates of association and dissociation of proteins with nanoparticles. They claimed that SPR is a suitable technique for detecting protein binding and affinity with nanoparticles [44].

In the present study, we have also evaluated the affinity of different proteins that existed in serum with both SWNT types by using SDS-PAGE. Generally, the serum contains large amount of proteins with different rates of hydrophobicity and hydrophilicity [30]. Therefore, it is logical to consider that serum proteins have a different affinity to SWNTs. The quantity of these proteins in serum defines their interaction efficacy with different nanomaterials. Here, albumin was the most abundant protein in the corona composition of all the samples. Albumin as a small molecule was able to bind to the surfaces of SWNTs and CO_2-SWNTs, whether pre-coated with FN or not [30, 45]. It was demonstrated by confocal microscopy and flow cytometry that both SWNTs coronas had low cellular uptake after FN coating. It was reported in a study that SWNT coronas could reduce their cytotoxicity; however, SWNT coronas did not affect increasing their cellular uptake [46]. Our obtained results also supported this observation. It is hypothesized that the functional motifs on the surface of FN for binding on cell receptors may be covered by proteins existed in coronas and this may hinder the optimal uptake of the samples. Moreover, if FN motifs were accessible after corona formation, it would promote the cellular internalization. Therefore, the uptake of samples in the absence of protein corona was higher than those in the presence of corona. Taken together, corona can have an impact on the biological properties of SWNTs but might reduce the cellular uptake of nanomaterials. Formation of SWNTs and Co_2-SWNTs coronas are more likely not a property of the proteins alone existed in serum but the surface properties of both SWNT types defined the corona composition. The attempts performed in the present study have somehow explained the nature of SWNTs coronas before and after protein coating. However, more caution should be considered for detecting the nature of interactions and corona formation.

Conclusions

In the present study, a strategy was designed to better understand the complexity of bulk SWNTs and functionalized SWNTs protein corona composition after surface modification with FN. From the obtained results, it is clear that the biological properties of FN play an important role in changing the surface properties and consequently the biological activities of different SWNT derivatives. These parameters consequently affected the biomolecular interface interaction of SWNTs with serum proteins. However, it is difficult to assess the nature of the interactions among the proteins competing for attachment to the surface of the SWNTs. Certainly, there are many indications that the presented results are collected in a rational process, but it will be difficult to describe based on individual protein binding studies. Thus, further investigations are needed to explain particular protein binding with SWNTs and protein corona composition.

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Conflict of Interests

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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