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Formulation and Characterization of Methotrexate Loaded Nanotubes for Rheumatoid Arthritis

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ABSTRACT
The goal of this work was to developed and characterize multiwall carbon nanotubes conjugated with dexamethasone. Fourier transform infrared was used to confirm that the dexamethasone was conjugated to amidated multi-walled carbon nanotubes (MWCNTs). Both pristine and functionalized MWCNTs were loaded with methotrexate, which was then thoroughly described in vitro. Dexamethasone conjugated MWCNTs were shown to have a high drug entrapment efficiency. It was discovered that the in vitro drug release from the formulation of pristine MWCNTs and dexamethasone coupled MWCNTs was 57.81 ±1.2 and 65.23 ± 1.4% in 24 hours, respectively, in PBS (pH 7.4).

Keywords: *Functionalization, Nanotubes, Amidated, Internalization, Biocompatibility.*

1. INTRODUCTION

Symmetric discomfort and swelling in the hands, wrists, feet, and knees are hallmarks of rheumatoid arthritis (RA), a prevalent systemic autoimmune illness that affects quality of life and physical functions [1]. Although the exact cause of RA is unknown, its clinical incidence ranges from 0.5% to 1% . It is a painful and incapacitating disorder that can cause a significant loss of movement and bodily functioning if left untreated [2]. Glucocorticoids (GCs), nonsteroidal anti-inflammatory drugs (NSAIDs), and disease-modifying anti-RA therapies (DMARDs), which include biologics and small molecule medications, are currently the primary chemotherapy options for treating RA . The preferred medication for treating RA in its early stages is usually methotrexate (MTX). At low dosages (15–25 mg/week), it can effectively treat RA [3]. Clinically, MTX is linked to side effects such as neurotoxicity, mucosal skin damage, gastrointestinal distress, and bone marrow suppression. As a result, frequent observation is necessary while administering. Additionally, the kidneys absorb 80% of MTX, which can result in sepsis, severe neutropenia, nephrotoxicity, and progressive renal failure [4, 5]. Drugs that target the joints are currently a new trend in the treatment of RA.

Nanomedicine has emerged as a successful therapeutic approach for RA due to its ability to distribute drugs efficiently. Anti-RA medications are frequently delivered by a variety of nanocarriers, such as liposomes [6], polymer micelles [7], dendrimers [8], and gold nanoparticles (GNPs) [9], either actively or passively through targeted systemic administration [10]. For the treatment of RA, the nanotubes make great drug carriers. Nanotubes offer distinct optical characteristics, sizes and shapes, facile surface modification, good biocompatibility, and simple binding to active ligands via chemical bonds. They are also basically nontoxic, durable, and dependable in vivo [11]. By conjugation of different ligands to promote immune system protection, targeting, cell internalization, and transport of therapeutic payloads, GNPs' therapeutic potential can enhance the effectiveness of existing RA treatments [12, 13].

2. MATERIAL AND METHOD

Platonac Nanotech Pvt. Ltd. in Jharkhand, India, supplied the MWCNTs, which were produced by chemical vapor deposition (CVD) and had a diameter × length of 10-15 nm × 2-10 µm with a content of >99% carbon. We bought methotrexate from Triveni Interchem Pvt. Ltd. in Gujarat, India, and dexamethasone from Kwality Pharmaceutical Ltd. in Amritsar, Punjab, India.Every solvent and reagent was bought from a commercial supplier and used exactly as supplied.

Purification of raw MWCNTs :- Following a 5-hour reaction with strong hydrochloric acid under magnetic agitation, 100 mg of purchased unpurified MWCNTs were filtered using a 0.45 µm polytetrafluoroethylene (PTFE) filter. The unpurified MWCNTs were treated with acid to remove amorphous and catalytic impurities [14, 15]. To eliminate the amorphous carbon, acid-purified MWCNTs were placed in an oven set to 530° C for 30 minutes.

Cutting and Carboxylation of MWCNTs:- To cut and create a carboxylic group on MWCNTs, 100 mg of acid-purified MWCNTs were treated with a solution of H_2SO_4 (98%)/HNO_3 (68%) (3:1 v/v) for four hours at 60 ± 2° C. Following PTFE filtering, deionized water washing to pH 7, vacuum oven drying, and characterization, these carboxylated MWCNTs were examined [16].

Acylation and amidation of MWCNTs :- Following the acylation of MWCNTs, they were centrifuged and rinsed five times with anhydrous tetrahydrofuran (THF) following 50 mg carboxylated MWCNTs were agitated in a volume ratio of 21:1 mixture of thionyl chloride (SOCL2) and N,Ndimethylformamide (DMF) at 70 ± 2° C for 24 hours. The residual solids were vacuum-dried. For two days, the acylated MWCNTs were exposed to an excess of ethelyne diamine at 100 ± 2° C. To get rid of the extra ethylene diamine, the MWCNTs were cooled to room temperature and then rinsed five times with ethanol. Ultimately, the black solids were described and allowed to dry overnight at room temperature (Figure 1) [17].

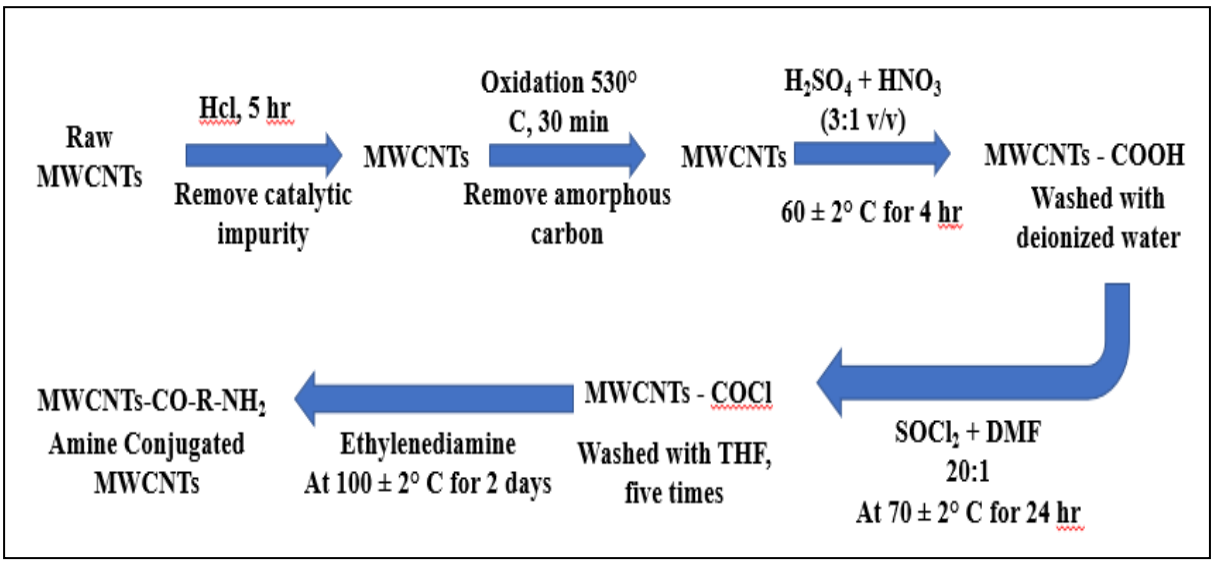

Figure 1:- Functionalization of MWCNTs

Conjugation of dexamethasone on Amide-MWCNTs:- 5 mL of anhydrous dimethyl sulfoxide (DMSO) was used to dissolve 2.5 mg of 2-iminothiolane and 10 mg of dexamethasone. Separately, 5 mL of anhydrous DMSO was used to disperse 5 mg of NH2-terminated MWCNTs. For 24 hours at room temperature, both solutions were combined while being constantly stirred, and excess deionized water was added to stop the process. In a dialysis tube (MWCO 5–6 kDa), the suspension was dialyzed for two days at room temperature before being filtered and dried [18, 19].

Encapsulation of Methotrexate into f-MWCNTs:- To aid in drug encapsulation, 20 mg of methotrexate was dissolved in phosphate buffer saline (pH=7.4), then f-MWCNTS was added. The mixture was then swirled overnight. The drug-loaded f-MWC Ts were separated from the solution by ultracentrifugation after encapsulation. Using a spectrophotometer the amount of methotrexate entrapped in f-MWCNTs-based systems was estimated spectrophotometrically [20, 21].

Calibration curve of methotrexate in Phosphate Buffer Saline (pH 7.4:- In accordance with the Indian Pharmacopeia 2010, PBS (0.1 M, pH 7.4) was made with purified water (1000 ml), sodium chloride (8.0 g), potassium dihydrogen phosphate (0.19 g), and disodium hydrogen phosphate (2.38 g). Ten milligrams of precisely weighed methotrexate were put into a 100 milliliter volumetric flask and dissolved in a tiny quantity of PBS. To achieve a standard stock solution of 100 µg/ml, the volume was increased to 100 ml using PBS. In a 10-ml volumetric flask, aliquots of 0.2, 0.4,…… up to 2.0 ml were collected, and the volume was increased to 10 ml using PBS. The concentration that resulted varied between 2 and

20 µg/ml. In a Cintra 10 GBC UV visible spectrophotometer, the absorbance of each concentration was measured at 258.5 nm in comparison to PBS. Figure 2 shows the standard curve plotted between absorbance and concentration.

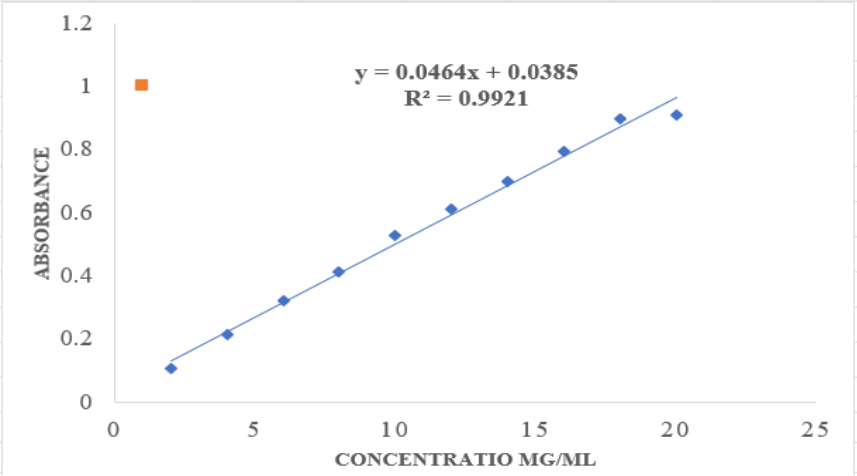

Figure 2:- Calibration curve of Methotrexate in PBS (pH 7.4)

Entrapment Efficiency:- A 10 ml volumetric flask was filled with 1 ml of the ultracentrifuge tube solution, and the volume was adjusted with fresh DDW. The unentrapped medication was assessed using the absorbance, which was measured at 258 nm. Additionally, the amount of drug entrapped was assessed. The formula (Figure 3) was used to compute the drug encapsulation efficiency in different f-MWCNTs [22].

$$\text{Entrapment Efficiency (EE \%)} = \frac{\text{Weight of encapsulated drug}}{\text{Weight of encapsulated drug + Free Drug}} \times 100$$

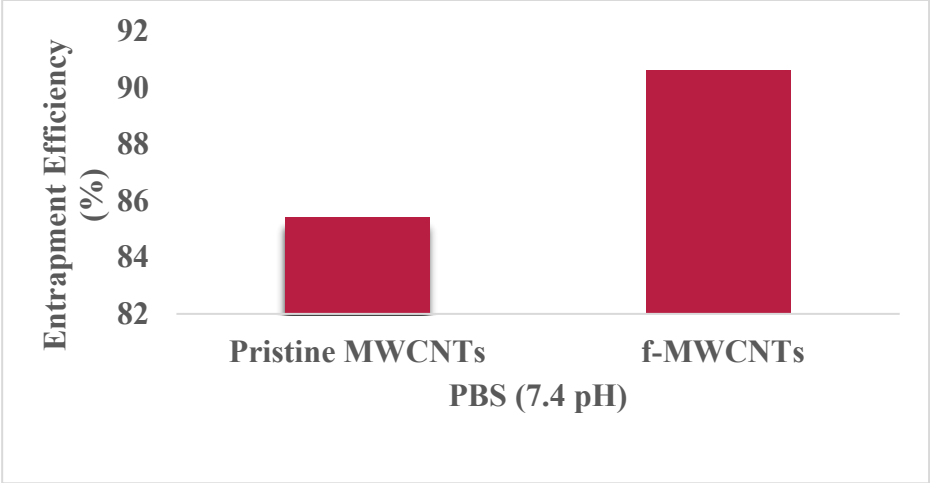

Figure 3:- Entrapment Efficiency (n=3)

***In-vitro* release of Methotrexate from functionalized and pristine MWCNTs:-** At phosphate buffer saline (pH=7.4), the in vitro release of the medication from two different formulations (Pristine and f-MWCNTs) was assessed. For the release study, a dialysis membrane (MWCO, 2000 Da, Himedia, India) was chosen. After being hermetically tied and immediately suspended in aqueous receptor medium, five milligrams of the formulation were put into the dialysis sac. The in vitro drug release experiment was conducted in the receptor compartment at 37±2°C with continuous stirring under sink conditions. In short, a dialysis bag containing 5 mg of drug-loaded pristine and functionalized MWCNTs was dialyzed against 100 mL of release media at room temperature while being stirred at 100 rpm using a magnetic stirrer (Remi, India). At regular intervals, one milliliter of the sample was taken out and replaced with an equivalent volume of the new medium. The medication was measured using spectrophotometry (UV/Vis Shimadzu 1601, Japan) at 258 nm after the proper dilutions. Refilling 1 milliliter

of sink solution from the receptor medium preserved the receptor compartment's volume showed in figure 4 [23, 24].

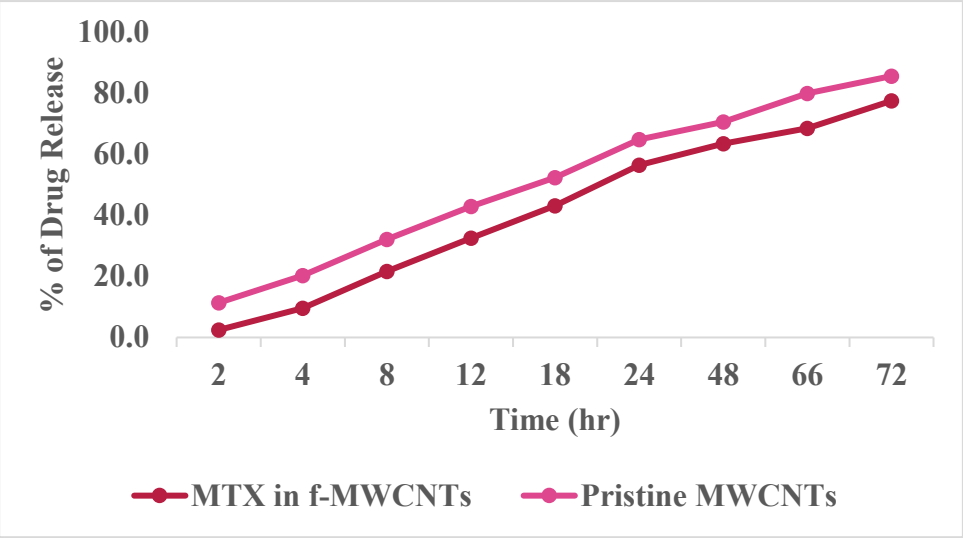

Figure 4:- Cumulative percentage drug release from pristine & f-MWCNTs (n=3)

Haemolytic toxicity of drug MWCNTs system:- Hi-Anticlot blood collection containers (Hi media, India) were used to collect whole human blood. RBCs were isolated at the tube's bottom after this was centrifuged for 15 minutes at 3000 rpm using Remi equipment, India. Normal saline (0.9 percent w/v) was used to wash the RBCs until a clear, colorless supernatant was obtained, indicating that the cells were above the cell mass. reconstituted in a standard saline solution [20]. 5 ml of distilled water were added to one milliliter of RBC suspension in a centrifuge tube, which was thought to result in 100% hemolysis. In a similar manner, 1 ml of RBC suspension was mixed with 5 ml of regular saline in a separate tube to prevent hemolysis and serve as a blank. A solution of methotrexate-loaded f-MWCNTs (0.5 ml) was mixed to 4.5 ml of normal saline and l ml of RBC suspension. The formulation had previously been dialyzed to remove the unentrapped medication. Likewise, 4.5 ml of regular saline and 1 ml of RBC suspension were combined with 0.5 ml of medication solution (10% w/v) in a different tube. The medicine and MWCNTs were ingested in different amounts in different tubes so that, in each of the aforementioned examples, the ultimate concentration of the drug and MWCNTs was equal. The tubes were shaken while they stood for an hour. The amount of the MWCNTs-Dexamethasone conjugate drug complex was calculated so that the final drug concentration, or f-MWCNTs complex, was equal to that of the pristine MWCNTs system. This will make it possible to compare the drug's hemolysis data with those of the pristine MWCNTs-drug complex and the Dexamethasone-CNT-drug complex. The tubes were centrifuged for 15 minutes at 3000 rpm using Remi equipment from India. The absorbance was measured at 540 nm in comparison to a supernatant of normal saline that had been diluted identically to the blank after the supernatants had been taken and diluted with an equal volume of normal saline. Each sample's percentage of hemolysis was determined by using the water absorbance as a 100% hemolytic sample (Figure 5).

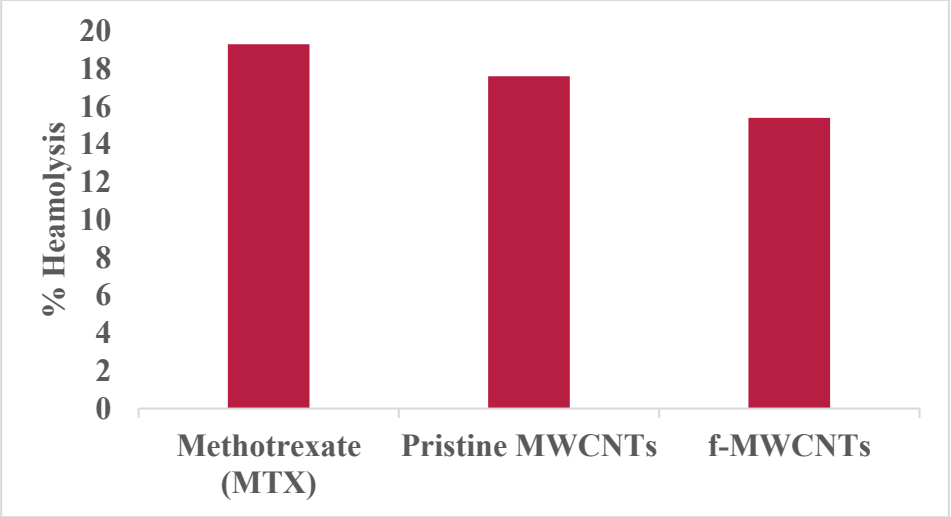

Figure 5:- Haemolytic toxicity (n=3)

Dispersion:- Nanotubes' intrinsic insolubility non the majority of solvents is a significant issue. Dispersibility was assessed after 5 mg of f-MWCNTs were dissolved in 10 ml of distilled water and then sonicated for 15 minutes using a sonicator (Soniweld, Mumbai). the distribution of f-MWCNTs and pure MWCNTs. In order to determine the quantitative outcome, the dispersibility of MWCNTs is typically assessed visually by examining the dispersion's blackness [25].

Stability Studies: - Functionalized MWCNTs loaded with methotrexate were stored in hermetically sealed glass vials. For five weeks, the samples were stored in amber-colored and colorless vials at 4° C, room temperature (25° C), and 50° C in a controlled oven. The samples were examined for precipitation, turbidity, crystallization, color changes, and consistency at the beginning and weekly intervals for a maximum of five weeks. The information gathered was utilized to analyze any chemical or physical deterioration under storage circumstances and to ascertain the necessary storage precautions [26].

3. RESULT & DISCUSSION

At least two goals guided the design of the drug characterisation study. First, the acquired drug would be verified by comparing some of its experimentally determined properties with those listed in pharmacopeia. Second, early research on a drug's characteristics would be beneficial for a more thorough experimental setup. It was found that the medication methotrexate was a crystalline powder with a similar physical appearance, yellow to orange in color, and odorless. comparable findings were made with the medicine dexamethasone, which was found to be a white to off-white powder with a comparable physical appearance and no smell.

At ambient temperature (25° C), the solubility profile of both medications in various solvents showed that they were soluble in phosphate buffer saline (PBS 7.4 pH). Additionally, these results aligned with the usual observation. The presence of distinct groups was confirmed by the FTIR spectra of dexamethasone and methotrexate. The different peaks seen in the infrared spectrum corresponded to the infrared spectrum listed in the official publications. Because of the stretching of the carbon nanotube's backbone, the FTIR spectra of virgin MWCNTs only displays one peak at 2936.7 cm. There is compelling evidence that pure MWCNTs are devoid of any impurities. The attachment of dexamethasone to MWCNTs that contained aromatic rings was suggested by the FTIR spectra of f-MWCNTs, which showed a peak at 3341.9 due to presence of O-H group, medium peak at 2934.2 which may bwe due to presence of trans alkenes showed in figure 6.

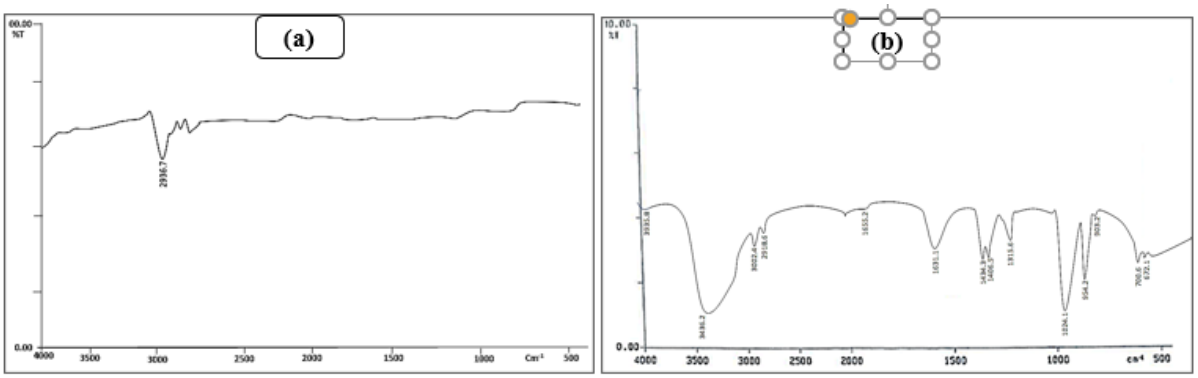

Figure 6 IR Spectrum (a) Raw MWCNTs (b) f-MWCNTs

PBS (pH 7.4) served as the dispersion medium for the drug loading tests, which were conducted at room temperature. Because Pristine MWCNTs originate in a bundled state, which reduces surface area and consequently lowers drug loading, poor drug loading was noted in this instance. Functionalized MWCNTs, on the other hand, are open structures that have the potential to gather more drug on their surface. Since the drug is mostly found in a unionized state at pH 7.4, the fundamental loading criterion in each of these situations is the drug-MWCNT interaction (Figure 3).

In a 24-hour period, the drug release from pristine MWCNTs was 65.2±1.3, whereas in a formulation of f-MWCNTs, it was 57.8+1.4. Controlled release medication administration was suggested by the drug's release from the formulation in PBS (pH 7.4) (Figure 4). Its utility as a drug delivery device was limited by the hemolytic toxicity of both virgin MWCNTs and drug-loaded formulations. Because there were fewer free medicines available, the FA affixed to the MWCNT surface significantly decreased the (%) hemolysis of RBC (Figure 5). The formulation was tested for stability under a range of conditions, including temperature (4°C, R.T., and 50°C), after being stored for five weeks in both light (colorless bottles) and dark (amber-colored bottles). It was found that the formulation was best stable around 40° C in the dark.

4. CONCLUSION

Carbon nanotubes have attracting a great deal of attention in pharmaceutical and biomedical fields. Significant progress has been made in drug delivery and targeting in the management of various diseases including cancer, AIDS/HIV, malaria and

macrophages targeting. Carbon nanotubes have broken new ground for the drug delivery scientist for exploring its role in biomedical field as well as in drug delivery. The present work on MWCNTs includes their purification, subsequent functionalization and finally dexamethasone conjugation. The various successful data proved that functionalization has been carried out.

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