Conjugation of Amphotericin B to Carbon Nanotubes via Amide-Functionalization for Drug Delivery Applications

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***Abstract*— Carbon nanotubes are among promising components for a wide range of applications, due to their unique properties; Specifically, They are a new alternative and efficient tool for transporting and translocating therapeutic molecules since they can translocate easily into the cytoplasm or nucleus of a cell through its membrane without generating an immunogenic response and toxic effects. While pristine carbon nanotubes are insoluble in all solvents, the organic functionalisation of carbon nanotubes is the prerequisite for their solubility profiles and their manipulation into biological systems. For this purpose, in this study, we first report the Amide-functionalization of CNTs which a direct coupling of ethylene-diamine with the carboxylic groups to introduce amino groups via amide formation. Finally, these functionalized carbon nanotubes will conjugate to Amphotericin B, the most effective antibiotic though toxic in the treatment of chronic fungal infections, via a two step process of diimide–activated amidation. The whole process is characterized by FTIR, TEM, UV-vis and Kaiser test.**

***Index Terms*— Carbon nanotubes; Amphotericin B; Functionalisation; Drug delivery; Conjugation**

I. INTRODUCTION

Targeted drug delivery is the most important goal of pharmaceutical research which is to delivery therapeutic molecules to targeted cells in a safe and efficient manner [1, 2]. All drug delivery systems should include continuous regulation of drug levels within the therapeutic range, effective targeted delivery, reduction in the amount of drug needed and, as a consequence, a decrease in toxicity and side effects [3,4].

Manuscript received July 21 ,2008 .

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Nanotechnology has nearly limitless potential in biomedical applications [5, 6]. Nanoparticles, among all available drug carriers gain more attractive attention; since they can be so manipulated that remains in circulation system to reach the diseased site and can penetrate through the cell membrane [5-7]. Above all, carbon nanotubes possess the capacity of penetrating the blood-brain barrier without causing the death of a living cell or without inflicting other damage. Carbon nanotubes, originally discovered by Iijima in 1991, are structures made up of thin sheets of benzene ring carbons rolled up into the shape of a seamless cylinder and are often capped on at least one end by a spherical buckyball structure [8,9].In order to modify carbon nanotubes which increase their biocompatibility and solubility profile, different functionalization approaches will apply to CNTs [8]. Therefore, functionalization of carbon nanotubes is a key step for further their application [9]. The oxidative reactions are widely employed for chemical modification of CNTs which generate carboxylic groups to the tips and defect sites of CNTs [10, 11]. These functional groups can further attached to other reactive groups or biological molecules such as drugs and used to deliver their cargos to cells and organs [12, 13]. As a therapeutic molecule antibiotic amphotericin B, which is used in the treatment of fungal infections, can be delivered by the means of carbon nanotubes. One major obstacle in traditional delivery of this drug lies in the fact that it has a low solubility and causes membrane leakage in eukaryotic cells [13]. Herein, we report the functionalization of Multi walled-carbon nanotubes through the amide formation which introduce the amine groups to the carboxylic groups of the tips and defect sites of carbon nanotubes. TEM and FTIR and UV-vis measurements clearly confirmed the functionalization and the attachment process.

II. EXPERIMENTAL SECTION

A. Materials

Amphotericin B, N-ethyl–N'-(3-dimethyl–aminopropyl) carbodimide hydrochloride (EDAC), N-hydroxysuccinimide(NHS),2-(N-morfolino)ethanesulfonic acid (MES) and $H_2SO_4$and HNO_3 were purchased from Merek chemicals. Purified Carbon nanotubes Multiwall produced by CVD process was purchased from Tehran University of Technology.

B. Oxidation of Carbon Nanotubes

The as-received purified CVD MWCNTs were first treated with a 3:1 mixture of concentrated sulfuric and nitric acid .This mixture was then sonicated to introduce carboxylic acid groups on the MWCNT surface. To establish the effect of acidic treatment on both the length of the CNTs and the number of carboxylic groups present, we prepared a series of oxidized multiwalled carbon nanotubes (MWNTs) by applying different acid conditions. Upon completion, the mixture was washed with cold distilled water to remove the residual acid and then centrifuged untill the supernatant of the mixture present the ph=7 which exhibit the no acidity in the suspension.The sample was then dried in a vacuum oven at 80°C for 4 h.

C. Functionalization of Carbon Nanotubes with Ethylenediamine

10 milligrams of the oxidized nanotubes was dispersed by sonication in 5 mL of ethylenediamine. 0.5 milligram of the EDAC was then added and sonicated for 4 h. The product was then diluted with excess methanol and centrifuged to remove excess materials. The functionalized SWCNTs were then dried in a vacuum oven at 80 °C for 4 h.

D. Two step conjugation process of Amphotericin B molecules to Carbon nanotubes

In the first step, 1 ml of a 250µg ml^{-1} Amphotericin B solution was dispersed in 0.2 ml 500 mM MES buffer .o.46 ml of a 50 mg ml^{-1}NHS aqueous solution were added to the above suspension and mixed. Under fast stirring, 024 ml fresh EDAC aqueous solution (10 mg ml^{-1}) was added quickly, and the mixture was continually stirred at room temperature for 30 min.In the second step, the amide -functionalized carbon nanotubes were redispersed in 9.0 ml of a 50 mM MES buffer solution (pH~6.1) and the above solution was added. After shaking the mixture on a platform shaker at 150 rpm at room temperature for 1 h, the nanotube suspension was centrifuged and washed with 50 mM MES buffer solution (pH ~ 6.1) three times to remove unbound Amphotericin B.

III. INSTRUMENTAL METHODS AND CHARACTERIZATION

A. FTIR spectroscopy

Utilizing FTIR, functional groups that may be present on particle can be identified.FIIR measurements were obtained on a pure sample of Carbon nanotubes and the Carbon nanotubes-functionalised particles before the conjugation process.

B. Transmission Electron Microscopy (TEM)

The CNT-length distribution was assessed by TEM, whereas the loadings were calculated the number of amino groups was measured with a quantitative Kaiser test.

C. Uv-vis spectrophotometer

The UV/Vis spectrum of pure MWNTs and MWNTs-AmB and unbounded drug molecules were obtained.

IV. RESULTS AND DISCUSSIONS

Fig 1a, 1b shows the TEM and SEM images of as-received multiwalled- carbon nanotubes. Also Fig 2.a Represent the FTIR of pure MWNTs. and broad band at 3400 cm^{-1} is attributed to the presence of O-H groups on the surface of the as-received SWCNTs and is believed to result from either ambient atmospheric moisture tightly bound to the SWCNTs or oxidation during purification of the raw material. In the FTIR spectrum of the oxidized SWCNT (Figure 2b), the peak at _1720 $cm^{-1}$is attributed to the C=O stretch of the carboxylic (COOH) group. The IR spectrum of the amide-functionalized MWCNT samples, MWCNT-CO-NH (CH2)2NH2 (Figure
2c), shows the disappearance of the band at 1720 cm^{-1} and a corresponding appearance of a band with lower frequency.(1661 cm^{-1}) assigned to the amide carbonyl (C=O) stretch. In addition, the presence of new bands at 1573 and 1223cm^{-1}, corresponding to N-H in-plane and C-N bond stretching, respectively, further confirms the presence of the amide functional group.

Fig.1.a

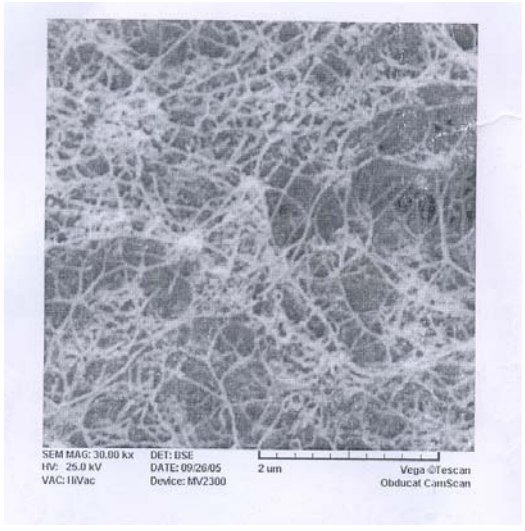

Fig 1.b.

Fig. 1 (a) Representative TEM micrographs of pure Multiwall carbon nanotubes (b) representative SEM micrographs of pure Multiwall carbon nanotubes.

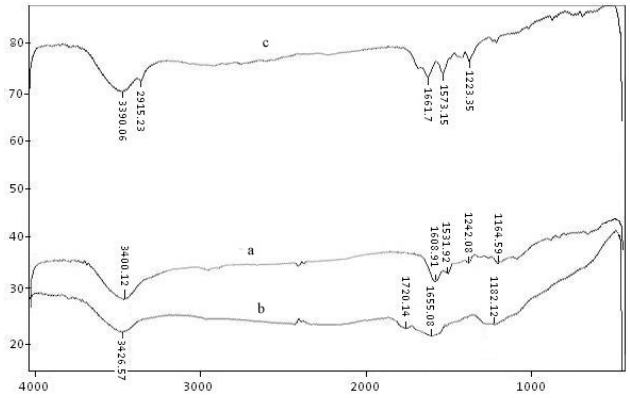

Fig . 2. FTIR spectra of (a) the as-received carbon nanotubes

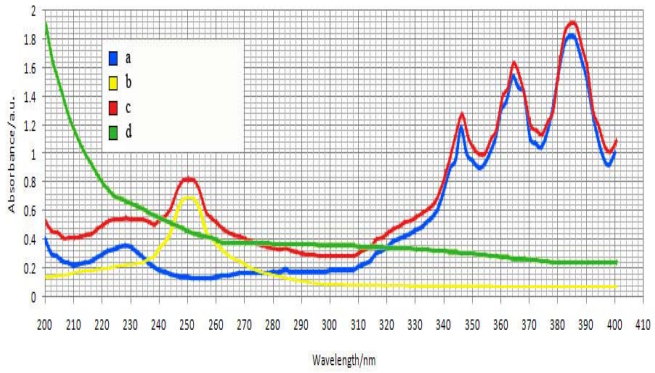

Fig.3. UV/vis spectrum of (a) Amphotericin B (b) f-CNTs (c)Amphotericin B conjugated to Multiwall carbon nanotubes(d) unbounded Amphotericin B

Table1. Chemico physical properties of MWNTs after treatment with strong acid and derivatization of the carboxylic acid functions. the result of TEM and Kaiser test for the distribution of the length and loading of functionalized carbon nanotubes.

t (h)	Length [nm]	Loading [mmolg^{-1}]
2	1200-3000	0.12
7	200-920	0.22
16	160-600	0.26
24	140-450	0.36

Table 1 also shows the Chemico physical properties of MWNTs after treatment with strong acid and derivatization of the carboxylic acid functions and also Kaiser test was used to determine the loading of amine groups. TEM analysis indicated that the lengths of f-MWNTs are consistent with the data reported in Table 1. Evidently, the length and the loading of the MWNTs strongly depend on the duration of the acid treatment. As expected, the tube length decreases and loading increases with an increase in the duration of oxidation. For our purpose, As expected, the tube length decreases and loading increases with an increase in the duration of oxidation. For our purpose, we selected MWNTs treated for 7 h as they exhibited the most convenient length and loading. The UV/Vis spectrum of Amphotericin B exhibit the typical absorption band of AmB in the 340-400nm which can be also observed in MWNTs conjugate to Amphotericin B. (Fig 3a,3c)This confirms the successful attachment of drug molecule to carbon nanotubes. For further confirmation, we observe the UV/Vis spectrum of the supernatant of the centrifuged solution which represents the unbound Amphotericin.(fig.3d) As a result, No absorption band was observed in the 340-400-nm range.

V. Conclusion

CNT is oxidized using strong acids, resulting in the reduction of their length while generating carboxylic groups, which increase their dispersibility in aqueous solutions. According to the results the best length and the loading of the carbon nanotubes was achieved for 8 h as they exhibited the most convenient length and loading. We attached the drug molecule to carbon nanotubes via a two-step process of diimide-activated amidation between the amine groups on MWNTs and the carboxylic acid groups on Amphotericin B. The carboxylic acid groups were activated by EDAC, a coupling agent, to form a highly reactive O-acylisourea active intermediate, which is unstable in aqueous solution, and does not have a sufficient lifetime for the two-step conjugation procedure. However, in the presence of N-hydroxysuccinimide (NHS), a more stable active ester (succinimidyl intermediate) can be formed. The active ester undergoes nucleophilic substitution reaction with the amine groups on Carbon nanotubes, resulting in the attachment of carbon nanotubes to Amphotericin B. Thus, the process can guarantee homogenous attachment of Drug molecule onto carbonnanotubes.As evidence, UV/vis studies showed that Amphotericin B molecules successfully conjugated to the surface of amide-functionalized Carbon nanotubes. This approach provides an efficient method to conjugate therapeutic proteins like Amphotericin B molecules to Carbon nanotubes for further delivery purposes.

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