

Surface Modification of Carbon Nano tubes with Nystatin for Drug Delivery Applications

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ABSTRACT

The present study is an approach to describe the increased antifungal efficacy of Nystatin by conjugation with functionalized multi-walled carbon nano tubes. Functionalized Multi-walled carbon nano tubes are acts as nano vectors for the delivery of therapeutic molecules by conjugation. Structural properties of carbon nano tubes have emerged as a new alternative and efficient tool for transporting and translocating therapeutic molecules to target moieties. Carbon nano tubes are easily cross cell membranes delivered drug molecules into fungal cells. Nystatin, most effective antibiotic in the treatment of fungal infections. Carbon nano tubes oxidized using strong acids (3:1 Ratio of Concentrated Sulphuric and Nitric acid), resulting in the reduction of their length while generating carboxylic groups, which increase their dispersibility in aqueous solutions. Oxidised CNTs are then functionalized by direct coupling of Ethylene-diamine with carboxylic groups cited at external walls to introduced amino group via amide formation. Nystatin, most effective antibiotic is conjugated with CNTs via two step process of diamide-activated amidation. Fourier-transform infrared spectroscopy (FT/IR), UV-Visible Spectroscopy, Scanning Electron Microscopy (SEM), & Transmission Electron Microscopy (TEM) measurements clearly confirmed the functionalization & conjugation steps.

Key words: Multi-walled carbon nanotubes, Nystatin, Antifungal, Surface Modification, Conjugation.

INTRODUCTION

The search for new and effective drug delivery systems is a fundamental issue of continuous interest. CNTs (Carbon nano tubes) are unique, one-dimensional macromolecules.¹ SWCNTs (Single-walled carbon nano tubes) are constructed of a single sheet of graphite (diameter 0.4-2 nm), while MWCNTs (multi-walled carbon nano tubes) consist of multiple concentric graphite cylinders of increasing diameter of (2-100 nm). One of the major areas of CNTs research is the field of biomedical materials and devices. Many applications for CNTs have been proposed including biosensors, drug and vaccine delivery vehicles and novel biomaterials.² Nanotechnology has nearly limitless potential in biomedical applications.^{3,4} Nano particle, 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.³⁻⁵ Carbon nano tubes possess the capacity of penetrating the blood-brain barrier without causing the death of a living cell or without inflicting other damage. Carbon nano tubes, 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 bucky ball structure.^{6,7} In order to modify carbon nano tubes which increase their biocompatibility and solubility profile, different fictionalization approaches will apply to CNTs.⁶ Therefore,

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fictionalization of carbon nano tubes is a key step for further their application.⁷ The oxidative reactions are widely employed for chemical modification of CNTs which generate carboxylic groups to the tips and defect sites of CNTs.^{8,9} 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.^{10,11}

Experimental

Materials

The following chemicals were obtained from commercial suppliers and used as received: Nystatin (Maneesh Pharmaceuticals Pvt. Ltd., Mumbai.), carbon nano tubes (Applied science Innovations Pvt. Ltd., Pune.), N-ethyl-N²-(3-dimethyl-aminopropyl) Carbodimide hydrochloride (EDAC) (Sisco Research Lab. Pvt. Ltd., Mumbai.), N-hydroxysuccinimide (NHS) (Sisco Research Lab. Pvt. Ltd., Mumbai.), 2-(N-morfolino) ethanesulfonic acid (MES) (Sisco Research Lab. Pvt. Ltd., Mumbai.), Ethylene-diamine (Thermo Fisher Scientific India Pvt. Ltd., Mumbai.), Concentrated H₂SO₄ (Hexon Laboratories Pvt. Ltd., Pune.) and Concentrated HNO₃ (Qualigens Fine Chemicals, Mumbai.).

Method

Oxidation of carbon nano tubes

MWCNTs expose to oxidative conditions, i.e. 150 mg of MWCNTs were treated with 3:1 mixture of concentrated sulphuric acid & nitric acid. This mixture was then sonicated (Ultrasonic Probe Sonicator, DP-120) simultaneously irradiated with varying amplitudes of ultrasonic energy up to 10 hrs. A probe has a tip diameter of 13 mm and is immersed 5 mm in the liquid. The device operates at a fixed wavelength of 20 kHz and is capable of inducing a maximum power output of 750 W. Upon completion, the mixture was washed with cold distilled water to remove the residual acid and then centrifuged at 3000 rpm, 4°C (Cryocentrifuge 2810R, Eppendorf, USA) until the supernatant of the mixture represents the pH=7 which exhibit absence of acid residues in the suspension. Sample was then dried in vacuum (Krishna Technocreates, Basic) and kept in oven at 80°C for 4 hr.

Functionalization of carbon nano tubes with ethylene-diamine

150 mg of the oxidized nano tubes was dispersed by sonication in 75 mL of ethylenediamine. 7.5 mg of the EDAC was then added and sonicated (Ultrasonic Probe Sonicator, DP-120) for 4 h. The product was then diluted with excess methanol and centrifuged

(Centrifuge Apparatus, RM-E2) to remove excess materials. The functionalized CNTs were then dried in a vacuum (Krishna Technocreates, Basic) oven at 80°C for 4 hr.

Two step conjugation process of Nystatin to carbon nano tubes

a. Step - I

10 ml of 250 µg ml⁻¹ Nystatin solution was re-dispersed in 4 ml of 500 mM MES buffer solution followed by addition of 9.2 ml of 50 mg ml⁻¹ NHS solution and mixed with fast stirring (Mechanical Stirrer, Omega Scientific Industries). Then add 4.8 ml of 10 mg ml⁻¹ fresh EDAC aqueous solution quickly. Solution or mixture was continually stirred at room temperature for 30 min (Figure 1).

b. Step - II

Amide functionalized CNTs were re-dispersed in 90 ml of MES 50 mM buffer solution (pH=6.1) then above solution was added. After addition of above solution, nano tubes mixture was kept on platform shaker (Orbital Shaker, RIS-24BL) at 120 rpm at room temperature for 90 minutes. After shaking the mixture, the nano tubes suspension was then centrifuged (Centrifuge Apparatus, RM-E2) and wash with 50 mM MES buffer solution (pH = 6.1) three times to remove unbound Nystatin (Figure 1).

Characterization of conjugated MWCNTs with Nystatin

Fourier-transform infrared (FTIR) Spectroscopy

IR spectra were measured with a Bruker Equinox 55S IR spectrometer, in the region of 400-4000 cm⁻¹ with a resolution of 4 cm⁻¹, equipped with a DTGS detector. Each spectrum was the average of 200 scans collected at 2 cm⁻¹ resolution.

UV-Vis Spectroscopy

The UV-Visible Spectrum of Pure Nystatin, f-MWCNTs, Nystatin conjugated to MWCNTs & unbound MWCNTs molecules were obtained in mixture of Glacial acetic acid & methanol (1:1 ratio). Samples were scanned over the range of 200-400 nm by using UV-Visible spectrophotometer (Jasco-1800).

Scanning Electron Microscopy (SEM)

Electron micrograph of Nystatin conjugated MWCNTs was obtained using a scanning electron microscope (JEOL JSM-5200) operating between 5 and 24 kV.

Transmission Electron Microscopy (TEM)

The size and morphology of the conjugated MWCNTs were characterized by a Philips CM-100 TEM equipped with Hamamatsu Digital Camera ORCA-HR operated using AMT software (Advanced Microscopy Techniques Corp, Denver, MA).

Ex-vivo Study: Minimum Inhibitory Concentration

Carbon nano tubes conjugated Nystatin was dissolved in dimethyl sulfoxide (DMSO)/absolute ethanol (at a ratio of 3:2), to an initial concentration of 10,000 µg/ml and stored at -20°C until used.

Bacterio static and bactericidal activities of nystatin towards *Candida albicans*, *Candida parapsilosis* and *Candida neoformans* were determined by the broth dilution technique as per McGinnis and Rinaldi (1996). Briefly, 50 µl of the Carbon nano tubes conjugated Nystatin was two-fold serially diluted with Sabouraud-dextrose broth in a microtitre plate. An equal volume of yeast suspension (9×10^7 cells/ml) was added and mixed with the drug. DMSO/absolute ethanol (3:2 ratio) without nystatin was used as the control. The minimum inhibitory concentration (MIC) was determined visually following 24 h incubation at 37°C for 2-3 days. The MIC was defined as the lowest concentration of the drug that inhibited growth of yeast cells, as indicated by the absence of turbidity (optically clear). Subculturing from the optically clear/turbid wells was performed to determine the minimum fungicidal concentration (MFC). The subcidal concentration of nystatin for all strains, used as positive control, in this study was the concentration slightly below the MFC of the drug.

All experiments were repeated on two separate occasions, with duplicate determinations on each occasion.

RESULTS AND DISCUSSION

The IR spectrum of the pure MWCNTs (Figure 2A) showed the characteristic stretching vibrations of O-H groups at 3400 cm^{-1} . Peak at 1720 cm^{-1} appears in the spectrum of the oxidised MWCNTs (Figure 2B) is attributed to the C=O stretch of the carboxylic group (COOH) after the oxidation process on the surface of the oxidised MWCNTs. The FT/IR spectrum of a amide-functionalized MWCNTs (Figure 2C) shows the disappearance of a band at 1720 cm^{-1} & appearance of a band with a lower frequency at 1661 cm^{-1} attributed the amide carbonyl (C=O) stretch. Also, presence of new band at 1523 cm^{-1} assigned to an N-H in-plane & at 1223 cm^{-1} arise from C-N band stretching. This confirms the presence of the amide functional group.¹²

Figure 3 shows the UV-visible spectra of the pure Nystatin, functionalized MWCNTs, conjugated MWCNTs and unbound Nystatin. The pure Nystatin display absorption peaks in range of 275 nm to 325 nm (Curve A), which is typical for Nystatin. These characteristic absorption peaks (Curve C) at range of 275 nm to 325 nm was also appear in the spectrum of conjugated MWCNTs confirms the conjugation of Nystatin with MWCNTs. The UV-visible spectra (Curve D) of supernatant of centrifuged solution which contains unbound Nystatin further confirms modification. From the UV/Vis Result, this confirmed that successful conjugation of nystatin molecule to MWCNTs. This implies that the Nystatin functionalization on MWCNTs significantly increases the water solubility, which is essential for them to be used for biological applications

The morphology of the Pure MWCNTs was characterized by SEM (Figure 4A). The clear contrast between Pure MWCNTs and MWCNTs conjugated with Nystatin indicate completion of conjugation process. Moreover, the SEM image of Pure MWCNTs shows a transparent thin film whereas after conjugation process the image (Figure 4B) obtained was not very clear due to the chemical modification of the MWCNTs.

TEM analysis (Figure 5) confirms the oxidation process (Treatment of strong acid in 3:1 ratio, $\text{H}_2\text{SO}_4:\text{HNO}_3$) by measuring the length of MWCNTs. Length & loading of the MWCNTs strongly depends on the duration of acid treatment. The tube length decreases & loading increase with an increase in the duration of oxidation.

From the Table 1, it is shows that physic-chemical properties of MWCNTs after oxidation process (Treatment of strong acid in 3:1 ratio, $\text{H}_2\text{SO}_4:\text{HNO}_3$) & derivatization of the carboxylic acid functions get changed.¹⁷ Kaiser test was used to determine the loading of amino groups. Firstly, prepared three test solutions, (A) Ninhydrin (5% w/v) in ethanol, (B) Phenol (4:1, w/v) in ethanol & (C) Potassium cyanide (2%, v/v, of a 1 mmole/liter aqueous solution) in pyridine. The test was carried out by adding 2 drops of A, 1 drop of B and 1 drop of C to the test sample contained in a small glass test tube and then heating the tube for 2-5 minutes on a hot plate.

We selected MWCNTs treated for 10 hr for further study as they exhibited the most convenient length & loading (Table 1).

From the antifungal activity (MIC and MFC) (Table 2 and 3), amide f-MWCNTs, free of Nystatin, were inactive up to the maximal concentration (80 µg mL^{-1}) used against all the microorganisms tested.

Conversely, Conjugation of MWCNTs-Nystatin was highly effective; indicating that activity of the Nystatin was not prevented by its covalent bonding to CNTs.

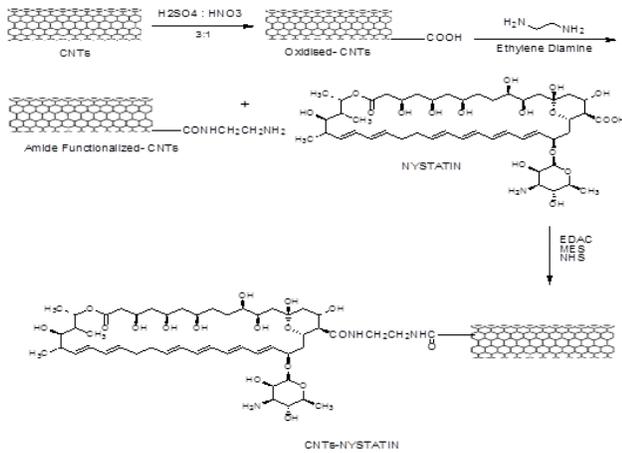


Figure 1: Schematic presentation of conjugation process of Carbon nanotubes with Nystatin.

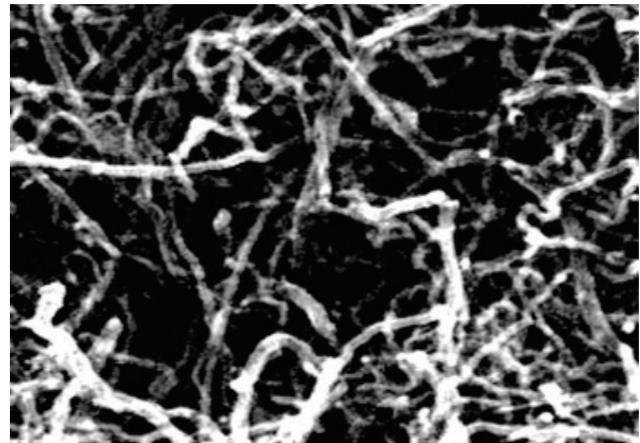


Figure 4(A): SEM Images of Pure MWCNTs.

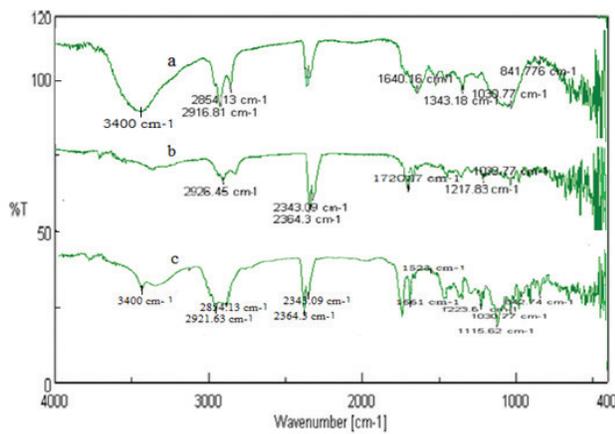


Figure 2: FT/IR Spectra of (a) Pure MWCNTs (b) Oxidised-MWCNTs (c) functionalised-MWCNTs.

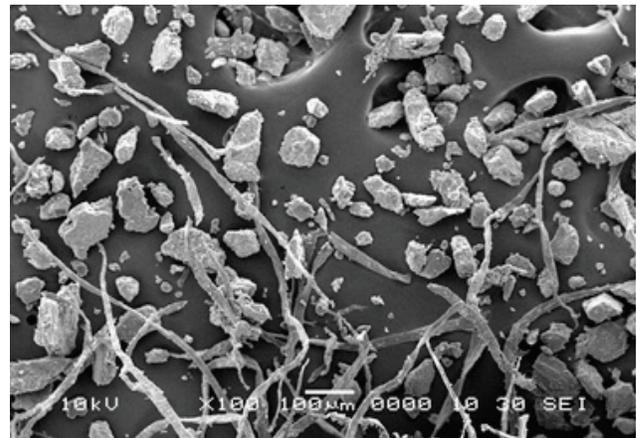


Figure 4(B): SEM Images of MWCNTs conjugated with Nystatin.

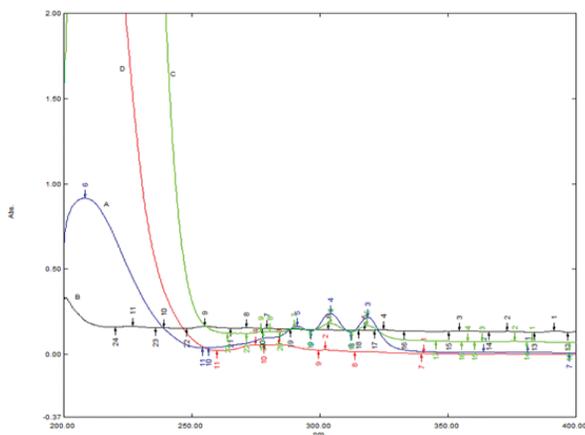


Figure 3: UV/Vis spectrum of (A) Nystatin (B) f-CNTs (C) Nystatin conjugated to MWCNTs (D) unbound Nystatin.

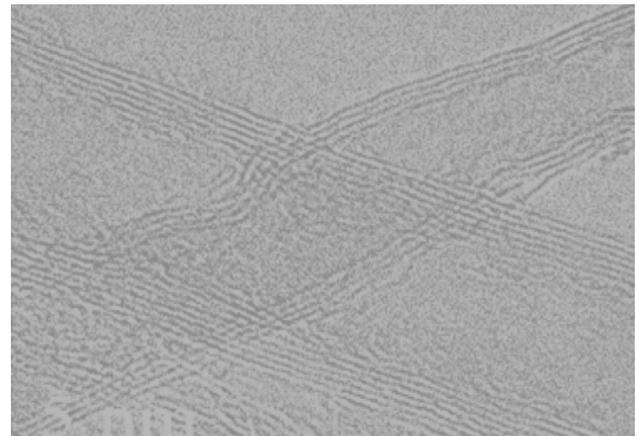


Figure 5(A): TEM images of Pure MWCNTs.

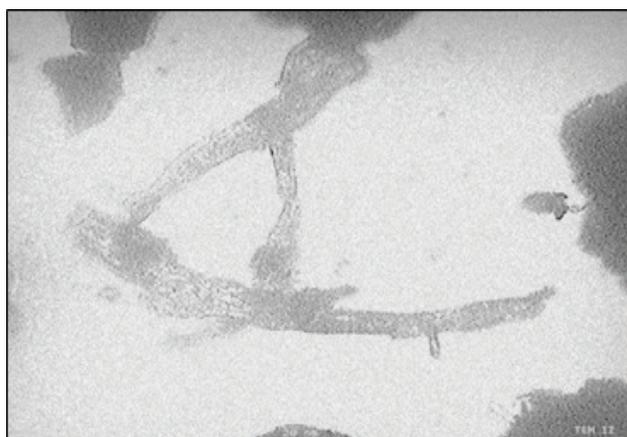


Figure 5(B):TEM images of MWCNTs after acid treatment.

Table 1: 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 (hr.)	Length (nm)	Loading (mmolg ⁻¹)
1	1400-4000	0.09
2	1000-2000	0.16
4	600-1400	0.22
6	200-1000	0.28
8	180-600	0.38
10	160-550	0.40
24	140-400	0.46

Table 2: Antifungal activity: Minimum Inhibitory Concentration (MIC)

CNTs	Minimum Inhibitory Concentration (MIC)		
	<i>Candida albicans</i> (NCIM NO-3471)	<i>Candida parapsilosis</i> (NCIM NO-3471)	<i>Candida neoformans</i> (NCIM NO-3471)
Nystatin (µg/ml)	8	6	4
Amide f-CNTs (µg/ml)	>80	>80	>80
MWCNTs-Nystatin (µg/ml)	6	4	2

Table 3: Antifungal activity: Minimum Fungicidal Concentration (MFC)

CNTs	Minimum Fungicidal Concentration (MFC) (µg/ml)		
	<i>Candida albicans</i> (NCIM NO-3471)	<i>Candida parapsilosis</i> (NCIM NO-3471)	<i>Candida neoformans</i> (NCIM NO-3471)
Nystatin	25	21	18
Amide f-CNTs	>80	>80	>80
MWCNTs-Nystatin	16	14	11

When the equal amounts of free & bound drug are considered, conjugated Nystatin is definitely more potent than the free drug against the candida species.¹⁰ The efficacy may improve because of modification in penetration ability of fragmented and conjugated MWCNT's with antifungal drug.

CONCLUSION

MWCNTs were oxidised using 3:1 ratio of H₂SO₄:HNO₃ acids, resulting in the reduction of MWCNTs length while generating carboxylic groups. According to the results, the best length & loading of MWCNTs was achieved for 10 hr as they exhibited

the best length & loading. We conjugated Nystatin to MWCNTs. i.e. Amino group of MWCNTs & Carboxylic acid group of Nystatin. From the above results, UV-Visible Spectroscopy & Ex-vivo antifungal studies show that Nystatin successfully conjugated to the amide-functionalized Carbon nano tubes. By using CNTs (Conjugated with Nystatin), efficacy of Nystatin was increased. CNTs can be used as carrier for the Delivery of Nystatin. Results of *Ex-vivo* study (MIC and MFC), clearly shows that the efficacy of Nystatin was increased. The covalent linkage of Nystatin to the CNTs is an approach that may be used to modulate the therapeutic action of the Nystatin.

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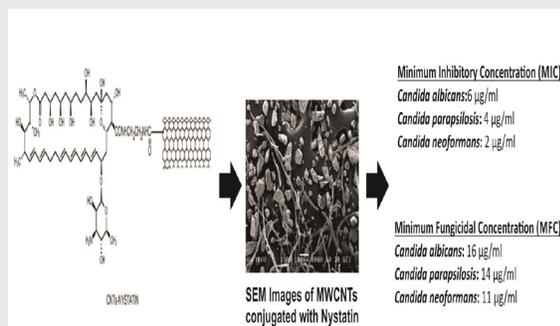
CONFLICT OF INTEREST

The authors report no conflicts of interest.

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PICTORIAL ABSTRACT



- Synthesized conjugate characterized by Fourier-transform infrared spectroscopy (FT/IR), UV-Visible Spectroscopy, Scanning Electron Microscopy (SEM), & Transmission Electron Microscopy (TEM).
- MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) results shows improved Antifungal activity.

SUMMARY

- Major concern of study is to improve antifungal efficacy of Nystatin which significantly improved after conjugation with functionalized multiwall carbon nanotubes.
- Functionalization of Multiwall carbon nanotubes (CNTs) are acts as nano vectors to efficient transport and translocation of therapeutic molecules to target moieties.
- Nystatin, most effective antibiotic is conjugated with CNTs via two step process of diamide-activated amidation.