Preparation, characterization and toxicity evaluation of Co$_3$O$_4$ and NiO-filled multi-walled carbon nanotubes loaded to chitosan

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**Highlights**

- Chitosan loaded to Co$_3$O$_4$ and NiO-filled MWNTs were synthesized and confirmed by FT-IR XRD.
- Cytotoxic effects of Co$_3$O$_4$ and NiO-filled MWNTs were studied in the presence of Chitosan.
- Functionalized MWNTs with Chitosan are able to reduce cytotoxic effects and improve biocompatibility.

**Abstract**

Multi-walled Carbon nanotubes (MWCNTs) have been extensively explored for a variety of biomedical and tissue engineering applications. Their high surface area provides tunable multiple attachment sites for acquiring appropriate biological and biomechanical properties. Despite, the cytotoxicity of MWCNTs is dubious with or without surface functionalization. In the present study, two different kinds of multi-walled carbon nanotubes (MWCNTs) in the presence of cobalt oxide (Co$_3$O$_4$) and nickel oxide (NiO) nanoparticles have been prepared through a simplistic and effective method, and thus, the Co$_3$O$_4$/MWNT and NiO/MWNT structures are loaded to chitosan in methanol solution at 65 °C. Results upon the synthesis and structural characterization were obtained by Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM), respectively. It has been shown that the tuned functionalized MWCNTs are able to reduce cytotoxic effects and improve biocompatibility and be more appropriate for potential future clinical applications.

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**1. Introduction**

Chitosan (CTS) is known as a natural polysaccharide, consisting of D-glucosamine and N-acetyl glucosamine unit which found in shrimps, crab, lobster, coral, jellyfish, butterfly, ladybug,
mushroom and fungi [1]. Over the past two decades, CTS has been developed remarkably in biomedical applications owing to its high biocompatibility, biodegradability, porous structure, suitability for cell ingrowth, osteoconduction and intrinsic antibacterial nature [2–4], CTS offers a wide range of applications, including cartilage tissue engineering, wound healing, and orthopedic applications [5–8]. In addition CTS can be easily modified on the surface of nanostructures [9–11]. Multi-walled Carbon nanotubes (MWCNTs) have been extensively explored for a variety of biomedical and tissue engineering applications [12–15]. Recently, carbon nanotubes (CNT) have been functionalized with chitosan as promising biomaterials for bone tissue engineering because of high mechanical strength and electrical conductivity of this nanomaterial [16]. Li et al. reported multi-walled carbon nanotube (MWCNT)-chitosan nanoparticle (CS-NP) hybrids are biocompatible at the concentrations up to 100 µg mL\(^{-1}\) for 24 h incubation [17]. Also, they found that MWCNT-CS NP hybrids can improve the bovine serum albumin (BSA) immobilization efficiency 0.8 times and simultaneously reduce the cellular toxicity by about 50% compared to carboxylated MWCNT. Arias and co-workers used glassy carbon electrodes modified with CNT dispersed in chitosan as analytical applications for sensing DNA-methylene blue interaction [18]. Application of a Cu-chitosan/MWCNT film-modified electrode to determine the presence of rutin in fruits with satisfactory results was investigated by Gholivand and co-workers [19]. Their results indicated that the Cu-chitosan/MWCNT has good selectivity, stability, and reproducibility in the presence of rutin. In this research we studied the multi-walled carbon nanotube loaded to Co\(_2\)O\(_4\) and NiO nanoparticles in the presence of chitosan. Co\(_2\)O\(_4\) and NiO nanoparticles are known as an important p-type metal oxide semiconductor (MOS) with its potential applications in drug delivery system and also as chelating biomolecules [20–22]. Ding et al. [23] fabricated Co\(_2\)O\(_4\) nanofibers by a two-step procedure consisting of electrospinning with subsequent calcinations and further applied it to construct a non-enzymatic sensor for glucose detection in alkaline solution. Lu et al. [24] synthesized layered Co\(_2\)O\(_4\) nanoflakes with spongy nanostructure as an immobilization matrix to entrap hemoglobin. In the present paper, we describe the preparation and toxicity evaluation of Co\(_2\)O\(_4\) and NiO-filled multi-walled carbon nanotubes loaded to chitosan. Since we introduce the hybrid MWCNT filled with Co\(_2\)O\(_4\) and NiO nanoparticles as protein delivery carrier.

2. Materials and methods

2.1. Materials

Chitosan powder (CTS), with a deacetylation degree of 85% and the molecular weight of chitosan was 2.6 × 10\(^4\) as determined by a viscometric method, was bought from the Sigma-Aldrich. Multi-walled carbon nanotubes (MWCNTs, purity > 98%, mean diameter from 10–15 nm and length of 50–100 nm) was provided from the Research Institute of the Petroleum Industry (Tehran, Iran). Methanol, ethanol, nitric acid, and sulfuric acid were purchased from Merck (Schuchardt, Germany). Deionized water was used for the preparation of all the solutions in this research.

2.2. Preparation of MWCNT-Co\(_2\)O\(_4\) and MWCNT-NiO nanocomposites

Co\(_2\)O\(_4\) and NiO nanoparticles used in the paper were prepared according to the procedure described in the literature [25,26]. Firstly, 0.03 g of Co\(_2\)O\(_4\) nanoparticle was dispersed in 25 mL of methanol by ultrasonication to form a red solution into a 250 mL balloon flask. Then, 0.03 g of acid treated MWCNT [27] was dispersed into the above mixture by ultrasonication for 30 min, while the temperature of the water in the ultrasonic bath was kept below 50 °C.

The reaction mixture was kept reacting for 2 h under reflux at 65 °C. After that, the products were washed with deionized water repeatedly to remove any impurities and dried. Then, a chitosan solution of 1% (w/v) was prepared by dissolving chitosan in a 1% (v/v) of 1% acetic acid aqueous solution followed by filtering to remove the impurity. Afterwards, to obtain Co\(_2\)O\(_4\)-CS/MWCNT and NiO-CS/MWCNT, 0.03 g of Co\(_2\)O\(_4\)/MWCNT and NiO/MWCNT products were added to chitosan solution and heated with reflux 65–70 °C for 6 h and then filtered and washed with deionized water and finally dried overnight at 40 °C. Fig. 1 show analysis of the oxidize MWCNT by scanning electron microscopy (a), transmission electron microscopy (b), and Raman spectroscopy (c).

2.3. Cell culture

Cell culture media RPMI1640 and Fetal Bovine Serum (FBS) were purchased from Gibco, NY, USA. Human breast MCF-7 cell line was obtained from the Pasteur Institute (Tehran; IRAN). Cells were cultured in RPMI 1640 medium respectively supplemented with 10% FBS and 1% antibiotic (penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5% CO\(_2\). After third passage, the cells were separated from the flasks using trypsin and used for further experiments.

2.4. Cell toxicity assay

Cell toxicity MTS assay was performed with a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) provided by a Promega Kit (CellTiter 96\(^\text{⃝}\) Aqueous One Solution Cell Proliferation Assay)]. MCF-7 cells were seeded at 1 × 10\(^4\) cells per well in 96-well plate in a final volume of 200 µl/well approximately 24 h before the assay. Cells were exposed to treatments; F-MWCNT, F-MWCNT-Chitosan, R-MWCNT, R-MWCNT-Chitosan with different concentrations (0–250 microgram/ml). At the end of the incubation time (24 h) 20 µl/well MTS reagent was added to each sample and the plate was incubated for 2.5 h at 37 °C in standard culture conditions. Finally, the absorbance was recorded at 490 nm using ELISA reader. Cell viability was estimated as a percentage of the value of the untreated control cells.

3. Results and discussion

3.1. FT-IR spectra for Co\(_2\)O\(_4\)-MWCNT/chitosan

Fourier transform infrared (FT-IR) spectroscopy is considered to determine the functional elements absorbed by CNTs. In the measured FT-IR absorbance spectrum in Fig. 2 shows two dominate peak at 1610 and 3441 cm\(^{-1}\) which are associated with O–H groups and observed another at 1350 cm\(^{-1}\) (associated with C≡C). Ososwald et al. indicated two significant peaks; 1600 and 3450 cm\(^{-1}\) (associated with O–H) and represent absorbance peak another at 1445 cm\(^{-1}\) [27]. Pristine CNT exhibited two significant signals at 1315 and 1590 cm\(^{-1}\), representing the disorder sp\(^3\) mode (D band) and the tangential mode (G band) of CNT, respectively [28]. FT-IR spectrum represents a C=O stretching vibration bands at 528 and 626 cm\(^{-1}\) that was proposed on the formation of spinel structure of Co\(_2\)O\(_4\) nanoparticle inside the MWCNT. Khalaji et al. synthesized Co\(_2\)O\(_4\) nanoparticle and reported two sharp peaks of this nanoparticle at 571 and 665 cm\(^{-1}\) due to u(C=O) [29]. In another report, FT-IR spectrum shows two new strong peaks at 661 and 565 cm\(^{-1}\) belongs to the Co\(_2\)O\(_4\) nanoparticle [30,31]. After the interaction of chitosan with Co\(_2\)O\(_4\)-MWCNT, a broad peak is observed in the region of 3443 cm\(^{-1}\) owing to –OH stretching vibration and a sharp peak at 2860 cm\(^{-1}\) was assigned to CH\(_3\) in amide group. The C=O stretching vibration in chitosan interacting
with Co$_3$O$_4$-MWCNT indicated two peaks at 997 and 1057 cm$^{-1}$ and peaks at 1629 and 1573 cm$^{-1}$ were owing to $\text{C=O}$ stretching (amide I) and NH stretching (amide II). FT-IR spectra showing a band at around 2910 cm$^{-1}$ reveal presence of C–H stretch.

### 3.2. FT-IR spectra for NiO-MWCNT/chitosan

In the FT-IR spectra of the NiO-filled MWCNT complex, a strong adsorption band exhibits the characteristic peak of the Ni–O stretch at 633 cm$^{-1}$, which is close to the results mentioned by Rahman and co-workers [32]. The peak at 3415 cm$^{-1}$ which is associated with O–H group and the two peaks at 2846 and 2917 cm$^{-1}$ are owing to the C–H stretching modes of the NiO-MWCNT. The absorption bands within the 2857–2906 cm$^{-1}$ region confirmed the presence of C–H stretching. The weak peaks at 1532 and 1598 cm$^{-1}$ in the FT-IR spectrum is from the stretch modes of the aromatic C–C bonds in the NiO-MWCNT (see Fig. 3). The adsorption peaks corresponding to Ni–O stretching and O–H stretching were observed in the region of 625 and 3402 cm$^{-1}$ that resulted from chitosan loaded on NiO-MWCNT complex [33–35]. In the chitosan loaded on the NiO-MWCNT, the broad peak at 1126–1054 cm$^{-1}$ is distributed to the combination vibration of C–O and C–C.

### 3.3. XRD analysis

The X-ray diffraction (XRD) patterns of all the synthesized materials have been shown in Fig. 4. Also the standard JCPDS patterns of NiO (no. 004-0835), Co$_3$O$_4$ (no. 009-0418) and C (no. 001-0640) have been shown in this figure. Both XRD patterns in Fig. 4a, consistent with the powder diffraction files of JCPDS, display the existence of the (111), (200), and (220) major lattice planes
and the (002), (100), (101) and (004) planes confirm the formation of cubic structure of NiO and hexagonal structure of carbon nanotubes, respectively [36]. Moreover comparison of the both XRD patterns in Fig. 4a exhibits an extra diffraction peak at 2θ = 20.3° which is consistent with the chitosan structure [37–39]. No additional peaks are observed in both patterns due to absence of any impurities. In Fig. 4b, for both XRD patterns, the presence of the (220), (311), (400), (511) and (440) lattice planes shows that the only cobalt-containing crystalline phase is Co₃O₄ (JCPDS: 009-0418) with cubic structure [40]. Additionally, four peaks indexed as (002), (100), (101) and (004) Bragg reflections are related to the presence of carbon nanotubes with hexagonal structure. The appearance of a peak at 2θ = 20.0° in the XRD pattern of Co₃O₄–MWCNT-chitosan sample confirms the presence of chitosan [36–38]. It is clearly seen that no characteristic peaks of the impurities are observed in both patterns of Fig. 1b, implying that the desired phases have been synthesized. These results can indicate that the nucleation and growth of both nanoparticles inside of the MWCNT increase in the presence of chitosan matrices, especially for Co₃O₄ nanoparticles [41].

### 3.4. TEM analysis

The surface morphologies of NiO (a) and Co₃O₄ (c) nanoparticles loaded to MWCNTs were investigated by TEM micrographs, as shown in Fig. 5. TEM analysis revealed the MWCNT attached with NiO (a) and Co₃O₄ (c) nanoparticles have an average diameter of 30 and 33 nm, respectively. From Fig. 5(a) we can observe the average length and width of NiO nanoparticle filled within MWCNT is calculated to be about 15 and 6 nm, respectively. Whereas we observe from Fig. 5(c), the size of Co₃O₄ nanoparticle filled within MWCNT has an average length and width of 13 and 11 nm, respectively. Fig. 5 also presents TEM micrographs of chitosan dispersed in NiO–MWCNT (b) and Co₃O₄–MWCNT (d) structures. The chitosan chains interacting with these structures indicate layer thickness of about 10 nm could be seen covering on Co₃O₄–MWCNT and NiO–MWCNT surfaces.

### 3.5. Cell toxicity

The MTS results were assessed as relatively non-toxic at 24 h of all the treatments including NiO–MWCNT (F–MWCNT), NiO–MWCNT–chitosan (F–MWCNT–chitosan), Co₃O₄–MWCNT (R–MWCNT), Co₃O₄–MWCNT–chitosan (R–MWCNT–chitosan) on MCF7 cells. None of the treatments can reach to the IC₅₀ concentration (Half maximal inhibitory concentration) (see Fig. 6). No significant toxicity was observed for complexes F–MWCNT, F–MWCNT–chitosan, R–MWCNT, R–MWCNT–chitosan until 250 microgram/ml concentration. Many investigations revealed more biocompatible and less toxic properties of functionalized MWCNT [42–46]. Li et al. results show toxicity of MWCNT at higher concentrations [47]. In an interesting study, Sahoo et al. evaluated functionalized carbon nanomaterials as nanocarriers for drug delivery and showed that after conjugation of biocompatible poly(vinyl alcohol) (PVA) to MWCNT, even at a high concentration of 500 mg/L, there is not significant toxicity (>80% cell viability) for MDA-MB-231 cells [46]. Therefore, observed anticancer properties of drug conjugated to MWCNT–PCA are related to the drug and not the nanocarrier. Our finding is in accordance with Sahoo and Li which proved that after conjugation of chitosan to MWCNT, cell viability increases significantly [46,47]. There is cell viability above 80% over all concentration tested, indicating that all of the complexes are bio-compatible with MCF7 cells.

### 4. Conclusion

Co₃O₄ and NiO nanoparticles trapped within the MWCNTs were successfully synthesized by the ultrasonic method, and then, these structures modified chitosan under reflux condition. The presence of peaks corresponding to M–O stretching in the FT-IR spectra of the products confirmed the preparation of NiO/Co₃O₄–MWCNT compounds. This research shows that all studied graft complexes decrease toxicity and safe to use in biomolecule delivery systems in comparison with MWCNT. For near future, it seems that simultaneously decreasing the cytotoxicity effects and improving biocompatibility of functionalized MWCNTs will extend the potential exploitation of nanotubes for clinical applications.

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Fig. 5. The TEM images of the (a) NiO/MWCNT, (b) NiO/MWCNT-Chitosan, (c) Co$_3$O$_4$/MWCNT, and (d) Co$_3$O$_4$/MWCNT-chitosan.

Fig. 6. Cell toxicity evaluation of different complexes on MCF7 cancer cell line.

References


