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Functionalization, 5-Fluorouracil Loading and Evaluation of Multi Walled Carbon Nanotubes for New Era of Cancer Treatment

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ABSTRACT

Purpose: Carbon nanotubes owning minimum toxicity, safety and biocompatibility properties, used for diagnosis and treatment of carcinoma in the current days. MWCNTs are conjugated with different molecules for delivery of therapeutic molecules. In the present research work MWCNTs is functionalized non- covalently by PEG polymer to improve the solubility of MWCNTs, preserve and optimize the properties of MWCNTs for the release of drug in controlled manner for cancer therapy. Methods: Preparation carried out in two steps, firstly pristine MWCNTs functionalized by non covalent process using PEG, then drug binded to PEG functionalized MWCNTs. The prepared formulations were evaluated by SEM studies, FT-IR studies, particle size, zeta potential, drug entrapment efficiency, and drug *in-vitro* release study. Results : SEM images clearly showed increase in size of MWCNTs, FT-IR studies confirms the presence of functional group, particle size increased both in PEG functionalized MWCNTs and drug binded MWCNTs compare to pristine MWCNTs, zeta potential shows negative, all formulations revealed good average drug entrapment efficiency. Conclusion: The present study exhibited that PEGlyation of MWCNTs helps to get better the drug entrapment and drug release in controlled manner. All formulations manifested good percentage of entrapment from 57.49 % to 75.67 % and gave better average drug release performance up to 12 h.

Keywords
5- Fluorouracil, PEGlyation, Non-covalent functionalization, Multi walled carbon nanotubes, Carcinoma

INTRODUCTION

Recently nanobiotechnology based developed formulations are used either diagnostic or therapeutic tools. The important nanotechnological based formulations are polymeric nanoparticles, liposomes, dendimers, nanoshells, carbon nanomaterials, superparamagnetic nanoparticles conjugated with DNA, RNA interference (RNAi) and antisense oligonucleotides (ASO) [1]. Especially carbon nanotubes and graphene were much considered in the scientific trials because of their physicochemical properties which could be also encouraging in many biomedical areas. One of the drawbacks of these is cytotoxicity. However, this can be managed to low cytotoxicity when properly functionalized. Functionalization additionally helps to increase the possibility to binding and release of multiple molecules from the carbon nanotubes, particularly suitable for anticancer drug delivery system[2]. Cancer is one of the major diseases, which will cause worldwide death. At present treatment for cancer, one can follow the surgery, radiotherapy or chemotherapy prominent sources of death worldwide. Treatment of cancer necessitates a cautious selection of one or more intervention, such as surgery, radiotherapy, and chemotherapy[3]. Anticancer drug bound to carbon nanotubes with surface engineering were under trials by several research groups[4]. Carbon nanotubes (CNTs) are allotropes of carbon, made of graphite and constructed in cylindrical tubes with nanometer in diameter and several millimeters in length. Carbon nanotubes are seamless tubes of graphite sheets with nanosized diameter based on layer of graphite sheets and includes, Single walled carbon nanotubes (SWNTs) and Multi walled carbon nanotubes (MWNTs). The terminal parts of some nanotubes are open and the others are closed with full fullerene caps. Carbon nanotubes are also named as 'King of Nanomaterials'. Depending on sheet direction and diameters, it may be either metallic or semi-conducting in nature. Carbon nanotubes have highest theoretical strength when compared with all kinds of natural materials. It is 100 times stronger than steel, although their specific gravity are only one sixth that of the latter. Carbon nanotubes enjoy special advantage in the field of absorbing electro-magnetic radiation, field emission, thermal conducting, hydrogen storing, adsorbing and catalyzing[5- 8]. Carbon nanostructure is a one atom that is densely packed in honey comb crystal lattice. There are various techniques which can be used for the synthesis of CNTs. These include the arc-discharge method, chemical vaporize deposition (CVD), the laser ablation method, and the sol gel method [9, 10]. The main problem with the majority of popular synthetic methods is that they produce samples yielding a mixture of various diameters and chiralities of nanotubes that are normally contaminated with metallic and amorphous impurities and less water solubility. CNTs severely limited in use due to,

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poor interfacial interaction, vander Waals interaction, between CNTs and polymer matrix. To resolve those problems, it has been directed towards developing methods to modify surface properties of CNTs by functionalization process. These approaches can be simply divided into chemical (covalent) and physical (noncovalent) functionalization as interactions between active materials and CNTs [11]. In our research work we preferred the non - covalent functionalization because it an alternative method for tuning the interfacial properties of nanotubes, does not destroy the conjugated system of the CNTs sidewalls, while improving their solubility quite and therefore it does not affect the final structural properties. The CNTs are functionalized non-covalently by aromatic compounds, surfactants, and polymers, employing π-π stacking or hydrophobic interactions[12-14]. PEG polymer is preferred for non covalent functionalization of MWCNTs in our studies. PEGylation is also a widely-used pharmaceutical formulation strategy in clinical settings, improves water dispersibility, as it reduces immunogenicity and non-specific interactions with plasma proteins, while concomitantly prolonging blood circulation times[15]. The current, study is concentrated on the Non-covalent functionalization of pristine MWCNTs by using PEG and preparation and characterization of PEG functionalized MWCNTs-5-Fluorouarcil complex. The produced complex were analyzed by various characterization techniques Scanning Electron Microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), Particle size, Zeta potential, Drug loading and drug release studies.

MATERIALS:

Multiwalled carbon nanotube (outer diameter 10- 30 nm, number of walls 5-15, length 1- 10μm) was procured from Nano Wings Private limited, Telangana. 5- Fluorouracil was kind gift of Nantong Jinghua Pharmaceutical co.,ltd. China. Poly ethylene glycol 4000, 1N Ammonium hydroxide, and Hydrogen peroxide etc... All chemicals used were of laboratory grade and they were kind gift from Samarth life science private limited. Formulations were prepared at Samarth life science private limited, Tumkur. SEM studies (ModelESEM QUANTA 200) were done at advanced facility for microscopy and microanalysis, Indian institute of science, Bengaluru. Infrared spectra were recorded on Perkin Elmer spectrum at Bangalore testing laboratories pvt.Ltd. Particle size and zeta potential was found by using Malvern zetasizer ZS at Malvern-Aimil application centre, Bengaluru.

METHODS:

Functionalization of MWCNTs with PEG [16-20]

500 mg of pristine MWCNT were dispersed in 1 g of PEG in 10 ml of distilled water with the assistance of a fast clean ultra sonic bath sonicator for 15 min. Removal of unbound and agglomerated MWCNTs was accomplished by centrifugation at 7000 r/5m where only the supernatant was kept. Disposal of agglomerates of unbound MWCNT was then performed by centrifugation and filtration done through 0.2 micro filters (Millipore) through vaccum filter and dried.

Preparation of PEG-MWCNTs -5- Fluorouracil: [21- 23]

PEG functionalized MWCNTs was dispersed in 5 Fluorouracil solution [5-Fluorouracil in 25-28%1N ammonium hydroxide solution (50 mg/ml)] in tarson tubes as shown in figure 2 according to given in table 1 and sonicated for 30 min. Successively, the dispersion is rotated for 24 h by using rotor to facilitate loading of 5 Fluorouracil. Subsequently, the mixture was subjected to centrifugation at 5000 r/m for 15 min and then washed with methanol and followed by deionized water three times and centrifuged to remove free unbound 5 Fluorouracil. Whereas the solid sample was dried at 30 °C in a vacuum oven for 24 h to obtain PEG functionalized -MWCNTs- 5 Fluorouracil complex. PEG functionalized - MWCNTs- 5 Fluorouracil complex was stored at room temperature in a vacuum desiccator for further use of studies.

Formulations	PEG – MWCNTs (mg)	5-Fluorouracil (mg)
F1	50	50
F2	100	100
F3	150	150
F4	200	200
F5	250	250

Table. 1: Formulation of PEG - Functionalized MWCNTs loaded with 5 –Fluorouracil

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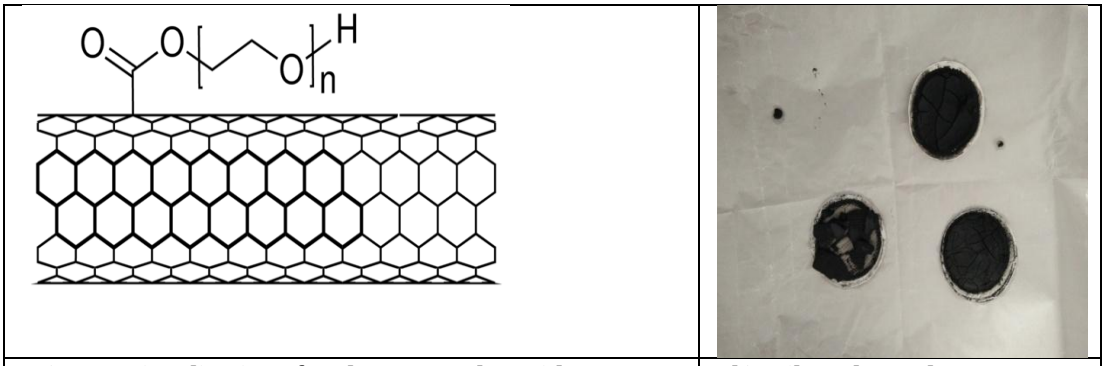

a) Functionalization of carbon nanotubes with PEG | **b) Filtered sample**

Figure. 1: PEG functionalized MWCNTs

Figure.2 : Dispersion of PEG – MWCNTs in to 5 Fluorouracil solution.

Evaluation of PEG - MWCNTs -5- Fluorouracil formulations:

SEM studies [24, 25]

Sample having agglomerates were exposed for sonication. Micro-pipette were used to put to a small drop of sample on SEM grid and the solvent present in the evaporated, SEM analysis were used for dry sample analysis. Samples are retained in vacuum chamber operated at a pressure of -1×E-3 Pa gaseous environments. SEM technique completed established on irradiation of the sample by an electron source. An electron beam is scanned across the sample, the back scattered electrons are detected to produce image of the morphology or topography of the sample.

FT-IR studies [26, 27]

Fourier- transform spectroscopy is a method used to catch an infrared absorption spectrum or emission of a solid, liquid or gas. Withdrawn 5 mg of sample, blended with 100 mg of potassium bromide to prepare pellet, sample holder was cleaned using acetone and wipe by using Kimwipes, Prepared sample was kept in sample holder, adjust the scan range 400- 4000 cm^{-1}, and resolution to 4. Monitor the pressure applied to the sample, and check IR spectra.

Particle size [28, 18]

Dynamic light scattering method is used for particle size determination. Dynamic light scattering techniques are used for characterizing nanomaterial due to its speed. The dispersion of sample shaken vigorously to break up loose aggregate. Take 1 ml sample in four sided transparent disposable cuvette, keep in cuvette holder, measured by right angle scattering for three repeats of two minutes each.

Zeta potential [29, 18]

The zeta potential is indicates the stability of colloidal dispersion. The zeta potential indicates the degree of electro static repulsion between adjacent similarly charged particles in dispersion. Take sample sonicate for 15 min at 90 Hz,

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then the dispersion was settled at room temperature for 24 h, 1 ml sample in disposable folded capillary zeta potential cuvette was taken and measured the zeta potential.

Drug Entrapment Efficiency [22, 23]

Drug entrapment efficiency done to evaluate the drug entrapment on MWCNTs. Take all formulations 5 mg separately in 100 ml of tarson tubes add 10 ml of phosphate buffer pH 7.4 mix thoroughly heated to 37 ºC on water bath, cool it, and then subject to centrifugation for 1 h at 10,000 r/m to remove entrapped drug from the formulations. Take 1 ml supernatant solution from centrifuged drug solution, dilute suitably to determine the drug concentration using by UV spectrophotometer at 266 nm.

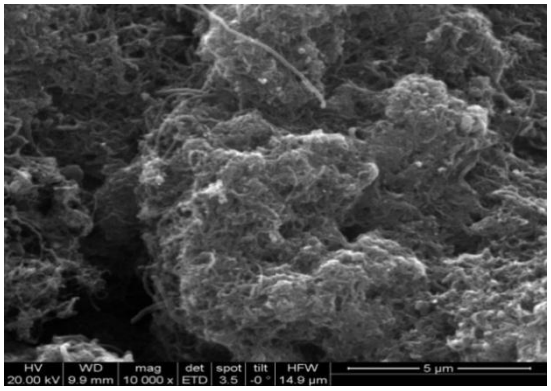
***In vitro* release studies** [22, 23, 30]

The *in vitro* drug release of all the formulations was conducted through a dialysis membrane -70 (HIMEDIA). Freshly prepared phosphate buffer pH 7.4 was used as a dissolution medium. Accurately weighed amount of using all formulation equivalents to 25 mg of drug was taken, soaked overnight in phosphate buffer. Place the drug containing buffer solution in approximately 1.2 inch in length dialysis tube and tie the ends of tube to form a pouch shape. All the dialysis tube containing drug solutions were placed separately in conical flasks containing 100 ml of phosphate buffer pH 7.4, maintained at 37ºC in shaking water bath with a frequency of 50 shakings per min. Aliquots, 1ml of volume, were withdrawn at regular intervals and replace with equal volume of phosphate buffer into conical flask. Taken aliquots were suitably diluted and analyzed by UV- spectrophotometer at 266 nm.

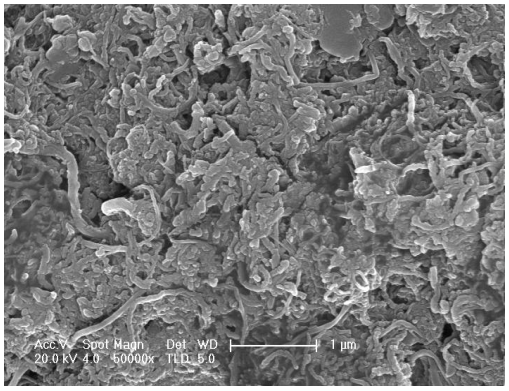
RESULTS AND DISCUSSION:

SEM studies:

SEM studies done at Indian institute of science, SEM images of PEG functionalized MWCNTs in figure 3a, 3b shows a layer of uniform polymer is clear on the sidewall of the nanotubes and diameters of samples are slightly increased, SEM images of PEG functionalized MWCNTs compared with to PEG functionalized - MWCNTs - 5 Fluorouracil composite, the PEG functionalized -MWCNTs- 5-Fluorouracil conjugate structures are quite different from those of PEG functionalized- MWCNTs, in which the tube surface and diameters of nanotubes are increased as depicted in figure 4a and 4b.



a) PEG Functionalized MWCNTs at 5 µm



b) PEG Functionalized MWCNTs at 1 nm

Figure.3: SEM studies of PEG Functionalized MWCNTs

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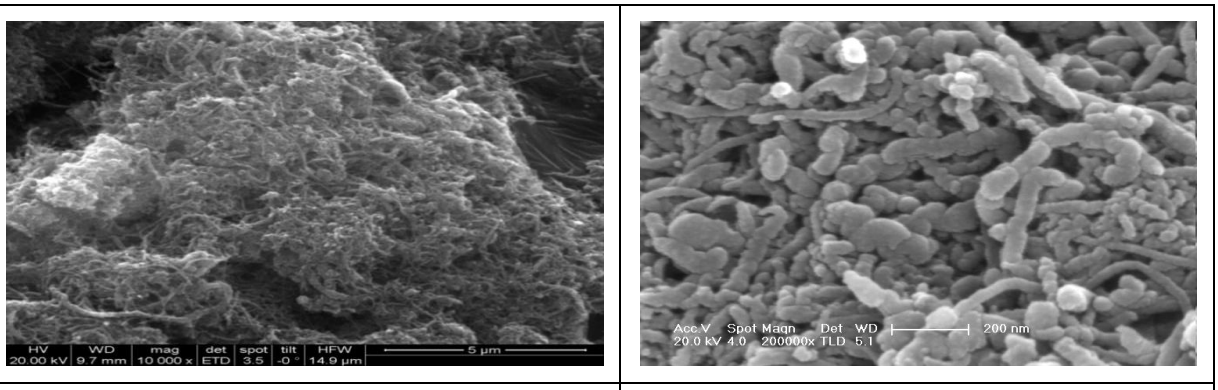
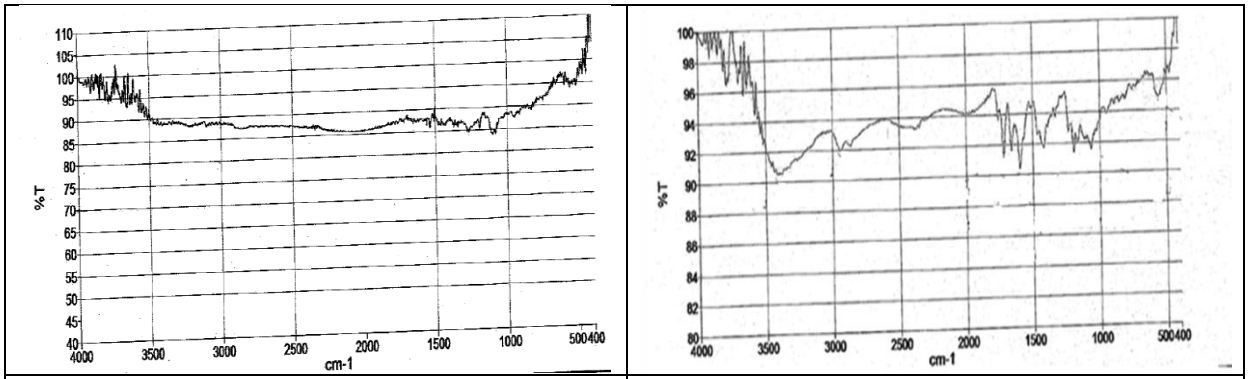


a) SEM studies of formulation at 5 µm | **b) SEM studies of formulation at 200 nm**

Figure.4 : SEM studies of Formulation PEG Functionalized MWCNTs 5-Fluorouracil composite

FT-IR Studies:

The optimized MWCNTs were then characterized by the use of FTIR spectroscopy (Perkin Elmer Spectrum II) at Bangalore testing laboratories Pvt.ltd. The samples were recorded in the 4000 to 400 cm^{-1}. The FTIR spectra for MWCNTS were given in the figure 5a, PEG functionalized -MWCNTs- 5 Fluorouracil composite FT-IR was signified in figure 5b**.** The peaks observed are from the FT-IR graph is evident that the absorption bands at 1661.51cm^{-1}, 1449.89cm^{-1}, 3136.40cm^{-1} , 1430.70cm^{-1} and 1246.87cm^{-1}sign posts the presence of C=O, C=C, N-H, C-F and C-N stretching vibrations corresponding to 5-Fluorouracil, the peak at 1349.35cm^{-1} refers to vibration of pyrimidine compound endorsing 5-Fluorouracil. The strong PEG absorption spectra at around 2886 cm^{-1} for CH_2CH_2streching, and the presence of saturated carbon at 3748 cm^{-1}.



a) FT-IR graph Of MWCNTs | **b) FT -IR graph of Formulation PEG Functionalized MWCNTs 5-Fluorouracil composite.**

Figure. 5: FT-IR graph Of MWCNTs and PEG Functionalized MWCNTs 5-Fluorouracil composite.

Particle size:

The particle size and zeta potential of the PEG functionalized MWCNTs and PEG functionalized -MWCNTs- 5 fluorouracil conjugate was characterized by a laser particle size analyzer at Aimil limited, Bengaluru. Figure 6a presents the particle size of PEG fuctionalized MWCNTs in three trials, figure 6b presents the particle size of PEG functionalized -MWCNTs- 5 fluorouracil composite in three trails. The average particle size diameter of PEG functionalized MWCNTs and PEG functionalized -MWCNTs- 5 fluorouracil conjugate is 191 nm, 432 nm respectively. The purchased MWCNTs particle size is about 30nm, after functionalization process the MWCNTs particle size increased to 191 nm, drug binded composite particle size increased up to 432 nm.

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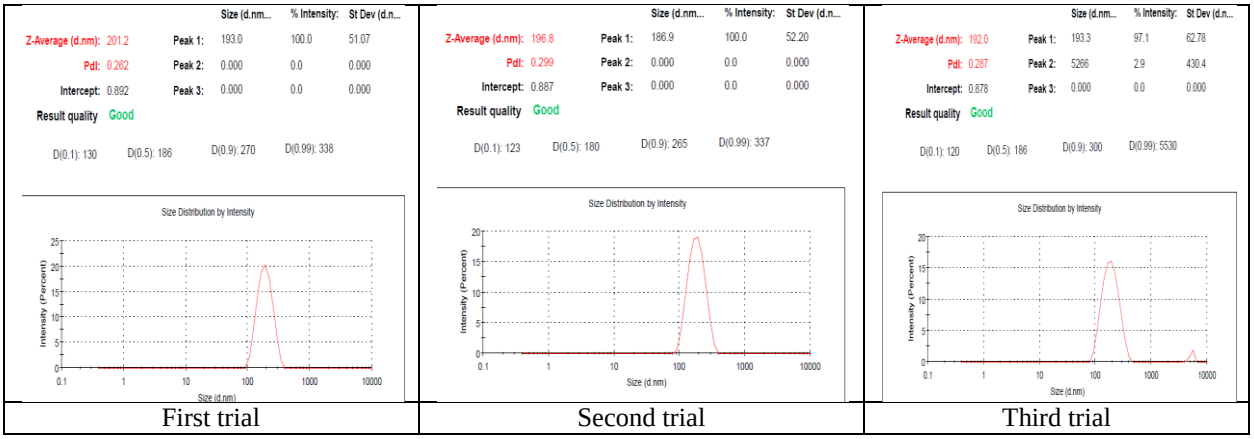
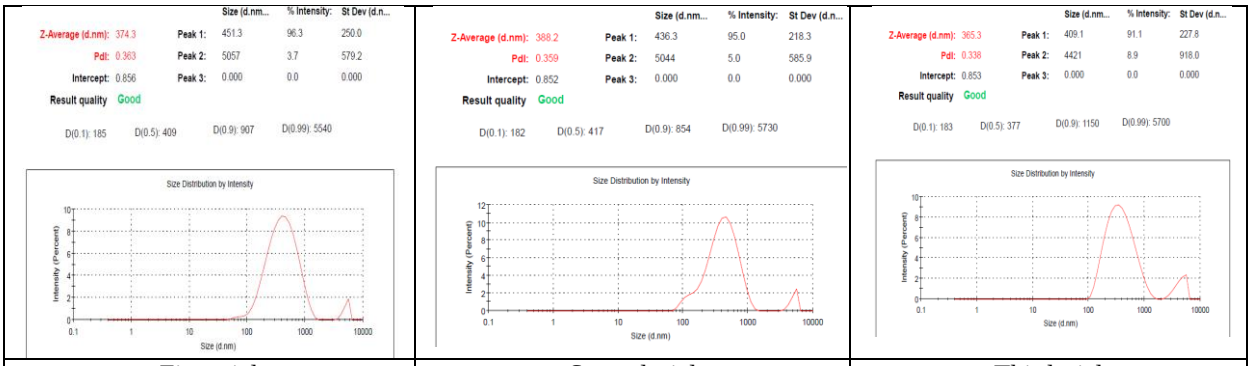


First trial	Second trial	Third trial

Figure 6a: Particle size distribution of PEG functionalized MWCNTs

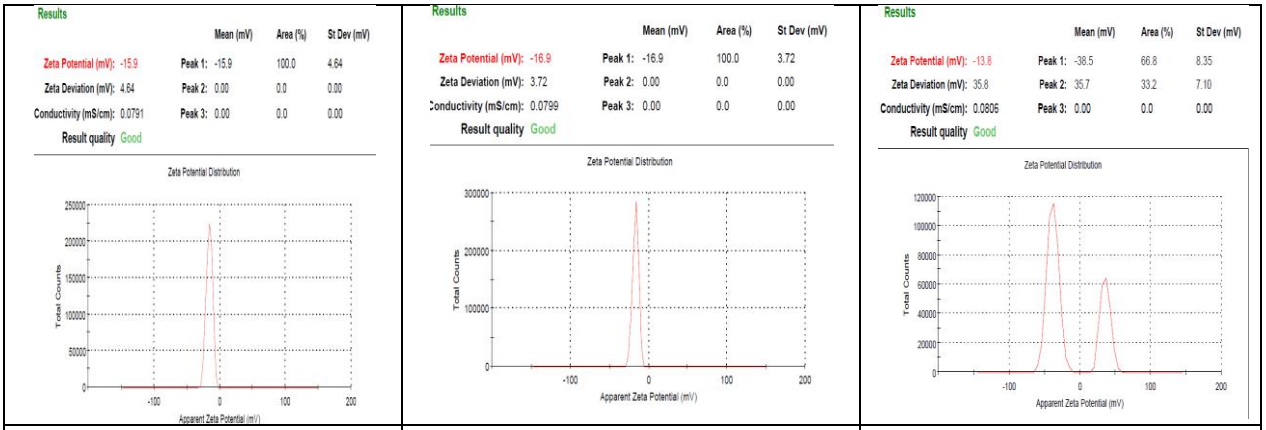


First trial	Second trial	Third trial

Figure. 6b: Particle size distribution of Formulation PEG Functionalized MWCNTs 5-Fluorouracil composite.

Zeta potential:

Figure 7a, 7b presents the zeta potential of PEG functionalized MWCNTs and PEG functionalized -MWCNTs- 5-fluorouracil composite correspondingly in three trials. The average zeta potential of PEG functionalized MWCNTs and PEG functionalized -MWCNTs- 5 fluorouracil conjugate is -15.53 mV, -23.4mV respectively.



First trial	Second trial	Third trial

Figure. 7a: Zeta potential of PEG functionalized MWCNTs.

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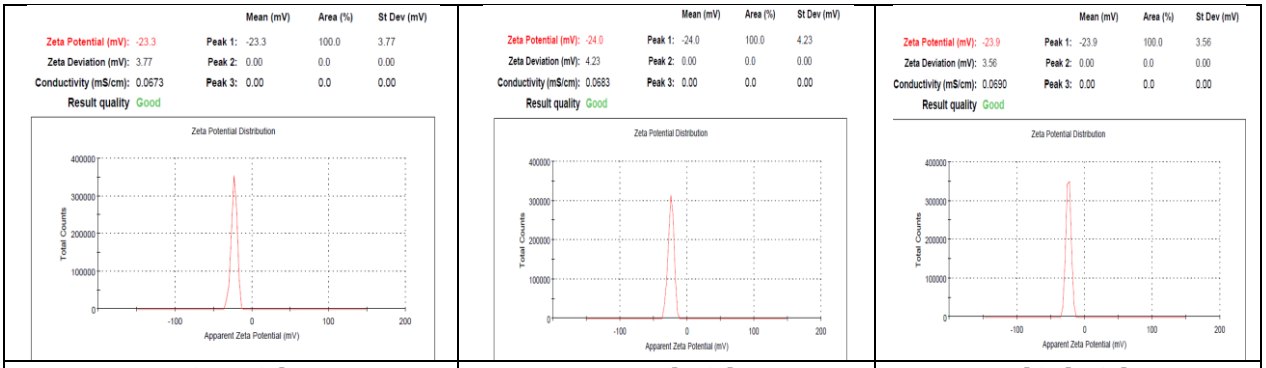


First trial	Second trial	Third trial

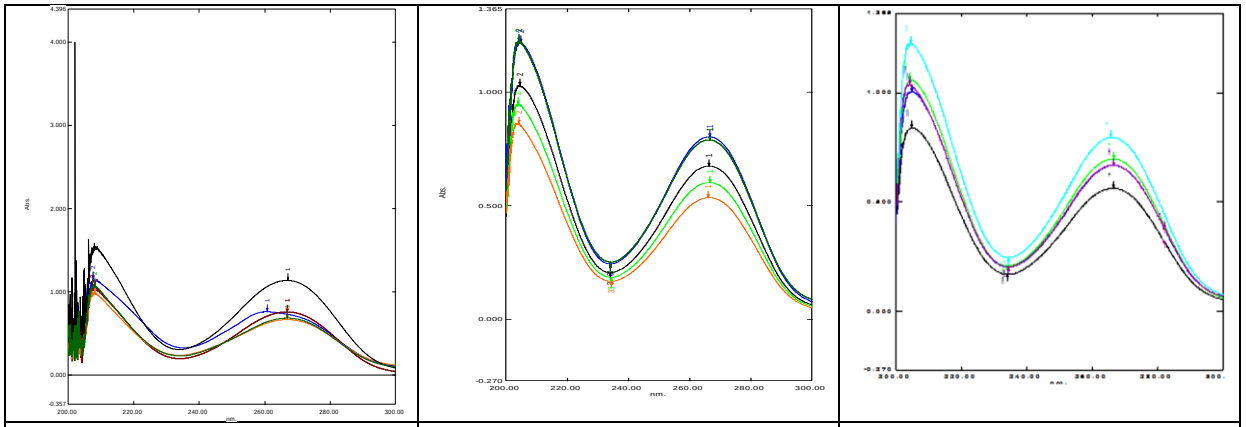
Figure. 7b: Zeta potential of Formulation PEG Functionalized MWCNTs 5-Fluorouracil composite

Drug entrapment efficiency:

Figure 8 depict the UV graph of 5- Fluorouracil, drug entrapment efficiency of all five formulations F1, F2, F3, F4, F5 done in 3 trials given in table 2 reveals traditionally good average percentage of entrapment efficiency. Drug entrapment efficiency and standard deviation of all five formulations 73.52%, 57.49%, 63.41%, 66.08%, 75.67% and 2.38, 0.711, 2.972, 0.20, 2.43 respectively.

DRUG ENTRAPMENT STUDIES										
1 st TRIAL			2 nd TRIAL			3 rd TRIAL			AVERAGE	STANDARD DEVIATION
FORMULATIONS	Uv absorbance	% of drug entrapment to MWCNTs	FORMULATIONS	Uv absorbance	% of drug entrapment to MWCNTs	FORMULATIONS	Uv absorbance	% of drug entrapment to MWCNTs		
F1	0.758	73.16	F1	0.788	76.06	F1	0.739	71.33	73.52	2.38
F2	0.604	58.30	F2	0.593	57.23	F2	0.590	56.95	57.49	0.71
F3	0.665	64.18	F3	0.623	60.13	F3	0.683	65.92	63.41	2.97
F4	0.683	65.92	F4	0.687	66.31	F4	0.684	66.02	66.08	0.20
F5	0.756	72.97	F5	0.791	76.35	F5	0.805	77.70	75.67	2.43

Table.2 :Average drug entrapment studies along with standard deviation



First trial	Second trial	Third trial

Figure.8: Drug entrapment UV graph of 5- Fluorouracil stacked of all formulations.

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Drug *in-vitro* release:
Drug *in-vitro* release of all five formulations is 60.10%, 50.74%, 56.81%, 57.76%, 63.08% respectively. Bar chart of drug *invitro* release of all formulations shown in figure 9. The comparative *in-vitro* drug release profile of all the five batches exhibited the controlled drug release, the formulation F5 shows better percentage of drug release in 12 h release study. It is clearly clinched that formulation F5 is the superlative among the five formulations when compared to other formulations.

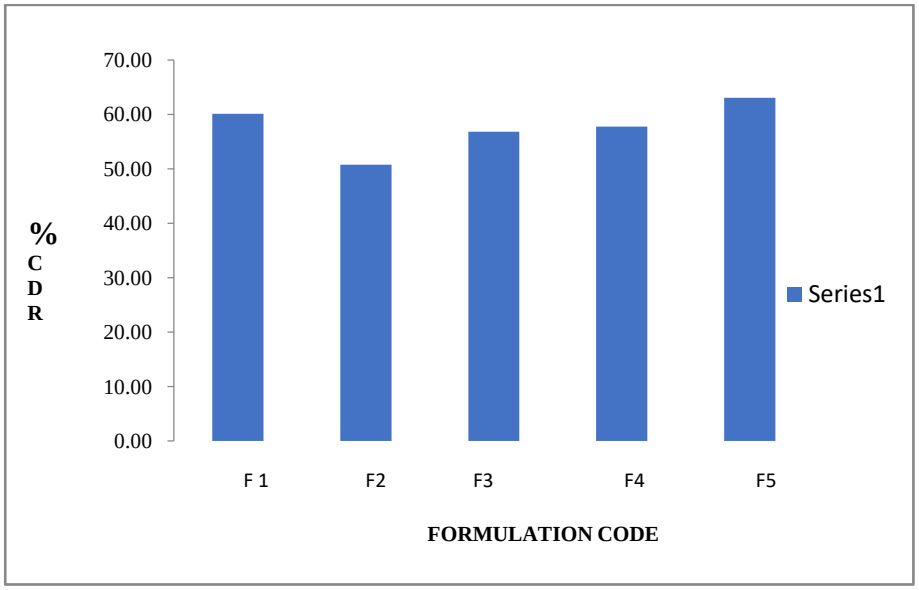

Figure 9: Bar chart for average of all formulations of drug release studies

CONCLUSION

In this present work, attempt is done for drug loading to nanotubes with strong specificity for the cancer treatment, Non covalent functionalization of MWCNTs was carried out by coating MWCNTs with polymer , poly ethylene glycol (PEG), shortening of length due to the sonication employed in the functionalization process followed by centrifugation, filtration and drying methods. The physical properties of CNTs are essentially preserved by the non covalent approach, the non-covalent functionalization of CNT is much simpler method preserving nanotubes's SP^2 aromatic structure and their electronic characteristics and binding of drug to functionalized MWCNTs were carried out effectively. Preparation of MWCNTs-5 Fluorouracil composite is simple, commodious and come up with enhanced development in medical use. Prepared formulations characterised by microscopic studies, good percentage drug entrapped to MWCNTs, the obtained results from unique nanotube drug delivery system clearly validate that drug release is enriched and diminish the side effects by PEGlyation process. CNTs could be used for drug delivery research for limited number of years, in upcoming application prospect of nanotubes has also been researched and discussed.

CONFLICT OF INTEREST:

There is no conflict of interest with authors.

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