International Journal for Multidisciplinary Research (IJFMR)
E-ISSN : 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Preparation and Statistical In-vitro Evaluation of Covalent Functionalized Multi Walled Carbon Nanotube 5-Fluorouracil Conjugates

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Abstract

In 21st century, carcinoma is predicted to be a remarkable health problem. The condition of the cancer patients will be critical when it comes to its remedy by chemotherapy using artificial anticancer molecules with various chemo side effects. In the field of nanotechnology, the nanomaterials are rapidly developing. Nanomaterials become admirable and necessary in lot of areas of human interest because of their exclusive size - dependent properties, chemical reactivity, conductivity, and enlarged strength. Carbon nanotubes (CNT) are referred to as nanomaterials. In this present world of medical research and the bio sensing technology has the capability to transform the CNT in the field of efficient medicine delivery, biosensor strategies for ailment treatment and for the health tracking. Our present work is an strive to probe the loading and release of 5- Fluorouracil from Multi Walled Carbon Nanotubes (MWCNTs) to cancer tissues. MWCNTs were subjected to covalent functionalization by using the strong acids, the oxidised MWCNTs are obtained in covalent functionalization process. Through this operation, carboxy (-COOH) groups are set up at the open sides (tips) and at the defects on the sidewalls of MWCNTs and then covalent functionalized MWCNTs were loaded with 5- Fluorouracil to prepare covalent functionalized MWCNTs-5-Flourouracil conjugate. Covalent functionalized MWCNTs-5-Flourouracil conjugate were subjected for SEM studies, TEM studies, particle size, zeta potential, drug entrapment efficiency and in vitro drug release. The release of drug shows longer than 40 h from covalent functionalized MWCNTs-5-Flourouracil conjugate and thus to manage the release of drug in controlled manner. The present research work presents esteemed, novel, and easy to prepare formulation of MWCNTs with superior drug entrapment efficiency with increased hydrophilic property of MWCNTs, and thus increases the bioavailability at selected site with narrow systemic toxicity.

Keywords: Multi walled carbon nanotubes, Nanomaterials, 5- Fluorouracil, Taguchi method, Covalent Functionalization, Orthogonal array

Introduction

Cancer is a deadliest malady in the globe, in this 21st century cancer is one of the health fret, killing millions of human beings. Cancer is a group of affliction involves unrestricted rise of atypical cells by overlooking the normal cell division (Jogi *et al.,* 2018; Zhang *et al.*, 2011). In our lifestyles carbon is the basic element. In the history of carbon, the discovery of carbon nanotubes (CNT) is a great breakthrough. In 1991, Sumio Iijima found the carbon nanotubes (CNT). In the field of nanotechnology, carbon

nanotubes are having novel property that make them prospective beneficial in many applications. The three major methods are used in the production of CNT (a) arc discharge technique (b) laser vaporization and (c) chemical vapour deposition (CVD) (Varshney 2014; Ruhal et al., 2012; Farahani et al., 2016; Brhane et al., 2016).

Carbon nanotubes built in cylindrical tubes with several millimetres in length and nanometer in diameter. Because of mass and nano size CNT possess structural, mechanical, electronic properties, mechanical potency, excessive electric and thermal conductivity. CNT were correctly executed in the medical field for the delivery plenty of diagnostic and therapeutic agents (medicines, fluorescent agent, genes, antibodies, vaccines, etc) because of their high surface area, (He et al., 2013; Meng et al., 2008; Sánchez et al., 2014). The nanotubes will be chiral and non-chiral depending on the manner of enfold of graphene sheets as regard to the shape of the edges, and the nanotubes will end up fullerene hemispheres. (Alpturk et al., 2018; Kalwat et al., 2018). Nanotubes are similar to graphite, they composed of sp^2 chemical bonding, and this bonding construction are stronger than the sp^3 bonds determined in diamonds, presents the molecules with their particular energy, by using Vander Waal's forces nanotubes align themselves into "ropes" (Basu et al., 2014; Saeed et al., 2013). Carbon Nanotubes can be broadly classified as Single Walled Carbon Nanotubes (SWCNTs) and Multi Walled Carbon Nanotubes (MWCNTs) (Karimi *et al.*, 2015; Liu *et al.*, 2009; Sadegh *et al.*, 2015).

CNTs needs to be functionalized. Functionalization is process of altering the physical and chemical properties of CNTs by attaching or introducing the molecules such as drugs, proteins, polymers *etc* on the surface of CNTs to build as a drug delivery system. Through the process of functionalization of carbon nanotubes, it possible to decrease cytotoxicity, impurity and increases the solubility of CNTs in a aqueous solution. Graphene sheets coupled with the strong π-π interactions it leads to aggregation of CNTs in to bundles. In functionalization process nanotubes surfaces will leads to modify so reduces the aggregation of CNTs in to bundles (Komane *et al.*, 2018; Tan *et al.*, 2014).

The functionalization of CNTs can be classified in to main four groups: Covalent functionalization, Non-Covalent functionalization, External decoration with inorganic materials, and endohedral filling.

In the present work the designs of experiments was done by Taguchi method using statistical software tool Minitab 17.0. Pristine MWCNTs were functionalized prior to drug binding. MWCNTs covalent functionalization is normally procured by oxidation with strong acids. In functionalization procedure, CNTs are oxidized by introducing the COOH and OH groups at the open sides (tips) and at the defects on the sidewalls of MWCNTs. Chemical treatment on nanotubes will leads to the presence of charge on surface and allows the adsorption of charged molecules on the surface through ionic interaction. (Das *et al.*, 2016; Garnica-Gutiérrez *et al.*, 2018; Le *et al.*, 2013; Cendrowski *et al.*, 2018). Covalent functionalized MWCNTs 5-Flourouracil conjugate was prepared by loading the 5- Fluorouracil in to Covalently Functionalized MWCNTs. 5- Fluorouracil is a pyrimidine analogue and anti-cancer action executed in different techniques, but mainly it acts as a thymidylate synthase inhibitor. It leads to DNA damage due to variation of deoxythymidine monophosphate and (dUMP) level will increase so quickly splitting neoplastic cells go through thymine less death (cell death).

All prepared formulations Covalent functionalized MWCNTs 5-Flourouracil conjugates were evaluated. Functionalization and drug loading were assessed and confirmed by Scanning Electron Microscopic technique and Transmission Electron Microscopic technique. Particle size, Zeta potential,

Fourier-transform spectroscopy studies, Thermo gravimetric Analysis. Drug Entrapment Efficiency and *in vitro* drug release were assessed.

Materials and Methods

Multi walled carbon nanotubes were purchased from Nano Wings Private limited, Telangana. Drug 5-Fluorouracil was kind gift of Nantong Jinghua Pharmaceutical Co., Ltd. Solvents and chemical reagents like Methanol, Hydrochloric acid, Sulphuric acid, Nitric acid, Ammonium hydroxide (25 %), Hydrogen peroxide (30 %) *etc* are laboratory grade, they were kind gift from Samarth life science private limited**.** Formulations were prepared at Samarth life science private limited, Tumkur. SEM studies were done at Advanced facility for microscopy and microanalysis (Model ESEM QUANTA 200), Indian institute of science, Bengaluru, and TEM studies were done at Centre for Nano Science and Engineering (Model-M3000), Indian institute of Science, Bengaluru. Infrared spectra were recorded on Perkin Elmer spectrum at Bangalore testing laboratories Pvt. Ltd. The purity of samples was evaluated via thermogravimetric analysis (TGA) by using Mettler Toledo analyser at Indian institute of sciences, Bangaluru. Particle size and zetapotential was found by using Malvern zetasizer ZS at Malvern-Aimil application centre, Bengaluru.

Covalent Functionalization

Pristine MWCNTs were subjected to Covalent functionalization by the following three methods

Initial acidic treatment followed by treatment with hydrochloric acid: Covalent functionalized MWCNTs are produced by this method. The initial acidic treatment done by using HNO_3 and H_2SO_4, which produces oxidized MWCNTs and then same oxidized MWCNTs are treated with HCl and produces carboxylated MWCNTs. Take 250 mg of MWCNTs in flask add 100 ml mixture of 98 % H_2SO_4 and 65 % HNO_3 (V:V = 3:1) and subjected to 12 h agitation at room temperature. Obtained oxidised MWCNTs were washed with fresh water and immersed in HCl, keep 24 h for reflux, then filtered to obtain carboxylated MWCNTs and wash with fresh water until to neutral pH. The collected products are dried overnight at 40 ^oC in vacuum.

Treatment with concentration Hydrochloric acid : Treatment with conc. hydrochloric acid is used to purify the CNTs. Take 250 mg of MWNCTs and 100 ml of HCl in a 500 ml beaker. Beaker containing mixture was subjected to stirring for 2 h using magnetic stirrer, then add more water, filter it, wash with fresh water and then dry overnight in vacuum at 40 ^oC.

Initial basic treatment followed by treatment with hydrochloric acid: Initial basic treatment followed by treatment with hydrochloric acid method is used to fabricate covalent functionalized MWCNTs. The basic treatment done by using ammonium hydroxide and hydrogen peroxide to obtain oxidized MWCNTs and then the oxidized MWCNTs were mixed with HCl to produce carboxylated MWCNTs. Take 250mg of MWCNTs and add 12.5 ml blend of ammonium hydroxide (25 %) and hydrogen peroxide (30%) (V: V=1:1) in a round bottom flask and connected with a condenser. The oxidized MWCNTs were heated at 80 ^oC, after 5h disperse in HCl and reflux for 12 h, dilute the treated MWCNTs in water and filter. Then until neutral pH the residue was washed with fresh water and dry the sample overnight in vacuum at 40 ^oC. (Ghoshal et al., 2014; Ntim et al., 2011; Jeon et al., 2011; Vardharajula et al., 2012).

Formulations Prepared by Covalent Functionalized MWCNTs and 5- Fluorouracil (Loading of 5-Fluorouracil Onto Covalent Functionalized MWCNTs)

Covalent functionalized MWCNTs were dispersed in to 5-Fluorouracil solution in methanol (1 mg / ml) at varied concentrations as depicted in Fig.1 and subjected to sonication for 30 min. Later, the MWCNTs dispersion is rotated for 24 h by using rotor to accelerate 5-Fluorouracil loading to MWCNTs. Subsequently, the dispersion was centrifuged at 7000 r/min for 15 min, wash with methanol and once again wash with deionized water for three times, again centrifuged to detach the unconfined drug. The supernatant was discarded, collect the sediment, whereas the solid sample was dried at 30 ^{o}C in a vacuum oven for 24 h to obtain covalent functionalized-MWCNTs-5-Fluorouracil conjugate. The covalent functionalized -MWCNTs- 5-Fluorouracil conjugate were stowed in a vacuum desiccators at room temperature for further studies. (Wang and Li et al., 2016; Ghoshal et al., 2015)

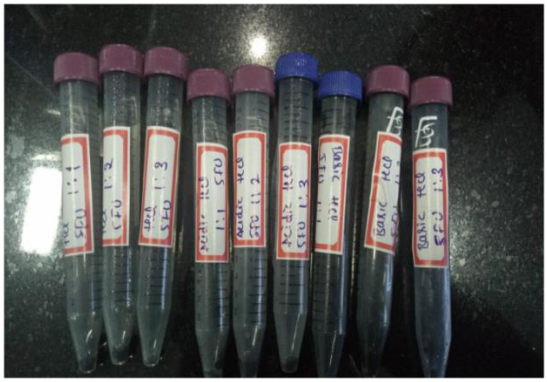

Fig. 1 Covalent functionalized MWCNTs was dispersed in 5-Fluorouracil solution

Scanning Electron Microscopy Analysis

Samples are allowed to be observed in pressure of -1×E-3 Pa gaseous environments in vacuum chamber, sample should be in dry condition for SEM analysis, an electron beam is scanned across the specimen and the back scattered electron are detected to generate an image of the morphology or topography of the sample.

Transmission Electron Microscopy Analysis

TEM studies were done at Indian institute of science, Bangalore. Ethanol is the solvent used for the preparation of MWCNTs sample, 4 to 5 drops of sample were kept on TEM grids made of copper, and the excess liquid on the sample was removed by the filter paper by touching the edges of the grid.

FT-IR studies

FT-IR Studies done at Merieux Nutrisciences Bangalore pvt .ltd. Prepare the pellet by using 5 mg of MWCNTs sample and 100 mg of potassium bromide, the sample holder was cleaned by using acetone with Kim wipes and keep in pellet in sample holder. The resolution is adjusted to 4 and the samples were recorded in the scan range limit 4000- 400 cm^{-1}.

Thermo gravimetric Analysis

In STAR e software (Mettler Toledo) allocate the sample name, Purge gas: N2, Heating temperature: RT to 1000, Heating rate: 10K/min, 1-20 mg sample loaded into a empty weight of aluminium oxide crucible (70 micro litre). The filled crucible are stored on board and auto sampler installed them in to the furnace on microbalance. When everything is set then start the experiment, the details will be given by a simple thermo gram.

Measurement of Particle size

Particle Size and Zeta Potential were analysed by instrument called Malvern zetasizer ZS at Malvern-Aimil application centre, Bengaluru.

Drug Entrapment Efficiency

50 mg of covalent functionalized -MWCNTs- 5- Fluorouracil conjugate was mixed with 100 ml of phosphate buffer pH 7.4, heated at 37 ^{o}C and then centrifuged at 10,000 r/min for 1 h to remove entrapped drug from MWCNTs in the formulation, 1ml supernatant solution is suitably diluted to determine the drugs concentration using by UV spectrophotometer at 266 nm.

***In- vitro* Drug Release Studies**

According to DOE *In-vitro* release studies done, the data obtained for all nine formulations were given in Table 2. Dialysis membrane-70 (HIMEDIA) was used to study the drug release patterns, freshly prepared phosphate buffers pH 6.3, 7.4, and 8.0 are taken as dissolution medium. All formulations are weighed precisely equivalent to 25 mg of drug, soak in the dissolution medium for overnight and then filled in dialysis tube (approx. 1.2 inch in length), the tube ends tied to form pouch shape. Take 100ml of phosphate buffer pH 6.3, 7.4, and 8.0 in different conical flask place the drug filled pouches, keep it on shaking water bath maintained at a temperature of 37 ^{o}C. 1ml volume of aliquots were taken at regular intervals accordingly designed in DOE given in Table 2, each time replace 1ml of dissolution medium into conical flask, The aliquots diluted with buffers suitably and analyzed in UV-Vis spectrophotometer at 266 nm.

Statistical Analysis

Taguchi is a one of statistical method established based on orthogonal array (OA). To carry out the experiments there are different orthogonal arrays depends on process parameters and levels. S/N ratio will be sorted out by the experiment process response. The equation 1 represents for higher the better S/N ratio. Excellent amalgamation of process parameters are attained by the combination of S/N ratio and ANOVA analysis.

The confirmation experiment was performed to verify the optimal process parameters.

$$S/N = -10*\log(\Sigma(1/Y^2)/n) \quad \text{..... (1)}$$

Where y_1, y_2, y_3, ...y_n, are the outcome of the drug release behavior studied, n is the number of observations. The details of the experiments and their levels as per the Taguchi L18 array were tabulated in the Table 1. In this study 18 tests were conducted and all parameters were varied for 6, 3, 3 and 3 levels respectively.

Table 1: Process parameters and levels

Level	Drug release time (hr)	Covalent Functionalized CNT	Drug Conc.(ratio)	Buffers (PH)
1	2	Method 1	1	6.3
2	8	Method 2	2	7.4
3	18	Method 3	3	8.0
4	28			
5	38			
6	48			

Results and Discussion

Scanning Electron Microscopy

SEM images of Covalent Functionalized -MWCNTs- 5 Fluorouracil conjugate were shown in Fig. 2a. SEM images of covalent functionalized -MWCNTs- 5 Fluorouracil conjugate structures are quite different from those of pristine MWCNTs in which the tube surface and diameters of Nanotubes are slightly increased.

Transmission Electron Microscopy

TEM image of Covalent Functionalized- MWCNTs- 5 Fluorouracil conjugate are presented in Fig. 2b. It shows that the drug molecules are placed within the tubes and with a larger inner diameter. TEM images revealed MWCNTs were strongly interacting with drug via formation of supramolecular cluster.

a) **b)**

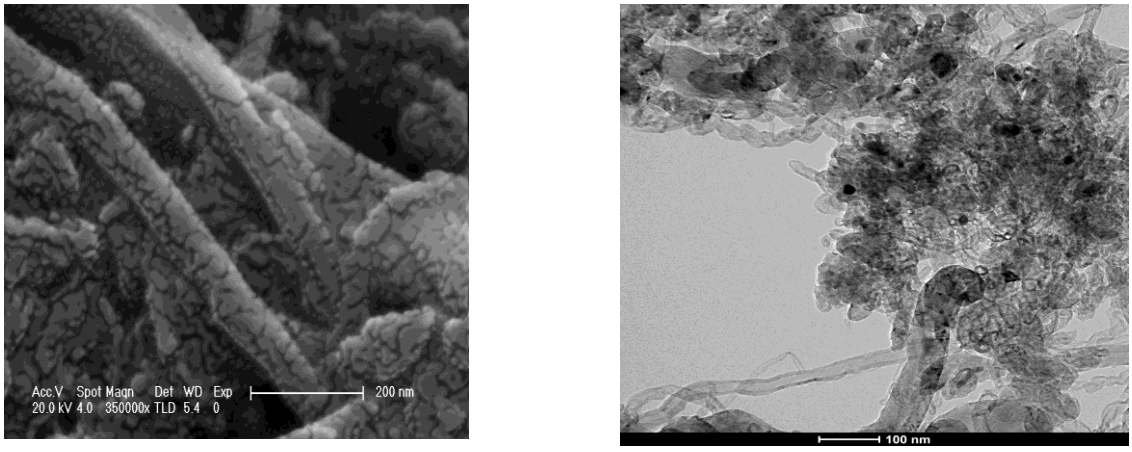

Fig.2. a) SEM images of F- MWCNTs -5 Fluorouracil conjugate, b) TEM images of covalent functionalized MWCNTs 5-Flourouracil conjugate.

Characterization of FT-IR spectra

The Covalent Functionalized -MWCNTs- 5 Fluorouracil conjugate FT-IR spectra was shown in the Fig.3. The -COOH and -OH group peaks were observed at 1709 cm^{-1} (range 1740-1700 cm-1) and 2800 cm^{-1} (range 3300- 2500 cm-1) respectively, and the functional groups are clearly indicated. The observed apparent peaks are evident, that the absorption bands at 1656.07cm^{-1}, 1439.74cm^{-1}, 3068.15cm^{-1} ,1428.61 cm^{-1} and 1246.42cm^{-1} clearly indicates the existence of C=O, C=C, N-H, C-F and C-N respectively, these stretching vibrations corresponds to 5-Fluorouracil. The peak observed at 1349.35 cm^{-1} confess to pyrimidine compound vibration, confirming 5-Fluorouracil.

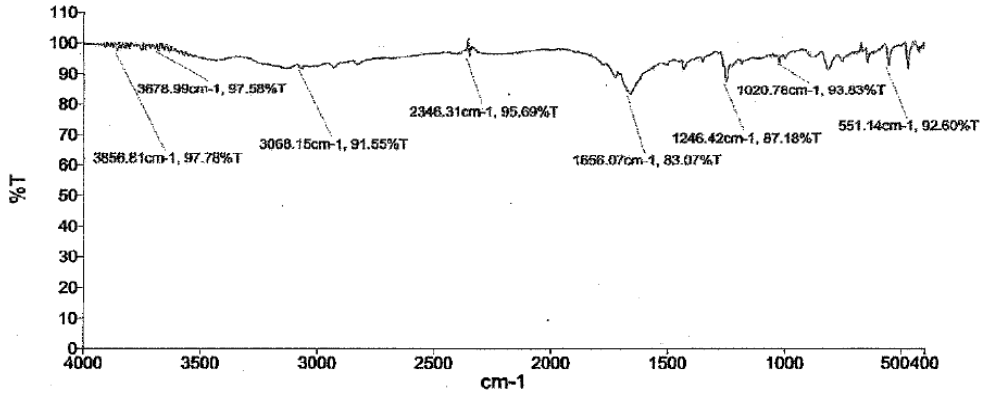

Fig. 3 FT-IR graph of covalent functionalized MWCNTs 5-Fluorouracil conjugate.

Thermo gravimetric Analysis

TGA analysis of Covalent Functionalized -MWCNTs- 5 Fluorouracil conjugate presented in Fig. 4 The solid lines correspond to Thermo gravimetric (TG) curve. These results revealed that, the initial burning starts around the temperature of 250 ^{o}C because of the existence of amorphous carbon in MWCNTs mixture, and around a temperature of 280 ^{o}C MWCNTs mixture start burning/disintegrating themselves. TGA performance was carried out up to 750 ^{o}C. After performing the TGA , the sample remainder observed in the furnace, due to the presence of drug found in the MWCNTs.

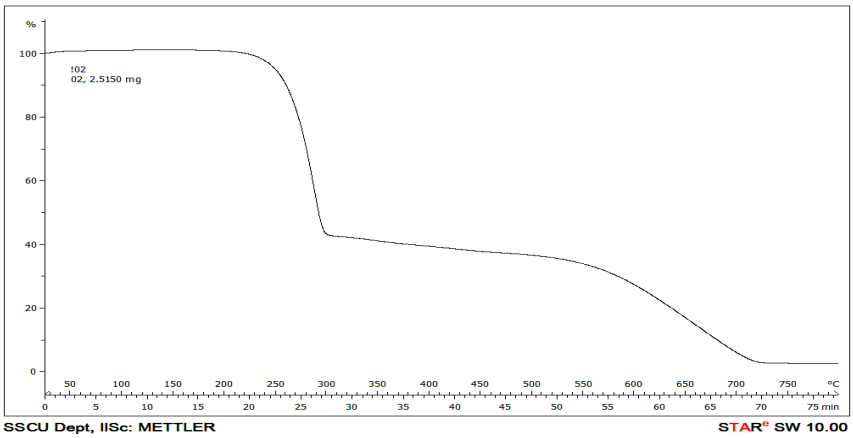

Fig. 4 TGA analysis of F- MWCNTs -5 Fluorouracil conjugate

Characterization of covalent functionalized -MWCNTs- 5- Fluorouracil conjugate

The particle size of Covalent Functionalized -MWCNTs- 5 Fluorouracil conjugate is 402 (d.nm). The zeta potential of Covalent Functionalized -MWCNTs- 5 Fluorouracil conjugate is -4.21 mV.

Drug entrapment efficiency

Drug entrapment efficiency of all nine formulations F1, F2, F3, F4, F5, F6, F7, F8 and F9 shown reasonably good percentage of entrapment efficiency with 76.33%, 82.71%, 84.64%, 68.60%, 61.06%, 78.26%, 79.52%, 78.16% and 77.39 % respectively.

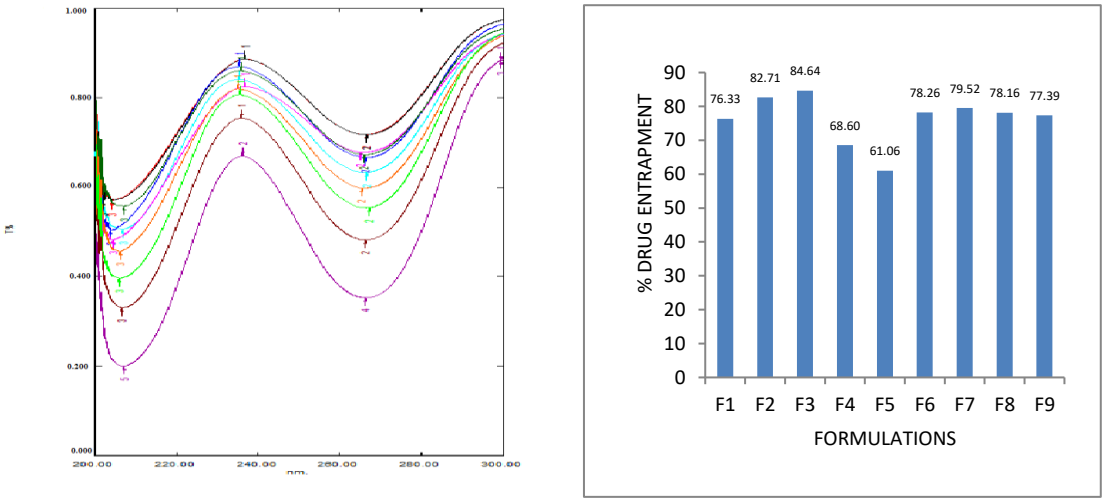

Fig.5 a) Uv graph overlay of drug entrapment efficiency b) bar chart for drug entrapment efficiency for all nine formulations.

Statistical Analysis of *In vitro* release data

The obtained different parameter sequences for S/N ratio and drug release data conducted according to L18 orthogonal array reported in Table 1 and Table 2. MINITAB 17 software used to scrutinize the measured results. The impact of process parameters (drug release time, functionalized CNTs, drug and buffers) were evaluated by S/N ratio. The response table for this experiment is given in Table 4, it is clearly describes that time is a predominant parameter on drug release followed by covalent functionalized CNT, different pH buffers and drug concentration.

Table 2: Result of orthogonal array of Taguchi for drug release

Drug release time (Hours)	Functionalized MWCNTs (methods)	Drug (ratio)	Buffers (pH)	Drug release (mg)	S/N ratio
2	1	1	6.3	2	6.0206
2	2	2	7.4	5	13.9794
2	3	3	8.0	5	13.9794
8	1	1	7.4	8	18.0618
8	2	2	8.0	10	20.0000
8	3	3	6.3	15	23.5218
18	1	2	6.3	15	23.5218
18	2	3	7.4	25	27.9588
18	3	1	8.0	25	27.9588
28	1	3	8.0	36	31.1261
28	2	1	6.3	37	31.3640
28	3	2	7.4	40	32.0412
38	1	2	8.0	56	34.9638
38	2	3	6.3	45	33.0643
38	3	1	7.4	70	36.9020
48	1	3	7.4	79	37.9525
48	2	1	8.0	70	36.9020
48	3	2	6.3	61	35.7066

Table 3: Analysis of variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Drug release time	1	9532.4	9532.42	321.00	0.000
Functionalized CNT	1	70.1	70.08	2.36	0.148
Drug ratio	1	21.3	21.33	0.72	0.412
Buffers	1	87.1	87.06	2.93	0.111
Error	13	386.0	29.70		
Total	17	10096.9			

Table 4: Response table for signal to noise ratios

Level	TIME	CNT	DRUG	BUFFER
1	11.33	25.27	26.20	25.53
2	20.53	27.21	26.70	27.82
3	26.48	28.35	27.93	27.49
4	31.51			
5	34.98			
6	36.85			
Delta	25.53	3.08	1.73	2.28
Rank	1	2	3	4

Table 2 shows each experiment S/N ratio for time. The evident value of experiment no.16 results are optimal, the formulation F3 (MWCNTs functionalized by using method 1 initial acidic treatment followed by treatment with hydrochloric acid and drug concentration (ratio3)) shows better percentage of drug release in buffer pH 7.4 at 48 h. It is clearly concluded that formulation F3 is the best among the nine batches when compared to other formulations.

Response table for SNR's shown in Table 4. The rank values gives the level of influence of input factor on response variable. The drug release mainly depends on time to a maximal intensity later the functionalized CNTs, Buffers and Drug ratio has the uttermost effect. Table 3 also represents the same and also shows the ANOVA results, the P- value indicates the factors which are significant on drug release. It's P-value is less than 0.05. R^2value access the identity, describes the experiment fitness degree and also explain the factors which affects the performance characteristics. The R^2value is 96.18%. Thus, our experiment model represents satisfactory, and explains the correlation between input factors and response variable. Fig.6b represents S/N ratio main effect plot. It demonstrates as increase in all the factors S/N ratio will increases. Always better to have S/N ratio maximum, if higher it is better choice…. But change in slope is very high in Time V/s SN ratio value graph hence to conclude optimum value for time at this point. Before drawing conclusions, it is necessary to check the assumptions of ANOVA. Normal probability plot presents the line dividing the points into two equal half, hence experimental data meets the normality assumption. From the standardized residual versus the fits plot, Randomly scattered residuals materialized to be zero, no proof of unusual structures, terms missing, outlier exists, non-constant variance, it specify that experimental data roughly satisfies the independent / randomization assumption.

Constant variance - The Fig.6a indicates plot of residuals v/s order values don't specify any pattern and hence constant variance assumption is satisfied.

a) **b)**

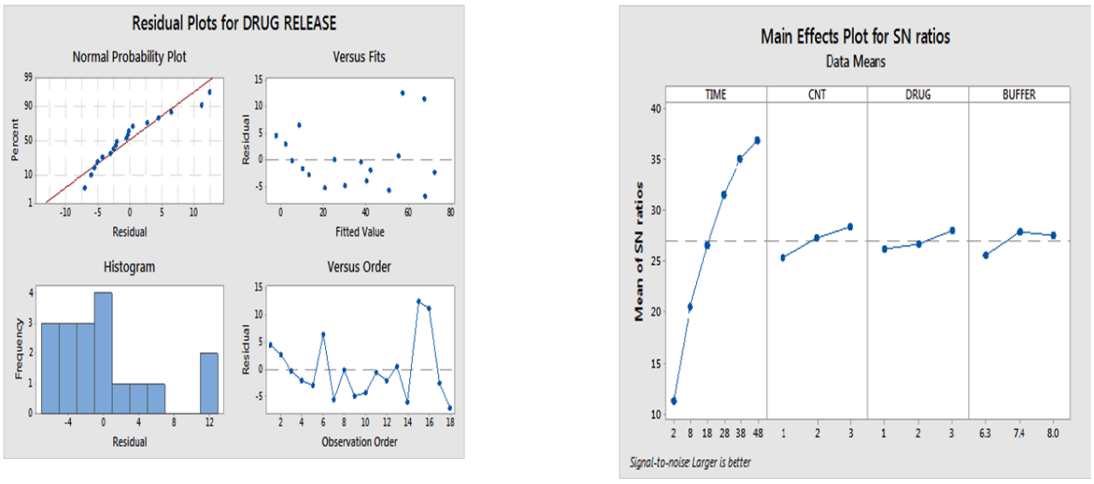

Fig.6 a) Residual plot for drug release, b) Main effects plots for SN ratio.

Conclusion

The facile three strategy methods were used for the covalent functionalization of MWCNTs were systematically reported. Covalent functionalized MWCNTS 5- Fluorouracil conjugate was prepared by loading 5- Fluorouracil to covalent functionalized MWCNTS by using a simple preparation method. covalent functionalized MWCNTS 5- Fluorouracil conjugate characterization was done by FTIR method, microscopic (SEM and TEM) studies showed that drug loaded to functionalized MWCNTs by increase in the diameter of pristine MWCNTs. All prepared formulations showed better drug entrapment efficiency, results also confirmed by thermo gravimetric analysis. The particle size of covalent functionalized MWCNTs 5- Fluorouracil conjugate as found to be 402 (d.nm). Compare to pristine MWCNTs, the covalent functionalized MWCNTs 5- Fluorouracil conjugate size was increased. Zeta potential is found to be measure the electro static interactions between colloidal particles and stability of colloidal dispersion.

In our work a new strategy to maximize the effects of 5- Fluorouracil along with reducing the systemic toxicity by functionalization. We have demonstrated with effective drug release by using different pH buffers from the prepared formulations. In future the purposed nanotubes formulations can potentially lead to further studies in drug targeting.

Acknowledgement

This research work was supported by Samarath Life Sciences Pvt. Ltd, KIADB Industrial area, Vasanthnarasarapura, Tumkur.

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