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Original Article

Drug Loading and Release from Functionalized Multiwalled Carbon Nanotubes Loaded With 6-Mercaptopurine Using Incipient Wetness Impregnation Method

Saurav Ghoshal*[1], Swatantra Kumar Singh Kushwaha[2], Mayank Srivastava[1] and Pallavi Tiwari[1]

[1]Shambhunath Institute of Pharmacy, Jhalwa, Allahabad, Uttar Pradesh, India 211012
[2]Pranveer Singh Institute of Technology, Bhauti, Kanpur, Uttar Pradesh, India 209305

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Address for Correspondence
Shambhunath Institute of Pharmacy, Jhalwa, Allahabad, Uttar Pradesh, India 211012.

Tel. +91-9026298672.
E-mail: saurav.ghoshalpharma@gmail.com

ABSTRACT

The present work was an attempt to investigate the potentialities of multi-walled carbon nanotubes (MWCNT) as a carrier for targeting 6 Mercaptopurine to cancer tissues. MWCNTs were carboxy functionalized and then loaded with 6 Mercaptopurine (6MP) using the Incipient Wetness impregnation method to produce 6MP loaded CNTs. The conjugate was characterized for drug loading efficiency, *in vitro* drug release and release kinetics. The result indicated that a maximum of about 65% entrapment was achieved. The loaded nanotubes were shown to release the drug for more than 20 hours and thus controlling the release. The release was found to follow the Zero Order and Hixson Crowell release pattern. Our work established a novel, easy to prepare formulation of MWCNTs with better drug loading efficiency and increased dispersibility of CNTs and thus improved bioavailability at cancer site with reduced systemic toxicity.

Keywords: Multiwalled carbon nanotubes, 6-Mercaptopurine, Anticancer, Incipient wetness impregnation method.

INTRODUCTION

Cancer is amongst the top three killers in modern society, next to heart and cerebrovascular diseases. Treating cancer has always been a challenge because cancer chemotherapeutic agents are cytotoxic and cannot differentiate cancer cells from normal cells. This leads to the destruction or impairment of vital organs particularly those that have high rate of cell division like the liver, GI lining, hair and skin; in addition to killing of the cancer cells, if their bio-distribution is not properly controlled and the therapeutic agents not targeted towards the cancer cells or tissues. Thus targeting continues to be the Holy Grail in anticancer therapy[1].

Discovery of Carbon Nanotubes in 1991 by Ijiama provided a ray of hope in this field. Carbon nanotubes (CNTs) are described as hollow cylinders formed by rolling single layer (single-walled CNTs; SWNTs) or multiple layers[1,2] (multi-walled CNTs; MWNTs) of graphene sheets into seamless cylinders. In recent years, it has been demonstrated that CNTs can not only be loaded with drugs[3-7], nucleic acids andpeptides[8] by forming stable covalent bonds or supramolecular assemblies based on noncovalent interactions, but also have capacity to penetrate into the cells to promote the cellular uptake of therapeutic molecules[9], which has offered new opportunities for their applications in nanobiotechnology and nanomedicine. Although most of the existing anticancer drugs are very potent small molecules, their efficacy is constrained not only by their systemic toxicity, narrow therapeutic window, low drug loading, size control, scale up, cost of formulation[10] but also as a result of drug resistance and limited cellular entry. For this reason, the development of efficient delivery systems with the ability to enhance cellular uptake of existing potent drugs is needed. The high aspect ratio of CNTs offers great advantages over existing delivery vectors, because the high surface area provides multiple attachment sites for drugs[11]. Functionalization of CNTs not only makes it more soluble/dispersible in water but also provides active sites for attachment of drugs, ligands and other agents like PEG to achieve long blood circulation half-life helping to impede in-vivo opsonization and reduced reticulo-endothelial system uptake[12]. In addition, many oxygen containing groups, mainly carboxyl and hydroxyl, have been found to decorate the surface of CNTs oxidized with strong acids[13].

Several targetted anticancer delivery systems containing 6-Mercaptopurine (6MP) have been reported[14-18]. In the present study, 6MP (6, 7-dihydro-3H-purine-6-thione) (Fig 1) loaded CNTs were developed by slight modification in incipient wetness impregnation method[19]. Non-covalent functionalization of Multiwalled Carbon Nanotubes (MWCNTs) was achieved using initial basic treatment followed by treatment with HCl. Functionalized MWCNT were attached with 6MP by sonication in a suitable media (pH 7.4). The formulations were characterized for drug entrapment, *in vitro* drug release and release kinetics.

EXPERIMENTAL

Materials

Multiwalled carbon nanotubes with 10-15 nm outer diameter, 2-6 nm inner diameter and 0.1-10 µm length were purchased from the Redex Technologies, Pvt. Ltd., Noida (UP) India. 6 Mercaptopurine was received as a gift sample from Dabur Pharmaceuticals, Baddi (H.P.), India. All other chemicals were of analytical grade purchased from local suppliers.

Method

Functionalization of Carbon Nanotubes

The Carbon Nanotubes were covalently functionalized by subjecting them to three types of treatments[20].

(a) Treatment with conc. Hydrochloric acid

This method simply purified the CNTs. In this method 500mg of MWNCTs was placed in a 500 ml round bottom flask and 200 ml of HCl was added. The mixture was stirred using magnetic stirrer for 2 h, then diluted in water, filtered, washed with ultrapure water and then dried in vacuum at 40^0C overnight.

(b) Initial acidic Treatment followed by treatment with hydrochloric acid

It was used to produce covalently functionalized MWCNTs. In this method the initial acidic treatment with nitric acid and sulphuric acid produced oxidized MWCNTs and then the treatment with hydrochloric acid produced carboxylated MWCNTs. 500 mg of MWCNTs were added to a 200ml mixture of 98% H_2SO_4 and 65% HNO_3 (V:V = 3:1) and agitated for 12 h at room temperature. The MCWNTs were thoroughly washed with ultrapure water and dispersed in HCl and refluxed for 24 h, then collected by filtration and washed with ultrapure water to neutral pH. The product was then dried in vacuum at 40^0C overnight.

(c) Initial basic treatment followed by treatment with hydrochloric acid

It was used to produce covalently functionalized MWCNTs. In this method the initial basic treatment with ammonium hydroxide and hydrogen peroxide produced oxidized MWCNTs and then the treatment with hydrochloric acid produced carboxylated MWCNTs. 500mg of MWCNT was dispersed in 25 ml of the mixture of ammonium hydroxide (25 %) and hydrogen peroxide (30%) (V: V=1:1) in a 100 ml round bottom flask equipped with a condenser and the dispersion was heated to 80^0C and kept for 5 h. After that, the resulting dispersion was diluted in water and filtered. Then the resulting residue was washed with ultrapure water up to neutral pH and the sample was dried in vacuum at 40^0C overnight.

Selection of the best method for functionalization

This selection was made on the basis of dispersion stability. For this 10 mg of functionalized nanotubes were dispersed into 10ml of phosphate buffer solution pH 7.4 by sonication for 2 minutes and these dispersions were then kept in sealed vials, the dispersion stability was visually analyzed after a period of 15 days.

Functionalization of MWCNTs by the Optimized method

After the visual evaluation of MWCNTs it was found that the initial basic treatment followed by HCl treatment was the best method for functionalization and this was chosen for functionalization of the MWCNTs for the preparation of drug loaded MWCNTs and 3gms of MWCNTs were then functionalized by the basic treatment followed by treatment with HCl. The optimized CNTs were then characterized by the use of FTIR spectroscopy (Perkin Elmer Spectrum II).

Preparation of drug loaded carboxylated MWCNTs

A concentrated solution of 6MP (100mg in 10ml) was prepared in two different solvents (01.N NaOH and ethanol: water 1:9). The solvent combination was chosen depending upon the solubility of 6 MP which is highly soluble in 0.1 N NaOH and Ethanol, Ethanol alone was not chosen as it corrodes the filtration membrane, hence it was diluted with water. This solution was

added to carboxylated-MWCNTs. Continuous agitation, using ultra sonicator, was applied during the addition of the 6MP solution. The solution thus obtained was stirred for 2 hrs and then the dispersion obtained was filtered using vacuum filtration assembly fitted with membrane filter (0.5µm, Sigma Aldrich), and then the residue was washed with ultrapure water. The products were dried at 40^0C for 24 hours.

Evaluation of the Drug loaded CNTs

Prepared formulations were evaluated by following tests:-

- Entrapment
- *In vitro* release studies
- Drug Release Kinetics studies

Drug Entrapment

All the formulations were subjected to the determination of drug entrapment studies. The entrapment was determined by dispersing accurately weighed quantity of formulation (containing amount of drug equivalent to 50 mg), into 100 ml of phosphate buffer pH 7.4 and heating upto 37.0^0c, to ensure the release of the entrapped drug. Aliquot of 1 ml was withdrawn and further diluted to 10 ml with buffer, 6 mercaptopurine concentration was then determined at 320 nm by using UV-Vis spectrophotometer(UV-1700 Pharma Spec, Schimadzu).

In vitro Release Studies

The *in vitro* release of 6 mercaptopurine from all the formulations was studied through a dialysis membrane (molecular weight cut off 12000, Sigma Aldrich). The dissolution medium used was freshly prepared Phosphate buffer pH 7.4. An accurately weighed amount of formulation equivalent to 25mg of drug was calculated and placed in the dialysis tube (approximately 1.2 inch in length), previously soaked overnight in the dissolution medium and the ends were tied to form a pouch. The dialysis tube pouches were then placed in conical flasks containing 100 ml of phosphate buffer pH 7.4, placed in the shaking water bath (HICON, New Delhi) and maintained at 37^0c with a frequency of 50 shakings per minute. Aliquots, each of 5 ml volume, were withdrawn at regular intervals and replaced by an equal volume of the dissolution medium. The aliquots were then suitably diluted (10 times) and analyzed by UV-Vis spectrophotometer at 320 nm.

Drug Release Kinetics studies

The Drug release data obtained from all the formulations were fitted into various mathematical models given below in order to determine the Drug release kinetics of prepared formulations:

- Cumulative percent drug released V/s. Time [Zero order rate kinetics].
- Log percent drug remaining to be released V/s. Time [First order rate kinetics].
- Cumulative percent drug released V/s. Root Time [Higuchi matrix].
- (Amount remaining to be released) $^{1/3}$ V/s. Time [Hixson-Crowell erosion equation].

To find out the mechanism of drug release, 60 % drug of release data was first fitted in the Korsmeyer-Pappas model. Where Log of cumulative percent drug released was plotted against Log Time. The model was used to study the drug release mechanism by analyzing 'n' as the diffusion exponent. According to this model if 'n' is below 0.45 then Fickian mechanism governs drug release, if between 0.45 to 0.89 then Non-Fickian mechanism governs drug release and if n is 0.89 or greater than 0.89, then release mechanism is governed by case-II transport or super case II transport mechanism respectively[21].

RESULT AND DISCUSSION

Functionalization of CNTs and Selection of the best method for functionalization

The CNTs were functionalized as per the three methods namely; treatment with conc. Hydrochloric acid, initial acidic Treatment followed by treatment with hydrochloric acid, initial basic treatment followed by treatment with hydrochloric acid. This selection was made on the basis of dispersion stability. For this 10 mg of functionalized nanotubes were dispersed into 10ml of phosphate buffer solution pH 7.4 by sonication for 2 minutes and these dispersions were then kept in sealed vials, the dispersion stability was visually analyzed after a period of 15 days. The best method was found to be the initial basic treatment followed by treatment with hydrochloric acid, as shown in Fig.1 and then the CNTs were functionalized according to this method itself and were used to prepare the formulations.

Characterization of the functionalized MWCNTS

This was done with the help of FTIR spectroscopy and the FTIR spectra of the functionalized MWCNTs was shown in Fig. 2(b). This shows the peaks for carboxy group at 1661cm^{-1}(range 1740-1700 cm^{-1}) and hydroxyl group at 3432 cm^{-1} (range 3300-2500 cm^{-1}) which were absent in pristine MWCNT Fig 2(a), thus proving that the MWCNTs were now carboxy functionalized[12].

Incorporation of drug by Incipient Wetness Impregnation Method

300mg of the drug was incorporated into the MWCNTs to prepare the formulations according to the formulation design table (table 1) and then the prepared formulations were filtered using vacuum filtration assembly.

Evaluation

Entrapment

Fig. 3 shows the percent entrapment of drug for the formulations. The entrapment was found to be quite low, with the maximum at around 65%. Entrapment for the formulations F1, F2, F3, F4, F5 and F6 was found to be 42.73%, 56.81%, 63.46%, 49.78%, 54.32% and 65.66% respectively.

In vitro Release Studies

The release profile for the formulation predicts how a delivery system might function and gives valuable insight into its *in vivo* behavior. The various formulations of 6 Mercaptopurine monohydrate were subjected to *in vitro* release studies. These *in vitro* release studies were carried out using phosphate buffer pH 7.4 as the dissolution medium.

The average cumulative drug release data obtained in triplicate (n=3) with respect to time for the various formulations were given in Table 2 and shown in Fig 5.

It was found that cumulative percent drug release for F1, F2, F3, F4, F5 and F6, was 96.0%, 95.3%, 91.3%, 95.1%, 96.1% and 92.1% respectively after 20 hours.

All the formulations showed similar release patterns with very slight differences. All formulations showed an initial burst release, which may be attributed to the drug which may be loosely attached to the surface of CNTs or held within the CNTs. Overall these 6 formulations prepared by the Incipient Wetness Impregnation method released almost all the drug content within 20 hours and thus were found to be more suitable for controlled release.

The prolonged release in the later stage could be attributed to the slow release of the drug form the CNTs. The *in vivo* drug release conditions may vary from those likely to be encountered within the body (particularly the extent of agitation and other

factors such as sink conditions). The bioavailability may also be lower than the values suggested in *in vitro* release because of the fast metabolism of the 6 MP in blood. However, the results clearly show that the formulations, particularly the ones prepared by the Incipient Wetness Impregnation method have the ability to release the drug for prolonged period of time as compared to formulations prepared by other methods such as the Solvent Method[22] and thus providing controlled release along with targeting.

Drug Release Kinetics Studies

Plots of zero order, first order, Higuchi matrix, Korsmayer Pappas and Hixson Crowell models for the formulations were plotted. The regression coefficient (r^2) values of zero order, first order, Higuchi matrix, Hixson-Crowell, Korsmayer Pappas and the 'n' values for Korsmayer Pappas were tabulated in Table 3.

Table 3 shows that for these formulations, the best fit model was Zero order for formulations F2 and F5, while for formulations F1, F3, F4 and F6 the best fit model was Hixson Crowell. The 'n' exponent value, for Pappas model, for formulations F1, F2 and F5 is greater than 0.45 indicating that formulation is released by Non Fickian diffusion mechanism. While for formulations F3, F4 and F6 the 'n' exponent value for Pappas model is less than 0.45 indicating that formulation is released by Fickian diffusion mechanism.

CONCLUSION

Anticancer drug delivery by using Carbon nanotubes is a new strategy with the potential to maximize the anticancer effect of a drug and reduce systemic toxicity. In this study, we have demonstrated the effectiveness of targeting of the anticancer agent 6 Mercaptopurine using CNTs, thus increasing bioavailability at cancer site and reduction of systemic toxicity due to tumour targeting using CNTs has been demonstrated. However some further studies are needed to confirm the in-vivo bioavailability of these products and this provides an avenue for further research.

Our work established a novel, easy to prepare formulation of MWCNTs with better drug loading efficiency and improved dispersibility of CNTs in water and provides new directions for preparation of efficient drug carriers.

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Table 1. Formulation design for preparation of drug loaded MWCNTs by the Incipient Wetness Impregnation Method

Contents	Quantity (w/w)					
	F1	F2	F3	F4	F5	F6
Drug (6MP)	1	3	7	1	3	7
C-MWCNT	1	2	3	1	2	3
0.1 N NaOH	10ml	10ml	10ml	-	-	-
Ethanol: water (1:9)	-	-	-	10ml	10ml	10ml

Table 2. Cumulative Drug Release with time of the prepared formulations

Time (hrs)	Formulation					
	Mean % cumulative drug release					
	F1	F2	F3	F4	F5	F6
0.5	2.1%	1.5%	1.4%	0.7%	1.3%	0.5%
1	9.2%	5.7%	7.9%	5.2%	4.8%	3.4%
2	17.9%	9.9%	11.4%	10.3%	8.2%	9.9%
3	22.7%	18.3%	18.2%	19.7%	14.2%	14.2%
4	28.1%	23.1%	24.1%	24.7%	20.1%	23.1%
6	32.9%	27.8%	28.7%	31.6%	25.4%	28.7%
8	46.7%	34.5%	38.2%	42.2%	35.1%	37.8%
10	57.2%	46.8%	48.5%	54.3%	43.2%	48.8%
12	68.8%	54.2%	56.8%	64.3%	51.9%	58.3%
14	80.1%	63.9%	67.3%	75.4%	63.2%	66.4%
16	91.2%	73.2%	74.2%	85.2%	73.0%	76.9%
18	95.3%	82.2%	84.2%	92.3%	82.1%	84.5%
20	96.0%	95.3%	91.3%	95.1%	96.1%	92.1%

Table 3. Regression coefficients for various models for the prepared formulations

Formulation	r^2				Korsmayer		Best Fit model	Release mechanism
	Hixsonc rowell	Zero	Higuchi	First	r^2	N		
F1	0.981	0.952	0.976	0.949	0.973	0.583	Hixson Crowell	Non fickian
F2	0.967	0.991	0.978	0.893	0.990	0.495	Zero	Non fickian
F3	0.991	0.981	0.985	0.964	0.975	0.433	Hixson Crowell	Fickian
F4	0.988	0.964	0.986	0.961	0.965	0.406	Hixson Crowell	Fickian
F5	0.956	0.995	0.970	0.867	0.994	0.497	Zero	Non fickian
F6	0.993	0.978	0.987	0.966	0.966	0.282	Hixson Crowell	Fickian

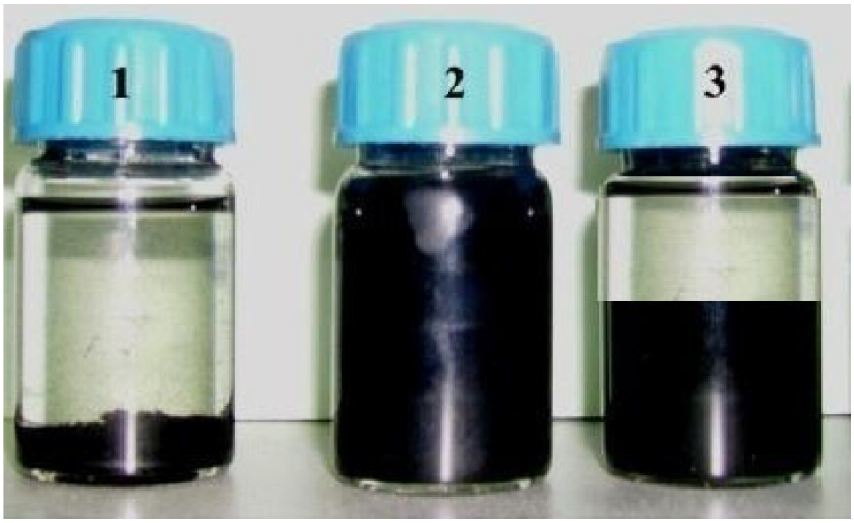

Figure 1. Dispersions of CNTs functionalized by different methods in phosphate buffer pH 7.4 (1=HCl treatment, 2=basic treatment and 3=acidic treatment) (picture taken after 15 days)

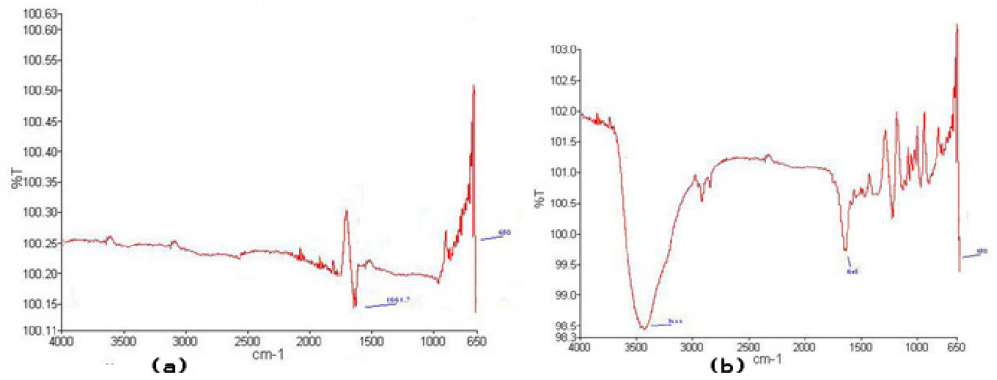

Figure 2. FTIR spectra of (a) pristine (untreated) MWCNT and (b) carboxylated MWCNT

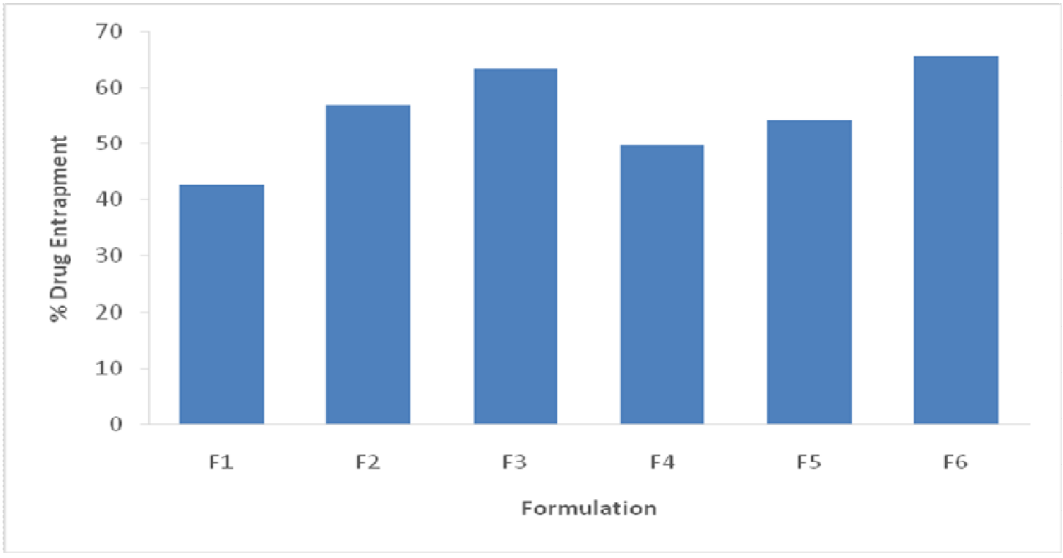

Figure 3. Percent Drug Entrapment of the prepared formulations

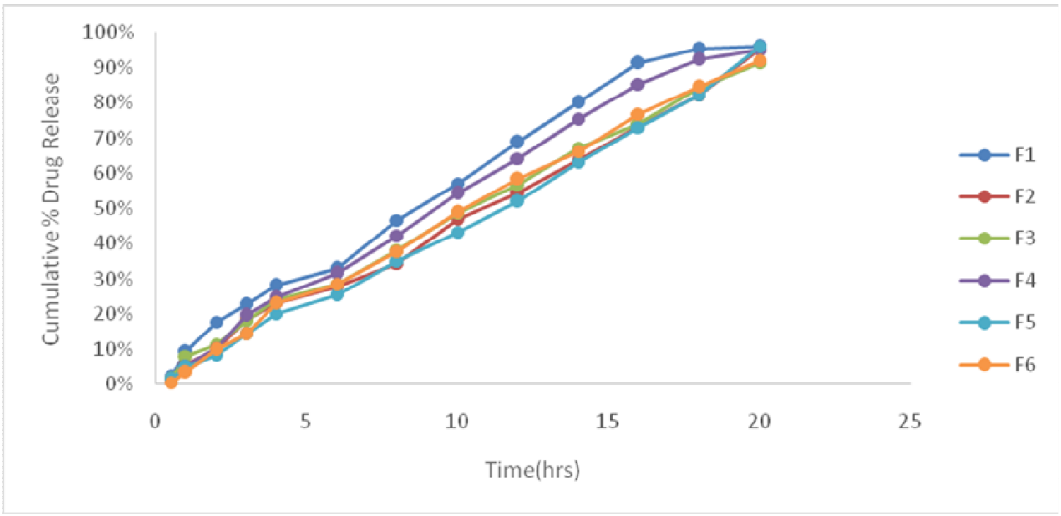

Figure 4. Release pattern of the prepared formulations